PHYTOCHEMICAL AND BIOASSAY SCREENING FOR FRUITS OF *SARCOCOCCA SALIGNA (D.DON) MUEL* AND AERIAL PARTS OF *ASTRAGALUS ANISACANTHUS*

Saima Naz

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

CENTRE FOR BIOTECHNOLOGY AND MICROBIOLOGY
UNIVERSITY OF PESHAWAR
2016



In the Name of

Allah,

The Most Merciful and Compassionate the Most Gracious and Beneficent Whose help and guidance we always solicit at every step, at every moment.

PHYTOCHEMICAL AND BIOASSAY SCREENING FOR FRUITS OF *SARCOCOCCA SALIGNA (D.DON) MUEL* AND AERIAL PARTS OF *ASTRAGALUS ANISACANTHUS*

This dissertation is submitted by Saima Naz as partial Fulfillment of the requirements for the Degree of Doctor of Philosophy in Biotechnology

Approved By:

1. _____
Prof. Dr. Bashir Ahmad
Research Supervisor

2. _____
Dr. Sadiq Azam
Co- Supervisor

3. _____
External Examiner

4. _____
Director
Centre for Biotechnology and Microbiology

CENTRE FOR BIOTECHNOLOGY AND MICROBIOLOGY

UNIVERSITY OF PESHAWAR

SESSION: 2011-2012

CERTIFICATE OF APPROVAL

This thesis titled "*PHYTOCHEMICAL AND BIOASSAY SCREENING FOR FRUITS OF SARCOCOCCA SALIGNA (D.DON) MUEL AND AERIAL PARTS OF ASTRAGALUS ANISACANTHUS*" submitted by

Saima Naz is hereby approved and recommended as partial fulfillment for the award of Degree of Doctor of Philosophy in Biotechnology

1. Supervisor _____
2. Co- Supervisor _____
3. External Examiner _____
4. Director
   Centre for Biotechnology &
   Microbiology, University of Peshawar _____

CENTRE FOR BIOTECHNOLOGY AND MICROBIOLOGY

UNIVERSITY OF PESHAWAR

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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, Parents and Friend who taught the things I would never have known otherwise"

AUTHOR'S DECLARATION

I solemnly declare that the research work presented in this thesis was carried out in accordance with the requirement of the University of Peshawar's regulation for research Degree programs. The author has not been submitted for any other academic award. The work is original and of author's own data. While work done in collaboration with, or with assistance of, other is indicated as such. The views expressed in the thesis, belongs to the authors.

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University of Peshawar

Deputy Director (QE)
University of Peshawar

Director
Quality Enhancement
University of Peshawar

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SUMMARY

Crude methanolic extract (Crd. MeOH Ext.) and different fractions of *S. saligna* fruits were investigated for various pharmacological / biological activities; Antimicrobial (Antibacterial and Antifungal) Brine-shrimp lethality, Phytotoxic, Heamagglutination, Insecticidal, Anti-termites, Diphenyl Picryl Hydrazine (DPPH) free radical scavenging assay, Acute toxicity, Analgesic, locomotr, Antidepressant and Gastro Intestinal Tract Motility (GIT). These pharmacological activities revealed that the plant possess good antibacterial, anti-termite, analgesic activity and GIT Motility. Moderate phytotoxic activity was shown by the test samples at higher concentration (1000µg/ml). Low antifungal, insecticidal, cytotoxic, locomotor and antidepressant activity was shown by various fractions of this plant. A concentration dependent DPPH free radical scavenging activity was shown by the test samples and moderate EC_{50} (58.61µg/ml). Fruits of the plant lack phytolectin as no heamagglutination activity was observed against human erythrocytes. The *S. saligna* fruits extract significantly inhibited the germination of wheat seeds (20g/72hrs). *S. saligna* fruits showed the presence of carbohydrates, proteins, alkaloids, sterols and triterpenoids, phenols, flavonoids , tannins and saponins. Elemental analysis showed the presence of Sodium (Na), Magnesium (Mg), Zinc (Zn), Potassium (K), Nickel (Ni), Copper (Cu), Lead (Pb), Manganese (Mn), Cobalt (Co), Cadmium (Cd), Iron (Fe) and Chromium (Cr). The proximat analysis showed carbohydrate, proteins, fibers, fats and moisture content in fairly large amounts. The Scanning Electron Microscopy (SEM) technique was used to determine the morphology of the *S. saligna* fruits powder. The Energy Dispersive X-rays (EDX) technique was utilized to determine elemental composition. Maximum number of oil component were identified through GC-MS analysis.

Part B describes extraction, isolation and structure elucidation of isolated compounds, Pharmacological and Biological investigations of the Crd. MeOH Ext. and various fractions of *A. anisacanthus*. The aerial parts of *A. anisacanthus* possess significant anti-termite, cytotoxic and percent radical scavenging activity. Good antibacterial, Analgesic, GIT Motility, Locomotor, Antidepressant and Insecticidal activity was shown by the Crd. MeOH Ext. and various fractions of *A. anisacanthus*. Moderate antifungal and phytotoxic activity was shown by the test sample. The plant showed moderate haemagglutination activity against O+ive and A+ive respectively. Toxicological studies showed that plant is safe for humman use (1000 mg/kg). *A. anisacanthus* aerial parts showing the presence of carbohydrates, proteins, alkaloids, sterols and triterpenoids, phenols, flavonoids , tannins and saponins. The nutritional analysis of *A. anisacanthus* showing moisture contents 9.5%, ash 7. 4%, fats 7%, fibers 23.21%, protein 8% and carbohydrates 48.73% and gross energy 51.27 cal/100g. Elemental analysis showed the presence of Zn, K, Ni, Cu and Pb, Mn, Co, Cd, Fe and Cr. The SEM technique was used to determine the morphology of the synthesized *A. anisacanhus* aerial parts. The EDX technique was utilized to determine the amount as well as to identify elementary composition of *A. anisacanhus* aerial parts. The EDX pattern of spectrum recorded high signals for C and oxygen O_2. During isolation and characterization of chemical constituents Octadecan-1-ol (Stearyl Alcohol), 4-(methoxy methyl) benzoic acid (Phenyl methanoic acid), Heptadecanoic acid (Margaric acid) Di-hydroxy benzoic acid, Kaempferol-3-β-D-glucopyranoside (astragalin) and stigma sterol was isolated in pure form.

Chapter 1

1.1 GENRAL INTRODUCTION

Plants either medicinal or non-medicinal have an important role in a country's economy. Much effort has been made to promote the traditional medicine especially; World Health Organization (WHO) is encouraging its use because low income South Asian countries rely mostly on it [1]. Out of 8,000 medicinal plant species exist in South Asia, more than 2,000 are exist in Pakistan, widely distributed in northern regions [2]. Our country has a rich store of medicinal flora which is still undiscovered. This huge store of medicinal flora attains much more importance when matters come to country like Pakistan where there is poverty. By the year 2050, it is being expected that the global market would rise to 5.0 trillion [3].

Plants are the richest source of drugs by bringing new chemotherapeutic agents. Traditional medicines are the economical source of therapy to the population [4]. On micro level in Pakistan the import bill of medicinal plants is just US $ 6.0 million. On the macro level, 30% of the total medicines are being prepared from herbs. It is the need of the day to let the medicinal flora play its role in the local and global market. Therefore, the medicinal plants of northern area of Pakistan have great potential in local and global market and it is important to explore the natural resources of phytomedicine of this area.

Relationship between man, life and nature is very old and time tested. Human being right from the beginning had three basic requirements. They need food to eat, a place to live and to remain fit i.e. to cope successfully with all the ailments disease and injuries. So we can say that man needed food shelter and treatment of diseases right from the dawn of human history.

These basic needs force human being to achieve them. As man is inquisitive in nature and wants to know about everything. Therefore this nature of man helped him to

fulfill these basic needs. It was the surrounding, that man had, upon which it started pondering. So plants were the first source that man found to fulfill these basic needs. The first human activity regarding to the nature was the study of plants and animal particularly for food. By constant observation man was able to know that not all plants are beneficial but some of them are harmful. So man was able to distinguish between nutritional and poisonous plants. Some plants were found to have specific pharmacological properties such as cathartic, purgative, analgesic. Consequently these plants were grouped into specific classes in various pharmacopeias. Thus, drugs like henbane, poppy and garlic were in use since 6000 B.C. This was how knowledge of drugs came into being and propagated [5]. One of the basic reasons of this fact is that the natural products have a very good potential of having good pharmacological activities. There are certain reasons behind it that plant is used as a major source of drugs, firstly plants produce various bioactive and secondary metabolites, secondly plants provides plate form for synthetic chemist to discover new molecules or modify the existing molecules into more effective drugs. So plants form a basis for a large number of pharmaceuticals [6, 7]. A strong part played by natural product is that many life saving drugs such as digoxin, morphine aspirin and emetine are still used with the same vigour as used at the time of it discovery. There are some drugs which have been derived from plants but still they have not been synthesised as given in Table 1 shows various plant species that showing different therapeutic activities under different groups [8].

Table 1.1 Plant species with therapeutic value under different plant groups

S. no	Name	Number
1	Thalophytes	230
2	Bryophytes	39
3	Pteridophytes	382
4	Gymnosperm	382
5	Angiosperm:	
5a	a) Monocotyledones	676
5b	b) Dicotyledones	3495
5c	Total	4877

When natural products are defined it is found that anything which is obtained either from plants or animals. In other words we can say which is natural, not artificial, which is pure and good as the name natural indicates [9]. Nowadays many stuffs are included in natural products such as herbs, plant preparation, extracts, tincture, dietary supplements and Chinese herbal medicines [10]. Natural products (NP) chemistry has been continuously developed during the past 4 decades. The first commercial natural product familiarized for therapeutic use is generally considered to be the narcotic, morphine, marketed by Merck in 1826 [11]. The first semi-synthetic pure drug based on a natural product, aspirin, was introduced by Bayer in 1899 [12].

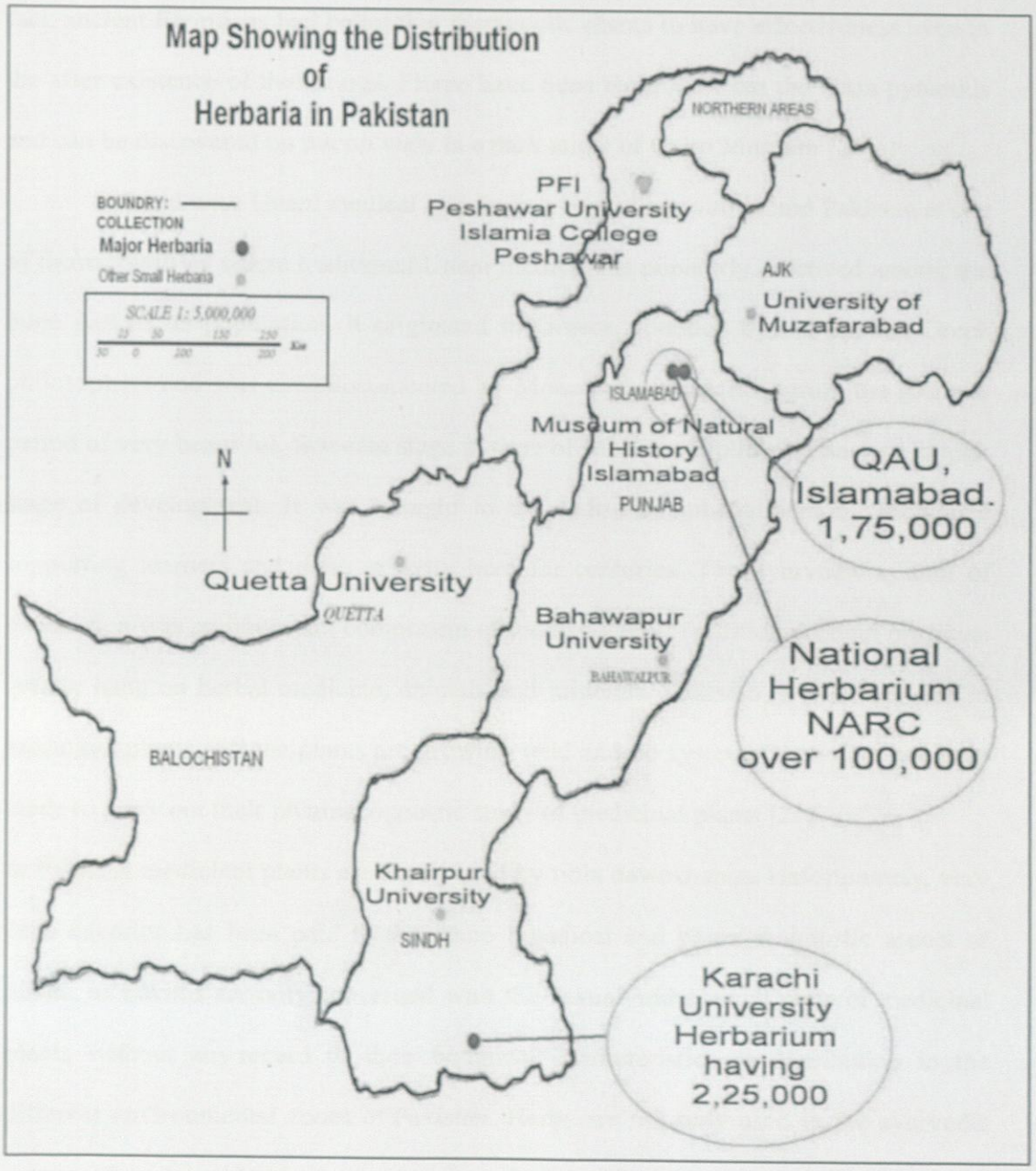
1.1.2 Medicinal plants

The plants which having potentially active constituents used to cure illness or pain are called medicinal plants [13]. Plants are considered to play very important role in health care system. That s why in many developing countries herbal medicines are

still in practices for curing diseases and complications. In spite of their importance, medicinal plants are rarely handled within an organize manner and most of them are broken little for the future [14]. By recording the use of medicinal plants it is considered that there is a continuous increase both for pharmaceutical industry and traditional uses. Medicinal plants provide opportunities for biological screening and trends and method used in the pharmacological and phytochemical investigations of natural plants constituent [15]. There is a need to discover new drugs by using plants; we need to explore the biological potential of plants, medicinal plants are important for the livings of poor populations all over the world [16]. In the world, Pakistan is the 9th country where transitional system of medicine is practiced as a major health care system. The widely used system of medicine in Pakistan is called Greek medicine or Unani system of medicine [17].

Even though the medicinal plants are worldwide in distribution and herbal medicines are the world primary source of therapeutics to fight against the diseases, a large number of medicinal flora remain unexplored still now. Only 35,000-70,000 plant species out of 400,000 higher plants have been use as medicine [18].

From a survey of naturally occurring plants of Pakistan which distributed in Malakand, Kuram Agencies, Hazara, Muree Hill, Azad Kashmir and in Northern areas or on the farm land of Punjab, Sindh and Baluchistan, there is rich floral diversity in Pakistan due to the unique geography. There are 39,584 hakims, 130,000 homoeopaths and 455 vaids registered in Pakistan where about 457 Tibia dispensaries and clinics provide medication to the public. Regional department of Local Bodies and Rural Development have been established 95 dispensaries. Pakistan is one of the leading countries distributing pharmaceutical plants [19].



1.1.3 History of medicinal plant

The people have used plants as medicines, since the beginning of human civilization. Perhaps as early as Neanderthal man (extinct species of human), herbal plants have therapeutic power. The most primitive uses are found in Babylonian around 1770 BC in the code of Hammurabi and in ancient Egypt around 1550 BC. In

fact, ancient Egyptians had belief that therapeutic plants to have effectiveness even in the after-existence of their kings. Florae have been recorded from the Gaza pyramids and can be discovered on put on view in a dark angle of Cairo Museum [20].

Old and wise Unani medical activity is a part of our values and Pakistan is one of those countries where traditional Unani medicine is popularly practiced among the huge part of its population. It originated in Greece, founded by old ancient Greek philosophers and was used/documented by Mohamed supporters during the glorious period of very beautiful, first-rate stage in time of Mohamed following society in high stage of development. It was brought to the Indo-Pak subcontinent by Mohamed supporting learners and done expertly here for centuries. The ayurvedic system of medication was an important component of hindu society. Traditional Unani medicine greatly hang on herbal medicine, animals and minerals. Pakistan is relatively rich in medicinal plants. All the plants are growing wild and no systematic attempt has been made to carry out their pharmacognostic study of medicinal plants [21].

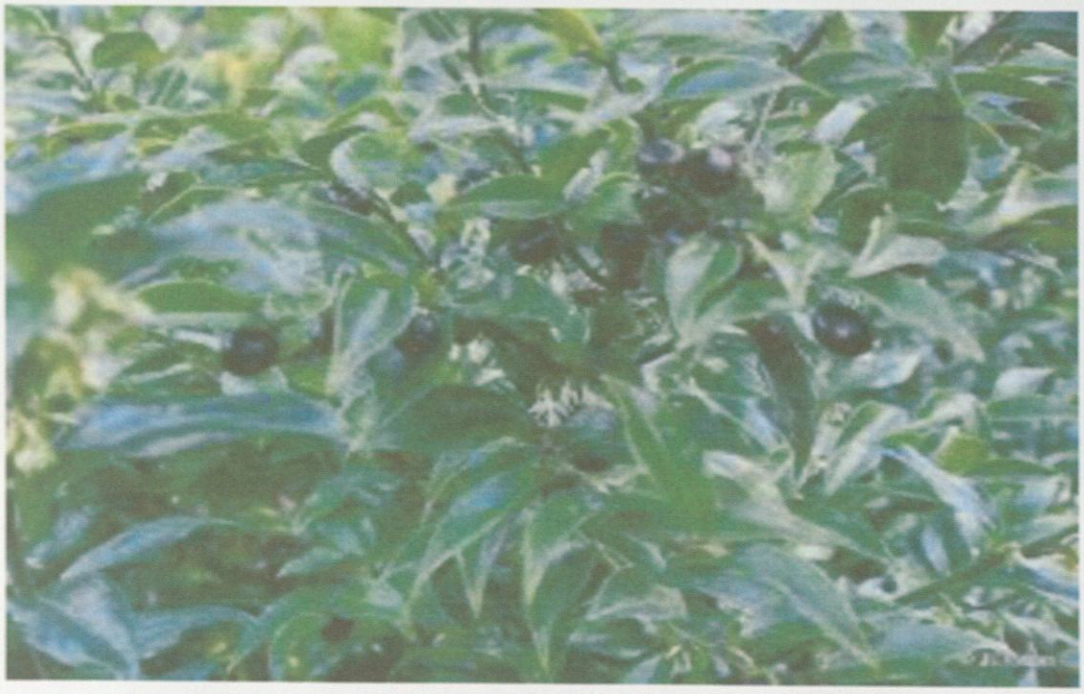
In Pakistan medicinal plants are firstly used by tibia dawakhanas. Unfortunately, very little devotion has been paid to the ethno botanical and pharmacognostic aspect of plants, as hakims are only concerned with the sexual and asexual parts of medicinal plants without any regard to their botanical characteristics or distribution in the different environmental zones of Pakistan. Herbs are not only used in the ayurvedic system of treatment but in the preparation of many allopathic and homeopathic drugs, no wonder these herbs are now being commercially exploited for the extraction of numerous constituents [22].

Hakims or Tabibs are using medicinal plants without any scientific rationale, therefore it is extremely important to provide scientific backgrounds to these treatments. Besides the pharmacological screening of medicinal plants using different

in-vivo and in-vitro protocols, the isolation of therapeutically active molecules is also required to augment pharmacological screening and to discover lead compounds.

In Pakistan major research activities on medicinal plants are conducted at institutional levels. These activities are mainly of two type i.e. pharmacological screenings and isolation of pure compounds. The pharmacological screening is totally dependent on the folkloric uses of these medicinal plants. Different localities of Pakistan have different type of medicinal plants and the peoples of those localities have centuries old information about these herbal remedies. These ethnomedicinal knowledge transfers from generation to generation. The researcher working on the screening of indigenous plants for various biological activities have totally ignored those plants which have no known folkloric uses. A large number of plants have proved good remedy for the treatment of various ailments, due to such research effort on a large number of plants [23, 24].

During the current study, biological/pharmacological and phytochemical investigations were carried out for the selected plants, *S. saligna* and *A. anisacanthus* members of Buxaceae and Febaceae family, respectively.



SARCOCOCCA SALIGNA

1.2 BUXACEAE (FAMILY)

1.2.1 Description

Buxaceae is a small family, it consist of 4-5 genera, 90-120 flower producing species. The members of this family are mainly herbs, shrubs, small trees and rarely perennial herbs, monoecious and diecious. The fifth genus (Notobuxus) of this family is included within the Buxus now. This family is also known as Box family [25]. The leaves are simple, alternate / opposite and spiral. Inflorescence is axillary. Flowers are unisexual, male flowers are smaller than female flowers, actinomorphic and sessile. Petals are absent in flowers, stamens and sepals are equal in numbers. Carpels are syncarpus and 2-4 in numbers. Fruits of this family when ripe having persistant style, smooth and blackish in colour seeds are 2-3 in numbers [26].

1.2.2 Distribution

This family is distributed in Kashmir and throughout the northern region of Pakistan at the altitude of 5000-9000 fts [27].

1.2.3 Importance

The plants of Buxaceae family are good source of a large number of new steroidal alkaloids [26]. The species of this family have potential for treatment of different diseases; fever, rheumatism, liver diseases, hyperactive states of the gastrointestinal tract, infections, syphilis, pain and inflammation [28]. The wood of this family is well known throughout as diaphoretic in native countries and used for treatment of venereal disease. The plant of this family is used as narcotic and sedative in full dose. The wood of the plant of this family used for curing leprosy. The volatile oil distiled from plants are used in cases of epilepsy. The oil has been employed for piles and also for toothache. Powdered Box leaves used for bot-worm in horses. The

active ingredients of this plant used for the bite of mad dogs [29]. Most of the plants of this family used as anti acne agents by the tribal and non tribal people of the India. Most of the plants of this family are used for cholinesterase inhibition and inflamation treatment [30].

1.3 *SARCOCOCCA* (GENUS)

Sarcococca is a genus of Buxacea family. It is also known as sweet box or Christmas box. It is flower producing genus of this family. *Sarcococca* is a small shrub having scally bud. Racemose type of inflorecense is present. Male and female flowers are towards the apex and base, respectively. Stamen and sepals are 4, free, inserted on receptacle around vestiges of ovary, exerted, oblong anthers and recurved. Ovary is 2-3 loculed with 2 ovules per locule, syncarpous. It is an ever green and slow budding shrub. Leaves of this genus are alternate, range in size 3-12cm in length and 1-4cm broad. In winter it produces sweet smelling flowers. Its fruits are red or black drupe with 2-3 seed [31].

1.3.1 Distribution

The genus is native to Eastern and South Eastern Asia and Himalaya. Afghanistan through Indo-Pakistan sub-continent to Central China and Sri Lanka (Ceylon) to South-East Asia and Philippine Isles [32].

1.3.2 Importance

This genus yields about 200 steroidal alkaloid. This genus is mostly used for ornamental purposes than that of medicinal, although it has potent for curanine like action and is useful in tuberculosis [33]. Many alkaloid isolated from this genus has been used as antileishmanial agent [34]. Genus Sarcococca possesses different chemical constituents including steroids, alkaloids, glycoside, triterpenoids, sugar and their alcohol. Crude extract, fractions and isolated secondary metabolites of genus Sarcococca have shown a wide range of pharmacological activities including antibacterial, antifeedant, antifungal and cholinesterase inhibiting activities [35].

1.4 *SARCOCOCCA SALIGNA*

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliatae

Order: Euphorbiales

Family: Buxaceae

Genus: *Sarcococca*

Specie: *Saligna*

Plant: *Sarcococca saligna*

S. saligna is a shrub having rough buds with scale. It has alternate leaves and glabrous petiole. This plant show axillary racemes or clusters inflorescence, with male and female flowers. The female flowers at the base and male towards apex. Calyx consists of 4 free sepals. Androecium consist of 2-3 stamens, anthers are oblong in shape and dehiscent. Gynoecium consists of bi or tri carpilary ovary, syncarpus with 2 ovules in each and 2-3 styles which are free. Fruits are fleshy and somewhat dry with 1-2 seeds, it is globose in shape. Seeds are hemispherical and without caruncle [36].

1.4.2 Distribution

The plant is distributed in shady and moist places in Himalayas from Afghanistan to Kumaon [37].

1.4.3 Importance

Local people use the dried branches of the plants in the roof as suthra. Locally, *S. saligna* leaves aqueous extract is used as anti-pyretic, calmative and have probable effect against rheumatism, pain and fever [38]. The plant is used for syphilis treatment, pain, liver disorders, the gastrointestinal tract hyperactive states, infection and inflammation [36, 37]. The plant has also been reported for its antiulcer,

antitumor, anti-diarrheal, acetyl cholinesterase inhibitory and anti-secretory activities [39].

The plant possesses blood purifying, laxative and muscular pain relief potential. The methanolic extracts have potential to block ganglion by lowering the nicotine's effect on blood pressure. The different alkaloids obtained from *S. saligna* suggesting discovery for nervous system disorder [40].

1.5 Aims and objectives

Aims and objectives of the current study are;

- To screen the Crd. MeOH Ext. and various fractions for pharmacological activities.
- ***In-vitro* pharmacological activities**: The selected plant species will be screened for; antibacterial, antifungal, phytotoxic, heamagglutination, cytotoxic, antitermite, allelopathy insecticidal and antioxidant activities.
- ***In-vivo* pharmacological activities**: The selected plant species will be screened for; analgesic, acute toxicity, locomotor, gastrointestinal tract motility and antidepressant activity.
- To screen the plant material for nutritional analysis, Scanning Electron Microscopy, Energy Dispersive X-rays, for determination of the concentration of the elements and chemical characterization of test sample and preliminary phytochemical analysis.
- To subject the active fraction(s) of the selected plants to column chromatography for isolation of active constituents. The structure of the isolated compound(s) will be identified / elucidated by using analytical techniques such as TLC, IR, UV, GC/MS and NMR, where applicable [40-43].

Chapter 2

2.1.3.2 Thin-layer Chromatography (TLC)

Pre-coated silica cards (E-Merck PF-254, Type-60) of 20x20cm and 0.5mm thickness were used for to perform preparative TLC

2.1.4 Spot Locating Reagents

For visualization of spots on TLC cards, various spot locating reagents (vanillin-phosphoric acid, dragendorff, iodine and ceric-sulphate-sulphuric acid) were used.

2.1.4.1 Vanillin-Phosphoric Acid (VPA)

1g vanillin was dissolved in 50% aqueous phosphoric acid. When the TLC cards sprayed with VPA and heated upto 100°C, terpenes and steroids gave intense blue or light pink and purple color, respectively. Spots of terpenoidal and steroidal glycosides also appear pink in color after spray and heating.

2.1.4.3 Dragendorff's Reagent

(i) 8g of potassium iodide dissolved in distilled water (20 ml).

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

(iii) For stock solution the above (i and ii) were mixed in 1:1.

(iv) For working reagent 10% (V/V) acetic acid was used to dilute the stock solution. The presence of alkaloid is indicated by appearance of light brown, light pink, dark blackish or brown color. Appearance of light pink to light yellow confirms the presence of highly oxygenated terpenoids and steroids.

2.1.4.4 Iodine Solution

Few crystals of iodine were placed in TLC tank and warmed upto 45°C for 4-5 minutes. Appearance of respective spots will appears for secondary metabolites after placing the TLC in tank.

2.2.1 Collection of plant

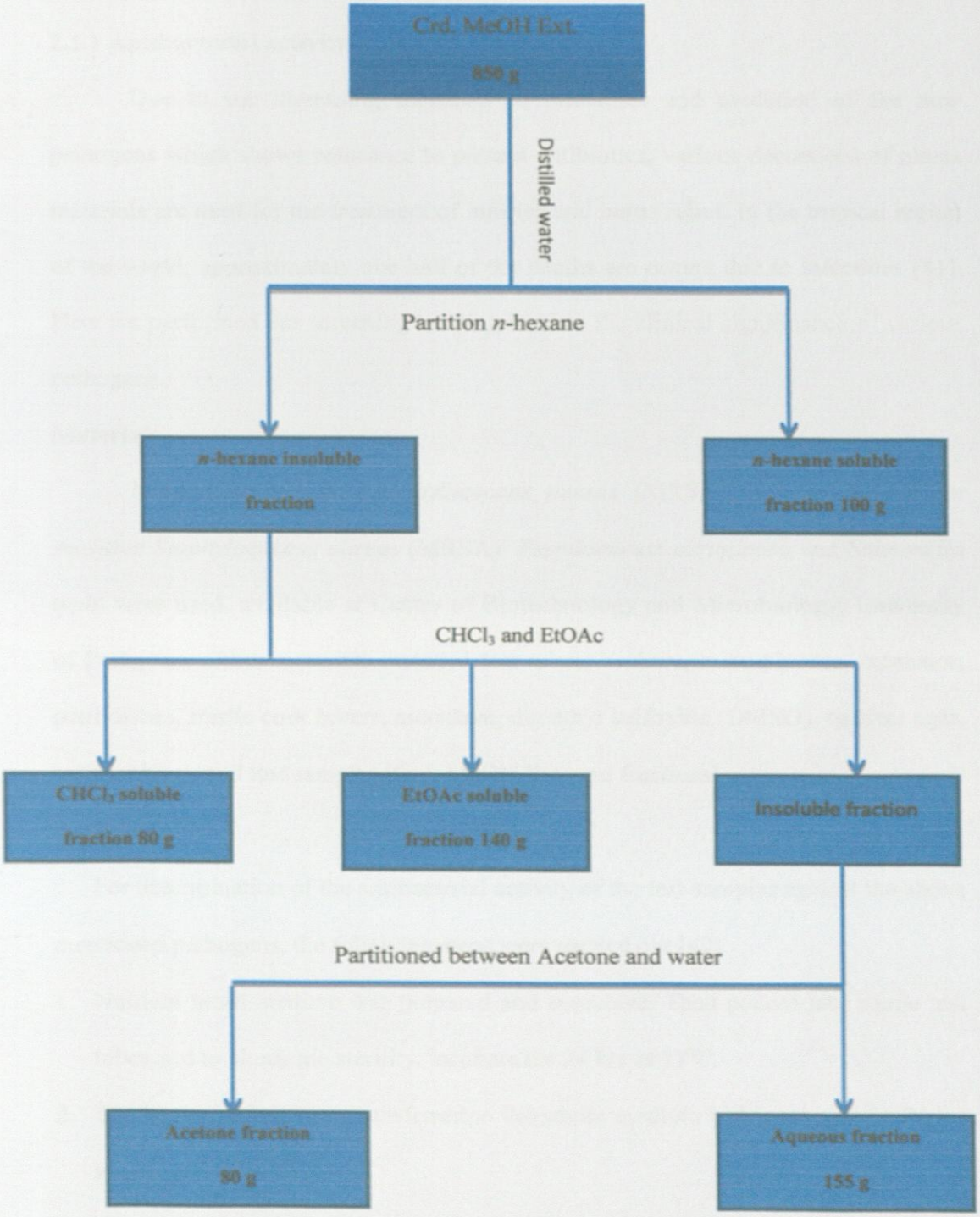
The fruits of *S. saligna* were collected from Abbottabad, Khyber Pakhtunkhwa (KP), Pakistan, in the month of September 2011. The collected fruits were identified by Prof. Dr. Farrukh Hussain, plant taxonomist, Department of Botany, UOP.

2.2.2 Extraction

The collected fruits of *S. saligna* were shade dried and grounded to fine powdered material with the help of grinder. The fine powdered materials (10Kg) were soaked in commercial grad MeOH for 10 days, two times at 27±1°C with vigorous shaking. After 10 days the MeOH soluble material was filtered off and the filtrate was concentrated at 40°C (under vacuum) by using a rotary evaporator. A blackish Crd. MeOH Ext. of 1kg was obtained.

2.2.3 Fractionation

The Crd. MeOH Ext. (850g) was suspended in distilled water (400ml) and partitioned with *n*-hexane (3 x 400ml), $CHCl_3$ (3 x 400ml), EtOAc (3 x 400ml) and acetone (3 x 400ml) to yield; *n*-hexane (10g), $CHCl_3$ (80g), EtOAc (140g), acetone (80g) and aqueous (155g) fractions (scheme-1). About 50g of Crd. MeOH Ext. was reserved for other pharmacological/biological screenings.



Scheme 1. Fractionation of Crd. MeOH Ext. of *S. saligna* fruits

2.3 PHARMACOLOGICAL / BIOLOGICAL INVESTIGATIONS (*In-vitro*)

2.3.1 Antibacterial activity

Due to the increasing incidents of infections and evolution of the new pathogens which shows resistance to present antibiotics, various decoctions of plants materials are used for the treatment of injuries and burns relief. In the tropical region of the world, approximately one half of the deaths are occurs due to infections [41]. Here we performed our screening keeping in view the clinical significance of various pathogens.

Materials

Methicillin resistant *Staphylococcus aureus* (MRSA), *E. coli*, Methicillin sensitive *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* and *Salmonella typhi* were used, available at Center of Biotechnology and Microbiology, University of Peshawar. Other materials included test tubes, beakers, micropipettes, incubator, petri dishes, sterile cork borers, autoclave, dimethyl sulfoxide (DMSO), nutrient agar, nutrient broth and test samples (Crd. MeOH Ext. and fractions)

Procedure

For determination of the antibacterial activity of the test samples against the above mentioned pathogens, the following steps were carried out [42].

1. Nutrient broth medium was prepared and autoclave. Then poured into sterile test tubes and to check the sterility, incubate for 24 hrs at 37°C.
2. The test organism were transferred to the sterile medium and incubated for 24 hrs at 37°C.
3. On the same day of experiment nutrient agar medium was prepared and poured into the sterilized petri dishes. For sterility test these plates were incubated overnight at 37°C.

4. After sterility check, 18-24 hrs old test culture from the nutrient broth, under laminar air flow, were transferred to each plate, to prepare bacterial lawn.
5. After introducing the test culture to plates, well (6mm) were made, using a sterile borer.
6. 3mg of the test sample was dissolved in 1 ml of DMSO (≤1%) for preparing the stock solution.
7. 100µl of test sample from the stock solution was transferred to the respective well and incubated at 37°C for 24 hrs.
8. DMSO (<1%) and Amoxicillin were also transferred to wells along with test sample as negative and positive control respectively.
9. The following formula was used to determine the percent zone of inhibition after incubation.

$$\text{Percent Inhibition} = \frac{\text{Zone of inhibition of test sample}}{\text{Zone of inhibition of standard}} \times 100$$

After measurement of the percent inhibition we preceded the experiment to determine the MIC_{50} according to the procedure of salmon *et al,* [43].

2.3.1.1 Minimum Inhibitory Concentration (MIC_{50})

With slight modification, following steps were carried for determining the MIC_{50}.

1. Transfer 4ml of the prepared nutrient broth into the sterile test tube and incubate at 37°C for 24 hrs for sterility check.
2. After 24 hrs the test bacteria was transferred to the test tubes.
3. 900, 700, 500 and 300µl from the stock solution were transferred to the test tubes containing test bacteria.
4. After incubation at 37°C for 24 hrs the results were recorded.

2.3.1.2 Minimum Bactericidal Concentrations (MBCs)

Due to low inhibitory effect of the test samples and higher MIC_{50} values obtained against the test pathogens the MBCs were not calculated.

2.3.2 Antifungal activity

Materials

Antifungal screening was performed against various fungal species; *Aspergillus oryzae, Fusarium fumigatus, Fusarium solanum, Aspergillus flavus, Aspergillus niger* and *Penicillium notatum*.

Other materials included test tubes, micro pipettes, magnetic stirrer, autoclave, incubator, Sabouraud Dextrose Agar (SDA), dimethyl sulfoxide (DMSO), test samples (Cr. MeOH Ext. and fractions) and Miconazole.

Procedure

The test samples were screened for anti-fungal activity, using tube dilution method [46]. 24mg of the test samples was dissolved in 1ml to prepare the stock solution. Sabouraud dextrose agar (SDA) medium, for the growth of fungal specie, was prepared as per reported procedure [44]. 4 ml of the sterilized medium was poured to test tubes and allowed to cool upto 50°C. From the stock solution, 66.7µl of the test samples was transferred through a micro-pipette and ten allowed solidifying in slanting position with final concentration of 400µg/ml SDA. Fresh fungal culture was inoculated to test tubes. Other test tubes supplemented with negative (DMSO) and Positive (standard drugs) control and incubated at 27±1°C for 3-7 days. The non-mycelial visible growth of the test fungi was noted on day 7th and percent growth inhibitions were calculated by comparison with positive control.

