Pharmacological Basis of Selected Medicinal Plants used in Muslim Ethnobotanic Culture

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A thesis submitted in partial fulfillment of the requirement for the degree of

Doctor of Philosophy (Ph.D) in **Applied Biosciences**

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بسم الله الرحمن الرحيم

The Prophet (ﷺ) said, "There is no disease that Allah has created, except that He also has created its treatment."

Sahih al-Bukhari 5678

Dedicated to Almighty ALLAH and the Holiest man ever born on earth PROPHET MOHUMMAD (peace be upon him), my beloved parents and my dear Husband and Motherland, Pakistan

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I am proud of being a Muslim.

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ABSTRACT

Medicinal plants have been a major source of biologically active natural products and are used as medicines to cure disases in all cultures since ancient times. *Cassia angustifolia* (senna) and *Trigonella foenum-graceum* (fenugreek), are medicinal herbs, recommended by Prophet Muhammad (Peace be upon him), used in Muslim ethnobotanical culture. These herbs have traditionally been used against liver diseases, constipation, typhoid, cholera, cancer, diabetes, high cholesterol, ulcer, and inflammations etc. The present study describes isolation and identification of biologically active compounds, selected based on their bioactivity, from *C. angustifolia* and *T. foenum-graceum*. The pharmacological activities; antibacterial, antioxidant, anticancer, neuroprotective and hepatoprotective effects of *C. angustifolia* and *T. foenum-graceum* were investigated from their aqueous and organic (methanol, ethanol, acetone and ethyl acetate) extracts. Phytochemical screening revealed the presence of steroids, alkaloids, terpenoids, flavonoids, coumarins, cardiac glycosides, saponins, anthraquinones, phenols and tannins in both plant extracts. Methanol and ethyl acetate extracts of both plants possess antibacterial, antioxidant, anticancer, neuroprotective and hepatoprotective activities which led to the isolation and identification of active compounds. A novel compound (proposed name "Irizoflavan") and three known flavonoids i.e. Quercimeritrin, Scutellarein, and Rutin were isolated from *C. angustifolia*. Two known flavonoids i.e. Amurensin and Cosmosiin were isolated from *T. foenum-graecum*. These compounds were isolated and reported for the first time from these plants. The present work provides basis for the in depth understanding of the molecular pathways associated with these isolated compounds and for the development of plant based drugs.

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List of Abbreviations

Abbreviations	**Description**
WHO	World Health Organization
US	United States
C. angustifolia	*Cassia angustifolia*
T. foenum-graecum	*Trigonella foenum-graecum*
N. sativa	*Nigella sativa*
Ca	Circa
BCE	Before Common Era
BC	Before Christ
AD	Anno Domini
CTM	Chinese traditional medicines
CTHM	Chinese traditional herbal medicines
IHM	Indian herbal medicines
TAIM	Traditional Arabic and Islamic Medicine
Rs	Rupees
mg	Miligram
g	Grams
Kg	Kilograms
L	Liter
C	Centigrade
mg/kg	Milligram/kilogram
mg/g	Miligram/gram
mL	Milliliter
µg	Micrgram
µL	Microliter
h	Hours
s	Second
min	Minute
µm	Micrometer

nm	Nanometer
mm	Millimeter
UV	Ultraviolet
OD	Optical density
QE	Quercetin equivalents
rpm	Revolution per minute
FDA	Food and Drug Administration
CD1	Cluster of differentiation 1
PRA	Plaque reduction assay
TPA	12-O-tetradecanoylphorbol-13-acetate
NS2-NS3	Non-structural 2-Non-structural 3
E. coli	*Escherichia coli*
K. pneumoniae	*Klebsiella pneumoniae*
S. shinga	*Shigella shinga*
S. aureus	*Staphylococcus aureus*
A. flavus	*Aspergillus flavus*
A. alternta	*Alternaria alternta*
C. dematium	*Collatotrichium dematium*
F. roseum	*Fusarium roseum*
C. lunata	*Curvularia lunata*
HepG2	Hepatocellular carcinoma
MCF7	Michigan Cancer Foundation-7
HeLa	Human cervical adenocarcinoma cell line
HBV	Hepatitis B Virus
HCEC	Human corneal epithelial cells
RPMI	Roswell Park Memorial Institute medium
FBS	Fetal bovine serum
PFA	Parafarmaldehyde
MTT	3-(4, 5-dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide
TCP	T-cell lymphoma
BCP	B-cell lymphomas
FRO	Thyroid Papillary carcinoma

KG-1	Human acute myeloblastic leukemia cell lines
DMBA	7,12 dimethylbenz(α)anthracene
ER	Estrogen receptor
THP-1	Human monocytic cell line
w/v	Weight/Volume
Bax	Bcl-2-associated X protein
p53	Tumor supressor protein
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
CCL_4	Carbon tetra chloride
GAE	Gallic acid equivalents
$NaNO_2$	Sodium Nitrite
NaOH	Sodium Hydroxide
DMSO	Dimethyl sulfoxide
L.B	Luria Bertani
NaCl	Sodium Chloride
DPPH	2,2-diphenyl-1-picrylhydrazyl
IC_{50}	Inhibitory Concentration 50
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
cDNA	Complementary deoxyribonucleic acid
PCR	Polymerase chain reaction
Ct	Comparative threshold
HPLC	High pressure liquid chromatography
MS	Mass Spectrometry
LC	Liquid Chromatography
NMR	Nucleic Magnetic Resonance
2D-NMRS	Two-dimensional nuclear magnetic resonance spectroscopy
FID	Free induction decay
DQF-COSY	Cosy Double Quantum Filter
HSQC	Heteronuclear single quantum coherence spectroscopy

HMBC	Heteronuclear Multiple Bond Correlation
MHz	Megahertz
Hz	Hertz
J	J coupling
s	Singlet
dd	Doublets of doublets
ddd	Doublets of doublets of doublets
dt	Doublets of triplets
br.d	Broad douplet

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1. Introduction

1.1 Overview and significance

Medicinal plants have been used to cure number of diseases since ancient times and are regarded as the sources of vital drugs such as taxol, digoxin, atropine, quinine, morphine, codeine etc. From the past century, with the advent of conventional medicines, traditional or folk medicines faced much challenges due to lack of scientific evidences despite of having long history of effective uses. Although, great advancements have been made in pharmaceutical research and development, but due to the side effects of conventional drugs, antibiotic resistance, lack of control on cancer progression and other chronic diseases, medicinal plants are gaining much attention as it provides slow recovery with lesser side effects and lower antimicrobial resistance. In the late twentieth century, the use of medicinal plants gained much interest in developed and industrialized countries. According to the World Health Organization (WHO), about 80% of the world population is relying on the traditional system of medicines to cure their primary health related problems. To date, it has been estimated that about 350,000 plant species exist worldwide (Pan *et al.,* 2014). Out of which 287,655 plant species have been identified and 35,000 to 70,000 species of plants have been explored and used as medicaments against different diseases (Mamedov, 2012). It has been reported that more than 50 major conventional drugs used commercially have their origin from medicinal plants (Padula, 1999). Plant based medicines/drugs producing a great deal of profit in international market generating a revenue of US$ 14 billion in China, US$ 5 billion in western Europe, and US$ 140 million in Brazil (WHO, 2008).

1.2 Historical prospective

People from different origins, traditions or cultures used plant parts (seeds, bark, flower, leaves, fruit, berries) and plant extracts (organic and aqueous) for the healing and treatment of different diseases and these plants took the place and became the part of their traditional or folk medicines. The medicinal products from plants, referred to as phytomedicine, botanical medicine or phytotherapy, are the active ingredients of the plant responsible for various pharmacological activities. The basis of these medicinal products from plants dated back to the early history with the beginning of life on earth, when man used plants for shelter, food and medicine. This interaction of man with plants over time gives insight to the local communities to study and explore the beneficial aspects linked with plants. The term ethnobotany is referred to as the study of useful plants by indigenous and ancient people. Although the term ethnobotany is relatively a new term but the study of useful plants is one of the oldest science. Plants are not only vital for all ecosystems but also important for humans of all societies. Different forms of healing methods from plants were used in different traditions throughout the world. Local people from different communities started documented the uses of traditional plants in their sculpture soon after they learned to write. Well documented record on the medicinal uses of thyme, caraway, and laurel were established by Sumerians, 5,000 years ago. (Falodun, 2010). Archeological studies revealed that in Iraq, local people were practicing medicinal plants against different diseases ca. 60,000 years ago, and ca. 8,000 years ago in China (Gourhan, 1975).

Egyptians recorded many detailed descriptions of traditional uses of medicinal plant in their manuscripts and on the temple walls. The Kahun Gynaecological Papyrus is the oldest form of ancient Egyptian medicine, dated to 1800 BCE. It contains the recipes related to the uses of medicinal plants including *Ceratonia siliqua* and describes in detail the diseases related

to women health such as contraception, pregnancy and fertility (Quirke, 2002). Ebers Papyrus, 1550 BCE, an oldest preserved medical text, contains 700 medicinal formulas and remedies (Glesinger, 1954). It prescribed remedies from ochre (medicinal clay), senna, pomegranate, aloe, fig, castor oil plant, garlic, dates, cucumber flower, acacia, elderberry for diseases like diabetes mellitus, cancer, contraception, gastro-intestinal, optical and urological disorders (Tucakov, 1964).

1.3 Traditional system of Medicines

Based on different ethnobotanical cultures throughout the world, different traditional medicine system was developed to improve the quality of life. Some of these ethnobotanical cultures introduced Chinese traditional medicines, Indian traditional medicines, western traditional medicine, traditional Arabic or Islamic medicines.

1.3.1 Chinese Traditional Medicines

Chinese traditional medicines (CTM) comprised of Chinese traditional herbal medicine (CTHM) which have been used by Chinese ca. 5,000 years ago. This term was initially used by Shen-Nong (2697-2737 BCE), legendary Chinese leader, who selected and described the properties of 70 herbs that are suitable for different remedies after testing hundreds of herbs (Xu and, Yang 2009; Lu *et al.,* 2004; Kuang, 2000). After his remarkable efforts, people started using herbs for their primary health care (Dharmananda, 2013). Shen-Nong-Ben-Cao-Jing, is the first compendium written by authors in 202–220 BCE. This user guide described the recipes and therapeutic effects of 365 herbal preparations from Chinese herbs, which includes preparation from 252 types of plant parts, 67 types of animal parts and 49 types of minerals (Pan *et al.,* 2014). It was documented that between 1405-1433, large number of herbs including cinnamon, ginseng, taurine, poria, rhubarb and angelica were exported by China to other

southeast asian countries. Forty different dosage forms of herbal formulations are available in CHM in the form of pastes, injections, granules, decoction, tablets, tincture, ointments, powder, liniments, and pills (Tang and Ling, 2007; Zhang 2006; Ding and Hong, 2007). CHM are different from natural drugs and plant drugs because the process of preparing CHM includes stir-frying, steaming, calcining, boiling etc. These procedures change the structure and composition of active ingredients which is responsible for therapeutic effect of CHM (Zhao*et al.,* 2010). China have ca. 31,000 of plant species, out of which 5,000 plant species are the part of CHM, which accounts for ca. one fifth of the Chinese pharmaceutical market (li, 2000). *Panax quinquefolium,* also known as Chinese or American ginseng, was first discovered in China in 18th century. It is also native to USA and Canada (Assinewe *et al.,* 2003). Recently, studies have shown that it possesses various pharmacological properties like antinociceptive, anticancer, antidiabetic, antioxidant, neuroprotective and cardioprotective (Qi *et al.,* 2011; Kuhle*et al.,* 2011). Artemisinin, an antimalarial compound from native Chinese plant *Artemisia annua,* commonly known as wormwood, first isolated in 1972 by Chinese chemist Tu Youyou (Mamedov N; Craker, 2008). In 2015, she has been awarded with the Nobel prize in discovering antimalarial drug which saves 100,000 lives in Africa annually. *Artemisia annua* is the herb from CHM and was first prescribed in 4th century to treat fever. Later, it was revealed that it kills the malarial parasite found in the blood (Guo, 2016).

1.3.2 Indian Traditional Medicines

Indian-subcontinent is rich in vast varieties of medicinal plants which are used in Indian traditional medicines. Ayurveda is the Indian traditional herbal medicine, which means “Science of life”. It is the most ancient living traditional system of medicine in Indian ethnobotanical culture originate between 2500 and 500 BC (Subhose*et al.,* 2005). India has vast repository of natural flora, comprises 315 out of 400 families of flowering plant in the

world. There are 3,500 plant species that are of medicinal value. Out of which 500 medicinal plants are the part of ayurvedic medicine (Singh, 2006). About 70% of the Indian population depends upon the traditional ayurvedic medicine. 80% of the herbal medicines, 40% of the Unani medicine, 33% of the allopathic medicine was formulated by medicinal plants which are available from the western Himalayan region (Baragi*et al.,* 2008). British pharmacopoeia comprises 50% of the plant based drugs that are grown in this region (Dev, 1997). Approximately 25,000 plant based medicines are the part of Indian folk or traditional medicines which are being used as remedies against chronic diseases (Joy, 2013). Out of which 7,500 are being used as diuretics, antirheumatics, hepatoprotectants, expectorants, aphrodisiacs, antipyretics and antimalarial (Mukherjee and Wahile, 2006; Aggarwal *et al.,* 2011). Indian herbal medicines (IHM) were extracted from whole plant or from different plant parts such as leaves, seeds, fruit, flower, or bark. IHM are also derived from animal, minerals and from plant excretory products such as latex, resins and gums (Sharma, 2007). IHM are delivered in crude dosage formulations containing alcoholic and acidic fermented components in the form of poultice, infusions, essentials oils, powders, or pills (Singh and Chaudhary, 2011). The extraction of active components from medicinal plants were carried out by self-generated alcohol. This form of extraction makes IHM different from other preparations as it has excellent therapeutic efficacy, quick absorption and action, and longer shelf life (Chaudhary*et al.,* 2011). These formulations are co-administered orally with other vehicle material such as milk, juice, sugar, honey, jiggery, ghee or some other herbs. These drug vehicles act as early antidote which increases the absorption and improves the palatability of ayurvedic medicine (Pan *et al.,* 2014). Indian herbal industry exports herbal product worth of US$ 1 billion, out of which 60% herbal products are the unique Indian products, while 30% and 10% are herbal extracts and ayurvedic products, respectively (Singh and Swanson, 2013). In 2014, India had exported more than

INR 36 billion worth of raw herbal products and materials and its herbal industry has a value of INR 70 billion (Sharma*et al.,* 2008). According to the report, the worth of Indian herbal industry increased to a greater extent from INR 75 billion to INR 150 billion, in 2015 (Saini and Singh, 2011).

Commiphora mukul Engl., commonly known as guggul, is used in ayurvedic medicine. It is a major ingredient in immune and joint care. It has immunomodulatory, antinociceptive and antihyperlipidemic activity (Sarup *et al.,* 2015). Another important Indian medicinal plant used in ayurvedic medicine is *Garcinia cambogia,* commonly known asgarcinia. Its active ingredient hydroxycitric acid has antihyperlipidemic activity by inhibiting the synthesis of fatty acids and lipids. Garcinia is a rich source of vitamin C and is used as a heart tonic (Mahendran *et al.,* 2002). Also, *Glycyrrhiza glabra L.,* also used in Chinese and Indian traditional system; is a versatile medicinal plant possessing anticancer, antioxidant, antimutagenic and immunodulating activities (Kataria and Hemraj, 2012).

1.3.3 Western traditional medicines

The concept of traditional herbal medicines was introduced by Greece and Rome to Europe, where it has been transferred to North and South America (Azaizeh, 2010). The term traditional medicines were replaced by complementary/integrative and alternative medicines in western countries (Awang, 1997). In the late 20th century, people started practicing herbal products by consuming dietary supplements with the concept of being natural rather than synthetic origin, having lesser side effects as compared to conventional drugs. In USA, it has been reported that after the approval of Dietary Supplements Health and Education Act in 1994, ca. 50% of the population consumed herbal products worth of US$ 4 billion which was increased to US$ 14 billion in the year 2000 (Angell, 1998). Medicinal plant research in USA

led to the discovery of alkaloid from *Catharanthus roseus*, commonly known as Madagascar periwinkle, which is used in the treatment of Hodgkin's disease and leukemia as a chemotherapeutic agent.

In 2004, survey was conducted in Canada on 2500 individuals, of which 38% (which was increased from 28% in 1999), were using herbal remedies against different diseases (Berger, 2001). The most popular herbal remedies used by Canadian population include valerian, tea tree oil, ginkgo biloba, camomile, echinacea, garlic, and ginseng (Sibbald, 1999).

Herbal medicine market in Europe had a worth of US$ 2.8 billion at retail selling prices which was increased upto US$ 5.6 billion in 1995. The investment made by herbal markets in UK was far less than Germany, which had the business of £1400 million compared to £88 million in 1994 (Shaw, 1998). The Beneficial effects of *Digitalis purpera* commonly known as foxglove, an European traditional herbal medicine, was discovered by British physician William Withering in 18th century. It was used for the treatment of dropsy. In 20th century, research on foxglove revealed the presence of 30 cardiac glycosides which includes digoxin and digitoxin (Balick and Cox, 1997), being used as cardioprotective agents (Zhu, 1998). It has been estimated that about 200 kg of digitoxin and 1500 kg of digoxin is prescribed to patients having heart problems worldwide (Balick and Cox, 1997; Shankar, 2004).

1.3.4 Traditional Islamic or Arabic Medicines

The birth of Traditional Arabic and Islamic Medicine (TAIM) took place in the 7th century. TAIM started off by using traditional Arabic medical practices which were derived from Persian, Chinese, Graeco-Roman, and Ayurvedic theories and practices. These traditional Arabic practices were then combined with Prophetic medicines, Islamic teachings that were further advanced by Muslim scholars and physicians who made remarkable discoveries in the

field of medicine (Oumeish, 1998). After decades of rigorous research Muslim scholars compiled their findings into a comprehensive system of medicine (Bhikha, 2007). TAIM has become a system of healing which is comprised of applied therapies, spiritual healings, mind-body therapies, dietary practices and medicinal herbs. Many of these practices share a deep connection to both Islamic medicine and prophetic teachings. Many researchers took great interest in studying the origins and practices of TAIM tobetter understand the origins of various modern medical practices that are used today.

Arab countries including Egypt, Morocco, Syria, and Yemen took great interest in studying TAIM herbs and practices. It was deduced that there were two main development phases for Islamic medicines. The first phase took place in the 8th century. It brought forth the translation of philosophical works by Aristotle and Plato, medical works of Galen and Hippocrates, and numerical illustrations of Archimedes and Euclid into Arabic. These researches of Muslim scholars led to a new age of medicine in the Arab world in which medical schools and hospitals were flourished. Among distinguished scholars, Al-Razi (Rhazes, 846-930 AC) and Ibn Sina (Avicenna, 980-1037AC), were influential in memorializing this historical phase as the golden period (Saad *et al.,* 2005). The final phase starts from 12th century, when the development of Arabic medicines began and European scholars started studying Arabic works and translated it into latin (The remarkable examples are the translation of Rhazes book 'The Comprehensive' and Avicenna's book named 'Canon of Medicine'. These researches were the part of medicinal studies in Europe until the 16th century (Al-Rawi and Michael, 2012).

Many new ideas were developed to enhance the medical efficacy and safety of the herbal products. As an example, Al-Rhazi discover the basis of small pox & stated that it can only be acquired only one time in one's life. He has important role in the discovery of immune

system; its existence and working. He started clinical trials on animals to test the efficacy and safety of isolated active compounds from plants. Monkey was the first animal used to examine the effectiveness of mercury on the body. Avicenna separates medicines from pharmacological sciences and he used ice first time in the treatment of fever. For general and local anesthetization, anesthetic compounds from local herbs were discovered by Jaber-Bin-Hayan. He developed procedures for the isolation and purification of different compounds including royal acid, sulfuric acid, nitric acid, alcohol etc (Saad *et al.,* 2005).

The innovations mentioned above were not the only great discoveries made by scholars in medicine. Arab scholars had taken a great role in many fields of medicine. One of the field of medicine where the Arab scholars and physicians had made the greatest contribution is Phytotherapy. One example of Arab scholar who contributed in phytotherapy would that of Daoud Al-Antaki. He was a Syrian physician and published a detailed document briefly explaining the knowledge of his predecessors on medicinal herbs whuch they used to treat patients. Another great example would be Spanish pharmacist Ibn Al-Bitar. Around new 350 plant species was introduced by him which were used as medicinal herbs to treat various human ailments. Another notable contribution would be by Abu Al-Abbas, a herbalist who published several books and dictionaries on the uses of each plant part of medicinal plants, the treatment procedure and various protocols of herbal preparations used as remedy against several diseases (Syed, 2003).

Phytotherapy was not the only branch of medicine in which Arabian people contributed greatly. Another great contribution by Arabs in medicine took place in the field of pharmacology in the 10th century. Al-Zahrawi (Abulcasis) was another physician who contributed greatly to the world of pharmacology by describing many methods and explaining the procedures of manufacturing complex as well as simple drugs. However, despite Al-

Zahrawi's work being revolutionary, He was the first physician Shapur-Ibn-Sahl, who started publishing pharmacopoeia. His book described a wide range of drugs with various treatment of human diseases (Saad, 2014). Another great contribution in pharmacology was at the hands of Al-Biruni, who wrote the book Saydanah fit-Tib. It contained comprehensive information on the pharmacology of drugs and highlighted the role of pharmacy and the duties and functions of the pharmacist. Another great contribution was made by Al-Kindi who revolutionized medicine by introducing the field of mathematics into the field of medicine, mainly in the pharmacology field (O'Malley, 1970).

Due to these researches, currently in TAIM there are now approximately 350-450 plant species that were used in the treatment of different human ailments (Said *et al.,* 2002). These remedies were administered by practitioners with the macerated plant parts, inhalations of essential oils, infusion in water or oil, aqueous extracts. The plant formulations were also taken in the form of milky sap, syrup, fresh salad or fruit, oil, paste and poultice (Azaizeh *et al.,* 2006).

1.3.4.1 Prophetic medicines

Despite TAIM being such an ancient form of medicine, there are still many aspects, principles and herbs related to TAIM that are being used in modern day medicine. Treatments based on traditional Arabic medicines, with the collaboration of physicians have been tested and prescribed to patients routinely in Europe and in Mediterranean countries. Most of these traditional Arabic medicines include herbs from the holy book Quran and Prophetic medicines. Prophetic medicines (also called tibb-e- nabawi) includes medicinal herbs recommended by Prophet Muhammad (Peace be upon him) for the treatment of different diseases.

One commonly herb used in Prophetic medicine, *Nigella sativa* (L.), habat al-suwda in Aabic, kalonji in urdu and black seed in english, (Fig. 1.1) traditionally been consumed as a

spice, oil and herb for thousands of years as folk medicines in Middle east and south-east Asia. This plant considered as a miracle herb with many of its compounds active against many human diseases (Al-Ghamdi, 2001). Prophet Muhammad (Peace be upon him) recommended the use of *Nigella sativa* in daily routine as it has the property to combat every disease except death as narrated in the following hadith. “Hold onto the use of the black seeds for in it is healing for all diseases except death” (Sahih Bukhari). It is involoved in wide range of thereapeutic effects including gastric ulcer healing, antimicrobial, anti-cancer activity, cardiovascular disorders, gastroprotective and antioxidant activity, immunomodulatory, anti-inflammatory and anti-tumor effects, anti-anxiety effect, anti-asthmatic effect, anti-helicobacter activity, anti-fungal activity, anti-viral activity against cytomegalovirus, hepatoprotective activity have been reported for this medicinal plant (Ahmed *et al.,* 2013). Even Avicenna in his book Canon of Medicine, mentioned *N. sativa* as the seed that helps recovery from fatigue and stimulates the body’s energy.

Figure 1.1. *Nigella sativa* flower and seeds.
Image taken from https://www.alibaba.com/showroom/nigella-sativa.html

Prophetic medicines also include various dietary practices which include the usage of specific kinds of foods in daily routine including honey. Honey is known to involve in health

promoting activities which includes promoting circulation, provides relief in intestinal and stomach pain, and acts as a topical antibiotic ((Nagamia, 2003; Oumeish, 1999).

Another commonly known prophetic medicine is *Olea europaea*, the Olive. The olive (like *N. sativa*) is another Prophetic recommended remedial herb throughout the Mediterranean. Leaves of olives (Fig. 1.2) have been used in various culture against different diseases. Olive oil is used as an ointment and to flavor food. Moreover, olive is known to possess various biological activities such as antimicrobial, immunostimulator, antioxidants and anti-aging (khan *et al.,* 2007). It has been proven scientifically that Olive leaf extracts have antidiabetic and antihypertensive effect. It is mentioned in the Quran as the holy tree and Prophet (Peace be upon him) said: ***"Eat olive oil and massage it over your bodies since it is a holy tree"*** (Sunan al-Tirmizi).

Senna is another herb that was recommended by the Prophet (Peace be upon him). At-Tirmidhi and Ibn Majah narrated that the Prophet (peace be upon him) ***"If there is a cure that prevents death, it would be Senna (Cassia angustifollia). "***(Ibne Majja (Ravi: Abu bin Um-e-Haram). It is known as *C. angustifolia*, (Fig. 1.3) a plant best known for its medicinal properties. Originally it is belong to Yemen, Egypt, and Saudi Arabia. Its pods and leaves used as decoctions and infusions have anti-helminthic activity aginst intestinal worms. Different pharmacological activities of *C. angustifolia* have been reported which includes anti-pyretic in anemia, laxative, cholera, splenic enlargements, genotoxicity and hepatotoxicity induced by *Escherichia coli* (Laghari *et al.,* 2014).

Figure 1.2. *Olea europaea* fruit and oil.
Image taken fromhttp://athanasopoulos.farm/en/products/organic-green-oliveoil

Another prophetic medicine is *Trigonella foenum-graecum*, named as Hulbah in Arabic, fenugreek in English and methi dana in Urdu (Fig. 1.4). It is native to Middle east, Asia and belongs to the family Leguminosea. It has been investigated because of its various remedial properties in different ethnobotanic cultures which includes hypocholestroleamistic, anticancer, febrifugal, carminative, galactogoguic, lactation-stimulating immunomodulatory, antibacterial, antidiabetic, hepatoprotective and anti-inflammatory (Haque*et al.,* 2011). Biologically active compounds have been identified from *Trigonella foenum-graecum* seeds which includes trigonelline, yamogenin, protodioscin, and diosgenin (Shabbeer*et al.,* 2009). Fenugreek is another herb that was favored by the Prophet Muhammad (Peace be upon him) and herbalists for thousands of years. Prophet Muhammad (PBUH) recommended the use of fenugreek, He said: ***"If my nation knew what good is in Fenugreek*** (***Trigonella foenum-graecum***)***, they would buy it worth for gold'*** (Majma al-Zawa'id).

Figure 1.3. *C. angustifolia* leaves, flowers and powder
Image taken form http://www.herbalhills.in/senna-powder.html

Based on the traditional, religious and scientific evidences, research study was formulated to investigate the pharmacological basis of *C. angustifollia* and *Trigonella foenum-graecum*, taken from the prophetic medicines. The findings of the present study will provide bases for further investigations on herbal medicine and will pave the way for the plant drived novel drugs with various pharmacological activities against different diseases.

Figure 1.4. *Trigonella foenum*-graecum (fenugreek) leaves and seeds.
Image taken from http://www.si-seeds.com/en/home/fenugreek-seeds-trigonella-foenum-graecum.html

1.4 Hypothesis

The *Cassia angustifolia* and *Trigonella foenum-graceum,* are medicinal herbs, recommended by Prophet Muhammad (Peace be upon him), used in Muslim ethnobotanical culture. They must have pharmacological basis in the form of active compounds which are responsible for their biological activities.

1.5 Objectives

The study has been designed with the following objectives:

1) Qualitative and quantitative screening of *C. angustifolia* and *T. foenum-graecum* for secondary metabolites.

2) Investigation of pharmacological activities of *C. angustifolia* and *T. foenum-graecum* i.e. antibacterial, antioxidant, anticancer, neuroprotective and hepatoprotective effects etc.

3) Isolation and identification of active compounds, based on their bioactivity, from *C. angustifolia* and *T. foenum-graecum.*

2. Review of Literature

2.1 Overview

Man's first tools for preventing death and disease were medicinal plants. These plants have been used over the centuries due to their fewer side effects, better efficacy and lower antimicrobial resistance as compared to commercially available drugs. Most of modern day medicine was born with the help of herbal medicine.

Herbal medications serve as a source of valuable pharmacological agents. These pharmacological agents tend to perform various biological activities which helps kill pathogens in a human body. Due to these biological activities, ethnobotanical community takes great interest in medicinal plants and intends on using these to treat life threatening and chronic diseases. Therefore, pharmacologists and ethnobotanists are exploring nutraceuticals from plant materials for validating indigenous knowledge regarding herbal medicine. ***Cassia angustifolia*** and ***Trigonella foenum-graecum*** are important medicinal plants used in oldest religious and medical texts. These plants belongs to Islamic medicines and are recommended in regular use because these are considered as the greatest forms of healing medicines available.

In the book of Hadith, it is stated that Ut-Tirmidhi and Ibn-e-Majah narrated that the Prophet Muhammad (Peace be upon him) said: ***"If there is a cure that prevents death, it would be Senna (C. angustifollia)"*** (Ibne Majja Ravi: Abu bin Um-e-Haram). It was narrated by Imam al-Haythami that the Prophet Muhammad (Peace be upon him) said: "***If my nation knew what good is in Fenugreek (T. foenum-graecum), they would buy it for gold"***. By keeping these hadith in mind, Muslims have used these two plants in various remedies for centuries and have become a key part of Muslim ethnobotanical culture. Based on their traditional and religious importance, systematic review was carried out to study their pharmacological basis.

