Chapter-4

EXPERIMENTAL INVESTIGATIONS

Chapter – 4

Chapter – 4. Experimental Investigation

4.1 Plant processing:

4.1.1 Plant collection

The fresh seeds of *Nigella sativa* (NS) were obtained in the month of January from the botanical gardens of RIPER of Anantapuramu district, Andhra Pradesh, India and were dried up in the absence of sunlight. The coarse powder of seeds was prepared using mixer.

4.1.2 Authentification

The plant sample was recognized and legitimated by Dr. C. Sudhakar, M.Sc, Ph.D Department of Botany, S K University, Anantapuramu, Andhra Pradesh. A voucher specimen numbered (riper-06/10) is conserved in Department of Pharmacology, RIPER, Anantapuramu.

4.1.3 Preparation of extracts

Extracts using different solvents with dissimilar polarity were prepared by subjecting the formerly organized powders to maceration followed by drying (exposure to air). The extraction was carried out with different solvents of increasing polarity like petroleum ether (Pet ether), chloroform, methanol and water successively in a maceration apparatus (earthen pot). The menstrum containing extracts were concentrated on rotary flash evaporator and air dehydrated to get the concentrated extract. The % yield of seed extracts of different solvents was calculated.

4.1.4 Macroscopical Characterization:

Macroscopic studies related to morphology and organoleptic characters of seeds were done by bare eye. Shape, Colour, odour of seed were determined and represented in the results.

4.1.5 Microscopical Characterization:

1 gram of seeds was positioned in a beaker with water 20 folds to that of seeds. The seeds container was then heated gradually for 10 minutes. The seeds then were separate from the seeds container and placed on a glass petri plate for developing transverse sections of the seeds employing vegetable cutting technique. Thinnest sections were chosen and detached from the group using soft hair brush. Then the sections were stained with phloroglucinal-HCL (1:1) for 5 minutes. After staining, the sections were positioned on a glass slide, a single drop of glycerine was placed on it and the preparation was sealed using cover slip.

Parallel method was applied in case of crushed (Powder) of seeds of *Nigella sativa* with tiny changes. The complete seeds were substituted with powder of the seeds while carrying out powder microscopy.

4.1.6 Photomicrography:

Seeds of *Nigella sativa* were subjected to both crossways section (T.S) and powder microscopic assessment; the results are submitted in the form of photographs at different magnifications which were taken using Lab photo microscopic unit. All the photos were taken against the bright light for clear visibility.

4.1.7 Physicochemical Evaluation:

Physicochemical parameters of *Nigella sativa* seeds were determined and reported as total ash, Alkali insoluble ash, water insoluble ash, Alcohol insoluble ash, Acid insoluble ash. Water, Alcohol, Chloroform and pet ether soluble extractive to determine the amount of

solvent soluble components present. The moisture content was also determined.

4.1.8 Preliminary Phytochemical Screening:

The course powder of seeds of *Nigella sativa* was subjected to cold macerated solvent extraction. 50 grams of seeds were added with 200 ml of different solvents including Pet ether, Chloroform, Alcohol, water and was allowed to stand for 7 days. After 7 days, the extracts were separated by filtration and were concentrated under reduced pressure and, then after the concentrated extracts were subjected to various chemical tests to detect the presence of different phytoconstituents.

4.1.9 Animals

Albino rats of either sex weighing 200-250g were procured from Raghavendra Enterprises, Bangalore. Animals were housed under standard husbandry conditions i.e. 22 ± 2 ^{0}C temperature and 12 hours light/dark cycle. The animals had free access to standard commercial diet (VRK Nutrition solution, Maharashtra Pvt Ltd. India) and water *ad libitum.*

4.1.10 Ethical approval

The Institutional Animal Ethics Committee (878/ac/05/CPCSEA/007/2012) of RIPER, Anantapuramu, Andhra Pradesh, India has approved the animal experimental protocol. All the experiments were conducted according to the norms of the CPCSEA.

4.1.11 Acute Oral Toxicity (AOT) Study

Acute oral toxicity study work was designed and performed as per Organisation for Economic Cooperation and Development (OECD) –

guidelines no 423 (acute toxic class method).[88] Mice of either sex or preferably female were chosen randomly and segregated into seven different groups (n = 6). Group I which received vehicle (2% gum acacia). The seeds extract is dosed at 2000mg/kg, body weight through the most preferred route i.e. orally to II group. The animals were observed continuously for 2 hr, and then intermittently for 6 hr. At the end of 24 hours, the number of deaths was noted to determine LD_{50} of the extract. Animals were also observed for behavioural, neurological and autonomic profiles simultaneously.

If no 50% death rate is observed in the animal group. The dose of the test extract was increased to 2500mg/kg body weight and subsequently increased till half of the animals in a group dies so that it facilitates in the easy calculation of LD_{50}.

4.1.12 Preliminary Phytochemical Investigation (PPI)[89]

4.1.12.1 Detection of Alkaloids

About 50 mg of solvent-free extract was treated with 10 ml of dilute HCL - hydrochloric acid and separated through filtration. The filtrate shall be tested for the presence of alkaloids using the following tests.

a) Mayer's test – To the 1 ml of filtrate taken gently add few drops of Mayer's reagent which bears 'Potassium mercuric iodide solution' down the side of the test tube and wait for the development of white or creamy sediment, which shows the existence of alkaloids in the test extract.

b) Hager's test – Take 1 ml of filtrate and add few drops of Hager's reagent which bears 'Picric acid' and observe for development of

prominent yellow precipitate, which indicates positive test for the presence of alkaloids.

c) Dragendorff's test - Take 1 ml of filtrate and add few drops of Dragendorff's reagent which bears 'Potassium bismuth iodide solution' and observe for the formation of prominent precipitate. If reddish brown precipitate is formed it directly indicates the existence of alkaloids in the extract.

d) Wagner's test – To the 1 ml of filtrate taken in a test tube, few drops of Wagner's reagent which bears 'Iodine-Potassium iodide solution' must be added by the side of the glass test tube and observed. If reddish brown precipitate is developed, it clearly indicates the presence of alkaloids in the extract.

4.1.12.2 Detection of Carbohydrates

The test solution was prepared by dissolving 50 mg of test extract with water, hydrolyzed with 2N HCl and subjected to following tests.

a) Molisch's test – To the 1 ml of the test solution mix few drops of α–naphthol in alcohol (Molisch's reagent). The mixture was shaken and 1 ml of concentrated H_2SO_4- sulphuric acid must be added gradually from the wall (side) of the test tube. Ice cooled test tube after standing for a while should be observed for development of a violet ring at the junction. If yes the violet ring is formed the test stands positive for the presence of carbohydrates.

**b) Barfoed`s test -** 1ml of test solution was heated with 1ml of Barfoed`s reagent on water bath and observed for red cupric oxide formation which indicates the presence of monosaccharides.

4.1.12.3 Detection of Amino acids

The test solution was prepared by dissolving 50 mg of test extract with water, hydrolyzed with 2N HCl and subjected to following tests.

**a)Millon`s test -** To the test solution, add 2ml of millon`s reagent. white precipitate formation indicates the existence of amino acids.

b) Ninhydrin test - To 3 ml of filtrate, three drops of 5% Ninhydrin reagent was added and placed in a boiling water bath for 10 minutes. Formation of a characteristic purple colour indicates the presence of amino acids.

4.1.12.4 Detection of Proteins

The test solution was prepared by dissolving 50 mg of test extract with water, hydrolyzed with 2N HCl and subjected to following tests.

a) Biuret test - Take 3 ml of filtrate, add two drops of 4% NaOH and further treated with few drops of 1% $CuSO_4$ solution. If pink colour is developed then there is existence of proteins.

b) Hydrolysis test - Ninhydrine was added to the test solution and boiled. Formation of violet colour indicates the presence of proteins.

4.1.12.5 Detection of Steroids and Terpenoids

a) Liebermann – Burchard's test - nearly 50 mg of the test extract shall be mixed with 2 ml of acetic anhydride in a glass test tube continue addition of 2 ml of chloroform and heat to the boiling point and cool the mixture. Then add 1 ml of concentrated H_2SO_4- sulphuric acid by the sides of the test tube and wait for the development of colour at the junction. If red, pink or violet colour any of the above mentioned colour

develops at the junction of the liquids it means that there is a presence of steroidal triterpenoids in the extract.

b) Salkowski test - The extract was treated with few drops of concentrated sulphuric acid. Red colouration at the lower layer proves the existence of steroids and development of yellow colouration at lower layer shows the existence of triterpenoids.

