Pharmacological & Toxicological Evaluation of
*Nelumbo nucifera* Fruit

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In the Name of ALLAH

The Most Beneficent and The Most Merciful
CERTIFICATE

This thesis is accepted in its present form by the Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences in the partial fulfillment of the requirements for the Degree of PhD in Pharmacology

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This thesis is dedicated to my wonderful parents.
For their support, encouragement and endless love.
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Dr. Muhammad Ali
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<td>GABA</td>
<td>Gama amino butyric acid</td>
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<td><em>N. nucifera</em></td>
<td><em>Nelumbo nucifera</em></td>
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<td>aPTT</td>
<td>Activated partial thromboplastin time</td>
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<tr>
<td>ASA</td>
<td>Acetyl salicylic acid (Aspirin)</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular diseases</td>
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<td>EPM</td>
<td>Elevated plus maze test</td>
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<td>FST</td>
<td>Forced swimming test</td>
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<tr>
<td>SNRIss</td>
<td>Serotonin/norepinephrine reuptake inhibitors</td>
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<tr>
<td>MAOIs</td>
<td>Monoamine oxidase inhibitors</td>
</tr>
<tr>
<td>PLT</td>
<td>Platelet</td>
</tr>
<tr>
<td>PO</td>
<td>Per oral</td>
</tr>
<tr>
<td>PT</td>
<td>Prothrombin time</td>
</tr>
<tr>
<td>Rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SSRIss</td>
<td>Selective serotonin reuptake inhibitors</td>
</tr>
<tr>
<td>ECVP</td>
<td>Echis carinatus venom product</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator activated receptor</td>
</tr>
<tr>
<td>TCAs</td>
<td>Tricyclic antidepressants</td>
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<tr>
<td>VLDL</td>
<td>Very low density lipoproteins</td>
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<td>LDL</td>
<td>Low density lipoproteins</td>
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AEM aqueous extract of *Myrtus communis* L
DVT deep venous thrombosis
PE pulmonary embolism
HCD high cholesterol diet
ALT alanine aminotransferase
AST IP GFR aspartate aminotransferase Intraperitoneal
COX glomerular filtration rate cyclo-oxygenase
NSAIDS non-steroidal anti-inflammatory drugs
GIT gastrointestinal tract
Recently use of herbal therapies and diet rich in flavonoids and vitamins has augmented significantly to manage minor to modest illnesses. But further studies are necessary to assess the pharmacological and toxicological effects of plants.

Hence current study was intended to assess the pharmacologic and toxic effects of *Nelumbo nucifera* fruit ethanol extract. The qualitative phytochemical screening of the seed pods of the *N. nucifera* fruit extract exposed the existence of flavonoids, saponins, alkaloids, tannins and terpenoids in it. The acute toxicity study of the *N. nucifera* fruit extract revealed its LD$_{50}$ value to be greater than 5000 mg/kg.

*N. nucifera* fruit have exhibited strong anxiolytic, antidepressant and antiepileptic activities and that is perhaps both segments of the *N. nucifera* fruit (seed and seed pod) are rich in significant phytochemical constituents e.g. flavonoids and alkaloids. Flavonoids, saponins and tannins have also reported inhibitory effects on the arachidonic acid metabolism in various studies, hence it can be stated that the analgesic and anti-inflammatory effects of *N. nucifera* fruit may be due to the presence of these secondary metabolites in it.

Present study has also revealed lipid lowering and antithrombotic effects of *N. nucifera* fruit. The inhibitory effect on lipid auto-oxidation and free radical scavenging activity of flavonoids, tannins and procyanidin might be a possible mechanism of its lipid lowering activity, whereas inhibitory effects of flavonoids and alkaloids (neferine and liensinine) on thromboxane A$_2$ formation and platelet aggregation may be a reason of its antithrombotic effect. Present study also conducted sub-chronic toxicity studies which showed no substantial hematological, biochemical and histopathological changes in hepatic and renal tissues of most animals of treated groups, however hepatic and renal sections of few animals showed reversible mild to moderate inflammatory changes which were in agreement with biochemical alterations. Hence it can be concluded that *N. nucifera* fruit has enormous therapeutic potential and is highly safe for short term use but may be used in moderate doses if required for prolonged period; however more investigations are required in this field to confirm these findings.
خلاقانی

حالیہ سٹیشن یوپن کو سطح ایجادات اور معاشرتی افراد علمی اور هنری عالم کے سفر کے خطے کے لئے لی اہلیا کی ہندی میں نظر انداز میں مطالعہ کی ہوئی ہے۔ پچھلے دو سالوں کے سفر کے معاشرتی اور اخلاقی اساتذہ کی کامیابی کرے ہے اور یہ تمام مطالعہ کی ہوئی ہے۔

خلاقانی کی یوپن کو معاشرتی فن کی ہندی میں ایک لئے تعلیم اور تحقیق کی کہانی ہے کہ ہوئے کا کہانی ہے۔

خلاقانی کی یوپن کو سطح ایجادات اور معاشرتی افراد علمی اور هنری عالم کے سفر کے خطے کے لئے لی اہلیا کی ہندی میں نظر انداز میں مطالعہ کی ہوئی ہے۔ پچھلے دو سالوں کے سفر کے معاشرتی اور اخلاقی اساتذہ کی کامیابی کرے ہے اور یہ تمام مطالعہ کی ہوئی ہے۔

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INTRODUCTION
1.1  Traditional medicine

Traditional medicine, alternative medicine or complementary medicine are interchangeable terms and can be explained as the over-all aggregate of skills, knowledge and practices established on the beliefs, concepts and experiences subject to diverse cultures that are used in the prevention and management of physical and mental disorders (Assad and Morse, 2013).

Since ancient times, natural products have been the integral component of traditional system but their application as isolated and characterized compounds to advance drug discovery started only in the 19th century. Plant derived drugs have a long history, for the prevention and cure of various disorders and for better patient tolerance, acceptance and low health care cost (Khan et al., 2013).

The first drug introduced by Merck in 1826 for civilian use was a pure natural product, named morphine, and the first semi-synthetic agent aspirin was introduced by Bayer in 1899. This led to the discovery and isolation of many significant drugs such as codeine, digitoxin, quinine, pilocarpine, paclitaxel and artemisinin which are effective for cardiovascular disorders, infection and for cancers. Currently about 122 clinically significant drugs is derived from 94 plant species and is employed worldwide for the management of human illnesses (Fabricant and Fransworth, 2001). The World Health Organization (WHO) has given its estimation that more than 2/3rd of the global population nowadays depends on alternative sources of treatment to fulfill the basic healthcare requirements and this most importantly embroils the usage of plant products. This means that nearby 2 thirds of the people globally trust on plants as a reliable way of their medication (Fransworth, 1983).

1.1.1  Herbal medicine

An herb can be defined as any plant carrying flowers, seeds and leaves used for medicine or flavoring food or, any seed bearing plant devoid of woody stem and dies down to the ground after flowering. A medicine is a manufactured article prepared according to
officially permitted technical procedures and is used for the diagnosis and management of medical disorders and has been scientifically characterized in terms of its efficacy, safety and quality (WHO, 1992).

Herbal Medicine or Herbalism is the practice or art of employing herbs and herbal preparations in order to remain healthy and also for the treatment and improvement in prognosis of diseases (Arias, 1999). These days vigorous research is ongoing to discover nontoxic and beneficial herbs. Several anti-anxiety, anti-depressant, anti-epileptic, anti-inflammatory, anti-hyperlipidemic and antithrombotic agents are present in the medical stores none the less most of them have associations with various unwanted effects. That’s why more people are switching towards safer alternatives, specially derived from plants with fewer side effects (Rajput et al., 2012).

Herbal plants contain secondary metabolite like alkaloids, glycosides, terpene, steroids, flavonoids, tannins, and so forth. Some chemical substances are phenols or their oxygen substituted derivatives such as tannins while some may contain nitrogen or sulphur that are biologically active and useful for the prevention of disease and treatment of ailment and preserve well-being in humans and animals. Polyphenol exhibit antioxidant activity which may lead to many health benefits (Devkota et al., 2015).

Herbalism is getting more popular and is frequently practiced nowadays for the cure of hypertension, arrhythmias, pain, asthma, chronic hepatitis, climacteric disorders, headache, common cold, constipation, autonomic instability, diabetes mellitus, gastritis, bowel disorders, allergic rhinitis and even cancers (Teraswa, 1986).

The researchers and scientists are actively working to explore new and effective drugs from plants all over the world (Fransworth, 1988). A huge population all over the world is now relying on plant products for preventive and therapeutic purposes (Mentz and Schenkel, 1989). However further research is necessary to establish the significant and toxic effects of plants, thus current research was focused to ascertain the pharmacological and toxicological prospective of *Nelumbo nucifera* fruit. This study would surely help in
the exploration of a novel agent from the plant source and therefore will propose the applications of complimentary medicines in humans (Ahmed et al., 2015).

1.1.2 *Nelumbo nucifera* plant

*N. nucifera* belongs to family *Nymphaeaceae*, and is frequently named as bean of India, Chinese water lily, Indian lotus or simply lotus and synonyms (*N. speciosum, Nelumbium nucifera, N. speciosa, and Nymphaea nucifera*). The two most prevalent species of Nelumbo are *N. lutea* Wild and *N. nucifera*. According to Linnaean classification *N. nucifera* Gaertn, is at present documented name of plant species and is widely cultivated in the tropical regions of Pakistan, India, China, Thailand and Australia while *N. lutea*, water chinquapin or the American lotus, grows in regions of southern and eastern North America and is considered as a subspecies of *N. nucifera* (Mukherjee et al., 2009).

This plant is water loving perennial herb and in good conditions its seeds may survive for decades. The roots of *N. nucifera* are planted in the soil of the pond or river bottom, while the leaves float on water surface and are positioned well above it. The flowers are usually found on thick stems rising several centimeters over the leaves. The plant usually grows up to a height of 150 cm and a horizontal spread of up to 3 meters. The seeds, leaves, flowers and roots (rhizomes) are all safe to eat and are marketed (Mukherjee et al., 1996).

*N. nucifera* has spiritual importance in many religions of the world and has played historic part in holy and enlightening activities and is considered as a sign of divine beauty, purity, perfection and enlightenment. In addition to its use as an ornamental plant, it is also used as a salutary herb in regions of eastern parts of Asia specially China where its cultivation is more than 1000 years old (Paudel and Panth, 2015).

All segments of the *N. nucifera* plant such as stem, leaves, flowers, roots, seedpods and seeds have produced significant amounts of bioactive constituents like alkaloids and flavonoids (Bhat et al., 2008). Traditionally, the whole plant of *N. nucifera* is utilized as astringent, emollient and diuretic, while also used in the management of diarrhea, tissue inflammation and homeostasis. Extracts obtained from various segments of the
N. nucifera plant have demonstrated a bunch of therapeutic effects for instance free radical scavenging, anti-oxidant, immune-modulatory and anti-inflammatory (Kuo et al., 2005; Kashiwada et al., 2005).

1.1.3 Nelumbo nucifera fruit

This fruit is commonly known as Paban in native language of Sindh province of Pakistan and is cultivated especially in the water gardens, ponds and lakes of Gotki, Thatta, Larkana and Matiari districts of interior Sindh. In Muzaffergharh regions of southern Punjab, it is called as ‘Pubun de tikee’. It is also found abundantly in the lakes and streams of Azad Jammu Kashmir (AJK), Pakistan.

Fruit consists of seed pods, also known as lotus bulbs, are green in color and provides attachment to the seeds, which are black in color, hard in nature, ovoid, oblongish or roundish in shape, up to 1.5 cm broad and 1.0 cm long and are organize in whorls (Sridhar and Rajeev, 2007). The seeds are the edible portion and are studded in the pod or bulb and have to be peeled individually before they are eaten. The seed is perhaps the strongest source of protein in the plant (Carlo et al., 2013).

1.1.4 Phytochemistry of Nelumbo nucifera fruit

The N. nucifera seeds are loaded with fat, amino acid, protein, asparagines, unsaturated fatty acids, minerals, starch, saponins and tannins. The seed has three main components; cotyledons, plumule and integuments, which occupies approximately 93.23, 3.03 & 3.74% in terms of its mass correspondingly. The average burden of hundred seeds is approximately 87.35 g. A huge amount of glutathione is present in cotyledons (164g/cotyledon) and also in plumule (13g/plumule) of N. nucifera seeds. The seeds also contain substantial percentage of various minerals such as calcium (22.10%), potassium (28.5%), magnesium (9.2%), sodium (1.0%), iron (0.19%), chromium (0.004%), manganese (0.35%), zinc (0.08%) and copper (0.046%). Other significant dietary elements include fat (72.17%), proteins (2.7%), total powdery residue (4.5%), wetness (10.50%) and raw fiber (10.60%). The seed are highly nutritious and possesses 348.45 cal per 100 g (Indrayan et al., 2005).
The chief secondary metabolites present in *N. nucifera* seeds are alkaloids, particularly lotusine, liensinine, isoliensinine, dauricine, nuciferine, pronuciferine, roemerine, procyanidin, neferine and armepavine. Procyanidin was also identified and sequestered from the pods of *N. nucifera* fruit. The seeds also have carbohydrates, Gallic acid and isoquininolinol (Mukherjee et al., 2009). The polysaccharides found in seeds are primarily composed of L-arabinose, D-galactose, D-mannose, and D-glucose (Das et al., 1992). In-source pyrolysis-mass spectroscopy and $^{13}$C-NMR assessment established that the seed coat along with the fruit wall of *N. nucifera* is comprised of a complex of polysaccharides which is supported principally on insoluble tannins along with mannose and galactose units (Bergen et al., 1997).

1.1.5 Traditional & reported pharmacological effects of *N. nucifera* fruit

Traditionally fruits of *N. nucifera* are consumed as a healthy foodstuff in Asia and for the cure of numerous conditions such as fever, inflammation, insomnia, palpitation, hypertension, arrhythmia, enteritis, chronic diarrhea, spermatorrhea, leucorrhoea, halitosis, skin diseases, leprosy and menorrhagia. It is also used as an antiemetic, antidote, refrigerant and diuretic (Chopra et al., 1956; Vershney and Rzoska, 1976).

*N. nucifera* seedpods are occasionally utilized as an alternative medicine for improving hemostatic function (Ling et al., 2005). The grinded seed assorted with honey is beneficial in suppressing cough (Khare, 2004). Embryos of seeds are frequently utilized in complementary Chinese medicine to cure CNS disorders, insomnia, elevated fever plus restlessness and heart complaints (Chen et al., 2007). Condensed tannin and procyanidin separated from the pods of *N. nucifera* possesses plentiful therapeutic effects such as lipoxygenase antagonist, lipid auto-oxidation and free radical scavenging similar to butylated hydroxytoluene which is approximately 0.1% (Ling et al., 2005).
Figure-1
*N. nucifera* fruit

Figure-2
*N. nucifera* seed pods (dried and grinded form)
1.2 Purpose of study

Plant derived medicines are being used globally and extensive research is underway to explore more and more pharmacologically valuable plants with minimal toxic effects. *N. nucifera* fruit is widely grown and marketed and has many traditional uses but very limited literature is available regarding its pharmacological and toxicological effect.

Hence present study aims to evaluate:

1. Phytochemical screening along with the anxiolytic, antidepressant, antiepileptic, analgesic, anti-inflammatory, hypolipidemic and anticoagulant, activities of *N. nucifera* fruit.

2. Acute and sub-chronic toxicity studies along with the hematological, biochemical analysis and histopathology of organs.
1.3 Anxiety

Anxiety is defined as a state of extreme nervousness, uncertainty and fear resulting from anticipation of a future threat. Remaining anxious throughout life has many adverse implications on subjective wellbeing and physical health (O’Donovana et al., 2013). Anxiety is the most studied psychiatric field in humans as globally 1/8th of the populace experience anxiety disorders. Hypothetically, this is an adaptive sentiment that develops by physiological and behavioral changes in order to face stressful situation and resolving it by fighting or escaping. Nevertheless, if pathological variants of anxiety occur then that could be deleterious for those affected. Most of the disabilities caused by these disorders can be prevented or cured by early diagnosis and effective treatment (Rauniar et al., 2007; Rajput et al., 2015).

Even in this advanced era of research scientist are looking for traditional remedies to find an appropriate cure for these mind disturbing conditions. The incidence of pathologic anxiety over all is increasing and is linked with lot of morbidity. Life time prevalence of anxiety in men is 19.2% and in women its 30.5% (Calvin, 2005). Hence it is very important to find effective remedies to alleviate this problem.

1.3.1 Anti-anxiety agents

Anxiolytic agents also known as sedative-hypnotics are amongst the most frequently prescribed medicines as anxiety is one of the most prevalent of the psychiatric diseases in the society (Rauniar et al., 2007).

Anxiolytic agents by definition should decrease anxiety by exerting as small effect as possible on cognitive functions. Nevertheless, various agents used for anxiety causes dose associated suppression of the CNS that extent to tranquilizing effects and probable anesthesia (Mohler et al., 2002).

1.3.2 Mode of action of anxiolytics

Many anti-anxiety agents potentiate or prolong the effect of GABA including benzodiazepines and barbiturates. GABA stands as the most important inhibitory neurotransmitter in the CNS which binds to GABA<sub>A</sub> receptor in the brain which has pentameric structure (having five subunits). GABA<sub>A</sub> is a subtype of GABA receptor and possesses allosteric attachments for benzodiazepines and drug groups e.g.
barbiturates and ethanol. Benzodiazepines facilitate the actions of GABA_A by enhancing the frequency of chloride ion channel opening which results in hyperpolarization and decreased excitability of neurons. Barbiturates on the other hand increase the duration of chloride ion channel opening (Clayton, 2007).

1.3.3 Adverse effects (Chouinard, 2004) Effects are largely dose dependent:

Low doses of S-H may cause impaired judgment, drowsiness and diminished motor skills. In toxic doses, suppression of the vasomotor center takes place especially with barbiturates. Rarely hypersensitivity reactions, including skin rashes can occur. Tolerance and dependence (physical and psychological) can occur with long term use and are more common with benzodiazepines. Teratogenicity has been reported and is associated with fetal deformation with the use of piperidinediones and certain benzodiazepine during pregnancy.

