MATERIALS AND METHODS

Collection of Plant material

The fresh plant materials of whole plant of *Rauwolfia densiflora* (Wall.) Benth. ex Hk.f. and tubers of *Stephania wightii* (Arn.) Dunn. were collected from Karaiyar, Agasthiarmalai Biosphere Reserve, Western Ghats, Tamil Nadu (Plate - I). The plants were identified with the help of local flora and authenticated in Botanical Survey of India, Southern Circle, Coimbatore, Tamil Nadu, India. A voucher specimen of collected plants was deposited in the Department of Botany Herbarium (SMCH), St.Mary’s College (Autonomous), Thoothukudi District, Tamil Nadu. The collected plant materials were cut into small fragments and shade dried until the fragments are uniform and smooth. The dried plant materials were granulated or powdered by using a blender and sieved to get uniform particles by using sieve No. 60. The final uniform powder was used for the extraction of active constituents of the plant materials.

Physicochemical constituents

Chemicals

All the chemicals and reagents used in the experiments were of analytical grade and were obtained from BDH (England and India), E.Merck (Germany), Sigma Chemical Company (U.S.A.), Sarabhai, M. Chemicals (India), LOBA - Chemie Indo Austranol Co., (India), whenever necessary the solvents were redistilled before use.
Ash values (Anonymous, 2002)

**Determination of total ash**

Three grams of the powdered drug was accurately weighed in a silica crucible, which was previously ignited and weighed. The powdered drug was spread as a fine layer on the bottom of the crucible. The crucible was incinerated at a temperature not exceeding 450°C until free from carbon. The crucible was cooled and weighed. The procedure was repeated to get the constant weight. The percentage of total ash was calculated with reference to the air dried powder.

**Determination of water soluble ash**

The ash obtained in the determination of total ash was boiled for 5 minutes with 25 ml of water. The insoluble matter was collected on an ashless filter paper and washed with hot water. The insoluble ash was transferred into a pre-weighed silica crucible and ignited for 15 minutes at a temperature not exceeding 450°C. The procedure was repeated to get the constant weight. The weight of the insoluble matter was subtracted from the weight of the total ash. The difference in weight was considered as the water soluble ash. The percentage of water soluble ash was calculated with reference to the air dried powder.

**Determination of sulphated ash**

A silica crucible was heated to redness for 10 minutes and cooled in a desiccator and weighed. Transferred to the crucible 1 gram of sample and weighing the crucible and the contents accurately. Ignite, gently at first, until the substance was thoroughly charred. The residue was cool moistened with 1 ml of sulphuric acid, heat gently until the white fumes are no longer evolved and ignite at 800°C ± 25°C until all
black particles disappear. Conduct the ignition in a place protected from air currents. Allow the crucible to cool, add a few drops of sulphuric acid and heat. Ignite as before, allow to cool and weigh. Repeat the operation until two successive weighings do not differ by more than 0.5 mg.

**Determination of acid insoluble ash**

The ash obtained as described in the determination of total ash was boiled with 25 ml of 2N HCl for 5 minutes. The insoluble ash was collected on an ashless filter paper and washed with hot water. The insoluble ash was transferred into a pre-weighed silica crucible. The procedure was repeated to get constant weight. The percentage of acid insoluble ash was calculated with reference to the air dried drug.

**Extractive values (Anonymous, 1996)**

Extractive values of crude drugs are useful for their evaluation especially when the constituents of a drug cannot be readily estimated by any other means. These values also indicate the value of the constituents present in a crude drug. The extractive values were determined as per the standard procedures.

**Petroleum ether soluble extractive**

Four grams of air dried coarse powder of samples of whole plant of *Rauwolfia densiflora* and tubers of *Stephania wightii* were macerated with 100 ml of petroleum ether in a closed flask for 24 hours, shaking frequently during first 6 hours and allowed to stand for 18 hours. It was then filtered rapidly taking precautions against loss of the solvent. 25 ml of the filtrate was evaporated to dryness in a tarred flat-bottomed shallow dish dried at 105°C and weighed. The percentage of petroleum ether soluble extractive with reference to the air dried drug was calculated.
**Benzene soluble extractive**

Four grams of air dried coarse powder of samples of selected plants were macerated with 100 ml of benzene in a closed flask for 24 hours, shaking frequently during first 6 hours and allowed to stand for 18 hours. It was then filtered rapidly taking precautions against loss of the solvent. 25 ml of the filtrate was evaporated to dryness in a tarred flat-bottomed shallow dish dried at 105°C and weighed. The percentage of benzene soluble extractive with reference to the air dried drug was calculated.

**Chloroform soluble extractive**

Four grams of air dried coarse powder of samples of selected plant were macerated with 100 ml of 95% chloroform in a closed flask for 24 hours, shaking frequently during first 6 hours and allowed to stand for 18 hours. It was then filtered rapidly taking precautions against loss of the solvent. 25 ml of the filtrate was evaporated to dryness in a tarred flat-bottomed shallow dish dried at 105°C and weighed. The percentage of chloroform soluble extractive with reference to the air dried drug was calculated.

**Acetone soluble extractive**

Four grams of air dried coarse powder of samples of selected plants were macerated with 100 ml of acetone in a closed flask for 24 hours, shaking frequently during first 6 hours and allowed to stand for 18 hours. It was then filtered rapidly taking precautions against loss of the solvent. 25 ml of the filtrate was evaporated to dryness in a tarred flat-bottomed shallow dish dried at 105°C and weighed. The
percentage of acetone soluble extractive with reference to the air dried drug was calculated.

**Methanol soluble extractive**

Four grams of air dried coarse powder of samples of selected plants were macerated with 100 ml of methanol in a closed flask for 24 hours, shaking frequently during first 6 hours and allowed to stand for 18 hours. It was then filtered rapidly taking precautions against loss of the solvent. 25 ml of the filtrate was evaporated to dryness in a tarred flat-bottomed shallow dish dried at 105°C and weighed. The percentage of methanol soluble extractive with reference to the air dried drug was calculated.

**Ethanol soluble extractive**

Four grams of air dried coarse powder of samples of selected plants were macerated with 100 ml of 95% ethanol in a closed flask for 24 hours, shaking frequently during first 6 hours and allowed to stand for 18 hours. It was then filtered rapidly taking precautions against loss of the solvent. 25 ml of the filtrate was evaporated to dryness in a tarred flat-bottomed shallow dish dried at 105°C and weighed. The percentage of ethanol soluble extractive with reference to the air dried drug was calculated.

**Water soluble extractive**

Four grams of air dried coarse powder of samples of the selected plants were macerated with 100 ml of water : chloroform (95 ml of water + 5 ml of chloroform) in a closed flask for 24 hours, shaking frequently during the first 6 hours and allowed to stand for 18 hours. It was then filtered rapidly taking precautions against loss of the
solvent. 25 ml of the filtrate was evaporated to dryness in a tarred flat-bottomed shallow dish dried at 105° C and weighed. The percentage of water soluble extractive with reference to the air dried drug was calculated.

**Fluorescence analysis (Chase and Pratt, 1949)**

The drug powders were treated with acids like 1N HCl, 50% H₂SO₄, nitric acid, acetic acid and alkaline solutions like aqueous sodium hydroxide, alcoholic sodium hydroxide and other solvents like acetone, benzene, lead acetate, ferric chloride and NH₃. They were subjected to fluorescence analysis in daylight and in UV light (254 nm and 365 nm).

**Preparation of extracts for Preliminary phytochemical screening, GC-MS analysis and Pharmacological studies**

**Hot maceration method using Soxhlet apparatus**

Freshly collected plant materials were dried in shade and then coarsely powdered in a blender. The coarse powder (100g) was extracted successively with petroleum ether, chloroform, methanol and ethanol each 250 ml in a Soxhlet apparatus for 24 hrs. All the extracts were filtered through Whatman No. 41 filter paper. All the extracts (petroleum ether, chloroform, methanol and ethanol) were subjected to qualitative tests for the identification of various phytochemical constituents as per the standard procedures (Brindha et al., 1981; Anonymous, 1990; Lala, 1993). The ethanol extracts were concentrated in a rotary evaporator. The concentrated ethanol extracts were used for GC-MS analysis and pharmacological studies.
Preliminary phytochemical screening of different extracts (Brindha et al., 1981; Anonymous, 1990; Lala, 1993)

The chemical tests for various phytoconstituents in the extracts were carried out as described below:

**Test for Alkaloid**

The test solution was mixed with little amount of dilute hydrochloric acid and Mayer’s reagent. Formation of a white precipitate indicates the presence of alkaloids.

**Test for Anthraquinone**

A few drops of magnesium acetate solution was added to the test solutions. Pink colour formation shows the presence of anthraquinones.

**Test for Catechin**

To the test solution, a few drops of Ehrlich reagent and concentrated hydrochloric acid were added. Appearance of pink colour indicates the presence of catechins.

**Test for Coumarin**

To 2 ml of the test solution, a few drops of alcoholic sodium hydroxide were added. Appearance of yellow colour indicates the presence of coumarins.

**Test for Flavonoid (Shindo’s test)**

To the test solution, a few magnesium turnings and a few drops of concentrated hydrochloric acid were added and boiled for five minutes. Appearance of red or orange red colour indicates the presence of flavonoids.
**Test for Phenol**

To the test solution, a few drops of ferric chloride solution were added. Bluish green or red colour indicates the presence of phenols.

**Test for Quinone**

The test solution was treated with a few drops of concentrated sulphuric acid or aqueous sodium hydroxide solution. Colour formation indicates the presence of quinoid compounds.

