4.0 INTRODUCTION AND LITERATURE REVIEW

4.1 RHAMNACEAE (Family)

4.1.1 Description

This family consists of 50 genera and more than 900 species [122]. Trees or shrubs with simple, usually stipulate leaves. Inflorescence Cymose. Flowers small, green, yellow or blue, sometime unisexual. Calyx tubular, 4-5 lobed, lobes valvate in bud. Petals 4-5, sometimes 0, small, inserted at mouth of calyx-tube and often hooded. Stamens 4-5, opposite the petals and often ± enclosed by them; anthers versatile. Ovary 2-4-celled, free or sunk in disk; ovules solitary, basal, erect, anatropous. Fruits are often fleshy [123].

4.1.2 Distribution

This family has a worldwide distribution but is more common in the tropical and subtropical regions of the world [123].

4.1.3 Importance

Several species of *Rhamnaceae* notably *R. cathartica* and *R. frangula* have been used as laxatives. Drugs as well as yellow and green dyes have been obtained from different species of *Rhamnus*. Timber of *Colubrina*, *Alphitonia*, *Ziziphus* and *Hovenia* species is used for fine furniture, construction, carving, musical instruments and lathwork [124]. *Hovenia dulcis* is important for its fleshy, edible inflorescence stalks. Species of *Rhamnus, Hovenia* and *Paliurus*, are cultivated as ornamentals [124]. *Rhamnus procumbens* have anticonvulsant, anti-inflammatory and anti-cancerous properties. This specie contains 'emodin' which exhibit properties as cardiac and intestinal stimulant, central nervous system depressant and analgesic in experimental animals [125]. The bark of *Rhamnus purshiana* is used as stool softener, non-habit

forming stimulant, laxative and pancreatic stimulant. It is also used for dyspepsia and habitual constipation [125]. The dried bark of *Rhamnus frangula* and dried ripe berries of *Rhamnus catharticus* are also used for constipation [125]. The bark of *Rhamnus triquetra* is used a tonic, astringent and deobstruent [125]. The fruit, pounded and macerated in vinegar, is prescribed for the treatment of herpes [125]. The ripe fruit of *Rhamnus virgatus* is purgative and emetic while the bark of *Rhamnus wightii* is astringent and deobstruent [125].

4.2 ZIZYPHUS (GENUS)

4.2.1 Description

Zizyphus is genus of 40 species. The members are trees or erect or climbing shrubs, usually armed with sharp, straight and hooked thorns, which are transformed stipules. Thorns solitary or in pairs, usually one straight, the other curved. Leaves alternate, subdistichous, 3-5 ribbed. Flowers small, greenish or yellowish, in axillary fascicles or in sessile or peduncled cymes. Calyx with broadly obconic tube and 5 triangular acute lobes keeled within, lobes valvate. Petals 5, or rarely 0, osucullate and deflexed. Stamens 5, opposite to and enclosed in the petals and usually longer than them. Disk 5-10 lobed, flat or pitted, the margin free. Ovary sunk in or adnate at the base to the disk, 2-4 celled; style 2-3, rarely 4, free or connate; stigma small papillose. Fruit a globose or oblong drupe, with a woody or bony 1-4 celled and seeded stone. Seed plano-convex; albumen 0 or scanty; cotyledons thick; radical short [126].

4.2.2 Distribution

This genus is mostly Indo-Malayan, however some species are found in Africa, Australia and America. The members of this genus are also found in the sub-continent region of South Asia. The medicinally important species of this genus are *Zizyphus jujuba*, *Ziziphus glaberata*, *Ziziphus mauritiana* and *Ziziphus rugosa* [126].

4.2.3 Importance

Many *Ziziphus* species are popular for their edible fruit; among them, *Z. mauritiana* (Indian jujube) and *Z. jujuba* (Chinese jujube) are cultivated on a commercial scale [124]. The members of this family are utilized for a variety of purposes. Some are important for their wood to be used in furniture and other ornamental purposes, some for food while others are important for their medicinal value. For example the fruits of *Ziziphus funiculosa* are edible. The fruits of *Z. glaberata* are well known for their pectoral and emollient properties. The fruits when matured are sour but the dried ones are sweet and delicious [127]. For blood purification and venereal diseases, decoction of the leaves is used [127]. The wood of *Z. mauritiana* is reddish in color and hard in quality and is therefore used in agricultural implements. It is also a source of charcoal and fuel [127]. Fruit acts as a medicine in stomatche, astringency, indigestion, biliousness, laxative, blood purification, scabies, nausea, vomiting and throat troubles. The fruit also has pectoral and emollient properties. The bark of *Z. mauritiana* is used in diarrhea and astringency. The powdered bark of *Z. mauritiana* is used in ulcers and wound's dressing. Root is helpful in curing delirium, fever, gout, rheumatism and is purgative. Tendered leaves and twigs are helpful in curing abscesses, boils, and carbuncles [128]. The fruit of *Ziziphus oenoplia* is edible while its bark is used for tanning [129]. The wood of *Z. rugosa* is mainly used for fuel. The fruits are consumed by people and leaves as fodder by animals. The powder of bark when mixed with ghee is used as medicine in mouth's ulcer and swelling in cheek [130].

4.3 ZIZYPHUS JUJUBA (PLANT)

4.3.1 Description

Botanical Name: *Zizyphus jujuba*

Family: *Rhamnaceae*

Genus: *Zizyphus*

Species *jujuba*

Common Names: Red Date, Chinese date

Local name: Bera (In Pushto language)

A small sub-deciduous tree with dense spreading crown, commonly 0.6 m girth and 6 m high. Bark blackish to grey or brown, rough, regularly and deeply furrowed, the furrows about 1.2 cm apart. Blaze 9-13 mm, short fiber and pink, with or without paler streaks. The juice turning purplish black on the blade of a knife. Branches usually armed with spines, mostly in pairs, one straight and the other one curved. Young shoots more or less densely pubescent. Leaves 3-6.3 by 2.5-5 cm, oblong or ovate, usually minutely serrulate or apex distinctly toothed, obtuse, base oblique and 3-nerved, nerves depressed on the glabrous shining upper surface, densely clothed beneath with white or buff tomentum. Petiole 2.5-10 mm long. Flowers 3.8-5 mm diameter, greenish, in dense axillary, tomentose cymes or fascicles 1.2-1.9 cm long. Drupe 1.2-2.5 cm diameter, globose, first yellow then orange and finally reddish brown, containing a single stone surrounded by fleshy pulp [126].

4.3.2 Distribution

Z. jujuba is indigenous and naturalized throughout India, Burma, Pakistan and Ceylon, in the outer Himalaya up to 4,500 fts. This specie is also found in China, Afghanistan, Africa and Australia [126].

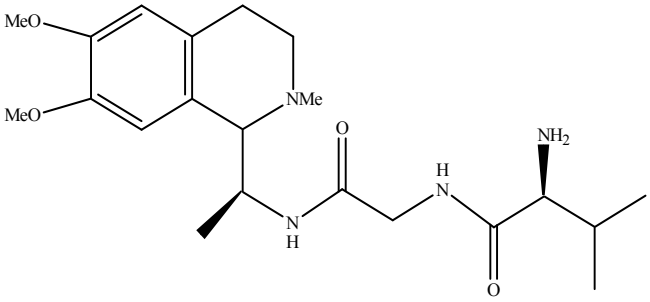
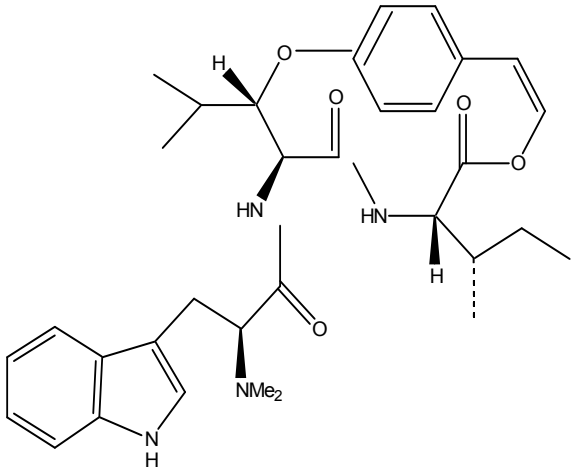
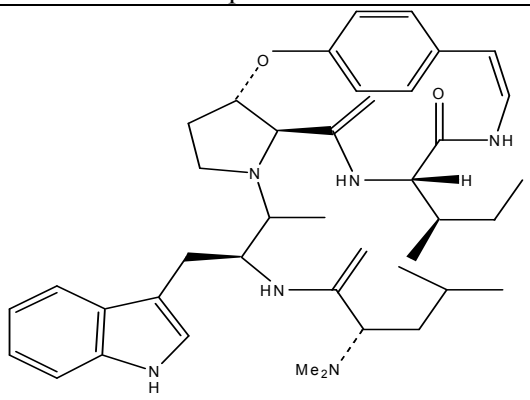
4.3.3 Ethno-botanical uses

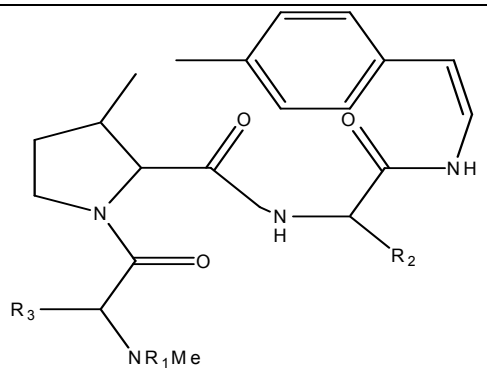
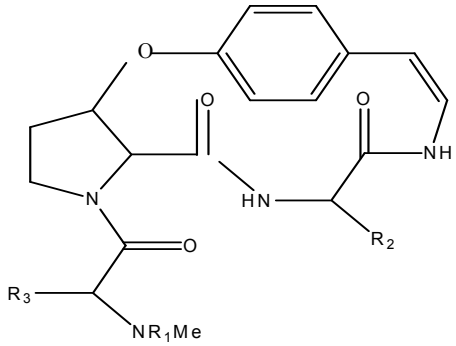
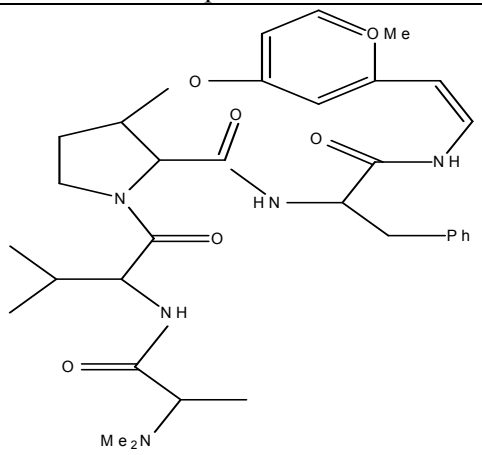
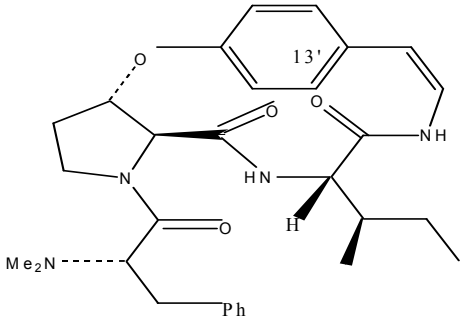
Z. jujuba commonly called, Red Date or Chinese date or Bera (Pushto), belonging to family *Rhamnaceae*, is used primarily for its fruits. Jujube, a delicious fruit, is an effective herbal remedy improving stamina and muscular strength and aids weight gain [131]. It strengthens liver function and increases immune system resistance [131]. It functions as antidote, diuretic, emollient and expectorant [132-133]. The leaves are febrifuge, astringent and said to promote the hair growth [134]. In the treatment of strangury they are used to form a plaster [135]. The dried fruits are anticancer, anodyne, refrigerant, sedative, styptic, pectoral, tonic and stomachic [134]. They help in digestion and blood purification [136]. They are used internally to treat loss of appetite, chronic fatigue, hysteria, diarrhea, irritability and anemia [136-137]. The seed is sedative, stomachic, hypnotic, tonic and narcotic [133-137]. It is used internally to treat insomnia, nervous exhaustion, palpitations, excessive perspiration and night sweats [133, 137]. For the treatment of dyspepsia and fevers, root is used [134-135]. The powdered root is applied to old ulcers and wounds [135]. The plant is a folk remedy in China as a treatment for burns, anemia, nephritis, hypertonia and nervous diseases [134]. The seeds have been used as tranquilizer, analgesic, convulsant in oriental countries like Korea and China [138]. Ziziphin, a compound in the leaves of the jujube, suppresses the ability to perceive sweet taste in humans [139]. The mucilaginous nature of the fruit of jujube makes them a candidate in pharmacy to treat sore throats. *Z. jujuba* extracts exhibited a protection against hydroquinone induced cytogenesis [140]. Extracts of *Z. jujuba* fruits and seeds exhibited moderate activity against *Lycoriella ingenua* and *Coboldia fuscipes*, which are important mushroom pests [141].

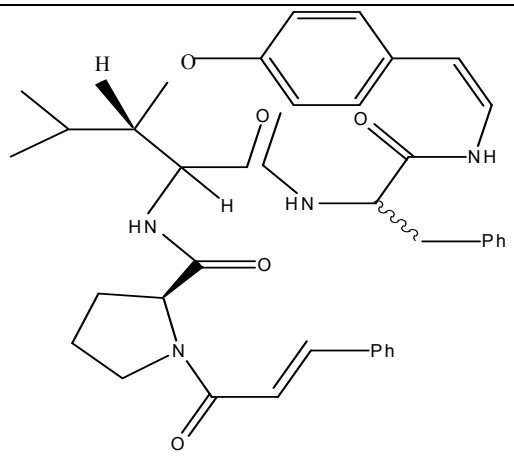
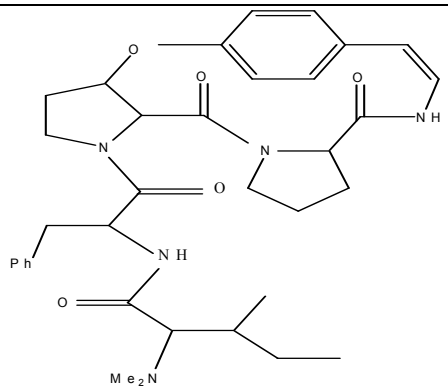
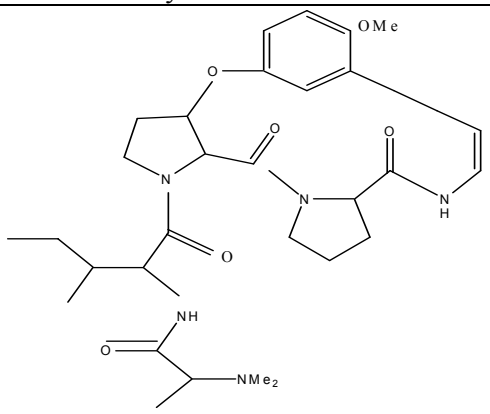
4.4 PHYTOCHEMISTRY OF GENUS *ZIZYPHUS*

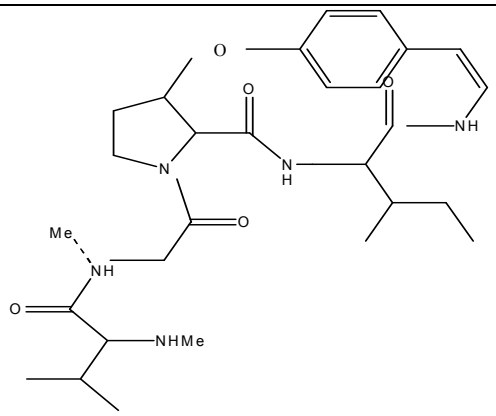
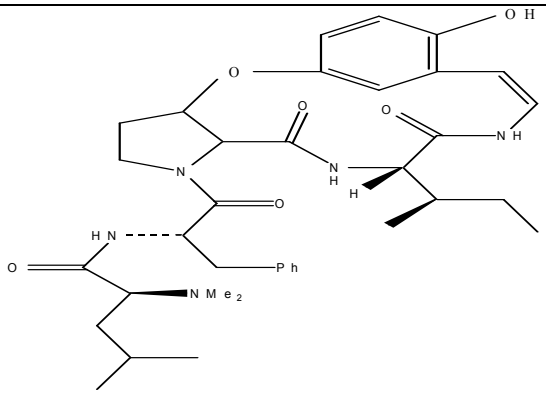
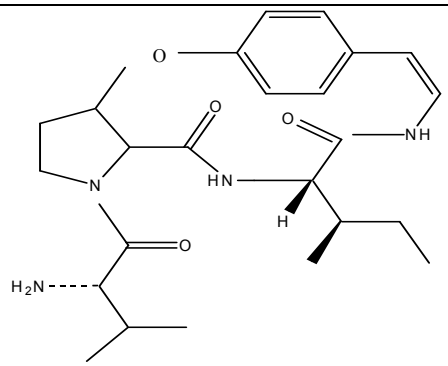
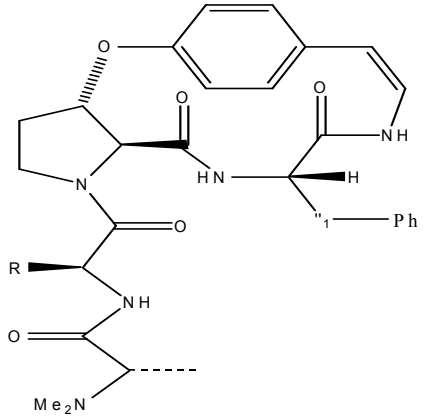
The literature shows that this genus is a rich source of alkaloids, triterpenes and flavonoids. **Table 4.1** shows the phytochemical investigation of genus *Zizyphus*.

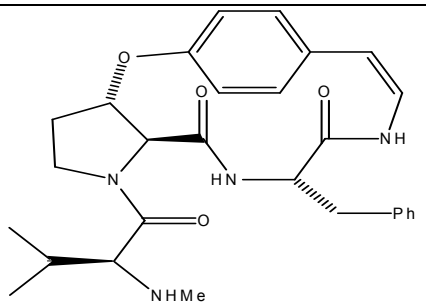
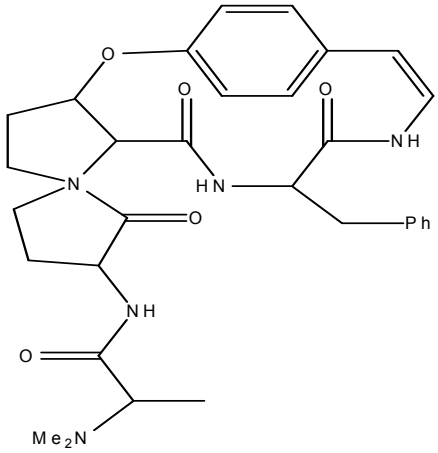
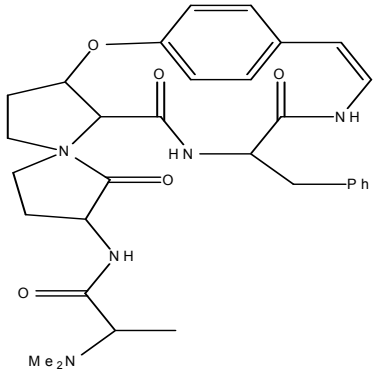
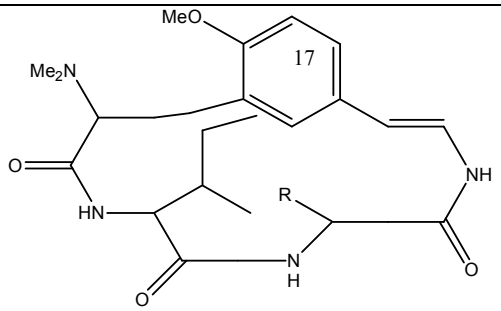
Table 4.1 Phytochemical constituents from genus *Zizyphus*

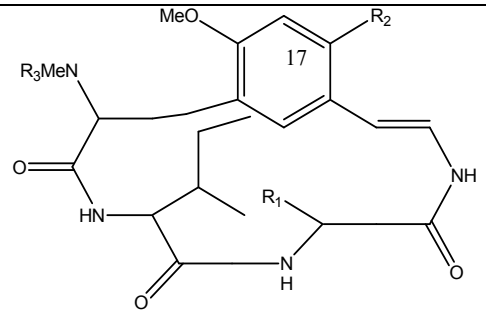
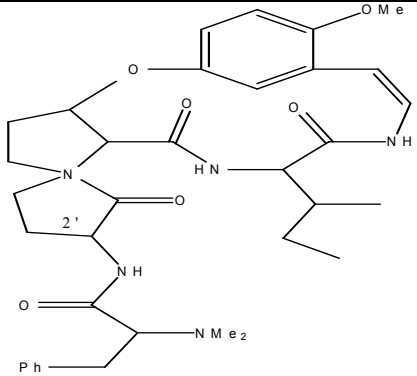
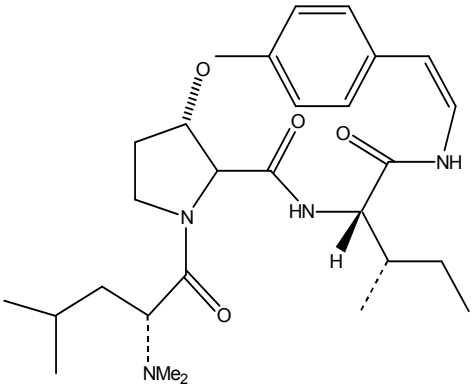
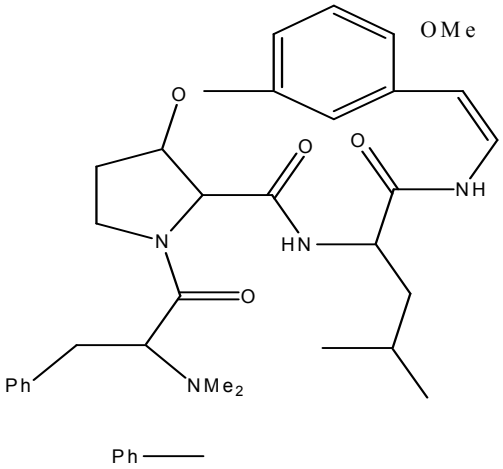
S.NO	Mol. Formula/ Mol. Wt/ Plant name	Compound structure/ Name	Reference
1	$C_{21}H_{34}N_4O_4$/ 406.524 *Zizyphus nummularia*	 Amphibine I	Tschesche R et al., Chem. Ber. 1974, 107, 1329
2	$C_{33}H_{43}N_5O_4$/ 573.734 *Zizyphus amphibia*	 Amphibine A	Tschesche R et al., Phytochemistry. 1974, 13, 1633
3	$C_{38}H_{50}N_6O_5$/ 670.85 *Zizyphus amphibia*	 Amphibine E	Tschesche et al., Phytochemistry. 1974, 13, 1633

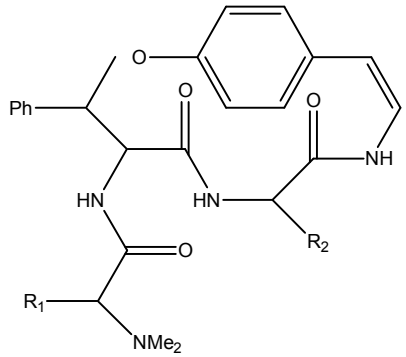
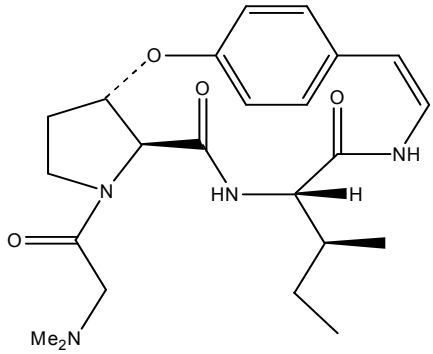
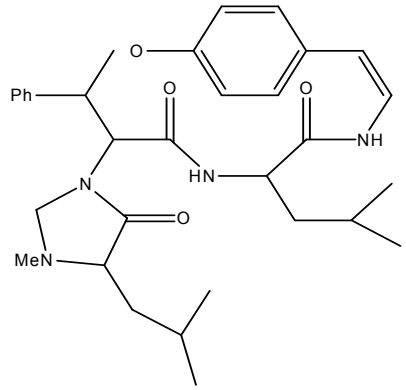
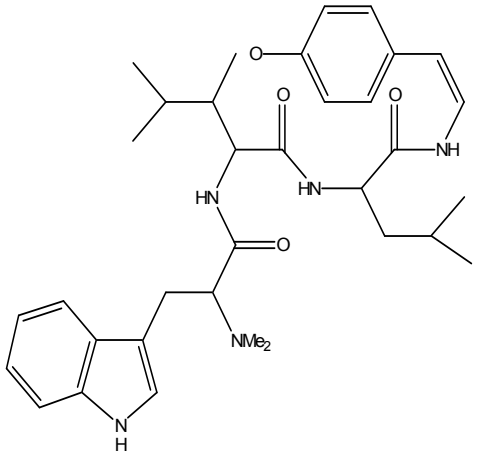
4	$C_{29}H_{36}N_4O_4$/ 504.628 *Zizyphus amphibia*	 Amphibine F	Tschesche R et al., Chem. Ber. 1974, 107, 686
5	$C_{32}H_{39}N_5O_4$/ 557.691 *Zizyphus nummularia*	 Amphibine G	Tschesche R et al., Chem. Ber. 1974, 107, 686
6	$C_{33}H_{43}N_5O_6$/ 605.733 *Zizyphus amphibia*	 Amphibine H	Tschesche R et al., Chem. Ber. 1974, 107, 686; 3180
7	$C_{30}H_{38}N_4O_4$/ 518.655 *Zizyphus lotus*	 Lotusine A	Ghedira K et al., Phytochemistry. 1993, 32, 1591-1594

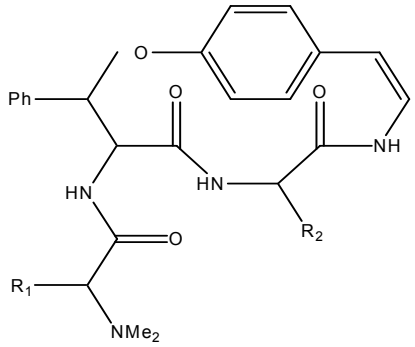
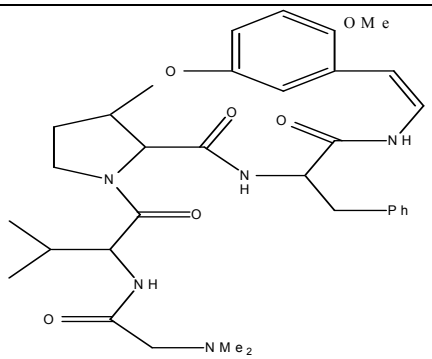
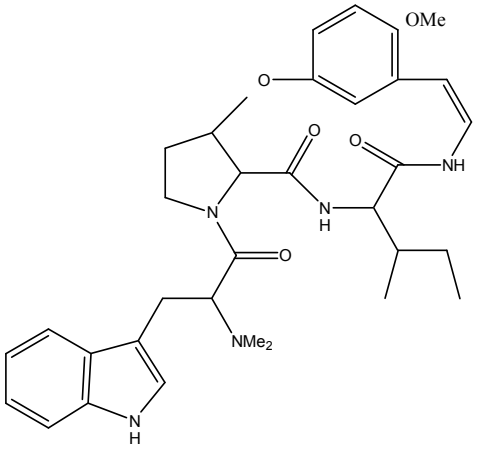
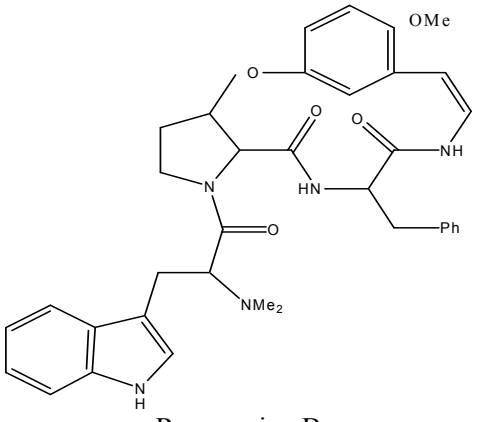
8	$C_{37}H_{40}N_4O_5$/ 620.747 *Zizyphus lotus*	 Lotusanine B	Abu-Zarga M et al., J. Nat. Prod. 1995, 58, 504-511
9	$C_{35}H_{45}N_5O_5$/ 615.771 *Zizyphus hutchinsonii*	 Hysodricanine A	Tschesche R et al., Phytochemistry. 1977, 16, 1025-1028
10	$C_{30}H_{43}N_5O_6$/ 569.7 *Zizyphus hutchinsonii*	 Hysodricanine B	Khokhar I et al., Sci.Int (Lahore). 1993, 5, 37-39

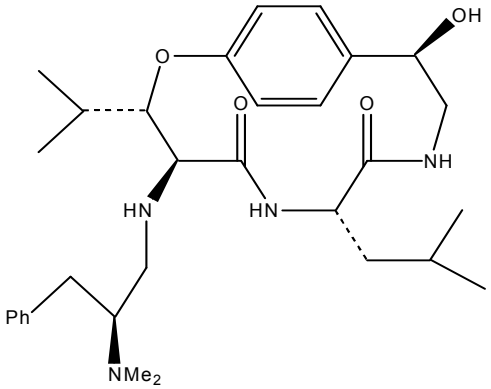
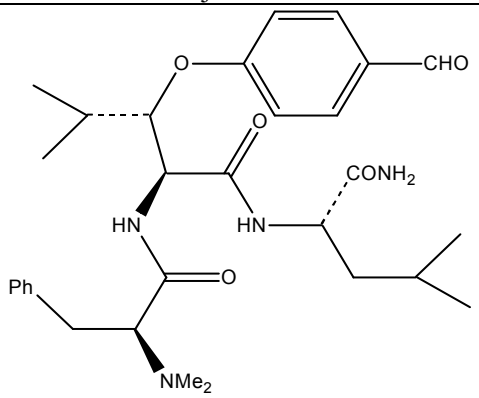
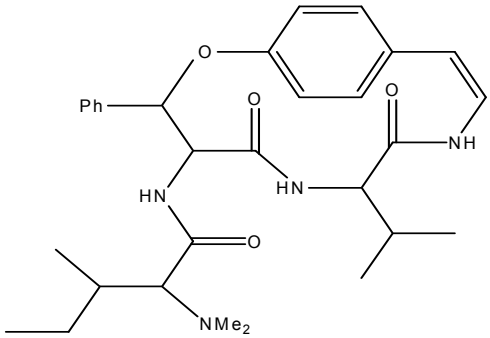
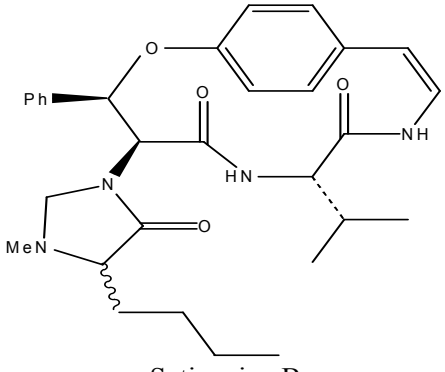
11	$C_{35}H_{47}N_5O_5$/ 617.787 *Zizyphus lotus*	 Lotusine C	Ghedira K et al., Phytochemistry. 1995, 38, 767
12	$C_{36}H_{49}N_5O_6$/ 647.813 *Zizyphus lotus*	 Lotusine E	Ghedira K et al., Phytochemistry. 1995, 38, 767-772
13	$C_{24}H_{34}N_4O_4$/ 442.557 *Zizyphus lotus*	 Lotusine G	Le Croueour G et al., Fitoterapia. 2002, 73, 63-68
14	$C_{32}H_{41}N_5O_5$/ 575.706 *Zizyphus nummularia*	 Mauritine A	Tschesche R et al., Tet. Lett. 1972,2609-2612

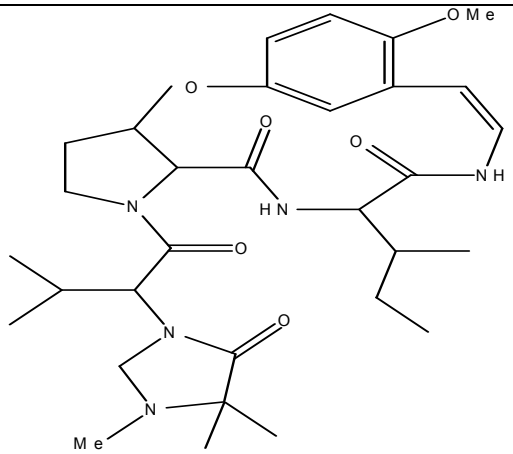
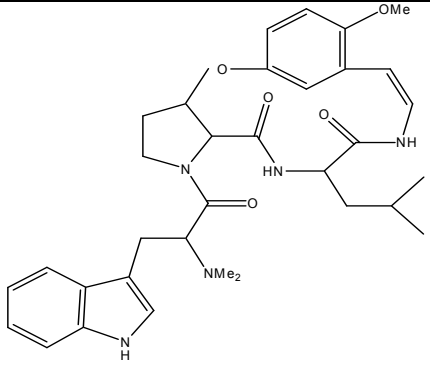
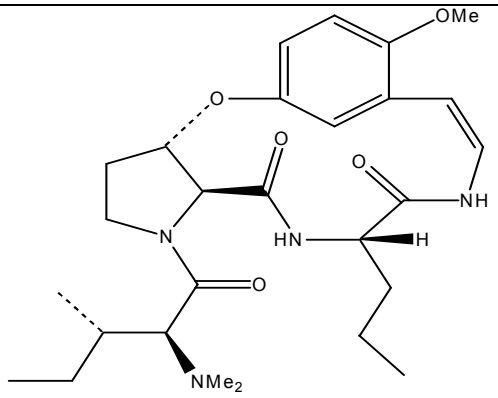
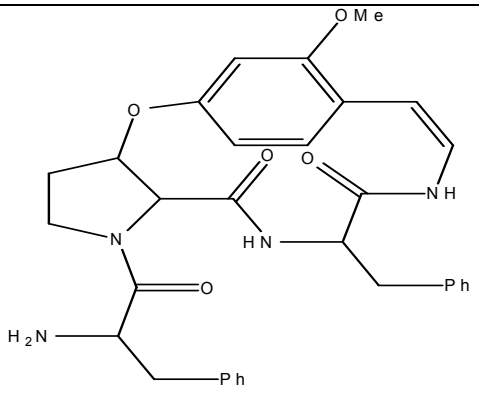
15	$C_{28}H_{34}N_4O_4$/ 490.601 *Zizyphus nummularia*	 Mauritine C	Tschesche R et al., Annalen. 1974,1694-1701
16	$C_{33}H_{51}N_5O_5$/ 597.796 *Zizyphus nummularia*	 Mauritine D	Tschesche R et al., Annalen. 1974,1694-1701
17	$C_{33}H_{43}N_5O_5$/ 589.733 *Zizyphus nummularia*	 Mauritine H	Tschesche R et al., Phytochemistry. 1977, 16, 1025-1028
18	$C_{29}H_{38}N_4O_4$/ 506.644 *Zizyphus mucronata*	 Mucronine A	Fehlhaber HW et al., Annalen. 1972, 759, 195

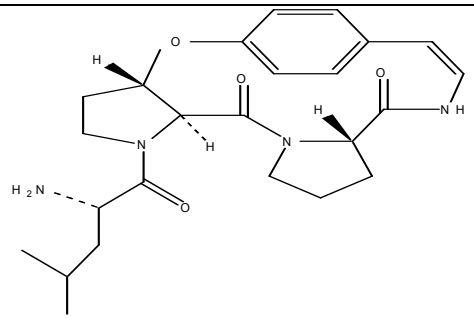
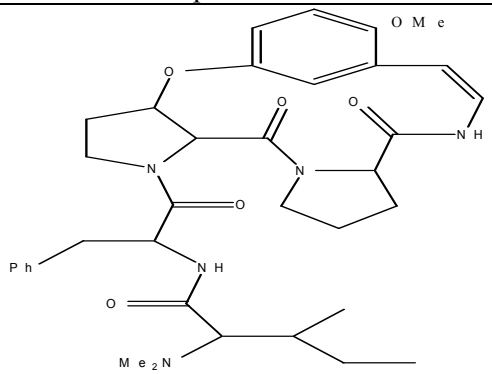
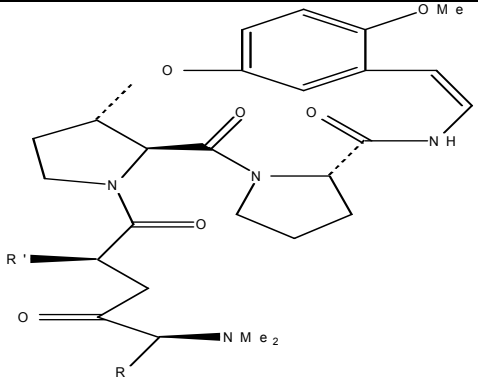
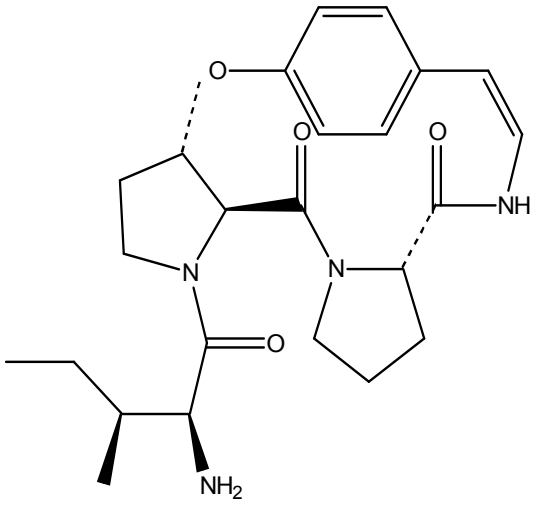
19	$C_{26}H_{40}N_4O_4$/ 472.626 *Zizyphus mucronata*	 Mucronine C	Fehlhaber HW et al., Annalen. 1972, 759, 195-207
20	$C_{37}H_{51}N_5O_6$/ 661.84 *Zizyphus mucronata*	 Mucronine D	Tschesche R et al., Chem. Ber. 1972, 105, 3106-3114
21	$C_{27}H_{40}N_4O_4$/ 484.637 *Zizyphus mucronata*	 Mucronine J	Auvin C et al., I. Nat. Prod. 1996, 59, 676- 678
22	$C_{31}H_{40}N_4O_5$/ 548.681 *Zizyphus nummularia*	 Nummularine C	Tschesche R et al., Chem. Ber. 1974, 107, 3180

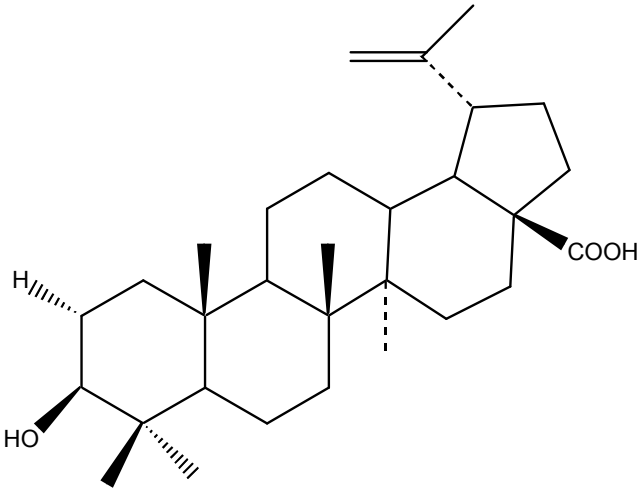
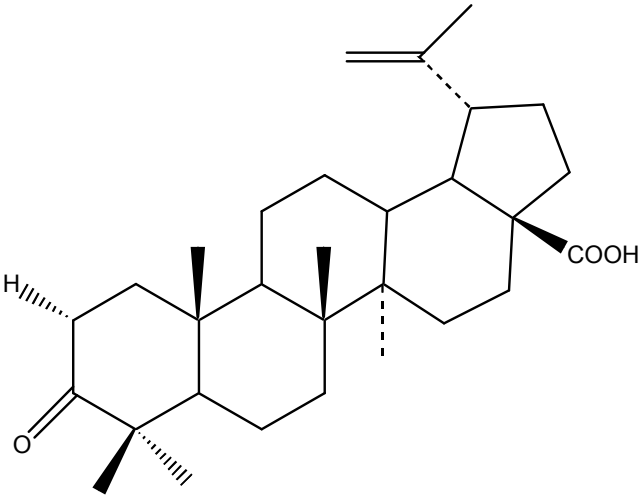
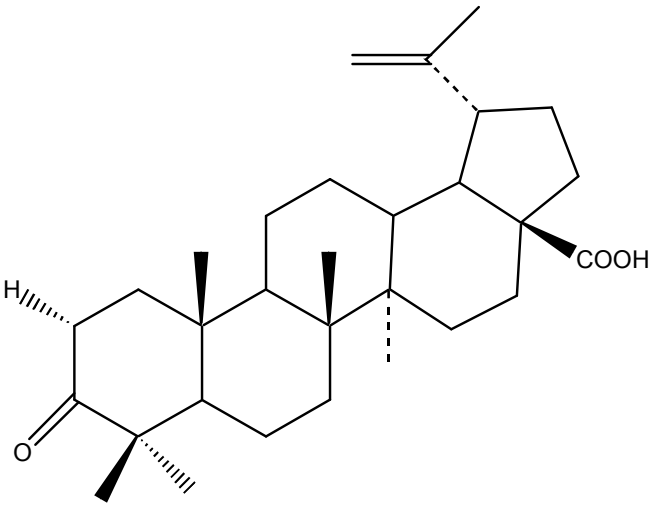
23	$C_{29}H_{38}N_4O_5$/ 522.643 *Zizyphus nummularia*	 Nummularine E	Tschesche R et al., Tetrahedron. 1975, 31, 2944-2947
24	$C_{23}H_{32}N_4O_4$/ 428.53 *Zizyphus nummularia*	 Nummularine F	Tschesche R et al., Tetrahedron. 1975, 31, 2944-2947
25	$C_{31}H_{40}N_4O_4$/ 532.681 *Zizyphus nummularia*	 Nummularine G	Tschesche R et al., Chem. Ber. 1977, 110, 2649
26	$C_{33}H_{43}N_5O_4$/ 573.734 *Zizyphus nummularia*	 Nummularine K	Tschesche R et al., Chem. Ber. 1977, 110, 2649

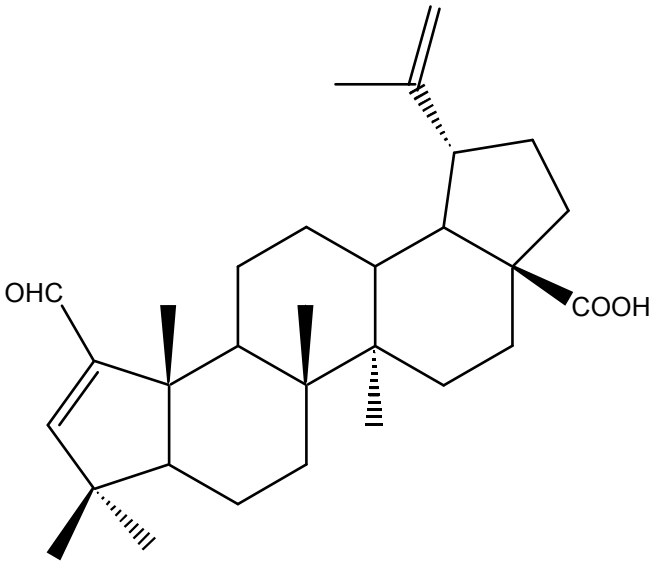
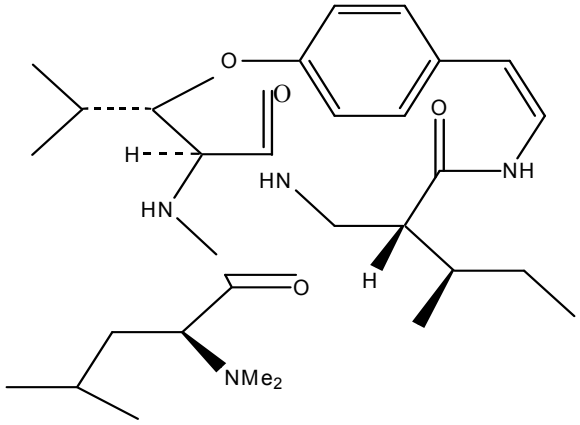
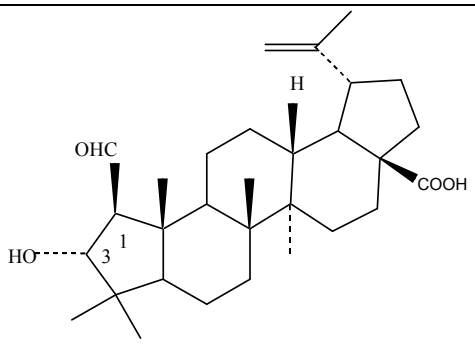
27	$C_{31}H_{42}N_4O_4$/ 573.734 *Zizyphus nummularia*	 Nummularine M	Pandey VB et al., Phytochemistry. 1984, 23, 2118
28	$C_{31}H_{41}N_5O_6$/ 591.706 *Zizyphus nummularia*	 Nummularine N	Pandey VB et al., Phytochemistry. 1984, 23, 2118
29	$C_{33}H_{41}N_5O_5$/ 587.717 *Zizyphus nummularia*	 Nummularine R	Devi S et al., Phytochemistry. 1987, 26, 3374-3375
30	$C_{36}H_{39}N_5O_5$/ 621.735 *Zizyphus rugosa*	 Rugosanine B	Tripathi YC et al., Phytochemistry. 1989, 28, 1563

