Biological Evaluation of *Nepeta laevigata*, *Nepeta kurramensis* and *Rhynchosia reniformis*

Department of Plant Sciences
Faculty of Biological Sciences

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Biological Evaluation of *Nepeta laevigata*, *Nepeta kurramensis* and *Rhynchosia reniformis*

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Declaration

I hereby declare that the research work presented in this thesis is my own effort except where others acknowledged. No part of the thesis has previously been presented for any other degree.

Nisar Ahmad

I certify that the above statement is correct.

Prof. Dr. Zabta Khan Shinwari
Supervisor


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“With Him are the keys of the unseen, the treasures that none knoweth but He. He knoweth whatever there is on the earth and in the sea. Not a leaf doth fall but with His knowledge. There is not a grain in the darkness (or depths) of the earth, nor anything fresh or dry (green or withered) but is (inscribed) in a record clear (to those who can read).”

(VI. 59)

TO MY GREAT

PARENTS, HONORABLE

TEACHERS AND

BELOVED SPOUSE

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Nisar Ahmad

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LIST OF ABBREVATIONS

AA	Arachidonic acid
ABTS	2, 2-Azinobis (3-ethylbenzothiazoline-6-sulfonate)
AIDS	Acquired immune deficiency syndrome
APS	Ammonium per sulfate
ATP	Adenosine triphasphate
AGEs	Advanced glycation end products
BB	Broad band
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
CC	Column chromatography
COSY	Correlation spectroscopy
DAD	Diode array detectors
DEET	N, N-diethyle-meta-toluamide
DEPT	Distortionless enhancement polarization techniques
DCM	Dichloromethane
DCHF-DA	Dichlorodihydrofluorescein diacetate
DNA	Deoxyribonucleic acid
DPPH	2-2-Diphenyl-1-picrylhydrazyl
DTPA	Diethylenetriaminepentaacetic acid
ESI-MS	Electrospray ionisation mass spectrometry
FAB	Fast atom bombardment
FC	Flash chromatography
GAE	Gallic acid equivalent
GCMS	Gas chromatography mass spectrometry
GLC	Gas-liquid chromatography
HEJ-RIC	Haji ebraheem jamal research institute of chemistry
HMBC	Heteronuclear multibond correlation
HMQC	Heteronuclear multibond quantum correlation
HPLC	High performance liquid chromatography
HPTLC	High performance thin-layer chromatography

HR-EI-MS	High resonance electron impact mass spectrometry
IR	Infra Red
LCMS	Liquid chromatography mass spectrometry
LLC	Liquid-liquid chromatography
LC	Liquid chromatography
LDH	Lactate dehyrogenase
MDA	Melondialdehyde
MS	Mass spectrometry
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
MHz	Megahertz
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PAF	Platelet activating factor
PRP	Platelet rich plasma
PPP	Platelet poor plasma
ROS	Reactive oxygen species
RSA	Rapid susceptibility assay
SDA	Sabouraud dextrose agar
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TBHQ	T-butyl hydroxyquinone
TLC	Thin layer chromatography
TCA	Trichloro acetic acid
UV	Ultra violet
WHO	World health organization

Abstract

The methanol extracts and solvent soluble fractions of three selected plant species [*Nepeta laevigata, Nepeta kurramensis* and *Rhynchosia reniformis*] were evaluated for their antimicrobial, antiglycation, antiplatelet aggregation, antioxidant, cytotoxicity, phytotoxic activities, proximate compositions and isolation of natural products, for the intention of standardization and proper manage of bioactive principles in such heterogonous botanicals and encourage drug finding work with plants.

The antibacterial results of *Nepeta laevigata* showed that the *n*-butanol fraction exhibited potential activity (85 % inhibition) against *Escherichia coli* and *Proteus morganii* (83 % inhibition), while in *Nepeta kurramensis* chloroform fraction exhibited promising activity (89 % inhibition) against *Streptococcus cricetus*, and *Micrococcus flavas* (84 % inhibition). In *Rhynchosia reniformis,* only crude extract exhibited 100% inhibition against *Streptococcus cricitus* while ethyl acetate fraction showed (99 % inhibition) against *Micrococcus flavas*, *Streptococcus cricitus* (95 % inhibition), and *Proteus morganii* (90 % inhibition). In antifungal activities; chloroform and ethyl acetate fractions of *Nepeta laevigata* as well as chloroform fraction of *Nepeta kurramensis* were promising; while in *Rhynchosia reniformis* chloroform, *n*-hexane and methanolic extracts were significant inhibitors as compared to rest of fractions.

The fractions *n*-hexane and ethyl acetate of *Nepeta laevigata* demonstrated significant antiglycation activity with 71.26 % and 74.02 % inhibition and for *Nepeta kurramensis* 67.24 % inhibition was shown only by the *n*-hexane fraction. Among *Rhynchosia reniformis* fractions, ethyl acetate and chloroform displayed a significant antiglycation profile with 70.27 % and 76.02 % inhibition against protein glycation, respectively, while *n*-hexane fraction illustrated a moderate 64.06 % inhibition.

In antiplatelet actions of *Rhynchosia reniformis* the water fraction was only dynamic against platelet activating factor **(**PAF) stimulate human platelet aggregation. Methanolic and *n*-butanol fractions exhibited potential activities against arachidonic acid (AA) and PAF while other fractions were insignificant and in platelet aggregation activity of

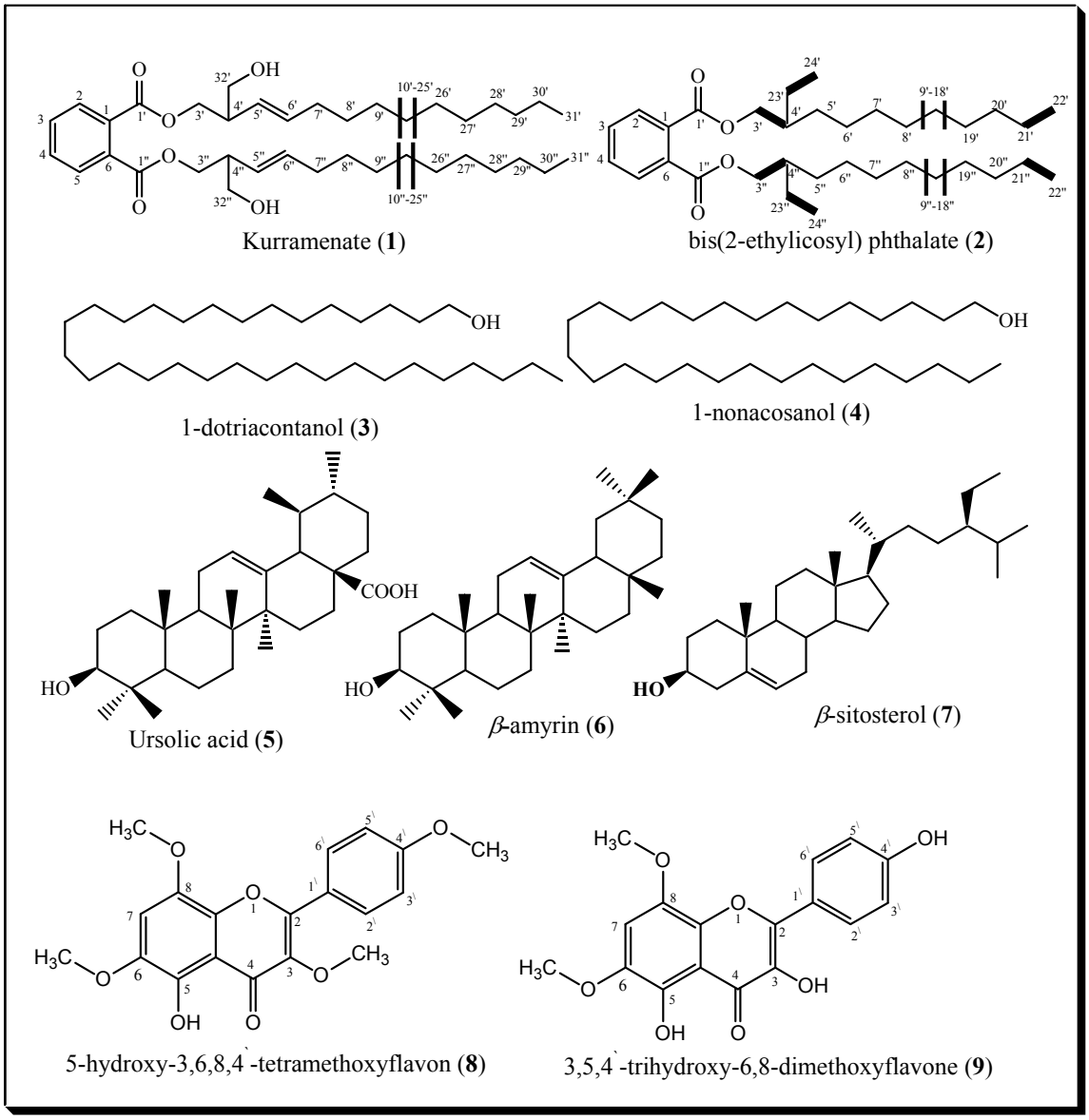
Nepeta plants crude extract, chloroform and *n*-hexane fractions showed significant activity.

The antioxidant potential of the crude extract and various fractions of *Rhynchosia* was assessed by using free radicals such as hydroxyl ($^{\bullet}OH$) radical, total reactive oxygen species (ROS) and to scavenge authentic nitric oxides ($ONOO^-$). The Chloroform-soluble fraction was noted to contain a maximum amount of poly phenolic compounds acting as antioxidants (with IC_{50} values of 30.24±0.07, 93.89±0.09 and 23.50±0.02, for scavenge authentic $ONOO^-$, total ROS and to $^{\bullet}OH$ radical, respectively) and established to be more efficient than crude extract and the other successive fractions. The ethyl acetate fraction of *Nepeta laevigata* exhibited stronger antioxidative profile with IC_{50} values of 88.37±0.05 and 30.42±0.04, 55.97±0.09 for total ROS, $^{\bullet}OH$ radical and to scavenge authentic $ONOO^-$, respectively. Similar antioxidant profile was observed in *Nepeta kurramensis.*

The fraction *n*-butanol of *Rhynchosia reniformis* showed potential cytotoxic activities while rests of the fractions were found to be inactive. No lethal activities were exhibited by *Nepeta* plants fractions. Surprisingly none of the fraction of all three plants under investigation exhibited phytotoxic activities.

The proximate composition of the selected medicinal plants was assessed and analyzed according to AOAC methods. All the selected species were found to be a good source of ash, proteins and fats which can contribute greatly towards nutritional requirements and adequate protection against microorganism and other diseases.

As a result of phytochemical investigation of *Nepeta kurramensis* seven pure compounds (**1-7**) were isolated out of which compound (**1**) is a new isolate and is named as Kurramenate after the plant species name. Two known flavonoids (**8** and **9**) have been provided by *Rhynchosia reniformis*. The compounds structures were confirmed on the basis of preliminary chemical tests and nuclear magnetic resonance (NMR) studies.



Compounds **1, 2, 8** and **9** exhibited moderate antimicrobial and antioxidative activities. The biologically active crude extracts, fractions and pure compounds can be used for the curing of microbial diseases, glycation, artery and oxygen stress allied syndromes. However, further *in vivo* examination of crude extracts, fractions and pure compounds will discover its potential pharmaceutical actions.

INTRODUCTION chapter 1

Plant Kingdom is one of the valuable offerings from Almighty ALLAH. Ever since long time man is constantly using plants for food, shelter and health preservations. The humanity has frequently emergent methodical and profitable significance in healing plants, owing to their massive monetary potential and prevalent artistic adequacy of plant based product (Shinwari *et al*., 2006). Medicinal plants recommend a prosperous foundation of structural biodiversity in the shape of diverse bioactive natural products, which has contributed a superior task in the sighting of drugs [Choudhary and Rehman, 2004]. The plants used in pills hold ample array of substances that can be used in the treatment of persistent as well as infectious diseases. Those substances which impede the development of microorganisms or eradicate them are precise entrant for emergent novel drugs for treatment of diverse carcinogenic diseases. In the rural areas of various developing countries the medicinal plants are usually used as a medicine [Martinez *et al*., 1996**].**

The nature has been a source of therapeutic agents for thousands of years and a striking number of standard drugs have been obtained from usual sources, many based on their use in conventional medicines. Diverse curative plants have been used for years in every day life to treat disease all over the planet. The extensive use of herbal remedies and healthcare measures, such as those described in prehistoric texts like the Vedas and the Bible, has been noted to the incidence of natural products with healing properties. In reality, plants create a diverse choice of bioactive molecules, making them an affluent resource of diverse types of medicines [Shinwari *et al.,* 2013]. The plants, as sources of remedial compounds, have sustained to engage in creating a leading role in the safeguarding of human physical condition since primeval times [Faromb, 2003]. Above 50% of all current experimental medicines are natural products and their derivatives [Stuffness and Douros, 1982]. Natural products play a central role in drug improvement programs in the pharmaceutical industries [Baker *et al*., 1995]

The affiliation stuck between plants, human and drugs resulting from plants portray the past of mankind. Plants are essential basis of natural drugs. Primal man ongoing to differentiate dietary and pharmacologically vigorous plants for his endurance. The chronological analyses of conventional medicines expose that man has always been in search of cure against disease and anxiety. Hence, therapeutic plants have been in use for the elimination of human sufferings since long. In the light of their reputable beneficial value, the pharmaceutical industries started to use crude extracts of medicinal plants for sorting and making medicines. As such plants can be described as the chief starting place of medicines [Rehman and Chaudhry, 2001]. The preparation of food substance, coloring matters, fibers, toxins, medicines and stimulants are the examples of actions as old as mankind. Medicinal plants contain substances which are known to modern and ancient civilizations for their vigorous medicinal properties [Pillipson, 1999].

Medicinal plants are known as a key foundation of drugs for the healing of different vigor disorders. There are sums of some 300000 species of advanced plants in the world. The plants have massively contributed to the health requests of man when no synthetic medicines were accessible. Approximately 25% of all approved medicines in the developing world contain ingredients resulting from medicinal plants. The world has witnessed the emergent scientific and marketable benefit in medicinal plants, principally owing to their huge financial prospective and extensive intellectual adequacy of plant-based products. A catalog of medicinal plants compile by world health organization (WHO) on the basis of literature from 91 countries, including the traditional manuscript on Ayurvedic and Unani medicine, lists 21000 species of medicinal plants. According to the pharmacognosist Norman Farnsworth of the University of Illinois, USA, there are 89 plant derivative drugs set in the industrialized world [Shinwari *et al.,* 2006].

According to WHO approximation, 80-85% of the world's 5.76 billion population in the developing countries relies on herbal remedies for their vital fitness concern requirements. In the last 100 years, several imperative discoveries were done in the recently promising skill of chemistry. Medicinal plants were examined cautiously, and numerous substances were extracted from special parts of plants. From the dawn of the

human survival, many generations of people have carefully experimented with and revealed a broad range of plants that alleviate a variety of diseases [Leak and Thomas, 1975]. This is how the discoveries of drugs from nature were developed.

In the middle of 20th century, scientists started to use synthetic medicines over natural medicines for curing various diseases. However due to emergence of various side effects of synthetic drugs, trend to use medicinal plants to cure various diseases is becoming popular [Awal *et al*., 2004; Jiang *et al*., 2006]. Natural products from medicinal plants are known to be chemically balanced, effective and least injurious with none or much reduced side effects as compared to synthetic medicines. As per description of WHO, in subcontinent, plant producing medicines have been used widely since long time [Shinwari *et al*., 2006]. According to assessment accomplished by WHO, traditional healers treat 65 % patients in Srilanka, 60 % in Indonesia, 75 % in Nepal, 85 % in Mayanmer, 80 % in India, and 90 % in Bangladesh. In Pakistan, 60 % of the people, particularly in villages are receiving health care by traditional practitioners (Hukama), who stipulate herbal measures [Ahmed *et al*., 2004].

Plants are natural factories which persistently developed valuable chemicals in the mainly outlay proficient technique and with defined supreme selectivity. While the hub of the 19th century plentiful bioactive constituents have been isolated and structurally characterized by the logical and hi-tech methods. In addition to these bioactive principals some are being used as the active ingredients of the modern drug for the healing of assorted diseases. Several of these medicinally useful usual constituents have served as replica systems, or escort compounds, for synthetic organic chemists to intend and create a multiplicity of parallel synthetic compounds in laboratories. Attempts to characterize the bioactive principals have just gained universal impetus in various pharmaceutical/ neutraceutical formulations and food dispensation applications [Hamburger and Hotettmann, 1991, Cowan, 1999, Bussmann and Sharon, 2006].

The edible medicinal plants widely recognized as the neutraceutical or nutrimedicines assist imperative part in the cure or manage of a range of disorders. Still, their approach of deed, scale of triumph and general curb of their therapeutic supremacy depend on their chemical structures, which literally utter the functionality at the cellular point. The utilization of medicinal plants for the treatment of different diseases is not new. Several dynamic constituents in the chemically synthesized medicines were previously resulting from medicinal plant species. Those plants, which hold healing properties, have been used for human safety since the dawn of the culture. Many herbal remedies independently or in amalgamation with divergent formulation such as leaf grind, pastes, decoction, mixture are used for particular ailments [Klayman *et al*., 1984, Zafar and Mujeeb, 2000].

Plants are imperative supply of standard drugs. The chronological psychoanalysis of usual medicine reveals that man has for eternity been in explore of therapy beside disease and distress. Consequently, medicinally important plant species have been in use for the purge of creature sufferings since primordial period. In the glow of their reputable salutary worth, the pharmaceutical industries ongoing to use crude extracts of curative plants for industrialized drugs. As such plants can be considered as the chief source of drugs [Ali and Azhar, 2000]. The import of medicines is rising day by day and receiving attractiveness right through world [Jia and Zhang, 2007].

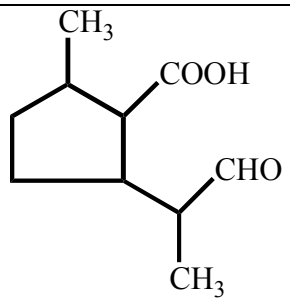
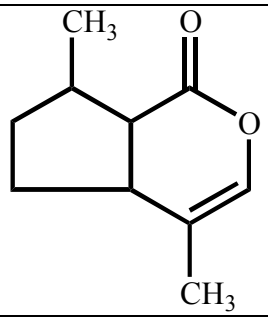
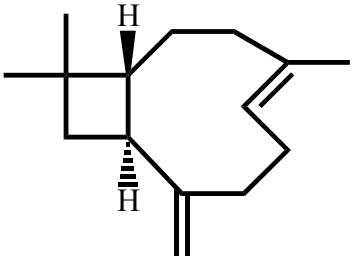
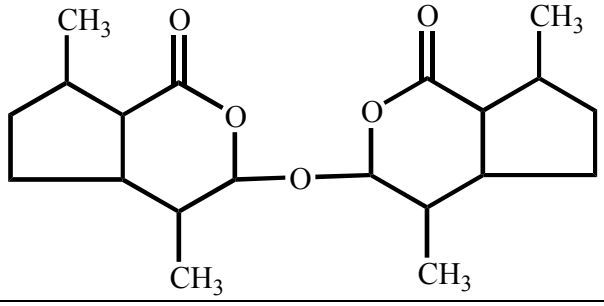
The awareness regarding plant metabolites and to acquire synthetic medicines for the curing of different diseases has guided the purification and isolation of chemical products with beneficial indications. The majority of the conventionally used healing plants have vigorous compounds that retort with hereditary material and whose carcinogenic properties is blown up by individual differences in the dose reaction relation, cell spoil recover and metabolic squalor [Morton, 1980]. The vital oils and extracts of numerous plant species have turned into trendy in current years. Crucial oils and rudimentary extracts of a number of medicinal plants showed fascinating biological actions such as insecticidal, antibacterial and antifungal, anticonceptive, spasmolytic, antiplasmodial, and antioxidant [Lemos *et al*., 1990, Ferdous et al., 1992, Fouzia *et al*.,

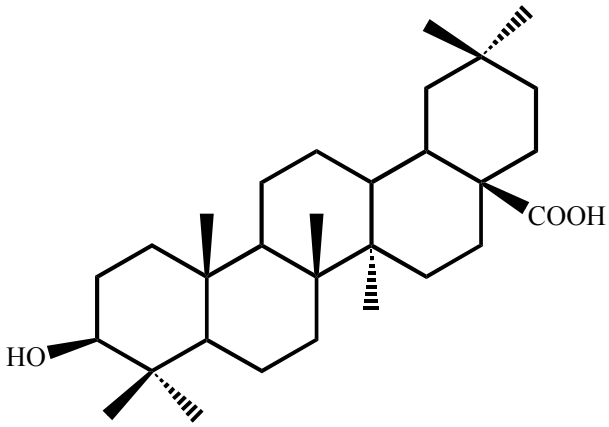
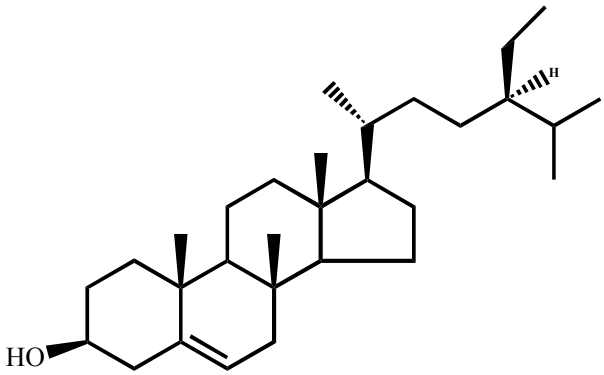
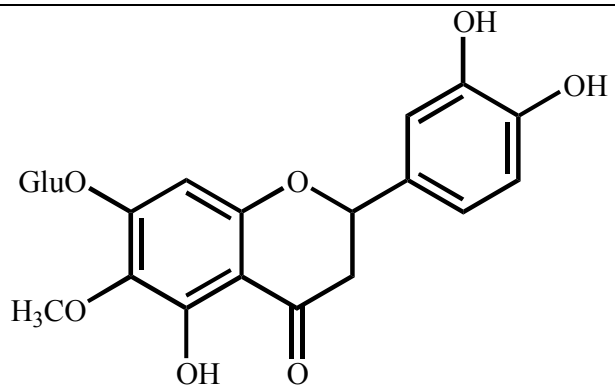
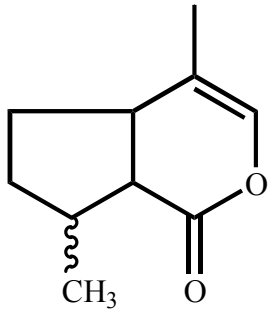
1993 and Santos *et al*., 1998]. Environment is a munificent supplier of a huge quantity of compounds displaying enthralling structural multiplicity with diverse biological actions. Civilization appreciates this contribution from nature but the pleasure with these medications frequently fallout in a number of undesired side effects as well as resistance of multi-drugs. Thus, it left over significant to augment the expansion of new drugs with fewer side effects and enhanced activity against a range of diseases [Passarella *et al*., 2006].

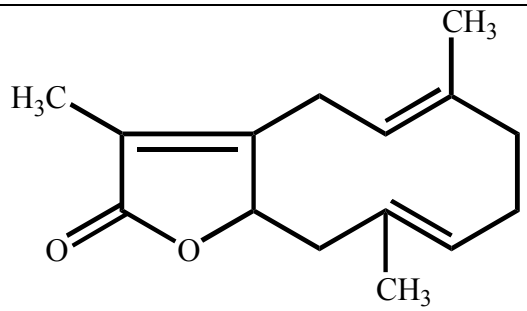
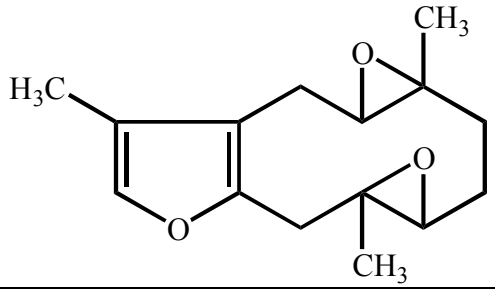
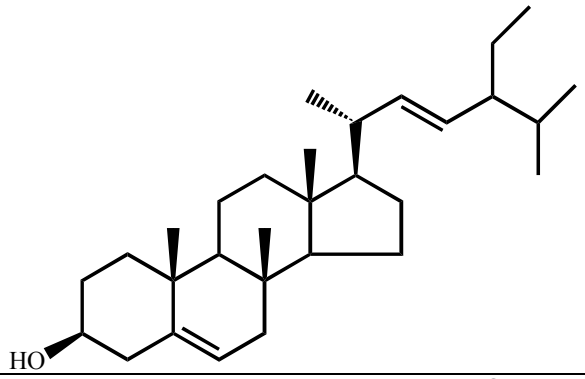
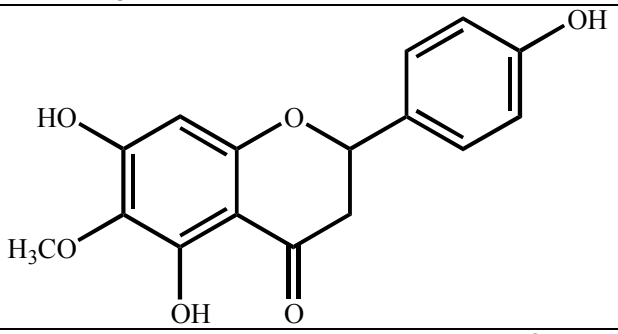
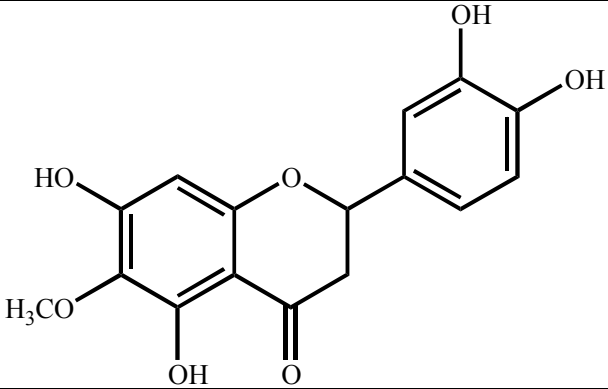
The medicinal properties of plants have been probe in the light of up-to-date scientific expansion all over the world, due to their heady pharmacological tricks and stumpy toxicity [Sharma *et al*., 1992; Vaquero *et al*., 2010]. Medical plants are extensively used in the cure of assorted diseases. Plant extracts and their diverse formulations in the healing and easing of several diseases in folk remedy have been dated back to the primeval times. Further, some natural products also subsist in vegetables, fruits and beverages [Oz, 2010].

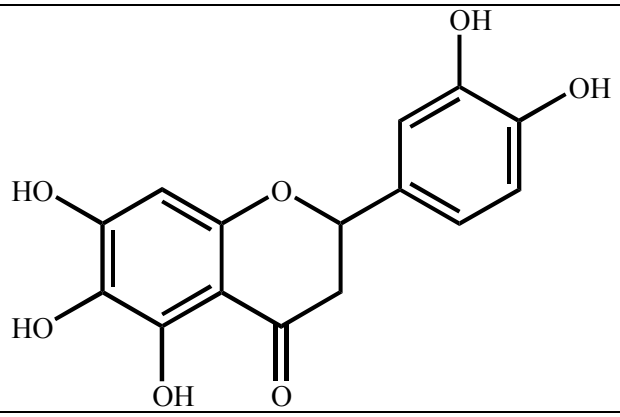
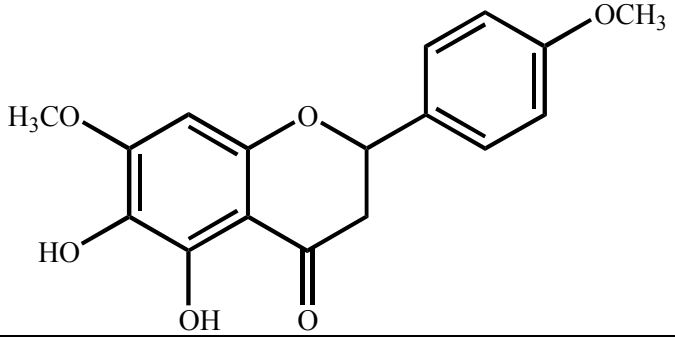
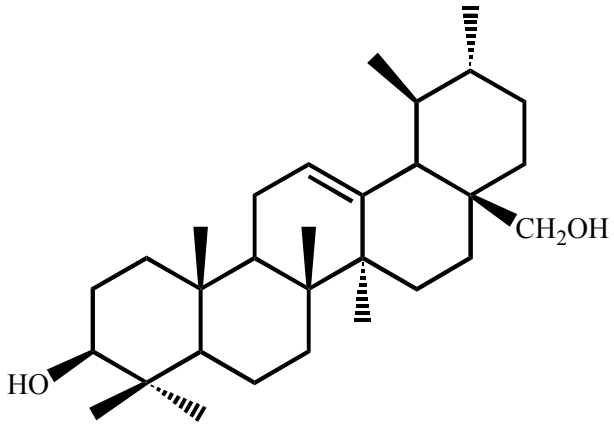
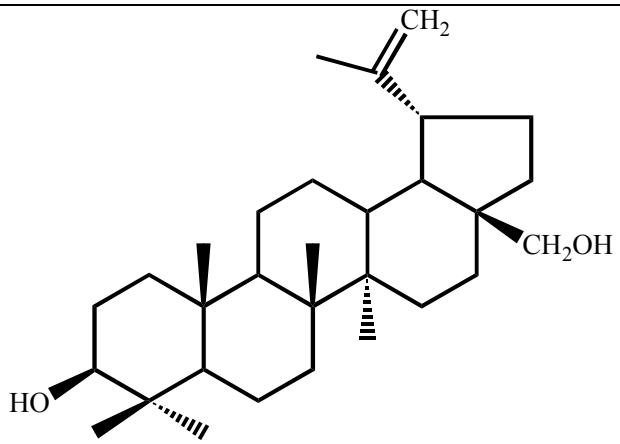
The isolation of compounds from therapeutic plant was not started until the 19th century. The first isolation was carried out by French scientists Caventou and Pelletier revealed quinine from *Cinchona* bark. Such discoveries opened the importance of plants that scientist takes interest to isolate lots of compounds from plants to treat various illness. Day by day man discovered million of compounds that it becomes a cosmic theme. Natural products, such as extracts from plant, either as pure compounds or as raw extracts, give infinite opportunities to discover novel medicine because of the supreme accessibility of chemical range [Cosa *et al.,* 2006, Ikan, 1991].The structures of some therapeutic compounds isolated from genus *Nepeta* and *Rhynchosia* are given in table (Table **1.1**).

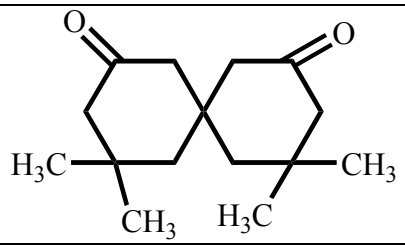
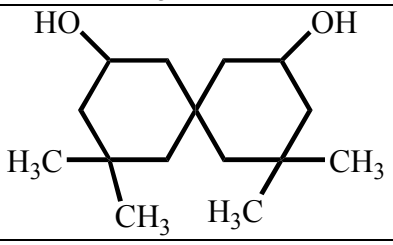
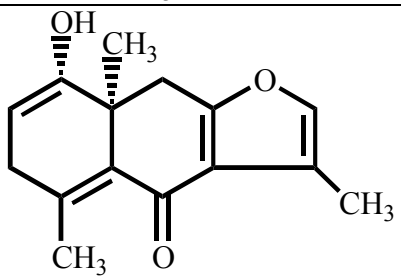
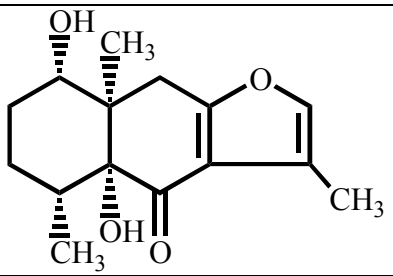
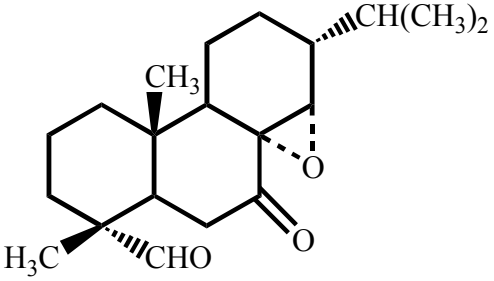
Table 1.1: List of chemical compounds, structure and their sources isolated from the genus *Nepeta* and *Rhynchosia*.

