IN THE NAME OF ALLAH, THE BENEFICENT
THE MERCIFUL

Read! And thy Lord is Most Honorable and Most Benevolent,
Who taught (to write) by pen, He taught man that which he knew not

(Surah Al-Alaq 30: 3-5)
Al-Quran

PHYTOCHEMICAL, PHARMACOGNOSTIC AND PHARMACOLOGICAL INVESTIGATIONS OF *VITEX AGNUS CASTUS* AND *MYRSINE AFRICANA*

Ph.D Thesis
By

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CENTRE FOR BIOTECHNOLOGY AND MICROBIOLOGY
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PHYTOCHEMICAL, PHARMACOGNOSTIC AND PHARMACOLOGICAL INVESTIGATIONS OF *VITEX AGNUS CASTUS* AND *MYRSINE AFRICANA*

Sadiq Azam

A thesis submitted to the University of Peshawar in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biotechnology and Microbiology

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This dissertation is submitted by Sadiq Azam as partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Biotechnology and Microbiology

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CERTIFICATE OF APPROVAL

This thesis titled "Phytochemical, Pharmacognostic and Pharmacological Investigations of "*Vitex agnus castus* and *Myrsine africana*" submitted by Sadiq Azam is hereby approved and recommended as partial fulfillment for the award of Degree of Doctor of Philosophy in Biotechnology and Microbiology

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Dedication

"I wish to dedicate this work to Holy Prophet Muhammad (Peace be upon him) and his companions who laid the foundations of Modern civilization and paved the way for Social, Moral, Political, Economical, Cultural and Physical revolution, my Supervisor and parents who taught the things I would never have known otherwise"

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ACKNOWLEDGEMENTS

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Summary

The present Ph.D dissertation comprises of two parts, **part A** and **part B**.

The **part A** describes extraction, isolation and structure elucidation of isolated compounds. Pharmacological and biological activities of crude extract, different fractions thereof and some compounds isolated in reasonable quantity from *Vitex agnus castus* have been investigated.

As regarded phytochemical study three new compounds namely vitexcarpan (01) vitexcarpan 1 (02) and Vitexoide (03) were isolated from the aerial parts of the plant in amount of 10.0, 16.4 and 5.0 mg respectively. Other known compounds (04, 05 and 06) were also isolated from this plant along with.

Crude methanol extract, different fractions thereof and some compounds isolated in fair quantity were investigated for various pharmacological / biological activities including, Antibacterial, Antifungal, Brine-shrimp lethality, Phytotoxic, Heamagglutination, Insecticidal, Anti-termites, Anti-inflammatory, Spasmolytic, Nitric oxide free radical scavenging assay and Enzyme inhibition assay.

The Pharmacological / Biological investigations revealed that the plant possess significant Antibacterial, Anti-inflammatory and Spasmolytic activity. The plant materials showed Good Phytotoxicity (at higher concentrations), Anti-termites and Nitric oxide free radical scavenging activities. Moderate insecticidal and Brine shrimp lethality were also observed. The plant was also found to exhibit no Antifungal and Heamagglutination activity. Moderate and low Enzyme inhibition and Anti-inflammatory activities were observed for the isolated compounds.

Part B describes extraction, isolation and structure elucidation of isolated compounds, Pharmacological and Biological investigations of the crude methanolic extract and various fractions of *Myrsine africana*.

A total of five compounds were isolated from *Myrsine africana*, including two new compounds Myrsinene (07) and Myrsigenin (08), along with compound (09), isolated for the first time from the plant specie. Two known compounds (10 and 11), already reported from the plant.

Crude methanol extract and different fractions of the plant were investigated for various pharmacological / biological activities including, Antibacterial, Antifungal, Brine-shrimp lethality, Phytotoxic, Heamagglutination, Insecticidal, Anti-termites, Anti-inflammatory, Spasmolytic and Nitric oxide free radical scavenging assay.

Biological / Pharmacological investigations revealed that the crude methanolic extract of the plant shows significant Spasmolytic activity, the plant was found to possess good and moderate Antibacterial activity against certain strains of bacteria, the *n*-hexane fraction of the plant shows good Brine shrimp lethality at higher dose while rest of the fractions including crude extract shows low activity. Moderate Anti-inflammatory activity was shown by the plant crude extract and fractions against human neutrophils. Moderate and week Heamagglutination effect was observed for the test samples against red blood cells. The plant also possesses good Anti-termite and Nitric oxide free radical scavenging assays. Similarly the plant shows low phytotoxic effect against *Lemna minor* at various concentrations. However the plant was found inactive against most of the Fungi used and has also a non-significant Insecticidal effect against tested insect.

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

Throughout the ages, human race have fulfilled its basic needs such as production of foodstuffs, fertilizers, fragrances, clothing, shelters, flavors and means of transportation from nature. Progressively they started to use parts of plants (leaves, stems, roots and rhizomes), some animal parts and minerals for the cure of different diseases. The modern medicine or allopathy is a result of scientific and observational development of traditional medicine and practices.

The main sources of traditional medicine system, existing for thousands of years, are plants. Nearly all civilization, from ancient times, has used plants as a source of medicine. Among the Egyptian medicine (2900 BC), the well known Egyptian pharmaceutical documentation is “Ebers Papyrus” (1500 BC). This document include seven hundred drugs mostly (plants, though animal organs were included together with some minerals) including formulae i.e. poultices, infusions, gargles, pills, snuffs and ointments, with beer, wine, milk and honey commonly used as solvents [1].

The first record from Mesopotamia (2600 BC), written on hundreds of clay tablets in cuneiform; amongst the approximately 1000 plants derived substances which they used were *Cedrus* species (cedar), *Commiphora* species (myrth), *Cupressus sempevirens* (cypress), *Papaver somniferum* (poppy juice) and *Glycyrrhiza glabra* (licorice) oils. All of these are still in use for the handling of inflammation and parasitic infections to colds and coughs [1].

Ayurveda, an ancient system of medicine, has a sound experimental and philosophical basis is still widely practiced in South Asia. The two main classes of Ayurveda, Atharvaveda (around 1200 BC), *Charak samhita* and *Sushrut Samhita* (500-1000 BC), give a detailed description of over seven hundred herbs [2].

The Chinese *Materia Medica* extensively documented over the centuries. With the first documentation (1100 BC) Wu Shi Er Bing Fang with 52 prescriptions followed by works such as Shennong Herbal (~100 BC) and Tang Herbal (659 AD) containing 365 and 850 drugs, respectively [1].

In the prehistoric western world, the Greeks contributed considerably to the rational development of the use of herbal drugs. Theophrastus (~300 BC), a natural scientist and philosopher, in his book (History of Plants), dealt with the medicinal properties of herbs and noted that through cultivation their medicinal characteristics can be changed. A Greek physician, Dioscorides (100 AD), accurately documented the collection, storage and use of the medicinal herbs during his travels with Roman armies throughout the "Known world". Galen (130-200 AD), known for his complex prescription and formulae used in compound drugs, practiced and taught pharmacy and medicine in Rome and published upto 30 books on these subjects [1].

In many developing countries, people are still depending on traditional medicine for their healthcare. In developed countries too, people are turning to herbal remedies for multiple reasons. Many of the modern medicines still depends on substances of plant origin and the knowledge gained from them.

According to an estimate of the World Health Organization (WHO), about 80 % of people rely almost entirely on traditional medicines for their primary healthcare needs, living in developing countries. Over three billion people in the developing countries utilize plant based medicines on a regular basis. Natural pharmaceuticals (Naturaceuticals), nutraceuticals and cosmecuticals have enormous significance as a pool of chemical diversity which has used in different types of drug discovery such as antimicrobial, cardiovascular, immunosuppressive and anticancer. According to the WHO survey, the international market of herbal products is estimated to be US$ 62 billion per year. It has been estimated to grow

upto US$ 5 trillion by the year 2050. The US and European nutraceutical marketplaces are estimated to be US$ 10-12 and 9 billion, respectively and expanding at a rate of more than 20 % annually [2, 3].

Natural products chemistry actually began with the isolation of morphine from opium (*Papaver somniferum*) by Serturner. This was followed by isolation of quinine from *Cinchona* tree in 1860. Cocaine, responsible for paralyzing of the nerve endings responsible to transmit pain, was isolated by a German chemist Carl Koler. Reserpine, an anti-hypertensive alkaloid was isolated from *Rauwolfia serpentine* [2].

Secondary metabolites which are produced by living organisms (microbes and plants) for the purpose of their own survival have huge importance in drug discovery. This group has been providing a large number of structurally novel bioactive molecules and life saving drugs. Secondary metabolites with therapeutic indications include cyclosporine (immunosuppression), mevinolin (hypercholesterolaemia), avermectin (parasitic disease), artemisinin (malaria), vinblastine, vincristine and taxol (cancer).

In present time discovery and development of drugs from natural sources competes with combinatorial chemistry, which recommends a significant complementary way for drug development. In a single experiment it can produced thousands of compounds, which are immediately available for bio-assay screening.

On the other hand, plants and microorganisms are capable for the production of unusual chemical structures in unpredictable ways. The genes and pathways by which plants and microbes used to produce novel skeleton are started to understand and it has been used in drug discovery. The secondary metabolites biosynthesis in plants and microbes also represents combinatorial chemistry. Natural product biosynthesis is mostly carried by enzymes, which represents an immense gene pool, evolved over billions of years [4].

Recent improvements in instrumentation, robotics and bioassay technology have increased the speed of bioassay-guided isolation and structure elucidation of natural products significantly. These improvements have allowed natural product research to be more competitive and cost effective with synthetic compound screening.

Since 2005, thirteen natural product (NP) derived drugs have been lunched in the market (Table-1.1), in addition to the 37 late stage clinical development NP-based candidates (6 in registration and 31 in phase III). The traditional strengths of NPs in oncological and transmittable diseases is still continuing with 19 (51%) compounds being evaluated for the treatment of cancer and 10 (27 %) treat bacterial infections. The remaining compounds are for the treatment of metabolic diseases (4), pain (2) and multiple sclerosis (2). The large number of NP-derived compounds are in various stages of clinical development indicates that natural products are still a feasible source of new drug candidates. In the absence of these natural products, there would be a significant therapeutic shortage in important clinical areas like cardiovascular disease, immune-inflammatory diseases such as rheumatoid arthritis, neurodegenerative disease and most solid tumors [5].

During the current study, bioprospecting studies including biological/pharmacological and phytochemical investigations were carried out for the selected plants, *Vitex agnus castus* and *Myrsine africana*, members of *Verbenaceae* and *Myrsinaceae* family, respectively.

Table 1.1 Natural Product derived drugs launched since 2005 **[5]**.

Year	Generic name (trade name)	Lead compound	Classification	Disease area
2005	Dronabinol / Cannabidol (Sativex ®)	Dronabinol / Cannabidol	NPs	Pain
2005	Fumagilin (Flisint®)	Fumagillin	NP	antiparasitic
2005	Doripenem (Finibax ® / Doribax™)	Thienamycin	NP-derived	antibacterial
2005	Tigecycline (Tygacil®)	Tetracycline	Semi-synthetic NP	antibacterial
2005	Ziconotide (Prialt®)	Ziconotide	NP	pain
2005	Zotarolimus (Endeavor ™ stent)	Sirolimus	Semi-synthetic NP	Cardiovascular surgery
2006	Anidulafungin (Eraxis ™ / Ecalta ™)	Echinocandin B	Semi-synthetic NP	antifungal
2006	Exenatide (Byetta ™)	Exenatide-4	NP	diabetes
2007	Lisdexamfetamine (Vyuanse ™)	amphetamine	NP-derived	ADHD
2007	Retapamulin (Altabax ™/Altargo ™)	pleuromutilin	Semi-synthetic NP	antibacterial
2007	Temsirolimus (Torisel ™)	Sirolimus	Semi-synthetic NP	oncology
2007	Trabectedin (Yondelis ™)	Trabectedin	NP	oncology
2007	Ixabepilone (Ixempra ™)	Epothilone B	Semi-synthetic NP	oncology

1.2 VERBENACEAE (FAMILY)

1.2.1 Description

Verbenaceae is a large family of about 75 genera and 2500 species includes several ornamental and medicinal plants in addition to teak which is highly prized for its wood. Members of this family are mainly herbs, shrubs and trees, and rarely woody climbers; branches and twigs tetragonal, not prominently nodose or articulated. Leaves opposite, decussate, rarely whorled or alternate, exstipulate, simple, rarely compound. Inflorescence definite and centrifugal or indefinite and centripetal, spicate, racemiform or panicled to umbellate or a head, very rarely solitary and axillary. Flowers are usually zygomorphic, bisexual and hypogynous. Calyx gamosepalous, mostly tubular or campanulate, (2-) 4 (-7)-lobed or toothed to subentire, persistent, sometimes enlarging and concealing the fruit. Corolla gamopetalous, often with as many lobes as the calyx and somewhat bilabiate, with unequal lobes above and tubular below. Stamens mostly 4 and didynamous, inserted on the corolla tube and alternating with the lobes; staminodes often present; anthers dithecous, mostly dehiscing longitudinally. Gynoecium 2(4-5), carpellary, syncarpous, somewhat 4-lobed and 2-4-loculed, with usually one ovule in each apparent cell on axile placentation; style simple, terminal. Fruits usually schizocarpic or drupaceous with ± hard endocarp, indehiscent or dehiscent into 1-2(-1O)-celled pyrenes; seeds are few [6].

1.2.2 Distribution

The family is native to South Europ, cultivated widely and now naturalized in most of the eastern and South United States and in the tropics and warm temperate regions of both hemispheres [7]. In Pakistan this family is mainly distributed in tropical and sub-tropical regions, represented here by 17 genera and 35 species.

1.2.3 Importance

Members of this family have shown a wide range of medicinal applications, berries, flowers and leaves are used in various decoction, syrup, traditional tincture, elixir, cider vinegar tincture and can also simply eaten as medicinal food. Berries of the plant are considered a tonic for the reproductive system of both male and female [8, 9]. *Clerodendron squamatum* Vahl and *Siphonanthus indicus* give non-specific agglutinating reactions while, other species of the family i.e. *Vitex divaricata* Sw, *Stachytarpheta jamaicensis* (L.) Vahl and *Clerodendrum speciosissinum* Paxt give no agglutinating reactions with the human Red Bloods Cells [10]. *Callicarpa japonica* oil was selectively phytotoxic, with 80–100% growth reduction to bentgrass compared to lettuce seeds [11]. The fruit seeds of *Vitex negundo* possess antifungal activity [12] and the plant is used in various preparation for skin diseases [13], anti- inflammatory and anti-androgenic [14]. The CO2 extract from *Vitex agnus castus* seeds, can be used as mosquitoes repellent, tick mosquitoes biting flies and fleas from animals and humans [15].

1.3 *VITEX* (GENUS)

1.3.1 Description

Members of the genus may be shrubs or trees, rarely climbing, often whitish pubescent or tomentose. Leaves opposite or ternate, palmately (1-) 3-5 (-7)-foliolate, petiolate; leaflets mostly petiolulate, unequal, entire to dentate. Inflorescence terminal or axillary, cymose, often in racemiform or spicate panicles, usually elongated and tapering above. Flowers small, white, blue, violet or yellowish, bracteates. Calyx usually campanulate, truncate, with 5-fid or -toothed apices, slightly enlarged in fruit. Corolla-tube cylindrical, short, straight or slightly curved; limb ± 2-lipped. with 5, unequal lobes, oblique; upper lip usually erect, 2-fid; lower lip 3-fid, with larger middle lobe. Stamens 4, didynamous, often exserted, inserted on corolla-tube; anther cells parallel, pendulous, becoming divaricate and

somewhat twisted later. Ovary 4-locular with laterally attached 1 ovule in each locule; style fili form with slightly 2-fid stigma. Fruit drupaceous, small, globose or ovoid, fleshy, invested below by somewhat enlarged calyx, endocarp hard and horny; seeds oblong or oblong-obovate, exalbuminous [6]

1.3.2 Distribution

Vitex genus is a large genus, represented by approximately 225 species, mainly distributed in the tropical, subtropical and warm temperate regions. In Pakistan the genus is represented by three species i.e. *Vitex trifolia*, *Vitex negundo* and *Vitex agnus castus*. Other species of the genus are; *Vitex divaricata* Sw, *Vitex glabrata* R. Br., *Vitex parviflora* Juss and *Vitex rotundifolia* L.

1.3.3 Importance

The members of *Vitex* genus have significant medicinal importance and use in the traditional system. Traditionally, *Vitex* is used as anthelmintic, expectorant, for menstrual problems due to deficiency of corpus luteum, spasmodic dysmenorrhoea, febrifuge, anti-inflammation, sprains and expectorant etc. *Vitex peduncularis* leaves and bark are used in malarial and black water fever. *Vitex trifolia* extract possesses antimicrobial activity against *Mycobacterium tuberculosis* and roots of the plant are used as poultice in rheumatism [16]. The aerial parts (leaves, fruits and stem) of *Vitex trifolia* are used as emmenagogue, diuretic, antipyretic, antitusive, antiseptic, vermifuge and carminatives. The roots of the plant have medicinal role in headache, eczema, spleen, rheumatism, swelling, constusion, sprains and anodyne. Fruits and roots of *Vitex trifolia* are also used to cure rheumatism, contusion and fever also used as diuretic. Decoction of the leaves of *Vitex negundo* are considered to be vermifuge and tonic, given along with long paper in catarrhal fever, extracts from the leaves and roots is important and sold as drug, leaf extract is used in Unani and Ayurvedic systems of medicine and anti-inflammatory, anti-histamine and analgesic activities are reported for

the water extracts of the mature fresh leaves [17]. The methanol extract from the root of *Vitex negundo* exhibit significant snake venom neutralizing capacity [18].

1.4 *VITEX AGNUS CASTUS* (PLANT)

1.4.1 Description

Botanical Name: *Vitex agnus castus*

Family: *Verbenaceae*

Genus: *Vitex*

Species *agnus castus*

Vitex agnus castus is an aromatic shrub or low tree belong to *Verbenaceae* family. Branches are densly short- puberulent, 5-9 digitate and velvety leaves and 5-7 leaflets which are mostly unequal, the central one is largest, the lowermost pair smallest, the three largest piteolates, 2-4 smallest usually sessile and narrow elliptical, the central one 4.5-11.5 cm long and 9-21 mm wide. Attenuate or acuminate at both ends, pulverulent or glabrate above. Petiole 1.5-2.5 cm long are densly puberulent and resinous granular. Flowers are pale purple or violet in color, in interrupted spikes, found in groups of several. Drupes are small, 4-celled, globose and exceeding the calyx [7].

1.4.2 Distribution

The plant grows in sandy (light) and loamy (medium) soils and in nutritionally poor soil cannot grow in the shade. The plant prefers basic (alkaline), neutral, acidic and moist or dry soils. The plant is native to Southern Europ and the orient it is widely cultivated. The plant is now naturalized in most of the Eastern and Southern United States and in the tropics and warm temperate regions of the both hemispheres [7]. In Pakistan the plant is present in Hazara, Mansehra regions of Khyber Pakhtunkhwa province.

1.4.3 Importance

The plant, *Vitex agnus castus*, known as women`s herb, used for thousands of years in treatment of female associated problems, hormonal cycle. The Hippocrates (father of medicine) first mentioned *Vitex agnus castus* in 450 B.C. since then traditional herbalist recommends the plant for ailments including headaches, fever, promote urination and to dispel wind. The seeds and fruits of the plant are aphrodisiac, anaphrodisiac, ophthalmic, stomachic, galactogogue and sedative. The prolong usage of the plant prove to restore carpus luteum function.

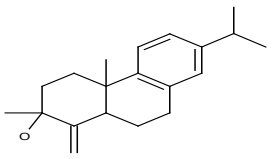
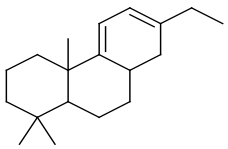
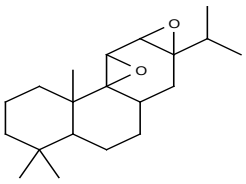
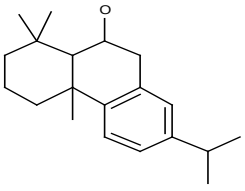
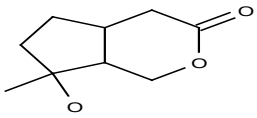
The dried seeds are used as emmenagogue and lactogenic and taken orally. Hot water extracts are used as contraceptive and in Unani medicine the whole plant is inhaled as an emmenagogue, by fumigation [19]. Fruits are taken orally and the hot water extracts from the leaf and flowering top are used as an antispasmodic, sedative and anaphrodisiac [20]. For antiestrogenic and antispasmodic effect the hot water extract of the fruits are used [21]. The plant is source for clinically important dopaminergic compounds, used for the improvement of premenstrual mastodynia and other symptoms of premenstrual syndrome [16].

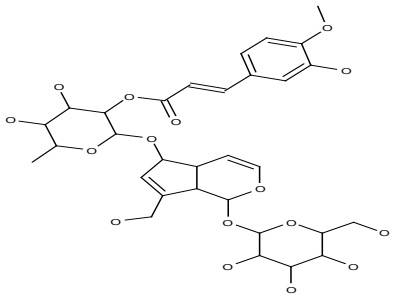
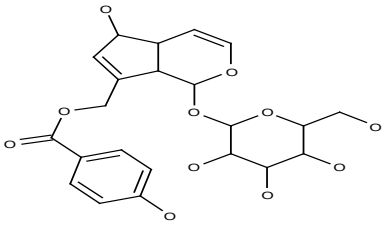
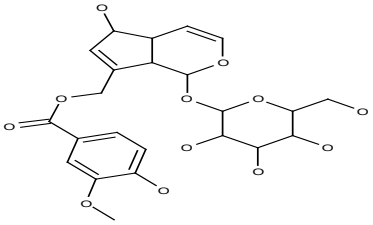
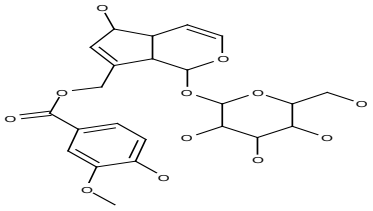
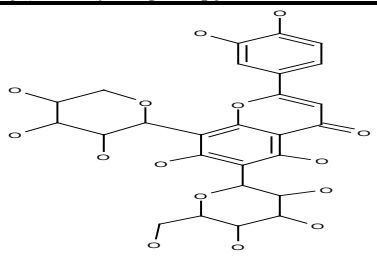
PHYTOCHEMISTRY OF GENUS *VITEX*

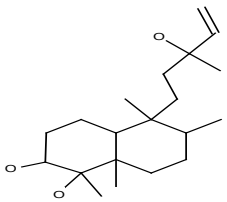
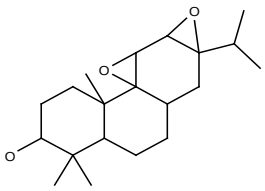
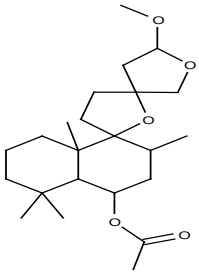
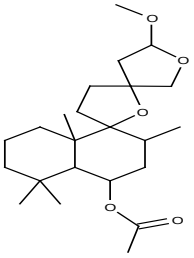
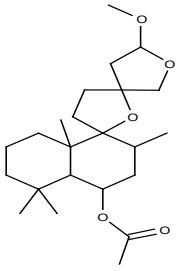
1.5 PHYTOCHEMISTRY OF THE GENUS *VITEX*

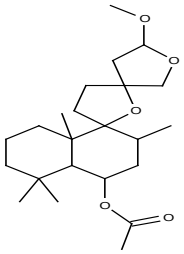
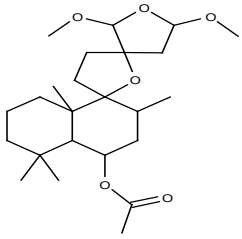
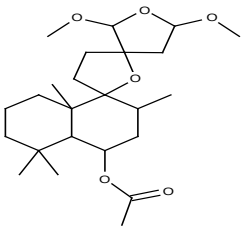
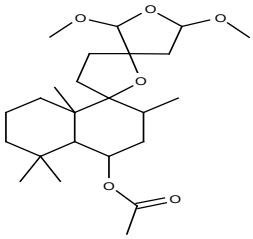
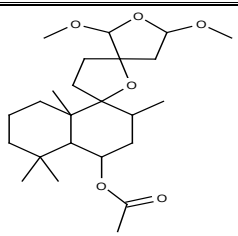
Literature reports volatile oils, sesquiterpenes, diterpenes, steroids, iridoids, lignins and stilbene derivatives from the genus *Vitex*. The results of the previous phytochemical investigations on the genus are presented in the following table.

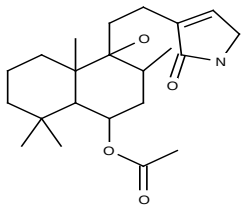
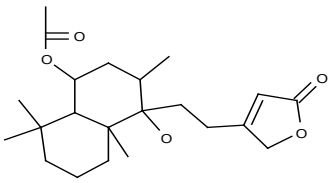
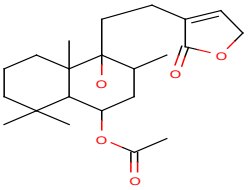
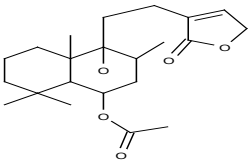
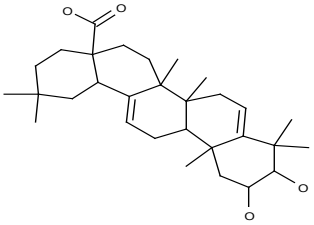
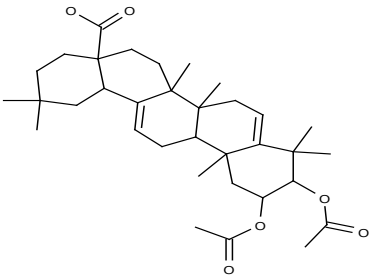
Table 1.2. Phytochemical constituents from genus Vitex.

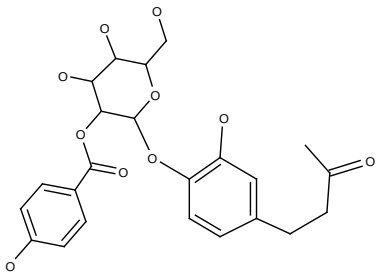
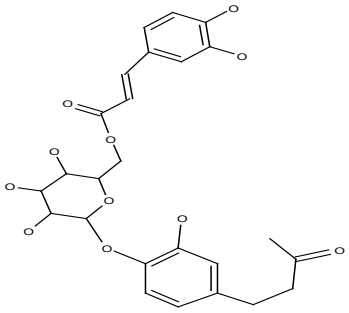
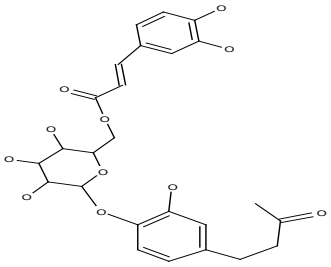
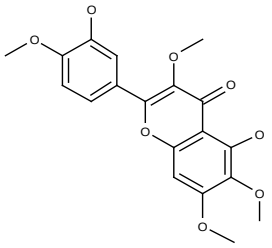
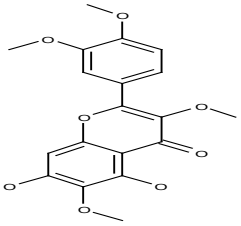
S.NO	Mol. Formula / Mol. wt	Compound Structure / Name	Reference
1	$C_{20}H_{28}O$ / 284	 Vitexifoline C	Ono, M. et al., J. Nat. Prod., 2002, 65, 537-541
2	$C_{20}H_{32}$ / 272	 9(11), 12-Abietadieane	Sakurai, A. et al., Nippon Kagaku Kaishi, 1999, 207-211; CA, 130, 349730k
3	$C_{20}H_{32}O_2$ / 304	 9α, 11α: 12α, 13α-Diepoxide	Sakurai, A. et al., Nippon Kagaku Kaishi, 1999, 207-211; CA, 130, 349730k
4	$C_{20}H_{30}O$ / 286	 8,11,13-Abietatrien-6-ol;(5β,6α)-form	Chawla, A.S. et al., Indian J. Chem., Sect. B, 1991, 30, 773
5	$C_9H_{14}O_3$ / 170	 Argyol: (+) form	Zhao, W. et al., Phytochemistry, 1996, 41, 1553-1555

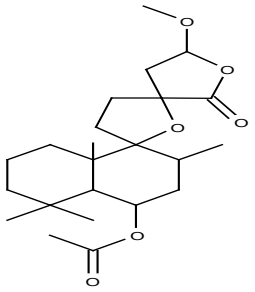
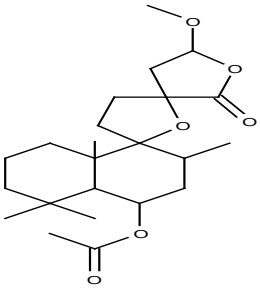
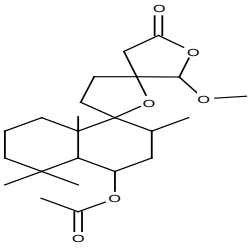
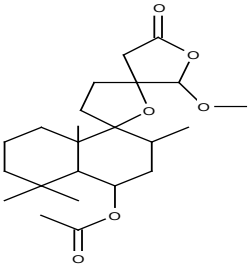
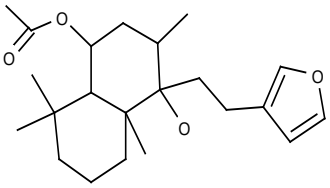
6	$C_{31}H_{40}O_{16}$ / 668	 Aucubigenin;1-*O*-β-D-glucopyranoside ,6-*O*-[3hydroxy-4-methoxycinnmoyl-(→2)- α-L-rhamnopyranoside	Seifert, K. et al., Helv. Chim. Acta, 1982, 65, 1678-1685
7	$C_{22}H_{26}O_{11}$ / 466	 Aucubigenin;10-*O*-(4-Hydroxybenzyl) ,1-*O*- β-D-glucopyrano-side	Ahmad, I. et al., Heterocycles, 2004, 63, 1875-1881
8	$C_{23}H_{28}O_{11}$ / 480	 Aucubigenin;10-*O*-(4-methoxybenzyl) ,1-*O*- β-D-glucop-yranoside	Suksamrarn, S. et al., Planta Med., 1999, 65, 392; 2002, 68-72
9	$C_{23}H_{28}O_{12}$ / 496	 Aucubigenin; 10-*O*-(4-hydroxy-3-meth oxybenzyl),1-*O*- β-D-glucopyranoside	Suksamrarn, S. et al., Planta Med., 1999, 65, 392; 2002, 68-72
10	$C_{26}H_{28}O_{15}$ / 580	 Carlinoside	Seikel, M.K. et al., Phytochemistry, 1966, 5, 439

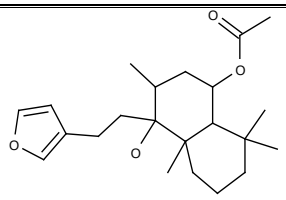
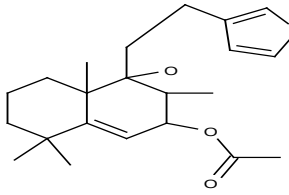
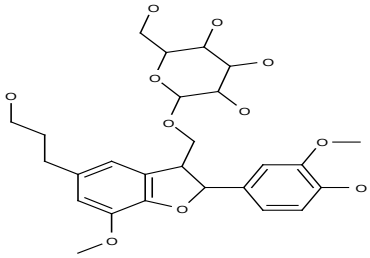
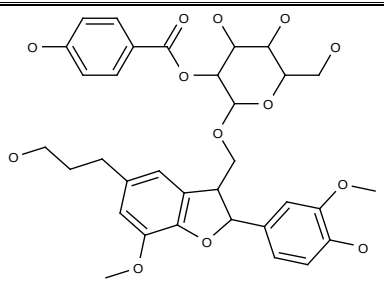
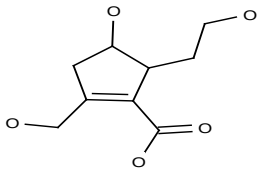
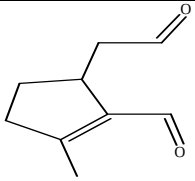
11	$C_{20}H_{36}O_3$ / 324	 14-Clerodine-3,4,13-triol; (3β,4α,5α,8 α,13€)-form	Ono, M. et al., J. Nat. Prod., 2002, 65, 537-541
12	$C_{20}H_{32}O_3$ / 320	 9,11:12,13-Diepoxy-3-Abietanol ; (3β,9α,11α,12α,13α)-form	Ono, M. et al., Phytochemistry, 2000, 55, 873-877
13	$C_{23}H_{38}O_5$ / 394	 9,13:15,16-Diepoxy-6,15-labdanediol; (6β,8α,9α,13R,15R) -form, 15-Me-ether, 6-Ac	Ono, M. et al., J. Nat. Prod., 1999, 62, 1532-1537
14	$C_{23}H_{38}O_5$ / 394	 9,13:15,16-Diepoxy-6,15-labdanediol; (6β,8α,9α,13R,15*S*) -form, 15-Me-ether, 6-Ac	Ono, M. et al., J. Nat. Prod., 1999, 62, 1532-1537
15	$C_{23}H_{38}O_5$ / 394	 9,13:15,16-Diepoxy-6,15-labdanediol; (6β,8α,9α,13*S*,15R) -form, 15-Me-ether, 6-Ac	Ono, M. et al., J. Nat. Prod., 1999, 62, 1532-1537

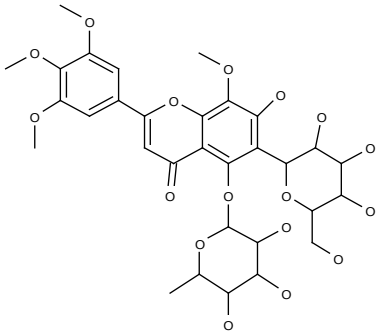
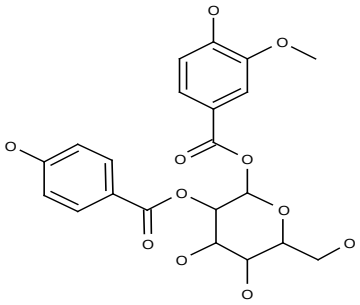
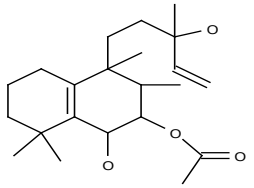
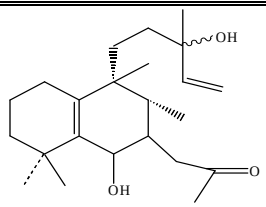
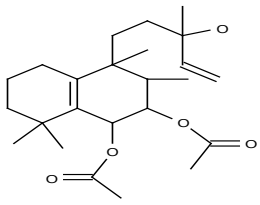
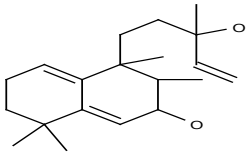
16	$C_{23}H_{38}O_5$ / 394	 9,13:15,16-Diepoxy-6,15-lab-danediol; (6β,8α,9α,13*S*,15*S*)-form, 15-Me-ether, 6-Ac	Ono, M. et al., J. Nat. Prod., 1999, 62, 1532-1537
17	$C_{24}H_{40}O_6$ / 424	 9,13:15,16-Diepoxy-6,15-labdanediol; (6β,8α,9α,13*S*,15R) -form, 15 ,16-di-Me-ether, 6-Ac	Ono, M. et al., J. Nat. Prod., 1999, 62, 1532-1537
18	$C_{24}H_{40}O_6$ / 424	 9,13:15,16-Diepoxy-6,15-labdanetriol; (6β,8α,9α, 13*S*,15R, 16S)-form, 15-Me-ether, 6-Ac	Ono, M. et al., J. Nat. Prod., 1999, 62, 1532-1537
19	$C_{24}H_{40}O_6$ / 424	 9,13:15,16-Diepoxy-6,15-labda-netriol; (6β,8α,9α,13*S*,15*S*,16R) form, 15,16-Me-ether, 6-Ac	Ono, M. et al., J. Nat. Prod., 1999, 62, 1532-1537
20	$C_{24}H_{40}O_6$ / 424	 9,13:15,16-Diepoxy-6,15-labdanetriol; (6β,8α,9α,13*S*,15*S*, 16*S*)-form, 15,16 Me - ether,6-Ac	Ono, M. et al., J. Nat. Prod., 1999, 62, 1532-1537

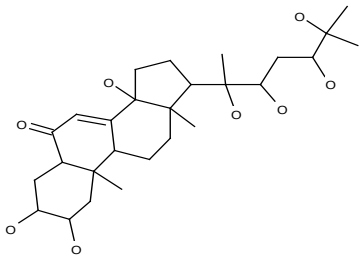
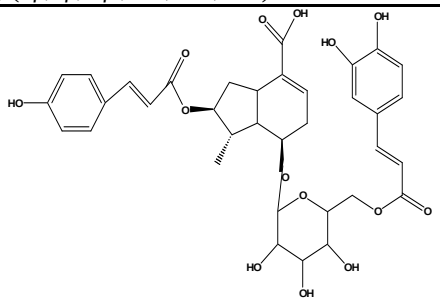
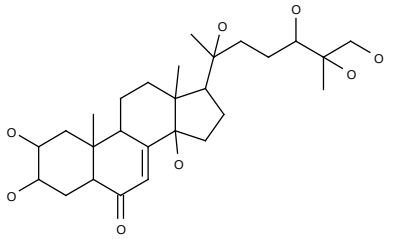
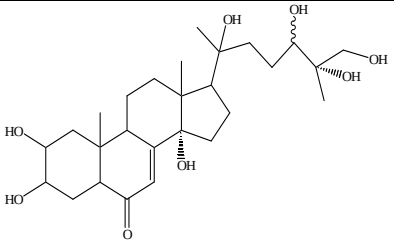
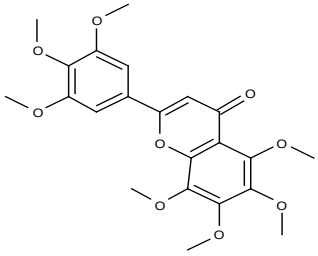
21	$C_{22}H_{35}O_4$ / 377	 6,9-Dihydroxy-13Labden-15,16-lactam ; (6β,8α,9β)-form, 6-AC	Li, S.-H. et al., Tet. Lett., 2002, 43, 5131-5134
22	$C_{22}H_{34}O_5$ / 378	 6,9-Dihydroxy-13Labden-15,16-olide; (6β,8α,9α)-form, 6-AC	Taguchi, H.Chem. Pharm. Bull., 1976, 24, 1668
23	$C_{22}H_{34}O_5$ / 378	 6,9-Dihydroxy-13Labden-16, 15-olide; (6β,8α,9β)-form, 6-AC	Ono, M. et al., Chem. Pharm. Bull., 2001, 49, 82-86
24	$C_{22}H_{34}O_5$ / 378	 6,9-Dihydroxy-13Labden-16,15-olide; (6β,8α,9α)-form, 6-AC	Ono, M. et al., Chem. Pharm. Bull., 2001, 49, 82-86
25	$C_{30}H_{46}O_4$ / 470	 2,3-Dihydroxy-5,12-oleanadien-28-oic acid; (2α,3α)-form	Chawla, A.S. et al., J. Nat. Prod., 1992, 55, 163-167
26	$C_{34}H_{50}O_6$ / 554	 2,3-Dihydroxy-5,12-oleanadien-28-oic acid; (2β ,3α)-form, di-AC	Ulubelen, A. et al., Nat. Prod. Lett., 1992, 1, 141-147

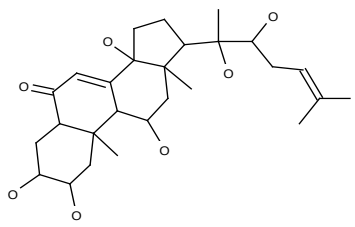
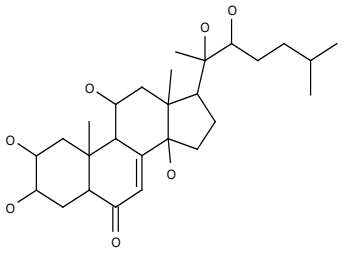
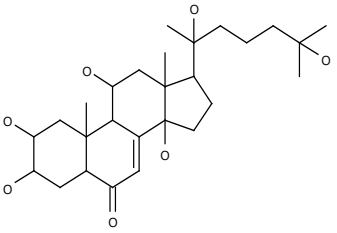
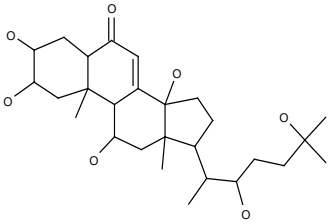
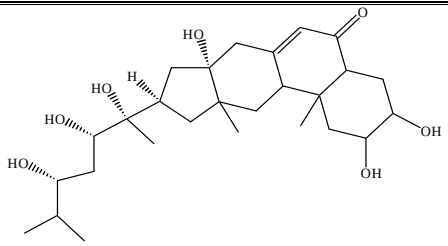
27	$C_{23}H_{26}O_{10}$ / 462	 4-(3,4-Dihydroxyphenyl)-2-butanone; 4-*O*-[4-Hydroxybenzoyl-(→2)- β-D-glucopyranoside	Okuyama, E. et al., Chem. Pharm. Bull., 1998, 46, 655-662
28	$C_{25}H_{28}O_{11}$ / 504	 4-(3,4-Dihydroxyphenyl)-2-butanone; 4-*O*-[3,4-dihydroxy-*E*-cinnamoyl(→6)-β-D-glucopyranoside	Okuyama, E. et al., Chem. Pharm. Bull., 1998, 46, 655-662
29	$C_{25}H_{28}O_{11}$ / 504	 4-(3,4-Dihydroxyphenyl)-2-butanone;4-*O*-[3,4-dihydroxy-*Z*-cinnamoyl(→6)-β-D-glucopyranoside	Okuyama, E. et al., Chem. Pharm. Bull., 1998, 46, 655-662
30	$C_{19}H_{18}O_8$ / 374	 3`,5-Dihydroxy-3,4`,6,7-tetramethoxy flavone	Flavonoids: Chemistry and Biochemistry, (ed. Andersen, O.M. et al)
31	$C_{19}H_{18}O_8$ / 374	 5`,7-Dihydroxy-3,3`,4`,6-tetramethoxy flavone	Nair, A.G.R. et al., Curr. Sci., 1975, 44, 214

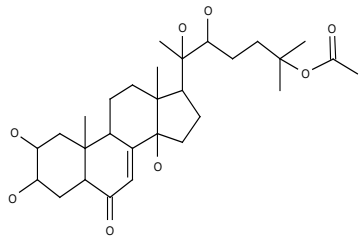
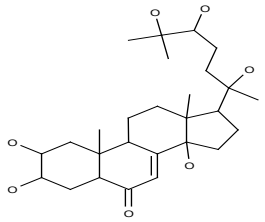
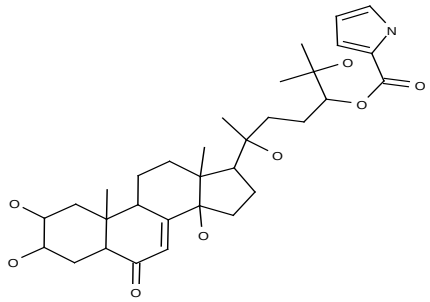
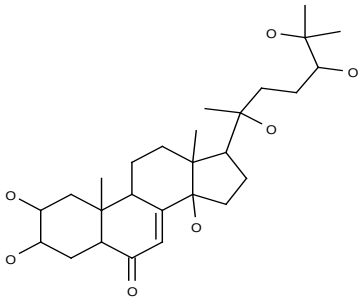
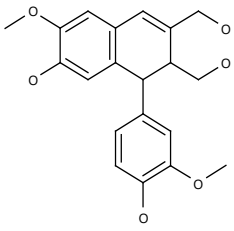
32	$C_{23}H_{36}O_6$ / 408	 9,13-Epoxy-6,15-dihydroxy-16,15-labdanolide; (6β,8α,9α,13*R*,15€)-form, 15-Me ether, 6AC	Ono, M. et al., Chem. Pharm. Bull., 2001, 49, 82-86
33	$C_{23}H_{36}O_6$ / 408	 9,13-Epoxy-6,15-dihydroxy-16,15-labdanolide; (6β,8α,9α,13*S*,15€)-form, 15-Me ether, 6AC	Ono, M. et al., Chem. Pharm. Bull., 2001, 49, 82-86
34	$C_{23}H_{36}O_6$ / 408	 9,13-Epoxy-6,15-dihydroxy-16,15-labdanolide; (6β,8α,9α,13R,16*S*)-form, 15-Me ether, 6AC	Ono, M. et al., Chem. Pharm. Bull., 2001, 49, 82-86
35	$C_{23}H_{36}O_6$ / 408	 9,13-Epoxy-6,15-dihydroxy-16,15-labdanolide; (6β,8α,9α,13*S*,16*S*)-form, 15-Me ether, 6AC	Ono, M. et al., Chem. Pharm. Bull., 2001, 49, 82-86
36	$C_{22}H_{34}O_4$ / 362	 15,16-Epoxy-13(16),14-labdadiene 6,9-diol; (6α,8α,9α)-form, 6Ac	Ono, M. et al., Phytochemistry, 2000, 55, 873-877

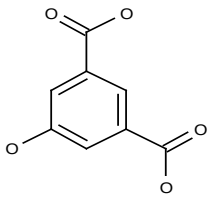
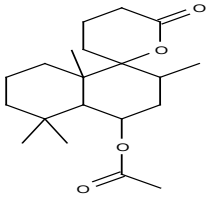
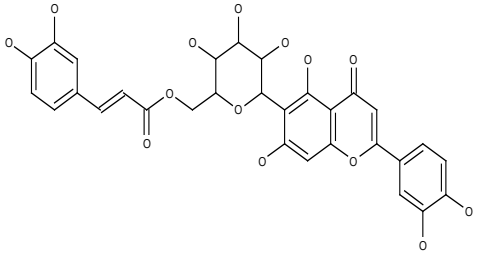
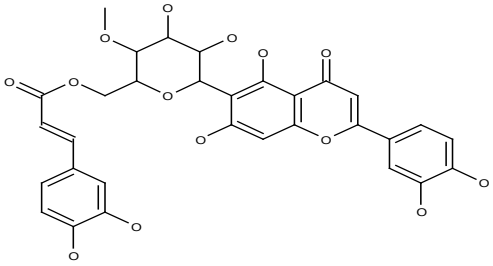
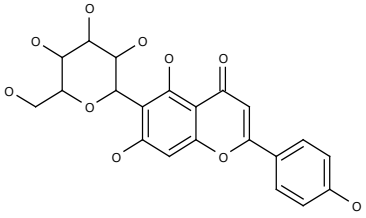
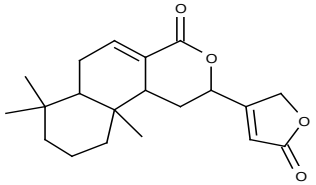
37	$C_{22}H_{34}O_4$ / 362	 15,16-Epoxy-13(16),14-labdadiene 6,9-diol;(6 β,8α,9α)-form, 6Ac	Ono, M. et al., Phytochemistry, 2000, 55, 873-877
38	$C_{22}H_{32}O_4$ / 360	 15,16-Epoxy-5,13(16),14-labdatriene-6,9-diol;(7β,8α,9α)-form, 6Ac	Ono, M. et al., Phytochemistry, 2000, 55, 873-877
39	$C_{26}H_{34}O_{11}$ / 522	 4,7`-Epoxy-3`,4`,5,9,9`-lignanpentol; (7`*S*,8`R)-form, 3`,5-di-Me ether,9`-*O*- β-D-glucopyrano-side	Okuyama, E. et al., Chem. Pharm. Bull., 1998, 46, 655-662
40	$C_{33}H_{38}O_{13}$ / 642	 4,7`-Epoxy-3`,4`,5,9,9`-lignanpentol; (7`€,8`€)-form, 3`,5-di-Me ether,9`-*O*- [4-hydroxybenzoyl-(→2)-β-D-glu-copyranoside	Okuyama, E. et al., Chem. Pharm. Bull., 1998, 46, 655-662
41	$C_9H_{14}O_5$ / 202	 Eucommiol; 1-carboxylic acid	Ono, M. et al., Chem. Pharm. Bull., 1997, 45, 1094-1096
42	$C_9H_{12}O_2$ / 152	 2-formyl-3-Methyl-2-cyclopentene-1acetaldehyde; (R)-form	Watanabe et al., 1998

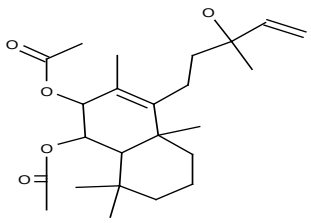
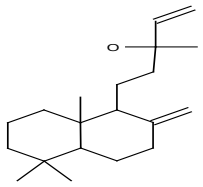
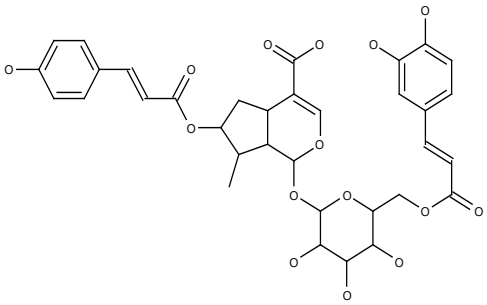
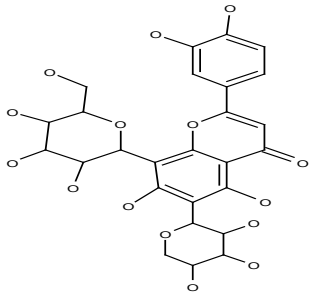
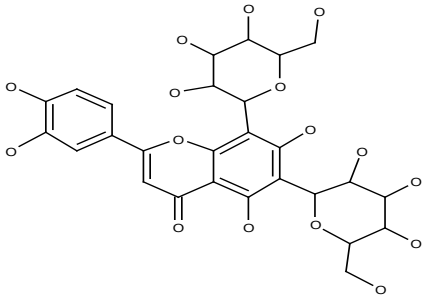
43	$C_{31}H_{38}O_{17}$ / 682	 6-glucopyrano-syl-3`,4`,5,5`, 7, 8-tetra Me ether, 5-*O*-α-L-rhamnopyranoside	Subramanian, P.M. et al., J. Nat. Prod., 1979, 42, 540
44	$C_{21}H_{22}O_{11}$ / 450	 Glucose- β-D-pyranose- form,2-*O*-(4-hydroxy-benzyl), 1-*O*-(4-hydroxy-3-methoxy-benzoyl)	Okuyama, E. et al., Chem. Pharm. Bull., 1998, 46, 655-662
45	$C_{22}H_{36}O_4$ / 364	 Vitetrifolin F	Ono, M. et al., Chem. Pharm. Bull., 2001, 49, 1220-1222
46	$C_{22}H_{36}O_4$ / 364	 Vitetrifolin E	Ono, M. et al., Chem. Pharm. Bull., 2001, 49, 1220-1222
47	$C_{24}H_{38}O_5$ / 406	 Vitetrifolin D	Ono, M. et al., Chem. Pharm. Bull., 2001, 49, 1220-1222
48	$C_{20}H_{32}O_2$ / 304	 Vitetrifolin G	Ono, M. et al., Chem. Pharm. Bull., 2001, 49, 1220-1222

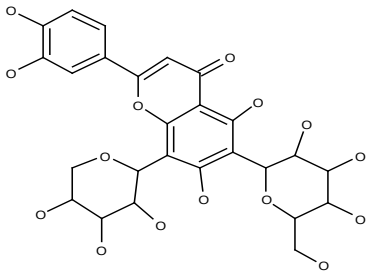
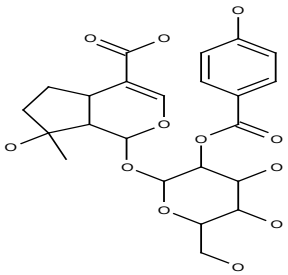
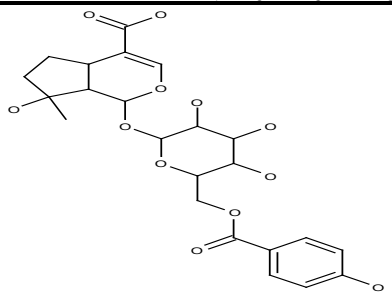
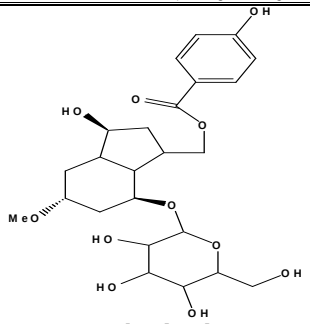
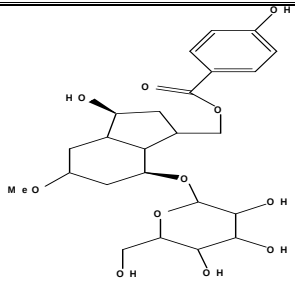
49	$C_{27}H_{44}O_8$ / 496	 2,3,5,14,20,22,25-Heptahydrox-ycholest-7-ene-6-one; (2β,3β, 5β ,14α,20R,22R)-form	Suksamrarn, A. et al., Phytochemistry, 1997, 45, 1149-1152
50	$C_{27}H_{44}O_8$ / 496	 2,3,14,20,22,24,25-Heptahydroxycholest-7-ene-6-one; (2β,3β, 5β, 14α,22R,24R)-form	Suksamrarn, A. et al., Phytochemistry, 1997, 45, 1149-1152
51	$C_{27}H_{44}O_8$ / 496	 2,3,14,20,24,25,26-Heptahydr-oxycholest-7-ene-6-one; (2β, 3β, 14α,24€, 25R)-form	Dos Santos, T.C. et al., Fitoterapia, 2001, 72, 215-220
52	$C_{27}H_{44}O_8$ / 496	 2,3,5,14,20,22,25-Heptahydroxy -cholest-7-ene-6-one; (2β,3β, 5β, 14α,24€,25*S*)-form	Dos Santos, T.C. et al., Fitoterapia, 2001, 72, 215-220
53	$C_{22}H_{24}O_9$ / 432	 3`,4`,5,5`,6,7,8-Heptahydroxy- flavone; Hepta-Me-ether	Ngo, L.-V. et al., Phytochemistry, 1979, 18, 1859; 1863

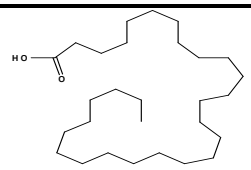
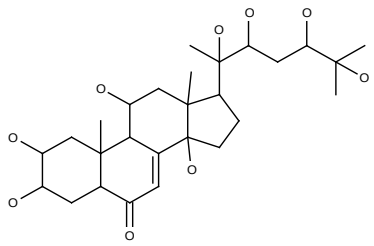
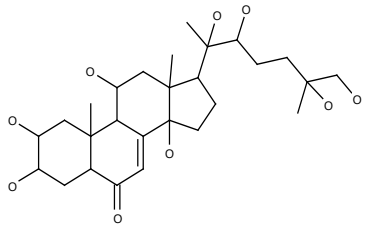
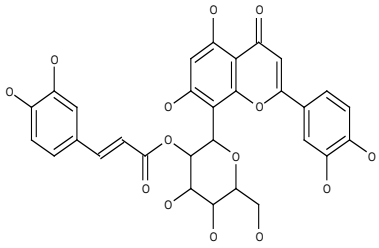
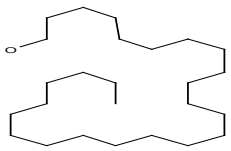
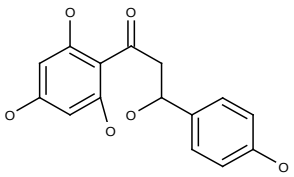
54	$C_{27}H_{42}O_7$ / 478	 2,3,11,14, 20,22-Hexahydroxy cholest-7,24 dien-6-one;(2β,3β, 5β,11α,14α, 20*R*,22*R*)-form	Kubo, I. et al., J. Chem. Ecol., 1990, 16, 2581
55	$C_{27}H_{44}O_7$ / 480	 2,3,11,14,20,22-Hexahydroxy cholest-7-ene-6-one;(2β,3β,5β, 11α,22*R*)-form	Chapman and Hall, 1998
56	$C_{27}H_{44}O_7$ / 480	 2,3,11,14,20,25,-Hextahydroxy cholest-7,24-dien-6-one; (2β,3β, 5β,11α,14α,20*S*)-form	Suksamrarn, A. et al., J. Nat. Prod., 2002, 65, 1690-1692
57	$C_{27}H_{44}O_7$ / 480	 2,3,11,14,22,25,Hextahydroxy cholest-7,ene-6-one; (2β,3β,5β, 11α,α,20*S*)form	Zhang, M. et al., Phytochemistry, 1992, 31, 247
58	$C_{27}H_{44}O_7$ / 480	 2,3,11,14,22,24-Hexahydroxy cholest-7-ene-6-one;(2β,3β,5β, 14α,20R, 22R,24*S*)-form	Chapman and Hall, 1998

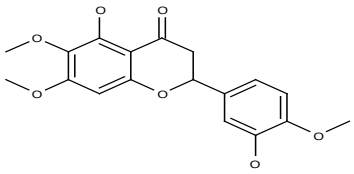
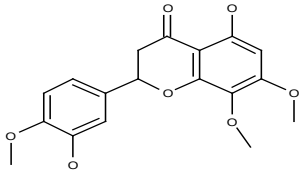
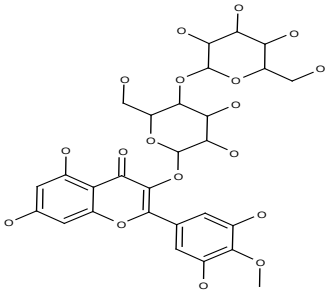
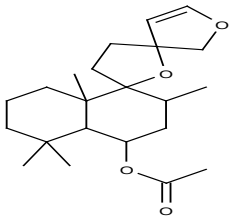
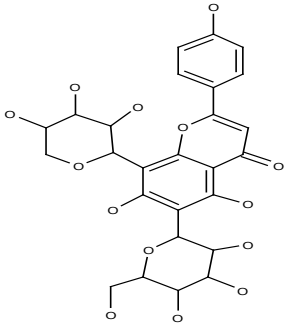
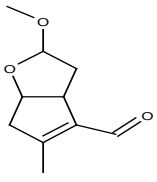
59	$C_{29}H_{46}O_8$ / 522	 2,3,14,20,22,25-Hexa-hydroxy-cholest-7-ene-6-one; (2 β,3β,5β, 14α, 20 R,22R)-form ,25-Ac	Politova, N.K. et al., Khim. Prir. Soedin., 1997, 33, 74-78; Chem. Nat. Compd.
60	$C_{27}H_{44}O_7$ / 480	 Pinnatasterone	Suksamrarn, A. et al., Phytochemistry, 1993, 32, 303
61	$C_{32}H_{47}O_8$ / 573	 Canescensterone	Suksamrarn, A. et al., Phytochemistry, 1995, 38, 473
62	$C_{27}H_{44}O_7$ / 480	 24-Epipinnata-sterone	Suksamrarn, A. et al., J. Nat. Prod., 2002, 65, 1690-1692
63	$C_{20}H_{20}O_6$ / 356	 3`,4,4`,5,9,9`-Hexa-hydroxy-2, 7`-cyclolignan; (7`S,8R,8`R)-form, 7,8-Dihy-dro,9-aldehyde, 3`,5-di-Me ether	Azhar-ul-Haq, et al., Chem. Pharm. Bull., 2004, 52, 1269-1272

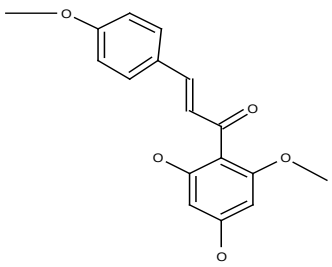
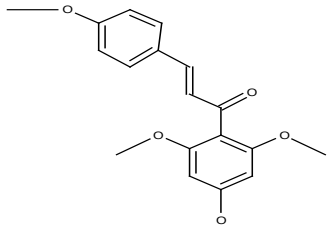
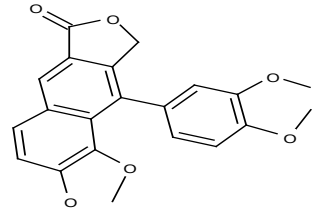
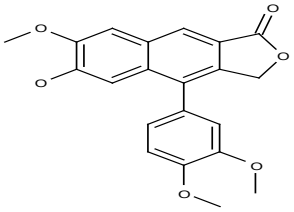
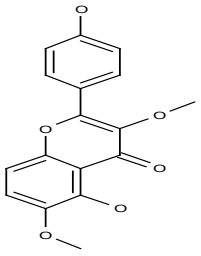
64	$C_8H_6O_5$ / 182	 5-Hydroxy-1,3-benzenedi-carboxylic acid	Gupta, G.S. et al., J. Indian Chem. Soc., 1973, 50, 367-368
65	$C_{20}H_{32}O_4$ / 336	 Vitexifolin E	Ono, M. et al., J. Nat. Prod., 2002, 65, 537-541
66	$C_{30}H_{26}O_{14}$ / 610	 Isoorientin;6``-*O*-(3,4-Dihydroxy-*E*-cinnamoyl)	Hirobe, C. et al., Phytochemistry, 1997, 46, 521-524
67	$C_{31}H_{28}O_{14}$ / 624	 Isoorientin;4``Me ether 6``-*O*-(3,4 Dihydroxy cinnamoyl) (*E*)	Hirobe, C. et al., Phytochemistry, 1997, 46, 521-524
68	$C_{21}H_{20}O_{10}$ / 432	 Isovitexin	Borodin, L.I. et al., Khim. Prir. Soedin., 1970, 6, 19; Chem. Nat. Compd
69	$C_{20}H_{26}O_4$ / 330	 7,13-Labdadiene-15,16:17,12-diolide;(ent 12α*H*)-form	Aphaijitt, S. et al., Aust. J. Chem., 1995, 48, 133

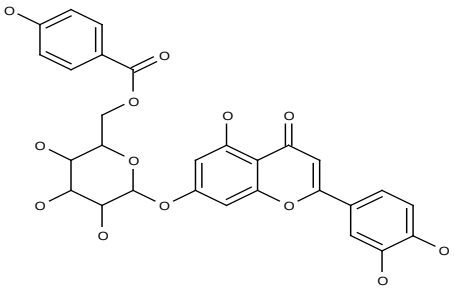
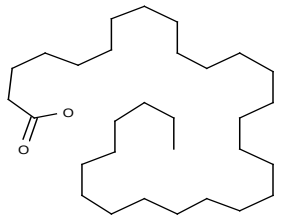
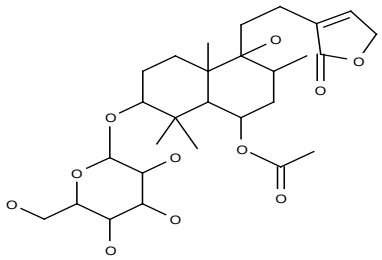
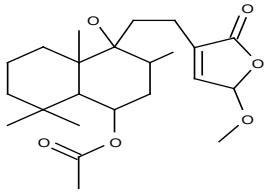
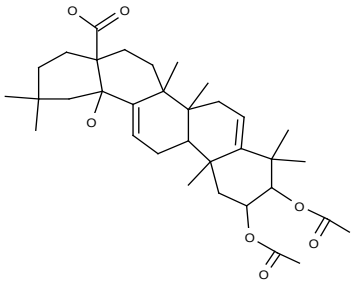
70	$C_{24}H_{38}O_5$ / 406	 7,13-Labdadiene-6,7,13-triol;6β,7 β,13*R*)-form, 6,7-Di-Ac	Hoberg, E. et al., Phytochemistry, 1999, 52, 1555-1558
71	$C_{20}H_{34}O$ / 290	 Vitexifolin A	Ono, M. et al., J. Nat. Prod., 2002, 65, 537-541
72	$C_{34}H_{36}O_{15}$ / 684	 Agnucastoside C	Kuruüzüm-Uz, A. et al., Phytochemistry, 2003, 63, 959-964
73	$C_{26}H_{28}O_{15}$ / 580	 Lucenin 1	Seikel, M.K.Tet. Lett., 1965, 1105
74	$C_{27}H_{30}O_{16}$ / 610	 Lucenin 2	Seikel, M.K.Tet. Lett., 1965, 1105