2.3.3 Heamaggulatination activity

Materials

Red blood cells (RBC's) of all blood groups, Disodium hydrogen phosphate (Na_2HPO_4), Potassium dihydrogen phosphate (KH_2PO_4), centrifuge, test tubes, phosphate buffer (pH 7.0) and incubator.

Procedures

Heamagglutination activity of Cr. MeOH Ext. and various fractions of plant performed according to reported procedure [45].

First the phosphate buffer (pH 7) was prepared by dissolving KH_2PO_4 (0.453g) and Na_2HPO_4 (0.47g) in 50ml of distilled water and were mixed in 3:7 (KH_2PO_4: Na_2HPO_4 v/v). A stock solution of test sample was prepared by dissolving 1mg of test sample in 1ml of DMSO and phosphate buffer was used to make different dilution; 1:2, 1:4, 1:8, 1:16. From a healthy person, blood samples were collected on the day of experiment and centrifuged. The red blood cells (RBC's) were collected for the next steps and the plasma was discarded. In the phosphate buffer 2% of RBC's suspension was prepared. From each dilution 1ml of sample was taken in a test tube and 1ml of RBC's suspension was transferred to it. The tubes were incubated at 37°C for 30 minutes. After incubation the positive and negative results were examined by rough and smooth button formation.

2.3.4 Phytotoxic activity

Materials

Phytotoxic activity of the test samples (Crd. MeOH Ext. and fractions) was tested against *Lemna minor*. Growth chamber, flasks, micro-pipettes, methanol and test samples were used in the experiments.

Procedure

The test samples were screened for phytotoxic activity according to Prof. Mc Laughlin protocol [46]. Following steps were followed during experiment.

1. Stock solutions were prepared by dissolving 20mg of test sample in 1ml of methanol. E-medium was prepared for the growth of *L. minor*.
2. 10, 100, 1000µg/ml from the stock solutions were poured to flask and allow to evaporate the organic solvent at room temperature.
3. After evaporation, 20ml of E-media was poured to all flasks.
4. Sixteen healthy *L. minor* plants were selected and put in respective flasks and incubated for 7 days at 27±1°C in growth chamber
5. Results were recorded after 7 days of incubation.

2.3.5 Insecticidal activity

Requirement

Different fractions of *S. saligna* fruits were screened for insecticidal activity against; *Tribolium castaneum*, *Rhizopherta dominica* and *Callosbruchus analis* organic solvent (methanol), growth chamber, brush, petri dishes (9 cm), glass vials standard insecticidal drug permethrine (copex), filter paper and micro-pipette (1000 µl).

Preparation of Test Samples

Test sample (200mg) was dissolved in 3ml of methanol to prepare stock solution.

Rearing Technique

The above mentioned test insects were reared in breading media using sterile bottles, providing optimized temperature and humidity level. Insects of uniform size and age were selected for experimental work.

Procedure

For determining the insecticidal activity, the contact toxicity assay [47] was performed as following.

On first day petri plates were sterilized and filter papers were cut according to the size of petri dishes. The filter papers were kept in the petri dishes and stock solutions of test samples were introduced using micropipette and left till evaporation of methanol. After evaporation, from selected species, healthy insects of equal size (16) were transferred to plates using a clean brush. The plates were incubated in growth chamber for 24hrs at 27°C (50% relative humidity). Permethrine (Copex) was used as positive control.

The results were noted by counting the number of survivals in plants and using following formula the mortality (percent) was calculated:

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

2.3.6 Diphenyl Picryl Hydrazine (DPPH) radical-scavenging activity

Procedure

Antioxidant activity of the Crd. MeOH Ext. and various fractions of *S. saligna* fruits were performed by using the method of Blois with slight modification [48].

1. From Stock Solutions (25mg/50ml) different concentrations (20, 40, 60, 80 and 100ppm) were prepared by using tube dilution method.
2. 1ml of the 0.001M DPPH solution was added to 2ml test sample, control containing no sample.
3. The absorbance was measured at 517nm after 30 min. (SP-3000 PLUS Spectrophotometer, Optima, Japan)
4. Percent result were calculated by using following formula

$$\text{Percent RSA} = \frac{\text{Control absorbance}}{\text{Control absorbance}} - \text{Sample absorbance} \times 100$$

The EC_{50} values for each test sample were calculated from the percent RSA graph.

2.3.7 Brine shrimp lethality bioassay

Materials

Test sample, sea salt, *Artemia salina* (test organism), pasture pipette, syringes (5.0 and 0.5 ml, 100 and 10µl), plastic dish (shallow rectangular), distilled water (double), magnifying glass, organic solvent, glass vials, organic solvent, aluminum foil and Etoposide.

Procedure

The test samples were screened for their cytotoxic effect as per reported procedure, against *A. salina* [49]. Sea water is a very good media for hatching of *A. salina*. Sea water (artificial) was prepared in double distilled water using commercial salt mixture and filled in an unequally divided (larger and smaller compartment) plastic dish for hatching purpose. Brine shrimps (50mg) were introduced into plastic dish and kept at room temperature for two days to hatch and mature. After maturation, the naupli were collected using a Pasteur pipette. Test samples (20mg) were dissolved in 2ml of volatile organic solvent, serving as stock solution. From the stock solution; 5, 50 and 500µl were transferred to vials (3vials/concentration). The organic solvent was completely evaporated in fume hood. To each vial, larvae (10) and sea water (1ml) were added. The final volume of each vial was adjusted to 5ml with sea water. The vials were incubated under illumination at 26±1°C for 24hrs. Etoposide and methanol were used as positive and negative control, respectively. After incubation, brine shrimps that survived were counted using a magnifying glass.

2.3.8 Anti-termite activity

Procedure

The test samples were screened for anti-termite potential, using reported procedure of Saliha *et al*., [50].

Requirements

The following were required to carry out the anti-termite activity of the test samples. Magnifying glass, blotting paper, petri dishes, beaker, cutter, *Heterotermes indicola* culture and test samples.

Procedure

The blotting paper was sterilized and cut according to the size of Petri plates. The test samples (2mg/ml) were prepared in respective solvents then the blotting papers were dipped in the respective test sample, evaporate the organic solvent. 25 termites were transferred to each petri plate and after each 24 hrs of incubation the termites killed were observed using magnifying glass, till all termites were dead. Respective solvents were also used as negative control i.e. with methanolic fraction, methanol served as a negative control and so on. All the experiments were performed 3 times and the average number of termites killed each day was noted.

IN-VIVO PHARMACOLOGICAL SCREENING

For the screening of various *in-vivo* biological activities, the Crd. MeOH Ext. obtained from fruits of *S. saligna* was used.

Experimental animals

Young BALB/C mice of either sex aged 4-5 weeks, average weight 20-25g were used for the experiment. The mice were purchased from the animal Research Branch National Institutes of Health (NIH).

The animals were kept in standard environmental condition for one week for acclimation after their purchase. The set of rules followed for animal experiment were approved by the institutional animal ethical committee [51].

2.3.9 Acute toxicity

Procedure

The acute toxicity was determined by using Crd. MeOH Ext *S. saligna* fruits various doses ranging from 500 mg/kg to 1000 mg/kg b. w (body weight of mice). The mice were distributed equally in groups. Negative control group was treated with distilled water at a dose of 10 ml/kg b. w. After treatment with test doses, the animals were kept under observation for 24 hrs. The animals were observed for acute toxicity effect in the first 4 hrs and the percent death was calculated after 24 hrs [52].

2.3.10 Analgesic activity

The test samples were screened for analgesic effect as per following protocol [56].

Requirement

Acetic acid (Merck), Tween-80, Normal saline solution, Diclofenac sodium and BALB/C mice.

2.3.10.1 Acetic acid induced writhing

For the determination of analgesic effect Crd. MeOH Ext. was used as test sample. For the experimental work BALB/C mice of both sex and 18-25g body weights (b.w) were selected. The experimental animals were divided into five different groups (n=6) out of which two groups (I and II) served as negative and positive control. Normal saline (negative control) and diclofenac sodium (positive control) at 10ml or mg/kg of b.w was administered to Group I and Group II, respectively and were fed according to recommended guidelines and 2 hrs before starting the experiment, food supply was stopped [53]. The remaining groups (III-V) were administered with Crd. MeOH Ext. at a dose of 500, 700 and 1000mg/kg b. w. Acetic acid (1%) was administered to the mice after half hour of the above treatment, through intra-peritoneal (i.p) route. The abdominal constrictions (writhes) were started after 5 min of acetic acid injection and count for next 10 min. The following formula was used to calculate the percent analgesic effect of the test samples.

$$\text{Analgesic activity (\%)} = 100 - \frac{\text{No. of writhing (tested animals)}}{\text{No. of writhing (control animals)}} \text{ X } 100$$

2.3.11 Gastrointestinal tract (GIT) Motility

Requirements

BALB/C mice, normal saline, castor oil and charcoal suspension.

2.3.11.1 Charcoal meal procedure

The Crd. MeOH Ext. was screened for possible effect(s) on the motility of GIT. BALB/C mice (25-30g) were selected and divided into five different groups, out of which two groups (I and II) served as negative and positive control [54]. Normal saline (negative control) and castor oil (positive control) at 10 and 0.1ml/kg b.w was administered to group I and II, respectively. The remaining groups (III-V) were treated through i.p rout, with Crd. MeOH Ext. at a dose of 1000, 700 and 500mg/kg b. w. After 15 min of the above treatments, aqueous suspension of charcoal at 0.3 ml dose was administered to each experimental animal. After 30 min, the test animals were killed by cervical dislocation and the small intestine was removed to measure / check the distance covered by charcoal. The following formula was used for calculating the percent GIT motility of the test samples.

GIT motility (%) = 100 - distance covered / total intestine length × 100

2.3.12 Antidepressant Activity

Requirements

Water tub, normal saline, Fluoxetine and stop watch.

2.3.12.1 Using Forced Swimming Test (FST) Model

To evaluate the antidepressant effect of the test sample, FST model was used. A water container with dimensions of 42x19 x19cm was used for this purpose. The mice were divided into 5 different groups (n=6), out of which two groups (I and II) served as negative and positive control.

Normal saline (negative control) and fluoxetine (positive control) at 10ml and 15mg /kg b.w dose was administered to group I and II through i.p, respectively [59].

The remaining groups (III-V) were treated with Crd. MeOH Ext. at a dose of 1000, 700 and 500mg/kg b.w. The experimental animal were allowed to swim in the water tub one day before performing the experiment. The water temperature was maintained at 25±2°C. The experimental animals were handed as per recommended procedure(s) and the standard laboratory environments conditions were also maintained during experimental work. All groups were treated with above stated doses of Crd. MeOH Ext. and drug. After 30 min each mouse was allowed to swim in water tub for 360second while immobility time was noted during last 240second.

2.3.13 Locomotor activity

Requirements

Wooden apparatus, BALB/C mice, normal saline and diazepam.

Procedure

The test samples were screened for locomotor activity, using a wooden device of 150cm in length. The wooden device was divided into 19 equal squares.

The experimental animals were divided into five different groups (n=6), out of which two groups (I and II) served as negative and positive control. The negative control (group I) was treated with normal saline (10 ml/kg) and positive control (group II) was injected diazepam at a dose of 0.5 mg/kg b. w (i. p) [59]. The remaining groups (III-V) were treated with Crd. MeOH Ext. at a dose of 1000, 700 and 500mg/kg b.w through i.p rout. After 30 min of administration of test sample, positive and negative controls, the experimental animals were observed for 10 min and the number of lines crossed were recorded [55, 56].

2.3.14 Qualitative Phytochemistry

Various phytochemical constitutes were identified preliminary in crude methanolic extract of the fruits of *S. saligna* following recommended methods [57-61].

2.3.14.1 Carbohydrate

Molisch's test

The extracts (1g) were treated with alpha-anpthol and then concentrated H_2SO_4 (2ml) was added. The purple to violet color at the link indicates the presence of carbohydrates.

Benedict'test

Few drops of Benedict's reagent (alkaline solution containing cupric citrate complex) was added to extract and boiled in water bath. The appearance of reddish brown precipitate indicates the presence of reducing sugar.

Fehling's test

Equal volume of Fehling's A (Copper Sulphate) and B (Potassium tartarate and Sodium hydroxide) were treated with few drops of sample solution and boiled. Presence of reducing sugar was confirmed by a brick red precipitate of cuprous oxide formation.

2.3.14.2 Proteins and amino acids

Ninhydrin Test

The presence of proteins was identified using ninhydrine test (the extract solutions were treated with 0.2% ninhydrine solution (indane 1, 2, 3 trione hydrate) and boiled, the appearance of violet color identifies the presence of proteins.

2.3.14.3 Alkaloid

Following qualitative test(s) were performed to determine the presence of alkaloid in the selected plant fruits.

Mayer's test

The Mayer's reagent (potassium mercuric iodide solution) was added to the plant extract (2.5g). The alkaloid contents were represented by white creamy precipitate.

Wagner's test

A few drops of Wagner's reagents (iodine solution in potassium iodide) were added to the plant extract (2.5g). The presence of alkaloids was represented by reddish brown precipitate.

Hager's test

The test sample (2.5g) was treated with saturated solution of picric acid (Hager's reagent), the yellow precipitate appearance indicates the alkaloid's presence.

2.3.14.4 Sterols and Triterpenoids

Sakowski's Test

The concentrated sulphuric acid was added to 1g of test extract, shake vigorously and then allowed to stand for some time. The red or yellow appearance at the lower layer indicates the presence of sterols or triterpeniods, respectively.

2.3.14.5 Phenolic compounds

Ferric chloride test

2ml of ferric chloride solution was added to 2g of test sample, the appearance of deep bluish green color reflects the phenolic compound's presence.

2.3.14.6 Flavonoids

Alkaline reagent test

1g of test sample was treated with NaOH solution and the disappearance of intense yellow color on additions of dilute CH_3COOH, confirm the presence of flavonoids.

2.3.14.7 Tannins

Gelatin Test

To 1g test sample, 1% gelatin solution was added containing NaCl. White precipitate appearance indicates the tannin's presence.

Ferric Chloride Test

The test extract was reacted with ferric chloride and tannin presence was confirmed by blue green color appearance.

2.3.14.8 Saponins

Frothing Test

The presence of saponins wasconfirmed by the frothing test, 5 ml of extract was shaken vigorously in a test tube. Formation of froth and it's persistence for some time showed the presence of saponins.

2.3.15 Nutritional Analysis

2.3.15.1 Determination of Ash

Requirements

Desiccators, burner, muffle furnace, china dish and electric balance.

Procedure

Hold a clean flat bottom china dish in a hot burner flame for 1 min, transferred desicator then weight (W) after cooling. Take a suitable amount of test sample(s) (W1), gently heat on bunsen burner then charred in suitable condition for transfer to muffle furnace (550°C) [62].

Continue the heating till all the carbon burnt away and after complete burning transfer the ash and dish to desiccator, weight after cooling (W_2).

Calculations

$$\text{Percent Ash} = \frac{W_1 - W_2}{\text{Wt of the sample}} \times 100$$

Weight (empty dish) = W

Weight (empty dish + Sample) = W_1

Weight (empty dish + Ash) = W_2

2.3.15 .2 Determination of the Moisture

Requirements

Petri plates, electric oven, desiccators and electric balance

Procedure

1-2g of the test sample(s) was weighed in a clean petri plate (W_1) and place in oven at 105°C for 4-6 hrs till a constant weight obtained. The plates were then transferred to a desiccator for 30 min to cool it. Weight of the plates was measured after cooling (W2).

Calculations

$$\text{Percent Moisture} = \frac{W_1 - W_2}{\text{Wt of the sample}} \times 100 \qquad [63]$$

2.3.15.3 Determination of proteins by "Macrojeldahl distillation method

Reagents Apparatus

H_2SO_4, boric acid, NaOH, K_2SO_4, $CuSO_4$, HCl, Kjeldahl flask, digestion and distillation apparatus.

Mixed indicator

Dissolve methyl red (0.016g) and bromo-cresol green (0.03g) in 100 ml of alcohol.

Macro-kjeldhal method was used for measurement of protein (% N x 6.25) as following.

a. Digestion

Take 0.5g of dry ground sample in a kjeldahl flask. Add 18ml of H_2SO_4, 1g of $CuSO_4$ and 20-25 ml of conc. H_2SO_4. Digest it in kjeldahl digestion unit (kjeldatherm) for 6 hrs so that the complete release of SO_2 occurs and the solution become clear or green then cool the mixture.

b. Distillation

Transfer 50ml of 4% boric acid solution to a receiving flask and add 3-5 drops of mixed indicator and place it under the condenser of Kjeldahl. Distillation unit making sure that the condenser tube extends beneath the surface of the acid, in the flask, now add 50 ml water and 60 ml of 32% NaOH solution to Kjeldahl flask. After distillation the flask was removed.

c. Titration

Take HCl (0.1N) in burette and titrate the contents. Note down the reading and determine the percentage of protein in it as follows.

Calculation: Percent Protein = % Nitrogen x 6.25. [63]

2.3.15.4 Determination of Fat or Ether

Equipment and Glassware

Petroleum ether extraction thimbles, soxhlet extraction apparatus.

Determination of fat was carried out by soxhlet extraction using petroleum ether in Soxhlet system H.T (Tecator). The procedure is given below.

Procedure

For extraction of crude fat, soxhelt's apparatus was used; first weigh 2-4g of extraction thimble. The thimble was plugged with absorbent cotton wool and placed in extraction chamber. A previously clean and dried 250ml round bottom flask was weighed, filled 1/3 with solvent and connect to extraction tube. The sample was placed over filter paper and turns on the flow of tap water and continues for 5-6 hrs. Tapping occurs after 5-6 min at 3-4 drops/second condensation rate. After completion of process, remove the thimble from the extractor and heat the flask so that all the solvent can be collected for future. The flask was then dried at 105°C, cooled and weighed. The fat contents were calculated by using following formula.

Calculation

$$\text{Percent fats content} = \frac{W_1 - W_2}{W_o} \qquad [63]$$

2.3.15.5 Determination of Crude Fiber by Fibertec

Equipment and Glassware

Crude fiber extraction apparatus (Fiber Tec System M. Tecator), oven, muffle furnace and suction pump.

Reagents

N-octanol, potassium hydroxide (KOH) solution and water.

Procedure

Weigh and transfer an exact quantity of test sample (1.0g = W_o) to a clean filter crucible and fix in a crucible stand. The foam formation was prevented by adding few drops of octanol and boiled for 30 min. Filter the sample and wash three times with hot water spray device (30 ml of water) and dry.

Add 150 ml of KOH solution to each sample preheated in the second reagent system. Add a few drops of octanol and boil as above for another period of 30 min. the percent crude fiber was calculated by following formula.

Calculations

$$\text{Percent Crude fibers} = \frac{W_1 - W_2}{W_o} \times 100 \qquad \text{[AOAC]}$$

2.3.15.6 Carbohydrates Contents

Carbohydrate content(s) were determined by subtracting the amount of fat, protein, crude fiber, moisture and ash from 100 [63].

Formula

Carbohydrates contents = (100 – ash + moisture + fibers+ fats+ protein).

Gross energy

Gross energy of proteins, fats, fibers and carbohydrates were find out by reported protocol (AOAC 2000). Following formula was used to calculate the total gross energy of proteins, fats, fibers and carbohydrates in a particular plant parts. We can find the gross energy individually as well for each of the nutrients.

Formula:

Energy value (Kcal/100g = (2.62×%protein)+(8.37×% fat)+(4.2×% carbohydrate)+(4.6×fibers) [68].

2.3.16 Elemental analysis

Reagents and Equipments

Double distilled water, H_2SO_4, Nitric acid (HNO_3), Hydrogen per oxide (H_2O_2), Hydrogen fluoride (HF), Perchloric acid ($HClO_4$) and HCl (Analytical grade). Lead (Pb), cadmium (Cd), cobalt (Co) and manganese (Mn), copper (Cu), zinc (Zn) and iron (Fe). Glass wares and plastic equipment were thoroughly washed with water, followed by rinsing with distilled water prior to use.

Sample preparation

Samples were prepared by wet digestion method. For this purpose, in a conical flask, 1g of powder drug was taken and 10ml of 67% conc. HNO_3 was added. The flask was kept overnight at room temperature and add 4ml of 67% conc. $HClO_4$. The flasks were then heated till a clear solution of approximately 1ml was left. After cooling, the final volume adjusted to 100ml by addition of double distilled water and filtered through whatman's filter paper and the filtrate serves as stock solution and stored in air tight containers for elemental analysis [61]. Each sample was then analyzed by flame atomic absorption spectrophotometer and flame photometer, in triplicate. Calibration standard of each metal was prepared by appropriate dilution of the stock solutions [64 -68].

2.3.17 Allelopathy

Procedure

The Allelopathic potential of *S. saligna* fruits was tested against wheat seeds. The experiment was designed in a completely randomized manner at Centre of Biotechnology and Microbiology, University of Peshawar [69]. Wheat seeds were tested for 48 and 72 hrs against different concentrations (10 and 20g/100ml). Distilled water was used as positive control. Petri dishes were properly washed and sterilized. The experiments were performed in triplicates and 10 seeds were added to each plate and incubated for 72hrs at 30°C. After 72 hrs, radical and plumule length and fresh weight, was recorded with the help of digital balance and ruler. After measuring the above parameter(s) the seeds were incubated in oven for 72 hrs at 60°C for determination of moisture contents and dry weight. The collected data was analyzed through Anova test [70].

2.3.18 Scanning Electron Microscopic (SEM) Analysis

Sample was prepared in the form of thin films on a copper grid carbon coated by taking a small quantity of *S. saligna* fruit powder in distilled water and then on the SEM grid that layer was kept for dryness through hot air of 50-60°C for 5min [71].

2.3.19 Energy Dispersive X-Ray (EDX)

The *S. saligna* fruits powder was prepared by grinding. The elemental composition study was carried out by EDX coupled with SEM JSM-5910. The dispersed particles were sprinkled over a double sided sticky tape which was mounted on a microscopic stub of aluminum [72].

2.3.20 Gas Chromatography-Mass Spectrometry (GC-MS)

A gas chromatograph from Shimadzu connected to mass spectrometer equipped with auto-injector and sampler was used. Helium was used as carrier gas. All chromatographic separations were performed on a capillary column (TRB-FFAP; Technokroma) with specifications; length (30m), internal diameter (0.35mm), thickness (0.250μm). 1μl of sample and standard were injected to the column. Injector was operated in a split mode with a split ratio 1:50. Injection temperature was 240°C. The column temperature started at 50°C for 1 min and was raised to 150°C at the rate of 15°C/min. The oven temperature was raised to 175°C with 2.5°C/min interval and hold for 5 min the temperature was then raised to 220°C with 2.5°C/min interval for 3 min. The total elution time was 43 min and the MS scanning was performed at 85-380m/z. The constituent(s) of the test sample were identified by comparing the mass spectra of test and standard obtained from the National Institute of Science and Technology library (NIST 05).

Chapter 3

3.1 Bioassay screening

3.1.1 Antibacterial activity

One of the major problem faces by the science in current situation is resistance to antimicrobial drugs. Finding new antimicrobial agent can help us to get rid of such problems [73]. Crd. MeOH Ext. and various fractions of *S. saligna* fruits were screened for antimicrobial activity against *E. coli,* MRSA, MSSA, *P. aeruginosa* and *S. typhi*. The results are summarized in table 3.1-3.2 and presented in figure 3.1-3.4

The order of antibacterial activity against *E. coli* was; E=L (48%) > A=D=J=K (37%) > L (33.3) > C=I (26%) and B (22.2%). G and H fractions were found inactive against, *E. coli*. Against *MRSA*; A=D=G=I (53.3%) > B=E=H (46.6%) > Cr. Ext. (33.3%) > J=K=L (26.6%) > F (20.0%) > C (13.5%). Against MSSA; F (65.3) > A (58%) > D=E=K=L (57%) > B=C (53.8%) > J (50%) > H=I (46%) > G (38.4%). Against *P. aeruginosa;* H (59.2%) > A=D=L (55.5%) > C (52%) > K (51.85%) > I (44.4%) > E=J (37%) and B, F and G fractions were found inactive. Against *S. typhi*; H (59.2%) > A (55.5%) > C (52%) > K (51%) > F (48.1%) > D=G=I (44.4%) > E (40%) > L (37%).

The above results revealed that all fractions except G and H, showed low antibacterial activity against *E. coli.* A, B, D, E, G, H and I fractions showed moderate activity while rest of the fractions possess low antibacterial activity against MRSA. Fraction F showed good antibacterial inhibition while other fractions were not that much active against MSSA. Fractions A, C, D, H, K and L were moderately active and the other fractions showed low activity or found inactive against the *P. aeruginosa.* Fraction C showed moderate while fractions A, C, H, F, D, G, I and E showed low activity against *S. typhi.*

Aerial parts of *S. saligna* showed significant antimicrobial activity. *S. ruscifolia* extracts has been reported for good antimicrobial activity [74]. The afore mentioned results indicate that *S. saligna* contains antibacterial agents. The fractions can be subjected to activity guided isolation of the natural active constituents

Minimum Inhibitory Concentration (MIC_{50})

The MIC_{50} values indicate the potency of a test sample, lower the MIC_{50} higher will be the potency. MIC_{50} values (mg/ml) of the various fractions of *S. saligna* (with moderate antibacterial activities) were calculated by using Microsoft XL sheet from three different reading as shown in table 3.3 and figure 3.5-3.7.

Minimum Bactericidal Concentration (MBC)

Due to higher MIC_{50} values the MBCs for the test samples were not calculated against test pathogen.

Table 3.1 Antibacterial activity of Crd. MeOH Ext. and various fractions of *S. saligna* fruits

Name of Bacteria	Zone of Inhibition (mm)													
	+ive Control	Crd. Ext	A	B	C	D	E	F	G	H	I	J	K	L
E. coli	27 (AMC)	7	10	6	7	10	13	9	0	0	7	10	10	13
MRSA	15 (CEF)	5	8	7	2	8	7	3	8	7	8	4	4	4
MSSA	26 (CEF)	9	15	14	14	15	15	17	10	12	12	13	15	15
P. aeruginosa	27 (AMC)	10	15	0	14	15	10	0	0	16	12	10	14	15
S. typhi	27 (AMC)	10	12	14	15	12	11	13	12	13	13	13	14	10

Table 3.2 Percent antibacterial activity of Crd. MeOH Ext. and various fractions of *S. saligna* fruits

Name of Bacteria	Zone of Inhibition (Percent)												
	Crd. Ext	A	B	C	D	E	F	G	H	I	J	K	L
E. coli	26.0	37.0	22.2	26.0	37.0	48.0	33.3	0.0	0.0	26.0	37.0	37.0	48.0
MRSA	33.3	53.3	46.6	13.5	53.3	46.6	20.0	53.3	46.6	53.3	26.6	26.6	26.6
MSSA	34.6	58.0	53.8	53.8	57.6	57.6	65.3	38.4	46.0	46	50.0	57.6	57.6
P. aeruginosa	37.0	55.5	0.0	52.0	55.5	37.0	0.0	0.0	59.2	44.4	37.0	51.0	55.5
S. typhi	37.0	51.8	55.5	63.0	44.4	40.0	48.1	44.4	48.0	48.0	48.0	51.8	37.0

IC. 14718

Table 3. 3 MIC_{50} values (mg/ml) of the Crd. MeOH Ext. and different fractions of *S. saligna* fruits

Test organism	Cr. Ext	A	B	C	D	E	F	G	H	I	J	K	L
E.coli	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	6.9
MRSA	NA	7.2	8.6	NA	7.0	8.9	NA	7.2	8.2	7.1	NA	NA	NA
MSSA	NA	6.1	6.6	6.7	6.5	6.6	5.9	NA	7.5	7.4	6.9	6.4	6.6
P. aeruginosa	NA	5.1	NA	5.6	5.2	NA	NA	NA	6.7	5.9	NA	7.3	5.9
S. typhi	NA	6.2	2.5	6.9	6.7	6.1	3.2	5.9	5.6	5.8	5.7	7.5	NA

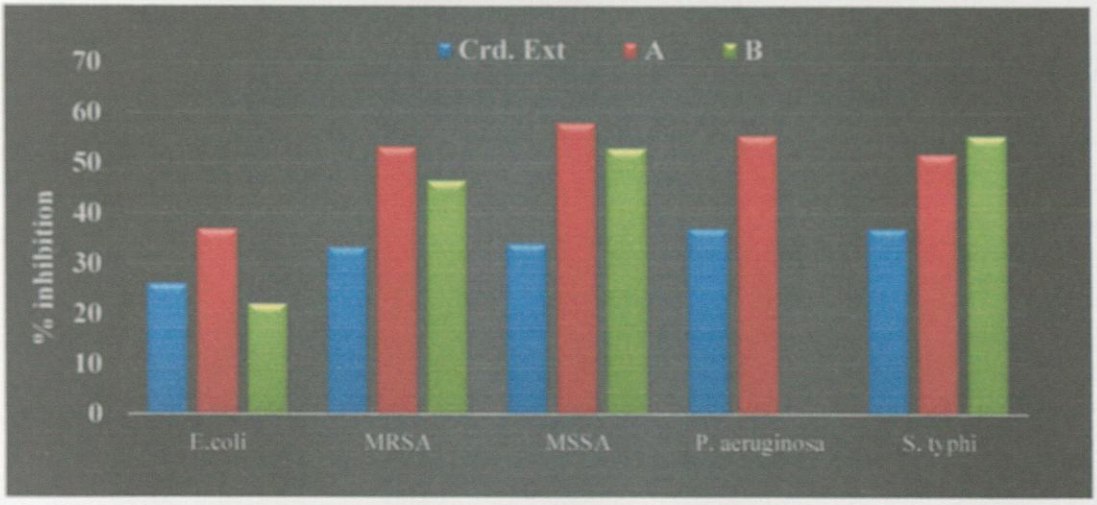


Fig. **3.1** Antibacterial activity of Crd. MeOH Ext, A and B fractions of *S. saligna* fruits

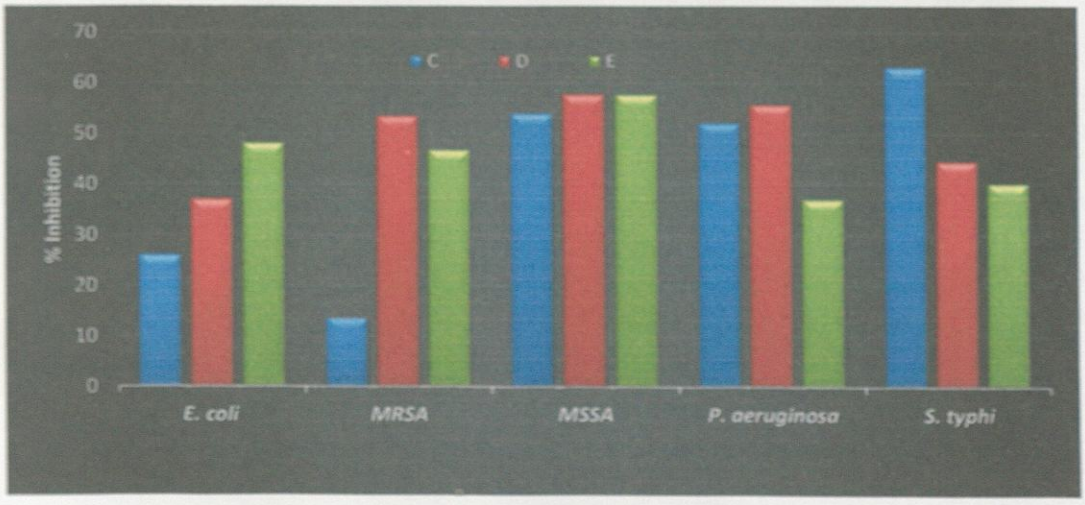


Fig 3.2 Antibacterial activity of fractions C, D and E of *S. saligna* fruits

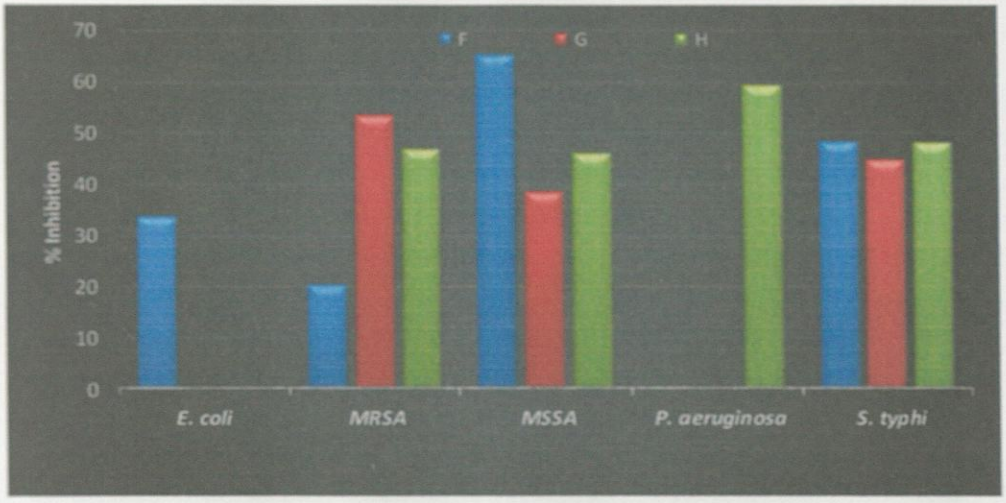


Fig 3.3 Antibacterial activity of fractions F, G and H of *S. saligna* fruits

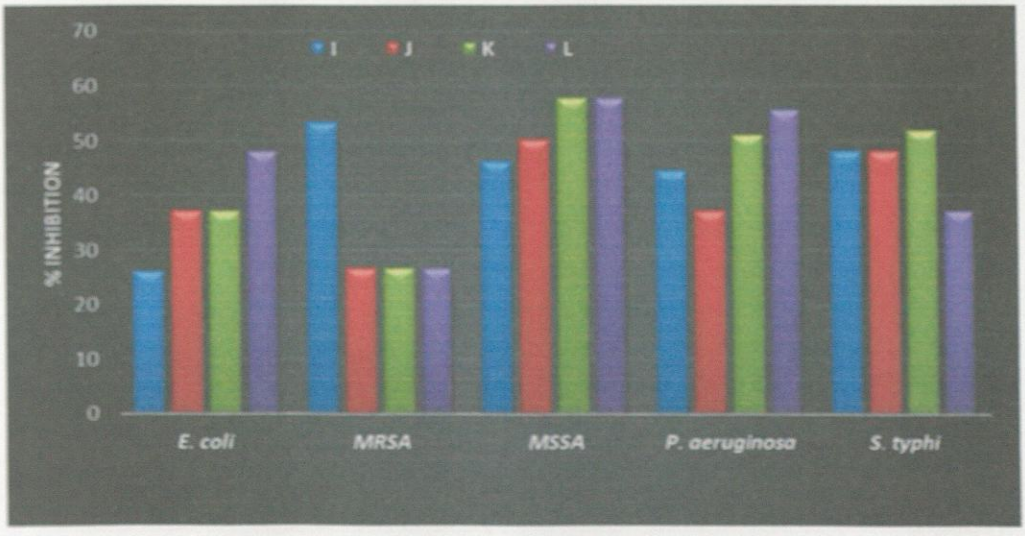


Fig 3.4 Antibacterial activity of fractions I, J, K and L of *S. saligna* fruits

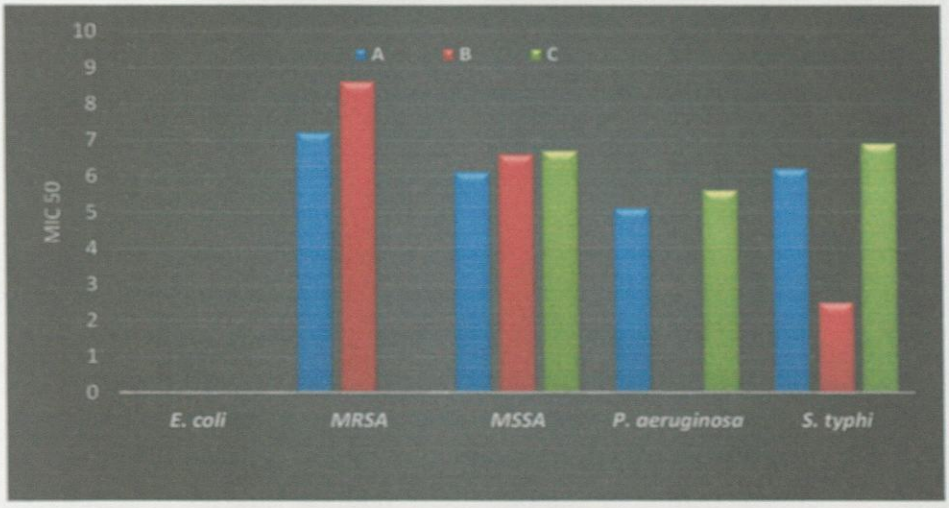


Fig 3.5 MIC_{50} of fractions A, B and C of *S. saligna* fruits

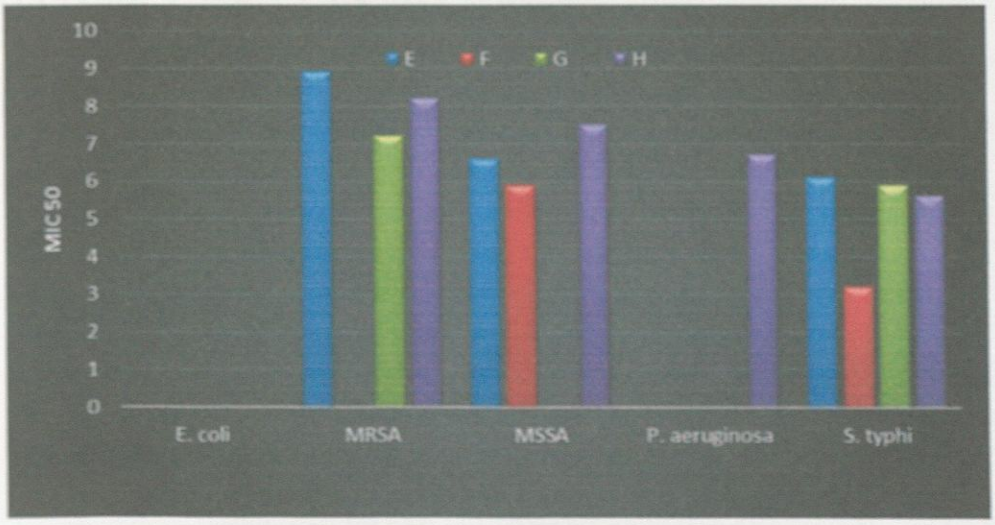


Fig 3.6 MIC_{50} of fractions E, F, G and H of *S. saligna* fruits

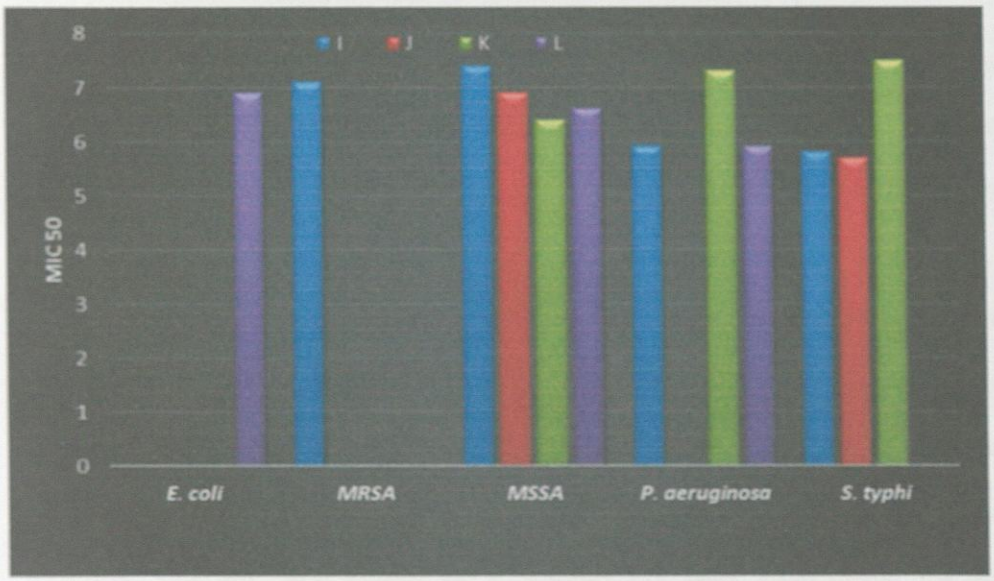


Fig 3.7 MIC_{50} of fraction I, J, K and L of *S. saligna* fruits

3.1.2 Antifungal activity

All the test samples of were screened for the antifungal activity against *A. oryzae, F. fumigatus, F. solani, A. flavus, A. niger* and *P. notatum*. The results are shown in table 3.4 and presented in fig 3.8. Miconazole and Amphotericin-B and used as standard drug. The Crd. MeOH Ext. D, H and J fractions showed low percent inhibition (20%) against *F. fumigatus*, while the rest of the fractions were inactive against *F. fumigatus*. All the fractions were inactive against *A. oryzae, F. solani, A. flavus, A. niger* and *P. notatum*. The results indicate that *S. saligna* fruits contain no antifungal agents. Major drawback in chemotherapy is microbial resistance of fungal infection. The ethanolic extract of *S. saligna* has been investigated for antifungal activity of against clinical isolates and showed good results [75].