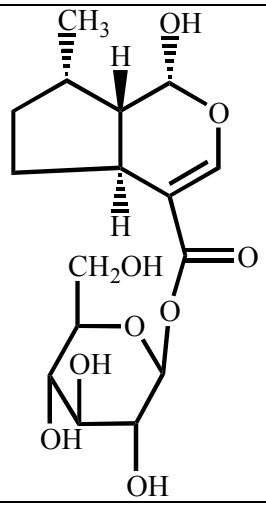
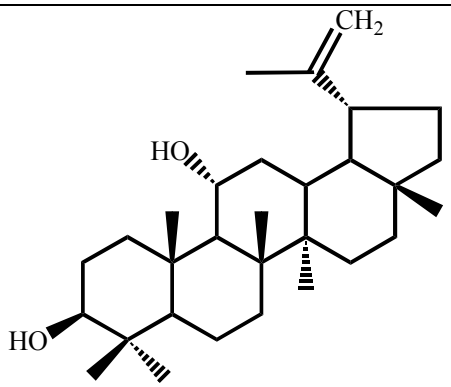
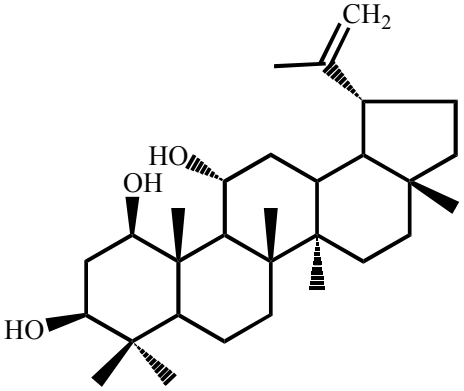
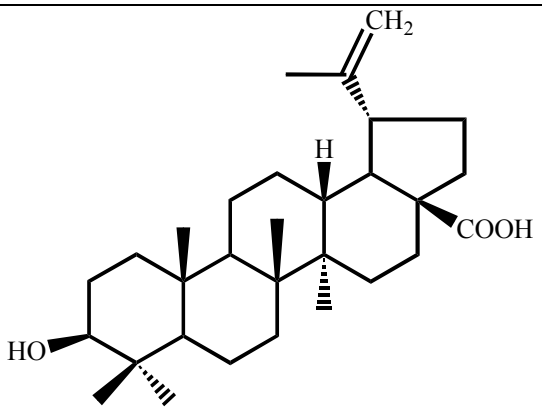
Name of the compound	Structure	S/No.
Nepetalic acid Source: *N. cataria* (McElvain *et al.,* 1941)		1
Nepetalactone Source: *N. cataria* (McElvain *et al.,* 1941)		2
β-caryophyllene Source: *N. cataria* (McElvain *et al.,* 1942)		3
Nepetallic anhydride Source: *N. cataria* (McElvain *et al.,* 1942)		4

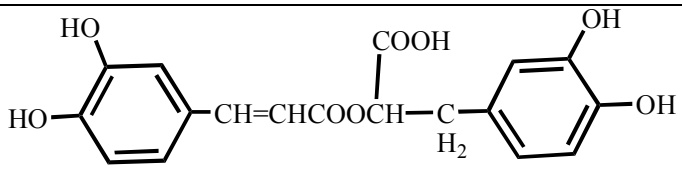
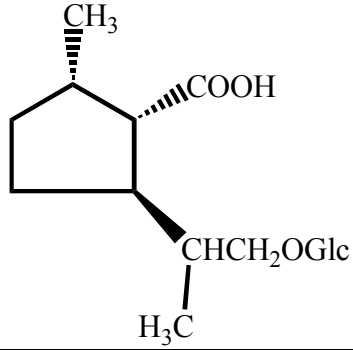
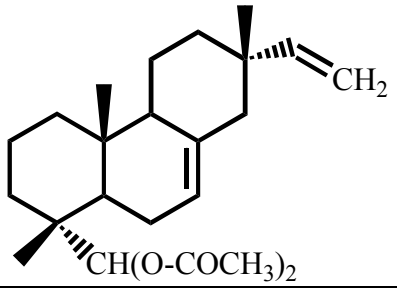
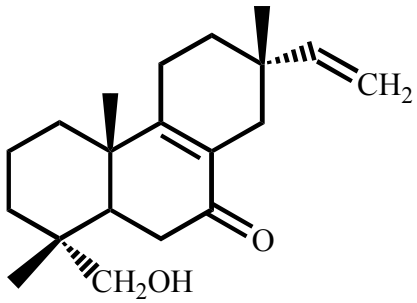
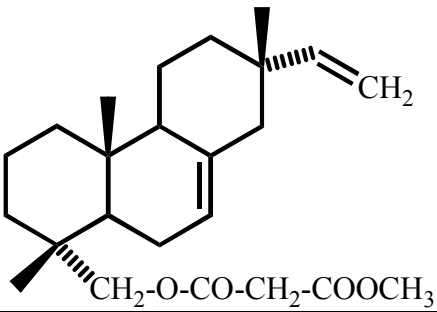
Oleanolic acid Source: *N. hindostana* (Gopinath *et al.,* 1962)		5
β- Sitosterol Source: *N. hindostana* (Siddiqui and Ahsan 1967)		6
Aglucone nepetin Source: *N. hindostana* (Krishnaswamy *et al.,* 1970)		7
Epinepatalactone Source: *N. cataria* (Sastry *et al.,* 1972)		8

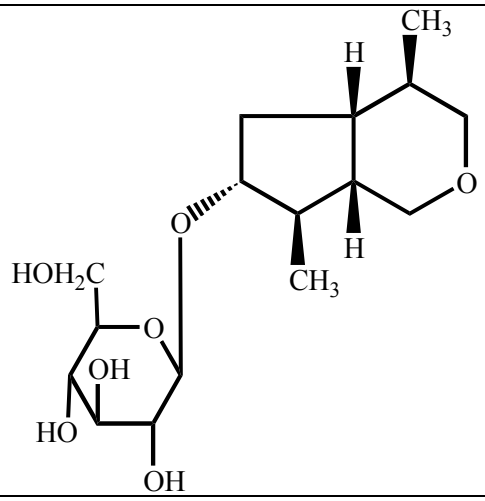
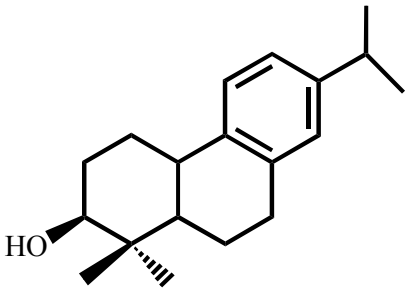
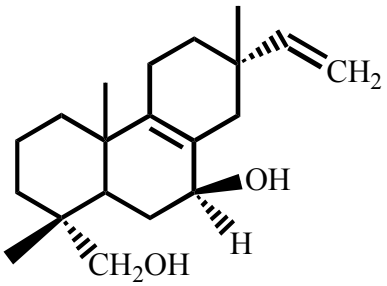
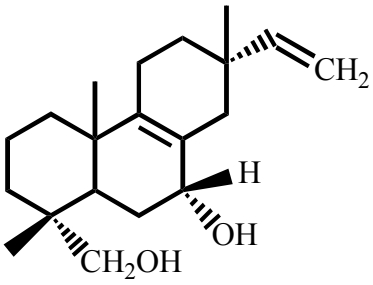
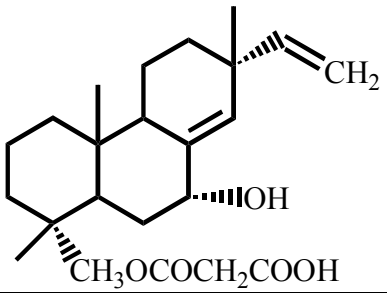
Glechomanolide Source: *N. hederacea*. (Stahl and Datta 1972)		9
Glechomafuran Source: *N. hederacea*. (Stahl and Datta 1972)		10
Stigmasterol Source: *N. hederacea*. (Seshadri and Sharma 1973; Hussain *et. al.,*2010)		11
Dinatin Source: *N. hederacea*. (Seshadri and Sharma 1973)		12
Nepetin Source: *N. hederacea*. (Seshadri and Sharma 1973)		13

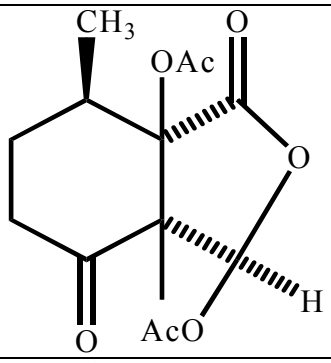
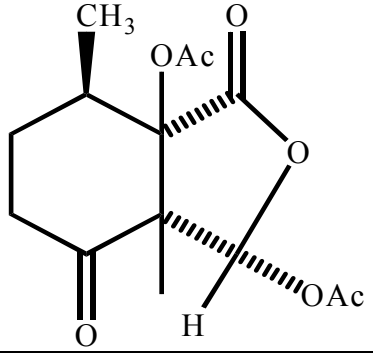
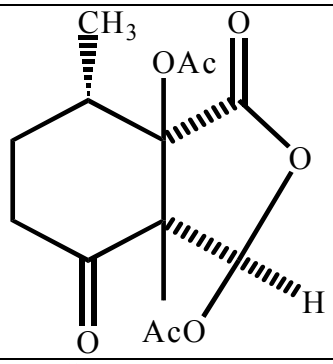
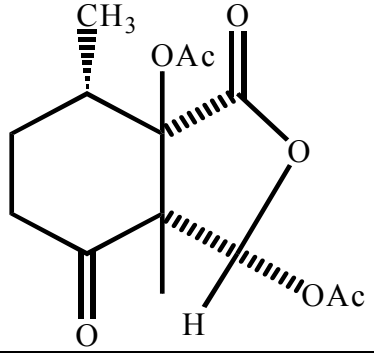
6- hydroxyluteolin Source: *N. hederacea*. (Seshadri and Sharma 1973)		14
Name: 7, 4'-*O*-dimethylscutellarein Source: *N. hederacea*. (Seshadri and Sharma 1973)		15
Uvaol Source: *N. aragonensis* (Voncastenn *et al.,* 1973)		16
Betulin Source: *N. aragonensis* (Voncastenn *et al.,* 1973)		17

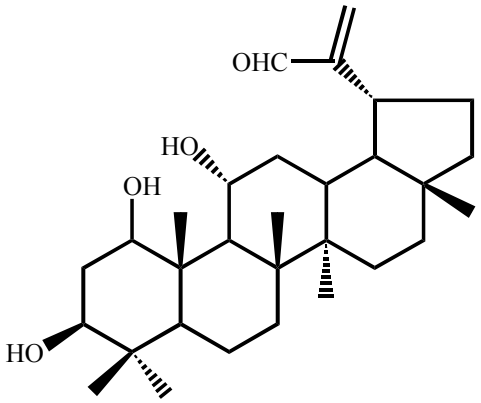
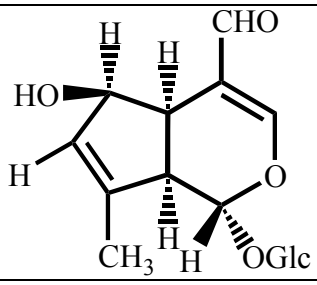
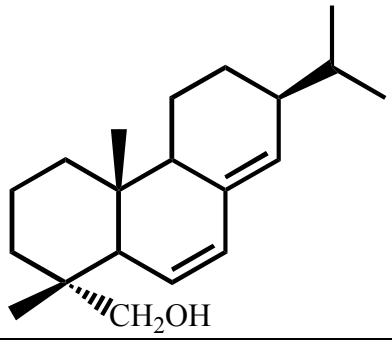
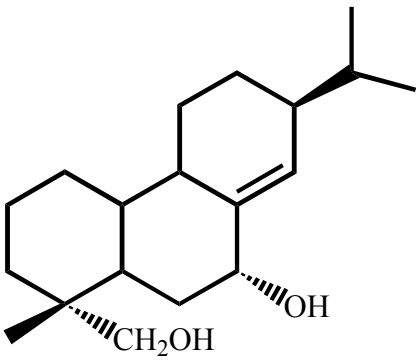
2, 2, 8, 8 tetramethylspiro [5, 5] undecane-4, 10-dione Source: *N. teydea* (Gonzalez *et al.,* 1974)		18
2, 2, 8, 8 tetramethylspiro [5, 5] undecane-4, 10-diol Source: *N. teydea* (Pyrek *et al.,* 1976)		19
Nehipetol Source: *N. hindostana* (Pyrek *et al.,* 1976)		20
Nehipediol Source: *N. hindostana* (Pyrek *et al.,* 1976)		21
Epoxy ketone Source: *N. granatensis* (Sundararaman and Herz 1977)		22

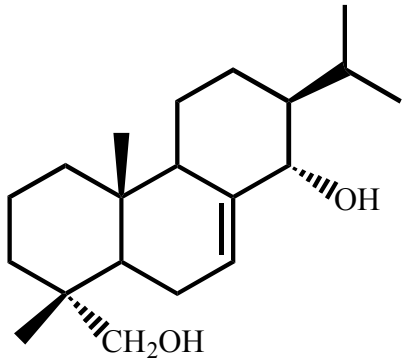
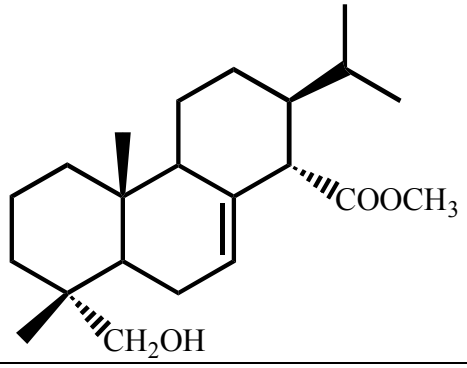
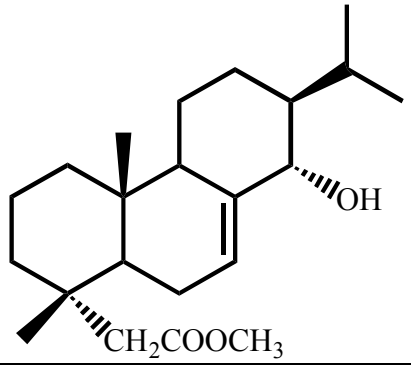
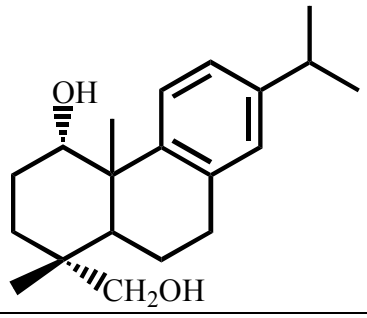
Nepetolglucosyl ester Source: *N. cataria* (Tagawa and Murai 1980)		23
Nepeticin Source: *N. hindostana* (Ahmad *et al.,* 1981)		24
Nepetidin Source: *N. hindostana* (Ahmad *et al.,* 1982)		25
Betulinic acid Source: *N. transcaucasia* (Tomas-Barberan *et al.,* 1982)		26

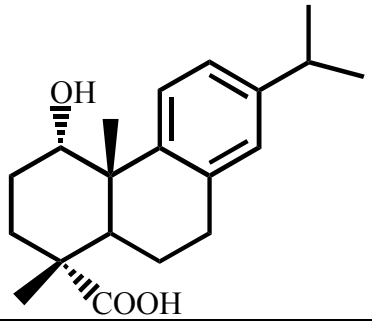
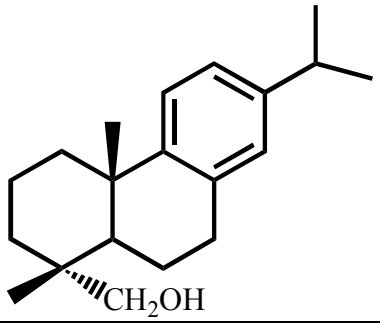
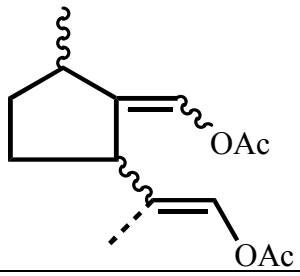
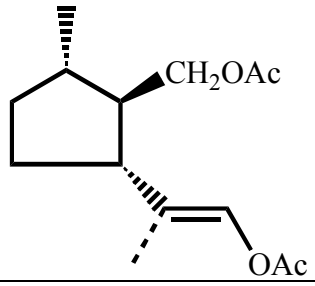
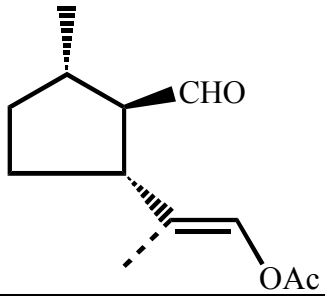
Rosmarinic acid Source: *N. hedaracea* (Okuda *et al.,* 1986)		27
Nepetariaside Source: *N. cataria* (Mural *et al.,* 1987)		28
Diisopimaryl malonate Source: *N. tuberosa* ssp. *reticulata* (Teresa *et al.,* 1987)		29
7-Oxo-isopimara-8, 15-dien-18-ol Source: *N. tuberosa* ssp. *reticulata* (Teresa *et al.,* 1987)		30
Isopiymaryl methyl malonate Source: *N. tuberose* ssp. *reticulata* (Teresa *et al.,* 1987)		31

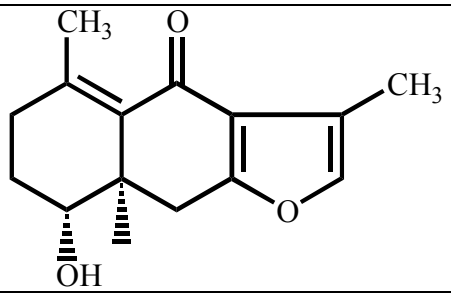
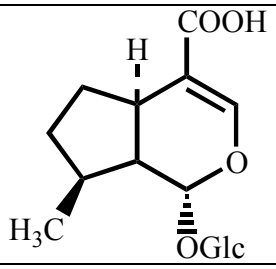
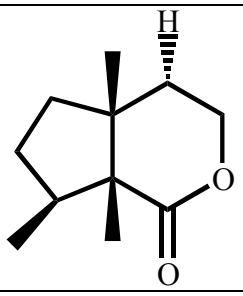
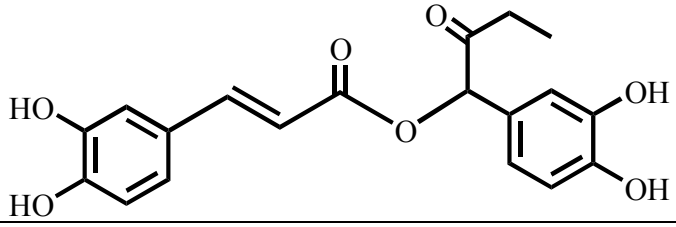
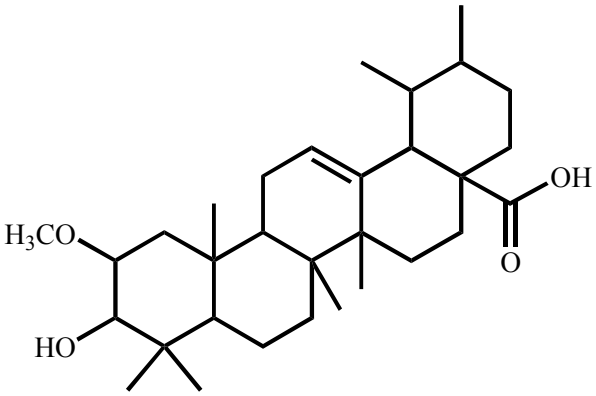
Nepetaside Source: *N. cataria* (Xie *et al.,* 1988)		32
Abietatrien-3*β*-ol Source: *N. tuberosa* ssp. *reticulata* (Urones *et al.,* 1988)		33
Isopimara-8, 15-dien-7*β*, 18-diol Source: *N. tuberosa* ssp. *reticulata* (Urones *et al.,* 1988)		34
Isopimara-8, 15-dien-7α, 18-diol Source: *N. tuberosa* ssp. *reticulata* (Urones *et al.,* 1988)		35
7α-Hydroxy-isopimara-8 (14), 15- dien- 18-yl malonate Source: *N. tuberosa* (Urones *et al.,* 1988)		36

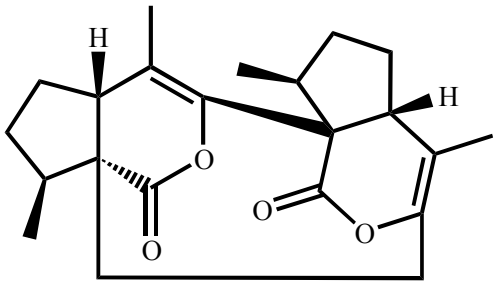
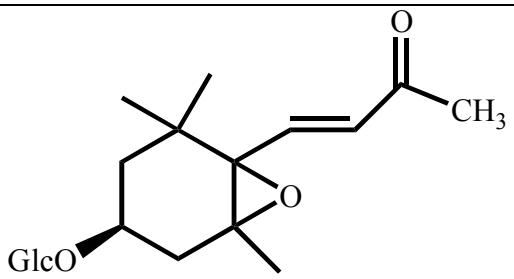
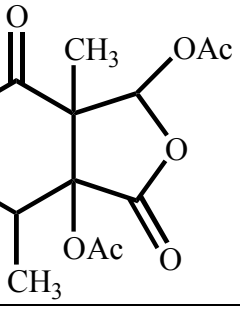
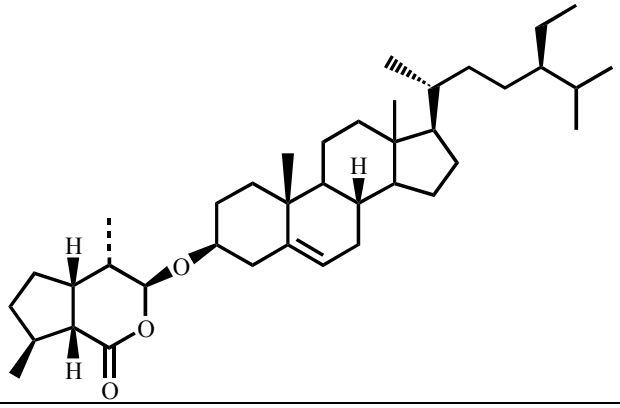
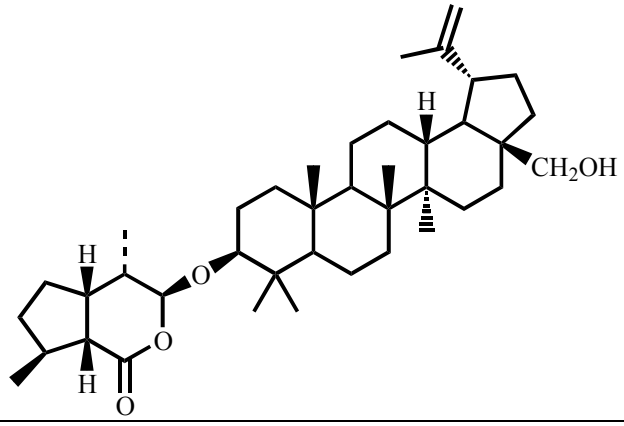
1, 5-Dioxo-2-oxa-3(R), 9(R)-diacetyl 4(R), 8(R)-dimethylhexahydroindane Source: *N. tuberosa* ssp. *reticulata* (Urones *et al.,* 1988)		37
1, 5-Dioxo-2-oxa-3(S), 9(R)-diacetyl-4(R), 8(R)-dimethylhexahydroindane Source: *N. tuberosa* ssp. *reticulata* (Urones *et al.,* 1988)		38
1, 5-Dioxo-2-oxa-3(R), 9(R)-diacetyl-4(R), 8(S)-dimethylhexahydroindane Source: *N. tuberosa* ssp. *reticulata* (Urones *et al.,* 1988)		39
1, 5-Dioxo-2-oxa-3(S), 9(R)-diacetyl-4(R), 8(S)-dimethylhexahydroindane Source: *N. tuberosa* (Urones *et al.,* 1988)		40

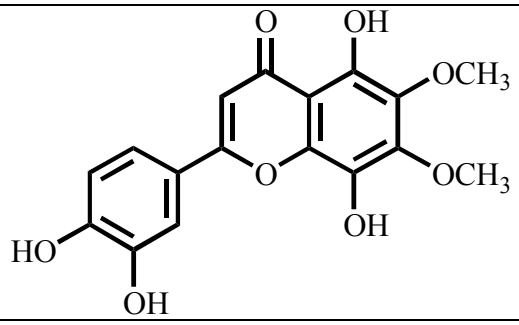
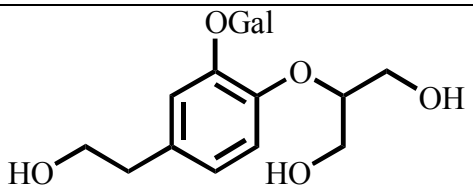
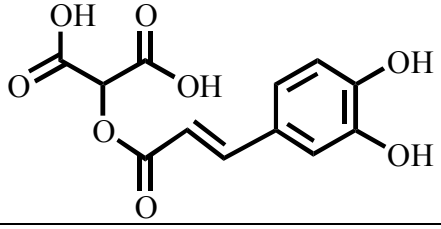
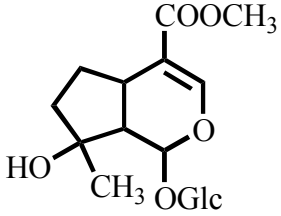
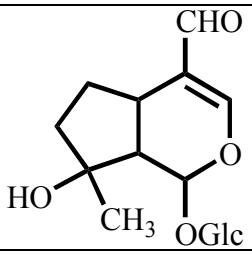
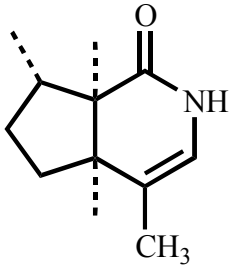
Nepehinal Source: *N. hindostana* (Ahmad *et al.,* 1993)		41
Nepetacilicioside Source: *N. cilicia* (Takeda *et al.,* 1996)		42
Netiol Source: *N. teydea* (Fraga *et al.,* 1998)		43
Netidiol A Source: *N. teydea* (Fraga *et al.,* 1998)		44

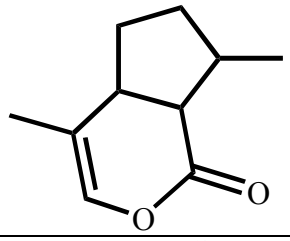
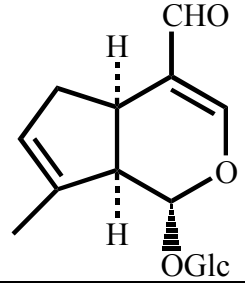
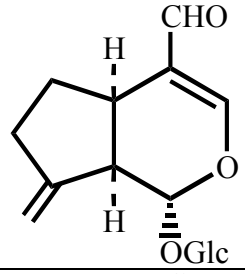
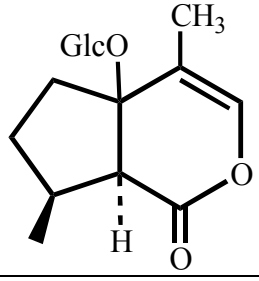
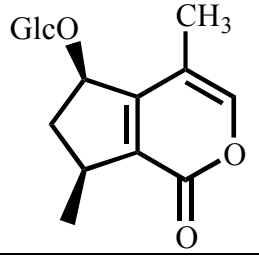
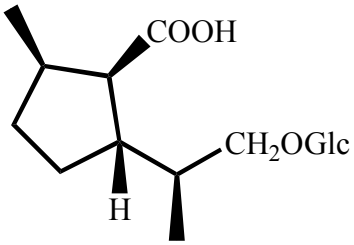
Netidiol B Source: *N. teydea* (Fraga *et al.,* 1998)		45
Netidiol B, 14α acetate Source: *N. teydea* (Fraga *et al.,* 1998)		46
Netidiol B, 18 acetate Source: *N. teydea* (Fraga *et al.,* 1998)		47
Teideadiol Source: *N. teydea* (Ulubelen and Topc 1992)		48

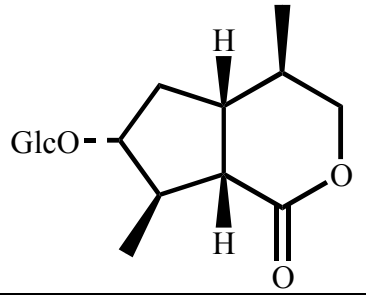
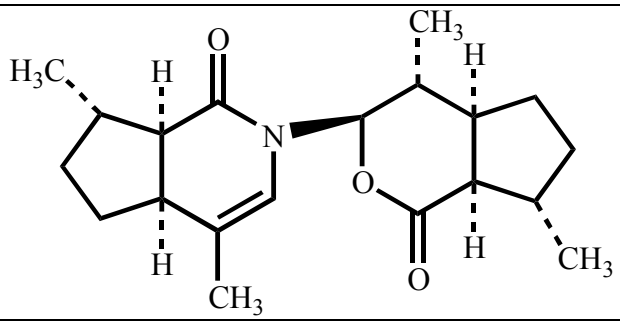
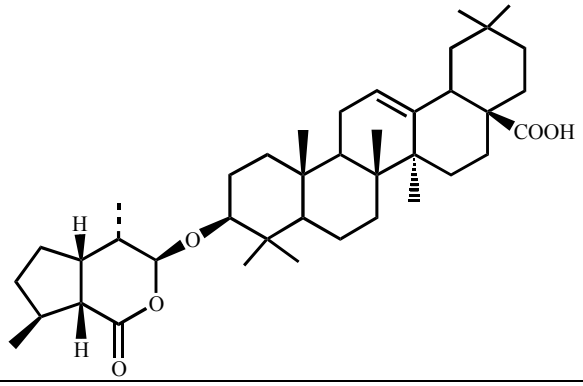
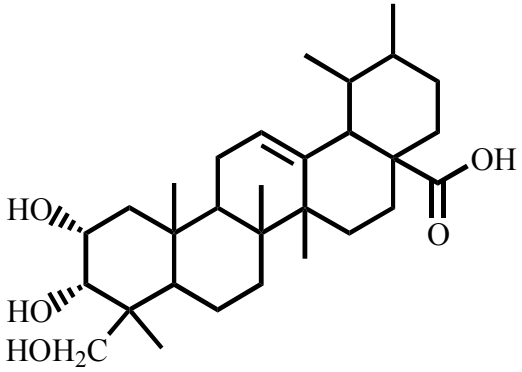
Teidic acid Source: *N. teydea* (Fraga *et al.,* 1994)		49
Pomiferin A Source: *N. teydea* (Ulubelen *et al.,* 1992; Fraga *et al*., 1994)		50
Iridodial dienol diacetate Source: *N. leucophylla* (Bottini *et al.,* 1992)		51
Dihydroiridodial diacetate Source: *N. leucophylla* (Bottini *et al.,* 1992)		52
Iridodial *β*-monoenol acetate Source: *N. leucophylla* (Bottini *et al.,* 1992; Saxena and Mathela 1996)		53

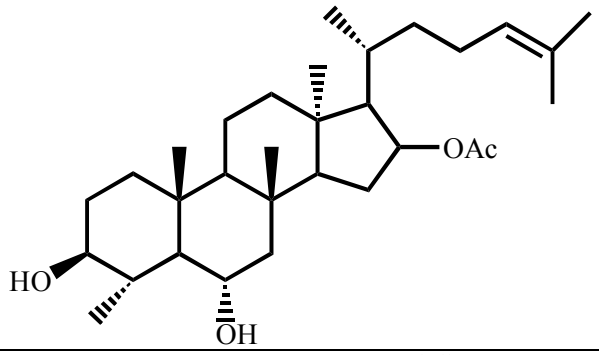
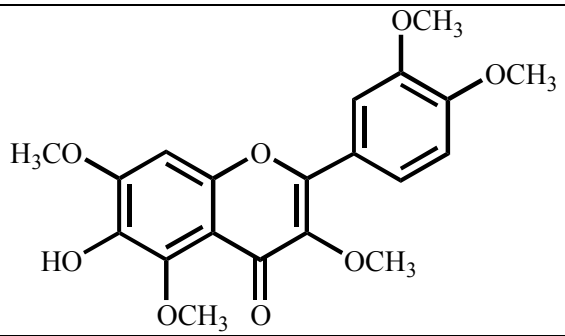
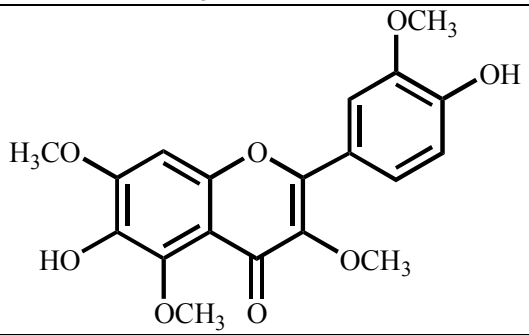
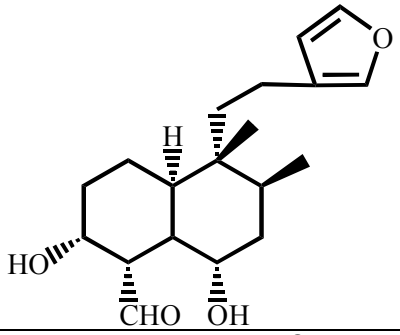
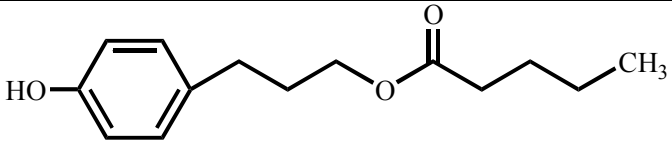
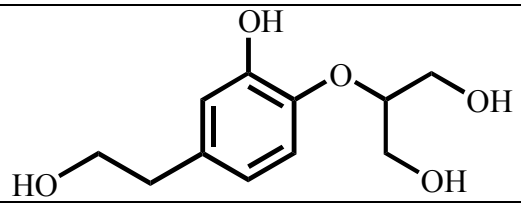
Curcolone Source: *N. hindostana* (Hikino 1968)		54
1,5,9-epideoxyloganic acid Source: *N. cataria* (catnip) (Tagawa et al., 1980, Guvenalp *et al.,* 2009, Nakamura *et al.,* 2000)		55
Dihydronepetalactone Source: *N. cataria* (catnip) (Regnier *et al.,* 1967; Ishii *et al.,* 2004)		56
Ethyl rosmarinate Source: *N. prattii* (Hou *et al.,* 2006;Zhou *et al.,* 2006, Zhang *et al.,* 2006)		57
Nepetoic acid Source: *N. eriostachia* (Bhandari *et al.,* 1990; Kokdil *et al.,* 1999)		58

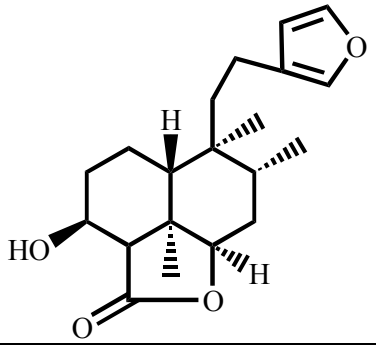
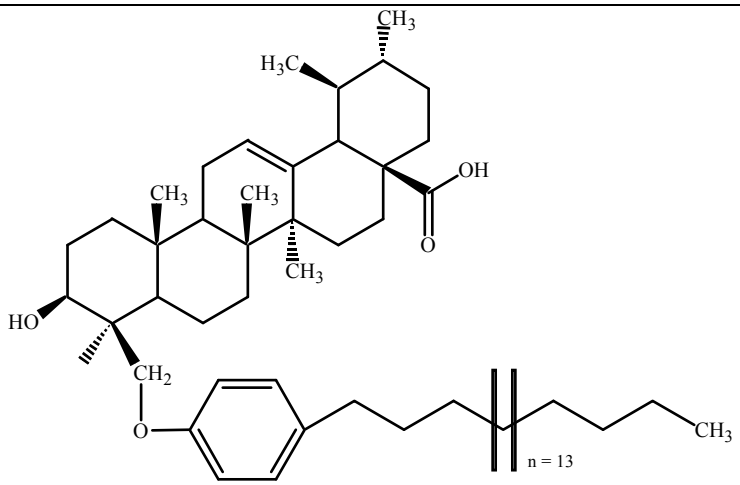
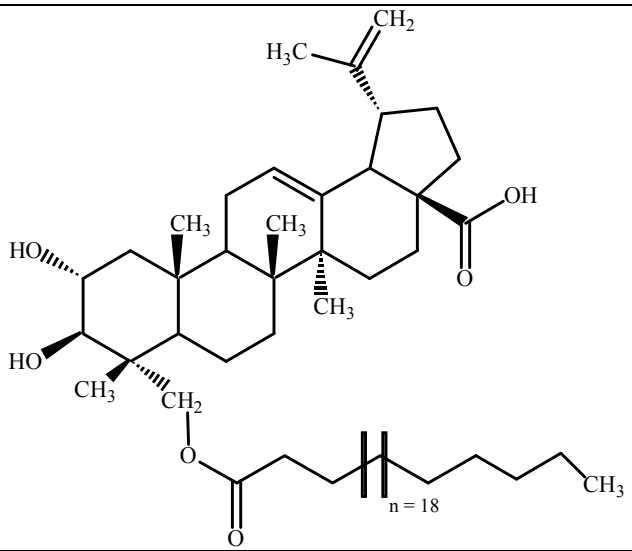
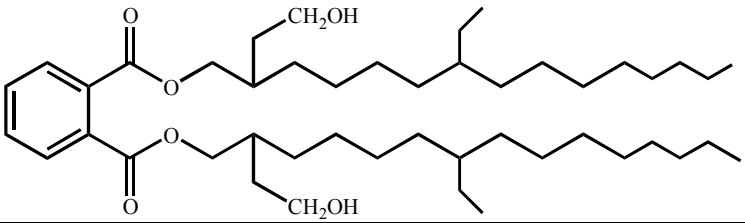
3, 9' : 3', 9-Dinepetalactone Source: *N. nuda* (Kokdil *et al.,* 1999)		59
Icariside B_2 Source: *N. cadmea* (Urones *et al.,* 1988; Takeda *et. al*., 1988)		60
3,7a Diacetoxyhexahydro-2-oxa-3a,7-dimethyl-1,4-indanedione Source: *N. tuberose* (Urones *et al.,* 1988)		61
Hexahydro-4,7-dimethyl-3-(stigmast-5-en-3-yloxy)-cyclopenta[c]pyran-1(3H)-one Source: *N. caesarea* (Heller *et al.,* 1992)		62
Hexahydro-3-(28-hydroxy-20(29)-lupen-3-yl)-4,7-dimethylcyclopenta[c]pyran-1(3H)-one Source: *N. caesarea* (Heller *et al.,* 1992)		63

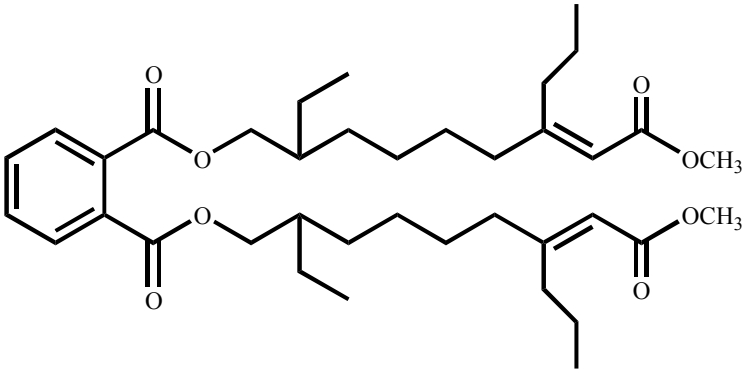
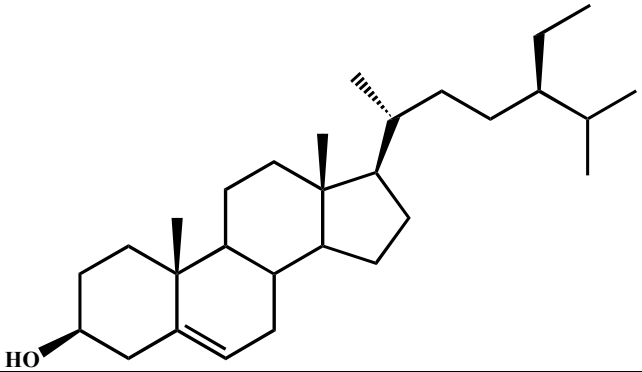
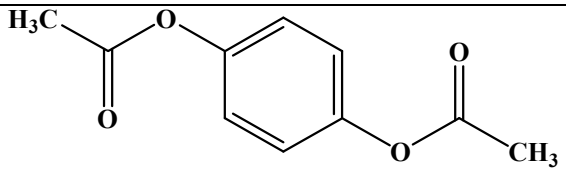
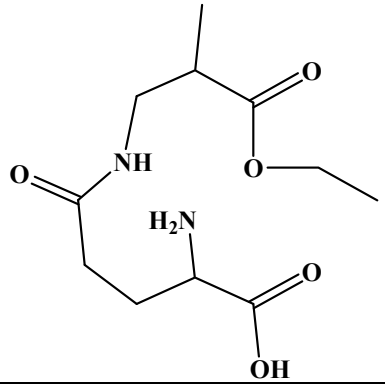
Leucantho flavones Source: *N. cataria* (catnip) (Hou *et al.,* 2002)		64
2'-O-D Galactopyranoside Source: *N. cataria* (catnip) (Hou *et al.,* 20002)		65
2-O-Caffeoyltartronic acid Source: *N. cataria* (Snook *et al*., 1993; Hou *et al.,* 2002, Chang 2000)		66
Nepetanudoside Source: *N. nuda* (Koganov and Tsorin 1999, Kanchanapoom and Yamasaki 2001)		67
Velpetin Source: *N. velutina* (Zapesochnaya *et. al.,* 1991; Kuruuzum *et al.,* 2003*;* Suksamran *et al.,* 2003)		68
Nepetalactam Source: *N. cataria* (Krawczyk *et al.,* 2004)		69