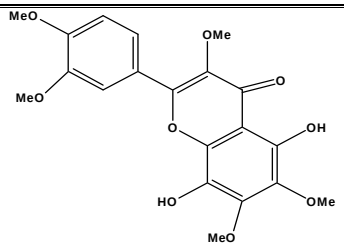
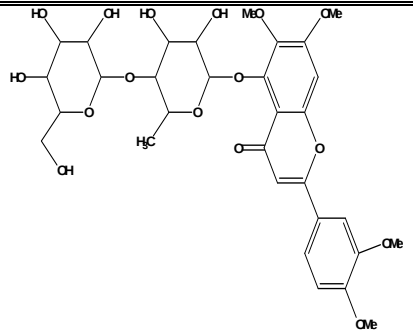
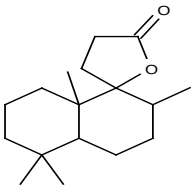
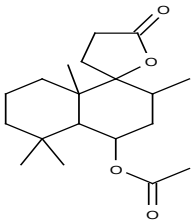
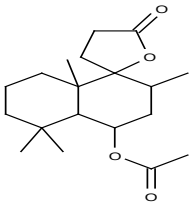
75	$C_{26}H_{28}O_{15}$ / 580	 Lucenin 3	Seikel, M.K.Tet. Lett., 1965, 1105
76		Structure not reported Lucenin 4	Seikel, M.K.Tet. Lett., 1965, 1105
77	$C_{23}H_{28}O_{12}$ / 496	 Mussaenisidic acid; 2`-(4-Hydroxybenzoyl)	Sehgal, C.K. et al., Phytochemistry, 1982, 21, 363-366; 1983, 22, 1036-1038
78	$C_{23}H_{28}O_{12}$ / 496	 Mussaenisidic acid; 6`-(4-Hydroxybenzoyl)	Sehgal, C.K. et al., Phytochemistry, 1982, 21, 363-366; 1983, 22, 1036-1038
79	$C_{23}H_{30}O_{12}$ / 498	 Nishindaside	
80	$C_{23}H_{30}O_{21}$ / 498	 Nishindaside;3-Epimer	

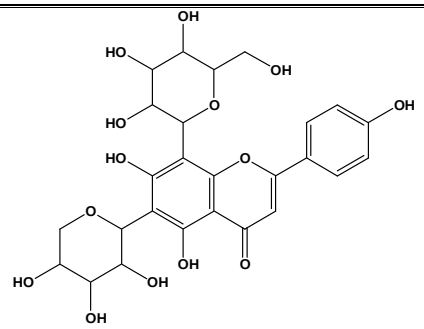
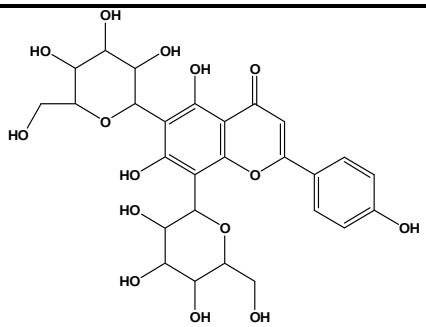
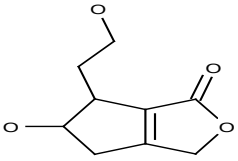
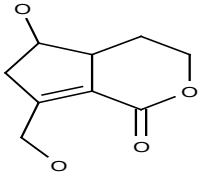
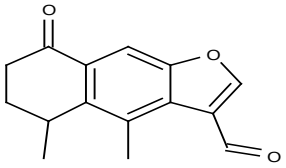
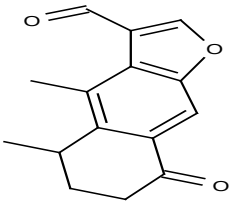
81	$C_{15}H_{21}NO$ / 231	Structure not reported Nishindne	Basu, N.K. et al., Q. J. Pharm. 1947, 20, 136-13
82	$C_{28}H_{56}O_2$ / 424	 Octacosanoic acid	
83	$C_{27}H_{44}O_9$ / 512	 2,3,11,14,20,22,24,25-Octahydroxy-cholest-7-ene-6-one;(2β,3β,5β,11α,14 α,20R,22R,24R)-form	Suksamrarn, A. et al., Phytochemistry, 2000, 53, 921-924
84	$C_{27}H_{44}O_9$ / 512	 2,3,11,14,20,22,25,26-Octahydroxy-cholest-7-ene-6-one;(2β,3β,5β,11α,14 α,20R,22R,25€)-form	Suksamrarn, A. et al., Phytochemistry, 2000, 53, 921-924
85	$C_{30}H_{26}O_{14}$ / 610	 Orientin;2``-*O*-(3,4-Dihydroxy cinnamoyl)	Leitao, S.G. et al., Phytochemistry, 1998, 49, 2167-2169
86	$C_{25}H_{52}O$ / 368	 1-pentacosanol	Cocker, W. et al., JCS, 1962, 5194
87	$C_{16}H_{16}O_6$ / 304	 β,2`,4,4`,6`,pentahydroxy-dihydrochalcone;(-)-form,2`,Me ether	Thuy, T.T. et al., Phytochemistry, 1998, 49, 2603-2605

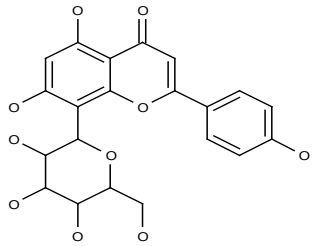
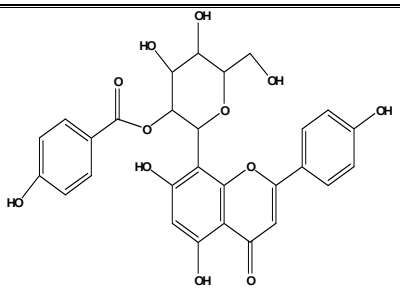
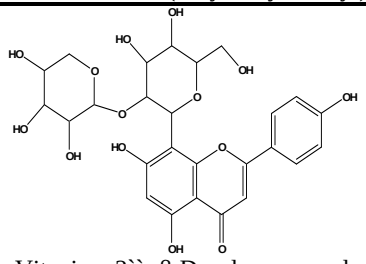
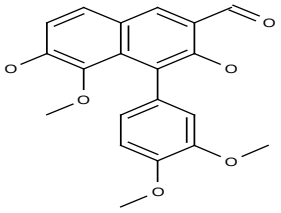
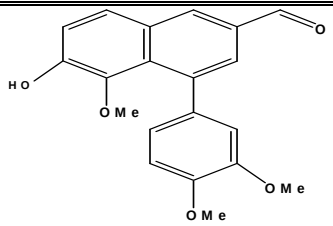
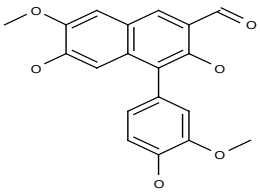
88	$C_{18}H_{18}O_7$ / 346	 3`,4`,5,6,7-pentahydroxy flavanone;(*S*)-form 4`,6,7-tri-Me-ether	Achari, B. et al., Phytochemistry, 1984, 23, 703
89	$C_{18}H_{18}O_7$ / 346	 3`,4`,5,7,8-pentahydroxy flavanone;(*S*)-form 4`,7,8-tri-Me-ether	Achari, B. et al., Phytochemistry, 1984, 23, 703
90	$C_{28}H_{32}O_{18}$ / 656	 3,3`,5,5`,7-pentahydroxy-4`-methoxy-flavone;3-*O*-[β-D-Galactopyranosil-(1→4)-β-D-Galactopyranoside]	Misra, G.S. et al., Planta Med., 1980, 38, 155-160
91	$C_{22}H_{34}O_4$ / 362	 perotundifuran	Asaka, Y. et al., Chem. Lett., 1973, 937
92	$C_{16}H_{28}O_{14}$ / 564	 Schaftoside; 2```,3```,4```-Triepimer	Bouillant, M.L. et al., C. R. Hebd. Seances Acad. Sci. Ser. C, 1971, 273, 1759
93	$C_{10}H_{14}O_3$ / 182	 Tarumal	Dos Santos, T.C. et al., J. Braz. Chem. Soc., 2001, 12, 763-766

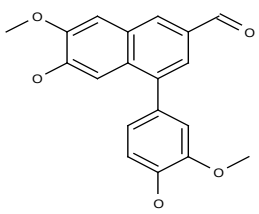
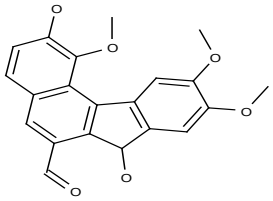
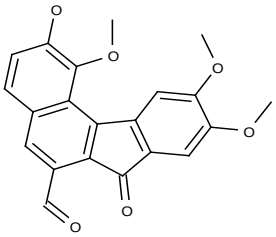
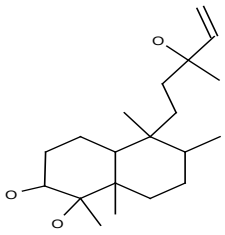
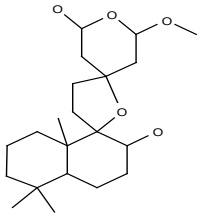
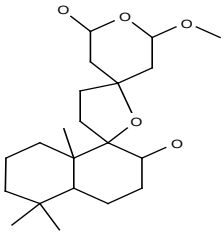
94	$C_{17}H_{16}O_5$ / 300	 2`,4,4`,6`-terahydroxy chalcone ; 2`,4-Di-Me ether	Thuy, T.T. et al., Phytochemistry, 1998, 49, 2603-2605
95	$C_{18}H_{18}O_5$ / 314	 2`,4,4`,6`-terahydroxychalcone;2`,4,6`-Tri-Me-ether	Thuy, T.T. et al., Phytochemistry, 1998, 49, 2603-2605
96	$C_{21}H_{18}O_6$ / 366	 3,3`,4,4`-Tetrahydroxy-2,7`-Cycloligna-7,7`-dien-9,9`-olide; 3,3`,4`-Tri-Me ether	Kawazoe, K. et al., J. Nat. Prod., 2001, 64, 588-591
97	$C_{21}H_{18}O_6$ / 366	 3`,4,4`,5-Tetrahydroxy-2,7`-Cycloligna-7,7`-dien-9,9`-olide; 3`,4`,5-Tri-Me ether	Kawazoe, K. et al., J. Nat. Prod., 2001, 64, 588-591
98	$C_{17}H_{14}O_6$ / 314	 3,4`,5,6-tetrahydroxyflavone; 3,6-Di-Me ether	Sahu, N.P. et al., Planta Med., 1984, 50, 527

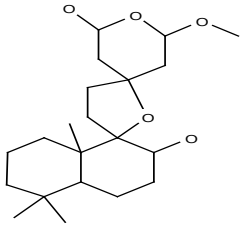
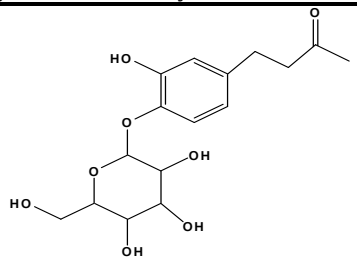
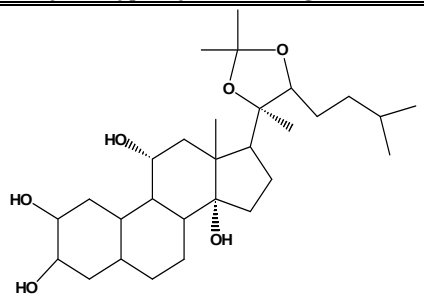
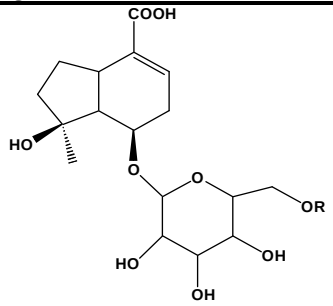
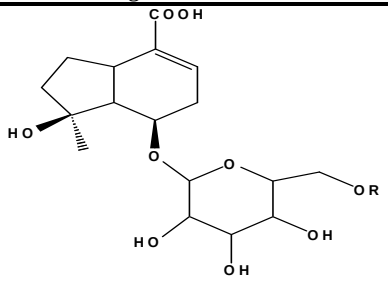
99	$C_{28}H_{24}O_{13}$ / 568	 3`,4`,5,7-tetrahydroxyflavone;7-*O*-[4-Hydroxy-benzoyl-(→6)-β-D-Glucopyranoside]	Hirobe, C. et al., Phytochemistry, 1997, 46, 521-524
100	$C_{30}H_{60}O_2$ / 452	 Triaconanoic acid	Cocker, W. et al., JCS, 1962, 5194
101	$C_{28}H_{44}O_{11}$ / 556	 Viteoside A	Ono, M. et al., Phytochemistry, 1998, 48, 207-209
102	$C_{23}H_{36}O_6$ / 408	 6,9,15-Trihydroxy-13-Labden-16,15-olide; (6β,9α, 15€)-form,15-Me-ether, 6-Ac	Ono, M. et al., Chem. Pharm. Bull., 2001, 49, 82-86
103	$C_{34}H_{50}O_7$ / 570	 2,3,18-Trihydroxy-5,12-Oleanadien-28-Oic acid; (2α, 3β,18β)-for-2,3-Di-Ac	Chawla, A.-S. et al., J. Nat. Prod., 1992, 55, 163-167

104	$C_{20}H_{20}O_8$ / 348	 Artemetin	Chawla, A.-S. et al. 1991
105	$C_{32}H_{40}O_{17}$ / 696	 3,6,7,3`,4`-pentamethoxy-5-*O*-glucopyranosil rhamnoside	Misra et al., 1980
106	$C_{17}H_{28}O_2$ / 264	 14,15,16-Trinor-13,19-labdanolide; (8α,9 α)-form	Ono, M. et al., J. Nat. Prod., 2002, 65, 537-541
107	$C_{19}H_{30}O_4$ / 322	 Vitexifolin D	Ono, M. et al., J. Nat. Prod., 2002, 65, 537-541
108	$C_{19}H_{30}O_4$ / 322	 14,15,16-Trinor-13,19-labdanolide; (8α,9 α)-form, 6β-Acetoxy	Ono, M. et al., J. Nat. Prod., 2002, 65, 537-541

109	$C_{26}H_{28}O_{14}$ / 564	 Vicenin 1	Seikel, M.K. et al., Phytochemistry, 1966, 5, 439
110	$C_{27}H_{30}O_{15}$ / 594	 Vicenin 2	Seikel, M.K. et al., Phytochemistry, 1966, 5, 439
111	$C_9H_{12}O_4$ / 184	 Viteolide I	Ono, M. et al., Chem. Pharm. Bull., 1997, 45, 1094-1096
112	$C_9H_{12}O_4$ / 184	 Viteolide II	Ono, M. et al., Chem. Pharm. Bull., 1997, 45, 1094-1096
113	$C_{15}H_{14}O_3$ / 242	 Viteralone;(*R*)-form	Vishnoi, S.P. et al., Phytochemistry, 1983, 22, 597
114	$C_{15}H_{14}O_3$ / 242	 Viteralone;(*S*)-form	Tada, H. et al., Heterocycles, 1984, 22, 2203

115	$C_{21}H_{20}O_{10}$ / 432	 Vitexine	Tomczyk, M. et al., Z. Naturforsch., C, 2002, 57, 440-444
116	$C_{28}H_{24}O_{12}$ / 552	 Vitexine; 2``,-*O*-(4-hydroxybenzoyl)	Horowitz, R.M. et al., Chem. Ind. (London), 1966, 625
117	$C_{26}H_{28}O_{14}$ / 564	 Vitexine; 2``,-β-D-xylopyranosyl	Horowitz, R.M. et al., Chem. Ind. (London), 1966, 625
118	$C_{20}H_{18}O_6$ / 354	 Vitrofolal B	Kawazoe, K. et al., Phytochemistry, 1999, 52, 1657-1659
119	$C_{20}H_{18}O_5$ / 338	 Vitrofolal A	Kawazoe, K. et al., Phytochemistry, 1999, 52, 1657-1659
120	$C_{19}H_{16}O_6$ / 340	 Vitrofolal B;5-Demethoxy,7-Methoxy,4`-*O*-de-Me	Kawazoe, K. et al., Phytochemistry, 2001, 52, 1657-1659

121	$C_{19}H_{16}O_5$ / 324	 Vitrofolal B ;5-Demethoxy,7-Methoxy, 3-deoxy, 4`-*O*-de-Me	Kawazoe, K. et al., Phytochemistry, 2001, 52, 1657-1659
122	$C_{21}H_{18}O_6$ / 366	 Vitrofolal C	Kawazoe, K. et al., Phytochemistry, 1999, 52, 1657-1659
123	$C_{21}H_{16}O_6$ / 364	 Vitrofolal C; 7-Ketone	Kawazoe, K. et al., J. Nat. Prod., 2001, 64, 588-591
124	$C_{20}H_{36}O_3$ / 324	 Vitexifolin B	Ono, M. et al., J. Nat. Prod., 2002, 65, 537-541
125	$C_{22}H_{38}O_4$ / 366	 (5*S*,8*R*,9 *R*,10 *S*,13*S*,15*S*,16*R*)-9,13:15,16-Diepoxy-15,16-dimethoxy labdane	Ono, M. et. al,2001
126	$C_{22}H_{38}O_4$ / 366	 (5*S*,8*R*,9 *R*,10 *S*,13*S*,15*R*,16*S*)-9,13:15,16-Diepoxy-15,16-dimethoxy labdane	Ono, M. et. al,2001

127	$C_{22}H_{38}O_4$ / 366	 (5*S*,8*R*,9 *R*,10*S*,13*S*,15*R*,16*R*)-9,13:15,16-Diepoxy-15,16-dimethoxy labdane	Ono, M. et. al,2001
128	$C_{16}H_{22}O_8$ / 342	 3`,4`-Dihydro-xyphenyl butanone glucoside	Kouno, et al., 1988
129	$C_{30}H_{48}O_7$ / 520	 Ajugasterone C-20,22-monoacetonoid	Zhang et al., 1992
130	$C_{26}H_{38}O_{12}$ / 542	 Agnocastoside A	Kurruzum Uz et al., 2003
131	$C_{26}H_{40}O_{12}$ / 544	 Agnocastoside B	Kurruzum Uz et al., 2003

As flavonoids are the main subject matter of this portion an account of their types and biosynthesis are discuss in the proceeding section.

1.6 FLAVONOIDS

1.6.1 Introduction

Flavonoids are polyphenolic compounds, isolated from a wide variety of plants and is one of the most diverse and widespread groups of natural products. The name "flavonoid" is derived from Greek word "flavus" (yellow). These are biologically important active plant ingredients and about 2% of all the carbon photosynthesized is converted into flavonoids [22]. Flavonoids are the coloring co-pigments [23], which are also called anthoxanthins. The wide range of colors and shades in flowers are principally due to the presence of flavonoids. These usually occur as pigments in the cells of flower tissues [24].

Flavonoids occur in a variety of structural forms. All contain fifteen carbon atoms in their parent nucleus and share a common structural feature of two phenyl rings linked by a three-carbon chain (diphenyl propane derivatives). The compounds possessing a 1, 3-diphenyl-propane skeletons are regarded as chalconoids (Figure 1.1). The three carbon chain may be cyclized into a five or six membered ring through an oxygen atom with one of the pre-existing phenyl rings, generating a tricyclic system. The tricyclic compounds possessing a five membered heterocyclic ring, referred to auronoids, whereas those possessing a six membered heterocyclic ring are designated as flavonoids (Figure 1.1).

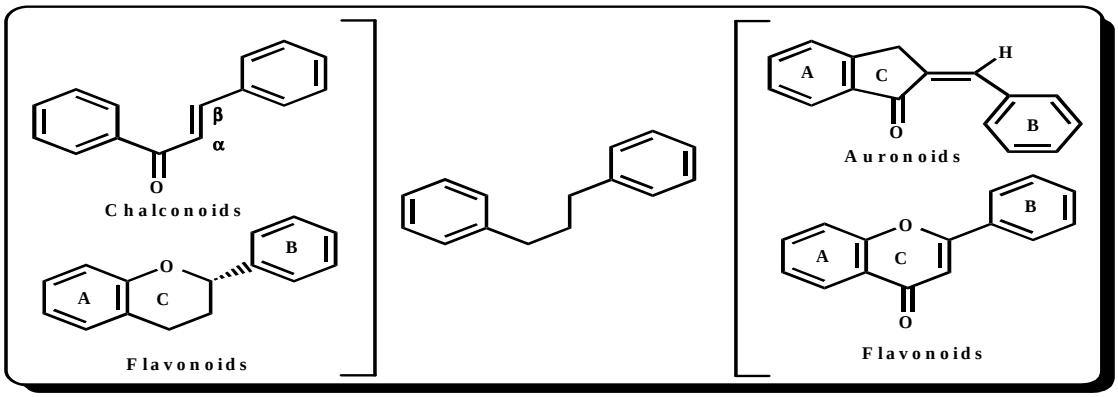


Figure: 1.1. Flavonoids having 1, 3-diphenylpropane skeleton

From 1, 2-diphenylpropane system the tricyclic compounds, isoflavonoids and 3-phenylcoumarins are derived (Figure 1.2), while those derived from 1, 1-diphenylpropane are called neoflavonoids (Figure 1.3).

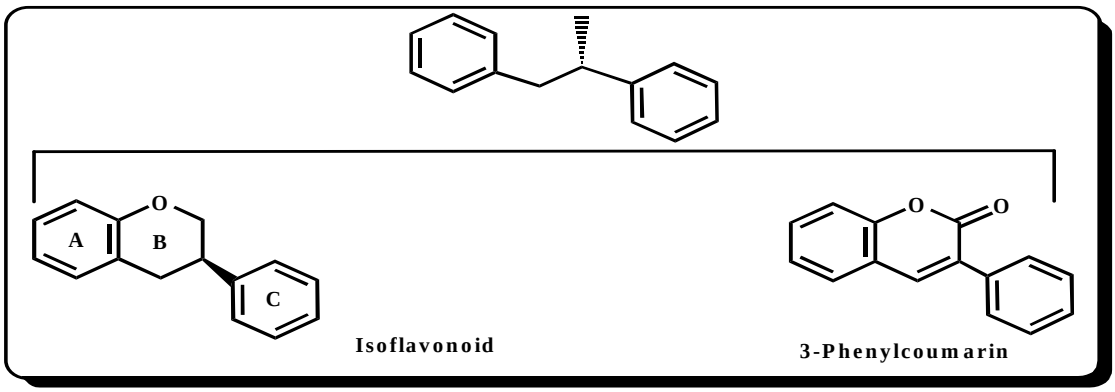


Figure: 1.2. Flavonoids having 1, 2-diphenylpropane skeleton.

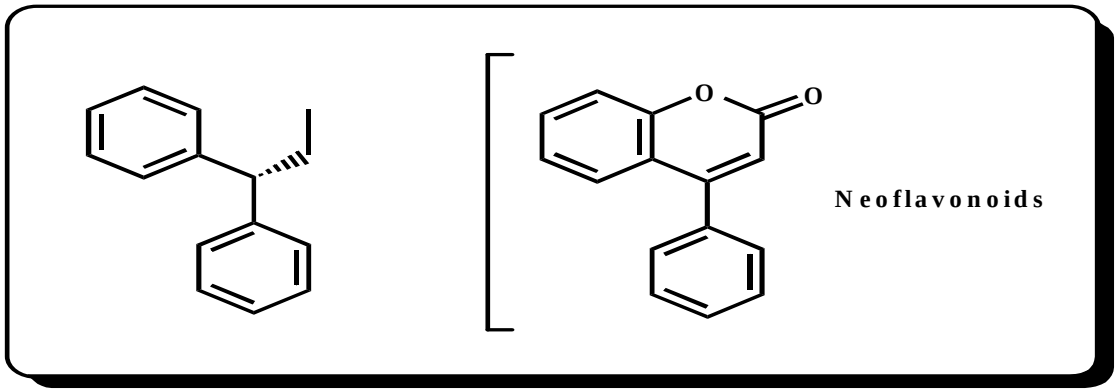


Figure: 1.3. Flavonoids having 1, 1-diphenylpropane skeleton

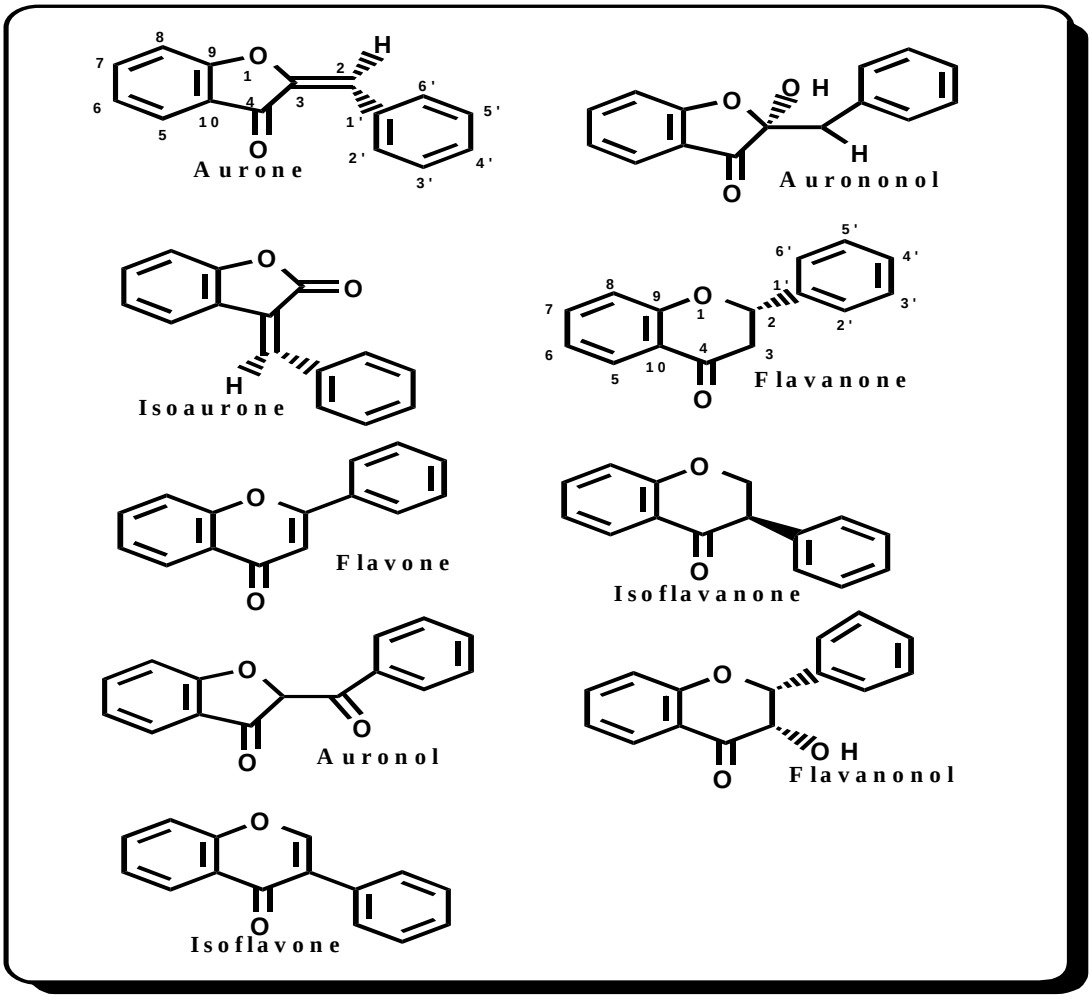


Figure: 1.4. Basic skeleton and numbering patterns in flavonoids

In flavonoid, auronoid and isoflavonoid types, the rings are labeled as A, B and C. The individual carbon atoms are referred by a numbering system which utilizes ordinary numerals for the ring A and C and primed numerals for the ring B (Figure 1.4).

The homo-flavonoids contain an additional carbon in their skeleton which is designated as C-11 (Figure 1.5). Most of the flavonoids occur as *O*-glycosides in which one or more of the hydroxyl groups are bind to a sugar(s) through an acid labile hemiactal bond. In flavonoid with *C*-glycosides, the link is acid resistant [25-26].

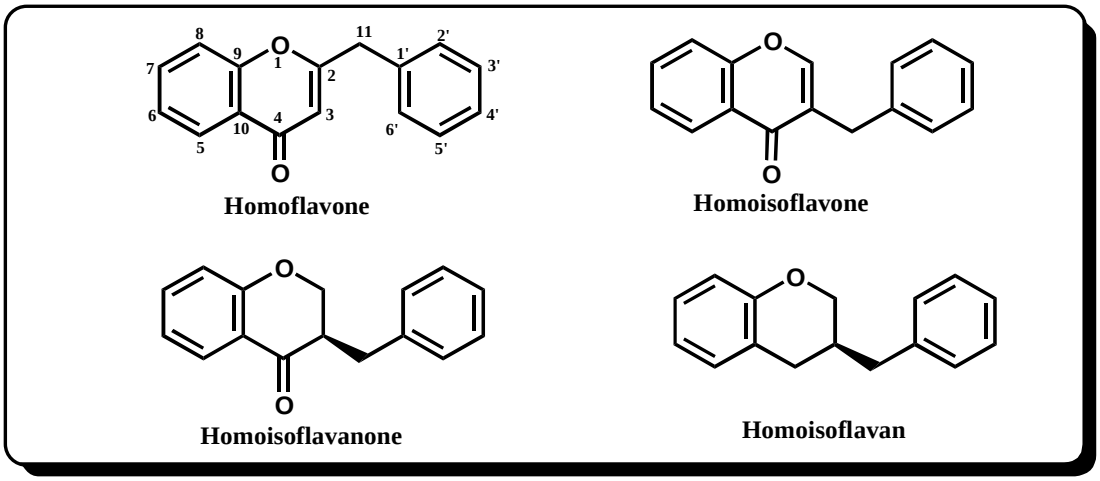


Figure: 1.5. Basic skeleton and numbering patterns in homoflavonoids

1.6.2 Medicinal Importance

A variety of classes of flavonoids have been screened for different physiological activities. No general function has yet been established for the flavones or flavonols in plants, although these are the most common and widely distributed flavonoids. On the other hand many functions in individual plants or plant groups have either been established or anticipated [27].

Willaman *et al*., [28] listed thirty three different manifestations of activity under the heading "Bioflavonoids" while reviewing the biological possessions of the flavonoids. Rutine and hesperidin (permeability factors or vitamin P) are used to cure various diseases like; diabetes, allergic manifestation, capillary fragility, capillary bleeding, increased and hypertention. Bioflavonoids isolated from the citrus have been used for the treatment of common cold [29]. The anti-protozoal activities of a number of flavonoids and chalcones have been reported [30]. Phenolic substances are well known to have anti-inflammatory activity. At non-toxic concentration, flavonoids like myrcetin and kaempferol-3-glucoside have an anti-HIV-I potency [31 - 32].

Medicinal properties of bioflavonoids are also affected by substitutions in position of hydroxyl group. The two *ortho* or *para* hydroxyl group, of flavonols, in the phenyl ring-C

have anti-oxidant properties, while at the 5, 7-positions the free hydroxyl group have a pro-oxidant effect [33]. Apigenin and genkwanin are supposed to have diuretic and anthelmintic properties, are present in the Chinese drug “Yuen-hua". Increasing the number of OH groups, these actions become more distinct. Flavones along with their glycosides play a vital role in the cardiac stimulation and vasoconstriction [33].

Some flavones have the ability to protect from anaphylactic shock, against X-rays and cure from frostbite [28]. The oesterogenic, insecticidal, anti-fungal and anti-bacterial activities of simple isoflavones and coumestans, rotenoids, isoflavonoid phytoalexins respectively, are reported [34]. Some minor flavonoids have very interesting anti-microbial, anti-fungal and cytotoxic properties [27]. Tricin plays an important role in the smooth muscle movement [35]. Anthocyanins, peonin and isorhamnetin, from *Chlamydomonas* are highly potent sex determining hormones [36]. Due to the lack of toxicity, flavones are suitable for further pharmacological investigation and as a result they have a number of therapeutic applications.

In fruits, vegetables and beverages, the flavonoid contents are measured, which is very important for the Dutch diet. The reported daily dietary intake of flavonoids is ~26 mg for Dutch population. Kaempferol, luteolin, quercetin, apigenin and myricitrin are the constituents of the flavonoid diet. The two compounds, quercetin (63%) and kaempferol (32%) make 95 % of total dietary flavonoids [37]. The polyphenolic contents in the plant based foods vary to a great extent even among cultivars of specie therefore, accurate informations are not available about the dietary intake on polyphenols. Environmental conditions and genetic factors are involved in variations of polyphenolic contents of the plants. Other factors which influence the plant phenolic contents are; germination, degree of ripening, variety, processing and storage [38].

Antioxidants, inhibit lipid peroxidation and other free radical-mediated processes, act as radical scavengers and thus protect the human body from several oxidative diseases. The phenolic antioxidants like flavonoids, tannins, coumarins, xanthenes, and recently, procyanidins found to show free radical scavenging activity in a dose-dependent manner and are viewed as potential therapeutic drugs for free radical pathologies [39].

Casticin (flavonoid), isolated from *Vitex agnus castus* (aerial parts), found to exhibit a significant inhibitory effect on monocyte oxidative burst in a dose dependent manner. At higher concentration it showed a significant suppressive effect against chemotaxic action on fMLP (10 (-8) m) stimulated neutrophils. A potent suppressive effect on PHA stimulated T-cell (PMBC) was shown by casticin [40]. Casticin also possesses a noticeable lipid peroxidation inhibitory effect [41].

Four new flavonoids; luteolin *6-C-(4"-methyl-6"-O-trans-caffeoylglucoside),* luteolin 6-C-(6"-O*trans-caffeoylglucoside),* luteolin *6-C-(2"-O-trans-caffeoylglucoside),* and luteolin 7-O-(6" – *p* - benzoyl glucoside) have been reported from *Vitex agnus castus* (root bark). Along with these other known flavones have also been reported from the root bark of the plant; 5, 4'-dihydroxy-3, 6, 7, Y-tetramethoxy-flavone, luteolin, artemetin and isorhamnetin [42]

The flavonoid glycosides have also a vital pharmacological role. 5-*O*-methyl-genistein-7-*O*-β-D-glucopyranoside isolated from *Ulex europaeus* L. posses anti-viral activity [43]. Similarly, prenyl-flavonol glycoside, epimedokoreanoside, isolated from the *Epimedium koreamum* (Berberidaceae) showed a hypotensive activity [44].

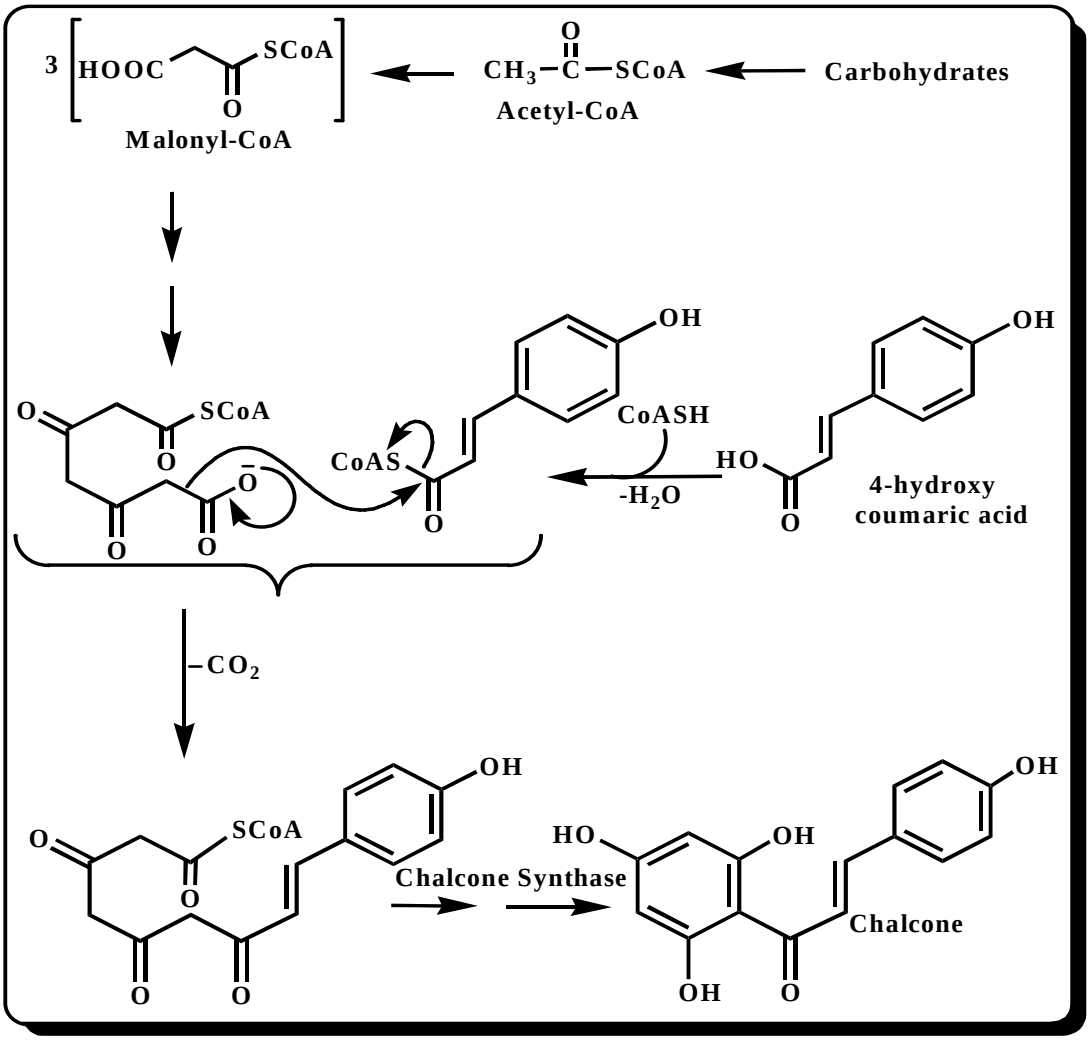
Bioflavonoids function as UV filters. They function as guard on the leaf from insect and microbial attack. They inhibit adhesion of blood platelets and block inflammatory effects. Quercetin, rutin and related bioflavonoids also act as heart stimulant [45].

1.6.3 BIOSYNTHESIS OF FLAVONOIDS

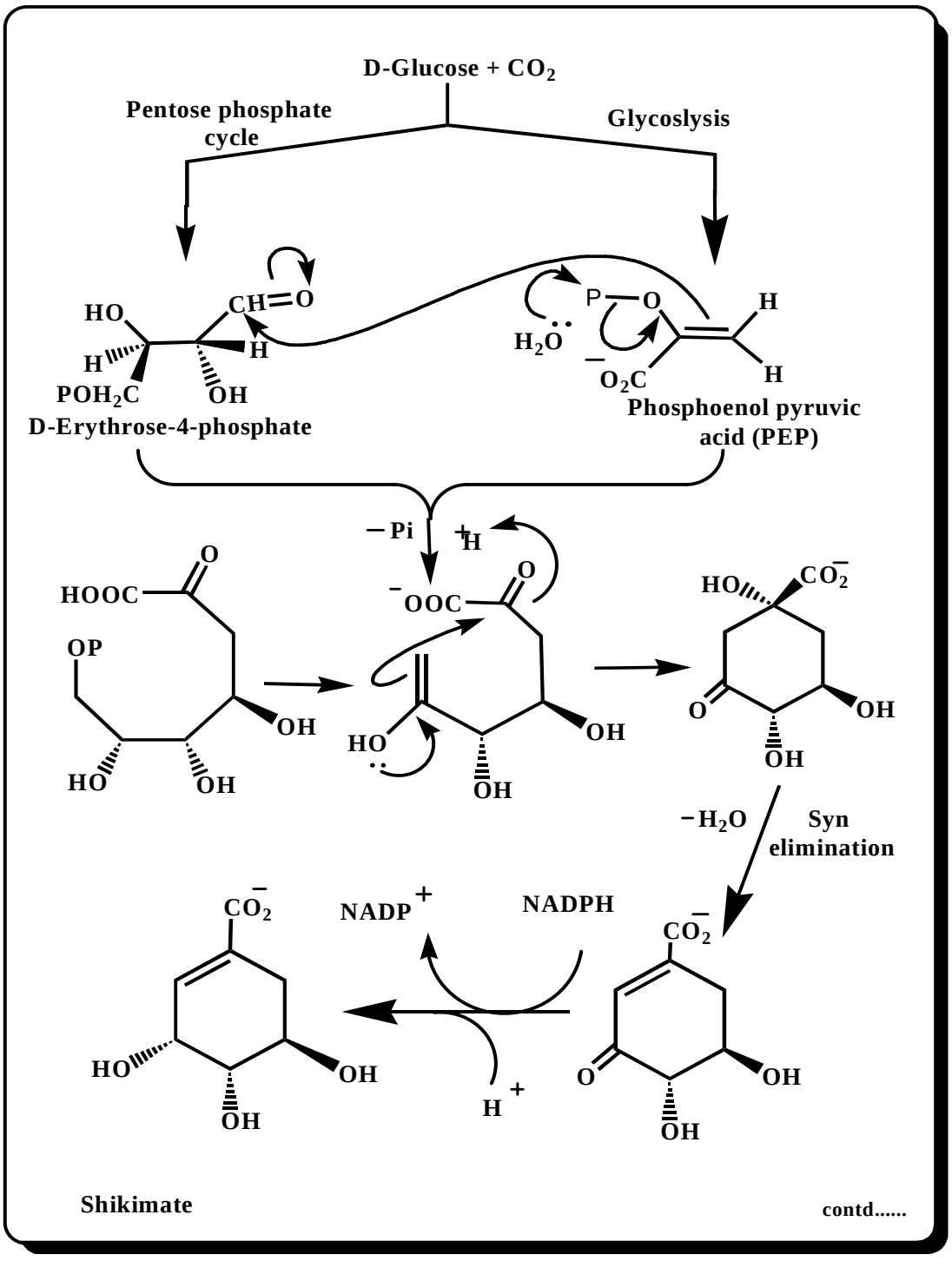
Flavonoids comprise a miscellaneous family of aromatic molecules, derived from Phenyl and malonyl-CoA (through fatty acid pathway). There are six major subgroups of flavonoids found in higher plants i.e. chalcones, flavones, flavonols, flavondiols, anthocyanins and condensed tannins. The aurones (7^{th} group) is widespread, but not common. Isoflavonoids, a specialized form of flavonoids, also synthesize by some of the leguminous and a small number of non-leguminous plant species. 3-deoxyanthocyanins, another class of flavonoids synthesized by few plant species i.e. *Sorghum bicolor*, *Zea mays* and *Sinningia cardinalis* [46].

1.6.3.1 Flavonoids Biosynthesis

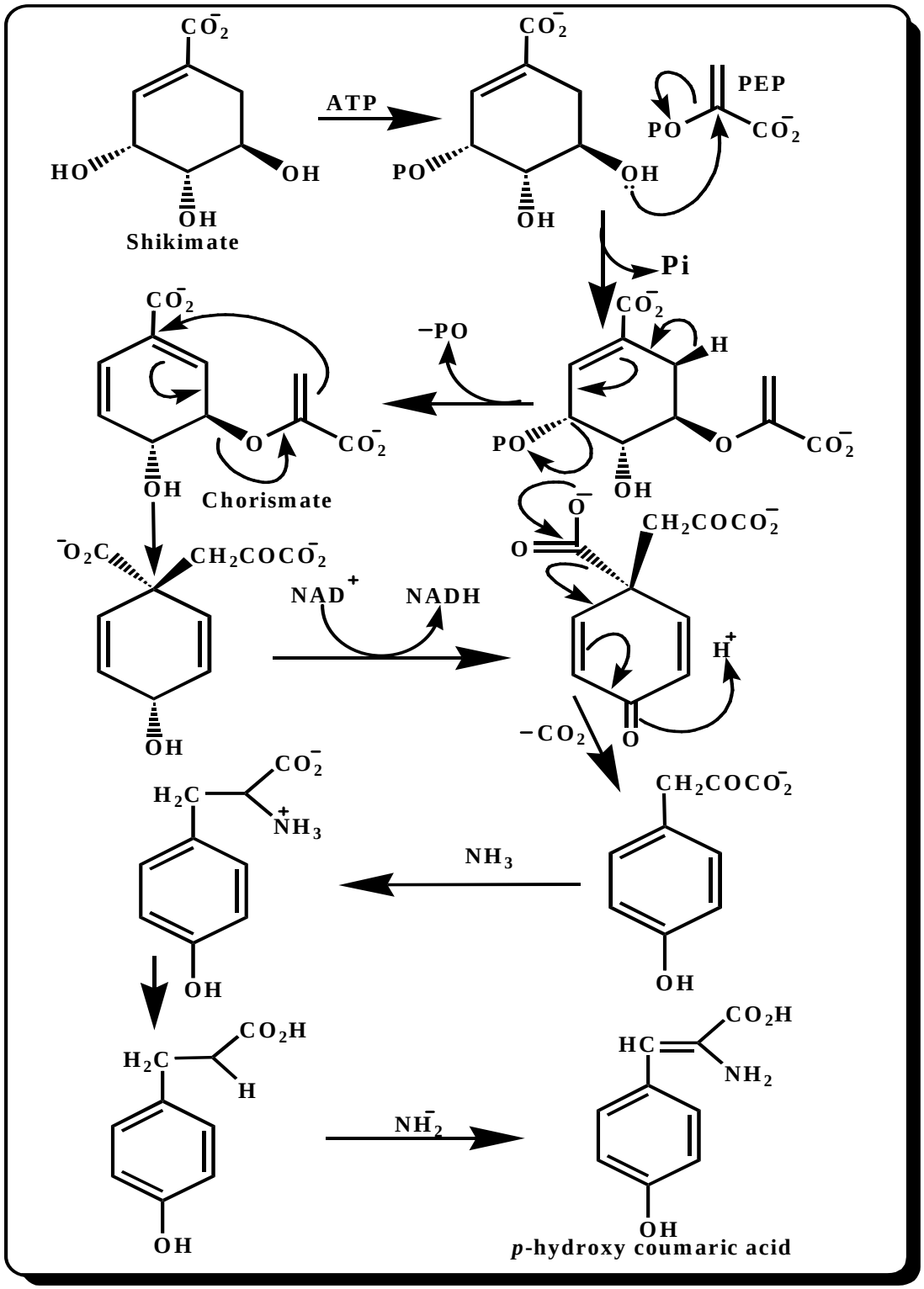
Chalcone, a central 15 carbon intermediates involved in the biosynthesis of all flavonoids and its role in the biosynthesis of flavonoids has been confirmed in a number of investigations [46-47]. The enzyme involved in biosynthesis of flavonoid is chalcone synthase, catalyzes formation of chalcone, for which the precursors are malonyl-CoA and 4-coumaroyl-CoA (hydroxycinnamic acid CoA ester), both of which are derived from carbohydrates. Malonyl-CoA is synthesized from an acetyl-CoA (glycolysis intermediate) and carbon dioxide, this reaction is catalyze by acetyl-CoA carboxylase **(Scheme 1),** while the 4-coumaroyl-CoA, synthesis of which is more complex and it involves the shikimate pathway **(Scheme 2)**, the main course to aromatic amino acid, phenylalanine and tyrosine in higher plants. The condensation of D-erythrose-4-phosphate and phosphoenolpyruvic acid is the starting point for the biosynthesis of shikimic acid **(Scheme 2)** [48].



Scheme 1: Biosynthesis of chalcones through malonyl-CoA [47].



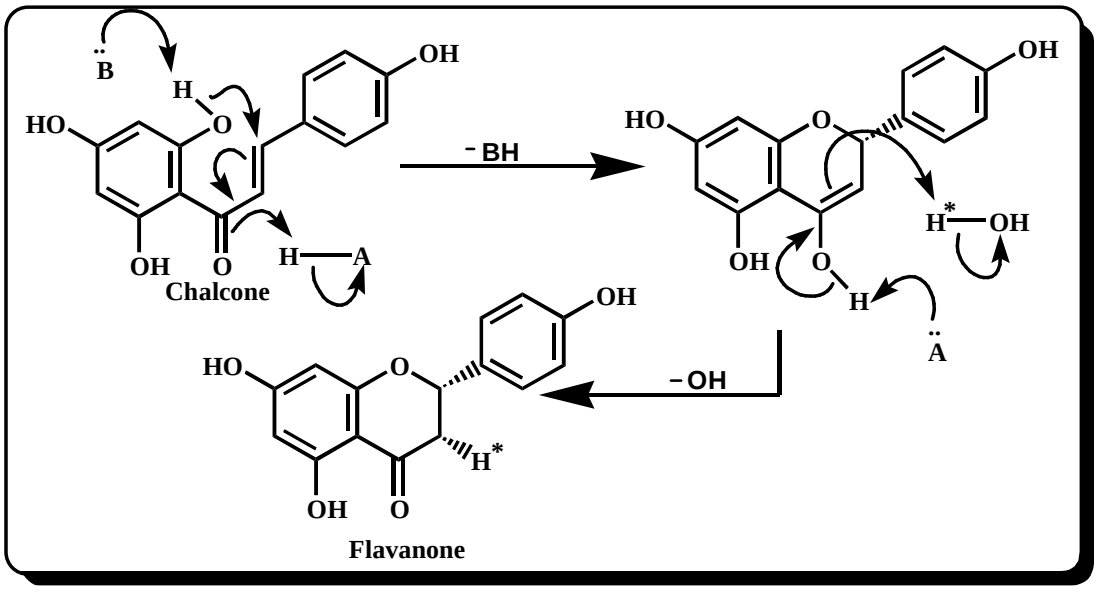
Scheme 2. Biosynthesis of *p*-hydroxy coumaric acid through Shikimate pathway [48]



Scheme 2. Biosynthesis of *p*-hydroxy coumaric acid through Shikimate pathway [48]

1.6.3.2 Flavanone Biosynthesis

Isomerization of chalcones result in flavanones and this interconversion is catalyzed by chalcone isomerase enzyme, *in-vivo*. There is a good evidence for the *in-vitro* and *in-vivo* existence of equilibrium between flavanones and the corresponding chalcones [48]. The stereospecificity of this enzymatic reaction is perceptible in the (*S*) chirality of C-2 in flavanone derivative. Therefore, it is not inadvertent that all the flavanones found in nature have the *(S)* pattern at C-2 and are levorotatory. In an aqueous solution, the equilibrium is rapidly and completely shifted to flavanone, when chalcones having at least two free hydroxyl groups at C-2 and C-6 **(Scheme 3)**. The interconversion rate and position of equilibrium is greatly influenced by the stabilization energy of strong H-bond between the carbonyl and *O*-phenolic hydroxyl groups. The system tends to remain in the open (chalcone) form when only 1 OH group is accessible, either for hydrogen bonding or cyclization [49].



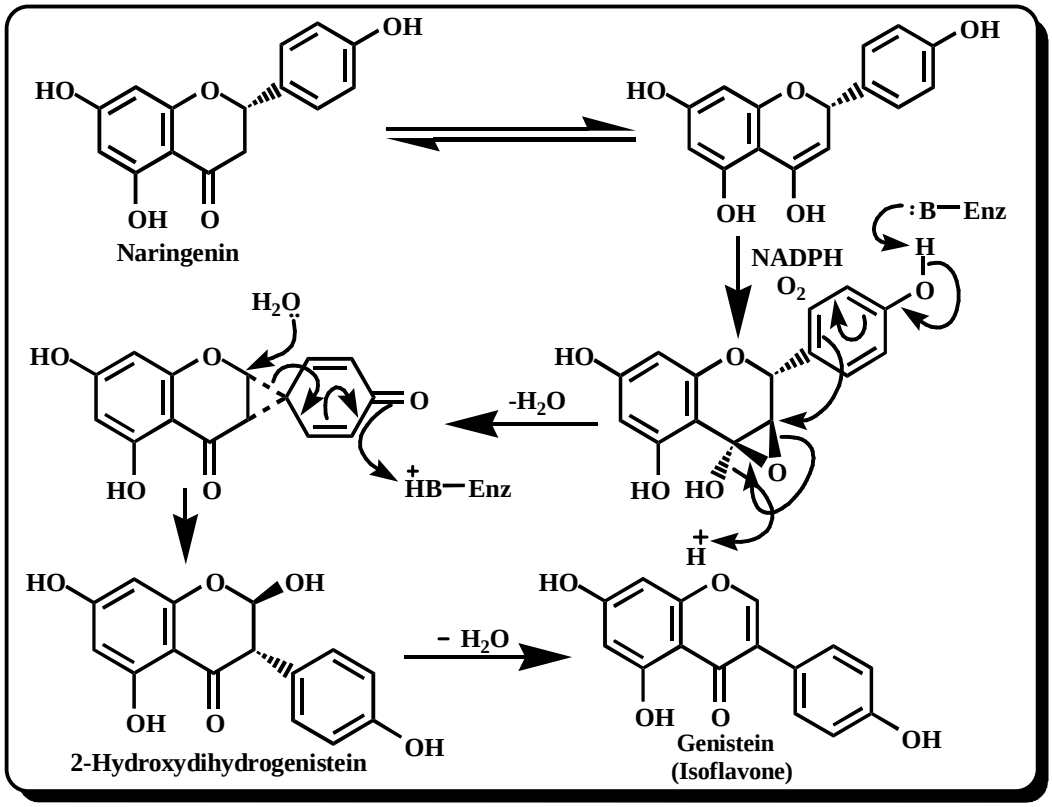
Scheme 3. Biosynthesis of flavanones.

1.6.3.3 Isoflavone Biosynthesis

The migration of 2, 3 aryl side chain of a flavanone intermediate (chalcone) is the key step in isoflavone formation (**Scheme 3)**. It was recently found that the microsomal preparations from elicitor challenged soybean cell suspension cultures, contains an enzyme, catalyzing the transformation of *(2S)*-naringenin (flavanone) into genistein (isoflavone) **(Scheme 4)**.

This transformation involve two enzymatic steps, the first step involve oxidation and rearrangement of naringenin to 2-hydroxy-2, 3-dihydrogenistein, strictly NADPH and molecular oxygen dependent. In the second step water is eliminated from 2-hydroxy-isoflavanone. The enzyme catalyzing this step has been isolated but has not yet been characterized [50].

An epoxidation step initiates the sequence of events. Protonation and succeeding cleavage of the epoxide would provide a positive charge to the ring B. Keto enol tautomerism, as indicated by proton swap at C-3 in flavanones [51], allows homoallylic interaction between C-3 and C-1′ and rearrangement of the structure then takes place. Addition of OH to C-2 leads to the 2-hydroxy isoflavanone intermediate, which is transformed to the isoflavone by eradication of water molecule.

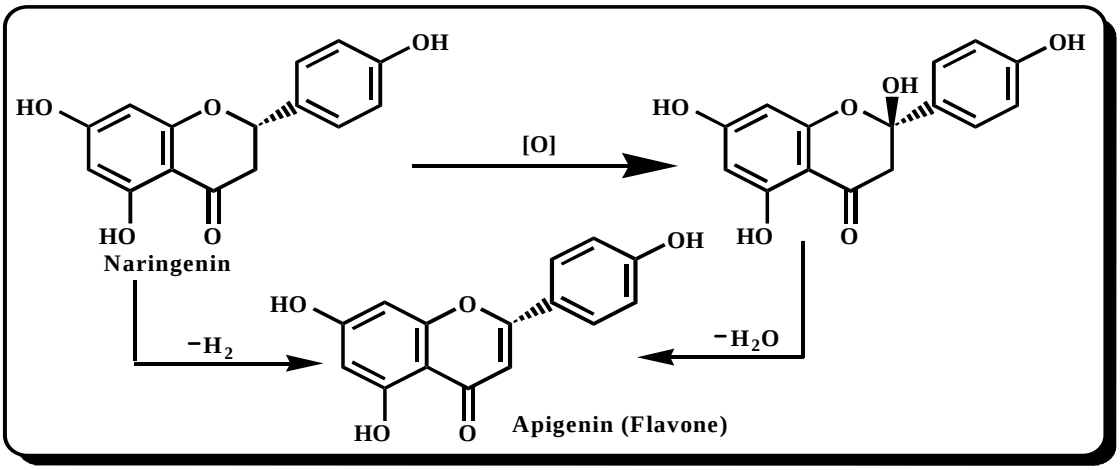


Scheme 4. Biosynthesis of isoflavones [50].

1.6.3.4 Flavone Biosynthesis

In parsley plants, for the first time the *in-vitro* conversion of flavanones to flavones was observed and this reaction was studied in parsley cell suspension cultures and *Antirrhinum* flowers, in more detail [52-53]. 2-oxoglutarate and Fe^{2+} are required for the parsley enzyme along with ascorbate as co-factors, due to this co-factors necessity the enzyme would be classified as 2-oxoglutarate dependent dioxogenase. The co-factor stimulates this enzyme and other 2-oxoglutarate dependent dioxygenases enzymes of the flavonoid pathway and it also exhibits a notable stabilizing effect on the enzyme activity [54]. Both, parsley and *Antirrhinum* flower enzymes catalyzed the conversion of *(2S)*-naringenin (flavanone) to apigenin (flavone) **(Scheme-5)**. The mechanism of double bond formation is still not clear. It has been suggested that 2-hydroxy flavanone is formed in the first step and water is then eliminated *via* a dehydratase [52, 53]. However, no such 2-hydroxy intermediate has yet

been isolated even with a nearly homogenous enzyme protein [54]. On the other hand, 2-hydroxy flavones certainly exist as plant metabolites and they are indeed, the substrates in *C*-glycosyl flavones formation [55].

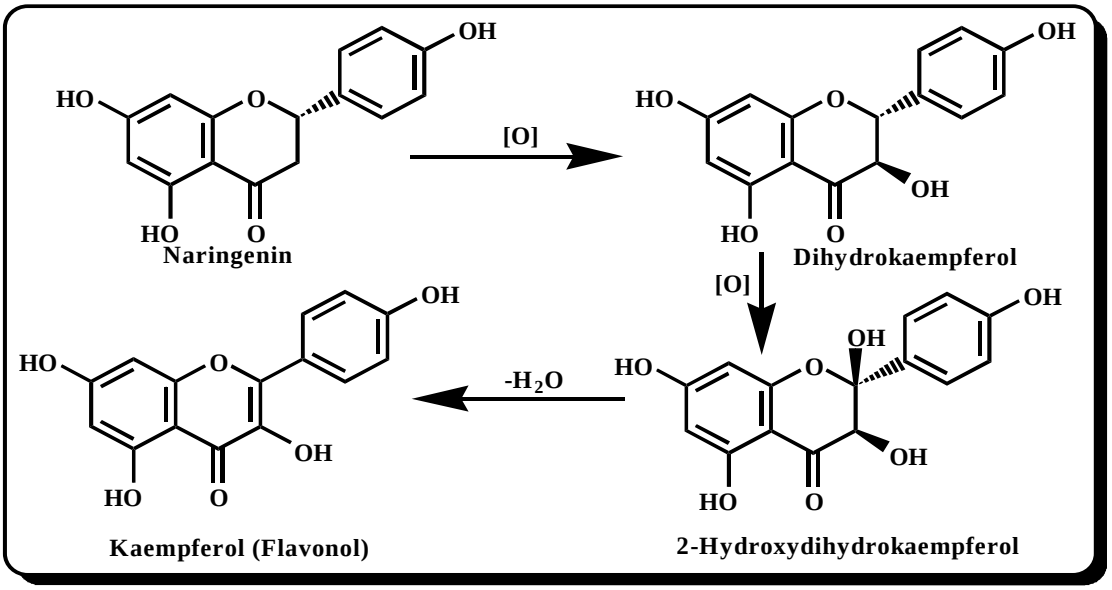


Scheme 5. Biosynthesis of flavones [52, 53].

1.6.3.5 Flavonol Biosynthesis

During preparation of enzyme from parsley cell suspension cultures, the enzymatic conversion of dihydro flavonols to flavonols was observed for the first time [52]. Flavonols synthesis was found to be catalyzed by a soluble 2-oxoglutarate reliant oxygenase.

Flavonol synthesis, most probably, proceeds *via* a 2-hydroxy intermediate such as; 2-hydroxy dihydrokaempferol with succeeding dehydration, giving rise to the particular flavonols [56] **(Scheme 6)**. In flower extracts of *Matthiola* and *Petunia*, flavonol synthase has also been identified [57-58]. Like parsley, flavonol biosynthesis is catalyzed by a soluble 2-oxoglutarate dependent dioxygenase, in these flowers.



Scheme 6. Biosynthesis of flavonols [56].

1.6.3.6 Glycosylation of Flavonoids

In nature an enormous number of flavonoid glycosides are present which indicates the occurrence of a range of glycosyltransferases with varying substrate specificities [59-60]. During enzyme preparations from tulip anthers, *Pisum* flowers, *Chrysosplenium* shoots and *Anethum* cell cultures, the novel flavonol *O*-glycosyltransferases enzymes were demonstrated [61 - 64]. These enzymes display a distinct specificity with regard to the substrate, position and sugar transferred. Similarly, high specificity was observed for isoflavone 7-*O*-glucosyltransferase, isolated from *Cicer* [65]. From *Chrysosplenium* extracts the enzymes were isolated which glycosylate the B-ring of highly methoxylated flavonols in the 2′ and 5′ positions [66]. A meticulous motivating situation has been found during extensive genetic and biochemical studies of the glycosylation of isovitexin (6-*C*-glucosylapigenin) in *Silene paratensis* and *Silene dioica.* Eleven functional alleles, spread over six loci, have now been identified. These codes for different glycosyltransferases which catalyzed glucosylation, galactosylation and xylosylation of the 7-hydroxyl group or glucosylation, rhamnosylation, xylosylation and arabinosylation of the 2-hydroxyl group of the carbon bound glucose of isovitexin [67].

1.6.3.7 Methylation of Flavonoid

Transfer of methyl group (methyl transferase) from *S*-adenosyl methionine to various OH groups of flavonoid substrates is catalyzing by various enzyme [68]. Some of these enzymes need Mg^{+2} as obligatory co-factor. *S*-Adenosyl homocysteine, formed in the reaction, is an inhibitor of these enzymes; an 8-hydroxyflavonol-8-*O*-methyltransferase was reported from *Lotus corniculatus* flowers [69-70]. *Lotus* flowers also contain 3- and 3′-*O*-methyltransferases, which have been shown to catalyze the methylation of the flavones (luteolin) and flavonols (quercetin, myricitrin and isorhamnetin) in the relevant positions [70].

1.7 Aims and Objectives

Based on the reported literature, traditional and medicinal importance of *Vitex agnus castus* and *Myrsine africana*, we set our objective for the present work as following;

1. ***Biological / Pharmacological investigations***

The crude methanolic extracts and various fractions of the selected plants will be screen, *in-vitro* for possible biological / pharmacological activities; antibacterial, antifungal, insecticidal, anti-termite, phytotoxicity, brine shrimp lethality, haemagglutination, anti-inflammatory, effects on rabbit's jejunum preparations (spasmolytic activity), enzyme (chymotrypsin and urease) inhibitory and nitric oxide free radical scavenging assay.

2. ***Phytochemical investigations***

a. ***Screening for different groups of compounds.*** After screening the crude methanolic extracts and various fractions of the selected plants for the above mentioned biological/pharmacological activities, the crude methanolic extracts of the plants will be screened for occurrence of various groups of natural products like; alkaloids, flavonoids, tannins and saponins.

b. ***Isolation of bioactive compounds / constituents.*** Various fractions of these plants will be subjected to column chromatography for isolation of bioactive compounds. The structures of the fair quantity of isolated pure chemical constituents will be determined by modern instrumental techniques; UV, IR, GC/MS, EI/MS, FAB/MS, ESI/MS, HR-EIMS, ^{1}H-NMR, ^{13}C-NMR, COSY, NOSY, HMBC, HMQC and X-ray crystallography (when applicable).

c. ***Biological / pharmacological screening of isolated compounds.*** The isolated compounds (fair quantity) will also be screened for different biological activities like chymotrypsin and urease inhibitory and anti-inflammatory activities.

2.0 EXPERIMENTAL

2.1 General Experimental Conditions

All biological, pharmacological, chemical and instrumental analysis were carried out at the Centre for Biotechnology and Microbiology (COBAM), University of Peshawar, International Centre for Chemical and Biological Studies (ICCBS) Karachi, University of Karachi and department of Pharmacy, University of Malakand. Analytical (Merck) and commercial grade chemicals were used while performing different experiments.

2.1.1 Physical Constants

For determination of melting points Buchi 535 apparatus and JASCO DIP-360 digital Polari meter was used for measuring optical rotations of the compounds.

2.1.2 Spectroscopy

UV spectra were recorded on Hitachi UV 3200 spectrophotometer in methanol. IR spectra were recorded on a JASCO A-302 IR spectrophotometer in $CHCl_3$. The EI-MS were recorded on a double focusing mass spectrometer (Varian MAT 311 A) coupled with PDP 11/34 computer system. Jeol JMS HX-110 mass spectrometers was used to measure HR-EI-MS and FAB (positive and negative) mass. Bruker AMX-400 and AMX-500 MHz instruments were used to measure the ^{1}H-NMR spectra, while ^{13}C-NMR spectra were measured at same instruments at 75, 100, 125 and 150 MHz. Multiplicities of carbon signals were determined by using DEPT 90^{o} and 135^{o} experiments. COSY 45^{o} experiments were used to determine the homonuclear ^{1}H-^{1}H connectivities. HMQC experiments were done to determine one-bond ^{1}H-^{13}C connectivities. For the determination of two and three-bond ^{1}H-^{13}C connectivities HMBC experiments were performed. The coupling constants (*J*) were measured in Hz from ^{1}H-NMR chemical shifts, reported in δ (ppm).

2.1.3 Isolation and Purification of Compounds

Various chromatographic techniques, for isolation and purification of different components of plant extract, were employed.

2.1.3.1 Column Chromatography

In column chromatography the stationary phase was silica gel-GF_{254}, 60 of E. Merck (Art. 7734, 70-230 mesh) while different organic solvents (*n*-hexane, chloroform, di-chloromethane, acetone, ethyl acetate, methanol) were used as mobile phase.