2.2 Ethnobotanical and ethnopharmacological relevance of *C. angustifolia* vahl.

2.2.1 Ethnobotany-Origin and History

C. angustifolia Vahl., belongs to the family Caesalpiniaceae and class Mabnoliopsida, traditionally being used in eastern ethnobotanic culture from ancient times. It is known by many names as senna, senna makkai, Arabian senna, Indian senna, or Tinnevelly senna. The height of the plant is ca. 5-8 meters tall and rapidly growing shrub and has been widely cultured for its leaves and fruit (Fig. 2.1). Senna is an Arabian name and it is native to Hadramaut province of Southern Arabia, Nubian region of Egypt, Khartoum in Sudan, opposite coast of Somalia and Yemen (Raju *et al.,* 2011; Tripathi, 1991). *C. angustifolia* was introduced to South Asia and western Europe by Arabian physician in early 11th century (Tripathi, 1991). Senna grew in the hot arid areas of Punjab and Sindh states of Pakistan (Franz, 1993; Laghari *et al.,* 2011). It was also domesticated in different parts of the world including; Western Europe, Northern Brazil, California state of USA, North Africa (Selvaraj and Chander, 1978).

2.2.2 Ethnobotany-Medical Uses

It is reputed drug in unani medicine and is traditionally being used in different medical systems including homeopathic, allopathic, ayurvedic and in Chinese medicines. It is documented in USA and British pharmacopoeias (Dave and Ledwani, 2012). Both pods and leaves of the plant are used in the form of powder, decoctions and confections. *C. angustifolia* pods and leaves are a source of powerful purgatives; Sennoside A, Sennoside B, Sennoside C, Sennoside D, which is approved by world health organization (WHO) and FDA as a non-prescription laxative (Ramchander *et al,* 2017).

It is traditionally being used to cure constipation, ringworm infection and snake bite, psoriasis (skin disorder), hemoglobin disorders, splenomegaly, hepatomegaly, indigestion, Jaundice, and leprosy. Senna is considered as a useful and safe drug for lactating mothers, adults and children. Leaves of the plant is used to improve liver functions by removing toxins from the blood (Ahmed *et al.,* 2016). The mixture of *C. fistula* and *C. angustifolia* seeds were used to treat ringworm and snake bite. *C. angustifolia* leaves and pods are used in the condition of splenomegaly, hepatomegaly and jaundice to stimulate intestinal peristalsis. . Its pods and leaves used as decoctions and infusions have anti-helminthic activity aginst intestinal worms. In middle east, Europe and South Asia, plant leaves are administered as a tea to treat constipation and bowel evacuation before colonoscopy. Whereas in China, the powdered leaves in vinegar is being used as ointment to treat skin diseases. Furthermore, Senna tea helps in the production of red blood cells and improves the absorbance of oxygen in the lungs by removing mucous from the respiratory tract. Some of ethnobotanical uses of *C. angustifolia* are summarized in Table 2.1.

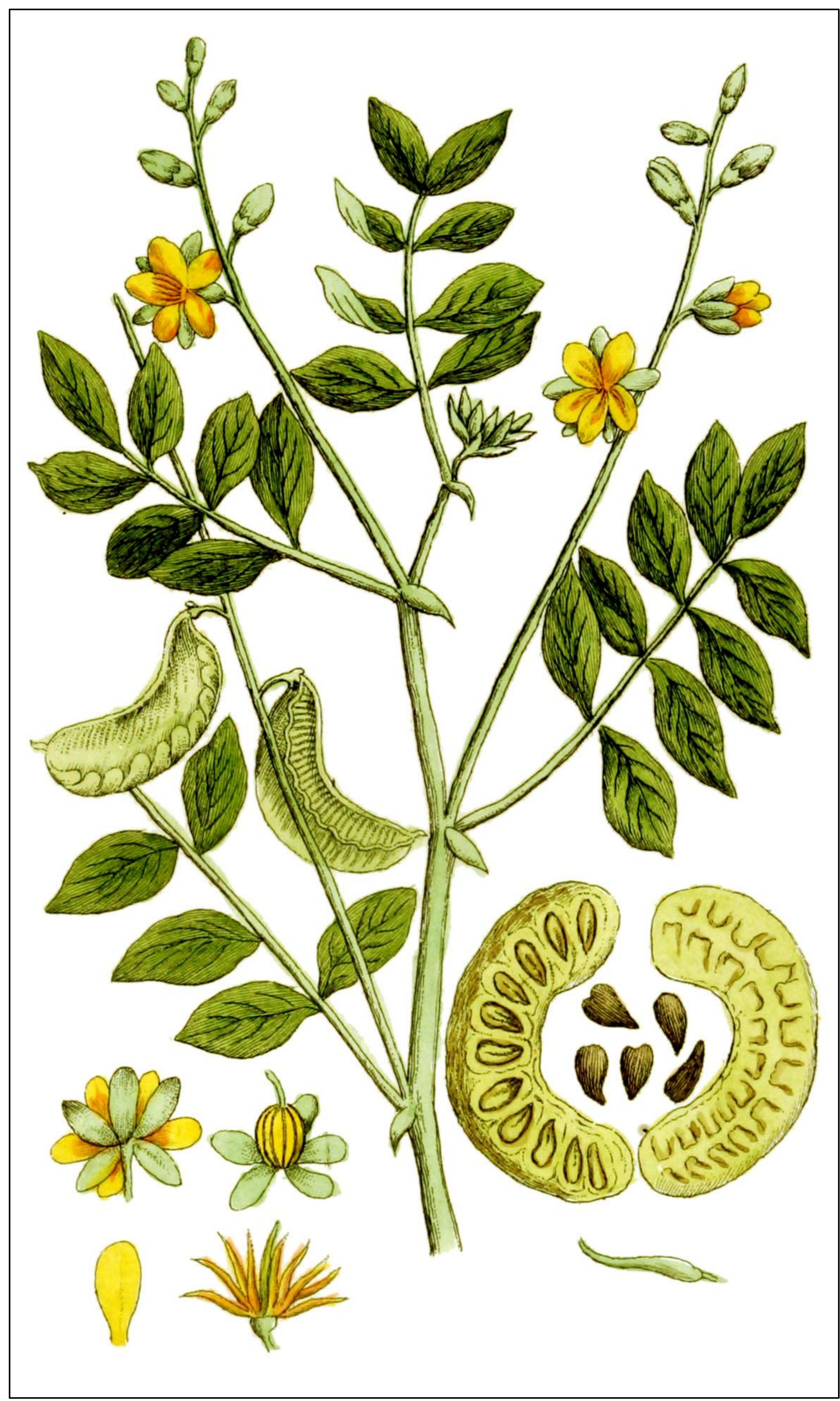


Figure 2.1. *C. angustifolia* (Senna) plant.
Image adapted fromhttps://en.wikipedia.org/wiki/Senna_(plant).

Table 2.1. Ethnobotanical data of *C. angustifolia*

Sr. no	Disease cure	Country region	Mode of use	References
1.	Constipation	Middle east, South Asia Europe	The leaves were brewed into a tea and administered as a strong laxative.	Heaton and Cripps, 1993
2.	Ring worm and snake bite	India	Mixture of *C. angustfolia* and *C. fistula* seed powder mixed in curd were used as ointment	Anton and Haag-Berrurier, 1980; Singh *et al.,* 2013
3.	Psoriasis (Skin disorder)	Europe China India	Paste from the leaves applied along with vinegar to the affected part of the body.	Ceuellar *et al.,* 2001
4.	Blood purifier	India	Leaves of the plant taken daily in a dose of 500 mg removes toxins from the blood	Ramchander *et al*, 2017
5.	Splenomegaly, Hepatomegaly and Jaundice	India	Leaves and pods of the plants were used as intestinal peristalsis stimulant.	Ramchander *et al,* 2017
6.	Leprosy	Africa	Dried leaves of the plant were used	Anton and Haag-Berrurier, 1980

2.2.3 Ethnopharmacology

C. angustifolia has been found to possess antibacterial, antifungal, antioxidant, laxative, antitumor, antiviral, anti-inflammatory, anti-diabetic, hepatoprotective properties. It also acts as an anti-pyretic in anemia, laxative, cholera, splenic enlargements, genotoxicity and hepatotoxicity induced by *E. coli*. Pharmacological data with detailed conditions are shown in Table 2.2.

Bameri *et al.* (2013) employed broth microdilution method to evaluate the bactericidal activity of *C. angustifolia* acetone, methanol, ethanol, and aqueous extracts. All the extracts were found to be active against *E. coli, K. pneumoniae, S. shinga, S. aureus*.

The methanol extract of *C. angustifolia* when tested against different pathogenic fungal strains was found to be active against *A. flavus, A. alternta, C. dematium, F. roseum, C. lunata* (Khan and Srivastava 2009).

The hepatoprotective effect of alcoholic extract of the leaves of *C. angustifolia* on carbon tetrachloride induced rat liver toxicity was studied by Ilavarasan *et al*. (2001). Different biochemical parameters including glutamate oxaloacetate, total protein, and total bilirubin, glutamate transaminase pyruvate was determined in the serum, whereas glutathione, lipid peroxidase and histopathological changes were studied in liver tissues. The alcoholic extract of the plant produced significant hepatoprotection.

In another study, laghari *et al.* (2011) reported that the *C. angustifolia* petroleum ether, ethanol and aqueous extracts exhibited strong antioxidant activity. It has been observed that the prescence of flavonoids in the extracts contributed to the antioxidant potential that acts as free radical scavengers.

Table 2.2. Ethnopharmacological data of *C. angustifolia*

Sr. no	Extract	Pharmacological activity	Tested material/Assay used	References
1.	Acetone, Methanol, Ethanol, Aqueous	Antibacterial	*Escherichia coli, Klebsiella pneumoniae, Shigella shinga Staphylococcus aureus*	Singanboina *et al.,* 2014; Bameri *et al* 2013
2.	Methanol	Antifungal	*Aspergillus flavus, Alternaria alternta, Collatotrichium dematium, Fusarium roseum, Curvularia lunata*	Khan and Srivastava 2009
3.	Petroleum ether, Aqueous, Ethanol	Antioxidant	DPPH radical scavenging assay	Laghari *et al.,* 2014
4.	Ethanol	Antidengue	Inhibitory activity against Dengue NS2B-NS3.	Rothan *et al.,* 2014
5.	Aqueous	Laxative	Mesenteric vascular beds of rats fed with senna	Ralevic *et al.,* 1990
6.	Alcoholic	Hepatoprotecetive	Carbon tetrachloride induced liver damage in rats	Ilavarasan *et al.* 2001
7.	Aqueous	Antitumor	CD1 mice transformed by solid sarcoma-180	Muller *et al.* 1998
8.	Hot glycerin	Antiviral	Antiviral activity against Herpes Simplex Virus type1 by a plaque reduction assay (PRA) with monolayer cultures of Vero cells grown in MEM.	Sydiskis *et al.,* 1991
9.	Ethanol	Anti-inflammtory	Edema induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) in female swiss mice.	Ceuellar *et al.,* 2001
10.	Aqueous	Anti-diabetic	Streptozotocin induced Diabetes in male rats.	Osman *et al.,* 2017

It was reported that C. angustifolia ethanol extract have significant anti-inflammatory activity. Ceuellar *et al.* (2001) induce edema in mice ear by 12-O-tetradecanoylphorbol-13-acetate (TPA) at a single dose of 2.5 µg/ear and multiple dose of 1 mg/ear daily. They have noted that the extract is active and inhibited edema induced by TPA.

Muller *et al.* (1998) isolated polysaccharides from *C. angustifolia* which were tested for antitumor activity against solid sarcoma-180 tumor cells subcutaneously inoculated in female CD-1 mice. Isolated polysaccharides showed significant antitumor activity by suppressing the growth of solid sarcoma-180 cells 51%.

Rothan *et al.* (2014) evaluated the anti-dengue activity of ethanol extract of *C. angustifolia* against Dengue NS2-NS3 protease. It was shown that the extracts exhibited significant protease inhibitory activity without causing cytopathic effect to Vero cells.

Sydiskis *et al.* (1991) in a different experiment, evaluated the antiviral activity of hot glycerin extracts of *C. angustifolia* against HSP1. The extract was effective and inactivated the virus in Vero cells. In a same experiment, anthraquinones were separated as active constituent, through thin layer chromatography, found to be directly virucidal to Herpes simplex virus type 1.

A recent study was carried out by Osman *et al.* (2017) to determine the antidiabetic activity of *C. angustifolia* aqueous extract. Streptozotocin at a quantity of 60 mg/kg body was intraperitoneally injected to induce diabetes in male rats. It was showed that aqueous extract of *C. angustifolia* increased insulin, restored body weight loss and liver function, and ameliorated hyperglycemia caused by streptozotocin.

2.2.4 Phytochemistry

Phytochemical investigation of *C. angustifolia* revealed the presence of different classes of natural products with diverse pharmacological activities. The structure and class of chemical compounds are listed in Table 2.3 and the bioactivities of major compounds *in-vivo* and *in-vitro* are shown in Table 2.4.

C. angustifolia leaves and pods are a rich source of anthraquinones, flavonoids, sterols, carbohydrates, saponins etc. Anthraquinone derivatives are the most frequent active constituent present in the leaves of senna. It contains rhein, aloe-emodin, physcion, chrysophanol and their glycoside compounds. The most important anthrquinones are sennoside A and sennoside B which were isolated in crystalline form from senna leaves by Stoll *et al.,* (1949). The leaves also contain sennoside C and sennoside D which are hetero-dianthrones with the respective aglycones rhein & aloe emodin (Ramchander *et al.,* 2017). All these sennosides contribute to the laxative effect. It has been reported that sennosides increase the fluid secretion and colon motility in small intestine thus promoting the enhanced laxative action. (Vanderperren *et al.,* 2005). In a series of different studies, Senna drug has been used as a safe laxative to treat constipation in psychiatric patients, elderly patients, spinal cord injury patients and in splenomegaly, jaundice, hepatomegaly and pregnancy (Passmore *et al.,* 1993; Pahor *et al*., 1995; Cornell*et al*., 1973; Tripathi, 1999).

Flavonoids are most active phytochemical in plant based medicines. The flavonoids isolated from *C. angustifolia* includes epicatechin, vidalenolone, kaempferol, kaempferol 3-O-rutinoside, kaempferol 3-O-sulphate-7-O-c-arabinopyranoside, Isorhamnetin, Calyxanthone, 14-Hydroxyartonin E, Apigenin-6,8-di-C-glycoside (Vicenin-2), (2S)-7,8, bis-3’,4’-(2,2-dimethyl-chromano)-5-hydroxy- flavanone, 3,7-dihydroxy-4’,8 dimethoxyflavone. All these flavonoids exert their physiological influence on the pharmacological activity of the senna.

Table 2.3. Biological active compounds from *C. angustifolia*

Sr. no	Chemical class	Compound	Structure	Molecular formula	Molecular weight (g/mol)	Reference
1.	Flavonoid	Epicatechin		$C_{15}H_{14}O_6$	290.271	Laghari *et al.,* 2011
		Vidalenolone		$C_{13}H_{14}O_4$	234.25	Laghari *et al.,* 2011
		Kaempferol		$C_{15}H_{10}O_6$	286.23	Singanboina *et al*., 2014
		Kaempferol 3-O-sulphate-7-O-c-arabinopyranoside		$C_{20}H_{18}SO_{13}$	498.41	Laghari *et al.,* 2014
		Isorhamnetin		$C_{16}H_{12}O_7$	316.265	Laghari *et al.,* 2014

	3,7-dihydroxy-4',8-dimethoxyflavone		$C_{17}H_{14}O_6$	314.28	Laghari *et al.,* 2014
	Calyxanthone		$C_{15}H_{10}O_6$	286.236	He *et al.,* 2007
	14-Hydroxyartonin E		$C_{25}H_{24}O_8$	452.46	Laghari *et al.,* 2014
	Apigenin-6,8-di-C-glycoside (Vicenin-2)		$C_{27}H_{30}O_{15}$	594.522	Wu *et al.,* 2007
	(2S)-7,8,bis-3',4'-(2,2-dimethyl-chromano)-5-hydroxyflavanone		$C_{25}H_{28}O_5$	408.49	Laghari *et al.,* 2014
Anthraquinone	Aloe-emodin		$C_{15}H_{10}O_5$	270.24	Dave and ledwani, 2012

Emodin-8-O-Beta-D-glucopyranoside		$C_{21}H_{20}O_{10}$	432.4	Dave and ledwani, 2012
Chrysophanol		$C_{15}H_{12}O_4$	254.24	Dave and ledwani, 2012
Rhein		$C_{15}H_8O_6$	284.22	Mehta and Laddha, 2009
Sennoside A		$C_{42}H_{38}O_{20}$	862.74	Dave and ledwani, 2012
Sennoside B		$C_{42}H_{38}O_{20}$	862.74	Srivastava *et al.,* 2006; Hayashi *et al.,* 1980

		Sennoside C	$C_{42}H_{40}O_{19}$	848.763	Srivastava *et al.,* 2006; Hayashi *et al.,* 1980
		Sennoside D	$C_{42}H_{40}O_{19}$	848.763	Srivastava *et al.,* 2006; Hayashi *et al.,* 1980
		Physcion	$C_{16}H_{12}O_5$	284.26	Mehta and Laddha, 2009
		6-hydroxymusizin glycoside	$C_{20}H_{24}O_8$	392.396	Lemli *et al*., 1981
3.	Naphthalene Glycoside	Tinnevellin glycoside	$C_{19}H_{22}O_9$	394.37	Franz, 1993

4.	Sterol	Beta-sitosterol		$C_{29}H_{50}O$	414.71	Franz, 1993
5.	Saponin	3-O-{-D-glucuronopyranosyl-(1→4)-[β-D-galactopyranosyl-(1→2)]-β-D-xylopyranosyl-(1→3)-β-D-glucopyranosyl}-2, 16α-dihydroxy-4, 20-hydroxy methyl olean-12-ene-28-oic acid (Triterpenoid glycoside)		$C_{53}H_{84}O_{27}$	1152.0	Khan and Srivastava, 2009
6.	Coumarin	Warfarin		$C_{19}H_{16}O_4$	308.33	Soyuncu *et al.*, 2008

Table 2.4. Pharmacological activities of bioactive compounds from *C. angustifolia.*

Sr. no.	Compounds	Pharmacological activities	References
1.	Epicatechin	Antioxidant, antimutagenic, antidiabetic, insulinogenic	Geetha *et al.,* 2004; Chakravarthy *et al.,* 1981, Ahmed *et al*., 1989
2.	Vidalenolone	Anticancer	Yoo *et al.,* 2002
3.	Kaempferol	Cardioprotective, antioxidant, anti-viral, anti-bacterial, anti-diabetic, anti-osteoporotic, estrogenic/antiestrogenic, anxiolytic, analgesic and antiallergic.	Calderon-Montano *et al.,* 2017
4.	Kaempferol 3-O-rutinoside	Hepatoprotective, antiglycation	Wang *et al.,* 2015; Lal-Shyaula *et al.,* 2012
5.	Aloe-emodin	Anti-neuroectodermal tumor, Antioxidant and scavenging, esterogenic, anticancer (Suppress breast cancer cells), antiviral	Pecere *et al.,* 2000; Vargas *et al.,* 2004; Huang *et al.,* 2013
6.	Chrysophanol	Anti-inflammatory, antibacterial, antidiabetic, antimutagenic, anticancer, antiviral, hepatoprotective	Kim *et al.,* 2010; Coopoosamy and Magwa, 2006; Semple *et al.,* 2001; Yuenyongsawad *et al*., 2014; Qian *et al*., 2011
7.	Rhein	Anti-inflammatory, nephroprotective, anticancer, antimicrobial hepatoprotective, antioxidant	Zhou *et al.,* 2015
8.	Sennosides	Laxative, Cathartic, gastroprotective, bovine serum monomine oxidase activity	Hwang and Jeong, 2015
9.	Physcion	Antifungal and antitumor	Agarwal *et al.*2000; Kuo *et al.,* 1997
10.	Isorhamnetin	Antitumor, anti-tuberculosis, anti-inflammatory, antiviral, Nerve growth factor	Jnawali *et al.,* 2016; Dayem *et al.,* 2015; Xu*et al*., 2012
11.	Apigenin-6,8-di-C-glycoside (Vicenin-2)	Antithrombotic, antiplatelet, anti-inflammatory	Lee and Bae, 2015; kang *et al.,* 2015
12.	Beta-sitosterol	Hypercholesterolemia, immunomodulator, anticancer (cervical cancer), anti-arthritis, antimicrobial, prevention against benign prostatic hyperplasia.	Saeidnia *et al.,* 2014
13.	Triterpenoid glycoside	Antifungal	Khan and Srivastava, 2009

Muller *et al.,* (1989) reported four water soluble polysaccharides; L-arabinose, L-rhamnose, D-galacturonic acid and D-galactose from *C. angustifolia* reported to exhibited anticancer activity against in CD mice transformed with solid Sarcoma-180.

In addition to these compounds, β-sitosterol, one naphthalene glycoside named tinneveline glycoside, and a triterpenoid isolated form the leaves of *C. angustifolia*. Tripathi *et al.,* (1999) reported the presence of other chemical compounds from senna leaves which includes sennapicrin, salicylic acid, myricyl alcohol cathartomannite, sennacrol, resin, mannitol, surose, mucilage, sodium potassium tertarate, phytosterolin, and chrysophanic acid.

2.3 Ethnobotanical and ethnopharmacological relevance of *T. foenum-graecum*

2.3.1 Ethnobotany

T. foenum-graceum, commonly known as fenugreek, belongs to the class Magnoliopsida and family Fabaceae. It is one of the oldest medicinal plant growing annualy with various pharmacological properties. It is native to Asia, and Middle East and now commercially cultivated in Pakistan, Afghanistan, Iraq, Iran, India, Spain, Turkey, Egypt, Morocco, and Argentina (Flammang *et al.,* 2004). It is named as Hulba in Arabic, Methi dana in Urdu and Hindi, Dari in Persian, Moshoseitaro in Greek (Ahmed *et al.,* 2016).

It is used as a spice in different countries to enhance the colour, flavor and texture of the food. The fenugreek seeds and leaves (Fig. 2.2) are used in the form of powder and decoctions to treat various diseases. It is traditionally being used to treat stomach and intestinal problems, lung infections, skin diseases, circulatory and immune system problems, liver disorders, heart burn, diabetes, antifertility, allergies etc. In Egypt and china, fenugreek tea is used by lactating mothers to increase milk flow and to treat abdominal pains (Snehlata and Payal, 2012). Infusions of fenugreek seed were used by practitioner in India to treat intestinal and skin disorders (Toppo *et al.,* 2009). In Iran, the leaves of fenugreek were used to treat splenomegaly, cough, backache and hepatitis due to its dry and warm nature. Infusions of fenugreek seed mixed with honey is used to treat asthma and inflammations. Fenugreek seeds are widely being used to treat diabetes by lowering blood glucose and urine sugar level. In Saudi Arabia, fenugreek seeds were used to treat kidney disorders by decreasing the level of calcium oxalate level in kidneys. In china, the fenugreek seeds are used to treat kidney diseases and cervical cancer. Ethnomedicinal data of fenugreek are listed in Table 2.5.

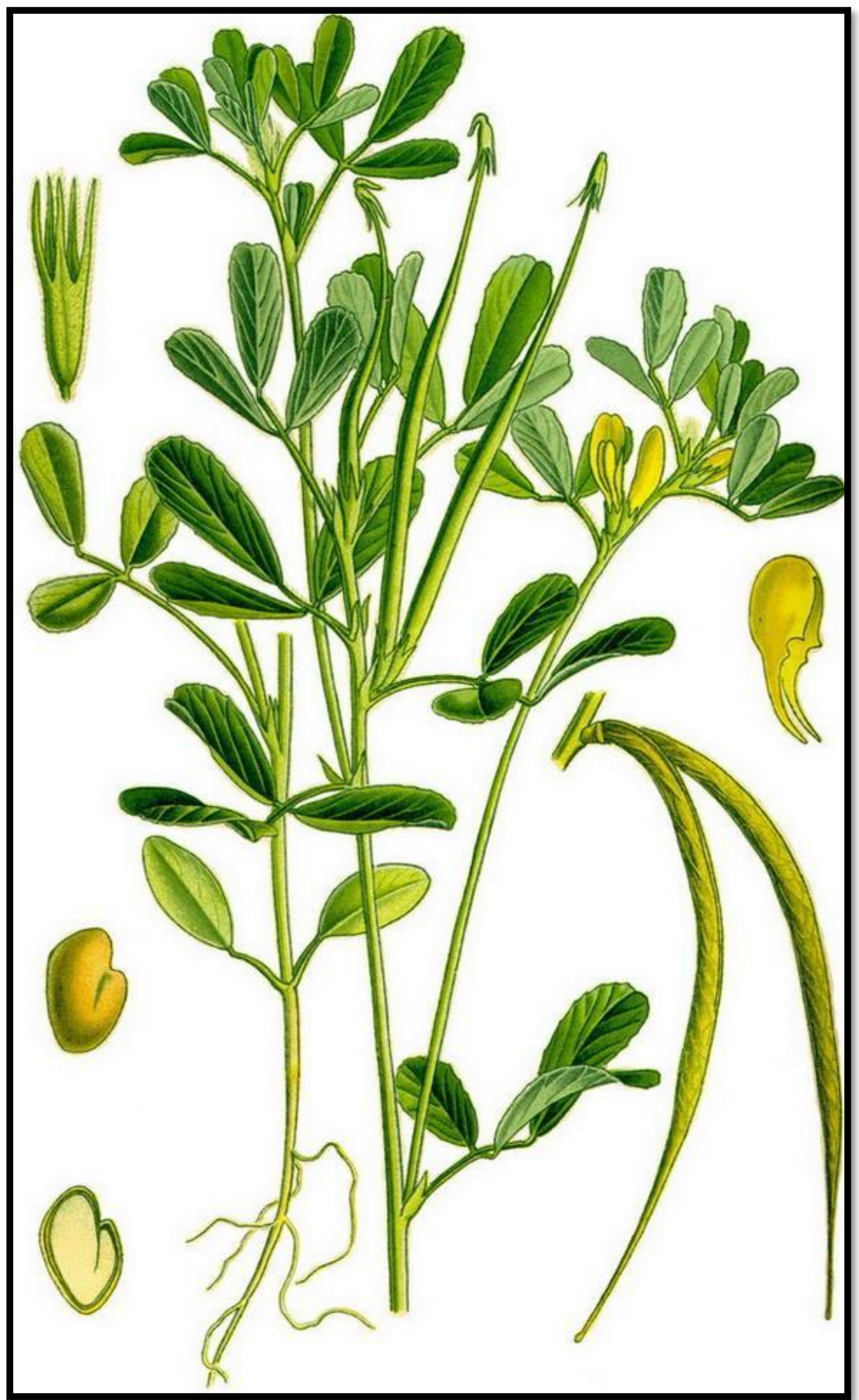


Figure 2.2: *Trigonella foenum-graecum* (fenugreek) plant
Image adapted fromhttp://medicalency.com/trigonella-foenum-graecum.htm

Table 2.5. Ethnobotany of *Trigonella foenum-graecum* (Fenugreek).

Sr. no	Disease cure	Country region	Traditional recipe/plant part used	References
1.	Dysentery	India	Fenugreek seeds were toasted and infusions were made by practitioners	Toppo *et al.,* 2009
2.	Small pox	Konkan, India	Fenugreek seed infusion were used as cooling drink	Toppo *et al.,* 2009
3.	Asthma and internal edemas	Iran	Fenugreek seed infusion mixed with honey which nourishes the body against ailment	Basch *et al.,* 2003
4.	Hepatitis, cold cough, bladder cooling reflex splenomegaly and backache	Iran	Fenugreek leaves were used as detox against different disease.	Basch *et al.,* 2003
5.	Breast milk stimulant	Egypt, China	Fenugreek seed powder boiled in water and taken as tea helps to increase milk production in nursing mothers.	Snehlata and Payal, 2012
6.	Diabetes	Iran	Fenugreek seeds were soaked in water overnight and taken orally to lower blood glucose and urine sugar level	Bu-Ali-Sina, 1988; Analava and Debaprasad, 2004
7.	Cervical cancer	China	Fenugreek seeds were used as decoction	Korand and Moradi, 2013
8.	Gastroenteritis	Middle east	Aerial part of the plant was used to treat abdominal disorders	Korand and Moradi, 2013
9.	Kidney disorders	Saudi Arabia	Fenugreek seeds were taken orally which decreased calcium oxalate level in kidneys	Korand and Moradi, 2013

2.3.2 Ethnopharmacology

Fenugreek seeds have been extensively studied due to its pharmacological properties. It possesses therapeutic properties such as antibacterial, antioxidant, antifungal, anticancer, antimutagenic, antineoplastic, analgesic, neuropharmacological, antidiabetic, estrogenic, chemo-preventive, antiulcer, immunomodulatory, anti-inflammatory, anti-melanogenic, hepatoprotective, antipyretic, antihyperlipidemic, antiadhesive, hypoglycemic effects. Pharmacological data with detailed conditions are shown in Table 2.6.

Out of all the listed pharmacological properties, *T. foenum-graecum* extensively been used by diabetic patients and studied for its antihyperglycemic and hypoglycemic effect. Hannan *et al.* (2007) demonstrated the antidiabetic effect of *T. foenum-graecum* in type 2 diabetic mice. After oral administration of *T. foenum-graecum* for 28 days, it significantl reduces the level of blood glucose and increases liver glycogen content were observed.