1.3.4 Clinical uses of sedatives-hypnotics (Mohler et al., 2002)

Drugs Indications

1. Alprazolam Panic, phobias & anxiety
2. Diazepam Preoperative sedation, muscle relaxation& anxiety
3. Lorazepam Preoperative sedation, status epilepticus & anxiety
4. Midazolam Preoperative sedation, anesthesia
5. Trizolam Insomnia
6. Temazepam Insomnia
7. Thiopental Anesthetic
8. Phenobarbital Seizures
9. Buspirone Generalized anxiety states
10. Zolpidem Insomnia
11. Zaleplon Insomnia
1.3.5 Recent studies on anti-anxiety activity

Rajput et al., (2015) evaluated the anti-anxiety activity of *Trachyspermum ammi*. L extract by using hole-board and passive avoidance response test. A population of 21 male albino mice and 21 male Wister rats for each hole-board and passive avoidance response test were used respectively. For each experiment animals were respectively placed into three categories; vehicle administered with 2% gum tragacanth 10 ml/kg, standard given diazepam 1mg/kg whereas test group was given 50mg/kg extract of *Trachyspermum ammi*. L. The data was analyzed by assessing average and standard error to the average using one sample t-test. *Trachyspermum ammi*. L revealed a decrease of 17.43 counts/3 minutes in no. of hole poking activities in hole-board test as compared to control, which was almost comparable to diazepam. Decrease in compartment change time (passive avoidance response test) of *Trachyspermum ammi*. L treated animals was 107.2 seconds as compared to control. All of the changes were statistically highly significant. *Trachyspermum ammi*. L methanol extract exhibited anxiolytic effect which may possibly be due to the presence of high concentration of thymol. The latter has the mechanism very much identical to benzodiazepines. It may also be due to the presence of alpha-pinene in it which has shown anxiolytic and central nervous system depressant effects in other studies on different plant species. But further studies are needed to reach a final conclusion.

Shivaraj et al., 2016 conducted research studies to evaluate the anxiolytic activities of *Punica granatum* L. fruit juice in different authenticated animal models of anxiety in mice. For assessing the anxiolytic activity, models like elevated plus maze and hole-board were used. Diazepam was utilized as a reference in EPM model. Low, moderate and high doses of PGFJ and diazepam 2 mg/kg had significantly augmented no. of entrances as well as time consumed in open arms and decreased the no. of entrances and time consumed in closed arms. As far as hole-board test was concerned medium and high doses i.e. 200 and 400mg/kg but not the low dose i.e. 100mg/kg of PGFJ substantially raised the no. of head dips, latency of first head dip and no. of rearing.
1.4 Depression

Depression can be defined as a neurotic or psychotic condition characterized by an inability to concentrate, alteration in sleep pattern and feelings of extreme sadness, rejection and hopelessness. Depression is ranked as one of the top most prevalent psychiatric conditions and at any specific time, nearly five percent of the population is depressed, and about ten percent of individuals could get depressed at some stage in their live span. The indicators of depression frequently remain silent and unrecognizable by the patient & the physician (William and Leo, 2007).

1.4.1 Classification of depression (Gillman, 2007).

The classification of depression is centered on its assumed origin and is as under:

- Short-term reaction or secondary depression (most common)
- Depression associated with bipolar affective disorder (Manic-depressive)
- Melancholic and recurrent depression, a genetically determined biochemical disorder manifested by an inability to experience ordinary pleasure or to cope with ordinary life events.

1.4.2 Antidepressant agent

Antidepressants were first developed in 1950’s and are the third most commonly prescribed agents in the United States. These drugs relieve the symptoms of depression. Antidepressants include many different families of drug groups, among them two most common groups are tricyclic antidepressants (TCAs) and selective serotonin reuptake inhibitors (SSRIs). Most of the psychotropic medications including TCAs were the result of chance observation. The TCAs were developed in 1950’s as a result of an ineffective attempt to improve the antipsychotic efficacy of phenothiazine (drugs used in the treatment of Schizophrenia) but its molecular modification led to the development of imipramine, the first clinically significant TCA (Montgomery and Djarv, 1996).
TCAs are labeled after the drug’s molecular configuration, which comprises of three rings of atoms. Prior to the development of SSRIs in 1987, TCAs were the standard treatment of depression. However, SSRIs have substituted TCAs and are the preferred agents now for depressive disorders, chiefly because of their better safety and tolerability (William and Leo, 2007).

1.4.3 Classification of antidepressants (Arnold, 1997; Asberg and Martensson, 1993).

*MAOIs:*

Hydrazine: phenelzine & isocarboxazid Non-hydrazine: tranylcypromine

*TCAs:*

Imipramine, amitriptyline, nortriptyline (active metabolite of amitriptyline), lofepramine, clomipramine

*SNRIs:*

Venlafaxine, duloxetine

*SSRIs:*

Fluoxetine, fluvoxamine, paroxetine, citalopram, escitalopram, sertraline

*Atypical Antidepressants:*

Bupropion, mirtazapine, nefazodone & trazodone
1.4.4 Mode of action of anti-depressants

Briefly TCAs blocks the reuptake of norepinephrine and serotonin from the synaptic cleft and improves adrenergic and serotonergic neurotransmission, whereas SSRIs selectively block the reuptake of 5HT and improves serotonergic neurotransmission (Delgado, 2004).

1.4.5 Adverse effects

TCAs exert four effects identical to phenothiazine which include muscarinic receptor blockade, alpha receptor blockade, sedation and seizure provoking effects especially in predisposed epileptic patients. The triad of 3Cs includes convulsions, cardio toxicity and coma. 15% mortality rate with overdose is reported. SSRIs in contrast have fewer side effects, which include anxiety, agitation, bruxism, sexual dysfunctions and seizures in overdose. SSRIs can also cause serotonin syndrome (hyperthermia, hyperkalemia, muscle rigidity), when use in combination with MAOIs (Anderson, 2000).

1.4.6 Clinical uses of TCAs & SSRIs

*TCAs* (Gillman, 2007) Neuropathic pain Major depressions Nocturnal enuresis

Obsessive compulsive disorder Phobic and panic anxiety states *SSRIs* (Geddes *et al.*, 2000) Major depressions

Premenstrual dysphoric disorders Anxiety states

Bulimia Alcoholism
1.4.7 Recent studies on anti-depressant activity

Antidepressant activity of photo oxidized *Echiscarinatus* venom was investigated to evaluate its effects on stress, learning and memory. Venom can be described as an extremely intoxicating complex mixture of chemicals formed or released from the venom gland and when instilled can paralyze or digest the prey. Snake venom has several properties including pharmacological & physical for instance the neurotoxins are beneficial in the cure of Alzheimer’s disease, stroke and brain injury. In order to evaluate its effects photochemical detoxification was done to generate photo oxidized *Echiscarinatus* venom product (POECVP), and in the existence of methylene blue, the venom was exposed to ultraviolet radiations. The treated group showed prolonged survival time in contrast to the group given *Echiscarinated* venom product (ECVP) following intracerebral and intraperitoneal administration. The POECVP lengthened sleep onset and shortens the period of phenobarbitone provoked hypnosis in mice. It was also noticed that POECVP considerably reduced the time required to arrive at food in T-maze, decreased immobility time in FST & reduced transfer latency in EPM. So finally it was concluded that POECVP can be employed in the management of chronic degenerative and depressive conditions as a non-synthetic and non-herbal substitute for patients unresponsive to conventional remedies, but additional studies would be beneficial and handy to reach a final outcome (Reddy and Gawade, 2006).

Another study on the behavioral action of ethanol was conducted by using Porsolt’s forced swimming test. Ethanol has many pharmacological effects including anxiolytic (sedative/hypnotic), motor in coordination and anticonvulsant action with GABA<sub>A</sub> receptor modulators like barbiturates and benzodiazepines. The forced swimming test paradigm was used to depict the role of 3alpha, 5alpha-THP in relation with the administration of ethanol. Its acute administration led to the antidepressant mimicking effect, where as its chronic utilization produces effect which results in enhanced behavioral despair. The 3alpha,5alphaTHP and neurosteriogenic drugs which are muscimol, GABA<sub>A</sub> receptor agonist, potentiates antidepressant like effect of ethanol, similarly inhibition of biosynthesis of endogenous neurosteroids by drugs like finasteride, 5alphareductase inhibitor, trilostane, indomethacin, 3Beta-hydroxysteriod dehydrogenase inhibitor, 3alpha-hydroxysteriod dehydrogenase inhibitor and GABA<sub>A</sub> receptor antagonist, biculline inhibited the antidepressant mimicking activity of.
ethanol, moreover its withdrawal after chronic consumption displayed enhances behavioral misery. Moreover, 3alpha, 5alpha THP, fluoxetine but not imipramine at sub-antidepressant doses opposes the depression associated ethanol withdrawal representing sensitization to their antidepressant mimicking activity (Hirani et al., 2002).

1.5 Epilepsy

A mental or neurologic disorder that encircles wide range of conditions which results in dysfunction of brain and spinal cord (Dennis et al., 2003). It’s one of the essential medical problems of today and approximately thirty thousand people around the globe suffer from epilepsy each year. This problem hits around 1 in 20 individuals at any time in their live span and there is approximately 20-70 fresh cases of epilepsy found every year in 100,000 individuals (Shporvan, 1990).

At a minimum 1% of the people around the globe are having this misery and it holds second spot in the list of most prevalent neurologic disorders. Evidence proposed that inequity between excitatory and inhibitory synaptic transmission within CNS is a major cause of epilepsy in both experimental and clinical situations (Mar’ia, 2008).

There are various classes of anti-epileptic’s that have clinical effectiveness and have shown fine prognosis in controlling convulsions in majority of patients (Cockeral et al., 1995). Regardless of that lot of patients with epilepsy never managed with conventional agents. Furthermore, the high rate of occurrence of undesirable effects, contraindications and possible interactions with the use of established antiepileptic is also a matter of distress for individuals who utilize them consistently (Perucca et al., 1993).
1.5.1 Definition & causes of epilepsy

The name epilepsy is originated from a Greek term epilambanein, meaning to seize upon or to get hold of or to attack (Ojewole, 2005).

Epilepsy is defined as a state of recurrent seizures due to chronic underlying process. It is due to sudden, excessive depolarization of some or all cerebral neurons. It may be localized and termed as focal or partial seizure or spread to cause a secondary generalized seizure or may affect all cortical neurons simultaneously as in primary generalized seizure (Fisher et al., 2005).

Seizures can elicit from some vascular event in the brain like stroke, hemorrhage and arteriovenous malformations. Infections like meningitis, abscess and encephalitis can provoke it. Penetrating trauma, autoimmune diseases, metabolic disorders, neoplastic and psychiatric illnesses can cause seizures as well. Seizures can also be idiopathic (Sander, 2004).

1.5.2 Classification of anti-epileptic agents (French and Pedley, 2008)

<table>
<thead>
<tr>
<th>Primary Agents</th>
<th>Newer Agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenytoin</td>
<td>Felbamate</td>
</tr>
<tr>
<td>Valproic acid</td>
<td>Lamotrigine</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>Topiramate</td>
</tr>
<tr>
<td>Ethusuximide</td>
<td>Gabapentine</td>
</tr>
<tr>
<td>Clonazepam</td>
<td>Tiagabine</td>
</tr>
<tr>
<td>Lorazepam, diazepam</td>
<td>Vigabatrin</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td></td>
</tr>
</tbody>
</table>

1.5.3 Mode of action of anti-epileptics (Czapinski et al., 2005; Paramdeep et al., 2014)

Convulsion or seizures results from sustained high frequency, repetitive firing of cerebral neurons, which is associated with prolonged depolarization, followed by delayed hyperpolarization. The aim of treatment is to restore the normal patterns of...
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electrical activity and that can be accomplished by:

1) Increasing the inhibitory tone by facilitating GABA-mediated hyperpolarization e.g. benzodiazepine and barbiturate.

2) Decreasing presynaptic entry of calcium through T-type calcium channels in thalamic neurons e.g. valproate and ethosuximide.

3) Decreasing neuronal transmission by preventing sodium entry through fast sodium channels e.g. phenytoin and carbamazepine; also at high doses valproate and barbiturate.

4) Decreasing effect of glutamic acid e.g. lamotrigine and topiramate.

1.5.4 Adverse effects (Roger and Brian, 2007)

Specific adverse effects of few frequently used antiepileptics are as follow:

a. Gingival overgrowth, hirsutism and hematotoxicity (phenytoin).

b. Megaloblastic anemia, seizures in OD and hematotoxicity (carbamazepine).

c. GI distress, hepatotoxicity and alopecia (valproic acid).
1.5.5 Clinical uses of anti-epileptics (Wheless and Bourgeois, 2004; Wheless and Venkataraman, 1999)

<table>
<thead>
<tr>
<th>Seizure Type</th>
<th>Drugs of Choice</th>
<th>Back up Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Partial simple, complex</td>
<td>Valproic acid, Phenytoin and Carbamazepine</td>
<td>Phenobarbital pregnancy</td>
</tr>
<tr>
<td>Generalized Tonic, Clonic</td>
<td>Valproic acid, Phenytoin, Carbamazepine</td>
<td>Phenobarbital Pregnancy</td>
</tr>
<tr>
<td>Myoclonic</td>
<td>Valproic acid</td>
<td>Clonazepam, Felbamate</td>
</tr>
<tr>
<td>Absence</td>
<td>Ethosuximide , Valproic acid and Clonazepam</td>
<td>Lamotrigine</td>
</tr>
<tr>
<td>Status epilepticus</td>
<td>Lorazepam, Diazepam, Phenytoin</td>
<td>Phenobarbital (long term)</td>
</tr>
</tbody>
</table>

1.5.6 Recent studies on anti-epileptic activity

This study was demonstrated by Rajput et al., 2013 to explore the epilepsy improving capability of *Trachyspermum ammi*. L methanol extract. Test was performed utilizing a strychnine induced seizure model of epilepsy after administration of *Trachyspermum ammi*. L extract for 14 days orally. A populace of twenty one rats was separated into three groups; control group was given gum tragacanth 2%, reference group was given diazepam 1 mg/kg and test group was given *Trachyspermum ammi*. L 50mg/kg. *Trachyspermum ammi*. L exhibited anticonvulsant activity, since extremely noteworthy delay was recorded in the commencement of fits in comparison to control. On the other hand the proportion of rats that remain protected or avoided convulsions was also better in comparison to control. Nonetheless, the period of convulsions was substantially raised with diazepam and *Trachyspermum ammi*. L in contrast to control. *Trachyspermum ammi*. L extract revealed anticonvulsant potential, which could be owing to the occurrence of thymol in it, which perhaps acts via mechanism which is almost identical to group of drugs that belongs to benzodiazepines and that makes it potentially efficient agent in epileptic condition.

Following study assessed the antiepileptic capability of *Morinda citrifolia* fruit against convulsions provoked by maximal electro shock (MES) method in rats. *Morinda citrifolia* Linn also called as noni plant cultivates extensively throughout the
pacific and is one of the most important sources of alternative medicine. It is used in conjunction with herbal preparation. Its juice is used in medicines for different kind of ailments including hypertension, diabetes, arthritis, muscle aches and pains. It can also be used for gastric ulcers, headaches, aids, cancers, drug dependence, depression, senility, indigestion, menstrual abnormalities and atherosclerosis (Wang et al., 2002). *Morinda citrifolia* fruit ethyl acetate extract in a dose of 200 and 400mg/kg was given to rats for 15 days and on completion of dosing period seizures were induced by electro-Convulse meter by applying 60 HZ alternating current of 150 mA strength and then the period of diverse phases of convulsions were recorded and compared against control. A noteworthy decline in the time taken for righting reflex was noted. The concentration of biogenic amines inside forebrain was also noted and the initial level of reinstatement was observed in the treated animals, mitigating the utility of this medicinal fruit in the management of convulsions (Muralidharan and Srikanth, 2010).

1.6 Hyperlipidemia

Hyperlipidemias may essentially be classified as either familial, which is also known as primary and caused by specific genetic abnormalities, or it can be acquired which is also known as secondary and occurs as a consequence of any underlying ailment which results in alteration of lipid level in plasma and its metabolism. It may also be idiopathic i.e. devoid of any underlying reason (Brookes et al., 2009).

1.6.1 Lipid lowering agents

Cholesterol has an essential part in supporting the cell membrane integrity as well as normal body functions which significantly includes synthesis of steroid hormones. In contrast, elevated levels of cholesterol are often connected with diseased states, such as atherosclerosis (Libby et al., 2010).
1.6.2 Statins

Statins are lipid soluble or non-polar agents such as atorvastatin, lovastatin, simvastatin and fluvastatin or water soluble or polar agents such as rosuvastatin and pravastatin. Statins reduce cholesterol synthesis via blocking the rate controlling enzyme HMG-CoA reductase (HMGCR). Transformation of HMG-CoA to mevalonic acid by HMGCR is the fundamental step in the mevalonate pathway of cholesterol synthesis. In hepatocytes, inhibiting HMGCR follows reduced synthesis of cholesterol along with enhanced formation of LDL receptors, directing raised LDL clearance out of the bloodstream (Ginter and Simko, 2009; Brookes et al., 2009).

Statins are useful in the treatments of all kinds of hyperlipidemias but can cause biochemical abnormalities in liver function. Myopathy and rhabdomyolysis are rare side effects (Pasha et al., 2006).

1.6.3 Fibrates

Fenofibrate and gemfibrozil stimulate PPAR alpha and thereby enhance the activity of lipoprotein lipase, which causes a reduction in triglyceride levels. LDL converts from small, dense shape to large, buoyant particles that are more readily eliminated from the liver. PPAR alpha activation also enhances HDL synthesis (Mohiuddin et al., 2009).

The fibrates are beneficial for the treatment of type IV & V hyperlipidemias as well as type III dysbetalipoproteinemia. Fibrates can cause GIT disturbances but are usually mild. Increased biliary cholesterol excretion leads to gall stones. Also cause myositis and muscle weakness (Wu et al., 2009).

1.6.4 Bile acid sequestrants

This group includes colestipol, colestimide, colesevelam and cholestyramine which are basically non soluble polymers that entrap bile acids in the lumen of intestine and encourage elimination in feces. In consequence, the drugs reduce the hepatic cholesterol pool and enhance the expression of LDL receptors in the liver. These agents are considered as the safest lipid-lowering drugs even though have few undesirable GIT effects like constipation, flatulence and impaired absorption of fat soluble vitamins. These agents are drugs of choice for the treatment of Type II A, II B
and hyperlipidemias. Bile acid sequestrants can relieve pruritus caused by the accumulation of bile acids in patients with biliary obstruction (Ascaso, 2010).

1.6.5 Niacin

This agent reduces blood LDL levels by lowering the VLDLs which are the predecessor of LDL. The drug also enhances HDL by facilitating synthesis of apolipoprotein predecessors that produce HDL constituent part. It also reduces plasma concentrations of triacylglycerol and cholesterol, that’s why it is significant in the management of familial hyperlipidemias. Moreover it is a potent elevator of plasma HDL. Most common side effect is intense cutaneous flush and itching. It also blocks uric acid secretion from kidney tubules and thus predisposes to gout. Hepatotoxicity and impaired glucose tolerance are also documented side effects (Bays and Rader, 2009).

