EVALUATION OF ANTIBACTERIAL, ANTIFUNGAL, ANTIOXIDANT ACTIVITIES AND PHYTOCHEMISTRY OF SELECTED SPECIES BELONGING TO FAMILIES PINACEAE, SOLANACEAE AND GUTTIFERAE

By

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PAKISTAN

(December 2009)

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By

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INSTITUTE OF CHEMICAL SCIENCES
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DECLARATIONS

This is to certify that this dissertation prepared by ***Mr.Mumtaz Ali*** *entitled "****Evaluation of Antibacterial, Antifungal, Antioxidant activities and Phytochemistry of Selected Species Belonging to Families Pinaceae, Solanaceae and Guttiferae****" is accepted in the present form by the Institute of Chemical Sciences University of Peshawar as fulfilling this part of the requirements for the degree*

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This Effort is

DEDICATED

To

My Parents & Family members

For

Their Love, Sacrifices, Prayers and

Encouragement

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ABSTRACT

The subject matter of the present dissertation deals with isolation, characterization and evaluation of biological activities of selected species belonging to families Solanaceae, Guttiferae and Pinaceae. The enclosed research data of the thesis is divided into following parts.

PART A: Phytochemical Studies of the Selected Species of Family Solonaceae

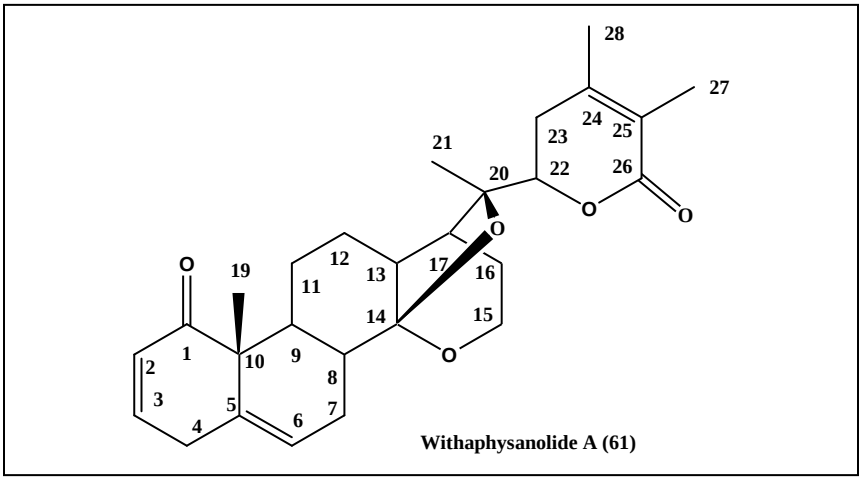
PART B: Phytochemical Studies of the Selected Species of Family Guttiferae

PART C: Phytochemical Studies of the Selected Species of Family Pinaceae

PART D: Evaluation of Biological Activities

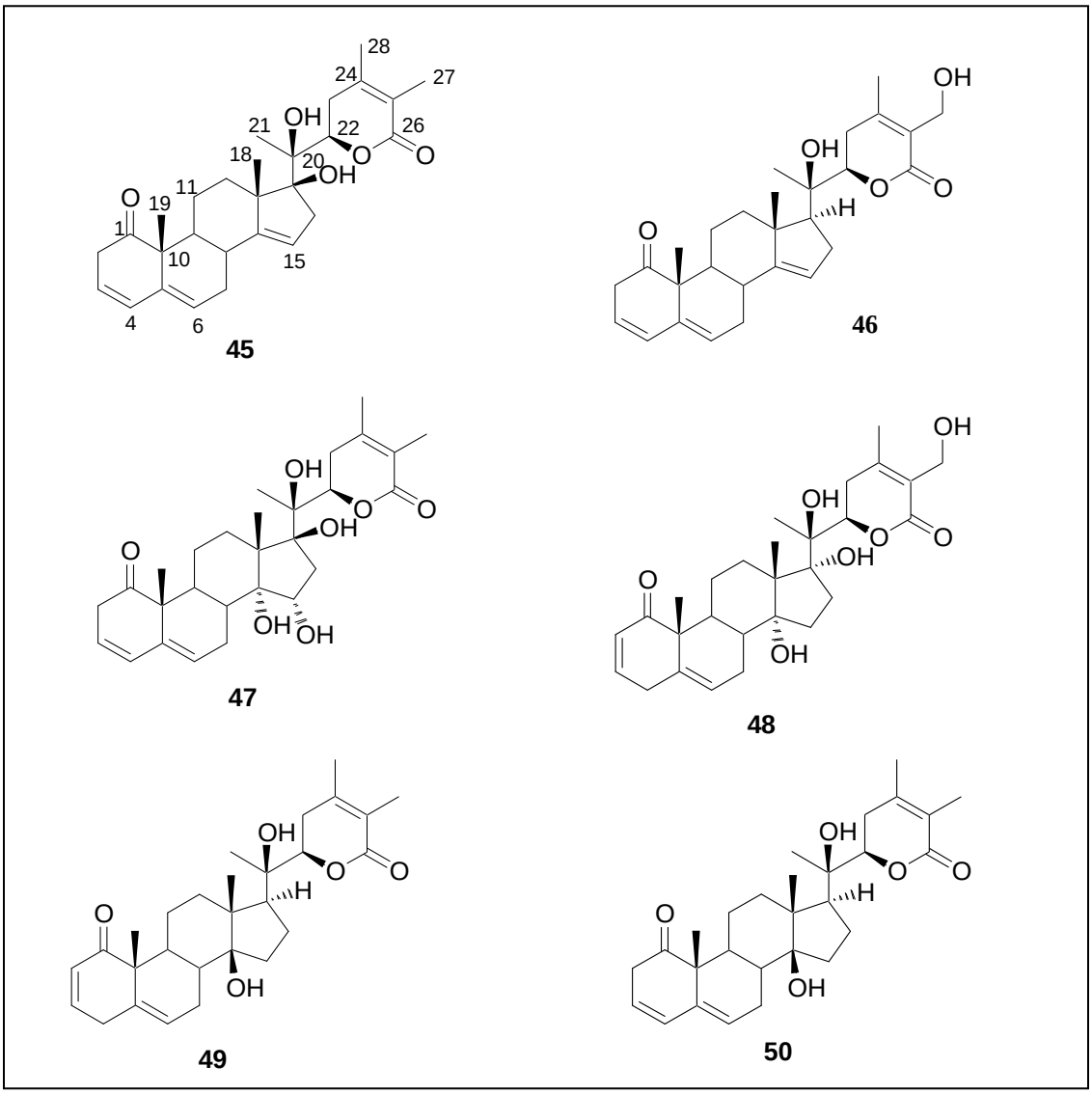
PART A

Part A describes the phytochemical investigation on *Witahinia coagulans* and *Physalis divericata* (Solanaceae). Six new (**45-50**) and ten known (**51-60**) withanolides have been isolated from *W. coagulans* of Pakistani origin, whereas withaphysanolide A (**61**), a novel withanolide together with five known physalins (**62-66**) and four withaphysalins (**67-70**) were isolated from the *P. divericata.* Various experimental techniques and extensive spectroscopic studies were used for the structural elucidation of the compounds. The isolated withanolides were evaluated for inhibition activity on lipopoly-saccharide (LPS) induced B-cell, concanavalin A (ConA)-induced T-cell proliferation and against human colorectal carcinoma HCT-116 and human lung cancer NCI-H460 cells



Withaphysanolide A (61)

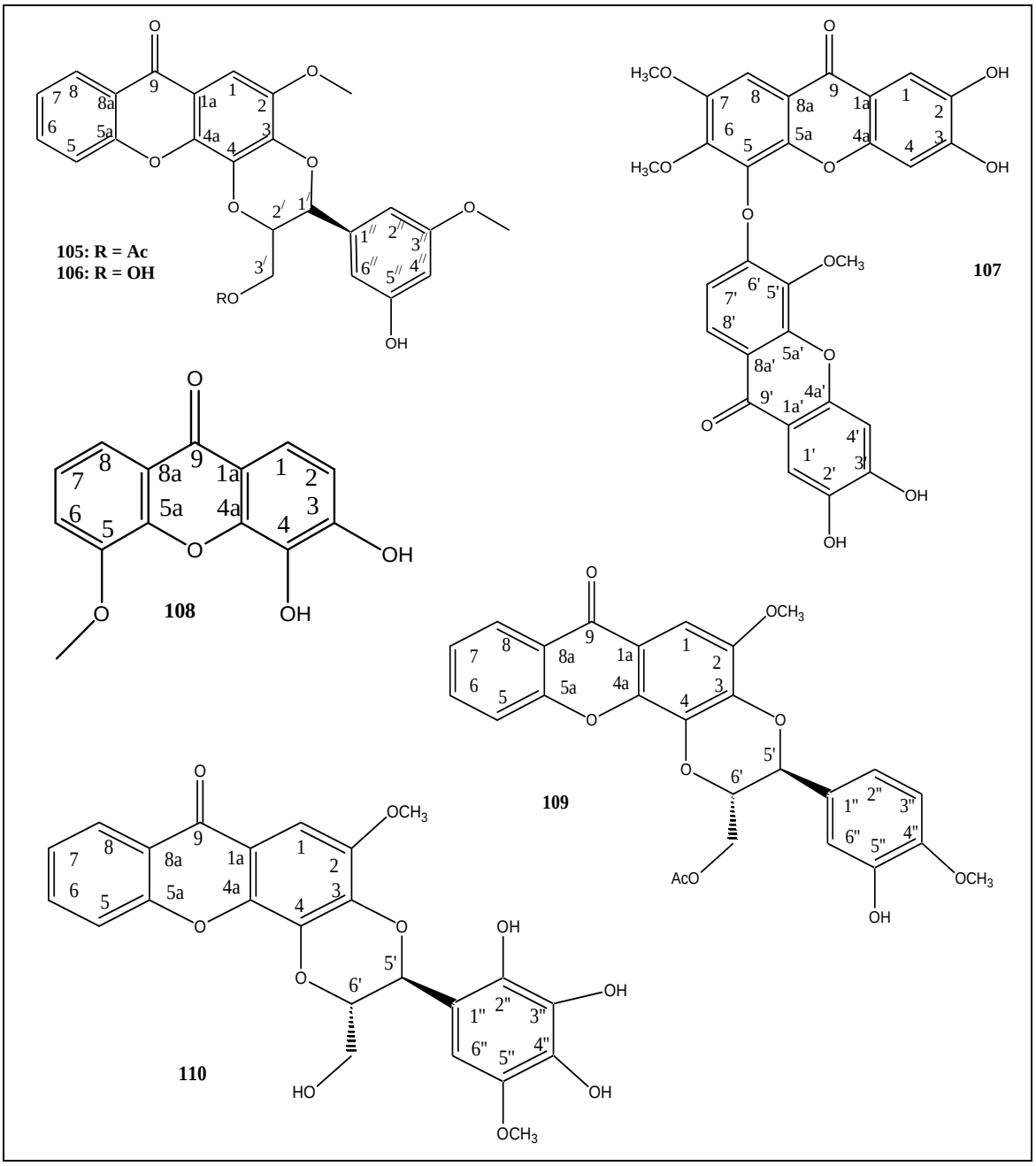
A novel withanolide from *Physalis divericata*
Tetrahedron Letters **2007**, *48 449–452*



New withanolides from *Withania coagulans*
Chemistry and Biodiversity **2009**, *6*, 1415-26

PART B

Part B includes the isolation and characterization of constituents from *Hypericum* species (Guttiferae). Six new (**105-110**) and fourteen known xanthones (**111-124**) along with nine other compounds (**125-133**) have been isolated from *H. oblongifolium*, while six known compounds (**134-139**) were isolated from *H. dyeri.* These components were evaluated for respiratory burst inhibitory (anti-inflammatory), enzyme inhibitory and antioxidant activities.



New xanthones from *Hypericum oblongifolium*

Planta Medica (Accepted)

Phytochemistry (Submitted)

PART C

Part C contains the the GC and GC-MS analysis of various extracts from conifers belonging to family Pinaceae. The amount and composition of lipophilic and hydrophilic extractives as well as proanthocyanidins in the bark of seven Pakistani conifers were

analyzed. The bioactive polyphenols and other known compounds were found interesting in order to find a potential value-added use of local tree species. Gravimetrically these extracts were analysed for lipophilic and hydrophilic extractactives. The predominant lipophilic extractives were common fatty and resin acids, fatty alcohols, and sterols. Different known lignans, stilbenes, ferulates, and flavonoids were generally predominant among the hydrophilic extractives. *Pinus* species e.g. *P. wallichiana, P. gerardiana* and *Picea smithiana* showed large amounts of lipophilic and hydrophilic extractives compared to the other examined conifers. *Pinus roxburghii* was found different from the other pine species having smaller amounts of both types of extractives. *A. pindrow* and *T. fuana* were also found to have the smallest amount of hexane extracts. The proanthocyanidin content and composition revealed that especially *Pinus wallichiana* and *Abies pindrow* could be rich sources of such compounds.

PART D

Part D is concerned with evaluation of biological activities of crude extracts, fractions, semi-pure and pure constituents. Different solvents soluble fractions of the selected plants belonging to family Guttiferae (*H. perforatum, H. oblongifolium, H. monogynum, H. choisianum* and *H. dyeri*), Pinaceae (bark and knotwood of *Picea smithiana, Abies pindrow, Pinus wallichiana, P. geradiana, P. roxburghii* and *Cedrus deodara)* and *Taxus fauna* from the north west of Pakistan were screened for their possible antioxidant activity. Anticancer (anti-proliferative) and enzyme inhibitory activities of *Hypericum* species as well as the cytotoxic, anti-inflammatory and urease inhibitory activities of pure compounds isolated from *Hypericum, Physalis* and *Withania* species were also studied. Four complementary test systems, namely phenolic compounds, free-radical scavenging capacity, measuring of reducing power and total antioxidant activities by Phospho-molybdenum method were used for analysis. We report here for the first time the antioxidant and antimicrobial potential of the various extracts and fractions of the listed plants for the first time except *Hypericum perforatum* which has been the subject of many investigations. The objectives of this study were to explore the biological and medicinal value of the extract/fractions of the above mentioned plants.

LIST OF ABBREVIATIONS

µL	Microlitre
^{13}C-NMR	Carbon-13-Nuclear Magnetic Resonance
^{1}H-NMR	Hydrogen-1-Nuclear Magnetic Resonance
AChE	Acetylcholine Esterase
Ar	Aryl
AcO	Acetate
BB	Broad Band
BHA	Butylated Hydroxyanisole
BHT	Butylated Hydroxytoluene
COSEY	Correlation spectroscopy
CVD	Cardio Vascular Diseases
D	Deuterium
DCM	Dichloromethane
DEPT	Distortionless Enhancement by Polarization Transfer
DMF	Dimethylformamide
DMSO	Dimethysulfoxide
DNA	Deoxyribonucleic Acid
DPPH	2,2-Diphenyl Picryl Hydrazide
EI-MS	Electron Impact-Mass spectrometer
Et	Ethyl
FAB-MS	Fast Atom Bombardment-Mass Spectrometer
FTC	Ferric Thiocyanate
GAE	Gallic Acid Equivalent
GPX	Glutathione Peroxidase
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Coherence
HR-EI-MS	Electron Impact-Mass spectrometer
HIV	Human Immunodeficiency Virus
Hz	Hertz

IC_{50}	Inhibitory Concentration
IR	Infrared
J	Coupling Constant
Ki	Dissociation constant
Km	Michaelis constant
M.P.	Melting Point
mM	Millimole
m/z	Mass to Charge ratio
Me	Methyl
MeO	Methoxy
MIC	Minimum Inhibitory Concentration
MOA	Monoamine Oxidase
NOE	Nuclear Overhauser effect
NOSEY	Nuclear Overhauser effect spectroscopy
OD	Optical Density
P	Probability
PBS	Phosphate Buffer Solution
R	Alkyl
ROS	Reactive Oxygen species
RSA	Radical Scavenging Activity
r.t.	Room Temperature
SDA	Sabouraud Dextrose Agar
TBA	Thiobarbituric Acid
TCA	Trichloroacetic Acid
ν	Wave Number
WST	Water soluble Tetrazolium salt

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Chapter: 1

GENERAL INTRODUCTION

Allah almighty has blessed the nature with enormous number of precious gifts, kingdom plantae being one of them. Basic need of life like food, medicines and shelter were made possible by interaction between human beings and plants. The use of plants for medicinal purposes has no historical record but the present era uses medicinal plants in different ways like Ayurvedic, Chinese, allopathic, homeopathic and many more, each one with a different philosophy.

Medicinal plants have been in use as a source of medication and today they are tested for their biological activities. The medicinal plants have provided a basis for the development of modern drug system. Plants are continuously producing chemical of medicinal value in a mode that has no parallel. A number of bioactive principles have been obtained and structurally identified with the help of various chemical and physical methods. These principles are now the active molecules in modern medicines. Also the synthetic chemists use these active molecules as a model in their synthetic schemes. Various projects are designed to synthesize the active principles present in natural products in laboratories. One such project for example is “Drug discovery and Design” which search for more effective, cheaper and globally available medicinal agents.

Different cultures of the world have been using medicinal plants for treatment of various diseases. Not only man but even animals have the instinct to use various parts of different plants for their treatment. For example sick dogs use different grasses to produce emesis and purgation. Female chimpanzees use *Aspilia* in large quantities. Later on it was found that two diterpenes kaurenoic acid and grandiflorenic acid are present in *Aspilia* which are strong contractors of the uterine walls.

Almost 80% of the world's population particularly that lives in the rural areas depends on traditional medicines for the management of their health. These folk treatments are largely based on the use of medicinal plants. Early in this century, the greater part of medical therapy in the industrialized countries was also dependent on medicinal plants. Even today 25% of all prescriptions contained plant extracts or active principles obtained from higher plants. The World Health Organization notes that of the 119 plant-derived pharmaceuticals medicines, about 74 percent are used in modern ways that correlate directly with their traditional uses as plant

medicines by native cultures.

The use of drugs can be divided broadly into periods. The early period covers the Greeks, Indian, Chinese, Sumerian, Egyptian and Assyrian civilizations followed by the Roman, Arabian, Medieval and Modern periods. The earliest mention of the medicinal use of plants in the India is found in the *Rig Veda* claimed to have been written between 4500 and 1600 B.C.[1] Charaka gave 50 groups of important herbs and Raja of Banaras Deodas Kashiraja, had a great amount of work on the Indian *Materia medica (Niganta).*

Greek civilization was an era of Science and Philosophy. They had made a large contribution in pharmaceutical science. Aristotle, for instance has described 500 drugs in the History of Plants [1]. Hipocrates (460-377 BC), the father of allopathic medicine had described nearly 400 medicinal substances of plant origin. One of the most popular pharmacological compilations of Greeks was the "Authoritative text of Discordies" and "Natural History" (23-70 AD). Similarly the contribution of Galen (129-199 AD) was also countable, who studied and prepared vegetable drugs called *Galenicals* and wrote around 300 books.

The Chinese system of medicines has its own features. Chinese medicine use of complex poly pharmaceutical preparation called fongs. FuIlis (2953 BC) is being considered as the pioneer of Chinese system, which was later developed by the emperor Hong Ti (2953 BC).The written documents of Chinese traditional medicine can be traced back to Shen Nong Beu Cao Jing (22-250 A.D). Li Shizen a great Chinese physician and naturalist had written a more comprehensive pharmacopoeia Ben Cao Gang Mu, which was published in 1596 had 1894 prescriptions and is still in use as a reference and guide for research and teaching in China and in other countries. The Chinese had developed centuries ago the treatment of some common diseases, like leprosy, asthma, high blood pressure, etc. Some common present day drugs like rhubarb, castor oil, kaolin, aconite, camphor, cannabis are all of Chinese origin.

Medicinal science was given another dimension by Arabs. Islam provided the rules of hygienic way of life [2] which are mainly based on *Al Quran and Sunnah* and are called *Tibb Al Nabi.* Arab medicine emerged as the successors of Greek medicine. Many Muslim scientists are also famous for their remarkable contributions in this field. Among the famous names of this period was Ali lbn Rabban al Tabri (782-855 AD) and his book *Firdous al Hikmat* [1], comprised seven parts shad one part

pecialized for drugs and poisons. Abu Bakr Mohammad Bin Zakarya (835-932 AD) was a prominent surgeon and one of the pioneers of Arab medicine, credited with having written nearly 250 works, some of which were on pharmaceutical subjects. His most famous book is *Kitab Al Hawi, a* good collection of Greek, Arabic and Indian plants and medicines. His book *Kitab Al Mansoor* is in 10 volumes and describes the Unani medicine extensively. Besides, he was the first one to use opium as an anesthetic. Abu Ali Al-Hussain lbn Abdullah lbn Sina (Avicenna, 980-1037 AD) was the founder cf the Greeco-Arabic school of medicine [3] and was a great astronomer, mathematician, philosopher and physician of his time. His book *Cannon* was considered as a text book on medicine in Europe and described more than 1000 drugs. His book *Kitab Ash Shifa* was considered as a scientific Encyclopedia. Another well-known scientist Al-ldrisi (1099-1166 A.D.) is famous for his contributions in medicinal plants. He wrote several books on medicinal plants : specially the *Kitab al Jamili Ashiat al Nabatat* was famous for plant origin drugs, described in six different languages.

In the west many herbal preparations were described by many other authors, including such well-known personalities as Discordies and Galen in the first and second centuries till Culpeper in the 17th century. Benzoic acid was the first compound isolated from plants in 1560. The German chemist Karl Wilhelm Scheele (1742-1786) extracted some simple compounds like, oxalic, lactic, citric, tartaric acids and also Glycerol from various organic sources, both vegetables and animals.

There has been a tradition of using Unani (Greco Arab) medicine in the Indo Sub Continent. Hakim Syed Mohammad Hussain is regarded as the father of Unani medicine in India. He is the author of *Makhzamul-Advia* in which he described 1500 drugs in his different research papers. Hakim Raza Ali Khan wrote *Tadhkirat-uI-Hiud* about 150 years ago describing the Sanskrit names of the herbs according to his analysis and experience. Hakim Mohammad Azam Khan wrote several treatises like his famous *Muheei-1-Azam* in which several thousand drugs including those used in allopathic medicine were described in four volumes. Similarly Hakim Mohammad Najmul Ghani Khan in 1915 wrote *Khazaemul advia* in which some 2500 drugs of plant origin were described.

With an increase in the knowledge of herbal drugs Hakim Ajmal Khan was motivated to establish Ayurvedic and Unani Tibbi College at Delhi in 1920. Here a detailed study was carried out for the physiologically active constituents of a number

of drugs used for the treatment of various ailments in the traditional system of medicines. A host of important discoveries have been based on the isolation of active principles from natural products. Quinine from *Cinchona* bark and resperine from *Rauwolfia serpentina* are the two outstanding examples, which had been acclaimed as effective drugs against malaria and mental ailment/high blood pressure, respectively.

The control of diseases through antibiotics and some other new drugs is a great achievement. In the last two decades, several compounds such as cyclosporin A, clavulanic acid, mevinolin and ivermectin were discovered by natural product screening approaches. These discoveries stimulated an expansion of natural product-based drug discovery efforts in the pharmaceutical industry. A review on natural products as a source of new drug has been presented by Clark in 1996 [4] while a review related to anticancer and anti-infective natural drugs has been contributed by Crag et al (1997) [5]. The central role of natural products in the discovery and development of new pharmaceuticals has been summarized in these reviews. Some of the recent publications citing the current literature in bioorganic chemistry [6,7], seeking drugs in natural products [8,9], new technologies and approaches in natural product drug discovery [10] and natural products for the improvement of the quality of life [11] are important in undemanding the progress in the field of natural products.

Thousands of compounds have been isolated from plants. These secondary metabolites mainly comprise of chemical constituents such as coumarins, flavonoids, isoflavonoids, lignin, withanolides, saponins, glycosides, terpenoids, alkaloids, essential oils, fatty acids, resins, gum etc. Natural products Chemists have a compelling curiosity to discover those bioactive compounds in a plant extract used as a remedy which are responsible for the therapeutic effects. Of the estimated, 250,000-500,000 plant species of the world, more than two third found in the tropical forests of developing, countries. Only a small percentage of these plants have been investigated phytochemically and subjected to biological or pharmacological screening. Since each plant may contain hundreds or even thousands of metabolites each with diverse biological activity, there is currently an interest in the plant kingdom as a possible source of new lead compounds for introduction into therapeutically screening programs. To do this it is necessary to isolate pure compounds by different chromatographic techniques such as TLC, HPLC, column chromatography, Chromatotron and GLC etc. and elucidate their structures. Similarly

chemists can also synthesize those compounds which are present in relatively small quantities in plants to meet the requirements. The combination of phytochemical investigations and chemical synthesis has resulted in the discovery of drugs and the development of pharmaceutical industries all over the world.

In continuation with the our ongoing efforts [12-16] to investigate the indigenous renewable natural resource (flora), endowed with many useful, yet to be explored wealth of valuable chemicals with potential uses in medicine, food, cosmetics and new materials, the proposed research was focused on evaluation of bioactive potentials of some indigenous plant species belonging to the families Solanaceae, Guttiferae and Pinaceae collected from the flora of N.W.F.P**.** Pakistan

The first family is Solanaceae with more than 100 genra and several thousand species. The name Solanaceae is derived from the Latin word "*solari*", meaning "soothing". This would presumably refer to alleged soothing pharmacological property of some of the psychoactive species found in the family. The family is also informally known as the nightshade or potato family. The family Solanaceae is characteristically ethnobotanical that is extensively utilized by humans. It is an important source of food, spice, medicines and even poison. The members of family Solanaceae are rich in alkaloidal glucosides that can range in their toxicity to humans and animals from mildly irritating to fatal in small quantities[17]. *Physalis* and *Withania* are considered as medicinally important genera. The whole plant of *P. philadelphica* has been used for the treatment of gastro-intestinal disorders in Guatemala [18] and for treating leprosy, purifying the blood and as a poison antidote in Mexico [19]. The fruits of *P. Philadelphica* known commonly as tomatillos are used as an ingredient in foods such as enchiladas and salsas in some countries in Latin America. They are also used in some North American sauces and relishes as a substitute of tomatoes[20]. *P. peruviana* is widely used medicinal herb for treating cancer, malaria, asthma, hepatitis, dermatitis and rheumatism[21]. Withanolides, the natural steroidal lactones produced mainly by plants in the Solanaceae**,** have been evaluated for their antimicrobial, antitumor, anti-inflammatory, patoprotective, immunomodulatory activity, antibacterial and insect antifeedent properties. Work on *P. peruviana* has focused on the isolation and characterization of several bioactive withanolides from the whole plant[21], leaves[22], roots[23] and berries with the surrounding calyx [24]. The fruit of this plant is used as an excellent source of vitamins A and C as well as minerals. The former name for *P. Philadelphica* is *P. ixocarpa* Brot [25] and the withanolides

ixocarpalactone A ixocarpalactone B, ixocarpanolide, physalin B and withaphysacarpin have been isolated from the leaves and epigeal parts of this plant [26] . Three withanolides i.e 2.3-dihydro-3-methoxywithaphysacarpin, dihydrowithanolide D and withaphysacarpin have shown significant induction of quinone reductase in hepalclc7 cells were isolated from the fruits and edible parts of *P. Philadelphica* [27]. They are also cancer chemo-preventive agents[28]. Two new 17-hydroxywithanolides, (philadephic- alactones A and B), one new spiro-acetal withanolide (ixocarpalactone B), four known withanolides, one new and two known cermides as well as the known porphyrin derivative (chlorophyllide) are reported from the leaves and stems of *P. Philadelphica*. These compounds were evaluated for their potential cancer chemo preventive properties in a cell-based quinone reductase induction assay [29] and a murine epidermal JB6 cell transformation assay[30,31].

The second family Guttiferae includes about 50 genera and 1200 species of trees and shrubs often with milky sap and fruits or capsules around the seeds. Guttiferae is of pharmaceutical importance because of St John's wort which in the last decade of the 20th century became one of the most important medicinal plants in Western medicine. Plants of the Guttiferae contain several constituents with diverse biological activities[32,33]. The genus *Hypericum* contains about 400 species have been long used in folk medicine. The genus contains compounds with properties like anti-septic, diuretic, digestive, expectorant, vermifugal, anti-depressive[33] and have received attention due to antiviral action of hypericin and pseudohypericin on lipid enveloped and non-enveloped DNA and RNA viruses. These polycyclic quinines were isolated from *Hypericum perforatum*, a well known species of the genus which is widely used as antidepressant[34,35]. The antidepressant activity of *H. perforatum* (St. John's wort) has resulted in the widespread interest in the study of the *Hypericum* genus [36]. The most common compounds isolated from plants of this genus are xanthones [37], flavonoids [38], phloroglucinol and licinic acid derivatives[39]. Among the approximately 20 native *Hypericum* species from South Brazil, only *H. brasiliense* has been investigated. From this plant xanthones and phloroglucinol derivatives were isolated and its extracts have been found to inhibit monoamine oxidases (MAO) enzymes which are important in the regulation of levels of some physiological amines and are thought to contribute to the management of depression[38]. Benzopyrans are isolated from the aerial parts of *H. polyanthemum*[40] and benzophenones are reported from the aerial parts of *H. carinatum,* native to southern Brazil with cytotoxic and

anti-HIV activities. It has been reported that some benzophenones (i.e.garcinol) possess free radical scavenging abilities[41]. *H. erectum* is a traditional Chinese herb used as an anti-haemorrhagic agent, astringent and antibiotic agent has been reported to contain some antiviral prenylated phloroglucinol derivatives[42] and two anti-haemorrhagic compounds otogirin and otogirone[43]. Phytochemical analysis of *H. perforatum* L shows that it is a rich source of flavonoids and much of its antioxidant activities are attributed to these compounds. However research on this plant has focussed mainly on its antidepressant activity. A flavonoid-rich extract of *H. perforatum L* was prepared and its antioxidant activity was determined by a series of models in vitro[44] and investigated the hypo- cholesterolemic effects of this flavonoid-rich extract by observing its effects on serum lipid levels and antioxidant enzyme activity in rats fed a cholesterol-rich diet [45]. The genus *Hypericum* is a rich source of antibacterial metabolites of which hyperforin from *H. perforatum* (St. Johns Wort) is an exceptional example. Minimum Inhibitory Concentration (MIC) values for this natural product range from 0.1 to 1 g/ml against penicillin-resistant *Staphylococcus aureus* (PRSA) and methicillin-resistant *S. aureus* (MRSA) strains[46]. These results substantiate the use of St. Johns Wort in several countries as a treatment for burns and wounds that heal poorly[46,47]. An investigation into the antibacterial properties of *H. foliosum* has led to the isolation of a new bioactive acylphloroglucinol natural product and this metabolite was evaluated against a panel of multi drug-resistant strains of *Staphylococcus aureus* and minimum inhibitory values ranged from 16 to 32 g/ml[47].

The third family, Pinaceae is a commercially important family with useful plants such as cedars, firs, hemlocks, larches, pines and spruces. It is the second largest family after Cupressaceae with 220-250 species in 11 genera and in geographical range found mostly in the Northern Hemisphere with the majority of the species in temperate climates but ranging from subarctic to tropical. There are four genera (*Pinus, Abies, Picea, Cedrus)* and nine species of this family are reported in Pakistan. Most of the species are trees which are often excellent sources of lumber, wood products, timber, paper, resins and are cultivated for forestation as well as ornamentals. Genus *Pinus* is the largest genus of this family with 120 species. The diverse nature of this genus can be witnessed in the mountains of southwest China, central Japan, California and Mexico[48]. The members of Pinaceae are prolific producers of resin defense which is a mixture of monoterpenoids, sesquiterpenoids and diterpenoids[49]. Chemical constituents of some species including *P. abies*[50] *P.*

glauca[51] and *P.glehni* [52] have been studied. These components contain lignans, flavonoids and their glucosides as well as diterpenoids of abietane-type diterpenes and norabietane derivatives[53]. Lignans are a class of phenolic compounds possessing 2, 3-dibenzylbutane skeleton with phenylpropane dimers enzymatically coupled through β,β-linkages between the propane chains. The oligomeric lignans consist of three or more β,β- linked phenyl propane units in addition to non-optically active oligolignols[54]. They are widely distributed in plants and occur in different parts of the plant (roots, leaves, stem, seeds and fruits) but usually in small amounts. Knots of *Picea abies* contain extremely large amounts of lignans (6-24% w/w) with hydroxymatairesinol (HMR) comprising 65–85% of them [55]. Hydroxymatairesinol, like many other lignans has several positive biological and physiological effects. It has been shown to metabolize primarily to enterolactone which thus has antitumor activity and antioxidant properties [56]. Due to its good availability and its biological properties, HMR has been proposed as a chemo preventive agent against cancer, hormone dependent diseases and cardiovascular diseases [57]. The effect of dietary HMR on growth of LNCaP human prostate cancer xenografts in athymic nude mice was studied. LNCaP is an androgen-sensitive adenocarcinoma cell line[58,59]. Recent research has revealed that knots of *Picea abie* i.e. the branch bases inside tree stems commonly contain 5–10% (w/w) of lignans [55]. Some of the species of spruce also contain HMR as the main lignan while some species have also other dominating lignans. Most firs *(Abies*) species contain secoisolariciresinol and lariciresinol as the main lignans. Lignans occur also in knots of pines *(Pinus)* although in lower amounts than in spruces and firs. Knots of Scots pine *(Pinus silvestris)* were found to contain 0.4–3% lignans with nortrachelogenin as the main lignan[60]. Heartwoods also contain lignans but flavonoids are more abundant. Several synthetic routes have been devised to synthesize lignans such as matairesinol, secoisolariciresinol, lariciresinol and cyclolariciresinol starting from hydroxymatairesinol by applying fairly straight-forward chemical transformations [61].

Free radicals are species with one or more unpaired electrons in the outer orbit such as super oxide anion ($O_2^{\bullet-}$), hydroxyl ($HO^{\bullet}$), peroxyl ($ROO^{\bullet}$), alkoxyl ($RO^{\bullet}$) and nitric oxide. Free radicals have been regarded as the fundamental cause of different kinds of diseases, including aging, coronary heart disease, inflammation, stroke, diabetes mellitus, rheumatism, liver disorders, renal failure, cancer and neuro

degeneration[62]. The modern theories of Reactive Oxygen Species (ROS) explain how they play a dual role in an organism. They are strong lipid peroxidizers as well as causes the deterioration of food, cellular injuries and also initiate peroxidation of polyunsaturated fatty acids in biological membranes. The tissue injury caused by ROS includes DNA and protein damage and oxidation of enzymes in the human body [63]. Antioxidants such as α-tocopherol are capable of mitigating free radical damage through scavenging ROS[63]. Some natural cellular enzymatic antioxidants are superoxide dismutase (SOD) catalase and glutathione peroxidase (GPX), whereas non-enzymatic antioxidants comprise α-tocopherol, carotene, carotenoids, chlorophylls, flavonoids, tannin and certain micronutrients *e.g.* zinc and selenium[63]. Extensive studies on antioxidant derived from plants can be correlated with oxidative stress and age-dependent diseases. Flavonoids are abundant in fruits, teas, vegetables, and medicinal plants and have been investigated extensively, since they are highly effective free radical scavengers and are assumed to be less toxic than synthetic antioxidants such as BHA and BHT, which are suspected of being carcinogenic and may cause liver damage [64]. The presence of these antioxidants in the cellular system is known to prevent oxidative damage. Phytochemicals in fruits, vegetables, spices and traditional herbal medicinal plants have been found to play protective role against many human chronic diseases including cancer and cardiovascular diseases (CVD). These diseases are considered to be associated with oxidative stresses caused by excess free radicals and other reactive oxygen species. Antioxidant phytochemicals exert their effect by neutralizing these highly reactive radicals [65]. An inverse relationship has been shown between dietary intake of antioxidant rich foods and the incidence of a number of human diseases [66]. Thus the search and research for natural antioxidant sources and their antioxidant potential is becoming more and more important. A number of antioxidants have been derived from plants such as *Physalis peruviana*,[63], *Hypericum perforatum, Hypericum androsaemum, Hypericum triquetrifolium, Hypericum hyssopifolium*[64,67,68] *Pinus pinaster, Pinus nigra* and *Pinus* morrisonicola[69-71].

The plant extracts and plant products of higher plants have been screened for antimicrobial activity showing promising results[72,73]. During recent past a sharp increase in drug resistance has been observed in human pathogenic organisms as well as the appearance of undesirable side effects of certain antibiotics and the emergence of previously uncommon infections [74,75]. Antimicrobial properties have been reported

more frequently in a wide range of plant extracts and natural products in an attempt to discover new chemical classes of antimicrobial agents.

The subject matter of the present dissertation relates to the aspect discussed above and deals with isolation, characterization as well as evaluation of biological activities of selected species of the above mentioned families. The thesis is divided into following parts

PART A: Phytochemical Studies of the Selected Species of Family Solanaceae

PART B: Phytochemical Studies of the Selected Species of Family Guttiferae

PART C: Phytochemical Studies of the Selected Species of Family Pinaceae

PART D: Evaluation of Biological Activities

PART A

Phytochemical Studies of the Selected Species of Family Solanacea

Chapter: 2

INTRODUCTION (PART A)

2.1: Solanaceae

The Solanaceae, a family of flowering plants, having more than 85 genera and 3000 species[76,77] spread all over the world in tropical and temperate regions of both the hemispheres but mainly in tropical America. They are herbs, shrubs and trees. Many of these species are very important for mankind because of their value as food (Potatoes, tomatoes, peppers, etc.,) while others are considered poisonous because of their alkaloid properties (tobacco, deadly nightshade, Thornapple, henbane, mandrake, etc.) and as garden plants. Some of the impartant genera of the family Solanaceae include *Withania, Physalis Solanum, Atropa, Brugmansia, Capsicum, Datura, Hyoscyamus, Lycopersicon, Nicotian* and *Petunia*. In Pakistan, this is represented by 14 genera and 52 species, of these 27 are native, 6 naturalized, and the others either exclusively cultivated or found occasionally[77].

2.1.1: Biological Importance

The name of the family originates from the Latin verb "*solari*", meaning "soothing". This would presumably refer to alleged soothing pharmacological properties of some of the psychoactive species found in the family. The family is also informally known as the nightshade or potato family. The family Solanaceae is characteristically ethno-botanical that is extensively utilized by humans. It is an important source of food, spice and medicine. The Solanaceae are also the third most important plant taxon economically and the most valuable in terms of vegetable crops and of agricultural utility, as they include the tuber-bearing potato, a number of fruit-bearing vegetables (tomato, eggplant and peppers), ornamental plants (*Petunias* and *Nicotiana*), plants with edible leaves (*Solanum aethiopicum* and *S. macrocarpon*) and medicinal plants (*Datura, Capsicum, Wathania* and *Physalis*)

Members of the family Solanaocae are widely used in ancient system of medicine. An atropine which is commonly found in *Atropa belladonna* L., belonging to family Solanaocae: is used in ophthalmology as a dilator of the pupil of eye. Similarly leaves *of Datura* species are used as bronchodilator for asthmatic patients whereas

Datura metel is used for hair care and dandruff. The flowers of the *W. somnifera* are also used as hair remedy[78]. Various pharmacological properties have been attributed to the species of *Withania* genus. *W. somnifera* Dunal, which is commonly known as "Ashwagandha" or "Indian Genseng" well known for its therapeutic use in the Ayurveda medicine and used as dietary supplement throughout the world.[79]. *W.coagulans.* Dunal is extensively used in the Indian indigenous systems of medicine in North-West India and neighboring countries. It has a prominent position in Ayurvedic and ancient Indian system of medicine[80,81]. *Physalis* is also considered as medicinally important genus. The whole plant of *P. philadelphica* have been used for the treatment of gastro-intestinal disorders in Guatemala[18] and for treating leprosy, purifying the blood and as a poison antidote in Mexico[19]. The fruits of *P. Philadelphica* known commonly as tomatillos are used as an ingredient in foods such as enchiladas and salsas in some countries in Latin America. They are also employed in some North American sauces and relishes as an acid source in place of tomatoes[20]. *P. peruviana* is widely used medicinal herb for treating cancer, malaria, asthma, hepatitis, dermatitis and rheumatism[63].

Solanaceae plants are also extensively used in biotechnology, biosynthesis and molecular biology research such as tobacco, tomato, potato and petunia.etc.

2.2: *Withania coagulans*. Dunal

Withania coagulans Dunal, a plant specie of the genus *Withania* belonging to family Solanaceae. *Withania* is a small genus of shrubs and has six species which are distributed in East of the Mediterranean region, North Africa, South Europe and extend to South Asia[82]. There are two species found in Pakistan, *W.coagulans* and *W. somnifera*[77,83].

W. coagulans is small ever green shrub of a 60-120 cm high plant which widely found in the drier parts of south Asian sub continent[83]. Leaves of the plant are usually lanceolate-oblong, clothed with a persistent, grayish toinentum on both sides, base narrowed into a stout petiole; flowers are yellow in axillaries cymose clusters and usually appear in November-April. The berries of *W. coagulans* are globose and red or brownish in color and ripen during January-May. These are smooth and enclosed in leathery calyx. The seeds of plant are dark brown ear-shaped and glabrous. The pulp is brown having fruity and nauseous odour.[82]

2.2.1: Pharmacological Importance

W. coagulans Dunal is extensively used in the Indian indigenous systems of medicine in North-West India and neighboring countries. It has a prominent position in Ayurvedic and ancient Indian system of medicine[80,81]. The fruits of the plant are used for milk coagulation[84]. Coagulation of milk is suggested to be due to an enzymatic action of the plant under optimum conditions[85]. Statistically one part of concentrated enzyme coagulates 90,000 parts of milk in half an hour. It has been estimated that 1 oz. of the fruit of *W.coagulans* and liter of boiling water make a combination, one table spoonful of which will coagulate a gallon of warm milk in about half an hour [86]. A proteolytic enzyme has also been isolated from the berries of the same plant and used for preparing cheese and dahi[81].

Fresh fruits of the plant are commonly used as an emetic and in smaller doses as a remedy for flatulent colic, dyspepsia and intestinal disorders[84,87], while the dried fruits posses diuretic, sedative and in some cases for chronic liver complications[81]. The red fruits of the shrub are employed for the treatment of asthma, stranguary, biliousness and are reported to be sedative, emetic, alterative and diuretic as well as blood purifier. The smoke of the plant is inhaled for relief in toothache whereas twigs are chewed for cleaning teeth[85]. The twigs are also prescribed as a tonic in Pakistan. The leaves of *W. caagulans* are used as a vegetable and as a fodder for camels and sheeps. They are also employed as a febrifuge bitter tonic, stomachic and growth promoter for infants[82]. The use of the plant for the treatment of ulcer and rheumatism is also reported[80]. Essential oil of the plant was active against *Micrococcus pyogenes* var. *aureus* and *Escherichia coli* [88].The crude extracts showed antifungal activity, CNS depressant activity and hypotensive activity[89].

Various pharmacological properties have been attributed to the species of *Withania* genus. *W.somnifera* Dunal, which is commonly known as “Ashwagandha” or “Indian Genseng”,well known for its therapeutic use in the Ayurveda medicine and used as dietary supplement throughout the world[79]. It is mentioned in Vedas as a health food and herbal tonic and an official drug in Indian Pharmacopoeia (1985). The commercially available drug consists of dried root powder. The chemical composition, therapeutic and pharmacological efficacy have been established [90,91]. It is a basic component of several marketed formulations prescribed for the relief of various

disorder like strain, stress, pain, fatigue, skin diseases, gastrointestinal diseases, diabetes, epilepsy, rheumatoid arthritis and debility as well as a supplement and nerve tonic on nutritional side[92]. Several preparations containing *W. somnifera* have been used for the treatments in advanced malignancies [92].

The leaves of the plant are used as antitumor and anti-inflammatory, while the roots of this plant have been used as an adaptogen and to treat arthritis, asthma, dyspepsia, hypertension, rheumatism and syphilis. Other pharmacological activities like antioxidant, immunomodulatory and tumor cell proliferation inhibitory activities of the plant are also reported[76]. The leaves are also used for relief of fever, painful swelling, ulcer and opthalmistic [78]. The plant is used as a remedy against intestinal parasites in Basutoland. The fresh juice of the leaves is applied to anthrax pustules and also for the preservation of meat. The use *W.somnifera* is also reported for the treatment of mental diseases, inflammation, infections, fever, tuberculosis, sexual disorders, asthma, arthritis and tumors. In the last decade, it has been extensively evaluated for radio-sensitizing and antitumor activity[93]. The plant also has antibacterial, antifungal and cytotoxic activities [79]. *W. somnifera* of Iraqi origin was found to have inhibitory activity against ganuloma-tissue formation[94]. *W. somnifera* along with other plants have been used by traditional healers in the central and southern parts of Somalia[95].

The aqueous suspensions of an Indian drug Ashwagandha *(W. somnifcra)* showed anti-stress activity as well as anabolic activity with significant results[96] and also proved to have analgesic activity[97].The alcoholic extract from the root powder showed growth inhibitory effect on transplanted Sarcoma-180 in the mouse[93]. The extract of the plant also have effect on arterial blood pressure in dogs and induces a significant decrease in the arterial and diastolic blood pressures in dogs[83,98]. The methanolic extract *W. somnifcra* is active against aging, obesity, hyperlipidemia and other patho-physiological as well as cardio vascular problems[79]. The roots extract of the plant is used as a dietary supplement in the United States and has received worldwide attention for its pharmacological activities[99].

2.2.2: Previous Phytochemical Investigation

The genus *Withania* along with other genera of Solanaceae are known to elaborating C-28 ergostane lactone derivative with structural diversity and biological activities. After the genus these compounds were given the name withanolide. Withaferin-A (**2**), the first member of this group which was isolated by Lavie from *W.somnifera*[100] had received considerable attention due to its antibiotic and anti-tumor activities. It can inhibit the growth of various gram-positive bacteria and fungi .The structural novelty and excellent biological activities of this compound has led to the chemical investigation of various plant species and numerous compounds of similar features were isolated[101]. Literature indicates that more than 200 withanolides had been isolated out of which 130 were reported from *W. somnifera* (Table 2.1) and 29 were from *W. coagulans* (Table 2.2)

Table 2.1: Withanolides from *Withania somnifera* (Solanaceae)

S.No	M. Mass	Formula	Name	Ref.
1	974	$C_{56}H_{79}O_2S$	Ashwagandhanolide	76
2	480	$C_{28}H_{40}O_5$	27-Acetoxy-3-oxowitha-1,4,24-trienolide	102
3	488	$C_{28}H_{40}O_7$	2,3-Dihydro-3β-hydroxywithanone	102
4	452	$C_{28}H_{36}O_5$	27-Deoxy-16-ene-withaferin A	102
5	568	$C_{28}H_{40}O_{10}S$	2,3-Dihydro-withanone-3β-O-sulfate	102
6	600	$C_{33}H_{48}O_9$	Glucosomniferanolide	103
7	784	$C_{40}H_{64}O_{15}$	24,25-Dihydrowithanoside V	104
8	486	$C2_8H_{38}O_7$	5,6-Epoxy-4,17,27-trihydroxy-1-oxowitha-2,24-dienolide	79
9	488	$C_{28}H_{40}O_7$	6,7-Epoxy-3,5,20-trihydroxy-1-oxowith-24-enolide	79
10	778	$C_{40}H_{62}N_2O_{13}$	Withanamide A	104
11	754	$C_{40}H_{62}N_2O_{13}$	Withanamide B	104
13	754	$C_{38}H_{62}N_2O_{13}$	Withanamide C	104
14	782	$C_{40}H_{66}N_2O_{13}$	Withanamide D	104
15	782	$C_{40}H_{66}N_2O_{13}$	Withanamide E	104
16	780	$C_{40}H_{66}N_2O_{13}$	Withanamide F	104
17	752	$C_{38}H_{60}N_2O_{13}$	Withanamide G	104
18	774	$C_{40}H_{58}N_2O_{13}$	Withanamide H	104
19	941	$C_{46}H_{72}N_2O_{18}$	Withanamide I	104
20	554	$C_{33}H_{46}O_7$	4-Dimethyloxocyclopropy1-2,3-dihydrowithaferin A	.99
21	668	$C_{34}H_{52}O_{13}$	5β,6β-Epoxy-1α,3β,4β,16β,27-pentahydroxy-24-enolide-3-O-β-D-Glucopyranoside	99
22	673	$C_{34}H_{53}O_{13}$	4β,16β-Hydroxy-5β,6β-Epoxyphysagulin D	99
23	669	$C_{34}H_{57}O_{13}$	27-O-β-D-Glucopyranosyl-Viscosalactone B	99
24	650	$C_{34}H_{50}O_{12}$	5β,6β-Epoxy-3β-4β,27-trihydroxy-1-oxowitha-24-enolide-27-O-β-D-Glucopyranoside	99
25	783	$C_{40}H_{63}O_{15}$	27-O-β-D-Glucopyranosyl-physalin D	99

26	488	$C_{28}H_{40}O_7$	3α,6 α-Epoxy-4β-5β,27-trihydroxy-1-oxowitha-24-enolide	105
27	502	$C_{28}H_{38}O_8$	14,17-Dihydroxywithanolide R	106
29	620	$C_{34}H_{52}O_{10}$	Withanoside XI	105
30	782	$C_{40}H_{62}O_{15}$	Withanoside X	105
31	110	$C_{52}H_{82}O_{25}$	Withanoside IX	105
32	944	$C_{46}H_{72}O_{20}$	Withanoside VIII	105
33	782	$C_{40}H_{62}O_{15}$	Withanoside VII	107
34	782	$C_{40}H_{62}O_{15}$	Withanoside VI	107
35	766	$C_{40}H_{62}O_{14}$	Withanoside V	107
36	782	$C_{40}H_{62}O_{15}$	Withanoside IV	107
37	652	$C_{34}H_{52}O_{12}$	Withanoside III	107
38	798	$C_{40}H_{62}O_{16}$	Withanoside II	107
39	636	$C_{34}H_{52}O_{11}$	Withanoside I	107
40	782	$C_{50}H_{42}O_{15}$	1α,3α-Dihydroxy-5,24-withadienolide-3-O-[β-D-Glucopyranosyl-(1-6)-α-D-glucopyranoside]	107
41	504	$C_{29}H_{44}O_7$	5β,6β -Epoxy-4β,20β-dihydroxy-3β-methoxy-1-oxowithanolide	108
42	766	$C_{40}H_{62}O_{14}$	1α,3α-Dihydroxy-5,24-withadienolide-3-O-[β-D-Glucopyranosyl-(1-6)-β-D-glucopyranoside]	108
43	468	$C_{28}H_{36}O_6$	Somniferanolide	109
44	470	$C_{28}H_{36}O_6$	Withasomniferanolide	109
45	470	$C_{28}H_{36}O_6$	Somniferawithanolide	109
46	470	$C_{28}H_{36}O_6$	Withasomnilide	109
47	486	$C_{28}H_{38}O_7$	Somniwithanolide	109
48	470	$C_{28}H_{36}O_6$	27-Hydroxywithanolide B	110
49	470	$C_{28}H_{38}O_7$	Withasomniferol	110
50	472	$C_{28}H_{40}O_6$	Withasomniferol B	110
51	486	$C_{28}H_{38}O_7$	Withasomniferol A	110
52	470	$C_{28}H_{36}O_6$	Withanolide A	110
53	486	$C_{29}H_{42}O_6$	Quresimine B	110
54	502	$C_{29}H_{42}O_7$	Quresimine A	110
56	488	$C_{28}H_{40}O_7$	2,3-Dehydrosomnifericin	111
57	490	$C_{28}H_{42}O_7$	Somnifericin	111
58	502	$C_{28}H_{38}O_8$	Withaoxylactone	111
59	452	$C_{28}H_{36}O_5$	Withanolide U	112
60	470	$C_{28}H_{36}O_6$	Withanolide D	100,113
61	438	$C_{28}H_{38}O_4$	27-Hydroxy-3-oxowitha-1,4,24-tetraenolide	84
62	458	$C_{28}H_{42}O_5$	Dunawithagenin	114
63	470	$C_{28}H_{36}O_6$	Sominolide	115
64	520	$C_{28}H_{39}ClO_7$	Withanolide C	116
65	454	$C_{22}H_{38}O_5$	Withasomniferin A	117
66	454	$C_{28}H_{38}O_5$	5-Deoxywithanolide R	117
67	454	$C_{28}H_{38}O_5$	27-Deoxywithaferin A	101
68	454	$C_{28}H_{38}O_5$	17α,27-Dihydroxy-1-1oxowitha-2,5,24-trienolide	=
69	632	$C_{34}H_{48}O_{11}$	Sitoindoside IX	118
70	870	$C_{50}H_{78}O_{12}$	Sitoindoside X	118
71	486	$C_{28}H_{38}O_7$	Withanolide Y	119
72	458	$C_{28}H_{42}O_5$	Pubesenolide	120
73	452	$C_{28}H_{36}O_5$	14α,20β-Dihydroxy-1-oxowitha-2,5,16,20-tetraenolide	121
74	522	$C_{28}H_{41}ClO_7$	4-Deoxyphysalolactone	122
75	532	$C_{30}H_{44}O_8$	5α-Ethoxy-6α,14α,17β-20β-tetrahydroxy-1-oxo-with a-2,24-dienolide	123.
76	470	$C_{28}H_{36}O_6$	14α,20β,27-trihydroxy-1-oxowitha-3,5,24-trienolide	123
77	474	$C_{28}H_{38}O_7$	6α,7 α-Epoxy-1,3β,5α-trihydroxy-24-enolide	124
78	486	$C_{28}H_{38}O_7$	6α,7 α-Epoxy-5α,14α,17α-trihydroxy-1-oxowitha-2,24-	124

			dienolide	
79	486	$C_{28}H_{38}O_7$	6β,7β -Epoxy-5α,14α,17α-trihydroxy-1-oxowitha-2,24-dienolide	124
80	486	$C_{28}H_{38}O_7$	14β-Hydroxywithanone	124
81	486	$C_{28}H_{38}O_7$	20β –Hydroxywithanone	124
82	452	$C_{28}H_{36}O_5$	14α,20β-Dihydroxy-1-oxowitha-2,4,6,24-tetraenolide	124
83	507	$C_{28}H_{39}ClO_6$	Withanolide D chlorohydrins	124
84	507	$C_{28}H_{39}ClO_6$	Withaferin A chlorohydrins	124
85	468	$C_{28}H_{36}O_6$	5β, 6β-Epoxy-4β,20 β-dihydroxy-1-oxowitha-24-enolide	125
86	468	$C_{28}H_{36}O_6$	5β, 6β-Epoxy-20β-dihydroxy-1, 4-dioxowiha-2,24-dienolide	125
87	470	$C_{28}H_{36}O_6$	5β, 6β-Epoxy-4β,20 β-dihydroxy-1,4-dioxowitha-2-enolide	125
88	436	$C_{28}H_{36}O_4$	20β-Hydroxy-1-oxowitha-2,5,14,24-tetraenolide	126
89	452	$C_{28}H_{36}O_5$	Withanolide U	127
90	486	$C_{28}H_{38}O_7$	Withanolide T	127
91	470	$C_{28}H_{36}O_6$	Withanolide F	128
92	486	$C_{28}H_{38}O_7$	14α-Hydroxywithanone	128
93	504	$C_{28}H_{38}O_8$	4-Deoxywithaperuvin	128
94	486	$C_{28}H_{38}O_7$	Withanalide E	128
95	502	$C_{28}H_{38}O_8$	4β-Hydroxywithanolide E	129
96	486	$C_{28}H_{38}O_7$	17-Isowithanolide E	do
98	486	$C_{28}H_{38}O_7$	17α-Hydroxywithanolide D	do
99	454	$C_{28}H_{38}O_5$	Withanolide P	128
100	502	$C_{28}H_{38}O_8$	Withanolide S	128
101	470	$C_{28}H_{38}O_7$	Withanolide WSI	130
102	470	$C_{28}H_{38}O_7$	Withanolide R	131
103	470	$C_{28}H_{38}O_7$	Withanolide Q	131
104	452	$C_{28}H_{36}O_5$	Withanolide O	132
105	452	$C_{28}H_{36}O_5$	Withanolide N	132
106	470	$C_{28}H_{36}O_6$	5β, 6β-Epoxy-20β-dihydroxy-1-oxowiha-2,24-dienolide	133
107	398	$C_{28}H_{46}O$	Ergosta-5,24-dien-3-o1	134
108	412	$C_{28}H_{48}O$	Stigmasta-5,24-dien-3-o1	134
109	468.	$C_{28}H_{36}O_6$	Withanolide M	135.
110	452.	$C_{28}H_{36}O_5$	Withanolide L	135
111	470.	$C_{28}H_{38}O_7$	Withanolide K	126,135, 136
112	470.	$C_{28}H_{38}O_7$	Withanolide J	-do-
113	454.	$C_{28}H_{38}O_5$	Withanolide I	-do-
114	470.	$C_{28}H_{38}O_7$	Withanolide H	-do-
115	454.	$C_{28}H_{38}O_5$	Withanolide G	-do-
116	470.	$C_{28}H_{38}O_7$	17β-Hydroxywithanolide K	-do-
117	452.	$C_{28}H_{36}O_5$	5β,6β-Epoxy-4β-hydroxy-1-oxowitha-2,14,24-trienolide	137.
118	452.	$C_{28}H_{36}O_5$	5β,6β-Epoxy-4β-hydroxy-1-oxowitha-2,14,24-trienolide	-do-
119	454.	$C_{28}H_{38}O_5$	5α,17α-Dihydroxy-1-oxowitha-2.6.24-troemplode	-do-
120	454.	$C_{28}H_{38}O_5$	7α,27-Dihydroxy-1-oxowitha-2.5.24-trienolide	-do-
121	454.	$C_{28}H_{38}O_5$	17α,27-Dihydroxy-1-oxowitha-2.5.24-trienolide	-do-
12	470.	$C_{28}H_{36}O_6$	5β, 6β-Epoxy-4β-17α-dihydroxy-1-oxowiha-2,24-dienolide	-do-
123	470	$C_{28}H_{38}O_6$	Wihanone	-do-
124	472	$C_{28}H_{40}O_6$	5β,6β-Epoxy-4β,20β-dihydroxy-1- oxowitha-24-enolide	-do-
125	472	$C_{28}H_{40}O_6$	5β,6β-Epoxy-4β,27-dihydroxy-1- oxowitha-2-enolide	138
126	486	$C_{28}H_{38}O_7$	14α-Hydroxywithanolide D	-do-
127	456	$C_{28}H_{40}O_5$	5β, 6β-Epoxy-4-β-hydroxy-1-oxowitha-2-enolide	-do-
128	470	$C_{28}H_{36}O_6$	27-Deoxy-14 α-hydroxywithaferin A	138,139
129	472	$C_{28}H_{40}O_6$	2,3-Dihydrowithaferin A	138,140
130	470	$C_{28}H_{36}O_6$	Withaferin A	141

Table 2.2: Withanolides from *Withania coagulans* (Solanaceae)

S.No	M. Mass	M.Formulla	Name	Ref.
1	452	$C_{28}H_{36}O_5$	Withacoagulin	85
2	438	$C_{28}H_{38}O_4$	20-Hydroxy-1-oxowitha-2,5,24-trienolide	85
3	452	$C_{28}H_{36}O_5$	14,20-Epoxy-17-hydroxy-1-oxowitha-3,5,24-trienolide	85
4	538	$C_{28}H_{42}O_{10}$	Coagulin S	142
5	470	$C_{28}H_{38}O_6$	Coagulin R	80
6	620	$C_{34}H_{52}O_{10}$	Coagulin Q	80
7	632	$C_{34}H_{48}O_{11}$	Coagulin P	80
8	634	$C_{34}H_{50}O_{11}$	Coagulin O	89
9	648	$C_{34}H_{48}O_{12}$	Coagulin N	89
10	488	$C_{28}H_{40}O_7$	Coagulin M	89
11	650	$C_{34}H_{50}O_{12}$	Coagulin L	82
12	616	$C_{34}H_{48}O_{10}$	Coagulin K	82
13	470	$C_{28}H_{38}O_6$	Coagulin J	82
14	486	$C_{28}H_{38}O_7$	Coagulin I	82
15	520	$C_{28}H_{40}O_9$	Coagulin H	82
16	468	$C_{28}H_{36}O_6$	Coagulin G	87
17	452	$C_{28}H_{36}O_5$	Coagulin F	87
18	436	$C_{28}H_{36}O_4$	Coagulin E	83
19	436	$C_{28}H_{36}O_4$	Coagulin D	83
20	452	$C_{28}H_{36}O_5$	Coagulin C	83
21	452	$C_{28}H_{36}O_5$	Coagulin B	83
23	468	$C_{28}H_{36}O_6$	4,15-Epoxywithanolide I	136
24	470	$C_{28}H_{36}O_6$	17-hydroxywithanolide K	136
25	468	$C_{28}H_{36}O_6$	Coagulin	84
26	454	$C_{28}H_{38}O_5$	Withacoagin	143
27	454	$C_{28}H_{38}O_5$	Withnolide I	144
28	488	$C_{28}H_{40}O_7$	3,14,17,20-Tetrahydroxy-1-oxo-5,24-withadienolide	145
29	488	$C_{28}H_{40}O_7$	3-hydroxy-2,3-dihydrowithanolide F	146

2.3: *Physalis divericata* D.Don

Physalis divericata D.Don, a plant of the genus *Physalis* belongs to family Solanaceae. The genus *Physalis* have more than 100 species, commonly found in Mexico, South and North America, while some of the species are also reported from Europe as well as Southeastern and Central Asia. In Pakistan it is represented by three species, *P. divericata*, *P. peruviana* and *P. alkekengi*[77,147].

P. divericata D.Don is a diffuse annual from 15-45 cm tall, subglabrous to pubescent perennial herb found in Pakistan, Afghanistan and eastward to Nepal. It is a common field weed in the monsoon season, found from 610-981 m. Leaves are ovate, sinuate, repand or sinuate-dentate to subentire, acute or acuminate, base cordate to oblique. Flowers are yellow in solitary axillary form and usually appear from August-October. Fruits are globose berries, surrounded by the inflated calyx .They are 10 mm broad, orange and ripen in October-December. Seeds are subreniform, minutely reticulate-undulate, compressed and brownish-yellow in colour[77].

2.3.1: Pharmacological importance

Plants belonging to *Physalis* genus have attracted the attention of human being since ancient times. *Physalis* species such as *P. angulata, P. philadelphica*, *P. chenopodifolia, P. peruviana*, *P.grisea*, and *P. coztomatl*, are cultivated as food and for their edible fruits[148] as well as used in the folk medicine of various countries of Central and South America and Southeast Asia[149,150].

P. minima L. known as “Xiaosuanjiang” in China, is a medicinal herb distributed throughout the world in tropical and subtropical regions and used in various countries in folk medicine as diuretic, purgative, anticancer, antimyco bac-terail, tonic and remedy for spleen disorders [151,152].

P. alkekengi L. known as ”Kuzhi” in china, is well known for its use in traditional Chinese medicine due to its ethno-pharmacological properties including anti-inflammatory,, antitissue, diuretic, anti-cough, anti-cold, expectorant, cytotoxic, anti-leukemic, antipyretic, anticancer, antimycobacterail, antifungal, immuno-modulatory as well as used in the treatment of different diseases like asthma, malaria, dermatitis, hepatitis and rheumatism. Moreover it is also used for dilatory purpose such as in preparation of jams and beverages[148,153]. Fresh berries of the plant are used as analgesic.

P. angulata is also popular in various countries as folk medicine and is one of the most common solanacious plant in Taiwan used in folk medicine as antipyretic, diuretic and antitumor[154,155]. The leaves of *P. latifolia* are used as dirutic, antipyretic, anti-inflammatory and emmenagogue in Morocco and Sardina[156].

P. peruviana L commonly called Cape ghooseberry native to tropical America also found in Pakistan, is a widely used medicinal herb for treating cancer, malaria, asthma, hepatitis, dermatitis and rheumatism. diuretic and juice of the plant leaves are given in bowl and worms complaints while hot leaves are used as poultice[63,157]. Fruits are also used as food in the form of pies, cakes, compotes and jams. The dried berries are used as substitute of raisin. It is also assumed that the fruits of the plant are rich source of vitamin A, C and B complex[158].

P. philadelphica have been used for treatment of leprosy, purifying the blood and as a poison antidote in Mexico as well as in gastro-intestinal disorders in Guatemala[159]. Tomatillos, the fruits of *P. Philadelphica* are used as an ingredient in foods such as salsas and enchiladas in some countries of Latin America whereas the fruits of *P. philadelphica* and *P. coztomatl* are used in the preparation of sauces and other dishes in Mexico. They are also utilized as sauces and relishes as an acid source in place of tomatoes in North American[159,160].

P. coztomatl was used as an antipyretic, antidiarrheic, diuretic as well as in the treatment of cataracts, liver spots on the face, nose abscess, flatulence, asthma, and stomach pains in Mexico. The use of the plant in Mexican folk medicine has been described in the Florentine codex and properly documented since the sixteenth century. *P. coztomatl* is still used in the treatment of stomach pains, pulpitis and as an antidiarrheic in Oaxaca[160].

The crude MeOH extract of the *P. viscosa L.* was found to have antibacterial activity against *Staphylococcus aureus* and *Streptococcus pneumonia*[161].The aqueous extract of *P. alkekengi* also showed antibacterial activity[154]. Saline extract of the leaves of *P. peruviana* showed positive activity against *Staphylococcus*[162]. Hot extract of *P.peruveriana* is used in preparation of health beverages. Hot water and alcoholic extract of the plant also showed antioxidant activities[63].

2.3.2: Previous Phytochemical Investigations

The genus *Physalis* along with other genera of Solanaceae are known to elaborating C-28 ergostane lactone derivative with structural diversity and biological activities. *Physalis* is one of major source of withanolides, specifically Withaphysalins and Physalins [163]. Phytochemical study on this genus started since 1852 when first plant of this genus, *P. alkekengi* L was studied chemically. A bitter amorphous substance with the empirical formula $C_{28}H_{30}O_9$ was isolated from the leaves of the plant and called physalin [164]. After more than a century later, lactone moiety was established on the basis of IR spectrum[165]. The structural determination of Physalin A from *P. alkekngi* and making use of physiochemical methods including X-ray structural analysis for elucidation of the structure of Physalin A was also studied[165,166]. Similarly Physalin B and C were also isolated from the same plant and their structure was also confirmed [166-168]. The ongoing phytochemical study on *Physalis* has led to the isolation of Phyalin D-K from *P.angulata* and *P.lancifolia* [166,169,170]. Thus, the isolation of these physalins (A-K) opened up a peculiar series of withasteroids (Withanolides). Structural novelty and broad spectrum biological activities exhibited by withanolides led to the undiminishing interest in them and thus till date more than 130 withanolides isolated from the geuns *Physalis*. A list of withanolides isolated from this genus is summarized in table 2.3.

Table 2.3: Withanolides isolated from plants of genus *Physalis* (Solanaceae)

S.No	M. Mass	M. Formula	Name	Source
1	524	$C_{30}H_{36}O_8$	Withangulatin I	*P. angulata*[171]
2	542	$C_{29}H_{34}O_{10}$	Physalin I	*P. alkekengi*[172]
3	542	$C_{29}H_{34}O_{10}$	Physalin II	*P. alkekengi*[172]
4	528	$C_{28}H_{32}O_{10}$	Physalin Y	*P. alkekengi*[172]
5	526	$C_{28}H_{30}O_{10}$	Physalin Z	*P. alkekengi*[172]
6	518	$C_{28}H_{38}O_9$	Withangulatin B	*P. angulata*[153]
7	534	$C_{29}H_{42}O_9$	Withangulatin C	*P. angulata*[153]
8	552	$C_{29}H_{45}O_{10}$	Withangulatin D	*P. angulata*[153]
9	518	$C_{29}H_{42}O_8$	Withangulatin E	*P. angulata*[153]
10	470	$C_{28}H_{38}O_6$	Withangulatin F	*P. angulata*[153]

11	536	$C_{28}H_{40}O_{10}$	Withangulatin G	*P. angulata*[153]
12	534	$C_{29}H_{42}O_9$	Withangulatin H	*P. angulata*[153]
13	482	$C_{28}H_{34}O_7$	Withaphysalin P	*P.minima*[152]
14	526	$C_{30}H_{38}O_8$	18-O-Acetylwithaphysalin C	*P.minima* [152]
15	568	$C_{32}H_{40}O_9$	14,18-Di-O-acetylwithaphysalin C	*P.minima*[152]
16	514	$C_{30}H_{42}O_7$	Withaphysalin Q	*P.minima*[152]
19	514	$C_{29}H_{40}O_7$	Withaphysalin R	*P.minima*[152]
20	514	$C_{30}H_{42}O_7$	5-O-Methoxywithaphysalin R	*P.minima*[152]
21	516	$C_{29}H_{40}O_8$	Withaphysalin S	*P.minima*[152]
22	526	$C_{28}H_{30}O_{10}$	Physalin W	*P. alkekengi*[173]
23	526	$C_{28}H_{30}O_{10}$	Physalin X	*P. alkekengi*[173]
24	510	$C_{30}H_{38}O_7$	Physagulin L	*P. angulata*[174]
25	528	$C_{30}H_{40}O_8$	Physagulin M	*P. angulata*[174]
26	488	$C_{28}H_{40}O_7$	Physagulin N	*P. angulata*[174]
27	544	$C_{30}H_{40}O_9$	Physagulin O	*P. angulata*[174]
28	530	$C_{30}H_{42}O_8$	Physacoztolide A	*P.coztomatl*[160]
29	488	$C_{28}H_{40}O_7$	Physacoztolide B	*P.coztomatl*[160]
30	512	$C_{30}H_{40}O_7$	Physacoztolide C	*P.coztomatl*[160]
31	544	$C_{30}H_{40}O_9$	Physacoztolide D	*P.coztomatl*[160]
32	512	$C_{30}H_{40}O_7$	Physacoztolide E	*P.coztomatl*[160]
33	562	$C_{30}H_{42}O_{10}$	Physanolide A	*P. angulata*[175]
34	558	$C_{29}H_{34}O_{11}$	Physalin U	*P. angulata*[175]
35	554	$C_{30}H_{34}O_{10}$	Physalin V	*P. angulata*[175]
36	470	$C_{28}H_{38}O_6$	Cinerolide	*P.cinerasces*[176]

37	506	$C_{28}H_{42}O_8$	24,25-Dihydrowithanolide S	*P.cinerascens*[176]
40	562	$C_{30}H_{42}O_{10}$	Physachenolide A	*P.chenopodifolia*[177]
41	578	$C_{30}H_{42}O_{11}$	Physachenolide B	*P.chenopodifolia*[177]
42	544	$C_{30}H_{40}O_9$	Physachenolide C	*P.chenopodifolia*[177]
43	528	$C_{30}H_{40}O_8$	Physachenolide D	*P.chenopodifolia*[177]
44	544	$C_{30}H_{40}O_9$	Physachenolide E	*P.chenopodifolia*[177]
45	526	$C_{30}H_{38}O_8$	Physagulin H	*P. angulata*[178]
46	530	$C_{30}H_{42}O_8$	Physagulin J	*P. angulata*[178]
47	546	$C_{30}H_{42}O_9$	Physagulin K	*P. angulata*[178]
48	504	$C_{28}H_{40}O_8$	Philadelphicalactone B	*P.philadelphica*[179]
49	536	$C_{29}H_{44}O_9$	2,3-Dihydro-3-methoxyixocarpalactone A	*P.philadelphica*[180]
50	504	$C_{28}H_{40}O_8$	2,3-Dihydroixocarpalactone B	*P.philadelphica*[180]
51	534	$C_{29}H_{40}O_9$	2,3-Dihydro-3β-methoxyixocarpalactone B	*P.philadelphica*[180]
53	472	$C_{28}H_{40}O_6$	4,7,20-Trihydroxy-1-oxowitha-2,5-dienolide	*P.philadelphica*[180]
54	546	$C_{28}H_{34}O_{11}$	Physalin T	*P. alkekengi*[181]
55	632	$C_{34}H_{48}O_{11}$	14,20-Epoxy-3,17-dihydroxy-1-oxowitha-5,24-dienolide -3-O- β -D-Glucopyranoside	*P.peruviana*[182]
56	486	$C_{28}H_{38}O_7$	5,6-Epoxy-4,14,15-trihydroxy-1-oxowitha-2,24-dienolide	*P.peruviana*[157]
57	506	$C_{28}H_{42}O_8$	5,6,14,20,27-Pentahydroxy-1-oxowith-24-enolide	*P.peruviana*[157]
58	650	$C_{34}H_{50}O_{12}$	3-O—β-D-Glucopyranoside	*P.peruviana*[183]
59	678	$C_{36}H_{54}O_{12}$	3-O--D-Glucopyranoside, 1-Ac	*P.peruviana*[183]
60	662	$C_{36}H_{54}O_{11}$	1-Ac, 3-O-β-D-glucopyranoside	*P.peruviana*[183]
61	452	$C_{28}H_{36}O_5$	14,20-Epoxy-17-hydroxy-1-oxowitha-3,5,24-trienolide (14 α,17 **β**,20S,22R)-form	*P.peruviana*[162]

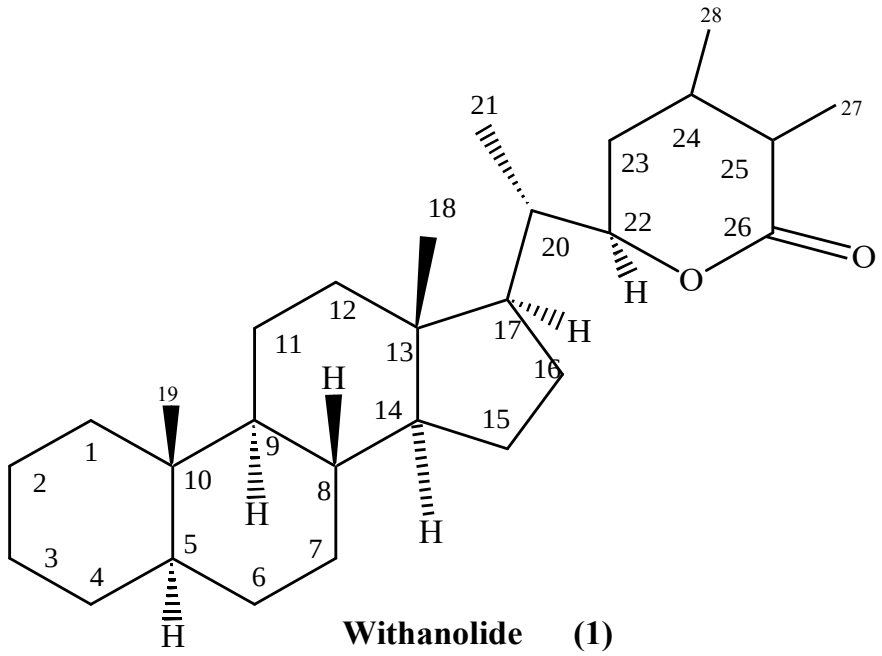
62	502	$C_{28}H_{38}O_8$	28-Hydroxywithanolide E	*P. angulata*[184]
63	562	$C_{28}H_{31}O_{10}C$	Physalin H: 5a-chloro-6β-hydroxy-5,6-dihydrophysalin B	*P. angulata*[185]
64	528	$C_{28}H_{32}O_{10}$	Physalin S: 3a,5a-cyclo-6β-hydroxy-2,3,5,6-tetrahydroxyphysalin B	*P. alkekengi var. franchati*[186]
65	510	$C_{28}H_{30}O_9$	Physalin R: 15a-hydroxy-11β,15β-cyclo-15-deoxphysalin B	*P. alkekengi var. franchati*[186]
66	542	$C_{28}H_{30}O_{11}$	Physalin Q: 2β,5β-epidoxy-6β-hydroxy-3-4-didehydro-2,3,5,6-tetrahydrophysalin B	*P. alkekengi var. franchati*[187]
67	542.	$C_{28}H_{30}O_{11}$	Physalin K: 2a,5a-epidioxy-6β-hydroxy-3,4-didehydro-2,3,5,6-tetrahydrophysalin B	*P. alkekengi var. franchati* [187]
68	508	$C_{28}H_{28}O_9$	4,7-didehydroneophysalin B	*P. alkekengi var. franchati* [188]
69	510	$C_{28}H_{30}O_9$	25 27-dihydro-4,7-didehydro-7.deoxyneophysalim A	*P. alkekengi var. franchati*[188]
70	510	$C_{28}H_{30}O_9$	Isophysalin B	*P. alkekengi* [188]
71	526	$C_{28}H_{30}O_{10}$	Isophysalin G	*P. alkekengi*[188]
72	602	$C_{34}H_{50}O_9$	Physapruin B	*P. alkekengi*[189]
73	526	$C_{28}H_{30}O_{10}$	Physalin P	*P. alkekengi*[190]
74	528	$C_{28}H_{32}O_{10}$	Physalin O	*P. alkekengi*[191]
75	526	$C_{28}H_{30}O_{10}$	Physalin N	*P. alkekengi*[191]
77	722	$C_{36}H_{50}O_{15}$	Physagulin G	*P. angulata*[192]
78	544	$C_{30}H_{40}O_9$	Physagulin F	*P. angulata*[192]
79	706	$C_{36}H_{50}O_{14}$	Physagulin E	*P. angulata*[192]
80	620	$C_{34}H_{52}O_{10}$	Physagulin D	*P. angulata*[193]
81	510	$C_{30}H_{38}O_7$	Physagulin A	*P. angulata*[193]
82	546	$C_{30}H_{39}ClO_7$	Physagulin B	*P. angulata*[193]
83	542	$C_{30}H_{38}O_9$	Physagulin C	*P. angulata*[194]
84	526	$C_{30}H_{38}O_8$	Withangulatin A	*P. angulata*[155]
85	522.634	$C_{28}H_{42}O_9$	Physangulide: 5β,6β-epoxy-3β,4β,20R,24S,25R-pentahydroxy-1-oxo-22S-withanolide	*P. angulata*[195]
86	578	$C_{30}H_{42}O_9S$	Withaperuvin H:	*P. peruviana*[196]
87	512	$C_{28}H_{32}O_9$	Physalin M	*P. alkekengi*[197]
88	482	$C_{28}H_{34}O_7$	Withaphysalin E: 18,20R-epoxy-6β, 14-dihydroxy-1, 18-dioxo- 22R-witha-2,.4,24-trienolide	*P. minima var. indica*[198]
89	502	$C_{28}H_{38}O_8$	Withaperuvin G: 2β,3β,5β,6β-diepoxy-14,17β,,r20R-trihydroxy-l –oxo-22R-with-24-enolide	*P. peruviana*[199]

90	502	$C_{28}H_{38}O_8$	Withaperuvin F: 3a,6a-epoxy-4β,17β,20R-tetrahydroxy-l-oxo-22R-with a-14,24-dienolide	*P. peruviana*[199]
91	528.	$C_{30}H_{40}O_8$	Withaminimin: 15a-acetoxy-5a,6β,14-trihydroxy-l-oxo-20S.22R-with a-2,16,24-trienolide	*P. minima*[192,200]
92	472	$C_{28}H_{40}O_6$	Vamonolide; 6,7 -epoxy-5, 14-dihydroxy-1 -oxo-2-withanolide	*P. angulata*[201]
93	528	$C_{28}H_{32}O_{10}$	Physalin L	*P. alkekeng*[166]
94	486	$C_{28}H_{38}O_7$	24S,25S-epoxywithanolide D	*P. angulata*[202]
95	518	$C_{28}H_{38}O_9$	Visconolide: 5,6β-epoxy-4β,l4β,17β,20β,28-pentahydroxy-l-oxo-22R-with a-2,24-dienolide	*P. viscose*[202,203]
96	488	$C_{28}H_{40}O_1$	l4a-Hydroxycarpanolide: 6a,7a-epoxy-5a,14a,20/R-trihydrxy-l-oxo-22R.24S,25R-wilh-2-enolide	*P. anguIata*[202,204]
97	472	$C_{28}H_{40}O_6$	laxocarpanelide: 6α,7a-epoxy-5,20R-dihydroxy-l-oxo-5a,22R,24S;25R-with-2-enolide	*P. ixocarpa-*[206]
98	502.	$C_{28}H_{38}O_8$	28-Hydroxywithaperuvin C: (20S,22R)-6β,14a,17β,20,28-pentahydroxy-1-oxo-2,4,24-withatrienolide	*P. vtscosa*[203]
99	458	$C_{28}H_{42}O_5$	Pubesenolide:1a,3β,27-trihydroxywilha-5,24-dienolide (Sominone)	*P. pubescen*[120]
100	474	$C_{28}H_{42}O_6$	Pubescenol: *24S,25S-e*poxy-4a,7a-dihydroxy-1 -oxo-withanolide	*P. pubescen*[205]
101	528	$C_{30}H_{40}O_8$	Physapubenolide: 15a-acetoxy5β,6β-epoxy-4β,14β-dihydroxy-1-oxo-20S,22R-witha-2,24-dienolide	*P. pubescen*[206]
102	502	$C_{28}H_{38}O_8$	28-Hydroxywithaphysanolide: 3,14,17β,20R,28-pentahydroxy-1-oxo-22R-witha2,5,24-trienolide	*P. viscose*[207]
103	466	$C_{28}H_{34}O_6$	Withaphysalin D:	*P. minima*[208]
104	500	$C_{28}H_{36}O_8$	Withaperuvin E	*P. peruviana*[209]
105	521	$C_{28}H_{37}O_7$	Physalolactone C	*P. peruviana*[210]
106	520	$C_{28}H_{40}O_9$	Withaperuvin D*:* 4β,5a,14a,17β,20S-pentahydroxy-3a,6a-oxido-1-oxo-24-ergosten-26,22R-olide	*P. peruviana*[211]
107	662	$C_{36}H_{54}O_{11}$	Physalolactone B-3 -O -β- D-ghucopyranoside	*P. peruviana*[212]
108	486	$C_{28}H_{38}O_7$	Withaperuvin C: (20S,22R) 6β,l4.17β.20-tetrahydroxy-l-oxowitha-2,4,24-trienolide	*P .peruviana*[213]
109	520	$C_{28}H_{40}O_9$	Withaperuvin B: *4β,5β,6a,l4a* 17β,20R-hcxahydroxy-1-oxowitha-2,24-dienolide	*P .peruviana*[213]
110	488	$C_{28}H_{40}O_7$	Perulactone B: 1 -oxo-14,17,20,22-tetrahydroxy2,5ergostadien-26,28-olide	*P. peruviana*[214]
111	520	$C_{28}H_{40}O_9$	Withaperuvin: 4β,5β,6a,l4a,l7β,20R-hexahydroxy-l-oxowitha-2,24-dienolide	*P. peruviana*[215]
112	488	$C_{28}H_{40}O_7$	Viscosalactone B	*P. viscose*[216]
113	486,604	$C_{28}H_{38}O_7$	Viscosalactone A	*P. viscose*[216]
114	486	$C_{28}H_{38}O_7$	Physangulide: 15a-acetoxy-5β,6β-epoxy-4β-dihydroxy-1-oxo-20S,22R-with a-2,24-dienolide	*P. viscosa*[217]
115	500	$C_{30}H_{44}O_6$	Physalotactone B	*P. peruviana*[218]
116	523	$C_{28}H_{39}O_7$	4-DeoxyphysaIolactone	*P. peruviana*[215]

117	486	$C_{28}H_{38}O_7$	Wtlhaphysanolide : 4β,14,17β,20R-tertrahydroxy-1-oxo-22R-with a-2,5,23-trienolide	*P. viscose*[219]
118	530	$C_{30}H_{42}O_8$	Physapiibescin: 15-acetoxy-5,6:22,26:24,25-triepoxy-4,26-dihydroxyergost-2-en-1-one	*P. pubescens*[220]
119	542	$C_{28}H_{30}O_{11}$	Physalin K	*P. l ancifolia* *P.angulata*[221]
120	526	$C_{28}H_{30}O_{10}$	Physalin G	*P. angulata*[221]
121	558	$C_{28}H_{34}O_{11}$	Physalin I	*P .angulata*[221]
122	544	$C_{28}H_{32}O_{11}$	Physalin D	*P. angulata* *P. minima*[221]
124	518	$C_{30}H_{46}O_7$	Perulactone	*P. peruviana*[222]
123	502	$C_{28}H_{38}O_8$	Ixocirpalndone B. 5**β**,6β16β,23S-diepoxy-4β,20R(,22S-trihydroxy-l-oxo-errgost-2-eno-26,23S-lacdone	*P. ixocarpa*[223]
124	504	$C_{28}H_{40}O_8$	Ixocarpalactone A; 5,6-cpoxy-4,16,20,22,23-pentahydroxy-l-oxoergost-2-en-26-oicacid,y-lactone	*P. ixocarpa*[223]
125	539	$C_{28}H_{39}O_8$	Physalolactone: 6a-chloro-4β,5β, 14. 17β.20S'-pentahydroxy -22R-witha-2.24-dienolide	*P. peruviana*[224]
125	544	$C_{28}H_{32}O_{11}$	Physalin E	*P. lancifolia* *P. angulata*[169]
126	526	$C_{28}H_{40}O_8$	Physalin F	*P. angulata* *P.lancifolia*[170]
127	526	$C_{28}H_{30}O_{10}$	Physalin H	*P. angulata* *P. lancifolia folia*[170]
128	526	$C_{28}H_{30}O_{10}$	Physalin J	*P. angulata*[170]
129	48S	$C_{28}H_{40}O_7$	2,3-Dihydrowithanolide E: *5β,6β-epoxy*-l4a,17β.20S-trihydroxy-1-oxo-22R-with-24-enolide	*P. peruviana*[128]
130	516	$C_{29}H_{40}O_8$	Physalactone: 5,6β-epoxy-4β.17a,20S-trihydroxy-3β-methoxy-l-oxo-22R-with-8(14).24-dienolide	*P. viscosa*[207,225]
131	484	$C_{28}H_{36}O_7$	Withaphysalin C: 13β,14β,18£,20R-diepoxy-1-oxo-13,14-seco-22R-with a-2,5,24-trienolide	*P. minima*[226]
132	502	$C_{28}H_{38}O_8$	4β-Hhdroxywithanolide E: 5β,6β-epoxy-4β,14a,17β,20S-tetrahydroxy-1-oxo-22R-with a-2,24-dienolide	*P. peruviana*[129,227]
133	468	$C_{28}H_{36}O_6$	Withaphysalin B: 5β.6β,18£,20R-diepoxy-18-hydroxy-1-oxo-22R-with a-2,24-dienolide	*P .minima*[150]
134	466	$C_{28}H_{34}O_6$	Withaphysalin A: 18,20R-epoxy-14-hydroxy-1,18-dioxo-22R-with a-2,5,24-trienolide	*P. minima*[150]
135	488	$C_{28}H_{40}O_7$	Withaphysacarpin: 5β,6β-epoxy-4β,16β,20R-trihydroxy-1-oxo-22R,24R,25R-with-2-enolide	*P. ixocarpa*[228]
136	526	$C_{28}H_{30}O_{10}$	Physalin A	*P. alkekengi*[167]
137	510	$C_{28}H_{30}O_9$	Physalin B	*P. alkekengi* *P. minima*[167]
138	510	$C_{28}H_{30}O_9$	Physalin C	*P. alkekengi*[168]

2.4: Withanolides

The withanolids are group of naturally occurring steroids based on an ergostan skeleton in which C-22 and C-26 are oxidized in order to form lactone ring [101]. They are chemically characterized by a lactone containing side chain which is made of nine carbons and different oxygen substituents particularly in the ring A, B[87]. Withanolides are generally poly-oxygenated and it is assumed that plants producing such type of compounds possess an enzyme system which oxidizes all carbon atoms in steroids nucleus. A side chain with lactone or lactol ring is the characteristic feature of withanolides. The basic skeleton of withanolides (**1**) is actually a rearranged ergostan framework, which may be defined as the 22-hydroxyergostan-26-oic acid lactone. In the Chemical Substances Index (CAS) the compounds are catalogued as ergosterooids deravatives[101]. Withaferin-A (**2**), the first member of this group was isolated by Lavie from *W. somnifera* [100] and because of the genus these compounds were given the name withanolide. The structural novelty and excellent biological activities of this compound led to chemical investigation of various plant species and numerous compounds of similar feature were isolated[101].

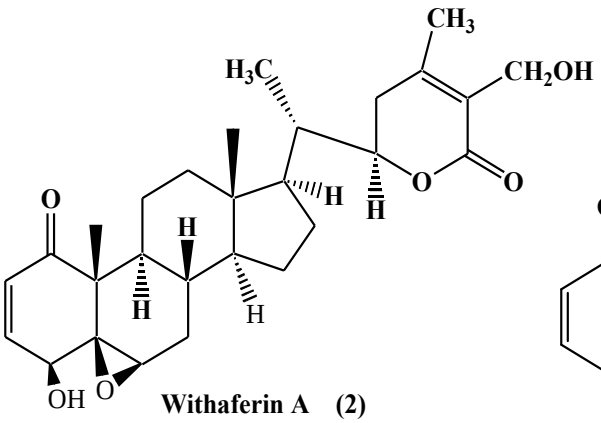


Withanolide (1)

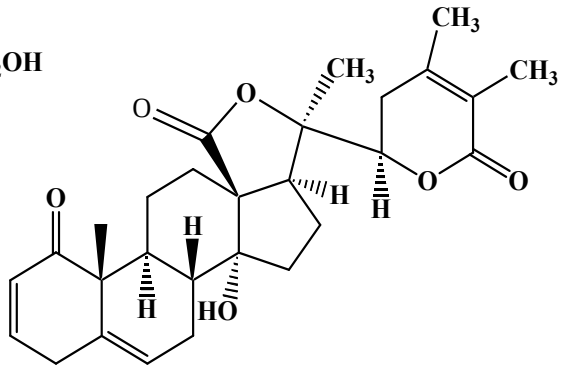
Although withanolides are the monopoly of Solanaceous plants, yet they are not present in all members of Solanaceae. So for sixteen genera have produced withanolides which are *Withania, Physalis, Jaborasa, Datura, Acnistus, Lycium, Exodeconus, Deprea, Ichroma, Nicandra, Salpichroa, Discopodium, Dunalia, Trechonaetes, Tubocapsicum* and *Witheringia.*[152]. However they are not restricted to Solanaceae and recently were also reported from the plants of Taccaceae and Leguminosae as well as from some marine organisms[79]. Most of the Withanolids exhibited antitumor, antibacterial, anti-inflammatory, cytotoxic, insecticidal, antifeedant, anti- fungal hepat-protective and immune suppressive activities [82,101].

2.4.1: Classification of withanolides

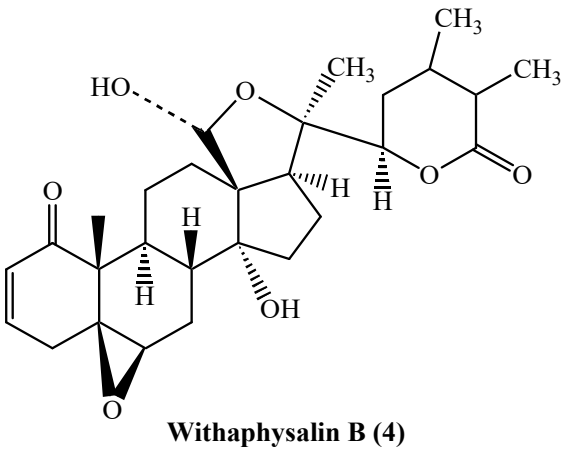
On the basis of oxygen substituents, formation of new bonds, aromatization of rings and several natural modification of the crabocyclic skeleton as well as of side chain, the resulting compounds with complex structural features are classified as Physalin, Withaphysalin, Jaborols, Nicandrenones, Ixocarpalactones, Acnistins and Withajaridin[152].



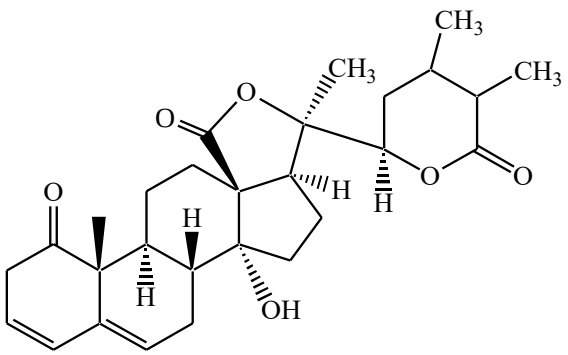
Withaferin A (2)
2,4-Dihydrowithaferin A (2a)



Withaphysalin A (3)



Withaphysalin B (4)



Withaphysalin D (5)

I) Physalins

Physalins are ergosteroids deravatives, modified particularly by oxidative cleavage of 13,14 –bond forming nine member ring, formation of new six member carbocyclic ring between C-16 and C-24 and oxidation of 13-methyl to carboxylic acid followed by lactonization at C-18,20[150] e.g. Physalin A (**6**) and Physalin B (**7**) [167].

II) Withaphysalins

Withaphysalins are modified by an additional lactone ring, formed between C-18-oic acid and C-20 hydroxy group e.g Withaphysalin A (**3**), B (**4**) [150] and Withaphysalin D (**5**)[208].

III) Jaborols (Ring A aromatic withanolides)

Jaborols are withanolides in which modification occur by aromatization of ring A. These are generally found in *Jaborosa* species.e.g Jaborol(**10**) from *Jaborosa magellanica*[229].

IV) Nicandrenones (Ring D aromatic withanolides)

Withasteroids modified by aromatization of D, are unique compounds isolated naturally from the only source *Nincandra Physaloides*. Nicandrenone **(11)** was named first time in 1964 while later on Crombei reproposed its structure by X-ray analysis and renamed as Nic-1[101].

V) Ixocarpalactones

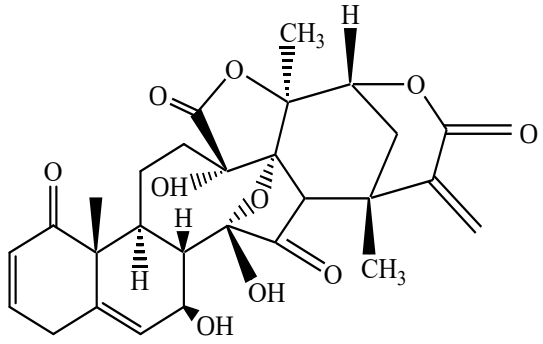
Ixocarpalactones are withnolides with a modified side chain, mainly characterized by the presence of C-23, 26-lactone.e.g Ixocarplacton A(**12**) isolated from *P. ixocarpa* [223].

VI) Acnistins

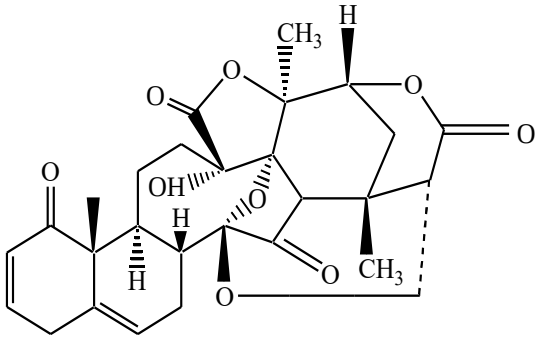
The structure of Acnistins type withanolides has a modified bicyclic system at C-17 Acnistin E (**13**). They are reported from genus *Dunalia* [230].

VII) Withajardins

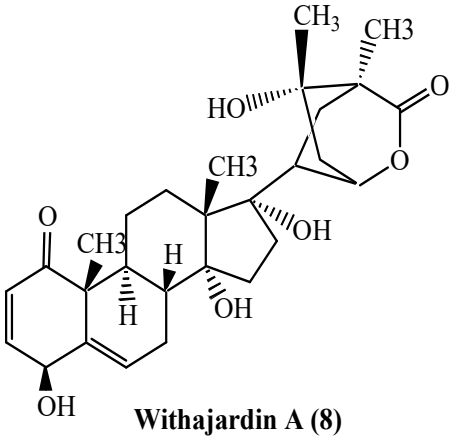
Withajardins are modified withanolides having a bicyclic side chain formed by linkage of C-21 and C-23 e.g.Withajardin A (**8**) isolated from *Deprea orinocensis* [231].



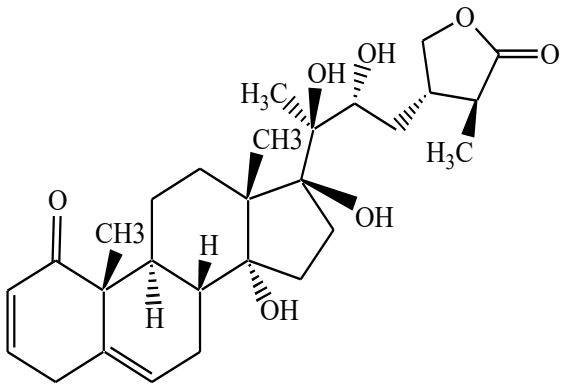
Physalin A (6)



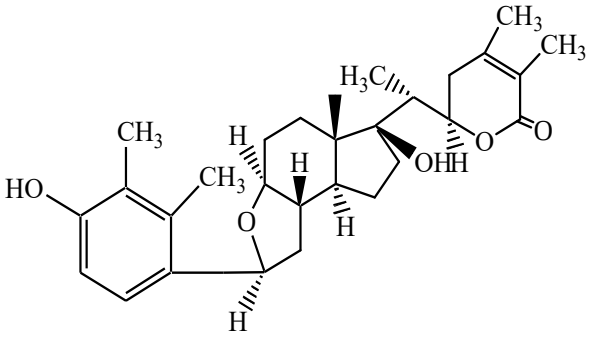
Physalin B (7)



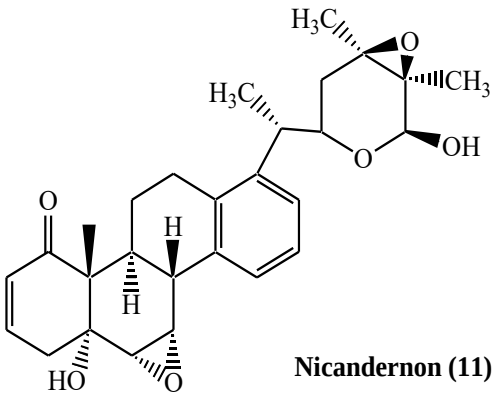
Withajardin A (8)



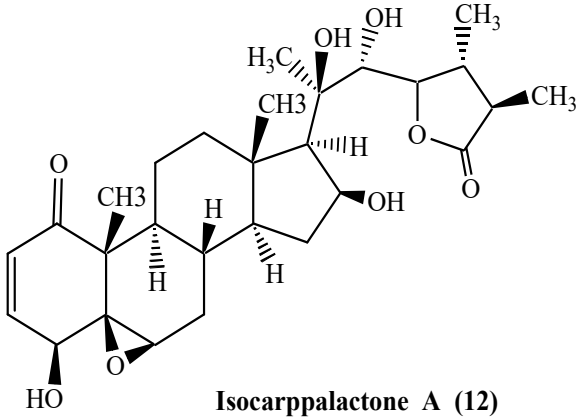
Perculacton B (9)



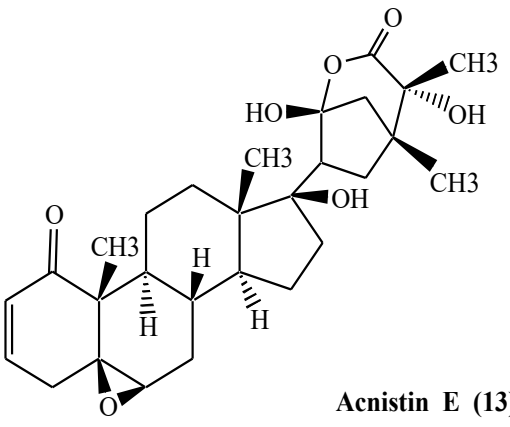
Jabrol (10)



Nicandernon (11)



Isocarppalactone A (12)



Acnistin E (13)

2.4.2: Pharmacological Importance of withanolides

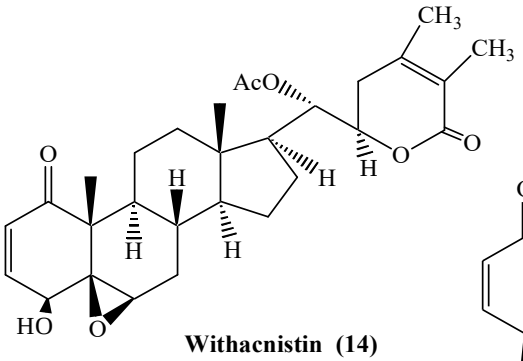
Withanolides are the most important group of naturally occurring compounds. They are interesting as many of them exhibit variety of pharmacological activities such as antitumor, antibacterial, anti-inflammatory, cytotoxic, hepatoprotective and immunosupp-ressive activities[82,101].

Withaferin-A (**2**), the first isolated withanolide from *W. somnifera* [100] had received considerable attention due to its antibiotic and anti-tumor activities. It can inhibit the growth of various gram-positive bacteria and fungi like *Aspergillus flavus. Epidermophyton floccosum* and *Cladosporum herbarm* [232]. It is assumed that C-26 carbon is responsible for this antibacterial activity [233]. Withaferin-A (**2**) and withacnistin (**14**) exhibited cytotoxicity against KB cells cultures derived from human carcinoma of the nasophyranx[101,234]. Inhibitory activity of of comound (**1**) against sarcoma 180 tumor in mice and walker intramuscular carcinosacima 256 in rats are also reported[101,235].

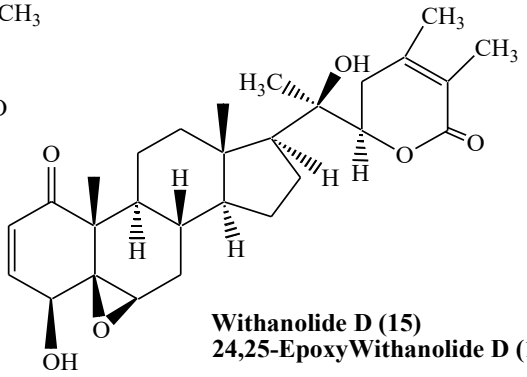
Withaferin-A (**2**), withanolide D (**15**) and 4β-hydroxy withanolide E (**26**) were active against mouse leukemia L5178 Y cells *in vitro* while Withaferin-A (**2**) and its 6α-chloro-5β-hydroxy-derivative exhibit cytotoxic activity against HeLa 229 cells in cultures[101,236,237]. The relationship between chemical structure and activity was also studied and it was assumed that the essential requirement in withanolides for antitumor and anti-proliferative activities were considered to be epoxide, enone functionality in A,B ring and unsaturated lactone in the side chain[236]. Withanolide E (25) and 4β-hydroxy withanolide E (**26**) were preclinicaly investigated by National Cancer Institute in USA on L-1210 leukemia and B-16 melanoma. However their activity was inferior as required for clinical investigations [101]. Similarly 4β-hydroxy withanolide E (**26**) showed life span enhancing activity against L1210- leukemia[129].

Withanolides of *W.sominifera*, withanolide A (**19**), withanoside IV (**21**), withanoside VI (**22**) and coagulin Q (**23**) showed significant neuritis outgrowth activity at low concentration of a human neuroblastoma SH-SY5Y cell line[105]. Ashwagandhanolide (**24**) showed growth inhibition against human gastric (AGS), breast (MCF-7), central nervous system (SF-268), colon (HCT-116), and lung (NCI H460) cancer cell lines[76]. Antiproliferative activity of withanolide on NCI-H460 (Lung), HCT-116 (Colon), SF-268 (Central Nervous System; CNS and MCF-7

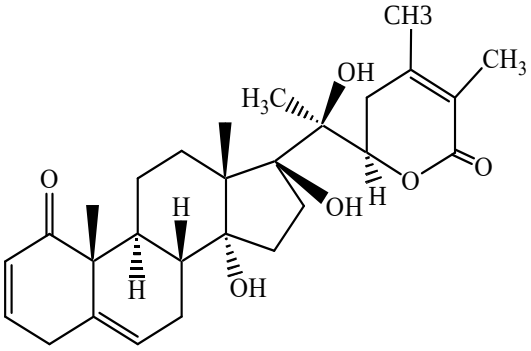
(Breast) human tumor cell line were also reported[99]. Recently our collaborative research group has studied cytotoxic withaphysalins from *P. minmia* [152].



Withacnistin (14)

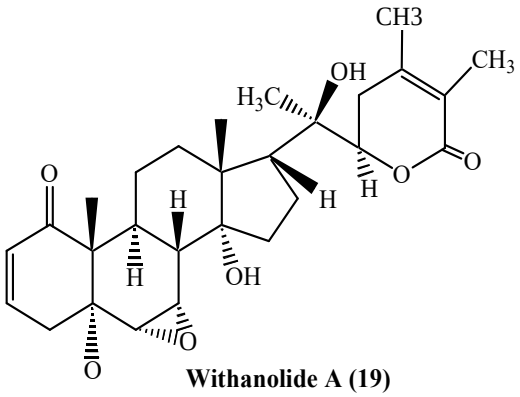


Withanolide D (15)
24,25-EpoxyWithanolide D (16)

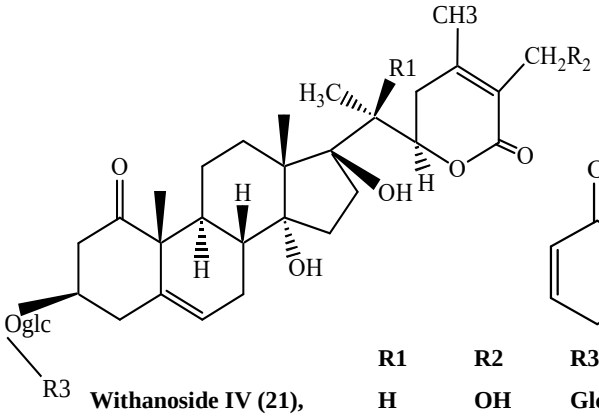


Withanolide F (17)

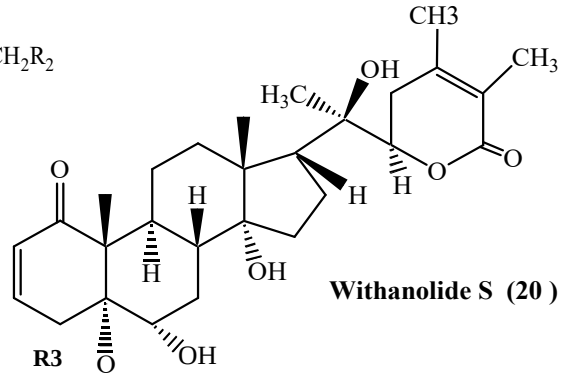
3β-Hydroxy-2,3-dihydrowithanolide F(18



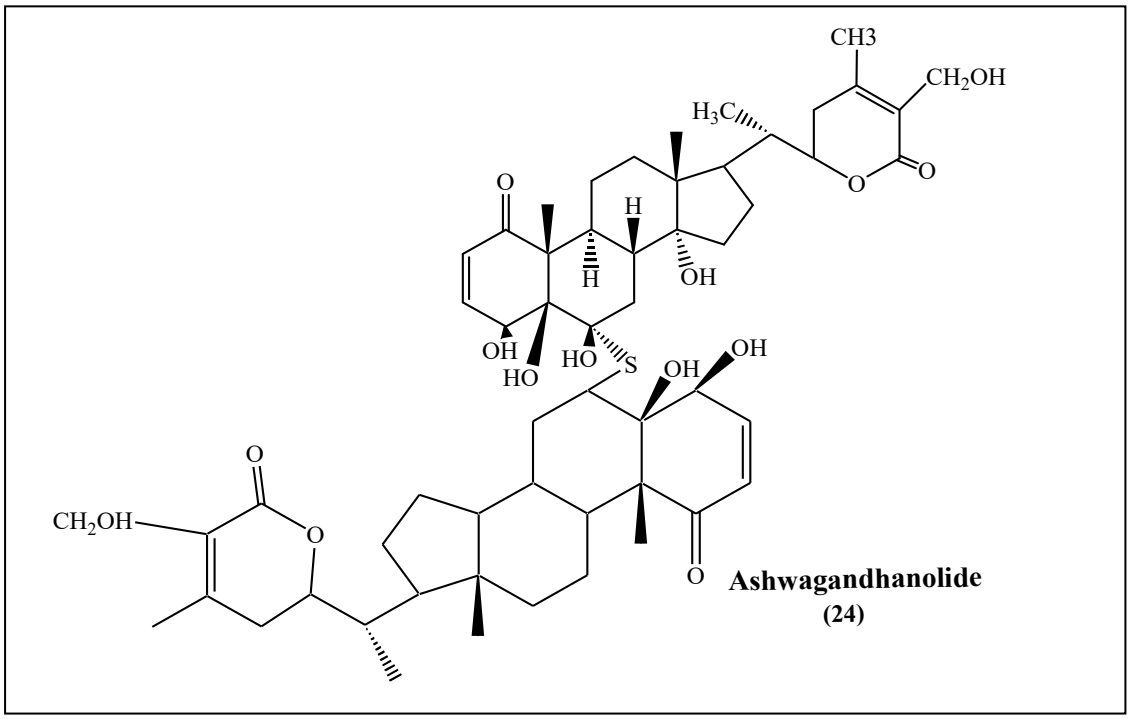
Withanolide A (19)



	R1	R2	R3
Withanoside IV (21),	**H**	**OH**	**Glc**
Withanoside VI (22)	**OH**	**H**	**Glc**
Coagulin Q (23)	**OH**	**H**	**H**



Withanolide S (20)

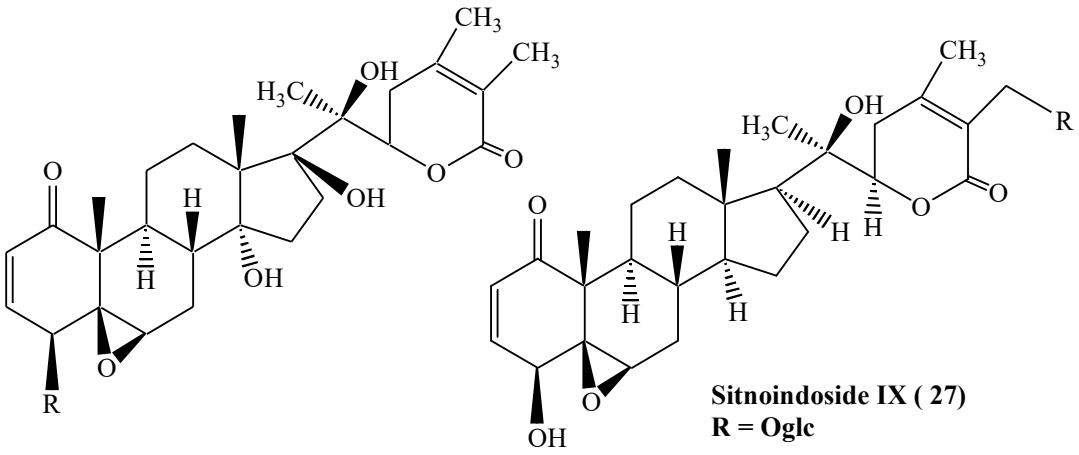


Ashwagandhanolide
(24)

The withanolides have shown to possess both immunosuppressive and immunostimulating properties [238]. Withaferin-A (**2**) and withanolide E (25) were shown to have immunosuppressive activity on human B and T Lymphocytes inhibition of the growth of Ehrlich ascites carcinoma in mice and complete disappearance of tumor cells by withaferin A as well as resistance of the cured mice to rechallenge with Ehrlich ascites tumor cells[239] indicate the immuno-activatmg property of withaferin A.

The anti-inflammatory and hapato-protective activities of withanolide were also studied. 3β-Hydroxy-2,3-dihydrowithanolide F **(18)** has protective effect in hepatoxcity in adult rats as well as produce a moderate fall of blood pressure in dogs[240], While 3β-Hydroxy-2,3-dihydroxy withanolide F (**18**), 24,25-epoxy withanolide D (**16**) and Physangulide **(32**) have significant anti-inflammatory effect, both in exudative and proliferative types of experimentally induced inflammation[101,240]. The immunosuppressive effect of Lycium substance A was also studied[241]. Bahr et al[241] demonstrated its ability to inhibit proliferation of murine spleen cell cultures. Adaptogenic and immuno-stimulatory activity of glycowithanolides, sitoindoside IX (**27**) and sitoindoside X **(28**) were studied. Both compounds resulted in significant anti-stress activity in albino mice and rats[118,242].

Physalin class of withanolides has also been evaluated for their biological activities. Physalin B (**7**) and 5α,6-αepoxyphysalin B (**7a**) have shown cytotoxicity against 9 KB cells while physalin D (**29**) against B-16 melanocarcinoma. The activity of and 5α, 6αepoxyphysalin B was found more prominent than physalin B [164,243]. Physalin A (**6**) showed moderate *in vitro* cytotoxicity against Mela cells.

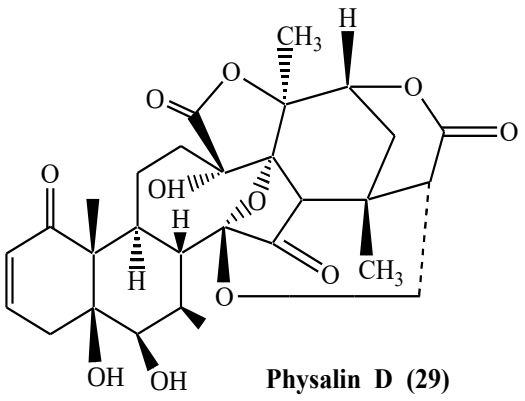


Withanolide E (25) R = H

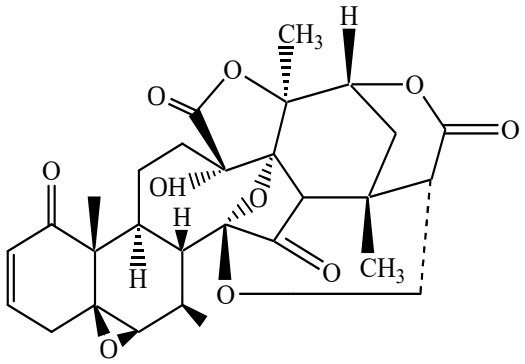
4 β-Hydroxywithanolide E (26) R = OH

Sitnoindoside IX (27)
R = Oglc

Sitnoindoside X (28)
R = Oglc(6-pamitoyl)



Physalin D (29)

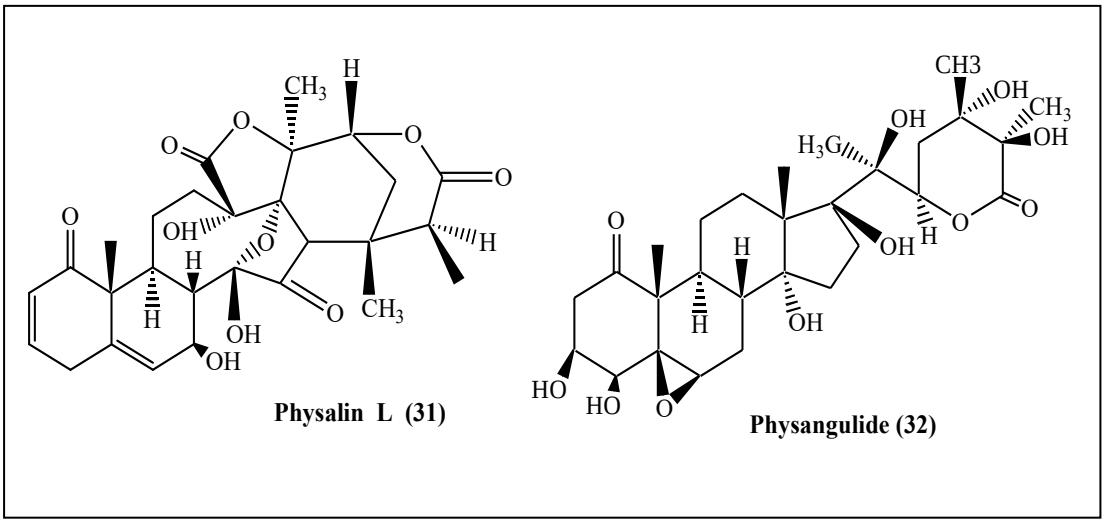


Physalin F (30)

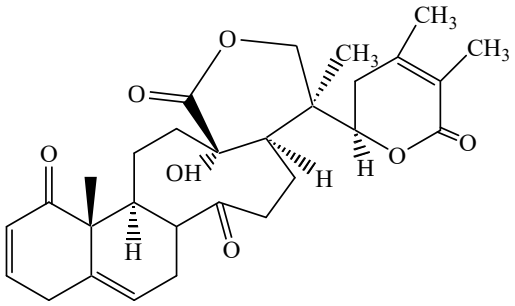
Physalin F (**30**) and physalin B (**7**) were found to be more active than physalin A (**6**). Physalin L (**31**) was remained inactive and the absence of cyclohehanone moiety is considered to be responsible for this inactivity. The abortive activity of physalin X has been reported which obtained by modifying the physalins from *P. minima* [244]. Physalin B, D and F exhibited inhibitory activity against *Mycobatrium tuberculosis* [245]. Physalin from *P. minima* also showed potent lishminicidal activity[246].

Physalins D (**29**) and F (**30**) displayed potent cytotoxic activity against a panel of human cancer cell lines [153] while Physalins B (7) and F (**30)** showed inhibitory activities on a human T cell leukemia Jurkat cell line[247].

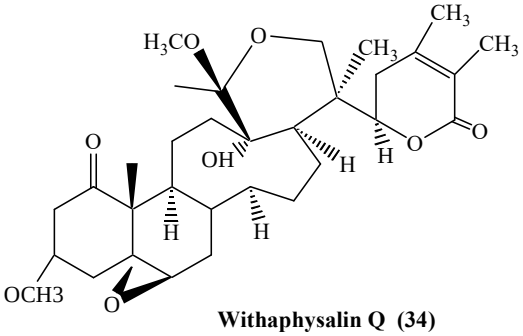
Several withanolides have shown insecticidal and insect antifeedant properties. Nic-1 (**11**), Withanolide E (**25**) and 4β-hydroxy withanolide E (**26**) were evaluated as antifeedant. Withanolide E is potent antifeedant while the later two are poor antifeedant and the most active compound in this regard is nicobolin A.[101,196,248].



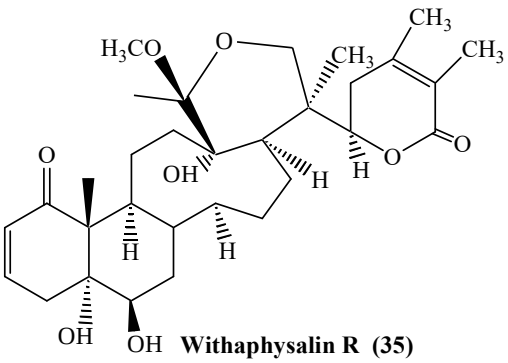
Enzyme inhibitory activities of withanolides are also reported. Choudhary et al [79] studied the butyrylcholinesterase and acetylcholinesterase inhibitory activities of withanolides from *W.somnifera***.** Withaferin-A (**2**) along with other withanolide were found active against acetylcholinesterase while its derivative 2,3-dihydrowithaferin A (**2a**) and two other compounds have inhibitory potential against butyryl cholin-esterase. Similarly withanolodes form *Ajuga bractosa* have also exibibited cholin-esterase inhibitory activity[249], whereas ashwagandhanolide (**24**) inhibited lipid peroxidation and the activity of the enzyme cyclooxygenase-2 in vitro[76].