In another study, Kannappan and Anuradha (2009) studied the insulinogenic activity of fenugreek polyphenol extracts in male Wistar mice. The insulin resistance in mice was induced by feeding high fructose diet for 60 days. Fenugreek polyphenol extracts were orally consumed at a quantity of 200 mg/kg along with fructose. After 60 days, fenugreek polyphenol extracts showed improved insulin signaling when compared to standard drug metformin.

Table 2.6. Ethnopharmacology of *Trigonella foenum-graceum.*

Sr. no	Extracts	Pharmacological activity	Tested material/ Assay used	References
1.	Polyphenolic	Cytoprotective	Ethanol induced liver damage in mice.	Kaviarasan *et al.,* 2006
2.	Ethanol	Antibacterial	*Pseudomonas aeruginosa*, *Staphylococcus aureus.*	Premanath *et al.,* 2011
3.	Ethanol Methanol Chloroform	Antioxidant	DPPH, superoxide, nitric oxide hydroxyl, hydrogen peroxide, total peroxyl radical scavenging assay. Ferrous reducing power assay	Premanath *et al.,* 2011; Subhashini *et al.,* 2011, Bukhari *et al*., 2008
4.	Methanol Ethanol Aqueous	Anticancer	Hepatocellular carcinoma (HepG2),Thyroid Papillary carcinoma (FRO), B-cell lymphomas (BCP), T-cell lymphoma (TCP), MCF7 cell line.	Khalil *et al.,* 2015; Alsemari *et a*l., 2014; Al-Daghri *et al*., 2012
5.	Aqueous	Antimutagenic	Salmonella microsomal assay.	Chatterjee *et al.,* 2013
6.	Hydroalcoholic	Antineoplastic	Human acute myeloblastic leukemia cell lines (KG-1).	Alizadeh *et al.,* 2009
7.	Aqueous	Chemopreventive	Breast cancer induction by 7,12 dimethylbenz(α)anthracene (DMBA) in rats.	Amin *et al.,* 2005
8.	Methanol	Analgesic	Tail absorption test, Acetic acid induced writhing in mice.	Akter *et al.,* 2011
9.	Methanol	Neuropharma-cological	Hole cross, Open field test on mice.	Akter *et al.,* 2011
10.	Ethanol Aqueous	Antidiabetic	Alloxan induced diabetic rats, Streptozotocin-induced diabetic rats.	Abdel-Barry *et al*., 1997; Xue *et al.,* 2007; Hannan *et al.,* 2007.
11.	Chloroform	Estrogenic	Estrogen receptor (ER) positive breast cancer cells MCF-7.	Sreeja *et al.,* 2010, Allred *et al.,* 2009
12.	Methanol Aqueous	Antiulcer	Aspirin induced gastric ulcer in rats, Ethanol-induced gastric ulcer in rats,	Al-dalain *et al.,* 2008; Anand *et*

			Cold restraint stress-induced ulcers in rats, water immersion restraint stress induced ulcer model in rats.	*al.,* 2012., Sumanth, 2011
13.	Aqueous	Immunomodulatory	Male swiss albino mice	Bin-hafeez *et al.,* 2003
14.	Diethyl ether Methanol Aqueous	Anti-inflammatory	Formalin-induced edema in rats, lambda-carrageenan induced inflammation in Wistar albino rats, Human monocytic THP-1 cell line.	Hassan *et al.,* 2006, Ahmadiani *et al.,* 2001; Kawabata *et al.,* 2011
15.	Methanol	Anti-melanogenic	Murine melanoma B16F1 cells	Kawabata *et al.,* 2011
16.	Diethyl ether Methanol	Hepatoprotective	CCl_4 induced toxicity in Wistar albino rats.	Said *et al.,* 2011
17.	Aqueous	Antipyretic	Induction of hyperthermia by injecting 20% (w/v) aqueous suspension of brewer's yeast intraperitonealy in mice.	Ahmadiani *et al.,* 2001
18.	Methanol Aqueous	Antihyperlipidemic	European strain albino rats fed with pure cholesterol, Diet-induced hypercholesterolemia in guinea pigs	Sharma and Chaudhry, 2012
19.	Aqueous	Antiadhesive	Helicobacter pylori	O'Mahony *et al.,* 2005

Ramesh *et al.* (2010) observed similar results by analyzing the effect of fenugreek seed extracts on pancreas of alloxan induced diabetic rats. Histopathologically, it was observed that Islets of Langerhans were restored in the mice treated with Fenugreek seed extracts.

In an experiment, Sharma and Chaudhry (2012) showed that aqueous extract of fenugreek seeds has the property to regulate the lipid level in hyperlipidemic rabbits. Hyperlipidemia was induced by feeding rabbits with pure cholesterol for 4 weeks. Significant reduction in the level of triglycerides and total cholesterol were observed in the serum of hyperlipidemic rabbits.

Khalil *et al.* (2015) demonstrated the anticancer activity of fenugreek methanol extract in HepG2 cell lines. It was observed that fenugreek methanol extract induces apoptosis in hepatocellular carcinoma cell line by increasing the gene expression level of *p53*, *Bax* and proliferating cell nuclear antigen.

Alsemari *et al*. (2014) in another study evaluated the selective cytotoxic anticancer activity of fenugreek aqueous extract against various cancer and normal cell lines. The results displayed that fenugreek aqueous extract specifically cytotoxic to transformed cell lines including FRO, TCP, and MCF-7 sparing normal cells *in-vitro*.

Bin-hafeez *et al.* (2003) reported the immunomodulatory effect of t. foenum-graecum aqueous extract in male swiss albino mice. Fenugreek aqueous extract was fed at three different doses of 50, 100, 250 mg/kg for 10 days. Enhanced immune functions were observed in mice treated with fenugreek aqueous extract.

A study was conducted by Said *et al.* (2011) in rats to investigate the hepatoprotective effect of fenugreek. Carbon tetra chloride was used to induce hepatotoxicity in wistar albino rats. Fenugreek aqueous extracts at a quantity of 250 mg/kg was administered orally for 14 days. Biochemical parameters like ALT, AST, total bilirubin was determined in serum. It was

observed that fenugreek aqueous extract possesses significant hepatoprotective activity against CCl_4 induced hepatotoxicity.

2.3.3 Phytochemistry

Phytochemical investigation of *T. foenum-graecum* revealed the presence of different classes of natural products with diverse pharmacological activities. Table 2.7 summarizes the structure and class of chemical compounds and the associated bioactivities of important compounds *in-vivo* and *in-vitro* are shown in Table 2.8.

The main class of phytochemical constituents of *T. foenum-graecum* consists of alkaloids, flavonoids, steroidal sapinogens, mucilage, proteins etc. Steroidal sapinogens are the most important active constituent of fenugreek seeds. It consists of diosgenin, dioscin, protodioscin, yamogenin, sarsasapogenin, tigogenin, neotigogenin, gitogenin, yuccagenin (Bahmani *et al.,* 2016). Diosgenin and protodioscin are major steroidal sapinogens known to involve in various pharmacological activities. Diosgenin possess anti-infectious, anticancer, anti-inflammatory, anticoagulant and antithrombotic, osteoprotective, anti-oxidant, hepatoprotective activities (Taylor *et al.,* 2000). Whereas, protodioscin are reported to have aphrodisiac activity, anticancer, antihyperglycemic, antihyperlipidemic, hepatoprotective effects (Yum *et al.,* 2010).

Table 2.7. Biological active compounds from *Trigonella foenum-graecum.*

Sr. no	Chemical class	Compound	Structure	Molecular formula	Molecular weight (g/mol)	Reference
1.	Alkaloids	Trigonelline		$C_7H_7NO_2$	137.138	Yoshinari and Igarashi, 2010
		Choline		$C_5H_{14}NO$	104.17	Kaviarasan *et al.,* 2007
		Carpaine		$C_{28}H_{50}N_2O_4$	478.70	Patil and Jain, 2014
		Gentianine		$C_{10}H_9NO_2$	175.187	Kokate *et al.,* 2007
		Neurine		$C_5H_{13}NO$	103.16	Sultana *et al.,* 2016
		Betaine		$C_5H_{11}NO_2$	117.148	Sultana *et al.,* 2016

2.	Flavonoids	Quercetin	$C_{15}H_{10}O_7$	302.236	Ahmad *et al.*, 2016
		Rutin	$C_{27}H_{30}O_{16}$	610.52	Ahmad *et al.*, 2016
		Apigenin	$C_{15}H_{10}O_5$	270.24	Ahmad *et al.*, 2016
		Vitexin	$C_{21}H_{20}O_{10}$	432.38	Ahmad *et al.*, 2016
		Saponaretin (Isovitexin)	$C_{21}H_{20}O_{10}$	432.38	Ahmad *et al.*, 2016
		Luteolin	$C_{15}H_{10}O_6$	286.24	Dietz *et al.*, 2016

	Vicenin-1		$C_{26}H_{28}O_{14}$	564.5	Ahmad *et al.*, 2016
	Naringenin		$C_{15}H_{12}O_5$	272.26	Shang *et al.*, 1998
	Tricin		$C_{17}H_{14}O_7$	330.29	Khalil *et al.*, 2015
	Tricine		$C_6H_{13}NO_5$	179.17	Khalil *et al.*, 2015
	Kaempferol		$C_{15}H_{10}O_6$	286.23	Gikas *et al.*, 2011
	Orientin		$C_{21}H_{20}O_{11}$	448.38	kor *et al.*, 2013

		Isoorientin (Homoorientin)		$C_{21}H_{20}O_{11}$	448.38	Ahmad *et al.*, 2016
		Biochanin A		$C_{16}H_{12}O_5$	284.27	Wang *et al.*, 2010
		Formononetin		$C_{16}H_{12}O_4$	268.26	Wang *et al.*, 2010
		Irilone		$C_{16}H_{10}O_6$	298.24	Wang *et al.*, 2010
		Daidzein		$C_{15}H_{10}O_4$	254.23	Wang *et al.*, 2010
		Calycosin		$C_{16}H_{12}O_5$	284.267	Wang *et al.*, 2010
3.	Steroidal Sapinogen s	Diosgenin		$C_{27}H_{42}O_3$	414.63	Taylor *et al.*, 2000
		Dioscin		$C_{45}H_{72}O_{16}$	869.055	Yum *et al.*, 2010

Protodioscin		$C_{51}H_{84}O_{22}$	1049.10	Hibasami *et al.,* 2003
Yamogenin		$C_{27}H_{42}O_3$	414.62	Hardman *et al.,* 1971
Sarsasapogenin		$C_{27}H_{44}O_3$	416.646	Taylor *et al*., 2000
Tigogenin		$C_{27}H_{44}O_3$	416.64	Doshi *et al.,* 2012
Neotigogenin		$C_{27}H_{44}O_3$	416.64	Doshi *et al.,* 2012
Gitogenin		$C_{27}H_{44}O_4$	432.64	Madhu and Mala, 2015

	Yuccagenin		$C_{27}H_{42}O_4$	430.629	Taylor *et al*., 2000
Saponins	*Fenugreekine*		$C_{21}H_{27}N_7O_{14}P_2$	663.43	Ouzir *et al.*, 2016
Coumarin	Scopoletin		$C_{10}H_8O_4$	192.17	Ouzir *et al.*, 2016
	7-Acetoxy-4-Methylcoumarin		$C_{12}H_{10}O_4$	218.21	Raju *et al.*, 2001

Table 2.8. Pharmacological activities of bioactive compounds from *T. foenum-graceum.*

Sr.no.	Compounds	Pharmacological activities	References
1.	Trigonelline	Antidiabetic, antioxidant, neuroprotective, antimigrane, antibacterial, antiviral, antitumor.	Salim and Hamadi, 2012
2.	Carpaine	Cardiovascular effect	Rapoport *et al.,*1953
3.	Gentianine	Antiamoebic, anti-bacterial, anti-convulsant, diuretic, anti-histaminic, hypotensive, anti-inflammatory, antimalarial, antipsychotic and sedative	Shaani *et al.,* 1953
4.	Quercetin	Antioxidant, anti-inflammatory, anti-tumor, neuroprotective	Ansari *et al*., 2009; Zhang *et al.,* 2012; Murota and Terao 2003
5.	Rutin	Ani-inflammatory, Anti-tumor, anti-asthmatic, antioxidant, neuroprotective, antinociceptive effects.	Ganeshpurkar and Saluja, 2017
6.	Apigenin	anxiolytic, sedative, antidepressant	Losi *et al.,* 2004
7.	Vitexin	Anti-oxidant, anti-cancer, anti-inflammatory, anti-hyperalgesic, and neuroprotective effects	He *et al.,* 2016
8.	luteolin	Hepatoprotective, anti-angiogenic, antithrombotic, anti-oxidant, anti-inflammatory, anti-allergic, anticancer	Lin *et al.,* 2008; Ambasta *et al.,* 2015
9.	Naringenin	Antidepressant, anti-viral, anticancer, radioprotective	Yi *et al.,* 2013; Gao *et al.,* 2006
10.	Tricin	Chemopreventive and anticancer	Zhou *et al.,* 2010
11.	Kaempferol	Cardioprotective, antioxidant, anti-viral, anti-bacterial, anti-diabetic, anti-osteoporotic, estrogenic/antiestrogenic, anxiolytic, analgesic and antiallergic.	Calderon-Montano *et al.,* 2017
12.	Orientin	Anti-adipogenesis, antinociceptive, radioprotective, vasodilatation and cardioprotective, anti-viral, anti-aging, antioxidant, neuroprotective, antibacterial, anti-inflammation	Lam *et al.,* 2016
13.	Biochanin A	Antimutagenic, antineoplastc, anticancer	Zhang and Morris, 2003
14.	Diosgenin	Anti-infectious, anticancer, anti-inflammatory, anticoagulant and antithrombotic, Osteoprotective, anti-oxidant, hepatoprotective	Abdelgawad *et al.,* 2012
15.	Dioscin	Desensitization, anti-tumor, anti-inflammatory, lipid-lowering, hepatoprotective, anti-viral, diuretic	Xu *et al.,* 2015

16.	Protodioscin	Aphrodisiac activity, anticancer, antihyperglycemic, antihyperlipidemic, hepatoprotective	Guo *et al.,* 2016
17.	Yamogenin	Anti-hyperlipidemic	Moriwaki *et al.,* 2014
18.	Scopoletin	Regulation of blood pressure, anticholinesterase, bacteriostatic, anti-asthmatic, anti-inflammatory and regulation of serotonin hormone	Patil and Jain, 2014

Fenugreek seeds contains fairly high number of flavonoids which includes quercetin, rutin, apigenin, vitexin, saponaretin (isovitexin), luteolin, vicenin-1, naringenin, tricine, kaempferol, orientin, isoorientin (homoorientin), biochanin A, formononetin, irilone, daidzein, calycosin.

Trigonelline is the most important alkaloid reported to possess antidiabetic, antioxidant, neuroprotective, antimigrane, antibacterial, antiviral and antitumor activities. Other alkaloids include choline, carpaine, gentianine, neurine, and betaine. Ahmed *et al.,* (2016) reported the presence of nutritional constituents from fenugreek seeds which includes; proteins (lecithin, globulin, albumin), aromatic compounds (3-isobutyl-2-methoxypyrazine, 4-dihydro-2(5H)-furanone, 1-octene-3-one, eugenol, diacetyl, isovaleric acid), lipids (Triacylglycerols, diacylglycerols, monoacylglycerols, phosphotidylcholine, phosphotidyl ethanolamine, phosphatidyl inositol, free fatty acids), vitamins (Vitamin A, B_1, B_2, B_6, A, niacin, nicotinic acid, β-carotene, thiamine, riboflavin, folic acidnicotinic acid) and minerals (Potassium, magnesium, calcium, zinc, manganese, cupper, iron).

3. Materials and Methods

3.1 Collection of plant material

Fresh leaves of *Cassia angustifolia* and seeds of *Trigonella foenum-graecum* were purchased from a local herb shop in Islamabad, Pakistan and verified by taxonomist Dr. Muhammad Qasim Hayat from ASAB, NUST, Islamabad. Both the plants were kept in Medicinal plant research laboratory for future records.

3.2 Formulation of plant crude extracts

The leaves of *C. angustifolia* and seeds of *T. foenum-graecum* were dried under mild sunlight and pulverized into powder in a grinding machine. Extracts were formulated by maceration in triplicate batches. The finely ground powder of mesh size 40-50 was subjected to aqueous, methanol, ethanol, acetone, and ethyl acetate solvents separately in flasks with the ratio 1:10 and placed in the dark at 37 ℃ for 48-72 h with shaking at 4 h intervals. Centrifugation was performed for 15 min at 2000 rpm. Supernatants were filtered with Whatman filter paper no.1. The filtrate was transferred to a round-bottom flask and the solvent was rotary evaporated. The dry extract was stored at 4 °C.

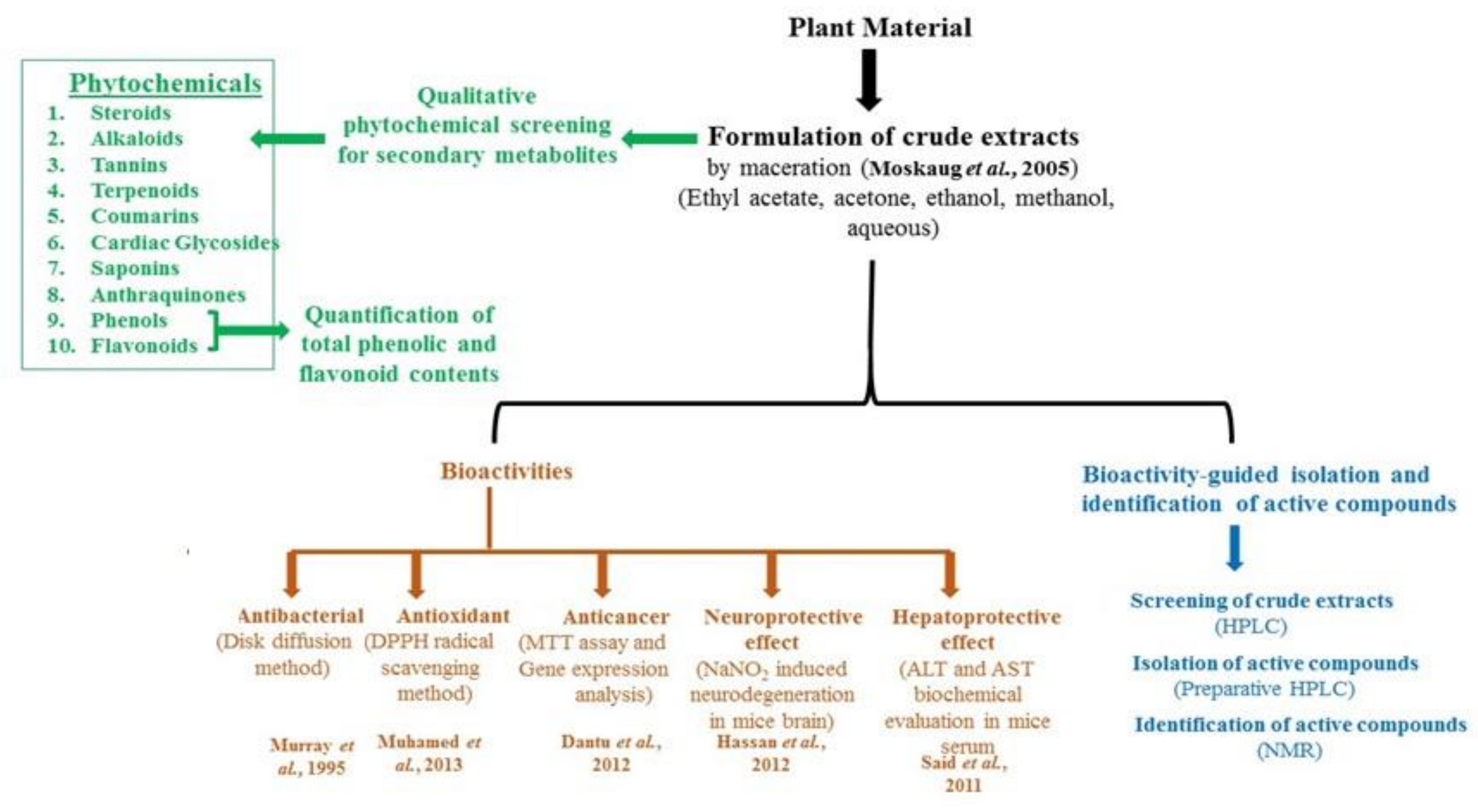


Figure 3.1. Schematic representation of methodology

3.3 Qualitative phytochemical secreening for secondary metabolites

C. angustifolia and *T. foenum-graecum* extracts were screened for phytochemical screening for the prescence of secondary metabolites. Qualitative phytochemical analysis for the presence of steroids, alkaloids, tannins, flavonoids, coumarins, cardiac glycosides, saponins, diterpenes, anthraquinones, and phenols were carried out by standard protocols (Harborne, 1973; Trease *et al*., 1989; Sofowra, 1993). The results of phytochemical screening are shown in Table 3.1.

3.4 Quantitative determination of total phenolics and flavonoids

3.4.1 Determination of total phenolic contents

Total phenolic contents of the organic and aqueous extracts of *C. angustifolia* and *T. foenum-graecum* were determined by the Folin-Ciocalteu method with slight modifications (Khatoon *et al*., 2013). Breifly, gallic acid was taken as a standard and the phenolic contents of the extracts were determined from a gallic acid calibration curve expressed as mg/g gallic acid equivalents (GAE). The gallic acid calibration curve was used for the evaluation of phenolic concentrations in the extracts. To prepare a calibration curve, 0.3 mL aliquots of concentrations 50, 100, 150, 200, 250 and 300 μg/mL of gallic acid-methanol solution were added to 1.5 mL of 10 times diluted Folin-Ciocalteu reagent and 1.5 mL of sodium carbonate (75 g/L). A blue color was produced because of reduction of samples containing polyphenols by Folin-Ciocalteu reagent. The same procedure was used for different extracts of *C. angustifolia* and *T. foenum-graecum*. After incubation at room temperature for 30 min, the absorbance was calculated at 700 nm using a UV spectrophotometer. The calibration curve between absorbance and concentration was constructed. All the experiments were performed in triplicate.

3.4.2 Determination of total flavonoid contents

The total flavonoid contents of *C. angustifolia* and *T. foenum-graecum* extracts were determined by the aluminum chloride colorimetric method (Mohammed *et al.,* 2013) with some modifications. Quercetin was used as a standard and the flavonoid contents of the extracts were estimated from the quercetin calibration curve which was expressed as mg/g quercetin equivalents (QE). Each extract of *C. angustifolia* and *T. foenum-graecum* (0.5 mL) was mixed with distilled water (2 mL) and then with 0.15 mL of a 10% $NaNO_2$ solution. After 5 min at room temperature, 10% aluminum chloride (0.15 mL) was added followed by 1% NaOH (1 mL). The solution was thoroughly shaken and absorbance was measured at 510 nm using a UV spectrophotometer. A calibration curve for quercetin was prepared similarly for the concentrations 50, 100, 150, 200, 250, and 300 μg/mL.

3.5 Pharmacological activities of *C. angustifolia* and *T. foenum-graecum*

3.5.1 Antibacterial assay

3.5.1.1 Bacterial strains

Acinetobacter junii IARS2, *Serratia mercescens* IARS6, *Enterobacter cloacae* IARS7, *Pseudomonas aeroginosa* IARS8 and *Salmonella typhi* ATCC 14079 were used for antibacterial assay. Glycerol stocks of all the bacterial strains were maintained in controlled conditions and subcultured on Mueller Hinton agar for 24 h before antibacterial assay.

3.5.1.2 Disk diffusion assay

Organic and aqueous extracts of *C. angustifolia* and *T. foenum-graecum* were screened for antibacterial potential by disk diffusion assay (Murray *et al.,* 1995). Briefly, sterile Mueller Hinton agar plates were prepared with standardized inoculums maintaining the bacterial culture count at $1x10^8$ cells/mL under aseptic conditions. Whatman No. 1 filter paper discs of 6 mm

diameter were prepared and sterilized. 10 µL of the extracts with concentrations (1.25, 2.5, 5 and 10 mg/mL) were placed separately on a 6-mm filter paper disk with a micropipette. The standard antibiotic disks for Tigecycline, Amikacin, and Cefepime served as positive controls and filter paper discs soaked in DMSO and solvent were used as negative controls. The plates were incubated for 8-12 h at 37 °C after which the zone of inhibition was measured. The experiments were performed in triplicate. The minimum inhibitory concentrations of the extracts that inhibit bacterial growth were recorded.

3.5.2 Antioxidant assay

3.5.2.1 DPPH radical scavenging activity

The antioxidant activities of the organic and aqueous extracts of *C. angustifolia, T. foenum-graecum* and gallic acid were evaluated with the DPPH method as explained by Muhamed *et al.* (2013) with slight modifications. Briefly, 100 µL of different extracts of *C. angustifolia* and *T. foenum-graecum* (methanol, ethanol, acetone, ethyl acetate, aqueous) at different concentrations (100, 200, 300, 400, 500 µg/mL) were added to 900 µL of DPPH in methanol solution having 0.299 ± 0.02 OD. The solution was placed in the dark for 30 min at room temperature. After incubation, absorbance was measured at 517 nm on a UV spectrometer. The radical scavenging activities of the extracts and standards were evaluated on the basis of percentage DPPH radicals scavenged, by the formula:

$$\%\ \text{scavenging activity} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

where $A_{control}$ is absorption of control without the test sample and A_{sample} is absorption of the standard or tested extract.

IC_{50} is the inhibitory concentration which was defined as the concentration (µg/mL) of the extract inhibiting 50% of DPPH radicals. It was calculated from the calibration curve of the standards. Gallic acid was taken as a positive control. Experiments were conducted in triplicate.

3.5.3 Anticancer assay

3.5.3.1 Cell culture

The human cervical adenocarcinoma cell line (HeLa) was provided by HBV (Hepatitis B Virus) lab, ASAB, NUST. The Human epithelial type 2 cell line (Hep2), Michigan Cancer Foundation-7 (MCF-7) and normal human corneal epithelial cells (HCEC) was provided by the Institute of Biotechnology and Genetic Engineering (IB & GE), Abdul Qadeer Khan Research Laboratory (KRL) Hospital, Islamabad. All the cell lines were grown in RPMI-1640 media which contained 10% fetal bovine serum (FBS). All cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

3.5.3.2 MTT assay

Anticancer assays of *C. angustifolia* and *T. foenum-graecum* extracts on HeLa, Hep2, MCF-7 and HCEC were carried out by the 3-(4, 5-dimethylthiazolyl)-2, 5-diphenyl tetrazolium bromide (MTT) colorimetric assay (Dantu*et al.,* 2012). The cells were cultured in RPMI-1640 media supplemented with 10% fetal bovine serum and 1% pencillin-streptomycin. The number of viable cells was calculated by the trypan blue dye exclusion method. The cells with a density of ca. $5x10^4$ were seeded in 96 well plates for 24-48 h with *C. angustifolia* extracts (100, 150, 200 and 250 μg/μL). After 24-48 h, MTT (10 μL; 5 mg/mL in 1X phosphate buffer saline) was added to each well. The plate was shaked gently to ensure equal mixing and kept at 37 ºC in a 5% CO_2 incubator for 24 h. After incubation, the medium from each well was removed and 100 μL of solublization solution was added to dissolve the purple formazan crystals. The plates were placed into the microplate reader to calculate the optical density of the solution at wavelength 540 nm. The percentages of viable and non-viable cells were calculated by the formulas:

% cell viability = 100 x (absorbance of test sample/ absorbance of control)

% cell death = [100 – (absorbance of test sample/ absorbance of control)] x 100

All the experiments were performed in triplicate. The IC_{50} value of each tested extract was calculated by percentage cell death at various concentrations using line regression test (Hasan *et al.,* 2014).

3.5.3.3 Gene expression analysis

The expression analysis of *p53*, *K-ras* and *β-actin* gene was carried out by real time PCR. RNA from MCF-7 cells was extracted by trizol method. Breifly, trizol was added into the cells and homogenized through ultrasonication. Chloroform was added and allowed to stand at room temperature for 10 min. Centrifugation was carried out for 15 min at 12000 rpm at 4 °C. Transparent top layer was collected. Isopropanol was added into the recovered layer and allowed to stand at room temperature after shaking vigorously. Centrifugation was carried out for 10 min at 12000 rpm at 4 °C. Supernatant was discarded and 75% ethanol was added into the pellet. Centrifugation was carried out again at 7500 rcf for 2 min at 4 °C. Ethanol was removed and nuclease free water was added into the RNA pellet. Reverse transcription was carried out by using oligo dt primer. After the cDNA was synthesized, SYBR green based real time PCR was carried out by using specific primers on Applied biosystems 7000 real time PCR system. Primers used are as follows: Actin, a house keeping gene was used as an internal control: Forward 5'-GCCTTCCTTCTTGGGTATGG-3', Reverse 5' CAGCT CAGTAACAGTCCGC-3'; *p53*: Forward 5' TAACAGTTCCTGCATGGGCGGC-3', Reverse 5' AGGACAGGCACAAACACCCACC-3'; *K-ras*: Forward 5' -TCTTGCC TCCCTACCTTCCACAT-3', Reverse 5'-CTGTCAGATTC TCTTGAGCCCTG-3'. Thermal profile used are as follows: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s, 60°C for 1 min and 60°C for 20 s. All the reactions were carried out in duplicate. Comparative

threshold (Ct) method was used to analyze the data. The relative expression of the genes was calculated by comparison with the untreated samples.

3.5.4 Neuroprotective effect

3.5.4.1 Animals

Sixty male adult Balb/c mice weighing 40-50 g were used in the experimental analysis. They were kept in propylene cages under hygienic conditions with free access to standard mice diet and water at 25 °C, in 12 hrs light and dark cycle. All the experiments were carried out according to international laws and policies of NIH (National Institutes of Health). Animal studies was approved by the Internal Review Board (IRB) ethical committee reference no: IRB-73 for research on animals at ASAB, NUST, Islamabad, Pakistan.