4.1.12.6 Detection of Tannins

a) Ferric chloride test - Nearly 50 mg of test extract should be mixed in 2 ml of distilled water. Then mix few drops of neutral 5% ferric chloride ($FeCl_3$) solution and wait for the formation of blue, green or violet colour. If so, it shows the presence of phenolic compounds. Test solution treated with few drops of ferric chloride solution gives dark colour.

4.1.12.7 Detection of Fats and fixed oils

The test solution was prepared by dissolving 50 mg of test extract with water, hydrolyzed with 2N HCl. To the sample, 1ml of 1% cupric sulphate solution was added. Then 10% NaOH was added. A clear blue solution formation indicates the presence of fats and fixed oils.

4.1.12.8 Detection of Glycosides

Test A - 200mg of drug was extracted with 5ml of dilute sulphuric acid by warming on water bath, then filter it. The acidic extract should be neutralised by the addition of 5% soln of NaOH and Fehling's solution A and B at a conc of 0.1 ml till it becomes basic. Heat it on a water jacket for 2 minutes. Amount of red precipitate produced should be compared with that of test B.

Test B - 200mg of drug was extracted with 5ml of water. After boiling, 5ml of water was added. Fehling's solution A and B must be added at a concentration of 0.1 ml until it becomes alkaline and heated on water bath for 2 minutes. If the sedimentation in test A is superior than in test B, then glycosides are existing in the test extract.

4.1.12.9 Detection of Inulin

The test solution was prepared by dissolving 50 mg of test extract with water, hydrolyzed with 2N HCl. To it, a solution of alpha-napthol and sulphuric acid was added. Formation of brownish red colour indicates the presence of inulin.

4.1.12.10 Detection of Flavonoids

The test solution was prepared by dissolving 50 mg of test extract with water, hydrolyzed with 2N HCl. To the test extract containing solution, few drops of NaOH- sodium hydroxide solution was added. Development of strong yellow colour on standing becomes colourless when few drops of dilute acid are added. This type of reaction shows the presence of flavonoids.

4.1.12.11 Detection of Lignin

A thin section of drug should be treated with concentrated hydrochloric acid and phloroglucinol at a ratio of 1:1 in the mixture. Development of pink colour shows the existence of lignin.

4.1.12.12 Detection of Waxes

The test solution was prepared by dissolving 50 mg of test extract with water, hydrolyzed with 2N HCl. To the test solution, alcoholic alkali solution was added. Waxes get saponified.

4.2 Anticancer activity:

4.2.1 MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay

Cell line used: HepG-2 (Hepatocellular carcinoma cells), MCF-7 (Human breast adenocarcinoma cells)

HepG2 and MCF-7 cell cultures were procured from National Centre for Cell Sciences (NCCS), Pune, India. The cells were cultured in minimum essential medium (DMEM) growth medium supplemented with 10% heat inactivated Fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin-B (5 µg/ml) in a humidified atmosphere of 5% CO2 at 37°C until confluent. The cells were trypsinized with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm2 flat bottles and the studies were carried out in 96 well microtitre plates.[16,17]

a) MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay:

Cells were plated in 96 well flat bottom microtitre plate at a density of 1 × 104 cells per well and cultured for 24 h at 37^0C in 5 % CO2 atmosphere to allow cell adhesion. After 24 h, when partial monolayer was formed, medium was removed and cells were treated with different concentration of standard drug (Doxorubicin) and sample compounds for 48 h. Microscopic examination was carried out and observations recorded every 24 h. After the treatment, the solutions in the wells were discarded and 50 µl of freshly prepared MTT (2 mg/ml, prepared in PBS) was added to each well. The plates were shaken gently and incubated for

3 h at 37ºC in 5% CO2 atmosphere. After 3 h, the supernatant was removed and the formazan crystals formed in the cells were solubilized by addition of 50 µl of iso-propanol. Finally, the absorbance was read using a Micro-plate reader (Bio-Tek, ELX-800 MS) at a wavelength of 540 nm.[16,17]

The percentage growth was calculated using the formula below:

$$\% \text{ growth inhibition} = \frac{\text{Control absorbance} - \text{Test absorbance}}{\text{Control absorbance}} \times 100$$

% growth inhibition v/s concentration graph was plotted and from curve IC50 (Concentration of drug required to kill 50% of cells in exponentially growing cultures after a 48 h exposure to the drug) values can be calculated.

b) Nuclear Staining studies:

50,000MCF-7 cells were placed in every well of 24 well plates along with culture medium bearing 10 % FBS. After 24 h, cells were treated with drugs for 48 h. The plates were incubated at 37^0C in 5% CO_2 atmosphere. After exposure of cells with drugs below CTC_{50} standards cells were fixed with 1 ml of methyl alcohol (90%) at (minus) -20°C for 20 min. Later the methyl alcohol is removed and dried in air. Fixed cells were washed/rinsed using ice cold PBS 2 to 3 times. Plate was washed/rinsed with PBS 2 to 3 times and 200 µl of acridine orange/ethidium bromide (10 µg/ml in PBS pH-7.4) was added and incubated at a temperature of 37°C for 20 min. The plate must be washed three times with PBS and to observe under luminous/fluorescent microscope for some nuclear changes.[90]

4.3 Antiosteoporotic activity

a) Ovariectomised model

Rats were selected randomly, anaesthetized with ketamine (75mg/kg, i.p.) and two-sided ovariectomy was done in aseptic surroundings. The surgery done rats were prophylactically administered with amoxicillin (25 mg/kg, i.p.) and superficially povidone - iodine solution was applied at the site of incision locally for a time period of seven days. All the rats were separated into seven groups.

b) Heparin induced Osteoporosis model

After one week of acclimatisation, the rats were obtained from quarantine. Randomly and grouped in to seven groups with six animals in each group. Neither anaesthetic nor any antibiotic was used as treatment for animals. Only Heparin was used as toxicant because it causes decrease in alkaline phosphatase which is a main precursor for the development oand formation of bone tissue. The treatment duration initially preferred was three months. But reduced to 30 days because of the unfavourable physiological changes in the rats.

Table 4.1 Treatment schedule of Ovariectomised model

Normal	Negative control	Standard	Test Group I	Test Group II	Test Group III	Test Group IV
Distilled water	Ovariectomised + Distilled water	Ovariectomised + Estradiol (0.15mg/kg p.o.) for four weeks	Ovariectomised + Pet ether seeds extract of NS (600mg/kg p.o) for four weeks	Ovariectomised + Chloroform seeds extract of NS (600mg/kg p.o) for four weeks	Ovariectomised + Ethanolic seeds extract of NS (600mg/kg p.o) for four weeks	Ovariectomised + Aqueous seed extract of NS (600mg/kg p.o) for four weeks

Table 4.2 Treatment schedule of Heparin induced Osteoporosis model

Normal	Negative control	Standard	Test Group I	Test Group II	Test Group III	Test Group IV
Distilled water	Heparin (2IU/g s.c.) for four weeks	Heparin (2IU/g s.c.) + Alendronate (10mg/kg p.o) for four weeks	Heparin (2IU/g s.c.) + Pet ether seeds extract of NS (600mg/kg p.o) for four weeks	Heparin (2IU/g s.c.) + Chloroform seeds extract of NS (600mg/kg p.o) for four weeks	Heparin (2IU/g s.c.) + Ethanolic seeds extract of NS (600mg/kg p.o) for four weeks	Heparin (2IU/g s.c.) + Water seeds extract of NS (600mg/kg p.o) for four weeks

4.3.1 Evaluation of Parameters

4.3.2 Biochemical parameters in serum and urine

a) Phosphorous

Principle:

Ammonium molybdate under acidic conditions reacts with phosphorous to form a complex known as phosphomolybdate, which is converted to blue colored complex in presence of metol. The intensity of colour produced is directly proportional to the elemental phosphorous concentration.

Procedure: Modified metol method

Wave length: 680nm (red filter)

Incubation temperature: Normal room temperature

Linearity: 15mg%

Reaction type: End point with standard

Standard: 5 mg% elemental phosphorous

Reaction slope: Increasing

Table 4.3 Patterns of reagents for the identification of phosphorous

	Blank (B)	Standard (S)	Test (T) (Serum or urine)
Catalyst reagent	1ml	1ml	1ml
Molybdate reagent	1ml	1ml	1ml
Deionised water	0.1ml	-	-
Standard	-	0.1ml	-
Serum/dilute urine	-	-	0.1ml
Metol reagent	1ml	1ml	1ml

Serum phosphorous in mg% = A of Test/A of Standard x 5

Urine phosphorous in mg% = A of Test/A of Standard x 0.5

A = Absorbance

b) Calcium

Principle:

Calcium forms a violet complex with o-cresolphthalein complexone in an alkaline medium.