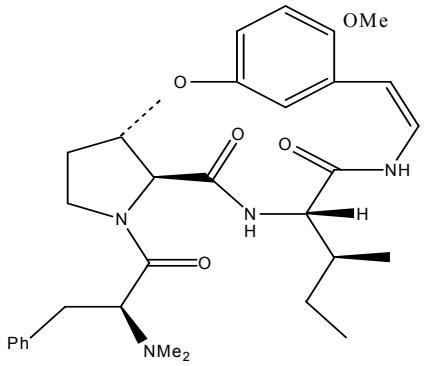
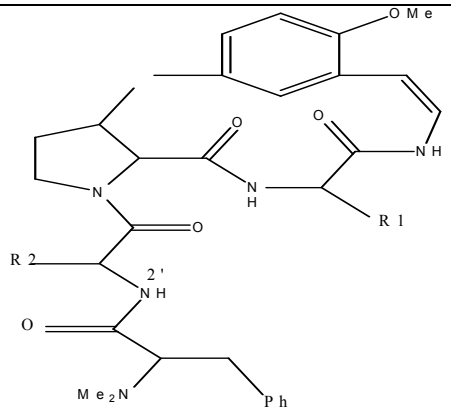
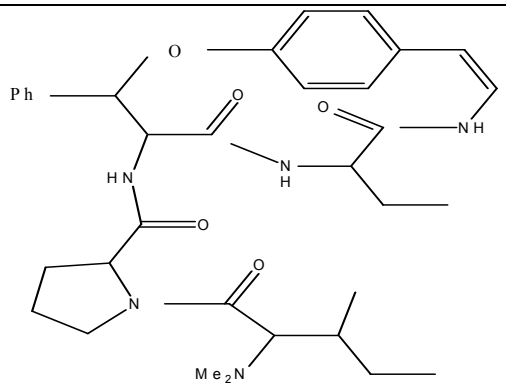
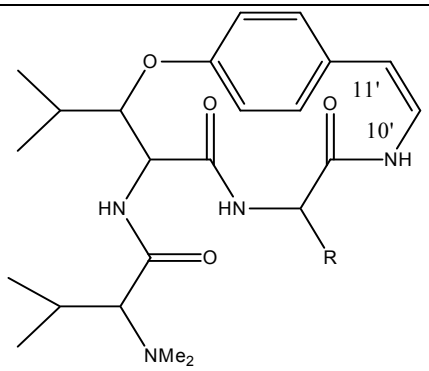
31	$C_{31}H_{44}N_4O_5$/ 552.712 *Zizyphus spinosa*	 Sanjoinine G_1	Han BH et al., Phytochemistry. 1990, 29, 3315-3319
32	$C_{30}H_{42}N_4O_5$/ 538.686 *Zizyphus spinosa*	 Sanjoinine G_2	Han BH et al., Pure Appl. Chem. 1989, 61,443-448
33	$C_{30}H_{40}N_4O_4$/ 520.67 *Zizyphus sativa*	 Sativanine A	Tschesche R et al., Phytochemistry. 1979, 18, 702
34	$C_{30}H_{38}N_4O_4$/ 518.655 *Zizyphus sativa*	 Sativanine B	Tschesche R et al., Phytochemistry. 1979, 18, 702

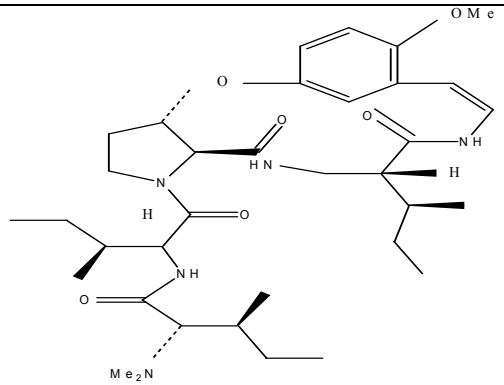
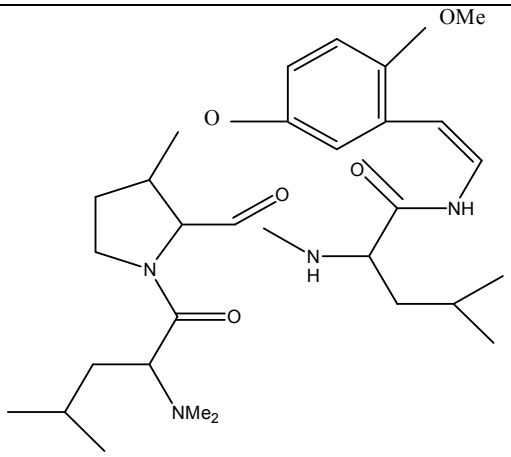
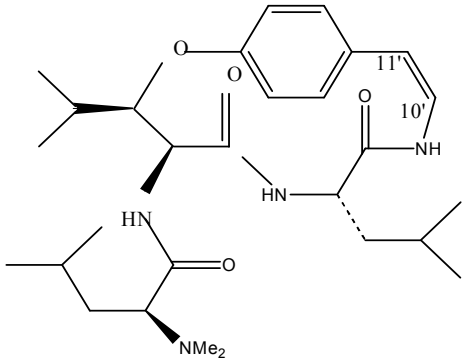
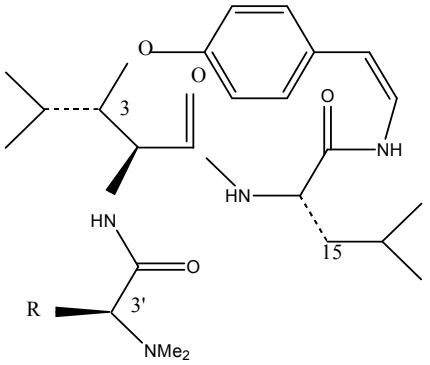
35	$C_{30}H_{43}N_5O_6$/ 569.7 *Zizyphus sativa*	 Sativanine D	Shah AH et al., Phytochemistry. 1985, 24, 2765
36	$C_{33}H_{41}N_5O_5$/ 587.717 *Zizyphus sativa*	 Sativanine E	Shah AH et al., J. Nat. Prod. 1985, 48, 555
37	$C_{28}H_{42}N_4O_5$/ 514.664 *Zizyphus sativa*	 Sativanine G	Shah AH et al., Phytochemistry. 1984, 23, 2120-2121
38	$C_{32}H_{34}N_4O_5$/ 554.644 *Zizyphus sativa*	 Sativanine O	Singh S et al., J. Asian Nat. Prod. Res. 2006, 8, 733-737

39	$C_{24}H_{32}N_4O_4$/ 440.541 *Zizyphus spina-christi*	 Spinanine A	Abdel-Galil FM et al., Phytochemistry. 1991, 30, 1348
40	$C_{36}H_{47}N_5O_6$/ 645.797 *Zizyphus oenoplia*	 Zizyphine I	Khokhar I et al., Pak. J. Sci. 1993, 45, 54
41	$C_{33}H_{49}N_5O_6$/ 611.78 *Zizyphus oenoplia*	 Zizyphine A	Menard EL et al., Helv. Chim. Acta. 1963, 46, 1801
42	$C_{24}H_{32}N_4O_4$/ 440.541 *Zizyphus oenoplia*	 Zizyphine G	Tschesche R et al., Tet. Lett. 1974, 15, 2941-2944

43	$C_{30}H_{46}O_4$/ 471.4 *Zizyphus jujuba*	 Alphitolic acid	Sang et al., Planta medica, 2003, 69, 1051-1054
44	$C_{30}H_{46}O_4$/ 455.5 *Zizyphus jujuba*	 Oleanolic acid	Sang et al., Planta medica, 2003, 69, 1051-1054
45	$C_{30}H_{46}O_4$/ 453.6 *Zizyphus jujuba*	 Oleanonlic acid	Sang et al., Planta medica, 2003, 69, 1051-1054

46	$C_{30}H_{46}O_4$/ 451.5 *Zizyphus jujuba*	 Zizyberenalic acid	Sang et al., Planta medica, 2003, 69, 1051-1054
47	$C_{28}H_{44}N_4O_4$/ 500.68 *Zizyphus jujuba*	 Adouetine X	Otsuka H etal., Phytochemistry. 1974, 13, 2016
48	$C_{30}H_{46}O_4$/ 470.691 *Zizyphus jujuba*	 Colubrinic acid	Lee SS et al., Phytochemistry. 1997, 46, 549-554

49	$C_{31}H_{40}N_4O_5$/ 548.681 *Zizyphus jujuba*	 Paliurine E	Lin HY et al., J. Nat. Prod. 2000, 63, 1338-1343
50	$C_{40}H_{49}N_5O_6$/ 695.857 *Zizyphus jujuba*	 Jubanine A	Tschesche R et al., Phytochemistry. 1976, 15, 541-542
51	$C_{39}H_{47}N_5O_5$/ 665.831 *Zizyphus jujuba*	 Jubanine C	Tripathi M et al., Fitoterapia. 2001, 72, 507-510
52	$C_{27}H_{42}N_4O_4$/ 486.653 *Zizyphus jujuba*	 Melonovine A	Kapadia G. J et al., Phytochemistry. 1977, 16, 1431

53	$C_{34}H_{53}N_5O_6$/ 627.823 *Zizyphus jujuba*	 Daechunine S3	Han BH et al., Pure Appl. Chem. 1989, 61, 443-448
54	$C_{28}H_{42}N_4O_5$/ 514.664 *Zizyphus jujuba*	 Daechunine S7	Han BH et al., Pure Appl. Chem. 1989, 61, 443
55	$C_{28}H_{44}N_4O_4$/ 500.68 *Zizyphus jujuba*	 Franganine	Tschesche R et al.,Tet. Lett. 1968, 2993; 3817
56	$C_{31}H_{42}N_4O_4$/ 534.697 *Zizyphus jujuba*	 Frangufoline	Mascaretti O.A et al., Phytochemistry. 1972, 11, 1133-1137

4.5 FLAVONOIDS

As flavonoids were the main subject matter of above portion, they will be discussed in the proceeding section.

4.5.1 Introduction

Flavonoids or bioflavonoid are low molecular weight polyphenolic compounds and are one of the major classes of compounds occurring in the plant kingdom. They are the secondary metabolites of the plant in that they have no direct role in the growth or development of plant. Over 8000 naturally occurring flavonoids have been described, mostly from the higher plants [142]. The word "flavonoid" is derived from Greek word "flavus" (yellow). These are important ingredients of the plant and about 2% photosynthesized carbon is converted into flavonoids [143]. Flavonoids most prominent in the petals of the flowers, give flower its color and it is this property of the flower (color) which attracts pollinators [144]. They are also present in the leaves but hidden by the ubiquitous green color of the chlorophyll [144].

C_6-C_3-C_6 flavone form the basic skeleton of flavonoids in which oxygen commonly cyclizes the three-carbon bridge between the phenyl groups. There are several classes of flavonoids based on the degree of oxidation and unsaturation of the three-carbon segment. Vacuoles of plant cells accumulate them and are mostly glycosides of a relatively small number of flavonoid aglycons [145]. There are six classes of flavonoids (flavones, flavanones, flavonols, anthocyanins, flavans and isoflavonoids) which vary in their heterocyclic oxygen ring [146]. Those compounds which contain a 1, 3-diphenylpropane skeletons are called chalconoids **(Fig 4.1)**. Cyclization of the three carbon chain with an oxygen atom may result into a five or six membered ring, with one of the preexisting phenyl rings, forming a tricyclic system. Those tricyclic compounds which

contain a five membered heterocyclic ring are referred to as auronoids, while those possessing a six membered heterocyclic ring are termed as flavonoids **(Fig 4.1)**.

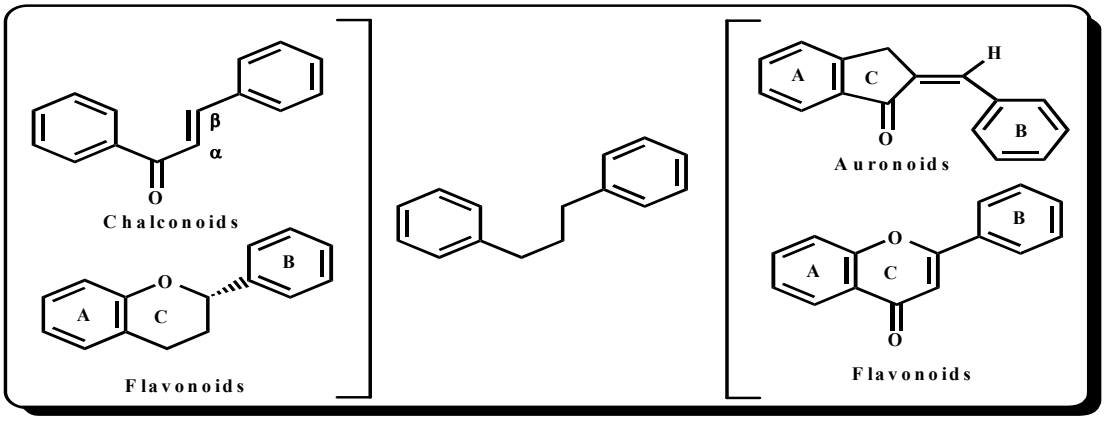


Fig. 4.1 Flavonoids having 1, 3-diphenylpropane skeleton

From 1, 2-diphenylpropane system the tricyclic compounds, 3-phenylcoumarins and isoflavonoids are derived **(Fig 4.2)**, while Neoflavonoids are derived from 1, 1-diphenylpropane **(Fig 4.3)**.

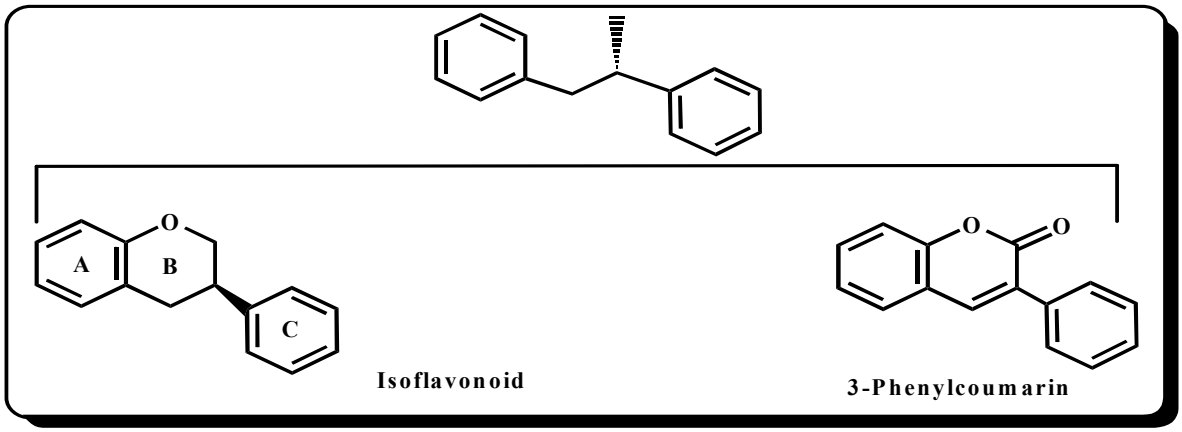


Fig 4.2 Flavonoids having 1, 2-diphenylpropane skeleton

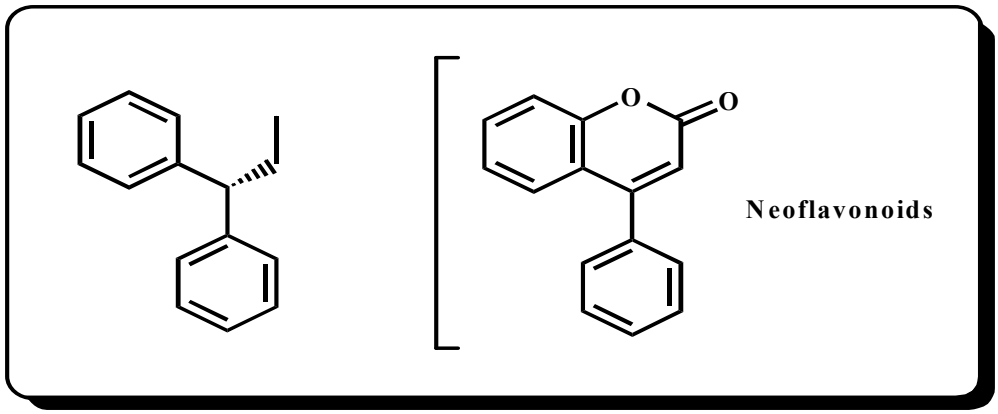


Fig 4.3 Flavonoids having 1, 1-diphenylpropane skeleton

The rings of auronoid and isoflavonoid types of flavonoids are labeled as A, B and C. Ordinary numerals are used to number the individual carbon atoms of ring A and C while primed numerals for the ring B **(Fig 4.4)**.

The homoflavonoids contain an additional carbon in their skeleton which is designated as C-11 **(Fig 4.5)**. Naturally many flavonoids are conjugated with sugars as monoglycosidic, diglycosidic etc. D-glucose, L-rhamnose, glucorhamnose, arabinose or galactose may be the carbohydrate unit; the glycosidic linkage being at position 3 or 7 [147]. In flavonoid with *C*-glycosides, the link is acid resistant [148].

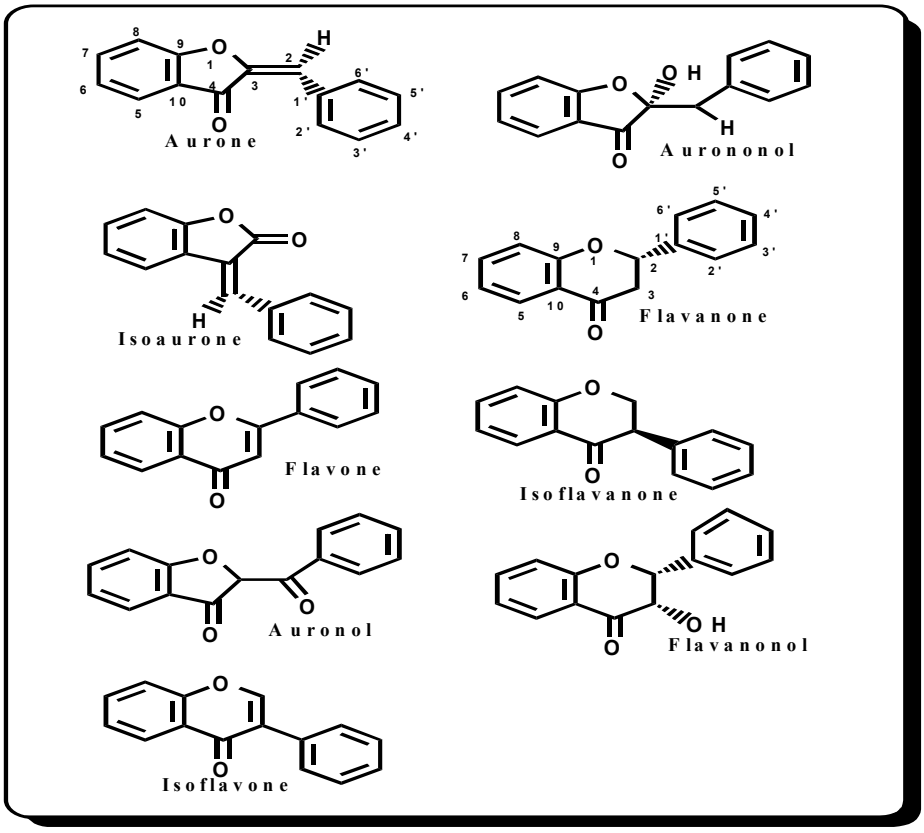


Fig 4.4 Basic skeleton and numbering patterns in flavonoids

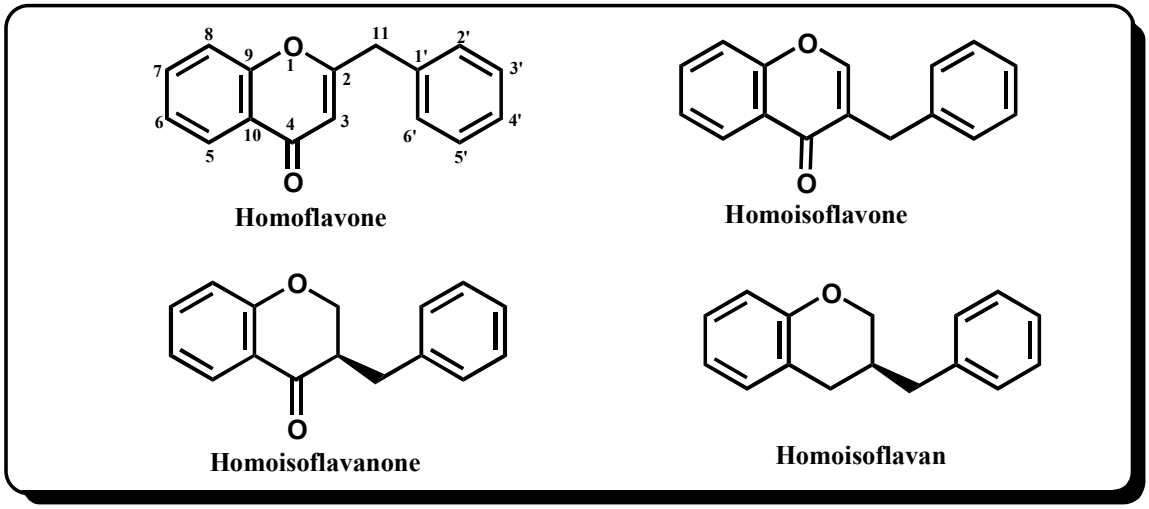


Fig 4.5 Basic skeleton and numbering patterns in homoflavonoids

4.5.2 Importance of flavonoids

One of the most ubiquitous plant phenolics are flavonoids. They are called 'nutraceuticals' because of their vital pharmacological roles in the mammalian body. Nutraceuticals are defined as "A food or parts of food providing medical or health benefits, including the prevention and treatment of disease". This may be dietary supplements or processed products such as soups, cereals, herbal products and beverages [149].

Flavonoids make up the major nutraceuticals ingredients of plants. The most important property of flavonoids is their ability to act as antioxidant. The production of reactive oxygen species (ROS) and free radicals takes place during metabolism or may be induced by exogenous factors, which are a continuous threat to the body cells and tissues. Flavonoids can protect the body from the damaging effect of ROS due to its antioxidant activity [150-151]. Different flavonoids have been checked for their antioxidant activity. Some of the examples include myrcetin, quercetin, rhamnetin, morin and catechin [152]. Flavonoids also possess antibacterial activity. The growth of *S. aureus* has been completely inhibited by quercetin. Sugarless flavonones exhibited antimicrobial activities whereas flavonolignans and flavonols were inactive against the test microorganisms [153]. Flavonoids of peelings of tangerine orange were tested against *Deuterophoma tracheiphila* for their fungistatic activity. Langeritin and Nobiletin displayed weak and strong activities, respectively. Hesperidin stimulated the fungal growth slightly. Strains of *Aspergillus candidus* produced chloroflavonin, which was antifungal antibiotic of chlorine-containing flavonoid-type. Viruses are sensitive to morin, quercetin, dihydroquercetin (taxifolin), rutin, catechin, hesperidine and apigenin [154]. Nonglycosidic compounds appears to have antiviral activity and prerequisite is hydroxylation at the 3-position. Against *Herpes simplex* virus type 1, flavonols are more active than flavones and the order of importance was

galangin>kaempferol>quercetin [155]. The spread of HIV is a great problem throughout the world since 1980 and flavonoid is one of the candidates for its treatment. Several reports have been published on the anti-HIV activity of the flavonoids. In selective inhibition of immunodeficiency virus infections like HIV-1 and HIV-2, flavans were generally more effective than flavononones and flavones [156]. Hesperidin, apigenin, quercetin and luteolin have been reported to have anti-inflammatory activity [157-158].

Hesperidin has also been reported for its analgesic effect [157]. The property of flavonoids to inhibit the activity of cyclo-oxygenase (COX) and lipooxygenase (LO) is responsible for their anti-inflammatory and antiallergic properties [159]. Quercetin, rutin, and Kaempferol, in a dose-dependent manner, decreased the gastric damage produced by acidified ethanol in rats [160]. Rutin and venoruton have been reported for its regenerative and hepatoprotective effects in experimental cirrhosis [161]. Quercetin has been reported for its antidiabetic activity [162]. Flavonoids could play a role in the treatment of noninsulin- dependent diabetes because they can stimulate insulin release and enhanced Ca^{++} uptake from isolated islets cell [163-164]. In the development of cardiovascular diseases, endothelial dysfunction represents a critical event [165]. Flavonoids consumption plays a key in prevention from endothelial dysfunction [166-167] and prevents a number of cardiovascular diseases like hypertension [168-169].

Willaman *et al* [170] while reviewing the biological possessions of the flavonoids, listed thirty-three different manifestations of activity under the heading “Bioflavonoids. For the treatment of different diseases like allergic manifestation, capillary bleeding, capillary fragility and diabetes, these bioflavonoids are used. Citrus bioflavonoids have been used for the treatment of cold [171]. A number of flavonoids and chalcones have been reported for its anti-protozoal activities [172]. Substitutions in position have an effect on the medicinal properties of the bioflavonoids.

The two *ortho* or *para* hydroxyl group, of flavonols, in the phenyl ring-C have anti-oxidant properties, while at the 5, 7-positions the free hydroxyl group have a pro-oxidant effect. Diuretic and anthelmintic properties of apigenin and genkwanin become more distinct with an increase in the number of OH groups [173]. An inverse correlation between plasma cholesterol concentrations and flavonoid intake has been reported and so flavonoid uptake is helpful in reducing the chances of atherosclerosis [174].

Because of the complexity of flavonoid metabolism in the human system and their possible interactions with other substances, more research is still needed [175].

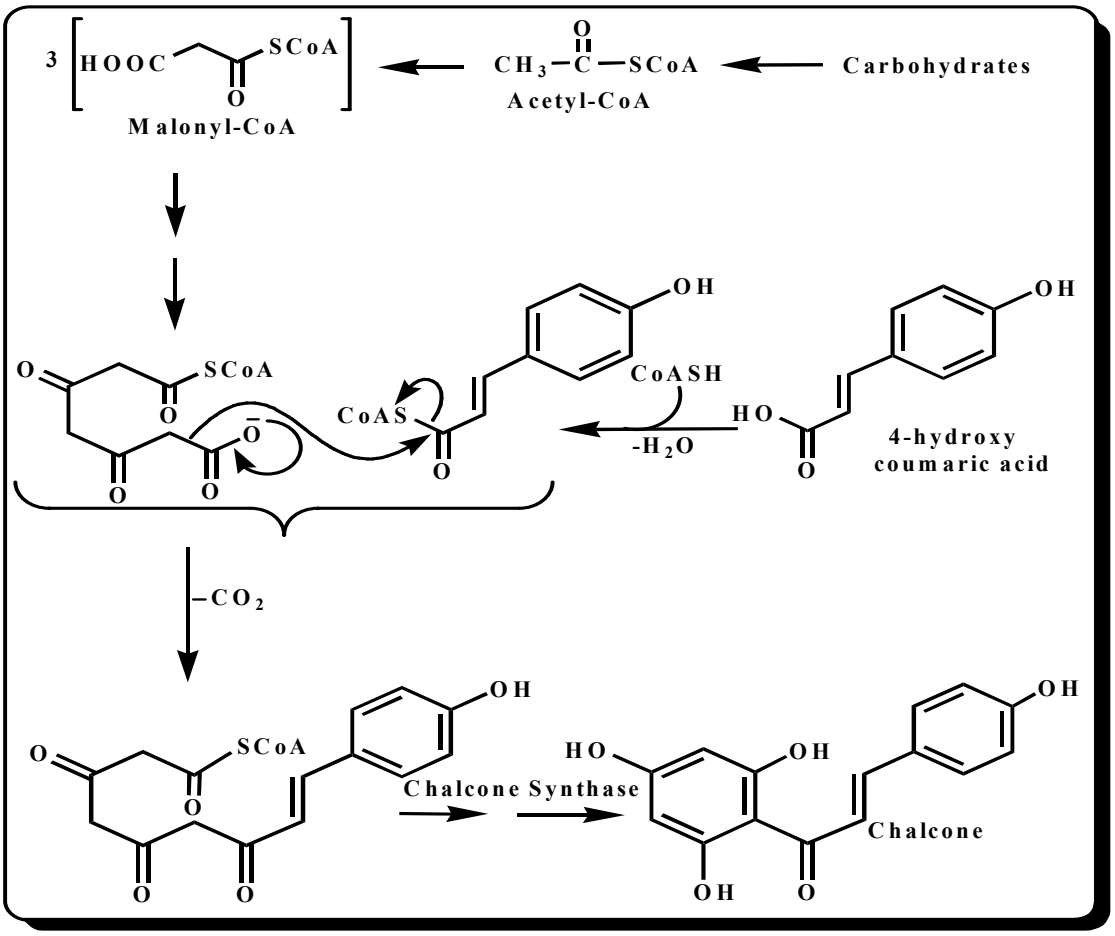
4.5.3 Biosynthesis of Flavonoids

Flavonoids, low molecular weight polyphenolic compounds, found mostly in higher plants are derived from Phenyl and malonyl-CoA through fatty acid pathway. There are six major subgroups of flavonoids found in higher plants i.e. flavandiols, chalcones, anthocyanins, flavones, flavonols and condensed tannins. Some of the leguminous and a small number of non-leguminous plant species produce a special form of flavonoid, called as Isoflavonoids. [176].

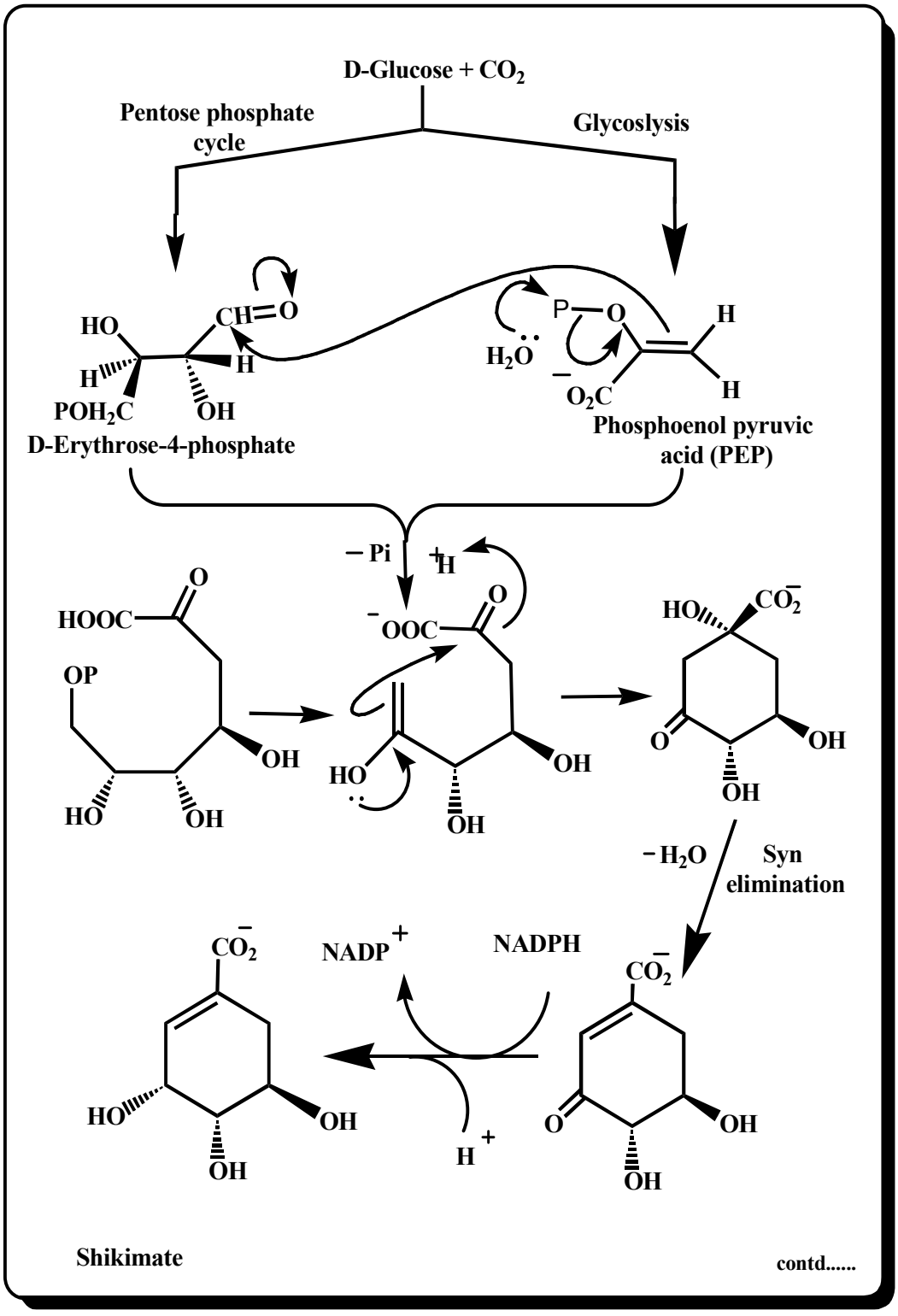
4.5.3 .1 Flavonoids Formation

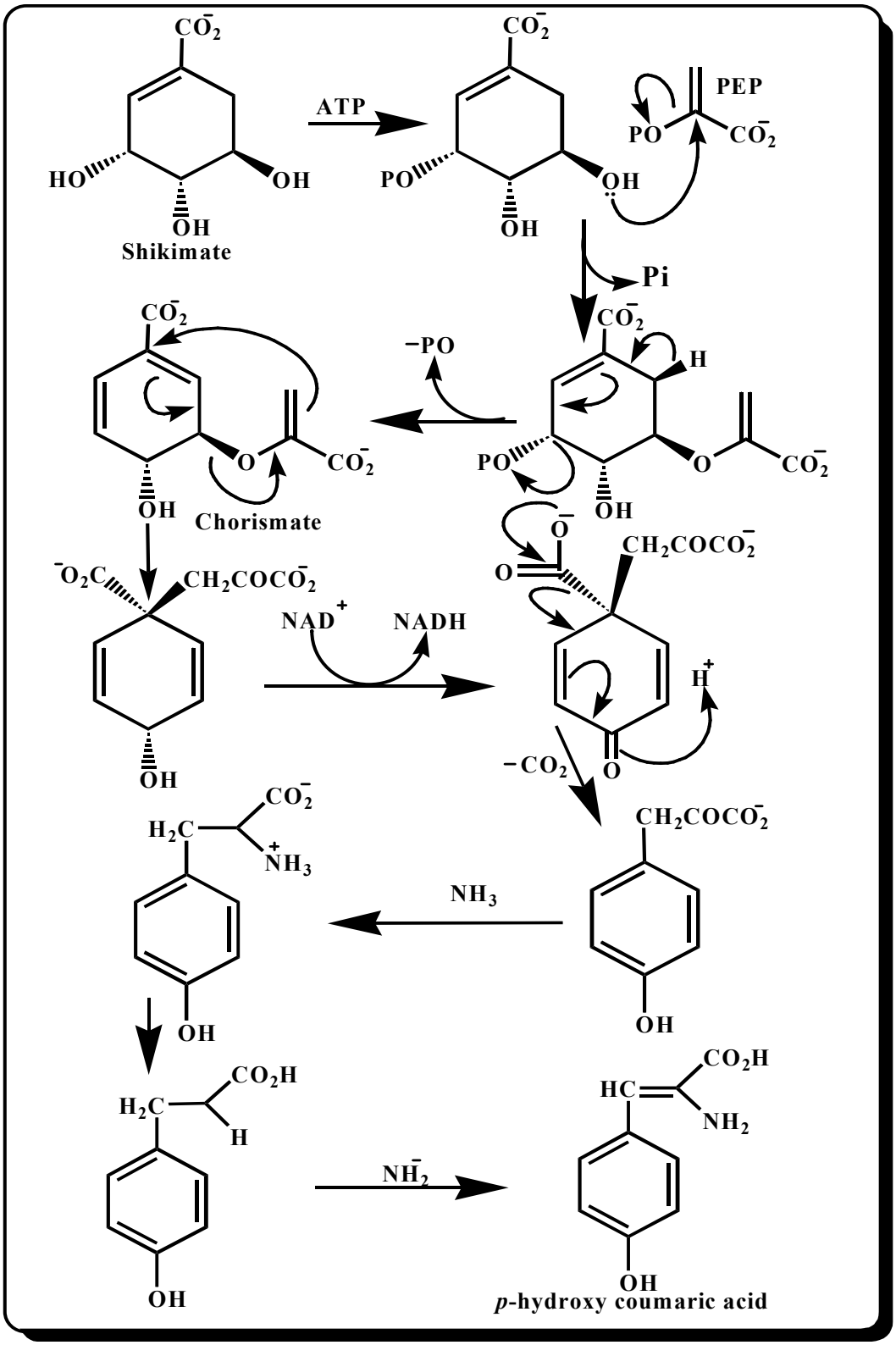
A central 15 carbon intermediates 'chalcone', is involved in the biosynthesis of all flavonoids and its role in the biosynthesis of flavonoids has been confirmed in a number of investigations [176-177]. The precursors for the synthesis of chalcone are malonyl-CoA and 4-coumaroyl-CoA (hydroxycinnamic acid CoA ester) both of which are derived from carbohydrates. The enzyme involved in the formation of chalcone is chalcone synthase. Malonyl-CoA is synthesized from an acetyl-CoA (glycolysis intermediate) and carbon dioxide and its formation is catalyze by acetyl-CoA carboxylase **(Scheme-14),** while the synthesis of 4-coumaroyl-CoA is more complex and it involves the shikimate pathway **(Scheme-15)**, the main course to aromatic amino acid,

phenylalanine and tyrosine in higher plants. The condensation of phosphoenolpyruvic acid and D-erythrose-4-phosphate is the starting point for the biosynthesis of shikimic acid **(Scheme-15)** [178].



Scheme-14 Biosynthesis of chalcones through malonyl-CoA

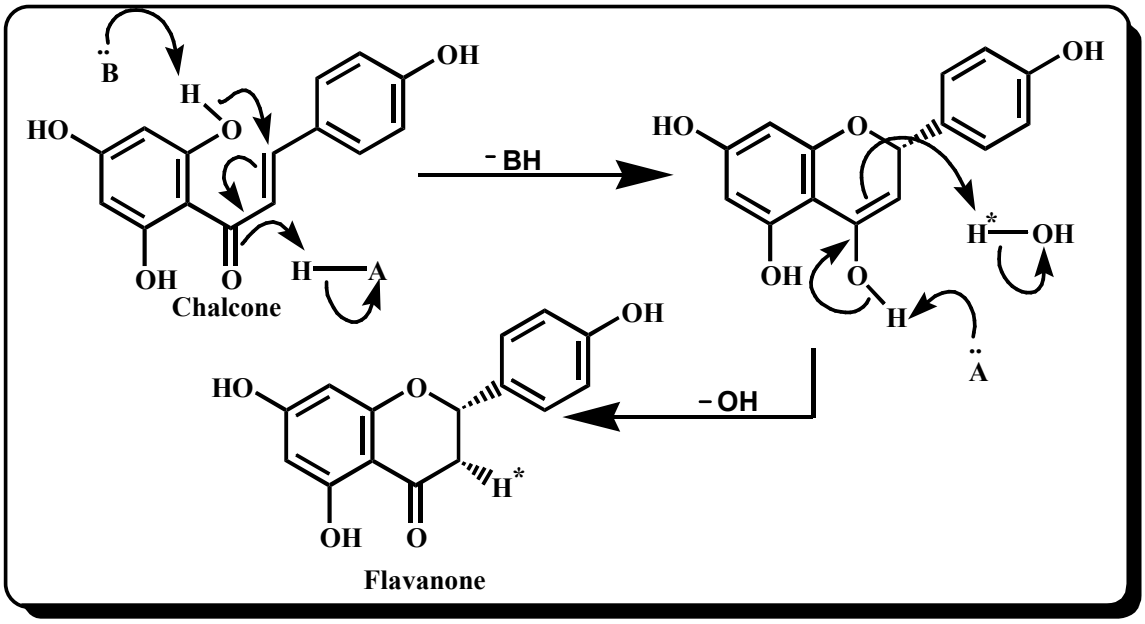

D-Glucose + CO_2
Pentose phosphate cycle
Glycoslysis
D-Erythrose-4-phosphate
Phosphoenol pyruvic acid (PEP)
− Pi
+ H
− H_2O
Syn elimination
NADP +
NADPH
+ H
Shikimate
contd.....



Scheme-15 Biosynthesis of *p*-hydroxy coumaric acid through Shikimate pathway

4.5.3.2 Flavanone Formation

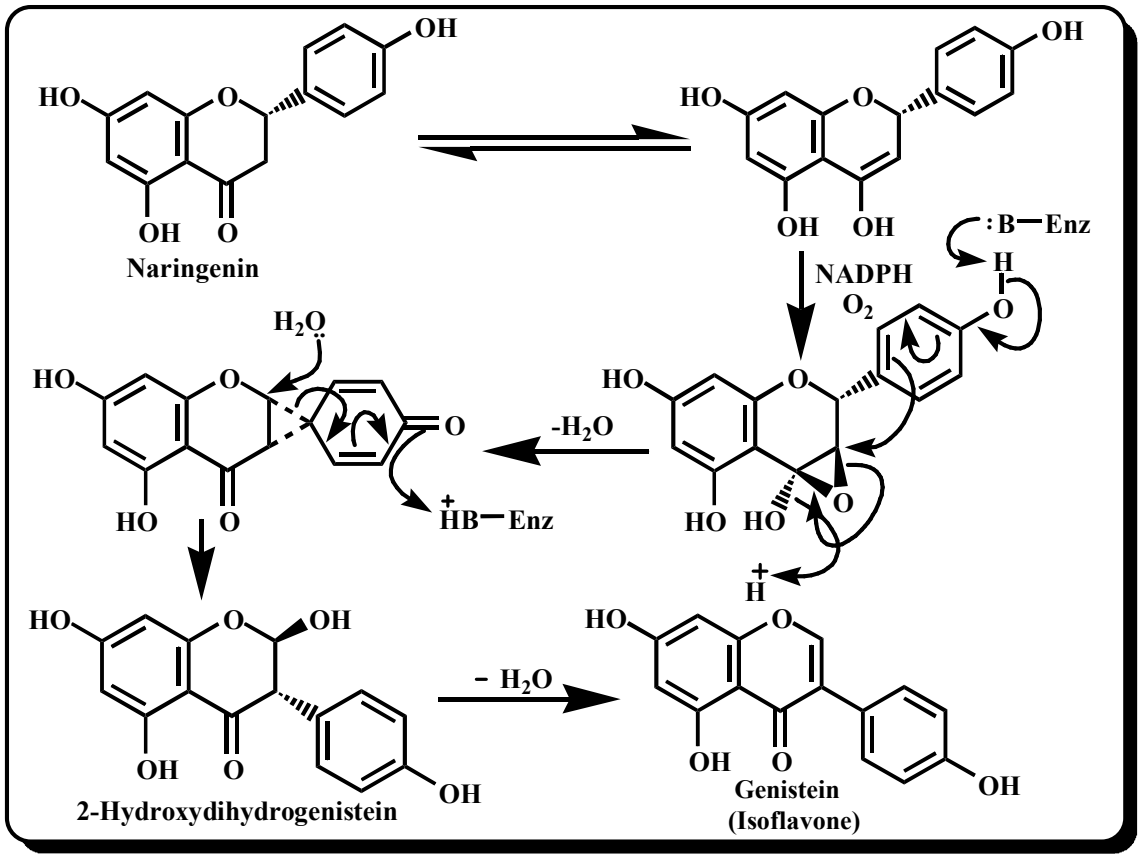
Chalcone isomerase enzyme is responsible for the isomerization of chalcones into flavanones. Evidences for the *in vitro* and *in vivo* existence of equilibrium between flavanones and the corresponding chalcones have been reported in the literature [178]. The stereospecificity of this enzymatic reaction is perceptible in the (S) chirality of C-2 in flavanone derivative. Therefore, it is not inadvertent that all the flavanones found in nature have the *(S)* pattern at C-2 and are levorotatory. When chalcones are having at least two free hydroxyl groups at C-2 and C-6, the equilibrium is rapidly and completely shifted to flavanone in an aqueous solution **(Scheme-16)**. The stabilization energy of strong H-bond between the carbonyl and *O*-phenolic hydroxyl groups is greatly influencing the interconversion rate and position of equilibrium. The system tends to remain in the open (chalcone) form when only 1 OH group is accessible, either for hydrogen bonding or cyclization [179].



Scheme-16 Biosynthesis of flavanones

4.5.3.3 Isoflavone Formation

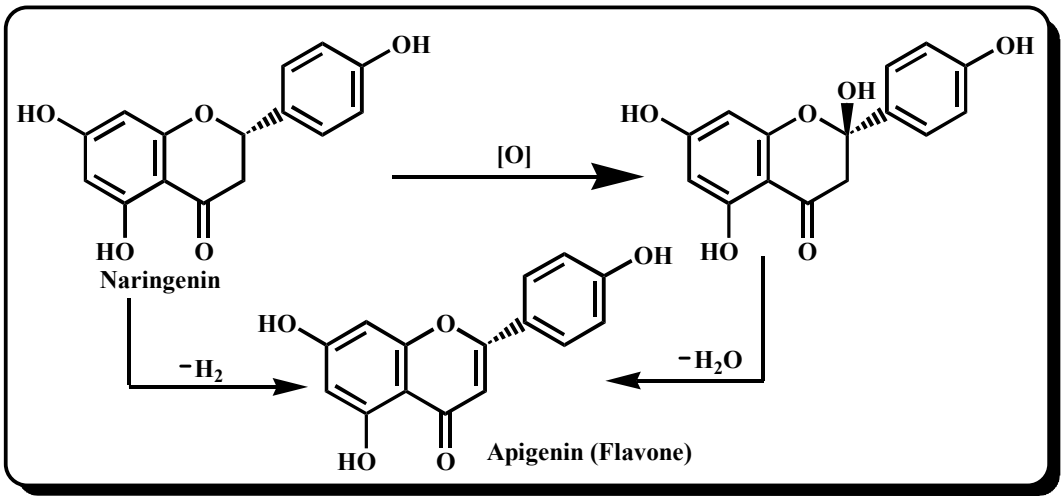
The key step in isoflavone formation is the migration of 2, 3 aryl side chain of a flavanone intermediate (chalcone) (**Scheme-17)**. It was recently found that soybean cell suspension cultures contains an enzyme, catalyzing the transformation of *(2S)*-naringenin (flavanone) into genistein (isoflavone) **(Scheme-17)**. Two enzymatic steps are involved in this transformation, the first step involves oxidation and rearrangement of naringenin to 2-hydroxy-2, 3-dihydrogenistein and this reaction is strictly NADPH and molecular oxygen dependent. In the second step water is eliminated from 2-hydroxy-isoflavanone. The enzyme catalyzing this step has been isolated but has not yet been characterized [180].