Table 3.4. Antifungal activity of the Crd. MeOH Ext and various fractions of *S. saligna* fruits

Names of Fungi	Percent linear growth inhibition														
	-ive Control	+ive Control	Crd. Ext	A	B	C	D	E	F	G	H	I	J	K	L
A. oryzae	-	100	-	-	-	-	-	-	-	-	-	-	-	-	-
F. fumigatus	-	100	20	-	-	-	10	-	-	-	20	-	10	-	-
F. solani	-	100	-	-	-	-	-	-	-	-	-	-	-	-	-
A. flavious	-	100	-	-	-	-	-	-	-	-	-	-	-	-	-
A. niger	-	100	-	-	-	-	-	-	-	-	-	-	-	-	-
P. notatum	-	100	-	-	-	-	-	-	-	-	-	-	-	-	-

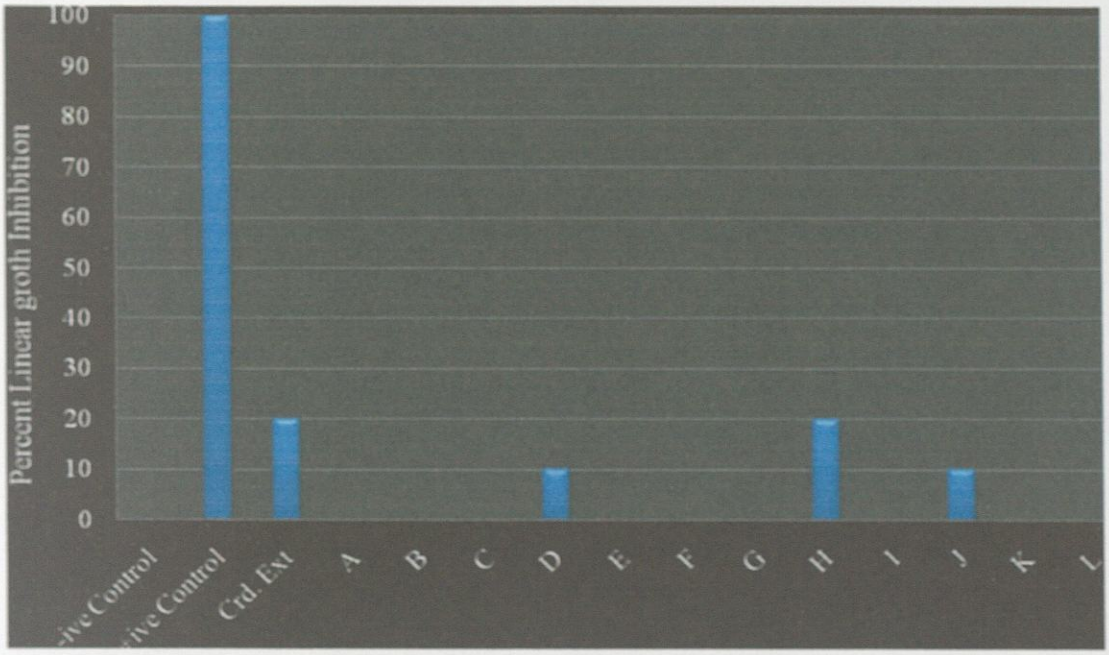


Fig 3. 8. Antifungal activity of Crd. MeOH Ext and various fractions of *S. saligna* fruits

3.1.3 Phytotoxic activity

The herbicides which are obtained from plants are often environment friendly. Therefore it was sensible to search for the herbicides which are plant's origin. *L. minor* is sensitive to bioactive compound, that's why it is used to detect the phytotoxic compounds. Phytotoxic compounds are accepted antitumor and plant growth stimulant [76]. All the test samples of were screened for possible phytotoxic activity against the *L. minor*. The results are given in table 3.5 and 3.5a and presented in figure 3.9.

Fractions D, C, G, H, K and L showed moderate phytotoxic activities (50, 43.75, 50, 56, 43.75 and 56.25 %), respectively at higher concentration (1000µg/ml).

Low phytotoxic activity was observed for all fractions at lower concentration (100µg/ml). Fractions C, D, F, G, H, J and L possess low phytotoxic effect at lower concentration of (10µg/ml).

Table 3.5. Phytotoxic activity of the Crd. MeOH Ext. and various fractions of *S. saligna* fruits

Concentration of sample (µg/ml)	Number of frond killed													
	Crd. Ext	A	B	C	D	E	F	G	H	I	J	K	L	Control
1000	4	4	5	8	7	3	6	8	9	5	6	7	9	16
100	2	1	2	5	4	1	3	6	6	3	3	3	5	16
10	0	0	0	1	1	0	1	2	2	0	1	0	1	16

Table 3. 5a. Percent growth regulation of *L. minor*

Conc of sample (µg/ml)	Percent growth regulation													
	Crd. Ext	A	B	C	D	E	F	G	H	I	J	K	L	Control
1000	25	25	31.25	50.0	43.75	18.75	37.5	50.0	56.25	31.25	37.5	43.7	56.25	100
100	12.5	6.25	12.5	31.25	25	6.25	18.75	37.5	37.5	18.75	18.75	18.75	31.25	100
10	0	0	0	6.25	6.25	0	6.25	12.5	12.5	0	6.25	0	6.25	100

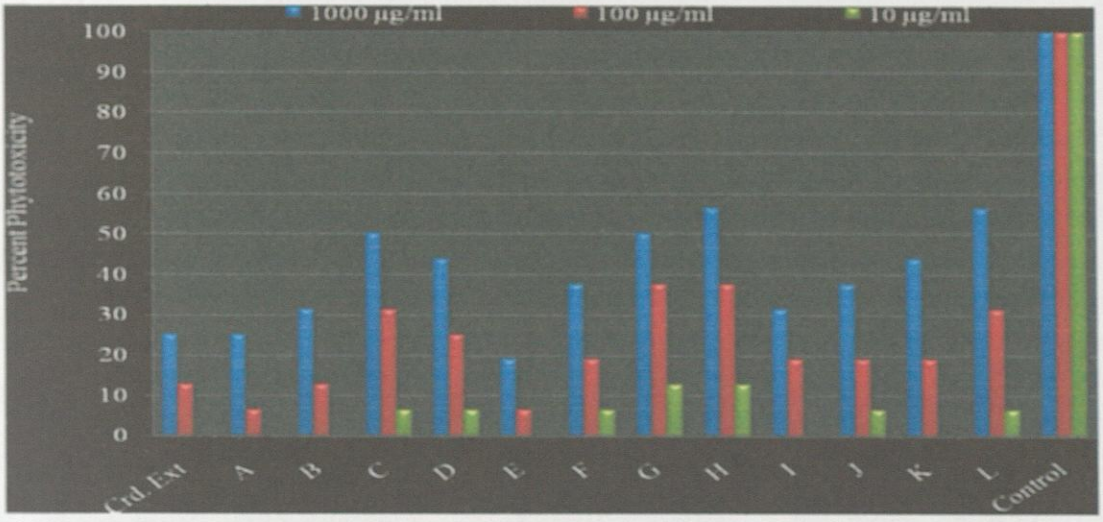


Fig 3.9. Percent growth regulation of Crd. MeOH Ext. and various fractions of *S. saligna* fruits

3.1.4 Heamagglutination activity

Certain plants, in their different parts, contain heamagglutinins, which can be used as blood typing reagent in the same way as agglutinin from animal source. By using lectins we can study the sugar components on the cancerous and normal cell surface [76]. Lectins are one of the most important toxin and show resistance to absorption when ingested [77]. By different mechanism, it can negatively affects the nutrient utilization. The plants source agglutinins are available in large quantities and are economical therefore it has advantages our animal source.

S. saligna fruits were tested for heamagglutination activity against RBC's of all blood group. The results are given in table 3.6. The Crd. MeOH Ext. and fraction A showed weak agglutination against O^{-ive} blood group at 1:2 dilutions. Similarly, rest of the fractions showed no activity against the human erythrocytes. The above results indicate that *S. saligna* fruits contain no lectins.

Table 3.6 Heamagglutination activity of the Crd. MeOH Ext and various fractions of *S. saligna* fruits

Blood	Crd. Ext				A				B				C				D				E				F			
Group	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16
AB^-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B^-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A^-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O^-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AB^+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B^+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A^+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O^+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3.6 Heamagglutination activity of the various fractions of *S. saligna* fruits

Blood	G				H				I				J				K				L			
Groups	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16
AB^-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B^-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A^-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O^-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AB^+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B^+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A^+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O^+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(-) No activity and (+) Weak.

3.1.5 Insecticidal activity

The Crd. MeOH Ext. and various fractions of *S. saligna* fruits were tested for insecticidal activity against *C. analis*, *R. dominica* and *T. castaneum*. Results are given in table 3.7 and figure 3.11.

These results revealed that fractions E, F, G, H, I, K and L showed low insecticidal activity (<40%) against *T. castaneum*, while rest of the fractions and Crd. MeOH Ext. was found inactive against the same insect specie. Fractions C, F G, H, I, K and L showed low activity (<40%), while Crd. MeOH Ext and rest of the fractions showed no insecticidal activity against *R. dominica.* All the fractions and Crd. MeOH Ext showed low insecticidal activity (<20%) against *C. analis* except A, B, G, H and J which were found inactive. Permethrine (Copex) was used as positive and organic solvent as negative control. Positive control showed 100% while negative control showed no insecticidal activity. Above results show that *S. saligna* fruits have low insecticidal property.

Table 3.7. Insecticidal activity of Crd. MeOH Ext. and various fractions of *S. saligna* fruits

Name of Insect	% Mortality													
	+ive Control	Crd. Ext	A	B	C	D	E	F	G	H	I	J	K	L
T. castaneum	100	0	0	0	0	0	20	20	20	40	40	30	20	40
R. dominica	100	0	0	0	20	6	6	20	40	40	40	0	40	40
C. analis	100	0	0	0	20	20	20	20	0	0	20	0	20	20

*Permethrine at concentration 235.90 $\mu g/cm^2$ act as standard drug

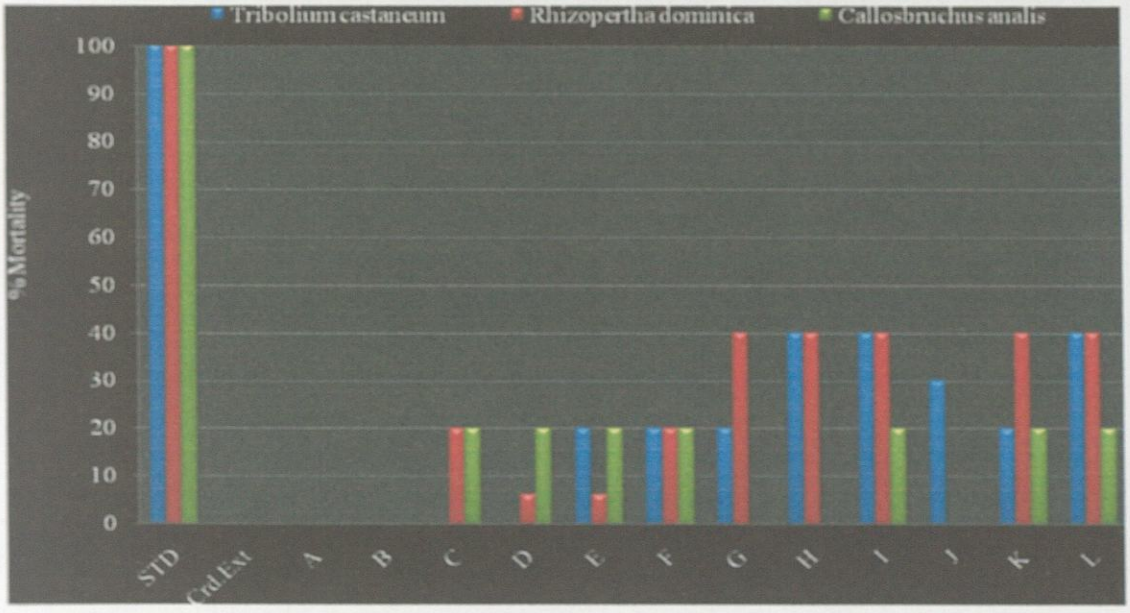


Fig.3.11. Insecticidal activity Crd. MeOH Ext. and various fractions of *S. saligna* fruits

3.1.6 Diphenyl Picryl Hydrazine (DPPH) radical-scavenging activity

Due to the importance of antioxidant activity, the test samples were screened for DPPH free radical scavenging activity at different concentration; 20, 40, 60, 80, 100ppm. The results are given in table 3.8-3.8a and are presented in figure 3.12-3.13.

A good scavenging potential was observed for fraction L (73%), I (71%), D (70%), J (69%), F (65%), H (63%), C (62%) and B (61%). Moderate activity was observed for fractions E (56%), K (55%), A (53%) along with Crd. MeOH Ext. (50%) Fractions D, L and J possess moderate activity (>40% and <43%). D, J, L, I, G, B, H and C fractions showed moderate while remaining fractions along with Crd. MeOH Ext. showed low scavenging effect.

Low EC_{50} values was obtained for J(40.50µ/ml), I(40.74µg/ml), D(42.574µg/ml), H(45.48µg/ml), L(45.5µg/ml), G(56.61µg/ml) and B(58.61µg/ml).

Table 3.8. DPPH free radical scavenging assay of Crd. MeOH Ext. and various fractions of *S. saligna* fruits

Concentration (ppm)	Crd. Ext	A	B	C	D	E	F	G	H	I	J	K	L	Standard (vit C)
20	10	10	22	14	39	18	12	15	21	29	32	14	27	38
40	20	22	34	26	43	25	22	30	34	39	40	25	41	53
60	29	32	45	40	57	39	32	47	43	51	55	38	53	68
80	30	45	58	54	60	48	45	63	54	60	65	48	65	80
100	50	53	61	62	70	56	65	73	63	71	69	55	73	83

Table 3. 8a. EC50 Values of the Crd. MeOH Ext. and various fractions of *S. saligna* fruits

Test Sample	Cr. Ext	A	B	C	D	E	F	G	H	I	J	K	L
EC50 (µg/ml)	90	84.5	58.4	73.5	42.5	76.6	77.5	56.6	45.5	40.7	40.5	78.6	45.5

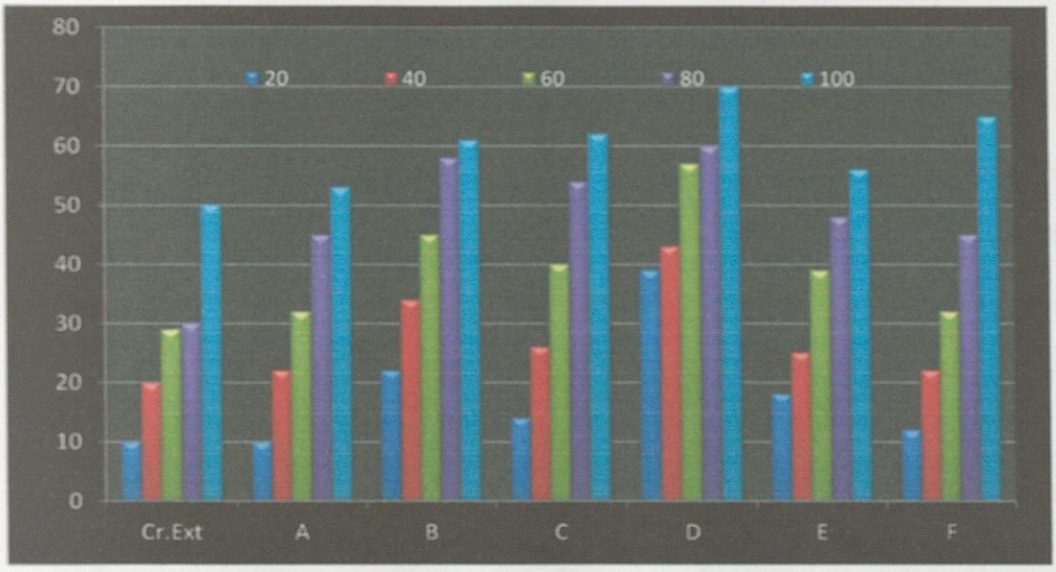


Fig.3.12. EC50 values of the Crd. MeOH Ext and various fractions of *S. saligna* fruits

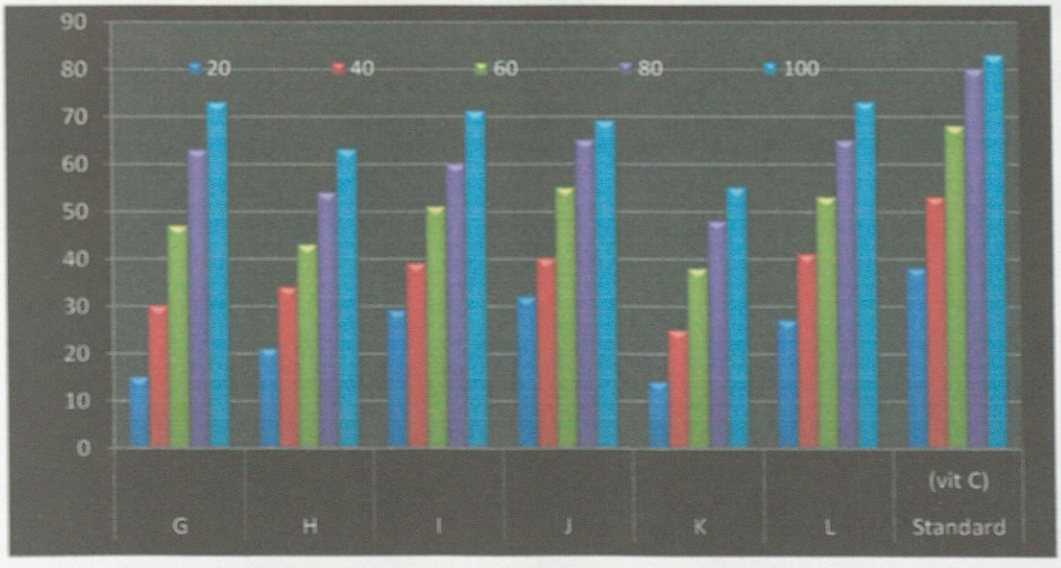


Fig. 3.13 EC50 values of the various fractions of *S. saligna* fruits

3.1.7 Brine shrimp lethality bioassay

Brine shrimp lethality bioassay was used to determine the cytotoxic effect of the test samples. The results indicated that the Crd. MeOH Ext showed low cytotoxicity (16.6%) at 1000µg/ml. At 100 and 10µg/ml, cytotoxicity was 13.3 and 6.66%, respectively. The fraction A showed low cytotoxicity (16.6%) at 1000µg/ml and low activity (10 and 6.66%) was also observed at concentration of 100 and 10µg/ml, respectively. The fractions B and C showed low toxicity of 20 and 16.6% at 1000 and 100µg/ml, respectively. The fraction D, E and F showed low brine shrimp lethality at 1000µg/ml (33.4, 23.3 and 20%), 100µg/ml (20, 13.3 and 10%) and 10µg/ml (20, 10 and 6.6%), respectively. The G, H and I fractions showed low brine shrimp lethality (16.6, 33.3 and 40%) at 1000µg/ml, respectively. The same fractions showed low lethality at 100 and 10µg/ml. A moderate cytotoxic effect was shown by fractions J (40%) and K (40%) at higher concentration while at low concentration, low activity was recorded for these fractions. The L fraction showed low cytotoxicity (36.66%) at higher and lower concentrations. Results are given in table 3.9 and 3.9a and presented in figure 3.14.

Table 3.9. Cytotoxic activity of Crd. MeOH Ext. and various fractions of *S. saligna* fruits

Dose (µg/ml)	Control (No. of shrimps)	Crd. Ext		A		B		C		D		E		F	
		Survivor	% killed	Survivor	% killed	survivor	% killed	survivor	% killed	survivor	% killed	survivor	% killed	survivor	% killed
100	30	26	13.3	25	16.6	24	20	24	20	20	33.3	24	20	24	20
100	30	25	16.6	27	10	25	16.6	25	16.6	23	17	26	13.3	27	10
10	30	28	6.6	28	6.6	27	10	26	13.3	24	20	27	10	28	6.6

Table 3. 9a. Cytotoxic activity of Crd. MeOH Ext. and various fractions of *S. saligna* fruits

Dose (µg/ml)	Control (No. of shrimps)	G		H		I		J		K		L		Standard Drug	
		Survivors	% killed	Survivors	% killed	Survivors	% killed	Survivors	% killed	Survivors	% killed	Survivors	% killed	Survivors	% killed
100	30	25	16.6	20	33.3	18	40	18	40	18	40	19	36.6	0	100
100	30	26	13.3	23	23.3	25	16.6	25	16.6	23	23.3	20	33.3	0	100
10	30	27	10	27	10	28	6.6	27	10	27	10	23	23.3	0	100

Etoposide (7.4625 µg/ml) was used as a standard drug

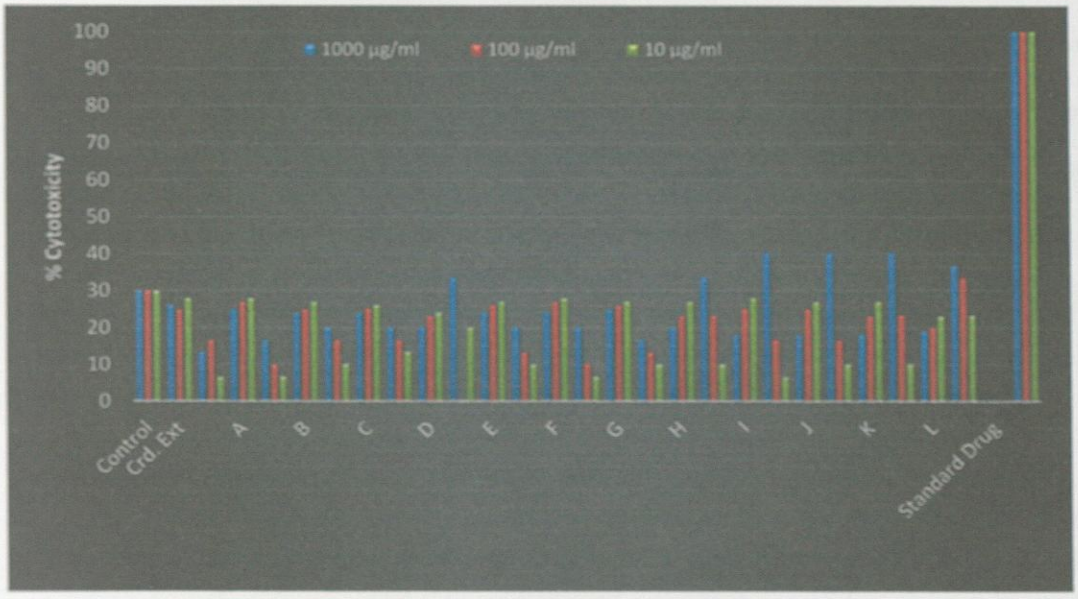


Fig 3.14: Cytotoxic activity of Crd. MeOH Ext. and various fractions of fruits of *S. saligna*

3.1.8 Anti-termite Assay

The anti-termite activity of Crd. MeOH Ext. and different fraction of *S. saligna* fruits were carried out at "Termites lab, Nuclear Institute of Food and Agriculture (NIFA), Peshawar, KPK, Pakistan. All the experiments were repeated 3 times. The results are shown in the table 3.10.

Termites play an important role for the recycling the plant resources. The burrowing effort of termites help in soil exposure to air. They are mainly found in tropical and subtropical regions. They cause great damage of economy by destroying wooden product besides being beneficial [78].

The Crd. MeOH Ext. showed significant activity against *H. indicola*. The experiment extended for next two days. On 1st day 13 and on 2nd day 20 termites were dead on an average, no termite survived on 3rd day. On 1st day A, B, C and D fractions killed an average of 13, 16, 20 and 22 termites, respectively. On the 2nd day of the experiment 20, 21, 22 and 23 termites were killed on an average on 3rd day all the termite were killed by fraction A but for B, C and D fractions 24, 23 and 22 termites were killed, respectively. The experiments for the fractions; E, F, G, H, I, J, K and L was completed in three days. On average; 21, 18, 17, 18, 20, 20 and 21 termites were killed on 1st day, respectively. On the 2nd day of the experiment, 22, 20, 20, 22, 22, 22, 23 and 23 termites were killed on an average, respectively. On 3rd day, no termite survived for fractions G, I, K and L but for the fractions E, F, H and J; 23, 22, 23 and 24 termites were killed, respectively.

Table 3.10 Anti-termite activity of the Crd. MeOH Ext. and various fractions of *S. saligna* fruits

Sample	No. of Termite used	Days	Average termite killed	Sample	No. of Termite used	Days	Average termite killed
Crd. Ext	25	1	13	G	25	1	20
		2	20			2	20
		3	25			3	25
A	25	1	16	H	25	1	21
		2	20			2	22
		3	25			3	23
B	25	1	20	I	25	1	20
		2	21			2	21
		3	24			3	25
C	25	1	22	J	25	1	22
		2	22			2	22
		3	23			3	24
D	25	1	20	K	25	1	20
		2	23			2	23
		3	23			3	25
E	25	1	21	L	25	1	21
		2	22			2	23
		3	23			3	25
F	25	1	18				
		2	20				
		3	22				

3.1.9 Acute toxicity

The result of acute toxicity for different doses (500, 700 and 1000 mg) of *S. saligna* fruits are given in table 3.11.

The Crd. MeOH Ext. was administrated at a dose of 500, 700 and 1000 mg/kg b.w intra-peritonialy (n=6). After administration the animal were observed for 24 hrs for mortality, but no dose was found to be toxic and all doses were found safe therefore the test samples are considered to be relatively safe.

The *in-vivo* studied showed that the *S. saligna* is safe upto a dose of 500-1000mg/kg body weight [79]. *S. salisifolia* alkaloidal steroids are safe upto a dose of 300-1000mg/kg b.w [80]. To investigate the effects of *n*-butanol extract and total alkaloids of *S. ruscifolia* a traditional Chinese medicine, it is safe upto a dose of 1.65×10~ (-5) and 4.3×10~ (-6) g/L, respectively, for rats [81]. The EtOAc fraction of the leaf of *S. saligna* is safe upto 250mg/kg b.w [82].

Table 3.11 Acute toxicity of the Crd. MeOH Ext. of *S. saligna* fruits

Test Samples	Dose (mg/kg)	Gross effect after 4 hrs	Mortality after 24 hrs
Test sample	500	-	-
	700	-	-
	1000	-	-

3.1.10 Analgesic activity

The Crd. MeOH. Ext. of *S. saligna* fruits was evaluated for its analgesic effect using chemical and thermal induced pain model. Analgesic is a group of drugs used as to relief pain or analgesia. Analgesic drugs are distinct from the anesthic which eliminate the sensation it act on various way on central nervous system [83].

Acetic acid induce writhing test

This study showed that the Crd. MeOH Ext. of *S. saligna* fruits possesses analgesic activity. The results showed that the pain release was achieved in a dose dependent manner as shown in table 3.12. The significant ($P<0.01$) analgesic effect was shown at all the applied doses, while the maximum inhibition was found at 1000mg/kg dose of the test sample as shown in table 3.12. The percent inhibition of writhing is shown in Figure 3.15. Diclofenac sodium was more than the high dose of test sample.

The experiment showed that the Crd. MeOH Ext. possess analgesic effect. This is a common, sensitive, quick and easy method for the determination of exterior analgesic effect of natural extract and other drugs as well [84]. The local receptor (peritoneal) in mouse is responsible for occurrence of abdominal contraction [85]. It is also reported that there is increase sensitization of peritoneal receptors (nociceptive) to prostaglandins. Generally it is assumed that with acetic acid induced pain model, the synthesis (production) of prostanoids like PGF2α and PGE2 and lipo-oxygenase derivatives increased in the peritoneal liquids and served as pain mediators [86]. These substances are produced by cyclo-oxygenase (COX) pathway derived from arachidonic acid, which is liberated from phosphatides of inflamed abdominal tissue [87]. These chemicals produced in peritoneal fluids which cause pain and appear in the form of belly

contractions. Writhing inhibition by different materials may be involved in inhibition of prostanoid, which is considered for pain inhibition through peripheral mechanism [88].

It is suggested that the test sample showed analgesic effect through inhibition of abdominal receptors (nociceptive), it may be due to decrease in synthesis or inhibition of prostanoids production. The active chemical(s) present in test sample are responsible to reduce the abdominal contraction due to analgesic effect.

Table 3.12 Analgesic activity of Crd. MeOH Ext. of *S. saligna* fruits

S. No.	Treatment	Dose (ml or mg/ kg)	No. of writhing (10 min) (Mean+SEM)
1	Normal saline	10	52.50 ± 4.28
2	Test sample	500	48.33 ± 1.78
		700	39.83 ± 1.22**
		1000	31.67± 1.24***
3	STD (Diclofenac sod.)	10	19.50 ± 1.02***

Values are reported as mean ± S.E.M. (n=6). ANOVA followed by Dunnett's test was applied on the data to find the level of significance in comparison with negative control. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

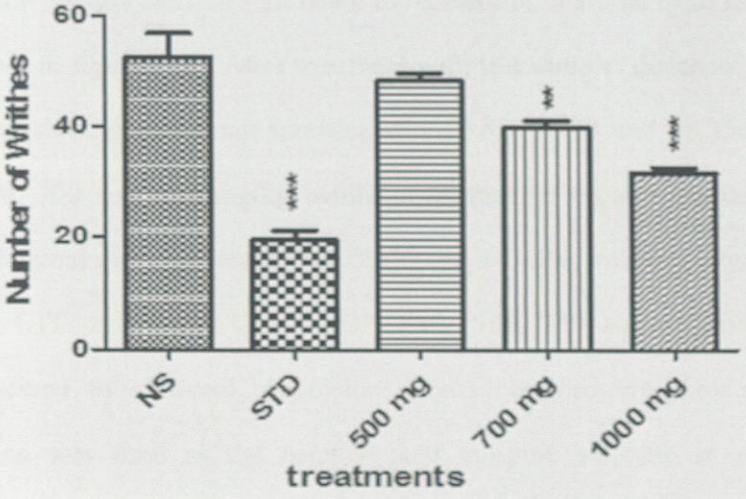


Fig 3.15: % analgesic activity of Crd. MeOH Ext. of *S. saligna* fruits

ANOVA followed by Dunnett's test was applied on the data to find the level of significance in comparison with negative control, $*P<0.05$, $**P<0.01$ and *** $P<0.001$.

3.1.11 Anti-diarrheal effect

Charcoal meal test (Anti GIT motility test)

The test sample cause a significant movement of charcoal meal in the intestine of mice as shown in figure 3.16. After treatment with test sample, distance (%) covered by charcoal meal through the small intestine was 20.58, 16.98 and 08.35cm at the tested doses of 500, 700 and 1000mg/kg, while in normal saline and charcoal meal treated groups the charcoal meal movement was 33.21 and 54.44%, respectively.

The percent GIT motility of Crd. MeOH Ext. (500, 700 and 1000mg) and atropine sulphate assessed by charcoal propulsion results obtained are shown in table 3.13. Normal saline was used as the negative and atropine sulphate as positive control. Multiple comparison tests revealed that test sample at concentration of 1000mg/kg b.w and atropine sulphate significantly reduced the GIT motility.