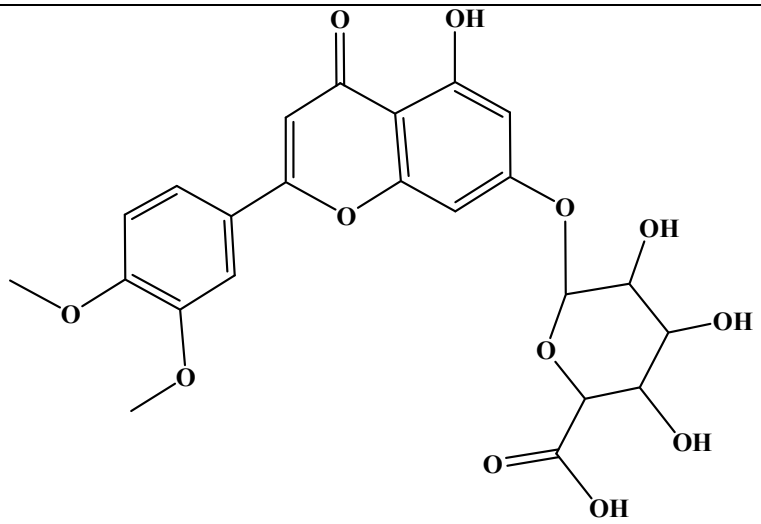
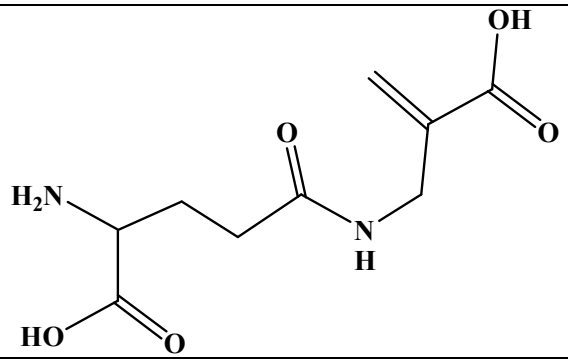
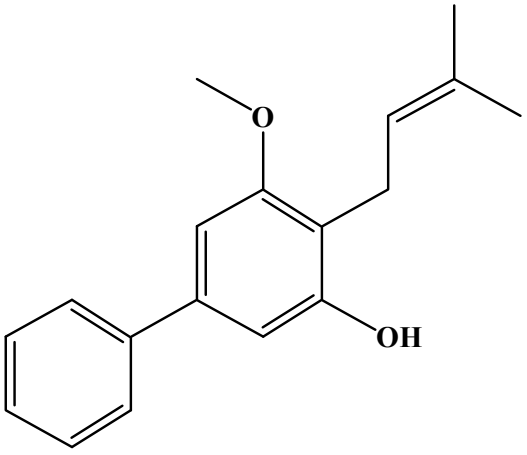
Epinepetalactone Source: *N. cataria* (catnip) (Nangia *et al.,* 1997, Liblikas 2005)		70
Nepetanudoside C Source: *N. nuda* (Takeda *et al.,* 1996)		71
Nepetanudoside D Source: *N. nud*a (Takeda *et al.,* 1996)		72
Nepetaracemoside A Source: *N. racemosa* (Takeda *et al.,* 1999)		73
Nepetaracemoside B Source: *N. racemosa* (Takeda *et al.,* 1999)		74
Nepetariaside Source: *N. cataria* (catnip) (Murai *et al.,* 1987)		75

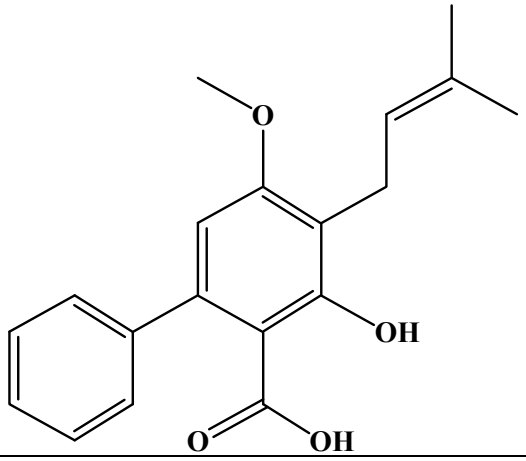
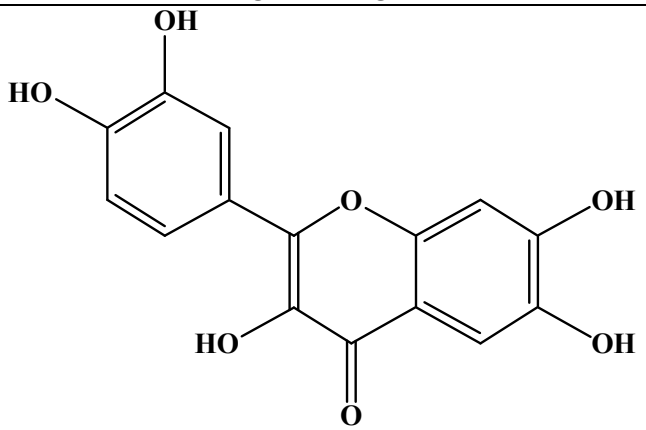
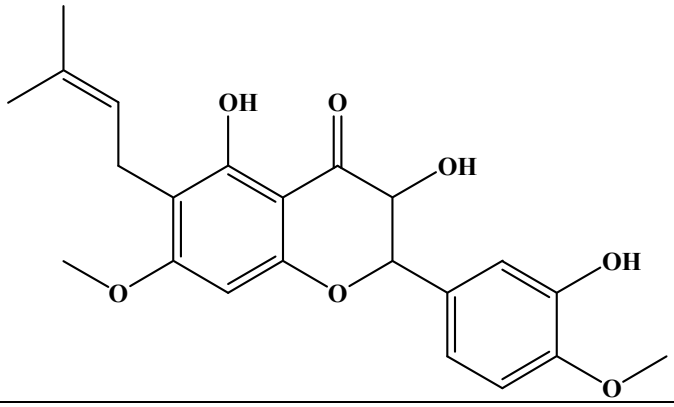
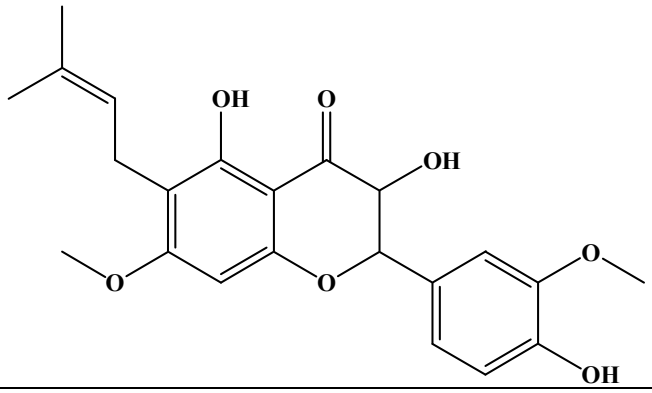
Nepetaside Source: *N. cataria* (catnip) (Xie *et al.,* 1988)		76
2-[Octahydro-4,7-dimethyl-1-oxocyclopenta[c]pyran-3-yl]nepetalactam Source: *N. cataria* (Eisenbraun *et al.,* 1988)		77
3-(Octahydro-4,7-dimethyl-1-oxocyclopenta[c]pyran-3-yloxy)-12-oleanen-28-oic acid Source: *N. caesarea* (Topcu *et al.,* 2000)		78
Esculentic acid Source: *N. hindostana* (Jiang *et al.,* 2005, Adnyano *et al.,* 2000)		79

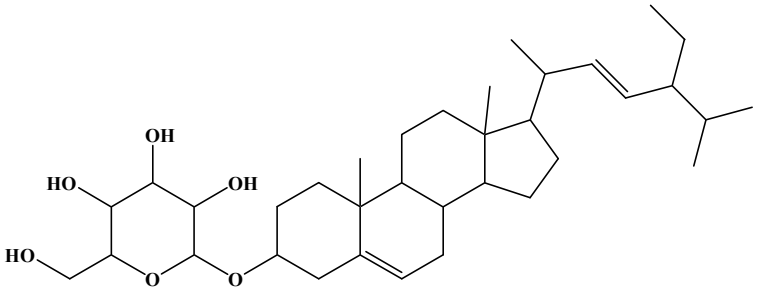
Triterpene ester Source: *N. suavis* (Hussain *et al.,* 2009)		80
Artemetin Source: *N. suavis* (Hussain *et al.,* 2009)		81
Jaceidin Source: *N. suavis* (Hussain *et al.,* 2009)		82
Nepetanal Source: *N. juncea* (Hussain *et al.,* 2009)		83
Nepetanoate Source: *N. juncea* (Hussain *et al.,* 2009)		84
Nepatanol Source: *N. distans* (Hussain *et al.,* 2010)		85

Nepetolide Source: *N. suavis* (Hussain *et al.,* 2008)		86
Kirmanoic acid Source: *N. clarkei* (Hussain *et al.,* 2012)		87
Kurramanoic acid Source: *N. clarkei* (Hussain *et al.,* 2012)		88
Nepethalate A Source: *N. clarkei* (Hussain *et al.,* 2011)		89

Nepethalate B Source: *N. clarkei* (Hussain *et al.,* 2011)		90
Beta-sterol Source**:** *R. minima* (Besson *et al.,* 1977; Ahmed *et al.,* 1992)		91
1,4-Benzenediol diacetate Source**:** *R. minima* (Krishnamurty *et al.,* 1975)		92
N-(2-Carboxypropyl) Glutamine Source**:** *R. albiflora* (Wathelet *et al.,* 1988)		93

5,7-Dihydroxy-3,4-dimethoxyflavone; 7-O- Beta-D- Glucophyranoside Source**:** *R. beddomei* (Adinarayana *et al.,* 1980)		94
N-Glutamyl-(3-amino-2-methayleneproponoic acid Source**:** *R. albiflora* (Wathelet *et al.,* 1988)		95
3-Hydroxy-5-methoxy-4-(3-methayl 2-butenyl) biphenyl Source**:** *R. suaveolens* (Khan *et al.,* 1984)		96

3-Hydroxy-5-methoxy-4-(3-methayl 2-butenyl) biphenyl-2-Carboxy Source: *R. suaveolens* (Khan *et al.,*1984)		97
3,3`,4`,6,7-Pentahydroyflavone Source**:** *R. beddomei* (Khan *et al.,*1984)		98
3,3`,4`, 5,7-Pentahydroxy-6-prenyleflavanone;(2R,3R-form,4`,7,Di-Me ether Source**:** *R. cyanosperma* (Adinarayana *et al.,* 1980)		99
3,3`,4`, 5,7-Pentahydroxy-6-prenyleflavanone;(2R,3R-form,3`,7,Di-Me ether Source**:** *R. densiflora* (Rao *et al.,* 1988)		100

Stigmasta-5,22-dien-3-ol; (3β,22*E*,24*S*)-form, 3-*O*-β-D-Galactopyranoside Source**:** *R. minima* (Ahmed *et al.,* 1982)		101

1. Drug discovery strategy

Modern strategies for drug discovery emphasize on accessibility of some easy and economical biological assays to assess medicinal prospective of plant species. Current research exposes a modern strategy with blend of local and modern information for estimation of biological activity of medicinal plant species. The core steps of this tactic are as following.

1.1. Selection of plant material
1.2. Preparation of plant extracts
1.3. Biological screening of plant extracts by simple bioassays
1.4. Bioassay guided isolation of natural products and their structure elucidation
1.5. Identification of components of plant extracts by analytical scale HPLC and NMR equipped.

1.1 Selection of plant material (Plants flora)

In the current effort the selected curative plants *Nepeta laevigata, Nepeta* kurramensis and *Rhynchosia reniformis* from the flora of Pakistan is screened for potential pharmaceutical activities, proximate composition, bioassay guided isolation and characterization of natural products. Plants were chosen on the basis of their ethnomedical histories.

1.1.1 Description of genus *Nepeta*

Nepeta is a genus of about 250 species of flowering plants in the family Lamiaceae. Most of the species are herbaceous perennial plants, but some are annuals. They have sturdy stems with opposite heart-shaped, green to grayish-green leaves. The flowers are white, blue, pink or lilac and occur in several clusters toward the tip of the stems. The flowers are tubular and spotted with tiny purple dots. The members of this group are known as catnip or catmint because of their famous effect on cats. *Nepeta* pleasantly stimulates cats' pheromonic receptors, typically resulting in temporary euphoria. It is also reported to induce mild euphoria in humans. The genus is native to Asia, Europe, and Africa. It is now also common in North America [Nasir, 1990]. The study was performed on the following two species.

- *Nepeta laevigata*
- *Nepeta kurramensis*

1.1.2 *Nepeta laevigata*

Nepeta laevigata is a perennial herb. Stems erect, 30-80 cm, usually branched, quadrangular, glabrous to minutely pilose with retrorse eglandular hairs, sometimes densely pilose-villous below inflorescence axis, leafy. Leaves are green, triangular-ovate to elliptic, 30-60 x 20-30 mm, regularly crenate to senate, broad truncate to cordate, acute, abaxially with a thin indumentum of short eglandular hairs and with sessile oil globules; petiole up to 4 cm on lowermost leaves, decreasing above. Inflorescence of mainly terminal continuous congested oblong spikes up to 8 x 2 cm, subtended by small uppermost stem leaves. Outer bracts up to 4 mm broad, often purplish and showy, sometimes yellowish, broad ovate or trullate, long attenuate; innermost bracts linear-filiform as long as calyx. Calyx 6-8 mm, slender, tubular, almost glabrous to finely glandular-papillose, with or without oil globules; throat slightly oblique, villous within; teeth unequal, linear triangular, aristate, as long as tube. Corolla mauve to lilac-blue or white, 12-14 mm; tube exserted, curved; upper lip ± straight; lower lip shorter than upper. Nutlets (few seen) 1.3 x 0.9 mm, brown, broad oblong, rounded, minutely granular, matt with a transverse basal areole. Flowering period is June to August. The plant species is cosmopolitan in Afghanistan, Pakistan, Kashmir, North West India, Nepal and South West China [Nasir, 1990].

1.1.3 *Nepeta kurramensis*

Though in leaf profile and indumentum's and in calyx lettering, *Nepeta kurramensis* has apparent similarities to *Nepeta floccose* but vary from *Nepeta floccose* in the scale-like lowermost leaves (cataphylls), the short-petiolate cauline leaves, the plodding lessening in leaf range up the stem, the retiring terminal, oblong-ovoid inflorescence head and the lanceolate-subulate bracts which equal the calyx length. The species is endemic to Kurram agency Pakistan and Afghanistan [Nasir, 1990].

1.1.4 Medicinal uses of *Nepeta*

The plant is used medicinally as a diaphoretic. Oil isolated from catnip by steam distillation is a repellent against insects, in particular mosquitoes, cockroaches and termites. Research suggests that in a test tube, distilled nepetalactone, the active ingredient in catnip, repels mosquitoes ten times more effectively than N, N-diethyle-meta-toluamide (DEET), the active ingredient in most insect repellents, but that it is not as effective a repellent when used on the skin. *Nepeta* species are used for folk treatment of many diseases such as antispasmodic, sedative, antiasthmatic, astringent, carminative, diuretic, diaphoretic, tonic, refrigerant, stimulant, stimulate sweating, reduce fever, used for miscarriage and disorder of nervous, respiratory and gastrointestinal systems [Schultz *et al,*. 2006].

1.1.5 Description of genus *Rhynchosia*

Rhynchosia, commonly known as snout bean, is a member of the legume family Fabaceae [Lackey 1981]. The genus consists of approximately 200 species and occurs in both the eastern and western hemisphere in warm temperate and tropical regions [Grear, 1978]. Fourteen species and two infraspecific taxa of *Rhynchosia* have been reported from the United States (US). Of these, ten species and one infraspecific taxon have been reported from the southeastern US [Isely, 1990]. In Pakistan, the genus is represented by 7 species [Jahan *et al*., 1994].

Roots are perennial. Stem is herbaceous, trailing, twining, or erect, simple or branched, glabrous to pubescent. Leaves are unifoliate or pinnately trifoliate; petioles 1-90 mm long; leaflets entire, elliptic to rhomboid, 10-70 mm long, glabrous to pubescent, glandular punctuate with yellow, dome-shaped, resin glands. Inflorescence pseudoracemes, axillary or short terminal; peduncles 1-90 mm long. Calyx 2.5-12.0 mm long; corolla yellow, some with purple to brown veins, 4-10 mm long; stamens 10, diadelphous (9 + 1); styles glabrous; ovaries glabrous to pubescent; ovules 1-2. Fruits 10-20 mm long, dehiscent, short and broad, asymmetrically ovate to oblong to falcate-oblong, laterally compressed, short-beaked, glandular-punctate, pubescent. Flowering stage is May to September. [Jahan *et al*., 1994, Nasir and Ali 1977]

1.1.6 *Rhynchosia reniformis*

Roots are perennial. Stem is erect, simple or branched. Leaves are 40-60 mm long, unifoliate, upper one rarely trifoliate; petioles 20-45 mm long; leaflets reniform or subcordate, 25-50 mm long, strigose above, hirsute beneath, especially along the veins. Inflorescence short and subsessile; peduncles 5-20 mm long. Calyx 7-10 mm long, lobes longer than tube; corolla yellow, 6.0-9.5 mm long, sub equal to calyx. Fruits shortly oblong or elliptic-oblong, 12-18 mm long, villous especially along the sutures. The plant is distributed in the sandy area of Pakistan [Jahan *et al*., 1994]

1. 1.7 Medicinal uses

Leaves are abortifacient [Mali *et al.,* 2006]. Seeds are astringent and poisonous [Patil, 2003]. The leaves contain isovitexin and apigenin derivatives. Aerial parts give steroidal glycosides, along with ergosterol peroxide, stigmasterol and lupeol; bergapten, isopimpinellin, umbelliferone and beta-sitosterol have also been isolated [Ahmed *et al.,* 1992]. The seed coat and pericarp contained gallic and protocatechuic acid, prodelphinmidine and hydroquinone diacetate and *C*-glycosyl flavones [Besson *et al.,* 1997]. The *Rhynchosia* species are traditionally used as antidiebatic, abortifacient, antibacterial, antifungal, antihelminthic, antiasthmatic, pills and healing of wounds, liver protective [Mali *et al.,* 2006; Tarafdar, 1983]. The extract of seeds shows agglutinating activity with certain type of human red blood cells [Patil, 2003].

In sight of the above, comprehensive biological studies were proposed to be undertaken. An efficient bioassay costing of the plants established a scientific platform for the use of selected plants as medicinal plants.

1.2 Preparation of plant extracts

Preparation of plant extracts involves an appropriate organic solvent. Solvents can be grouped into polar and non-polar solvents. Both classes have the ability to dissolve polar and non-polar constituents from raw extracts. A variety of approaches have been used formerly for the preparation of plant extracts from plant material.

1.2.1 Organic solvents for preparation of plant extracts

A range of organic solvents including methanol, dichloromethane (DCM), ethyl acetate, petroleum ether, acetone, chloroform, *n*-butanol, ethanol and *n*-hexane etc have been used earlier for preparation of plant extracts.

1.2.2 Procedures used for preparation of plant extracts

a. Plant material

Fresh plants of *Nepeta laevigata, Nepeta kurramensis* and *Rhynchosia reniformis* were collected randomly from Kuram Agency and Karak, Khyber Pakhtunkhwa, Pakistan. The taxonomic identity of these plants were determined by plant taxonomist at Department of Botany, Kohat University of Science and Technology, Pakistan and the voucher specimen has been kept for future reference with voucher no. PLS/Herb 1015, 1016, 1017. Fresh plant materials were washed under running tap water, airs dried and then were powdered.

b. Extraction and fractionation

The shade-dried whole plant material of *Nepeta laevigata* was chopped and soaked in methanol for 10 days, extracted three times at room temperature in the same solvent and filtered. The filtrate was evaporated *in vacuo* to give a dark-greenish residue (extract), which was further suspended in water and partitioned successively with *n*-hexane, chloroform, ethyl acetate and *n*-butanol to obtain *n*-hexane-soluble, chloroform-

soluble ethyl acetate-soluble and *n*-butanol soluble fractions, respectively. Same extraction and fractionation procedure was adopted for *Nepeta kurramensis* and *Rhynchosia reniformis*. The crude plant extracts and subsequent solvents soluble fractions were subjected to various bioassay and isolation techniques (Figure **2.1**) [Khan *et al*., 2011].

1.3 Biological screening of plant extracts

Biological activities of plant extracts to estimate their potential for diverse activities required the development of simple, quick and cheap biological assays. Potential plant extracts then can further be fractionated for isolation and characterization of biologically active natural products.

The frequently used biological assess can generally be illustrated in the following method.

1.3.1Antimicrobial assays

(a) Antibacterial assay

(b) Antifungal assay

1.3.2. Antiglycation

1.3.3 Antiplatelet aggregation

1.3.4 Antioxidant

1.3.5 Toxicity assays

(a) Phytotoxicity assay

(b) Brine shrimp cytotoxicity assay

1.3.1 Antimicrobial assays

The news of antimicrobial bustle of numerous plant extracts has been available from many regions in the humanity. It is projected, though, that of the 250,000–500,000 species originate on Earth, and only 1-10 % have been calculated for their pharmaceutical prospective [Melendez & Capriles, 2006]. Research on plants used usually for the handling of systemic and topical infections has publicized that many restrain the escalation of a thick array of microorganisms. Such plants may be full of antiparasitic,

antifungal, antibacterial and antihistamine compounds [Jones *et al*., 2000]. Some of these conventionally used plants may pilot to development of new antimicrobial agents which are in increasing demand due to resistance to conventional drugs [Omar *et al*., 2000].

Antimicrobial activity of herbs has been branded and described for various centuries [Begamboula *et al*., 2003]. Many logically stirring compounds establish in edible and remedial plants, herbs, and spices have been exposed to own antimicrobial functions and could dish up as a font of antimicrobial agents touching bacteria and fungi [Deans and Ritchie, 1987; Janssen *et al*., 1985; Kim *et al*., 1995]. A number of studies have keen out the leeway to use indispensable oils and their machinery in health check and plant pathology as well as in the food commerce for the manage of microorganisms pathogenic to clients and liable for food spoilage [Cantore *et al*., 2009].

The approval of standard medication as an alternative form of dynamism source and the progress of microbial resistance to the orthodox antibiotics led researchers to examine the antimicrobial action of a number of therapeutic plants [Maoz *et al*., 1998; Hammer *et al*., 1999, Al-Bakri and Afifi, 2007]. During few modern decades and frequently as a upshot of their multiplicity, flexibility and security in contrast with the artificial resources, natural products from plants have attained extraordinary curiosity among educational and modern scientific communities [Colegate and Molyneux, 2008; Ebrahimabadi *et al*., 2010]. Nowadays we determined that the essential oils and a range of plant extracts have a wide band activity against the Gram-positive and Gram-negative pathogenesis and they also execute the antifungal action [Kotzekidou *et al*., 2008; Sartoratto *et al.*, 2004].

In order to ensure antimicrobial activities of sanitized antibiotics, crude extracts or pure compounds following procedures can be used

(a) The disc diffusion method

(b) Agar well diffusion method

(c) Dilution method

(d) Serum Killing power test

(e) Automated bioassays methods

(a) The disc diffusion method

In the disc diffusion method, or Kirby-Bauer method, a standard quantity of the causative is equally spread over an agar plate. Then numerous filter paper discs permeated with definite concentrations of chosen chemotherapeutic mediators are located on the agar surface. At last, the culture with the antibiotic discs is incubated. A fresh version of the diffusion test, called an E test uses a plastic strip restrained a gradient of concentration of antibiotic. [Rahman and Rashid 2008].

(b) Agar well diffusion method

In this system, wells are made in seeded agar and the test sample is then putted directly into these wells. After incubation the diameter of the obvious zones around each well is calculated and evaluated against zone of inhibition formed by solution of known concentration of typical antibiotics. Five or six samples may be tested concurrently by the diffusion technique. [Shinwari *et al*., 2013].

(c) Dilution method

In this process a stable quantity of microbial inoculum's (specimen) is introduced into a string of broth cultures containing declining concentrations of a chemotherapeutic agent. After incubation (for 16 to 20 hours) the tubes or wells are observed, and the lowly concentration of the agent that averts visible growth (indicated by turbidity or colony forming units) is noted. This concentration is the minimum inhibitory concentration (MIC) for an exacting agent acting on a particular microorganism. Samples from tubes that illustrate no growth but that might contain introverted organisms can be used to

inoculate broth that contains no therapeutic agent. In this test, the lowest concentration of the therapeutic agent that yields no growth following this second inoculation, or subculturing, is the minimum bactericidal concentration (MBC).[Riesselman *et al*., 2000].

(d) Serum killing power test

This test is executed by obtaining a sample of a patient's blood while the patient is getting an antibiotic. A bacterial deferment is added to a recognized quantity of the patient's serum (blood plasma minus the clotting factors). Growth (turbidity) in the serum after incubation means that the antibiotic is futile. Inhibition of growth recommended that the drug is effective, and more quantitative determinations can be complete to categorize the lowest concentration that still presents serum killing power. [Chowdhury *et al*., 2004].

(e) Automated bioassays methods

Automated techniques are now accessible to recognize the pathogenic organisms and to conclude which antimicrobial agents will successfully combat them. One such scheme uses primed trays with small wells into which a calculated quantity of inoculums is automatically dispensed. Trays are also obtainable to determine the compassion of organisms to a mixture of antimicrobial agents. The trays are interleaving into a machine that measures microbial expansion. Some machines do this by using a beam of light to determine turbidity. Others utilize media containing radioactive carbon. Organisms growing on such media liberate radioactive carbon dioxide into the air, and a sampling appliance automatically detects it [Black, 2005].

Microbial agents

(a) Bacteria

(b) Fungi

(a) Bacteria

In 1884, a Danish researcher Hans Christian Gram developed the most commonly used discrepancy stain, which now stands his name. Bacteria are grouped on the origin of Gram staining of bacterial cell wall into two main types.

1. Gram-negative bacteria
2. Gram-positive bacteria

The bacterial strains used in finding the antibacterial profile of our selected plants are briefly discussed. *Pseudomonas testosteroni* has been mostly ignored as a potential pathogen in humans. The organism was most often found in association with anatomic abnormalities of the gastrointestinal tract. Perforation of the appendix is the commonest abnormality. *Klebsiella* is a type of Gram-negative bacteria that can cause different types of healthcare-associated infections, including pneumonia, bloodstream infections, wound or surgical site infections, and meningitis. *Streptococci* are Gram-positive spherical bacteria that divide in one plane. They are characterized by their ability to infect the mouth. *Proteus morganii* is a gram-negative rod commonly found in the environment and in the intestinal tracts of humans, mammals, and reptiles. Despite its wide distribution, it is an uncommon cause of community-acquired infections. *Micrococcus* is generally thought to be a saprotrophic or commensal organism, though it can be an opportunistic pathogen, particularly in hosts with compromised immune systems, such as HIV patients. It can be difficult to identify *Micrococcus* as the cause of an infection, since the organism is a normally present in skin microflora, and the genus is seldom linked to disease. Micrococci have Gram-positive spherical cells ranging from about 0.5 to 3 micrometers in diameter and typically appear in tetrads [Andreopoulos *et al*., 2000]. *Bacillus subtilis* is a ubiquitous bacterium commonly recovered from water, soil, air, and decomposing plant residue. It is not considered pathogenic or toxigenic to humans, animals, or plants. *B. subtilis* could be expected to temporarily inhabit the skin and gastrointestinal tract of humans, but it is doubtful that this organism would colonize other sites in the human body [Edberg, 1991]. *Escherichia coli* are known to cause urinary infection, wound

infection and gastroenteritis *Staphyllococcus aureus is* causative agent of pneumonia, meningitis and food poisoning in human [Fosse *et al*., 1985].

(b) Fungi

Fungal infections are common in human. It can also infect animal and plants. In nature there are 1.5 million species of fungi. To treat infections caused by fungi noval and effective fungicidal agents are needed. It is believed that plants or products derived from plants can be a cheep and effective source for new drugs to tackle fungal infections in human. A range of pathogenic strains of fungi are found. Here we discussed some of the important species of fungi that were been used in antifungal assays of our selected plants. *Aspergillus* includes over 185 species. Around 20 species have so far been reported as causative agents of opportunistic infections in human. Among these, *Aspergillus fumigatus* is the most commonly isolated species, followed by *Aspergillus flavus* and *Aspergillus niger*. These are well-known to play a role in three different clinical settings in man: (i) opportunistic infections; (ii) allergic states; and (iii) toxicoses. Immunosuppression is the major factor predisposing to development of opportunistic infections. These infections may present in a wide spectrum, varying from local involvement to dissemination and as a whole called aspergillosis. Among all filamentous fungi, *Aspergillus* is in general the most commonly isolated one in invasive infections. It is the second most commonly recovered fungus in opportunistic mycoses following *Candida* [Crissy *et al*., 1995]. *Fusarium* species as well as being common plant pathogens are also causative agents of superficial and systemic infections in humans. Infections due to *Fusarium* species are collectively referred to as fusariosis. The most virulent species is *Fusarium solani* [Mayayo *et al*., 1999]. Trauma is the major predisposing factor for development of cutaneous infections due to *Fusarium* strains. Disseminated opportunistic infections, on the other hand, develop in immunosuppressed hosts, particularly in neutropenic and transplant patients [Austen *et al*., 2001, Boutati and Anaissie, 1997, Girmenia *et al*., 1999, Vartivarian *et al*., 1993, Venditti *et al*., 1988]. *Fusarium* infections following solid organ transplantation tend to remain local and have a better outcome compared to those that develop in patients with hematological malignancies and bone marrow transplantation patients [Sampathkumar and Paya, 2001].

Rhizoctonia solani is a plant pathogenic fungus with a wide host range and worldwide distribution. It frequently exists as thread-like growth on plants or in culture, and is considered a soil-borne pathogen. *Rhizoctonia solani* is best known to cause various plant diseases such as collar rot, root rot, damping off and wire stem. *Pseudallescheria boydii* is a saprophytic fungus frequently isolated from agricultural and industrial soil and from polluted water. *P. boydii* has been the leading cause of Madura foot in the US and Europe. More recently colonization of the lungs of patients with cystic fibrosis is becoming a significant clinical syndrome. Disseminated and invasive infections with this organism are seen primarily in immunocompromised hosts and include pneumonitis, osteomyelitis, endophthalmitis, meningitis and prosthetic valve endocarditis. *Microsporum canis* is an organism that can cause tinea capitis (ringworm of the hair) in humans, and simple ringworm in pets.The organism's major reservoir in companion animals is within domestic cats and dogs (Girmenia *et al*., 1999). *Candida albicans* infect oral and genital system in human (Enfert and Hube 2007).

1.3.2 Antiglycation

In diabetes, most of the anon phase complications are primarily due to a procedure called glycation of proteins, resulted from the large concentrations of sugar present in the blood streams of patients for a prolonged time. Glycation is post translational modifications in the structures of proteins. This is a non-enzymatic reaction amid plummeting sugars such as glucose and free amino residues of proteins. Glycation alters the structures and natal properties of proteins and begin their squalor procedure. Glycation of protein is also recognized as browning reaction due to the development of brown color products called advanced glycation end products (AGEs), which are luminous materials. Hyperglycaemia, the medical stamp of feebly controlled diabetes, is famous to cause fast protein glycation and making of AGEs in the body. Numerous studies have been conducted to learn the noteworthy function of glycation in the development of normal aging and pathogenic age linked diseases such as diabetic complications i.e. retinopathy, neuropathy, cataract formation, atherosclerosis, end stage renal diseases, rheumatoid arthritis and neurodegenerative diseases. It is thus a substantial attention in the finding of plant extracts and natural compounds, which can either, avoid

the pattern of AGEs or can sluggish down this procedure. [Ahmed, 2005]. Estimation of non-enzymatic protein glycation can be measured by using following three methods.

a. Polyacrylamide gel electrophoresis technique

Polyacrylamide gel electrophoresis (PAGE) is perhaps the most familiar analytical technique used to split and portray proteins. A solution of acrylamide and bisacrylamide is polymerized. Acrylamide alone shapes linear polymers. The bisacrylamide launch cross links between polyacrylamide chains. The 'pore size' is gritty by the ratio of acrylamide to bisacrylamide, and by the concentration of acrylamide. A lofty ratio of bisacrylamide to acrylamide and a high acrylamide concentration cause squat electrophoretic mobility. Polymerization of acrylamide and bisacrylamide monomers is provoked by ammonium persulfate (APS), which impulsively crumbles to form free radicals. TEMED, a free radical stabilizer, is usually included to endorse polymerization [Atta-ur-Rahman *et al.,* 2007].

b. Electrospray ionisation mass spectrometry technique

Electrospray ionisation mass spectrometry (ESI-MS) which has become an increasingly important technique in the clinical laboratory for structural study or quantitative measurement of metabolites in a complex biological sample. The capability of ESI-tandem-MS in measuring bio-molecules sharing similar molecular structures makes it particularly useful in screening for inborn errors of amino acid, fatty acid, purine, pyrimidine metabolism and diagnosis of galactosaemia and peroxisomal disorders. Electrospray ionisation is also efficient in generating cluster ions for structural elucidation of macromolecules. The Electrospray Ionization (ESI) is a soft ionization technique extensively used for production of gas phase ions (without fragmentation) of thermally labile large supramolecules [Atta-ur-Rahman *et al.,* 2007].

c. Fluorescence Spectrophotometery

Spectrophotometery of glycation can be done through fluorescence spectrophotometers which are intended to identify and quantify fluorophores. These are fluorescent molecules that, when exposed to light in a fluorescence spectrophotometer,

take in photons at a characteristic wavelength. Consequently, they then release photons at a diverse and somewhat longer characteristic wavelength. Fluorescence spectrophotometers contain excitation and emission filters for using different fluorophores. Monitoring the actions of molecules taged with fluorophores is vital to many kinds of research in cell biology, molecular biology and genetics, pharmaceuticals, and forensics, among many others. Emanated light may be noticed by photomultiplier tubes or photodiodes within the spectrofluorometer, depending on the model. In addition, users of spectrofluorometers now have a wide choice of high-tech fluorophores from which to decide to complement the features of a fluorometer, also referred to as fluorimeter. Modern fluorometers and fluorimeters are proficient of revealing as low as 1 part per trillion [Atta-ur-Rahman *et al.,* 2007].

1.3.3 Antiplatelet

In normal physiological circumstances, platelets strewn liberally in the blood at a concentration of 150 to 300 × 10^8 / L and do not stick to each other or to the vessel wall. The major task of platelets is to guarantee haemostasis, which means the protection of blood vessel veracity and the swift termination of bleeding in the occasion of vascular damage. The same mechanisms are drawn in when platelets are activated at the spot of an atherosclerotic plaque shatter, leading to vessel occlusion and, depending on the vascular bed concerned, ischemic complications such as myocardial infarction, stroke, or tangential artery disease [Yamguchi *et al*., 2000]. Platelets are themselves concerned in the succession of the atherosclerotic lesions as well as in angiogenesis through release of provocative mediators and growth. Platelets have also been revealed to take part in a cancer metastasis. Therefore, this skinny cell unit has a central function in physiology and is involved in the pathogenesis of several diseases, of which atheroscleros -is and associated thrombosis is the foremost cause of transience and morbidity in most cases [Carmelite, 2003]. Antiplatelet aggregation can easily be measured by methodology as used by Saeed *et al.*, (2007).

1.3.4 Antioxidant assays

The potentially toxic and beneficial properties of pro-oxidants and antioxidants have made them the focus of many studies. Pro-oxidants may represent a threat to health, whereas antioxidants may counteracts these effects by scavenging pro-oxidants. Antioxidants are very important in industrial processes as well as in biological systems. They are known to possess antiinflammatory, anticardiovascular disease, antineurogenerative and anticancer properties. Imbalances between pro-oxidants and antioxidants in favor of the pro-oxidants may result in oxidative stress, resulting in oxidative damage of cellular components in the form of lipid peroxidation, protein denaturation or DNA conjugation. Oxidative stress has been associated with many diseases namely cancer, post-ischemic and neural degradation, Parkinson's and Alzheimer disease, AIDS, aging and cardiovascular diseases [Kool *et al*., 2007].

Antioxidants, which forage dynamic oxygen species (free radicals), were establishing in a range of products and are generally referred to as scavengers [Beckman *et al.,* 1990]. Numerous oxidants are plant based and play significant role in shielding plants that are exposed to sunlight and live beneath stern oxygen stress. Antioxidants also play a vital job in human fitness because the biologic defense system cannot maneuver under severe oxygen stress. According to topical research, activated oxygen is considered to be chief aspect in aging, hardening of the arteries, diabetes, cancer and tissue in injury skin [Ito and Hirose, 1989]. Certainly around 90% of age associated diseases are allied to activated oxygen.

The aerobic life on earth depends upon oxygen but it is also implicated in a number of noxious chemical reactions. Auto-oxidation happens when any organic glimmer retorts with atmospheric oxygen. Auto-oxidation of food substance results in oxidation of lipids and rancidity of food. Person physiology also engrosses a number of oxidation reactions. Syndromes such as atherosclerosis, cancer and tissue damage in rheumatoid arthritis involve oxidative anxiety [McDonald *et al*., 2001, Halliwell, 1994, Basu *et al*., 1999]. Antioxidants are compounds dependable for harmonizing oxidation procedures in our body. These compounds are generally phenolic compounds which are oxidized very hastily and decrease the consequence of oxidants.