1.6.6 Recent studies on hypolipidemic agents

Citrus (lemon) fruit is assumed as a rich source of nutritious and nourishing diet. This is now highly acceptable and documented that various nutrients in lemon facilitate health and provide shield against chronic ailments. This research work projected the cholesterol reducing abilities of citrus juice in rabbits following administration of diet enriched with cholesterol for 30 days. Juice of citrus lemon in a dose of 1ml/kg/day exhibited a considerable reduction in LDL, triglycerides and total cholesterol levels and resulted in subsequent rise in HDL. Hence, the results exposed the cholesterol lowering activity of lemon juice which could be owing to its antioxidant activity (Khan et al., 2010).

The following research article proposed the hypolipidemic and thrombosis limiting effects of Myrtus communis. L fruit aqueous extract. Serum levels of cholesterol and parameters representing coagulation are possibly the leading factors which determine the prognosis of cardiovascular and brain diseases. Hypercholesterolemia was induced after continuous feeding of cholesterol rich diet for forty five days. Animals belonging to treated group were given the extract daily for thirty and forty five days in a dose of 50 mg/kg and during this study period first biochemical test was performed on 31st day and 2nd on 46th day. Myrtus communis. L fruit demonstrated decline in serum TG and LDL whereas elevation in thrombin & fibrinogen time was
also noted. Results projected that the extract displayed hypolipidemic activity and also influences blood coagulation parameters and could be of significance in the treatment of stroke & CVDs. Still, additional studies are mandatory to evaluate the exact mode of action of these effects (Khan et al., 2014).

1.7 Thrombosis

Coagulation of blood represents complicated bunch of physical, biochemical and cellular events which leads to the formation of thrombus (Rajput et al., 2012; Brummel et al., 2002). The formation of thrombus has a considerable part in the development as well as in the progression of ischemic heart and brain diseases (Boos and Lip, 2006; Srivastava et al., 1994; Viles-Gonzalez et al., 2006). Various modes of anticoagulation in humans and animals are well documented (Brummel et al., 1999).

Thrombosis occurs after intravascular insult which causes noteworthy disruption of blood flow. Many elements predispose it such as oozing of blood from veins which occurs in prolonged bed ridden or immobilized patients, biventricular failure, polycythemia, carcinomas, sickle cell disease and oral contraceptive utilization in conjunction with cigarette smoking (Srivastava et al., 1994).

1.7.1 Basic pharmacology of anti-thrombotic

The antithrombotic agents by definition prevent thrombosis and minimize reperfusion injury, however allow regular response to tissue injury. Coagulation occurs by conversion of soluble fibrinogen in to insoluble fibrin. Circulating proteins interact in a cascade, whereas clotting factors go through limited proteolysis to become active serine proteases. Platelets also have a basic role in homeostasis, both for development of clot as well as for activation of coagulation proteins. Anticoagulants lower the formation of fibrin clots (Colman, 2006).
1.7.2 Mode of action of heparin and warfarin

Heparin is a large polysaccharide and water soluble in nature. Heparin blocks the action of a number of activated clotting factors particularly factor IIa and Xa through stimulation of anti-thrombin III. Heparin attaches to anti-thrombin III and increases its serine protease inhibiting activity which results in fast inactivation of factors IIa, IXa, Xa, X1a and XIIa. Heparin’s activity may be monitored by aPTT. Warfarin is a lipid soluble derivative of vitamin K. It decreases the hepatic synthesis of vitamin K dependent clotting factors II, VII, IX and X (Hirsh et al., 2008).

1.7.3 Adverse effects

Toxicities associated to Heparin therapy includes, redness, bruising or itching at injection site, bleeding and Heparin induced thrombocytopenia (HIT). Toxicities associated to warfarin therapy include alopecia, anorexia, abdominal cramps, nausea, vomiting, diarrhea, hemorrhage/bleeding, necrosis, purple toe syndrome. It is contraindicated in pregnancy as it crosses placenta and causes fetal hemorrhagic disorder (James, 2007).

1.7.4 Clinical uses of anti-coagulant therapy (Mariamma, 2001)

1. Prevent blood clotting during heart/vascular surgery
2. During blood transfusion.
3. Prevent stroke
4. During hemodialysis prevent/treat DVT/PE
5. Prevention of recurrent thromboembolism
6. In atrial fibrillation

1.7.5 Recent studies on anti-thrombotic activity

The study carried out by Rajput et al., 2012 detected the activity of *Trachyspermum ammi* L seeds on coagulation parameters i-e PT and aPTT in order to establish its pharmacological usefulness. Anti-thrombotic tests PT and aPTT were conducted after multiple dosing of *Trachyspermum ammi* on rats using standard kits and reagents, which were performed on Humaclot duo Germany. The results revealed that *Trachyspermum ammi* extract did not show any significant effect on aPTT whereas
increase in PT was extremely significant (P < 0.001). The results suggested that the methanol extract of *Trachyspermum ammi* seeds lengthened PT which was comparable to warfarin, proposing its probable impact on the extrinsic system, whereas aPTT did not altered substantially. Hence the study depicted the potential of *Trachyspermum ammi* which has mild antithrombotic effect and that can prove worthy in treating CVDs and thrombotic states, however, nothing can be said definitely; hence more studies in this field would be encouraged.

Another research work was carried out to illustrate the activity of diallyl-trisulfide enriched garlic oil on coagulation of blood and plasma activity of anticoagulation factors in rats. Garlic has a very essential part in the deterrence of CVDs and is studied comprehensively since last 10 years. Various reports have described the inhibitory action of garlic and aqueous extract of garlic on platelet clumping through several methods; hence it may be regarded as herb with anti-thrombotic activity. In this study DAT enriched garlic was given to 36 male rats, weighing 250–300mg, divided in to three groups of twelve rats each and then the effect of DAT enriched garlic was evaluated on bleeding time, clotting time and anticoagulation factors on two levels, low 5mg garlic oil group and high 50mg garlic oil group. Garlic oil supplement at both concentrations extensively lengthened bleeding time and thrombin time and improved anticoagulant ability of factors e.g. anti-thrombin III and protein C. The mechanism behind it is the inhibition or inactivation of thrombin. The outcomes also proposed that the garlic oil enriched with DAT also aided blood coagulation factors, which may promote the aversion and the progression of thrombus development. In addition the garlic oil at high dose considerably augmented plasma fibrinogen level and also influence the concentration of various hematologic parameters e.g. erythrocyte count, hemoglobin and platelets. Furthermore it could adversely influence the haemostatic balance at high doses, so it must be carefully measured in its applications. Garlic oil at 5mg/kg/BW showed significant coagulation inhibiting activity in (Chan *et al.*, 2007).
MATERIALS AND METHODS
2.1 Experimental design & methodology

Research work was executed utilizing the laboratory facilities of Pharmacology department and the Research Institute of Pharmaceutical Sciences, University of Karachi, following approval from the Board of Advance Studies & Research (BASR), and departmental research & ethical committee.

2.1.1 Selection of animals

Albino mice weighing 20-25 g, Wister rats weighing 180-220 g and white rabbits weighing 1000 to 1400 g were used in present study.

Animals used for acute toxicity study were 12 male albino mice weighing 20-25 g. The study was conducted in two phases i.e. Phase I and II. In phase I, nine mice were equally divided in to three groups i.e. $n=3$. Phase II involved the use of 3 animals which were distributed in three groups of one animal each i.e. $n=1$.

35 male albino mice was used for forced swimming test, and was divided in to five treatment groups; control, reference and 3 test groups. 7 mice were placed in each group.

49 albino mice of either sex were used for tail flick test, and were divided in to seven treatment groups; control, 3 reference and 3 test groups. 7 mice were placed in each group.

49 albino mice of either sex were used for acetic acid induced writhing test, and were divided in to five treatment groups; control, 3 standard and 3 test groups. 7 mice were placed in each group.

35 male Wister rats were used for elevated plus maze test and were divided in to five treatment groups i.e. control, reference and 3 test groups. 7 rats were kept in each group.

35 male Wister rats was used for the evaluation of antiepileptic activity and was divided in to five treatment group’s i.e. control, reference and 3 test groups. 7 rats were kept in each group.
35 male Wister rats were used for carrageenan induced paw edema method, and were divided in to five treatment group’s i.e. control, reference and 3 test groups. Seven animals were kept in every group.

Antithrombotic activity was determined in 35 male Wister rats and was divided in to five groups; control, reference and 3 test groups and 7 rats were placed in each group.

28 male Wister rats were used for sub chronic studies and were divided in to four groups i.e. control and 3 test groups, 7 rats were placed in each group.

Hypolipidemic effect was evaluated in 28 white rabbits and was divided in to four groups; control and 3 test groups. 7 rabbits were placed in each group.

2.1.2 Animal housing

Animals were placed in plastic cages with preservation of room temperature at 23±2°C and moisture kept at 50 to 60% in a substituting 12-h light/dark succession. Every animal was provided with normal diet prepared in laboratory plus water as desired. The animals were shifted to the laboratory approximately an hour before the commencement of experiments. All experiments were performed during day time. Prior to dose administration, overall fitness of animals were assessed throughout the adaptation phase utilizing the laboratory conditions for a week particularly observing lack of activity, edema, diarrhea and ulceration.

The research committee Department of Pharmacology permitted the use of animals for these experiments in agreement with the guidelines of NIH (The National Institute of Health Guide for the Care and Use of Laboratory Animals, 2010) and NAClark (National Advisory Committee for Laboratory Animals Research, 2004).

2.1.3 Preparation of drugs

Diazepam, imipramine, aspirin, warfarin tablets and 0.5% Corn oil were purchased from local medical stores in Karachi, while gum tragacanth, strychnine and cholesterol powder were supplied by Merck. Carrageenan and acetic acid were obtained from Sigma Aldrich.
The suspending agent (*gum Tragacanth* powder) was consumed to prepare suspensions of the control, reference (diazepam, imipramine & aspirin) and 3 doses of test group i.e. (*N. nucifera* fruit extract 50, 100 & 200 mg/kg). It was administered to control group as placebo in the dose of 10ml/kg per oral. Every time fresh suspensions were made for the purpose of dosing (Madhu *et al*., 2009; Rajput *et al*., 2013).

Diazepam5mg tablets were trampled to powder and were then suspended in 2% gum tragacanth, administered orally in a dose of 1mg/kg (Martinez *et al*., 2006) with the help of orogastric tube.

Imipramine 25mg tablets were trampled and suspended in gum tragacanth (2%) and it was then administered orally in a dose of 25mg/kg with the help of orogastric tube (Riaz and khan, 2014).

The dilution of strychnine was made in distilled water and then was given IP in a dose of 4mg/kg for provoking seizures (Duraisami *et al*., 2009).

Aspirin 300mg tablets were trampled and suspended in gum tragacanth (2%) which was then administered per oral through orogastric tube, in a dose of 50, 100 and 200 mg/kg in tail flick test and acetic acid induced writhing test, whereas in rat hind paw edema method, aspirin was administered in a dose of 150 mg/kg orally as standard agent with orogastric tube (Shadab *et al*., 2015).

0.7% acetic acid in the dose of 10ml/kg was administered IP (Bukhari *et al*., 2010).

Carrageenan suspension 0.1 ml of 1% w/v prepared in 0.9% saline, injected under the planter Apo neurosis of right hind paw was used to induce hind paw edema in rats (Ocete *et al*., 1989).

High cholesterol diet (HCD) i.e. 0.125gm/kg cholesterol in 0.5% corn oil was given to rabbits for 30 days in order to derange lipid profile (Khan *et al*., 2010).

Warfarin Sodium5mg tablets were pulverized and then diluted in distilled water and was given to animals in a dose of 0.54mg/kg PO (Zacchigna *et al*., 2004).
2.1.4 Plant material and preparation of extract

*N. nucifera* fruits were obtained from the local fruit & vegetable market of Qasimabad, Hyderabad, Sindh, Pakistan. The *N. nucifera* fruits were identified and authenticated in the Department of Pharmacognosy. The receipt sample no NNF-03 was placed in the section of Pharmacognosy, University of Karachi.

Grounding of crude extract followed cold extraction procedure (Hossain *et al*., 2010). 6 kg fruits were washed and the seeds were peeled off from the seed pods of the fruits manually and were then chopped. After that chopped seeds were dried under shade for 6 days. The dried material was ground to coarse powder. The seed pods on the other hand were dried under shade for 3 days and then chopped to fine powder. The seed powder along with the seed pod powder was macerated in 10 liters of 98% ethanol for 30 days with occasional shaking and stirring until the color of the solvent becomes black.

The solvent was filtered with the help of Whatman No. 1 filter paper. Afterwards the extract was evaporated under reduced pressure in a rotary evaporator at 40°C to 45°C followed by freeze drying at -30°C. The solid Lyophilized material so obtained was saved at -20°C until further use in different doses. The final quantity of the extract obtained was 400g of dry mass.
Figure-3
Rotary Evaporators used for Extraction HEJ, Research Institute of Chemistry

Figure-4
Freeze Dryer used for drying of Extract HEJ, Research Institute of Chemistry
2.1.5 Phytochemical screening

2 kg of *N. nucifera* fruit was washed and seeds were peeled off manually. Seed pods were then dried under shade for 3 days and chopped to fine powder which was then soaked in 4 liters of 98% ethanol for 30 days. Soaked material was then filtered through (Whatman No. 1) filter paper. Afterwards the extract was evaporated under reduced pressure in a rotary evaporator at 40°C to 45°C followed by freeze drying at -30°C (Hossain *et al*., 2010). The solid Lyophilized plant material so gained was saved at -20°C until further use for the assessment of qualitative phytochemical screening.

Qualitative phytochemical screening of the seed pods of the *N. nucifera* fruit extract was conducted at Industrial Analytical Center (IAC), HEJ Research Institute of Chemistry, University of Karachi, Karachi, sample code # IAC/TR/7008, dated 08.25.2016, in order to evaluate the presence or absence of flavonoids, alkaloids, saponins, tannins and terpenoids in accordance with the method described in earlier studies (Edeoga *et al*. 2005; Sofowora, 1993).

2.2 Experimental procedures

2.2.1 Acute toxicity study

Animals used for this study were 12 male albino mice weighing 20-25 gm and were bred at the animal house of the department of Pharmacology, University of Karachi. Before the commencement of drug administration, noticeable health of these animals during the conditioning period under the laboratory environment was observed for a week and then the LD$_{50}$ i.e. the dose of a drug that causes death in 50% of animals within 24 hours was calculated as described by the Lorke, 1983.

The study was performed in two phases i.e. Phase I and II. In phase I, nine mice were equally divided in to three groups 3 in each group. Each group was administered different dose (10, 100 and 1000mg/kg) of ethanol extract of *N. nucifera* fruit and then animals were monitored for 7 days for any acute toxicity feature i.e. behavioral feature and mortality. Phase II encompasses the use of 3 animals which were placed in three groups of one animal each. The animals were administered higher doses of extract i.e. 1600, 2900 and 5000mg/kg and were again monitored for 7 days for signs of behavioral toxicity as well as mortality.
2.2.2 Elevated plus maze test

It is an extensively used assay to determine anxiety like behavior in rodents. It is also helpful in assessing the anti-anxiety effects of pharmacological agents and to define mechanism underlying anxiety (Walf and Frye, 2007).

A population of 35 male Wister rats weighing 200-220 g was equally divided into 5 groups i.e. 7 animals in every group. Group I was kept as control and given 10 ml/kg of vehicle (Gum tragacanth). Group II, III, IV served as treated groups and were administered 50, 100 and 200 mg/kg of *N. nucifera* fruit extract and group V served as reference and was administered diazepam 1 mg/kg for 15 days through orogastric tube. The EPM comprises of two open arms (50cm long and 10cm wide) and two closed arms (50 cm long, 10 cm wide and 38 cm high with open roof) positioned opposite to one another around 10×10 cm platform. Each animal was exposed to the maze for 5 minutes and then no. of entries in open and closed arms was noted and time spent in open and closed arms was noted through stop watch (Riaz and Khan, 2014).

2.2.3 Light and dark test

The light and dark test was carried out as designed by Gong et al., 2006 with slight changes. The animals used in EPM underwent testing in light and dark box just 30 min after EPM. The equipment used in the test consisted of a dark safe cubicle and a light aversive cubicle. Dimensions of the cubicle are commonly 1/3 for the dark compartment and 2/3 for the light compartment with an external size of 46×27×30 cm. During the test, the rats were positioned at the midpoint of the light compartment with their back towards dark compartment. The percentage of time spent in the light compartment was noted for 5 minutes for each animal through stop watch. After 5 minutes; rats were removed from the box by the base of their tails and returned to their home cage. The maze was then cleaned with a solution of 10% ethanol and allowed to dry between tests. Anxiety is thought to be high if percentage of time spent in the light compartment is less than 50% (Michel and Martine, 2003).
2.2.4 Forced swimming test

Modified Porsolt et al., 1977 method was used. A population of 35 male albino mice weighing 20-25 g was equally divided into 5 groups i.e. 7 animals in each group. Group I was kept as control and given 10 ml/kg of vehicle (Gum tragacanth). Group II, III, IV served as treated groups and were administered 50, 100 and 200 mg/kg of N. nucifera fruit extract and group V served as reference and was administered imipramine 25 mg/kg for 14 days. All drugs were given orally through orogastric tube. Study duration was fifteen days and 14 doses were given (from 2\textsuperscript{nd} to 15\textsuperscript{th} day).

This method consists of two sessions, the pretest and the test session, where as in old Porsolt’s method, the animals were subjected to direct immersion after injecting drugs 40 minutes before test session. In the pretest session on 1\textsuperscript{st} day, the animals were allowed to swim separately for 15 minutes in a glass tank (height: 40 cms, diameter, 24 cms) containing 15 cms water at 25 ± 2° C, after which the animals were removed, dried and returned to home cages. On the 2\textsuperscript{nd} day test session was performed for single dose studies and each mouse was forced to swim for 5 minutes and variable measured was duration of immobility i.e the time at which animal halts swimming, except for those movements which keeps its head above water was recorded through stop watch. On day 15\textsuperscript{th} same procedure was performed after completion of 14 doses (Reddy and Gawade, 2006).

2.2.5 Strychnine induced seizures

Populations of 35 male rats were divided in five groups i.e. control, reference and 3 test groups. Each comprising of 7 animals and were given 2% Gum tragacanth (control), Diazepam 1mg/kg PO (reference) and N. nucifera fruit 50, 100 and 200mg/kg PO (test)OD for 15 days. On 15\textsuperscript{th} day of study, Strychnine in a dose of 4mg/kg IP was administered to rats 40 minutes after the administration of vehicle, Diazepam and fruit extract at all specified doses and then time of onset along with duration of convulsions and the percentage of animals avoided or remain alive after strychnine administration was recorded.