**Test for Saponin**

The test solution was shaken with water. Copious lather formation indicates the presence of saponins.

**Test for Steroid (Libermann - Burchard test)**

To 2 ml of the test solution, few drops of chloroform, 3-4 drops of acetic anhydride and one drop of concentrated sulphuric acid were added. Appearance of purple colour, which changes to blue colour or green colour, shows the presence of steroids.

**Test for Tannin**

The test solution was mixed with basic lead acetate solution. Formation of a white precipitate indicates the presence of tannins.

**Test for Terpenoid (Noller’s test)**

The test solution was warmed with a piece of tin and a few drops of thionyl chloride. Violet or purple colouration indicates the presence of terpenoids.
Test for Sugar

The test solution was mixed with equal volumes of Fehling’s solution A and B and heated. Formation of red colour is the indication of the presence of sugars.

To the test solution, very small quantity of anthrone and few drops of concentrated sulphuric acid were added and heated. Green to purple colouration indicates the presence of sugars.

Test for Glycoside

The extract was mixed with a little anthrone on a watch glass. One drop of concentrated sulphuric acid was added and made into a paste and warmed gently over the water bath. The presence of glycosides was identified by dark green colouration.

Test for Xanthoprotein

To the test solution, a few drops of concentrated nitric acid and few ml of ammonia were added. Appearance of a red precipitate indicates the presence of xanthoproteins.

Test for Fixed oil (Spot test)

A small quantity of powder/extract is pressed between the filter papers. Formation of grease spot indicates the presence of fixed oils and fats.

HPTLC Analysis (Mohana Rao et al., 2005)

In the past few decades, compounds from natural sources have been gaining importance because of vast chemical diversity that they offer. This leads to the phenomenal increase in the demand of herbal medicine and herbal drug. Phytochemical evaluation is one of the tools for quality assessment, which includes
preliminary phytochemical screening, chemo profiling and marker compound analysis using modern analytical techniques. HPTLC (High Performance Thin Layer Chromatography) is one of the techniques for the qualitative, semi-qualitative and quantitative phytochemical analysis of herbal drugs and formulations. This includes developing TLC fingerprint profiles and estimation of chemical markers and biomarkers. The major advantage of HPTLC is that several samples can be analyzed simultaneously using a small quantity of mobile phase (Mohana Rao et al., 2005).

**Alkaloid profile**

**Samples code**

Sample 1 - *Rauwolfia densiflora* ethanolic extract  
Sample 2 - *Stephania wightii* ethanolic extract  
NIC - Nicotine standard as a reference compound

**Procedure**

**Extraction and Test solution preparation**

The dried plant materials (5 gm) were extracted with ethanol in Soxhlet apparatus for 3 hrs. The content was cooled, filtered and concentrated using vacuum flash evaporator. The content was dissolved in 1ml ethanol and centrifuged at 3000 rpm for 5 min. These solutions were used as test solutions for HPTLC analysis.

**Sample application**

1.5 µl of test solutions (two samples) and 3 µl of standard solution were loaded at 5 mm band length in the 3 x 10 Silica gel 60F_{254} TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument.
Spot development

The samples loaded plate was kept in TLC twin trough developing chamber (after saturated with solvent vapour) with respective mobile phase (alkaloids) and the plate was developed in the respective mobile phase upto 90 mm.

Photo-documentation

The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at white light, UV 254 nm and UV 366 nm.

Derivatization

The developed plate was sprayed with respective spray reagent (alkaloids) and dried at 100°C in hot air oven. The plate was photo-documented in day light and UV 366 nm mode using photo-documentation (CAMAG REPROSTAR 3) chamber.

Scanning

Before derivatization, the plate was fixed in scanner stage and scanning was done at 254 nm. The peak table, peak display and peak densitogram were noted.

Analysis details

Mobile phase

n-Butanol-acetic acid-water (4 : 4 : 1)

Spray reagent

Dragendorff’s reagent followed by 10% ethanolic sulphuric acid reagent and dried at 100°C for 2 minutes.
Detection

Orange-brown coloured zone at day light mode was present in the standard track, it was observed from the chromatogram after derivatization, which confirmed the presence of Alkaloids in the given standard and brown coloured zone at day light mode was present in the sample track, it was observed from the chromatogram after 10% ethanolic sulphuric acid reagent derivatization, which confirmed the presence of Alkaloids in the given sample.

Glycoside profile

Samples code

    Sample 1   - Rauwolfia densiflora ethanolic extract
    Sample 2   - Stephania wightii ethanolic extract
    STE        - Stevioside standard as a reference compound

Procedure

Extraction and Test solution preparation

The dried plant materials (5 gm) were extracted with ethanol in Soxhlet apparatus for 3 hrs. The content was cooled, filtered and concentrated using vacuum flash evaporator. The content was dissolved in 1ml ethanol and centrifuged at 3000 rpm for 5 min. These solutions were used as test solutions for HPTLC analysis.

Sample application

1.5 µl of test solution and 5 µl of standard solution were loaded at 5 mm band length in the 3 x 10 Silica gel 60F_{254} TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument.
**Spot development**

The samples loaded plate was kept in TLC twin trough developing chamber (after saturated with solvent vapour) with respective mobile phase (glycosides) and the plate was developed in the respective mobile phase upto 90 mm.

**Photo-documentation**

The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at white light, UV 254 nm and UV 366 nm.

**Derivatization**

The developed plate was sprayed with respective spray reagent (glycosides) and dried at 100°C in hot air oven. The plate was photo-documented in day light mode using photo-documentation (CAMAG REPROSTAR 3) chamber.

**Scanning**

After derivatization, the plate was fixed in scanner stage and scanning was done at 500 nm. The peak table, peak display and peak densitogram were noted.

**Analysis details**

**Mobile phase**

Ethyl acetate-ethanol-water (8 : 2 : 1.2)

**Spray reagent**

Libermann Burchard reagent and dried at 100°C for 2 minutes.
Detection

Bluish brown coloured zone at day light mode were present in the standard and sample track, it was observed from the chromatogram after derivatization, which confirmed the presence of Glycosides in the given sample.

Steroid profile

Samples code

Sample 1  -  *Rauwolfia densiflora* ethanolic extract  
Sample 2  -  *Stephania wightii* ethanolic extract  
SGL       -  Stigmasterol standard as a reference compound

Procedure

Extraction and Test solution preparation

The dried plant materials (5 gm) were extracted with ethanol in Soxhlet apparatus for 3 hrs. The content was cooled, filtered and concentrated using vacuum flash evaporator. The content was dissolved in 1ml ethanol and centrifuged at 3000 rpm for 5 min. These solutions were used as test solutions for HPTLC analysis.

Sample application

1.5 µl of test solution and 3 µl of standard solution were loaded at 5 mm band length in the 3 ×10 Silica gel 60F254 TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument.
**Spot development**

The samples loaded plate was kept in TLC twin trough developing chamber (after saturated with solvent vapour) with respective mobile phase (steroids) and the plate was developed in the respective mobile phase upto 80 mm.

**Photo-documentation**

The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at white light, UV 254 nm and UV 366 nm.

**Derivatization**

The developed plate was sprayed with respective spray reagent (steroids) and dried at 100\(^0\)C in hot air oven. The plate was photo-documented in day light and UV 366 nm mode using photo-documentation (CAMAG REPROSTAR 3) chamber.

**Scanning**

After derivatization, the plate was fixed in scanner stage and scanning was done at 500 nm. The peak table, peak display and peak densitogram were noted.

**Analysis details**

**Mobile phase**

Toluene-acetone (9 : 1)

**Spray reagent**

Anisaldehyde sulphuric acid reagent and dried at 100\(^0\)C for 2 minutes.
Detection

Blue, violet coloured zone at day light mode were present in the standard and sample track, it was observed from the chromatogram after derivatization, which confirmed the presence of Steroids in the given sample.

Coumarin profile

Sample 1  - *Rauwolfia densiflora* ethanolic extract
Sample 2  - *Stephania wightii* ethanolic extract
CMN       - Coumarin standard as a reference compound

Procedure

Extraction and Test solution preparation

The dried plant materials (5 gm) were extracted with ethanol in soxhlet apparatus for 3 hrs. The content was cooled, filtered and concentrated using vacuum flash evaporator. The content was dissolved with 1ml ethanol and centrifuged at 3000 rpm for 5 min. These solutions were used as test solutions for HPTLC analysis.

Sample application

1.5 µl of test solution and 1 µl of standard solution were loaded at 5 mm band length in the 3 x 10 Silica gel 60F₂₅₄ TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument.

Spot development

The samples loaded plate was kept in TLC twin trough developing chamber (after saturated with solvent vapour) with respective mobile phase (coumarins) and the plate was developed in the respective mobile phase upto 90 mm.
Photo-documentation

The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at white light, UV 254 nm and UV 366 nm.

Derivatization

The developed plate was sprayed with respective spray reagent (coumarins) and dried at 100°C in hot air oven. The plate was photo-documented in UV 366 nm mode using photo-documentation (CAMAG REPROSTAR 3) chamber.

Scanning

Before derivatization, the plate was fixed in scanner stage and scanning was done at UV 254 nm. The peak table, peak display and peak densitogram were noted.

Analysis details

Mobile phase

Ethyl acetate-formic acid-glacial acetic acid-water (100 : 11 : 11 : 26)

Spray reagent

5% ethanolic KOH reagent and dried at 100°C for 2 minutes.