2.1.3.2 Thin-layer Chromatography (TLC)

For performing preparative TLC, preparative pre-coated silica plates (20x20 cm, 0.5 mm thickness, E-Merck $PF_{254,}$ Type 60) were used while pre-coated silica plates were used to perform TLC (PF_{254}, Merck, 0.25 mm).

2.1.4 Spot Locating Reagents

Different locating reagents were used to visualize the spots on TLC plates. These were; ceric sulphate-sulphuric acid, vanillin-phosphoric acid, iodine solution and dragendorff's reagents.

2.1.4.1 Vanillin-Phosphoric Acid

Vanillin (1g), dissolved in aqueous phosphoric acid **(**50 %**)** [71]. Steroids and terpenes gave intense purple and blue or light pink color, respectively, after spraying the vanillin-phosphoric acid and subsequent heating at 100-110^{o}C. Steroidal and Terpenoidal glycosides also gave pink color after spray and heating.

2.1.4.2 Ceric Sulphate-Sulphuric Acid

For visualizing the spots of oxidizable substances, saturated solution of Ceric Sulphate in sulphuric acid (65%) [71] was used as spray on TLC plates. Pink color, upon heating reveals the presence of terpenoids, while light yellow or blackish color without heating reveals the presence of alkaloids.

2.1.4.3 Dragendorff's Reagent

(i) Potassium iodide (8 gm) dissolved in 20 ml of distilled water.

(ii) Basic bismith nitrate (0.85 gm) was dissolved in a mixture of acetic acid (10 ml) and water (40 ml).

(iii) (i) and (ii) were mixed in 1:1 which gave stock solution.

(iv) 5 ml from the stock solution was dilute with 10 ml of acetic acid and 90 ml of distilled water [71]. Light pink, light brown, dark brown or blackish color indicate the presence of alkaloids and light yellow to light pink appearance upon spraying indicate the presence of highly oxygenated terpenoids and steroids.

2.1.4.4 Iodine Solution

In this test, few iodine crystals were placed in TLC tank and warmed for few minutes at temperature rage 40-50^{o}C. Spots will appear when the TLC plates are placed inside the TLC tank [71].

2.2 PHYTOCHEMICAL INVESTIGATIONS

2.2.1 Plant material

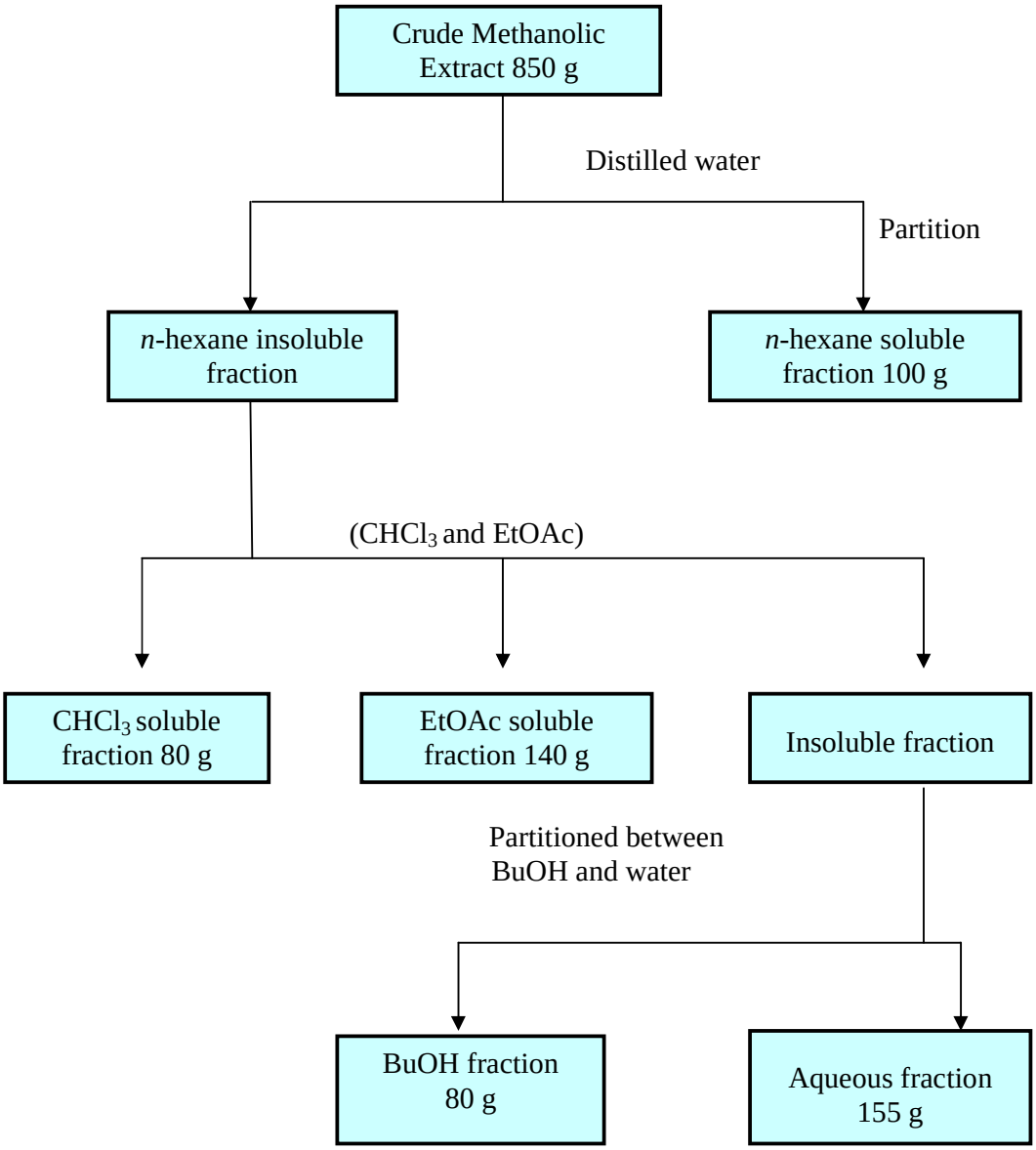
Vitex agnus castus (*Verbenaceae*), aerial parts (leaves and stem) were collected from Hazara division, Khyber Pakhtunkhwa, Pakistan, in December 2007- January 2008 and were identified by Professor Dr. Habib Ahmad, Plant Taxonomist, Hazara University, Khyber Pakhtunkhwa, Pakistan.

2.2.2 Extraction

The shade dried plant material was chopped into small pieces and ground to fine powder using electric grinder. The powdered plant material of *Vitex agnus castus* (9 kg) was soaked in commercial grade methanol for 15 days at room temperature with occasional shaking. After 15 days of soaking, methanol soluble material was filtered off. All filtrates were combined and concentrated, under vacuum at 40°C using rotary evaporator, blackish crude methanolic extract (900 g) was obtained.

2.2.3 Fractionation

The crude methanolic extract (850 g) of *Vitex agnus castus* was suspended in distilled water (400 ml) and partitioned with *n*-hexane (3 x 400 ml), $CHCl_3$ (3 x 400 ml), EtOAc (3 x 400 ml) and BuOH (3 x 400 ml) to yield; *n*-hexane (100 g), $CHCl_3$ (80 g), EtOAc (140 g), BuOH (80 g) and aqueous (155 g) fractions (**scheme 7)**. About 50 g of crude methanolic extract was reserved for other pharmacological/biological screenings.



Scheme 7. Fractionation of methanolic crude extracts of *Vitex agnus castus*.

2.2.4 Screening of crude extract for Different classes of Compounds

By refluxing the plant materials, in the soxhelet apparatus with hydrous methanol, extracts were obtained.

Alkaloids

Crude methanolic extract (2.5 g) in HCl (5 ml, 2N) was heated on boiling water bath till it dissolved. The mixture was then filtered, cooled and divided into four parts. These parts were treated as follow:

Mayer's reagent was used to treat one part of the mixture and Wagner's reagent was used to treat other parts of the mixture. Precipitation or turbidity in the test sample(s) was then observed. A (+) score was recorded for slight opaqueness, (++) for definite turbidity without flocculation and (+++) for a definite turbidity along with heavy floccules or precipitates [72].

Flavonoids

Dissolve 1 g of plant material in 5.0 ml methanol then treated with few drops of concentrated HCL and 0.5 g of magnesium. The appearance of pink or magenta red colors within 3 minutes indicates the presence of flavonoids in the sample [73].

Saponin

Plant material (2.5 g) was extracted with boiling water and then allowed to cool to room temperature followed by vigorous shaking to form the froth and then allowed for 15-20 minutes to stand and the results were recorded as:

No forth (-) negative, froth less than 1cm (+) weakly positive, froth up to 1.2 cm and more than 2 cm (++) positive and (+++) strongly positive, respectively [74, 75].

Tannins

Plant material (1 g) extracted with methanol was further partitioned with hot normal saline solution (10 ml). The saline extract was filtered off and divided into three equal parts. Each portion was treated as following:

Sodium chloride solution was added to the first portion, serve as blank.

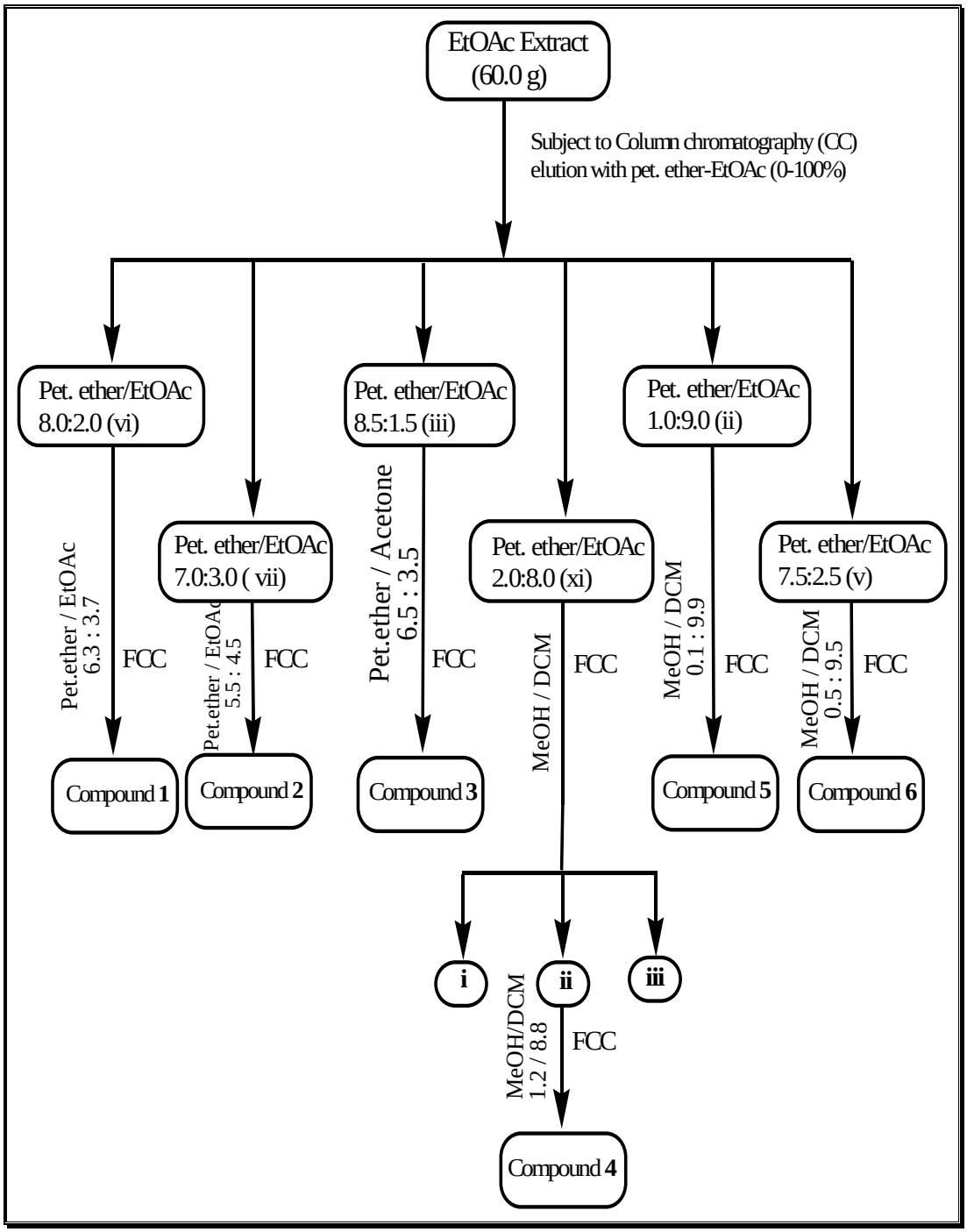
Gelatin (1%) and gelatin-salt solution were added to second portion and third portion, respectively. Tannins presence was indicated by formation of precipitate in second or in both second and third portions. Presence of tannins was further confirmed by the appearance of characteristic blue-black, blue, blue-green or green color, that was precipitated by adding ferric chloride ($FeCl_3$) solution to the test sample(s) [76].

2.2.5 Compounds isolated from the *Vitex agnus castus*

Ethyl acetate (EtOAc) fraction (60 g) of *Vitex agnus castus* was subjected to column chromatography and sequentially sub-fractionated in increasing order of polarity *n*-hexane / EtOAc (9.5/0.5 (i), 9.0/1.0 (ii), 8.5/1.5 (iii), 8.0/2.0 (iv), 7.5/2.5 (v), 7.0/3.0 (vi), 6.0/4.0 (vii), 5.0/5.0 (viii), 4.0/6.0 (ix), 3.0/7.0 (x), 8.0/2.0 (xi), 9.0/1.0 (xii) and (xiii) 100 % EtOAc).

From the above, sub-fractions were then selected and subjected to Flash Column Chromatography (FCC) and isolate the following compounds [**Scheme 8**]. Before subjecting the samples to FCC different solvent systems (EtOAc / *n*-hexane, acetone / *n*-hexane and MeOH / DCM) were checked for good separation on TLC plate.

Compound **1** was obtained from the sub-fraction (vi) eluting with solvent system *n*-hexane: EtOAc (6.3:3.7). Compound **2** was isolated from sub-fraction (vii) eluting with solvent system of *n*-hexane:EtOAc (5.5:4.5). Compound **3** was afforded from the sub-fraction (iii) by subjecting to FCC eluting with solvent system of *n*-hexane:acetone (6.5:3.5). The sub-fraction (xi) was subjected to FCC and further fractionated to different fractions (i, ii and iii) eluting with solvent system of MeOH and DCM in increasing order of polarity. The compound **4** was afforded from the fraction ii eluting with solvent system of DCM:MeOH (8.8:1.2). Compound **5** was obtained from sub-fraction (ii) eluting with solvent system of DCM:MeOH (9.9:0.1). Similarly compounds **6**, was isolated from sub-fraction (v), subjecting to FCC and eluting with solvent system of DCM:MeOH (9.5:0.5).



Scheme 8. Isolation of compounds from EtOAc fraction of *Vitex agnus castus*.

2.2.6. CHARACHTERIZATION OF COMPOUNDS

2.2.6.1. Characterization of Vitexcarpan (1)

Yield: 9 mg from EtOAc fraction.

Physical state: white crystals.

M.P: 123-128 ^{o}C

M. wt: ($C_{20}H_{20}O_4$) 324 a.m.u.

$[\alpha]_D^{25}$: 17.230 (c = 0.26, MeOH)

UV (MeOH) nm (log ε): 309, 291 and 286.0 nm

IR ($CHCl_3$) V_{max} cm^{-1}: OH (3361), C ═ C (1609) and C − O (1138).

EIMS *m/z* (%): 324 (100), 268 (94), 251(06), 211 (08), 197 (10), 147 (35), 134 (20), 91 (18), 55 (25).

^{13}C-NMR (C_3D_6O, 100 MHz): δ:159.7 (C-10), 158.9 (C-8a), 157.4 (C-4a), 155.8 (C-13a), 133.1 (C- 12), 125.0 (C-6), 118.2 (C-12a), 117.0 (C-6a), 113.0 (C-13b), 110.4 (11), 109.1 (C-5), 103.9 (C-9), 79.3 (C-12b), 93.9 (C-4), 79.1 (C-11a), 74.5 (C-3), 67.2 (C-7), 40.8 (C-6b), 32.5 (C-2), 26.9 (C-14), 26.9 (C-15), 17.6 (C-1).

^{1}H-NMR (C_3D_6O, 400 MHz): See table 3.2

HMBC: See figure 3.2

NOESY: See figure 3.1

COSY: See figure 3.1

2.2.6.2. Characterization of Vitexcarpan 1 (2)

Yield: 14 mg from EtOAc fraction.

Physical State: White crystals.

M.P: 121-125 ^{o}C

M. wt: ($C_{16}H_{12}O_5$) 284 a.m.u.

$[\alpha]_D^{25}$: 40.76 (c = 0.6, MeOH)

UV (MeOH) nm (log ε): 309, 291 and 286.0 nm

IR (KBr) V_{max} cm^{-1}: OH (3382), C═C (1622) and C−O (1138)

EIMS *m/z* (%): 284 (100), 241 (10), 215 (15), 197 (18), 162 (35), 134 (40), 115 (20), 77 (20), 69 (45).

^{13}C-NMR (CD_3OD, 100 MHz): δ:157 (C-9), 153.3 (C-10a), 149.5 (C- 4a), 147.0 (C-3), 143.8 (C-2), 133.0 (C-7), 114.0 (C-6b), 112.4 (11b), 110.0 (C-8), 105.8 (C-10), 105.1 (C-1), 93.9 (C-4), 79.1 (C-11a), 66.9 (C-6), 41.0 (C-6a), 102.1 (OCH_2O).

^{1}H-NMR (CD_3OD, 400 MHz): See table 3.3

HMBC: See figure 3.4

NOESY: See figure 3.3

COSY: See figure 3.3

2.2.6.3. Characterization of Vitexoide (3)

Yield: 11 mg from EtOAc fraction

Physical State: White powder.

M.P: 98-102 ^{o}C

M. wt: ($C_{24}H_{40}O_4$) 393 a.m.u.

$[\alpha]_D^{25}$: - 74.8 (c = 0.20, MeOH)

IR (KBr) V_{max} cm^{-1}: 3517, 1008 (OH), 1721, 1669 (C =O).

EIMS *m/z* (%): 392.0 (35), 374.2 (05), 356.2 (14), 302.2 (04), 273.2 (22), 255.2 (32), 201.2 (06), 159.1 (08), 121.1 (18), 93.1 (36), 55.0 (100).

FAB +ve *m/z*: 392.0

HR-ESI-MS *m/z*: Pseudo M^+ peak at *m/z* 410.3300; corresponding to formula $C_{24}H_{40}O_4$ (Cacld. for $C_{24}H_{40}O_4$ +NH_4 =410.3270).

^{13}C-NMR (C_3D_6O, 100 MHz): δ: 178.3 (C-24), 74.1 (C-16), 72.6 (C-3), 57.1 (C-17), 49.3 (C- 9), 48.2 (C-14), 43.7 (C-5), 42.6 (C-13), 39.0 (C-7), 37.3 (C-12), 37.3 (C-23), 37.2 (C-4), 36.4 (C-1), 36.3 (C-15), 35.3 (C-10), 34.8 (C-8), 31.1 (C-20), 29.9 (C-22), 28.6 (C-2), 28.4 (C-6), 24.8 (C-11), 23.7 (C-21), 17.5 (C-18), 13.2 (C-19).

^{1}H-NMR (C_3D_6O, 400 MHz): See table 3.4

HMBC: See figure 3.5

COSY: See figure 3.6

NOESY: See figure 3.7

2.2.6.4. Characterization of Compound (4)

Yield: 60 mg from EtOAc fraction.

Physical State: White powder.

M. wt: ($C_{19}H_{26}O_3$) 466 a.m.u.

M.P: 156-159 ^oC.

UV (MeOH) nm (log ε): 217, 275.

IR (KBr) V_{max} **cm^{-1}:** 3421, 1704, 1636, 1450

EI-MS *m/z* (%): 224.2 (3), 166.1 (6), 148.1 (12), 138.0 (46), 121.0 (100), 94.1 (73), 73.0 (34), 65.0 (61).

ESI-MS *m/z*: 950.3311, 484.1885, 449.1510, 123.1204.

^{13}C-NMR (CD_3OD 150MHz): δ:167.8 (C-1”), 163.7 (C-5”), 142.9 (C-8), 141.7 (C-3),132.9 (C-3”), 132.9 (C-7”), 132.4 (C-7), 122.1 (C-2”), 116.3 (C-4”), 116.3 (C-6”), 105.5 (C-4), 100.2 (C-1’), 97.9 (C-1), 82.9 (C-6), 78.3 (C-3’), 77.9 (C-5’), 74.7 (C-2’), 71.5 (C-4’), 63.7 (C-10), 62.7 (C-6’), 48.6 (C-9), 46.3 (C-5),

^{1}H-NMR (CD_3OD, 600 MHz): See table 3.5

HMBC: See figure 3.8

NOESY: See figure 3.9

2.2.6.5. Characterization of Compound (5)

Yield: 35 mg from EtOAc fraction.

Physical State: White crystals

Mp: 122 – 123^{o}C

UV (CD_3OD) nm (log ε): 228 (3.62), 272 (3.77), 300 (4.01) nm.

IR (KBr) υ_{max} cm^{-1}: 3260 - 2610 (COOH), 1705 (C=O).

HREIMS *m/z*: 122.0367 (calcd. for $C_7H_5O_2$, 122.0360).

^{1}H-NMR ($CDCl_3$, 400 MHz): δ 11.92 (1H, s, O-H), 8.12 (2H, t, J= 8.5Hz, H- 2, 6), 7.60 (1H, t, J = 8.5 Hz, H-4), 7.46 (2H, t, J = 8.5 Hz, H- 3, 5).

^{13}C-NMR ($CDCl_3$, 400 MHz): 181.0 (C-7), 134.5 (C- 4), 131.1 (C-1), 130.7 (C-2, 6), and 129.7 (C-3, 5).

2.2.6.6. Characterization of Compound (6)

Yield: 20 mg from EtOAc fraction.

Physical State: White crystals.

MP: 122-123 ^{o}C

UV (MeOH) nm (log ε): 285 (3.22), 326 (3.01).

HR-EIMS *m/z*: 148.9701 (calcd. For $C_9H_8O_2$, 128.9703).

EIMS *m/z* (%): 148 (70), 131 (70), 103 (68), 91 (50), 77 (75), 63 (40), 51 (100).

^{1}H-NMR ($CDCl_3$, 300 MHz): δ: 7.52 (1H, 15.9 Hz, H-1`), 6.2 (1H, d, 15.9 Hz, H-2`), 7.36 (m), 7.29 (m) and 7.21 (m).

^{13}C-NMR (CDCl3, 75 MHz): δ: 125.2 (C-1), 115.9(C-2), 117.0 (C-5), 122 (C-6), 124.6 (C-4), 115.5 (C-3), 175.0 (C-1`), 123.5 (C-2`), 156.0 (C-3`),

2.3 PHARMACOLOGICAL INVESTIGATIONS

2.3.1 ANTIBACTERIAL ACTIVITY

Various decoctions of plants materials, in folk medicine, are used to treat infections. These plant materials are used for the treatment of wounds or for relief from burns in the form of poultice(s) or household remedies. Infectious diseases cause approximately one half of all deaths, especially, in the tropical regions of the world [77]. We performed our screening against different pathogens while keeping in view their clinical significance.

Materials

Escherichia coli, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella typhi*, *Enterobacter aerogenes*, *Bacillus Pumilus*, *Klebsella pneumoniae*, *Streptococcus pneumoniae* and *Staphylococcus epidermidis* species were used, available at Centre of Biotechnology and Microbiology, University of Peshawar. Organic solvents used in the experiments were of analytical grade. Others materials include nutrient broth, sterile cork borers, petri dishes (14 cm), micropipettes, incubator, standard antibiotics (amoxicillin), test samples (plant Cr. Ext. and fractions).

Procedure

The crude extract and fractions of the plant were screened for possible antibacterial activity against the above mentioned pathogens. The experiment was performed by following steps as per our reported procedure [78].

1. Nutrient broth medium was prepared, poured into sterile test tubes and incubate for 24 hours at 37 ^{o}C to check its sterility.
2. After incubation for 24 hours, the test organisms were inoculated to the sterile nutrient broth and incubated for 24 hours at 37 ^{o}C.
3. Nutrient agar medium was prepared on the same day of the experiment and poured into sterile petri dishes. The plates were incubated overnight for sterility test at 37 ^{o}C.

4. After sterility test of the medium, test cultures (18-24 hours old) from the nutrient broth were transferred and evenly distributed over the plates under the laminar air flow.
5. After introducing the test culture to plates, wells (6 mm) were made with a sterile borer.
6. Sterile DMSO (< 1%) was used for preparation of stock solutions (test samples) at a concentration of 3 mg/ml.
7. From the stock solution, 100 µl of the test samples was poured into the respective wells, using a sterile micropipette.
8. Amoxicillin was used positive control in DMSO while DMSO less than 1% was used as negative control. For better diffusion of the test samples to media the plates were allowed undisturbed for two to three hours inside the laminar air flow.
9. The zones of inhibitions were noted after overnight incubation at 37 ^{o}C.

After recording the zone of inhibitions, we proceed the experiments to determine the MIC_{50} according to the procedure of Banso [79] with slight modifications. Following steps were carried for determining the MIC_{50}.

1. Nutrient broth medium was prepared, poured 4 ml of medium into each sterile test tube and incubate for 24 hours at 37 ^{o}C to check the sterility.
2. On the next day 18-24 hours old culture of the test pathogen was inoculated to the test tubes containing sterilized medium.
3. From the stock solution, prepared for the determination of zone of inhibition, 300, 500, 700 and 900 µl were poured to the test tubes containing test pathogens.
4. Results were recorded after 24 hours of incubation at 37 ^{o}C.

2.3.2 ANTIFUNGAL BIOASSAY

Materials

Test fungi: *Aspergillus niger, Aspergillus flavus, Penicillium notatum, Fusarium oxysporum, Triticum harzianum* and *Rhizopus stolonifer,* Sabouraud Dextrose Agar (SDA), autoclave, incubator, micropipettes, magnetic stirrer, dimethyl sulfoxide (DMSO), screw test tubes, standard antifungal drugs such as Miconazole and test samples (Cr. Ext. and fractions).

Procedure

The anti-fungal activity of the test samples was performed as per our reported procedure [78]. Stock solutions (24 mg/ml) of Cr. Ext. and fractions were prepared in DMSO.

Sabouraud dextrose agar (SDA) medium, for the growth of fungal specie, was prepared as per our reported procedure [78]. 4 ml aliquots and 66.6 µl from the stock solution were transferred to screw test tubes that were sterilized using moist steam sterilization method. The test tubes were allowed to cool in slanting position. The final concentration became 400 µg/ml of SDA. Fresh culture of each specie was inoculated to test tubes. Other test tubes supplemented with DMSO and standard drugs were used as negative and positive control, respectively. All the test tubes were incubated for 3-7 days at 27±1ºC. On day 7^{th}, the visible non mycelial linear growths (mm) of the microorganisms were measured and percent linear growth inhibitions of fungal strains were calculated by comparison with reference drug.

2.3.3 INSECTICIDAL ACTIVITY

Material

Test insects (*Tribolium castaneum, Rhyzopertha dominica* and *Callosobruchus analis*), organic solvent (methanol), growth chamber, test sample, standard insecticidal drug (Permithrine), petri dishes (9 cm diameter), micropipette (1000 µl), filter paper, glass vials and brush.

Preparation of Test Sample

200 mg of the plant's crude methanolic extracts and various fractions were dissolved in 3 ml of volatile solvent for preparation of the stock solution.

Rearing Technique

The stored grain pests were reared in plastic bottles containing breeding media (sterile), under controlled conditions required for the rearing of pests. The insects, selected for the experimental work, were of uniform size and age.

Procedure

The contact toxicity assay [80] was performed as following:

On first day, the filter paper was cut, equal in size and placed it in petri dishes of about 9 cm or 90 mm in size. Sample solutions of the crude methanolic extracts and fractions were loaded to the petri dishes using a sterile micropipette. The plates were left overnight to evaporate the organic solvents from the samples completely.

After complete evaporation of organic solvents from the petri dishes, on second day, 10 healthy insects small in size from each species were selected and transfered with the help of a clean brush to each plate including test samples and control. The plates containing samples and insects were incubated for 24 hours at 27°C in growth chamber with 50% relative humidity.

After incubation, the results were recorded by counting the number of survived insects in each plate. The percent mortality of insects treated with the test samples were calculated according to the formula:

$$\text{Percentage Mortality} = 100 - \frac{\text{No. of insects alive in test}}{\text{No. of insects alive in control}} \times 100$$

Control

Standard insecticidal drug (Permithrine) was used as positive control while the organic volatile solvent (methanol) was used as negative control.

2.3.4 ANTI-TERMITES BIOASSAY

Materials

The plant materials were screened against termites according to the procedure of salihah et al., [81]. Termite culture, test samples (crude methanolic extract, EtOAc and aqueous fractions), blotting paper, sterile petri dishes, desiccators and magnifying glass with stand were used during the experimental work.

Procedure:

The experiment was performed according to the following steps:

1. Blotting paper and petri dishes were sterilized before using in the experiments.
2. Blotting paper was cut uniformly according to the base size of the petri dishes.
3. Stock solutions (2 mg/ml) of the plant materials were prepared in respective solvents.
4. The blotting paper was soaked in solution of the test samples and leave for some time at room temperature to evaporate the excess solvent and then place them in respective petri dish.
5. 25 termites were transferred to each petri dish and placed the plates in desiccators containing water at the base to maintain the humidity.
6. The plates were observed after 24 hours, till all of the termites were dead.
7. Negative controls (MeOH and EtOAc) were also run along with test samples.

All the experiments were performed three times and the average termites killed each day were noted.

2.3.5 **PHYTOTOXIC ACTIVITY**

Materials

The test samples (Cr. Met. Ext. and fractions) were tested for phytotoxic bioassay against *Lemna minor*. Micro-pipettes, flasks, growth chamber, test samples and organic solvent (methanol) were used in the experiments.

Procedure

The plant extracts were screened for phytotoxic bioassay according to McLaughlin protocols [82]. The experiment was performed by following steps.

1. Stock solutions (20 mg/ml) of the test samples were prepared in methanol and E-medium was prepared for the growth of *L. minor*.
2. From the stock solution, test sample at concentration of 10, 100 and 1000 µg/ml were poured to flasks and left at room temperature to evaporate the organic solvent.
3. After evaporation of the organic solvent, 20 ml of the E-media was added to all flasks.
4. Sixteen healthy *L. minor* plants were selected, with a rosette of three fronds and put in respective flasks.
5. The flasks containing *L. minor* and E-media were incubated at $27\pm1^{o}C$ in growth chamber for seven days. Results were noted after seven days of incubation.

2.3.6. BRINE SHRIMP LETHALITY BIOASSAY

Materials

Artemia salina, sea salt, shallow rectangular plastic dish, pasture pipette, syringes; 5.0 ml, 0.5ml, 100µl and 10µl, glass vials, magnifying glass. Organic solvents (methanol, dichloro-methane, chloroform, DMSO), double distilled water, aluminum foil and test sample (crude extract and fractions).

Procedure

Artemia salina (brine-shrimp eggs) was used to determine the cytotoxicity of the plant materials as per our reported procedure [83]. The eggs of brine shrimp (*Artemia salina*) are easily hatched when placed in the sea water. A shallow rectangular plastic dish of 22x32 cm in size containing the artificial sea water prepared with a commercial salt mixture (Instant Ocean, Aquarium System, Inc., Mentor, OH, USA) and double distilled water. A perforated device was used for unequal partition in the plastic dish. To the larger compartment, which was darkened, eggs of about 50 mg were sparged. The smaller compartment of the plastic dish was kept open to ordinary light. This whole system was kept at room temperature for 48 hours and allowed the eggs to hatch and mature then using a Pasteur pipette the nauplii were collected from the lighten side. From the stock solution (20 mg/2 ml) of the test samples 5, 50 and 500 µl were transfer to vials (3 vials/concentration) with final concentration of 10, 100 and 1000 µg/ml, respectively. The vials were allowed over-night or placed in the hood for half an hour to evaporate the organic solvents completely. 1 ml of sea water was added to each vial and placed 10 larvae per vial with the help of Pasteur pipette. The final volume of each vial was 5 ml, adjusted with sea water. The vials were incubated under illumination at 26±1ºC for 24 hours. Other vials were supplemented with respective organic solvent as negative control and reference cytotoxic drug as positive control. After incubation period the survived shrimps were counted using a magnifying glass.

2.3.7 HEAMAGGLUTINATION ACTIVITY

Materials

Materials required for heamagglutination are; all blood group samples, KH_2PO_4, Na_2HPO_4, test tubes, centrifuge, incubator and phosphate buffer (pH 7.0).

Procedure

Haemagglutination activity of test samples was performed according to procedure of Naqvi et al., [84]. Phosphate buffer (pH 7.0) was prepared by dissolving 0.453g of KH_2PO_4 and 0.47g of Na_2HPO_4 each in 50 ml of distilled water. The dissolved KH_2PO_4 and Na_2HPO_4 were mixed in ratio of 3:7 (V/V). Stock solution (1 mg/ml of DMSO) was prepared and different dilutions i.e. 1:2, 1:4, 1:8 and 1:16 were made in phosphate buffer, from the stock solution. Fresh blood samples, from healthy persons, were collected on the same day of the experiment and centrifuged, the plasma was discarded and red blood cells were collected for the next steps. 2% RBC's suspension was prepared in the phosphate buffer. From each dilution 1 ml of sample was taken in a test tube and then adds 1 ml of the RBC's suspension to the sample. Incubate the test tubes for 30 minutes at 37^oC. After incubation the test tubes were examined for the button formation, indicating positive results.

2.3.8 IN VITRO ANTI-INFLAMMATORY ACTIVITY

Inflammation, a self-protective retort, induces physiological adaptations to remove pathogenic infections and reduce tissue damage [85]. Reactive oxygen species (ROS) are formed following to the assemblage and activation of NADPH oxidase (phagocyte-specific enzyme). The process initiates, during respiratory burst of non-mitochondrial oxygen uptake by NADPH oxidase system, by production of superoxide anion (O^{-2}) [86].

A water soluble tetrazolium salt (WST-1) was used in this study for the measurement of superoxide production by neutrophils which will be activated by opsonized zymosan, induces phagocytic activation of neutrophils. This is a more sensitive and consistent technique among other existing techniques [87].

Isolation of human neutrophils

A modified method of Siddiqui et al., [88] was employed for the isolation of human neutrophils. Briefly; from healthy volunteers fresh heparinized blood was collected and diluted with an equal volume of modified Hank's solution (MHS) at pH 7.4. The upper leukocyte layer was collected, after leaving at room temperature for 20 minutes, layered over Ficoll paque. The pellets obtained after centrifugation (1500 rpm) were resuspended with MHS. The cells obtained were counted using an improved Neubaur chamber. Trypan Blue method was employed to determine the viability of cells, was above 97%.

Respiratory burst assay

A modified assay [89] was used to determine the anti-inflammatory activity of the test samples. This *in-vitro* assay was based on the reduction of WST-1 in the presence of activated neutrophils. A total volume of 200 µl MHS (pH 7.4), containing neutrophils (1.0-104 /ml), WST-1 (250 µM) and test samples (various concentrations) was used to determine the anti-inflammatory activity. Neutrophils, buffer solution and WST-1 were added to the control. At 37°C the samples were equilibrated and by addition of opsonized zymosan A (15

mg/ml), prepared by mixing with human pooled serum, the reaction was initiated. The pellet obtained after centrifugation (3000 rpm) was resuspended in PBS buffer. The absorbance of the reaction mixture was determined at 450 nm. For positive control the non-steroidal anti-inflammatory drugs like aspirin and indomethacin were used which are widely used for the treatment of several inflammatory diseases. IC_{50} values of were measured with reference to the DMSO (blank) and expressed as percent inhibition of the production of superoxide anions. The percent inhibitory activity showed by plant extracts was determined against blank and calculated as following:

% Inhibition = 100 - {(OD test compound/OD control) x100}.

2.3.9 EFFECTS ON RABBIT'S JEJUNUM PREPARATIONS

Our present studies were done on isolated rabbit's jejunum tissues to explore the plant extract for possible spasmolytic activity.

Drugs, standards and solution preparation

Analytical grade chemicals, Acetylcholine (BDH Chemicals, Poole, England), Atropine and rest of the chemicals (E. Merck, Germany) were used in the experiments. On the same day of the experiments all solution(s) were freshly prepared in distilled water. Kobayashi et al., [90] method was used for the preparation of Normal Tyrode solution (Table 2.1).

Stock solutions of Acetylcholine (10^{-2} µM) and KCl were prepared. The acetylcholine solution was diluted upto 10^{-4} µM by serial dilution of the stock solution and final concentration of the KCl solution was made 80 mM in bath solution. The sample (crude methanolic extract) solution was prepared in distilled water by suspending 300 mg/ml and diluted upto 30 mg/ml and 3 mg/ml by diluting serially the stock solution.

Table 2.1 Composition of Tyrode solution used.

Normal Tyrode's Solution Constituents	**Constituents in gram / liter**
NaCl	8.0 gm
KCl	0.2 gm
$MgCl_2$	0.1 gm
NaH_2PO_4	0.5 gm
Glucose	1.0 gm
$NaHCO_3$	1.0 gm
$CaCl_2$	0.2 gm

Animals and data recording

Rabbits either male or female (local breed; weight in Kg 1.0-1.4) were used in the experiments. The rabbits were maintained at the "Animal House of University of Malakand" according to the standards mentioned in the Animals (Scientific Procedures) Act 1986, UK and "Animals Bylaws 2008 of the University of Malakand (Scientific Procedures Issue-1)". All of the rabbits were fed with standard diet and tap water. They were given only water, 24 hours prior to start of the experiments. The tissue responses were noted by Teaching Force Transducer (Model No: MLT 0210/A Pan Lab S.I.) attached with Power lab (Model No: 4/25 T) AD Instruments, Australia. Range for recording the data was 20 mv, Low pass 5 Hz x 10 gain using input 1 at rate 40/s.

Rabbit's jejunum preparations

The tissue preparation for the experiments was carried out through following procedure [91].

1. The rabbits maintained at animal house were sacrificed and the jejunum portion(s) of the rabbits were isolated. The carbogen gas (5% CO_2 and O_2 mixture) was used to aerate these isolated tissues placed in tyrode's solution. The constituents and their concentrations (mM) used in tyrode's solution were: KCl 2.68, NaCl 136.9, $MgCl_2$ 1.05, $NaHCO_3$ 11.90, NaH_2PO_4 0.42, $CaCl_2$ 1.8 and glucose 5.55.
2. 1.5 cm pieces of jejunum, maintained in the tyrode's, were mounted in 10 ml tissue bath maintained at 37^{o}C in tyrode's solution aerated with carbogen gas.
3. Pressure (one gram) was put on over tissue. Earlier, by giving the sub-maximal doses of acetylcholine (0.3 μM) to the tissue to keep the tissues undisturbed and stabilized for an equilibrium period of 30 minutes and to produce a reproducible response (normal response).

4. The plant crude methanolic extract at concentration(s) of 0.01, 0.03, 0.1, 0.3, 1.0, 5.0 and 10 mg/ml were tested for the possible spasmolytic activity throughout experiments.

Spasmolytic activity

The highly concentrated K^+ solution (80 Mm) was used to treat tissues in order to depolarize it and to get them in position of sustained concentration [92]. The test samples were then applied on those pre-treated tissues mounted in tissue bath, in cumulative manner, to obtain a dose response curve and their relaxation was articulated as percent of the K^+ induced contractions [93].

2.3.10 NITRIC OXIDE FREE RADICAL SCAVENGING ASSAY

Sodium Nitroprusside in aqueous solution at physiological pH, spontaneously generate nitric oxide, to produce nitrite ion. These nitrite ions on reaction with sulphanilic acid produce a *p*-diazonium salt. Napthylethylenediaminedihydrochloride (0.1% w/v) on reaction with *p*-diazonium salt, form a pink complex of Azo dye and the absorbance of the pink chromophore is measured at 570 nm.

Requirements

96-well microtiter plate reader (UV region), 96-well microtiter plate, multi-chamber micropipettes, test samples (crude extracts and its various fractions), Sodium Nitroprusside (10 mM) (Sigma Co), sulphanilic acid (0.33% in 20% Acetic acid) (Sigma Co), [N-(1-Napthyl) Ethylenediaminedihydrochloride] (0.1% in H2O) (Sigma Co)), Phosphate buffer (10 mM) (pH = 7.4), Vitamin C as positive control and DMSO as negative control.

Procedure:

The NO free radical scavenging activity was performed by following the procedure.

Stock solution (3 mg/ml) of the test samples was prepared in DMSO. From this stock solution, by dilution, different concentration of the test samples like 0.3, 0.6, 0.9, 1.2 and 1.5 mg/ml were prepared. 10 µl from each concentration of the test sample were taken in microtiter plate and add 20 µl of phosphate buffer to it. Then add 70 µl of sodium Nitroprusside. The microtiter plate is then incubated at 20-25^{o}C for one and half hour. The plate is well shaked after incubation and adds 50 µl of sulphanilic acid. Take the absorbance (pre-absorbance) at 570 nm. After pre-reading add 50 µl of [N-(1-Napthyl) Ethylenediaminedi-hydrochloride], shake well and take the final reading. Similarly vitamin C and DMSO were run as positive control and blank, respectively.

2.3.11 ENZYME INHIBITORY ASSAYS

2.3.11a Chymotrypsin Inhibitory Activity

The chymotrypsin inhibitory activity of the compounds was performed by following method [94]. Chymotrypsin (9 units/ml of 50 mM Tris–HCl buffer pH 7.6; Sigma Chemical Co. USA) was preincubated with the compounds at 25^{o}C for 20 minutes. Substrate solution (1 mg *of N*-succinyl-phenylalanine-*p*-nitroanilide/ml of 50 mM Tris–HCl buffer, pH 7.6) of 100 µl was added to start the enzymatic reaction. The *p*-nitroaniline released during the reaction, was continuously monitored by measuring the absorbance at 410 nm until we achieved a significant change in color. In the reaction mixture the final concentration of DMSO was 7.0%. The percent inhibitory activity for the compound was found out using the formula; $(E - S) / E \times 100$

Where *E* and *S* are the activity of the enzyme without and in the presence of test compound, respectively.

2.3.11 b Urease Inhibition Assay

The urease inhibition activity was performed according to the procedure of Akhtar et al., [95]. A solution containing 25 µl of Jack bean Urease, 100 mM urea and 55 µl of buffer, were incubated with 5 µl (0.5 mM conc.) of the test compounds for 15 min at 30^{o}C in a 96 well microtiter plate. For the determination of urease inhibition activity, release of ammonia was measured using indophenol. To each well the alkali reagents (70 µl, 0.5% w/v sodium hydroxide and 0.1% NaOCl) and phenol reagents (45 µl, 1 % w/v phenol and 0.005 % w/v sodium nitroprusside) were added. After 50 minutes a microplate reader (Molecular Device, USA) was used to measure the increasing absorbance at 630 nm. The results were processed using Soft-Max Pro software (Molecular Device, USA) after measuring change in absorbance per minute. The experiments were performed in triplicate at pH 8.2 (0.01 M

$K_2HPO_4.3H_2O$, 1.0 mM EDTA and 0.01 M $LiCl_2$). The percent inhibition activity was calculated using formula:

$$100 - (OD_{\text{test well}} / OD_{\text{control}}) \times 100.$$

The standard inhibitor thiourea was used as positive control.

3.0 RESULTS AND DISCUSSION

3.1 PHYTOCHEMICAL INVESTIGATIONS

3.1.1 Screening for Different Groups of Compounds

The presence of different groups of natural products, produced by plants such as Flavonoids, Alkaloids, Tannins and Saponins etc. has directly linked with the medicinal values of a particular plant. These natural products are used as medicines for treatment of various diseases, after isolation and formulation, in various pharmaceutical dosage forms.

The plant *Vitex agnus castus* was screened for occurrence of various groups of natural products. The results for the phytochemical screening are shown in table 3.1; reveal that alkaloids give positive result with confirmation of precipitate formation, settled down at the bottom. The development of pink color was observed in test for flavonoid, confirms that the plant also contains flavonoid group of natural products. Result for saponins was also positive by the forth formation of about 1.2 cm as observed. Negative test result was observed for tannins showing the absence of tannins in the plant.

Table 3.1 Phytochemical screening for *Vitex agnus castus*

S. No.	TEST	Results	Remarks
1	Alkaloids	+	Positive
2	Flavonoids	++	Positive
3	Saponins	+	Positive
4	Tannins	-	Negative

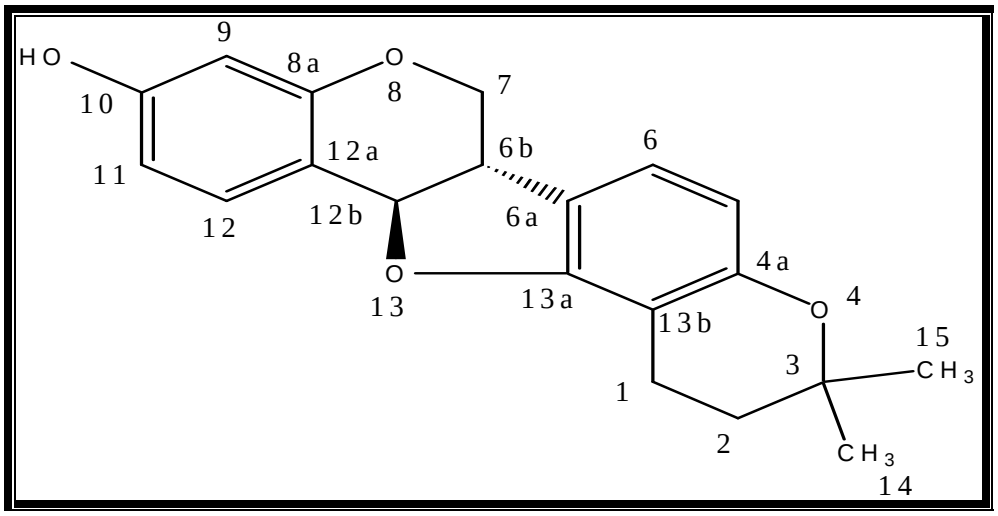
3.1.2 Structural Elucidation of Isolated Compounds

3.1.2.1 Structural Elucidation of Vitexcarpan (01)

The Vitexcarpan (01) was obtained as a white crystal from sub-fraction (vi) (8.0/2.0 Pet. Ether/EtOAc) eluting with solvent system of 6.3/3.7 (Pet. Ether/EtOAc) on silica gel column, of the EtOAc fraction of the *Vitex agnus castus*. Its optical rotation, {$[\alpha]_D^{25}$ = +17.230 (MeOH, c = 0.26)} indicated the presence of chiral center in molecule. The HREI-MS displayed M^+ at 324.1460 which is consistent with the molecular formula $C_{20}H_{20}O_4$ (calcd for $C_{20}H_{20}O_4$ = 324.1452) with eleven degrees of unsaturation. The IR max (KBr) cm^{-1}; 3461(O-H stretches), 1609 (C=C stretches) and 1138 (C–O stretches) compared with reported values [96].

The ^{1}H-NMR spectrum (table 3.2) showed four proton signals at δ 3.51 (1H, dd, $J_{7ax,7eq}$ = 10.4 Hz, $J_{7ax,6b}$ 9.0 Hz), 4.23 (1H, dd, $J_{7eq,7ax}$ = 10.4 Hz, $J_{7eq\ ,\ 6b}$ = 4.5 Hz), 3.50 (1H, ddd, $J_{6b,\ 7ax}$ = 9.0 Hz, $J_{6b,\ 12b}$ = 7.0 Hz, $J_{6b,\ 7eq}$ = 4.5 Hz) and 5.48 (1H,d, $J_{12b,\ 6b}$ = 7.0 Hz) characteristic of the H-7_{ax}, H-7_{eq}, H-6b and H-12b, -O-CH2-CH-CH-O- unit forming the rings B and C of a pterocarpan nucleus [97]. The ^{1}H-NMR spectrum further revealed the five proton signals in the aromatic region, at δ 6.24 (1H, d, $J_{5,6}$ = 8.4 Hz), 7.02 (1H, d, $J_{6,5}$ = 8.4 Hz), 6.34 (1H, d, $J_{9,\ 11}$= 2.4 Hz), 6.54 (1H, dd, $J_{11,12}$ = 8.4 Hz , $J_{11,9}$ = 2.4 Hz), and 7.31 (1H, d, $J_{12,11}$ = 8.4 Hz) were assigned to the H-5, H-6, H-9, H-11 and H-12 respectively. Presence of signals on ^{1}H-NMR spectrum at δ 2.55 and 2.61 (1H each, m), 1.74 (2H, t, $J_{2,1}$ = 6.8 Hz), 1.34 (3H, s), 1.36 (3H, s) were attributed to the methylene protons of C-1, methylene protons of C-2, methyl protons of C-14 and methyl protons of C-15 respectively. This indicated the presence of dimethyl dihydropyran ring. The placement of dimethyl dihydropyran substituent was deduced by the help of splitting pattern of the protons of the aromatic rings, ^{13}C-NMR values and HMBC correlations.

Strong NOESY correlation (figure 3.1) between H-12b and H-7_{ax} revealed that , H-12b is axially oriented, coupling constant value of H-12b with H-6b (7.0 Hz) indicated that they are trans (both are axially oriented) to each other. *Trans* ring fusion at carbon number 6b and 12b was further confirmed by the comparison of its optical rotation $\{[\alpha]_D^{25} = + 17.70\}$ and negative cotton effect at 342 nm on CD spectrum, with reported pterocarpan skeleton which has *cis* ring fusion with negative optical rotation and positive cotton effect [98]. Proton carbon connectivities were determined by HSQC while long range proton carbon interactions were predicted by using HMBC interactions (figure 3.2). ^{13}C-NMR spectrum broad band and DEPT (table 3.2) displayed twenty carbon signals, including two methyl, three methylene, seven methine and eight quaternary carbons. From the above spectral discussion the structure of compound is (6b R, 12b R) - 3, 3 - dimethyl - 2, 3, 6b, 12b – tetrahydro - 1H, 7H - chromeno{6',5': 4,5}furo{3,2-c}chromen-10-ol, which is trivially named vitexcarpan.



Vitexcarpan

Table 3.2 ^{1}H and ^{13}C-NMR chemical shift values of Vitexcarpan (ppm, C_3D_6O, 400 and 100 MHz, respectively)

C. No	Multiplicity [DEPT]	δ ^{13}C (^{13}C-NMR)	δ ^{1}H (^{1}H-NMR)
1	CH_2	17.6	2.55 m, 2.61 m
2	CH_2	32.5	1.74 t (6.8)
3	C	74.5	-------
4a	C	157.4	-------
5	CH	109.1	6.24 d (1H, d, $J_{5,6}$ = 8.4 Hz)
6	CH	125.0	7.02 (1H, d, $J_{6,5}$ = 8.4 Hz)
6a	C	117.0	-------
6b	CH	40.8	3.50 (1H, ddd, $J_{6b,7ax}$ = 9.0 Hz, $J_{6b,12b}$ = 7.0 Hz, $J_{6b,7eq}$ = 4.5 Hz)
7	CH_2	67.2	3.51 (1H, dd, $J_{7ax,7eq}$ = 10.4 Hz, $J_{7ax,6b}$ 9.0 Hz), 4.23 (1H, dd, $J_{7eq,7ax}$ = 10.4 Hz, $J_{7eq,6b}$ = 4.5 Hz)
8a	C	158.9	-------
9	CH	103.9	6.34 (1H, d, $J_{9,11}$= 2.4 Hz)
10	C	159.7	-------
11	CH	110.4	6.54 (1H, dd, $J_{11,12}$ = 8.4 Hz , $J_{11,9}$ = 2.4 Hz)
12	CH	133.1	7.31 (1H, d, $J_{12,11}$ = 8.4 Hz)
12a	C	118.2	-------
12b	CH	79.3	5.48 (1H,d, $J_{12b,6b}$ = 7.0 Hz)
13a	C	155.8	-------
13b	C	113.0	-------
14	CH_3	26.9	1.34 s
15	CH_3	26.9	1.36 s

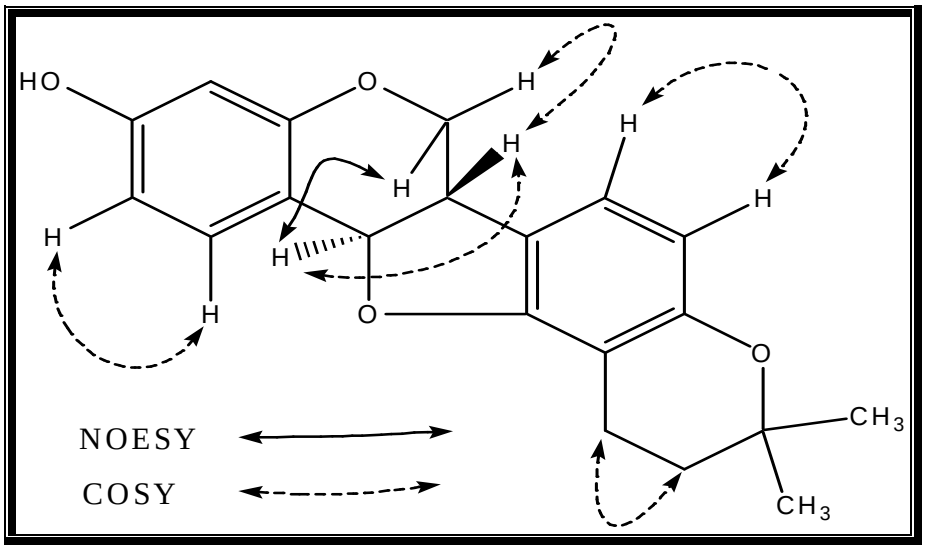


Figure: 3.1. Key COSY and NOESY interactions of Vitexcarpan **(01)**.

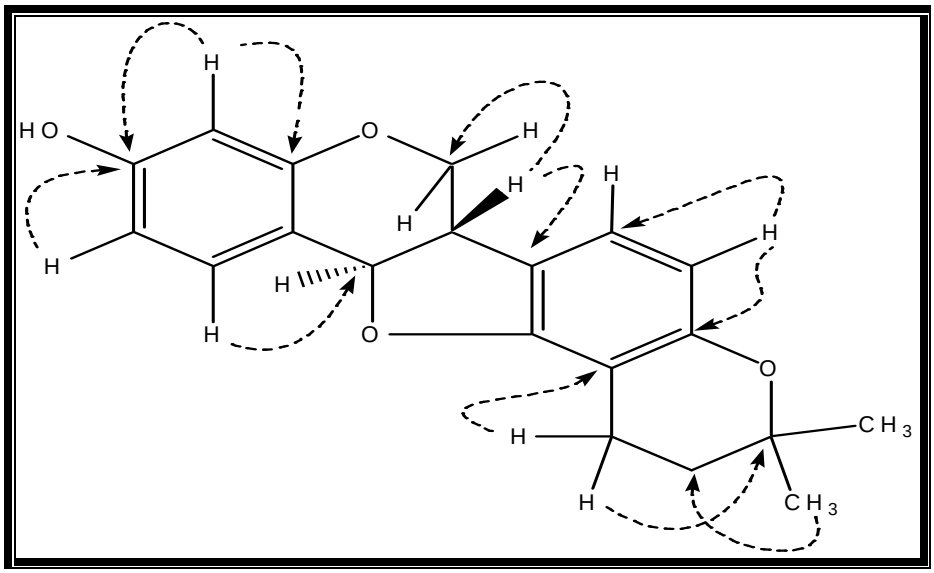


Figure: 3.2. Key HMBC interactions of Vitexcarpan **(01)**.

3.1.2.2 Structural Elucidation of Vitexcarpan (02)

The **Vitexcarpan 1 (02)** was isolated as white amorphous material from the EtOAc fraction of the plant, its optical rotation $\{[\alpha]_D^{25}$ = + 40.76 (MeOH, c = 0.6) indicated the presence of chiral center in molecule. An intense negative Cotton effect ($\Delta\varepsilon$ = -11.7) at 285 nm was observed for the compound on CD spectrum. The EI-MS exhibited the M^+ peak at *m/z* 284. HREI-MS displayed M^+ at 284.1213 which is consistent with the molecular formula $C_{16}H_{12}O_5$ (calcd = 284.1216).The IR max (KBr) cm^{-1} indicated OH at (3382), C ═ C at (1622) and C−O at (1138) functionalities. The characteristic absorptions showed by UV spectra were at 309, 291 and 286 nm which conform that the compound skeleton is flavonoid [99-100].

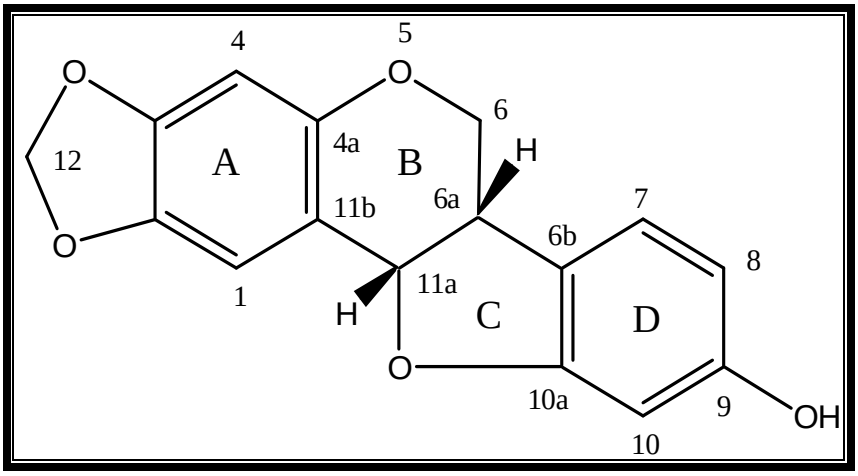
^{1}H-NMR (Table-1) exhibited resonance at δ 3.53 (1H, ddd $J_{6a,6ax}$ = 10.0 Hz, $J_{6a,11a}$ = 7.0 Hz, $J_{6a,6eq}$ = 3.6 Hz) was assigned to H-6a, its axial orientation was predicted on the basis of coupling constant values as it showed with H-6$_{ax}$ (10.0 Hz), while a signal at δ 3.61 (1H, t, $J_{6ax,6a\,/\,6ax,6eq}$ = 10.0 Hz) and at δ 4.25 (1H, dd, $J_{6eq,6ax}$ = 10.0 Hz, $J_{6eq,6a}$ = 3.6 Hz) were assigned for H-6$_{ax}$ and H-6$_{eq}$ protons, respectively. While signal at δ 5.48 (1H, d, $J_{11a,6a}$ = 7.0 Hz) was assigned to the H-11a. This revealed that compound has pterocarpan as basic skeleton [101]. Resonances at δ 5.89 (1H d, *J* = 14.0 Hz) and 5.92 (1H d, *J* = 14.0 Hz) were assigned to the oxymethylene (O-CH_2-O) protons. Two aromatic signals at δ 6.88 (s) and 6.38 (s) were assigned to H-1 and H-4, respectively, whereas H-7 was appeared at δ 7.28 (1H, d, $J_{7,8}$ = 8.5 Hz) while H-8 resonated at δ 6.53 (1H, dd, $J_{8,7}$ = 8.5 Hz and $J_{8,10}$ = 2.0 Hz). A meta coupled signal at δ 6.38 (1H, d, $J_{10,8}$ = 2.0 Hz) was attributed to H-10. ^{13}C-NMR (broad band and DEPT) displayed resonances for sixteen carbon signals including two methylene, seven methine and seven non hydrogenated carbons (table-1). ^{1}H and ^{13}C-NMR inferred that compound has pterocarpan type basic skeleton, with oxymethylene and

hydroxyl group as substituent, which was further confirmed by using 2D-NMR spectroscopy such as COSY, HSQC and HMBC.

Different spin system was revealed with the help of COSY spectrum. H-11a (δ 5.48) showed cross peaks on COSY spectrum with H-6a (δ 3.53) which in turn showed correlation with H_2-6 (δ 3.61 and 4.25). Key COSY correlations in compound are shown in figure 1. Different spin system and functionality were assembled by using HMBC interactions. Careful study of HMBC interactions revealed the fact that oxymethylene was attached to ring A. H-1 (δ 6.88) showed HMBC interactions with C-11b (δ 112.4), C-2 (δ 143.8) and C-3, similarly H-4 (δ 6.38) displayed interactions with C-3 (δ 147.0) and C-4a (δ 149.5), this further confirmed that oxymethylene is attached to the ring A. Furthermore H-11a showed HMBC interactions with H-11b (δ 112.4), H-1 (δ 105.1) and H-6a (δ 41.0), while H-6a (δ 3.53) displayed HMBC correlations with C-6 (66.9), C- 6b (δ 114.0) and C-11a (δ 79.1). Oxymethylene protons (H_2-12) displayed HMBC interactions with C-2 (δ 143.8) and C-3 (δ 147). Key HMBC interactions in compound are shown in figure 2.

Stereochemistry at carbons C-6a and C-11a were inferred by the help of coupling constant values and it was confirmed by NOESY interaction. H-11a proton appeared at δ 5.48 has a NOESY correlation (figure 1) with H-1 (δ 6.88), Which confirm that H-11a is equatorially oriented, *Cis* ring fusion between ring B and C (at carbon no 6a and 11a) was further confirmed by the comparison of coupling constant, ^{13}C-NMR chemical shift values and CD data with reported pterocarpan skeleton which has *cis* ring fusion [101-104].

From the above spectral discussion the structure of compound is, 6a, 11a- dihydro- 6H-[1] benzofuro [3, 2-c] [1, 3] dioxolo [4, 5-g] chromen-9-ol.