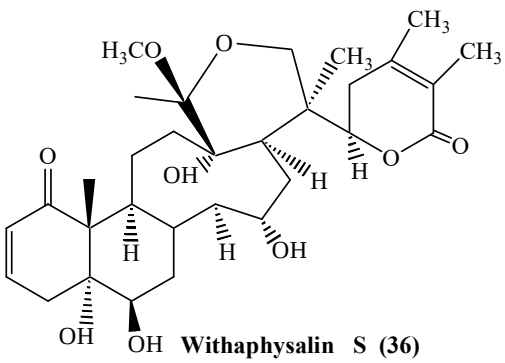
Withaphysalin P (33)



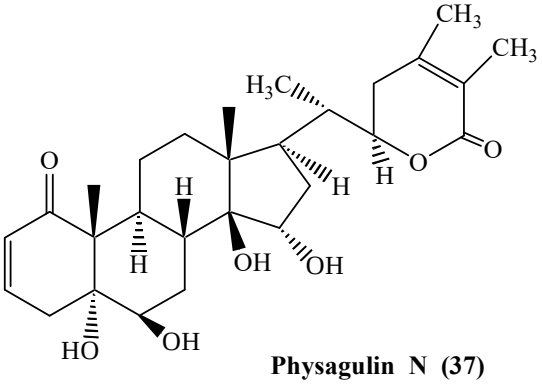
Withaphysalin Q (34)



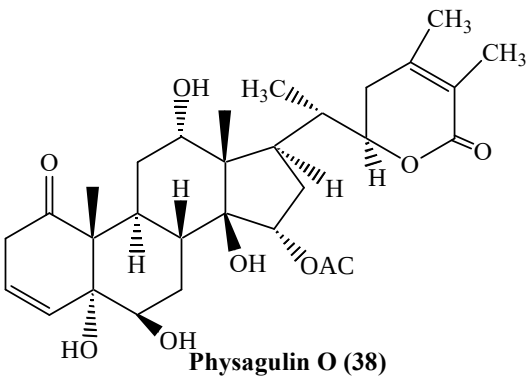
Withaphysalin R (35)



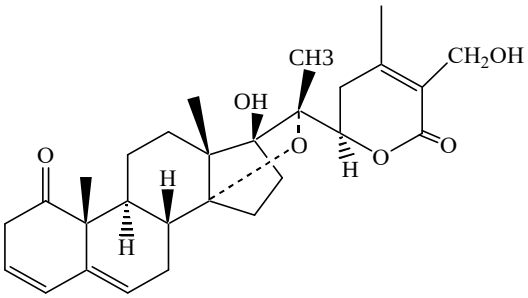
Withaphysalin S (36)



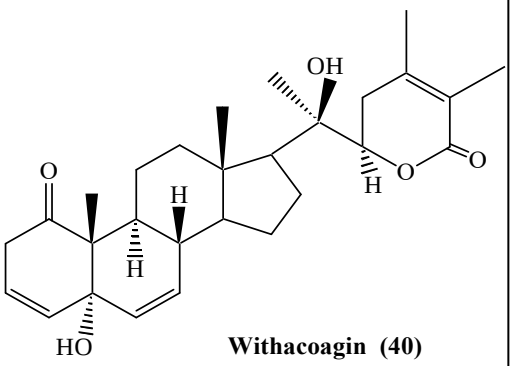
Physagulin N (37)



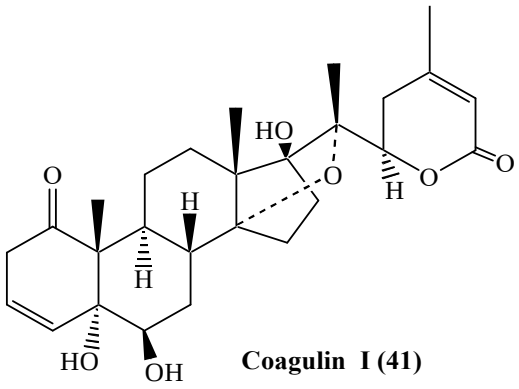
Physagulin O (38)



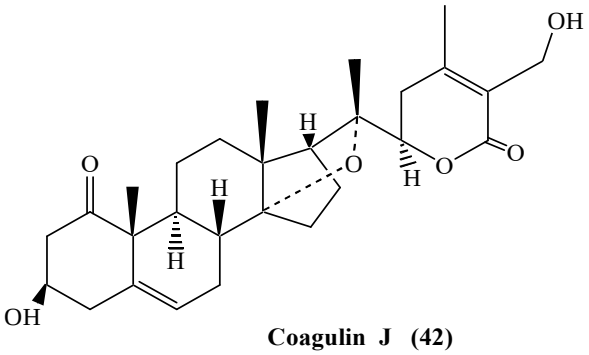
Coagulin (39)



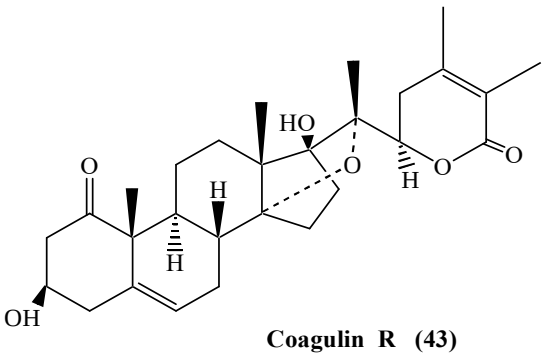
Withacoagin (40)



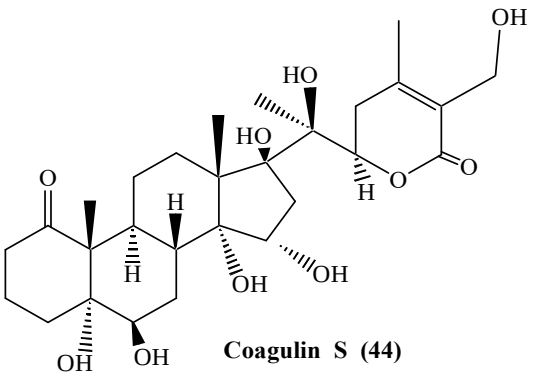
Coagulin I (41)



Coagulin J (42)



Coagulin R (43)



Coagulin S (44)

Chapter: 3

RESULTS AND DISCUSSION (PART A)

3.1: Withanolides isolated from *Withania coagulans*

Six new and ten known withanolides have been isolated from *W coagulans* of Pakistani origin. Various experimental techniques and extensive spectroscopic studies were used for the structural elucidation of these compounds. Most of the isolated withanolides showed inhibition activity on lipopolysaccharide (LPS) induced B and Concanavalin A (ConA)-induced T cell proliferation. The results of this study are discussed in this chapter. The extraction and isolation procedures are discussed in detail in the experimental section (Chp.4; Sec.4.2).

The aerial parts of *W. cogulans* were collected from the Khyber agency area near Peshawar during August 2006. The dried powdered plant materials (5 kg) were extracted with ethanol. The ethanolic extract was then filtered and concentrated under vacuum to give dark residue (850 g) after evaporation. The residue was subjected to polyamide CC (EtOH/H_2O, 3:7 & 7:3) to provide two fractions (A & B). These fractions were further fractionated on silica gel CC and then subjected to RP-18 CC to obtained sixteen pure compounds. Compounds **(45-50)** were identified as new withanolides and compound **(51-60)** were proved as reported withanolides.

3.1.1: New Withanolides isolated from *Withania coagulans*

3.1.1.1: Withacoagulin A (45)

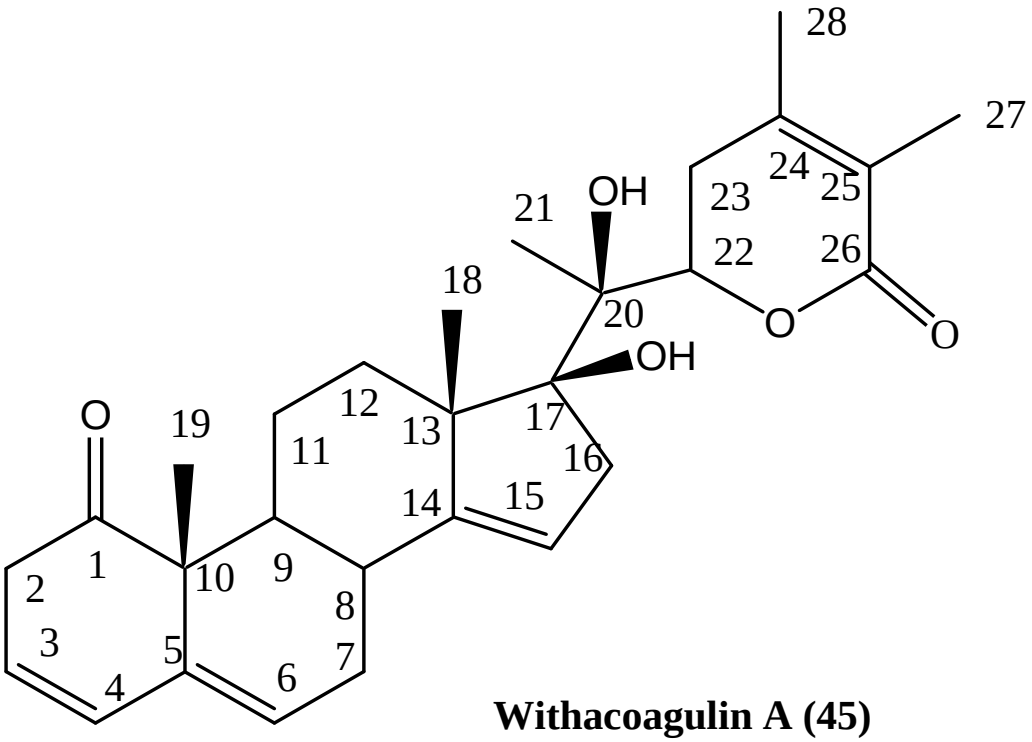
Fraction A was loaded on silica gel CC (hexane/acetone 15:1, 10:1, 5:1, 2:1, and 1:1) to provide six sub-fractions (A1-6). Sub-fraction A1 was further subjected to RP-18 CC (MeOH/water 6.50:3.50), yielging an optically active colorless solid (**45**, 23 mg). Fractionation scheme is given in experimental section (Fig. 4.1). The UV spectrum showed a characteristic absorption at 221nm, indicating α,β-usaturated lactone chromophore [250]. The bands at 1684 and 1712cm^{-1} in IR spectrum indicating α,β-unsaturated lactone and six-membered cyclic ketone functionalities. [176,250]. The molecular formula was established as $C_{28}H_{36}O_5$ by its HR-ESI-MS from the $[M+Na]^+$ and $[M+HCOO]^-$ signals at *m/z* 475.2455 (positive, calc. 475.2460) and 497.2542

(negative mode, calc.497.2539) respectively.

The ^{I}H NMR and ^{13}C NMR spectra of **(45)** showed characteristic peaks (Table 3.1) for the steroidal structure of withanolides[132,135]. In the ^{I}H NMR, five methyl peaks (δ1.19, 1.30, 1.35, 1.87, 1.94) were observed due to the protons H_3C (l8), H_3C (21), H_3C (l9), H_3C (27) and H_3C (28) respectively. The lowfieled chemical shifts of the C-27 and C-28 methyl singlets indicated that they both are substituted on a double bond. These two methyl and the characteristic H-22 signal δ 4.65 (dd, *J* = 13.1, 3.4 Hz) showed the presence of the α,β-unsaturated lactone moiety typical for withanolides. The C-21 methyl singlet and the multiplicity of H-22 *(dd)* suggested that C-20 should be a quaternary carbon. In the low field of ^{I}H NMR, two mutually coupled olefinic protons at δ 5.61 and 6.05 were assigned to vicinal protons (H-3) and (H-4) respectively. The olefinic signal resonating at δ 5.66 showing 3*J* couplings to carbons at δ 30.5 (C-8) and 52.1 (C-10) in the HMBC spectrum was assigned to the olefinic proton of C-6, while another olefinic signal resonating at δ 5.25 displaying 3*J* correlations to carbons at δ 53.6 (C-13) and 88.0 (C-17) in the HMBC spectrum was attributed to the olefinic proton H-C (15).

The ^{I3}C and DEPT NMR spectral data (Table 3.1) of compound **(45)** disclosed 28 carbons, including five methyls, six CH_2, seven CH and ten quaternary carbons. The downfield signal at δ 210.2 was due to C-l ketonic function, while the signal at δ 165.5 was attributed to C-26 of the lactone moeity. The peaks at olefinic region δ 117.0, 121.4, 126.5 and 129.1 were due to the unsaturated carbons C-15, C-3, C-6 and C-4, respectively. The signals at δ 121.0, 140.1, 150.3 and 151.1 were assigned to th**e** quaternary unsaturated carbons C-26, C-5, C-25 and C-14, respectively. The signals at δ 75.8, 80.2 and 88.0 were assigned to oxygen containing carbons C-20, C-22 and C-17 respectively. The above chemical shift assignments were confirmed by HMBC data (Fig.3.1).

Further information was also obtained from HMBC spectrum (Fig. 3.1). For instance, the H-2 (δ 2.75, 3.29) showed correlations with the carbon resonating at δ 210.2 (C-l), 121.4 (C-3) and 129.1 (C-4), while (H-7) (δ 2.03, 2.40) displayed couplings with the carbons at δ 140.1 (C-5) and 126.5 (C-7). Similarly, the H-16 (δ 2.24, 2.98) showed interaction with the carbon resonating at δ 151.1 (C-14) and 117.0 (C-15), while H-C(23) (δ 2.36, 2.64) displayed correlations with the carbons at δ 75.8 (C-20) and 121.0 (C-25). All of the HMBC data further confirmed the structure of **45**.



Withacoagulin A (45)

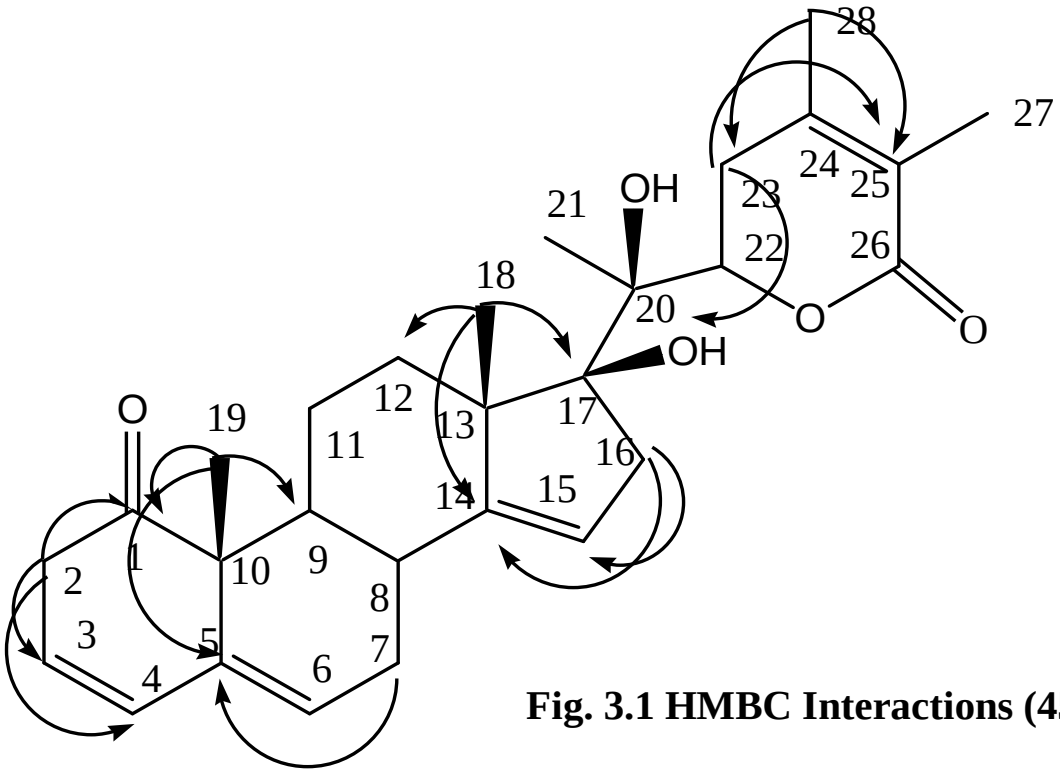


Fig. 3.1 HMBC Interactions (45)

Table-3.1: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound (45) in CDCl3

C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR(δ) Coupling Constants J_{HH} (Hz)[cd]
1	210.2	C	-
2	39.5	CH_2	2.75 (dd, *J2a,2b* = 20.2, *J2a,3* =4.5)
			3.29 (dd, *J2a,2b* = 20.1, *J2b,3* = 2.5)
3	121.4	CH	5.61 – 6.64 (*m*)
4	129.1	CH	6.05 (d, *J4,3*= 8.1)
5	140.1	C	-
6	126.5	CH	5.66 (dd, *J6,7a* = 5.3, *J6,7b* = 2.0)
7	28.9	CH_2	2.01 – 2.05 (m)
			2.39 – 2.42 (*m*)
8	30.5	CH	2.46 – 2.50 (*m*)
9	39.5	CH	1.78 – 1.81 (*m*)
10	52.1	C	-
11	22.8	CH_2	1.39 – 1.43 (*m*)
			1.82 – 1.85 (*m*)
12	30.4	CH_2	1.54 – 1.58 (*m*)
			2.20 – 2.24 (*m*)
13	53.6	C	-
14	151.1	C	-
15	117.0	CH	5.52 (*br. s*)
16	39.8	CH_2	2.22 – 2.26 (*m*)
			2.98 (dd, *J16a, 16b* = 17.4, *J16a,15* = 3.1)
17	88.0	CH	-
18	21.1	CH_3	1.19 (*s*)
19	19.9	CH_3	1.35 (*s*)
20	75.8	C	-
21	19.4	CH_3	1.30 (*s*)
22	80.2	CH	4.65 (dd, *J22, 23a* = 13.1, *J22, 23b* = 3.4)
23	31.8	CH_2	2.35 – 2.37 (*m*)
			2.62 – 2.66 (*m*)
24	150.3	C	-
25	121.0	C	-
26	165.5	C	-
27	12.2	CH_3	1.87 (*s*)
28	20.5	CH_3	1.94 (*s*)

a: Broad band; b: DEPT; c: ^{1}H NMR; d: HMQC interaction

It has been reported that when C-22 has (S)-configuration then CH (22) resonated as a broad singlet with $W_{I/2}$≈5 Hz, while in the (22R)-isomer, it appeared as a *dd* with two coupling constants characteristic for axial-axial and axial-equatorial interactions with protons of C-23[195]. In the case of compound **(45),** HC (22) resonated as a double doublet *(J* = 13.1, 3.4 Hz), revealing the R-configuration at C-22. The β-configuration of OH-C (17) could be deduced from the characteristic pyridine-induced downfield shift for both Me-18 and Me-21, as had been observed with the 17-hydroxywithanolides[116,251]. Based on these observations, the structure 17 β, 20 β - dihydroxy-l-oxo-(20S, 22R)-witha-3,5,14,24-tetraenolide was assigned to compound **(45),** which was named withacoagulin A.

Withacoagulin A **(45)** showed relatively good activities (IC_{50}< 20 μM) on the inhibition of both on lipopolysaccharide (LPS) induced B and oncanavalin A (ConA)-induced T cell proliferation (Sec.11.7)

3.1.1.2: Withacoagulin B (46)

Sub-fraction A4 was further subjected to reverse phase chromatography (RP-18 CC) and eluted with MeOH/H_2O (5.7:4.3), resultin in compound **(46),** an optically active amorphous powder (24 mg). Fractionation scheme is given in experimental section (Fig. 4.1) A characteristic absorption of α,β-usaturated lactone chromophore at 224nm was observed in UV spectrum [250]. The bands at 1684 and 1712cm^{-1} in IR spectrum indicated α,β-unsaturated lactone and six-membered cyclic ketone functionalities. [176,250]. The molecular formula was established as $C_{28}H_{36}O_5$ by its HR-ESI-MS from the $[M+Na]^+$ and $[M+HCOO]^-$ signals at *m/z* 475.2455 (positive, calc. 475.2460) and 497.2542 (negative mode, calc.497.2539), respectively.

The ^{I}H and ^{I3}C NMR spectral data (Table 3.2) of compound **46** were close to those of withacoagulin **51** [85]. The NMR spectra indicated that the main difference between them was found in ring A. The ring A of **(51)** contains a 2,5-diene-1-one system, while the spectra of ring A in **(46)** was characteristic of the 3,5-diene-1-one system of withanolides

The ^{1}H-NMR spectrum (Table 3.2) of compound **46** showed four methyl singlets at δ 1.40 (C-18H), 1.36 (C-19H), 1.45 (C-21H) and 1.92 (C-28H). The singlet at C-21 methyl indicated that the adjacent carbon C-20 has no proton. The lowfield chemical shift (δ 1.45) of C-21 methyl suggested that an oxygen function may be present on C-

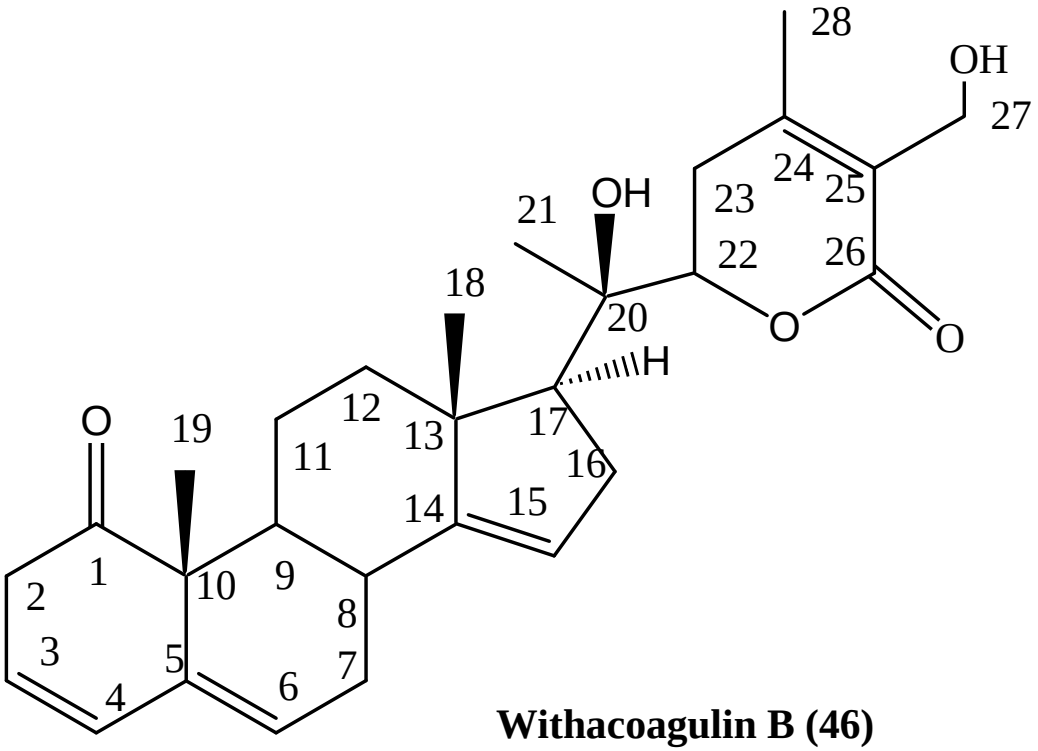
20. The lowfieled chemical shifts of the C-27 and C-28 methyl singlets indicated that they both are substituted on a double bond. The downfield signals at δ 5.58 (d, *J3, 4* = 9.2Hz), 6.08 (d, *J3, 4* = 9.2Hz), 5.67 (*br, s*) and 5.25 (*br, s*) represented four vinylic protons H-3, H-4, H-6 and H-15 respectively. A downfield methine doublet of doublet at δ 4.49 (*J22, 23a*=13.6 Hz, *J22, 23b*=3.3Hz) was attributed to the proton of lactone moiety at C-22.

The ^{13}C NMR and DEPT spectra of compound **46** (Table 3.2) indicated that there are 28 carbons resonance including four methyl (C-18, C-19, C-21 & C-28), seven CH_2 (C-2, C-7, C-11, C-12, C-16, C-23 & C-27), eight CH (C-3, C-4, C-6, C-8, C-9, C-15, C-17, & C-22) and nine quaternary carbons (C-1, C-5, C-10, C-13, C-14, C-20, C-24, C-25 & C-26). The lowfield peaks at δ166.1 (C-26) and 209.8 (C-1) were due to lactone carbonyl and ketone carbons respectively. The olefinic signals at δ 122.3, 129.4, 126.9 & 118.8 were assigned to unsaturated methine carbons (C-3, C-4, C-6 & C-15 respectively),while the peaks at δ 140.7, 153.2, 154.0 and 127.2 were due to the quaternary usaturated carbons (C-5, C-1, 4 C-24 and C-25 respectively). The peak at δ 56.0 was assigned to oxygen containing CH_2 at C-27 and thus showed absence of methyl signal as in compound **45**. Similarly signal at δ 82.1 was assigned to the oxygen-bearing methine (C-22), while the peak appearing at δ 74.2 was assigned to quaternary carbon having hydroxyl group (C-20).

The methine protons at C-15H (δ 5.25) showed correlations with C-13 (δ 48.2), C-17 (δ 57.7) and C-16 (δ 42.1) in HMBC spectrum. Similarly C-17H (δ 1.91) methine proton also showed HMBC corelation with the C-13, C-14 and C-16 (48.2, 153.2 &_ 42.0) respectively, which establishes the position of the double bond between C-14/C-15. The doublets at δ 4.73 and 4.87 (*J* = 11.7 Hz) for the C-27H hydroxymethylenic protons exhibited direct coupling with C-27 (δ 56.0). These protons also showed couplings with C-28 (δ 20.1) in the HMBC spectrum. The methyl protons at C-28H (δ 1.92) showed relation with C-24 olefinic (δ 154.0) and C-25 (δ 127.2), whereas hydroxymethylenic protons at C-27H (δ 4.73 and 4.82) have long-range correlation with the olefinic C-25 (δ 127.2). The entire above chemical shift assignments were confirmed by HMBC data (Fig. 3.2) and by comparing to the spectra of **(45)**. Compound **46,** was therefore assigned the structure 20β, 27-dihyd-roxy-l-oxo-(20R,22R)-witha-3, 5,14,24-tetraenolide and named as withacoagulin B.

Withacoagulin B **(46)** was found to be an inhibitior of both ConA-induced T cell proliferation (IC_{50} = 35.4 μM) and LPS-induced B cell proliferation (IC_{50} = 27.7 μM)

(Sec. 11.7).



Withacoagulin B (46)

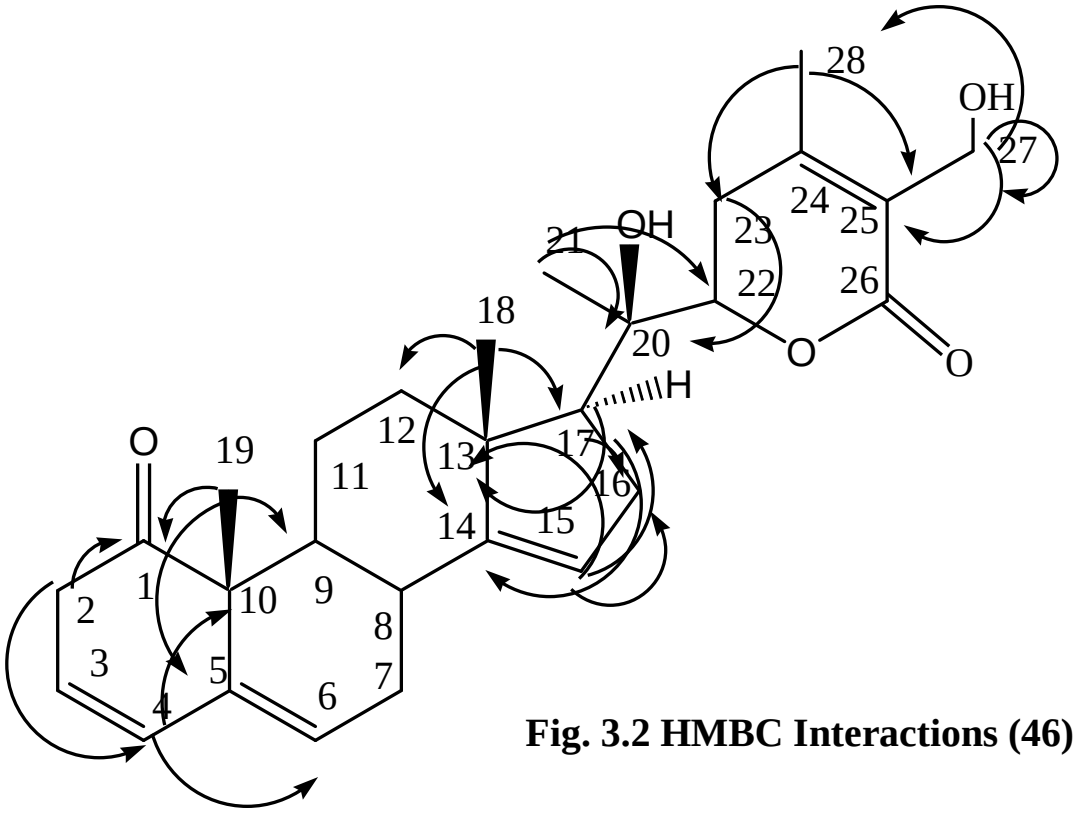


Fig. 3.2 HMBC Interactions (46)

Table-3.2: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound **(46)** in C_5D_5N

C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	209.8	C	-
2	39.9	CH_2	2.77 (*m*)
			3.29 (*d, J2b,3* = 20.1)
3	122.3	CH	5.58 (*d, J3,4 = 9.2)*
4	129.4	CH	6.08 (*d, J4,3 = 9.2)*
5	140.7	C	-
6	126.9	CH	5.67 (*br. s*)
7	29.3	CH_2	1.96 – 2.00 (*m*)
			2.25 – 2.29 (*m*)
8	30.5	CH	2.20 – 2.23 (*m*)
9	41.0	CH	2.01 – 2.04 (*m*)
10	52.6	C	-
11	23.6	CH_2	1.91 – 1.97 (2H, *m*)
12	30.9	CH_2	2.22 – 2.25 (*m*)
			2.79 – 2.82 (*m*)
13	48.2	C	-
14	153.2	C	-
15	118.8	CH	5.25 (*br. s*)
16	42.1	CH_2	1.54 – 1.58 (*m*)
			2.03 – 2.07 (*m*)
17	57.7	CH	-
18	19.4	CH_3	1.40 (*s*)
19	20.1	CH_3	1.36 (*s*)
20	74.2	C	-
21	20.8	CH_3	1.45 (*s*)
22	82.1	CH	4.49 (*dd, J22,23a* = 13.0, *J22,23b* = 3.2)
23	32.0	CH_2	2.30 – 2.34 (*m*)
			2.55 – 2.58 (*m*)
24	154.0	C	-
25	127.2	C	-
26	166.1	C	-
27	56.0	CH_2	4.73 (*d, J27a,27b* = 11.7)
			4.87 (*d, J*27b,27a = 11.7)
28	20.1	CH_3	1.92 (*s*)

a: Broad band; b: DEPT; c: ^{1}H NMR; d: HMQC interaction

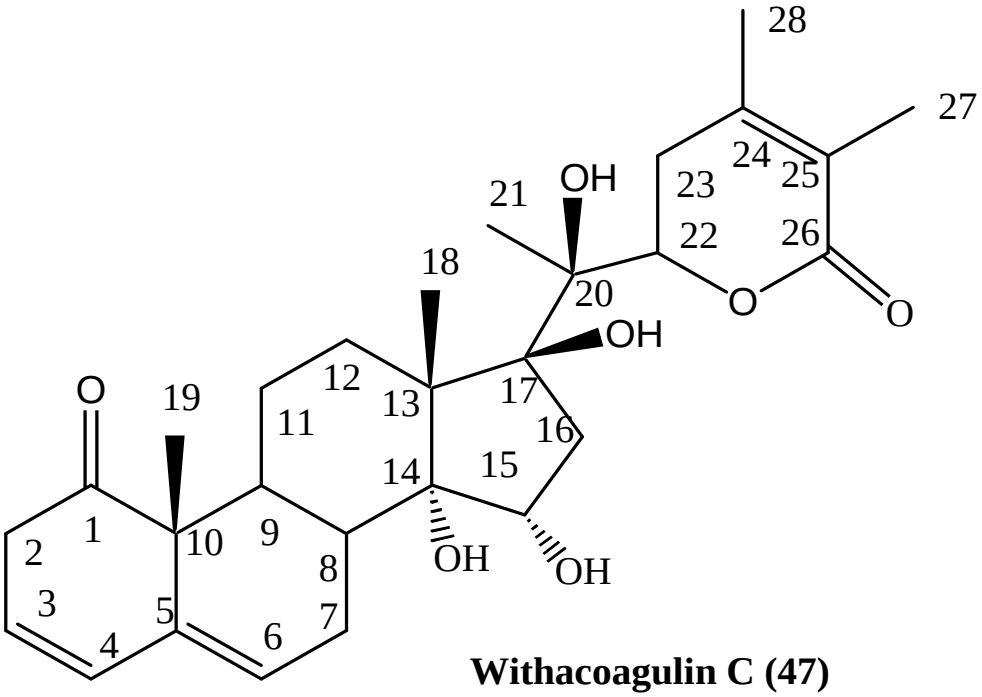
3.1.1.3: Withacoagulin C (47)

As fraction A was loaded on silica gel CC and eluted with hexane/acetone 15:1, 10:1, 5:1, 2:1, 1:1) to provide six sub-fraction (A1-6). Sub-fraction A5 was further loaded on RP-18 CC and eluted with MeOH/water (5.5:4.5) which afforded an optically active compound **(47)**. Details of isolation are given in experimental section (Sec. 4.2.2; Fig.4.1). A characteristic absorption at 222nm was shown by compound **47** in UV spectrum which indicating α,β-usaturated lactone chromophore [250]. The bands at 1684 and 1712cm^{-1} in IR spectrum indicating α,β-unsaturated lactone and six-membered cyclic ketone functionalities [176,250]. The molecular formula was established as $C_{28}H_{38}O_7$ by its HR-ESI-MS from the $[2M+Na]^+$ and $[M+HCOO]^-$ signals at *m/z* 995.5136 (positive, calc. 995.5132) and 531.2591 (negative mode, calc. 531.2594), respectively.

Its ^{1}H and ^{13}C NMR spectra (Table 3.3) were also similar to those of withacoagulin **51**[85]. The NMR spectra indicated that the chief difference between them was found in ring A and D. The ring A of **(51)** contains a 2,5-diene-1-one system, while the spectra of ring A in **(47)** was characteristic of the 3,5-diene-1-one system of withanolides. The absence of 14-en in ring D as well as sign of two more hydroxyl group in compound **(47)** showed further difference. The ^{1}H-NMR spectrum (Table 3.3) of compound (**47**) showed five methyl singlets at δ 1.96 (C-18H), 1.44 (C-19H), 1.82 (C-21H), 1.92 (C-27) and 1.71 (C-28H). The C-21 methyl gave a singlet at δ 1.82 which indicated that the adjucent C-20 has no proton. The downfield chemical shifts of the C-28H and C-29H (δ 1.92 & 1.71) methyl singlets were the sign of their substitution on a double bond. The lowfield shift (δ 1.82) of C-21 methyl showed that oxygen functionality may be present on C-20 and hence indicating the lactone moiety. Three downfield signals at δ 5.59 (*m*), 6.08 (d, *J3,4* = 19.6Hz) and 5.71 (*m*) represented three protons of olefinic nature (H-3, H-4 and H-6) respectively. The peak at δ 4.49 (dd, *J22, 23a* = 13.2 Hz, *J22, 23b* = 3.2Hz) was assigned to the CH (methine proton) of lactone moiety at C-22. The downfield signal (δ 4.51) at C-15 and no proton at C-14 is the sign of substitution hydroxy group at C-14 and C-15 which is confirmed by ^{13}C NMR spectrum.

The ^{13}C NMR and DEPT spectra of compound **(47)** (Table 3.3) like other withanolide isolated, indicated that there are 28 carbons resonance including five

methyls (C-18, C-19, C-21, C-27 & C-28), six CH_2 (C-2, C-7, C-11, C-12, C-16 & C-



Withacoagulin C (47)

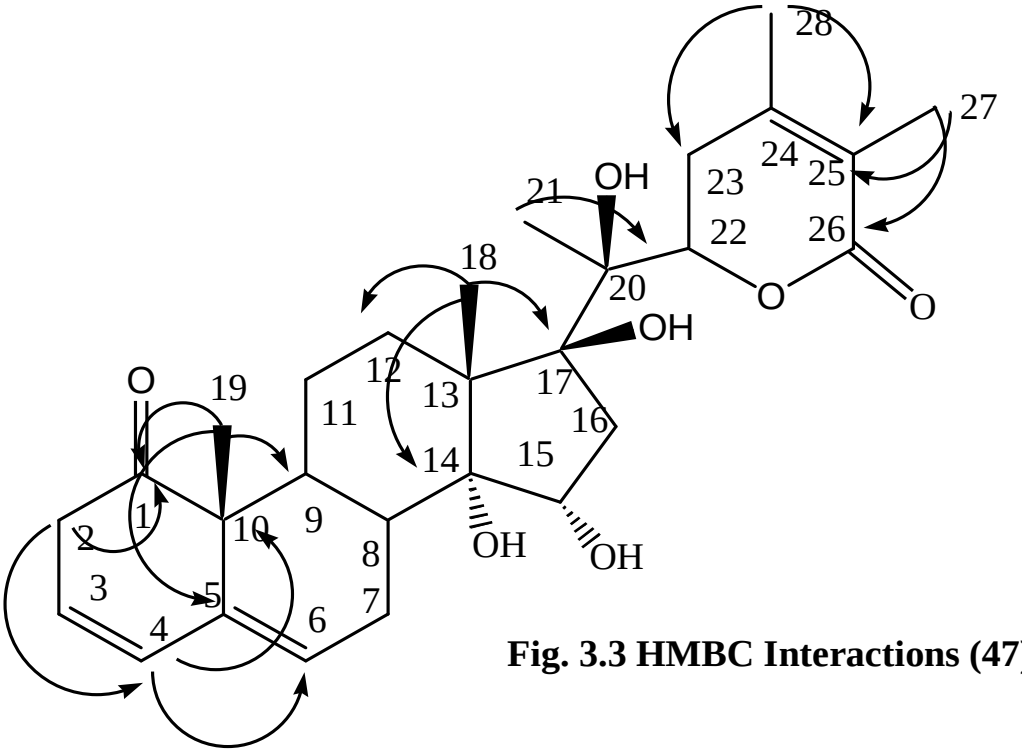


Fig. 3.3 HMBC Interactions (47)

23), seven CH (C-3, C-4, C-6, C-8, C-9, C-15, & C-22) and ten quaternary carbons (C-1, C-5, C-10, C-13, C-14, C-17, C-20, C-24, C-25 & C-26). The lowfield peaks at δ 166.8 (C-26) and 210.5 (C-1) were assigned to carbonyl carbons of lactone and ketone respectively. The downfield signal δ 82.8 and 89.2 were assigned to hydroxyl bearing quaternary carbons (C-14 & C-17 respectively) while δ 76.2 was assigned to carbon (C-15) having hydroxyl group and signal at δ 81.7 was assigned to the oxygen-bearing methine (C-22). While the peak appearing at δ 79.4 was assigned to quaternary carbon having hydroxyl group (C-20). The olefinic signals at δ 121.8, 129.7 & 128.9 were attributed to vinylic methine carbons (C-3, C-4 & C-6 respectively) in ring A & B, while the peaks at δ 141.2, 150.8 & 121.4 were assigned to the quaternary vinylic carbons (C-5, C-24 and C-25) respectively.

The C-2 protons (δ 2.24) of compound **(47)** showed correlation with carbon at δ 210.5 (C-1) and δ 129.7 (C-4) in HMBC spectrum. The C-4 proton (δ 6.05) in turn showed HMB correlation with carbon resonating at δ 128.7 (C-6) and 53.0 (C-10). The methyl protons at C-28 (δ 1.71) showed connectivities with C-24 olefinic (δ 150.8) and C-25 (δ 121.2),whereas methyl protons at C-27 (δ 1.92) exhibited long-range correlation with the olefinic C-25 (δ 121.2) and C-26(δ 166.8). The entire above chemical shift assignments were confirmed by HMBC data (Fig. 3.3). By comparing to the spectra of **45** and **46**, the structure of compound **(47)** was assigned as 14*α*,15*α*,17*β*,20*β*-tetrahydroxy-1-oxo-(20S,22R)-witha-3,5,24-trienolide and named as Withacoagulin C.

Withacoagulin C **(47)** showed relatively good activities on the inhibition of both ConA-induced T cell proliferation (IC_{50}< 20 μM) and LPS-induced B(IC_{50}< 22 μM) cell proliferation (Sec. 11.7).

Table-3.3: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound **(47)** in C_5D_5N

C.No	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	210.5	C	-
2	40.1	CH_2	2.7 (*m*)
			3.30 (d, *J2b,3* = 19.8)
3	121.8	CH	5.52 – 5.54 (*m*)
4	129.7	CH	6.05 (d, *J4,3* = 19.7)
5	141.2	C	-
6	128.7	CH	5.70 – 5.73 (*m*)
7	26.2	CH_2	2.61 – 2.64 (*m*)
			2.78 – 2.82 (*m*)
8	32.8	CH	2.76 – 2.80 (*m*)
9	34.6	CH	3.15 (dt, *J9,8* = 12.0, *J9,7* = 5.3)
10	53.0	C	-
11	22.4	CH_2	1.68 – 1.70 (*m*)
			2.20 – 2.23 (*m*)
12	32.8	CH_2	1.57 – 1.60 (*m*)
			2.92 – 2.97 (*m*)
13	54.6	C	-
14	82.8	C	-
15	76.2	CH	4.51 (d, *J15,16a* = 6.4)
16	48.1	CH_2	2.27 (d, *J16a,16b* = 15.6)
			3.56 (dd, *J16a,17* =15.5, *J16a,15* = 6.4)
17	89.2	CH	-
18	20.9	CH_3	1.96 (*s*)
19	20.2	CH_3	1.44 (*s*)
20	79.4	C	-
21	20.1	CH_3	1.82 (*s*)
22	81.7	CH	5.35 (dd, *J22,23a* = 13.2, *J22,23b* = 3.1)
23	35.2	CH_2	2.71 – 2.75 (*m*)
			3.00 – 3.03 (*m*)
24	150.8	C	-
25	121.4	C	-
26	166.8	C	-
27	12.5	CH_3	1.92 (*s*)
28	20.1	CH_3	1.71 (*s*)

a: Broad band; b: DEPT; c: ^{1}H NMR; d: HMQC interaction

3.1.1.4: Withacoagulin D (48)

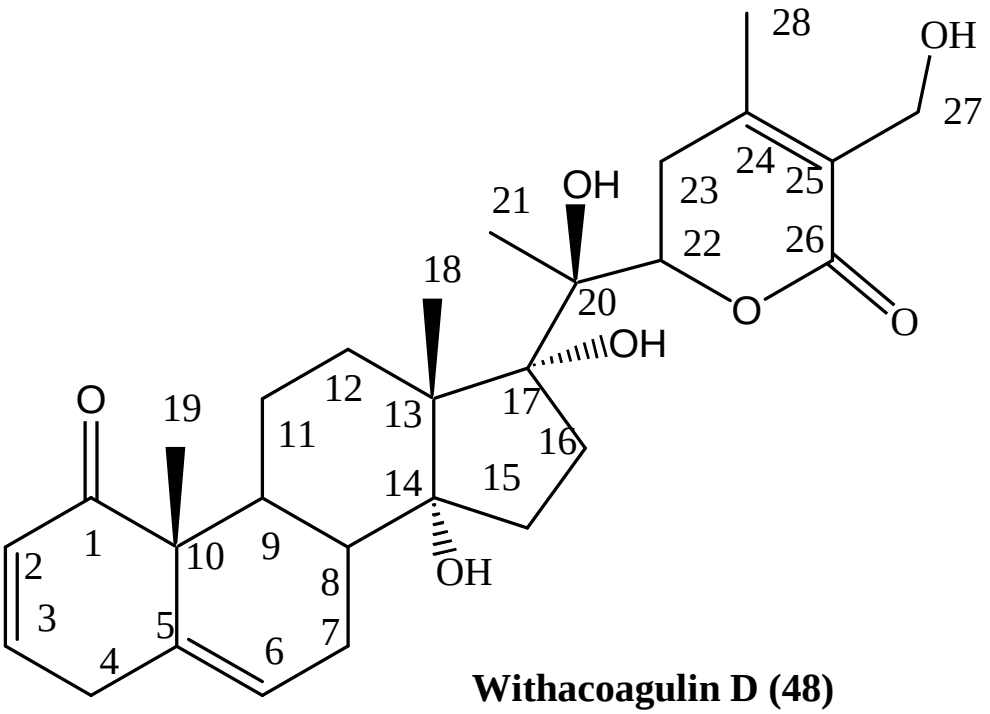
Fraction A was loaded on silica gel CC (hexane/acetone 15:1, 10:1, 5:1, 2:1 and 1:1) to provide six sub-fractions (A1-6). Compound **48** (18 mg) was purified as amorphous powder from Sub-fraction A5 by RP-18 CC (MeOH/H2O 5.5:4.5). Fractionation and isolation scheme is given in experimental section (Fig.4.1)

The UV spectrum showed a characteristic absorption at 221nm, indicating α,β-usaturated lactone chromophore[250]. The IR spectrum disclosed peaks at 3488 and 3419 cm^{-1} for hydroxyl group whereas 1654 and 1689 cm^{-1} indicating and unsaturated lactone and six-membered cyclic ketone respectively[176,250]. The molecular formula of the compound **(48)** was established as $C_{28}H_{38}O_7$ by its HR-ESI-MS from the $[2M+Na]^+$ and $[M-H]^-$ signals at *m/z* 995.6131 (positive, calc. 995.5132) and 485.2540 (negative mode, calc. 485.2539), respectively.

The ^{I}H and ^{I3}C NMR spectra (Table 3.4) of compound **(48)** were also showed similarities with those of withacoagulin **51**[85]. The NMR spectra indicated that the main difference between them was the absence of 14-en and addition of two more hydroxy groups in compound **48**. Like **51** the H-NMR spectrum (Table 3.4) of compound **48** also showed four methyl singlets at δ 1.46 (C-18H), 1.24 (C-19H), 1.56 (C-21H) and 1.97 (C-28H). The singlet at C-21 methyl is the sign of no proton on the neighboring C-20. The signal of C-21 methyl at δ 1.56 suggested that oxygen of lactone moiety is present on the adjacent carbon, C-20. The chemical shifts (δ 1.92) of the C-28 methyl singlets indicated it is substituted on unsaturated carbon. Three downfield signals at δ 5.98 (dd, *J* 2, 3 = 10.1, 2,4 = 1.8 Hz), 6.68 (ddd, *J*2,3 =10.1 Hz, *J*2,4a = 4.7 Hz, *J*2,4b = 2.3 Hz) and 5.53 (d *J6,7* = 5.6 Hz) were due to three olefinic nature protons H-2, H-3 andH-6 respectively. The peak at δ 4.49 (dd, *J*22,23a = 13.2 Hz, *J*22, 23b = 3.2Hz) was assigned to the CH (methine proton) of lactone moiety at C-22.

Similarly the ^{13}C NMR and DEPT spectra of compound **48** (Table 3.4) indicated that there are 28 carbon resonances including four methyl (C-18, C-19, C-21 & C-28), eight CH_2 (C-4, C-7, C-11, C-12, C-15, C-16, C-23 & C-27), six CH (C-2, C-3, C-6, C-8, C-9, C-15, & C-22) and ten quaternary carbons (C-1, C-5, C-10, C-13, C-14, C-17, C-20, C-24, C-25 & C-26). The lowfield peaks at δ 204.8 (C-1) and 166.4 (C-26) were due to ketone and lactone carbonyl carbons respectively. The olefinic signals at δ 127.9, 145.7 & 125.2 were assigned to unsaturated carbons (C-2, C-3 &

C-6respectively),while the peaks at δ 134.9, 155.0 and 126.7 were assigned to



Withacoagulin D (48)

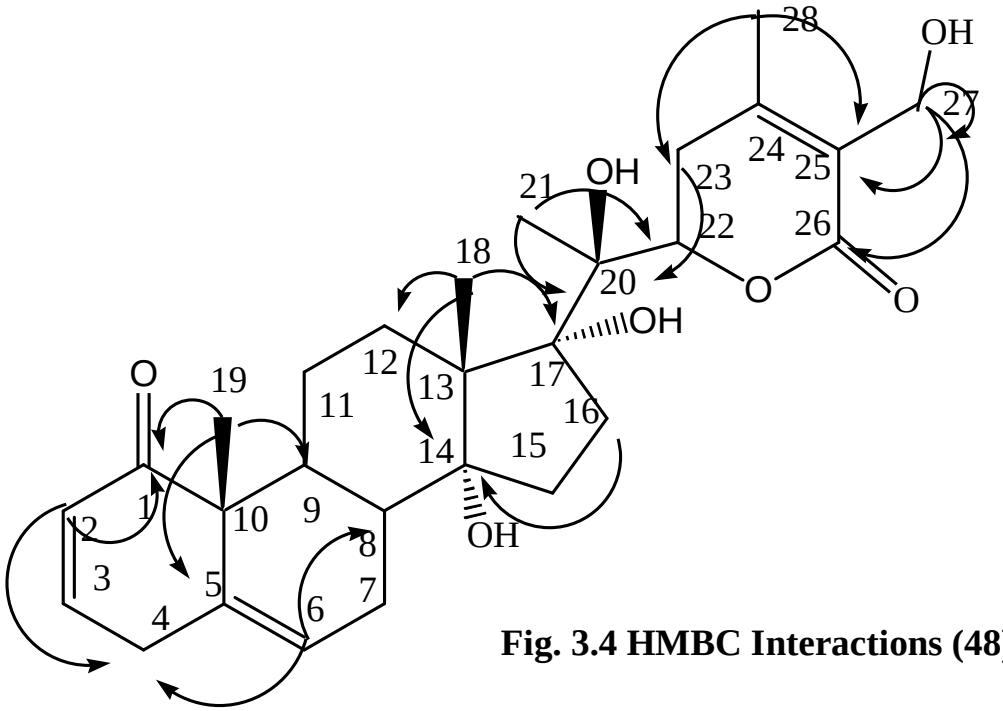


Fig. 3.4 HMBC Interactions (48)

the quaternary carbons (C-5, C-24 and C-25) respectively. The signal at δ 56 was assigned to methylene at C-27 next to oxygen and hence showed absence of methyl signal as in compound **(47)**. Similarly signal at δ 80.9 was due to the methine (C-22) having oxygen, while the peak appearing at δ 77.7 was assigned to quaternary carbon having hydroxyl group (C-20).

The C-2 proton (δ 5.98) of compound **48** showed correlation with carbon at δ 210.5(C-1) and 33.6 (C-4) in HMBC spectrum. The C-6 proton (δ 5.53) in turn showed HMBC correlation with carbon resonating at δ 33.6 (C-4) and 36.6 (C-8). The methyl protons at C-28H (δ 1.93) showed correlation with C-24 olefinic (δ 155.0) and C-25 (δ 126.7), whereas hydroxymethylenic protons at C-27 (δ 4.68 and 4.80) exhibited long-range HMBC corelation with the olefinic C-25 (δ 126.7). The entire above chemical shift assignments were confirmed by HMBC data (Fig. 3.4). By comparing to the spectra of **(45)** and **(46)**, the structure of **(48)** was identified as 14*α*,17*β*,20*β*,27-tetrahydroxy-1-oxo-(20S,22R)-witha-2,5,24-trienolide and named as withacoagulin D.

Withacoagulin D **(48)** was found good inhibitor of both ConA-induced T cell (IC_{50}< 20 μM) and LPS-induced B (IC_{50}< 22 μM) cell proliferation (Sec. 11.7).

3.1.1.5:. Withacoagulin E (49)

Sub-fraction A1 was subjected to reverse phase chromatography (RP-18 CC) and eluted with (MeOH/water 6.5:3.5), resulting in compound **(49)**, an optically active amorphous powder (31 mg). Fractionation scheme is given in experimental section (Fig. 4.1).

The UV indicating the presence of α,β-usaturated lactone chromophore by displaying absorption at 224 as mentioned above. The IR spectrum displayed bands at 1689 cm^{-1} indicated six-membered cyclic ketone. The molecular formula was determined as $C_{28}H_{38}O_5$ by its HR-ESI-MS from the $[2M+Na]^+$ and $[M+HCOO]^-$ signals at *m/z* 931.5331 (positive, calc. 931.5336) and 499.2683 (negative mode, calc. 499.2695), respectively.

Its ^1H and ^{13}C NMR spectra (Tables 3.5) were close to those of Withanolide G [252]. The only difference in ^{13}C NMR spectra was the appearance of carbon resonatings at δ 41.9 (C-12) and 55.2 (C-17) of **(49)** instead of resonating at δ_C 32.5 (C-12) and 49.4 (C-17) as observed in case of Withanolide G

Table-3.4: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound **(48)** in C_5D_5N

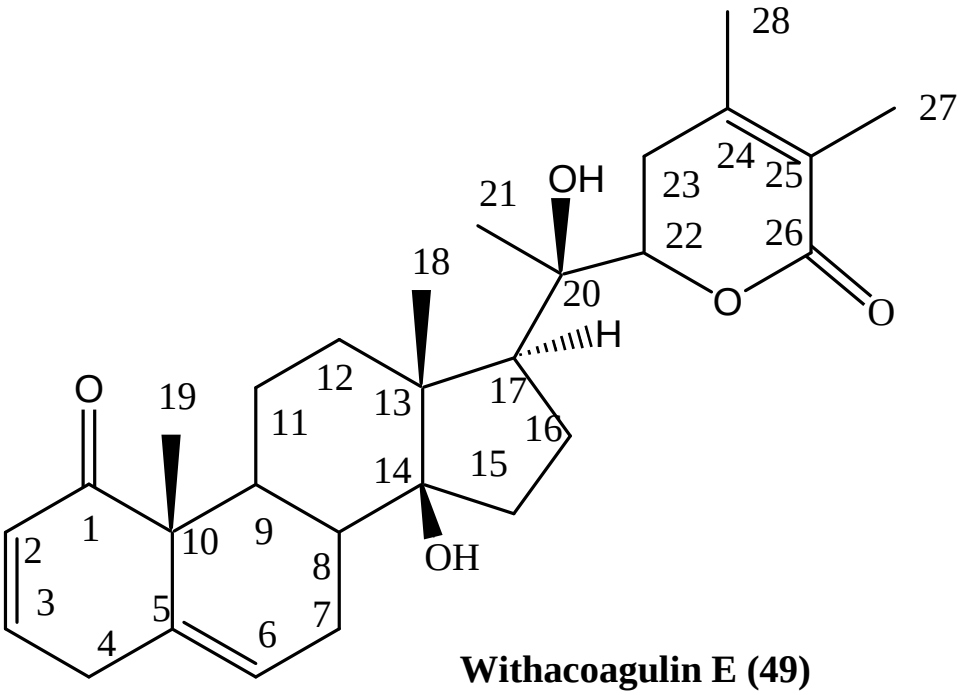
C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	204.0	C	-
2	127.9	CH	5.98 (dd, *J2,3* = 10.0, *J2,4* = 1.9)
3	145.7	CH	6.68 (ddd, *J3, 2*= 10.0, *J3,4a*= 4.8, *J3,4 b* = 2.3)
4	33.6	CH_2	2.66 – 2.75 (*m*)
			3.22 (d, *J4,3* = 21)
5	134.9	C	-
6	125.2	CH	5.53 (d, *J6,7* = 5.6)
7	25.4	CH_2	1.80 – 1.84 (*m*)
			2.36 – 2.40 (*m*)
8	36.6	CH	1.91 – 1.94 (*m*)
9	36.5	CH	2.73 – 2.77 (*m*)
10	51.2	C	-
11	22.7	CH_2	1.85 – 1.87 (*m*)
			2.62 – 2.66 (*m*)
12	27.6	CH_2	1.80 – 1.84 (*m*)
			2.80 – 2.83 (*m*)
13	52.0	C	-
14	86.1	C	-
15	33.6	CH_2	1.71 – 1.75 (*m*)
			2.00 –2.03 (*m*
16	34.5	CH_2	2.30 – 2.34 (*m*)
			3.36 (t, *J15,16* = 12.8)
17	88.4	C	-
18	19.0	CH_3	1.46 (*s*)
19	18.9	CH_3	1.24 (*s*)
20	77.7	C	-
21	19.9	CH_3	1.56 (*s*)
22	80.9	CH	5.12 (dd, *J22, 23a* =12.4, *J22, 23b* = 4.0)
23	33.0	CH_2	2.61-2.77 (2H, *m*)
24	155.0	C	-
25	126.7	C	-
26	166.4	C	-
27	56.0	CH_2	4.68 (*d*, 11.7)
			4.80 (*d*, 11.7)
28	20.0	CH_3	1.93 – 1.97 (*m*)

a: Broad band; b: DEPT; c: ^{1}H NMR; d: HMQC interaction

The carbon shift of C-14 at δ 83.8 indicated a hydroxyl quaternary carbon. Since a 14α-OH group would shield C-12 through a γ effect while a 14β-OH did not have such an effect[206], the lack of shielding of C-12 in compound **(49)** and by camparing the carbon shifts in 14-unsubstituted withanolides and 14β-OH substituted withanolides[206], indicated a 14β-OH group. The β-orientation of 14-OH was confirmed by pyridine-induced solvent shifts of C-18 methyl group. In compound **(49)**, there was a strong solvent effect on the signal of H-18 (δ 1.60 in pyridine solvent and δ 1.31 in $CDCl_3$). Such effects can be explained only by assuming a β-orientation of the 14-OH group. The chemical shift of C-17 (δ 55.2) was very near to that of 14β-OH withanolides [206].

The H-NMR spectrum (Table 3.5) of compound **49** showed like **47** five methyls singlets at δ 1.31 (C-18H), 1.22 (C-19H), 1.43 (C-21H), 1.88 (C-27) and 1.94 (C-28H). C-21 methyl displayed singlet at δ 1.43 which is the sign of no proton on neighboring carbon (C-20) and hence confirmed by ^{13}C NMR. The chemical shifts of the C-27H and C-28H (δ 1.88, 1.94) methyl singlets indicated the methyl groups attached to the olifinic functionality. The lowfield shift (δ 1.43) of C-21 methyl is the sign of an oxygen function present on C-20. Three downfield signals at δ 5.98 (dd, *J2,3* = 10.1 Hz, *J2,4* = 1.8Hz), 6.68 (ddd, *J*2,3 =10.1 Hz, *J*2,4a = 4.7 Hz, *J*2,4b = 2.3 Hz) and 5.53 (d *J6,7* = 5.6 Hz) were due to three protons H-2, H-3 and H-6 respectively. The peak at δ 4.49 (dd, *J*22, 23a = 13.2 Hz, *J*22, 23b = 3.2 Hz) was assigned to the CH (methine proton) of lactone moiety at C-22. The downfield signal (δ 1.55) at C-15 and no proton at C-14 is the sign of substituent like hydroxy group which is also confirmed by ^{13}C NMR.

The ^{13}C NMR spectra including broad band and DEPT spectra of compound **(49)** (Table 3.5) like other withanolide isolated, indicated that there are 28 carbon resonances including five CH_3 (C-18, C-19, C-21, C-27 & C-28), seven CH_2 (C-4, C-7, C-11, C-12, C-15, C-16 & C-23), seven CH (C-2, C-3, C-6, C-8, C-9, C-17, & C-22) and nine quaternary carbons (C-1, C-5, C-10, C-13, C-14, C-20, C-24, C-25 & C-26). The lowfield peaks at δ 204.5(C-1) and δ 166.0 (C-26) were due to the carbonyl carbons of ketone and lactone respectively. The downfield signal δ 83.8 and 75.5 were attributed to hydroxyl bearing quaternary carbon (C-14 & C-20) respectively while signal at δ. 81.1 was assigned to the oxygen-bearing methine (C-22)



Withacoagulin E (49)

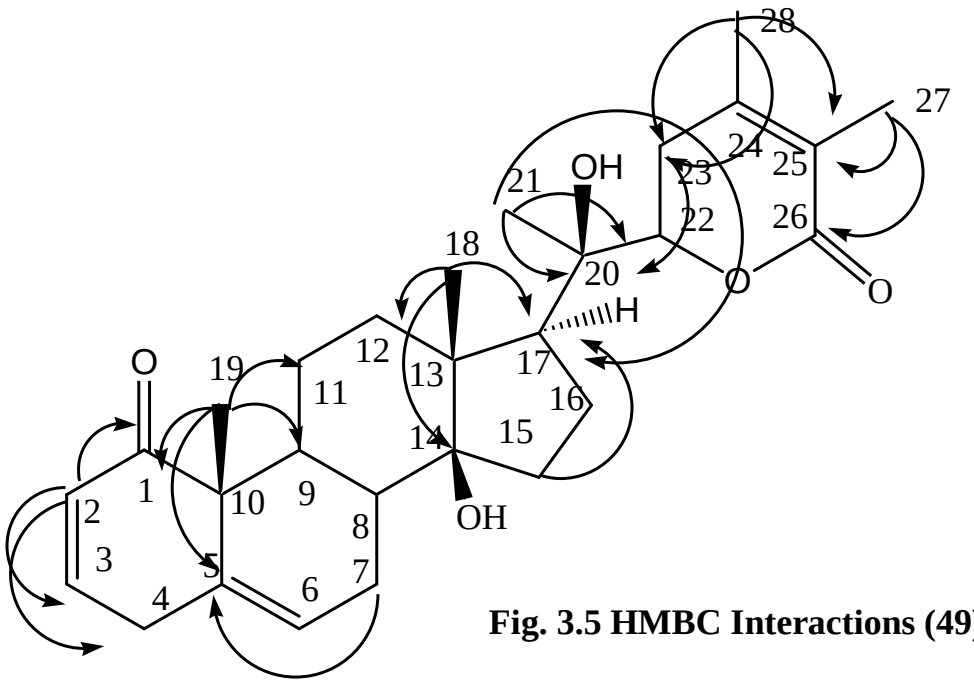


Fig. 3.5 HMBC Interactions (49)

The downfield resonance at δ 127.7, 145.7 & 125.5 were attributed to unsaturated carbons (C-2, C-3 & C-6) in ring A & B, while the peaks at δ 134.3, 148.8 & 121.9 were assigned to the quaternary carbons (C-5, C-24 and C-25) respectively. The methyl proton appearing at δ 1.31(C-18) exhibited coupling with C-12 (41.9) and C-17 (δ 55.2) as well as long range with C-14 appear at δ 83.8. The entire above chemical shift assignments were confirmed by HMBC data (Fig. 3.5). The C-4 proton (δ 2.24) of compound **(49)** showed correlation with carbon appearing at δ 127.7(C-3) and in long range with carbon resonating at δ 129.7(C-4) in HMBC spectrum. The methyl proton appear at 1.22 (C-19) showed correlation with carbons resonating at δ 204.5,38.4 & 23.3(C-1,C-9 & C-11 respectively) as well as in long range exhibited connectivity with quaternary carbon (C-5) resonating at δ 134.3. The HMBC spectrum also showed heteronuclear correlation of methyl protons of C-28 (δ 1.94) with olefinic carbon C-24(δ 148.8) and C-23 (δ 31.7), whereas methyl protons at C-27 (δ 1.88) exhibited long-range heteronuclear connectivity with the olefinic C-25 (δ 121.9) and C-26 (δ 166.4). Hereby, compound **49** was assigned the structure 14*β*,20*β*-dihydroxy-1-oxo-(20*R*,22*R*)-witha-2,5,24-trienolide and named withacoagulin E. Withacoagulin E **(49)** also showed good inhibitory activities of both ConA-induced T (IC_{50}< 20 μM) and LPS-induced B (IC_{50}< 23 μM) cell proliferation (Sec. 11.7)

3.1.1.6: Withacoagulin F (50)

Fraction A was loaded on silica gel CC and eluted with petroleum ether/acetone (15:1, 10:1, 5:1, 2:1, 1:1) yielding six sub-fraction (A1-6). Sub-fraction A3 was further purified with RP-18 CC (MeOH/water 5.5:4.5), resulted in a colorless compound **50** (35 mg). Fractionation scheme is given in Experimental section (Fig. 4.1). The UV spectrum showed a characteristic absorption at 220 nm, indicating α, β-usaturated lactone chromophore as mentioned earlier. The molecular formula was founded as $C_{28}H_{38}O_5$ by its HR-ESI-MS from the $[2M+Na]^+$ and $[M+HCOO]^-$ signals at *m/z* 931.5344 (positive, calc. 931.5336) and 499.2690 (negative mode, calc. 499.2695), respectively.

The ^1H and ^{13}C NMR spectra (Tables 3.6) were much closed to those of **(49)**. The NMR spectra indicated that the main difference between them was observed in the ring A. The spectra of ring A in **(50)** were characteristic for the 3,5-diene-1-one system of withanolides. In the ^{13}C NMR of **(50)**, the carbon resonating at δ 210.1 (C-

Table-3.5: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound **(49)** in $CDCl_3$

C.No.	^{13}C NMR(δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR(δ) Coupling Constants J_{HH} (Hz)[cd]
1	204.5	C	-
2	127.7	CH	5.85 (dd,*J2,3* = 10.0, *J2,4*= 2.1)
3	145.7	CH	6.79 (ddd, *J3,* = 10.0, *J3,4a*= 4.9, *J3,4b*= 2.4)
4	33.3	CH_2	2.83 (dd, *d,J4,3* = 11.4, *J4,2* = 5.0)
			3.29 (dd, *J4,3* = 11.2, *J4,2* = 2.3)
5	134.3	C	-
6	125.5	CH	5.61 (d, *J6,7*= 6.2)
7	26.3	CH_2	1.76 – 1.80 (*m*)
			2.25 – 2.29 (*m*)
8	37.0	CH	1.75 – 1.77 (*m*)
9	38.4	CH	1.90 – 1.93 (*m*)
10	50.8	C	-
11	23.2	CH_2	1.48 – 1.51 (*m*)
			2.02 – 2.06 (*m*)
12	41.9	CH_2	1.46 – 1.50 (2H, *m*)
13	48.8	C	-
14	83.8	C	-
15	32.5	CH_2	1.55 – 1.57 (*m*)
			1.91 – 1.96 (*m*)
16	21.8	CH_2	1.65 – 1.68 (*m*)
			1.90 – 1.95 (*m*)
17	55.3	CH	1.62 – 1.65 (*m*)
18	17.9	CH_3	1.31 (*s*)
19	19.0	CH_3	1.22 (*s*)
20	75.5	C	-
21	21.5	CH_3	1.43 (*s*)
22	81.1	CH	4.47 (dd, *J22, 23a*=13.3, *J22,2 3b* = 3.4)
23	31.7	CH_2	2.10 – 2.14 (*m*)
			2.34 – 2.38 (*m*)
24	148.8	C	-
25	121.9	C	-
26	166.0	C	-
27	12.4	CH_3	1.88 (*s*)
28	20.5	CH_3	1.94 (*s*)

a: Broad band; b: DEPT; c: ^{1}H NMR; d: HMQC interaction

1) indicated a saturated carbonyl carbon. The olefinic signals at δ 121.9 and δ 129.6 were assigned to C-3 and C-4 respectively

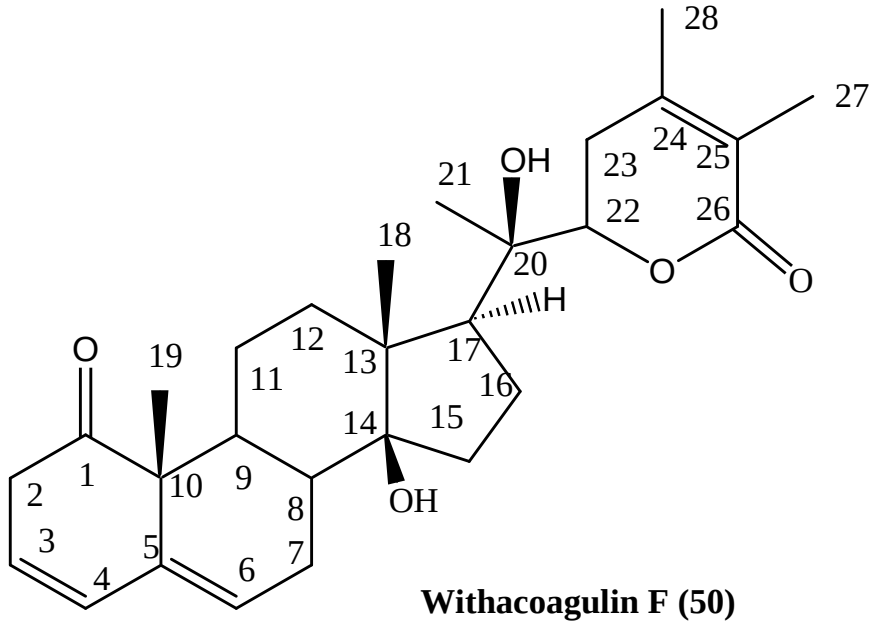
The H-NMR spectrum (Table 3.6) of compound **50** showed five methyl singlets at δ 1.56 (C-18H), 1.35 (C-19H), 1.56 (C-21H) 1.91 (C-27H) and 1.75 (C-28H) as observed in compound **(49)**. The singlet at C-21 methyl indicated that the C-20 has no proton and hence confirmed by ^{13}C NMR. The downfield signals of the C-27H and C-28H (δ 1.91, 1.75) methyl singlets indicated the methyl groups are attached to the olifinic functionality. The lowfield shift (δ 1.43) of C-21 methyl is the sign of an oxygen function present on C-20. Three downfield signals at δ 5.58 (*m*), 6.07 (d, *J*3, 4 = 9.5 Hz) and 5.66 (d, *J*6, 7 = 3.2) showed three vinylic protons H-2, H-3 and H-6 respectively. A downfield signal of doublet of doublet at δ 4.55 (*J*22, 23a = 13.2Hz, *J*22, 23b = 3.5 Hz) was assigned to the proton of lactone moiety at C-22. The signal (δ 1.70) at C-15H and no proton at C-14 is indicating the substitution of hydroxy group which is confirmed by ^{13}C NMR.

The ^{13}C NMR spectra including broad band and DEPT spectra of compound **50** (Table 3.6) is also similar to that of compound **(49)** and indicated that there are 28 carbons including methyl (C-18, C-19, C-21, C-27 & C-28), methylene (C-2, C-7, C-11, C-12, C-15, C-16 & C-23), methane (C-3, C-4, C-6, C-8, C-9,C-17, & C-22) and quaternary carbons (C-1, C-5, C-10, C-13, C-14, C-20, C-24, C-25 & C-26). The lowfield peaks at δ 210.5 (C-1) and δ 166.4 (C-26) were due to the carbonyl carbons of ketone and lactone respectively. The downfield signal δ 83.9 and 75.1 were atrributed to hydroxyl bearing carbon (C-14 & C-20 respectively), while signal at δ 82.1 was assigned to methine (C-22) having oxygen. The downfield signal at δ121.9, 129.6 & 128.3 were attributed to olefinic methine carbons (C-3, C-4 & C-6 respectively) in ring A & B, while the peaks at δ140.4, 149.2 & 121.8 were assigned to the quaternary olefinic carbons (C-5, C-24 and C-25 respectively).

In HMBC spectrum the C-2 methylene protons (δ 2.8 & 3.34) of compound **(50)** showed correlation with quaternary carbon appearing at δ 210.1 (C-1) and with carbon resonating at 129.6 (C-4). Similarly the olefinic proton at 5.66 (C-6) exhibited HMBC correlation with carbon resonating at δ 129.6 (C-4).The methylen protons at δ 2.0 (C-7) have connectivity with quaternary carbon C-5 (δ 140.4). The methyl proton appear at δ 1.35 (C-19) showed correlation with carbons resonating at δ 210.1, 37.0 & 22.6 (C-1, C-9 & C-11 respectively) and long range coupling exhibited connectivity with quaternary carbon (C-5) resonating at *δ* 140.4. The HMBC spectrum also

showed hetero- nuclear correlation of methyl protons of C-28 (δ 1.75) with olefinic carbon C-24 (δ 149.2) and C-23 (δ 32.2),whereas methyl protons at C-27 (δ 1.91) exhibited long-range hetero- nuclear connectivity with the olefinic C-25 (δ 121.8) and C-26 (δ 166.4). The methyl protons appearing at 1.56 (C-18) exhibited coupling with C-12 (41.5) and C-17 (δ 56.5) and long range coupling with C-14 appear at 83.9. The entire above chemical shift assignments were confirmed by HMBC data (Fig. 3.6). Therefore, the structure of compound **(50)** was assigned as 14*β*, 20*β*-dihydroxy-1-oxo-(20*R*, 22*R*)-witha-3,5,24-trienolide and named as Withacoagulin F.

Withacoagulin F **(50)** was found inhibitior of both ConA-induced T cell proliferation (IC_{50} = 29.2 μM) and LPS-induced B cell proliferation (IC_{50} = 42.7 μM) (Sec. 11.7).



Withacoagulin F (50)

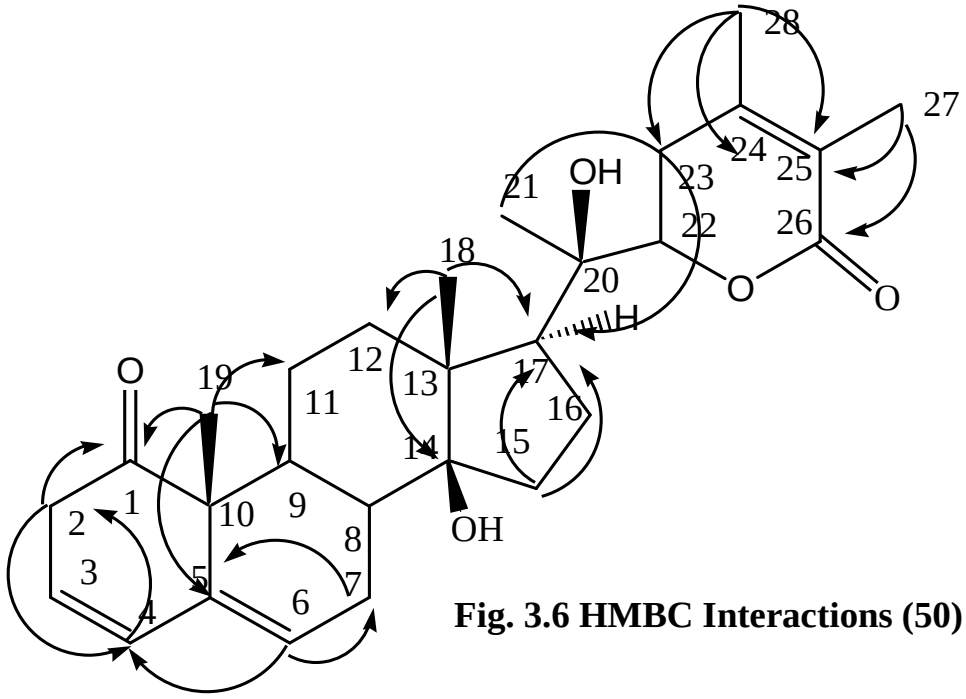


Fig. 3.6 HMBC Interactions (50)

Table-3.6: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound **(50)** in C_5D_5N

C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	210.1	C	-
2	40.0	CH_2	2.80 (dd, *J2a, 3* = 19.8, *J2a, 2 b*= 4.5)
			3.34 (d, *J2a,3*= 19.8)
3	121.9	CH	5.58 (*m*)
4	129.6	CH	6.07 *(*d, *J4,3*= 9.5*)*
5	140.4	C	-
6	128.3	CH	5.66 (d, *J6, 7a* = 3.2)
7	27.4	CH_2	2.00 – 2.03 (*m*)
			2.71 – 2.75 (*m*)
8	36.2	CH	2.11 – 2.14 (*m*)
9	37.0	CH	2.26 – 2.29 (*m*)
10	52.8	C	-
11	22.6	CH_2	1.45 – 1.47 (*m*)
			1.83 – 1.87 (*m*)
12	41.5	CH_2	1.48 – 1.51 (2H, *m*)
13	49.5	C	-
14	83.9	C	-
15	32.6	CH_2	1.70 – 1.74 (*m*)
			1.93 – 1.97 (*m*)
16	22.1	CH_2	1.58 – 1.72 (*m*)
			2.10 – 2.14 (*m*)
17	56.5	CH	1.80 – 1.85 (*m*)
18	18.4	CH_3	1.56 (*s*)
19	20.4	CH_3	1.35 (*s*)
20	75.1	C	-
21	21.7	CH_3	1.56 (*s*)
22	82.1	CH	4.55 (dd, *J22, 23a* = 13.1, *J22, 23b*= 3.4)
23	32.2	CH_2	2.22 – 2.26 (*m*)
			2.39 – 2.42 (*m*)
24	149.2	C	-
25	121.8	C	-
26	166.4	C	-
27	12.6	CH_3	1.91 (*s*)
28	20.1	CH_3	1.71 (*s*)

a: Broad band; b: DEPT; c: ^{1}H NMR; d: HMQC interaction

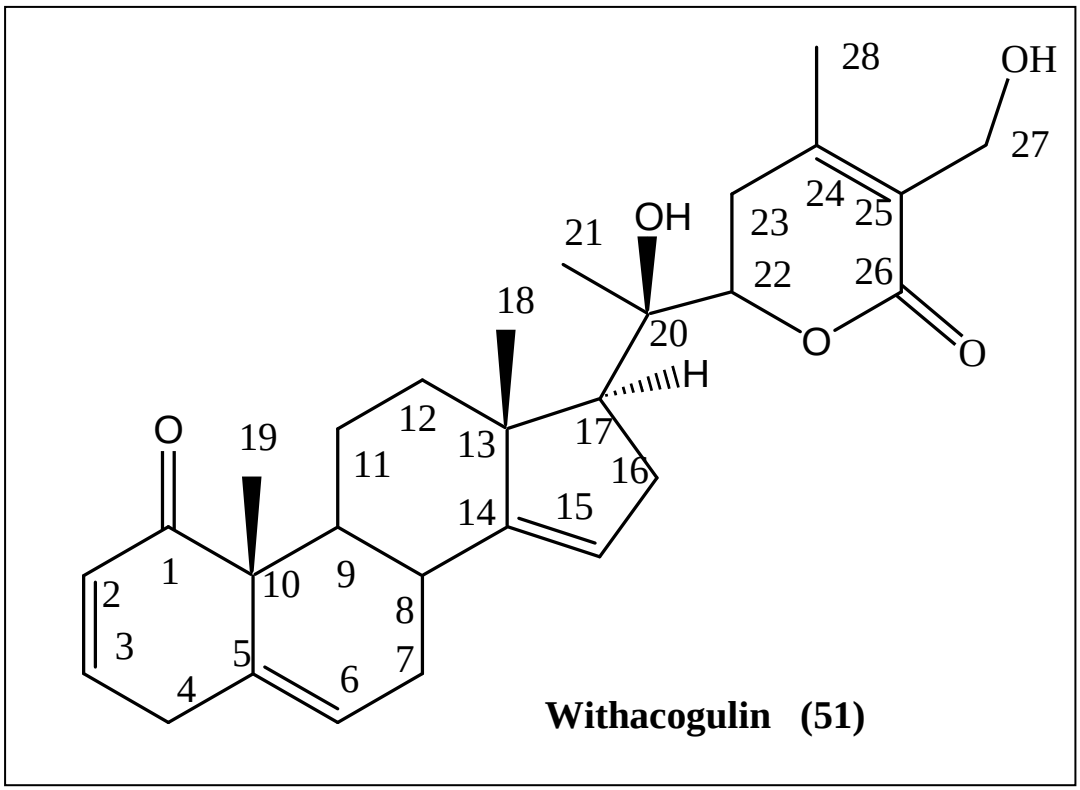
3.1.2: known withanolides isolated from *Withania coagulans*

3.1.2.1: Withacoagulin (51)

Fraction A was loaded on silica gel CC (hexane/acetone 15:1, 10:1, 5:1, 2:1, 1:1) to provide six sub-fractions (A1-6). Sub-fraction A4 was further subjected to RP-18 CC (MeOH/water 5.3:4.7), yielding compound **51** an optically active amorphous powder (93 mg). Fractionation scheme is given in experimental section (Fig. 4.1)

The UV spectrum exhibited a characteristic absorption at 215nm, indicating α,β-unsaturated lactone chromophore.The IR spectrum gives signal at 3583, 1706and 1684 cm^{-1} indicating, hydroxyl, six-membered cyclic ketone and a, α,β -unsaturated lactone respectively as mentioned earlier. The molecular formula was established as $C_{28}H_{36}O_5$ by its HR-ESI-MS from the $[M+Na]^+$ and $[M+HCOO]^-$ signals at *m/z* 475.2455 (positive, calc. 475.2460) and 497.2542 (negative mode, calc.497.2539), respectively.

Its ^1H and ^{13}C NMR spectra (Table 3.7) were closed to those of withacoagulin B **(45)**. The NMR spectra indicated that the only difference between them was found in the ring A. The ring A of **51** contains a 2, 5-diene-1-one system, while the spectrum of ring A in **45** was characteristic of the 3, 5-diene-1-one system of withanolide.



Withacogulin (51)

Table-3.7: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound **(51)** in C_5D_5N

C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	203.6	C	-
2	127.9	CH	5.83 (dd, *J2, 3* = 9.8, *J2, 4a* = 2.3)
3	145.2	CH	6.74 (dd *J3, 4a* =9.9, *J3,4b* = 3.1)
4	33.4	CH_2	3.28 (ddd, *J4a 4b = 21.3, J4a, 3* = 2.3, *J4a, 4b* = 3.21) 2.85 (dd, *J4a, 4b* = 21.3, *J4a, 3* = 4.8)
5	135.4	C	-
6	124.3	CH	5.57 (d, *J* = 6.1)
7	30.0	CH_2	2.75 *m*
8	31.9	CH	1.55 *m*
9	42.4	CH	1.65 *m*
10	50.1	C	-
11	28.2	CH_2	1.50 *m*
12	26.3	CH_2	1.35 *m*
13	47.9	C	-
14	152.4	C	-
15	118.0	CH	5.18 *br.s*
16	42.0	CH_2	2.0–2.1 *m*
17	57.3	CH	1.90 *m*
18	18.7	CH_3	1.13 *s*
19	18.8	CH_3	1.25 *s*
20	74.6	C	-
21	20.0	CH_3	1.31 *s*
22	81.7	CH	4.28 (dd, *J22a, 23a* = 13.3, *J22a, 23b* = 3.5)
23	31.7	CH_2	2.52 *m* 2.17 *m*
24	153.4	C	-
25	125.9	C	-
26	165.7	C	-
27	57.4	CH_2	4.38, 4.34 (*AB* d, *J* = 12.3)
28	20.5	CH_3	2.03 s

In the ^{13}C NMR of **51**, the carbon resonating at δ 209.8 (C-1) indicated a saturated carbonyl carbon. The olefinic signals at δ 122.3 and 129.4 were attributed to C-3 and C-4 respectively. The methylene peak at δ_C 39.9 was attributed to C-2. The H-NMR spectrum (Table3.7) of compound **(51)** showed four methyl singlets at δ 1.13 (C-18H), 1.25(C-19H), 1.31(C-21H) and 2.03(C-28H). The lowfield shift (δ 1.31) of C-21 methyl is the due to the oxygen function present on C-20. The downfield signal (δ 2.03) of the C-28 methyl singlets indicated it is substituted on a double bond. The downfield signals at δ 5.83 (dd, *J2, 3* = 9.3 Hz, *J2, 4a* = 2.3 Hz), 6.74 *(*dd *J3, 2* = 9.8, *J3, 4a* = 3.2 Hz*)*, 5.58 *(*d, *J* = 6.0 Hz*)*and 5.18 *br.s* represented four protons of olefinic nature H-2, H-3, H-6 and H-15 respectively. A lowfield methine doublet of doublet at δ 4.49 (*J*22, 23a = 13.3 Hz, *J*22, 23b = 3.3 Hz) was assigned to the proton of lactone moiety at C-22. The ^{13}C NMR spectra of compound (22) showed resonance for all 28 carbons including, methyl, methylen, methine and quaternary carbons.1H NMR and 13C NMR splitting is given in Table (3.7). On the interpretation of above mentioned spectroscopic tehniqes, the compound **(51)** was identified as known compound, Withacoagulin previously isolated from the same palnt[85].

Withacoagulin **(51)** showed good inhibitory activities of both ConA-induced T (IC_{50}< 20 μM) and LPS-induced B (IC_{50}< 20 μM) cell proliferation (Sec. 11.7).

3.1.2.2: Withanoilde F (52)

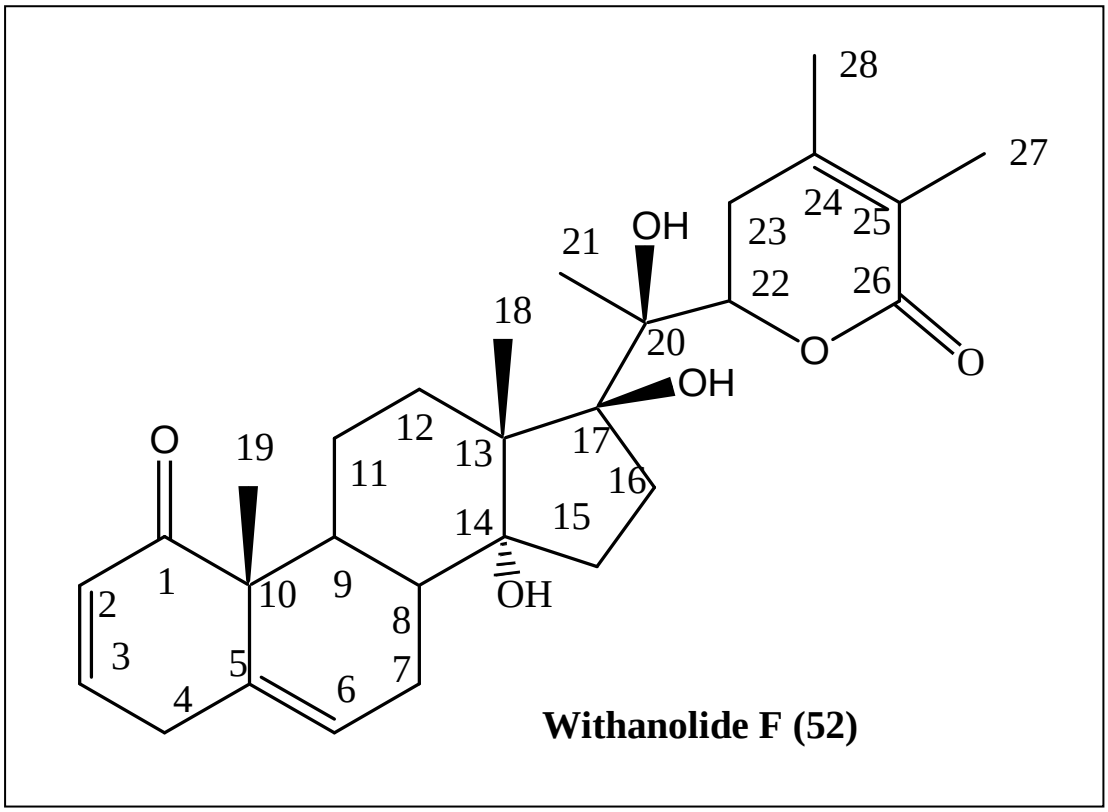
Fraction A was loaded on silica gel CC (hexane/acetone 15:1, 10:1, 5:1, 2:1, 1:1) to provide six sub-fraction (A1-6). Optically active solid **(52)**, was purified from Sub-fraction A3 on similar way as mentioned earlier. Fractionation scheme is given in experimental section (Fig.4.1)

The characteristic absorption at 226nm was observed in UV spectrum and indicating α,β-unsaturated lactone chromophore as previously stated. The IR spectrum showed peaks at, 3424 and 1684 cm^{-1} indicating, hydroxyl and a, α, β -unsaturated lactone functionalities respectively as stated before. The molecular formula was established as $C_{28}H_{36}O_6$ by its HR-ESI-MS from the molecular ion peak $[M+Na]^+$ at 493.2583.

The 1H and ^{13}C NMR spectra of **52** (Table 3.8) showed similarities with those of compound (**58**). The main difference between them was the presence of one more hydroxyl group assigned to C-15 in compound **(58)**. The ^{13}C NMR of **52** showed all

the 28 carbons of steroidal skeleton. The carbon resonating at δ 203.1 (C-1) indicated a saturated carbonyl carbon. The olefinic signals at δ 127.3 and 146.4 were attributed to C-2 and C-3, respectively. The peak at δ_C 32.9 was attributed to C-4 (methylene). The ^{1}H-NMR spectrum (Table 3.8) of **52** showed five methyl singlets. The lowfield shift (δ 1.31) of C-21 methyl indicating an oxygen function present on C-20. The downfield signal (1.70) of the C-28 methyl singlets indicated its location on a double bond. The downfield signals at δ 5.74 (*m*), 6.88 *(*ddd, *J3, 2* = 9.8, *J3, 4b* = 4.9, *J3, 4a* = 2.2 Hz*)* and 5.58 *m* was assigned to protons of olefinic nature H-2, H-3 and H-6 respectively. A lowfield doublet of doublet at δ 4.49 (*J*22, 23a =13.4, *J*22, 23b = 3.3Hz) was assigned to the proton of methane in lactone moiety at C-22.^{1}H NMR and ^{13}C NMR data is given in table 3.8. After interpreting the UV, IR,NMR and mass spectra,the compound was identified as withanolid F **(52),** previously reported from *W. adpressa*[252].

Withanolide F **(52)** was found the inhibitior of both ConA-induced T cell proliferation (IC_{50} = 36.8 μM) and LPS-induced B cell proliferation (IC_{50} = 39.5μM) (Sec. 11.7).



Withanolide F (52)

Table-3.8: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound (**52)** in C_5D_5N

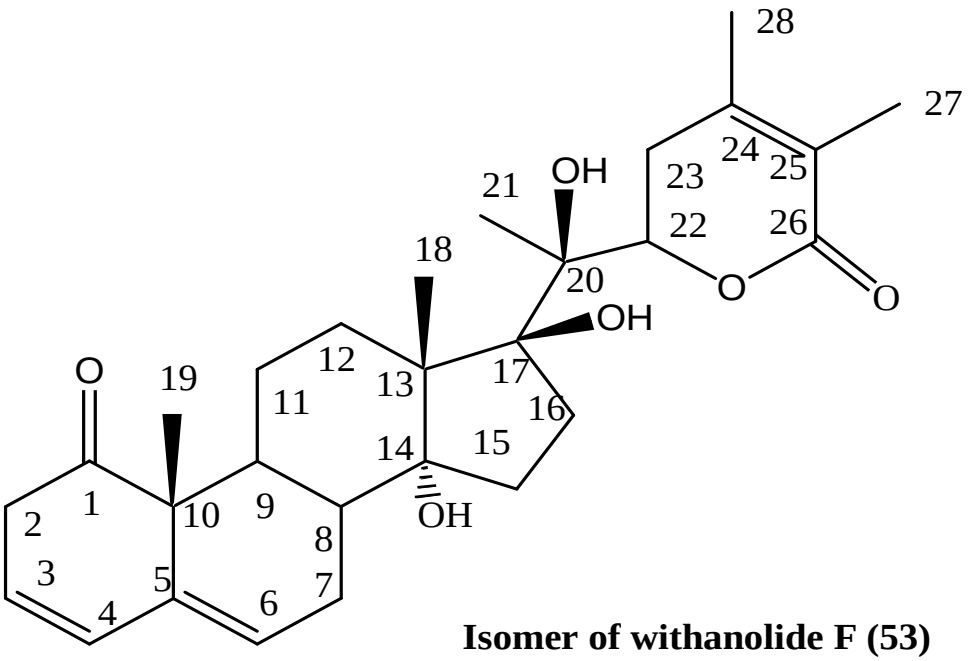
C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	204.1	C	-
2	127.0	CH	5.74 *m*
3	146.4	CH	6.88 (ddd, *J3, 2* = 9.8, *J3,4b* = 4.9, *J3, 4a* = 2.2)
4	32.9	CH_2	2.23 *m*
5	135.1	C	-
6	125.1	CH	5.58 *m*
7	25.5	CH_2	2.0 *m*
8	32.9	CH	1.70 *m*
9	34.4	CH	1.90 *m*
10	50.9	C	-
11	22.2	CH_2	2.0 *m*
12	31.3	CH_2	1.15 m
13	53.9	C	-
14	82.1	C	5.76 *s*
15	74.5	CH_2	1.32 *m*
16	46.0	CH_2	2.3–2.4 *m*
17	88.3	C	4.64 *br.s* (OH)
18	19.7	CH_3	1.05 *s*
19	18.8	CH_3	1.13 *s*
20	78.6	C	6.85 *br.s* (OH)
21	20.0	CH_3	1.31 *m*
22	80.7	CH	4.58 (dd, *J22, 23a* = 13.4, *J22, 23b* = 3.3*)*
23	34.7	CH_2	2.51 *m*
24	150.4	C	-
25	120.9	C	-
26	165.7	C	-
27	20.4	CH_3	1.85 *s*
28	12.5	CH_3	1.70 s

3.1.2.3: Δ^3-isomer of withanolide F (53)

Fraction A was loaded to silica gel CC and eluted with hexane/acetone (15:1, 10:1, 5:1, 2:1, 1:1) to afford six sub-fraction.(AI-6). Sub-fraction A3 was further subjected to reverse phase chromatography (RP-18 CC) and eluted with MeOH/$H_2$0 (6:4), afforded an optically active amorphous solid **(53).** Fractionation scheme is

given in experimental section (Fig.4.1). The characteristic absorption at 216nm was observed in UV spectrum and indicating α, β-unsaturated lactone chromophore. Similarly the IR spectrum of compound **(53)** displayed the same bands as stated earlier. The molecular formula was found as $C_{28}H_{36}O_6$ by its HR-ESI-MS from the molecular ion peak $[M+Na]^+$ at 493.2583. The ^1H and ^{13}C NMR spectral data (Table 3.9) of **(53)** were much closed to its isomer **(52),** the only diference was observed in dien system. The ring A of **(52)** contains a 2, 5-diene-I-one system, while the spectra of ring A in **(53)** was characteristic of the 3, 5-diene-I-one system of withanolides.

The ^{13}C NMR data (Table 3.9) of compound **(53)** resonating all the 28 carbons of steroidal skeleton. The carbon resonating at δ 211.1 was assign to carbonyl carbon (C-1). The olefinic signals at δ 127.9, 129.5, 140.5, 121.1, 150.4 and 121.5 were assigned to C-3 to C-6, C-24 and C-25 respectively. Five methyl singlets were also observed in the ^1H NMR spectrum. The lowfield shift (δ 1.43) of C-21 methyl showed the presence of oxygen function on C-20. The methyl singlet at (δ 1.87 & 193) was the sign of its location on a unsaturated carbon. The signals at δ 5.62 (d, *J3,4* = 10.3 Hz), 6.06 (d, *J4, 3* = 10 Hz) and 5.70 (m) were assigned to protons of olefinic nature H-3, H-4 and H-6 respectively.The ^1H NMR and ^{13}C NMR data is presented in table (3.9). On the basis of spectroscopic techniques such as UV, IR,NMR and mass spectra, the compound was identified as Δ^3-isowithanolide F **(53)** previously reported from *W. coagulans*[253]. Compound **(53)** showed good inhibitory activities (IC_{50}< 20 μM) of both ConA-induced T and LPS-induced B cell proliferation (Sec. 11.7).



Isomer of withanolide F (53)

Table-3.9: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound (**53)** in C_5D_5N

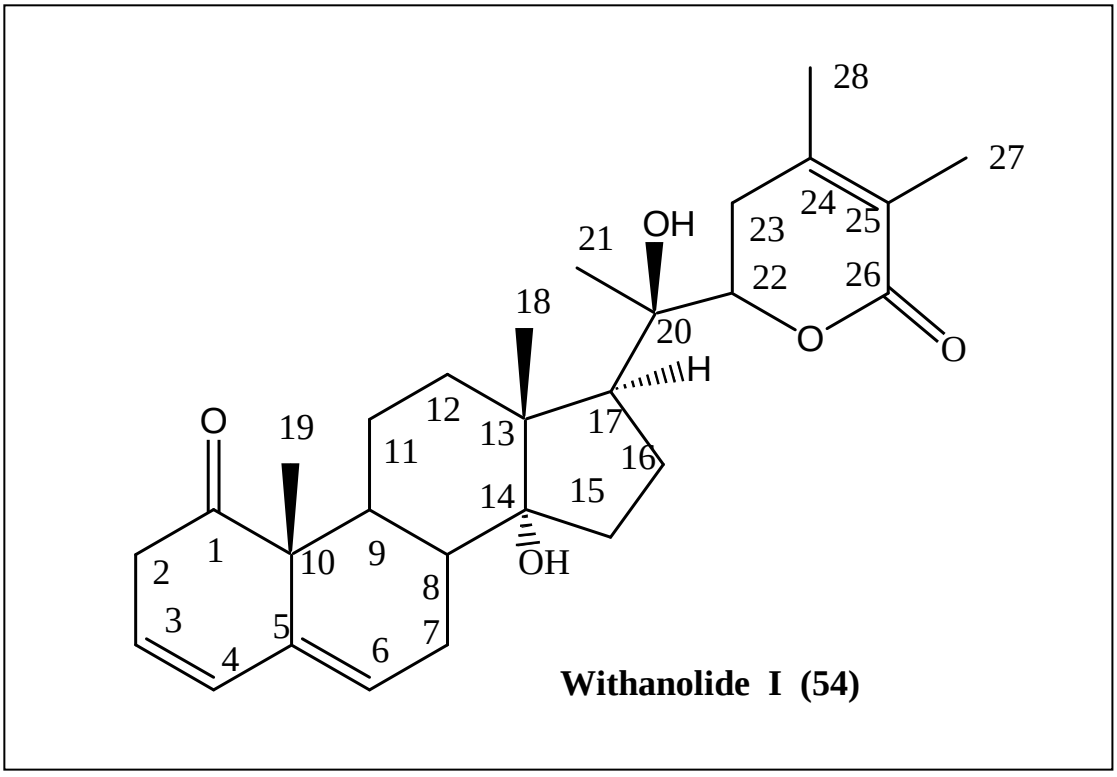
C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	210.4	C	-
2	39.7	CH	2.76 *m*
3	127.8	CH	5.62 (d, *J3, 4* = 10.3)
4	129.1	CH_2	6.06 (d, *J4, 3* = 10)
5	140.5	C	-
6	121.1	CH	5.70 *(m)*
7	25.1	CH_2	2.0 *m*
8	33.9	CH	2.1 *m*
9	36.2	CH	2.17*m*
10	52.1	C	-
11	21.8	CH_2	2.08 *m*
12	34.3	CH_2	2.15 m
13	53.9	C	-
14	83.4	C	-
15	30.5	CH_2	3.65 *m*
16	37.0	CH_2	2.73 *m*
17	87.3	C	2.0 *m*
18	20.7	CH_3	1.13 *s*
19	20.3	CH_3	1.37 *s*
20	79.1	C	-
21	19.0	CH_3	1.43 *s*
22	79.7	CH	4.93 (dd, *J22, 23a* = 9.7, *J22,23b* = 7.0)
23	32.7	CH_2	2.15 m
24	150.4	C	-
25	121.9	C	-
26	165.4	C	-
27	12.1	CH_3	1.87 *s*
28	20.1	CH_3	1.93 s

3.1.2.4: Withanoilde I (54)

Fraction A was further fractionated on silica gel CC and got six sub-fractions (AI-6). Sub-fraction A2 was further subjected to RP-18 CC (MeOH/H20 6.0:4.0) and afforded compound **54,** an optically active amorphous solid (13 mg). Fractionation scheme is given in experimental section (Fig.4.1). The characteristic absorption at 227nm was observed in UV spectrum and indicating α, β-unsaturated lactone

chromophore. Similarly the IR spectrum showed absorption peaks at 3375 (O-H), 1705, 1695 cm^{-1} (lactone carbonyl and ketone carbonyl) as described above. The molecular formula was determined as $C_{28}H_{38}O_5$ by its HR-ESI-MS from the molecular ion peak $[M+H]^+$ at *m/z* 455.25.

Its ^1H and ^{13}C NMR spectra (Table 3.10) also showed similarities with those of Withacoagulin E (**49**) and difference between them was in diene system of ring A. The ring A of **(54)** contains a 3, 5-diene-1-one system, while the spectra of ring A in **49** was characteristic of the 2,5-diene-1-one system of withanolides. The ^{13}C NMR of compound **(54)** showed the resonance peaks of all 28 carbons of the steroidal skeleton having methyl, methylene, methine and quaternary carbons. The signals at δ 127.9 and 140.5 were attributed to C-3 and C-5, respectively which confirming the 3, 5-diene-1-one system. The H-NMR spectrum (Table 3.10) of compound (**54)** showed five methyl singlets. The methyl singlet at downfield chemical shifts (δ 1.78& 1.90) was the sign of its location on a double bond. The downfield signals at δ 5.55 *m*, 6.08 (dd, *J4, 3* = 9.7, *J4, 2* = 2.4 Hz) and 5.81 (dd, *J6, 7a* = 5.1, *J6, 7b* =2.4 Hz) were assigned to the protons of olefinic nature H-3, H-4 and H-6 respectively. The ^1H NMR and ^{13}C NMR data is presented in table 3.10. On the basis of spectroscopic techniques such as UV, IR,NMR and mass spectra, the compound **(54)** was identified as withanolide I previously reported from *W. somnifera*[135]. Withanolide I **(54)** was found the inhibitior of both ConA-induced T cell proliferation (IC_{50} = 38.8 μM) and LPS-induced B cell proliferation (IC_{50} = 41.5μM), (Sec. 11.7)



Withanolide I (54)

Table 3.10: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound (54) in C5D5N

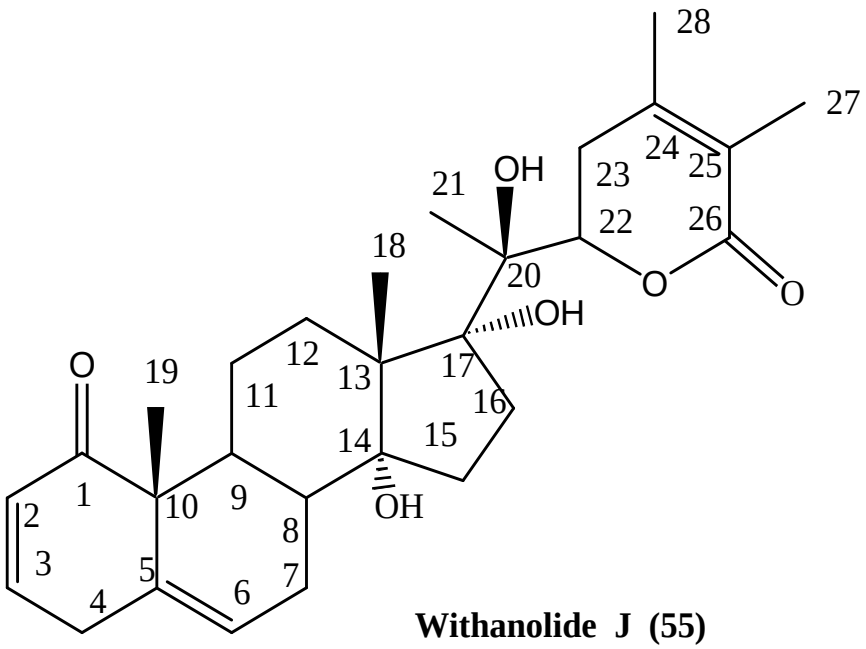
C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	210.1	C	-
2	39.7	CH_2	3.2 *m*
			2.7*m*
3	127.8	CH	5.55 *m*
4	127.8	CH	6.08 (dd, *J4, 3* = 9.7, *J4, 2* = 2.4)
5	140.4	C	-
6	121.1	CH	5.81 (dd, *J6, 7a* =5.1, *J6,7b* = 2.4)
7	32.5	CH_2	2.62 *m*
8	35.2	CH	2.10 *m*
9	32.4	CH	1.70 *m*
10	52.9	C	-
11	21.2	CH_2	1.55 *m*
12	25.3	CH_2	1.58 m
13	53.9	C	-
14	82.1	C	5.76 *s*
15	29.5	CH_2	2.1 *m*
			1.81 *m*
16	37.0	CH_2	1.3–1.4 *m*
17	88.3	C	-
18	19.7	CH_3	1.45 *s*
19	20.8	CH_3	1.71 *s*
20	78.6	C	-
21	20.0	CH_3	1.41 *s*
22	79.7	CH	5.22 (dd, *J22, 23a* = 13.7 *J22, 23b* = 3.5)
23	30.7	CH_2	2.75-3.01 *m*
24	151.4	C	-
25	120.9	C	-
26	164.7	C	-
27	12.4	CH_3	1.78 *s*
28	20.5	CH_3	1.90 s

3.1.2.5: Withanoilde J (55)

Fraction A was loaded to silica gel CC (hexane/acetone 15:1, 10:1, 5:1, 2:1, 1:1) to provide six sub-fractions (A1-6). Sub-fraction A2 was purified by RP-18 CC (MeOH/water 5.5:4.5) and afforded an optically active amorphous solid (70 mg). Fractionation scheme is given in Experimental section (Fig.4.1). The UV spectrum of

55 like withaolide F (**52**) showed a characteristic absorption at 226 nm, indicating α, β-unsaturated lactone chromophore. Similarly the IR spectrum of Withanolid J **(55)** displayed bands at 3426, 16843 and 1663 cm^{-1} indicating, hydroxyl, α,β -unsaturated lactone and olefinic functionalities respectively as stated in case of withanolide F. The molecular formula of compound **(55)** was determined as $C_{28}H_{36}O_6$ by its HR-ESI-MS from the molecular ion peak $[M+Na]^+$ at 493.2583.

The ^1H and ^{13}C NMR spectral data of compound **(55)** (Table 3.11) showed similarity with those of compound (**58**) and difference between them was the presence of one more hydroxyl group in compound **(58)**. The ^{13}C NMR of compound **(55)** gave the peaks of all 28 carbons of the steroidal skeleton having methyl, methylene, methine and quaternary carbons either. The carbon resonating at δ 203.1(C-1) indicated a saturated carbonyl carbon. The olefinic signals at δ 127.3 and 146.4 were attributted to C-2 and C-3, respectively. The ^1H-NMR spectrum (Table 3.11) of compound **(55)** showed five methyl singlets. The lowfield shift (δ 1.31) of methyl (C-21) is indicating the presence of an oxygen function on C-20. The methyl singlet at downfield signals (δ 1.88 & 173) was the sign of its location on usaturated carbon. The downfield signals at δ 5.74 (dd *J2, 3* = 9.80, *J2, 4a* = 2.40 Hz), 6.89 (ddd, *J3, 2* = 9.71, *J3,4b* = 4.9 *J3, 4a* = 2.2 Hz) and 5.58 *m* was assigned to protons of olefinic nature H-2, H-3 and H-6 respectively.^1H NMR and ^{13}C NMR data of compound **(55)** is presented in table 3.11. The UV, IR, NMR and mass spectra of the compound **(55),** identified it as reported one, Withanolide **J** [135,252]. Withanolid J **(55)** also showed good inhibitory activities of both ConA-induced T (IC_{50}< 20 μM) and LPS-induced B (IC_{50}< 22 μM) cell proliferation (Sec. 11.7).



Withanolide J (55)

Table-3.11: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound **(55)** in C_5D_5N

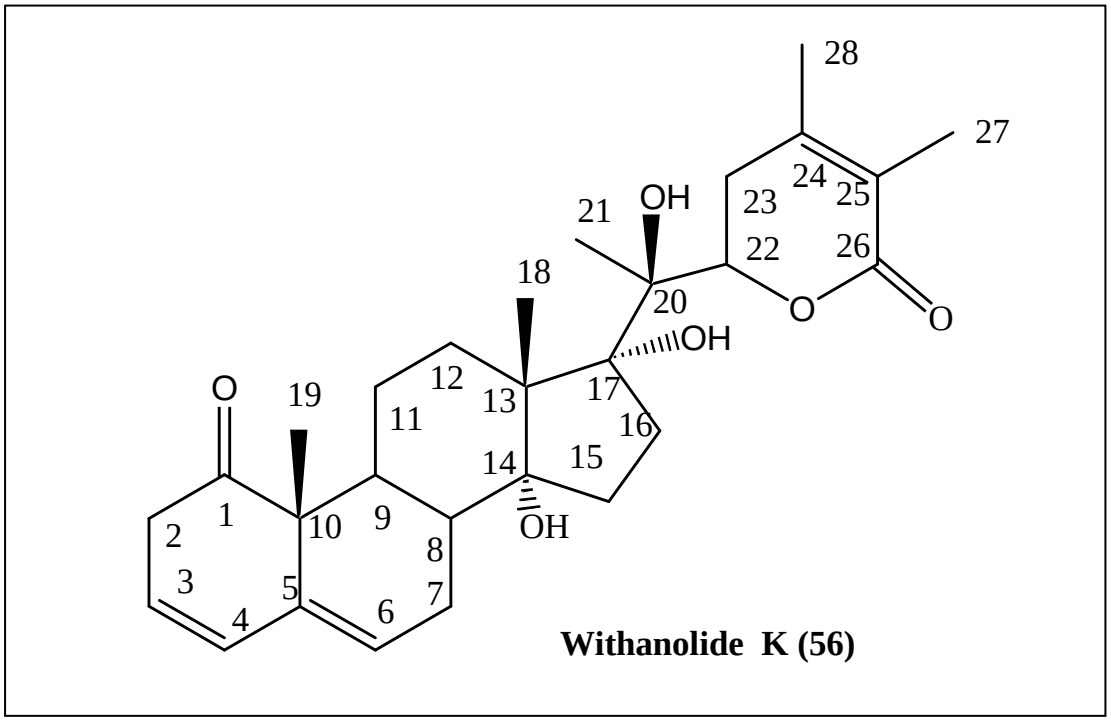
C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	203.1	C	-
2	127.0	CH	5.74 (dd, *J2, 3* = 9.8, *J2, 4a* = 2.4*)*
3	146.4	CH	6.88 (ddd, *J3, 2* =10.*3, J3, 4a* = 4.9, *J3,4b* = 2.2)
4	32.1	CH_2	2.23 *m*
5	135.5	C	-
6	124.1	CH	5.58 *m*
7	25.1	CH_2	2.0 *m*
8	36.9	CH	2.1 *m*
9	35.2	CH	1.78 *m*
10	50.1	C	-
11	22.8	CH_2	2.08 *m*
12	30.3	CH_2	2.15 m
13	53.9	C	-
14	81.4	C	-
15	31.5	CH_2	1.51 *m*
16	35.0	CH_2	2.63 (ddd, *J16a, 16b* = 14.2, *J16a, 15a* = 11.9, *J16a, 15b* = 1.9)
17	83.3	C	-
18	20.7	CH_3	1.05 *s*
19	18.3	CH_3	1.13 *s*
20	78.1	C	-
21	19.0	CH_3	1.16 *s*
22	81.7	CH	4.58 (dd, *J22, 23a* = 12.3, *J22, 23b* = 3.5)
23	34.7	CH_2	2.31 *m*
24	150.4	C	-
25	120.9	C	-
26	165.7	C	-
27	20.4	CH_3	1.88 *s*
28	12.5`	CH_3	1.73 s

3.1.2.6: Withanoilde K (56)

Fraction A was loaded on silica gel CC (hexane/acetone 15:1, 10:1, 5:1, 2:1, 1:1) to provide six sub-fraction (A1-6). Compound **(56,** 65 mg) was purified from Sub-fraction A2 on similar way as mentioned earlier. Fractionation scheme is given in experimental section (Fig.4.1). The UV absorption at 223 nm, indicating α,β-

unsaturated lactone chromophore as previously stated. The IR spectrum displayed of compound **(56)** bands at, 3420, 1690 and 1675 cm^{-1} indicating hydroxyl, six member keton and α,β -unsaturated lactone respectively. The molecular formula of compound **(56)** was determined as $C_{28}H_{36}O_6$ by its HR-ESI-MS from the molecular ion peak $[M + H]^+$ ion at *m/z* 469.

Its ^1H and ^{13}C NMR spectra (Table 3.12) showed similarities with those of Withanolid J **(55)**. The NMR spectra indicated that the main difference between them was observed in ring A. The ring A of **(55)** contains a 2, 5-diene-1-one system, while the spectra of ring A in **(56)** was characteristic of the 3, 5-diene-1-one system of withanolides. The ^{13}C NMR of **(56)** showed all the 28 carbons resonance of steroidal skeleton. The carbon resonating at δ 210.1 (C-1) indicated a saturated carbonyl carbon. The olefinic signals at δ 121.6, 129 and 140.4 were attibuted to C-3, C-4 and C-5, respectively. The methylene peak at δ_C 40.1 was attributed to C-2. The H-NMR spectrum (Table 3.12) of compound **(56)** showed five methyl singlets. The lowfield shift (δ 1.56) of methyl (C-21) is indicating the presence of an oxygen function on C-20. The downfield peaks at (δ 1.81, 1.19) of the methyl singlets (C-27 and C-28) are the sign of their attachment on double bond. The protons H-3, H-4 and H-6 have given the signals at δ 5.58 (*m*), 6.07 (dd, *J4, 3* = 9.7, *J4, 2* = 2.4 Hz) and 5.66 (dd, *J6, 7a* = 5.1, *J6, 7b* = 2.4 Hz) respectively. A downfield methine doublet of doublet at δ 4.55 (dd, *J22, 23a* = 12.2, *J22 23b* = 4.2 Hz) was assigned to the proton of lactone moiety at C-22.^1H NMR and ^{13}C NMR data is given in table(3.12) On the basis of above spectroscopic study, the compound **(56)** was identified as known compound, Withanolid **K** already reported fom *Withania spp.*[135,136]



Withanolide K (56)

Table-3.12: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound **(56)** in C_5D_5N

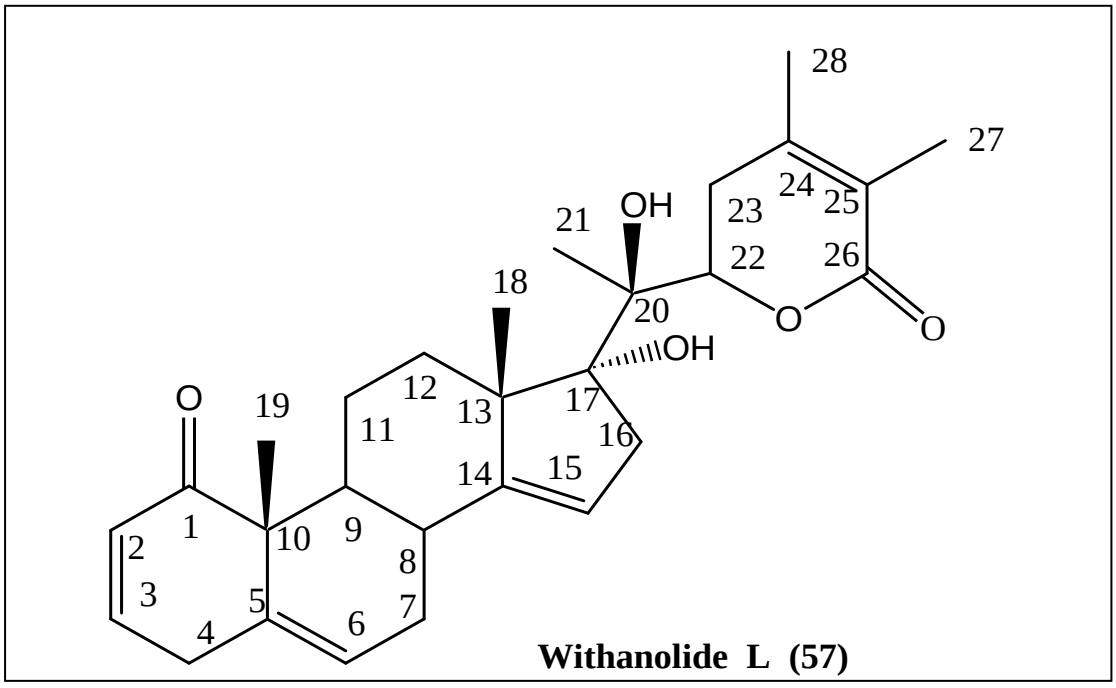
C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	210.1	C	-
2	40.0	CH_2	2.80 *m*, 3.34 *m*
3	121.9	CH	5.58 *m*
4	129.6	CH	6.07 (dd, *J4, 3* = 9.7, *J4, 2* = 2.4)
5	140.4	C	-
6	128.3	CH	5.66 (dd, *J6, 7a* = 5.1, *J6,7b* = 2.4)
7	27.4	CH_2	2.00 – 2.13 (*m*), 2.61 – 2.65 (*m*)
8	36.2	CH	2.21 – 2.24 (*m*)
9	37.0	CH	2.16 – 2.19 (*m*)
10	52.8	C	-
11	22.6	CH_2	1.55 – 1.57 (*m*), 17 – 1.67 (*m*)
12	41.5	CH_2	1.48 – 1.51 (2H, *m*)
13	49.5	C	-
14	83.9	C	-
15	32.6	CH_2	1.70 – 1.74 (*m*), 1.93 – 1.97 (*m*)
16	22.1	CH_2	1.58 – 1.72 (*m*), 2.10 – 2.14 (*m*)
17	88.5	C	-
18	18.4	CH_3	1.56 (*s*)
19	20.4	CH_3	1.35 (*s*)
20	75.1	C	-
21	21.7	CH_3	1.56 (*s*)
22	82.1	CH	4.55 (dd, *J22, 23a* = 12.2, *J22, 23b* = 4.2)
23	32.2	CH_2	2.22 – 2.26 (*m*),2.39 – 2.42 (*m*)
24	149.2	C	-
25	121.8	C	-
26	167.4	C	-
27	*12.6*	CH_3	1.81 (*s*)
28	20.1	CH_3	1.91 (*s*)

3.1.2.7: Withanoilde L (57)

Fraction A was further fractionated on silica gel CC and got six sub-fractions (AI-6). Sub-fraction A1 was further subjected to RP-18 CC (MeOH/water 6.5:3.5) and afforded an optically active amorphous solid (**57,** 50 mg). Fractionation scheme is given in experimental section (Fig 4.1). The UV spectrum of compound **57**, like

withanolide K (**56**) also showed a characteristic absorption at 220 nm, indicating α, β-unsaturated lactone chromophore a. Similarly the IR spectrum showed absorption at, 1698 cm^{-1} indicating α, β -unsaturated lactone group. The molecular formula of compound **(57)** was determined as $C_{28}H_{36}O_5$ by its HR-ESI-MS from the molecular ion peak $[M+H]^+$ at 453.25.