There are two kinds of antioxidants existing
(a). Synthetic antioxidants
(b). Natural antioxidants

(a) Synthetic antioxidants

Antioxidants which are manufactured under laboratory conditions are identified as synthetic antioxidants. For inhibition/delaying of inception of oxidation procedures in food material there is wide use of synthetic antioxidants like butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and t-butyl hydroxyquinone (TBHQ) in food commerce.

(b) Natural antioxidants

Antioxidants produced naturally by living organisms are called natural antioxidants. Toxicity of artificial antioxidants is palpable so there is reduced use of man-made antioxidants in food industries. Assessment of herbal extracts for naturally occurring antioxidants principally phenolic phyto-chemicals is need of the current medicine and food industries. Now numerous natural antioxidants like ascorbic acid, caffeic acid, gallic acid, quercetin and crude herb and spice-derived extracts can be obtainable in the souk and observed as safe, naturally occurring antioxidants [Kosar *et al*., 2003].

Determination of total phenolic contents

The entire phenolic contents in crude extracts can be determined precisely by Folin-Ciocalteu reagent by using the system of Singleton and Rossi (1965). Folin-Ciocalteu reagent is molybdotungs to phosphoric heteropolyanion reagent that can decrease phenols exclusively. A blue color is developed which can be detected at a wave length of 765 nm by using spectrophotometer. Intensity of blue color depends upon application of phenolic compounds in the test substance. Gallic acid is usually used as standard and standard curve is used to reckon gallic acid equivalent (GAE) value as mg of gallic acid per gram of crude extract. Phenolic standards other than gallic acid can also be used. Estimation of natural and synthetic antioxidants needs antioxidant assays.

Antioxidant assays can be described in two systems

(a) Antioxidant assays in aqueous system

(b) Antioxidant assays in lipid system

(a) Antioxidant assays in aqueous system

In aqueous system three assays can be carried out.

1. DPPH assay
2. $ABTS^{+}$ assay
3. DNA protection assay

1. DPPH (2-2-Diphenyl-1-picrylhydrazyl) assay

This assay calculates tumbling capability of antioxidants towards DPPH radicals. DPPH radical is obtainable commercially in deep blue color. Antioxidant propensity can be considered by measuring the decline in absorbance of methanolic solution of DPPH at 517 nm. Reaction can easily be monitored by spectrophotometer [Prior *et al*., 2005]. The percentage of antioxidant aptitude is summarized by using the following formula.

% age of antioxidant capacity = (Ac – As/Ac) × 100

Where

Ac = Absorbance of negative control at 517 nm

As = Absorbance of sample at 517 nm

2. $ABTS^+$ assay (2, 2-Azinobis (3-ethylbenzothiazoline-6-sulfonate)

In this test, ABTS+ is oxidized by peroxyl radicals or other oxidants to its radical cation, $ABTS^+$, which is deeply colored (dark green), and antioxidant activity is measured as the capability of the test compounds to decrease the color reacting freely with the $ABTS^+$ radical [Prior *et al*., 2003]. Results of test compounds are expressed against to trolox. Decline in absorbance by test compound and control is measured at 415 nm by using spectrophotometer.

3. DNA protection assay

Antioxidant/pro-oxidant action of crude extracts or pure compounds can also be investigated by another assay in aqueous method. The assay is based upon Fenton reaction. In a Fenton reaction, Fe_2^+ reacts with H_2O_2, follow-on in the making of hydroxyl radical, which is measured to be the most injurious radical to bio- molecules [Meneghini, 1997]. Fe_2^+ is oxidized to Fe3+ in the Fenton reaction [Tian and Hua, 2005]. With the assault of ^-OH spawned from the Fenton reaction, super twisting plasmid DNA is broken into three shapes, including super coiled (SC), open circular (OC) and linear form (Linear). The degree of DNA defense can be reviewed by the percentage of super coiled form in gel electrophoresis, and the antioxidant or pro- oxidant effect of tested sample is presented by the ratio of super coiled percentage of tested sample to that of the control (DNA treated with $FeSO_4$ and H_2O_2).

(b) Antioxidant assays in lipid system

The lipid oxidation of food results in food spoilage. Lipid oxidation is concerned in a number of physiological conditions so estimation of antioxidant impending of natural and synthetic compounds needs an assay in lipid system as well.

Thiobarbituric acid reactive substances assay

The most universally used analyze in lipid system is thiobarbituric acid reactive substances (TBARS) assay. Linoleic acid is applied to oxidation in the presence of copper chloride as an effect of which lipid oxidation products are formed. Melondialdehyde (MDA) is one of the chief products of lipid oxidation. These products react with thiobarbituric acid (TBA) and give pink color which can be calculated at 532 nm by spectrophotometer. Presence of any antioxidant of lipid in this system result in fewer products of lipid oxidation and consequently less pink color is created [McDonald *et al*., 2001].

Percentage inhibition of oxidation can be measured by using the following formula.

% age of antioxidant capacity = (Ac – As/Ac) × 100
Where,
Ac = Absorbance of negative control at 532 nm
As = Absorbance of sample at 532 nm

1.3.5 Toxicity assays

(a) Cytotoxicity assay

Cytotoxicity is the eminence of being noxious to cells. Examples of lethal agents are a chemical substance, an immune cell or some types of poison. Treating cells with a cytotoxic compound can result in a variety of cell fates. The cells may undergo necrosis in which they lose membrane integrity and die rapidly as a result of cell lysis. The cells can stop actively growing and dividing (a decrease in cell viability), or the cells can activate a genetic program of controlled cell death (apoptosis). Cells undergoing necrosis

typically exhibit rapid swelling, lose membrane integrity, shut down metabolism and release their contents into the environment. Cells that undergo rapid necrosis *in vitro* do not have sufficient time or energy to activate apoptotic machinery and will not express apoptotic markers [Promega Corporation 2006]. Apoptosis is characterized by well defined cytological and molecular events including a change in the refractive index of the cell, cytoplasmic shrinkage, nuclear condensation and cleavage of DNA into regularly sized fragments [Promega Corporation 2007]. Cells in culture that are undergoing apoptosis eventually undergo secondary necrosis. They will shut down metabolism, lose membrane integrity and lyse [Riss and Moravec 2004].

The prognostic worth of *in vitro* cytotoxicity tests is based on the thought of 'basal' cytotoxicity that poisonous chemicals influence vital functions of cells which are frequent to all cells, and that the toxicity can be deliberate by assessing cellular spoil. The progress of *in vitro* cytotoxicity assays has been ambitious by the demand to swiftly appraise the latent toxicity of great numbers of compounds, to edge animal trialing whenever potential, and to lug out tests with small quantities of compound. Confirmation for the usefulness of *in vitro* cytotoxicity tests has lead numerous pharmaceutical industries to monitor compound libraries to eliminate potentially lethal compounds early in the drug finding method. Early detection of toxic property can help plan teams prioritize a mid chemical sequence and categorize structure toxicity relationships to ease cost downstream [Davila *et al*., 1990,Barile *et al.,* 1994, Todd *et al.,* 1999].

There are three fundamental criteria upon which the toxicity dimensions are based. The primary test category is the measurement of cellular metabolic actions. An early signal of cellular break is a lessening in metabolic bustle. Tests which can calculate metabolic function gauge cellular adenosine triphasphate (ATP) levels or mitochondrial actions. Another parameter frequently experienced is the capacity of membrane reliability. The cell membrane forms a functional fence around the cell and passage into and out of the cell is extremely synchronized by transporters, receptors and secretion pathways. When cells are injured, they become 'spongy' and this forms the root for the second type of assess. Membrane veracity is gritty by measuring lactate dehyrogenase

(LDH) in the extracellular medium. This enzyme is usually there in the cytosol, and cannot be calculated extracellularly except cell spoil has occurred. Second assays compute the uptake of fluorescent color (ethidium bromide) usually barred from unbroken cells. It has been revealed that changes in metabolic bustle are superior indicators of early cell damage, and that effects on membrane integrity are analytic of further stern injury, leading to cell bereavement [Barile et al., 1994]. The third type of assay is the shortest measure of cell number, since departed cells in general disengage from a culture plate, and are washed away in the medium. Cell number can be measured by through cell counting, or by the measurement of whole cell protein or DNA, which are proportional to the number of cells [Todd *et al.,* 1999].

Brine shrimp cytotoxicity assay is very simple bench-top assay. It can be used to measure cytotoxicity of plant extracts as well as synthetic compounds. Brine shrimp eggs are available commercially, and being used as fish food. Evaluation of natural products and synthetic compounds by using brine shrimp cytotoxicity assay describes not only cytotoxicity but also anticancer, antiviral, insecticidal and pesticidal potential [Sheikh *et al*., 2004]. A good correlation has been found between brine shrimp cytotoxicity and cytotoxicity against KB cells [McLaughlin, 1991]. Awal *et al*., (2004) has demonstrated toxicity of leaf and seed extracts of *Cassia alata* by using brine shrimp cytotoxicity assay while in another study by Mongelli *et al*., (2003), cytotoxic evaluation of components of *Bolax gummifera* was demonstrated by using brine shrimp cytotoxicity assay. Brine shrimp assay has also been used by Chowdhury *et al*., (2004) while describing cytotoxic potential of extracts and purified components of *Stachytarpheta urticaefolia.*

(b) Phytotoxicity assay

Phytotoxicity is a phrase damaged to portray the venomous consequence of a compound on plant development. Such harm may be caused by a wide range of compounds, including trace metals, pesticides, salinity or allelopathy, which is the procedure used by a plant to liberate deadly chemicals into the soil to slay neighboring plants. Phytotoxicity is common in the application of urea due to the limited ability for soil to fully convert the fertilizer to ammonium in order for mass flow uptake to occur.

Organic compost enables more effective uptake of nitrogen due to higher prevalence of aerobic microbial activity.

Phytotoxicity can effect in a range of symptoms and may be more brutal under firm production situations. The erroneous selection of pesticide, whether due to vigorous ingredient, hauler chemical, or rate applied, can produce plant damage. Using pesticide susceptible plants, such as Schefflera can cause in plant wound more often. The practice of cistern amalgamation pesticides, fertilizers, and purveyor stickers can substantiate detrimental to plants as can the relevance of pesticides during unpleasant ecological or cultural circumstances. Still diverse chemicals such as algicides and disinfestants used in the industrial location, though not released by plants, can cause phytotoxicity. Phytotoxicity can shrink plant eminence and augment time required to create salable plants. Phytotoxicity may articulate itself in an array of behavior, some palpable and some slight. Symptoms vary from trivial leaf speckling to plant fatality. One of the most ordinary phytotoxicity symptoms is chlorosis. Symptoms usually emerge on the new growth and can be perplexed with fertilizer deficiencies. Occasionally chlorosis is trivial and barely obvious while at other times the tissue turns white within a few weeks of submission. In other case, the leaves stay green while the petioles or stems become chlorotic [Chase and Simone, 1985].

Another frequent symptom of phytotoxicity is necrosis or blazing. Fiery can happen on leaf tips, borders, interveinal tissue or merely speckled athwart the leaf surface. Burning is the most clear phytotoxicity symptom petite of plant death. Burning usually appears within a week of pesticide appliance, but may take as long as six to eight weeks to come into view if manifold applications are necessary and the pesticide is applied only once a month. Smoldering may be caused in numerous ways. Contact burns are characterized by a universal speckling of the leaf surface with acne found wherever spray droplets landed. Some burns occur at spots on the leaf where spray accumulates and originate restricted injury. Other burns take place when the pesticide is engrossed through leaves or roots and is redistributed regularly appearing on leaf margins where the pesticide store. In conclusion, some burns occur on juvenile plant tissue which is not

entirely developed at the time of revelation. This damage can occur on leaf tips or edges which were exposed, while the rest of the leaf which was rolled left over with out any symptoms [Chase and Simone, 1985].

Warp of new growth or termination of growth is occasionally hard to analyze as phytotoxicity except plants are observed strictly. Deformation of new leaves on quickly growing plants can take place within a week of pesticide appliance. This symptom is rarely attributed to a viral infection since it also can cause buckle of new leaves. Growth misrepresentation symptoms owing to pesticide phytotoxicity ought to be more unvaryingly strewn all over a bed or variety of plants than symptoms caused by a viral disease. Sometimes a blend of deformation and stunting occurs, which may cause ending of plant growth. Tips of some hazy leaves become visible chlorotic or necrotic and internodes may be stunted [Chase and Simone, 1985].

Herbicidal properties of plant extracts can be evaluated by using phytotoxic assays. Chemicals used to kill weeds in crops are known as herbicides. Herbicides can be obtained from natural resources or synthesized chemically in the laboratory which can be used to improve the quality and yield of crops. Due to side effects of chemically synthesized herbicides and their environmental hazards, herbicides from natural resources are preferred [Turker and Camper 2002]. Site of action of herbicide, their translocation in the plant and metabolism are keys, used to select proper herbicide for a specific crop. Turker and Camper (2002) have described lemmna minor and radish seed phytotoxicity assay as a general prescreening assay for phytotoxic evaluation of plant extracts. Moreover phytotoxic evaluation is necessary in case of plant antitumor agents because those with growth inhibitory properties cannot be used as antitumor agents against *A. tumefaciens* induced tumors.

1.4 Bioassay guided fractionation of selected plant extracts using semi-preparative HPLC

Crude plant extracts selected for a biological activity can be fractionated in various ways. Semi-preparative HPLC is one of the best methods which can be used to get various fractions in sufficient quantity. Method development requires various steps. Several factors including solvent system, type of column, concentration of crude extract, flow rate of solvent, injection volume and time required for a single run are very important.

The best method would require less time span for a single run by using high concentration of injection volume of crude extract. In this way, sufficient quantity of fractions, in less number of runs can be obtained. Fractions obtained from semi-preparative HPLC can further be tested for biological activity so that potent fraction for a biological activity can be identified.

1.5 Identification of components of plant extracts by analytical scale HPLC equipped with UV-DAD and LC-MS

Chemical analysis of crude plant extracts include two very interesting techniques i.e. analytical scale HPLC equipped with UV-DAD and LC-MS. These techniques can help to identify various components in crude plant extracts.

1.5.1 Chromatography

Chromatography can be defined as a physical process whereby components (solutes) of a sample mixture are separated by their differential distribution between stationary and mobile phases [Ullman and Burtis, 2006]. There are two forms of chromatography.

1. Planar chromatography: In planar chromatography, the stationary phase is coated on a sheet of paper (paper chromatography) or bound to a solid surface (thin layer chromatography) [Gocan *et. al.,* 2002].

a. **Paper chromatography:** In paper chromatography, the stationary phase is a layer of water or a polar solvent coated onto the paper fibers.

b. Thin layer chromatography: In thin layer chromatography (TLC), a thin layer of particles of a material such as silica gel is spread uniformly on a glass plate or a plastic sheet. When the thin layer consists of particles with small diameters (4.5 μm), the technique is termed as high-performance thin-layer chromatography (HPTLC) [Gocan *et. al.,* 2002].

2. Column chromatography: Depending upon type of mobile phase column chromatography can either be gas chromatography or liquid chromatography In column chromatography, the stationary phase can be pure silica or polymer, or it can be coated onto, or chemically bonded to, support particles. The stationary phase may be packed into a tube, or it is coated onto the inner surface of the tube. When the stationary phase in the liquid chromatography consists of small-diameter particles, the technique is called high-performance liquid chromatography (HPLC) [Meyer, 2004].

Chromatogram

In analytical scale gas chromatography and liquid chromatography, the mobile phase, or eluent, exits from the column and passes through a detector or series of detectors that produces a series of electronic signals that are plotted as a function of time, distance or volume. The resulting graphical display is called a chromatogram.

Separation mechanisms

Chromatographic separations are classified by the chemical or physical mechanisms used to separate the solutes. They can be classified as follows.

Ion-exchange chromatography

Ion-exchange chromatography is based on an exchange of ions between a charged stationary surface and mobile phase of the opposite charge. Depending upon the conditions, solutes are either cations (positively charged) or anions (negatively charged). [Acıkara *et. al.,* 2013].They are separated depending on the differences in their ionic charge or the magnitude of their ionic charges [Scott, 2003]

Partition chromatography

The differential distribution of solutes between two immiscible liquids is the basis for separation by partition chromatography. Operationally, one of the immiscible liquids serves as the stationary phase. To prepare this phase, a thin film of the liquid is adsorbed

or chemically bonded onto the surface of support particles or onto the inner wall of a capillary column. Separation is based on differences in the relative solubility of solute molecules between the stationary and mobile phases.

Partition chromatography is categorized as either GLC (gas-liquid chromatography) or liquid-liquid chromatography (LLC).

Liquid-liquid chromatography is further categorized as either normal phase or reverse phase.

Normal phase liquid-liquid chromatography

For normal phase LLC a polar liquid is used as the stationary phase, and a relatively non polar solvent or solvent mixture is used as the mobile phase.

Reversed-phase partition chromatography

In reversed-phase partition chromatography, the stationary phase is non-polar and the mobile phase is relatively polar. When particles of small diameter are used as the stationary-phase support, the technique is HPLC. Because column efficiency is inversely related to the column packing particle size and pressure drop is related to the square of the particle diameter, relatively high pressures are required to pump liquids through efficient HPLC columns. Consequently the technique has also been referred to as high pressure liquid chromatography.

1. Adsorption chromatography

The basis of separation by adsorption chromatography is the differences between the adsorption and desorption of solutes at the surface of a solid particle. Electrostatic, hydrogen-bonding, and dispersive interactions are the physical forces that control this type of chromatography.

2. Affinity chromatography

In affinity chromatography the unique and specific biological interactions of the analyte and ligand is used for the separation. The specificity resulting from enzyme substrate, hormone-receptor, or antigen-antibody interactions has been used in this type of chromatography.

3. **Size-exclusion chromatography**

Separation is based mainly on exclusion effects such as differences in molecular size and/or shape or charge. Size-exclusion chromatography also known as gel-filtration, gel-permeation, stericexclusion, molecular-exclusion or molecular-sieve chromatography separates solutes on the basis of their molecular sizes. Molecular shape and hydration are also factors in the process.

Basic components of a liquid chromatograph

a. Column

Advances in column technology have improved the selectivity, stability, and reproducibility of liquid chromatography (LC) analytical columns. For example, analytical columns are packed with a variety of stationary phases, providing enormous versatility in the separation process.

b. Guard columns

A guard column is placed between the injector and the analytical column. It is packed with the same or similar stationary phase as the analytical column. It collects particulate matter and any strongly retained components from the sample and thus conserves the life of the analytical column. After a predetermined number of separations, a guard column is routinely replaced.

c. Solvent reservoir

Solvents used as the mobile phase are contained in solvent reservoirs. In their simplest forms, the reservoirs are glass bottles or flasks into which "feed lines" to the pump are inserted. To remove particles from solvents, inline filters are placed on the inlets of the feed lines.

d. Pump

Both constant pressure and constant displacement pumps are used in liquid chromatographs. The HPLC pump is operated in either an isocratic or gradient mode.

In the isocratic mode, the mobile phase composition remains constant throughout the chromatographic run. This mode is usually used for simple separations and separations of those compounds with similar structures and/or retention times. Gradient elution is used for more complex separations. In this mode, mobile phase composition is changed during the run in either a stepwise or continuous fashion.

e. Injector

To initiate an LC separation, an aliquot of sample (e.g. 0.2 to 50 µl) is first introduced into the column via an injector. The most widely used type of injector is the fixed-loop injector. In the fill position, an aliquot of sample is introduced at atmospheric pressure into a stainless steel loop. In the inject mode, the sample loop is rotated into the flowing stream of the mobile phase, and the sample is swept into the chromatographic column. These injectors are precise, function at high pressures, and can be programmed for use in automated systems.

f. Detectors

Examples of the detectors used in HPLC include UV photometer (fixed wavelength), UV photometer (variable wavelength), diode array, fluorometer, refractometer and electrochemical detectors [Kupiec, 2004].

Most commonly used detectors as HPLC detectors are diode array detectors.

Diode array detectors (DAD)

Diode arrays are used as HPLC detectors because they rapidly yield spectral data over the entire wavelength range of 190 to 600 nm in about 10 milliseconds. During operation the diode array detector passes polychromatic light through the detector flow cell. The transmitted light is dispersed by a diffraction grating and then directed to a photodiode array, where the intensity of light at multiple wavelengths in the spectrum is measured. Such detectors have been helpful in the identification of drugs in urine and serum [Meyer, 2004; Lai *et. al.,* 1997].

g. Computers

The incorporation of computer technology into HPLC instrumentation has resulted in cost effective, easy-to-operate automated systems with improved analytical performances. In these systems, a computer provides both system control and data processing functions.

Qualitative and quantitative analyses by HPLC

Chromatography is basically a separation technique. However it can be used for both qualitative (identifying the analytes of interest) and quantitative analyses.

Analyte identification

The retention time or volume at which an unknown solute elutes from a column is often matched to that of a reference compound.

Analyte quantification

The electronic signals from the detector are used to produce quantitative information's. Both external and internal calibrating techniques can be used. With external calibration, reference solutions containing known quantities of analytes are processed in a manner identical to the samples containing the analyte. A calibration curve of peak height, peak are versus calibrator concentration is constructed and used to calculate the concentration of the analyte in the samples. With internal calibration, also called internal standardization, reference solutions of known analyte concentrations are prepared, and a constant amount of a different compound, the internal standard, is added to each reference solution and each sample. By plotting the ratio of the peak height (or area under curve) of the analyte to the peak height or area of the internal standard versus the concentration of the analyte, a calibration curve that corrects for systematic losses is constructed. This curve is then used to compute the analyte concentration in the samples by interpolation.

2. Liquid chromatography-mass spectrometry (LC-MS)

Several interface techniques have been developed for coupling a liquid chromatograph to a mass spectrometer, which has allowed HPLC-MS and HPLCMS/ MS to be successfully applied to a wide range of compounds. In theory, as long as a compound can be dissolved in a liquid, it can be introduced into an HPLC-MS system. Thus polar and non-polar analytes and large molecular weight compounds, such as proteins, can be monitored using this technique [Annesley *et al*., 2006; Halcopek *et. al.,* 2012].

Other clinically relevant compounds that are amenable to HPLC-MS analysis include all of the major immunosuppressants, antiretrovirals, homocysteine, biogenic amines, methylmalonic acid, and hemoglobin variants. Even LC-MS or LC-ESI-MS has become the most efficient is widely used in pharmaceutical development via the process of "high throughput screening". To most efficiently use available resources, research labs

synthesize large sets of diverse organic compounds or derivatives of starting core structures. This is often called combinatorial chemistry. The same process of identification is also applied to natural products as potentials pharmaceutical agents. These vast set of compounds must be screened for unique structure and evaluated for potential as agents for further study. This requires high throughput screening of large mixtures or isolates containing many compounds. MS has become the most efficient technique to identify these types of compounds. One example is the use of HPLC-MS to identify new taxanes in botanical extracts as effective anticancer reagents [Madhusudanan *et. al.,* 2002].

1.6 Scope of the present work

Keeping in mind the significance of therapeutic plants as medication for illness and as a source of medicinal lead compounds, there has constantly been a need for their apposite and organized phytochemical explorations. The present research scheme endeavors at the analysis of bioassays guided isolation of potential compounds from medicinal plants, *Nepeta laevigata, Nepeta kurramensis* and *Rhynchosia reniformis* collected from the flora of Pakistan, and thus to sustain the conventional uses of these plants and to afford promising novel leads for drug discovery. The outcomes of the phytochemical, biological and proximate analysis research on above cited medicinal plants are presented in this thesis.

MATERIALS AND METHODS chapter 2

PHASE 1: ISOLATION OF NATURAL PRODUCTS

Attempts to characterize the bioactive principals have recently gained worldwide momentum in many pharmaceutical/neutraceutical formulations and food processing applications, thus the findings of this study will hopefully add more positive effects in these fields. With the increasing acceptance that the chemical diversity of natural products is well suited to provide the core scaffolds for future drugs, there will be further developments in the use of novel natural products and chemical libraries based on natural products in drug discovery campaigns.

2. Materials and methods

All chemical and instrumental analysis was performed at the Kohat University of Science and Technology, Kohat with collaboration of Haji Ebraheem Jamal (HEJ) Research Institute of Chemistry (RIC), Agha Khan University Karachi, Pakistan, DARIS Centre, University of Nizwa, Sultanate of Oman. The commercially available solvents were distilled and used for different chromatographic techniques.

2.1 Chromatography

2.1.1 Column chromatography

Merck silica gel having 70-230 mesh was used for column chromatography (CC), while silica gel having 230-400 mesh was used for flash chromatography (FC).

2.1.2 Thin layer chromatography

The thin layer chromatography (TLC) was performed on DC- Micro-Cards SIF 5×10 cm (silica gel on aluminum cards with layer thickness of 0.2 mm, UV light-254 nm), Riedael de Haens, Art no. 37360. Purity was checked by TLC with different solvent systems using methanol, acetone, ethyl acetate, chloroform and *n*-hexane giving single

spot for pure compounds. Spots detections were observed by spraying with 10% solution of Ce $(SO_4)_2$ in 2N H_2SO_4 and heating with hot air gun.

2.2 General instrumentations

2.2.1 Infra red spectroscopy

The IR spectra were recorded on a Shimadzu IR-460 (Shimadzu Corporation, Tokyo, Japan) instrument.

2.2.2 Ultra violet spectroscopy

The UV spectra were recorded on a Shimadzu UV-240 (Shimadzu Corporation, Tokyo, Japan) spectrophotometer.

2.2.3 Mass spectrometry

EI-MS and fast atom bombardment (FAB) MS spectra were recorded on a JMS-HX-110 spectrometer, with a data system.

2.2.4 NMR spectroscopy

The 1H-NMR spectra were recorded at 400 and 500 MHz on Bruker AM-400 and AM-500 spectrometers at 400 and 500 data system. The ^{13}C-NMR (BB and DEPT) spectra were recorded on the same instruments at 100 and 125 MHz, respectively. The distortionless enhancement polarization techniques (DEPT) experiments were carried out with 45°, 90 ° and 135 °. The quaternary carbons were determined by the subtraction of these spectra from the broad band (BB), ^{13}C-NMR spectrum. The chemical shifts are expressed as ppm (δ) and coupling constants (*J*) are in Hz. Tetra methyl silane (TMS) is used as an internal standard. HMBC, HMQC and COSY spectra were run on Bruker spectrometers operating at 500 and 400 MHz.

2.2.5 Spray reagents

Ceric sulphate reagent was used for the detection of compounds and aniline phthalate reagent for detection of reducing sugar.

2.2.6 Ceric sulphate

Ceric sulphate (0.1g) and trichloroacetic acid (1g) were dissolved in 4 mL distilled water. The solution was boiled and conc. H_2SO_4 was added drop-wise until the disappearance of turbidity.

2.2.7 Aniline phthalate

This spraying regent can be prepared from aniline hydrogen phthalate 99%; phthalic acid monoaniline salt; aniline phthalate 1-butanol solution; phthalic acid, compound with aniline (1:1).

2.2.8 Iodine reagent

A few iodine crystals were placed in a TLC tank and warmed for few minutes on a water bath (40-50 ºC). Spots appeared on the TLC plate when placed in the tank for one minute. Spots turn tan-brown in color.

2.3 Extraction and fractionation of *Nepeta kurramensis*

The complete plant of *N. kurramensis* was dried in murky, chopped and ground to powder. The pulverized plant material (7.5 kg) was primarily extracted with methanol (10 L) thrice for two weeks at room temperature. The collective methanol extract was vanished under reduced pressure leaving behind a greenish residue (280.5 g).

The crude extract was suspended in 0ne liter water and then *n*-hexane was added to it with dynamic shaking in a separating funnel. The mixture was permitted to split in two layers. The *n*- hexane layer (upper) was removed. The extraction with *n*-hexane was repeated twice more. All of the *n*-hexane layers were combined and evaporated on rotary evaporator to give the *n*-hexane fraction (47.8 g). The other separated layer (lower) was taken in a separating funnel; chloroform was added to it and separated as above. The chloroform layer (lower) was collected three times and evaporated on rotary evaporator to give the chloroform fraction (65 g). The other layer (upper) was again taken into a separating funnel; ethyl acetate was added to it, separated and evaporated with rotary evaporator to obtain the ethyl acetate fraction (55 g). Same practice was done with *n*-

butanol to acquired *n*- butanol fraction (23.5 g).The enduring lower layer was collected and evaporated to find the aqueous fraction (89 g) as presented in (Figure **2.1**). Same extraction and fractionation procedure was adopted for *Nepeta laevigata* and *Rhynchosia reniformis*.

2.4 Isolation and purification from *Nepeta kurramensis*

Since chloroform soluble fraction of *Nepeta kurramensis* was found to be most active in different tested biological activities, it was chromatographed over a silica gel column for further isolation and purification. The elution was conceded out with a incline of escalating polarity in the order of *n*-hexane, hexane- chloroform, pure chloroform, chloroform- methanol and lastly with pure methanol.

All the eluted fractions were experienced on silica gel card plates. The compounds on the plates were perceived with ceric sulphate reagent pursued by gradual heating. The fractions viewing the similar TLC summaries were mixed and seven fractions were acquired after anthology i.e. NS1, NS2, NS3, NS4, NS5, NS6 and NS7. These combined fractions were independently subjected to recurring column chromatography on silica gel.

Fraction NS2: It was gained through elution with chloroform-hexane (10:90) and was subjected to column chromatography with the solvent system (chloroform-hexane). This fraction afforded for acknowledged compounds from **(3**) to **(4)** (Figure **2.2**).

Fraction NS3: This fraction was obtained through elution with chloroform-*n*-hexane (50:50) and was subjected to column chromatography with the solvent system (chloroform-hexane). This fraction released one new novel compound Kurramenate (**1**) and one commonly known compound bis (2-ethylicosyl) phthalate **(2)** (Figure **2.2**).

Fraction NS4: It was getting through elution with chloroform-*n*-hexane (60:40) and was subjected to column chromatography with the solvent system (chloroform-*n*-hexane). This fraction afforded two known compounds **(6)** and **(7)** (Figure **2.2**).

Fraction NS6: It was attained through elution with chloroform-hexane (70:30) and was subjected to column chromatography with the solvent system (chloroform-hexane). This fraction gives only one known compound **(5)** (Figure **2.2**).

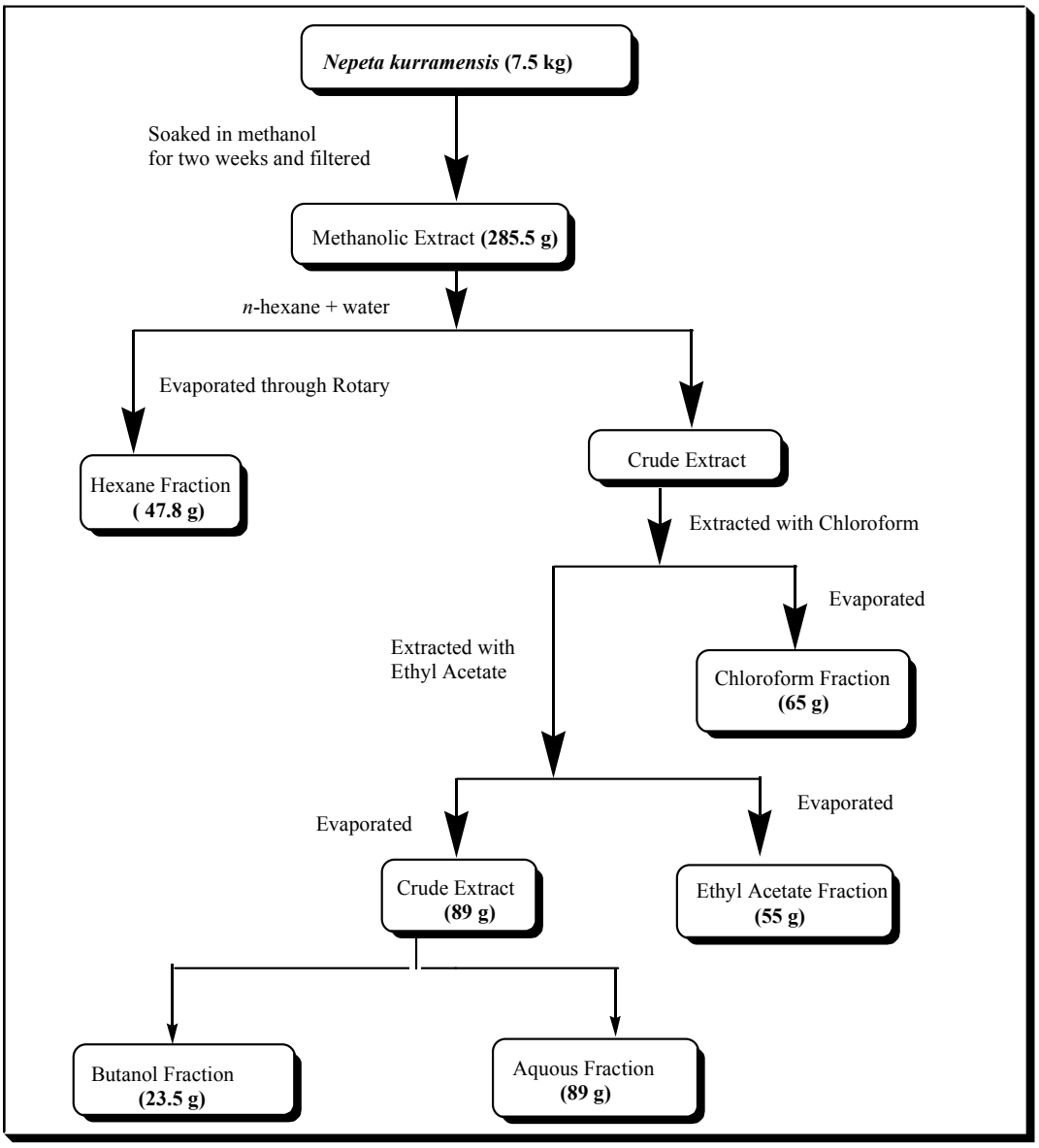


Figure 2.1: Schematic diagram for the preparation of *N. kurramensis* fractions.

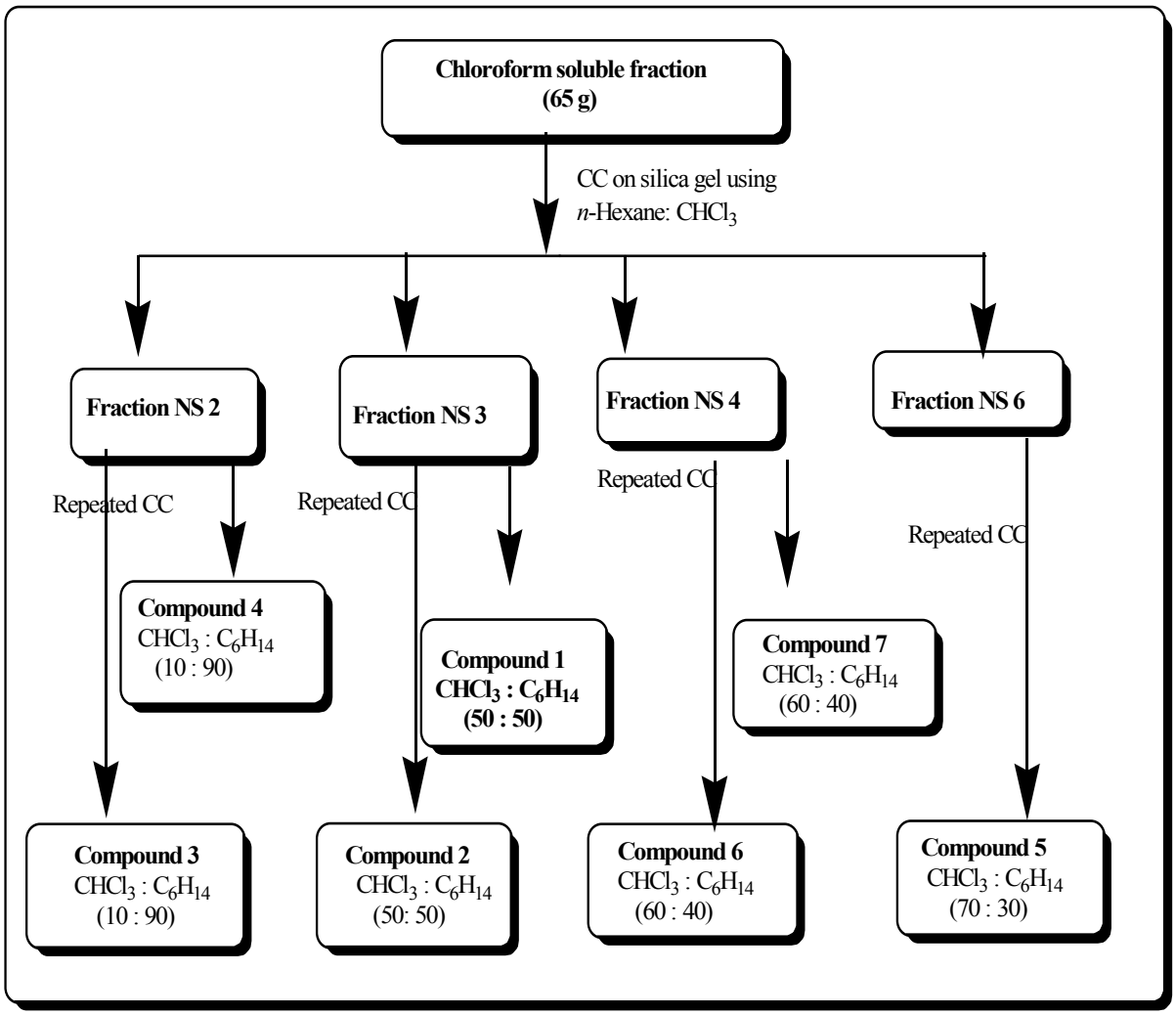


Figure 2.2: Schematic diagram for the isolated compounds from chloroform fraction of *Nepeta kurramensis*.