3.5.4.2 Experimental Design

Adult male mice were divided into ten groups. Six mice were placed in each group and treatment was carried out for consecutive 15 days. Group I received distilled water and standard mice feed. Group II received $NaNO_2$ (300 mg/kg) in drinking water with standard mice feed. Group III received *C. angustifolia* methanol extract (200 mg/g) in mice feed with normal drinking water. Group IV received $NaNO_2$ (300 mg/kg) in drinking water and *C. angustifolia* methanol extract (200 mg/g) in mice feed. Group V received *C. angustifolia* ethyl acetate extract (200 mg/g) in mice feed with normal drinking water. Group VI received $NaNO_2$ (300 mg/kg) in drinking water and *C. angustifolia* ethyl acetate extract (200 mg/g) in mice feed. GroupVII received *T. foenum-graecum* methanol extract (200 mg/g) in mice feed with normal drinking water. Group VIII received $NaNO_2$ (300 mg/kg) in drinking water and *T. foenum-graecum* methanol extract (200 mg/g) in mice feed. Group IX received *T. foenum-graecum* ethyl acetate extract (200 mg/g) in mice feed with normal drinking water. Group X received

$NaNO_2$ (300 mg/kg) in drinking water and *T. foenum-graecum* ethyl acetate extract (200 mg/g) in mice feed.

3.5.4.3 Sample collection

After 15-days of treatment, the animals were divided equally for two parallel experiments. For histopathology, half of the animals were anesthetized followed by transcradial perfusion with 60-80 mL of 0.9% normal saline and paraformaldehyde (PFA). For Liver function test, half of the animals were sacrificed by cervical decapitation. Blood samples were collected in the gel tubes.

3.5.4.4 Histopathology

After perfusion, the dissected brain sections were fixed in 10% formalin. The hippocampus and cortex regions of the brain were processed for paraffin embedding and sectioned at 3-5 µm. Cresyl violet staining was carried out for histopathological examination.

3.5.5 Hepatoprotective effect

3.5.5.1 Liver function test

For liver function test, serum was separated from collected blood by centrifugation at 300 rpm for 5 min. The level of liver enzymes alanine aminotransferase and aspartate transaminase was determined by commercially available kits.

3.6 Screening and isolation of active compounds

The *C. angustifolia* extracts were submitted to HPLC-MS analysis for screening of active compounds. A Shimadzu preparative HPLC, equipped with an LC-20AD pump (the make-up pump), two LC-8A pumps (the gradient pumps), an SPD-20A UV detector and a CTC analytics PAL sample injector and fraction collector were used for the isolation of compounds.

The fractions were collected based on UV @ 214 nm. The column used was a Phenomenex Gemini-NX C18 (30 x 50 mm i.d., 5 µm particle size, 110 A). The injection volumes were 800 to 1200 µL. The gradient was 5% to 70% acetonitrile over 25 min with a flow rate of 35 mL/min. The modifier was 0.1% formic acid, used primarily for ionization.

3.7 Identification of active compounds

NMR data were acquired at 25 °C on a Varian Inova-600 spectrometer operating at a 1H frequency of 599.7 MHz, with a cryogenic (25K) HCN 1H-observe probe, except for 13C spectra which were obtained at 25 °C on a Bruker DRX-500 spectrometer using a broadband observe (BBO) probe. All 2D spectra were acquired using 375 t1 increments (750 FIDs) with 1024 complex data points in t2 (520 points for HSQC). Gradient-enhanced 2D-NOESY spectra were acquired using 24 scans for each FID, with a mixing time of 0.5 s. Gradient-selected DQF-COSY spectra used 8 scans per FID, gradient-selected edited 13C-decoupled HSQC used 16 scans per FID, and gradient-selected magnitude-mode HMBC used 32 scans per FID. HSQC and HMBC used adiabatic shaped pulses for 13C refocusing.

3.8 Statistical analysis

All the experiments were carried out in triplicate and the data is presented as mean±SD. Student's t-test was performed to determine statistical significance. Differences were considered statistically significant when $p \leq 0.05$, 0.01 and 0.001. Microsoft Excel 2010 was used for the statistical and graphical evaluations

4. Results

4.1 Identification of plant material

C. angustifolia and *T. foenum-graecum* were purchased from local herb shop in G-9 markaz, Islamabad, Pakistan. *C. angustifolia* was identified by comparing with the voucher specimen no P03088812 of Herbier museum Paris (http://mediaphoto.mnhn.fr/media/1441330963480e0yHg eqQ0CSk9mGW). The local botanical description of *C. angustifolia* is also available at flora of Pakistan (http://www.tropicos.org/Name/13028414?projectid=32) and original plant material was kept at MPRL (Medicinal Plant Research Laboratory), ASAB, NUST for future references. *T. foenum-graecum* seeds were deposited in Plant Genetic Resources Institute (http://www.parc.gov.pk/index.php/en/about-narc/101-narc/pgri/567-pgri-scientificstaff) National Agricultural Research Center, Park Road, Islamabad (Pakistan) under accession no. 03660. The Herbarium voucher of the specimen is available at Pakistan Natural History Museum (http://www.pmnh.gov.pk), Islamabad (Pakistan), the no. is 2910.

4.2 Qualitative phytochemical analysis of different extracts of *C. angustifolia* and *T. foenum-graecum*

4.2.1 Phytochenical analysis of different extracts of *C. angustifolia*

Preliminary qualitative phytochemical screening of different extracts of *C. angustifolia* revealed the presence of steroids, alkaloids, tannins, terpenoids, flavonoids, coumarins, cardiac glycosides, saponins, anthraquinones, and phenols which contributed to the antimicrobial,

Table 4.1. Phytochemical screening for secondary metabolites of different solvent extracts of *Cassia angustifolia*.

Phytochemical	Extracts				
	Aqueous	**Methanol**	**Ethanol**	**Acetone**	**Ethyl acetate**
Steroids	+	+	+	+	+
Alkaloids	-	+	+	-	+
Tannins	+	+	+	-	+
Terpenoids	+	+	+	+	+
Flavonoids	+	+	+	+	+
Coumarins	+	+	+	-	-
Cardiac Glycosides	+	+	+	+	+
Saponins	+	+	+	+	+
Anthraquinones	+	+	+	-	-
Phenols	+	+	+	+	+

Present= +, Absent= -

antioxidant and anticancer activities of the plant (Table 4.1). Steroids, terpenoids, flavonoids, cardiac glycosides, saponins and phenols were detected in all the tested extracts of *C. angustifolia*. Alkaloids were only detected in methanol, ethanol and ethyl acetate extracts. Tannins were not present in acetone extract. Whereas, anthraquinones and coumarins were not detected in acetone and ethyl acetate extracts.

4.2.2 Phytochenical analysis of different extracts of *T. foenum-graecum*

Qualitative pytochemical screening of different extracts of *T. foenum-graecum* were carried out (Table 4.2). The aqueous extract contains steroids, tannins, terpenoids, flavonoids, coumarins, cardiac glycosides, saponins, phenols. The methanol extract contains steroids, tannins, terpenoids, flavonoids, cardiac glycosides, saponins, anthraquninoes, phenols. The ethanol extract contains steroids, alkaloids, tannins, terpenoids, flavonoids, cardiac glycosides, saponins, anthraquninoes, phenols. The acetone extract conatins alkaloids, terpenoids, flavonoids, cardiac glycosides, anthraquninoes, phenols. Ethyl acetate extracts contains alkaloids, flavonoids, cardiac glycosides, anthraquninoes and phenols.

4.3 Quantitative screening of Phytochemicals from *C. angustifolia* and *T. foenum-graecum*

4.3.1 Total phenolic contents in organic and aqueous extracts of *C. angustifolia*

Total phenolic contents in the aqueous and organic extracts of *C. angustifolia* were evaluated by plotting a standard curve using different concentrations of GAE (Gallic acid equivalent) with their respective absorbance at 700 nm (Fig. 4.1). The linear regression equation [y =0.504x (R2 = 0.991)] for the calibration curve was used to evaluate the total amount of phenolic contents present in each extract which was expressed as mg of GAE/g (Table 4.3). The analysis showed that a considerable amount of phenolic contents was present

Table 4.2. Phytochemical screening for secondary metabolites of different solvent extracts of *T. foenum-graecum*.

Phytochemical	Extracts				
	Aqueous	**Methanol**	**Ethanol**	**Acetone**	**Ethyl acetate**
Steroids	+	+	+	-	-
Alkaloids	-	-	+	+	+
Tannins	+	+	+	-	-
Terpenoids	+	+	+	+	-
Flavonoids	+	+	+	+	+
Coumarins	+	-	-	-	-
Cardiac Glycosides	+	+	+	+	+
Saponins	+	+	+	-	-
Anthraquinones	-	+	+	+	+
Phenols	+	+	+	+	+

Present= +, Absent= -

in organic and aqueous extracts of *C. angustifolia* ranging from 0.53±0.02 to 2.32±0.03 mg of GAE/g of extract. The methanol and ethanol extracts have the most phenolic contents followed in order by the acetone, ethyl acetate, and aqueous extracts (Fig. 4.3).

4.3.2 Total flavonoid contents in organic and aqueous extracts of *C. angustifolia*

The total flavonoid contents were determined by plotting a standard curve using different concentrations of QE (Quercetin equivalent) with their absorbance at 510 nm (Fig. 4.2). The linear regression equation [y =0.040x (R2 = 0.975)] of the calibration curve was used to evaluate the total amount of flavonoid contents present in each extract which is expressed as mg of quercetin equivalents per gram QE/g (Table 4.3). Methanol extracts of *C. angustifolia* showed higher content of flavonoids than the other solvent extracts (Fig. 4.3).

4.3.3 Total phenolic contents in organic and aqueous extracts of *T. foenum-graecum*

Total phenolic contents in the aqueous and organic extracts of *T. foenum-graecum* were evaluated by the same method as described for *C. angustifolia* by plotting graph for gallic acid equivalent (Fig. 4.1). Results revealed that all the tested extracts have considerable amount of phenolic contents ranging from 0.22±0.01 to 2.26±0.03mg of GAE/g (Table 4.4). Methanol extracts have significantly ($P \leq 0.01$) higher level of phenolic contents (2.26±0.003) with ethyl acetate being slightly moderate phenlic contents (1.23±0.005) followed by ethanol (0.23±0.001), acetone (0.23±0.004) and aqueous extracts (0.22±0.00) (Fig. 4.4).

4.3.4 Total flavonoid contents in organic and aqueous extracts of *T. foenum-graecum*

The total flavonoid contents of *T. foenum-graecum* extracts in term of Quercetein eqviualent (Fig. 4.2) were found to exist between the range of 0.28±0.03 to 1.24±0.03. Methanol extract contains significantly ($P \leq 0.001$) highest amount of flavonoid contents (1.24±0.03) followed by ethyl acetae (1.04±0.12), ethanol (0.64±0.09), aqueous (0.422±0.323) and acetone (0.28±0.03) extracts (Fig. 4.4).

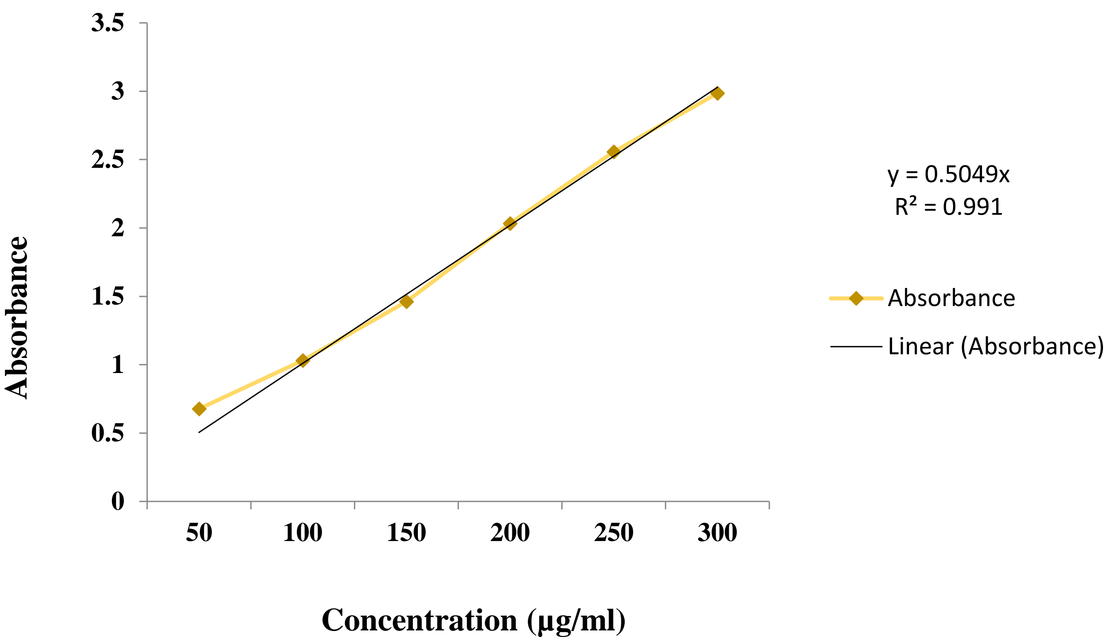


Figure. 4.1. Showing calibration curve of different concentrations of gallic acid and their respective absorbance at 700 nm. Each point represents the mean of three experiments.

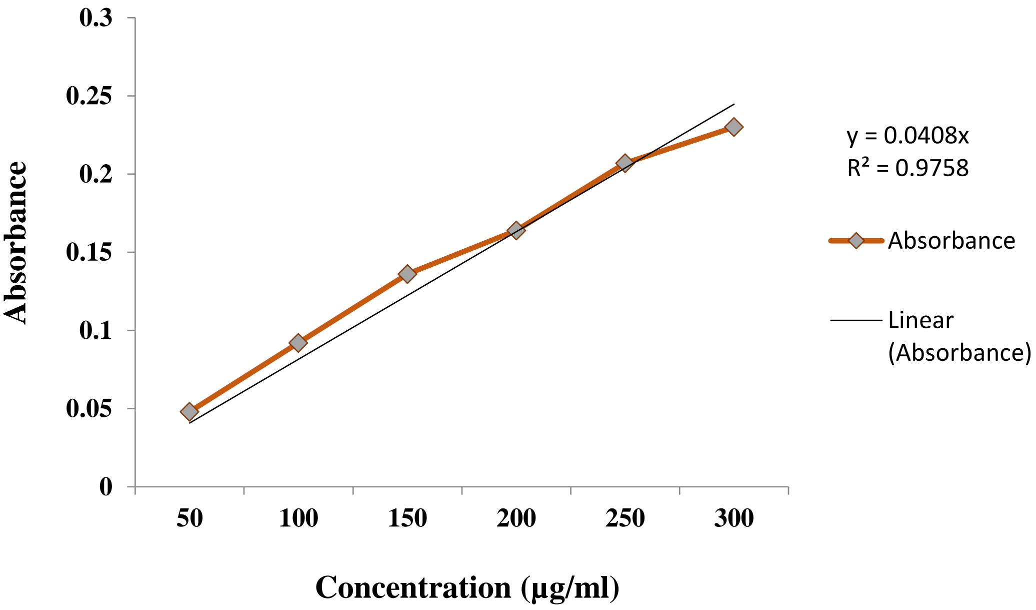


Figure. 4.2. Showing calibration curve of different concentrations of quercetin and their respective absorbance at 510 nm. Each point represents the mean of three experiments.

Table 4.3. Quantification of phenolics and flavonoids in different solvent extracts of C. *angustifolia*

S.No	Plant extracts/ chemicals (all 400µg/mL)	Total phenolic contents ([a]mg of GAE/g of extract)	Total flavonoid contents ([b]mg QE/g dried extract)
1	**Aqueous**	0.53±0.02***	1.29±0.03***
2	**Methanol**	2.32±0.03**	5.00±0.04***
3	**Ethanol**	1.76±0.01**	3.70±0.08***
4	**Acetone**	1.53±0.04***	3.31±0.06***
5	**Ethyl acetate**	1.31±0.02***	3.73±0.04***

Values are expressed as mean ± standard deviation mg of the extracts (observations of three replicates of each sample extract). (**), and (***), significant at level P ≤ 0.01 and 0.001, respectively. ([a]) Gallic Acid Equivalent per gram of dry weight (mg GAE/gm). ([b]) Quercetin Equivalent per gram of dry weight (mg QE/gm).

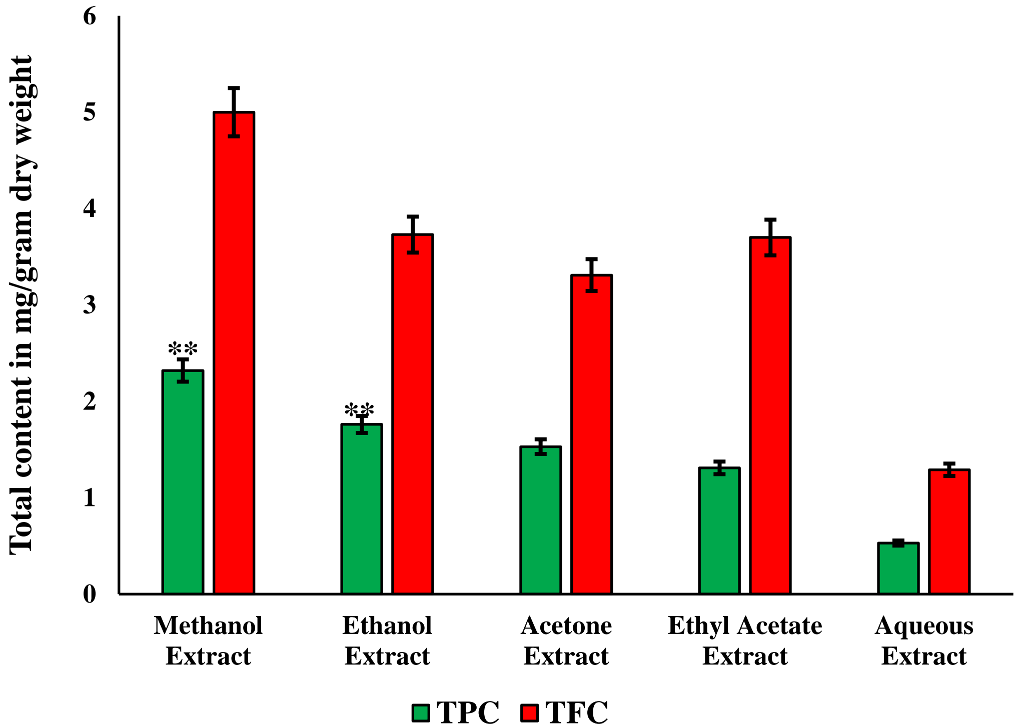


Figure. 4.3. Total phenolic and flavonoid contents of different *C. angustifolia* extracts. The total phenolic contents are expressed as mg of gallic acid equivalent (GAE) per gram of extract and the total flavonoid contents are expressed as mg of Quercetin equivalent (QE) per gram of extract. TPC=Total phenolic content, TFC=Total flavonoid content. Values expressed are mean ± standard deviation (n = 3). All the values are significant at P ≤ 0.001 except (**) indicated values are significant at P ≤ 0.01.

Table 4.4. Quantification of phenolics and flavonoids in different solvent extracts of *T. foenum-graecum*

S.No	Plant extracts/ chemicals (all 400µg/mL)	Total phenolic contents ([a]mg of GAE/g of extract)	Total flavonoid contents ([b]mg QE/g dried extract)
1	**Aqueous**	0.22±0.01***	0.42±0.323***
2	**Methanol**	2.26±0.03**	1.24±0.03***
3	**Ethanol**	0.23±0.01***	0.64±0.09***
4	**Acetone**	0.23±0.04***	0.28±0.03***
5	**Ethyl acetate**	1.23±0.05***	1.04±0.12***

Values are expressed as mean ± standard deviation mg of the extracts (observations of three replicates of each sample extract). (**), and (***), significant at level $P \leq 0.01$ and 0.001, respectively. ([a]) Gallic Acid Equivalent per gram of dry weight (mg GAE/gm). ([b]) Quercetin Equivalent per gram of dry weight (mg QE/gm).

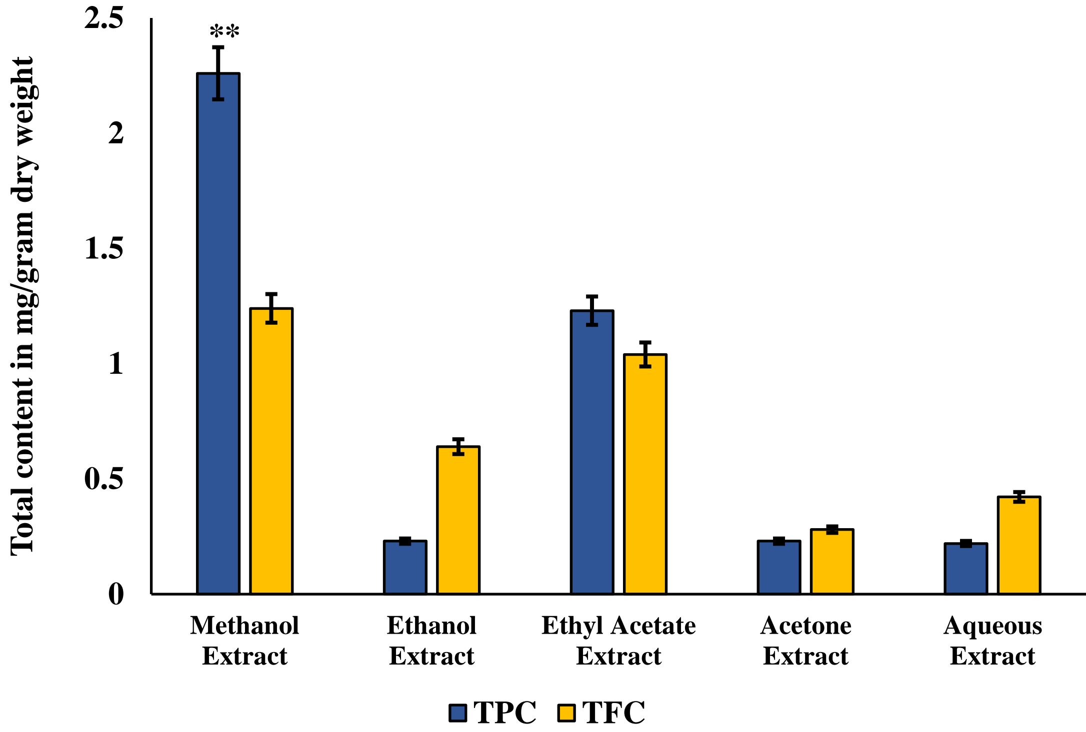


Figure 4.4. Total phenolic and flavonoid contents of different *T. foenum-graecum* extracts. The total phenolic contents are expressed as mg of gallic acid equivalent (GAE) per gram of extract and the total flavonoid contents are expressed as mg of Quercetin equivalent (QE) per gram of extract. TPC=Total phenolic content, TFC=Total flavonoid content. Values expressed are mean ± standard deviation (n = 3). All the values are significant at $P \leq 0.001$ except (**) indicated values are significant at $P \leq 0.01$.

4.4 In-vitro antimicrobial activity of *C. angustifolia* and *T. foenum-graecum*

Aqueous, methanol, ethanol, acetone and ethyl acetate extracts of *C. angustifolia* and *T. foenum-graecum* were tested against *A. junii* IARS2, *S. mercescens* IARS6, *E. cloacae* IARS7, *P. aeroginosa* IARS8, *S. typhi* ATCC 14079 for antibacterial activities.

4.4.1 Inhibitory activity of *C. angustifolia* extracts against bacterial strains

The antibacterial activities of different extracts of *C. angustifolia* and controls are shown in Tables 4.5 and 4.6. All the tested pathogenic bacterial strains were sensitive to *C. angustifolia* extracts. Extracts of *C. angustifolia* showed variable degrees of bactericidal activity, with inhibitory effects of methanol extracts being observed against all of the selected bacterial strains. The highest bactericidal activity of the ethyl acetate extract was recorded against *S. mercescens*, with a 10.5±0.76 mm zone of inhibition at 1.25 mg/mL. It was observed that *A. junni*, *E. cloacae* and *P. aeroginosa* were resistant to aqueous extract. The ethanol extract showed no antibacterial activity against *E. faecalis* and *P. aeruginosa*, while it showed its best antibacterial activity against *S. mercescens* with a 9.0±0.50 mm zone of inhibition at 1.25 mg/mL. *A. junii*, *S. mercescens*, *E. cloacae*, and *S. typhi* were sensitive to acetone extract. Tigecycline, amikacin and cefepime were used as standard antibiotic drugs (Table 4.6).

4.4.2 Inhibitory activity of *T. foenum-graecum* extracts against bacterial strains

The antibacterial activity of *T. foenum-graecum* were evaluated by employing disk diffusion method Table 4.7 and 4.8. It was observed that different extracts of *T. foenum-graecum* showed different extent of activity against different pathogenic bacterial strains. Methanol extract showed significant antibacterial activity against all the tested bacterial strains except *E. cloacae.* Maximum activity was observed against *S. typhi* with a mean inhibition of zone

Table 4.5. Zone of inhibition of *C. angustifolia* extracts against pathogenic bacterial strains

Microrganisms		Extracts	Zones of Inhibition (mm)			
			1.25 mg/mL	2.5 mg/mL	5 mg/mL	10 mg/mL
Bacterial strain	***Acinetobacter junii***	**Methanol**	N.D	N.D	7.5±0.1**	10±0.2**
		Acetone	N.D	N.D	7.9±0.1**	9.5±0.1**
		Ethyl acetate	N.D	8.0±0.2**	10.5±0.2**	11±0.36**
		Ethanol	N.D	N.D	8.0±0.36**	10±0.2**
		Aqueous	N.D	N.D	N.D	N.D
	Serratia mercescens	**Methanol**	9.5±0.36**	9.7±0.17**	10.5±0.60*	12.8±1**
		Acetone	8.5±0.2**	8.8±0.3**	10.8±0.26*	11.6±0.2**
		Ethyl acetate	9.0±0.60**	10.0±0.17**	12.1±.95*	13.3±0.51**
		Ethanol	10.5±0.26*	10.8±0.1**	11.6±1.2*	12.5±1.1*
		Aqueous	7.3±0.33**	7.8±0.57**	9.0±0.60**	10.0±0.20**
	Enterobacter cloacae	**Methanol**	7.3±0.26**	7.5±0.20**	8.6±0.36**	10.6±0.20**
		Acetone	N.D	N.D	7.5±0.3**	8.5±0.1**
		Ethyl acetate	7.8±0.78*	8.5±0.1**	10.5±0.26**	12.5±0.50**
		Ethanol	N.D	N.D	N.D	N.D
		Aqueous	N.D	N.D	N.D	N.D
	Pseudomonas aeruginosa	**Methanol**	7.5±0.20**	8.5±0.1**	10.0±0.20**	12.1±0.43**
		Acetone	N.D	N.D	N.D	N.D
		Ethyl acetate	N.D	N.D	N.D	N.D
		Ethanol	N.D	N.D	N.D	N.D
		Aqueous	N.D	N.D	N.D	N.D
	Salmonella typhi	**Methanol**	7.8±0.3**	8.8±0.52**	10.8±0.65**	12.17±0.43**
		Acetone	7.1±0.6*	7.8±0.30**	8.2±0.36**	8.9±0.43**
		Ethyl acetate	8.8±0.28**	9.3±0.45**	10.0±0.20**	12.5±0.62**
		Ethanol	7.1±0.26**	8.0±0.34**	10.5±0.50**	11.8±0.40**
		Aqueous	7.6±0.40**	8.8±0.40**	9.5±0.43**	10.0±0.34**

N.D= Not detected. Each value represents the mean ± standard deviation of three replicates (n = 3). (*), and (**), significant at level $P \leq 0.01$ and 0.001, respectively.

Table 4.6. Zone of inhibition of positive and negative controls against pathogenic bacterial strains.

Controls	Chemicals	Zone of Inhibition (mm)				
		A. junii	***S. mercescens***	***E. cloacae***	***P. aeroginosa***	***S. typhi***
Positive	Tigecycline	12.1±0.28	15.0±0.50	16.1±0.28	12.5±0.50	16.0±0.50
	Amikacin	±0.20	13.3±0.28	20.1±0.28	12.0±0.50	20.1±0.29
	Cefepime	N.D	N.D	19.8±0.28	N.D	17.0±0.50
Negative	DMSO[(a)]	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

Values are expressed as mean± SD (n = 3). ([a]) Dimethylsulfoxide.

diameter 12.36±1.47 mm at a dose of 10 mg/mL. Ethanol, acetone and ethyl acetate extract were only active significantly ($P \leq 0.05, 0.01$) against *S. mercescens* and *S. typhi.* Aqueous extract was not active against all the tested bacterial strains except it only inhibit the growth of *S. typhi* with a mean inhibition zone of diameter 15.0±1.74 at a dose of 10 mg/mL. Furthermore, among tested bacterial strains *E. cloacae* was found to be resistant against all the tested extracts. While, *P. aeruginosa* and *A. junii* were only sensitive to methanol extracts among all the tested extracts with a zone of inhibition 10.1±0.76 and 9.4±0.45 respectively.

4.5 Antioxidant potential of *C. angustifolia* and *T. foenum-graecum*

The antioxidant activities of the aqueous and organic extracts of *C. angustifolia, T. foenum-graecum* and gallic acid were evaluated by the free radical DPPH scavenging assay on the basis of IC_{50} values. IC_{50} values are the inhibitory concentrations required for 50% scavenging of DPPH free radicals. The smaller the IC_{50} values, the higher the antioxidant potential of the plant constituents. The absorbance values of different extracts of both the plants and standards were measured at the wavelength 517 nm.