Strychnine exerts its convulsant action by blocking directly the inhibitory CNS (spinal cord and brain stem) reflexes of glycine and thus enhances spinal nerve reflexes.
(Biggio et al., 1992). Rats that escape convulsions thirty minutes after strychnine administration are considered protected (Duraisami et al., 2009).

2.2.6 Tail flick test

Analgesia is the loss of ability to feel or react to painful stimulus such as chemical, thermal or mechanical (Ahmed et al., 2015). In current study central analgesic activity in mice was assessed by tail flick test and tail flick latency difference (TFLD) i.e. the time in seconds taken by mouse to remove its tail clearly out of water was noted as the reaction time (Luiz et al., 1998).

The test was conducted on 49 white albino mice of either sex which were equally placed in seven groups i.e. (n=7). Group control was administered gum tragacanth as vehicle; three groups served as test groups and were given N. nucifera fruit extract at a dose of 50, 100 and 200 mg/kg and three groups served as reference groups and were given aspirin 50, 100 and 200 mg/kg. All drugs were administered PO. Mice were kept in a particular restrainer with only the tail extending out then 1/3rd of the tail was submerged in a digital constant temperature water tank maintained at 51ºC. The 1st reading was taken straightaway prior to the administration of drugs then after 30, 60, 90, 120, 150 & 180 minutes. Animals which did not remove their tail in 10 seconds were discarded from the experiment in order to avoid tissue damage (Dashti et al., 2007). Data acquired was calculated utilizing average values of every group and average increase in latency following dosing was measured. Lastly % time elongation of tail with respect to the control group was evaluated using the following formula as previously described (Islam et al., 2014).

\[
\% \text{ Time elongation of tail} = \frac{\text{Average tail flick time for sample} - \text{Average tail flick time for Control}}{\text{Average tail flick time for sample}} \times 100
\]
2.2.7  Acetic acid induced writhing test

Acetic acid induced writhing test is a valuable test conducted for the assessment of systemic analgesic effect in mice by already described method (Koster et al., 1959). A population of 49 white albino mice of both sex were equally placed in seven groups i-e (n=7). The group control was administered gum tragacanth as vehicle; three groups served as test groups and were given N. nucifera fruit extract at a dose of 50, 100 and 200 mg/kg and three groups served as reference groups and were given aspirin in a dose of 50, 100 and 200 mg/kg. All drugs were given orally. 30 minutes after the administration of drugs, 0.7% acetic acid 10ml/kg was given IP. Mice were placed immediately in a plastic transparent box (13 cm height×12 cm width×23 cm length) and no. of wretches which includes abdominal muscle contraction, periodic arching of body, stretching and drawing up of hind limbs were counted for twenty minutes and lastly percent inhibition of wretches was assessed using the following formula (Ahmed et al., 2015):

% Inhibition= Vc–Vt/Vc × 100

Where

Vc = Average no. of wretches for control animals

Vt = Average no. of wretches for treated animals

2.2.8  Hind paw edema method

It is the widely used method, performed to assess the anti-inflammatory potential of N. nucifera fruit extract by inducing edema in the right hind paw of rat by carrageenan (Winter et al., 1989). Generally carrageenan is used as phlogistic agent i.e. a substance that induces inflammation or edema (Khan et al., 2016).
The test was performed on 35 Wister rats which were equally placed in five groups i.e. n=7. One group served as control and was given 2% gum tragacanth; one group served as reference group and was given aspirin 150 mg/kg and three groups represented as test groups and were given N. nucifera fruit 50, 100 and 200 mg/kg. All drugs were administered PO one hour prior the administration of carrageenan injection. Carrageenan suspension 0.1 ml of 1% w/v prepared in saline was injected under the planter Apo-neurosis of right hind paw of rats and edema was induced (Occente et al., 1989).

The paw edema was measured using volume displacement method by plethysmometer (UGO Basile 7140, Italy), which is perhaps the most efficient method to measure anti-inflammatory response of a drug. Plethysmometer is basically a volume meter, composed of water filled Perspex cell in to which hind paw of rat was immersed and the transducer recorded small changes in water level caused by volume displacement and its digital meter displayed the exact volume of water being displaced by edema in hind paw of rats. The swelling was quantified in terms of ml of edema at different time interval i.e. paw volume prior the administration of carrageenan (time=0 baseline) and after 1, 2, 3, 4 and 5 hour was noted. The difference in paw volume assessed before and after injection of phlogistic agent points towards the severity of edema (Khan et al., 2016). Lastly percent inhibition of paw inflammation was assessed in terms of % inhibition (Palanichamy et al., 1990).

\[
\text{% Inhibition} = \frac{A - B}{A} \times 100
\]

Where

A= the average paw volume for test groups  
B= the average paw volume for control group
2.2.9 Effect of *N. nucifera* fruit on lipid profile

A population of 28 white healthy rabbits of either sex was divided into four groups. Control group was given gum tragacanth and 3 groups served as test groups and were given *N. nucifera* fruit extract 50, 100 and 200 mg/kg for 45 days. Initially animals of all four groups received high cholesterol diet (HCD) i.e. 0.125 g/kg cholesterol in 0.5% corn oil for 30 days. All agents were given PO (Khan *et al.*, 2010). Blood samples were taken thrice from the ear vein of animals, 1st after 24 hours of thirty days of HCD then again after 24 hours of thirty and forty five days dosing of *N. nucifera* fruit extract i.e. on 31st and 46th day of experiment.

Blood samples (5 ml) were collected in gel tube. Serum was instantly separated by centrifuging blood samples on 14KHumax centrifuge at 3000 rpm for 15 min. Lipid profile was investigated on Humalyzer 3000 (semi-automatic chemistry analyzer, Model #16700) (Human Germany) using standard kits supplied by Human. TC and LDL-C was assessed by CHOD-PAP method; TG by GPO-PAP methods (Trinder, 1969), and HDL-C according to the method of Friedwald *et al.* 1972.

2.2.10 Effect of *N. nucifera* fruit on coagulation parameters

A population of 35 male Wister rats were equally placed in five groups, control, reference and 3 test groups; each comprising of 7 animals. This study was conducted for fifteen days and on day 15th blood samples were taken. Each group of animals were treated either with a test drug i.e. *N. nucifera* fruit 50, 100 and 200 mg/kg PO, vehicle at a dose of 10ml/kg PO and reference drug i.e. warfarin at a dose of 0.54mg/kg PO with orogastric tube.

Blood sample (3ml) was collected in coagulation tubes; plasma was separated by centrifugation at 3000 rpm for 15 minutes by 14k Humax centrifuge. Prothrombin time, Activated Partial Thromboplastin time, Thrombin time and Fibrinogen were evaluated through Humaclot duo Germany, utilizing standard reagent kits obtained from Human (Chan *et al.*, 2007).
2.2.11 **Sub-chronic toxicity study**

28 male Wister rats were divided in four groups; each comprising of 7 animals. This study was conducted for 90 days. One group served as control and received 2% gum tragacanth and 3 groups served as test groups and were given orally 50, 100 and 200 mg/kg *N. nucifera* fruit ethanol extract suspended in 2% gum tragacanth.

**Assessment of toxicities**

The gross examination of animals was carried out at every week following administration of drugs precisely noticing average weight variation, loss of hair, skin ulceration, lack of interest in food, loss of activity, hematuria, lacrimation, salivation, vomiting, diarrhea, edema, muscle tone, tremor, and aggressive behavior. Weekly body weight was also noted. At the end of dosing the rats were sacrificed under anesthetic condition. Autopsy was performed by random selection at the completion of dosage and blood samples were collected for biochemical analysis.

**Sample collection**

7ml blood sample was collected from each animal by cardiac puncture at the completion of dosing period of 90 days.

**Hematological examination**

Blood samples were collected under 10% EDTA at 7.2pH and then hemoglobin concentration, red blood cells, white blood cells and platelet counts were measured on Humacount plus a fully automated hematology analyzer (Human Germany).

**Biochemical evaluation**

Blood samples were immediately centrifuged (Heraeus, Christ Labofuge A) at 4000 rpm for 8 minutes to collect serum, which were then analyzed within 3 hours on Vita Lab eclipse automatic analyzer (Merck) using standard reagent kits obtained from Merck.
Renal parameters

Total protein

Serum protein was analyzed according to biuret technique using Vita Lab Eclipse in which proteins and peptides produce a violet color complex with copper ions in presence of sodium hydroxide (Thomas, 1998). Sample absorbance was checked against reagent blank at 546 nm within 60 min. on Vitalab that directly gives the total protein concentration in g/dl.

Urea

Urea is determined enzymatically. The decrease in NADH absorbance per unit time is proportional to the Urea concentration. Absorbance was measured at 340 nm in Vitalab that directly gives the urea levels in mg/dl.

Creatinine

Serum creatinine activity was evaluated by a colorimetric reaction known as Jaffe reaction (Seeling and Wuest 1969). Creatinine forms a yellow-orange compound in alkaline solution with picric acid. The concentration of the dye over a certain reaction time is a measure of the creatinine concentration (Bartels et al, 1972). Absorbance of sample and standard were read at 492 nm on Vitalab that directly gives serum creatinine activity in mg/dl.

Hepatic parameters

Alkaline phosphatase

The concentration of enzyme alkaline phosphate in serum was assessed photometrically, with the help of Ecoline 125 AP supplied by Merck. The rate of increase in 4-nitrophenolate was evaluated photometrically, which was directly proportional to the activity of enzyme alkaline phosphatase present in sample serum (Deutsche, 1972). The escalation in absorbance was calculated at 405nm every minute for 3 min, on Vitalab, that directly gives the concentration of enzyme alkaline phosphatase in U/l.
Alanine transaminase (ALT)

The level of enzyme ALT in serum was assessed by utilizing Ecoline 125, ALATTris of Merck. The rate of NADH utilization was measured photometrically, which was directly proportional to the ALT activity in the sample. The reduction in absorbance was measured at 340 nm every minute for 3 minute on Vita Lab that directly gives the level of the enzyme in U/l.

Aspartate transaminase (AST)

The level of enzyme AST in serum was assessed by utilizing Ecoline 125, ASAT supplied by Merck. The rate of NADH consumption was measured photometrically, which is directly proportional to the AST activity in the sample. The reduction in absorbance was measured at 340 nm, every minute for 3 minutes on Vitalab that directly gives the level of the enzyme AST in U/l.

**Histopathological examination**

Specimens of hepatic and renal tissues were conserved in 10% buffered formalin till further processing at Basic Medical Sciences Institute, Jinnah Post Graduate Medical Center, Karachi-75510, Pakistan. Appropriate blocks of these organs were taken, fixed and the sections were cut for microscopic examination.

Representative blocks from various regions of these organs were cut from each sample after separating the fat from respective organs. The blocks were processed in an automatic tissue processor (Gilford 101 system) and after that embedded in paraffin at 56° C in hot air oven. These paraffin embedded tissues were then sectioned with microtome (3 to 4 micron thickness) and stained with hematoxylin and eosin stain and were evaluated histologically with light microscope under lens 10x & 40x.

**2.3 Statistical analysis**

The data was subjected to analysis by taking average and standard error to the average utilizing two sample student T- test and values of P< 0.05 were considered as noteworthy and P<0.005 as extremely noteworthy. All statistical techniques were executed using SPSS software version 20 (Walpole, 1982).
RESULTS
3.1 Phytochemical screening

The qualitative phytochemical analysis of the ethanol extract of the seed pods of *N. nucifera* fruit demonstrated the presence of flavonoids, alkaloids, terpenoids, saponins and tannins as summarized in Table 1.
Table-1

Qualitative phytochemical analysis of the seed pods of *N. nucifera* fruit extract

<table>
<thead>
<tr>
<th>Test performed</th>
<th>Observations</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavanoids Defatted extract + ethanol and filter; filterate + AlCl₃</td>
<td>Yellow color</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids Dragendroff’s test</td>
<td>Orange red precipitates/turbidity</td>
<td>+</td>
</tr>
<tr>
<td>Terpenoids Decolorized extract residue+ chloroform+ acetic anhydride + conc: H₂SO₄</td>
<td>Brown precipitates formed</td>
<td>+</td>
</tr>
<tr>
<td>Saponins Extract shaken vigorously in a test tube for 2 min</td>
<td>Frothing occurred</td>
<td>+</td>
</tr>
<tr>
<td>Tannins FeCl₃ test</td>
<td>Dark greenish precipitate formed</td>
<td>+</td>
</tr>
</tbody>
</table>

“+” present
3.2  **Acute toxicity study**

All animals in both phases tolerated doses of *N. nucifera* fruit extract up to 5000mg/kg body weight as observed for 24 to 48 hours and then for 7 days. There were no signs of behavioral toxicity including salivation, lacrimation, defecation, urination, over activity, aggressiveness, piloerection, twitches, tremors and convulsions and no deaths were recorded during this period.

3.3  **Elevated plus maze test**

Table-2 revealed the comparison of anxiolytic effect of *N. nucifera* fruit extract and diazepam with control in rats by elevated plus maze test. No. of entries in open arms and time spent in open arms was significantly augmented whereas no. of entries in closed arms and time spent in closed arms was significantly decreased at dose of 50mg/kg as compared to control. No. of entries in open arms and time spent in open arms was increased highly significantly at doses of 100 and 200mg/kg whereas time spent in closed arms was decreased highly significantly as compared to control. However, no significant change in no. of entries in closed arms was observed at extract dose of 100 mg/kg. Diazepam 1mg/kg exhibited highly significant rise in no. of entries in open arms and time spent in open arms whereas no. of entries in closed arms and time spent in closed arms was highly significantly decreased as compare to control.
### Table-2

**Anxiolytic effect of *N. nucifera* fruit extract and diazepam in elevated plus maze**

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of entries in open arms</th>
<th>Time spent in open arms (s)</th>
<th>No. of entries in closed arms</th>
<th>Time spent in closed arms (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 10ml/kg</td>
<td>2.7±0.29</td>
<td>127.5±3.30</td>
<td>5.6±0.37</td>
<td>172.4±3.30</td>
</tr>
<tr>
<td><em>N. nucifera</em> 50mg/kg</td>
<td>3.8±0.34*</td>
<td>138.3±2.90*</td>
<td>5.0±0.31*</td>
<td>161.6±2.90*</td>
</tr>
<tr>
<td><em>N. nucifera</em> 100mg/kg</td>
<td>5.3±0.42**</td>
<td>150.6±0.79**</td>
<td>4.8±0.40</td>
<td>149.3±0.79**</td>
</tr>
<tr>
<td><em>N. nucifera</em> 200mg/kg</td>
<td>7.1±0.51**</td>
<td>175.8±1.90**</td>
<td>3.1±0.40**</td>
<td>124.1±1.90**</td>
</tr>
<tr>
<td>Diazepam 1mg/kg</td>
<td>5.0±0.53**</td>
<td>168.3±1.80**</td>
<td>4.1±0.40**</td>
<td>131.6±1.80**</td>
</tr>
</tbody>
</table>

n=7  
Values are Mean ± S.E.M  
*p< 0.05 noteworthy in comparison to control  
**p< 0.005 extremely noteworthy in comparison to control
3.4 Light and dark test

Table-3 has demonstrated the comparison of anxiolytic effect of *N. nucifera* fruit extract and diazepam with control in rats by using light and dark test and percentage of time spent in light compartment was recorded. There was highly significant increase in percentage of time spent in light compartment at extract doses 50, 100 and 200 mg/kg as compare to control. Diazepam 1mg/kg also exhibited extremely noteworthy increase in percentage of time spent in light compartment as compared to control.
### Table-3

**Anxiolytic effect of *N. nucifera* fruit extract and diazepam in light and dark test**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Percentage of time spent in light compartment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 10ml/kg</td>
<td>36.2± 1.50</td>
</tr>
<tr>
<td><em>N. nucifera</em> 50mg/kg</td>
<td>45.4± 0.67**</td>
</tr>
<tr>
<td><em>N. nucifera</em> 100mg/kg</td>
<td>51.8± 0.89**</td>
</tr>
<tr>
<td><em>N. nucifera</em> 200mg/kg</td>
<td>58.3± 0.64**</td>
</tr>
<tr>
<td>Diazepam 1mg/kg</td>
<td>52.8± 1.10**</td>
</tr>
</tbody>
</table>

n=7
Values are Mean ± S.E.M
**p<0.005 extremely noteworthy in comparison to control**
3.5 Forced swimming test

Table-4 has investigated the comparison of antidepressant effect of *N. nucifera* fruit extract and imipramine with control in mice using forced swimming test and immobility time was recorded. *N. nucifera* fruit has demonstrated substantial decline in the duration of immobility in mice at doses 100 and 200 mg/kg on 2\textsuperscript{nd} day of test session in comparison with control. Duration of immobility was also decreased extremely noteworthy in comparison with control at the same doses of extract on 15\textsuperscript{th} day of experiment. On the other hand imipramine 25 mg/kg decreased highly significantly the duration of immobility on 2\textsuperscript{nd} and 15\textsuperscript{th} day as compared to control.
### Table-4

**Antidepressant effect of *N. nucifera* fruit and imipramine in forced swimming test**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day</th>
<th>Immobility duration (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>197.5±0.85</td>
</tr>
<tr>
<td></td>
<td>15&lt;sup&gt;th&lt;/sup&gt;</td>
<td>181.1±0.87</td>
</tr>
<tr>
<td><em>N. nucifera</em></td>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>196.6±0.69</td>
</tr>
<tr>
<td>50 mg/kg</td>
<td>15&lt;sup&gt;th&lt;/sup&gt;</td>
<td>180.2±0.89</td>
</tr>
<tr>
<td><em>N. nucifera</em></td>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>195.2±0.64*</td>
</tr>
<tr>
<td>100 mg/kg</td>
<td>15&lt;sup&gt;th&lt;/sup&gt;</td>
<td>174.6±1.20**</td>
</tr>
<tr>
<td><em>N. nucifera</em></td>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>194.3±0.61*</td>
</tr>
<tr>
<td>200 mg/kg</td>
<td>15&lt;sup&gt;th&lt;/sup&gt;</td>
<td>169.8±3.20**</td>
</tr>
<tr>
<td>Imipramine</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>152.1±0.97**</td>
</tr>
<tr>
<td>25 mg/kg</td>
<td>15&lt;sup&gt;th&lt;/sup&gt;</td>
<td>120.5±0.90**</td>
</tr>
</tbody>
</table>

n=7
Values are Mean ± S.E.M
*p* < 0.05 noteworthy in comparison to control
**p* < 0.005 extremely noteworthy in comparison to control
3.6  Strychnine induced seizures