Scheme-17 Biosynthesis of isoflavones

4.5.3.4 Flavone Formation

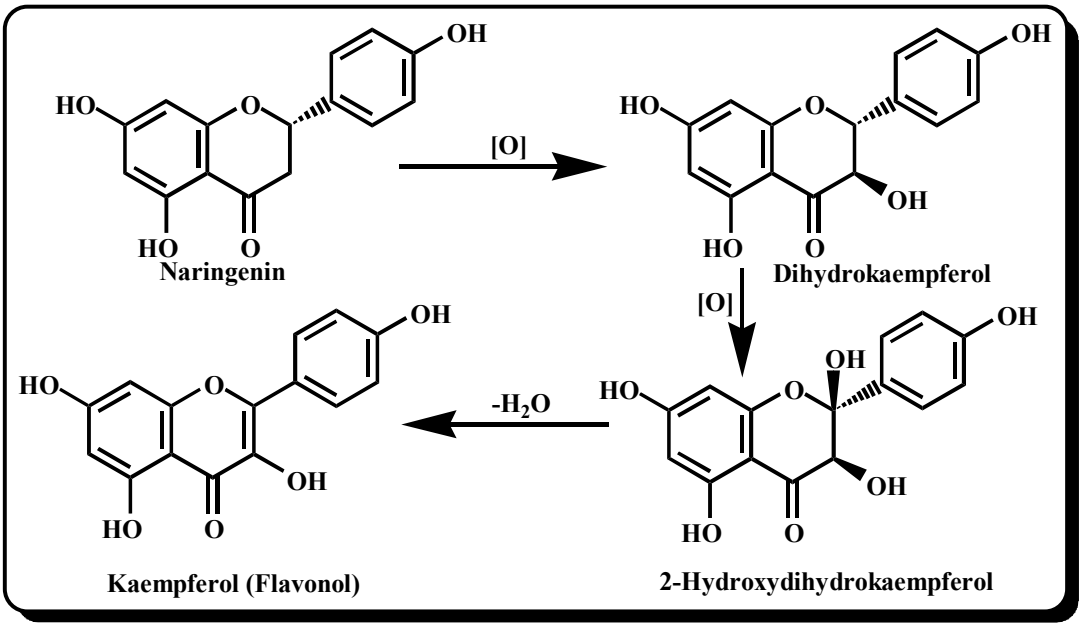
The *in vitro* conversion of flavanones to flavones was observed in *parsley* cell suspension cultures and *Antirrhinum* flowers [181-182]. The parsley enzyme requires 2-oxoglutarate and Fe^{++} along with ascorbate as co-factors. The co-factor (ascorbate) is important for the stimulation of this enzyme and other 2-oxoglutarate dependent dioxygenases enzymes of the flavonoid pathway and also has a stabilizing effect on the enzyme activity [183]. Flower enzymes of both, *parsley* and *Antirrhinum* catalyzed the conversion of *(2S)*-naringenin (flavanone) to apigenin (flavone) **(Scheme-18)**. The mechanism of double bond formation is still unclear. It has been suggested that 2-hydroxyflavanone is formed in the first step, and water is then eliminated *via* a dehydratase [182-183]. However, no such 2-hydroxy intermediate has yet been isolated even with a nearly homogenous enzyme protein [184]. On the other hand, 2-hydroxyflavones certainly exist as plant metabolites and they are indeed, the substrates in *C*-glycosylflavones formation [185].



Scheme-18 Biosynthesis of flavones

4.5.3.5 Flavonol Formation

The stereospecific 3ß-hydroxylation of (2S)-flavanones to dihydroflavonols is catalyzed by Flavanone 3-hydroxylase (F3H). Flavonol synthesis, most probably, proceeds *via* a 2-hydroxy intermediate such as; 2-hydroxydihydrokaempferol with succeeding dehydration, giving rise to the particular flavonols [185] **(Scheme-19)**.



Scheme-19 Biosynthesis of flavonols

2.0 MATERIALS AND METHODS

2.1 General Experimental conditions

Pharmacological, biological, chemical and instrumental analysis were carried out at the Centre of Biotechnology and Microbiology (COBAM), University of Peshawar, Department of Pharmacy, University of Malakand and International Centre for Chemical and Biological Studies (ICCBS), University of Karachi, Karachi. Commercial and analytical (Merck) grade solvents were utilized for different experiments.

2.1.1 Physical Constants

Melting points of the compounds were determined by Buchi 535 apparatus. For determining the optical rotations of the compounds, JASCO DIP-360 digital Polari meter was used.

2.1.2 Spectroscopy

UV Spectra; A fully automated Hitachi U-3200 spectrophotometer was used for the determination of UV spectra. **IR Spectra;** In chloroform or potassium bromide (KBr) pellet, Infrared spectra was determined using Infrared (IR) Spectrometer, JASCO 302-A. **Mass Spectra;** MAT 311A mass spectrophotometer was used for recording of low-resolution electron impact mass spectra, coupled with PDP 11/34 computer system. For the measurement of High resolution (HR) mass and Fast Atom Bombardment (FAB positive and FAB negative) mass, Jeol JMS HX 110 mass spectrometer was used. **Nuclear Magnetic Resonance (NMR);** For the measurement of ^{1}H-NMR spectra, Bruker AM-300, AM-400 or AMX-500 nuclear magnetic resonance spectrometer was used. The spectra were recorded at 300, 400 or 500 MHz using TMS as an internal reference. In deutorated solvents like, CH_3OD or $CDCl_3$, ^{13}C-NMR spectra were recorded. For determination of CH, CH_2, and CH_3 groups, DEPT experiments (Distortionless Enhancement by Polarization Transfer) were carried out, at 90^{o} and 135^{o} and by subtracting the

signals of these spectra from broad band (BB) ^{13}C-NMR spectrum, the quaternary carbons were determined. **Gas Chromatography and Gas Chromatography-Mass Spectrometry;** Using GC and GC-MS, qualitative and quantitative data of the oils were determined respectively. The oils were injected into a GC-17A system (Shimadzu), equipped with a splitless / split injector and AOC - 20i autosampler. BD-5 (Optima -5) column was used in the GC, 30.0 m, 0.25 mm i.d., 0.25 μm df, using diphenyl (5%) and polydimethylsiloxane (95%) as solvents. The oven temperature was operated with oven temperature programme, as following; 50^{o}C for 1 minute, the temperature was raised to 250^{o}C with an increase of 3^{o}C per minute and then held for 5 minutes at 250^{o}C. The temperature was then raised to 280^{o}C with an increase of 2^{o}C per minute and held for 3 minutes at 280^{o}C. Injection volume and temperature was 1.0 μl and 250^{o}C, respectively; nitrogen at 30 cm / s linear velocity and 99.8 KPa inlet pressure was used as a carrier gas, the flow rate of hydrogen was 50 ml / min and temperature of the detector was 280^{o}C. The flow rate of air and make-up (H_2 / air) was 400 ml / min and 50 ml / min respectively; sampling rate was 40 ms. GC solution software (Shimadzu) was used for acquiring the data. **Gas Chromatography – Mass Spectrometry;** VG analytical 70-250s double focusing Mass spectrometer was interfaced with GC, Agilent 6890 N. Carrier gas was Helium. Conditions for the MS operating were; Temperature was 250^{o}C and ionization voltage 70eV. The GC column was a DB-5 coated, capillary silica column of 30m-0.32mm of size. The operating parameters for GC-MS were the same as for GC analysis.

2.1.3 Isolation and Purification of Compounds

Using different chromatographic techniques, different compounds were isolated from various fractions of the plant.

2.1.3.1 Column Chromatography (CC)

In column chromatography the stationary phase was Silica gel-GF_{254}, E. Merck (Art. 7734, 70-230 mesh) while different organic solvents (*n*-hexane, chloroform ($CHCl_3$), di-chloromethane (DCM), ethyl acetate (EtOAc) and methanol) were used as mobile phase.

2.1.3.2 Thin-layer Chromatography (TLC)

Preparative TLC was performed on preparative pre-coated silica gel plates (20 x 20 cm, 0.5 mm thickness, Merck $PF_{254,}$ Type 60). Pre-coated silica gel TLC plates (PF_{254}, Merck, 0.25 mm) were utilized for TLC.

2.1.4 Spray Reagents for Visualization of Spots

For the visualization of spots on TLC plates, different spraying reagents were used such as vanillin-phosphoric acid, ceric sulphate-sulphuric acid, Dragendorff's reagent and iodine solution.

2.1.4.1 Vanillin-Phosphoric Acid

In 100 ml of 50% aqueous Phosphoric acid, 1 gm vanillin was dissolved [62]. Terpenes give blue or light pink color while steroids give intense purple color after spray and subsequent heating at 100-110^{o}C. After spray and heating, terpenoidal and steroidal glycosides also give pink color.

2.1.4.2 Ceric Sulphate-Sulphuric Acid

Saturated solution of ceric sulphate was made in 65 % H_2SO_4 [62] that was used to spray on TLC plates. Terpenoids give pink color, after spraying and subsequent heating. Alkaloids give light yellow or blackish color without heating.

2.1.4.3 Dragendorff's Reagent

The following procedure was employed for the preparation of Dragendorff's reagent.

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

(ii) A mixture of water and acetic acid containing 40 ml water and 10 ml acetic acid was used to dissolve 0.85 gm of basic bismith nitrate.

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

(iv) 10 ml of acetic acid and 90 ml of distilled water was used to dilute 5 ml of the stock solution. [63]. Alkaloids give light brown, light pink, dark brown or blackish color, upon spraying. Steroids and terpenoids, upon spraying, give light yellow to light pink color.

2.1.4.4 Iodine Solution

Few crystals of iodine were placed in a TLC tank and warmed at temperature of 40-50^{o}C for few minutes. Spots will appear when TLC plates are placed inside the TLC tank [62].

2.2 PHYTOCHEMICAL INVESTIGATIONS

2.2.1 Plant material

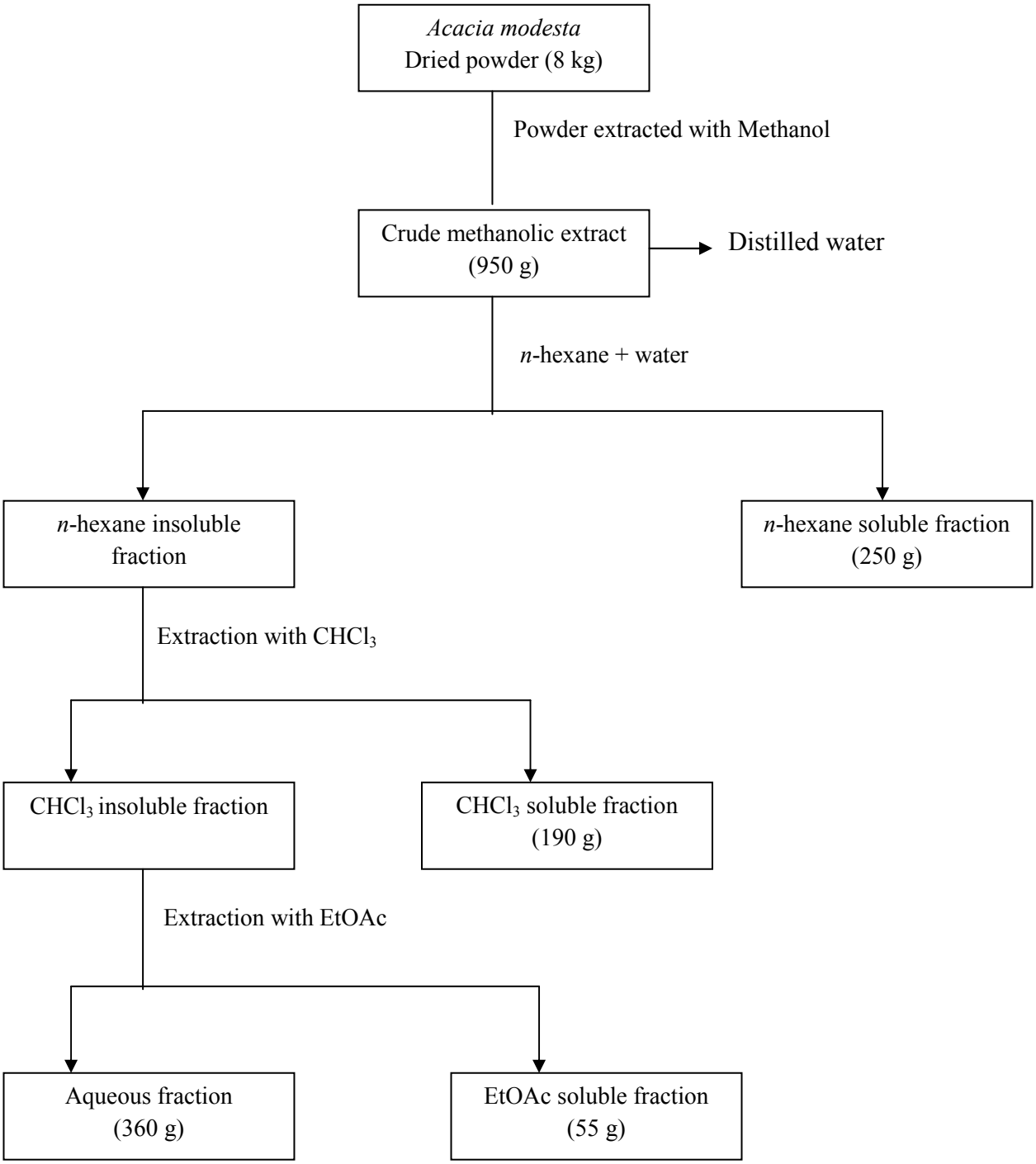
Acacia modesta (aerial parts) was collected from Northern region of Khyber PukhtoonKhwa, Pakistan and identified by Prof. Dr. Abdur-Rasheed, plant taxonomist, Department of Botany, University of Peshawar, Khyber PakhtunKhwa, Pakistan.

2.2.2 Extraction

The plant material was kept in shade for drying. After drying they were chopped into small pieces and ground to powder, using an electric grinder. Soaking of the powdered material (8 kg) was performed in commercial grade methanol for 15 days, twice, at room temperature, with occasional shaking. Each time the material was filtered. All the filtrates were combined and concentrated below 40^{o}C under vacuum using rotary evaporator which gave a blackish crude methanolic extract of 950 g.

2.2.3 Fractionation

In distilled water (500 ml), the crude methanolic extract (855 g) was dissolved and partitioned with *n*-hexane (3 x 500 ml), $CHCl_3$ (3 x 500 ml) and EtOAc (3 x 500 ml) yielding the *n*- hexane (250 g), $CHCl_3$ (190 g), EtOAc (55 g) and aqueous (360 g) fractions, respectively. The scheme of fractionation is depicted in **Scheme 12**. 95 g of the crude methanolic extract was reserved for biological/pharmacological activities.



Scheme 12 Fractionation of crude methanolic extracts of *Acacia modesta*

2.2.4 Screening for Different Groups of Compounds

In the Soxhelet apparatus using hydrous methanol, the plant material was refluxed and the plant extracts were obtained.

Alkaloids

Methanol was used to extract the plant material (2.5 g). The dried material was heated on a water bath with 5 ml of HCL (2N). The mixture was filtered after heating on boiling water bath, cooled and divided into four parts. One part of the mixture was treated with Mayer's reagent and other parts of the mixture were treated Wagner's reagent. Precipitation or turbidity of the test samples was then observed. A (+) score was recorded for slight opaqueness, (++) for definite turbidity without flocculation and (+++) for a definite turbidity along with heavy floccules or precipitates [63].

Flavonoids

To prepare the sample for the test of flavonoids, 1 g of plant material was dissolved in 5.0 ml of methanol. The mixture was then treated with few drops of concentrated HCL and 0.5 g of magnesium. Appearance of pink or magenta red colors indicated the presence of flavonoids in the sample [64].

Saponins

Water was used to extract 2.5 g of plant material on boiling. The extract was kept at room temperature to cool it and then vigorously shaken to form the froth. The extract was allowed to stand for 15-20 minutes and the results were recorded as:

No froth (-) negative, for froth less than 1cm (+) weakly positive, for froth up to 1.2 cm and more than 2 cm (++) positive and (+++) strongly positive, respectively [65-66].

Tannins

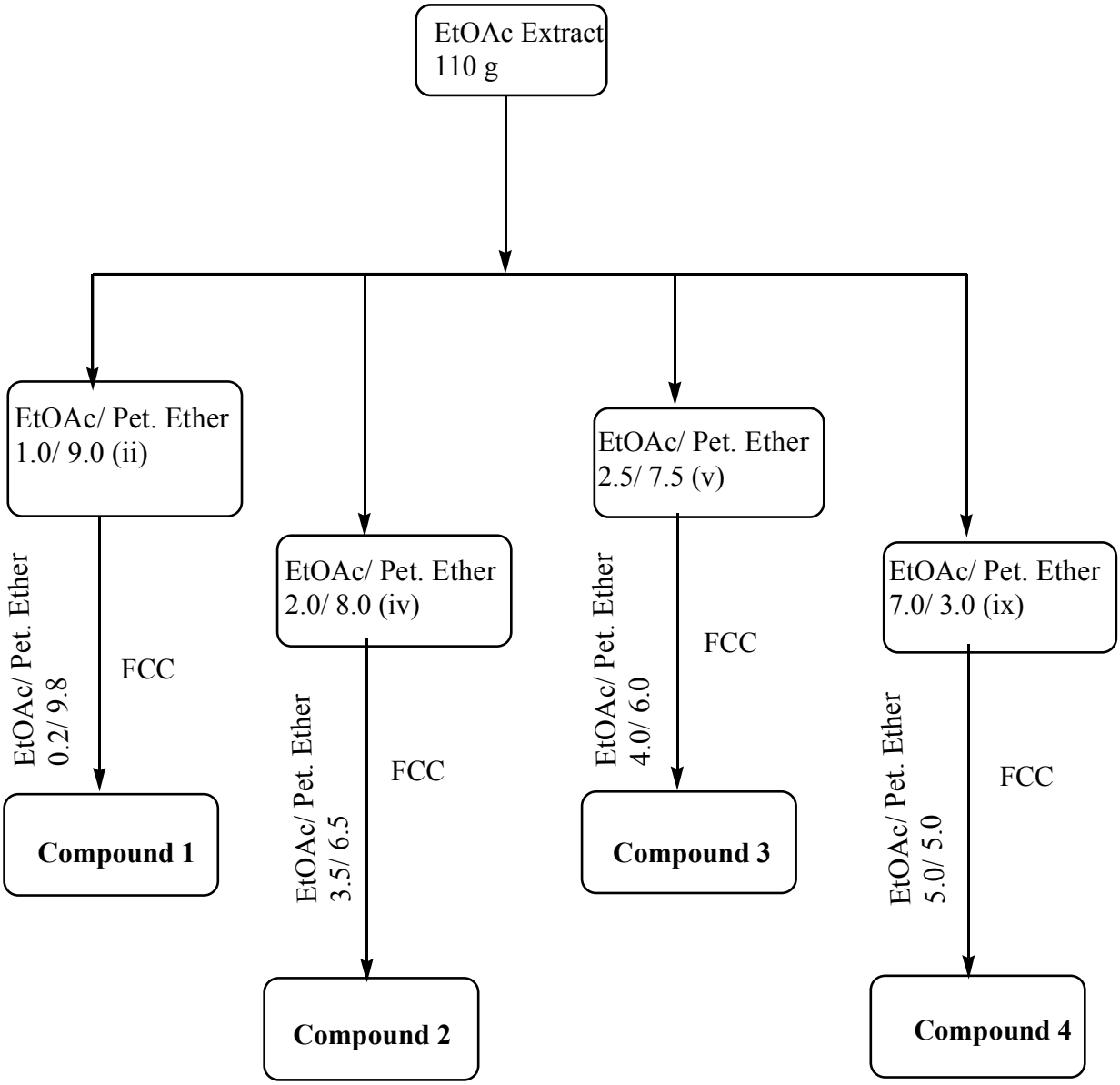
Methanol was used to extract the plant material (1 g). The extract was allowed to evaporate to dryness. With 10 ml of hot normal saline solution the residues was again extracted. The extract was then filtered and divided into three equal parts. One portion was treated with sodium chloride solution, which served as blank. To the second and third portion, Gelatin (1%) and gelatin-salt solution were added. The formation of precipitate indicates the presence of tannins. The appearance of characteristic blue, blue-black, green or blue-green color, further confirm the presence of tannins, that were precipitated by adding ferric chloride ($FeCl_3$) solution to the test sample(s) [67].

2.2.5 Compounds isolated from *Acacia modesta*

The EtOAc fraction (55 g) of *A. modesta* was subjected to Column Chromatography (CC) and sequentially sub-fractionated with solvent system of pet ether and EtOAc in increasing order of polarity (i) 0.5 / 9.5, (ii) 1.0 / 9.0, (iii) 1.5 / 8.5, (iv) 2.0 / 8.0, (v) 2.5 / 7.5, (vi) 3.5 / 6.5, (vii) 4.0 / 6.0, (viii) 5.0 / 5.0, (ix) 7.0 / 3.0, (x) 9.0 / 1.0 and 100% EtOAc **(Scheme-13)**.

From the above sub fractions, those fractions that showed good separation pattern of compounds on TLC plate using a given solvent system were subjected to Flash column chromatography (FCC) for separation of compounds.

Compound **1** was obtained from the sub fraction (ii) eluting with the solvent system EtOAc / pet. ether (0.2: 9.8). Compound **2** was obtained from the sub fraction (iv) eluting with solvent system EtOAc / pet. ether (3.5: 6.5). Compound **3** was obtained from the sub fraction (v) eluting with solvent system EtOAc / pet. ether (4.0: 6.0). Compound **4** was obtained from the sub fraction (ix) eluting with solvent system EtOAc / pet. ether (5.0: 5.0).



Scheme 13 Isolation of compounds from EtOAc fraction of *Acacia modesta*

2.2.6 CHARACTERIZATION OF COMPOUNDS

2.2.6.1 Characterization of Compound (1)

Yield: 11 mg from EtOAc fraction

Physical state: Yellow buff colored amorphous solid

M.P: 62-64^{o}C

R_f: 0.60 ($CHCl_3$- pet. Ether, 7:3)

UV: λ_{max} 243, 275 (*sh*) nm

IR (KBr): ν_{max} 3398 (OH), 2916 (CH_3), 2848 (CH_2), 1470, 1462 (C-O), 1061, 729, 719 cm^{-1}

^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.82 (3 H, m, *CH_3*-29), 1.25 (54 H, brs, 27 x *CH_2*-2-28), 3.32 (2H, dd, *J* = 6.0 Hz, -*CH_2*-OH)

EIMS (probe) 70 eV, *m/z* % (rel. int): 426 (38), 419 (35), 411(17), 257 (10), 218 (67), 207(45), 189 (52), 135 (50)43 (82).

2.2.6.2 Characterization of Compound (2)

Yield: 14mg from EtOAc fraction

Physical state: White amorphous solid

M.P: 82-83^{o}C

IR (KBr): V_{max} = 2915, 2847, 1700 (C=O), 1462 (-CH2-), 773, 718 cm^{-1}

EI-MS: *m/z* (rel int) = 450 [M+ (6)], 281 (7), 239 (100), 255 (24), 221 (4), 183 (7), 156 (5), 125 (9), 96 (14), 81 (17), 57 (9)

^{1}H-NMR ($CDCl_2$): δ = 2.38 (t, *J* = 7.4 Hz, H-15), 1.55 (m, H-14), 1.25 (bs, H-2-H-13), 0.88 (t, *J* = 7.2 Hz, H-1).

^{13}C-NMR ($CDCl_3$); δ = 14.1 (C-1), 22.7 (C-2), 31.9 (C-3), 29.3-29.7 (C-4- C-13), 23.9 (C-14), 42.8 (C-15) and 211.7 (C-16).

EIMS: *m/z*: 450 (6), 281 (7), 239(100), 255 (24), 221 (4), 183(7), 156 (5), 125 (9), 96 (14), 81(17), 57(9)**.**

2.2.6.3 Characterization of Compound (3)

Yield: 17 mg from EtOAc fraction

Physical state: Colorless needles

MP: 213-214^{o}C

UV (CD_3OD) λ_{max} log ϵ: 222 (3.88), 310 (3.92) nm

IR (KBr) ν_{max} cm^{-1}: 3510 (O-H), 3330-2720 (carboxylic OH), 1705 (C=O)

^{1}H NMR ($CDCl_3$, 400 MHz): δ 11.92 (1H, s, O-H), 7.92 (2H, d, *J* = 10.68, *J* = 8.5 Hz, H-2, 6), 6.72 (1H, d, *J* = 8.5 Hz, H-3, 5).

^{13}C NMR (CD_3OD, 100 MHz): δ 180.0 (C-7), 160.3 (C-4), 131.5 (C-2, 6), 122.4 (C-1) and 135.5 (C-3, 5)

HREIMS *m/z*: 138.0316 (calcd. For $C_7H_6O_3$, 138.0309)

2.2.6.4 Characterization of Compound (4)

Yield: 25 mg from EtOAc fraction

Physical state: White powder

MP: 215-216^{o}C

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

HR-EIMS *m/z*: 426.7189 (calcd. For C30H50O, 426.7194)

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

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

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

2.3 PHARMACOLOGICAL INVESTIGATIONS

2.3.1 ANTIBACTERIAL ACTIVITY

Currently there are increasing incidents of infections due to evolution of new pathogens and resistance of the present pathogens to the existing antibiotics e.g. multi-drug resistant tuberculosis (MDR-TB) is now resistant to isoniazid and rifampicin [68]. Plants are rich sources of bioactive compounds. 12 of the world's 25 best selling pharmaceutical agents are natural products derived. So the current study was carried out in hope of finding new antimicrobial agents from the plant sources.

MATERIALS

The test organisms used in the current research for crude methanolic extract and various fractions were; *Pseudomonas aeruginosa, Escherichia coli*, *Staphylococcus aureus, Staphylococcus epidermidis, Salmonella typhi, Bacillus Pumilus, Klebsiella pneumoniae, Enterobacter aerogenes* and *Streptococcus pneumonia*. The test organisms used for oils were: *E. coli*, *Shigella flexenari*, *Bacillus subtilis*, *S. aureus*, *P. aeruginosa* and *S. typhi*. All these test organisms were available at the Centre of Biotechnology and Microbiology, University of Peshawar, Khyber PakhtunKhwa, Pakistan. Analytical grade organic solvents were used in the experiments. The other materials utilized were: nutrient broth, nutrient agar, 6mm borer, Petri dishes, incubator, micropipettes, standard antibiotic (Amoxicillin), Dimethyl sulfoxide (DMSO), test samples (crude methanolic extract and fractions of the plant) and autoclave.

Procedure

The following steps were followed for the determination of antibacterial activity of test samples against the above mentioned pathogens. The experiment was performed as per our reported procedure [69].

Nutrient broth and Nutrient agar media were prepared and incubated for 24 hours at 37^{o}C to check its sterility. On the second day, a loop dipped in the broth containing the bacterial culture was transferred to fresh broth for dilution. From this broth culture, 1ml was transferred to nutrient agar plates and was uniformly distributed on it by moving it round to make bacterial lawn. With the help of a 6mm borer, wells were made in the nutrient agar plates and were labeled. 3mg of the test samples dissolved in 1ml of DMSO which served as stock solutions. From stock solutions 100µl was introduced in the respective well and incubated for 24hours at 37^{o}C. DMSO was used as negative and Amoxicillin as positive control. Zone of inhibition was measured on the next day.

Percent zone of inhibition was measured in comparison with positive control using the following formula

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

After the recording the zone of inhibition, the experiments were proceeded to determine the minimum inhibitory concentration (MIC_{50}) following Salmon et al [70].

MEASUREMENT OF MININUM INHIBITORY CONCENTRATION (MIC)

Requirements

Test tubes, Nutrient broth, Test samples, Micropipettes and test pathogens (*Pseudomonas aeruginosa, Escherichia coli*, *Staphylococcus aureus, Staphylococcus epidermidis, Salmonella*

typhi, Bacillus Pumilus, Klebsiella pneumoniae, Enterobacter aerogenes and *Streptococcus pneumoniae*).

Procedure

The following steps were performed to find MIC_{50}.

Nutrient broth media was prepared in distilled water. Nutrient broth media and test tubes were autoclaved. After autoclaving 10ml of media was added to each test tube. To check sterility of the media, the tubes were incubated at 37^{o}C for 24 hours. Cultures of bacteria were introduced into the test tubes. From the stock solution prepared for the measurement of zone of inhibition, 300, 500, 700 and 900µl were introduced into the respective labeled tubes. The tubes were incubated at 37^{o}C for 24 hours and results were taken with naked eye, checking out the turbidity of the tubes in comparison with negative control. Negative control in this case was nutrient broth media.

2.3.2 ANTIFUNGAL BIOASSAY

The requirements for the antifungal activity were the following.

The test organisms used in the current research for crude methanolic extract and various fractions were; *Aspergillus niger*, *Aspergillus flavus*, *Penicillium notatum*, *Fusarium oxysporum*, *Trichoderma harzianum* and *Rhizopus stolonifer* provided by the Khyber PakhtunKhwa, Agriculture University Peshawar, Khyber PakhtunKhwa, Pakistan. The test organisms used for oils were: *A. flavus*, *Candida albicans*, *Candida glaberata, Fusarium solani* and *Microsporum canis*. The other requirements include: Sabouraud Dextrose Agar (SDA)**,** autoclave**,** incubator**,** micropipettes, magnetic stirrer**,** DMSO**,** screw cap test tubes**,** standard antifungal drugs (Miconazole and Amphotericin B) and test samples (crude methanolic extract and fractions of the plant).

Procedure

For determining the antifungal activity, the procedure of Bashir et al., was used [69].

Stock solutions were prepared from the test samples by dissolving 24 mg / ml in sterile DMSO. SDA media was prepared, autoclaved, introduced into Petri plates and incubated for 24 hrs at 28±1^{o}C for sterility check. The sterilized plates were used to refresh the above mentioned fungal species. The fungal species were inoculated into plates and incubated for 5-7 days at 25±1^{o}C, as we need to use a 5-7 days old culture in our experiments. 4 ml of SDA was introduced into the test tubes. The media was then autoclaved and when the temperature of the media was about 50^{o}C, 66.6 µl of test samples were added from each stock solution into respective test tube. The test tubes were kept in slanted position to make slants and incubated for 24 hrs at 28±1^{o}C for sterility check. After 24 hrs, the seven days old fungal culture was introduced into labeled test

tubes and incubated at 25±1°C for seven days in growth chamber. For negative and positive controls, DMSO and Miconazole were used.

The results were taken on day 7 by measuring the linear growth on the slanted test tubes in comparison with negative control using the following formula.

$$\% \text{ inhibition} = \frac{\text{Linear growth in test (mm)}}{\text{Linear growth in standard (mm)}} \times 100$$

2.3.3 PHYTOTOXIC ACTIVITY

Requirements

The following materials were required for phytotoxic activity

Flasks, E-media, *Lemna minor* L (test plant), test samples (crude methanolic extract and various fractions), growth chamber, magnifying glass and beakers

Procedure

The phytotoxic bioassay of the test samples were carried out according to Bashir et al., [71].

L. minor L was collected from the Department of Botany, University of Peshawar, Khyber PakhtunKhwa, Pakistan. Test samples were prepared at concentration of 20mg/ml of methanol serving as stock solutions. From the stock solutions 10, 100 and 1000µl were introduced into the respective flask with the help of micropipette. The flasks were left overnight for evaporation of methanol. E-media (for the growth of *L. minor* L) (**Table 2.1)** was prepared and 20ml of it was introduced into each flask after the evaporation of methanol. 16 healthy plants were selected and introduced into each flask. The flasks were incubated in growth chamber at $28\pm1^{o}C$ for seven days. Results were taken after seven days of incubation by counting the number of damaged plants.

Table 2.1 Composition of essential medium for phytotoxic activity

Constituents	**gm/L**
Potassium di-hydrogen phosphate	0.68
Potassium nitrate	1.515
Calcium nitrate	1.180
Magnesium sulphate	0.492
Boric acid	0.00286
Manganese chloride	0.00362
Ferric chloride	0.00540
Zinc sulphate	0.00022
Copper sulphate	0.00022
Sodium molybdate	0.00012
Ethylene diamine tetra-acetic acid	0.01120

2.3.4 INSECTICIDAL ACTIVITY

Requirements

Test insects (*Rhyzopertha dominica, Tribolium castaneum* and *Callosobruchus analis*) were provided by the HEJ, University of Karachi, Pakistan. The other requirements were: Organic solvent (methanol, Merck), growth chamber, test samples (crude methanolic extract and various fractions), standard insecticidal drug (Permethrin), Petri dishes (9 cm diameter), micropipette (1000 µl), filter paper, glass vials and brush

Test Samples Preparation

For preparation of the stock solutions, 200 mg of the test samples were dissolved in 3 ml of methanol.

Rearing Technique

In breeding media (sterile) the above mentioned pests were reared in plastic bottles in the laboratory, under controlled conditions of humidity and temperature. Insects of uniform size and age were selected for experimental work.

Procedure

For determining the insecticidal activity, the contact toxicity assay [72] was used. The assay was performed as following:

On first day Petri plates (9 cm or 90 mm) 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. The Petri plates were left overnight for methanol evaporation.

On the second day, 10 healthy insects of small and equal size from each specie were selected and transferred to the labeled plates using a clean brush. For 24 hours at 27°C with 50% relative humidity in growth chamber, incubation of the plates was done.

After incubation for 24 hrs, results were recorded by counting the number of survivals in each plate. The percent inhibition or mortality was calculated by using the following formula:

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

Permethrin (235.9 μg / cm^2) served as positive while methanol as negative control.

2.3.5 BRINE SHRIMP LETHALITY BIOASSAY

Materials

The requirements for this bioassay were: *Artemia salina* (test organism), sea salt, Pasture pipette, syringes; 5.0 and 0.5 ml, 100 and 10 µl, shallow rectangular plastic dish, glass vials, magnifying glass, organic solvent (methanol, Merck), double distilled water, aluminum foil, test samples (crude methanolic extract and fractions) and standard drug (Etoposide).

Procedure

The cytotoxic effect of the test samples was carried out against *A. salina* (brine-shrimp eggs) as per our reported procedure [73]. Sea water is a very good media for hatching of *A. salina*. Artificial sea water prepared with double distilled water and a commercial salt mixture; was used in a plastic dish for the hatching purpose. Eggs (50 mg) were introduced into unequally divided [Larger compartment (darkened) and smaller compartment (lightened)] plastic dish. This whole setup was kept for two days at room temperature and eggs were allowed to hatch and mature. After maturation, the nauplii were collected using a Pasteur pipette. Test samples (20 mg) were dissolved in 2 ml of volatile organic solvent, serving as stock solution. 5, 50 and 500 µl of the stock solution were transferred to vials (3 vials / concentration). The vials were placed in the hood for half an hour or allowed overnight so that the organic solvent evaporates completely. To each vial, larvae (10) and sea water (1 ml) were added. The final volume of each vial was adjusted to 5ml with sea water. The vials were incubated under illumination at 26±1ºC for 24 hrs. Organic solvents was used as negative control while reference cytotoxic drug, Etoposide (7.4625 µg / ml), as positive control. After incubation period brine shrimps that survived were counted using a magnifying glass. Finney computer program (Probit analysis) was used to analyze the data and determine LD_{50} values with 95% confidence interval.

2.3.6 HAEMAGGLUTINATION ACTIVITY

Requirements

Blood samples (all blood groups from volunteers), test tubes, centrifuge, phosphate buffer, Potassium dihydrogen phosphate (KH_2PO_4), Disodium hydrogen phosphate (Na_2HPO_4) and test samples (crude methanolic extract and fractions)

Procedure

Haemagglutination activity of test samples was performed as per our reported procedure [71]. Phosphate buffer was prepared by dissolving 0.453g/50ml of KH_2PO_4 and 0.47g/50ml of Na_2HPO_4 in distilled water and mixing KH_2PO_4 and Na_2HPO_4 in 3:7. In 1 ml of DMSO, 1 mg of the test samples was dissolved which served as stock solutions. Different dilutions i.e. 1:2, 1:4, 1:6, 1:8, 1:10 and 1:12 were made in phosphate buffer from stock solutions. Blood was taken from healthy volunteer individuals on the day of experiment and centrifuged. 2% RBC's suspension was prepared in phosphate buffer. From each dilution 1 ml of sample was taken in a test tube and then adds 1ml of the RBC's suspension to the sample. At 37^oC for 30 minutes, the tubes were incubated. After incubation, the tubes were centrifuged and looked for agglutination. Positive and negative results were indicated by rough granules and smooth button formation. Extent of deposition determined the intensity of positive result.

2.3.7 ANTI-TERMITE ACTIVITY

The procedure of Salihah et al [74] was employed for determining the anti-termite activity

Requirements

The following were required to carry out the anti-termite activity of the test samples.

Blotting paper, Petri dishes, scissors, beakers, magnifying glass with stand, *Heterotermes indicola* culture and test samples (crude methanolic extract, $CHCl_3$ and aqueous fractions).

Procedure

Petri plates and the blotting papers were sterilized and were cut according to the size of Petri plates (Petri plates were of uniform size). The test samples were prepared in respective solvents at a concentration of 2mg / ml. The blotting papers were dipped in the respective test sample, held for some time to remove the excess test sample and were then kept in the Petri plates. The Petri plates were left overnight so that the solvent evaporate from the blotting paper. Termites (25 in number) were then transferred to each Petri plate and observed with the help of magnifying glass after 24 hours, till all the termites were dead. Negative control was also applied with the test sample i.e. with methanolic fraction, methanol served as a negative control and so on. All the experiments were performed three times and the average number of termites killed each day was noted.

2.3.8 NITRIC OXIDE (NO) FREE RADICAL SCAVENGING ASSAY

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

Requirements

The following materials were required for the assay. 96-wells micro titer plate, 96-wells microtiter plate reader, micropipettes, test samples (crude methanolic extract and various fractions), Sodium nitroprusside (10mM), Sulphanilic acid (0.33% in 20% Acetic acid), [N-(1-Napthyl) Ethylenediaminedihydrochloride] (0.1% in H2O), Phosphate buffer (10mM, pH=7.4), DMSO and positive control (Vitamin C).

Procedure

The nitric oxide (NO) free radical scavenging assay was performed following Bashir, 2010 [75]. Stock solutions of test samples were prepared by dissolving 3mg / ml in DMSO. Different dilutions (0.3, 0.6, 0.9, 1.2 and 1.5 mg / ml) of test samples were prepared in DMSO using stock solutions. 10 µl of each dilution was introduced to microtiter plate and 20 µl of phosphate buffer was added to it. 70 µl of Sodium Nitroprusside was then added and incubated for 90 minutes at 20-25^{o} C. After incubation, microtiter plate was shacked and 50 µl of sulphanilic acid was added. Absorbance (pre-read) was taken at 570nm. 50 µl of [N-(1-Napthyl) Ethylenediaminedihydrochloride] was then added and shacked. End point was taken at 570nm. Vitamin C and DMSO were used as a positive control and blank respectively.

2.3.9 EFFECTS ON RABBIT'S JEJUNUM PREPARATIONS

For possible spasmolytic activity of the crude methanolic extract of plant, isolated rabbit's jejunum tissues were used.

Drugs, standards and solution preparation

Analytical grade chemicals, Acetylcholine and Atropine (E. Merck, Germany) were used in the experiments. Fresh solutions were prepared in distilled water on the day of the experiment. Normal Tyrode solution was prepared following Kobayashi method [76]. The ingredients of this solution are shown in **Table 2.2.**

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

Table 2.2 Composition of Tyrode solution used

Normal Tyrode Solution's constituents	**Concentration (gram /liter)**
NaCl	8.0
KCl	0.2
$MgCl_2$	0.1
NaH_2PO_4	0.5
Glucose	1.0
$NaHCO_3$	1.0
$CaCl_2$	0.2

Animals and data recording

Rabbits weighing from 1.0 - 1.4 kg were used in the experiments. The rabbits were maintained according to the international standards (Scientific Procedures) Act 1986, UK and Animals Bylaws 2008 of the University of Malakand (Scientific Procedures Issue- 1). All of the rabbits were fed with standard diet. No water was provided to the rabbits, 24 hours prior to the experiments. Teaching Force Transducer attached with Power lab was used to record tissue responses. Voltage used for recording the data was 20 mv.

Rabbit's jejunum preparations

The tissue preparation for the experiments was carried out using the following procedure [77].

For the isolation of the jejunum portion(s) of the rabbits, the rabbits maintained at animal house were sacrificed. Isolated tissues were placed in Tyrode's solution aerated with carbogen gas (5% CO_2 and O_2 mixture). At 37^oC, in Tyrode's solution aerated with carbogen gas, 1.5 cm piece of jejunum was mounted in 10 ml tissue bath. Pressure (one gram) was applied over the tissue. The tissue was maintained undisturbed and stabilized for a period of 30 minutes by giving the sub-maximal doses of acetylcholine (0.3 μM). The plant crude methanolic extract at concentrations of 0.01, 0.03, 0.1, 0.3, 1.0, 5.0 and 10 mg / ml were tested for the possible spasmolytic activity.

Spasmolytic activity

The highly concentrated K^+ solution (80 mM) was used to treat tissues in order to depolarize it and to get them in position of sustained concentration [78]. The test samples were then applied, in cumulative manner, on those pre-treated tissues mounted in tissue bath, to obtain a dose response curve [79].

5.0 MATERIALS AND METHODS

5.1 GENERAL EXPERIMENTAL CONDITIONS

The same experimental conditions were followed for Part B, as discussed in Part A.

5.2 Phytochemical investigation

5.2.1 Plant material

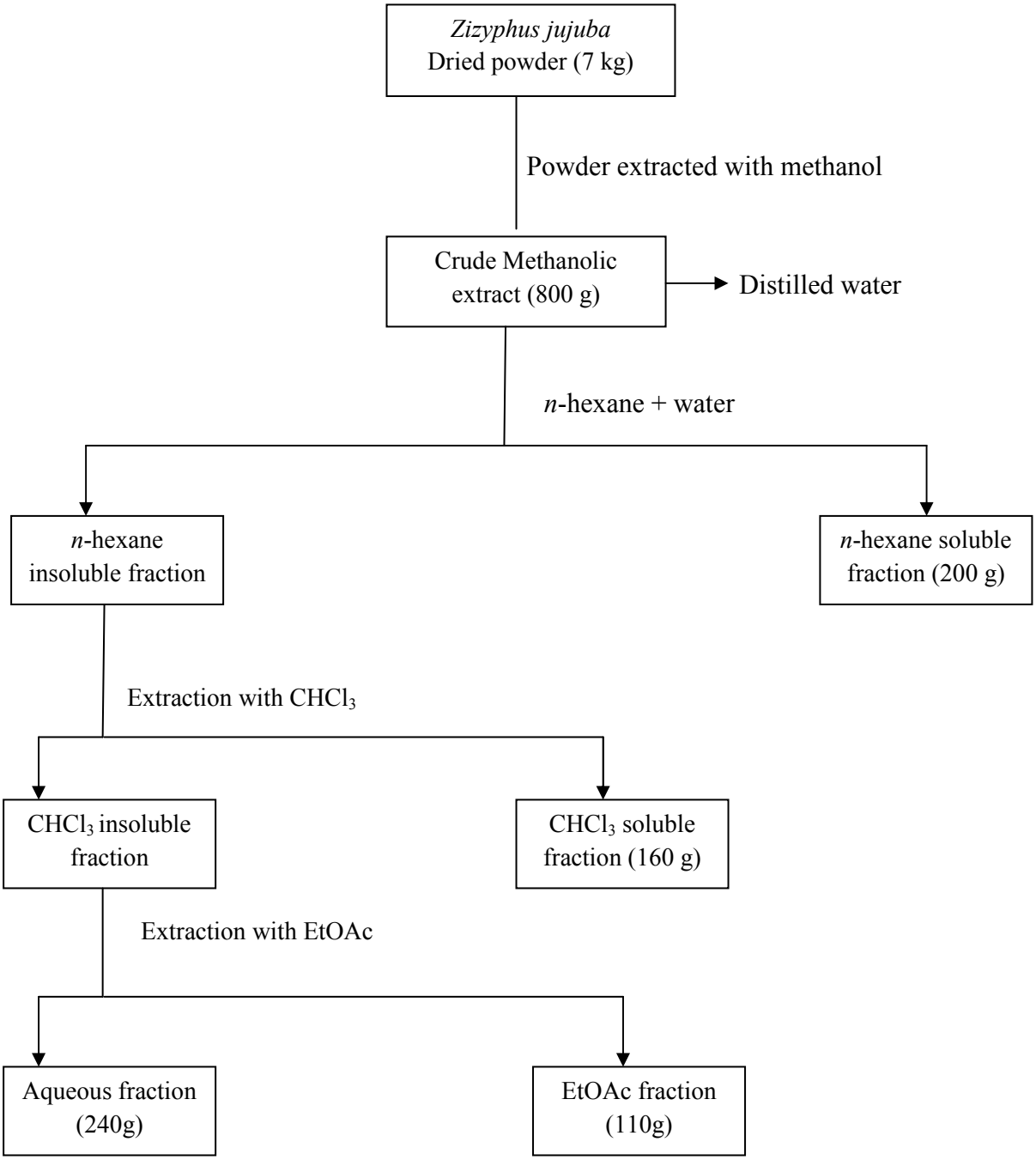
The aerial parts of *Zizyphus jujuba* were collected from the native Northern region of Khyber PakhtunKhwa Pakistan. The sample was very kindly identified by Prof. Dr. Abdur-Rashid, Department of Botany, University of Peshawar, Khyber PakhtunKhwa, Pakistan.

5.2.2 Extraction

The shade dried plant material was chopped into small pieces and grounded to powder, using an electric grinder. The powdered material of *Z. jujuba* (7 kg) was soaked in methanol for 15 days, twice, at room temperature, with occasional shaking. Each time the material was filtered and the filtrate was concentrated below 40^{o}C under vacuum using rotary evaporator. A blackish crude methanolic extract (800 g) of *Z. jujuba* was obtained.

5.2.3 Fractionation

In distilled water (500 ml), the crude methanolic extract of *Z. jujuba* (710 g) was suspended and partitioned with *n*-hexane (3 x 500ml), $CHCl_3$ (3 x 500ml) and EtOAc (3 x 500 ml) respectively yielding *n*- hexane (200 g), $CHCl_3$ (160 g), EtOAc (110 g) and aqueous (240 g) fractions, respectively. 90g of the crude methanolic extract of *Z. jujuba* was left for biological / pharmacological activities **(Scheme-20)**. All the fractions will only carry their particular compounds based on the solubility from the crude methanolic extract. For example the *n*- hexane fraction will contain only those compounds which are non-polar, and so on.



Scheme 20 Extraction of crude methanolic extract and preparation of various fractions of *Zizyphus jujuba*

5.2.4 Screening for Different Groups of Compounds

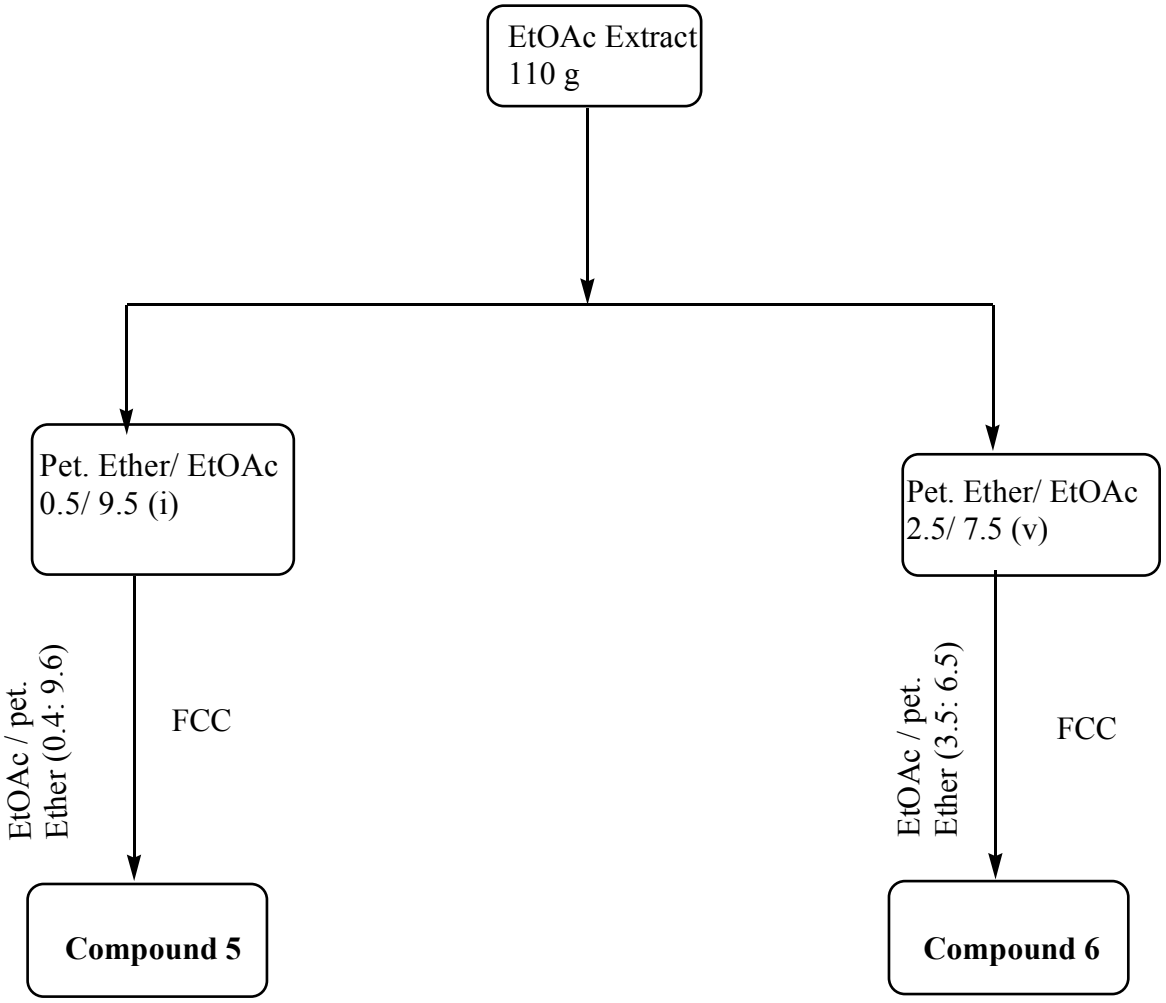
Tests for the presence of different compounds groups i.e. flavonoids, alkaloids, saponins and tannins were carried as discussed in ***Part-A***.