2.5 Isolation and purification from *Rhynchosia reniformis*

The bioassays guided isolation from the chloroform soluble fraction of *R. reniformis* resulted in isolation of two flavonoids (**8** and **9**). Chloroform soluble fraction was subjected to column chromatography and four sub fractions (NS A, NS B, NS C and NS D) were obtained. The sub fractions NS B and NS C provided compound **8** and **9** with repeated column chromatography using solvent system chloroform:*n*-hexane (50:50) and chlorofrom:hexan (60:40), respectively.

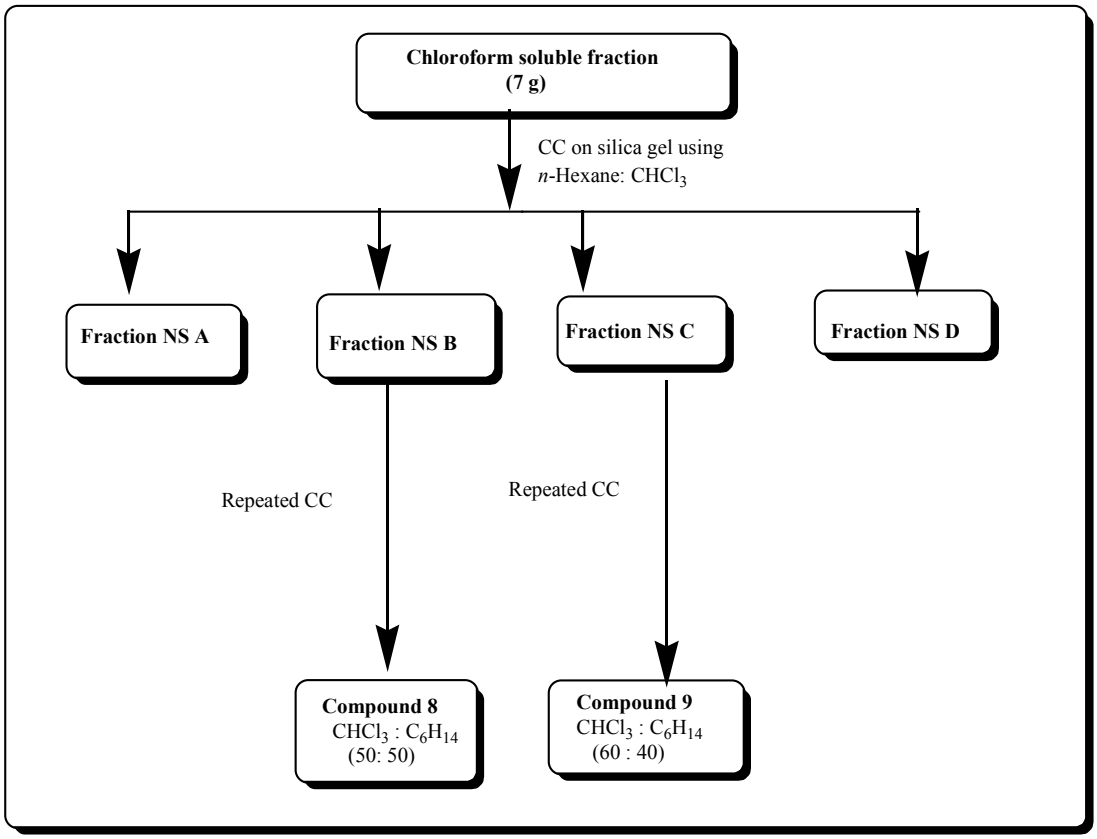
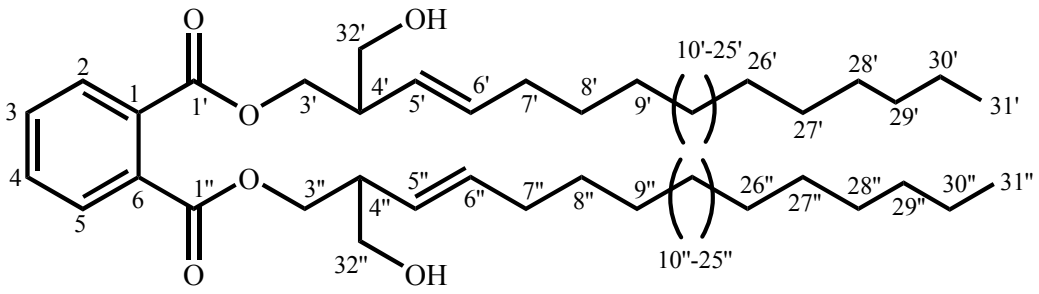


Figure 2.3: Schematic diagram for the isolated compounds from chloroform fraction of *Rhynchosia reniformis*.

2.6 Physical and spectral data of the compounds

2.6.1 Characterization of Bis (E)-2-(hydroxymethyl) nonacos-3-enyl) phthalate (1)

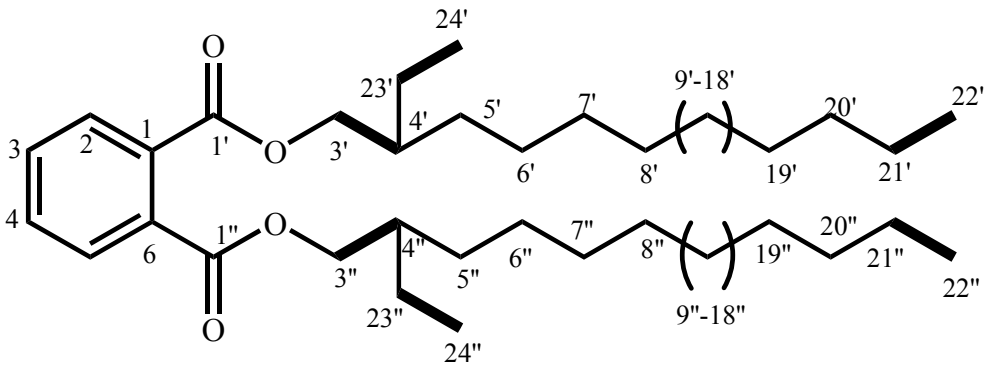
Molecular formula:	$C_{68}H_{122}O_6$
Physical state:	yellowish oil
M.P	207-209 °C
HR- EI-MS (*rel. int.* %) *m/z*:	1034.924
EIMS (*rel. int.* %) *m/z*:	451, 421, 351, 281, 149, 113, 85 and 57.
IR ν_{max} ($CHCl_3$) cm^{-1}:	3345 (OH), 2814 (C-H), 1725 (C=O), 1611, 1575, 1445, 745 cm^{-1} (aromatic).
^{1}H-NMR and ^{13}C data:	see **Table 3.1**



Kurramenate (1)

2.6.2 Physical data for compound Bis (2-ethylicosyl) phthalate (2)

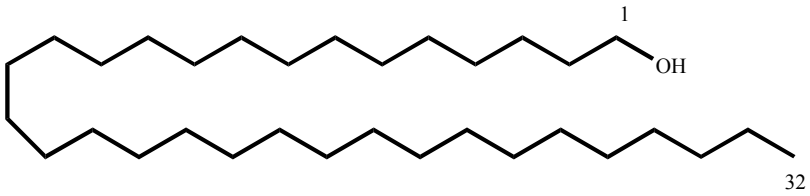
HR-EI-MS (*rel. int.* %) *m/z:*	782.7148 (calcd for $C_{52}H_{94}O_4$, 782.7152).
EIMS (*rel. int.* %) *m/z*:	353, 325, 295, 281, 239, 197, 149, 113, 85 and 57.
IR ν_{max} ($CHCl_3$) cm^{-1}:	3030, 2810 (C-H), 1732 (CO), 1600–1580 (aromatic C=C), 1450, 1405, 950 cm_2
^{1}H-NMR and ^{13}C data:	see **Table 3.2**



bis (2-ethylicosyl) phthalate (2)

2.6.3 Physical Constants and Spectral Data 1-dotriacontanol (3)

Molecular formula:	$C_{32}H_{66}O$
Physical state:	Colorless solid
M.P	204-206 °C
EI-MS (*rel. int.* %) *m/z*:	466 $[M]^+$, 392, 167, 125, 97 and 57.
^{1}H-NMR ($CDCl_3$, 400 MHz):	δ 3.63 (αH, H-1), 0.88 (3H, H-32), 1.53 (2H, C-2), 1.23 (2H, C-4-31).
^{13}C-NMR ($CDCl_3$, 100 MHz):	δ 63.4 (C-1), 32.6 (C-2), 31.7 (C-3), 29.1-29.6 (C-4-31, 14.1 (C-32).



1-dotriacontanol

2.6.4 Characterization of 1-nonacosanol (4)

Molecular formula: $C_{29}H_{60}O$

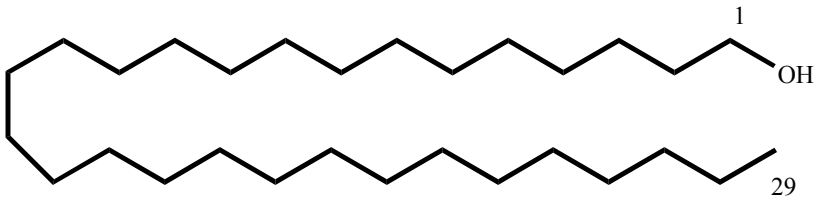
Physical state: Colorless solid

M.P 204 °C

EI-MS (*rel. int.* %) *m/z*: 424 $[M]^+$, 392, 167, 125, 97 and 57.

^{1}H-NMR ($CDCl_3$, 400 MHz): δ 3.63 (αH, H-1), 0.87 (3H, H-30), 1.53 (2H, C-2), 1.23 (2H, C-4-29).

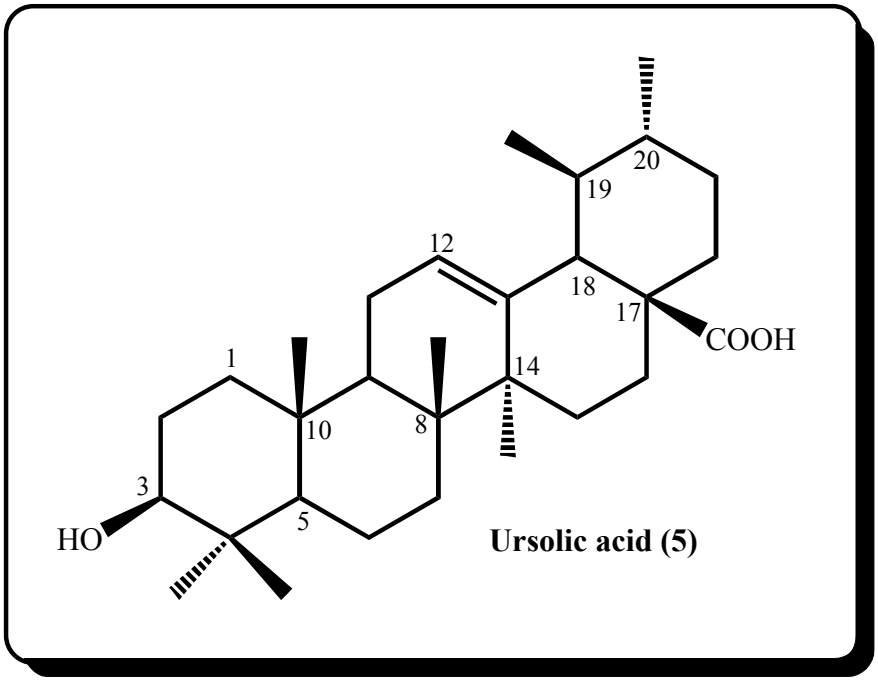
^{13}C-NMR ($CDCl_3$, 100 MHz): δ 63.1 (C-1), 32.8 (C-2), 31.9 (C-3), 29.0-29.3 (C-4-29, 14.1 (C-30).



1-nonacosanol (4)

2.6.5 Physical constants and spectral data Ursolic acid (5)

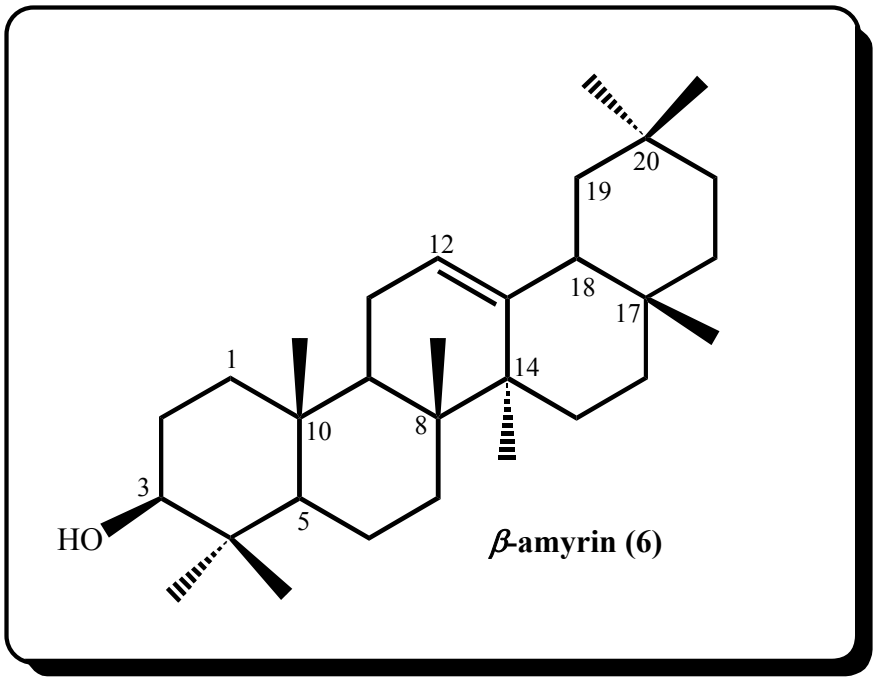
Molecular formula:	$C_{30}H_{48}O_3$
Physical state:	Colorless needles **(**22 mg).
M.P	283-285 °C
IR ν_{max} ($CHCl_3$) cm^{-1}:	3510 (OH), 3050, 1697 (C=O), 1635 and 820.
EI-MS (*rel. int.* %) *m/z*:	456 $[M]^+$, 411, 393, 248, 207, 203, 133,119 and 55.
^{1}H-NMR and ^{13}C data:	see **Table 3.3**



Ursolic acid (5)

2.6.6 Characterization of *β*-amyrin (6)

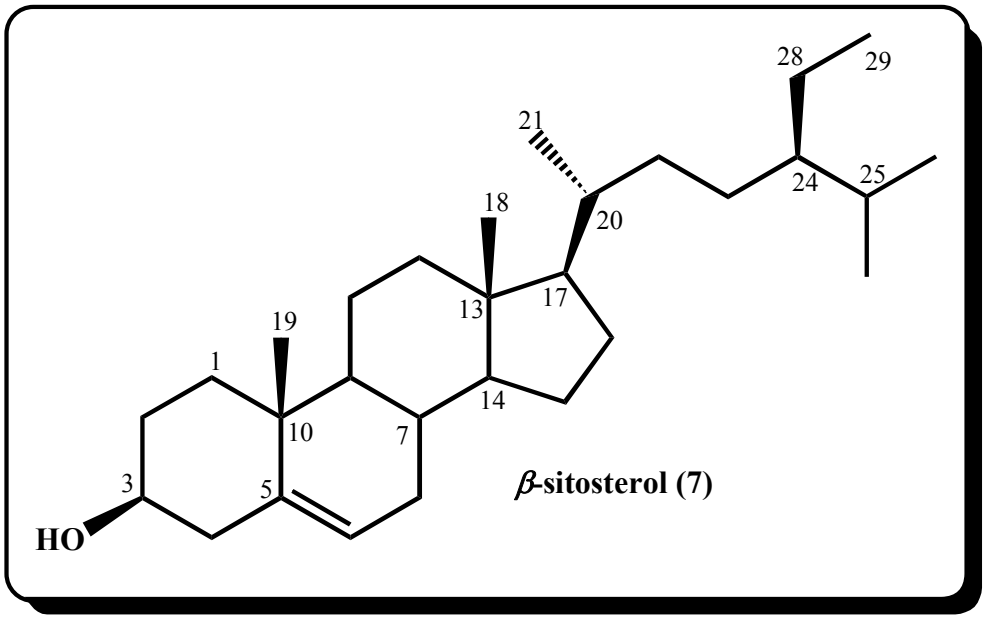
Molecular formula:	$C_{30}H_{50}O$
Physical state:	Colorless crystalline solid (15.2 mg).
M.P	197-198 °C
IR ν_{max} ($CHCl_3$) cm^{-1}:	3510, 3055, 1635 and 820.
EI-MS (*rel. int.* %) *m/z*:	426 $[M]^+$, 408, 393, 257, 218, 203, 189 and 85.
^{1}H-NMR and ^{13}C data:	see **Table 3.4**



***β*-amyrin (6)**

2.6.7 Characterization of *β*-sitosterol (7)

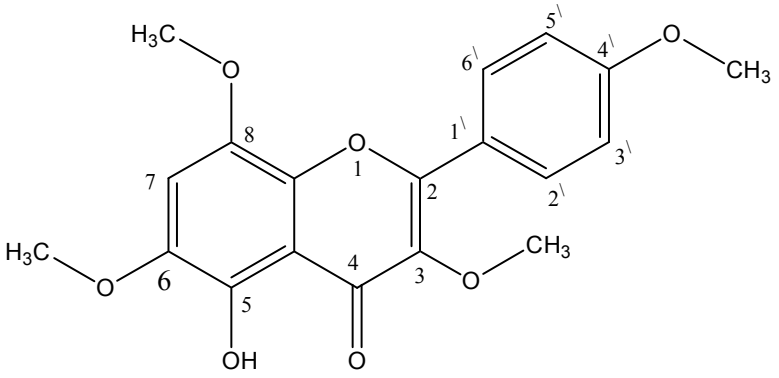
Molecular formula:	$C_{29}H_{50}O$
Physical state:	Colorless needles
M.P	135 °C
IR ν_{max} ($CHCl_3$) cm^{-1}:	3446 (OH), 3050 (C=C), 1650, and 815.
EI-MS (*rel. int.* %) *m/z*:	414 $[M]^+$, 396, 381, 329, 371, 303, 273, 255, 99 and 85
^{1}H-NMR and ^{13}C data:	see **Table 3.5**



***β*-sitosterol (7)**

2.6.8 Physical and spectral data of 5-hydroxy-3,6,8,4′-tetramethoxyflavon (8)

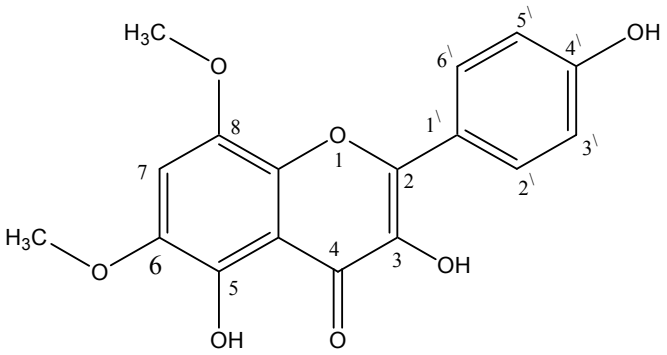
Molecular formula:	$C_{19}H_{18}O_7$
Physical state:	Yellow powder
[α] $_{25}$ ^{D} :	+93.6 ^{0} (C = 0.01 MeOH)
MP:	193 ^{0}C
UVλ $_{max}$ (MeOH) nm (log ε):	273 (4.00), 345 (4.31)
IR (KBr) cm^{-1}:	3410 (OH), 2925 (OCH3), 1690 (α, β-unsaturated C=0), 1620 (aromatic ring)
^{1}H-NMR and ^{13}C data:	see **Table 3.6**



5-hydroxy-3,6,8,4\-tetramethoxyflavon (8)

2.6.9 Physical and spectral data of 3,5,4′-trihydroxy-6,8-dimethoxyflavone (9)

Molecular formula:	$C_{17}H_{14}O_7$
Physical state:	Yellow powder
$[\alpha]_{25}^{D}$:	$+96.4^{0}$ (C = 0.01 MeOH)
MP:	$180\ ^{0}C$
UVλ_{max} (MeOH) nm (log ε):	272 (4.76), 338 (4.53)
IR (KBr) cm^{-1} :	3412 (OH), 2923 (OCH3), 1691 (α, β-unsaturated C=O), 1621 (aromatic ring)
^{1}H-NMR and ^{13}C data:	see **Table 3.7**



3,5,4\-trihydroxy-6,8-dimethoxyflavone (9)

PHASE 2: BIOLOGICAL ASSAYS

2.7 Antibacterial methodology

For antibacterial review agar diffusion procedure as recognized by Khan, *et al.,* (2011) and Rahman *et al.,* (2008) was espoused with slight adjustment**.** In this scheme, wells were prepared in petriplates and the solution of veteran plants extort were poured in these wells and after incubation of 24 hours, the precincts made around these wells were calculated and evaluated with the zones made around the customary antibiotic used.

2.7.1 Preparation of stock solution

The test solution was prepared by adding 150 mg of the plant extaract in 10 ml of the pure autoclaved refined water to obtain 15 mg/ml.

2.7.2 Pathogenic microbes used

Seven bacterial species and eight fungal strains were used for antibacterial and antifungal analysis. The bacteria were *Bacillus subtilis, Pseudomonas testosteroni, Eschericha coli, Klebsiella pneumonia, Streptococcus cricetus, Proteus morganii,* and *Micrococcus flavus* and fungal species were *Aspergillus niger, Aspergilus fumigatus, Aspergilus flavus, Fusarium solani, Candida albicans, Rhizoctonia solani, Pseudallescheria boydii and Microsporum canis*. All the pathogens were managed from Microbiology Department, Kohat University of Science and Technology, Kohat, Pakistan. The microbes were kept at 4 ^{o}C on nutrient agar media.

2.7.3 Preparation of inoculums

Two or three colonies of bacteria were taken from 24 hours old culture and the colonies were assorted in 10 ml nutrient broth media in a test tube. The test tubes were placed in incubator at 37 ^{o}C for 24 hours. An autoclaved briny solution was sundry in the test tubes including bacterial cultures and their turbidity were accreted until harmonized with turbidly of McFarland 0 .5 $BaSO_4$ typical. This inoculum's was set for seeding nutrient agar plates.

2.7.4 Preparation of the seeded agar plates

The 20 g nutrient agar was taken in one liter distilled water and autoclaved to organize nutrient agar media. The media was then permitted to chill up to 45 ^{o}C. 75 ml media was dispensed into each petriplate and allowed to congeal. Bacterial strains from broth media were scrubbed by an inoculating sphere or swab. Fissure or wells were made in each plate by a cork borer (8mm).

2.7.5 Pouring of test solutions, incubation and measurement of zone of inhibition

The 100 µl of the test solutions, and identified concentration of antibiotic (chloramphenicol) were placed in their relevant wells. Antibiotic was used as positive control while the filter-sterilized solvents without extracts were used as negative controls. The plates were then incubated at 37 ^{o}C in paraffin oven. The measurement of the length of the zones around wells were measured and then evaluated with the diameter of the zones produced by antibiotic.

2.8 Antifungal activity

The rapid susceptibility assay (RSA) is based on substrate utilization by fungi in the presence of antifungal drugs. Substrate uptake is determined by a colorimetric method, which can be scored by analysis of data obtained from a microplate reader. Variables evaluated in the development of the RSA included inoculum size, incubation period, and efficacy with different classes of antifungal drugs and different yeast isolates. With the rapidly available and quantitative end points of the RSA, correlation of minimal drug concentration (MICs) and therapeutic drug doses can be evaluated more successfully. Strains were subcultured twice on plates of Sabouraud dextrose agar (SDA), with the Emmons modification and each subculture was grown for 23 to 25 h in a humidified incubator with atmospheric air at 37 °C. The use of a single temperature for the subcultures helped limit possible subsequent variability in the preparation of inoculum suspensions. Stock suspensions of the strains were maintained in sterile deionized water at room temperature. To prepare the inoculum, isolated colonies from the second-subculture plates were suspended in RPMI-0.2G to achieve a turbidity

approximating a 0.5 McFarland standard. This suspension was used to prepare the inoculum dilutions for the RSA assay.

The RSA was performed with minor modifications [Riesselman *et al.,* 2000; Chowdhury *et al.,* 2004]. The 0.5 McFarland standard inoculum suspensions was diluted 1:4 in test medium, and 100 µl of the dilute suspension was added to each of the drug wells and the positive control wells in quadruplicate. The negative control well received 100 µl of test medium. Once inoculated, plates were placed in a humidified incubator under atmospheric air at 37 °C and incubated for 6 to 7 hours. To determine the amount of glucose left in each well, an enzyme substrate color mix was added to each well with an eight-channel repeating pipette and color development was allowed to proceed for 20 min. The color mix was always added beginning with the negative control column and progressing sequentially to the column with the highest drug concentration. After incubation, the relative amount of glucose in each well was determined with a colorimetric glucose oxidase assay [Riesselman *et al.,* 2000] and the percent residual glucose for each drug concentration relative to the level in the growth control was calculated by using the following formula:

$$[(A_{drug} - A_{growth\ control})/(A_{neg\ control} - A_{growth\ control})] \times 100$$

where A is the average absorbance at a wavelength of 540 nm, Adrug is the value for the wells containing the drug, Agrowth control and Aneg control are the values for the growth control and the negative control, and (Aneg control − Agrowth control) is the glucose consumption dynamic range.

2.9 Materials and methods antiglycation

The bovine serum albumin (BSA) solution (10 mg/mL) was prepared in 67 mM phosphate buffer of pH 7.4. Anhydrous glucose 50 mg/mL solution was also prepared in 67 mM phosphate buffer of pH 7.4, Sodium azide (3 mM) was added in required quantity of phosphate buffer to inhibit the bacterial growth. Plant extract\fractions were dissolved in DMSO and 1mg/mL concentration was used. 96-well plate containing 60 mL of the test sample per well in triplicate was used. Each well contained 20 mL of BSA, 20 mL of glucose and 20 mL of plant extract. A blank sample containing only BSA dissolved in

phosphate buffer and positive control sample having BSA and glucose, were prepared and incubated for a week at 37 °C. After incubation in 96- well plate for a week, samples were removed and cooled at room temperature. Then 6 μL of 100 % trichloro acetic acid (TCA) was added to each well. Supernatant containing unbounded glucose, inhibitor and interfering substances were removed after centrifugation at 14,000 rpm for 4 minutes, pellets were obtained at the bottom of the wells. Then supernatant was removed from each well and 60 mL of PBS (pH 10) was added to dissolve the pellets for screening. The comparison of fluorescence intensity at 370 nm excitations and emission at 440 nm was obtained by using spectrofluorimeter [Nakagawa *et al.,* 2002, Matsuda *et al*., 2002, Matsuura *et al*., 2002, Duraisamy *et al.,* 2003]. Rutin, a standard inhibitor, showed 82.5% inhibition of glycation at 3 mM concentration [Atta-ur-Rahman *et al.,* 2007].

2.10 Antiplatelet materials and methods

Antiplatelet assay was performed by same methodology as used by Saeed *et al.*, (2007**).** Briefly, platelet effects were studied in human platelets by taking blood via venipuncture from normal human volunteers reported to be free of medication for 7 days. Blood sample were mixed with 3.8% (w/v) sodium citrate solution (9:1) and centrifuged at 260 × g for 15 min at 20 °C to obtain platelet-rich plasma (PRP). The remaining blood samples were centrifuged at 1200 × g for 10 min to obtain platelet-poor plasma (PPP). Platelet count was determined by phase contrast microscopy and all aggregation studies were carried out at 37°C with PRP having platelet counts between 2.5 and 3.0 × 108 ml–1 of plasma. Aggregation was monitored using dual-channel Lumi-aggregometer (Model 400 Chronolog Corporation, Chicago, USA) using 0.45 ml aliquots of PRP [Saeed *et al*., 2007]. The final volume was made up to 0.5 ml with the test fraction. Aggregation was induced by AA (1.7 mM) or PAF (0.8 μM). The antiplatelet effects of test fractions were studied by pretreatment of PRP with each fraction for 2 min followed by addition of platelet agonist. The resulting aggregation was recorded for 5 min after challenge by the change in light transmission as a function of time. After establishing the anti-platelet activity against various agonists, dose-response curves were constructed to calculate the $1C_{50}$ values.

2.11 Evaluation of antioxidative activity

Measurement of the inhibition of hydroxyl radical generation

Extracts or compounds that were dissolved in 10% EtOH (final concentration: 0.4%) were added to 1 mM H_2O_2 and 0.2 mM $FeSO_4$ (Fisher Scientific, Fair Lawn, N.J.) and incubated at 37 °C for 5 minutes. Esterase-treated 2 M DCHFDA (Molecular Probes Inc., Eugene, Oregon) in 100% EtOH was then added, and the changes in fluorescence were monitored on a microplate fluorescence spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT), with excitation and emission wavelengths of 460 and 530 nm, respectively, for 30 min [Ahmad *et al.,* 2008, Nagao *et al*., 1999]. Trolox being an effective standard oxidant was used as a positive control.

Measurement of the inhibition of total reactive oxygen species (ROS) generation

Rat kidney homogenates prepared from the kidneys of freshly killed male Wistar rats, weighing 130-180 g, were mixed with or without the suspension of extracts or compounds, which were dissolved in 10% EtOH (final concentration: 0.4%). The mixtures were then incubated with 12.5 mM 2',7'- dichlorodihydrofluorescein diacetate (DCHF-DA, Molecular Probes Inc., Eugene, Oregon), which was dissolved in 100% EtOH (final concentration: 0.2%), at 37 °C for 30 minutes. A 50 mM phosphate buffer (Wako Pure Chemical Industries, Osaka, Japan) solution at pH 7.4 was also used. DCHF-DA is a stable compound, which is hydrolyzed by intracellular esterase to yield a reduced, nonfluorescent compound, 2', 7'-dichlorodihydrofluorescein (DCHF). The ROS produced by the homogenates oxidizes the DCHF to highly fluorescent 2',7'-dichlorofluorescein (DCF). The fluorescence intensity of the oxidized DCF was monitored using a microplate fluorescence spectrophotometer (Bio- Tek Instruments Inc., Winooski, VT) with excitation and emission wavelengths of 460 and 530 nm, respectively [Label and Bondy, 1990]. Trolox being an effective standard oxidant was used as a positive control.

Measurement of nitric oxides ($ONOO^-$) scavenging activity

The ONOO- scavenging activity was measured by monitoring the oxidation of dihydrorhodamine 123 (DHR 123, Molecular Probes Inc., Eugene, Oregon) using a slight

modification of the method reported by Kooy *et al.,* (1994). DHR 123 (5 mM) in DMF, which was purged with N_2, was stored as a stock solution at 80 ^{0}C. This solution was then placed on ice and kept in the dark prior to the study. The buffer consisted of 90 mM NaCl, 50 mM Na_3PO_4, 5 mM KCl at pH 7.4, and 100 M diethylenetriaminepentaacetic acid (DTPA), each of which was prepared with high-quality deionized water and purged with N_2. The final concentration of DHR 123 was 5 M. The background and final fluorescent intensities were measured 5 min after treatment with and without the authentic ONOO-. DHR 123 was oxidized rapidly by the authentic ONOO-, and the final fluorescent intensity of the oxidized DHR 123 was measured using a FL 500 microplate fluorescence reader (Bio-Tek Instruments Inc., Winooski, VT) at excitation and emission wavelengths of 480 and 530 nm, respectively. The results are expressed as the mean ± standard error (n = 3) for the final fluorescence intensity minus background fluorescence. The effects are expressed as the percent inhibition of DHR 123 oxidation, and the standard oxidant, DL-penicillamine was used as a positive control. The IC_{50} was defined as the concentrations of sample showing 50 % scavenging activity and were calculated from a triplicate experiment in all of the three scavenging tests.

2.12 Cytotoxicity materials and methods

Using the protocol of Meyer *et al*., (1992), brine shrimp (*Artemia salina*) eggs were hatched in a shallow rectangular plastic dish, filled with artificial seawater, which were prepared by mixing a commercial salt mixture (Instant Ocean, Aquarium System, Inc., Mentor, OH, USA) with double distilled water. Unequal partitions were made in the plastic dish with the help of a perforated device. An approximately 50 mg of eggs were sprinkled into the large compartment, which were darkened while the smaller compartments were opened to ordinary light. After two days a pipette were collected, naupil from the lighted side. Samples of the test fraction were prepared by dissolving 20 mg of each fraction in 2 ml of methanol. From this stock solution, 10,100 and 1000 µg/mL was transferred to 12 vials; three for each dilution and three vials were kept as control having 2 ml of methanol only. The solvent was allowed to evaporate overnight. After two weeks, when shrimp larvae was ready, one ml of sea water was added to each vial along with 10 shrimps and the volume was adjusted with sea water to 5ml per vial.

After 24 hrs, the numbers of surviving shrimps were counted. Data was analyzed by a Finney computer program to determine the LD50 [Meyer *et al*., 1992].

2.13 Phytotoxicity materials and methods

This test was performed according to the modified protocol of McLaughlin *et al.,* (1988). The test fractions were incorporated with sterilized E-medium at different concentrations i.e. 10, 100, 1000 µg/mL in methanol. Sterilized conical flasks were inoculated with fractions of desired concentrations prepared from the stock solution and were allowed to evaporate overnight. Each flask was inoculated with 20 ml of sterilized E-medium and added ten *Lemna minor* each containing a rosette of three fronds. Other flasks were supplemented with methanol serving as negative control and reference inhibitor i.e. Parquet serving as positive control. Treatments were replicated three times and the flasks were incubated at 30 °C in Fisons Fi-Totron 600H growth cabinet for seven days, 9000 lux intensity, 56+10 rh (relative humidity) and 12 hours day length. Growths of Lemna minor in fraction containing flasks were determined by counting the number of fronds per dose and growth inhibitor calculated with reference to negative control [McLaughlin *et al.,* 1988, Finney 1971].

PHASE 3: PROXIMATE ANALYSIS

2.14 Materials and methods

Proximate analysis of a plant sample determines the total protein, fat, carbohydrate, ash, and moisture reported as the percentage composition of the product. All the samples were milled using mortar and piston and powdered samples were used for proximate analysis.

AOAC methods were applied to carryout proximate analysis of the samples for moisture, ash, crude fibers, crude fats, proteins and carbohydrates. The nitrogen was determined by micro Kjeldahl method described by Pearson (1976) and the nitrogen content was converted to protein by multiplying by a factor of 6.25. Carbohydrate was determined by difference. All the proximate values were reported in percentage [AOAC, 2000; AOAC, 2003].

2.14.1 Determination of moisture

Three gram of plant material was taken in a petri dish and placed in the oven at 105 ºC for 12 hours. It was then removed, cooled in a desicator and weighed. The sample was heated again in the oven for another two hours and the process was repeated, till a constant weight was obtained. The moisture content was calculated using the following formula. The process was repeated two times more for getting a more precise data. The apparatus used in the moisture determination were oven, desiccators and analytical balance [AOAC, 2000, AOAC, 2003].

Calculation

$$\text{Moisture Content (\%)} = \frac{(B-A) - (C-A)}{(B-A)} 100$$

Where:

W_1= A = weight of clean, dry petri dish (g)

w_2 = B = weight of Petri dish + wet sample (g)

w_3 = C = weight of Petri dish + dry sample (g)

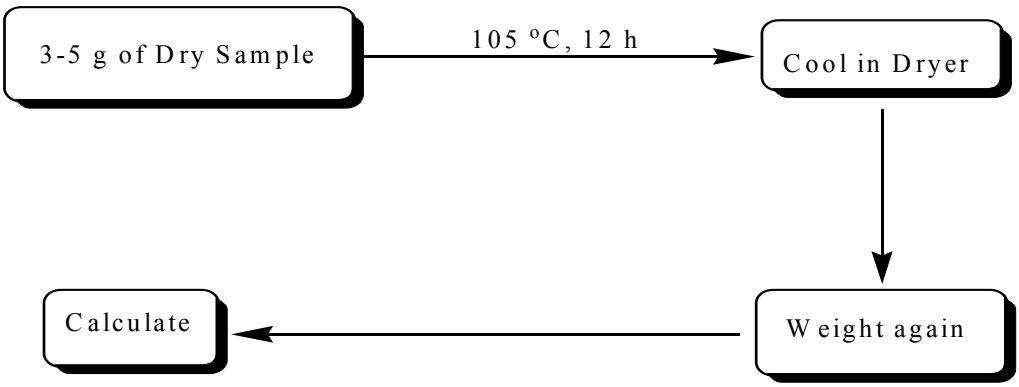


Fig 2.4: Diagrammatical presentation of humidity content in feed ingredients

2.14.2 Determination of ash

One gram dried sample was taken in a crucible. The sample was charred over a low flame. The crucible was then kept in a muffle furnace set at 550 ^{o}C and left until white ash was obtained. The ash was moistened with water, dried on steam and then on hot plate. The crucible was again placed in the muffle furnace at 550 ^{o}C, till a constant weight was obtained. The percent ash was calculated by the formula. The apparatus used in the ash determination were muffle furnace, hot plate, and desiccators [AOAC, 2000; AOAC, 2003]

Calculations:

$$\text{Ash (\%)} = \frac{W_1 \times 100}{W_2}$$

W_1 = Weight of sample after ashing

W_2 = Weight of sample

2.14.3 Crude fat

In this method, the fats are extracted from the sample with petroleum ether and evaluated as a percentage of the weight before the solvent is evaporated.