Procedure: (OCPC o-cresolphthalein complexone) method

Wave length: Hg 578 nm

Temperature: 20^0 C – 30^0 C

Table 4.4 Patterns of reagents for the identification of Calcium

	Reagent blank	Standard	Sample (Serum or urine)
Reagent 2	0.50ml	0.50ml	0.50ml
Reagent 2	0.50ml	0.50ml	0.50ml
Standard	-	0.02ml	-
Sample	-	-	0.02ml

Serum or Urine concentration in mg/100ml = A of Sample/A of Standard x 8

A = Absorbance

4.3.3 Biochemical parameters in serum

a) Alkaline phosphatase

Principle:

Para Nitrophenyl Phosphate reacts with water in the presence of ALP to form phosphate and Para Nitrophenol.

Procedure: kinetic photometric test method

Wave length: Hg 405nm

Temperature: 30^0 C

Measurement: Against air

Substrate start

Table 4.5 Mixing pattern for ALP

Sample	20 µl
Reagent 1	1000 µl
Reagent 2	250 µl

From the retro-orbital plexus of rat, blood samples were withdrawn by plexus puncture and serum was obtained by subjecting the blood sample to centrifugation (5000rpm for 20 min). Reagent 2 was added after mixing Reagent 1 with Sample and incubated for 1 minute. After adding Reagent 2 and mixing well, the absorbance was measured after one minute. Again the absorbance was measured after one, two and three minutes (QDX).

ALP activity (U/l) = Absorbance/minute x 3433

4.3.4 Physical characters study of animals

a) Weight of the animals

The weight of animals was noted every week using balance until the specified period of treatment.

b) Length of the bone

After four weeks of treatment, all groups of rats were sacrificed by cervical dislocation. The femur bones of both the legs were isolated. The length of the each bone was measured using a ruler.

c) Diameter of the bone

After sacrificing the rats by cervical dislocation and isolation of femur bones, by using a thread the circumference of each bone was measured. Using the formula circumference = $2\pi r$, the radius was calculated. By substituting the value of radius in the formula $d = 2r$, we get the diameter of the bone.

d) Density of the bone

The rats were sacrificed by cervical dislocation and the femur bones were isolated by using a thread the circumference of each bone

was measured. Using the formula circumference = $2\pi r$, the radius was calculated. By substituting the value of radius in the formula $\pi r^2 h$, the volume of each bone was calculated. Further, substituting the volume of the bone in the formula D = M/V, we can calculate the density of the bone.

e) Weight of the bone

The isolated femur bone was weighed using a balance.

f) Weight of the heart

All groups of animals were sacrificed by cervical dislocation. The heart of each animal was separated carefully and weighed.

g) Weight of the kidney

By cervical dislocation all the animals were sacrificed, both the kidneys were separated from each animal and weighed.

h) Ash value of the bone

The femur bones of both the legs were isolated by sacrificing the animal by cervical dislocation. Each bone was placed in a crucible. The crucible was placed in a muffle`s furnace which was maintained at 600±20^{o}C. The crucible was heated until the bone became colourless. The weight of the resulting bone was noted.

4.3.5 Histopathological study of the bone

All groups of rats were sacrificed by cervical dislocation on the last day of the treatment i.e. 30th day. Bones of each rat was then collected, fixed in 10% formalin and sectioned. Histopathological changes in the bones were observed under light microscope.

4.4 Anti urolithiasis:

4.4.1 Invitro study:

a) Kinetic Study

The consequence of the test substance on kinetics of crystals of calcium oxalate (CaOx) shall be found by the period course measurement of turbidity alterations because of the initiation and grouping of crystal formation soon after combining the metastable solutions mixtures of Calcium (Ca++) and Oxalate (Ox). Pre prepared stock solutions of $CaCl_2$ (8.5 mM) and $Na_2C_2O_4$ (1.5 mM), bearing 200 mM of NaCl and 10 mM of sodium acetate be in synchronised to a pH 5.7.[32] An aggregometer (particle grouping device) (Chrono-Log Corporation, USA) designed and developed for platelet aggregation studies based on the study of optical density exactly at 620 nm shall be preferred to study the episode of CaOx crystallization.[33] The CaCl2 solution (0.5 ml) should be circularly stirred continuously both in the nonexistence and company of dissimilar concentrations of the test/study/reference material: potassium citrate at 37°C. Past getting the steady base line, crystal formation shall be started by the adding up of $Na_2C_2O_4$ solution (0.5 ml) to get the last concentration of Ca++ as 4.25 and Ox as max to be 0.75 mM. The period route measurement of turbidity was concurrently beginned on a chart, moving at the pace of 30 mm/h and sustained for 15 mins with steady circular stirring of the solutions. All procedures are to be run in thrice to conclude the effect. The slopes of nucleation (SN) and aggregation stages (SA) are considered through linear regression analysis. By means of the slopes, the % inhibition can be calculated as [(1-Sm/Sc) x 100], where

Sm is slope in the existence of modifier and SC is slope of the absence of modifier. (control experiment).

The effect of test extract on CaOx crystal formation shall be found by the period route measurement of turbidity differences due to the crystal nucleation and aggregation in the artificial urine on adding up of 0.01M sodium oxalate. The deposition of calcium oxalate at 37°C and pH 6.8 has been calculated by the calculation different turbidity at 620 nm. A spectrophotometer UV/Vis (Shimadzu 1800) shall be engaged to measure the turbidity of the development of calcium oxalate.[34]

b) Incubation Study

To establish the consequence of incubation on CaOx crystallisation, prepared stock solutions of $CaCl_2$ and $Na_2C_2O_4$ parallel to those in the above mentioned kinetic study shall be used. $CaCl_2$ solutions, bearing dissimilar amount of the test extract, material or potassium citrate, were mixed and placed in the flat-7 - bottomed tubes. The volume of aliquoted solution should not be greater than 0.5 ml in a 24 well plate (Iwaki Micro plate along with lid, Asahi Techno Glass limited, Japan). To every well of these tubes $Na_2C_2O_4$ solution (0.5 ml) shall be added.[34] Every concentration of the test substance must be prepared three times. The plates must be then incubated in a vibration water jacket at 90 vibrations/ min at a temperature of 37°C for 45 min. Quantity and the external appearance of the crystals in each and every tube was then noticed underneath inverted microscope (Nikon Corporation limited, Tokyo, Japan)

c) Artificial urine preparation

The artificial urine (AU) shall be organized corresponding to the method of Burns and Finlayson and with the subsequent content. The AU must be freshly prepared every day and pH accustomed to 6.0. the concentration of different chemical components are given below in the table no 4.6.

Table 4.6 Composition of artificial urine

Sodium chloride	105.5 mmol/1
Sodium oxalate	0.32 mmol/1
Sodium citrate	3.21 mmol/1
Magnesium sulphate	3.85 mmol/1
Ammonium hydroxide	17.9 mmol/1
Potassium chloride	63.7 mmol/1
Ammonium chloride	0.0028 mmol/1
Sodium phosphate	32.3 mmol/1
Calcium chloride	4.5 mmol/1
Sodium sulfate	16.95 mmol/1

d) Study with inhibitor

Extracts were suspended in distilled and deionised water (25 mg/ml), passed through a 0.22 ml filter, and preferably selected at an ultimate concentration of 1/16th mg, 1/8th mg, 1/4th mg, 1/2nd mg and 1.0 mg/ml urine, taking in account of dose-response curve. A combination of 1 ml of artificial urine and 0.5 ml of test extract/substance solution is placed in the cell. A plain (Blank) reading was noted and then amount of half (0.5) ml of 0.01M sodium oxalate was mixed and the measurement is at once started for a time of 10 min. For each testing, six alike preparations were taken. The percentage of inhibition shall be calculated using the following formula:

$$\%\text{inhibition} = \{1-[Si / Sc]\} X\ 100$$

Here; Si - slope of graph in the existence of inhibitor (test extract), Sc - slope in the absence of inhibitor (Control)

4.4.2 In vivo study: On animal model of urolithiasis

Antiurolithic activity of the test substance was screened by means of animal replica of CaOx Urolithiasis. Male Wistar rats weighing approximately 180-220 gms were alienated with matching body Weights into seven groups of 6 animals each, which were then at random selected to take delivery of various treatments for preventive work.

Induction of Renal calculi in rats

There are three procedures for inducing calcium oxalate crystal formation in rats.

Method - I Ethylene glycol treatment induced Urolithiasis model.

Method - 2 Ethylene glycol-Ammonium chloride treatments induced urolithiasis model.