The GIT motility of test sample was best determine by this protocol [86]. The different plants of buxaceae are famous for its antispasmodic effect [89]. M3 receptor of small intestine cause activation of the relaxation, acetylcholine cause activation of M3 which increase the abdominal crump. The Crd. MeOH Ext. may cause relaxation of intestine [90]. The effect of antispasmodic agents is to relieve the symptoms of abdominal pains. The Crd. MeOH Ext. reduces GIT motility and can be used for the management of diarrhoea and in abdominal spasm.

Table 3.13 Effect of Crd. MeOH Ext. of *S. saligna* fruits on GIT Motility

Treatment	Dose (ml or mg/kg)	Total length of intestine (cm)	Mean charcoal movement (cm)	% GIT
Normal saline	10	61.00 ± 2.182	33.21 ± 2.781	54.44
Test sample	500	55.00 ± 2.131	20.58 ± 2.658***	37.41
	700	52.00 ± 1.915	16.98 ± 2.245***	32.62
	1000	59.00 ± 2.357	08.35 ± 2.305***	14.15

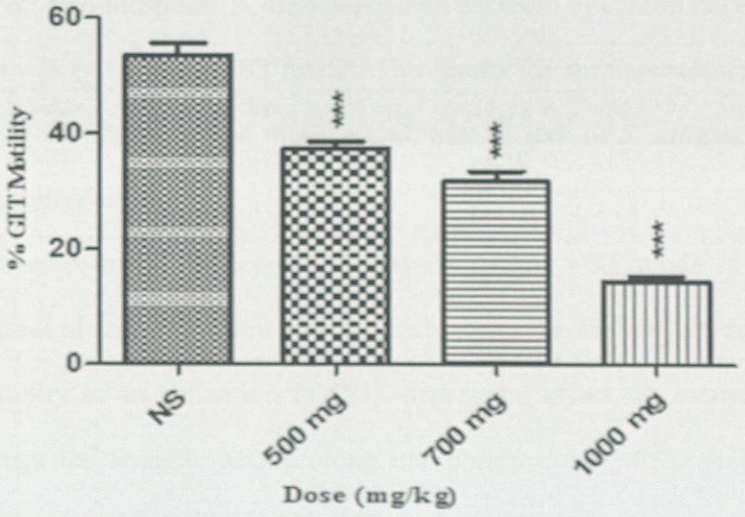


Fig. 3.16: Effect of Crd. MeOH Ext. of *S. Saligna* fruits on GIT Motility.

Data were presented as mean ± SEM (n=6). *P< 0.05, **P< 0.01 and ***P < 0.001.

3.1.12 Antidepressant Activity

The antidepressant activity of the test sample was determined by using forced swimming test (FST) model. It is active in behavioural models predictive of antidepressant potential. The Crd. MeOH Ext. was investigated and in almost all cases, the extracts showed activity. Normal saline was used as negative and fluoxetine as positive control. It is clear from table 3.13 that moderate antidepressant effect was observed for the test sample at 500mg tested dose. With the increase of test doses, the antidepressant effect also increased. A dose dependent decrease of central nervous system (CNS) depression was observed in FST model. The results for antidepressant activity are given in the table 3.14 which showed that the Crd. MeOH Ext. of *S. saligna* fruits have potential for the antidepressant.

For the assessment of antidepressant activity mostly FST mode is used [91]. Antidepressant effect in animal is shown by the increase in the mobility in the water tub and decrease mobility is an indication of CNS depressant effect. In comparison with antidepressant drugs the animals with prolong immobility during FST shows that the animals in the state of tiredness, sleepiness and grief. These symptoms are only found in the patient suffering from depression [92, 93]. The results of our experiment showed immobility caused by Crd. MeOH Ext. is associated with relaxing effect, may be due to CNS depression.

Table 3.13 Antidepressant activity of Crd. MeOH Ext. of *S. saligna* fruits

Treatments	**Dose (mg or ml/ kg)**	**Immobility time (s)**
Normal Saline	10	118 ± 2.70
Crd. MeOH Ext.	500	160 ± 2.46
	700	141 ± 3.50
	1000	121 ± 3.11
Fluoxetine	15	23 ± 2.01

Data were presented as mean ± S.E.M (n=6).

3.1.13 Locomotor activity

Open field effect

The *S. saligna* fruit Crd. MeOH Ext. (500, 700 and 1000g/kg) were tested for locomotor (sedative) effect using open field test.

It is clear that in open field test the Crd. MeOH Ext. decrease the number of line crossing. The total lines cross by mice in open field was 41, 29 and 20 at the dose of 500, 700 and 1000 g/kg, respectively. The decrease in number of the positive control lines crossing shows the soothing effect of the test samples. The locomotor effect of diazepam was most significant ($P<0.001$) than the test doses. Normal saline used as negative and Diazepam as positive control. The results obtained showed a dose dependent response as given in table 3.14. It is concluded from the results that *S. saligna* fruits have potential for locomotor activity.

Table: 3.14 Locomotor activity of Crd. MeOH Ext. *S. saligna* fruits

Treatments	Dose (mg or ml/kg)	Number of lines crossed in 10 min
Normal saline	10	115 ± 2.44
Test sample	500	41 ± 3.11
	700	29 ± 2.43
	1000	20 ± 1.80
Diazepam	0.5	06 ± 0.81

3.1.14 Phytochemical screening

Plants contain a wide range of natural products; tannin, flavonoids, alkaloids and saponin these are associated with the therapeutic values of that specific plant. These natural products on isolation and preparation can be used in various pharmaceutical drug to treat various diseases.

The Crd. MeOH Ext. of *S. saligna* fruits were screened for the presence of phytochemical constituents; carbohydrates, proteins, alkaloids, sterols, terpenes, phenols, flavonoids, tannins and saponin. In alkaloid test precipitate formation shows the presence of alkaloid. The appearance of pink color showed flavonoids confirmation in flavonoid test. For saponin presence the positive result was shown by froth formation and precipitate and later appearance of bluish green color indicate presence of tannins in the plant. The existence of phenols and tannin were also confirmed by the appearance of green or dark green colour after dissolution of extract in aqueous $FeCl_3$. The presence of terpenes and sterols were determined by change of colour from pink to purple and green to pink, respectively. Observation are shown in table 3.15.

Table 3.15 Preliminary phytochemical profile

S. No	Phytochemical Constitutes	Tests	Results
1	Carbohydrate	Molisch's test	+
		Benedict's test	+
		Fehling's test	+
2	Proteins	Ninhydrin test	+
3	Alkaloids	Mayers's test	+
		Wagner's test	+
		Hager's test	+
4	Sterols and Triterpenoids	Sakowski's test	+
5	Phenols	$FeCl_3$ test	+
6	Flavonoids	Alkaline Reagent test	+
7	Tannins	Gelatin test	+
		Ferric Chloride test	+
8	Saponins	Frothing test	+

3.1.15 Nutritional Analysis

This is the process which is used for determining the nutritional content of food stuff. A standard human nutrition is consists of water, vitamin, protein, lipid, organic and inorganic element. Human fitness always depend on balance contents in the body. Balanced mineral diet have an important role in human health, any disorder in balance results in the irregularities of human health [94]. The food information is needed to label on parcel to do trade with general public food in the form of food taking board. In the nearby years, many people have started labelling food material [95].

Table 3.16 revealed the nutritional analysis of *S. saligna* fruits, showing moisture contents 9.6%, ash 5.7%, fats 5.7%, fibers 17.07%, protein 6.2% and carbohydrates 54.93% and gross energy 19.72 cal/ 100g.

Five of the top ten causes of death in the United States have been linked to diet, by using this plant species one can get rid of it. The proteins in body responsible for making structure, tissues and antibodies that compete infection. Proteins are present in every cell of body, muscles, bones, hair, nail, hormone, enzymes, DNA and RNA. For the normal growth of foetus during pregnancy protein play an important role and secondary source of energy [96]. Fats are essential for transport of fat soluble vitamin A, D, E and K. Water for joints lubrication, eyes tissues, nose and muscle function and prevents constipation, transport of nutrients to the different cells and excretion of waste from the body. Fibre is an indigestible form of carbohydrates found in *S. saligna* fruits helps to keep normal bowel movement that reduces risk of haemorrhoids, constipation and diverticulosis and colon cancer [97].

Table 3.16 Nutritional analysis of *S. saligna* fruits

Moisture contents = B-C/D×100					
Sample	Empty dish weight	Dish + sample weight(B)	Sample weight (D)	After drying weight (C)	% Moisture contents
S. saligna	16.8282	17.9574	1.1292	17.8498	9.6
Ash contents = B-A/C×100					
Sample	Empty dish weight (A)	Dish + sample weight	Sample weight (C)	After heat dish + Ash weight (B)	% Ash
S. saligna	17.9039	18.5294	1.3255	17.9800	5.7
Fats = A-B/C×100					
Sample	Paper weight	Paper + sample weight (A)	After extraction weight (B)	Sample weight (C)	% Fats
S. saligna	0.5875	1.7200	1.4960	1.1325	6.5
Fibers $W_1 - W_2/W_0 \times 100$					
Sample	Sample weight (W0)	Cavit + dry sample (W1)	Cavit+Ash (W2)	% Fibers	
S. saligna	1.3098	31.4767	31.2531	17.07	
Proteins = (Titre×N×14×100×25/weight of the sample×1000)					
Sample	Sample weight	Titre	Blank	% Proteins	
S. saligna	1.2581	9.3	9.0	6.2	

Carbohydrates Formula = 100 - Moisture + Ash + Fibres + Fats + Proteins

Carbohydrates Formula = 100 - 9.6 + 5.7 + 6.2 + 17.07 + 6.5 + 6.2= 48.73

Gross energy (Cal/100g) = (%Fats) + (% Proteins) + (% carbohydrates) = 51.27

3.1.16 Elemental Analysis

Elemental analysis is a process where a test sample (waste, soil, water, minerals, body liquid and compound) is analysed for its elemental composition. This analysis may be qualitative or quantitative. Actually the element observation is the part of analytical chemistry, which is used for the determination of elements in a sample. This data is essential to determine the structure and pureness of any sample [97]. Minerals analysis of *S. saligna* fruits are shown in the table 3.17 and presented in figure 3.18. Na was 5.817 ppm and the concentration of Mg was determined to be 3.760 ppm. Zn contents were recorded 0.498 ppm followed by K (0.476), Ni (0.016), Cu (0.042) and Pb (0.038) ppm. The *S. saligna* fruits were also evaluated for presence of Mn and Co (0.009 ppm) each, Cd and Fe (0.008) each, found up to detection limit. Cr was not detected. Most of the other members of buxaceae such as *S. pruniformis* and *Buxus wallichiana* were also analysed for trace elements like Ni, Cu, Co and Mg [98].

Table 3.17 Elemental analysis of *S. saligna* fruits

S. No	Elements	Concentration (ppm)	S. No	Elements	Concentration (ppm)
1	Zinc	0.498	7	Copper	0.042
2	Manganese	0.009	8	Lead	0.038
3	Cobalt	0.009	9	Iron	0.008
4	Nickel	0.016	10	Magnesium	3.760
5	Cadmium	0.008	11	Potassium	0.476
6	Chromium	0.00	12	Sodium	5.817

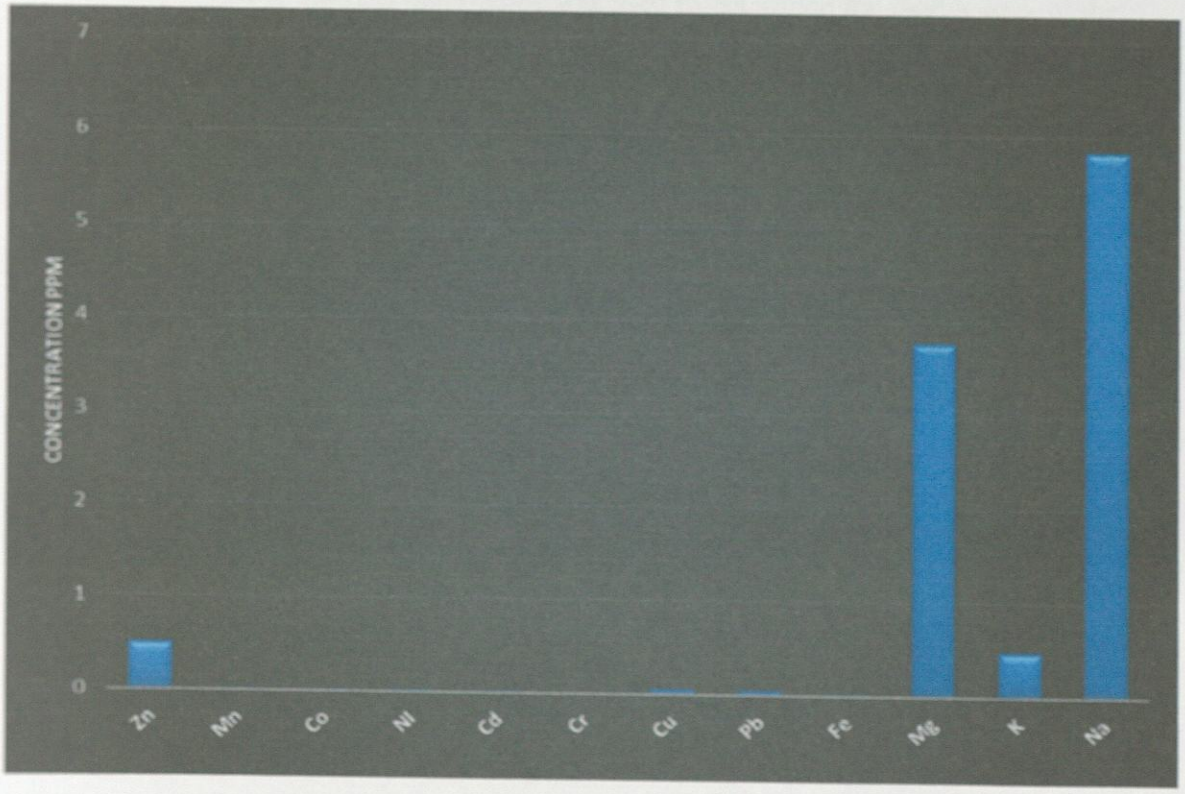


Fig. 3.16 Elemental analysis of *S. saligna* fruits

3.1.18 Scanning Electron Microscopy (SEM) Analysis

The scanning electron microscope was used to determine the morphology of the *S. saligna* fruits powder. Figures 3.17, 3.18 and 3.19 shows the SEM image of plant fruits powder. Three images were taken with three different magnification powers (X150, 500 and 1000). The image of the sample was visible more clearly upon increasing magnification power. The images showed that the *S. saligna* fruits powder showed number of masses and also individual. It was also seen that aggregate are bigger round individual shape. It was also observed that sample was not in direct contact with the aggregation, showed the presence of protein capping agent in plant sample. The SEM analysis indicate that the *S. saligna* fruits powder were spherical in shapes. The pollen morphology and the degree of viability of *Buxus sirindhornina* was first reported species of buxus family [99].

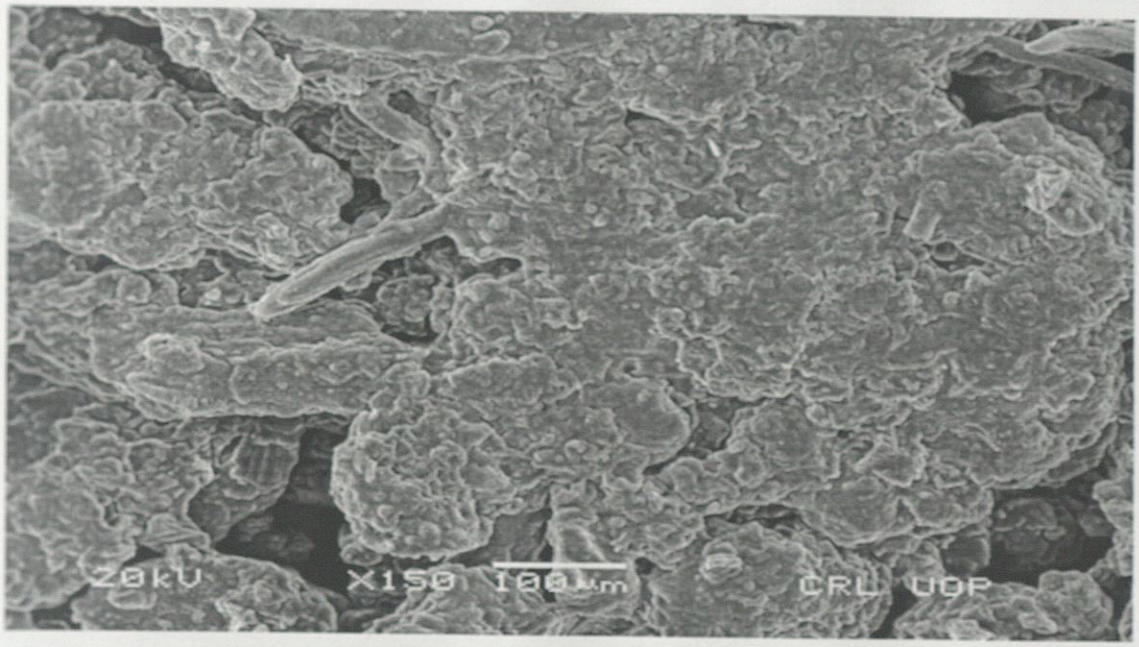


Figure 3.17 SEM micrograph of *S. saligna* fruits powder

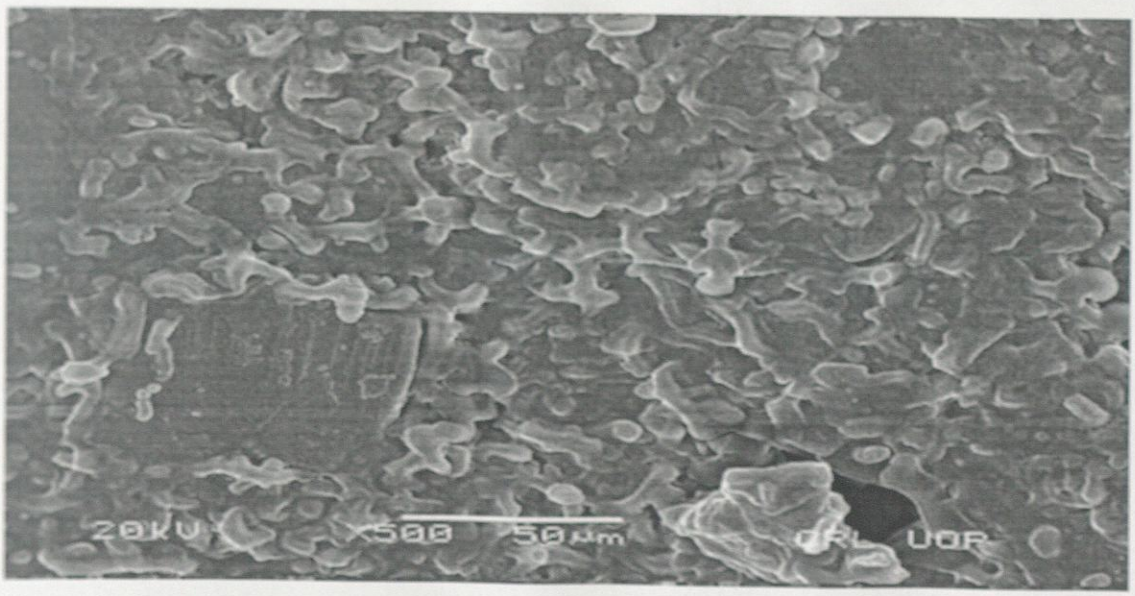


Fig 3.20 SEM micrograph of *S. saligna* fruits powder

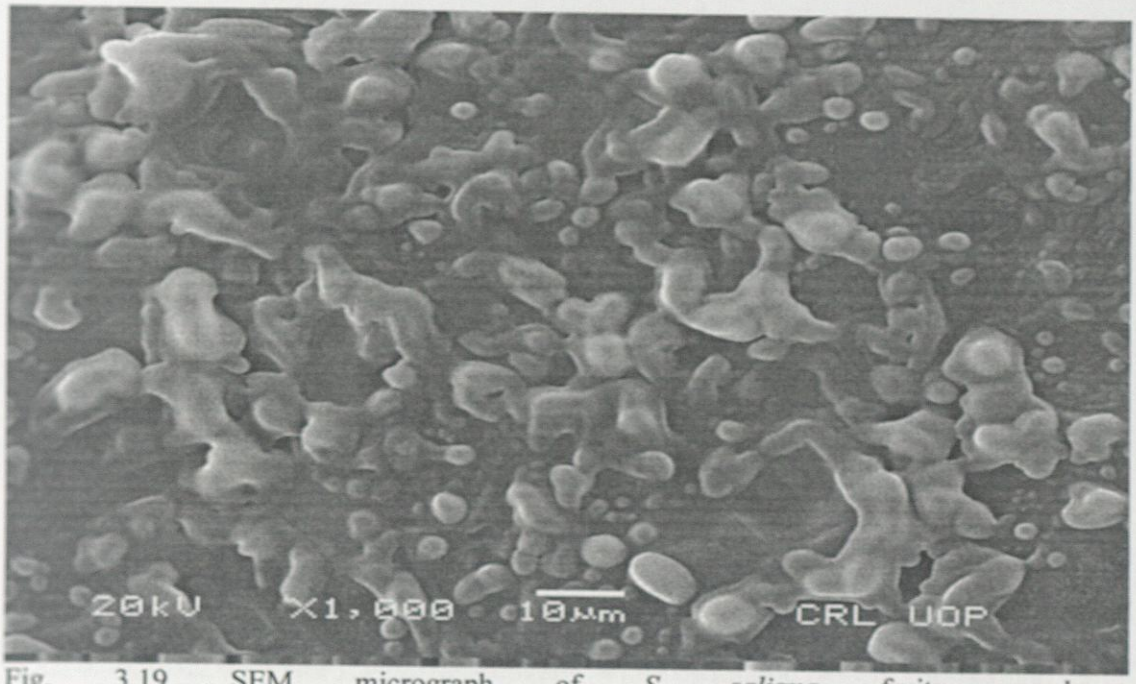


Fig. 3.19 SEM micrograph of *S. saligna* fruits powder

3.1.19 Energy Dispersive X-rays Analysis

The Energy Dispersive X-rays (EDX) technique was utilized to determine the amount as well as to identify elementary composition of *S. saligna* fruits. Anatomy and ultrastructure in cell wall and ratio of lignin concentration can be determined with EDX [100]. High signal were recorded for carbon and oxygen in EDX pattern of spectrum. Elemental composition of *S. saligna* fruits showed that carbon, oxygen other elements such as Mg, P, S, K, Cl and Ca were also detected. The counts of X-ray is showing on vertical while energy (keV) on the parallel axis. Figure 3.20 and 3.21 shows the elemental composition of plant extract. The carbon composition revealed that by weight it was 64.84 and 72.47%. Similarly O, Mg, P, S, Cl, K and Ca by weight were 30.86, 0.32, 0.56, 0.52, 0.40 and 1.54%, respectively. While by atomic percent composition the O, Mg, P, S, Cl, K and Ca were 25.89, 0.18, 0.24, 0.22, 0.15, 0.53 and 0.32%, respectively. *Buxus sempervirens* analyse an obsidian surface of about 0.5 mm, which were determined from polished sections by energy-dispersive X-ray [101].

Table 3.18 Percent atomic weights of the elements present in *S. saligna* fruits

Element (*K)	Weight%	Atomic%
C	64.84	72.47
O	30.86	25.89
Mg	0.32	0.18
P	0.56	0.24
S	0.52	0.22
Cl	0.40	0.15
K	1.54	0.53
Ca	0.96	0.32

*K alfa radiation are used for XRD which emitted from Cu element.

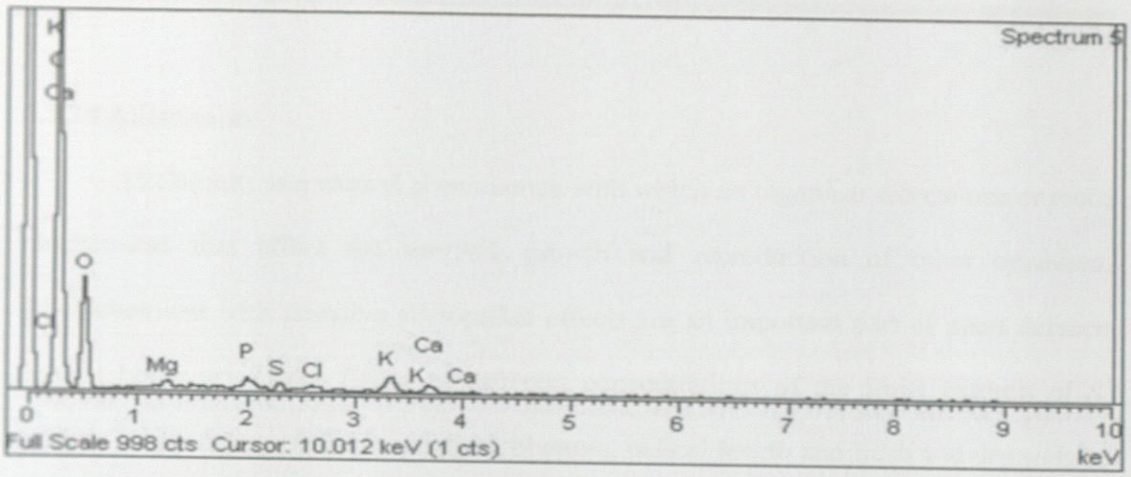


Fig 3. 20 Energy Dispersive X-Rays spectrum for the fruits of *S. saligna* fruits



Fig 3. 21 Percent weight of the elements in *S. saligna* fruits powder

3.1.20 Allelopathy

Allelopathy is a natural phenomenon with which an organism secrete one or more biochemical that effect the survival, growth and reproduction of other organism. Allelochemical with negative allelopathic effects are an important part of plant defence against herbivory [102]. Effect of different concentrations of the fruits extracts of *S. saligna* on percent germination, radical, plumule, radical length and fresh and dry weight, moisture contents of *T. aestivum* seeds was determine. The *S. saligna* fruits extract significantly inhibited the germination of wheat seeds. The extract and concentration interaction was not significant. Concentration for percent germination showed decreased trend and analysis of variance (ANOVA) showed that the maximum germination of *T. aestivum* was recorded in control (60%), the percent germination of *T. aestivum* seeds recorded for extract was 56.67%. The extract reduced the plumule length of wheat seedlings. The concentrations was highly significant while extracts and concentrations interaction was non-significant as shown in table 3.19. ANOVA revealed that maximum plumule growth was reported from control. Aqueous extract induce the growth of plumule at different concentrations and significantly reduced the radical length of wheat seedlings. The test sample significantly reduced fresh weight and dry weight of wheat as shown in table 3.19. The allelochemicals were extracted from the leaves of *Buxus sinica* and *Buxus ichangensis* [102]. *Buxus sempervirens* (Family Buxaceae) showed significant alkaloid-mediated allelopathy against higher plants in ecosystems [103].

Table.3.19 The effect of the aqueous fraction of *S. saligna* fruits on *T. aestivum* seed

Replicates	Germination %	Mean Radicles length (mm)	Mean Plumule length(mm)	Fresh weight (g)	Dry weight(g)	Moisture Content
R1	50	0.16	0.24	0.3	0.25	20.00
R2	60	0.13	0.21	0.32	0.22	45.45
R3	60	0.13	0.18	0.31	0.26	19.23
Average	56.67	0.14	0.21	0.31	0.24	28.22

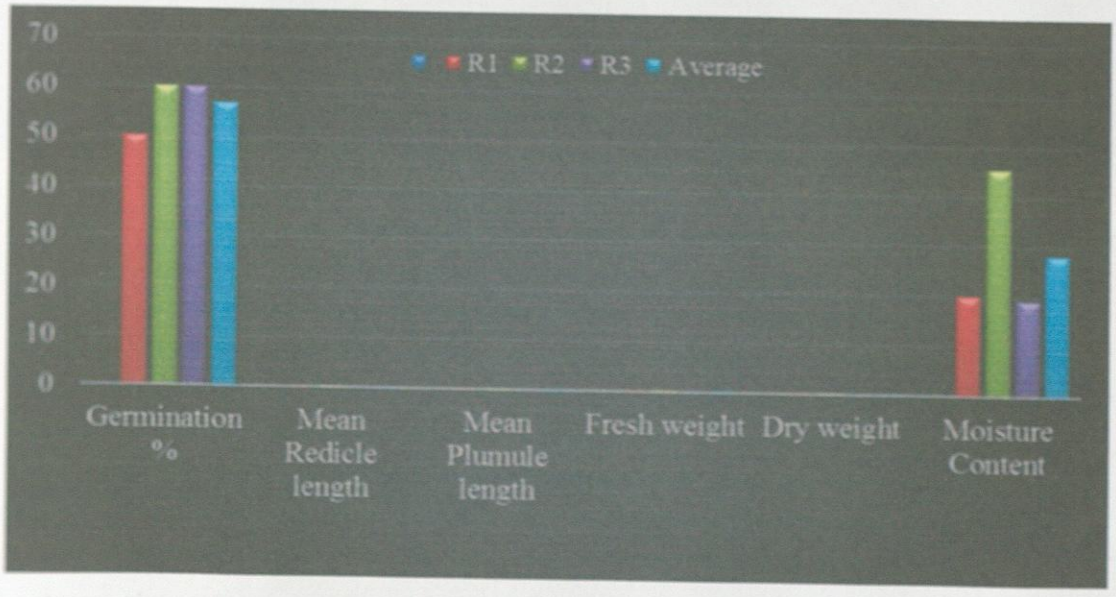


Fig 3.22 Effect of the aqueous fraction of *S. saligna* fruits on *T. aestivum* seed

3.1.21 Gas chromatography-mass spectrometry (GC-MS)

Most of the food industries are in search of new methods that stop the growth of microbes instead stabilizers. One of the new approach of the modern world is to get the essential oils from plants source. Because of the antifungal, antioxidant and antibacterial activities essential oils are gaining popularity in scientific and industrial research and can be used in foods as natural flavours. Fungi causes major lose in food production. For example *Aspergillus species* causes spoilage of mangoes while Fusarium spp. causes spoilage during food production. The aflatoxins of *A. flavus* and *A. parasiticus* pollute corn, peanuts, cotton seed and tree nuts in reaping [104]. Barley and wheat is commonly effected by *Fusarium,* head scar caused by *F. oxysporum*. This result in economic losses i.e. decrease of ounce quality and crop. The kernels polluted by the mycotoxin have low market rate [105-106]. *P. xylostella* (Plutellidea: Lepidoptera) is a universal disastrous herbal organism that causes infection for several groups in a year. The Samson an active components isolated from agitated product of *P. cicadae* (Miquel) play an important role in environmental and ecological defence against the pest. In recent years many medicinal activities have been reported for *P. cicadae* [107]. Because of the therapeutic importance of essential oils, the *n*-hexane fraction of the *S. saligna* was subjected to column chromatography for the extraction of essential oils. The GC-MS analysis revealed that these oils contain following 22 components.

The oil sample contains Palmitic acid (3.08), Stearic acid (1.42), linoleic acid (1.18) and oleic acid (5.18) in highest concentration. Hexanoic acid, Caprylic acid, ,lauric acid, Meristic acid, Margaric acid, Stearic acid, Elaidic acid, linoleic acid and Arachidic acid are also present in lowest concentration as given in the table 3.20.

Table 3.20 Essential oil of *S. saligna* fruits analysed by GC-MS

S.no	Name	R. Time	Area	Conc. (%)
1.	Palmitic acid	14.62	20068	0.04
2.	Stearic acid	19.634	4789	0.01
3.	Oleic acid	20.158	5136	0.05
4.	Linoleic acid	21.351	5124	0.05
5.	Octadecanoic acid	21.73	1515	0.01
6.	Hexanoic acid	3.034	75790	0.62
7.	Caprylic acid	4.936	17157	0.08
8.	Capric acid	6.779	10591	0.04
9.	Undecanoic acid	7.669	1436	0.00
10.	Lauric acid	8.530	17612	0.06
11.	Myristic acid	10.962	11949	0.04
12.	Pentadecanoic acid	12.626	3584	0.01
13.	Palmatic acid	14.645	1145295	3.08
14.	Margaric acid	16.940	6183	0.02
15.	Stearic acid	19.657	448383	1.42
16.	Oleic acid	20.209	433502	5.18
17.	Elaidic acid	20.419	10299	0.15
18.	Linoleic acid	21.380	97362	1.18
19.	Octa decadienoic acid	21.749	12288	0.14
20.	Arachidic acid	27.238	37350	0.12
21.	Eicosadienoic acid	29.030	22183	0.36
22.	Bhenic acid	34.380	23094	0.08
23.	Tetracosanoic acid	40.734	21193	0.07



ASTRAGALUS ANISACANTHUS

4.1 FABACEAE (FAMILY)

4.1.1 Description

Among the flowering plants fabaceae is the 3rd main family, it contains 731 genera and 18,400 species. This family consist herb and shrubs or trees. Simple or usually compound leaves, frequently three foliate, occasionally end in a stem. Papilionate flowers, regularly erect, adaxial petal, two adjacent petal (wings) and two inferior petals usually +connate by their inferior borders (keel); standard outer. Sepals five, + connate into a tube. Stamens ten, one free and nine fused (diadelphous) or all fused (monadelphous) or rarely all free. Fruit usually dehiscent. Seeds often large; endosperm or very scanty [108].

4.1.2 Distribution

The members of fabaceae are distributed both in tropical and temperate areas. In Pakistan, it is represented by 90 genera and 435 species [107].

4.1.3 Importance

The Fabaceae, also called Leguminoceae family. Several plants of this family are agriculturally and economically important. Legumes are collected as crops for human being and animal intake as well as for oils, fuel, fiber, timber, fertilizers, medicines, chemicals and as agricultural crops and vegetables [108]. Several member of the family like barrel medic (*Medicago truncatula*), pea (*P. sativum*) are studied as genetic model systems [108].

For 1000 of years man and animal both have been using the legume. In North and South America (more than three thousand years) legumes have been used as a source of food and the roman used it for soil improvement. Febaceae family account for 27 % of the world's chief crop production as a vital food source not only for humans but also for farm animals [109]. For poultry and animal protein industries,

legumes are a rich source of nutritional protein and 34% of the dietary protein "Nitrogen" need of humans are achieved by the grain pulses. It is also responsible for atmospheric nitrogen fixation. They form mutual link with the soil rhizobia and fixing inorganic nitrogen for the soil fertility [110]. Industries using legumes for production of oils, biodegradable oil, dyes and biodiesel and also play an important role in the field of pharmaceutic. Legume are a common source of flavones and phytoestrogens use to reduce risk of cancer and postmenopausal hormone replacement therapy. Legumes are recommended food for diabetics [111].

Chapter 4

4.2 Astragalus (Genus)

4.2.1 Description

The common name given to this genus *Astragalus* is Bunge. The member of *Astragalus* can survive under extreme condition. Most of the member of febaceae family are herbs and shrub. Leaves are paripinate or imparipinate, uncommonly trifoliate or unifoliate, stipules absent. Inflorescence racemose, spicat, peduncle or axillary. Flowers bracteoles are absent or present, sessile, red or violet and dark yellow. Sepals are tubular and teeth sub equal. Vexillum oval, narrower end, rectangle, round ends or sometimes appendiculate. Keel and wings generally with long claws. One stamen is free and nine are fused, ovary is sessile, style and stigma generally terminal. Fruits are sessile with intrusive membrane, seed often reniform [112].

4.2.2 Distribution

Astragalus is one of the largest Angiospermous genera with about 1600 species widely distributed in the old and the New World. In Pakistan it is represented by about 135 species. The genus is mainly distributed in the mild heat and alpine fields of the Himalayas at heights ranging from 3000-6000m. Distribution in tropical and subtropical regions of Punjab and Hryanaa region. The greatest point being different of the species lies in the dry cold deserts of Leh and Ladakh (Jammu and Kashmir) and Lahul spiti (Himachal pradesh) in the North-West Himalaya. On the other hands, the more moist and humid East Himalaya harbors only 16 Species of *Astragalus* [113].