Table-5 has revealed the comparison of antiepileptic effect of *N. nucifera* fruit extract and diazepam with control using strychnine induced seizure model in rats and time of commencement of fits or convulsions, period of convulsions and percentage of animals remain protected or avoided convulsions was recorded. *N. nucifera* fruit ethanol extract at dose 200 mg/kg exhibited extremely noteworthy delay in the inception of convulsions in comparison with control while period or duration of convulsions was also increased significantly at the same dose of test drug as compared to control but the intensity of convulsions was reduced which resulted in better percentage of animal survived i.e. 42.85% from seizures. *N. nucifera* fruit extract at dose 100 mg/kg also showed noteworthy delay in the commencement of convulsions as compared to control without exhibiting any significant change in the period or duration of convulsions in comparison to control which resulted in 14.28% of animals survived or protected from seizures. The time for the commencement of convulsions was also deferred and period of convulsions was also increased with *N. nucifera* fruit extract 50 mg/kg but not to a noteworthy level. On the other hand diazepam 1 mg/kg considerably delayed the commencement of convulsions in comparison with control; while period of convulsions was also significantly increased as compared to control and the animal survival rate was 57.14%.
### Table-5

Comparative effect of *N. nucifera* fruit and diazepam on strychnine induced convulsions in rats

<table>
<thead>
<tr>
<th>Groups/Doses</th>
<th>Commencement of convulsions (minutes)</th>
<th>Period of convulsions (minutes)</th>
<th>Animals survived or protected (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 10 ml/kg</td>
<td>0.13±0.01</td>
<td>0.904±0.41</td>
<td>0</td>
</tr>
<tr>
<td><em>N. nucifera</em> 50 mg/kg</td>
<td>0.393±0.15</td>
<td>1.289±0.35</td>
<td>14.28</td>
</tr>
<tr>
<td><em>N. nucifera</em> 100 mg/kg</td>
<td>1.84±0.42*</td>
<td>1.576±0.719</td>
<td>14.28</td>
</tr>
<tr>
<td><em>N. nucifera</em> 200 mg/kg</td>
<td>2.836±0.27**</td>
<td>1.653±0.22*</td>
<td>42.85</td>
</tr>
<tr>
<td>Diazepam 1 mg/kg</td>
<td>2.80±0.69*</td>
<td>2.40±0.45*</td>
<td>57.14</td>
</tr>
</tbody>
</table>

n=7
Values are Mean ± S.E.M
* p< 0.05 noteworthy in comparison to control
** p< 0.005 extremely noteworthy in comparison to control
3.7 Tail flick test

Table-6 has depicted the analgesic activity of *N. nucifera* fruit and aspirin using tail flick test. The table has summarized the results of *N. nucifera* fruit ethanol extract at doses 50, 100 and 200 mg/kg against control and also with similar doses of aspirin. It was revealed that *N. nucifera* fruit extract at dose 50 mg/kg exhibited extremely noteworthy analgesic effects at 30, 60 and 90 minutes and noteworthy effects at 120 and 150 minutes in comparison to control. Whereas *N. nucifera* fruit extract at doses 100 and 200 mg/kg exhibited highly noteworthy analgesic effects from 30 to 180 minutes as compared to control. Aspirin in contrast revealed extremely noteworthy analgesic effects at 50, 100 and 200 mg/kg from 30 to 180 minutes as compared to control. Table-7 has demonstrated % time elongation of tail at 90 minutes after administration of *N. nucifera* fruit and aspirin with respect to control. *N. nucifera* fruit extract showed highest % of tail elongation time at 200 mg/kg dose i-e 82% followed by 76% and 42% for extract doses 100 and 50 mg/kg. Aspirin on the other hand exhibited % tail elongation time of 81, 84 and 95 % at doses 50, 100 and 200 mg/kg.
Table 6
Analgesic effect of *N. nucifera* fruit and aspirin in tail flick test demonstrating tail flick latency difference in mice

<table>
<thead>
<tr>
<th>Groups/ Doses</th>
<th>Analgesia TFLD or Average Increase in Latency After Drug Administration ±S.E.M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-drug</td>
</tr>
<tr>
<td>Control 10 ml/kg</td>
<td>0.9± 0.01</td>
</tr>
<tr>
<td><em>N. nucifera</em> 50 mg/kg</td>
<td>1.0± 0.01</td>
</tr>
<tr>
<td><em>N. nucifera</em> 100 mg/kg</td>
<td>1.0± 0.02</td>
</tr>
<tr>
<td><em>N. nucifera</em> 200 mg/kg</td>
<td>0.9± 0.04</td>
</tr>
<tr>
<td>Aspirin 50 mg/kg</td>
<td>1.0± 0.03</td>
</tr>
<tr>
<td>Aspirin 100 mg/kg</td>
<td>1.0± 0.02</td>
</tr>
<tr>
<td>Aspirin 200 mg/kg</td>
<td>1.104± 0.14</td>
</tr>
</tbody>
</table>

n=7
Values are Mean ± S.E.M
*p< 0.05 noteworthy in comparison to control
**p< 0.005 extremely noteworthy in comparison to control
Table-7
Analgesic effect of *N. nucifera* fruit and aspirin in tail flick test demonstrating % elongation time of tail in mice

<table>
<thead>
<tr>
<th>Groups/Doses</th>
<th>% Elongation at 90 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 10 ml/kg</td>
<td>-</td>
</tr>
<tr>
<td><em>N. nucifera</em> 50 mg/kg</td>
<td>42</td>
</tr>
<tr>
<td><em>N. nucifera</em> 100 mg/kg</td>
<td>76</td>
</tr>
<tr>
<td><em>N. nucifera</em> 200 mg/kg</td>
<td>82</td>
</tr>
<tr>
<td>Aspirin 50 mg/kg</td>
<td>81</td>
</tr>
<tr>
<td>Aspirin 100 mg/kg</td>
<td>84</td>
</tr>
<tr>
<td>Aspirin 200 mg/kg</td>
<td>95</td>
</tr>
</tbody>
</table>
3.8 Acetic acid induced writhing test

Table-8 revealed the analgesic effects of *N. nucifera* fruit and aspirin using acetic acid induced writhing test. Number of writhes were highly significantly reduced at 50, 100 and 200 mg/kg doses of *N. nucifera* fruit ethanol extract but maximum effects were observed at extract dose of 200 mg/kg i.e. 11.42±0.57 as compared to control 22.14±0.46 (indicating 48.41 % inhibition of writhes). On the other hand aspirin also decreased highly significantly number of writhes at doses 50, 100 and 200 mg/kg (representing 65.8, 72.89 and 79.35 % inhibition of writhes) as compared to number of writhes in control animals.
Table-8
Analgesic effect of *N. nucifera* fruit and aspirin in acetic acid induced writhing test in mice

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Dose</th>
<th>No. of Writhes</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10 ml/kg</td>
<td>22.1±0.46</td>
<td>-</td>
</tr>
<tr>
<td><em>N. nucifera</em></td>
<td>50 mg/kg</td>
<td>16.3±0.29**</td>
<td>26.46</td>
</tr>
<tr>
<td><em>N. nucifera</em></td>
<td>100 mg/kg</td>
<td>12.5±0.20**</td>
<td>43.22</td>
</tr>
<tr>
<td><em>N. nucifera</em></td>
<td>200 mg/kg</td>
<td>11.4±0.57**</td>
<td>48.41</td>
</tr>
<tr>
<td>Aspirin</td>
<td>50 mg/kg</td>
<td>7.5±0.20**</td>
<td>65.8</td>
</tr>
<tr>
<td>Aspirin</td>
<td>100 mg/kg</td>
<td>6.0±0.31**</td>
<td>72.89</td>
</tr>
<tr>
<td>Aspirin</td>
<td>200 mg/kg</td>
<td>4.5±0.20**</td>
<td>79.35</td>
</tr>
</tbody>
</table>

n=7
Values are Mean ± S.E.M
*p< 0.05 noteworthy in comparison to control
**p< 0.005 extremely noteworthy in comparison to control
3.9 Hind paw edema method

Table 9 and 10 demonstrated that the carrageenan injection produced a localized edema at the right hind paw of rats which reached to its maximum at the 3\textsuperscript{rd} hour after carrageenan administration and then gradually reduces after this period. *N. nucifera* fruit ethanol extract at doses 50, 100 and 200 mg/kg highly significantly reduced the paw edema volume from 3\textsuperscript{rd} to 5\textsuperscript{th} hour as compared to control with maximum percent reduction in edema was noted at a dose of 100 mg/kg (73.92\%) at 5\textsuperscript{th} hour after administration of carrageenan. The initial phase of the edema i.e. 1\textsuperscript{st} and 2\textsuperscript{nd} hour was not affected by fruit extract. On the other hand aspirin 150 mg/kg highly significantly reduced paw edema volume as compared to control from 1\textsuperscript{st} to 5\textsuperscript{th} hour with maximum percent inhibition of edema (88.17\%) was noted at 5\textsuperscript{th} hour after administration of carrageenan.
# Table-9

**Anti-inflammatory effect of N. nucifera fruit & aspirin by hind paw edema method in rats**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Average Paw Size (ml) ± S.E.M</th>
<th>Average Increase in Paw Volume (ml) ± S.E.M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-drug</td>
<td>1 h</td>
</tr>
<tr>
<td>Control</td>
<td>2.0±0.02</td>
<td>2.8±0.10</td>
</tr>
<tr>
<td>N. nucifera 50 mg/kg</td>
<td>2.1±0.03</td>
<td>3.3±0.21</td>
</tr>
<tr>
<td>N. nucifera 100 mg/kg</td>
<td>2.0±0.01</td>
<td>2.8±0.10</td>
</tr>
<tr>
<td>N. nucifera 200 mg/kg</td>
<td>1.9±0.02</td>
<td>2.8±0.18</td>
</tr>
<tr>
<td>Aspirin 150 mg/kg</td>
<td>1.8±0.04</td>
<td>1.9±0.05**</td>
</tr>
</tbody>
</table>

n=7
Values are Mean ± S.E.M
**p< 0.005 extremely noteworthy in comparison to control**
Table-10

Percent inhibition of edema in rats treated with *N. nucifera* fruit and aspirin

<table>
<thead>
<tr>
<th>Groups</th>
<th>% Inhibition of Edema</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 h</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
</tr>
<tr>
<td><em>N. nucifera</em> 50mg/kg</td>
<td>51.29</td>
</tr>
<tr>
<td><em>N. nucifera</em> 100mg/kg</td>
<td>55.12</td>
</tr>
<tr>
<td><em>N. nucifera</em> 200mg/kg</td>
<td>52.15</td>
</tr>
<tr>
<td>Aspirin 150 mg/kg</td>
<td>78.44</td>
</tr>
</tbody>
</table>

n=7
3.10 Effects of *N. nucifera* fruit on lipid profile

Table 11 and 12 gives the comparative effect of total serum cholesterol (TC), triglycerides, high density lipoproteins (HDL-C) and low density lipoproteins (LDL-C) in animals of control and 3 extract groups after 30 and 45 days. No deaths of animal were recorded in any group all through the entire period of study. *N. nucifera* fruit ethanol extract at a dose of 50 mg/kg exhibited considerable lowering effects on TC after 30 days and extremely noteworthy effects after 45 days in comparison to control. The effects of the same dose of extract highly significantly lowered TG and LDL-C after 30 and 45 days whereas HDL-C was raised significantly after 45 days.

*N. nucifera* fruit ethanol extract at a dose of 100 mg/kg revealed highly significant lowering effects on TC after 30 and 45 days as compared to control, whereas the TG and LDL-C lowering effects were also highly significant after 30 and 45 days in comparison to control. The HDL-C was highly significantly increased after 45 days but was not significantly altered after 30 days in comparison to control.

*N. nucifera* fruit ethanol extract 200 mg/kg revealed highly noteworthy TC lowering effects after 30 and 45 days in comparison to control, whereas the TG and LDL-C lowering effects were also highly significant after 30 and 45 days in comparison to control. The HDL-C was significantly increased after 30 days and extremely considerably raised after 45 days as compared to control.
### Table-11

**Effect of *N. nucifera* fruit on lipid profile after 30 days**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cholesterol</th>
<th>Triglycerides</th>
<th>HDL-C</th>
<th>LDL-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 10 ml/kg</td>
<td>139.0±1.30</td>
<td>249.8±1.80</td>
<td>32.7±0.68</td>
<td>179.8±1.10</td>
</tr>
<tr>
<td><em>N. nucifera</em> 50 mg/kg</td>
<td>133.8±0.40*</td>
<td>223.1±0.40**</td>
<td>32.1±0.74</td>
<td>170.3±0.68**</td>
</tr>
<tr>
<td><em>N. nucifera</em> 100 mg/kg</td>
<td>118.0±0.65**</td>
<td>180.5±1.30**</td>
<td>33.8±0.34</td>
<td>152.0±0.82**</td>
</tr>
<tr>
<td><em>N. nucifera</em> 200 mg/kg</td>
<td>90.8±0.86**</td>
<td>142.0±0.82**</td>
<td>36.5±1.1*</td>
<td>129.3±1.0**</td>
</tr>
</tbody>
</table>

n=7
Values are Mean ± S.E.M
*p< 0.05 noteworthy in comparison to control
**p< 0.005 extremely noteworthy in comparison to control
Table-12

Effect of *N. nucifera* fruit on lipid profile after 45 days

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cholesterol</th>
<th>Triglycerides</th>
<th>HDL-C</th>
<th>LDL-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 10 ml/kg</td>
<td>90.4±1.0</td>
<td>187.8±0.80</td>
<td>32.0±0.72</td>
<td>144.43±1.2</td>
</tr>
<tr>
<td><em>N. nucifera</em> 50 mg/kg</td>
<td>83.8±0.74**</td>
<td>172.0±0.82**</td>
<td>35.7±1.2*</td>
<td>133.1±0.91**</td>
</tr>
<tr>
<td><em>N. nucifera</em> 100 mg/kg</td>
<td>71.3±0.89**</td>
<td>151.4±0.90**</td>
<td>39.8±0.40**</td>
<td>112.4±1.10**</td>
</tr>
<tr>
<td><em>N. nucifera</em> 200 mg/kg</td>
<td>54.3±0.97**</td>
<td>131.0±1.60**</td>
<td>43.0±0.53**</td>
<td>92.43±0.87**</td>
</tr>
</tbody>
</table>

n=7

Values are Mean ± S.E.M

*p< 0.05 noteworthy in comparison to control

**p< 0.005 extremely noteworthy in comparison to control
3.11 Effects of *N. nucifera* fruit on coagulation parameters

Table-13 revealed the comparison of *N. nucifera* fruit and warfarin on coagulation parameters. *N. nucifera* fruit extract at dose of 200mg/kg significantly prolonged PT and TT, whereas fibrinogen level was highly significantly reduced as compare to control. Fibrinogen level was also reduced highly significantly with *N. nucifera* fruit extract dose of 100 mg/kg in comparison to control without affecting other parameters of coagulation i.e. aPTT, PT and TT. Conversely warfarin at a dose of 0.54 mg/kg highly significantly prolonged PT and TT and significantly prolonged aPTT as compared to control, whereas fibrinogen level was not affected significantly with warfarin in comparison to control.
Table-13
Outcome of *N. nucifera* fruit and warfarin on coagulation parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control 10ml/kg</th>
<th><em>N. nucifera</em> 50 mg/kg</th>
<th><em>N. nucifera</em> 100 mg/kg</th>
<th><em>N. nucifera</em> 200 mg/kg</th>
<th>Warfarin 0.54 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>aPTT sec</td>
<td>8.4±0.20</td>
<td>8.8±0.34</td>
<td>9.1±0.26</td>
<td>8.8±0.34</td>
<td>9.8±0.34*</td>
</tr>
<tr>
<td>PT sec</td>
<td>5.5±0.76</td>
<td>5.4±0.30</td>
<td>6.1±0.40</td>
<td>6.7±0.29*</td>
<td>8.5±0.37**</td>
</tr>
<tr>
<td>TT sec</td>
<td>9.3±0.30</td>
<td>9.0±0.31</td>
<td>9.7±0.29</td>
<td>10.5±0.37*</td>
<td>12.3±0.30**</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>429.4±1.90</td>
<td>428.5±2.80</td>
<td>409.0±2.10**</td>
<td>356.5±1.50**</td>
<td>431.1±0.91</td>
</tr>
</tbody>
</table>

n=7
Values are Mean ± S.E.M
*p< 0.05 noteworthy in comparison to control
**p< 0.005 extremely noteworthy in comparison to control
3.12 Sub-chronic toxicity study

*Physical examination*

Animals in any group did not reveal any significant toxicities and gross anomalies during the total period of study i.e. 90 days. There was no skin ulceration, loss of hair, loss of activity, vomiting, diarrhea, hematuria, edema, salivation, tremor and aggressive behavior. The data also showed no significant difference in the average weight variation.