Detection

Blue-green, blue coloured fluorescent zones at UV 366 nm mode were present in the track, it was observed from the chromatogram after derivatization, which confirmed the presence of Coumarins in the given sample.
**Phenol profile**

Sample 1  - *Rauwolfia densiflora* ethanolic extract  
Sample 2  - *Stephania wightii* ethanolic extract  
QUER  - Quercetin standard as a reference compound

**Procedure**

**Extraction and Test solution preparation**

The dried plant materials (5 gm) were extracted with ethanol in soxhlet apparatus for 3 hrs. The content was cooled, filtered and concentrated using vacuum flash evaporator. The content was dissolved with 1ml ethanol and centrifuged at 3000 rpm for 5 min. These solutions were used as test solutions for HPTLC analysis.

**Sample application**

1.5 µl of test solution and 3 µl of standard solution were loaded at 5 mm band length in the 3 x 10 Silica gel 60F$_{254}$ TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument.

**Spot development**

The samples loaded plate was kept in TLC twin trough developing chamber (after saturated with solvent vapour) with respective mobile phase (phenolics) and the plate was developed in the respective mobile phase upto 90 mm.
Photo-documentation

The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at white light, UV 254 nm and UV 366 nm.

Derivatization

The developed plate was sprayed with respective spray reagent (phenolics) and dried at 100°C in hot air oven. The plate was photo-documented in day light mode using photo-documentation (CAMAG REPROSTAR 3) chamber.

Scanning

Before derivatization, the plate was fixed in scanner stage and scanning was done at UV 254 nm. The peak table, peak display and peak densitogram were noted.

Analysis details

Mobile phase

Toluene-acetone-formic acid (4.5 : 4.5 : 1)

Spray reagent

Fast blue B salt reagent and dried at 100°C for 3 minutes.

Detection

Orange-red, reddish brown coloured zones at day light mode were present in the tracks, it was observed from the chromatogram after derivatization, which confirmed the presence of Phenols in the given sample.
GC-MS Analysis (Hema et al., 2010)

GC-MS analysis of these extracts was carried out by following the method of Hema et al. (2010). GC-MS analysis were performed using a Perkin-Elmer GC clauses 500 system and Gas Chromatograph interfaced to a Mass Spectrometer (GC-MS) equipped with a Elite-I fused silica capillary column (30m × 0.25mm ID × 1µdf), composed of 100% Dimethyl polysiloxane). For GC-MS detection, an electron ionization system with ionizing energy of 70eV was used. Helium gas (99.999%) was used as the carrier gas at constant flow rate 1 ml/min. and an injection volume of 2 µl was employed split ratio of 10:1 injector temperature 250°C; ion-source temperature 280°C. The oven temperature was programmed from 110°C (isothermal for 2 min.) with an increase of 10°C/min. to 200°C, then 5°C/min. to 280°C, ending with a 9 min. isothermal at 280°C. Mass spectra were taken at 70eV; a scan interval of 0.5 seconds and fragments from 45 to 450 Da. Total GC running time was 36 minutes. The relative % amount of each component was calculated by comparing its average peak area to the total areas, software adopted to handle mass spectra and chromatograms was a Turbo mass.

Interpretation on mass spectrum GC-MS was conducted using the database of National Institute of Standard and Technology (NIST) having more than 62000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.
PHARMACOLOGICAL STUDIES

DPPH Radical Scavenging (Blois, 1958)

The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant component. This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen donating antioxidant due to the formation of the non-radical form DPPH-H (Blois, 1958).

The free radical scavenging activity of all the extracts was evaluated by 1,1-picyrryl-hydrazyl (DPPH) according to the previously reported method (Blois, 1958). An 0.1 mm solution of DPPH in ethanol was prepared, and 1 ml of this solution was added to 3 ml of all extracts in methanol at different concentrations (125, 250, 500 and 1000 µg/ml). The mixtures were shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbances were measured at 517 nm using a UV-VIS spectrophotometer (Genesys 10 UV : Thermoelectron Corporation). Ascorbic acid was used as the reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability to scavenging DPPH radical was calculated by using the following formula:

\[
\text{DPPH scavenging effect (\% inhibition) } = \left( A_0 - A_1 \right) \times 100/A_0
\]

Where, \( A_0 \) is the absorbance of the control reaction and \( A_1 \) is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

Acute toxicity study (OECD, 2002)

Acute oral toxicity study was performed as per OECD - 423 guidelines (acute toxic class method), Wistar albino rats (n = 6) of either sex selected by random
sampling were used for acute toxicity study (OECD, 2002). The animals were kept fasting for overnight and provided only with water, after which the extracts were administered orally at 5 mg/kg body weight by gastric incubations and observed for 14 days. If mortality was observed in two out of three animals, then the dose administered was assigned as toxic dose. If mortality was observed in one animal, then the same dose was repeated again to confirm the toxic dose. If mortality was not observed, the procedure was repeated for higher doses such as 50, 100 and 2000 mg/kg body weight. All the experimental protocol was approved by the institutional animal ethics committee.

**Anticancer activity**

**Animals**

Male adult Swiss Albino mice (20-25 gm) were procured from Animal Experimental Laboratory of Raja Muthaiya Medical College, Annamalai University, Chidambaram, Tamil Nadu and used throughout the study. They were housed in microlon boxes in a controlled environment (temperature 25±2°C) and 12 hours dark/light cycle) with standard laboratory diet (SaiDurga Animal Feeds, Bangalore, India) and water *ad libitum*. The study was conducted after obtaining institutional animal ethical committee’s clearance. As per the standard practice, the mice were segregated based on their gender and quarantined for 15 days before the commencement of the experiment. They were fed on healthy diet and maintained in hygienic environment in our animal house.

**Tumour cells**

Dalton ascites lymphoma (DAL) cells were obtained under the courtesy of Department of Biochemistry, Adaiyar Cancer Institute, Chennai, India. The freshly
drawn ascitic fluid was diluted in phosphate buffer solution pH (6.8) and aliquot of (1 x 10^6 cells 0.25 ml) of the diluted solution were injected intraperitoneal inoculation to mice belonging to age group of 5 to 6 weeks and weight (20 to 25 gms).

**Antitumour activity (Gothoskar and Ranadive, 1971; Mazumdar et al., 1997)**

After acclimatization, mature male Swiss albino mice were divided into six groups (n=6). All the groups except Group I, were injected with DAL cells (1×10^6 cells/mouse. i.p.). This was taken as day 0, Group I served as normal saline control (1 ml/kg, p.o.) and Group II served as DAL bearing control. On day 1, the ethanol extracts of *R. densiflora* and *S. wightii* at a dose of 200 mg/kg each to Group III and IV were administered orally and continued for 30 consecutive days respectively. Group V served as tumour induced animal administered with vincristine (80 mg/kg body weight) for 30 consecutive days. On day 31, five mice of each group were sacrificed 24 hours after the last dose and the rest were kept with food and water *ad libitum* to check the increase in the life span of the tumour hosts. The effect of ethanol extract of *R. densiflora* and *S. wightii* on tumour growth and host's survival time were monitored by studying parameters like tumour volume, tumour cell count, viable tumour cell count, nonviable tumour cell count, mean survival time and increase in life span.

**Determination of tumour volume**

The mice were dissected and the ascitic fluid was collected from the peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube and packed cell volume determined by centrifuging at 1000 g for five minutes.
**Determination of tumour cell count**

The ascitic fluid was taken in a haematocrit (micro) tube and diluted 1000 times. Then a drop of the diluted cell suspension was placed on the Neubauer counting chamber and the cells in 64 small squares were counted.

**Estimation of viable and non-viable tumour cell count**

The cells were then stained with 0.4% trypan blue in physiological saline. The dye was counted as viable and nonviable cell count. The cells that did not take up the dye were viable and those that took the stains were non-viable. These viable and non-viable cells were counted.

\[
\text{Cell count} = \frac{\text{No. of cells} \times \text{Dilution}}{\text{Area} \times \text{Thickness of liquid film}}
\]

**Percentage of increase life span (% ILS)**

The effect of ethanol extracts of *R. densiflora* and *S. wightii* tumour growth was monitored by recording the mortality daily and percentage increase in the life span (% ILS) was calculated.

\[
\% \text{ ILS} = \frac{T - C}{C} \times 100
\]

Where,

\[T = \text{Mean survival time in days of treated group and}\]
\[C = \text{Mean survival time in days of control group}\]

Mean survival = Day of 1\textsuperscript{st} death + Day of last death \[\div 2\]
Haematological studies

Red blood cell count (RBC), haemoglobin content and white blood cell (WBC) counts were measured from freely flowing tail vein blood. WBC differential count was carried out from Leishman stained blood smears. Protein concentration was estimated by Lowry's method (Lowry et al., 1951). One millilitre of peritoneal fluid was withdrawn and centrifuged at 3000 rpm for 30 min. according to the method described by Dacie and Lewis (1958).

Antidiabetic activity

Experimental induction of diabetes in rats

Three months old male Wistar albino rats weighing 180-240g were obtained from the animal house of the laboratory of Agricultural University, Trissur, Kerala. All animals were kept in an environmentally controlled room with a 12hours light/12hours dark cycle. The animals had free access to water and standard rat diet. The rats were injected alloxan monohydrate dissolved in sterile normal saline at a dose of 150 mg/kg body weight, intraperitoneally. Since alloxan is capable of producing fatal hypoglycemia as a result of massive pancreatic insulin release, rats were treated with 20% glucose solution intraperitoneally after 6 h. The rats were then kept for the next 24h on 5% glucose solution bottles in their cages to prevent hypoglycemia (Dhandapani et al., 2002). After a fortnight, rats with moderate diabetes having glycosuria (indicated by Benedict’s test for urine) and hyperglycemia with blood glucose range of 200 - 260 mg/100 ml were used for the experiment.
Experimental design

In the present investigation, non-diabetic control rats and diabetic induced rats were used. Diabetics were induced in rats 2 weeks before starting the treatment. The rats were divided into five groups as follows after the induction of diabetics. Each group consists of 6 number of rats.