4.2.3 Medicinal Importance

This genus is of slight importance for economics point of view. Some members of *Astragalus* are the source of gum production. The stem and roots of *A.*

gummifer, *A. microcephalus* and many other Asiatic species of *Astragalus*. *A. verus* are used for water soluble gum production, it is the gum producing species in India, and however, there is no evidence in recent time showing commercial utilization of this species. The maximum amount of gum produce by Iran, which is used for pharmaceutical industries as well as in different food industries [114]. Bunge is a familiar drug as "Rudravanti" used for T.B, skin infection, cold and blood purifier. Since Asian times this drug is used in India. In 2006 the survey conducted in Gangotri gaumak showed that saint of local people of this region take aerial parts of plants for making paste and soaking in water for 30 or 60 days during the plants season for purifying the blood. The paste of *Astragalus* also used for sugar patient to reduce sugar level in blood [115]. The current research was carried out on this species which shows potential for its anti-diabetic activitiy. Usually *A. candolleanus* is known as 'Rudanti' or 'Rudravanti' in Indian literature. However, during field survey it was noticed that in Gangotri gaumukh region *A. malacophyllus* is called 'Rudanti' locally. Some of the species like *A. multiceps, A. ribuloides, A. chlorostachys* and *A. himalayanus* are also used therapeutically for many purposes in ethnobotanical uses [116]. However, no reliable information are presented. Since the species of *Astragalus* produce with shallow root system and horizontal branch, they protect the soil from erosion and also act as ground cover. Some of the species are also used as forage. *Astragalus* root is a well-known drug in traditional Chinese medicine since quite a long time. It is ethnobotanicaly used as a stimulant and for treatment of diabetes and nephrites [117].

Roots are used in treating tumors of eyes, liver, throat, chest and back pain. They are also used for tissues regeneration and wound healing. The Indian women chewed the root to stimulate lactation. *Astragalus* root is also used in herbal

medicines in the US to enhance the immune system of the body after cancer therapy treatment. The rhizoids are also used for "human immunodeficiency virus" (HIV). In china the cooked seeds used for coffee in China [118]. A large number of *Astragalus* species poisonous both for livestock (cattle and sheep) and environment in the North America. The poisonous species of *Astragalus* have been placed in 3 groups.

1. **Nitro toxin producing species:**

   Majority of the species produce 3-nitro propanol which disturb the function of CNS which cause paralysis.

2. **Swains nine producing species:**

   Some species produce alkaloid swains nine or that stops cellular enzyme production that eventually lead to reduce the weight.

3. **Selenium storing species:**

   Some species store Se from soil and act as Se indicator [119]. It may be move in the milk. Upon feeding, the animals produce a different kind of actions which is described as "crazy". Due to this *Astragalus* is also known as "Locoweed". The green plants are more toxic than dried. But Se content is not affected by drying. It has been observed that animals may become habitual to *Astragali* even when other fodder are available [120].

4.3 ASTRAGALUS ANISACANTHUS (PLANT)

Family: Leguminosae

Sub family: Papilionoideae

Genus: Astragalus

Species: *Anisacanthus*

Botanical Name: *Astragalus anisacanthus*

Astragalus anisacanthus belongs to the subfamily Papilionoideae in the family Leguminoceae considered a remarkable example of adaptive radiation. The stem of this species erect and covered with hairs. Leaves are peripinnate compound and leaflets are broad. Stipules are long and pubescent. Axillary raceme type of inflorescenc, peduncle. Calyx gets enlarge in fruits. Fruit 5-6 mm long, pubescent, unilocular, 2-seeded [122]

4.3.1 Distribution

Distribution: Pakistan (Baluchistan, KPK); Afghanistan and Persia. The genus *Astragalus* has about 1000 species [123].

4.3.2 Importance

This genus is used for curing skin problems, cough, cold, fever, throat infection, joint, abdominal disorders, respiratory infection, body pain and gynecological disorders [124]. Economic importance of this genus is not of considerable importance, but some of its species in Iran and China are especially famous for marketable gum tragacanth making. In India, *A. candollenus* is a familiar drug as "Rudanti" used for tuber-closis, coughs and as blood purifier [125].

4.4 Phytochemistry

Literature reports terpenes, diterpenes, oils, steroids, iridoids, lignin and stilbene derivities from the genus Astragulus. The earlier phytochemical studies on this genus are shown in the following table 4.1. Flavenoids, saponin and polysaccharides are the principle active components of Astragalus [126]. It also includes constituents such as anthraquinones, amino acid, alkaloids, β-sitosterol and metallic elements. Just like many other herbs, Astragalus genus plants are also a rich source of flavonoids.

Table 4.1 Phytochemical constituents from genus Astragulus

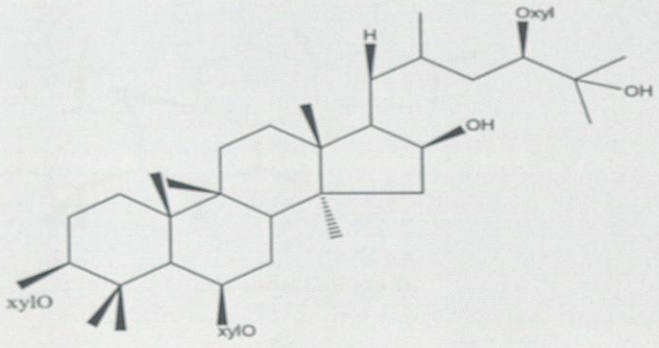
S. No	Compound's Name	Species Resource	Parts Used	Reference
1	*β* - D-glucuronopyranosyl	*A. Erinaceus*	Whole plant	[127]
2	*β* - D-glucopyranosyl	*A. Erinaceus*	Whole plant	[127]
3	Hareftoside A	*A. Hareftae*	Whole plant	[129]
4	Hareftoside B	*A. Hareftae*	Whole plant	[129]
5	Cycloquivinoside A	*A. Hareftae*	Whole plant	[129]
6	Cyclocanthogenin	*A. Chivensis*	Aerial parts	[130]
7	Cyclocanthoside E	*A. Caucasicus*	Leaves	[131]
8	Cyclochivinoside B	*A. Chivensis*	Aerial parts	[130]
9	Cyclochivinoside C	*A. Chivensis*	Aerial parts	[132]
10	Caspicuside I	*A. Caspicus*	Roots	[133]
11	Oleifoliosides A	*A. Oleifolius*	Lower stem	[134]
12	Oleifoliosides B	*A. Oleifolius*	Lower stem	[134]
13	Cicerosides A	*A. Cicer*	Aerial parts	[135]
14	Cicerosides B	*A. Cicer*	Aerial parts	[135]
15	Cycloascidoside	*A. Ernestii*	Roots	[136]
16	Eremophiloside A	*A. Eremophilus*	Aerial parts	[137]

17	Eremophiloside B	*A. Eremophilus*	Aerial parts	[137]
18	Cycloascidoside A	*A. Mucidus*	Aerial parts	[138]
19	Cyclounifoliside C	*A. Unifoliolatus*	Epigeal parts	[139]
20	3-*O*-[α-L-Arabino-pyranosyl(1→2)-β-enosyl] - 3β, 6α, 16β, 20(*S*), 24(*R*), 25 – hexahdroxycycloartane	*A. Stereocalyx*	Roots	[140]
21	Cyclomacrogenin B	*A. Macropus*	Root	[141, 142]
22	Cyclomacroside E	*A. Macropus*	Root	[143]
23	Cyclomacroside B	*A. Macropus*	Root	[143]
24	Cyclomacroside D	*A. Macropus*	Root	[142]
25	Mongholicoside B	*A. membrance*	Root	[144]
26	Mongholicoside A	*A.membrance*	Root	[144]
27	Askendoside K	*A. taschkendicus*	Root	[145]
28	Askendoside H	*A. taschkendicus*	Root	[146]
29	Cycloorbicoside D	*A. orbiculatus*	Aerial parts	[147]
30	Cycloorbigenin C	*A. eremophilus*	Roots	[146, 147]
31	Eremophiloside D	*A. eremophilus*	Aerial parts	[139]
32	Bicusposide F	*A. bicuspis*	Whole plant	[147]
33	Bicusposide F	*A. bicuspis*	Whole plant	[147]
40	Kahiricoside II	*A. kahiricus*	Aerial parts	[148]
41	Kahiricoside III	*A. kahiricus*	Aerial parts	[148]
42	Kahiricoside IV	*A. kahiricus*	Aerial parts	[148]
43	Kahiricoside V	*A. kahiricus*	Aerial parts	[148]
44	Secomacrogenin B	*A. macropus*	Aerial parts	[149]
45	Orbicoside	*A. orbiculatus*	Aerial parts	[146]
46	Orbigenin	*A. orbiculatus*	Aerial parts	[147]
47	Cyclosiversioside F	*A. oldenburgii*	Aerial parts	[150]
48	Astramembranosides A	*A. membarnaceus*	Roots	[148]
49	Astraverrucin IV	*A. oldenburgii*	Aerial parts	[150]
50	Astragaloside VII	*A. oldenburgii*	Aerial parts	[150]

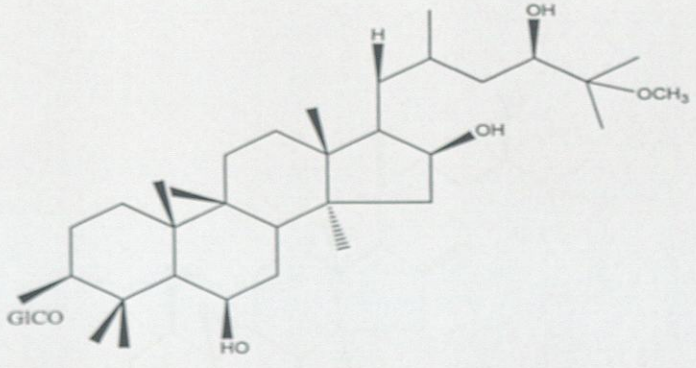
51	CyclolehmanosideC	*A. lehmannianus*	Aerial parts	[151]
52	Acetylastragalosid I	*A. baibutensis*	Roots	[152]
53	Astragaloside III	*A. illyricus*	Roots	[153]
54	Cyclounifolioside B	*A. illyricus*	Roots	[153]
56	Astraverrucin I	*A. illyricus*	Roots	[153]
57	Cycloorbigenin	*A. orbiculatus*	Roots	[147]
58	Cycloorbigenin B	*A. orbiculatus*	Roots	[147]
59	Cycloorbicoside A	*A. orbiculatus*	Roots	[147]
60	Cycloorbicoside C	*A. orbiculatus*	Roots	[147]
60	Cycloorbicoside G	*A. orbiculatus*	Aerial parts	[147]
61	Tomentoside I	*A. tomentosus*	Aerial parts	[154]
62	Deacetyltomentoside I	*A. tomentosus*	Aerial parts	[154]
63	Cycloorbicoside G	*A. tomentosus*	Aerial parts	[154]
64	Tomentoside III	*A. tomentosus*	Aerial parts	[154]
65	Tomentoside IV	*A. tomentosus*	Aerial parts	[154]
66	Huangqiyenin E	*A. membranaceus*	leaves	[155]
67	Huangqiyenin III	*A. membranaceus*	leaves	[155]
68	Huangqiyenin F	*A. membranaceus*	leaves	[155]
69	Huangqiyenin IV	*A. membranaceus*	leaves	[155]
70	Trideacetylhuangqiyegenin III	*A. membranaceus*	leaves	[155]
71	Narcissin	*A. cruciatus*	Aerial parts and root	[156]
72	Nicotiflorin	*A. verrucosus*	Aerial parts	[156]
73	Kaempferol	*A. cruciatus*	Aerial parts	[156]
74	Microcephalin I	*A. microcephalus*	leaves	[157]
75	Microcephalin II	*A. microcephalus*	leaves	[157]
76	Kaempferol-3-*O*-α-L-rhamnoxyloside	*A. microcephalus*	leaves	[157]
77	Quercetin	*A. asper*	Aerial parts	[158]
78	Quercimeritrin	*A. asper*	Aerial parts	[158]
79	Rutin	*A. asper*	Aerial parts	[159]

80	Kaempferol	*A. asper*	Aerial parts	[159]
81	Soyasapogenol B	*A. caprinus*	Whole plant	[160]
82	Astragaloside VIII	*A. flavescens*	Root	[161]
83	Quercetin-3-*O*-galactoside	*A. corniculatus*	Aerial parts	[162]
84	Flagaloside C	*A. galegiformis*	Leaves	[163]
85	Apigenin	*A. verrucosus*	Aerial parts	[164]
86	Luteolin	*A. bombycinus*	Roots	[164]
87	Salvigenin	*A. propinquus*	Roots	[169]
88	Apigenin-7-*O*-β-D-glucopyranoside	*A. bombycinus*	Whole plant	[165]
89	Calycosin	*A.membranaceus*	Roots	[166]
90	Ononin	*A.membranaceus*	Roots	[166]
91	Formononetin	*A.membranaceus*	Roots	[166]
92	Daidzen	*A. bombycinus*	Whole plant	[148]
93	Isomucronulatol	*A.membranaceus*	Root	[167]

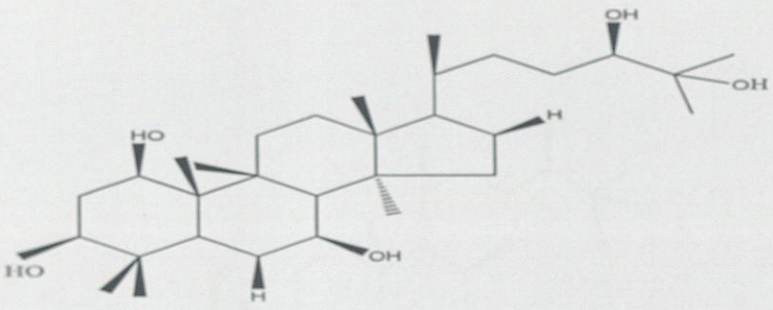
The structures of some important compounds obtained from the *Astragalus* genus



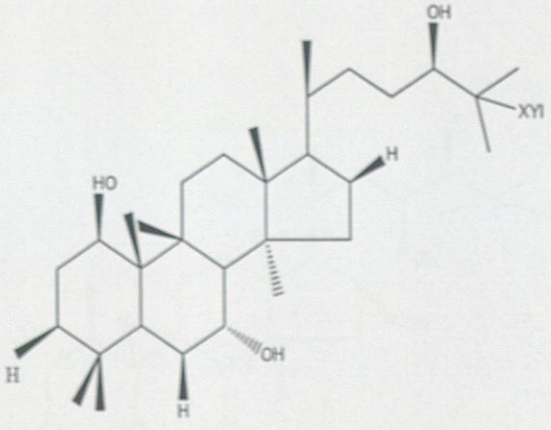
3 Hareftoside A



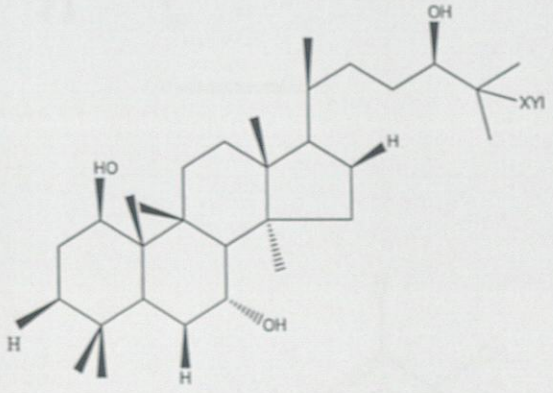
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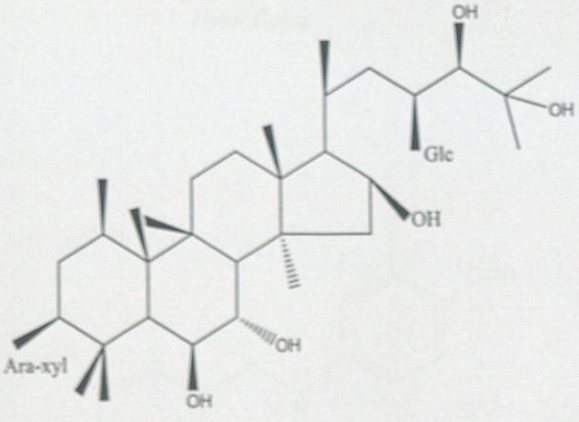
Cyclomacrogenin B



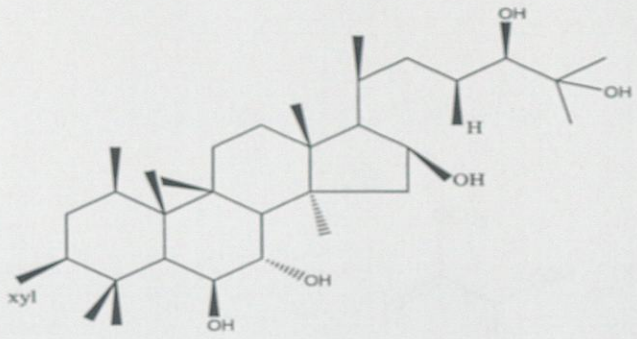
Cyclomacrogenin E



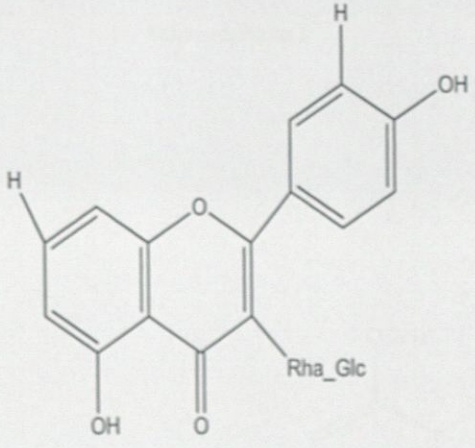
Mongholicoside A



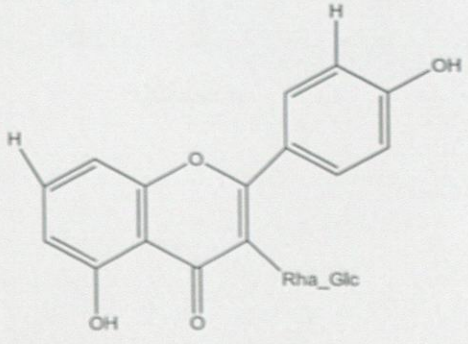
Askendoside H



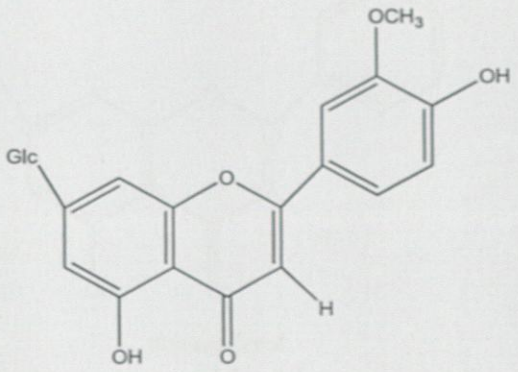
Cycloorbicoside D



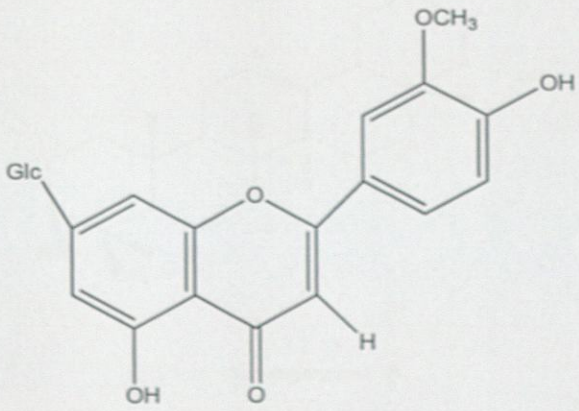
Nicotiflorin



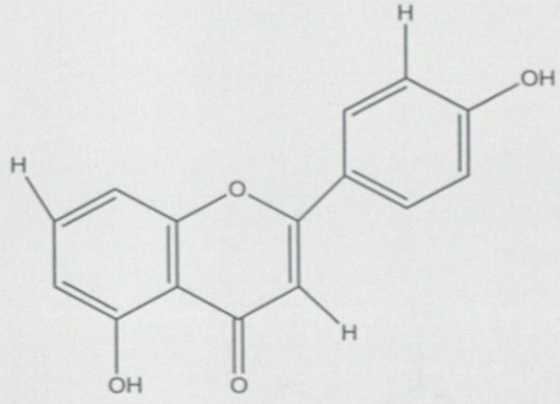
Nicotiflorin



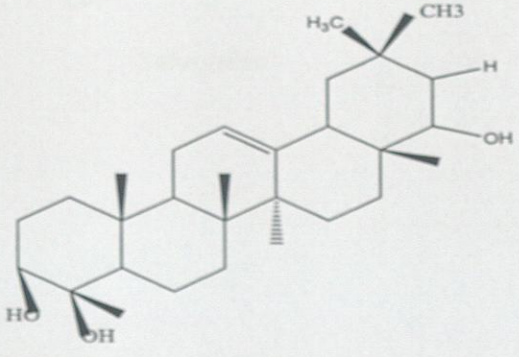
Microcephalin I



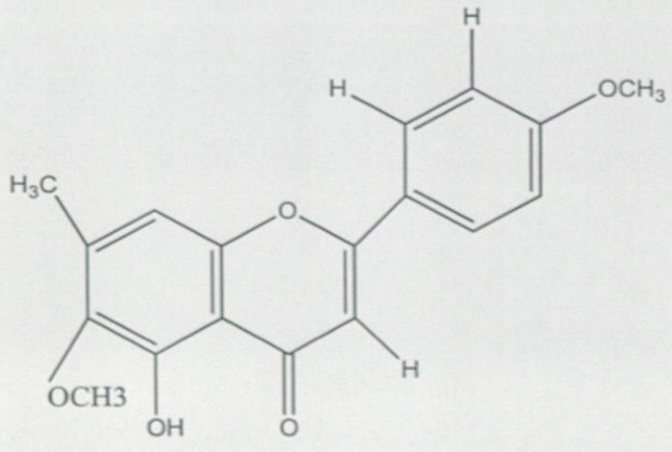
Quercetin



Kaempferol



Soyasapogenol B



Salvigenin

Chapter 5

2.1 GENRAL EXPERIMENTAL CONDITION

The experimental conditions followed for ***Part-B*** were same as discussed in ***Part-A*** section 2.2.

2.2 Phytochemical Investigation

2.2.1 Plant material

A. anisacanthus plant was collected from Hazara division, in the month of June 2013 and identified by Prof. Dr. Farrukh Hussain, plant taxonomist, Department of Botany; University of Peshawar, KPK.

2.2.2 Extraction

The collected aerial parts of *A. anisacanthus* were shade dried, cut into pieces of small size and ground to fine powdered material, using an electrical grinder. The powdered materials (7.6Kg) was soaked in commercial grade MeOH for 15 days, twice at room temperature, with occasional shaking. After 15 days the soluble material were filtered and the filtrate was concentrated at 40°C under vacume by using a rotary evaporator. Crd. MeOH. Ext. (800g) was obtained.

2.2.3 Fractionation

The Crd. MeOH Ext. (750g) of *A. anisacanthus* was suspended in distilled water (400ml) and partitioned with *n*-hexane (3x400ml), $CHCl_3$ (3x400ml), EtOAc (3x400ml) and Acetone (3x400ml) to yield *n*-hexane (50g), $CHCl_3$ (45g), EtOAc (55g), Acetone (190g) and aqueous (210g) fractions. About 50g of Crd. MeOH. Ext. was store for other biological/ pharmacological activities.

5.1 GENRAL EXPERIMENTAL CONDITION

The experimental conditions followed for ***Part-B*** were same as discussed in ***Part-A*** section 2.2.

5.2 Phytochemical Investigation

5.2.1 Plant material

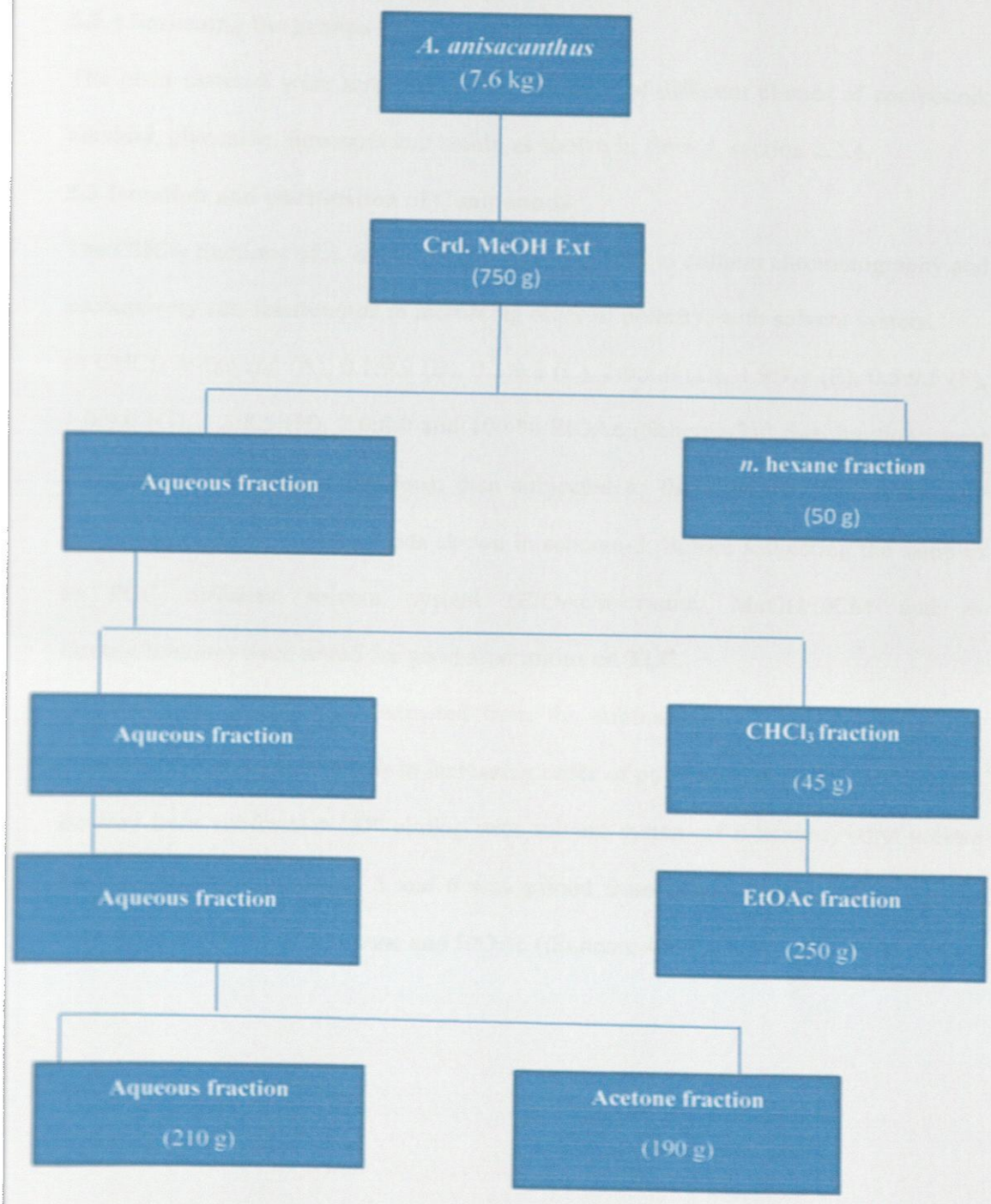
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5.2.3 Fractionation

The Crd. MeOH Ext. (750g) of *A. anisacanthus* was suspended in distilled water (400ml) and partitioned with *n*-hexane (3x400ml), $CHCl_3$ (3x400ml), EtOAc (3x400ml) and Acetone (3x400ml) to yield *n*-hexane (50g), $CHCl_3$ (45g), EtOAc (255g), Acetone (190g) and aqueous (210g) fractions. About 50g of Crd. MeOH. Ext. was store for other biological/ pharmacological activities.



Scheme 2: Extraction and fractionation of *A. anisacanthus*

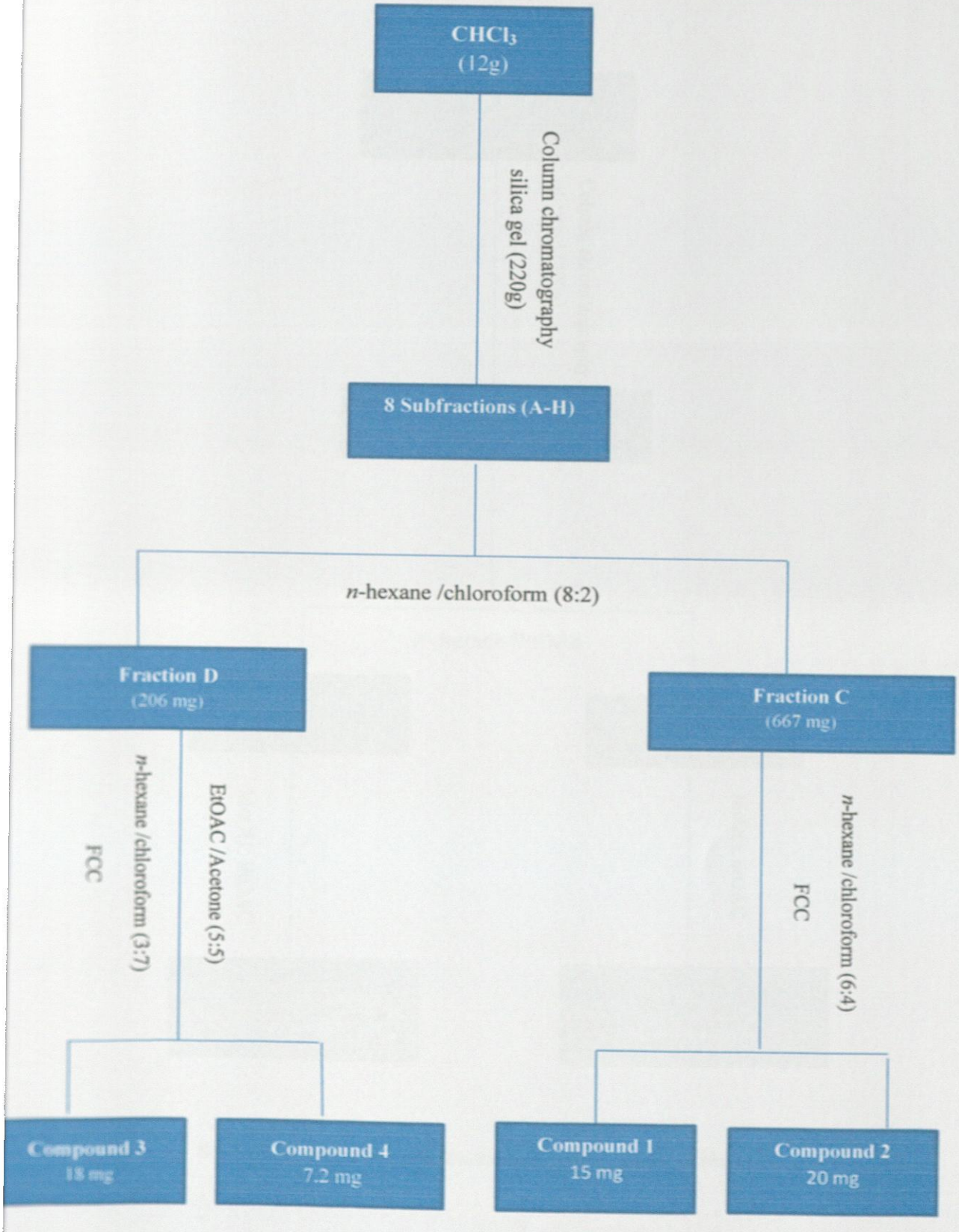
5.2.4 Screening for groups of Compound

The plant material were screened for the presence of different classes of compound; alkaloid, glycoside, flavonoid and tannin as shown in *Part-A*, section 2.2.4.

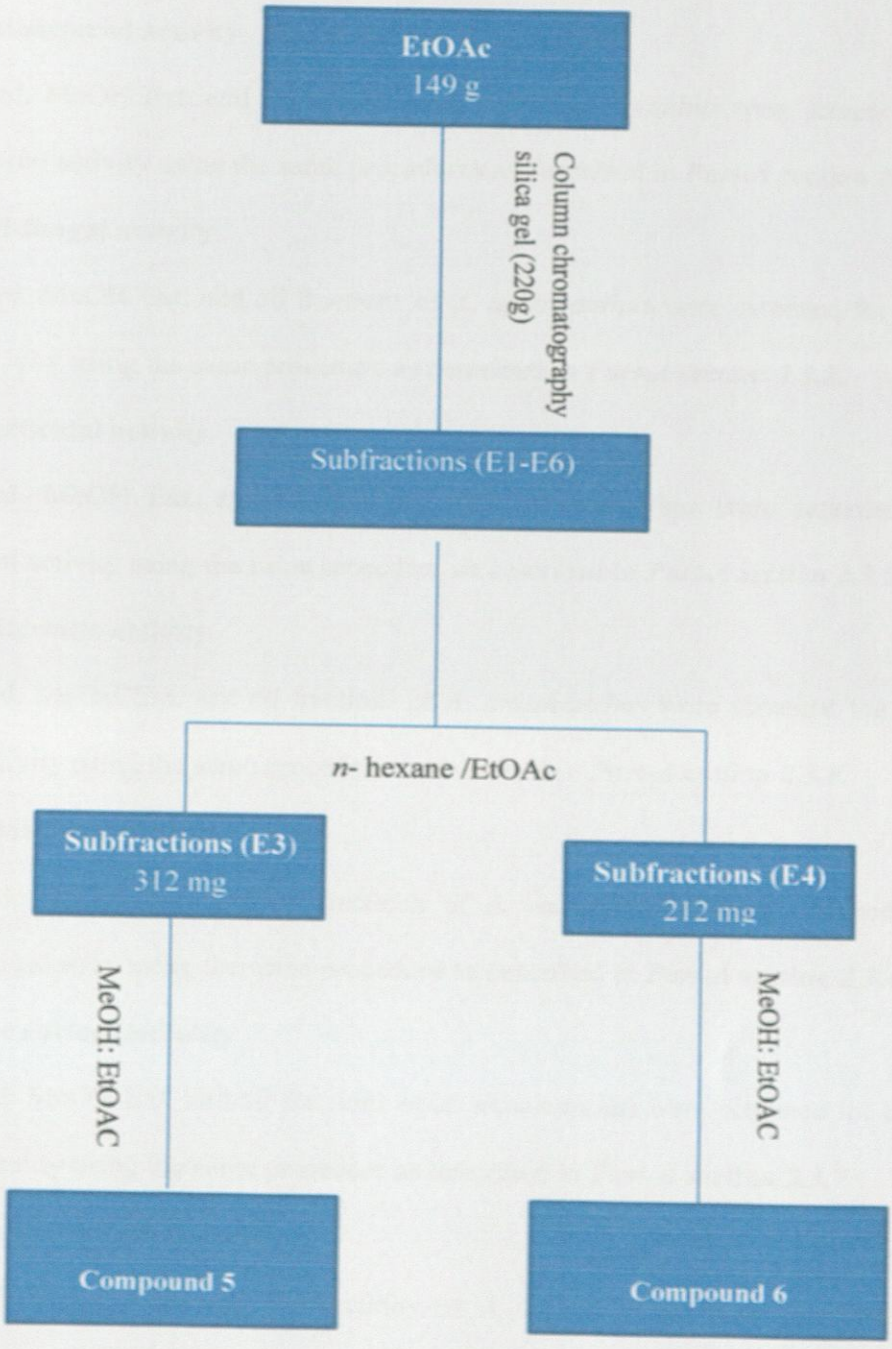
5.3 Isolation and purification of Compounds

The $CHCl_3$ fractions of *A. anisacanthus* was subjected to column chromatography and successively sub-fractionated in increasing order of polarity, with solvent system. of $CHCl_3$ /*n*-hexane (A), 0.1:9.9 (B), 0.2:9.8 (C), 2.0:8.0 (D), 1.9:7.9 (E), 0.5:9.5 (F), 1.0:9.0 (G), 1.5:8.5 (H), 2.0:8.0 and 100 % EtOAc (Scheme-3). Sub fractions were selected from the above fractions, then subjected to flash column chromatography (FCC) and isolate the compounds shown in scheme-2. Before subjecting the samples to FCC different solvent system (EtOAc/n-hexane, MeOH/DCM and *n*-hexane/acetone) were tested for good separations on TLC.