Vitexcarpan 1

Table 3.3 ^{1}H and ^{13}C-NMR chemical shift values of Vitexcarpan 1 (ppm, CD_3OD, 400 and 100 MHz, respectively)

C. No	**Multiplicity [DEPT]**	**δ ^{13}C (^{13}C-NMR)**	**δ ^{1}H (^{1}H-NMR)**
1	CH	105.1	6.88 s
2	C	143.8	-
3	C	147.0	-
4	CH	93.9	6.38 s
4a	C	149.5	-
6	CH2	66.9	4.25 dd ($J_{6eq,6ax}$= 10.0, $J_{6eq,6a}$ 3.6) 3.61 t ($J_{6ax,6a/6ax,6eq}$ = 10.0)
6a	CH	41.0	3.53 ddd ($J_{6a,6ax}$ = 10.0, $J_{6a,11a}$ =7.0, $J_{6a,6eq}$ = 3.6)
6b	C	114.0	-
7	CH	133.0	7.28 d ($J_{7,8}$ = 8.5)
8	CH	110.0	6.53 dd ($J_{8,7}$ = 8.5, $J_{8,10}$ = 2.0)
9	C	157.0	-
10	CH	105.8	6.38 d ($J_{10,8}$ = 2.0)
10a	C	153.3	-
11a	CH	79.1	5.48 d ($J_{11a,6a}$ = 7.0)
11b	C	112.4	-
OCH_2O	CH2	102.1	5.89 d (J = 14.0)
			5.92 d (J = 14.0)

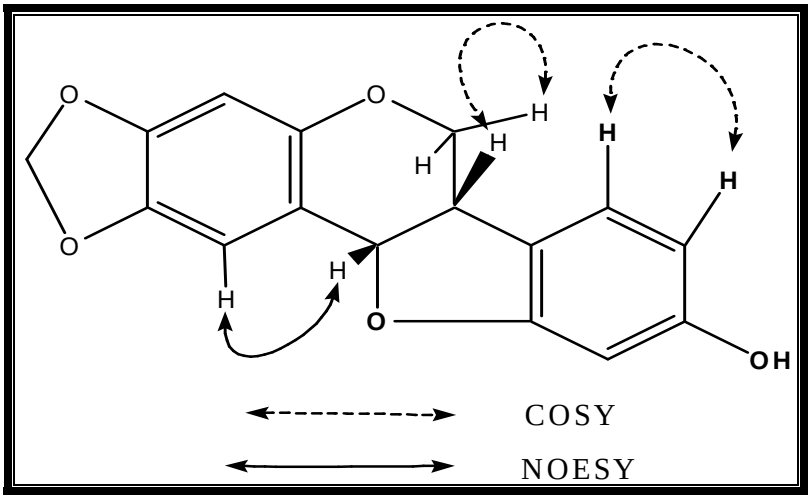


Figure: 3.3. Key COSY and NOSY interactions of Vitexcarpan 1 **(02)**

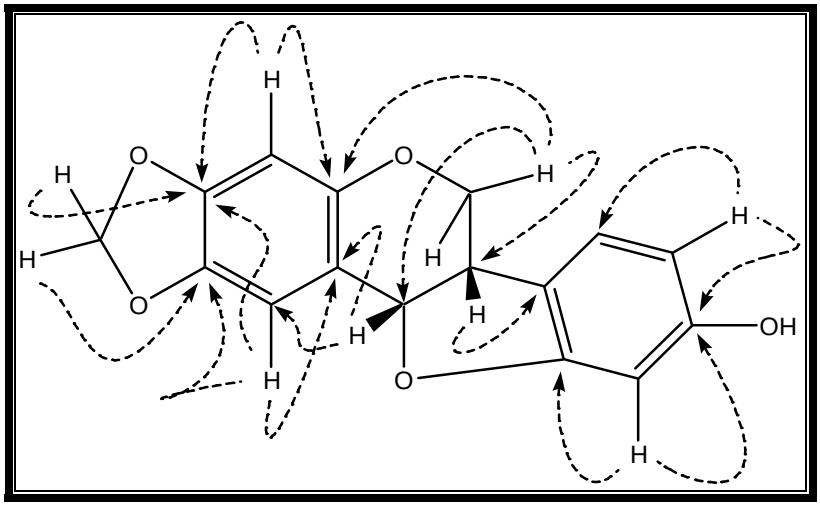


Figure: 3.4. Key HMBC Interactions of Vitexcarpan 1 **(02)**

3.1.2.3 Structural Elucidation of Vitexoide (03)

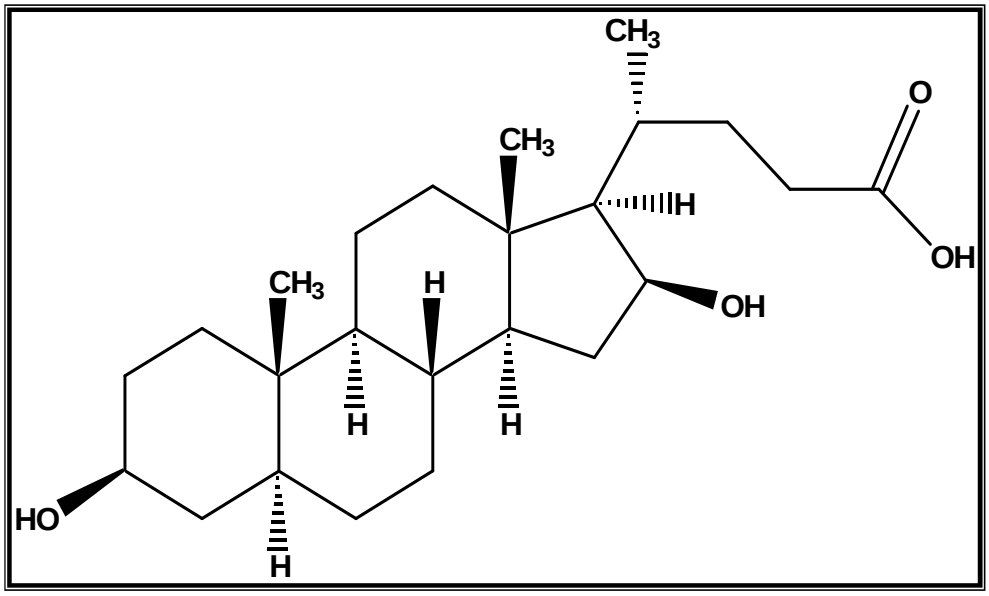
The **Vitexoide (3)** was isolated as amorphous material from sub-fraction (iii) (1.5/8.5 EtOAc/*n*-hexane) eluting with solvent system of 3.5/6.5 (Acetone/*n*-hexane) on silica gel column, of EtOAc fraction of the plant. Its optical rotation, $\{[\alpha]_D^{25}$ = - 74.8 (MeOH, c = 0.20)} indicated the presence of chiral center in molecule and negative cotton effect at 388 nm on CD spectrum. EIMS of compound showed M^+ ion peak at *m/z* 392. Which was further conformed by FAB [$(M+H)^+$ = 393]. HR-ESI-MS showed pseudo molecular ion peak at *m/z* 410.3300, corresponding to the formula $C_{24}H_{40}O_4$ (Cacld. for $C_{24}H_{40}O_4 + NH_4$ = 410.3270).

The ^{1}H and ^{13}C-NMR spectrum clearly indicated that compound is steroidal type [101, 102]. The ^{1}H-NMR spectrum exhibited three methyl signals at δ 0.70 s, 0.92 s and 0.99 d ($J_{21,20}$ = 6.5 Hz) which were assigned to the methyl protons of C-19, C-18 and C-21 respectively. A downfield signal at δ 3.51 (br, m $w_{1/2}$ = 18.5 Hz) was assigned to the C-3 methine proton its axial orientation was inferred on the basis of its $w_{1/2}$ value, while another signal at δ 3.94 (br, s) was attributed to the H-16.

^{13}C-NMR spectrum (BB and DEPT) display 24 carbons including, three methyl, ten methylene, eight methine and three non-hydrogenated carbons.

Structure of compound was further conformed by 2D-NMR spectra (HMBC, HMQC, COSY (figure 3.6) and NOESY). Position of the functional group was determined by the help of ^{13}C-NMR values and HMBC interactions (figure 3.5). H-16 (δ 3.94 br s) displayed HMBC correlation with C-15 (δ 36.3) and C-17 (δ 57.1), methyl proton of C-21, showed HMBC correlation with C-17 (δ 57.1). Shifting of C-17 value to lower field and both H-16 (δ 3.94) and H-21 (δ 0.99) showed HMBC correlations with C-17 clearly indicated that hydroxyl group is substituted at C-16.

Stereochemistry of the compound **(03)** was determined with the help of biogenesis basis [105], coupling constant values and literature values, which were further conformed by using NOESY spectrum. NOESY cross peak observed between H-3 and H-5, H-5 and H-9, H-18 and H-8, H-14 and H-16, H-8 and H-19. Correlation H-16 with H-14 clearly indicated that H-16 is in pseudo axially oriented. Key NOESY correlations are shown in figure 3.7.



Vitexoide (03)

Table 3.4 ^{1}H- and ^{13}C- NMR chemical shift values of Vitexoide (ppm, C_3D_6O, 400 and 100 MHz, respectively)

C. No	δ_C	Multiplicity [DEPT]	δ_H (*J*, Hz)
1	36.4	CH_2	1.63, 1.75 m
2	28.6	CH_2	1.31, 1.71 m
3	72.6	CH	3.51 (br, m, $w_{½}$ = 18.5)
4	37.2	CH_2	1.34, 1.62 m
5	43.7	CH	1.26 m
6	28.4	CH_2	1.17, 1.39 m
7	39.0	CH_2	1.23, 1.41 m
8	34.8	CH	1.80 m
9	49.3	CH	1.54 m
10	35.3	C	--------
11	24.8	CH_2	1.91, 1.60 m
12	37.3	CH_2	1.36, 1.52 m
13	42.6	C	--------
14	48.2	CH	1.75 m
15	36.3	CH_2	1.69, 1.73 m
16	74.1	CH	3.94 br s
17	57.1	CH	1.55 m
18	17.5	CH_3	0.92 s
19	13.2	CH_3	0.70 s
20	31.1	CH	1.51 m
21	23.7	CH_3	0.99 d ($J_{20,21}$ = 6.5 Hz)
22	29.9	CH_2	1.41, 1.59m
23	37.3	CH_2	2.33, 2.19 m
24	178.3	C	--------

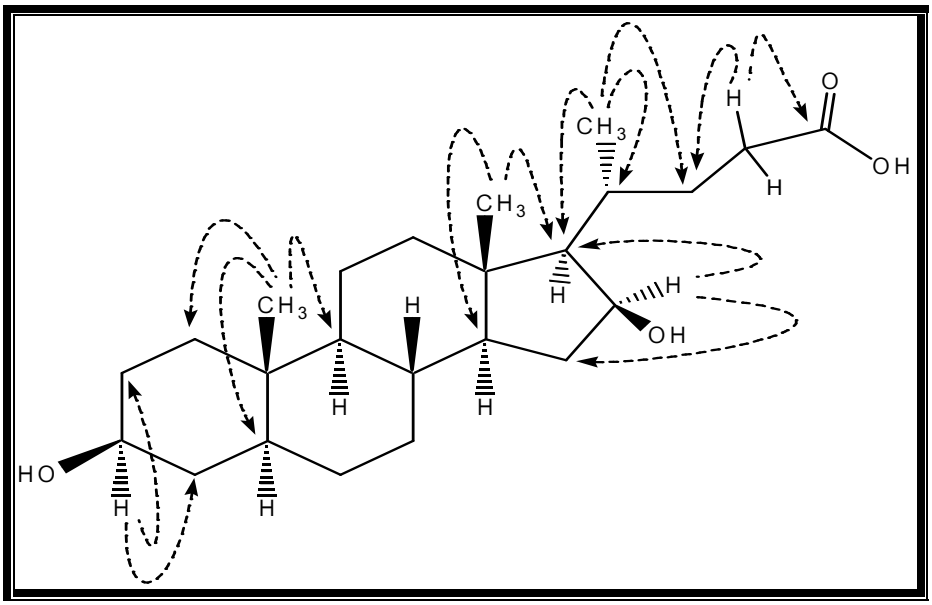


Figure: 3.5. Key HMBC correlations of Vitexoide **(03)**

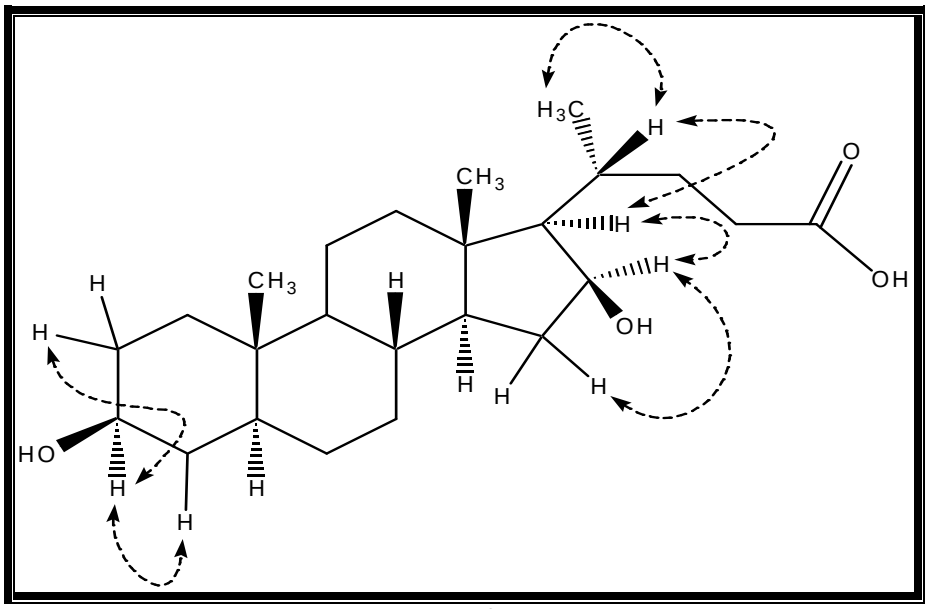


Figure: 3.6. Key COSY correlations of Vitexoide **(03)**.

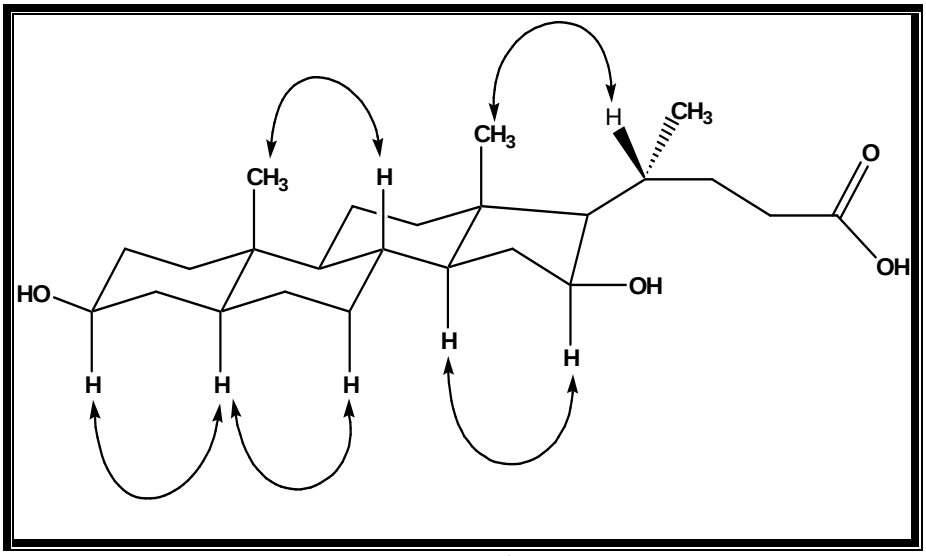
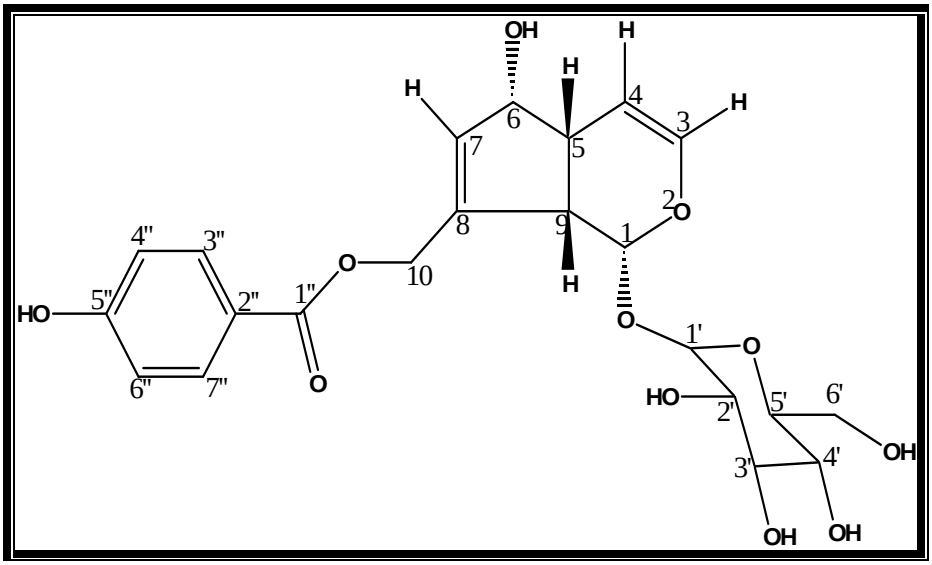


Figure: 3.7. Key NOESY correlations of Vitexoide **(03)**

3.1.2.4 Structural Elucidation of compound (04)

The compound (04) was isolated in non-crystallized form, from sub-fraction xi (8/2 EtOAc/*n*-hexane) with solvent system of MeOH/DCM (1.2/8.8), of EtOAc fraction of the plant. The compound did not give the M^+ in the EI-MS. The ESI-MS showed peak at 950.3 for $2M^+$+NH4, a peak at 484.2 for M^++ NH4 and a peak at 449.1 for M^+- H2O + H. The molecular weight of compound was deduced to be 466.4. The UV spectrum exhibited maxima at 217 and 275 nm and IR spectrum gives absorptions at 3421, 1704, 1636, and 1450 cm^{-1} indicating OH and ester C=O functionalities and aromatic C=C bonds.

The ^{1}H-NMR spectrum of compound showed three olefinic peaks at δ 5.11 (1H, dd J = 3.6, 6.0Hz), 5.82 (1H, s) and 6.33 (1H, dd J = 1.8, 6.0 Hz), respectively. The spectrum also showed five hydroxyl peaks, four for the sugar moiety and the fifth at C-6. The ppm values for these hydroxyl protons appeared at δ 4.70 (1H, d J = 7.8 Hz, C-1'), 3.23 (1H, m, C-2'), 3.26 (1H, m, C-3'), 3.30 (1H, m C-4'), 3.37 (1H, m C-5'), 3.65 (1H, dd J = 5.4, 12.0, C-6'), 3.84 (1H, dd J = 12.0, 1.8, C-6') and 4.45 (1H, br. s, C-6), respectively. The stereochemistry at C-1 was confirmed by the presence of NOESY interaction of H-1 with H-9. Both H-5 and H-9 were showing NOESY interactions and were assigned as β. The H-6 also displayed NOESY interactions with H-5, suggesting that the H-5 is oriented β to the plan. NOESY correlations are shown in figure 3.9. Aromatic protons for the disubstituted benzene ring appeared at δ 6.83 (2-H, d J = 9.0 Hz) and 7.91 (2H, d J = 9.0 Hz). The ^{13}C-NMR spectrum showed four olefinic peaks at δ 105.5, 132.4, 142.9 and 141.7, respectively. The carbons of the sugar moiety appeared at δ 100.2, 74.7, 78.3, 71.5, 77.9 and 62.7, respectively. The aromatic region displayed peaks at δ 122.1, 132.9, 116.3 and 163.7, respectively. The key HMBC correlations are shown in figure 3.8.



Compound (04)

Table 3.5 ^{1}H and ^{13}C-NMR chemical shift values of compound **(04)** (ppm, CD_3OD, 600 and 150 MHz respectively).

C. NO	Multiplicity[DEPT]	$\delta^{13}C$ ($^{13}CNMR$)	$\delta^{1}H$ (^{1}H-NMR)
1	CH	97.9	4.97 d ($J_{1,9}$ = 7.8 Hz)
3	CH	141.7	6.33 dd ($J_{3,5}$ = 1.8, $J_{3,4}$ = 6.0 Hz)
4	CH	105.5	5.11 , dd ($J_{4,5}$ = 3.6, $J_{4,3}$ = 6.0 Hz)
5	CH	46.3	2.69 brs $w_{1/2}$ = 21.4
6	CH	82.9	4.45 brs $w_{1/2}$ = 12.5
7	CH	132.4	5.82 s
8	C	142.9	-
9	CH	48.6	2.97 t ($J_{9,1}$= 7.2 Hz)
10	CH_2	63.7	4.90 m, 5.08 m
1'	CH	100.2	4.70 d ($J_{1',2'}$ = 7.8 Hz)
2'	CH	74.7	3.23 m
3'	CH	78.3	3.26 m
4'	CH	71.5	3.30 m
5'	CH	77.9	3.37 m
6'	CH_2	62.7	3.65 dd ($J_{6',5'}$ = 5.4 Hz, $J_{6',6'}$ = 12.0 Hz, 3.84) dd ($J_{6',5'}$ = 1.8 Hz, $J_{6',6'}$ = 12.0 Hz)
1"	C	167.8	-
2"	C	122.1	-
3"	CH	132.9	7.91 d ($J_{3",4"}$ = 9.0 Hz)
4"	CH	116.3	6.83 d ($J_{4",3"}$= 9.0 Hz)
5"	C	163.7	-
6"	CH	116.3	6.83 d ($J_{6",7"}$ = 9.0 Hz)
7"	CH	132.9	7.91 d ($J_{7",6"}$ = 9.0 Hz)

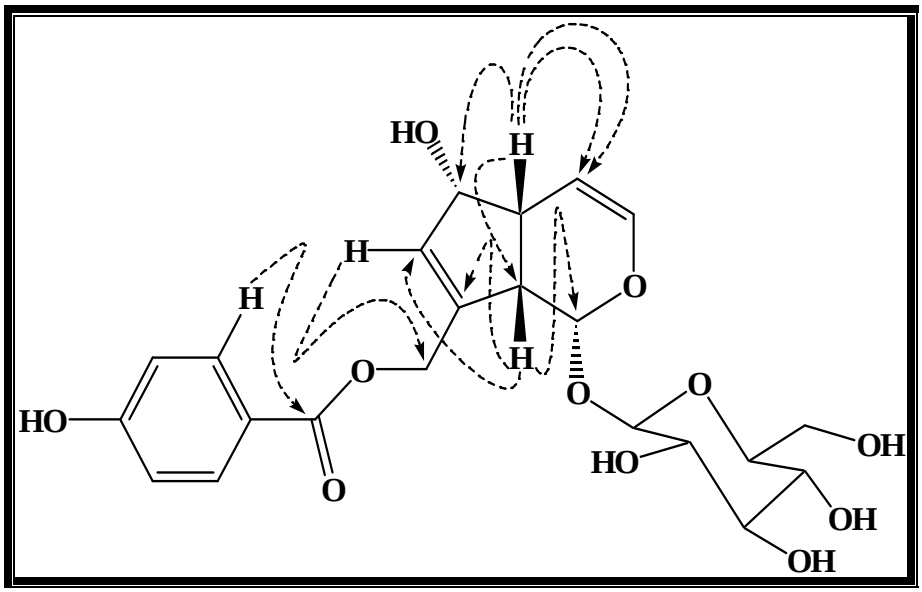


Figure: 3.8. Key HMBC correlations of compound **(04)**

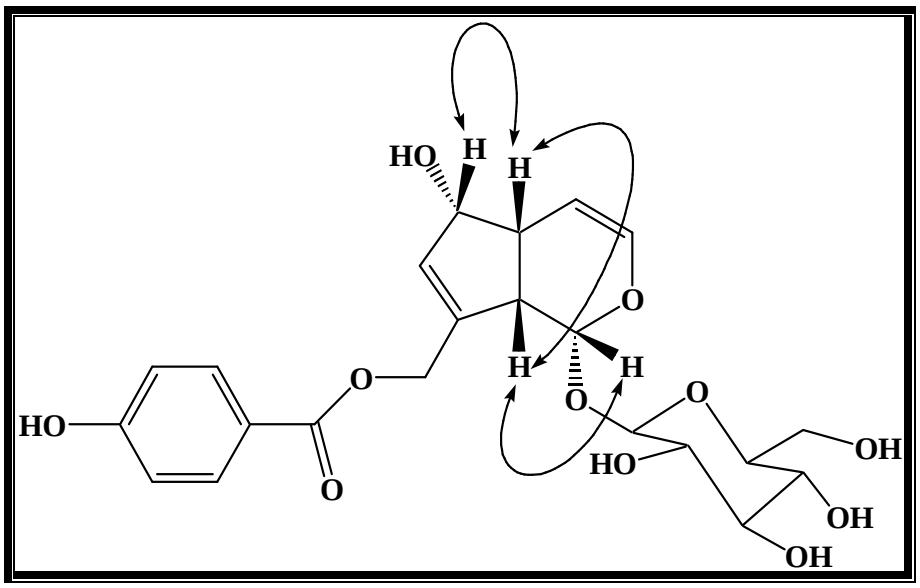
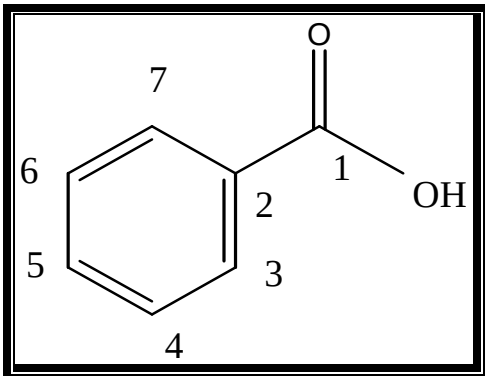


Figure: 3.9. Key NOESY correlations of compound **(04)**

3.1.2.5 Structural Elucidation of Compound (05)

The compound **(05)** was isolated from the ethyl acetate fraction of the aerial parts of *Vitex agnus castus.*

The structure was determined by combined interpretation of ^{1}H NMR, ^{13}C NMR, HMBC and HMQC spectroscopic techniques. Its UV absorption at 272 and 300 nm was characteristic of an aromatic carboxylic acid group. The ^{1}H NMR spectrum showed aromatic protons signals at δ 8.12 (2H, t, *J* = 8.5Hz), 7.60 (1H, t, *J* = 8.5 Hz), 7.46 (2H, t *J* = 8.5 Hz) for positions (H-2, H-6) and (H-4), (H-3, H-5) respectively. A chelated OH was also displayed at δ 11.92 in the proton NMR spectrum. The ^{13}C NMR spectrum Broad Band (BB, DEPT) of the compound showed seven carbon signals: five methane and two quaternary carbon atoms in the molecules of compound. The ^{1}H-^{13}C correlations were determined by HMQC. In the spectrum the signals appeared at δ 180.0, 134.5, 131.1, 130.7 and 129.7 for C=O, (C-4), (C-1), (C-2, 6) and (C-3, 5), respectively. The structure was established as benzoic acid.



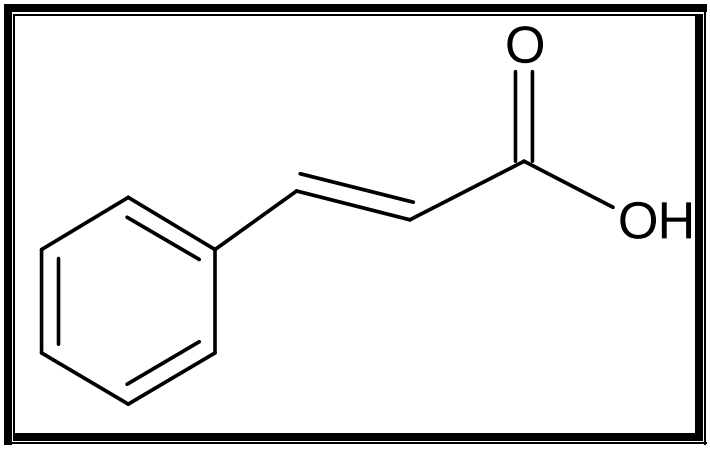
Benzoic acid

3.1.2.6 Structural Elucidation of Compound (06)

The HR-EIMS of the compound showed $[M]^+$ peak at *m/z* 148.0701 (calcd for $C_9H_{10}O_2$) consistent with the molecular formula $C_9H_{10}O_2$. Beside the $[M]^+$ other fragments were observed at *m/z* 77.0 $[C_6H_5]^+$, 91.1 $[C_7H_7]$ and $[C_8H_7]^+$.

The ^{1}H-NMR spectra of the compound corroborate the presence of three aromatic protons, observed at δ 7.21 m, 7.23 m and 7.36 m. The oleifinic protons resonate at δ 6.2 (d, *J* = 15.9 Hz) and 7.52 (d, *J* = 15.9 Hz). The larger coupling constant of the oleifinic protons were in the conformity to *E* configuration.

The ^{13}C-NMR spectrum (BB and DEPT) of the compound showed nine carbon atom signals comprising seven methine and two quaternary carbons. The physical and spectral data of the compound correspond to reported Cinnamic acid.



Cinnamic acid

3.2 PHARMACOLOGICAL INVESTIGATIONS

3.2.1 ANTIBACTERIAL ACTIVITY

Antimicrobial resistance has become a global problem. Strategies to improve the current situation include research in finding new and innovative antimicrobials [104**]**. Antibiotics and the chemotherapeutic agents have been of value in controlling many infections but they depend on judicious use to minimize the incidence of resistance [107]**.**

Crude methanolic extract and various fractions of *Vitex agnus castus* were screened against the said test pathogens for possible antibacterial activity. The results are summarized in table 3.6 and presented in figures 3.10 to 3.15. Order of the antibacterial activity of the crude methanolic extract and fractions, against *E. coli* was *n*-hexane = BuOH (69.56%) > crude extract (60.80%) > EtOAc = aqueous (56.52%) > $CHCl_3$ fraction (52.17%). Against *S. epidermidis*, EtOAc (62.96%) > *n*-hexane (57.69%) > $CHCl_3$ (53.85%) > crude extract = BuOH (50.00%) > aqueous (42.30%). Against *S. typhi*, $CHCl_3$ (62.96%) > EtOAc = BuOH (51.85%) > crude extract (59.25%) > aqueous (57.69%) while *n*-hexane showed no activity. Against *S. pneumoniae,* aqueous (48.27%) > BuOH (44.82%) > crude extract = *n*-hexane = $CHCl_3$ (41.37%) > EtOAc (37.93%). Against *S. aureus*, BuOH (53.85%) > $CHCl_3$ (53.84%) *n*-hexane = aqueous (50.00%) > EtOAc (43.15%) > crude extract (42.30%). The activity against *P. aeruginosa* was EtOAc (62.96%) > BuOH (59.25%) > $CHCl_3$ (57.69%) > aqueous (51.85%) > crude extract (48.18%) > *n*-hexane (44.44%). Against *K. pneumoniae*, $CHCl_3$ (80.95%) > *n*-hexane = EtOAc = BuOH (61.90%) > crude extract (57.14%) > aqueous (52.38%). Against *B. pumilus* EtOAc (52.00%) > BuOH (48.00%) > crude extract = *n*-hexane (44.00%); and no activity was shown by $CHCl_3$ and aqueous fractions. The activity of the test samples against *E. aerogenes* was in order of, *n*-hexane (51.72%) > crude (48.27%) > $CHCl_3$ (44.82%) > BuOH (41.37%) > EtOAc = aqueous (37.93%).

From the above results it is revealed that $CHCl_3$ fraction of *Vitex agnus castus* showed significant antibacterial activity against *K. pneumoniae* (zone of inhibition = 80%, MIC_{50} = 2.19 mg/ml), while rest of the fractions and crude methanolic extract showed moderate and low activity except *n*-hexane, $CHCl_3$ and aqueous fractions, exhibit no activity against *S. typhi* and *B. pumilus*, respectively. The lower the MIC_{50} values, the higher will be its potency and vice versa. The MIC_{50} values (mg/ml) of the crude methanolic extract and all fractions of *Vitex agnus castus* was calculated from three separate reading by using a Microsoft XL sheet and are measured as given in table 3.7 and summarized in figures 3.16 to 3.21. In a comparative study with some therapeutically used antibiotics, different extracts of *Vitex negundo* leaves were investigated for its antimicrobial activity on five bacterial species, among all extracts water-ethanol (50:50) extract showed maximum antimicrobial activity against all tested species [108]. The methanolic extracts of stem bark of *Vitex doniana* were screened against *S. typhi, Shigella dysentariae* and *E. coli.* The extracts were able to inhibit the growth pattern of the tested microorganisms. In all cases *S. dysentariae* showed the highest sensitivity [109]. Five African *Vitex* species were screened for antimicrobial activity, mostly good antimicrobial inhibition was evident against Gram positive bacteria (0.02–8.00 mg/ml) and lower activity against the Gram negative bacteria and the yeast (0.50–8.00 mg/ml) [110]. The results confirm that the plant specie could be a potential target for activity guided isolation of the bioactive constituents against the Gram positive pathogens.

Table 3.6 Antibacterial activities (zone of inhibition and percent) of crude extract and various fractions of *Vitex agnus castus*.

Name of Bacteria	Zone of inhibition of standard (amoxicillin) 10µg/disc	Crude Extract		*n*-hexane		$CHCl_3$		EtOAc		BuOH		Aqueous	
		Zone of Inhibition (mm)	Inhibition (%)	Zone of Inhibition (mm)	Inhibition (%)	Zone of Inhibition (mm)	Inhibition (%)	Zone of Inhibition (mm)	Inhibition (%)	Zone of Inhibition (mm)	Inhibition (%)	Zone of Inhibition (mm)	Inhibition (%)
E. coli	23	14	60.80	16	69.56	12	52.17	13	56.52	16	69.56	13	56.52
S. epidermidis	26	13	50.00	15	57.69	14	53.85	17	69.23	13	50.00	11	42.30
S. typhi	27	16	59.25	00	00.00	17	62.96	14	51.85	14	51.85	15	57.69
S. pneumoniae	29	12	41.37	12	41.37	12	41.37	11	37.93	13	44.82	14	48.27
S. aureus	26	11	42.30	13	50.00	14	53.84	12	43.15	14	53.85	13	50.00
P. aeruginosa	27	13	48.18	12	44.44	15	57.69	17	62.96	16	59.25	14	51.85
K. pneumoniae	21	12	57.14	13	61.90	17	80.95	13	61.90	13	61.90	11	52.38
B. pumilus	25	11	44.00	11	44.00	00	00	13	52.00	12	48.00	00	00
E. aerogenes	29	14	48.27	15	51.72	13	44.82	11	37.93	12	41.37	11	37.93

Table 3.7 MIC_{50} values (mg) of crude extract and fractions of *Vitex agnus castus* against tested pathogens.

Name of Bacteria	Crude extract	*n*-hexane	$CHCl_3$	EtOAc	BuOH	Aqueous
E. coli	1.82	1.65	1.9	1.95	1.68	1.65
S. epidermidis	2.18	2.7	1.7	1.8	1.76	1.87
S. typhi	2.4	5.1	1.89	2.22	2.65	2.1
S. pneumoniae	2.23	1.95	2.19	2.48	2.43	1.7
S. aureus	2.38	2.1	3.15	2.21	2.57	1.6
P. aeruginosa	4.8	1.96	2.53	2.24	2.25	1.9
K. pneumoniae	2.59	1.83	2.19	2.4	1.72	1.8
B. Pumilus	1.83	2.45	1.72	1.98	2.94	1.7
E. aerogenes	2.7	2.08	2.49	2.38	2.5	2.29

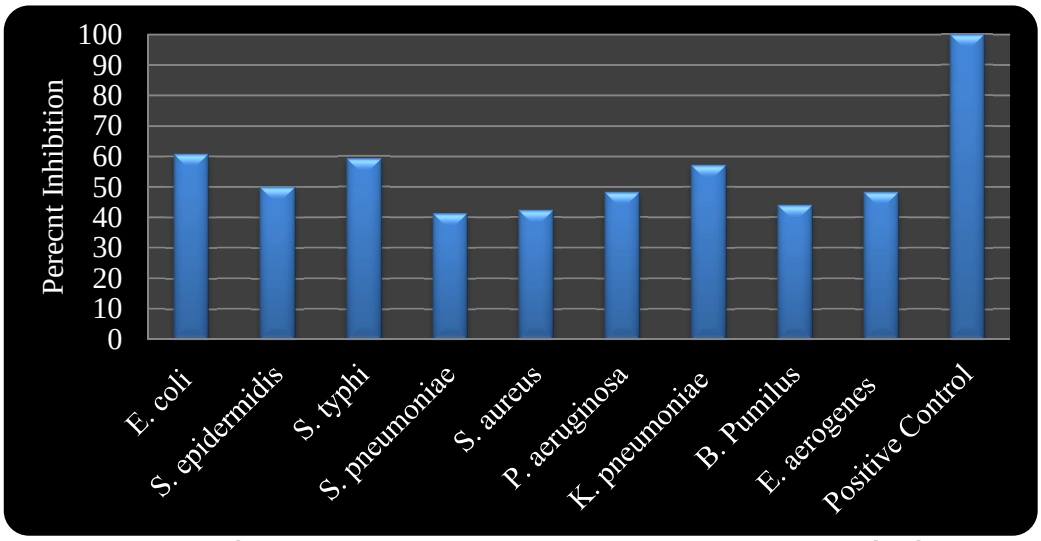


Figure: 3.10. Antibacterial activity of the crude extract of *V. agnus castus* against tested pathogens.

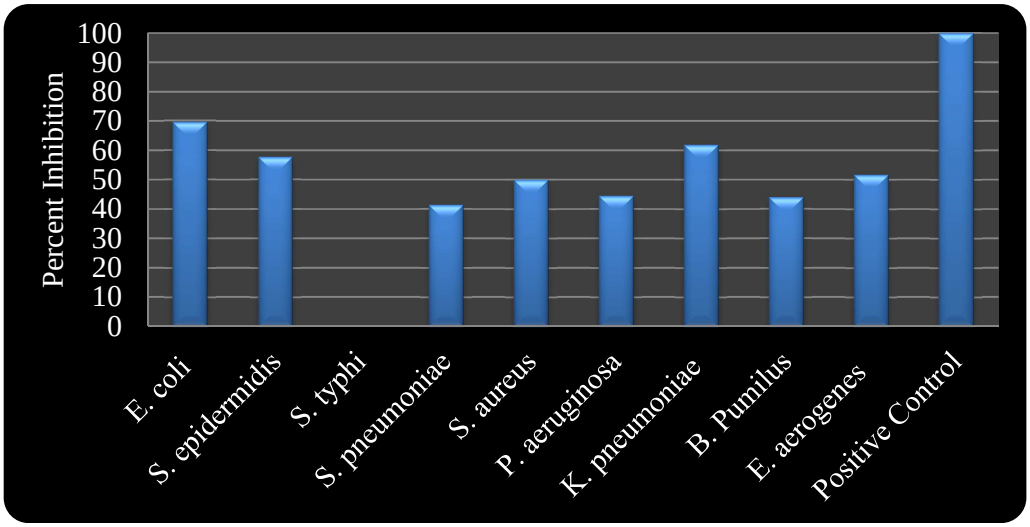


Figure: 3.11. Antibacterial activity of *n*-hexane fraction of *V. agnus castus* against tested pathogens.

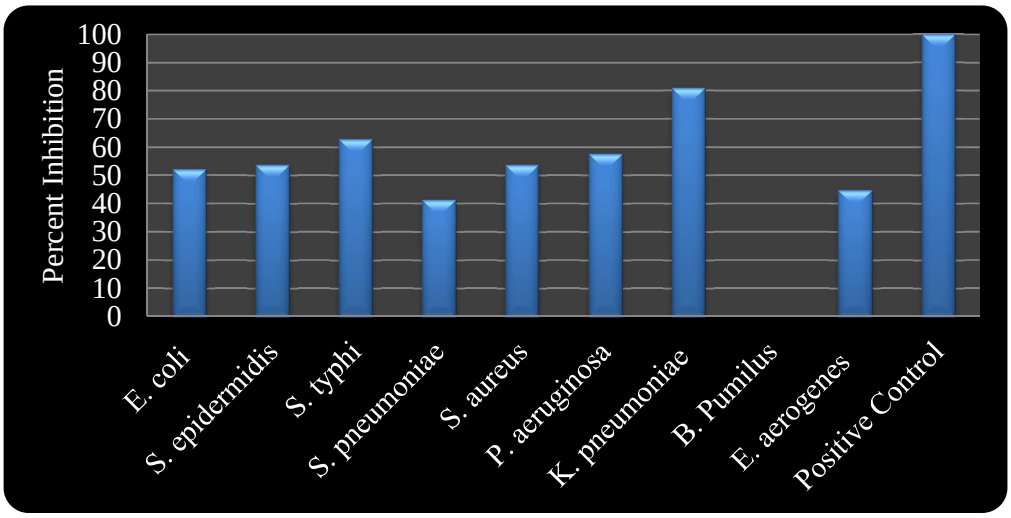


Figure: 3.12. Antibacterial activity of $CHCl_3$ fraction of *V. agnus castus* against tested pathogens.

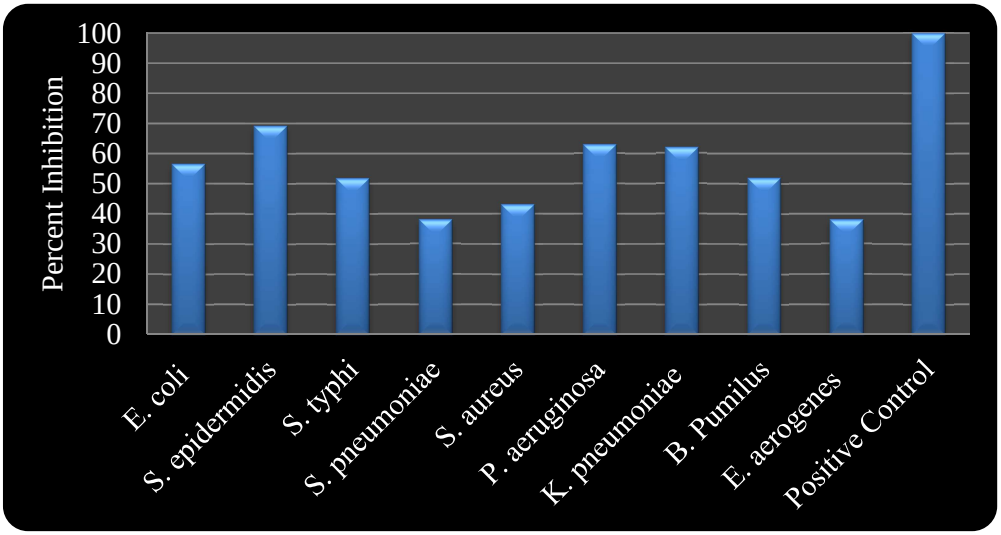


Figure: 3.13. Antibacterial activity of EtOAc fraction of *V. agnus castus* against tested pathogens.

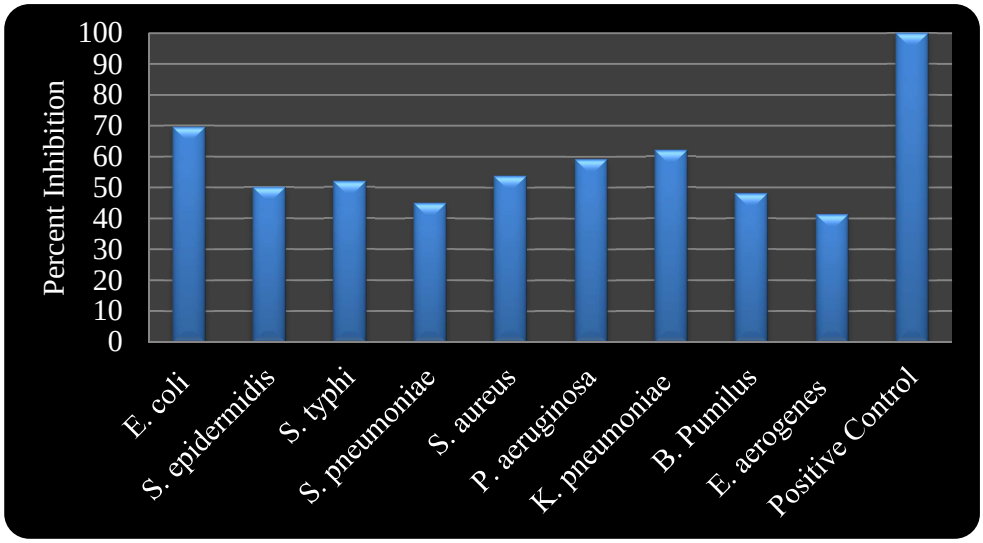


Figure: 3.14. Antibacterial activity of *n*-butanol fraction of *V. agnus castus* against tested pathogens.

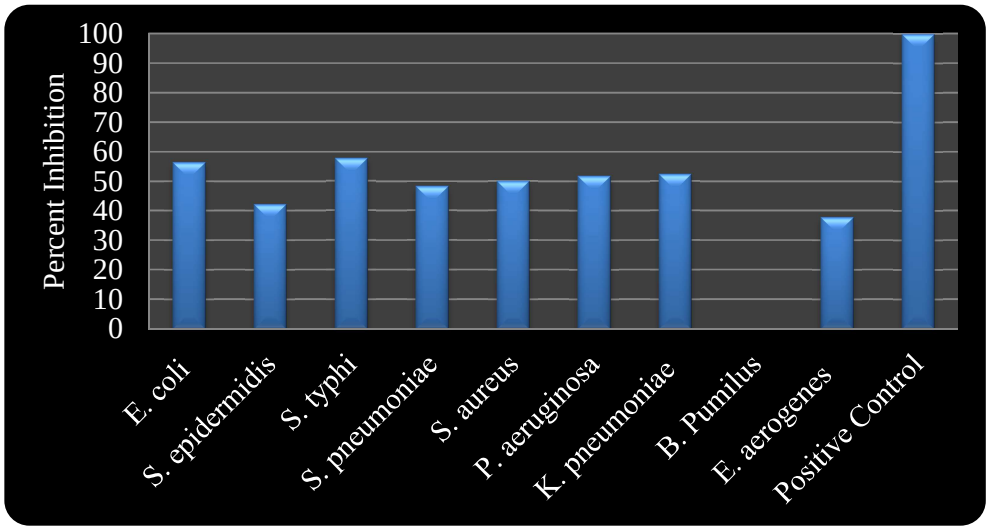


Figure: 3.15. Antibacterial activity of aqueous fraction of *V. agnus castus* against tested pathogens.

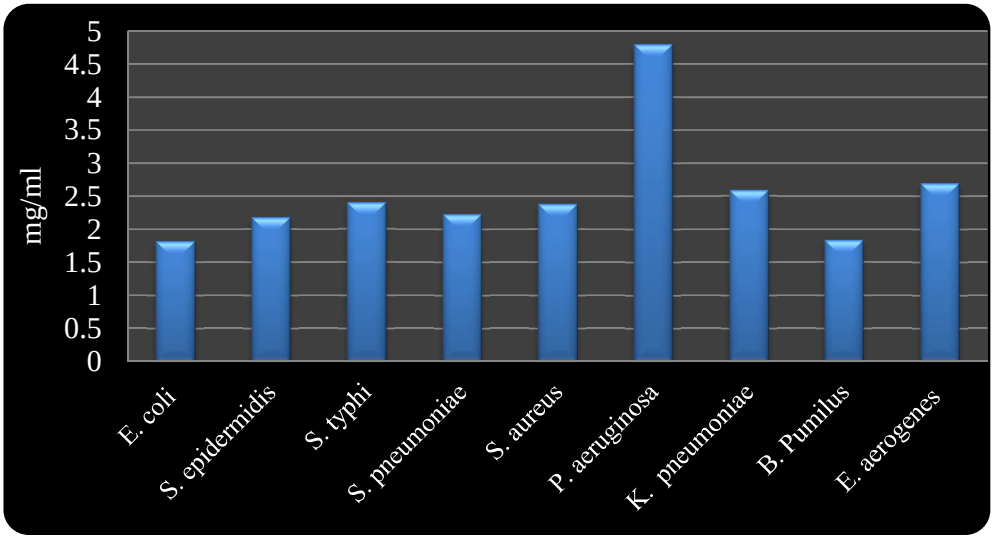


Figure: 3.16. MIC_{50} values of crude extract of *V. agnus castus* against tested pathogens.

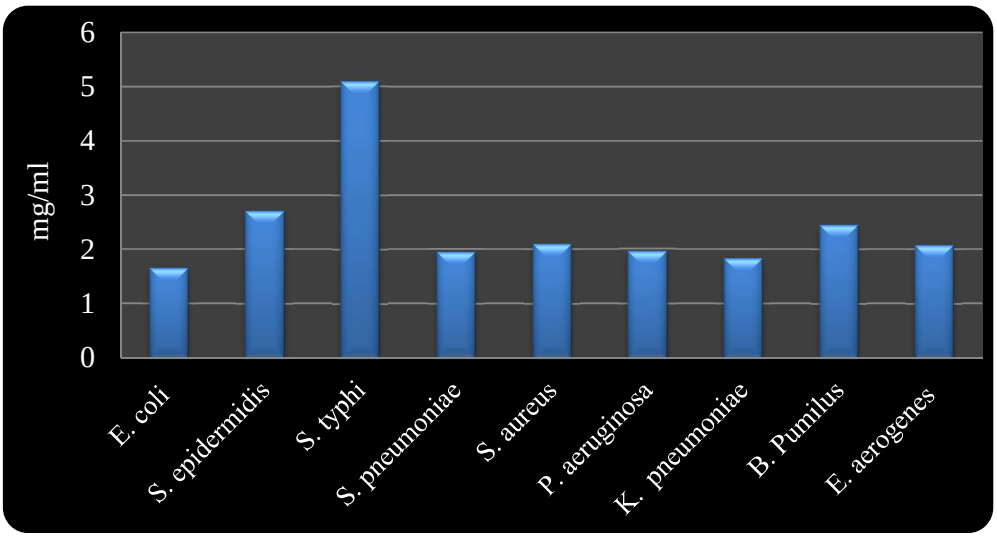


Figure: 3.17. MIC_{50} values of *n*-hexane fraction of *V. agnus castus* against tested pathogens.

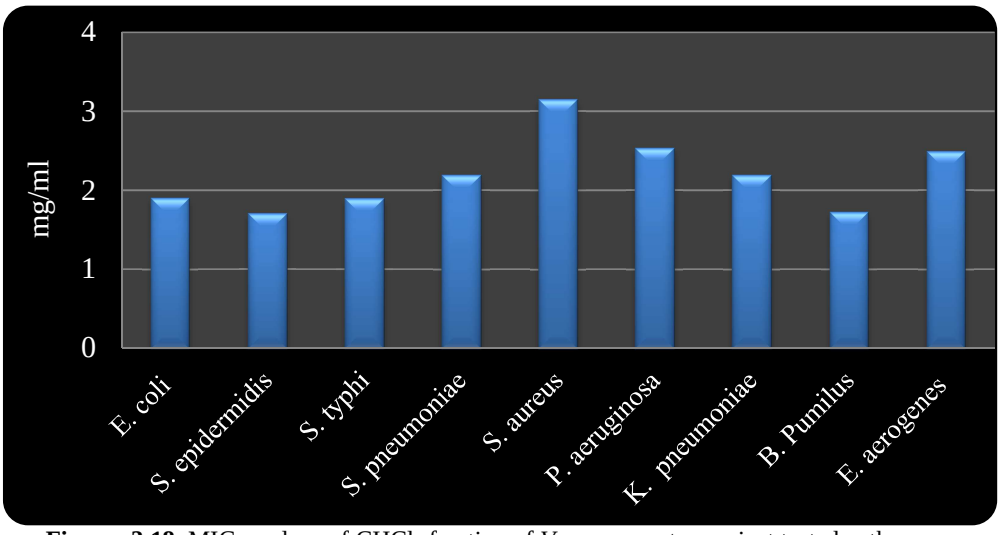


Figure: 3.18. MIC_{50} values of $CHCl_3$ fraction of *V. agnus castus* against tested pathogens.

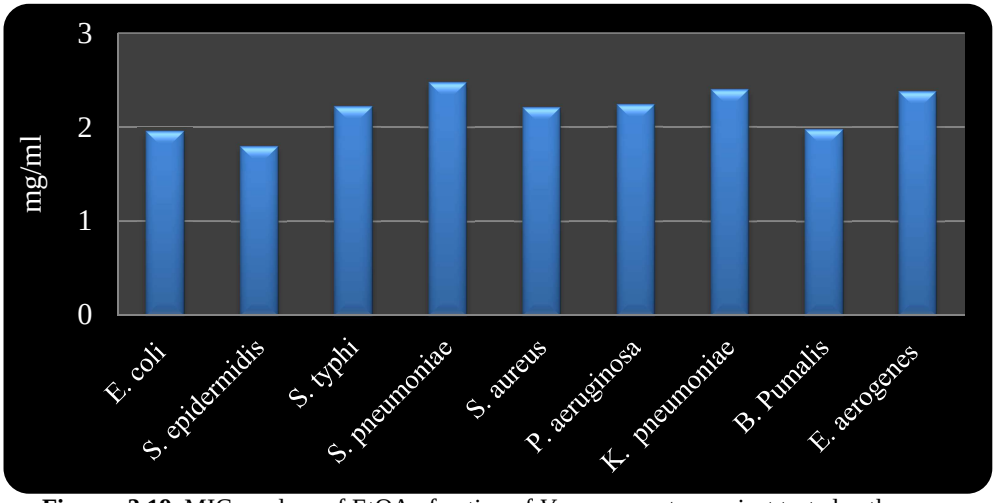


Figure: 3.19. MIC_{50} values of EtOAc fraction of *V. agnus castus* against tested pathogens.

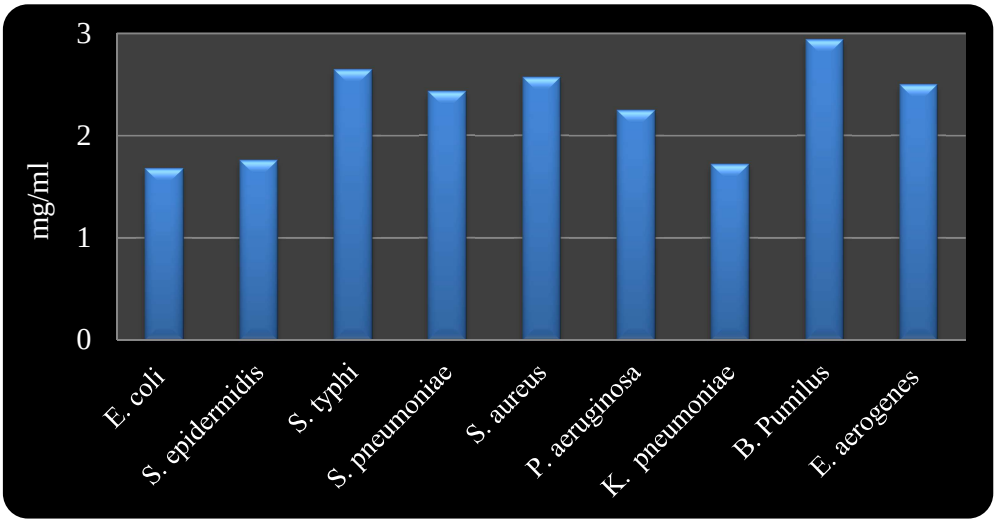


Figure: 3.20. MIC_{50} values of *n*-butanol fraction of *V. agnus castus* against tested pathogens.

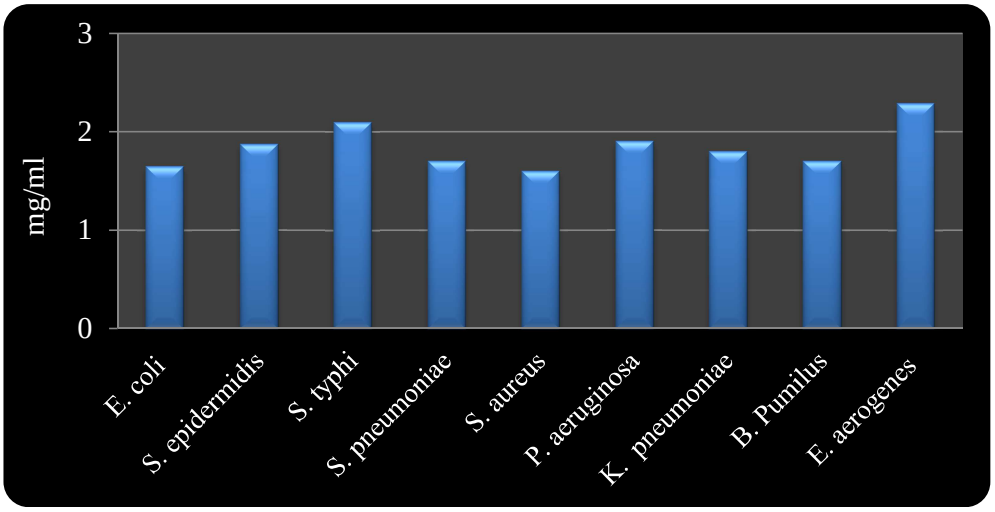


Figure: 3.21. MIC_{50} values of aqueous fraction of *V. agnus castus* against tested pathogens.

3.2.2 ANTIFUNGAL ASSAY

The plant materials were screened for antifungal activity against *Aspergillus niger, Aspergillus flavus, Penicillium notatum, Fusarium oxysporum, Triticum harzianum* and *Rhizopus stolonifer.* The results were recorded as percent growth inhibition, compared with positive control. Amphotericin-B and Miconazole were used as standard drugs.

The *n*-hexane (20%), $CHCl_3$ (15%) and BuOH (10%) fractions of the plant showed low activity while the crude extract and EtOAc fraction were found inactive against *A. niger*. Similarly, the $CHCl_3$ fraction showed low inhibiting effect (15%) for *A. flavus* and rest of the fractions along with crude extract showed no inhibitory activity. The crude extract, *n*-hexane, $CHCl_3$ and EtOAc fractions showed low antifungal activity, 20, 10, 20 and 10% respectively, against *P. notatum* and rest of the fractions failed to produce any inhibitory effect against *P. notatum*. Similarly, in the antifungal experiments the BuOH and aqueous fractions of the plant showed low inhibitory effect against *F. oxysporum*, while rest of the fractions and crude extract were found inactive against the tested specie. The test samples were also screened against *T. harzianum* and low antifungal effect was observed for crude extract and $CHCl_3$ fraction, 10% each and BuOH 15% while rest of the fractions were found to be inactive against the tested pathogen. Similarly 20% growth inhibition was shown by BuOH fraction against *R. stolonifer* and crude extract along with other fractions doesn't show any antifungal activity. The results are summarized in table 3.8 and presented in figures 3.22 - 3.27.

The above results reveals that the crude extract and various fractions from the aerial parts of the plant were showed no significant antifungal activity against the tested organisms.

Table 3.8. Antifungal activity of the crude extract and fractions of *Vitex agnus castus.*

Name of Fungi	**Percent Inhibition**							
	Negative control	**Positive control**	**Crude Extract**	***n*-hexane**	**$CHCl_3$**	**EtOAc**	**BuOH**	**Aqueous**
A. niger	0	100	0	20	15	0	10	0
A. flavus	0	100	0	0	15	0	0	0
P. notatum	0	100	20	10	20	8	0	0
F. oxysporum	0	100	0	0	0	0	15	15
T. harzianum	0	100	10	0	10	0	15	0
R. stolonifer	0	100	0	0	0	0	20	0

$$\% \text{ inhibition of fungal growth} = 100 - \frac{\text{linear growth in test (mm)}}{\text{linear growth in control (mm)}} \times 100$$

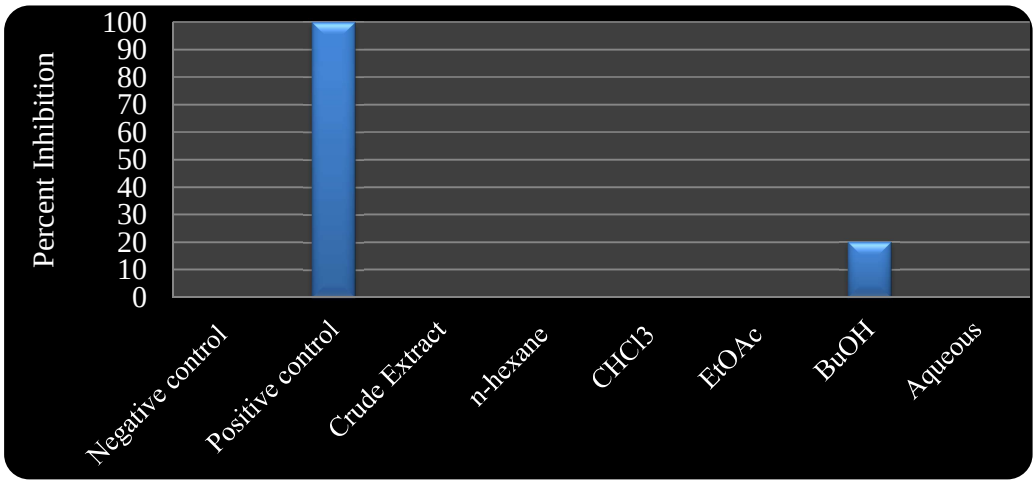


Figure: 3.22. Antifungal activity of crude extract and fractions of *V. agnus castus* against *A. niger*.

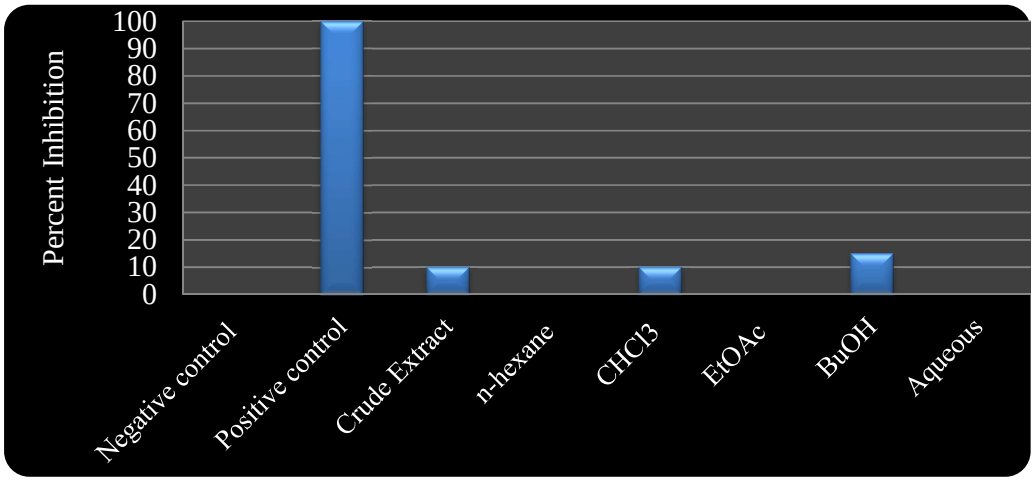


Figure: 3.23. Antifungal activity of crude extract and fractions of *V. agnus castus* against *A. flavus.*

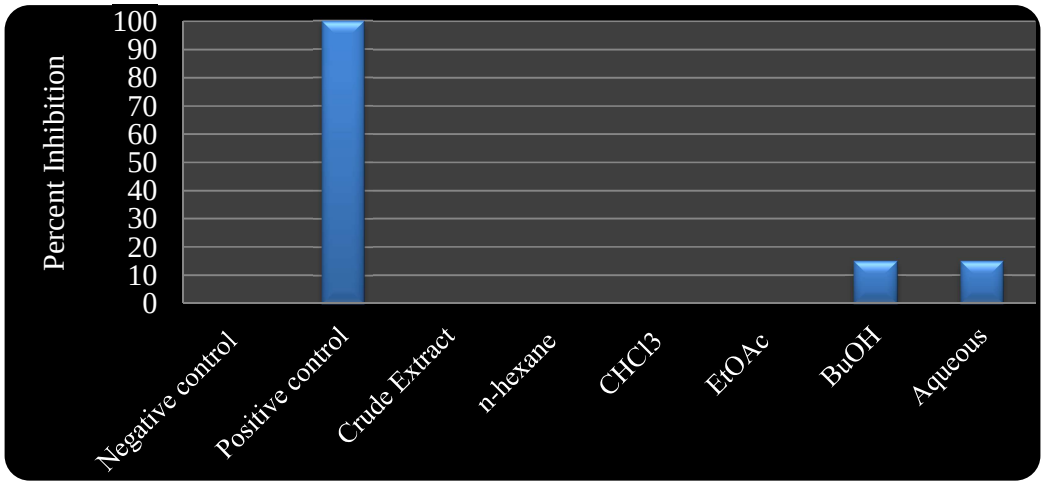


Figure: 3.24. Antifungal activity of crude extract and fractions of *V. agnus castus* against *P. notatum.*

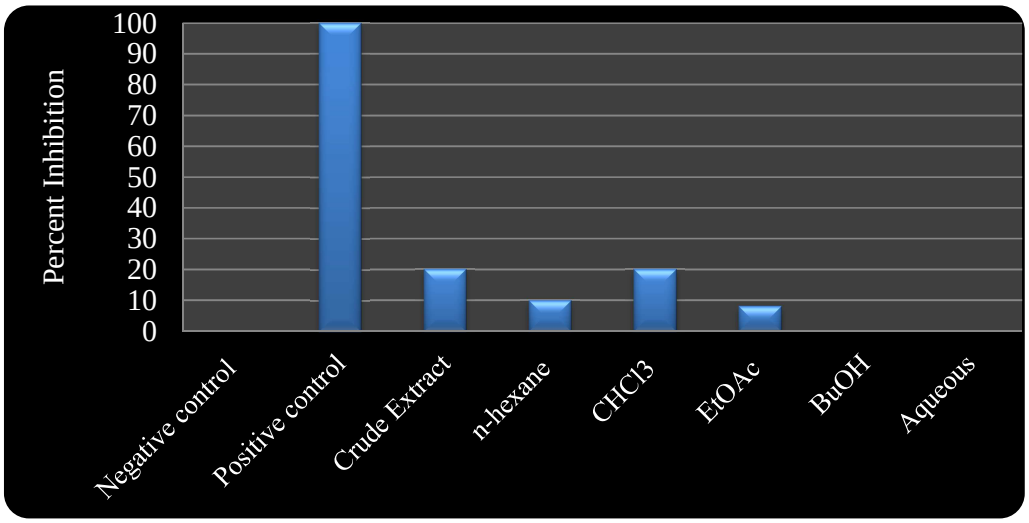


Figure: 3.25. Antifungal activity of crude extract and fractions of *V. agnus castus* against *F. oxysporum.*

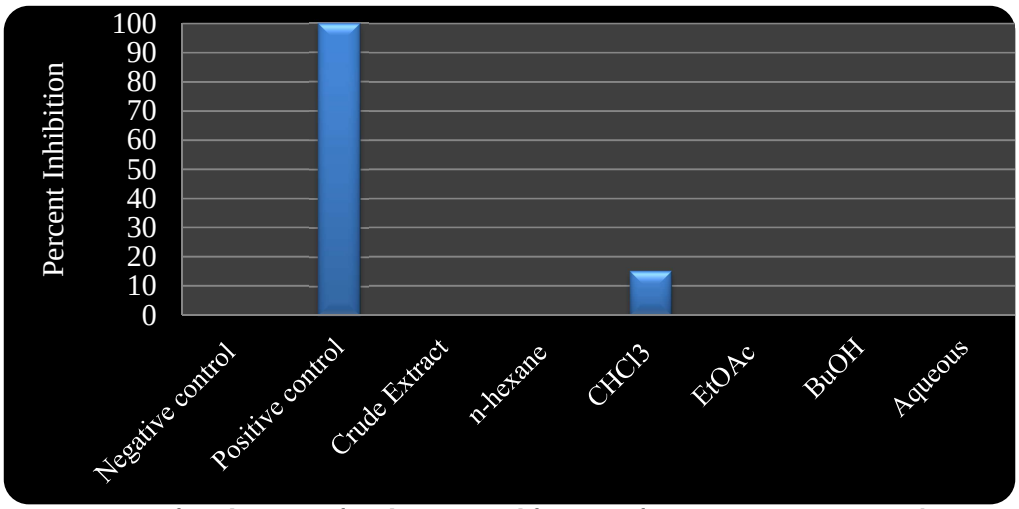


Figure: 3.26. Antifungal activity of crude extract and fractions of *V. agnus castus* against *T. harzianum.*

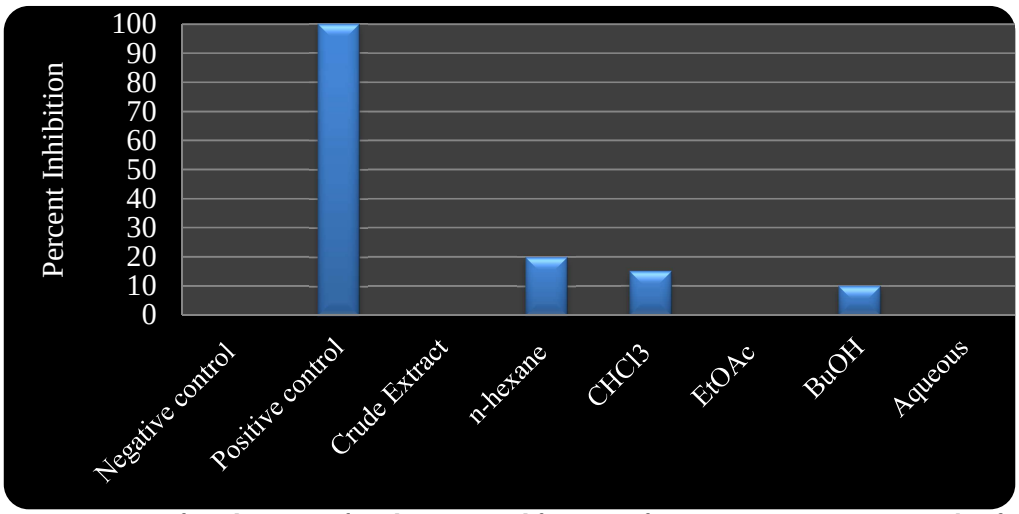


Figure: 3.27. Antifungal activity of crude extract and fractions of *V. agnus castus* against *R. stolonifer*.