Method - 3 Sodium oxalate treatment induced urolithiasis.

a) Ethylene Glycol model

Ethylene Glycol is used to induce renal stones. The major metabolic end product of Ethylene Glycol is oxalates. These oxalates react with the calcium and shape in to insoluble complex. Generally 0.75% ethylene glycol (through drink water) is used for 28 days to induce stones.

4.4.2.1 Anti-Urolithiatic assay

Ethylene glycol induced urolithiasis was selected to measure the ant-urolithiatic action of test extrats in albino rats.[35,36] The

acclimatized animals were alienated into 7 groups namingly Group I to Group VII, each bearing 6 wister rats and urolithiasis (stones in urinary system) was induced in all the groups excluding Normal.

Table 4.7 Treatment schedule of anti - urolithiatic assay

Normal Group I	Negative control Group II	Standard Group III	Group IV (Test Group)	Group V (Test Group)	Group VI (Test Group)	Group VII (Test Group)
Distilled water	1% ethylene glycol in drinking water for 28 dys	1% ethylene glycol in drinking water + Cystone (750 mg/kg)	1% ethylene glycol in drinking water + Aqueous extract of NS at a dose of 600 mg/kg body weight	1% ethylene glycol in drinking water + Alcoholic extract of NS at a dose of 600 mg/kg body weight	1% ethylene glycol in drinking water + Chloroform extract of NS at a dose of 600 mg/kg body weight	1% ethylene glycol in drinking water + Pet ether extract of NS at a dose of 600 mg/kg body weight

4.4.2.2 Collection and analysis of urine

Animals were placed separately in different metabolic cages and the samples of urine of 24 h were obtained on 28th day. A few drops of concentrated hydrochloric acid were added to the urine prior to storage at 40˚C. Urine would be analysed for the existence of calcium, phosphate, oxalate, protein, creatinine, magnesium and also uric acid in it. [91,92,93]

4.4.2.3 Serum analysis

After the test period, blood was obtained from the puncture of retro-orbital plexus under the effect of anaesthetic agent and the animals were killed by cervical decapitation. Serum was isolated by centrifugation at a rate of 10,000 rpm for 10 min and estimated for the presence of calcium, phosphate, oxalate, creatinine, magnesium and uric acid content.

4.4.2.4 Estimation of Parameters

a) Body weight

The change of body weight in grams of each animal was recorded at day 0, 7, 14 21 and 28 using ACCULAB digital balance, (Model No. ALC-310.3, Sartorius Mechatronics India Pvt. Ltd., Bangalore, India).

$$\% \text{ change in body weight} = \frac{\text{Body weight on day 1} - \text{Body weight on day 28}}{\text{Body weight on day1}} \times 100$$

4.4.2.5 Estimation of biochemical parameters

The separated serum and urine samples were used for the determination of Ca, oxalates, P, electrolytes, total protein, blood urea nitrogen (BUN) and creatinine levels with semi auto analyzer (Erba). The measurements were mg/dl for Ca, P, BUN, creatinine and UA. In urine samples, Ca, oxalates, P, electrolytes, total protein, and creatinine while in

serum, Ca, oxalates, P, electrolytes, total protein, BUN and creatinine were estimated with the help of kit based methods.

a) Estimation of calcium

Principle

Calcium in alkaline medium reacts with o-cresopthalein complex - 1 to form a purple colored compound. Its absorbance is directly proportional to the calcium strength. Intervention due to double valence elements like magnesium and iron is separated by means of 8-hydroxyquinoloine.

Procedure

Pipette into clean dry tubes labelled blank (B) standard (S) test (T)

Table 4.8 Pattern of reagents for the identification of Calcium

Reagents	Blank	Standard	Test
Buffer solution (1)	2.0 µL	2.0 µL	2.0 µL
Color reagent	2.0 µL	2.0 µL	2.0 µL
Deionized water	0.02 µL	---	---
Standard	---	0.02 µL	---
Serum	---	---	0.02 µL

Mix well and keep warm at room temperature for about five minutes. Determine the absorbance of Standard (S) preparation and Test (T) preparation against Blank (B) preparation either on a yellow filtered photo colorimeter or on a spectrophotometer at 570 nm, within or lessthen 30 minutes.

Calculations

$$\text{Calcium in mg \%} = \frac{\text{A of (T)}}{\text{A of (S)}} \times 10 \text{ (std. Conc.)}$$

b) Estimation of oxalic acid

Oxalic acid in urine was determined by the in direct method.

Principle of the method: Precipitation of oxalate

Oxalate was precipitated from prepared urine with excess calcium ions and ethanol. Unless otherwise stated, our standard method of precipitation of oxalate was: add 0.4 ml of 2% (w/v) $CaCl_2$ to 3 ml of urine (p^H 5), then add 12 ml of ethanol. After mixing, the solutions were allowed to precipitate by standing over night at room temperature or for at least 3 h in a refrigerator. The calcium oxalate precipitate (COP) was collected by centrifugation at 1500 rpm for 10 min, followed by decantation of the superficial fluid. Interferences in COP were removed by washing thrice, each with 4 ml of washing solution (0.1 M acetic acid). To obtain a reproducible result, all experiments were carried on in special 15 ml conical centrifuge tubes and the COP was mixed thoroughly with washing solution at each step. The supernatant was easily decanted, after centrifugation, without disturbing the COP.

Calcium determination

After the last washing and leaving the walls of the centrifuge tubes to dry (for about 1 h), 1 ml of 1 M HCl was added and mixed thoroughly to dissolve the COP. Finally, 5 ml of deionized water were added and the calcium content was determined using an semi auto analyser.

Relationship of calcium content in COP and original oxalate conc^n

Varying amounts of 0.1 M sodium oxalate solution were added to the AU solutions to give a final concentration ranging from 0 – 2.00 mM and were used as media for the induction of COP in our study. After washing, the COP was dissolved and determined for its calcium content then plotted

against the original concentration of oxalate in AU. The concentration of oxalate was determined from slope of the curve.

c) Estimation of inorganic phosphate

Inorganic phosphate ($PO4^{-2}$) excretion was determined by the molybdenum blue reaction (Daly JA & Ertingshausen G, 1972) Molybdate solution was prepared by dissolving 2.0 g of ammonium Molybdate in 1 liter of H_2SO_4 (0.6 mol/liter). 100 ml of Molybdate solution was mixed with 0.9 ml of the diluted tween 80 (one volume of tween 80 mixed with two volumes of double distilled water) to prepare Working reagent which was to be used after 30 min. To 0.1 ml of appropriately diluted urine Samples, 4 ml of the working reagent was added. The solutions were allowed to stand for 15 min. The absorbance was then read at 340 nm against reagent blank. The concentration of inorganic phosphate was determined by using standard.

d) Estimation of sodium

Principle

The elemental sodium, proteins and the amino acids are sedimented concurrently by way of reagent bearing magnesium uranyl acetate bearing alcohol. The sediment is removed by centrifugation. The sodium content is calculated from the decrease in amount of magnesium uranyl acetate in the reagent mixture in relationship to a standard sodium solution treated correspondingly. The remaining quantity of magnesium uranyl acetate is determined by forming dark brown ferrous uranyl acetate, which shall be read calorimetrically.

Procedure

Step-I Precipitation of sodium and proteins

Pipette out standard(S) and test (T) solutions into two clean dry prelabelled test tubes. Mix well with vortex for 1 min and remain for 5 mins at normal room temperature. Centrifuge for 1 min at 3000 rpm.

Table 4.9 Pattern of reagents for the identification of Na and Proteins

Reagents	Standard	Test
Sodium PPT Reagent (1)	1.0 ml	1.0 ml
Standard Sodium / potassium (2)	0.02 ml	-
Serum	-	0.02 ml

Step II – Colour development

Pipette out blank (B), Standard (S) and test (T) into three clean dry prelabelled test tubes.

Table 4.10 Pattern of reagents for the colour development (Na assay)

Reagents	Blank	Standard	Test
Distilled Water	3 ml	3 ml	3 ml
Supernatant from Step I	-	0.05 ml	0.05 ml
Sodium PPT Reagent (1)	0.05 ml	-	-
Sodium Color Reagent (3)	0.2 ml	0.2 ml	0.2 ml

Mix well and let it to stand at normal room temperature for 5 mins. Then compute absorbance of Blank, Standard and Test next to distilled water on a photo colorimeter at 540 nm within 10 minutes.