4.5.1 DPPH radical scavenging activity of *C. angustifolia* extracts

All the extracts have dose dependent antioxidant activities, i.e., the scavenging activities of the extracts increased with the respective increase in the concentrations (Fig. 4.5). According to the results, all the extracts have potential antioxidant activities. The methanol extracts exhibited maximum DPPH free radical scavenging activity (93±0.12%) at a concentration of 500 µg/mL with an IC_{50} value of 2.41±0.02 µg/mL, whereas the aqueous extract showed poor DPPH scavenging activity (68±0.01%) at 500 µg/mL with IC_{50} values of 3.03±0.04 µg/mL (Table 4.9). The other *C. angustifolia* extracts showed moderate DPPH scavenging activities (Fig. 4.5).

Table 4.7. Zone of inhibition of *T. foenum-graecum* extracts against pathogenic bacterial strains.

Microrganisms		Extracts	Zones of Inhibition (mm)			
			1.25 mg/mL	**2.5 mg/mL**	**5 mg/mL**	**10 mg/mL**
Bacterial Strain	***Acinetobacter junii***	**Methanol**	7.6±0.4***	8.46±0.4***	9.03±0.1***	9.4±0.45***
		Acetone	N.D	N.D	N.D	N.D
		Ethyl acetate	N.D	N.D	N.D	N.D
		Ethanol	N.D	N.D	N.D	N.D
		Aqueous	N.D	N.D	N.D	N.D
	Serratia mercescens	**Methanol**	7.5±0.5**	8.6±0.6**	9.1±0.5**	10.46±0.0**
		Acetone	10.3±1.5**	13.43±2.3*	13.16±1.6**	13.5±0.9**
		Ethyl acetate	10.2±0.26*	11.3±1.6**	14.5±1.0**	16.2±1.4**
		Ethanol	6.9±1.0**	7.9±1.8*	8.6±.60**	8.9±1.3**
		Aqueous	N.D	N.D	N.D	N.D
	Enterobacter cloacae	**Methanol**	N.D	N.D	N.D	N.D
		Acetone	N.D	N.D	N.D	N.D
		Ethyl acetate	N.D	N.D	N.D	N.D
		Ethanol	N.D	N.D	N.D	N.D
		Aqueous	N.D	N.D	N.D	N.D
	Pseudomonas aeruginosa	**Methanol**	7.0±1.1**	8.76±1.0**	9.46±0.64**	10.1±0.76**
		Acetone	N.D	N.D	N.D	N.D
		Ethyl acetate	N.D	N.D	9.5±0.97**	10±0.2***
		Ethanol	N.D	N.D	N.D	N.D
		Aqueous	N.D	N.D	N.D	N.D
	Salmonella typhi	**Methanol**	10.23±1.59**	12.83±1.80**	13.66±1.5**	15.0±1.74**
		Acetone	10.95±1.1**	12.6±1.5**	13.2±1.96**	15.13±2.2**
		Ethyl acetate	11.23±0.6**	13.16±1.30**	14±1.4**	15.3±1.17**
		Ethanol	7.76±0.86**	8.46±0.5**	11.3±0.75**	11.6±2.27**
		Aqueous	8.4±0.5**	9.7±0.8**	10.46±1.0**	12.36±1.47**

N.D= Not detected.Each value represents the mean ± standard deviation of three replicates (n = 3). (*), (**), (***) significant at level $P \leq 0.05$, 0.01 and 0.001, respectively.

Table 4.8. Zone of inhibition of positive and negative controls against pathogenic bacterial strain.

Controls	Chemicals	Zone of Inhibition (mm)				
		A. junii	***S. mercescens***	***E. cloacae***	***P. aeroginosa***	***S. typhi***
Positive	Tigecycline	12.1±0.2	15.0±0.50	16.1±0.28	12.5±0.50	16.0±0.50
	Amikacin	8±0.20	13.3±0.28	20.1±0.28	12.0±0.50	20.1±0.29
	Cefepime	N.D	N.D	19.8±0.28	0.0±0.0	17.0±0.50
Negative	DMSO[(a)]	N.D	N.D	N.D	N.D	N.D

Values are expressed as mean± SD (n = 3). ([a]) Dimethylsulfoxide.

Table 4.9. IC_{50} of free radical scavenging activity of standards and different extracts of *C. angustifolia.*

Test material		DPPH radical IC_{50} in µg/mL
Plant extracts	Methanol	2.41±0.02**
	Ethanol	2.74±0.01**
	Acetone	3.03±0.04***
	Ethyl acetate	2.49±0.02**
	Aqueous	3.06±0.04***
Standards	Gallic acid	2.52±0.02
	Ascorbic acid	2.54±0.00

Each value represents the mean ± standard deviation of three replicates (n = 3).
**, significant at level P ≤ 0.01
***, significant at level P ≤ 0.001, respectively.

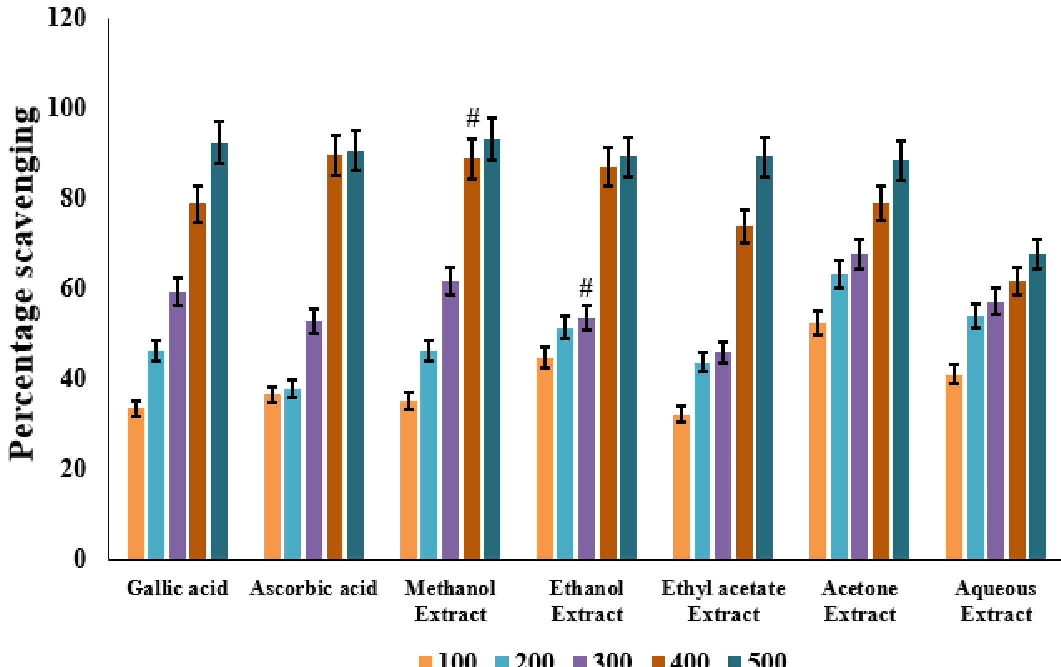


Figure. 4.5. Graph showing DPPH radical scavenging activity of different extracts of *C. angustifolia*. Each point represents the mean of three experiments. Data are expressed as mean ± standard deviation. (#) indicated values are not significant at $P \leq 0.01$.

4.5.2 DPPH radical scavenging activity of *T. foenum-graecum* extracts

The extracts of *T. foenum-graecum* showed the DPPH radical scavenging activity in concentration dependent manner i.e. the activity increases with the relative increase in concentration of the extracts. Thus, in this respect ethyl acetate extract showed significantly ($P \leq 0.001$) higher percentage scavenging activity (68±0.33%) with lower IC_{50} value (3.5±0.017µg/mL). Methanol extract possess maximum radical scavenging activity (66.44±0.19%) at the concentration of 500 mg/mL with IC_{50} value of 3.65±0.00 µg/mL followed by ethanol, acetone and aqueous extract with the IC_{50} values of 4.1±0.01, 4.1±0.03, 4.94±0.043 µg/mL respectively. The IC_{50} values of the extracts were summarized in the table 4.10 and the percentage scavenging of DPPH radical were shown in Fig. 4.6.

4.6 In-vitro anticancer activity of *C. angustifolia* and *T. foenum-graecum*

Anticancer activities of *C. angustifolia* and *T. foenum-graecum* aqueous and organic extracts was determined by evaluating its toxicity against HeLa, Hep2, MCF-7 and normal HCEC cell lines *in vitro* by employing the MTT colorimetric method. In addition, the molecular pathway associated with the anticancer effect induce by both the plants were determined by analyzing the real-time expression of *p53* (tumor suppressor) and *k-ras* gene in MCF-7 cell line by RT-PCR.

4.6.1 Cytotoxic potential of *C. angustifolia* extracts against cancer cell lines

The *C. angustifolia* aqueous, methanol, ethanol, acetone and ethyl acetate extracts were investigated first time for their anticancer potential against Hep2, HeLa, MCF-7 and normal HCEC cell lines. The anticancer activity showed by *C. angustifolia* extracts were summarized in the Table 4.11.

Table 4.10. IC_{50} of free radical scavenging activity of standards and different extracts of *T. foenum-graecum.*

Test material		**DPPH radical IC_{50} in µg/mL**
Plant extracts	Methanol	3.65±0.00**
	Ethanol	4.1±0.01***
	Ethyl acetate	3.5±0.017***
	Acetone	4.1±0.03***
	Aqueous	4.94±0.043***
Standards	Gallic acid	2.52±0.020
	Ascorbic acid	2.54±0.00

Each value represents the mean ± standard deviation of three replicates (n = 3). (***), significant at level $P \leq 0.001$, respectively

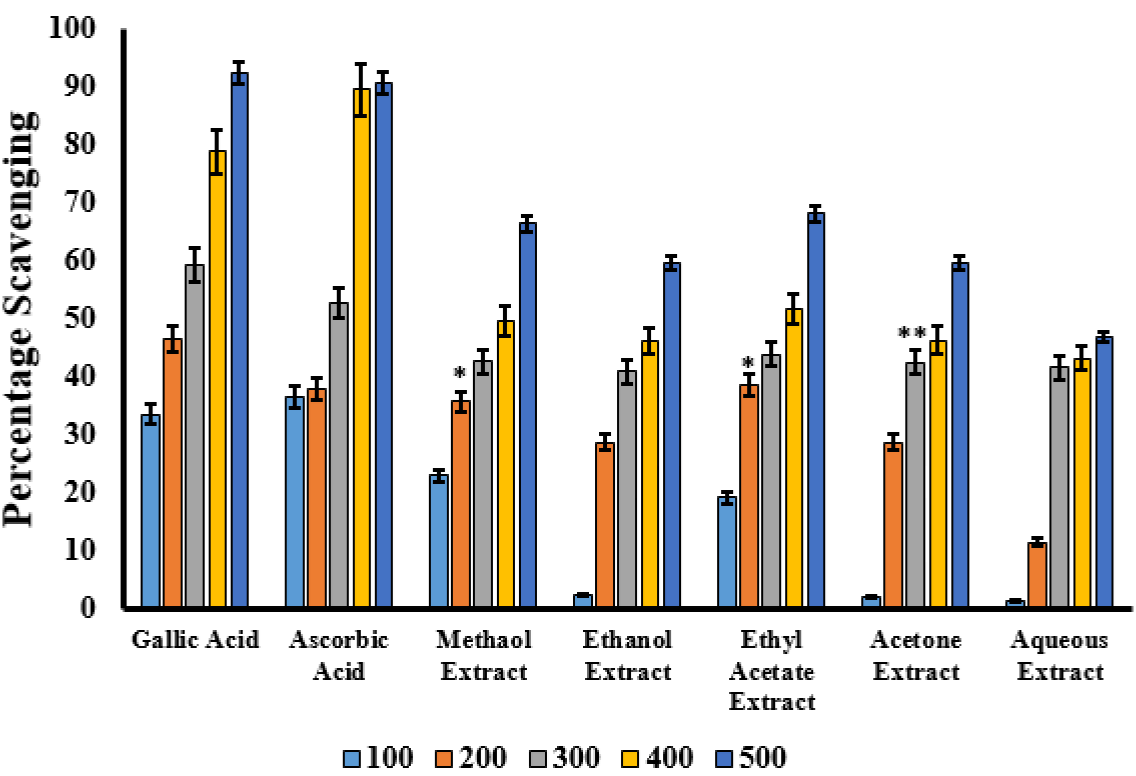


Fig. 4.6. Graph showing DPPH radical scavenging activity of different extracts of *T. foenum-graecum*. Each point represents the mean of three experiments. Data are expressed as mean ± standard deviation. All the values are significant at $P \leq 0.001$ except (*), (**) indicated values are significant at $P \leq 0.05$ and 0.01 with respect to ascorbic acid.

Only the methanolic extract significantly ($P \leq 0.01$-0.001) exhibited anticancer activity, with 28% death in Hep2 cells with an IC_{50} of 7.28 µg/µL. The IC_{50} values are used to find the potency of drugs, lower IC_{50} values mean more potent the drugs. In HeLa cell lines, Methanolic and ethanolic extracts showed 33% and 23% cell death with IC_{50} values of 5.45and 8.18 respectively. Furthermore, methanolic and ethanolic extracts significantly ($P \leq 0.01$-0.001) exhibited 43% and 23% cell death in MCF-7 cell lines (Table 4.11). It was observed that IC_{50} value of methanolic extract is 5.45 µg/µL against HeLa cells and 4 µg/µL against MCF-7 cells which are far less than the IC_{50} value of standard anticancer drug Taxol 6.07 µg/µL and Tamoxifen 6.4 µg/µL. Taxol and Tamoxifen are standard anticancer drugs taken as positive controls. Extracts were further examined for cytotoxicity against the normal cell line to answer whether it is selective towards cancerous cells. For this, normal HCEC cells were incubated with different concentrations of extracts (100, 150, 200 and 250 µg/µL) to analyze cell viability. The data showed that HCEC cells were unaffected by exposure to extracts. It showed ca. 100% cell viability against different concentrations of extracts. These results showed that methanolic, ethanolic and ethyl acetate extracts have the potential to inhibit the proliferation of Hep2, HeLa, and MCF-7 cells. These cancerous cells were more sensitive to inhibition by *C. angustifolia* extracts compared to normal HCEC cell lines. To further investigate the anticancer potential of *C. angustifolia* extracts, the real-time expressions of *p53* and *k-ras* gene were analyzed in MCF-7 cell line (Fig. 4.7). The IC_{50} value of methanolic extract (4.0 µg/µL) and ethanolic extract (8.79 µg/µL) were used to study their effect on gene expression. It was observed that methanolic extract increased the expression of *p53* gene 1.71±0.79 folds and significantly ($P \leq 0.05$) decreased the expression of *K-ras* gene 3.65E-06±9.2E-07 folds as compared to standard anticancer drug tamoxifen in MCF-7 cells. The ethanolic extract significantly ($P \leq 0.05$) decreases the expression of *K-ras* gene 1.23E-07 ±1.4E-08 folds without increasing the expression of *p-53* gene which suggested that it does not have the role

Table 4.11. Anticancer activity of *C. angustifolia* extracts against HCEC, Hep2, HeLa, and MCF-7cell lines.

Cell Lines	Extracts	% Cell death				I.C$_{50}$
		100 µg/µL	**150 µg/µL**	**200 µg/µL**	**250 µg/µL**	**µg/µL**
HCEC	**Aqueous**	N.D	N.D	N.D	N.D	N.D
	Methanol	2.3±0.76	1.83±0.76	3.43±0.50*	13.7±2.42*	21.09
	Ethanol	1.2±0.52	6.23±1.02*	9.20±0.81*	12.8±1.01*	16.23
	Acetone	N.D	N.D	N.D	N.D	N.D
	Ethyl acetate	N.D	N.D	N.D	N.D	N.D
	Taxol	N.D	N.D	N.D	N.D	N.D
	Tamoxifen	14.5±0.5	19.3±0.7	26±0.5	26.8±0.7	6.29
Hep2	**Aqueous**	N.D	N.D	N.D	N.D	N.D
	Methanol	6.06±0.49***	11.83±0.65***	21.0±0.90**	28.26±0.25**	7.28
	Ethanol	N.D	N.D	N.D	N.D	N.D
	Acetone	N.D	N.D	N.D	N.D	N.D
	Ethyl acetate	N.D	N.D	N.D	N.D	N.D
	Taxol	11.46±0.40	19.7±0.51	24.63±0.50	30.56±0.45	6.07
HeLa	**Aqueous**	N.D	N.D	N.D	N.D	N.D
	Methanol	16.7±0.70***	22.56±0.40***	27.73±0.2***	32.46±0.61***	5.45
	Ethanol	5.46±0.40***	15.53±0.37***	18.43±0.4***	22.9±.075***	8.18
	Acetone	N.D	N.D	N.D	N.D	N.D
	Ethyl acetate	N.D	N.D	N.D	N.D	N.D
	Taxol	26.5±0.5	31.76±0.66	35.53±0.25	45.16±0.60	3.97
MCF-7	**Aqueous**	N.D	N.D	N.D	N.D	N.D
	Methanol	23.33±0.7***	26.23±0.25***	42.4±0.36***	43.6±0.45***	4.0
	Ethanol	6.8±0.52**	11.5±0.37***	16.1±0.1***	23.56±0.45***	8.79
	Acetone	N.D	N.D	N.D	N.D	N.D
	Ethyl acetate	N.D	N.D	N.D	N.D	N.D
	Tamoxifen	12.46±0.40	17.36±0.55	24.63±0.50	28.56±0.45	6.4

N.D= Not detected.
Values represents the mean ± standard deviation of three replicates (n = 3). (*) (**), and (***), significant at level $P \leq 0.05$, 0.01 and 0.001, respectively

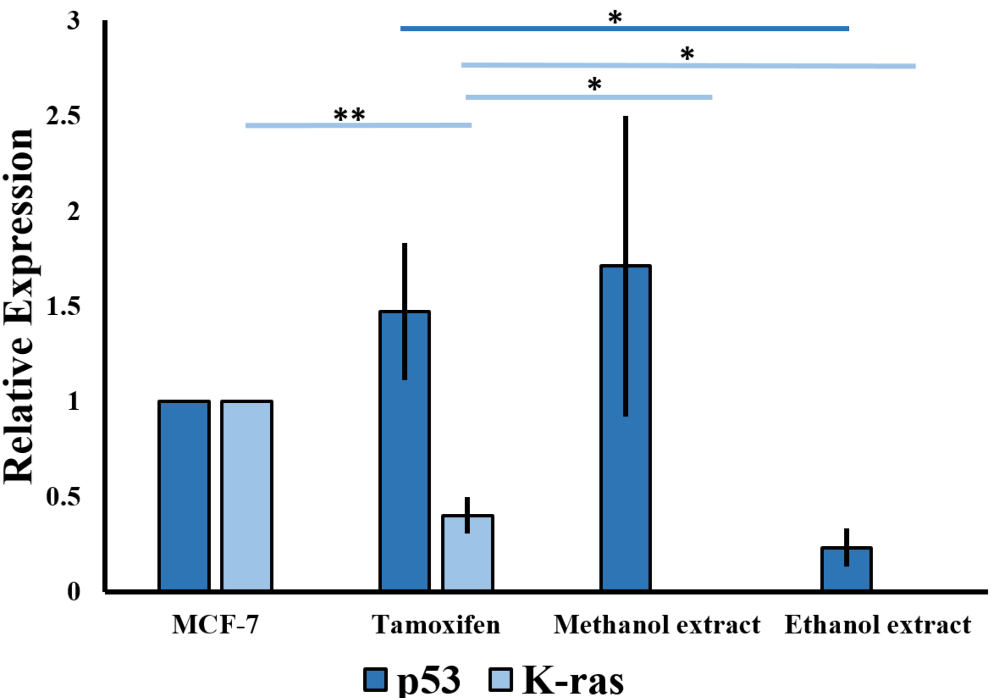


Figure 4.7. Graphical representation of the effect of *C. angustifolia* methanol and ethanol extracts on the gene expression of *p53* and *K-ras* in MCF-7 cells. Relative expression levels were obtained using the comparative Ct (ΔΔCt) method. Each point represents the mean of three experiments. Data are expressed as mean ± standard deviation (*), (**) significant at level $P \leq 0.05$, and 0.01 respectively.

in regulating the expression of p53 gene. Whereas, methanolic extract induce apoptosis in *p53*-dependent pathway by up-regulating *p53* gene in MCF-7 cells.

4.6.2 Cytotoxic potential of *T. foenum-graecum* extracts against cancer cell lines

The effect of anticancer from *T. foenum-graecum* extracts on cancer cell line Hela, Hep-2 and MCF-7 and normal cell line HCEC were evaluated through MTT colorimetric assay, with the results shown in Table 4.12. Methanol extract exhibited significant ($P \leq 0.01$-0.001) cytotoxicity against Hep2 and MCF-7 cell lines with IC_{50} ranging from 2.85 to 3.14 µg/µL. In HeLa cell lines, aqueous, ethyl acetate, methanol extracts showed 48%, 23% and 29% cell death with IC_{50} values of 3.36, 6.33, 8.03, respectively. It was also noticed that high cytotoxicity against Hep2 and MCF-7 was shown by methanol extract is far high then cytotoxicity (IC_{50}= 6.07 and 6.4 µg/µL) shown by standard drug taxol and tamoxifen, respectively. *T. foenum*-graecum methanol and ethyl acetate extracts were screened for cytotoxicity against normal cell line HCEC. Except aqueous extract which was slightly cytotoxic to normal cells, the extracts showed no cytotoxicity against healthy cells. Effect of *T. foenum-graecum* ethyl acetate, methanol and ethanol extracts on the gene expression of *p53* and *K-ras* in MCF-7 cells showed that *T. foenum-graecum* ethyl acetate extract inhibits the cellular proliferation and viability by up-regulating the expression of *p53* 1.98 ± 1.14 folds and down-regulating the expression of *K-ras* gene by 8.2E-05 ± 9.3E-06 folds (Fig. 4.8). Furthermore, it was observed that *T. foenum-graecum* methanol extract exhibit anticancer potential in *p53*-independent and *k-ras* dependent pathyway in MCF-7 cell lines. Whereas, the expression of *p53* gene was unaffected by the treatment of ethanolic extract.

Table 4.12. Anticancer activity of *T. foenum-graecum* extracts against HCEC, Hep2, HeLa, and Mcf-7cell lines

Cell Lines	Extracts	% Cell death				I.C50
		100 µg/µL	150 µg/µL	200 µg/µL	250 µg/µL	µg/µL
HCEC	Aqueous	8.32±1.4*	13.54±3.08*	17.92±2.7**	19.12±1.71**	9.05
	Methanol	0.87±0.26	2.8±0.26*	5.73±0.20	9.79±0.33**	24.08
	Ethanol	N.D	N.D	N.D	N.D	N.D
	Acetone	N.D	N.D	N.D	N.D	N.D
	Ethyl acetate	0.73±0.39	4.85±0.26*	7.37±0.54*	9.58±0.41*	21.18
	Taxol	N.D	N.D	N.D	N.D	N.D
	Tamoxifen	14.5±0.5	19.3±0.7	26±0.5	26.8±0.7	6.29
Hep2	Aqueous	N.D	N.D	N.D	N.D	N.D
	Methanol	34.53±0.45***	47.55±0.42***	53.88±0.14***	58.6±0.55***	2.85
	Ethanol	N.D	N.D	N.D	N.D	N.D
	Acetone	N.D	N.D	N.D	N.D	N.D
	Ethyl acetate	7.34±0.34***	18.62±0.34**	23.64±0.45***	31.22±0.23***	6.24
	Taxol	11.46±0.40	19.7±0.51	24.63±0.50	30.56±0.45	6.07
HeLa	Aqueous	39.92±1.73**	42.2±2.1**	43.52±2.18*	47.85±2.1	3.36
	Methanol	7.55±0.50***	15.96±2.2**	18.54±1.90**	22.92±2.1**	8.03
	Ethanol	N.D	N.D	N.D	N.D	N.D
	Acetone	N.D	N.D	N.D	N.D	N.D
	Ethyl acetate	18.78±2.4**	19.77±1.60**	20.42±1.6*	29.32±2.1**	6.33
	Taxol	26.5±0.5	31.76±0.66	35.53±0.25	45.16±0.60	3.97
MCF-7	Aqueous	N.D	N.D	N.D	N.D	N.D
	Methanol	15.39±0.25**	29.56±0.40***	49.38±0.42***	48.38±0.50***	3.14
	Ethanol	N.D	N.D	N.D	N.D	N.D
	Acetone	N.D	N.D	N.D	N.D	N.D
	Ethyl acetate	34.57±0.4***	43.68±0.34***	48.64±0.45***	52.2±0.23***	2.61
	Tamoxifen	12.46±0.40	17.36±0.55	24.63±0.50	28.56±0.45	6.4

N.D= Not detected.
Taxol and Tamoxifen are standard anticancer drugs taken as positive controls. Values represents the mean ± standard deviation of three replicates (n = 3). (*) (**), and (***), significant at level $P \leq 0.05$, 0.01 and 0.001, respectively.

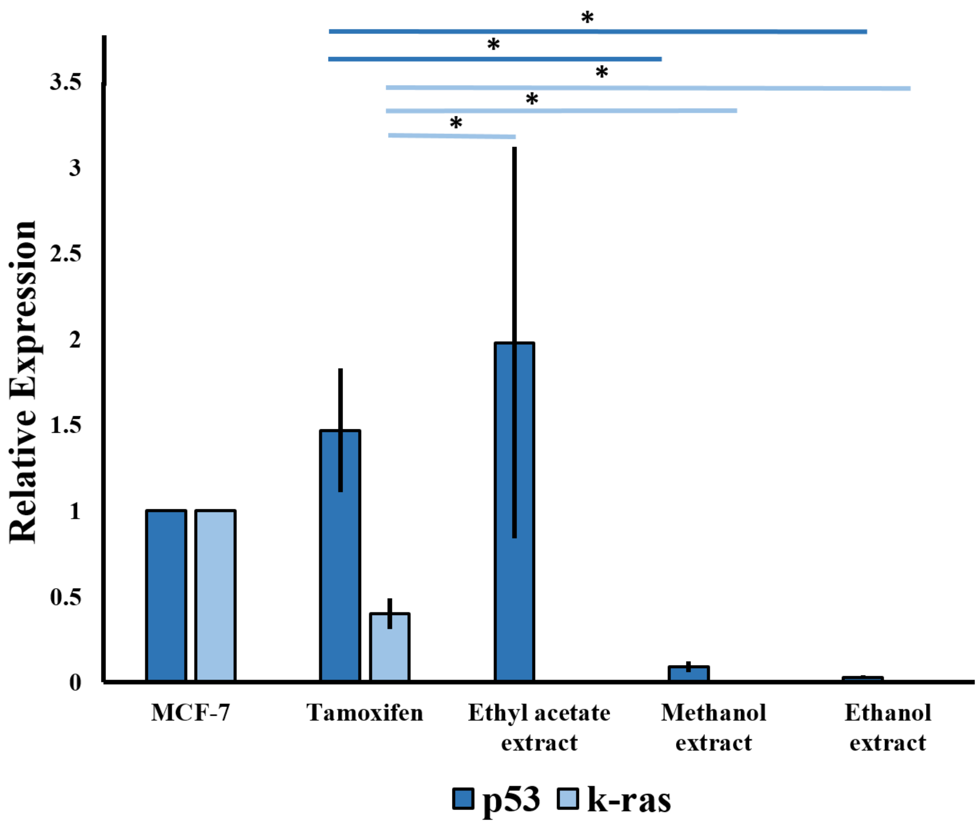


Figure 4.8. Graphical representation of the effect of *T. foenum-graecum* ethyl acetate, methanol and ethanol extracts on the gene expression of *p53* and *k-ras* in MCF-7 cells. Relative expression levels were obtained using the comparative Ct (ΔΔCt) method Each point represents the mean of three experiments. Data are expressed as mean ± standard deviation (*) significant at level $P \leq 0.05$.

4.7 Neuroprotective effect of *C. angustifolia* and *T. foenum-graecum* extracts against $NaNO_2$ induced neurodegeneration in mice brain

$NaNO_2$ induced neurodegeneration in mice was used as a model to study the neurodegeneration in different brain regions. The extent of neurodegeneration in hippocampus and cortex was histopathologically examined by cresyl violet staining. It was found that the continuous oral administration of $NaNO_2$ to experimental groups of mice for 15 days induced pathological hallmarks of neurodegeneration in the cortex and hippocampus regions of the brain. Histopathological examination showed significant degeneration and vacuolation of the cells in the cortex and scanty neurofibrillary tangles and reduced pyramidal cells in the hippocampus when compared to the control group.

4.7.1 Neuroprotective effect of *C. angustifolia* extracts against $NaNO_2$ induced neurodegeneration in mice brain

Neuroprotection conferred by methanolic and ethyl acetate extracts of *C. angustifolia* has been observed in the cortex and hippocampal regions of the mice brain. The images of histopathology stained by cresyl voilet were shown in Fig. 4.9. Histopathological examination of cortex and hippocampal sections of the control group, fed with standard mice feed, showed normal neuronal architecture with intact pyramidal cells and vesicular nuclei. Exessive neurodegeneration with shrunken neuronal cell bodies were observed in $NaNO_2$ treated group. Metahnolic and ethyl acetate extracts at a dose of 200 mg/kg co-administered with $NaNO_2$ amelioerate the neuronal damage back to normal. In comparison with methanolic extract, ethyl acetate extract showed more protection against $NaNO_2$ neurotoxicity evidenced by reduction in the neurodegeneration, vacuolation of the cells in the cortex and hippocampus.