3.2.3 INSECTICIDAL ASSAY

Due to the toxic effects of the synthetic insecticides to the environment, more interests are developed in the environment friendly insecticides from the natural resources [111]. The insecticidal activity of crude methanolic extract and various fractions of *Vitex agnus castus* were determined against *Tribolium castaneum, Rhyzopertha dominica* and *Callosbruchus analis* pests. The results are summarized in table 3.9 and presented in figure 3.28. Crude methanolic extract showed moderate (40%) and $CHCl_3$ and *n*-hexane fractions showed low insecticidal activities (25 and 20%) against *C. analis* respectively. The BuOH fraction showed weak insecticidal activity against *T. castaneum* (20%) and *R. dominica* (20%). Rest of the test samples showed no activity against the tested insects. The CO_2 extract from the *Vitex agnus castus* is used as insect repellent for animals [10].

Table 3.9 Insecticidal activity of crude extract and various fractions of *V. agnus castus* by contact toxicity method.

Name of Insects	% Mortality							
	Control Positive*	Control Negative	Crude Extract	*n*-hexane	$CHCl_3$	EtOAc	BuOH	Aqueous
Tribolium castaneum	100	0	0	0	0	0	0	20
Rhyzopertha dominica	100	0	20	0	0	0	0	20
Callosbruchus analis	100	0	40	0	0	25	20	0

* Permethrin at concentration 235.9 $\mu g/cm^2$ was used as standard drug (positive control).

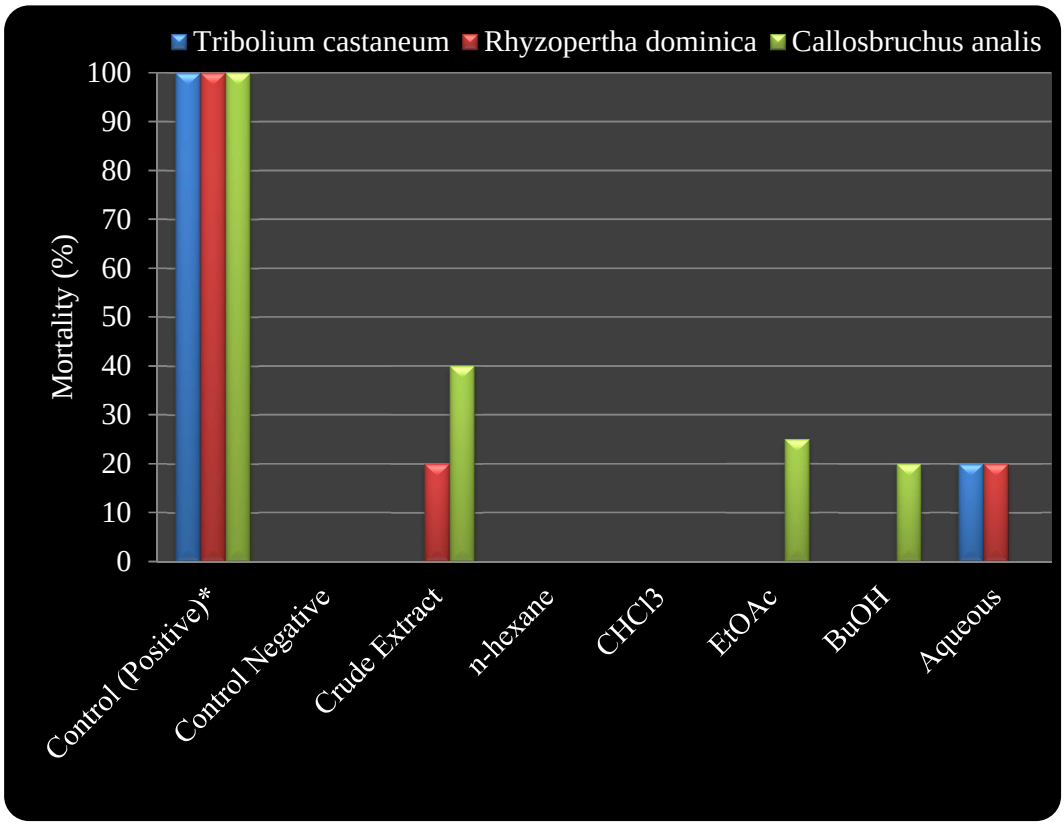


Figure: 3.28. Insecticidal activity of crude extract and fractions of *V. agnus castus.*
* Permethrin at concentration of 235.9 µg/cm^2 was used as standard drug .

3.2.4 ANTI-TERMITE ACTIVITY

The results of anti-termite activity of crude methanolic extract, EtOAc and aqueous fractions of *Vitex agnus castus* are mentioned in table 3.10.

The results indicated that average of the termites killed on day 1st was 10, on second day the average was found to be 18 and on day three all the termites were found dead. Similarly, the EtOAc fraction was screened for anti-termite activity. The results indicated that average of the termites killed on day 1st was 10. On second day the average was found to be 18. On day three all the termites were found dead. The results obtained, for the aqueous fraction, showed that average of the termites killed on day 1st was 2. On second day the average was found to be 14 and on day three all the termites were dead. In the control average of the termites killed on 1st and 2nd day was 0 and only two termites were found dead on day three. The results indicated that the EtOAc fraction of *Vitex agnus castus* showed significant activity against termites.

Table 3.10. Anti-termites activity of crude extract, EtOAc and Aqueous fractions of *V. agnus castus*

Test Sample	**No. of Termites**	**Day**	**Average termites killed by –ive controls**			**Average termites killed by *Positive Control**	**Average termites killed by sample**
			MeOH	**EtOAc**	**H_2O**		
Crude extract	25	1	0	NA	NA	23	10
		2	0	NA	NA	25	18
		3	2	NA	NA	-----	25
EtOAc		1	NA	0	NA	22	10
		2	NA	0	NA	25	18
		3	NA	2	NA	--------	25
Aqueous		1	NA	NA	0	23	2
		2	NA	NA	0	25	14
		3	NA	NA	2	--------	25

* Termisolve B-PRO was used as positive control

3.2.5 PHYTOTOXIC ACTIVITY

Herbicides of the plants origin are friendly to the environment that necessitate for their search. *L. minor* is miniature aquatic monocot, sensitive to bioactive compounds and *Lemna* assay is used to detect natural anti-tumor, phytotoxic compounds and may be useful to detect new plant growth stimulants [112]. The phytotoxic results of the crude methanolic extract and various fractions of *Vitex agnus castus* against *L. minor* are summarized in table 3.11 (a) and 3.11 (b) and presented in figure 3.29. The methanolic crude extract showed (50 and 6.25%), *n*-hexane (62.5 and 12.5%), $CHCl_3$ (43.75 and 25.0%), EtOAc (43.75 and 25.0%), BuOH (37.5 and 18.75%) and aqueous (37.5 and 18.75%) growth inhibition at concentrations 1000 and 100 µg/ml, respectively. At concentration 10 µg/ml, no activity was observed.

Table 3.11 (a). Phytotoxic activity of crude extract and fractions of *V. agnus castus.*

Name of plant	**Concentration of sample (µg/ml)**	**No. of fronds**							**Concentration of standard drug * (µg/ml)**
		Crude extract	***n*-hexane**	**$CHCl_3$**	**EtOAc**	**BuOH**	**Aqueous**	**Control**	
Lemna minor	1000	08	06	09	09	10	10	16	0.015
	100	15	14	12	12	13	13	16	
	10	16	16	14	15	14	15	16	

Table 3.11 (b) Percent growth regulation of the *Lemna minor.*

Concentration of sample (µg/ml)	**Percent growth regulation**						
	Crude methanolic extract	***n*-hexane**	**$CHCl_3$**	**EtOAc**	**BuOH**	**Aqueous**	**Standard**
1000	50	62.5	43.75	43.75	37.5	37.5	100
100	6.25	12.5	25.0	25.0	18.75	18.75	100
10	00	00	12.5	6.25	12.5	6.25	100

*Paraquat was used as standard drug

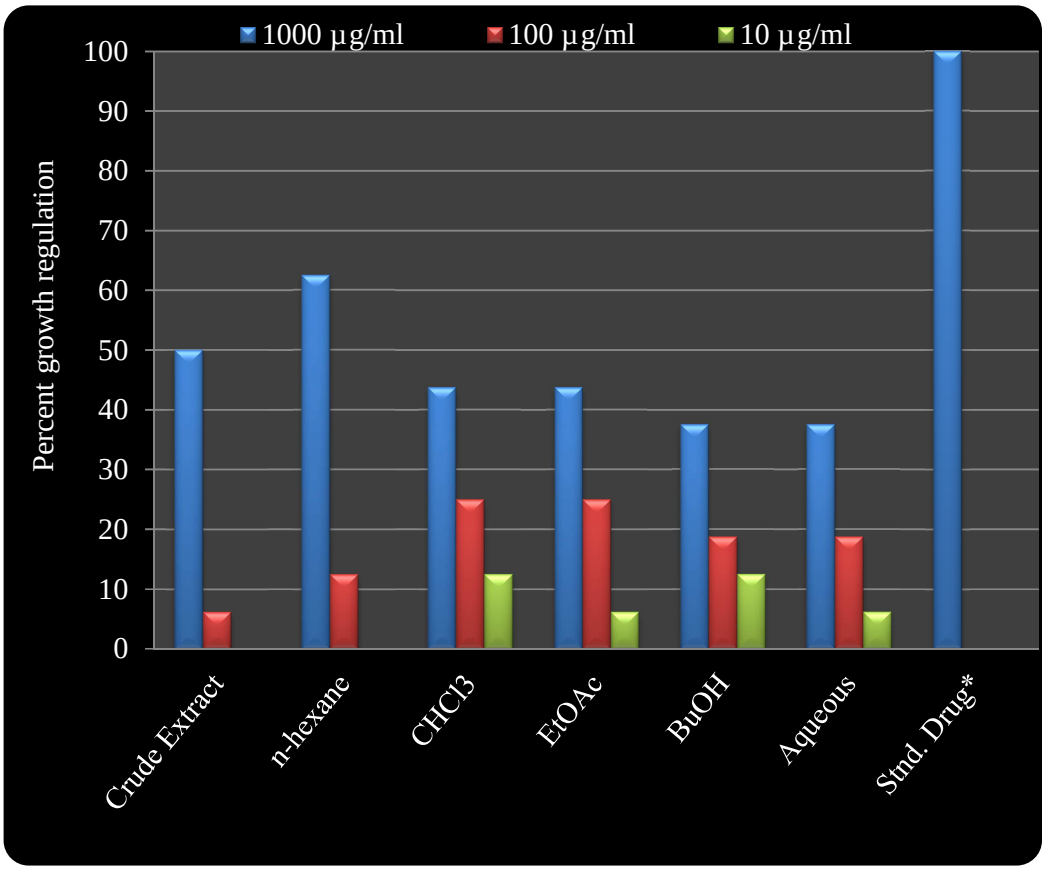


Figure: 3.29. Phytotoxic activity of the crude extract and fractions of *V. agnus castus* against *L. minor*

*** Standard.** Paraquat at a concentration 0.015 µg/ml was used as standard drug

3.2.6 BRINE SHRIMP CYTOTOXICITY

The higher the mortality of the sample(s) in the shrimps test, the lower will be the safety of the drug and vice versa. The results shown in table 3.12 (figure 3.30), reveals that crude extract of the plant showed 23% cytotoxicity at higher dose with LD_{50} value 20683 µg/ml, the lower and upper toxic concentration limits were 1.31175 and 1960.53 with G value 0.7513. The cytotoxic activity showed by *n*-hexane fraction was 26.66% at higher dose with LD_{50} value 26757 µg/ml. The upper and lower limits were 1746.6 and 1.70141 respectively with G value 0.8962.16% cytotoxicity was observed for the $CHCl_3$ fraction having LD_{50} value of 4733740 µg/ml, upper and lower limits toxic concentrations limits were 5249.78 and 0.0001, respectively, with G value of 2.9128. EtOAc fraction of the plant shows 23% cytotoxicity at higher dose with LD_{50} value 4263428 µg/ml, upper and lower toxic concentration limits were 0.0015 and 11002.63 with G value 5.1060. The cytotoxicity observed for the BuOH fraction was 13% at higher concentration with LD_{50} value 11277070 µg/ml, upper and lower limits were 5792 and 0.0005, respectively, with G value of 2.001. Aqueous fraction of the plant killed 33% of the shrimps vs. control having an LD_{50} value 0.0016 µg/ml. The lower and upper toxic limits (concentrations) for the aqueous were 2.3067 µg/ml and 1755340.0 µg/ml, respectively G value was 3.4278, at temperature 28±1^{o}C. The crude ethanolic and methanolic extracts of ripened fruits of *Vitex agnus castus* showed significant cytotoxic effect against various human cancerous cells [113], similarly *Vitex pubescens* Vahl. member of *Verbenaceae* also possess cytotoxic effect against cancerous cell lines [114].

Table 3.12. Brine shrimps cytotoxicity of crude extract and various fractions of *V. agnus castus.*

Dose (µg/ml)	***Standard drug**		**Crude extract**		***n*-hexane**		**$CHCl_3$**		**EtOAc**		**BuOH**		**Aqueous**	
	Survivors	% Killed	Survivors	% Killed	Survivors	% Killed	Survivors	% Killed	Survivors	% Killed	Survivors	% Killed	Survivors	% Killed
1000	0	100	23	23	22	26	25	16	23	23	26	13	20	33
100	0	100	27	10	26	13	26	13	26	13	28	6	25	16
10	0	100	29	3	28	6	28	6	28	6	29	3	27	10

* Etoposide at concentration of 7.4625 µg/ml was used as standard drug.

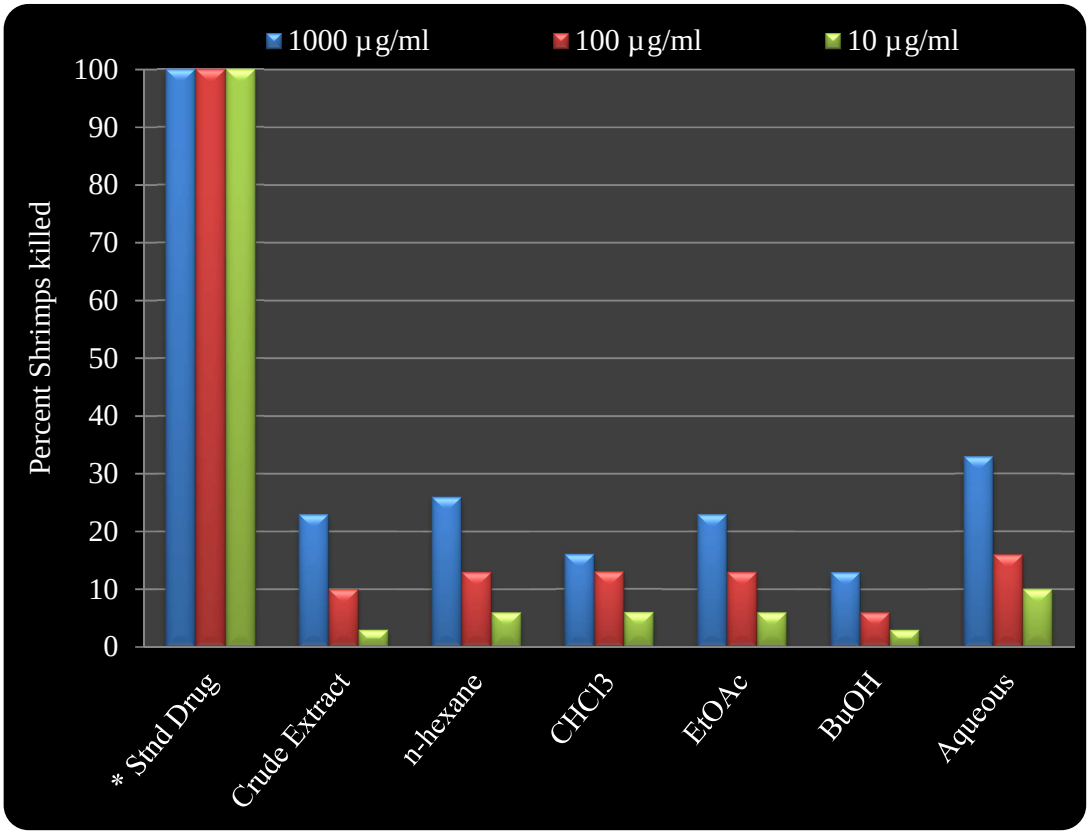


Figure: 3.30. Brine shrimp cytotoxicity of crude extract and fractions of *V. agnus castus.*
* Etoposide at concentration of 7.4625 µg/ml was used as standard drug.

3.2.7 HAEMAGGLUTINATION ACTIVITY

Certain plants have been found to contain agglutinins in their different parts, which can be extracted and used for the production of blood typing reagents in the same way as agglutinins from animal sources. Lectins (carbohydrate-binding proteins) are one of the most important toxic, anti-nutritional and resistant to digestion when ingested [115]. In addition to their ability to agglutinate the erythrocytes of numerous animal species, lectins also clump certain bacteria and precipitate glycogen and starch from solutions. Lectins also negatively affect nutrient utilization by different mechanisms. The plant source agglutinins have advantage over the animal sources because they are economical and available in large quantities.

Each test sample dilutions of *Vitex agnus castus* were screened for haemagglutination activity against human RBCs of all blood groups. In neither of the cases agglutination was shown by the test samples, results are shown in table 3.13. The present investigation reveals that *Vitex agnus castus* contains no phytolectins.

Table 3.13. Haemagglutination activity of the crude extract and fractions of *V. agnus castus* at various dilutions.

Blood groups	**AB^{-ive}, AB^{+ive}, O^{+ive}, O^{-ive}, A^{-ive}, A^{+ive}, B^{-ive}, B^{+ive}**			
Dilutions	1:2	1:4	1:8	1:16
Crude extract	-	-	-	-
n-hexane	-	-	-	-
$CHCl_3$	-	-	-	-
EtOAc	-	-	-	-
BuOH	-	-	-	-
Aqueous	-	-	-	-

No activity = (-)

3.2.8 ANTI-INFLAMMATORY ACTIVITY

Crude extract and fractions

Inflammation is a protective attempt to remove the injurious stimuli and also to initiate the healing process for the tissue. It is a complex biological response of vascular tissues to harmful stimuli like pathogens, damaged cells or irritants [116]. Results are shown in table 3.14 and presented in figure 3.31. Indomethacin, a clinically used anti-inflammatory drug, was used as positive control. At concentration of 500 μg/ml the crude extract showed significant anti-inflammatory activity (90%). EtOAc and the $CHCl_3$ fractions of the plant exhibited moderate activity 43 and 41%, respectively. Other fractions; *n*-hexane, BuOH and aqueous fractions showed low anti-inflammatory activity (table 3.14). The leaves, fruits, roots and seeds of *Vitex negundo* have anti-inflammatory activity [117-119]. As the crude extract of the plant exhibited significant anti-inflammatory activity, thus it can be used as a source for isolations of natural anti-inflammatory constituents.

Table 3.14 Percent inhibition of reduction of WST-1 by NADPH oxidase *via* Super oxides in presence of test samples and positive control, using freshly isolated human neutrophils.

Test sample	**Percent Inhibition at 500 µg/ml**
Crude extract	90.01
n-hexane	35.30
$CHCl_3$	43.43
EtOAc	41.32
BuOH	32.18
Aqueous	24.35
*Indomethacin	98.0

* Standard drug (positive control)

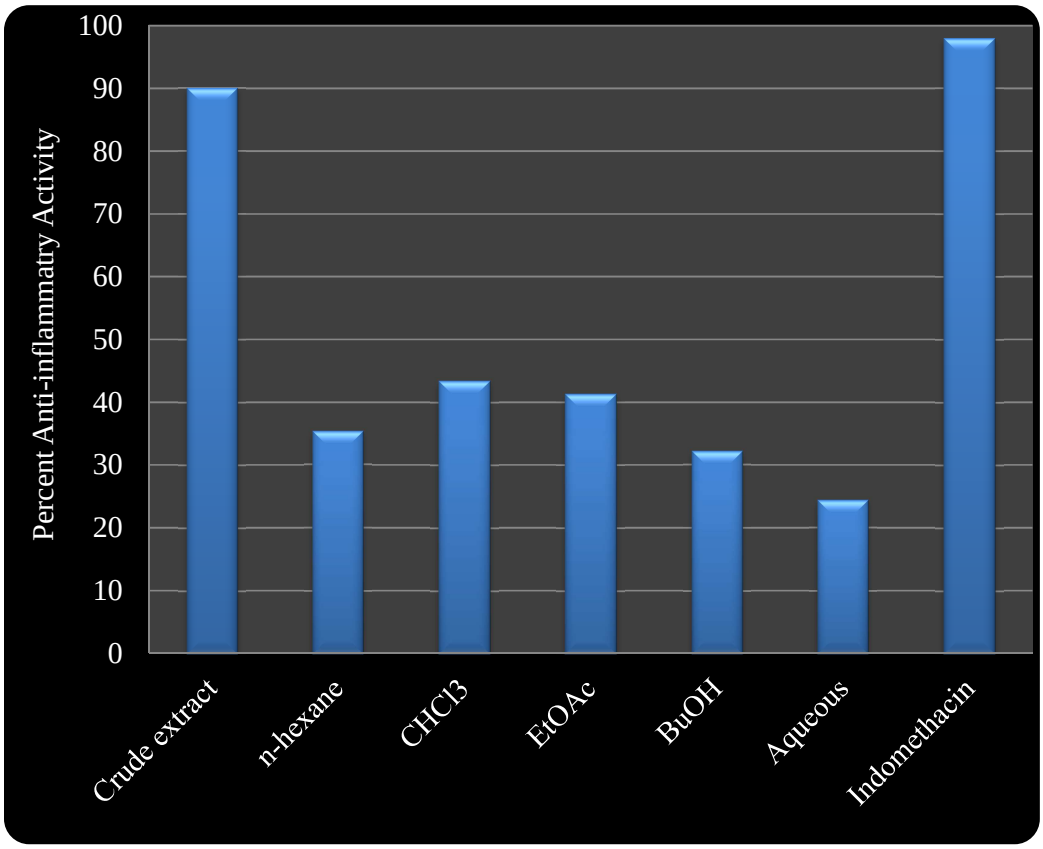


Figure: 3.31. Anti-inflammatory activity of crude extract and fractions of *V. agnus castus*.

Isolated compounds

Different compounds i.e. Casticin, *p*-hydroxybenzoic acid, methyl 3, 4-dihydroxy-benzoate, 3, 4-dihydroxybenzoic acid, isolated from the aerial parts of the *Vitex agnus castus* possessed significant anti-inflammatory activity with MIC_{50} in the range of 156-400 µg [120]. The compounds isolated from the aerial parts of the plant were screened for the anti-inflammatory activity. The compound **1** and **2** showed moderate (41.3, 48%) activity while low (37.2 and 28.5%) anti-inflammatory activity was observed for the compounds **3** and **4** as shown in table 3.15 and presented in figure 3.32.

Table 3.15 Anti-inflammatory activity of isolated compounds from *Vitex agnus castus*.

Test Compound	Anti-inflammatory activity (%)
1	41.3
2	48.0
3	37.8
4	28.5
Indomethacin*	98.0

* Standard drug (positive control)

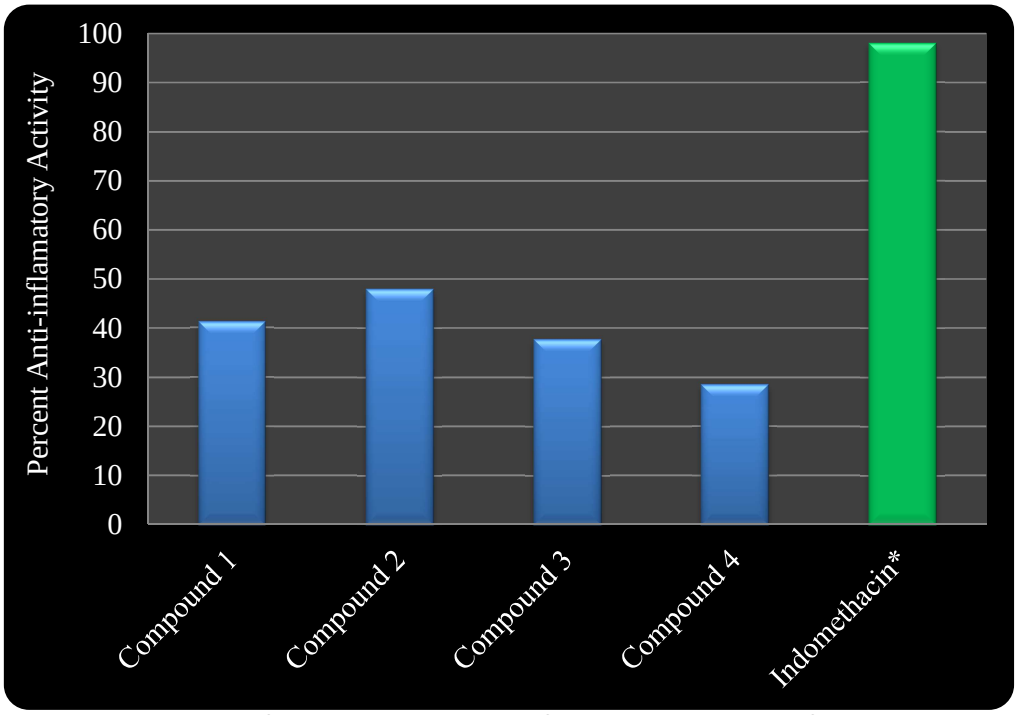


Figure: 3.32. Anti-inflammatory activities of isolated compounds from *V. agnus castus*.
* Standard drug (positive control)

3.2.9 EFFECTS ON RABBITS JEJUNUM PREPARATIONS

Spasmolytic activity

According to the figure 3.33, crude methanolic extract produced concentration dependent relaxation on spontaneous rabbit's jejunum preparations. Maximum relaxation was obtained at concentration 3 mg/ml that 100% relaxed the tissue. In another series of experiments, the extract relaxed the tissue by 74% (figure 3.34) at higher concentration of 10.0 mg/ml.

For comparison, we plotted a combined graph (figure 3.35), the changes are clear as the curves are not superimposable and therefore have different EC_{50} values. This explains that the relaxation is not only through the calcium channel and some other mechanisms are involved that requires further work to explain the mixed action for spasmolytic activity.

Table. 3.16. Spasmolytic activities of crude extract of *V. agnus castus*

Concentration of crude extract (mg/ml)	**Percent effect of control max. (Mean ± S.D, n= 3)**
0.03	97.0
0.1	91.1
0.3	76.4
1.0	38.2
3.0	0.00

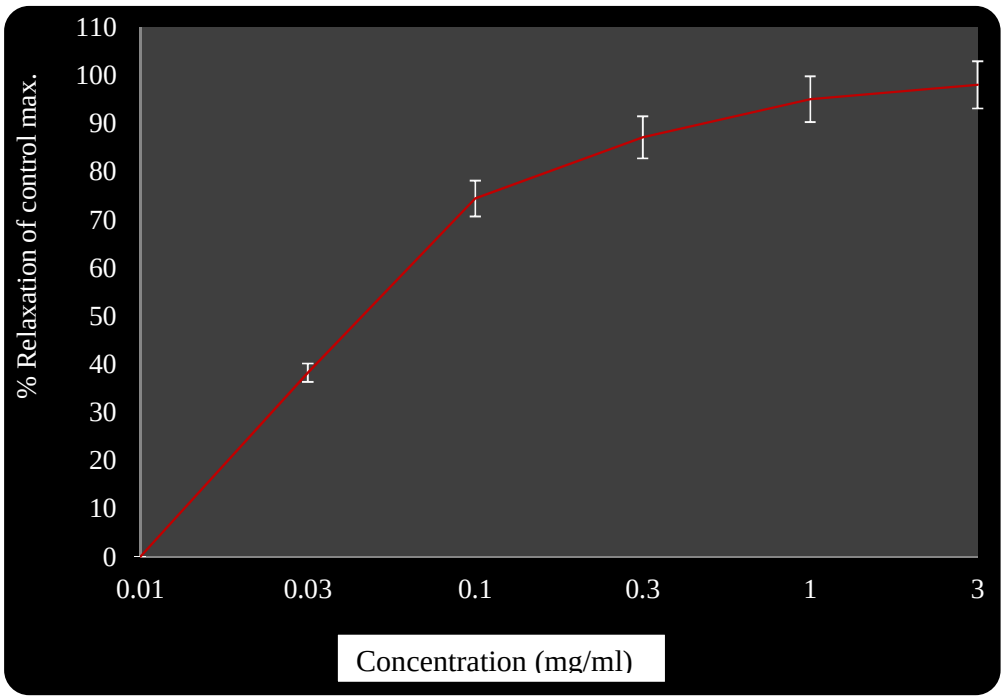


Figure: 3.33. Spasmolytic activity of *V. agnus castus* on spontaneous rabbit's jejunum preparations.

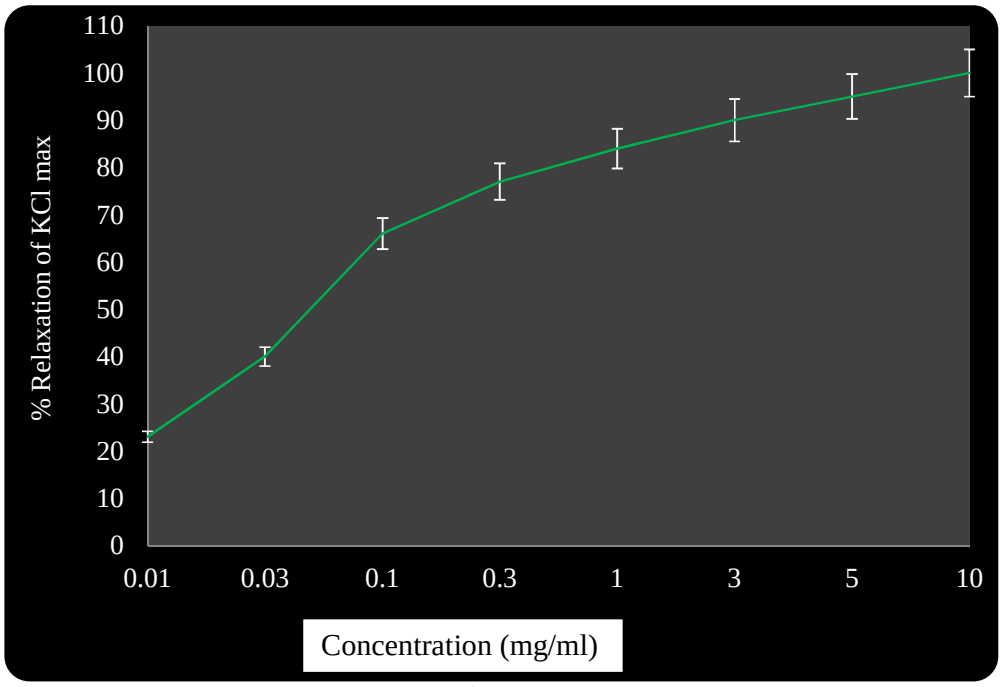


Figure: 3.34. Spasmolytic activity of *V. agnus castus* on spontaneous rabbit's Jejunum preparations.

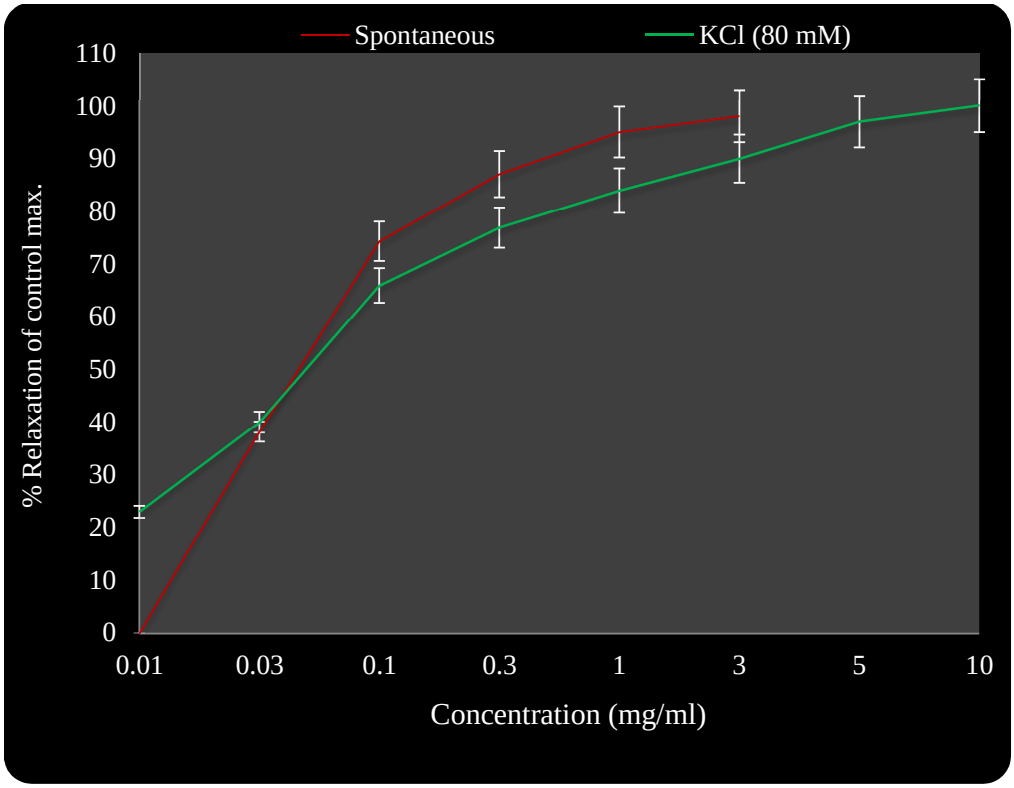


Figure: 3.35. Spasmolytic activity of *Vitex agnus castus* on spontaneous rabbit's jejunum preparations.

3.2.10 NITRIC OXIDE (NO) FREE RADICAL SCAVENGING ASSAY

The role of NO can be attributed to the various physiological and pathological states [121]. NO is an important second messenger, thereby acting as a neurotransmitter, which has a role in the defense against pathogens and in control blood pressure. Various types of cells like neurons, endothelial cells and neutrophils produces NO, by three isoforms of NO synthase enzyme, which are encoded by a unique gene, from the nitrogen of guanidine group of l-arginine and from molecular oxygen [122]. The formation of hazardous radicals i.e. hydroxyl radical and per-oxynitrite anion, are due to the interaction of NO with other radicals. In fact, the reaction of NO with superoxide is very rapid than the latter dose with superoxide dismutase. The plant crude extract and fraction were screened for NO free radical scavenging assay at different concentrations as shown in table 3.17. Results revealed (fig 3.36) that at concentration of 0.3 mg/ml the crude extract and $CHCl_3$ fractions showed moderate NO free radical scavenging activity (30.5 and 30.69%, respectively) while other test samples at this concentration exhibit low activity. Results obtained for the assay at concentration of 0.6 mg/ml for the test samples showed that crude extract and $CHCl_3$ fraction exhibited moderate activity (33.66 and 38.11%, respectively). The rest of fraction showed low activity at this concentration; results are summarized in figure 3.37. Similarly the crude extract and $CHCl_3$ fraction showed moderate (37.31 and 43.12%, respectively). The other test samples showed low NO free radical scavenging activity at concentration of 0.9 mg/ml (figure 3.38). At concentration of 1.2 mg/ml moderate activity was observed for methanolic crude extract (41.47%), *n*-hexane (37.63 %) and $CHCl_3$ (46.64%) fractions while other fractions showed low activity as shown in figure 3.39. As we increased the concentration to 1.5 mg/ml, all of the test samples showed moderate NO radical scavenging activity as shown in figure 3.40. Crude extract (48.47%), *n*- hexane (45.09%), $CHCl_3$ (51.54 %), EtOAc (30.23%) and Aqueous (30.56%)

while the BuOH fraction of the plant showed low (26.69%) NO free radical scavenging activity at this concentration.

The specificity of the NO free radical scavenging assay has been questioned as nitrite is one of the final product between the NO and oxygen reaction, through intermediates such as NO_3, N_2O_4 and N_2O_3 [123] therefore, due to interaction of extract with other nitrogen could decrease the production of nitrite. The above results indicated that the crude fractions of *Vitex agnus castus* showed free radical scavenging activity as the concentration increased. Therefore, further studies are needed to recover the important bioactive compounds responsible for the free radical scavenging activity.

Table. 3.17. Nitric Oxide free radical scavenging assay of the crude extract and fractions of *V. agnus castus*

Concentration of samples (mg/ml)	**Percent Nitric Oxide free radical scavenging assay**					
	Cr. Met. Extract	***n*-hexane**	**$CHCl_3$**	**EtOAc**	**BuOH**	**Aqueous**
0.3	30.5	23.1	30.69	7.00	3.19	4.67
0.6	33.66	24.58	38.11	11.45	8.54	12.35
0.9	37.31	27.22	43.12	18.84	13.15	17.41
1.2	47.41	37.63	46.34	23.56	19.7	24.18
1.5	48.47	45.09	51.54	30.23	26.69	30.56

Standard: Vitamen C was used as standard at concentration of 47.78 µg/ml.

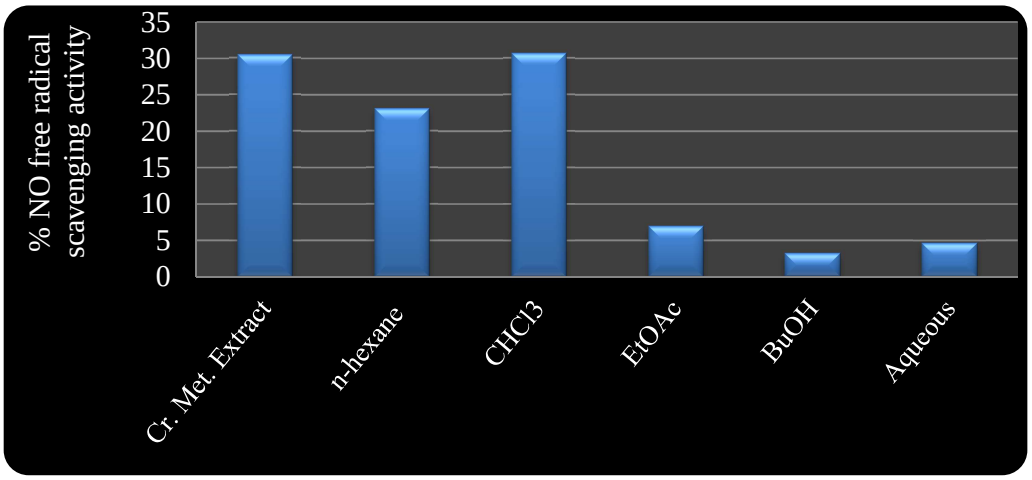


Figure: 3.36. NO free radical scavenging assay of crude and fractions of *V. agnus castus* at concentration of 0.3 mg/ml.

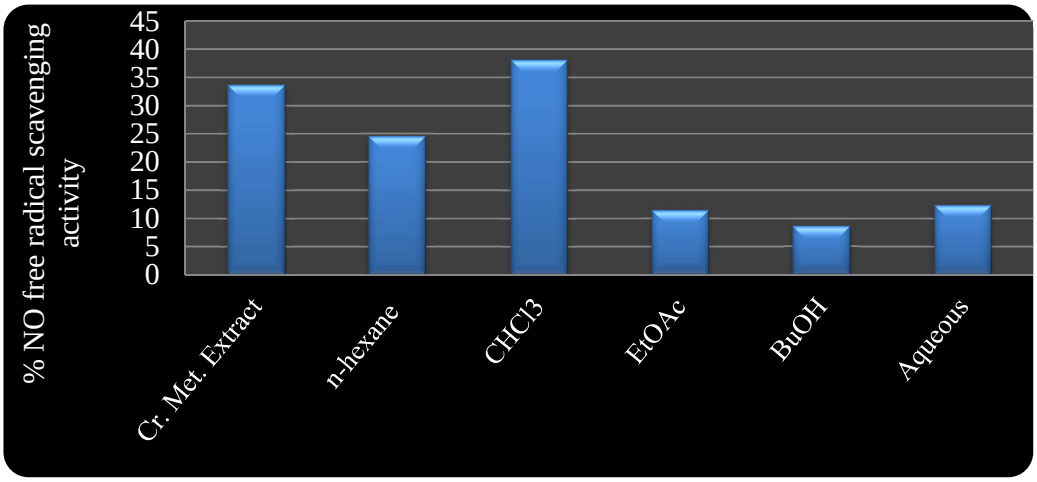


Figure: 3. 37. NO free radical scavenging assay of crude and fractions of *V. agnus castus* at concentration of 0.6 mg/ml.

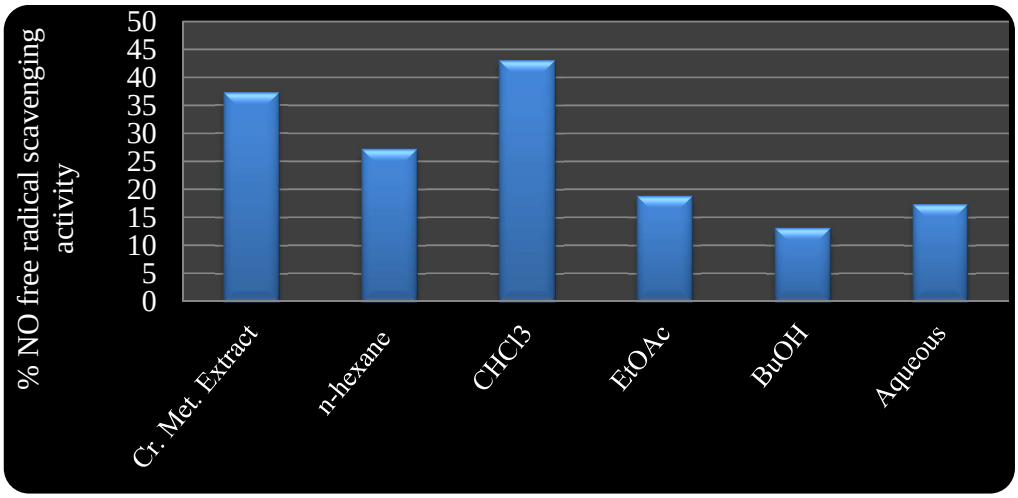


Figure: 3.38. NO free radical scavenging assay of crude and fractions of *V. agnus castus* at concentration of 0.9 mg/ml.

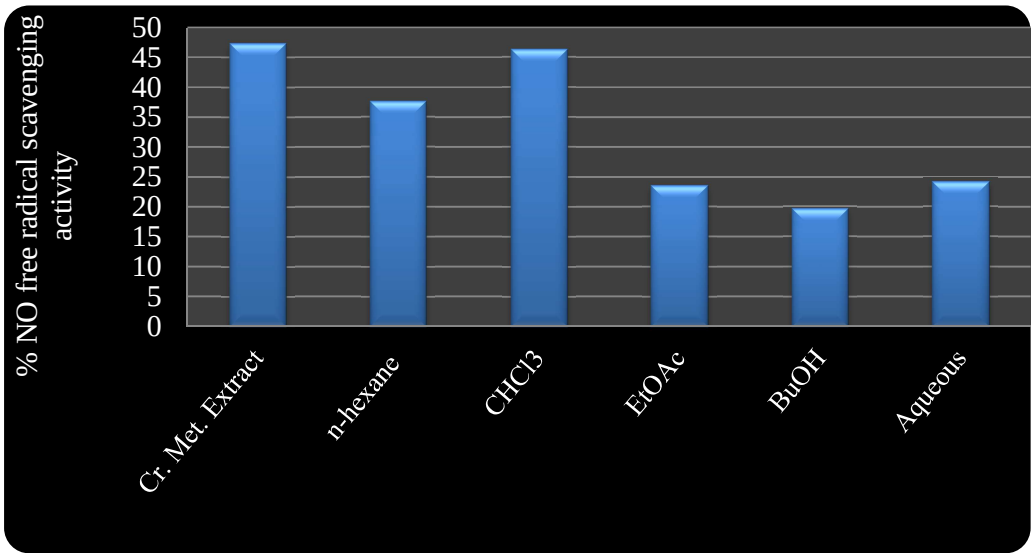


Figure: 3.39. NO free radical scavenging assay of crude and fractions of *V. agnus castus* at concentration of 1.2 mg/ml.

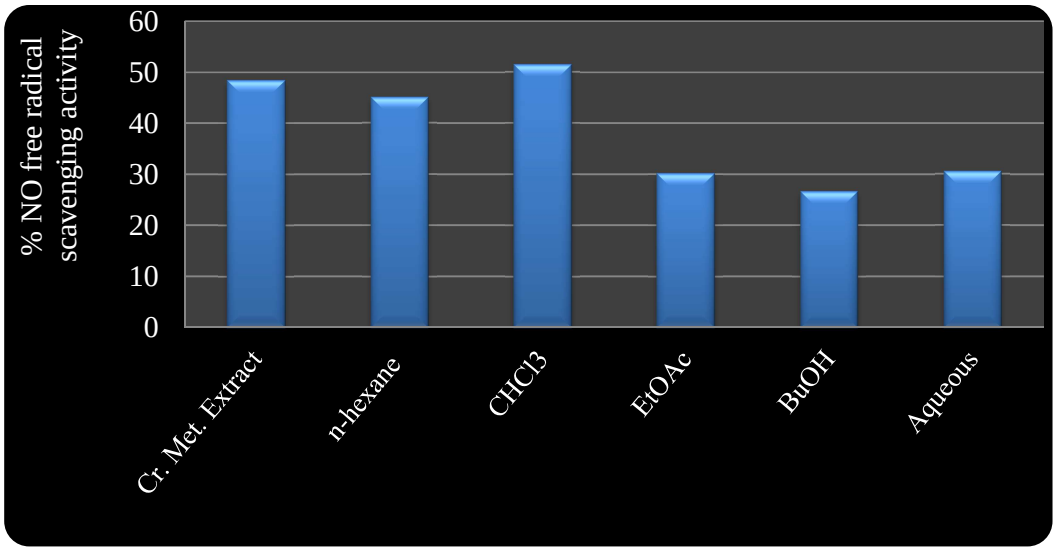


Figure: 3.40. NO free radical scavenging assay of crude and fractions of *V. agnus castus* at concentration of 1.5 mg/ml.

3.2.11 ENZYME INHIBITORY ASSAYS

Urease

Urease, that serves as a powerful immunogen is the most important protein component of the *Helicobacter Pylori* [124-127]. Patients, which have active gastritis due to *H. pylori* infection shows significantly, elevated IgG and IgA levels along with urease level in the serum as compared to the normal levels [128]. In this study, the isolated new compounds were screened against urease enzyme. Compound **1-3** showed moderate (**1** = 32.0%, **2** = 43.3% and **3** = 39.6%) urease inhibitory activity, while the compound **4** showed low (23.0%) urease inhibitory activity. Results are shown in table 3.18 and presented in figure 3.41.

Chymotrypsin

The physiological role of serine proteases inhibitors (SPI) has undoubtedly been recognized. It has been predicted that SPI are the part of plant's natural defense system by inhibiting insect proteinases against insect predation. Due to their inhibitory action, they have gained attention as possible sources of engineered resistance against harmful pests and pathogens for transgenic plants expressing heterologous inhibitors [129-131]. The lignans isolated from the roots of *Vitex negundo* Linn. were found to be active and noncompetitive and competitive inhibitors for α-chymotrypsin, respectively [132]. The compounds isolated from the aerial parts of the plant were screened for chymotrypsin inhibitory activity. Moderate (39.8 and 31.4%) inhibitory activity were shown by compound **2** and **1**, respectively, while low (29.7 and 21.3%) inhibitory activity was shown by Compounds **3** and **4,** against the chymotrypsin enzyme. Results are shown in table 3.18 (figure 3.41).

Table 3.18 Enzyme inhibitory activities of compounds (**1-4**) isolated from *Vitex agnus castus*.

Test Compound	Urease inhibition (%)	Chymotrypsin inhibition (%)
1	32.0	31.4
2	43.3	39.8
3	39.6	29.7
4	23.0	21.3

*Thiourea was used as the standard inhibitor for urease inhibition.
*Chymostatin was used as the standard inhibitor in chymotrypsin inhibition.

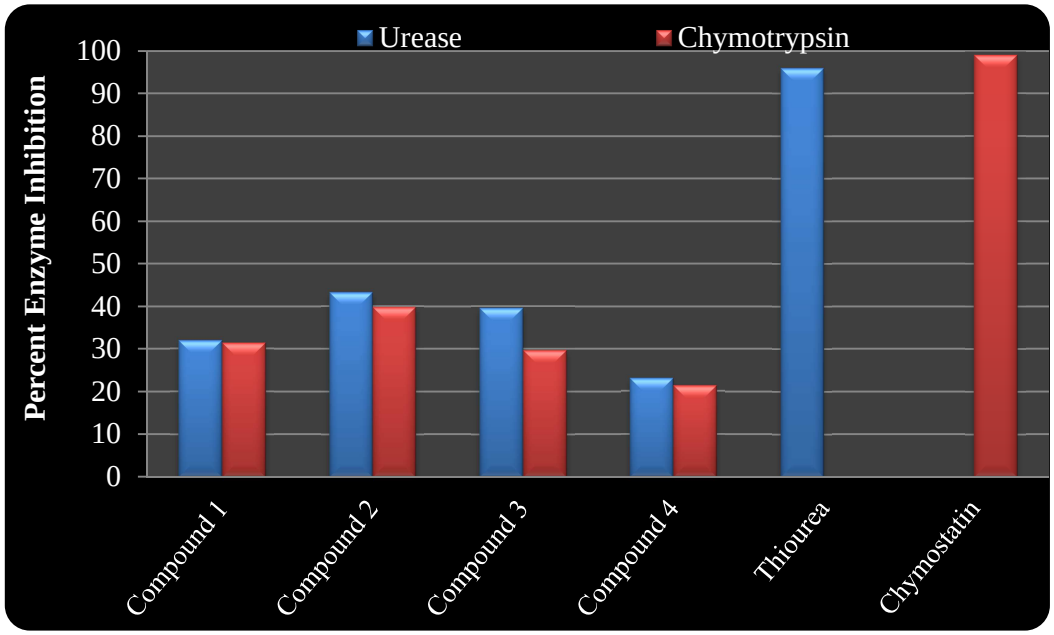


Figure: 3.41. Percent enzyme inhibition of compounds (**1-4**) isolated from *V. agnus castus*.

4.0 INTRODUCTION AND LITERATURE REVIEW

4.1 MYRSINACEAE (FAMILY)

4.1.1 Description

Myrsinaceae, a large family of about 35 genera and upto 1000 species, mainly distributed in the tropical and subtropical regions. Members of the family include trees or shrubs, sometimes scandent. Leaves are simple, alternate, exstipulate, usually coriaceous and glandular. Inflorescence racemose or short fascicled. Flowers are small, 4-5-merous and bi or uni-sexual. Sepals 4-5, free or basally connate, persistent. Corolla 4-5-lobed, with very short to conspicuous tube. Stamens 4-5, epipetalous, opposite to corolla-lobes; staminodes in flowers sometimes quite large; anthers 2-celled, introrse. Ovary superior, rarely semi-inferior, 1-locular with few to many ovules on a basal or free-central placenta, often sunk in placental tissue; style and stigma simple or stigma lobed. Fruits a drupe or a berry, 1 to many-seeded; seeds are globose with fleshy or horny endosperm [133].

4.1.2 Distribution

Myrsine africana is widely distributed from the Himalayas, China and the Azores to eastern and southern Africa and found throughout South Africa, Siberia, Japan, Mexico and Florida and South to New Zealand, South America and South Africa, common in the summer and winter rainfall areas, growing naturally on rocky krantzes, in fynbos and forests. In Pakistan the family is represented by 7 species and 5 genera, found in tropical and subtropical regions [133].

4.1.3 Importance

The Myrsinaceae are well established ethno-anthelmintic and anti-bacterial. A few genera; *Myrsine, Ardisia*, *Lysimachia* and *Cyclamen* are cultivated as ornamental plants, especially *Myrsine africana* and *Ardisia crispa*. *Ardisia japonica* is one of the fifty fundamental herbs in traditional Chinese medicine.

4.2 MYRSINE (GENUS)

4.2.1 Description

Shrubs or small trees, with closely placed, small, coriaceous leaves. Flowers small, in short axillary fascicles, polygamous, often dioecious. Calyx 4-5-lobed, persistent. Corolla 4-5-lobed, rotate. Stamens 4-5, inserted at the base of corolla-lobes; filaments very short to obsolete; anthers short, dithecous. Ovary superior, ovoid-orbicular, 1-locular with several ovules in several rows, on free-central placentae; style very short or obsolete with erect or spreading stigma. Fruit small, globose, subfleshy or dry, often red and smooth, 1-seeded [133].

4.2.2 Distribution

Myrsine is a small genus of about 7 species, mainly distributed in tropical and subtropical regions. In Pakistan the genus *Myrsine* is represented by 2 species which are *Myrsine africana* and *Myrsine semiserrata* [133].

4.2.3 Importance

The *Myrsine*, a small genus of few species have both medicinal and economic importance. Extracts of leaves and fruits of *M. africana* and fruits of *R. melanophloeos* were not efficacious against *Haemonchus contortus* in sheep [134]. Anti-fertility activity of *Embelia ribes* is also reported [135]. The seed extract of the *Rapanes guinensis* Aubl gave nonspecific agglutinating reactions with human blood [136]. From the stem bark of *Myrsine pellucida* 3-O-(6'-O-palmitoyl) beta-D-glucopyranosyl stigmasterol was isolated for the first time [137]. Embelin isolated from *Rapanea melanophloeos* acts as an anti-feedant to *Schistocerca gregaria* and is larvicidal to *Aedes egyptii* larvae [138]. Saponin isolated from the leaves of *M. lanceolata* has shown virucidal, haemolytic and anthelmintic activities [139-140].

4.3 MYRSINE AFRICANA (PLANT)

4.3.1 Description

Botanical Name: *Myrsine africana*

Synonym: *Myrsine retusa* - Aiton

Family: *Myrsinaceae*

Genus: *Myrsine*

Species *Africana*

Myrsine africana, a small and slow-growing evergreen pubescent shrub, sometime soft, stiff and upright shape when old, 1 to 2 m high over time. Thin and dark brown bark with large lenticels. Moderately hard light brown wood. Braclets and pitioles ferrungenous-pubescent. Small oval-shaped leaves, glossy dark green colour, nearly sessile, midrib prominent, shorply toothed, lanceolate and ½ - in. Upper half of the leaf edge is slightly cut with fine teeth; older leaves are tough and leathery, while newly growth is soft with a lovely deep red color. Flowers are nearly sessile, small axillary fascicles; in cluster form, diameter less than ½ in. 4 lobed Calyx and Carolla, 5 merous. 4 Stamens. Anthers exceeding corolla, style short; Stigma capitate, covered by minute prutoberances. Red glands dotted berries, smooth, usually solitary, says Kanjilal; 1/10 in. diameter and swelled when fully ripe. Berry says Clarke, 1 / 8-1 / 6 in. diameter; style branches 2-4, speculate [141].

4.3.2 DISTRIBUTION

Outer Himaliyas from Kashmir and the salt range to Nepal and in khasi hills 300 - 2,700 m. The plant is found in the hilly areas of Hazara regions of Pakistan [142].

4.3.3 IMPORTANCE

Traditionally the plant is used as fragrance in tea, spices, carminative, appetizer and flavoring agent. Its fruits are edible and locally used as an anthelmintic [143-146], for the treatment of diarrhea, rheumatism, toothache, pulmonary tuberculosis and relieving hemorrhage [147].

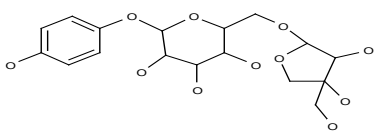
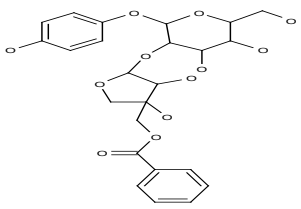
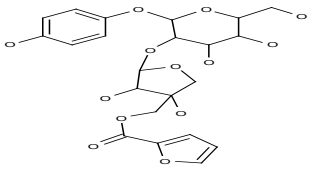
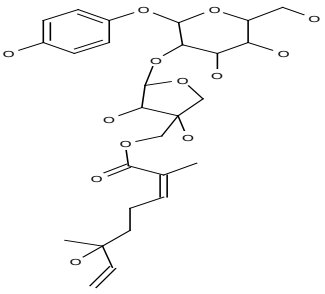
Fruits of the plant are taken as anthelmintics spatially for tapeworm [141], purgative, externally used against ringworm and other skin infections. The aerial parts of the plant are, antifertility and abortifacient [142]. The alcoholic extract of twigs and leaves of the plants possesses significant inhibitory activities against walker intramuscular carcinosarcoma in rates [148]. Emodin and 2-hydroxychrysophanol have been isolated from ethanolic extract of the roots of the plants [149]. Preparations from the mixture of dried fruits and leaves of *M. africana* in water has shown 77% efficacy against *Haemonchus*, *Trichostrogylus* and *Oesophagostomum* spps. [150]. The ethanolic extract of the fruits of *Myrsine africana* showed lethal action against tape worms while ethereal, alcoholic and aqueous extracts had a marked purgative activity in albino rats [151]. Myrsininone A and Myrsininone B, the two novel flavonoids were isolated from the stem of *Myrsine africana* possesses very strong antibacterial activities [152].

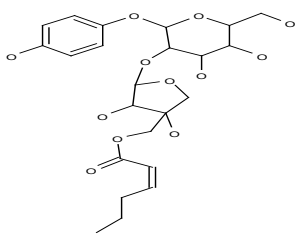
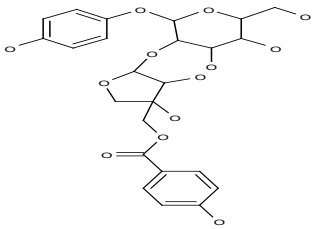
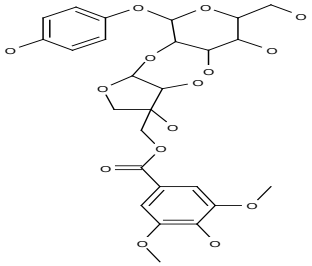
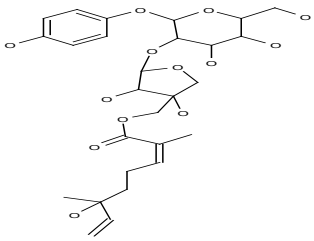
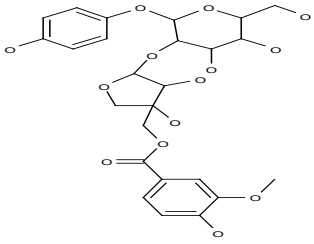
PHYTOCHEMISTRY OF THE GENUS MYRSINE

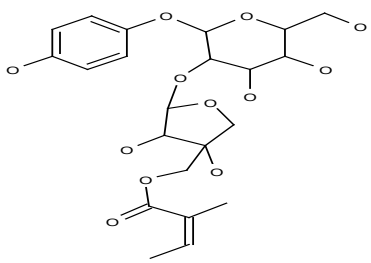
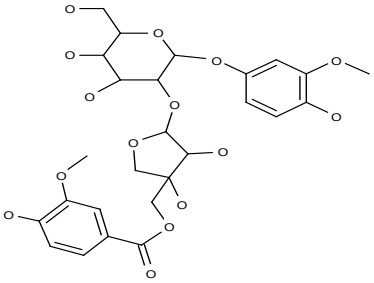
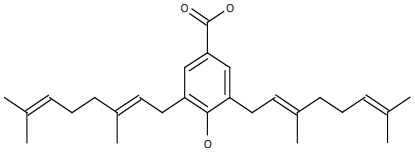
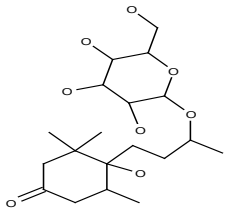
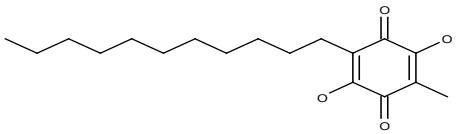
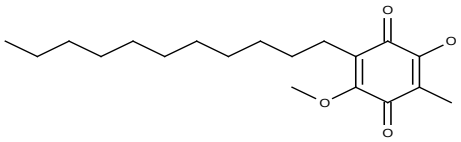
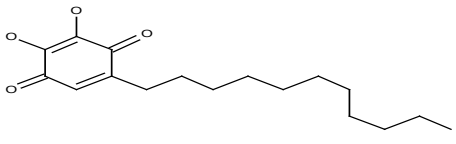
4.4 PHYTOCHEMISTRY OF THE GENUS MYRISINE

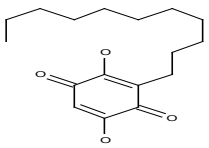
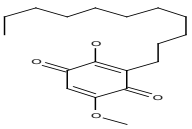
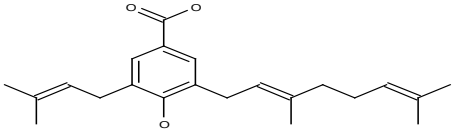
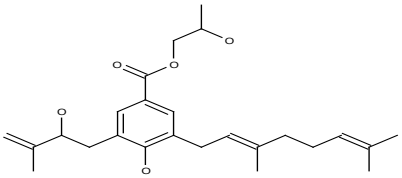
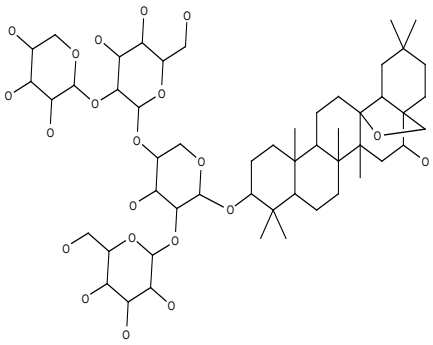
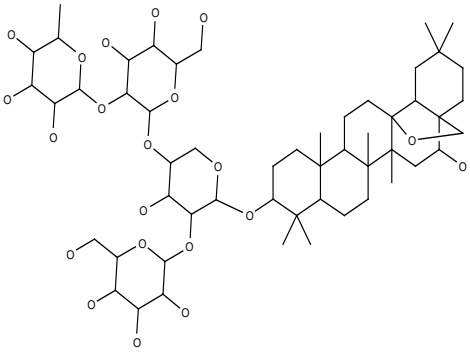
Literature survey revealed that Volatile oils, terpenes, steroids, saponins are present in the genus *Myrsine*. The results of the previous phytochemical investigations on the genus are presented in the following table.