Calculation

$$\text{Sodium in m.Mol/L} = \frac{\boldsymbol{Absorbance\ of\ B-T}}{\boldsymbol{Absorbance\ of\ B-S}} \times 150 \text{ (Standard Concentration)}$$

e) Estimation of potassium

Principle

Potassium concentration can be determined by numerously different methods. Directly it can be estimated by flame photometric method or colorimetric method. It may also be calculated by the help of ion selective electrode. The technique is based on the amount of turbidity developed because of the reaction mixture bearing Sodium Tetra phenyl Boron, basic EDTA, Formaldehyde and sample/test bearing standard potassium salt. The technique precise in the concentration of 2.0 to 7.0 m.M per Liter. There is a fine union with flame photometry.

Procedure

Pipette out Standard (S) and Test (T) solutions into two clean dry prelabelled test tubes.

Table 4.11 Pattern of reagents for the identification of potassium

Reagents	Standard	Test
Potassium Reagent (4)	1.0 ml	1.0 ml
Standard Sodium / potassium (2)	0.05 ml	-
Serum	-	0.05 ml

Mix softly and stay for 5 mins at normal room temperature and compute the absorbance of standard and test next to distilled water in a photo colorimeter at 620 nm before 10 minutes.

Calculation

Potassium in m.Mol/L = $\frac{\boldsymbol{Absorbance\ of\ T}}{\boldsymbol{Absorbance\ of\ S}} \times 5$ (Standard Concentration)

f) Estimation of chloride

Principle

Chloride ions form a colored complex soon after the reaction with mercury (II) thiocynate solution. The strength of the colour is directly proportional to the chlorine ion concentration.

Procedure

Pipette out Blank (B), Standard (S) and Test (T) into three clean dry prelabelled test tubes.

Table 4.12 Pattern of reagents for the identification of chlorine

Reagents	Blank	Standard	Test
Chloride Reagent (5)	1.0 ml	1.0 ml	1.0 ml
Standard Chloride (6)	-	0.005 ml	-
Serum	-	-	0.005 ml

Mix well stay for 2 mins at normal room temperature and compute the absorbance of blank, standard and test next to distilled water on a photo colorimeter at 505 nm before 10 minutes.

Calculation

$$\text{Chloride in mMol/L} = \frac{\text{Absorbance of Test} - \text{Absorbance of Blank}}{\text{Absorbance of Standard} - \text{Absorbance of Blank}} \times 100$$

g) Estimation of uric acid

Principle

Uric Acid + O_2 + $2H_2o$ $\xrightarrow{\text{Uri case}}$ allantoine + CO_2 +H_2O

$2H_2o$ + ESPAS +4- AAP $\xrightarrow{\text{POD}}$ purple quinoneimine + 4 H_2O_2

Procedure

Pipette out Blank (B) standard(S) and test (T) in to clean, dry prelabelled tubes and mix with the reagents in the following order.

Table 4.13 Pattern of reagents for the identification of Uric acid

Reagents	Blank	Standard	Test
Enzyme Reagent	1000 µL	1000 µL	1000 µL
Distilled Water	100µL	---	---
Standard	---	100µL	---
Serum/plasma	---	---	100µL

Calculations

$$\text{Uric acid mg/dl} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 5$$

h) Total Protein

Principle

The peptide bonds of proteins act in response through copper II cation in basic solution to form blue-violet coloured complex, (biuret reaction) each copper cation complexing with 5 to 6 peptide bonds. Tartarate is added which acts as a stabilizer whereas iodide is used to avoid self reduction of the basic copper cationic complex. The colour produced is directly proportional to the protein amount and is computed/observed at 546nm.

Assay procedure

Table 4.14 Pattern of reagents for the identification of proteins

Reagent	Blank	Standard	Test
Working reagent	1000 µL	1000 µL	1000 µL
Distilled water	20 µL	--	--
Standard	--	20 µL	--
Test	--	--	20 µL

Incubate for 10 mins at 37^0c. Interpret absorbance of the standard and every test mixture at 546 nm next to blank reagent (Erba diagnostics, Mallaauster, Mannheim/Germany).

Calculations

$$\text{Total protein mg/dl} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 26.5$$

I) Estimation of creatinine-k

Creatinine was estimated by Jaffe's Method (Kinetic & End Point Method)

Principle

Creatinine reacts with basic picrate to generate a crimson red colored complex; the speed of crimson red coloured complex development is directly proportional to the creatinine amount.

Procedure

Pipette out standard (S) and test (T) in to a clean, dry and prelabelled test tubes.

Table 4.15 Pattern of reagents for the identification of creatinine

Reagents	Standard	Test
Working reagent	1000 µL	1000 µL
Standard	100µL	---
Serum/diluted urine	---	100µL

Mix well and allow it to stand at retention time of 5 mins. interpret absorbance for standard as AS_o and test as AT_o next to distilled water at 520 nm (or) green filter in spectrophotometrically.

Acid reagent	50 µL	50 µL

Calculations

$$\Delta AS = AS_O - AS_1$$

$$\Delta AT = AT_O - AT_1$$

a) Serum creatinine = $\frac{\Delta\, AT}{\Delta\, As} \times 2$ (std.Conc.)

b) Urine creatinine in mg% = $\frac{\Delta\, AT}{\Delta\, As} \times 2$ (std.Conc.)

c) Urine creatinine in mg /24 hours = (b) 24 hours urine volume in litres.

4.4.3 Histopathological Studies

Kidney after isolation were weighed and fixed with 10% formalin bearing pH 7.4. A section of fixed kidney in using paraffin was equipped and stained using haematoxylin (basic dye) and eosin (acidic dye) and was observed for Histopathological changes.

Tissue parameters

The abdomen was cut open to isolate both kidneys from each and every animal. Separated kidneys were made free of extraneous tissue and conserved in 10% v/v neutral formalin solution. The kidneys then were dried at a temperature of 80^0C in a hot air oven. A sample fraction of 100 mgs of the predried kidney was subjected to boil in 10 ml of 1 N hydrochloric acid (HCL) for a period of 30 mins and homogenized. The obtained homogenate was subjected to centrifuge at 2,000 rpm for 10 mins and the superficial solution was separated. The calcium, phosphate and oxalate substances in kidney homogenate were determined as per methods described in the earlier sections.[94,95]

4.5. Antihyperglycemic activity: [38,96,97,98]

Elevated glucose levels were obtained by intraperitoneal introduction of Alloxan at a dose of 140mg/kg in two separate divided doses (i.e. 100mg/kg body weight and 40mg/kg bodyweight) in rats in order to reduce the death percentage of rats as a whole and within 4 - 14 days of time the drug induced hyperglycemia was developed successfully. The hyperglycaemic rats with glucose level > 250 mg/dl were isolated and separated into dissimilar groups where each group bears 6 rats each for the anti-diabetic

study. All the four extracts basing upon their LD_{50} values were administered for the study. And the sequence of administration of extracts is explained as follows,

- ❖ Biochemical parameters include :
  - Fasting blood glucose levels.
  - Body weight measurement.

Table 4.16: Treatment pattern for anti hyperglycaemic study

Group I	Normal Control: Administered with only 2% gum acacia
Group II	Alloxan (toxicant) + glibenclamide at a dose of 180µg/ml (standard drug) and served as Standard.
Group III	Alloxan (toxicant) + Aqueous extract at a dose of 600 mg/kg, per oral (Test extract)
Group IV	Alloxan (toxicant) + Alcoholic extract at a dose of 600 mg/kg, per oral (Test extract)
Group V	Alloxan (toxicant) + Chloroform extract at a dose of 600 mg/kg, per oral (Test extract)
Group VI	Alloxan (toxicant) + Petether extract at a dose of 600 mg/kg, per oral (Test extract)

Animals were fasted overnight (12 hrs) before administering the test drug. The calculated doses of the test extracts were administered and blood was regularly withdrawn at a time intervals of 0 hr, 1 hr, 2 hr, 4 hr, 8 hr, 12 hr, 18 hr and 24 hr and estimated for the effect of test drugs on the blood glucose levels of animals.

4.5.1 Estimation of fasting blood glucose: [99,100]

Method: Endpoint Colorimetric

Principle:

Glucose Oxidase is an enzyme which oxidises Glucose and converts in to Gluconic acid along with hydrogen peroxide concomitantly. In a succession peroxidise also catalyses the reaction, the oxygen produced is received by the chromogen integral colour developing system to grant a red coloured quinoneimine compound. The red coloured quinoneimine so developed must be computed at 505 nm using semi auto analyser and the intensity of colour developed is directly proportional to the concentration of Glucose moiety.

Procedure:

Pipette out 1 ml of glucose oxidizing reagent and peroxidising reagent (GOD/POD reagent) into pre tarred test tubes and then very soon blend it with 10µl of fresh separated serum or plasma. Nurture the test tube for about 15 mins and then find out the absorbance values at a constant range of 505 nm.