**Group I** : Rats received normal saline were served as a normal control by using an intragastric catheter tube (IGC).

**Group II** : Diabetic rats received 2.5 ml/kg body weight of normal saline 14 days by using an IGC and served as diabetic control.

**Group III** : Diabetic rats received *R. densiflora* whole plant extract at the dose of 200 mg/kg body weight for 14 days by using an IGC.

**Group IV** : Diabetic rats received *S. wightii* tuber extract at the dose of 200 mg/kg body weight for 14 days by using an IGC.

**Group V** : Diabetic rats received glibenclamide (600 µg/kg/body weight/day) orally for 14 days by using an IGC.

All the plant drug treatments were given between 9.30 to 10.00 hours in the morning. All rats were sacrificed on the morning of the respective experimental day, by decapitation. Blood was collected, sera separated by centrifugation at 3000 x g for 10 min. and stored at -20°C until used for enzyme and biochemical assays.
Estimation of Glucose (Sasaki et al., 1972)

Principle

Ortho toludine reacts with glucose in hot acetic acid solution to produce blue colour which is measured at 630 nm.

Reagents

1. Ortho toludine boric acid reagent: This reagent consists of 2.5g of thiourea and 2.4g of boric acid in 100 ml of a mixture of water, acetic acid and ortho toludine (distilled) in the ratio of 10:75:15.
2. Standard glucose: 100 mg of glucose in 0.1% benzoic acid. 10 ml of the above solution was diluted to 100 ml to give 100 µg of glucose ml.

Procedure

To 0.2 ml of serum, 0.8 ml of 10% TCA was added, mixed well and centrifuged. 0.5 ml of the supernatant was taken. To this, 2.0 ml of ortho toludine reagent was added and heated in a boiling water bath for 15 min. along with standard solution containing 20-100 µg of glucose. The blue colour developed was read at 640 nm. The result was expressed as mg/dl in serum.

Estimation of Insulin (Anderson et al., 1993)

Principle

UBI MAGIWEL™ Insulin is a solid phase Enzyme-Linked Immuno Sorbant Assay (ELISA). The wells are coated with monoclonal antibody with higher activity for insulin. When the samples and controls are incubated in the wells with enzyme conjugate, which is another antibody linked to horse radish peroxidase to form a sandwich complex bound to the well. Unbound conjugate are then washed off with
wash buffer. The amount of bounded peroxidase is proportional to the concentration of the insulin present in the sample. Upon addition of the substrate and chromogen, the intensity of the colour developed is proportional to the concentration of insulin in the samples.

**Assay procedure**

- Secured the designed number of coated wells in the holder. The data sheet was marked with sample identification.
- 25 µl of serum sample, control and reference were dispensed into the assigned wells.
- Then 100 µl of enzyme conjugate was dispensed into each well and mixed for 5 secs.
- The mixture was incubated for 30 min. at 25°C.
- The incubation mixture was removed and rinsed the wells five times with washing buffer.
- 100 µl of solution A and then 100 µl of solution B were dispensed into each well.
- Then incubated for 15 min. at room temperature.
- The reaction was stopped by adding 50 µl of 1N sulphuric acid or 2N HCl to each well and read OD at 450 nm with a micro well reader.

**Estimation of Urea (Varley, 1976)**

**Principle**

Diacetyl monoxime, in the presence of acid, hydrolyse to produce the unstable compound diacetyl. This reacts with urea to produce a yellow diazone derivative. The colour of this product becomes pink by addition of thiosemicarbazide which is measured colorimetrically at 520 nm.
Reagents

- TCA, 10%
- Stock Diacetylmonoxime, 25 g/L
- Stock Thiosemicarbazide, 2.5 g/L
- Acid ferric chloride solution: Added 1.0 ml sulphuric acid to 100 ml of ferric chloride solution containing 50 g/L in water.
- Acid reagent: Added 10 ml of ortho phosphoric acid, 80 ml sulphuric acid and 10 ml of ferric chloride solution to 1 litre of water and mixed.
- Color reagent: To 300 ml acid reagent, added 200 ml water, 10 ml stock diacetylmonoxime and 2.5 ml thiosemicarbazide.
- Stock urea standard: 5, 10, 15, 20, 30, 40 and 50 mM/L (30, 60, 90, 120, 180, 240 and 300 mg/100 ml).

Procedure

To 0.2 ml of serum, 1.0 ml water and 1.0 ml of 10% TCA were added, mixed well and centrifuged. 0.2 ml of the supernatant was taken and added 3.0 ml of colour reagent. The test tubes were kept in water bath for 20 min., cooled to room temperature and read the colour developed at 520 nm within 15 min. The result was expressed as mg/dl in serum.

Estimation of Creatinine (Owen et al., 1954)

Principle

Creatinine forms a coloured complex with picrate in alkaline medium. The rate of formation of the complex is measured at 540 nm.
Reagents

- Picric acid: 8.02 g/L
- Sodium hydroxide: 12.8 g/L
- Standard creatinine: Dissolved 100 mg of creatinine in 100 ml of distilled water.
- Working standard: 2.0 ml of stock solution was diluted to 100 ml with distilled water. This contains 20 μg of creatinine/ml.
- Reagent mixture: Mixed one part by volume of diluted NaOH with one part by volume of picric acid at least 30 minutes before the assay.

Procedure

0.2 ml of the serum and 2.0 ml of the reagent mixture were pipetted into a cuvette. Simultaneously, a blank was set up with the reagent mixture and distilled water. Mixed well and the change in absorbance was measured after 30 sec. which was taken as A₁ and exactly after 2 min., the absorbance was read as A₂ at 490 nm. Sets of standards were also treated in the same manner. A₁-A₂ gives the change in absorbance, which was the measure of the creatinine present in the sample. The result was expressed as mg/dl in serum. The values were expressed as mg of creatinine/dl.

Estimation of Glycosylated Haemoglobin (HBA₁C) (Karunanayake and Chandrasekharan, 1985)

At the end of the experimental period, animals were killed and blood samples (5 ml) were collected in heparinized tubes by cardiac puncture. Plasma was separated and cells were washed twice (0.154 M saline) and stored at -20°C until HBA₁C
concentration were determined by the method of Karunanayake and Chandrasekharan (1985).

**Estimation of Protein (Lowry et al., 1951)**

**Principle**

The blue colour developed by the reduction of the phosphomolybdic phosphotungstic components in the Folin-Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the biurette reaction of the protein with the alkaline cupric tartarate are measured at 660 nm.

**Reagents**

- 1.2% Sodium carbonate in 0.1N NaOH (Reagent A).
- 0.5% Copper sulphate in 1% potassium sodium tartrate (Reagent B).
- Alkaline copper reagent: Mixed 50 ml of A and 1.0 ml of B prior to use.
- Folin-Ciocalteau reagent: Mixed 1 part of reagent with 2 parts of water.
- Stock standard: Weighed 50 mg of bovine serum albumin and made upto 50 ml in a standard flask with saline.
- Working standard: Diluted 10 ml of the stock to 50 ml with distilled water. 1.0 ml of this solution contains 200 µg of protein.

**Procedure**

0.2 ml to 1.0 ml working standard solution was pipetted into test tubes. 0.1 ml of the sample was taken. The volume in all the tubes was made upto 1.0 ml with distilled water. 5.0 ml of alkaline copper reagent to each tube was added and mixed well and allowed to stand for 10 min. Then added 0.5 ml of Folin-Ciocalteau reagent,
mixed well and incubated at room temperature for 30 min. A reagent blank was also prepared. After 30 minutes, the blue colour developed was read at 660 nm. The result was expressed as g/dl in serum.

Serum albumin is determined by quantitative colorimetric method by using bromocresol green reagent. The test was performed by adding 10 µl of serum to 1 ml of albumin reagent. This test did not require any pretreatment. This reagent forms a coloured complex specifically with albumin. The intensity of the colour measured at 620 nm is directly proportional to the albumin concentration in the serum. The total protein minus the albumin gives the globulin (James et al., 2007).

Estimation of Serum Glutamate Pyruvate Transaminase (SGPT) (Reitman and Frankel, 1957)

Principle

The enzyme catalyses the following reaction:

L-Alanine + α-oxoglutarate $\rightarrow$ Pyruvate + L-glutamate

The pyruvate is measured by the reaction with 2,4-dinitrophenylhydrazine giving a brown coloured hydrazone after the addition of sodium hydroxide. The colour developed was read at 520 nm.

Reagents

- Phosphate buffer: 0.1 M, pH 7.5
- Substrate: 146 mg of α- ketoglutarate and 17.8 g of L-alanine were dissolved in 1 N NaOH with constant stirring and the pH was adjusted to 7.4 and made up to 1000 ml with phosphate buffer.
Standard pyruvate, 2 mM: 22 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer (0.2 ml of standard contained 0.4 μM of sodium pyruvate).

Dinitrophenyl hydrazine (DNPH) reagent, 1 mM/L : 200 mg/L in 1 mol/L HCl.