Compounds 1 and 2 were extracted from the subfraction "C" by eluting solvent system of *n*-hexane and $CHCl_3$ in increasing order of polarity. Compound 3 and 4 was isolated from subfraction "D" eluting with solvent system of *n*-hexane, ethyl acetate and chloroform. Compound 5 and 6 was gained from fraction ethyl acetate eluting with solvent system of *n*-hexane and EtOAc ((Scheme-4).



Scheme 3: Extraction and fractionation of *A. anisacanthus*



Scheme 4: Extraction and fractionation of *A. anisacanthus*

5.4 PHARMACOLOGICAL / BIOLOGICAL INVESTIGATIONS

5.4.1 Antibacterial activity

Crd. MeOH Ext. and various fractions of *A. anisacanthus* were screened for anti-bacterial activity using the same procedures as described in ***Part-A* section *2.3.1.***

5.4.2 Anti-fungal activity

Crd. MeOH Ext. and all fractions of *A. anisacanthus* were screened for anti-fungal activity using the same procedure as described in ***Part-A section 2.3.2.***

5.4.3 Insecticidal activity

Crd. MeOH Ext. and all fractions of *A. anisacanthus* were screened for insecticidal activity using the same procedure as described in ***Part-A section 2.3.5.***

5.4.4 Antitermite activity

Crd. MeOH Ext. and all fractions of *A. anisacanthus* were screened for anti-termite activity using the same procedure as described in ***Part-A section 2.3.8.***

5.4.5 Phytotoxic activity

Crd. MeOH Ext. and all fractions of *A. anisacanthus* were screened for phytotoxic bioassay using the same procedure as described in ***Part-A* section *2.3.4.***

5.4.6 Brine shrimp lethality

Crd. MeOH Ext. and all fractions of *A. anisacanthus* were screened for brine shrimp lethality using the same procedure as described in ***Part-A* section *2.3.7.***

5.4.7 Heamagglutination activity

Crd. MeOH Ext. and all fractions of *A. anisacanthus* were screened for heamagglutination activity using the same procedure as described in ***Part-A* section *2.3.3.***

5.4.8 Diphenyl Picryl Hydrazine (DPPH) radical scavenging activity

Crd. MeOH Ext. and all fractions of *A. anisacanthus* were screened for nitric oxide free radical scavenging activity using the same procedure as described in ***Part-A* section *2.3.6.***

In-vivo Pharmacological activities

5.4.9 Acute toxicity

Crd. MeOH Ext. of *A. anisacanthus* was screened for acute toxicity using the same the procedure as described in ***Part-A section 2.3.9.***

5.4.10 Analgesic activity

Crd. MeOH Ext. of *A. anisacanthus* were screened for analgesic activity using the same procedure as described in ***Part-A section 2.3.10.***

5.4.11 Locomotor activity

Crd. MeOH Ext. of *A. anisacanthus* was screened for locomotor activity using the same procedure as described in ***Part-A section 2.3.11.***

5.4.12 Antidepressant activity

Crd. MeOH Ext. of *A. anisacanthus* was screened for antidepressant activity using the same procedure as described in ***Part-A section 2.3.12.***

5.4.13 Gastro-Intestinal Tract (GIT) Motility

Crd. MeOH Ext. of *A. anisacanthus* was screened for GIT motility by using the same procedure as described in ***Part-A section 2.3.13.***

5.4.14 Screening for different groups of compound

The plant materials were screened for various compound group; alkaloids, flavonoid, protein, saponin and tannin as per ***Part-A* section 2.3.14.**

5.4.15 Nutritional analysis

The nutritional analysis of plant material for different component; ash, moister content, protein, carbohydrates and fats as per **Part-*A section 2.3.15.***

5.4.16 Elemental analysis

The plant materials were screened for the different elements as per **Part-*A section 2.3.16.***

5.4.17 Scanning Electron Microscope (SEM) analysis

The scanning electron microscope analysis of plant material was performed as per **Part-*A section 2.3.17.***

5.4.18 Energy Dispersive X-ray (EDX)

The elemental composition study was carried out with EDX as per **Part-*A section 2.3.18.***

5.4.19 Gas Chromatography-Mass Spectrometry

GC-MS was performed as per reported procedure **Part-*A section 2.3.19.***

5.5.1 Characterization of Isolated Compound (1)

Physical status: White needle crystals

Yield: 1.98 g

M.P: 162-165 °C

EI-MS: 412 amu

Mol: formula: $C_{29}H_{48}O$

^{1}H -NMR and ^{13}C -NMR (table 6.22)

5.5.2 Characterization of Isolated Compound (2)

Physical status: Yellowish amorphous powder

Yield: 1.97 g

M.P: 59.8 °C

EI-MS: 270 amu

Mol: formula: $C_{18}H_{38}O$

^{1}H -NMR and ^{13}C -NMR (table 6.23)

5.5.3 Characterization of Isolated Compound (3)

Physical status: white crystal

Yield: 2.96 g

M.P: 88-92 °C

EI-MS: 167.8 amu

Mol: formula: $C_9H_{10}O_3$

^{1}H -NMR (MeOD, 400MHz): ^{13}C-NMR (MeOD, 400MHz) (Table 6.24)

5.5.4 Characterization of Isolated Compound (4)

Physical status: yellowish amorphous

Yield: 2.96g

M.P: 59-61 °C

EI-MS: 410 amu

Mol: formula: $C_{27}H_{54}O_2$

^{1}H NMR and ^{13}C -NMR (Table 6.24)

5.5.5 Characterization of Isolated Compound (5)

Physical State: White amorphous powder

Yield: 16 mg

Melting point: 200-201°C

IR (MeOH) cm-1: 3390, 3315, 1710 and 1627

EI-MS: 155

^{1}H-NMR and ^{13}C-NMR: Table 6.25

5.5.6 Characterization of Isolated Compound (6)

Physical State: White amorphous powder

Yield: 16 mg

Melting point: 212-215°C

IR (MeOH) cm-1: 3390, 3315, 1710 and 1627

EI-MS: 448.377

Mol: Formula: $C_{21}H_{20}O_{11}$

UV: 267.5, 297, 351; +$AlCl_3$: 274; +CH_3COONa: 276, 371; +CH_3O : 276, 398

IR: 3440–3290 (OH), 1670 (C = O γ-pyrone), 1100–1000 (C-O glucosides)

^{1}H-NMR and ^{13}C-NMR: Table 6.26

Chapter 6

6.0 RESULTS AND DISCUSSION

6.1 Phytochemical screening

Bioactive compounds of medicinal plants play a very important role for curing various human diseases. Natural compounds have two types constituents; primary and secondary. Primary constituents proteins, chlorophyll, carbohydrates and amino acids. Secondary constituents contain alkaloids and terpenoid [168]. The Crd. MeOH Ext. of *A. anisacanthus* was screened for the presence of several phytochemical components as presented in Table 6.1 showed that alkaloids give positive result with confirmation of precipitate formation, at the lowermost part. The test for flavonoids with pink color appearance, confirms that the plant contain flavonoids. Result for saponins was also positive by the froth formation of about 1.2cm. The Crd. MeOH Ext. gives strongly positive results for carbohydrates, proteins, steroids, sterols and terpenes.

Commercially the phytochemical analysis of the plant has great importance in pharmaceutical industries for the preparation of new medicines and treatment of several diseases. The phytochemical screening showed that *A. anisacanthus* can be used for preparation of various drugs.

Table 6.1 Qualitative phytochemical screening of Crd. MeOH Ext. of *A. anisacanthus*

S. No	Phytochemical constitutes	Tests	Results
1	Carbohydrate	Molisch's test	++
		Benedict' test	++
		Fehling's test	+
2	Proteins	Ninhydrin Test	+
3	Alkaloid	Mayer's test	++
		Wagner's test	++
		Hager's test	++
4	Sterols and triterpenoids	Sadoski's test	+++
5	Phenol	Ferric chloride test	+
6	Flavonoids	Alkaline reagent test	++
7	Tannins	Gelatin test	+
		Ferric chloride test	+
8	Saponin	Frothing test	++

key: + = weak positive, ++ = moderately positive, +++ = strongly positive

6.2 Pharmacological Investigation

6. 2. 1 Antibacterial activity

Crd. MeOH Ext. and various fractions of aerial parts of *A. anisacanthus* were screened for antibacterial activity. The result Crd. MeOH Ext. and different fractions were compared with amoxicillin clavulanate and cephalothin (10µg/disc) and standard antibacterial agent and percent growth inhibition were calculated as mentioned in Table 6.2.

Crd. MeOH Ext. showed good antibacterial activity against *MSSA* (69%) and *P. aeruginosa* (70%), moderate effect showed against *MRSA* (46%), *E. coli* (57%) and *S. typhi* (59%). *n*-hexane fraction showed moderate antibacterial effect against *E. coli* (59%), *MRSA* (53%), *P. aeruginosa* (55%), *MSSA* (53%) and *S. typhi* (59%). $CHCl_3$ fraction showed good antibacterial activity against *E. coli* (70%) and *MRSA* (60%), moderate effect was shown against *MSSA* (46%) and *S. typhi* (51%) while low effect was shown against *P. aeruginosa* (37%). EtOAc fraction showed moderate antibacterial effect against *E. coli* (59%), *MRSA* (40%), and *S. typhi* (44%), respectively while low effect was shown against *P. aeruginosa* (33%) and *MSSA* (38%). Acetone fraction showed good activity against *MSSA* (65%) and moderate activity was observed against *E. coli* (55%) and *S. typhi* (44%), while *MRSA* (33%) and *P. aeruginosa* (37%) showed low activity, respectively.

Aqueous fraction showed moderate activity against *E. coli* (48%), *MRSA* (40%), *P. aeruginosa* (40%), *MSSA* (57%) and *S. typhi* (44%) respectively. The above results showed that the Crd. MeOH Ext. and various fractions are rich source of antibacterial activity and can be used in several infections. The results obtained from *A. anisacanthus* support the medicinal uses of this plant in various diseases like

bronchitis and nasal infection [169-170]. *A. siculus* showed significant result against Gram positive and negative bacteria [171].

6. 2. 1. 1 Minimum inhibitory concentration (MIC_{50})

The MIC_{50} values showed the potency of a test sample against various test organism, low MIC_{50} values (mg/ ml) showed higher potency of the test samples of *A. anisacanthus* (with moderate antibacterial activities). The test samples with moderate antibacterial activity were calculated by using Microsoft XL sheet from three different reading as shown in table 6.3. *In vitro* studies of *A. radix*, root extract indicate that the MICs and minimum bactericidal concentration (MBC) are linked to the level of active substance [172].

6. 2. 1. 2 Minimum bactericidal concentration (MBC)

Due to higher MIC_{50} values the MBCs for the test samples were not calculated against test pathogen.

Table 6.2. Antibacterial activity of Crd. MeOH Ext. and various fractions of aerial parts of *A. anisacanthus*

Name of Bacteria	Zone of Inhibition (mm)						
	+ive Control	Crd. Ext	*n*-hexane	$CHCl_3$	EtOAc	Acetone	Aqueous
E. coli	27(AMC)	15	16	19	16	14	13
MRSA	15(CEF)	7	8	9	6	5	6
MSSA	26 (CEF)	18	14	12	10	17	15
P. aeruginosa	27(AMC)	19	15	10	9	10	12
S. typhi	27(AMC)	16	12	14	15	12	11

Table 6. 3. Antibacterial activity (percent) of Crd. MeOH Ext and various fractions of *A. anisacanthus*

Name of Bacteria	% Zone of Inhibition					
	Crd. Ext	*n*-hexane	$CHCl_3$	EtOAc	Acetone	Aqueous
E. coli	57	59	70	59	55	48
MRSA	46	53	60	40	33	40
MSSA	69	53	46	38	65	57
P. aeruginosa	70	55	37	33	37	40
S. typhi	59	44	51	55	55	44

Table 6. 4. MIC_{50} values (mg /ml) of the Crd. MeOH Ext and different fractions of *A. anisacanthus*

Name of Bacteria	Crd. Ext	*n*-hexane	$CHCl_3$	EtOAc	Acetone	Aqueous
E. coli	6.8	6.7	5.7	6.7	5.8	NA
MRSA	5.9	7.2	NA	NA	NA	NA
MSSA	6.9	7.2	NA	NA	6.5	6.5
P. aeruginosa	6.8	5.8	NA	NA	NA	NA
S. typhi	6.7	NA	NA	5.5	NA	NA

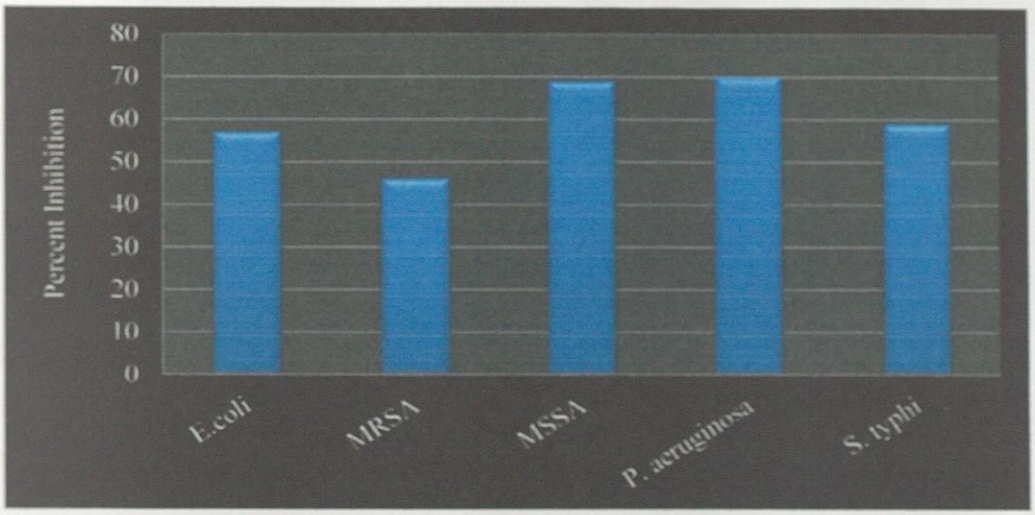


Fig. 6.1. Antibacterial activity of the Crd. MeOH Ext of *A. anisacanthus*

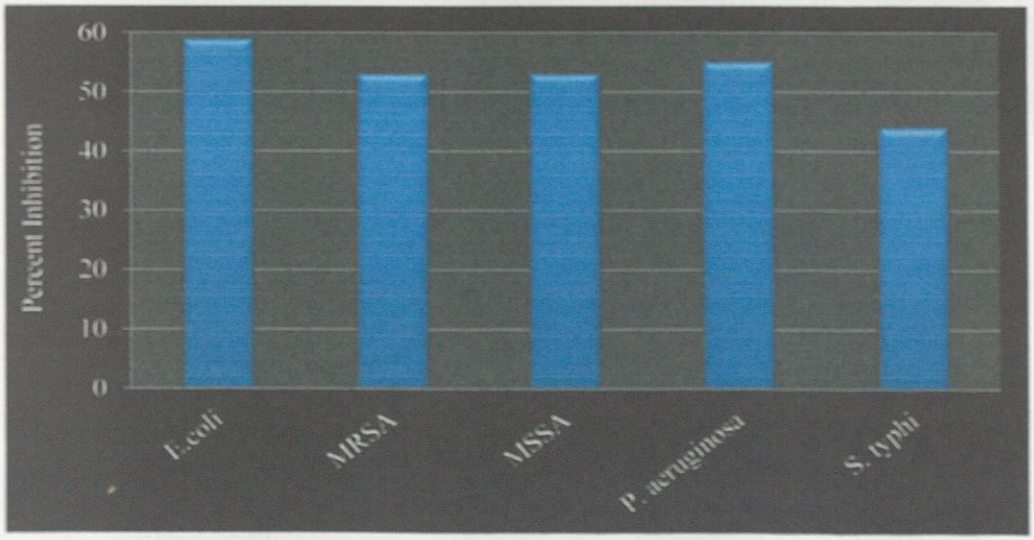


Fig. 6. 2. Antibacterial activity of the *n*-hexane fraction of *A. anisacanthus*

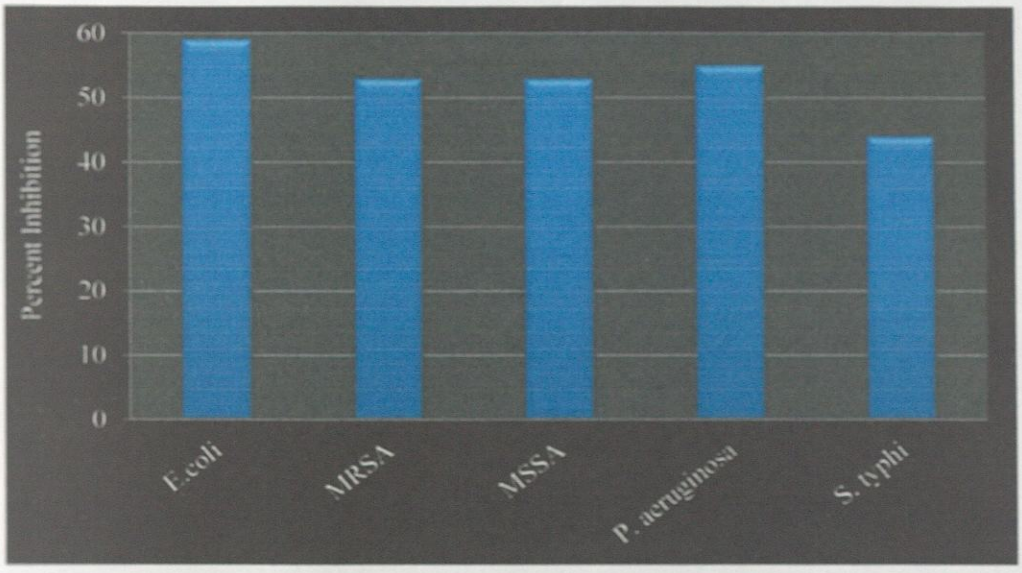


Fig: 6. 3.Antibacterial activity of the $CHCl_3$ fraction of *A. anisacanthus*

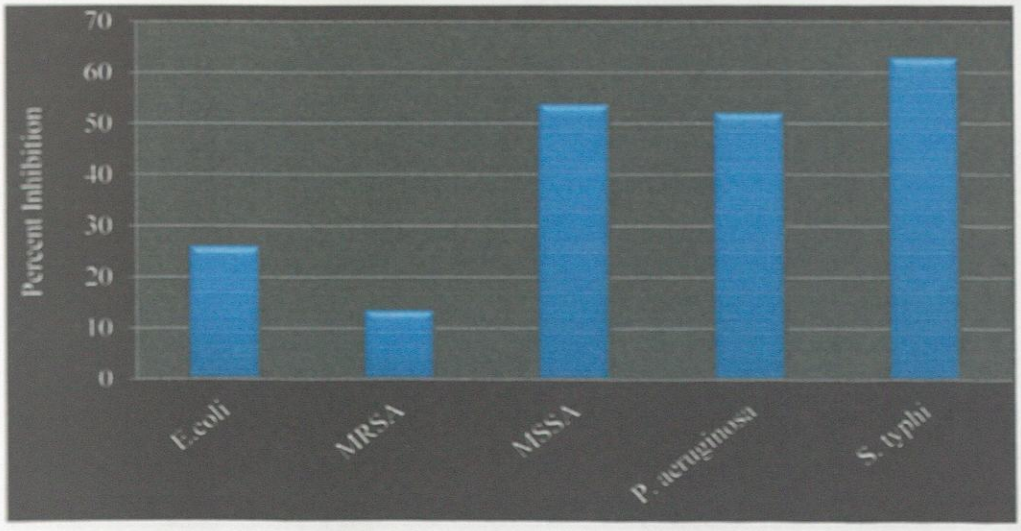


Fig: 6. 4. Antibacterial activity of the EtOAc fraction of *A. anisacanthus*

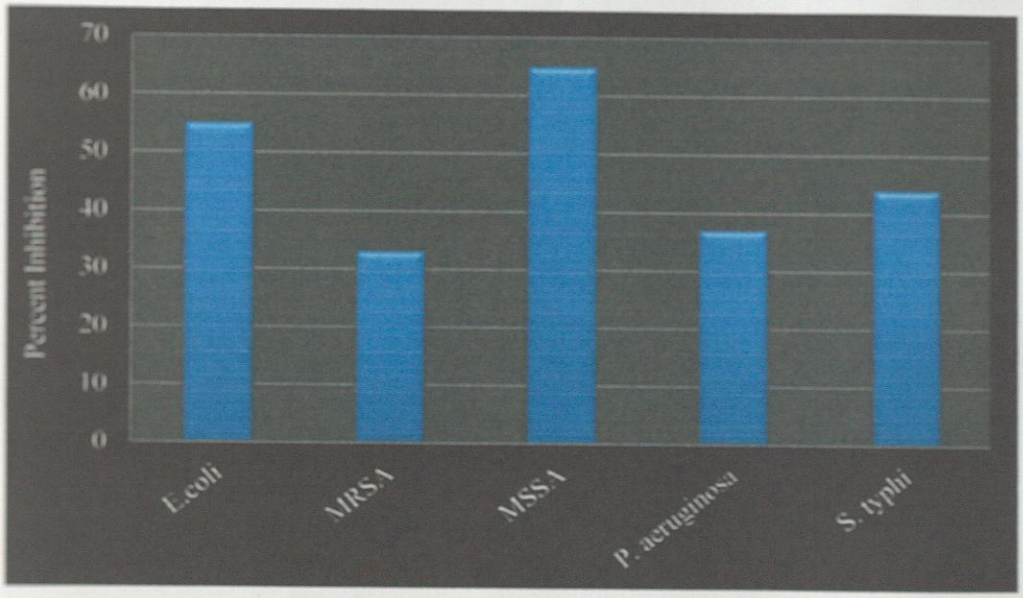


Fig: 6. 5. Antibacterial activity of the acetone fraction of *A. anisacanthus*

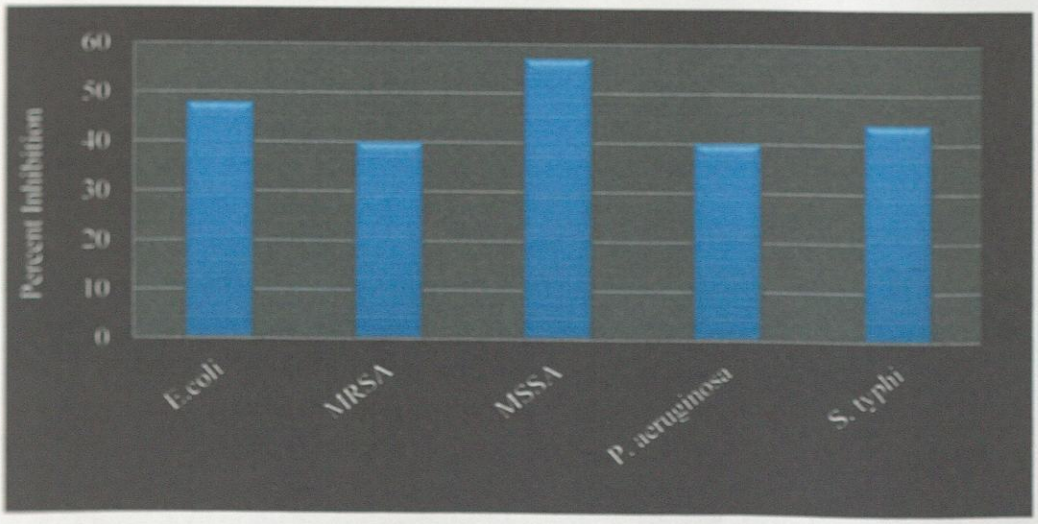


Fig: 6. 6. Antibacterial activity of the aqueous fraction of *A. anisacanthus*

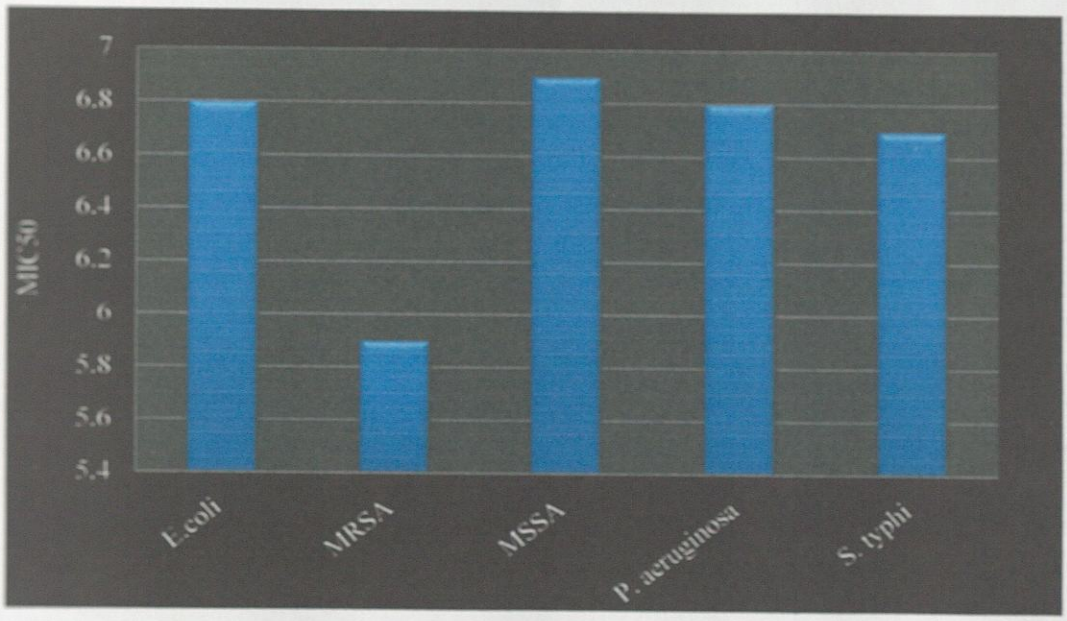


Fig: 6. 7. MIC_{50} (mg/ml) values of the Crd. MeOH Ext of *A. anisacanthus*

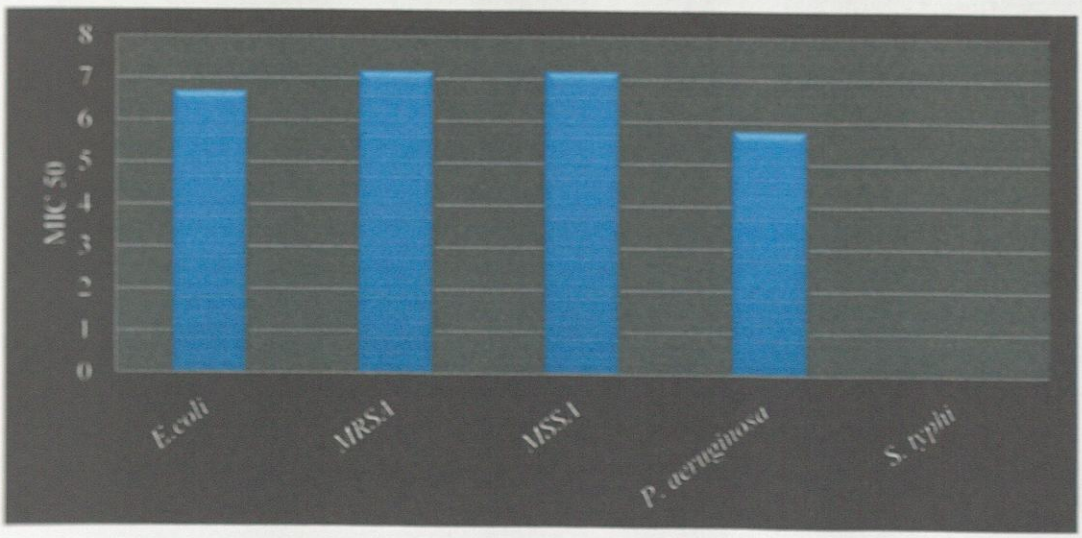


Fig: 6. 8. MIC_{50} (mg/ ml) values of the *n*-hexan fraction of *A. anisacanthus*

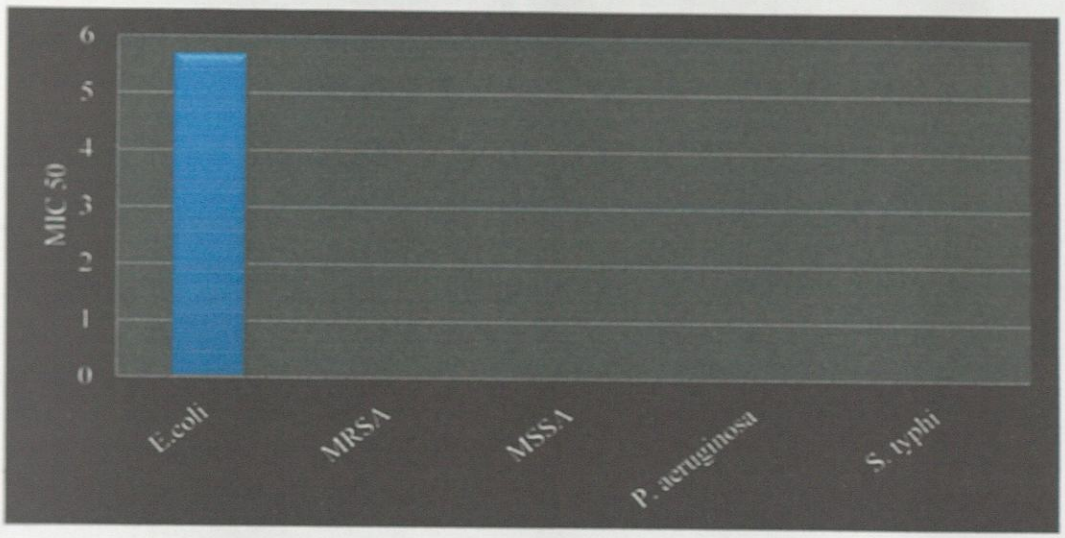


Fig: 6. 9. MIC_{50} (mg/ ml) values of the $CHCl_3$ fraction of *A. anisacanthus*

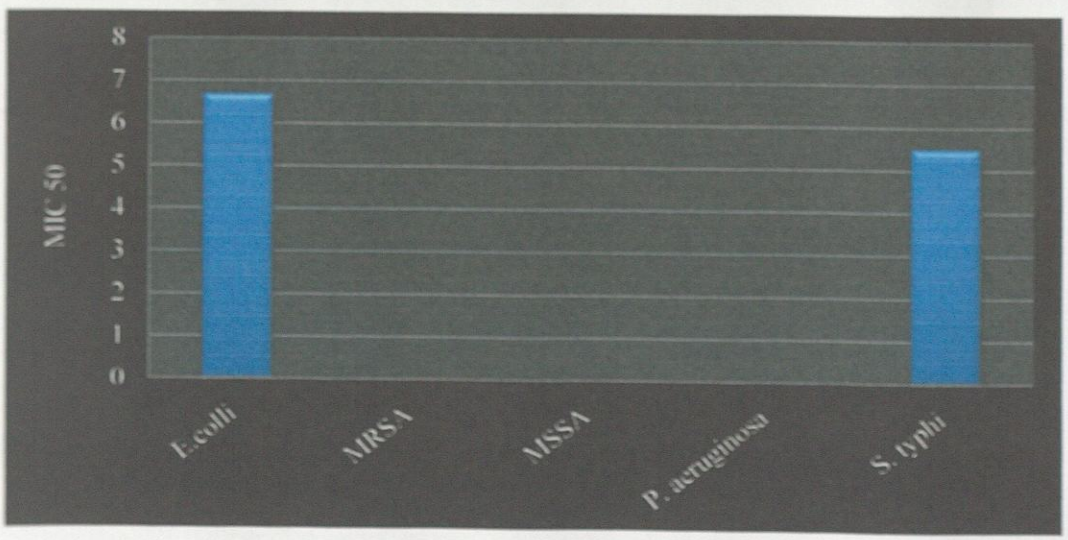


Fig: 6. 10. MIC_{50} (mg/ml) values of the EtOAc fraction of *A. anisacanthus*

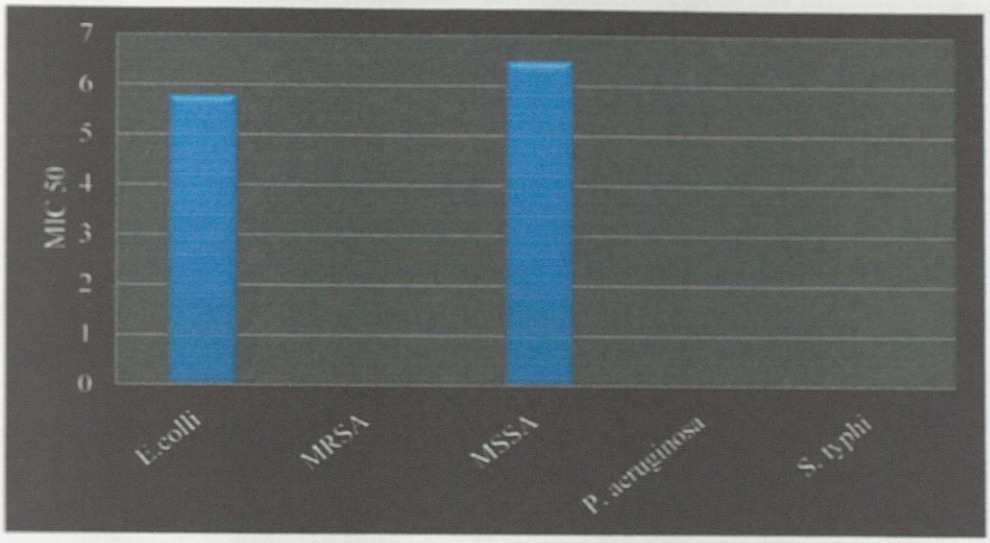


Fig: 6. 11. MIC_{50} (mg/ml) values of the Acetone fraction of *A. anisacanthus*

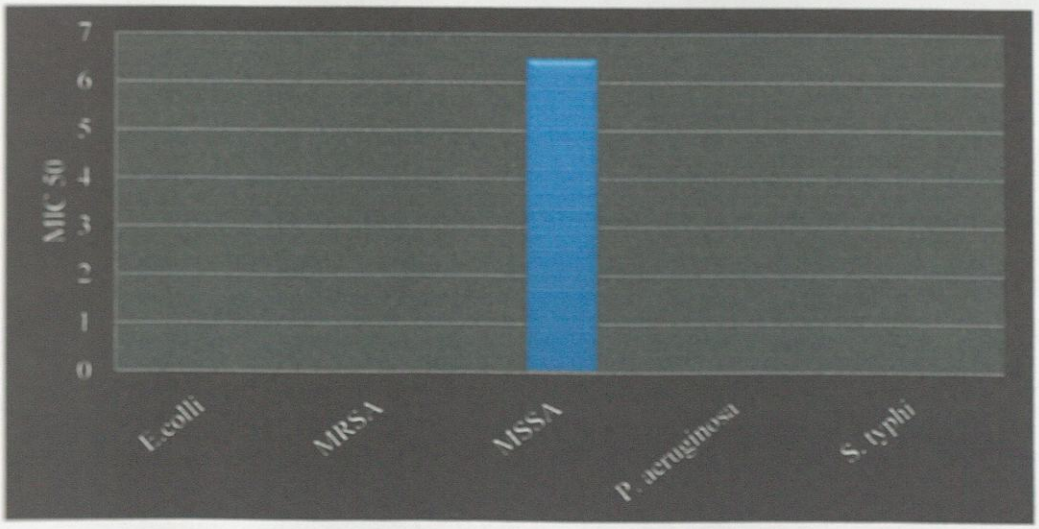


Fig: 6. 12. MIC_{50} (mg/ ml) values of the aqueous fraction of *A. anisacanthus*

6. 2. 2 Antifungal activity

In case of immune compromised patients fungal infection become an important cause of sickness and death [173]. Mainly the fungal toxicities are caused by *Candida* and *Aspergillus* species. Currently the prevalence of disease have pointed out a shift towards infection caused by non albicans *Candida* and *Aspergillus* [174]. All the test samples of *A. anisacanthus* aerial parts were screened for the antifungal activity against *A. oryzae, A. fumigatus, F. solanum, A. flavus, A. niger* and *P. notatum*. The results are shown in tables 6. 5. Crd. MeOH Ext. showed low percent inhibition against *A. flavious* (20%) and *A. oryzae* (30%), and in active against *A. fumigatus, F. solanum, A. niger* and *P. notatum*. *n*-hexane fraction showed low activity (17%) against *A. flavious* and showed no activity for *A. fumigatus, F. solani, A. niger* and *P. notatum*. $CHCl_3$ fraction showed low activity against *A. flavious* (20%) and *A. niger* (7%) and no activity against the other tested organism. EtOAc fraction showed low activity against *A. fumigatus* (30%), *A. flavious* (30%) and *A. niger* (10%). Acetone fraction is inactive against the tested organism. Aqueous fraction showed moderate percent inhibition of (40%) against *A. fumigatus* and *A. flavious*.