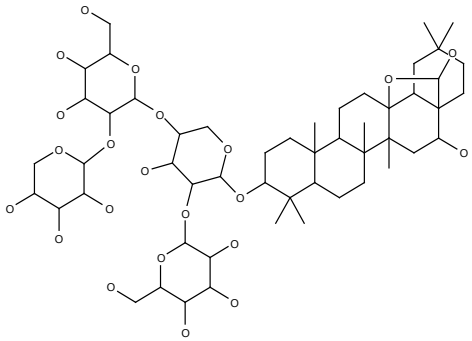
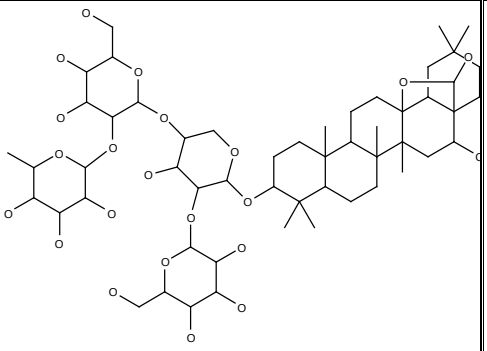
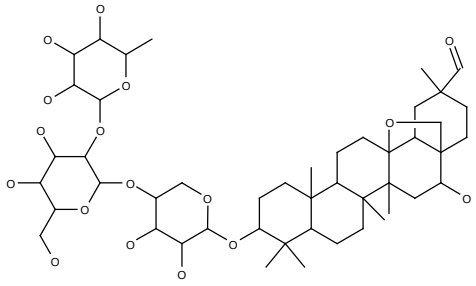
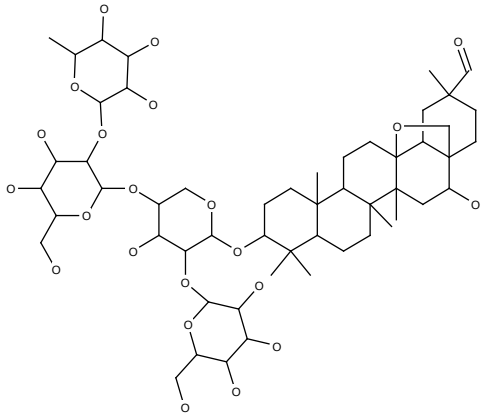
Table 4.1 Chemical constituents from the genus *Myrsine*.

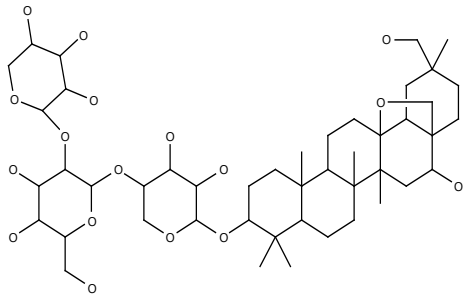
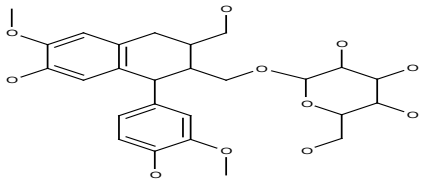
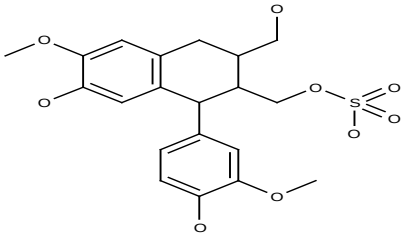
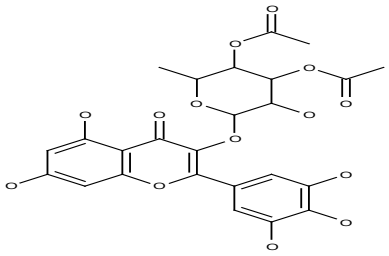
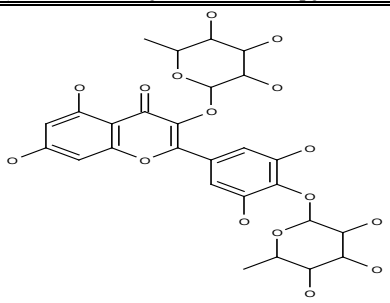
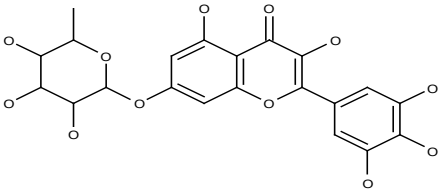
No	Formula/Mol. wt	Compound structure / Name	Reference
1	$C_{17}H_{24}O_{11}$ / 404	 2-O-β-D-Apiofuranosyl	Zhong, XN. et al., Phytochem 1998. 49: 2149 - 2153;
2	$C_{24}H28O_{12}$ / 508	 2-O-(5-O-Benzoyl-β-D-apiofuranosyl	Sakar MK. et al., Fitoterapia, 1991, 62, 176
3	$C_{22}H_{26}O_{13}$ / 498	 2-O-[(2-Furancarbonyl)-(→5)-β-D-apiofuranosyl	Zhong, XN et al., Phytoc. 1999. 52: 923 - 927
4	$C_{27}H_{38}O_{13}$ / 570	 2-O-[6ξ-Hydroxy-2,6-dimethyl-2E,7-octadienoyl-(→5)-β-D-apiofuranosyl	Zhong, XN. et al., Phytochem 1999, 52, 923 -927

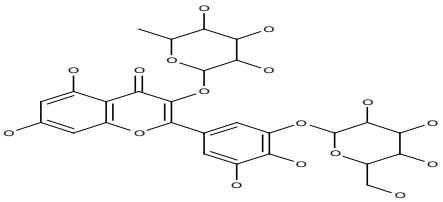
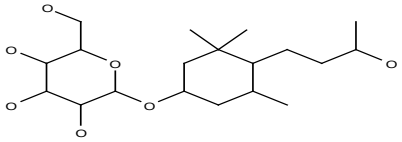
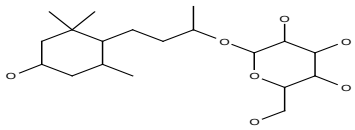
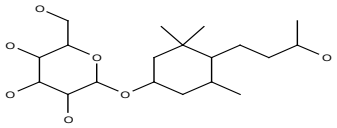
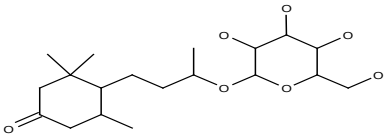
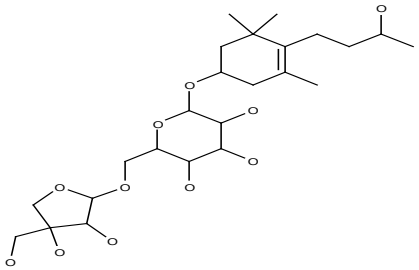
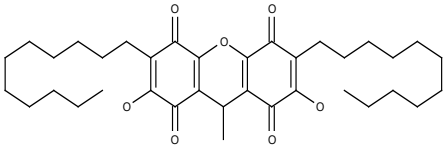
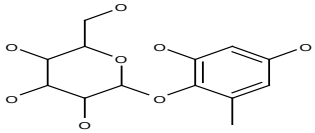
5	$C_{23}H_{32}O_{12}$ / 500	 2-O-[2E-Hexenoyl-(→5)-β-D-apiofuranosyl	Zhong XN et al., Phytochem 1999. 52: 923 -927
6	$C_{24}H_{28}O_{13}$ / 524	 2-O-[(4-Hydroxybenzoyl)-(→5)-β-D-apiofuranosyl	Zhong XN et al., Phytochem 1999. 52: 923 -927
7	$C_{26}H_{32}O_{15}$ / 584	 2-O-[(4-Hydroxy-3,5-dimethoxybenzoyl)-(→5)-β-D-apiofuranosyl	Zhong XN et al., Phytochem 1998. 49: 2149 - 2153
8	$C_{27}H_{38}O_{13}$ / 570	 2-O-[6ξ-Hydroxy-2,6-dimethyl-2E,7-octadienoyl-(→5)-β-D-apiofuranosyl	Zhong XN et al., Phytochem 1999. 52: 923 -927
9	$C_{25}H_{30}O_{14}$ / 554	 2-O-[(4-Hydroxy-3-methoxybenzoyl)-(→5)-β-D-apiofuranosyl	Zhong XN et al., Phytochem 1998. 49: 2149 - 2153

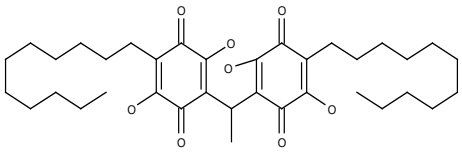
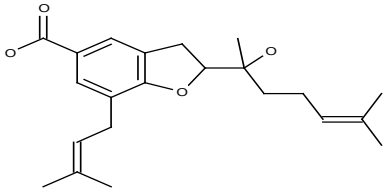
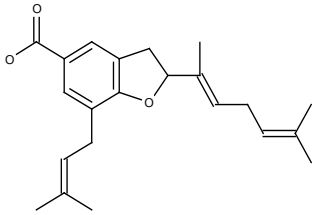
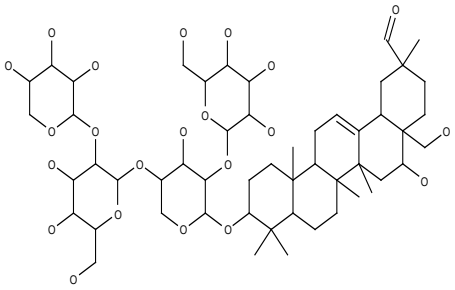
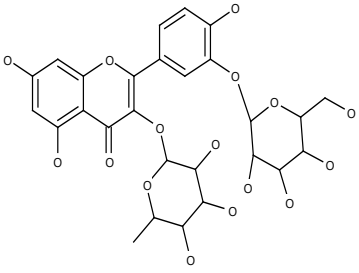
10	$C_{22}H_{30}O_{12}$ / 486	 2-O-(5-O-Tigloyl-β-D-apiofuranosyl	Zhong XN et al., Phytochem 1998. 49: 2149 – 2153
11	$C_{26}H_{32}O_{15}$ / 584	 2-Me ether, 4-O-[4-hydroxy-3-methoxybenzoyl-(→5)-β-D-apiofuranosyl-(1→2)-β-D-glucopyranoside	Zhong XN et al., Phytochem 1999. 52: 923 -927
12	$C_{27}H_{38}O_3$ / 410	 Myrsinoic acid E	Makabe, H. et al., Biosci., Biotechnol., Biochem., 2003, 67, 2038-2041
13	$C_{19}H_{34}O_8$ / 390	 Myrsinionoside B	Otsuka, H. et al., Chem. Pharm. Bull., 2001, 49, 1093-1097
14	$C_{18}H_{28}O_4$ / 308	 2,5-Dihydroxy-3-methyl-6-undecyl-1,4-benzoquinone	Midiwo, J.O. et al., Nat. Prod. Lett., 1996, 8, 11-14
15	$C_{19}H_{30}O_4$ / 322	 2-Hydroxy-5-methoxy-3-methyl-6-undecyl-1,4-benzoquinon	Midiwo, J.O. et al., Nat. Prod. Lett., 1996, 8, 11-14
16	$C_{17}H_{26}O_4$ / 294	 2,3-Dihydroxy-5-undecyl-1,4-benzoquinone	Midiwo, J.O. et al., Bull. Chem. Soc. Ethiop., 1992, 6, 15

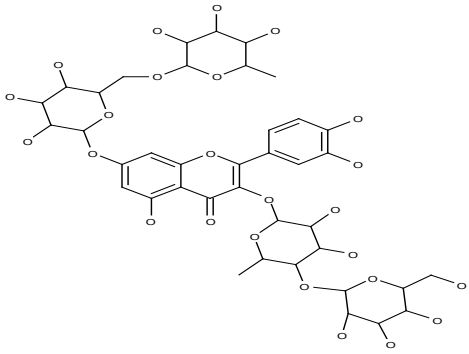
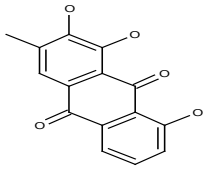
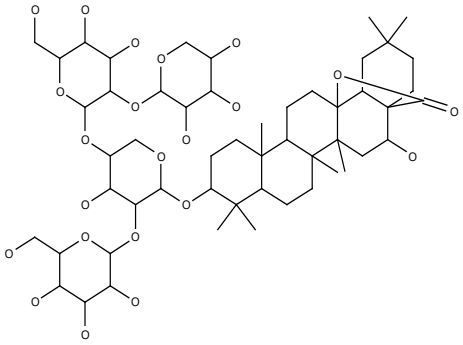
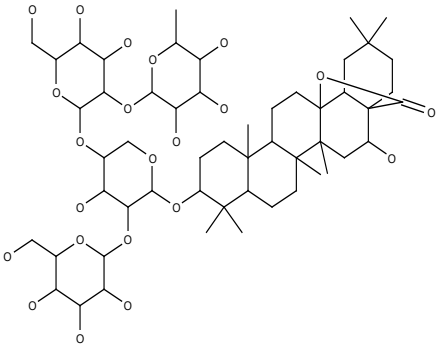
17	$C_{17}H_{26}O_4$ / 294	 2,5-Dihydroxy-3-undecyl-1,4-benzoquinone	Merian, M. et al., Helv. Chim. Acta, 1948, 31, 2237
18	$C_{18}H_{28}O_4$ / 308	 2-Hydroxy-5-methoxy-3-undecyl-1,4-benzoquinone	Merian, M. et al., Helv. Chim. Acta, 1948, 31, 2237
19	$C_{22}H_{30}O_3$ / 342	 3-Geranyl-4-hydroxy-5-prenylbenzoic acid	Hirota, M. et al., Biosci., Biotechnol., Biochem., 1999, 63, 1650-1653
20	$C_{25}H_{36}O_5$ / 416	 Myrsicorianol	Hirota, M. et al., Biosci., Biotechnol., Biochem., 2002
21	$C_{52}H_{86}O_{21}$ / 1047	 3-O-[β-D-Xylopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→2)]-α-L-arabinopyranoside	Kohda, H. et al., Chem. Pharm. Bull., 1989, 37, 3304-3305
22	$C_{53}H_{88}O_{21}$ / 1061	 3-O-[β-D-Rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→2)]-α-L-arabinopyranoside	Kohda, H. et al., Chem. Pharm. Bull., 1989, 37, 3304-3305

23	$C_{52}H_{86}O_{22}$ / 1063	 3-O-[β-D-Xylopyranosyl-(1→2)-β-D-gluco-pyranosyl-(1→4)-[β-D-glucopyranosyl-(1→2)]-α-L-arabinopyranoside	Bloor, S.J. et al., Journal of natural product, 1994, 57, 1354-1360
24	$C_{53}H_{88}O_{22}$ / 1077	 3-O-[β-D-Rhamnopyranosyl-(1→2)-β-D-gluco-pyranosyl-(1→4)-[β-D-glucopyranosyl-(1→2)]-α-L-arabinopyranoside	Bloor, S.J. et al., Journal of natural product, 1994, 57, 1354-1360
25	$C_{47}H_{76}O_{17}$ / 913	 30-Aldehyde, 3-O-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-α-L-arabinopyranoside	Lavaud, C. et al., Photochem, 1994, 37, 1671
26	$C_{53}H_{86}O_{22}$ / 1075	 30-Aldehyde, 3-O-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→2)]-α-L-arabinopyranoside	Lavaud, C. et al., Photochem, 1994, 37, 1671

27	$C_{46}H_{76}O_{17}$ / 901	 3-O-[β-D-Xylopyranosyl-(1→2)-β-D-gluco-pyranosyl-(1→4)-α-L-arabinopyranoside	Lavaud, C. et al., Photochem, 1994, 37, 1671
28	$C_{26}H_{34}O_{11}$ / 522	 3′,5-Di-Me ether, 9′-O-β-D-glucopyranoside	Latté, K.P. et al., Phytochem, 2008, 69, 820-826 (Isolariciresinol 9′-glucoside
29	$C_{20}H_{24}O_9S$ / 440	 3′,5-Di-Me ether, 9′-O-sulfate	Zhong, X.-N. et al., Phytochem, 1998, 49, 1777 - 1778 (Isolariciresinol 9′-sulfate
30	$C_{25}H_{24}O_{14}$ / 548	 3-O-(3,4-Di-O-acetyl-α-L-rhamnopyranoside	Timbola, A.K. et al., Fitoterapia, 2002, 73, 174-176
31	$C_{27}H_{30}O_{16}$ / 610	 3,4′-Di-O-α-L-rhamnopyranoside	He, H.-P. et al., CA, 2001, 135, 270054n
32	$C_{21}H_{20}O_{12}$ / 464	 7-O-α-L-Rhamnopyranoside	He, H.-P. et al., CA, 2001, 135, 270054n

33	$C_{27}H_{30}O_{17}$ / 626	 3-O-α-L-Rhamnopyranoside, 3′-O-β-D-glucopyranoside	David, J.M. et al., J. Braz. Chem. Soc., 1996, 7, 115-148
34	$C_{19}H_{36}O_7$ / 376	 3-O-β-D-Glucopyranoside	Otsuka, H. et al., Chem. Pharm. Bull., 1995, 43, 754-759
35	$C_{19}H_{36}O_7$ / 376	 9-O-β-D-Glucopyranoside	Otsuka, H. et al., Chem. Pharm. Bull., 2001, 49, 1093-1097
36	$C_{19}H_{36}O_7$ / 376	 3-O-β-D-Glucopyranoside	Otsuka, H. et al., Chem. Pharm. Bull., 2001, 49, 1093-1097
37	$C_{19}H_{34}O_7$ / 374	 3-Ketone, 9-O-β-D-glucopyranoside	Otsuka, H. et al., Chem. Pharm. Bull., 2001, 49, 1093-1097
38	$C_{24}H_{42}O_{11}$ / 506	 3-O-[β-D-Apiofuranosyl(1→6)-β-D glucopyranoside]	Otsuka, H. et al., Chem. Pharm. Bull., 2001, 49, 1093-1097
39	$C_{36}H_{52}O_7$ / 596	 Methylanhydrovilangin	Arot Manguro, L.O. et al., Phytochem, 2003, 64, 855-862
40	$C_{13}H_{18}O_8$ / 302	 Myrsinoside B	Pettersson, G.Acta Chem. Scand., 1965, 18

41	$C_{36}H_{54}O_8$ / 614	 Methylvilangin	Arot Manguro, LO et al., Phytochem, 2003, 64, 855-862
42	$C_{22}H_{30}O_4$ / 358	 Myrsinoic acid B	Januário, AH et al., Phytochem, 1991, 30, 2019 - 2023
43	$C_{22}H_{28}O_3$ / 340	 1′-Deoxy, 1′,2′-didehydro(E-)	Blunt, S.B. et al., J. Nat. Prod., 1998, 61, 1400-1403
44	$C_{52}H_{84}O_{22}$ / 1061	 30-Aldehyde, 3-O-[β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→2)]-α-L-arabinopyranoside]	Lavaud, C. et al., Phytochem, 1994, 37, 1671
45	$C_{27}H_{30}O_{16}$ / 610	 3-O-α-L-Rhamnopyranoside, 3′-O-β-D-glucopyranoside	Zhong, X.N. et al., Phytochem, 1997, 46, 943-946

46	$C_{39}H_{50}O_{25}$ / 918	 3-O-[β-D-Glucopyranosyl-(1→4)-α-L-rhamnopyranoside], 7-O-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside]	Zhong, X.N. et al., Phytochem, 1997, 46, 943-946
47	$C_{15}H_{10}O_5$ / 270	 1,2,8-Trihydroxy-3-methylanthraquinone	Takido, M.Chem. Pharm. Bull., 1958, 6, 397
48	$C_{52}H_{84}O_{22}$ / 1061	 28→13 Lactone, 3-O-[β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→2)]-α-L-arabinopyranoside]	Bloor, S.J. et al., J. Nat. Prod., 1994, 57, 1354-1360 (*Myrsine australis* saponins)
49	$C_{53}H_{86}O_{22}$ / 1075	 28→13 Lactone, 3-O-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→2)]-α-L-arabinopyranoside]	Bloor, S.J. et al., J. Nat. Prod., 1994, 57, 1354-1360 (*Myrsine australis* saponins)

TRITERPENOIDS

4.5 TRITERPENOIDS

Triterpenoids, characterized by a carboxylic ring system and extended carbon chains oxidized to unstable degrees to alcohol or carbonyl group, form a bulky group of natural products. Essential oils are low molecular weight terpenes, volatile in nature, are used in perfume industry, pheromones in insects, employed as solvents in paints, artificial flavoring agents and preservatives for various food stuffs. In both plant and animal kingdoms the terpenoids are widely distributed. The pentacyclic triterpenes are more widespread in plants while the tetracyclic triterpenoids, in particular lanosterol and its derivatives are more frequent in animals, these may be found free or may be in conjugated forms, with one or more molecules.

The five carbon unit of terpenes called isoprene. The isoprene rule states that, “Terpenes are derived from integral number of biological equivalents of isoprene which are joined together in a head-to-head or head-to-tail” fashion. The role of isoprene as the “monomer” of terpenes is reflected in the term of isoprenoids, which is used to describe this class of natural products.

4.5.1 Importance

Triterpenoids, oleanolic and ursolic acids are found to possess strong activity against human lymphoma and leukemia cell lines. The derivatives of these triterpenoids possess significant anti-inflammatory and anti-carcinogenic activities. Anti-proliferative effect for the members of cycloartane, lupine, ursane, oleanane, friedelane (especially quinine methides), dammarene, cucurbitacin and limonoid terpenoids, have been demonstrated.

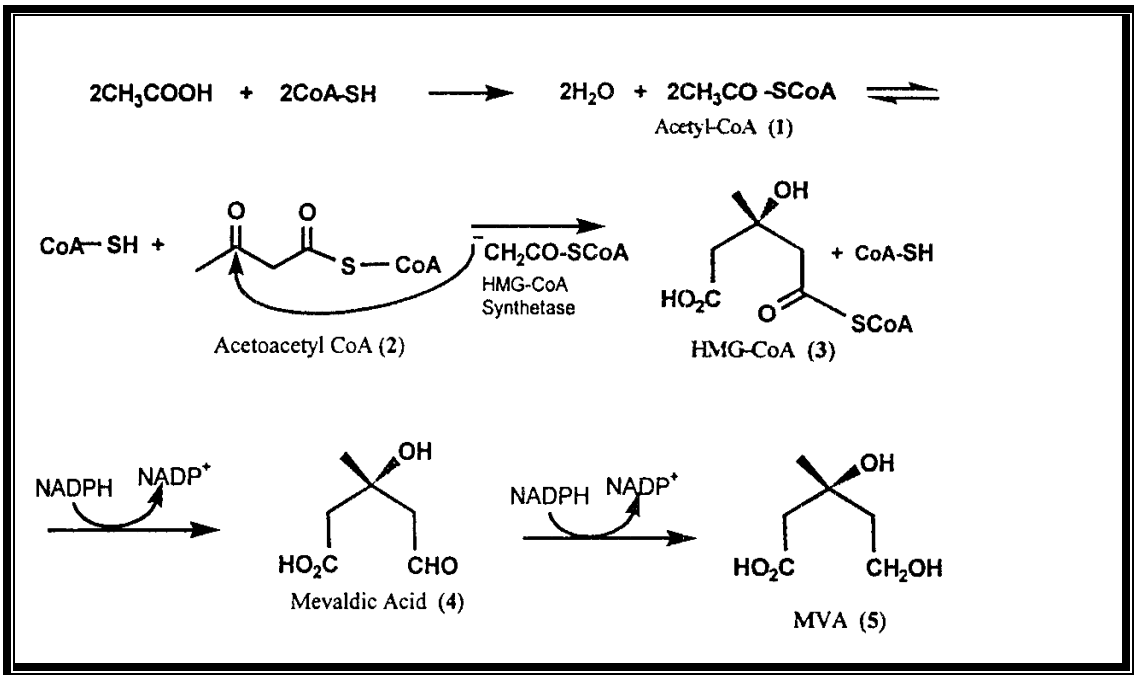
4.5.2 Biosynthesis of Triterpenoids

The following three steps are involved in the biosynthesis of triterpenoids;

1. The isoprene unit formation from acetate.

2. The isoprene unit's condensation to form acyclic terpenoids.
3. The conservation of acyclic terpenoids into terpenoids and the introduction of functional groups.

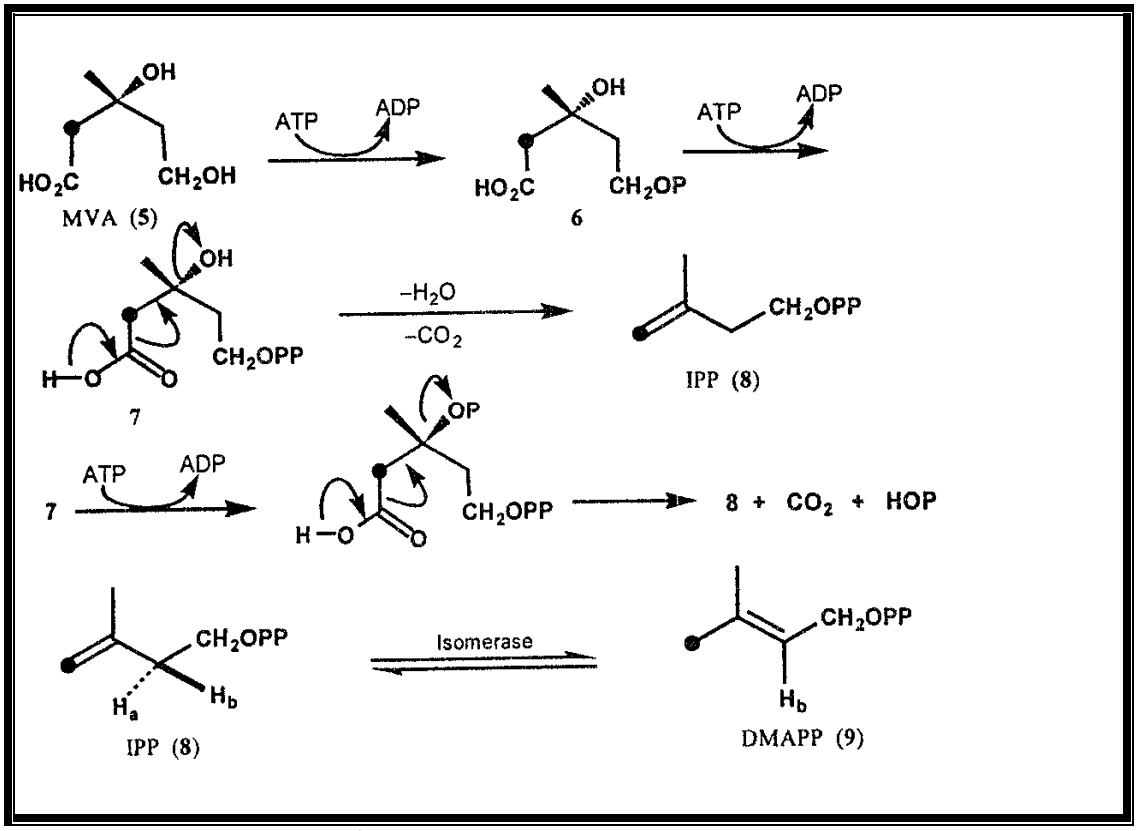
According to the following **Scheme (9)**, the triterpenoids are resulting from mevalonic acid (5), through squalene and 2,3-epoxysqulene (15) and from Acetyl Coenzyme A (Acetyl CoA) (1) the formation of Mevalonic acid (5) takes place. Acetyl CoA (1) (Two molecules) give rise to acetoacetyl CoA (2) through Claisen condensation reaction and through aldol condensation reaction with another molecule of acetyl CoA, acetoacetyl-CoA (2) produce 3-hydroxy-3-methylglutaryl CoA (3) (HMG-CoA).



Scheme 9: Biosynthesis of Mevalonic Acid **(5)**

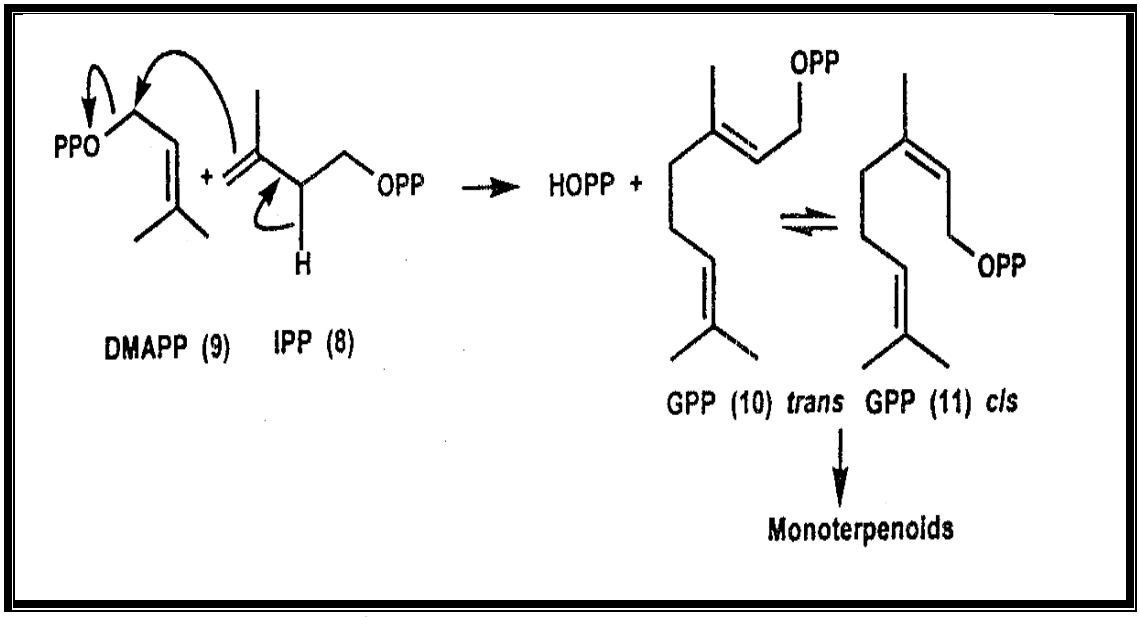
After reduction of HMG-CoA (3) with NADPH the mevalonic acid (5) is produced [153-154] through melvaldic acid (4). The catalyzing enzyme for this reaction is hydroxy-methyl-glutaryl CoA reductase **(Scheme 9)**. The mevalonic acid (5) is converted into 5-phosphomevalonic acid (6) through an ATP-dependent mevalonate kinase enzyme, catalyze the reaction, the resulting 5-phosphomevalonic acid (6) is again phosphorylated to yield 5-

phosphosphatemevalonic acid (7) and then by the loss of one water molecule MVA 5-pyrophosphate (7) form 3-methylbut-3-enyl (isopentenyl) pyrophosphate (IPP, 8). The IPP is a biological "isoprene unit" which then undergoes isomerization to yield dimethyl-allyl pyrophosphate (DMAPP, 9) [155], isopentenyl diphosphate-isomerase is the catalyzing enzyme for the reaction **(Scheme- 10)**.



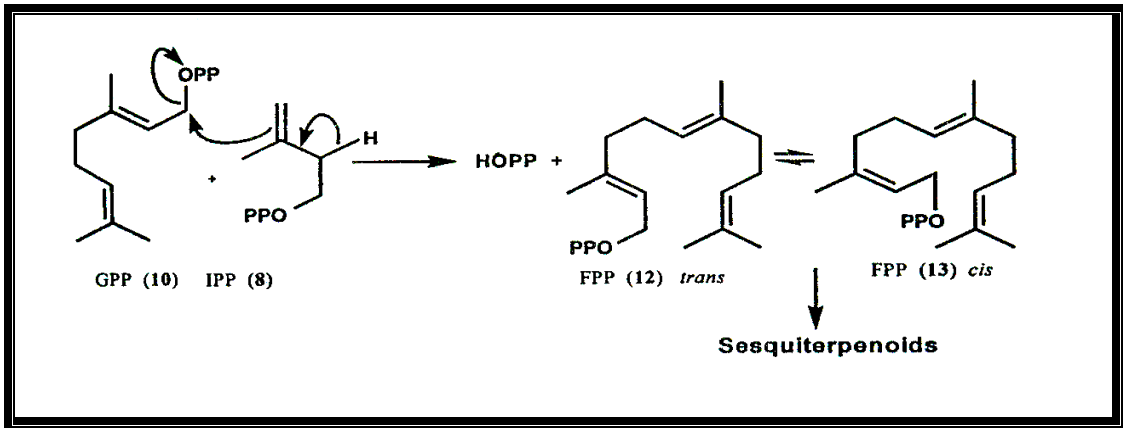
Scheme 10: Biosynthesis of dimethylallyl pyrophosphate **(9).**

Then by the combination of IPP (8) and DMAPP (9) geranyl pyrophosphate (GPP, 10, 11) is produced, IPP act as nucleophilic reagent, while GPP as electrophilic reagent which give rise to head-to-tail union **(Scheme 11)**.



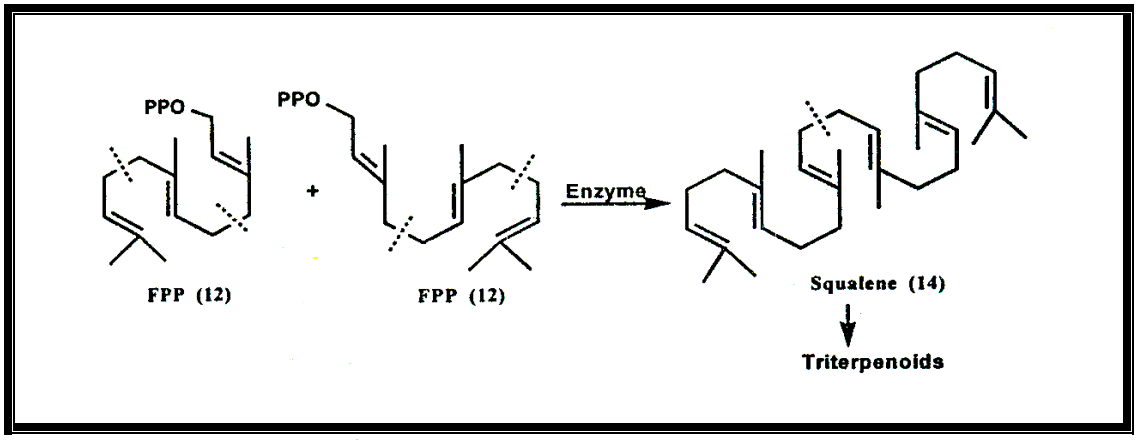
Scheme 11: Biosynthesis of geranyl pyrophosphate **(10)**

The *cis* and *trans* isomers of the 15-carbon compounds, farnesyl pyrophosphate (FPP, 12, 13), is produced when GPP (10) reacts with IPP (8) which extend the chain by another five-carbon unit **(Scheme 12)** [156, 157].



Scheme 12: Biosynthesis of farnesyl pyrophosphate **(12)**

A thirty carbon molecule known as squalene, an intermediate biological precursor for all triterpenoids, is formed when two farnesyl pyrophosphate (FPP, 12) molecules are joined in a tail-to-tail dimerization process in the presence of a suitable enzyme [158].

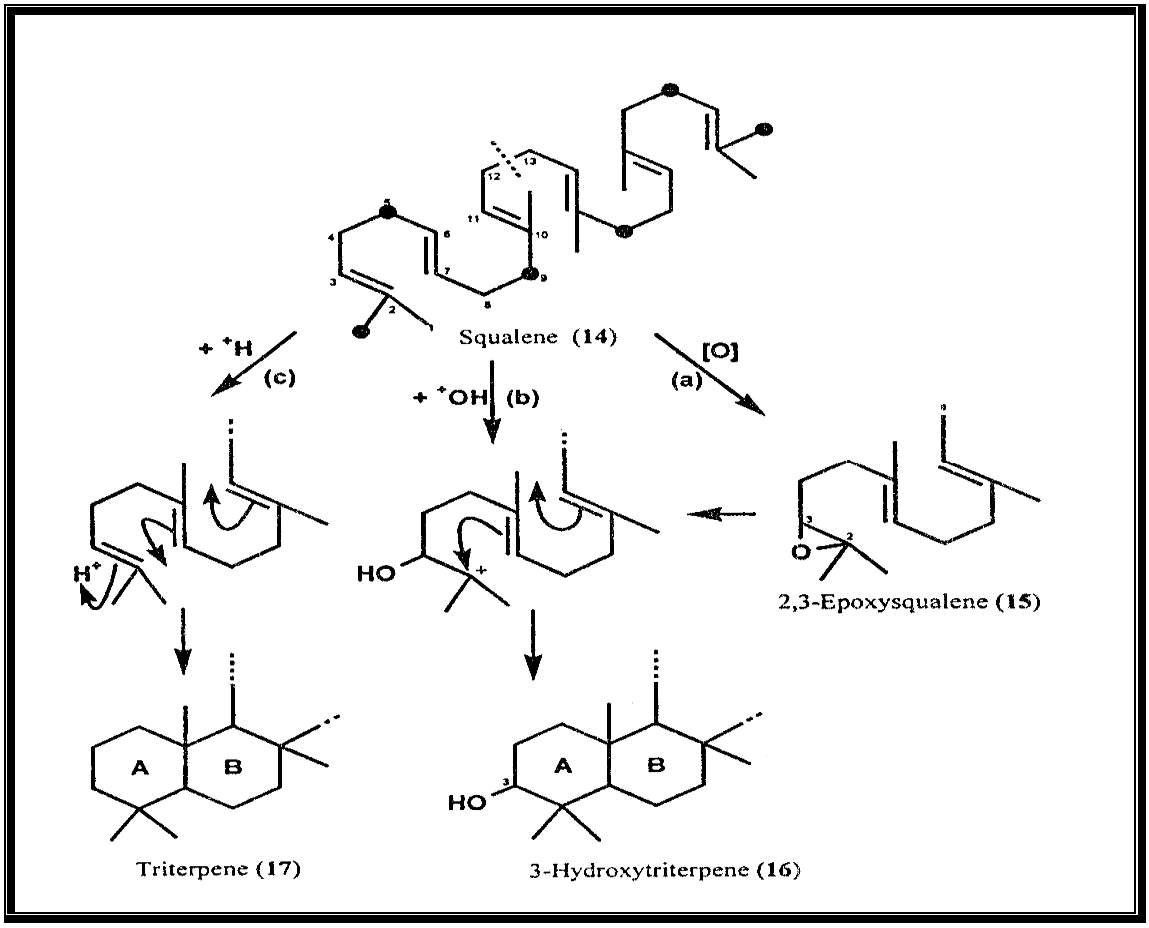


Scheme13: Biosynthesis of squalene **(14)**

Squalene is a symmetrical, unsaturated hydrocarbon and can undergo multiple cyclizations because of its six double bonds. There are more than twenty skeletal types of triterpenoids which are derived from squalene.

4.5.3 Cyclization of Squalene (14)

The process of cyclization of Squalene, promoted either by oxidative or non-oxidative agents, starts with the formation of a carbocation at the tertiary carbon of the end double bond of squalene (14) **(Scheme 14)**.



Scheme 14: Oxidative (a, b) and non-oxidative (c) pathways for the biosynthesis of triterpenoids **(17)**

In the oxidative route, cyclic compounds with ring A, bearing a OH group at carbon number three by the renovation of squalene into 2,3- epoxy squalene then followed by protonation and Markowniokoff's opening of the epoxy ring (route a), or OH^+ (protonated oxene) attack on the C-2, C-3 double bond (route b). Protonation of the double bond followed by cyclization (rout c), initiate the non-oxidative cyclization processes. The fundamental or primary triterpenoids are directly derived from the cyclization of squalene or its 2, 3-epoxide and are further divided into three subgroups according to their derivation i.e. 1; Those arising from, oxidative cyclization starting from the squalene terminal part, 2; Non-oxidative cyclization, resulting from the terminal part of squalene, 3; Both oxidative and non-oxidative cyclization independently, involving both the terminal double bonds of squalene.

4.5.4 Oxidative Cyclization of Squalene or 2, 3-Epoxy Squalene

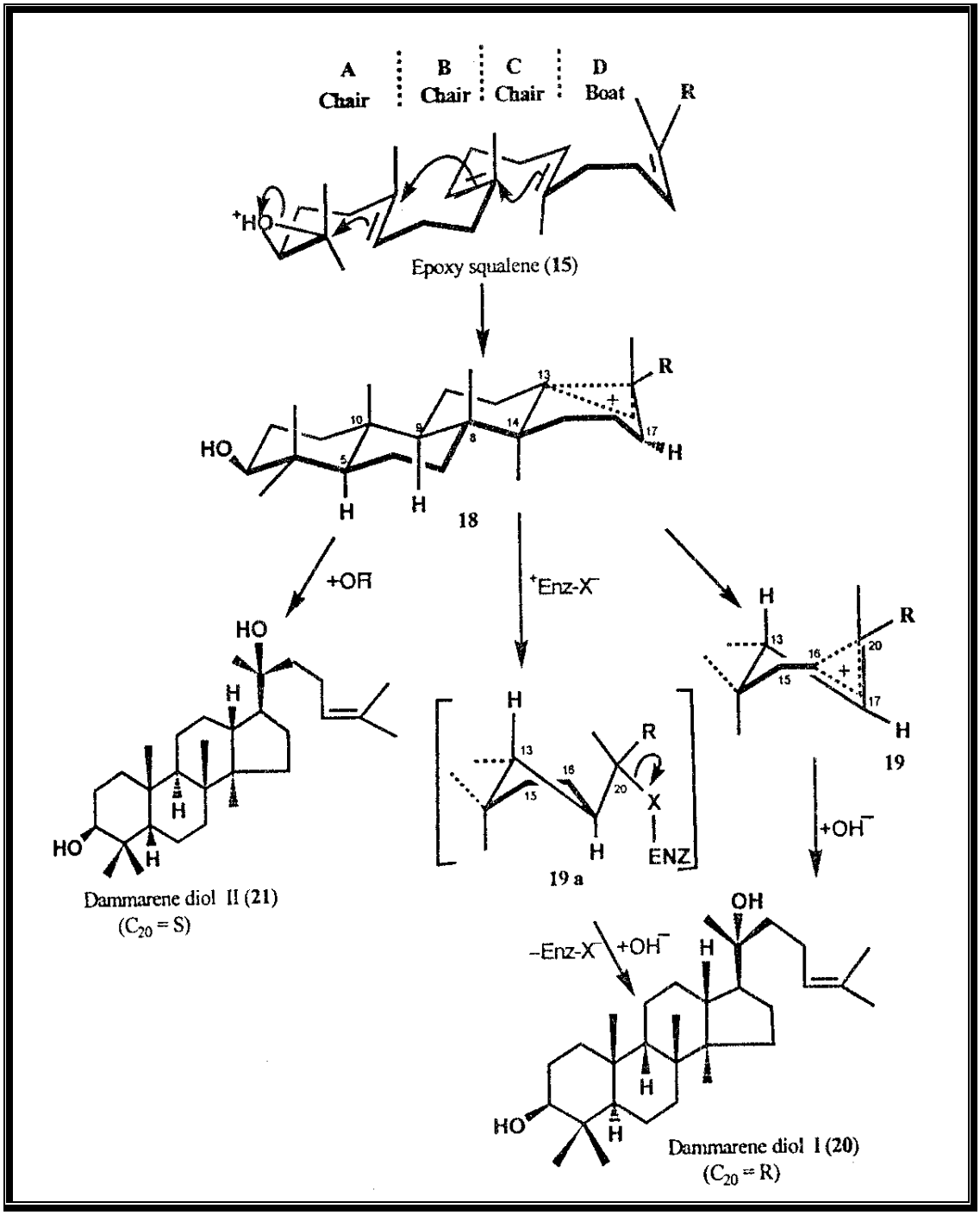
Formation of various types of tetra and penta cyclic triterpenoids depends on the conformation that squalene (epoxide) adopts, presumably on enzyme surface prior to cyclization. Each squalene stereospecifically leads to a particular cyclization product following the principles of the biogenetic rule.

4.5.5 Cyclization of Squalene Epoxide in Chair-Chair-Chair-Boat Sequence

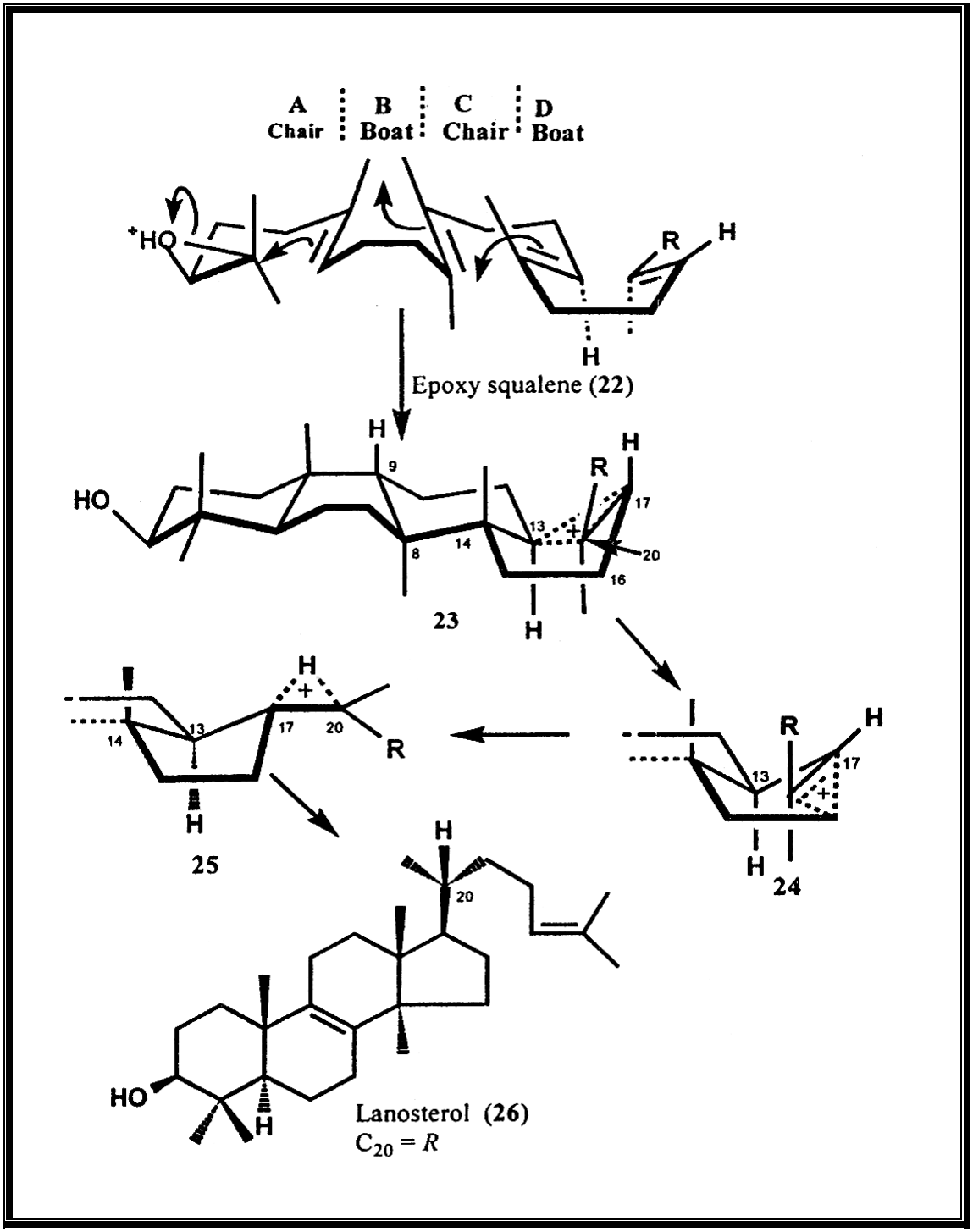
From 2,3-epoxy squalene (15), the dammarnediols (20, 21) [159-160] are derived, all with *trans* conformation and held at the active site of the enzyme in a chair-chair-chair-boat conformation **(Schem-15)**. Isomerization of the non-classical cation (18) into the non-classical (19) is responsible for the formation of dammarenediol I (20), but not dammarenediol II (21).

4.5.6. Cyclization of Squalene Epoxide in the Chair-Boat-Chair-Boat Sequence

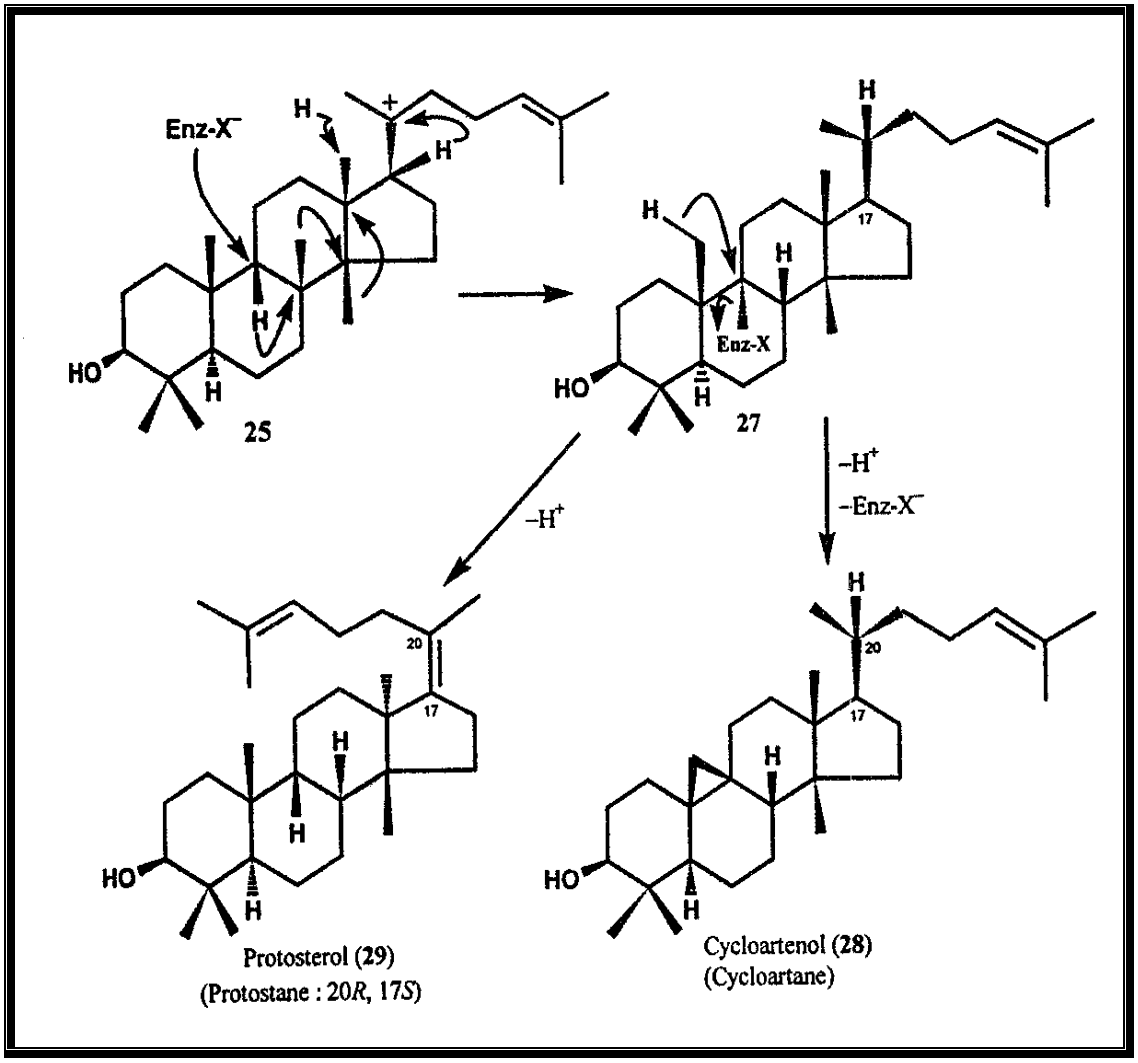
From the chair – boat – chair - boat conformation of squalene epoxide (22), lanosterol (26) and cycloartenol (28) are derived. The intermediate (23) into (24) by isomerization, produces an R configuration at C-20 of lanosterol and cycloartenol. The cyclization of squalene epoxide (22) leads initially to a bridged cation (23) and then *via* (23), either directly to protosterol (29) or by a sequence of 1,2-hydride and methyl shifts to lanosterol (26) and cycloartenol (28) (**Scheme 16, 17)** [161].



Scheme-15: Biosynthesis route to the Dammarenes **(20, 21)**

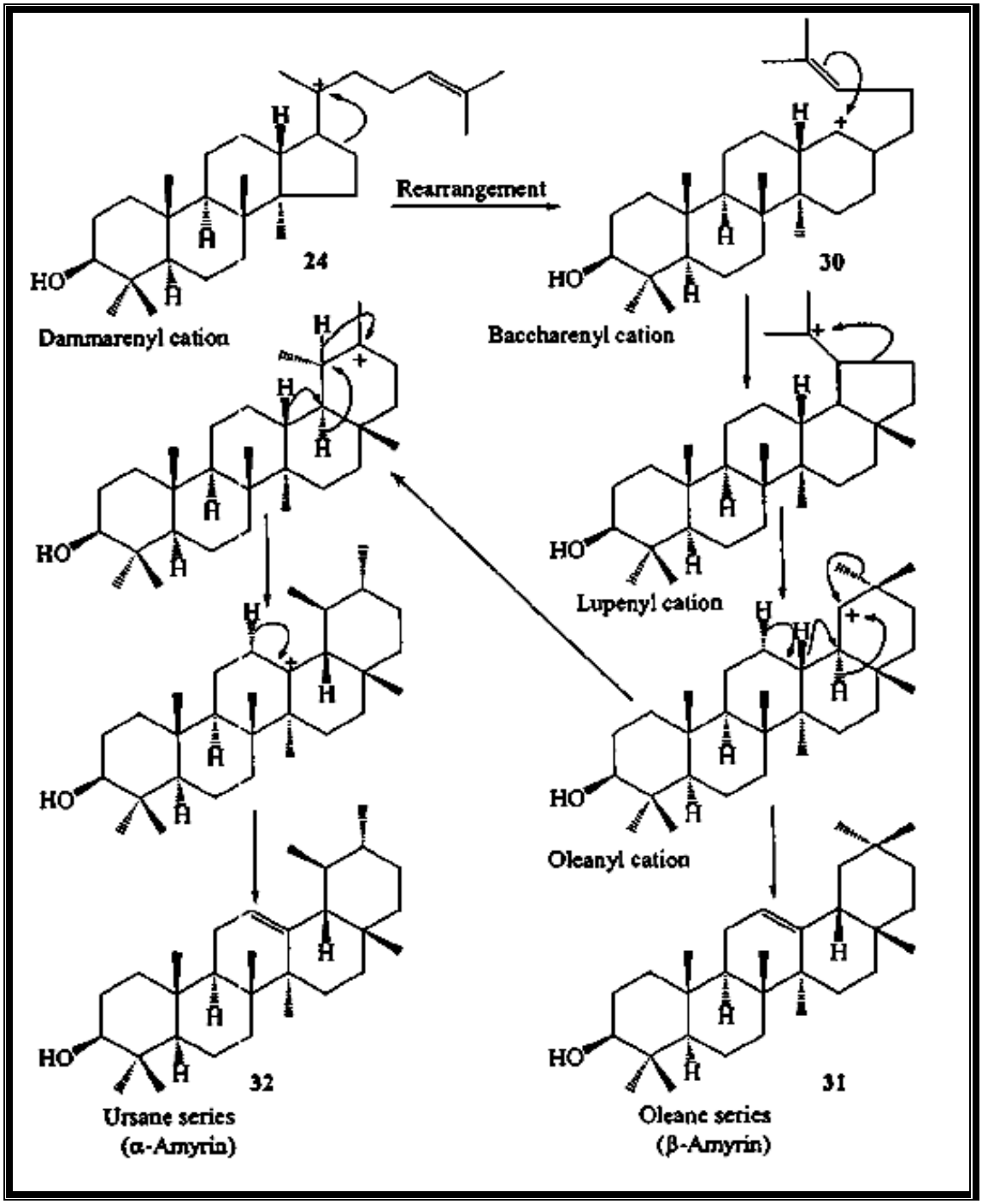


Scheme 16: Biosynthesis route to lanoserol **(26)**

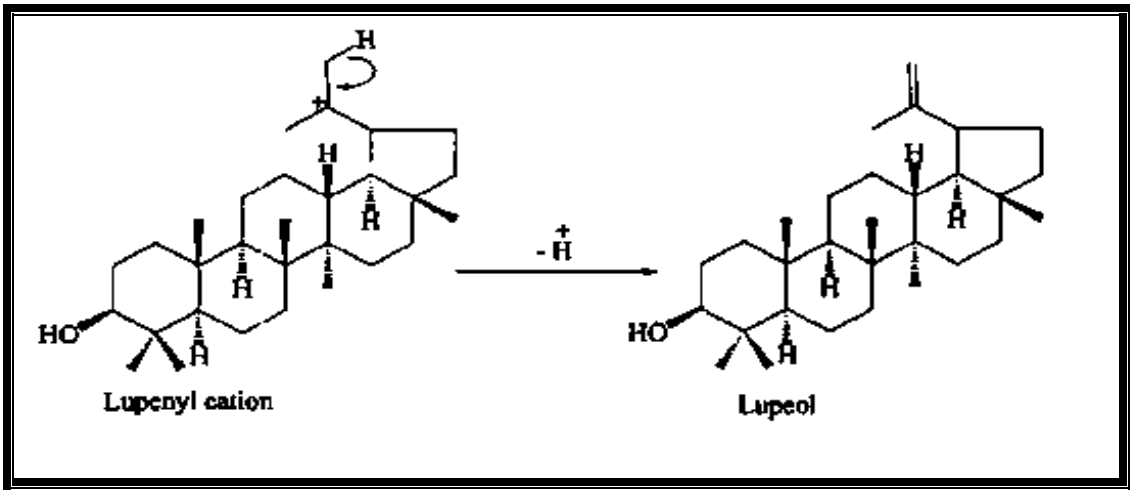


Scheme 17: Biosynthesis route to the cycloartanes **(28)** and protosterols **(29)**

Oxidative cyclization of (22) to dammarenyl cation followed by rearrangement to give baccharenyl cation (30), which gives lupenyl cation that can collapse in two ways, yielding β-amyrin (oleane) (31) or α-amyrin (ursane) (32). A series the intermediate lupenyl cation may reduce to Lupeol (33) during the biogenesis pathway of oleane or ursane **(Scheme 18**, **19)** [161-162].



Scheme 18: Biosynthetic route for β-amyrin **(31)** and α-amyrin **(32)**



Scheme 19: Formation of Lupeol **(33)** from Lupenyl cation.

5.0 EXPERIMENTAL

5.1 GENERAL EXPERIMENTAL CONDITIONS

The general experimental settings followed for Part B were the same as discussed in ***Part-A*** section 2.1.

5.2 PHYTOCHEMICAL INVESTIGATIONS

5.2.1 Plant material

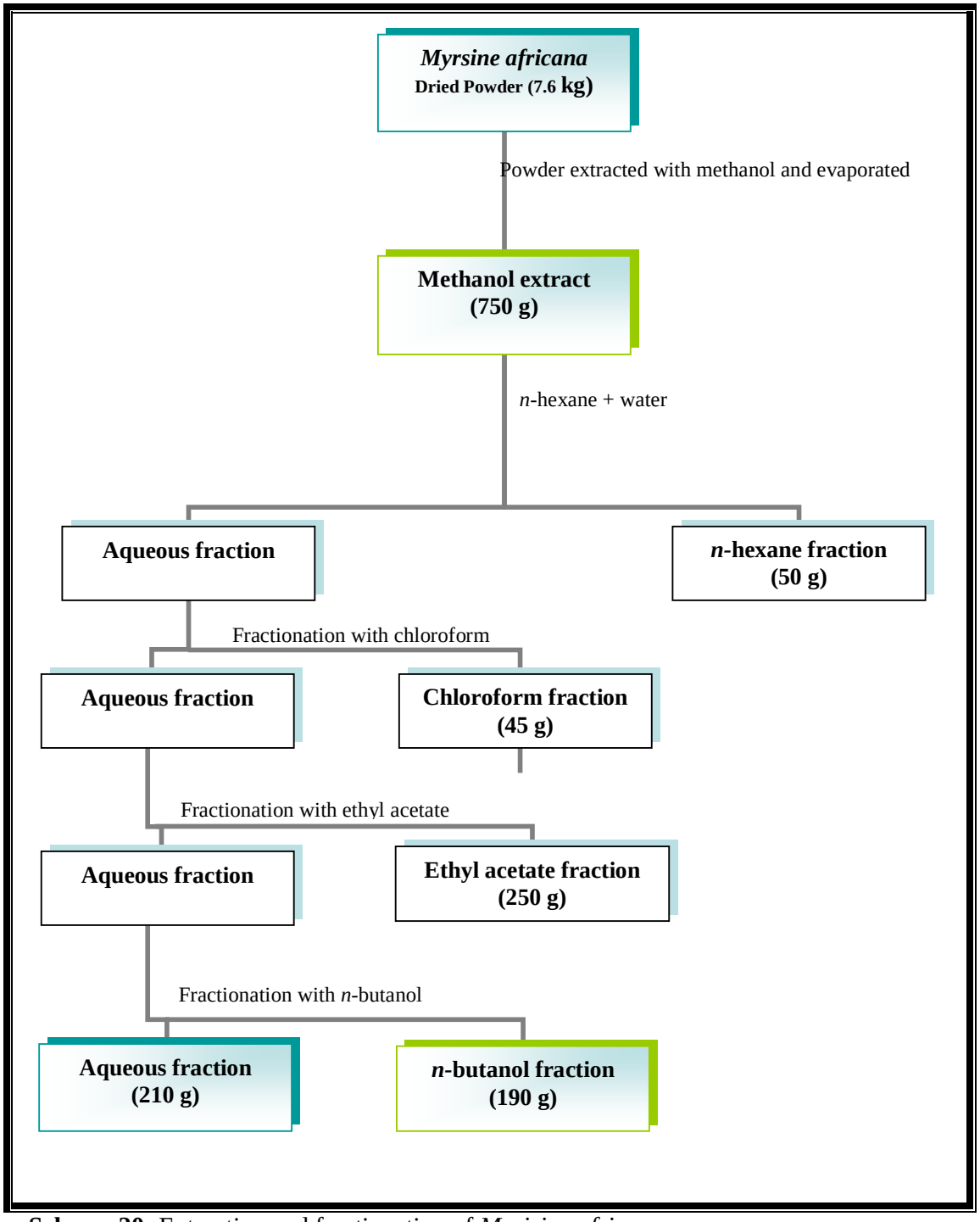
Myrsine africana (*Myrsinaceae*), aerial parts were collected from Hazara division, in December 2007 - January 2008 and identified by Prof. Dr. Habib Ahmad, Plant Taxonomist, Hazara University, Khyber Pakhtunkhwa, Pakistan.

5.2.2 Extraction

The shade dried plant material was chopped into small pieces and ground to fine powder using electric grinder. The powdered plant material of *Myrsine africana* (7.6 kg) was soaked in commercial grade methanol (MeOH) for 15 days at room temperature with occasional shaking. After 15 days the MeOH soluble material were filtered off. The filtrates, obtained after filtration, were combined and concentrated under vacuum at 40°C using rotary evaporator. A blackish MeOH extract (800 g) was obtained. **(Scheme 20).**

5.2.3 Fractionation

The crude MeOH extract (750 g) of *Myrisine africana* was suspended in distilled water (400 ml) and partitioned with *n*-hexane (3 x 400 ml), $CHCl_3$ (3 x 400 ml), EtOAc (3 x 400 ml) and BuOH (3 x 400 ml) to yield *n*-hexane (50 g), $CHCl_3$ (45 g), EtOAc (255 g), BuOH (190 g) and aqueous (210 g) fractions. About 50 gram of crude MeOH extract was reserved for other pharmacological/biological screenings **(Scheme 20).**



Scheme 20: Extraction and fractionation of *Myrisine africana*.