0.4 N NaOH: 16 g of NaOH dissolved in 1000 ml water.

Procedure

0.2 ml of sample and 1.0 ml of the buffer substrate were incubated for 30 min. at 37°C. To the control tubes, enzyme was added after arresting the reaction with 1.0 ml of DNPH and the tubes were kept at room temperature for 20 min. Then 10 ml of 0.4N NaOH was added. A set of standard pyruvate was also treated in a similar manner. The colour developed was read at 520 nm. The enzyme activities were expressed as units/L in serum and units/protein in tissues.

Estimation of Serum Glutamate Oxalo Transaminase (SGOT or AST)

(Reitman and Frankel, 1957)

Principle

The enzyme catalyses the following reaction:

\[
\text{L-Aspartate} + \alpha\text{-oxoglutarate} \rightarrow \text{oxaloacetate} + \text{L-glutamate}
\]

The oxaloacetate is measured by the reaction with 2, 4-dinitrophenylhydrazine giving a brown coloured hydrazone after the addition of sodium hydroxide. The colour developed is read at 520 nm.

Reagent

- Phosphate buffer, 0.1 M, pH 7.5
Solution A: 0.1 M solution of monobasic sodium phosphate (13.9 g/l).

Solution B: 0.1 M solution of dibasic sodium phosphate (6.8 of Na$_2$PO$_4$.7H$_2$O g/l)

16 ml of A and 84 ml of B, diluted to a total of 200 ml.

- Substrate: 146 mg of α-ketoglutarate and 13.3 g of aspartic acid were dissolved in 1N NaOH with constant stirring and the pH was adjusted to 7.4 and made upto 1000 ml with phosphate buffer.

- Standard pyruvate, 2 mmol/L: 22 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer (0.2 ml of standard contained 0.4 µM of sodium pyruvate).

- Dinitrophenylhydrazine (DNPH) reagent, 1 mM/L: 200 mg in 1 mol/L HCl.

- 0.4N NaOH: 16g of NaOH dissolved in 1000 ml water.

**Procedure**

0.2 ml of sample and 1.0 ml of the buffer substrate was incubated for 60 min. at 37°C. To the control tubes, enzyme was added after arresting the reaction with 1.0 ml of DNPH and the tubes were kept at room temperature for 20 min. Then 10 ml of 0.4N NaOH was added. A set of standard pyruvate was also treated in a similar manner. The colour developed was read at 520 nm. The enzyme activity was expressed as units/L in serum and units/protein in tissue.

**Estimation of Alkaline Phosphatase (ALP)** (King and Armstrong, 1934)

**Principle**

The method used was that of King and Armstrong (1934) in which disodium phenyl phosphate is hydrolysed with the liberation of phenol and inorganic phosphate. The liberated phenol is measured at 700 nm with Folin-Ciocalteau reagent.
Reagent

- Sodium carbonate - sodium bicarbonate buffer, 100 mM/L: Dissolved 6.36 g anhydrous sodium carbonate and 3.36 g sodium bicarbonate in water and made to a litre.
- Disodium phenyl phosphate solution, 100 mM/L: Dissolved 2.18 g Disodium phenyl phosphate in water, heated to boil, cooled and made to a litre. Added 1.0 ml of chloroform and stored in the refrigerator.
- Buffer substrate: Prepared by mixing equal volume of the above two solutions. This has a pH of 10.
- Folin-Ciocalteau reagent: Mixed 1.0 ml of reagent with 2.0 ml of water.
- 15% Sodium carbonate solution: Dissolved 15 g of anhydrous sodium carbonate in 100 ml of water.
- Standard phenol solution: Dissolved 1 g pure crystalline phenol in 100 mM/L HCl and made to a litre with the acid.
- Working standard solution: Added 100 ml dilute phenol reagent to 5.0 ml of stock standard and diluted to 500 ml with water. This contained 10 µg phenol/ml.

Procedure

4.0 ml of the buffer substrate was pipetted into a test tube and incubated at 37°C for 5 min. 0.2 ml of serum or tissue homogenate was added and incubated further for 15 min. Immediately added 1.8 ml of diluted phenol reagent. At the same time, a control was set up containing 4.0 ml buffer substrate and 0.2 ml sample to which 1.8 ml phenol reagent was added immediately, mixed well and centrifuged. To 4.0 ml of supernatant, added 2.0 ml of sodium carbonate. 4.0 ml of working standard solution
was taken and 3.2 ml of water for blank and added 0.8 ml of phenol reagent. Then added 2.0 ml of sodium carbonate. All the tubes were incubated at 37°C for 15 min. and the absorbance was read at 700 nm. The activity was expressed as units/L in serum and units/protein in tissue.

**Estimation of Total Cholesterol (TC) (Parekh and Jung, 1970)**

**Principle**

Cholesterol reacts with ferric chloride in the presence of concentrated sulphuric acid to give a pink colour. The intensity of colour developed is directly proportional to the amount of cholesterol present and is read at 540 nm in a colorimeter.

**Reagents**

- **Stock ferric chloride:** 840 mg of pure dry ferric chloride was weighed and dissolved in 100 ml of glacial acetic acid.
- **Ferric chloride precipitation reagent:** 10 ml of stock ferric chloride reagent was taken in 10 ml of standard flask and made upto the mark with pure glacial acetic acid.
- **Ferric chloride diluting reagent:** 8.5 ml of stock ferric chloride is diluted to 100 ml with pure glacial acetic acid.
- **Standard cholesterol solution:** 100 mg of cholesterol was dissolved in 100 ml of glacial acetic acid.
- **Working standard:** 10 ml of stock was dissolved in 0.85 ml of ferric chloride precipitation reagent and made upto 100 ml with glacial acetic acid. The concentration of working standard is microgram/ml.
**Procedure**

To 0.1 ml of the serum, added 4.9 ml of ferric chloride precipitating reagent. The mixture was centrifuged and to 2.5 ml of supernatant and added 2.5 ml of ferric chloride diluting reagent and added 4.0 ml of concentrated sulphuric acid. A blank was prepared simultaneously by taking 5.0 ml of diluting reagent and 4.0 ml of concentrated sulphuric acid. A set of standards (0.5-2.5 ml) were taken and made upto 5.0 ml with ferric chloride diluting reagent. Then added 4.0 ml of concentrated sulphuric acid. After 30 min., the intensity of colour developed was read at 540 nm against a reagent blank. The amount of cholesterol in the sample was expressed as mg/dl.

**Estimation of Tri Glycerides (TG) (Rice, 1970)**

**Principle**

The glycerol moiety is oxidized to formaldehyde and the later condensed with ammonia and 2,4-pentanedione (acetyl acetone) to produce 3,5-diacetyl 1,4-dihydrrotoludine, which is yellow in colour and has absorption at 450 nm.

**Reagents**

- Chloroform - methanol mixture (2:1).
- Activated silicic acid: It was activated by washing silicic acid with 4N or 2N HCl and then with water until the washings become natural. After drying, ether was added. Silicic acid was then dried at 60°C and activated at 100°C over night prior to use.
- 0.2 N H₂SO₄.
Saponification reagent: Dissolved 5 g of KOH in 60 ml water and added 40 ml of isopropanol.

Sodium metaperiodate reagent: To 77 g of anhydrous ammonium acetate in 700 ml water, added 60 ml acetic acid and 650 mg of sodium metaperiodate. Dissolved and diluted into 1 litre with distilled water.

Acetyl acetone reagent: Added 0.75 ml of acetyl acetone to 20 ml of isopropanol and mixed well followed by 80 ml of distilled water and mixed.

Tripalmitin standard containing 100 µg/ml in chloroform.

**Procedure**

0.1 ml of the serum or dried lipid extract was taken. The volume was made up to 4.0 ml with isopropanol, mixed well and added 400 mg of silicic acid. The mixture was placed in a mechanical shaker and centrifuged.

To 2.0 ml of the supernatant, added 0.6 ml of saponification reagent and incubated at 60-70°C for 15 min. After cooling, added 1.0 ml of sodium metaperiodate and mixed well. Then added 0.5 ml of acetyl acetone reagent and mixed again. The tubes were incubated at 50°C for 30 min. After cooling, read the colour at 405 nm. Standard tripalmitin (20-100 µg) were taken in tubes and treated similarly. Triglycerides were expressed as mg/100 ml in serum.

**Estimation of High Density Lipoprotein - Cholesterol (HDL-C) (Warnick et al., 1985)**

**Principle**

Cholesterol reacts with hot solution of ferric perchlorate, ethyl acetate and sulphuric acid (cholesterol reagent) and gives a lavender coloured complex which is measured at 560 nm.
High density lipoproteins (HDL) are obtained in the supernatant after centrifugation. The cholesterol in the HDL fraction is also estimated by this method.

**Determination of Low Density Lipoprotein - Cholesterol (LDL-C) and Very Low Density Lipoprotein - Cholesterol (VLDL-C) (Friedwald et al., 1972)**

LDL cholesterol and VLDL-cholesterol levels in serum were calculated by Friedwald *et al.* (1972) formula.

**Determination of Phospholipids (Takagama et al., 1977)**

Lipids were extracted from 2 ml of the serum by following a modification in the procedure of Folch *et al.* (1957) using a chloroform-methanol mixture in the proportion of 2:1 (v/v) containing 15 mg of butylated hydroxyl toluene (BHT). This lipid extract was used for the estimation of the phospholipids by using the method of Takagama *et al.* (1977).

**Estimation of Lipid Peroxidation (LPO) (Uchiyama and Mihara, 1978)**

**Principle**

Malondialdehyde has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid to give a red colour absorbing at 535 nm.