Calculations:

$$\% \text{ Blood-Glucose reduction (mg/dl)} = \frac{\text{Initial reading (reading of first blood withdrawn)} - \text{Test reading (at standard gap of time)}}{\text{Initial reading (at 0'time)}} \times 100$$

4.5.2 Histopathological studies:[101]

Processing of Isolated Pancreas:

The animals were sacrificed and their pancreas was isolated. The isolated pancreas were made into small pieces devoid of extra tissues and sealed and fixed in 10% formalin solution. Then the isolated pancreas were washed in running water for about 10 minutes. The dehydration of isolated tissue was done with the use of Isopropyl alcohol of increasing

concentrations ranging about 70%, 80% and 90% for about 12 hours each and the final step of dehydration was done by means of absolute alcohol.

Clearing of the tissue was performed by using chloroform with two changes at about 15 to 20 minutes each. After clearing the organ pieces were placed in L moulds followed by paraffin infiltration in self tissue processing unit.

Hard paraffin which was premelted must be poured in to L-shaped moulds. The pieces of pancreas were then placed into the molten paraffin preparation carefully, quickly and the whole set up was allowed to cool.

Sectioning:

The square blocks solidified paraffin with tissue inside it was cut into minute sections using microtome. The sections obtained will be of thickness of 5μ. The sections were taken on a micro glass slide preapplied egg albumin (sticking substance). The sections were placed in an hot air oven maintained at 60^0C for about 1 hour. As a result Paraffin melts and egg albumin solidified or denatured, which fixes the tissues to the slide on which they are placed.

Staining:

Eosin which is an acid stain stains all the cellular components which are basic naturally to pink colour like RNA, Cytoplasm whereas, Haematoxylin which is a basic stain stains all the cellular constituents naturally acidic in to blue like DNA of the nucleus.

4.6 Anti hyperlipidaemic activity[39,40,41]

The animals kept in cages (6 per cage) with 12:12 hr light and dark cycle at 22 ± 2°C. The animals were maintained on the standard diets with

adequate supply of water. Animals were divided into seven groups of 6 animals each. The hyperlipidaemia was induced by allowing the animals to feed on standard high fat diet which is explained below specifically for 8 days. Standard and test drugs were administered to all the test animals and on the end of eight day, blood of all the animals were obtained through retro orbital puncture and analysed for the biochemical constituents following the standard procedures.

Treatment

Group I (control)	:	High fat diet
Group II (Standard)	:	High fat diet + standard drug (Atoravastatin 1.2mg/kg B.wt)
Group III	:	High fat diet + Petroleum ether extract of Seeds of NS administered 600mg/kg Body weight
Group IV	:	High fat diet + Chloroform extract of Seeds of NS administered 600mg/kg Body weight
Group V	:	High fat diet + Alcoholic extract of Seeds of NS administered 600mg/kg Body weight
Group VI	:	High fat diet + Aqueous extract of Seeds of NS administered 600mg/kg b/wt

The two different diets which were preferably given to all the test animals are explained below:

Control diet animals were administered with a mixture of various ingredients required to be supplied for the maintenance of normal health are tabulated below[9],

Table 4.17 Ingredients used in the preparation of control diet

roasted Bengal gram powder	60%
coconut oil	9%
vitamin & choline mixture	0.5%
refined oil	4%
Wheat flour	22.5%
skimmed milk powder	5%
salt mixture with starch	4%
casein	4%

High fat diet animals were administered with a mixture of various ingredients required to be supplied for the maintenance of normal health are tabulated below,

Table 4.18 Ingredients used in the preparation of high fat diet

roasted Bengal gram powder	52.6%
coconut oil	9%
vitamin & choline mixture	0.5%
refined oil	4%
Wheat flour	20.5%
skimmed milk powder	5%
salt mixture with starch	4%
casein	4%
Cholesterol	0.4%%

Group I was administered with only 2% gum acacia solution where as Group II to Group VI were administered with required amount of standard and test drugs premixed with 2% Gum acacia respectively by oral intubation [10]. Animals were given required care as recommended by the institutional animal ethical committee.

Biochemical assessment

By the end of 8th day, blood was withdrawn through retro orbital sinus puncture under the influence of placid ether anaesthesia. The withdrawn blood samples were centrifuged and serum was separated, which was used for various biochemical parameters assessment. The separated plasma samples were then analysed for estimation of total cholesterol, triglycerides, S.G.O.T, S.G.P.T levels by the use of Boehringer Mannaheim kits (Erba smart lab analyser).

4.7 Antiparkinson's Activity: Include EEG analysis.

Table 4.19 Drugs and Chemicals for anti Parkinson's study

Drugs & Chemicals	Manufactures
Chloroform	Hi-Media Pvt. Ltd.
Methanol	Hi-Media Pvt. Ltd.
DTNB	Hi-Media Pvt. Ltd.
Tris – HCL	Hi-Media Pvt. Ltd.
EDTA	Hi-Media Pvt. Ltd.
Hydrogen peroxide	Hi-Media Pvt. Ltd.
TCA	Hi-Media Pvt. Ltd.
TBA	Hi-Media Pvt. Ltd.

Table 4.20 Instruments for anti Parkinson's study

Instrument	Company
Analytical UV -Visible spectrophotometer	2200/Systronics
Centrifuge	REMI RM – 12C
Homogenizer	Remi motor/Remi Electro Technik Ltd.
Rotarod apparatus	INCO Ltd., India
Swim test apparatus	INCO Ltd., India
Actophotometer	INCO Ltd., India

Table 4.21 Treatment schedule of different groups in anti Parkinson's

S.No.	Group	Reserpine antagonism
1	Group I Normal	Distilled water 10ml/kg p.o.,/5 days
2	Group II	Reserpine (5mg/kg, i.p.,)/5 consecutive days

	Negative control	
3	Group III Test group	Reserpine (5mg/kg, i.p.,)/5 consecutive days + NS Pet ether extract (600mg/kg, p.o.,)/5 days
4	Group IV Test group	Reserpine (5mg/kg, i.p.,)/5 consecutive days + NS Chloroform extract (600mg/kg, p.o.,)/5 days
5	Group IV Test group	Reserpine (5mg/kg, i.p.,)/5 consecutive days + NS Alcoholic extract (600mg/kg, p.o.,)/5 days
6	Group IV Test group	Reserpine (5mg/kg, i.p.,)/5 consecutive days + NS Aqueous extract (250mg/kg, p.o.,)/5 days

4.7.1 Estimation of behavioural parameters

1) Locomotor activity

a) Actophotometer

This test estimates the investigation and the intended locomotion inside a closed region. The purpose value for the natural motor movement shall be obtained through a photoactometer (INCO Ltd., India). The test animal was positioned alone in a 30 × 30 cm black metal compartment with a panel floor and a light-tight closure. Six separate beams of red light were purposefully focused 2 cm higher than the floor in to specialised photocells on the contrary side. Every beam disturbance was registered as a happening on the outer digital counter. The breakage in the light beams were counted continuously for 5mins.

b) Openfield test

The open field apparatus consists of a big square area 76x76 with walls 42 cm high. The floor was divided into 25 equal squares. To determine activity, an animal was placed at the corner of a square of the open field and

immediately after the placement the number of squares crossed was scored for 5 minutes.[102]

2) Motor coordination

a) Rotarod test

The rotarod apparatus consists of a motor rod with a drum of 7.0 cm diameter. It was adjusted to a speed of 12 revolutions/min during the test session. The latency to fall in a test session of 180 s was taken as a measure of motor coordination.

b) Despire swim test

Each animal was introduced into a pool (45cm long; 22cm wide diameter and 20cm high) filled with 10cm deep water. The animals were allowed to make rotations. The number of rotations made per 3mins was recorded.

3) Cataleptic behavior

Bar test

Catalepsy is defined as a decreased capability to start movement and not a successful effort to rectify uncharacteristic position, was calculated by help of the bar test method. To test for catalepsy, one by one all the animals were placed in such a way so that their rear legs were on the counter and their forelimbs were rested on a 1cm width flat bar, 6-9cm higher than the counter. The time to which the animal maintained this arrangement was noted by the use of stopwatch to a ceiling limit of 180 s (likewise, three consecutive trials must be conducted at an interval of 1 min between each trial). Animals were declared to be cataleptic if they remain in this arrangement for 30 s or more.

4.7.2 Estimation of biochemical parameters

1) Oxidative parameters in brain tissue homogenate

Tissue processing

Reagents

1. Phosphate buffered Saline (P^H – 7.4): and the ingredients are

1.38g of DSEDTA (disodium ethylene diamine tetra acetic acid), 0.19g of potassium dihydrogen phosphate ($K_2H_2PO_4$) and 8g of sodium chloride (NaCl) were dissolved in 900ml of distilled water and PH was adjusted using dilute HCl. The volume was made upto 1000ml.