*Hematological evaluation*

Table-14 revealed the comparison of hematological parameters i.e. red blood cell count, white blood cell count, platelet count and hemoglobin following 90 days oral administration of *N. nucifera* fruit in three doses i.e. 50, 100 and 200 mg/kg in different groups against control. There were no noteworthy variations noted in any of the parameters at any dose in comparison to control.
Table-14
Effect of different doses of *N. nucifera* fruit on hematological parameters in rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>N. <em>nucifera</em> 50 mg/kg</th>
<th>N. <em>nucifera</em> 100 mg/kg</th>
<th>N. <em>nucifera</em> 200 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cells (×10^6/µl)</td>
<td>7.27±0.01</td>
<td>6.94±0.19</td>
<td>6.76±0.22</td>
<td>6.77±0.21</td>
</tr>
<tr>
<td>White blood cells (×10^3/µl)</td>
<td>6.47±0.09</td>
<td>6.31±0.08</td>
<td>6.40±0.082</td>
<td>6.68±0.05</td>
</tr>
<tr>
<td>Platelet (×10^3/µl)</td>
<td>371.11±0.51</td>
<td>369.63±0.88</td>
<td>369.34±0.95</td>
<td>370.60±0.64</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>9.50±0.19</td>
<td>9.77±0.20</td>
<td>9.80±0.20</td>
<td>9.11±0.04</td>
</tr>
</tbody>
</table>

n=7
Values are Mean ± S.E.M
Biochemical evaluation

Table-15 revealed the effect of *N. nucifera* fruit following its oral administration in three doses i.e. 50, 100 and 200 mg/kg against control on biochemical parameters i.e. total protein, urea, creatinine, alkaline phosphates, ALT and AST. No significant biochemical changes were observed and recorded in groups treated with 50 and 100 mg/kg dose of *N. nucifera* fruit in comparison to control, but the group treated with *N. nucifera* fruit ethanol extract at a dose of 200 mg/kg showed significant increase in blood creatinine and ALT levels in comparison to control, though the changes were in normal physiological limits.
Table-15
Effect of different doses of *N. nucifera* fruit on biochemical parameters in rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th><em>N. nucifera</em> 50 mg/kg</th>
<th><em>N. nucifera</em> 100 mg/kg</th>
<th><em>N. nucifera</em> 200 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Protein g/dl</td>
<td>6.6±0.08</td>
<td>6.3±0.11</td>
<td>6.4±0.11</td>
<td>6.8±0.14</td>
</tr>
<tr>
<td>Urea mg/dl</td>
<td>29.0±1.7</td>
<td>25.5±1.20</td>
<td>27.3±0.64</td>
<td>26.0±1.90</td>
</tr>
<tr>
<td>Creatinine mg/dl</td>
<td>0.7±0.03</td>
<td>0.71±0.02</td>
<td>0.7±0.02</td>
<td>0.8±0.03*</td>
</tr>
<tr>
<td>AST U/l</td>
<td>18.5±1.10</td>
<td>22.1±2.20</td>
<td>19.8±1.50</td>
<td>19.14±1.20</td>
</tr>
<tr>
<td>ALT U/l</td>
<td>22.8±1.70</td>
<td>21.3±0.68</td>
<td>24.7±1.40</td>
<td>28.5±1.50*</td>
</tr>
<tr>
<td>ALP U/l</td>
<td>55.8±3.30</td>
<td>62.8±1.30</td>
<td>64.7±6.30</td>
<td>63.8±2.60</td>
</tr>
</tbody>
</table>

n=7
Values are Mean ± S.E.M
*p*< 0.05 noteworthy in comparison to control
**Histopathological examination of liver and kidneys**

Gross examination of vital organs e.g. liver and kidneys did not reveal any macroscopic changes in any group. Table-16 has represented histopathological changes in liver tissues treated with different doses of *N. nucifera* fruit.
Table-16

**Histopathological changes in hepatic tissues treated with *N. nucifera* fruit**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Fatty change</th>
<th>Vacuolar Degeneration</th>
<th>Necrosis</th>
<th>Portal Inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>N. nucifera</em> 50 mg/kg</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>N. nucifera</em> 100 mg/kg</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>N. nucifera</em> 200 mg/kg</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

(-) Absent, (+) Mild, (++) Moderate

The animals treated with 50 and 100 mg/kg *N. nucifera* fruit ethanol extract showed focal areas of ballooning (Vacuolar) degeneration and mild inflammatory changes around the portal tract (figure 5 and 6).
The animals treated with 200 mg/kg of *N. nucifera* ethanol extract in addition to mild areas of vacuolar degeneration and moderate inflammatory changes also showed areas of necrosis at lesser extent (figure 7&8). However there were no microscopic changes in liver architecture, hepatocytes and central vein in control group (figure 9).
Figure-8:
Hepatic tissue showing few areas of necrosis with ballooning 40x

Figure-9:
Normal hepatic tissue showing intact hepatocytes 40x

Table-17 has represented histopathological changes in renal tissues treated with different doses of *N. nucifera* fruit.
Table-17

Histopathological changes in renal tissues treated with *N. nucifera* fruit

<table>
<thead>
<tr>
<th>Features</th>
<th>Control</th>
<th><em>N. nucifera</em> 50 mg/kg</th>
<th><em>N. nucifera</em> 100 mg/g</th>
<th><em>N. nucifera</em> 200 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerular Disruption</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Tubular Disruption</td>
<td>_</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Tubular Necrosis</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>+</td>
</tr>
<tr>
<td>Interstitial Inflammatory Infiltrates</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Edema</td>
<td>_</td>
<td>_</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

(-) Absent, (+) Mild, (++) Moderate

Group treated with *N. nucifera* fruit 50 mg/kg showed focal areas of tubular derangement (figure 10).
Animals treated with *N. nucifera* fruit ethanol extract 100 mg/kg showed few areas of tubular vacuolization (figure 11).

Animals treated with *N. nucifera* ethanol extract 200 mg/kg revealed areas of moderate tubular disruption and few foci of tubular necrosis along with moderate interstitial edema (figure 12&13).

Animals treated with *N. nucifera* fruit ethanol extract 100 mg/kg showed few areas of tubular vacuolization (figure 11).

Animals treated with *N. nucifera* ethanol extract 200 mg/kg revealed areas of moderate tubular disruption and few foci of tubular necrosis along with moderate interstitial edema (figure 12&13).
Figure-13:
Renal tissue showing congestion, tubular disruption and interstitial edema 40x

No microscopic changes were observed in renal tissues of control group (figure 14)

Figure-14:
Normal renal tissue 40x
DISCUSSION AND CONCLUSION
Recently, use of herbal remedies and dietary supplements rich in flavonoids and vitamins have increased to treat health related problems (Riaz and Khan, 2014). Although many drugs are available in the market to treat cardiovascular, central nervous system, inflammation, blood lipid and coagulation disorders but all of them are associated with various side effects. Hence there is a substantial inclination in the demand of plant derived medications (Rajput et al., 2015). Thus extensive studies are necessary to investigate the pharmacologic and toxic effects of plants, which would facilitate in the discovery of novel drugs from herbal sources and thus recommend the applications of complementary drugs in mankind (Ahmed et al., 2015).

There are studies available which have reported phytochemical analysis of *N. nucifera* seeds (Mukherjee et al., 2009), but very limited literature was available before this study to validate the chemical constituents present in the seed pods of *N. nucifera* fruit, so present study conducted the qualitative phytochemical analysis in order to explore the significant constituents present in the seed pods of *N. nucifera* fruit, and many significant constituents like alkaloids, saponins, tannins, terpenoids and flavonoids were found in it. Acute toxicity study of *N. nucifera* fruit extract was also evaluated and LD$_{50}$ values were noted to be greater than 5 g/kg body weight of the animal.

Anxiety is the most studied psychiatric field in humans (Rauniar et al., 2007; Rajput et al., 2015) whereas various depressive disorders are also increasing intensively in society (William and Leo, 2007). Since research in humans is restricted, animal models have been established for anxiety and depression (Riaz and Khan, 2014).

Current study also illustrated the anti-anxiety activity of *N. nucifera* fruit extract through EPM and light and dark tests. In EPM test comparison of anxiolytic effect of *N. nucifera* fruit extract with control group revealed substantial rise in no. of entries in open arms and time spent in open arms at dose 50 mg/kg whereas no. of entries in closed arms and time spent in closed arms were considerably decreased as compared to control. No. of entries in open arms and time spent in open arms was raised highly significantly at doses of 100 and 200mg/kg whereas time spent in closed arms was tremendously reduced in comparison to control at same doses. Diazepam 1mg/kg exhibited highly substantial increase in no. of entries in open arms and time spent in open arms whereas no. of entries...
in closed arms and time spent in closed arms was decreased highly significantly as compare to control.

Fear of height provokes anxiety in animal when positioned in the EPM. The sign of fear and anxiety in animal is shown by decline in the motor activity and preference to stay at protected places. Anti-anxiety agents are expected to increase the motor activity which is estimated by the time spent in the open arms by the animals. Hence a general stimulatory and anti-anxiety behavior can be speculated by open arm entries (Mansouri et al., 2014). The highly significant rise in open arm entries and time spent in open arms by *N. nucifera* fruit extract at doses 100 and 200 mg/kg were rather similar to standard agent diazepam so it may be assumed that *N. nucifera* fruit extract has strong anxiolytic activity.

Present study also demonstrated the anxiolytic effect of *N. nucifera* fruit extract and diazepam in rats by using light and dark test. The light and dark test is used to assess the activity of anxiolytic agents (Michel and Martine, 2013). There was highly noteworthy rise in percentage of time spent in light compartment at all specified doses of extract as compared to control. Diazepam 1mg/kg also exhibited extremely noteworthy increase in percentage of time spent in light compartment in comparison to control. It was also observed that percentage of time spent in the light compartment by the animal at extract doses 100 and 200 mg/kg was > 50% i.e. 51.8±0.89 and 58.3±0.64, so after treatment with the extract, the level of anxiety was greatly reduced in experimental animals. Hence *N. nucifera* fruit extract has demonstrated strong anxiolytic effect at light and dark box test.

In the CNS various flavones binds to the benzodiazepine site on the GABA<sub>A</sub> receptor producing anxiolytic, sedative and anticonvulsant effects. Flavonoids with anti-anxiety activity have been designated in many plant species used in traditional medicine e.g. *Passiflora coerulea* (Kumar and Sharma, 2005).

In previous study, neferine which is a bisbenzyl isoquinoline alkaloid present in the seeds of *N. nucifera* fruit has exhibited anti-anxiety activity in the EPM without involving
muscle coordination as demonstrated in the rota rod test whereas in the same study diazepam showed anti-anxiety and muscle relaxant effects as well (Sugimoto et al., 2008).

In another study GABA was found to be involved in anxiolytic activity of saponins isolated from *Albizzia Lebbeck* leaves and suggested that saponins act by modifying GABAergic mechanisms (Kumar and Sanjay, 2005). Similarly saponins extracted from *Anilaecapanax quinque folium* L. were studied in male mice using EPM test, light and dark test and hole board test. The finding of this study suggested that saponins from PQS may be a possible nominee to use as an anxiolytic agent (Wei et al., 2007). In addition some terpenes and also various terpene-derived compounds have potentiated the GABAergic responses (Kessler et al., 2014).

Present study also investigated the antidepressant activity of *N. nucifera* fruit at doses 50, 100 and 200 mg/kg by FST. There was a significant decline in the period of immobility of mice at doses 100 and 200 mg/kg on 2nd day of experiment and highly significant decrease was noticed on 15th day of experiment at the same doses. No considerable change was observed at dose 50 mg/kg of extract at 2nd and 15th day of experiment.

*N. nucifera* fruit is a rich source of significant constituents e.g. flavonoids, alkaloids, saponins, and terpenoids which have shown anxiolytic activity in various studies, so it may be suggested that *N. nucifera* fruit exerts its anxiolytic activity through synergistic action of all of these constituents.

FST is the established method employed to assess behavioral sadness in rodents. Despondency is established as a frequent trait of depression in humans and is impersonated in rodents by the paradigm of learned helplessness. Thus FST is considered as a useful animal model for evaluating behavioral dejection in human since the behavioral immobility of animals during forced swimming has been reported to emulate some features of human despair (Porsolt et al., 1977; Wilner, 1984; Yoshimura and Yamakawa, 2000). Diminution in duration of immobility corresponds to antidepressant effect (Taiwoo et al., 2012). Hence *N. nucifera* fruit showed remarkable antidepressant
effect more or less similar to imipramine at doses 100 and 200 mg/kg after continuous administration for 14 days i.e. up to 15th day of experiment.

Nefereine, liensinine and isoliensinine are alkaloids found in the seeds of *N. nucifera* fruit have demonstrated antidepressant activities in previous studies and the central effects of these alkaloids are most likely linked to serotonergic neurotransmission particularly involving 5HT1A receptors in mice (Sugimoto *et al.*, 2010; Sugimoto *et al.*, 2015).

*N. nucifera* fruit have exhibited strong anxiolytic and antidepressant activities and that is perhaps both segments of the *N. nucifera* fruit (seed and seed pod) are rich in significant phytochemical constituents e.g. flavonoids and alkaloids. Moreover the anxiolytic and antidepressant activities of *N. nucifera* fruit were largely comparable to diazepam and imipramine. Hence *N. nucifera* fruit seems to have an immense potential for therapeutic applications especially in the management of CNS disorders, such as anxiety and depression and thus encourage more preclinical and clinical trials in this field.

Epilepsy is the second most commonly encountered neurological disorder affecting around 70 million people worldwide, out of which approximately 80% are in developing countries (Yemedje *et al.*, 2011). Several shortcomings appeared with the use of conventional antiepileptic drugs (AEDs) like, inadequate seizure control, side effects, cost and potentiation of epilepsy-induced co-morbidities such as depression, memory deficits and mood disorders, which limit their use as comprehensive therapy. Therefore, satisfactory treatment of epilepsy not only demands the suppression of abnormal neuronal discharge but also control of epileptic co-morbidities associated with it along with wide margin of safety and low cost (Paramdeep *et al.*, 2014).

Present study revealed the antiepileptic effect of *N. nucifera* fruit extract and diazepam using strychnine induced seizure model in rats and time of commencement/onset of seizures, period/duration of seizures along with percentage of animals remain protected/avoided convulsions was recorded. *N. nucifera* fruit ethanol extract exhibited pronounced antiepileptic activity at a dose of 200 mg/kg which resulted in highly significant delay in the commencement of seizures whereas period of convulsions was
also increased significantly at the same dose of test drug but the intensity of convulsions was reduced which resulted in better percentage of animal survived i.e. 42.85% from seizures.

In the central nervous system various flavonoids have been found to inhibit almost all the mechanisms involved in seizures generation in epilepsy e.g. inhibition of voltage gated Na$^+$ channels results in decrease sodium ion influx into the neuronal cell (Nicholson et al., 2010); activation of Ca$^+$ activated K$^+$ channels results in increased potassium ion outflow from the neuronal cell (Engelborghs et al., 2000); activation of inhibitory GABAergic receptors through direct action on GABA or through benzodiazepine receptor results in increased chloride ion influx causing neuronal hyperpolarisation (Hanrahan et al., 2011); inhibition of opioid receptors leading to proconvulsant or anticonvulsant effects (Engelborghs et al., 2000); Inhibition of NMDA receptor, results in decrease entry of calcium into the neuronal cell (Subash and Subramanian, 2009); and antioxidant effects of flavonoids leading to increased seizure threshold (Paramdeep et al., 2014).

Progression of epilepsy is also dependent on the development of free radicals. Raised levels of free radicals are coupled with low level of antioxidants, which results in the neuronal damage. It is already documented that development of free radicals provoke convulsions through inhibiting enzyme glutamine synthase which leads to accumulation of stimulatory neurotransmitter, glutamate or by decreasing glutamate decarboxylase, thus causing decrease in GABA turnover (Devi et al., 2008). Considering this, flavonoids are a bioactive metabolite of choice for the treatment of oxidative stress induced diseases like, epilepsy. Flavonoids, due to their antiepileptic and neuroprotective effects have been found to ameliorate the co-morbidities. They interact with critical protein and lipid kinase signaling cascades e.g. phosphatidylinositol-3 kinase (PI3K)/Akt, protein kinase C and mitogen-activated protein kinase in the brain, leading to inhibition of apoptosis activated by neurotoxic species and promote neuronal endurance and synaptic plasticity. Moreover, flavonoids exert positive impact on the vascular system which results inmodification in
cerebral blood supply and are able enough to speed up, angiogenesis, neurogenesis and transformations in neuronal morphology (Vauzour et al., 2008).

Procyanidin and tannins present in the seed pod of *N. nucifera* fruit have demonstrated antioxidant effects which may also contribute in reducing the oxidative stress by lowering the levels of free radicals in the brain, responsible for neuronal damage (Ling et al., 2005). Similarly terpenoids in various studies have exhibited alteration in GABA mediated system and diminution in neuronal excitability by jamming the voltage reliant sodium channels (De et al., 2008). Saponins loaded fraction of *ficus platyphyla* stem bark also exhibited anticonvulsant activity on strychnine induced and PTZ seizures (Chindo et al., 2009).

Hence it can be stated that the *N. nucifera* fruit exerts antiepileptic effect mainly due to the constituents present in it specially flavonoids which have established anticonvulsant effects. Moreover current studies have also demonstrated antidepressant activity of this fruit; hence it can be postulated that *N. nucifera* fruit may be useful in managing the co-morbidities associated with epilepsy such as depression.

Lots of individuals who experience severe, inexorable and excruciating pain, for instance that resulting from cancer or injury has to rely on morphine, in spite of its established adverse outcomes. Similarly unceasing anti-inflammatory conditions such as rheumatoid arthritis and osteoarthritis are mostly treated with non-steroidal anti-inflammatory agents. Although these synthetic agents are dominating the market but issue of toxicity with prolonged use of these agents cannot be ruled out, the most frequent being GIT bleeding and ulcers (Yesilada et al., 1997; Corley et al., 2003). Hence there is a need to develop new, safe and effective analgesic and anti-inflammatory agents with minimum toxicity profile (Singh et al., 2010).

In current study analgesic effects of *N. nucifera* fruit ethanol extract was evaluated utilizing couple of animal models against aspirin as it is a remarkably well recognized analgesic agent, produces analgesia via non selective and irreversible inhibition of cyclo-oxygenase enzyme (COX) which in turn decreases the synthesis of prostaglandins and
reduction of prostaglandins reduces pain, inflammation and fever (Besson and Chaouch, 1987). Despite the fact that aspirin does have a central component of action but it primarily produces analgesia via its peripheral action (Barsante et al., 2005).

Tail flick test is efficient in guesstimating the potency and efficacy of centrally acting analgesics (Brownlee, 1950). In tail flick latency difference N. nucifera fruit exhibited highly noteworthy analgesic action at all doses; nevertheless the effects were especially intense at doses 100 and 200 mg/kg. The peak anti-nociceptive effect at all doses of fruit extract was observed at 90 minutes after which gradual decline in analgesic activity was recorded which was highly similar in pattern with aspirin. The percentage of tail elongation time was highest at a dose of 200 mg/kg i.e. 82% at 90 minutes. The higher the % elongation of the group the greater is the group’s central analgesic effect (Islam et al., 2014). Hence it can be stated that N. nucifera fruit extract at dose of 200 mg/kg possesses strong central analgesic activity at 90 minutes after its administration.

Acetic acid induced writhing test is a widely used method for the evaluation of visceral pain model in rodents (Moloney et al., 2015). It is also known as the abdominal constriction response, and is very perceptive in detecting anti-nociceptive activities of agents at dose levels that may appear inactive in other procedures (Bentley et al., 1981; Bentley et al., 1983).

Present study depicted and confirmed analgesic effects of N. nucifera fruit using acetic acid induced writhing test. No. of writhes were highly significantly reduced at doses of 50, 100 and 200 mg/kg of N. nucifera fruit, but maximum effects were observed at extract dose of 200 mg/kg in comparison to control (indicating 48.41 % inhibition of writhes).

Local presence of peritoneal receptors is assumed to be partially involved in abdominal constriction reaction (Derardt et al., 1980). This method is linked with increased levels of PGE2 and PGF2α as well as lipoxygenase products in abdominal fluid (Levini et al., 1984; Dhara et al., 2000). Acetic acid intraperitoneal administration also causes the release of inflammatory mediators such as bradykinin and histamine which in turn excites nerve
fibers responsible for transmitting signals to the higher centers of the brain and spinal cord which amalgamate and modulate nociception (Evan and Andrew, 2004).