5.2.5 Compounds isolated from *Zizyphus jujuba*

The Ethyl acetate (EtOAc) fraction (55 g) of *Z. jujuba* was subjected to Column Chromatography (CC) and sequentially sub-fractionated in increasing order of polarity. The increasing order of polarity was EtOAc / Pet ether (i) 0.5 / 9.5, (ii) 1.0 / 9.0, (iii) 1.5 / 8.5, (iv) 2.0 / 8.0, (v) 2.5 / 7.5, (vi) 3.5 / 6.5, (vii) 4.0 / 6.0, (viii) 5.0 / 5.0, (ix) 7.0 / 3.0, (x) 9.0 / 1.0 and 100 % EtOAc **(Scheme-21)**.

From the above sub fractions, those fractions that showed good separation pattern of compounds on TLC plate using a given solvent system were subjected to Flash column chromatography (FCC) for separation of compounds.

Compound **5** was obtained from the sub fraction (i) by eluting with the solvent system EtOAc / Pet ether (0.4: 9.6). Compound **6** was obtained from the sub fraction (v) by eluting with the solvent system EtOAc / Pet ether (3.5: 6.5). Compound **7** was obtained from the chloroform ($CHCl_3$) fraction by eluting with the solvent system $CHCl_3$ / Pet ether (2.0: 8.0). Compound **8** was obtained from *n*-hexane fraction by eluting with the solvent system EtOAc / Pet ether (0.3: 9.7).



Scheme-21 Isolation of compound form EtOAc fraction of *Zizyphus jujuba*

5.2.5.1 Characterization of Compound (5)

Yield: 23 mg from EtOAc fraction

Physical state: White amorphous solid

MP: 280^{o}C

UV (MeOH), λ_{max} nm: 297

IR (KBR) ***V*** **max cm^{-1}**: 3417-2600, 1710, 1670 and 1007

FDMS ***m/z***: 254.45 [M^{+}]

HREIMS ***m/z*** **(formula; Calcd value):** 254.45 ($C_{16}H_{14}O_{3.}$ 254.45)

^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.89 (d. 1H, $J_{2.0}$ Hz, H-29a), 5.01 (1-H. d, $J_{2.0}$ Hz, H-3"), 5.21 (1H, m, H-2b"), 6.22 (3-H, s, H-2"), 6.48 (6-H, s, H-2), 5.99 (3-H, s, H-5)

5.2.5.2 Characterization of Compound (6)

Yield: 34 mg from EtOAc fraction

Physical state: White amorphous solid

MP: 282^{o}C

UV (MeOH), λ_{max} nm: 200

IR (KBR) ***V*** **max cm^{-1}**: 3400-2600, 1705, 1615 and 1245

FDMS ***m/z***: 456 [M^{+}]

EIMS ***m/z*** **(rel. int. %):** 456 [M^{+}.12], 438 (12), 411 (6), 248 (45), 228 (52), 207 (66), 203 (238), 189 (100)

HREIMS ***m/z*** **(formula; Calcd value):** 456. 3601 ($C_{30}H_{48}O_{3.}$ 456.36.03)

^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.95 (d. 1H, $J_{2.0}$ Hz, H-29a), 4.78 (1-H. d, $J_{2.0}$ Hz, H-29b), 3.55 (1H, m, H-3), 1.80 (3-H, s, H-30), 1.23 (6-H, s,H-23), 1.08 (3-H, s, H-27), 1.07 (3-H, s,H-25), 1.02 (3-H, s,H-26), 0.82 (3-H, s, H-24)

^{13}C NMR: See **Table 6.2**

5.2.5.3 Characterization of Compound (7)

Yield: 14 mg from $CHCl_3$ fraction

Physical state: Colorless crystalline solid

M.P: 170^oC

$[\alpha]^{25}_D$ - 51.5^o (c = 0.28, $CHCl_3$)

IR ($CHCl_3$) V_{max} **cm^{-1}** 3432 (OH), 1648 (C=C)

EIMS *m/z* (rel. int. %) $[M]^+$ 412 (8), 396 (12), 394 (20), 379 (27), 369 (35), 351 (71), 327 (60), 301 (18), 300 (67), 273 (30), 270 (24)

HREI-MS *m/z* 412.3920 (calcd. for $C_{29}H_{48}O_3$ 412.3926)

^{1}H-NMR ($CDCl_3$, 400MHz): δ 5.33 (1H, m, H-6), 5.15 (1H, dd, $J_{15.2,\ 8.4}$ Hz, H-22), 5.02 (1H, dd, $J_{15.2,\ 8.6}$ Hz, H-23), 3.28 (1H, m, H-3), 0.90 (3H, d, $J_{6.5}$ Hz, Me-21), 0.83 (3H, d, $J_{6.6}$ Hz, Me-26), 0.84 (3H, t, $J_{7.0}$ Hz, Me-29), 0.81 (3H, d, $J_{6.5}$ Hz, Me-27), 0.80 (3H, s, Me-19), 0.65 (3H, s, Me-18)

^{13}C-NMR ($CDCl_3$, 100 MHz): See **Table 6.3**

5.2.5.4 Characterization of Compound (8)

Yield: 27 mg from *n*-hexane fraction

Physical state: Needle like crystals

M.P. 142^{o}C

IR (KBR) νmax: 3402, 2901, 1641 cm^{-1}

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

HREIMS m/z: 414.3857 (calcd. 414.3861 for C29H50O)

^{1}H-NMR (CDCl3, 400 MHz) δ: 5.32 (1H, m, H-3α), 0.92 (3H, s, CH3-19), 0.88 (3H, d, J 21,20 = $_{6.5}$ Hz, CH3-21), 0.83 (3H, d, J26,25 = $_{6.5}$ Hz, CH3-26), 0.81 (3H, d, J27,25 = $_{6.5}$ Hz, CH3-27), 0.77 (3H, t, J 29,28 = $_{7.0}$ Hz, CH3-29), 0.63 (3H, s, CH3-18)

^{13}C-NMR (CDCl3, 100 MHz): See **Table 6.4**

5.3 PHARMACOLOGICAL INVESTIGATION

5.3.1 ANTIBACTERIAL ACTIVITY

To determine the antibacterial activity of the crude methanolic extract and fractions of *Z. jujuba* the same procedure as described in ***Part-A,*** was followed.

5.3.2 ANTI FUNGAL ACTIVITY

Same procedure as described in ***Part-A,*** was followed for antifungal activity of the crude methanolic extract and fractions of *Z. jujuba*.

5.3.3 PHYTOTOXIC ACTIVITY

Same procedure as described in ***Part-A*** was followed for phytotoxic activity of crude methanolic extract and fractions of *Z. jujuba.*

5.3.4 INSECTICIDAL ACTIVITY

Same procedure as described in ***Part-A*** was followed for insecticidal activity of the crude methanolic extract and fractions of *Z. jujuba.*

5.3.5 BRINE SHRIMP LETHALITY BIOASSAY

Using the same procedure as described in ***Part-A,*** brine shrimp lethality of the crude methanolic extract and fractions of *Z. jujuba* were determined.

5.3.6 HEAMAGGLUTINATION ACTIVITY

The procedure for determining the haemagglutination activity of crude methanolic extract and fractions of *Z. jujuba* was the same as described in ***Part A***.

5.3.7 ANTI-TERMITE ACTIVITY

The anti-termite activity of the crude methanolic extract and fractions of *Z. jujuba* were determined using the same procedure as described in ***Part-A*.**

5.3.8 NITRIC OXIDE FREE RADICAL SCAVENGING ASSAY

For determining the nitric oxide free radical scavenging activity of the crude methanolic extract and fractions of *Z. jujuba*, same procedure as described in ***Part-A,*** was followed.

5.3.9 EFFECTS ON RABBIT'S JEJUNUM PREPARATIONS

Spasmolytic activity

The cholinomimatic activity of the crude methanolic extract of *Z. jujuba* was determined as described in ***Part-A.***

3.0 RESULTS AND DISCUSSION

3.1 PHYTOCHEMICAL INVESTIGATIONS

3.1.1 Screening for Different Groups of Compounds

Plants contain a variety of natural products like flavonoids, alkaloids, tannins and saponins that have direct link with the medicinal values of that particular plant. These natural products, after isolation and formulations in various pharmaceutical dosage forms, are used to treat various diseases.

Acacia modesta was screened for presence of various groups of natural products. The data in the **table 3.1** reveal that during the test for alkaloids, precipitate formation was observed indicating the presence of alkaloids in this plant. The development of pink color observed in test for flavonoids confirm that the plant also contains flavonoids group of natural products. Result for the presence of saponins was negative as there was no froth formation. The formation of precipitate and subsequent appearance of blue green color indicated presence of tannins in the plant.

Table 3.1 Phytochemical Screening for *Acacia modesta*

S. No.	TEST	Results	Remarks	Page (Material and Methods)
1	Alkaloids	+	Positive	45
2	Flavonoids	+	Positive	45
3	Saponins	-	Negative	45
4	Tannins	+	Positive	46

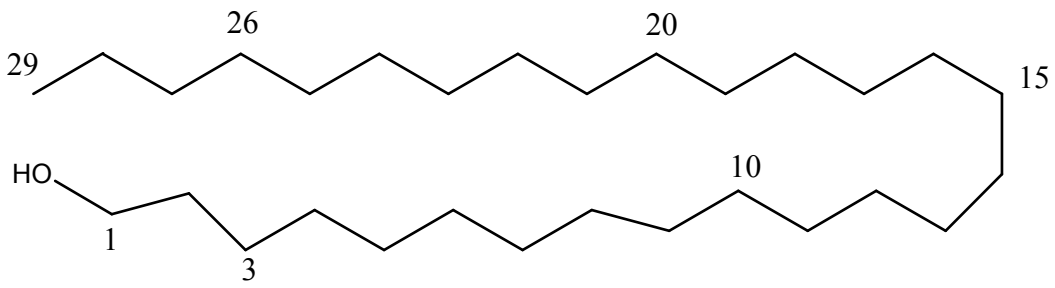
3.1.2 STRUCTURAL ELUCIDATION OF ISOLATED COMPOUNDS

3.1.2.1 Structural Elucidation of Compound (1)

Compound **1** was isolated from the EtOAc fraction of *Acacia modesta* eluting with EtOAc / Pet ether (0.2: 9.8) as mobile phase on flash silica column. M^+ at *m/z* 424 was shown by EI MS of compound **1** which in combination of ^{13}C NMR determined the formula $C_{29}H_{60}O$. Bands at 3442 and 1057 cm^{-1} in IR spectrum of compound **1** shows hydroxyl group and aliphatic nature of the molecule. The ^1H NMR spectrum exhibit a triplet at δ 0.82 (3H, $J_{7.2}$ Hz), which was assigned to the protons of terminal methyl group. A downfield triplet at δ 3.55 (2H, $J_{6.6}$ Hz) was attributed to the methylene protons attached to the hydroxyl. All the MS and NMR values of the compound unambiguously matched with the reported compound in the literature as Nonaeicosanol [80].

Table 3.2 ^{13}C and ^{1}H NMR chemical shift assignment of compound **1** ($CDCl_3$, ppm, 75 and 300 MHz respectively)

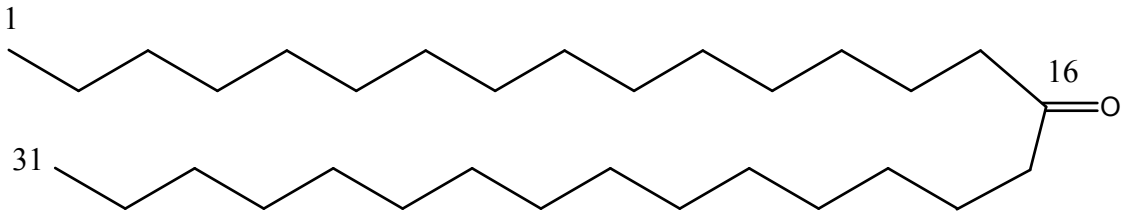
C. No.	δ_C	δ_H (*J*, Hz)
1	62.76	3.55 t (6.6)
2	32.6	2.26 m
3	25.7	1.25 m
4	29.3	1.24 m
5	29.4	1.12 m
6-26	29.5	1.19 overlapped
27	31.8	1.50 m
28	22.6	1.23 overlapped
29	14.0	0.82 t (7.2)



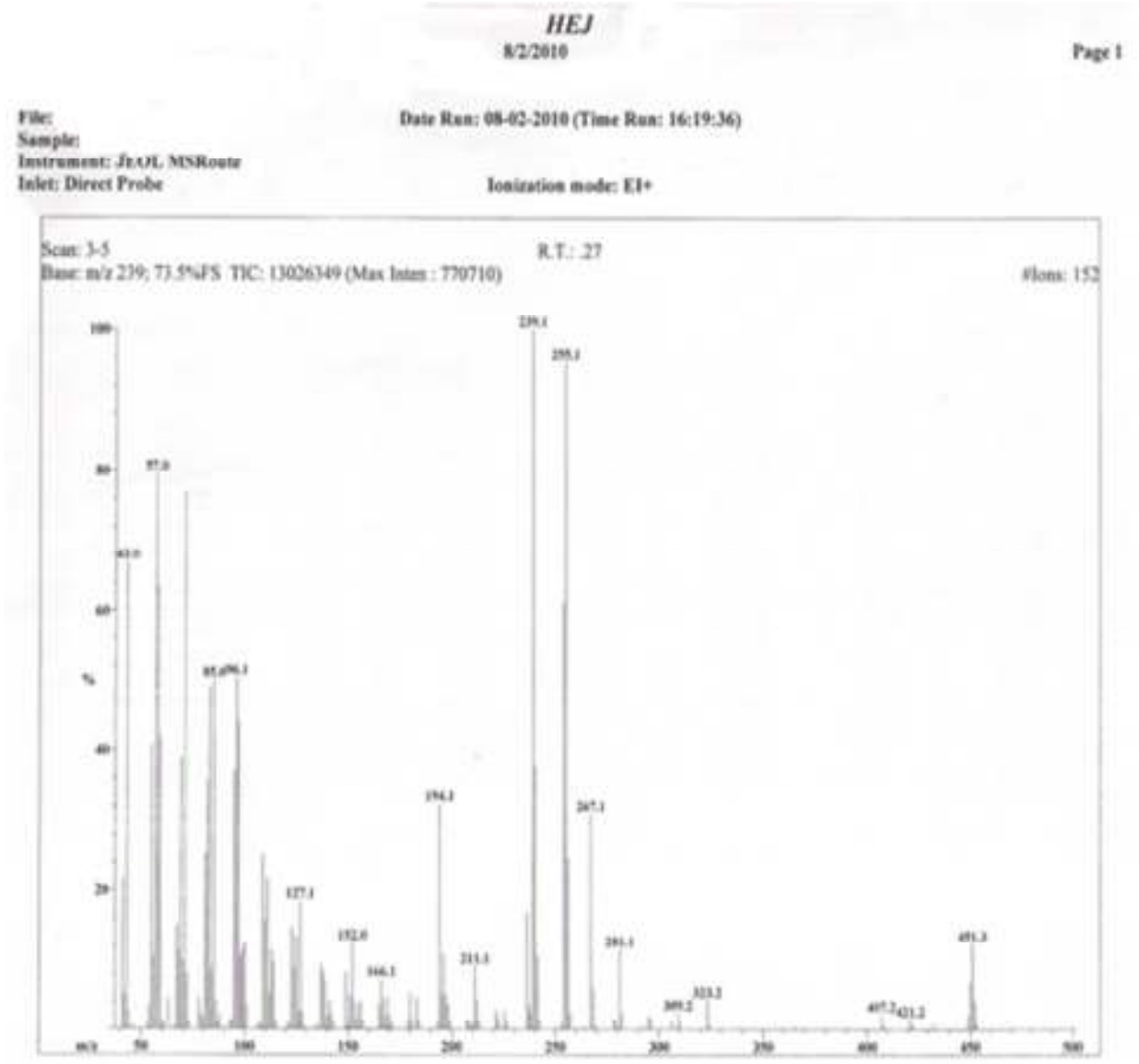
Nonaeicosanol (1)

3.1.2.2 Structural Elucidation of Compound (2)

The compound **2** was isolated from EtOAc fraction of *A. modesta* eluting with EtOAc / Pet ether (3.5: 6.5) as mobile phase on flash silica column. M^+ at *m/z* 450 in EI MS and other prominent peaks at *m/z* 239 and 211 indicated that compound is palmitone. Its IR spectrum displayed presence of ketone functionality (1723 cm^{-1}). ^{13}C NMR spectrum displayed signals for one carbonyl carbon at δ 211.0 (C-16), signal for terminal methyl at δ 13.8 (C-1 and C-31). Methylene carbons adjacent to the carbonyl group were shown by signal at δ 42.0. The NMR values unambiguously matched with the reported compound in literature as palmitone [81]. Palmitone is anticonvulsant in nature [82].



Palmitone (2)



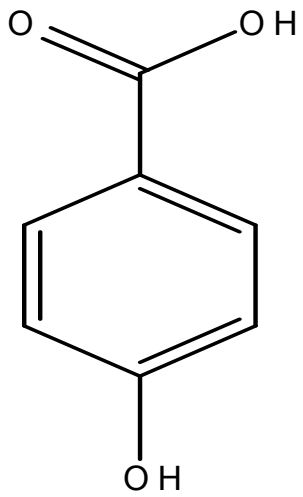
Spectra 1: EI MS spectra of palmitone

3.1.2.3 Structural Elucidation of Compound (3)

The compound **3** was isolated from EtOAc fraction of *A. modesta* eluting with EtOAc / Pet ether (4.0: 6.0) as mobile phase on flash silica column. Molecular ion peak of compound **3** at *m/z* 138.0316 in HREI-MS showed the molecular formula $C_7H_6O_3$ (calcd. for $C_7H_6O_3$, 138.0309). Absorption bands at 3510 (OH), 1705 (C=O) and 1626 (aromatic) were present in IR spectrum. The ^{1}H-NMR spectrum of compound **3** displayed two resonances in the aromatic region at δ 7.92 (2H, d, $J_{8.5}$ Hz, H-3, 5), 6.72 (2H, d, $J_{10.68}$ and 8.5 Hz, H-2, 4) and a carboxylic proton at δ 11.92 (2H, br s).

The presence of seven signals for four methine and three quaternary carbons were confirmed in ^{13}C-NMR (BB and DEPT) spectrum of compound **3**. The downfield signal at δ180.0 and 160.3 were assigned to acid carbonyl and aromatic oxygenated quaternary carbon atoms, whereas other signals in the aromatic region at δ 131.5, 116.5 and 122.4 were assigned to aromatic methines and aromatic quaternary carbon atoms.

The compound **3** was identified as 4-hydroxybenzoic acid on the basis of reported literature values [83] and the above evidences.



4-hydroxybenzoic acid (3)

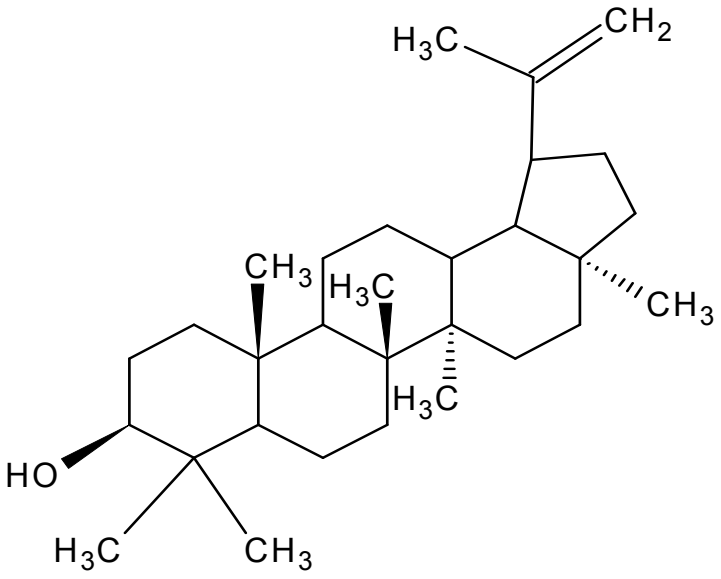
3.1.2.4 Structural Elucidation of Compound (4)

Compound **4** was isolated from the EtOAc fraction of *A. modesta* eluting with EtOAc / Pet ether (5.6: 4.4) on flash silica column. Molecular ion peak at *m/z* 426.3835 in HREI-MS of compound **4** confirmed the molecular formula $C_{30}H_{50}O$ (calcd. for $C_{30}H_{50}O$, 426.3861). It showed characteristic band at V_{max} 3450 cm^{-1} for hydroxyl group in IR spectrum. The terminal bond presence was confirmed by bands at V_{max} cm^{-1} 3070, 1650 and 880 cm^{-1}.

Beside $[M^+]$, other fragments occurring at m/z 385 $[M- 41]^-$, 220 $[M- C_{15}H_{26}]^+$, 218 $[M- C_{14}H_{20}]^+$, and 207 $[M- C_{16}H_{27}]^+$ matched the characteristics of lupine series [84].

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

On the basis of above evidences and literature review [85], the compound was designated as Lupeol.



Lupeol (4)

3.1.2.5 GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS) OF ESSENTIAL OILS

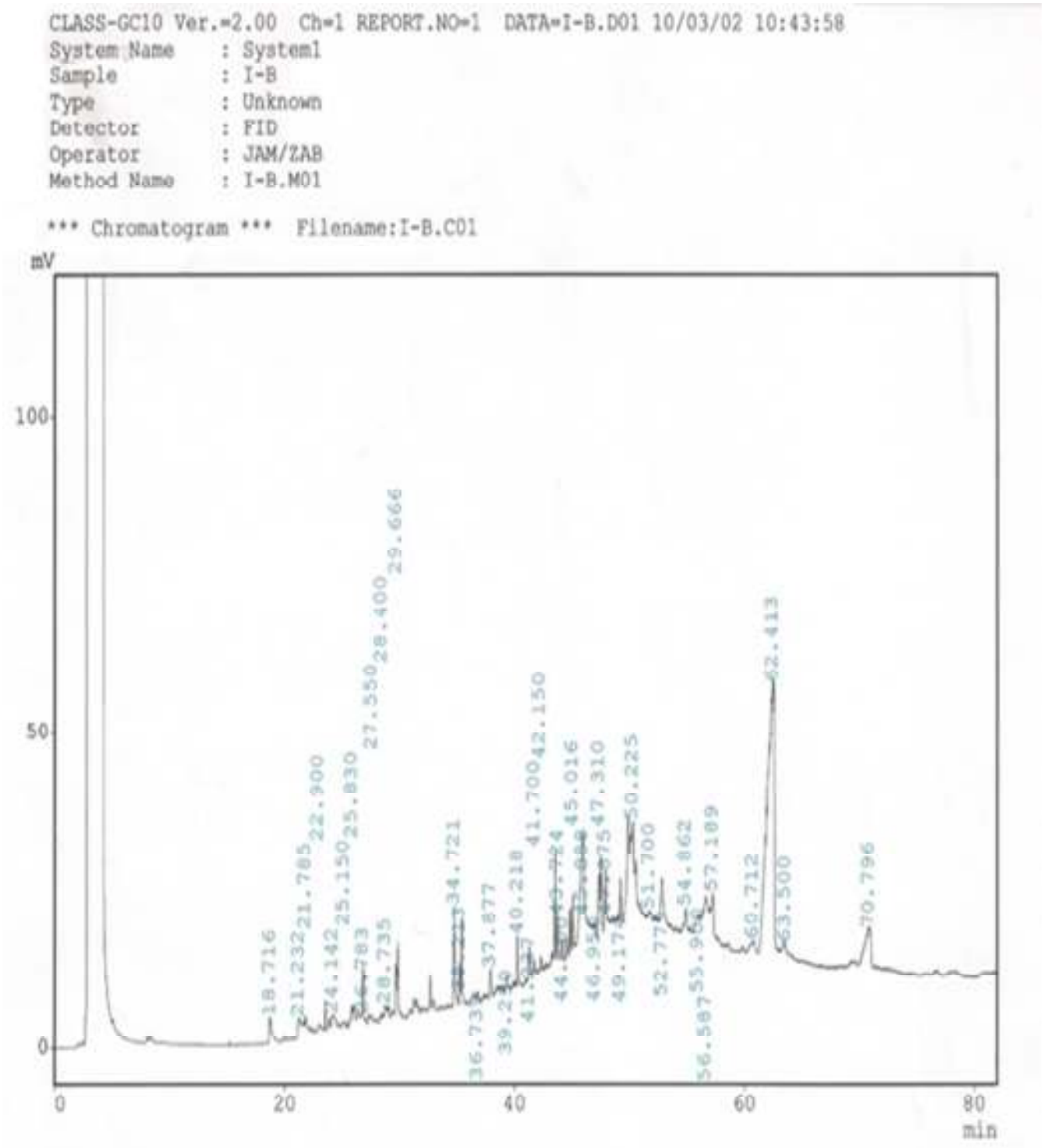
The food industry is in search of news ways to prevent the growth of food borne and spoiling microbes instead of chemical preservatives. The use of essential oils from plant's origin is one of the new approaches. Essential oils are gaining popularity both in scientific research and industry because of their antifungal, antioxidant and antibacterial activities and could be used in foods as natural additives [86]. Major losses in agriculture production are caused by fungi. For example *Aspergillus* spp. causes spoilage of mangoes while *Fusarium* spp. causes spoilage during food production. The aflotoxins of *Aspergillus flavus* and *A. parasiticus* contaminate corn, cotton seed, peanuts and tree nuts during storage or harvesting [87]. Wheat and barley is commonly attacked by Fusarium head blight caused by *Fusarium oxysporum*. This results in economic losses i.e. reduction of grain quality and yield. The seeds contaminated by the mycotoxins have low market value [88]. The identification of novel antifungal drugs is need due to increase of fungal diseases in human, animals and plants. The scientific community is focusing on medicinal plants to combat fungal infectious diseases [89]. The development of highly effective and selective biopesticides that are non-toxic to humans and animals has increased as a result of awareness of ecology [90]. *Plutella xylostella* (Lepidoptera: Plutellidae) is a global, catastrophic vegetable pest that causes infection for several generations in a year. The active components isolated from fermented products of *Paecilomyces cicadae* (Miquel) Samson have played a key role in ecological and environmental protection against the pest [91]. Multiple therapeutic activities have been reported for *P. cicadae* in recent years [92-96].

Keeping in view the importance of essential oils, the *n*-hexane fraction of the aerial parts of *A. modesta* was subjected to column chromatography for the extraction of essential oils. Silica

gel was used as stationary phase while *n*-hexane served as mobile phase. The results are given in **Table 3.3.** The GC-MS analysis revealed that these oils contain thirty eight components.

Table 3.3 Essential oil components of aerial parts of *Acacia modesta*

S.NO	Component	Retention Time	Factor
1	Benzyl Alcohol	5:41.3	66.8
2	Benzyl urethane	8:44.6	62.9
3	Tridecane	12:41.2	66.9
4	Benzenepropanoic acid, å-hydroxy-, methyl ester	15:36.7	71.1
5	L-Phenylalanine, N-[N-(trifluoroacetyl)-L-alanyl]-, methyl ester	15:39.3	61.6
6	Tetradecane	15:53.6	74.6
7	5-Benzofuranacetic acid, 6-ethenyl-2,4,5,6,7,7a-hexahydro-3, 6-dimethyl-å-methylene-2-oxo-, methyl ester	17:37.6	60.3
8	Pentadecane	18:58.2	73.1
9	Phenol, 2,4-bis (1, 1-dimethylethyl)-	20:25.2	77.3
10	1-Decanol, 2-hexyl-	21:47.1	81.7
11	Propanoic acid, 2-methyl-, 1- (1,1-dimethyl)-2-methyl-1,3-propanediyl ester	21:54.9	79
12	5-Benzofuranacetic acid, 6-ethenyl-2,4,5,6,7,7å-hexahydroxy-3,6-dimethyl-å-methylene-2-oxo-, methyl ester	23:24.6	59.5
13	Colchicine, N-desacetyl-N-retinoyl	27:13.4	64.2
14	9-Hexadecenoic acid	27:14.7	72.5
15	Acetamide, N-methyl-N-[4-[4-methoxy-1-hexahydropyridyl]-2-butynyl]-	27:16	72.7
16	Acetamide, N-methyl-N-[4-[4-methoxy-1-hexahydropyridyl]-2-butynyl]-	27:17.3	73.7
17	11-Octadecenal	27:19.9	72.6
18	10,13-Eicosadienoic acid, methyl ester	28:28.8	65.9
19	8,11-Eicosadienoic acid, methyl ester	28:30.1	66.4
20	Ethanol,2- (9-octadecenyloxy)-,(Z)-	28:31.4	69.1
21	Pentadecanoic acid,14-methyl-, methyl ester	30:53.1	75.6
22	Cyclopropanepropionic acid, 2- [(2-decylcyclopropyl) methyl ester	32:21.5	60.3
23	3', 8,8'-Trimethoxy-3-piperidyl-2,2'-binaphtthyl-1,1',4,4'-tetrone	32:24.1	62.9
24	Cyclopropaneoctanoic acid, 2-[(2-pentylcyclopropyl)methyl]-, methyl ester, trans, trans-	32:25.4	61.8
25	4H-1-Benzopyran-4-one,5,7-dihydroxy-2-(4-hydroxyphenyl)3-methoxy-	32:30.6	65.2
26	Oxiraneoctanoic acid, 3-octyl-, cis	32:31.9	64
27	9, 15-Octadecadienoic acid, methyl ester, (Z,Z)-	34:57.4	70.9
28	9,15,octadecadienoic acid, methyl ester, (Z,Z)-	35:01.3	70.4
29	7,10,octadecadienoic acid, methyl ester	35:06.5	70.7
30	10-H-Phenothiazine, 2-chloro-6-methoxy-	35:09.1	68
31	Nonanoic acid, 9-(3-hexenylidenecyclopropylidene)-2-hydroxy-1-(hydroxymethyl) ethyl ester	35:11.7	68.2
32	4H-1-Benzopyran-4-one-,2(3,4-dihydroxyphenyl)-6,8-di-å-D-glucopyranosyl-5,7-dihydroxy-	35:16.9	68.1
33	Methyl 9,12-epithio-9,11-octadecanoate	35:18.2	68.3
34	Thiocyanic acid, (2-benzothiazolythio) methyl ester	37:07.4	60
35	1,2-Benzenedicarboxylic acid, diisooctyl ester	47:37.8	78.4
36	Card-20 (22)-enolide, 3-[(4,6-dideoxy-3-O-methylhexopyranos-2-ulos-1-yl) oxy]-6, 11, 14- trihydroxy-12-oxo-, (3å, 11å)-	51:35.7	67.4
37	1' H-Androst-16-eno [17,16-b] indol-3-ol, 1'-methyl-, acetate (ester), (3å, 5å)-	51:38.3	53.8
38	1H-2,8a-Methanocyclopenta [a] cyclopropa [e] cyclodecen-11-one, 5, 6-bis (acetoloxy)-1-[(acetoloxy) methyl]- 1a, 2,3,4,5,5a, 6,9,10,10a-decahydro-5a-hydroxy-1,4,7,9,tetramethyl-	51:42.2	52



Spectra 2: Gas chromatogram of oils of *Acacia modesta*

3.2 PHARMACOLOGICAL INVESTIGATIONS

3.2.1 ANTIBACTERIAL ACTIVITY

Crude methanolic extract and fractions

One of the major problems faced by medical science these days is antibiotic resistance. Finding new and innovative antimicrobials will help us to tackle this problem [97]. *Staphylococcus aureus* has become resistant to several antibiotics to which it was previously susceptible. Some of the antibiotics to which it is now resistant are pencillin G, macrolides, lincosamides, tetracyclines and gentamicin [98].

With the intent of exploring new bioactive compounds from plant origin, the test samples were tested against the selected pathogens. **Table 3.4 and Figures 3.1-3.5** show the results of the antibacterial activity. The crude methanolic extract showed moderate activity against *E. coli* (40.74%), *P. aeruginosa* (40.74%) and *B. pumalis* (40%), low against *S. epidermidis* (34.61%), *S. typhi* (22.22%), *S. pneumoniae* (27.58%) and *E. aerogens* (31.03%) and no activity against *S. aureus* and *K. pneumoniae.* The *n*-hexane fraction was significantly active against *K. pneumoniae* (66.66%) and moderately active against *E. coli* (48.14%), *S. typhi* (51.85%), *P. aeruginosa* (51.85%) and *B. pumalis* (40%). It showed low activity against *S. epidermidis* (34.61%), *S. pneumoniae* (20.68%), *S. aureus* (38.46%) and *E. aerogens* (34.48%). Chloroform fraction was moderately active against *K. pneumoniae* (57.14%), *S. typhi* (48.14%), *E .aerogens* (41.37%) and *B. pumalis* (40%) and low active against *S. aureus* (38.46%), *P. aerugenosa* (37.03%), *S. epidermidis* (34.61%), *E. coli* (33.33%) and *S. pneumoniae* (24.13%). Significant activity was shown by the EtOAc fraction against *K. pneumoniae* (61.90%), moderate against *S. typhi* (48.14%), *E. coli* (44.44%), *P. aeruginosa* (44.44%) and *B. pumalis* (40%) while low activity against *E. aerogens* (37.93%), *S. aureus* (34.61%), *S. epidermidis* (34.61%) and *S.*

pneumoniae (24.13%). The aqueous fraction showed moderate activity against *S. epidermidis* (53.84%), *B. pumalis* (44%), *E. coli* (48.14%) and *S. typhi* (40.74%), low activity against *K. pneumoniae* (38.09%), *P. aeruginosa* (37.03%), *E. aerogens* (37.93%), *S. aureus* (34.61%) and no activity against *S. pneumoniae.*

The above results indicate that crude methanolic extract have low activity against most of the test pathogens. The *n*-hexane and EtOAc fractions exhibited significant while $CHCl_3$ fraction showed moderate activity against *K. pneumoniae*. The aqueous fraction showed low activity against the majority of test pathogens.

Potency of a drug is inversely related to its MIC_{50} value. The MIC_{50} values of the test samples are presented in **Table 3.5 and Figures 3.6-3.10.** These values were calculated from three separate readings using Microsoft XL sheet. The MIC_{50} of the test samples range from 2.4-3.9 mg / ml.

Essential oils

The antimicrobial plant products are gaining popularity as a candidate to fight antimicrobial resistance [99] and as an alternative to synthetic food additives that are harmful to human health [100]. Among these plant derived antimicrobial products, essential oils have an important role as an antioxidant, anti-inflammatory and antimicrobial agents. These essential oils many consist of sesquiterpenes, monoterpenes and their oxygenated derivatives (aldehydes, alcohols, ethers, esters, ketones, oxides and phenols) [101].

Screening of essential oils of *A. modesta* for antibacterial activities was carried out against *E. coli*, *Shigella flexenari*, *B. subtilis*, *S. aureus*, *P. aeruginosa* and *S. typhi*. **Table 3.6** comprises the results. The results indicate that the oils of *A. modesta* were inactive against all the test organisms.

Table 3.4 Antibacterial activity of crude methanolic extract & various fractions of *Acacia modesta*

Name of Bacteria	Zone of Inhibition of standard (Amoxicillin) 10µg / Disc	Crude Extract		*n*-hexane		$CHCl_3$		EtOAc		Aqueous	
		Zone of Inhibition (mm)	Inhibition (%)	Zone of Inhibition (mm)	Inhibition (%)	Zone of Inhibition (mm)	Inhibition (%)	Zone of Inhibition (mm)	Inhibition (%)	Zone of Inhibition (mm)	Inhibition (%)
E. coli	27	11	40.74	13	48.14	9	33.33	12	44.44	13	48.14
S.epidermidis	26	9	34.61	9	34.61	9	34.61	9	34.61	14	53.84
S.typhi	27	6	22.22	14	51.85	13	48.14	13	48.14	11	40.74
S.pneumoniae	29	8	27.58	6	20.68	7	24.13	7	24.13	0	0
S.aureus	26	0	0	10	38.46	10	38.46	9	34.61	9	34.61
P.aeruginosa	27	11	40.74	14	51.85	10	37.03	12	44.44	10	37.03
K. pneumoniae	21	0	0	14	66.66	12	57.14	13	61.90	8	38.09
B.pumalis	25	10	40	10	40	10	40	10	40	11	44
E.aerogens	29	9	31.03	10	34.48	12	41.37	11	37.93	11	37.93

Table 3.5 MIC_{50} values (mg / mL) of crude methanolic extract and various fractions of *Acacia modesta*

Name of Bacteria	Crude extract	*n*-hexane	$CHCl_3$	EtOAc	Aqueous
E. coli	3.2	2.7	3.5	2.9	2.7
S. epidermidis	3.5	3.2	3.5	3.4	2.9
S. typhi	3.7	2.7	2.9	2.7	3.2
S. pneumoniae	3.7	3.9	3.9	3.5	0
S. aureus	0	3.2	3.2	3.5	3.8
P. aeruginosa	3.2	2.7	3.2	3	3.7
K. pneumoniae	0	2.4	2.5	2.4	3.5
B. Pumalis	3.2	3.2	3.2	3.2	2.9
E. aerogenes	3.9	3.6	3.2	3.9	3.9

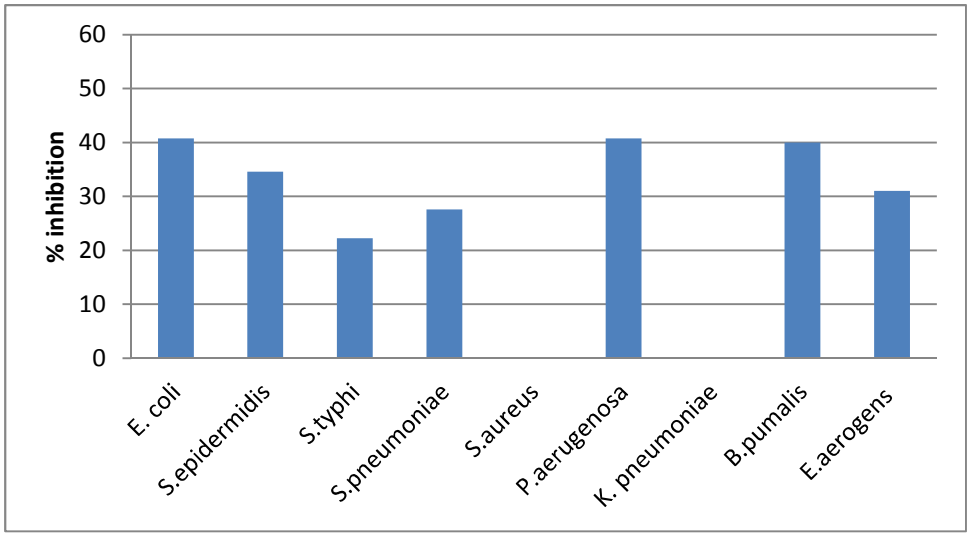


Fig 3.1 Antibacterial activity of the crude methanolic extract of *Acacia modesta*

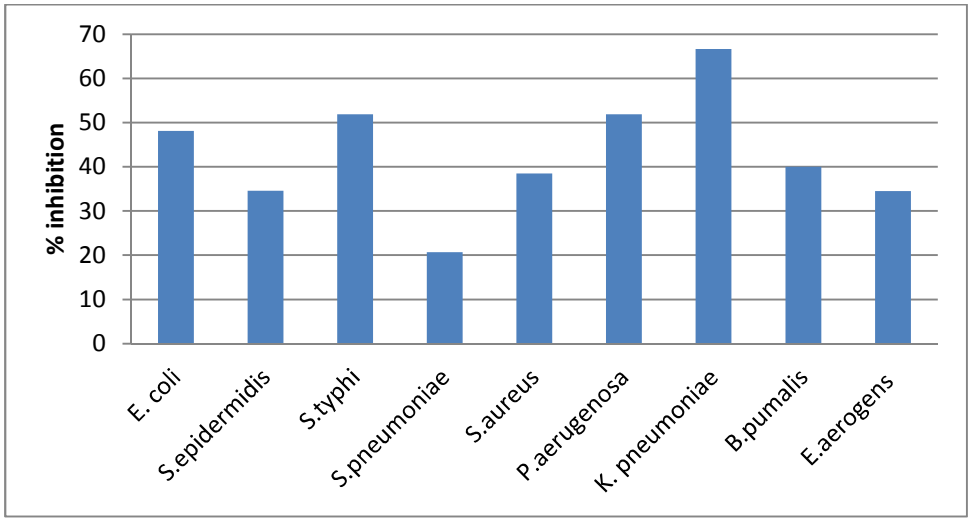


Fig 3.2 Antibacterial activity of *n*-hexane fraction of *Acacia modesta*

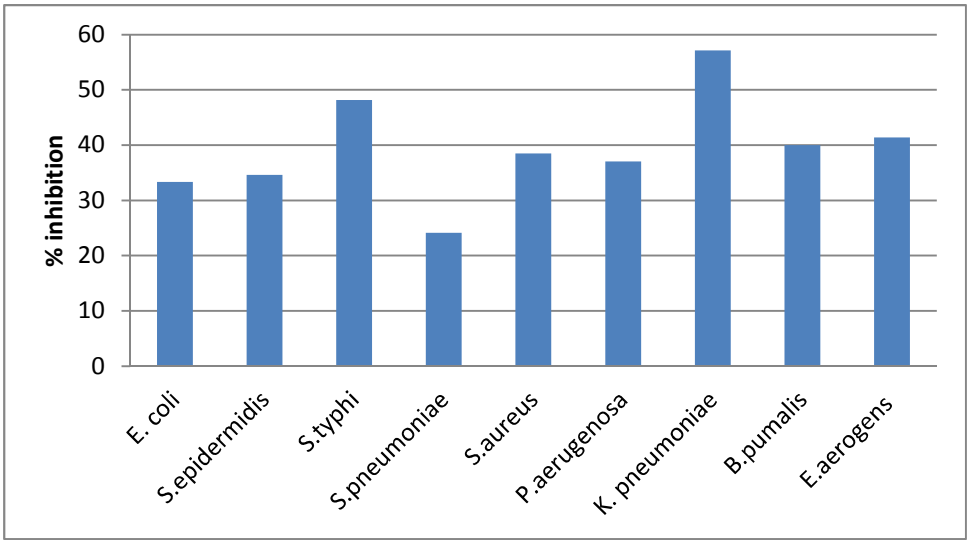


Fig 3.3 Antibacterial activity of $CHCl_3$ fraction of *Acacia modesta*

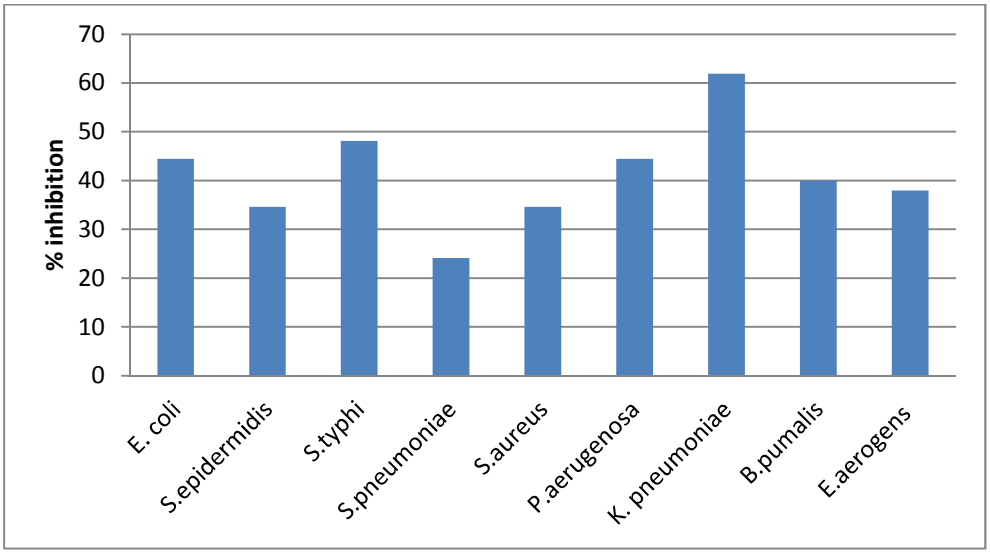


Fig 3.4 Antibacterial activity of EtOAc fraction of *Acacia modesta*

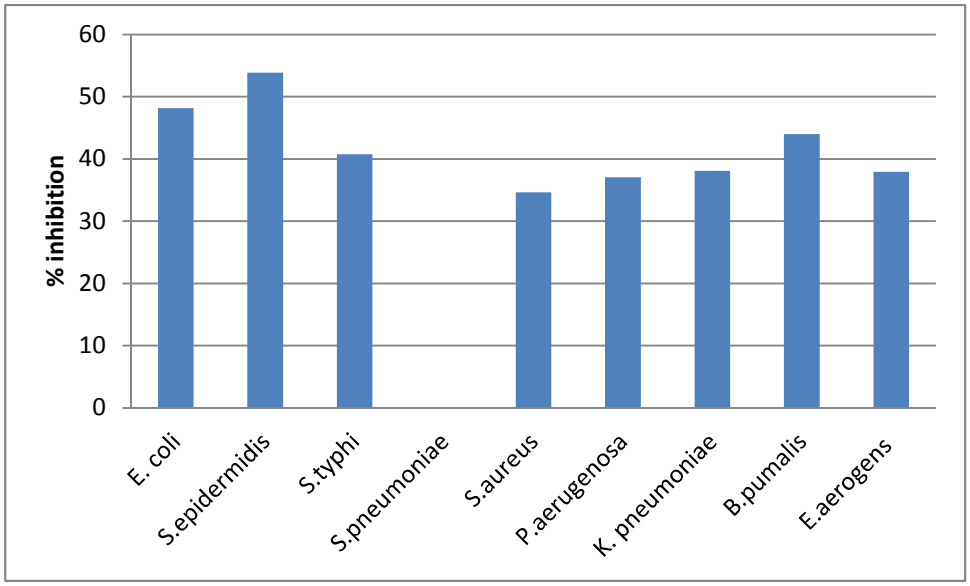


Fig 3.5 Antibacterial activity of aqueous fraction of *Acacia modesta*

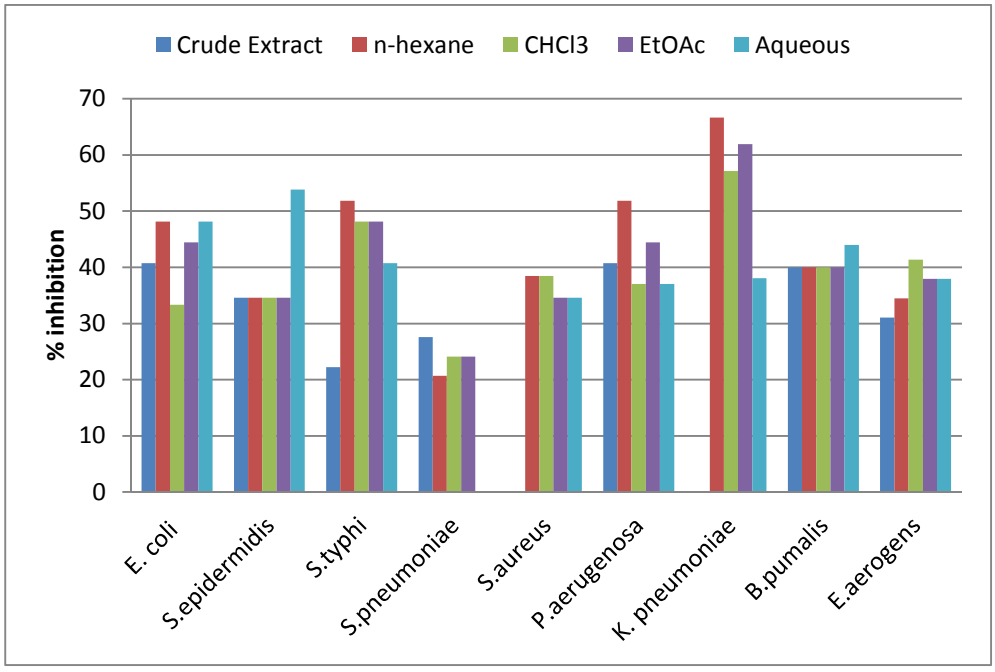


Fig 3.6 Antibacterial activity of crude methanolic extract and various fractions of *Acacia modesta*