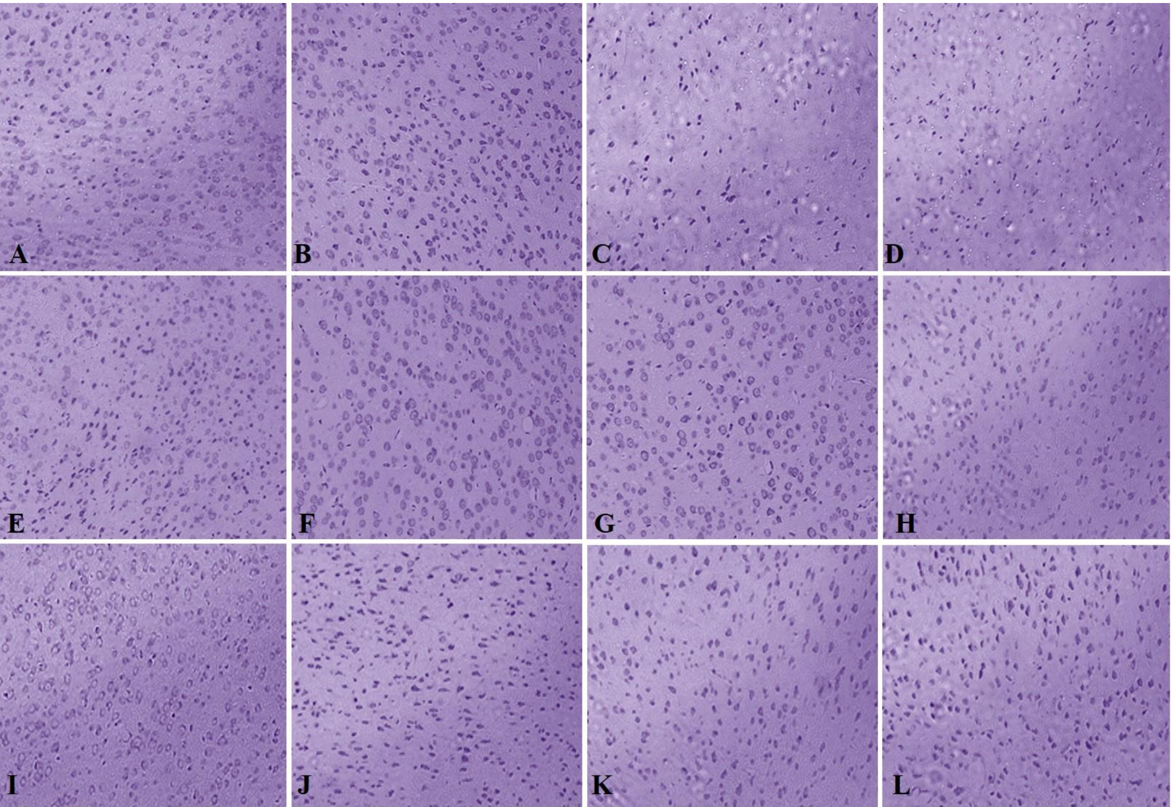


Figure 4.9: Cresyl violet staining of cortex and hippocampus regions of the brain (20X). (A) Control cortex, (B) Control hippocampus, (C) $NaNO_2$ (300 mg/kg)-treated cortex, (D) $NaNO_2$ (300 mg/kg)-treated hippocampus, (E) *C. angustifolia* methanol extract (200 mg/kg)-treated cortex, (F) *C. angustifolia* methanol extract (200 mg/kg)-treated hippocampus, (G) $NaNO_2$ + *C. angustifolia* methanol extract (200 mg/kg)-treated cortex, (H) $NaNO_2$ + *C. angustifolia* methanol extract (200 mg/kg)-treated hippocampus, (I) *C. angustifolia* ethyl acetate extract (200 mg/kg)-treated cortex, (J) *C. angustifolia* ethyl acetate extract (200 mg/kg)-treated hippocampus, (K), $NaNO_2$ + *C. angustifolia* ethyl acetate extract (200 mg/kg)-treated cortex, (L) $NaNO_2$ + *C. angustifolia* ethyl acetate extract (200 mg/kg)-treated hippocampus.

4.7.2 Neuroprotective effect of *T. foenum-graecum* extracts against $NaNO_2$ induced neurodegeneration in mice brain

Oral administration of methanolic or ethyl acetate extracts (100 & 200 mg/kg) of *T. foenum-graecum* retrograded the pathological hallmarks back to normal when compared to the $NaNO_2$ treated group. It was also observed that the experimental group treated with oral administration of methanolic and ethyl acetate extract (200 mg/kg) resembled the control group with compactly arranged round pyramidal cells, vesicular nuclei, without any nuclear distortion and condensation in hippocampus and cortex. The methanolic extract showed a substantial neuroprotective effect as compared to the ethyl acetate extract by the deteriorating effect of $NaNO_2$ (Fig. 4.10).

4.8 Hepatoprotective effect of *C. angustifolia* and *T. foenum-graecum* extracts against $NaNO_2$ induced hepatotoxicity in mice liver

$NaNO_2$ induced hepatotoxicity in mice was used as a model to study the hepatotoxicity in mice liver. The hepatotoxicity was observed by estimating the levels of liver enzymes (ALT and AST) in mice blood (Fig 4.11 and 4.12). It was found that the continuous oral administration of $NaNO_2$ to experimental groups of mice for 15 days significantly ($P \leq 0.001$) induced hepatotoxicity in mice liver when compared to the control group.

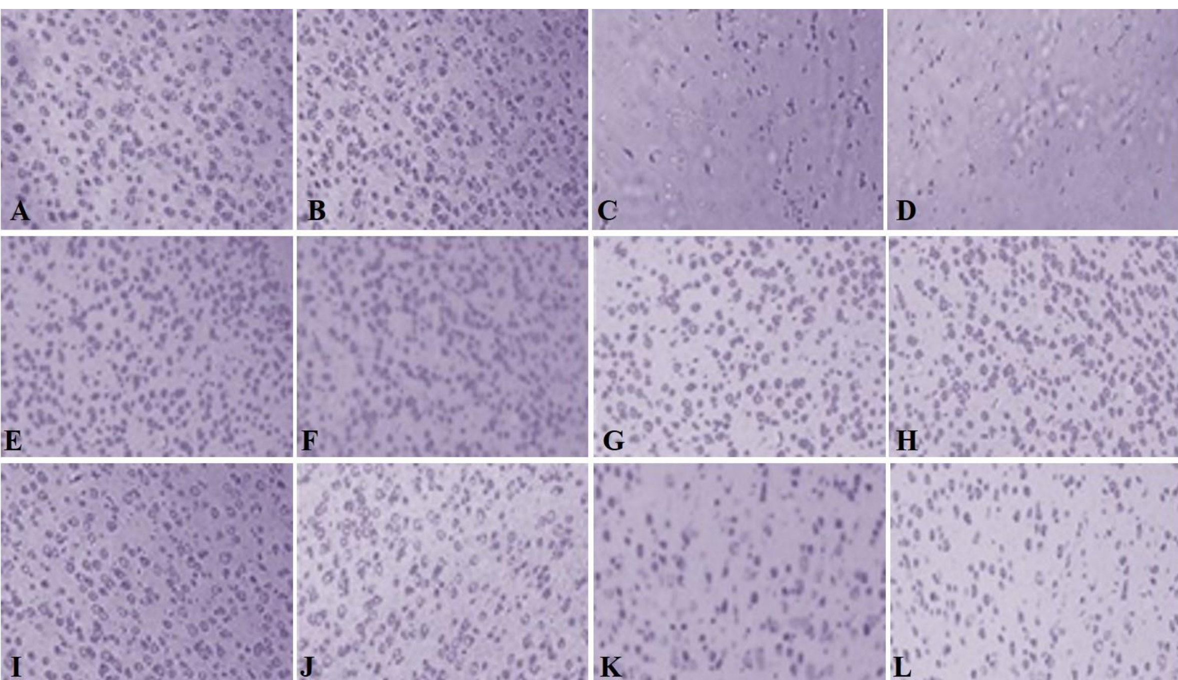


Figure 4.10: Cresyl violet staining of cortex and hippocampus regions of the brain (20X). (A) Control cortex, (B) Control hippocampus, (C) $NaNO_2$ (300 mg/kg)-treated cortex, (D) $NaNO_2$ (300 mg/kg)-treated hippocampus, (E) *T. foenum-graecum* methanol extract (200 mg/kg)-treated cortex, (F) *T. foenum-graecum* methanol extract (200 mg/kg)-treated hippocampus, (G) $NaNO_2$ + *T. foenum-graecum* methanol extract (200 mg/kg)-treated cortex, (H) $NaNO_2$ + *T. foenum-graecum* methanol extract (200 mg/kg)-treated hippocampus, (I) *T. foenum- graecum* ethyl acetate extract (200 mg/kg)-treated cortex, (J) *T. foenum-graecum* ethyl acetate extract (200 mg/kg)-treated hippocampus, (K) $NaNO_2$ + *T. foenum-graecum* ethyl acetate extract (200 mg/kg)-treated cortex, (L) $NaNO_2$ + *T. foenum-graecum* ethyl acetate extract (200 mg/kg)-treated hippocampus.

4.8.1 Hepatoprotective effect of *C. angustifolia* extracts against $NaNO_2$ induced hepatotoxicity in mice liver

Oral administration of methanol and ethyl acetate extracts (200 mg/kg) of *C. angustifolia* decreases the level of liver enzymes when compared to the $NaNO_2$ treated group. In $NaNO_2$ treated group, the activities of ALT and AST enzymes were significantly ($P \leq 0.001$) increased upto 103.66 ± 2.5, and 88.33 ± 1.52 respectively, which were decreased in mice treated with methanol (72.33 ± 2.5, 61.66 ± 2.8) and ethyl acetate (90.66 ± 4.0, 76.00 ± 3.6) extracts of *C. angustifolia.* In comparison with the group treated with ethyl acetate extract, it was observed that the group treated with methanol extract has the highest effect in reducing the toxic effect of $NaNO_2$ by lowering the levels of ALT and AST. It was also observed that the experimental group treated with methanol and ethyl acetate extract of *C. angustifolia* showed similar results when compared to control group by not elevating the level of liver enzymes to toxic levels (Fig 4.11).

4.8.2 Hepatoprotective effect of *T. foenum-graecum* extracts against $NaNO_2$ induced hepatotoxicity in mice liver

T. foenum-graecum methanol and ethyl acetate extracts at a dose of 200 mg/kg significantly ($P < 0.05$) reduced the $NaNO_2$-induced hepatotoxicity in mice by lowering the ALT and AST levels. *T. foenum-graecum* methanol extract markedly decreases the level of ALT (83.66 ± 5.8) and AST (71.6 ± 5.7) when compared with $NaNO_2$ (ALT (103.66 ± 2.5) and AST (88.33 ± 1.52)) treated group. *T. foenum-graecum* ethyl acetate extract reduced the elevated levels of ALT (95.33 ± 4.7) and AST (81.3 ± 7.2) to minimum level when compared with *T. foenum-graecum* methanol extract. Furthermore, *T. foenum-graecum* methanol extract showed the comparable effect with the control group. The normal and elevated levels of liver enzymes in blood serum of mice were shown in Fig. 4.12.

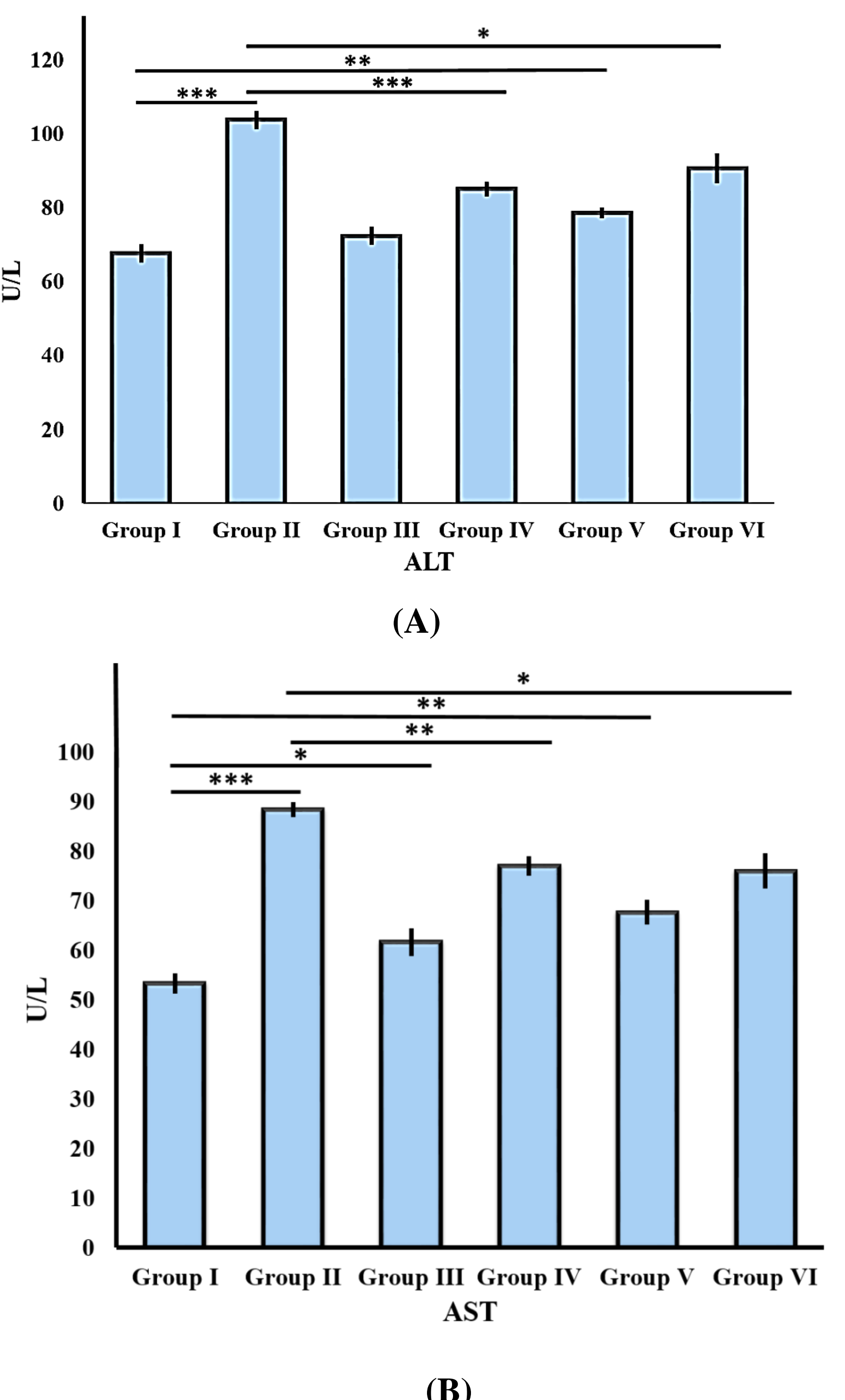
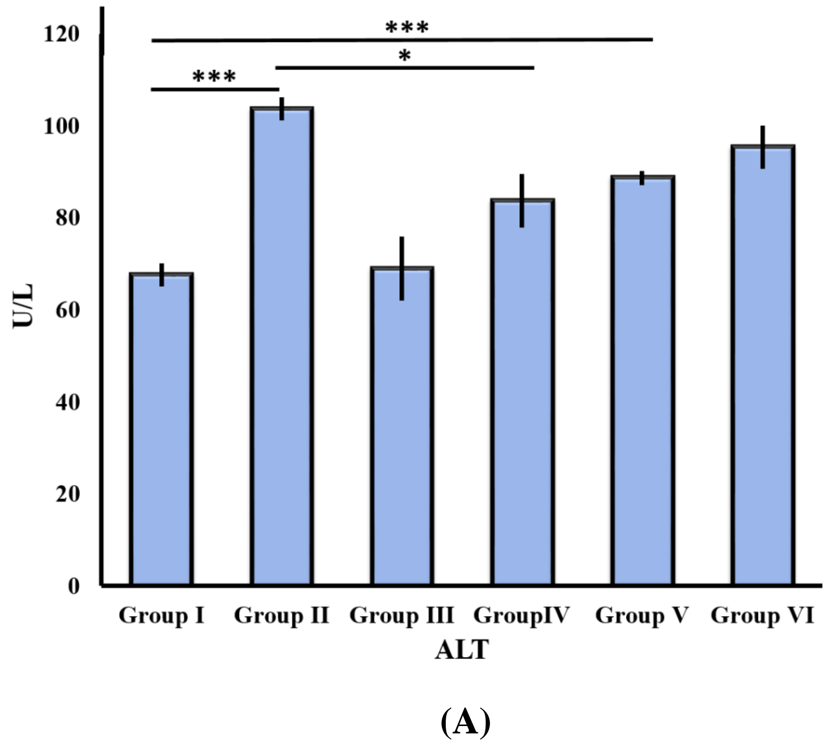
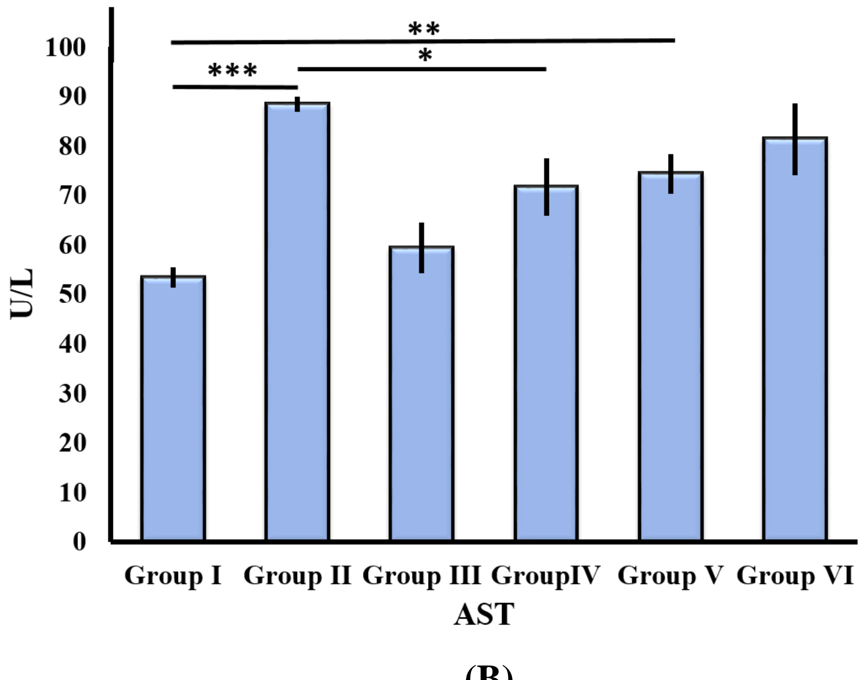


Figure 4.11. Graph showing the effect *C. angustifolia* methanol and ethyl acetate extracts on the biochemical parameters of $NaNO_2$-induced hepatotoxic mice liver. (A) ALT (Alanine Amino transferase). (B) AST (Aspartate Aminotransferase). Group I: Control group, Group II: $NaNO_2$ treated group, Group III: *C. angustifolia* methanol extract treated group, Group IV: $NaNO_2$ + *C. angustifolia* methanol extract treated group, Group V: *C. angustifolia* ethyl acetate extract treated group, Group VI: $NaNO_2$ + *C. angustifolia* ethyl acetate extract treated group. Each point represents the mean of three experiments. Data are expressed as mean ± standard deviation (*), (**), (***) significant at level $P \leq$ 0.05, 0.01 and 0.001, respectively.



(A)



(B)

Figure 4.12. Graph showing the effect *T. foenum-graecum* methanol and ethyl acetate extracts on the biochemical parameters of $NaNO_2$-induced hepatotoxic mice liver. (A) ALT (Alanine Amino transferase). (B) AST (Aspartate Aminotransferase). Group I: Control group, Group II: $NaNO_2$ treated group, Group III: *T. foenum-graecum* methanol extract treated group, Group IV: $NaNO_2$ + *T. foenum-graecum* methanol extract treated group, Group V: *T. foenum-graecum* ethyl acetate extract treated group, Group VI: $NaNO_2$ + *T. foenum-graecum* ethyl acetate extract treated group Each point represents the mean of three experiments. Data are expressed as mean ± standard deviation (*), (**), (***) significant at level $P \leq 0.05$, 0.01 and 0.001, respectively.

4.9 Bioactivity guided Screening of active compounds from *C. angustifolia* and *T. foenum-graecum*

4.9.1 HPLC-MS screening of active compounds from *C. angustifolia*

Bioactivity-guided screening of *C. angustifolia* methanol, ethyl acetate extracts by HPLC-MS revealed the presence of one novel compound named Irizoflavan, and three known bioactive compounds: Quercimeritrin, Scutellarein, and Rutin. HPLC-MS chromatograms of *C. angustifolia* extracts and compounds were shown in Fig. 4.13, 4.14, 4.15, 4.16, 4.17.

4.9.2 HPLC-MS screening of active compounds from *T. foenum-graecum*

Bioactivity-guided screening of *T. foenum-graecum* methanol and ethyl acetate extracts by HPLC-MS revealed the presence of two bioactive compounds: Amurensin, Cosmosiin.HPLC-MS chromatograms of *T. foenum-graecum* extracts and compounds were shown in Fig. 4.18, 4.19, 4.20.

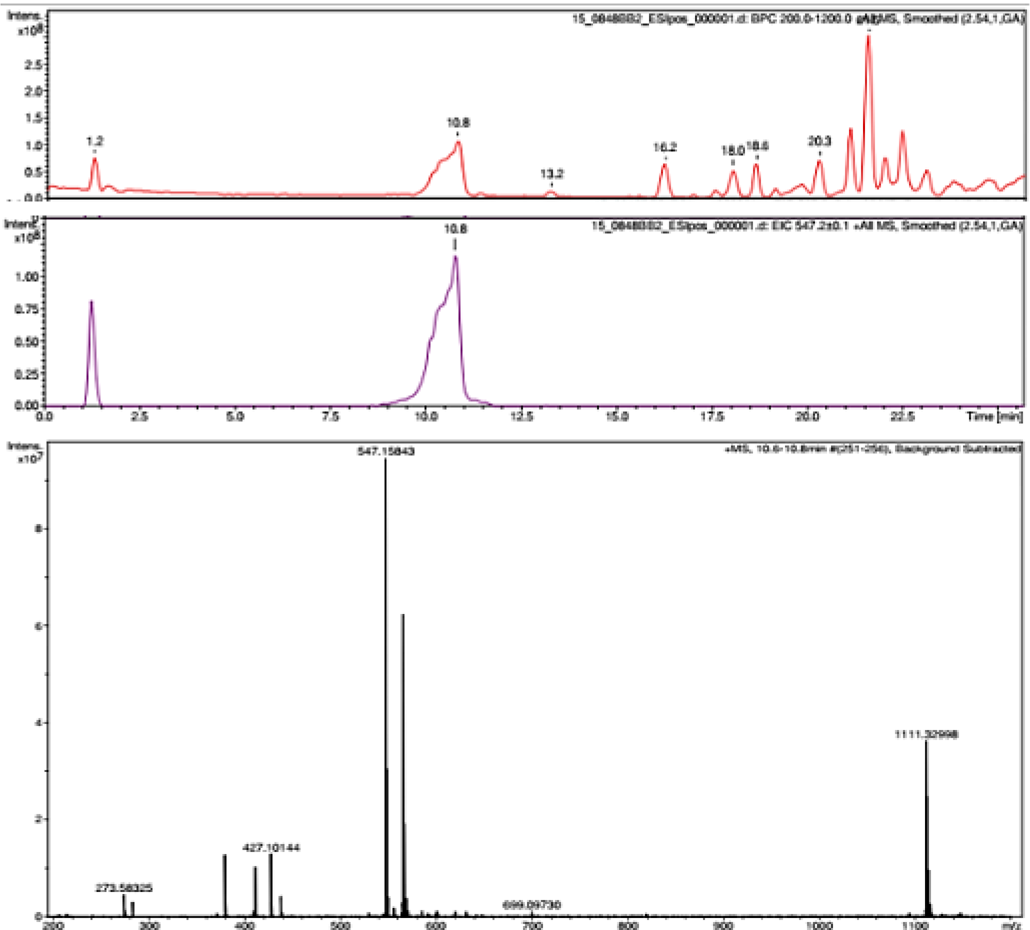


Figure 4.13. HPLC-MS chromatogram of *C. angustifolia* ethyl acetate extract and Irizoflavan.

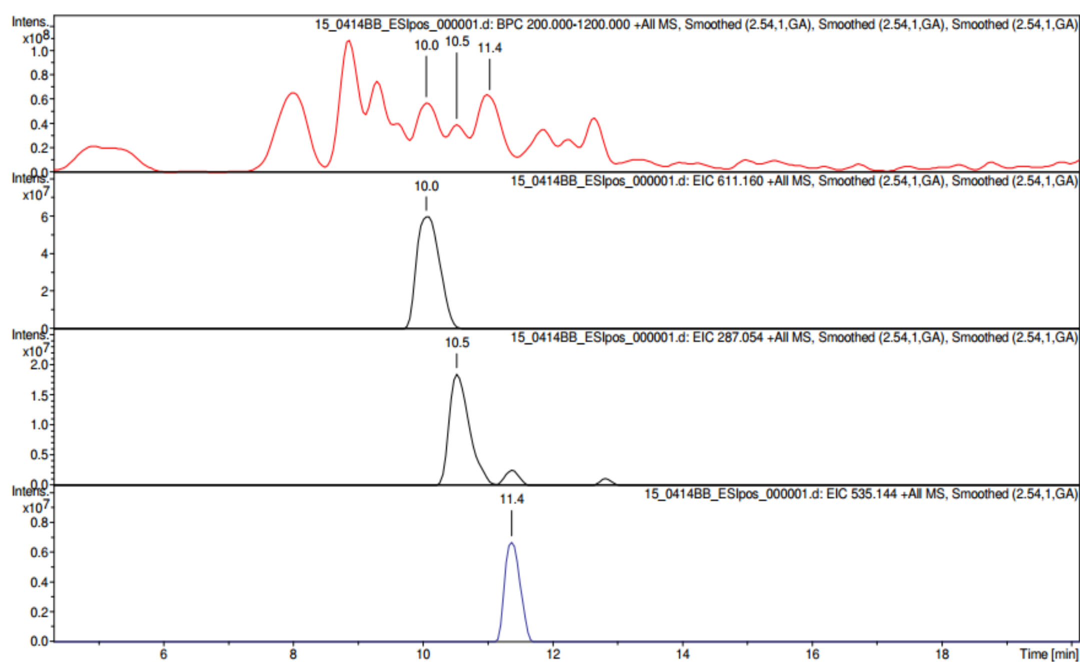


Figure 4.14. HPLC chromatogram of *C. angustifolia* methanol extracts

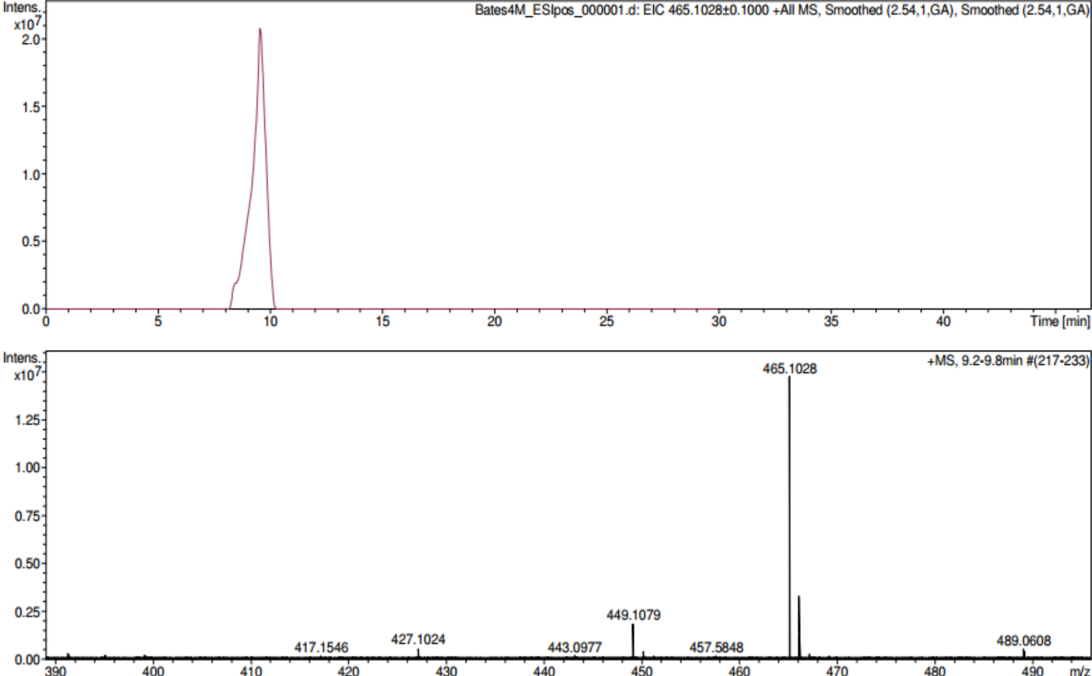


Figure 4.15. HPLC-MS chromatogram of Quercimeritrin.