The ^1H and ^{13}C NMR spectral data of compound **(57)** (Table 3.13) of **(57)** also showed similarities with those of Withacoagulin A (**45**) and the difference between them was observed in diene system of ring A. The ring A of **(45)** contains a 3, 5-diene-1-one system, while the spectra of ring A in **(57)** was characteristic of the 2,5-diene-1-one system of withanolides. The ^{13}C NMR of compound **(57)** showed the resonance of the steroidal skeleton having methyl, methylene, methine and quaternary carbons as well. The carbon resonating at δ 204.5 (C-1) was indicated carbonyl carbon. The olefinic signals at δ 127.7 and 140.7 were attributed to C-2 and C-5, respectively which confirming the 2, 5-diene-1-one system. The ^1H-NMR spectrum (Table 3.13) of compound **(57)** showed five methyl singlets. The methyl singlets at downfield chemical shifts (δ 1.77 & 1.84) were the sign of its location on a double bond. The downfield signals at *δ* 5.75 (dd, *J2a, 2b* = 10.0, *J2a, 3* = 2.1 Hz), 6.09 (ddd, *J3, 4a*= 19.8, *J3, 4b* = 4.9, *J3,2* = 2.4 Hz) and 5.66 (dd, *J6, 7a* = 5.3, *J6,7b* = 2.0) were assigned to protons H-2, H-3 and H-6 respectivel.[1] H NMR and ^{13}C NMR data is of compound **(57)** presented in table (3.13). On the basis of spectral techniques such as Mass UV, IR and NMR, the compound **(57)** was identified as Withanolid **L** previously reported from *W. somnifera* [135]. Withanolid **L (57)** showed excellent inhibitory activities (IC_{50}< 15 μM) of both ConA-induced T cell proliferation and LPS-induced B cell proliferation (Sec.11.7)



Withanolide L (57)

Table-3.13: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound **(57)** in C_5D_5N

C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	204.5	C	-
2	127.7	CH	5.75 (dd, *J2,3* = 10.0, *J2,4* = 2.1)
3	145.7	CH	6.09 (ddd, *J3,2* = 19.8, *J3, 4a* = 4.9, *J3,4b*= 2.4)
4	33.3	CH_2	2.83 (dd, *J4a, 3* = 11.4, *J4a, 2* = 5.0)
			3.29 (dd, *J4b, 3* = 11.2, *J4b, 2* = 2.3)
5	140.1	C	-
6	126.5	CH	5.66 (dd, *J6, 7a* = 5.3, *J6,7b* = 2.0)
7	28.9	CH_2	2.11 – 2.15 (m)
			2.29 – 2.32 (*m*)
8	30.5	CH	2.56 – 2.60 (*m*)
9	39.5	CH	1.68 – 1.71 (*m*)
10	52.1	C	-
11	22.8	CH_2	1.38 – 1.42 (*m*)
			1.81 – 1.85 (*m*)
12	30.4	CH_2	1.54 – 1.58 (*m*)
			2.20 – 2.24 (*m*)
13	53.6	C	-
14	151.1	C	-
15	117.0	CH	5.52 (*s*)
16	39.8	CH_2	2.22 – 2.26 (*m*)
			2.98 (dd, *J16a*, *16b* = 17.4, *J16a*, *15* = 3.1)
17	88.0	CH	-
18	21.1	CH_3	1.19 (*s*)
19	19.9	CH_3	1.35 (*s*)
20	75.8	C	-
21	19.4	CH_3	1.30 (*s*)
22	80.2	CH	4.65 (dd, *J22, 23a* = 13.1, *J22, 23b* = 3.4)
23	32.8	CH_2	2.35 – 2.37 (*m*)
			2.62 – 2.66 (*m*)
24	151.3	C	-
25	120.0	C	-
26	165.5	C	-
27	13.2	CH_3	1.77 (*s*)
28	19.5	CH_3	1.84 (*s*)

3.1.2.8: (22R)-14α, 15α, 17β, 20β-Tetrahydroxy-1-oxowitha-2, 5, 24-trien-26, 22-olide (58) a known withanolide

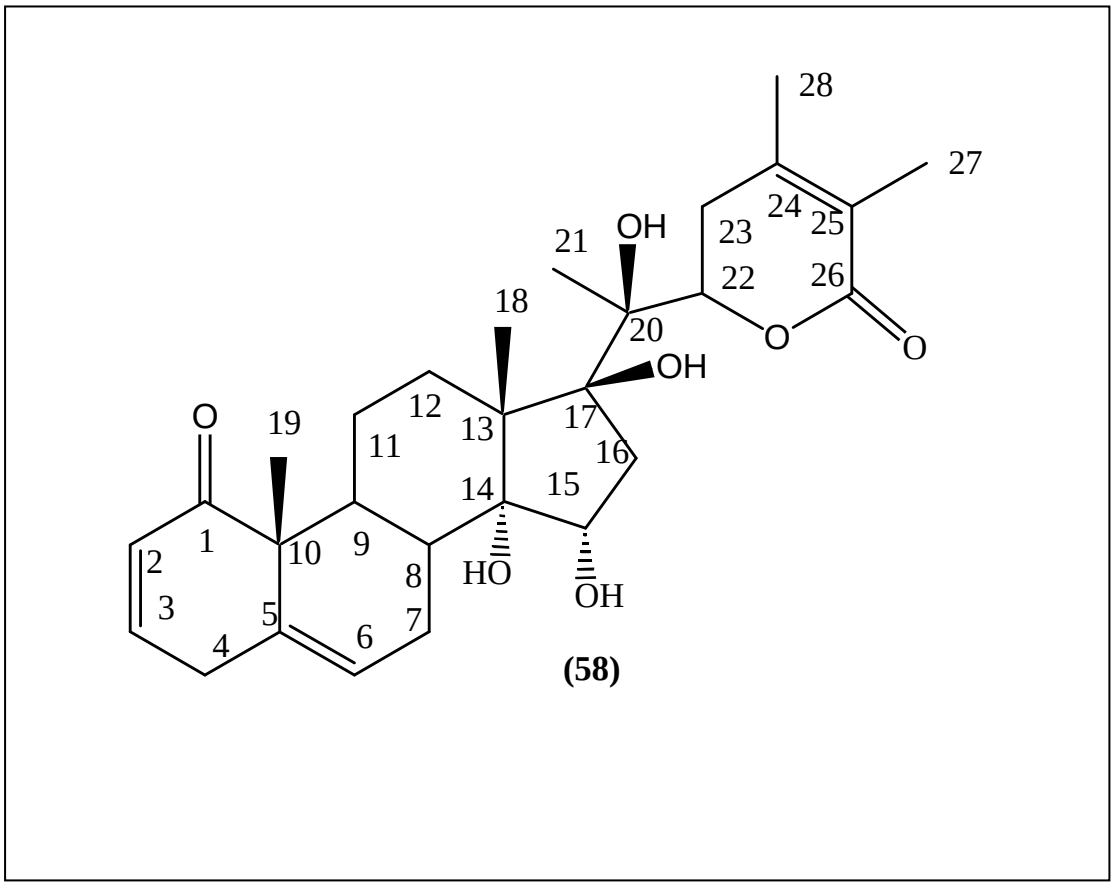
Fraction A was loaded on silica gel CC and eluted with hexane/acetone in increasing order of polarity (15:1, 10:1, 5:1, 2:1, 1:1) to yield six sub-fractions (AI-6). Sub-fraction A6 was further subjected to reverse phase chromotagraphy (RP-18 CC) and eluted with MeOH/H_20 (5.5:4.5) has afforded compound **58,** an optically active amorphous solid (120 mg). Fractionation scheme is given in Experimental section (Fig 4.1). A characteristic absorption at 226 nm as observed in UV spectrum and indicating α,β-unsaturated lactone chromophore. The IR spectrum displayed bands at,3 424, 1684 and1660 cm^{-1} indicating, hydroxyl, α, β -unsaturated lactone and double bond functionalities respectively as mentioned ealier. The HR-ESI-MS showed molecular ion peak $[M+Na]^+$ at 509.2517 (calc.509.2516) which corresponded to molecular formula as $C_{28}H_{36}O_6$. The ^1H and ^{13}C NMR spectra of compound **58** (Table 3.14) were foud similar to the withanolid F **(52)** discussed above. The only difference between them found was the presence of one more hydroxyl group at C-15 in compound **58**.

The ^{13}C NMR of **(58)** showed all the 28 carbons of different multiplicity of steroidal lactone. The carbon resonating at δ 203.1(C-1) indicated a saturated carbonyl carbon. The signals at δ 127.0 and 146.8 were attributed to the unsaturated carbon, C-2 and C-3 respectively. The peak at δ 32.9 was attributed to C-4 and hence indicating 2, 3-diene system in ring A. The ^1H-NMR spectrum (Table 3.14) of compound **(58)** showed five methyl singlets. The lowfield shift (δ 1.31) of the methyl (C-21) is indicated that that oxygen function may be present on C-20. The downfield peaks (δ 1.70) of the C-28 methyl singlets indicated its attachment to unsaturated carbon. The lowfield signals at δ 5.74 (*m*), 6.88 (ddd, *J3, 2* = 9.81, *J3, 4b* = 4.9, *J3, 4a* = 2.21 Hz) and 5.58 *m* was assigned to protons of olefinic nature H-2, H-3 and H-6 respectively. A lownfield doublet of doublet at δ 4.49 (*J22, 23a*=13.25 Hz, *J22, 23b*=3.23 Hz) was assigned to the methane proton of lactone moiety at C-22. The ^1H NMR and ^{13}C NMR data of compound (**58**) is given in table 3.14. On the basis of spectroscopic techniques such as UV, IR, NMR and mass spectra, the compound **58** was identified as (22R)-14α, 15α, 17β, 20β-tetrahydroxy-1-oxowitha-2,5,24- trien-26,22-olide (**58**) previously reported from *W. adpressa* [252]. Compound **(58)** was found the inhibitior of both ConA-induced T cell proliferation (IC_{50} = 31.4 μM) and LPS-induced B cell

proliferation (IC_{50} = 32.4μM) (Sec 11.7).

Table-3.14: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound **(58)** in C_5D_5N

C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	204.1	C	-
2	127.0	CH	5.76 (dd *J2, 3* = 9.91, *J2, 4a* = 2.31)
3	146.8	CH	6.88 (ddd, *J3,2 = 10.3, J3, 4b = 4.9, J3, 4a* = 2.2)
4	33.1	CH_2	3.23 *m*
5	135.5	C	-
6	124.1	CH	5.58 (d, *J6, 7* = 5.2)
7	24.1	CH_2	2.0 *m*
8	35.9	CH	2.1 *m*
9	36.2	CH	2.17*m*
10	51.1	C	-
11	22.8	CH_2	2.08 *m (qd superimposed)*
12	27.3	CH_2	2.15 m *(td superimposed)*
13	50.9	C	-
14	85.4	C	5.77 *br.s(OH)*
15	82.5	CH	3.65 *(*d, *J15,16a=5.8)*
16	33.0	CH_2	2.73 (ddd, *J16a, 16b* = 14.2, *J16a,15a* = 11.9, *J16a, 15b* = 1.9)
17	87.3	C	4.64 *br.s* (OH)
18	18.7	CH_3	1.22 *s*
19	18.3	CH_3	1.15 *s*
20	77.1	C	6.64 *br.s* (OH)
21	19.0	CH_3	1.22 *s*
22	80.7	CH	4.58 *(*dd, *J22, 23a* = 12.7, *J22, 23b* = 3.5)
23	31.7	CH_2	2.31 (d, *J23a, 23* = 16)
24	151.4	C	-
25	120.9	C	-
26	166.7	C	-
27	20.4	CH_3	1.88 *s*
28	12.1	CH_3	1.75 s



(58)

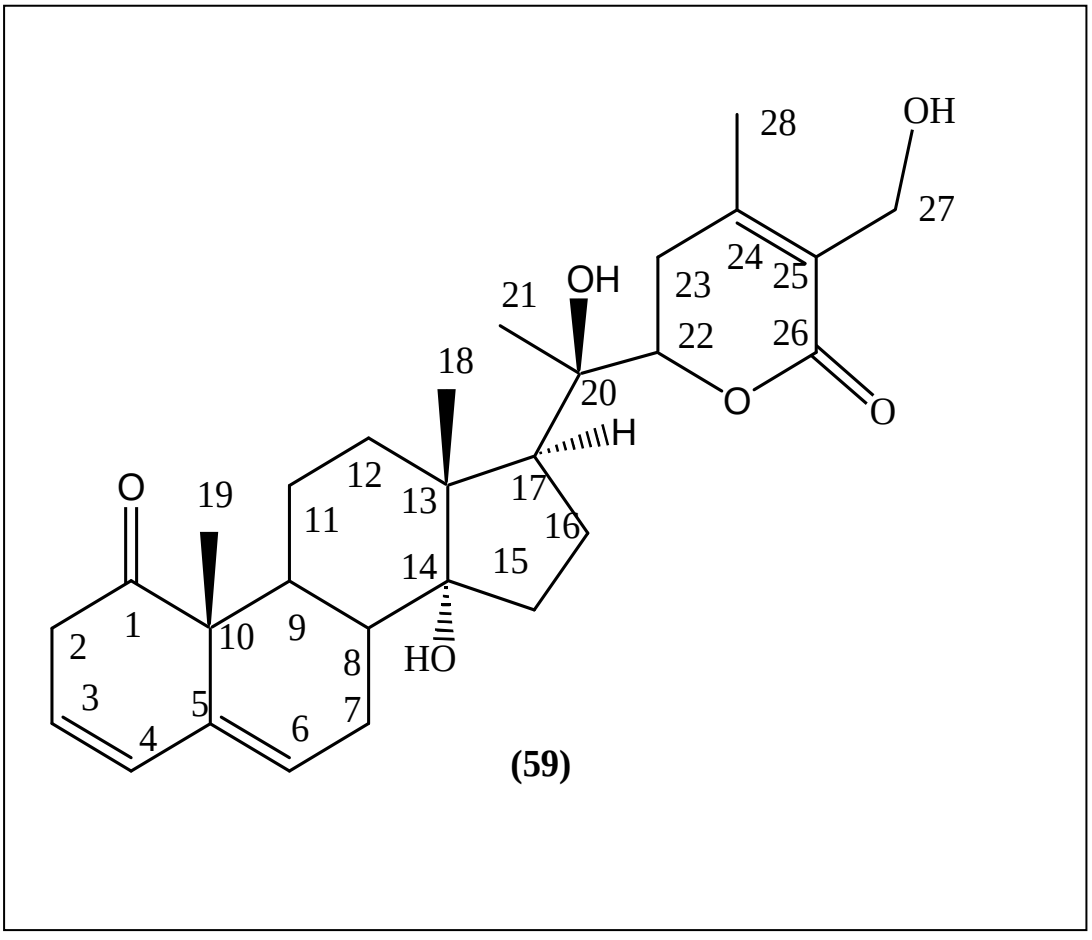
3.1.2.9: 1-Oxo-14, 20α,27-trihydroxy-20R,22R-witha-3,5,24- trienolide (59) a known Withanolide

Fraction A was loaded on silica gel CC (hexane/acetone 15:1, 10:1, 5:1, 2:1, 1:1) and obtained six fractions (AI-6). Sub-fraction A6 was further subjected to RP-18 CC (MeOH/water 5.7:4.3), resulted an optically active amorphous solid (**59**). Fractionation scheme is given in experimental section (Fig 4.1). The UV spectrum of the compound showed a characteristic absorption at 226 nm, indicating α, β-unsaturated lactone chromophore as stated earlier. Similarly the compound (**59**) showed absorption bands in IR spectrum at, 3400, 1700 and 1684 cm^{-1} indicating, hydroxyl, a α,β -unsaturated lactone and cyclic ketone respectively. The molecular formula of the compound (**59**) was determined as $C_{28}H_{38}O_6$ by its HR-ESI-MS from the molecular ion peak $[M-H]^-$ at 469.2583.

The ^{13}C NMR of compound (**59**) showed the resonance all the 28 carbons of steroidal skeleton having methyl, methylene, methine and quaternary carbons as well. The olefinic signals at δ 121.3, 130.1, 139.5, 128.1, 149.4 and 120.9 were assigned to C-3 to C-6, C-24 and C-25 respectively. The H-NMR spectrum (Table 3.15) of

compound (**59**) showed four methyl singlets. The lowfield shift (δ 1.31) of C-21 methyl proved the presence of an oxygen function on C-20. The downfield signals at δ 5.62 (ddd, *J3, 2* = 10.3, *J3, 4b* = 4.9, *J3, 4a* =2.2 Hz), 6.06 (d, *J4, 3* = 10 Hz) and 5.58 (d, *J6, 7* = 5.2 Hz) were assigned to protons of olefinic nature H-3, H-4 and H-6 respectively.^{1}H NMR and ^{13}C NMR data of the compound (**59**) is presented in Table (3.15). On the basis of the above modern techniques such as UV, IR,NMR and mass spectra, the compound (**59**) was identified a 1-oxo-14α,, 20β, 27-trihydroxy-20R, 22R-witha-3,5,24- trienolide, previously reported from *W. somnifera* [123]

Compound **(59)** showed potent inhibitory activities of both ConA-induced T (IC_{50}< 11 μM) and LPS-induced B (IC_{50}< 12 μM) cell proliferation (Sec. 11.7)



(59)

Table 3.15: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compounds **(59)** in C_5D_5N

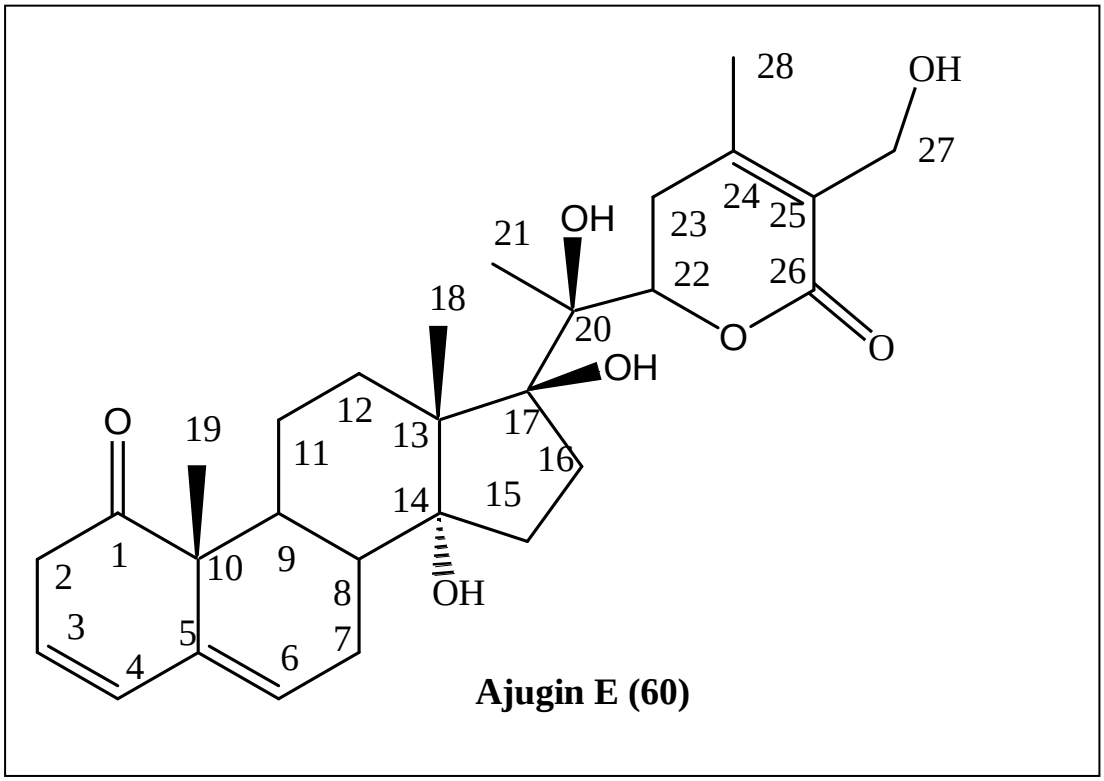
C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	210.1	C	-
2	40.0	CH_2	2.76 (dd *J2, 3* = 9.91, *J2, 4a* = 2.31)
3	121.8	CH	5.62 (ddd, *J3,2* = 10.1, *J3,4b = 4.9*, *J3, 4a* = 2.2)
4	130.1	CH	6.06 (d, *J4, 3* = 10.0)
5	139.5	C	-
6	128.1	CH	5.58 (d, *J6,7* = 5.2)
7	27.1	CH_2	2.0 *m*
8	36.9	CH	2.1 *m*
9	37.2	CH	2.17*m*
10	52.1	C	-
11	22.8	CH_2	2.08 *m*
12	41.3	CH_2	2.15 m
13	49.9	C	-
14	83.4	C	-
15	32.5	CH_2	3.65 *m*
16	22.0	CH_2	2.73 *m*
17	56.3	CH	2.0 *m*
18	18.7	CH_3	1.22 *s*
19	20.3	CH_3	1.15 *s*
20	75.1	C	-
21	21.0	CH_3	1.22 *s*
22	82.7	CH	4.93 (dd, *J22, 23a* = 12.7, *J22, 23b* = 3.5)
23	32.7	CH_2	2.15 m
24	149.4	C	-
25	120.9	C	-
26	166.4	C	-
27	56.4	CH_2	4.37 *s*
28	20.1	CH_3	1.94 s

3.1.2.10: Ajugin E (60)

Fraction A was loaded on silica gel CC and on elution with hexane/acetone (15:1, 10:1, 5:1, 2:1, 1:1) afforded six fractions.(AI-6). Sub-fraction A5 was further purified by RP-18 CC (MeOH/water 5.5:4.5) has resulted an optically active amorphous solid (**60**). Fractionation scheme is given in experimental section (Fig 4.1)

The UV spectrum of the compound showed a characteristic absorption at 223 nm, indicating α,β-unsaturated lactone chromophore as stated earlier. Similarly the The IR spectrum displayed bands at,3425, 1720 and1660 cm^{-1} indicating, hydroxyl, unsaturated lactone and double bond functionalities respectively at. The molecular formula was established as $C_{28}H_{38}O_7$ by its HR-ESI-MS from the molecular ion peak $[M+H]^+$ at 487.2583.

The ^1H NMR and ^{13}C NMR spectral data of **(60)** showed close resembelence with withanolide F (**52)** and its isomer **(53)** and the difference in ^{13}C NMR appeared at the missing of methyl group at C-28 (δ 20.1 which was replaced by hydroxy methlene group (δ 56.1).The ^{13}C NMR of compound (**60**) showed the resonance all the 28 carbons of steroidal skeleton having methyl, methylene, methine and quaternary carbons as well. The carbon resonating at *δ* 210.6 was assign to carbonyl (C-1). The H-NMR spectrum (Table 3.15) of compound (**60**) showed four methyl singlets. The downfield olefinic signals at δ 5.82 *(m)* and 5.96 (d, *J4, 3* = 9.8 Hz) were assigned to vinylic vicinal protons H-3, H-4 and H-6 respectively. The ^1H NMR and ^{13}C NMR data of the compound (**60**) is presented in table 3.16. On the basis of the above modern techniques such as UV, IR,NMR and mass spectra, the compound (**60**) was identified as Ajugin E, previously reported from *Ajuga parviflora* [254]. Ajugin E **(60)** was found the weak inhibitior of both ConA-induced T cell proliferation (IC_{50} = 49.2μM) and LPS-induced B cell proliferation (IC_{50} = 45.1μM), (Sec. 11.7).



Ajugin E (60)

Table 3.16: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compounds **(60)** in C_5D_5N

C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	210.6	C	-
2	39.0	CH_2	2.52 (dd *J2, 3* = 9.91, *J2, 4a* = 2.31)
3	127.8	CH	5.82 *(m)*
4	129.1	CH	5.96 (d, *J4, 3* = 9.8)
5	140.5	C	-
6	121.1	CH	5.62 (d, *J6, 7* = 5.1)
7	25.1	CH_2	2.1 m
8	34.9	CH	2.2 m
9	35.2	CH	2.01 m
10	53.1	C	-
11	21.8	CH_2	1.98 m
12	34.3	CH_2	1.96 m
13	51.9	C	-
14	83.4	C	-
15	32.5	CH_2	3.85 m
16	37.0	CH_2	2.53 m
17	86.3	CH	2.15 m
18	18.7	CH_3	1.15 s
19	20.3	CH_3	1.26 s
20	78.1	C	-
21	20.0	CH_3	1.22 s
22	80.7	CH	4.93 (dd, *J22, 23a* = 12.7, *J22, 23b* = 3.5)
23	32.7	CH_2	2.15 m
24	154.4	C	-
25	125.9	C	-
26	166.4	C	-
27	56.4	CH_2	4.27 s
28	20.1	CH_3	2.04 s

3.2: Withasteroids from *Physalis divericata*

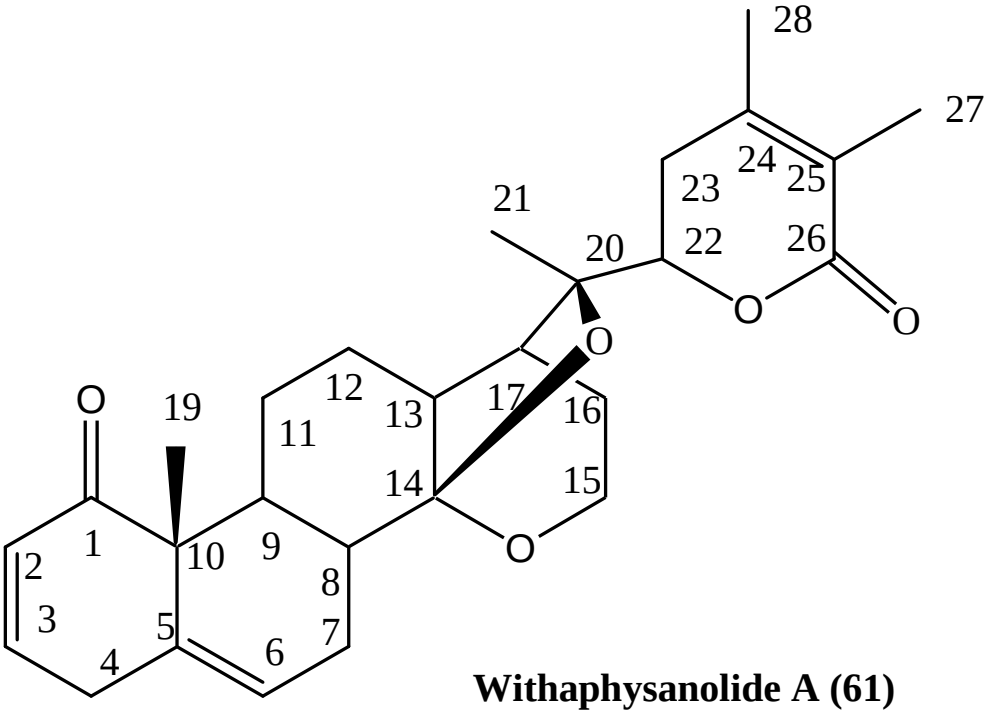
Withaphysanolide A, a novel withanolide, together with five known physalins and four withaphysalins were isolated from the whole plant of *P. divericata* plant of Pakistani origin. Various experimental techniques and extensive spectroscopic studies were used for the structural elucidation of these compounds. Most of the isolated withanolides showed inhibition activity against human colorectal carcinoma HCT-116 and human non-small cell lung cancer NCI-H460 cells. The isolation, structural elucidation of these compounds is discussed in this chapter. The extraction and isolation procedures are discussed in detail in the experimental section while cytotoxicity discussed in bioactivity section.

The aerial parts of *P. divericata* were collected from the Swat District of N.W.F.P during September 2005. The dry powdered plant materails (5 kg) of the same plant were extracted with ethanol. The ethanolic extract was then filtered and concentrated under vacuum, resulted a crude residue (1.8 kg) after evaporation. The residue was suspended in water and chroform. The chloroform fraction (197 g) was loaded on CC (D-101 porous resin) and eluted with ethanol/water 1:3, 3:1, 9.5:0.5) to provide three fractions (A-C). Successive fractionation and purification of fraction B resulted ten pure compounds, withaphysanolide A **(61)** together with five known physalins **(62-66)** and four known withaphysalins **(67-70).**

3.2.1: New Withasteroids from *Physalis divericata*

3.2.1.1: Withaphysanolide A (61), a novel withanolide

Fraction B was subjected to CC (MCI gel CHP 20P) and eluted with water/acetone (1:1) which afforded four sub-fractions (B1-4). Sub-fraction B3 was further subjected to RP-18 CC (MeOH/$H_2$0 6.0:4.0), resulting in Withaphysanolide A **(61),** an optically active colorless crystal (9 mg). Fractionation scheme is given in experimental section (Fig 4.2). Withaphysanolide A (**61**), a novel withanolide was isolated in the form of colorless cubic crystals. The molecular formula of **61** was determined as $C_{27}H_{34}O_5$ from its HREI MS ([M]+ at *m/z* 438.25. UV showed absorption at 224nm, indicating presence α,β-usaturated lactone chromophore[250]. The IR spectrum showed absorption at 1706 cm^{-1} and 1683 cm^{-1} indicating the presence of carbonyl group of lactone and α,β -unsaturated ketone moieties[176,250].



Withaphysanolide A (61)

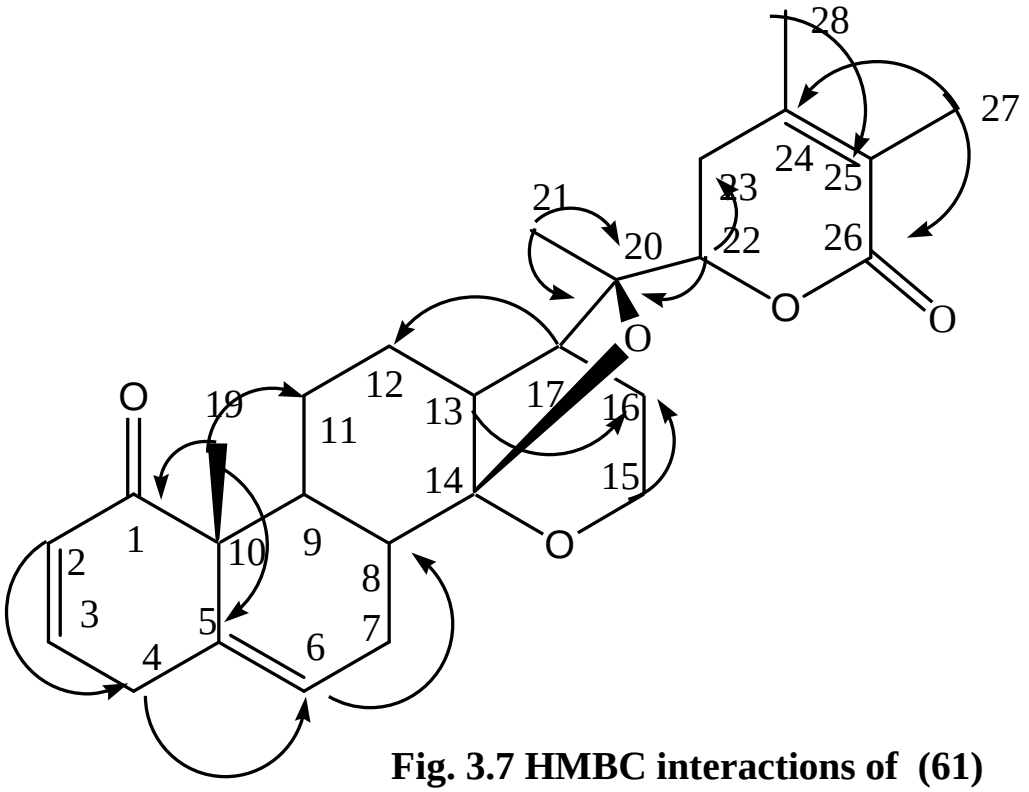


Fig. 3.7 HMBC interactions of (61)

The of ^{13}C NMR and DEPT spectral data of the compound **61** (Table 3.17), showed the presence of four CH_3 groups, seven CH_2, and eight CH and eight quaternary carbons. The signals at δ 127.80, 145.40, and 125.30, were the assign to the olefinic methines (C-2, C-3 & C-6 respectively) and at *δ* 78.2 (C-22) was to one oxygenated methine. Out of eight quaternary carbon atoms three were olefinic (Sp2 hybridized) giving peaks at δ 134.13, 147.40 and 122.6 (C-5, C-24 & C-25 respectively), two were carbonyl carbons resonating at *δ* 165.8 (C-26) and 204.1 (C-1), showed the presence of an α, β -unsaturated lactone and ketone functionalities and signal appearing at δ 83.8 was assigned to the quaternary carbon having oxygen function (C-20). The methine signal at δ 46.6 (C-13) indicated the absence of methyl (C-18) on that position and hence showing novel skeleton of C-27 norwithasteroid.

The ^{1}H NMR spectrum of the compound **61** (Table 3.17) diclosed the signals for four methyl groups at δ 1.12, 1.37, 1.77 and 1.84 (C-19, C-21, C-27 & C-28 respectively). The downfield signals δ 1.89 and 1.93 of methyls (C-27 & C-28) is the sign of their location on olefinic carbons (C-24 & C-25). The peaks at δ 5.84 (d, *J* = 10.1 Hz) and 6.77 (ddd, *J* = 10.10, 5.0, and 2.4 Hz) were attributed to the H-2 and H-3 olefinic vicinal protons, respectively. The ^{1}H NMR spectrum also disclosed a methylene hydrogen (H-4) by giving signal at δ 3.26 (dd, *J* = 21.41, 2.5 Hz) and 2.82 (dd, *J* = 21.4, 5.1 Hz). The peak at δ 5.58 (dd, *J* = 5.2, 2.4 Hz) was assigned to the last olefinic proton (H-6). This showed the presence of\ 2,5-dien-1-one system at the A/B ring moiety. A double doublet at δ 4.83 (*J* = 13.21 and 3.11 Hz), the lowfieled signals of two methyls at δ 1.93 (C-28) and 1.89 (C-27) in the ^{1}H NMR spectrum and signal at δ 165.8 (C-26) in ^{13}C NMR spectrum indicated the presence of an α,β -unsaturated lactone side chain of the withasteroids.

By comparing the withaphysanolide A with typical withanolide skeleton, the main differences were observed in rings C and D. The signal for methyl (C-18) could not be found in the specctra and hence disappeared, which might have been decarboxylated during biosynthesis from a withaphysalin structure (Scheme 3.1). The proton of methyl (C-21) which gives a singlet at (δ 1.37), showed a HMBC correlation (Fig 3.7) with the C-20 appeared at δ 83.9 and the correlation of H-15 (δ 3.85) with C-14 (δ 104.9) indicated a novel ring D possessing the C(14)–O–C(15) moiety. The ring D found as 4H-pyran ring instead of a typical five-membered ring found in withaphysalin skeleton was further confirmed through the HMBC correlations between between H-21 and C-17, H-15 and C-16 and between H-17 and C-12. These

assignments were also confirmed by X-ray diffraction analysis (Fig.3.8). Biogenetically, the chiral center at C(10) of withanolides was R onfiguration[231] so the molecule has several asymmetric centers with the following configurations: C-8 R, C-9 S, C-10 R, C-13 R, C-14 S, C-17 S, C-20 R, C-22 R. Further evidences for unambiguous structural assignment came through proposing a possible biogenetic pathway for withaphysanolide A **(61)** as shown in scheme 3.1. Withaphysanolide A **(61)** might be derived by sequential cleavage via C(14)–C(15) and C(18)–O–C(20), decarboxylation of C (18) and cyclization via C(14)–O–C(20) bridge and C(14)–O–C(15) bridge formation from the biogenetically acceptable withaphysalin A **(67),** which was isolated from the same plant. Consequently, the structure of **(61)** was unambiguously established based on evidences from spectroscopic data, X-ray crystallographic analysis and the proposed biogenetic pathway and the novel compound was named as withaphysanolide A.

Compound **61** showed moderate cytotoxicity against human against two tumer cell lines, the and human non-small cell lung cancer NCI-H460 and colorectal carcinoma HCT-116 cells (Section *12.6*)

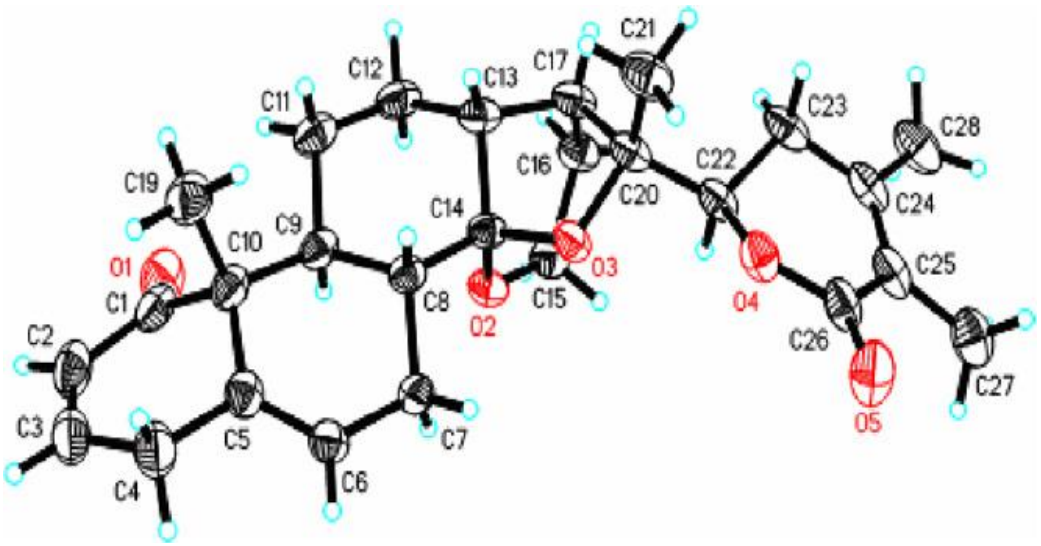
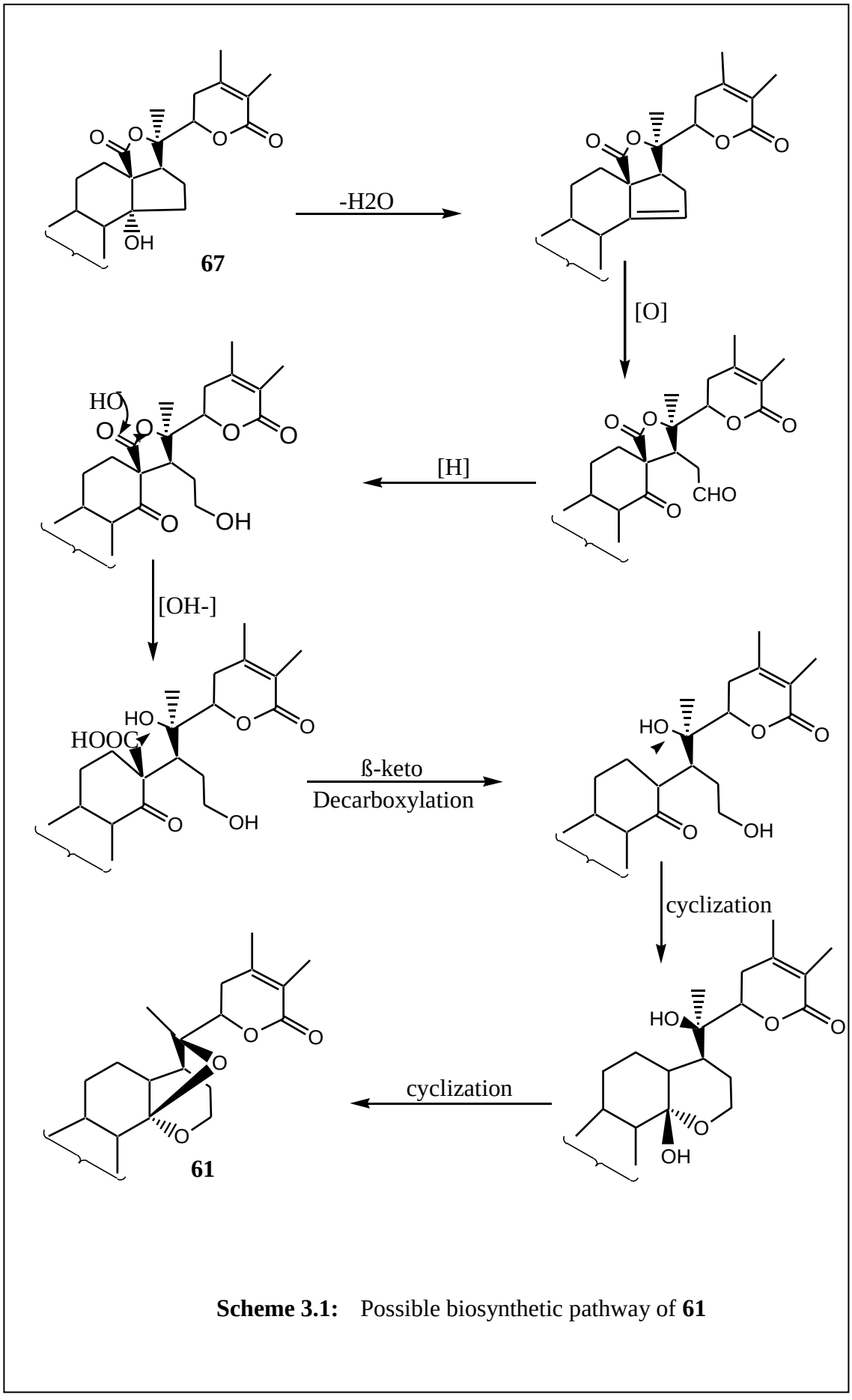


Fig. 3.8: X-ray structure of **61** showing relative configuration



Scheme 3.1: Possible biosynthetic pathway of **61**

Table-3.17: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound **(61)** in $CDCl_3$

C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	204.1	C	-
2	127.8	CH	5.85 (d, *J2 3* = 10.0)
3	145.4	CH	6.77 (ddd, *J3*, *4a* = 10, *J3*, *4b* = 5.0, *J3*,*2* = 2.4)
4	33.4	CH_2	2.81 (dd, *J4a*,*3* = 21.3, *J4a*, *2*= 5.0)
			3.29 (dd, *J4b*, *3* = 21.3, *J4b*, *2* = 2.3)
5	134.1	C	-
6	125.5	CH	5.56 (dd, *J6*, *7a* = 5.3, *J6*, *7b* = 2.4)
	23.0	CH_2	2.11 – 2.18 (m)
			1.59 – 1.66 (*m*)
8	43.5	CH	1.46 – 1.53 (*m*)
9	38.5	CH	2.06 – 2.14 (*m*)
10	50.4	C	-
11	28.3	CH_2	2.58 – 2.91 (*m*)
			1.21 – 1.27(*m*)
12	22.9	CH_2	2.00 – 2.05 (*m*)
			1.83 – 1.89 (*m*)
13	46.1	CH	1.58 – 2.06 (*m*)
14	104.9	C	-
15	59.3	CH_2	3.83 – 3.89 (*m*)
16	21.4	CH_2	1.98 – 2.06 (*m*)
			1.34 – 1.41 (*m*)
17	42.1	CH	1.98 – 2.06 (*m*)

19	18.7	CH_3	1.12 (*s*)
20	83.8	C	-
21	20.6	CH_3	1.37 (*s*)
22	78.2	CH	4.83 (dd, *J22*,*23a* = 13.1, *J22*, *23b* = 3.1)
23	32.8	CH_2	2.18 – 2.23 (*m*)
			2.48 – 2.54 (*m*)
24	147.4	C	-
25	122.6	C	-
26	165.5	C	-
27	12.5	CH_3	1.88 (*s*)
28	20.3	CH_3	1.94 (*s*)

a: Broad band; b: DEPT; c: ^{1}H NMR; d: HMQC interaction

3.2.2: Known Withasteroids from *Physalis divericata*

3.2.2.1: Physalin A (62)

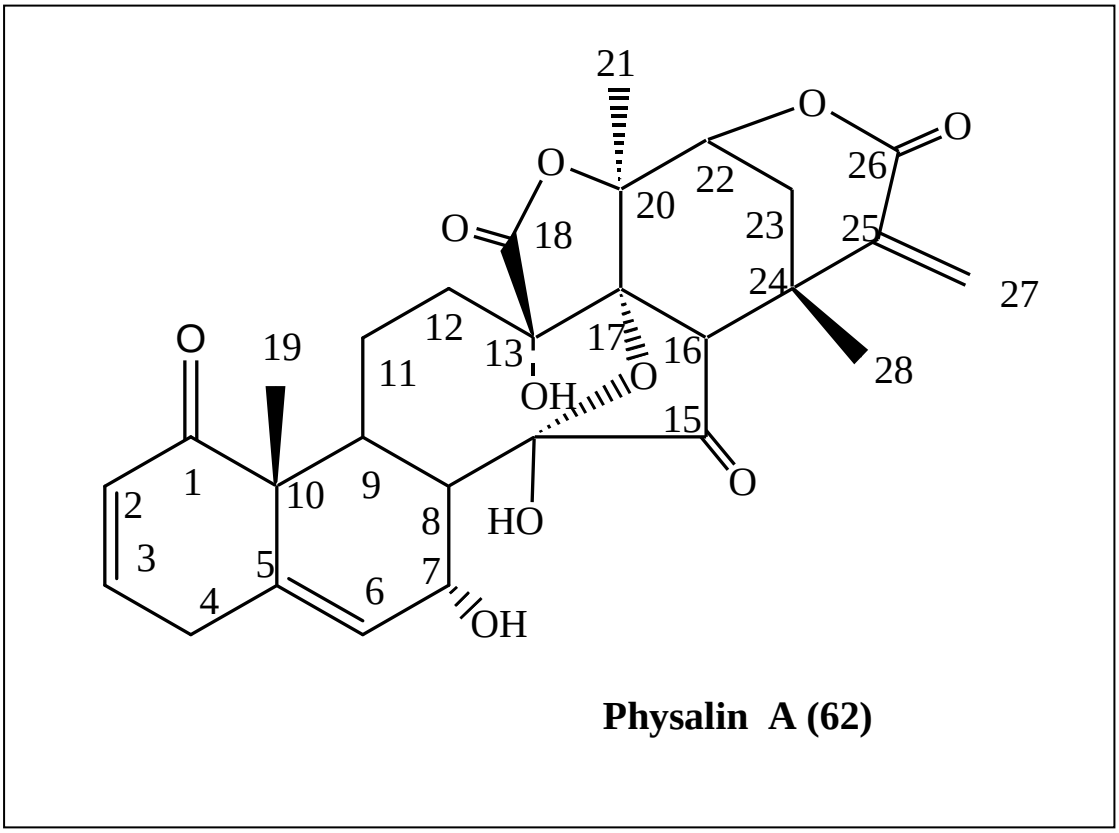
Fraction B was subjected to CC (MCI gel CHP 20P) and eluted with water/acetone (1:1) which afforded four sub-fractions (B1-4). Sub-fraction B2 was further rpurified on RP-18 CC (MeOH/H_2O 5.0:5.0) to yield Physalin A **(62)** an optically active amorphous solid (19 mg) along with other compounds. Fractionation scheme is given in Experimental section (Fig 4.2). The UV spectrum of **(62)** like other withanolide showed a characteristic absorption at 221 nm, indicating α, β-unsaturated lactone chromophor. Similarly the IR absorption bands at 3426, 1721 and 1663 cm^{-1} indicating, hydroxyl, a, α,β-unsaturated lactone and olefinic functionalities respectively as the characteristic peaks of withanolides[250]. The molecular formula $C_{28}H_{30}O_{10}$ was established by HR-ESI-MS from the molecular ion peak $[M]^+$ at 527.2583.

The ^1H and ^{13}C NMR spectral data of compound **62** (Table 3.18) showed similarities with those of Physalin B (**63**) and difference between them was the peak at δ 61.3 (C-7) in ^{13}C NMR spectrum indicated the presence of hydroxyl group in compound (**62**) and characteristic peak of olefinic methylene H_2C-27 (δ_C 132.6 and δ_H 5.58 *s*) The ^{13}C NMR of compound (**62**) showed the resonance of all 28 carbons of the steroidal skeleton having methyl, methylene, methine and quaternary carbons as well. The signal at δ 201.12 (C-1) and 213.32 (C-15) indicated ketonic carbonyl carbons whereas the signals at δ 171.8 and 161.5 (C-18 & C-26 respectively) showing carbonyl carbons of lactone moiety. The signals at δ 126.3 and 146.4 were assigned to olefinic carbons C-2 and C-3 respectively. The ^1H-NMR spectrum of **62** (Table 3.18) disclosed three methyl singlets. The low field shift (δ 1.71) of methyl (C-21) indicating the presence of oxygen on C-20. The downfield signals at δ 5.84 (dd, *J2, 3* = 9.99, *J2, 4a* = 2.02 Hz), 6.90 (ddd, *J3, 2* = 9.92, *J3, 4b* = 4.92, *J3, 4a* = 2.22 Hz) and 5.68 (dd, *J6, 7* = 6.35, *J6, 8* = 1.85 Hz) were attributed to proton of olefinic region H-2, H-3 and H-6 respectively. The^1H NMR and ^{13}C NMR data of compound **(62)** is presented in table (3.18). The above spectroscopic techniques such as Mass, UV, IR and NMR spectra disclosed the structure of the compound **(62)** and identified as Physalin A previously reported from *P. alkekengi* [165,188]. Compound **62** also showed strong

cytotoxicity against human two against two tumer cell lines, the human non-small cell lung cancer NCI-H460 cells and colorectal carcinoma HCT-116 cells (Section *12.6*)

Table-3.18: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound **(62)** in $CDCl_3$

C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	201.1	C	-
2	126.8	CH	5.84 (dd, *J2, 3* = 10.0, *J2, 4b* = 2.0)
3	146.4	CH	6.93(ddd, *J3, 2* = 10, *J3, 4a* = 5.0, *J3, 4b*= 2.4)
4	31.4	CH_2	2.90 (dd, *J4a, 4b* = 20.3, *J4a, 3* = 5.0)
			3.29 (dd, *J4b, 4a* = 21.3, *J4b, 3* = 2.3)
5	139.1	C	-
6	127.5	CH	5.69 (dd, *J6, 7* = 6.32, *J6, 8* = 1.83)
7	61.4	CH	4.47 (t, *J = 5.0)*
			5.0 (d, *J7,*OH = 5.0)
8	46.5	CH	1.92 (dd, *J8, 9* = 12.3, *J8, 11* = 1.3)
9	28.5	CH	2.98 (dd, *J9, 8* = 12.3, *J9, 11b* =9.0)
10	53.4	C	-
11	24.3	CH_2	2.08 (*m*)
			1.10(*m*)
12	29.9	CH_2	2.27 (*m*)
			1.45 (*m*)
13	81.1	C	5.58 (*s*)(OH)
14	102.9	C	6.28 (*s*)(OH)
15	213.3	C	-
16	52.4	CH	2.98 (*s*)
17	79.1	C	-
18	171.8	C	
19	13.7	CH_3	1.02 (*s*)
20	81.8	C	-
21	21.6	CH_3	1.7 1(*s*)
22	75.2	CH	4.83 (dd, *J22, 23a* = 13.1, *J22, 23b*= 3.1)
23	30.8	CH_2	2.09 (*m*)
			2.14 (*m*)
24	35.5	C	-
25	137.5	C	-
26	161.5	C	-
27	132.6	CH_2	5.58 (*s*)
28	26.3	CH_3	1.56(*s*)



Physalin A (62)

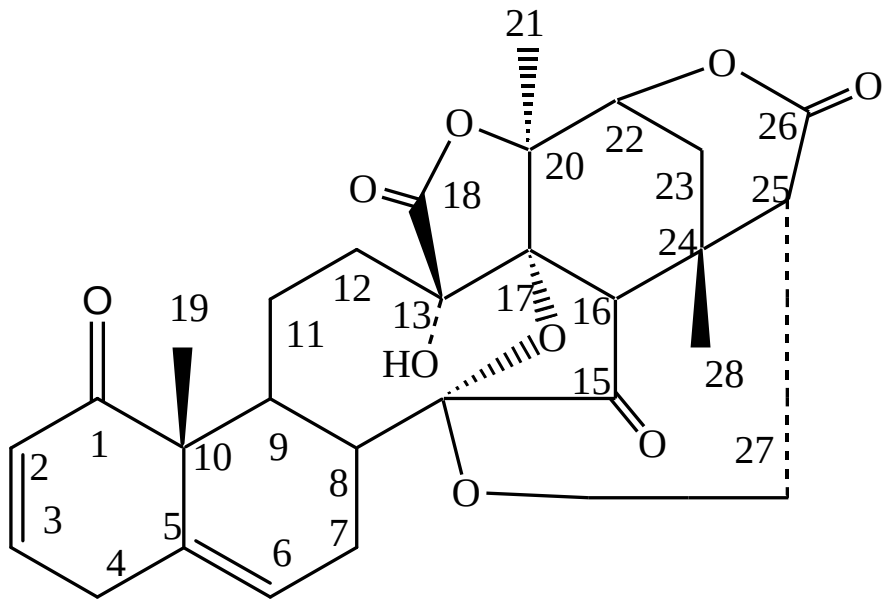
3.2.2.2: Physalin B (63)

Fraction B was loaded on CC (MCI gel CHP 20P) and eluted with water/acetone (1:1) which afforded four sub-fractions (B1-4). Sub-fraction B4 was subjected to CC (Sephadex LH-20; MeOH) to yield Physalin B **(62)** an optically active amorphous solid (120 mg) along with other compounds. Fractionation scheme is given in experimental section (Fig 4.2). The characteristic UV absorption at 225 nm was observed in the spectrum of **63**. The IR spectrum of **(63)** displayed bands a complicated absorption between 1600 and 1800 cm^{-1} showing existence of several carbonyl functions including five and six member lactone rings. The molecular formula of compound **63** was found as $C_{28}H_{30}O_9$**,** established by its HR-ESI-MS from the molecular ion peak $[M]^+$ at 511.2583.

The ^1H and ^{13}C NMR spectral data of compound (**62**) and (**63**) (Tables 3.18 & 3.19) showed much closed similarities. The difference between them was due to the absence of oxygen bearing methine in compound (**63)** giving peak at δ 61.45 (C-7) in ^{13}C NMR spectrum and also replacement of olefinic methylene (C-27) by saturated one giving peaks at δ 3.60 (dd, *J27a, 27b* = 14.0, *J27a, 25*= 4.0 Hz) and 4.26 (dd, *J27b, 27a* = 14.0, *J27b, 25* = 4.0 Hz) in^1H NMR. The ^{13}C NMR of compound (**63**)

showed the resonance of all 28 carbons of the steroidal skeleton having methyl, methylene, methine and quaternary carbons as well. The peaks at δ 202.1 (C-1) and 209.3 (C-15) indicated ketonic carbonyl carbons whereas the signals at δ 171.8 and 167.5 (C-18 & C-26 respectively) showing carbonyl carbons of lactone moiety. The quaternary carbon (C-14) resonating at δ 106.9 indicating its attachment to more than one oxygen functions. The signals at δ 126.8 and 146.4 were due to the olefinic carbons (C-2 and C-3, respectively). The H-NMR spectrum (Table 3.19) of compound **(63)** also disclosed three methyl singlets as discussed ealier. The lowfield shift (δ 1.37) of methyl (C-21) indicating the presence of oxygen function on C-20. The downfield signals at δ 5.82 (dd, *J2, 3* = 10.06, *J2, 4b* = 2.06 Hz), 6.87 (ddd, *J3, 2* = 10.7, *J3, 4a* = 5.04, *J3, 4b* = 2.44 Hz) and 5.69 (dd, *J6, 7* = 6.34, *J6, 8* = 1.84 Hz) were assigned to protons of olefinic center H-2, H-3 and H-6 respectively.[1]H NMR and [13]C NMR data is presented in table (3.19). The above mentioned spectroscopic techniques such as Mass, UV, IR and NMR spectra disclosed the structure of the compound **(63)** was identified as Physalin B previously reported from *P. alkekengi* [167,170]

Compound **63** also showed remarkable cytotoxicity against human two against two tumer cell lines, the human non-small cell lung cancer NCI-H460 cells and colorectal carcinoma HCT-116 cells (section *12.6*)



Physalin B (63)

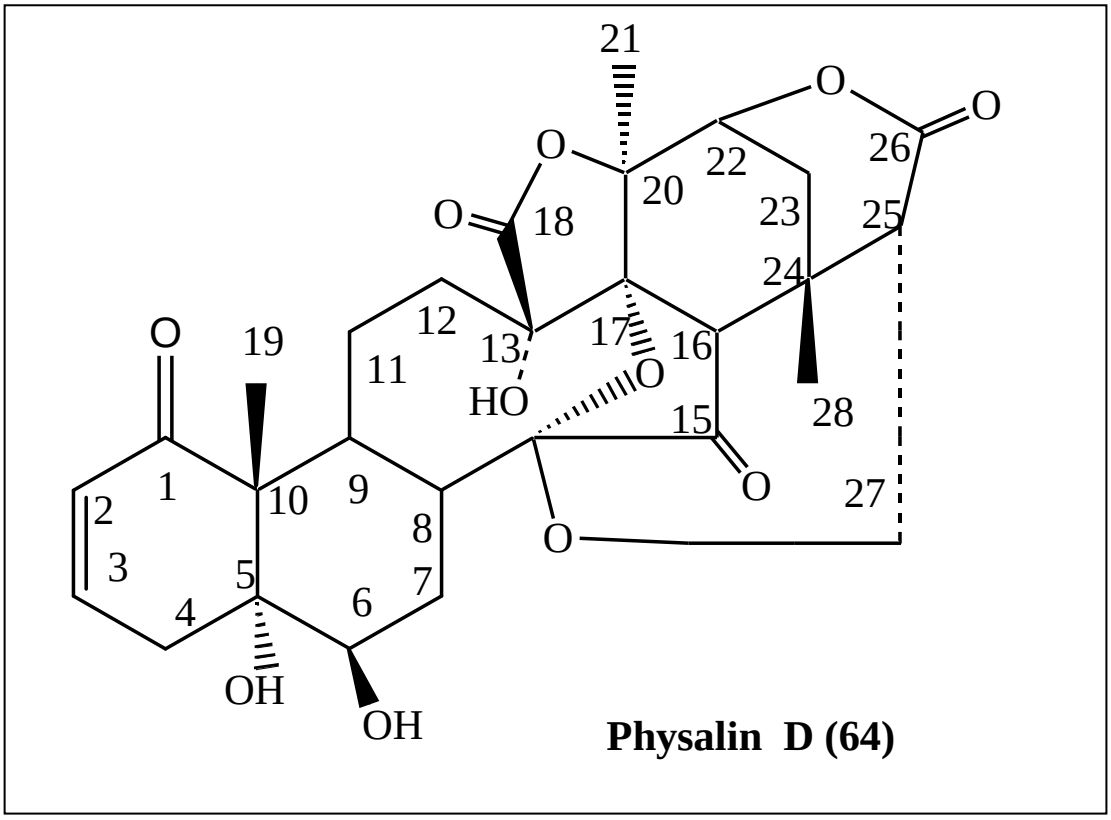
Table-3.19: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound **(63)** in $CDCl_3$

C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	202.1	C	-
2	126.8	CH	5.80 (dd, *J2, 3* = 10.0, *J2, 4b* = 2.0)
3	146.4	CH	6.89 (ddd, *J3, 2* = 10, *J3, 4a* = 5.0, *J3, 4b*= 2.4)
4	32.4	CH_2	2.89 (dd, *J4a, 4b* = 21.3, *J4a, 3* = 5.0)
			3.29 (dd, *J4b, 4a* = 21.3, *J4b, 3* = 2.3)
5	135.1	C	-
6	123.5	CH	5.59 (d, *J6, 7b* = 6.3)
7	24.4	CH_2	1.97 (m)
			2.21 (*m*)
8	40.5	CH	1.92 (*m*)
9	33.5	CH	2.92 (dd, *J9,8* = 11.0, *J9,11b* = 9.0)
10	52.4	C	-
11	24.3	CH_2	2.18 (*m*)
			1.10 (*m*)
12	25.9	CH_2	2.17 (*m*)
			1.45 (*m*)
13	78.1	C	6.28 (*s*)(OH)
14	106.9	C	-
15	209.3	C	-
16	54.4	CH	2.86 (*s*)
17	80.1	C	-
18	171.8	C	
19	16.7	CH_3	1.12 (*s*)
20	80.8	C	-
21	21.6	CH_3	1.37 (*s*)
22	76.2	CH	4.83 (dd, *J22, 23a* = 13.1, *J22, 23b* = 3.1)
23	31.8	CH_2	1.91 (*m*)
			2.14 (*m*)
24	30.5	C	-
25	49.5	CH	2.88 (d, *J25, 27b* = 4)
26	167.5	C	-
27	60.6	CH_2	3.60 (dd, *J27a, 27b*= 14.0, *J27a, 25* = 4.0)
			4.26 (dd, *J27b, 27a*= 14.0, *J27b, 25* = 4.0)
28	20.3	CH_3	1.1 6 (*s*)

3.2.2.3: Physalin D (64)

Fraction B was subjected to CC (MCI gel CHP 20P) and eluted with water/acetone (1:1) which afforded four sub-fractions (B1-4). Sub-fraction B1 was further purified by RP-18 CC (MeOH/$H_2$0 4.0:6.0) to yield Physalin D **(64)** an amorphous solid (14 mg) along with other compounds. Fractionation scheme is given in experimental section (Fig 4.2)

The characteristic UV absorption at 225 nm was observed in the spectrum of **64** and indicating α,β-unsaturated lactone chromophore. The IR spectrum of **64** displayed a complicated absorption between 3400, 1790, 1757, 1732 and 1640 cm^{-1} showing existence of several carbonyl function including five and six membered lactone rings. The molecular formula of compound **63** was found as $C_{28}H_{32}O_{11}$**,** established by its HR-ESI-MS from the molecular ion peak $[M]^+$ at *m/z* 544 .2583. The ^1H and ^{13}C NMR spectral data of compound **64** and **63** (Table 3.20) showed much closed similarities. The difference between them was observed in ring B where the olefinic signals were replaced by oxygen bearing quaternary carbon C-5 (δ 82.3) and methine C-6 (δ 78.6). Furthermore broad singlets in ^1H NMR at δ 4.25 and 5.74 were assigned to hydroxyl groups attached to C5 and C-13 respectively.



Physalin D (64)

Table-3.20: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound **(64)** in $CDCl_3$

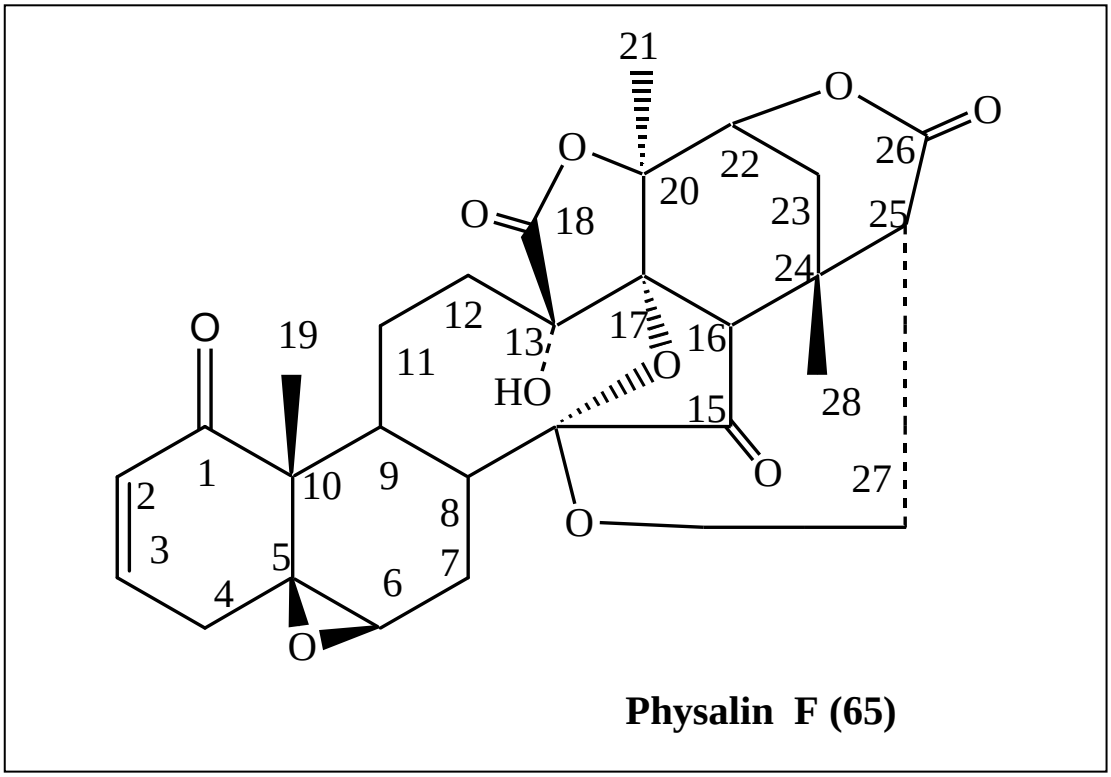
C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	205.1	C	-
2	126.8	CH	5.77 (dd, *J2, 3* = 10.0, *J2, 4b* = 2.5)
3	148.4	CH	6.78 (ddd, *J3, 2* = 10.0, *J3, 4a* = 5.0, *J3,4b* = 2.4)
4	33.4	CH_2	1.96 (dd, *J4a, 4b* = 20.3, *J4a, 3*= 5.0) 3.41 (d, *J4b, 4a*= 20.3)
5	82.3	C	4.25 (*s*) (OH)
6	78.6	CH	3.58 (*m)*
7	28.4	CH_2	1.97 (*m*) 2.21 (*m*)
8	38.5	CH	2.20 (*m*)
9	33.5	CH	3.11 (*m*)
10	52.4	C	-
11	26.3	CH_2	2.28 (*m*)
12	25.9	CH_2	1.09 (*m*) 2.15 (*m*)
13	77.1	C	5.7 4 (*s*) (OH)
14	107.9	C	-
15	208.3	C	-
16	53.4	CH	2.72 (*s*)
17	81.4	C	-
18	172.8	C	
19	16.7	CH_3	1.12 (*s*)
20	81.8	C	-
21	22.6	CH_3	1.82 (*s*)
22	78.2	CH	4.57 (dd, *J22, 23a* = 13.1, *J22, 23b* = 3.1)
23	31.8	CH_2	1.98 (*m*) 2.14 (*m*)
24	31.5	C	-
25	48.5	CH	2.87 (d, *J25, 27b* = 4.5)
26	166.5	C	-
27	61.6	CH_2	3.55 (dd, *J27a, 27b* = 13.0, *J27a, 25* = 1.0) 4.26 (dd, *J27b, 27a* = 13.0, *J27b, 25* = 4.5)
28	19.5	CH_3	1.17 (*s*)

The ^{13}C NMR data (Table 3.20) of compound (**64**) showed the resonance of all 28 carbons of the steroidal skeleton having methyl, methylene, methine and quaternary carbons as well. The signal at δ 205.1 (C-1) and 208.3 (C-15) indicated ketonic carbonyl carbons whereas the signals at δ 172.8 and 166.5 (C-18 & C-26 respectively) showing carbonyl carbons of lactone moiety. The quaternary carbon (C-14) resonating at δ 107.9 indicating its attachment to more than one oxygen functions. The signals at δ 126.8 and 148.4 were due to the olefinic carbons (C-2 and C-3 respectively). The ^{1}H-NMR spectrum (Table 3.20) of compound **(64)** disclosed the peaks of three methyls as singlet. The lowfield shifts (δ 1.82) of C-21 methyl indicating the presence of oxygen function at C-20. The downfield signals at δ 5.77 (dd, *J2, 3* = 10.0, *J2, 4b* = 2.5 Hz) and 6.78 (ddd, *J3, 2* = 10.0, *J3, 4a* = 5.0, *J3, 4b* = 2.4 Hz) were assigned to vinylic protons H-2 and H-3 respectively. The ^{1}H NMR and ^{13}C NMR data of compound **(64)** is given in table (3.20). The above mentioned spectroscopic techniques such as Mass, UV, IR and NMR spectra disclosed the structure of the compound **(64)** was identified as Physalin D a known compound[221]. Compound **64** showed good cytotoxicity against human two against two tumer cell lines, the human non-small cell lung cancer NCI-H460 cells and colorectal carcinoma HCT-116 cells (Section *12.6*)

3.2.2.4: Physalin F (65)

Fraction B was subjected to CC (MCI gel CHP 20P) and eluted with water/acetone (1:1) which afforded four sub-fractions (B1-4). Sub-fraction B1 was further purified by RP-18 CC (MeOH/H_2O 4.0:6.0) to yield Physalin F **(65)** an amorphous solid (37mg). Fractionation scheme is given in Experimental section (Fig 4.2). The characteristic UV absorption at 225 nm was observed in the spectrum of **65** and indicating α,β-unsaturated lactone chromophore. The IR spectrum of **(65)** displayed bands a complicated absorption between 1600 and 1800 cm^{-1} showing existence of several carbonyl functions including five and six member lactone rings. The molecular formula of compound **63** was found as $C_{28}H_{30}O_{10}$**,** established by its HR-ESI-MS from the molecular ion peak $[M]^+$ at *m/z* 527.2583. The ^{1}H and ^{13}C NMR spectral data of compound (**64**) and (**65**) (Tables 3.20, 3.21) showed much closed similarities. The only difference between them was the absence of hydroxyl peak at δ 4.25 in (**65**). The ^{13}C NMR (Table 3.21) of compound (**65**) showed the presence of all

28 carbons of the steroidal skeleton having methyl, methylene, methine and quaternary carbons, resonating in the same manner as shown by (**64)**. The lowfield shift (δ 1.37) of C-21 methyl is indicating that an oxygen function present on C-20. The downfield signals at δ 5.89 (dd, *J2, 3* = 10.0, *J2, 4b* = 2.0 Hz) and 6.99 (ddd, *J3, 2* = 10.0, *J3, 4a* = 5.0, *J3,4b* = 2.4 Hz) were assigned to vinylic protons H-2 and H-3 respectively.^{1}H NMR and ^{13}C NMR data of compound **(65)** is given in table(3.21). The above mentioned spectroscopic techniques such as Mass, UV, IR and NMR spectra disclosed the structure of the compound **(65)** and identified as Physalin F a known compound[150,170]. Compound **65** exibited strong cytotoxicity against human two against two tumer cell lines, the human non-small cell lung cancer NCI-H460 cells and colorectal carcinoma HCT-116 cells (Table 12.17)



Physalin F (65)

3.2.2.5: Physalin H (66)

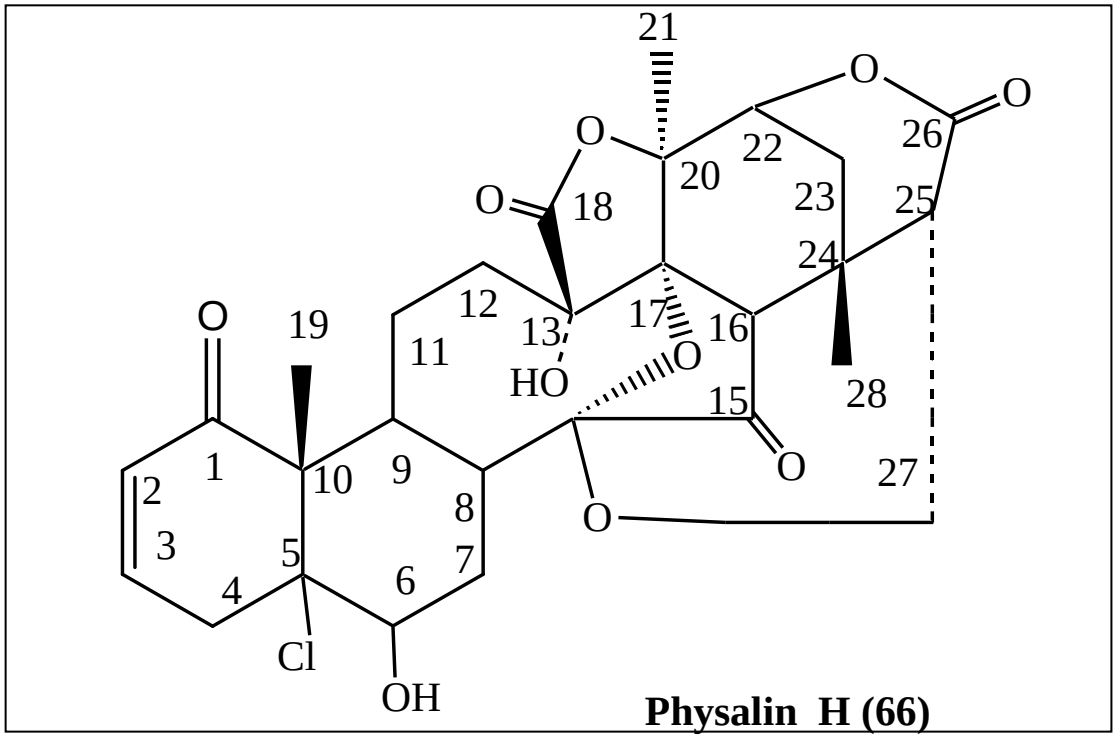
Fraction B was subjected to CC (MCI gel CHP 20P) and eluted with water/acetone (1:1) which afforded four sub-fractions (B1-4). Sub-fraction B1 was further purified by RP-18 CC (MeOH/$H_2$0 4.0:6.0) to yield Physalin H **(66),** an amorphous solid (40 mg) along with other compounds. Fractionation scheme is given in experimental section (Fig 4.2)

Table-3.21: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound **(65)** in $CDCl_3$

C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	202.1	C	-
2	126.8	CH	5.89 (dd, *J2, 3* = 10.0, *J2, 4b* = 2.0)
3	146.4	CH	6.99 (ddd, *J3, 2* = 10, *J3, 4a* = 5.0, *J3, 4b* = 2.4)
4	40.4	CH_2	3.09 (dd, *J4a, 4b*= 21.3, *J4a,3*= 5.0)
			3.29 (dd, *J4b, 4a*= 21.3, *J4b,3*= 2.3)
5	-	C	-
6	-	CH	5.59 (d, *J6, 7b* = 6.3)
7	24.4	CH_2	1.97 (m)
			2.21 (*m*)
8	40.5	CH	1.92 (*m*)
9	33.5	CH	2.92 (dd, *J9, 8* = 11.0, *J9, 11b* = 9.0)
10	52.4	C	-
11	24.3	CH_2	2.18 (*m*)
			1.10 (*m*)
12	25.9	CH_2	2.17 (*m*)
			1.45 (*m*)
13	78.1	C	6.28 (*s*)(OH)
14	106.9	C	-
15	209.3	C	-
16	54.4	CH	2.86 (*s*)
17	80.1	C	-
18	171.8	C	
19	16.7	CH_3	1.12 (*s*)
20	80.8	C	-
21	21.6	CH_3	1.37 (*s*)
22	76.2	CH	4.83 (dd, *J22, 23a* = 13.1, *J22, 23b* = 3.1)
23	31.8	CH_2	1.91 (*m*)
			2.14 (*m*)
24	30.5	C	-
25	49.5	CH	2.88 (d, *J25, 27b* = 4)
26	167.5	C	-
27	60.6	CH_2	3.60 (dd, *J27a, 27b* = 14.0, *J27a, 25* = 4.0)
			4.26 (dd, *J27b, 27a* = 14.0, *J27b, 25* = 4.0)
28	20.3	CH_3	1.16 (*s*)

The UV spectrum of **(66)** showed a characteristic absorption of α, β-unsaturated lactone at 226 nm.The IR spectrum of **(66)** displayed a complicated absorption between 3400, 1795, 1760, 1730, and 1670 cm^{-1} showing carbonyl function of ketones and lactones ring. The molecular formula of compound **63** was found as $C_{28}H_{31}O_{10}Cl$**,** established by its HR-ESI-MS from the molecular ion peak $[M]^+$ at *m/z* 562 .258. The ^1H and ^{13}C NMR spectral data of compound (**66**) and (**64**) (Tables 3.22 & 3.20) showed much closed similarities. The only difference between them was replacement of hydroxyl peak at δ 4.25 by chlorine at C-5 in (**66**).

The ^{13}C NMR (Table 3.22) of compound **66** diclosed the signals of all 28 carbons of the steroidal skeleton having methyls, methylenes, methines and quaternary carbons. The peaks ^{13}C NMR of (**66**) was almost similar to that observed in (**64**). The ^1H NMR spectrum (Table 3.21) of compound **(66)** displayed the signal of three methyls as singlets. The downfield signals at δ 5.83 (dd, *J2, 3* = 10.05, *J2, 4b* =2.55 Hz) and 66.78 (ddd, *J3, 2* = 10.1, *J3, 4a* = 5.05, *J3, 4b* = 2.4 Hz) were aasigned to the vinylic protons H-2 and H-3 respectively.^1H NMR and ^{13}C NMR data is given in table(3.22). The above mentioned spectroscopic techniques such as Mass, UV, IR and NMR spectra disclosed the structure of the compound **(66)** and identified as Physalin H a known compound[169,185]. Compound **66** showed strogest cytotoxicity against human two against two tumer cell lines, the human non-small cell lung cancer NCI-H460 cells and colorectal carcinoma HCT-116 cells (Section *12.6*). This migh be attributed to the presence of chlorine and hydroxyl moiety.



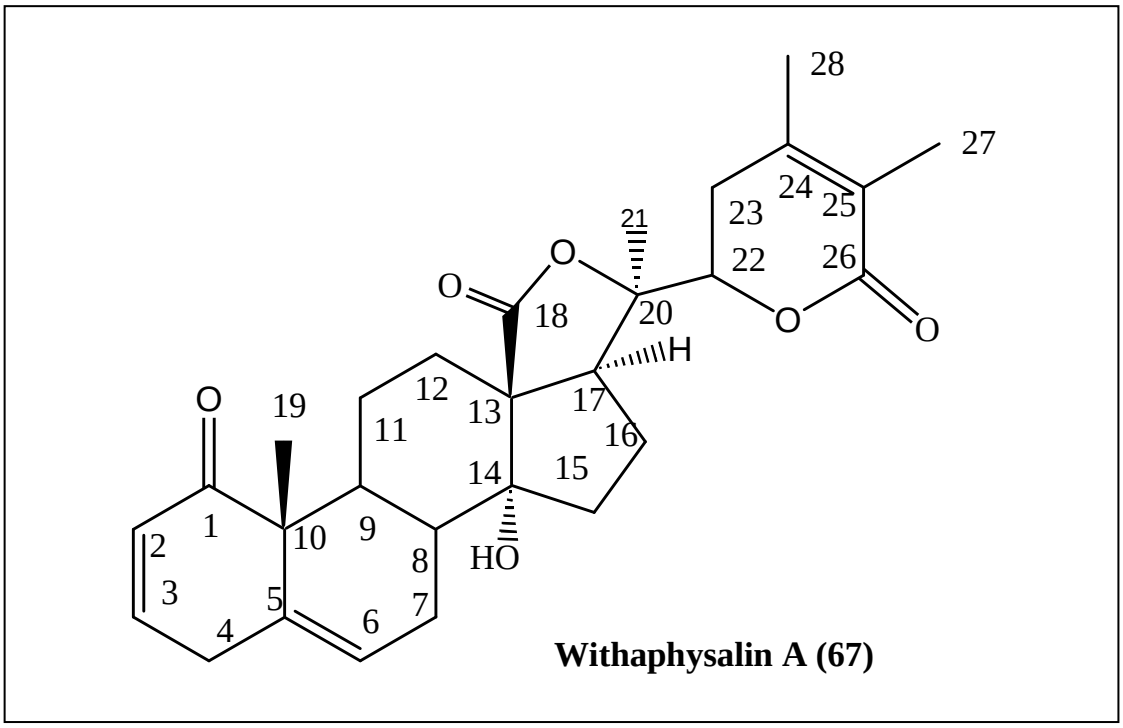
Physalin H (66)

Table-3.22: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound **(66)** in $CDCl_3$

C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	203.1	C	-
2	127.8	CH	5.83 (dd, *J2, 3* = 10.05, *J2, 4b* = 2.55)
3	142.4	CH	6.78 (ddd, *J3, 2* = 10, *J3, 4a* = 5.0, *J3,4b* = 2.4)
4	36.4	CH_2	2.46 (dd, *J4a, 4b* = 21.3, *J4a, 3* = 5.0)
			3.48 (dd, *J4b, 4a* = 21.3, *J4b, 3* = 2.3)
5	82.3	C	-
6	72.6	CH	3.88 (dt, *J6, OH* = 5.3, *J6, 7a* = 3.0, *J6, 7b* = 3.0)
			5.66 (d, *J6-OH* = 5)
7	26.4	CH_2	1.97 (m)
			2.21 (*m*)
8	39.5	CH	2.27 (*m*)
9	31.5	CH	3.55`(dd, *J9, 8* = 11.0, *J9, 11b* = 8.0)
10	53.4	C	-
11	24.3	CH_2	2.18 (*m*)
			1.00 (*m*)
12	25.9	CH_2	1.89 (*m*)
			1.45 (*m*)
13	78.1	C	6.04 (*s*) (OH)
14	106.9	C	-
15	209.3	C	-
16	52.4	CH	2.82 (*s*)
17	80.4	C	-
18	171.8	C	
19	15.7	CH_3	1.22 (*s*)
20	80.8	C	-
21	21.6	CH_3	1.87 (*s*)
22	76.2	CH	4.57 (dd, *J22, 23a* = 13.1, *J22, 23b* = 3.1)
23	31.8	CH_2	1.88 (*m*)
			2.14 (*m*)
24	30.5	C	-
25	49.5	CH	2.89 (dd, *J27a, 27b* = 4.5 *J7a, 25 = 1.0*)
26	167.5	C	-
27	60.6	CH_2	3.65 (dd, *J27a, 27b* = 13.0, *J27a, 25* = 1,0)
			4.26 (dd, *J27b, 27a* = 13.0, *J27b, 25* = 45)
28	20.5	CH_3	1.17 (*s*)

3.2.2.6: Withaphysalin A (67)

Fraction B1 was further purified by RP-18 CC (MeOH/H_20 4.0:6.0) to yield Withaphysalin A **(67)** an amorphous solid (104 mg) along with other compounds. Fractionation scheme is given in experimental section (Fig 4.2). The UV spectrum of compound **(67)**, like other withanolides showed a characteristic absorption at 224 nm, indicating α, β-unsaturated lactone chromophore a. Similarly the IR spectrum also displayed absorption peak at, 3350, 1755 and 1684cm^{-1} indicating hydroxyl, carbonyl of keton and α, β -unsaturated lactone respectively. The molecular formula of compound **67** was found as $C_{28}H_{34}O_6$**,** established by its HR-ESI-MS from the molecular ion peak $[M+H]^+$ at *m/z* 467.25.Its ^1H and ^{13}C NMR spectra (Table 3.23) also showed similarities with those of compound (**69**) and difference between them seem in diene system of ring A. The ring A of (**69**) contains a 3, 5-diene-1-one system, while the spectra of ring A in (**67)** was characteristic of the 2, 5-diene-1-one system of withanolides. The ^{13}C NMR of compound (**67)** showed the resonance of all 28 carbons of the steroidal skeleton having methyl, methylene, methine and quaternary carbons. The peak at δ 204.5 (C-1) indicated carbonyl of ketone functionality whereas at δ 177.8 (C-18) and 166.5 (C-26) indicated carbonyl carbon of lactone moiety. The lowfield signals at δ 127.2 and 146.4 were aattributed to olefinic centre C-2 and C-5, respectively which confirming the 2, 5-diene-1-one system The H-NMR spectrum (Table 3.23) of compound (**67**) also displayed four singlets showing four methyls.



Withaphysalin A (67)

Table-3.23: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound **(67)** in $CDCl_3$

C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	204.1	C	-
2	127.2	CH	5.82 (ddd, *J2, 3* = 10, *J2, 4a = 2.5, J2, 4b* = 1.4)
3	146.4	CH	6.88 (ddd, *J3, 2* = 10, *J3,4a* =5.0, *J3, 4b* = 2.4)
4	33.8	CH_2	2.23 *m*
5	135.5	C	-
6	124.5	CH	5.59 (d, *J6, 7* = 5.5)
7	26.4	CH_2	2.07 (m) 2.31 (*m*)
8	39.5	CH	1.81 – 1.90 (*m*)
9	37.5	CH	2.63 – 2.77 (*m*)
10	51.4	C	-
11	23.3	CH_2	2.28 (*m*) 1.10 (*m*)
12	35.9	CH_2	2.27 (*m*) 1.55 (*m*)
13	60.1	C	-
14	83.9	C	5.48 (*s*) (OH)
15	34.7	CH_2	1.81 – 1.85 (*m*) 2.05 –2.13 (*m*
16	24.4	CH_2	1.45 – 1.59 (*m*) 1.97 – 2.06 (*m*)
17	55.2	CH	1.71 – 1.78 (*m*)
18	177.8	C	-
19	18.7	CH_3	1.29 (*s*)
20	82.8	C	-
21	26.6	CH_3	1.50 (*s*)
22	78.2	CH	4.50 (dd, *J22, 23a* = 13.1, *J22, 23b* = 4.1)
23	31.8	CH_2	1.90 (*m*) 2.14 (*m*)
24	148.5	C	-
25	122.5	C	-
26	166.5	C	-
27	12.6	CH_3	1.88 (*s*)
28	20.3	CH_3	1.94 (*s*)

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The methyl singlet at downfield chemical shifts (δ 1.88 & 1.94) was the sign of teir location on a double bond. The downfield signals at δ 5.82 (ddd, *J2, 3* = 10, *J2, 4a* = 2.5, *J2, 4b* = 1.4), 6.87 (ddd, *J3, 2* = 10.03, *J3, 4a* = 5.03, *J3, 4b* = 2.43 Hz) and 55.59 (d. *J6, 7* = 5.50 Hz) were assigned to protons of olefinic carbons H-2, H-3 and H-6 respectively. The^{1} H NMR and ^{13}C NMR data of compound (**67** is presented in table (3.23). Thus the compound (**67**) was identified as known compound Withaphysalin A previously reported from *P. minima* [150].

3.2.2.7: Withaphysalin C (68)

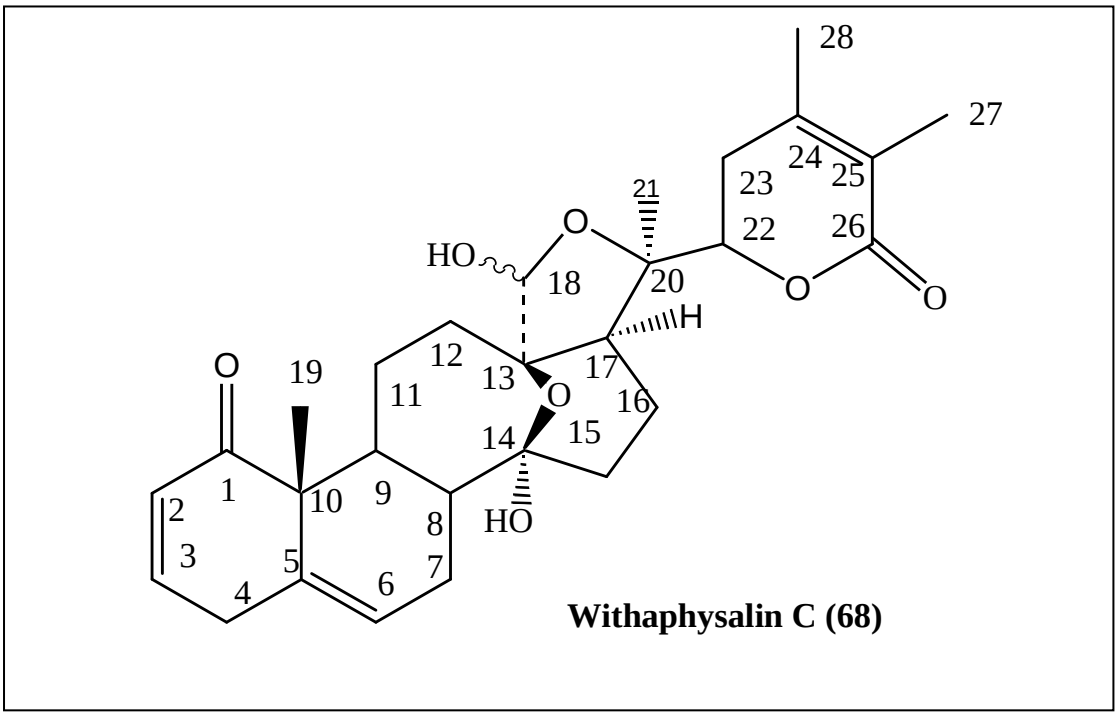
Fraction B was subjected to CC (MCI gel CHP 20P) and eluted with water/acetone (1:1), afforded four sub-fractions (B1-4). Sub-fraction B2 was further rpurified on RP-18 CC (MeOH/$H_2$0 5.0:5.0) to yield Withaphysalin C **(68)** an optically active amorphous solid (42 mg). Fractionation scheme is given in experimental section (Fig 4.2). The UV spectrum of compound **(68)**, like other withanolides showed a characteristic absorption at 225 nm, indicating α, β-unsaturated lactone chromophore. Similarly the IR spectrum also displayed absorption peak at 3350, 1751 and 1694 cm^{-1} indicating hydroxyl, carbonyl of keton and α, β -unsaturated lactone respectively. The molecular formula of compound **68** was found as $C_{28}H_{34}O_7$**,** established by its HR-ESI-MS from the molecular ion peak $[M+H]^+$ at 485.25.

Its ^{1}H and ^{13}C NMR spectra (Tables 3.24) showed similarities with those of compound (**67**) in ring A and B. The difference observed in ring D and E where the epoxide between C-13 and C14 was found in (**68**). Furthermore the lactone signal appeared at *δ* 177.8 (C-18) in (**67**) was reduced to alcohol *δ* 81.8 (C-18) in (**68**). The ^{13}C NMR of compound (**68**) showed the resonance of all 28 carbons of the steroidal skeleton having methyl, methylene, methine and quaternary carbons. The carbon resonating at δ 204.1 (C-1) indicated the carbonyl of ketone functionality whereas at δ 166.5 (C-26) indicated lactone moiety. The signals at δ 127.2 and 146.4 were assigned to olefinic carbons C-2 and C-5 respectively which confirming the 2, 5-diene-1-one system. The ^{1}H-NMR spectrum (Table 3.24) of compound (**68)** also displayed four singlets sindicating four methyls. The methyl singlet at downfield (δ 1.88 & 1.94) was the sign of its location on a double bond. The downfield signals at δ 5.92 (ddd, *J2, 3* = 10.04, *J2, 4a* = 2.54, *J2, 4b* = 1.44 Hz), 6.81(ddd, *J3,2* = 10.01, *J3, 4a* = 5.01, *J3,4b* = 2.41

Hz) and 5.69 (d. *J6, 71* = 5.51 Hz) were atributed to olefinic protons H-2, H-3 and H-6 respectively. The ^{1}H NMR and ^{13}C NMR data of compound **(68)** is given in table (3.24). Thus the compound **(68)** was identified as a known compound Withaphysalin C previously reported from *P. minima* [150].

Table-3.24: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound **(68)** in $CDCl_3$

C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	204.1	C	-
2	127.2	CH	5.92 (ddd, *J2,3* = 10, *J2, 4a* = 2.5, *J2, 4b* = 1.4)
3	146.4	CH	6.81 (ddd, *J3, 2* = 10, *J3, 4a* = 5.0, *J3, 4b* = 2.4)
4	33.8	CH_2	2.21 *m*
5	135.5	C	-
6	124.5	CH	5.69 (d, *J6, 7* = 5.5)
7	26.4	CH_2	2.17 (m) 2.36 (*m*)
8	39.5	CH	1.71 – 1.98 (*m*)
9	37.5	CH	2.69 – 2.79 (*m*)
10	51.4	C	-
11	23.3	CH_2	2.25 (*m*) 1.17 (*m*)
12	35.9	CH_2	2.88 (*m*) 2.23 (*m*)
13	78.1	C	-
14	102.9	C	6.28 (*s*) (OH)
15	34.7	CH_2	1.81 – 1.85 (*m*) 2.05 –2.13 (*m*
16	24.4	CH_2	1.45 – 1.59 (*m*) 1.97 – 2.06 (*m*)
17	55.3	CH	1.71 – 1.76 (*m*)
18	81.8	CH	4.28 (d, *J18,OH* = 5.3) 6.26 (d, *JOH,18* = 5.3)
19	18.7	CH_3	1.29 (*s*)
20	82.8	C	-
21	26.6	CH_3	1.50 (*s*)
22	78.2	CH	4.40 (dd, *J22, 23a* = 13.1, *J22, 23b* = 4.1)
23	31.8	CH_2	1.9 (*m*) 2.14 (*m*)
24	148.5	C	-
25	122.5	C	-
26	166.5	C	-
27	12.6	CH_3	1.88 (*s*)
28	20.3	CH_3	1.94 (*s*)



Withaphysalin C (68)

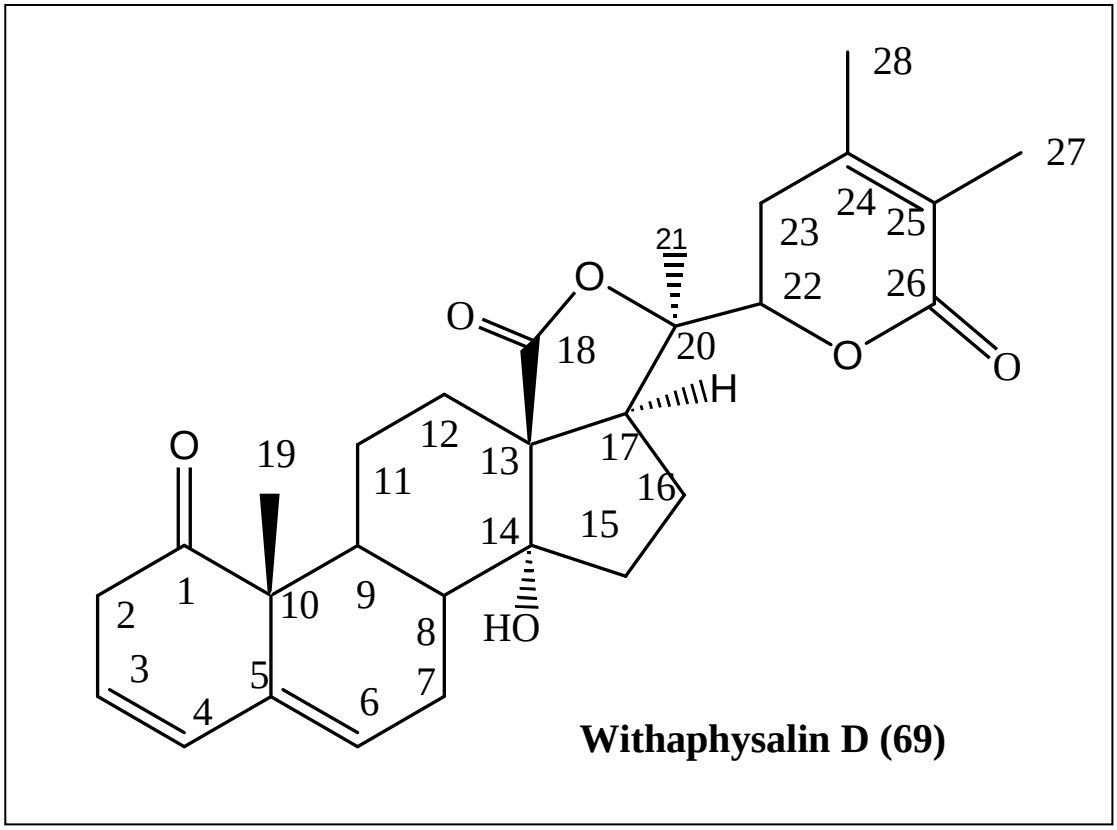
3.2.2.8: Withaphysalin D (69)

Fraction B was loaded on CC (MCI gel CHP 20P) and eluted with water/acetone (1:1) which afforded four sub-fractions (B1-4). Sub-fraction B4 was subjected to CC (Sephadex LH-20; MeOH) to yield Withaphysalin D **(69)** an optically active amorphous solid (14 mg) along with other compounds. Fractionation scheme is given in experimental section (Fig 4.2). The UV spectrum of compound **(69)**, like other withanolide showed a characteristic absorption at 228 nm, indicating α,β-unsaturated lactone chromophore. The IR spectrum bands attributed to hydroxl (3350 cm^{-1}), ketone (1755 cm^{-1}) and lactone (1694 cm^{-1}) fuctions. The molecular formula of compound **(69)** was found as $C_{28}H_{34}O_6$, established by its HR-ESI-MS from the molecular ion peak $[M+H]^+$ at 467.25. The ^1H and ^{13}C NMR spectra (Tables 3.22, 3.25) of (**69**) and (**67**) showed much closed similarities with with each other and the difference between them was found in the diene system of ring A. The ring A of (**69**) contains a 3, 5-diene-1-one system, while the spectra of ring A in (**67)** was characteristic of the 2, 5-diene-1-one system of withanolides. The ^{13}C NMR of compound (**69**) showed the resonance of all 28 carbons of the steroidal skeleton having methyl, methylene, methine and quaternary carbons. The ^{13}C NMR and ^1H-NMR resonating pattern of compound (**69**) was as such as discussed in case of Withaphysalin (**67)**. The ^1H NMR and ^{13}C NMR data of compound (**69**) is presented

in table (3.25). Thus the compound (**69**) was identified as a known compound Withaphysalin D previously reported from *P. minima* [208].

Table-3.25: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of **(69)** in $CDCl_3$

C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	202.10	C	-
2	39.8	CH_2	-
3	126.4	CH	6.08 (ddd, *J3, 4* = 10, *J3, 2a* = 5.0, *J3, 2b*= 2.4)
4	129.8	CH	5.72 (ddd, *J4, 3* = 10, *J4, 2a* = 2.5, *J4,2b*= 1.4)
5	139.5	C	-
6	121.5	CH	5.59 (*m*)
7	26.4	CH_2	1.97 (m)
			2.21 (*m*)
8	38.5	CH	1.91 – 1.94 (*m*)
9	36.5	CH	2.73 – 2.77 (*m*)
10	53.4	C	-
11	22.3	CH_2	2.18 (*m*)
			1.10(*m*)
12	35.9	CH_2	2.17 (*m*)
			1.45 (*m*)
13	60.1	C	-
14	83.9	C	5.28 (*s*)(OH)
15	34.3	CH_2	1.71 – 1.77 (*m*)
			2.01 –2.04 (*m)*
16	24.4	CH_2	1.65 – 1.69 (*m*)
			1.90 – 1.95 (*m*)
17	57.1	CH	1.62 – 1.65 (*m*)
18	177.8	C	-
19	20.7	CH_3	1.42 (*s*)
20	82.8	C	-
21	26.6	CH_3	1.47 (*s*)
22	78.2	CH	4.57 (dd, *J22, 23a* = 13.1, *J22, 23b* = 4.1)
23	31.8	CH_2	1.9 (*m*)
			2.14 (*m*)
24	148.5	C	-
25	122.5	C	-
26	165.5	C	-
27	12.6	CH_3	1.89 (*s*)
28	20.3	CH_3	1.95 (*s*)



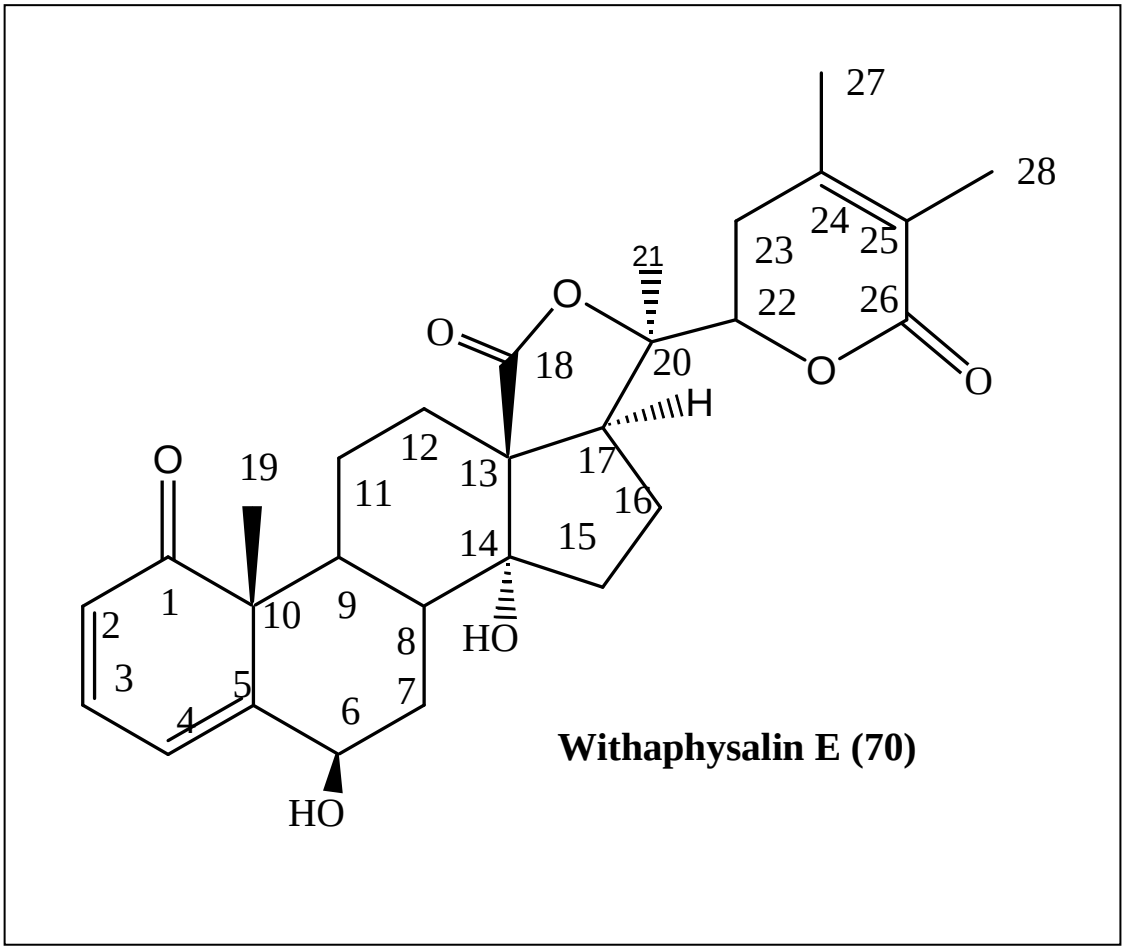
Withaphysalin D (69)

3.2.2.9: Withaphysalin E (70)

Fraction B2 was purified by RP-18 CC (MeOH/water 5.0:4.0) to yield Withaphysalin E **(70)** an amorphous solid (27 mg) along with other compounds. Fraction- ation scheme is given in experimental section (Fig 4.2). The UV spectrum of compound **(70)**, like other withanolide showed a characteristic absorption at 228nm, indicating α, β-unsaturated lactone chromophore a. Similarly the IR spectrum also displayed absorption peaks at, 3450, 1745, 1699 and 1645 cm^{-1} indicating hydroxyl, carbonyl of ketone and α, β -unsaturated lactone functionalities. The molecular formula was found as $C_{28}H_{34}O_{7,}$ established by its HR-ESI-MS from the molecular ion peak $[M\text{-}H_2O]^+$ at *m/z* 464.25.

Its ^1H and ^{13}C NMR spectra (Table 3.26) showed similarities with those of compound (**69**) and (**70**) . The difference between them seems in diene system of ring A. The ring A of (**69**) contains a 3,5-diene-1-one system, while the spectra of ring A in (**67)** was characteristic of the 2,5-diene-1-one system but the ring A of compound **(70)** contains 2,4 diene-1-one system within the same ring. The ^{13}C NMR of compound **(70)** showed the resonance of all 28 carbons of the steroidal skeleton

having methyl, methylene, methine and quaternary carbons. The carbon resonating at δ 205.5(C-1) indicated the presence carbonyl of ketone functionality whereas at δ 177.5 (C-18) and 165.5 (C-26) indicated carbonyl of lactone moiety. The olefinic signals at δ 117.2 and 123.4 were assigned to C-2 and C-4 respectively which confirming the 2, 4-diene-1-one system. The ^{1}H-NMR spectrum (Table 3.26) of compound (**67**) also have four methyl singlets. The methyl singlet at downfield chemical shifts (δ 1.86 & 1.98) was attributed to their attachment to double bond. The downfield signals at δ 5.98 (d, *J3, 2*= 10 Hz), 6.98 (dd, *J2, 3* = 10, *J3, 4* = 6.5 Hz) and 6.14 (d, *J3, 2* = 6.5 Hz) were assigned to protons of olefinic center H-2, H-3 and H-4 respectively. The ^{1}H NMR and ^{13}C NMR data of compound **(70)** is given in table (3.26). Thus the compound (**70**) was identified as known compound Withaphysalin E previously reported from *P. minima* [255]



Withaphysalin E (70)

Table-3.26: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound **(70)** in $CDCl_3$

C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	205.1	C	-
2	117.2	CH	5.98 (d, *J3,2* = 10)
3	142.4	CH	6.98 (dd, *J2,3* = 10, *J3,4* =6.5)
4	123.8	CH	6.14 (d,*J3,2*= 6.5)
5	155.5	C	-
6	73.5	CH	3.88 (dt, *J6,OH* = 5.3 *J6, 7a*= 3.0, *J6,7b* = 3.0) 5.66 (d, *J6-OH* = 5)
7	36.4	CH_2	1.97 (m) 2.21 (*m*)
8	39.5	CH	1.81 – 1.90 (*m*)
9	47.5	CH	2.68 – 2.79(*m*)
10	53.4	C	-
11	24.3	CH_2	2.23 (*m*) 1.18 (*m*)
12	37.9	CH_2	2.21 (*m*) 1.59 (*m*)
13	60.1	C	-
14	83.2	C	5.48 (*s*)(OH)
15	34.7	CH_2	1.76 – 1.80 (*m*) 2.15 –2.23 (*m*
16	25.4	CH_2	1.55 – 1.59 (*m*) 1.96 – 2.04 (*m*)
17	56.13	CH	1.74– 1.81 (*m*)
18	177.5	C	-
19	20.7	CH_3	1.39 (*s*)
20	82.8	C	-
21	26.6	CH_3	1.55(*s*)
22	78.2	CH	4.50 (dd, *J22, 23a* = 13.1, *J22, 23b* = 3.1)
23	30.8	CH_2	1.98 (*m*) 2.12 (*m*)
24	148.5	C	-
25	122.5	C	-
26	165.5	C	-
27	12.6	CH_3	1.86 (*s*)
28	20.3	CH_3	1.98 (*s*)

.

Chapter 4:

EXPERIMENTAL (PART A)

4.1: General Experimental Conditions

4.1.1: Physical constants

Melting points (corrected and uncorrected) were determined by Buchi 535 melting point apparatus using glass capillary tubes. Optical rotations were measured on Schmidt Haensch Polartronic D polarimeter. X-Ray diffraction data was collected on Brucker diffractometer equipped with SMART APUX CCU area detector using Mo Kc radiations (0.71073 A).

4.1.2: Spectroscopic techniques

Ultraviolet (UV) spectra were taken in methanol on a Hitachi U-3200 IV spectrophotometer. Infrared (IR) spectra were measured using KBr disc by Perkin-Elmer 16 PC FT-IR spectrophotometer. Electron impact mass spectra (ESI-MS) were measured on Esquire 3000 plus_01005 spectrometer in *m/z*. The ^{1}H NMR (400MHz), ^{13}C NMR (100MHz) and two dimensional NMR (HMQC, HMBC & COSEY) spectra were measured on Bruker AMX-400 spectrometers using deutrated solvents (C_5D_5N and $CDCl_3$). The chemical shifts (δ) were reported in ppm relative to tetramethyl silane ($SiMe_4$) as an internal standard. The coupling constants (*J*) were measured in Hz.

4.1.3: Chromatographic techniques

Chromatographic separations were carried out using polyamide (30-60 mesh, Sinopharm Chemical Reagent Co. Ltd, Shanghai, P. R. China), silica gel H (220–300 mesh; Qingdao Marine Chemical Ltd., Qingdao, P. R. China), D-101 porous Resin, MCI gel CHP 20P, Sephadex LH-20 (Sinopharm Chemical Reagent Co. Ltd, Shanghai, P. R. China) and RP-18 (20 – 45 μm; Fuji Silysia Chemical Ltd.). Thin-layer chromatography (TLC) was performed on silica gel GF254 (Yantai Huiyou Inc., Yantai, P. R. China). Purity of the samples were also checked on the same pre-coated plates. All solvents and reagents used were of analytical grade (Shanghai Chemical Plant, Shanghai, P. R. China)

4.1.4: Detection of compounds:

TLC plates were studied under Ultraviolet light at 254 nm for fluorescence quenching spots and at 366 nm for fluorescent spots. Dragendorff's solution, ceric sulphate, stabnum chloride solution and other spraying reagents were used to detect the spots on TLC plates.

4.2: *Withania coagulans*

4.2.1: Plant material

The plant materails of *W. cogulans* were collected from the Shagai post (Khyber agency) near Peshawar during August 2006 and was identified by Professor Dr. Abdul Rashid, Center of Biodiversity, University of Peshawar, Pakistan. A voucher specimen (Mumtaz-15-PUP) was deposited in the herbarium at the Department of Botany, University of Peshawar, Pakistan

4.2.2: Extraction and isolation

The dried powdered plant materails (5 kg) were extracted with ethanol. The ethanolic extract was then filtered and concentrated under vacuum to give dark residue (850 g) after evaporation. The 350 g of the residue was subjected to polyamide CC and eluted with EtOH/H_2O (30:70, 70:30) to provide two fractions [*Fr. A* (90 g) and *B* (123 g)]. Fr. A was loaded on silica gel CC and on elution with hexane/acetone (15:1, 10:1, 5:1, 2:1, 1:1) to provide six subfractions (*Fr. A1-6*). *Fr. A1* was further subjected to RP-18 CC (MeOH/H_2O 65:35) to afford **45** (23 mg), **49** (31 mg) and **51** (50 mg). *Fr. A2* was subjected to RP-18 CC (MeOH/H_2O 60:40) to afford **52** (65 mg), **53** (70 mg) and **54** (13 mg). *Fr. A3* was also subjected to RP-18 CC (MeOH/H2O 60:40) to yield **50** (35mg), **55** (140 mg) and **56** (216 mg). *Fr. A4* was purified similarly (RP-18; Methanol/water 57:43) to provide **46** (24 mg) and **57** (93 mg). *Fr. A5* was purified similarly (RP-18; MeOH/H_2O 55:45) to afford **58** (38 mg), **47** (87 mg) and **48** (18 mg). *Fr. A6* was also purified similarly (RP-18; MeOH/H_2O 50:50) to yield **59** (120 mg) and **60** (64 mg).

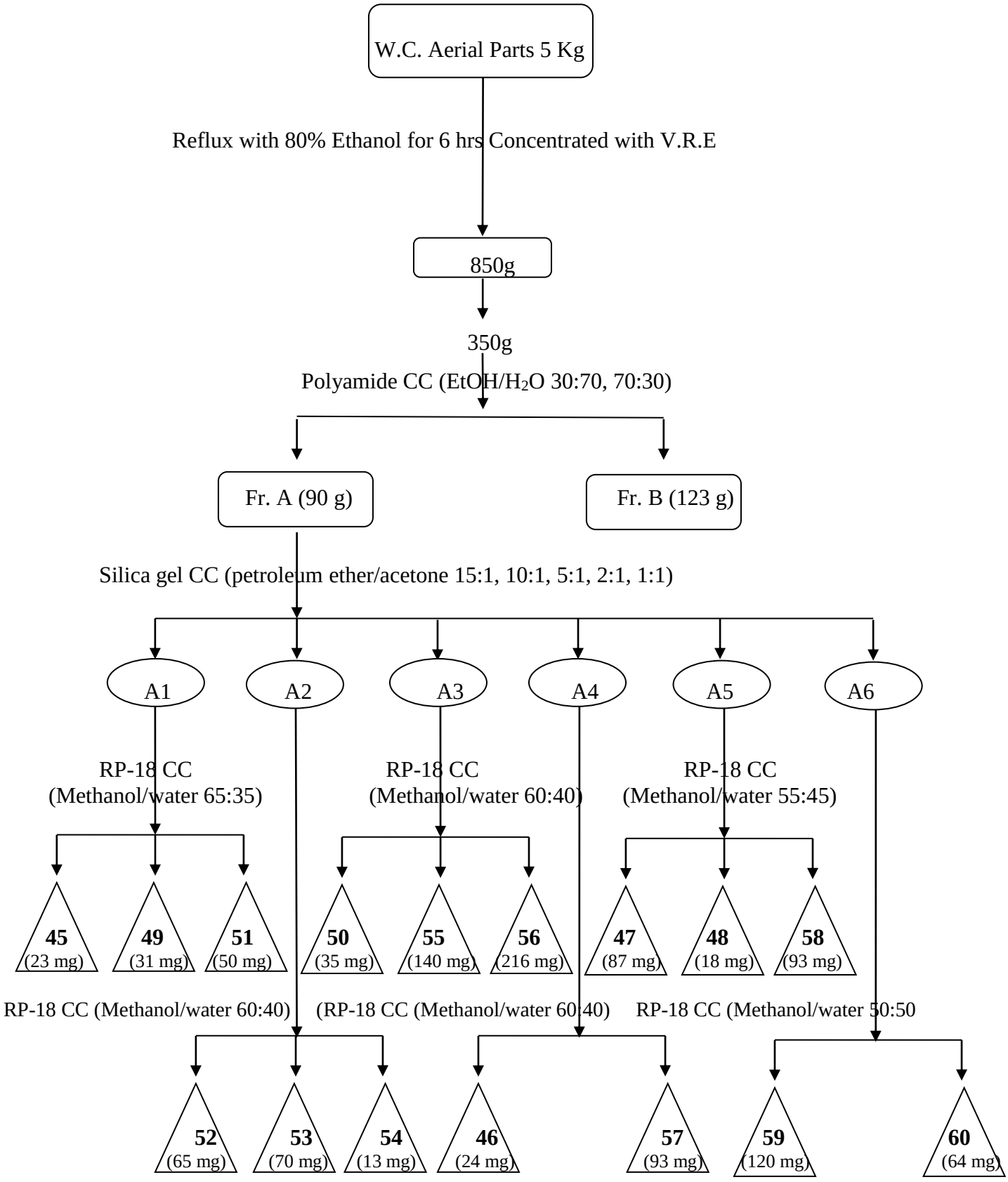


Fig.4.1: Extraction, fractionation and isolation of Withanolides from *Withania Coagulans*

4.2.3: Experimental data of new withanolides from *Withania coagulans*

4.2.3.1: Withacoagulin A (45)

IUPAC name: 17*β*, 20*β*-dihydroxy-1-oxo-(20*S*,22*R*)-witha-3,5,14,24-tetraenolide

Physical state: White amorphous solid

Yield: 23 mg (4.6 x 10^{-4} %)

Optical rotation $[\alpha]_D^{20}$: +5° (c = 0.09, $CHCl_3$)

IR (KBr): 3453, 2933, 1712, 1684, 1452, 1382, 1319, 1135, 597 cm^{-1}

HR-ESI-MS (pos.): 475.2455 ($[M+Na]^+$, $C_{28}H_{36}NaO_5^+$; calc. 475.2460)

HR-ESI-MS (neg.): 497.2542 ($[M+COOH]^-$, $C_{29}H_{37}O_7^-$; calc. 497.2539).

^1H NMR (400 MHz, $CDCl_3$): Given in **Table 3.1**

^{13}C NMR (100 MHz, $CDCl_3$): Given in **Table 3.1**

HMQC (100 MHz, $CDCl_3$): Given in **Table 3.1**

4.2.3.2: Withacoagulin B (46)

IUPAC name: 20*β*,27-dihydroxy-1-oxo-(20*R*,22*R*)-witha-3,5,14,24-tetraenolide

Physical state: White amorphous powder

Yield: 24 mg (4.8 x 10^{-4} %)

Optical rotation $[\alpha]_D^{20}$: +29° (c = 0.14, CH_3OH)

IR (KBr): 3423, 2839, 1704, 1684, 1390, 1326, 1137, 1001, 595 cm^{-1}

HR-ESI-MS (pos.): 475.2455 ($[M+Na]^+$, $C_{28}H_{36}NaO_5^+$; calc. 475.2460)

HR-ESI-MS (neg.): 497.2542 ($[M+COOH]^-$, $C_{29}H_{37}O_7^-$; calc. 497.2539).

^1H NMR (400 MHz, C_5D_5N): Given in **Table 3.2**

^{13}C NMR (100 MHz, C_5D_5N): Given in **Table 3.2**

HMQC (100 MHz, C_5D_5N): Given in **Table 3.2**

4.2.3.3: Withacoagulin C (47)

IUPAC name: 14*α*, 15*α*, 17*β*, 20*β*-tetrahydroxy-1-oxo-(20S,22R)-witha-3,5,24-trienolide Physical state: Amorphous powder

Yield: 87 mg (1.74 x 10^{-3} %)

Optical rotation $[\alpha]_D^{20}$: +22° (c = 0.18, CH_3OH)

IR (KBr): 3409, 2944, 1691, 1390, 1322, 1139, 1091, 1022, 732 cm^{-1}

HR-ESI-MS (pos.): 995.5136 ($[2M+Na]^+$, $C_{56}H_{76}NaO_{14}^+$; calc. 995.5132)

HR-ESI-MS (neg.): 531.2591 ($[M+COOH]^-$, $C_{29}H_{39}O_9^-$; calc. 531.2594).

^1H NMR (400 MHz, C_5D_5N): Given in **Table 3.3**

^{13}C NMR (100 MHz, C_5D_5N): Given in **Table 3.3**

HMQC (100 MHz, C_5D_5N): Given in **Table 3.3**

4.2.3.4: Withacoagulin D (48)

IUPAC name: 14*α*, 17*β*, 20*β*, 27-tetrahydroxy-1-oxo-(20S,22R)-witha-2,5,24-trienolide Physical state: Amorphous powder

Yield: 18 mg (3.6 x 10^{-4} %)

Optical rotation $[\alpha]_D^{20}$: +60° (c = 0.21, CH_3OH)

IR (KBr): 3488, 3419, 2966, 1689, 1654, 1392, 1322, 1143, 1026, 1006, 810, 644 cm^{-1}

HR-ESI-MS (pos.): 995.5131 ($[2M+Na]^+$, $C_{56}H_{76}NaO_{14}^+$; calc. 995.5132)

HR-ESI-MS (neg.): 485.2540 ($[M-H]^-$, $C_{28}H_{37}O_7^-$; calc. 485.2539).

^1H NMR (400 MHz, C_5D_5N): Given in **Table 3.4**

^{13}C NMR (100 MHz, C_5D_5N): Given in **Table 3.4**

HMQC (100 MHz, C_5D_5N): Given in **Table 3.4**

4.2.3.5: Withacoagulin E (49)

IUPAC name: 14*β*, 20*β*-dihydroxy-1-oxo-(20*R*, 22*R*)-witha-2,5,24-trienolide

Physical state: Amorphous powder

Yield: 31 mg (6.2 x 10^{-4} %)

Optical rotation $[\alpha]_D^{20}$:+179° (c = 0.21, CH_3OH)

IR (KBr): 3415, 2941, 1689, 1384, 1319, 1124, 962, 761 cm^{-1}

HR-ESI-MS (pos.): 931.5331 ($[2M+Na]^+$, $C_{56}H_{76}NaO_{10}^+$; calc. 931.5336)

HR-ESI-MS (neg.): 499.2683 ($[M+COOH]^-$, $C_{29}H_{39}O_7^-$; calc. 499.2695).