Since flavonoids, saponins and tannins are important secondary metabolites of *N. nucifera* fruit and have exhibited inhibitory effects on arachidonic acid metabolism and interfere with prostaglandin synthesis (Ahmadiani *et al.*, 1998; Ahmadiani *et al.*, 2000; Chindoet *et al.*, 2010). Therefore, the results of the tail flick test and acetic acid-induced writhing test strongly recommend that the mode of action of *N. nucifera* fruit may be connected with the inhibition of arachidonic acid metabolism.

Present study also revealed anti-inflammatory activity of *N. nucifera* fruit which incredibly decreased the paw edema volume at all doses from 3rd to 5th hour as compared to control with maximum percent reduction of edema was calculated at a dose of 100 mg/kg i.e. 73.92% at 5th hour after administration of carrageenan. The first phase of the edema i.e. 1st and 2nd hour was not affected by fruit. Acute inflammation induced by carrageenan is one of the most appropriate techniques to screen agents with anti-inflammatory activity. The time course of edema development in carrageenan-induced hind paw edema model in rats is frequently characterized by a biphasic curve (Vinegar *et al.*, 1969). The initial phase of inflammation arises within an hour of carrageenan injection and is somewhat because of trauma at the injection site and also due to histamine and serotonin element (Crunkhorn and Meacock, 1971). The second phase of edema primarily starts from 3rd hour and is sensitive to COX inhibitors such as NSAIDS (Singh *et al.*, 2010). Hence, in present study there was no inhibition of histamine and serotonin at the first phase of the test but cyclooxygenase pathway was effectively inhibited at the second phase of the test.

Flavonoids exert anti-inflammatory effects by several mechanisms; one of them is their proposed capability to diminish neutrophil degranulation. This represents the shortest possible way to inhibit the release of arachidonic acid by neutrophils and other immune cells. Neutrophils containing lipoxygenase produce chemotactic factors from arachidonic
acid which also stimulate the release of cytokines (Hoult et al., 1994; Tordera et al., 1994).

An additional anti-inflammatory characteristic is the potential of flavonoids to reduce the level of eicosanoid synthesis (Damas et al., 1985). Eicosanoids e.g. prostaglandins are implicated in diverse immunologic responses and are the end products of the cyclooxygenase and lipoxygenase pathways (Hoult et al., 1994). Flavonoids also diminish both cytosolic and membranal tyrosine kinase (Friesenecker et al., 1995). Integral membrane proteins e.g. tyrosine 3-monooxygenase kinase is involved in performing variety of functions for instance enzyme catalysis, transport across membrane sand transduction of signals that function as receptors of hormones and growth factors and energy transfer in ATP synthesis. Inhibition of such proteins results in reduction of uncontrolled cell growth and proliferation. Tyrosine kinase substrates appear to play chief role in the signal transduction pathway that controls cell proliferation (Hoult et al., 1994; Tordera et al., 1994).

Certain flavonoids are capable of reducing complement activation, thus diminishing the sticking together of inflammatory cells to the endothelium and overall results in a reduced inflammatory response (Friesenecker et al., 1995).

Saponins and tannins have also reported inhibitory effects on the arachidonic acid metabolism (Ahmadiani et al., 2000; Chindo et al., 2010). Hence it can be stated that the anti-inflammatory effects of N. nucifera fruit may be due to the presence of flavonoids, saponins and tannins which synergistically exert inhibitory effects on the arachidonic acid metabolism, neutrophil degranulation and on enzyme systems which promote cell growth, proliferation and regulates complement cascade.

Hypercholesterolemia or high levels of cholesterol in the blood are primarily a metabolic derangement that can contribute to many diseases such as CVD and stroke. Hypercholesterolemia or hyperlipidemia may occur due to abnormalities in lipoproteins, the particles that bring cholesterol to the blood stream. Hyperlipidemia has a strong association with atherosclerosis (Allen et al., 1996). LDL cholesterol gets deposited in
the blood vessel walls and plays major role in the formation of atherosclerotic plaque. These pathological processes can be reversed by lowering the serum LDL level (Ross, 1993). Moreover increase in serum HDL level has shown to reduce the progression of atherosclerosis (Stein, 1999; Nofer, 2002).

Current study was distinctively planned to assess the hypolipidemic activities of *N. nucifera* fruit after 30 and 45 days in rabbits kept on high cholesterol diet for 30 days. The results showed remarkable cholesterol lowering effects of *N. nucifera* fruit extract at doses 50, 100 and 200 mg/kg but were particularly dominant at 200 mg/kg at which fruit extract highly significantly reduced TC, TG and LDL-C after 30 and 45 days and increased HDL-C significantly after 30 days and highly significantly after 45 days.

Previous studies have established an inverse relationship between flavonoids intake and total lipid concentration in plasma. Diet rich in flavonoids protects against coronary artery diseases (Arai *et al.*, 2000; Hertog *et al.*, 1995). Similarly tannins and procyanidin, significant component of the seed pods of *N. nucifera* fruit have demonstrated lipid auto-oxidation and free radical scavenging activity (Ling *et al.*, 2005). In several studies saponins have been shown to inhibit the intestinal absorption of cholesterol and there by reduces plasma lipid levels in variety of animal models (Harwood *et al.*, 1993; Sauvaire *et al.*, 1991).

Previous studies on anti-obesity and hypolipidemic activities of *N. nucifera* seed resulted in inhibition of lipid accumulation and reduced expression of peroxisome proliferator activated receptor gamma (PPAR), leptin in cultured human adipocytes and glucose transporter (GLUT 4) indicating the inhibitory effect on the differentiation of pre- adipocytes in to adipocytes (You *et al.*, 2013). Moreover flavonoids, saponins, tannins and procyanidin are significant constituents of *N. nucifera* fruit, hence it can be postulated that lipid lowering effects of *N. nucifera* fruit can be due to the presence of all of these constituents which can contribute in lowering the risk of cardiovascular diseases and stroke.
Abnormalities of coagulation are frequently present in seriously sick patients and usually result in disability and death hence requires prompt diagnosis and treatment (Marcel and Steven, 2006). Acute platelet thrombus formation leads to the development of atherosclerosis, followed by embolization of stenosed vessels (Lou et al., 1989). Platelets and thrombin are interdependent; since thrombin induced activation of platelets is as important as platelets availability for thrombus formation (Riaz et al., 2009).

Results of the current study revealed that N. nucifera fruit extract at dose of 200mg/kg significantly prolonged PT and TT, whereas fibrinogen level was highly significantly reduced as compared to control. Fibrinogen level was also reduced highly significantly with N. nucifera fruit extract dose of 100 mg/kg as compared to control without affecting other parameters of coagulation i.e. aPTT, PT and TT.

Neferine, an alkaloid and one of the constituents of N. nucifera seed has exhibited antithrombotic effects by inhibiting platelet activation, adhesion and aggregation (Zhou et al., 2013). Similarly liensinine which is also an isoquinoline alkaloid like neferine has significantly inhibited platelet aggregation and prolonged PT, aPTT and TT and thus exhibited strong effects against thrombus formation (Wang et al., 2010).

Previous studies have demonstrated, powerful antithrombotic effects of flavonoids and is thought to be by its inhibitory effect on thromboxane A₂ formation. Thromboxane A₂ increases the expression of glycoprotein 11b/111a receptor complex on the membranes of the platelets and thus stimulates platelet aggregation. Circulating fibrinogen sticks to these platelets and give further strength to the clot (Tzeng et al., 1991). Since flavonoids and alkaloids (neferine & liensinine) are significant constituents present in N. nucifera fruit, so it can be stated that antithrombotic effects of N. nucifera fruit may be due to the presence of these constituents in it.

The uses of herbal drugs are becoming progressively more popular as they are supposed to be natural, advantageous and lack unwanted effects (Leonardo et al., 2000). Mostly the plant derived drugs are taken randomly by local population for the treatment of various diseases without having adequate information on the safety and toxicity related with their
use. However the accompanying adverse effects are usually mild and affect small number of people. Hence for proper guidance of the general population, especially users of these natural products, there is a need to document the safety and toxicity profile of these medicinal plants (Agbaje et al, 2009).

Sub-chronic study was also a significant component of present study in order to evaluate the safety profile of N. nucifera fruit after its administration to rats in 3 doses i.e. 50, 100 and 200 mg/kg for 90 days and then the hematological, biochemical and histopathological parameters were compared to control group.

Hematological parameters were not altered significantly with any dose of N. nucifera fruit extract as compared to control. In addition to this, biochemical changes were also not exposed in groups treated with 50 and 100 mg/kg N. nucifera fruit but significant increase was noted in ALT and creatinine of group treated with 200 mg/kg of N. nucifera fruit as compared to control, however the rise in both parameters were in normal physiological limits.

Certain enzymes, not originally produced in serum, but in fact leak in to the serum at some point of tissue damage, are a valuable source in clinical diagnosis. They are informative on the effect and nature of pathological loss to any tissue. ALT & AST, sensitive markers of hepatocellular loss, can provide a quantitative assessment of certain degree of damage to liver (Will's et al., 1985; Al-Habori et al., 2002).

Generally, ALT levels are constantly higher than AST and the reason for this is that body cells generate little higher ALT in contrast to AST (Mayne, 1996). Approximately 80% of ALT is originated in mitochondria while AST solely resides in cytoplasm; as a result ALT emerges in greater concentrations from various tissues such as liver, kidneys, heart and pancreas and is released gradually as compared to AST. Though AST is confined primarily to cytosol of hepatocytes but is believed to be a more sensitive indicator of hepatocellular injury than ALT (Saritha and Anilakumar, 2010).

Similarly urea and creatinine are sensitive indicators of renal function (Odoula et al., 2007). Damage to glomeruli causes substantial decline in the GFR and may raise the
levels of both markers in the blood which subsequently results in chronic renal failure (Ramakrishnan and Swami, 1995).

In present study gross inspection of hepatic and renal tissues did not show any noteworthy macroscopic variations. Microscopic examination of hepatic and renal tissues also showed no substantial changes in animals of control and extract treated groups of 50 and 100 mg/kg. In addition to this most of the hepatic and renal tissue sections of animals treated with *N. nucifera* fruit 200 mg/kg did not show any noteworthy histopathological change, however mild to moderate inflammatory changes in hepatic and renal cells of few animals were noted, however such changes in general are reversible. The results of present study suggested that *N. nucifera* fruit maybe used in moderate doses if required for prolonged period however, more investigations are required on large and higher groups of animals to confirm these findings.

Hence it may be concluded that *N. nucifera* fruit ethanol extract has enormous therapeutic potential to be used in CNS disorders such as anxiety, depression and epilepsy and also in inflammatory conditions. Its lipid lowering and anticoagulant ability may be of value in managing CVDs and stroke. It is highly safe for short term use but may be used in moderate doses if required for prolonged period however, more investigations are required in this field to confirm these findings.

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ANNEXURE
Phytochemical screening, acute toxicity, anxiolytic and antidepressant activities of the Nelumbo nucifera fruit

Muhammad Ali Rajput & Rafeeq Alam Khan

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Abstract Recently use of herbal therapies and diet rich in flavonoids and vitamin C have increased significantly to treat minor to modest anxiety disorders and various forms of depression. But further research and studies are necessary to evaluate the pharmacological & toxicological effects of plants. Hence present study was designed to conduct phytochemical screening, acute toxicity study, anxiolytic and antidepressant activities of the ethanol extract of Nelumbo nucifera fruit in order to ascertain its therapeutic potential. The qualitative phytochemical screening of the seed pods of the N. nucifera fruit extract exposed the existence of flavonoids, saponins, alkaloids, tannins and terpenoids in it. The acute toxicity of the N. nucifera fruit extract in mice revealed its LD$_{50}$ value to be greater than 5000 mg/kg. Antianxiety activity was determined by elevated plus maze and light and dark test using 35 male Wister rats weighing 200–220 g which were equally divided in to 5 groups. The animals used in EPM underwent testing in light and dark box just 30 min after EPM. The antidepressant effect was assessed by forced swimming test using 35 male albino mice weighing 20–25 g equally divided in to 5 groups. In elevated plus maze, N. nucifera fruit extract exhibited sub-stantial rise in number of open arm entries and time spent in open arms at dose 50 mg/kg while highly noteworthy increase in both parameters were observed at extract doses 100 and 200 mg/kg as compared to control. In light dark test highly significant increase in the percentage of time spent in light compartment was observed as compared to control. In forced swimming test highly noteworthy decline in duration of immobility was recorded at doses 100 and 200 mg/kg on 15th day i-e after administration of 14 doses, as compared to control; whereas same doses demonstrated significant decrease as compared to control in duration of immobility after single dose administration i-e on 2nd day of experiment. Thus N. nucifera fruit have exhibited strong anxiolytic and antidepressant effects and proved to have a great potential for therapeutic applications such as anxiety and depression and thus encourage more preclinical and clinical trials in this field.

Keywords Nelumbo nucifera · Anxiolytic · Elevated plus maze · Light and dark test

Introduction

Anxiety is defined as a state of extreme trepidation, uncertainty and a fear resulting from anticipation of a future threat. Remaining anxious throughout life has many ad-verse implications on subjective wellbeing and physical health (O’Donovan et al. 2013). Depression can be de-fined as a neurotic or psychotic state characterized by an inability to concentrate, alteration in sleep pattern and feel-ings of extreme sadness, rejection and hopelessness (William and Leo 2007) however, since research in humans is restricted, animal models of depression have been established (Riaz and Khan 2014).

Herbal Medicine is the practice or art of consuming herbs and herbal remedies to maintain health and to avoid, relieve, or cure disease. Today active research is underway to discover the safe and pharmacologically active herbs. A number of anxiolytics and antidepressants are currently available in the market but nearly all are linked with some restrictions. Hence there is a substantial rise in demand for medicinal plants (Rajput et al. 2012).
N. nucifera belongs to the mono-generic family Nymphaeaceae and is known by some common names like bean of India, Indian lotus, Chinese water lily and sacred lotus or simply lotus (Nelumbium nelumbo, N. speciosa, N. speciosum and Nymphae nulumbo). The Linnaean binomial N. nucifera Gaertn, is currently recognized name of this species and is widely cultivated in the tropical regions of Pakistan, India, China, Thailand and Australia (Mukherjee et al. 2009).

N. nucifera fruit consists of seed pods, also known as lotus bulbs, are green in color and provides attachment to the seeds, which are black in color, hard in nature, ovoid, oblong or roundish in shape, up to 1.0 m long and 1.5 cm broad and are organize in whorls. The seeds are the edible portion and are studded in the pod or bulb and have to be peeled individ-ually before they are eaten. The seed is possibly the most popular source of protein in the plant (Sridhar and Bhat 2007).

The seeds of N. nucifera are rich in asparagin, fat, protein, starch, saponins and tannin. The main secondary metabolites present in lotus seeds are alkaloids, particularly dauricine, lotusine, nuciferine, pronuciferine, liensinine, isoliensinine, roemerine, neferine, armepavine and procanadin. Seeds also have gallic acid, isoquininolinol, saponins and carbohydrates (Mukherjee et al. 2009).

Traditionally the seeds of N. nucifera fruit are generally employed as a healthy food in Asia and also use to treat various ailments including skin diseases, tissue inflammation, cancer, leprosy, poison antidote, astringent, emollient and diuretic (Paudel and Panth 2015; Mukherjee et al. 2009).

Due to limited literature on the chemical constituents present in the seed pods of N. nucifera fruit, present study conducted the qualitative phytochemical analysis in order to explore the significant constituents present in the seed pods of N. nucifera fruit, followed by acute toxicity study and evaluation of anxiolytic and antidepressant effects of the ethanol extract of N. nucifera fruit using elevated plus maze test, light and dark test and forced swimming test.

Materials and methods

All experiments were performed in the Department of Pharmacology, University of Karachi, Karachi, after approval from Board of Advance Studies & Research (BASR), and departmental research committee.

Animal housing

Animals were kept in plastic cages in natural day and night cycle with room temperature 23 ± 2 °C and moisture (50 to 60%) in an alternating 12-h light/dark cycle. All animals were given standard diet made in laboratory and water ad labitum. The animals were moved to the laboratory at least one hour before starting the experiment. All experiments were complet-ed in day time.

The research committee Department of Pharmacology per-mitted the use of animals for these experiments in accordance with the guidelines of NACLAR (National Advisory Committee for Laboratory Animal Research 2004) and NIH (The National Institute of Health Guide for the Care and Use of Laboratory Animals 2010).

Drugs

Gum tragacanth was obtained from Merck, while diazepam 5 mg and imipramine 25 mg tablets were procured from local medical store in Karachi. 2% gum tragacanth in the dose of 10 ml/kg was administered orally to control animals as place-bo, while it was also used as suspending agent to formulate suspension of the test drug (N. nucifera fruit) and standard drugs i.e. diazepam and imipramine (Rajput et al. 2013). Diazepam was administered in a dose of 1 mg/kg after suspending in 2% gum tragacanth with the help of orogastric tube (Rajput et al. 2015).

Imipramine was also given through orogastric tube pow-dered as 2% gum tragacanth suspension in a dose of 25 mg/kg to mice (Riaz and Khan 2014).

Plant material and preparation of extract

The N. nucifera fruits were purchased from a local fruit & vegetable market of Qasimabad, Hyderabad, Sindh, Pakistan. The N. nucifera fruits were identified and authenti-cated in the Department of Pharmacognosy. The voucher specimen no NNF-03 was placed in the department of Pharmacognosy, University of Karachi.

The preparation of crude extract followed cold extraction process (Hossain et al. 2010). 6 kg fruits were washed and the seeds were peeled off from the seed pods of the fruits manually and were then chopped. After that chopped seeds were dried under shade for 6 days. The dried material was ground to coarse powder. The seed pods on the other hand were dried under shade for 3 days and then chopped to fine powder. The seed powder along with the seed pod powder was macerated in 10 l of 98% ethanol for 30 days with occasional shaking and stirring until the color of the solvent becomes black.

The solvent was filtered through filter paper (Whatman No. 1). After filtration the extract was evaporated under reduced pressure in a rotary evaporator at 40 °C - 45 °C, followed by freeze drying at -30 °C. The solid Lyophilized fruit extract so gained was saved at -20 °C until further use in different doses for the determination of acute toxicity and anxiolytic activity of N. nucifera fruit. The resultant yield of the extract was 400 g of dry weight.
Phytochemical screening

2 kg of N. nucifera fruit was washed and seeds were peeled off manually. Seed pods were then dried under shade for 3 days and chopped to fine powder which was then soaked in 4 l of 98% ethanol for 30 days. Soaked material was then filtered through (Whatman No. 1) filter paper. After filtration extract was evaporated under reduced pressure in a rotary evaporator at 40 °C - 45 °C, followed by freeze drying at -30 °C (Hossain et al. 2010). The solid Lyophilized plant material so gained was saved at -20 °C until further use for the assessment of qualitative phytochemical screening.