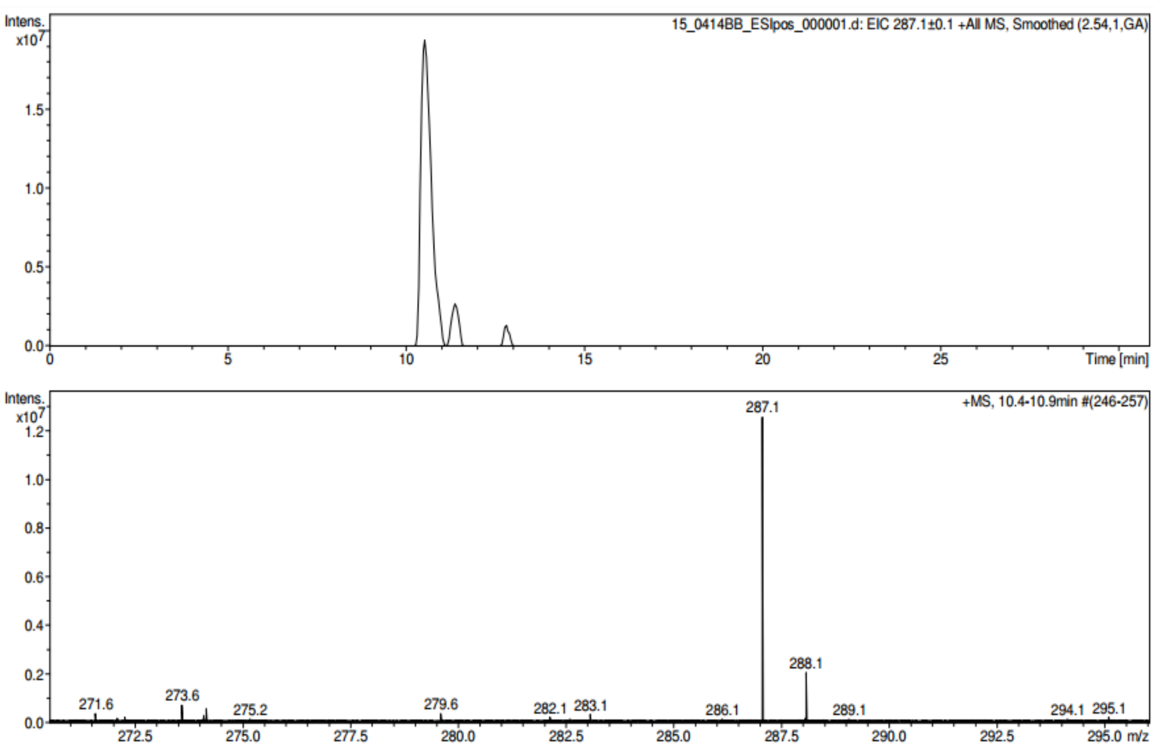


Figure 4.16. HPLC-MS chromatogram of Scutellarein.

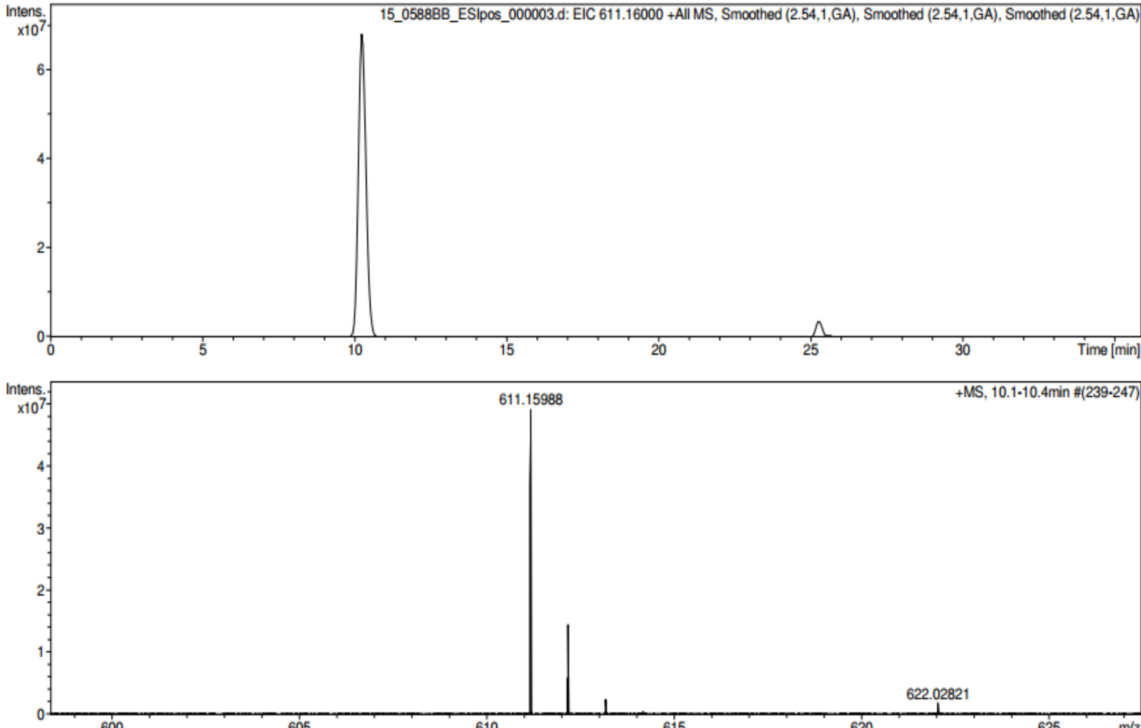


Figure 4.17. HPLC-MS chromatogram of Rutin.

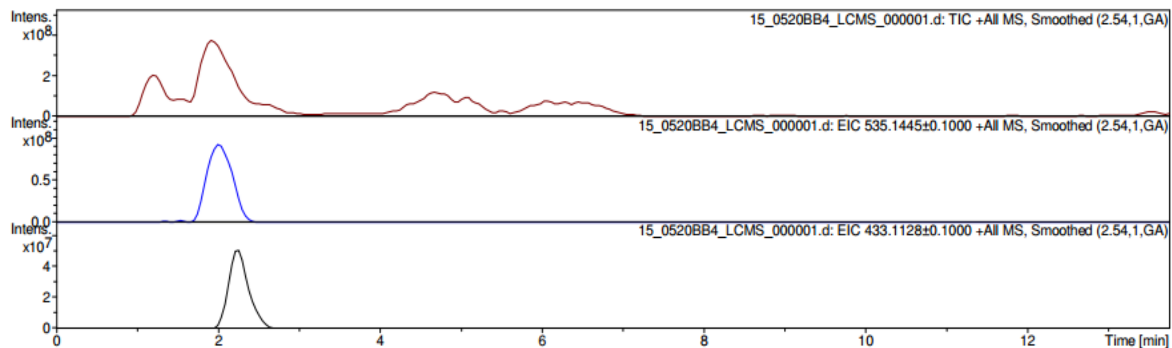


Figure 4.18. HPLC chromatogram of *T. foenum-graecum* ethyl acetate extract.

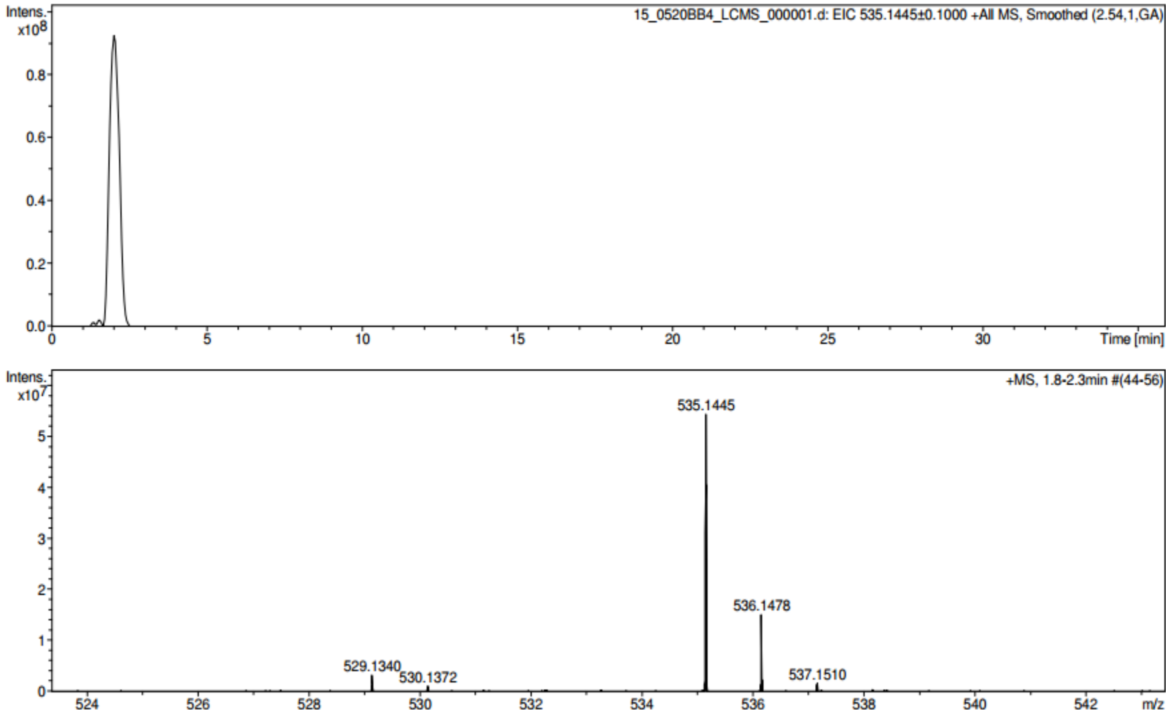


Figure 4.19. HPLC-MS chromatogram of Amurensin.

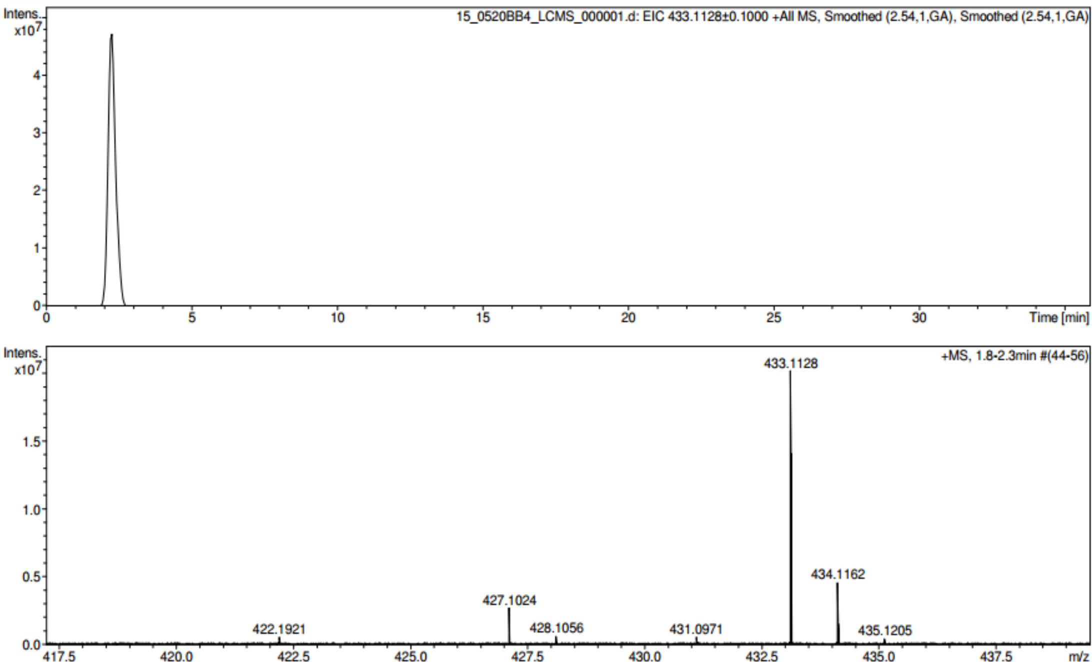


Figure 4.20. HPLC-MS chromatogram of Cosmosiin.

4.10 Identification of active compounds from *C. angustifolia* and *T. foenum-graecum*

4.10.1 ^{1}H NMR Investigation of active compounds from *C. angustifolia*

Figure 4.21: Structure of Irizoflavan

4.10.1.1 IrizoFlavan

The high-resolution ESI mass spectrum gave an $[M+H]^+$ ion with m/z 547.1584 (calc. 547.1604 for C30H27O10$^+$). The molecular formula and initial examination of ^{1}H NMR spectra suggested a dimer of catechin ($C_{15}H_{14}O_6$), with carbon 4 of ring C of one catechin moiety bonded to carbon 8 of ring A of the other.

Examination of the 1H, 13C, DQF-COSY, HSQC, HMBC and NOESY NMR data in CD_3OD solvent at 600 MHz showed the presence of two very similar molecules, 1a and 1b, each with the following structural fragments:

1) An aliphatic chain CH-CH2-CH-O. The left-hand CH has an unusually downfield 1H shift of 4.681 ppm (1a) and 4.775 ppm (1b), considering the upfield 13C shifts of 32.78

and 32.96 ppm, respectively. This suggests the magnetic anisotropy effects of two aromatic rings connected to this CH.

2) A p-hydroxyphenyl group with HMBC and NOE connections to the right-hand CH group of fragment 1.

3) A second aliphatic chain CH2-CH(-O)-CH-O;

4) A 3,4,5-hydroxyphenyl group with HMBC and NOE connection to the right-hand CH group of fragment 3. One singlet 1H peak is observed for this fragment at 6.035 ppm (1a) and 6.41 ppm (1b) with an HMBC correlation to its own (HSQC-correlated) 13C (107.91 and 107.27 ppm, respectively). This HMBC "self peak" indicates symmetry in a benzene ring. HMBC and NOE correlations are observed between this ring and the right-hand CH group of fragment 3.

5) A 1, 2, 4 - trisubstituted benzene ring (spin system CH=CH-Cq=CH) with 3J coupling of 8.3 Hz and 4J coupling of 2.5 Hz, with HMBC correlation and J coupling to the left-hand CH of fragment 1.

6) A singlet aromatic proton at 6.055 ppm (1a) and 5.91 ppm (1b), the only proton in an aromatic ring, with HMBC correlations to both aliphatic fragments 1 and 3.

^{1}H NMR (400 MHz, CD_3OD) δ: 1.787 (C3β, ddd, J=13.2, 6.1, 2.1Hz), 2.44 (F4, dd, J=16.2, 8.8 Hz), 2.64 (C3α, dt, J=13.2, 11.8 Hz), 2.85 (F4', dd, J=16.2, 5.6), 3.649 (F3, dt, J=5.9, 8.4 Hz), 4.24 (F2, br.d, J=8.1 Hz), 4.681 (C4, ddd, J =11.9, 6.2, 1.2 Hz), 6.03 (E2/6, d, J =6.6 Hz), 6.05 (D6, s),6.18 (A8, d, J =2.5 Hz), 6.24 (A6, dd, J =8.3, 2.5 Hz), 6.61 (A5, dd, J =8.5, 1.1 Hz), 6.69 (B3/5, AA' J =8.3, 2.5, 0.3 Hz), 6.99 (B2/6, BB' J =8.3, 2.5, 0.3 Hz) (Fig. 4.22).

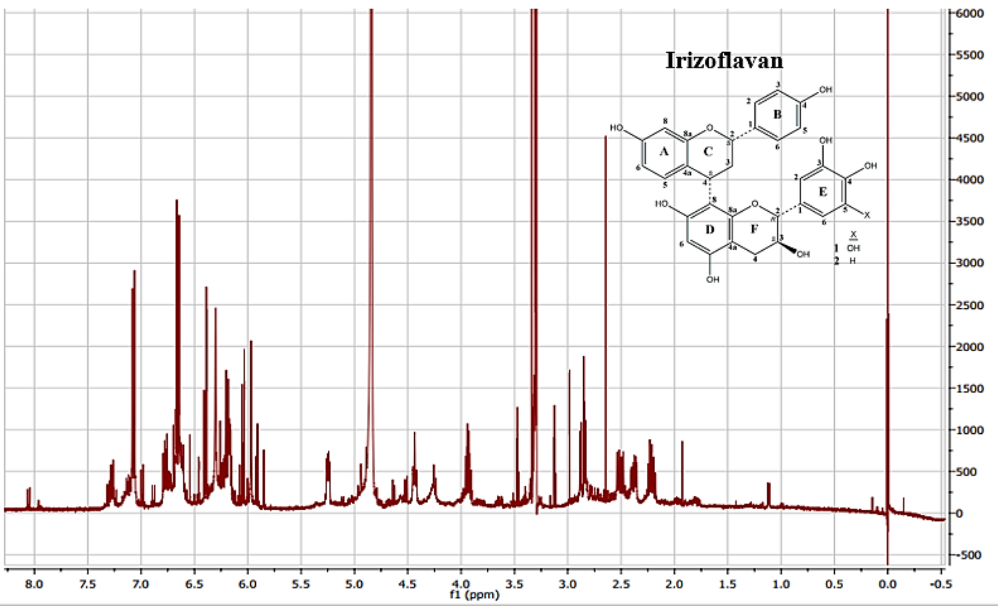


Figure 4.22.^{1}H NMR spectrum of isolated Irizoflavan from the *C. angustifolia* ethyl acetate extract

4.10.1.2 Quercimeritrin

$C_{21}H_{20}O_{12}$, yellow amorphous powder; ^{1}H NMR (400 MHz, CD_3OD) δ: 3.48-3.98 (6H, m, H-2”, H-3”, H-4”, Ha-5”, Hb-5”), 5.25 (1H, d, J=8 Hz, H-1”), 6.25 (1H, d, J=2 Hz, H-8), 6.48 (1H, d, J=2 Hz, H-6), 6.86 (1H, d, J=8.5 Hz, H-3’), 7.63 (1H, dd, J = 8.5, 2.5 Hz, H-6’), 7.67 (1H, d, J = 2.0 Hz, H-2’). ESIMS m/z 464.38 $[M]^+$, 465.38 $[M+H]^+$, 487.38$[M+Na]^+$, 463.37 $[M-H]^-$ (Fig. 4.23)

4.10.1.3 Scutellarein

$C_{15}H_{10}O_6$, reddish-brown crystals; ^{1}H NMR (400 MHz, CD_3OD) δ: 6.22 (1H, s, H-3), 6.76 (2H, d, J=8, H-3’,5’), 7.09 (2H, d, J=8, H-2’,6’), 7.11(1H, s, H-8). ESIMS m/z 286.15 $[M]^+$, 287.15 $[M+H]^+$, 309.15 $[M+Na]^+$, 285.14 $[M-H]^-$(Fig. 4.24).

4.10.1.4 Rutin

$C_{27}H_{30}O_{16}$, yellowish-brown crytals; ^{1}H NMR (400 MHz, CD_3OD) δ: 1.15 (3H, d, J=6, H-6’”), 3.27 (1H, m, H-4’”), 3.45 (1H, m, H-5’”), 3.56 (1H, dd, J=9.5/3.5 Hz, H-3’”), 3.65 (1H, dd, J=3.5/1.5, H-2’”), 3.26-3.53 (6H, m, H-2”, H-3”, H-4”, Ha-5”, Hb-5”), 5.13 (1H, d, J=7.8 Hz, H-1”), 6.24 (1H, d, J=1.8 Hz, H-6), 6.43 (1H, d, J=2.2 Hz, H-8), 6.90 (1H, d, J=8.0 Hz, H-5’), 7.65 (1H, dd, J= 8.0/1.8, H-2’), 7.69 (1H, d, J=1.8 Hz, H-6’). ESIMS m/z 610.16 $[M]^+$, 611.16 $[M+H]^+$, 633.15 $[M+Na]^+$, 609.15 $[M-H]^-$(Fig. 4.25).

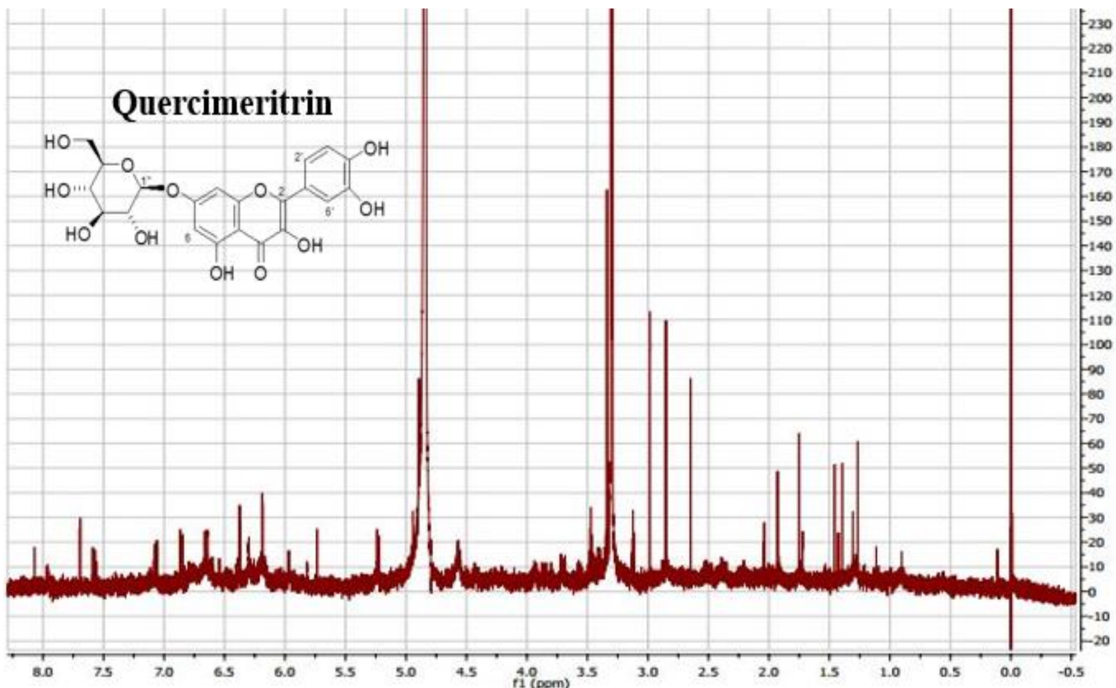


Figure 4.23.^{1}H NMR spectrum of isolated Quercimeritrin from the *C. angustifolia* methanol extract.

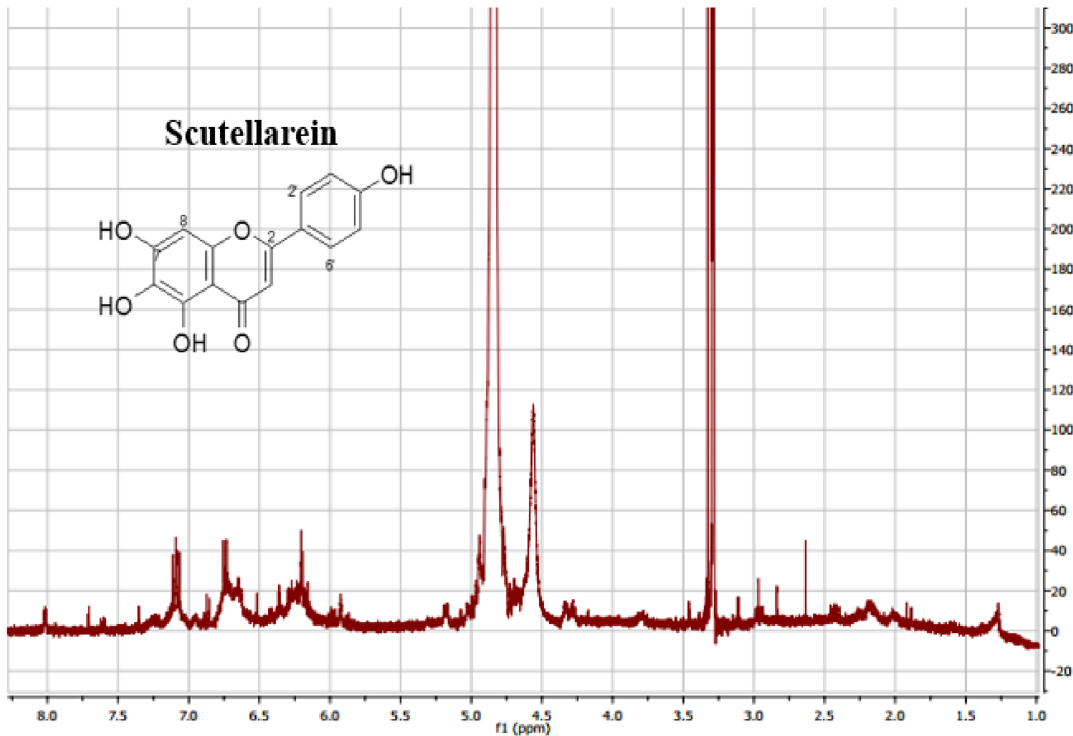


Figure 4.24.^{1}H NMR spectrum of isolated Scutellarein from the *C. angustifolia* methanol extract.

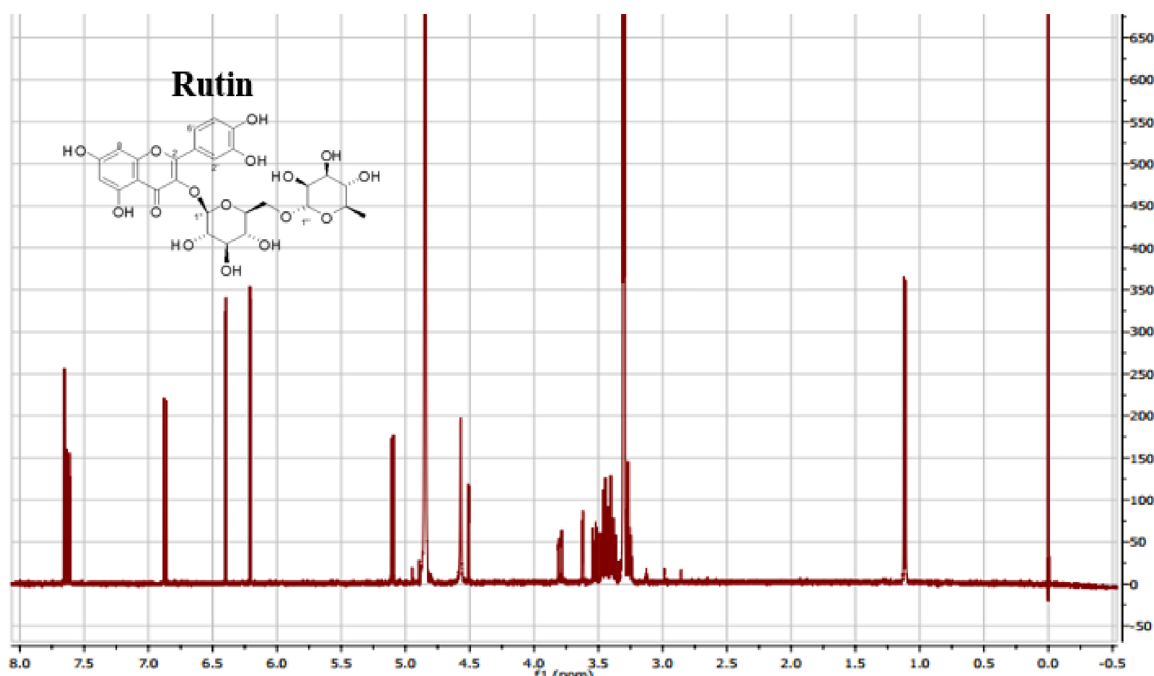


Figure 4.25.^{1}H NMR spectrum of isolated Rutin from the *C. angustifolia* methanol extract.

4.10.2 ^{1}H NMR Investigation of active compounds from *T. foenum-graecum*

Amurensin was isolated from methanol and ethyl acetate extract as amorphous yellow powder.

4.10.2.1 Amurensin

Molecular formula $C_{26}H_{30}O_{12}$ was assigned based on ESIMS m/z ESIMS m/z 534.15 $[M]^+$, 535.15 $[M+H]^+$, 557.16 $[M+Na]^+$, 533. 15 $[M-H]^-$. ^{1}H NMR (400 MHZ, CD_3OD) δ: 1.35 (3H, s, H-4”), 1.95 (3H, s, H-5”), 3.70 (2H, m, H-1”), 5.30 (1H, br t, J= 6.8, H-2”), 5.13 (1H, d, J=7.8 Hz, H-1”’), 3.4-4.10 (6H, m, sugar protons), 6.67 (1H, s, H-6), 7.0(2H, d, J=8.8 Hz, H-3’, H-5’), 7.8 (2H, d, J= 8.8 Hz, H-2’, H-6’) (Fig. 4.26).

Cosmosiin, an apigenin flavone glucoside, was isolatedas an amorphous yellow powder.

4.10.2.2 Cosmosiin

Molecular formula $C_{21}H_{20}O_{10}$ was assigned by ESIMS m/z 432.387 $[M]^+$, 433.387 $[M+H]^+$, 455.387 $[M+Na]^+$, 431.387 $[M-H]^-$. 1H NMR (400 MHZ, CD_3OD) δ: 5.00 (1H, s, H-3), 5.20 (1H, d, J=8 Hz, H-1”), 6.20 (1H, d, J=2 Hz, H-8), 6.61 (1H, d, J=2 Hz, H-6), 6.96 (2H, d, J=8, H-3’,5’), 7.75 (2H, d, J=8, H-2’,6’) (Fig. 4.27).