^1H NMR (400 MHz, C_5D_5N): Given in **Table 3.5**

^{13}C NMR (100 MHz, C_5D_5N): Given in **Table 3.5**

HMQC (100 MHz, C_5D_5N): Given in **Table 3.5**

4.2.3.6: Withacoagulin F (50)

IUPAC name: 14*β*, 20*β*-dihydroxy-1-oxo-(20*R*,22*R*)-witha-3,5,24-trienolide

Physical state: Amorphous powder

Yield: 35 mg (7.0 x 10^{-4} %)

Optical rotation $[\alpha]_D^{20}$:+52° (c = 0.40, CH_3OH)

IR (KBr): 3419, 2942, 1700, 1456, 1386, 1319, 1143, 1126, 960, 761 cm^{-1}

HR-ESI-MS (pos.): 931.5331 ($[2M+Na]^+$, $C_{56}H_{76}NaO_{10}^+$; calc. 931.5336)

HR-ESI-MS (neg.): 499.2683 ($[M+COOH]^-$, $C_{29}H_{39}O_7^-$; calc. 499.2695).

^1H NMR (400 MHz, C_5D_5N): Given in **Table 3.6**

^{13}C NMR (100 MHz, C_5D_5N): Given in **Table 3.6**

HMQC (100 MHz, C_5D_5N): Given in **Table 3.6**

4.2.4: Experimental data of known withanolides from *Withania coagulans*

4.2.4.1: Withacoagulin (51)

IUPAC name: 20*β*, 27-dihydroxy-1-oxo-(22*R*)-witha-2,5,14,24-trienolide

Physical state: Amorphous powder

Yield: 50 mg (1.0 x 10^{-3} %)

Optical rotation $[\alpha]_D^{20}$:+37° (c = 0.0081, CH_3OH)

IR (KBr): 3583, 2942, 1706, 1682, 1456, 1386, 1319, 1143, 1126, 960, 761 cm^{-1}

HR-ESI-MS (pos.): 475.2455 ($[M+Na]^+$, $C_{28}H_{36}NaO_5^+$; calc. 475.2460)

HR-ESI-MS (neg.): 497.2542 ($[M+COOH]^-$, $C_{29}H_{37}O_7^-$; calc. 497.2539).

^1H NMR (400MHz, C_5D_5N): Given in **Table 3.7**

^{13}C NMR (100MHz, C_5D_5N): Given in **Table 3.7**

4.2.4.2: Withanoilde F (52)

IUPAC name: 14β, 17β, 20S-Trihydroxy-1-oxo-22R-witha-2,5,24-trienolide

Physical state: Amorphous solid

Yield: 65 mg (1.3 x 10^{-3} %)

Optical rotation $[\alpha]_D^{20}$:+57° (c = 0.081, CH_3OH)

IR (KBr): 3424, 2942, 1706, 1685, 1450, 1386, 1319, 1141, 1092 cm^{-1}

HR-ESI-MS (pos.): 959.5331 ($[2M+Na]^+$, $C_{56}H_{72}NaO_{12}^+$; calc. 959..5336)

HR-ESI-MS (neg.): 513.2683 ($[M+COOH]^-$, $C_{29}H_{37}O_8^-$; calc. 513.2695).

^1H NMR (400MHz, C_5D_5N): Given in **Table 3.8**

^{13}C NMR (100MHz, C_5D_5N): Given in **Table 3.8**

4.2.4.3: Δ^3-isomer of withanolide F (53)

IUPAC name: 14, 17, 20S-Trihydroxy-1-oxo-22R-witha-3,5,24-trienolide

Physical state: Colorless Solid

Yield: 70 mg (1.4 x 10^{-3} %)

Optical rotation $[\alpha]_D^{20}$:+67° (c = 0.18, CH_3OH)

IR (KBr): 3423, 2942, 1700, 1685, 1456, 1386, 1319, 1141, 1092 cm^{-1}

HR-ESI-MS (pos.): 959.5331 ($[2M+Na]^+$, $C_{56}H_{72}NaO_{12}^+$; calc. 959.5336)

HR-ESI-MS (neg.): 513.2683 ($[M+COOH]^-$, $C_{29}H_{37}O_8^-$; calc. 513.2695).

^1H NMR (400 MHz, C_5D_5N): Given in **Table 3.9**

^{13}C NMR (100 MHz, C_5D_5N): Given in **Table 3.9**

4.2.4.4: Withanoilde I (54)

IUPAC name: 14α, 20R-Dihydroxy-1-oxo-22R-witha-3,5,24-trienolide

Physical state: Amorphous solid

Yield: 65 mg (2.6 x 10^{-4} %)

Optical rotation $[\alpha]_D^{20}$:+113° (c = 0.81, CH_3OH)

IR (KBr): 3375, 2942, 1705, 1695, 1450, 1386, 1319, 1141, 1092 cm^{-1}

HR-ESI-MS (pos.): 931.5331 ($[2M+Na]^+$, $C_{56}H_{76}NaO_{10}^+$; calc. 931.5336)

HR-ESI-MS (neg.): 499.2683 ($[M+COOH]^-$, $C_{29}H_{39}O_7^-$; calc. 499.2695).

^1H NMR (400 MHz, C_5D_5N): Given in **Table 3.10**

^{13}C NMR (100 MHz, C_5D_5N): Given in **Table 3.10**

4.2.4.5: Withanoilde J (55)

IUPAC name: 14α,17α,20S-Trihydroxy-1-oxo-22R-witha-2,5,24-trienolide

Physical state: Colorless solid

Yield: 65 mg (2.8 x 10^{-3} %)

Optical rotation $[\alpha]_D^{20}$:+316° (c = 1, CH_3OH)

IR (KBr): 3426, 2955, 1705, 1686,1450, 1386, 1319, 1141, 1092 cm^{-1}

HR-ESI-MS (pos.): 959.5331 ($[2M+Na]^+$, $C_{56}H_{72}NaO_{12}^+$; calc. 931.5336)

HR-ESI-MS (neg.): 513.2683 ($[M+COOH]^-$, $C_{29}H_{37}O_8^-$; calc. 513.2695).

^1H NMR (400 MHz, C_5D_5N): Given in **Table 3.11**

^{13}C NMR (100 MHz, C_5D_5N): Given in **Table 3.11**

4.2.4.6: Withanoilde K (56)

IUPAC name: 14α,17α,20S-Trihydroxy-1-oxo-22R-witha-3,5,24-trienolide

Physical state: Colorless solid

Yield: 65 mg (4.32 x 10^{-3} %)

Optical rotation $[\alpha]_D^{20}$:+42° (c = 0.018, CH_3OH)

IR (KBr): 3420, 2955, 1690, 1675,1450, 1386, 1319, 1141, 1092 cm^{-1}

HR-ESI-MS (pos.): 959.5331 ($[2M+Na]^+$, $C_{56}H_{72}NaO_{12}^+$; calc. 959.5336)

HR-ESI-MS (neg.): 513.2683 ($[M+COOH]^-$, $C_{29}H_{37}O_8^-$; calc. 513.2695).

^1H NMR (400 MHz, C_5D_5N): Given in **Table 3.12**

^{13}C NMR (100 MHz, C_5D_5N): Given in **Table 3.12**

4.2.4.7: Withanoilde L (57)

IUPAC name: 17α, 20S-dihydroxy-1-oxo-22R-witha-2,5, 14,24-tetraenolide

Physical state: Amorphous solid

Yield: 93mg (1.8 x 10^{-3} %)

Optical rotation $[\alpha]_D^{20}$:+55° (c = 0.018, CH_3OH)

IR (KBr): 3455, 2955, 1698, 1450, 1386, 1319, 1141, 1092 cm^{-1}

HR-ESI-MS (pos.): 475.2455 ($[M+Na]^+$, $C_{28}H_{36}NaO_5^+$; calc. 475.2460)

HR-ESI-MS (neg.): 497.2542 ($[M+COOH]^-$, $C_{29}H_{37}O_7^-$; calc. 497.2539).

^1H NMR (400MHz, C_5D_5N): Given in **Table 3.13**

^{13}C NMR (100MHz, C_5D_5N): Given in **Table 3.13**

4.2.4.8: (22R)-14α, 15α, 17β, 20β-Tetrahydroxy-1-oxowitha-2,5, 24-trien-26,22-olide(58)

UPAC name: 14α, 15α, 17β,20β-tetrahydroxy-1-oxowitha-2,5,24-trien-26,22-olide

Physical state: Colorless solid

Yield: 93 mg (1.8 x 10^{-3} %)

Optical rotation $[\alpha]_D^{20}$:+103° (c = 1, CH_3OH)

IR (KBr): 3416, 2978,1685,1660,1450, 1386, 1319, 1141, 1092 cm^{-1}

HR-ESI-MS (pos.): 995.5136 ($[2M+Na]^+$, $C_{56}H_{76}NaO_{14}^+$; calc. 995.5132)

HR-ESI-MS (neg.): 531.2591 ($[M+COOH]^-$, $C_{29}H_{39}O_9^-$; calc. 531.2594.

^1H NMR (400 MHz, C_5D_5N): Given in **Table 3.14**

^{13}C NMR (100 MHz, C_5D_5N): Given in **Table 3.14**

4.2.4.9: 1-oxo-14,20α,27-trihydroxy-20R,22R-witha-3,5,24- trienolide (59)

UPAC name: 1-oxo-14, 20α,27-trihydroxy-20R,22R-witha-3,5,24- trienolide

Physical state: Colorless solid

Yield: 120 mg (2.4x 10^{-3} %)

Optical rotation $[\alpha]_D^{20}$:+98° (c = 0.12, CH_3OH)

IR (KBr): 3550, 3400, 2978, 1700, 1684, 1660, 1450, 1386, 1319, 1141, 1092 cm^{-1}

HR-ESI-MS (pos.): 963.5136 ($[2M+Na]^+$, $C_{56}H_{76}NaO_{12}^+$; calc. 963.5132)

HR-ESI-MS (neg.): 515.2591 ($[M+COOH]^-$, $C_{29}H_{39}O_7^-$; calc. 515.2594.

^1H NMR (400MHz, C_5D_5N): Given in **Table 3.15**

^{13}C NMR (100MHz, C_5D_5N): Given in **Table 3.15**

4.2.4.10: Ajugin E (60)

IUPAC name: 14α,17β,20β,27-tetrahydroxy-1-oxo-(20R, 22R)-witha-3,5,24-trienolide

Physical state: White amorphous solid

Yield: 64 mg (2.6 x 10^{-4} %)

Optical rotation $[\alpha]_D^{20}$:+125° (c = 0.051, CH_3OH)

IR (KBr): 3455, 2942, 1715, 1699, 1450, cm^{-1}

HR-ESI-MS (pos.): 487.2695 $[M+H]^+$, $C_{28}H_{39}O_7^+$; calc. 487.5336)

^1H NMR (400 MHz, C_5D_5N): Given in **Table 3.16**

^{13}C NMR (100 MHz, C_5D_5N): Given in **Table 3.16**

4.3: *Physalis divericata*

4.3.1: Plant material

The aerial parts of *P. divericata* were collected from the Swat District of N.W.F.P during September 2005 and was identified by Professor Mehboob Ahmad. A voucher specimen was deposited in the herbarium at the Department of Botany Jahanzeb College Swat Pakistan

4.3.2: Extraction and isolation

The air dried and powdered aerial parts (5 kg) of *P. divericata* were extracted with ethanol/water (80:20) by reflux for 6 hrs,, which afforded a dark residue (1.8 kg) after evaporation under reduced pressure. The residue was partitioned between chloroform and water. The organic layer obtained was concentrated with v.r.e under reduced pressure. The residue i.e. chloroform fraction(197g) was subjected to CC (D-101 porous resin) and eluted with EtOH/H_2O in increasing order of ethanol in water (25:75, 75:25, 95:5), affording three fractions (Fr. A–C). Subsequent CC (MCI gel CHP 20P) of Fr. B (24.4 g) using solvent system H_2O/Me_2CO (1:1) resulted in four sub-fractions (Fr. B.1–B.4). Fr. B.1 was re-subjected to reverse phase CC (RP-18) and eluted with MeOH/H_2O (40:60) to afford physalin D (**64**, 37 mg), physalin F (**65**, 14 mg), and physalin H (**66**, 40 mg). Fr. B.2 was purified similarly on (RP-18) using same solvent system with different ratio (MeOH/H_2O 50:50) to yield withaphysalin C (**68**, 42 mg), withaphysalin E (**70**, 27 mg), and physalin A (**62**, 19 mg). Fr. B.3 was similarly subjected to CC (RP-18) and eluted with MeOH/H_2O (60:40) to afford withaphysalin D (**69**, 24 mg), withaphysalin A (**67**, 104 mg), and withaphysanolide A (**61**, 9 mg). Finally the Fr. B.4 was subjected to CC (Sephadex LH-20) and eluted with MeOH to yield withaphysalin D (**69**, 14 mg) and physalin B (**63**, 120 mg).

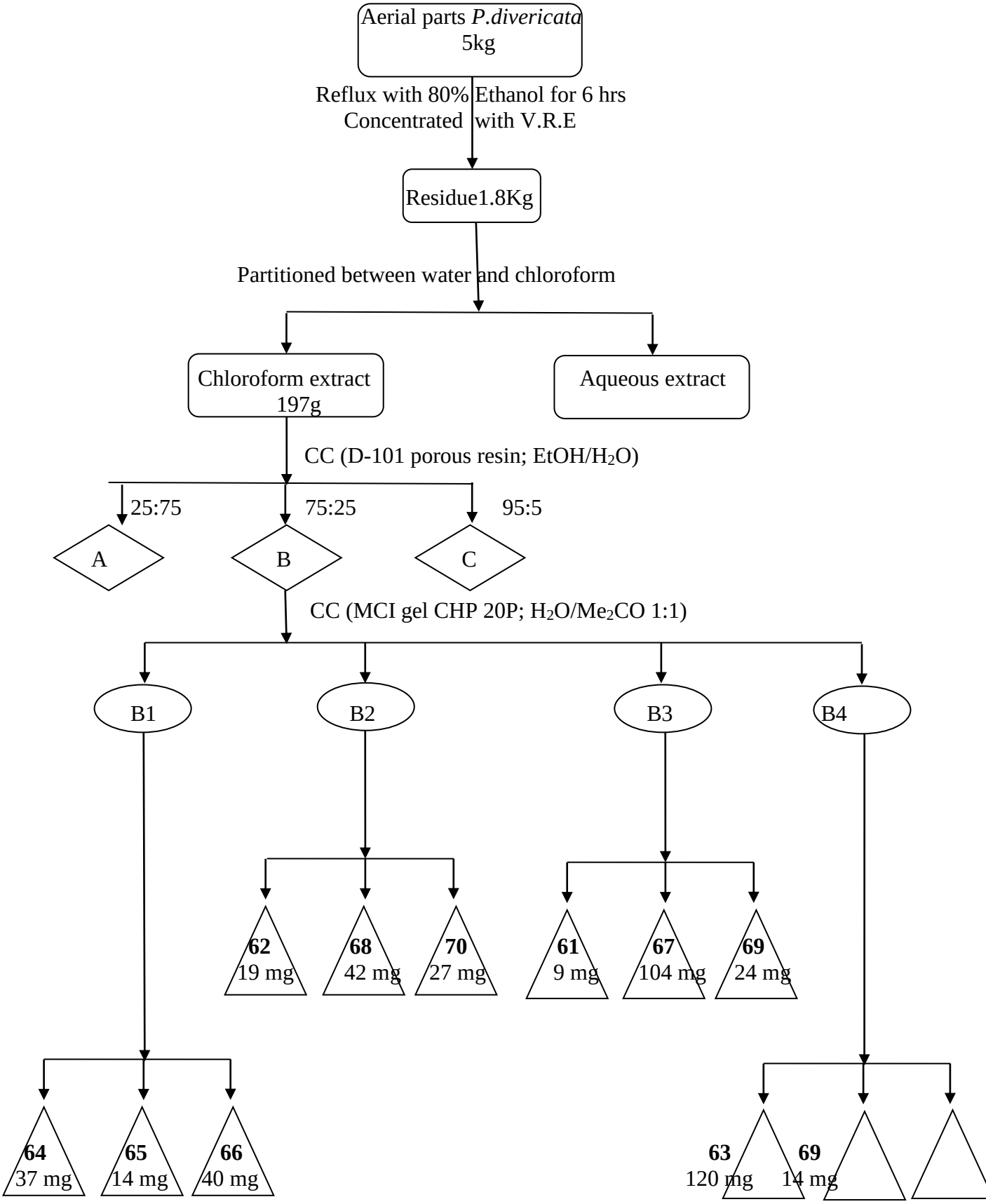


Fig.4.2: Extraction, fractionation and isolation of Withanolides from *P. divericata*

4.3.3: Experimental data of new withasteroids from *Physalis divericata*

4.3.3.1: Withaphysanolide A (61)

Physical state: Colorless cubic crystal

Yield: 9 mg (1.8×10^{-4} %)

Melting point: 221-222 C°

Optical rotation $[\alpha]_D^{20}$: +103° (c = 0.35, $CHCl_3$)

UV (MeOH) λmax ($\log_{\varepsilon}$): 224 nm (4.10)

IR (KBr): 3553, 2953, 1706, 1683, 1452, 1382, 1329, 1155, 550 cm^{-1}

HR-ESI-MS (pos.): 438.2405 ($[M]^+$, $C_{27}H_{34}O_5^+$; calc. 438.2406)

^1H NMR (400 MHz,$CDCl_3$): Given in **Table 3.17**

^{13}C NMR (100 MHz, $CDCl_3$): Given in **Table 3.17**

HMQC (100 MHz, CDCl3): Given in **Table 3.17**

4.3.4: Experimental data of known withasteroids from *Physalis divericata*

4.3.4.1: Physalin A (62)

Physical state: Colorless solid

Yield: 19 mg (3.8×10^{-4} %)

Melting point: 262-266 C°

Optical rotation $[\alpha]_D^{20}$:-173° (c = 0.32, $CHCl_3$)

UV (MeOH) λmax ($\log_{\varepsilon}$): 221 nm (4.00)

IR (KBr): 3426, 2928, 1721, 1663, 1452, 1382, 1319, 1135, 478 cm^{-1}

HR-ESI-MS (pos.): 526.2235 ($[M]^+$, $C_{28}H_{30}O_{10}^+$; calc. 526.2236)

^1H NMR (400 MHz, $CDCl_3$): Given in **Table 3.18**

^{13}C NMR (100 MHz, $CDCl_3$): Given in **Table 3.18**

4.3.4.2: Physalin B (63)

Physical state: Colorless solid

Yield: 120 mg (2.4×10^{-3} %)

Melting point: 269-272 C°

Optical rotation $[\alpha]_D^{20}$: Not studied

UV (MeOH) λmax (log$_{\acute{\varepsilon}}$): 222 nm (4.00)

IR (KBr): 3403, 2955, 1780, 1758, 1740 1655, 1452, 1382, 1334, 1135, cm^{-1}

HR-ESI-MS (pos.): 510.2235 ($[M]^+$, $C_{28}H_{30}O^-$, calc. 510.2236)

^1H NMR (400 MHz, $CDCl_3$): Given in **Table 3.19**

^{13}C NMR (100 MHz, $CDCl_3$): Given in **Table 3.19**

4.3.4.3: Physalin D (64)

Physical state: Colorless solid

Yield: 24 mg (4.8 x 10^{-4} %)

Melting point: 286-287 C^o

Optical rotation $[\alpha]_D^{20}$:-68° (c = 0.30, MeOH)

UV (MeOH) λmax (log$_{\acute{\varepsilon}}$): 225 nm (4.00)

IR (KBr): 3400, 2925, 1790, 1757, 1732, 1640, 1452, 1382, 1319, 1140, 478 cm^{-1}

HR-ESI-MS (pos.): 544.2235 ($[M]^+$, $C_{28}H_{32}O_{11}^+$; calc. 544.2236)

^1H NMR (500 MHz, $CDCl_3$): Given in **Table 3.20**

^{13}C NMR (100 MHz, $CDCl_3$): Given in **Table 3.20**

4.3.4.4: Physalin F (65)

Physical state: Amorphous powder

Yield: 14 mg (2.8 x 10^{-4} %)

Melting point: 262-264 C^o

Optical rotation $[\alpha]_D^{20}$:-60° (c = 0.17, $CHCl_3$)

UV (MeOH) λmax (log$_{\acute{\varepsilon}}$): 225 nm (4.10)

IR (KBr): 3400, 2925, 1775, 1740,1650, 1452, 1382, 1334, 1135, 522 cm^{-1}

HR-ESI-MS (pos.): 526.2235 ($[M]^+$, $C_{28}H_{30}O_{10}^+$; calc. 526.2236)

^1H NMR (400MHz, $CDCl_3$): Given in **Table 3.21**

^{13}C NMR (100MHz, $CDCl_3$): Given in **Table 3.21**

4.3.4.5: Physalin H (66)

Physical state: Colorless solid

Yield: 40 mg (8.0 x 10^{-4} %)

Melting point: 238-240 C^o

Optical rotation $[\alpha]_D^{20}$:-70° (c = 0.17, $CHCl_3$)

UV (MeOH) λmax ($\log_{\varepsilon}$): 226nm (3.80)

IR (KBr): 3400, 2933, 1795, 1760, 1670, 1452, 1382, 1367, 1098, 499 cm^{-1}

HR-ESI-MS (pos.): 544.2235 ($[M]^+$, $C_{28}H_{32}O_{11}^+$; calc. 544.2236)

^1H NMR (400 MHz, $CDCl_3$): Given in **Table 3.22**

^{13}C NMR (100 MHz, $CDCl_3$): Given in **Table 3.22**

4.3.4.6: Withaphysalin A (67)

IUPAC name: 18, 20R-Epoxy-14-hydroxy-1,18-dioxo-22R-witha-2,5,24-trienolide

Physical state: Amorphous solid

Yield: 104 mg (2.0×10^{-3} %)

Melting point: 222-223 C°

Optical rotation $[\alpha]_D^{20}$: +43° (c = 0.18, $CHCl_3$)

UV (MeOH) λmax ($\log_{\varepsilon}$): 224 nm (5.26)

IR (KBr): 3447, 2927, 1755, 1694, 1452, 1382, 1347, 1134, 545 cm^{-1}

HR-ESI-MS (pos.): 466.4567 ($[M]^+$, $C_{28}H_{34}O_6^+$; calc. 466.4567)

^1H NMR (400MHz, $CDCl_3$): Given in **Table 3.23**

^{13}C NMR (100MHz, $CDCl_3$): Given in **Table 3.23**

4.3.4.7: Withaphysalin C (68)

IUPAC name: 13β, 14β:18,20R-Diepoxy-14-hydroxy-1-oxo-13,14-seco-22R-witha-2,5,24-trienolide

Physical state: Amorphous powder

Yield: 24 mg (4.8×10^{-4}%)

Melting point: 202-203 C°

Optical rotation $[\alpha]_D^{20}$: +33° (c = 0.15, $CHCl_3$)

UV (MeOH) λmax ($\log_{\varepsilon}$): 225 nm (5.2)

IR (KBr): 3350, 2965, 1715, 1684, 1452, 1382, 1319, 1135, 597 cm^{-1}

HR-ESI-MS (pos.): 484.4567 ($[M]^+$, $C_{28}H_{36}O_7^+$; calc. 484.4568)

^1H NMR (400 MHz, $CDCl_3$): Given in **Table 3.24**

^{13}C NMR (100 MHz, $CDCl_3$): Given in **Table 3.24**

4.3.4.8: Withaphysalin D (69)

IUPAC name: 18,20R-Epoxy-14-hydroxy-1,18-dioxo-22R-witha-3,5,24-trienolide

Physical state: Colorless powder

Yield: 14 mg (2.8 x 10^{-4}%)

Melting point: 202-203 C^{o}

Optical rotation $[\alpha]_D^{20}$: +43° (c = 0.15, $CHCl_3$)

UV (MeOH) λmax (log$_{\acute{\varepsilon}}$): 228 nm (5.8)

IR (KBr): 3350, 2943, 1755, 1694, 1452, 1382, 1329, 1150, 555 cm^{-1}

HR-ESI-MS (pos.): 466.4567 ($[M]^+$, $C_{28}H_{34}O_6^+$; calc. 466.4568)

^{1}H NMR (400 MHz, $CDCl_3$): Given in **Table 3.25**

^{13}C NMR (100 MHz, $CDCl_3$): Given in **Table 3.25**

4.3.4.9: Withaphysalin E (70)

IUPAC name: 18, 20R-Epoxy-6,14-dihydroxy-1,18-dioxo-22R-witha-2,4,24-trienolide

Physical state: Colorless amorphous powder

Yield: 27 mg (4.83 x 10^{-4}%)

Melting point: 311-312 C^{o}

Optical rotation $[\alpha]_D^{20}$: +61° (c = 0.18, $CHCl_3$)

UV (MeOH) λmax (log$_{\acute{\varepsilon}}$): 228 nm (4.1)

IR (KBr): 3350, 2956, 1745, 1699, 1645, 1452, 1382, 1360, 1189, cm^{-1}

HR-ESI-MS (pos.): 482.4567 ($[M]^+$, $C_{28}H_{34}O_7^+$; calc. 482.4569)

^{1}H NMR (400 MHz, $CDCl_3$): Given in **Table 3.26**

^{13}C NMR (100 MHz, $CDCl_3$): Given in **Table 3.26**

References

(1) Zhuang, L. G.; Otto, S.; Hildeberl, W. *Phytochemistry* **1983**, *11*, 617.

(2) Ronlan, A.; Wickberk, B. *Tetrahedron lett.* **1970**, 4261.

(3) Kupchan, S. M.; Baxter, R. L. *Science* **1975**, *187*, 652.

(4) Clark, A. M. *Pharmaceut. Res.* **1996**, *13*, 1133.

(5) Cragg, G. M.; Newman, D. J.; Snador, K. M. *J.Nat.Prod.* **1997**, *60*, 52.

(6) Hill, R. A.; Pitt, A. R. *Nat. Prod. Rep.* **1997**, *14*, iii.

(7) Rasyid, A.; Lelo, A. *Aliment. Pharmacol. Ther.* **1999**, *13*, 249.

(8) Rouhi, A. M. *Chem.and Eng. News* **1997**, *75*, 14.

(9) Rajbhandari, M.; Schopke, T. *Pharmazie* **1999**, *3*, 232.

(10) Stead, P. *Drug Discovery Today* **1997**, *2*, 256.

(11) Mahidol, C.; Prawat, H.; Ruchirawat, S. *Pure Appl. Chem.* **1997**, *69*, 655.

(12) Ulubelen, A.; Mericli, H.; Mericli, F.; Kolak, U.; Arfan, M.; Ahmad, M.; Ahmad, H. *Hetrocycles* **2000**, *53*, 2279.

(13) Rehman, I.; Arfan, M. *J. Chem.Soc. Pak.* **1997**, *19*, 240.

(14) Arfan, M.; Akhtar, G.; Naheed, A. *J. Chem. Soc. Pak.* **1996**, *18*, 170.

(15) Ahmad, K. D.; Khan, L.; Kifayatullah, Q.; Arfan, M. *J. Chem. Soc. Pak.* **1994**, *16*, 269.

(16) Khan, L.; Ahmad, K. D.; Kifayatullah, Q.; Arfan, M. *J.Pharmacol.* **1994**, *33*, 344.

(17) Arcy, D.; William, G. *Solanaceae*; Columbia University Press, 1986.

(18) Caceres, A.; Torres, M. F.; Ortiz, S.; Cano, F. *J. Ethnopharmacol.***1993**, *39*, 73

(19) Dimayuga, R. E.; Virgen, M.; Ochoa, N. *Pharm. Biol.* **1998**, *36*, 33.

(20) Bock, M. A.; Sanchez, P. *J. Plant Foods Hum. Nutr.* **1995**, *48*, 127.

(21) Sue-Jing, W. U.; Lean-Teik, N. G.; Yuan-Man, H. U. A. *Biol. Pharm. Bull.* **2005**, *28*, 963.

(22) Ray, A. B.; Ali, A.; Sahai, M.; Schiff, P. L.; Knapp, L.*Chem. Ind.* **1981**,*62*, 62.

(23) Neogi, P.; Sahai, M.; Ray, A. B. *Phytochemistry* **1987**, *26*, 243.

(24) Baumann, T. W.; Meier, C. M. *Phytochemistry* **1993**, *33*, 317.

(25) Waterfall, U. T. *Rhodora* **1967**, *69*, 203.

(26) Abdullaev, N. D.; Vasina, T. *Chem. Nat. Compd.* **1986**, *22*, 300.

(27) Kennelly, E. J.; Gerhauser, C.; Song, L. L.; Graham, J. G.; Pessuto, J. M.;

Kinghorn, A. D. *J. Agric. Food Chem.* **1997**, *45*, 3771.

(28) Kinghorn, A. D.; Fong, H. H. S.; Farnsworth, N. R.; Mehta, R. G.; Moon, R. C.; Moriatry, R. M.; Pessuto, J. M. *Curr. Org. Chem.* **1998**, *2*, 597.

(29) Gerhauser, C.; You, M.; Liu, J.; Moriatry, R. M.; Hawthorne, M.; Mehta, R. G.; Moon, R. C.; Pessuto, J. M. *Cancer Res.* **1997**, *57*, 272.

(30) Bao-Ning, S.; Rosana, M. *Tetrahedron* **2002**, *58*, 34.

(31) El Sayed, K. A.; Hamann, M. T.; Waddling, C. A.; Jensen, C.; Lee, S. K.; Dunstan, C. A.; Pessuto, J. M. *J. Org. Chem.* **1998**, *63*, 7449.

(32) Fuller, R. W.; Westegaard, C. K.; Collins, J. W.; Cardelina II, J. H.; Boyd, M. R. *J. Nat. Prod.* **1999**, *62*, 67.

(33) McKee, T. C.; Covington, C.; Fuller, R. W.; Bokesch, L. R.; Young, S.; Cardellina II, J. H. *J. Nat. Prod.* **1998**, *61*, 1252.

(34) Sommer, H. *J.Ger. Psych.Neuropharmacol.* **1994**, S9.

(35) Vorbach, E. U.; Hubner, W. D.; Arnoldt, K. H. *J.Ger. Psych.Neuropharmacol.* **1994**, S19.

(36) Hu, L. H.; Sim, K. Y. *Tetrahedron* **2000**, *56*, 1379.

(37) Bennett, G. J.; Lee, H. H. *Phytochemistry* **1989**, *28*, 967.

(38) Rocha, L.; Marston, A.; Potterat, O.; Kaplan, M. A. C.; Stoeckli Evans, H. *Phytochemistry* **1995**, *40*, 1447.

(39) Jayasuriya, H.; Clark, A. M.; McChesney, J. D. *J. Nat. Prod.* **1991**, *54*, 1314.

(40) Alexandre, B. F.; Ferraz, S., A. L; Bordignon, C. S.; Schripsema, J.; Von Poser, G. L. *Phytochemistry* **2001**, *57*, 1227.

(41) Bernardi, A. P. M.; Ferraz, A. B. F.; Albring, D. V.; Bordignon, S. A. L.; Schripsema, J.; Bridi, R.; Dutra-Filho, C. S.; Henriques, A. T.; Von Poser, G. L. *J.Nat.Prod.* **2005**, *68*, 784.

(42) Tada, M.; Chiba, K.; Yamada, H.; Maruyama, H. *Phytochemistry* **1991**, *30*, 2559.

(43) Kosuge, T.; Ishida, H.; Satoh, T. *Chem. Pharm. Bull.* **1985**, 33, 202.

(44) Zou, Y. P.; Lu, Y. H.; Wei, D. Z. *J. Agric. Food Chem.* **2004**, *52*, 5032.

(45) Zou, Y. P.; Lu, Y. H.; Wei, D. Z. *J. Agric. Food Chem.* **2005**, *53*, 2462.

(46) Reichling, J.; Weseler, A.; Saller, R. *Phytochemistry* **2001**, *58*, s116.

(47) Simon, G.; Elisabeth, M.; Sebastian, H.; Michael, S.; Eileen, S.; Christopher, C. *Phytochemistry* **2005**, *66*, 1472.

(48) Farjon, A. *World Checklist and Bibliography of Conifers, Kew* U.K, 1998.

p.300,

(49) Raffa, K. F.; Smalley, E. B. *Oecologia* **1995**, *102*, 285.

(50) Slimestad, R.; Andersen, F. M.; Francis, G. W.; Marston, A.; Hostettmann, K. *Phytochemistry* **1994**, *35*, 1537.

(51) Kraus, G.; Spiteller, G. *Phytochemistry* **1997**, *44*, 59.

(52) Nabeta, K.; Hirata, M.; Ohki, Y.; Samaraweera, S. W. A.; Okuyama, J. *Phytochemistry* **1994**, *37*, 409.

(53) Yueh-Hsiung, K. U. O. *Chem. Pharm. Bull.* **2004,** *52*, 861.

(54) Ayres, D. C.; Loike, J. D. *Lignans Chemical, Biologicaland Clinical Properties*; Cambridge University Press: Cambridge 1990, p.275–278.

(55) Willfor, S.; Hemming, J.; Eckerman, C. *Holzforsch.* **2003**, *57*, 27.

(56) Saarinen, N. M.; Warri, A.; Makela, S. I.; Eckerman, C.; Ahotupa, M.; Salmi, S. M.; Franke, A. A.; Kangas, L.; Santti, R. *Nutr.Cancer* **2000**, *36*, 207.

(57) Taskinena, A.; Eklundb, P.; Sjoholmb, R. *Theochem.* **2004**, *677*, 113.

(58) Lim, D. J.; Liu, X. L.; Sutkowski, D. M. *J .Agri. Food.Chem.* **1993**, *22*, 109.

(59) Horoszewicz, J. S.; Leong, S. S.; Kawinski, E.; Karr, J. P.; Rosenthal, H.; Chu, T. M.; Mirand, E. A.; Murphy, G. P. *Cancer Res.* **1983**, *43*, 1809.

(60) Willför, S.; Hemming, J.; Reunanen, M.; Holmbom, B. *Holzforsch.* **2003a**, *57*, 359.

(61) Eklund, P.; Lindholm, A.; Mikkola, J. P.; Smeds, A.; Lehtila, R.; Sjoholm, R. *Org. Lett.* **2003**, *5*, 491.

(62) Cheng, H. Y.; Lin, T. C.; Yu, K. H.; Yang, C. M.; Lin, C. C. *Biol. Pharm. Bull.* **2003**, *26*, 1331.

(63) Sue, J. W.; Lean, T. N.; Yuan, M. H.; Doung, L. L.; Shyh, S. W.; Shan, N. H.; Chun, C. L. *Biol. Pharm. Bull.* **2005**, *28*, 963.

(64) Yanping, Z.; Yanhua, L.; Dongzhi, W. *J. Agric. Food Chem.* **2004**, *52*, 5032.

(65) Tsao, R.; Deng, Z. *J.Chromatog.B* **2004**, *812*, 85.

(66) Lu, Y.; Foo, Y. *Food Chem.* **2000**, *68*, 81.

(67) Valentao, P.; Fernandes, E.; Carvalho, F.; Andrade, P. B.; Seabra, R. M.; Bastos, M. L. *Biol. Pharm. Bull.* **2002**, *25*, 1320.

(68) Filomena, C.; Giancarlo, A. S.; Rosa, T.; Francesco, M.; Peter, H. *Fitoterapia* **2002**, *73*, 479.

(69) Hsu, T. Y.; Sheu, S. C.; Liaw, E. T.; Wang, T. C.; Lin, C. C. *Phytomedicine* **2005**, *12*, 663.

(70) Pinelo, M.; Rubilar, M.; Sineiro, J.; Núñez, M. *J. Food Chem.* **2004**, *85*, 267.

(71) Gülçin, I.; Büyükokuroglu, M. E.; Oktay, M.; Küfrevioglu, Ö. I. *J. Ethnopharmacol.* **2003**, *86*, 51.

(72) Rabanal, R. M.; Arias, A.; Prado, B.; Hernandez-Perez, M.; Sanchez-Mateo, C. C. M. **2002**, *81*, 287.

(73) Srinivasan, D.; Nathan, S.; Suresh, T.; Perumalsamy, L. P. *J. Ethnopharmacol.* **2001**, *74*, 217.

(74) Poole, K. *J.Pharma. Pharmacol.* **2001**, *53*, 283.

(75) Marchese, A.; Schito, G. C. *Int. J. Antimicrob. Ag.* **2000**, *16*, s25.

(76) Subbaraju, G. V.; Vanisree, M.; Rao, C. V.; Sivaramakrishna; C.; Sridhar, P.; B.Jayaprakasam; Nair, M. G. *J. Nat. Prod.* **2006**, *69*, 1790.

(77) Nasir, E.; Ali, S. I. *Flora of Pakistan*; Shamim Printig Press Karachi Karachi, 1987; Vol. 178-186.

(78) Prajpati, P.; Kumar, S. *A Hand Book of medicinal Plants*; Agrobios (India): Dehli, 2003.

(79) Choudhary, M. I.; Yousuf, S.; Nawaz, S. A.; Ahmed, S.; Attu-ur-Rahman *Chem. Pharm. Bull.* **2004**, *52*, 1358.

(80) Atta-ur-Rehman; Shahbir, M.; Yousuf, M.; Qureshi, S.; Dur-e-Shahwar; Naz, A.; Choudhan, M. I. *Phytochemistry* **1999**, *52*, 1361.

(81) Budhiraj, R. D.; Bala, S.; Garge, K. N. *Planta Med.* **1977**, *32*, 154.

(82) Atta-Ur-Rahman.; Yousaf, M.; Gul, W.; Qureshi, S.; Choudhary, M. I.; Voelter, W.; Hoff, A.; Jens, F.; Naz, A. *Heterocycles* **1998c**, *48*, 1801.

(83) Atta-ur-Rahman.; Shabbir, M.; Dur-e-Shahwar.; Choudhary, M. I.; Voelter, W.; Hohnholz, D. *Heterocycles* **1998b**, *47*, 1005.

(84) Atta-ur-Rahman.; Abbas, S.; Dur-e-Shahwar.; Jamal, A. S.; Choudhary, M. I. *J. Nat. Prod.* **1993**, *56*, 1000.

(85) Atta-ur-Rahman.; Dur-e-Shahwar.; Naz, A.; Choudhary, M. I. *Phytochemistry* **2003**, *63*, 387.

(86) Kirtika, R. *Indian Medicinal Plants*; Allahbaj, 1981.

(87) Atta-ur-Rahman.; Choudhary, M. I.; Qureshi, S.; Gul, W.; Yousaf, M. *J. Nat. Prod.* **1998**, *61*, 812.

(88) Israili, A. H.; Siddiqui, H. H. *Ind.J.Pharm.* **1965**, *27*, 178.

(89) Atta-Ur-Rahman.; Choudhary, M. I.; Yousaf, M.; Gul, W.; Qureshi, S. *Chem. Pharm. Bull.* **1998a**, *46*, 1853.

(90) Agarwal, R.; Diwanay, S.; Patki, P.; Patwardhan, B. *J.Ethnopharmacol.* **1999**, *67*, 27.

(91) Ziauddin, M.; Phansalkar, N.; Patki, P. S.; Diwanay, S.; Patwardhan, B. K. *J. Ethnopharmacol.* **1996**, *50*, 69.

(92) Prakash, J.; Gupta, S. K.; K.Dinda, A. *Nut.and Cance.* **2002**, *42*, 91.

(93) Umadvi, P.; Akagi, K.; Ostapenko, V.; Tanaka, Y.; Sugahara, T. *Int.J.Radiat. biol.* **1996**, *69*, 193.

(94) Alhindawi, M. K.; Alkhafaji, S. H.; addul Nabi, M. H. *J.Ethnopharmacol.* **1992**, *37*, 113.

(95) Samuelson, G.; Farah, M. H.; Claeson, P.; Hagos, M.; Thulin, M.; Hedberg, O.; Warfa, A. M. A.; Hassan, O.; Elmi, A. H.; Abdurahman, A., D; Elmi, A., S; Abdi, Y. A.; Alin, M. H. *J.Ethnopharmacol.* **1993**, *38*, 7.

(96) Grandhi, A.; Mujumdar, A. M.; Patwadhan, B. *J.Ethnopharmacol.* **1994**, *44*, 131.

(97) Sahni, Y. P.; Srivastava, D. N.; Parasar, G. C. *Ind.Vet.J.* **1995**, *72*, 1035.

(98) Mehtha, A. K.; Binkely, P.; Gandhi, S. s.; Ticku, M. K. *ind. J.Med.Res.Sect.B* **1991**, *94*, 312.

(99) Jayaprakasam, B.; Zhang, Y.; Seeram, N. P.; Nair, M. G. *Life Sci.* **2003**, *74*, 125.

(100) Lavie, D.; Kirson, I.; Glotter, E. *Isr.J.Chem* **1968**, *6*,, 671.

(101) Glotter, E. *Nat. Prod. Rep.* **1991**, *8*, 415.

(102) Misra, L.; Lal, P.; Sangwan, R. S.; Sangwan, N. S.; Uniyal, G. C.; Tuli, R. *Phytochemistry* **2005**, *66*, 2702.

(103) Kumar, A.; Ali, M.; Mir, S. R. *I. J. Chem.* **2004**, *43B*, 2001.

(104) Jayaprakasam, B.; Strasburg, G. A. N. P.; Nair, M. G. *Tetrahedron* **2004**, *60*, 3109.

(105) Zhao, J.; Nakamura, N.; Hattori, M.; Kuboyama, T.; Thoda, C.; Komastsu, K. . *Chem. Pharm. Bull.* **2002**, *50*, 760.

(106) Abou-Douth, A. M. *Arch. Pharm.* **2002**, *6*, 267.

(107) Matsuda, H.; Murakami, T.; kishi, A.; yoshikawa, M. *Bioorg. Med. Chem.* **2001**, *9*, 1499.

(108) Kuroyanagi, M. L.; Shibata, K.; Umeharo, K. *Chem. Pharm. Bull.* **1999**, *47*, 1646.

(109) Ali, M.; Shuaib, M.; Ansar, S. H. *Phytochemistry* **1997**, *44*, 1163.

(110) Anjaneyulu, A. S. R.; Rao, D. S. *Ind. J Chem.* **1997**, *36B*, 424.

(111) Choudhary, M. I.; Abbas, S.; Jamal A.S.; Rahman, A. *Hetrocycles* **1996**, *42*, 555.

(112) Singh, K.; Miyagawa, M.; Yahara, S.; Mohara, T. *Chem. Pharm. Bull.* **1993**, *41*, 1873.

(113) Silva, G. L.; Pacciaroni, A.; Oberti, J. C.; Velerio, A. S.; Burton, G. *Phytochemistry* **1993**, *34*, 871.

(114) Lischewski, M.; Hang, N. T. B.; prozel, A.; Adam, G.; Massiot, G.; Lavaud, C. *Phytochemistry* **1992**, *31*, 930.

(115) Attaur-Rahman ; Jamal, S. A.; Choudhary, M. I. *Heterocycles* **1992**, *34*, 689.

(116) Bessalle, R.; Lavie, D. *Phytochemistry* **1992**, *31*, 3648.

(117) Attaur-Rahman.; Jamal, S. A.; Choudhary, M. I.; Asif, P. *Phytochemistry* **1991**, *30*, 3824.

(118) Ghosal, S.; Kaur, R.; Srivastava, R.; Radhary, S. *Ind. J. Nat. Prod.* **1988**, *4*, 12.

(119) Bessalle, R.; Lavie, D.; Frolow, F. *Phytochemistry* **1987**, *26*, 1797.

(120) Sahai, M. *J.Nat.Prod.* **1985**, *48*, 474.

(121) Velde, V. V.; Lavie, D. *Phytochemistry* **1982**, *21*, 731.

(122) Frolow, F.; Ray, A. B.; Sahai, M.; Glotter, E.; Gottlieb, I. E.; Kirson, I. *J.Chem.Soc. Perkin 1,* **1981**, 1029.

(123) Velde, V. V.; Lavie, D. *Phytochemistry* **1981**, *20*, 1359.

(124) Nittala, S. S.; Lavie, D. *Phytochemistry* **1981**, *20*, 2741.

(125) Eastwood, F. W.; Kirson, I.; Lavie, D.; Abraham, A. *Phytochemistry* **1980**, *19*, 1503.

(126) Kirson, I.; Gottlieb, E. H. *J. Chem.Res., Synop.* **1980**, 338.

(127) Kirson, I.; Abraham, A.; Lavie, D. *Isr. J. Chem.* **1977**, *16*, 20.

(128) Glotter, E.; Abraham, A.; Guenzberg, G.; Kirson, I. *J.Chem.Soc. Perkin 1* **1977**, 341.

(129) Sakurai, K.; Jshii, H.; Kobayashi, S.; Iwao, T. *Chem. Pharm. Bull.* **1976**, *24*, 1403.

(130) Kundu, A. B.; Mukherjee, A.; Dey, A. K. *Indian J. Chem. Sect. B* **1976**, *14B*, 434.

(131) Kirson, I.; Cohen, A.; Abraham, A. *J. Chem. Soc.Perkin l* **1975**, 2136.

(132) Abraham, A.; Kirson, I.; Lavie, D.; Glotter, E. *Phytochemistry* **1975**, *14*, 189.

(133) Chakraborti, S. K.; De, B. K.; Bandyophadayay. *Experientia* **1974**, *30*, 852.

(134) Lockley, W. J. S.; Robert, D. P.; Ress, H.; Goodween, T. W. *Tett. Lett.* **1974**, 3773.

(135) Glotter, E.; Kirson, I.; Abraham, A.; Lavie, D. *Tetrahedron* **1973**, *29*, 1353.

(136) Choudhary, M. I.; Dur-e-Shahwar.; Parveen, Z.; Jabbar, A.; Ali, I.; Atta-ur-Rahman. *Phytochemistry* **1995**, *40*, 1243.

(137) Lavie, D.; Kirson, I.; Glotter, E. *J. Chem. Soc (C).* **1971**, *11*, 2032.

(138) Kirson, I.; Glotter, E.; Abraham, A.; Lavie, D. *Tetrahedron* **1970**, *26*, 2209.

(139) Glotter, E.; Waitman, R.; Lavie, D. *J.Chem.Soc. (C)* **1966**, *19*, 1765.

(140) Lavie, D.; Glotter, E.; Shvo, Y. *J. org. Chem.* **1965**, *30*, 1774.

(141) Kupehan, S. M.; Anderson, W. K.; Bollinger, P.; Doskotch, R. W.; Smith, R. M.; Renaold, A. S.; Schenose, H. K.; Burlingance, A. L.; Smith, D. H. *J. Org. Chem.* **1969**, *34*, 3858.

(142) Nur-e-Alam, M.; Yousaf, M.; Qureshi, S.; Baig, I.; Nasim, S.; Atta-ur-Rahman.; Choudhary, M. I. *Helv. Chim. Acta.* **2003**, *86*, 607-614.

(143) Neogi, P.; Kawai, M.; Butsugan, Y.; Mori, Y.; Suzuki, M. *Bull. Chem. Soc. Jpn.* **1988**, *61*, 4479.

(144) Ramaiah, P. A.; Lavie, D.; Budhiraja, R. D.; Sudhir, S.; Garg, K. N. *Phytochemistry* **1984**, *23*, 143.

(145) Vande, V. V.; Lavie, D.; Budhiraja, R. D.; Sudhir, S.; Garg, K. N. *Phytochemistry* **1983**, *22*, 2253.

(146) Budhiraja, R. D.; Sudhir, S.; Garg, K. N. *Ind. J. Physio. Pharmacol.* **1983**, *27*, 129.

(147) Ahmada, S.; Malik, A.; Yasminb, R.; Nisar-Ullah.; Gul, W.; Khan, P. M.; Nawaz, H. R.; Afza, N. *Phytochemistry* **1999**, *50*, 647.

(148) Tong, H.; Liang, Z.; Wang, G. *Carbohyd. Polym.* **2008**, *71*, 316.

(149) Alluri, R. R.; Miller, R. J.; Shelver, W. H.; Khalil, S. K. W. *Ltoydia* **1976**, *39*, 405.

(150) Glotter, E.; Kirson, I.; Abraham, A.; Seihi, P. D.; Subramanian, S. S. *J. Chem. Soc.Perkin Trans. l* **1975**, 1370.

(151) Sinha, S.C.; Ali, A.; Bagchi, A.; Sahai, M.; Ray, A.B. *Planta Med.***1987**,*53*,55.

(152) Ma, L.; Gan, X. W.; He, Q. P.; Bai, H. Y.; Arfan, M.; Lou, F. C.; Hu, L. H. *Helv. Chim. Acta* **2007**, *90*, 1406.

(153) Damu, A. G.; Kuo, P.; Su, C.; Kuo, T.; Chen, T.; Bastow, K. F.; Lee, K.;

T.Wu *J. Nat. Prod.* **2007**, *70*, 1146.

(154) Basey, K.; McGaw, B. A.; Woolley, J. G. *Phytochemistry* **1992**, *31*, 4173.

(155) Chen, C.; Chen, Z.; Hsieh, C.; Li, W.; S, W. *Heterocycles* **1990**, *31*, 1371.

(156) Huang, A. J.; Viaetingk. *Fitoterapia* **1998**, *69*, 469.

(157) Ahmad, S.; Malik, A.; Yasminb, R.; Nisar-Ullah.; Gul, W.; Khan, P. M.; Nawaz, H. R.; Afza, N. *Phytochemistry* **1999**, *50*, 647.

(158) Mayorga, H.; Knapp, H.; Winterhalter, P.; Duque, C. *J. Agric. Food Chem.* **2001**, *49*, 1904.

(159) Su, B.; Rosan, M.; E.J.Park; Santarsiero, B. D.; Mesecar, A. D.; Fong, H. H. S.; Pezzuto, J. M.; Kinghorn, A. D. *Tetrahedron* **2002**, *58*, 3453.

(160) Castorena, A. P.; Oropeza, R. F.; Vazquez, A. R.; Martı´nez, M.; Maldonad, E. *J. Nat. Prod.* **2006**, *69*, 1029.

(161) Ovenden, S. P. B.; Yu, J.; Bernays, J.; Wan, S. S.; Christophidis, L. J.; Sberna, G.; M.Tait, R.; Wildman, H. G.; Lebeller, D.; Lowther, J.; Walsh, N. G.; Meurer-Grimes, B. M. *J.Nat.Prod.* **2005**, *68*, 282.

(162) Ahmad, S.; Malik, A.; Muhammad, P. *Fitoterapia* **1998**, *69*, 433.

(163) Maldonado, E.; Torres, F. R.; Martınez, M.; Perez-Castorena, A. L. *J.Nat. Prod.* **2006**, *69*, 1511.

(164) Vasina, O. E.; Maslennikova, V. A.; Abubakirov, N. K. *Khim. Priro. Soedi.* **1986b**, *22*, 263.

(165) Matsuura, T.; Kawai, M.; Nakashima, R.; Butsugan, Y. *Tetrahedron Lett.* **1969**, *12*, 1083.

(166) Kawai, M.; Matsuura, T.; Kyuno, S.; Matsuki, H.; Takenaka, M.; Katsuoka, T.; Butsugan, Y.; Saito, K. *Phytochemistry* **1987**, *26*, 3313.

(167) Matsuura, T.; Kawai, M.; Nakashima, R.; Butsugan, Y. *J. Chem. Soc.C* **1970**, 664.

(168) Kawai, M.; Matsuura, T. *Tetrahedron* **1970**, *26*, 1743.

(169) Row, L. R.; Sarma, N. S.; Matsuura, T.; Nakashima, R. *Phytochemistry* **1978**, *17*, 1641.

(170) Row, L. R.; Sarma, N. S.; Reddy, K. S.; Matsuura, T.; Nakashima, R. *Phytochemistry* **1978a**, *17*, 1647.

(171) Lee, S. W.; Pan, M. H.; Chen, C. M.; Chen, Z. T. *Chem. Pharm. Bull.* **2008**, *56*, 234.

(172) Qiu, L.; Zhao, F.; Jiang, Z. H.; Chen, L. X.; Zhao, Q.; Liu, H. X.; Yao, X. S.;

Qiu, F. *J.Nat.Prod.* **2008**, *71*, 642.

(173) Chen, R.; Liang, J. Y.; Liu, R. *Helv. Chim. Acta* **2007**, *90*, 963.

(174) He, Q. P.; Ma, L.; Luo, J. Y.; He, F. Y.; Lou, L. G.; Hu, L. H. *Chem.Biodiver.* **2007**, *4*, 443.

(175) Kuo, P. C.; Kuo, T. H.; Damu, A. G.; Su, C. R.; Lee, E. J.; Wu, T. S.; Shu, R.; Chen, C. M.; Bastow, K. F.; Chen, T. H.; Lee, K. H. *Organic Lett.* **2006**, *14*, 2953.

(176) Maldonado, E.; Alvarado, V. E.; Torres, F. R.; Martinez, M.; Perez-Castorena, A. L. *Planta Med.* **2005**, *71*, 548.

(177) Maldonado, E.; Alvarado, V. E.; Torres, F. R.; Martinez, M.; Perez-Castorena, A. L. *Planta Med.* **2004**, *70*, 59.

(178) Nagafuji, S.; Okabe, H.; Akahane, H.; Abe, F. *Biol. Pharm. Bull.* **2004**, *27*, 193.

(179) Su, B. N.; Gu, J.-Q.; Kang, Y. H.; Park, E. J.; Pezzuto, J. M.; Kinghorn, A. D. *Mini-Reviews Org. Chem.* **20004**, *1*, 115.

(180) Gu, J. Q.; Li, W.; Kang, Y. H.; Su, B. N.; Fong, H. H. S.; Breemen, R. B. V.; Pezzuto, J. M.; Kinghorn, A. D. *Chem. Pharm. Bull.* **2003**, *50*, 530.

(181) Kawai, M.; Yamamoto, T.; Makino, B.; Yamamura, H.; Araki, S.; Butsugan, Y.; Saito, K. *J. Asian Nat.Prod. Res.* **2001**, *3*, 199.

(182) Ahmad, S.; Malik, A.; Afza, N.; Yasmin, R. *J.Nat.prod.* **1999a**, *62*, 493

(183) Ahmad, S.; Yasmin, R.; Malik, A. *Chem. Pharm.Bull.* **1999b**, *47*, 477.

(184) Dinan, L. N.; Sarker, S. D.; Sik, V. *Phytochemistry* **1997** *44*, 509.

(185) Makino, B.; Kawai, M.; Ogura, T.; Nakanishi, M.; Yamamura, H.; Butsugan, Y. *J.Nat.Prod.* **1995**, *58*, 1668.

(186) Makino, B.; Kawai, M.; Kito, K.; Yamamura, H.; Butsugan, Y.; *Tetrahedron* **1995a**, *51*, 12529.

(187) Makino, B.; Kawai, M.; Kito, K.; Yamamura, H.; Butsugan, Y. *Bull.Chem. Soc.Jpn.* **1995b**, *68*, 219.

(188) Sunayama, R.; Kuroyanagi, M.; Umehara, K.; Ueno, A. *Phytochemistry* **1993**, *34*, 529.

(189) Shingu, K.; Miyagawa, M.; Yahara, S. N.; Toshihiro. *Chem. Pharm. Bull.* **1993**, *41*, 1873.

(190) Kawai, M.; Matsumoto, A.; Makino, B.; Mori, H.; Ogura, T.; Butsugan, Y.; Ogawa, K.; Hayashi, M. *Bull.Chem.Soc.Jpn.* **1993**, *66*, 1299.

(191) Kawai, M.; Ogura, T.; Makino, B.; Matsumoto, A.; Yamamura, H.; Butsugan, Y.; Hayashi, M. *Phytochemistry* **1992**, *31*, 4299.

(192) Shingu, K.; Yahara, S.; Okabe, H.; Nohara, T. *Chem. Pharm. Bull.* **1992a**, *40*, 2448.

(193) Shingu, K.; Yahara, S.; Okabe, H.; Nohara, T. *Chem. Pharm. Bull.* **1992b**, *40*, 2088.

(194) Shingu, K.; Marubayashi, N.; Ueda, I.; Yahara, S.; Nohara, T. *Chem. Pharm. Bull.* **1991**, *39*, 1591.

(195) Vasina, O. E.; Abdullaev, N. D.; Abubakirov, N. K. *Khim. Prir. Soedin.* **1990**, *27*, 366.

(196) Oshima, Y.; Hikino, H.; Sahai, M.; Ray, A. B., *J.Chem.Soc. Chem. Comm.* **1989,** 628.

(197) Kawai, M.; Ogura, T.; Nakanishi, M.; Matsuura, T.; Butsucan, Y.; Mori, Y.; Harada, K.; Suzuki, M. *Bull.Chem.Soc.Jpn.* **1988**, *61*, 2696.

(198) Sinha, S. C.; Ray, A. B.; Oshima, Y.; Bagchi, A.; Hikino, H. *Phytochemistry* **1987**, *26*, 2115.

(199) Neogi, P.; Sahai, M.; Ray, A. B. *Phytochemistry*, *26*, 243.

(200) Gottlieb, H. E.; Cojocaru, M.; Sinha, S. C.; Saha, M.; Bagchi, A.; Ali, A.; Ray, A. B. *Phytochemistry* **1987**, *26*, 1801.

(201) Vasina, O. E.; Abdullaev, N. D.; Abubakirov, N. K. *Chem.Nat.Compd.* **1987**, *23*, 712.

(202) Vasina, O. E.; Maslennikova, V. A.; Abdullaev, N. D.; Abubakirov, N. K. *Khim. Priro. Soedi.* **1986a**, *22*, 596.

(203) Abdullaev, N. D.; Vasina, O. E.; Maslennikova, V. A.; Abubakirov, N. K. *Khim. Priro. Soedi.* **1985**, *21*, 657.

(204) Abdullaev, N. D.; Vasina, O. E.; Maslennikova, V. A.; Abubakirov, N. K. *Khim. Priro. Soedi.* **1986a**, *22*, 300.

(205) Reddy, K. S.; Row, L. R.; Matsuura, T. *J.Chem.Soc.Perkin I* **1985**, 419.

(206) Glotter, E.; Sahai, M.; Kirson, I.; Gottlieb, E. H. *J.Chem.Soc.Perkin I* **1985**, 2241.

(207) Abdullaev, N. D.; maslennikova, V. A.; Tursonova, R. N.; Abubakirov, N. K.; Yagudae, M. R. *Khim. Priro. Soedi.* **1984**, *20*, 182.

(208) Sahai, M.; Kirson, I. *J.Nat.Prod.* **1984**, *47*, 527.

(209) Bagchi, A.; Neogi, P.; Saha, M.; Ray, A. B.; Oshima, Y.; Hikino, H.

Phytochemistry **1984**, *23*, 853.

(210) Ali, A.; Saha, M.; Ray, A. B.; Salakin, D. J. *J.Nat.Prod.* **1984**, *47*, 648.

(211) Sahai, M.; Ali, A.; Ray, A. B.; Slatkin, D. J.; Kirson, I. *J.Chem.Res.Synop.* **1983**, 152.

(212) Kirson, I.; Glotter, E.; Ray, A. B.; Ali, A.; Gottlieb, E. H.; Saha, M. *J.Chem.Res.Synop.* **1983**, 120.

(213) Sahai, M.; Neogi, P.; Ray, A. B.; Oshima, Y.; Hikino, H. *Heterocycles* **1982**, *19*, 37.

(214) Sahai, M.; Gottlieb, E. H.; Ray, A. B.; Ali, A.; Glotter, E.; Kirson, I. *J.Chem.Res.Synop.* **1982a**, 346.

(215) Frolow, F.; Ray, A. B.; Sahai, M.; Glotter, E.; Gottlieb, E. H.; Kirson, I. *J.Chem.Soc.Perkin I* **1981**, 1029.

(216) Pletter, S. W.; Gebeyehu, G.; Nowacki, J.; Mody, N. V. *Heterocycles* **1981**, *15*, 317.

(217) Tursonova, R. N.; Maslennikova, V. A.; Abubakirov, N. K. *Khim. Priro.Soedi.* **1981**, *17*, 187.

(218) Ray, A. B.; Sahai, M.; Schiff, P. L.; Knapp, H.; Slatkin, D. J. *Chem.Ind.* **1981**,, 62.

(219) Maslennikova, V. A.; Tursonova, R. N.; Scitanidi, K. L.; Abubakirov, N. K. *Khim. Priro. Soedi.* **1980**, *16*, 214.

(220) Kirson, I.; Gottlieb, E. H.; Glotter, E. *J.Chem.Res.Synop.* **1980a**, -, 125.

(221) Row, L. R.; Reddy, K. S.; Sarma, N. S.; Matsuura, T.; Nakashima, R. *Phytochemistry* **1980**, *19*, 1175.

(222) Gottlieb, E. H.; Kirson, I.; Glotter, E.; Ray, A. B.; Saha, M.; Ali, A. *J.Chem.Soc.Perkin I* **1980**, -, 2700.

(223) Kirson, I.; Cohen, A.; Greenberg, M.; Gottlieb, E. H.; Varenne, P.; Abraham, A. *J.Chem.Res.Synop.* **1979**, -, 103.

(224) Ray, A. B.; Saha, M.; Das, B. C. *J.Indian.Chem.Soc.* **1978**, *55*, 1175.

(225) Maslennikova, V. A.; Tursonova, R. N.; Abubakirov, N. K. *Khimiya Prirodnykh Soedinenii* **1977**, *13*, 443.

(226) Kirson, I.; Zaretskii, Z.; Glotter, E. *J.Chem.Soc.Perkin I* **1976**, 1244.

(227) Kirson, I.; Abraham, A.; Sethi, P. D.; Subramanian, S. S.; Glotter, E. *Phytochemistry* **1976**, *15*, 340.

(228) Subramanian, S. S.; Sethi, P. D. *Ind. J. Pharm.* **1973**, *35*, 36.

(229) Fajardo, V.; Podesta, F.; Shamma, M.; Feryer, A. J. *J.Nat.Prod.* **1991**, *54*, 554.

(230) Luis, J. G.; F. Echeverri.; González, A. G. *Phytochemistry* **1994a**, *36*, 1297.

(231) Luis, J. G.; Echeverri, F.; Quiñones, W.; González, A. G.; Torres, F.; Cardona, G.; Archbold, R.; Perales, A. *Tetrahedron* **1994b**, *50*, 1217.

(232) Sethi, P. D.; Kosha, R. L. *Curr.Sci.(Indian)* **1975**, *44*, 867.

(233) Shanazbano, S. S.; Babu, V. L. A.; Dhanapal, R. *Asian J.Chem.***2006**, *18*, 1243.

(234) Kupehan, S. M.; Doskotch, R. W. G.; Bollinger, P.; McPhail, A. T.; Sim, G. A.; Renauld, J. A. S. *J. Am. Chem. Soc.* **1965**, *87*, 5805.

(235) Gill, H. K.; Smith, R. W.; Whiting, D. A. *J.Chem. Soc.Chem.Commun.* **1986**, 1459.

(236) Yoshida, M.; Hoshi, A.; Kurentani, K.; Ishiguro, M. *J. Pharm. Dyn.***1979**,*2*,92.

(237) Shohat, D.; Kirson, I.; Lavie, D. *Biomedicine* **1978**, *28*, 18.

(238) Budhiraj, R. D.; Sudhir, S. *J. Scientific Ind. Res.* **1987**, *46*, 488.

(239) Shohat, D.; Gitter, S.; Lavie, D. *int.J.Cancer* **1970**, *5*, 244.

(240) Budhiraj, R. D.; Garg, K. N.; Sudhir, S.; Arora, B. *Planta Med.* **1986**, *52*, 28.

(241) Bahr, V.; Hanset, R. *Planta Med.***1982**, *48*, 32.

(242) Bales, R. B.; Eckert, D. J. *J.Am.Chem.Soc.* **1972**, *94*, 8258.

(243) Antoun, M. D.; Abramson, D.; Tyson, R.; Chang, C.; Melaughlin, J. L.; Peck, G.; Cassady, J. M. *J.Nat.Prod.* **1981**, *44*, 579.

(244) Mohana, K.; Uma, R.; Purushothaman, k. K. *Indian J. Exp.Biol.* **1979**, *17*, 690.

(245) Januario, A. H.; Filho, E. R.; Pietro, R. C. L. R.; Kashima, S.; Sato, D. N.; France, S. C. *Phytother. Res.* **2002**, *16*, 445.

(246) Choudhary, M. I.; Yousuf, S.; A, S.; Shakil;; Atta-Ur-Rahman. . *Nat. Prod. Res.,Part A* **2007**, *21*, 877.

(247) Herrera, N. J. J.; Bremner, P.; Marquez, N.; Gupta, M. P.; Gibbons, S.; Munoz, E.; Cascade, M. H. *J.Nat.Prod.* **2006**, *69*, 328.

(248) Rosana, I. M.; Juan, C. O. *J.Nat.Prod.* **1996**, *59*, 66.

(249) Riaz, N.; Malik, A.; Aziz-ur-Rehman; Nawaz, A. S.; Muhammad, P.; Choudhary, M. I. *Chem. Biodiver.* **2004**, *1*, 1289.

(250) Pavia, D. L.; Lampman, G. M.; Kriz, G. S. *Introduction to spectroscopy*; Saunders College Publishing Co.,Philadelphia p.64, 1979.

(251) Monteagudo, E. S.; Burton, G.; Gonzalez, C. M.; Oberti, J. C.; Gros, E. G.

Phytochemistry **1988**, *27*, 3925.

(252) Abdeljebbar, L. H.; Humam, M.; Christen, P.; Jeannerat, D.; Vitorge, B.; Amzazi, S.; Benjouad, A.; Hostettmann, K.; Bekkouche, K. *Helv. Chim. Acta.* **2007**, *90*, 346.

(253) Velde, V. V.; Lavie, D.; Budhiraja, R. D.; Sudhir, S.; Garg, K. N. *Phytochemistry* **1983**, *22*, 2253.

(254) Nawaz, H. R.; Malik, A.; Khan, P. M.; Ahmed, S. *Phytochemistry* **1999**, *52*, 1357.

(255) Sinha, S. C.; Ray, A. B.; Oshima, Y.; Bagchi, A.; Hikino, H. *Phytochemistry* **1987**, *26*, 2115.

PART B

Phytochemical Studies of the Selected Species of Family Guttiferae

Chapter: 5

INTRODUCTION (Part B)

5.1: Guttiferae

Family Guttiferae belongs to the order Malpighiales, comprised of about 50 genera and 1200 species. They are found mainly in tropical regions and also in the northern temperate regions. They are trees, shrubs or herbs often with milky sap. A number of useful timbers, drugs, dyes, gums, pigments, and resins are derived from the members of the family[1]. A peculiarity of the Guttiferae family is that the leaves contain glands, containing oil and sometimes a pigment, which appear as translucent spots when held against a source of light, or as black dots on the surface[2]. This family is widely distributed in East Asia and North East America. The subfamily Hypericoideae is sometimes treated as a separate family, Hypericaceae. There is some disagreement as to the plant's family, some placing *Hypericum* in the segregate family Hypericaceae, while others place it in the family Guttiferae. However, most researchers now think that the morphological and chemical differences of the two families are insufficient to justify separating them[3,4].

Plants of the Guttiferae are characterized by the occurrence of xanthones[5] and several other constituents with diverse biological activities.[6,7]. Guttiferae is of pharmaceutical importance because of St John's wort, which in the last decade of the 20th century became one of the most important medicinal plants in Western medicine. The genus *Hypericum* has about 400 species. Many of them are used in folk medicine as anti-septic, diuretic, digestive, expectorant, vermifugal, anti-depressive and as other remedies[7].

5.2: Genus *Hypericum*

Hypericum is a large genus and represented by 400 species worldwide however, absent from arctic regions and rare in Australasia and lowland tropical regions[8]. *Hypericum* species are found as trees, shrubs or perennial to annual herbs, glabrous, with translucent, red or black glands. In Pakistan this genus is represented by the following nine species[8].

1. *Hypericum perforatum*
2. *Hypericum monogynum*
3. *Hypericum oblongifolium*
4. *Hypericum choisionum*
5. *Hypericum dyeri*
6. *Hypericum uralum*
7. *Hypericum scabrum*
8. *Hypericum elodeoides*
9. *Hypericum Nepalese*

Shrubs of *H. oblongifolium* are widely found in Hazara, Swat and Buner while *H.dyeri* is rarely found in Swat and Hazara Districts of N.W.F.P. The plant materials of *H. oblongifolium* and *H.dyeri* were collected during its trimming period (July, 2005 and August, 2006 respectively) from their respective locations for the research work presented here.

5.2.1: *Hypericum oblongifolium* Wall.

H*ypericum oblongifoliun* Wall. is an erect evergreen shrub 1—2 m high, usually is common on Khasia Hill at an altitude of 1800-3600 m in China and in the Himalaya Hills[9]. Stems are spreading; branches 4-lined and flattened at first, eventually trite. Leaves with petiole 2-4 mm long; lamina 25-88 mm long, 10-42 mm broad, ovate to lanceolate or oblong-lanceolate, flowers are 4-7 cm in diameter and yellow in colour. Capsule are 4-19 mm long, ovoid, without vitae or vesicles. Seeds are 0.7-1 mm long, carinate or slightly winged; testa shallowly linearreticulate[8]. It is being used in traditional Chinese herbal medicine for the treatment of hepatitis, bacterial diseases, nasal hemorrhage and as a remedy for dog bite and the sting of bees.[10]



Hypericum oblongifolium

5.2.2: *Hypericum dyeri* Rehder.

Hypericum dyeri Rehder is an erect evergreen shrub, 0.6—1.2 m high, found on cliffs and rocky slopes at an altitude of 1500-2400 m in China and in the Himalaya Hills[8]. Stems arching; branches 2-4-lined and flattened at first, soon 2-lined to trite. Leaves with petiole 1-2 mm long; lamina 10-60 mm long. 5-35 mm broad, ovate to lanceolate or elliptic-lanceolate, apex acute or apiculate to rounded, base cuneate to rounded, venation laxly or scarcely reticulate. Flowers 1.5-3.5 cm in diameter. Capsule 7-10 mm long, subglobose, without vittae or vescicles. Seeds 0.9-1 mm long, apiculate, carinate; testa laxly reticulate[8].



Hypericum dyeri

5.2.3: Pharmacological importance of *Hypericum* species

Hypericum (Guttiferae) is large genus found as herbs and shrubs. They are used as medicinal plants in various parts of the world in traditional folk medicines[11]. Several species have been used in folk medicines and a number of species have been found to possess various biological properties. Plants belonging to the genus *Hypericum* have been used in traditional medicine for the treatment of trauma, rheumatism, neuralgia, gastroenteritis, ulcers, hysteria, bedwetting, burns, bruises, inflammation, swelling and anxiety as well as bacterial and viral infections [12,13].

Hypericum is of pharmaceutical importance because of St John's wort (*Hypericum perforatum)*. Dioscorides, the famous Greek herbalist recommended four species of *Hypericum* namely *H uperikon, H.askuron, H.androsaimon* and *H.koris* as herbal remedy for sciatica.[14]. Theophrastus recommended *H lanuginosum* for external

applications, while Pliny prescribed its usage in wine against poisonous reptiles. Another Greek specie *H.eoris*, was mentioned by Hippocrates and Pliny in their traditional medicines. The Indians and American used several indigenous species of *Hypericum* as an antidiarrheal, abortifacient, hemostat, dermatological aid, febrifuge, snakebite remedy and as general health tonic. European settlers introduced St. John's wort and used it for similar conditions[15]. Oil from *Hypericum* was used for the treatment of wounds, and bruises and even used by the surgeons to clean foul wounds, and was official in the first London Pharmacopoeia as Oleum

H.perforatum (St. lohn's wort) is one of the most often used species in herbal remedies. *H. perforatum* has been used a medicinal plant since ancient time. It is used for the treatment of a range of ailments for more than 2000 years[16]. It is one of the medicinal plant traditionally used in European countries for the treatment of melancholia, abdominal and urinogenetal pain, ulcerated burns[17], skin injuries and neuralgia. Recently, it has already gained a considerable international recognition and now successfully establishing the status as a standard antidepressant therapy[17]. In Europe various preparations of *H. perforatum* are commercialized for the treatment of various depressive diseases[18]. It is widely used as healing and anti-inflammatory agent in traditional medicine. Some preparations of the extract of this plant are used for their anti-viral and anti-depressive properties[19,20]. Alcoholic extracts from the flowers of *H. perforatum* are widely used as antidepressant[21]. *H. perforatum* is also reported for its antiviral activity against human immunodeficiency virus (HIV) and hepatitis C virus[22].

H.grandifolium used for skin infections in traditional Canadian medicines[23]. *H.hookerianum* has been used widely in India as a wound healing agent as an ointments prepared from the dried extracts of the leaves and stems of this plant[24]. The dried whole plant of *H. japonicum* was used for the treatment of scrofula, contusions, abscesses, wounds, skin diseases, and leech bites in traditional Chinese medicine[25]. *H. erectum* is another important herb used in Chinese medicine as anti-hemorrhagic antibiotic and astringent[26]. *H. sampsonii* is used in Chinese herbal medicine for the treatment of backache, burns, diarrhea, snakebites, blood status, hepatitis, hematoma as well as detoxifying agent and as remedy for swelling and as an antitumor in Taiwan[27-29]. The southern Brazilian *Hypericum* species *H.brasiliensis* and *H.connatum* are popularly used for relief of disorders such as angina, cramps, oral and pharyngeal inflammations[30]. *H. japonicum*, *H.*

geminiflorum and *H. Patulum* are used as herbal medicine for the treatment of several diseases caused by bacteria, infectious hepatitis, gastrointestinal disorder, nasal hemorrhage and tumors[31-33]. *H. papuanum* are used in folk medicines for the treatment of sores. *H. ascyron* L. used in Chinese herbal medicine for the treatment of numerous disorders like boils, abscesses, headache, nausea and stomach ache[34]. *H. scabrum* is used in the treatment of heart diseases, rheumatism and cystitis in Uzbakistan[35]. A crude lipophilic extract of *H. caprifoliatum* and its constituents showed antidepressant action and antinociceptive effect[36]. Recently antifungal, antibiotic, anticancer and antiviral constituents were isolated from the species of this genus[32].

Various *Hypericum* species have been reported for their biological potentails. *H. caprifoliatum, H. piriai* and *H. polyanthemum* extracts showed monoamine oxidase A-inhibitory activity[37]. *H. caprifoliatum* and *H. polyanthemum* extract showed antinociceptive effects[38]. Plant extracts of *H. mysorence* and *H. hookerianum* exhibited significant antiviral activity[39]. The methanolic extract of *H. capilatum* exhibited antiviral activity against HSV[40]. Methanolic extracts of the aerial parts of both *H. mysorense* and *H. hookerianum* displayed significant effects in anxiety and inflammation[39].The alcoholic extract of *H. calycinum* have also shown antidepressant activity which is almost equal to the extract prepared from St. John's wort, (*H perforatum)*[41]. The methanolic extract of *H. hookerianum* and *H. patulam* was found to have wound healing potential[42,43]. The methanolic extract of *H. empertrifolium* exhibited anti-inflammatory activity[44]. Flower extracts of *H .perforatum, H. hirsutum, H. patulum* and *H. olympicum* efficiently inhibited binding of [3H] flumazenil to rat brain benzodiazepine binding sites of the GABA-receptor *in vitro*[45].

Among the approximately 20 native *Hypericum* species from south Brazil, only *H. brasiliense* has been investigated. Xanthones and phloroglucinol derivatives were isolated from this plant and its extracts have been found to inhibit monoamine oxidases (MAO) enzymes important in the regulation of levels of some physiological amines and which are thought to contribute to the management of depression[46]. benzopyrans are isolated from the aerial parts of *H. polyanthemum*[47 50] and benzo-phenones are isolated from the aerial parts of *H carinatum.*, native to southern Brazil, with cytotoxic and anti-HIV activities. It has been reported that some benzophenones (i.e.,garcinol) possess free radical scavenging abilities[48].[51] *H. erectum*, a traditional

Chinese herb used as an anti-hemorrhagic agent and antibiotic agent[23,52] has been reported to contain some antiviral prenylated phloroglucinol derivatives[49,53] and two anti-hemorrhagic compounds, otogirin and otogirone[50].[54] Phytochemical analysis of *H perforatum* L shows that it is a rich source of flavonoids, and much of its antioxidant activities are attributed to these compounds. However, research on this plant has focussed mainly on its antidepressant activity. A Flavonoid-rich Extract of *H. perforatum L*. (FEHP) was prepared and its antioxidant activity was determined by a series of models *in vitro*[51.] The hypocholesterolemic effect of (FEHP) was observed by determining the serum lipid level and antioxidant enzyme activity in rats fed a cholesterol-rich diet[52,56]. The genus *Hypericum* is a rich source of antibacterial metabolites of which hyperforin isolated from *H perforatum* is an exceptional example. Minimum inhibitory concentration (MIC) values for this natural product range from 0.1 to 1 g/ml against Penicillin-Resistant *Staphylococcus aureus* (PRSA) and Methicillin-Resistant *S. aureus* (MRSA) strains[53, 57]. These results substantiate the use of *H perforatum* in several countries as a treatment for super burns and wounds that heal poorly. An investigation into the antibacterial properties of *H foliosum* has led to the isolation of a new bioactive acylphloroglucinol. It was tested against a panel of multi drug-resistant strains of *Staphylococcus aureus* and the minimum inhibitory concentration (MIC) ranged from 16 to 32 µg/ml.[5459]

5.2.4: Reported phytochemical investigations

The genus *Hypericum* which contains about 400 species has been long used in folk medicine as traditional medicinal plants in various parts of the world since long.[55] Most of these species have been used for a long time as treatment of external wounds and gastric ulcer and also as sedative, antiseptic and antispasmodic in folk medicine[55]. Some of the chemicals isolated from this genus have exhibited anti-septic, anxiolytic, diuretic, digestive, expectorant, vermifugal, anti-depressive[55]. Hypericin and pseudohypericin were studied for their antiviral activity on lipid enveloped and non-enveloped DNA and RNA viruses. These polycyclic quinines were isolated from *H.perforatum*, the most well known specie widely employed as for its anti- depressive action[56,57]. The antidepressant activity of *H. perforatum* (St. John's wort) has resulted in the widespread interest in the study of the *Hypericum*

genus[58] and has led to the isolation of more than 300 compounds (Table 5.1 and 5.2). The most common compounds isolated from plants of this genus are xanthones[59], flavonoids[46], phloroglucinol, licinic acid derivatives[60], benzopyrans[47] as well as benzophenones[48]. However, according to our knowledge xanthones were found abundantly in the plants belonging to family Guttiferae and more than 80 xanthones were isolated from *Hypericum* (Table 5.1). List of chemical constituents isolated from the various species of *Hypericum* is given table 5.1 and 5.2.

Table 5.1: List of chemical constituents (other than xanthones) isolated from the various species of *Hypericum*

S.No	M. Formula	M. mass	Name	Source
1.	$C_{19}H_{18}O_4$	310.34	Cariphenone A	*H.carinatum*[48]
2.	$C_{19}H_{18}O_4$	310.34	Cariphenone B	*H,carinatum*[48]
3.	$C_{31}H_38O_4$	474.63	Biyouyanagin A	*H.chinense*[61]
4.	$C2_1H_{30}O_5$	362.46	4-Deoxyadhumulone 2',3-Epoxide	*H.foliosum*[62]
5.	$C_{17}H_{16}O_6$	316.31	4-Hydroxy-3-methoxyphenyl ferulate	*H.hookerianum*[63]
6.	$C_{35}H_{50}O_4$	534.77	Hypersampsone A	*H. sampsonii*[29]
7.	$C_{35}H_{52}O_4$	536.77	Hypersampsone B	*do*
8.	$C_{32}H_{46}O_4$	530.77	Hypersampsone C	*do*
9.	$C_{38}H_{50}O_4$	570.81	Hypersampsone D	*do*
10.	$C_{38}H_{50}O_4$	570.81	Hypersampsone E	*do*
11.	$C_{38}H_{48}O_4$	568.81	Hypersampsone F	*do*
12.	$C_{19}H_{20}O_8$	376.36	Hyperinone	*H. styphelioides*[64]
13.	$C_{20}H_{28}O_4$	332.43	3,4-Dihydro-5,7-dihydroxy-2-methyl-2-(4-methyl-3-pentenyl)-6-(2-methylpropanoyl)-2H-1-benzopyran	*H.jovis*[65]
14.	$C_{20}H_{28}O_4$	332.43	Hyperjovinol B	*do*
15.	$C_{31}H_{46}O_5$	498.70	Hyperibone J	*H.scabrum*[35]
16.	$C_{33}H_{40}O_4$	500.67	Hyperibone K	*do*
17.	$C_{29}H_{36}O_4$	448.60	Hyperibone L	*do*
18.	$C_{33}H_{42}O_4$	502.69	7-Epiclusianone	*do*
19.	$C_{21}H_{40}O_2$	324.54	5-Methyl-5-(4,8,12-trimethyltridecyl)dihydro-2(5H)-furanone	*H.perforatum*[66]
20.	$C_{11}H_{10}O_4$	206.19	5-Hydroxy-7-methoxy-3-methyl-4H-1-benzopyran-4-one. 5-Hydroxy-7-methoxy-3-methylchromone	*do*
21.	$C_{21}H_{30}O_4$	346.46	3,4-Dihydro-5,7-dihydroxy-2-methyl-8-(2-methylbutanoyl)-2-(4-methyl-3-pentenyl)-2H-1-	*H.amblycalyx*[67]

			benzopyran	
22.	$C_{20}H_{28}O_4$	332.43	3,4-Dihydro-5,7-dihydroxy-2-methyl-2-(4-methyl-3-pentenyl)-8-(2-methylpropanoyl)-2H-1-benzopyran	*do*
23.	$C_{25}H_{36}O_4$	400.55	Hypercalyxone A	*do*
24.	$C_{25}H_{36}O_4$	400.55	Hypercalyxone A	*do*
25.	$C_{26}H_{38}O_4$	414.58	Hypercalyxone B	*do*
26.	$C_{38}H_{66}O_4$	586.93	Nonacosyl caffeate	*H. laricifolium*[68]
27.	$C_{25}H_{36}O_4$	400.55	Hyperatomarin	*H.atomarium*[69]
28.	$C_{31}H_{44}O_4$	480.68	Erectone A	*H.erectum*[70]
29.	$C_{31}H_{44}O_4$	480.68	Erectone B	*do*
30.	$C_{21}H_{28}O_4$	344.45	Erectquione A	*H.erectum*[71]
31.	$C_{29}H_{40}O_6$	484.63	Erectquione B	*do*
32.	$C_{25}H_{34}O_6$	430.54	Erectquione C	*do*
33.	$C_{33}H_{42}O_5$	518.69	Hyperibone I	*H.scabrum*[72]
34.	$C_{33}H_{42}O_5$	518.69	Hyperibone A	*do*
35.	$C_{33}H_{42}O_5$	518.69	Hyperibone B	*do*
36.	$C_{33}H_{42}O_6$	534.69	Hyperibone C	*do*
37.	$C_{33}H_{42}O6$	534.69	Hyperibone D	*do*
38.	$C_{33}H_{42}O7$	550.69	Hyperibone E	*do*
39.	$C_{33}H_{42}O6$	534.69	Hyperibone F	*do*
40.	$C_{33}H_{42}O5$	518.69	Hyperibone G	*do*
41.	$C_{33}H_{42}O6$	534.69	Hyperibone H	*do*
42.	$C_{10}H_8O4$	192.17	5,7-Dihydroxy-3-methylchromone	*H.annulatum*[73]
43.	$C_{19}H_{20}O_{10}$	408.36	Annulatophenonoside	*do*
44.	$C_{21}H_{22}O_{11}$	450.39	Acetylannulatophenonoside	*do*
45.	$C_{20}H_{26}O_4$	330.42	Hyperguinone A	*H. papuanum*[74]
46.	$C_{21}H_{28}O_4$	344.45	Hyperguinone B	*do*
47.	$C_{26}H_{38}O_4$	414.58	Hyperpapuanone	*do*
48.	$C_{26}H_{36}O_4$	412.56	Papuaforin A	*do*
49.	$C_{26}H_{36}O_4$	412.56	Papuaforin B	*do*
50.	$C_{27}H_{38}O_4$	426.59	Papuaforin C	*do*
51.	$C_{32}H_{46}O_4$	494.71	Papuaforin D	*do*
52.	$C_{31}H_{44}O_4$	480.68	Papuaforin E	*do*
53.	$C_{24}H_{30}O_8$	446.49	Hypertricone	*H.geminiflorum*[75]
54.	$C_{16}H_{20}O_4$	276.33	7-Hydroxy-6-isobutyryl-5-methoxy-2,2-dimethylchromene	*H.polyanthemum*[76]
55.	$C1_6H_{20}O_4$	276.33	5-Hydroxy-6-isobutyryl-7-methoxy-2,2-dimethylchromene	*do*
56.	$C_{17}H_{22}O_4$	290.35	6-Isobutyryl-5,7-dimethoxy-2,2-dimethylchromene	*do*
57.	$C_{21}H_{30}O_5$	362.46	Enaimeone A	*H. papuanum*[77]
58.	$C_{21}H_{30}O_5$	362.46	Enaimeone B	*do*
59.	$C_{22}H_{32}O_5$	376.49	Enaimeone C	*do*
60.	$C_{21}H_{30}O_5$	362.46	1'-Hydroxyialibinone A	*do*
61.	$C_{21}H_{30}O_5$	362.46	1'-Hydroxyialibinone B	*do*
62.	$C_{22}H3_2O_6$	376.49	1'-Hydroxyialibinone D	*do*

63.	$C_{21}H_{30}O_6$	378.46	Furonewguinone A	*do*
64.	$C_{19}H_{20}O_{11}$	424.36	Hypericophenonoside	*H.annulatum*[78]
65.	$C_{14}H_{12}O_6$	276.24	Annulatophenone	*do*
66.	$C_{35}H_{50}O_4$	534.77	Pyrano[7,28-b]hyperforin	*H.perforatum*[79]
67.	$C_{35}H_{50}O_4$	534.77	Pyrohyperforin	*H.perforatum*[80]
68.	$C_{21}H_{28}O_4$	344.45	Ialibinone A	*H. papuanum*[81]
69.	$C_{21}H_{28}O_4$	344.45	Ialibinone B	*do*
70.	$C_{22}H_{30}O_4$	358.47	Ialibinone C	*do*
71.	$C_{22}H_{30}O_4$	358.47	Ialibinone D	*do*
72.	$C_{18}H_{24}O_4$	304.38	Ialibinone E	*do*
73.	$C_{14}H_{18}O_5$	266.29	1-(3-Acetyl-2,4,6-trihydroxy-5-methylphenyl)-2-methyl-1-butanone	*H. japonicum*[82]
74.	$C_{33}H_{42}O_8$	566.69	Sarothralen B	*do*
75.	$C_{35}H_{52}O_6$	568.79	33-Hydroperoxyfurohyperforin	*H.perforatum*[83]
76.	$C_{35}H_{52}O_5$	552.79	8-Hydroxyhyperforin 8,1-hemiacetal	*Do*
77.	$C_{35}H_{52}O_5$	552.79	Oxepahyperforin	*Do*
78.	$C_{33}H_{44}O_9$	584.70	Sarothralen C	=
79.	$C_{38}H_{50}O_5$	586.81	Sampsonione K	*H. sampsonii*[58]
80.	$C_{33}H_{42}O_5$	518.69	Sampsonione L	*do*
81.	$C_{38}H_{50}O_5$	586.81	Sampsonione M	*do*
82.	$C_{35}H_{52}O_5$	552.79	Furohyperforin	*H.perforatum*[84]
83.	$C_{30}H_{28}O_9$	532.54	Gemichalcone C	*H.geminiflorum*[85]
84.	$C38H_{50}O_5$	586.81	Sampsonione C	*H.geminiflorum*[86]
85.	$C_{38}H_{48}O_4$	568.79	Sampsonione D	*do*
86.	$C_{35}H_{42}O_5$	542.71	Sampsonione E	*do*
87.	$C_{38}H_{50}O_5$	586.81	Sampsonione F	*do*
88.	$C_{33}H_{42}O_5$	518.69	Sampsonione G	*do*
89.	$C_{35}H_{44}O_4$	528.73	Sampsonione H	*do*
90.	$C_{38}H_{48}O_5$	584.79	Sampsonione I	*H. sampsonii*[87]
91.	$C_{38}H_{48}O_5$	584.79	Sampsonione J	*do*
92.	$C_{38}H_{50}O_5$	586.81	Sampsonione A	*H. sampsonii* [28]
93.	$C_{33}H_{42}O_5$	518.69	Sampsonione B	*do*
94.	$C_{21}H_{18}O_7$	382.36	2-(3,4-Dihydroxyphenyl)-5-hydroxy-3-methoxy-8,8-dimethyl-4H,8H-benzo[1,2-b':3,4-b']dipyran-4-one	*H. japonicum*[88]
95.	$C_{18}H_{22}O_9$	382.36	8-Glucosyl-5,7-dihydroxy-2-isopropyl-4H-1-benzopyran-4-one	*do*
96.	$C_{19}H_{24}O_9$	396.39	8-Glucosyl-5,7-dihydroxy-2-(1-methylpropyl)chromone	*do*
97.	$C_{18}H_{26}O_5$	322.40	Japonica acid	*H. japonicum* [89]
98.	$C_{23}H_{26}O_4$	366.45	Paglucinol	*H.patulum*[90]
99.	C20H18O6	354.35	3',4',5,7-Tetrahydroxy-6-prenylflavone	H.perforatum[90a]
100	$C_{30}H_{28}O_8$	51566.	Gemichalcone A	*H.geminiflorum*[91]

101	$C_{29}H_{26}O_7$	486.54	Gemichalcone B	*do*
102	$C_{29}H_{26}O_7$	486.52	Isogemichalcone B	*do*
103	$C_{16}H_{16}O_5$	288.29	Japonicumone B	*H. japonicum*[92]
104	$C_{33}H_{44}O_8$	568.72	Hyperbrasilol A	*H.brasiliense*[93]
105	$C_{32}H_{40}O_8$	552.66	Hyperbrasilol B	*do*
106	$C_{32}H_{40}O_8$	552.66	Isohyperbrasilol B	*do*
107	$C_{32}H_{42}O_8$	554.67	Hyperbrasilol C	*do*
108	$C_{21}H_{24}O_8$	404.41	Albaspidin AA	*H.brasiliense*[46]
109	$C_{28}H_{34}O_8$	498.57	Isouliginosin B	*do*
110	$C_{12}H_{14}O_2$	190.24	Naphthalenone	*H.erectum*[94]
111	$C_{14}H_{18}O_4$	250.29	Hyperolactone A	*H. chinense*[95]
112	$C_{13}H_{16}O_4$	236.26	Hyperolactone B	*do*
113	$C_{16}H_{14}O_4$	270.28	Hyperolactone C	*do*
114	$C_{16}H_{16}O_4$	272.34	Hyperolactone D	*do*
115	$C_{16}H_{16}O_5$	288.29	Saropyrone	*H. japonicum*[96]
116	$C_{16}H_{16}O_4$	272.32	Hyperbrasilone	*H.brasiliense*[97]
117	$C_{21}H_{18}O_7$	382.36	Sarothranol	*H.japonicum*[98]
118	$C_{21}H_{28}O_6$	376.44	Hyperireflexolide A	*H. reflexum*[99]
119	$C_{21}H_{28}O_6$	376.44	Hyperireflexolide B	*do*
120	$C_{36}H_{54}O_4$	550.82	Adhyperforin	*H.perforatum*[100]
121	$C_{20}H_{30}O_5$	350.45	Hyperjovinol A	*H.jovis*[101]
122	$C_{21}H_{30}O_4$	346.46	Otogirin	*H.erectum*[49]
123	$C_{23}H_{34}O_5$	390.51	Otogirone	*do*
124	$C_{28}H_{32}O_8$	496.55	Drummondin D	*H.drummondii*[102]
125	$C_{28}H_{34}O_8$	498.57	Drummondin E	*do*
126	$C_{28}H_{34}O_8$	498.57	Drummondin F	*do*
127	$C_{28}H_{32}O_8$	496.55	Isodrummondin D	*do*
128	$C_{18}H_{14}O_5$	310.30	Sarolactone	*H.japonicum*[103]
129	$C_{26}H_{30}O_8$	470.51	Drummondin A	*H.drummondii*[104]
130	$C_{25}H_{28}O_8$	456.49	Drummondin B	*do*
131	$C_{24}H_{26}O_8$	442.46	Drummondin C	*do*
132	$C_{27}H_{40}O_5$	444.61	Chinensin I	*H.chinense*[105]
133	$C_{26}H_{38}O_5$	430.58	Chinensin II	*do*
134	$C_{16}H_{20}O_4$	276.33	5,7-Dihydroxy-8-isobutyryl-2,2,6-trimethylchromene	*H.revolutum*[106]
135	$C_{17}H_{22}O_4$	290.35	1-(5,7-Dihydroxy-2,2,6-trimethyl-2H-1-benzopyran-8-yl)2-methyl-1-butanone	*do*
136	$C_{18}H_{14}O_5$	310.30	Hypericanarin	*H.canariensis*[107]
137	$C_{17}H_{20}O_4$	288.34	Mysorenone A	*H. mysorense*[108]
138	$C_{15}H_{18}O_2$	230.30	Mysorenone B	*do*
139	$C_{15}H_{16}O_3$	244.29	Mysorenone C	*do*
140	$C_{16}H_{16}O_3$	256.30	Hyperenone B	*do*
141	$C_{17}H_{20}O_5$	304.32	Methyl phenacyl 1,1-dimethyl-2-propenylmalonate	*H. mysorense*[109]
142	$C_{17}H_{18}O_3$	270.32	Hyperenone A	*do*
143	$C_{30}H_{16}O_8$	504.45	Hypericin	*H. perforatum*[110]
144	$C_{30}H_{20}O_8$	508.48	Hypericodehydrodianthrone	*do*

5.3: Xanthones

Xanthone is a class of organic compounds having molecular formula $C_{13}H_8O_2$ of the basic skeleton. It can be prepared by the heating of phenyl salicylate[111]. In 1939, xanthone was introduced as an insecticide. Xanthone is one of the major classes of natural product commonly found in a few higher plant families, fungi and lichen and their high taxonomic value in such families as well as their pharmacological properties have provoked great interest [112,113]. The symmetrical nature of the xanthone nucleus, coupled with its mixed biogenetic origin in higher plants necessitates that the carbons numbered according to a biosynthetic convention, Carbons 1-4 are assigned to the acetate-derived ring A, and carbons 5-8 to the shikimate-derived ring B. The numbering system is based on xanthene-9-one (Fig. 5.1) as the basic skeleton and in cases where only ring B is oxygenated the lowest numbers are used. Xanthones are classified into five major groups: simple oxygenated, xanthone glycosides, xanthonolignoids prenylated xanthones and miscellaneous[113].

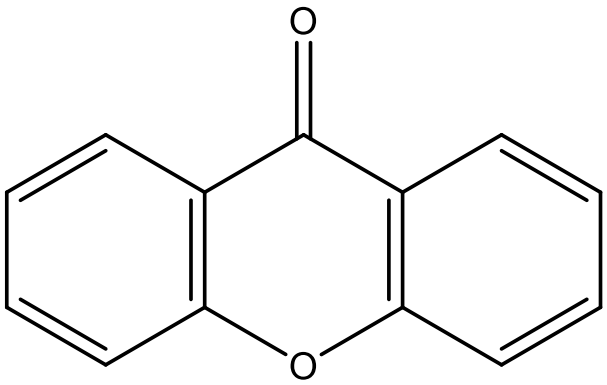


Fig. 5.1 Xanthone skeleton

5.3.1: Pharmacological importance of Xanthones

Xanthone is one of the major classes of natural product commonly found in a few higher plant families, fungi, lichen and are also found in the genus *Hypericun* (Guttiferae)[113]. The inhibitory effects (*in vivo)* of xanthones on PAF-Induced Hypotension from Guttiferae plants have been reported[114]. Ctotoxic xanthones from *H. hookrianum* has been also noted[115]. Xanthones are known to have various biological activities, such as cytotoxicity, antiviral, antimicrobial, antiulcer, antitumor, antidepressant, activities and inhibition of lipidperoxidase[113]. Recently, various bioactivities of xanthones that have been described include cytotoxic and antitumour activity, anti-inflammatory, antifungal activities and enhancement of choline acetyltransferase activity[116]. Xanthone currently finds uses as ovicide for coddling moth eggs and as a larvicide[117]. It is also used in the preparation of xanthydrol, used in the determination of urea levels in the blood. Xanthones and their glycosides have an anti-tubercular and antidepressant activities. Choleretic, antimicrobial, diuretic, antiviral and cardiotonic action of some xanthones has also been established[118-120]. Inhibition of monoamine oxidase by xanthones were also observed[113].

The ethanolic extract of *Psorospermum febrifugum* has shown significant antitumor and cytotoxic activities. Garciniaxanthone B was found to have choline acetyltransferase activity on a cultured neuronal cell of foetal rat brain hemisphere[121]. 1,7-dihydroxy-3-methoxyxanthone, isolated from *Swertia davida,* is used for treatment of hepatitis and enteritis[122]. Polyhydroxy-xanthones have to posses tuberculostatic activities, 1,3,7-trihydroxyxanthone showed the highest activity[113,123]. The excellent biological activities of xanthones led to chemical investigation of various plant species and numerous compounds of similar feature were isolated[113]. Literature indicates that more than 300 xanthones had been isolated out of which above 80 were reported from the genus *Hypericum* (Table 5.2).

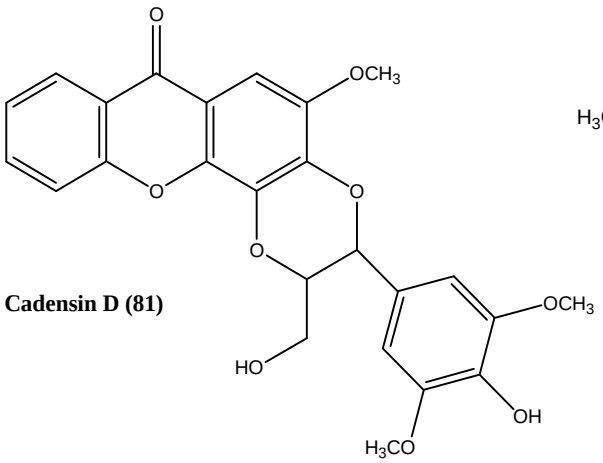
Table 5.2: List of Xanthones isolated from various species of *Hypericum*

S.No	M. Formula	M. mass	Name	Source
1.	$C_{15}H_{12}O_6$	288.0723	4,6-dihydroxy-2,3-dimethoxyxanthone	*H.chinense*[124]
2.	$C_{15}H_{12}O_6$	288.0734	2,6-dihydroxy-3,4-dimethoxyxanthone	*do*
3.	$C_{16}H_{14}O_6$	302.6723	6-hydroxy-2,3,4-trimethoxyxanthone	*do*
4.	$C_{15}H_{12}O_6$	288.0745	3,6-dihydroxy-1,2-dimethoxyxanthone	*do*
5.	$C_{15}H_{12}O_6$	288.0767	4,7-dihydroxy-2,3-dimethoxyxanthone	*do*
6.	$C_{15}H_{12}O_6$	288.0789	3,7-dihydroxy-2,4-dimethoxyxanthone	*do*
7.	$C_{24}H_{24}O1_4$	536.3009	1,6-dihydroxyisojacereubin-5-O-□-D-glucoside	*H.japonicum*[125]
8.	$C_{14}H_{10}O_5$	258.0907	3,6,7-tri-hydroxy-1-methoxy-xanthone	*do*
9.	$C_{18}H_{14}O_6$	326.3400	1,3,7-trihydroxy-2-(2-hydroxy-3-methyl-3-butenyl)-xanthone	*H.chinense*[126]
10	$C_{18}H_{16}O_6$	328.0956	1,7-dihydroxy-2,3-[2"-(1-hydroxy-1-methylethyl)-dihydrofurano]-xanthone	*do*
11	$C_{14}H_{10}O_6$	274.0477	1,3,7-trihydroxy-5-methoxyxanthone	*do*
12	$C_{15}H_{12}O_6$	288.0656	1,7-dihydroxy-5,6-dimethoxyxanthone	*do*
13	$C_{15}H_{12}O_6$	288.0611	4,5-dihydroxy-2,3-dimethoxyxanthone	*do*
14	$C_{15}H_{12}O_6$	288.0623	1,3-dihydroxy-2,4-dimethoxyxanthone	*do*
15	$C_{36}H_{30}O_{13}$	670.6244	Bijaponicaxanthone C	*H. japonicum*[127]
16	$C_{16}H_{14}O_6$	302.2856	1-Hydroxy-5,6,7-trimethoxyxanthone	*H.perforatum*[128]
17	$C_{15}H_{12}O_5$	272.2578	1-Hydroxy-6,7-dimethoxy-9H-xanthen-9-one	*do*
18	$C_{19}H_{18}O_6$	342.3490	Hyperxanthone	*H.sampsonii*[27]
19	$C_{18}H_{16}O_7$	344.3223	Hyperxanthone A	*H. scabrum*[35]
20	$C_{18}H_{16}O_8$	360.3245	Hyperxanthone B	*do*
21	$C_{18}H_{16}O_7$	344.3254	Hyperxanthone C	*do*
22	$C_{18}H_{16}O_6$	328.3264	Hyperxanthone D	*do*
23	$C_{18}H_{16}O_6$	328.3257	Hyperxanthone E	*do*
24	$C_{14}H_{10}O_8S$	338.2948	1,3-Dihydroxy-5-methoxyxanthone-4-	*H.sampsonii*[129]

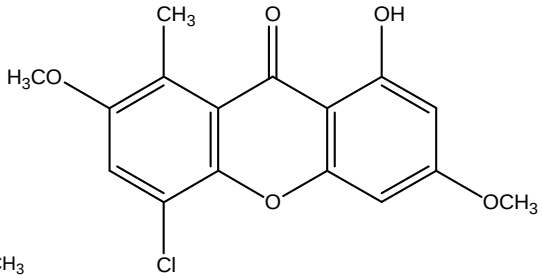
			87sulfonic acid	
25	$C_{19}H_{18}O_{13}S$	486.4055	1,365-Dihydroxy 5-O--D-Gluco34pyranoside	*do*
26	$C_{23}H_{24}O_5$	380.4477	5-O-Dem23ethyl-6-deoxypaxanthonin	*H. styphelioides*[64]
27	$C_{36}H_{28}O_{13}$	668.6067	Jacarelhyperol A	*H. japonicum*[130]
28	$C_{36}H_{28}O_{12}$	652.6198	Jacarelhyperol B	*do*
29	$C_{28}H_{32}O_6$	464.5534	5-(1,1-Dimethyl-2-propenyl)-3,6,8-trihydroxy-1,1-bis(3-methyl-2-butenyl)-1H-xanthene-2,9-dione	*H. erectum*[26]
30	$C_{16}H_{14}O_7$	318.2822	2,3-Dihydroxy-1,6,7-trimethoxyxanthone	*H.geminiflorum*[131]
31	$C_{16}H_{14}O_7$	318.2833	3,6-Dihydroxy-1,5,7-trimethoxyxanthone	*do*
32	$C_{15}H_{12}O_6$	288.2545	2,7-Dihydroxy-3,4-dimethoxyxanthone	*H. subalatum*[132]
33	$C_{26}H_24O_{11}$	512.4678	Gemixanthone A	*H.geminiflorum*[133]
34	$C_{15}H_{12}O_6$	288.2554	6,7-Dihydroxy-1,3-dimethoxyxanthone	*do*
35	$C_{13}H_8O_5$	244.2032	1,2,4-Trihydroxyxanthone	*do*
36	$C_{19}H_{18}O_{11}$	422.3444	Patuloside A	*H.patulum*[32]
37	$C_{25}H_{28}O_{15}$	568.4856	Patuloside B	*do*
38	$C_{15}H_{12}O_6$	288.2578	3,6-Dihydroxy-1,7-dimethoxyxanthone	*H.ascyron*[34]
39	$C_{15}H_{11}ClO_5$	306.7012	Vinetorin	*do*
40	$C_{36}H_{28}O_{13}$	668.6034	Bijaponicaxanthone	*H. japonicum*[134]
41	$C_{18}H_{16}O_6$	328.3223	Deprenylrheediaxanthone B	*do*
42	$C_{18}H_{16}O_6$	328.3254	1,3,5,6-Tetrahydroxy-4-prenylxanthone	*do*
43	$C_{18}H_{14}O_5$	310.3065	6-Deoxyisojacareubin	*do*
44	$C_{24}H_{20}O_8$	436.4178	Kielcorin	*do*
45	$C_{23}H_{24}O_6$	396.4398	Patulone	*H.patulum*[135]
46	$C_{19}H_{18}O_6$	342.3412	5-O-Methyldeprenylrheediaxanthone B	*H.roeperanum*[136]
47	$C_{14}H_{10}O_4$	242.2334	5-Hydroxy-2-methoxyxanthone	*do*
48	$C_{23}H_{24}O_6$	396.4365	Calycinoxanthone D	*do*
49	$C_{23}H_{24}O_6$	396.4309	5-O-Demethylpaxanthonin	*do*
50	$C_{14}H_{10}O_5$	258.2353	1,2,5-Trihydroxyxanthone	*do*
51	$C_{28}H_{32}O_6$	464.5545	Roeperanone	*do*

52	$C_{19}H_{16}O_6$	340.3367	5-O-Methylisojacareubin	*do*
53	$C_{23}H_{20}O_6$	392.408	Padiaxanthone	*H.patulum*[137]
54	$C_{23}H_{22}O_6$	394.4277	Paxanthone B	*H.patulum*[138]
55	$C_{18}H_{16}O_6$	328.3200	2,3,6,8-Tetrahydroxy-1-prenylxanthone	*do*
56	$C_{24}H_{26}O_6$	410.4623	Paxanthonin	*H.patulum*[139]
57	$C_{19}H_{16}O_6$	340.3312	Paxanthone	*H.paturum*[140]
58	$C_{18}H_{14}O_6$	326.3011	Isojacareubin	*H. japonicum* [141]
59	$C_{19}H_{18}O_6$	342.3435	Morusignin D	*H.patulum*[142]
60	$C_{18}H_{14}O_4$	294.3054	Hyperireflexin	*H.reflexum*[112]
61	$C_{25}H_{22}O_9$	466.4465	6-Methoxykielcorin	*do*
62	$C_{15}H_{12}O_6$	288.2577	2,4-Dihydroxy-3,6-dimethoxyxanthone	*do*
63	$C_{16}H_{14}O_6$	302.2809	4-Hydroxy-2,3,6-trimethoxyxanthone	*do*
64	$C_{14}H_{10}O_5$	258.2307	3,6-Dihydroxy-2-methyoxyxanthone	*do*
65	$C_{18}H_{14}O_4$	294.3006	Hypericanarin B	*H.canariensis*[143]
66	$C_{14}H_8O_4$	240.2155	2,3-Methylenedioxyxanthone.	*H.mysorense*[144]
67	$C_{24}H_{20}O_9$	452.4145	Subalatin	*H.subalatum*[145]
68	$C_{25}H_{22}O_9$	466.4423	Cadensin D	*H.canariensis*[146]
69	$C_{13}H_8O_4$	228.2044	2,5-Dihydroxyxanthone	*H.canariensis*[147]
70	$C_{18}H_{14}O_5$	310.3076	Hyperxanthone	*H.sampsonii*[148]
71	$C_{15}H_{12}O_5$	272.2556	2-Hydroxy-3,4-dimethoxyxanthone	*do*
72	$C_{14}H_{10}O_4$	242.2311	1-Hydroxy-7-methoxyxanthone	*H.mysorense*[149]
73	$C_{15}H_{12}O_4$	256.2534	1,2-Dimethoxyxanthone	*do*
74	$C_{16}H_{14}O_6$	302.2800	7-Hydroxy-2,3,4-trimethoxyxanthone	*H.ericoides*[150]
75	$C_{13}H8O_5$	244.2009	2,3,5-Trihydroxyxanthone	*H.androsaemum*[151]
76	$C_{28}H_{30}O_6$	462.5405	Maculatoxanthone	*H.maculatum*[152]

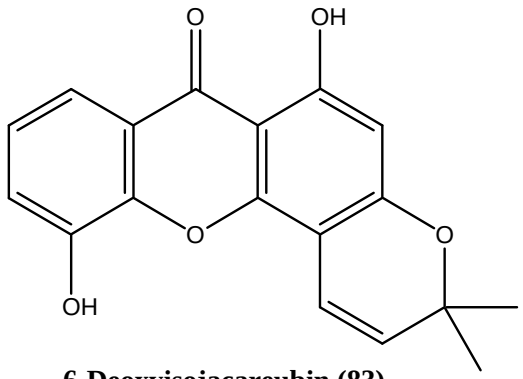
5.3.1: Structures of some common Xanthones isolated from *Hypericum*



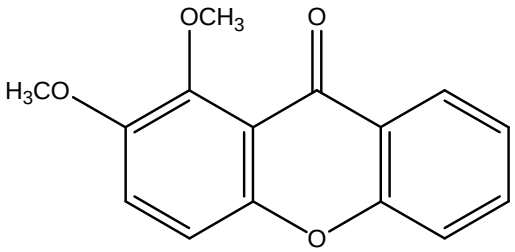
Cadensin D (81)



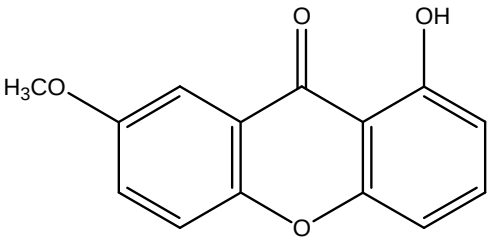
Vinetorin (82)



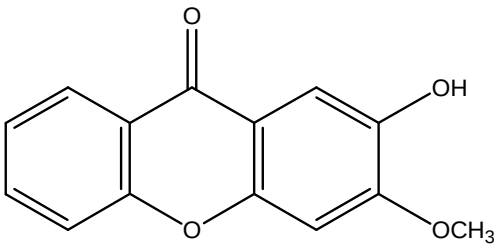
6-Deoxyisojacareubin (83)



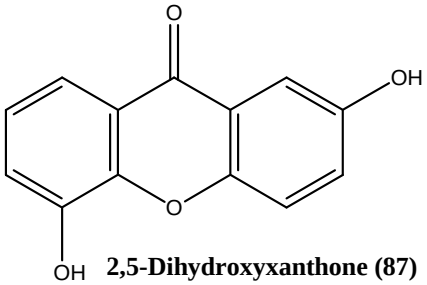
1,2-Dimethoxyxanthone (84)



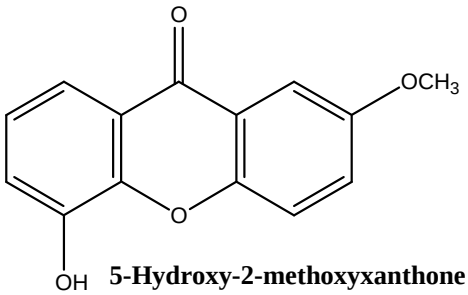
1-Hydroxy-7-methoxyxanthone (85)



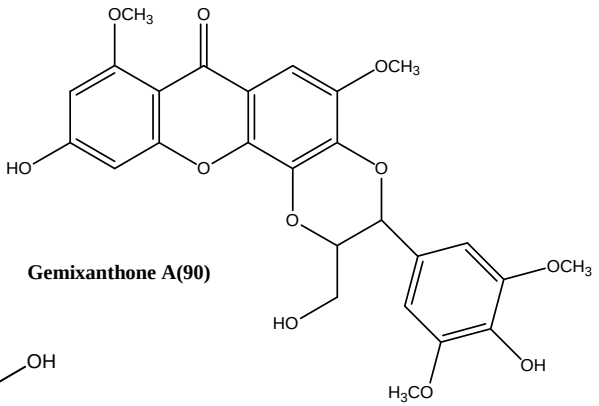
2-Hydroxy-3-methoxyxanthone (86)



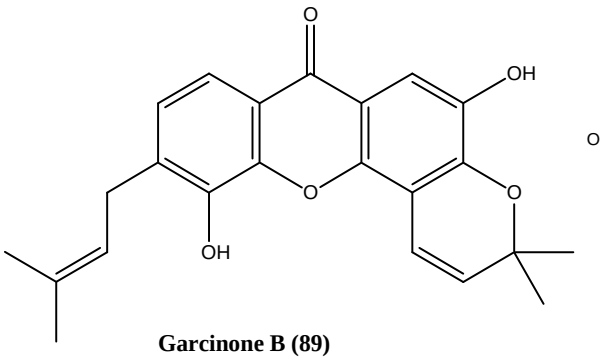
2,5-Dihydroxyxanthone (87)



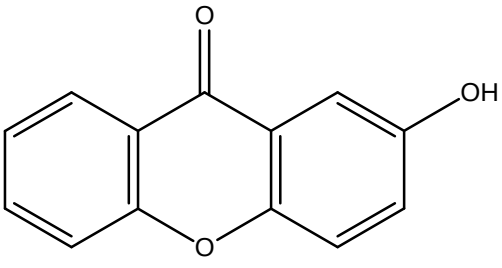
5-Hydroxy-2-methoxyxanthone (88)



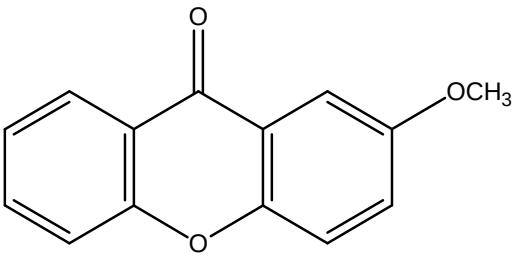
Gemixanthone A(90)



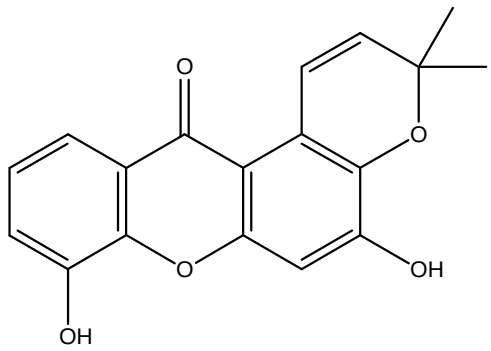
Garcinone B (89)



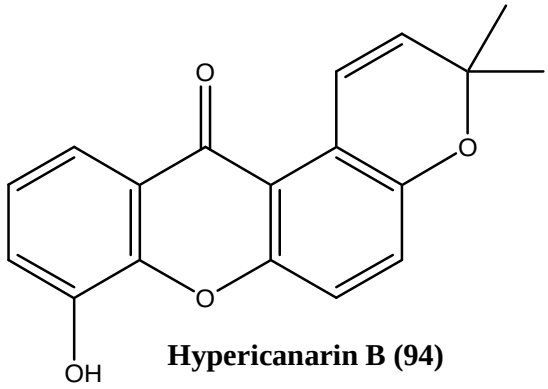
2-Hydroxyxanthone(91)



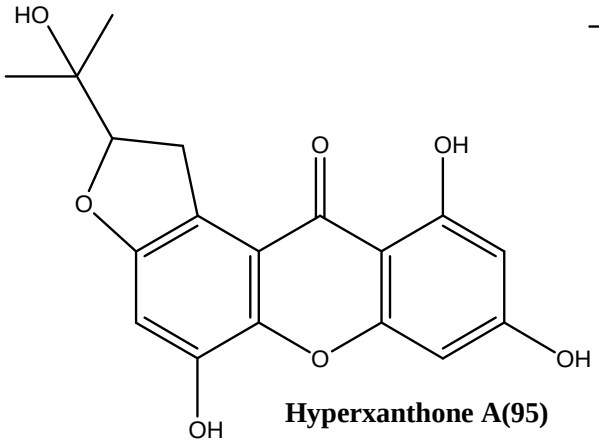
2-Methoxyxanthone (92)



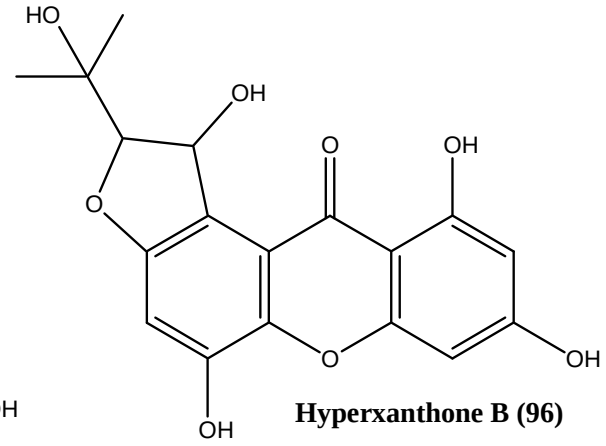
Hypericanarin (93)



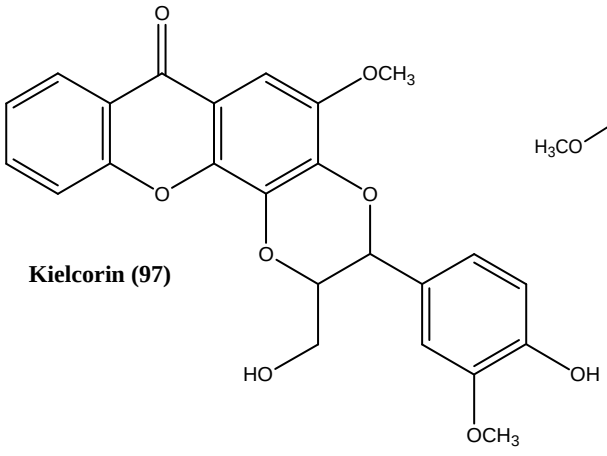
Hypericanarin B (94)



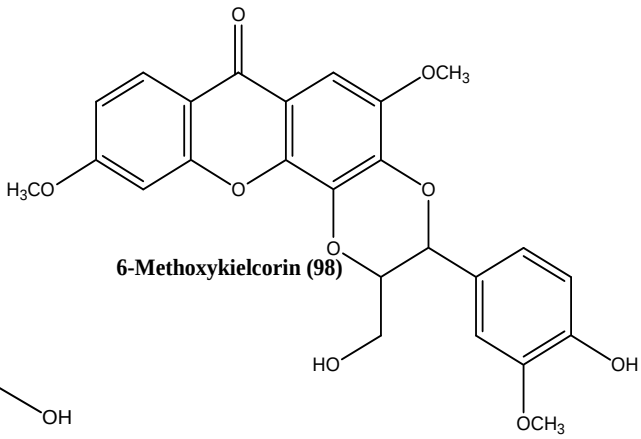
Hyperxanthone A(95)



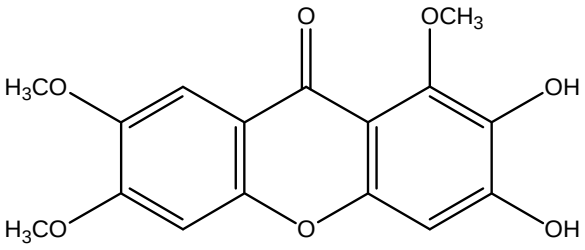
Hyperxanthone B (96)



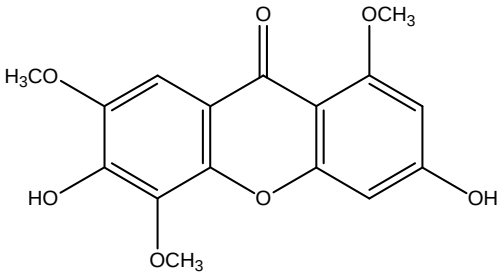
Kielcorin (97)



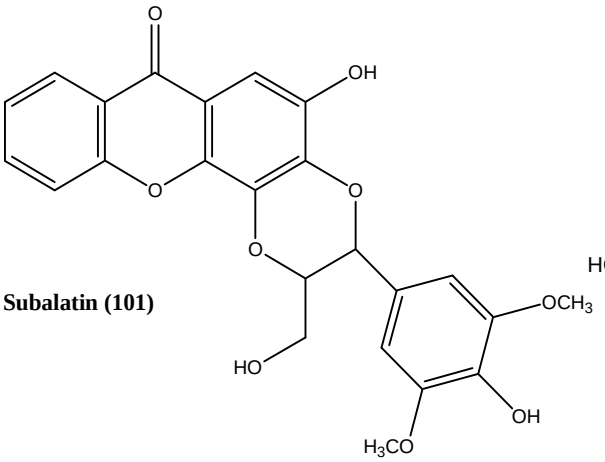
6-Methoxykielcorin (98)



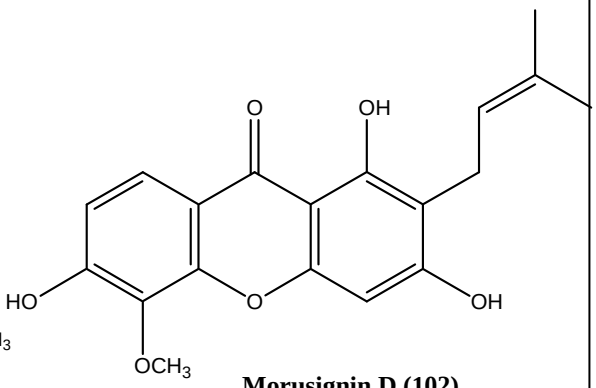
2,3-Dihydroxy-1,6,7-trimethoxyxanthone (99)



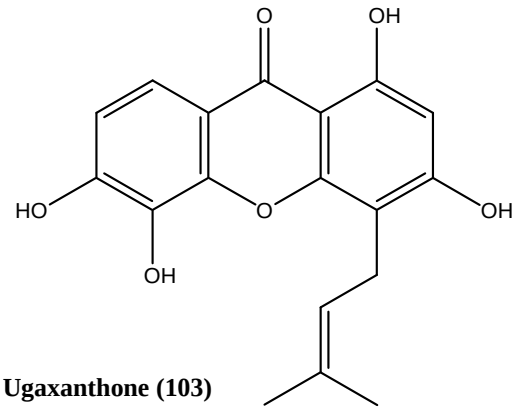
3,6-Dihydroxy-1,5,7-trimethoxyxanthone (100)



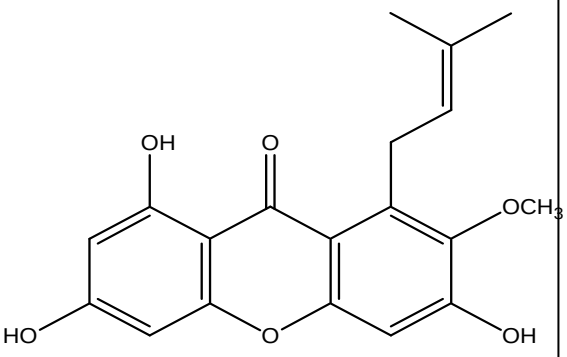
Subalatin (101)



Morusignin D (102)



Ugaxanthone (103)



Dulxanthone D (104)

Chapter: 6

RESULTS AND DISCUSSION (Part B)

6.1: Compounds isolated from *Hypericum oblongifolium*

Six new and fourteen known Xanthones along with ten other compounds were isolated from *Hypericum oblongifolium* of Pakistani origin. Various experimental techniques and extensive spectroscopic studies were used for the structural elucidation of these compounds. Some of the isolated xanthones showed respiratory burst inhibitory and enzyme inhibitory activities. The results of these experimental studies are discussed in this chapter. The extraction and isolation procedures are discussed in detail in the experimental section.