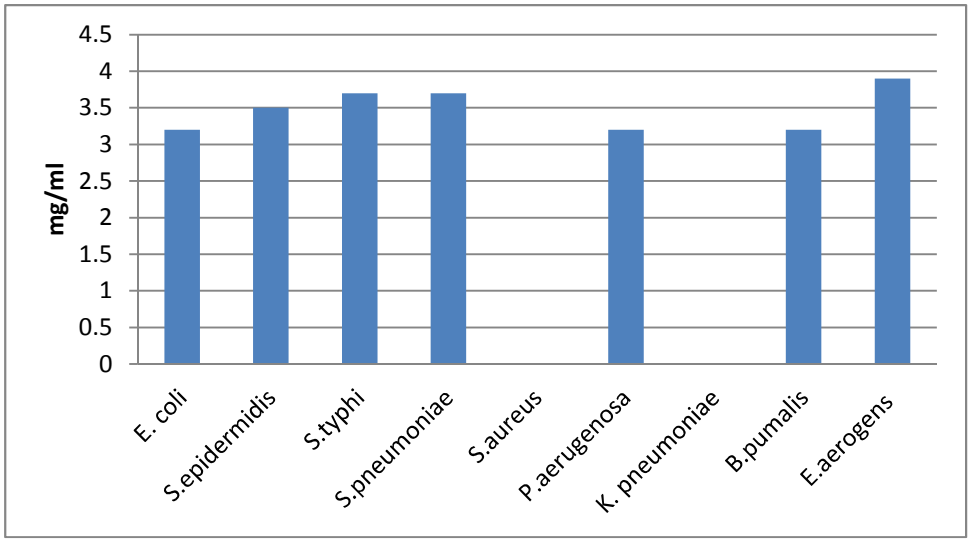


Fig 3.7 MIC_{50} values of the crude methanolic extract of *Acacia modesta*

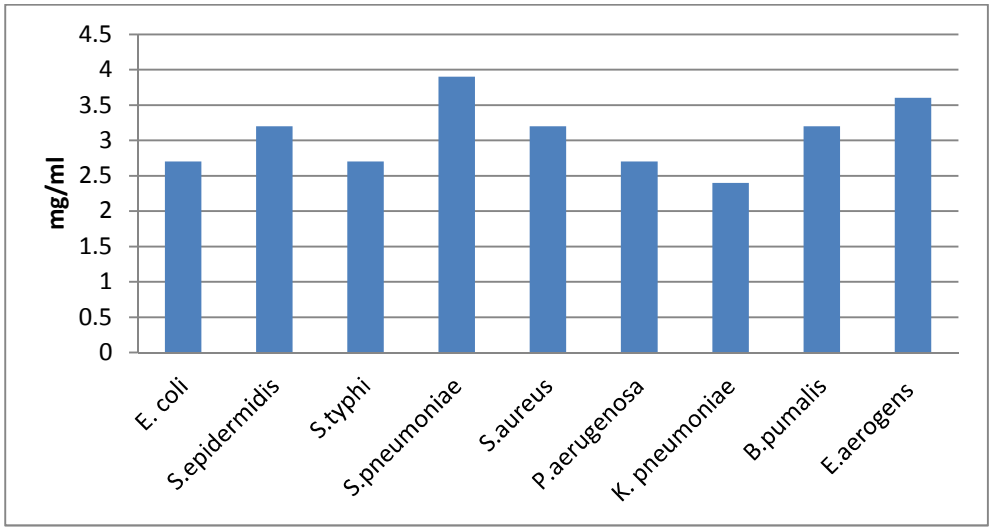


Fig 3.8 MIC_{50} values of *n*-hexane fraction of *Acacia modesta*

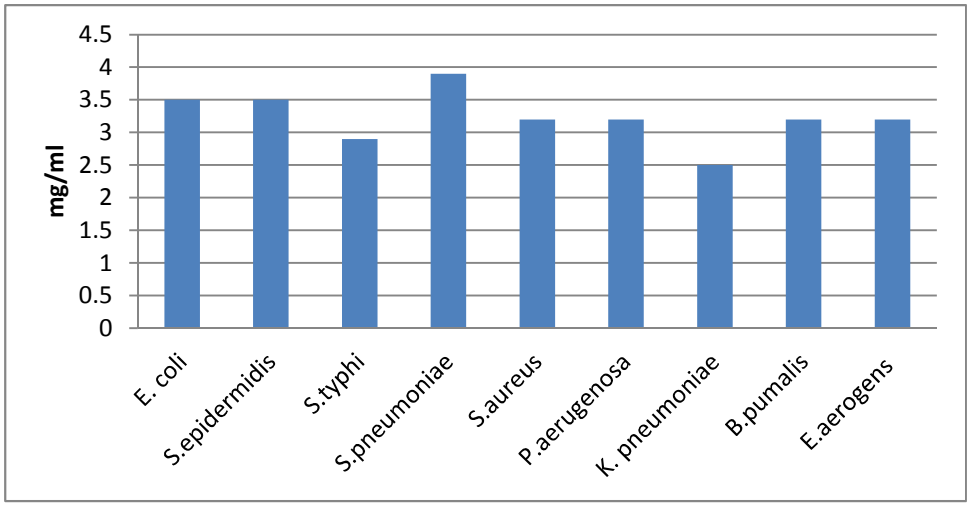


Fig 3.9 MIC_{50} values of $CHCl_3$ fraction of *Acacia modesta*

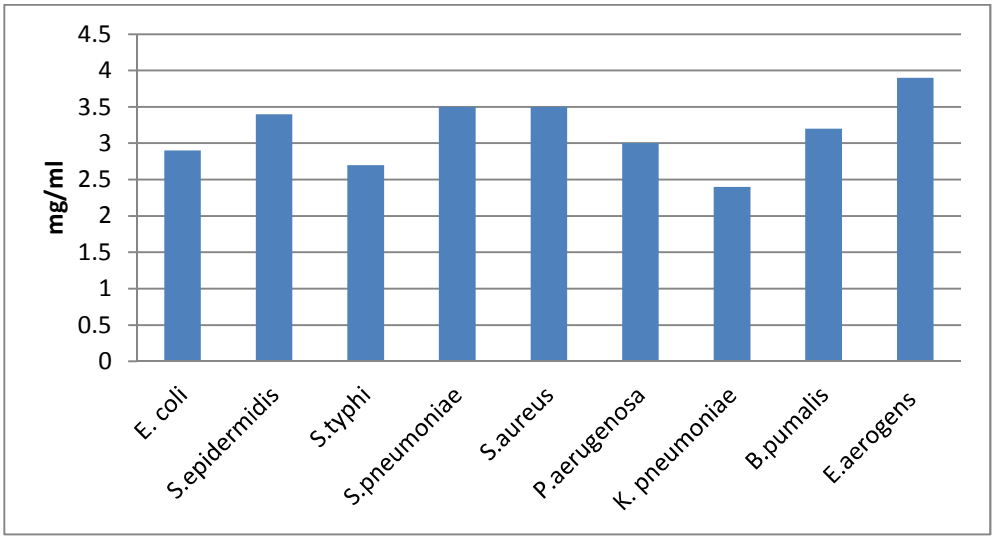


Fig 3.10 MIC_{50} values of EtOAc fraction of *Acacia modesta*

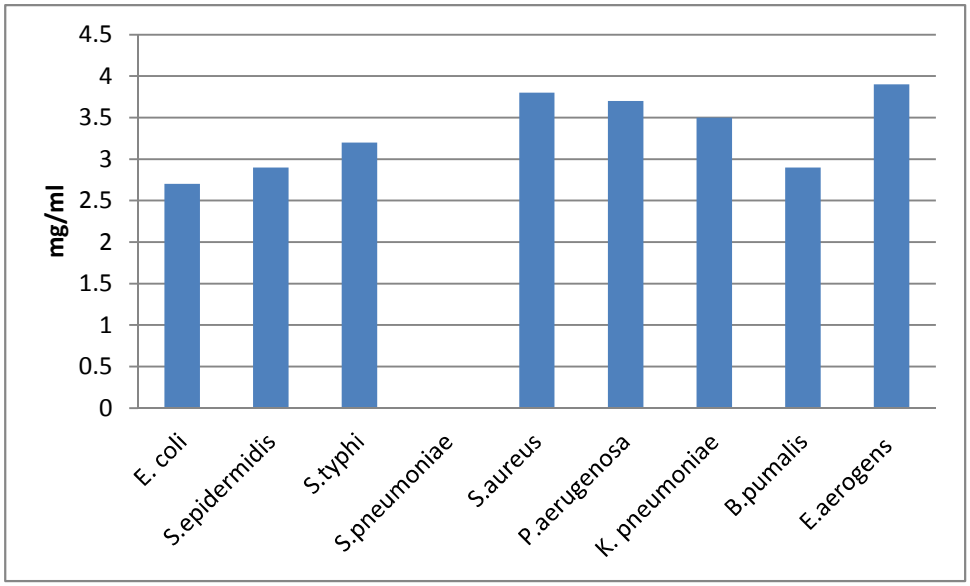


Fig 3.11 MIC_{50} values of aqueous fraction of *Acacia modesta*

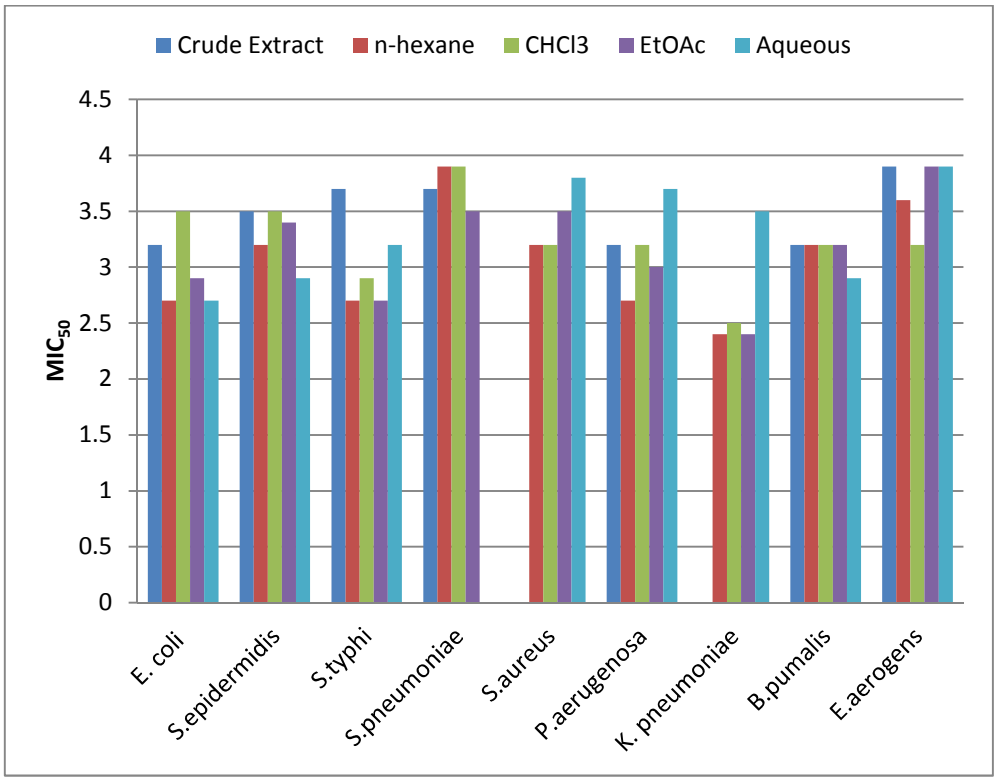


Fig 3.12 MIC_{50} values of crude methanolic extract and various fractions of *Acacia modesta*

Table 3.6 Antibacterial activity of the oils of *Acacia modesta*

Name of Bacteria	**Zone of inhibition of sample (mm)**	**Zone of inhibition of standard drug (mm)**
Escherichia coli	0	35
Bacillus subtilis	0	36
Shigella flexenari	0	35
Staphylococcus aureus	0	43
Pseudomonas aeruginosa	0	32
Salmonella typhi	0	40

3.2.2 ANTIFUNGAL ACTIVITY

Crude methanolic extract and fractions

The test samples were screened for their antifungal activity against *Aspergillus niger*, *Aspergillus flavus*, *Penicillium notatum*, *Fusarium oxysporum*, *Trichoderma harzianum* and *Rhizopus stolonifer*. The results are depicted in **Table 3.7 and Figures 3.13-3.17.** Amphotericin-B and Miconazole served as standard drugs.

All the test samples were inactive against *A. niger*, *P. notatum* and *R. stolonifer.* Against *A. flavus* the percent inhibition was: crude methanolic extract (5%), *n*-hexane (9%), Chloroform (10%) and EtOAc (3%). The aqueous fraction was inactive against *A. flavus*. The $CHCl_3$ and EtOAc fractions exhibited low activity of 15 and 5% respectively against *F. oxysporum* while rest of the fractions were inactive against it. All the fractions were inactive against *T. harzianum* except the crude methanolic extract which exhibited a low activity of 10%.

The above results indicate that *A. modesta* has no antifungal agents because neither the crude methanolic extract nor any of its fractions exhibited significant antifungal activity.

Essential oils

Many of the plant diseases like vascular wilt (*F. oxysporum),* fruit rot (*Fusarium solani*), rice sheath blight (*Rhizoctonia solani*) are caused by fungi [102-103]. Chemical fungicides are highly effective in controlling various diseases of fruits and vegetables. But they are not a suitable solution in long run because of the associated health and environmental hazards, tolerance development and residue persistence [104-105]. The essential oils of the plant contain different volatile compounds having fungicidal activities [106]. Wide acceptance and relative safety status of essential oils make them popular in consumers [107]. So one of the promising candidates as fungicide are essential oils of the plants.

The antifungal activity of the oils was determined against *A. flavus*, *C. albicans*, *C. glaberata, F. solani* and *M. canis*. **Table 3.8 and Fig 3.18** show the results. The sample showed moderate activity (40%) against *M. canis* and low activity against *F. solani* (25%) and *A. flavus* (5%). The sample was inactive against *C. albicans* and *C. glaberata*.

Table 3.7 Antifungal activity of the crude methanolic extract and various fractions of *Acacia modesta*

Name of Fungi	**Percent linear growth inhibition**						
	-ive control	**+ive control**	**Crude Met. extract**	***n*-hexane**	**$CHCl_3$**	**EtOAc**	**Aqueous**
A. niger	0	100	0	0	0	0	0
A. flavus	0	100	5	9	10	3	0
P. notatum	0	100	0	0	0	0	0
F. oxysporum	0	100	0	0	15	5	0
T. harzianum	0	100	10	0	0	0	0
R. stolonifer	0	100	0	0	0	0	0

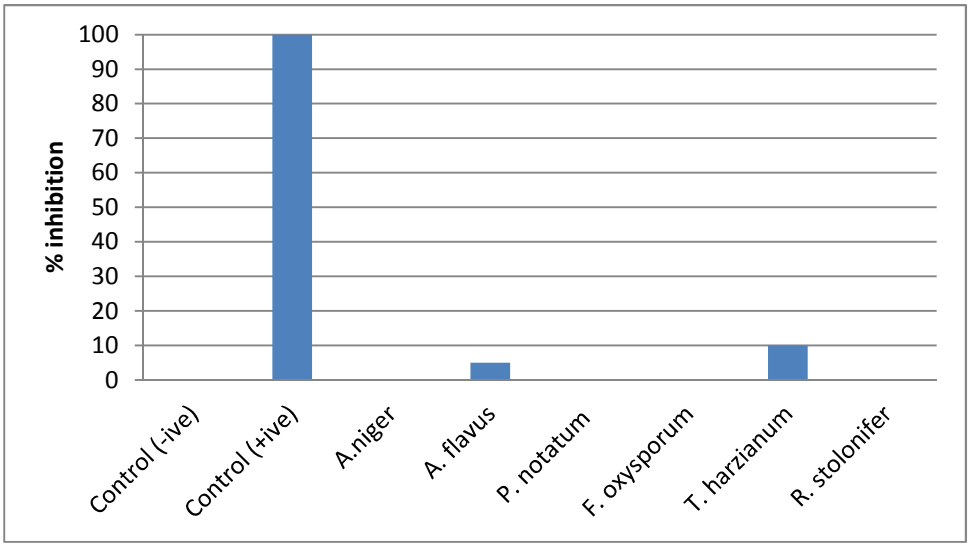


Fig 3.13 Antifungal activity of the crude methanolic extract of *Acacia modesta*

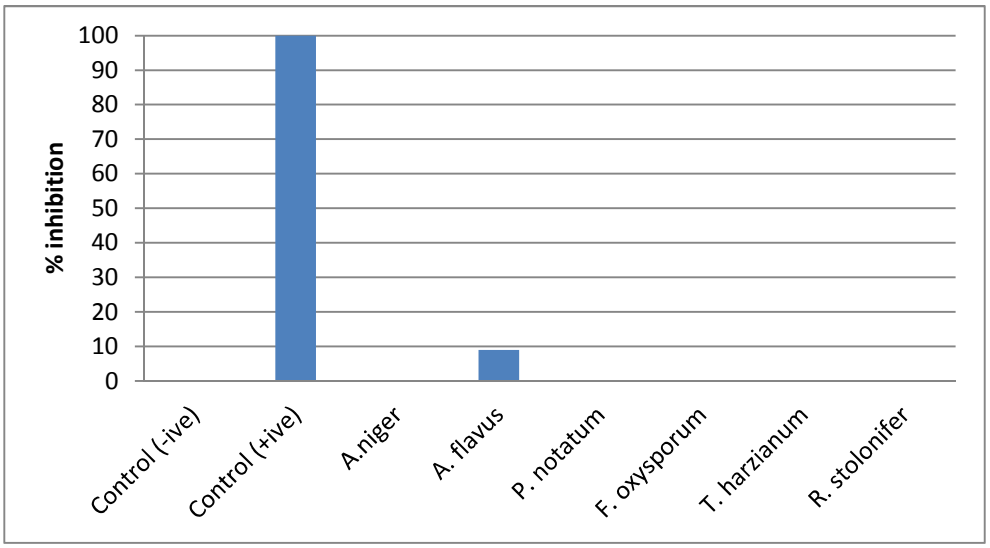


Fig 3.14 Antifungal activity of *n*-hexane fraction of *Acacia modesta*

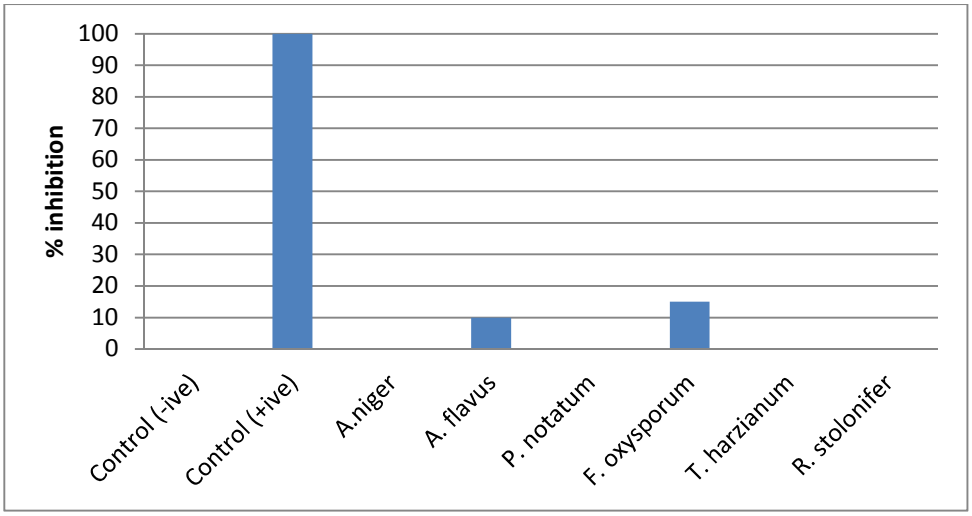


Fig 3.15 Antifungal activity of $CHCl_3$fraction of *Acacia modesta*

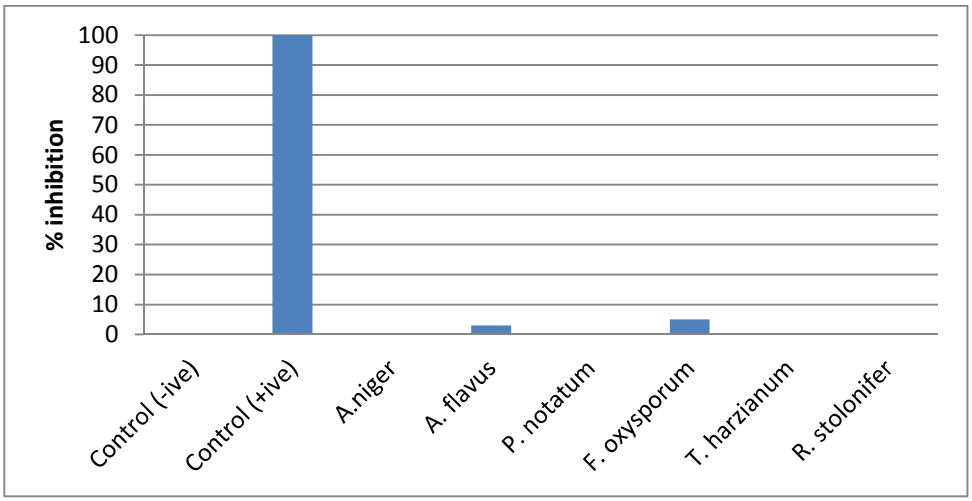


Fig 3.16 Antifungal activity of EtOAc fraction of *Acacia modesta*

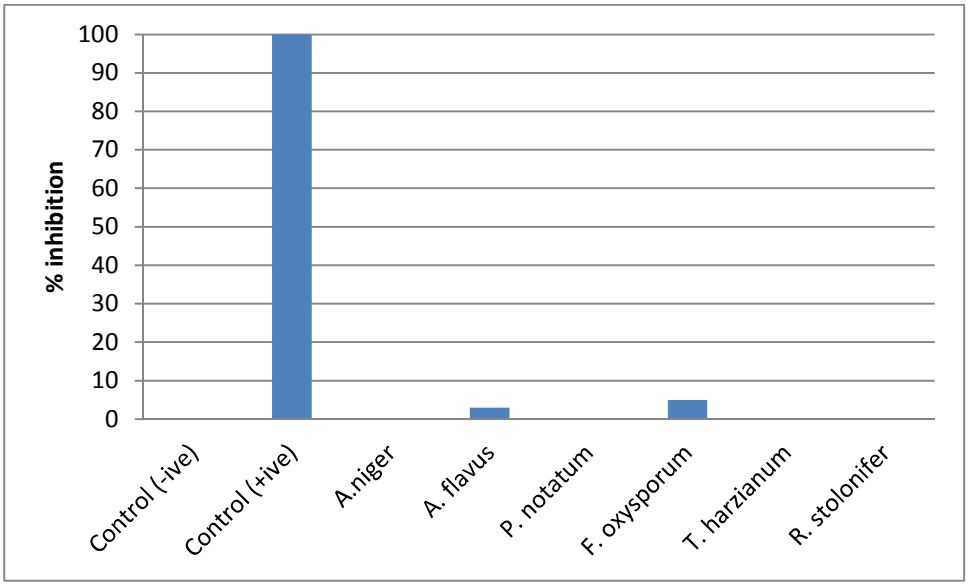


Fig 3.17 Antifungal activity of aqueous fraction of *Acacia modesta*

Table 3.8 Antifungal activity of the oils of *Acacia modesta*

Name of fungus	**Linear Growth (mm)**		**% inhibition**	**MIC of Standard drug (µg/ml)**
	Sample	**Control**		
C. albicans	100	100	0	Miconazole (110.8)
A. flavus	95	100	5	Amphotericin B (20.20)
M. canis	60	100	40	Miconazole (98.4)
F.solani	75	100	25	Miconazole (73.25)
C. glaberata	100	100	0	Miconazole (110.8)

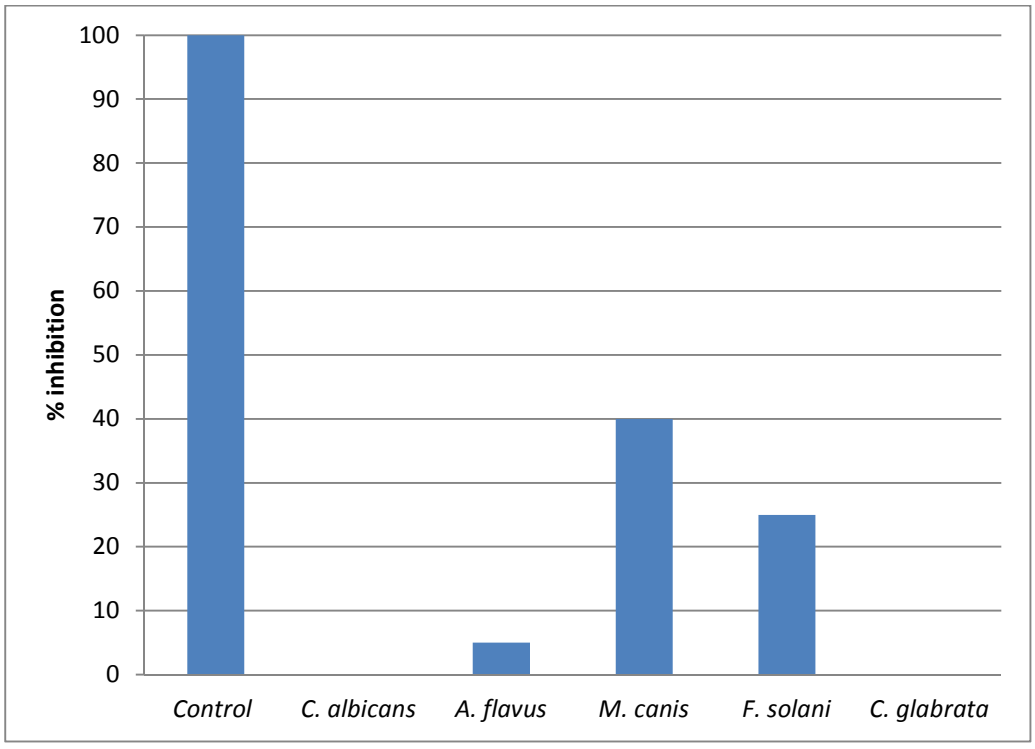


Fig 3.18 Antifungal activity of the oils of *Acacia modesta*

3.2.3 PHYTOTOXIC ACTIVITY

Crude methanolic extract and fractions

Herbicides, originating from plant's origin are often environment friendly. Therefore search for plant's origin herbicides, is sensible. *Lemna minor* L is small aquatic monocot, sensitive to bioactive compounds. Hence the plant is used as a model system to detect phytotoxic compounds, natural anti-tumor and detection of new plant growth stimulant in *Lemna* assay [106]. The results of the phytotoxic activity of the test samples using *Lemna* assay are given in **Table 3.9, 3.10 and Fig 3.19**. The crude methanolic extract, *n*-hexane, $CHCl_3$, EtOAc and aqueous fractions showed low activities of (25% and 6.25%), (31.25% and 25%), (37.5% and 18.75%), (31.25% and 18.75%) and (31.25% and 18.75%) at concentrations 1000 and 100 µg / ml, respectively. At concentration of 10 µg / ml no phytotoxic activity was observed.

The results indicated that *A. modesta* lacks phytotoxic agents.

Essential oils

The phytotoxic activity of the essential oils was determined using *Lemna* assay. The results are given in **Table 3.11 and Fig 3.20**.

At the concentration of 1000 and 100 µg / ml, the oils showed moderate activity of 50 and 40% respectively. At the concentration of 10 µg / ml, the sample showed low activity of 25%.

Table 3.9 Phytotoxic activity of crude methanolic extract and various fractions of *Acacia modesta*

Name of plant	**Concentration of sample (µgm/ml)**	**No. of fronds survived**						**Concentration of standard drug * (µg/ml)**
		Crude methanolic extract	***n*-hexane**	**$CHCl_3$**	**EtOAc**	**Aqueous**	**Control**	
Lemna minor	1000	12	11	10	11	11	16	0.015
	100	15	12	13	13	13	16	
	10	16	16	16	16	16	16	

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

Table 3.10 Percent reduction in growth regulation of the *Lemna minor*

Concentration of sample (µgm/ml)	**Percent growth regulation**				
	Crude methanolic extract	***n*-hexane**	**$CHCl_3$**	**EtOAc**	**Aqueous**
1000	25	31.25	37.5	31.25	31.25
100	6.25	25	18.75	18.75	18.75
10	0	0	0	0	0

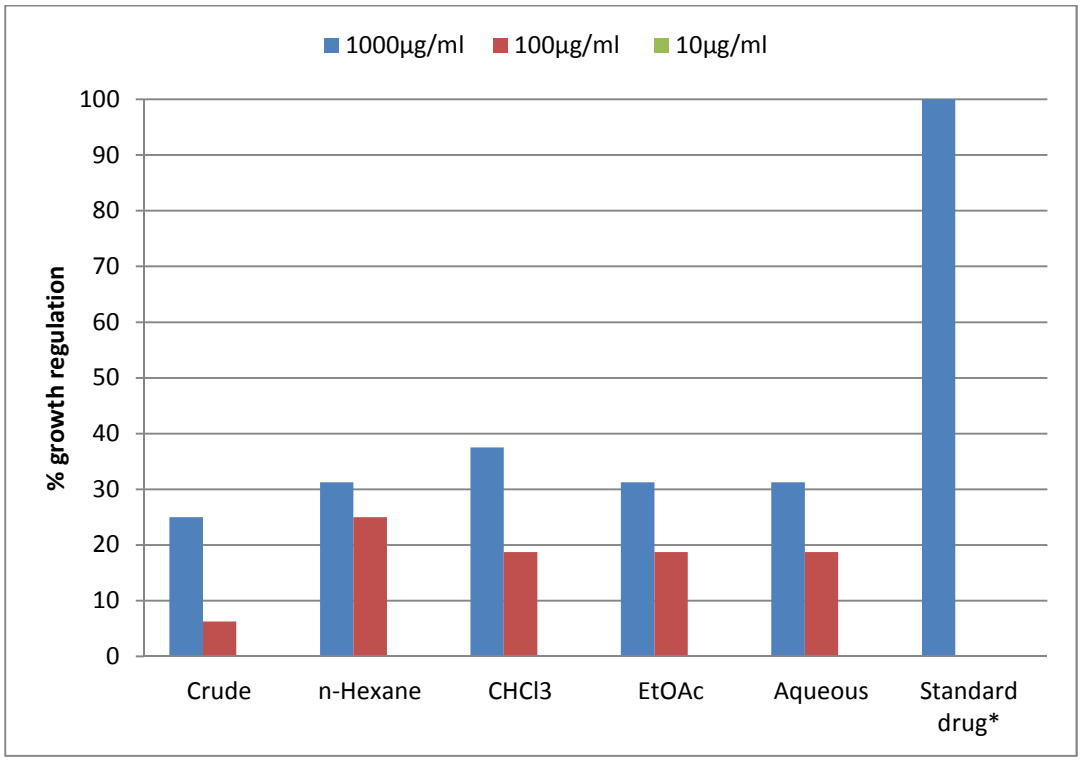


Fig 3.19 Phytotoxic activity of the crude methanolic extract and various fractions of *Acacia modesta*

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

Table 3.11 Phytotoxic activity of the oils of *Acacia modesta*

Name of plant	**Conc. of compound (µg/ml)**	**No. of Fronds survived**		**% Growth regulation**	**Conc. of Std. Drug* (µg/ml)**
		Sample	**Control**		
Lemna minor	1000	10	20	50	0.015
	100	12		40	
	10	15		25	

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

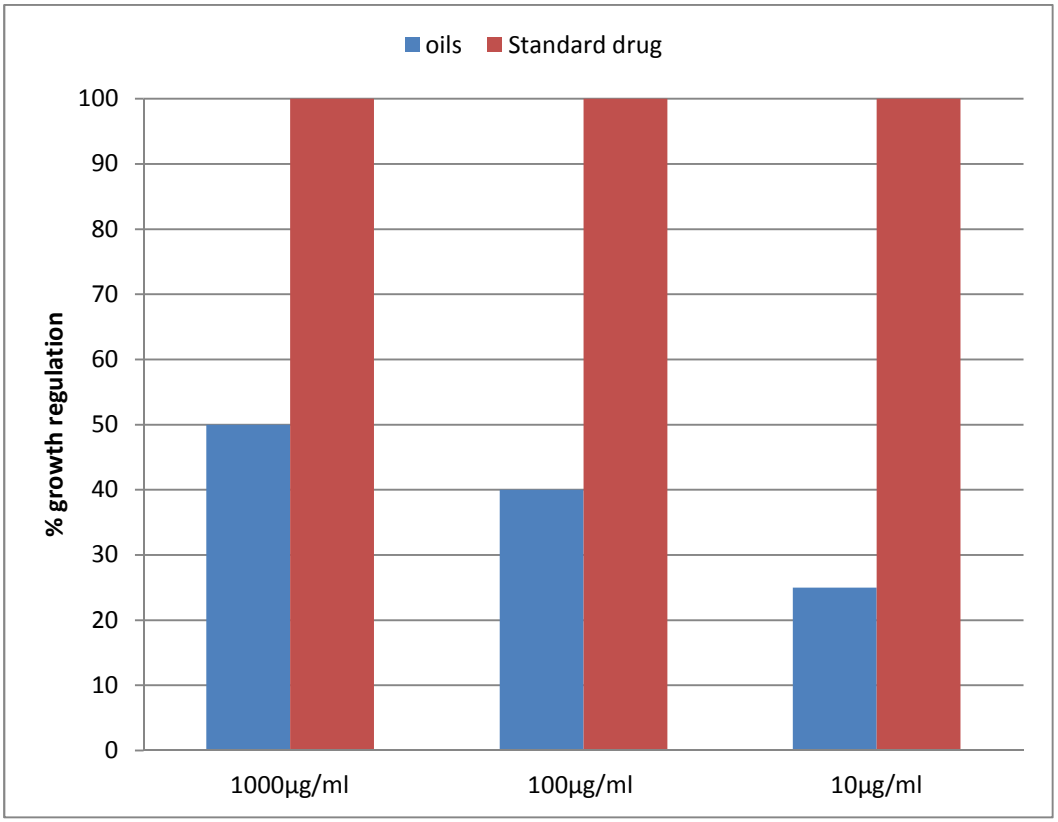


Fig 3.20 Phytotoxic activity of the oils of *Acacia modesta* against *Lemna minor*

3.2.4 INSECTICIDAL ASSAY

Crude methanolic extract and fractions

The synthetic insecticides are a great threat to the environment due to their toxic effect. Therefore environment friendly insecticides from the natural resources should be searched for to replace the harmful insecticides [109]. *Tribolium castaneum, Rhizopertha dominica* and *Callosbruchus analis* were used as model system to know the insecticidal activity of test samples. The results are mentioned in **table 3.12 and Fig 3.21**. The test samples were inactive against *T. castaneum.* Low activity of 20% was shown by the *n*-hexane and $CHCl_3$ fractions against *R. dominica*, while rest of the test samples were inactive against it. The *n*-hexane fraction showed good activity (60%) and EtOAc and aqueous fractions showed low activity (20%). The crude methanolic extract and $CHCl_3$ fractions were inactive against *C. analis*.

Essential oils

One of the approaches to treat human diseases is the use of essential oils. A huge amount of literature is available on the acute toxic effect of essential oils against insects [110]. Against tobacco cutworm (*Spodoptera litura*) the oils of *Thymus serpyllum*, *Satureia hortensis* and *Origanum* showed more than 90% larvicidal activity in 24 hrs at a dose of 100 µg per larva [111]. The oils of *A. modesta* were screened for insecticidal activity against *T. castaneum*, *R. dominica* and *C. analis*. The results are mentioned in **Table 3.13.** The oils obtained from *A. modesta* showed no activity against any of the test insects indicating that these oils lack insecticidal potential.

Table 3.12 Insecticidal activity of crude extract and fractions of *Acacia modesta* by contact toxicity method

Name of Insect	**% Mortality of test insects**						
	Control (Positive) *	**Control (Negative)**	**Crude Methanolic Extract**	***n*-hexane**	**$CHCl_3$**	**EtOAc**	**Aqueous**
Tribolium castaneum	100	0	0	0	0	0	0
Rhizopertha dominica	100	0	0	20	20	0	0
Callosbruchus analis	100	0	0	60	0	20	20

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

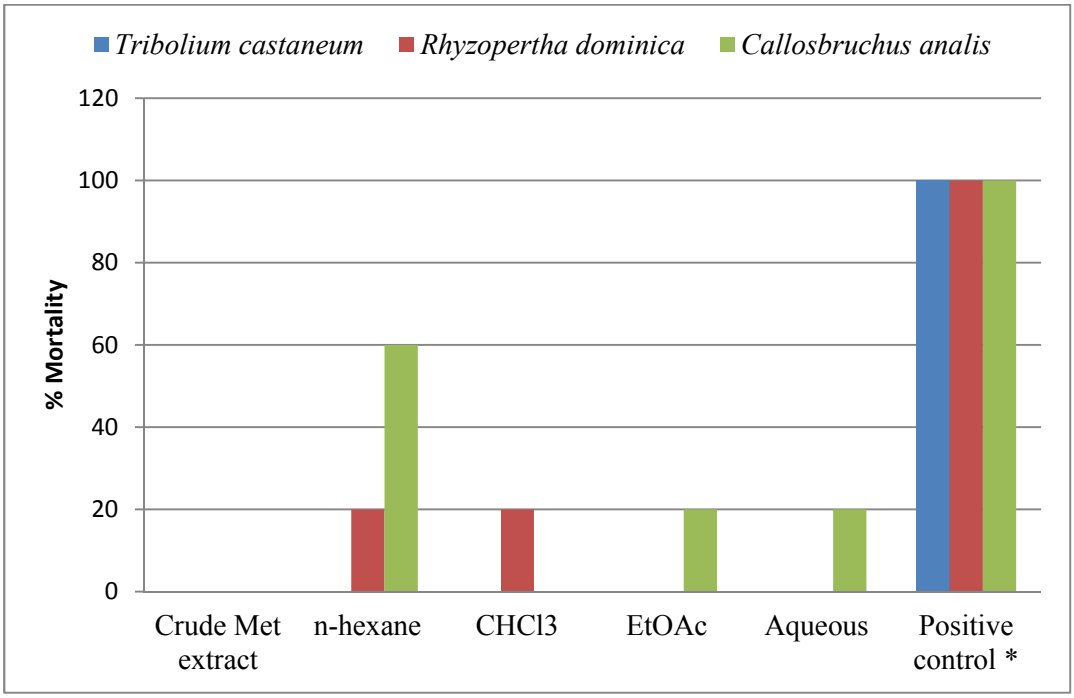


Fig 3.21 Insecticidal activity of crude methanolic extract and fractions of *Acacia modesta*

Table 3.13 Insecticidal activity of the oils of *Acacia modesta*

Name of the insect	**% Mortality of test insects**		
	Control (+ ve) *	**Control (- ve)**	**Sample**
Tribolium castaneum	100	0	0
Rhyzopertha dominica	100	0	0
Callosbruchus analis	100	0	0

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

3.2.5 BRINE SHRIMP LETHALITY BIOASSAY

Crude methanolic extract and fractions

Brine shrimp (*Artemia salina*) lethality bioassay was used to check the cytotoxic effect of the test samples. The results of the assay are mentioned in **Table 3.14 and Fig 3.22.** The results indicated that the crude methanolic extract showed low cytotoxicity (16.66%) at 1000 µg / ml. At 100 and 10 µg / ml, cytotoxicity was 10% and 6.66% respectively. The LD_{50} value was 4251653.0. The upper and lower limits were 0.000 and 5791.19 respectively while the G value was 2.6687. The *n*-hexane fraction showed a toxicity of 20% at 1000 µg / ml and 13.33% and 6.66% at 100 and 10 µg / ml respectively. The LD_{50} value recorded was 377166.8. The values of the upper limit, lower limit and G were 377166.8, 3362.08 and 1.8072 respectively for this fraction. The $CHCl_3$ fraction showed lethality of 40% at 1000 µg / ml. It showed lethality of 16.66% and 6.66% at 100 and 10 µg / ml. Upper and lower limit values were 486675.7 and 675.38. The G value for the $CHCl_3$ fraction was 0.4205. The EtOAc fraction showed 26.66%, 133.33% and 6.66% mortality at 1000, 100 and 10 µg / ml respectively. The G value was 0.8962, while the upper and lower limits were 1.701412 and 1746.599 respectively. The results of the aqueous fraction showed 40% mortality at 1000 µg / ml, 20% at 100 µg / ml and 10% at 10 µg / ml of the test samples. The upper and lower limits recorded were 236150 and 636.32 respectively. The G value was 0.5449.

Essential oils

The scientists are focusing on the development of drugs from natural origin because plants are rich source of drugs and much novel therapeutics has been isolated from plants origin. Many of the plant derived agents have cytotoxic activities [112-113]. The essential oils of

Heracleum transcaucasicum have moderate (IC_{50} values; 0.362–0.594 mg / ml) cytotoxic activities against three human cancer cell lines (HeLa, LS180 and Raji) [114]. Therefore, the essential oils of *A. modesta* were screened for cytotoxic activity against Brine shrimp (*Artemia salina*).

The results of cytotoxic activity of *A. modesta* oils are depicted in **Table 3.15 and Fig 3.23.** The results indicate that *A. modesta* oils are highly toxic at 1000 µg / ml. Out of 30 shrimps, not a single shrimp survived. At 100 µg / ml, only 2 shrimps out of 30 survived. Out of 30, 8 shrimps survived at 10 µg / ml. The LD_{50} value was 0.1887 µg / ml. The upper and lower limit was 0.7390 and 0.0002 respectively while the G value was 0.4974.

These results indicate that the oils obtained from *A. modesta* are highly cytotoxic. These should not be utilized by the human beings and the animals. On the other side they can be used as a cytotoxic agent, when required.

Table 3.14 Brine shrimp cytotoxicity of crude methanolic extract and various fractions of *Acacia modesta*

Dose (µg/ml)	**Control (No. of shrimps)**	**Crude methanolic extract**		***n*-hexane**		**$CHCl_3$**		**EtOAc**		**Aqueous**		**Standard drug***	
		Survivors	% killed	Survivors	% killed	Survivors	% killed	Survivors	% killed	Survivors	% killed	Survivors	% killed
1000	30	25	16.66	24	20	18	40	22	26.66	18	40	0	100
100	30	27	10	26	13.33	25	16.66	26	13.33	24	20	0	100
10	30	28	6.66	28	6.66	28	6.66	28	6.66	27	10	0	100

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

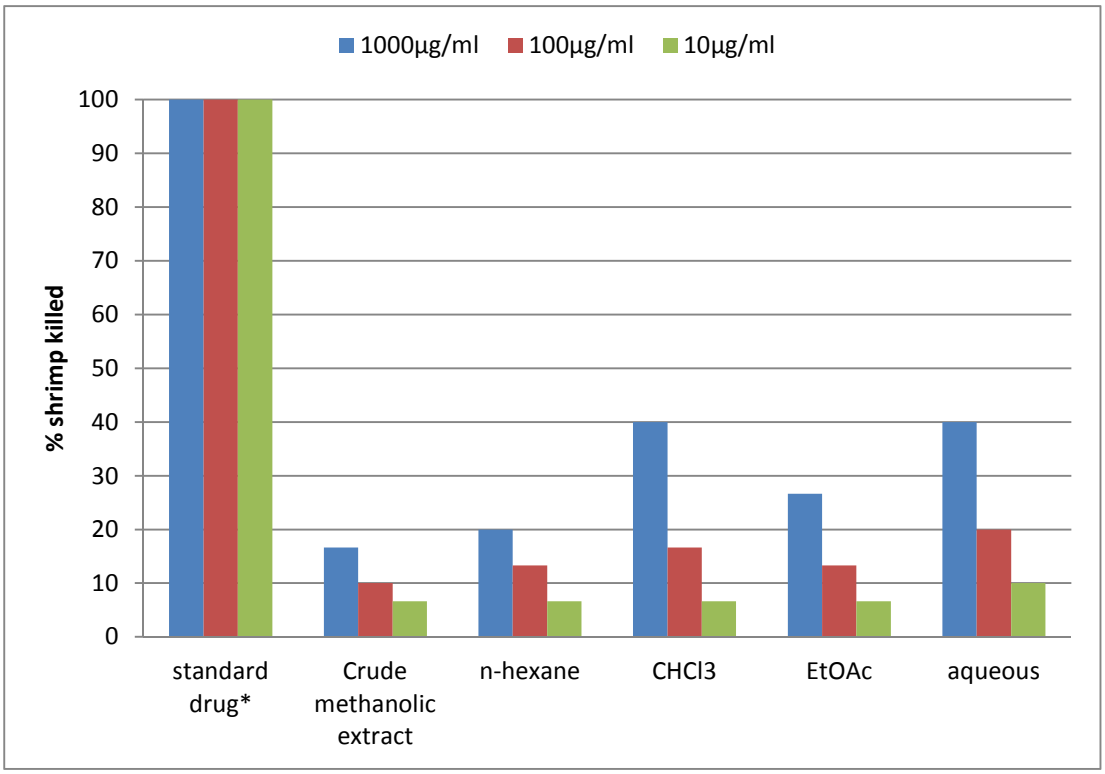


Fig 3.22 Brine shrimp cytotoxicity of crude methanolic extract and various fractions of *Acacia modesta*

* Standard drug: Etoposide at concentration of 7.4625 µg / ml.