5.2.4 Screening for Different Groups of Compounds

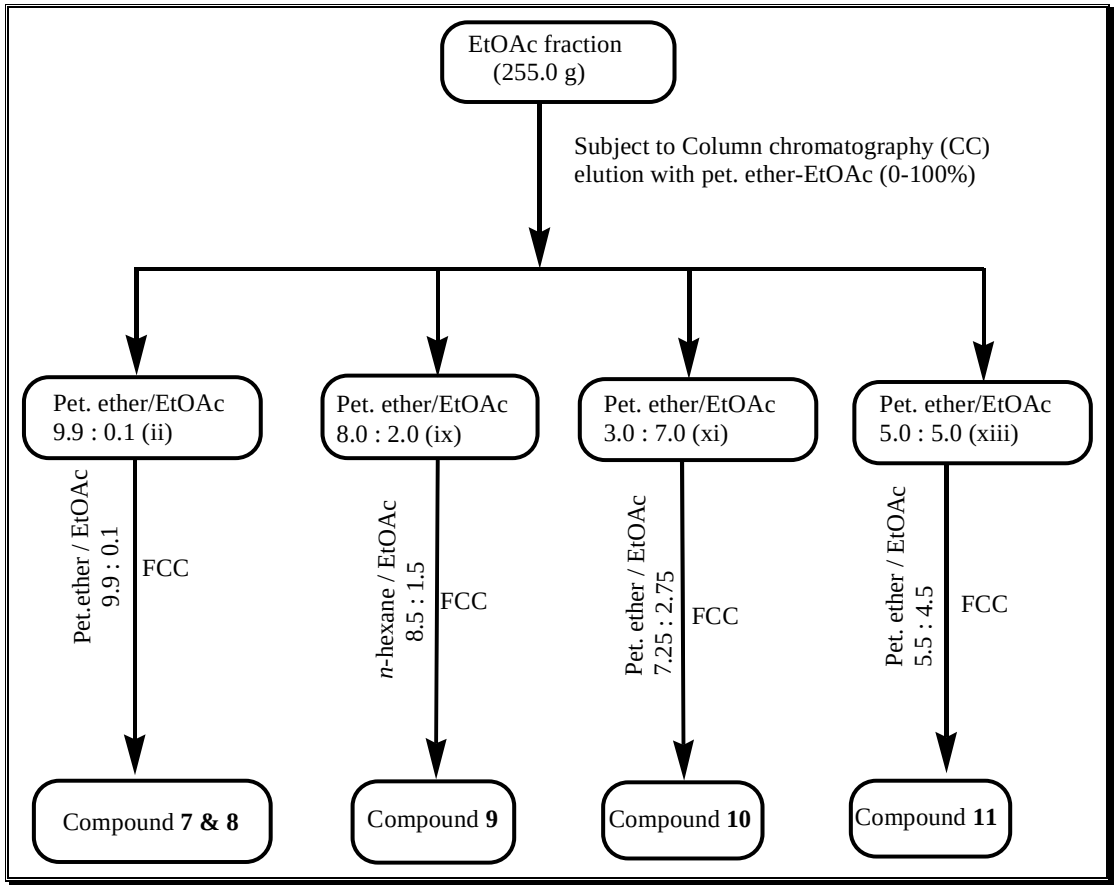
The plant materials were screened for the different compound groups i.e. alkaloids, flavonoids, glycosides and tannins as per ***Part-A***, section 2.2.4.

5.2.5 Compounds isolated from *Myrsine africana*

The EtOAc fractions of *Myrsine africana* were subjected to column chromatography and successively sub-fractionated in increasing order of polarity with solvent system of EtOAc/*n*-hexane (0.0:10 (i), 0.1:9.9 (ii), 0.2:9.8 (iii), 0.3:9.7 (iv), 0.4:9.6 (v), 0.5:9.5 (vi), 1.0:9.0 (vii), 1.5:8.5 (viii), 2.0:8.0 (ix), 2.5:7.5 (x), 3.0:7.0 (xi), 4.0:6.0 (xii), 5.0:5.0 (xiii), 6.0:4.0 (xiv), 7.0:3.0 (xv), 8.0:2.0 (xvi), 9.0:1.0 (xvii) and 100 % EtOAc (xviii) (**Scheme 21)**.

From the above, sub-fractions were then selected and subjected to Flash Column Chromatography (FCC) and isolate the following compounds (**Scheme 20**). Before subjecting the samples to FCC different solvent systems (EtOAc/*n*-hexane, acetone/*n*-hexane and MeOH/DCM) were checked for good separation on TLC plate.

Compound 7 and Compound 8 were obtained from the sub-fraction (ii) eluting with solvent system *n*-hexane (9.9) and EtOAc (0.1) in increasing order of polarity. Compound 9 was isolated from sub-fraction (ix) eluting with solvent system of *n*-hexane (1.5) and EtOAc (8.5). Compound 10 was obtained from sub-fraction (xi) eluting with solvent system of *n*-hexane (7.25) and EtOAc (2.75). Similarly compound 11 was isolated from sub-fraction (viii), subjecting to FCC and eluting with solvent system of *n*-hexane (5.5) and EtOAc (4.5) **(Scheme 21)**.



Scheme 21: Isolation of compounds from EtOAc fraction of *Myrsine africana*

5.3 CHARACHTERIZATION OF COMPOUNDS

5.3.1 Characterization of Myrsinene (07)

Yield: 23 mg from EtOAc fraction.

Physical State: White powder.

M.P: 199 - 201 ^{o}C

M. wt: ($C_{30}H_{50}O$) 426.3861 a.m.u.

$[\alpha]^{25}_{D}$: 39.1 (c = 0.4, MeOH)

IR (KBR) *V* max cm^{-1}: 3400 (OH), 2820 (CH), 1640, 885 (isoprenyle group), 1365, 1100, 992 cm^{-1}.

EIMS *m/z* (rel. int. %): [M^{+}] 426.2 (12), 392.3 (06), 302 (100), 287.2 (36), 231.2 (10), 204.1 (100), 161.0 (28), 135.1 (58), 95.0 (48), 69.0 (98).

HR-EIMS *m/z*: 426.3862 (Calcd for $C_{30}H_{50}O$. 426.3861).

^{13}C-NMR (100 MHz, C_5D_5N) δ: 35.3 (C-1), 28.0 (C-2), 78.1 (C-3), 37.7 (C-4), 55.9 (C-5), 17.8 (C-6), 33.3 (C-7), 39.4 (C-8), 158.4 (C-9), 38.2 (C-10), 117.0 (C-11), 33.9 (C-12), 49.1 (C-13), 39.2 (C-14), 29.9 (C-15), 36.8 (C-16), 36.0 (C-17), 49.5 (C-18), 41.6 (C-19), 28.9 (C-20), 37.9 (C-21), 38.2 (C-22), 30.0 (C-23), 16.4 (C-24), 21.5 (C-25), 26.1 (C-26), 15.6 (C-27), 28.6 (C-28), 33.3 (C-29), 30.0 (C-30).

^{1}H-NMR (500 MHz, C_5D_5N): See table 6.2.

HMBC: see figure 6.1.

NOESY: see figure 6.2.

5.3.2 Characterization of Myrsigenin (08)

Yield: 6 mg from EtOAc fraction.

Physical State: White powder.

M.P: 115-118^{o}C

M. wt: ($C_{27}H_{42}O_3$) 414.3212 a.m.u.

$[\alpha]^{25}_{D}$: - 16.1^{o} (c= 0.2, MeOH)

IR (KBR) V_{max} cm^{-1}: 3448, 2923, 980, 920, 900, 865 cm^{-1}

HRESI MS *m/z*: 415.3217 (calcd, $C_{27}H_{42}O_3$ + H = 415.3212).

EIMS *m/z* (rel. int. %): [M^+] 414 (04), 342 (08), 300 (14), 282 (18), 253 (06), 199 (04), 159 (06), 139 (100), 115 (18), 69 (54).

^{13}C-NMR (100 MHz, $CDCl_3$)δ: 38.5 (C-1), 32.3 (C-2), 72.4 (C-3), 43.0 (C-4), 140.8 (C-5), 121.8 (C-6), 32.8 (C-7), 31.8 (C-8), 51.7 (C-9), 36.5 (C-10), 22.0 (C-11), 40.9 (C-12), 41.3 (C-13), 57.8 (C-14), 32.7 (C-15), 82.2 (C-16), 63.7 (C-17), 19.8 (C-18), 14.9 (C-19), 29.9 (C-20), 17.5 (C-21), 32.2 (C-22), 110.5 (C-23), 42.9 (C-24), 32.4 (C-25), 67.9 (C-26), 16.8 (C-27).

^{1}H-NMR (500 MHz, $CDCl_3$): See table 6.3.

HMBC: See figure 6.3.

NOESY: See figure 6.4.

COSY: See figure 6.4.

5.3.3 Characterization of Compound (09)

Yield: 9 mg from EtOAc fraction.

Physical State: White powder.

M.P: 123-126^{o}C

M. wt: ($C_{28}H_{44}O_3$) 429.3369 a.m.u.

HR-EIMS *m/z*: 429.3348 (Calcd for C28H44O3. 429.3369).

EIMS *m/z* (rel. int. %): [M^+] 429 (100), 411 (30), 393 (24), 377 (14), 315 (08), 287 (08), 191 (14), 175 (20), 159 (12), 125 (12), 83 (10), 69 (34), 57 (10).

^{13}C-NMR (CD_3OD, 100 MHz)δ: 35.9 (C-1), 30.9 (C-2), 67.0 (C-3), 37.7 (C-4), 83.5 (C-5), 136.8 (C-6), 131.7 (C-7), 80.8 (C-8), 52.6 (C-9), 38.2 (C-10), 21.5 (C-11), 40.7 (C-12), 45.8 (C-13), 53.1 (C-14), 29.8 (C-15), 24.4 (C-16), 57.6 (C-17), 18.6 (C-18), 13.3 (C-19), 41.1 (C-20), 21.4 (C-21), 136.8 (C-22), 133.5 (C-23), 44.3 (C-24), 34.4 (C-25), 20.4 (C-26), 20.1 (C-27), 18.2 (C-28).

^{1}H-NMR (CD_3OD, 400 MHz): See table 6.4**.**

HMBC: See figure 6.5.

5.3.4 Characterization of Compound (10)

Yield: 10 mg from EtOAc fraction.

Physical State: Needle like crystals.

M. wt: ($C_{29}H_{50}O$) 414.3861 a.m.u.

M.P. 142-144^{o}C

IR (KBR) ν_{max}: 3402, 2901, 1641 cm^{-1}.

EIMS (rel. Int. %) *m/z*: 414 (100), 399(15), 396 (19), 329 (25), 303 (21), 273 (12), 213 (18), 161 (14), 145 (19), 135 (9), 119 (10), 107 (18), 95 (21).

HR-EIMS *m/z*: 414.3857 (calcd. 414.3861 for $C_{29}H_{50}O$).

^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 5.32 (1H, m, H-3α), 0.92 (3H, s, CH_3-19), 0.88 (3H, d, $J_{21,20}$ = 6.5 Hz, CH_3-21), 0.83 (3H, d, $J_{26,25}$ = 6.5 Hz, CH_3-26), 0.81 (3H, d, $J_{27,25}$ = 6.5 Hz, CH_3-27), 0.77 (3H, t, $J_{29,28}$ = 7.0 Hz, |CH_3-29), 0.63 (3H, s, CH_3-18).

^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 140.9 (C-5), 121.9 (C-3), 56.8 (C-14), 56.2 (C-17), 50.8 (C-9), 50.4 (C-24), 42.6 (C-13), 42.4 (C-4), 40.3 (C-12), 39.5 (C-20), 37.3 (C-1), 36.6 (C-10), 36.3 (C-20), 35.6 (C-8), 34.0 (C-22), 33.0 (C-6), 32.1 (C-7), 32.0 (C-8), 31.8 (C-2), 29.3 (C-23), 28.2 (C-16), 26.2 (C-25), 24.3 (C-15), 23.1 (C-28), 21.1 (C-21), 21.1 (C-11), 19.8 (C-27), 19.4 (C-19), 19.1 (C-21), 18.8 (C-26), 11.9 (C-29), 11.9 (C-18).

5.3.5 Characterization of Compound (11)

Yield: 17 mg from EtOAc fraction.

Physical State: White powder.

M. wt: ($C_{30}H_{50}O$) 426.3861 a.m.u.

MP: 215-216^{o}C

EIMS *m/z* (rel. int. %): [M^{+}] 426 (14), 411 (30), 207 (10), 206 (8), 205 (1), 204 (7), 203 (6), 189 (20), 133 (14).

HR-EIMS *m/z*: 426.7189 (calcd. For $C_{30}H_{50}O$, 426.7194).

IR (KBR) *V* max cm^{-1}: 3450 (OH), 3070, 1650 and 880 (C = CH_2)

^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.63 (2H, m, H2-29), 3.64 (1-H, dd, $J_{ax, ax}$ = 10.68, $J_{ax, eq}$ = 4.27 Hz, H-3), 1.65 (3-H, br s, Me 30), 1.05 (3-H, s, Me-26), 0.96 (6-H, s, Me-25, Me 27), 0.90 (3-H, s, Me-24), 0.85 (3-H, s, Me-28), 0.76 (3-H, s, Me-23).

13 C NMR (CDCl3, 100 MHz): δ; 150.6 (C-20), 109.2 (C-29), 78.8 (C-3), 55 (C-5), 50.5 (C-9), 48.2 (C-18), 47.9 (C-19), 42.9 (C-17), 42.8 (C-14), 40.9 (C-8), 39.9 (C-22), 38.8 (C-4), 38.7 (C-1), 38.0 (C-13), 37.2 (C-10), 35.5 (C-16), 34.2 (C-7), 29.8 (C-21), 28.0 (C-23), 27.4 (C-15), 27.4 (C-2), 25.1 (C-112), 20.9 (C-11), 19.3 (C-30), 18.4 (C-6), 18.1 (C-28), 16.1 (C-25), 15.9 (C-26), 15.4 (C-24), 14.5 (C-27).

5.4 PHARMACOLOGICAL INVESTIGATIONS

5.4.1 ANTIBACTERIAL ACTIVITY

Crude MeOH extract and all fractions of *Myrsine africana* were screened for anti-bacterial activity using the same procedure as described in ***Part-A,*** section **2.3.1**.

5.4.2 ANTI FUNGAL ACTIVITY

Crude MeOH extract and all fractions of *Myrsine africana* were screened for anti-fungal activity using the same procedure as described in ***Part-A,*** section **2.3.2**.

5.4.3 INSECTICIDAL ACTIVITY

Crude MeOH extract and all fractions of *Myrsine africana* were screened for insecticidal activity using the same procedure as described in ***Part-A,*** section **2.3.3**.

5.4.4 ANTITERMITE ACTIVITY

Crude MeOH extract and all fractions of *Myrsine africana* were screened for anti-termite activity using the same procedure as described in ***Part-A,*** section **2.3.4**.

5.4.5 PHYTOTOXIC ACTIVITY

Crude MeOH extract and all fractions of *Myrsine africana* were screened for phytotoxic bioassay using the same procedure as described in ***Part-A,*** section **2.3.5**.

5.4.6 BRINE SHRIMP LETHALITY

Crude MeOH extract and all fractions of *Myrsine africana* were screened for brine shrimp lethality using the same procedure as described in ***Part-A,*** section **2.3.6**.

5.4.7 HEAM-AGGLUTINATION ACTIVITY

Crude MeOH extract and all fractions of *Myrsine africana* were screened for heam-agglutination activity using the same procedure as described in ***Part-A,*** section **2.3.7**.

5.4.8 ANTI-INFLAMMATORY ACTIVITY

Crude MeOH extract and all fractions of *Myrsine africana* were screened for anti-inflammatory activity using the same procedure as described in ***Part-A,*** section **2.3.8**.

5.4.9 EFFECTS ON RABBIT'S JEJUNUM PREPARATIONS

Spasmolytic activity

Crude MeOH extract of *Myrsine africana* were screened for spasmolytic activity using the same procedure as described in ***Part-A,*** section **2.3.9**.

5.4.10 NITRIC OXIDE FREE RADICAL SCAVENGING ACTIVITY

Crude MeOH extract and all fractions of *Myrsine africana* were screened for nitric oxide free radical scavenging activity using the same procedure as described in ***Part-A,*** section **2.3.10**.

6.0 RESULTS AND DISCUSSION

6.1 PHYTOCHEMICAL INVESTIGATIONS

6.1.1 Screening for Different Groups of Compounds

The plant materials of *Myrsine africana* were screened for the presence of different types of compounds groups of natural products i.e. alkaloids, flavonoids, saponin and tannin. The results for phytochemical screening of the plant are summarized in table 6.1. There was negative test for alkaloids with no precipitation, showing the absence of alkaloids in *Myrsine africana.* The flavonoid test gave strongly positive result with the appearance of pink color, indicating the presence of flavonoids in relatively large quantity. A froth formation of more than 1.0 cm was observed showing that the plant contains saponin. Test for tannins gave negative results showing the absence of tannins in *Myrsine africana.*

Table 6.1. Phytochemical Screening for *Myrsine africana*

S.No	TEST	Results	Remarks
1	Alkaloids	+	Positive
2	Flavonoids	++	Positive
3	Saponin	++	Positive
4	Tannin	-	Negative

6.1.2 STRUCTURAL ELUCIDATION OF COMPOUNDS

6.1.2.1 Structural Elucidation of Myrsinene (07)

This compound was isolated as white powder from the ethyl acetate fraction of *Myrsine africana,* eluting with EtOAc/*n*-hexane (0.2:9.8) as mobile phase on flash silica column. EI-MS of the compond showed M^+ at *m/z* 426. Its optical rotation, {$[\alpha]_D^{25}$ = + 39.1 (MeOH, c = 0.4)} indicated the presence of chiral center in molecule. HR-EI-MS showed molecular ion peak at *m/z* 426.3862, corresponding to the formula $C_{30}H_{50}O$ (Cal. for $C_{30}H_{50}O$ = 426.3861).

^{1}H-NMR spectrum of the compound showed eight singlets due to methyl groups at δ 0.89, 0.94, 0.97, 0.98, 0.99, 1.06, 1.11and 1.23ppm, which are due to C-23, C-27, C-29, C-25, C-30, C-24, C-26, C-28 methyl protons, respectively (table 6.2, figure 6.1). A down field proton due to methine (H-3) at δ 3.43 (dd, $J_{3,2}$ = 10.5 Hz, $J_{3,2}$ = 5.0 Hz). The *J* values suggest the axial orientation of (H-3) proton. A downfield shift at δ 5.61 (dd, *J* = 8.0, 3.5 Hz) was assigned to the H-11 proton (table 6.2, figure 6.1). The ^{1}H-NMR data clearly indicated that compound is triterpene [163-164].

^{13}C-NMR spectrum (BB, DEPT) displayed 30 carbon resonance including eight methyl, ten methylene, five methine and seven quaternary carbons (table 6.2).

The structure of compound was further conformed from 2-D NMR spectroscopy (HSQC, HMBC, COSY and NOESY) data. Position of the double bond was assigned with the help of ^{13}C-NMR values and HMBC intaractions (figure 6.1). Methyl protons of C-25 and C-26 displayed HMBC correlations with C-9 (158.4), conformed the position of double bond at C-9 and C-11. CD spectrum indicates an intense positive cotton effect at 280 nm. ^{1}H-^{13}C connectivities were determined by using HSQC interactions. Stereochemistry of the compound was deduced by the help of biosynthetic pathway [159, 160], ^{1}H-^{1}H coupling constant values and NOESY correlations (figure 6.2).

Table 6.2. ^{1}H and ^{13}C-NMR and Chemical Shifts of Myrsinene (ppm, C_5D_5N, 500 and 100 MHz)

C. No	Multiplicity (DEPT)	^{13}C-NMR	^{1}H (J = Hz)
1	CH_2	35.3	1.12 m, 1.38 m
2	CH_2	28.0	1.21 m. 1.82 m
3	CH	78.1	3.43 (dd , J = 10.5, 5.0 Hz)
4	C	37.7	------
5	CH	55.9	0.81 m
6	CH_2	17.8	1.49 m, 1.61 m
7	CH_2	33.3	1.26 m, 1.37 m
8	C	39.4	------
9	C	158.4	------
10	C	38.2	------
11	CH	117	5.61 dd (J = 8.0, 3.5 Hz)
12	CH_2	33.9	1.51 m, 1.59 m
13	CH	49.1	1.15 m
14	C	39.2	------
15	CH_2	29.9	1.21 m, 1.83 m
16	CH_2	36.8	1.01 m, 1.34 m
17	C	36.0	------
18	CH	49.5	1.45 m
19	CH_2	41.6	1.36 m, 2.25 m
20	C	28.9	------
21	CH_2	37.9	1.69 m, 1.99 m
22	CH_2	38.2	1.53 m, 1.68 m
23	CH_3	30.0	0.89 s
24	CH_3	16.4	1.06 s
25	CH_3	21.5	0.98 s
26	CH_3	26.1	1.11 s
27	CH_3	15.6	0.94 s
28	CH_3	28.6	1.23 s
29	CH_3	33.3	0.97 s
30	CH_3	30.0	0.99 s

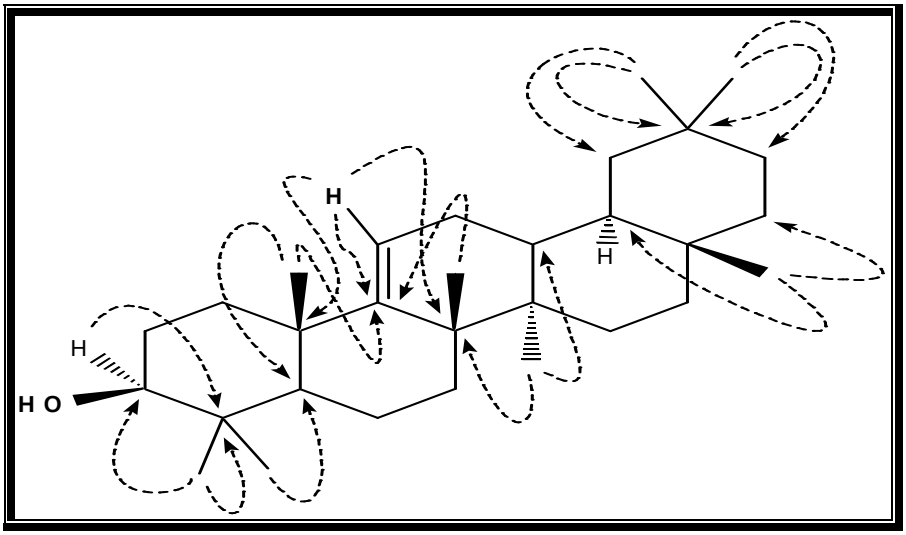


Figure: 6.1. Key HMBC Correlations in Myrsinene (**07**)

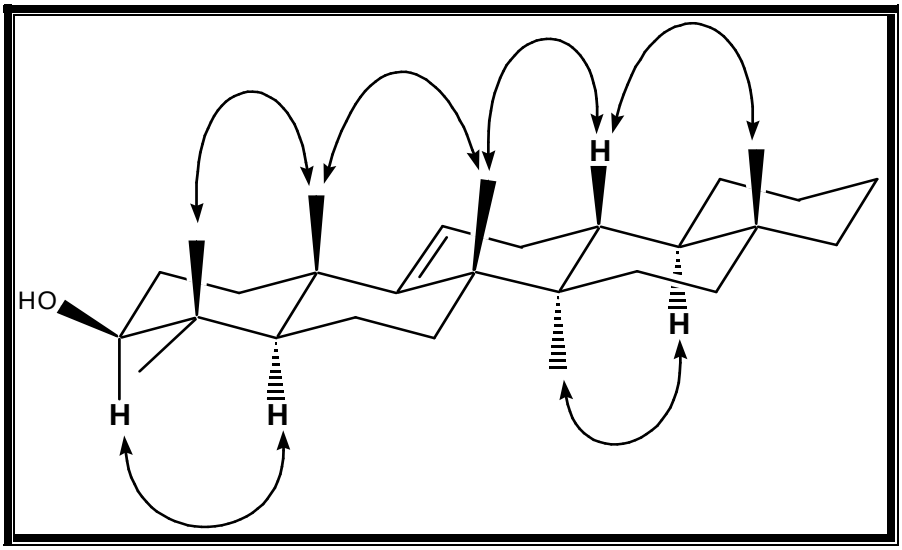
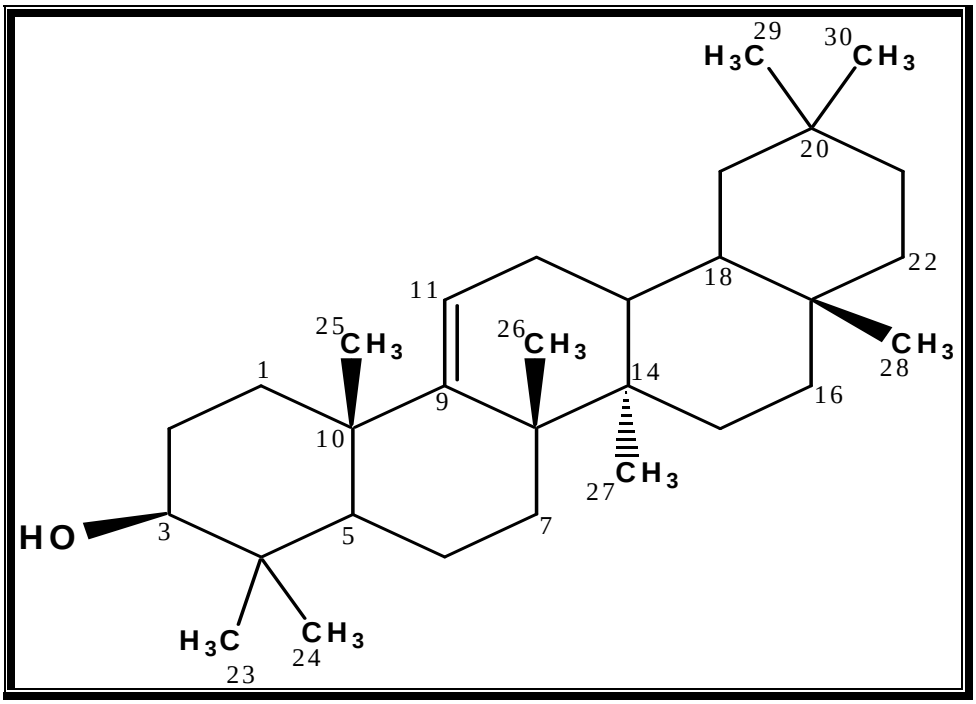


Figure: 6.2. Key NOESY correlations in Myrsinene (**07**)



Myrsinene (07)

6.1.2.2 Structure Elucidation of Myrsigenin (08)

This compound was isolated as white powder from ethyl acetate fraction of *Myrsine africana* eluting with EtOAc/*n*-hexane (0.3:9.7) as mobile phase on flash silica column. Its optical rotation, {$[\alpha]_D^{25}$ = - 16.1^o (MeOH, c = 0.2)} indicated the presence of chiral center in molecule. HR-ESI-MS displayed quasi molecular ion at *m/z* 415.3217, corresponding to the formula $C_{27}H_{42}O_3$ (cal. $C_{27}H_{42}O_3$ + H = 415.3212).

^{1}H-NMR spectrum exhibited resonances at δ 0.72 d ($J_{21, 20}$ = 6.5 Hz), 0.80 (s), 0.95 (d, $J_{27,24}$ = 6.5 Hz) and 1.06 ppm (s), which were assigned to the C-21, C-19, C-27 and C-18 methyl protons, respectively. Downfield methine protons at δ 3.44 (m, broad) ($W_{1/2}$ = 12.5 Hz) and 4.39 (q, J = 6.5 Hz) were attributed to the H-3 and H-16 respectively. Resonances at δ 3.35 (m) and 3.42 (m) were ascribed to the methylene protons of the C-27: this revealed the presence of oxygenated methylene carbon in molecule. A down field signal resonated at δ 5.33 br d ($J_{6, 7}$ = 5.0), was due to the H-6.

^{13}C-NMR spectrum (BB, DEPT) displayed twenty seven carbons including: four methyl, ten methylene, nine methine and four quaternary carbon atoms (table 6.3). ^{1}H and ^{13}C NMR clearly indicated that the compound has steroidal skeleton. ^{13}C NMR values at δ 67.9 (CH_2), 82.2 (CH) and 110.5 (q C), indicated the presence of dioxygenated spiro ring as a side chain of the molecule. Structure of compound was further confirmed with the help of two dimensional NMR spectroscopic techniques including: COSY, HSQC, HMBC and NOESY. COSY and HMBC revealed that ring E is a six membered, while the ring F is a five membered. H-16 showed cross peaks in COSY spectrum with H-17, which in turn showed correlation with H-20, this spin system extended up to the C-22 methylene protons. This clearly indicated that ring E is six membered. Similarly, H_2-26 showed cross peaks on COSY spectrum with H_2-25, which in turn showed correlation with H-24, this spin system extended up to C-27 methyl protons, this further supported that ring E is six membered and ring F is

five membered. Different spin systems, hetero atoms and functionalities were assembled using HMBC interactions. H-16, H_2-26 and H_3-27 displayed HMBC correlation with C-23. While H_3-21 exhibited correlation with C-22, C-20 and C-17. Key HMBC interactions in compound are shown in (figure 6.3).

Stereochemistry in compound was assigned on the basis of biogenesis pathway, coupling constant values and NOESY spectroscopy. H-16 displayed NOESY interactions with H-26, H-14 and H-17, this indicated that *cis* ring fusion between ring D and E, and H-16 and H-26 are close in space. Key NOESY interactions in compound are shown in (figure 6.4). CD spectrum indicates an intense positive cotton effect at 210 nm. This type of six membered rings E and five membered F ring spiro fused compound is not so common, this compound is believed to have been biosynthesized from C-27 [165].

Table 6.3. ^{1}H and ^{13}C-NMR and Chemical Shifts of Myrsigenin **(08)** (ppm, $CDCl_3$, 500 and 100 MHz, respectively)

C. No	Multiplicity (DEPT)	^{13}C-NMR	^{1}H (J = Hz)
1	CH2	38.5	1.71(m # of H), 1.93(m, #of H)
2	CH2	32.3	1.59 m, 1.83 m
3	CH	72.4	3.44 br m ($w_{1/2}$ = 12.5)
4	CH2	43.0	2.21 dd, 2.23 dd
5	C	140.8	------
6	CH	121.8	5.53 br d (5.0)
7	CH2	32.8	1.81 m , 1.97 m
8	CH	31.8	1.61 m
9	CH	51.7	0.95 m
10	C	36.5	------
11	CH2	22.0	1.01 m, 1.42 m
12	CH2	40.9	1.69 m, 1.40 m
13	C	41.3	------
14	CH	57.8	1.09 m
15	CH2	32.7	1.49 m, 1.63 m
16	CH	82.2	4.39 q like (7.5)
17	CH	63.7	1.75 m
18	CH3	19.8	1.06 s
19	CH3	14.9	0.80 s
20	CH	29.9	1.60 m
21	CH3	17.5	0.72 d ($J_{21,20}$ = 6.5)
22	CH2	33.2	1.2 dd (overlapped), 1.50 dd (overlapped)
23	C	110.5	------
24	CH	42.9	1.84 dd (5.5, 4.5)
25	CH2	32.4	1.65 m, 1.81m
26	CH2	67.9	3.35 m, 3.42 m
27	CH3	16.8	0.95 d ($J_{27,24}$ = 6.5)

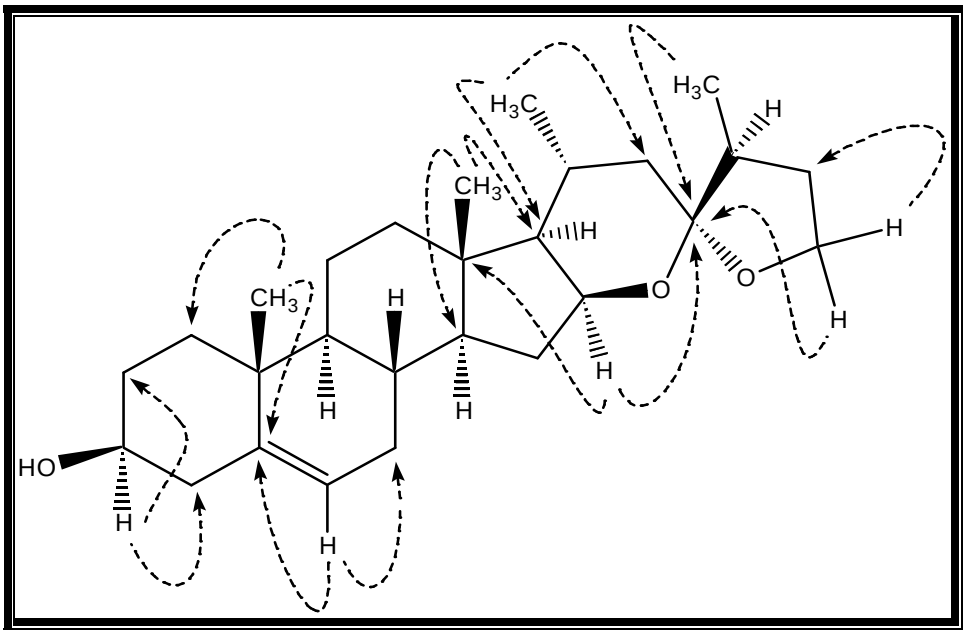


Figure: 6.3. Key HMBC interactions in Myrsigenin **(08)**

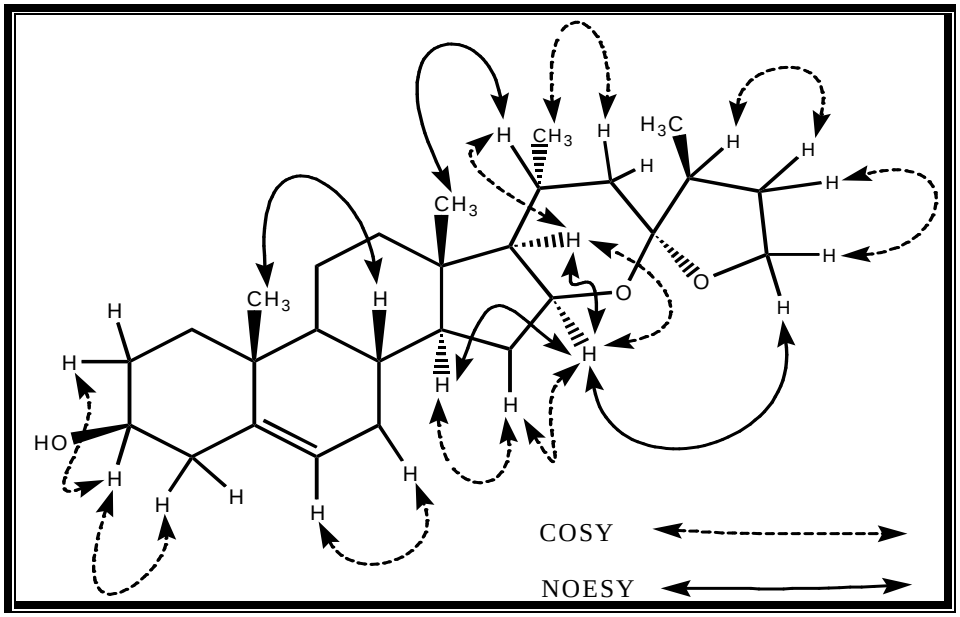
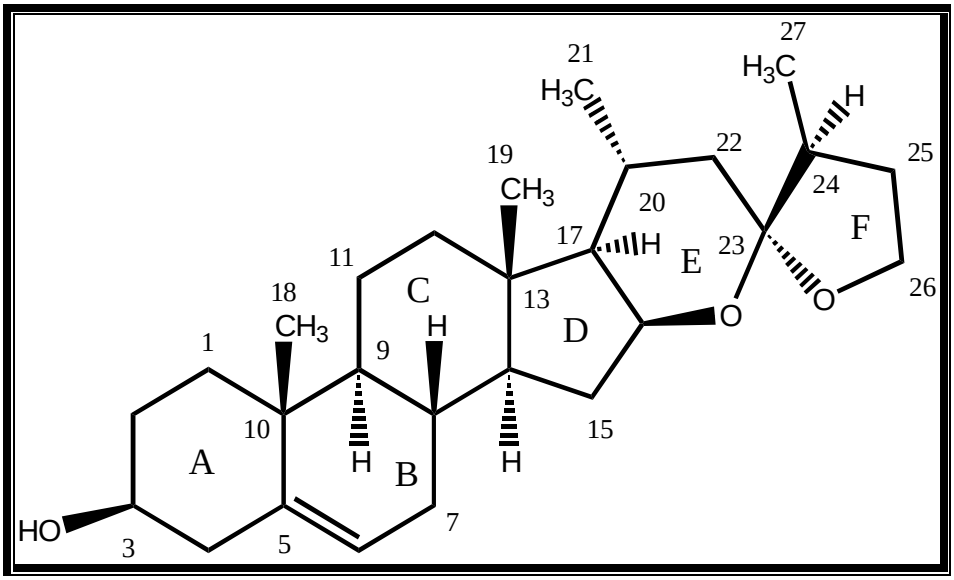


Figure: 6.4. Key NOESY and COSY intaractions in Myrsigenin **(08)**



Myrsigenin (08)

6.1.2.3 Structural Elucidation of Compound (09)

This compound was isolated as white powder from the ethyl acetate fraction of *Myrsine africana* eluting with EtOAc/*n*-hexane (0.25:9.75) as mobile phase on flash silica column.

The molecular formula of compound was determined by HR-ESI-MS and ^{13}C-NMR (BB and DEPT). HR-ESI-MS showed pseudo molecular ion peak [M^+] at *m/z* 429.3348 $[M+H]^+$ corresponding to the molecular formula $C_{28}H_{44}O_3$ [Cald. for $C_{28}H_{44}O_3$ + H = 429.3369].

^{1}H-NMR displayed the presence of two methyl singlets at δ 0.82 and 0.89, which were ascribed to C-19 and C-18 methyl protons: while four doublets at δ 0.84, 0.85, 0.92 and 1.02 were assigned to the C-26, C-27, C-28 and C-21 methyl protons respectively. A methine proton at δ 3.76 was assigned to the H-3, two downfield doublet at δ 6.24 ($J_{6,7}$ = 8.4 Hz) and δ 6.63 ($J_{7,6}$ = 8.4 Hz) were assigned to the H-6 and H-7 respectively. Two double doublets at δ 5.18 (*J* = 7.6, 14.4 Hz) and δ 5.21 (*J* = 7.6, 14.4 Hz) were assigned to the H-22 and H-23 respectively. ^{13}C-NMR spectrum (BB, DEPT) showed 28 resonances, including six methyl, seven methylene, eleven methine and four quaternary carbons. Structure was further conformed by 2D- NMR spectroscopy (HSQC, HMBC, COSY and NOESY). Key HMBC correlations are shown in figure 6.5. All the spectroscopic data of the compound **(09)** is unambiguously matches with reported compound [166]

Table 6.4. ^{1}H and ^{13}C-NMR and Chemical Shifts of Compound (**09**) (ppm, CD_3OD, 400 and 100 MHz, respectively).

C. No	Multiplicity (DEPT)	^{13}C-NMR	^{1}H (J = Hz)
1	CH_2	35.9	1.64, 1.89 m
2	CH_2	30.9	1.45, 1.61 m
3	CH	67.0	3.76 m
4	CH_2	37.7	1.62, 1.81 m
5	C	83.5	----------
6	CH	136.8	6.26 *d* ($J_{6,7}$ = 8.4Hz)
7	CH	131.7	6.63 d ($J_{7,6}$= 8.4Hz)
8	C	80.8	----------
9	CH	52.6	1.41 m
10	C	38.2	----------
11	CH_2	21.5	1.43, 1.52 m
12	CH_2	40.7	1.21, 1.02 m
13	CH_2	45.8	1.80 m
14	CH	53.1	1.59 m
15	CH_2	29.8	1.33, 1.71 m
16	CH_2	24.4	1.23, 1.58 m
17	CH	57.6	1.33 m
18	CH_3	18.6	0.89 s
19	CH_3	13.3	0.82 s
20	CH	41.1	2.09 m
21	CH_3	21.4	1.02 d (J = 6.4 Hz)
22	CH	136.8	5.18 dd (J = 7.6, 7.2 Hz)
23	CH	133.5	5.21 dd (J = 7.6, 7.2 Hz)
24	CH	44.3	1.82 m
25	CH	34.4	1.44 m
26	CH_3	20.4	0.84 d (J = 6.8 Hz)
27	CH_3	20.1	0.85 d (J = 6.8 Hz)
28	CH_3	18.2	0.92 d (J = 6.8 Hz)

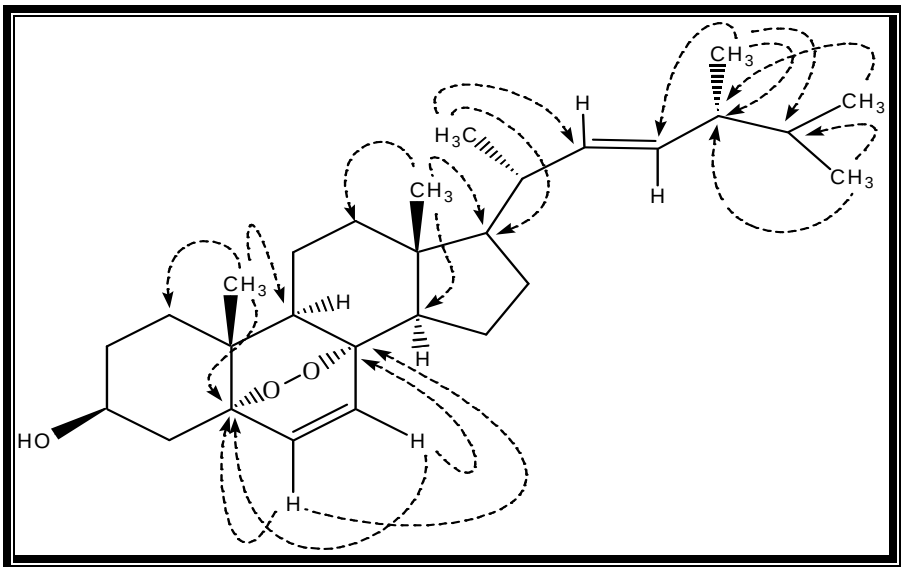
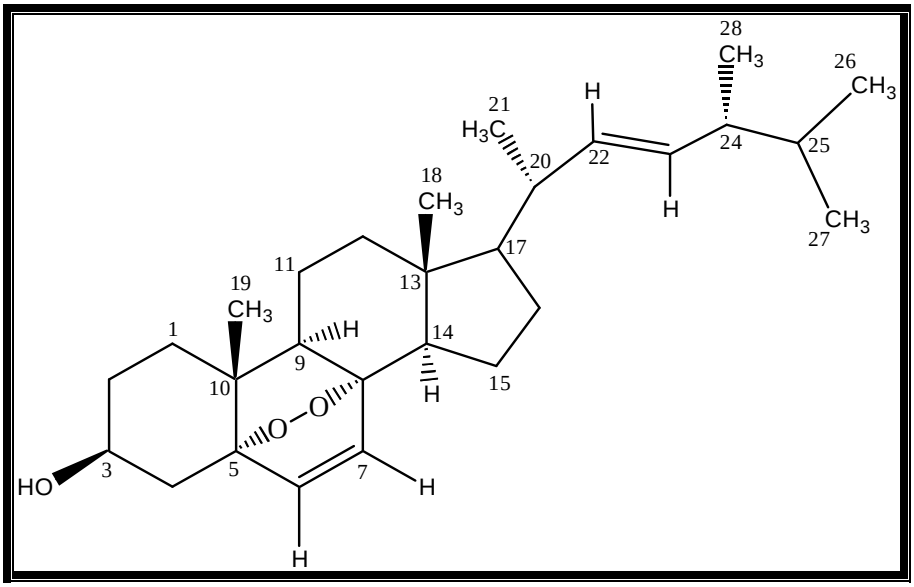


Figure: 6.5. Key HMBC correlations of compound **(09)**



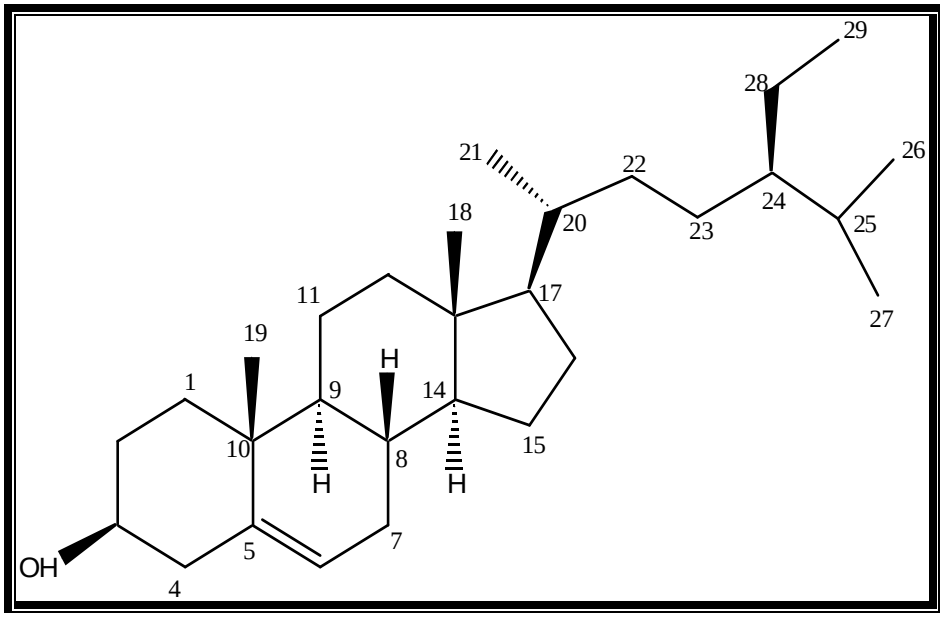
Compound (09)

6.1.2.4 Structural Elucidation of Compound (10)

This compound **(10)** was isolated as needle like crystals from ethyl acetate fraction of *Myrsine africana* eluting with EtOAc/*n*-hexane (7.25:2.75) as mobile phase on flash silica column. The EIMS of compound **(10)** showed the $[M]^+$ at *m/z* 414. The HR-EIMS showed the $[M]^+$ at *m/z* 414.3857 in agreement with the molecular formula $C_{29}H_{50}O$ (calcd. 414.3861 for $C_{29}H_{50}O$) corresponding to five degrees of unsaturation. Characteristic fragment ions were also observed at *m/z* 399, 381, 329 and 303 in the EIMS spectrum of the compound. The last two ions were diagnostic for sterols having β-unsaturation [167]. Other important fragments were observed at *m/z* 273 and 255 for [M-side chain] $^+$ and [M-side chain-H_2O]$^+$ respectively. The IR spectrum showed the bands at 3050, 1650 and 815 cm^{-1} due to trisubstituted double bonds and hydroxyl band at 3450 cm^{-1}.

^{1}H-NMR spectrum of compound **(10)** was typical for a sterol molecule. Due to quaternary CH_3-18 and CH_3-19, two singlets integrating for three hydrogens, were appeared at δ 0.63 and 0.92, respectively. Three doublets integrating for 3H at δ 0.88 ($J_{21, 20}$ = 6.5 Hz), 0.83 ($J_{26, 25}$ = 6.5 Hz) and 0.81 ($J_{27, 25}$ =6.5 Hz) were due to secondary CH_3-21, CH_3-26 and CH_3-27, respectively, while a triplet integrating for 3H at δ 0.77 ($J_{29, 28}$ = 7.0 Hz) was due to primary CH_3-29. The olefinic signals integrating for 1H signal at δ 5.32 (m) was assigned to H-6 of the β-bond. The chemical shift and splitting pattern of the signal integrating for 1H at δ 3.36 was consistent with the H-3α and H-3 *β* hydroxyl functions.

The comparative study of compound **(10)** NMR spectroscopic data with the reported data revealed its identity as Sitosterol [168].



***β*- Sitosterol**

6.1.2.5 Structural Elucidation of Compound (11)

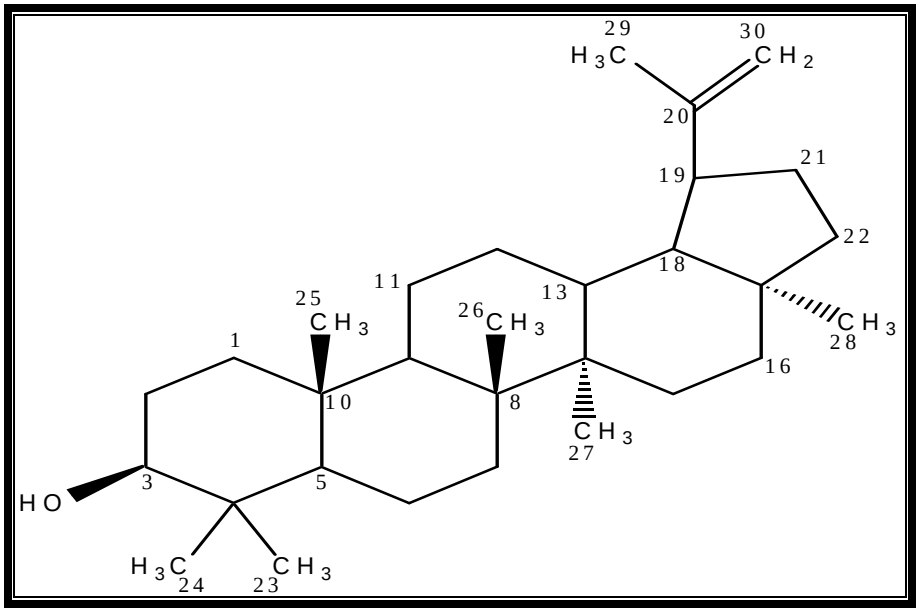
This compound was isolated as white powder from ethyl acetate fraction of *Myrsine africana* eluting with EtOAc/*n*-hexane (5.5:4.5) as mobile phase on flash silica column.

The HRMS of the compound **(11)** showed molecular ion peak at *m/z* 426.3835 consistent with the molecular formula $C_{30}H_{50}O$ (calcd. for $C_{30}H_{50}O$, 426.3861). It showed characteristic band at V_{max} 3450 cm^{-1} for hydroxyl group in IR spectrum. The terminal bond presence was confirmed by bands at V_{max} cm^{-1} 3070, 1650 and 880 cm^{-1}.

Beside $[M^+]$, other fragments occurring at m/z 385 $[M- 41]^-$, 220 $[M- C_{15}H_{26}]^+$, 218 $[M- C_{14}H_{20}]^+$, and 207 $[M- C_{16}H_{27}]^+$ were characteristics for lupine series [169].

The ^1H NMR spectrum of the compound **(11)** corroborated the presence of seven tertiary methyls at δ 0.77, 0.79, 0.85, 0.94, 0.97, 1.05 and 1.65 (all singlet). The carbonylic carbon resonance was centered at δ 3.21 as double doublet ($J_{ax, ax}$ = 9.9 Hz, $J_{ax, eq}$ = 4.5 Hz). Its chemical shift and coupling constant revealed *β* and equatorial configuration of the OH group at C-3. The olefinic proton resonance were observed at δ 4.62 and 4.75 (1H each, broad singlets), while a sextet of one proton at δ 2.36 (J = 10.5, 10.5 and 5.4 Hz) could be attributed to 19 *β*-H.

The physical and spectral data of the compound **(11)** complete agreement to those previously reported for Lupeol [170-171].


29
H3C
30
CH2
20
21
19
22
18
11
13
25
CH3
26
CH3
1
CH3
28
16
10
8
CH3
27
3
5
HO
H3C
24
23
CH3

Lupeol

6.2 PHARMACOLOGICAL INVESTIGATIONS

6.2.1 ANTIBACTERIAL ACTIVITY

Crude MeOH extract and various fractions of *Myrsine africana* were screened against the said test pathogens for possible antibacterial activity. The results are summarized in table 6.6 and the percent inhibition of each samples are presented in figures 6.6 - 6.11. Order of the antibacterial activity of the crude methanolic extract and fractions against *E.coli* is: crude extract (60.86%) *n*-hexane = EtOAc (52.17%) > BuOH (47.82%) > $CHCl_3$. Aqueous fractions showed no activity against *E.coli*. Against *S. epidermidis*: crude extract (39.39%) > *n*-hexane = aqueous (36.36%) > $CHCl_3$ = BuOH (33.33%) > EtOAc (30.30%). Against *S. typhi* crude extract (53.57%) > aqueous (52.57%) > BuOH (50.0%) > EtOAc (46.42%) > *n*-hexane (42.85%) > $CHCl_3$ (39.28%). Against *S. pneumoniae*: crude extract (57.69%) > $CHCl_3$ (53.84%) > *n*-hexane = EtOAc = BuOH = aqueous (50.00%). Against *S. aureus*: $CHCl_3$ (60.00%) > *n*-hexane (56.0%) > EtOAc (52.0%) > crude extract = aqueous (48.0%) > BuOH (44.0%). The activity against *P. aeruginosa* was: crude extract (60.0%) > $CHCl_3$ = BuOH (56.0%) > *n*-hexane (52.0%) > EtOAc = aqueous (48.0 %). Against *K. pneumoniae*: crude extract = $CHCl_3$ (68.42%) > *n*-hexane (63.15%) > EtOAc = aqueous (57.89%) > BuOH (47.36%). Against *B. pumilus*: crude extract (54.83%) > aqueous (51.61%) > BuOH (48.38%) > *n*-hexane = $CHCl_3$ (45.16%) while the EtOAc fraction of plant extract showed no activity. The activity of the test samples against *E. aerogenes* was in order of; crude extract (40.62%) > *n*-hexane = $CHCl_3$ (37.50%) > EtOAc (34.37%). The BuOH and aqueous fractions were found inactive against *E. aerogenes*. From the above results it is revealed that crude methanolic extract, $CHCl_3$ and *n*-hexane fractions show good antibacterial activity against *K. pneumoniae* while other fractions showed moderate to low and no activity against the tested pathogens. Extracts obtained from stem of *Myrsinaceae* plant showed antimicrobial effect against *E. faecalis* (MIC = 30µg/ml and MBC = 50 µg/ml) [172].

The lower the MIC_{50} values, the higher will be its potency and vice versa. The MIC_{50} values (mg/ml) of the crude methanolic extract and all fractions were calculated from three separate reading by using a Microsoft XL sheet that are mentioned in table 6.7 and presented in figures 6.12 - 6.17. Interestingly, highest activity 100% was displayed by the $CHCl_3$ fraction against *P. aeruginosa*, *n*-hexane fraction against *K. pneumoniae* and *n*-hexane fraction against *S. pneumoniae.* The research can further be proceeded to isolate the bioactive antibacterial compounds from the crude extracts and its fractions.

Table 6.5 Antibacterial activities of crude extract and various fractions of *Myrsine africana*.

Name of Bacteria	Zone of Inhibition of standard (Imipenem) 10µg /Disc	Crude Extract		*n*-hexane		$CHCl_3$		EtOAc		BuOH		Aqueous	
		Zone of Inhibition (mm)	Inhibition (%)	Zone of Inhibition (mm)	Inhibition (%)	Zone of Inhibition (mm)	Inhibition (%)	Zone of Inhibition (mm)	Inhibition (%)	Zone of Inhibition (mm)	Inhibition (%)	Zone of Inhibition (mm)	Inhibition (%)
E. Coli	23	14	60.86	12	52.17	00	00	12	52.17	11	47.82	00	00
S. epidermidis	33	13	39.39	12	36.36	11	33.33	10	30.30	11	33.33	12	36.36
S. typhi	28	15	53.57	12	42.85	11	39.28	13	46.42	14	50	15	52.57
S. pneumoniae	26	15	57.69	13	50.00	14	53.84	13	50.00	13	50.00	13	50.00
S. aureus	25	12	48.00	14	56.00	15	60.00	13	52.00	11	44.00	12	48.00
P. aeruginosa	25	15	60.00	13	52.00	14	56.00	12	48.00	14	56.00	17	48.00
K. pneumoniae	19	13	68.42	12	63.15	13	68.42	11	57.89	09	47.36	11	57.89
B. Pumalis	31	17	54.83	14	45.16	14	45.16	00	00.00	15	48.38	16	51.61
E. aerogenes	32	13	40.62	12	37.50	12	37.50	11	34.37	00	0.00	00	0.00

$$\text{Formula for \% Inhibition} = \frac{\text{Zone of Inhibition of Sample}}{\text{Zone of Inhibition of Standard}} \times 100$$

Table 6.6. MIC_{50} values (mg/ml) of the crude and fractions of *Myrsine africana*

Name of Bacteria	**MIC_{50} (mg/ml)**					
	Crude extract	***n*-hexane**	**$CHCl_3$**	**EtOAc**	**BuOH**	**Aqueous**
E. Coli	2.49	2.54	1.98	2.48	2.3	2.54
S. epidermidis	2.52	2.52	3.1	2.0	2.4	2.7
S. typhi	2.5	2.1	1.94	2.1	2.25	2.1
S. pneumoniae	1.9	1.9	2.3	2.3	2.7	2.53
S. aureus	2.7	2.2	2.2	2.38	2.3	2.48
P. aeruginosa	2.36	1.86	1.7	2.2	2.53	2.36
K. pneumoniae	2.45	1.8	2.1	2.4	1.96	1.89
B. Pumilus	2.53	2.58	1.96	2.4	2.59	2.7
E. aerogenes	2.45	2.7	2.48	2.2	2.5	2.59

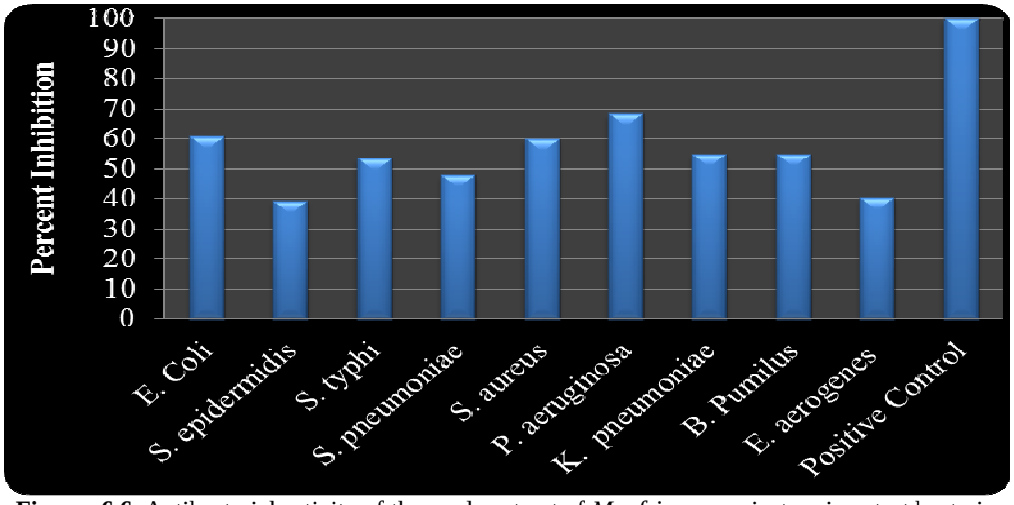


Figure: 6.6. Antibacterial activity of the crude extract of *M. africana* against various test bacteria.

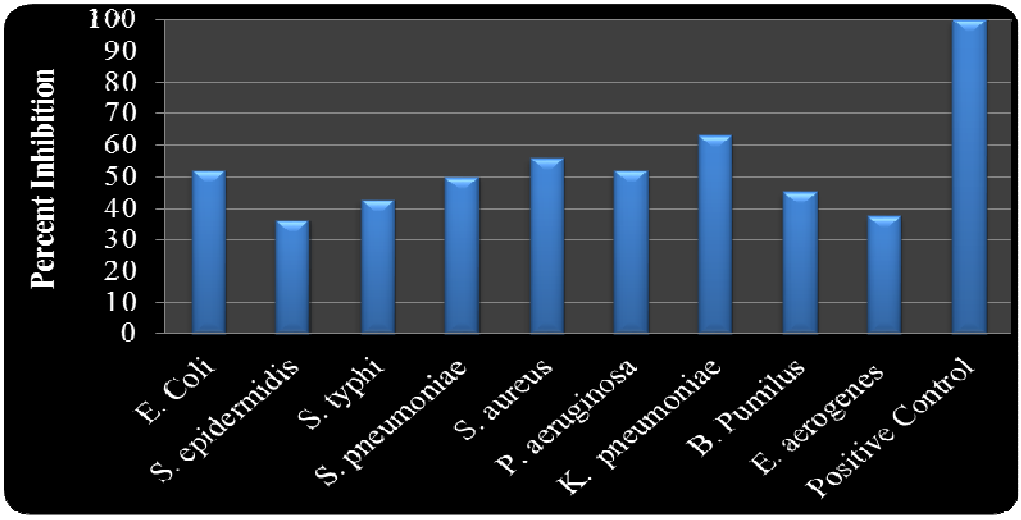


Figure: 6.7. Antibacterial activity of the *n*-hexane fraction of *M. africana* against various test bacteria.

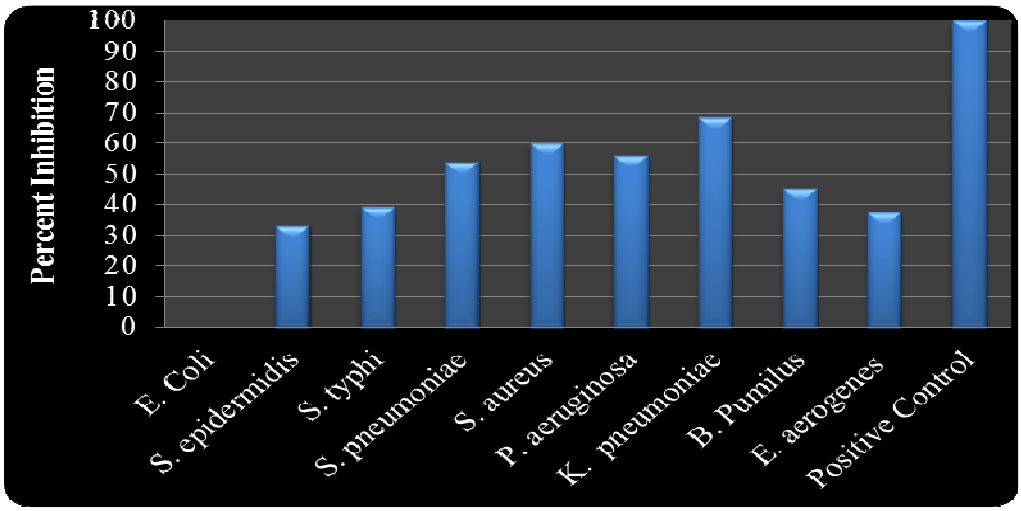


Figure: 6.8. Antibacterial activity of the $CHCl_3$ fraction of *M. africana* against various test bacteria.

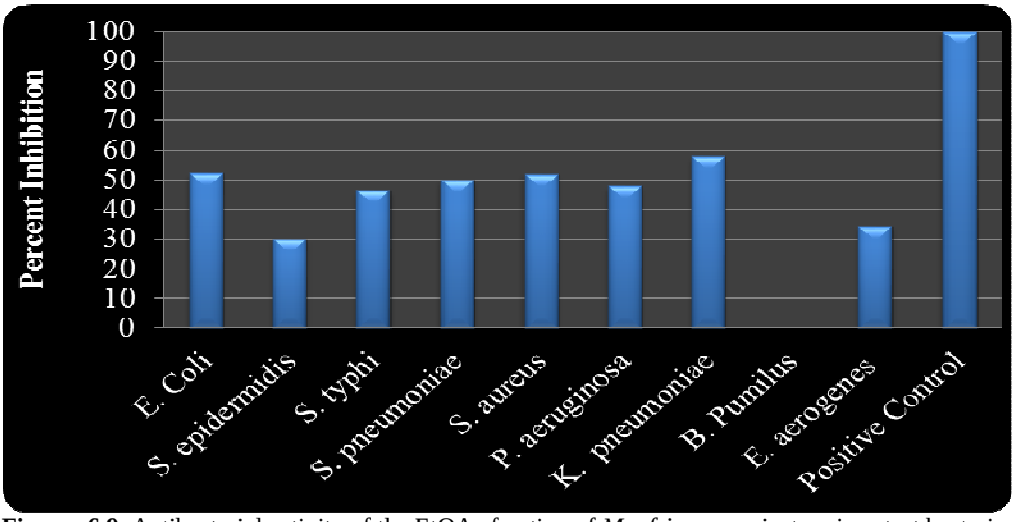


Figure: 6.9. Antibacterial activity of the EtOAc fraction of *M. africana* against various test bacteria.

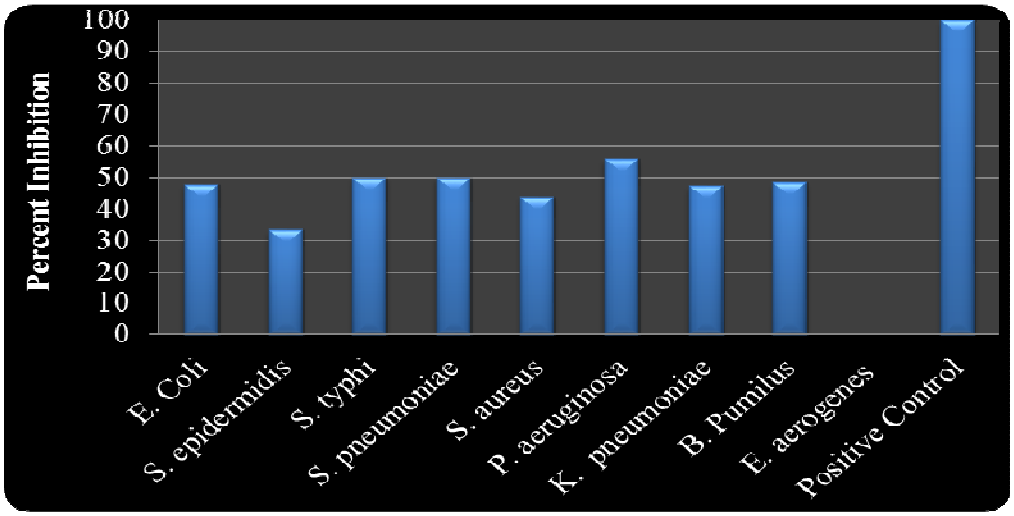


Figure: 6.10. Antibacterial activity of the BuOH fraction of *M. africana* against various test bacteria.