Qualitative phytochemical screening of the seed pods of the N. nucifera fruit extract was conducted at Industrial Analytical Center (IAC), HEJ Research Institute of Chemistry, University of Karachi, Karachi, sample code # IAC/TR/7008, dated 08.25.2016, in order to evaluate the pres-ence or absence of flavonoids, alkaloids, saponins, tannins and terpenoids according to the previously described method (Edeoga et al. 2005; Sofowora 1993).

Acute toxicity study

Animals used for this study were 12 male albino mice weighing 20–25 g and were bred at the animal house of the Department of Pharmacology, University of Karachi. Before the commencement of drug administration, evident health of the animals in the acclimatization period under the laboratory environment was observed for a week and then the LD50 was estimated as described by the Lorke 1983.

The study was conducted in two phases i.e. Phase I and II. In phase I, nine mice were equally divided in to three groups i.e (n = 3) and each group was administered different doses (10, 100 and 1000 mg/kg) of ethanol extract of N. nucifera fruit and then animals were monitored for 7 days for any acute toxicity feature i.e. behavioral feature and mortality. Phase II involves the use of 3 animals which were distributed in three groups of one animal each. The animals were administered higher doses of extract i.e. 1600, 2900 and 5000 mg/kg and were again monitored for a week for signs of behavioral toxicity as well as mortality.

Elevated plus maze test

It is a widely employed assay to determine anxiety like behav-ior in rodents. It is also helpful in assessing the antianxiety effects of pharmacological agents and to define mechanism underlying anxiety (Walf and Frye 2007).

35 male Wister rats weighing 200–220 g were equally divided into 5 group’s i.e. 7 animals in every group. Group I was kept as control and given 10 ml/kg of vehicle (Gum tragacanth). Group II, III, IV served as treated groups and were administered 50, 100 and 200 mg/kg of N. nucifera fruit extract and group V served as standard and was administered Diazepam 1 mg/kg for 15 days through orogastric tube. The EPM comprises of two open arms (50 cm long and 10 cm wide) and two closed arms (50 cm long, 10 cm wide and 38 cm high with open roof) placed opposite to each other around 10 × 10 cm platform. Each animal was exposed to the maze for 5 min and then No. of entries in open and closed arms and time spent in open and closed arms were noted through stop watch (Riaz and Khan 2014).

Light and dark test

The light and dark test was carried out as designed by Gong et al. 2006 with slight changes. The animals used in EPM underwent testing in light and dark box just 30 min after EPM. The equipment used in the test consisted of a dark safe cubicle and a light aversive cubicule. Dimensions of the cubicule are commonly 1/3 for the dark compartment and 2/3 for the light compartment with an external size of 46 × 27 × 30 cm. During the test, the rats were positioned at the midpoint of the light compartment with their back towards dark compartment. The percentage of time spent for each animal in the light compartment was noted for 5 min through stop watch. After 5 min, rats were removed from the box by the base of their tails and returned to their home cage. The maze was then cleaned with a solution of 10% ethanol and allowed to dry between tests. Anxiety is thought to be high if percentage of time spent in the light compartment is less than 50% (Michel and Martine 2003).

Forced swimming test

Modified Porsolt et al. 1977 method was used. 35 male albino mice weighing 20–25 g were equally divided in to 5 groups i.e. 7 animals in each group. Group I was kept as control and given 10 ml/kg of vehicle (Gum tragacanth). Group II, III, IV served as treated groups and were administered 50, 100 and 200 mg/kg of N. nucifera fruit extract and group V served as standard and was administered Imipramine 25 mg/kg for 14 days. All drugs were given orally through orogastric tube. Study duration was fifteen days and 14 doses were given (from 2nd to 15th day).

This method consists of two sessions, the pretest and the test session, where as in old Porsolt’s method, the animals were subjected to direct immersion after injecting drugs. 40 min before test session. In the pretest session on 1st day, the animals were allowed to swim separately for 15 min in a glass tank (height: 40cms, diameter: 24cms) containing 15cms water at 25 ± 2 °C, after which the animals were removed, dried and returned to home cages. On the 2nd day test session was performed for single dose studies and each mouse was forced to swim for 5 min and variable measured was duration of immobility i-e the time at which animal halts swimming.
except for those movements which keeps its head above water was recorded through stop watch. On day 15th, same procedure was performed after completion of 14 doses (Reddy and Gawade 2006).

Statistical analysis

The data was subjected to analysis by taking mean and standard error to the mean using two sample student T-test, values of $P < 0.05$ were regarded as significant and $P < 0.005$ as highly significant. All statistical methods were carried out through SPSS software version 20 (Walpole 1982).

Results

Phytochemical screening

The qualitative phytochemical screening of the ethanol extract of the seed pods of N. nucifera fruit demonstrated the presence of flavonoids, alkaloids, terpenoids, saponins and tannins as summarized in Table 1.

Acute toxicity study

All animals in both phases tolerated doses of extract up to 5000 mg/kg body weight as observed for 24 to 48 h. There were no signs of behavioral toxicity including salivation, lacrimation, defecation, urination, over activity, aggressiveness, piloerection, twitches, tremors and convulsions and no deaths were recorded during this period.

Table 1 Qualitative phytochemical examination of the seed pods of N. nucifera fruit extract

<table>
<thead>
<tr>
<th>Test performed</th>
<th>Observations</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoids</td>
<td>Yellow color</td>
<td>+</td>
</tr>
<tr>
<td>Effatted extract + ethanol and filter; AlCl$_3$</td>
<td>Orange red precipitates/ turbidity</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>Brown precipitates formed</td>
<td>+</td>
</tr>
<tr>
<td>Dragendorff’s test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Terpenoids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decolorized extract residue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ chloroform + acetic anhydride</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ conc: H$_2$SO$_4$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saponins</td>
<td>Frothing occurred</td>
<td>+</td>
</tr>
<tr>
<td>Extract shaken vigorously in a test tube for 2 min</td>
<td>Dark greenish</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FeCl$_3$ test</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$B^{+^\wedge}$ present

Elevated plus maze test

Table 2 reveals the comparison of anxiolytic effect of N. nucifera fruit extract and diazepam with control in rats by elevated plus maze test. No. of entries in open arms and time spent in open arms was significantly augmented whereas no. of entries in closed arms and time spent in closed arms was significantly decreased at dose of 50 mg/kg as compared to control. No. of entries in open arms and time spent in open arms was increased highly significantly at doses of 100 and 200 mg/kg whereas time spent in closed arms was decreased highly significantly as compared to control. However, no significant change in no. of entries in closed arms was observed at extract dose of 100 mg/kg. Diazepam 1 mg/kg exhibited highly significant rise in no. of entries in open arms and time spent in open arms whereas no. of entries in closed arms and time spent in closed arms was highly significantly decreased as compare to control.

Light and dark test

Table 3 demonstrates the comparison of anxiolytic effect of N. nucifera fruit extract and diazepam with control in rats by using light and dark box test and percentage of time spent in light compartment was recorded. There was highly significant increase in percentage of time spent in light compartment at extract doses 50, 100 and 200 mg/kg as compare to control. Diazepam 1 mg/kg also exhibited highly significant rise in percentage of time spent in light compartment in comparison to control.

Forced swimming test

Table 4 shows the comparison of antidepressant effect of N. nucifera fruit extract and Imipramine with control in mice using forced swimming test and immobility time was recorded. N. nucifera fruit has demonstrated substantial decline in the duration of immobility in mice at doses 100 and 200 mg/kg on 2nd day of test session in comparison with control. Duration of immobility was decreased highly significant as compared to control at the same doses of extract on 15th day of experiment. But did not show any significant effect on the duration of immobility on 2nd and 15th day as compare to control. On the other hand Imipramine 25 mg/kg decreased highly significantly the duration of immobility on 2nd and 15th day as compared to control.

Discussion

Anxiety is the most studied psychiatric field in humans as 1/8th of the population of the world suffers various anxiety disorders (Rauniar et al. 2007; Rajput et al. 2015) whereas
Table 2  Anxiolytic effect of N. nucifera fruit extract and diazepam in elevated plus maze

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of entries in open arms</th>
<th>Time spent in open arms (s)</th>
<th>No. of entries in closed arms</th>
<th>Time spent in closed arms (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 10 ml/kg</td>
<td>2.7 ± 0.29</td>
<td>127.5 ± 3.30</td>
<td>5.6 ± 0.37</td>
<td>172.4 ± 3.30</td>
</tr>
<tr>
<td>N. nucifera 50 mg/kg</td>
<td>3.8 ± 0.34*</td>
<td>138.4 ± 2.90*</td>
<td>5.0 ± 0.31*</td>
<td>161.6 ± 2.90*</td>
</tr>
<tr>
<td>N. nucifera 100 mg/kg</td>
<td>5.3 ± 0.42**</td>
<td>150.6 ± 0.79**</td>
<td>4.8 ± 0.40</td>
<td>149.3 ± 0.79**</td>
</tr>
<tr>
<td>N. nucifera 200 mg/kg</td>
<td>7.1 ± 0.51**</td>
<td>175.8 ± 1.90**</td>
<td>3.1 ± 0.40**</td>
<td>124.1 ± 1.90**</td>
</tr>
<tr>
<td>Diazepam 1 mg/kg</td>
<td>5.0 ± 0.53**</td>
<td>168.3 ± 1.80**</td>
<td>4.1 ± 0.40**</td>
<td>131.6 ± 1.80**</td>
</tr>
</tbody>
</table>

n = 7
Values are Mean ± S.E. M
*p < 0.05 significant as compared to control
**p < 0.005 highly significant as compared to control

Table 3 Anxiolytic effect of N. nucifera fruit extract and diazepam in light and dark test

<table>
<thead>
<tr>
<th>Groups</th>
<th>Percentage of time spent in light compartment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 10 ml/kg</td>
<td>36.2 ± 1.50</td>
</tr>
<tr>
<td>N. nucifera 50 mg/kg</td>
<td>45.4 ± 0.67**</td>
</tr>
<tr>
<td>N. nucifera 100 mg/kg</td>
<td>51.9 ± 0.89**</td>
</tr>
<tr>
<td>N. nucifera 200 mg/kg</td>
<td>58.3 ± 0.64**</td>
</tr>
<tr>
<td>Diazepam 1 mg/kg</td>
<td>52.8 ± 1.10**</td>
</tr>
</tbody>
</table>

n = 7
Values are Mean ± S.E. M
**p < 0.005 highly significant as compared to control

Present study was conducted to screen the phytochemical constituents present in the seed pods of N. nucifera fruit extract and many significant constituents like alkaloids, saponins, tannins, terpenoids and flavonoids were found in it. Acute toxicity study of N. nucifera fruit extract was also evaluated and LD₅₀ values were found to be more than 5 g/kg.

Present study also investigated the anxiolytic activity of N. nucifera fruit extract through elevated plus maze test and light and dark test. In EPM test comparison of anxiolytic effect of N. nucifera fruit extract with control rats revealed substantial rise in no. of entries in open arms and time spent in open arms at dose 50 mg/kg whereas no. of entries in closed arms and time spent in closed arms were considerably decreased as compared to control. No. of entries in open arms and time spent in open arms was raised highly significantly at doses of 100 and 200 mg/kg whereas time spent in closed arms was highly significantly decreased as compared to control at the same doses. Diazepam 1 mg/kg exhibited highly substantial increase in no. of entries in open arms and time spent in open arms whereas no. of entries in closed arms and time spent in closed arms was decreased highly significantly as compared to control.

Fear of height provokes anxiety in animal when placed in the elevated plus maze. The sign of fear and anxiety in an animal is shown by decrease in the motor activity and prefer-ence to remain at safer places. Anxiolytic agents are likely to raise the motor activity which was estimated by the time spent in the open arms by the animals. Hence a general stimulatory and anti-anxiety behavior can be speculated by open arm entries (Mansouri et al. 2014). The highly significant rise in open arm entries and time spent in open arms by N. nucifera fruit

Table 4 Antidepressant effect of N. nucifera fruit and imipramine in forced swimming test

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day</th>
<th>Immobility duration (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2nd</td>
<td>197.6 ± 0.85</td>
</tr>
<tr>
<td>N. nucifera 50 mg/kg</td>
<td></td>
<td>15th</td>
</tr>
<tr>
<td>N. nucifera 100 mg/kg</td>
<td>180.2 ± 0.89</td>
<td></td>
</tr>
<tr>
<td>N. nucifera 200 mg/kg</td>
<td>195.2 ± 0.64*</td>
<td></td>
</tr>
<tr>
<td>Diazepam 1 mg/kg</td>
<td>174.7 ± 1.20**</td>
<td></td>
</tr>
<tr>
<td>Diazepam 2 mg/kg</td>
<td>194.3 ± 0.61*</td>
<td></td>
</tr>
<tr>
<td>Diazepam 5 mg/kg</td>
<td>169.8 ± 3.20**</td>
<td></td>
</tr>
<tr>
<td>Imipramine 25 mg/kg</td>
<td>152.1 ± 0.97**</td>
<td></td>
</tr>
<tr>
<td>Imipramine 50 mg/kg</td>
<td>120.5 ± 0.90**</td>
<td></td>
</tr>
</tbody>
</table>

n = 7
Values are Mean ± S.E. M
*p < 0.05 significant as compared to control
**p < 0.005 highly significant as compared to control

Currently use of herbal remedies and dietary complement rich in flavonoids and vitamin C has augmented to treat mild to moderate anxiety disorders and depression (Riaz and Khan 2014). But further research and studies are necessary to evaluate the pharmacological & toxicological effects of plants. These investigations may benefit in the discovery of new drugs from the herbal sources and hence may recommend the use of traditional drugs in humans (Ahmed et al. 2015).

Fear of height provokes anxiety in animal when placed in the elevated plus maze. The sign of fear and anxiety in an animal is shown by decrease in the motor activity and prefer-ence to remain at safer places. Anxiolytic agents are likely to raise the motor activity which was estimated by the time spent in the open arms by the animals. Hence a general stimulatory and anti-anxiety behavior can be speculated by open arm entries (Mansouri et al. 2014). The highly significant rise in open arm entries and time spent in open arms by N. nucifera fruit. 
extract at doses 100 and 200 mg/kg were rather similar to standard agent diazepam so it may be assumed that N. nucifera fruit extract has strong anxiolytic activity.

Present study also demonstrated the anxiolytic effect of N. nucifera fruit extract and diazepam in rats by using light and dark test. The light and dark test is used to assess the activity of anxiolytic agents (Michel and Martine 2003). There was highly significant rise in percentage of time spent in light compartment at extract doses 50, 100 and 200 mg/kg as compare to control. Diazepam 1 mg/kg also exhibited high-ly significant increase in percentage of time spent in light compartment in comparison to control. It was also observed that percentage of time spent in the light compartment by the animal at extract doses 100 and 200 mg/kg was >50% i.e 51.9 ± 0.89 and 58.3 ± 0.64, so after treatment with the extract, the level of anxiety was greatly reduced in experimental animals. Hence N. nucifera fruit extract has demonstrated strong anxiolytic effect at light and dark box test.

In the Central Nervous System various flavonoids attach to the benzodiazepine site on the GABA A receptor producing anxiolytic, sedative and anticonvulsant effects. Flavonoids with anti-anxiety effects have been designated in many plant species used in traditional medicine e.g. Passiflora coerulea (Kumar and Sharma 2005).

In previous study, Neferine which is a Bisbenzyloisoquinoline alkaloid present in the seeds of N. nucifera fruit has exhibited anxiotxiety effects in the elevated plus maze test without affecting muscle coordination as shown in the rota rod test whereas in the same study diazepam showed anxiotxiety and muscle relax-ant effects as well (Sugimoto et al. 2008).

In another study GABA was found to be involved in anxiolytic activity of saponins isolated from Albizzia Lebbeck leaves and suggested that saponins act by modifying GABAergic mechanisms (Kumar and Sanjay 2005). Similarly saponins extracted from Anilaeaeca panax quinquefolium L. (PQS) were studied in male mice using ele- vated plus maze test, light and dark test and hole board test. The finding of this study suggested that saponins from PQS might be a potential candidate for use as an anxiolytic drug (Wei et al. 2007). Also some terpenes and also various terpene- derived compounds have potentiated the GABAergic responses (Kessler et al. 2014).

N. nucifera fruit is a rich source of all of these constituents e.g. flavonoids, alkaloids, saponins, tannins and terpenoids which have shown anxiolytic activity in various studies, so it may be suggested that N. nucifera fruit exerts its anxiolytic activity through synergistic action of all of these constituents.

Present study also investigated the antidepressant activity of N. nucifera fruit extract at doses 50, 100 and 200 mg/kg by forced swimming test. There was a significant decline in the duration of immobility of mice at doses 100 and 200 mg/kg on 2nd day of experiment and highly significant decrease was noticed on 15th day of experiment at the same doses. No significant change was observed at dose 50 mg/kg of extract at 2nd and 15th day of experiment.

Forced swimming test is the established method employed to assess behavioral sadness in rodents. Hopelessness, reported as a common trait of depression in humans, is mimicked in rodents by the paradigm of learned helplessness. Thus forced swimming test is considered to be useful for evaluating depressive state in human since the behavioral immobility of animals during forced swimming has been reported to emulate some aspects of human depression (Porsolt et al. 1977; Wilner 1984; Yoshimura and Yamakawa 2000). Reduction in duration of immobility represents antidepressant effects (Taiwo et al. 2012). Hence N. nucifera fruit showed remarkable antidepressant effects more or less similar to Imipramine at doses 100 and 200 mg/kg after continuous administration of extract for 14 days i.e. up to 15th day of experiment.

Neferine, liensinine and isoliensinine are alkaloids found in the seeds of N. nucifera fruit have demonstrated antidepressant activities in previous studies and the central effects of these alkaloids are most likely linked to serotonergic neurotransmission particularly involving 5HT1A receptors in mice (Sugimoto et al. 2010; Sugimoto et al. 2015).

Hence the anxiolytic and antidepressant activities of N. nucifera fruit were largely comparable to diazepam and Imipramine but its margin of safety makes it superior to ben-zodiazepines and conventional antidepressants. N. nucifera fruit seems to have great potential for therapeutic applications in the treatment of central nervous system disorders, such as anxiety and depression and thus encourage more investiga-tions in this field.

Conclusion

N. nucifera fruit has demonstrated anxiolytic and antidepres-sant activities and that is perhaps both segments of the N. nucifera fruit i.e. seeds and the seed pods are rich in sig-nificant phytochemical constituents e.g. flavonoids and alka-loids. Moreover the anxiolytic and antidepressant activities of N. nucifera fruit were largely comparable to Diazepam and Imipramine but its margin of safety makes it superior to both of these agents. Hence N. nucifera fruit seems to have great potential for therapeutic applications in the treatment of central nervous system disorders, such as anxiety and depression and thus encourage more preclinical and clinical trials in this field.

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