Table 3.15 Brine shrimp (*Artemia salina*) cytotoxicity of the oils of *Acacia modesta*

Dose (µg/ml)	**No. of Shrimps**	**No. of Survivors**	**% killed**	**LD_{50} (µg/ml)**	**Standard Drug**	**LD_{50} (µg/ml)**
1000	30	00	100	0. 1887	Etoposide	7. 4625
100	30	02	93.33			
10	30	08	73.33			

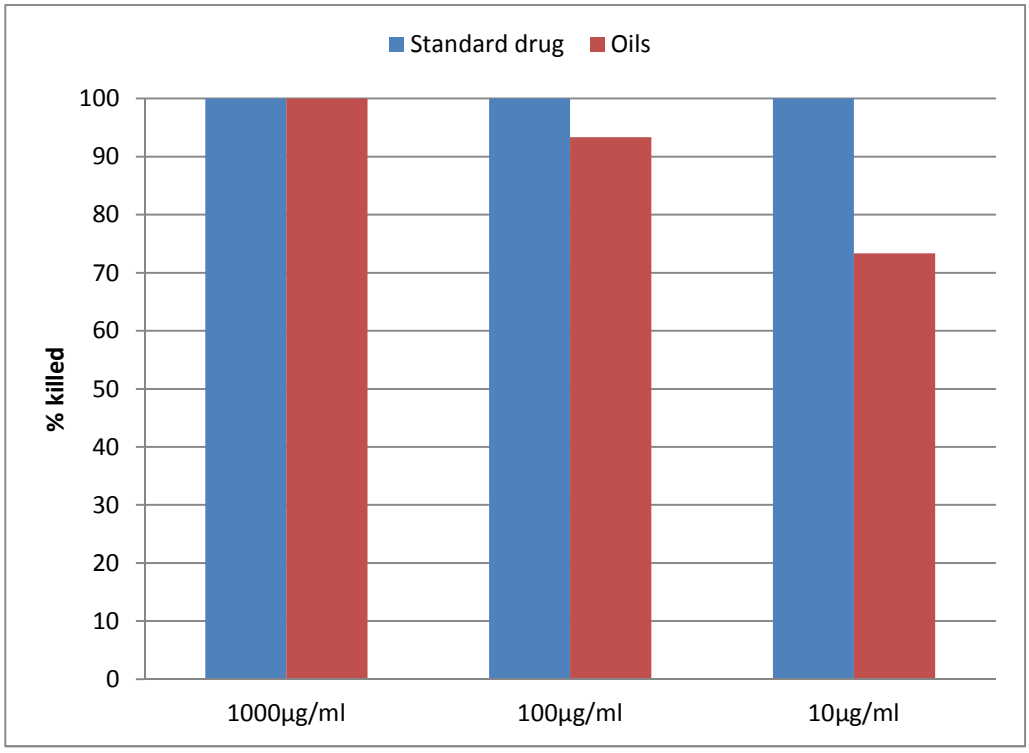


Fig 3.23 Brine shrimp (*Artemia salina*) cytotoxicity of the oils of *Acacia modesta*

3.2.6 HAEMAGGLUTINATION ACTIVITY

The structural and functional roles of cell surface carbohydrates have been demonstrated using lectin specificities [115]. The sugar components on cancerous and normal cell surfaces have also been studied using lectin specificities [116]. They have been used for the isolation and characterization of glycoconjugates [117] and to agglutinate erythrocytes [118]. Agglutinin from the plant sources are advantageous over animal sources because they are available in large quantities and are economical.

The results of the haemagglutination activity of the test samples were determined against human RBC's of all blood groups. The results are shown in **Table 3.16.** Low haemagglutination activity was shown by crude methanolic extract against blood groups A^+, B^- and O^+ at dilution of 1:2. The *n*-hexane fraction showed low activity against O^+ while aqueous fractions presented the same against A^+ at dilution of 1:2. EtOAc fraction showed moderate (++) and low (+) haemagglutination activity at dilution of 1:2 and 1:4 respectively against O^+. The results were negative at all concentration against AB^-, AB^+, O^- and B^+ blood groups.

Table 3.16 Haemagglutination activity of the crude methanolic extract and various fractions of *Acacia modesta*.

Blood group	Crude methanolic extract				*n*-hexane				$CHCl_3$				EtOAc				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
AB^-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B^-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
A^-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B^+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A^+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
AB^+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O^+	+	-	-	-	+	-	-	-	-	-	-	-	++ -	+	-			-	-	-
O^-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- = no agglutination, + = weak, ++ = moderate, +++ = strong

3.2.7 ANTI-TERMITE ACTIVITY

Against the *Heterotermes indicola*, the anti-termite activity of crude methanolic extract, $CHCl_3$ and aqueous fractions of *A. modesta* were carried out at "Termites lab at Nuclear institute of Food and Agriculture (NIFA), Peshawar, Khyber PakhtunKhwa, Pakistan. All the experiments were performed in triplicates. **Table 3.17** represents the result.

For the recycling of woody and other plant materials termites play an important role. The tunneling efforts of termites help in soil aeration. They are mainly found in tropical and subtropical regions. Apart from being beneficial they cause great economic loss by destroying wood and wooden products [109].

The crude methanolic extract showed significant activity against *H. indicola*. The experiment extended only for two days. On day 1, 22 termites were dead on an average and on the second day, no termite survived.

The experiment for the aqueous fraction lasted for three days. On average, 7 termites were killed on day 1. On the second day of the experiment, 22 termites were dead on an average and on the third day, no termite survived.

The experiment for the anti-termite activity of the $CHCl_3$fraction lasted for three days. On day 1 13 termite, on average, were killed. On the next days, 20 and on the last day of the experiment no termite was alive.

Table 3.17 Anti-termite activity of the crude methanolic extract, $CHCl_3$ and aqueous fraction of *Acacia modesta*

Sample	**No. of Termites used**	**Day**	**Average Termites killed**
Crude methanolic extract	25	1	22
		2	25
$CHCl_3$		1	13
		2	20
		3	25
Aqueous		1	7
		2	22
		3	25

3.2.8 NITRIC OXIDE (NO) FREE RADICAL SCAVENGING ASSAY

An important messenger molecule in many pathological and physiological processes within the mammalian body is Nitric oxide (NO). Nitric oxide has both beneficial and detrimental effect on the human health [119]. NO when produced in appropriate amount helps in the protection of organs for example liver from ischemic damage. A higher level of NO is toxic to tissue and contributes to many diseases like carcinomas, multiple sclerosis etc [120].

Keeping in view the importance of NO, the test samples were screened for NO free radical scavenging at different concentrations i.e. 0.3. 0.6, 0.9, 1.2 and 1.5 mg / ml. **Table 3.18 and Figures 3.24-3.28** represent the results. At 0.3 mg / ml, the $CHCl_3$ fraction exhibited moderate activity of 31.84%. The crude methanolic extract, *n*-hexane, EtOAc and aqueous fraction showed 29.62%, 20.54%, 14.04% and 6.16% activity at this concentration **(Fig 3.24)**. Results of the assay at 0.6 mg / ml **(Fig 3.25)** showed that the crude methanolic extract, *n*- hexane and $CHCl_3$ fractions have 33.56%, 30.13% and 40.58% NO free radical scavenging activity respectively. The EtOAc and aqueous fractions showed low activity (16.09% and 11.47%) at this concentration. The crude methanolic extract, *n*- hexane and $CHCl_3$ fractions showed NO free radical scavenging activity of 43.83%, 38.69% and 41.95% respectively at 0.9 mg / ml while EtOAc and aqueous fractions exhibited low activity of 23.28% and 18.32% (**Fig 3.26**). At 1.2 mg / ml, the results recorded showed that the NO free radical scavenging activity of crude methanolic extract, *n*-hexane, $CHCl_3$ and EtOAc fractions were 51.19%, 41.78%, 43.49% and 32.70% respectively while aqueous fraction showed low activity of 20.03%. At a concentration of 1.5 mg / ml, NO free radical scavenging activity was; crude methanolic extract (56.50%), *n*-hexane (53.25%), $CHCl_3$ (52.22%), EtOAc (42.29%) and aqueous fraction (33.90%) respectively.

The above results indicate that the crude methanolic extract, *n*- hexane and EtOAc fractions of *A. modesta* have concentration dependent NO free radical scavenging activity. Therefore, this plant can be searched for free radical scavenging compounds.

Table 3.18 Percent Nitric oxide free radical scavenging assay of the crude methanolic extract and fractions of *Acacia modesta*

Concentration of sample (mg/ ml)	**Percent activity**				
	Crude Met. Ext	**n-hexane**	**$CHCl_3$**	**EtOAc**	**Aqueous**
0.3	29.62	20.54	31.84	14.04	6.16
0.6	33.56	30.13	40.58	16.09	11.47
0.9	43.83	38.69	41.95	23.28	18.23
1.2	51.19	41.78	43.49	32.70	20.03
1.5	56.50	53.25	52.22	42.29	33.90

Standard: Vitamin C was used as a standard at concentration of 47.87 µg / ml

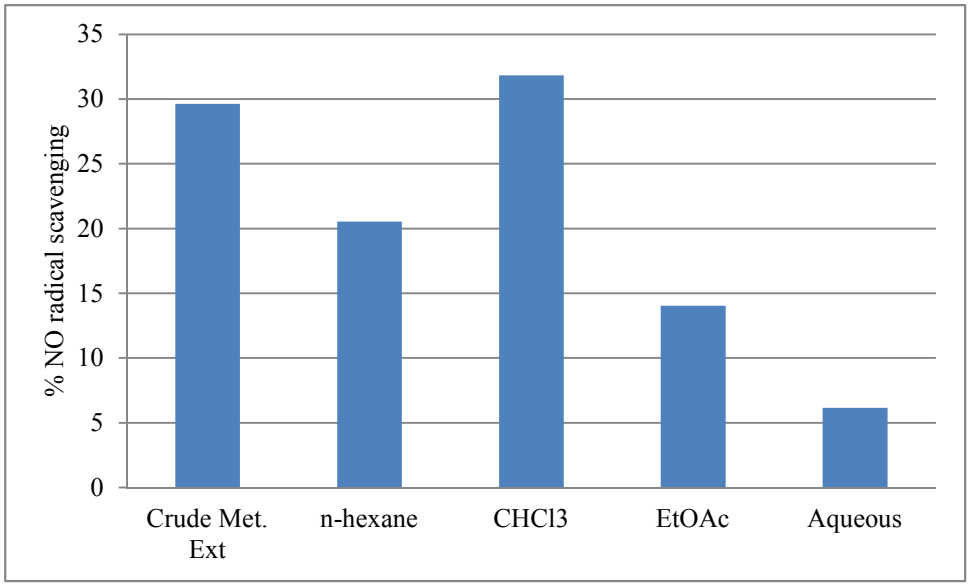


Fig 3.24 NO free radical scavenging assay of the crude methanolic extract and fractions of *Acacia modesta* at concentration of 0.3 mg / ml

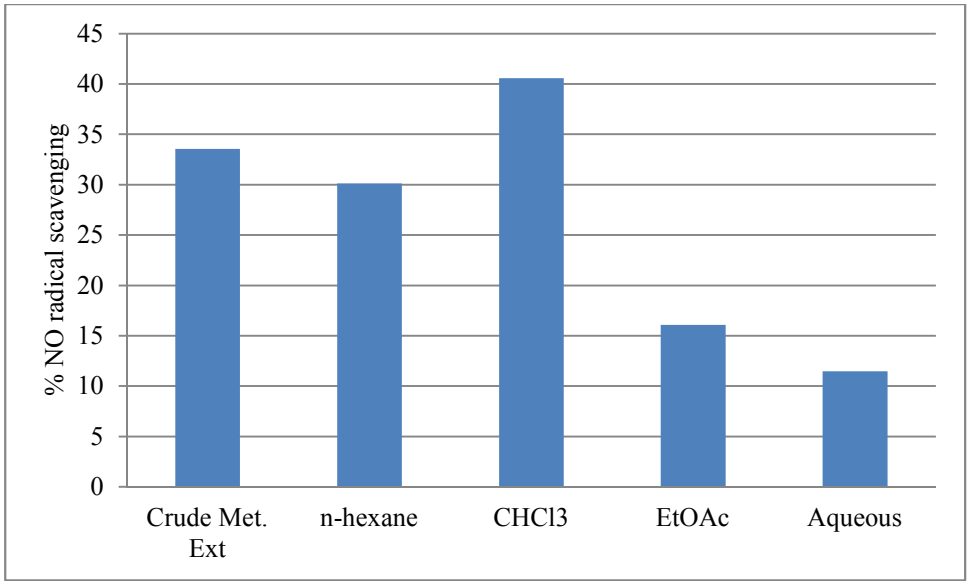


Fig 3.25 NO free radical scavenging assay of the crude methanolic extract and fractions of *Acacia modesta* at concentration of 0.6 mg / ml

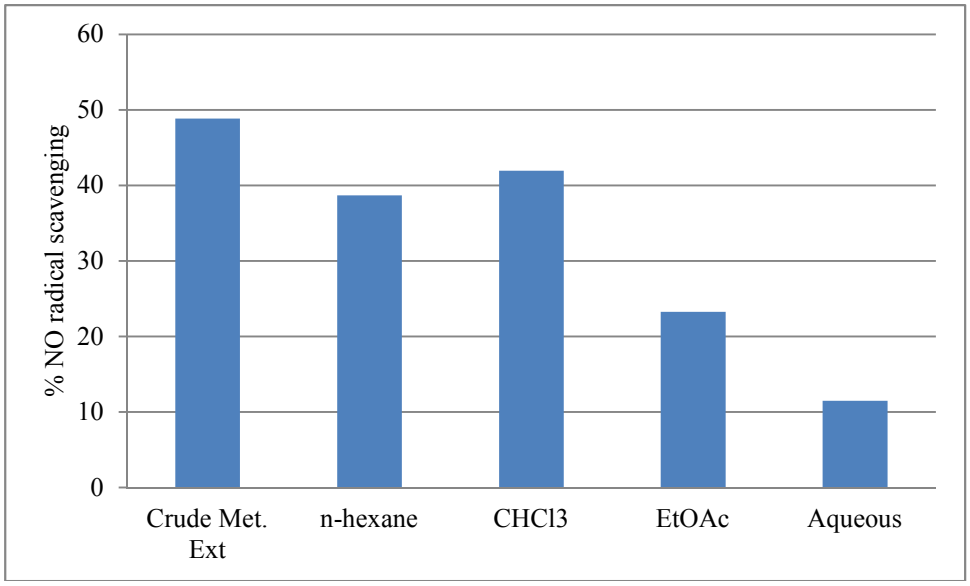


Fig 3.26 NO free radical scavenging assay of the crude methanolic extract and fractions of *Acacia modesta* at concentration of 0.9 mg / ml

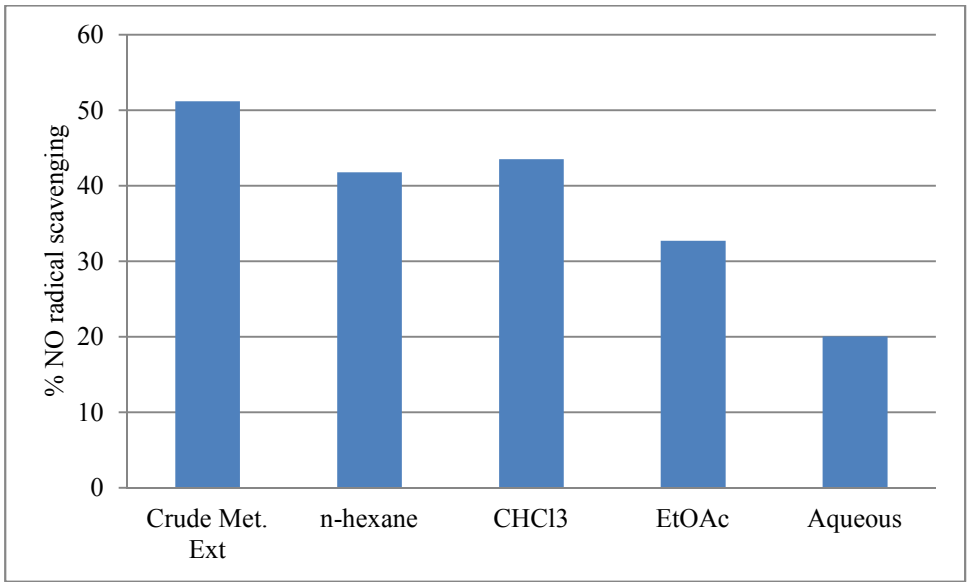


Fig 3.27 NO free radical scavenging assay of the crude methanolic extract and fractions of *Acacia modesta* at concentration of 1.2 mg / ml

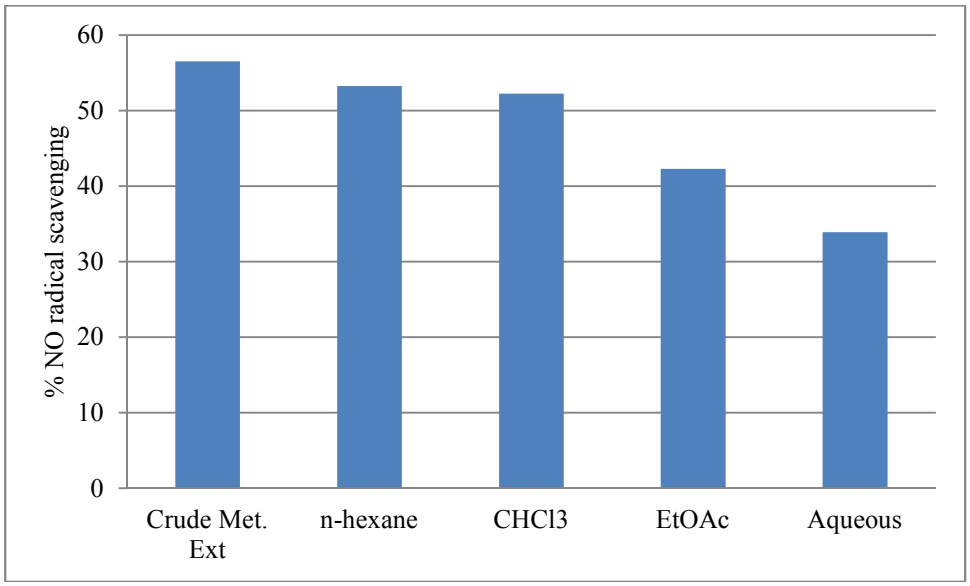


Fig 3.28 NO free radical scavenging assay of the crude methanolic extract and fractions of *Acacia modesta* at concentration of 1.5 mg / ml

3.2.9 EFFECTS ON RABBIT'S JEJUNUM PREPARATION

Spasmolytic activity

Effects of crude methanolic extract of *A. modesta* on rabbit's jejunum preparation are shown in **Figures 3.29-3.31**. There is dose dependent fall in spontaneous activity. Relaxing activity starts at dose of 0.3 mg / ml till 10 mg / ml with EC_{50} value of 5.9±1.15 (4.8-7.1).

At 10 mg / ml, it reduces the spontaneous activity up to 90% of the control maximum. Sustained contractions were produced by high concentration of KCl (80mM). The crude methanolic extract was tried in similar manners. Crude methanolic extract produced dose dependent relaxing effect from 0.1 mg / ml to 10 mg / ml with EC_{50} value of 6.8±0.53 (6.2-7.2). The relaxing effects on KCl induced contraction suggest the anti spasmodic activity of crude methanolic extract of *A. modesta* [121].

Table 3.19 Spasmolytic activity of crude methanolic extract of *Acacia modesta*

Concentration of Crude extract (mg/ ml)	Percent effect of control max. (Mean ± S.D, n= 3)
0.01	100
0.03	100
0.1	97.8
0.3	89.74
1	82.05
3	49
5	7.69
10	11

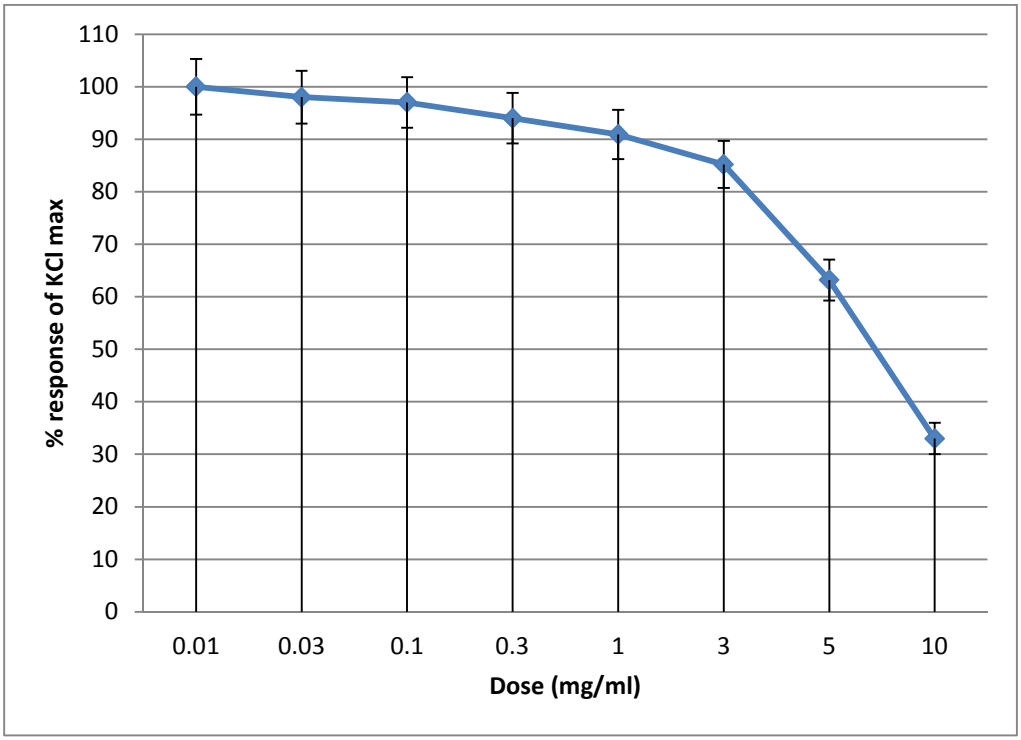


Fig 3.29 Spasmolytic activity of *Acacia modesta* on KCl (80 mM) induced rabbit's jejunum preparation

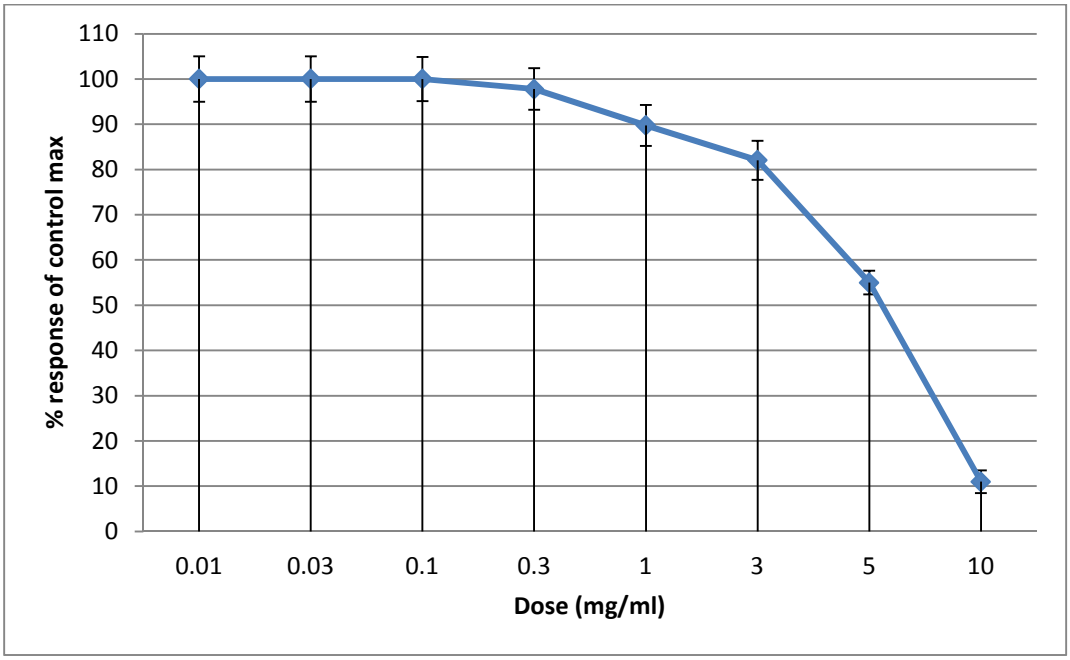


Fig 3.30 Spasmolytic activity of *Acacia modesta* on spontaneous rabbit's jejunum preparation

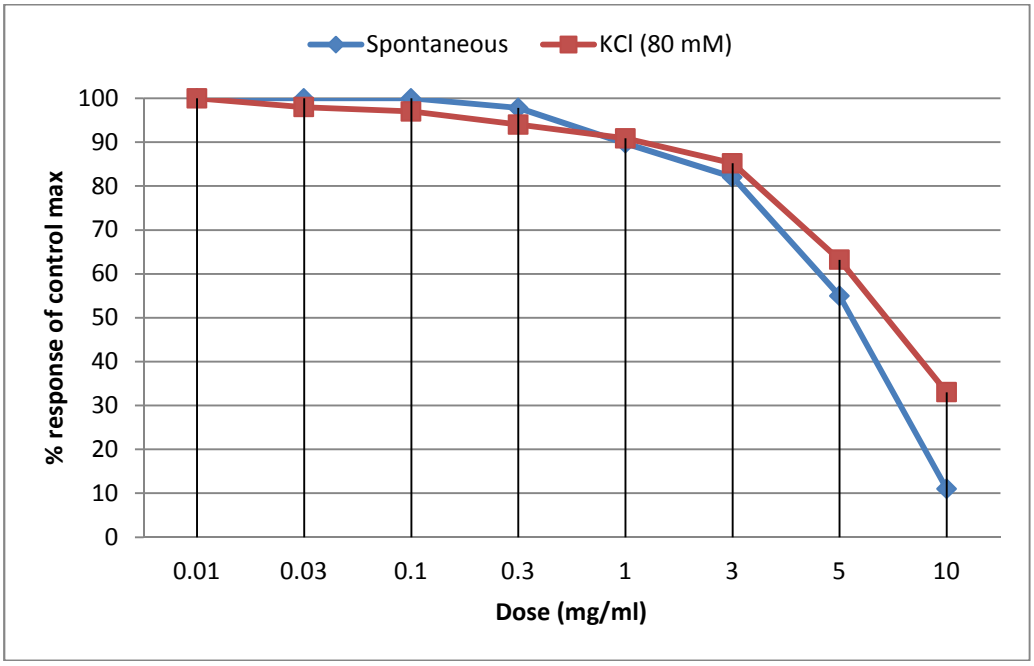


Fig 3.31 Spasmolytic activity of *Acacia modesta* on spontaneous and KCl induced rabbit's jejunum preparation

6.1 PHYTOCHEMICAL INVESTIGATIONS

6.1.1 Screening for Different Groups of Compounds

The medicinal values of a particular plant has a direct link with the presence of different types of groups of natural products, produced by that plant such as flavonoids, alkaloids, tannins and saponins. These natural products are used as medicines against various diseases, after isolation and formulation in various pharmaceutical dosage forms.

The plant *Zizyphus jujuba* was screened for presence of various groups of natural products. The results for the phytochemical screening are shown in **Table 6.1.** The precipitate formation, which settled down at the bottom, revealed that alkaloids are present in *Z. jujuba.* The development of pink color was observed in test for flavonoid, confirming its presence in *Z. jujuba*. Results for the presence of saponins were also positive by the froth formation of about 1.2 cm. The formation of precipitate and subsequent appearance of blue green color indicated presence of tannins in the plant.

Table 6.1 Phytochemical Screening for *Zizyphus jujuba*

S. No.	TEST	Results	Remarks	Page (Material and Methods)
1	Alkaloids	+	Positive	45
2	Flavonoids	++	Positive	45
3	Saponins	+	Positive	45
4	Tannins	++	Positive	46

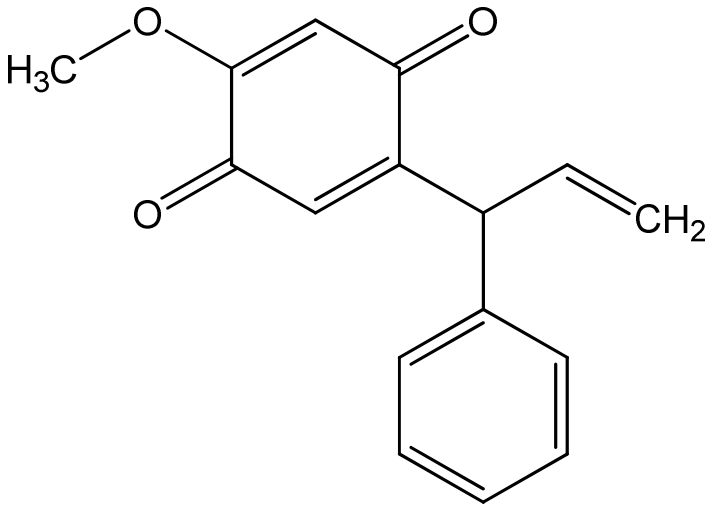
6.1.2 Structural elucidation of Compounds

6.1.2.1 Structural elucidation of Compound (5)

Compound **5** was isolated from the EtOAc fraction of *Z. jujuba* eluting with EtOAc / Pet ether (0.4: 9.6) as mobile phase on flash silica column. The presence of conjugated system in compound **5** was indicated by absorption in the UV spectrum (MeOH) values. Molecular ion peak at m/z 254.45 in EI mass spectrum was confirmed by ESI-MS. Through HREI-MS mass spectrum the molecular formula was established as $C_{16}H_{14}O_3$ corresponding to the molecular mass m/z 254.45.

The ^{1}H-NMR spectrums (C_3D_6O, 300 MH_Z) revealed the presence of methoxy group protons (3.82 ppm, S). Resonance at 4.89 (7.0, d) was assigned to methine proton H-1. Similarly resonances at 5.01 (10.5, Br d), 5.21 (15.5, Br d), 6.22 (7.0, m), 6.48 (2.0, d) and 5.99 (S) were assigned to olefinic protons H-3a", H-2b", H-2", H-2 and H-5 respectively. Resonances at 7.20-7.50 were assigned to aromatic protons.

The ^{13}C-NMR spectrum (C_3D_6O, 300 MH_Z) revealed the presence of 16 carbons, which comprised of one methyl, one methylene, nine methine and five quaternary carbons. The olefinic signals that appeared in the ^{13}C-NMR spectrum at 151.1 ppm, 132.2 ppm, 159.8 ppm, 108.5, 139.0 and 117.9 ppm were characteristics chemical shifts of C-1, C-2, C-4, C-5, C-2" and C 3"respectively. Resonance at 56.70 ppm was assigned to methoxy carbon. Signal at 48.2 ppm was assigned to C-1". Similarly signals of double integration at 129.3 and 129.4 were assigned to aromatic carbons (C-2', C-6') and (C-3', C-5') respectively. Resonances at 141.1 and 127.7 were assigned to C-1' and C-6'. The compound was identified as 4-methoxydalbergione by comparative study with reported data [186].



4-methoxydalbergione (5)

6.1.2.2 Structural elucidation of Compound (6)

Compound **6** was isolated from the EtOAc fraction of *Z. jujuba* eluting with EtOAc / Pet ether (3.5: 6.5) as mobile phase on flash silica column. The mass spectrum of compound **6** showed a weak molecular ion peak at *m/z* 456 that appeared at an exact mass at *m/z* 456.360 on high resolution mass measurement corresponding to molecular formula $C_{30}H_{48}O_3$. The other major fragments in the spectrum appeared at *m/z* 438 [M^+ -H_2O], 411 [M^+ -COOH], 248, 228, 207, 203 and 189.

The IR spectrum of the compound showed sharp absorption band at 1705 cm^{-1} (carbonyl region) indicating a carbonyl group in the molecule. Another strong absorption at 1615 cm^{-1} appeared due to olefinic functions in the molecule.

The broad absorptions in the region between 3400-2600 cm^{-1} were indicative of the carboxylic acid group. End absorption at λ_{max} 200 nm in UV spectrum indicated the absence of conjugated unsaturation in the molecule.

Two doublets integrating for one proton each resonated at δ 4.95 and δ 4.78 ($J_{2.0}$ Hz) in the olefinic region of the ^{1}H NMR spectrum of the compound. Both the doublets were correlated to the methylene at δ 109.7 in HMQC spectrum and were assigned to the terminal olefinic methylene in the structure and were fully in agreement with the olefinic absorption observed at 1615 cm^{-1} in the IR spectrum of the compound.

Six sharp singlets, of three proton integration each exhibited at δ1.80, 1.23, 1.08, 1.07, 1.02 and 0.82 in the ^{1}H NMR were ascribed to the protons of six tertiary methyl groups i.e. H-30, H-23, H-27, H-25, H-26 and H-24 respectively.

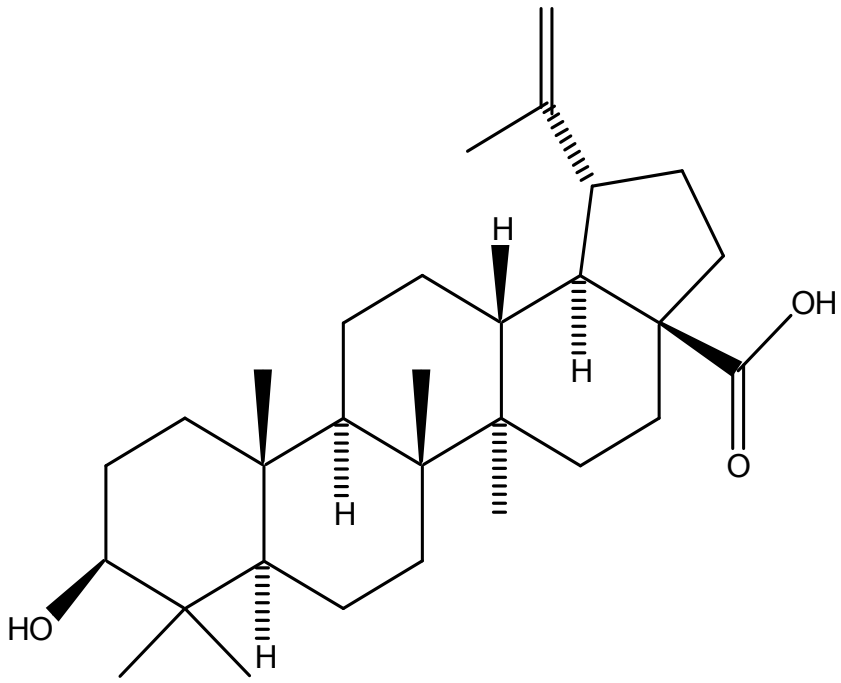
A one proton multiplet at δ 3.55 corresponding to the carbon appeared at δ78.8 showed the presence of hydroxyl group at C-3 in the structure. The appearance of a quaternary carbon signal

at a downfield value of δ180.3 in ^{13}C-NMR spectrum of the compound further confirmed the presence of carboxylic group in the molecule and was supported by the presence of the fragment at *m/z* 114 in the mass spectrum of the compound.

With the help of the spectral data discussed above and comparative literature report [187], the compound was identified as 3*β*-hydroxylup-20(29)-en-28-oic acid i.e. betulinic acid. It has anti-malarial, anti-retroviral and anti-inflammatory properties. It has recently been discovered that it has the potential to be an anti-cancer agent, by inhibiting topoisomerase [188].

Table 6.2 ^{13}C NMR chemical shifts and Multiplicity of Compound **6**

C.NO	**Multiplicity (DEPT)**	**δ, ppm**	**C.NO**	**Multiplicity (DEPT)**	**δ, ppm**
1	CH_2	38.7	16	CH_2	32.1
2	CH_2	27.4	17	C	56.3
3	CH	78.8	18	CH	46.7
4	C	38.4	19	CH	49.3
5	CH	55.3	20	C	150.3
6	CH_2	18.3	21	CH_2	29.7
7	CH_2	34.2	22	CH_2	37.0
8	C	40.7	23	CH_3	27.9
9	CH	50.5	24	CH_3	15.3
10	C	37.2	25	CH_3	16.0
11	CH_2	20.8	26	CH_3	16.1
12	CH_2	25.5	27	CH_3	14.7
13	CH	38.4	28	C	180.3
14	C	42.4	29	CH_2	109.7
15	CH_2	30.5	30	CH_3	19.4



Betulinic acid (6)

6.1.2.3 Structure elucidation of Compound (7)

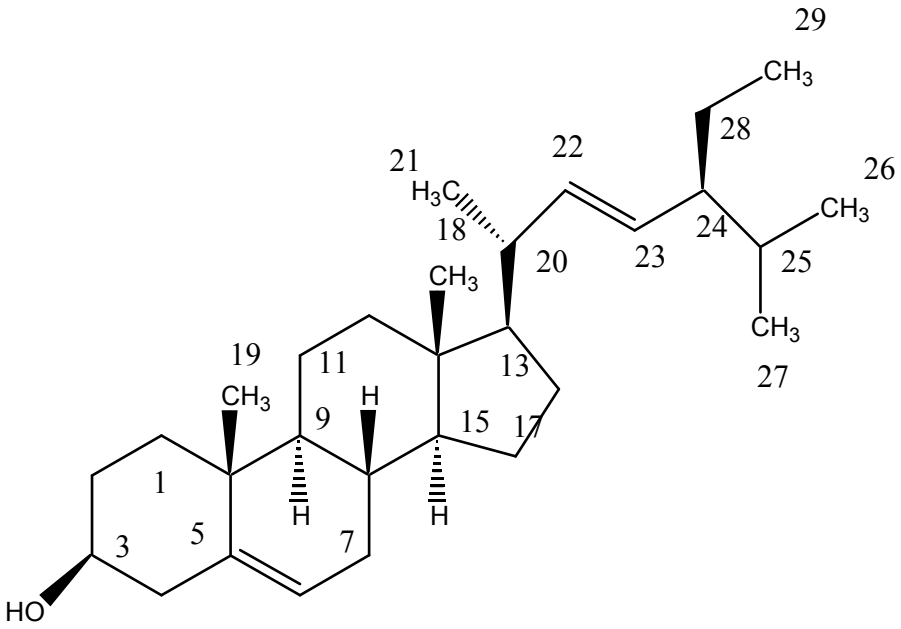
Compound **7** was isolated from the EtOAc fraction of *Z. jujuba* eluting with $CHCl_3$ / Pet ether (2.0: 8.0) as mobile phase on flash silica column. The molecular formula $C_{29}H_{48}O$ was established through HREIMS showing molecular ion peak $[M]^+$ at *m/z* 412.3920 (calcd. for $C_{29}H_{48}O$, 412.3926). The nature of oxygen in compound **7** was shown to be hydroxyl as indicated by IR spectrum (3432 cm^{-1}). The mass spectrum showed characteristics fragmentation pattern of Δ^5, Δ^{22} sterol [189].

The ^1H NMR spectrum of compound **7** completely corresponds to the data for stigmasterol [188]. It displayed signals for two tertiary methyl groups (δ 0.84, 0.65), two multiplet for carbonylic proton at δ 3.28.

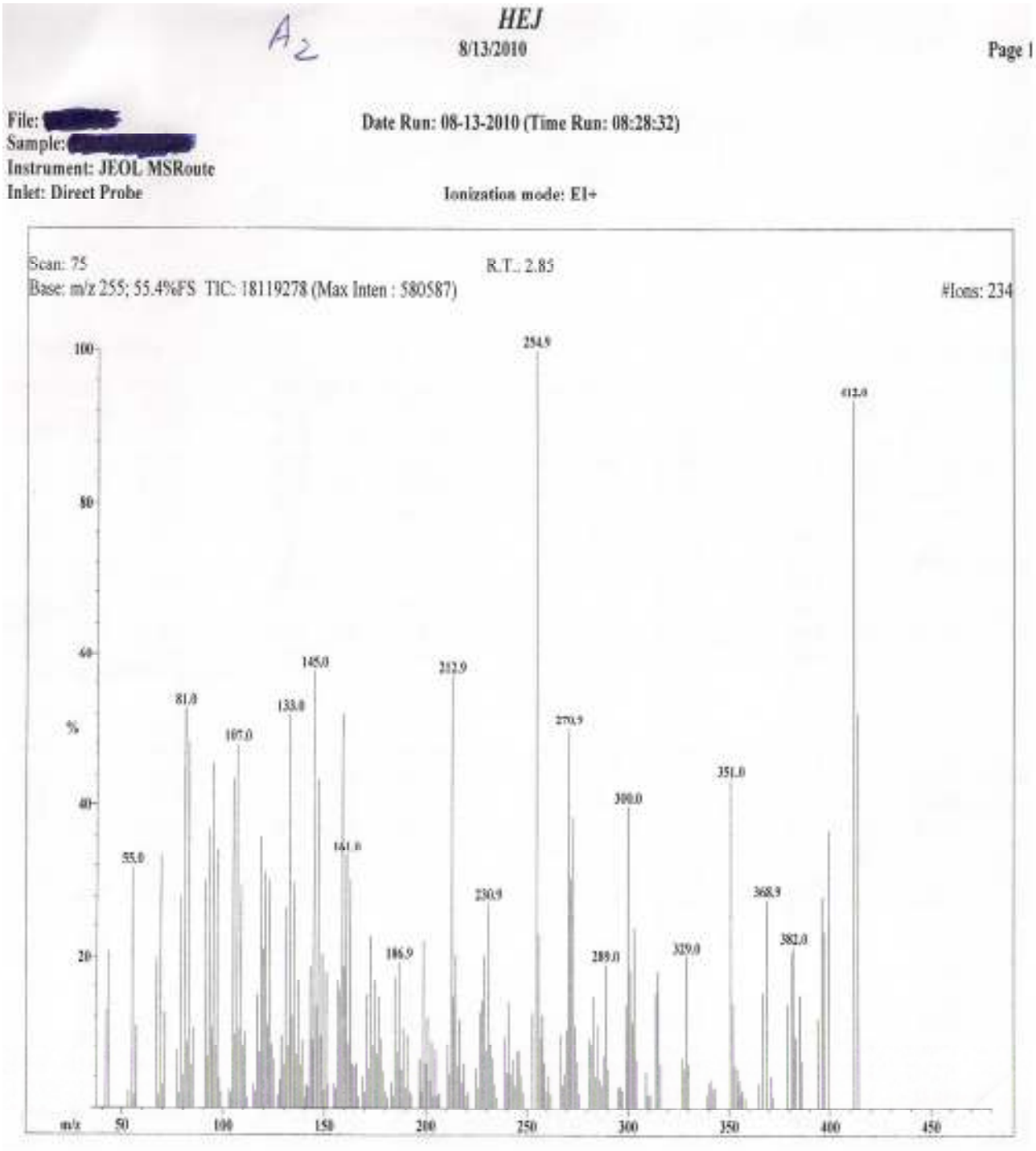
The ^{13}C NMR (BB and DEPT) spectrum of compound **7** disclosed the presence of twenty nine carbon signals and was resolved in DEPT spectra for six methyl, nine methylene, eleven methine and three quaternary carbon atoms. Comparison with the above data and literature showed the compound to be stigmasterol [190].

Table 6.3 ^{13}C-NMR ($CDCl_3$, 100 MHz) Chemical Shifts and Multiplicities of compound 7

C. NO	^{13}C- NMR	Multiplicity (DEPT)	C. NO	^{13}C- NMR	Multiplicity (DEPT)
1	37.5	CH_2	16	28.9	CH_2
2	31.9	CH_2	17	56.0	CH
3	71.9	CH	18	12.4	CH_3
4	42.2	CH_2	19	19.4	CH_3
5	140.9	C	20	40.5	CH
6	121.7	CH	21	21.1	CH_3
7	32.9	CH_2	22	138.4	CH
8	32.2	CH	23	129.4	CH
9	50.3	CH	24	51.3	CH
10	36.6	C	25	32.0	CH
11	21	CH_2	26	19.0	CH_3
12	39.7	CH_2	27	21.2	CH_3
13	42.5	C	28	25.4	CH_2
14	57.0	CH	29	12.0	CH_3
15	24.4	CH_2			



Stigmasterol (7)



Spectra 3: EI-MS spectra of Stigmasterol

6.1.2.4 Structure elucidation of Compound (8)

Compound **8** was isolated from the EtOAc fraction of *Z. jujuba* eluting with EtOAc / Pet ether (0.3: 9.7) as mobile phase on flash silica column. The $[M]^+$ at *m/z* 414 in EIMS of compound **8** was confirmed by $[M]^+$ at *m/z* 414.3857 in HREIMS which was in agreement with the molecular formula $C_{29}H_{50}O$ (calcd. 414.3861 for $C_{29}H_{50}O$) corresponding to five degrees of unsaturation. Bands at 3050, 1650 and 815 cm^{-1} in IR spectrum were due to trisubstituted double bonds and hydroxyl band at 3450 cm^{-1}. Characteristic fragment ions were also observed at *m/z* 399, 381, 329 and 303 in the EI-MS spectrum of the compound. The last two ions were diagnostic for sterols having Δ^5-unsaturation [89]. Other important fragments were observed at *m/z* 273 and 255 for $[M\text{-side chain}]^+$ and $[M\text{-side chain-}H_2O]^+$ respectively.