Method

First of all extraction flasks were taken from the kiln without touching them with the fingers then cool in a dryer and weighed (mg). After weighing 3 to 5 g of dry sample (mg) in an extraction thimble, handling it with tongs and placed in the extraction unit. Connected the flask containing petroleum ether at 2/3 of total volume to the extractor. Then boiled and adjust heat to obtain about 10 refluxes per hour (Fig. **2.5**). When finished, evaporated the petroleum ether by distillation or in a Rotavapor. Cooled the flasks in a dryer and weighed again. The length of the extraction will depend on the quantity of lipids in the sample. Very fatty materials will take 6 hours and the defatted sample can be used in determining crude fibers. The apparatus used in the determination of fat were heating mantle, soxhlet extraction, desiccators and oven [AOAC, 2000; AOAC, 2003].

Calculations

$$\text{Crude lipid content (\%)} = \frac{(B-A)}{C} 100$$

A = W_1 = weight of clean dry flask (g)

B = W_2 = weight of flask with fat (g)

C = W_3 = weight of sample (g)

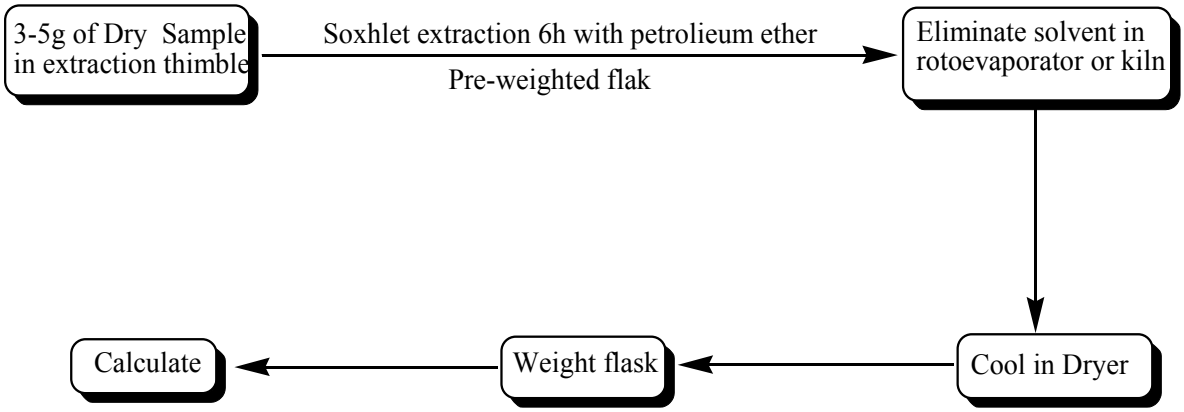


Fig 2.5: Diagrammatical presentation of lipids by Soxhlet's method

2.14.4 Determination of crude fiber

One gram of the defatted plant material was taken. The sample was placed in a beaker and boiled in 200 mL of 1.25% sulphuric acid for 30 minutes. After the contents were filtered through linen cloth in the fluted funnel and washed with distilled water to neutralize the contents. The contents were transferred again to the beaker and boiled in 200 mL of 1.25% sodium hydroxide for 30 minutes. After that, contents were again filtered, washed with distilled water for neutralization. A Gooch crucible was prepared with an asbestos mat and contents of beaker were placed on the mat and washed with 15 mL of ethyl alcohol. The crucible was dried in an oven at 110 ^{o}C to a constant weight. The crucible having crude fiber was cooled and weighed (W_1). The apparatus used in the determination of fiber were muffle furnace, oven and suction pump.

The contents of the crucible were ignited over a low flame until charred and then kept in a muffle furnace at 550 ^{o}C and weighed (W_2) [AOAC, 2000; AOAC, 2003].

Formula:

$$\text{Crude fiber (\%)} = \frac{W_1 - W_2 \times 100}{W_3}$$

Where

W_1 = Weight of sample before ignition

W_2 = Weight of sample after ignition

W_3 = Weight of sample

2.14.5 Determination of protein by micro Kjeldahl's method

The protein determination is divided into three steps:

(1) Digestion
(2) Distillation
(3) Titration

Digestion

Dried plant material (0.5 g) was taken and transferred to a digestion flask. To this about 1 g of the digestion mixture (copper sulphate, potassium sulphate and ferrous sulphate, 1:18:0.2) was added and about 20 mL of concentrated sulphuric acid was added. The solution was heated until the solution become clear and frothing ceased. Then it was boiled briskly for another 2 hours, cooled and to the digest about 50 mL water was added in 5ml portion with mixing. The digest was transferred to a 100 mL volumetric flask and made the volume up to the mark [AOAC, 2000; AOAC, 2003].

Distillation

The Parnas Wagner distillation assembly was arranged. About 100 mL of 4% boric acid was taken and a drop of methyl red indicator was added, and pink color appeared. Five milliliter of the digest was transferred to the distillation assembly and 10 mL of 50% sodium hydroxide solution was added to the digest in the assembly. The distillation was completed in 6 minutes, with change of color of boric acid to yellow due to the formation of ammonium borate.

Titration

The boric acid having trapped ammonia was titrated with 0.1N hydrochloric acid, the colour of boric acid having ammonia changed again to pink. The percent protein was calculated by the formula. The instrument used in the determination of protein was micro Kjeldahl, burette, hot plate and digestion.

Calculations:

$$\% \text{ Protein} = \left[\frac{(\text{volume used for sample} - \text{volume used for blank}) \times 1.4007 \times \text{E.N})}{\text{Weight of sample taken}}\right] \times 6.25$$

Where:

1.4007 = Weight of nitrogen expressed in gram in the formula

6.25 = Protein factor

E.N = Exact Normality

2.14.6 Determination of carbohydrate

Carbohydrate was determined by difference, using the following the formula:

Formula:

Carbohydrate (in grams) = 100 - (% Moisture + % crude fat + % Ash + % protein)

2.14.7 Determination of energy values

Energy Values was determined by using the following formula,

Formula:

K calories/ 100 gm: [(9× % crude fats) + (4 × % carbohydrates) + (4 × % proteins)]

PHASE 1: ISOLATION OF NATURAL PRODUCTS AND THEIR STRUCTURAL ELUCIDATION

Pakistan has a durable folklore of herbal remedies and like utmost developing countries; its rural population still depends primarily on the native system of medicine for their fitness related issues. It was, therefore, looked stimulating to evaluate systematically and determine the effectiveness of the medicinal plants. The aim of bioscreening is to realize the leeway of utilization of the samples (synthetic or natural) in future for humanity. The first step to drug discovery is the bioscreening of biologically active compounds. A total of seven compounds (**1-7**) have been isolated from the chloroform fraction of *Nepeta kurramensis* out of which compound **(1)** is identified as new isolate from plant origin and two known flavonoids (**8**, **9**) have been isolated and characterized for the first time from *Rhynchosia reniformis*.

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3.1 Bis((*E)*-2-(hydroxymethyl)nonacos-3-enyl)phthalate (1)

Compound (**1**) was isolated as yellowish oil from the chloroform fraction of *N. kurramensis* in the ratio (70:30, chloroform in hexane). The molecular formula of compound (**1**) was found to be $C_{68}H_{122}O_6$ by HR-EI-MS analysis [*m/z* 1034.924], which was supported by ^{1}H, ^{13}C, and DEPT NMR data. The mass spectrum exhibited signals at *m/z* 451, 421, 351, 281, 149, 113, 85 and 57 corresponding to the skeleton of phthalate derivatives (Figure 3.1). The UV spectrum of (**1**) showed the presence of conjugated system (276 nm) [Hussain, *et al.,* 2011]. The IR spectrum of (**1**) showed absorptions for hydroxyl (OH) 3345 cm-1, alkane (2814 cm-1, C–H stretch), carbonyl (1725 cm^{-1}), aromatic (1611, 1575, 1445, 745 cm^{-1}).

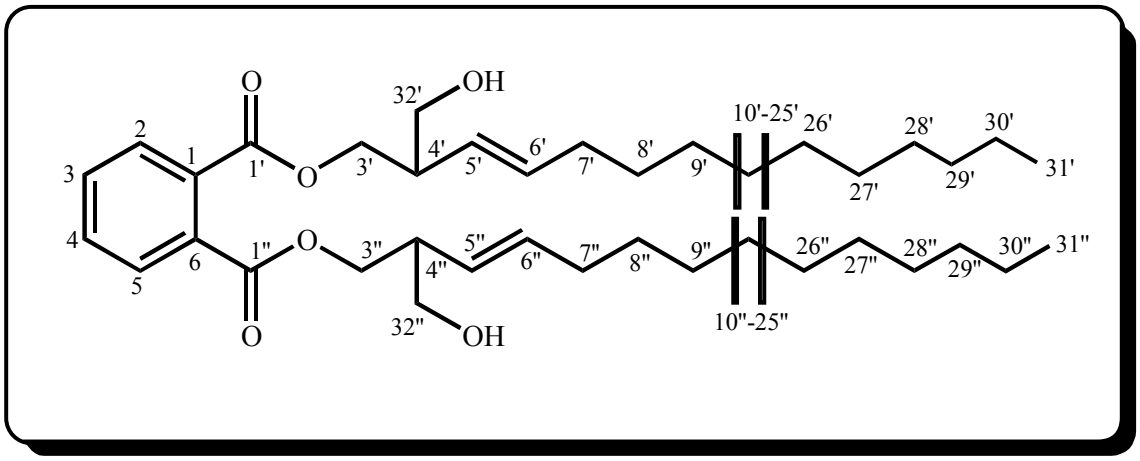

Figure 3.1: Kurraminate (1)

An AA′BB′ system existed in ^{1}H-NMR spectrum at δ 7.51 (2H, dd, *J* = 5.5, 3.5 Hz, H-2, H- 5), 7.69 (2H, dd, *J* = 5.5, 3.5 Hz, H-3, H- 4). These data accounted for all required the compound to have one benzene ring, and a side chain with two carbonyl groups [Kim *et al.,* 199; Yong *et al.,* 2008; Hussain *et. al.,* 2011]. The compound **1** showed one methine signal at δ 2.30 (2H, t, *J* = 7.5 Hz, 7.0 Hz, H-4′, 4″), one methylenes at δ 2.30 (2H, t, *J* = 7.5 Hz, 7.0 Hz, H-4′, 4″), and one methyl at 0.91 (6H, t, *J* = 7.5 Hz, H-31′, H-31″), one hydroxyl groups at 3.71 (2H, d, *J* = 7.0 Hz, H-32′, H-32″). The spectrum also designated multiplets at δ 1.23-1.27 (m, H-11′-24′, and H-11″-24″) for long chain methylenes in the molecule. These data indicated that the compound have one benzene ring (three double bonds and one ring, 4 degrees of unsaturation), and side chains with two carbonyl groups (2 degrees of unsaturation).

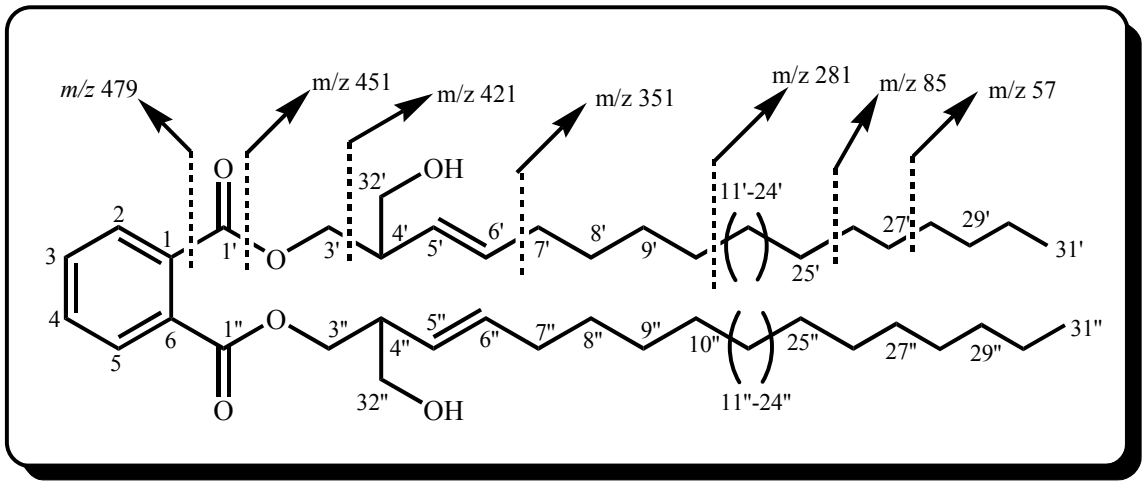

Figure 3.2: Mass fragmentation of compound (1)

The NMR data for the compound (**1**) was characteristic of a 1, 2-disubstituted benzene ring. Another important signals observed in the ^{1}HNMR at 5.32 (1H, br s, H-5′, H-5″), and 5.24 (1H, br s, H-6′, H-6″) were indicative of the double bond in the molecule. Analysis of the ^{1}H, ^{13}C, DEPT, and HMQC data of compound (**1**) showed the presence of one methyl group, twenty six methylene units (two O-bearing methylene), two sp^2 aromatic carbons, three methines, one sp^2 quaternary carbon, and one carbonyl carbon for half of the molecule.

In the ^{13}C-NMR spectrum, the resonances at δ 173.2, 132.4, 130.8, 129.8, and 62.07 indicated the nature of phthalate moiety, while the signals at δ 68.13 were assigned to C-32′, and C-32″, which revealed the presence of hydroxyl group at this C-32, which was also confirmed by HMBC and HMQC. The signals observed at 38.7, 34.2, 32.5, 30.3, 27.2-29.7, 24.8, 23.7, 22.9, and 22.6 indicated the presence of methylene carbons with double intensity (Table 3.1), which was half of the number observed in the MS spectrum, indicating two identical parts in compound (**1**). Analysis of ^{1}H and ^{13}C-NMR spectral data and the coupling constants examine for four aromatic protons led to the determination of one *Ortho*-substituted aromatic ring. These assignments were further supported by HMBC correlations of compound (**1**).

Two aromatic protons at δ 7.51, 7.69 and HMBC correlation of H-3 with C-1, C-4 and C-1′, indicating that two carbonyl carbons were directly connected to aromatic sp^2 quaternary carbons (C-1 and C-6). Due to the ^{13}C-NMR signals of these two carbonyl carbons, and ^{1}H-NMR spectral data of two *Ortho*-bearing methylene units, the structure of compound (**1**) was suggested to be a phthalate derivative [Hussain *et. al.,* 2010 and Hussain *et. al.,* 2011]. HMBC and COSY correlations of compound (**1**) also assigned the connections of the one side chain moieties. The structure of compound (**1**) was established as phthalate on the basis of these data. HMBC and COSY spectral data of compound (**1**) revealed the key correlations, H-3/6 to C-1/6, 4/3, and C-1′/1″; H-32′/32″ to C-3′/3″, C-5′/5″ and C-6′/6″; H-5′/5″ to C-4′/4″, C-6′/6″; and H-3′/3″ to C-1′/1″ and H-31′/31″ to C-30′/30″. DEPT experiment determined the multiplicities of carbons and the complete structure were elucidated on the basis of COSY, HMQC, and HMBC techniques (Figure 3.3; Table 3.1), and IUPAC name of the compound was established as bis ((*E)*-2-(hydroxymethyl) nonacos-3-enyl) phthalate.

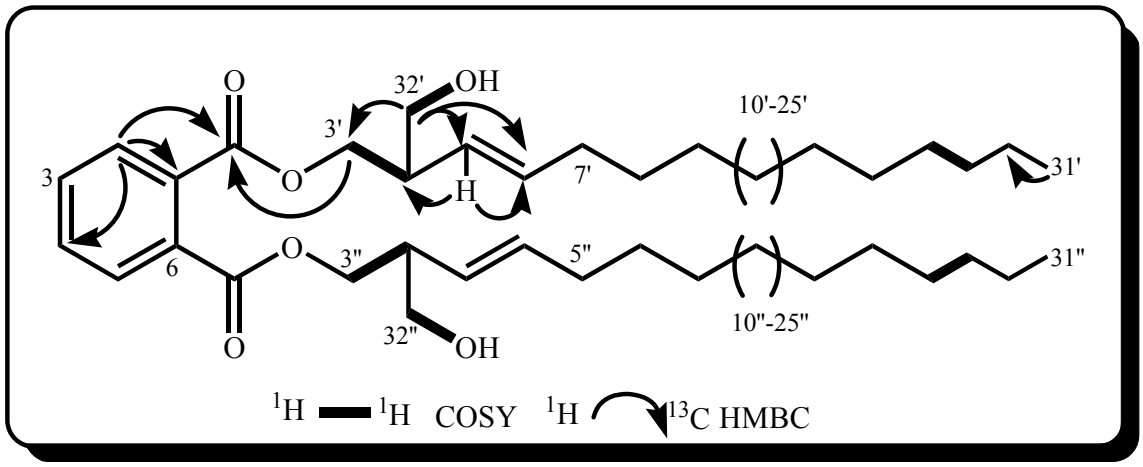

Figure 3.3: Key COSY and HMBC correlations for Bis((*E)*-2-(hydroxymethyl)nonacos-3-enyl)phthalate (**1**).

Table 3.1.^{1}H NMR (500 MHz $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) spectral data for compound (**1**)

Position	**^{1}H NMR**	**^{13}C NMR**
1,6	-	132.4
2,5	7.51 (2H, dd, *J* = 5.5, 3.5 Hz)	129.8
3,4	7.69 (2H, dd, *J* = 5.5, 3.5 Hz)	130.8 (s)
1′, 1″	-	173.2 (s)
3′, 3″	4.27 (4H, m)	62.07 (t)
4′, 4″	2.30 (2H, t, *J* = 7.5 Hz, 7.0 Hz)	38.7 (d)
5′, 5″	5.32 (1H, br s)	128.7 (s)
6″,6″	5.24 (1H, br s)	130.0 (s)
7′, 7″	1.58 (2H, d, br s)	34.2 (t)
8′, 8″	11.43 (8H, m)	32.5 (t)
9-30′, 9-30″	1.23-1.27 (remaining CH_2)	27.2-29.7
31′, 31″	0.91 (6H, t, *J* = 7.5 Hz)	14.1 (q)
32′, 32″	3.71 (2H, d, *J* = 7.0 Hz)	68.13

3.2 Bis (2-ethylicosyl) phthalate (**2**)

Compound (**2**) was achieved as light yellow oil. EI-MS of the compound (**2**) illustrates molecular ion peak at *m/z* 782.7148 ensuing to the molecular formula $C_{52}H_{94}O_4$ with other fragments at *m/z* 57, 85, 113, 149, 197, 239, 281, 295 and 353. The fragrant region of the ^{1}H NMR spectrum of **2** exhibited two gestures at δ 7.65 (2H, dd, *J* = 5.6, 2.2 Hz, H-2, 5) and 7.48 (2H, dd, *J* = 5.6, 2.2 Hz, H-3, 4) and oxygenated methylene at δ 4.12 (4H, m**,** H-3′, 3″), giving clue for the phthalate nature of the molecule [Satyan *et al.,* 1995; Al-Bari *et al.,* 2006; Saleem *et. al.,* 2009]. The spectrum further explained a methine signal at δ 1.62 (2H, m, H-4′, 4″), two methylenes at δ 1.42 (4H, m, H- 23′, 23″), and 0.93 (6H, t, *J* = 7.3 Hz, H-24′, 24″)**.** The same spectrum also confirmed multiplets about δ 1.22–1.37 for numerous methylenes in the molecule (Table **3.2)**.

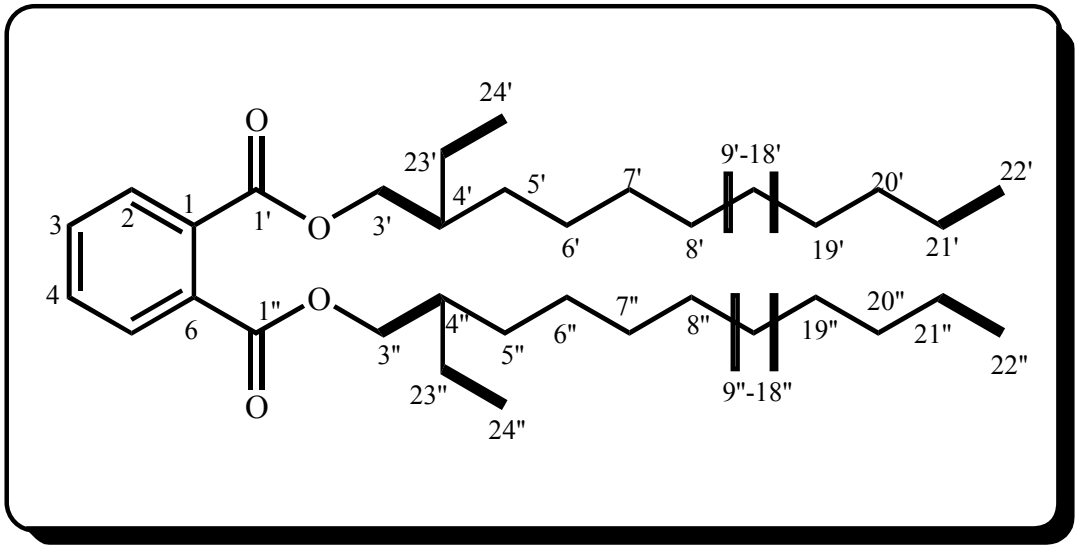

Figure 3.4: Bis(2-ethylicosyl)phthalate

The ^{13}C NMR spectrum (Broad Band (BB) and DEPT) was also very informative in the structural elucidation of compound (**2**). The signals observed between 22.7 and 22.9 also indicated the presence of long chain methylenes in the molecule. The ^{13}C-NMR spectrum demonstrated 25 carbon signals with double intensity which was half of the number pragmatic in the MS spectrum, signifying two identical parts in (**2**). The multiplicities of carbons were resolute by DEPT experiment and the absolute assignments were made on the basis of COSY-45, HMQC, and HMBC methods **(**Fig. **3.5)**. Thus, compound (**2**) was distinguished as bis (2-ethylicosyl) phthalate, earlier reported from Phyllanthus muellerianus [Satyan *et al.,* 1995; Saleem *et al.,* 2009].

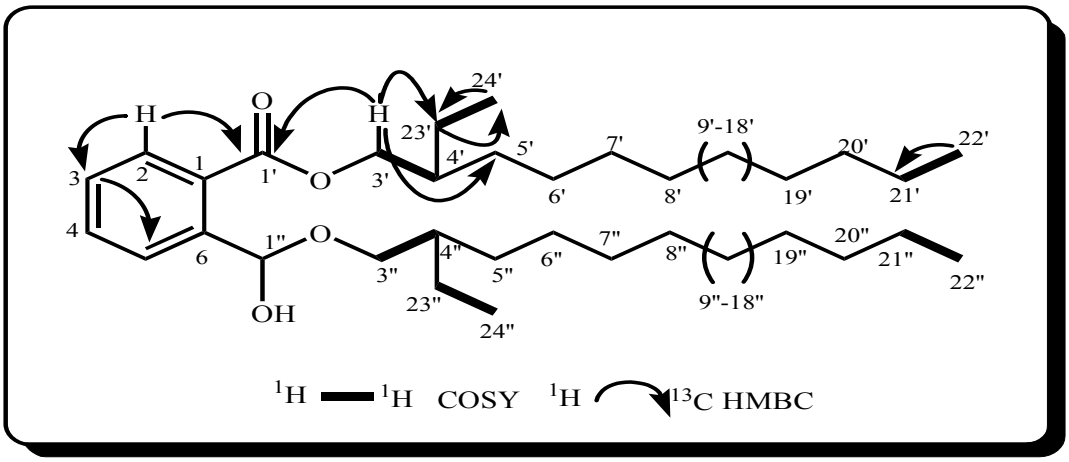

Figure 3.5: Key COSY and HMBC correlations for bis(2-ethylicosyl) phthalate (**2**).

Table 3.2.[1]H NMR (500 MHz $CDCl_3$) and [13]C NMR (125 MHz, $CDCl_3$) spectral data for compound (**2**)

Position	**^{1}H NMR**	**^{13}C NMR**
1, 6		132.5
2, 5	d 7.65 (2H, dd, J = 5.6, 2.2 Hz)	128.7
3, 4	7.48 (2H, dd, J = 5.6, 2.2 Hz)	130.9
1′, 1″		168.3
3′, 3″	4.12 (4H, m)	68.1
4′, 4″	1.62 (2H, m)	38.6
5′, 5″	1.42 (4H, m)	23.7
6′-21′,6″-21″	1.31–1.25 (remaining CH_2)	22.7- 22.9
22′, 22″	0.84 (6H, t, J = 6.7 Hz)	13.9
23′, 23″	1.42 (4H, m)	30.4
24′, 24″	0.93 (6H, t, J = 7.3 Hz)	10.9

3.3 1-Dotriacontanol (3)

The compound (**3**) was isolated as a colorless solid. The chloroform soluble fraction of *Nepeta kurramensis* was subjected to column chromatography over flash silica gel and eluted with chloroform: *n*-hexane (60:40) to give compound (**3**). The compound (**3**) was recognized on the basis of MS, 1D and 2D NMR spectra.

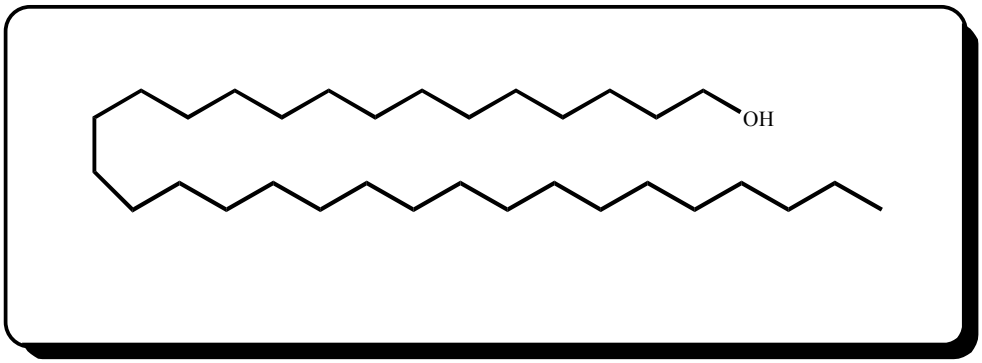

Figure 3.6: 1-dotriacontanol (**3**)

Mass spectrum confirmed molecular ion peak at *m/z* 466, signifying molecular formula $C_{32}H_{66}O$, with other fragments at 392, 167, 125, 97 and 57. The ^{1}H-NMR spectrum exhibited a triplet at δ 0.88 (3H) and a broad singlet at δ 1.23 communicated straight chain hydrocarbon. ^{1}H-NMR spectrum showed signals at δ 3.63 (αH, H-1) which illustrate the existence of hydroxyl at C-1. The ^{13}C-NMR spectrum (BB and DEPT) was also awfully edifying in the structure elucidation of compound (**3**). One oxygenated methylene echoed at δ 63.4 auxiliary confirmed the position of hydroxyl. The signals pragmatic between 29.1-29.6 specify the presence of a long chain hydrocarbon [The AOCS Lipid Library and Krishnan &Chandrasekaran,1989; Hussain *et al*., 2010].

3.4 1-nonacosanol (4)

The chloroform soluble fraction of *Nepeta kurramensis* was subjected to column chromatography over flash silica gel and eluted with chloroform-*n*-hexane (60:40) to give compound (**4**). Compound (**4**) was isolated as a monochrome solid. The molecular formula $C_{29}H_{60}O$ was recognized on the basis of MS, 1D and 2D NMR spectra.

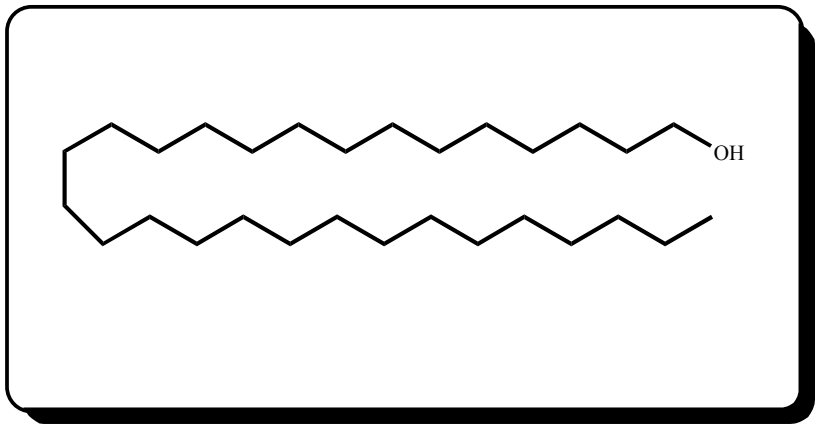

Figure 3.7: 1-nanacosanol (**4**)

Mass spectrum confirmed molecular ion peak at *m/z* 424, signifying molecular formula $C_{29}H_{60}O$. The ^{1}H-NMR spectrum exhibited a terminal CH_3 group at δ 0.87 and a broad singlet at δ 1.23 communicated straight chain hydrocarbon. ^{1}H-NMR spectrum showed signals at δ 3.63 (αH, H-1) which illustrate the existence of hydroxyl at C-1. The ^{13}C-NMR spectrum (BB and DEPT) was also awfully edifying in the structure elucidation of compound (**4**). One oxygenated methylene echoed at δ 63.1 auxiliary confirmed the position of hydroxyl at one position. The signals pragmatic between 29.0-29.3 specify the presence of a long chain hydrocarbon. The position of the hydroxyl group was also incorrigible by the HMBC experiments. The same compound was isolated prior from *Rhizophora apiculata* [***El-Seedi* et al.*, 2003; Pladio et. al., 2004***].

3.5 Ursolic acid (**5**)

Compound (**5**) was isolated as monotonous needles from the hexane soluble fraction of *Nepeta kurramensis* with chloroform and hexane (70:30). The IR spectrum revealed absorptions for hydroxyl group (3510 cm^{-1}) carbonyl group (1697 cm^{-1}) and trisubstituted double bond (1635 and 820 cm^{-1}). The molecular formula $C_{30}H_{48}O_3$ was established through MS showing molecular ion peak at *m/z* 456. Beside the molecular ion peak, the EI-MS displayed other indicative peaks at *m/z* 411 representing the loss of COOH group. Another high-flying peak at *m/z* 248 characterized retro diel alder fragmentation, distinctive of Δ^{12} ursane type triterpenes with COOH group at C-17 [Hussain *et al.,* 2008]. The base peak at *m/z* 203 was endorsed to the loss of COOH from the fragment at *m/z* 248.

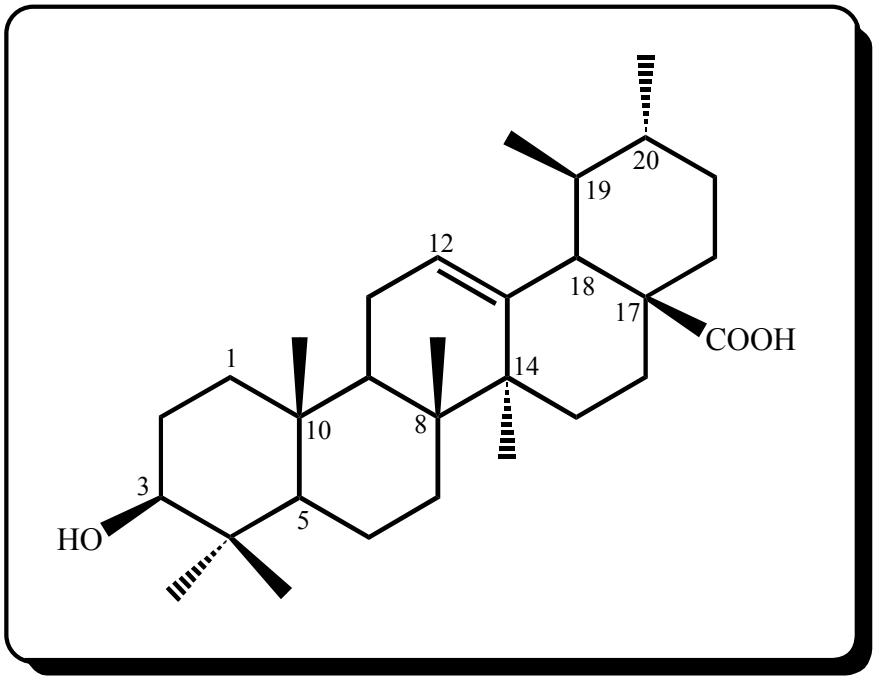

Figure 3.8: Ursolic acid (**5**)

The ^{1}H-NMR spectrum of compound (**5**) presents five tertiary methyl signals at δ 1.27, 1.25, 1.08, 1.05, 0.91 (each 3H, s, H-23, 27, 26, 24 and 25) and two secondary methyl signals at 1.02 (3H, d, *J* = 6.6 Hz, H-30), 0.97 (3H, d, *J* = 6.3 Hz, H-29) which were the inkling of ursane skeleton. The carbonylic proton that emerge at δ 3.46 (1H, dd, *J* = 10.3, 4.1 Hz) was in axial configuration as inveterate by a double doublet. The olefinic proton was experiential at δ 5.52 as a triplet (*J* = 3.5 Hz) (Table 3.3) [Seebacher *et al.,* 2003]. The association of ^{13}C-NMR spectrum with that of reported in the literature

acknowledged it as ursolic acid, previously reported from *Nepeta suavis* [Hussain *et al.,* 2008; Yokota *et al.,* 1987].

Table 3.3. ^{1}H and ^{13}C-NMR data for compound (**5**)

C. No.	^{13}C-NMR (δ)	DEPT	^{1}H-NMR (δ)
1	38.5	CH_2	
2	27.4	CH_2	
3	79.1	CH	3.46 (1H, dd, *J*= 10.3, *J* = 4.1, H-3*α*)
4	38.6	C	
5	52.4	CH	
6	18.3	CH_2	
7	33.2	CH_2	
8	39.6	C	
9	47.4	CH	
10	37.1	C	
11	23.9	CH_2	
12	125.8	CH	5.52 (m)
13	138.7	C	
14	42.0	C	
15	29.4	CH_2	
16	23.5	CH_2	
17	47.9	C	
18	55.2	CH	2.35 (d, *J* = 3.5 Hz)
19	30.5	CH	
20	30.3	CH	
21	27.5	CH_2	
22	37.0	CH_2	
23	24.0	CH_3	1.27 (s)
24	15.4	CH_3	1.05 (s)
25	15.9	CH_3	0.91 (s)
26	17.2	CH_3	1.08 (s)
27	24.5	CH_3	1.25 (s)
28	176.2	C	
29	22.4	CH_3	0.97 (3H, d, *J* = 6.3 Hz)
30	24.0	CH_3	1.02 (3H, d, *J* = 6.6 Hz)

3.6 *β*-amyrin (**6**)

Compound (**6**) was acquired as dull crystalline solid from the hexane soluble fraction of *Nepeta kurramensis.* The molecular formula $C_{30}H_{50}O$ was recognized through mass spectrum, which illustrated molecular ion peak at *m/z* 426.

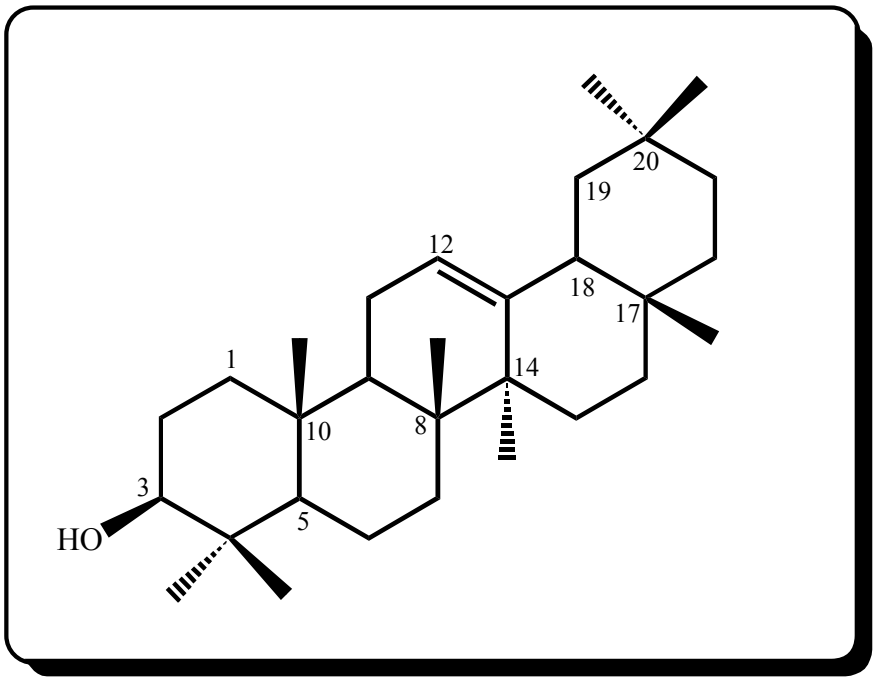

Figure 3.9: *β*-amyrin (**6**)

The EI-MS spectrum of compound (**6**) showed pinpointing peaks at *m/z* 257, 218, 207, 203 and 189, feature for amyrin skeleton with Δ^{12} unsaturation [Saleem *et al.,* 2010]. The ^{1}H-NMR spectrum of (**6**) flaunted eight tertiary methyls boomed at δ 1.08, 1.02, 1.01, 0.96, 0.93, 0.88, 0.85 and 0.80 (all singlets). The carbinylic proton resonated at δ 3.19 projected axial configuration as confirmed by a double doublet (dd, J = 10.0, J = 4.5 Hz), and a multiplet at δ 5.11 was investigative of the olefinic proton (Table 3.4). The ^{13}C-NMR spectrum (BB and DEPT) of (**6**) divulged thirty carbon signals including eight methyls, ten methylenes, five methines and seven quaternary carbon atoms. Evaluation of these data with those reported in literature identified compound (**6**) as *β*-amyrin [Saleem *et al.,* 2010], earlier reported from *Salvadora oleoides*.