Table 6. 5 Antifungal activity of the Crd. MeOH Ext. and various fractions of *A. anisacanthus*

Fungus spp	Percent linear growth inhibition							
	-ive Control	+ive Control	Crd. MeOH	*n*-hexane	$CHCl_3$	EtOAc	Acetone	Aqueous
A. oryzae	0	100	30	-	-	-	-	-
A. fumigatus	0	100	-	-	-	30	-	40
F. solani	0	100	-	-	-	-	-	-
A. flavious	0	100	20	17	20	30	-	40
A. niger	0	100	-	-	7	10	-	-
P. notatum	0	100	-	-	-	-	-	-

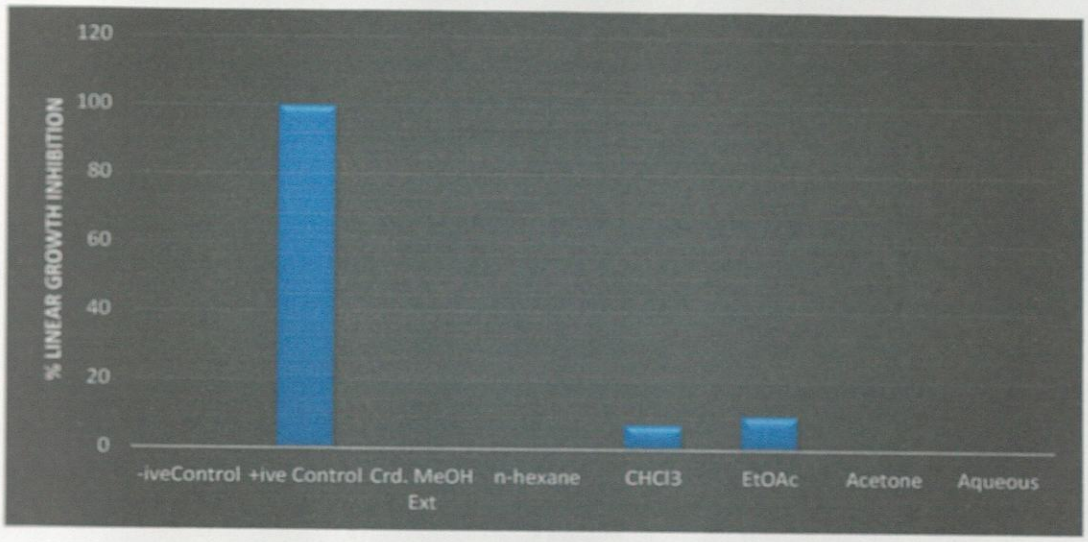


Fig: 6. 13. Antifungal activity of Crd. MeOH Ext. and various fractions of *A. nisacanthus* against *A. niger*

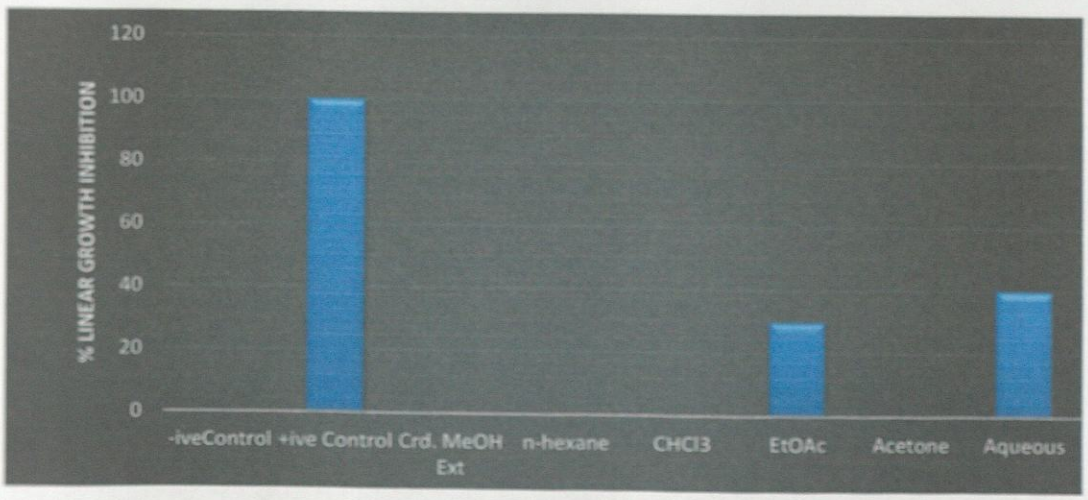


Fig: 6. 14. Antifungal activity of Crd. MeOH Ext. and various fractions of *A. anisacanthus* against *A. fumigatus*

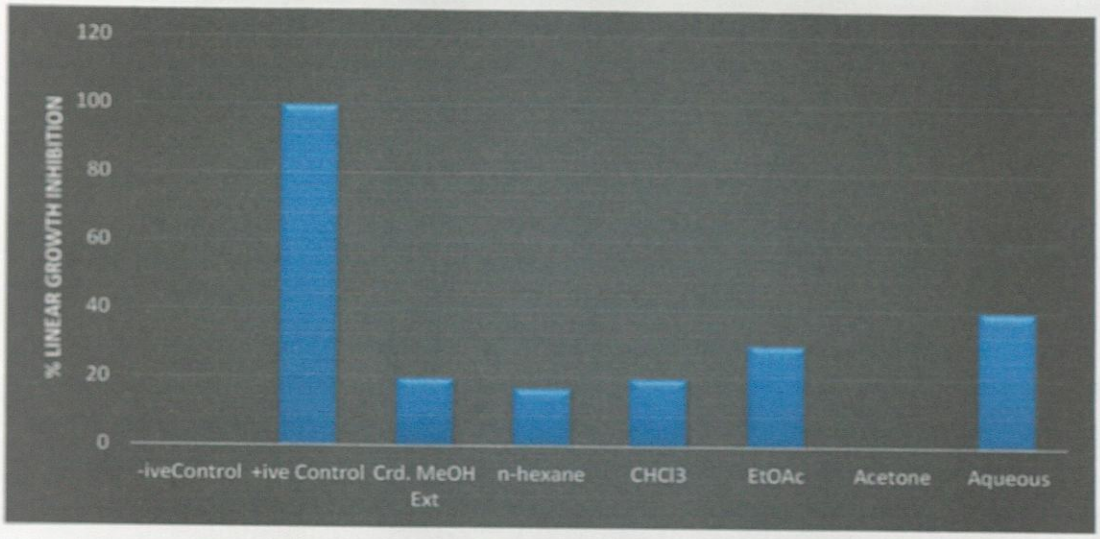


Fig: 6. 15. Antifungal activity of crude extract and various fractions of *A. anisacanthus* against *A. flavious*.

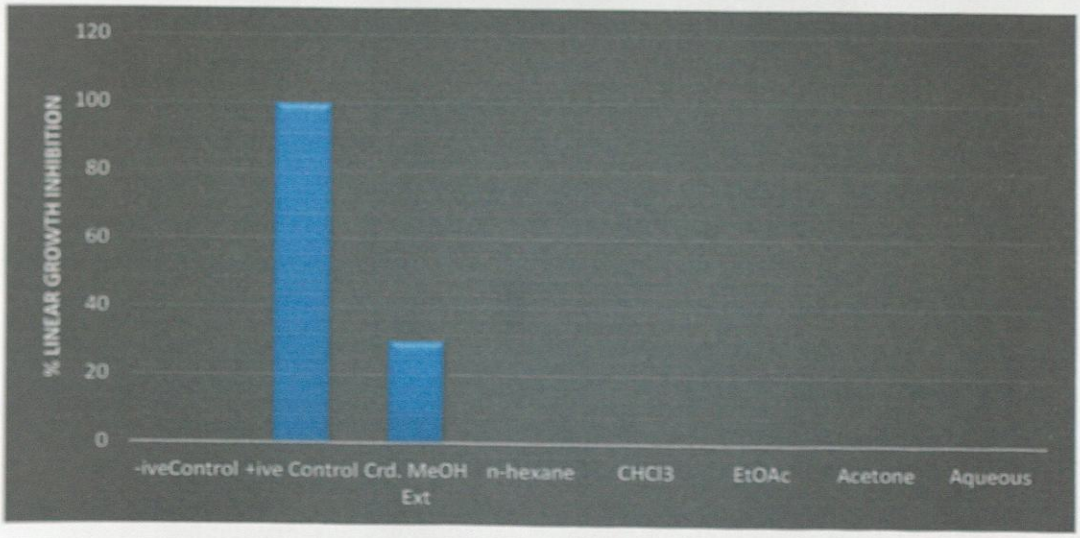


Fig: 6. 16. Antifungal activity of Crd. MeOH Ext. and various fractions of *A. anisacanthus* against *A. oryzae*

6. 2. 3 Phytotoxic activity

Phytotoxic compounds are considered as natural anti-tumor compounds that inhibit growth of *L. minor*. [175]. All the test samples of *A. anisacanthus* were screened for the possible phytotoxic activity against the *L. minor*. The results are given in table 6. 6 and 6.6a.

The Crd. MeOH Ext. showed moderate phytotoxic effect (56%, 50%) at concentration of 1000 and 100 µg/ml, respectively. *n*-hexane fraction showed moderate (50%, 43%) phytotoxic effect at 1000 and 100 µg/ml respectively, while low (12%) plant growth regulation was observed at 10µg/ml. $CHCl_3$ fraction (43%) showed moderate plant growth regulation (43%) at 1000µg/ml and low (31%, 18%) at 100 and 100µg/ml, respectively. EtOAc fraction showed moderate (56%) and low (37%, 25%) plant growth regulation at 100 and 10µg/m, respectively. Acetone fraction showed low (18%, 25%, 37%) activity at 10, 100 and 1000µg/m, respectively. Aqueous fraction showed good (62%) activity at 100µg/m, moderate (43%) and low (12%) growth regulation at 1000, 100 and 10 µg/ml. Parquet was used as standard in phytotoxic activity.

Table 6. 6. Phytotoxic activity of the Crd. MeOH Ext. and various fractions of *A. anisacanthus*

Conc of sample (µg/ml)	Number of frond killed						
	Crd. MeOH Ext	*n*-hexane	$CHCl_3$	EtOAc	Acetone	Aqueous	Control
1000	9	8	7	9	6	7	16
100	8	7	5	6	4	5	16
10	6	2	3	4	3	2	16

Table 6. 6a Percent growth regulation of *L. minor*

Conc of sample (µg/ml)	Percent growth regulation						
	Crd. MeOH Ext	*n*-hexane	$CHCl_3$	EtOAc	Acetone	Aqueous	Control
1000	56	50	43	56	37	43	100
100	50	43	31	37	25	62	100
10	37	12	18	25	18	12	100

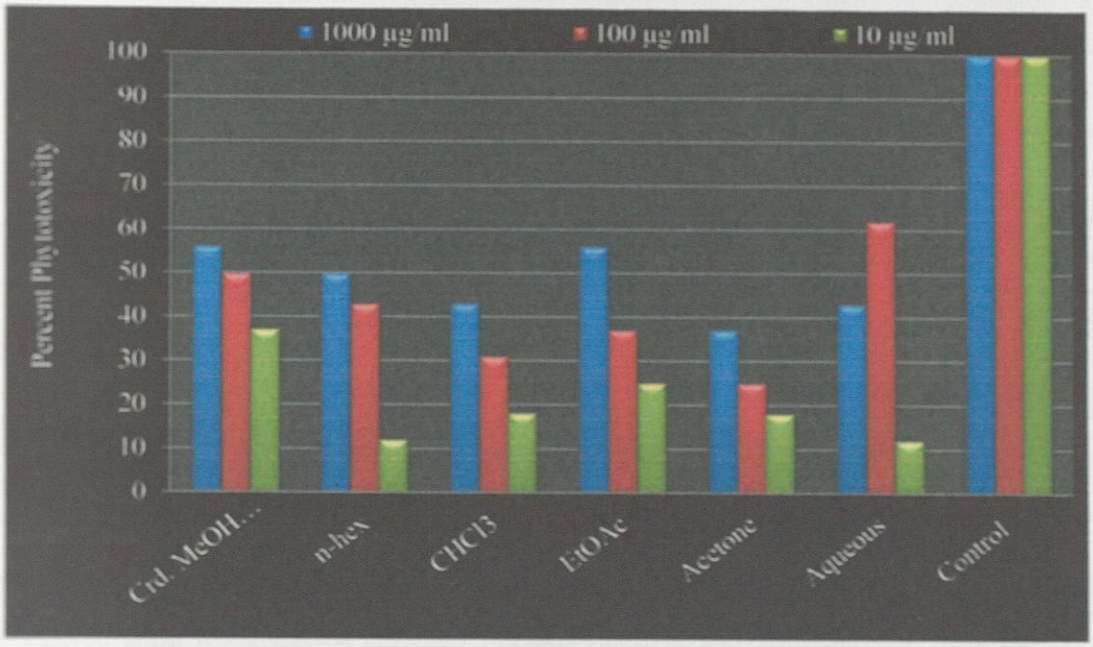


Fig: 6. 17. Phytotoxic activity of Crd. MeOH Ext. and various fractions of against *L. minor*.

6. 2. 4 Anti termite activity

In buildings "Termites" cause extraordinary damage to wooden structures, besides losses in agriculture and forestry. Synthetic termitcides have been used for controlling termites. At the start of termite control, preservation of the chemical was considered to be beneficial, as it provided safety for long time. Synthetic termiticid cause a great damage to surroundings, due to their residual influence [176]. The anti-termite activity of the various fractions of *A. anisacanthus* were carried out at "Termites lab, NIFA, Peshawar, KPK, Pakistan. All the experiments were performed in triplicates.

On 1stday Crd. MeOH Ext. (15), *n*-hexane = aqueous = EtOAc (18) fractions showed good while $CHCl_3$ (22) and Acetone (21) fractions showed significant activity against *H. indicola*. On 2ndday all fractions (Crd. MeOH Ext, $CHCl_3$, aqueous, EtOAc and *n*. hexane showed significant results and killed an average of 22 termites. On 3rd day Crd. MeOH Ext, EtOAc and *n*. hexane killed an average of 25 while $CHCl_3$ (23) and aqueous (24) fractions showed significant results as shown in table 6. 7.

Table 6.7 Anti-termite activity of the Crd. MeOH Ext. and various fractions of *A. anisacanthus*

Sample	No. of Termite used	Days	Average termite killed
Crd. MeOH Ext.	25	1	15
		2	22
		3	25
n-hexane	25	1	18
		2	22
		3	25
$CHCl_3$	25	1	22
		2	22
		3	23
EtOAc	25	1	18
		2	22
		3	25
Acetone	25	1	21
		2	22
		3	25
Aqueous	25	1	18
		2	22
		3	24

6. 2. 5 Haemagglutination activity

The structural and functional roles of cell surface carbohydrates have been demonstrated using lectin specificities [177]. They have been used for the isolation and characterization of glyconjugates (cell membrane) and to agglutinate erythrocytes [178, 179]. Agglutinin from the plant sources are advantageous over animal sources because they are available in large quantities and are economical. The results given in the table 6.8 shows the haemagglutination activity of the test samples against human RBC's of all blood groups. The *n*-hexane fraction showed low activity against O^{+ive} while aqueous fraction presented the same against A^{+ive} at dilution of 1:2, EtOAc fraction showed moderate (++) and low (+) haemagglutination activity at dilution of 1:2 and 1:4, respectively against O^{+ive}. The results for remaining fractions and Crd. MeOH Ext. were negative at all concentration against all blood groups.

Table 6. 8. Heamagglutination activity of the Crd. MeOH Ext. and various fractions of *A. anisacanthus*

Blood groups	Crd. MeOH Ext				*n*-hexane				$CHCl_3$				EtOAc				Acetone				Aqueous			
	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16
AB-ive	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B-ive	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A-ive	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O-ive	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AB+ive	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B+ive	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A+ive	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
O+ive	-	-	-	-	+	+	-	-	-	-	-	-	++	+	-	-	-	-	-	-	-	-	-	-

6. 2. 6 Insecticidal activity

The synthetic insecticides are of great threat to the environment due to their toxic effect. Therefore environment friendly insecticides from the natural resources should be searched to replace the harmful insecticides [180]. *T. castaneum, R. dominica* and *C. analis* were used to know the insecticidal activity of test samples. The results are mentioned in table 6.9. The $CHCl_3$ fraction showed good activity against *T. castaneum* (70%), *R. dominica* (60%) and *C. analis* (60%), respectively. Crd. MeOH Ext. Showed moderate activity against *T. castaneum* (40%), *R. dominica* (50%) and *C. analis* (50%). *n*-hexane fraction showed good activity for *T. castaneum* (60%), *R. dominica* (60%) *and C. analis* (70%), respectively. EtOAc fraction showed good activity against *T. castaneum* (60%), while moderate activity was observed for *R. dominica* (50%) and *C. analis* (40%). Aqueous fraction (60%) showed good activity against *T. castaneum*, while moderate activity against *R. dominica* (40%) and *C. analis* (30%). The insecticidal activity of the test samples showed that Crd. MeOH Ext. and various fraction of *A. anisacanthus* aerial parts can be used as insect repellent.

Table 6. 9. Insecticidal activity of Crd. MeOH Ext and various fractions of *A. anisacanthus*

Name of Insect	% Mortality						
	Control	Crd. MeOH Ext	*n*-hexane	$CHCl_3$	EtOAc	Acetone	Aqueous
T. castaneum	100	40	60	70	60	20	60
R. dominica	100	50	60	60	50	30	40
C. analis	100	50	70	60	40	50	30

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

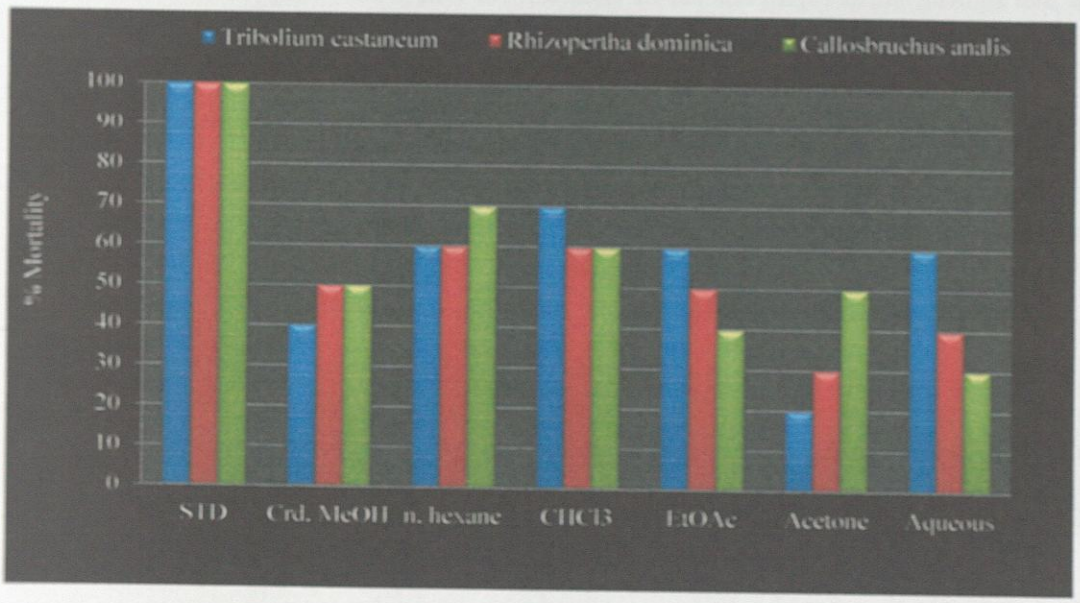


Fig 6. 18 Insecticidal activity of Crd. MeOH Ext. and various fractions of *A. anisacanhus*

6. 2. 6 Brine shrimp lethality bioassay

Brine shrimp (*Artemia salina*) lethality bioassay was used to check the cytotoxic effect of the test samples. 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 test samples (Crd. MeOH Ext and fractions) were used in concentration of 10, 100 and 1000 µg/ml. The results of the assay are mentioned in table 6.10.

The Crd. MeOH Ext (83%), EtOAc (100%), $CHCl_3$, acetone and aqueous fractions showed significant activity (90%) at 1000µg/ml, while *n*-hexane fractions showed good activity (76%) at same concentration. All the fractions showed moderate activity while EtOAc fraction (66.6%) and Crd. MeOH Ext. low activity at 100µg/ml. Low activity was shown by all the fractions at 10µg/ml. It is concluded from the results that *A. anisacanthus* may possess cytotoxic effect against cancerous cell lines.

Table 6. 10. Cytotoxic activity of Crd. MeOH Ext and various fractions of *A. anisacanthus*

Dose (µg/ml)	Control (No. of shrimps)	Crd. Ext		*n*-hexane		$CHCl_3$		EtOAc		Acetone		Aqueous	
		Survivor	%killed	Survivor	%killed	Survivor	%killed	Survivor	%killed	Survivor	%killed	Survivor	%killed
10	30	27	10	29	3.3	21	30	29	3.3	27	10	21	30
100	30	25	10	18	40	15	50	24	20	12	60	12	60
1000	30	5	83	7	76	3	90	21	30	3	90	3	90

Etoposide (7.4625 µg/ml) was used as a standard drug

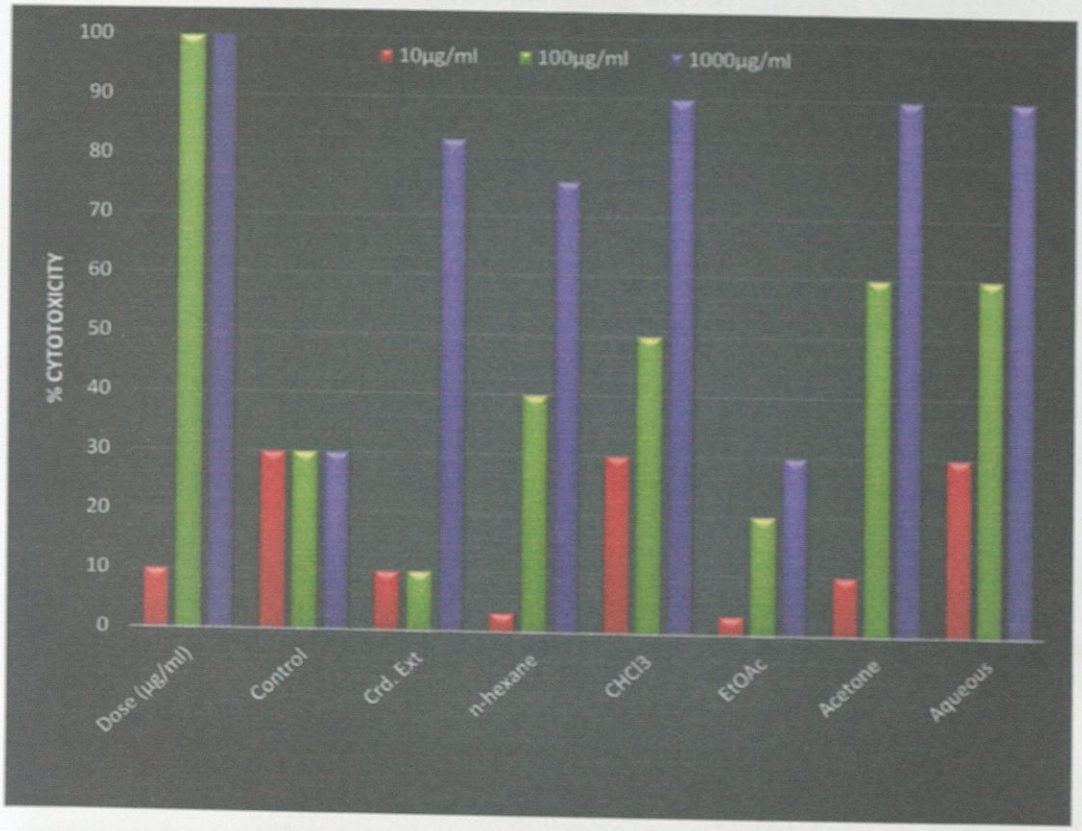


Fig. 6.19. Cytotoxic activity of Crd. MeOH Ext. and various fractions of *A. anisacanthus*

6. 2. 7 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 there by causing various diseases like cancer and atherosclerosis [181]. For reduction of these oxidative damages, many synthetic drugs are used but their side effects cannot be 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 [182-184]. Due to importance of the natural antioxidants, we screened the Crd. MeOH Ext and various fractions of *A. amsacanthus* at different concentrations (20, 40, 60, 80 and 100ppm) for possible NO free radical scavenging assay. The results obtained are given in table 6. 12 and presented in figures 6.20.

These results revealed that at concentration of 20ppm the EtOAc fraction showed moderate (48%) activity and rest of the samples Crd. MeOH Ext. (11%), $CHCl_3$ (17%), Acetone (18%), Aqueous (15%) and *n*-hexane (22%) showed low percent radical scavenging activity. At the concentration 40ppm various fractions were screened for antioxidant activity EtOAc fraction showed moderate (59%), while Crd. MeOH Ext. (22%), $CHCl_3$ (25%), Acetone (29%), Aqueous (22%) and *n*-hexane (35%) showed low percent radical scavenging activity. At the concentration 60ppm EtOAc fraction (67%) showed good, *n*-hexane (46%) and $CHCl_3$ (42%) showed moderate, Acetone (39%), Crd. MeOH Ext. (32%) and Aqueous (32%) showed low percent radical scavenging activity. At 80ppm good percent radical scavenging activity was shown by EtOAc fraction (78%), while *n*-hexane (57%), $CHCl_3$ (51%), Acetone (48%) Crd. MeOH

Ext. (46%) and Aqueous (46%) fractions showed moderate percent radical scavenging activity. At 100ppm significant activity was shown by the EtOAc fraction (85%), good result was observed for *n*-hexane (66%), $CHCl_3$ = Aqueous (65%), while Crd. MeOH Ext. (51%) showed moderate percent radical scavenging activity. The extract concentration showing 50% inhibition (EC_{50}) was calculated from the graph of percent RSA against extract concentration. EtOAc and Acetone fractions showed low EC_{50} values (43.5 and 40.5µg/ ml), while rest of the fractions Crd. MeOH Ext, *n*-hexane, $CHCl_3$ and Acetone showed high EC_{50} values (89, 74.5, 79.4, 72.5µg/ ml).

Table 6. 11 Nitric oxide free radical scavenging activity of the Crd. MeOH Ext and various fractions of *A. anisacanthus*

Conc (ppm)	Crd. Ext	*n*-hexane	$CHCl_3$	EtOAc	Acetone	Aqueous	Standard (vit C)
20	11	22	17	48	18	15	38
40	22	35	25	59	29	22	53
60	32	46	42	67	39	32	68
80	46	57	51	78	48	46	80
100	51	66	65	85	59	65	83

Table 6. 12 EC_{50} values of the Crd. MeOH Ext and various fractions of *A. anisacanthus*

Test Sample	Crd. MeOH Ext	*n*-hexane	$CHCl_3$	EtOAc	Acetone	Aqueous
EC_{50} (µg/ml)	89	74.5	79.4	43.5	72.5	40.5

Vitamin C was used as standard at concentration of (47.78µg/ml)

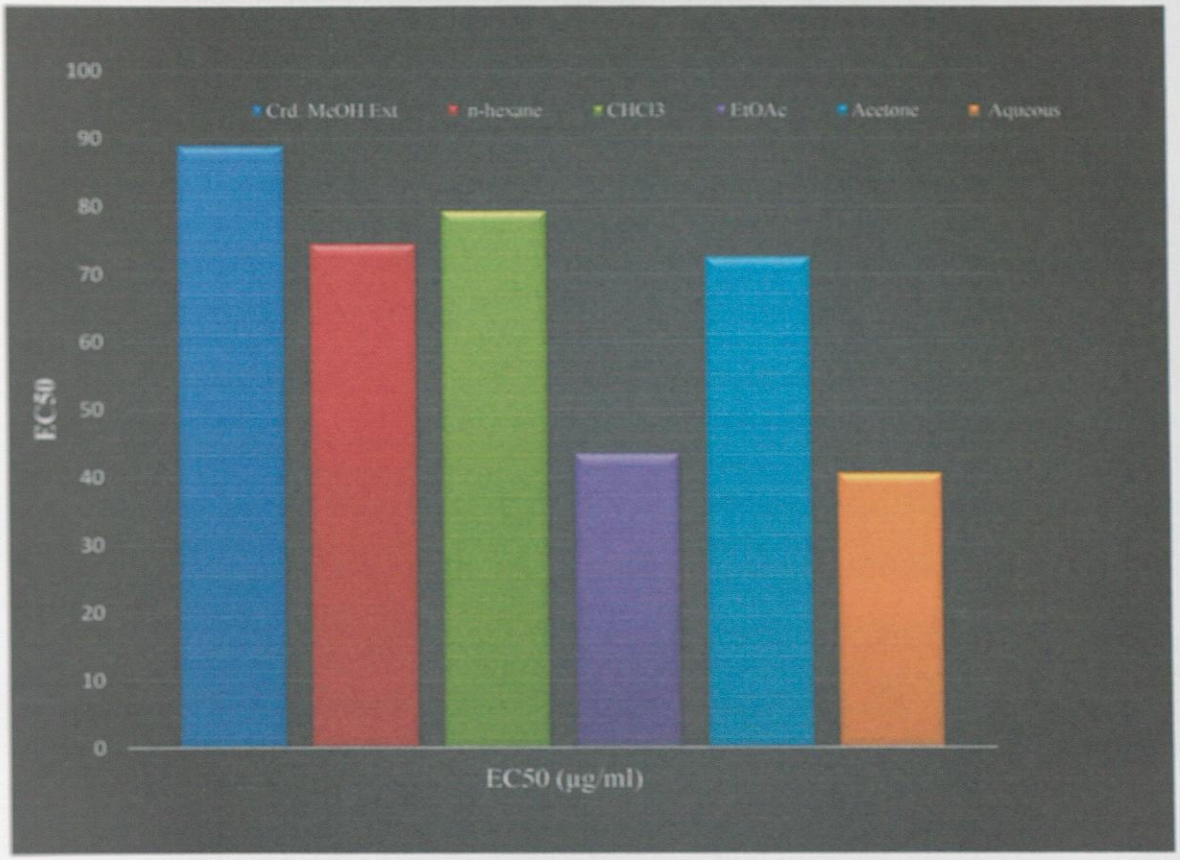


Fig: 6.19 EC_{50} values of the Crd. MeOH Ext. and various fractions of *A. anisacanthus*

IN-VIVO BIOLOGICAL ACTIVITIES OF CRUDE EXTRACT

6. 2. 8 Acute toxicity

The Crd. MeOH Ext. was tested for its toxic effect with different doses and the results obtained are given in table 6.13. BALB/C mice were used for this purpose. The mice were kept under observation for 24hrs and were observed for gross effect after 4hrs of test sample administration. Up to a dose of 1000mg/kg there was no harmful effect observed in the first 4hrs and no death was caused by test sample after 24hrs. It is concluded that the test sample is safe up to a dose of 1000mg/kg. Studies on acute toxicity of *A. anisacanthus* composition showed the maximal dosage (500mg/kg) showed no toxic effects [186]. *A. echidnaeform is* protecting mice from mortality at intraperitoneal doses between 3.2 and 100mgkg^{-1} day^{-1} treatments [187].

Table 6.13 Acute toxicity of Crd. MeOH Ext of *A. anisacanthus*

Treatment extract (ml or mg/kg)	% Death after 4hrs	% Death after 24hrs
500	-	-
700	-	-
1000	-	-

6. 2. 9 Analgesic activity

Crd. MeOH Ext. at different doses (500, 700 and 1000 mg/kg) showed decrease in the mean number of writhing in different test groups as shown in table 6.15. In normal saline treated group mean writhing was 60.00 ± 1.59. The low analgesic activity produced by different test doses of Crd. MeOH Ext. was 11.95% (500mg/kg) and 23.4% (700mg/kg), while moderate result observed was 40.30% (1000mg/kg). The effect produced by Crd. MeOH Ext. was dose dependent. The Diclofenac sodium (standard drug) at 10mg was maximum analgesic activity 76%, which is greater than the highest dose of Crd. MeOH Ext. (1000 mg/kg). The percent decrease in number of writhing by the standard and Crd. MeOH Ext. is represented by figure 6.20.

The experiments showed that the Crd. MeOH Ext. possess moderate analgesic effect. The acetic acid induced pain model is a common, rapid, sensitive and easy method for the determination of peripheral analgesic effect of plant extracts and other drugs as well [188]. Abdominal constriction occurrence in mice is considered to be involvement of local receptors (peritoneal) [189]. These chemicals produced in peritoneal fluids are responsible for pain which may appear in the form of abdominal constrictions. Writhing inhibition by various substances may be involved in the decreased production or inhibition of prostanoids, which is considered for pain inhibition through peripheral mechanism [190]. It is suggested that the Crd. MeOH Ext. shows its peripheral analgesic effect through inhibition of abdominal receptors (nociceptive), it may be due to decreased in the synthesis or inhibition of prostanoids production. The active chemical(s) present in Crd. MeOH Ext. produced analgesic effect in the form of

reduction in abdominal constrictions, suggesting the mechanism of action is linked with pain mediators.

Table 6. 14. Analgesic activity of Crd. MeOH Ext. of *A. anisacanthus*

S. No	Treatment	Dose ml or mg/kg	No. of writhing (10 min)	% inhibition of writhing
1	Normal saline	10	60.00 ± 1.59	5.56
2	Test sample	500	52.83 ± 1.40	11.95*
		700	46.00 ± 1.47	23.4*
		1000	35.83 ± 1.98	40.3**
3	STD (Diclofenac sod.)	10	16.67 ± 1.30	76***

*($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$) compared with negative control, ANOVA followed by Dunnett's test.

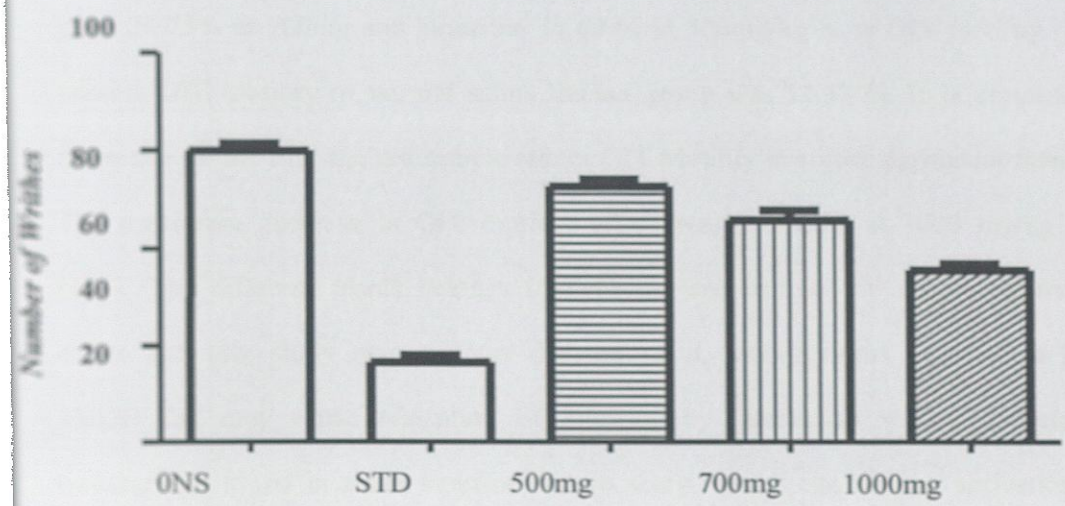


Fig. 6. 20 Percent analgesic activity of Crd. MeOH Ext. of *A. anisacanthus* *($P < 0.05$), **($P < 0.01$) and *** ($P < 0.001$) compared with negative control, ANOVA followed by Dunnett's test.