**Reagents**

- 15% KCl
- 1% phosphoric acid
- n-butanol
- 0.6% thiobarbituric acid
- 10 mM ferrous sulphate
0.2 mM ascorbate

Procedure

0.5 ml of aliquot of the serum was mixed with 3.0 ml of 1% phosphoric acid and 1.0 ml of 0.6% thiobarbituric acid. The mixture was heated for 45 min. in a boiling water bath and after addition of 4.0 ml of n-butanol vigorously vortexed and centrifuged at 2000 rpm for 20 min. The absorbance of the upper organic layer at 535 nm was measured in a spectrophotometer and compared with a standard of freshly prepared 1,1,3,3-tetraethoxy propane at concentration of 5.125, 10.25 and 20.5 nmol ml\(^{-1}\) or using an extinction coefficient of the chromophore 1.56 x 10\(^{-5}\) M\(^{-1}\) cm\(^{-1}\) and the results were expressed as n moles of MDA formed/mg protein.

Estimation of Glutathione Peroxidase (GPx) and Reduced Glutathione (GSH)
(Rotruck et al., 1984)

GPx activity was measured by the method described by Rotruck et al. (1984). Briefly, reaction mixture contained 0.2 ml of 0.4 mM phosphate buffer (pH 7.0), 0.1 ml of 10 mM sodium azide, 0.2 ml of serum, 0.2 ml of GSH and 0.1 ml of 0.2 mM H\(_2\)O\(_2\). The contents were incubated at 37°C for 10 min. The reaction was arrested by 0.4 ml of 10% TCA and centrifuged. Supernatant was assayed for GSH content by using Ellman’s reagent (19.8 mg of 5,5’-dithiobisnitro benzoic acid in 100 ml of 0.1% sodium nitrate). GSH was determined by the method of Ellman (1959). 1.0 ml of supernatant was treated with 0.5 ml of Ellman’s reagent and 3.0 ml of phosphate buffer (0.2 mM, pH 8.0). The absorbance was read at 412 nm. GPx activity was expressed as μg of GSH consumed/min/mg protein and reduced GSH as mg/dl.
Estimation of Superoxide Dismutase (SOD) (Das et al., 2000)

Principle

The method involves generation of superoxide radical of riboflavin and its detection by nitrite formation from hydroxylamine hydrochloride. The nitrite reacts with sulphanilic acid to produce a diazonium compound which subsequently reacts with naphthylamine to produce a red azo compound whose absorbance is measured at 543 nm.

Reagents

- 50 mM phosphate buffer, pH 7.4
- 20 mM L-Methionine
- 1% (v/v) Triton X-100
- 10 mM hydroxylamine hydrochloride
- 50 µM EDTA
- 50 µM Riboflavin
- Griess reagent: 1% sulphanilamide, 2% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride.

Procedure

1.4 ml aliquot of the reaction mixture was taken in a test tube. 100 µl of the sample was added followed by pre-incubation at 37°C for 5 min. 80 µl of riboflavin was added and the tubes were exposed for 10 min. to 200 W Philips fluorescent lamps. The control tube contained equal amount of buffer instead of sample. The sample and its respective control were run together. At the end of the exposure time, 0.1 ml of Greiss reagent was added to each tube and the absorbance of the colour
formed was measured at 543 nm. One unit of enzyme activity was defined as the amount of SOD capable of inhibiting 50% of nitrite formation under assay condition.

**Estimation of Catalase (CAT) (Sinha, 1972)**

**Principle**

Catalase causes rapid decomposition of hydrogen peroxide to water.

\[
2\text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \text{O}_2
\]

The method is based on the fact that, dichromate in acetic acid reduces to chromic acetate when heated in the presence of H\textsubscript{2}O\textsubscript{2} with the formation of perchloric acid as an unstable intermediate. The chromic acetate thus produced was measured colorimetrically at 610 nm. Since dichromate has no absorbancy in this region, the presence of the compound in the assay mixture does not interfere with the colorimetric determination of chromic acetate. The catalase preparation was allowed to split \text{H}_2\text{O}_2 for different periods of time. The reaction was stopped at specific time intervals by the addition of dichromate/acetic acid mixture and the remaining \text{H}_2\text{O}_2 was determined by measuring chromic acetate colorimetrically after heating the reagents.

**Reagents**

- 0.01 M phosphate buffer, pH 7.0
- 0.2 M hydrogen peroxide
- Stock dichromate/acetic acid solution: Mixed 5% potassium dichromate with glacial acetic acid (1:3 by volume).
- Working dichromate/acetic acid solution: The stock was diluted to 1:5 with water to make the working dichromate/acetic acid solution.
Procedure

The assay mixture contained 0.5 ml of H₂O₂, 10 ml of buffer and 0.4 ml water. 0.2 ml of the enzyme was added to initiate the reaction. 2.0 ml of the dichromate/acetic acid reagent was added after 0, 30, 60, 90 seconds of incubation. To the control tube, the enzyme was added and read at 610 nm. The activity of catalase was expressed as μ mole of H₂O₂ decomposed/min/mg protein.

Methodology for Tissue antioxidant

Preparation of tissue homogenate

After the experimental regimen, the animals were sacrificed under mild chloroform anaesthesia. Liver and kidney were excised, immediately washed with cold saline. The tissue was weighed and 10% tissue homogenate was prepared with 0.025 m Tris-HCl buffer, pH 7.5. After centrifugation at 10,000 × g for 10 min., the resulting supernatant was used for enzyme assays for the estimation of non-enzymatic and enzymatic antioxidants.

Estimation of lipid peroxidation (LPO) of rat liver and kidneys (Fraga et al., 1984)

Lipid peroxidation in liver and kidney was estimated colorimetrically by measuring thiobarbituric acid reactive substances (TBARS) using the method of Fraga et al. (1984). 0.1 ml of tissue homogenate was treated with 2 ml of TBA-trichloroacetic acid - HCl reagent (0.37% TBA, 0.25 M HCl and 15% TCA, 1:1:1 ratio), placed for 15 min. in a water bath and then cooled and centrifuged at 3500 × g for 10 min. at room temperature, the absorbance of clear supernatant was measured at 535 nm against a reference blank. Values were expressed as nmol mg⁻¹ tissue protein.
Estimation of Superoxide Dismutase (SOD) (Kakkar et al., 1984)

The activity of superoxide dismutase (SOD) was assayed by the method of Kakkar et al. (1984) based on the oxidation of epinephrine adrenochrome transition by enzyme. 0.5 ml of tissue homogenate was diluted with distilled water 0.5 ml. To this, chilled ethanol 0.25 ml and chloroform 0.15 ml were added. The mixture was shaken for 1 min. and centrifuged at 2000 × g for 10 min. From this, 0.5 ml was added with 1.5 ml PBS buffer (pH 7.2). The reaction initiated by the addition of 0.4 ml epinephrine and change in optical density OD (min\(^{-1}\)) was measured at 470 nm. SOD activity was expressed as μ/mg of protein. Change in OD (min\(^{-1}\)) at 50% inhibition to adrenochrome transition by the enzyme was taken as one enzyme unit.

Estimation of Catalase (CAT) (Sinha, 1972)

Catalase (CAT) was estimated by the method of Sinha (1972). The reaction mixture 1.5 ml volume contained 1.0 ml of 0.01M phosphate buffer (pH 7.0), 0.1 ml of tissue homogenate and 0.4 ml of 2 M H\(_2\)O\(_2\). The reaction was stopped by the addition of 2.0 ml dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio). Then the absorbance was measured at 530 nm; CAT activity was expressed as k/sec/mg protein.

Estimation of Glutathione peroxidase (GPx) (Rotruck et al., 1984)

Glutathione peroxidase (GPx) was measured by the method described by Rotruck et al. (1984). Briefly, the reaction mixture contained 0.2 ml of 0.4 M phosphate buffer (pH 7.0), 0.1 ml 10 mM sodium azide, 0.2 ml tissue extract, 0.2 ml reduced glutathione and 0.1 ml 0.2 mM hydrogen peroxide. The contents were incubated for 10 min at 37\(^{0}\)C, 0.4 ml 10% TCA was added to stop the reaction and
centrifuged at 3200 × g for 20 min. The supernatant was assayed for glutathione content using Ellman’s reagent (19.8 mg 5,5’-dithiobisnitrobenzoic acid [DTNB] in 100 ml 0.1% sodium nitrate). The activities were expressed as μg consumed/min/mg protein.

**Estimation of reduced glutathione (GSH) (Ellman, 1959)**

Reduced glutathione (GSH) was measured by the method of Ellman (1959). Briefly, an aliquot of tissue extract (720 μl) and 5% TCA were mixed well to precipitate the protein content of the supernatant. After centrifugation at 10,000 × g for 5 min., the supernatant was taken. DTNB (5,5’-dithio-bis (2-nitrobenzoic acid) and Ellman’s reagent were added to it and the absorbance was measured at 412 nm. A standard graph was drawn using different concentration of standard GSH solution. GSH contents were calculated in the rat liver and kidney and expressed as n mol/mg protein.

**Hepatoprotective activity**

**Experimental animal**

Mature adult male Wistar strain albino rats weighing about 180-200 gm body weight were selected for this work. They were maintained in a well-ventilated animal house with constant 12 hours of darkness and 12 hours of light schedule. Clean water and standard pellet diet (Hindustan Lever Ltd., India) were given to them ‘ad libitum’.

**Experimental protocol**

**Induction of hepatotoxicity**

Carbon tetrachloride (CCl₄) 2.5 ml/kg body weight was dissolved in 7.5 ml of paraffin and administered intraperitoneally.
**Grouping of animals**

The animals were randomly divided into seven groups, each group consists of six individuals.