Hypericum oblongifolium was authenticated by Dr. Habib Ahmad, Dean Faculty of Science, Hazara University, was collected at flowering period in June, 2006 from Buner District, NWFP. Voucher specimens (HUH-002) retained for verification in Department of Botany, Hazara University, NWFP, Pakistan. The air-dried, powdered twigs materials (12 Kg) and roots (4 kg) were exhaustively extracted with petroleum ether (hexane), ethyl acetate and methanol (3x25 L, each for 3 days) on cold peculation method at room temperature. The extracts were concentrated in a rotavapor and dried under reduced pressure to yield the residue. The ethyl acetate fractions of both twigs (260 g) and roots (70 g) were loaded on column chromatography over silica gel and eluted with solvent inncreasing order of polarity (*n*-hexane– ethyl acetate and ethyl acetate –methanol), resulted 200 and 180 fractions respectively. These fractions were combined according to the similarity on TLC profiles, afforded 30 and 20 major fractions respectively. These fractions were further subjected to silica gel and purified 35 compounds using different solvent system. Compounds **105** to **110** were identified as new xanthones whereas compounds **111** to **124 E** were proven as reported xanthones along with fifteen other reported compounds (**125-134**)

6.1.1: New Xanthones from the aerial parts (Twigs) of *Hypericum oblongifolium*

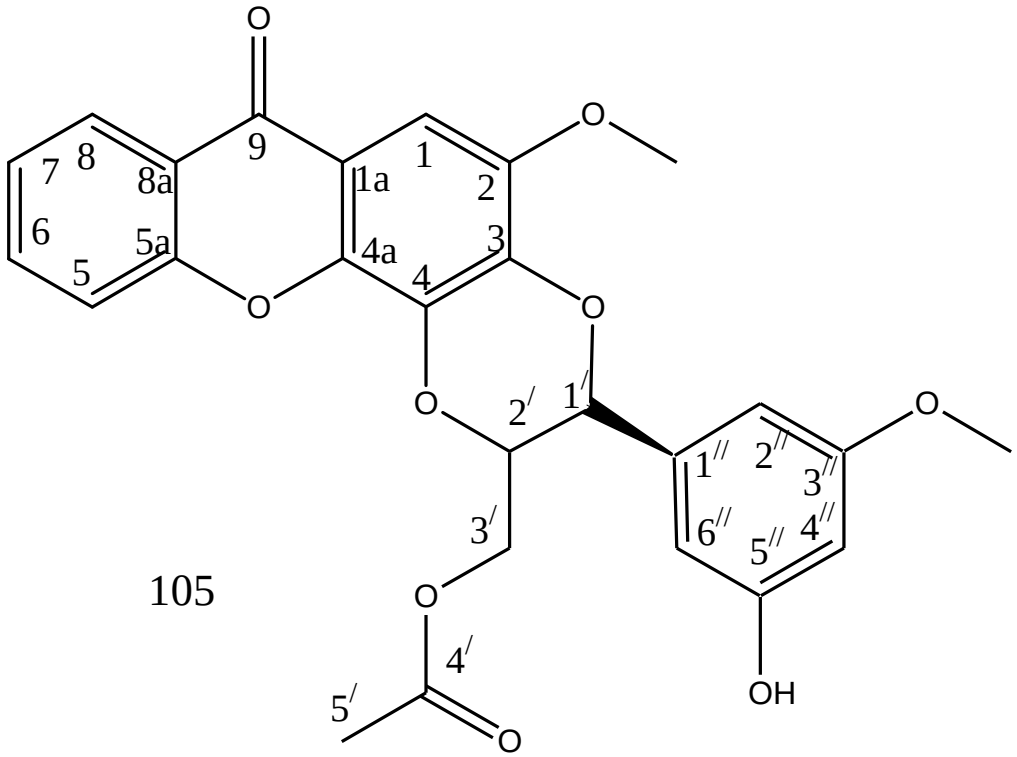
6.1.1.1: Hypericorin A (105)

The ethyl acetate fraction (260g) was subjected to column chromatography over silica gel eluting with *n*-hexane– ethyl acetate and ethyl acetate –MeOH in increasing order of polarity to afford 30 major fraction Fractions 22 and 23 were combined and subjected to flash silica gel CC (methanol/Chloroform 3:97, 4:96) and led to the isolation of **105** (15 mg) as white amorphous powder (See section 7.2.2.1). The molecular formula of compound **105** was determined as $C_{26}H_{24}O_9$ by HR-EI-MS giving molecular ion peak $[M]^+$ at *m/z* 478.23 (calcd. 478.1264). The UV spectrum exhibited characteristic absorption for xanthone at 248, 308 and 346 nm[153]. The IR spectrum displayed bands at 3462, 1648 and 1580 cm^{-1} indicating the presence of OH, conjugated carbonyl and aromatic ring respectively[153].

The ^{I}H NMR and ^{13}CNMR spectra of **105** (Table 6.1) showed characteristic peak of xanthone functionality[85,153a]. The ^{I}H NMR gives signal of five aromatic protons at δ 7.33, s (H-1), 7.52, d (*J* = 8.4 Hz, H-5), 7.63, td (*J* = 8.4, 1.6 Hz, H-6), 7.32, t (*J* = 8.3 Hz, H-7) and 8.2, dd (*J* = 8.3, 1.4 Hz, H-8). ^{I}H NMR of **105** also showed three aromatic protons singlets (δ 6.84, brs, 3H) in addition to above five signals which indicated another phenyl moiety attached. The ^{I}H NMR signals at δ 3.82, s and 3.83, s were assigned to two MeO attached to aromatic ring (MeO-Ar). Signal at δ 2.03, s was attributed to methyl attached to carbonyl functionality of ester type. The spectrum of **105** also showed *trans* diaxial dioxane proton signals at δ 4.33, dd (*J* = 7.8, 4.4, 3.5 Hz) and 4.92, d (*J* = 7.8 Hz). The deshielded doublet (δ 4.92) typical of a benzylic methylene substituted by oxygen and its typical *trans*–coupling (*J* =7.8 Hz) implied the existence of a *trans*–substituted 1,4-dioxane ring between the xanthone moiety and the phenyl ring . The EIMS shows significant peak at *m/z* 222 could be rationalized in term of retro-Diels-Alder reaction in a dioxane ring, while the ions at *m/z* 222, 180, 179 and 162 indicated that one acetyl group in phenyl propane unit while one hydroxyl and one methoxyl groups present in phenyl ring [107,154].

The ^{1}H NMR spectrum also showed two aliphatic proton signal of CH_2O-group at 4.4 (dd, *J* = 12.0, 2.8 Hz) and 4.05 (dd, *J* = 12.0, 4.3 Hz). On the basis of ^{I3}C NMR (BB and DEPT) spectral data (Table 6.1), compound **105** contained 26 carbons including three methyl, one CH_2, ten CH and twelve quaternary carbons. The downfield signal at δ 176.7 was due to C-9, the conjugated carbonyl of xanthone

skeleton, while the carbonylic signal at δ 170.6 was assigned to the ester moiety. The peaks at δ 114.9 and 120.8 were assigned to the quaternary aromatic carbons C-1a and C-8a while 147.8, 142.0, 142.4, 139.8 and 154.7 were attributed to the aromatic quaternary carbon attached to oxygen functionalities. The signals at δ 78.9 and 77.5 were assigned to oxygenated methine carbons at C-6$^{/}$ and C-5$^{/}$ respectively. Similarly the signal at δ 20.7 was assigned to acetyl methyl and δ 62.7 was attributable to methylene next to oxygen in the ester moiety. The chemical shift assignments were confirmed by HMQC and HMBC data (Fig.6.1).The proton appeared at δ 7.31, s (H-1) showed correlation with δ 114.9 (C-1a), 139.1 (C-4a), 141.9 (C-3), 147.0 (C-2) and 175.7 (C-9). On the basis of HMBC interactions it is suggested that one methoxyl group is attached at C-2 while 1,4- dioxane ring is fused with xanthone skeleton at C-3 and C-4. Similarly the proton appeared at δ 4.92 (H-5$^{/}$) having correlation in HMBC spectrum with δ 110.9 (C-6$^{//}$), 120.2 (C-2$^{//}$) and 126.6 (C-1$^{//}$), which indicate that meta substituted phenyl moiety is linked to 1, 4-dioxane at C-5$^{/}$ and C-1$^{//}$. Methyl at δ 2.03 shows cross peak with 170.6 (CH_2*CO*CH_3) confirmed the presence of acetyl group. Furthermore ,the HMBC spectrum confirmed that 1,4-dioxane ring was fused between xanthone framework and phenyl moiety and framed a xanthonolignoid skeleton. ^{I}H-^{I}H COSY spectrum also confirmed the same skeleton, by giving cross peaks between H-5/H-6, H-6/H-7, H-7/H-8 and H-2$^{//}$/H-4$^{//}$/H-6$^{//}$. The NOESY spectrum indicated correlation between H-1 and the methoxyl signal δ 3.83, the methoxyl signal at δ 3.83 and dioxane proton at δ 4.92 (H-5$^{/}$), the aliphatic methylene proton at δ 4.05 and the dioxane proton signal at δ 4.92 (H-5$^{/}$), similarly proton δ 4.30 (H-6$^{/}$) did not show NOESY interactions with δ 4.92 (H-5$^{/}$) conformed the trans-dioxane protons. The optical rotation of compound **1** was zero, $[\alpha]^{D} = 0^{o}$, with *trans* relative configuration, having both the 5$^{/}$R, 6$^{/}$R and 5$^{/}$S, 6$^{/}$S enantiomers. Based on the aforementioned spectroscopic methods the compounds was proposed to be Hypericorin A



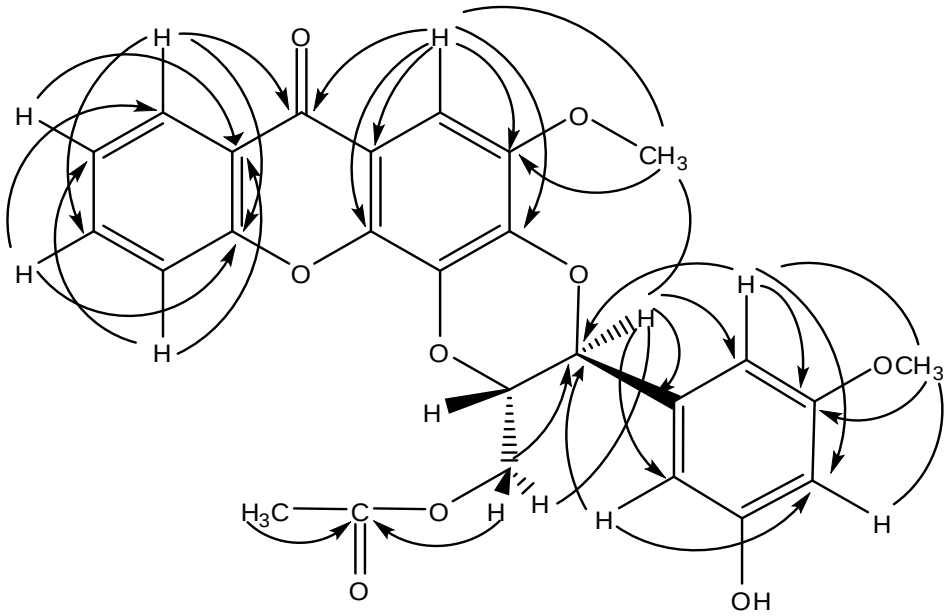


Fig.6.1: Important HMBC and NOE Interactions of **105**

Table-6.1: ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound (**105**) in $CD_3OD+CDCl_3$ (1:1)

C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	97.3	CH	7.3, s
1a	114.9	C	-
2	147.8	C	-
3	142.0	C	-
4	142.0	C	-
4a	139.8	C	-
5	118.8	CH	7.52, d (*J* = 8.3)
5a	154.7	C	-
6	134.5	CH	7.63, td (*J* = 8.4, 1.6)
7	123.3	CH	7.32, t (*J* = 8.3)
8	126.5	CH	8.22, dd (*J* = 8.3, 1.4)
8a	120.8	C	-
9	176.7	C	-
1′	78.9	CH	4.9, d (*J* = 7.8)
2′	77.5	CH	4.05, dd (*J* = 7.8, 4.4)
3′	62.7	CH_2	4.4, m
			4.3, m
4′	170.6	C	-
5′	20.7	CH_3	2.03, s
1″	126.6	C	-
2″	120.2	CH	6.84, s
3″	147.0	C	-
4″	115.8	CH	6.83, s
5″	147.3	C	-
6″	110.9	CH	6.85, s
MeO-2	56.1	CH_3	3.81, s
MeO-3″	55.8	CH_3	3.82, s

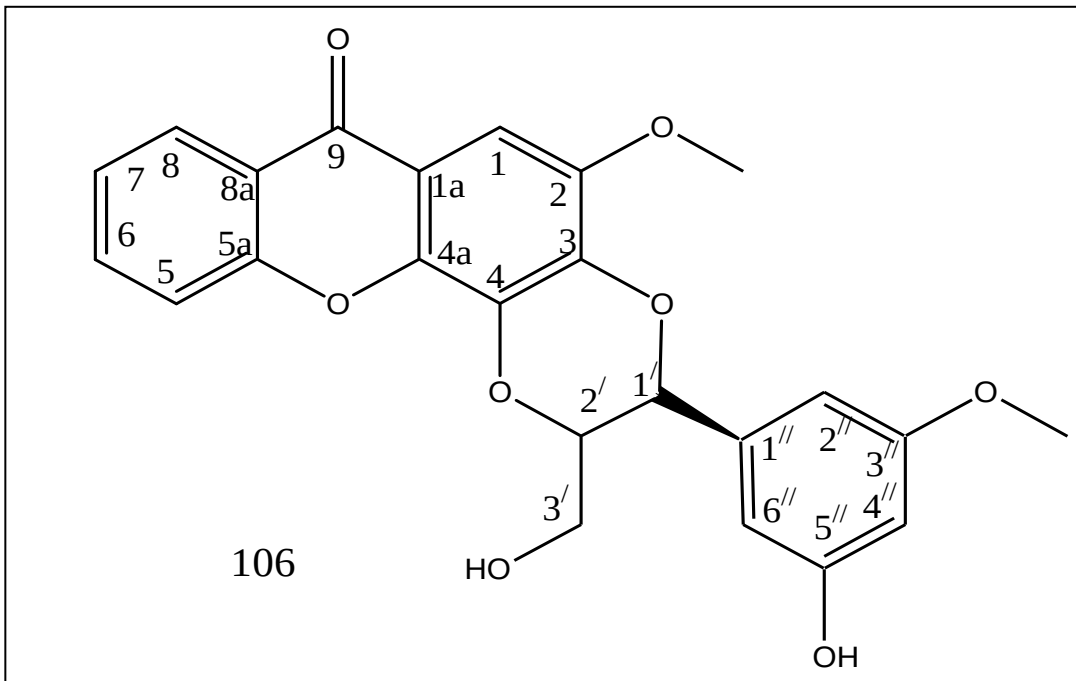
a: Broad band; b: DEPT; c: ^{1}H NMR; d: HMQC interaction

6.1.1.2: Hypericorin B (106)

As stated earlier, fractions 22 and 23 were combined and subjected to flash silica gel CC (methanol/Chloroform 3:97, 4:96) has led to the isolation of **106** (18 mg) as white powder (See section 7.2.2.1). The molecular formula of compound **106** was determined as $C_{24}H_{20}O_8$ by HR-EI-MS giving molecular ion peak $[M]^+$ at *m/z* 436 (calcd.436) The UV spectrum showed the presence of xanthone, giving absorption peaks at 254, 306 and 382 nm[153]. The IR spectrum displayed bands at 3592, 1642 and 1600 cm^{-1} indicating the presence of OH, conjugated carbonyl and aromatic ring respectively[153]. The ^{I}H and ^{I3}C NMR spectra of **106** (Table 6.2) were similar to those of **105**, except the acetyl signal was missing in the ^{I}H NMR spectra and the main difference between them was the lack of carbonyl and methyl peak of ester functionality in ^{I3}C NMR compound **106.** The EIMS of **106** also showed the peak at *m/z* 180, could be rationalized in terms of a retro-Diels-Alder reaction in the dioxane ring and ions at *m/z* 180,162, 137 and 124 respectively indicated that phenyl propane unit having only one hydroxyl and one methoxyl groups (absence of fragment peak at *m/z* 222 due to acetyl group).

Like **1** the ^{I}H NMR (Tables 6.2) of **106** also showed five signals for aromatic protons at δ 7.33, s (H-1), 7.52, d (*J* = 8.4 Hz, H-5), 7.69, td (*J* = 8.4, 1.6 Hz, H-6), 7.32, t (*J* = 8.3 Hz, H-7) and 8.2, dd (*J* = 8.3, 1.4 Hz, H-8) as well as the additional peaks of three aromatic proton singlets at δ 6.87 (1H) and 6.90 (2H). The ^{I}H NMR signals at δ 3.84, s and 3.89, s were attributed to two MeO-Ar. Signals at δ 5.06, d *(J* = 8.1) and 4.11, td (*J* = 7.7, 3.1 Hz) were assigned to oxygenated methine proton of 1,4-dioxane ring. On the basis of the interpretation of its ^{I3}C NMR (BB and DEPT) spectral data (Table 6.2), compound **106** contained 24 carbons, including two methyl, one methylene, ten methine and eleven quaternary carbons. The only one downfield signal at δ 176.4 (C-9) was due to the presence of conjugated carbonyl of xanthone skeleton. The signals at δ 78.5 and 76.9 were assigned to oxygenated methine carbons at C-5$^{/}$ and C-6$^{/}$ respectively like **105**. The chemical shift assignments were confirmed by HMQC and HMBC (Fig. 6.2). The proton appeared at δ 7.33, s (H-1) showed correlation with 139.8 (C-4a), 141.0 (C-3), 147.8 (C-2) and 176.4 (C-9). The HMBC spectrum also showed same set of correlation as observed in case of compound **105** and indicated that methoxyl group attached at C-2 while 1,4- dioxane ring was fused with xanthone skeleton at C-3 and C-4. Similarly the proton appeared at δ 5.06, d (*J* =

8.1 Hz) showed correlation in HMBC spectrum with 120.3 (C-$2^{//}$), 109.9(C-$6^{//}$) and 126.7(C-$1^{//}$), which suggest that another phenyl moiety was linked to 1, 4-dioxane at C-$5^{/}$ and C-$1^{//}$. Furthermore the HMBC, COSY and NOESY supported the structure of compound **106** named as Hypericorin B



106

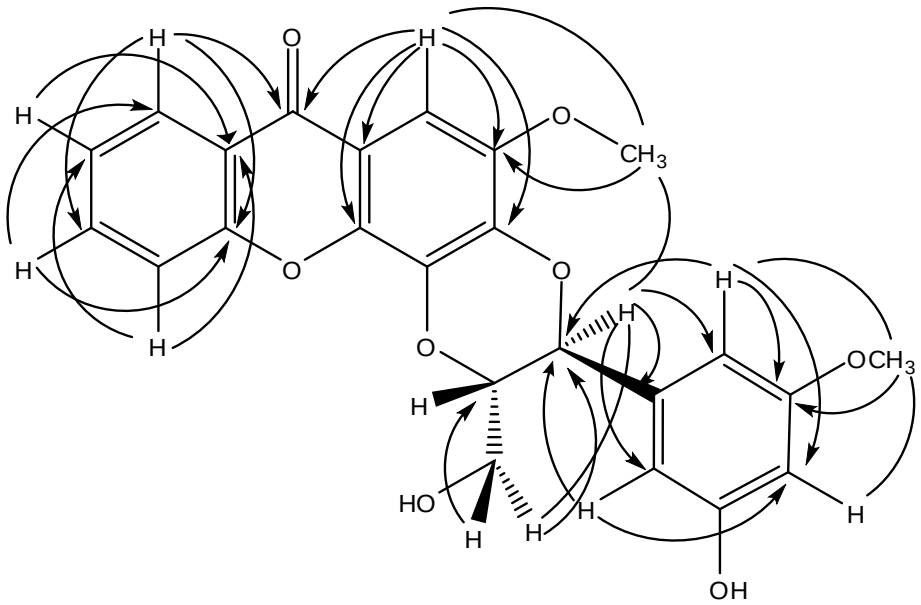


Fig.6.2: Important HMBC ⌒ and NOE ⌒ Interactions of **106**

Table-6.2: ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) Spectral Data of Compound (**106**) in $CD_3OD+CDCl_3$ (1:1)

C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	97.4	CH	7.33, s
1a	114.9	C	-
2	147.8	C	-
3	141.0	C	-
4	142.0	C	-
4a	139.8	C	-
5	117.8	CH	7.5, d (J = 8.4)
5a	154.9	C	-
6	134.5	CH	7.69, td (J = 8.4, 1.4)
7	123.3	CH	7.32, t (J = 7.3)
8	126.5	CH	8.21, dd (J = 7.3, 1.4)
8a	121.8	C	-
9	175.7	C	-
$1^{/}$	78.4	CH	5.1, d (J = 8.1)
$2^{/}$	77.3	CH	4.1,m
$3^{/}$	60.7	CH_2	3.5, m
			3.9, m
$1^{//}$	126.4	C	-
$2^{//}$	110.9	CH	6.9, s
$3^{//}$	147.0	C	-
$4^{//}$	146.3	C	-
$5^{//}$	114.8	CH	6.9, d (J = 1.5)
$6^{//}$	120.9	CH	6.8, d (J = 1.5)
MeO-2	56.1	CH_3	3.89, s
MeO-$3^{//}$	55.9	CH_3	3.84, s

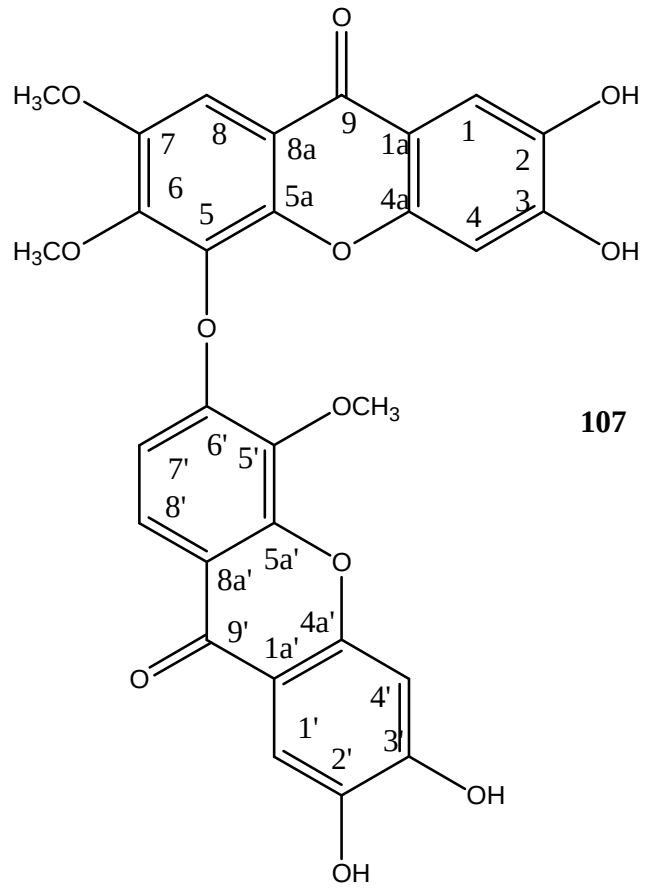
a: Broad band; b: DEPT; c: ^{1}H NMR; d: HMQC interaction

6.1.1.3: Bihyponicaxanthone A (107)

A yellowish amorphous solid (**107**, 13 mg) has been isolated from the combined fraction of 18 and 19 (See section 7.2.2.1). The UV spectrum showed the presence of xanthone giving absorption peaks at 250, 308 and 389 nm[153]. The IR spectrum displayed bands at 3582, 1648 and 1605 cm^{-1} for the presence of OH, conjugated carbonyl and aromatic ring respectively[153].

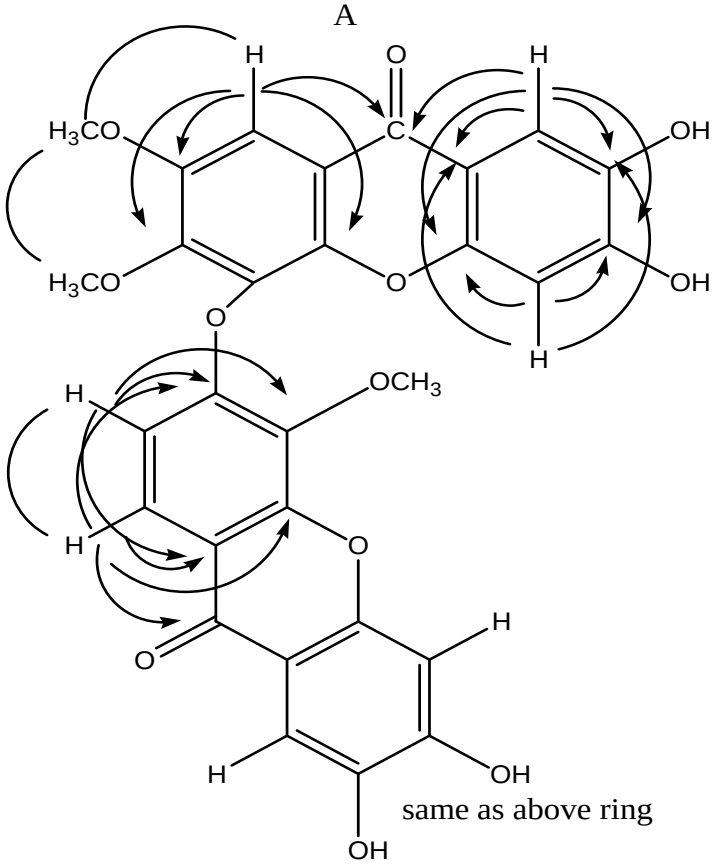
Compound **107** was looking dimer and the molecular formula was determined as $C_{29}H_{20}O_{12}$ by the fusion of two fragments A ($C_{15}H_{12}O_7$) and B ($C_{14}H_{11}O_5$) appeared in EI-MS as molecular ion peaks *m/z* at 304 and 259. The ^{1}H NMR (Table-6.3) of **107** have signals at δ 6.1 (2H, s, H-4, 4′), 6.32 (1H, s, H-1′) and 6.34 (1H, s, H-1) were also indicating the dimmer skeleton for **107** which was further supported by ^{13}C NMR signals appeared twice at each position and confirmed by HMQC and HMBC spectra showing correlation from proton to carbon. The protons appeared at δ 6.1 (2H, s H-4, 4′) showed correlation with carbons resonating at δ 103.1 (C-1a, 1a′), 164.5 (C-2, 2 ′), 166.2 (C-3, 3′). The ^{1}H NMR singlets at δ 3.92, 3.98 and 4.0 were attributed to MeO-Ar located at C-5′, C-7 and C-6 respectively. The position of methoxyl groups at C-7 and C-6 were confirmed by HMBC and H′-H′ COSEY correlations whereas the attachment of MeO-5′ was confirmed by HMBC interaction of δ 6.88 (H-7′) with δ 1.35 (C-5′).

On the basis of the interpretation of its ^{13}C and DEPT NMR spectral data (Table 6.3), compound **107** contained 29 carbons, including three MeO, seven CH and nineteen quaternary carbons. The two signals at δ 181 were due to C-9, 9′. The double peak each at δ 103 and 115.4 and 117.3 were assigned to the quaternary aromatic carbons C-1a, 1a′, C-8a and C-8a′ respectively. Similarly resonance at δ 142.4, 145.0, 149.8, and 152.4, two peaks each at δ 159, 164 and 166 were attributed to the aromatic quaternary carbon having oxygen functionalities**.** The EIMS shows significant peak at *m/z* 304 and 259 could be rationalized in term of retro-Diels-Alder reaction for two fragments (A & B), while the ions at *m/z* 289, 274 and 243 indicate the presence of methyl groups. The chemical shift assignments were confirmed by advanced two dimensional NMR techniques such as HMBC, HMQC and COSEY (Fig.6.3). The protons appeared at δ 6.3 (H-1, 1′) showed correlation with carbons resonating at δ 103.1 (C-1a, 1a′), 159.5 (C-4a, 4a′), 166.2 (C-3, 3′) and 181 (C-9, 9′).



107

A



same as above ring

Fig.6.3: Important HMBC ⌒ and NOE ⌒ Interactions of **107**

Similarly the proton at δ 7.25 (H-8) have shown relation with δ 142.0 (C-7), 145.0 (C-5), 148.8 (C-5a) and 181.3 (C-9) whereas H-8/ showed correlation with δ 17.3 (H-8a/), 135.9 (H-5/), 152.7 (H-5a/), 157.9 (H-6/), 181.1 (H-9/) in HMBC spectrum ^{I}H- ^{I}H COSEY spectrum also have a set of relation showing cross peaks between H-7/H-8/ and NOSEY cross peak between MeO-6/MeO-7, H-8/MeO-7, H-7/H-8/ and MeO-6/MeO-7 confirming their respective positions. Based on the aforementioned spectroscopic methods the compound was proposed to be Bihyponicaxanthone A

Table-6.3: ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) Spectral Data of Compound **(107)** in CD_3OD

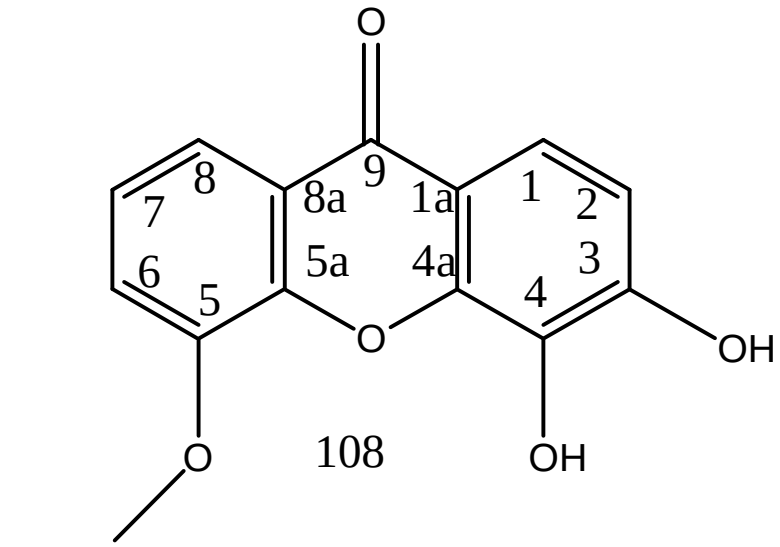
C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	95.1	CH	6.34, s
1/	94.9	CH	6.23, s
1a	103.5	C	-
1a/	103.1	C	-
2	164.6	C	-
2/	164.4	C	-
3	166.9	C	-
3/	166.9	C	
4	99.2	CH	6.1, s
4/	99.0	CH	6.1, s
4a	159.2	C	-
4a/	159.1	C	-
5	145.8	C	-
5/	135.9	C	-
5a	148.9	C	-
5a/	152.0	C	
6	148.7	-	-
6/	157.9	C	
7	I42.4	C	
7/	114.3	CH	6.88, d (*J* = 8.8)
8	105.2	CH	7.25, s
8/	122.6	CH	7.78, d (*J* = 8.8)
8a	115.8	C	-
8a/	117.3	C	
9	181.3	C	-
9/	181.1	C	
MeO-5/	61.6	CH_3	3.92, s
MeO-6	61.6	CH_3	4.01, s
MeO-7	62.2	CH_3	3.98, s

a: Broad band; b: DEPT; c: ^{1}H NMR; d: HMQC interaction

6.1.1.4: 3, 4-Dihydroxy-5-methoxyxanthone (108)

Whitish yellow compound (**108**, 13 mg) has been isolated from the same fraction mentioned in case of **107** (See section 7.2.2.1). The UV spectrum showed the presence of xanthone giving absorption peaks at 240, 258 and 376 nm[155]. The IR spectrum displayed bands at 3437, 1622 and 1595 cm^{-1} indicating the presence of OH, conjugated carbonyl and aromatic ring respectively [155]. The molecular formula was determined as $C_{14}H_{10}O_5$ by its EI-MS giving molecular ion peak $[M+]^+$ at *m/z* 258.

The ^{I}H NMR and ^{13}NMR spectra of **108** (Table-6.4) showed characteristic peak of xanthone functionality[85,153] and has similarity with **116** . The ^{I}H NMR also gives signal of five aromatic protons at δ 7.7, dd (*J* = 7.6, 1.5 Hz, H-8), 7.39, d (*J*= 9.1 Hz, H-2), 7.27,d (*J* = 9.1 Hz, H-1), 7.25, dd (*J* = 7.6, 1.5 Hz, H-6) and 7.19, t (H-7). The singlet at δ 3.83 was assigned to MeOAr positioned at C-5 which was confirmed by the cross peak between MeO-5/H-6 in NOSEY spectrum. The EIMS Spectrum also supported the substituted xanthone skeleton, giving characteristic peaks at *m/z* 240, 229, 215 for the loss of H_2O, CO and CH_3 respectively. On the basis of the interpretation of its ^{I3}C and DEPT NMR spectral data (Table 6.4), compound **108** also contained 14 carbons, including one methoxyl, six CH and seven quaternary carbons as observed in **116**. The downfield signal at δ 176.7 was due the conjugated carbonyl of xanthone skeleton. The peaks at δ 117.5 and 123.8 were assigned to the quaternary aromatic carbons C-1a and C-8a. Similarly the signals appeared at δ 151.8, 147.3, 146.8, 146.1 and 145.3 were assigned to five aromatic quaternary carbons attached to oxygen functionalities. The peak appeared at δ 62.1 was assigned to methoxy attached at C-4. These assignments were confirmed by advance 2D-NMR techniques e.g HMBC, HMQC and COSY (Fig 6.4). The proton appeared at δ 7.27 (H-1) showed correlation with carbons resonating at δ 117.1 (C-1a), 145.5 (C-4a), 147.2 (C-3) and 176,7 (C-9/). Similarly the proton at δ 7.39 (H-8) has shown relation with δ 142.0 (C-7), 145.0 (C-5), 148.8 (C-5a) and 181.3 (C-9) whereas H-8/ showed correlation with δ 17.3 117.1 (C-1a), 151.7 (H-4), 147.3 (H-3) in HMBC spectrum and thus confirming the position of two hydroxyl at C-3 and C-4. ^{I}H- ^{I}H COSEY relation showing cross peaks between H-7/H-8 and H-6/H-7 and the patron of splitting already discussed confirming the positions H-6, H-7and H-8. On the basis of spectroscopic and physical data the compound **108** was named as 3,4-Dihydroxy-5-methoxy xanthon, reported here for the first time as new compound from *H.oblongifolium* .



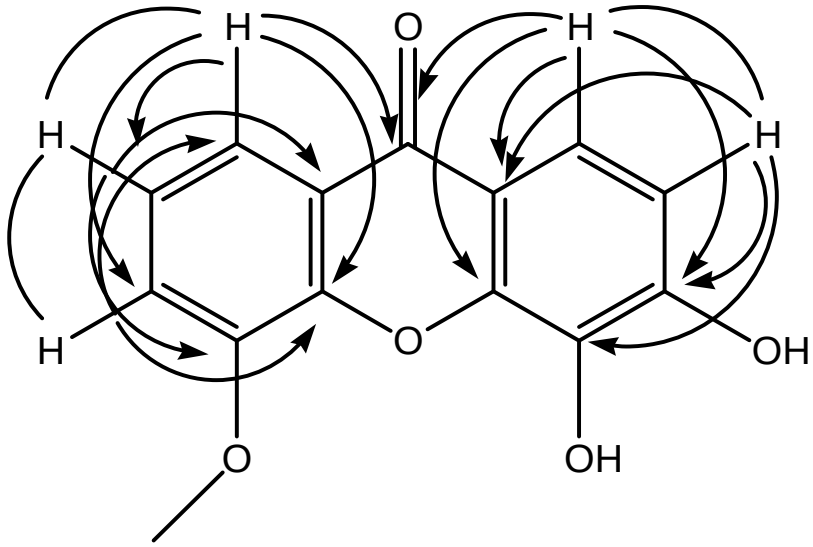


Fig. 6.4: Important HMBC and NOE Interactions of 108

Table-6.4: ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) Spectral Data of Compound (**108**) in $(CD_3)_2CO$

C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	114.6	CH	7.27, d (*J* = 9.1)
1a	117.5	C	-
2	124.3	CH	7.39, d (*J* = 9.1)
3	147.3	C	-
4	151.1	C	-
4a	145.3	C	-
5	146.8	C	-
5a	146.1	C	-
6	120.2	CH	7.25, dd (*J* = 7.6, 1.5)
7	124.3	CH	7.19, t (*J* = 7.8)
8	116.8	CH	7.75, dd (*J* = 7.6, 1.5)
8a	123.8	C	-
9	176.7	C	-
MeO-5	62.1	CH_3	3.83, s

a: Broad band; b: DEPT; c: ^{1}H NMR; d: HMQC interaction

6.1.2: New Xanthones from the Roots of *Hypericum oblongifolium*

6.1.2.1: Hypericorin C (109)

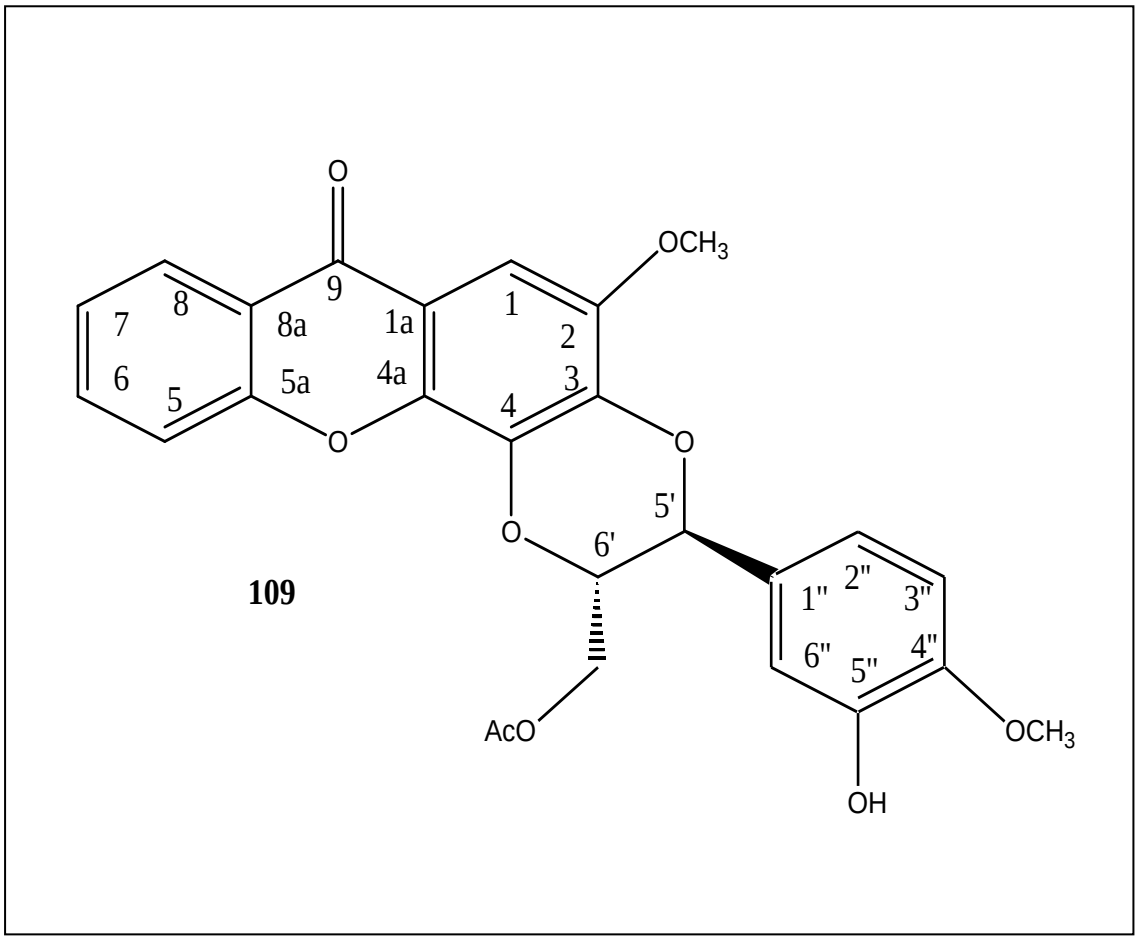
Fraction 17 obtained from the ethyl acetate fraction (**F2**) of the roots of *H. oblnogifolium* was subjected to column chromatography eluted with hexane: chloroform (80:20) to pure chloroform and then methanol: chloroform (1:99) to yield **109** (15 mg). The molecular formula of compound **109** was determined as $C_{26}H_{22}O_9$ by HR-EI-MS giving molecular ion peak $[M+1]^+$ at *m/z* 479.23 (calcd. 479.1264) and $[M+Na]^+$ *m/z* 501.23 (calcd. 501.131). The UV spectrum exhibited characteristic absorption for xanthone at 248, 308 and 346 nm [153]. The IR spectrum displayed bands at 3416, 1742, 1643 and 1608 cm^{-1} indicating the presence of OH, ester conjugated cyclic ketone and aromatic ring respectively [153].

The ^{I}H NMR and ^{13}CNMR spectra of **109** (Table 6.5) showed characteristic peaks of xanthone functionality [85,153]. The ^{I}H NMR, gives signal of five aromatic

protons at δ 7.28, s (H-1), 7.61, dd (*J* = 8.4, 1.0 Hz, H-5), 7.80, td (*J* = 8.4, 1.7 Hz, H-6), 7.45, td, (*J* = 7.9, 1.0 Hz, H-7) and 8.23, dd (*J* = 7.9, 1.4 Hz, H-8). ^{1}H NMR of **109** also showed three aromatic protons singlets at δ 6.9, d (*J* = 8.3 Hz, H-3$^{//}$), 7.02, dd (*J* = 8.3, 1.9 Hz, H-2$^{//}$) and 7.16 (*J* = 1.9 Hz, H-6$^{//}$) in addition to above five signals which indicated another phenyl moiety attached. The ^{1}H NMR signals at δ 3.86, s and 3.90, s was assigned to two MeO attached to aromatic ring (MeO-Ar). Signal at δ 2.03, s was attributed to methyl attached to carbonyl functionality of ester type. The spectrum of **109** also showed *trans* diaxial dioxane proton signals at δ 4.64, m and 5.1, d (*J* = 7.8 Hz). The deshielded doublet (δ 5.1) typical of a benzylic methylene substituted by oxygen and its typical *trans*–coupling (*J* = 7.8 Hz) implied the existence of a *trans*–substituted 1,4-dioxane ring between the xanthone moiety and the phenyl ring . The EIMS shows significant peak at *m/z* 225 could be rationalized in term of retro-Diels-Alder reaction in a dioxane ring, while the ions at *m/z* 222, 180, 179 and 162 indicated that one acetyl group in phenyl propane unit while one hydroxyl and one methoxyl groups present in phenyl ring [107,154]. The ^{1}H NMR spectrum also showed two aliphatic proton signal of CH_2O-group at δ 4.36 (dd, *J* = 12.0, 2.8 Hz) and 4.18 (dd, *J* = 12.0, 4.3 Hz).

On the basis of ^{13}C NMR (BB and DEPT) spectral data (Table 6.3), compound **109** contained 26 carbons, including, three methyl, one CH_2, ten CH and twelve quaternary carbons. The downfield signal at δ 174.9 was due to C-9, the conjugated carbonyl of xanthone skeleton, while the carbonylic signal at δ 169.8 was assigned to the ester moiety. The peaks at δ 114.9 and 121.1 were assigned to the quaternary aromatic carbons C-1a and C-8a while 156.7, 147.8, 140.5, 142.4, 139.8 and 132.3 were attributed to the aromatic quaternary carbon attached to oxygen functionalities. The signals at δ 77.0 and 75.5 were assigned to oxygenated methine carbons at C-6$^{/}$ and C-5$^{/}$ respectively. Similarly the signal at δ 19.7 was assigned to acetyl methyl and δ 62.5 was attributable to methylene next to oxygen in the ester moiety. The chemical shift assignments were confirmed by HMQC and HMBC data (Fig. 6.5). The proton appeared at δ 7.28, s (H-1) showed correlation with δ 141.3 (C-4a), 140.5 (C-3) and 174.9 (C-9). On the basis of HMBC interactions it is suggested that one methoxyl group is attached at C-2 while 1, 4- dioxane ring is fused with xanthone skeleton at C-3 and C-4. Similarly the proton appeared at δ 5.1 (H-5$^{/}$) having correlation in HMBC spectrum with δ 111.4 (C-6$^{//}$), 121.8 (C-2$^{//}$) and 126.8 (C-1$^{//}$), which indicate that meta substituted phenyl moiety is linked to 1,4-dioxane at C-5$^{/}$ and C-1$^{//}$. Methyl at δ 2.03

shows cross peak with 169.8 (CH_2COCH_3) confirmed the presence of acetyl group. Furthermore the HMBC spectrum confirmed that 1, 4-dioxane ring was fused between xanthone framework and phenyl moiety and framed a xanthonolignoid skeleton. ^{I}H-^{I}H COSY spectrum also confirmed the same skeleton by giving cross peaks between H-5/H-6, H-6/H-7, H-7/H-8 and H-2$^{//}$/H-4$^{//}$/H-6$^{//}$. The NOE spectrum indicated correlation between H-1 (δ 7.28, s) and the methoxyl (Meo-2) signal δ 3.90, when irradiated at δ 7.28 and vice versa. Similarly the position of second methoxyl (MeO-3$^{//}$) was also confirmed when proton appeared at δ 7.16 was irradiated, showed cross peak at δ 3.86 and also with dioxane proton at δ 5.1(H-5$^{/}$), the aliphatic methylene proton at δ 4.18 and the dioxane proton signal at δ 5.1 (H-5$^{/}$), similarly proton δ 4.64 (H-6$^{/}$) did not showed NOE interactions with δ 5.1 (H-5$^{/}$) conformed the trans-dioxane protons. The optical rotation of compound **109** was, $[\alpha]^{D}$ = + 0.33º (c, 0.01 acetone), with *trans* relative configuration, having 5$^{/}$R, 6$^{/}$R configuration. Based on the aforementioned spectroscopic methods the compound was proposed to be Hypericorin C.



109

Table-6.5: ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) Spectral Data of Compound (**109**) in $(CD_3)_2CO$

C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	97.4	CH	7.28, s
1a	114.8	C	-
2	147.8	C	-
3	140.5	C	-
4	132.2	C	-
4a	141.3	C	-
5	118.0	CH	7.61, dd (*J* = 8.4, 1.0)
5a	156.7	C	-
6	134.4	CH	7.80, td (*J* = 8.4,1.7)
7	124.0	CH	7.45, td (*J* = 7.9, 1.0)
8	126.1	CH	8.23, dd (*J* = 7.9, 1.3)
8a	121.1	C	-
9	174.9	C	-
5′	77.0	CH	5.1,d (*J* = 7.8)
6′	75.5	CH	4.64, m
$\underline{CH_2}O$	62.5	CH_2	4.18, dd (*J* = 12.0, 4.4) 4.36, dd (*J* = 12.0, 2.8)
$CH_2\underline{CO}CH_3$	169.8	C	-
$OCH_2CO\underline{CH_3}$	19.7	CH_3	2.03, s
1″	126.8s	C	-
2″	121.8	CH	7.02, dd (*J* = 8.3, 1.9)
3″	115.2	CH	6.9, d (*J* = 8.3)
4″	147.0	C	-
5″	147.3	C	-
6″	111.4	CH	7.16, d (*J* = 1.9)
MeO-2	55.6	CH_3	3.90, s
MeO-4″	55.8	CH_3	3.86, s

a: Broad band; b: DEPT; c: ^{1}H NMR; d: HMQC interaction

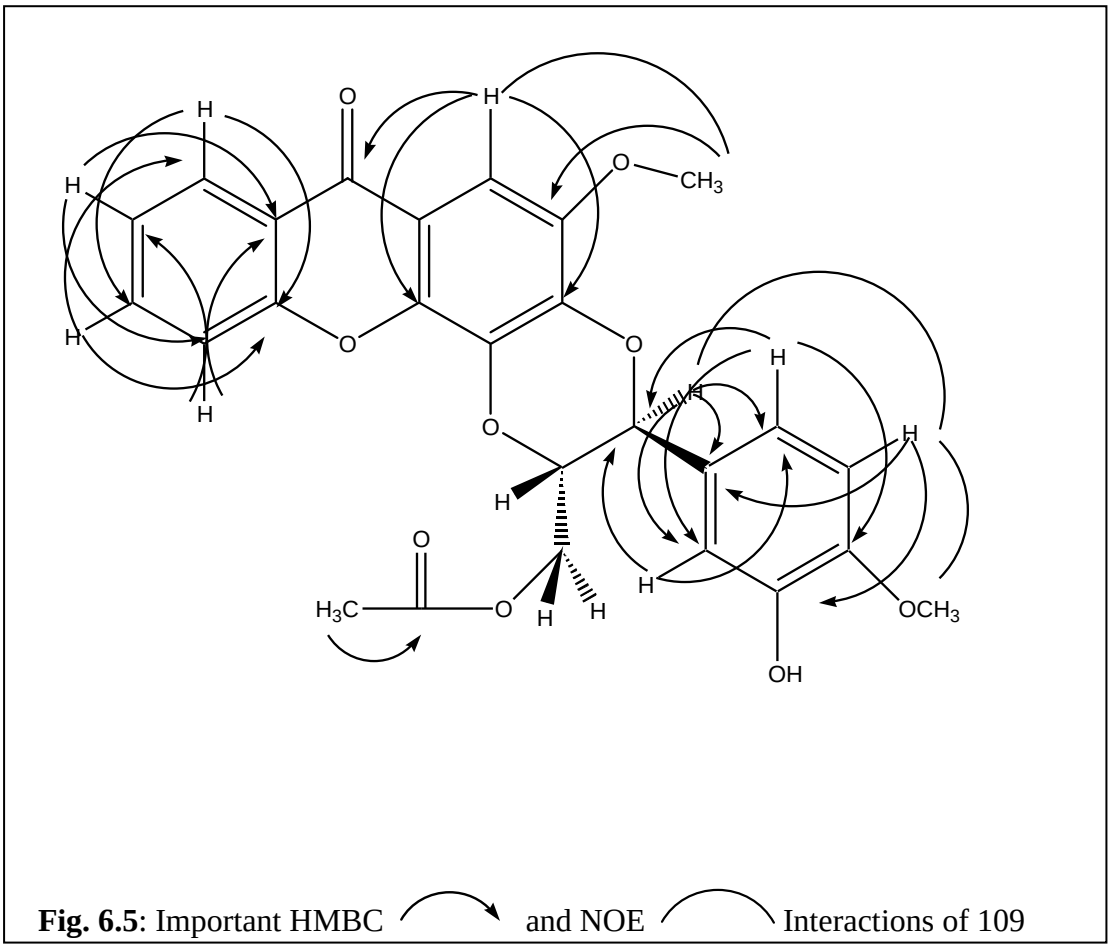


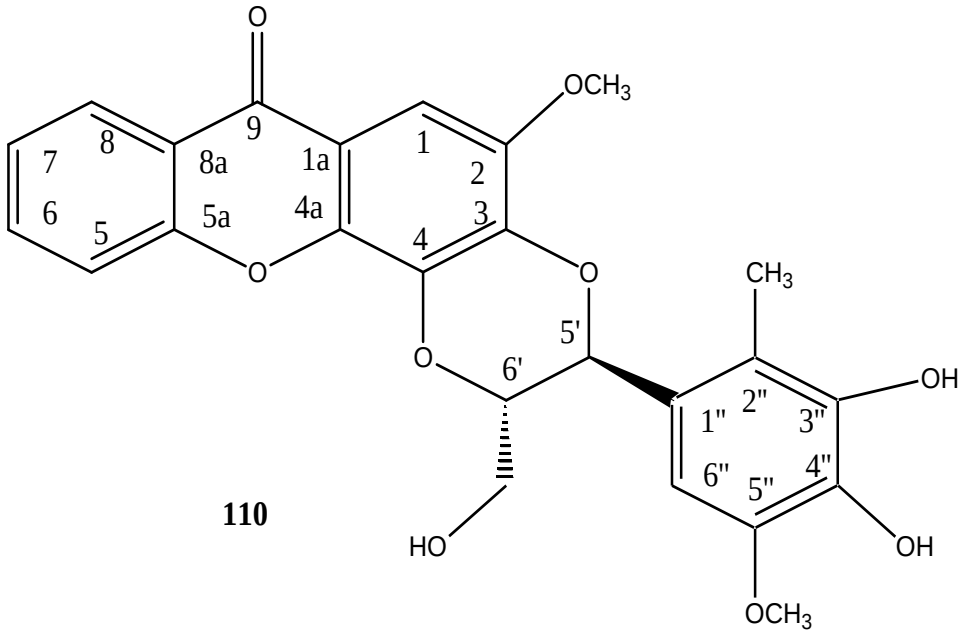
Fig. 6.5: Important HMBC and NOE Interactions of 109

6.1.2.2: Hypericorin D (110)

Fraction 19 obtained from the ethyl acetate fraction (**F2**) of the roots of *H. oblnogifolium* was subjected to column chromatography eluted with hexane: chloroform (80:20) to pure chloroform and then methanol: chloroform (1:99) to yield **110** (15 mg).The molecular formula of compound **110** was determined as $C_{25}H_{22}O_9$ by HR-EI-MS giving molecular ion peak $[M-1]^-$ at *m/z* 467 (calcd. 467.45). The UV spectrum showed the presence of xanthone giving absorption peaks at 250, 302 and 387 nm [153]. The IR spectrum displayed bands at 3384 br, 1639 and 1599 cm^{-1} indicating the presence of OH, conjugated carbonyl and aromatic ring respectively [153]. The ^{I}H and ^{I3}C NMR spectra of **110** (Table 6.6) were much similar to those of **109**, except the acetyl signal was missing in the ^{I}H NMR spectra and the main difference between them was the lack of carbonyl and methyl peaks of acetyl group in ^{I3}C NMR of compound **110.** The EIMS of **110** also showed the peak at *m/z* 173, could be rationalized in terms of a retro-Diels-Alder reaction in the dioxane ring and ions at

m/z 205,173, 156, 130 and 102 respectively indicated that phenyl propane unit having one methoxyl and hydroxyl groups (absence of fragment peak at *m/z* 222 due to acetyl group). Like **109** the ^{1}H NMR (Tables-1) of **110** also showed five signals for aromatic protons at δ 7.16, s (H-1), 7.66, d (*J* = 8.4 Hz, H-5), 7.8, t (*J* = 8.4 Hz, H-6), 7.46, t (*J* = 7.5 Hz, H-7) and 8.17, d (*J* = 7.5 Hz, H-8) as well as the additional peaks of one aromatic proton singlet at δ 6.78 (1H). The ^{1}H NMR signals at δ 3.8, s and 3.7, s were attributed to two MeO-Ar. Signals at δ 5.06, d (*J* = 7.8) and 4.42, m were assigned to oxygenated methine proton of 1,4-dioxane ring. Another signal at 2.46, s was attributed to the methyl group (Me-2$^{//}$) attached to phenyl ring.

On the basis of the interpretation of its ^{13}C NMR (BB and DEPT) spectral data (Table 6.6), compound **110** contained 24 carbons, including three methyl, one methylene, ten methine and eleven quaternary carbons. The only one downfield signal at δ 175.3 (C-9) was due to the presence of conjugated carbonyl of xanthone skeleton. The signals at δ 78.5 and 77.9 were assigned to oxygenated methine carbons at C-5$^{/}$ and C-6$^{/}$ respectively like **109**. The chemical shift assignments were confirmed by HMQC and HMBC (Fig. 6.6). The proton appeared at δ 7.16, s (H-1) showed correlation with 141.8 (C-4a), 140.0 (C-3), 146.4 (C-2) and 175.4 (C-9). The HMBC spectrum also showed same set of correlation as observed in case of compound **109** and indicated that methoxyl group attached at C-2 while 1,4- dioxane ring was fused with xanthone skeleton at C-3 and C-4. Similarly the proton appeared at δ 5.06, d (*J*=7.8) showed correlation in HMBC spectrum with 120.3 (C-2$^{//}$), 106.9 (C-6$^{//}$) and 126.4(C-1$^{//}$), which suggest that another phenyl moiety was linked to 1,4-dioxane at C-5$^{/}$ and C-1$^{//}$. Furthermore the HMBC, COSY and NOE supported the structure of compound **110**. The optical rotation of compound **110** was, $[\alpha]^{D}$ = + 0.58^{o} (c, 0.01 acetone), with *Trans* relative configuration, having 5$^{/}$R, 6$^{/}$R configuration. On the basis of above discussion the compound **110** tagged as Hypericorin **D**.



110

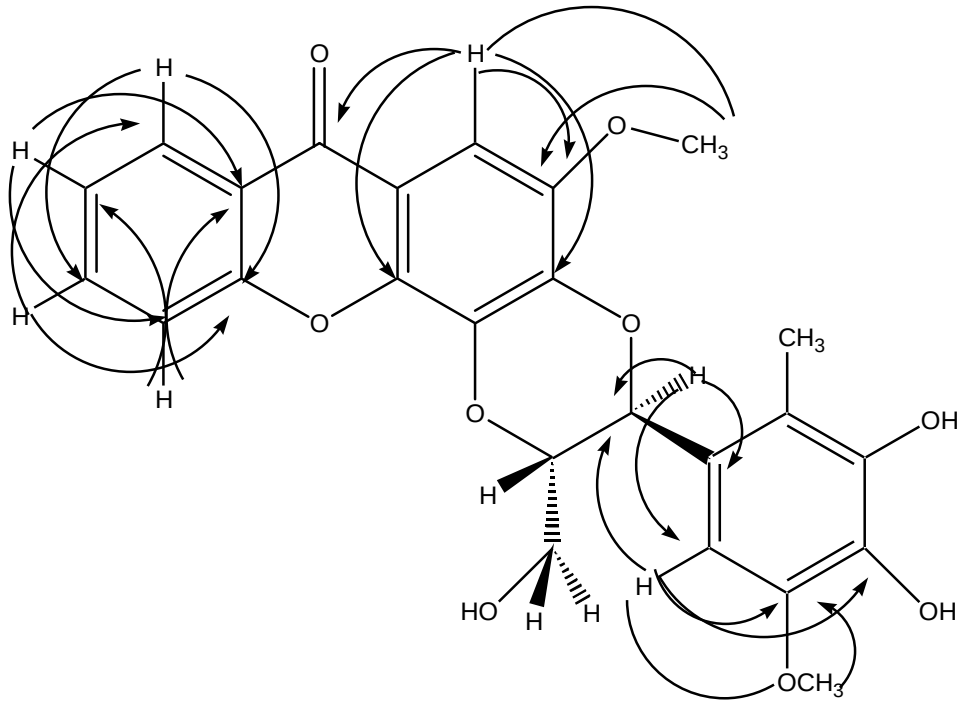


Fig. 6.6: Important HMBC and NOE Interactions of **110**

Table-6.6: ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) Spectral Data of Compound (**110**) in DMSO

C.No.	^{13}C NMR (δ)[a]	Multiplicity (DEPT)[bd]	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)[cd]
1	97.0	CH	7.16, s
1a	114.4	C	-
2	146.4	C	-
3	140.1	C	-
4	133.0	C	-
4a	141.8	C	-
5	118.6	CH	7.66, d (*J* = 8.4)
5a	155.8	C	-
6	135.4	CH	7.81, t (*J* = 8.4)
7	124.8	CH	7.46, t (*J* = 7.5)
8	126.4	CH	8.17, d (*J* = 7.5)
8a	121.2	C	-
9	175.3	C	-
5′	77.2	CH	5.05, d (*J* = 7.8)
6′	78.2	CH	4.42, td (*m*)
$\underline{CH_2}O$	60.4	CH_2	3.68, dd (*J* = 12.0, 4.6)
			3.38, dd (*J* = 12.0, 2.7)
1″	126.4	C	-
2″	137.8	C	-
3″	133.5	C	-
4″	136.8	C	-
5″	148.5	C	-
6″	106.2	CH	6.7, s
MeO-2	56.3	CH_3	3.8, s
MeO-5″	56.7	CH_3	3.7, s

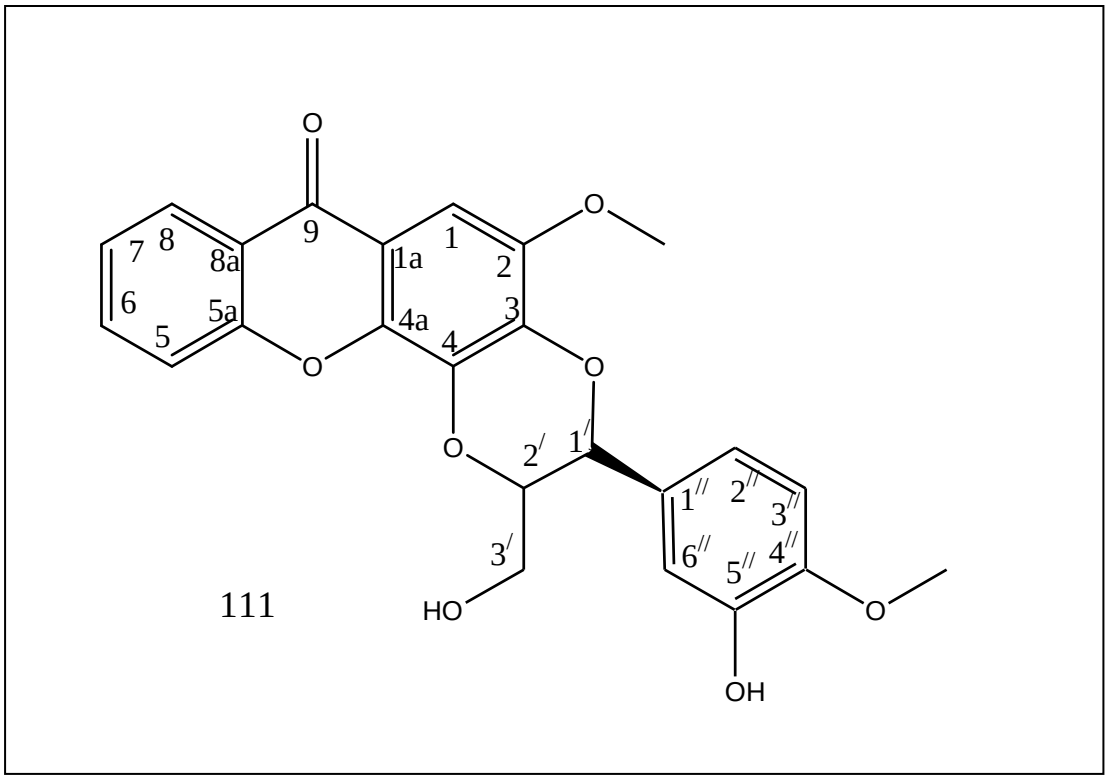
a: Broad band; b: DEPT; c: ^{1}H NMR; d: HMQC interaction

6.1.3: Known Xanthones from the aerial parts (Twigs) of *H. oblongifolium*

6.1.3.1: 3-(4-hydroxy-5-methoxyphenyl)-2-(hydroxymethyl)-5-methoxy-2H-[1,4] dioxino [2,3-c] xanthen-7 (3H)-one (111)

The ethyl acetate fraction (260g) was loaded on column chromatography over silica gel and eluted with solvent in increasing order of polarity (*n*-hexane– ethyl acetate and ethyl acetate –methanol) to afford 30 major fractions. Fraction 26 was purified on the same way to yield pure **111** (12 mg). Fractionation and isolation scheme is given in section 7.2. The molecular formula of compound **111** was found as $C_{24}H_{20}O_8$ established by its EI-MS giving molecular ion peak $[M+]^+$ at 436. The UV spectrum showed the presence of xanthone giving absorption peaks at 254, 306 and 382 nm[153]. The bands displayed in IR spectrum at 3592, 1642 and 1600 cm^{-1} indicating the presence of OH, conjugated carbonyl and aromatic ring respectively[153].

The IH and ^{I3}C NMR spectra of compound **111** (Table 6.7) were found much closed to those of **106** and Kielcorin[134]. The ^{I}H NMR (Tables-6.7) of **111** also have the signals of five aromatic protons at δ 7.33, s (H-1), 7.62,d (*J* = 8.40 Hz, H-5), 7.79,t,d (*J* = 8.40 Hz, 1.60 Hz, H-6), 7.42, t (*J* = 7.30 Hz, H-7) and 8.20, dd (*J* = 7.30, 1.40 Hz, H-8) as well as the additional three peaks of aromatic protons singlets δ 6.8, d (*J* = 6.0 Hz), 6.90, dd (*J* = 6.10, 1.40 Hz) and 7.04, d (*J* = 1.40 Hz). The ^{I}H NMR signals at δ 3.84, s and 3.89, s were attributed to two MeO-Ar. Signals at δ 5.1, d (*J* = 8.1 Hz) and 4.1, m were assigned to oxygenated methine proton of 1,4-dioxane ring.



111

Table-6.5: ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) Spectral Data of Compound **(111)** in CD_3OD + $CDCl_3$ (1:1)

C.No.	^{13}C NMR (δ)	Multiplicity (DEPT)	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)
1	98.3	CH	7.33, s
1a	114.5	C	-
2	146.40	C	-
3	142.00	C	-
4	142.00	C	-
4a	139.20	C	-
5	119.81	CH	7.62, d (*J* = 8.4)
5a	155.71	C	-
6	134.51	CH	7.79, td (*J* = 8.4, 1.4)
7	125.31	CH	7.42, t (*J* = 7.3)
8	126.51	CH	8.2, dd (*J* = 7.3, 1.4)
8a	121.81	C	-
9	175.71	C	-
$1^{/}$	78.42	CH	5.1, d (*J* = 8.1)
$2^{/}$	77.32	CH	4.1, m
$3^{/}$	60.73	CH_2	3.5,m, 3.9,m
$1^{//}$	132.42	C	
$2^{//}$	122.92	CH	6.9, dd (*J* = 6.1, 1.4)
$3^{//}$	116.81	CH	6.8, d (*J* = 6.2)
$4^{//}$	147.01	C	-
$5^{//}$	126.31	C	-
$6^{//}$	112.92	CH	7.04, d (*J* = 1.4)
MeO-2	56.13	CH_3	3.89, s.
MeO-$4^{//}$	55.95	CH_3	3.84, s

The ^{I3}C and DEPT NMR spectral data (Table 6.7) of compound **111** disclosed 24 carbons, including two MeO, one CH_2, ten CH and eleven quaternary carbons. The lowfield signal at δ 175.72 was due to C-9, the conjugated carbonyl of xanthone skeleton. The peaks at δ 114.5 and 121.8 were assigned to the quaternary carbons of aromatic ring C-1a and C-8a, 155.7, 146.8, 142.0, 142.0 and 139.81 were attributed to the aromatic quaternary carbon having oxygen functionalities as observed in compound **106.** The signals at δ 78.4 and 77.3 were assigned to oxygenated carbons of dioxane ring at C-$1^{/}$ and C-$2^{/}$ respectively. These assignments were confirmed by advance 2D-NMR techniques (HMBC, HMQC and COSY). the HMBC and ^{I}H- ^{I}H

COSEY spectrum also have the same set of relation as in compound **106** showing cross peaks between H-6/H-5, H-7/H-6, H-8/H-6 and H-5$^{//}$/H-6$^{//}$ and NOSEY cross peak between H-6$^{//}$/MeO-5$^{//}$ supported that the structure of Compound **111** was determined 3-(4-hydroxy-5-methoxyphenyl)-2-(hydroxymethyl)-5-methoxy-2H-[1,4]dioxino[2,3-c]xanthen-7(3H)-one.

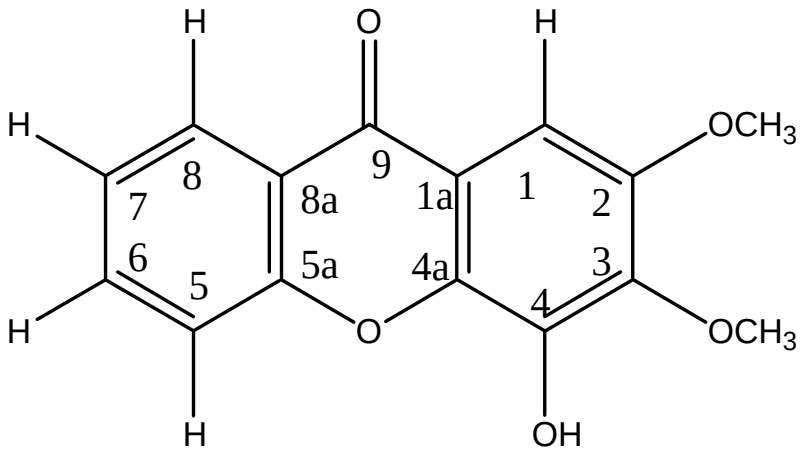
6.1.3.2: 4-Hydroxy-2, 3-dimethoxyxanthone (112)

Fractions 15 and 16 from the **F2** were mixed and loaded over flash silica gel CC (Chloroform/hexane 40:60, 50:50) to afford compounds **112** (9mg) and **113** (6mg). Fractionation and isolation scheme is given in Experimental section (Fig 7.1 and 7.2). A positive EI-MS of **112** showed an $[M]^+$ peak at *m*/*z* 272, and determined the molecular formula $C_{15}H_{12}O_5$. The IR, ^1H and ^{13}C NMR spectra of **112 (**Table 6.6**)** disclosed that a carbonyl carbon δ_C 178.05, *s*; 1641cm^{-1}(C═O), an aromatic OH group (3599 cm^{-1}), two methoxy groups δ_H 3.94 *s*, 3.96 *s*, each 3H; δ_C 56 (CH_3, 61.8 (CH_3) and five signal of aromatic proton in proton NMR. All these proved that it has Xanthone skeleton.

The HMBC spectrum showed direct relation between carbonyl (C-9) and also giving 1H doublet at δ 8.23 (J = 8.14 Hz) confirmed this as H-8. The COSEY spectrum then by its relation, permitting the assignment of a 1H triplet, doublet at δ 7.42 (J = 7.92, 0.6 Hz) to H-7. This correlation in the COSEY spectrum also allowed the assignment of 1H triplet, doublet at δ 7.79 (J = 8.53, 1.43 Hz) to H-6 as well as the position of methoxy groups were assigned as 3-OCH_3 and 2-OCH_3, respectively. The two methoxy in compound **112** were placed in the second aromatic ring at adjacent carbon (2 & 3) which was also confirmed by HMBC spectrum. A cross peak in the COESY spectrum between the methoxy (δ_H 3.94, δ_c 56.0) and the 1H singlet aromatic signal at δ 7.25 confirmed its position as H-1. The only hydroxyl group assigned to C-4 supported from both its up field position, relative to other aromatic carbons and correlation in the HMBC spectrum. The compound **112** is thus the 4-hydroxy-2,3-dimethoxy-9H-xanthen-9-one, reported here from *H. oblongifolium* . The physical and spectral data (Table 6.8) showed complete resemblance with reported[112].

Table-6.8: ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) Spectral Data of Compound **(112)** in CD_3OD + $CDCl_3$ (1:1)

C.No.	^{13}C NMR (δ)	Multiplicity (DEPT)	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)
1	97.0	CH	7.25, s
1a	118.0	C	-
2	151.8	C	-
3	143.71	C	-
4	144.02	C	-
4a	141.02	C	-
5	119.03	CH	7.64, d (J = 8.5)
5a	157.04	C	-
6	136.0 5	CH	7.79, td (J = 8.5, 1.45)
7	125.0 0	CH	7.42, td (J = 7.9, 0.6)
8	127.00	CH	8.25, dd (J = 7.9, 1.4)
8a	123.80	C	-
9	176.71	C	-
MeO-2	56.02	CH_3	3.94, s
MeO-3	61.83	CH_3	3.96, s

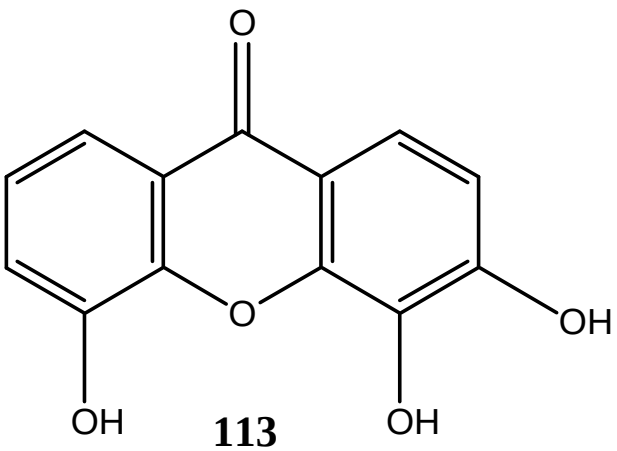


112

6.1.3.3: 3, 4, 5-Trihydroxyxanthone (113)

A yellow amorphous powder **(113,** 6 mg**)** was isolated from the combined fraction (15 & 16) as stated earlier. The UV spectrum showed the presence of xanthone giving absorption peaks at 252, 316 and 372 nm[153]. The bands in IR spectrum at 3592, 1641 and 1600 cm^{-1} indicating the presence of OH, conjugated carbonyl and aromatic ring respectively. The positive EI-MS of **113** disclosed a $[M]^+$ peak at *m*/*z* 244.0, confirming the molecular formula, $C_{13}H_9O_5$. The IR, ^1H and ^{13}C NMR spectra of **113 (**Table 6.7**)** were much closed to those of **112**, it also have the carbonyl carbon (δ_C 183.01 *s*; IR 1641 cm^{-1}). 1H proton resonances at δ 7.7, dd (*J* = 7.81, 1.45 Hz, H-8); 7.29, t (*J* = 7.92 Hz, H-7); 7.37, dd (*J* = 7.92, 1.42Hz, H-6) were also noted.

The HMBC spectrum disclosed the direct relation between the carbonyl resonance (C-9) and a 1H doublet at *δ* 6.96 (*J* = 8.95 Hz) and confirmed its position as H-1. The cross peak of COSEY spectrum intern confirming the assignment of a 1H doublet at *δ* 7.35(*J* = 8.91 Hz) to H-2. The difference, relative to **112**, was the substitution of two methoxy groups by hydroxyl as there was no signal for methoxy in the NMR spectra of **112** and have been replaced by the signals of OH (δ_H 12.4, s).The positioning of hydroxyl group (C-3, C-4 & C-5) were confirmed by the chemical shift value in ^{13}C NMR spectrum. This placement was also confirmed by a correlation in the COESY and HMBC spectra between the OH (5), and OH (4) proton signals. The compound **113** is thus 3, 4, 5-trihydroxy-9H-xanthen-9-one. The physical and spectral data (Table 6.9) showed complete resemblance with reported [156], isolated here for the first time from *H.oblongifolium*



113

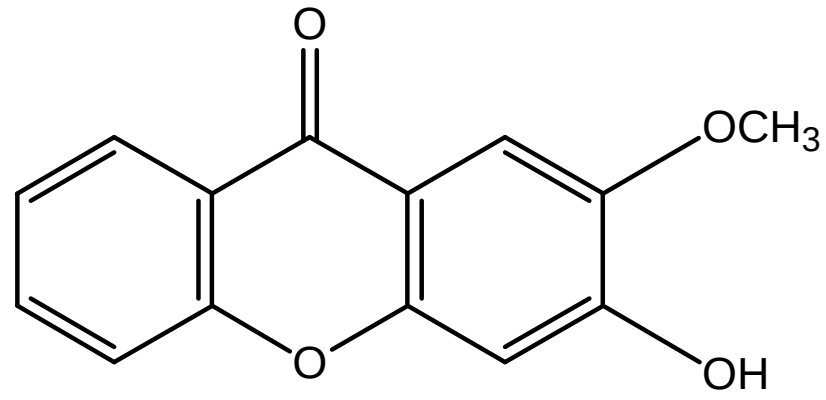
Table-6.9: ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) Spectral Data of Compound **(113)** in $(CD_3)_2CO$

C.No.	^{13}C NMR(δ)	Multiplicity (DEPT)	^{1}H NMR(δ) Coupling Constants J_{HH} (Hz)
1	107.1	CH	6.97 (d, 8.9)
1a	110.01	C	-
2	124.02	CH	7.35 (d, 8.9)
3	149.01	C	-
4	161.03	C	-
4a	141.05	C	-
5	148.02	C	-
5a	147.01	C	-
6	121.02	CH	7.39 (dd, 7.9, 1.45)
7	124.01	CH	7.28 (t, 7.9)
8	116.03	CH	7.70 (dd,7.9,1.4)
8a	122.04	C	-
9	183.02	C	-

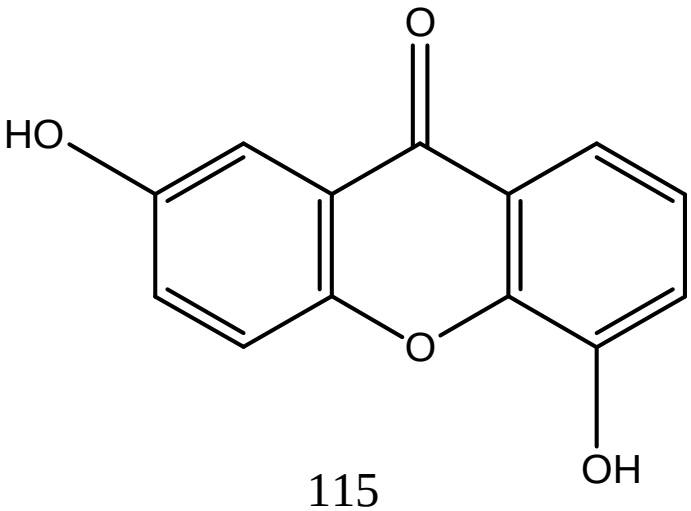
6.1.3.4: 3-Hydroxy-2-methoxyxanthone (114)

Fraction 14 was put over flash silica gel CC (Chloroform/hexane 10:90, 20:80, 30:70, and 40:60) and afforded the compounds the compounds **114** (10 mg), along with other compounds. Fractionation and isolation scheme is given in Experimental section (Fig 7.2 and 7.3). The positive EI-MS of **114** showed a $[M]^+$ peak at *m*/*z* 242.09, confirming the molecular formula, $C_{14}H_{10}O_4$ for the compound. The IR, ^{1}H and ^{13}C NMR spectra of **114 (**Table 6.10) were found much closed to those of 112 and disclosed peaks at δ_C 177.83 (C-9), δ_H 8.23 (dd, J = 7.87, 1 .43 Hz, H-8); δ_H 7.38 (ddd, J = 8.42, 7.92, 1.43 Hz H-7); δ_H 7.76 (td, J = 8.44, 1.45 Hz, H-6); δ_H 7.52 (d, J = 8.4Hz, H-5); δ_H 3.961, *s*, 3H; δ_C 56.53 (OCH_3), at C-2 δ_H 6.94. *s*, H-4; δ_H 6.3, *s*, H-1). However, the methyl singlet found in the NMR spectra of **110** was disappeared. The HMBC spectrum also disclosed the correlation between the C-4a and a H-4 (δ_H 6.97,

s) as well as NOESY correlation in the spectrum between the methoxy (δ_H 3.941, δ_c 56.04) resonance and aromatic signal at δ_H 7.62, s the methoxy was placed at C-2 while hydroxyl at C-3. The compound **114** is thus 3-hydroxy-2-methoxyxanthone reported here, for the first time, from *H.oblongifolium.* The physical and spectral data (Table 6.10) showed complete resemblance with reported[151].



114



115

Table-6.10: ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) Spectral Data of Compound **(114)** in CD_3OD + $CDCl_3$ (1:1)

C.No.	^{13}C NMR (δ)	Multiplicity (DEPT)	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)
1	106.01	CH	7.62, s
1a	114.80	C	-
2	156.01	C	-
3	103.03	C	-
4	118.02	CH	6.98, s
4a	154.01	C	-
5	148.08	CH	7.25, d (J = 8.4)
5a	157.06	C	-
6	135.01	CH	7.75, td (J = 8.4, 1.45)
7	124.01	CH	7.38, ddd (J = 8.4, 7.9, 1.4)
8	126.01	CH	7.82, dd (J = 7.87, 1.4)
8a	122.02	C	-
9	177.80	C	-
MeO-2	56.02	CH_3	3.94, s

6.1.3.5: 4, 7-Dihydroxyxanthone (115)

Yellowish amorphous solid **115** (11mg) along with other compound was purified from the fraction 20 using flash silica gel CC (See section 7.2.2.1). The UV spectrum of **115** showed the presence of aromatic ring giving absorption peaks at 240, 291 and 373nm[157]. The IR spectrum disclosed absorption bands at 3500, 1635 and 1595 cm^{-1} indicating the presence of OH, conjugated carbonyl and aromatic ring respectively[157]. The molecular formula was found as established by $C_{13}H_8O_4$, by its LR-EI-MS giving molecular ion peak $[M+]^+$ at *m/z* 228.34

The ^{l}H and ^{13}C NMR spectra of **115** (Table 6.11) showed characteristic peak of xanthone functionality. The ^{l}H NMR, gives signal of six aromatic protons at δ 7.68,

dd (*J* = 7.53, 1.23 Hz, H-1), 7.20, t (*J* = 7.73 Hz, H-2), 7.25, dd (*J* = 7.53, 1.23 Hz, H-3), 7.5,d (*J* = 6.4 Hz, H-5), 7.3, dd (*J* = 6.22, 2.92 Hz, H-6) and 7.5,d (*J* = 3.01 Hz, H-8). The ^{I3}C and DEPT NMR spectral data (Table-6.11) of compound **115** have shown 13 carbons including six CH and seven quaternary carbons. The lowfield signal at δ 179.72 was due to C-9, the conjugated carbonyl of xanthone skeleton. The peaks at δ 117.5 and 122.8 were assigned to the quaternary aromatic carbons C-1a and C-8a while 147.8, 147.3, 151.9, and 155.3 were attributed to the aromatic quaternary carbon attached to oxygen functionalities. These assignments were confirmed by advance 2D-NMR techniques (HMBC, HMQC and COSY). On the basis of spectroscopic and physical data the compound **115** was identified as 2,5-dihydroxy xanthone already reported[157], isolated here for the first time, from *H.oblongifolium.*

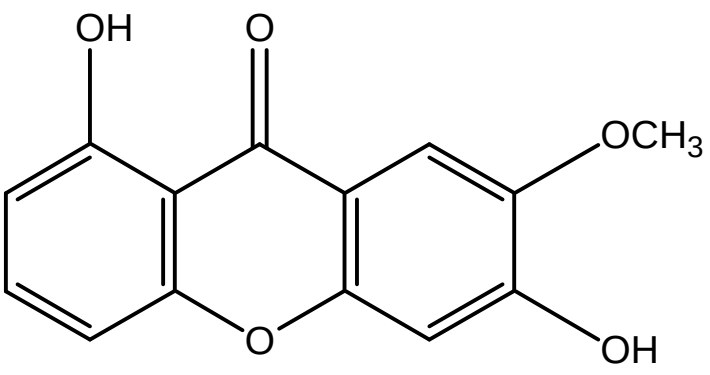
Table-6.11: ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) Spectral Data of Compound **(115)** in CD_3OD

C.No.	^{13}C NMR (δ)	Multiplicity (DEPT)	^{1}H NMR(δ) Coupling Constants J_{HH} (Hz)
1	117.5	CH	7.68, dd (*J* = 7.5, 1.2)
1a	123.5	C	
2	124.3	CH	7.20, t (*J* = 7.7)
3	121.1	CH	7.25, dd (*J* = 7.5, 1.2)
4	147.8	C	-
4a	147.3	C	-
5	121.8	CH	7.5, d (*J* = 6.4)
5a	151.9	C	-
6	125.5	CH	7.3, dd (*J* = 6.2, 2.9)
7	155.3	C	-
8	109.5	CH	7.5, d (*J* = 3.0)
8a	122.8	C	-
9	179.7	C	-

6.1.3.6: 1, 6-Dihydroxy-7-metoxyxanthone (116)

Yellowish amorphous solid **116** (14 mg) along with other compound was purified from the fraction 20 using flash silica gel CC (See section 7.2.2.1). The UV spectrum showed the absorption peaks at 250, 290 and 368 nm[158]. The IR spectrum giving absorption at 3550, 1647 and 1585 cm^{-1} showed the presence of OH, conjugated carbonyl and aromatic ring respectively[158]. The molecular formula was determined as $C_{14}H_{10}O_5$ by its LR-EI-MS giving molecular ion peak $[M+]^+$ at *m/z* 258.2

The ^{I}H and ^{13}C NMR spectra of **116** (Table-6.12) showed closed resemblances with class of xanthone[85,153]. The ^{I}H NMR, gives signal of five aromatic protons at δ 8.16, d (*J* = 7.61 Hz, H-7), 7.58, t (*J* = 7.53 Hz, H-6), 7.45, d (*J* = 7.54 Hz, H-5), 7.22, d (*J* = 4.3 Hz, H-4) and 7.16, s (H-1). The ^{I3}C and DEPT NMR spectral data (Table 6.4) of compound **116** disclosed 14 carbons, including one methoxy, six CH and seven quaternary carbons. The lowfield signal at δ 176.9 was due to C-9, the conjugated carbonyl of xanthone skeleton. The peaks at δ 113.5 and 121.8 were assigned to the quaternary aromatic carbons C-1a and C-8a while δ 155.8, 145.8, 142.3, 140.3, and 132.3 were attributed to the aromatic quaternary carbons attached to oxygen functionalities. The peak appeared at δ 55.6 was assigned to methoxy attached at C-2. These assignments were confirmed by advance 2D-NMR techniques (HMBC, HMQC and COSY). The spectroscopic and physical data of compound **116** showed complete resemblance with those available in literature as 1,6-dihydroxy-7-methoxy xanthone [158], reported here for the first time, from *H.oblongifolium* .



116

Table-6.12: ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) Spectral Data of Compound **(116)** in CD_3OD+ $CDCl_3$ (1:1)

C.No.	^{13}C NMR (δ)	Multiplicity (DEPT)	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)
1	155.8	C	7.16, s
1a	113.5	C	-
2	123.3	CH	7.45, d (*J* = 7.9)
3	133.5123.3	CH	7.58, t (*J* = 7.6)
4	126.3142	CH	8.16, d (*J* = 7.6)
4a	132.3	C	-
5	117.8	CH	7.22, s
5a	140.9	C	-
6	145.3	CH	7.58, t (*J* = 7.6)
7	145.3	CH	8.16, d (*J* = 7.6)
8	96.3	CH	7.16, s
8a	121.8	C	-
9	176.9	C	-
MeO-2		CH_3	3.8, s

6.1.3.7: 1, 3, 7-Trihydroxyxanthone (117)

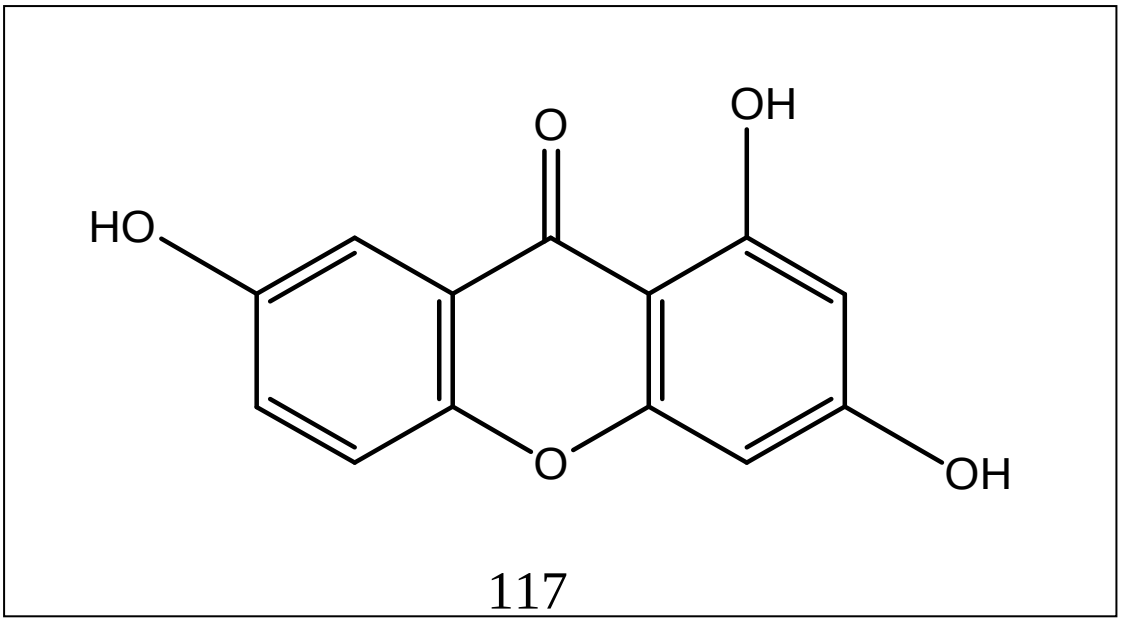
As mentioned above, The fractions 18 and 19 were mixed and treated on flash silica gel CC (Chloroform/hexane 60:40, 70:30, 80:20, 95:5), resulting in the isolation of **117** (15 mg) as yellow powder along with other compounds (Section 7.2.2.1).
The UV spectrum of **117** showed the absorption peaks at 244, 318 and 356 nm[159]. The IR spectrum displayed bands at 3519, 3502, 3442 (O-H), 2928, 2843, 1654 (C═O). The positive EI-MS of **117** showed a $[M]^+$ peak at *m*/*z* 244.0, corresponding to the molecular formula, $C_{13}H_8O_5$.

The IR, ^{1}H and ^{13}C NMR spectra of **117** (Table 6.13) were found much close to those of **113**, it also have the carbonyl carbon (δ_C 183.01 *s*; 1654 cm^{-1}). In ^{1}H NMR, 1H proton resonances at δ *7*.45 (d, *J* = 8.91 Hz) was assigned to H-8, 7.35 (d, *J* = 8.91 Hz) to H-5, 7.25 (dd, *J* = 8.92, 2.81 Hz) to H-6 and 6.39 (d, *J* = 1.9 Hz) to H-4. The difference relative to **113**, was observed in attachment of hydroxyl groups. The

interpretation of its ^{I3}C and DEPT NMR spectral data (Table 6.13), compound **117** also contained 13 carbons, including, five CH and seven quaternary carbons as observed in **113**. The downfield signal at δ 181.31 was assigned to the conjugated carbonyl of xanthone skeleton (C-9). The signals at δ 103.61 and 122.51 were attributed to the quaternary aromatic carbons C-1a and C-8a. The signal appeared at δ 167.3(C-3), 164.6 (C-1), 159.3 (C-4a), 155.8 (C-7), 151.0 (C-5a) were assigned to five aromatic quaternary carbons attached to oxygen functionalities. These assignments were confirmed by advance 2D-NMR techniques (HMBC, HMQC and COSY).The spectroscopic and physical data of compound **117** has closed similarities with those reported in literature as 1,3,7-trihydroxy-9H-xanthen-9-one[159], reported here for the first time from *H. oblongifolium.*

Table-6.13: ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound **(117)** in CD_3OD+ $CDCl_3$ (1:1)

C.No.	^{13}C NMR (δ)	Multiplicity (DEPT)	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)
1	164.6	C	-
1a	103.5	C	-
2	98.8	CH	6.19,d (*J* = .8)
3	167.3	C	-
4	96.9	CH	6.39, d (*J* = 1.8)
4a	159.3	C	-
5	119.8	CH	7.35, d (*J* = 8.9)
5a	151.1	C	-
6	125.2	CH	7.25, dd (*J* = 8.91, 2.8)
7	155.8	C	-
8	109.8	CH	7.45, d (*J* = 2.8)
8a	122.8	C	-
9	176.7	C	-

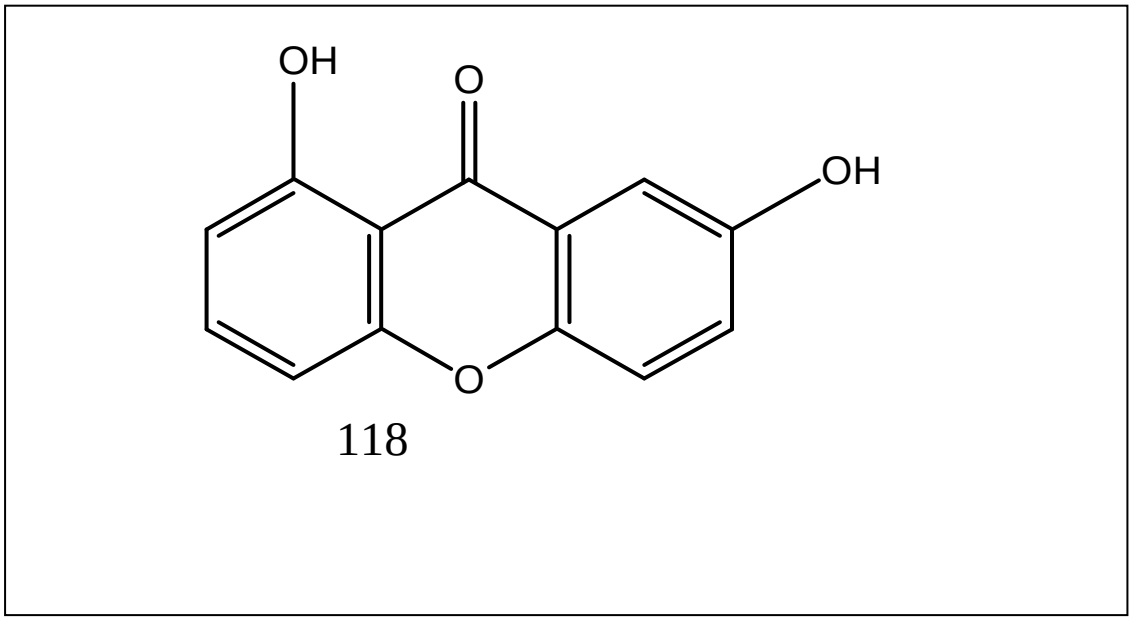


117

6.1.3.8: 1, 7-Dihydroxyxanthone (118)

As mentioned above, fraction 9 was applied to column chromatography using flash silica (Ethyl acetate/ hexane 5:95) to purified **118** (25 mg) as yellow solid (Section 7.2.2.1). The absorption peaks at 206, 238, 258, 319, 375 nm was observed in the UV spectrum of **118**. The IR spectrum disclosed absorption bands at 3500, 1635 and 1595 cm^{-1} indicating the presence of OH, conjugated carbonyl and aromatic ring respectively. The molecular formula was determined as $C_{13}H_8O_4$ by its EI-MS giving molecular ion peak $[M+]^+$ at *m/z* 228.

The ^{1}H and ^{13}C NMR spectra of **118** (Table-6.14) have shown characteristic peaks of xanthone functionality. The ^{1}H NMR, gives signal of six aromatic protons at δ 7.64, t (*J* = 8.3 Hz, H-6), 7.59, d (*J* = 3.01 Hz, H-1), 7.45, dd (*J* = 8.91, 2.91 Hz, H-3), 7.51, d (*J* = 8.81 Hz, H-4), 6.9, d (*J* = 8.31 Hz, H-5) and 6.7, d (*J* = 8.31 Hz, H-7). The interpretation of its ^{13}C and DEPT NMR spectral data (Table 6.14), of **118** disclosed 13 carbons, including six CH and seven quaternary carbons. The signal at δ 182.71 was due to the conjugated carbonyl of xanthone skeleton (C-9). The peaks at δ 110.8 and 121.5 were assigned to the quaternary aromatic carbons C-1a and C-8a while δ 162.3, 157.8, 154.1 and 151.3 were attributed to the aromatic quaternary carbon attached to oxygen functionalities. These assignments were also confirmed by advance 2D-NMR techniques (HMBC, HMQC and COSY). The detailed spectroscopic studies proposed the structure of compound **118** as 1,7-dihydroxy xanthone already reported[158], isolated here for the first time from *H.oblongifolium.*



118

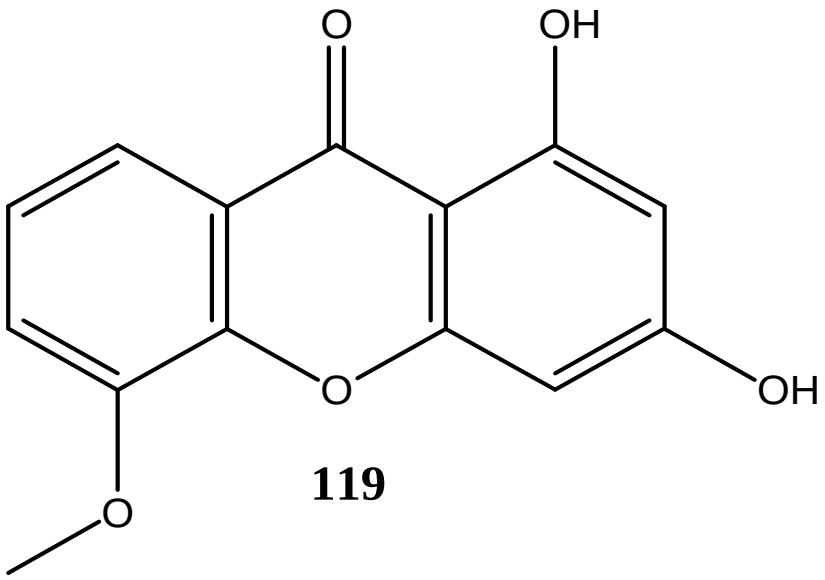
Table-6.14: ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) Spectral Data of Compound **(118)** in $(CD_3)_2CO$

C.No.	^{13}C NMR (δ)	Multiplicity (DEPT)	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)
1	109.6	CH	7.59, d (*J* = 3.0)
1a	121.5	C	-
2	157.3	C	-
3	126.1	CH	7.45, dd (*J* = 8.9, 2.8)
4	120.2	CH	7.51, d (*J* = 8.8)
4a	151.3	C	-
5	107.8	CH	6.9, d (*J* = 8.8)
5a	154.3	C	-
6	137.8	CH	7.65, t (*J* = 8.3)
7	110.1	CH	6.70, d (*J* = 8.3)
8	162.8	C	-
8a	110.5.8	C	-
9	182.9	C	-

6.1.3.9: 1, 3-Dihydroxy-5-methoxyxanthone (119)

As discussed earlier the fraction 14 was applied to column chromatography over flash silica gel CC (Chloroform/hexane 10:90, 20:80, 30:70, and 40:60) and afforded compound **119** (8 mg) along with other compounds (Section 7.2.2.1). The UV spectrum gives the absorption peaks at 207, 241 and 313 nm[129]. The bands in IR spectrum at 3433, 3219, 1645 and 1573 cm^{-1} indicating the presence of OH, conjugated carbonyl and aromatic ring respectively[129]. The molecular formula was determined as $C_{14}H_{10}O_5$ by its EI-MS giving molecular ion peak $[M+]^+$ at 258.4

The ^{I}H and ^{I3}C NMR spectra of **119** (Table-6.15) were much closed to that of **116** and **108**. The ^{I}H NMR signals appeared at δ 6.27, d (*J* = 1.9 Hz), 6.47, d (*J* = 1.9 Hz), 7.33, t (*J* = 8.0 Hz), 7.2, d (*J* = 9.11, H-1), 7.47, dd (*J* = 8.01, 1.31 Hz) and 7.74, dd (*J* = 8.01, 1.31 Hz) were attributed to H-2, H-4, H-7, H-6 and H-8 respectively. The peak at δ 3.83 was assigned to MeOAr. The signal at 12.91 was assigned the chelated hydroxyl group (OH-1). The interpretation of ^{I3}C and DEPT NMR spectral data (Table 6.15), compound **119** also displayed 14 carbons, including one methoxy, five CH and eight quaternary carbons as observed in **116**. The downfield signal at δ 188.7 was due to C-9, the conjugated carbonyl of xanthone skeleton. The peaks at δ 110.5 and 124.5 were assigned to the quaternary aromatic carbons C-1a and C-8a. The peak appeared at δ 56.1 was assigned to methoxy attached at C-5. These assignments were confirmed by advance 2D-NMR techniques (HMBC, HMQC and COSY). The structure was also established with help of X-Ray crystallography (Fig 6.7). The spectroscopic and physical data of compound **119** agree with those reported in literature as 1,3-Dihydroxy-5-methoxyxanthone[129].



119

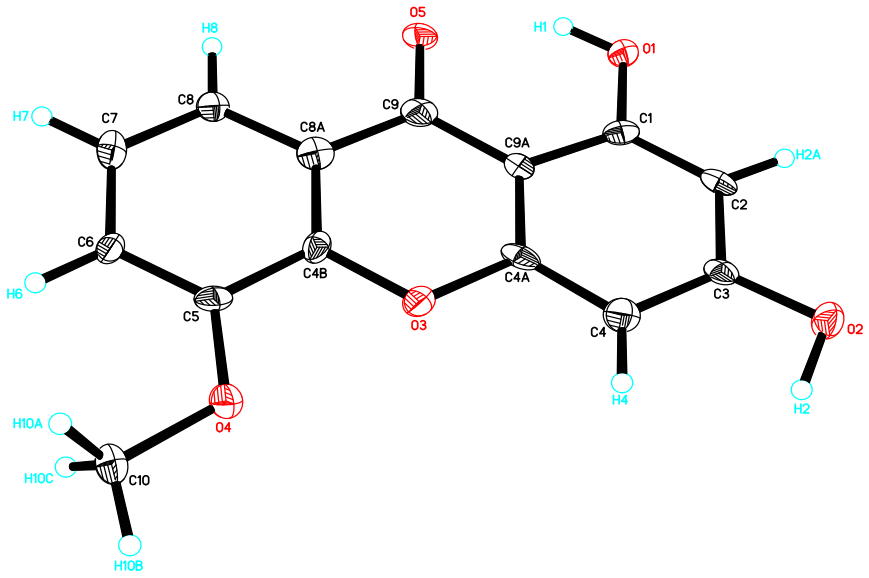


Fig.6.7: Crystal structure of **119**

Table-6.15: ^{1}H NMR (300 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound **(119)** in $(CD_3)_2CO$

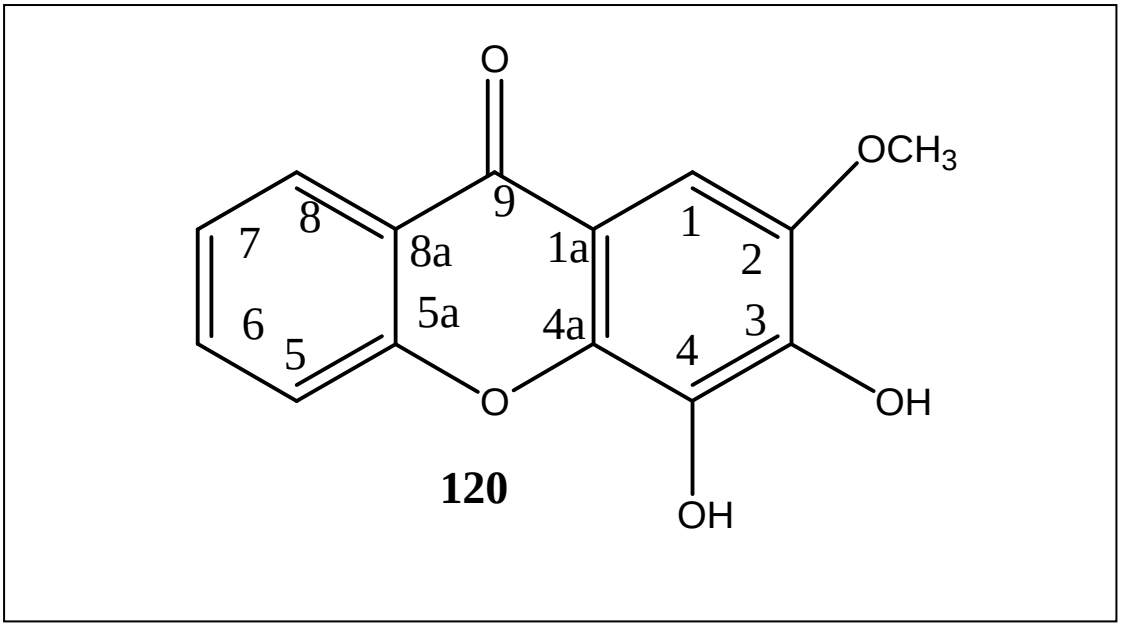
C.No.	^{13}C NMR(δ)	Multiplicity (DEPT)	^{1}H NMR(δ) Coupling Constants J_{HH} (Hz)
1	170.0	C	-
1a	124.5	C	-
2	99.4	CH	6.27, d (*J* = 1.9)
3	170.0	C	-
4	95.2	CH	6.47, d (*J* = 1.9)
4a	131.3	C	-
5	148.8	C	-
5a	141.1	C	-
6	117.2	CH	7.47, dd (*J* = 8.0, 1.3)
7	124.7	CH	7.33, t (*J* = 8.0)
8	117.3	CH	7.74, dd (*J* = 8.0, 1.3)
8a	110.8	C	-
9	188.7	C	-
OH-1	-	-	12.91, s
MeO-3	56.1	CH_3	3.83, s

6.1.3.10: 3, 4-Dihydroxy-2-methoxyxanthone (120)

Whitish Yellow amorphous solid (**120**) was purified from fraction 18 of the roots of *H. oblongifolium* by preparative TLC using Methanol: chloroform (7:93) as eluting system (Section 7.2.2.2). The UV spectrum of **120** showed the absorption peaks at 250, 290 and 368nm. The IR spectrum displayed bands at 3339, 1726 and 1605 cm^{-1} indicating the presence of OH, conjugated carbonyl and aromatic ring respectively[158]. The molecular formula was determined as $C_{14}H_{10}O_5$ by its LR-EI-MS giving molecular ion peak $[M+H]^+$ at *m/z* 259.

The ^{I}H and ^{I3}C NMR spectra of **120** (Table-6.16) showed closed resemblances with class of xanthone peaks [85,153]. The ^{I}H NMR, gives signal of five aromatic protons

at δ 8.22, dd (*J* = 8.3, 1.8 Hz, H-8), 7.40, dt (*J* = 8.3, 1.2 Hz, H-7), 7.76, dt (*J* = 8.3, 1.8 Hz, H-6), 7.55, dd (*J* = 8.3, 1.8 Hz, H-5) and 7.22, s (H-1). On the basis of the interpretation of its ^{I3}C and DEPT NMR spectral data (Table 6.16), compound **120** contained 14 carbons, including one methoxyl, six CH and seven quaternary carbons. The downfield signal at δ 175.1 was assigned to C-9, the conjugated carbonyl of xanthone skeleton. The peaks at δ 113.58 and 121.4 were assigned to the quaternary aromatic carbons C-1a and C-8a while δ 156.1, 145.8, 142.3 and 141.3 were attributed to the aromatic quaternary carbon attached to oxygen functionalities. The peak appeared at δ 55.6 was assigned to methoxyl attached at C-2. The position of methoxy group (MeO-2) was confirmed by NOE experiment, on irradiation it showed cross peak with 7.22, s (H-1). Various fragments ions *z/m* for the loss of OH, H_2O and CHO were also found in EI-MS. These assignments were confirmed by advance 2D-NMR techniques (HMBC, HMQC, COSY and NOE). The structure of **120** was confirmed as 3, 4-Dihydroxy-2-metoxyxanthone by spectroscopic data and with help of related literature. It had been reported in 1966 [160] and structure was confirmed by chemical transformation. After then it has been cited by a couple of authors [161,162], but has never been published its ^{13}C and ^{1}H NMR data. It is presented here with revised and additional data analyzed on latest techniques, reported here for the first time, from *H.oblongifolium* .



120

Table-6.16: ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) Spectral Data of Compound **(120)** in $(CD_3)_2CO$

C.No.	^{13}C NMR(δ)	Multiplicity (DEPT)	^{1}H NMR(δ) Coupling Constants J_{HH} (Hz)
1	96.29	CH	7.22, s
1a	113.8	C	-
2	145.8	C	-
3	142.5	C	-
4	141.0	C	-
4a	133.8	C	-
5	117.9	CH	7.55, dd (*J* = 8.3, 1.8)
5a	156.0	C	-
6	134.1	CH	7.76, dt (*J* = 8.3, 1.8)
7	123.7	CH	7.40, dt (*J* = 8.3, 1.2)
8	126.1	CH	8.22, dd (*J* = 8.3, 1.8)
8a	121.4	C	-
9	175.1	C	-
2-CH_3O	55.7	CH_3	3.93, s

6.1.4: Known Xanthones from the Roots of *Hypericum oblongifolium*

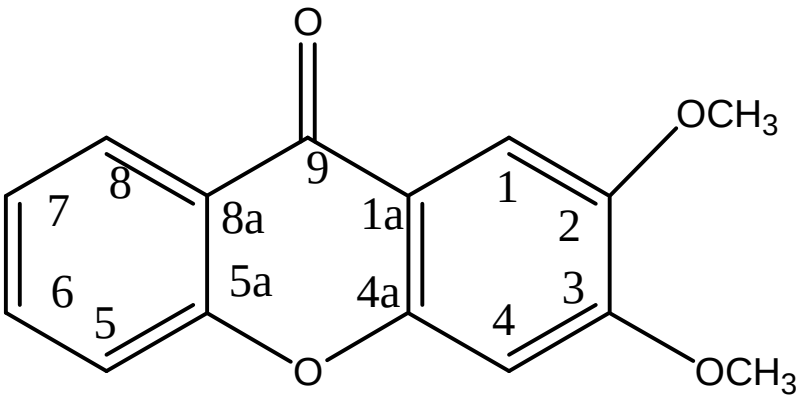
6.1.4.1: 2, 3-Dimethoxyxanthone (121)

Fraction 5 from the roots of *H. oblongifolium* was subjected to column chromatography eluted with hexane: chloroform in increasing order of polarity started at 80:20 and yield thee sub fractions (5.1-5.3), which were further purified by preparative TLC using chloroform as eluting solvent (Section 7.2.2.2) and purified **121** (4 mg) and **123** (3mg). A positive EI-MS of **121** showed an $[M+1]^+$ peak at *m*/*z* 257, corresponding to the molecular formula $C_{15}H_{12}O_4$. Inspection of the IR, ^{1}H and ^{13}C NMR spectra of **121 (**Table 6.17**)** showed it to possess a carbonyl carbon giving peaks at δ 176.2 *s*; 1642 cm^{-1}(C═O stretch), an aromatic OH group (3599 cm^{-1}), two methoxy groups (δ 4.0 *s* and 4.05, *s* each 3H) and five aromatic proton signals

suggested the Xanthone skeleton. A correlation in the HMBC spectrum between C-9 carbonyl resonance and a 1H doublet at δ 8.38 (J = 7.5 Hz) established this as H-8, with a correlation in the HMQC spectrum then permitting the assignment of a 1H td, at δ 7.36 (J = 7.5 Hz) to H-7. This signal, in turn, displays a correlation in the HMBC spectrum to 1H td, at δ 7.71 (J = 8.4 Hz) to H-6 whereas the methoxy groups were established as 3-OCH_3 and 2-OCH_3, respectively, by stepwise correlation. The two methoxyl in **121** were thus attached to the second aromatic ring at adjacent carbon (2 & 3) which was also confirmed by HMBC spectrum. The compound **121** is thus the 2, 3-dimethoxy-9H-xanthen-9-one, reported here, for the first time, from *Hypericum oblongifolium.* Its ^{1}H NMR had been reported in 1979 [163] in literature and also cited by a number of authors but according to our knowledge none of them had published ^{13}C NMR data. Here in, we report the same structure with additional data analyzed by modern spectroscopic techniques.

Table-6.17: ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) Spectral Data of Compound **(121)** in $CDCl_3$

C.No.	^{13}C NMR(δ)	Multiplicity (DEPT)	^{1}H NMR(δ) Coupling Constants J_{HH} (Hz)
1	105.5	CH	7.68, s
1a	115.0	C	-
2	146.8	C	-
3	152.5	C	-
4	97.5	CH	6.9, s
4a	155.5	C	-
5	117.7	CH	7.46, d (J=8.4)
5a	156.2	C	-
6	134.1	CH	7.71, t (J =8.4)
7	123.8	CH	7.36, t (J =7.5)
8	126.8	CH	8.36, d (J =7.5)
8a	121.6	C	-
9	176.2	C	-
CH_3O -2	56.6	CH_3	4.0, s
$\underline{CH_3}$ O-3	56.4	CH_3	4.0, s



121

6.1.4.2: 3, 5-Dihydroxy-1-methoxyxanthone (122)

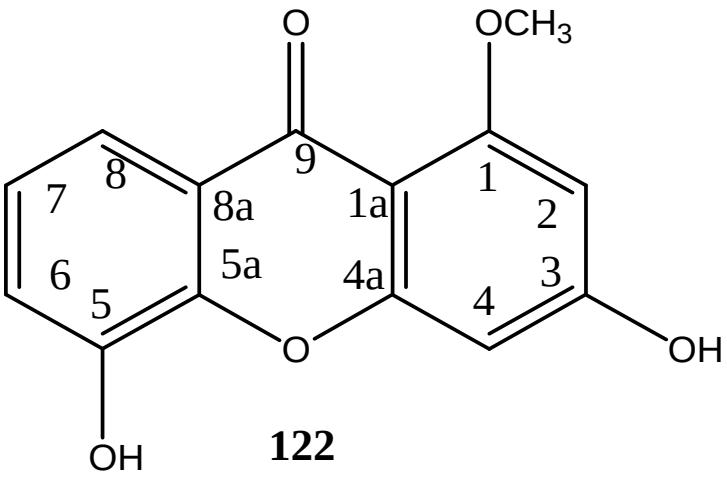
Compound **122** (6 mg) was purified from fraction 20 of the roots of *H. oblongifolium* as white amorphous powder by preparative TLC (See section 7.2.2.2). The molecular formula of **122** was determined as $C_{14}H_{10}O_5$ by its EI-MS giving molecular ion peak $[M-1]^-$ at 257. The IR spectrum displayed bands at 3433, 1655 and 1605 cm^{-1} indicating the presence of OH, conjugated carbonyl and aromatic ring respectively[129].

The ^{I}H and $^{1\ I3}C$ NMR spectra of **122** (Table 6.18) were much close to that of **120**. The ^{I}H NMR signals appeared at δ 6.23, d (J =2 .3) and 6.36 d (J = 2.3) were assigned to *meta* coupled aromatic proton at H-2 and H-4 respectively. The peak at δ 7.10 m suggesting an ABC system associated with H-6, H-7 and H-8 respectively. The singlet at δ 3.88 was assigned to MeOAr. The position of methoxy (C-1) was confirmed by NOE experiment, while irradiating at δ 3.88, it showed cross peak with δ 6.23 (H-2). The ^{I3}C and DEPT NMR spectral data (Table 6.14), compound **122** also showed 14 carbons resonance, including one methoxy, five CH and eight quaternary carbons as observed in **121**. The downfield signal at δ 175.7 was due to C-9, the conjugated carbonyl of xanthone skeleton. The peaks at δ 103.5 and 121.5 were assigned to the quaternary aromatic carbons C-1a and C-8a. The peak appeared at δ 56.1 was assigned to methoxy attached at C-1. Beside from molecular ion peak,

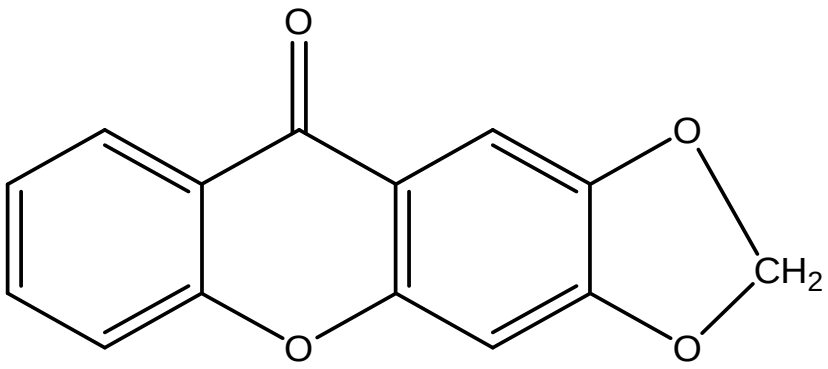
significant fragment ion peaks for the loss of OH, H_2O and CHO were also observed in Mass spectrum. These assignments were confirmed by advance 2D-NMR techniques (HMBC, HMQC and COSY). The structure was also established with help of modern spectroscopic techniques as 3,5-Dihydroxy-1-methoxyxanthone and being presented with additional study to already published data [164].