6.2. 10 Gastro-Intestinal Tract (GIT) Motility

The Crd. MeOH Ext. was screened for its effect on GIT motility. The Crd. MeOH Ext. decreased the percent GIT motility in a dose dependent manner results are given in table 6. 16. The Crd. MeOH Ext. showed significant 16.71% at 1000mg/kg, good 26.75% at 700mg and moderate 31.60 % at 500mg/kg b. w GIT motility. The percent GIT motility of normal saline treated group was 52.37 %. It is concluded from the result that the test sample reduce GIT Motility in a dose dependent manner. The maximum decrease in GIT motility effect was produced at 1000 mg/kg b.w [191]. The different plants belongs to Febaceae are famous for its antispasmodic effect and this study also support the use of *A. anisacanthus* [192]. The Crd. MeOH Ext. may cause relaxation of intestine by interaction with M3 receptor (muscarinic) found in small intestine, where acetylcholine can act and activation of M3 receptors will lead to increase contraction [193]. The effect of antispasmodic agents is to alleviate the symptoms of abdominal cramps. The plant extract reduces GIT motility and can be used for the management of diarrhea and in abdominal spasm.

Table 6. 16 GIT Motility of Crd. MeOH Ext of *A. anisacanthus*

Treatment	Dose	Mean Total length	Mean Charcoal movement (cm)	% GIT Motility
Normal saline	10	56.00 ± 1.218	29.33 ± 1.781	52.37
Crd. MeOH Ext.	500	55.00 ± 1.311	17.38 ± 1.600	31.60 *
	700	54.00 ± 1.718	14.45 ± 1.819	26.75 **
	1000	57.00 ± 1.988	09.53 ± 1.903	16.71 ***
Std Caster oil	0.1ml/kg	68.00 ± 1.997	05.05 ± 1.903	10.03 ***

*($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$) compared with negative control, ANOVA followed by Dunnett's test.

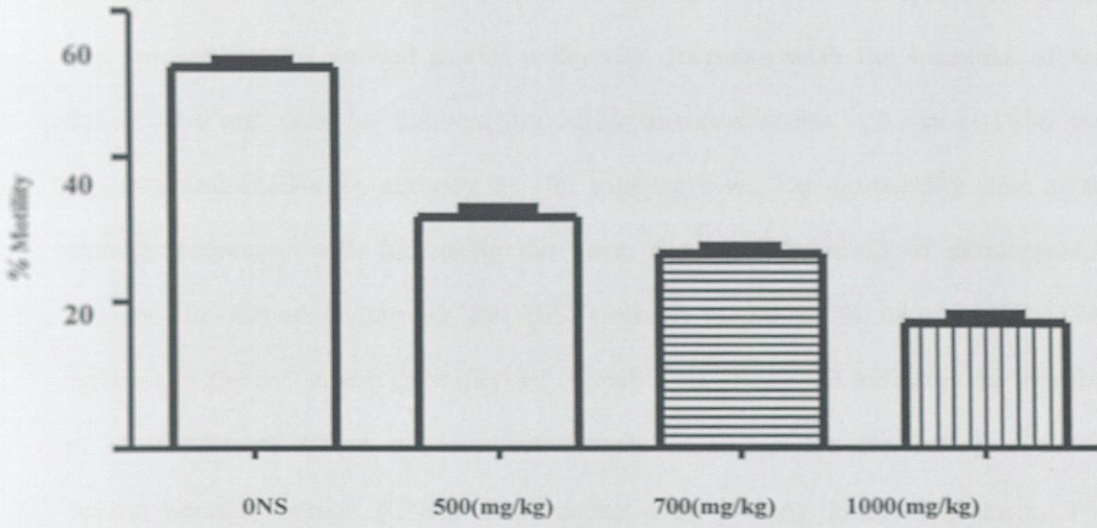


Fig 6. 21 GIT Motility of Crd. MeOH Ext. of *A. anisacanthus*

6. 2. 11 Antidepressant activity

The Crd. MeOH Ext. was evaluated for antidepressant potential. The results are given in the table 6.17, it is clear that test sample exhibited antidepressant activity. The immobility of animal in the water tub decrease with the increase of test doses. The test samples showed maximum antidepressant activity at 1000 mg/ kg b.w. and moderate activity at 100 mg/ kg b.w. The immobility time of the animals decreases with increasing the dose. For the evaluation of antidepressant activity the forced swimming test (FST) model in animal is widely used [194]. Increase in the movement (mobility) of animal in the water tub indicates the presence of antidepressant effect and decrease in movement indicate the involvement of central nervous system (CNS). The animals with prolong immobility during FST shows that the animals in the state of fatigue, tiredness and sadness. These symptoms are associated with depression, which is mostly found in patients suffering from depression [195]. The results of our test doses reflect that the plant extract produced antidepressant effect.

Table 6.18 Antidepressant activity of Crd. MeOH Ext. of *A. anisacanthus*

Treatment	Dose mg or ml/kg	Immobility time (s)
Saline	10	118 ± 2.70
Crd. MeOH Ext.	500	56 ± 2.86*
	700	41 ± 3.14*
	1000	31 ± 2.29**
Fluoxetine	15	23 ± 2.01***

*($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$) compared with negative control, ANOVA followed by Dunnett's test.

6. 2. 12 Locomotor activity

The open field test was used to screen locomotor (sedative) activity of Crd. MeOH Ext., results are given in table 16. 19. It is clear that the plant extract decrease the number of line crossing during open field test. The sedative effect of 500 mg/kg dose in comparison with negative control group was not significant (54 lines crossed). At 700mg/kg dose the number of lines crossed by mice was 35 and for 1000mg/kg it was 23. The decrease in number of lines crossed by mice was dose dependent which demonstrate the presence of sedative activity in extract. The standard drug (diazepam) exhibited significant activity in comparison with test doses.

From the present experiments we can conclude that Crd. MeOH Ext. was equally potent in reducing locomotor behaviour when injected in mice at high dose. Furthermore, as far as the mechanism of action, it is noteworthy that, while the effect of Crd. MeOH Ext. on locomotor behaviour was not blocked by an antagonist of (BZD) benzodiazepine receptors, so excluding an interaction with $GABA_A$ receptor (γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the central nervous system), the anxiolytic effect of Crd. MeOH Ext. to be related to BZD receptor activation.

In conclusion, the Crd. MeOH Ext. could be considered as partly responsible of the anxiolytic effect of *A. anisacanthus*, the anxiolytic effect of *A. anisacanthus* is better explained by the presence of benzodiazepine-like compounds in this plant [196]

Table 6. 19. Locomotor activity of Crd. MeOH Ext. of *A. anisacanthus*

Treatment	Dose mg or ml/kg	no. of line crossed in 10 min
Normal Saline	10	115 ± 2.45
Test sample	500	54 ± 2.71*
	700	35 ± 2.80**
	1000	23 ± 3.20**
Diazepam	0.5	06 ± 0.81***

*($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$) compared with negative control, ANOVA followed by Dunnett's test.

6. 2. 13 Nutritional Analysis

A normal human diet consists of water, vitamins, fats, proteins, carbohydrates and minerals. Results (Table 6. 20) revealed the nutritional analysis of *A. anisacanthus* showing moisture contents (9.5%), ash (7.4%), fats (7%), fibers (23.21%), protein (8%) and carbohydrates (48.73%) the gross energy was also calculated as mention in the table 6.17. The present analysis showed that this plant can be used as a good source of nutrient in case of shortage of nutrients. Effects of dietary *Astragalus* polysaccharide (APS) on growth performance and immune function in weaned pigs, this observation suggest that nutritive APS can be used as a potential immuno-modulating agent by affecting cellular immunity of weaned pigs [197]. From the gross energy calculation nutritional analysis it is concluded that *A. anisacanthus* is a good source of formation of a high energy phosphate bond, which is then available for tissue protein synthesis, muscle fibers and cellular respiration to body for resynthesizing glycogen from lactic acid. This plant can satisfy inland consumption of the geographical entity under consideration.

Table 6. 21 Nutritional analysis of *A. anisacanthus*

Sample Test sample	**Empty dish weight**	**Dish + sample weight(B)**	**Sample weight (D)**	**After drying weight (C)**	**% Moisture contents**
	16.7814	17.7926	1.0112	17.6963	9.5
Ash contents =B-A/C×100					
Sample	Empty dish weight (A)	Dish +sample	Sample weight (C)	After heat dish + Ash weight (B)	% Ash
Test sample	17.9879	19.0004	1.1125	18.0711	7.4
Fats =A-B/C×100					
Sample	Paper weight	Paper + sample	After extraction	Sample weight C)	% Fats
Test sample	0.3307	1.4590	1.1965	1.1283	7.0
Fibers =W1 – W2/W0×100					
Sample	Sample weight (W0)	Cavit + dry sample	Cavit + Ash (W2)	% Fibers	
Test sample	1.0013	28.9698	28.7373	23.21	
Proteins = (Titre×N ×14×100×6.25/weight of the sample×1000)					
Sample	Sample weight	Titre	Blank	% Proteins	
Test sample	1.0387	9.9	9.6	8.0	

Gross energy (Cal/100g) = (% Fats) + (% Proteins) + (% carbohydrates) = 51.27

Carbohydrates = 100- Moisture + Ash +Fibers + Fats + Proteins

= 100 - 7.4+9.5+23.21+23.21+7.0+8.0 = 48.73

6. 2. 14 Elemental Analysis

Minerals analysis of *A. anisacanthus* aerial parts are shown in the table 6. 18 and presented in figure 6.22. The analysis revealed that K contents were recorded in higher concentration (114.9ppm) followed by Fe (1.875 ppm) and Zn (0.511 ppm) as compared with other elements which are in lower concentration as Ni (0.072 ppm), Cu (0.096 ppm) and Pb (0.142 ppm). The plant was also evaluated for Mn (0.418 ppm) and Co (0.004 ppm), Cd (0.019ppm) and Cr (0.020 ppm), found up to detection limit.

Anisacanthus quadrifidus (Humming bird Bush) was evaluated for their respective salinity tolerance to increased levels of Na and Ca [198]. In Southern Arizona the seasonal diet seemed deficient in phosphorus, which may affect deer reproduction and general welfare. *A. thurberi* are deciduous plants and rich source of inorganic elements that normally influence during drought on diets and numbers of desert deer [199]. The objectives of elemental analysis is to analyse tissues for trace elements [200].

Table 6.22 Elemental analysis of *A. anisacanthus*

S. No	Elements	Concentration (ppm)
1	Zinc	0.511
2	Manganese	0.418
3	Cobalt	0.004
4	Nickel	0.072
5	Cadmium	0.019
6	Chromium	0.020
7	Copper	0.096
8	Lead	0.142
9	Iron	1.875
10	Potassium	114.9

6. 2. 15 Scanning Electron Microscopy (SEM) Analysis

The SEM technique was used to determine the morphology of the plant sample. figure 6.23-6.25 shows the SEM image of plant powder. Three images of plant powder were taken with three different magnification powers (X150, 500 and 1000). The images showed that there is number of aggregates and also individual identity. The test sample showed needle shape and trichrome like appearance. It is concluded that hairy and needle like structure on the surface of plant help it to survive during drought condition and prevent plant from damage or death due, which are too high or low temperature. Temperature effect the of physiological and biochemical processes, which generally (within limits) increasing with temperature. The morphological characteristics of infection root swellings (nodules) of *A. sinicus* were examined by SEM. The infection roots, epidermal cell walls and vascular bundles of the swellings were not altered when a swelling was treated with driselase (a plant cell wall degrading enzyme), although the cell walls of meristematic and bactericide-including zones were completely decomposed by the enzyme treatment. Some infection threads were funnel shaped at the site of attachment of the infection thread to the host cell wall [201].

The propagation response of these species to mechanical, physical and chemical scarification, applied for overcoming dormancy, has been studied with SEM. Observations with a SEM revealed a thicker palisade layer of the seed coat in *A. humosus*, which may explain the greater resistance of its seeds to chemical and physical scarification in hot water [202].

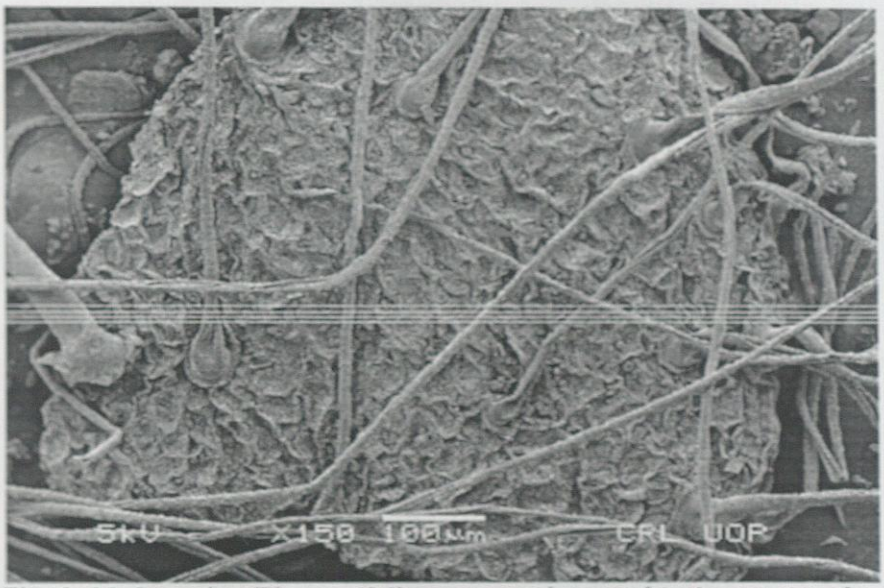


Fig 6. 23 Scanning Electron Microscopy micrograph *A. anisacanthus*

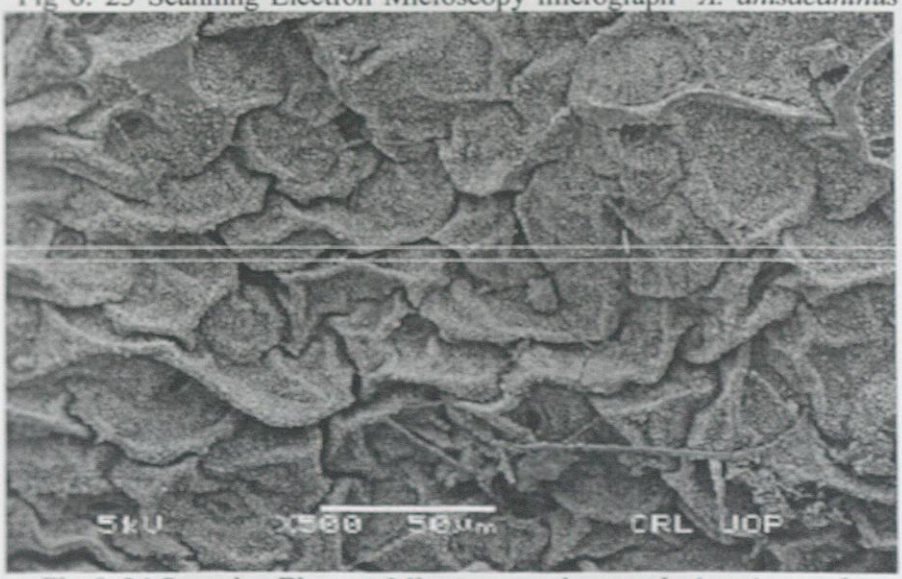


Fig 6. 24 Scanning Electron Microscopy micrograph *A. anisacanthus*

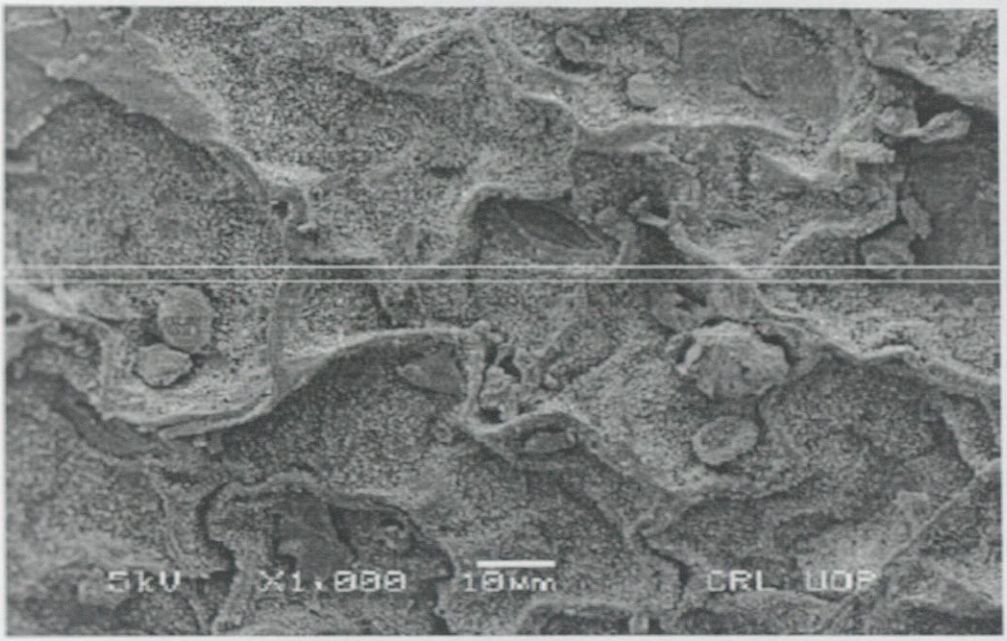


Fig. 6. 25 Scanning Electron Microscopy micrograph of *A. anisacanthus*

6. 2. 16 Energy Dispersive X-Rays Analysis

Inorganic elements, often at the trace level, play a significant role in pathology and physiology of living systems. Metalloproteomics, metallogenomic and metallomics are among the developing disciplines which are critically dependent on spatially resolved concentration maps of trace elements in a cell or tissue, information on chemical speciation and on metal-binding coordination positions [203]. The EDX technique was utilized to determine the amount as well as to identify elementary composition of plant. The EDX pattern of spectrum recorded high signals for carbon and oxygen. Elemental composition of *A. anisacanthus* showed that besides carbon and oxygen other elements; Mg, P, S, K, Cl and Ca were also detected. The counts of X-ray is showing on perpendicular while energy (Kev) on the parallel axis. Figure 6.26 and 6.27 shows the elemental composition of plant material. The carbon composition revealed that by weight it was 10.34% and 22.45% (by atomic weight/ concentration). Similarly O, Mg, P, S, Cl, K and Ca by weight were 44.86, 1.32, 1.56, 2.52, 0.30, 2.54 and 0.96%, respectively.

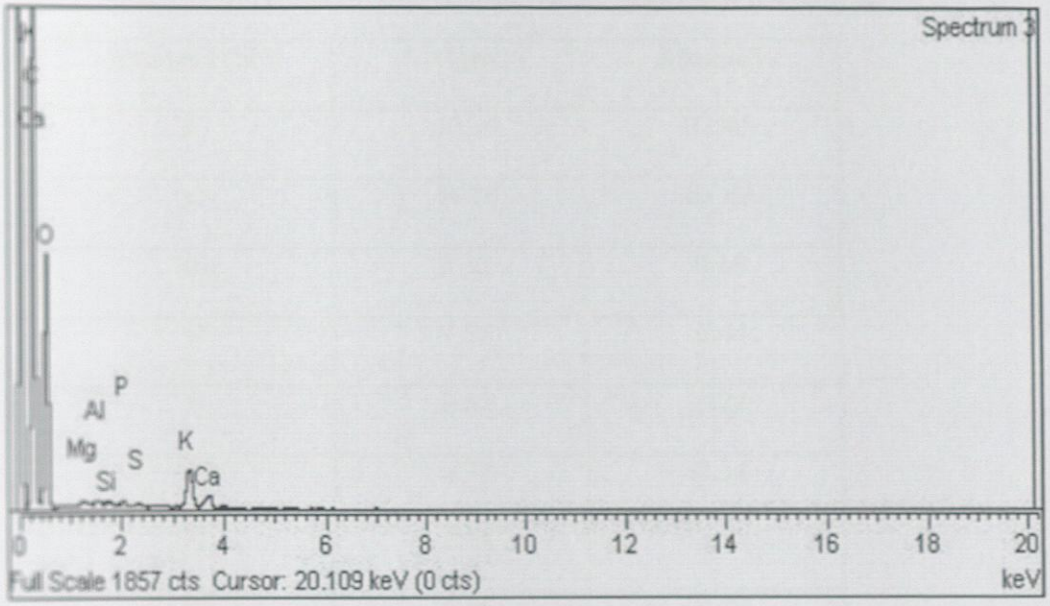


Fig 6. 26 Energy Dispersive X-Rays spectrum for the *A. anisacanthus*

Table 6.23 Percent atomic weights of the elements present in *A. anisacanthus*

Element (K*)	Weight%	Atomic%
C	10.34	22.45
O	44.86	65.89
Mg	1.32	0.19
P	1.56	0.44
S	2.52	1.22
Cl	0.30	0.14
K	2.54	1.53
Ca	0.96	0.32

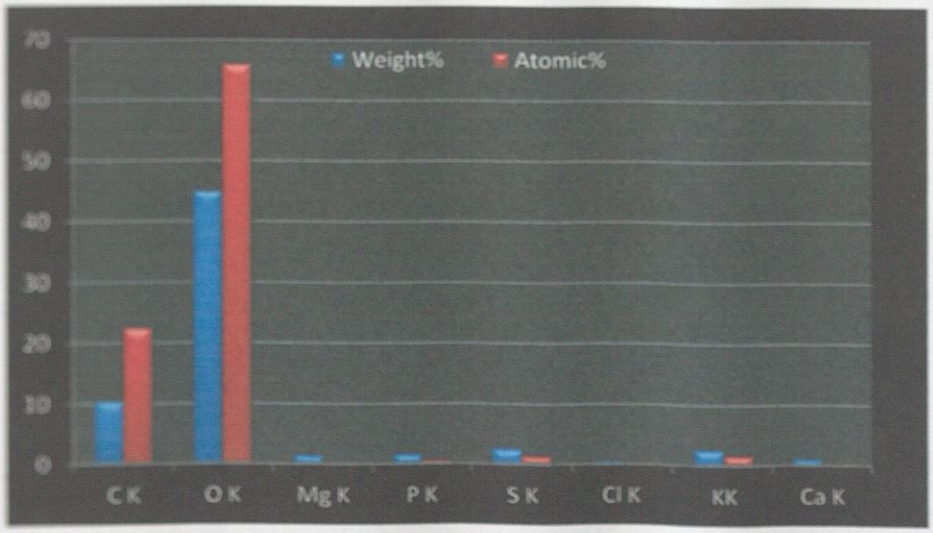


Fig 6. 27 Percent weight of the elements in *A. anisacanthus*

6. 2. 17 Structural Elucidation of Compounds

6. 2. 17.1 Structural Elucidation of Stigmasterol (1)

Stigmasterol was obtained as a white needle crystal from the $CHCl_3$ fraction of *A. anisacanthus*. It exhibited a molecular ions peak at *m/z* 412 a.m.u. which consistent with the molecular formula $C_{29}H_{48}O$. The IR spectrum (cm^{-1}) showed absorption bands at 3500 for (OH), 2965, and 2935 for CH stretching 2870, 1680 for double bond stretching. The UV spectrum showed absorptions peaks at 242 and 261 nm. The ^{1}H-NMR and ^{13}C-NMR spectra are given in table 6.24. The spectroscopic analysis of compound (1) matched with reported compound [204].

Table 6. 24 ^{1}H and ^{13}C-NMR and Chemical Shifts of Stigmasterol (**1**) (ppm, CD3OD, 400 and 100 MHz, respectively).

No	^{13}C-NMR δ_C	^{1}H-NMR δ_H (mult, *J*, HZ)	Types
1	37.3	1.07, dd, (*J*=3.5/6.11)	CH_2
2	31.8	1.23, d, (*J*=1.48)	CH_2
3	71.9	3.51, m	CH_2
4	42.3	2.00, t, (*J*=9); 1.99, d, (*J*= 3.12)	CH_2
5	140.8	-	C
6	121.8	5.34, d, (*J*=2.34)	CH
7	32.2	1.44, m	CH_2
8	31.9	1.42, m	CH
9	50.5	0.84, m	CH
10	37.1	-	C
11	20.9	1.49,m, 1.47 m	CH_2
12	42.3	2.19, d, (*J*=2.1); 2.25, dd, (*J*=2.1, 3)	CH_2
13	39.8	-	C
14	56.8	0.96, m	CH
15	24.3	1.56, m	CH_2
16	28.3	1.23, m	CH_2
17	56.1	1.07, dd, (*J*=7.8, 3.48)	CH
18	11.9	0.66, s	CH_3
19	19.4	0.99, s	CH_3
20	36.2	1.34, m	CH
21	21.4	1.20, d, (*J*=7)	CH_3
22	139.4	5.03, dd, (*J*=15, 8.5)	CH
23	129.6	1.14, dd, (*J*=15.1, 8.5)	CH
24	52.9	0.92, m	CH
25	30.2	1.25, m	CH
26	19.11	0.79, d, (*J*=6.8)	CH_3
27	19.90	0.81, d, (*J*=5.58)	CH_3
28	23.12	1.22, m	CH_2
29	12.06	0.82, t, (*J*=7.5)	CH_3

6. 2. 17.2 Structural Elucidation of Octadecan-1-ol (2)

The $CHCl_3$ fraction was chromatographed over silica column and eluted with mixture *n*-hexane and $CHCl_3$ (8:2) as mobile phase and the compound was isolated in pure form. The excess of solvent (mobile phase) was evaporated to give the pure product. The molecular formula is $C_{18}H_{38}O$ was determined from its EI-MS; *m/z*: 270 $[M]^+$: ^{1}HNMR and ^{13}C -NMR data was used to identify the structure of the compounds (table 6. 25). The spectroscopic analysis of compound (2) matched with reported compound [205].

Table 6. 25 ^{1}H and ^{13}C-NMR and Chemical Shifts of Compound (**2**) (ppm, CD3OD, 400 and 100 MHz, respectively).

No	Position of Carbon	^{13}C- NMR data δ_C	^{1}H-NMR data δ_H	Multiplicity
1.	1	63.08	4.83 (2H, s,)	CH2
2.	2	32.88	3.30 (2H, s)	CH2
3.	3	25.74	1.28 (2H, s)	CH2
4.	4-17	31.68	0.90(30, m)	CH2
5.	18	14.12	0.858 (3H, m)	CH3

octadecan-1- ol

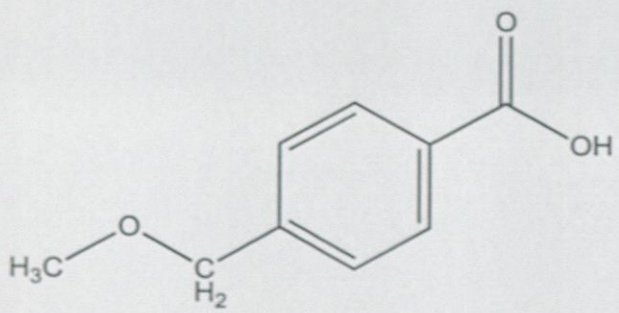


6.2.17.3 Structural Elucidation Of 4-(methoxymethyl) benzoic acid (3)

The $CHCl_3$ fraction was chromatographed over silica column and eluted with *n*-hexane and chloroform (2:8) as mobile phase and the compound was isolated in pure form. The excess of solvent (mobile phase) was evaporated to give the pure product. The molecular formula is $C_9H_{10}O_3$ was determined from its El-MS; *m/z*: (167.8) $[M]^+$ and ^{1}HNMR and ^{13}CNMR data was using to identify structure (table 6.26). The spectroscopic analysis of compound (4) matched with reported compound [206]

Table 6. 26 ^{1}H and ^{13}C-NMR and Chemical Shifts of Compound (**3**) (ppm, CD3OD, 400 and 100 MHz, respectively).

No	Position of Carbon	^{13}C- NMR data δ_C	^{1}H-NMR data δH	Multiplicity
1	1	124.92	-	C
2	2-3	115.78	7.85(2H, d, J= 8.8)	2CH
3	4	132.81	-	C
4	5-6	115.58	6.7 (2H, d, J=8)	2CH



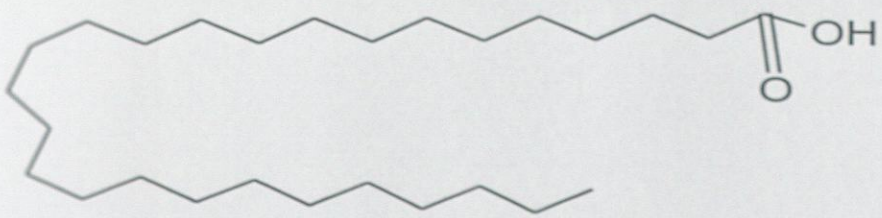
4-(Methoxymethyl) benzoic acid

6. 2. 17. 4 Structural Elucidation of Heptadecanoic acid (4)

The $CHCl_3$ fraction was chromatographed over FCC and eluted with ethyl acetate and acetone (5:5) as mobile phase and the compound was isolated in pure form. The excess of solvent (mobile phase) was allowed to evaporate for isolation of pure compound. The molecular formula of isolated compound was $C_{27}H_{54}O_2$ determined from its El-MS; *m/z*: (410) $[M]^+$ and ^{1}H-NMR and ^{13}CNMR data was using to identify structure (table 6. 27). The spectroscopic analysis of compound (4) matched with reported compound [207].

Table 6.27 ^{1}H and ^{13}C-NMR and Chemical Shifts of Compound (4) (ppm, CD3OD, 400 and 100 MHz, respectively).

No	Position of Carbon	^{13}C- NMR data	^{1}H-NMR data	Multiplicity
1.	1	176.34	-	C
2.	2	33.58	3.64(2H, s)	CH2
3.	3-25	28.93	2.264(2H, t, J=14.8)	CH2
4.	26	26.68	1.57 (2H,t, J=14)	CH2
5.	27	12.99	0.89 (3H, t, J=22)	CH3



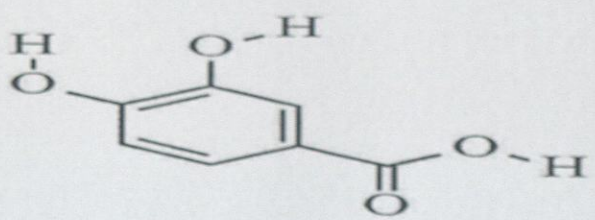
Heptadecanoic acid

6. 2. 17. 5 Structural Elucidation Of 3, 4-Dihydroxybenzoic acid (5)

The EtOAc fraction afforded compound (5) as white powder. The IR spectrum exhibited hydroxyl (3390 cm-1), C–H aromatic (3315 cm-1), carbonyl (1710 cm-1) and C=C aromatic (1627 cm-1) absorption bands. Its molecular formula ($C_7H_6O_4$) was determined by ^{1}H- and ^{13}C-NMR analysis and EI-MS which gave Molecular ion peak [M + H] + at m/z 155 and [M + Na]+ at 177 in ESI-positive mode and [M – H]- at m/z 153 in ESI-negative mode, suggesting a molecular mass 154 a.m.u. The NMR spectral data (Table 6.28) of compound (5) matched with reported compound [208].

Table 6.28 ^{1}H and ^{13}C-NMR and Chemical Shifts of Compound (5) (ppm, CD3OD, 400 and 100 MHz, respectively).

C. NO	^{13}C- NMR	^{1}H-NMR	Multiplicity
1.	123.6	-	C
2.	118.5	7.43, (d, 1.8)	CH
3.	145.6	-	C
4.	151.9	-	C
5.	116.8	6.74, (d, 8.2	CH
6.	124.7	7.48, (dd, 1.8, 8.2)	CH
7.	171.5	-	C



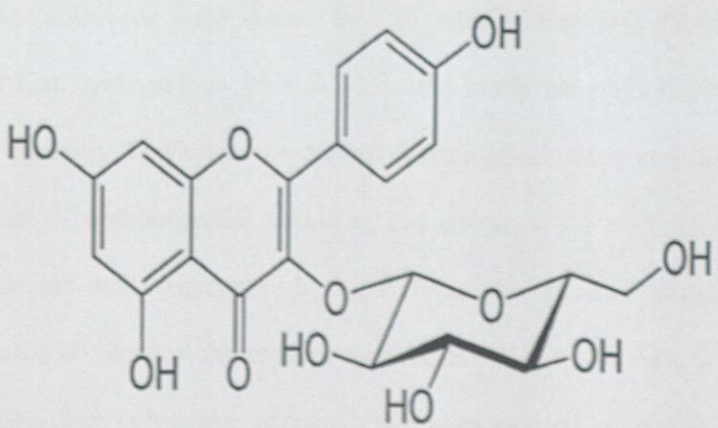
3, 4-Dihydroxybenzoic acid (5)

6.2.17.6 Structural Elucidation of Kaempferol-3-β-D-glucopyranoside (astragalin) (6)

Compound 6 was isolated as yellow needles. The molecular formula ($C_{21}H_{20}O_{11}$) was determined by ESI-HR-MS m/z 449.1074 a.m.u. calcd. 449.1070 a.m.u. The IR spectrum indicated presence of phenolic groups (3380 cm^{-1}), and a carbonyl carbon (1680 cm^{-1}) while UV spectrum was consistent with the presence of highly conjugated system (λ-max 356nm). The ^{1}H- and ^{13}C-NMR of compound 6 are identical with the spectral data of reported compound astragalin from literature. The ^{1}H-NMR and ^{13}C-NMR spectrum are shown in (Table 6. 29) molecular formula $C_{21}H_{20}O_{11}$. This compound closely matched with reported compound [209]

Table 6.26 ^{1}H and ^{13}C-NMR and Chemical shifts of compound (6) (ppm, CD3OD, 400 and 100 MHz, respectively).

C. NO	^{13}C- NMR data	^{1}H-NMR data	Multiplicity
2	158.8	-	C
3	135.4	-	C
4	179.2	-	C
5	162.9	-	C
6	100.8	6.14, d (J=1.8)	CH
7	95.4	-	C
8	158.4	6.32, d (J=1.8)	CH
9	104.9	-	C
10	123.1	-	C
1`	132.4	-	C
2`, 6`	115.9	8.04, d (J=8.5)	CH
3`, 5`	161.6	6.87, d (J=8.5)	CH
4`	104.3	-	C
1`	76.1	5.18, d (J=7.0)	CH
2`	77.9	3.42, t (J=6.5)	CH
3`	78.1	3.38, t (J=6.5)	CH
4`	79.8	3.1, m	CH
5`	71.3	3.53, dd (J=11.5, 5)	CH
6`	62.1	3.67,dd (J=11.5, 5)	CH



Kaempferol-3-β-D-glucopyranoside (astragalin) (6)

CONCLUSION

Based on the experimental work, it is concluded that, the Crd. MeOH Ext. of the fruits of *S. saligna* showed significant anti-termite activity (n=25), analgesic activity (31.67), GIT Motility (14.5%) and allelopathy (56.6%). Good antibacterial activity against *MSSA* and *S. typhi* and locomotor activity were shown by Crd. MeOH Ext. and various fractions. Moderate phytotoxic activity and NO free radical scavenging activity was shown by the test samples. Low insecticidal, antifungal cytotoxic and antidepressant activities were shown by Crd. MeOH Ext. and various fractions respectively. No heamagglutination activity was observed. The phytochemical screenings of *S. saligna* fruits showing the presence of carbohydrates, proteins, alkaloids, sterols and triterpenoids, phenols, flavonoids , tannins and saponins.. Elemental analysis showed the presence of Zn, K, Ni, Cu and Pb, Mn, Co, Cd, Fe and Cr. The EDX pattern of spectrum recorded high signals for C and O_2. Maximum number of oil component were identified through GC-MS analysis.

A. anisacanthus possess significant percent radical scavenging and cytotoxic activity. Good antibacterial, insecticidal, antitermite, analgesic, GIT Motility, locomotor and antidepressant activities were shown by Crd. MeOH Ext. and various fractions. The Crd. MeOH Ext. and various fractions showed moderate phytotoxic activity. The *n*-hexane and aqueous fractions showed low heamagglutination activity. Phytochemical screenings of *A. anisacanthus* showing the presence of carbohydrates, proteins, alkaloids, sterols and triterpenoids, phenols, flavonoids, tannins and saponins. Elemental analysis showed the presence of Zn, K, Ni, Cu, Pb, Mn, Co, Cd, Fe and Cr. The phytochemical screening revealed the presence of alkaloids, flavonoids and saponins in these plants. Total of six compounds were isolated during current studies from *A. anisacanthus* Octadecan-1-ol (Stearyl Alcohol), 4-(methoxymethyl) benzoic

acid (Phenylmethanoic acid), Heptadecanoic acid (Margaric), 3, 4 Dihydroxybenzoic acid, Kaempferol-3-β-D-glucopyranoside and stigma sterol was isolated in pure form. Our current work open 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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