Table 3.4 ^{1}H and ^{13}C-NMR data for compound (**6**)

C. No.	^{13}C-NMR (δ)	DEPT	^{1}H-NMR (δ)
1	39.0	CH_2	
2	27.4	CH_2	
3	78.8	CH	3.19 (dd, *J* = 10.0, 4.5 Hz)
4	39.1	C	
5	54.4	CH	
6	23.3	CH_2	
7	33.2	CH_2	
8	40.9	C	
9	47.7	CH	
10	37.0	C	
11	23.6	CH_2	
12	124.1	CH	5.11 (m)
13	144.3	C	
14	42.3	C	
15	26.3	CH_2	
16	26.5	CH_2	
17	34.0	C	
18	47.3	CH	
19	46.9	CH_2	
20	31.7	C	
21	32.9	CH_2	
22	41.6	CH_2	
23	28.2	CH_3	1.08 (s)
24	15.5	CH_3	0.85 (s)
25	15.6	CH_3	0.96 (s)
26	16.9	CH_3	1.01 (s)
27	26.0	CH_3	1.02 (s)
28	28.0	CH_3	0.93 (s)
29	33.3	CH_3	0.80 (s)
30	18.5	CH_3	0.83 (s)

3.7 *β*-sitosterol (**7**)

Compound (**7**) was isolated as white needles from the methanol extract of *Nepeta kurramensis*. The IR spectrum of the compound (**7**) explained the inclusion for hydroxyl group (3450 cm^{-1}) and trisubstituted double bonds (3446, 3050, 1650 and 815 cm^{-1}). The molecular formula $C_{29}H_{50}O$ was determined through EI-MS showing molecular ion peak at *m/z* 414. Fragments pragmatic at *m/z* 273 and 255, signifying the loss (M^+- side chain) and (M^+- side chain $–H_2O$), correspondingly were found to be the most important [Hasan *et al.,* 1991].

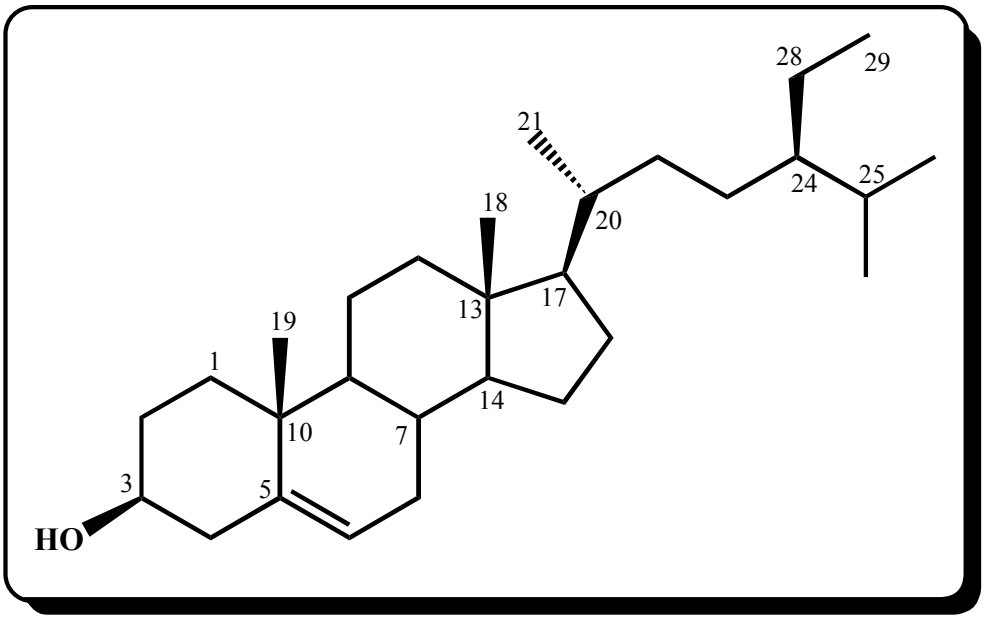

Figure 3.10: *β*-sitosterol (**7**)

The ^{1}H-NMR spectrum of (**7**) exhibited six methyl signals out of which two were tertiary (δ 0.68 and 1.01), three secondary (δ 0.92, 0.83 and 0.81) and one primary (δ 0.84). The signals for carbinylic and olefinic proton materialized at δ 3.32 (1H, m) and 5.23 (1H, m). The ^{13}C-NMR (BB, DEPT) spectrum of (**7**) revealed the presence of twenty nine carbon signals for six methyl, eleven methylene, nine methine and three quarternary carbon atoms. Compound (**7**) was recognized as *β*- sitosterol by the proportional study of

its NMR data with that of literature [Rubinstein *et al.,* 1976, Hasan *et al.,* 1991], earlier reported from *Nepeta hindostana* [Siddiqui and Ahsan 1967].

Table 3.5 ^{1}H and ^{13}C-NMR data for compound (**7**)

C. No.	^{13}C-NMR (δ)	DEPT	^{1}H-NMR (δ)
1	37.2	CH_2	
2	31.8	CH_2	
3	71.8	CH	3.32 (1H, m, H-3)
4	39.8	CH_2	
5	140.7	C	
6	121.7	CH	5.23 (1H, m)
7	29.6	CH_2	
8	31.9	CH	
9	50.1	CH	
10	36.1	C	
11	21.1	CH_2	
12	37.2	CH_2	
13	42.3	C	
14	56.7	CH	
15	24.1	CH_2	
16	28.2	CH_2	
17	56.0	CH	
18	11.9	CH_3	0.68 (3H, s)
19	19.4	CH_3	1.01 (3H, s)
20	33.9	CH	
21	19.0	CH_3	0.92 (3H, d, J = 6.2 Hz)
22	31.9	CH_2	
23	29.3	CH_2	
24	45.8	CH	
25	26.1	CH	
26	18.7	CH_3	0.83 (3H, d, J = 6.5 Hz)
27	19.8	CH_3	0.81 (3H, d, J = 6.5 Hz)
28	23.1	CH_2	
29	11.9	CH_3	0.84 (3H, t, J = 7.0 Hz)

3.8 Structural elucidation of 5-hydroxy-3,6,8,4′-tetramethoxyflavon (**8**)

The chloroform soluble fraction of *Rhynchosia reniformis* yielded compound (**8**) with a solvent system *n*-hexane:chloroform (50:50) through silica gel column chromatography. This compound was obtained as yellow powder with a melting point 193 ^{o}C. The molecular formula $C_{19}H_{18}O_7$ of the compound was confirmed through EIMS having molecular ion peak at *m/z* 358.

The IR spectral bands of the compound showed the presence of hydroxyl group (3410 cm^{-1}), α, β-unsaturated carbonyl group (1690 cm^{-1}) and aromatic ring (1620-1400 cm^{-1}). The UV spectral peaks were observed at λ_{max} 345 and 273 nm, which are characteristics of flavonoid skeleton.

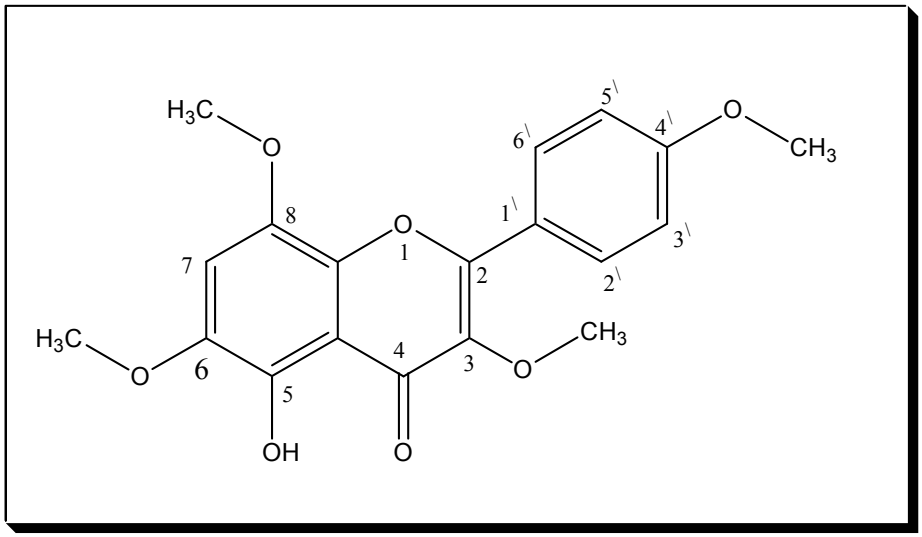

Figure 3.11: 5-hydroxy-3,6,8,4′-tetramethoxyflavon (**8**)

The ^{1}H-NMR of the compound showed two doublets at δ 7.95 (H-2′ *J* = 8.0 Hz) and 7.20 (H-3′ *J* = 8.0 Hz) respectively, which confirms para substituted aromatic ring. The aromatic proton signal resonating at δ 6.70 was assigned to H-7. The signals at δ 3.85, 3.80, 3.75 and 3.90 for the OCH_3 groups of carbon 3, 6, 8, and 4′ atoms. The most deshielded proton signal at δ 12.98 was assigned to the proton of hydroxyl group at C-5.

The ^{13}C-NMR spectral analysis of the compound confirmed nineteen carbon atoms. The resonance signal at δ 176 was allocated to C-4 a flavonoid carbonyl carbon, δ 153 for the olifinic C-2 and other olifinic carbon at δ 142. Signal at δ 142 was allocated

to C-8. Signals at δ 144, 114 and 145 were assigned to C-6, C-7 and C-5. Chemical shifts at δ 104 and 150 for C-9 and C-10. High field chemical shifts at δ 59, 58, 57 and 55 allocated to 3-OCH_3, 6-OCH_3, 8-OCH_3 and 4′-OCH3. The chemical shifts at δ 123 for C-1′ and 132, 115 for C-2′, 6′ and C-3′, 5′ respectively. From above data and with the help of literature the compound (**8**) was identified as 5-hydroxy-3,6,8,4′-tetramethoxyflavon [Hioki *et al*., 2008].

Table 3.6 ^{1}H-NMR and ^{13}C-NMR data of 5-Hydroxy-3,6,8,4′-tetramethoxyflavon (**8**)

C. No.	^{13}C-NMR (δ)	DEPT	^{1}H-NMR (δ)
2	153	C	
3	142	C	
4	176	C	
5	145	C	12.98 (s)
6	144	C	
7	114	CH	6.70 (s)
8	142	C	
9	104	C	
10	150	C	
1′	123	C	
2′	132	CH	7.95 (d)
3′	115	CH	7.20 (d)
4′	162	C	
5′	115	CH	7.20 (d)
6′	132	CH	7.95 (d)
3-OCH_3	59	CH_3	3.85 (s)
6-OCH_3	58	CH_3	3.80 (s)
8-OCH_3	57	CH_3	3.75 (s)
4′-OCH_3	55	CH_3	3.90 (s)

3.9 Structural elucidation of 3,5,4′-trihydroxy-6,8-dimethoxyflavone (**9**)

The compound (**9**) was isolated as pale yellow powder from the chloroform soluble fraction of *Rhynchosia reniformis* using solvent system *n*-hexane:chloroform (40:60). Its melting point is 180 C. The molecular formula of the compound was confirmed as $C_{17}H_{14}O_7$ through EIMS and ^{13}C-NMR (BB, DEPT) and molecular ion peak was observed at *m/z* 330. IR spectrum showed absorption bands at 3412 cm-1 for OH group, 1691 cm-1 for C=O group and 1621-1400 cm-1 for aromatic C=C stretching. The UV spectra was characteristic for flavonoid skeleton which showed λ_{max} at 338 and 272 nm.

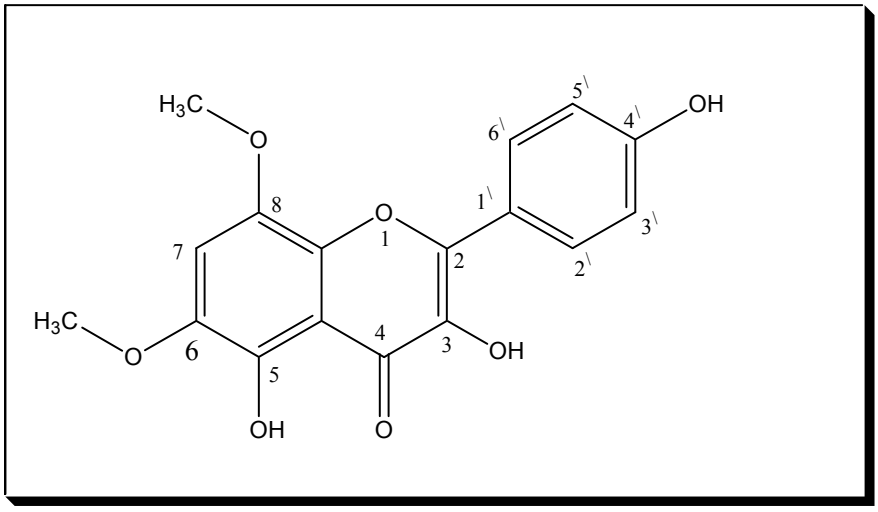

Figure 3.12: 3,5,4′-trihydroxy-6,8-dimethoxyflavone (**9**)

The ^{1}H-NMR of the compound confirmed the presence of para substituted aromatic ring which showed two doublets at δ 8.00 (H-2' *J* = 8.0 Hz) and 7.20 (H-3' *J* = 8.0 Hz), respectively. The aromatic proton signal resonating at δ 6.70 was assigned to H-7. The signals resonating at δ 3.80 and 3.85 for the OCH_3 groups of carbon 6 and 8. The proton signal at δ 12.98 was assigned to the proton of hydroxyl group at C-5.

The ^{13}C-NMR spectral analysis of the compound (**9**) confirmed seventeen carbon atoms. The resonance signal at δ 177 was allocated to C-4 a carbonyl carbon, δ 148 for the olifinic C-2 and other olifinic carbon at δ 138. Signal resonating at δ 143 was allocated to C-8. Signals at δ 144, 114 and 147 were assigned to C-6, C-7 and C-5.

Chemical shifts at δ 108 and 151 for C-9 and C-10. High field chemical shifts at δ 57 and 56 allocated to 6-OCH_3 and 8-OCH_3. The chemical shifts at δ 124 for C-1′ and 130, 116 for C-2′, 6′ and C-3′, 5′ respectively. By comparison of the above data with literature the compound (**9**) was confirmed as 3,5,4′-trihydroxy-6,8-dimethoxyflavone [Hioki *et al*., 2008].

Table 3.7 ^{1}H-NMR and ^{13}C-NMR data of 3,5,4′-Trihydroxy-6,8-dimethoxyflavone (**9**)

C. No.	^{13}C-NMR (δ)	DEPT	^{1}H-NMR (δ)
2	148	C	
3	138	C	
4	177	C	
5	147	C	12.98 (s)
6	144	C	
7	114	CH	6.70 (s)
8	143	C	
9	108	C	
10	151	C	
1′	124	C	
2′	130	CH	8.00 (d)
3′	116	CH	7.20 (d)
4′	159	C	
5′	116	CH	7.20 (d)
6′	130	CH	8.00 (d)
6-OCH_3	57	CH_3	3.80
8-OCH_3	56	CH_3	3.85

3.10 Preliminary phytochemical studies

The preliminary phytochemical screening *N. kurramensis, N. laevigata* and *Rhynchosia reniformis* was conducted using the extracts for different types of chemical constituents as per method described by Prabhu, (2009). The extracts were imperiled to preliminary phytochemical examination for revealing of flavonoids, alkaloids, glycosides, phenolic compounds, carbohydrates, fats and proteins and. Both species of *Nepeta* showed similar phytochemical profile. Presence and absence of different phyto-constituents in *Nepeta* and *Rhynchosia reniformis* are presented in tables (**3.8** and **3.9**).

Table 3.8 Preliminary phytochemical studies of *Nepeta kurramensis* and *Nepeta laevigata*

S. No	Phytochemical Tests	Methanol extract	Hexane fraction	Chloroform fraction	Ethyl acetate fraction	Butanol fraction	Water fraction
1	Phenolic compound	+	-	+	+	-	-
2	terpenes	+	+	+	+	+	+
3	Flavonoids	+	+	+	+	+	+
4	Alkaloid	+	-	+	+	-	+
5	Carbohydrates	+	-	-	-	-	+
6	Glycosides	+	+	+	+	+	+
7	Resin	-	-	-	-	-	-
8	fats	-	-	+	+	-	-
9	Acidic compounds	+	-	+	+	-	-
10	Mucilage	-	-	-	-	-	-
11	Proteins	+	-	-	-	-	+

Table 3.9 Preliminary phytochemical studies of *Rhynchosia reniformis*

S. No	Phytochemical Tests	Methanol extract	Hexane fraction	Chloroform fraction	Ethyl acetate fraction	Butanol fraction	Water fraction
1	Phenolic compound	+	+	+	-	-	-
2	terpenes	+	+	+	+	+	+
3	Flavonoids	+	-	+	+	-	+
4	Alkaloid	+	-	-	+	-	+
5	Carbohydrates	+	-	+	-	-	+
6	Glycosides	+	-	-	+	-	+
7	Resin	-	-	-	-	-	-
8	fats	+	+	+	-	-	-
9	Acidic compounds	+	-	-	-	-	-
10	Mucilage	-	-	-	-	-	-
11	Proteins	-	-	-	-	-	-

PHASE 2: BIOLOGICAL EVALUATION

3.11 Antimicrobial results

Plants have been used since ancient times for the treatment of various diseases from flu to cancer [Sarwat *et al*., 2012]. Approximately two thirds to three-quarters of the world's population rely on medicinal flora as their main source of medicines [Qayum *et al*., 2012]. The crude extract and fractions of *N. laevigata, N. kurramensis* and *R. reniformis* were screened against various human pathogens.

The antibacterial results of *N. laevigata* showed that the *n*-butanol fraction displayed promising activity against *E. coli* (85% inhibition), *P. morganii* (83% inhibition) and moderately active against *M. flavas* (73% inhibition), and *B. subtilis* (70% inhibition). However it was inactive against *P. testosteroni* and *K. pneumoniae*. The ethyl acetate fraction exhibited low activity against *P. testosterone and B. subtilis* with percentage inhibition 36% and 32% respectively while it was inactive against all other tested bacterial strains**.** The crude extract, *n*-hexane, chloroform and aqueous fractions were found to have insignificant against the tested pathogens (Table **3.10**).

In case of *N. kurramensis,* chloroform fraction was significantly active against *Streptococcus cricetus* (89% inhibition), and *Micrococcus flavas* (84% inhibition) against *P. testosteroni* (71% inhibition) and was active against *B. subtilis, E. coli, P. morganii*, *K. pneumonia.* The *n*-butanol fraction projected 60 % inhibition against *E. coli* and was also active against all pathogens used except *K. pneumonia.* The ethyl acetate fraction was active against all pathogens except *E. coli.* Water fraction showed low activity against *E. coli* and *P. morganii* only with 20 % and 16 % inhibition. The crude extract was active against *B. subtilis* (29 % inhibition), *K. pneumonia* (16 % inhibition) only and n-hexane gleaned low activity against *E. coli* (27 % inihibition), *S. cricetus* (27 % inihibition) (Table **3.11**).

Where in *R. reniformis* crude extract exhibited 100% inhibition against *Streptococcus cricitus,* 63 % inhibition against *B. subtilis*, 36 and 30% against *P. testosterone* and *E. coli* while was inactive against other bacterial species. The ethyl acetate fraction showed promising activity against *M. flavas* (99% inhibition)*, S. cricitus* (95% inhibition) and *P. morganii* (90% inhibition) with low activity against other tree pathogens. The chloroform fraction exhibited excellent activity against *S. cricitus* (90 % inhibition) followed by 60 % and 52 % inhibition against *P. testosteroni* and *B. subtilis*. The *n*-hexane fraction showed moderate activity against *P. testosteroni* (63 % inhibition) followed by 40 % inhibition against *B. subtilis* (Table **3.12**).

In this study, antifungal activity of the crude extract and various fractions of the plants under investigation were evaluated against fungal pathogens. The chloroform and ethyl acetate fractions of *N. laevigata* were active against *A. fumigatus, F. solanim, C. albicans, R. solani,* and *M. canis* (Table **3.13**) and chloroform fraction of *N. kurramensis* showed inhibition against *A. fumigatus, P. boydii,* and *C. albicans* (Table **3.14**), while in *R. reniformis,* chloroform, *n*-hexane and methanolic extracts were more active against most tested pathogens as compared to rest of fractions (Table **3.15**).

Antimicrobial screening of pure compounds

Since the chloroform soluble fraction of both *Nepeta kurramensis* and *Rhynchosia* were found as most active against different bioassays and were subjected to column chromatography for isolation and purification of compounds. The uncommon skeleton compounds (**1**, **2**, **8** and **9**) isolated from the plants were tested against various pathogens to check their antimicrobial potentials.

The isolated compounds **1**, **2** and **8**, **9** were subjected to antibacterial and antifungal activities. All the compounds **1**, **2** and **8**, **9** were active against all the tested bacterial and fungal pathogens (Table **3.16** and Table **3.17**). The result showed that compound (**1**) was more potent as compared to the all others tested compounds.

3.12 Antiglycation results

The crude extract\fractions of *Rhynchosia reniformis* were evaluated for their inhibitory potential against protein glycation *in vitro*. Among these fractions, chloroform and ethyl acetate fractions exhibited a significant antiglycation activity with 76.02 % and 70.27 % inhibition against protein glycation, respectively, while *n*-hexane fraction showed a moderate inhibition with 64.06 %. However, crude extract, *n*- butanol and water fractions were found to be inactive against protein glycation *in vitro* at a concentration 1mg/1000 µL (Table **3.18**).

Among *Nepeta laevigata* fractions, ethyl acetate and *n*- hexane fraction exhibited significant antiglycation activities with 74.02 % and 71.26 % inhibition respectively, while crude extract showed a moderate activity against protein glycation with 63.51 % inhibition and in case of *Nepeta kurramensis* only the *n*-hexane fraction exhibit 67.24 % inhibition against protein glycation (Table **3.18**).

3.13 Antiplatelet results

In antiplatelet activities of *Rhynchosia reniformis* the aqueous fraction was only active against platelet activating factor **(**PAF) induced human platelet aggregation, methanolic and *n*- butanol fractions show activities against arachidonic acid (AA) and PAF while others fractions were insignificant (Table **3.19**). In platelet aggregation studies of *Nepeta laevigata, n*-hexane fraction was effective against AA (IC_{50} 35 ug/ml) and PAF induced human platelet aggregation (IC_{50} 35 ug/ml), methanol fraction also inhibited PAF- induced human platelet aggregation (Table **3.19**). In *Nepeta kurramensis* only chloroform fraction inhibited AA and PAF- induced human platelet aggregation (IC_{50} 35 ug/ml) (Table **3.19**).

3.14 Antioxidant results

We also investigated the general antioxidative effects of the extract\fractions of *Rhynchosia reniformis* plant to inhibit $^{\bullet}OH$, DPPH radical, total ROS and to scavenge authentic $ONOO^-$ for the crude extract and solvent soluble fractions. The chloroform-

soluble fraction showed stronger antioxidative activity than that of the other fractions in all three scavenging tests with *IC50* values of 23.50±0.02, 93.89±0.09 and 30.24±0.07 for $^{\bullet}$OH radical, total ROS and to scavenge authentic $ONOO^-$, respectively (Table **3.20**). The ethyl acetate soluble fraction was also found to contain second maximum amount of polyphenolic compounds acting as antioxidants (38.23±0.08) and proved more effective then rest of the fractions (Table **3.20**).

The crude extract of the *Nepeta laevigata* showed moderate antioxidative activity in all three scavenging tests with IC_{50} values of 50.25±0.03, >400 and 77.13±0.07 for $^{\bullet}$OH radical, total ROS and to scavenge authentic $ONOO^-$, respectively (Table **3.21**). The EtOAc-soluble fraction of the plant showed stronger antioxidative activity than that of the other fractions in all the three scavenging tests followed by *n*-butanol fraction (Table 3.21). The *n*-hexane fraction was found to exhibit the least activity in all the solvent soluble fractions (Table **3.21**). A similar trend in antioxidative scavenging activity was also observed in the related species of the genus *N. Kurramensis* (Table **3.22**).

Antioxidative screening of pure compounds

The pure compounds **1, 2**, **8** and **9** were subjected to four different antioxidative tests. The compounds **1** and **2** were found to have moderate activities while the flavonoids **8** and **9** showed excellent results in the four different tests (Table **3.23**)

3.15 Cytotoxicity results

The methanolic extract and various solvent soluble fractions of *Rhynchosia reniformis, Nepeta laevigata* and *Nepeta reniformis* were screened for their cytotoxic (brine shrimp bioassay) activities using the standard protocol developed by Myer *et al*. It was observed that the *n*-butanol fraction of *Rhynchosia* displayed a significant cytotoxic activity against *Artemia saline (*brine shrimp) larvae while the methanolic extract and rest of fractions was inactive (Table **3.24**). Surprisingly none of the fraction of the both *Nepeta* species showed significant cytotoxic and phytotoxic activities.

3.16 Phytotoxicity results

The methanolic extract and various solvent soluble fractions of *Rhynchosia reniformis*, *Nepeta laevigata* and *Nepeta reniformis* were tested for their phytotoxic activities. The methanolic extract/fractions of the tested plants show insignificant phytotoxic effect on *Lemna* plant. The members of Laminiaceae family are suitable organism to investigate physiological process.

PHASE 3: RESULTS OF PROXIMATE ANALYSIS

3.17 Proximate results

The results of the proximate analysis of the plant species with their standard deviation are summarized in Table **3.25**. The moisture content was found to be highest in *N. kurramensis* (3.53%), whereas *R. reniformis* was found to be the lowest (2.35%). As a result of this study, *N. kurramensis* was found to contain the highest content of the moisture. The descending order of the moisture content was observed as *N. kurramensis* > *N. laevigata* > *R. reniformis*. The highest ash and protein value were observed in *N. laevigata* during analysis. Ash contents of *N. laevigata, N. kurramensis* and *R. reniformis* were found to have 18.55, 35.15 and 11.95% respectively. The results of ash content with standard deviation are presented in Table **3.25**.

N. kurramensis was found to be highest in its crude fat content (8.27%). However, *R. reniformis* contains crude fat (1.79%). *N. laevigata* were found to have 3.72% fat *Nepeta* species were found to be a good source of fats particularly *N. kurramensis*. Crude fiber of *R. reniformis* was found to have value of 31.59 %, followed by the *N. kurramensis* (10.326%) (Table 3.205). The protein content of the medicinal plants was calculated on the basis of the available nitrogen using Kjeldahl method and was observed in the range of 0.44- 10.22% with *N. laevigata* having the highest value (10.22%), followed by *N. kurramensis* (6.31%) and *R. reniformis* (0.44%) (Table**3.25**). The carbohydrate content of analyzed samples revealed that *N. kurramensis* had highest

amount of carbohydrates (53.02%), followed by the decreasing order of *R. reniformis* (51.88%) and *N. laevigata* (42.52%) (Table **3.25**)

According to the results of the energy calculations, based on the carbohydrates, fats, and protein contents, the highest value was found in the *N. kurramensis* (311.73 kcal/100g), while the *R. reniformis* was found to contain the lowest energy value (225.48 kcal/100g) (Table **3.25**). The correlation analysis of the selected parameters showed that similar parameters have highly significant correlation while among other parameters the correlation is either significant or non-significant, and in some cases moderate. Moisture with fats and energy value, ash with proteins, fats with energy value showed significant correlation. Ash with fibers and carbohydrates, fibers with proteins and proteins with carbohydrates displayed insignificant correlation (Table **3.26**).

Table 3.10: Antibacterial activity of crude extract and various fractions of *N. laevigata*

Name of Bacteria	**Crude extract** **Zone of inhibition (mm)**	***Inhibition percentage***	***n*-Hexane fraction** **Zone of inhibition (mm)**	***Inhibition percentage***	**Ethyl acetate fraction** **Zone of inhibition (mm)**	***Inhibition percentage***	**Chloroform fraction** **Zone of inhibition (mm)**	***Inhibition Percentage***	***n*-butanol fraction** **Zone of inhibition (mm)**	***Inhibition percentage***	**Water fraction** **Zone of inhibition (mm)**	***Inhibition Percentage***
B. subtilis	Nil	Nil	6	25	10	32	Nil	Nil	18	**70**	Nil	Nil
P. testosterone	8	29	11	36	11	36	Nil	Nil	Nil	Nil	3	15
E. coli	7	27	7	27	Nil	Nil	6	25	20	**85**	Nil	Nil
K. pneumoniae	8	29	6	25	Nil	Nil	Nil	Nil	Nil	Nil	7	27
S. cricetus	Nil	Nil	9	30	Nil	Nil	6	25	12	40	Nil	Nil
P. morganii	5	23	5	23	Nil	Nil	7	27	19	**83**	Nil	Nil
M. flavas	Nil	Nil	6	25	Nil	Nil	9	30	18	**73**	Nil	Nil
Antibiotic Agent Nulidixic Acid	*K. pneumoniae* 15	*63*	*B. subtilis* 19	*83*	*E. coli* 18	*76*	*P. testosteroni* 16	65	S. C 19	*81*	*M. flavas* 13	*39*
Antibiotic Agent Dixoycline	K. *pneumoniae* 17	*67*	*B. subtilis* 18	*76*	*E. coli* 16	*65*	*P. testosteroni* 17	67	S. C 24	*99*	*M. flavas* 17	67

The plates were inculated at a concentration of 3 mg/mL of DMSO

Table 3.11: Antibacterial activity of crude extract and various fractions of *N. kurramensis*

Name of Bacteria	**Crude extract** **Zone of inhibition (mm)**	***Inhibition percentage***	***n*-Hexane fraction** **Zone of inhibition (mm)**	***Inhibition percentage***	**Ethyl acetate fraction** **Zone of inhibition (mm)**	***Inhibition percentage***	**Chloroform fraction** **Zone of inhibition (mm)**	***Inhibition Percentage***	***n*-butanol fraction** **Zone of inhibition (mm)**	***Inhibition Percentage***	**Water fraction** **Zone of inhibition (mm)**	***Inhibition Percentage***
B. subtilis	8	29	Nil	Nil	6	25	11	36	9	30	Nil	Nil
P. testosteroni	Nil	Nil	Nil	Nil	6	25	17	**71**	5	23	Nil	Nil
E. coli	Nil	Nil	7	27	Nil	Nil	8	29	14	60	6	20
K. pneumoniae	4	16	Nil	Nil	5	23	10	32	Nil	Nil	Nil	Nil
S. cricetus	Nil	Nil	7	27	12	40	20	**89**	7	27	Nil	Nil
P. morganii	Nil	Nil	Nil	Nil	8	29	11	36	6	25	4	16
M. flavas	4	16	Nil	Nil	6	25	19	**84**	5	23	Nil	Nil
Antibiotic Agent Nulidixic Acid	*K. pneumoniae* 20	*90*	*B. subtilis* 18	*70*	*E. coli* 22	*95*	*P. testosteroni* 18	70	S. C 20	*90*	*M. flavas* 16	*65*
Antibiotic Agent Dixoycline	*K. pneumoniae* 18	*70*	*B. subtilis* 18	*70*	*E. coli* 17	*67*	*P. testosteroni* 19	71	S. C 22	*95*	*M. flavas* 15	*63*

The plates were inculated at a concentration of 3 mg/mL of DMSO

Table 3.12: Antibacterial activity of crude extract and various fractions of *R. reniformis*

Name of Bacteria	**Crude extract** **Zone of inhibition (mm)**	***Inhibition percentage***	**n-Hexane fraction** **Zone of inhibition (mm)**	***Inhibition percentage***	**Ethyl acetate fraction** **Zone of inhibition (mm)**	***Inhibition percentage***	**Chloroform fraction** **Zone of inhibition (mm)**	***Inhibition Percentage***	**n-butanol fraction** **Zone of inhibition (mm)**	***Inhibition Percentage***	**Water fraction** **Zone of inhibition (mm)**	***Inhibition percentage***
B. subtilis	15	63	12	40	9	30	17	52	13	38	6	25
P. testosteroni	11	36	15	63	12	35	14	60	10	32	Nil	Nil
E. coli	9	30	8	29	18	**70**	Nil	Nil	Nil	Nil	5	23
K. pneumoniae	Nil	Nil	Nil	Nil	7	27	6	25	Nil	Nil	Nil	Nil
S. cricetus	25	**100**	Nil	Nil	22	**95**	20	**90**	Nil	Nil	Nil	Nil
P. morganii	Nil	Nil	5	23	20	**90**	12	40	Nil	Nil	Nil	Nil
M. flavas	Nil	Nil	7	27	24	**99**	10	32	6	25	Nil	Nil
Antibiotic Agent Nulidixic Acid	*K. pneumoniae* 18	*70*	*B. subtilis* 17	*67*	*E. coli* 19	*71*	*P. testosteroni* 19	71	S. C 18	*70*	*M. flavas* 15	*63*
Antibiotic Agent Dixoycline	*K. pneumoniae* 17	*67*	*B. subtilis* 19	*71*	*E. coli* 20	*90*	*P. testosteroni* 19	71	S. C 19	*71*	*M. flavas* 13	*38*

The plates were inculated at a concentration of 3 mg/mL of DMSO

Table 3.13: Antifungal activities of crude extract and various fractions of *N. laevigata*
MIC* (mg/mL)

Fungal Strain	Methanol extract	*n*-Hexane fraction	Chloroform fraction	Ethyl acetate fraction	*n* –Butanol fraction	Aqueous fraction
A. *niger*	3	2.5	2	NA	NA	3
A. *fumigatus*	NA	2	3	3	2	2
A. *flavus*	2	3	2	2	NA	3
F. *solani*	2	3	3	2	2.5	NA
C. albicans	NA	NA	4	3	NA	NA
R. solani	2	NA	3	3	2	NA
P. boydii	NA	2	2	2.5	NA	NA
M. canis	NA	NA	3.5	3	NA	NA

MIC* : Minimum inhibitory concentration
NA* : Not active

The concentration of relent extract used was 24 mg/mL of DMSO

Table 3.14: Antifungal activities of crude extract and various fractions of *N. kurramensis*
MIC* (mg/mL)

Fungal Strain	Methanolic extract	*n*-Hexane fraction	Chloroform fraction	Ethyl Acetate fraction	*n* – Butanol fraction	Aqueous fraction
A. *niger*	NA	NA	2.5	NA	2	NA
A. *fumigatus*	NA	2.5	4	2	NA	NA
A. *flavus*	NA	NA	2	NA	NA	NA
F. *solani*	2	NA	2.5	2	NA	NA
C. albicans	NA	NA	3	2	NA	2.5
R. solani	NA	NA	2.5	NA	NA	NA
P. boydii	NA	2	4	NA	2	NA
M. canis	NA	NA	3.5	NA	NA	2

MIC* : Minimum inhibitory concentration
NA* : Not active

The concentration of relent extract used was 24 mg/mL of DMSO

Table 3.15: Antifungal activities of crude extract and various fractions of *R. reniformis*
MIC* (mg/mL)

Fungal Strain	Methanolic extract	*n*-Hexane fraction	Chloroform fraction	Ethyl acetate fraction	*n* – Butanol fraction	Aqueous fraction
A. niger	3	2	2	NA	3	3
A. fumigatus	2	3.5	2	3	2	2
A. flavus	3.5	2	2.5	2	NA	NA
F. solani	2.5	3	2	2.5	NA	NA
C. albicans	3	4	NA	NA	NA	NA
R. solani	3	3.5	NA	2	2	NA
P. boydii	2	3.5	2	NA	NA	NA
M. canis	2	3	2.5	NA	NA	NA

MIC* : Minimum inhibitory concentration
NA* : Not active

The concentration of relent extract used was 24 mg/mL of DMSO

Table 3.16: Zone of inhibition in mm of compounds **1**, **2, 8** and **9** against different bacteria

SNo	Compounds	*E.coli*	*P.testosteroni*	*S.cricitus*	*P.morganii*	*B.subtilis*	*K.pneumoniae*	*M.flavas*
1	**1**	18.4	19.7	19.9	10.1	16.3	17.3	11.6
2	**2**	16.8	15.4	18.6	8.3	15.7	15.8	14.6
3	**8**	10.2	7.4	8.7	4.3	13	7.2	8.2
4	**9**	11.1	7.8	8.2	4.1	11	7.8	8
5	Antibiotic Dixoycline	22.3	23.8	23.8	21.2	24.2	22.8	20.7

Table 3.17: MIC (µg/mL) values for compounds **1**, **2, 8** and **9** against different fungal strains

SNo	Compounds	*F.solani*	*A.flavus*	*R.solani*	*P.boydii*	*M.canis*	*C.albicans*	*A. niger*
1	**1**	45	37	50	53	42	39	34
2	**2**	60	63	54	59	48	44	40
3	**8**	68	70	59	63	70	58	55
4	**9**	65	72	62	60	68	60	51