Table-6.18: ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) Spectral Data of Compound **(122)** in CD_3OD

C.No.	^{13}C NMR(δ)	Multiplicity(DEPT)	^{1}H NMR(δ) Coupling Constants J_{HH} (Hz)
1	162.1	Cx	-
1a	103.4	C	-
2	97.5	C ^{I3}C	6.22, d (J = 2.3)
3	162.3	C	-
4	96.6	CH	6.36, d (J = 2.3)
4a	160.1	C	-
5	146.2	C	-
5a	144.6	C	-
6	118.9	CH	7.10, m
7	122.8	CH	7.10, m
8	115.2	CH	7.58, dd (J = 7.0,2.3)
8a	123.4	C	-
9	175.2	C	-
CH_3O -1	61.1	CH_3	3.88, s



122



123

6.1.4.3: 2, 3-Methylenedioxyxanthone (123)

As mentioned earlier, compound **123** was isolated as white crystalline solid from sub fraction 5.3 (See section 7.2.2.2). A positive EI-MS of **123** showed an $[M+1]^+$and $[M+Na]^+$ peaks at *m/z* 241 and 263 respectively, corresponding to the molecular formula $C_{13}H_8O_4$. The IR, ^1H and ^{13}C NMR spectra of **123 (**Table 6.19**)**

showed close resemblance to the spectral data of **121**, only difference was the disappearance of two singlets around δ 4.0 in ^{1}H NMR and around δ 60.0 in ^{13}C NMR. The appearance of peak at δ 6.11 (2H, s) in ^{1}H NMR and its corresponding resonance at δ 102 in ^{13}C NMR was indicated that the two adjacent methoxyl (C2 & C3) groups were condensed forming 2,3-methylene dioxide ring by the loss of one methyl group. The position and orientation of 2,3- methlene dioxide was confirmed by the appearance of two singlets at δ 6.9 and δ 7.5 attributable to C-4 and C-1 respectively. The rest of splitting in ^{1}H and ^{3}C NMR was almost closed to that already discussed in **121**. The physical and spectral data of **123** showed complete agreement with reported compound, 2, 3-Methylenedioxyxanthone [165].

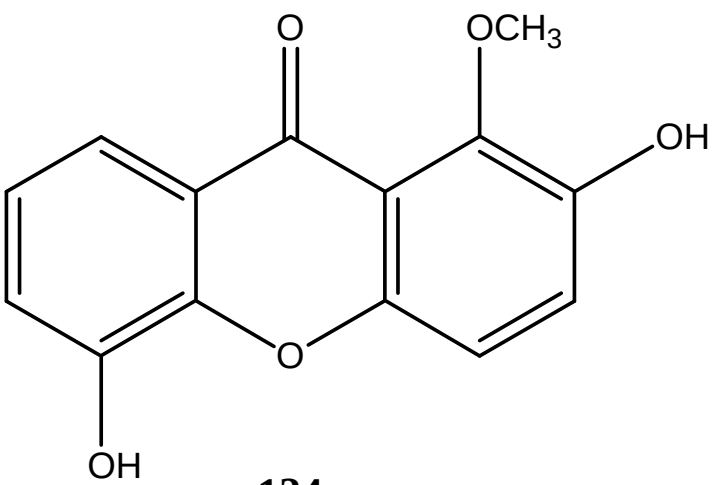
Table-6.19: ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) Spectral Data of Compound **(123)** in $CDCl_3$

C.No.	^{13}C NMR (δ)	Multiplicity (DEPT)	^{1}H NMR(δ) Coupling Constants J_{HH} (Hz)
1	103.3	CH	7.65, s
1a	116.5	C	-
2	145.4	C	-
3	153.5	C	-
4	98.0	CH	6.9, s
4a	153.8	C	-
5	117.7	CH	7.46, d (J = 8.4)
5a	156.1	C	-
6	134.1	CH	7.69, t (J = 8.4)
7	124.0	CH	7.37, t (J = 7.4)
8	126.6	CH	8.30, d (J = 7.5)
8a	121.6	C	-
9	176.0	C	-
OCH_2O	102.0	CH_2	6.11, s

6.1.4.5: 3, 5-Dihydroxy-1-methoxyxanthone (124)

Sub fraction 15.3 from the roots of *H. oblongifolium* was purified on preparative TLC to yield a yellowish white amorphous solid (**124)**. The molecular formula of **124** was determined as $C_{14}H_{10}O_5$ by its EI-MS giving molecular ion peak $[M-1]^-$ at *m/z* 257. The IR spectrum displayed bands at 3153, 1655 and 1600 cm^{-1} indicating the presence of OH, conjugated carbonyl and aromatic ring respectively [129]

The ^{I}H NMR signals appeared at δ 7.28, d (*J* = 9.1) and 7.38 d (*J* = 9.1) were assigned to the ortho coupled proton at H-2 and H-4 respectively. The peak at δ 7.19, t (*J* = 7.9), 7.24, dd (*J* = 7.9, 1.6) and 7.24, dd (*J* = 7.9, 1.6), suggesting an ABC system associated with H-7, H-6 and H-8 respectively. The singlet at δ 3.91 was assigned to MeOAr. The position of methoxy (C-1) was confirmed by NOE experiment, while irradiating at δ 3.91, did not show any cross peak. The ^{I3}C and DEPT NMR spectral data (Table 6.14), compound **124** also showed 14 carbons resonance, including one methoxy, five CH and eight quaternary carbons as observed in **121**. The downfield signal at δ 175.2 was due to C-9, the conjugated carbonyl of xanthone skeleton. The peaks at δ 113.5 and 116.5 were assigned to the quaternary aromatic carbons C-1a and C-8a. The peak appeared at δ 61.1 was assigned to methoxy attached at C-1. Beside from molecular ion peak, significant fragment ion peaks for the loss of OH, H2O and CHO were also observed in Mass spectrum. These assignments were confirmed by advance 2D-NMR techniques (HMBC, HMQC and COSY). The ^{1}H and ^{13}C NMR spectral data is given in table 6.20. The structure of **124** was established with help of modern spectroscopic techniques as 2,5-Dihydroxy-1-methoxyxanthone, already reported [166].



124

Table 6.20: ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) Spectral Data of Compound **(124)** in $(CD_3)_2CO$

C.No.	^{13}C NMR (δ)	Multiplicity (DEPT)	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)
1	145.1	CH	-
1a	113.4	C	-
2	146.	C	-
3	123.8	CH	7.38, d (J = 9.1)
4	99.8	CH	7.28, d (J = 9.1)
4a	149.8	C	-
5	146.2	C	-
5a	145.2	C	-
6	119.2	CH	7.24, dd (J = 1.6, 7.8)
7	123.1	CH	7.19, t (J = 1.5, 7.8)
8	116.4	CH	7.66, dd (J = 1.6, 7.8)
8a	121.4	C	-
9	175.2	C	-
CH_3O	61.1	CH_3	3.91, s

Six other xanthones **(124 A –F)** have also been isolated from the roots of *H. oblongifolium* and they were identified as

124 A: 4, Hydroxy-2,3-dimethoxyxanthone **(112)**

124 B: 3,4,5-Trihydroxy xanthone **(113)**

124 C: 3-Hydroxy-2-methoxyxanthone **(114)**

124 D: 1,3,7-Trihydroxyxanthone **(117)**

124 E: 1,7-Dihydroxyxanthone **(118)** and

124 F: 3,4-Dihydroxy-2-methoxyxanthone **(120)**.

All of them have already been discussed in section **(6.1.3)**

6.1.5: Other compounds from the aerial parts (Twigs) of *H.oblongifolium*

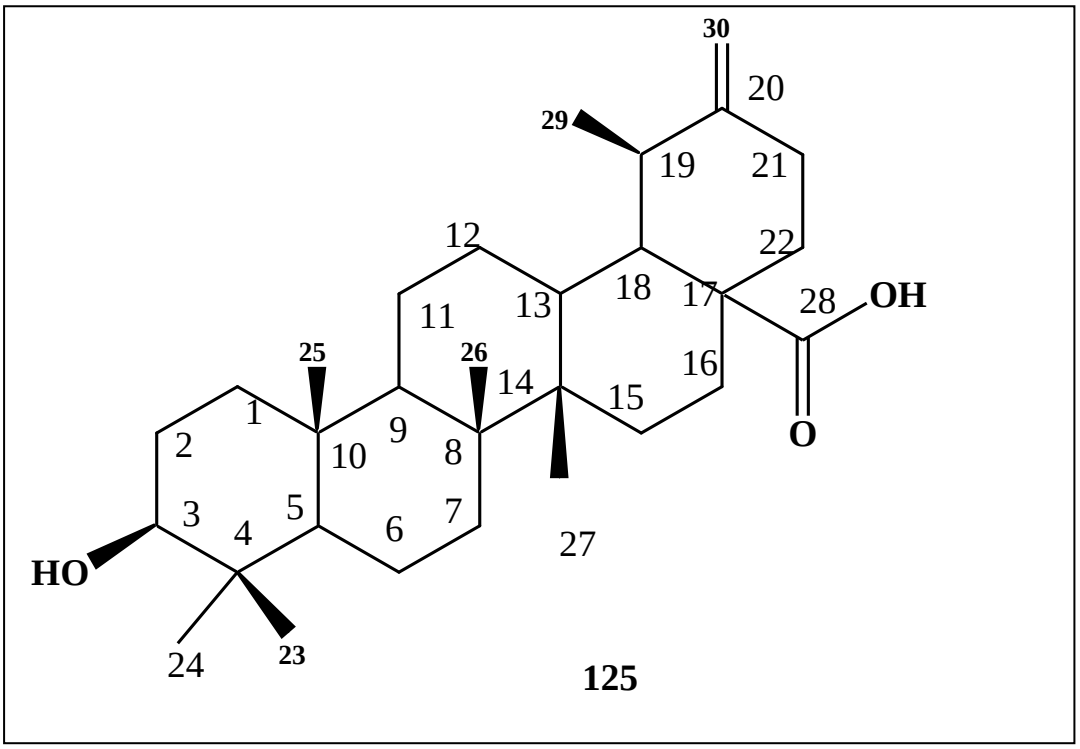
6.1.5.1: Zizyphursolic acid (125)

The ethyl acetate fraction (260g) was loaded on column chromatography over silica gel eluting with solvent in increasing order of polarity (*n*-hexane– ethyl acetate and ethyl acetate –MeOH) to afford 30 major fractions. The fraction 14 was applied to column chromatography over flash silica gel (Chloroform/hexane 10:90, 20:80, 30:70, and 40:60) and afforded **125** (32mg) along with other compounds (7.2.2.1).

Two singlets at δ 4.70 and 4.50 in the ^{1}H NMR were assigned to H-30 (exocyclic methylene) protons. A 1H broad signal at δ 3.0 (m) was ascribed to carbinol proton.H-3. A signal at δ 2.21, dd (J = 8.01, 11.21 Hz) was due to the H-18 proton, having association with C-13 and C-19 methine protons in the ursane-type carbon skeleton. The a singlets at δ 0.70, 0.82, 0.92, 0.96 and 0.97 were attributed to five methyls (CH_3-27, CH_3-24, CH_3-25, CH_3-26 and CH_3-23 respectively). The signal between δ 2.91 and 1.10 were attributable to the remaining methylene and methine protons (Table 6.21). The ^{13}C NMR spectrum of **125** (Table 6.21) disclosed the presence of 30 carbon atom[167]. The downfield signals at δ177.11, 150.21, and 109.51 were assigned to carboxylic (C-28) and olefinic carbons (C-20 and C-30) respectively. The peak appeared at δ 76.77 was assigned to carbinol carbon (C-3). A signal at δ 55.3 supported ursane-type carbon skeleton having 18 protons. These assignments were also confirmed by advance 2D-NMR techniques (HMBC, HMQC and COSY). The spectroscopic and physical data of compound **125** agreed with those reported in literature as zizyphursolic acid (18βH-urs-20 (30)-en-3β-ol-28-oic acid)[167].

Table-6.21: ^{1}H (300 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound **(125)** in $(CD_3)_2CO$

C.No.	^{13}C NMR (δ)	Multiplicity (DEPT)	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)
1	39.0	CH_2	2.3, m
2	26.5	CH_2	1.66, s
3	78.5	CH	3.0, m
4	45.7	C	-
5	56.4	CH	1.68, m
6	19.6	CH_2	1.39, m
7	35.4	CH_2	1.38, m
8	39.4	C	-
9	47.5	CH	1.56, m
10	41.4	C	-
11	21.5	CH_2	1.4, s
12	28.9	CH_2	1.4, s
13	39.4	CH	1.73, m
14	43.0	C	-
15	30.5	CH_2	1.42, m
16	31.3	CH_2	1.86, m
17	46.3	C	-
18	51.2	CH	2.22 (dd, *J* = 8.5, 11.2 Hz,)
19	50.8	CH	2.13 (d, *J* = 11.2 Hz)
20	151.9	C	-
21	32.3	CH_2	2.70, s
22	37.3	CH_2	2.68, s
23	28.5	CH_3	0.97, s
24	16.0	CH_3	0.82, s
25	16.5	CH_3	0.92, s
26	19.4	CH_3	0.96, s
27	15.5	CH_3	0.70, s
28	178.5	C	12.52 (1H, br s),
29	16.6	CH_3	1.18, m
30	109.9	CH_2	4.50, s 4.70, s

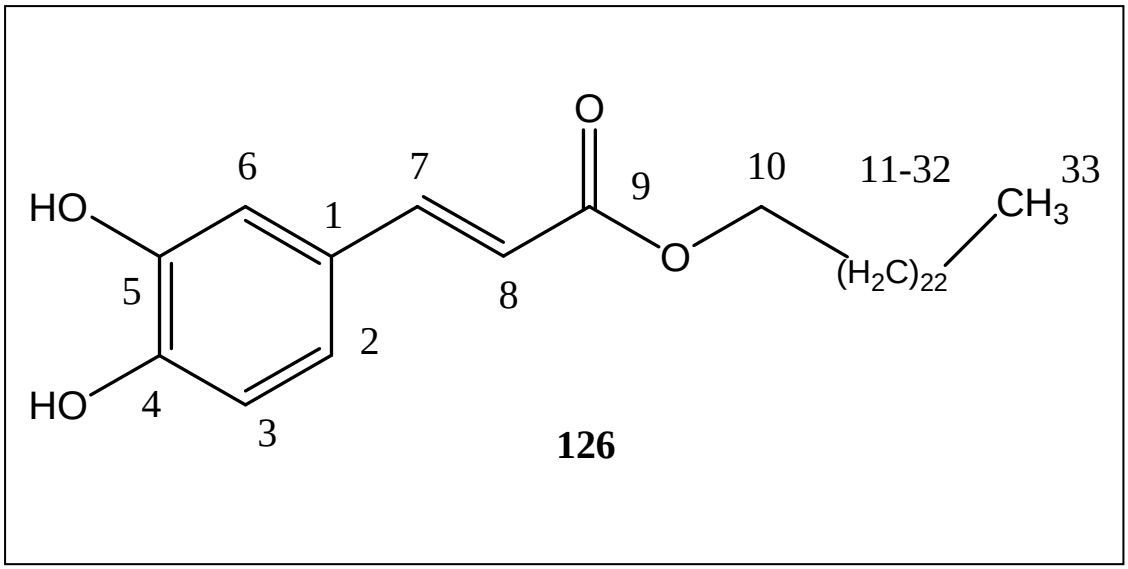


125

6.1.5.2: Tetracosyl 3-(3,4-dihydroxyphenyl) acrylate (126)

As described above the Fraction 14 was loaded to column chromatography over flash silica gel (Chloroform/hexane 10:90, 20:80, 30:70, and 40:60) and afforded **126** (22 mg) along with other compound (See section 7.2.2.1). The UV spectrum showed the presence of aromatic giving absorption peaks at 235nm [168]. The bands in IR spectrum at 3500,1700, 1670 and 1595 cm^{-1} indicating the presence of OH, conjugated carbonyl and aromatic ring respectively[168]. The molecular formula was established as $C_{33}H_{56}O_4$ by its LR-EI-MS giving molecular ion peak $[M+]^+$ at *m/z* 516.4. The ^{I}H NMR of **126** (Table 6.22), gives signal of three aromatic and two olefinic protons at δ 7.63 (1H, d, *J* = 15.03 Hz, H-7), 7.2 (1H, *s*, H-6), 7.1 (1H, d, *J* = 10.03 Hz, H-3), 6.98 (1H, d,*J* = 10.03 Hz, H-2), 6.26 (1H, d, *J* = 15.03 Hz, H-8). The singlet appeared at δ 0.97 was assigned to Me-33. On the basis of the interpretation of its ^{I3}C and DEPT NMR spectral data (Table 6.22), compound **126** contained 33 carbons, including one methyl, 23 CH_2, five CH and four quaternary carbons. The downfield signal at δ 168.9 was due to C-9, the conjugated carbonyl of ester. The peaks at δ 123.0 was assigned to the quaternary aromatic carbons C-1 while δ 148.3 and 146.8 were attributed to the aromatic quaternary carbon attached to oxygen functionalities. The peak appeared at δ 23.9 was assigned to methyl at C-25. These assignments were confirmed by advance 2D-NMR techniques (HMBC, HMQC and

COSY).The spectroscopic and physical data of compound **126** completely agreed with literature as Tetracosyl 3-(3,4-dihydroxyphenyl) acrylate [168].



126

Table-6.22: ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound **(126)** in $CDCl_3$

C.No.	^{13}C NMR (δ)	Multiplicity (DEPT)	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)
1	123.0	C	-
2	120.4	CH	6.98 (d,*J2,3* = 10.0)
3	118.6	CH	7.10 (d,*J2,3* = 10.0)
4	146.81	C	-
5	148.3	C	-
6	122.61	CH	7.2 (s)
7	147.4	CH	7.61 (d *J7,8* = 15.0)
8	119.5	CH	6.26 (d, *J8,7* = 15.0)
9	168.5	C	-
10	63.4	CH_2	4.1 (m)
11-32	24.3 -30	CH_2	1.18-1.30 (*m*) 1.00-1.10 (*m*)
25	23.9	CH_3	0.90-1.00 (*m*)

6.1.5.3: β-Sitosterol (127)

Compound **127** (51 mg) was isolated from the fraction (2-4) of the twigs of H. oblongifolium along with other compounds (See section 7.2.2.1). The IR spectrum of **127** displayed bands at 3408, 1628, 1379 and 1065 cm^{-1} presenting the presence of OH and unsaturation respectively[169]. The molecular formula was determined as $C_{29}H_{50}O$ by its LR-EI-MS giving molecular ion peak $[M+]^+$ at 414.

The ^1H NMR spectrum of **125** (Table 6.23) displayed multiplet at δ 5.35 which was assigned to H-6 exocyclic methylene protons. A 1H broad signal appeared at δ 3.51 (m) was due to carbinol proton (H-3). The six methyl substituents appeared at δ 1.01 (3H, s), 0.92 (3H, d, *J* = 6.81 Hz), 0.84 (3H, t, *J* = 6.91 Hz), 0.82 (3H, d, *J* = 6.51 Hz), 0.81 (3H, d, *J* = 6.51 Hz), 0.64 (3H, s, Me-18) were attributed to six methyls (CH_3-19, CH_3-21, CH_3-29, CH_3-26, CH_3-27 and CH_3-18) respectively. The remaining methylene and methine protons were appeared between δ 2.96 and 1.10 (Table 6.16). On the basis of the interpretation of its ^{I3}C and DEPT NMR spectral data (Table 6.18), compound **127** contained 29 carbons, including six methyl, 12 CH_2, eight CH and three quaternary carbons. The signal at δ 140.71 was assigned to C-5, the quaternary olefinic carbon. The peaks at δ 121.6 was assigned to the olefinic methine carbons C-6 while δ 36.50 and 42.3 were attributed to the aliphatic carbons (C-10 and C-13 respectively). The peaks appeared at δ 19.82 (C-18), 19.40 (C-21), 19.405 (C-27), 18.79 (C-26), 11.99 (C-29) and 11.82 (C-19) were assigned to methyl of the respective carbons. These assignments were confirmed by advance 2D-NMR techniques (HMBC,HMQC and COSY).The spectroscopic and physical data of compound **127** agreed with those reported in literature as β-Sitosterol[169]

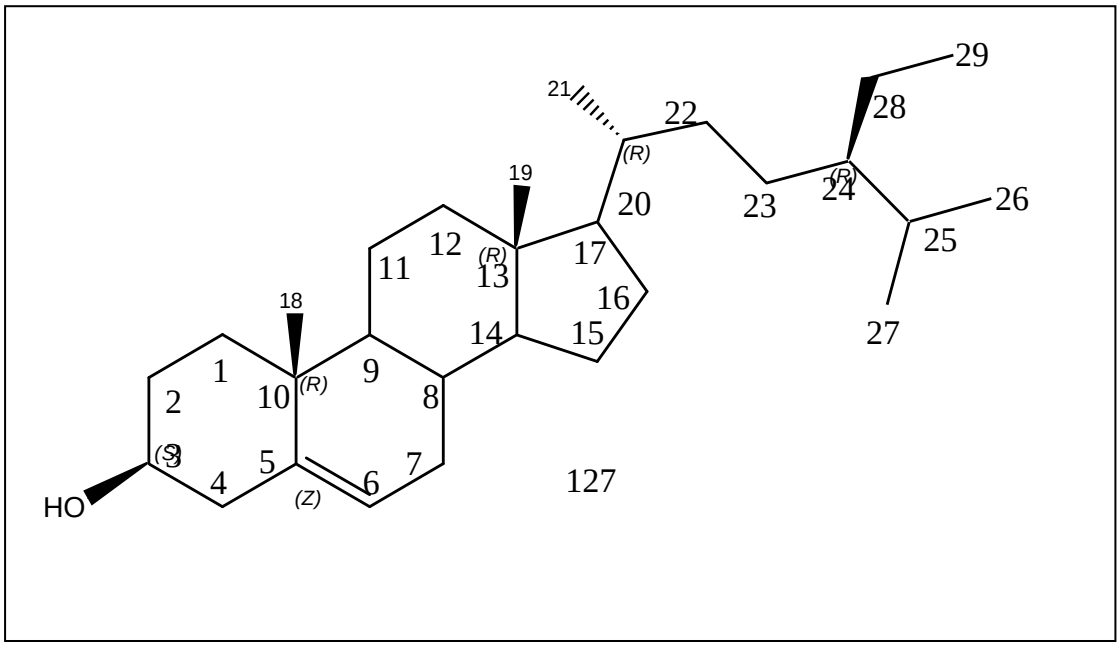


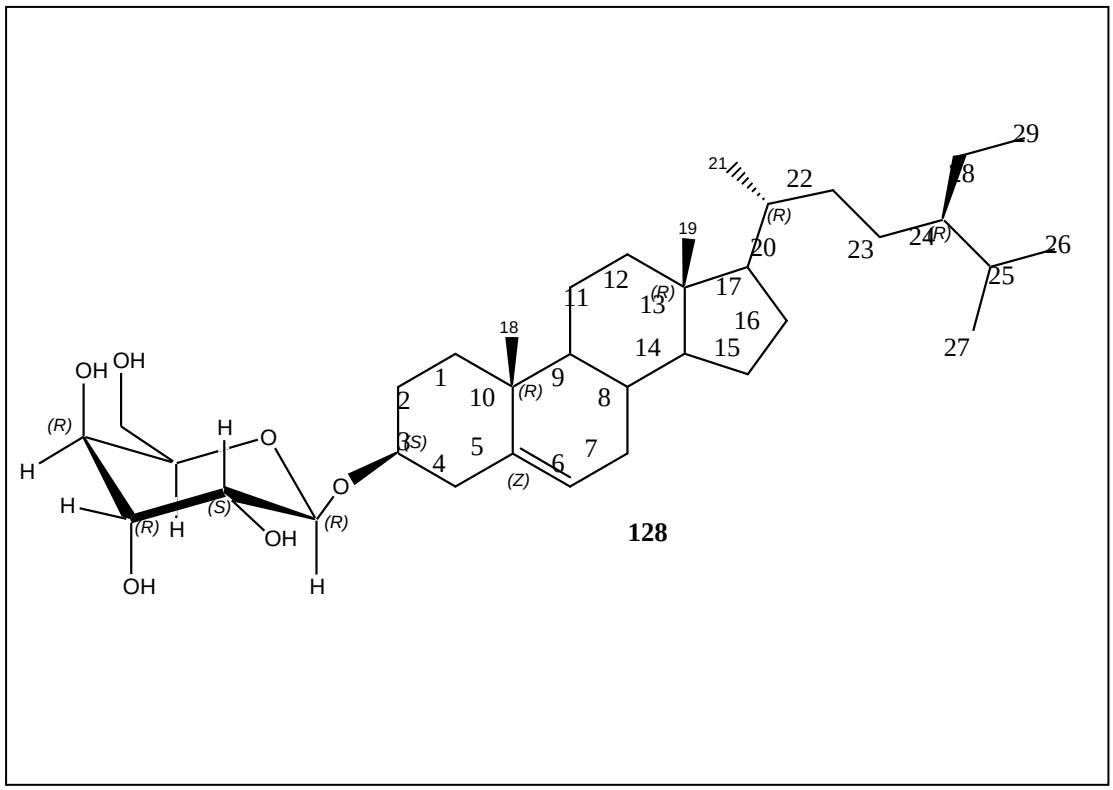
Table-6.23: ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of Compound **(127)** in $CDCl_3$

C.No.	^{13}C NMR (δ)	Multiplicity (DEPT)	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)
1	37.0	CH_2	1.20 (m)
2	29.70	CH_2	1.66 (s)
3	71.81	CH	3.51 (m)
4	42.3	CH_2	-
5	140.7	C	1.68 (m)
6	121.6	CH	5.35 (m)
7	31.70	CH_2	1.40 (m)
8	31.94	CH	-
9	50.16	CH	1.56 (m)
10	36.50	C	-
11	21.10	CH_2	1.40 (s)
12	39.8	CH_2	1.41 (s)
13	42.30	C	1.73 (m)
14	56.41	CH	-
15	24.30	CH_2	1.42 (m)
16	28.20	CH_2	1.86 (m)
17	37.20	C H_2	-
18	19.82	C H_3	0.64 (s)
19	11.87	C H_3	1.01 (m)
20	36.30	C H	-
21	19.40	C H_3	0.92, d (J = 6.81 Hz)
22	33.99	CH_2	1.68 (s)
23	26.12	C H_2	0.83 (s)
24	45.80	C H	0.82 (s)
25	29.10	CH	1.2 (m)
26	18.79	CH_3	0.82, d (J = 6.51 Hz)
27	19.05	CH_3	0.81, d (J= 6.51 Hz)
28	23.10	C H_2	1.34 (m)
29	11.99	CH_3	0.84, t (J= 6.91 Hz)

6.1.5.4: β-Sitosterol3-O-β-D-glucopyranoside (128)

Fraction 21 from the twigs of *H. oblongifolium* was loaded on flash silica gel CC (methanol/ Chloroform 1:99, 2:98) to afford **128** (51mg) and **129** (28 mg). The IR spectrum of **128** displayed bands at 3452, 1648, 1379 and 1065 cm^{-1} indicted the presence of OH and unsaturation respectively[169]. The molecular formula was determined as $C_{29}H_{50}O$ by its EI-MS giving molecular ion peak $[M+]^+$ at *m/z* 414 (as glycosides disappear in EI-MS).

The ^1H and ^{13}C NMR spectrum of **128** (Table 6.24) were found much closed with **127**. The main difference was the glycosides peak appeared in the spectra of 123. In ^1H NMR spectra the additional peaks of sugar moiety were found as δ 4.57 (3H, d, *J* = 7.51 Hz, H-1′), 3.85 (1H, dd, *J* = 11.81, 2.41 Hz, Ha-6′), 3.68 (1H, dd, *J* =11.87, 5.71 Hz, Hb-6′) and 3.24-3.45 (5H, m, Glc-H). While in ^{13}C NMR the peak of numeric carbon of sugar moiety was appeared at 101.1 (C-1′). The signals at 77.0, 76.6, 74.0, 70.7 and 62.1 were due to the C-5′, C-3′, C-4′, 2′, C-6′ other carbons of sugar moiety respectively. The spectroscopic and physical data of compound **128** agreed with those reported in literature as β-Sitosterol glycoside[169].



128

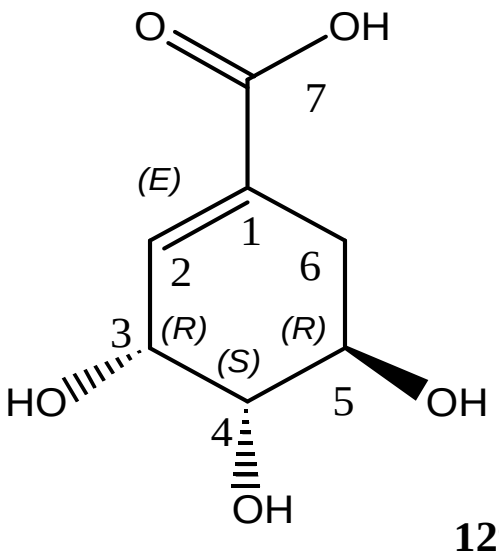
Table-6.24: ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) Spectral Data of Compound **(128)** in DMSO

C.No.	^{13}C NMR (δ)	Multiplicity (DEPT)	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)
1	37.0	CH_2	1.21 (m)
2	29.70	CH_2	1.66 (s)
3	71.81	CH	3.51 (m)
4	42.3	CH_2	-
5	140.7	C	1.68 (m)
6	121.6	CH	5.35 (m)
7	31.70	CH_2	1.40 (m)
8	31.94	CH	-
9	50.16	CH	1.56 (m)
10	36.50	C	-
11	21.10	CH_2	1.41 (s)
12	39.8	CH_2	1.41 (s)
13	42.30	C	1.73 (m)
14	56.41	CH	-
15	24.30	CH_2	1.42 (m)
16	28.20	CH_2	1.86 (m)
17	37.20	C H_2	-
18	19.82	C H_3	0.64 (s)
19	11.87	C H_3	1.01 (m)
20	36.30	C H	-
21	19.40	C H_3	0.92, d (J = 6.18 Hz)
22	33.99	CH_2	1.68 (s)
23	26.12	C H_2	0.83 (s)
24	45.80	C H	0.82 (s)
25	29.10	CH	1.22 (m)
26	18.79	CH_3	0.82, d (J = 6.51 Hz)
27	19.05	CH_3	0.81, d (J = 6.51 Hz)
28	23.10	C H_2	1.34 (m)
29	11.99	CH_3	0.84, t (J = 6.91 Hz)
$1^{/}$	101.1	C H	4.57, d (J = 7.51 Hz)
$2^{/}$	70.7	C H	3.14-3.20 (m)
$3^{/}$	76.6	C H	3.21-3.25 (m)
$4^{/}$	74.0	C H	3.24-3.28 (m)
$5^{/}$	77.0	C H	3.34-3.38 (m)
$6^{/}$	62.1	C H_2	3.85, dd (J = 11.8, 2.4 Hz) 3.68, dd (J = 11.8, 5.4 Hz)

\6.1.5.5: Shikimic acid (124)

The UV spectrum of **129** giving absorption peaks at 230nm[170]. The IR spectrum displayed bands at 3250-27000, 2940, 1705, 1610, 1595cm^{-1} indicating the presence of carboxylic OH, conjugated carbonyl and olefinic bond respectively[170]. The molecular formula was determined as $C_7H_{10}O_5$ by its EI-MS giving molecular ion peak $[M+]^+$ at *m/z* 174.

The ^{I}H NMR of **129** (See experimental section), gives signal of three carbinol protons at δ 5.67 (1H, *s*, H-3), 4.08 (1H, *m*, H-5), 3.96 (1H, dd, *J* = 6.52, 4.02 Hz, H-4) and two olefinic at δ 6.73 (1H, *s*, H-2), 5.69 (1H, *s*, H-3). The interpretation of its ^{I3}C and DEPT NMR spectral data (given in experimental section), compound **129** contained 7 carbons, including one CH2, four CH and two quaternary carbons. The signal at δ 168.53 was attributed to carboxylic carbon C-7. The peaks at δ 134.5 (C-2), and 127.4 (C-1) were assigned to the olefinic carbons. While δ 70.97 (C-4), 70.71 (C-5) and 68.71 (C-3) were attributed to the carbinol carbon attached to oxygen functionalities. These assignments were confirmed by advance 2D-NMR techniques (HMBC, HMQC and COSY).The spectroscopic and physical data of compound **129** agreed with those reported in literature as Shikimic Acid[170].

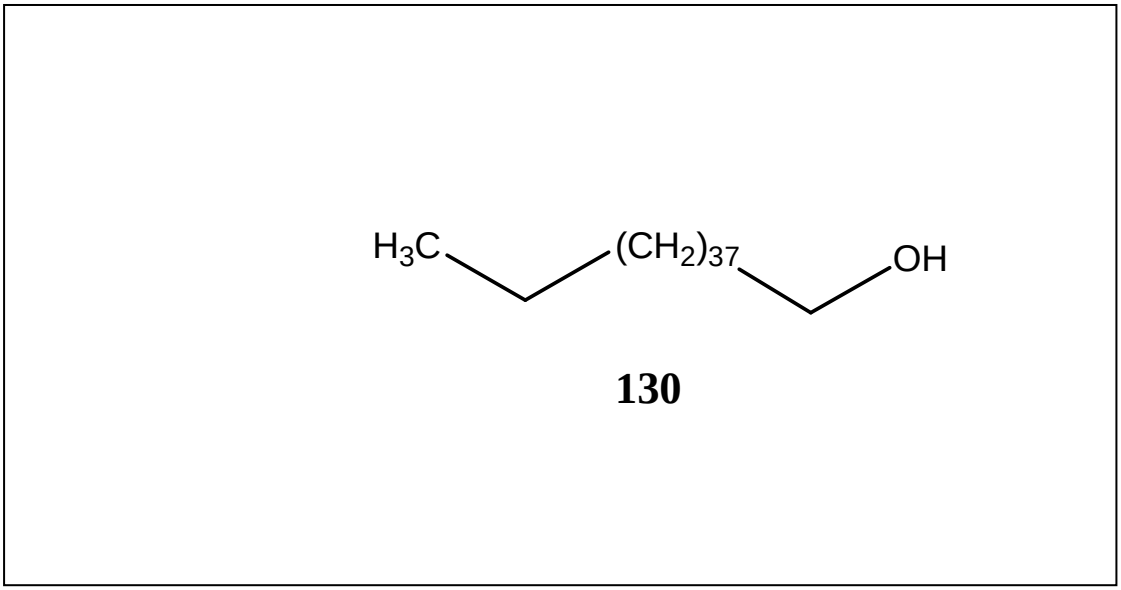


129

6.1.5.6: 1-Octatriacontanol (130)

Fraction 2-4 were combined and applied to column chromatography over flash silica gel (Chloroform/hexane 10:90, 20:80, 30:70), led to the isolation of compound **130** (15mg) with other compounds (See section 7.2.2.1). Compound **130** was isolated as white amorphous powder with m.p 63-66C^0. The IR spectrum displayed bands at 3461, 2928, 2843, 1740 cm^{-1} sowing the presence of OH and carbonyl functionality respectively[171]. The molecular formula was determined as $C_{38}H_{78}O$ by its LR-EI-MS giving molecular ion peak $[M+H]^+$ at *m/z* 551.

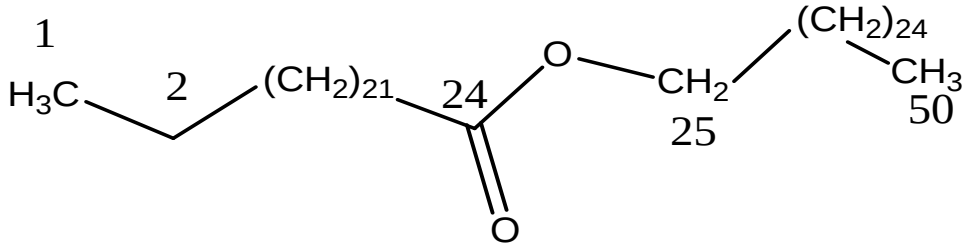
The ^{1}H NMR of **130** (See experimental section), gives signal at δ 3.62 assigned to CH_2 that attached to the OH group. A broad singlet at δ 1.23-1.32 was assigned as (68H, brs, CH_2-3-36). The protons of terminal methyl was appeared at δ 0.842 (3H, t, *J* = 6.91 Hz, CH_3-38). On the basis of the interpretation of its ^{13}C and DEPT NMR spectral data (given in experimental section), compound **130** contained 38 carbons, including one CH_3 and 37 CH_2. The signal at δ 62.52 was assigned to the carbinol carbon (C-1). The peak at δ 14.05 was assigned to the terminal methyl (C-38). The signals at δ 29.3-29.6 (32x CH_2-4-35) were assigned to a bunch of CH_2. The spectroscopic and physical data of compound **130** agreed with those reported in literature as 1-Octatriacontanol[171].



130

6.1.5.7: Hexacosyl tetracosanoate (131)

Compound **131** was isolated as white solid with having 79-81^{0}C from the the combined fractions (2-4). The bands in the IR spectrum at 2928, 2843, 1740cm^{-1} indicating the presence of carbonyl of ester functionality[172]. The molecular formula was established as $C_{50}H_{100}O_2$ by its LR-EI-MS giving molecular ion peak $[M+H]^+$ at 733. The ^{I}H NMR of **131** (See experimental section), gives signal at δ 4.02 (2H, t, *J* = *7.22* Hz, CH_2-25) was assigned to CH_2 that attached to the OCOR group while peak at 2.26,t (2H, t, *J* = *7.12* Hz, CH_2-23) to CH_2 next to COR. Abroad singlet at δ 1.23-1.34 was assigned as (86H,br s, CH_2-2-21,27-49). The protons of terminal methyls was appeared at δ 0.842 (6H, t, *J*= *6.93* Hz, CH_3-1, 51). The interpretation of its ^{I3}C and DEPT NMR spectral data (Given in experimental section), compound **131** contained 50carbons, including two CH_3, 47 CH_2 and one quaternary carbon. The signal at δ 173.5 was assigned to carbonyl carbon (C-24). The peak at δ 14.08 was assigned to the terminal methyls (C-, 50). The signala at δ 29.3-29.6 (41x CH_2-3-21, 27-48), were assigned to a bunch of CH_2. The spectroscopic and physical data of compound **131** agree with those reported in literature as Hexacosyl tetracosanoate[172]



131

6.1.6: Other compounds from the Roots of *H.oblongifolium*

6.1.6.1: Methyl betulinate -3-acetate (132)

A white crystalline compound **(132)**, soluble in chloroform having, M.P 262-264 ^{o}C was isolated from fraction 4 of the roots of *H.oblongifolium* through column chromatography using silica gel. The molecular formula was found $C_{32}H_{50}O_4$, showing molecular ion peak at 497 $[M-1]^-$. The ^{1}H NMR spectrum of **132** displayed two singlets at δ *m/z* 4.72 and 4.59 which are assigned to H_2-30 exocyclic methylene protons. A 1H broad signal at δ 4.47, dd (*J* = 10.2, 5.5 Hz) was ascribed to proton (H-3). A signal at d 3.0, td (*J* = 10.2, 5.5 Hz) was due to the H-19 proton. The five methyl substituent appeared as singlets at δ 0.81, 0.82, 0.93, 0.91 and 0.95 were attributed to CH_3-25, CH_3-23, CH_3-27, CH_3-24 and CH_3-26 methyl protons respectively and a three protons broad singlet at d 1.68 accounted to C-29 methyl protons, attached to saturated carbons. The remaining methylene and methine protons were appeared in between δ 2.96 and 1.10 (Table 6.25).

The ^{13}C NMR spectrum of **132** (Table 6.25) showed the presence of 30 carbon atom. The assignments of the chemical shifts were made by comparison with the values of the corresponding carbon atoms of ursane-type triterpenes[167]. The downfield signals at δ182.4, 150.5 and 109.8 were assigned to C-28 carboxylic and olefinic carbons (C-20 and C-30) respectively. The peak appeared at δ 81.0 was assigned to C-3 carbon. Methyl at δ 2.03, s shows cross peak with 171.8 (CH_2*CO*CH_3) confirmed the presence of acetyl group. These assignments were also confirmed by advance 2D-NMR techniques (HMBC, HMQC and COSY). The ^{1}H NMR and physical data of compound **132** agreed with those reported in literature as Methyl betulinate -3-Acetate [173]. Here in we report ^{13}C NMR data and other additional data which has not been published before.

6.1.6.2: Betulinic acid (133)

The molecular formula of **133** was established as $C_{30}H_{48}O_3$ showing molecular ion peak at 455 [M-1] EI-MS. The ^{1}H NMR spectrum of **133** showed complete resemblance with that of **132.** It also displayed two singlets at δ 4.7 and 4.8 which were assigned to H_2-30 exocyclic methylene protons.

Table-6.25: ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) Spectral Data of Compound **(132)** in $CDCl_3$

C.No	^{13}C NMR (δ)	Multiplicity (DEPT)	^{1}H NMR (δ) Coupling Constants J_{HH} (Hz)
1	34.2	CH_2	1.27, m, 1.40, m
2	23.8	CH_2	1.60, m, 1.93, m
3	81.0	CH	4.47, dd (*J* = 10.2, 5.5)
4	37.23	C	-
5	50.4	CH	1.28, m
6	18.2	CH_2	1.41, m
7	37.17	CH_2	1.48, m, 1.92, m
8	40.7	C	-
9	55.5	CH	0.76, m
10	37.9	C	-
11	20.9	CH_2	1.42, m
12	25.5	CH_2	1.26, m
13	38.5	CH	2.16, dt (*J* =11.9, 3.4)
14	42.25	C	-
15	29.8	CH_2	1.04, m, 1.60, m
16	32.2	CH_2	1.32, m, 2.27,td (*J* = 13.08, 3.5)
17	56.5	C	-
18	49.0	CH	1.67, m
19	47.3	CH	3.0, dt (*J* = 10.4, 5.5)
20	150.5	C	-
21	30.67	CH_2	1.36, m, 1.93, m
22	38.4	CH_2	1.41, m,1.95, m
23	28.06	CH_3	0.82, s
24	16.3	CH_3	0.91, s
25	16.5	CH_3	0.81, s
26	14.7	CH_3	0.95, s
27	16.1	CH_3	0.83, s
28	182.4	C	-
29	19.4	CH_3	1.68, s
30	109.8	CH_2	4.72, d (*J* = 1.6), 4.59 br s
CH_3 COO	171.2	C	-
CH_3 COO	21.4	CH_3	2.03, s

A 1H broad signal at δ 3.4, m was assigned to proton, H-3. The only difference was the missing of singlet at 2.03 in ^{1}H NMR for the methyl group of the acetyl group and peak at 171.2 in ^{13}C NMR spectrum for acetyl assignable to acetyl quaternary carbon as observed in of **132.** The ^{13}C NMR spectrum also showed the presence of 30 carbon atoms. These assignments were also confirmed by advance 2D-NMR techniques (HMBC, HMQC and COSY) The assignments of the carbon chemical shifts were made by comparison with the values of the corresponding carbon

atoms of ursane-type triterpenes [167]. The downfield signals at δ178.4, 150.5, and 109.8 were assigned to C-28 carboxylic and C-20, C-30, olefinic carbons, respectively. Table 6.26represents the ^{1}H and ^{13}C NMR data of the **133.** The ^{1}H NMR and physical data of compound **133** agreed with those reported in literature as betulinic acid [173]

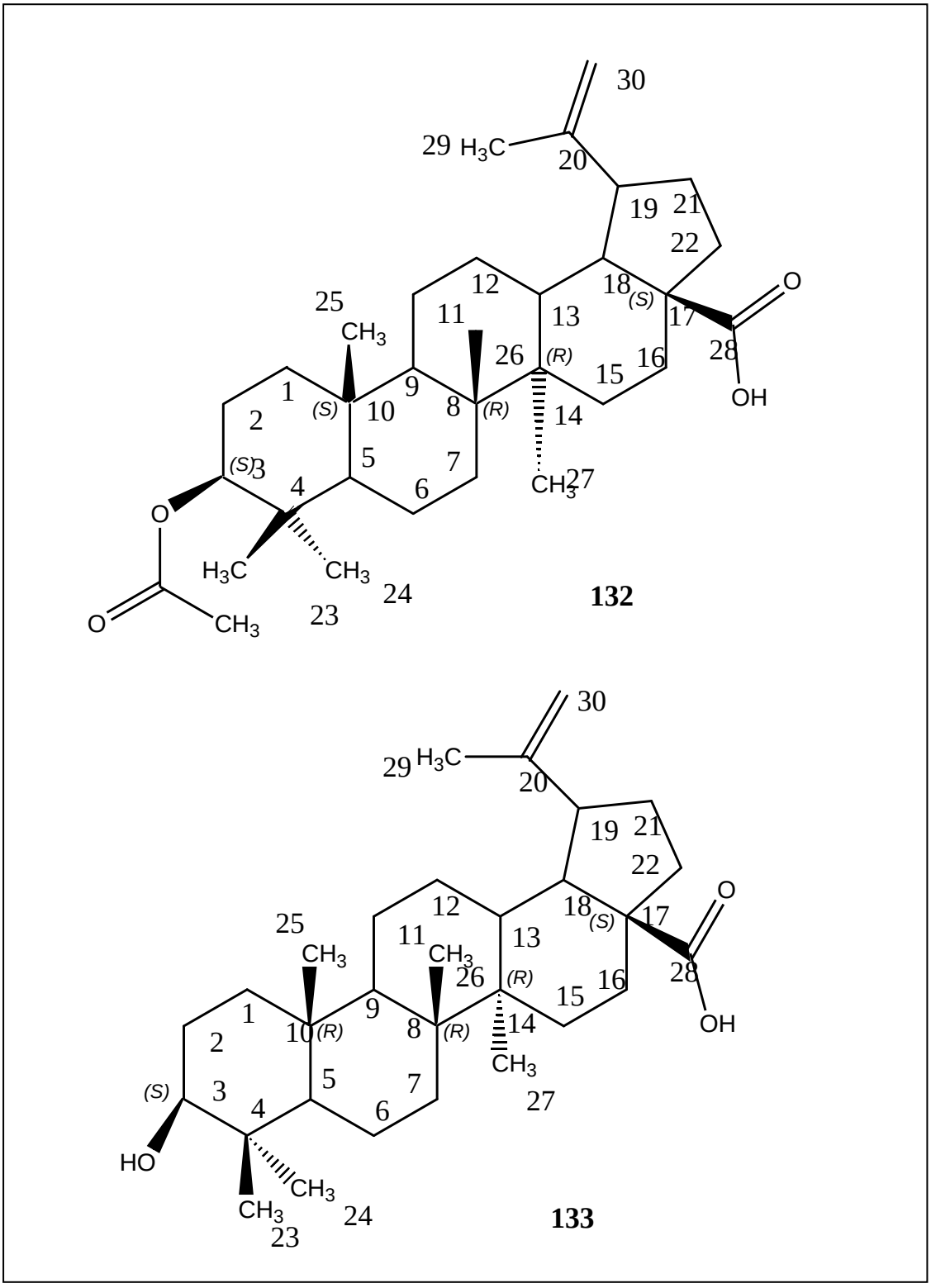


Table-6.26: ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) Spectral Data of Compound **(133)** in C_5D_5N

C.No	^{13}C NMR(δ)	Multiplicity (DEPT)	^{1}H NMR(δ) Coupling Constants J_{HH} (Hz)
1	34.3	CH_2	1.29, m, 1.46 m
2	21.8	CH_2	1.70, m, 1.80 m
3	77.9	CH	3.4, m
4	37.3	C	-
5	50.7	CH	1.29, m
6	18.6	CH_2	1.36, m
7	37.4	CH_2	1.48, m, 1.79 m
8	40.9	C	-
9	55.7	CH	1.76, m
10	38.4	C	-
11	21.3	CH_2	1.42, m
12	25.0	CH_2	1.31, m
13	39.1	CH	2.21, m
14	42.5	C	-
15	28.1	CH_2	1.04, m, 1.60, m
16	32.6	CH_2	1.32, m, 2.5, d (J= 13.08)
17	56.7	C	-
18	49.6	CH	1.67, m
19	47.6	CH	2.60, dt (J = 10.4, 5.5)
20	150.5	C	-
21	30.1	CH_2	1.36, m, 1.73 m
22	39.3	CH_2	1.41, m,1.78 m
23	28.5	CH_3	0.95, s
24	16.2	CH_3	1.01, s
25	16.3	CH_3	0.75, s
26	14.7	CH_3	0.98 s
27	16.1	CH_3	0.99 s
28	178.4	C	-
29	19.3	CH_3	1.78 s
30	109.6	CH_2	4.72, s, 4.89, br s

6.2: Compounds isolated from *H.dyeri*

Six known compounds have been isolated from *Hypericum dyeri* of Pakistani origin. Various experimental techniques and extensive spectroscopic studies were used for the structural elucidation of these compounds. The results of these experimental studies are discussed in this chapter. The extraction and isolation procedures are discussed in detail in the experimental section.

Hypericum dyeri was authenticated by Dr. Habib Ahmad, Dean Faculty of Science, Hazara University, was collected at flowering period in Sept., 2006 from Hazara District, NWFP. A voucher specimens (HUH-17) retained for verification purposes in Department of Botany, Hazara University,NWFP, Pakistan.

The air-dried, powdered Aerial parts (3kg) were exhaustively extracted with hexane, ethyl acetate and methanol (3 x7 L, each for 3 days) at room temperature. The extracts were concentrated under vacuum to yield the residue of fractions, **F1** (hexane) and **F2** (ethyl acetate). The methanolic fraction was suspended in water partioned with n-butanol to afford fractions, **F3** (butanol) and **F4** (Water). The ethyl acetate fraction (**F2**, 20g) was loaded on column chromatography over silica gel eluting with solvents in increasing order of polarity (*n*-hexane–chloroform and chloroform–MeOH) to afford 100 fractions which were combined according to the similarity on TLC profiles and get 11 major fractions. These fractions were further purified to afford six known compounds (**134-139**).

6.2.1: 1-Octatriacontanol (134)

Compound **134** showed complete resemblance to **130** on the basis of physical and spectroscopic data already discussed in section 6.5.5.6

6.2.2: Hexacosyl tetracosanoate (135)

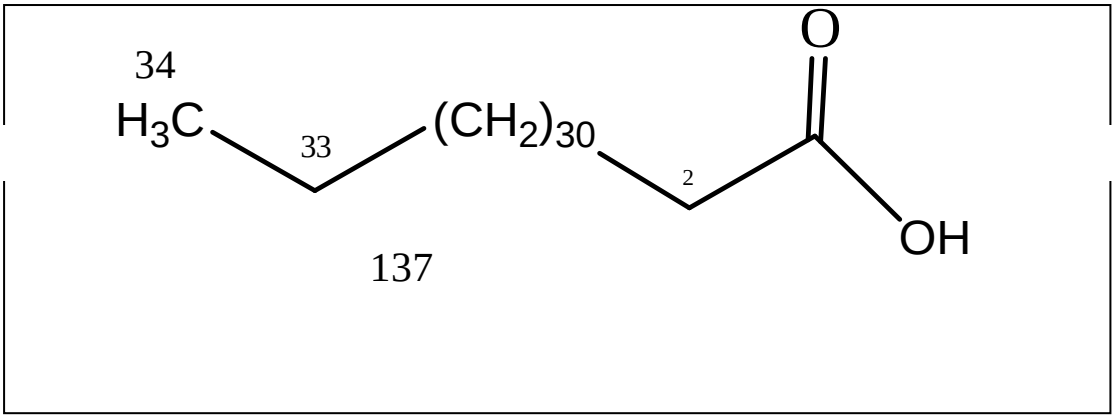
Compound **135** showed complete resemblance to **131** on the basis of physical and spectroscopic data, isolated from H.oblongifoium. It is discussed in section 6.1.5.7

6.2.3: β-Sitosterol (136)

Compound **137** also showed complete resemblance to **127** on the basis of physical and spectroscopic data already discussed in section 6.1.5.3

6.2.4: Geddic acid (137)

Compound **136** was isolated as white solid with m.p 75-77C^0. The IR spectrum gives absorption bands at 3300-2610, 1705cm^{-1} indicating the presence of carboxylic OH and carbonyl functionality[174]. The molecular formula was determined as $C_{34}H_{68}O_2$ by its LR-EI-MS giving molecular ion peak $[M+H]^+$ at *m/z* 507. The ^{I}H NMR of **136** (See experimental section), gives signal at δ 2.32, t (2H, t, *J* = 6.91 Hz, CH_2-2) was assigned to CH_2 that attached to the COOR. A broad singlet at 1.4-1.23 was assigned as (60H, br s, CH_2-4-33). The protons of terminal methyl was appeared at δ 0.843 (3H, t, *J*= 6.71 Hz, CH_3-34). The ^{I3}C and DEPT NMR spectral data (given in experimental section) of compound **136,** disclosed 34 carbons, including one CH_3, 32 CH_2 and one quaternary carbon. The signal at δ 173.2 was attributed to carbonyl carbon (C-1). The peak at δ 14.11 was assigned to the terminal methyl (C-34). The signals at δ 29.32-29.62 (28 x CH_2-4-31) were assigned to a bunch of CH_2. The spectroscopic and physical data of compound **136** agreed with those reported in literature as Geddic acid[174].



137

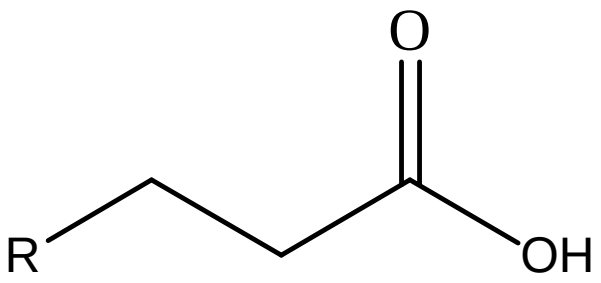
6.2.5: Octacosanoic acid (138)

Compound **138** was isolated as white solid. The bands in IR spectrum displayed at 3220-2540, 1720 cm^{-1} indicating the presence of carboxylic OH and carbonyl functionality[175]. The molecular formula was determined as $C_{28}H_{68}O_2$ by its LR-EI-MS giving molecular ion peak $[M+]^+$ at *m/z* 424.

The ^{I}H NMR of **138** (See experimental section), gives signal at δ 2.32 (2H, t, *J* = 6.71 Hz, CH_2-2) was assigned to CH_2 that attached to the COOR. A broad singlet at 1.4-1.23 was assigned as (48H, br s, CH_2-4-27). The protons of terminal methyl was appeared at δ 0.843 (3H, t, *J*= 6.61 Hz, CH_3-28). The ^{I3}C and DEPT NMR spectral data (given in experimental section), of compound **138** showed 28 carbons, including one CH_3, 26 CH_2 and one quaternary carbon. The peak raised at δ 179.2 was assigned to carbonyl carbon C-1. The peak at δ 14.09 was assigned to the terminal methyl (C-28). The signals at δ 29.3-29.6(28x CH_2-4-31) were assigned to a bunch of CH_2. The spectroscopic and physical data of compound **131** showed complete resemblance with those reported in literature as Geddic acid[175] .

6.2.6: Ceric acid (139)

Compound **132** was isolated as amorphous solid with m.p 89-91C^0. The IR bands at 3320-2620, 1705cm^{-1} showed the presence of carboxylic OH and carbonyl functionality[176]. The molecular formula was determined as $C_{28}H_{68}O_2$ by its LR-EI-MS giving molecular ion peak $[M+]^+$ at *m/z* 424. The ^{I}H NMR of **139** (See experimental section), gives signal at δ 2.32, t (2H, t, *J* = 6.71 Hz, CH_2-2) was assigned to CH_2 that attached to the COOR. Abroad singlet at 1.4-1.23 was assigned as (44H, br s, CH_2-4-25). The protons of terminal methyl was appeared at δ 0.843(3H, t, *J = 6.61* Hz, CH_3-28). The ^{I3}C and DEPT NMR spectral data (given in experimental section), compound **139** contained 28 carbons, including one CH_3, 26 CH_2 and one quaternary carbon. The signal appeared at δ 179.2 was assigned to carbonyl carbon (C-1). The peak at δ 14.09 was assigned to the terminal methyl (C-28). The signal at δ 29.3-29.6(20x CH_2-4-23), was assigned to a bunch of CH_2. The spectroscopic and physical data of compound **139** agreed with those reported in literature as Geddic acid[176].



138: R = C_{27} H_{55}

139: R = C_{25} H_{51}

Chapter 7:

EXPERIMENTAL (Part B)

7.1: General Experimental Conditions

7.1.1: Physical constants

Melting points (corrected and uncorrected) were determined in glass capillary tubes using Buchi 535 melting point apparatus. Optical rotations were measured on Schmidt Haensch Polartronic D polarimeter. X-Ray diffraction data was collected on Brucker diffractometer equipped with SMART APUX CCU area detector usinu Mo Kc radiations (0.71073 A).

7.1.2: Spectroscopic techniques

UV spectra were obtained on Optima SP3000 plus (Japan) using Chloroform or Methanol as solvent. IR spectra were analyzed on a Elmer Fourier-Transform spectrometer, using KBr plates. ^{1}H, ^{13C}-NMR and The 2D-NMR (HMQC, HMBC, NOSEY & COSEY) spectra were recorded on a JEOL ECA 600 (USA) and Bruker AV 500 (Germany) spectrometers. Tetramethylsilane (TMS) was ues as an internal standard and Chemical shifts (δ) were expressed in ppm relative to TMS. Coupling constants were measured in Hz. ^{1}H NMR and ^{13}C NMR spectra were referenced against the known peaks of solvents used. Mass spectra (EI and HR-EI-MS) were measured in an electron impact mode on MAT-312 and MAT-95XP spectrometers and ions are given in *m*/*z* (%).

7.1.3: Chromatographic techniques

Thin-layer chromatography (TLC) was performed on silica gel GF-254 (E.Merck). Purity of the samples was also checked on the same pre-coated plates; the detection was done at 254 nm and by spraying with ceric sulphate reagent. Chromatographic separations were carried out using Column silica gel (E. Merck, 70-230 mesh) and flash silica gel (E. Merck, 230-400 mesh) was used for column

chromatography. Redistilled commercial solvents and reagents of analytical grade were used.

7.1.4: Detection of compounds:

TL-C plates were viewed under Ultraviolet light at 254 nm for fluorescence quenching spots and at 366 nm for fluorescent spots. Dragendorff's solution, ceric sulphate, stabnum chloride solution and other spraying reagents were used to detect the spots on TLC plates.

7.2: *Hypericum oblongifolium*

7.2.1: Plant material

Hypericum oblongifolium was authenticated by Dr. Habib Ahmad, Dean Faculty of Science, Hazara University, was collected at flowering period in June, 2006 from Bunre District, NWFP. A voucher specimen (HUH-002) retained for verification purposes in Department of Botany, Hazara University, NWFP, Pakistan.

7.2.2: Extraction and isolation

7.2.2.1: Extraction and isolation from the aerial parts (Twigs) of *H. oblongifolium*

The air-dried, powdered twigs of *H. oblongifolium* (12Kg) were exhaustively extracted with hexane, ethyl acetate and methanol (3 x 25 L, each for 3 days) at room temperature (Fig. 7.1). The extracts were concentrated under vacuum to yield the residue of fractions, **F1** (hexane) and **F2** (ethyl acetate). The methanolic fraction was suspended in water partioned with n-butanol to get fractions, **F3** (butanol) and **F4** (Water). The ethyl acetate fraction (**F2**, 260g) was loaded on column chromatography over silica gel eluting with solvent in increasing order (*n*-hexane–ethyl acetate and ethyl acetate–MeOH) of polarity to afford 200 fractions which in turn resulted 30 sub-fractions on compilation. Fraction 2-4 were combined and applied to column chromatography over flash silica gel (Chloroform/ hexane 10:90, 20:80, 30:70) and led to the isolation of compounds **127** (20 mg), **130** (15 mg) and **131** (18 mg). Similarly fraction 9 was applied to column chromatography (Flash silica gel; Ethyl

acetate/ hexane 5:95) to purified **118** (25 mg). Fraction 14 was loaded to column chromatography using solvent system (Chloroform/hexane 10:90, 20:80, 30:70, and 40:60) to get the compounds **114** (10 mg), **119** (8 mg) **125** (32 mg) and **126** (12mg). The fractions 15 and 16 were mixed and loaded over flash silica gel CC (Chloroform/hexane 40:60, 50:50) to afford compounds **112** (9 mg) and **113** (6 mg). The fractions 18 and 19 were mixed and treated similarly on flash silica gel CC (Chloroform/hexane 60:40, 70:30, 80:20, 95:5), resulting in **107** (13 mg), **108** (17 mg), and **117** (15 mg). While **115** (11 mg), **116** (14 mg) and **120** (20 mg) were purified from the fraction 20 using flash silica gel CC (Chloroform/hexane 70:30, 80:20, 90:10, 100:0). Fraction 21 was loaded on flash silica gel CC (Methanol/ Chloroform 1:99, 2:98) to afford **128** (51 mg) and **129** (28 mg) whereas the fractions 22 and 23 were combined and subjected to flash silica gel CC (Methanol/Chloroform 3:97, 4:96) led to the isolation of **105** (15 mg) and **106** (18 mg). Fraction 26 was also purified on the same way to yield pure **111** (12 mg).

7.2.2.2: Extraction and isolation from the Roots of *H. oblongifolium*

The air-dried, powdered roots (4 Kg) were exhaustively extracted with hexane, ethyl acetate and methanol (3 x 7 L, each for 3 days) at room temperature. The extracts were concentrated in a rotary evaporator and dried under vacuum to yield the gummy residue (Fig. 7.1). The ethyl-acetate fraction (70g) was subjected to column chromatography over silica gel eluting with *n*-hexane–ethyl acetate and ethyl acetate–MeOH in increasing order of polarity to afford 180 fractions which were combined according to the similarity on TLC profiles and get 21 major fractions. Fraction 4 was purified through column chromatography (hexane: chloroform; 1:1) and yield 20 mg of pure compound **132.** Fraction 5 was also subjected to column chromatography eluted with hexane: chloroform in increasing order of polarity started at 80:20 and yield thee sub fractions (5.1-5.3), which were further purified by preparative TLC using chloroform as eluting solvent and purified **121** (4 mg) and **123** (3 mg). Compound **124 E** was purified by repeated recrystallization of fraction 6. Fraction 7 was subjected to column chromatography eluted with hexane: chloroform (50:50 to 10:90) yield **133** (100 mg). Fraction 11 was also subjected to column chromatography eluted with hexane: chloroform in increasing order of polarity started at 1:1 and yield five fractions (11.1-11.5), on further purification by preparative TLC using Methanol:

chloroform (5:95) as eluting solvents yield **124 C** (3 mg) and **124 B** (4 mg). Fraction 12 was also subjected to preparative TLC using Methanol: chloroform (5:95) as eluting system and got pure **124 A** (20 mg). Similarly Fraction 14 was also subjected to column chromatography eluted with hexane: chloroform in increasing order of polarity started at 2:3 and yield three sub fractions (14.1-14.3), these were further purified on preparative TLC using Methanol: chloroform (7:93) as eluting system and yield **124 A** (6 mg) and **124 D** (5 mg). Compounds **124 C** (7 mg) and **124** (6 mg) were also purified on the same way from fraction 15. Fraction 17 was subjected to column chromatography eluted with hexane: chloroform (80:20) to pure chloroform and then methanol: chloroform (1:99) to yield **109** (15 mg). Compound **120** was purified from fraction 18 by Preparative TLC using Methanol: chloroform (7:93) as eluting system from fraction 18. Fraction 19 was subjected to column chromatography eluted with hexane: chloroform (80:20 to pure chloroform and then methanol: chloroform 1:99) to yield **110** (17 mg). Finally **122** was purified from fraction 20 by preparative TLC (Methanol: chloroform 7:93). Isolation scheme is given in figure 7.2

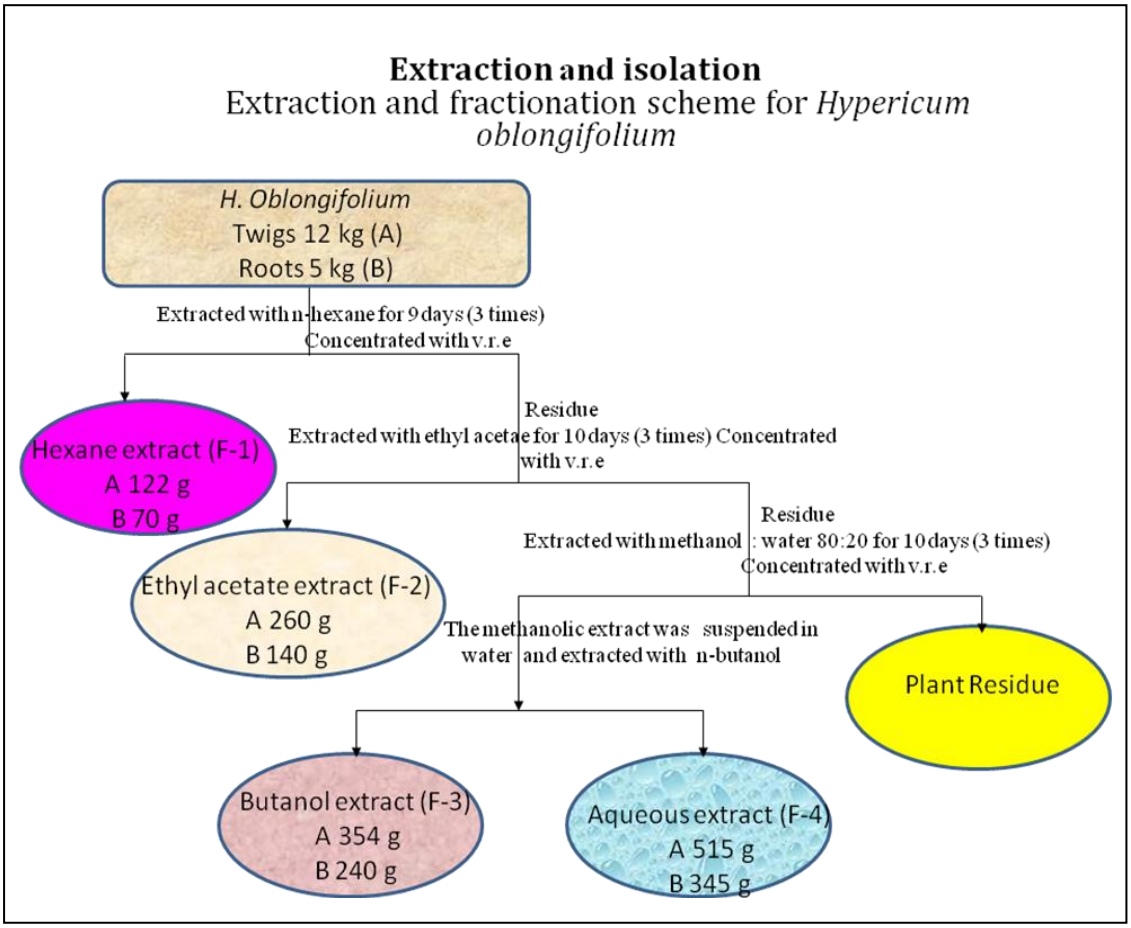


Fig. 7.1:Extraction and fractionation scheme for the Twigs and Roots of *H.oblongifolium*

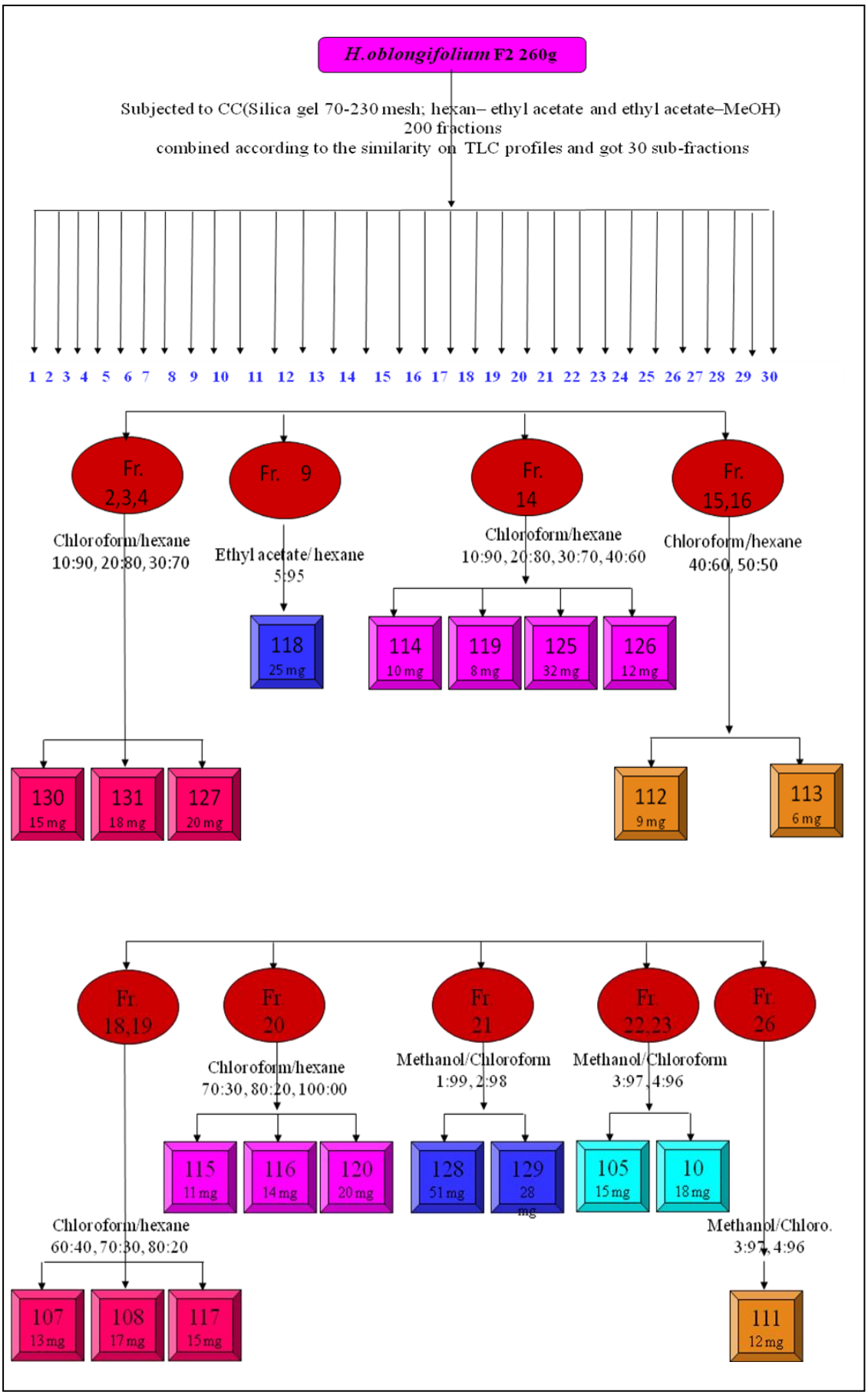


Fig.7.2. Isolation scheme of compound isolated from *Hypericum oblongifolium*

7.2.3: Experimental data of new Xanthones from the Twigs of *H. oblongifolium*

7.2.3.1: Hypericorin A (105)

IUPAC name: 3-(3-hydroxy-5-methoxyphenyl)-5-methoxy-7-oxo-3,7-dihydro-2H-[1,4] dioxino[2,3-c] xanthen-2-yl)methyl acetate

Physical state: White amorphous solid

Yield: 15 mg (1.26 x 10^{-4}%)

Melting point: 235-238C^0

λ_{max} (MeOH) nm (log ε): 205 (4.35), 286 (3.83), 316 (3. 86)

IR ν_{max} (KBr): 3453, 2933, 1648, 1452, 1382, 1319, 1135, 597 cm^{-1}

EI-MS (70.0 eV) : *m*/*z* 478.0 (calc. for $[C_{26}H_{24}O_9]^+$)

EIMS *m*/*z* (rel. int. %) : 478 (30), 418 (100), 258 (77), 243 ()38, 222 (71), 179 (35),

^{1}H NMR (400 MHz, CD_3OD+$CDCl_3$): Given in **Table 6.1**

HMQC and ^{13}C NMR (100 MHz, CD_3OD+$CDCl_3$): Given in **Table 6.1**

7.2.3.2: Hypericorin B (106)

IUPAC name: 3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-5-methoxy-2H-[1,4]dioxino[2,3-c]xanthen-7(3H)-one.

Physical state: White amorphous solid

Yield: 18 mg (1.32 x 10^{-4}%)

Melting point: 240-243C^0

λ_{max} ($CHCl_3$) nm (log ε): 203 (4.5), 236 (4.38), 286 (3.81), 315 (3.71)

IR ν_{max} (KBr): 3592, 2933, 1642, 1600, 1452, 1382, 1319, 1135, 597 cm^{-1}

EI-MS (70 .0eV) : *m*/*z* 436.0 (calc. for $[C_{24}H_{20}O_8]^+$)

EIMS *m*/*z* (rel. int. %) : 436 (33), 418 (29), 258 (100), 243 (50), 229 (9), 180 (66), 162 (28)

^{1}H NMR (500 MHz, CD_3OD+$CDCl_3$): Given in **Table 6.2**

HMQC and ^{13}C NMR (125 MHz, CD_3OD+$CDCl_3$): Given in **Table 6.2**

7.2.3.3: Bihyponicaxanthone A (107)

Physical state: Yellow amorphous solid

Yield: 13 mg (1.21 x 10^{-4}%)

Melting point: 239-245C^0

λ_{max} (MeOH) nm (log ε): 243 (4.11), 268 (3.9), 376 (3.5)

IR ν_{max} (KBr): 3437, 2900, 1648, 1642, 1595, 1473, 1345, 1315, 1241, 1173 cm^{-1}

EI-MS (70 eV): *m/z* at 304 and at 259 (calc. fragments A $[C_{15}H_{12}O_7]^+$ and B $[C_{14}H_{11}O_5]^+$)

EIMS *m/z* (rel. int. %): 304 (100), 289 (20), 274 (80), 259 (30), 243 (15), 231 (22).

^{1}H NMR (500 MHz, CD_3OD): Given in **Table 6.3**
HMQC and ^{13}C NMR (125 MHz, CD_3OD): Given in **Table 6.3**

7.2.3.4: 3, 4-Dihydroxy-5-methoxyxanthone (108)

IUPAC name: 3, 4-Dihydroxy-5-methoxyxanthone

Physical state: Yellow amorphous solid

Yield: 17 mg (1.3 x 10^{-4}%)

Melting point: 230-235 C^0

λ_{max} (MeOH) nm (log ε): 240 (4.32), 258 (4.37), 269 (4.45), 376 (3.58)

IR ν_{max} (KBr): 3437, 2900, 1622, 1585, 1470, 1455, 1345, 1310, 1245, 1215 cm^{-1}

EI-MS (70 eV): *m/z* 258.0 (calc. for $[C_{14}H_{10}O_5]^+$)

EIMS *m/z* (rel. int. %): 258 (39), 240 (54), 215 (92), 184 (83), 115 (43)

^{1}H NMR (500 MHz, $(CD_3)_2CO$): Given in **Table 6.4**
HMQC and ^{13}C NMR (125 MHz, $(CD_3)_2CO$): Given in **Table 6.4**

7.2.4: Experimental data of new xanthones from the Roots of *H. oblongifolium*

7.2.4.1: Hypericorin C (109)

IUPAC name: 3-(5-hydroxy-4-methoxyphenyl)-5-methoxy-7-oxo-3,7-dihydro-2H-[1,4] dioxino[2,3-c] xanthen-2-yl)methyl acetate

Physical state: White amorphous powder

Yield: 17 mg (2.28 x 10^{-4}%)

Melting point: 230-232 C^0

$[\alpha]^D$ = +0.33^o (0.01 acetone)

λ_{max} (MeOH) nm (log ε): 248 (4.34), 308 (3.83), 346 (3. 82)

IR ν_{max} (KBr): 3416, 3015, 2941, 1742, 1642, 1608, 1485, 1343, 1228, 1140, cm^{-1}

EI-MS (positive ion mode): *m/z* 479.0 (calc. for $[C_{26}H_{25}O_9]^+$)

EIMS *m/z* (rel. int. %): 479 (20), 360 (40), 338 (100), 258 (3), 243 (3), 222 (3), 180

^{1}H NMR (600 MHz, $(CD_3)_2CO$): Given in **Table 6.5**

HMQC and ^{13}C NMR (150MHz, $(CD_3)_2CO$): Given in **Table 6.5**

7.2.4.2: Hypericorin D (110)

IUPAC name: 3-(2,3,4-trihydroxy-5-methoxyphenyl)-2-(hydroxymethyl)-5-methoxy-2H- [1,4]dioxino[2,3-c]xanthen-7(3H)-one

Physical state: White amorphous powder.

Yield: 15 mg (2.26 x 10^{-4}%)

Melting point: 250-254 C^0

$[\alpha]^D$ = +0.58º (0.01 acetone)

λ_{max} (MeOH) nm (log ε): 250 (4.5), 286 (4.38), 302 (3.81), 387 (3.71)

IR ν_{max} (KBr): 3384, 3010, 2940, 1704, 1639, 1599, 1464, 1325, 1285, 1138 cm^{-1}

EI-MS (Negative-ion mode): *m/z* 467.0 (calc. for $[C_{24}H_{19}O_{10}]^-$)

EIMS *m/z* (rel. int. %): 467 (8), 437 (4), 338 (16), 283 (3), 245 (5), 215 (5), 173 (5),

^{1}H NMR (600 MHz, $(CD_3)_2SO$): Given in **Table 6.6**

HMQC and ^{13}C NMR (150MHz, $(CD_3)_2SO$): Given in **Table 6.6**

7.2.5: Experimental data of known xanthones from theTwigs of *H oblongifolium*

7.2.5.1: (109)

IUPAC name: 3-(5-hydroxy-4-methoxyphenyl)-5-methoxy-7-oxo-3,7-dihydro-2H-[1,4] dioxino[2,3-c] xanthen-2-yl)methyl acetate

Physical state: White amorphous powder.

Yield: 12 mg (1.0 x 10^{-4}%)

Melting point: 250-254 C^0

$[\alpha]^D$ = +0.58º (0.01 acetone)

λ_{max} (MeOH) nm (log ε): 255 (4.5), 280 (4.38), 316 (3.81), 387 (3.71)

IR ν_{max} (KBr): 3354, 3012, 2945, 1639, 1590, 1484, 1320, 1284, 1128 cm^{-1}

EI-MS (70 eV): *m/z* 258.0 (calc. for $[C_{14}H_{10}O_5]^+$)

EIMS *m/z* (rel. int. %): 258 (39), 240 (54), 215 (92), 184 (83), 115 (43)

^{1}H (500 MHz) and ^{13}C (125) NMR (CD_3OD+$CDCl_3$): Given in **Table 6.7**

7.2.5.2: 4-Hydroxy-2, 3-dimethoxyxanthone (112)

IUPAC name: 4-Hydroxy-2, 3-dimethoxy-9H-xanthen-9-one

Physical state: Yellowish white crystalline solid

Yield: 9 mg (7.46 x 10^{-5} % yield %)

Melting point: 220-223C^0

λ_{max} ($CHCl_3$) nm (log ε): 255 (4.34), 286 (3.83), 306 (3. 82), 353 (3.62).

IR ν_{max} (KBr): 3599, 2928, 2843, 1642, 1600, 1496, 1303, 1262, 1131, 1092 cm^{-1}

EI-MS (70 .0eV) : *m/z* 272.1 (calc. for $[C_{15}H_{12}O_5]^+$)

EIMS *m/z* (rel. int. %) : 272 (100), 257 (23.55), 229 (12.42), 214(19.39)

^{1}H (500 MHz) and ^{13}C (125) NMR (CD_3OD+$CDCl_3$) Given in **Table 6.8**

7.2.5.3: 3, 4, 5-Trihydroxyxanthone (113)

IUPAC name: 3, 4, 5-trihydroxy-9H-xanthen-9-one

Physical state: Yellow amorphous powder solid

Yield: 6 mg (5.16 x 10^{-5} %)

Melting point: 280-283C^0

λ_{max} ($CHCl_3$) nm (log ε): 240 (4.38), 308 (3.81), 346 (3.71)

IR ν_{max} (KBr): 3599, 3512, 3462, 3151, 2928, 2843, 1644, 1580, 1443, 1328, cm^{-1}

EI-MS (70 eV) : *m/z* 244.2 (calc. for $[C_{13}H_8O_5]^+$)

EIMS *m/z* (rel. int. %) : 244.2 (100), 215 (11.29), 121 (7.83), 108 (5.22)

^{1}H (500 MHz) and ^{13}C (125) NMR $(CD_3)_2CO$): Given in **Table 6.9**

7.2.5.4: 3-Hydroxy-2-methoxyxanthone (114)

IUPAC name: 3-Hydroxy-2-methoxy-9H-xanthene-9-one

Physical state: Yellowish white amorphous solid

Yield: 10 mg (9.86 x 10^{-5}%)

Melting point: 215-218C^0

λ_{max}($CHCl_3$) nm (log ε): 244 (4.22), 262 (4.44), 393 (3.58), 382

IR ν_{max} (Chloroform): 3464, 3150, 2928, 2843, 1665, 1594, 1439, 1324, 1270, cm^{-1}

EI-MS (70.0 eV) : *m/z* 242.0 (calc. for $[C_{14}H_{10}O_4]^+$)

EIMS *m/z* (rel. int. %) : 242 (100), 227 (61), 199 (24), 171 (28), 115 (15)

^{1}H (500 MHz) and ^{13}C (125) NMR (CD_3OD+$CDCl_3$): Given in **Table 6.10**

7.2.5.5: 4, 7-Dihydroxyxanthone (115)

IUPAC name: 2,5-Dihydroxy -9H-xanthene-9-one

Physical state: yellowish white amorphous solid

Yield: 11 mg (9.19×10^{-5}%)

Melting point: 288-290C^0

λ_{max}(MeOH) nm (log ε): 240 (4.32), 245(4.37), 256 (4.45), 291 (3.34, 373 (3.58

IR ν_{max} (KBr): 3500-3000, 1635, 1595, 1490, 1470, 1455, 1345, 1310, 1245 cm^{-1}

EI-MS (70.0 eV) : *m/z* 228.0 (calc. for $[C_{13}H_8O_4]^+$)

EIMS m/*z* (rel. int. %) : 228 (100), 200 (10.9, M), 172 (3.1), 171 (8.2), 144(5.5).

^{1}H (500 MHz) and ^{13}C (125) NMR ($CD_3OD+CDCl_3$): Given in **Table 6.11**

7.2.5.6: 1, 6-Dihydroxy-7-metoxyxanthone (116)

IUPAC name: 1, 6-dihydroxy-7-metoxy 9H-xanthene-9-one

Physical state: Yellow amorphous solid

Yield: 14 mg (1.18×10^{-4} %)

Melting point: 265-268C^0

λ_{max}(MeOH) nm (log ε): 250 (4.32), 261 (4.37), 269 (4.45), 290 (3.34), 368 (3.58)

IR ν_{max} (KBr): 3350, 2900, 1647, 1585, 1470, 1455, 1345, 1310, 1245, 1215, cm^{-1}

EI-MS (70.0 eV): *m/z* 258.0 (calc. for $[C_{14}H_{10}O_5]^+$)

EIMS: *m/z* (rel. int. %): 258 (100), 243 (45), 224 (8), 215 (26), 187 (19), 149 (4),

^{1}H (500 MHz) and ^{13}C (125) NMR ($CD_3OD+CDCl_3$): Given in **Table 6.12**

7.2.5.7: 1, 3, 7-Trihydroxyxanthone (117)

IUPAC name: 1, 3, 7-trihydroxy-9H-xanthen-9-one

Physical state: Yellow amorphous solid

Yield: 15 mg (1.25×10^{-4} %)

Melting point: 318-320C^0

λ_{max}($CHCl_3$) nm (log ε): 244 (4.38), 269 (4.31), 318 (3.81), 356 (3.571)

IR ν_{max} (KBr): 3519, 3502, 3442, 2928, 2843, 1654, 1580, 1443cm^{-1}

EI-MS (70.0 eV) : *m/z* 244.3 (calc. for $[C_{13}H_8O_5]^+$)

EIMS *m/z* (rel. int. %) : 244 (100), 215 (7), 187 (12), 108 (13)

^{1}H (400 MHz) and ^{13}C (100) NMR (CD_3OD+ $CDCl_3$): Given in **Table 6.13**

7.2.5.8: 1, 7-Dihydroxyxanthone. (118)

IUPAC name: 1, 7-dihydroxy-9H-xanthen-9-one

Physical state: Yellow amorphous solid

Yield: 25 mg (2.08 x 10^{-4} %)

Melting point: 258-259C^0

λ_{max}($CHCl_3$) nm (log ε): 238 (4.21), 259 (4.11), 319 (3.61), 375 (3.47)

IR ν_{max} (KBr): 3500, 3446, 2948, 1635, 1595, 1443 cm^{-1}

EI-MS (70.0 eV) : *m/z* 228.0 (calc. for $[C_{13}H_8O_4]^+$)

EIMS *m/z* (rel. int. %) : 228 (100), 200 (13), 171 (8), 144 (9), 115 (20)

^{1}H (500 MHz) and ^{13}C (125) NMR $(CD_3)_2CO$): Given in **Table 6.14**

7.2.5.9: 3, 5-Dihydroxy-4-methoxyxanthone (119)

IUPAC name: 3, 5-dihydroxy-4-methoxy-9H-xanthen-9-one

Physical state: Yellowish white needles

Yield: 8 mg (7.28 x 10^{-5} %)

Melting point: 228-229C^0

λ_{max} (MeOH) nm (log ε): 207(4.01), 241 (3.71), 313 (3.31), 375 (3.47)

IR ν_{max} (KBr): 3433, 3219, 2928, 2843, 1645, 1580, 1443cm^{--1}

EI-MS (70.0 eV), *m/z* 258.0 (calc. for $[C_{14}H_{10}O_5]^+$)

EIMS: *m/z* (rel. int. %): 258 (100), 243 (36), 215 (10), 187 (14), 129 (4)

^{1}H NMR (300 MHz, $(CD_3)_2CO$): Given in **Table 6.15**

^{13}C NMR (100 MHz, $(CD_3)_2CO$): Given in **Table 6.15**

7.2.5.10: 3,4-Dihydroxy-2-methoxyxanthone (120)

IUPAC name: 3,4-dihydroxy-2-methoxy-9H-xanthen-9-one

Physical state: Yellowish amorphous powder

Yield: 5 mg (1.1 x 10^{-4} %)

Melting point: 245-250C^0

λ_{max} (MeOH) nm (log ε): 232 (4.5), 276 (3.78), 367 (3.01)

IR ν_{max} (KBr): 33390, 3240, 2930, 1726, 1604, 1466, 1273 cm^{-1}

EI-MS (70.0 eV) : *m/z* 259.0 (calc. for $[C_{14}H_{11}O_5]^+$)

EIMS: *m/z* (rel. int. %): 259 (100), 244 (30), 216 (10), 164 (15), 145 (60)

^1H (600 MHz) and ^{13}C (150) NMR $(CD_3)_2CO$): Given in **Table 6.16**

7.2.6: Experimental data of known xanthones from the Roots of *H oblongifolium*

7.2.6.1: 2, 3-Dimethoxyxanthone (121)

IUPAC name: 2,3-Dimethoxy-9H-xanthen-9-one

Physical state: White crystalline solid

Yield: 4 mg (9.18×10^{-5} %)

Melting point: 145-150 C^0

λ_{max}(MeOH) nm (log ε): 242 (3.5), 272 (3.38), 307 (2.71)

IR ν_{max} (KBr): 1657, 1590, 1444, 1315, 1281, 1138, 1089 cm^{-1}

EI-MS (Positive-ion mode) *m/z* 257.0 (calc. for $[C_{15}H_{13}O_4]^+$)

EIMS: *m/z* (rel. int. %): 257 (100), 242 (24), 214 (16), 163 (5), 147 (5)

^1H (600 MHz) and ^{13}C NMR (150 MHz, $CDCl_3$): Given in **Table 6.17**

7.2.6.2: 3, 5-Dihydroxy-1-methoxyxanthone (122)

IUPAC name: 3,5-Dihydroxy-1-methoxy -9H-xanthen-9-one

Physical state: White amorphous solid

Yield: 6 mg (1.1×10^{-4} %)

Melting point: 320-25 C^0

λ_{max}(MeOH) nm (log ε): 242 (4.1), 278 (3.8), 307 (2.91)

IR ν_{max} (KBr): 3455, 2959, 1658, 1604, 1457, 1275, 1143, 1084 cm^{-1}

EI-MS (Negative-ion mode): *m/z* 257.0 (calc. for $[C_{14}H_9O_5]^-$)

EIMS: *m/z* (rel. int. %): 257 (100), 242 (22), 214 (22), 162 (5), 147 (6)

^1H (600 MHz) and ^{13}C NMR (150 MHz, CD_3OD): Given in **Table 6.18**

7.2.6.3: 2, 3-Methylenedioxyxanthone (123)

IUPAC name: 2, 3-Methylenedioxy-9H-xanthen-9-one

Physical state: White crystalline solid

Yield: 3 mg (6.0×10^{-5} %)

Melting point: 180-185 C^0

λ_{max}(MeOH) nm (log ε): 239 (4.`), 270 (3.68), 307 (3.21)

IR ν_{max} (KBr): 1640, 1604, 1480, 1089 cm^{-1}

EI-MS (Positive-ion mode): *m/z* 241.0 (calc. for $[C_{14}H_9O_4]^+$)

EIMS: *m/z* (rel. int. %): (60), 227(30), 216 (10), 173 (10)

^1H (600 MHz) and ^{13}C NMR (150 MHz, $CDCl_3$): Given in **Table 6.19**

7.2.6.4: 2, 5-Dihydroxy-1-methoxyxanthone (124)

IUPAC name: 2, 5-Dihydroxy-1-methoxy -9H-xanthen-9-one

Physical state: Yellowish amorphous powder

Yield: 6 mg (1.1 x 10^{-4} %)

Melting point: 315-320 C^0

λ_{max} (MeOH) nm (log ε): 242 (4.2), 256 (3.8), 310 (3.21)

IR ν_{max} (KBr): 3230, 1635, 1595, 1495 cm^{-1}

EI-MS (Negative-ion mode): *m/z* 257.0 (calc. for $[C_{14}H_9O_5]^-$)

EIMS: *m/z* (rel. int. %): 257 (75), 242 (100), 214 (15), 186 (15)

^1H (600 MHz) and ^{13}C NMR (150 MHz, $CD_3CO\ CD_3$): Given in **Table 6.20**

7.2.7: Experimental data of other compounds from the Twigs of *H.oblongifolium*

7.2.7.1: Zizyphursolic acid (125)

IUPAC name: 18βH-urs-20 (30)-en-3β-ol-28-oic acid

Physical state: White amorphous solid

Yield: 32 mg (2.68 x 10^{-4} %)

Melting point: 262-265C^0

λ_{max}($CHCl_3$) nm (log ε): 240 (5.1), 269 (4.31),

IR ν_{max} (KBr): 3100, 2955, 2870, 1695, 1640, 1455, 1380, 1235, 1045, 890 cm^{-1}

EI-MS (70.0 eV) : *m/z* 456.0 (calc. for $[C_{30}H4_8O_3]^+$)

EIMS *m/z* (rel. int. %) : 456 (32), 395 (3), 257 (4), 248 (35), 189 (100), 175 (32)

^1H (300 MHz) and ^{13}C NMR (100 MHz, $(CD_3)_2CO$): Given in **Table 6.2`1**

7.2.7.2: Tetracosyl 3-(3,4-dihydroxyphenyl) acrylate (126)

IUPAC name: Tetracosyl 3-(3,4-dihydroxyphenyl) acrylate (**2**)

Physical state: White amorphous solid

Yield: 12 mg (1.0 x 10^{-4} %)

Melting point: 202-205C^0

λ_{max}($CHCl_{3+}$) nm (log ε): 235 (4.12), 325 (4.04).

IR ν_{max} (KBr): 3500, 1700, 1670, 1600, 1510, 1460, 1280, 1160, cm^{-1}

EI-MS (70.0 eV) : *m*/*z* 516.0 (calc. for $[C_{33}H4_{56}O_4]^+$)

EIMS *m*/*z* (rel. int. %) : 516 (5), 488 (3), 248 (2), 180(62)

^{1}H (400 MHz) and ^{13}C NMR (100 MHz, $CDCl_3$): Given in **Table 6.22**

7.2.7.3: β-Sitosterol (127)

IUPAC name: 24-Ethylcholest-5-en-3-ol

Physical state: White crystal

Yield: 20 mg (1.66 x 10^{-4} %)

Melting point: 136-38C^0

λ_{max}($CHCl_3$) nm (log ε): 230 (4.1), 325 (3.67),

IR ν_{max} (KBr): 3408, 1628, 1379 and 1065cm^{-1}

EI-MS (70 eV) : *m*/*z* 414.0 (calc. for $[C_{29}H4_{50}O]^+$)

EIMS *m*/*z* (rel. int. %) : 414 (38), 381 (10), 329 (12), 273 (17), 255 (29), 213 (24)

^{1}H (400 MHz) and ^{13}C NMR (100 MHz, $CDCl_3$): Given in **Table 6.23**

7.2.7.4: β-Sitosterol3-O-β-D-glucopyranoside (128)

IUPAC name: 24-Ethylcholest-5-en-3-ol- glucopyranoside

Physical state: White amorphous solid

Yield: 51 mg (4.25x 10^{-4} %)

Melting point: 236-238C^0

IR ν_{max} (KBr): 3452, 1648, 1379 and 1065 cm^{-1}

EI-MS (70 eV) : *m*/*z* 414.0 (calc. for $[C_{29}H4_{50}O]^+$)

EIMS *m*/*z* (rel. int. %) : 396 (10), 381 (2), 255 (2), 173 (4), 145 (16), 60 (48), 57 (57)

^{1}H (500 MHz) and ^{13}C NMR (125 MHz, DMSO): Given in **Table 6.24**

7.2.7.5: Shikimic Acid (129)

IUPAC name: 3, 4, 5-trihydroxycyclohex-1-enecarboxylic acid

Physical state: White amorphous solid

Yield: 28 mg (2.33 x 10^{-4} %)

Melting point: 190-93C^0

λ_{max} ($CHCl_3$) nm (log ε): 230 (4.1), 325 (3.67),

IR ν_{max} (KBr): 3450, 2940, 1705, 1610, 1595 cm^{-1}

EI-MS (70 eV) : *m/z* 174.0 (calc. for $[C_7H4_{10}O_5]^+$)

^{1}H NMR (600MHz, CD_3OD): δ_H 6.75 (1H, *s*, H-2), 5.65 (1H, *s*, H-3), 4.03 (1H, *m*, H-5), 3.94 (1H, *dd*, *J* = 6.52, 4.05 Hz, H-4), 2.75 (1H, *d*, *J* = 18.06, H-6b), 2.29 (1H, *dd*, *J* = 18.04 Hz, 4.06, H-6a)

^{13}C NMR (150MHz, CD_3OD): δ_C 168.5 (C-7), 134.5 (C-2), 127.4 (C-1), 70.92 (C-4), 70.79 (C-5), 68.77 (C-3), 31.65 (C-6)

.

7.2.7.6: 1-Octatriacontanol (130)

IUPAC name: 1-Octatriacontanol

Physical state: White amorphous solid

Yield: 15 mg (1.23 x 10^{-4} %)

Melting point: 63-66C^0

IR ν_{max} (KBr): 3461, 2928, 2843, 1740, 1280, 1160 cm^{-1}

EI-MS (70.01 eV) : *m/z* 551.0 (calc. for $[C_{38}H_{78}O+H]^+$)

^{1}H NMR (400 MHz, $CDCl_3$) δ_H: 3.62 (2H, t, *J* = *7.11* Hz, CH_2-1); 1.53-1.57 (4H, m, CH_2- 2, 37); 1.23-1.292 (68H, m, CH_2-3-36); 0.842 (3H, t, *J* = 6.92 Hz, CH_3-38)

^{13}C NMR (100 MHz, $CDCl_3$)δ_C: 62 (CH_2-1), 32.9 (CH_2-2), 31.97(CH_2-3), 29.3-29.6(32x CH_2-4-35), 25.7 (CH_2-37), 22.5 (CH_2-36), 14.08 (CH_3-38).

The physical and spectral data showed complete agreement with those reported in literature[171].

7.2.7.7: Hexacosyl tetracosanoate (131)

IUPAC name: Hexacosyl tetracosanoate

Physical state: White amorphous solid

Yield: 18 mg (1.53 x 10^{-4} %)

Melting point: 79-81C^0

IR ν_{max} (KBr): 2928, 2843, 1740cm^{--1}

EI-MS (70.0 eV) : *m/z* 733.0 (calc. for $[C_{50}H_{100}O_2+H]^+$)

^{1}H NMR (400 MHz, $CDCl_3$) δ_H:4.02 (2H, t, *J* = 7.22 Hz, CH_2-25), 2.26, t (2H, t, *J* = 7.12 Hz, CH_2-23), 1.572 (4H, m, CH_2-22, 26); 1.23-1.322 (86H, br s, CH_2-2-21, 27-49), 0.84 (6H, t, *J* = 6.92 Hz, CH_3-1,50)

^{13}C NMR (100 MHz, $CDCl_3$), δ_C: 173 (C-24), 64 (CH_2-25), 34.4 (CH_2-23), 31.97 (2xCH_2-22,26), 29.3-29.6 (41x CH_2-3-21, 27-48), 25.7 (CH_2), 22.5 (2xCH_2-2, 49), 14.08 (2x CH_3-1, 50).

The physical and spectral data showed complete agreement with those reported in literature[172] and complete spectral data presented here for the first time.

7.2.7: Experimental data of other compounds from the Roots of *H oblongifolium*

7.2.7.1: Methyl betulinate -3-Acetate (132)

Physical state: White crystalline solid

Yield: 20 mg (5.03 x 10^{-4}%)

Melting point: 262-264 C^0

λ_{max}($CHCl_3$) nm (log ε): 240 (5.1), 269 (4.31)

IR ν_{max} (KBr): 2946, 1732, 1696, 1452, 1369, 1244, 1105, 1024 cm^{-1}

EI-MS (Negative-ion mode) *m/z* 497.0 (calc. for $[C_{32}H_{49}O_4]$-)

EIMS *m/z* (rel. int. %): 497 (100), 283 (3), 279 (5), 212 (50), 156 (5)

^{1}H (600 MHz) and ^{13}C NMR (150 MHz, $CDCl_3$): Given in **Table 6.25**

7.2.7.2: Betulinic acid (133)

Physical state: White crystalline solid

Yield: 100 mg (2.03 x 10^{-3} %)

Melting point: 295-300 C^0

$\lambda_{max}(CHCl_3)$ nm (log ε): 242 (4.1), 265 (3.31)

IR ν_{max} (KBr): 3412, 2943, 1686, 1451, 1373, 1234 cm^{-1}

EI-MS (Negative-ion mode) *m*/*z* 455.0 (calc. for $[C_{30}H_{47}O_3]$-)

EIMS *m*/*z* (rel. int. %): 455 (100), 283 (3), 279 (5), 212 (50), 156 (5)

^1H (600 MHz) and ^{13}C NMR (150 MHz, Pyridine-): Given in **Table 6.26**

7.3: *Hypericum dyeri*

7.3.1: Plant material

Hypericum dyeri was authenticated by Dr. Habib Ahmad, Dean Faculty of Science, Hazara University, was collected at flowering period in Sept.,2006 from Hazara District, NWFP. A voucher specimens (HUH-017) retained for verification purposes in Department of Botany, Hazara University,NWFP, Pakistan.

7.3.2: Extraction and isolation

The air-dried, powdered aerial parts of *Hypericum dyeri* (3kg) were extracted with hexane, ethyl acetate and methanol (3x7 L, each for 3 days) at room temperature (Fig 7.3). The extracts were concentrated vacuum to yield the residue of various fractions, **F1** (hexane) and **F2** (ethyl acetate). The methanolic fraction was further dissolved in water partitioned with n-butanol to afford fractions, **F3** (butanol) and **F4** (Water) The ethyl acetate fraction (**F2**, 20g) was loaded oncolumn chromatography over silica gel eluting solvent in increasing order of polarity (*n*-hexane– chloroform and chloroform–MeOH) to afford 100 fractions which produced according 11 major fractions on compilation. Fraction 1and 2 were combined and applied to flash silica gel CC (Chloroform/hexane 20:80, 30:70) and led to the isolation of compounds **134** (9mg) and **135** (7 mg). The Fraction 8 was loaded to column chromatography over flash silica gel (Chloroform/hexane 40:60) and afforded the compounds **136** (8 mg). Similarly the fraction 9 was loaded on CC (flash silica gel; Chloroform/hexane 35:65) to afford **137** (6 mg) while fractions 10 and 11 were treated similarly to obtained **138** (9 mg) and **139** (7 mg). Isolation scheme is given in figure 7.4.

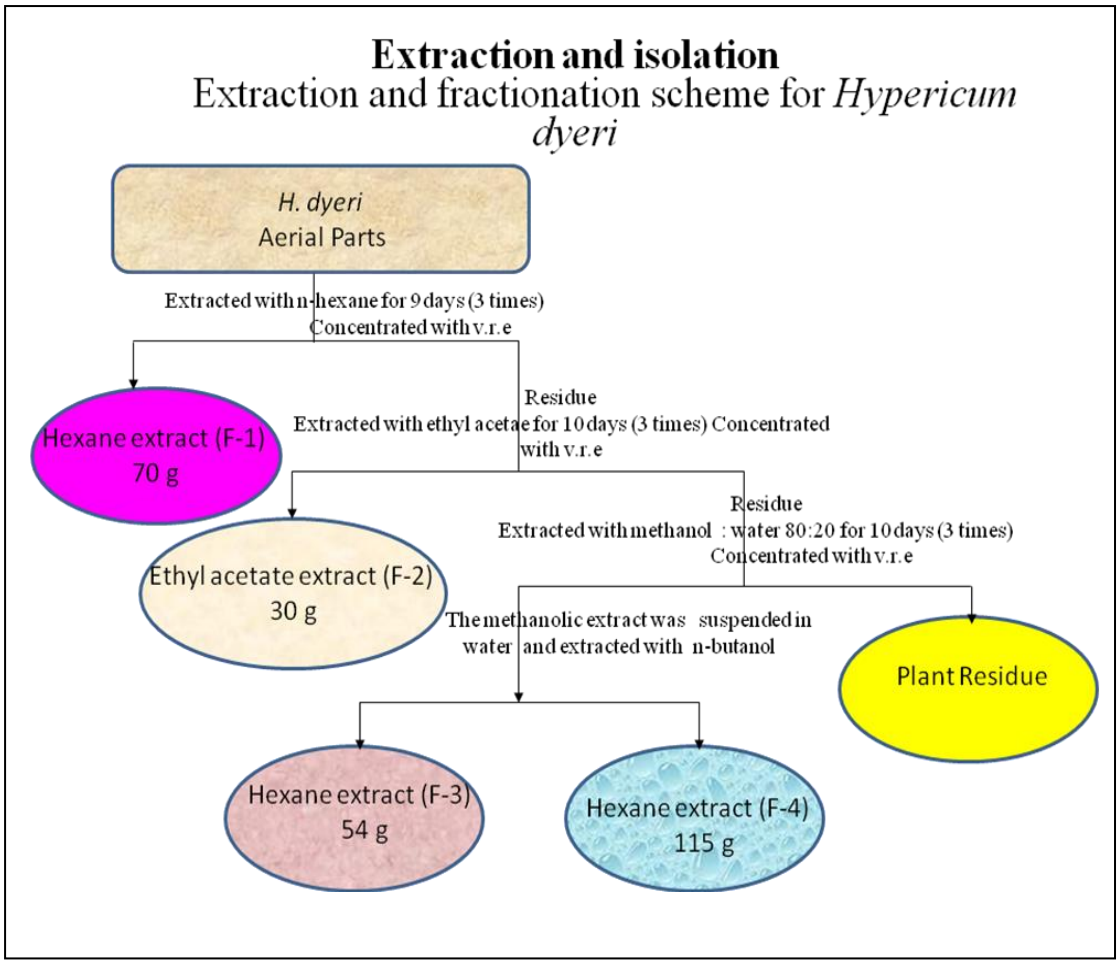


Fig. 7.3: Extraction and fractionation scheme for the aerial parts of *H. dyeri*

7.3.3: Experimental data of the compounds from the aerial parts of *H. dyeri*

7.3.3.1: 1-Octatriacontanol (134)

IUPAC name: 1-Octatriacontanol

Physical state: White amorphous solid

Yield: 9 mg (3.03×10^{-4} %)

Melting point: 63-66C^0

IR ν_{max} (KBr): 3461, 2928, 2843, 1740, 1280, 1160 cm^{-1}

EI-MS (70.0 eV): *m/z* 551.0 (calc. for $[C_{38}H_{78}O+H]^+$)

^{1}H NMR (400 MHz, $CDCl_3$) δ_H: 3.62 (2H, t, J = 7.13 Hz, CH_2-1); 1.53-1.563 (4H, m, CH_2, 2, 37); 1.23-1.293 (68H, m, CH_2-3-36); 0.842 (3H, t, J = 6.93 Hz, CH_3-38)

^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 62 (CH_2-1), 32.9 (CH_2-2), 31.97(CH_2-3), 29.3-29.6 (32x CH_2-4-35), 25.7 (CH_2-37), 22.5 (CH_2-36), 14.08 (CH_3-38).

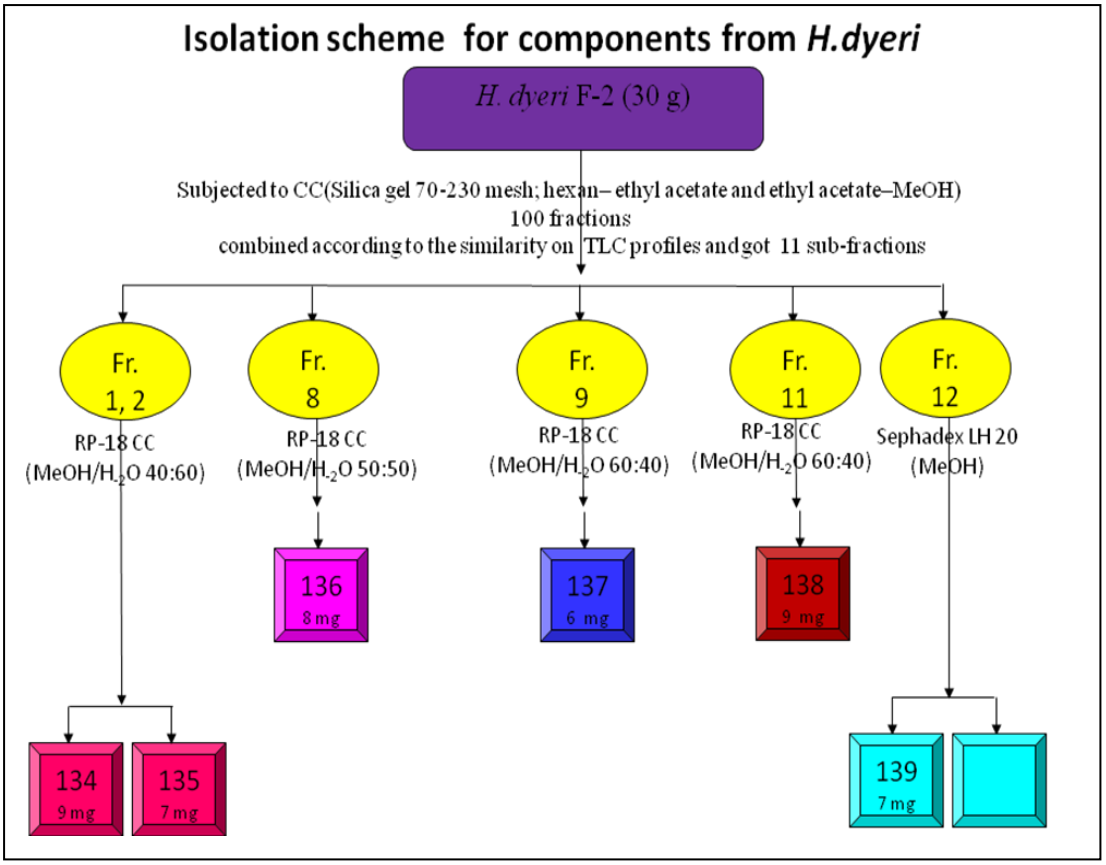


Fig.7.4. Isolation scheme of compound isolated from *Hypericum dyeri*

The physical and spectral data showed complete agreement with those reported in literature[171]

7.3.3.1: Hexacosyl tetracosanoate (135)

IUPAC name: Hexacosyl tetracosanoate

Physical state: White amorphous solid

Yield: 7 mg (2.23×10^{-4} %)

Melting point: 79-81C^0

IR ν_{max} (KBr): 2928, 2843, 1740cm^{-1}

EI-MS (70.0 eV) : *m/z* 733.0 (calc. for $[C_{50}H_{100}O_2+H]^+$)

^{1}H NMR (400 MHz, $CDCl_3$) δ_H:4.02 (2H, t, *J* = 7.22 Hz, CH_2-25), 2.26, t (2H, t, *J* = 7.12 Hz, CH_2-23), 1.57 (4H, m, CH_2-22, 26); 1.23-1.32 (86H, br s, CH_2-2-21, 27-49), 0.84 (6H, t, *J* = 6.92 Hz, CH_3-1,50)

^{13}C NMR (100 MHz, $CDCl_3$), δ_C: 173.9(C-24), 64 (CH_2-25), 34.4 (CH_2-23), 31.97 (2xCH_2-22, 26), 29.3-29.6 (41x CH_2-3-21, 27-48), 25.7 (CH_2), 22.5 (2xCH_2-2, 49), 14.08 (2x CH_3-1,50).

The physical data showed complete resemblance with those reported in literature[172] and complete spectral data presented here for the first time.

7.3.3.3: Geddic acid (136)

IUPAC name: Tetratriacontanoic acid

Physical state: White amorphous solid

Yield: 8 mg (2.67 x 10^{-4} %)

Melting point: 75-76C^0

IR ν_{max} (KBr): 3220-2540, 1720, 1160 cm^{-1}

EI-MS (70 .0eV) : *m/z* 507.0 (calc. for $[C_{34}H_{68}O_2+H]^+$)

^{1}H NMR (400 MHz, $CDCl_3$) δ_H: 2.32 (2H, t, J = 6.82 Hz, CH_2-2), 1.57-1.552 (2H, m, CH_2-3), 1.4-1.22 (60H, br s, CH_2-4-33), 0.843 (3H, t, J = 6.73 Hz, CH_3-34)

^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 173.29(C-1), 34.54 (CH_2-2), 31.77 (CH_2-3), 29.3-29.6 (28x CH_2-4-31), 24.8 (CH_2-33), 22.6 (CH_2-32), 14.11 (CH_3-34).

The physical data showed complete resemblance with those reported in literature[174] and complete spectral data presented here for the first time

7.3.3.4: β-Sitosterol (137)

IUPAC name: 24-Ethylcholest-5-en-3-ol

Physical state: White crystal

Yield: 6 mg (2.0x 10^{-4} %)

Melting point: 136-38C^0

λ_{max}($CHCl_3$) nm (log ε): 230(4.1), 325(3.67),

IR ν_{max} (KBr): 3408, 1628, 1379 and 1065cm^{-1}

EI-MS (70.0 eV) : *m/z* 414.0 (calc. for $[C_{29}H4_{50}O]^+$)

^{1}H (400 MHz) and ^{13}C NMR (100MHz, $CDCl_3$): Given in **Table 6.23**

7.3.3.5: Octacosanoic acid (138)

IUPAC name Octacosanoic acid acid

Physical state: White amorphous solid

Yield: 9 mg (3.03 x 10^{-4} %)

Melting point: 90-92C^0

IR ν_{max} (KBr): 3300-2610, 1705, 1160 cm^{-1}

EI-MS (70 eV) : *m/z* 424.0 (calc. for $[C_{28}H_{56}O_2+H]^+$)

^{1}H NMR (400 MHz, $CDCl_3$) δ_H: 2.352(2H, t, *J* = 6.72 Hz, CH_2-2), 1.57-1.555 (2H, m, CH_2-3), 1.4-1.23 (48H, s, CH_2-4-27), 0.845 (3H, t, *J* = 6.65 Hz, CH_3-28)

^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 179.96 (C-1), 33.9 (CH_2-2), 31.97 (CH_2-3), 29.3-29.6 (21x CH_2-4-25), 24.8 (CH_2-27), 22.6 (CH_2-26), 14.09 (CH_3-28)

The physical data showed complete resemblance with those reported in literature[175] and complete spectral data presented here for the first time.

7.3.3.6: Ceric acid (139)

IUPAC name: Hexacosanoic acid

Physical state: White amorphous solid

Yield: 7 mg (2.43 x 10^{-4} %)

Melting point: 88- 90C^0

IR ν_{max} (KBr): 3320-2620, 1705, 1160 cm^{-1}

EI-MS (70.0 eV) : *m/z* 396.0 (calc. for $[C_{26}H_{52}O_2+H]^+$)

^{1}H NMR (400 MHz, $CDCl_3$) δ_H: 2.12 (2H, t, *J* = 6.92 Hz, CH_2-2), 1.57-1.552 (2H, m, CH_2-3), 1.15 (44H, s, CH_2-4-25), 0.784 (3H, t, *J*= 6.82 Hz, CH_3-26)

^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 179.6 5(C-1), 33.95 (CH_2-2), 31.7 (CH_2-3), 29.3-29.6 (20x CH_2-4-23), 24.8 (CH_2-25), 22.6 (CH_2-24), 14.09 (CH_3-26)

The physical data showed complete resemblance with those reported in literature[176] and complete spectral data presented here for the first time.

References

(1) Mabberley, D. G. *The Plant-Book,A Pportable dictionary of the higher plants;*; Cambridge University Press: Cambridge, 1987.

(2) Rockit, H. W. *Wild flowr of the United States*; McGraw Hill Book Company: New York, 1966.

(3) Roboson, N. K. B. *Bull.Br.Mus.* **1977**, *5*, 293.

(4) Taskhtajan, A. L. *Bat.Rev.* **1980**, *46*, 225.

(5) Lslie, A. A.; Sinthamabay, B.; Vijaya, K. *Phytochemistry* **1979**, *18*, 182.

(6) Fuller, R. W.; Westegaard, C. K.; Collins, J. W.; Cardelina II, J. H.; Boyd, M. R. *J. Nat. Prod.* **1999**, *62*, 67.

(7) McKee, T. C.; Covington, C.; Fuller, R. W.; Bokesch, L. R.; Young, S.; Cardellina II, J. H. *J. Nat. Prod.* **1998**, *61*, 1252.

(8) Nasir, A.; Ali, S. I. *Flora of West Pakistan*; Shamim Printing Press: Karachi, 1973.

(9) Chopra, R. N.; Chopra, I. C.; Verma, B. S. *Supplement to Glossary of Indian Medicinal Plants* 1998.

(10) Ferheen, S.; Ahmed, E.; Malik, A.; Afza, N.; Lodhi, M. A.; Choudhary, M. I. *Chem. Pharm. Bull.* **2006**, *54*, 1088

(11) Yazaki, K.; Okada, T. *Biotech in Agri. Forest.* **1999**, *26*, 167.

(12) Anthansas, K.; Magiatis, P.; Fokialakis, N.; Skaltsounis, A. L.; Pratsinis, H.; Kletsas, D. *J. Nat. Prod.* **2004**, *67*, 973-977.

(13) Miller, N. D. *Altern. Med. Rev.* **1998**, *3*, 1826.

(14) Gunther, R. T. *The Greek Herbal of Dioscorides*; Hahner Pub.Co., 1933.

(15) Vogal, V. *Amirican Indian Medicines*; University of Oklahoma Press: Norman, 1970.

(16) Chavez, M. L. *Hosp.Pharma.* **1997**, *23*, 1621.

(17) Patocka, J. *J.Appl. Biomed.* **2003**, *1*, 61.

(18) El-Seedi, H. R.; Ringbom, T.; Torssell, K.; Bohlin, L. *Chem. Phar. Bull.* **2003**, *51*, 1439.

(19) Yin, Z. Q.; Wang, Y.; W.C.Ye; Zhao, S. X. *Biochem. Syst. Ecol.* **2004**, *32*, 521

(20) Dias, A. C. P.; Tomas-Barberan, F. A.; Fernandes-Ferreira, M.; Ferrers, F. *Phytochemistry* **1998**, *48*, 1165.

(21) Klingauf, P.; Beuerle, T.; Mellenthin, A.; El-Moghazy, S. A. M.; Boubakir, Z.; Beerhues, L. *Phytochemistry* **2005**, *66*, 139.

(22) Hudson, J. B.; Bazzocchi, I.; Tower, G. H. N. *Antivir.Res.* **1991**, *15*, 101

(23) Change, S. U. *Dictionary of Chinese Crude Druds*; Shamhgia Scientific Technological Publisher: Shanghi, 1977.

(24) William, I. P.; Francis, J. *J.Am.Chem.Soc.* **1968**, 4716-23.

(25) Chopra, R. M.; Chopra, J. C.; Handa, K. L.; kapur, L. D. *Chopra,s Indian genous Drugs*; U.N Dhur and sons Private Ltd: Calcutta, 1958.

(26) AN, T. Y.; HU, L. H.; CHEN, Z. L. *Chin. Chem. Lett.* **2002**, *13*, 623.

(27) Don, M.-J.; Huang, Y.-J.; Huang, R.-L.; Lin, Y.-L. *Chem. Phar. Bull.* **2004**, *52*, 866.

(28) Hu, L.-H.; Sim, K.-Y. *Tetrahedron Lett.* **1998**, *39*, 7999

(29) Lin, Y.-L.; Wu, Y.-S. *Helv.Chem.Act.* **2003**, *86*, 2156.

(30) Mentz, L. a.; Lutzember, L. C. *Cardin.Pharm.* **1997**, *15*, 25.

(31) Ishiguro, K.; Yamaki, M.; Kashihara, M.; Takagi, S. *Planta Med.* **1986**, *52*, 288.

(32) Ishigur, K.; Yamamoto, R.; Oku, H. *J. Nat. Prod.* **1999**, *62*, 906.

(33) Ql, W.; SP, W.; LJ, D.; JS, Y.; PG, X. *Phytochemistry* **1998**, *49*, 1395.

(34) Hu, L.-H.; Yip, S.-C.; Sim, K.-Y. *Phytochemistry* **1999**, *52*, 1371.

(35) Tanaka, N.; Takaishi, Y.; shikishima, Y.; Nakanishi, Y.; Bastow, K.; Lee, K.-H.; Honda, G.; Ito, m.; Takeda, Y.; Kodzhimatov, O. K.; Ashurmetov, O. *J. Nat.Prod.* **2004**, *67*, 1870.