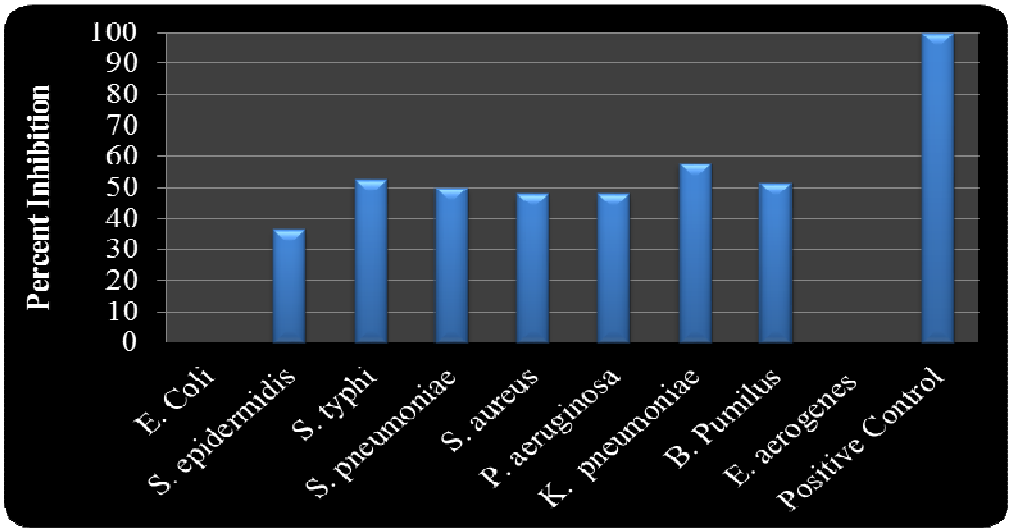


Figure: 6.11. Antibacterial activity of the Aqueous fraction of *M. africana* against various test bacteria.

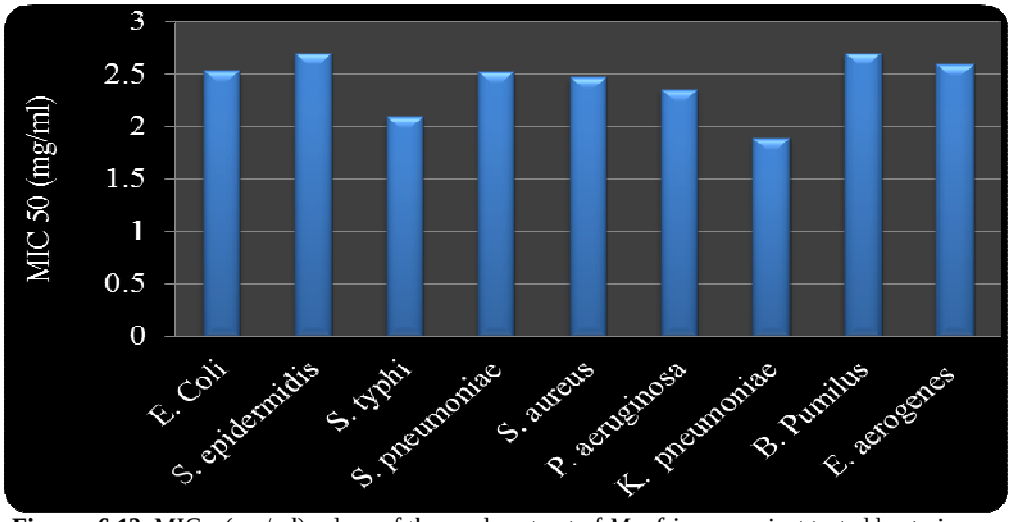


Figure: 6.12. MIC_{50} (mg/ml) values of the crude extract of *M. africana* against tested bacteria.

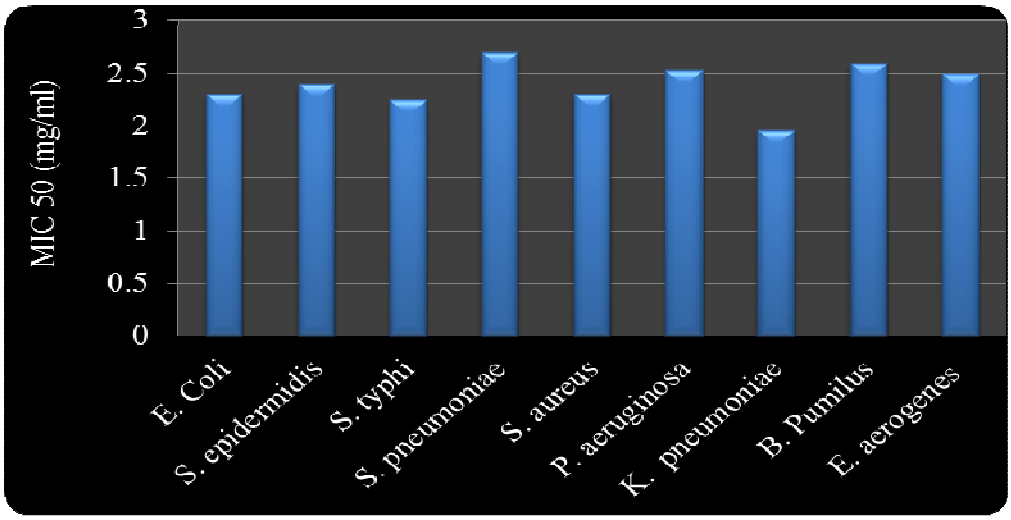


Figure: 6.13. MIC_{50} (mg/ml) values of the *n*-hexane fraction of *M. africana* against tested bacteria.

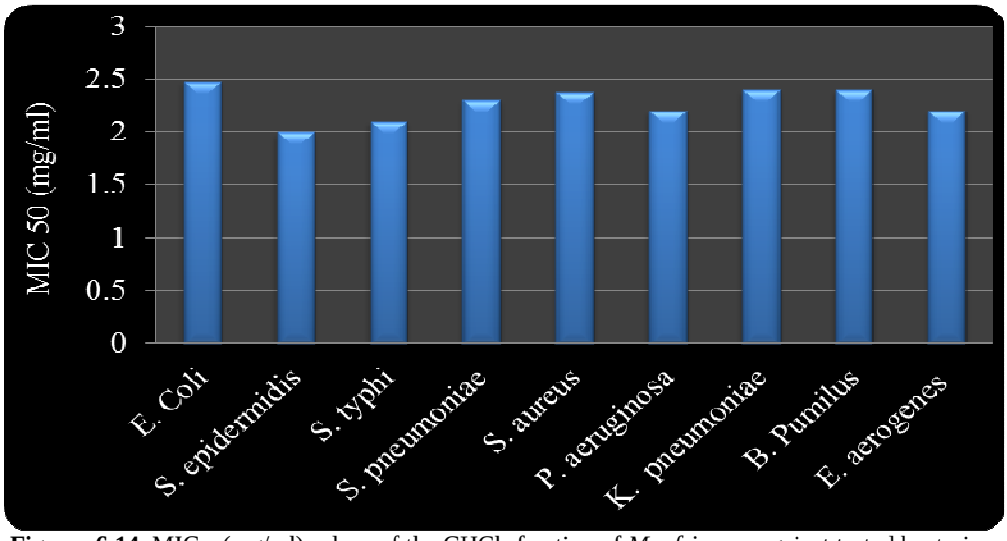


Figure: 6.14. MIC_{50} (mg/ml) values of the $CHCl_3$ fraction of *M. africana* against tested bacteria.

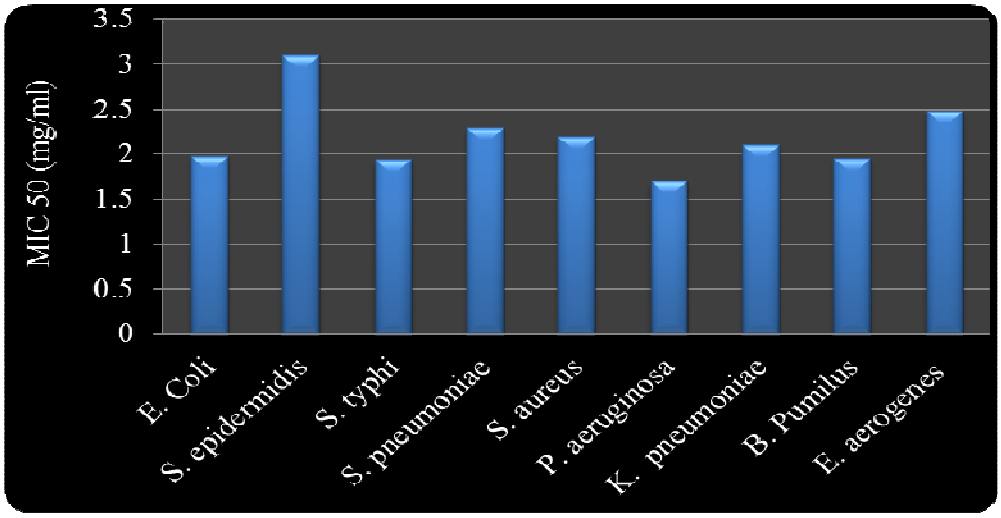


Figure: 6.15. MIC_{50} (mg/ml) values of the EtOAc fraction of *M. africana* against tested bacteria.

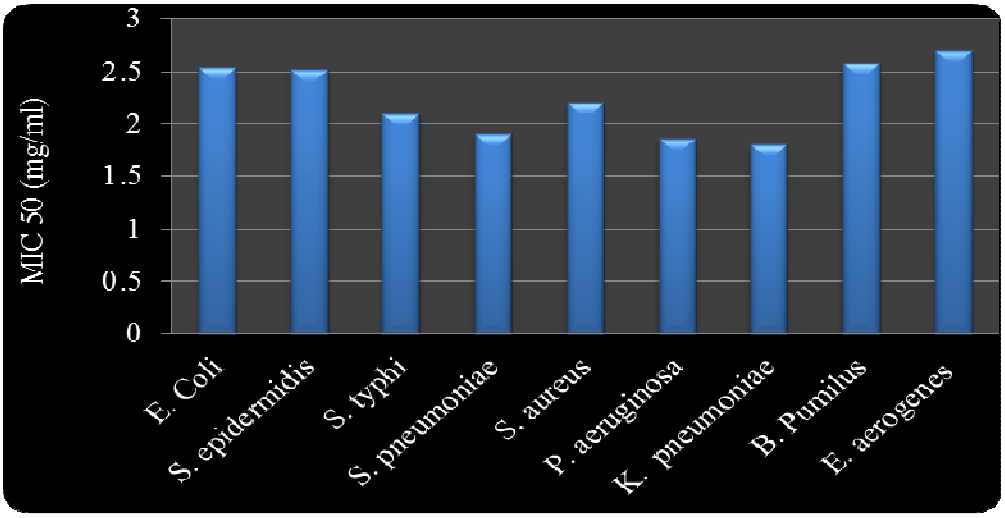


Figure: 6.16. MIC_{50} (mg/ml) values of the BuOH fraction of *M. africana* against tested bacteria.

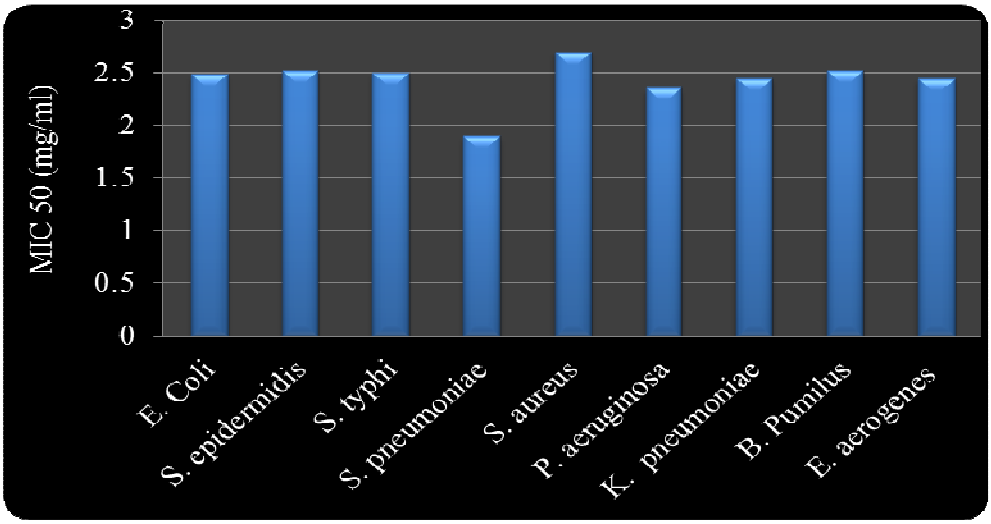


Figure: 6.17. MIC_{50} (mg/ml) values of the Aqueous fraction of *M. africana* against tested bacteria.

6.2.2 ANTIFUNGAL ACTIVITY

Persistent opportunistic fungal infections have become an important factor for morbidity and mortality in immune-compromised patients [173]. The majority of the fungal infections are mainly caused by the *Aspergillus* and *Candida* species. Recent trends in epidemiological trends have pointed out a shift towards infection caused by *Aspergillus* and non-albicans *Candida* [174].

The test samples were screened for antifungal activity against *A. niger, A. flavus, P. notatum, F. oxysporum, T. harzianum, R. stolonifer* using agar tube dilution method. Amphotericin B and Miconazole were used as standard drugs.

The crude methanolic extract and all fractions of plant were failed to show antifungal effect against *A. niger, P. notatum* and *R. stolonifer* as shown in table 6.7. Low antifungal activity was observed for crude extract (10%), *n*-hexane (10%), $CHCl_3$ (15%) and BuOH (5%) fractions against *A. flavus* as presented in figure 6.18. In addition, the $CHCl_3$ and aqueous fractions showed low inhibiting effect, 15% and 24% respectively, against *F. oxysporum*: while crude extract and other fractions were inactive against the tested fungal specie (figure 6.19). Similarly the *n*-hexane, $CHCl_3$ and BuOH fractions of the plant show low antifungal activity against *T. harzianum* (figure 6.20). Rest of the fractions and crude extract was unable to inhibit the growth of the tested fungal species. The results showed that *M. africana* has no significant antifungal activity against the tested fugal species.

A significant antifungal activity against *Candida albicans*, with MIC of 0.250 mg/ml have been reported for the crude methanolic extract of *Monoporus clusiifolius* (Myrsinaceae). The methanolic extract of *Labisia pumila* Benth also possesses significant antifungal activity against *Candida albicans* [175,176].

Table 6.7 Antifungal activity of the crude extract and various fractions of *Myrsine africana*.

Name of Fungi	Percent linear growth inhibition							
	negative control	positive control	crude extract	*n*-hexane	$CHCl_3$	EtOAc	BuOH	Aqueous
A. niger	0	100	0	0	0	0	0	0
A. flavus	0	100	10	10	15	0	5	0
P. notatum	0	100	0	0	0	0	0	0
F. oxysporum	0	100	0	0	15	0	0	24
T. harzianum	0	100	0	8	12	0	16	0
R. stolonifer	0	100	0	0	0	0	0	0

$$\% \text{ inhibition of fungal growth} = 100 - \frac{\text{linear growth in test (mm)}}{\text{linear growth in control (mm)}} \times 100$$

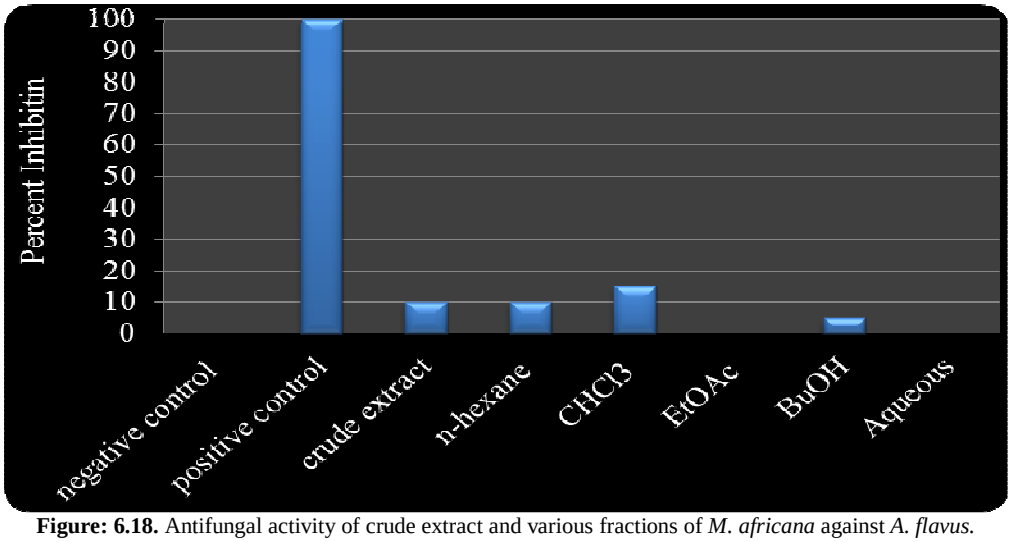


Figure: 6.18. Antifungal activity of crude extract and various fractions of *M. africana* against *A. flavus.*

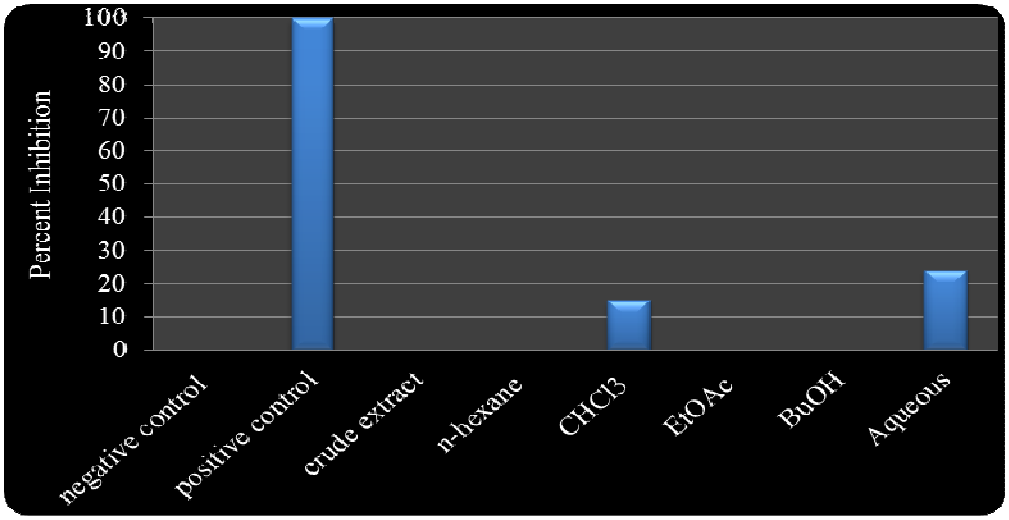


Figure: 6.19. Antifungal activity of crude extract and various fractions of *M. africana* against *F. oxysporum.*

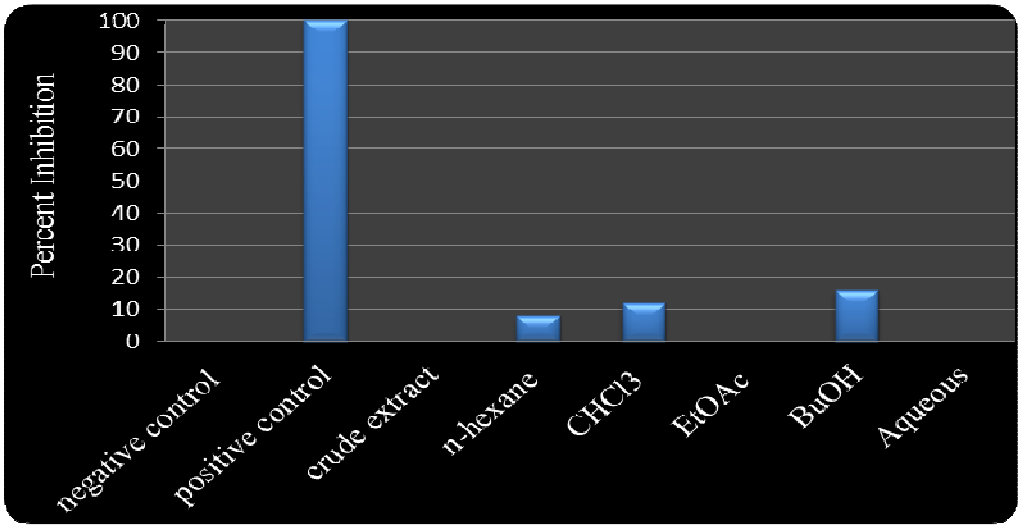


Figure: 6.20. Antifungal activity of crude extract and various fractions of *M. africana* against *T. harzianum.*

6.2.3 INSECTICIDAL ACTIVITY

The crude extract and fractions of *Myrsine africana* were screened for insecticidal activity, using impregnated filter paper method [177], against three different insect species i.e. *Tribolium castaneum, Rhizopertha dominica* and *Callosbruchus analis.* Results are summarized in table 6.8 and presented in figure 6.21, revealed that crude extract along with other fractions of the plant, showed no activity against the tested insects except $CHCl_3$ and aqueous fractions showed low insecticidal activity (20%, each) against *T. castaneum* and *R. dominica* respectively.

Permithrine (copex) was used as positive control while the organic solvent was treated as negative control. The positive control shows 100 % mortality while the mortality rate was 0% in case of negative control.

From the results it may be concluded that *M. africana* has no insecticidal property.

Table 6.8 Insecticidal activity of crude extract and various fractions of *Myrsine africana* by contact toxicity method.

Name of Insect	% Mortality							
	Control (Positive)*	Control Negative	Crude Extract	*n*-hexane	$CHCl_3$	EtOAc	BuOH	Aqueous
Tribolium castaneum	100	0	0	0	20	0	0	0
Rhizopertha dominica	100	0	0	0	0	0	0	20
Callosbruchus analis	100	0	0	0	0	0	0	0

* Permethrin at concentration 235.9 $\mu g/cm^2$ was used as standard drug in positive control.

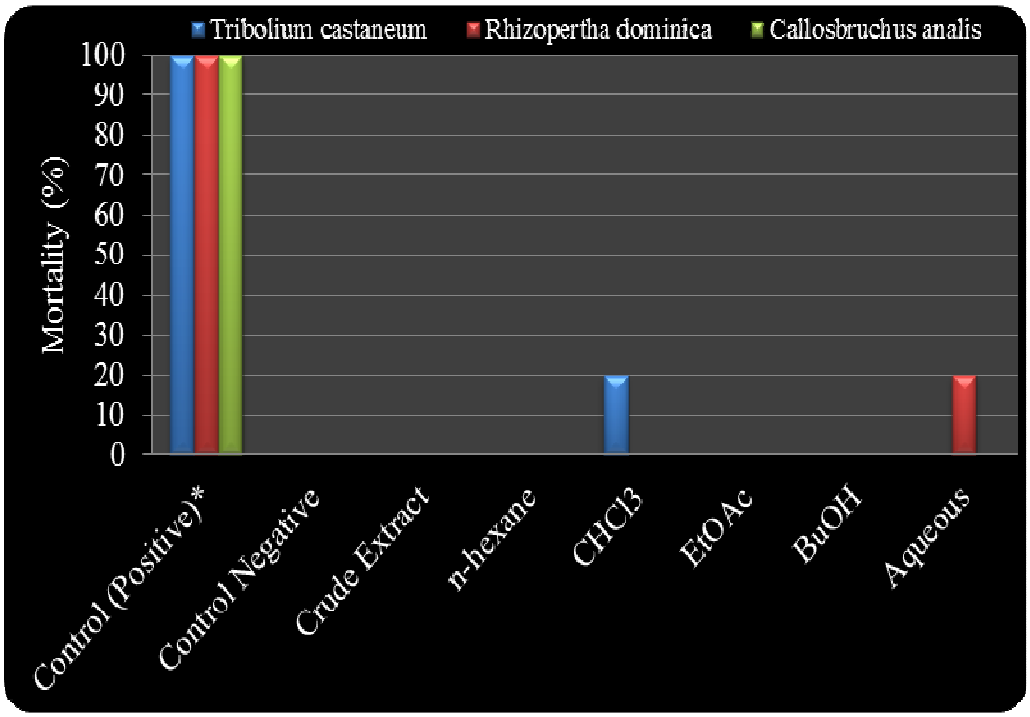


Figure: 6.21. Insecticidal activity of crude extract and various fractions of *M. africana* against the selected insects

* Permethrin at concentration 235.9 µg/cm^2 was used as standard drug in positive control

6.2.4 ANTI-TERMITE ACTIVITY

The crude methanolic extract, $CHCl_3$, aqueous fractions of *Myrsine africana* were screened for anti-termite activity in the "Termites lab of Nuclear Institute of Food and Agriculture (NIFA), Peshawar. All the experiments were performed in triplicates.

The anti-termite activity of the crude methanolic extract experiments extended for three days. Twenty five termites were transferred to petri dishes containing negative control and test samples, respectively. The results indicated that the average termites killed on day first were eight, on day the second average killed were 17, and on day third all the termites were found dead. Similarly, the aqueous fraction of the plant was screened for anti-termite activity. The results indicated that average of the termites killed by aqueous fraction on day first was six. On day second the average was found to be fifteen. On day third all the termites were found dead. Similarly, the EtOAc fraction of the plant was also screened. The results indicated that average of the termites killed on day first was eleven, on day second the average was found to be nineteen and on day third all the termites were found dead. In the negative control average of the termites killed on day first and second was zero and only two termites were found dead on day three. The above results (given in table 6.9) indicated that EtOAc fraction of *Myrsine africana* showed significant activity against termites.

Table 6.9 Anti-termites activity of crude extract and selected fractions of *M. africana*

Test Sample	**No. of Termites**	**Day**	**Average termites killed by –ive controls**			**Average termites killed by *Positive Control**	**Average termites killed by sample**
			MeOH	**EtOAc**	**H_2O**		
Crude extract	25	1	0	NA	NA	23	8
		2	0	NA	NA	25	17
		3	2	NA	NA	-----	25
EtOAc		1	NA	0	NA	22	11
		2	NA	0	NA	25	19
		3	NA	2	NA	--------	25
Aqueous		1	NA	NA	0	23	6
		2	NA	NA	0	25	15
		3	NA	NA	2	--------	25

6.2.5 PHYTOTOXIC ACTIVITY

Herbicides of the plants' origin are friendly to the environment that necessitates for their search. The phytotoxic results of crude extract and various fractions of *M. africana* against *Lemna minor* are summarized in table 6.10 - 6.11 and presented in figure 6.22. The methanolic extract showed (25 and 6.25%), *n*-hexane (25 and 12.50%), $CHCl_3$ (31.25 and 6.25%), EtOAc (18.75 and 6.25%), BuOH (25 and 18.75%) and aqueous (25 and 12.5%) growth inhibition at concentrations of 1000 and 100 µg/ml, respectively. While no phytotoxic activity was observed at concentration 10 µg/ml. Paraquat was used as standard plant growth inhibitor.

Table 6.10 Percent growth regulation of *Lemna minor*

Name of plant	Concentration of sample (µg/ml)	Number of fronds						
		Crude extract	*n*-hexane	$CHCl_3$	EtOAc	BuOH	Aqueous	Control
Lemna minor	1000	12	12	11	13	13	12	16
	100	15	14	15	15	15	14	16
	10	16	16	16	16	16	16	16

* Paraquat at a concentration 0.015 µgm / ml was used as standard drug

Table 6.11 Phytotoxic activity of *Myrsine africana* against *Lemna minor*

Concentration of sample (µg/ml)	Percent growth regulation						
	Crude extract	*n*-hexane	$CHCl_3$	EtOAc	BuOH	Aqueous	Control
1000	25.00	25.00	31.25	18.75	25.00	25.00	100
100	6.25	12.50	6.25	6.25	18.75	12.50	100
10	00	00	00	00	00	00	100

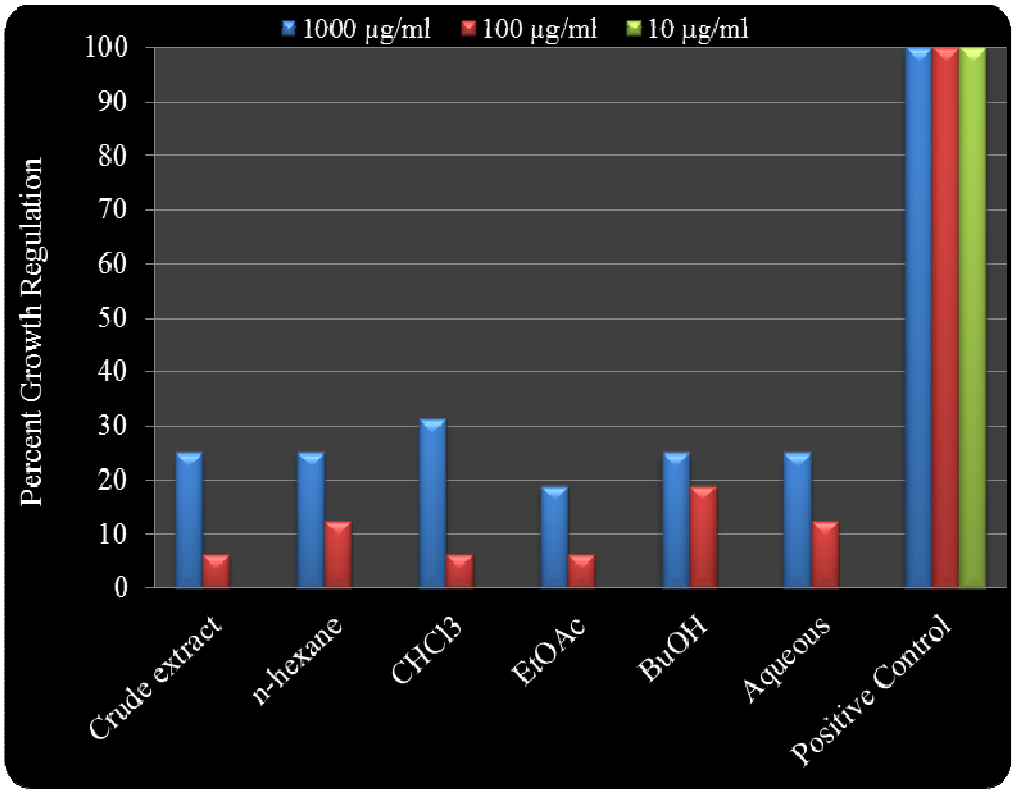


Figure: 6.22. Phytotoxic activity of crude extract and various fractions of *M. africana* against *L. minor*.

*Paraquat at a concentration 0.015 µg/ml was used as standard drug

6.2.6 BRINE SHRIMP BIOASSAY

The brine shrimp lethality test was performed to assess the toxicity of *M. africana*. The test samples (crude and fractions) were used in concentration of 10, 100 and 1000 µg/ml. Results for the lethality were noted in term of deaths of larvae, compared with Etoposide (standard drug). The calculated LD_{50} value for the standard drug was 7.4625 µg/ml.

The crude extract of the plant showed low cytotoxic activity 20.00, 13.33 and 6.66%, against experimental shrimps, at concentrations of 10, 100 and 1000 µg/ml respectively.

Different fractions exhibited various cytotoxic activities. The *n*-hexane fraction showed good cytotoxicity (66.66%) at concentration of 1000 µg/ml while low activity (33.33 and 13.33%) was observed at concentration of 100 and 10 µg/ml, respectively. The LD_{50} value for the fraction calculated as 30.344 µg/ml. The $CHCl_3$ showed low activity at all concentrations, 30.0% at 1000 µg/ml, 16.66% at100 µg/ml and 13.33% at 10 µg/ml. The EtOAc fraction showed low brine shrimp lethality at respective test concentrations that was; 26.66, 20.0 and 13.33% at 1000, 100 and 10 µg/ml, respectively. Similarly, the BuOH fraction of the plant showed low cytotoxic activity (30.0, 20.0 and 10.0%) at respective test concentrations. The aqueous fraction screened at the end of the experiment did not show any significant cytotoxic activity against the tested shrimps. The maximum cytotoxicity was 33.33% as shown by the aqueous fraction at higher concentration. The crude extract from the bark of *Aegiceras corniculatum* Blanco showed prominent cytotoxic effect against *Artemia salina* with LD_{50}=5.5 µg/ml and LC_{90}=9.3 µg/ml [178]. Cytotoxic triterpene saponins have been reported from several *Myrsine* species [179]. The cytotoxic results of the crude and all fractions are summarized in table 6.12, also presented in figures 6.23.

From the above results it is concluded that the *n*-hexane fraction the plant contains the cytotoxic constituents.

Table 6.12 Brine shrimps activity of crude methanolic extract and various fractions of *Myrsine africana.*

Dose (µg/ml)	*Standard drug		Crude extract		*n*-hexane		$CHCl_3$		EtOAc		BuOH		Aqueous	
	Survivors	% Killed	Survivors	% Killed	Survivors	% Killed	Survivors	% Killed	Survivors	% Killed	Survivors	% Killed	Survivors	% Killed
1000	0	100	24	20.00	10	66.66	21	30.00	22	26.66	21	30.00	20	33.33
100	0	100	26	13.33	20	33.33	25	16.66	24	20.00	24	20.00	24	20.00
10	0	100	28	6.66	26	13.33	26	13.33	26	13.33	27	10.00	26	13.33

* Etoposide at concentration of 7.4625 µg / ml was used as standard drug.

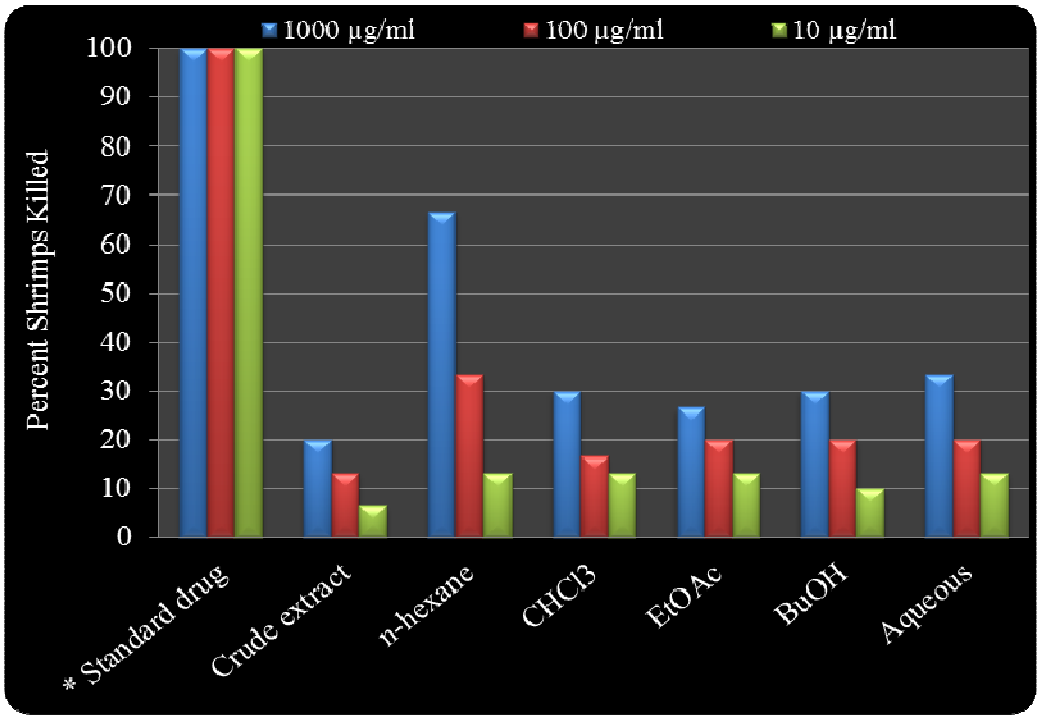


Figure: 6.23. Brine shrimp lethality of crude extract and various fractions of *M. africana.*

* Etoposide at concentration of 7.4625 µg / ml was used as standard drug.

6.2.7 HAEMAGGLUTINATION ACTIVITY

Certain plants have been found to contain hemagglutinins in their different parts, which can be extracted and used for the production of blood typing reagents in the same way as agglutinins from animal sources. The plant source agglutinins have advantage over the animal sources because they are economical and available in large quantities. Crude extract and various fractions of *M. africana* at different dilutions were screened for possible haemagglutination activity against human erythrocytes of all blood groups (ABO). The results showed that crude methanolic extract and $CHCl_3$ fraction possess moderate (++) haemagglutination effect against AB^- blood group. Aqueous fraction have moderate activity (++) against O^+ blood groups at higher concentrations (1:2). Weak activity (+) was also observed against the erythrocytes of other blood groups at dilution of 1:2 and 1:4. The *n*-hexane and EtOAc fractions of the plant showed no haemagglutination activity against human RBCs of all blood groups. Results are summarized in table 6.13. The present investigations revealed that the plant contains lectins and could be a useful source for phytolectins.

Table 6.13 Haemagglutination activity of the crude extract and various fractions of *Myrsine africana* at various dilutions

Blood groups	Crude extract				*n*-hexane				$CHCl_3$				EtOAc				BuOH				Aqueous			
	1:2	1:4	1:6	1:8	1:2	1:4	1:6	1:8	1:2	1:4	1:6	1:8	1:2	1:4	1:6	1:8	1:2	1:4	1:6	1:8	1:2	1:4	1:6	1:8
AB $^{-}$	++	+	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
B $^{-}$	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
A $^{-}$	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
B $^{+}$	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
A $^{+}$	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
AB $^{+}$	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	+	-	-
O $^{+}$	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-
O $^{-}$	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-

6.2.8 ANTI-INFLAMMATORY ACTIVITY

The main causes of the inflammation are the immune system reaction to infection, malignancy, cellular changes, environmental agents and injury. Inflammation is a key mark in autoimmune disease. In conditions like, Hashimoto's thyroiditis, the disease process is due to inflammation, while in some other conditions like Crohn's disease, the cause of inflammation is the diseased condition [180]. As the herbs possess anti-inflammatory characteristics, we screen crude extract and various fractions of the plant for possible anti-inflammatory activity. The results obtained are shown in table 6.14 and presented if figure 6.24 which reveals that EtOAc fraction of the plant showed moderate (41.8%) anti-inflammatory activity at concentration of 500 μg/ml. The rest of the fractions and crude methanolic extract of the plant showed low anti-inflammatory activity. Indomethacin was used as positive control. Myrsinoic acids B, C and F isolated from the methanolic extract of *Myrsine seguinii*, possess anti-inflammatory activity. The TPA-induced edema of mouse ear was suppressed by these compounds but the myrsinoic acid F was found most potent (77% at 0.56 μM). Another novel anti-inflammatory compound has been reported from the methanolic extract of the *M. seguinii* and its potency against TPA-induced inflammation of mouse ears was 59% at 1.4 μM [181, 182].

Table 6.14 Anti-inflammatory activity of the crude extract and various fractions of *Myrsine africana*

Test sample	Percent inhibition at 500 μg/ml
Crude extract	38.5
n-hexane	40.9
$CHCl_3$	37.6
EtOAc	44.8
BuOH	33.1
Aqueous	29.7
* Indomethacin	98.0

* Standard drug

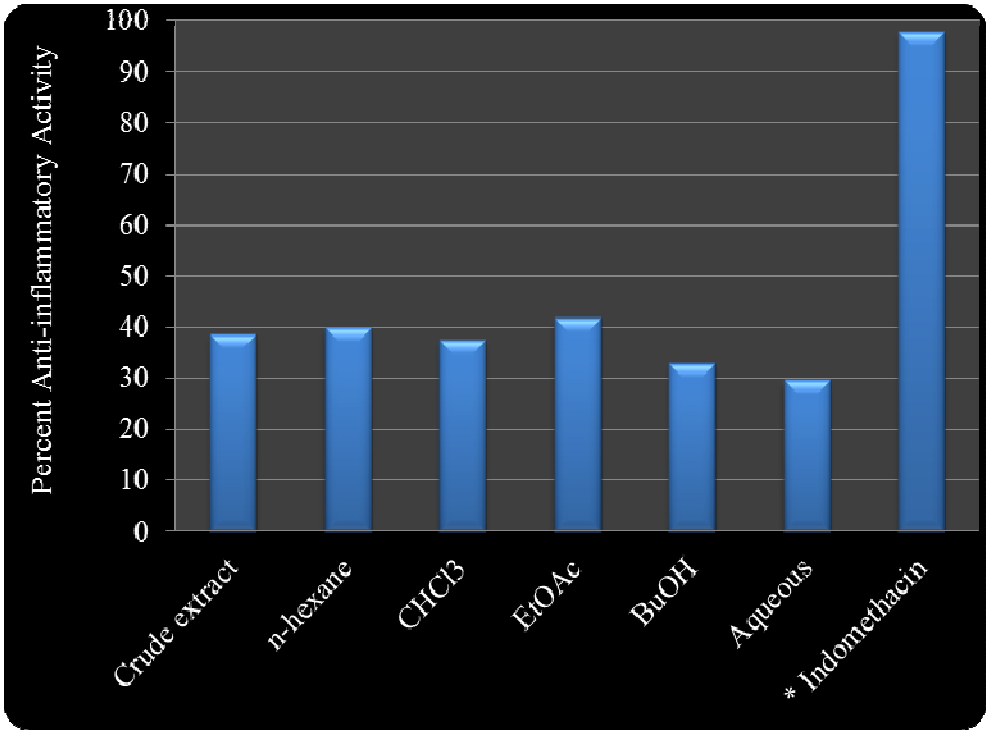


Figure: 6.24. Anti-inflammatory activity of crude extract and fractions of *M. africana*.

6.2.9 EFFECTS ON RABBIT'S JEJUNUM PREPARATIONS

Spasmolytic activity

It is evident from figure 6.25 that extract produced a concentration dependent fall in spontaneous rabbit's jejunum preparations. The extract totally abolished the spontaneous contractions of tissues at concentration 5.0 mg/ml. This confirms that the plant specie contains spasmolytic constituents and therefore has antispasmodic action.

In order to explain the possible mode of actions, we tried the extract on contractions produced by high potassium concentration (80.0 mM). The results are shown in figure 6.26. Interestingly, the extract produced similar type of actions in the KCl-induced contractions. The results of the higher concentration are suprimposible (figure 6.27). Thus the extract is having almost similar values of EC_{50} for the spontaneous contractions and KCl - induced contractions. The results suggest that the plant specie is having antispasmodic action possibly through the calcium channel blocking mechanisms [90].

Table 6.15 Spasmolytic activity of crude methanolic extract of *M. Africana*

Concentration of crude methanolic extract (mg/ml)	**Percent response of control max. (Mean ± S.D, n= 3)**
0.01	0.00
0.03	6.80
0.1	31.04
0.3	65.00
1.0	86.20
3.0	93.00
5.0	96.50
10.0	100

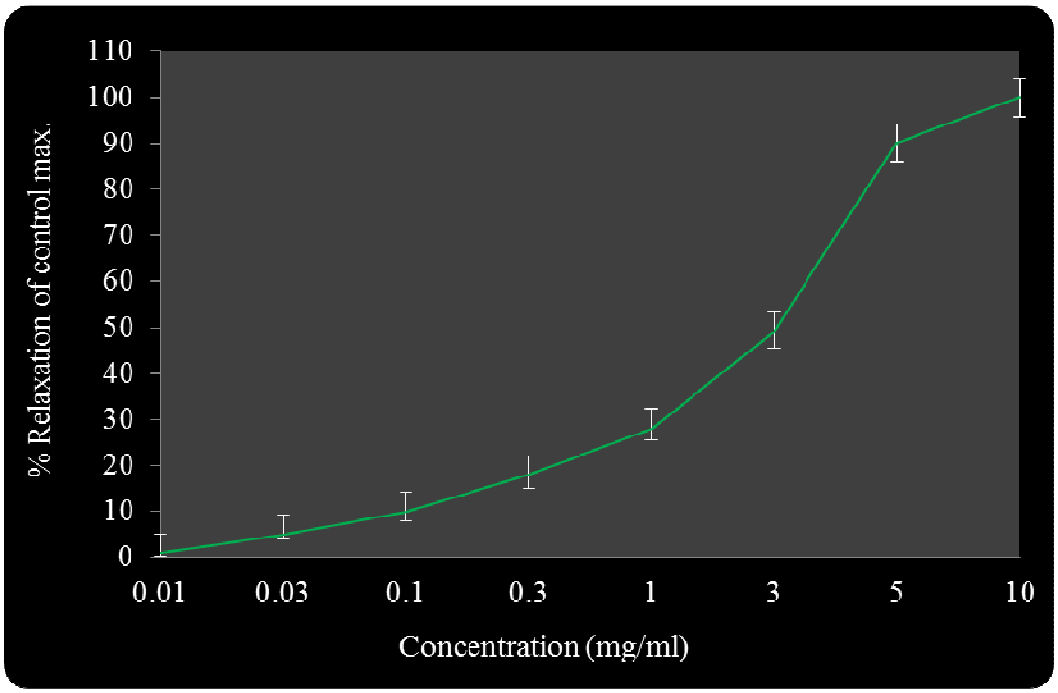


Figure: 6.25. Spasmolytic activity of *M. africana* on spontaneous rabbit's jejunum preparations.

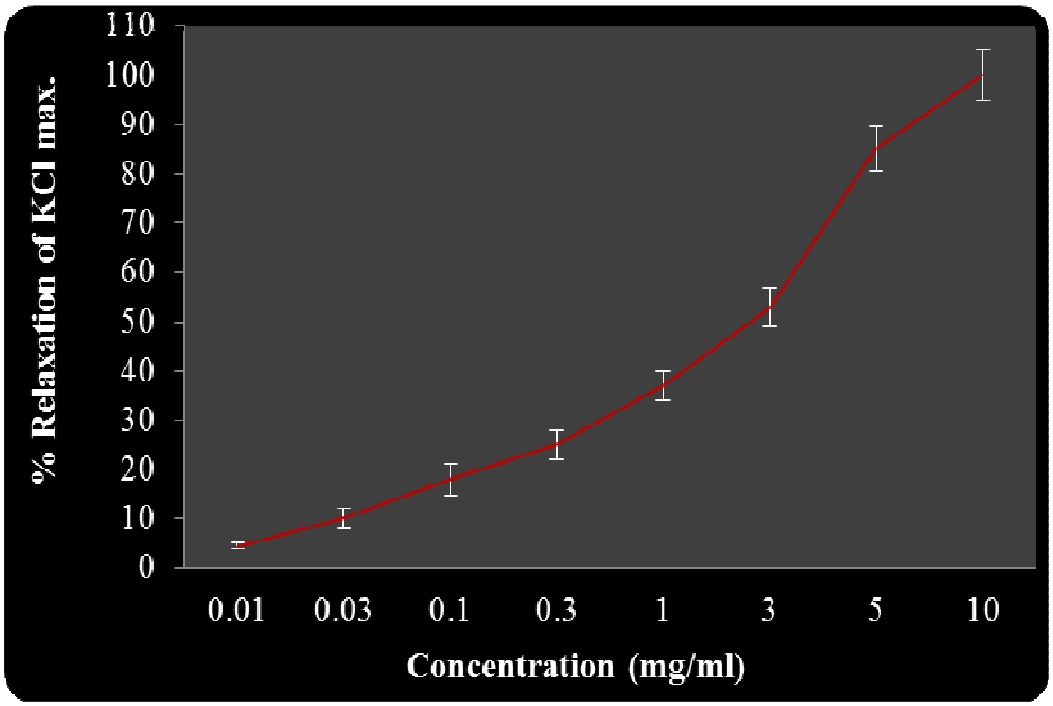


Figure: 6.26. Spasmolytic activity of *M. africana* on KCl (80 mM) induced contractions on rabbit's jejunum preparations.

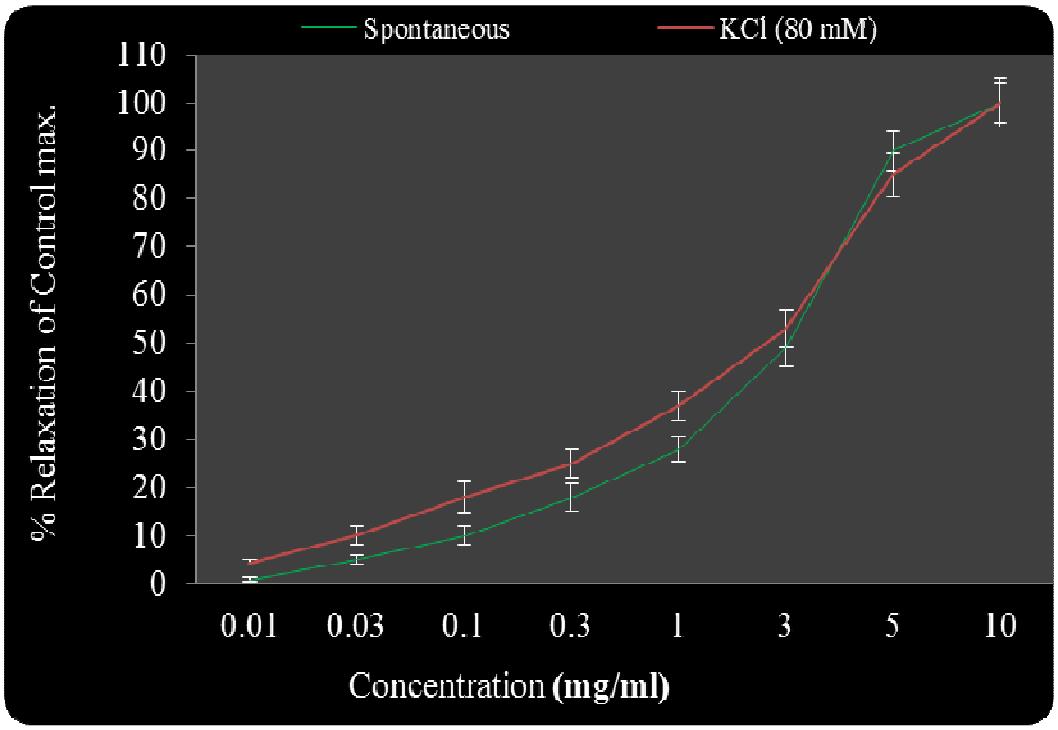


Figure: 6.27. Spasmolytic activity of *M. africana* on spontaneous and KCl (80 mM) induced contractions on rabbit's jejunum preparations.

6.2.10 NITRIC OXIDE (NO) FREE RADICAL SCAVENGING ACTIVITY

Free radicals are constantly produced in the living system, which can cause an extensive damage to biomolecules and tissues thereby causing various diseases like extensive lysis and degenerative diseases [183]. For reduction of these oxidative damages, many synthetic drugs are used but their side effects cannot ignore. The consumption of the natural food products and traditional medicines containing antioxidant constituents, are alternative source to avoid the side effect. Recently, from different natural sources various natural antioxidants have been reported [184-186].

Due to importance of the natural antioxidants, we screened the crude methanolic extract and various fractions of *Myrsine africana* at different concentrations (0.3, 0.6, 0.9, 1.2 and 1.5 mg) for possible Nitric Oxide free radical scavenging assay. The results obtained are shown in table 6.16 and presented in figures 6.28-6.32. Figure 6.28 indicates that crude extract (32.27%) and aqueous fraction (35.36%) of the plant posses moderate NO free radical scavenging activity. While low activity was observed for other fractions including *n*-hexane (23.49%), $CHCl_3$ (28.13%), EtOAc (22.03%) and BuOH (26.74%) at concentration of 0.3 mg/ml. The NO free radical scavenging activity was increased by increasing the concentration of the test samples and at 0.6 mg/ml the crude extract along with four other fractions showed moderate activity i.e. 38.04% for crude extract 34.47% for *n*-hexane, 34.30% for EtOAc, BuOH 30.24% and the aqueous fraction of the plant showed 45.28% activity. Results are given in figure 6.29. At concentration of 0.9 mg/ml all of the test samples showed moderate NO free radical scavenging activity the order of the activity of the test sample is: aqueous 49.91% > crude extract 45.69% > EtOAc 39.52% > $CHCl_3$ 36.82% > BuOH 33.98% > *n*-hexane 33.73% (figure 6.30). Similarly, the activity was performed at concentration of 1.2 mg/ml. Results summarized in figure 6.31, indicates that the crude extract along with other fractions showed moderate NO free radical scavenging activity with

order; aqueous 52.68% > crude extract 49.17% > EtOAc 43.69% > $CHCl_3$ 39.26% > BuOH 38.94% > *n*-hexane 36.17%. The activity was also performed at concentration of 1.5 mg/ml and the results are given in figure 6.32. The aqueous fraction and crude extract showed the highest activity (55.36 and 53.05%, respectively) and in rest of the fractions; EtOAc; 49.59%, $CHCl_{3;}$ 44.65%, BuOH; 43.65% and the *n*-hexane showed 41.94% NO free radical scavenging activity. From the above results it is concluded that the plant crude extract and fractions possess concentration dependent nitric oxide free radical scavenging activity that necessitates for further work to isolate the active molecules from *Myrsine africana*.

A concentration dependent anti-oxidant activity has been reported for the ethyl acetate extract of *R. melanophloeos*, with IC_{50} values were comparable with the standard used [187].

Table 6.16 Nitric oxide free radical scavenging activity of the crude extract and fractions of *Myrsine africana.*

Standard: Vitamen C was used as standard at concentration of 47.78 µg/ml.

Concentration of samples (mg/ml)	**Crude extract**	***n*-hexane**	**$CHCl_3$**	**EtOAc**	**BuOH**	**Aqueous**
0.3	32.27	23.49	28.13	22.03	26.74	35.36
0.6	38.04	27.47	34.47	34.3	30.24	45.28
0.9	45.69	33.73	36.82	39.52	33.98	49.91
1.2	49.17	36.17	39.26	43.69	38.94	52.68
1.5	53.05	41.94	44.65	49.59	43.65	55.36

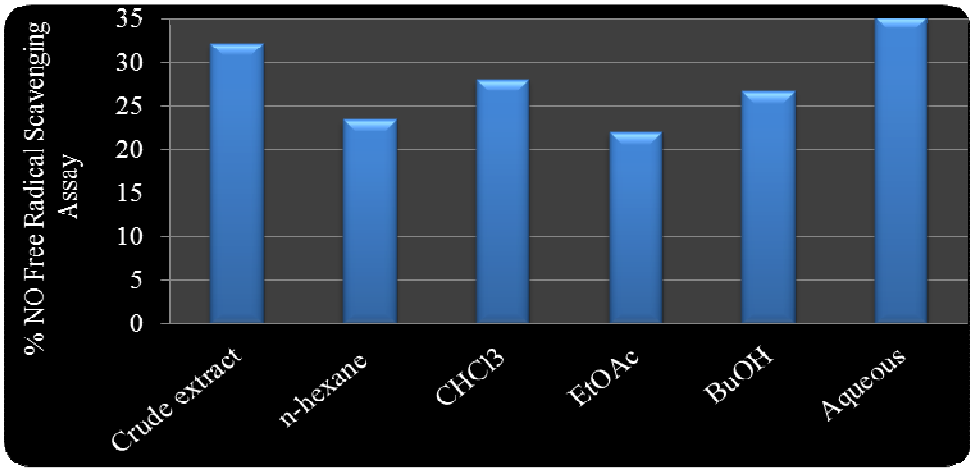


Figure: 6.28. NO free radical scavenging activity of crude and various fractions of *M. africana* at concentration of 0.3 mg/ml.

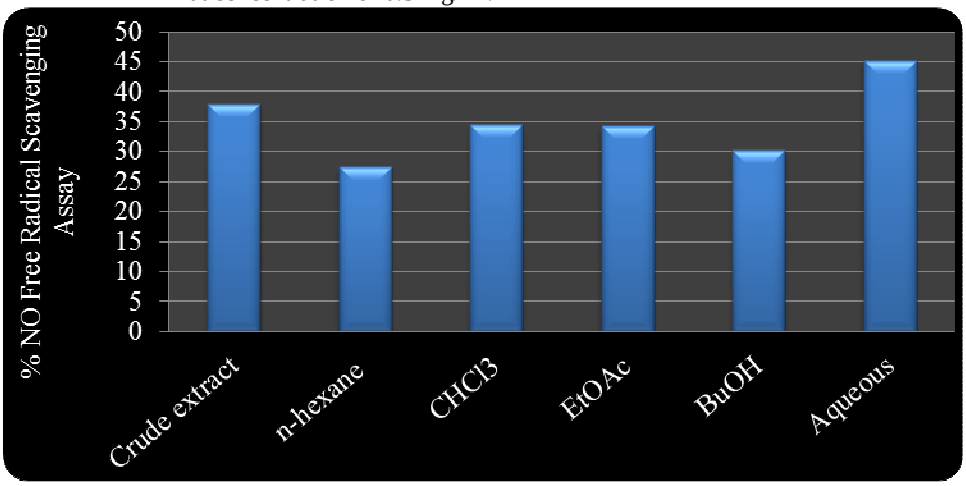


Figure: 6.29. NO free radical scavenging activity of crude and various fractions of *M. africana* at concentration of 0.6 mg/ml.

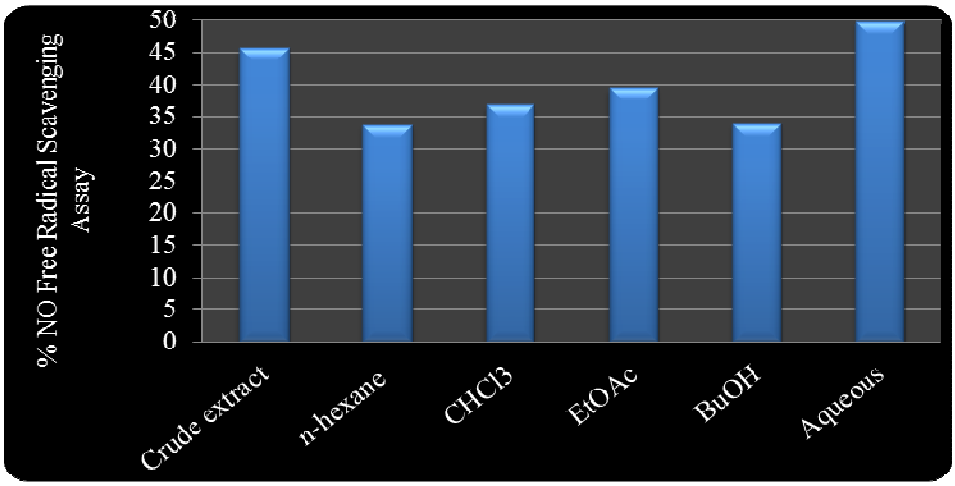


Figure: 6.30. NO free radical scavenging activity of crude and various fractions of *M. africana* at concentration of 0.9 mg/ml.

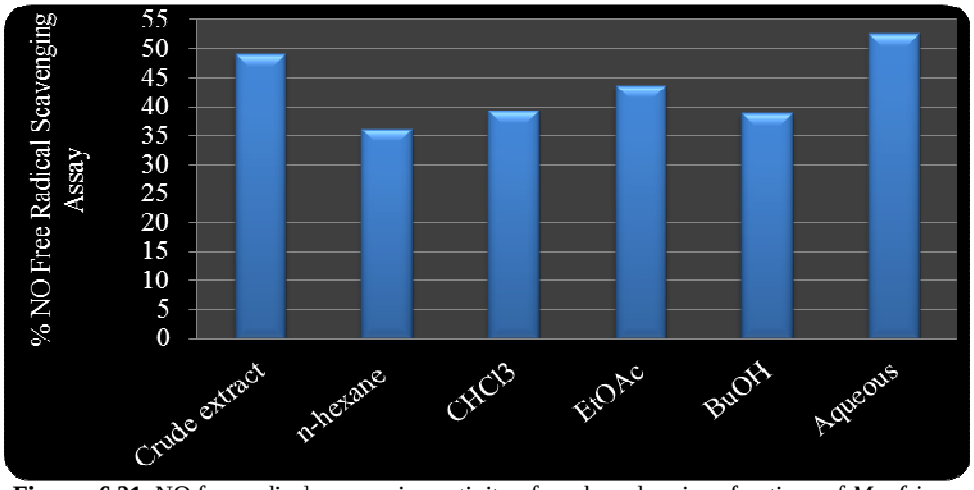


Figure: 6.31. NO free radical scavenging activity of crude and various fractions of *M. africana* at concentration of 1.2 mg/ml.

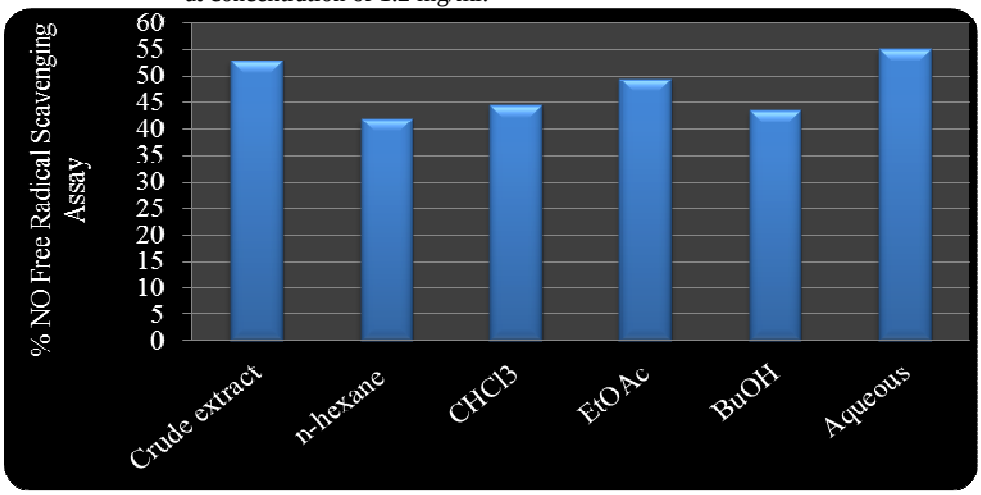


Figure: 6.32. NO free radical scavenging activity of crude and various fractions of *M. africana* at concentration of 1.5 mg/ml.

CONCLUSION

Based on the experimental work, it is concluded that, the $CHCl_3$ fraction of *Vitex agnus castus* exhibited significant (80.95% with MIC_{50}=2.19 mg) antibacterial, the methanolic extract of the plant also showed significant (90.01%) anti-inflammatory activity. The methanolic extract of the plant showed significant spasmolytic activity at concentration of 3 mg/ml that 100% relaxed the isolated tissue. Moderate insecticidal, anti-termites, phytotoxic, brine shrimp cytotoxic and NO free radical scavenging activities were shown by crude extract and various fractions of *Vitex agnus castus*. No haemagglutination and antifungal activities were observed during this work.

The crude methanolic extract of *Myrsine africana* exhibited significant spasmolytic activity as the extract completely abolish the spontaneous contractions of isolated tissues at 5.0 mg/ml. Good antibacterial, anti-termites, brine shrimp cytotoxicity (only by *n*-hexane fraction) was shown by extracts of this plant. Similarly moderate phytotoxic, brine shrimp lethality (at higher concentration), haemagglutination, anti-inflammatory and nitric oxide free radical scavenging assay were shown by crude extract and various fractions of the plant. The plant exhibited low insecticidal and no antifungal activity.

The phytochemical screening revealed the presence of alkaloids, flavonoids and saponins in these plants. A total of eleven compounds (six from *Vitex agnus castus* and five from *Myrsine africana*) were isolated during current studies from these plants. Five compounds were found new {three (Compound **1**-**3**) from *Vitex agnus castus* and two (Compound **7**-**8**) from *Myrsine africana*}, one (Compound **9**) for the first time from *Myrsine africana* and five known/common compound {three (Compound **4**-**6**) from *Vitex agnus castus* and two (Compound **10**-**11**) from *Myrsine africana*}. Compounds **1** and **3** exhibited moderate anti-inflammatory activity against freshly isolated human neutrophils. Our current work opens a

new window for researchers to further work on activity guided isolation from these species so that we may standardize the extract, and snatch share(s) from the global market.

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LIST OF PUBLICATIONS

1. Bashir A, **Sadiq A**, Bashir S, Khan I, Adhikari A, Choudhary MI (2010). Anti-inflammatory and enzyme inhibitory activities of a crude extract and a pterocarpan isolated from the aerial parts of *Vitex agnus castus.* Biotechnology Journal 5(11):1207-1215.
2. Bashir A, **Sadiq A**, Bashir S, Khan I, A. Adhikari, Choudhary MI (2010). Biological activities of a new compound isolated from the aerial parts of *Vitex agnus castus*. African journal of Biotechnology. 9: 9063-69**.**
3. Bashir A, **Sadiq A**, S. Bashir, I. Khan, MI Chaudhary (2010). Phytotoxic, Antibacterial and Heamagglutination activities of the aerial parts of *Myrsine africana*. African journal of Biotechnology. 10: 97-102**.**
4. Bashir A, **Sadiq A**, Bashir S, Ali N, Khan I, Chaudhary MI (2010). Insecticidal, brine shrimp cytotoxicity, antifungal and nitric oxide free radical scavenging activities of the aerial parts of *Myrsine africana* L. African journal of Biotechnology. 10(8): 1448-53.
5. **Sadiq A**, Shumaila B, Bashir A. Antispasmodic action of crude methanolic extract and a new compound isolated from aerial parts of *Vitex agnus castus*. BMC, Complementary and Alternative Medicine. 11:55.
6. Bashir A, **Sadiq A**, Bashir S, Adhikari A, Choudhary MI (2010). Anti-inflammatory activity of a new compound isolated from the aerial parts of the *Myrsine africana.* African journal of Biotechnology. **In press.**