**Group I** : Rats received normal saline were served as a normal control by using an intragastric catheter tube (IGC).

**Group II** : Liver injured rats received 2.5 ml/kg body weight of normal saline 14 days by IGC and served as a CCl₄ hepatic toxicity induced control.

**Group III** : Liver injured rats received *R. densiflora* whole plant extract at the dose of 100 mg/kg body weight for 14 days by using an IGC.

**Group IV** : Liver injured rats received *R. densiflora* whole plant extract at the dose of 200 mg/kg body weight for 14 days by using an IGC.

**Group V** : Liver injured rats received *S. wightii* tuber extract at the dose of 100 mg/kg body weight for 14 days by using an IGC.

**Group VI** : Liver injured rats received *S. wightii* tuber extract at the dose of 200 mg/kg body weight for 14 days by using an IGC.

**Group VII** : Liver injured rats received silymarin (100 mg/kg body weight/day) orally at the dose of 100 mg/kg body weight for 14 days by using an IGC.

All the treatments were given between 9.30 and 10.00 hour in the morning. After 24 hours of last treatment, the final body weight was recorded and the animals were sacrificed by decapitation. Blood was collected from each group of rats. Serum
from the blood was separated by centrifugation at 3000 x g for 10 minutes and stored at -20°C until used for various biochemical assays.

Estimation of alkaline phosphate (ALP), serum glutamate oxalo transaminase and serum glutamate pyruvate transaminase (AST and ALT), blood glucose and serum protein were already given in the diabetic methodology.

**Estimation of Total, Conjugated and Unconjugated Bilirubin**

Total bilirubin and conjugated bilirubin were determined as described by Balistrei and Shaw (1987). The unconjugated bilirubin concentration was calculated as the difference between total and conjugated bilirubin concentrations.

**Estimation of Liver MDA, SOD, CAT, GPx, GRD and GGTP**

Qualitative estimation of MDA formation was done by determining the concentration of thiobarbituric acid reactive substances (TBARS) in 10% liver homogenates by the method of Okhawa *et al.* (1979). Enzymatic antioxidants, superoxide dismutase (SOD) (Mishra and Fridowich, 1972), Catalase (Aebi, 1974), non enzymatic antioxidant glutathione peroxidase (GPx) (Pagila and Valentine, 1967), glutathione reductase (GRD) (Goldberg and Spooner, 1983) and gama glutamyl transpeptidase (GGTP) (Szasz, 1969) were also assayed in liver homogenates.

**Fertility activity**

**Experimental animal**

Mature adult male Wistar strain albino rats weighing about 180-200 gm body weight were selected for this work. They were maintained in a well-ventilated animal house with constant 12 hours of darkness and 12 hours of light schedule. Clean water...
and standard pellet diet (Hindustan Lever Ltd., India) were available to them ‘ad libitum’.

**Experimental design**

The animals were divided into five groups, each consisting of 5 animals.

**Group I** : Rats given normal saline daily for 14 days consequently orally by using an intragastric catheter tube (IGC).

**Group II** : Rats given *R. densiflora* whole plant extract at the dose of 100 mg/kg body weight, daily, orally for 14 days consequently by using an IGC.

**Group III** : Rats given *R. densiflora* whole plant extract at the dose of 200 mg/kg body weight, daily, orally for 14 days consequently by using an IGC.

**Group IV** : Rats given *S. wightii* tuber extract at the dose of 100 mg/kg body weight, daily, orally for 14 days consequently by using an IGC.

**Group V** : Rats given *S. wightii* tuber extract at the dose of 200 mg/kg body weight, daily, orally for 14 days consequently by using an IGC.

Suitable controls were maintained for each duration of treatment. However, as there was no obvious difference on any parameter among control groups, a common control was employed in the present study.

All the treatments were given between 8.00 and 11.00 hour in the morning. After 24 hours of last treatment, the final body weight was recorded and the animals were sacrificed by decapitation. Blood was collected, sera separated by centrifugation at 3000 x g for 10 minutes and stored at -20°C until used for various biochemical assays. Then testes, epididymis, vas deferens, seminal vesicle and ventral prostate
were dissected out, trimmed off extraneous tissues and weighed accurately on a torsion balance. The organs weights were expressed in terms of mg/100 gm body weight.

**Assessment of Sperm Motility**

Immediately after animals sacrifice, cauda epididymis was punctured using hypodermic needle, sperm suspension was collected and transferred into a droplet of physiological saline. The motility of the spermatozoa was observed under microscope at 100 x magnification. The distance traversed by the sperm was determined using an oculometer and expressed as micrometre (µm) traversed per minute.

**Sperm count determination (Zaneveld and Polakoski, 1977)**

**Collection of epididymal fluid**

Epididymal fluid (for sperm count) was collected from caput and cauda segments, separately, minced in 2 ml Sorenson’s buffer (pH 7.2) and passed through nylon mesh of 75 µ size. The separated fluid was taken for sperm count.

Sperm count was carried out by using Neubauer haemocytometer as described by Zaneveld and Polakoski (1977).

For the standard sperm analysis, a 20 fold dilution was made by mixing the epididymal fluid with Sorenson’s buffer. 0.1 ml epididymal fluid was added to 1.9 ml of Sorenson’s buffer. The preparation was thoroughly mixed and one drop was added to both side of a standard blood cell haemocytometer. The number of spermatozoa in the appropriate square of the haemocytometer was counted under the microscope at x 100. Both sides of the haemocytometer were counted and an average was taken.
Calculation

The sperm concentration refers to the number of spermatozoa per ml epididymal fluid. The haemocytometer is a grid containing 5 major squares called E1, E2, E3, E4 and the central small square is E5. While counting, all the spermatozoa within the designated squares and those that cross the lines at the top and right hand sides were included.

Major square E is 1 mm long, 1 mm wide and the thickness of the fluid between the cover slip and the haemocytometer is 0.1 mm. The total volume represented by major square is thus 0.1 mm$^3$ or $10^{-4}$ ml. The multiplication factor of corner square E is therefore $10^{-4}$ or 10,000. When all the spermatozoa in the major square E were counted, the number was multiplied with the multiplication factor 10,000 to get the number of spermatozoa per ml of the solution applied to the haemocytometer. When this was multiplied with a dilution factor (the amount of sperm dilution normally was 20 times), the concentration of spermatozoa in the original sample was obtained.

When the spermatozoa in small square E1, E2, E3, E4 and E5 were counted, the multiplication factor was 5 times greater than when the entire E square was counted and was thus 50,000. When all the major squares were counted as in the case of very low sperm concentration, the multiplication factor was 5 times smaller i.e., 2000.

The sperm concentration is expressed in counts x $10^6$/ml.

The basic formula that is applied to obtain the sperm concentrations is:

\[ \text{Sperm concentration} = \frac{\text{Number of spermatozoa}}{\text{multiplication factor} \times \text{dilution factor}} \]
Sperm motility, viability and counts

The rats were anaesthetized with 25% urethane at a dose of 0.6 ml/100g intraperitoneally. The caudal epididymis was then dissected. An incision (about 1 mm) was made in the caudal epididymis and drops of sperm fluid were squeezed onto the microscope slide and 2 drops of normal saline were added to mobilize the sperm cells. Epididymal sperm motility was then assessed by calculating motile spermatozoa per unit area and was expressed in percentage.

Epididymal sperm counts were also done by homogenizing the epididymis in 5 ml of normal saline. Counting was then done using the counting chamber in the haemocytometer. The sperm viability was also determined using Eosin/Nigrosin stain.

Fertility test

Fertility was estimated in adult male rats treated with ethanol extracts of whole plant of R. densiflora and tubers of S. wightii and in the control male counterparts. Each male was placed in an individual cage with two virgin untreated females of the same strain. They were left together for 10 days during which two estron cycles had elapsed (Rugh, 1968). One week after the removal of the exposed males, pregnant females were killed by cervical dislocation under light ether anesthesia and the number of implantation sites, the number of fetuses and the number of resorption sites were recorded.

Hormonal analysis

Blood removed from the animals by intracardiac method. Blood was centrifuged at 2000 rpm (Revolution per minute) to separate the serum for the measurement of FSH, LH, Testosterone and Estrogen. The quantitative determination
of hormones was done by using Enzyme Immuno Assay method (EIA). The EIA kit was obtained from immunometrics (London, UK) and contained a testosterone EIA enzyme label, testosterone EIA substrate reagent and EIA quality controlled sample. An enzyme-based immunoassay (EIA) system was used to measure all the hormones in the serum samples collected. A quality control was carried out at the beginning and the end of the assay to ascertain the acceptability with respect to bias and within batch variation. The EIA kit used had a sensitivity level of approximately 0.3-0.1 respective of all reproductive hormones. The intra- and inter-assay variations were 10.02%, 10.12%, 9.7% and 10.4% for Testosterone, FSH, LH and Estrogen respectively.

Antiinflammatory activity (Winter et al., 1962)

Acute toxicity study

For toxicity studies, two different groups of six albino rats of both sexes were administrated orally with the test substance in the range of doses 100 - 2000 mg/kg and the mortality rates were observed after 72 hours. The ethanol extracts of R. densiflora and S. wightii have shown no mortality at 2000 mg/kg. Therefore 2000 mg/kg dose was considered as LD$_{50}$ cutoff dose (safe dose). So, 1/10 and 1/5 of LD$_{50}$ dose were the selected (200 and 400 mg/kg) doses.