(36) Porsolt, R. D.; Anto, G. *Europ.J.Pharmacol.* **1978**, *47*, 379.

(37) Gnerre, C.; Van Poser, G. L. *J.Pharm.Pharmacol.* **2001**, *53*.

(38) Dulger, B.; Gonuz, A.; Bilen, S. *South Afric.J.Bot.* **2005**, *71*, 101.

(39) Vijayan, P.; Raghu, C. *Indian.J.Med.Res.* **2004**, *120*, 24.

(40) Atalay *Turk.J.Biol.* **2001**, *25*, 343.

(41) Oztruk, P. *Pharmacopsychaitry* **1997**, *30*, 125.

(42) Mukherjee, P. K.; Suesh, B. *J.Altern. Compl. Med.* **2002**, *6*, 61.

(43) Fraklin, M.; Reed, A.; Murk, H. *Sub-Chronic treatment with an extract of H.perforatum*; Oxford University Publication: Oxford, 2004.

(44) Trovato, A.; Raneri, E. *Farmaco.* **2001**, *56*, 455.

(45) Baureithel, K. H.; Butter, K. B.; Engesser, A.; Burkard, W. *Pharm. Acta. Helve.* **1997**, *72*, 153.

(46) Rocha, L.; Marston, A.; Potterat, O.; Kaplan, M. A. C.; Stoeckli Evans, H. *Phytochemistry* **1995**, *40*, 1447.

(47) Alexandre, B. F.; Ferraz, S., A. L; Bordignon, C. S.; Schripsema, J.; von Poser, G. L. *Phytochemistry* **2001**, *57*, 1227.

(48) Ana Paula, M. B.; Alexandre, B. F. F.; Daniela, V. A.; Sérgio, A. L. B.; Jan, S.; Raquel, B.; Carlos, S. D.; Amélia, T. H.; Gilsane, L. v. *J. Nat. Prod.* **2005**, *68*, 784.

(49) Tada, M.; Chiba, K.; Yamada, H.; Maruyama, H. *Phytochemistry* **1991**, *30*, 2559.

(50) Kosuge, T.; Ishida, H.; Satoh, T. *Chem. Pharm. Bull.* **1985**, 202.

(51) Zou, Y. P.; Lu, Y. H.; Wei, D. Z. *J. Agric. Food Chem.* **2004**, *52*, 5032.

(52) Zou, Y. P.; Lu, Y. H.; Wei, D. Z. *J. Agric. Food Chem.* **2005**, *53*, 2462.

(53) Reichling, J.; Weseler, A.; Saller, R. *Phytochemistry* **2001**, *58*, s116.

(54) Simon, G.; Elisabeth, M.; Sebastian, H.; Michael, S.; Eileen, S.; Christopher, C. *Phytochemistry* **2005**, *66*, 1472.

(55) Cakir, O. A.; Mavi, A.; Yildirim, A.; Duru, M. E.; Harmandar, M.; Kazaz, C. *J. Ethnopharmacol.* **2003**, *87*, 73.

(56) Sommer, H.; Harrer, G. *J.Geriat. Psych. Neurol.* **1994**, *7*, S9.

(57) Wagner, H.; Bladt, S. *J.Geriat. Psych. Neurol.***1994**, *7*, S65.

(58) Hu, L. H.; Sim, K. Y. *Tetrahedron* **2000**, *56*, 1379.

(59) Bennett, G. J.; Lee, H. H. *Phytochemistry* **1989**, *28*, 967.

(60) Jayasuriya, H.; Clark, A. M.; McChesney, J. D. *J. Nat. Prod.* **1991**, *54*, 1314.

(61) Tanaka, N.; Okasaka, M.; Ishimaru, Y; Takaishi, Y.; Sato, M.; Okamoto, M.; Oshikawa, T.;Ahmed, S.U.;Consentino, L. M.; Lee, K.H. *Org.Lett.***2005**, 2997

(62) Gibbons, S.; Moser, E.; Hausmann, S.; Stavri, M.; Smith, E.; Clennet, C. *Phytochemistry* **2005**, *66*, 1472.

(63) Wilairat, R.; Manosroi, J.; Manosroi, A.; Kijjoa, A.; Nascimento, M. S. J.; Pinto, M.; Silva, A. M. S.; Eaton, G.; Herz, W. *Planta Med.* **2005**, *71*, 680.

(64) Daylin, G.; Osmany, C.-R.; Sylvia, P.-G.; Francesco, D. S.; Siro, P.; Luca, R. *J. Nat. Prod.* **2004**, *67*, 869.

(65) Athanasas, K.; Magiatis, P.; Fokialakis, N.; Skaltsounis, A.-L.; Pratsinis, H.; Kletsas, D. *J. Nat, Prod.* **2004**, *67*, 973.

(66) Shan, M. D.; An, T. Y.; Hu, L. H.; Chen, Z. L. *Nat. Prod. Res.* **2004**, *18*, 15.

(67) Winkelmanna, K.; Sana, M.; Kypriotakisb, Z.; Skaltsac, H.; Bosilijd, B.; Heilmann, J. *Naturforsch* **2003**, *58c*, 527.

(68) Rushdey, E.S. H.; Therese, R.; Kurt, T.; Lars, B. *Chem. Phar. Bull.***2003**, *51*, 1439

(69) Savikin-Fodulovic, K.; Aljancˇic, I.; Vajs, V.; Menkovic, N. a.; Macura, S.; Gojgic, G.; Milosavljevic, S. *J. Nat. Prod.* **2003**, *66*, 1236.

(70) An, T.-Y.; Hu, L.-H.; Chen, Z.-L.; Sim, K.-Y. *Tetrahedron Lett.* **2002**, *43*, 163

(71) An, T.-Y.; Shan, M.-D.; Hua, L.-H.; Liu, S.-J.; Chen, Z.-L. *Phytochemistry* **2002**, *59*, 395.

(72) Matsuhisa, M.; Shikishima, Y.; Takaishi, Y.; Honda, G.; Ito, M.; Takeda, Y.; Shibata, H.; Higuti, T.; Kodzhimatov, O. K.; Ashurmetov, O. *J. Nat. Prod.* **2002**, *65*, 290.

(73) Nedialkov, P. T.; Kitanov, G. M. *Phytochemistry* **2002**, *59*, 867.

(74) Winkelmann, K.; Heilmann, J.; Zerbe, O.; Rali, T.; Sticher, O. *J. Nat. Prod.* **2001**, *64*, 701.

(75) Jing-Ru, W.; Mei-Ing, C.; Ming-Hong, Y.; Chun-Nan, L.; Ru-Rong, W. *Helv.Chem.Act.* **2001**, *84*, 1976.

(76) Ferraza, A. B. F.; Bordignonb, S. A. L.; Staatsa, C.; Schripsemac, J.; Posera, G. L. v. *Phytochemistry* **2001**, *57*, 1227.

(77) Winkelmann, K.; Heilmann, J.; Zerbe, O.; Rali, T.; Sticher, O. *Helv.Chem.Act.* **2001**, *84*, 3380.

(78) Kitanov, G. M.; Nedialkov, P. T. *Phytochemistry* **2001**, *57*, 1237.

(79) Shan, M. D.; An, T. Y.; Hu, L. H.; Chen, Z. L. *J. Nat. Prod.* **2001**, *64*, 127.

(80) Shan, M. D.; Hu, L. H.; Chen, Z. L. *Chin. Chem. Lett.* **2000**, *11*, 701.

(81) Winkelmann, K.; Heilmann, J.; Zerbe, O.; Rali, T.; Sticher, O. *J. Nat, Prod.* **2000**, *63*, 104.

(82) Hu, L.-H.; Khoo, C.-W.; Vittal, J. J.; Sim, K.-Y. *Phytochemistry* **2000**,*53*, 705.

(83) Verotta, L.; Appendino, G.; Jakupovic, J.; Bombardelli, E. *J. Nat. Prod.* **2000**, *63*, 412

(84) Verotta, L.; Appendino, G.; Belloro, E.; Jakupovic, J.; Bombardelli, E. *J. Nat. Prod.* **1999**, *62*, 770.

(85) Chung, M.-I.; Weng, J.-R.; Lai, M.-H.; Yen, M.-H.; Lin, C.-N. *J. Nat. Prod.* **1999**, *62*, 1033.

(86) Hu, L.-H.; Sim, K.-Y. *Tetrahedron Lett.* **1999**, *40*, 759.

(87) Hu, L.-H.; Sim, K.-Y. *Org.Lett.* **1999**, *1*, 879.

(88) Wu, Q. L.; Wang, S. P.; Du, L. J.; Zhang, S. M.; Yang, J. S.; Xiao, P. G. *Phytochemistry* **1998**, *49*, 1417.

(89) Wu, Q. L.; Wang, S. P.; Du, L. J.; Zhang, S. M.; Yang, J. S.; Xiao, P. G. *Phytother.Res.* **1998**, *12*, S164.

(90) Ishigro, K.; Nagareya, N.; Fukumoto, H. *Phytochemistry* **1998**, *47*, 1041.

(91) Chung, M. I.; Lai, M. H.; Yen, M.-H.; Wu, R.-R.; Lint, C.-N. *Phytochemistry* **1997**, *44*, 943.

(92) Wu, Q. L.; Wang, S. P.; Du, L. J.; Zhang, S. M.; Yang, J. S.; Xiao, P. G. *Chin. Chem. Lett.* **1996**, *7*, 1095.

(93) Rocha, L.; Marston, A.; Potterata, O.; Kaplanb, M. A. C.; Hostettmann, K. *Phytochemistry* **1996**, *42*, 185.

(94) Achenbach, H.; Schwinn, A. *Phytochemistry* **1995**, *38*, 1037.

(95) Aramak, Y.; Chiba, K.; Tada, M. *Phytochemistry* **1995**, *38*, 1419.

(96) Ishiguro, K.; Nagata, S.; Fukumoto, H.; Yamaki, M.; Isoi, K.; Yamagata, Y. *Phytochemistry* **1994**, *37*, 283.

(97) Rocha, L.; Marston, A.; Auxiliadora, M.; Kaplan, C.; Stoeckli-Evans, H.; Thull, U.; Testa, B.; Hostettmann, K. *Phytochemistry* **1994**, *36*, 1381.

(98) Ishiguro, K.; Nagata, S.; Fukumoto, H.; Yamaki, M.; Isoi, K.; Oyama, Y. *Phytochemistry* **1993**, *32*, 1583.

(99) Cardona, L.; Pedro, J. R.; Serrano, A.; Muñoz, M. C.; Solans, X. *Phytochemistry* **1993**, *33*, 1185.

(100) Maisenbacher, P. *Planta Med.* **1992**, *58*, 291.

(101) Rios, M. Y.; Delgado, G. *Phytochemistry* **1992**, *31*, 3491.

(102) Jayasuriya, H.; Clark, A. M.; McChesney, J. D. *J. Nat. Prod.* **1991**, *54*, 1314.

(103) Ishiguro, K.; Yamaki, M.; Kashihara, M.; Takagi, S.; Isoi, K. *Phytochemistry* **1990**, *29*, 1010.

(104) Jayasuriya, H.; Clark, A. M.; McChesney, J. D. *J. Nat, Prod.* **1989**, *52*, 325.

(105) Nagai, M. *Chem.Lett.* **1987**, 1337.

(106) Décostered, L. A.; Hostettmann, K.; Stoeckli-Evans, H.; Msonthi, J. D. *Helv.Chem.Act.* **1987**, *70*, 1694.

(107) Cardona, M. L.; Fernández, M. I.; Pedro, J. R.; Seoane, E.; Vida, R. *J. Nat, Prod.* **1986**, *49*, 95.

(108) Tohru, K.; Shigetoshi, K.; Satoko, M.; Tanaka, K. *Chem. Phar. Bull.* **1985**, *33*, 1969.

(109) Tohru, K.; Shigetosh, K.; Satoko, M.; Ken, T.; Tsuneo, N. *Chem. Phar. Bull.* **1985**, *33*, 557.

(110) Banks, H. J. *Aust.J.Chem.* **1976**, *29*, 1509.

(111) Coll, R. *Organic Syntheses*, 1941, Vol. 1; p.552

(112) Cardona, L.M.; Isabel, F.; Jose, P. R.; Angel., S. *Phytochemistry* **1990**,*29*,3003

(113) Peres, V.; Nagem, T. J. *Phytochemistry* **1997**, *44*, 191-214.

(114) Oku, H.; Ueda, Y.; Linuma, M.; Ishigro, K. *Planta Med.* **2005**, *71*, 90.

(115) Wilairat, R.; Manosroi, J.; Manosroi, A.; Kijjoa, A.; Nascimento, M. S. J.; Pinto, M.; Saliva, A. M. S.; Eaton, G.; Herz, W. *Planta Med.* **2005**, *71*, 680

(116) Iinuma, M.; Tosa, H.; Tanaka, T.; Yonemori, S. *Phytochemistry* **1994**, *35*, 527

(117) Steiner, L. F.; Summerland, S. A. *J.Econom. Entom.* **1943**, *36*, 435.

(118) Kitanov, G. M.; Blinova, K. F. *Chem.Nat.Comds* **1987**, *2*, 151.

(119) Suzuki, O.; Katsumata, Y.; Oya, M.; Chari, V. M.; Klapfenberger, R.; Wagner, H.; Hostettmann, K. *Planta Med.* **1980**, *39*, 19.

(120) Suzuki, O.; Katsumata, Y.; Oya, M.; Chari, V. M.; Klapfenberger, R.; Wagner, H.; Hostettmann, K. *Planta Med.* **1981**, *42*, 17.

(121) Fukuyama, Y., Kamiyama, A., Mima, Y.,Kodama, M. *Phytochemistry* **1991**, *30*, 3433.

(122) Xin-Fang, G., Chang-Piau, C. *ChungTsao Yo* **1980**, *11*, 200.

(123) Hambloch, H., Frahm, A. W. *Eur. J.Med. Chem. Chim. Ther.* **1985**, *20*, 71.

(124) Naonobu, T.; Yoshihisa, T. *Chem. Pharm. Bull.* **2007**, *55*, 19-21.

(125) Fu, P.; Zhang, W.-D.; Liu, R. H.; Li, T.-Z.; Shen, Y.-H.; Li, H.-L.; Zhang, W.; Chen, H.-S. *Nat. Prod. Res. Part A* **2006**, *20*, 1237.

(126) Naonobu, T.; Yoshihisa, T. *Phytochemistry* **2006**, *67*, 2146.

(127) Fu, P.; Zhang, W.-D.; Liu, R. H.; Li, T.-Z.; Shen, Y.-H.; Li, H.-L.; Zhang, W.; Chen, H.-S. *Chin. Chem. Lett.* **2005**, *16*, 771.

(128) Franco, F.; Gabriella, P.; Barbara, M.; Paola, C.; Bruno, B. *Nat. Prod. Res. Part A* **2005**, *19*, 171.

(129) Hong, D.; Yin, F.; Hu, L.-H.; Lu, P. *Phytochemistry* **2004**, *65*, 2595.

(130) Ishigro, K.; Nagareya, N.; Fukumoto, H. *Planta Med.* **2002**, *68*, 258.

(131) Mei-Ing, C.; Jing-Ru, W.; Jih-Pyang, W.; Che-Ming, T.; Chun-Nan, L. *Planta Med.* **2002**, *68*, 25.

(132) Morel, C. *Molecules* **2002**, *7*, 38.

(133) Mei-Ing, C.; Jing-R, W.; Mei-Hsun, L.; Ming-Hon, Y.; Chun-Nan, L. *J. Nat. Prod.* **1999**, *62*, 1033.

(134) Wu, Q. L.; Wang, S. P.; Du, L. J.; Zhang, S. M.; Yang, J. S.; Xiao, P. G. *Phytochemistry* **1998**, *49*, 1395.

(135) Ishiguiro, K.; Nagareya, N.; Suitani, A.; Fukumoto, H. *Phytochemistry* **1997**, *44*, 1065.

(136) Guido, R.; Olivier, P.; Stephen, M.; Kurt, H. *Phytochemistry* **1996**, *43*, 513-20.

(137) Kyoko, I.; Hisae, F.; Akiko, S.; Mariko, N.; Koichiro, I. *Phytochemistry* **1996**, *42*, 435.

(138) Kyoko, I.; Hisae, F.; Akiko, S.; Mariko, N.; Koichiro, I. *Phytochemistry* **1995**, *38*, 867.

(139) Kyoko, I.; Hisae, F.; Akiko, S.; Mariko, N.; Koichiro, I. *Phytochemistry* **1995**, *39*, 903.

(140) Kyoko, I.; Hisae, F.; Mariko, N.; Koichiro, I. *Phytochemistry* **1993**, *33*, 839.

(141) Kyoko, I.; Hisae, F.; Mariko, N.; Koichiro, I. *Phytochemistry* **1993**, *32*, 1583.

(142) Hano, Y. *Heterocycles* **1990**, *31*, 1345.

(143) Cardona, L.M.; Isabel, F.; Jose, P.R.; Angel., S. *Phytochemistry* **1989**,*29*,2297.

(144) Balachandran, S.; Vishwakarma, R.A.; Popli, S.P. *Ind. J.Chem.* **1988**,*27B*, 385

(145) Chen, M.-T. *Heterocycles* **1988**, *27*, 2589.

(146) Vishwakarma, R. A.; Kapil, R. S.; Popli, S. P. *Ind. J.Chem.* **1986**, *25B*, 1155.

(147) Cardona, M. L.; Fernandez, M. I.; Pedro, J. R.; Seoane, E.; Vidal, R. *J. Nat, Prod.* **1985**, *48*, 467

(148) Chen, M. T.; Chen, C. M. *Heterocycles* **1985**, *23*, 2543.

(149) Gunatilanka, A. A.; Leslie, D.; Silva, A. M. Y.; Jasmin, S. S. *Phytochemistry* **1982**, *21*, 1751.

(150) Cardona, L. M.; Eliseo, S. *J. Nat.Prod.* **1982**, *45*, 134.

(151) Nielsen, H.; Arends, P. *J. Nat. Prod.* **1979**, *42*, 303.

(152) Arends, P. *Tetrahedron Lett.* **1969**, 4869.

(153) Wu, C.-C.; Yen, M.-H.; Yang, S.-H.; Lin, C.-N. *J. Nat. Prod.* **2008**, *71*, 1027.

(154) Castelao, J. F.; Gottlieb, O. R.; De Lima, R. A.; Mesquita, A. A. L. *Phytochemistry* **1977**, *16*, 735.

(155) Zhang, Z.; ElSohly, H. N.; Jacob, M. R.; Pasco, D. S.; Walker, L. A.; Clark, A. M. *Planta Med.* **2002**, *68*, 49.

(156) Patanaik, M. *Acta.Chem. Scand.,Ser.B* **1987**, *41*, 210.

(157) Russell, G. K.; Richard, V.; Stacey, S. K. *J.Nat.Prod.* **1999**, *62*, 468.

(158) Tosa, H., Iinuma, M., Murakami, K.I., Ito, T., Chelladurai, V.S., *Phytochemistry* **1997**, *45*, 133.

(159) Nagem, T. J.; Silverira, J. C. *Phytochemistry* **1986**, *25*, 2681.

(160) Gottlieb, O. R.; Magalhães, M. T.; Camey, M.; Mesquita, A. A. L.; de Barros Corrêa, D. *Tetrahedron* **1966**, *22*, 1777.

(161) Cardona, M. L.; Fernandez, M. I.; Pedro, J. R.; Serrano, A. *Phytochemistry* **1990**, *29*, 3003.

(162) De M. Pinto, M. M.; Mesquita, A. A. L.; Gottlieb, O. R. *Phytochemistry* **1987**, *26*, 2045.

(163) Gunatilaka, A. A. L.; Balasubramaniam, S.; Kumar, V. *Phytochemistry* **1979**, *18*, 182.

(164) Ghosal, S.; Chauhan, R. B. P. S.; Biswas, K.; Chaudhuri, R. K. *Phytochemistry* **1976**, *15*, 1041.

(165) Balachandran, S.; Vishwakarma, R. A. *Indian. J. Chem.* **1988**, *27B*, 385.

(166) Minami, H.; Takahashi, E.; Kodama, M.; Fukuyama, Y. *Phytochemistry* **1996**, *41*, 629.

(167) Mukhtar, H. M.; Ansari, S. H.; Ali, M.; Naved, T.; Bhat, Z. A. *Pharmaceut. Biol.* **2005**, *43*, 392.

(168) Lee, T. H.; Chiou, J.L.; Lee, C.K.; Kuo, Y.H. *J. Chin. Chem. Soc.***2005**,*52*,833.

(169) Tariq, S.; Ferheen, S.; Moazzam, M.; Jabbar, A.; Riaz, N.; Saleem, M.; Afza, N.; Malik, A.; Tareen, R. B. *J.Chem.Soc.Pak.* **2008**, *30*, 762.

(170) Adam, K. P. *Phytochemistry* **1999**, *52*, 929.

(171) Singh, H.; Chawla, A. S.; K.Kapoor, V.; Kumar, N.; Piatak, D. M.; Nowicki, W. *J.Nat.Prod.* **1981**, *44*, 526.

(172) Chandra, G.; Clark, J.; McLean, J.; Pauson, P. L.; Watson, J.; Reed, R. I.; Tabrizi, F. M. *J.Chem.Soc.* **1964**, 3648.

(173) Otsuka, H.; Fujioka, S.; Komiya, T.; Goto, M.; Hiramatsu, Y.; Fujimura, H. *Chem. Pharm. Bull.* **1981**, *29*, 3099.

(174) Banerji, J.; Kadas, K.; Ghoshal, N.; Das, B. *Ind.J.Chem.,Sect,B* **1988**, *27*, 594

(175) Misra, G.; Voigt, D.; Nigam, S. K. *Planta Med.* **1975**, *28*, 165.

(176) Watanabe, S. *Z. Naturforsch.C* **1975**, *30*, 825.

PART C

Phytochemical Studies of the Selected Species of Family Pinaceae

Chapter: 8

INTRODUCTION (Part C)

8.1: Introduction

8.1.1: Family Pinaceae

The family Pinaceae (pine family) belongs to the order pinales and is a commercially important family with useful plants such as cedars, firs, hemlocks, larches, pines and spruces. It is the second largest family after Cupressaceae with 220-250 species in 11 genera. They are found mostly in the Northern Hemisphere with the majority of the species in temperate climates but ranging from subarctic to tropical. There are four genera (*Pinus, Abies, Picea,Cedrus)* and nine species of this family in Pakistan. Most of the species are trees which are often excellent sources of lumber, wood products, timber, paper, resins and are cultivated for forestation as well as ornamentals plants[1,1a]. The Genus *Pinus* is the largest genus of this family with 120 species. The diverse nature of this genus can be witnessed in the mountains of southwest China, central Japan, California and Mexico[2]. The members of Pinaceae are prolific producers of resin defense which is a mixture of monoterpenoids, sesquiterpenoids and diterpenoids [3]. The chemical constituents of some species including *P. abies* [4] *P. glauca* [5] and *P.glehni* [6]. *P. morrisonicola* have been studied. These components contain lignans, flavonoids and their glucosides as well as diterpenoids of abietane-type diterpenes and norabietane derivatives[7].

8.1.2: Pharmacological importance of family Pinaceae

The conifers family, Pinaceae has recently attracted a great deal of attention as a source of pharmacologically active procyanidins, one subclass of proanthocyanidins [8,9]. The use of procyanidins may be traced back to ancient traditional medicine in both the Old World and in the Americas. The bark of the pine has been used for more than 20 centuries. Pine bark was used for wound healing[10]. Essential oils derived through steam distillation of needles and bark of *Pinus* and *Picea* species are widely used as ointments,

bathing oils or inhaling drugs for curing a wide range of bronchial, skin, and muscle-disorders of infectious, rheumatic or neuralgic origin[11]. In the New World, Americans utilized the bark of the pine as beverage, food and also remedy for various conditions , such as inflamed wounds or ulcers, now recognized to have free radical involvement [10,12]. The procyanidins extracted from the bark of *P. pinaster* was used as nutritional supplement and remedy for cardiovascular diseases [8]. Turpentine from *P. nigra* has been used for several years in Turkish folk medicine as antiseptic particularly in respiratory and urinary diseases. Additionally, it is used for back pain as resin plaster and as stomachic, dermatological and analgesic drugs [13]. Various plant products, wood tar and resins exhibit antimicrobial effects against human bacteria, and might therefore become tools to treat human infections. Home-made resin salve from Norway spruce (*Picea abies*) is used to heal skin wounds and various skin infections is an example of folk medicine in Finland[14]. The leaves of *Abies webbiana* has been used by the people of west Bengal for the treatment of hyperglycemia, rheumatism and fever[15]. *Cedrus* wood has been used since ancient days in Ayurvedic medicine for the treatment of inflammations and rheumatoid arthritis[16]. The essential oil from *Cedrus atlantica* has been shown to possess antiinflammatory, antifungal and antimicrobial activities. It also proved to be useful in the treatment of hair loss in a combination of aromatherapy oils [17]. In Turkey, a kind of tar is obtained from resinous root and steam wood of *Cedrus libani* (Lebanese cedar) which is used for treating skin diseases in animals and for killing parasites, e.g. aphids, insects, ticks. Lebanese cedar wood oil and was even used to cure leprosy. Cones and leaves of the same plant also possess anti-microbial and anti-ulcerogenic activity[18].

Taxus is a genus of Yews, small coniferous trees or shrubs in the yew family Taxaceae which has recently attracted a great deal of attention as sources for an anticancer agent, Paclitaxel (Taxol), a unique diterpene taxoid originally extracted from the bark of the Pacific yew, *Taxus brevifolia* [19-21]. Taxol, is well-known worldwide as a powerful anticancer agent and clinically used as a therapeutic agent in the treatment of breast and ovarian cancers. In traditional medicine, Yew leaves are reported to be used as abortifacient, anti-malarial, anti-rheumatic and for bronchitis [22-24], while dried leaves and bark were used against asthma [25]. Wood of *Taxus yunnanesis* has been used in the treatment of kidney problem and dilater [26]. Leaves of a *Taxus fuana* have shown anti-

inflammatory, anticonvulsant and antmitotic activities [27]. *Taxus fuana* is a single specie native to Pakistan [28]. Literature revealed that this plant is used in traditional medicine for the treatment of high fever and acute painful conditions (Kaul, 1997). Leaves of the plant are used to make herbal tea for indigestion and epilepsy [29]. Its bark is locally used for the treatment of Hepatitis C in the folk herbal medicine.

Species of conifers have proved to be of special significance among phyto-chemists in recent years because they have been found to possess a number of biological activities. Antioxidant and analgesic activities of turpentine exudates from *Pinus nigra* were studied[13].Tree materials such as knotwood, heartwood, foliage, phloem, bark, and cork of several species have been found to be sources of natural phenolic antioxidants. Chemical constituents of some *Picea* species including *P. abies* [30,] *P. glauca* [31] and *P.glehni* [32] have been studied. The composition of essential oils from *Cedrus libani* [18] and active constituents of *C.deodara*[33] were also studied. *Pinus pinaster* and *P.radiata* were compared for the composition and antiradical activity of procyanidins.[8]. Lignans are widely distributed in the conifers and occur in different parts (Bark, roots, leaves, stem, kotwoods, seeds and fruits). Our research has revealed that knots i.e. branch bases inside tree stems, commonly contain 5-10 (w/w) of lignan whereas knots of *Picea abies* contain extremely large amounts of lignans (6-24% w/ w) with hydroxy matairesinol (HMR) comprising 65–85% of them [34-36]. Lignan are known to have remarkable biological activities, including antibacterial, antifungal, antiviral, antioxidant , anticancer, anti-inflammatory and analgesic effects[37]. The amount and composition of lipophilic and hydrophilic extractives are also having value in the industrial usage of trees and as source of potential bioactive substances as well as in the possible environmental impact. In continuation with the ongoing efforts [38]to investigate the composition of lipophilic and hydrophilic extractives of conifers , endowed with many useful, yet to be explored wealth of valuable chemicals with potential uses in medicine, food, cosmetics and new materials, herein we present the amount and composition of the extractives and proanthocyanidins in the bark of seven Pakistani coniferous tree species.

Chapter: 9

RESULTS AND DISCUSSION (Part C)

9.1: Extractives in bark of different conifer species growing in Pakistan

The amount and composition of lipophilic and hydrophilic extractives as well as proanthocyanidins in the bark of seven Pakistani conifers were analysed. The bioactive polyphenols and other known compounds were found interesting in order to find a potential value-added use of local tree species. However, this work should be taken as a first screening due to the limited number of trees sampled.

Gravimetrically these extracts were analysed for lipophilic and hydrophilic extractactives (Tables 9.3-9.5). *Pinus* species e.g *P wallichiana, P gerardiana* and *Picea smithiana* showed large amounts of lipophilic and hydrophilic extractives as compared to the other examined conifers. *Pinus roxburghii* was found different from the other pine species having smaller amounts of both types of extractives. *A. pindrow* and *T. fuana* were also found to have the smallest amount of hexane extracts. GC and GC-MS analyses could account for around 50 % of most of the extracts.

9.1.1: Lipophilic extractives

The amount and composition of the lipophilic extractives in the barks of the seven coniferious tree specieves have been studied (Tables 9.2, 9.4). The short-chain fatty acids were found dominant as compared to the long-chain fatty acids in all bark samples particularly the oleic acid (C18:1 acid) was the major fatty acid in all studied samples except in *Cedrus deodara,* where lignoceric acid (C24:0 acid) was the prominent fatty acid. Similarly the amount of free fatty acids was found higher than the amount of triglycerides in all cases except *Taxus fauna* whe*re* the amount of triglycerides was not determined. *Pinus wallichiana*, *Pinus gerardiana* and *Picea smithiana* showed the largest amounts of fatty acids and fatty alcohols whereas *P. roxburghii* showed the smallest amounts among the studied species.

The resin acid was found as the prominent constituent of the bark of *Pinus gerardiana* while *A. pindrow* and *T. fuana* showed the lowest as compared to the other species. *Cedrus deodara* and *Picea smithiana* contained considerable amount of resin acid. Free sterols was found approximately at the same level or larger than the amount of steryl esters in most samples (the amount of steryl esters was not determined for *T. fuana*). *P. gerardiana* contained clearly more steryl esters than free sterols and made an exception, whereas once again *P. roxburghii* was found to have the lowest amounts than the other pine species. Since, it is clear that the amount and composition of lipophilic extractives is quite common in the bark of the all studied conifers growing in our indigenous flora, and hence, it appears that the bark lipophilic extractives are not interesting from an exploitation point of view.

9.1.2: Hydrophilic extractives

The hydrophilic extractives of the seven conifer species have been studied and summarized in tables 9.3 and 9.4. *P. wallichiana*, and *P. gerardiana* were found to have the larger amounts of simple sugars and sugar alcohols, while *A. pindrow* and *T. Fauna* have the smaller amount of the same constituents. Simple acids were found absolutely in small amounts in the bark of all studied species. All samples also contained different ferulates (e.g. ferulic acid glucoside), which was also confirmed by the HPLC-ESI/MS analysis.

Table 9.2: Lipophilic extractives in mg/g dry bark analysed by gas chromatography for the six conifer species

	Abies pindrow	***Pinus wallichiana***	***Pinus roxburghii***	***Pinus gerardiana***	***Taxus baccata***	***Cedrus deodara***
			mg/g dry bark			
Σ Fatty acids	**1.77**	**12.4**	**1.30**	**14.7**	**1.39**	**3.19**
C14:0 acid	0.04	0.34	0.05	0.01	0.16	0.30
C16:0 acid	0.18	1.01	0.29	1.22	0.35	0.34
C17:0 acid	0.30	0.26	0.05	0.24	0.05	0.12
C18:1 acid	0.60	5.40	0.30	11.2	0.52	0.77
C18:2 acid	0.23	3.35	0.25	2.09	0.13	0.20
C18:3 acid	0.04	0.52	-	-	-	-
C20:3 acid	-	0.56	-	-	0.10	-
C22:0 acid	0.11	0.25	0.16	-	0.02	0.46
C24:0 acid	0.26	0.70	0.20	-	0.05	1.00
Σ α,Ω-fatty acids	**0.02**	**0.02**	**0.01**	**0.04**		**0.40**
1,22-dioic-22:acid	0.02	0.02	0.01	0.04	-	0.40
Σ Fatty alcohols	**0.09**	**1.85**	**0.23**	**2.99**	**0.02**	**0.63**
C22:0 alcohol	0.03	0.04	0.05	2.99	-	0.11
C24:0 alcohol	0.06	1.48	0.18	-	-	0.33
C26:0 alcohol	-	0.32	-	-	0.02	0.19
Σ Diterpens and diterpene alcohols	**0.05**	**0.23**	**0.02**	*****	**0.02**	*****
Thunbergene	0.03	0.02	0.01	-	0.01	*
Diterpene alcohol	0.02	0.21	0.01	*	0.01	-
Σ Resin acids	**0.69**	**5.85**	**4.96**	**31.1**	**0.38**	**6.96**
Pimaric acid	-	-	0.45	2.63	0.02	0.17
Sandaracopimaric acid	0.01	0.06	0.10	4.79	0.01	0.28
Isopimaric acid	0.24	2.78	1.54	5.69	0.12	3.09
Palustric acid	0.00	0.04	0.00	0.04	-	0.30
Dehydroabietic acid	0.08	1.26	1.21	8.80	0.06	1.73
Abietic acid	0.32	1.45	1.32	7.72	0.14	1.03
Neoabietic acid	0.04	0.27	0.18	0.79	0.03	0.31
x-hydroxy-dehydroabietic acid	-	-	0.15	0.26	-	0.06
8,15-isopimaridien-18-oic acid	-	-	-	0.36	-	-
Σ Monoglycerids	**0.05**	**0.06**	**0.05**	**0.02**		**0.13**
C24:0-monoglyceride	0.05	0.06	0.05	0.02	-	0.13
Σ Sterols and triterpyl alcohols	**0.99**	**2.47**	**1.61**	**2.76**	**1.96**	**2.26**
Campesterol	0.40	0.06	0.07	0.04	0.02	0.44
Sitosterol	0.59	0.92	0.52	0.36	0.29	0.77
Stigmasterol	-	-	-	-	0.01	0.06
Sitosterol-glucopyranoside**	-	1.49	1.01	2.36	1.63	0.99
Σ Steryl esters	**1.07**	**2.07**	**0.73**	**4.68**	*****	**1.81**
Σ Triglycerides	**0.44**	**2.31**	**0.33**	**4.05**	*****	**2.38**

- Not detected
*Trace amounts
** Overlapping with a taxifolin derivative and campesteryl-glucopyranoside
*** Not analysed

Lignans and lignan derivatives (identified or unidentified) have also been analyzed (Tables 9.3-9.4). Interestingly, the amounts of lignans were found in the bark of all species except *Picea smithiana*. The lagest amounts were found in *P. gerardiana* and *C. Deodara* whereas *P. willichiana* contained the smaller amount. Free stilbenes were found in all bark samples. Stilbenes have earlier been shown to be incorporated in the structure of bark tannins in spruce. However, the identified stilbenes were of the pinosylvin-type, which are not necessarily and the part of the tannin structure. Resveratrol glycoside was found exceptionally in large amount in the bark of *P. wallichiana* and was not identified in the other species. Although resveratrol is a highly interesting compound with promising bioactivity, the amounts (<0.1 % of the bark) are obviously not large enough for sufficient commercial exploitation.

Proanthocyanidin-related catechin and its derivatives were found in the barks of all species (Table 9.5). Similarly, taxifolin was found in exceptionally large amount in the bark of *C. deodara* and *P. roxburghii*. Taxifolin is a known and interesting bioactive compound having strong antioxidant and radical scavenging activity. The presence of cedeodarin in the *C. deodara* bark was first identified by HPLC-ESI/MS analysis (see next chapter) and later verified by GC-MS[39].

From an exploitation point of view, it is not likely that the common extractives in any of the studied species offer any business opportunity at this stage. The resveratrol glycoside and the taxifolin and its derivatives are interesting as bioactive compounds, but the amounts in the bark appear to be too small for a cost effective production to be feasible.

9.1.3: Proanthocyanidins

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Proanthocyanidin composition in the bark of the all species has been studied except for *Picea smithiana*. These contents were determined by using normal-phase HPLC-ESI/MS[39]. Proanthocyanidin monomers through decamers were identified based on mass spectral data consisting of characteristic deprotonated molecules and fragments ions[39-41]. ESI produced molecular ions (for proanthocyanidins from monomers to hexamers), a few fragment ions (for proanthocyanidin monomers, dimers, and trimers), and multiply charged ions ($[M–2H]^{2–}$ and $[M-3H]^{3–}$ ions for proanthocyanidins from

pentamers to decamers). No mass spectral data was obtained for proanthocyanidin polymers.

Table 9.3: Hydrophilic extractives in mg/g dry bark analysed by gas chromatography for the six conifer species

	Abies pindrow	*Pinus wallichiana*	*Pinus roxburghii*	*Pinus gerardiana*	*Taxus baccata*	*Cedrus deodara*
	mg/g dry bark					
Σ Sugars and sugar alcohols	**7.31**	**70.0**	**17.0**	**39.1**	**6.99**	**17.2**
Σ Simple acids	**0.65**	**0.66**	**0.53**	**0.17**	**-**	**0.52**
4-hydroxycinnamic acid	0.07	-	-	-	-	0.09
Vanillic acid	-	-	-	-	-	0.09
3,4-dihydroxybenzoic acid	0.07	0.66	0.22	0.17	-	0.34
3,4-dihydroxycinnamic acid	-	-	0.31	-	-	-
3,4,5-trihydroxy-1-cyclohexene-1-carboxylic acid	0.46	-	-	-	-	-
3,4,5-trihydroxybenzoic acid	0.04	-	-	-	-	-
Σ Ferulates	**1.12**	**4.78**	**1.44**	**2.94**	******	**3.64**
Σ Lignans	**0.67**	**0.14**	**0.72**	**7.21**	**0.81**	**6.15**
Matairesinol	0.29	-	-	-	-	-
Pinoresinol	0.25	-	0.35	-	-	-
Isolariciresinol	-	-	-	-	-	3.93
Secoisolariresinol	-	0.14	0.37	-	0.05	0.17
Unidentified lignan derivatives	0.13	-	-	7.21	0.76	2.05
Σ Stilbenes	**0.22**	**9.61**	**0.01**	**0.03**	**0.25**	**0.21**
Monomethyl pinosylvin	0.14	0.48	-	-	0.19	0.21
Dihydro-monomethyl pinosylvin	0.08	0.04	0.01	0.03	0.06	0.01
Resveratrol glycoside	-	9.09	-	-	-	-
Σ Flavonoids	**36.4**	**18.9**	**36.6**	**23.3**	**57.2**	**16.0**
Catechin	10.9	5.78	13.3	6.15	21.8	2.67
Gallocatechin	2.64	-	-	-	-	-
Taxifolin	-	-	2.65	0.54	-	5.98
Quercetin	-	-	0.09	-	-	-
Cedeodarin	-	-	-	-	-	3.23
Taxifolin derivative	-	2.11	-	-	-	0.11
Quercetin derivative	-	-	10.3	-	-	-
Catechin and gallocatechin derivatives*	22.8	11.0	10.3	5.96	35.4	3.97

- Not detected

* Partially overlapping with sitosterol-glucopyranoside

The peaks of polymers was identified according to thier long retention times and the characteristic flavanol-type UV spectrum [42]. Two typical HPLC traces of bark extracts are presented in Figure 9.1. All bark extracts contained exclusively B-type proanthocyanidin aglycones, and these proanthocyanidins were largely procyanidins (Table 9.5). Only bark extracts of *A. pindrow* and *T. fuana* contained also prodelphinidins

Table 9.4: Hydrophilic and lipophilic extractives in mg/g dry bark analysed by gas chromatography for the bark of *Picea smithiana*

Hydrophilic Extractives		**Lipophilic Extractives**	
Simple phenolic		Unknown sesquiterpene alcohol	1.58
1-Guaiacyl lignan glycerol	0.006	***Saturated fatty acids***	-
3,4–Dihydroxy benzoic acid	0.003	Palmitic acid (16:0)	0.569
Total simple phenolic	0.009	14-methyl hexadecanoic acid (17:0)	0.026
Pinosylvin		14-methyl hexadecanoic acid (17:0ai)	0.290
Pinosylvin dimethyl ether	tr	Stearic acid (18:0)	0.046
Hydroxy-pinosylvin dimethyl ether	0.015	Arachidic acid(20)	0.018
Pinosylvin monomethyl ether	0.024	***Monoenoic fatty acids***	
Pinosylvin	0.015	Oleic acid (9-18:1)	0.679
Total Pinosylvin	0.054	Vaccenic acid (11-18:1)	0.148
Lignans	-	***Dienoic fatty acids***	-
Enterolactone	-	Linoidleic ac (18:2)	0.490
Isoliovil	-	Hydroxy linoidleic acid	0.004
Todolactol	-	***Trienoic fatty acids***	-
Secoisolaricirsinol	-	Eicosatrienoic acid(20:3)	0.017
Secoisolaricirsinol actonids	-	**Total fatty acids**	**2.3**
allo-Hydroxymatairesinol	-	***Fatty alcohols and ferulates***	-
Hydroxymatairesinol	-	Docosanol	-
Matairesinol	-	Eicosanyl ferulate	-
α-Conidendrin	-	**Total Fatty alcohols and ferulates**	-
Conidendric acid	-	***Resin acids***	-
Laricirsinol	-	Sandaracopimaric acid	0.063
Cyclolaricirsinol (isolaricirsinol)	-	Isopimaric acid	0.319
Nortrachelogenin	0.012	Levopimaric acid	0.234
Lignan A		Palustric acid	0.580
Pinoresinol	0.037	Abietic acid	1.04
Unknown lignans	-	Neoabietic acid	0.279
Total lignans	0.049	Dehydroabietic acid	0.328
Sesquinoligans		Hydroxyresin acid	0.069
Dineolignans	tr	Secodehydroabietic acid	0.038
Higher oligolignan	0.1	**Total Resin acids**	3.0
sugars and sugar alcohol	0.62	**Sterols and triterphenyl alcohol**	-
		sitosterol	0.057
		Sitostanol	tr
		Campesterol	tr
		Campestanol	tr
		Cholesta -3,4 -diene	-
		Total Sterols and triterphenyl alcohol	0.057
		Total unknown compounds	0.25
		Steryl ester	1.0
		triglycerides	8.4

-Not detected
tr. Trace amount

Compositions of the proanthocyanidin content varied greatly, due the degree of polymerisation and the nature of flavan-3-ol units. *A. pindrow* and *P. wallichiana* contained proanthocyanidins from monomers up to polymers. But the oligomeric proanthocyanidins were found dominant in the *A. pindrow* extract, whereas polymeric proanthocyanidins in *P. wallichiana* extract (Figure 9.1). The entire series of procyanidins from monomers up to polymers were observed in the *P. wallichiana* and *P. gerardiana* extracts and contained only one monomeric procyanidin, i.e. catechin. Several other isomers were also detected as procyanidin dimers and hence showed that procyanidins were consisting of both catechin and epicatechin units. Similarly only mono- and oligomeric procyanidins were present in *P. roxburghii* extract. The same extract also contained two monomeric procyanidins which were detected in. The composition of the bark extracts were fairly simple as the number of individual isomers was measurable whereas the prodelphinidin compositions were instead rather complex. *A. pindrow* and *T. fuana* extracts contained four monomeric proanthocyanidins, i.e. epicatechin ($[M-H]^-$ ion at *m/z* 289), catechin ($[M-H]^-$ ion at *m/z* 289), epigallocatechin ($[M-H]^-$ ion at *m/z* 305) and gallocatechin ($[M-H]^-$ ion at *m/z* 305). Similarly, several molecular ions were found for each proanthocyanidin having different degree of polymerisation as observed in proanthocyanidin trimers, $[M-H]^-$ ions at *m/z* 865, 881, 897, and 913, corresponding to proanthocyanidins trimers consisting of three procyanidin units, two procyanidin and one prodelphinidin units, one procyanidin and two prodelphinidin units, and three prodelphinidin units, respectively.

Table 9.5: Proanthocyanidin content and composition in bark acetone extracts.

Tree species	Proanthocyanidin content	Procyanidins	Prodelphinidins	DP range
	mg/g			
Abies pindrow	405 ± 2	x	x	1-P
Pinus wallichiana	550 ± 19	x		1-P
Taxus baccata	296 ± 2	x	x	1-7
Pinus roxburghii	246 ± 4	x		1-7
Pinus gerardiana	148 ± 11	x		1-P
Cedrus deodara	72 ± 1	x		1-7, P

DP = degree of polymerisation. The '1-7' or '1-P' in the DP range indicates that monomers through heptamers or polymers were detected.

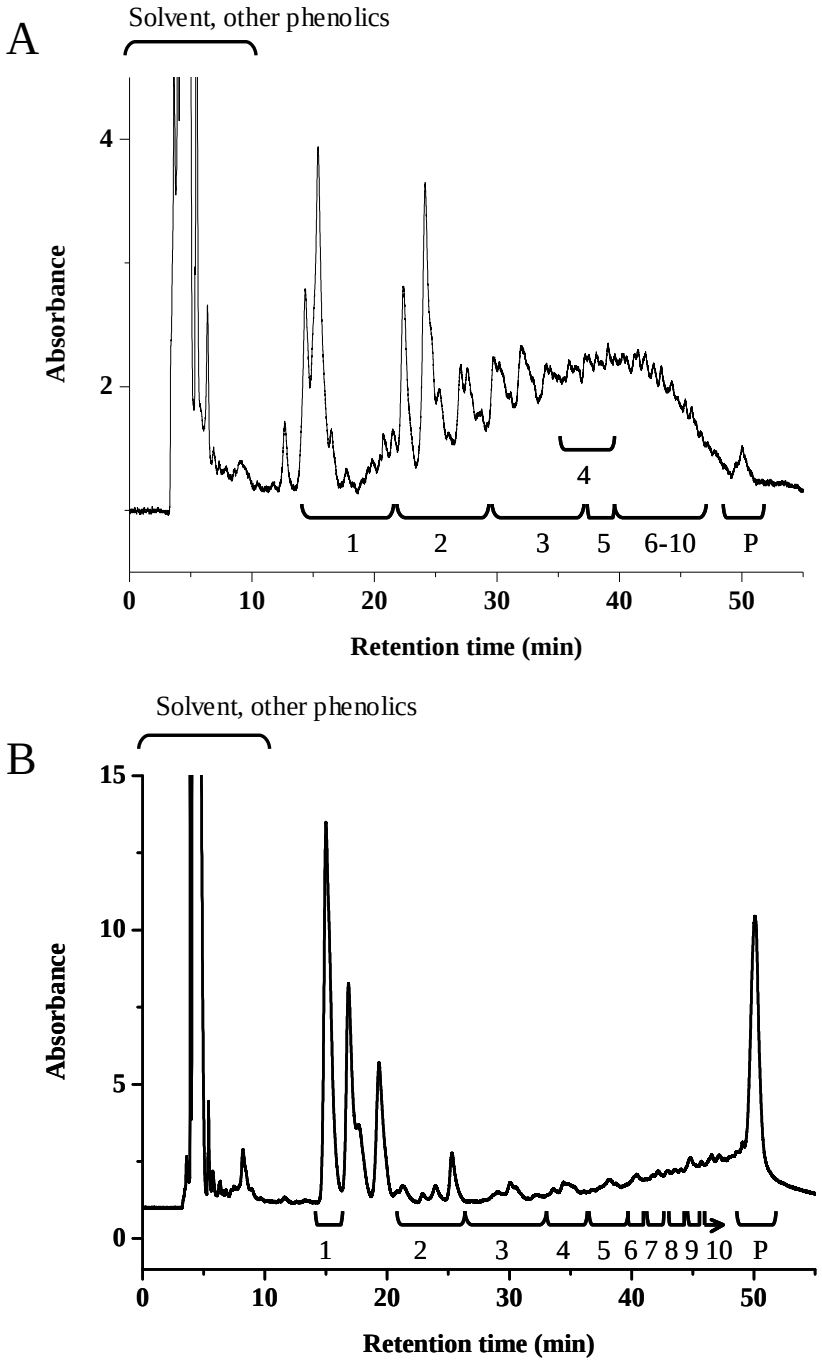


Figure 9.1: Normal-phase HPLC profile of (A) *Abies pindrow* and (B) *Pinus wallichiana* bark acetone extracts. Labels 1-10 indicate the degrees of polymerisation of proanthocyanidins in the peaks. Polymeric proanthocyanidins (P) eluted as a single peak at the end of the chromatogram.

If only the hydroxylation pattern (procyanidin or prodelphinidin) and the sequential order of the flavan-3-ol units and differences in stereochemistry at C2 and C3 (2*R*, 3*S* or 2*R*,3*R*) are taken into account, there is already 64 possible structures for proanthocyanidin trimmers and the number of alternative structures increases exponentially with the chain length. The complexity of proanthocyanidins and high number of isomers deteriorated separation in HPLC analysis and caused remarkable overlapping of peaks which can also be seen in HPLC traces of *A. pindrow* extract (Figure 9.1A). All bark extracts were found to be rich in proanthocyanidins (Table 9.5). The structural complexity and high number of isomers of oligomeric and polymeric proanthocyanidins prevented their direct chromatographic quantification. Therefore, proanthocyanidins were quantified as total proanthocyanidins by butanol-HCl assays. The total contents of proanthocyanidins varied from 72 mg/g extract (*C. deodara*) up to 550 mg/g extract (*P. wallichiana*); i.e. proanthocyanidins were significant components in bark extracts.

The qualitative and quantitative results for proanthocyanidins obtained here for Pakistani species are similar to European species [39,43]. The bark of European conifers and broad-leaved tree species contain mainly procyanidins with average degrees of polymerisation ranging from 3 to 8[43]. Normal-phase HPLC-ESI/MS analyses also revealed the presence of other phenolic compound in the bark extracts. Most of these compounds, for example the lignans, eluted in the beginning of the normal-phase chromatogram as an unseparated broad peak (Figure 9.1). All *Pinus* sp. and *C. deodara* contained taxifolin and its glucoside, which was confirmed by the GC-MS analyses. The *P. wallichiana* extract also contained several phenolic acids, such as ferulic acid glucoside, *β*-hydroxypropiophenone and *p*-coumaric acid, which were identified based on our previous work[44]. *C. deodara* contained also cedeodarin[45] which was identified based on ESI-MS data: *m/z* 635 ($[2M–H]^-$), 317 ($[M–H]^-$), 299 ($[M–H_2O]^-$), 177 ([M–140]$^-$, heterocyclic ring fission), 139 ([M–178]$^-$, heterocyclic ring fission).

The proanthocyanidins were abundant enough in all species except *C. deodara* to be interesting from an exploitation point of view. Especially, *P. wallichiana* and *A. pindrow* bark, containing 40-55% proanthocyanidins, could have huge potential as sources of bioactive extracts. A hydrophilic extract of *P. wallichiana* would also

contain some resveratrol glycosides and ferulates, which are known to have interesting effects and may also, induce promising synergistic effects. It is possible that the effects can be similar to Pycnogenol, which is a commercial product of *Pinus maritima* (syn. *Pinus pinaster*) bark extract. Packer et al. (1999) have reviewed the well-known antioxidative properties and chemical composition of pycnogenol[10]. The main constituents can be broadly divided into flavonoids (catechin, epicatechin, and taxifolin) and condensed tannins. Pycnogenol has been shown to possess greater biologic potency as a mixture than in the purified form, indicating that other components exert synergy. However, the effects of the specific extracts should be screened and studied more in detail before any definite conclusion about feasibility can be given.

Chapter: 10

EXPERIMENTAL (Part C)

10.1: Plant species

The collected healty and mature plant species belonging to family Pinaceae were authenticated by Dr. Habib Ahmad, Dean Faculty of Science, Hazara University and collected from the forest of Shawar, Swat, NWFP Pakistan. Voucher specimens have been retained at the herbarium, Department of Botany, Hazara University Pakistan. Voucher numbers and details of species collected are given in tables 10.

10.2: Sampling of bark specimens and preparation of wood extracts

Representative bark samples (200 g of each species) were air-dried, ground, and extracted with n-hexane followed by acetone/water (95% v/v) extraction using a Soxhlet apparatus. The solvents were removed under vacuum with rotaevapourator. Extracts were evaporated to dryness for shipping. The lipophilic extracts were then re-dissolved in hexane/acetone (1:1 v/v) and the hydrophilic extracts in acetone. The exact concentrations of the re-dissolved solutions were determined by drying 10 mL of the solution in a vacuum oven at 40°C and weighing.

10.3: Analysis of lipophilic and hydrophilic extractives

The extractives, after evaporation of the extract solutions and silylation of the extractives, analysed on a 25 m × 0.20 mm i.d. column coated with crosslinked methyl polysiloxane (HP-1, 0.11 μm film thickness). The method used was according to Willför et al. [36,46]. The practical limit of quantification of the individual compounds was about 1% of the internal standard amount in each sample, but compounds present in smaller amounts were identified and are reported as “trace amounts”. Ferulates, steryl esters, triglycerides, and flavonoid derivatives were quantified on a short (6 m × 0.53 mm i.d., 0.15 μm HP-1) column [47,48]. All results were calculated on a dry wood basis.

Identification of the individual components was performed by GC-MS analysis of the silylated components with an HP 6890-5973 GC-quadrupole- MSD instrument using a similar 25 m HP-1 GC column as above.

10.4: Analysis of proanthocyanidins

Characterisation of proanthocyanidins was performed by normal-phase HPLC-ESI/MS analyses of the acetone extracts according to Karonen et al [39]. The analysis were conducted using a Perkin-Elmer Sciex API 365 LC/MS/MS mass spectrometer connected to a Series 200 HPLC system with UV/VIS detector and Analyst Software 1.1 data system with a Merck LiChrospher Si 60 column (250 mm × 4 mm i.d., 5 µm). The mobile phase consisted of two solvents: (A) dichloromethane, methanol, water, and acetic acid (82:14:2:2, v/v) and (B) methanol, water, and acetic acid (96:2:2, v/v). The elution profile was: 0–30 min, 0–18% B in A (linear gradient); 30–45 min, 18–31% B in A (linear gradient); 45–50 min, 31–88 % B in A (linear gradient); and 50–60 min 88% B (isocratic). The flow rate was 1 mL/min, detection wavelength 280 nm and injection volume 5 µL. The ionization technique was an ionspray (pneumatically assisted electrospray). The mass spectrometer was operated in negative ion mode since proanthocyanidins are thereby better detected due to the acidity of phenolic protons. The spray needle voltage was 4200 V, orifice plate voltage 35 V and ring voltage 220 V. The heated nitrogen gas temperature was generally 310°C. The setting for nebulizer gas (purified air) flow was 10 and for curtain gas (nitrogen) 12. The flow of turbo ion spray gas was set at 7000 mL/min. Masses were generally scanned from *m/z* 100 to *m/z* 2800 in steps of 0.3 amu. The split ratio was 7:3 prior to introduction into the ionization chamber. Proanthocyanidin contents of the acetone extracts were determined by butanol-HCl assays according to[49]. A sample of bark extract (0.1 mL) and water (0.6 mL) were added to a 1-butanol:Hydrochloric acid (95:5, v/v) solution (6 mL). The reaction mixture was heated 2 h at 95°C and then cooled down to room temperature. The absorbance was measured at 555 nm using a Perkin-Elmer Lambda 12 UV/VIS spectrometer. The proanthocyanidin contents were quantified against purified mountain birch leaf proanthocyanidins, which contain both procyanidins and prodelphinidins.[50].

References

(1) Vascocelos, A. M. S. *J. Chromatog.* **2006**, *1105*, 191.

(2) Farjon, A. *World Checklist and Bibliography of Conifers,Kew* U.K,p.300, 1998.

(3) Raffa, K. F.; Smalley, E. B. *Oecologia* **1995**, *102*, 285-88.

(4) Slimestad, R.; Andersen, F. M.; Francis, G. W.; Marston, A.; Hostettmann, K. *Phytochemistry* **1994**, *35*, 1517.

(5) Kraus , G.; Spiteller, G. *Phytochemistry* **1997**, *44*, 59.

(6) Nabeta, K.; Hirata, M.; Ohki, Y.; Samaraweera, S. W. A.; Okuyama, J. *Phytochemistry* **1994**, *37*, 409.

(7) Yueh-Hsiung, K. U. O. *Chem. Pharm. Bull.* **2004**, *52*, 861.

(8) Jerez , M.; Selga , A.; Sineiro , J.; Torres , J. L.; Nunez , M. J. *Food Chem.* **2007**, *100*, 439.

(9) Escribano-Bailo´n, T.; Gutie´rrez-Ferna´ndez, Y.; Rivas-Gonzalo, J. C.; Santos-Buelga, C. *J.Agri.Food Chem.* **1992**, *40*, 1794.

(10) Packer, L.; Rimbach, G.; Virgili, F. *Free Radical Biol. Med.* **1999**, *27*, 704.

(11) Grassmann, J.; Hippeli, S.; Vollmann, R.; Elstner, F. *J. Agric. Food Chem.* **2003**, *51*, 7576.

(12) Chandler, F. R.; Freeman, L.; Hooper, S. N. *J. Ethnopharmacol.* **1979**, *1*, 49.

(13) Gülçin, I.; Büyükokuroglu , M. E.; Oktay , M.; Küfrevioglu, O. I. *J. Ethnopharmacol.* **2003**, *86*, 51.

(14) Rautio, M.; Sipponen, A.; Peltola, R.; Lohi, J.; Jokinen, J. J.; Papp, A.; Carlson, P.; Sipponen, P. *Apmis* **2007**, *115*, 335.

(15) Vishnoi , S. P.; Ghosh , A. K.; Debnath , B.; Samanta , S.; Gayen , S.; Jha, T. *Fitoterapia* **2007**, *78*, 153.

(16) Shinde , U. A.; Phadke, A. S.; Nair, A. M.; Mungantiwar, A. A.; Dikshit, V. J.; Saraf, M. N. *J. Ethnopharmacol.* **1999**, *65*, 21.

(17) Barrero , A. F.; Quı´lez del Moral, J. F.; Herrador , M. M.; Arteaga , J. F.; Akssira, M.; Benharref , A.; Dakir, M. *Phytochemistry* **2005**, *66*, 105.

(18) Loizzo, M. R.; Saab, A. M.; Statti, G. A.; Menichini, F. *Fitoterapia* **2007**, *78*, 323.

(19) Wani, M. C.; Taylor, H. L.; Wall, M. E.; Coggon, P.; McPhail, A. T. *J.*

Am. Chem. Soc. **1971**, *93*, 2325.

(20) Balo˘glu, E.; Kingston, D. G. I. *J.Nat. Prod.* **1999**, *62*, 1448.

(21) Virinder, S. P.; Amitabh , J.; Kirpal , S. B.; Poonam, T.; Sanjay, K. S.; Ajay, K.; Poonama; Rajni , J.; Carl , E. *Phytochemistry* **1999**, *50*, 1267.

(22) Ballero, M.; Fresu, I. *Fitoterapia* **1993**, *64*, 141.

(23) Esra , K.; Nurgün , E.; Erdem , Y.; Bilge , S. *J. Ethnopharmacol.***2003**, *89*, 265.

(24) Appendino, G. *Fitoterapia* **1993**, *64*, 5.

(25) Singh, V. *Fitoterapia* **1995**, *66*, 507.

(26) Junko, K.; Izumi, M.; Norihiro , K.; Keiichi , H.; Eriko , S.; Takahiro , N.; Shigetoshi , K. *Biol. Pharm. Bull.* **2006**, *29*, 2310.

(27) Mantle , D.; Eddeb, F.; Pickering, A. T. *J. Ethnopharmacol.* **2000**, *72*, 47.

(28) Shah, A.; Li, D. Z.; Möller, M.; Gao, L. M.; Hollingsworth, M. L.; Gibby, M. *Taxon* **2008**, *57*, 211.

(29) Nisar, M.; Khan, I.; U. Simjee, S.; Gilani, A. H.; Obaidullah; Perveen, H. *J. Ethnopharmacol.* **2008**, *116*, 490.

(30) Slimestad , R.; Andersen , F. M.; Francis , G. W. *Phytochemistry* **1994**, 550.

(31) Kraus, C.; Spiteller, G. **1997**, *44*, 59.

(32) Nabeta, K.; Hirata, M.; Ohki, Y.; Samaraweera, S. W. A.; Okuyama, H. *Phytochemistry* **1994**, *37*, 409.

(33) Tiwari, A. K.; Srinivas, P. V.; P.Kumar, S.; Rao, J. M. *J. Agric. Food Chem.* **2001**, *49*, 4642.

(34) Willför, S.; Hemming, J.; Reunanen, M.; Eckerman, C.; Holmbom, B. *Holzforsch.* **2003c**, *57*, 27.

(35) Willför, S.; Hemming, J.; Reunanen, M.; Holmbom, B. *Holzforschung* **2003a**, *57*, 359.

(36) Willför, S. M.; Ahoutupa, M. O.; Hemming, J. E.; Reunanen, M. H. T.; Eklund, P. C.; Sjoholm, R. E.; Eckerman, C. S. E.; Pohjamo, S. P.; Holmbom, B. R. *J. Agric. Food Chem.* **2003b**, *51*, 7600.

(37) Cosentino, M.; Marino, F.; Ferrari, M.; Rasini, E.; Bombelli, R.; Luini, A.; Legnaro, M.; Canne, M. G. D.; Luzzani, M.; Crema, F.; Paracchini, S.; Lecchini, S. *Pharmacol. Res.* **2007**, *56*, 140.

(38) Willfor, S.; Hafizoglu, H.; T¨umen, I.; Yazici, H.; Arfan, M.; Ali, M.; Holmbom, B. *Holz. Roh. Werkst* **2007**, *65*, 215.

(39) Agrawal, P. K.; Agarwal, S. K.; Rastogi, R. P. *Phytochemistry* **1980**, *19*, 893.

(40) Karonen, M.; Loponen, J.; Ossipov, V.; Pihlaja, K. *Anal. Chim. Acta.* **2004a**, *522*, 105.

(41) Friedrich, W.; Eberhardt, A.; Galensa, R. *Eur. Food Res. Technol.* **2000**, *211*, 56.

(42) Gu, L.; Kelm, M. A.; Hammerstone, J. F.; Beecher, G.; Holden, J.; Haytowitz, D.; Prior, R. L. *J. Agric. Food Chem.* **2003**, *51*, 7512.

(43) Karonen, M.; Ossipov, V.; Sinkkonen, J.; Loponen, J.; Haukioja, E.; Pihlaja, K. *Phytochem. Anal.* **2006**, *17*, 149.

(44) Matthews, S.; Mila, I.; Scalbert, A.; Donnelly, D.M.X. *Phytochemistry* **1997**, *45*, 405.

(45) Karonen, M.; Hämäläinen, M.; Nieminen, R.; Klika, K. D.; Loponen, J.; Ovcharenko, V. V.; Moilanen, E.; Pihlaja, K. *J. Agric. Food Chem.* **2004b**, *52*, 7532.

(46) Ekman, R.; Holmbom, B. *Nord. Pulp. Pap. Res.* **1989**, *4*, J16.

(47) Orsa, F.; Holmbom, B. *J. Pulp. Pap. Sci.* **1994**, *20*, J361.

(48) Willfor, S.; Reunanen, M.; Eklund, P.; Sjoholm, R.; Kronberg, L.; Fardim, P.; Pietarinen , S.; Holmbom, B. *Holzforsch.* **2004c**, *58*, 345.

(49) Ossipova, S.; Ossipov, V.; Haukioja, E.; Loponen, J.; Pihlaja, K. *Phytochem. Anal.* **2001**, *12*, 128.

(50) Karonen, M.; Leikas, A.; Loponen, J.; Sinkkonen, J.; Ossipov, V.; Pihlaja, K. *Phytochem. Anal.* **2007**, *18*, 378.

PART D

Evaluation of Biological Activities

Chapter 11

INTRODUCTION (Part D)

11.1: Biological screening of medicinal plants

Plants have been used as herbal medicines for thousands of years. These herbal prescription are taken in the form of crude drugs such as teas, tinctures, poultices, powder, and other herbal formulations[1,2]. The specific plants for particular purpose and the methods of application for particular use have passed down both in written documented form as well as passed on through observation and practice as folk traditional medicine. Recently the use of plants as medicines has involved the isolation of the bioactive compounds, starting from the isolation of morphine from opium in the early 19th century for the first time[1] and later on drug discovery from medicinal plants have led to the isolation of drugs such as codeine, digoxin, cocaine and quinine[1,2]. Isolation and characterization of pharmacologically active compounds from medicinal plants is still an important area of research around the world. Chemists have a compelling curiosity to discover those bioactive compounds in a plant extract used as a remedy which are responsible for the therapeutic effects. Of the estimated 250,000-500,000 plant species of the world, more than two third occur in the tropical forests of developing countries. Only a small percentage of these plants have been investigated phytochemically and only a fraction of that has been subjected to biological or pharmacological screening.

Drug discovery from medicinal plants has evolved to include several fields of inquiry and various methods of analysis. The process typically begins with a Botanist, Ethno-botanist, Ethno-pharmacologist or plant ecologist who collects and identifies the plant(s) of interest. Collection may involve species with known biological activity for which active compound(s) have not been isolated (e.g., traditionally used herbal remedies) or may involve taxa collected randomly for a large screening program. It is necessary to respect the intellectual property rights of a given country where plant (s) of interest are collected[2]. Phytochemists (natural product chemists) obtain extracts from the plant materials, subject these extracts to biological screening in pharmacologically relevant assays, and initiate the process of isolation and

characterization of the active compound (s) through bioassay-guided fractionation. Molecular biology has become essential to medicinal plant drug discovery through the determination and implementation of appropriate screening assays directed towards physiologically relevant molecular targets[2]. A large number of assays and protocols are developed for the screening of medicinal plants for their activities. Some of the important biological activities are discussed relevant to the research work documented in this dissertation.

11.2: Anticancer (anti-proliferative) activity

Nowadays, cancer is considered to be one of the most lethal diseases in human beings throughout the world and over ten million new cases of cancer, with over six million deaths, were estimated in the year 2000[3]. Since 1990 there has been a 22% increase in cancer incidence and mortality with the four most frequent cancers being lung, breast, colorectal and stomach and the four most deadly cancers being lung, stomach, liver, and colorectal[3]. Cancer is the second leading cause of death in the United States (U.S.), surpassed only by cardiovascular disease. Although these figures are disquieting, some progress has been made in cancer diagnosis and treatment as evident through the high incidence of breast, prostate, testicular, and uterine cancers as compared with their relatively lower mortality[2,3]. Drug discovery from medicinal plants has played an important role in the treatment of cancer and indeed, most new clinical applications of plant secondary metabolites and their derivatives over the last half century have been applied towards combating cancer. Of all the available anticancer drugs between 1940 and 2002, 40% were natural products or natural product-derived with another 8% being natural product mimics[2].

The investigations for finding new anticancer compounds are imperative and interesting. After taking into consideration the immense side effects of synthetic anticancer drugs, many researchers are making concerted efforts to find new and natural anticancer compounds [4]. Therefore, a number studies have been carried out on various medicinal plants including fruits and vegetables. The results of these research work showed that dietary patterns were significantly associated with the prevention of chronic diseases such as heart disease, cancer, diabetes and other fatal diseases [5,6]. The use of fruits and vegetables has been highly associated with the reduced risk of

cancer[5,7]. The levels of oxidants and antioxidants in humans are maintained in balance at normal metabolism, which is important for sustaining optimal physiological conditions[5,8]. Overproduction of oxidants species like super oxide anion ($O_2^{\bullet-}$), hydroxyl ($HO^{\bullet}$), peroxyl ($ROO^{\bullet}$), alkoxyl ($RO^{\bullet}$) and nitric oxide can cause an imbalance, leading to oxidative damage to large biomolecules such as lipids, DNA and proteins [9]. These oxidants have been regarded as the fundamental cause of different kinds of diseases, including aging, coronary heart disease, inflammation, stroke, diabetes mellitus, rheumatism, liver disorders, renal failure, cancer and neuro degeneration [10]. More and more evidence suggests that this potentially cancer-inducing oxidative damage might be prevented or limited by dietary antioxidants found in natural source. Phytochemicals in fruits, vegetables, spices and traditional herbal medicinal plants have been found to play protective role against many human chronic diseases including cancer and cardiovascular diseases (CVD). These diseases are associated with oxidative stresses caused by excess free radicals and other reactive oxygen species. Antioxidant phytochemicals exert their effect by neutralizing these highly reactive radicals[11].

11.3: Antioxidant activity

Free radical have been regarded as the fundamental cause of different kinds of diseases, including aging, coronary heart disease, inflammation, stroke, diabetes mellitus, rheumatism, liver disorders, renal failure, cancer and neuro degeneration[10]. The Modern theories of Reactive Oxygen Species (ROS) explain how they play a dual role in an organism. They are strong lipid peroxidizers as well causes the deterioration of food, cellular injuries and also initiate peroxidation of polyunsaturated fatty acids in biological membranes. The tissue injury caused by ROS include DNA and protein damage and oxidation of enzymes in the human body [12]. Antioxidants such as α-tocopherol are capable of mitigating free radical damage through scavenging ROS[12]. Some natural cellular enzymatic antioxidants are superoxide dismutase (SOD) catalase and glutathione peroxidase (GPX), whereas non enzymatic antioxidants comprise α-tocopherol, carotene, carotenoids, chlorophylls, flavonoids, tannin and certain micronutrients *e.g.* zinc and selenium[12]. Extensive studies on antioxidant derived from plants can be correlated with oxidative stress and age-dependent

diseases. Flavonoids are abundant in fruits, teas, vegetables, and medicinal plants and have been investigated extensively, since they are highly effective free radical scavengers and are assumed to be less toxic than synthetic antioxidants such as Butylated Hydroyanisole (BHA) and Butaylated Hydroxytoluene (BHT), which are suspected of being carcinogenic and may cause liver damage [13]. The presence of these antioxidants in the cellular system is known to prevent oxidative damage. Phytochemicals can have complementary and overlapping mechanisms of oxidative agents, stimulation of the immune system, regulation of gene expression in cell proliferation and apoptosis, hormone metabolism and antibacterial and antiviral effects[5,9]. An inverse relationship has been shown between dietary intake of antioxidant rich foods and the incidence of a number of human diseases [14]. Thus the search and research for natural antioxidant sources and their antioxidant potential is becoming more and more important. A number of antioxidants have been derived from plants such as *Physalis peruviana,*[12], *Hypericum perforatum, Hypericum androsaemum, Hypericum triquetrifolium, Hypericum hyssopifolium*[13,15,16] *Pinus pinaster, Pinus nigra* and *Pinus morrisonicola*[17-19].

11.4: Antimicrobial activity

The plant extracts and plant products of higher plants have been screened for antimicrobial activity showing positive results for the said activity [20,21]. An increase in drug resistance in human pathogenic organisms as well as the appearance of undesirable side effects of certain antibiotics and the emergence of previously uncommon infections have been observed during the recent past [22,23]. Antimicrobial properties are being reported more frequently in a wide range of plant extracts and natural products am to discover new chemical classes of antibiotics that are effective multidrug resistant microorganism resolve these problems. A detailed review describes the antifungal properties of natural products [24]. This include the cyclic lipopeptide echinocandins from *Aspergillus* sp., pneumocandins produced by directed-biosynthesis using the fungus *Zalerion arhohcola*[25], the cyclopeptide aureobasidins from the fungus *Aureobasidiiim pultutans* [24], the pradimicins from *Actiitomadiira hibisca*[26] and the inkkomycins from *Streptoniyces letnlae*[24]. New antibacterial agents were also discussed in this review [27] which clearly outlined the

important classes of known antibiotics in providing templates for chemical modification and listed novel targets against which natural products screening might be productive. Promising recent developments in this field include semi-synthetic glycopeptides antibiotics with greatly improved potency against vancomycin-resistant enterococci and ziracin, a novel oligosaccharide from *Microiuonospora carhonacea* var. *africann* which has good activity against drug-resistant bacteria and undergoing phase 1 clinical studies[28]. Screening plants for antiviral activities has resulted in the discovery of agents such as michellamine B and SP-303. Michellamine B inhibits HlV-induced cell killing by at least two distinct mechanisms and is currently in preclinical phases [29], SP-303 is a plant flavonoid discovered by an ethanobotanical approach which is currently being evaluated for use against influenza virus. Some recent studies have revealed the antimicrobial activity of various medicinal plants[30 31]

11.5: Pharmacological importance of the species belonging to families Guttiferae, Solanaceae and Pinaceae (See Section 2.1.2, 2.2.1, 5.2.3 and 8.1.2 respectively)

Chapter: 12

RESULTS AND DISCUSSION (Part D)

12.1: Biological screening of the selected species of Guttiferae, Pinaceae and Solonaceae

Different solvents soluble fractions of the plants belonging to family Guttiferae (*H. perforatum, H. oblongifolium, H. monogynum, H. choisianum* and *H.dyeri*), Pinaceae (bark and knotwood of *Picea smithiana, Abies pindrow, Pinus wallichiana, P. geradiana* and *P. roxburghii* and *Cedrus deodara)* and *Taxus fauna* from the north west of Pakistan were subjected to screening for their possible antioxidant activity. Anticancer (aniproliferative) and enzyme inhibition activities of *Hypericum* species while the cytotoxic, anti-inflammatory and urease inhibition activities of purified compounds isolated from *Hypericum, Physalis* and *Withania* species were also studied. Four complementary antioxidant test systems namely, phenolic contents, free-radical scavenging capacity (DPPH assay), reducing power and total antioxidant activities by Phosphomolybdenum method were used for analysis. Folin-Ciocalteu's phenol reagent was used to determine total phenolic content. The ferric compounds were used to find out the reducing power the samples. The DPPH radical scavenging was determined by measuring the decay in absorbance at 517 nm indicating its radical reduction. [32] We report here for the first time the antioxidant and antimicrobial potential of the various extracts and fractions of the listed plants except *Hypericum perforatum* which has been the subject of many investigations. The objectives of this study were to explore the biological and medicinal value the extracts/fractions of aerial parts, knotwood and bark of the above mentioned plants.

12.2: Biological screening of the *Hypericum* species

12.2.1: Antioxidant potential of the *Hypericum* species

12.2.1.1: Determination of total phenols

It has been reported that the phenolic contents in plant material have direct correlation with antioxidant activities[33]. In present study the content of total phenols

in ethanol **(F1),** aqueous **(F2),** ethyl acetate **(F3),** acetone **(F4),** and final residue **(F5),** of *H. perforatum (A), H. oblongifoilum (B), H. monogynum (C), H. choisianum (D)* and *H. dyeri (E)* were measured. The phenolic contents in different fractions/extracts and standard (expressed as gallic acid equivalents mg/g of sample) were given in table 12.1. There was no statistically significant difference observed among the phenolic contents of various fractions (P >0.05). The phenolic contents in crude extracts (**F1)** of above mentioned species were 71.6±1.75, 76.8 ±1.750, 72.7±0.98, 96.7±1.75, and 58.1 ± 1.778 mg/g of extract respectively. The aqueous (**F2)** of *H.choisianum* exhibited maximum and ethanolic fraction (**F1)** from *H. dyeri* showed minimum phenolic contents. The aqueous extracts of *Hypericum* species (A, B, C, D and E) contain 48.0±0.85, 54.1±0.850, 46.0±0.52, 92.7±1.32, and 61.0±2.166 mg/g of extract respectively. Generally the ethanolic extracts **(F1)** of the tested species contain higher phenolic contents than their aqueous fractions (**F2)**. The ethyl acetate fraction (**F3)** of all five species showed lower phenolic contents except *H. choisianum* (92.7±1.32). The acetone (**F4)** and final fraction (**F5)** of all plant analyzed except *H.dyeri* contained reasonable phenolic contents. The Folin–Ciocalteu method determined the phenols by giving different responses to different phenolic compounds, depending on chemical structures. A linear relation between antioxidant activity and the total phenolics was observed in most cases (Table 12.1).

12.2.1.2: DPPH radical-scavenging activity

The DPPH radical scavenging is one the most popular method used to evaluate the antioxidant potential. It is a stable organic free radical having purple color with adsorption band at 515-528 nm in UV. The absorption intensity decreases when accepting an electron or a free radical species, which results in a visually noticeable discoloration from purple to yellow[13]. The radical scavenging power of various extracts and fractions i.e ethanol **(F1),** aqueous **(F2),** ethyl acetate **(F3),** acetone **(F4),** and final residues **(F5)** of five *Hypericum* species (A, B, C, D and E) and standards were studied (Fig. 12.1 and Table 12.1 & 12.2). The scavenging activities were exhibited by all fractions even at the lower concentration (20*u*g/mL) and at higher concentration (100*u*g/mL) nearly touched the activity shown by standards. There was statistically significant difference observed among the same fractions (P <0.05) of

different plants except the final fraction **(F5)** of *H.dyeri* while no difference (P > 0.05) was observed in various fractions (**F1-F5**) of the same plant. Among the ethaolic extracts (**F1**) of five plant species, the highest activity (92.26 % at 100*u*g/mL and with 30.13 *u*g/mL EC_{50}) was observed in *H monogynum* while aqueous extract of *H. oblongifoilum* has strong activity (92.70 % at 100*u*g/mL and with 29.33 *u*g/mL EC_{50}). The ethyl acetate fraction of all species have lower activity except *H.dyeri* (80.25% at100*u*g/mL and with 57.75 *u*g/mL EC_{50}). A very good activity was shown by acetone fraction ranged from minimum (70.39 % at 100 *u*g/mL) in *H.dyeri* to maximum (92.70 % at 100*u*g/mL) of *H.oblongifolium.* The highest % DDPH activity (93.26 % at 100*u*g/mL) was observed in final fraction **(F5)** of *H.choisianum* while remarkable effective concentration (EC_{50}, 6.67*u*g/mL) was noted in final fraction **(F5)** of *H.perforatum*. The significant DPPH radical scavenging activities of fractions were due their corresponding phenolic contents, the fraction with higher phenolic contents showed remarkable radical scavenging activity (Table 12.1).

12.2.1.3: Reducing power

The reducing power is also used to evaluate the antioxidant capacity of an analyte using a iron (III) to iron (II) reduction assay. In this assay the reductants present in solution causes the reduction of the Fe3+/Ferricyanide complex to the ferrous form which changes the yellow color of solution to green or blue-green depending on the reducing power of reductants and can be monitored by measurement the absorption of the Perl's Prussian blue at 700 nm [13]. Reducing power of various extracts/fraction (**F1-F5**) of five *Hypericum* species and standards were studied (Fig.12.2, Table 12.1 & 12.2). All the tested samples have shown some degree of reducing power. However, as anticipated, their reducing power was less than standard. The reducing power of samples increased with increasing amount of concentration as observed in case of radical scavenging assay. There was statistically significant differences observed among the same fraction (P <0.05) of different plants except final fraction of *H.dyeri* while no difference (P > 0.05) was observed in various fractions of the same plant. The crude extract (**F1)** of *H. monogynum* showed highest reducing power (0.760 at 25*u*g/mL) among crude extracts of all five species. Among aqueous (**F2)** and acetone (**F3)** fractions the highest activity (0.843 and 0.823 at 25 *u*g/mL respectively) was observed in case of *H.oblongifolium.* The ethyl acetate

fractions (**F2)** all species have shown lower activity except *H.dyeri* (0.517 at 25*u*g/mL). The highest reducing power (0.907 at 25*u*g/mL) was observed in final fraction (**F5)** of *H.choisianum* while the lowest was in final fraction (**F5)** of *H.dyeri* among all fractions. The reducing power might be due to either phenolic compounds or some other reducing natural compounds present in plant. The linear correlation among phenolic contents, reducing power and DPPH radical scavenging activity was found in most cases (Table 12.1). The fractions with higher phenolic contents exhibited remarkable scavenging activity and reducing power.

12.2.1.4: Total antioxidant capacity

The total antioxidant capacity of the samples (extracts and fractions) was measured by phosphomolybdenum method. This method is based on the reduction mechanism. The reductants present in solution cause reduction of Mo (IV) to Mo (V) which results in formation of green phosphate/Mo (V) complex giving maximum absorption at 695 nm. The antioxidant capacity of various extracts/fractions (**F1-F5**) of five *Hypericum* species (A, B, C, D & E) and standards were compared (Table 12.1). The antioxidant activity was shown by all the tested fractions upto some degree but inferior to standards. There were no statistically significant difference among various fractions ($P > 0.05$). Same trend in activity was observed in most cases as already discussed in case of radical scavenging activity (RSA) and reducing power. The highest antioxidant activity (1410.03$\pm$60.20 *u*mol/mg) was observed in final fraction(**F5)** of *H.choisianum* followed by the antioxidant potential (1385.3 $\pm$140.5 *u*mol/mg) of aqueous fraction (**F2)** of *H.dyeri*. The crude extract (**F1)** of *H. monogynum*, ethyl acetate (**F3)** and acetone (**F4)** fractions of *H.oblongifolium* also exhibited good antioxidant capacity (1344.7$\pm$60.63, 1078.60$\pm$82.54 and 1306.7$\pm$69.3 *u*mol/mg respectively). The lowest antioxidant activity (95.57$\pm$16.87 *u*mol/mg) was in final fraction (**F5)** of *H.dyeri.* Phenolic compounds often have showed the best antioxidant activity; therefore correlation between activities and phenolic contents was noted in some cases (Table 12. 1).

Table 12.1: Antioxidant activities and total phenolic contents of various fractions of *Hypericum* species

Plant species	Fractions*/ Standards	[a]DPPH assay %RSA (100 *u*g/mL)	[b]Reducing Power (25*u*g/mL)	[c]Total Antioxidant Phosphomolybdate assay as Ascorbic acid equivalents (mg/g of extract)	[d]Total phenolic contents as gallic acid equivalents (mg/g of extract)
Hypericum perforatum **(A)**	F1	90.34±0 .750	0.644+0.008	762.01 ±51.82	71.6±1.75
	F2	88.25±0.734	0.464+0.012	613.27 ±49.69	48.0±0.85
	F3	66.88±1.092	0.303+0.005	954.63 ±82.10	52.3±1.32
	F4	90.86±0.411	0.533+0.010	962.29 ±50.56	82.3±0.52
	F5	91.85±0.349	0.894+0.010	1256.05±128.47	89.2±0.52
Hypericum oblongifolium **(B)**	F1	91.75±0.536	0.548±0.0070	1286.26±151.73	76.8±1.750
	F2	92.70±0.876	0.836±0.0177	1009.04±48.18	54.1±0.850
	F3	85.16±0.327	0.482± 0.004	1078.60±82.54	73.1±3.464
	F4	92.70±0.500	0.843±0.0062	1306.7 ±69.23	87.8±0.458
	F5	90.99±0.193	0.666±0.0081	888.46±181.43	73.1±2.676
Hypericum monogynum (**C)**	F1	92.26±1.298	0.760+0.011	1344.7 ±60.63	72.7±0.98
	F2	81.08±0.565	0.495+0.005	986.9 ±76.65	46.0±0.52
	F3	67.37±0.766	0.322+0.007	856.13 ±54.93	62.4±0.85
	F4	89.70±0.310	0.548+0.011	1287.12±88.73	72.5±1.80
	F5	89.20±0.610	0.505+0.001	651.89 ±42.66	56.1±0.5
Hypericum choisianum **(D)**	F1	92.11±0.505	0.620+0.0138	1050.8 ±158.8	96.7±1.75
	F2	91.07±0.171	0.571+0.008	911.8 ±79.4	92.7±1.32
	F3	45.46±0.761	0.252+0.0152	738.3 ±92.1	46.3±2.60
	F4	84.59±0.290	0.478+0.015	942.4 ±59.9	87.2±1.70
	F5	93.06±0.294	0.907+0.088	1410.03±60.20	104.±1.32
Hypericum dyeri **(E)**	F1	86.02±0.322	0.553+0.010	994.00 ±108.73	58.1±1.778
	F2	90.42±0.182	0.681+0.018	1385.3 ±140.5	61.0±2.166
	F3	80.25±0.445	0.517+0.010	758.14 ±56.18	48.0±2.166
	F4	70.39±0.476	0.438+0.008	610.30 ±44.58	46.0±2.166
	F5	35.05±0.838	0.245+0.010	95.57 ±16.87	21.2±2.641
Standards	Quercetein	98.28±0.257	1.638+0.024	2058.70±180.1	370.18±14.11
	Ascorbic acid	97.60±0.689	1.692+0.020	2470.30±146.8	----------
	Gallic acid	98.03±0.503	1.653+0.019	2173.50±194.6	322.66±22.2
	α-tocopherol	92.48±0.68	0.468+0.088	557.70 ±54.56	67.40±5.51

[a,b,c,d] *The assays were carried out in triplicate and the results are expressed as mean values ± standard deviations*

*Fractions: Ethanol **(F1),** aqueous **(F2),** ethyl acetate **(F3),** acetone **(F4)** and final residue **(F5)**

Table 12.2: EC_{50} values[a,b] (*u*g/mL) of various extracts (five *Hypericum* species) in reducing power and DPPH scavenging assays

Plant species	Fractions/ Standards	DPPH Radical scavenging assay (EC_{50}^{a})	Reducing Power (EC_{50}^{b})
Hypericum perforatum **(A)**	F1	33.10 ± 1.12	13.32± 1.12
	F2	32.30 ± 1.32	23.50± 1.50
	F3	50.50 ± 1.53	28.33± 1.52
	F4	35.12 ± 1.12	19.67± 1.53
	F5	6.667 ± 1.52	9.66± 1.50
Hypericum oblongifolium **(B)**	F1	31.32 ± 1.42	17.50± 1.50
	F2	29.33 ± 1.52	13.67± 1.53
	F3	51.15 ± 1.62	20.34± 1.52
	F4	40.24 ± 1.21	8.17± 1.28
	F5	34.66 ± 1.32	16.33± 1.52
Hypericum monogynum **(C)**	F1	30.13 ± 1.12	12.17± 1.04
	F2	58.33 ± 1.52	20.33± 1.53
	F3	61.67 ± 2.12	29.10± 1.02
	F4	34.42 ± 1.42	17.16± 0.764
	F5	38.33 ± 1.52	19.17± 1.26
Hypericum choisianum **(D)**	F1	39.33 ± 1.52	16.16± 1.041
	F2	42.34 ± 1.33	17.50± 0.50
	F3	112.25± 2.65	34.83± 1.76
	F4	44.43 ± 1.32	20.67± 1.52
	F5	21.33 ± 1.52	11.34± 1.258
Hypericum dyeri **(E)**	F1	50.55 ± 0.92	17.17± 0.764
	F2	43.45 ± 2.31	11.87± 1.02
	F3	57.75 ± 1.12	19.67± 1.52
	F4	71.73 ± 1.52	22.00± 2.12
	F5	125.33± 5.03	40.00± 2.65
Standards	Quercetin	4.12+ 1.27	1.88+ 0.032
	Ascorbic acid	6.20+ 1.67	3.31+ 0.041
	Gallic acid	4.75+ 1.24	1.20+ 0.025
	α-tocopherol	32.50+ 1.57	21.50+ 0.085

[a] *EC50 (mg/mL): effective concentration at which 50% of DPPH radicals are scavenged EC50 (mg/mL): effective concentration at which the absorbance is 0.4.*

*Fractions: Ethanol **(F1),** aqueous **(F2),** ethyl acetate **(F3),** acetone **(F4)** and final residue **(F5)**

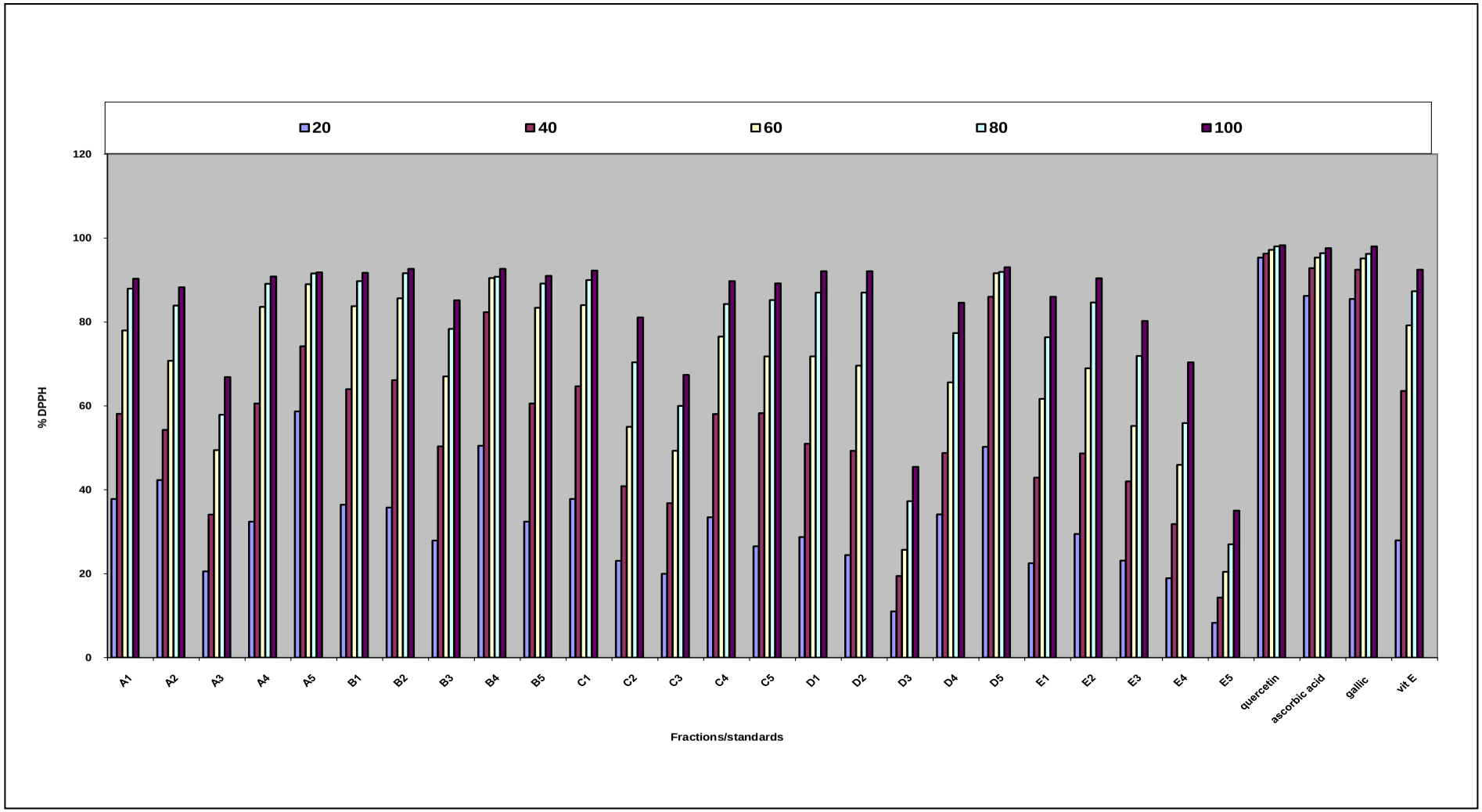


Fig.12.1. Free radical-scavenging capacities of various fractions of *Hypericum* species and standards measured in DPPH assay

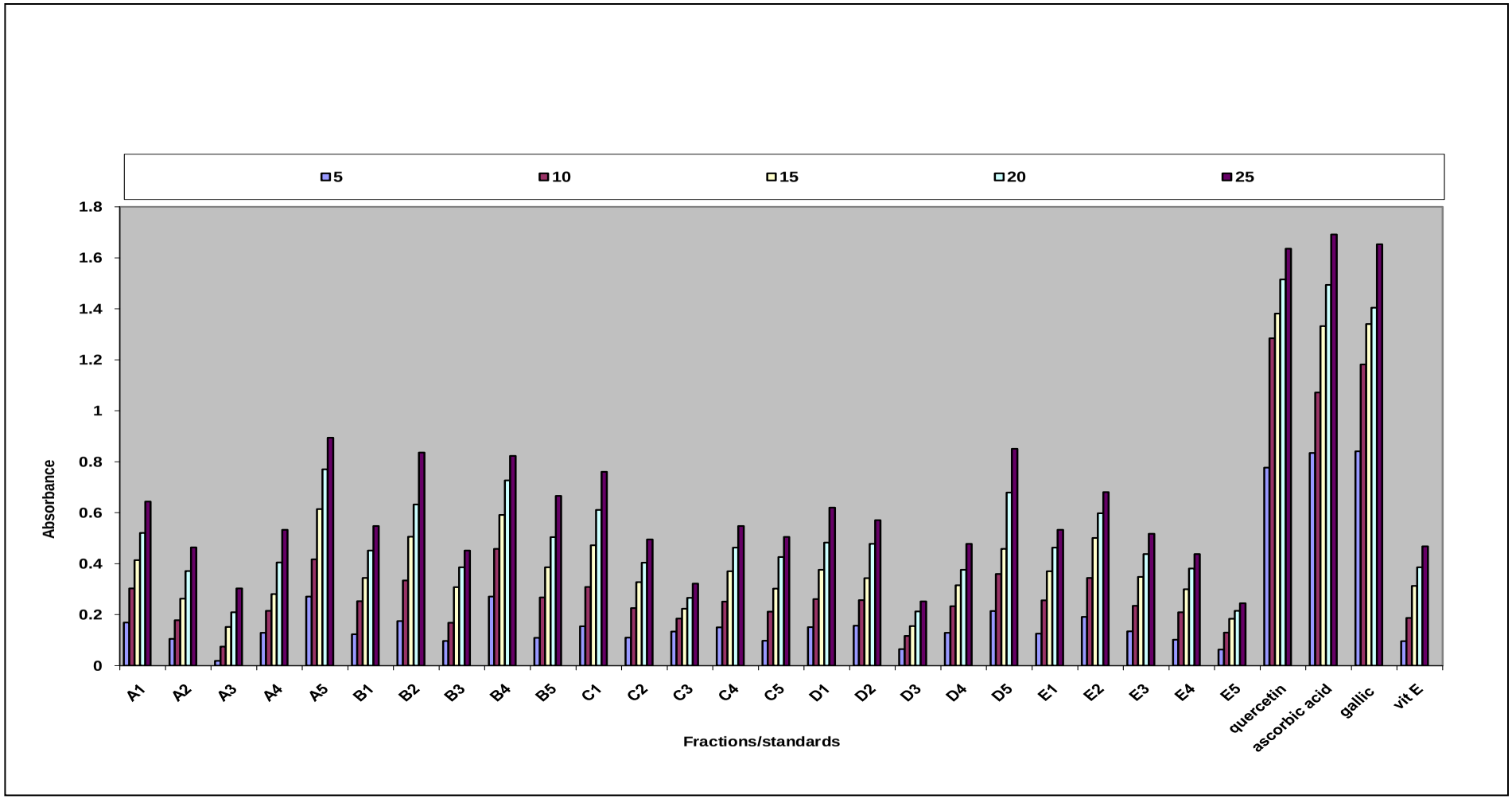


Fig.12.2. Reducing power of various fractions of *Hypericum* species & standards

12.2.2: Antimicrobial potential of the *Hypericum* species

12.2.2.1: Antibacterial activity

The antibacterial potential of various extracts from five *Hypericum* species were studied (Table 12.3). All the analyte showed antibacterial activity against all of the tested micro organisms to different extent, with the diameters of zone of inhibition ranging between 10 and 23 mm. The significant difference ($P < 0.05$) was found in the activity of various fractions ethanol **(F1),** aqueous **(F2),** ethyl acetate **(F3),** acetone **(F4)** and final residue **(F5)** against all tested stains. The most active fractions were crude **(F1)** and final residue **(F5)** obtained from *Hypericum dyeri* against *Escherichia coli.* The ethyl acetate **(F3)**, acetone **(F4)** and final fractions **(F5)** of *H. perforatum* whereas crude **(F1)**, ethyl acetate **(F3)** and acetone **(F4)** fractions of *H.oblongifolium* have shown good antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa.* Crude extracts **(F1)** of *H.monogynum and H.choisianum* were observed active against *Staphylococcus aureus* and *Pseudomonas aeruginosa.* Similarly the ethyl acetate **(F1)**, acetone **(F4)** and final fractions **(F5)** of *H. mnogynum* showed activity against *Pseudomonas aeruginosa.* None of the sample showed significant activity. All the bacterial strains in this study were found sensitive to streptomycin, specially *Staphylococcus aureus* and *Pseudomonas aeruginosa* were found most sensitive (inhibition zone values of 31and 32 mm respectively). *Escherichia coli* and *Salmonella typhi* were resistant to erythromycin and contrimoxazole.

12.2.2.2: Antifungal activity

The antifungal activities of various extracts from five *Hypericum* species were studied (Table 12.4). All the extract showed some antifungal activity against the entire tested organism to different extent (zone of % inhibition ranging between 10 and 52 %.). Significant difference ($P > 0.05$) was not found in the activities of fractions i.e ethanol **(F1),** aqueous **(F2),** ethyl acetate **(F3),** acetone **(F4)** and final residue **(F5)** against all tested stains. Crude **(F1)**, aqueous **(F2),** ethyl acetate **(F3)** and acetone fraction **(F4)** of *H.perforatum* were found most active against *Helminthosporium maydis.* Other fractions of the same plant showed moderate activity against tested fungal strains. Aqueous **(F2)**, ethyl acetate **(F3)**, acetone **(F4)** and final residue **(F4)**

of *H.oblongifolium* showed moderate activity against *Aspergillus niger, Helminthosporium maydis* and *Alternaria solani* and showed weak activity against *Aspergillus flavus.* Aqueous **(F2)**, ethyl acetate **(F2)** and acetone **(F3)** extracts of *H.monogynum* showed moderate activity against *Aspergillus niger* and *Alternaria solani* and have shown weak activities against *Helminthosporium maydis* and *Aspergillus flavus*. Relatively good activities were exhibited by crude **(F1)** and final **(F5)** fractions of the same plant against *Aspergillus niger*, *Helminthosporium maydis* and *Aspergillus flavus*. Activity was also exhibited by **F4** and **F5** fractions of *H.choisianum* against *Aspergillus niger*. Similarly aqueous **(F2)** and ethyl acetate **(F3)** fractions have shown activity against *Alternaria solani* and *Aspergillus niger*. Aqueous **(F2)** and ethyl acetate **(F3)** fractions of *H.dyeri* have shown against *Helminthosporium maydis*, *Alternaria solani* and *Aspergillus flavus.* All the organisms studied were found sensitive to fuconazole specialy the *Aspergillus niger* and *Alternaria solani* were the most sensitive (inhibition zone values of 76 and 74 % respectively) which is significant, however, none of the samples extracts studied showed significant activity. Most of them have moderate or weak activity as observed in antibacterial screening.

The data presented above indicated excellent antioxidant activity reaching up to 93% at 100*u*g/mL in DPPH radical scavenging activity which is almost close to that of the methanolic extract of *H . triquetrifolium*[34] and much more than that of the standardized extract of *H. perforatum*[35]. Silva et al, [36] evaluated the antioxidant activity with IC 50 (21*u*g/mL) of total extract of *Hypericum perforatum.* Yanping et al.[13] also evaluated DPPH radical quenching effect and observed its IC_{50} (10.63ug/mL) and comparable to the IC_{50} values of our samples ranges from 6.667 to 125.33*u*g/mL for final fractions (**F5**) of *H. perforatum* and *H.dyeri* respectively. The results in term of total antioxidant activity in our study ranged from 95 to 1410 umole/mg and coparable to the results obtained by Radulovic et al[37]. From the data and above discussion it is concluded that the excellent antioxidant activity observed in this study seems to be due to the presence of polyphenols. Mechanistically the reductants (polyphenols) donate their electrons to DPPH radical to convert them to more stable products and thus break down the free radical chain reactions.

Radulovic et al.[37]studied the antibacterial activities of nine *Hypericum* species which showed significant activity even at dose of 5*u*g/disc and zone of inhibition range from 12 - 42mm. In our findings the activities ranged between 10 and 23.8 mm

which is almost the same as previously reported [20]. As mentioned most of the active plants showed activity against Gram-positive strains and only few were active against Gram-negative bacteria[20]. Moreover, our findings showed an antimicrobial activity against the Gram-negative bacterium *Escherichia coli* and this micro-organism has been isolated from infected wounds of humans. These antioxidant and antimicrobial results revealed that *Hypericum* species contain some active constituents, which justify their use in traditional medicine. Results obtained also suggest that further work is required on the medicinal side in order to isolate and identify the active principles from the various extracts and thus such type of research could result in the discovery of lead compounds that could serve as a template for synthetic medicinal chemists.

Table 12.3: Antibacterial activities (diameter of growth inhibition zone) of ethanol **(F1),** aqueous **(F2),** ethyl acetate **(F3),** acetone **(F4)** and final residue **(F5)** fractions (10 mg/mL) of five *Hypericum* species

Plant species	Bacteria[a] tested zone of inhibition (mm)						
	Fractions/ Standards	*Ec*	*Sa*	*Ea*	*St*	*Pv*	*Pa*
Hypericum perforatum **(A)**	F1	11.3	13.2	15.5	10.6	10.3	11.2
	F2	11.2	14.5	11.4	11.2	11.1	12.1
	F3	10.6	16.4	11.2	10.1	11.4	18.3
	F4	11.2	19.1	11.1	10.5	11.3	15.5
	F5	11.2	18.2	10.3	11.6	10.5	15.4
Hypericum oblongifolium **(B)**	F1	10.2	18.8	10.6	11.4	12.2	16.6
	F2	10.1	11.5	10.3	11.2	12.1	14.3
	F3	11.2	19.2	11.1	10.8	11.2	17.2
	F4	11.5	19.3	10.4	11.6	12.5	18.1
	F5	12.8	12.2	10.8	12.2	10.1	12.3
Hypericum monogynum **(C)**	F1	12.8	21.1	11.7	10.1	13.4	20.3
	F2	13.1	14.2	11.2	15.4	10.8	13.6
	F3	11.4	14.3	11.5	15.5	11.5	13.4
	F4	11.7	14.6	11.4	13.6	11.4	14.1
	F5	14.6	11.6	13.3	12.2	14.2	13.2
Hypericum choisianum **(D)**	F1	10.3	15.2	11.2	12.2	11.3	16.5
	F2	10.2	10.3	11.2	11.1	12.6	13.7
	F3	11.1	12.4	11.1	11.3	12.8	16.6
	F4	11.4	10.2	10.1	12.4	13.5	14.3
	F5	10.1	11.1	11.2	12.2	13.6	17.1
Hypericum dyeri **(E)**	F1	19.7	11.3	10.3	12.6	12.3	12.5
	F2	23.1	12.6	11.5	12.3	14.5	13.4
	F3	11.6	12.5	11.8	10.2	11.2	12.2
	F4	11.1	13.2	12.4	14.2	12.5	14.2
	F5	23.2	11.3	12.1	14.2	11.2	13.3
	streptomycin	30.3	31.2	25.3	30.2	28.5	32.1

[a] Bacteria: *Sa, Staphylococcus aureus; Pa, Pseudomonas aeruginosa;St, Salmonella typhi;Ec, Escherichia coli;Pv, Proteus vulgaris;Ea, Enterobacter aerogenes*

Table 12.4: Antifungal screening (% growth inhibition) of ethanol **(F1),** aqueous **(F2),** ethyl acetate **(F3),** acetone **(F4)** and final residue **(F5)** fractions (400*u*g/mL) of five *Hypericum* species

Plant species	Fungi[a] tested zone of inhibition (%)				
	Fractions/ standards	*An*	*Hm*	*Af*	*As*
*Hypericum perforatum***(A)**	F1	35.29	47.29	35.29	27.18
	F2	29.41	51.76	23.53	23.53
	F3	41.18	47.06	31.76	29.41
	F4	35.29	52.94	23.53	29.41
	F5	27.06	40.00	28.76	25.88
*Hypericum oblongifolium***(B)**	F1	29.65	11.76	23.53	23.53
	F2	31.53	29.41	23.53	31.76
	F3	41.18	44.71	22.35	29.35
	F4	35.29	30.59	22.35	35.29
	F5	35.29	43.53	22.35	31.76
*Hypericum monogynum***(C)**	F1	29.41	30.59	21.18	29.41
	F2	30.53	11.76	21.18	27.06
	F3	40.00	17.65	28.24	29.41
	F4	29.41	17.65	22.35	41.18
	F5	35.29	35.29	22.35	29.41
*Hypericum choisianum***(D)**	F1	23.53	23.53	11.76	29.41
	F2	35.29	8.24	23.53	31.76
	F3	29.41	11.76	31.76	41.18
	F4	31.76	23.53	17.65	8.24
	F5	29.41	23.53	22.35	22.35
*Hypericum dyeri***(E)**	F1	41.18	31.76	22.35	23.53
	F2	15.29	43.53	41.18	30.59
	F3	38.82	11.76	28.24	27.06
	F4	35.29	35.29	22.35	35.29
	F5	35.29	11.76	8.24	21.18
	Fuconazole	76.47	70.59	72.94	74.12

[a]Fungi *:An,Aspergillus niger; Af, Aspergillus flavus Hm, Helminthosporium maydis; As, Alternaria solani*

12.2.3: Anti-proliferative activity of the *Hypericum* species

As stated in experimental section the air-dried and powdered materials of *Hypericum* species (*H. oblongfolium, H. monogynum, H. choisianum* and *H. dyeri*) were exhaustively extracted with hexane, ethyl acetate and methanol (3x25 L, each for 3 days) at room temperature (Fig. 13.2). The extracts were concentrated in a rotavapor and dried under vacuum to yield the residue of fractions, **F1** (hexane) and **F2** (ethyl acetate). The methanolic fraction was suspended in water extracted with n-butanol to afford fractions, **F3** (butanol) and **F4** (Water). These fractions (**F1, F2, F3** and **F4)** of *Hypericum* species were tested *in vitro* for their anti- proliferative (anticancer) activities on the different cell lines like human non-small cell lung carcinoma (NCI -H460), human colon adenocarcinoma cell (HT-29), human breast cancer (MCF-7), human ovarian adenocarcinoma (OVCAR-3), human renal cell carcinoma (RXF-393) using antineoplastic, etoposide as positive control. The results of these extract are given in table 12.4a. Among the various extracts/fractions of *H.oblongifolium* the **F1** showed relatively potent anti-proliferative activities (IC_{50}, 10.55 ± 4.19 µg/mL) on OVCAR-3 human ovarian adenocarcinoma cell growth almost equal to the activity of etoposide (IC_{50}, 9.42 ± 1.62 µg/mL). The anti-proliferative activities of various fractions were expressed in terms of IC_{50} (the concentration of extract required to inhibit the 50% of cell growth). Lower the IC_{50} value indicating higher potency. As can be seen from table 12.4a, the **F1** had significant activities (IC_{50}< 18 µg/mL) followed by the **F2**, which showed relatively good activity (IC_{50}< 40 µg/mL) on the inhibition of all five types cells lines tested. The **F3** showed activity (IC_{50}< 43 µg/mL) on the inhibition of four cells type except the human renal cell carcinoma (RXF-393) while the **F4** was found less active (IC_{50}> 90 µg/mL).

The antiproliferative activities of the various extracts/fractions of *H.dyeri* are also listed in table 114a. The **F1** of the same plant showed relatively potent anti-proliferative activity (IC_{50}, 17.20 ± 4.80µg/mL) on human non-small cell lung carcinoma (NCI-H460) cell growth. As can be seen from table 12.4a, the **F1** had significant activities (IC_{50}< 23 µg/mL) followed by the **F2**, which showed relatively good activity (IC_{50}< 26 µg/mL) on the inhibition of all five types cells line tested.

Table 12.4a: Antiproliferative activity (IC_{50} values µg/mL, means ± SDs of 3 determinations) of fractions of four *Hypericum species.*

	Cell lines				
Extracts*/positive control	**HT-29**	**NCI-H460**	**MCF-7**	**OVCAR-3**	**RXF-393**
H. choisianum					
F1	51.77 ± 9.60	49.10 ± 4.33	65.35 ± 1.27	48.48 ± 1.53	20.32 ± 4.63
F2	24.78 ± 7.16	17.63 ± 3.41	26.40 ± 4.68	32.00 ± 5.94	19.77 ± 5.62
F3	> 100	> 100	> 100	> 100	> 100
F4	> 100	> 100	> 100	> 100	> 100
H. dyeri					
F1	21.10 ± 0.70	17.20 ± 4.80	18.80 ± 4.10	23,18 ± 1.34	22.19 ± 0.36
F2	25.95 ± 0.49	24.70 ± 1.55	25.76 ± 2.70	26.50 ± 1.56	23.76 ± 2.10
F3	> 100	> 100	> 100	> 100	67.05 ± 0.49
F4	> 100	> 100	> 100	> 100	> 100
H. monogynum					
F1	13.08 ± 5.55	18.03 ± 0.34	15.01 ± 2.87	16.62 ± 2.69	19.71 ± 7.16
F2	26.31 ± 6.90	23.40 ± 8.48	25.96 ± 1.20	30.35 ± 7.57	23.16 ± 8.27
F3	> 100	> 100	> 100	> 100	> 100
F4	> 100	> 100	> 100	> 100	> 100
H. oblongifolium	15.85 ± 0.71	11.00 ± 2.26	14.14 ± 4.03	10.55 ± 4.19	18.01 ± 1.82
F1	39.30 ± 6.78	31.97 ± 3.28	40.15 ± 1.76	24.41 ± 7.09	22.86 ± 7.10
F2	89.50 ± 2.68	90.34 ± 8.71	> 100	> 100	> 100
F3	41.20 ± 7.43	43.25 ± 7.28	11.57 ± 4.75	11.97 ± 6.48	81.71 ± 2.98
F4					
Etoposide	1.22 ± 0.99	0.27 ± 0.02	3.42 ± 1.00	9.42 ± 1.62	13.77± 2.67

*Fractions: Hexane **(F1),** ethyl acetate **(F2),** butanol **(F3)** and aqueous **(F4)**

The **F3** showed weak activity (IC_{50}, 67.05 ± 0.49µg/mL) on the inhibition of only human renal cell carcinoma cell growth (RXF-393) while the rest were weakly active or almost inactive (IC_{50}> 100 µg/mL).

The antiproliferative activity of the two other species (*H.monogynum and H .choisianum*) were also evaluated **(**Table 12.4a**)**. The **F1** of *H.monogynum* showed significant activities (IC_{50}< 19 µg/mL) followed by **F2**, which showed relatively good activity (IC_{50}< 30 µg/mL) on the inhibition of all the five type cells tested mentioned above. The **F3** and **F3** showed weak activity or almost inactive (IC_{50}> 100 µg/mL). Among the various extracts of *H .choisianum* only **F2** showed good activity (IC_{50}< 32 µg/mL) and **F1** showed lower activity (IC_{50}, 65.05 ± 0.49µg/mL).

12.3: Biological screening of the family Pinaceae

12.3.1: Biological screening of the *Pinus* species

12.3.1.1: Antioxidant potential of the *Pinus* species

12.3.1.1.1: Determination of total phenolic content

The total phenolic content in crude ethanol (**F1**), aqueous (**F2**), ethyl acetate (**F3**), acetone (**F4**), and final residue (**F5**) of the bark and knotwood of *P. wallichiana* and *P. roxburghii* were measured (Table.12.5). The data in table 9.5 shows the phenolic contents in different fractions/extracts and standard in ug/mg of sample (Gallic Acid Equivalents). Statistically, no significant difference was observed among the phenolic contents of various fractions (P >0.05). Significant phenolic contents were observed in all fractions. Generally, the aqueous fraction of the bark and knotwood of both species showed higher phenolic contents. The highest phenolic contents were observed in the aqueous fraction of the bark of *P. roxburghii* whereas **F2** fraction of the bark of *P. wallichiana* showed lowest phenolic content of all. Interestingly, all the fractions showed higher phenolic compounds than one of the standards (α-tocopherol) except **F2** fraction of the bark of *P. wallichiana*. The Folin–Ciocalteu method determines the phenols by giving different responses to different phenolic compounds, depending on their chemical structures. Here we observed a linear relation between antioxidant activity and the total phenolics in most cases (Table 12.5).

12.3.1.1.2: DPPH radical scavenging activity

The DPPH radical scavenging activity of various extracts/fractions (**F1**, **F2, F3, F4** and **F5**) of the bark and knotwood of *P. wallichiana* and *P. roxburghii* along with standards were studied (Fig.12.3; Tables 12.5 and 12.6). A very good scavenging activity was shown by all fractions almost the same to the activity shown by the standards and interestingly more than that of α-tocopherol (standard) in some cases. There was statistically significant difference observed among the various fractions ($P < 0.05$) of the bark and knotwood .The highest activity (93.68 % at 100 *u*g/mL and with 1.5 *u*g/mL EC_{50}) was observed in the aqueous extracts of the bark of *P. roxburghii* while the aqueous fractions of bark and knotwood of both species showed excellent % RSA.The **F2** fraction of all four tested parts (bark and knotwood) have shown lower activity except the bark of *P. roxburghii.* The significant DPPH radical scavenging activities of fractions showed linear correlation with their corresponding phenolic contents, the fraction with higher phenolic contents showed higher scavenging activity (Table 12.5).

12.3.1.1.3: Reducing power

Comparative reducing power of various extracts/fractions (**F1**, **F2, F3, F4** and **F5**) of the bark and knotwood of *P. wallichiana* and *P. roxburghii* along with standards were studied (Fig.12.4; 12.5 and 12.6). All samples showed significant reducing power nearly equal to those shown by the standards and even more than that of α-tocopherol (standard). Like the scavenging activity, the reducing power also showed linear relation with concentrations of the samples. There was statistically significant differences observed among the same fraction ($P < 0.05$) of different parts (A, B, C and D). Generally aqueous extracts of the bark and knotwood of both plants showed high reducing power. Among various fractions the highest activity (1.14+0.01 at 25 *u*g/mL,with 8.5+0.9 *u*g/mL EC50) was observed in the aqueous fraction of the bark of *P. roxburghii*, whereas, the acetonic fraction of the knotwood of *P. wallichiana* exhibited the lowest activity. The reducing power might be due to either phenolic contents or some other reducing agents like tannins.