2. Sucrose Solution (0.25M):

85.85g of sucrose was dissolved in 200ml of water and diluted to 1000ml with distilled water.

3. Tris HCl buffer (10mM, P^H-7.4):

1.21g tris was added and dissolved in 900ml of pure distilled water and the P^H was made to 7.4 with 1 M HCl. The resultant solution was thinned (diluted) to 1000 ml using pure distilled water.

Procedure

The animals were euthanasiously sacrificed; the brain was quickly transferred to ice cold phosphate buffered saline (PBS P^H – 7.4). It was maintained free of blood and other tissue fluids and weighed

The cut off tissue was nicely chopped with surgical blade into small slices. Then the pieces were placed in ice-cold 0.25M sucrose solution and swiftly placed on a filter paper. The tissues were then crushed and homogenized in ice-cold tris HCl buffer of strength 10mM of P^H – 7.4 to a concentration of 10% w/v. long-standing homogenization under hypotonic state was intended to disrupt as far as achievable so that the structure of

cells can easily free soluble proteins. The homogenate obtained was centrifuged at 7000 rpm for 25 minutes under normal conditions using Remi (RM-12C) high speed centrifuge. The clear supernatant fluid was used for the purpose of different biochemical parameters estimation. The residue was resuspended in ice cold tris HCl buffer (10mM, P^H – 7.4) to get a ultimate concentration of 10%w/v and was used for cellular protein estimations.

a. Superoxide dismutase (SOD)

SOD was estimated by the method of Misra and Fridovich (1967).

Principle

Speed of self oxidation of epinephrine and the understanding of this relation between self oxidation to stoppage by SOD were amplified rise in the P^H from 7.8 – 10.2. Oxygen generated by xanthine oxidase mediated reaction which causes the oxidation of epinephrine to adrenochrome in the presence of pre yielded oxygen. The self oxidation of epinephrine moves forward by minimum of two separate pathways among those only one is free radical mediated chain reaction including oxygen and thus inhibit able by SOD.

Reagents

1. Carbonate buffer (0.05 M, pH 10.2): 16.8 grams of sodium bicarbonate ($NaHCO_3$) and 22 grams of sodium carbonate (Na_2CO_3) were dissolved in 500 ml of distilled water and final volume was made up to with the help of distilled water.
2. Ethylene diamine tetra acetic acid (EDTA) (0.49M): 1.82 g of EDTA was dissolved in 1000 ml of distilled water.
3. Epinephrine (3 mM): 9.9 mg of epinephrine bitartrate was dissolved in 10 ml of 1M HCl solution.
4. SOD standard: dissolve 1mg (1000 units/mg) of SOD from bovine liver in 100 ml of carbonate buffer.

Procedure

0.5ml of tissue homogenate obtained (brain) was diluted with 0.5 ml distilled water; to this 0.25 ml ethanol and 0.5 ml of chloroform (all pre chilled reagents) were to be added. The resultant mixture was shaken for about 1 min and centrifuged at a rate of 2000 rpm for 20 min. The enzymatic action of supernated fluid was found out by adding 0.05ml of carbonated buffer (0.05 M pH 10.2) and also 0.5 ml EDTA (0.49 M). The reactions were underway by the adding up of 0.4 ml epinephrine and the

changes in optical density/mm were regularly measured at 480 nm. SOD was shown with U/mg protein. A calibration curve was prepared using 10-125 units SOD.

$$SOD = \frac{(0.025 - Y)}{Y \text{ X } 50} X100$$

Y = Final reading – initial reading

b. Catalase (CAT)

Catalase was estimated by Hugo E. Aebi method, 1974

Principle

$$H_2O_2 \longrightarrow H_2+O_2$$

$$RDOH \longrightarrow H_2O + ROH + A$$

Decomposition of H_2O_2 = Decrease in absorbance at 240 nm

Reagents

1. Phosphate buffer (50mM, pH 7.0):

A) Dissolve 6.81 g KH_2PO_4 in pure distilled water and make the volume up to 1000 ml using pure distilled water.

B) Dissolve 8.9 g NaH_2PO_4. $2H_2O$ in pure distilled water and make the volume up to 1000 ml using pure distilled water.

Mix the solution A and B in proportion 1:15 (v/v)

2. Hydrogen peroxide (30 mM/l): Dilute 0.34 ml of 30% Hydrogen peroxide with phosphate buffer upto 100ml.

Dilute homogenate (kidney or brain) 20 times with Phosphate buffer pH 7.0.

Blank	Test
4 ml of tissue homogenate diluted with 2 ml of phosphate buffer pH 7, and take absorbance at 254 nm for 3 min. with 30 sec. interval.	2 ml of tissue homogenate diluted with 1 ml of H_2O_2 (8.5 micro lit. in 2.5 ml phosphate buffer (50mM/1. pH 7.0) and take the absorbance at 254 nm for 3 min. with 30 sec interval. (Add H_2O_2 just before taking O.D)

Calculation

Log (A / B) x 2297.3

Where, A: Initial absorbance, B: final absorbance (after 30 seconds)

Units = µmoles of H_2O_2 consumed / mg protein/min in brain

c. Reduced glutathione (GSH)

Reagents

1. TCA (10% w/v) solution: Precisely weigh 10 g of Tricarboxylicacid i.e. ascorbic acid was mixed in 100ml of distilled water.
2. Phosphate buffer (0.2 M, pH8) was used when it is required
3. DTNB reagent (0.6 M): 60 mg of 5,5 = dithio bis (2-nitro benzoic acid) was dissolved in 100 ml of 0.2 M sodium phosphate (pH 8).
4. Standard glutathione: Prepared by dissolving 10 mg of reduced glutathione is 100 ml of distilled water.

Procedure

To the 1 ml of tissue homogenate of either kidney or brain, 1 ml of 10% TCA i.e. ascorbic acid was supplemented. The precipitated fraction was separated by centrifugation and 0.5 ml of superficial fluid was taken to which 2 ml of DTNB was further added. The ultimate volume was made up to 3 ml with the help of phosphate buffer. The colour represented was read at 412 nm. The amount of glutathione was expressed in nmol of GSH/mg protein reduced glutathione was used as standard (100 µg/ml).

$$X = \frac{Y - 0.0046}{0.0034}$$

Y = Absorbance of test sample

d. Lipid peroxidation (LPO)

Reagents

1. Thiobarbituric acid: 0.67% w/v in 1 M tris hydrochloride pH – 7, 0.67 grams of thiobarbituric acid (TBA) was dissolved in 100ml of pure distilled water.
2. Trichloroacetic acid (TCA) (20% w/v): 20 g of TCA was to be dissolved in 100 ml of pure distilled water.
3. Standard malondialdehyde (0-25 n.mol)

A stock solution containing 50 mm/ml of 1,1,3,3-tetra ethoxy propane in tris hydrochloride buffer in pH – 7, of which 10 ml of stock/reserve solution was added with water to make final volume to 100 ml to obtain a working standard 50 nm malondialdehyde/ml. This was used for preparation of calilbration curves.

Procedure

1 ml of tissue homogenate (kidney or brain) was mixed with 2 ml of 20% TCA and kept in ice for a period of 15 min. The precipitate separation was done by centrifugation and 2 ml of sample of clear supernatant solution were mixed with 2 ml aq. 0.67% TBA solution. This mixture was heated on a boiling water bat for 10 min. It was cooled in ice for 5 min and the absorbance was calibrated at 535 nm. The obtained values were expressed as nmol of MDA formed/mg of protein values are normalized to protein content of tissues.

X = (Y + 0.002) / 0.0026086

Y = Absorbance differences of final (after 3 min) and initial reading of test sample.

e) Tissue parameters

Dopamine assay

Reagents

1. 0.4M HCl: 3.4ml of concentrated HCl was diluted 100ml using distilled water.
2. Sodium Acetate Buffer: 2.88ml of IM acetic acid and 27.33 ml of 0.3M sodium acetate were mixed and the volume was made upto 100ml with distilled water and the P^H was adjusted with NaOH solution.
3. 5M NaOH: 20g of NaOH pellets were dissolved in distilled water and the volume was made upto 100ml.
4. Sodium thio sulphate: 0.5grams of sodium thio sulphate was to be dissolved accurately in 2ml of pure distilled water and then 18ml of 5M NaOH was added.

5. 0.1M Iodine solution (in Ethanol): 4g of KI and 2.6g of iodine in ethanol and the volume was made upto 100ml.
6. 10M Acetic acid: to prepare this 57ml of glacial acetic acid must be dissolved in accurately 100ml of pure distilled water.