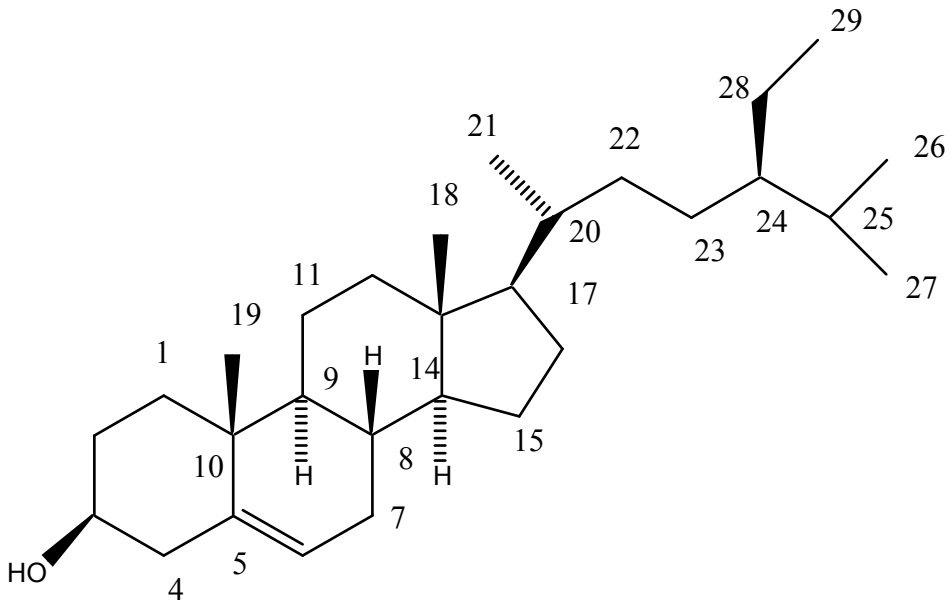
^1H NMR spectrum of compound **8** was characteristic of a sterol molecule. Two singlets each of three proton integration resonating at δ 0.63 and 0.92 were due to quaternary CH_3-18 and CH_3-19, respectively. Similarly, three doublets each of three proton integration at δ 0.88 0.83 and 0.81 having the coupling constant $J_{6.5}$ Hz, were assigned to the proton of secondary methyl, attached at C-2 and C-25 respectively. While a triplet of three proton integration at δ 0.77 having the coupling constant 7.0 Hz was assigned to the proton of primary methyl C-29.

The olefinic signals resonating at δ 5.32 was assigned to H-6 of the Δ^5-bond. The chemical shift and splitting pattern of the signal integrating for 1H at δ 3.36 was consistent with the H-3α and H-3*β* hydroxyl functions.

The comparative study of compound **8** NMR spectroscopic data with the reported data revealed its identity as *β* -sitosterol [191].

Table 6.4 ^{13}C-NMR ($CDCl_3$, 100 MHz) Chemical Shifts and Multiplicities of compound 8

C. NO	^{13}C-NMR	Multiplicity (DEPT)	C. NO	^{13}C-NMR	Multiplicity (DEPT)
1	37.3	CH_2	16	28.2	CH_2
2	31.8	CH_2	17	56.2	CH
3	121.9	CH	18	11.9	CH_3
4	42.4	CH_2	19	19.4	CH_3
5	140.9	C	20	36.3	CH
6	33.0	CH	21	19.1	CH_3
7	32.1	CH_2	22	21.1	CH_2
8	32.0	CH	23	29.3	CH_2
9	50.8	CH	24	50.4	CH
10	36.6	C	25	26.2	CH
11	21.1	CH_2	26	18.8	CH_3
12	40.3	CH_2	27	19.8	CH_3
13	42.6	C	28	23.1	CH_2
14	56.8	CH	29	11.9	CH_3
15	24.3	CH_2			



***β*- Sitosterol (8)**

6.2 PHARMACOLOGICAL INVESTIGATIONS

6.2.1 Antibacterial activity

Crude methanolic extract and fractions

The interest, regarding the research on medicinal plants has increased over the last few decades due to onset of new infections. These infections in particular are caused by *Enterococcus* and *Staphylococcus* species. These are agents of many intra-hospital infections and antibiotic resistant to available drugs e.g. *S. aureus* has become resistant to several antibiotics to which it was previously susceptible. Some of the antibiotics to which it is now resistant are pencillin G, macrolides, lincosamides, tetracyclines and gentamicin [98]. With the intent of exploring new bioactive compounds from plant origin, we selected *Z. jujuba* to screen it for antibacterial activity against the stated pathogens. The results of antibacterial activity of test samples of *Z. jujuba* are shown in **Table 6.5** and **Figures 6.1-6.5.** The crude methanolic extract showed moderate activity against *P. aeruginosa, B. pumilus* and *E. aerogenes* with 55.55%, 52% and 41.37% inhibition and low activity against *S. typhi*, *S. epidermidis*, *S. pneumoniae*, *S. aureus*, *K. pneumoniae* with 37.03%, 34.61%, 31.03%, 30.76% and 28.57% inhibition respectively. It was inactive against *E. coli.* The *n*-hexane fraction was significantly active against *B. pumilus* (60%), moderately active against *S. typhi, S. epidermidis*, and *E. aerogenes* with 55.55%, 53.84% and 44.82%. It showed low activity against *S. aureus*, *P. aeruginosa*, *E. coli* with 38.46%, 37.03% and 29.62% inhibition respectively. In contrary the *n*-hexane fraction presented no activity against *S. pneumoniae* and *K. pneumoniae.* The $CHCl_3$ fraction of the plant was moderately active against *S. typhi*, *S. aureus*, *P. aeruginosa* and *E. aerogenes* having percent inhibition of 51.85, 50, 44.44 and 41.37 respectively. Low activity was observed against *S. epidermidis*, *K. pneumoniae*, *B. pumilus*, *E. coli* and *S. pneumoniae* with 38.46, 38.09, 32, 29.62 and 27.58%

inhibition, respectively. The EtOAc fraction showed good activity against *B. pumilus*, *S. epidermidis*, *S. typhi* and *P. aeruginosa* with 72, 65.38, 62.96, and 62.96% inhibition, moderately active against *E. aerogenes* and *E. coli* with 55.17 and 48.14 percent inhibition respectively. It showed low activity against *S. aureus*, *K. pneumoniae* with 30.76 and 28.57 percent inhibition and inactive against *S. pneumoniae.* The aqueous fraction showed good activity against *P. aeruginosa,* 66.66%, moderate activity against *S. typhi*, *E. aerogens*, *S. pneumoniae, E. coli* and *B. pumilus* with 55.55, 44.82, 41.37, 40.74 and 40 percent inhibition respectively. It conferred low activity on *S. epidermidis*, *S. aureus* and *K. pneumoniae* with inhibition percentage of 38.46, 34.61 and 33.33 respectively. This work is continuation of our effort for exploring new bioactive compounds. Previously we utilized similar approach and screened various fractions of *Onosma griffithii* against *E. coli, B. subtilis, S. aureus, S. flexenari* and *S. typhi* [69]. Same strategy was followed by Ajaiyeoba, 2002, in which the *n*-hexane, EtOAc, ethanol and water extract of *Parkia bicolor* A. *Chev* were tested against *S. aureus, B. cereus, E. coli, P. aeruginosa, A. niger* and *C. utilis* [44]. The crude methanolic extracts of different plants were tested against gram-positive and gram-negative bacteria for new bioactive compounds [192]. MIC_{50} of the test samples ranged from 1.9-3.8 mg / ml. The *n*-hexane and EtOAc fractions of *Z. jujuba* were significantly active against *B. pumalis*, *S. epidermidis*, *S. typhi and P. aeruginosa* with low values of MIC_{50} (**Table 6.6** and **Figures 6.6-6.10**).

Essential oils

The essential oils from *Z. jujuba* were tested against *B. subtilis*, *E. coli*, *S. typhi, S. flexenari*, *S. aureus* and *P. aeruginosa*. The results are depicted in **Table 6.7.** The results indicate that the *Z. jujuba* oils were inactive against all the bacterial strains used in the research.

Table 6.5 Antibacterial activities of crude methanolic extract & various fractions of *Zizyphus jujuba*

Name of Bacteria	Zone of Inhibition of standard (Amoxicillin) 10µg /Disc	**Crude Extract**		***n*-hexane**		**CHCl3**		**EtOAc**		**Aqueous**	
		Zone of Inhibition (mm)	Inhibition (%)	Zone of Inhibition (mm)	Inhibition (%)	Zone of Inhibition (mm)	Inhibition (%)	Zone of Inhibition (mm)	Inhibition (%)	Zone of Inhibition (mm)	Inhibition (%)
E. coli	27	0	0	8	29.62	8	29.62	13	48.14	11	40.74
S.epidermidis	26	9	34.61	14	53.84	10	38.46	17	65.38	10	38.46
S.typhi	27	10	37.03	15	55.55	14	51.85	17	62.96	15	55.55
S.pneumoniae	29	9	31.03	0	0	8	27.58	0	0	12	41.37
S.aureus	26	8	30.76	10	38.46	13	50	8	30.76	9	34.61
P.aeruginosa	27	15	55.55	10	37.03	12	44.44	17	62.96	18	66.66
K. pneumoniae	21	6	28.57	0	0	8	38.09	6	28.57	7	33.33
B.pumilus	25	13	52	15	60	8	32	18	72	10	40
E.aerogenes	29	12	41.37	13	44.82	12	41.37	16	55.17	13	44.82

Table 6.6 MIC_{50} values of crude methanolic extract and various fractions of *Zizyphus jujuba*

Name of Bacteria	**Crude extract**	***n*-hexane**	**$CHCl_3$**	**EtOAc**	**Aqueous**
E. coli	0	4.0	3.8	3.0	3.2
S.epidermidis	3.7	2.9	3.2	2.4	3.2
S.typhi	3.2	2.9	3.0	2.4	2.9
S.pneumoniae	3.2	0	3.2	0	2.7
S.aureus	2.9	3.0	2.7	3.1	3.0
P.aerugenosa	2.5	3.0	2.7	2.3	1.9
K. pneumoniae	3.4	0	3.1	3.7	3.2
B.pumalis	2.5	2.1	3.2	2.1	3.0
E.aerogens	2.7	2.7	3.0	2.7	2.9

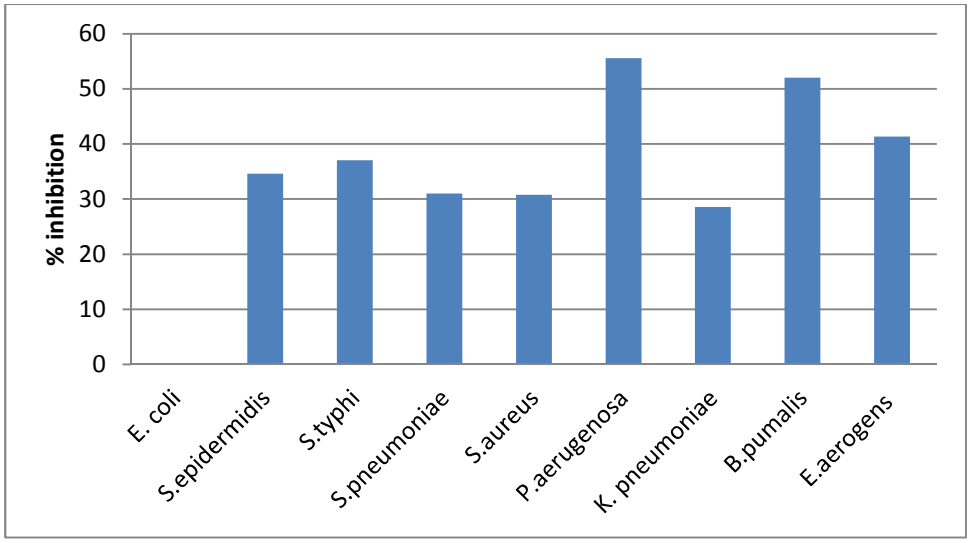


Fig 6.1 Antibacterial activity of the crude methanolic extract of *Zizyphus jujuba*

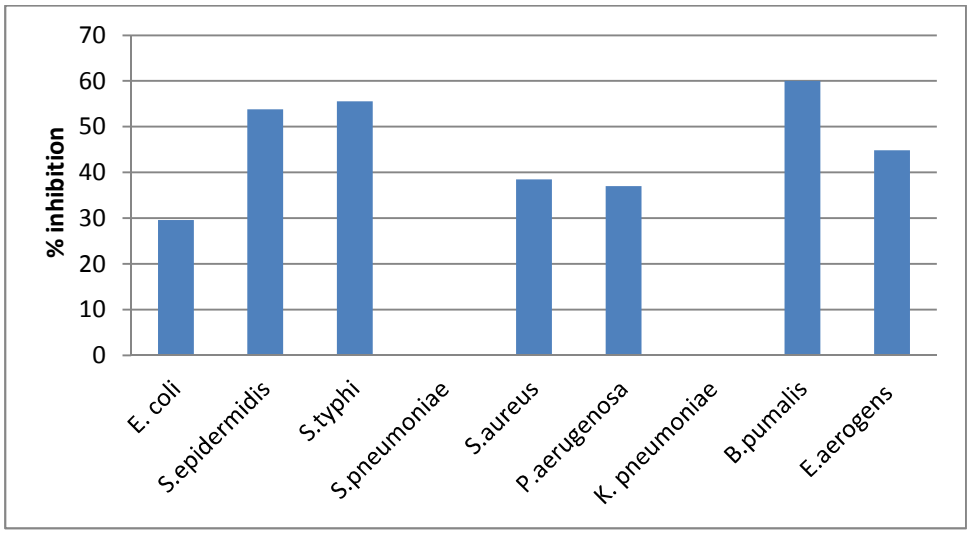


Fig 6.2 Antibacterial activity of the *n*-hexane fraction of *Zizyphus jujuba*

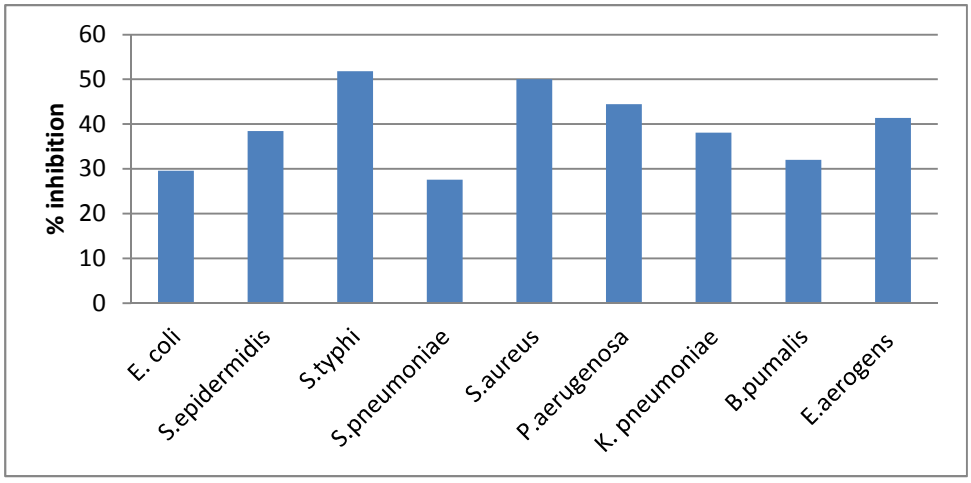


Fig 6.3 Antibacterial activity of the $CHCl_3$ fraction of *Zizyphus jujuba*

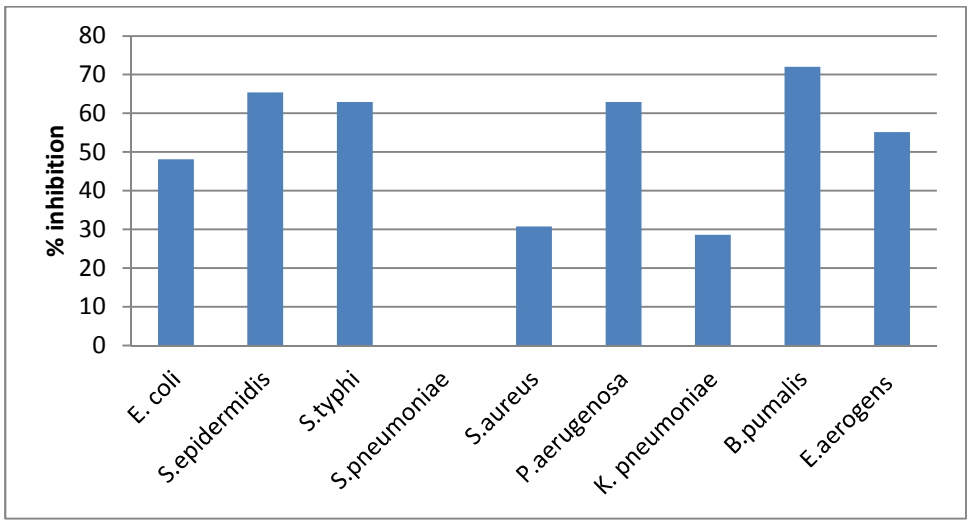


Fig 6.4 Antibacterial activity of the EtOAc fraction of *Zizyphus jujuba*

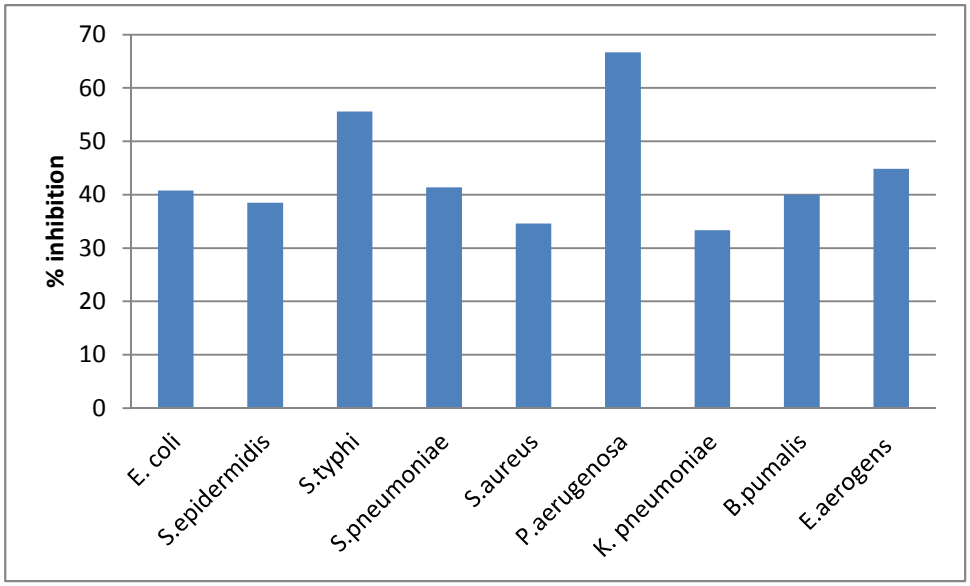


Fig 6.5 Antibacterial activity of the aqueous fraction of *Zizyphus jujuba*

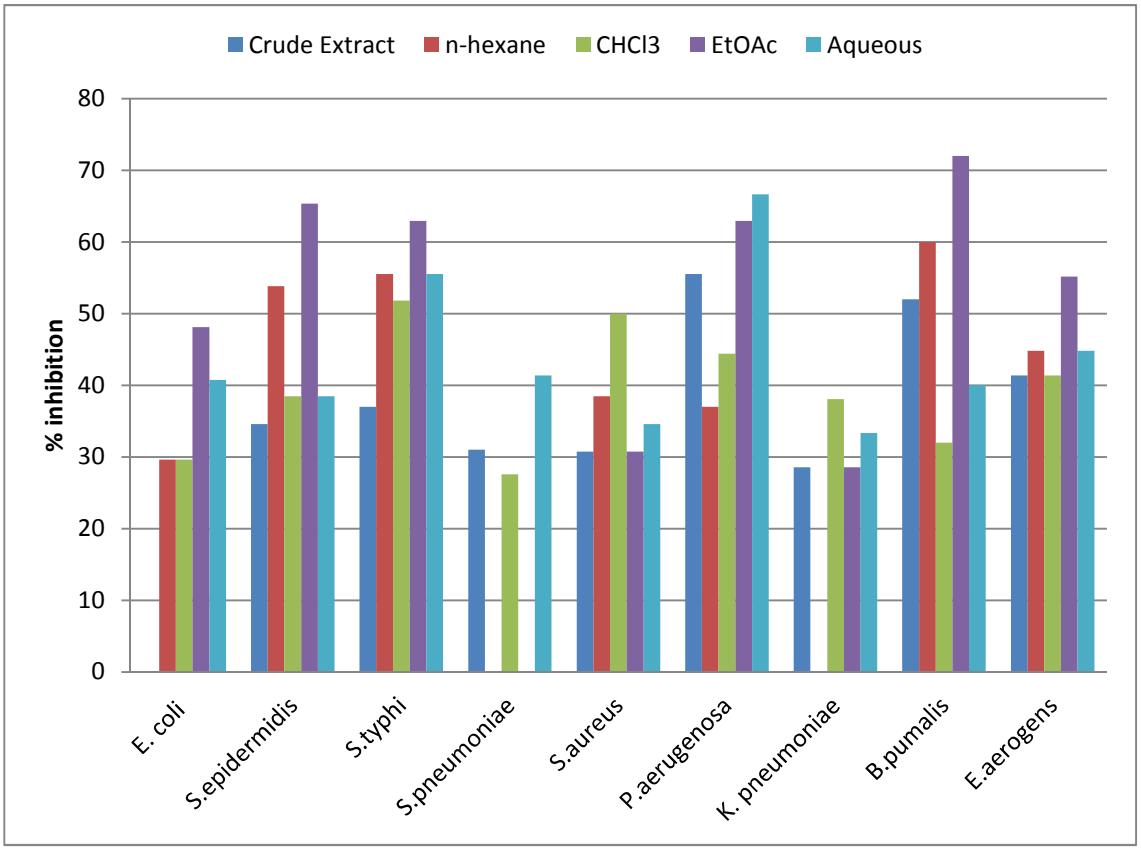


Fig 6.6 Antibacterial activity of the crude methanolic extract and various fractions of *Zizyphus jujuba*

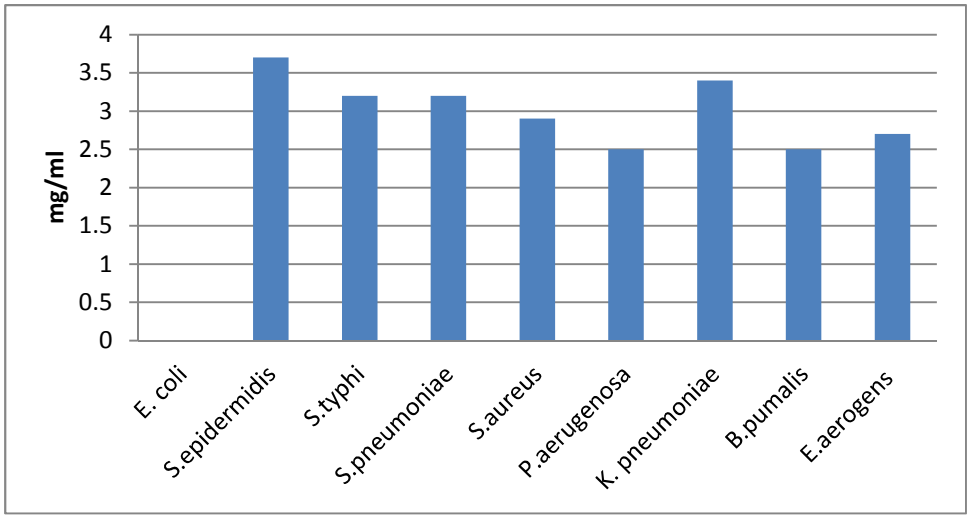


Fig 6.7 MIC_{50} of the crude methanolic extract of *Zizyphus jujuba*

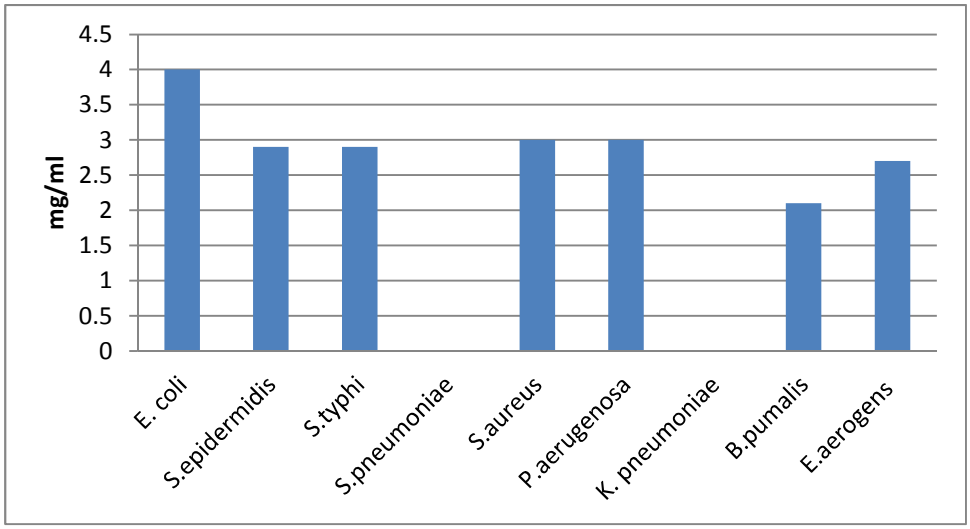


Fig 6.8 MIC_{50} of the *n*-hexane fraction of *Zizyphus jujuba*

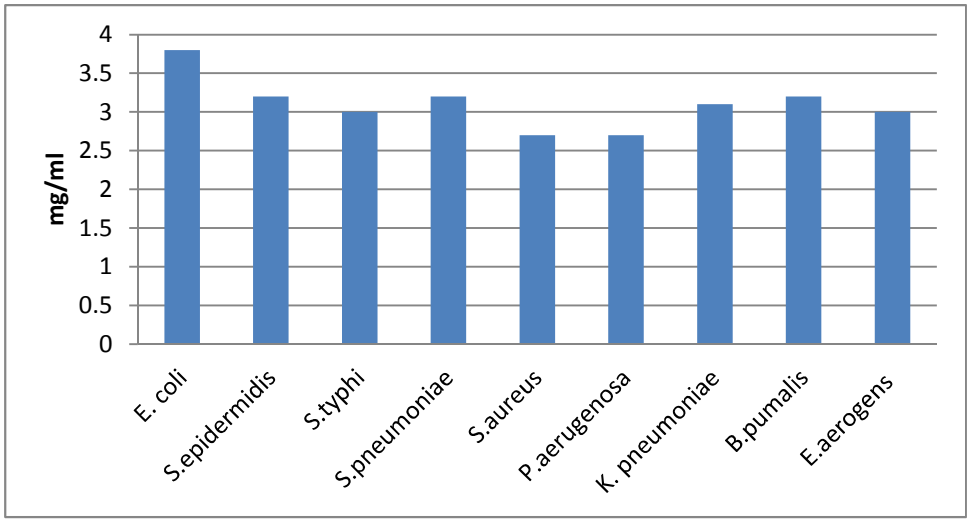


Fig 6.9 MIC_{50} of the $CHCl_3$ fraction of *Zizyphus jujuba*

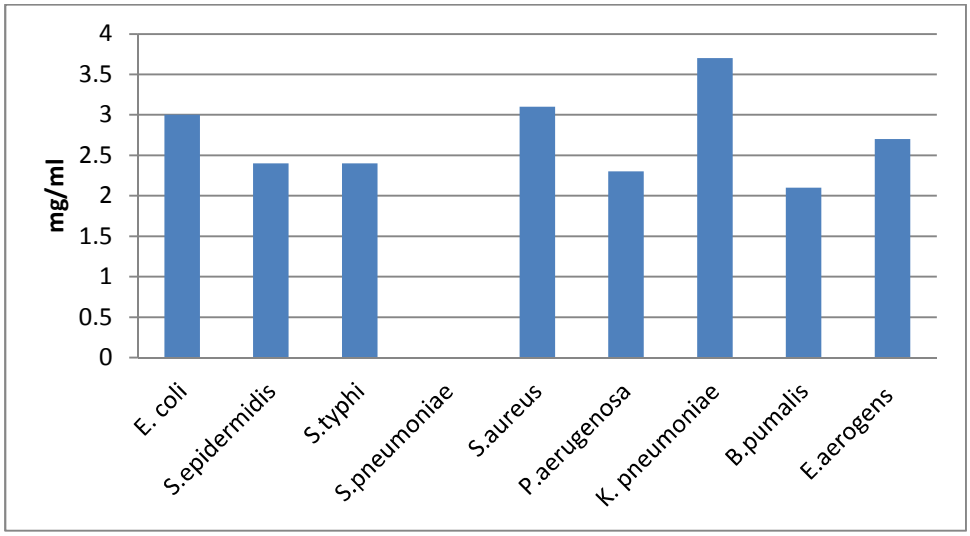


Fig 6.10 MIC_{50} of the EtOAc fraction of *Zizyphus jujuba*

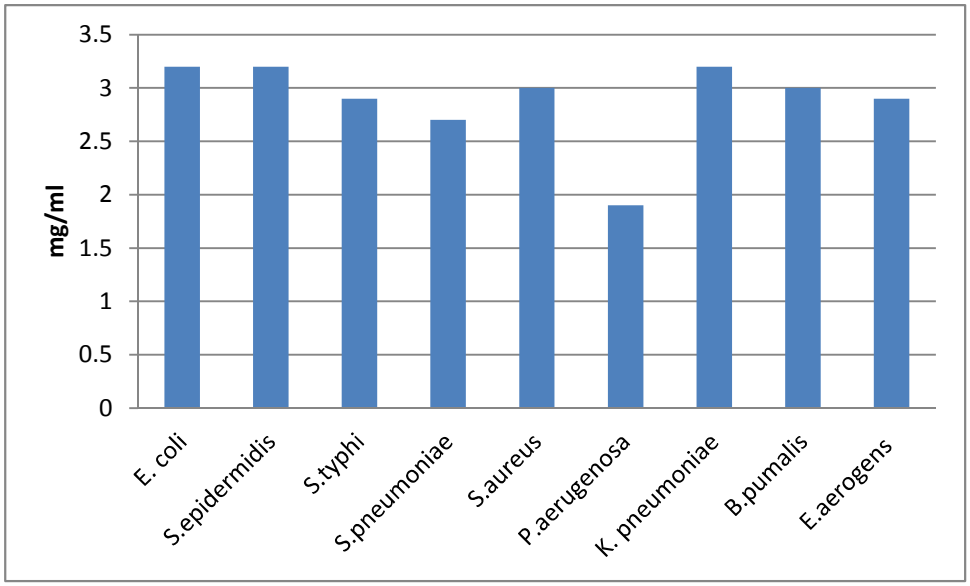


Fig 6.11 MIC_{50} of the aqueous fraction of *Zizyphus jujuba*

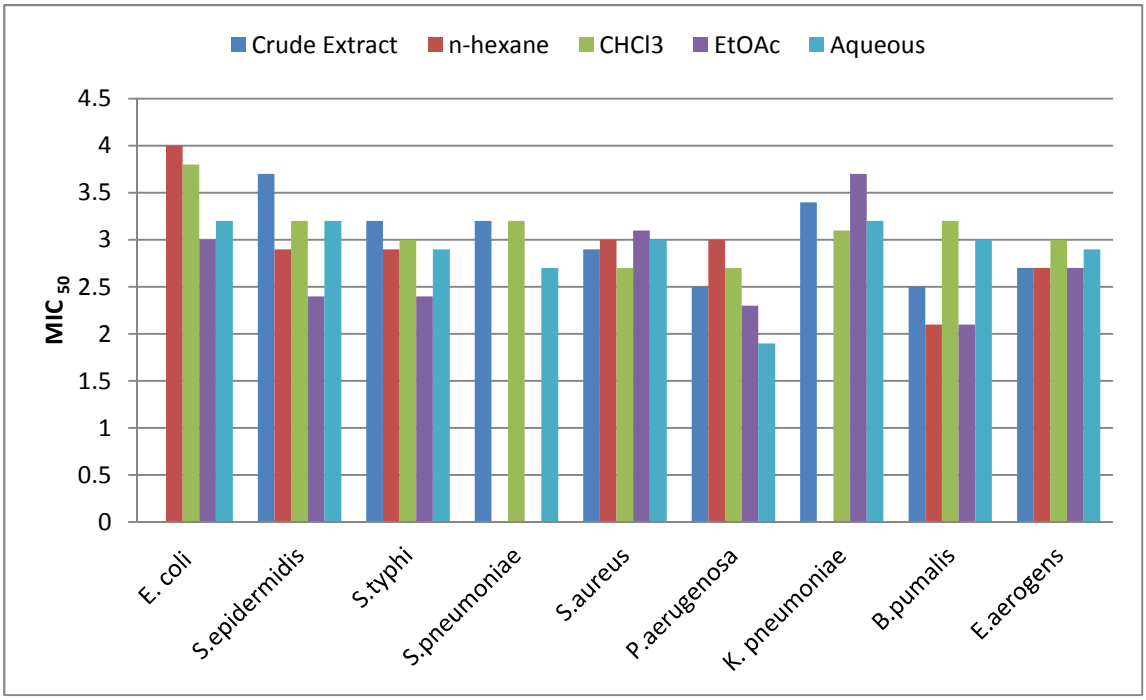


Fig 6.12 MIC_{50} of the crude methanolic extract and various fractions of *Zizyphus jujuba*

Table 6.7 Antibacterial activity of the oils of *Zizyphus jujuba*

Name of Bacteria	**Zone of inhibition of sample (mm)**	**Zone of inhibition of standard drug (mm)**
Escherichia coli	0	35
Bacillus subtilis	0	36
Shigella flexenari	0	35
Staphylococcus aureus	0	43
Pseudomonas aeruginosa	0	32
Salmonella typhi	0	40

6.2.2Antifungal activity

Crude methanolic extract and fractions

A number of secondary metabolites (mycotoxins) toxic to humans and animals are produced by fungi. Malformins, trichodermins etc are secondary metabolites that were reported to be produced by *A. niger* [193]. Chemical fungicides are highly effective in controlling various diseases of fruits and vegetables. But they are not a suitable solution in long run because of the associated health and environmental hazards, tolerance development and residue persistence [84-85]. As the fungicides from the plant origin are specific, therefore the crude methanolic extract and various fractions of *Z. jujuba* were screened against *A.niger, A.flavus, P. notatum, F. oxysporum, T. harzianum* and *R. stolonifer*. **Table 6.8** and **Figures 6.13-6.17** encompass the results.

The crude methanolic extract showed low activity against *P. notatum* (12%) and *T. harzianum* (10%) while no activity against *A. niger, A. flavus, F. oxysporum* and *R. stolonifer*. Low activity was shown by the *n*-hexane fraction against *A. niger* (10%), *P. notatum* (9%) and *T. harzianum* (13%). The *n*-hexane fraction was inactive against *A. flavus*, *F. oxysporum* and *R. stolonifer*. The $CHCl_3$ fraction exhibited low activity of 20% and 10% against *P. notatum* and *F. oxysporum* respectively while showing no activity against the rest of the test fungi. 12%, 14% and 11% inhibition was shown by the EtOAc fraction against *A. niger*, *P. notatum* and *T. harzianum*. Against *A. flavus*, *F. oxysporum* and *R. stolonifer* this fraction was inactive. The aqueous fraction exhibited low activity against *A. flavus* (15%), *F. oxysporum* (5%) and *T. harzianum* (17%). This fraction was inactive against the rest of the test fungi.

The results indicate that *Z. jujuba* is lacking potent antifungal agents.

Essential oils

The antifungal activity of the oils was determined against *A. flavus*, *Candida albicans*, *Candida glaberata*, *Microsporum canis* and *Fusarium solani*. The results are mentioned in **Table 6.9.** The oils of *Z. jujuba* were inactive against all the test fungi.

Table 6.8 Antifungal activity of the crude methanolic extract and various fractions of *Zizyphus jujuba*

Name of Fungi	**Percent inhibition**						
	-ive control	**+ive control**	**Crude Met. extract**	***n*-hexane**	**$CHCl_3$**	**EtOAc**	**Aqueous**
A. niger	0	100	0	10	0	12	0
A. flavus	0	100	0	0	0	0	15
P. notatum	0	100	12	9	20	14	0
F. oxysporum	0	100	0	0	10	0	5
T. harzianum	0	100	10	13	0	11	17
R. stolonifer	0	100	0	0	0	0	0

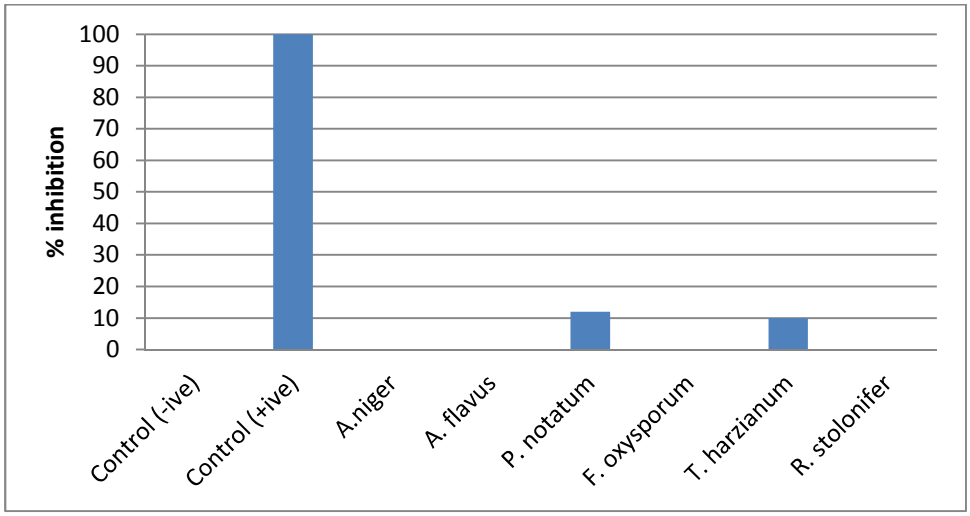


Fig 6.13 Antifungal activity of the crude methanolic extract of *Zizyphus jujuba*

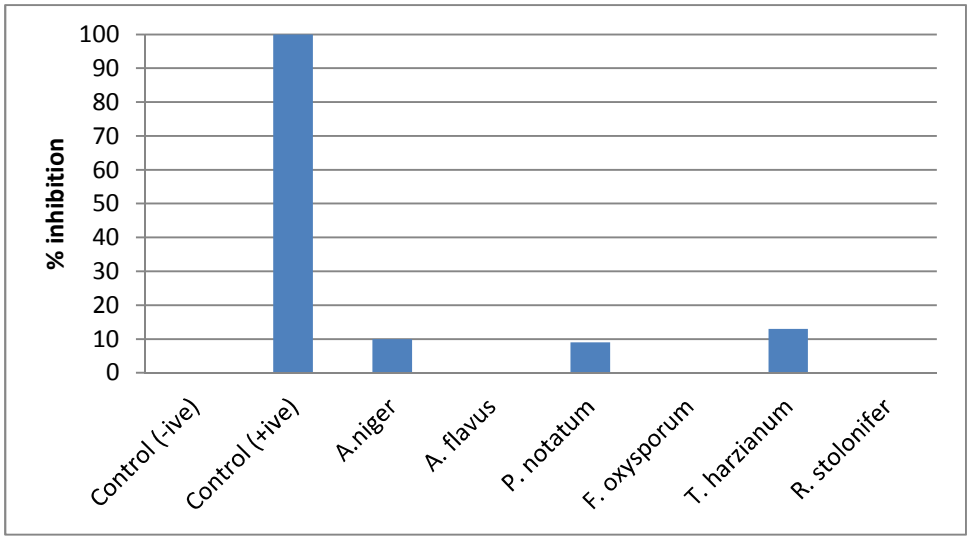


Fig 6.14 Antifungal activity of the *n*-hexane fraction of *Zizyphus jujuba*

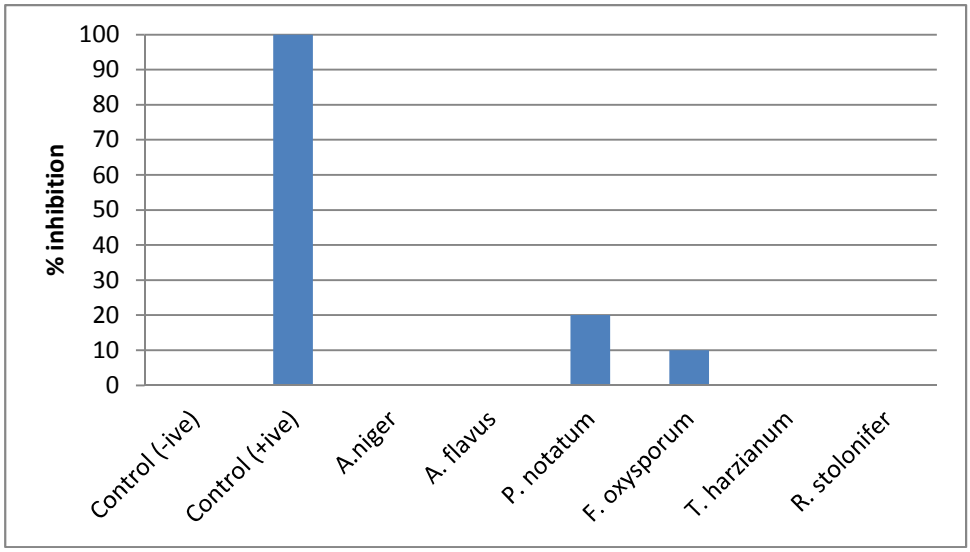


Fig 6.15 Antifungal activity of the $CHCl_3$ fraction of *Zizyphus jujuba*

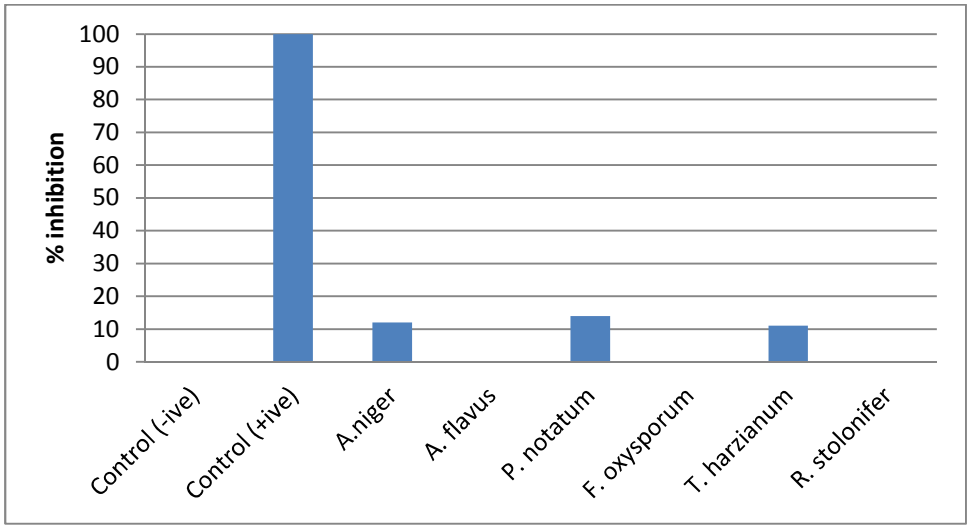


Fig 6.16 Antifungal activity of the EtOAc fraction of *Zizyphus jujuba*

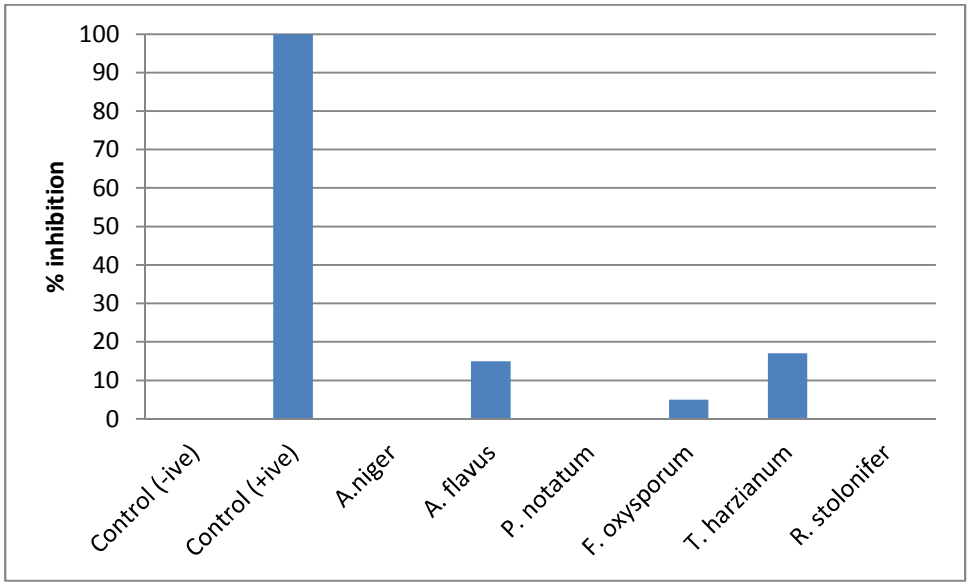


Fig 6.17 Antifungal activity of the aqueous fraction of *Zizyphus jujuba*

Table 6.9 Antifungal activity of the oils of *Zizyphus jujuba*

Name of fungus	**Linear Growth (mm)**		**% inhibition**	**MIC of Standard drug (µg/ml)**
	Sample	**Control**		
C. albicans	100	100	0	Miconazole (110.8)
A. flavus	100	100	0	Amphotericin B (20.20)
M. canis	100	100	0	Miconazole (98.4)
F.solani	100	100	0	Miconazole (73.25)
C. glaberata	100	100	0	Miconazole (110.8)

6.2.3 Phytotoxic activity

Crude methanolic extract and fractions

Lemna plants are miniature aquatic monocot, which are very sensitive to bioactive compounds. *Lemna* assay has been used to detect natural anti-tumor, phytotoxic compounds and new plant growth stimulants [108]. Previously we have studied the phytotoxicity of the crude methanolic extracts of *Rumex dentatus, Rumex hastatus, Rheum australe, Rumex nepalensis, Polygonum plebejum* and *Polygonum persicaria* (Family *Polygonaceae*) using *Lemna* bioassay. At 1000 µg / ml, all the extracts except *R. hastatus,* were significantly active [194]. Phytotoxic activity of test samples was carried out against *Lemna minor* L **(Tables 6.10, 6.11** and **Fig 6.18)**. The crude methanolic extract, *n*-hexane, $CHCl_3$ and aqueous fractions showed low activity of 12 and 0%, 25% and 6.25%, 18.75 and 12.5%, 31.25 and 12.55% growth inhibition at concentrations 1000 and 100 µg / ml, respectively. The $EtOA_c$ fraction showed moderate activity of 43.75 % at 1000 µg / ml and low activity of 18.75% at100 µg / ml. At concentration of 10µg / ml no phytotoxic activity was observed. However this time, the selected specie was not phytotoxic in most of the cases. Only $EtOA_c$ (43.75%) fraction was moderately active. Aqueous extracts of seven species of *Eleocharis* were tested for allelopathic activity using common duckweed lemna minor assay showing different results [195]. Our results indicate that this specie of *Rhamnaceae* is having no potent phytotoxic activity.

Essential oils

The phytotoxic activity of the essential oils was determined against *L. minor* L and **Table 6.12** and **Fig 6.19** represents the result. At the concentration of 1000 and 100 µg / ml, the oils showed low activity of 15 and5% respectively. At the concentration of 10 µg / ml, the sample showed no activity against *L minor* L.