Table 12.5: Antioxidant activities and total phenolic contents of various fraction of of knotwood and bark of *Pinus* species

Plant species	Part studied	Fractions/ Standards	[a]DPPH assay %RSA (100 *u*g/mL)	[b]Reducing Power (25ug/mL)	[c]Total Antioxidant Phosphomolybdate assay as gallic acid equivalents (*u*mole/mg of extract)	[d]Total phenolic contents as gallic acid equivalents(mg/ g of extract)
Pinus roxburghii	Bark (A)	F1	93.25+0.052	1.134+0.0214	1661.4±51.1	182.80+3.04
		F2	93.68+0.529	1.14+0.0100	2467.96±135.2	259.21+4.35
		F3	92.57+0.142	1.081+0.0362	1128.9±66.1	150.79+4.36
		F4	93.19+0.121	1.130+0.110	1110.44±81.0	141.27+4.76
		F5	90.74+0.233	0.497+0.047	734.14±58.2	113.30+3.46
	Knotwood (B)	F1	67.91+1.306	0.353+0.0166	819.34±69.3	57.07+1.730
		F2	83.28+0.211	0.488+0.0190	1207±130.1	91.39+3.60
		F3	42.39+0.756	0.287+0.0220	1080.62±96.8	53.61+2.29
		F4	43.65+1.272	0.243+0.0091	1074.94±109.8	51.02+1.730
		F5	46.23+0.937	0.334+0.0068	555.22±35.5	49.00+2.64
Pinus wallichiana	Bark (C)	F1	93.39+0.176	0.769+0.0121	972.7±53.5	104.36+3.50
		F2	93.55+0.176	0.911+0.0106	1432.78±76.5	121.09+4.33
		F3	31.93+0.942	0.192+0.0105	587.88±61.5	14.98+1.80
		C4	93.48+0.263	0.744+0.0166	796.62±49.1	67.74+2.17
		F5	85.89+1.095	0.395+0.0051	1001.1±79.4	47.56+1.730
	Knotwood (D)	F1	43.25+0796	0.351+0.0066	1833.22±83.5	95.14+4.33
		F2	77.43+0.678	0.394+0.0036	2395.54±53.3	131.76+2.64
		F3	33.86+0.680	0.255+0.0191	1417.16±74.1	53.04+1.80
		F4	31.52+2.02	0.169+0.0060	1661.4±160.8	53.61+1.501
		F5	31.52+2.02	0.230+0.0064	149.1±56.4	44.39+1.80
Standards		Quercetein	98.28±0.257	1.638+0.024	2058.70±180.1	370.18±14.11
		Ascorbic acid	97.60±0.689	1.692+0.020	2470.30±146.8	----------
		Gallic acid	98.03±0.503	1.653+0.019	2173.50±194.6	322.66±22.2
		α-tocopherol	92.48±0.68	0.468+0.088	557.70 ±54.56	67.40±5.51

[a,b,c,d] *A triplicate assays were carried and the result swere expressed as mean values ± standard deviations*

*Fractions: Ethanol **(F1),** aqueous **(F2),** ethyl acetate **(F3),** acetone **(F4)** and final residue **(F5)**

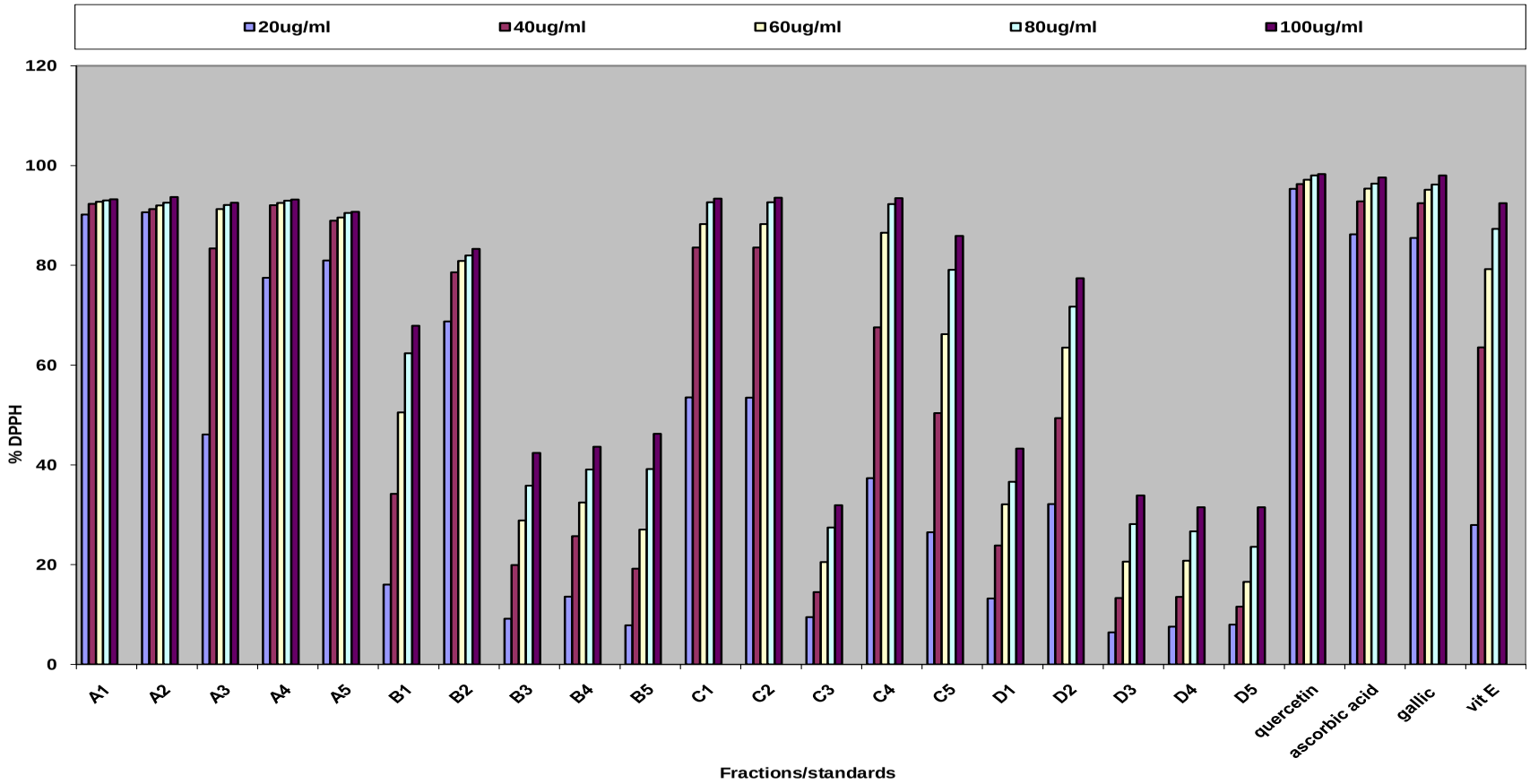


Fig. 12.3. Free radical-scavenging capacities of various fractions of knot wood and bark of *Pinus* species and standards measured in DPPH assay

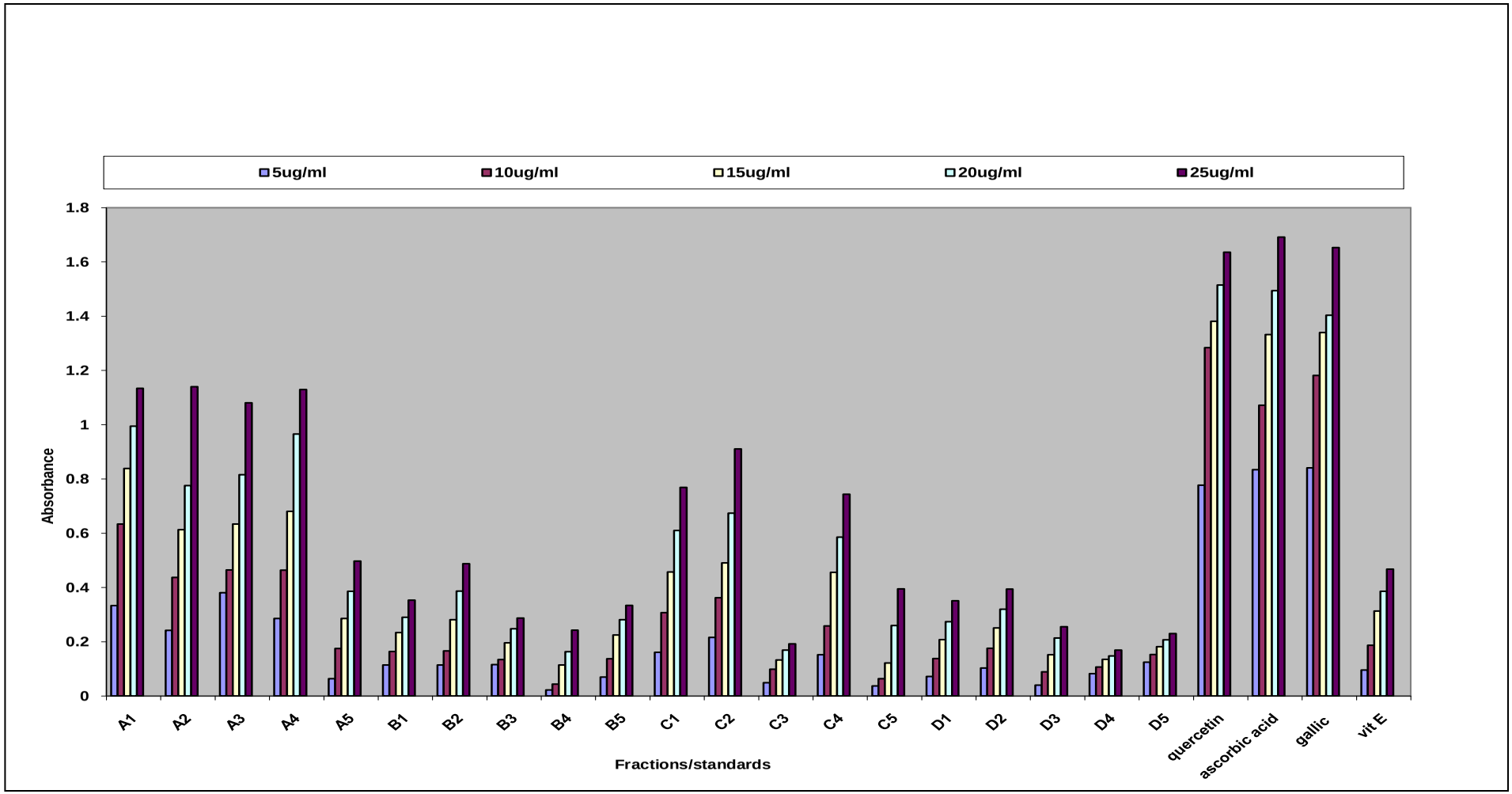


Fig.12.4.Reducing power of various fractions of the knot wood and bark of *Pinus* species & standards

12.3.1.1.4: Total antioxidant capicity

The total antioxidant capacity of various extracts/fractions (**F1**, **F2, F3, F4** and **F5**) of the bark and knotwood of *P. wallichiana* and *P. roxburghii* along with standards were compared (Table 12.5). All the fractions showed antioxidant activities nearly equal to the activity shown by the standards in some cases and even higher than that of α-tocopherol (standard). However, there were no statistically significant difference among various fractions (P >0.05). Same trend in activity was observed in most cases as in radical scavenging activity (RSA) and reducing power e.g the aqueous fraction of the bark of *P. roxburghii* showed highest antioxidant activity(2467.96+135.2 *u*mol/mg) whereas the lowest antioxidant activity (149.1±56.2 *u*mol/mg) was observed in **F5** of the knotwood of *P. wallichiana* . Higher phenolic contents show higher antioxidant activity, therefore correlation between total antioxidant capacity and the phenolic contents can be established from the data obtained (Table. 12.5)

12.3.1.2: Antimicrobial potential of the *Pinus* species

12.3.1.2.1: Antibacterial activity

The antibacterial tests were performed using agar-well diffusion assay [38-40]. The antibacterial potential of various extracts from the bark and knotwood of *P. wallichiana* and *P. roxburghii* was studied (Table 12.7). The extract showed remarkably good antibacterial activities against all of the tested Gram positive and Gram negative microorganisms to different extents having zones of inhibition ranging between 10 and 36 mm. Statistically, no significant difference (*P* >0.05) was found in the antibacterial activity of **F1**, **F2, F3, F4** and **F5** extracts against all the tested strains. The aqueous fraction of knotwood of *P. wallichiana* was found the most active fraction against all strains. All fractions from the bark of *Pinus roxburghii* and knotwood of *P. wallichiana* showed significant activity against *Escherichia coli, Pseudomonas aeruginosa* and *Salmonella typhi* while good activities were observed against *Staphylococcus aureus* and *Proteus vulgaris.* Fractions of knotwood of the *P. roxburghii* were found active against *Staphylococcus aureus* and *Pseudomonas aeruginosa* and aslo showed activity against *Proteus vulgaris* and *Salmonella typhi.*

Interestingly, all fractions from the knotwood of *P. wallichiana* were found more active against *Salmonella* typhi and *Pseudomonas aeruginosa* as compared to the standard (Steptomycin). All the bacterial strains in the study were found sensitive to streptomycin with *Staphylococcus aureus* and *Pseudomonas aeruginosa* being the most sensitive (inhibition zone values of 31and 32 mm respectively). *Escherichia coli* and *Salmonella typhi* were found resistant to other standards (erythromycin and contrimoxazole).

12.3.1.2.2: Antifungal activity

Antifungal activities of the extract was determined by the test tube dilution method [39,41]. Table 12.8 presents the antifungal activities of the extracts from the bark and knotwood of *P. wallichiana* and *P. roxburghii,* They showed excellent antifungal activities against all tested organisms upto different extent having % inhibition between 25 to 94 %. Statistically no significant differences ($P > 0.05$) were found in the antifungal activities of **F1**, **F2, F3, F4** and **F5** against all tested stains. **F2** and **F3** fractions from the knotwood of *P. wallichiana* were found most active against all the tested strains and interestingly their zones of inhibition (75-88%) were found higher than the standard. **F2** (87.5%), **F4** (75%) and **F5** (75%) fractions of the bark of *P. wallichiana* while **F2** (75%), **F4** (75%) fractions of knotwood of the same plant were significantly active against *Aspergillus niger* as compared to standard (fuconazole). All the fungi in the study were found sensitive to fuconazole specially *Aspergillus niger* and *Alternaria solani* were the most sensitive (inhibition zone values of 76and 74 % respectively) showing significant inhibition. In conclusion, some of the samples studied showed significant activities, however most of them have good or moderate activities.

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Table 12.7: Antibacterial activities (diameter of growth inhibition zone) of ethanol **(F1),** aqueous **(F2),** ethyl acetate **(F3),** acetone **(F4)** and final residue **(F5)** fractions (10mg/mL) of knotwood and bark of *Pinus* species

Plant species	Part studied	Bacteria[a] tested zone of inhibition (mm)						
		Fractions/ Standards	*Ec*	*Sa*	*Ea*	*St*	*Pv*	*Pa*
Pinus roxburghii	Bark (A)	F1	16	16	13	15	18	18
		F2	20	15	14	14	19	21
		F3	18	16	12	16	17	16
		F4	16	15	11	16	16	17
		F5	17	14	12	15	17	18
	Knotwood (B)	F1	12	18	12	16	12	32
		F2	13	24	15	15	13	27
		F3	11	20	12	18	15	28
		F4	12	18	12	15	16	32
		F5	17	14	12	15	17	18
Pinus wallichiana	Bark (C)	F1	13	14	11	12	20	18
		F2	15	18	11	12	17	18
		F3	11	15	12	11	16	16
		F4	11	14	11	12	16	15
		F5	12	11	11	12	15	16
	Knotwood (D)	F1	17	14	12	15	17	18
		F2	22	32	15	17	17	36
		F3	17	32	15	14	16	30
		F4	16	35	14	18	16	28
		F5	17	33	13	19	15	29
		streptomycin	30.3	31.2	25.3	30.2	28.5	32.1

[a] Bacteria: *Sa, Staphylococcus aureus; Pa,Pseudomonas aeruginosa;St, Salmonella* typhi;Ec, *Escherichia coli;Pv, Proteus vulgaris ;Ea,Enterobacter aerogenes*

Table 12.8: Antifungal screening (% growth inhibition) of ethanol **(F1),** aqueous **(F2),** ethyl acetate **(F3),** acetone **(F4)** and final residue **(F5)** fractions (400 ug/mL) of knotwood and bark of *Pinus* species.

Plant species	Part studied	Fungi[a] tested zone of inhibition (%)				
		Fractions/ standards	*An*	*Hm*	*Af*	*As*
	Bark (A)					
Pinus		F1	50.0	27.7	62.5	39.0
roxburghii		F2	31.3	39.8	25.0	93.9
		F3	25.0	88.0	75.0	39.0
		F4	25.0	27.7	50.0	26.8
		F5	50.0	39.8	50.0	26.8
	Knotwood (B)	F1	37.5	57.8	37.5	39.0
		F2	25.0	39.8	62.5	39.0
		F3	50.0	39.8	87.5	51.2
		F4	50.0	27.7	62.5	26.8
		F5	50.0	39.8	62.5	39.0
Pinus	Bark (C)	F1	25.0	33.7	50.0	39.0
wallichiana		F2	62.5	75.9	37.5	26.8
		F3	87.5	45.8	50.0	63.4
		F4	75.0	39.8	87.5	26.8
		F5	75.0	37.3	37.5	32.9
	Knotwood (D)	F1	62.5	75.9	87.5	32.9
		F2	50.0	51.8	75.0	39.0
		F3	75.0	88.0	87.5	69.5
		F4	75.0	75.9	87.5	75.6
		F5	37.5	9.6	56.3	57.3
		Fuconazole	76.47	70.59	72.94	74.12

[a]Fungi *:An,Aspergillus niger; Af, Aspergillus flavus Hm, Helminthosporium maydis; As, Alternaria solani*

Antioxidant activities of turpentine exudes from *P. nigra* have been studied[42].Similarly antioxidant and anticancer activities of phenolic extract from *P. massoinana* have also been investigated[43]. *P. pinea, P. brutia, P. radiata, P.*

halepensis, P. attenuata, P. nigra, P. densiflora, P. massoinana and essential oils from *P. mugo* was tested for their antioxidative capacity[44-47]. Our results obtained are comparatively similar to those reported in literature. The antimicrobial potential of the essential oils from the species of the family Pinaceae was well investigated [48], which showed activities and superior to our findings from the extracts rather than their essentials oils. *Pinus* species were evaluated for their antimicrobial activities. Knotwood and bark from 30 species along with pure compounds were assayed for their antimicrobial activities[49] which are also comparatively higher than our results. Results obtained suggest that further work is required on the medicinal side in order to detrmine the active principles from the various extracts and thus the findings could result in discovery of new compounds medicinal importance.

12.3.2: Biological screening of the *Picea smithiana, Abies pindrow* and *Cedrus deodara*

12.3.2.1: Antioxidant potential of the *Picea smithiana, Abies pindrow* and *Cedrus deodara*

12.3.2.1.1: Determination of total phenols

The contents of total phenols in ethanol **(F1),** aqueous **(F2),** ethyl acetate **(F3),** acetone **(F4)** and final residue **(F5)** fractions of the bark and knotwood of *Picea smithiana, Abies pindrow* and *Cedrus deodara* were measured by Follin Ciocalteu method. Table 12.9 shows the phenolic contents in different fractions/extracts and standard (expressed in ug/mg gallic acid equivalent). Statistically, no significant difference observed among the phenolic contents of various fractions ($P > 0.05$). Considerable amounts of phenolic contents were observed in all fractions. Generally the knotwood showed higher phenolic contents as compared to bark of the same plant. The highest phenolic contents (168.38+2.18) were observed in aqueous fraction **(F2)** of the bark of *Abies pindrow* whereas final fraction **(F5)** of the knotwood of *Picea smithiana* showed lowest phenolic contents of (23.05+2.49). Most of the fractions showed higher phenolic compounds than one of the standards (α-tocopherol).

12.3.2.1.2: DPPH radical scavenging activity

The DPPH radical scavenging activity of ethanol **(F1),** aqueous **(F2),** ethyl acetate **(F3),** acetone **(F4)** and final residue **(F5)** fractions of the bark and knotwood of *Picea smithiana, Abies pindrow* and *Cedrus deodara* along with standards were studied(Fig.12.5, Table 12.9 and 12.10). Considerable radical scavenging activities were shown by most of the fractions and almost similar to the activity shown by the standards. Interestingly, some of the fractions have higher activity than that of α-tocopherol (standard). There was no statistically significant difference observed among the various fractions ($P > 0.05$) of the bark and knotwood. Generally the bark showed higher RSA as compared to knotwood of the same specie. Bark of *Abies pindrow* and *Cedrus deodara* have comparatively high whereas knotwood of *Cedrus deodara* have low RSA while the other extracts showed moderate activity. The highest activity (94.22% at 100*u*g/mL and with 2.5*u*g/mL EC_{50}) was observed in the aqueous extracts **(F2)** for the bark *Abies pindrow*. The significant DPPH radical scavenging activities of fractions were due their corresponding phenolic contents, the fraction with higher phenolic contents showed higher radical scavenging activity (Table 12.9).

12.3.2.1.3: Reducing power

Comparative reducing power of ethanol **(F1),** aqueous **(F2),** ethyl acetate **(F3),** acetone **(F4)** and final residue **(F5)** fractions of the bark and knotwood of *Picea smithiana, Abies pindrow* and *Cedrus deodara* along with standards were studied (Fig.12.6, Table 12.9 and 12.10). All samples showed significant reducing power and some of them are closed to the activity shown by the standards. Interestingly, some of the fractions have shownt higher reducing power than α-tocopherol (standard). Like the scavenging activity, the reducing power of samples increased with increasing amount of concentration. There was statistically significant differences observed among the same fractions ($P < 0.05$) of different parts (bark and knotwood) of tested species. Generally, the bark showed high reducing power as compared to knotwood of the same species. Bark of *Abies pindrow* and *Cedrus deodara* have comparatively higher reducing power than knotwood of *Cedrus deodara* whereas all other extracts showed moderate activities . Among various fractions, the highest activity (1.260+

0.0100 at 25*u*g/mL; with 5.5+0.9 *u*g/mL EC50) was observed in the aqueous extracts **(F2)** of bark of *Abies pindrow* whereas the lowest (0.225+0.00451 with 45.5+1.2 *u*g/mL EC50) was found in final fraction **(F5)** of the knotwood of *Cedrus deodara*. The reducing power might be due to either phenolic contents or some other reducing agents present in the plant however correlation in phenolic contents, reducing power and DPPH radical scavenging activity observed in most cases (Table 12.9). The fraction with higher phenolic contents showed higher scavenging activity and reducing power.

12.3.2.1.4: Total antioxidant activity

The antioxidant capacity of ethanol **(F1),** aqueous **(F2),** ethyl acetate **(F3),** acetone **(F4)** and final residue **(F5)** fractions of the bark and knotwood of *Picea smithiana, Abies pindrow* and *Cedrus deodara* along with standards were compared (Table 9.9). All the fractions showed considerable antioxidant activities nearly closed to the activity shown by the standards in some cases and even higher than that of α-tocopherol (standard). However, there were no statistically significant difference among various fractions (P >0.05). Same trend in activity was observed in most cases as in reducing power and radical scavenging activity (RSA) e.g the aqueous fraction (**F2**) of bark of *Abies pindrow* showed maximum antioxidant activity (1907.1+ 160.0 *u*mole/mg) whereas the lowest antioxidant activity (92.3+27.4 *u*mole/mg) was obtained for the final fraction (**F5**) of the knotwood of *Picea smithiana*. Phenolic compounds often have showed the best antioxidant activity, therefore correlation between activities and phenolic contents was noted in some points (Table 12.9).

Table 12.9: Antioxidant activities and total phenolic contents of ethanol **(F1),** aqueous **(F2),** ethyl acetate **(F3),** acetone **(F4)** and final residue **(F5)** fractions of the knotwood and bark of *P. smithiana, A. pindrow* and *C. deodara*

Plant species	Part studied	Fractions/ Standards	[a]DPPH assay %RSA (100 *u*g/mL)	[b]Reducing Power (25ug/mL)	[c]Total Antioxidant Phosphomolybdate assay as gallic acid equivalents (*u*mole/mg of extract)	[d]Total phenolic contents as gallic acid equivalents (mg/g of extract)
Picea smithiana	Bark (A)	F1	87.97+1.97	0.973+0.01429	1828.96±85.2	152.52+4.36
		F2	91.12+0.150	0.651+0.00751	1782.1±107.9	73.51+0.865
		F3	89.83+0.618	0.766+0.01079	917.32±62.1	103.79+0.87
		F4	92.06+0.069	0.599+0.00721	1255.28±236	66.88+0.502
		F5	90.87+0.435	0.547+0.0060	1469.7±267	51.60+1.80s
	Knotwood (B)	F1	92.22+0.573	0.858+0.0083	2317.44+163.2	97.16+2.64
		F2	82.83+0.619	0.679+0.0225	1932.62+190	164.05+1.32
		F3	78.42+0.375	0.721+0.01531	1273.74+147	145.89+0.50
		F4	79.70+1.673	1.034+0.0170	1574.78+108	158.29+0.87
		F5	43.76+2.22	0.263+0.00200	92.3+27.4	23.05+2.49
Abies pindrow	Bark (C)	F1	92.62+0.177	0.925+0.00451	867.62+98.3	94.85+1.80
		F2	94.22+0.10	1.260+0.0100	1907.1+160.0	168.38+2.18
		F3	92.02+0.380	0.939+0.0278s	113.00+85	97.73+1.730
		F4	91.710.207	0.853+0.00520	1718.2+113.5	88.50+2.64
		F5	90.43+0343	1.230+0.0100	1256.7+101.8	123.11+2.64
	Knotwood (D)	F1	83.86+0.122	0.939+0.00361	1491+230	166.65+2.18
		F2	87.49+0.261	1.045+0.0225	1421.4+224	145.31+3.12
		F3	83.70+0.600	0.883+0.01058	1056.5+148.4	135.79+1.73
		F4	81.89+0.427	0.794+0.01518	1449.8+147.1	148.48+2.65
		F5	82.60+0.525	0.729+0.0176	1150.2+110.8	133.49+2.64
Cedrus deodara	Bark (E)	F1	93.25+0.115	1.211+0.0101	1499.52+111.0	134.35+2.78
		F2	93.99+0.023	1.252+0.0247	556.64+113.9	157.71+2.64
		F3	85.43+0.767	0.780+0.00902	948.56+98.5	69.76+2.17
		F4	92.35+0.160	0.792+0.01021	1218.36+73.9	91.10+3.90
		F5	93.41+0.108	0.655+0.01114	981.22+72.5	78.12+2.17
	Knotwood (F)	F1	29.86+1.334	0.279+0.00603	1326.28+42.7	49.29+1.730
		F2	36.44+0.391	0.471+0.01150	1456.92+53.4	123.97+2.49
		F3	27.51+0.599	0.287+0.00666	1331.96+45.9	38.91+1.730
		F4	27.51+0.599	0.248+0.01026	1373.14+102.6	48.71+2.18
		F5	32.75+0.574	0.225+0.00451	1131.74+90.4	38.91+1.730
Standards		Quercetein	98.28±0.257	1.638+0.024	2058.70±180.1	370.18±14.11
		Ascorbic acid	97.60±0.689	1.692+0.020	2470.30±146.8	----------
		Gallic acid	98.03±0.503	1.653+0.019	2173.50±194.6	322.66±22.2
		α-tocopherol	92.48±0.68	0.468+0.088	557.70 ±54.56	67.40±5.51

Table 12.10: EC_{50} values [a,b] (ug/mL) of various extracts fraction of of the knotwood and bark of *Picea smithiana,Abies pindrow* and *Cedrus deodara.* in reducing power and DPPH scavenging assays

Plant species	Part studied	Fractions/ Standards	DPPH Radical scavenging assay (EC_{50}[a])	Reducing Power (EC_{50}[b])
Picea smithiana	Bark (A)	F1	20.5+1.3	8.5+1.5
		F2	24.0+2.5	14.0+1.8
		F3	23.5+3.1	13.5+2.3
		F4	19.3+1.4	15.0+2.0
		F5	35.45+3.2	18.0+3.1
	Knotwood (B)	F1	21.5+1.5	9.5+1.5
		F2	26.5+3.0	13.5+2.1
		F3	39.0+2.5	19.9+1.5
		F4	35.5+3.4	9.0+1.3
		F5	110+3.5	35.5+3.5
Abies pindrow	Bark (C)	F1	18.5+2.4	9.5+1.8
		F2	2.5+0.5	5.5+0.9
		F3	14.5+1.2	8.5+1.9
		F4	16.5+2.1	11.5+1.4
		F5	3.5+1.8	4.5+1.5
	Knotwood (D)	F1	28.5+1.4	9.0+1.5
		F2	22.5+1.9	8.5+2.1
		F3	29.6+1.6	9.5+1.4
		F4	30.5+2.3	11.3+2.5
		F5	23.0+3.0	12.5+1.6
Cedrus deodara	Bark (E)	F1	3.5+0.9	7.6+1.5
		F2	2.5+0.6	6.5+1.2
		F3	49.0+2.4	13.0+1.6
		E4	21.5+3.0	11.0+1.8
		E5	22.5+0.537	17.5+0.4
	Knotwood (F)	F1	140.5+1.5	40.5+3.5
		F2	130.0+2.8	19.5+2.1
		F3	145.5+3.5	32.5+1.5
		F4	120.0+1.9	39.5+2.7
		F5	132.5+2.8	45.5+1.2
Standards		Quercetein	4.12+ 1.27	1.88+ 0.032
		Ascorbic acid	6.20+ 1.67	3.31+ 0.041
		Gallic acid	4.75+ 1.24	1.20+ 0.025
		α-tocopherol	32.50+ 1.57	21.50+ 0.085

[a] *EC50 (mg/mL): effective concentration at which 50% of DPPH radicals are scavenged.*
[b] *EC50 (mg/mL): effective concentration at which the absorbance is 0.4.*
*Fractions: Ethanol **(F1),** aqueous **(F2),** ethyl acetate **(F3),** acetone **(F4)** and final residue **(F5)**

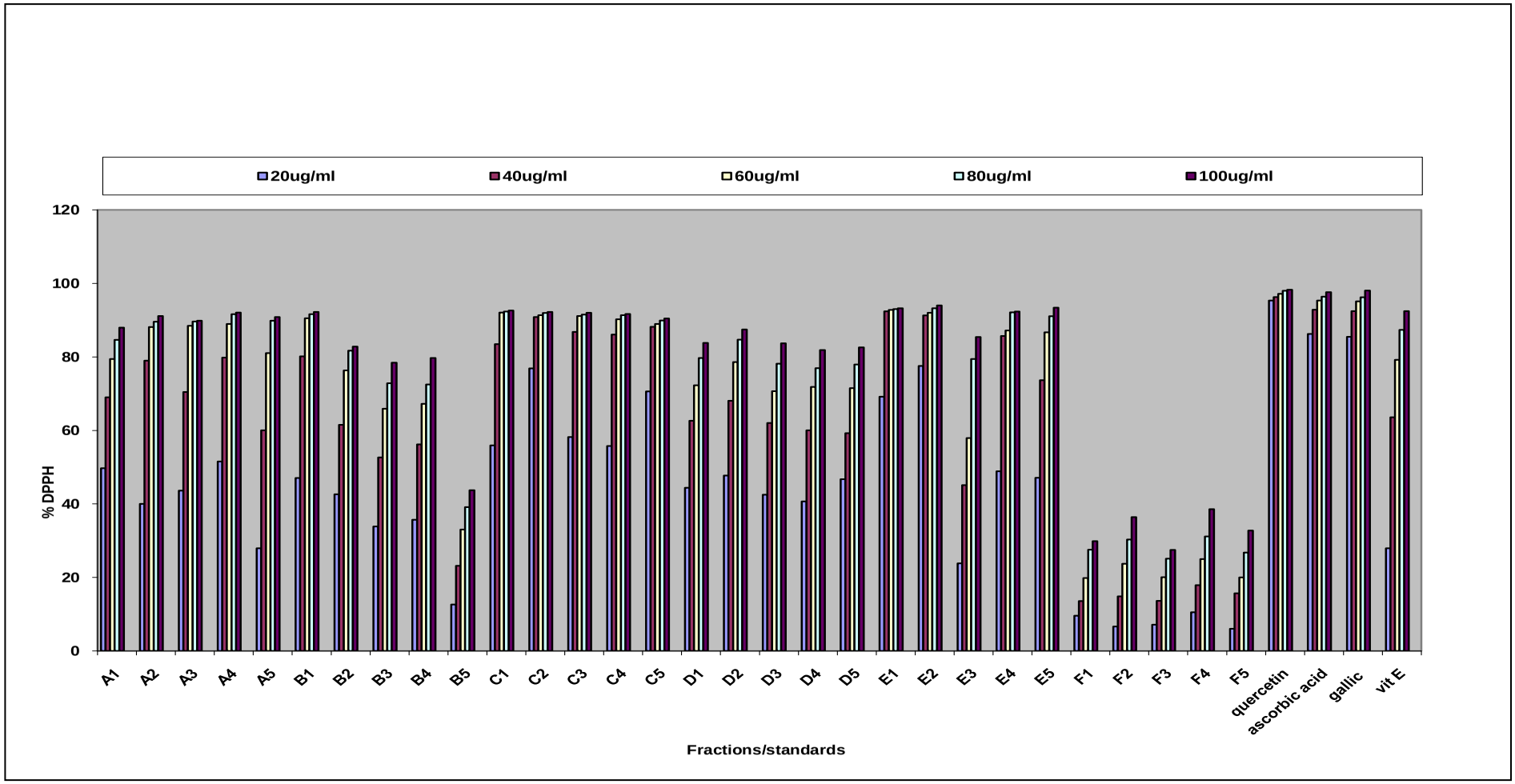


Fig.12.5. Free radical-scavenging capacities of various fractions of the knotwood and bark of *Picea smithiana, Abies pindrow* and *Cedrus deodara* and standards measured in DPPH assay

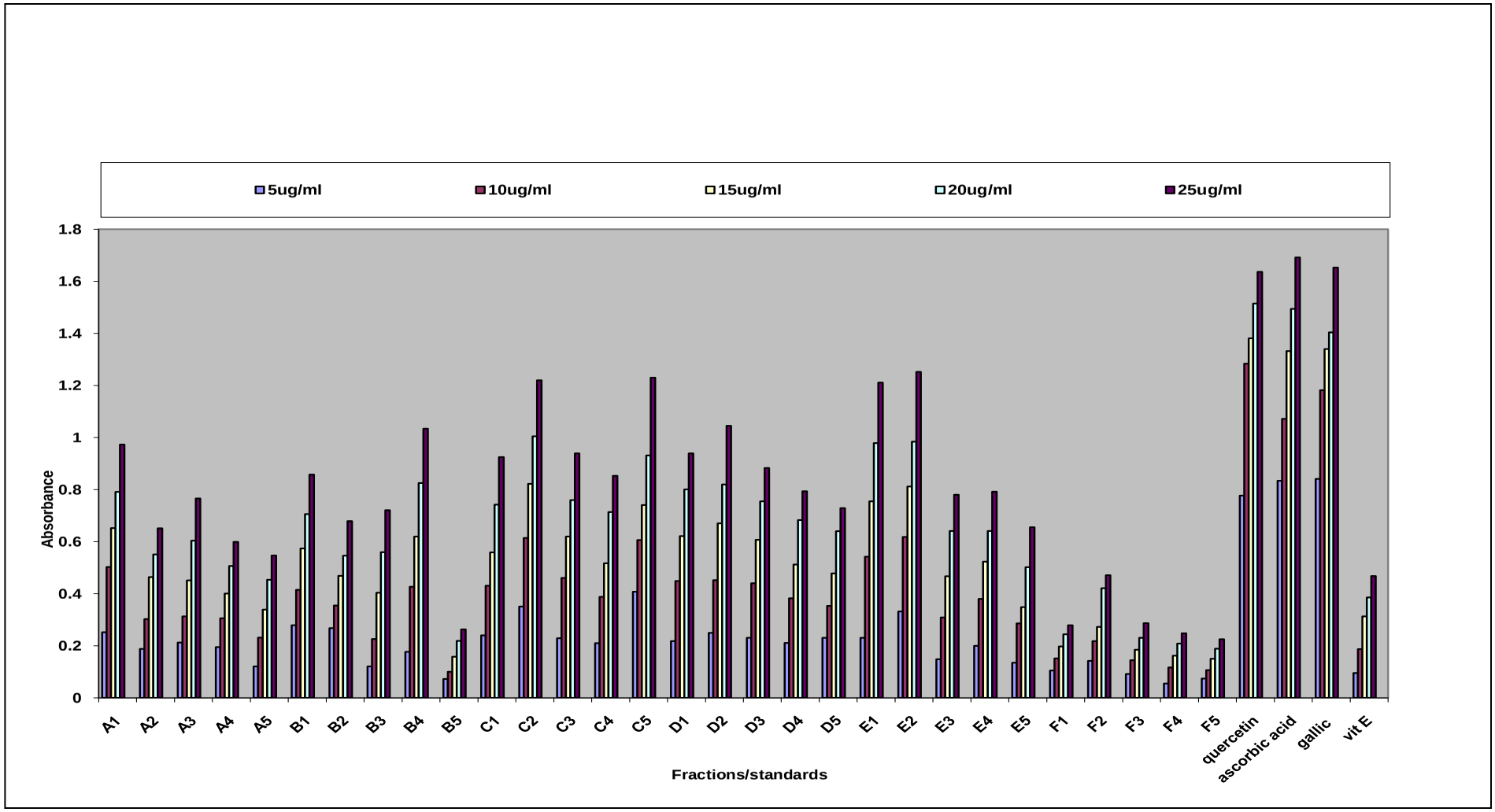


Fig.12.6.Reducing power of various fractions of the knotwood and bark of *Picea smithiana, Abies pindrow* and *Cedrus deodara* & standards

12.3.2.2: Antimicrobial potential of the *Picea smithiana, Abies pindrow* and *Cedrus deodara*

12.3.2.2.1: Antibacterial activity

The antibacterial activities of ethanol **(F1),** aqueous **(F2),** ethyl acetate **(F3),** acetone **(F4)** and final residue **(F5)** fractions from the bark and knotwood of *Picea smithiana, Abies pindrow* and *Cedrus deodara* were determined and listed in table 12.11. Various extracts showed antibacterial activity against all of the tested Gram positive and Gram negative microorganisms to different extent, having diameters of zone of inhibition ranging between 10 and 33mm. Statistically, no significant difference ($P > 0.05$) was found in the antibacterial activities of various extracts (**F1-F5**) against all tested strains. The ethyl acetate (**F3**), acetonic (**F4**) and final (**F5**) fractions of knotwood of *Picea smithiana* showed relatively good activity against all strains. All fractions obtained from the bark and knotwood of all species showed good antibacterial activity against *Pseudomonas aeruginosa.* Ethyl acetate fraction (**F3**) of the bark of *Picea smithiana* was active against *Salmonella typhi.* Ethanolic extract (**F1**) of the bark of *Abies pindrow* showed good activity against *Staphylococcus aureus, Escherichia coli, Proteus vulgaris* and *Pseudomonas aeruginosa.* The etanolic (**F1**), aqueous (**F2**) and ethyl acetate (**F4**) fractions of the knotwood of the same plant remained active *Escherichia coli, Enterobacter aerogenes and Pseudomonas aeruginosa.* All fractions of the bark of *Cedrus deodara* showed significant activities against *Staphylococcus aureus* while good activity against *Enterobacter aerogenes and Pseudomonas aeruginosa.* Interestingly, ethyl acetate (**F3**) fraction from the knotwood of *Picea smithiana* was found most active against *Escherichia coli and Staphylococcus aureus* as compared to standard (Steptomycin). All the bacterial strains in the study were found sensitive to streptomycin, specially the *Staphylococcus aureus* and *Pseudomonas aeruginosa* were the most sensitive (inhibition zone values of 31 and 32 mm respectively). *Escherichia coli* and *Salmonella typhi* were resistant to other standards (erythromycin and contrimoxazole).

12.3.2.2.2: Antifungal activity

The antifungal activities of ethanol **(F1),** aqueous **(F2),** ethyl acetate **(F3),** acetone **(F4)** and final residue **(F5)** fractions from the bark and knotwood of *Picea smithiana, Abies pindrow* and *Cedrus deodara* were determined and listed in table

12.11. Considerable activity was shown all tested extracts against the entire tested organism to different extent, having % inhibition ranging between 1.3 and 88 %. Statistically, no significant difference ($P > 0.05$) was found in the antifungal activity of various fractions (**F1-F5**) against all tested stains. Extracts obtained from *Cedrus deoera* showed higher antifungal activities, those obtained from *Abies pindrow* showed moderate activities while extracts from *Picea smithiana* showed lower activities against fungal strains. Ethyl acetate fraction (**F3)** from the bark of *Cedrus deoera* was found the most sensitive against all the tested strains and interestingly, tits zone of inhibition (75-88 %) was found higher than standard (fuconazole). **F2 (**62.5 %), and **F5 (**59 %) fractions of the bark of *Cedrus deoera* while **F1** (62.5 %), **F2** (60 %), **F3** (72.5 %), **F4** (67.5%) fractions of knotwood of the same plant were found significantly active against *Aspergillus niger*. All the strain in the study were found sensitive to fuconazole, specialy, *Aspergillus niger* and *Alternaria solani* were found most sensitive (inhibition zone values of 76and 74 % respectively). However, some of the samples (extracts) studied showed significant activity, most of them have good or moderate activity as observed in antibacterial screening.

Tiwari et al.,[50] studied free radical scavenging activity of components from heart wood of *Cedrus deodara*, similarly antioxidant activity and phenolic contents from knotwood of *Picea abies* were studied[51]. Antioxidant activities of turpentine exudes from *Pinus nigra are also reported*[42]. The antioxidant and anticancer activities of phenolic extract from *Pinus massoinana* has also been investigated[43]. Various species of family Pinaceae were tested for its antioxidative capacity [44-47]. Our findings were in conformity with previously reported results. The antimicrobial activity of essential oils from family Pinaceae have been investigated[48] which showed significant activities and are superior to our findings. *Abies webbiana* was evaluated for its antimicrobial activities[52] which is almost similar to the results obtained in our study. Knotwood and bark from 30 species along with pure compounds were assayed for their antimicrobial activities[49] which are also comparatively higher than our results. Results obtained in this study suggest that further work is required on the medicinal side in order to identify the active principles from the various extracts of the studied conifers. Potential of discovery of new compounds exist that may add to the existing arsenal of medicinal agent.

Table.12.11 Antibacterial activities (diameter of growth inhibition zone) of ethanol **(F1),** aqueous **(F2),** ethyl acetate **(F3),** acetone **(F4)** and final residue **(F5)** fractions (10mg/mL) of the knotwood and bark of *Picea smithiana, Abies pindrow* and *Cedrus deodara*

Plant species	Part studied	Bacteria[a] tested zone of inhibition (mm)						
		Fractions/ Standards	*Ec*	*Sa*	*Ea*	*St*	*Pv*	*Pa*
Picea smithiana	Bark (A)	F1	11	14	11	13	12	14
		F2	11	12	11	14	13	16
		F3	11	14	11	19	11	18
		F4	10	12	10	11	13	20
		F5	11	13	11	12	11	14
	Knotwood (B)	F1	25	14	16	11	10	17
		F2	10	25	12	12	11	23
		F3	30	33	28	25	11	12
		F4	11	27	15	16	22	17
		F5	32	19	20	21	18	17
Abies pindrow	Bark (C)	F1	16	18	13	12	16	18
		F2	14	17	14	16	17	18
		F3	12	14	13	12	13	17
		F4	13	12	12	20	15	18
		F5	11	13	10	15	15	16
	Knotwood (D)	F1	16	14	16	12	11	22
		F2	17	12	13	13	12	18
		F3	15	18	13	16	14	25
		F4	12	13	18	13	13	20
		F5	13	13	14	12	13	16
Cedrus deodara	Bark (E)	F1	11	21	18	12	12	17
		F2	11	22	15	12	15	18
		F3	11	21	12	11	13	16
		F4	12	23	14	13	13	20
		F5	10	19	14	15	14	15
	Knotwood (F)	F1	14	17	12	11	12	23
		F2	13	15	11	9	11	13
		F3	14	18	13	11	12	17
		F4	16	17	13	11	11	16
		F5	15	16	13	11	11	17
	Standard	streptomycin	30.3	31.2	25.3	30.2	28.5	32.1

[a] Bacteria: *Sa, Staphylococcus aureus; Pa,Pseudomonas aeruginosa;St, Salmonella typhi;Ec, Escherichia coli;Pv, Proteus vulgaris ;Ea,Enterobacter*

.

Table 12.12: Antifungal screening (% growth inhibition) of ethanol **(F1),** aqueous **(F2),** ethyl acetate **(F3),** acetone **(F4)** and final residue **(F5)** fractions (400ug/mL) of the knotwood and bark of *Picea smithiana, Abies pindrow* and *Cedrus deodara*

Plant species	Part studied	Bacteria[a] tested zone of inhibition (mm)				
		Fractions/ Standards	*An*	*Hm*	*Af*	*As*
Picea smithiana	Bark (A)	F1	12.5	18.1	6.3	12.2
		F2	37.5	13.3	15.0	17.1
		F3	15.0	3.6	15.0	8.5
		F4	12.5	12.0	18.8	8.5
		F5	6.3	21.7	10.0	32.9
	Knotwood (B)	F1	0.0	3.6	6.3	8.5
		F2	6.3	3.6	-1.3	14.6
		F3	15.0	15.7	0.0	2.4
		F4	8.8	30.1	13.8	26.8
		F5	2.5	9.6	6.3	2.4
Abies pindrow	Bark (C)	F1	37.5	45.8	25.0	45.1
		F2	37.5	27.7	37.5	32.9
		F3	50.0	51.8	25.0	26.8
		F4	25.0	27.7	37.5	39.0
		F5	50.0	27.7	37.5	39.0
	Knotwood (D)	F1	15.0	24.1	8.8	17.1
		F2	12.5	21.7	6.3	20.7
		F3	25.0	21.7	15.0	14.6
		F4	18.8	22.9	25.0	13.4
		F5	15.0	18.1	27.5	20.7
Cedrus deodara	Bark (E)	F1	43.8	39.8	25.0	51.2
		F2	62.5	33.7	12.5	63.4
		F3	87.5	75.9	62.5	87.8
		F4	0.0	33.7	50.0	63.4
		F5	58.8	45.8	68.8	39.0
	Knotwood (F)	F1	62.5	45.8	87.5	39.0
		F2	60.0	39.8	43.8	26.8
		F3	72.5	39.8	50.0	69.5
		F4	67.5	27.7	87.5	39.0
		F5	12.5	63.9	25.0	63.4
	Standard	Fuconazole	76.47	70.59	72.94	74.12

[a]Fungi *:An Aspergillus niger; Af, Aspergillus flavus Hm, Helminthosporium maydis; As, Alternaria solani*

12.4: Biological screening of the *Taxus fuana* Nan Li & R.R. Mill

12.4.1: Antioxidant potential of the *Taxus fuana* Nan Li & R.R. Mill

12.4.1.1: Determination of total phenols

In present study the contents of total phenols in ethanol (**F1**), aqueous (**F2**), ethyl acetate (**F3**), acetone (**F4**) and final residue (**F5**) of the bark (A) and knotwood (B) of *Taxus fuana* were measured (Table 12.13). The phenolic contents in **F1**, **F2, F3, F4** and **F5** of the bark (A) extract were 150.21+3.61 166.75+5.19, 53.04+2.78, 138.97+3.50 and 116.65+5.07, while that of knotwood (B) extract were 237.58 + 5.63, 172.41+5.07, 213.65+5.19, 216.53+8.88 and 177.89+5.19 respectively. Statistically no significant difference was observed among the phenolic contents of various fractions ($P > 0.05$). Higher phenolic contents were observed in all fractions which ranging close to that of standard. The highest phenolic contents were observed in **F1** of knotwood (B) whereas **F3** fraction of the bark showed lowest phenolic contents of all. The **F2** fraction of the bark also exhibited higher phenolic contents in among the extract obtained from the bark. All the fractions showed higher phenolic compounds than one of the standards (α-tocopherol) except **F3** fraction of bark (A).

12.4.1.2: DPPH radical scavenging activity

The DPPH radical scavenging activity of various extracts/fractions (**F1**, **F2, F3, F4 and F5**) of the bark (A) and knotwood (B) of *Taxus fuana* and standards were studied (Fig.12.7, Table 12.13 and 12.14). Excellent scavenging activity was shown by all fractions. The scavenging activities obtained for analtes were close to the standard used. The % Radical Scavenging Activity (%RSA) of the **F1**, **F2, F3, F4 and F5** of the bark (A) were 93.67+0.155, 94.05+0.164, 69.26+0.943, 93.3+0.23 and 93.42+0.269 while that of knotwood (B) were 94.89+1.12, 93.662.26, 94.58+ 0.122, 93.32+0.49 and 93.51+0.72 at 100 *u*g/mL respectively. There was statistically significant difference observed among the various fractions ($P < 0.05$) of the bark (A) and knotwood (B). The highest activity (94.89% at 100 *u*g/mL and with 21.2*u*g/mL EC_{50}) was observed in the **F1** of knotwood (B) while the **F2** fractions of bark (A) showed maximum % RSA (94.05+0.164 at 100 *u*g/mL and with 1.5+.05 EC_{50}) among

the extracts obtained from the bark. The significant DPPH radical scavenging activities of fractions were due their corresponding phenolic contents, the fraction with higher phenolic contents showed higher scavenging activity (Table 12.13).

12.4.1.3: Reducing power

Comparative reducing power of various extracts/fractions (**F1**, **F2, F3, F4 and F5**) of the bark (A) and knotwood (B) of *Taxus fuana* and standards were studied (Fig.12.8, Table 12.13 and 12.14). All samples showed significant reducing power almost equal to the activity shown by the standards and higherthan that of α-tocopherol one of the standard in most cases. The reducing power of samples increased with increasing amount of concentration. There was statistically significant differences observed among the same fraction ($P < 0.05$) of both parts (A & B) as well as in various fractions of the same plant except **F5** of knotwood (B). The reducing power of the **F1**, **F2, F3, F4** and **F5** of the bark (A) were 1.117+0.0160, 1.212+0.164, 0.424+0.0105, 0.953+ 0.0120, 0.655+ 0.0119 while that of knotwood (B) were 1.554+0.207, 1.251+ 0.095, 1.237+0.0122, 1.298+0.0246 and 0.288+0.0458 at 25 *u*g/mL respectively. The **F1** of knotwood (B) showed high reducing power (1.554+0.207 at 25*u*g/mL; with 2.0+0.1 *u*g/mL EC50). Among various fractions of the bark (A), the highest activity (1.212+0.164 at 25*u*g/mL; with 6.5+0.3*u*g/mL EC50) was observed in **F2** fraction. The reducing power might be due to either phenolic compounds or some other reducing agents present in the plants, however, correlation between phenolic contents, reducing power and DPPH radical scavenging activity was observed in most cases (Table 12.13). The fraction with higher phenolic contents showed higher scavenging activity and reducing power.

Table 12.13: Antioxidant activities and total phenolic contents of various fractions of the bark and knotwood of *Taxus fuana*

Plant species	Part Studied	Fractions*/ Standards	[a]DPPH assay* %RSA (100 *u*g/mL)	[b]Reducing Power * (25ug/mL)	[c]Total Antioxidant Phosphomolybdate assay as gallic acid equivalents** (umole/mg of extract)	[d]Total phenolic contents as gallic acid equivalents** (mg/g of extract)
Taxus fuana	Bark (A)	F1	93.67+0.154	1.117+0.0160	1150.2+89.7	150.21+3.61
		F2	94.05+0.164	1.212+0.164	2054.74+87.2	166.65+5.07
		F3	69.26+0.943	0.424+0.0105	930.1+81.2	53.04+2.78
		F4	93.37+0.238	0.953+0.0120	1117.5+69.34	138.97+3.50
		F5	93.42+0.269	0.655+0.0119	479.96+47.5	116.76+5.19
	Knotwood (B)	F1	94.89+1.12	1.554+0.207	5694.2+107.2	237.58+5.63
		F2	93.66+2.262	1.251+0.095	2029.18+87.2	172.41+5.07
		F3	94.58+0.122	1.237+0.0122	3248.96+188.0	213.65+5.19
		F4	93.32+0.49	1.298+0.0246	5596.22+138.7	216.53+8.88
		F5	93.51+0.72	0.288+0.0458	2449.5+108.7	177.89+4.35
	Standards	Quercetein	98.28±0.257	1.638+0.024	2058.70±180.1	370.18±14.11
		Ascorbic acid	97.60±0.689	1.692+0.020	2470.30±146.8	----------
		Gallic acid	98.03±0.503	1.653+0.019	2173.50±194.6	322.66±22.2
		α-tocopherol	92.48±0.68	0.468+0.088	557.70 ±54.56	67.40±5.51

[a,b,c,d] A triplicate aa carried out in triplicate and the results are expressed as mean values ± standard deviations *(P<0.5) and **(P>0.5)

*Fractions: Ethanol **(F1),** aqueous **(F2),** ethyl acetate **(F3),** acetone **(F4)** and final residue **(F5)**

Table 12.14: EC_{50} values[a,b] (ug/mL) of various extracts (*Taxus fuana*) in reducing power and DPPH scavenging assays

Plant species	Part Studied	Fractions*/ Standards	DPPH Radical scavenging assay (EC_{50}[a])	Reducing Power (EC_{50}[b])
Taxus fuana	Bark (A)	F1	2.50+0.5	7.5+0.5
		F2	1.5+0.15	6.5+0.3
		F3	61.5+1.5	24.5+1.5
		F4	18.5+0.9	8.5+0.9
		F5	21.5+1.0	17.5+0.8
	Knotwood (B)	F1	21.2+1.49	2.0+0.10
		F2	33.5+1.28	6.5+0.5
		F3	23.5+1.00	5.2+0.12
		F4	22.0+0.80	7.5+05
		F5	23.8+1.54	30.5+1.5
	Standards	Quercetein	4.12+ 1.27	1.88+ 0.032
		Ascorbic acid	6.20+ 1.67	3.31+ 0.041
		Gallic acid	4.75+ 1.24	1.20+ 0.025
		α-tocopherol	32.50+ 1.57	21.50+ 0.085

[a] EC50 (mg/mL): The concentration at which 50% of DPPH radicals are scavenged.

[b] EC50 (mg/mL): The concentration at which the absorbance is 0.4.

*Fractions: Ethanol **(F1),** aqueous **(F2),** ethyl acetate **(F3),** acetone **(F4)** and final residue **(F5)**

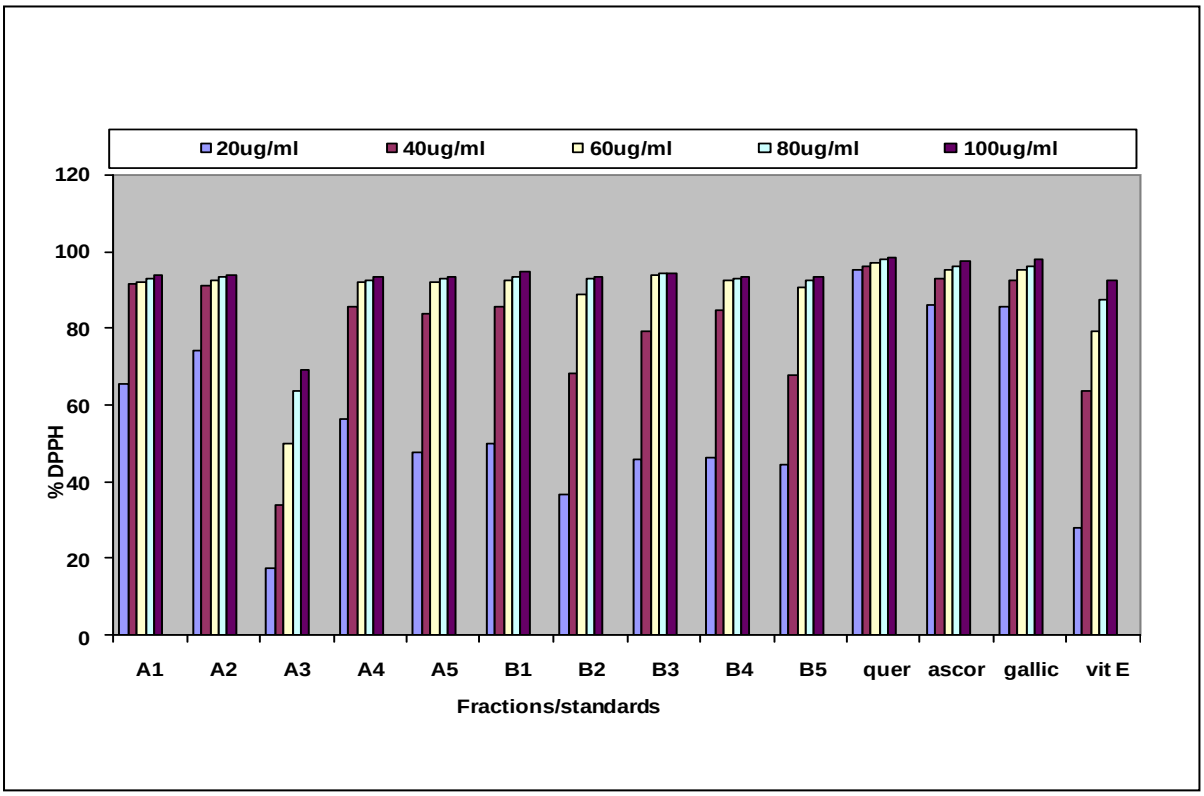


Fig. 12.7: Free radical-scavenging capacities of various fractions the bark and knotwood of *Taxus fuana* and standards measured in DPPH assay

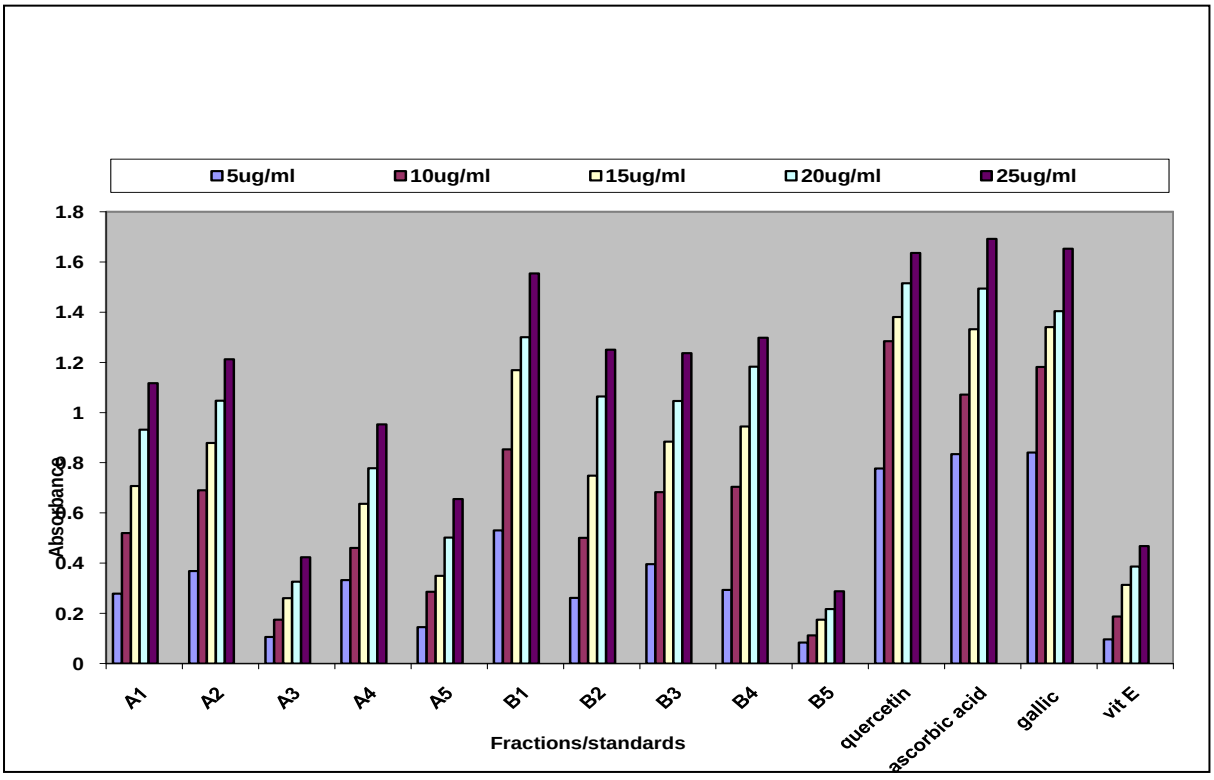


Fig.12.8: Reducing power of various fraction the bark and knotwood of *Taxus fuana* and standards

12.4.1.4: Total antioxidant capacity

The antioxidant capacity of various extracts/fractions (**F1**, **F2, F3, F4** and **F5**) of the bark (A) and knotwood (B) of *Taxus fauna* and standards were compared (Table 12.13).All the fractions showed remarkable activities almost closed to the activity shown by the standards and interestingly in some cases much more than that of standards. However there were no statistically significant difference among various fractions (P >0.05). The total antioxidant activity of **F1**, **F2, F3, F4** and **F5** of the bark(A) were, 1150.2+89.7, 2054.74+87.2, 930.1+81.2, 1117.5+69.34, 479.96 +47.5 while that of knotwood (B) were 5694.2+107.2, 2029.18+87.2, 3248.96+188.0, 5596.22+138.7 and 2449.5+108.7 *u*mole/mg respectively. Same trend in activity was observed in most cases as in radical scavenging activity(RSA) and reducing power e.g the **F1** of knotwood (B) showed maximum antioxidant activity (5694.2+107.2 *u*mol/mg) whereas the high antioxidant activity (2054.74$\pm$87.2 *u*mol/mg) in bark was observed in **F2** fraction. Phenolic compounds often have showed the best antioxidant activity. The correlation between activities and phenolic contents was noted in some points (Table 12.13)

12.4.2: Antimicrobail potential of the *Taxus fuana* Nan Li & R.R. Mill

12.4.2.1: Antibacterial activity

The antibacterial activitie of extracts from the bark (A) and knotwood (B) of *Taxus fauna* are shown in table 12.15. The extracts were found active against the entire tested Gram positive and Gram negative microorganisms to different extent, having zones of inhibitions ranging between 10 and 33 mm. The most active fraction was **F1** and **F5** obtained from bark (A) against all strains. All fractions of bark (A) showed significant antibacterial activity against *Escherichia coli* while good activity against *Proteus vulgaris, Staphylococcus aureus* and *Pseudomonas aeruginosa.* Fractions of knotwood (B) were significantly active against *Staphylococcus aureus* while showed good activity against *Proteus vulgaris* and *Salmonella typhi.* The significant difference ($P < 0.05$) was found in the antibacterial activities of **F1**, **F2, F3, F4** and **F5** against all tested stains Interestingly, **F5** f bark (A) was found more active than standard (Steptomycin). All the bacterial strains used in the study were found sensitive to streptomycin.

12.4.2.2: Antifungal activity

The antifungal activity was measured by test tube dilution method [39,41]. The extracts from the bark (A) and knotwood (B) of *Taxus fauna* showed significant antifungal activities against the entire tested organisms, with the % inhibition zones ranging between 20 and 94% (Table 12.16). No significant difference ($P > 0.05$) was found in the antifungal activity of **F1**, **F2, F3, F4** and **F5** against all tested stains. All fractions of bark (A) and Knotwood (B) showed significant antifungal activity against *Aspergillus niger* while good activity against, *Helminthosporium maydis*, *Alternaria solani* and *Aspergillus flavus*. **F1** and **F5** of bark (A) while **F2** of knotwood were found most active against all the four tested strains. Interestingly, **F3** (72%) and **F5** (93.75%) fractions of the bark (A) while **F1** (75%) and **F2** (93.75%) fractions of knotwood showed impressive activity against *Aspergillus niger* as compared to the standard fuconazole. The fungi used in the study were found sensitive to fuconazole with *Aspergillus niger* and *Alternaria solani* being the most sensitive (inhibition zone values of 76 and 74 % respectively). However, some of the samples (extracts) studied showed significant activity, most of them have good or moderate activities as observed in antibacterial screening.

Taxus is the most investigated genus for its biological activities and bioactive costituents. Taxoids from the needles of *Taxus wallichiana* showed significant anticancer and immunomodulatory properties [53]. Taxoids and lignans from the heart wood of *Taxus baccata* has been investigated for their anti-inflammatory and antinociceptive activities [54]. Hypoglycemic effect and antiallergic activity of the ethyl acetate and methanolic extract of the wood of *Taxus yunnanesis* were also reported [55,56]. Antioxidant activity of leaves and fruits of the Iranian *Taxus baccata* was evaluated[57] by ferric thiocyanate (FTC) and thiobarbituric acid (TBA) methods which showed excellent activity. The ethanolic extract of the heart wood of *Taxus baccata* showed significant antibacterial and antifungal activities[58] however, they are lower than our findings. Antitumor and antifungal activities in endophytic fungi isolated from *Taxus maire* have been studied [59] which are also lower than our antifungal results. From the above discussion it is clear that our findings of the antioxidant and antimicrobial studies on *Taxus fauna* are worth for the detail studies to isolate and characterize new bioactive chemical constituents.

Table 12.15: Antibacterial activities (diameter of growth inhibition zone) of ethanol **(F1),** aqueous **(F2),** ethyl acetate **(F3),** acetone **(F4)** and final residue **(F5)** fractions (10mg/mL) of the bark and knotwood of *Taxus fuana*

Plant species	Part Studied	Bacteria[a] tested zone of inhibition (mm)						
		Fractions/ Standards	*Ec*	*Sa*	*Ea*	*St*	*Pv*	*Pa*
Taxus fuana	Bark (A)	F1	27	18	17	16	19	16
		F2	21	15	11	14	15	16
		F3	25	17	10	12	17	18
		F4	24	17	13	15	16	18
		F5	33	17	16	16	18	19
	Knotwood (B)	F1	12	27	11	18	11	20
		F2	14	28	14	21	12	20
		F3	11	20	11	18	11	23
		F4	12	28	12	20	14	18
	Standard	F5	11	25	12	19	13	19
		Steptomycin	30	31	25	30	28	32

[a] Bacteria: *Sa, Staphylococcus aureus; Pa, Pseudomonas aeruginosa;St, Salmonella typhi;Ec, Escherichia coli;Pv, Proteus vulgari ;Ea, Enterobacter aerogenes*

Table 12.16: Antifungal screening (% growth inhibition) of ethanol **(F1),** aqueous **(F2),** ethyl acetate **(F3),** acetone **(F4)** and final residue **(F5)** fractions (400ug/mL) the bark and knotwood of *Taxus fuana*

Plant species	Part Studied	Fungi[a] tested zone of inhibition (%)				
		Fractions/ standards	*An*	*Hm*	*Af*	*As*
		F1	65.00	33.73	75.00	32.92
		F2	62.50	37.34	56.25	39.02
		F3	72.50	39.75	50.00	26.82
		F4	62.50	33.73	37.50	26.82
		F5	93.75	45.78	50.00	51.21
	Knotwood (B)	F1	75.00	39.75	37.50	39.02
		F2	93.75	51.80	56.25	51.21
		F3	50.00	27.71	50.00	39.02
		F4	62.50	45.78	50.00	20.73
		F5	65.00	39.75	50.00	39.02
	Standard	Fuconazole	76.47	70.59	72.94	74.12

[a]Fungi *:An,Aspergillus niger; Af,Aspergillus flavus Hm,Helminthosporium maydisAs,Alternaria solani*

12.6: Cytotoxic activities of withasteroids isolated from *Physalis divericata*

Compounds **61–70** were screened for *in vitro* cytotoxicity against two human against cancer cell lines, the colorectal carcinoma cells (HCT-116) and human non-small cell lung cancer cells (NCI-H460). The results are summarized in table 12.17. Compounds **62–64** and **66** exhibited a strong cytotoxicity, with IC_{50} values of 1.4, 1.2, 3.7, and 0.3 μM respectively against HCT-116 cell line, Physalin H (**66**) showed the highest cytotoxicity with IC_{50} value 0.3 ± 0.04 μM while the new compound withaphysanolide A (**61**) and the known compounds **65**, **67**, **68** and **70** showed moderate cytotoxicity against HCT-116 cell line. Compounds **62**, **63**, and **66** exhibited higher cytotoxicity (IC_{50} values, 1.4, 1.9, and 1.8 μM respectively) against NCI-H460 cell line. Compounds **64**, **65, 67**, **68** and **70** showed mild cytotoxicity (IC_{50} values, 1.9 ± 0.06, 20.8 ± 0.4, 24.4 ± 0.2, 15.3 ± 0.2 and 32.1 ± 0.8 respectively) against NCI-H460 cell line. As can be seen from table 12.17, compounds with withaphysalin structure (**61,67–70**) have shown moderate cytotoxicity against both cancer cell lines, while physalins (**62–66)** showed stronger cytotoxicity. All the above compounds isolated from this plant showed activities. The regulations in terms of structure-activity relationships (SAR) were concluded as follow;

(i) Comparison of the cytotoxic activities of physalins (**62-66)**, the physalin A,B, D and H (**62**–**64** & **66**) exhibited a strong cytotoxicity, indicated that Physalin with 2,5-diene-1-one systems in ring A and B as well as with substituent like hydroxyl and chlorine groups were likely to show stronger activities than other on both type of cell tested. Physalin H showed the highest activity due to the presence of chlorine at C-5.

(ii) Comparing the cytotoxicity of compounds, it is assumed that compounds with withaphysalin structure (**67**–**70**) have only shown a moderate cytotoxicity against the two tumor cell lines, while physalins (**62**–**66**) have shown stronger cytotoxicity. The stronger cytotoxic activities of physalins were attributed to the greater number of oxygen and carbonyl functionalities as compared to withaphysalins

Table 12.17: Cytotoxicities of **61**–**70** toward HCT-116 and NCI-H460 cells

Compounds	IC_{50}[a] (µM)	
	HCT116	**NCI-H460**
61	30.8 ± 0.5	>100
62	1.4 ± 0.05	1.4 ± 0.08
63	1.2 ± 0.04	1.9 ± 0.06
64	3.7 ± 0.06	10.2 ± 0.3
65	17.4 ± 0.3	20.8 ± 0.4
66	0.3 ± 0.04	1.8 ± 0.07
67	17.5 ± 0.2	24.4 ± 0.2
68	14.2 ± 0.3	15.3 ± 0.2
69	>100	>100
70	27.0 ± 0.6	32.1 ± 0.8
Topotecan[b]	0.026 ± 0.004	0.07 ± 0.005

[a] Mean ± SEM*n* = 3.

[b] Positive control

12.7: Antiproliferative activity of withanolides isolated from *Withania coagulans*

The cytotoxicity of the isolated withanolides from *Withania coagulans* were tested *in vitro* on murine spleen cells. The inhibition activity on lipopolysaccharide (LPS)-induced B and concanavalin A (ConA)-induced T cell proliferation, with cyclosporin A (CsA) as positive control was measured. The results of these compounds are given in table 3.16a. The immunosuppressive activity of each compound was expressed in terms of IC_{50} (the concentration of compound that inhibited T and B cell proliferation to 50% of the control value). The cell viability was also expressed as half inhibitive concentration (IC_{50}) which means the drug concentration when viability rate of cells reached 50%. The selective index (SI) value,

the ratio of IC_{50} of cell viability to IC_{50} of inhibition of proliferation, was used to evaluate the bioactivity of compounds.

As can be seen from table 12.18, compounds **45**, **47**, **48**, **49**, **51**, **53**, **55**, **56**, **57**, **59** had relatively good activities (IC_{50}< 20 μM) on the inhibition of both ConA-induced T and LPS-induced B cell proliferation, among which compound **56** had the strongest activity (IC_{50} = 1.66 μM) and the best SI value (25.5). Compound **47** also exhibited a satisfactory SI value.From the preliminary immunosuppressive evaluation, it was observed that the ethanol extract of the aerial part of *W. coagulans* showed strong activities in inhibiting the T and B cell proliferation, and their mechanism of action in treatment of rheumatism might be attributed to the inhibition of T and B cell functions. The major effective components of this herb were withanolides. All withanolide derivatives isolated from this plant showed good activities. The regulations in terms of structure-activity relationship (SAR) were concluded as followings:

(i) Comparison of the activities of compounds **53**, **56**, **57**and **59** with **52**, **55**, **46** and **47** respectively, indicated withanolides with 2,5-diene-1-one systems in ring A and B were likely to show stronger acitivities than those with 3,5-diene-1-one systems.

(ii) Comparing the inhibitory activities of compounds **55**, **56** and **58** with **48**, **52** and **60**, respectively, it appeared that 17*β*-OH enhanced the inhibition of T cell proliferation.

(iii) The comparison of compounds **53**, **54** and **55** with **48**, **58** and **60** respectively, showed that the activities of withanolides with a 27-Me were stronger than those substituted with a hydroxymethyl group at C-27.

(iv) A 14α-OH or a double-bond between C-14 and C-15 did not show any significant effect when activities of compounds **53**, **55** and **56** with **57**, **45** and **46** were compared.

(v) Comparative activities of compounds **47** and **59** with **55** and **56** showed that a 15*α*-OH would retard the activities.

Table 12.18: Inhibitory Effects of CsA (positive control), and Compounds **45-60** on Spleen Lymphocyte Proliferation Induced by Mitogens *in Vitro*

Compounds[a]	IC_{50} (μM) [SI][b]		
	Cell viability IC_{50} (μM)	Inhibition of ConA-induced T cell proliferation	Inhibition of LPS-induced B cell proliferation
CsA	26.7	0.40 [66.8]	0.47 [56.8]
45	53.2	14.0 [3.8]	10.1 [5.3]
46	50.1	35.4 [1.4]	27.7 [1.8]
47	182.3	11.3 [16.1]	15.4 [11.8]
48	60.3	19.0 [3.2]	15.3 [3.9]
49	45.5	13.8 [3.3]	10.1 [4.5]
50	51.3	29.2 [1.7]	42.8 [1.2]
51	55.6	12.7 [4.4]	9.09 [6.1]
52	48.9	36.8 [1.3]	39.5 [1.2]
53	47.1	10.0 [4.7]	11.8 [4.0]
54	49.4	38.8 [1.3]	41.7 [1.2]
55	44.4	11.5 [3.9]	7.19 [6.2]
56	42.3	1.66 [25.5]	1.66 [25.5]
57	48.5	12.7 [3.8]	8.61 [5.6]
58	54.9	31.4 [1.7]	32.4 [1.7]
59	42.9	10.4 [4.1]	9.73 [4.4]
60	52.2	49.2 [1.1]	45.1 [1.2]
Ethanolic extract	50.0 (μg/mL)	8.6 [5.8](μg/mL)	10.6 [4.7] (μg/mL)

[a]*The compounds tested for immunosuppressive activity were consistent with the description in the Experimental Section;* [b] *Selectivity index [SI] is determined as the ratio of the concentration of the compound when viability rate cells reached 50% (IC_{50}) to the concentration of the compound needed to inhibit the proliferation to 50% (IC_{50}) of the control value.*

12.8: Urease inhibitory activity of extracts and Xanthones from *H.oblongifolium*

The bioassay-guided fractionation of *H. oblongifolium* led to the isolation of potent urease inhibitors. Various fractions (**F1**, **F2**, **F3**, and **F4)** were obtained from the air-dried, powdered twigs of *H. oblongifolium* (see fractionation scheme in "Experimental" section). The fractions **F1**, **F2**, **F3**, and **F4** were tested *in vitro* for their urease inhibition activity. Among the fractions **F2** and **F4** showed significant activity with IC50 140.37 ± 1.93 and 167.43 ± 3.03 μM respectively. Therefore **F**2 was subjected to column chromatography over silica gel, eluting with n-hexane–ethyl acetate and ethyl acetate–MeOH in increasing order of polarity, to afford compounds **105–108**, **111-120** and **125-132** (see section 6.2 and 7.2). All these compounds were evaluated for urease inhibitory activity. The IC_{50} values with percent inhibition of urease by various fractions and compounds are summarized in table 12.19. Compound **126** showed potent activity (IC50 20.96 ± 0.93 μM), which is comparatively higher than that for standard thiourea (IC50 21.01 ± 0.51 μM). Compounds **113** and **108** showed significant activity, with IC50 37.95 ± 1.93 and 92.6±.41 μM, respectively, whereas **107** and **117** exhibited good activity. The activities of compounds can be attributed to their co-ordinating capabilities with the metallocenter (i.e. nickel) of the enzyme[60]. The greater activity of compound **113** can be conceived to be due to the presence of two aromatic hydroxyl groups and α,β unsaturated carboxylic in the backbone of the molecule, which can strongly bind to the active sites of the enzyme[61].

Compounds **113** and **126** inhibited the urease enzymes (Figure 12.9) in a concentration-dependent manner, with Ki (Dissociation constant) value of 31 ± 0.010 and 18 ± 0.014 mM against the jack bean ureases, respectively. Lineweaver–Burk plots and their replots indicated that **126** is a mixed type of inhibitor of jack bean urease, as a change in both Vmax and affinity (Km value) of urease toward the substrate (urea) was observed. On the other hand, compound **113** showed a competitive type of inhibition (Figure 12.9), causing an increase in Km (Michalas constan) without affecting the V_{max} value. Mechanistic studies of both compounds are expected to provide useful information about the design of new inhibitors of jack bean urease.

Table 12.19: The IC_{50} values and percent inhibition of urease by the fractions and compounds

Compound	% inhibition at 1000ug/mL	IC_{50} (µM) ±SEM
F1	26.9	-
F2	68.3	140. 37±1.93
F3	23.7	-
F4	67.5	167.50±3.8
107	65.5	150.3±1.25
108	76.12	92.60±.41
113	99.3	37.50±0.94
117	71.4	138.46±1.25
126	96.96	20.96±0.47
Thiourea	98.82	21.01 ± 0.51

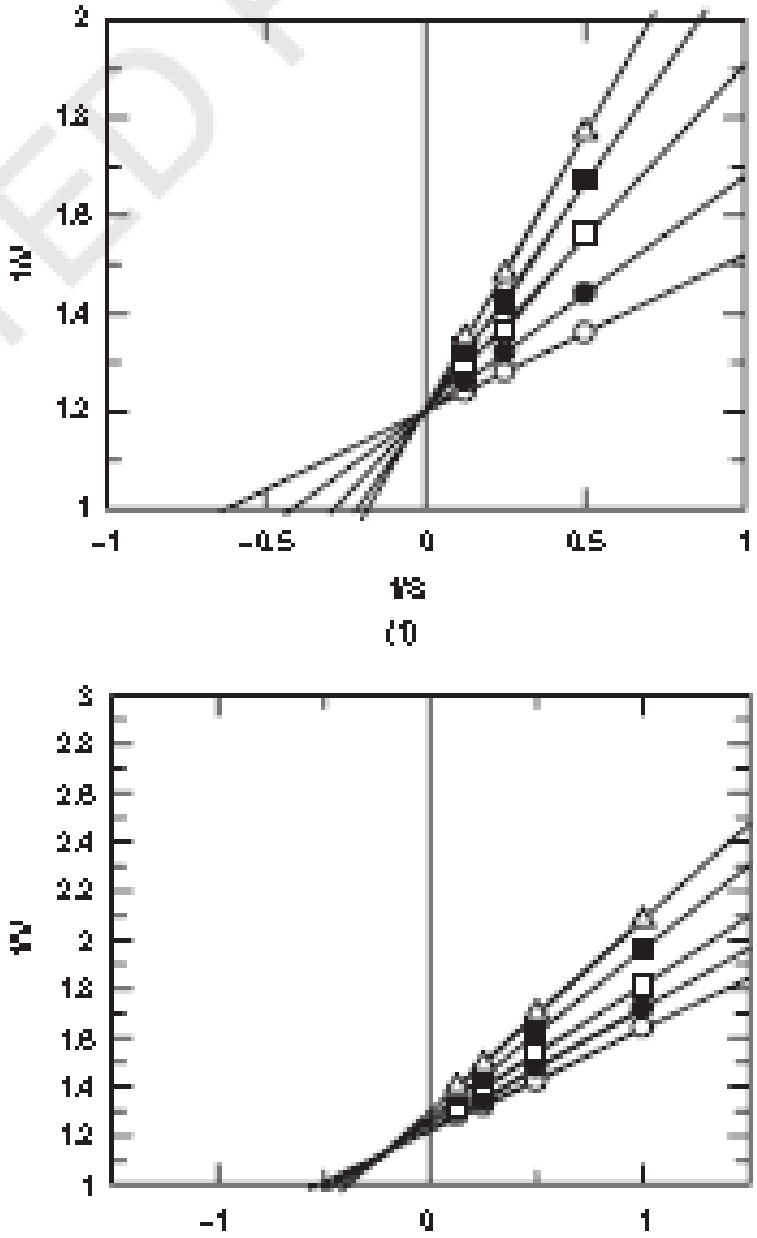


Figure 12.9: Inhibition of jack bean urease by compounds 113 and 126.Lineweaver–Burk plots of the reciprocal of initial velocities vs. reciprocal of four fixed substrate concentrations in absence (○) and presence of 100 mM (▲), 80 mM (△), 60 mM (■), 40 mM (□), 20 mM (●).

12.9:Anti-inflammatory activity of extracts and Xanthones from *H.oblongifolium*

Xanthones are commonly found in few families of higher plants. fungi and lichens[62]. Xanthones from *Calophyllum inophylum* have shown CNS depressant activity whereas xanthones from *Garcina* genus showed useful pharmacological activities[62]. The medicinally important compounds have been reported from genus *Hypericum*. The xanthones isolated from the leaves of *H.brasilienes* showed MAO inhibitory activities[62]. Similarly antitumor activity of the chemical constituents of *H. sampsonii* has also been reported[62]. The bioassay-guided fractionation of *H. oblongifolium* led to the isolation of potent anti-inflammatory. Various fractions (**F1**, **F2**, **F3**, and **F4)** were obtained from the air-dried, powdered twigs of *H. oblongifolium* (see fractionation scheme in "Experimental" section). The ethyl acetate fraction (**F2**) from the twigs of *H. oblongifolium* was subjected to column chromatography over silica gel, eluting with n-hexane–ethyl acetate and ethyl acetate–MeOH in increasing order of polarity, to afford compounds **105–108**, **111-120** and **125-132** (see section 6.2 and 7.2). All of these compounds were evaluated for respiratory burst inhibiting activity (anti-inflammatory) using a standard contemporary methods[63]. Table 12.9 summarizes the IC_{50} values (the concentration of a compound at which superoxide production was suppressed up to 50%) and percent inhibition of reduction of water soluble tetetrazollium salt (WST) compared with positive control. It can be seen from table 12.9, that compound **105, 106, 111, 113** and **117** showed significant activity while the rest are either less active or almost inactive. The activity of **105, 106** and **111** would be attributed to the presence of 1,4-dioxane ring and hydroxyl phenyl substituents, whereas the activity of **113** and **117** were attributable to the presence greater phenolic character. The rest of compounds were either showd less activity or inactive

Table 12.20: IC_{50} Values and percent inhibition of reduction of WST-1 by NADPH oxidase, via superoxidase in presence of test compounds and positive controls, using freshly isolated human neutrophils.

Compound	% inhibition at 1000ug/mL	IC_{50} (um) ±SEM
105	73.5	816.23±73.3
106	70.1	985.20±55.8
111	75.1	965.214±65.8
113	71.1	907.20±50.8
117	68.7	975.20±81.05
119	21.4	2500.85±50.5
Indomethacin	58.82	757.99±5.9
Aspirin	70.45	279.44±4.42

Chapter:13

EXPERIMENTAL (Part D)

13.1: Plant material

The collected plant species belonging to famailie Guttiferae, Solanaceae and Pinaceae were authenticated by Dr. Habib Ahmad, Dean Faculty of Science, Hazara University and collected from swat, NWFP Pakistan. Voucher specimens have been retained at the herbarium, Department of Botany, Hazara University Pakistan. Voucher numbers and details of species collected are given in tables 10.1-10.4.

13.2*:* Preparation of extracts and fractions

The collected species were extracted with ethanol by soxhlet apparatus (for antioxidant and antimicrobial activities). The respective extracts were filtered and dried under reduce pressure below 50 ^{o}C. Various fractions were obtained (Fig. 12 .1) by solvent extraction. Details of fractions are given in table 12.1-12.4. The extracts and fraction of *Hypericum* species for antiproliferative and enzyme inhibition studies were also made on cold perculation extraction methods (Fig 13.2)

13.3*:* Antioxidant activities

13.3.1: Chemicals

All the chemicals used were of analytical grade, Gallic acid and quecetein were purchased from Acros(USA), 1,1- Diphenyl-2-picryl hydrazyl radical (DPPH) from Fluka (Germany), α-tocopherol from E.Merck (Germany), trichloro acetic acid from Riedal–deHaen (Germany) sodium phosphate from Panreac(Spain),ammonium molybdate from ABSCO(UK), sodium carbonate, Folin–Ciocalteu's phenol reagent (FCR), ascorbic acid, potassium ferricyanide, ferric chloride,sulfuric acid and the other reagents were got from Merck (Germany). Spectra were recorded on a SP-3000 PLUS Spectrophotometer (Optima, Japan) and the commercial solvents used for extraction were re-distilled.

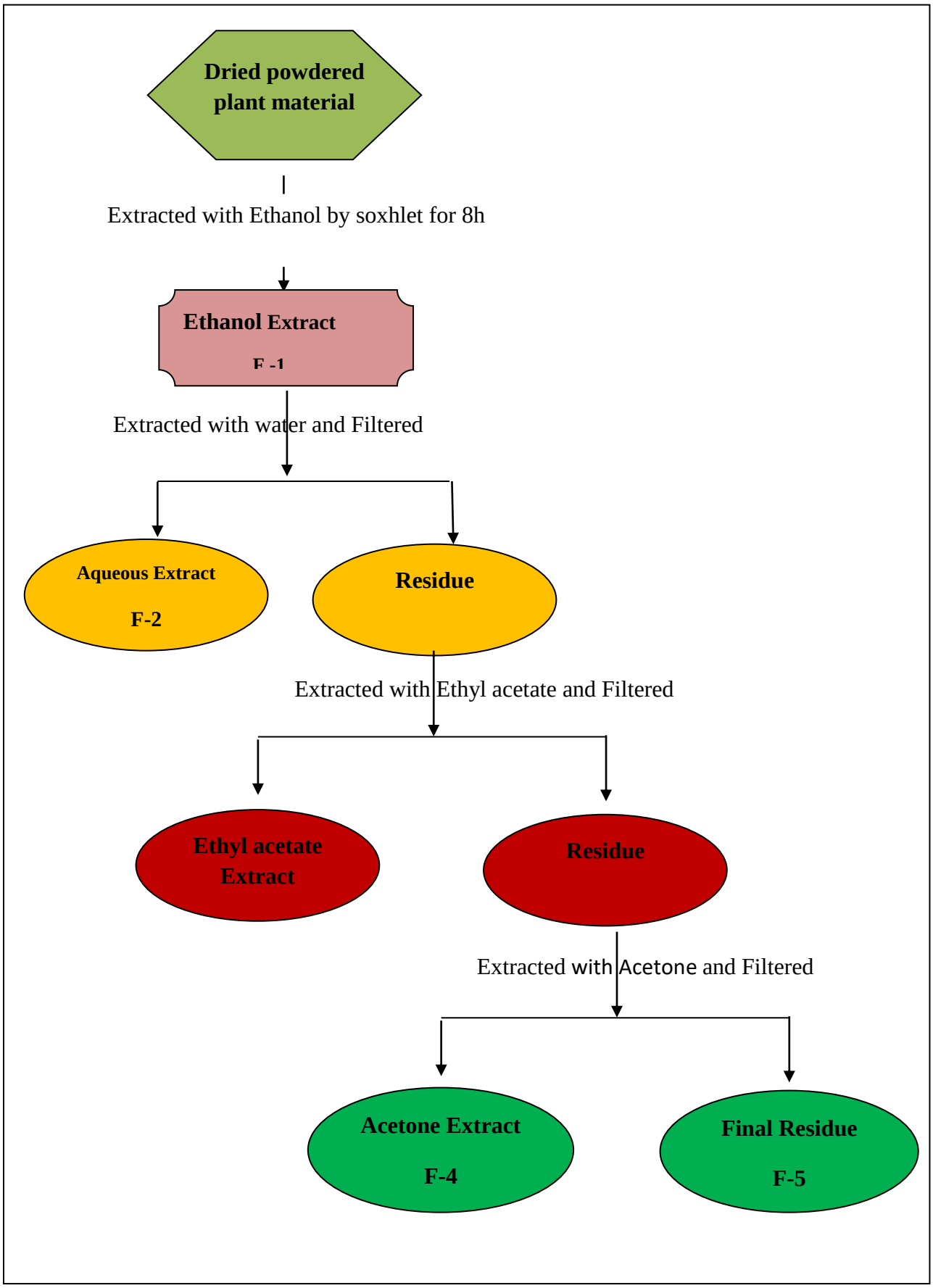


Fig 13.1. General scheme of the plants material extraction and solvent fractionation for antioxidant and antimicrobial activities.

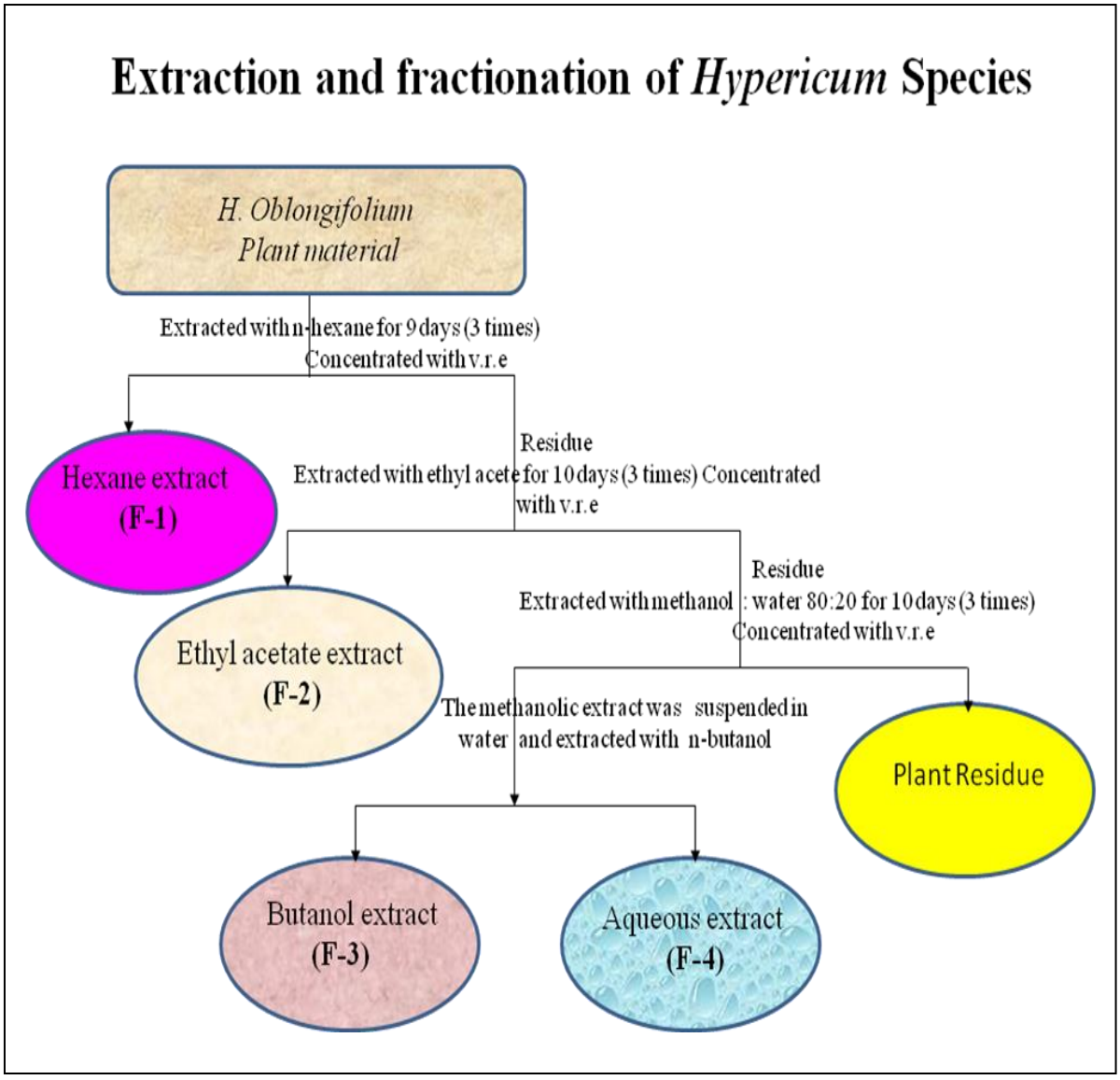


Fig. 13.2: General scheme of the extraction of *Hypericum* species for antiproliferative and enzyme inhibition studies

Table 13.1: Relevant data on the studied of *Picea smithiana*, *Abies pindrow*, *Cedrus deodara* and the yields of the crude extracts and fractions.

Plant species	Vouccher No	Collection Period	Locality	Part studied	Fractions	Code	Wt. of Extract/ fraction	yield (% w/w)
Picea smithiana	HUH-001	September, 2005	Swat, NWFP Pakistan	Bark (A)	Ethanol	A1	7.00g	14.0%
					Aqueous	A2	4.20g	8.4%
					Ethylacetate	A3	0.25g	0.50%
					Acetone	A4	1.30g	2.6%
					Final residue	A5	0.50	1.0%
				Knotwood (B)	Ethanol	B1	2.30g	4.6%
					Aqueous	B2	0.085g	0.17%
					Ethylacetate	B3	0.455g	0.91%
					Acetone	B4	1.20g	2.4%
					Final residue	B5	0.450g	0.90%
Abies pindrow	HUH-009	September, 2005	Swat, NWFP Pakistan	Bark (C)	Ethanol	C1	9.25g	18.50%
					Aqueous	C2	4.275g	8.55%
					Ethylacetate	C3	1.65g	3.3%
					Acetone	C4	1.535g	3.07%
					Final residue	C5	0.565g	1.130%
				Knotwood (D)	Ethanol	D1	12.25g	24.50%
					Aqueous	D2	0.275g	0.55%
					Ethylacetate	D3	4.165g	8.33%
					Acetone	D4	5.74g	11.48%
					Final residue	D5	0.865g	1.73%
Cedrus deodara	HUH-011	September, 2005	Swat, NWFP Pakistan	Bark (C)	Ethanol	E1	7.50g	15.0%
					Aqueous	E2	3.42g	6.82%
					Ethylacetate	E3	0.410g	0.842%
					Acetone	E4	1.122g	2.244%
					Final residue	E5	0.750g	1.50%
				Knotwood (D)	Ethanol	F1	6.00g	12.0%
					Aqueous	F2	0. 07g	0.14%
					Ethylacetate	F3	4.105g	8.21%
					Acetone	F4	0.684g	1.368%
					Final residue	F5	0.075g	0.15%

Table 13.2: Relevant data on the studied *Hypericum* species from Pakistan and the yields of dry extracts

Plant species	Voucher No	Collection period	Locality	Fractions	Code	Wt.of Extract/ fraction	yield (% w/w)
Hypericum	HUH-	June, 2005	Swat,	Ethanol	A1	5.00g	10.0%
perforatum	003		NWFP	Aqueous	A2	3.05g	6.1%
(A)			Pakistan	Ethyl acetate	A3	1.00g	2.0%
				Acetone	A4	0.50g	1.0%
				Final residue	A5	0.250g	0.5%
Hypericum	HUH-	June, 2005	Hazara and	Ethanol	B1	4.00g	8.0%
oblongifolium	002		Buner	Aqueous	B2	1.72g	3.44%
(B)			NWFP	Ethyl acetate	B3	1.00g	2.0%
			Pakistan	Acetone	B4	0.40g	0.8%
				Final residue	B5	0.150g	0.3%
Hypericum	HUH-	June, 2005	PeshawarN	Ethanol	C1	5.00g	10.0%
monogynum	004		WFP	Aqueous	C2	2.20g	4.4%
(C)			Pakistan	Ethyl acetate	C3	1.00g	2.0%
				Acetone	C4	0.650g	1.3%
				Final residue	C5	0.150g	0.3%
Hypericum	HUH-	July, 2006	Besham,	Ethanol	D1	8.00g	16.0%
Coisianum ***(D)***	013		shangla	Aqueous	D2	3.45g	6.9%
			NWFP	Ethyl acetate	D3	3.20g	6.4%
			Pakistan	Acetone	D4	0.50g	1.0%
				Final residue	D5	0.250g	0.5%
Hypericum	HUH-	Sept,2006	Dnga gali,	Ethanol	E1	9.00g	18.0%
Dyeri ***(E)***	017		Hazara	Aqueous	E2	4.65g	9.30%
			NWFP	Ethyl acetate	E3	1.60g	3.2%
			Pakistan	Acetone	E4	0.55g	1.1%
				Final residue	E5	1.95g	3.9%

Table 13.3: Relevant data on the studied of *Pinus species* and the yields of the crude extracts and fractions.

Plant species	Voucher No	Collection Period	Locality	Part studied	Fractions	Code	Wt. of Extract/ fraction	yield (% w/w)
Pinus roxburghii	HUH-007	September, 2005	Swat, NWFP Pakistan	Bark (A)	Ethanol	A1	18.00g	36.0%
					Aqueous	A2	5.37g	10.74%
					Ethyl acetate	A3	0.11g	0.22%
					Acetone	A4	6.25g	12.50%
					Final residue	A5	5.15g	10.30%
				Knotwood (B)	Ethanol	B1	6.00g	12.0%
					Aqueous	B2	0.05g	0.1%
					Ethyl acetate	B3	3.95g	7.90%
					Acetone	B4	6.74g	13.48%
					Final residue	B5	0.13g	0.26%
Pinus wallichiana	HUH-008	September, 2005	Swat, NWFP Pakistan	Bark (C)	Ethanol	C1	12.00g	24.0%
					Aqueous	C2	7.15g	14.3%
					Ethyl acetate	C3	1.25g	3.5%
					Acetone	C4	1.42g	2.84%
					Final residue	C5	0.65g	0.130%
				Knotwood (D)	Ethanol	D1	6.00g	12.0%
					Aqueous	D2	0.105g	0.21%
					Ethyl acetate	D3	4.165g	8.33%
					Acetone	D4	0.584g	1.09%
					Final residue	D5	0.155g	0.301%

Table 13.4: Relevant data on the studied of *Taxus fuana* and the yields of the crude extacts and fractions.

Plant specie	Medicinal uses	Voucher No	collection Peroid	Locality	Part Studied	Fractions	Code	Wt.of fraction	yield % w/w
Taxus fuana	For the treatment high fever, epilepsy and Hepatitis C	HUH-003	Sept. 2005	Swat, NWFP Pakistan	Bark (A)	Ethanol	A1	12.00g	24.0%
						Aqueous	A2	8.95g	17.9%
						Ethylacetate	A3	3.05g	6.1%
						Acetone	A4	1.54g	3.08%
						Final residue	A5	0.185g	0.37%
					Knotwood (B)	Ethanol	B1	7.00g	14.0%
						Aqueous	B2	0.105g	3.1%
						Ethylacetate	B3	0.105g	3.1%
						Acetone	B4	5.74g	11.48%
						Final residue	B5	0.355g	0.71%

13.3.2: DPPH radical-scavenging activity

The hydrogen atom or electron donation abilities of the corresponding extracts/fractions and standards were measured from the bleaching of the purple-coloured methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH.) Experiments were carried out according to the method of Blois [64,65] with a slight modification. Briefly, a 1mM solution of DPPH radical solution in methanol was prepared and 1ml of this solution was mixed with 3ml of sample solutions in ethanol (containing 20-100*u*g/mL) and control (without sample). After 30 min, the absorbance at 517 was measured. An increase of the DPPH radical-scavenging activity is correlated with decreasing of the DPPH solution absorbance. Scavenging of free radicals by DPPH as percent radical scavenging activities (%RSA) was calculated as follow.

$$\% \text{ RSA} = \frac{\text{control absorbance - sample absorbance}}{\text{control absorbance}} \times 100$$

The assays were carried out in triplicate and the results are expressed as mean values ± standard deviations. The EC_{50} (extract concentration showing 50% inhibition) was calculated from the graph of % RSA against extract concentration and quercetin, ascorbic acid, Gallic acid and α-tocopherol used as standards.

13.3.3: Determination of reducing power

The reducing power was determined according to the method of Oyaiz et al.[66] Briefly 1 mL of various concentrations (in such a way that final concentration remain 5-25ug/mL) of extracts and 1ml of solvent without extract for control was mixed with 2.5ml of phosphate buffer (6.60) and 2.50 mL of potassium ferricyanide solution (10g/l), then the mixture was incubated at 50 C^0 for 30 min. Afterwards, 2.5ml of trichloroacetic acid (100g/mL) was put into the mixture, which was then centrifuged at 650 rpm for 10 min. Then 2.5 mL of the upper was mixed with 2.5 mL of distilled water and 0.5 mL of ferric chloride (1g/l). The absorbance of greenish solution was measured at 700 nm. Higher absorbance at the same wave length means higher reducing power. The assays were carried out in triplicate and the results are expressed

as mean values ± standard deviations. The EC_{50} (extract concentration providing 0.4 of absorbance) was calculated from the graph of absorbance at 700 nm.

13.3.4: Evaluation of total antioxidant capacity

The total antioxidant capacity of the extracts/fractions was evaluated by the method of Prieto et al. [67] with slight modification. An aliquot of 0.3 mL of sample solution in methanol was combined in an Eppendorf tube with 2.7 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The effective concentration of the sample was 50 *u*g/mL in the reaction mixture. For the blank, 0.3 mL ethanol was mixed with 2.7 mL of the reagent. The tubes were capped and incubated in water bath at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. For samples of unknown composition, water-soluble antioxidant capacity was expressed as equivalents of ascorbic acid μmole/mg of extract .The assays were carried out in triplicate and the results are expressed as mean values ± standard deviations.

13.3.5: Determination of total phenolic compounds

Total phenoilc content was determined by Follin Ciocaltea method[68]. Briefly, 1ml of each of the already prepared extract solution (500*u*g) was poured in to 50-ml volumetric flask and distilled water was added in such a way that the volume was adjusted to 46 mL. The Folin-Ciocalteu Reagent (1 mL) was added into this mixture and 3ml of Na_2CO_3 (2%) was added by 3 min. Subsequently, mixture was shaken for 2 h at room temperature and then absorbance was measured at 760 nm. Estimation of the phenolic compounds was carried out in triplicate. The results were mean values ± standard deviations and expressed as mg of gallic acid equivalents *u*g/mg of extract (GAEs) by using an equation that was obtained from the standard curve, is given as:

A =1156C +0189

Where A is the absorbance and C is the gallic acid equivalent (mg/g). In this assay, 500*u*g of dried extracts were added to test samples, and final volumes were 50ml.

13.4: Antimicrobial activities

13.4.1: Test organisms for bioassays

Gram-positive bacteria: *Staphylococcus aureus,* Gram-negative bacteria: *Pseudomonas aeruginosa*, *Salmonella typhi*, *Escherichia coli*, *Proteus vulgaris, Enterobacter aerogenes*. and fungal strain: *Aspergillus niger, Aspergillus flavus, Alternaria solani, Helminthosporium maydis*. Pure bacterial and fungal cultures (clinical isolates) were obtained from Centre of Biotechnology and Microbiology University of Peshawar. Different bacterial and fungal strains were kept on nutrient agar (NA, Oxide, UK) and Sabouraud dextrose agar (SDA, Oxide, UK) respectively. Bacterial cultures were prepared by inoculation of two to three colonies into a tube containing 20 mL nutrient broth (Oxide, UK) and grown overnight at 37 ◦C while fungal cultures were prepared in SDA . Fresh cultures suspensions at McFarland 0.5 density (10^8 CFU/mL) were used for inoculation.

13.4.2: Antibacterial screening

The antibacterial tests were performed using agar-well diffusion assay [38-40]. Petri dishes were prepared from the sterile nutrient agar (Oxide, UK). Standardized bacterial cultures were evenly spread onto the surface of the agar dishes with help of sterile swab sticks. Five wells (6mm diameter) were bored in each plate with sterile borer.100 *u*l of ethanol, aqueous, ethyl acetate, acetone and final extracts (10 mg/mL) of the bark and knotwood of the investigated plants were added in each well. 100 *u*l of absolute alcohol per well was used as a negative and control. For positive control 100 *u*l of streptomycin (2mg/mL) was used. The agar plates were then covered with lids and incubated at 37 ◦C for 24 h. The plates were observed for the presence of inhibition of bacterial growth that was indicated by a clear zone around the wells. The size of the zones of inhibition was measured and the antibacterial activity expressed in terms of the average diameter of the zone inhibition in millimeters. The absence of a zone inhibition was interpreted as the absence of activity.

13.4.3: Antifungal activity assay

For the antifungal activity test tube dilution method was used [39,41]. Five mL of medium (SDA) was added to each screw-capped test tube and they were autoclaved at $121C^0$ for 15 min. Tubes having 5 mL sterile SDA were added ethanol, aqueous, ethyl acetate, acetone and final extracts (400µg/mL) of the bark and knotwood of the investigated plants and fuconazole (200µg/mL) in absolute alcohol. Tubes were kept in the salutation position overnight for checking the sterility. Next day the tubes were inoculated with fungal culture on the salutation position and all the test tubes were kept for ten days at 27-30C incubation. Each compound or extract was tested against four fungal cultures. The negative control tubes contained 5 mL SDA, 1.0 mL absolute alcohol and 0.1 mL of fungal culture, Positive control tube contained 5 mL SDA, (200µg/mL) of fuconazol in 1 mL absolute alcohol and fungal cultures. After 10 days, the results were recorded according to formula:

$$\% \text{ growth inhibition} = \frac{\text{Linear growth of negative control-Linear growth sample}}{\text{Linear growth of negative control}} \times 100$$

The degree of activity was recorded in four grades according to the % inhibition of growth: inactive (0), low (0-30 %), moderate (30-50 %), Good (50-70 %) and significant (70% & above).

13.5: Anti-proliferative assay

13.5.1: Tumor cell line maintenance

The cell lines HT-29 human colon adenocarcinoma, NCI-H460 human non-small cell lung carcinoma, MCF-7 human breast cancer, OVCAR-3 human ovarian adenocarcinoma and RXF-393 human renal cell carcinoma, were obtained from American Type Culture Collection (Rockville, MD, USA). All the cell lines were maintained in RPMI 1640 culture medium, supplemented with 10% fetal bovine serum, at 37 ^oC and in a humidified atmosphere of 5% CO_2 in air.

13.5.2: Cell growth inhibition studies

For the assay of antiproliferative activity, the samples were dissolved in dimethylsulfoxide (DMSO; not exceeding the concentration of 0.01%), and further diluted in cell culture. The cell lines were inoculated into 96-well microplates. After 24 h, triplicate cultures were treated for 72 h with the biflavones in final volumes of 200 µL per well. Untreated control wells received only maintenance medium. The antineoplastic agent etoposide was used as a positive control. Cellular responses were colorimetrically assessed by sulforhodamine B (SRB) assay [69]. Briefly, the cells were fixed with 50% (w/v; 50µL/well) trichloroacetic acid and stained with 0.4% SRB. Later the cell-bound SRB was solubilized by the addition of 10 mM Trizma base. The latter was colorimetrically assessed with an ELISA microplate reader (Multiskan Ex, Labsystems, Finland) at a wavelength of 540 nm. The fractions were tested at concentrations ranging from 0 to 100µg/mL. Cell growth inhibition was expressed in terms of percentage of untreated control absorbance following subtraction of mean background absorbance. Compounds were considered to have potent growth inhibitory activity when the reduction in SRB absorbance was more than 25% compared to untreated control cells[69]. The IC_{50} concentration (50% inhibition of cell growth values) was calculated from the dose-response curves.

13.6: Evaluation of cytotoxicity

13.6.1: Biological materials

Stock solutions of compounds were prepared with 100% dimethylsulfoxide (DMSO, Sigma) and diluted with RPMI 1640 medium containing 10% fetal bovine serum (FBS). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl -tetrazolium bromide), Cyclosporin A (CsA), concanavalin A (ConA), and lipopolysaccharide (LPS) were purchased from Sigma.

*Animals***.** BALB/c mice, used at 6-8 weeks of age were purchased from Shanghai Experimental Animal Center and were housed in a controlled environment (12 h of light/12 h of dark photoperiod, 22 ± 1 °C, 55% ± 5% relative humidity). All husbandry and experimental contacts made with the mice were conducted under specific pathogen-free conditions. All mice were allowed to acclimatize in our facility

for 1 week before any experiment started. All experiments were carried out according to the NIH Guide for Care and Use of Laboratory Animals.

13.6.2: Preparation of spleen cell from Mice.

Fresh lymphocyte suspensions wereprepared before each experiment from male BALB/c mice. They were exsanguinated, and then spleens were immediately excised aseptically. A single cell suspension was prepared after cell debris, and clumps were removed Erythrocytes were lysed with Gey's reagent for 5 min [32]. Cells were then washed with sterile phosphate buffered saline (PBS). Mononuclear lymphocytes were isolated by buoyant density centrifugation (5 min at 1000 rpm). After that, the isolated splenic lymphocytes were resuspended at 1×10^6 cells/mL in RPMI-1640 medium (Sigma Co.) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (GIBCO Co.). Cells were cultured in 96 well tissue culture plates with 2×105 lymphocytes per well [33]. They were incubated at 37°C in a humidified atmosphere of 5% carbon dioxide (CO_2) for the indicated period. The recovered cells were typically＞98% viable as assessed by Trypan blue exclusion, and the heterogeneous mononuclear cell suspension mainly consisted of 40% B cells and 60% T cells.

12.6.3: T cell and B cell function assay.

Splenocytes were seeded into a 96-well flat-bottom microtiter plate (Nunc) at 1×107 cell/mL in 100 μL of complete medium; thereafter, Con A (final concentration 5 mg/mL), or LPS (final concentration 10 mg/ mL) or RPMI 1640 medium with CsA and these compounds separately, was added to give a final volume of 200 μL (quadruplicate wells). The plate was incubated at 37° in a humidified atmosphere with 5% CO_2. After 44 h, 20 μL of MTT (5 mg/mL) was added to each well and incubated for further 4 h. The plates were centrifuged (3000×g, 5 min) and the untransformed MTT was removed carefully by pipetting. 200 mL of DMSO was added to each well, and the absorbance was evaluated in an ELISA reader at 570 nm with a 630 nm reference after 15 min.

13.6.4: Cell viability assay

Isolated splenocytes (100 µL /well) were cultured in 96 well tissue culture plates for 48 h in the presence or absence of four concentrations of compounds separately. MTT was added after 48 h at a final concentration of 500µg/mL and incubated for 4 h. After removal of MTT, the formazan precipitate was solubilized in DMSO (100 µg /well) and measured on a Bio-rad model 550 micro plate reader at the absorbance of 570 nm and reference of 630 nm. The experiment was repeated three times. The percentage of all dead cells was measured using the following formula: Viability rate of cells = average OD of test group/ average OD of control group×100%. Half inhibitive concentration (IC_{50}) here meant the drug concentration when viability rate of cells reached 50%.

References

(1) Samuelsson, G. *Drugs of Natural Origin:a Textbook of Pharmacognosy* 5th ed.; Swedish Pharmaceutical Press: Stockholm, 2004.

(2) Balunas, M. J.; Kinghorn, A. D. *Life Scienc.* **2005**, *78*, 431.

(3) Parkin, D. M. *Lancet Oncology* **2001**, *2*, 533.

(4) Zandi, K.; Farsangi, M. H.; Nabipour, I.; Soleimani, M.; Khajeh, K.; Sajedi, R. H.; Jafari, S. M. *Afric.J. Biotech.***2007**, *6*, 1280.

(5) Sun, J.; Chu, Y.-F.; Wu, X.; Liu, R. H. *J.Agric.Food.Chem.* **2002**, *50*, 7449.

(6) Willett, W. C. *Science* **2002**, *296*, 695.

(7) Doll, R. *Proc. Nutr. Soc.* **1990**, *49*, 119.

(8) Thompson, L. U.; . *Crit. Rev. Food Sci. Nutr.* **1994**, *34*, 473.

(9) Liu, R. H. *Food Technol.Int.* **2002**, *1*, 71.

(10) Cheng, H. Y.; Lin, T. C.; Yu, K. H.; Yang, C. M.; Lin, C. C. *Biol. Pharm. Bull.* **2003**, *26*, 1331.

(11) Tsao, R.; Deng, Z. *J. Chromat. B* **2004**, *812*, 85.

(12) Sue, J. W.; Lean, T. N.; Yuan, M. H.; Doung, L. L.; Shyh, S. W.; Shan, N. H.; Chun, C. L. *Biol. Pharm. Bull.* **2005**, *28*, 963.

(13) Yanping, Z.; Yanhua, L.; Dongzhi, W. *J. Agric. Food Chem.* **2004**, *52*, 5032.

(14) Lu, Y.; Foo, Y. *Food Chem.* **2000**, *68*, 81.

(15) Valentao, P.; Fernandes, E.; Carvalho, F.; Andrade, P. B.; Seabra, R. M.; Bastos, M. L. *Biol. Pharm. Bull.* **2002**, *25*, 1320.

(16) Filomena, C.; Giancarlo, A. S.; Rosa, T.; Francesco, M.; Peter, H. *Fitoterapia* **2002**, *73*, 479.

(17) Hsu, T. Y.; Sheu, S. C.; Liaw, E. T.; Wang, T. C.; Lin, C. C. *Phytomedicine* **2005**, *12*, 663.

(18) Pinelo, M.; Rubilar, M.; Sineiro, J.; Núñez, M. *J. Food Chem.* **2004**, *85*, 267.

(19) Gülçin, I.; Büyükokuroglu, M. E.; M.Oktay; Küfrevioglu, Ö. I. *J.Ethnopharmacol.* **2003**, *86*, 51.

(20) Rabanal, R. M.; Arias, A.; Prado, B.; Hernandez-Perez, M.; Sanchez-Mateo, C. C. *J.Ethnopharmacol.* **2002**, *81*, 287.

(21) Srinivasan, D.; Nathan, S.; Suresh, T.; Perumalsamy, L. P. *J.Ethnopharmacol.* **2001**, *74*, 217.

(22) Poole, K. *J.Ethnopharmacol.* **2001**, *53*, 283.

(23) Marchese, A.; Schito, G. C. *Int. J. Antimicrob. Agt.***2000**, *16*, s25.

(24) Turner, W. W.; Roderiguez, M. J. *Curr.Pharm.Des.* **1996**, *2*, 209.

(25) Schwartz, R. E.; Sesin, D. F.; Joshua, H.; Wilson, K. E.; Goklen, K. A.; Kuehner, D.; Gailliot, P.; Gleason, C.; White, R. *J.Antibio.* **1992**, *45*, 1853.

(26) Prous, J. *Drugs Future* **1996**, *21*, 881.

(27) Chu, D. T. W.; Plattner, J. J.; Katz, L. *J.Med.Chem.* **1996**, *39*, 3853.

(28) Patel, M. In *Third IBC Intrnational Conference on Natural products Drug Discovery Coronado, California* 1997.

(29) Cragg, G. M.; Newman, D. J.; Snador, K. M. *J.Nat.Prod.* **1997**, *60*, 52.

(30) Hammer, K. A.; Carson, C. F.; Piley, T. V. *Antimicrob. Agt. Chemother.***1999**, *43*, 196.

(31) Dur-e-Shahwar, University of Karachi, Ph.D Thesis, 1999.

(32) Amarowicz, R.; Pegg, R. B.; Rahimi-Moghaddam, P.; Barl, B.; Weil, J. A. *Food Chem.* **2004**, *84*, 551.

(33) Velioglu, Y. S.; Mazza, G.; Gao, L.; Oomah, B. D. *J Agr. Food Chem.* **1998**, *46*, 4113.

(34) Conforti, F.; Statti, G. A.; Tundis, R.; Menichini, F.; Houghton, P. *Fitoterapia* **2002**, *73*, 479.

(35) Bendi, J.; Arroyo, R.; Rommero, C.; Martin-Aragon, S.; Villar, A. M. *Life Sci.* **2004**, *75*, 1263.

(36) Silva, B. A.; Ferreres, F.; Malva, J. O.; Dias, A. C. P. *Food Chem.* **2005**, *90*, 157.

(37) Radulovic, N.; Stankov-Jovanovic, V.; Stojanovic, G.; Smelcerovic, A.; Spiteller, M.; Asakawa, Y. *Food Chem.* **2007**, *103*, 15.

(38) Mathabe, M. C.; Nikolova, R. V.; Lall, N.; Nyazema, N. Z. *J.Ethnopharmacol.* **2006**, *105*, 286.

(39) Taous, K.; Mansoor, A.; Hamayun, K.; Mir, A. K. *Afric.J. Biotech.* **2005**, *4*, 1313.

(40) Atta-ur-Rahman.; Choudary, M. I.; Thomsen, J. W. *Manual Bioassay Techniques for Natural Product*; Research Harward Academic press.: Amsterdam 1999.

(41) Paxton, J. D. *Assay for antifungal activity.* ; In: Hostemann, K. ed.; Academic Press, Harcourt Brace Jovanovich Publishers.: London, 1991; Vol. 6.

(42) Gülçin, I.; Büyükokuroglu, M. E.; Oktay, M.; Küfrevioglu, O. I. *J.Ethnopharmacol.* **2003**, *86*, 51.

(43) Yu, L.; Zhao, M.; Wang, J. S.; Cui, C.; Yang, B.; Jiang, Y.; Zhao, Q. *Innov.Food Sci. Emerg. Technol.* **2008**, *9*, 122.

(44) Guri, A.; Kefalas, P.; Roussis, V. *Phytother. Res.* **2006**, *20*, 263.

(45) Jung, M. J.; Chung, H. Y.; Choi, J. H.; Choi, J. S. *Phytother. Res.* **2003**, *17*, 1064.

(46) Saleem, A.; Kivelä, H.; Pihlaja, K. *Z. Naturforsch. C* **2003**, *58*, 351.

(47) Grassmann, J.; Hippeli, S.; Vollmann, R.; Elstner, F. *J. Agric. Food Chem.* **2003**, *51*, 7576.

(48) Motiejūnaitė, O.; Pečiulytė, D. *Medicina* **2004**, *40*, 787-794.

(49) Välimaa, A.; Honkalampi-Hämäläinen, U.; Pietarinen, S.; Willför, S. H., B; Wright, A. *Int.J. Food Microbiol.* **2007**, *115*, 235.

(50) Tiwari, A. K.; Srinivas, P. V.; P.Kumar, S.; Rao, J. M. *J. Agric. Food Chem.* **2001**, *49*, 4642.

(51) Willför, S. M.; Ahoutupa, M. O.; Hemming, J. E.; Reunanen, M. H. T.; Eklund, P. C.; Sjoholm, R. E.; Eckerman, C. S. E.; Pohjamo, S. P.; Holmbom, B. R. *J. Agric. Food Chem.* **2003b**, *51*, 7600.

(52) Vishnoi, S. P.; Ghosh, A. K.; Debnath, B.; Samanta, S.; Gayen, S.; Jha, T. *Fitoterapia* **2007**, *78*, 153.

(53) Sunil, K. C.; Anirban, P.; Prakas, R. M.; Tanpreet, K.; Ankur, G.; Suman, P. S. K. *Bioorg.Med.Chem. Lett.* **2006**, *16*, 2446.

(54) Esra, K.; Nurgün, E.; Erdem, Y.; Bilge, S. *J.Ethnopharmacol.***2003**,*89*, 265.

(55) Banskotaa, A. H.; Nguyena, N. T.; Tezukaa, Y.; Nobukawab, T.; Kadotaa_, S. *Phytomedicine* **2006**, *13*, 109.

(56) Junko, K.; Izumi, M.; Norihiro, K.; Keiichi, H.; Eriko, S.; Takahiro, N.; Shigetoshi, K. *Biol. Pharm. Bull.* **2006**, *29*, 2310.

(57) Emami, S. A.; Asili, J.; Mohagheghi, Z.; Hassanzadeh, M. K. *Advance Access Publication* **2007**, *4*, 313.

(58) Erdemoglu, N.; Sener, B. *Fitoterapia* **2001**, *72*, 59.

(59) Yaojian, H.; Jianfeng, W.; Guiling, L.; Zhonghui, Z.; Wenjin, S. *FEMS Immunol. Med. Microbiol.* **2001**, *31*, 163.

(60) Tanaka, T.; Kawase, M.; Tani, S. *Life Sci.* **2003**, *73*, 2985.

(61) Xiao, Z.P.; Shi, D.H.; Li, H.Q.; Zhang, L.N.; Xu, C.; Zhu, H. L. *Bioorg. Med. Chem.***2007**, *15*, 3703.

(62) Peres, V.; Nagem, T. J. *Phytochemistry* **1997**, *44*, 191.

(63) Tan, A. S.; Berridge, M. V. *Journal of Immunological Methods* **2000**, *238*, 59.

(64) Blois, M. S. *Nature* **1958**, *26*, 1199.

(65) Yıldırım, A.; Mavi, A.; Kara, A. A. *J. Sci. Food Agric.* **2003**, *83*, 64.

(66) Oyaizu, M. *Jpn. J.Nutr.* **1986**, *44*, 307.

(67) Prieto, P.; Pineda, M.; Aguilar, M. *Anal. Biochem.* **1999**, *269*, 337.

(68) Singleton, V. L.; Orthofer, R.; Lamuela-Raventos, R. M. *Method. Enzymol.* **1999**, *299*, 152.

(69) Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; Mcmahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Nat.Cancer Inst.* **1990**, *82*, 1107.

LIST OF PUBLICATIONS

1. Reija Harlamow**, Mumtaz Ali**, Maarit Karonen, , Mohammad Arfan, Markku Reunanen, Stefan Willför. Extractives in bark of different conifer species growing in Pakistan. *Holzforschung, 63,* (2009), 545-50
2. Chao-Feng Huang, Lei Ma, Li-Juan Sun, **Mumtaz Ali**, Mohammad Arfan, Jian-Wen Liu, and Li-Hong Hu Immunosuppressive Withanolides from *Withania coagulans*. *Chemistry Biodiversity,6,* (2009), 1415-26
3. Lei Ma, **Mumtaz Ali**, Mohammad Arfan et al. Withaphysanolide A, a novel nor withanolide skeleton and other cytotoxic Compounds from *physalis divericata., Teterahedran letters,48*, (2007), 449-452
4. S. Willför H. Hafizoglu, I. Tümen, H. Yazici, M. Arfan, **M. Ali** and B. Holmbom Extractives of Turkish and Pakistani Tree Species. *Holz.Roh. Werkstoff, 65*, (2007), 215-221
5. Mohammad Arfan, **Mumtaz Ali**, Habib Ahmad, Itrat Raza, Raza Shah, Mohammad Ajmal and Mohammad Iqbal Choudhary., Urease inhibitors from *Hypericum oblongifoliun* Wall. Accepted in *Journal of Enzyme Inhibition And Medicinal Chemistry.*
6. Mohammad Arfan , **Mumtaz Al**i, Habib Ahmad, Itrat Raza ,Raza Shah,Irfan Qadir and Choudhary Mohammad Iqbal., Bioactive xanthones from *Hypericum oblongifolium.* Accepted in *Planta Medica.*
7. Mohammad Arfan , **Mumtaz Ali**, Khair Zaman, Habib Ahmad, Itrat Raza and Raza Shah, Antiproliferative activity and chemical constituents of *Hypericum dyeri.* Submitted
8. Mohammad Arfan , **Mumtaz Ali**, Khair Zaman, Habib Ahmad, Itrat Raza and Raza Shah Antiproliferative activity and chemical constituents of Hypericum oblongifolium.Submitted.
9. Mohammad Arfan, **Mumtaz Ali**, Habib Ahmad, Khair Zaman, Farhatullah and Ryszard Amarowicz. Comparative antioxidant and antimicrobial activities of phenolic compounds extracted from five *Hypericum* species. Accepted in Food technology and Biotechnology.

10. Mohammad Arfan, **Mumtaz Ali**, Habib Ahmad and Hazrat Amin, Comparative antioxidant and antimicrobial activities of phenolic compounds extracted from *Pinus* species, Submitted
11. Mohammad Arfan, **Mumtaz Ali** and Habib Ahmad, Antioxidant and Antimicrobial activities of *Taxus fuana*, Submitted
12. Mohammad Arfan, **Mumtaz Ali**, Habib Ahmad and Khair Zaman, Comparative antioxidant and antimicrobial activities of plants belonging to family Pinaceae, Submitted
13. Mohammad Arfan, Mumtaz **Ali,** M. Luisa M. Serralheiro, Lina Falcão, M. Eduarda M. Araújo. Antioxidant, antiplatelet aggregation and acetyl cholinesterase inhibition activities of *Hypericun* species from Pakistan. Submitted