Animals

Albino rats of Wistar strain of 150 g average body weight were used. The animals were kept individually in individual cages at room temperature of 25 ± 2$^{\circ}$C and a relative humidity of about 55%.
Drugs (Synthetic antiinflammatory agents)

The reference antiinflammatory drug used in our study is Indomethacin (commercial name). Its chemical name is 1-(4-Chlorobenzoyl)-(methoxy-2-methylindole-3-yl) acetic acid obtained from Pharmco Pharmaceuticals Company.

Chemicals used for induction of inflammation

Carrageenan, type IV (Sigma, USA). Carrageenan is a polysaccharide isolated from two species, Gigartina aciculaire and G. pistillata which grow together in the sea.

Rats were maintained on laboratory stock diet and fasted for 16 hours before starting the experiment and divided into six groups each comprised of five rats.

**Group I** : Control animals received normal saline 0.5 ml/kg.

**Group II** : Animals received ethanol extract of *R. densiflora* whole plant at the dose of 200 mg/kg body weight.

**Group III** : Animals received ethanol extract of *R. densiflora* whole plant at the dose of 400 mg/kg body weight.

**Group IV** : Animals received ethanol extract of *S. wightii* tuber at the dose of 200 mg/kg body weight.

**Group V** : Animals received ethanol extract of *S. wightii* tuber at the dose of 400 mg/kg body weight.

**Group VI** : Standard Indomethacin 10 mg/kg body weight was used as reference drug.
Paw oedema was induced by injecting 0.1 ml of 1% W/V carrageenan in physiological saline into the subplantar tissues of the left hind paw of each rat (Winter et al., 1962). The ethanol extracts of whole plant of *R. densiflora* and tubers of *S. wightii* were administered orally 30 min. prior to carrageenan administration. The paw volume was measured at intervals of 60, 120, 180 and 240 min. by the mercury displacement method using a plethysmograph. The percentage inhibition of paw volume in drug treated group was compared with the carrageenan control group.

\[
\text{Percentage inhibition} = \frac{\text{Control (% increase in paw volume in 3rd hour)} - \text{Test (% increase in paw volume in 3rd hour)}}{\text{Control (% increase in paw volume in 3rd hour)}} \times 100
\]

**CNS activity**

**Experimental animals**

Studies were carried out using Swiss albino mice (20 - 25g) and Wistar albino rats (150 - 180g) of either sex. They were obtained from the animal house, PSG Institute of Medical Science, Coimbatore, India. The animals were grouped and housed in polyacrylic cages (38 \times 23 \times 10 \text{ cm}^3) with not more than eight animals per cage, and maintained under standard laboratory conditions (temperature 25\pm2^\circ\text{C}) with dark and light cycle (14/10 hour). They were allowed free access to standard dry pellet diet (Saidurga Animal Feeds, Bangalore, India) and water *ad libitum*. The mice were acclimatized to laboratory condition for 10 days before commencement of experiment.
Drugs

The following drugs were used: Diazepam (Lupin Laboratories Ltd., India), Phenobarbitone sodium (Rhone-Poulenc India Ltd., India), Cocaine (M.M. Pharma, New Delhi, India) and Carboxy methyl cellulose (SRL Laboratories Ltd., India).

General behavioral profiles (Dixit and Varma, 1979)

Evaluation of general behavioral profiles was performed by the method of Dixit and Varma (1979). Forty adult albino mice were divided into five groups (n = 5). Ethanol extract of R. densiflora and S. wightii and 5% CMC was administered for the first three groups of animals at the doses of 50, 100 and 150 mg/kg p.o. 1 ml of 5% CMC as a vehicle control for 15 days respectively. While the last group were administered diazepam (5 mg/kg, i.p.) on the test day as a drug control. The animals were under observation for their behavioral changes, if any, at 30 min. intervals in the first one hour and at the hourly intervals for the next 4 hours for the following parameters.

Awareness, alertness and spontaneous activity

The awareness and alertness were recorded by visual measure of the animal’s response when placed in a different position and its ability to orient itself without bumps or falls. The normal behaviour at resting position was scored as (-), little activity (+), moderate flexibility (+ +), strong response (+ + +) and abnormal restlessness as (+ + + +). The spontaneous activity of the mice was recorded by placing the animal in a bell jar. It usually shows a moderate degree of inquisitive behaviour. Moderate activity was scored as (+ +) and strong activity as (+ + +). If there is little motion, the score was (+), while if the animal sleeps, the score was (−).
Excessive or very strong inquisitive activity like constant walking or running was scored as (+ + + +). A similar test was performed with the same scoring, when the animals are removed from the jar and placed on a table.

**Righting reflex**

Groups of mice were treated with the test compounds on the test day. After 15, 30 and 60 min., each mouse was placed gently on its back on an undulated surface made of white iron and kept at 30°C. If the animal remained on its back for 30 sec., it was considered as a loss of righting reflex.

**Pinna reflex (Turner, 1965)**

The reflex is examined by touching the center of pinna with a hair or other fine instrument. The unaffected mouse withdraws from the irritating hair.

**Grip strength**

The grip strength test is used to assess muscular strength or neuromuscular function in rodents. It was measured by allowing the animal to grasp a pencil in the horizontal position and noting the time taken by the animal to drop the pencil on the table.

**Touch response**

The touch response was recorded by touching the mice with a pencil or forceps at various parts of the body (i.e., on the side of the neck, abdomen and groin).

**Pain response**

The pain response was graded when a small artery clamp was attached to the base of the tail and response was noted.
Sound response

Swiss albino mice normally utter no sound so that vocalization may indicate a noxious stimulus.

The righting reflex, pinna reflex, grip strength, touch, pain and sound responses were carried out to study the depressant action of *R. densiflora* and *S. wightii* extracts at various doses. The depressant action was scored as no effect (-), slight depression (+), moderate depression (+ +), strong depression (+ + +), very strong depression (+ + + +). A trained observer unaware of the experiment assigned the score for the general behavioral studies.

Cocaine-induced hyperactivity experiments in rats (In-Won Chung *et al*., 2002)

Male wistar albino rats were divided into four groups of five in each. The animals were removed from the holding room and randomly assigned to treatment groups. Animals received either the vehicle or ethanol extract of *R. densiflora* and *S. wightii* (50, 100 and 150 mg/kg) for fifteen days and were placed in the activity cages. Following the 30 min. of habituation period on the test day, the animals received cocaine (40 mg/kg, i.p.) and were returned to the activity cages for a further 90 min. Activity was measured as light beam interruptions per 10 min. period.

Effect of phenobarbitone sodium-induced sleeping time (Dandiya and Collumbine, 1956)

Swiss albino mice were divided into four groups of five in each. On the test day, animals received 40 mg/kg (i.p.) phenobarbitone sodium 30 min. after the administration of ethanol extract of *R. densiflora* and *S. wightii* at the doses of 50, 100 and 200 mg/kg and vehicle control 1 ml of 5% CMC. The sleeping time was recorded
and measured as the time interval between the loss and regaining of the righting reflex.

**Exploratory behaviour**

This was performed by Y-maze and head dip tests.

**Y-maze test (Rushton et al., 1961)**

Y-maze test is used to measure the exploratory behaviour in mice. This was performed in the groups of 5 albino mice at 30, 60, 90 and 120 min. after administration of either CMC, ethanol extract of *R. densiflora* and *S. wightii* (50, 100 and 150 mg/kg, p.o.) or diazepam (5 mg/kg, i.p.) respectively on the test day. The mice were placed individually in a symmetrical Y-shaped runway (33cm × 38cm × 13cm) for 3 min. and the number of the mazes with all 4ft. (an ‘entry’) were counted.

**Head dip test (Tomkiewicz et al., 1971)**

The evaluation of certain components of behaviour of mice such as curiosity or exploration has been attempted in Head dip test. Five groups of albino mice (n = 5) were placed on top of a wooden box with 16 evenly spaced holes, 30 min. after administration of the ethanol extract of *R. densiflora* and *S. wightii* (50, 100 and 150 mg/kg), vehicle (1 ml of 5% CMC) and diazepam (5 mg/kg) respectively. The number of times that each animal dipped its head into the holes was counted for the period of 3 min.

**Muscle relaxant activity**

The effect of extracts on muscle relaxant activity was studied by the traction test and Rota rod test.
**Traction test (Rudzik et al., 1973)**

The forepaws of the mice were placed in a small twisted wire rigidly supported above the bench top, and the screening of animal was performed for traction test. Normally, the mice grasp the wire with the forepaws and place at least one hind foot on the wire without 5 sec. when allowed to hang free. The test was conducted on five groups of animals (n = 5) that were previously screened, on the 15th day 30 min. after the administration of ethanol extract of *R. densiflora* and *S. wightii* (50, 00 and 150 mg/kg, p.o.), CMC (5%) and diazepam (5 mg/kg, i.p.), the test was carried out. Inability to put up at least one hind foot is considered as failure in the traction test.

**Rotarod test (Dunham and Miya, 1957)**

The test is used to evaluate the activity of drugs interfering with motor coordination. Fresh mice were placed on a horizontal wooden rod (32mm diameter) rotating at a speed of 16 rpm (Model 7600; UgoBasile). The mice capable of remaining on the top for 3 min. or more, in three successive trials were selected for the study. The selected animals were divided into five groups (n = 5). After the administration of doses on test day, each group of animals was then placed on the rod at an interval of 30, 60, 90, 120 and 150 min. The animals failed more than once to remain on the rotarod for 3 min. were considered as passed the test.

**Statistical analysis**

All values were expressed as mean ± standard error of mean (S.E.M) and comparison between the groups were made by Analysis of Variance (ANOVA). The Data were analysed using the statistical analysis system SPSS (SPSS Software for windows release 10.0; SPSS Inc., Chicago IL, USA).