Table 6.10 Phytotoxic activity of crude methanolic extract and various fractions of *Zizyphus jujuba* against *Lemna minor* L

Name of plant	**Concentration of sample (µg/ml)**	**No. of fronds survived**						**Concentration of standard drug * (µg/ml)**
Lemna minor		**Crude methanolic extract**	***n*-hexane**	**$CHCl_3$**	**EtOAc**	**Aqueous**	**Control**	
	1000	14	12	13	9	11	16	0.015
	100	16	15	14	13	14	16	
	10	16	16	16	16	16	16	

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

Table 6.11 Percent growth regulation of the *Lemna minor*

Concentration of sample (µg/ml)	**Percent growth regulation**				
	Crude methanolic extract	***n*-hexane**	**$CHCl_3$**	**EtOAc**	**Aqueous**
1000	12.5	25	18.75	43.75	31.25
100	0	6.25	12.5	18.75	12.5
10	0	0	0	0	0

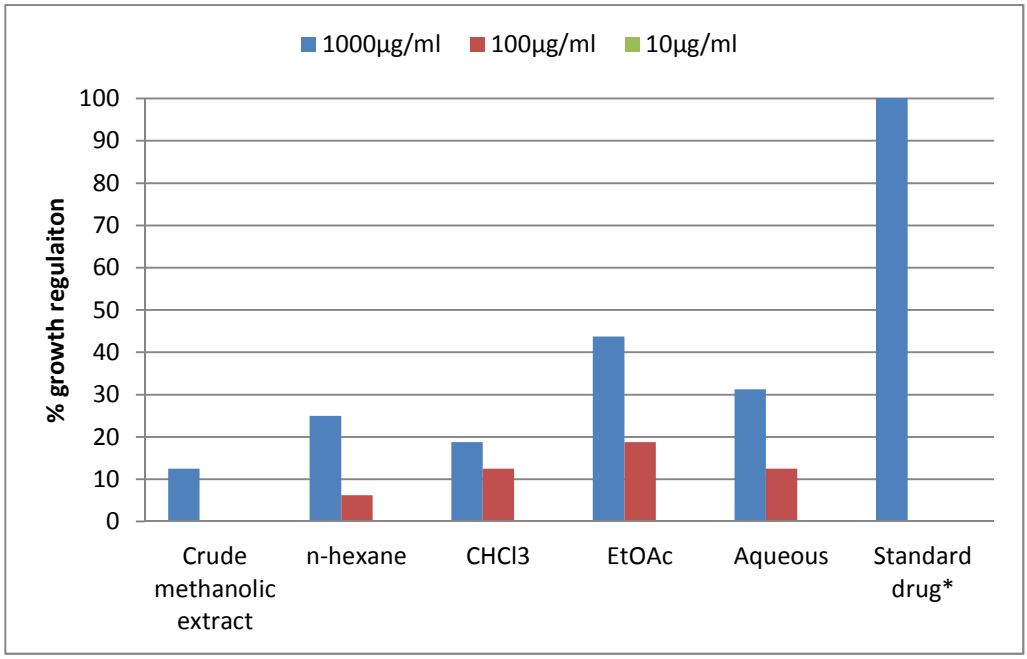


Fig 6.18 Phytotoxic activity of the crude methanolic extract and various fractions of *Zizyphus jujuba*

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

Table 6.12 Phytotoxic activity of the oils of *Zizyphus jujuba*

Name of plant	**Conc. of compound (µg/ml)**	**No. of Fronds**		**% Growth regulation**	**Conc. of Std. Drug* (µg/ml)**
		Sample	**Control**		
Lemna minor	1000	17	20	15	0.015
	100	19		5	
	10	20		0	

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

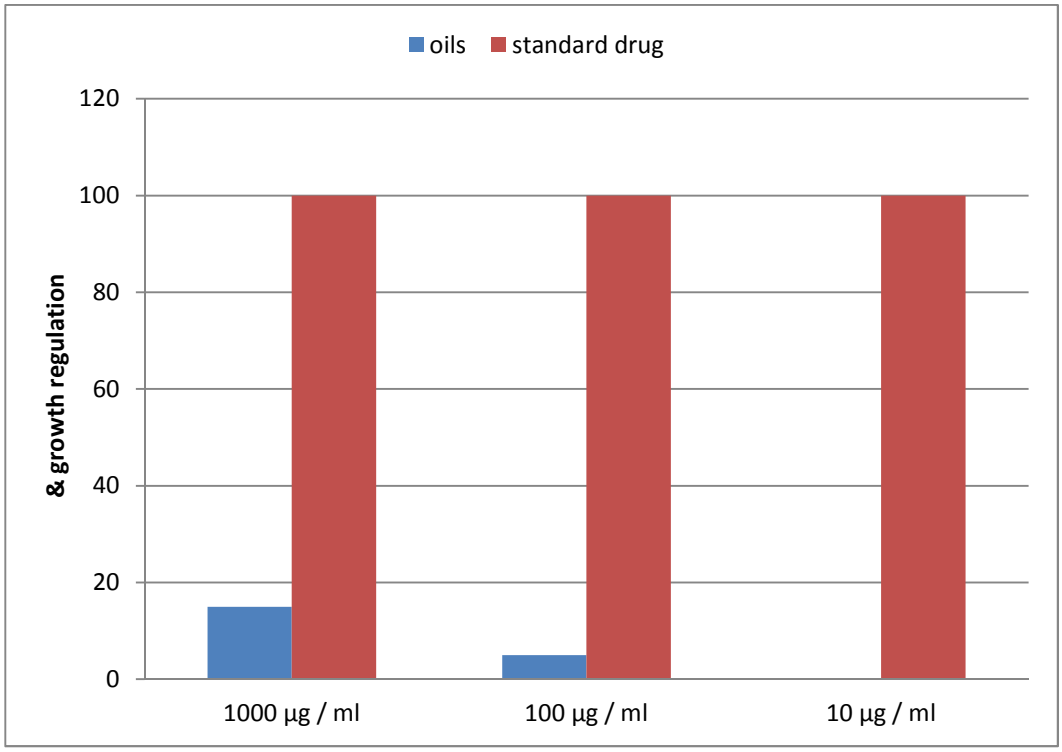


Fig 6.19 Phytotoxic activity of the oils of *Zizyphus jujuba*

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

6.2.4 Insecticidal assay

Crude methanolic extract and fractions

Many ecological problems have been created by the indiscriminate use of synthetic insecticides, including harm to mammals and the accumulation of harmful residues in the environment [196-197]. Therefore, insecticides most efficiently controlling the pests without serious effects on the environment are clearly required.

The test samples of *Z. jujuba* were screened for insecticidal activity against *Tribolium castaneum, Rhizopertha dominica* and *Callosbruchus analis*. The results are mentioned in **Table 6.13** and **Fig 6.20.** All the test samples except *n*-hexane showed low activity (20%) against *T. castaneum*. Against *R. dominica* only the *n*-hexane fraction showed low activity of 20% while rest of the test samples were inactive against it. The $CHCl_3$ and *n*-hexane fractions exhibited 40% and 20% activity respectively against *C. analis*. Crude methanolic extract, EtOAc and aqueous fractions were inactive against *C. analis.*

Essential oils

The insecticidal activity of the oils of *Z. jujuba* was carried out against *T. castaneum*, *R. dominica* and *C. analis*. The results are mentioned in **Table 6.14** and **Fig 6.21.**

The oils showed low activity (20%) against *T. castaneum* and *R. dominica*. The oils were inactive against *C. analis*.

Table 6.13 Insecticidal activity of crude extract and fractions of *Zizyphus jujuba* by contact toxicity method

Name of insect	**% Mortality of the insects**					
	Control (Positive) *	**Crude methanolic extract**	***n*-hexane**	**$CHCl_3$**	**EtOAc**	**Aqueous**
Tribolium castaneum	100	20	0	20	20	20
Rhizopertha dominica	100	0	20	0	0	0
Callosbruchus analis	100	0	20	40	0	0

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

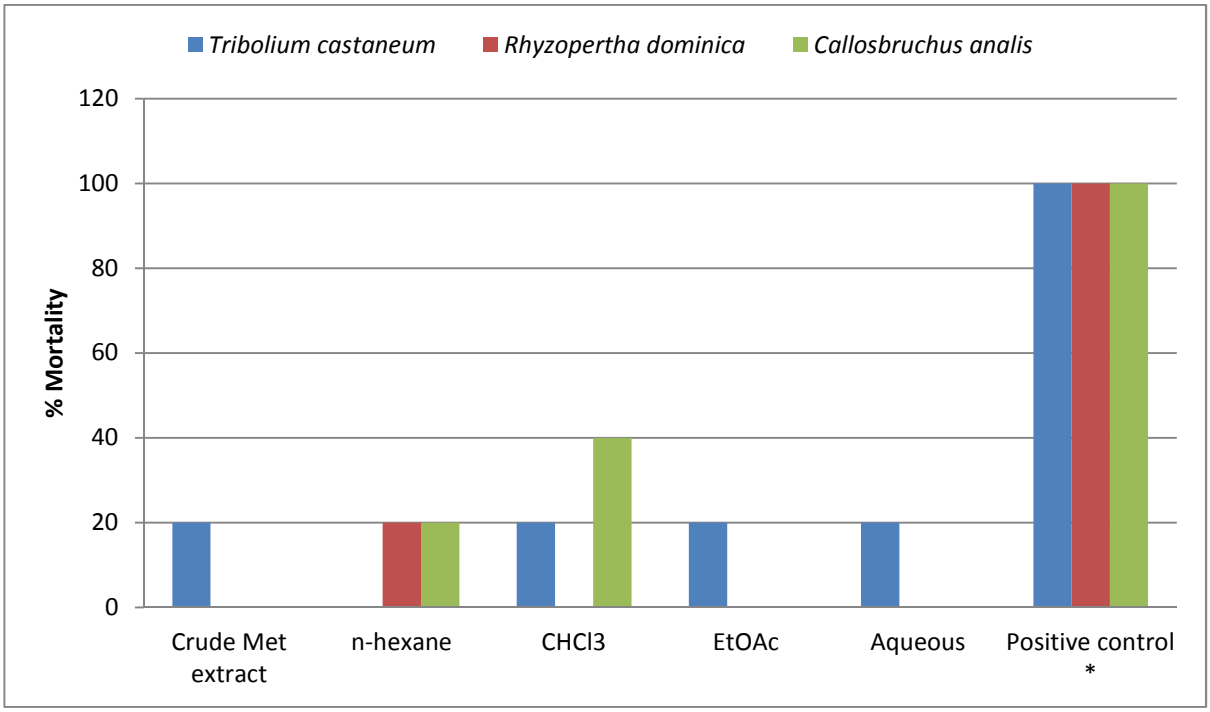


Fig 6.20 Insecticidal activity of crude methanolic extract and fractions of *Zizyphus jujuba*

Table 6.14 Insecticidal activity of the oils of *Zizyphus jujuba*

Name of the insect	**% Mortality of the insects**		
	Control (+ ve) *	**Control (- ve)**	**Sample**
Tribolium castaneum	100	0	20
Rhyzopertha dominica	100	0	20
Callosbruchus analis	100	0	0

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

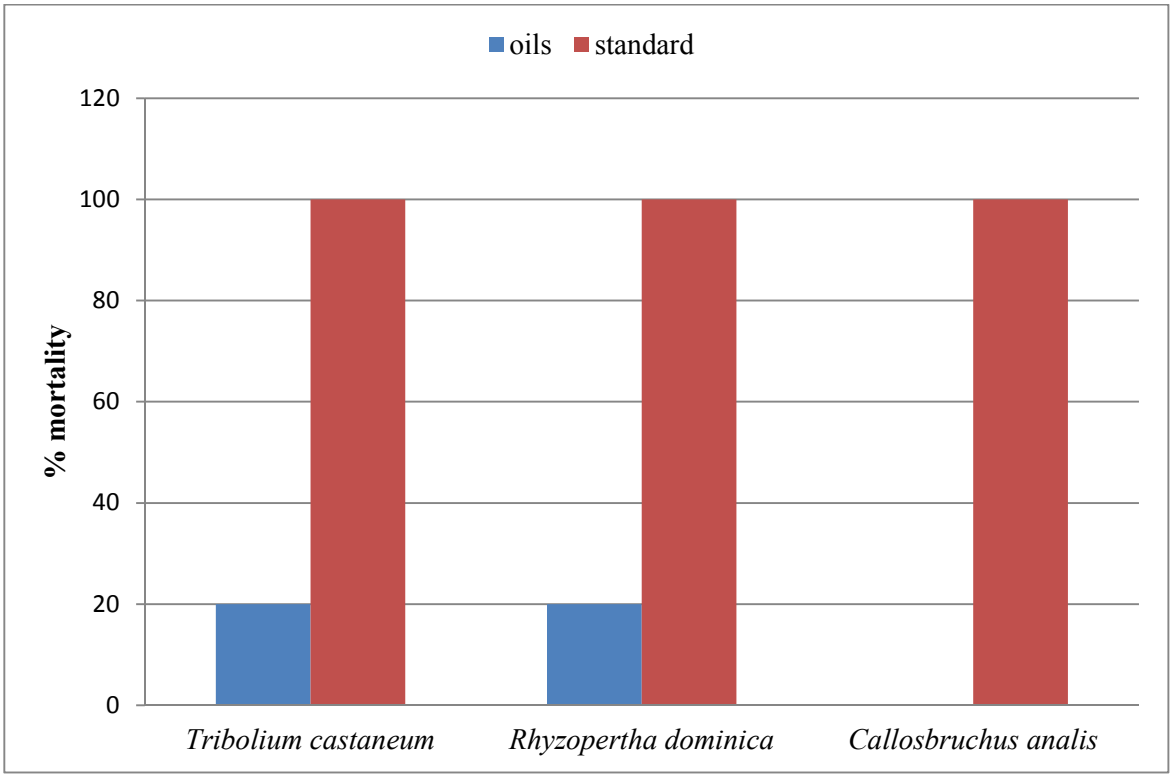


Fig 6.21 Insecticidal activity of the oils of *Zizyphus jujuba*

6.2.5 Brine shrimp lethality bioassay

Crude methanolic extract and fractions

Brine shrimp (*Artemia salina*) lethality bioassay is used to check the cytotoxic effect of a sample. The results are presented in **Table 6.15** and **Fig 6.22.** The results showed that the crude methanolic extract is highly toxic (73.33%) at 1000 µg / ml. At the concentration of 100 and 10µg / ml, it showed 26.66% and 13.33% cytotoxic effect respectively. The values of the upper limit and G were 128.40 and 768.45 respectively. The *n*-hexane fraction showed cytotoxic effect of 13.33% at 1000 µg / ml, 10% at 100 µg / ml and 6.66% at 10 µg / ml. The value of upper limit was 0.0019 and lower limit was 10389.93. The G value was 5.3815. The $CHCl_3$ fraction showed 40% lethality at 1000 µg/ml. 26.66% and 13.33% mortality was recorded at the concentration of 100 µg / ml and 10 µg / ml respectively. The values of the upper limit, lower limit and G were 285727, 529.185 and 0.7137 respectively. The EtOAc fraction showed toxicity of 13.33% at 1000 µg / ml, 10% at 100 µg / ml and 6.66% at 10 µg / ml. The value of the upper limit was 0.0019, lower limit was 10389.9 and G was 5.3815. The cytotoxic effect of the aqueous fraction was 26.66% at 1000 µg / ml, 16.66% at 100 µg / ml and 6.66% at 10 µg / ml. Upper limit value was 0.00. The value of the lower limit was 6.2341 and G was 0.9453.

Essential oils

The results of cytotoxic activity of the oils of *Z. jujuba* are depicted in **Table 6.16** and **Fig 6.23.** The results indicate that the oils of *Z. jujuba* are moderately toxic at 1000 µg / ml. Out of 30 shrimps, 17 shrimp survived. At 100 µg / ml, 23 shrimps out of 30 survived. 29 shrimps out of 30, survived at 10 µg / ml. The LD_{50} value was 1387.20 µg / ml. The upper and lower limit was 1.20273 and 486.88 respectively while the G value was 0.4974. These results indicate that the oils obtained from *Z. jujuba* are moderately cytotoxic at higher concentration.

Table 6.15 Brine shrimp cytotoxicity of crude methanolic extract and various fractions of *Zizyphus jujuba*

Dose (µg/ml)	**Control**	**Crude methanolic extract**		**n-hexane**		**$CHCl_3$**		**EtOAc**		**Aqueous**	
		Survivors	% killed	Survivors	% killed	Survivors	% killed	Survivors	% killed	Survivors	% killed
1000	30	8	73.33	26	13.33	18	40	26	13.33	22	26.66
100	30	22	26.66	27	10	22	26.66	27	10	25	16.66
10	30	26	13.33	28	6.66	26	13.33	28	6.66	28	6.66

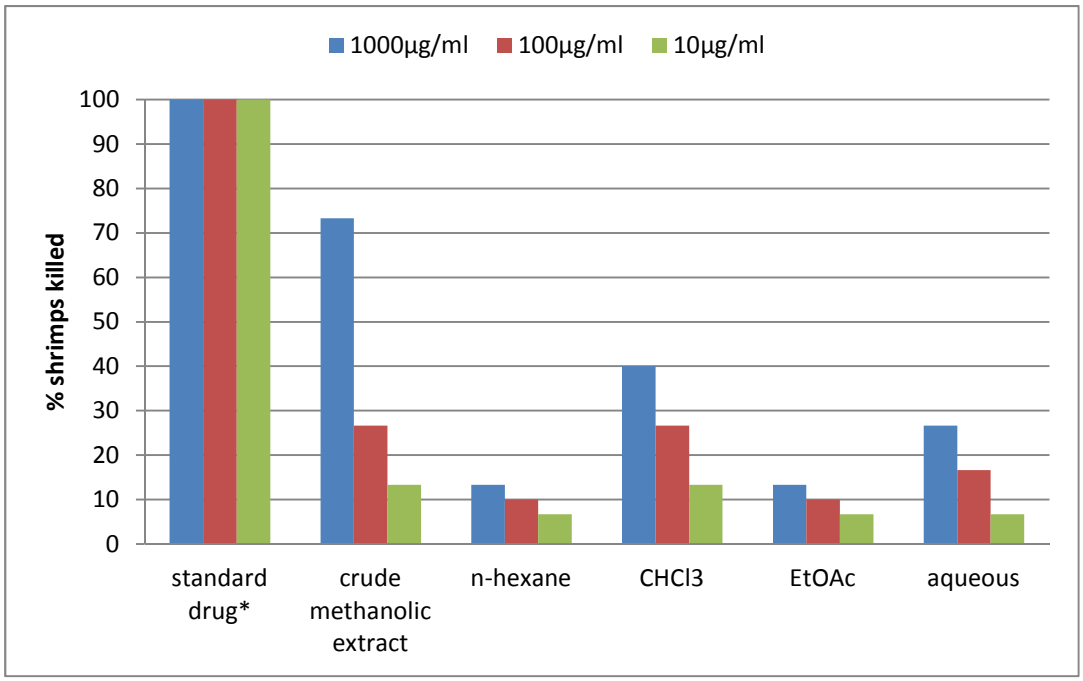


Fig 6.22 Brine shrimp cytotoxicity of crude methanolic extract and various fractions of *Zizyphus jujuba*

Table 6.16 Brine shrimp (*Artemia salina*) cytotoxicity of the oils of *Zizyphus jujuba*

Dose (µg/ml)	**No. of Shrimps**	**No. of Survivors**	**% killed**	**LD_{50} (µg/ml)**	**Standard Drug**	**LD_{50} (µg/ml)**
1000	30	17	43.33	1387.20	Etoposide	7. 4625
100	30	23	23.33			
10	30	29	3.33			

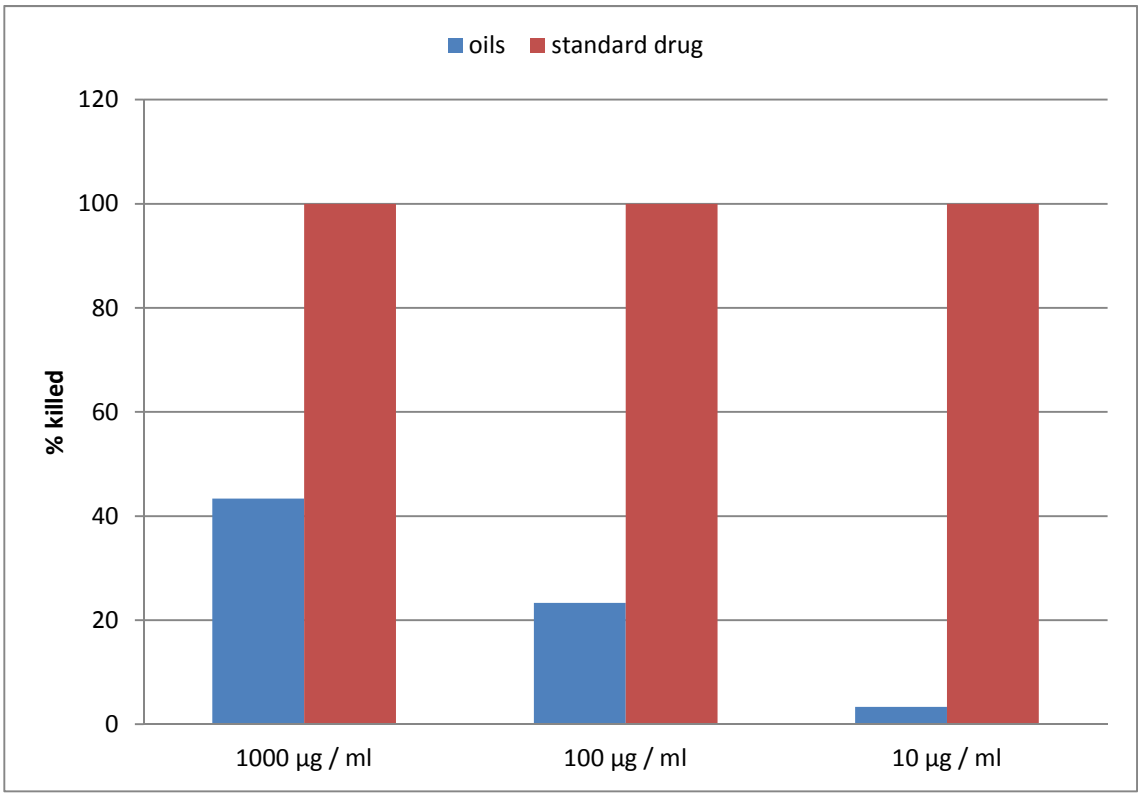


Fig 6.23 Brine shrimp (*Artemia salina*) cytotoxicity of the oils of *Zizyphus jujuba*

6.2.6 Haemagglutination activity

The lectins are found ubiquitously in plants and other organisms. Their ability to differentiate between different carbohydrates moiety on cell surfaces and in solution, have promoted speculations on their physiological role. They are used for mutants isolation, characterization and isolation of glycoconjugates [117] and for agglutination of erythrocytes to find the blood type and can be used for an estimation of the number of virus particles [118]. Keeping in view the diverse role of lectins, haemagglutination activity of *Z. jujuba* was carried against RBC's of all blood groups of humans **(Table 6.17)**. All dilutions of the test samples showed no haemagglutination activity against any blood group. The extracts from *Z. jujuba* were unable to agglutinate RBC's of the human blood, indicating that this specie of *Rhamnaceae* lack phytolectins.

Table 6.17 Haemagglutination activity of the crude methanolic and various fractions of *Zizyphus jujuba* against ABO blood groups

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

- = no agglutination, + = weak, ++ = moderate, +++ = strong

6.2.7 Anti-Termite Activity

Against the *Heterotermes indicola*, the anti-termite activity of crude methanolic extract, $CHCl_3$ and aqueous fractions of *Z. jujuba* were carried out at "Termites lab at Nuclear Institute of Food and Agriculture (NIFA), Peshawar, Khyber PukhtoonKhwa, Pakistan. All the experiments were performed in triplicates. The results are given in **Table 6.18.**

Termites are playing an important role in aeration of soil and decomposition of wood. But can also cause economic loss when they destroy wood and wooden products of human homes, building materials, forests etc [109].

The crude methanolic extract of *Z. jujuba* took 3 days to kill all termites of the experiment. On day 1, 20 and on day 2, 23 termites were killed by this fraction, on average.

The experiment for the anti-termite activity of the $CHCl_3$ fraction extended for three days. Day 1 results indicated that 12 termite, on average, was dead. On the next days, 16 and on the last day of the experiment no termite was alive.

The experiment for the aqueous fraction extended for four days. On day 1, an average of 5 termites was killed. On days 2, 7 and day 3, 16 termites were killed. On the last day of the experiment all termites were found dead.

Table 6.18 Antitermite activity of the crude methanolic extract, chloroform and aqueous fraction of *Zizyphus jujuba*

Sample	**No. of Termites**	**Day**	**Average Termites killed**
Crude methanolic extract	25	1	20
		2	23
		3	25
$CHCl_3$		1	12
		2	16
		3	25
Aqueous		1	5
		2	7
		3	16
		4	25

6.2.8 NITRIC OXIDE FREE RADICAL SCAVENGING ASSAY

During the metabolism in the living system free radicals are produced which can cause damage to biomolecules like DNA, proteins and tissues leading to various diseases like extensive lyses and degenerative diseases [198]. Many synthetic drugs are used to reduce these oxidative damages but these drugs are not free from side effects. The other option to combat with these damages is the consumption of natural food products and traditional medicines which contains antioxidants constituents. Recently various natural antioxidants have been reported from different natural sources [199-201].

As the importance of natural antioxidants cannot be ignored, we screened the test samples for possible Nitric Oxide (NO) radical scavenging at different concentrations (0.3, 0.6, 0.9, 1.2 and 1.5 mg / ml). The results are displayed in **Table 6.19** and **Figures 6.24-6.28.**

At the concentration of 0.3 mg / ml and 0.6 mg / ml, as shown in **Fig 6.24** and **Fig 6.25**, all the test samples showed low NO free radical scavenging activity. At 0.9 mg / ml, the *n*-hexane fraction showed 34.93% activity and rest of the test samples exhibited low activity at this concentration (**Fig 6.26**). As we increased the concentration to 1.2 mg / ml, the crude methanolic extract, *n*-hexane and $CHCl_3$ fraction exhibited activity of 32.53, 38.69 and 30.30% respectively. The EtOAc and aqueous fractions showed low activity of 27.05 and 22.60% respectively (**Fig 6.27**). Moving to 1.5 mg / ml the order of activity of the test samples were: *n*-hexane 46.57% > crude methanolic extract 35.95% > $CHCl_3$ 33.39% > EtOAc 32.87% > aqueous 26.88% (**Fig 6.28**).

From the above results it is concluded that the NO free radical scavenging activity of the crude methanolic extract and various fractions of *Z. jujuba* is concentration dependent.

Table 6.19 Nitric oxide free radical scavenging activity of the crude methanolic extract and various fractions of *Zizyphus jujuba*

Concentration of sample (mg/ ml)	Crude Met. Ext	n-hexane	$CHCl_3$	EtOAc	Aqueous
0.3	13.52	15.92	11.4	12.67	9.7
0.6	17.63	22.9	22.06	16.95	16.26
0.9	20.71	34.93	24.31	20.54	18.15
1.2	32.53	38.69	30.30	27.05	22.60
1.5	35.95	46.57	33.39	32.87	26.88

Standard: Vitamin C was used as a standard at concentration of 47.87 µg / ml

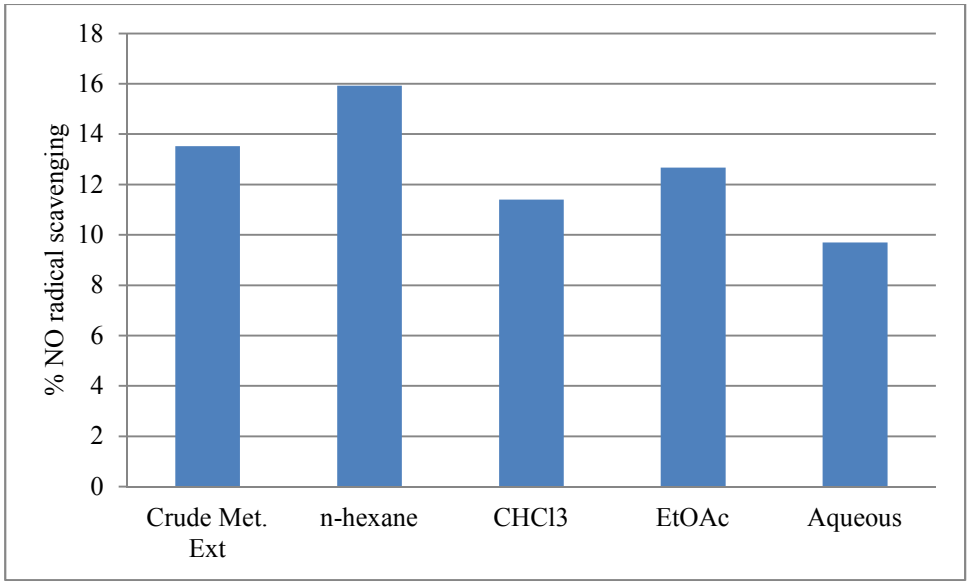


Fig 6.24 NO free radical scavenging assay of the crude methanolic extract and fractions of *Zizyphus jujuba* at concentration of 0.3 mg / ml

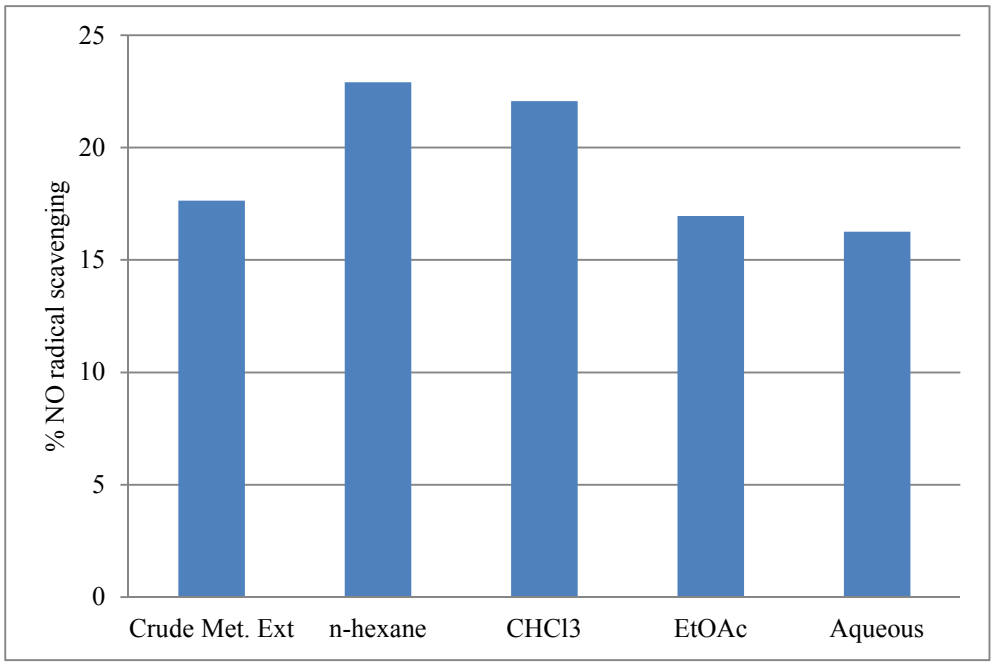


Fig 6.25 NO free radical scavenging assay of the crude methanolic extract and fractions of *Zizyphus jujuba* at concentration of 0.6 mg / ml

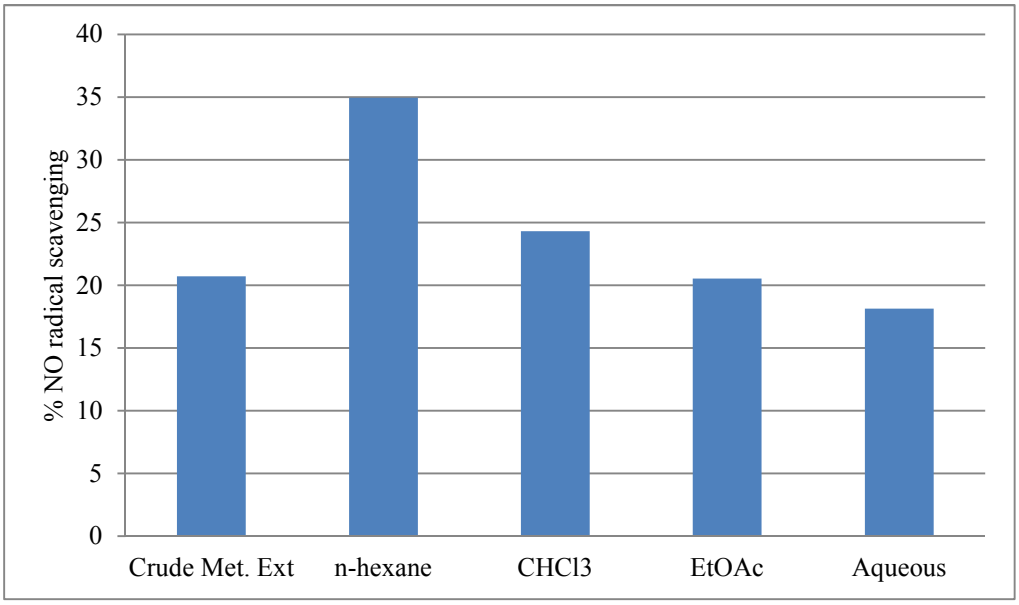


Fig 6.26 NO free radical scavenging assay of the crude methanolic extract and fractions of *Zizyphus jujuba* at concentration of 0.9 mg / ml

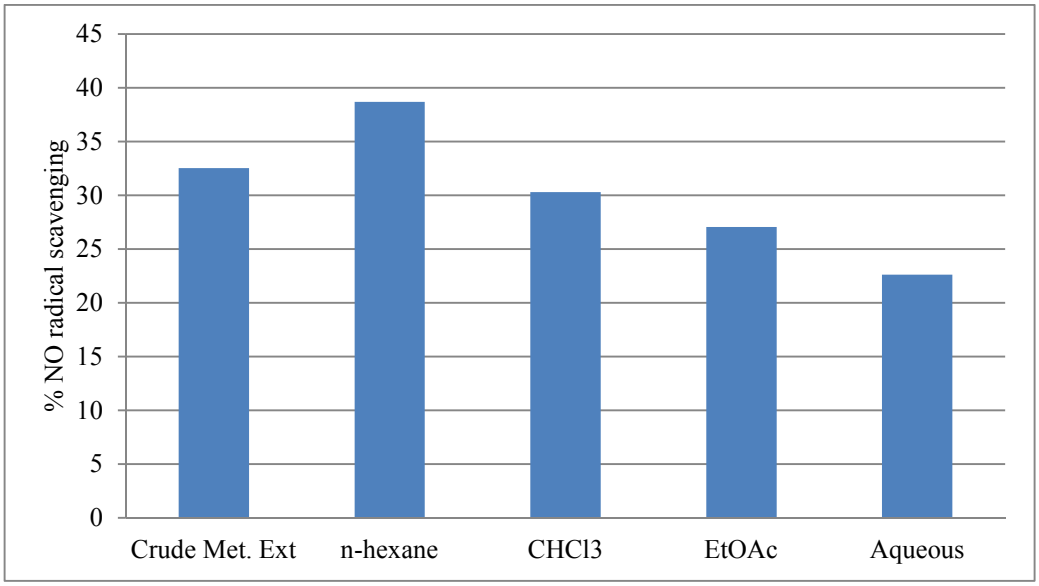


Fig 6.27 NO free radical scavenging assay of the crude methanolic extract and fractions of *Zizyphus jujuba* at concentration of 1.2 mg / ml

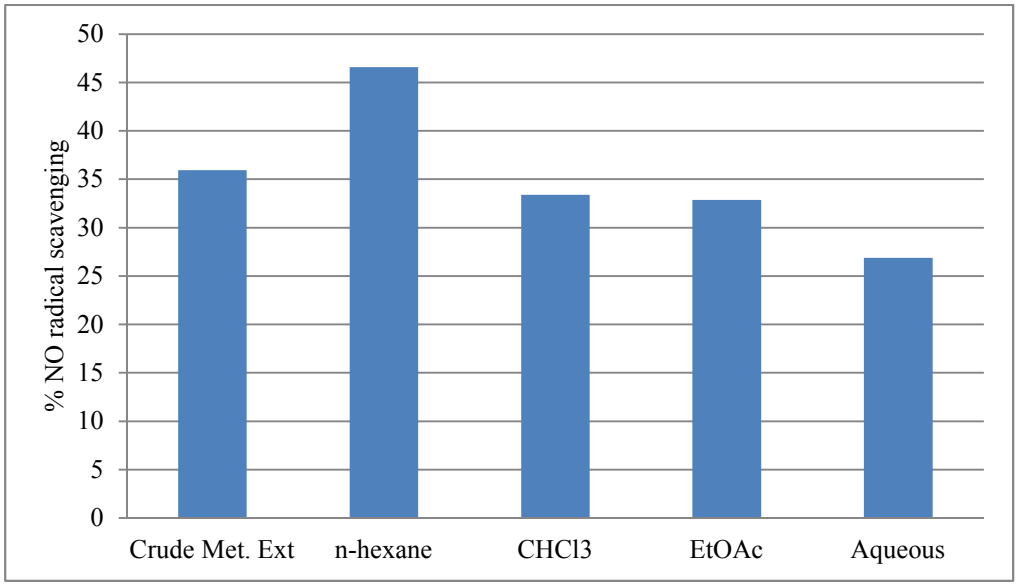


Fig 6.28 NO free radical scavenging assay of the crude methanolic extract and fractions of *Zizyphus jujuba* at concentration of 1.5 mg / ml

6.2.9 EFFECTS ON RABBIT'S JEJUNUM PREPARATION

Spasmolytic activity

Effects of crude methanolic extract of *Z. jujuba* on spontaneous contraction of rabbit's jejunum preparation are summarized in **Figures 6.29-6.31**. There is dose dependent fall in spontaneous activity starting from 0.03 mg / ml – 10 mg / ml. The crude methanolic extract relaxed the spontaneous activity up to 91% at dose of 10 mg / ml with EC_{50} value of 5.2$\pm$1.07 (4.7-6.7). Similar results are shown by the crude methanolic extract on KCl (80 mM) induced contraction with EC_{50} value of 5.1$\pm$0.77 (4.7-6.2). This suggests that the relaxing (anti-spasmodic) effects may be through calcium channel blocking mechanism that requires further work for confirmation [79].

Table 6.20 Spasmolytic activity of crude extract of *Zizyphus jujuba*

Concentration of Crude extract (mg / ml)	Percent effect of control max. (Mean ± S.D, n= 3)
0.01	100
0.03	98
0.1	95
0.3	90
1	85.71
3	76.91
5	52.38
10	9.52

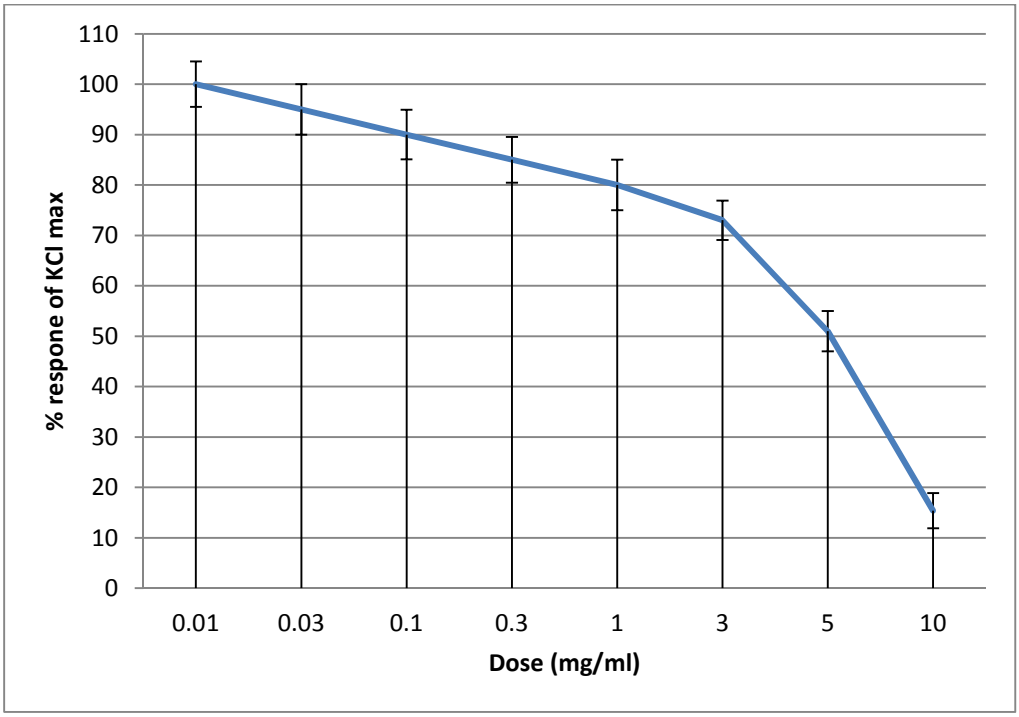


Fig 6.29 Spasmolytic activity of *Zizyphus jujuba* on KCl (80 mM) induced contraction on rabbit's jejunum preparation

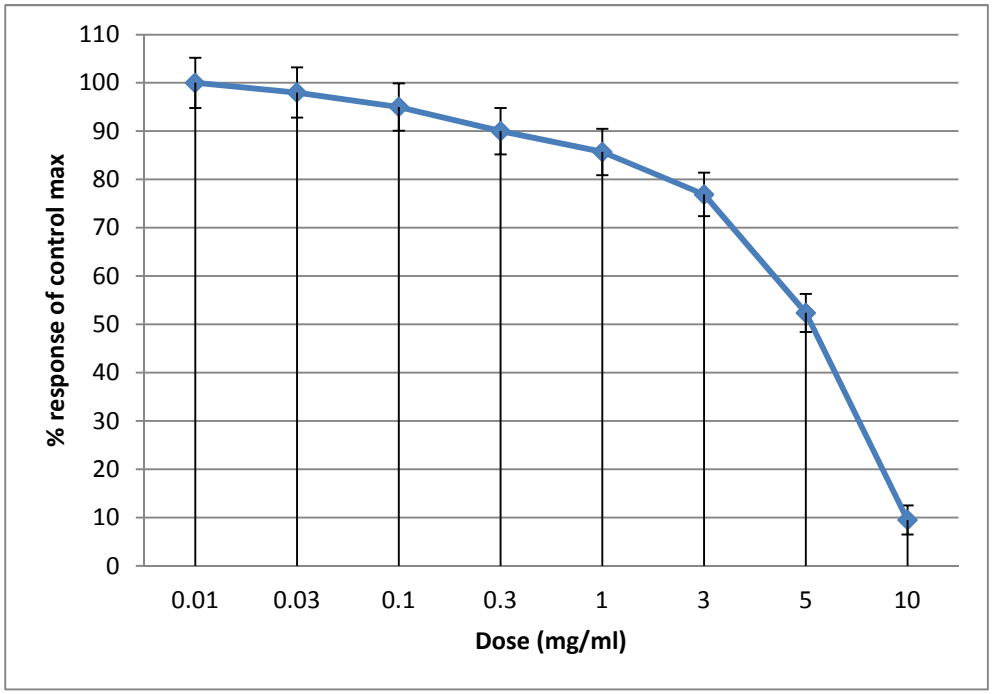


Fig 6.30 Spasmolytic activity of *Zizyphus jujuba* on spontaneous rabbit's jejunum preparation

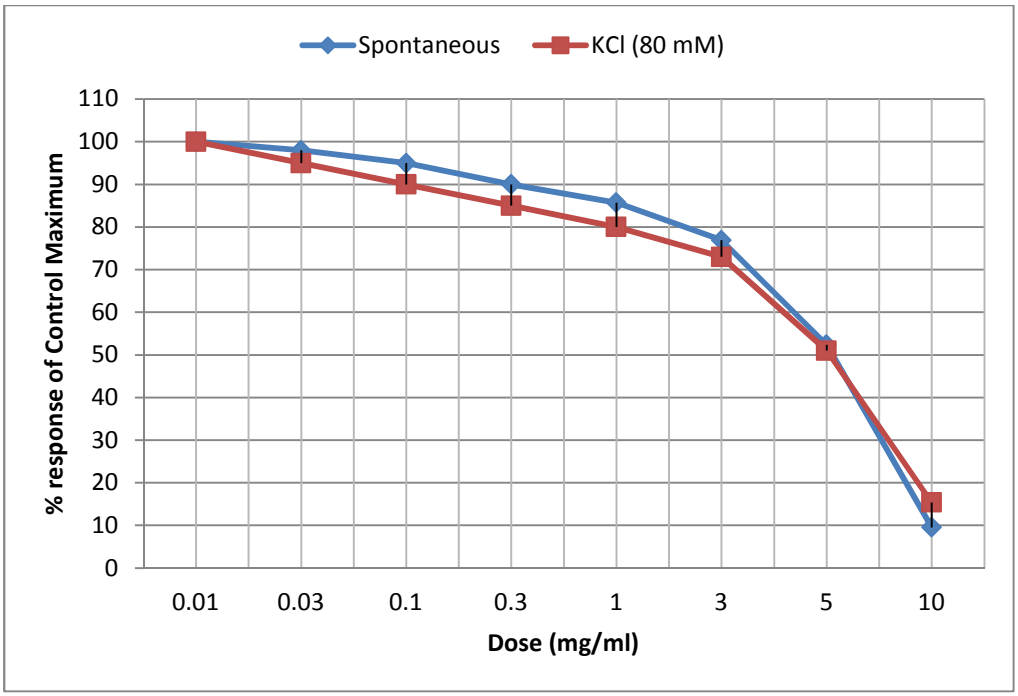


Fig 6.31 Spasmolytic activity of *Zizyphus jujuba* on spontaneous and KCl (80 mM) induced rabbit's jejunum preparation

Conclusions

The pharmacological / biological investigations of *Acacia modesta* revealed that the plant possesses significant antibacterial, insecticidal and anti-termite activities. Moderate activity was observed in case of nitric oxide (NO) free radical scavenging activity and haemagglutination. The plant showed low antifungal, phytotoxic, brine shrimp lethality and spasmolytic activities.

Four compounds i.e. Nonaeicosanol (**1**), Palmitone (**2**), 4-hydroxybenzoic acid (**3**) and Lupeol (**4**) were isolated from the ethyl acetate fraction. *A. modesta* was a new source for Nonaeicosanol (**1**) and Palmitone (**2**). The oils isolated from the *n*-hexane fraction were subjected to GC-MS analysis revealing **38** components.

The oils of *A. modesta* possessed significant brine shrimp lethality, moderate antifungal and phytotoxic and no antibacterial and insecticidal activity.

The pharmacological / biological investigations of *Zizyphus jujuba* revealed that the plant possesses significant antibacterial and anti-termite activities. Moderate phytotoxic, insecticidal and brine shrimp lethality activities were observed. The plant showed low antifungal, NO free radical scavenging and spasmolytic activities. No haemagglutination activity was shown by the crude methanolic extract and various fractions of the plant.

Two compounds i.e. 4-methoxydelbergeione (**5**) and betulinic acid (**6**) were isolated from the ethyl acetate fraction of the plant. Stigmasterol (**7**) was isolated from the chloroform and *β* –sitosterol (**8**) from the *n*-hexane fraction of the plant.

The oils of the plant possessed moderate brine shrimp lethality and low phytotoxic activities but did not showed antibacterial, antifungal and insecticidal activities.