Procedure

Preparation of tissue extract

Weighed quantity of tissue was to be homogenized in around 3ml HCl-Butanol mixture in a precooled setting. The sample was then subjected to centrifugation for 10 minutes at a rate of 2000 rpm. After the centrifugation 0.8ml of supernatant fluid was removed and mixed with an eppendorf reagent tube bearing 2ml of heptanes and 0.25ml of 0.1M HCl. After 10mins, shake the tube gently and continue centrifugation under same conditions and environment to separate two phases again. The superior organic phase must be discarded and the lower aqueous phase was preferably used for dopamine assay.

Assay Procedure

To 0.02ml of the HCl acid phase, 0.4 ml HCl and 0.01 m1 of EDTA/ sodium acetate buffer (P^H 6.9) must be added, following this 0.01ml iodine solution was also added for oxidation. The reaction was blocked soon after 2mins of start by the adding 0.ml of sodium thio sulphate in 5M sodium hydroxide solution. Also 10M acetic acid was added after 1.5 min to the previous reaction. The solution after then was heated to 100^{o}c for 6mins continuously. Allow the samples to reach to room temperature. When the samples reach room temperature soon excitation following emission spectra was read at a wavelength of 330-375nm in a spectrofluorimeter. Compare

the values of tissue extracts obtained (fluorescence of tissue extract v/s fluorescence of tissue blank) with an interior reagent standard (fluorescence of internal standard v/s fluorescence of internal reagent blank). Tissue blanks required for the assay were determined by the addition of reagents required for the oxidation step in overturn/reverse order (iodine after sodium thio sulphate). Preparations with Internal reagent standards were conputed by adding 0.005ml of twice distilled water and 0.1ml of HCl Butanol to 20 nano grams of dopamine standard (Schlumpf et al.,). It is expressed in picograms/mg protein.

f) Histopathology

Light microscopy:

After the experiment rats were sacrificed by cervical dislocation. Their brains were removed and weighed individually. Thereafter, cerebellum was dissected out for biochemical analysis. For the evaluation of histopathological changes in cerebellum, small section of the tissue was immediately fixed with formalin. Thereafter, the specimens were embedded in paraffin, sectioned at 5 µm and stained with hematoxyline and eosin.

4.8 In vitro antioxidant activity

Principle

An antioxidant is a natural or synthetic molecule which bears the capacity of outpacing or abolishing the free radical mediated oxidation of other vital molecules. Oxidation reactions are capable of producing free radicals, which can start nonstop chain reactions that are capable of damaging the live cells. Antioxidant action falls into two main distinct

categories, those whose role is to avoid the production of free radicals and those that cut off any radicals that are being generated.[51,52,53]

Free radicals are chemical group possessing one or more free electrons and frequently craft a particle more reactive than the matching non-radical. The fragment acts as an electron acceptor and fundamentally 'Steals' electrons from erstwhile molecules. Reactive free radicals are considered as oxidizing agents (not required) as they root other molecules to give their electrons. They are produced endlessly in cells, either as unintentional by products of metabolism or intentionally. The most widespread cellular oxygen free radicals are superoxide free radical (O_2-), hydroxy free radical (OH^-), nitric acid free radical (NO) and other related molecules, such as hydrogen peroxide (H_2O_2) capable of forming super oxides and peroxynitrate (ONOOO) are not conventional free radicals but can lead to the generation of free radical through different chemicals reactions.[50]

Table 4.22 Drugs and Chemicals of Invitro antioxidant activity

Drugs	Manufacturers
DPPH	Hi-Media Pvt Ltd.
FTC	Hi-Media Pvt Ltd.
TBA	Hi-Media Pvt Ltd.
Petroleum Ether	Hi-Media Pvt Ltd.
Chloroform	Hi-Media Pvt Ltd.

Table 4.23 Instruments of Invitro antioxidant activity

Instrument	Company
Analytical UV-Visible spectrophotometer	2200/Systronics
Electronic balance	LC/GC
Centrifuge	13REMI
Automated Hematology Analyzer	Chem7/Erba

The antioxidant activity of *Nigella sativa* was evaluated by both qualitative and quantitative methods.

The antioxidant activity of the NS plant extracts was tested using three well known methods: DPPH-method, ferric thiocynate (FTC) method and thiobarbituric acid (TBA) method. The preferred FTC method was selected to evaluate the quantity of peroxide development before the onset of the lipid peroxidation, in which ferric ion is formed after the reaction of ferric chloride with peroxides. The ferric ion formed then joins with ammonium thiocynate and produce ferric thiocynate. The resultant substance is red in colour. The intensity of the colour is directly proportional to the absorbance and concentration. Whereas the TBA methods accounts for the free radicals generated after peroxide oxidation. Whereas DPPH method was employed to estimate the degree of free-radical scavenging potency.

4.8.1 Qualitative methods

a. FTC (Ferric thiocyanate) method

To 4mg of plant extract, add 4ml of absolute alcohol (ethanol), and also 4.1ml of linolenic acid (2.5%) an essential fatty acid in absolute ethanol along with precisely 8ml of accurate 0.05M phosphate buffer (P^H 7) and make the final volume by adding 3.9ml of water. The sample solution was placed in a vial with screw cap and kept in an oven at 40c in dark for half an hour. To 0.1ml of above solution, 9.7ml of 75%ethanol, 0.1ml of 30%ammonium thiocyanate and 0.1ml of 0.02M ferric chloride in 3.5% Hydrochloric acid was also added. The absorbance must be measured at

500nm at regular intervals of 24 hours until standard (control) absorbance reaches maximum.[46,47,48,49]

b. TBA (Thiobarbituric acid) method

This was performed on final day of FTC method. To 1ml of above sample solution, 2ml of 20% trichloroacetic acid, 2ml of 0.67% Thiobarbituric acid was added. The mixture was placed in boiling water bath for ten minutes. After ten minutes, the mixture was removed, cooled and centrifuged at 3000rpm for 20 minutes. The absorbance was measured at 552nm. [46,47,48,49]

c. DPPH Free redical scavenging activity

1. 1ml of different concentrations (25 µg, 50 µg, 100 µg, 200 µg, 400 µg, 800 µg) of the test extracts of NS was prepared in methanol (medium) and was added precisely with 3ml of 0.1milli Molar methanol containing solution of DPPH.
2. The solution in the tubes were shaken dynamically and permitted to stand for 3mins at normal room temperature in dark.
3. Absorbance of different samples was measured precisely at 517 nm.
4. Control absorbance reading was obtained using methanol.

Free radical scavenging potency of the test substances was expressed as inhibition percentage using the below given formula. [46,47,48,49]

$$\% \text{ Inhibitation} = \frac{[AT - A0]}{AT} \times 100$$

Where,

A_o is the absorbance of the control, A_r is the absorbance of test sample

4.8.2 Quantitative method

Suitable mobile phase was prepared. Plant extract was placed on the TLC plate and kept in mobile phase for the chromatogram to develop. After

the development, the whole plate was sprayed with 0.15%w/v DPPH solution using an automiser. The change in colour was observed. [46,47,48,49]

Statistical analysis

The data was expressed as mean ± SEM and statistically analysed using DUNNET test.

4.9 Pharmacovigilance:[87]

It is a non-randomized, prospective, observational study carried out at a primary care hospital at Kurnool district of Andhra Pradesh under the supervision of ayurvedic physicians. The present study was approved by Institutional human ethical committee of Raghavendra institute of Pharmaceutical education and research (RIPER/IRB/005/2012). The data collection and analysis of data was done for a period of 6 months After explaining the purpose, protocol, risk and benefit of the present study the inform consent were taken from the study subjects. Subjects those who are aged between 18 – 60 years with or without concominent diseases were included in the study and bears inclusion criteria. Drugs were measured and prescribed by the Ayurvedic physician and dispensed and counselled by the qualified pharmacist.

Nigella sativa Seed:

The seeds were washed, dried, powdered and 500 mg of powdered drug was selected as dose for the common ailments like Infections, Fever, Arthritis etc. [21]

A total of 250 were registered, among which 60 were excluded from the study as they were not fulfilling the inclusion criteria. Remaining 190 patients were included in the study.

Well designed proforma was used as a data collection form which includes the details of demography, diagnostic and all details about medication (Dose, Frequency, Dosage form etc). The patients were monitored and complaints of ADRs were noted through direct interview once every week.

The causality of reaction was assessed by using Naranjo's algorithm[103] and WHO probability scale.[104] The severity and preventability of adverse drug reaction was assessed by modified Hartwig and Seigel scale[105] and Shumock and Thornton scale.[106]