Table 3.18: Antiglycation profile of *Nepeta laevigata, Nepeta kurramensis* and *Rhynchosia reniformis*

Sample	**Concentration**	**% Inhibition**		
		(Nepeta laevigata)	*(Nepeta kurramensis)*	(*Rhynchosia reniformis)*
Crude extract	1mg/1000 μL	63.51	41.24	60.05
n-Hexane	1mg/1000 μL	71.26	67.24	64.06
$CHCl_3$	1mg/1000 μL	12.03	17.07	76.02
EtOAc	1mg/1000 μL	74.02	16.15	70.27
n-BuOH	1mg/1000 μL	14.21	12.02	16.21
H_2O	1mg/1000 μL	8.52	7.23	9.02
Rutin (standard)	3mM	82.50	82.50	82. 50

Table 3.19: IC_{50} value (µg/ml) of different fractions against AA (1.8mM) or PAF (0.8µM)-induced human platelet aggregation of *Nepeta laevigata, Nepeta kurramensis* and *Rhynchosia reniformis.*

Fractions	IC_{50} value (µg/ml)					
	(Nepeta laevigata)		*(Nepeta kurramensis)*		(*Rhynchosia reniformis)*	
	PAF	**AA**	**PAF**	**AA**	**PAF**	**AA**
Crude extract	9	NA	NA	NA	78	44
n-Hexane	66	19	NA	NA	NA	NA
$CHCl_3$	NA	NA	63	16	NA	NA
EtOAc	NA	NA	NA	NA	NA	NA
n-BuOH	NA	NA	NA	NA	282	185
H_2O	NA	NA	NA	NA	56	NA

Table 3.20: Antioxidative activity of the crude extract and solvent fractions of *Rhynchosia reniformis*

Sample	IC_{50} [$\mu g/mL$] [a]		
	$^{\bullet}OH$ [b]	Total ROS [c]	$ONOO^{-}$ [d]
Crude extract	66.30± 0.06	>600	91.37± 0.04
n-Hexane	55.16± 0.09	>600	97.85± 0.02
$CHCl_3$	43.49± 0.03	>500	77.31± 0.05
EtOAc	23.50± 0.02	93.89± 0.09	30.24± 0.07
n-BuOH	38.23± 0.08	>400	67.45± 0.03
H_2O	96.11± 0.05	>600	99.79± 0.06
Trolox [e]	5.79±0.06	35.30±0.05	-
DL-Penicillamine [f]	-	-	1.07±0.04

[a] Values of OH, total ROS, and $ONOO^-$ are expressed as the mean ± standard error of triplicate experiments.
[b] Inhibitory activity of hydroxyl radical generation in 1.0 mM H_2O_2 and 0.2 mM $FeSO_4$.
[c] Inhibitory activity of total ROS generation in kidney postmicrosomal fraction.
[d] Inhibitory activity of authentic peroxynitrite.
[e] Trolox was used as a positive control.
[f] DL-Penicillamine was used as a positive control

Table 3.21: Antioxidative activityies of the crude extract and solvent fractions of *Nepeta laevigata*

Sample	IC_{50} [$\mu g/mL$] [a]		
	$^{\bullet}OH$ [b]	**Total ROS [c]**	**$ONOO^{-}$ [d]**
Crude extract	50.25± 0.03	>400	77.13± 0.07
n-Hexane	70.79± 0.05	>600	98.37± 0.06
$CHCl_3$	61.14± 0.07	>600	84.55± 0.02
EtOAc	30.42± 0.04	88.37± 0.05	55.97± 0.09
n-BuOH	33.19± 0.06	>500	70.45± 0.07
H_2O	89.29± 0.03	>600	98.67± 0.05
Trolox [e]	5.61±0.07	34.81±0.03	-
DL-Penicillamine [f]	-	-	1.03±0.02

[a] Values of OH, total ROS, and $ONOO^-$ are expressed as the mean ± standard error of triplicate experiments.
[b] Inhibitory activity of hydroxyl radical generation in 1.0 mM H_2O_2 and 0.2 mM $FeSO_4$.
[c] Inhibitory activity of total ROS generation in kidney postmicrosomal fraction.
[d] Inhibitory activity of authentic peroxynitrite.
[e] Trolox was used as a positive control.
[f] DL-Penicillamine was used as a positive control

Table 3.22: Antioxidative activities of the crude extract and solvent fractions of *Nepeta kurramensis*

Sample	IC_{50} [µg/mL] [a]		
	•OH[b]	Total ROS[c]	$ONOO^-$[d]
Crude extract	59.57± 0.09	>400	75.24± 0.04
n-Hexane	75.12± 0.06	>600	99.55± 0.09
$CHCl_3$	70.31± 0.02	>600	88.32± 0.02
EtOAc	37.16± 0.05	93.45± 0.07	60.13± 0.05
n-BuOH	44.29± 0.09	>500	77.41± 0.09
H_2O	96.55± 0.06	>600	99.59± 0.07
Trolox [e]	5.61±0.07	34.81±0.03	-
DL-Penicillamine [f]	-	-	1.03±0.02

[a] Values of OH, total ROS, and $ONOO^-$ are expressed as the mean ± standard error of triplicate experiments.
[b] Inhibitory activity of hydroxyl radical generation in 1.0 mM H_2O_2 and 0.2 mM $FeSO_4$.
[c] Inhibitory activity of total ROS generation in kidney postmicrosomal fraction.
[d] Inhibitory activity of authentic peroxynitrite.
[e] Trolox was used as a positive control.
[f] DL-Penicillamine was used as a positive control

Table 3.23: Antioxidative activities of compounds **1**, **2**, **8** and **9**

Compound	IC_{50} [μM] [a]			**DPPH** IC_{50} ± SEM[a] [*m*M]
	•OH[b]	**Total ROS**[c]	**$ONOO^-$**[d]	
1	41.31± 0.04	90.53± 0.02	25.70± 0.05	2.45± 0.03
2	34.03± 0.05	81.40 ± 0.06	19.25± 0.03	1.97± 0.04
8	14.02± 0.05	39.03± 0.03	9.20± 0.04	0.070± 0.05
9	13.05± 0.04	39.68± 0.05	10.03± 0.05	0.83± 0.03
Trolox [e]	2.85±0.05	30.15±0.08	-	-
DL-Penicillamine [f]	-	-	1.09±0.06	-
3-t-butyl-4-hydroxyanisole (BHA)[g]	-	-	-	0.049±0.03

[a] Values of •OH, total ROS, $ONOO^-$ and DPPH are expressed as the mean ± standard error of triplicate experiments.
[b] Inhibitory activity of hydroxyl radical generation in 1.0 mM H_2O_2 and 0.2 mM $FeSO_4$.
[c] Inhibitory activity of total ROS generation in kidney postmicrosomal fraction.
[d] Inhibitory activity of authentic peroxynitrite.
[e] Trolox was used as a positive control.
[f] DL-Penicillamine was used as a positive control.
[g] positive control used in DPPH assays.

Table 3.24: Cytotoxic activity of the crude extract and solvent fractions of *Rhynchosia reniformis*

Samples	**Lethality against Brain Shrimp larvae Concentration [5-25*mg/mL*] LD_{50}**
Crude extract	Nil
n-Hexane	Nil
CHCl3	Nil
EtOAc	Nil
n-BuOH	5.5990 ±1.00(SD)
H2O	Nil

Table 3.25: Proximate Values of the Selected Medicinal Plants with Standard Error (%)

Species name	Moisture	Ash	Fats	Fibers	Proteins	Carbohydrate	Energy value
R. reniformis	2.35 ± 0.14	11.95 ± 0.17	1.79 ± 0.02	31.59 ± 0.89	0.44 ± 0.16	51.88 ± 0.81	225.48 ± 3.14
N. laevigata	2.63 ± 1.10	35.15 ± 0.38	3.72 ± 0.33	5.77 ± 0.73	10.22 ± 0.81	42.52 ± 2.08	244.39 ± 5.44
N. kurramensis	3.53 ± 0.68	18.55 ± 0.89	8.27 ± 0.42	10.33 ± 0.88	6.31 ± 0.17	53.02 ± 0.85	311.73 ± 6.19

Table 3.26: Correlation Matrix of Proximate Parameters

Parameters	Moisture	Ash	Fats	Fibers	Proteins	CHO	E.V
Moisture	1						
Ash	-0.02	1					
Fats	0.99	0.05	1				
Fibers	-0.55	-0.82	-0.60	1			
Proteins	0.33	0.94	0.40	-0.97	1		
Carbohydrates	0.39	-0.93	0.32	0.56	-0.74	1	
Energy value	0.99	-0.04	0.99	-0.54	0.32	0.40	1

CHO = Carbohydrates, E.V = Energy value

DISCUSSION chapter 4

Since long plants are used as an important source for development of new pharmaceutical products. Scientific community is giving a lot of attention to use ethnobotanical knowledge in medicinal plant research. Screening of medicinal plants for obtaining biodynamic compounds is of great therapeutic value. Plants extracts are used in traditional medical practices to treat different types of ailments since long. Crude extracts from nature and compounds purified from these extracts can serve as better drug sources as herbal medicines and have no or minimum side effects, biofriendly and also have benefit due to the combination of medicinal ingredients with vitamins and minerals [Saetung *et al.,* 2005]. Activity guided fractionation and isolation of compounds is the starting point for drug discovery. Bioassays are helpful and simplest tools for testing the activity of plant extracts and on the basis of these activities extracts are preceded for phytochemical studies to isolate novel therapeutic agents. Plants and plant fractions are used for medication since prehistory. As reported by WHO about 80 to 85% of the world's population depends mainly on conventional medicines that involve the use of plant extracts [WHO, 1993; Shinwari *et al*., 2006]. The use of medicinal plants on synthetic drugs is preferred due to their less or no side effects, low cost of treatment and easy availability. Medicinal plants are good source to obtain a wide range of drugs in view of the fact that, a single plant can be used to treat more than one ailment. In fact, in recent years, traditional medicines are of extensive use. Food scientists and nutrition specialists consent that plants, characterized as huge source of natural antioxidants, contributes to reduce risks of certain diseases, like cancer, hepatitis, arthrosclerosis, inflammation and cardio-vascular diseases. In this context, concerned plants are supposed to have a significant position in the prevention of these diseases. Therefore, the rationale of present study was to explore the pharmacological status of plant samples.

Systematic exploration of plant components follows a rational pathway. Plants are collected either casually or by following leads delivered by local healers in geographical areas where the plants are found. Preliminary screening of plants for promising biological screening naturally begins by using crude, aqueous or alcohol extraction and can be

followed by numerous organic extraction procedures. Since nearly all of the identified components from plants are active against microorganisms are aromatic or saturated organic compounds, they are often obtained through initial ethanol or methanol extraction [Vilegs *et al*., 1997]. Therefore, in the present study, for the preparation of all the crude extracts (figure **2.1**) methanol was used as the extraction solvent. Duraipandiyan *et al.,* (2006) studied the antibacterial activity of methanol and *n*-hexane extracts of 18 plants; in which methanol extract showed significant activity against tested organisms. Previously the methanol extract of the stem bark of *Tridesmostemon omphalocarpoides* was found to be active on the nine pathogens studied [Kuete *et al.,* 2006]. In another study, Shahidi *et al.,* (2004) investigated the antibacterial and antifungal activities of 221 methanol plant extracts. Eighty one samples showed the antimicrobial activities against at least one of the tested organism. Methanol extracts of aerial parts of *Seseli libanotis* showed antibacterial activity against different bacterial strains [Ozturk and Ercisli, 2006], while in another study by Falahati *et al.,* (2005) methanol leaf extracts of *Eucalyptus camaldulensis* presented antifungal activity. Beside antibacterial components, methanol extracts have been used for identification of other active components as well. In one study the methanolic crude extracts of 12 traditionally used medicinal plants were screened for their antioxidant, antiglycation, cytotoxic, phytotoxic and phytochemicals. The tested plant extracts showed promising pharmaceutical activities [Hussain *et al*., 2010].

Since synthetic drugs are quite expensive and have associated side effects therefore the development of new effective and safe products for the treatment of different diseases caused by human pathogens is highly desirable [Victor et al., 2004]. The plants under investigation showed significant biological activities which support the traditional use of these plants to treat various diseases. Similar studies were conducted on other indigenous species [Shinwari *et al*., 2009]. Therefore these plant species could be an excellent natural source for the treatment of diseases and might be potential targets for the activity guided isolation of its active constituents.

Discovery of drugs, ethnobotany, and traditional and indigenous medicines have long been the components of medicinal plant research. For a long time ago, natural compounds have been a wealthy source of lead molecules in the development of drug discovery and most of the natural product and their derivatives have been developed for clinical and pharmaceutical uses, in addition to that sixty one (61 %) percent of all new products introduced worldwide as drugs during 1981-2002. Their structural and stereo chemical characteristics make them valuable for exploring novel molecules [Dewick, 2002, Mahidol *et al.,* 1998].

Pharmaceutical activities of plant extracts/fractions are due to the presence of major phytocompounds, including terphinoids, fatty acids, carotenes, phenolics, alkaloids, glycosides, flavonoids, tannins [Aqil *et al*., 2006]. Mohamed (2001) isolated the phenylpropanoid glucosides from the methanol extracts of aerial parts of *Chrozophora oblique*. Three plants *Nepeta laevigata*, *Nepeta kurramensis* and *Rhynchosia reniformis* were used in the present study. Methanol extracts and solvent soluble fractions of these plants were prepared to investigate their pharmacological activities including antimicrobial, antiglycation, antiplatelet, antioxidant cytotoxicity and phytotoxic activities leading towards the isolation and structure elucidation of compounds.

4.1 Phytochemical screening and isolation of compounds

Natural products and its derivatives have provided leads for many effective drugs including taxol, scopolamine, digitaline, vinblastine, aspirin, vincristine, amoxycillin, ceftriaxone, cefaclor, lovastatin, and had a huge impact on the pharmaceutical industry. Many of these compounds are in use for decades and still have important uses. In 1950's, two antileukemic agents: vinblastine and vincristine were derived from *Catharanthus roseus* by the scientists and developed semisynthetic anti-cancer agents, etoposide and teniposide, as derivatives of podophyllotoxin, a natural lignan isolated from *Podophyllum peltatum* [Taylor *et al.,* 1996]. Some of natural products like terpenoids and flavanoids have not only been used as a source of pharmacological activities but are also used as

coloring agents in food additives, similarly essential oils and terpenes derived from the plants, are used in flavoring and perfume industries [Stojanovic *et al.,* 2005].

Chemical substances of plants are the real cause of the medicinal value of plants and they produce their effects by interacting with human physiology [Hill, 1952]. Phytochemical screening is carried out to allow isolation of novel components with potential activities at the earliest stages thus economically very important. Present study exposed the naturally occurring substances such as fatty acids, carotenes, phthalates, alkaloids, flavonoids, saponins, tannins and other bioactive metabolites in various fractions of plant samples. Methanol extracts/fractions of the tested plant samples showed maximum number of plant constituents. Bioactive substances possess anti- disease wealth particularly minimizes the risk of oxidative injuries [Etuk *et al*., 2009]. In literature, many medicinal plants indicated their strength through antimicrobial, cytotoxic and antioxidant behavior that was endorsed with high concentration of flavonoids and alkaloids [Miller and Rice-Evans, 1997; Sharififar *et al*., 2009].

Flavonoids, a large group of phenolics also described as nature loving drugs, owned various biological actions like anti-inflammatory aptitude flavonoid containing Chinese medicine [Jiang *et al*., 2008; Wu *et al*., 2008]. Thereby, the presence of flavonoids in various fractions of plants can verify their folkloric use against hepatitis, skin infections and rheumatism. In modern medicine, alkaloids are used against nervous system disorders, like Alzheimer's disease [Maelicke *et al*., 2001] while, saponins are reported as nephroprotective agent [Jeong *et al.*, 1996]. Tannins, as polymeric phenolic substances having astringent property, are important in pharmaceutical preparations [Cowan, 1999]. In addition, antifungal activities [Adekunle and Ikumapayi, 2006] and anticarcinogenic activities in tannins of tea polyphenols were also observed [Chung *et al*., 1998; Kaur *et al*., 1998]. Aguinaldo *et al*., (2005) reported cytotoxic and/or antineoplastic activities of tannins. Therefore, presence of tannins in the tested plant samples may confirm their traditional use as potential medicine.

The present study supports the tested plants as large sources of bioactive chemicals specifically with reference to carotenes, fatty acids, anthraquinones, coumarins, alkaloids, flavonoids, resin, mucilage, saponins and tannins that ought to be isolated and monitored for biological activities as reported in traditional and therapeutic utilization. Summing preliminary screening tests, it is believable that detection of bioactive secondary metabolites may lead to the drug discovery and development. However, the qualitative analysis alone may not ascertain the pharmacological action of the plant.

The phytochemicals detected in our extracts are well known for various pharmacological activities. For example alkaloids are common antibacterial, antimalarial, cytotoxic and anticancerous agents [Wirasathien *et al.,* 2006]. Similarly saponins have the insecticidal, antibiotic, fungicidal properties [Sparg *et al.,* 2004]. Anthraquinones are antibacterial, antifungal and cytotoxic agents, while terpenoids are antimalarial and antibacterial agents [Kanokmedhakul *et al.,* 2005]. Flavonoids have been shown to have antibacterial, anti-inflamatory, antiallergic, antineoplastic, antiviral, anti-thrombotic antioxidant and vasodilatory activities [Miller, 1996]. Tannins have shown potential antiviral [Lin *et al.,* 2004], antibacterial [Akiyama *et al.,* 2001] and antioxidant activity [Yokozawa *et al.,* 1998]. Fifty-one tannins isolated from oriental medicinal herbs have been evaluated for their antioxidant ability with a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-generating system. The results showed that tannins are potential free-radical scavengers [Yokozawa *et al.,* 1998]. Hydrolyzable tannins could cause both doublestrand and single-strand breakages in DNA [Shirahata *et al.,* 1985]. In the past few years, tannins have also been studied for their potential effects against cancer through different mechanisms [Bhagavathi, 1999]. Cardiac glycosides have the cytotoxic properties and the Na__K_-ATPase inhibitory properties **[**Joseph *et al.,* 2005]. These compounds are known to have pharmacological activities and therefore are commonly found in medicinal plants.

In another study hydrolysable tannins were isolated from *Quercus* species [Nishimura *et al.,* 1984]. Therefore, the present investigation is in accordance with other reports as Mojab *et al.,* (2003**)** studied fifty five Iranian plants and found alkaloids in 39

plants while flavonoids, tannins and saponins were reported in 37, 20 and 44 plants respectively. Hadi and Bremner (2001) studied 100 plant species for alkaloid and found 23% positive for alkaloids. The differences in observations could be because of different species studied or because of environmental factors.

Presence of the active constituents in some other species of *Nepeta* is reported earlier too. [Hussain *et al*., 2012]. Therefore, this plant was selected for further purification and isolation of chemical constituents responsible for potential activities. Such strategy has been used previously, for example extracts of separate part of *Prismatomeris malayana* were analyzed for the anti inflammatory activity. The leaf extract was then subjected to bioassay guided fractionation (solvent partitioning and column chromatography) and isolation based on anti inflammatory activity [Nor Hayati, 2007]. Biological activities of the plant extracts and fractions gave clue for the further isolation studies. Fractions with active potential activities were further proceeded for isolation studies. For this purpose chloroform fraction of *Nepeta kurramensis* were investigated. Normal phase column chromatography, reverse phase column chromatography along with thin layer chromatography were used as isolation techniques and resulted in the isolation of seven compounds from chloroform fraction. A total of seven compounds have been isolated in this research from the chloroform fraction of *Nepeta kurramensis* out of which compound (**1)** [Bis ((*E)*-2-(hydroxymethyl) nonacos-3-enyl) phthalate] is a new isolate, first time reported from plant origin while six were commonly known compounds [Bis (2-ethylicosyl) phthalate **(2)**, 1-Dotriacontanol **(3**), 1-Nonacosanol **(4),** Ursolic acid **(5),** *β*-Amyrin **(6),** *β*-Sitosterol **(7**)] reported from the *N. kurramensis* and earlier isolated from other plants species and two known flavonoids **5-**hydroxy-3,6,8,4′-tetramethoxyflavon (**8**),3,5,4′-trihydroxy-6,8-dimethoxyflavone (**9**) have been isolated and identified for the first time from *Rhynchosia reniformis.* Column chromatography is a very simple chromatographic technique and has extensively been used for the purification of compounds from plant extracts [Jhade *et al.,* 2008]. TLC is another simple and cheap method used for the detection of various chemicals present in plants as it is reproducible, easy to run and require simple instrument [Marston *et al*., 1997]. Techniques used for structure elucidation were mass spectrometry (GC-MS and LC-MS), NMR and IR. Mass

spectrometry is most important method of molecular analysis and gives information of the molecular weight as well as structure of isolated compounds by producing fragments of varied length [Wolfender and Hostettmann, 1995]. IR spectroscopy gives clue about bonding and functional groups of the isolated compound. NMR is most powerful spectroscopic technique so far that help in obtaining detailed structural information about organic compounds [Albert, 1995].

Hussain *et al.,* (2010) reported five flavonoids (5-demethylnobiletin, nornobiletin, artemetin, chrysosplenetin, chrysoeriol), one lignin (yangambin), four triterpens (isopimara-8,15-dien-7alpha-18-diol, nepetidin, *β*-amyrin, ursolic acid), one diterpene (lupeol) and two steroids (*β*-sitosterol, stigmasterol) from *Nepeta clarkei.* In another study Hussain *et al.,* (2011) isolated two new phthalate bis (2-ethylundecyle) phthalate A and bis (2-ethyltridecyle) Phthalate (B) along with one known compound *β*-amyrin from *Nepeta clarkei.* From *Nepeta distant* Hussain *et al.,* (2010) screened one new phenolic compound nepatanol and eight known compounds which have been isolated from the first time from the stated plant species. One new tetracyclic triterpene ester and two known compouns (Artemetin, Jaceidin) have been isolated from *Nepeta suavis* by Hussain *et al.,* (2009). In addition to that a number of flavonoids have been isolated from different species of *Nepeta* by different scientists. Luteolin was isolated from *N. cataria, N. argolica, N. asterotricha* by Modnicki *et al.,* (2007) and Jamzad *et al.,* (2003). Apigenin was obtained from *N. cataria* by Modnicki *et al.,* (2007). Apigenin 7-0-B-glucopyranoside from *N. crispa* by Jamzad *et al.,* (2003).

4.2 Biological assays of plant extracts

Bioassays guide the discovery of natural drugs. A bioassay plays a very important role in the discovery of natural products. First, bioassays are used to detect the bioactivity of the crude extracts and the results guide the selection of extracts for further study. Then the selected crude extracts are fractionated and screened for biological activities. A combination of chemical screening with biological screening is the fastest way to arrive at new lead compounds from plants. For many plants there is no relevant literature

available so, biological activity must be evaluated using more direct methods such as pharmacological testing or screening. Generally, extracts must be active in at least in one of the bioassays adopted in the screening to be used for further studies.

In the present study, different bioassays were used to characterize the medicinal plants. The ethnobotanical approach offers strong clues to the biological activity of plant samples. The high percentage of positive results assures their biological activity. The results obtained in the course of present study demonstrated that different bioactive compounds like flavonoids and phenolics as reported in the phytochemical screening may have apt for antimicrobial potential. Numbers of authors have focused on antibacterial and antifungal potency of flavonoids [Bruneton, 1999; Cowan, 1999; Kuete *et al*., 2006]. Antimicrobial activity may be attributed to plant bioactive compounds to make complex with bacterial cell wall [Cowan, 1999] and thus inhibiting the microbial growth. The present study provides basis for the use of bioactive fractions from the tested plants for the treatment of infections associated with the studied microorganisms.

Antibacterial activities of our selected plants may be, because of the presence of the fatty acid esters in the extract. Preethi *et al.,* (2010) suggested that plants having fatty acid esters in their extract are more potent antimicrobial agents. Similar type of finding is illustrated by Gohar *et al.,* (2010) in characterization of marine antibacterial agents, where they isolated hexadecanoic acid ethyl ester and other components from marine bacteria and found out their antibacterial potential against different pathogens. Although they observed antibacterial activity of isolated compound but at the same time reported that activity of crude ethanol extract was more than isolated compounds. GCMS, LCMS, NMR and IR experiments were conducted on the compounds isolated from chloroform fraction of *Nepeta kurramensis* and *Rhynchosia reniformis* to determine their structures.

Antibacterial assay of methanol extracts and solvent soluble fraction of tested plants showed that *n*- butanol fraction of *N. laevigata* exhibited excellent activity against *E. coli* and *P. morganii* (Table **3.10**) while chloroform fraction of *N. kurramensis* displayed significant inhibition against *S. cricetus* and *M. flavas*. (Table **3.11**)The crude

extract of *R. reniformis* showed 100% inhibition. Ethyl acetate fraction of *R. reniformis* also exhibited promising activity against *M. flava, S. cricetus* and *P. morganii.*(Table **3.12**) The chloroform and ethyl acetate fractions of *N. laevigata* also exhibited promising antifungal activity (Table **3.13**), while chloroform fraction of *N. kurramensis* showed moderate activity against all fungal strains (Table **3.14**). In case of *R. reniformis*; chloroform, *n*- hexane and methanolic extract were more active as compared to other fractions (Table **3.15**). Results of proximate analysis revealed that all the selected species are a good source of ash, proteins and fats and can contribute greatly towards nutritional requirements (Table **3.26**).

There have been some reports on the antibacterial and antifungal activities of several species of *Nepeta* [Hussain *et al.,* 2010]. It is being confirmed that the *Nepeta* extract had wide antibacterial activities against both gram positive and gram negative. In another study it has been suggested that the antibacterial activity of *Nepeta* species against the strains could allow its use in the treatment of microbial infections [Voravuthikunchai *et al.,* 2006]. In a previous study, Satish *et al.,* (2007) tested aqueous extract of 52 plants for their antifungal potential against eight important species of *Aspergillus*. Out of 52 plant species, only twelve plant extracts showed the antifungal activities.

Despite the availability of the current therapies to prevent glycation, platelet dysfunctions, atherothrombotic diseases and oxygen stress related diseases continue to pose a threat to human health. The search for new and more effective antiglycation, anti-platelet aggregation agents of natural origin is rather timely and appropriate. Literature reports that plants such as *Urtica dioica* [El Haouari *et al.,* 2006], *Ocimum basilicum* [Amrani *et al.,* 2009] and *Nepeta juncea* [Hussain *et al.,* 2009] have antiglycation, anti-platelet aggregation activities.

The results obtained from this study suggest that the medicinal plants (*Nepeta laevigata, Nepeta kurramensisand* and *Rhynchosia reniformis*) possess the ability to exhibit a significant antiglycation activity (Table **3.18**) and also inhibit arachidonic acid (AA) and platelet activating factor (PAF)-induced human platelet aggregation (Table

3.18). Clearly there are many questions regarding the mode of action of these extracts\fractions against anti-platelet aggregation and glycation activity that need to be answered and these answers of these questions will play an important role in the development of future generations of these inhibitors. For that reason we have isolating pure compounds from these bioactive extracts\fractions to be evaluated against anti-platelet aggregation and glycation *in vitro* through STD NMR and molecular dynamics simulation and kinetics studies to establish a detailed mechanism of inhibition of these compounds. Free radicals have been reported to stimulate platelet aggregation and glycation by interfering with several key steps of platelet functions [Bakdash and Williams 2008]. Furthermore, researchers [Lin and Hsieh 2010] have linked the anti-platelet aggregation and glycation activity of some plants with their antioxidant activity. The crude extracts and solvent soluble fractions of *Nepeta laevigata, Nepeta Kurramensis* and *Rhynchosia reniformis* significantly inhibited •OH, total ROS and to scavenge authentic ONOO- . (Tables **3.20**, **3.21** and **3.22**)

The DPPH test is a non-enzymatic method currently used to provide basic information on the ability of extracts to scavenge free radicals. In the present study tested plants displayed potential antioxidant profile. Previously, free radical scavenging activity of twelve traditionally used medicinal plants was evaluated using diphenyl picryl hydrazyl (DPPH) radicals. Of the 12 plants tested, 7 plants showed more than 70% scavenging activity [Aqil *et al.,* 2006]. In our results *Nepeta laevigata*, *Nepeta kurramensis* and *Rhynchosia reniformis* presented significant DPPH scavenging activity. This supports previous reports of plant species containing antioxidant components. Previously comparable IC50 values are reported for crude extracts of plants. Nia *et al.,* (2004) assessed the antioxidant activity of methanol extracts of *Sphenocentrum jollyanum* organs using DPPH. The results indicated the stem bark as the most active organ with an IC50 of 1.80 ppm. Hydroxyl radical is the most reactive radical known. It can attack and damage almost every molecule found in living cells [Halliwell, 1996]. Numerous vitamins, minerals and other phytochemicals may protect against the damage caused by reactive oxygen species (ROS). Several studies have demonstrated that plants produce potent antioxidants and represent an important source of natural antioxidants [Santos-

Gomes *et al.,* 2003; Couladis *et al.,* 2003; Es-Safi *et al.,* 2005]. It has been reported that several chemopreventive agents that are antioxidants at some concentrations become prooxidant at other concentrations [Lee and Park, 2007]. Therefore, dose selection may be very important in the application of antioxidants and the Fenton reaction was also proved to be possible *in vivo* [Meneghini, 1997]. Prooxidant and antioxidant effect of chemopreventive agents on DNA may be due to the balance of two activities, free radical-scavenging activity and reducing power on iron ions. It seems that the predomination of reducing power on iron ions over free radicals scavenging activity can results in the prooxidant effect on DNA. Ascorbic acid may also have prooxidant properties in the presence of iron. Several mechanisms of ascorbic acid induced oxidant stress have been suggested. As an electron donor, ascorbic acid can maintain iron in a reduced state that may then react with H_2O_2 to form OH [Samuni *et al.,* 1983].

Generally the antioxidant scavenging activity depends upon the presence of maximum amount of polyphenolic compounds in an extract or fractions. This trend was observed in case of *Nepeta laevigata,* as the polarity of the solvent soluble fractions increase gradually from *n*-hexane to EtOAc-soluble fractions, the antioxidant activity increases as the possibility of number of polyphenolic moieties increase gradually. A slight decrease in activity was observed in respect of *n*-butanol fraction compared to EtOAc-soluble fraction. This may be due to the possible glycosidation of phytochemicals in the *n*-butanol fraction, thus reducing the number of free polyphenolic groups and hence reduced the activity. A similar trend in antioxidative scavenging activity was also observed in the related species of the genus, *Nepeta Kurramensis*. (Tables **3.21**, **3.22**) This similar trend in activities of the both species may be because of the presence of similar groups of phytochemicals in a genus. None of the fraction of the *Nepeta* species under investigation showed significant anticancer and allelopathic potential.

Beneficial biological activity may be indicated from selected bioassay results, however it is important to know the level of general toxicity of plant extracts as well. The brine shrimp assay can be used to screen the plant extracts for their general toxicity [McLaughlin *et al.,* 1991]. Two of the three tested plants presented nil cytotoxicity while

the *n*-butanol fraction of *Rhynchosia reniformis* exhibited significant cytotoxicity (Table **3.24**). A number of previous studies indicated potent cytotoxicity in case of methanol extracts/fractions of several plant species, as Quignard *et al.,* (2003) identified several cytotoxic plants when 226 methanolic and water extracts were screened for cytotoxicity towards brine shrimp larvae. Lamina minor has been used in general toxicity studies because of their sensitivity to phytotoxic compounds and is a standard assay in alleopathic studies [McLaughlin *et al.,* 1988]. All extracts/fractions of our research plants exhibited no phytotoxicity.

4.3 Proximate analysis

The results of the proximate analysis of the plant species with their standard deviation are summarized in Table 3.25. The moisture content was found to be highest in *N. kurramensis* (3.53 %), whereas *R. reniformis* was found to be the lowest (2.35 %). As a result of this study, *N. kurramensis* was found to contain the highest content of the moisture. The descending order of the moisture content was observed as *N. kurramensis* > *N. laevigata* > *R. reniformis* (Table **3.25**). The moisture content of plant species were found lower than *N. sauvis* (8.44%) reported by Hussain *et al*, (2011). The highest ash and protein value were observed in *N. laevigata* during analysis. Ash contents of *N. laevigata, N. kurramensis* and *R. reniformis* were found to have 18.55, 35.15 and 11.95 % respectively. The results of ash content with standard deviation are presented in Table 3.25. In comparison, ash content was found higher than the *N. sauvis* (7.91%) [Hussin *et al*., 2011]. The ash content of *N. kurramensis* was found similar to that of *Datura alba* (18.80 %), *Phlomis cashmeriana* (17.66 %) and *Calotropis procera* (17.62 %), while ash values of *R. reniformis* was also in good agreement with *Dalbergia sisso* (12.33 %), *Phlomis bracteosa* (10.83 %), and slightly lower than *Avera javanica* (14.23 %) [Hussain *et al*., 2011; Hussain *et al*., 2010].

N. kurramensis was found to be highest in its crude fat content (8.27%). However, *R. reniformis* contains crude fat (1.79 %) similar to that of *Aerva javanica* having 1.15 % fat [Hussain *et al*., 2011]. *N. laevigata* were found to have 3.72% fat and showed close similarity with *Rhiza stricta* (3.98 %) and *Dalbergia sisso* (3.35 %) [Hussain *et al*.,

2010]. *Nepeta* species were found to be a good source of fats particularly *N. kurramensis* (Table **3.25**). Crude fiber of *R. reniformis* was found to have value of 31.59 %, followed by the *N. kurramensis* (10.326%), (Table **3.25**). In comparison with other medicinal plants *R. reniformis* showed close resemblance with *Aerva javanica* (29.186 %) and *Calotropis procera* (29.49 %) [Hussain *et al*., 2010]. A high intake of dietary fiber improves glycemic control, decreases hyperinsulinemia, and lowers plasma lipid concentrations [Chandalia *et al*., 2000]. Therefore, dietary guidelines for patients with diabetes should emphasize an overall increase in dietary fiber through the consumption of unfortified sources, rather than the use of fiber supplements.

The protein content of the medicinal plants was calculated on the basis of the available nitrogen using Kjeldahl method and was observed in the range of 0.44- 10.22 % with *N. laevigata* having the highest value (10.22 %), followed by *N. kurramensis* (6.31 %) and *R. reniformis* (0.44 %) (Table **3.25**). The *N. laevigata* showed very close value to *Phlomis bracteosa* (10.61 %) and *Phlomis cashmeriana* (9.51%) belong to the family Labiateae [Hussain *et al*., 2010].

The carbohydrate content of analyzed samples revealed that *N. kurramensis* had highest amount of carbohydrates (53.02 %), followed by the decreasing order of *R. reniformis* (51.88 %) and *N. laevigata* (42.52 %) (Table **3.25**). The contribution of the carbohydrates to the energy in a food ration recommended by Anon., (1990) is from 55 to 75 %. The carbohydrates of *N. kurramensis* and *R. reniformis* fall in the acceptable range set by WHO. Thus these 2 plant species can be used as a source of energy contribution in a food ration.

According to the results of the energy calculations, based on the carbohydrates, fats, and protein content, the highest value was found in the *N. kurramensis* (311.73 kcal/100g), while the *R. reniformis* was found to contain the lowest energy value (225.48 kcal/100g) (Table **3.26**). The energy value of the *Nepeta* species were found comparatively close to the reported values of some Nigerian leafy vegetables (248.8-307.1 kcal/100g) (Isong *et al*., 1999), some Ghanian green leafy vegetables like

Corchorus tridens (283.1 kcal/100g) and sweet potato leaves (288.3 kcal/100g) (Asibey & Tavie, 1999). However, the energy value of *N. kurramensis* was observed to have similar value to that of *Calotropis procera* (312.41 kcal/100 g) and *Datura alba* (308.10 kcal/100 g) reported by Hussain *et al*. (2011).

Conclusion

This study provides new scientific information about *Nepeta* and *Rhynchosia* plants based on its biological potential and phytochemical analysis that has never been reported earlier. All the plants under investigation showed promising antimicrobial, antiglycation, antiplatelet and antioxidant activities. Thus the study authenticated the pharmacological use of the tested plant samples with special reference to most promising fractions.

Solvent partitioning of *Nepeta kurramensis* revealed six partitioned fractions i.e., methanol, *n*-hexane, chloroform, ethyl acetate, *n*-butanol and water (Figure **2.1**). Chloroform fraction was selected on the basis of results of biological assays and phytochemical analysis for further fractionation through column chromatography techniques. Fractionation of chloroform partitioned fraction revealed seven fractions NS1, NS2, NS3, NS4, NS5, NS6 and NS7 (Figure **2.2**). The structures of the active compounds (**1-9**) were characterized/ assigned on the basis of H, C NMR spectra including 2D NMR techniques such as COSY, HMQC and HMBC experiments and compared with the literature data. The compound (**1)** is different from already known natural products of plants and is a newly identified compound while compounds **(2-9**) are known, previously reported from other sources of plant origin.

Future research

The compounds isolated from *Nepeta kurramensis* and *Rhynchosia reniformis* can be studied in detail for their mechanism of action, clinical trials and finger printing. Furthermore a number of valuable compounds would have been available for the use in the manufacture of new drugs. The elucidated structure of the new compounds would have added information to the natural product chemistry and open new areas of research in this field. In addition to that, the same plant species will be collected from different locations to extract compounds identified in this study. It would help to study any geographical effect of sample collection on the active constituents of our selected research plants.

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