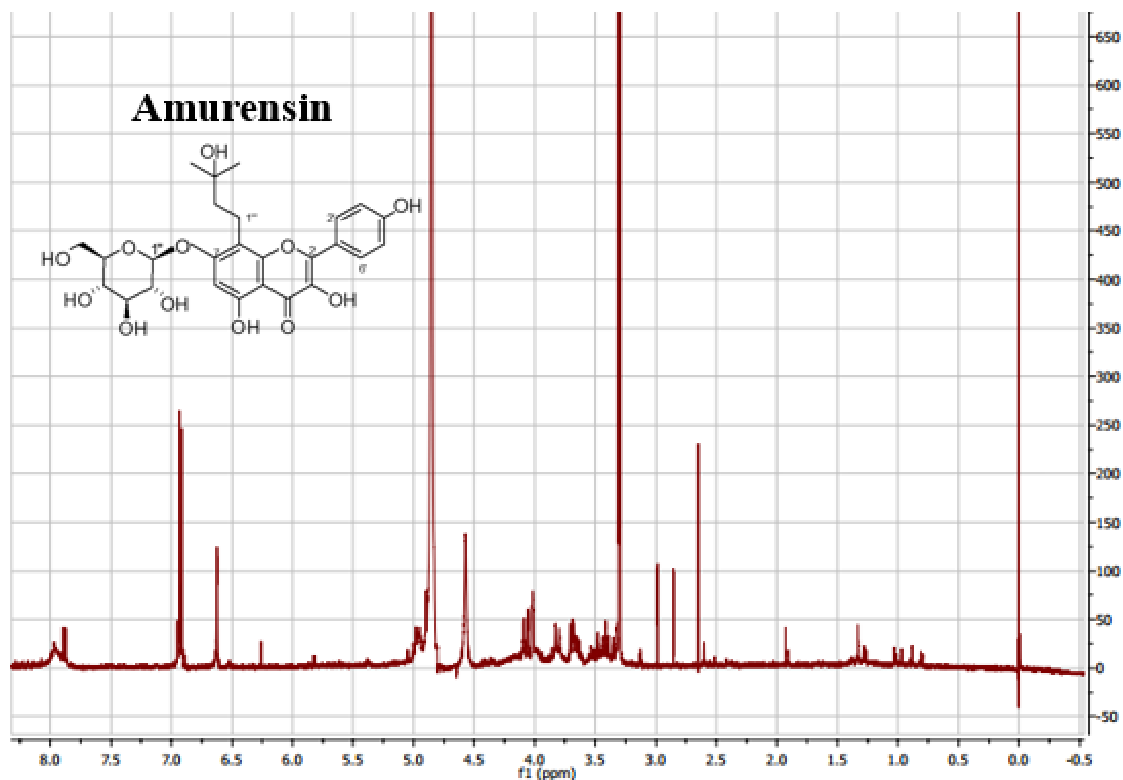


Figure 4.26.^{1}H NMR spectrum of isolated Amurensin from the *T. foenum-graecum* ethyl acetate extract.

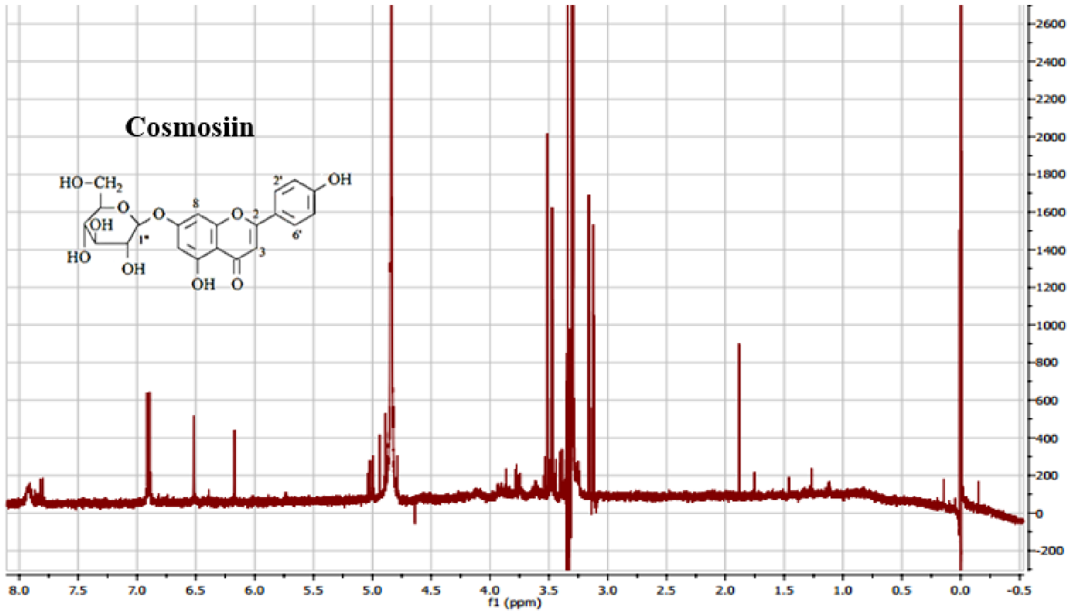


Figure 4.27.^{1}H NMR spectrum of isolated Quercimeritrin from the *T. foenum-graecum* ethyl acetate extract.

5. Discussion

Medicinal plants have been a major source of biologically active natural products and are used as medicines to cure disases in all cultures since ancient times (Ahmed *et al.,* 2014; 2015). In the present study, two medicinal plants i.e. *C. angustifolia* and *T. foenum-graecum* were taken from Prophetic medicines to explore their pharmacological basis.

The qualitative phytochemical screening of *C. angustifolia* and *T. foenum-graecum* aqueous and organic extracts revealed the presence of secondary metabolites such as steroids, alkaloids, terpenoids, anthraquinones, saponins, cardiac glycosides, coumarins, flavonoids, phenols and tannins responsible for therapeutic and physiological effects on human health. It was observed from the presence study that, in *C. angustifolia,* steroids were detected in the ethyl acetate, acetone, ethanol, methanol, and aqueous extracts, whereas, in *T. foenum-graecum*, steroids were only detected in aqueous, methanol and ethanol extracts. Steroids are the most important secondary metabolites with four carbon rings, forming the backbone of the structure. Different chemical compounds attached to the carbon backbone forms different types of the steroidal compounds such as steroidal alkaloids, brassinosteroids, sapogenins or steroidal saponins, cucurbitacins, bufadienolides, withasteroids etc. (Patel and Savjani, 2015). Steroids are also known to be involved in various pharmacological activities such as hepatoprotective, immunosuppressive, antihelminthic, antitumor, antibacterial, and cardiotonic activity (Alexei *et al.,* 2009). The current results of phytochemical screening of *C. angustifolia* are in accordance with the study conducted by Singanaboina *et al.,* 2014. Their results showed that steroids were detected in methanol, ethanol, acetone, chloroform and petroleum ether extracts of *C. angustifolia*. On the other hand, it was reported that *T. foenum-graecum* is also an important biological source for the isolation of steroidal saponins. Presence of steroidal saponins such as diosgenin, dioscin, protodioscin, yamogenin, sarsasapogenin, tigogenin,

neotigogenin, gitogenin, and yuccagenin in hydroalcoholic extracts indicates the therapeutic significance of *T. foenum-graecum* (Taylor *et al*., 2000).

Similarly, phytochemical screening revealed the presence of alkaloids in ethyl acetate, ethanol, and methanol extracts of *C. angustifolia,* whereas the ethyl acetate, acetone, and ethanol extracts of *T. foenum-graecum* showed high concentration of alkaloids which may be responsible for various pharmacological activities. Alkaloids are derived from amino acids containing nitrogen atom as a building block. Kaur *et al.* (2015) reported that approximately 12,000 alkaloids have been isolated from different species of medicinal plants, which are known to involve in various biological activities. They have been reported to possess antimalarial, antispasmodic, antibacterial, anti-inflammatory, and anticancer activities (Augusto *et al.,* 2011; Dua *et al.,* 2013; Benbott *et al.,* 2012). Mandegary *et al.* (2012), revealed the presence of alkaloids in the alkaline chloroform fraction of *T. foenum-graecum* which exhibited antinociceptive activity. Different alkaloids with promising pharmacological activities such as trigonelline, choline, carpaine, gentianine neurine, betaine have been isolated from *T. foenum-graecum* (Yoshinari and Igarashi, 2010; Kaviarasan *et al.,* 2007; Patil and Jain, 2014; Kokate *et al.,* 2007; Sultana *et al.,* 2016).

Preliminary phytochemical analysis showed that terpenoids were observed in all the extracts of *C. angustifolia* and *T. foenum-graecum* except ethyl acetate extract. Terpenoids are one of the largest class of natural compounds consisting approximately 40,000 active compounds (Goto *et al.,* 2010). Classic example of terpene-based drugs are taxol and artimesinin. Terpenoids consist of two five carbon building blocks, classified as monoterpenes, diterpenes, sesquiterpenes and sesterterpenes. They have been widely consumed as remedy against various human diseases because of their anticancer, anti-inflammatory, antiseptic, antimalarial, and antimicrobial activities. Therefore, the detected terpenoids in all the extracts could contribute their therapeutic properties to plants.

Moreover, coumarins and saponins were detected in the ethanol, methanol, and aqueous extracts of *C. angustifolia*. Previously, it has been reported that Warfarin, an active compound, isolated as coumarin from *C. angustifolia* have anti-coagulant activity (Soyuncu *et al.,* 2008). Khan and Srivastava, 2009 isolated from the butanolic extracts of *C. angustifolia,* a novel triterpenoid saponin called oleanen which exhibited antifungal activity against *Colletotrichium dematium*. Hence, based on accumulated evidences it may be hypothesized that *C. angustifolia* may play its role as anti-coagulant activity due to the presence of coumarins and saponins. On the other hand, coumarins were only detected in aqueous extract and saponins were detected in aqueous, methanol and ethanol extracts of *T. foenum-graecum*. Scopoletin, a coumarin known to possess anticholinesterase, anti-asthmatic, anti-inflammatory, bacteriostatic activities, has been isolated from *T. foenum-graecum*. In another study, Ouzir *et al.* (2016), reported a saponin fenugreekine from *T. foenum-graecum.* Therefore, the isolated compounds as coumarins and saponins impart potential pharmacological properties to *T. foenum-graecum.*

Current findings revealed the prescence of cardiac glycosides in all the understudy ethyl acetate, acetone, ethanol, methanol, and aqueous extracts of *C. angustifolia* and *T. foenum-graecum*. Cardiac glycosides comprise large family of natural products. It consists of steroidal framework, forming the central moiety of the structure, responsible for various pharmacological actions. They are involved in the treatment of cancer, atrial fibrillation, flutter and congestive heart failure (Prassas and Diamandis, 2008).

C. angustifolia aqueous, methanol, and ethanol extracts are rich source of anthraquinones. Anthraquinone derivatives are the most frequent active constituent present in the leaves of *C. angustifolia*. It contains rhein, aloe-emodin, physcion, chrysophanol and their glycoside compounds. The most important anthrquinones are sennoside A and sennoside B which were isolated in crystalline form from the leaves of *C. angustifolia* by Stoll *et al.* (1949). The leaves also contain sennoside C and sennoside D which are hetero-dianthrones with the

respective aglycones rhein & aloe emodin (Ramchander *et al.,* 2017). In *T. foenum-graecum*, anthraquinones were detected in methanol, ethanol, acetone and ethyl acetate extracts accounting for various biological activities. Khalil *et al.* (2015), reported the presence of anthrquinone obtusifolin in the GC-MS analysis of methanolic extract of *T. foenum-graecum.*

It was observed that Phenols were detected in all the understudy extracts of *C. angustifolia* and *T. foenum-graecum.* The findings showed that the TPCof ethyl acetate and methanol extracts were higher than those of ethanol, acetone and aqueous extracts. It may due to the high polarity of phenolic compounds extracted in all the solvents. Phenolic compounds contributed therapeutic values to the plant which in human bodies produce specific physiological actions. Different medicinal properties linked to phenolic compounds includes antimutagenic, anticarcinogenic, anti-inflammatory, antiartherogenic, antithrombotic, antimicrobial, and antioxidant activities (Nakamura *et al*., 2003; Tapiero, 2002; Middleton *et al.,* 1998). Phenolic compounds are characterized by aromatic hydroxyl groups and function as antioxidants by quenching free radicals by donating protons (Khan *et al.,* 2012; Kaur and Mondal, 2014; Choudhary and Swarnkar, 2011).

Flavonoids found in all parts of the plant. It consists of two benzene rings with fifteen carbon atoms link through heterocyclic pyrane ring. Based on the position of hydroxyl ring and functional group on benzene ring flavonoids are classified as flavan, flavones, flavonols, flavanones and others (Middleton, 1998). Accumulated evidence has discovered the therapeutic effcts associated with naturally occuring flavonoids. Wide range of biological activities are associated with flavonoids including anti-inflammatory, anti-allergic, antithrombotic, anti-microbial, anti-tumor, antioxidant, neuroprotective and hepatoprotective (Kumar and Pandey, 2013). Phytochemical screening revealed the presence of flavonoids in all the extracts of *C. angustifolia* and *T. foenum-graecum.* It has also been observed in the current study that the methanol extracts of *C. angustifolia* and *T. foenum-graecum* have significantly

($P \leq 0.001$) higher content of total flavonoid content i.e. 5.00±0.04 and 1.24 ±0.03 respectively, as compared to the other extracts. The lowest flavonoid content was observed in aqueous extracts of *C. angustifolia* and *T. foenum-graecum* (1.29 ± 0.03 and 0.422 ± 0.323). It was reported earlier by Esmaeili *et al.* (2015) that the extraction yield of flavonoid contents was observed more in methanol extracts because flavonoid compounds are more soluble in methanol as compared to any other solvents. Furthermore, screening for bioactive compounds revealed the presence of one novel compound Irizoflavan, and three known flavonoids from *C. angustifolia* i.e. Quercimeritrin, Scutellarein, Rutin and two known flavonoids from *T. foenum-graecum* i.e. Amurensin and Cosmosiin reported first time from these plants.

After qualitative phytochemical screening, both plant extracts were investigated to find out their different pharmacological activities such as antibacterial, antioxidant, anticancer, neuroprotective and hepatoprotective effects.

In recent years, there has been an alarming increase in the antibiotic resistance to a broad range of human pathogenic bacterial strains which contribute to the recurrence of infectious diseases. Urinary tract, bloodstream, and respiratory organs are some of the common sites of infections caused by antibiotic resistance microbes throughout the world (Sharma *et al.,* 2005). Multidrug-resistant bacterial strains are the main cause of hospital acquired infections which reduce the efficacy of drugs and are ultimately responsible for treatment failure (Djeussi *et al.,* 2013). This situation has created a need to find more effective drugs. Natural products from microorganisms have been the primary source of antibiotics, and with the increasing acceptance of herbal medicines, the screening of medicinal plants for new active compounds has become a very important source of novel antibiotics (Adedapo *et al.,* 2008). Bactericidal potential of *C. angustifolia* and *T. foenum-graecum* extracts were investigated against five pathogenic bacterial strains. Several studies reported that *A. junii*, *S. mercescens*, *E. cloacae*, *P. aeroginosa*, and *S. typhi* have been implicated in the pathogenesis of various

infectious diseases (Sanches *et al.,* 1998). Among the extracts, the methanolic extract of *C. angustifolia* and *T. foenum-graecum* displayed the broad-spectrum antibacterial activity against all the pathogenic bacterial strains except against *E. cloacae*. It was reported that the bactericidal activity was due to the presence of flavonoids found in the methanol extract (Das *et al.,* 2014). Bameri *et al.* (2013) reported that methanol and ethanol extracts of *C. angustifolia* possess antibacterial activity against *E. coli*, *Klebsiella pneumoniae* and *Shigella shinga*. In another study, it was reported that the n-butanol extract of *C. angustifolia* showed maximum antibacterial potential against *S. aureus* and *typhi* with 13 and 15 mm zones of inhibition, respectively (Gnanavel *et al.,* 2012). However, similar study conducted by Sharma *et al.* (2017) confirms the antibacterial activity of *T. foenum-graecum* aqueous, methanol and acetone extracts against *E. coli* and *Staphyloccocus*. The findings of the present study suggested that methanol, ethanol and ethyl acetate extracts are rich in flavonoids which are responsible for antibacterial activities. Flavonoids including rutin have been reported to have antimicrobial activities against resistant bacterial strains (Priya and Anil, 2013). The aqueous extract of *C. angustifolia* and *T. foenum-graecum* was not active at the highest concentrations tested against *A. junni*, *E. cloacae*, S. *typhi* and *P. aeroginosa.* It was reported that this was due to the lower extraction of antimicrobial compounds into the aqueous extract or to minimum availability of the aqueous extract to the microorganism (Ishtiaq *et al.,* 2013). The present study revealed that *A. junii*, *S. mercescens* and *P. aeroginosa* show resistance against the standard antibiotics amikacin and cefepime, while these bacterial strains showed sensitivity to *C. angustifolia* and *T. foenum-graecum* extracts.

There has been a greater interest in the natural antioxidants, present in fruits, vegetables, juices and medicinal plants. The antioxidant activity of plant extracts is largely due to the presence of secondary metabolites which can scavenge free radicals or reactive oxygen species, which in human body responsible for several chronic degenerative diseases like heart diseases,

liver diseases and cancer (Hoye *et al.,* 2008). Natural antioxidants of plant origin are usually more potent and beneficial than synthetic antioxidants such as propylgallate (PG), butylated hydroxy toluene (BHT), t-butyl hydroxy toluene (TBH) and butylated hydroxyanisole (BHA) (Sherwin, 1990). It was also reported that synthetic antioxidants were the cause of carcinogenesis and liver damage in laboratory animals (Kumar and Singh, 2011). Thus, there is a need to explore and develop antioxidants of natural origin with greater efficacy and fewer side effects. In the present study, antioxidant activities of *C. angustifolia* and *T. foenum-graecum* extracts were evaluated by DPPH assay. DPPH (α,α-diphenyl-β-picrylhydrazyl) assay is based on the electron donating capability of natural antioxidant to donate electron or hydrogen radical to the DPPH radical. It is deep violet colored solution when it reacts with antioxidant agent, it gets reduced to α,α-diphenyl-β-picrylhydrazine, as it is scavenged by the antioxidant the solution decolorized into light yellow color. The degree of change in color is directly proportional to the concentration of antioxidant which is measured spectrophotometrically (Nunes *et al.,* 2012). Lower absorbance means greater scavenging potential of the antioxidant (Krishnaiah *et al.,* 2011). In the present study, among the tested *C. angustifolia* extracts, methanol, ethanol, ethyl acetate and acetone extracts exhibited significantly ($P \leq 0.001$) higher scavenging percentage and are positively correlated with total phenolic and flavonoid contents. Phenolic and flavonoid compounds are derived from tyrosine and phenylalanine which contributes towards antioxidant activities (Montoro *et al.,* 2005). Isolated flavonoids from *C. angustifolia* i.e. quercimeritri, scutellarein, and rutin reported to have significant antioxidant activities against oxidative stress (Siraichi *et al.,* 2013; Jianxiong *et al.,* 2008; Yokozawa *et al.,* 1998). Note that all three have 1,2-dihydroxybenzene groups which are readily oxidized to orthoquinones, making them strong antioxidants. The observed antioxidant activity of *C. angustifolia* extracts might due to the presence of these active compounds. Similar findings were observed when compared with the study conducted by

Laghari *et al.* (2011). There findings showed that *C. angustifolia* leaf and flower extracts exhibited strong antioxidant activities. On the other hand, *T. foenum-graecum* ethyl acetate and methanolic extracts possess relatively higher antioxidant activities as compared to other extracts. Study conducted by Bukhari *et al.* (2008) confirms the antioxidative activity of *T. foenum-graecum* methanol, ethanol and ethyl acetate extracts by DPPH assay. In another study, it was reported that *T. foenum-graecum* protects cellular damage caused by oxidative stress due to its antioxidant activity by scavenging hydroxyl radical (-OH) (Kaviarasan *et al*., 2007). Amurensin, a flavanol derivative of kaempferol 7-O-glucoside, has been reported to show antioxidant properties through DPPH radical scavenging activity (Singh *et al.,* 2014; Leu *et al.,* 2006). Cosmosiin an apigenin flavone glucoside, exhibited significant antioxidant activities through DPPH and ABTS radical scavenging activity (Wang *et al.,* 2010; Mikhaeil *et al.,* 2004; Petrichenko *et al.,* 2004). From the results, it was also observed that the aqueous extracts of both the plants showed poor DPPH-scavenging activity. This is because the flavonoids and phenols responsible for antioxidant activity are poorly extracted into the aqueous extract (Ao *et al.,* 2008).

Cancer is the second largest cause of death worldwide (Desai *et al.,* 2007). Although great advancements have been made in the treatment and control of cancer progression, significant deficiencies and room for improvement remains. Several undesired side effects sometimes occur during chemotherapy. Natural therapies, such as the use of plant-derived products in cancer treatment, may reduce adverse side effects. There are many natural products including phytochemicals and dietary compounds from vegetables, plants, spices and herbs that have been used for the treatment of cancer throughout history due to their safety, low toxicity, and general availability (Elkady *et al.,* 2012). Reserveratol, taxol, vincristine, lovastatin, calceorioside, paclitaxel, vitamins A and C, and curcumin are some of the compounds isolated from medicinal plants which possess anticancer activities (Ali *et al.,* 2012).

The anticancer activity through MTT assay of aqueous and organic extracts of *C. angustifolia* and *T. foenum-graecum* in Hep2, Hela, MCF-7, and HCEC cell lines were evaluated in the present study. No detailed anticancer study of *C. angustifolia* has been reported earlier. It was revealed that only methanol and ethanol extracts of *C. angustifolia* exhibit anticancer properties. On the other hand, anticancer activity of *T. foenum-graecum* extracts revealed that the methanol and ethyl acetate extracts of *T. foenum-graecum* inhibit the proliferation of cancer cell lines. These observations were supported by previous studies which reported the anticancer activity of *T. foenum-graecum* oil against T-cell and B-cell lymphomas, breast cancer (MCF-7), Thyroid Papillary carcinoma (FRO) (Abdulaziz *et al.,* 2014). In another study, the cytotoxic potential of *T. foenum-graecum* methanol extract was reported against HepG2 cell line (Mahmoud *et al.,* 2015). It was reported that secondary metabolites like flavonoids can be responsible for anticancer activities (Priya and Anil, 2013). Based on these findings, it was assumed that the anticancer activities of *C. angustifolia* and *T. foenum-graecum* extracts was due to the presence of isolated flavonoids. It was investigated earlier that scutellarien, which in present study isolated from *C. angustifolia*, extracted from *Scutellaria lateriflora,* possesses anticancer activity by significantly suppressing the proliferation of HT1080 human fibrosarcoma cells through induction of apoptosis (Didem *et al.,* 2010). In a similar study, it was revealed from the in vivo experiment that that the size and weight of the tumor was reduced after treatment with scutellarein (Xiujuan *et al.,* 2015). Moreover, rutin (**3**) has been reported as anticancer agent by inducing apoptosis and cell cycle arrest in murine leukemia WEHI-3 cells (Lin *et al.,* 2012). It was also reported that rutin (**3**) had the potential to kill the breast cancer cells in MDA-MB-231 cell line (Aliye *et al.,* 2014). On the other hand, cosmosiin has the potential to inhibit the growth of cancer cells in HepG2 and MCF-7 cell lines (Chiu *et al.,* 2005; Yao *et al.,* 2011). In an empirical study, molecular docking revealed that cosmosiin have high binding affinity with Bcl-X_L with a dissociate constant (Kd) values < 10 mM, which

contributed toward its anticancer activity (Chen *et al.,* 2014). According to CHMIS-C, cosmosiin is considered as an active anticancer compound having $GI_{50} < 1$ µm, when screened against NCI's human cancer cell lines (Fang *et al.,* 2005).

Many natural and synthetic food additives have deleterious effects on human health and are not easily excreted from the body (Hassan *et al.,* 2009). $NaNO_2$ is an inorganic salt used as a color fixative, food additive, and preservative in canned foods. Although the wide use of $NaNO_2$ in food industry improves the food quality, shelf life, taste and texture but it also has hazardous effects on human health. It was documented that $NaNO_2$ on reaction with amines and amides in food produces free radicals, nitrosamines and nitrosamide which are associated with cancer, nephrotoxicity, hepatotoxicity, dysregulation of inflammation and tissue injury, and endocrine disturbance (Choi *et al.,* 2002). Dietary intake of natural compounds could serve as a source of prevention against biochemical alterations and diseases associated with free radicals like hepatic and neurological impairment. Therefore, the hazardous effects of $NaNO_2$ on human health elevates the importance of studying its role in the induction of oxidative stress in mice leading to neuronal degeneration and hepatotoxicity, in addition to evaluating the protective role of *C. angustifolia* and *T. foenum-graecum* extracts in amelioration of neuronal and hepatic damage. The observations of the present study are in consistent with the earlier studies carried out to determine the negative effects of $NaNO_2$ on cell membrane by inhibiting membrane bound enzymes in rat brain (Zargar, 2014). It was also reported that $NaNO_2$ caused liver damage by increasing the level of liver enzymes 35 to 40 times compared to normal level (Sumi and Miyakawa *et al.,* 1983). Oral administration of $NaNO_2$ for 15 days induced neuronal and hepatic damage in the mice, indicated by histopathological and biochemical examination compared to the control group. Significant increase in the level of liver enzymes i.e. ALT, AST and neurodegeneration in the cortex and hippocampus section of mice brain was observed in $NaNO_2$ treated group. $NaNO_2$ affects glial cells and neurons in the brain, leading to

programmed cell death (Hassan *et al.,* 2009), the pathological hallmark of many human neurodegenerative disorders. It was reported that the significant increase in cytochrome C activity (responsible for oxidative stress and apoptosis) was associated with $NaNO_2$ toxicity (Al-Gayyar *et al.,* 2014). Oral administration of methanol or ethyl acetate extracts of *C. angustifolia* and *T. foenum graecum* retrograded the pathological hallmarks back to normal when compared to the $NaNO_2$ treated group. Administration of *C. angustifolia* was previously reported to protect the neuronal cells and tissue in rat model of Parkinson's disease. Several studies conducted on the neuroprotective effect of *T. foenum graecum* in different neurotoxic models are in agreement with the present study. Moghadam *et al.* (2013) reported the protective effect of *T. foenum-graecum* in pyridoxine induced peripheral neuropathy in mice. In another study by Kumar *et al.* (2012) *T. foenum-graecum* showed neuroprotective effect in diabetic female wistar rats. Oral administration of *T. foenum-graecum* aqueous and organic extracts showed neuroprotective effect in phenobarbitone induced neurotoxicity in albino wistar rats (Natarajan *et al.,* 2007).

To investigate the hepatoprotective activity in the present study, treatment of *C. angustifolia* and *T. foenum-graecum* methanol and ethyl acetate extracts were co-administered with $NaNO_2$. Significant decrease in the elevated levels of ALT and AST to normal level as compared to $NaNO_2$ treated group were observed. The hepatoprotective effect of *C. angustifolia* agree with the previous study, proving that *C. angustifolia* alcoholic extract showed protective effect against Carbon tetra chloride induced hepatotoxicity (Ilavarasan *et al.,* 2001). *T. foenum-graecum* has hepatoprotective role in mice under lithogenic conditions (Reddy and Srinivasan, 2011) against thioacetamide induced hepatotoxicity in rats (Das, 2014), and against CCl_4 induced toxicity in wistar albino rats (Zargar, 2014). The *in-vivo* and *in-vitro* neuro and hepatoprotective effect of *C. angustifolia* and *T. foenum-graecum* may be associated

with the isolated flavonoid compounds present in methanol and ethyl acetate extracts which have been known to have hepato and neuroprotective effects.

Future Prospects

The present work provide basis for the development of plant based drugs from *Cassia angustifolia* and *Trigonella foenum-graecum* based on their activities against different diseases. Additionally, the identification of novel compound (Irizoflavan) will lead to screen its pharmacological activities and its underlying mechanism of action at molecular level.

Conclusion

It is concluded that organic extracts of medicinal plants *C. angustifloia* and *T. foenum-graecum,* taken from Prophetic medicines, exhibited strong antibacterial, antioxidant, anticancer, neuroprotective and hepatoprotective activities. These activities are correlated with the traditional use of these plants as laxative, antimicrobial, antioxidant, antidiabetic, antihyperlipidemic. A novel compound (proposed name "Irizoflavan") and three known flavonoids i.e. Quercimeritrin, Scutellarein, and Rutin were isolated from *C. angustifolia*. Two known flavonoids i.e. Amurensin and Cosmosiin were isolated from *T. foenum-graecum*.

It is also concluded that the continuous exposure of $NaNO_2$ induces neurodegeneration in brain sections, and hepatotoxicity in mice liver. The effect of $NaNO_2$ was compensated by methanol and ethyl acetate extracts of *C. angustifloia* and *T. foenum graecum.* This suggests that these extracts have the potential for providing significant neuroprotection and hepatoprotection in $NaNO_2$-induced neurodegeneration and hepatotoxicity

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APPENDICES

Appendix I (Solutions for Perfusion)

8 % Parafarmaldehyde

Paraformaldehyde	48 g
Distilled H_2O	300 ml

Monobasic

NaH_2PO_4	6 g
Distilled H_2O	250 ml

Dibasic

Na_2HPO_4	14.2 g
Distilled H_2O	500 ml

Appendix II (Solutions for MTT assay)

Solublization solution

Sodium dodecyl sulphate	10%
HCL	0.01 M

MTT

MTT	5 mg
1X Phosphate Buffer saline	1 ml

Appendix II (Publications)

Based on the study conducted for the present thesis, the following manuscripts have been published:

1. **Ishtiaq, S.,** Hayat, M. Q., Tahir, M., Mansoor, Q., Ismail, M., Keck, K., and Bates, B.R. (2016) Pharmacologically active flavonoids from the anticancer, antioxidant and antimicrobial extracts of *Cassia angustifolia* Vahl. BMC Complementary and Alternative Medicine, 16:40.
2. **Ishtiaq, S.,** Hayat, M. Q., Tahir, M., Mansoor, Q., Ismail, M., Keck, K., and Bates, B.R. (2017). Isolation of Amurensin and cosmosiin from Neuroprotective and anticancer extracts of *Trigonella foenum graceum* extracts. Tropical Journal of Pharmaceutical Sciences, 16(6): 1391-1398.