Materials and Methods

The present study was carried out to evaluate the phytochemical profile and bioactive potential of *Bacolepis nervosa* belonging to the family Periploceae.

**Study area**

The Nilgiri Biosphere Reserve (NBR) is a part of Southern Western Ghats and a place of incredible diversity in landscape and life. It lies between 10°50’N - 12°16’N latitude and 76°00’E - 77°15’E longitude with a total area of 5,520 km² spread across the three states of Karnataka, Kerala and Tamil Nadu. Altitude within the NBR varies from 250 - 2670 m and the reserve encompasses a diversity of vegetation types, ranging from tropical evergreen to thorny scrub (Chandrashekara *et al.*, 2005). NBR is one of the hot spots of the world with many rare, endemic and threatened plants (Fyson, 1932; Nayar, 1996).

**Collection of plant material**

The aerial parts (stem and leaf) of *Bacolepis nervosa* (Wight & Arn.) Decne. ex Moq. were collected from Kothagiri, Nilgiri Biosphere Reserve, Western Ghats, Tamil Nadu, India. The plant sample was identified with the help of local flora and authenticated by Botanical Survey of India, Southern Circle, Coimbatore, Tamil Nadu, India. A voucher specimen of collected plants was deposited in the Ethnopharmacology Unit, PG & Research Department of Botany, V.O. Chidamabaram College, Thoothukudi District, Tamil Nadu, India.
Microscopic studies

Care was taken to select healthy plants. The required samples of different organs were cut and removed from the plants and fixed in FAA solution (Formalin - 10 ml, Acetic acid - 5 ml and 70% Ethyl alcohol - 85 ml). After 24 hrs of fixing, the specimens were dehydrated with graded series of Tertiary Butyl Alcohol (TBA) as per the schedule given by Sass (1940). Infiltration of the specimens was carried by gradual addition of paraffin wax (melting point 58° - 60°C) until TBA solution attained super saturation. The plant materials were cast into paraffin blocks.

Sectioning

The paraffin embedded specimens were sectioned with the help of rotary microtome. The thickness of the section was 10 to 12 µm. Dewaxing of the section was done by customary procedure (Johansen, 1940). The sections were stained with Toluidine blue as per the method published by O’ Brien et al. (1964). Since Toluidine blue is a polychromatic stain, the staining results were remarkably good. The dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc. Wherever necessary, sections were also stained with Safranin and Fast - green.

For studying the stomatal morphology, venation pattern and trichome distribution, paradermal sections (sections taken parallel to the surface of leaf) as well as clearing of leaf with 5% sodium hydroxide or epidermal peeling by partial maceration employing Jeffrey’s maceration fluid (Sass, 1940) were prepared. Glycerin mounted temporary preparations were made for macerated / cleared materials.
Photomicrographs

Microscopic descriptions of tissues were supplemented with micrographs wherever necessary. Photographs of different magnifications were taken with Nikon Labphot 2 microscopic unit. For normal observations bright field was used, for the study of crystals polarized light was employed. Since these structures have birefringent property, under polarized light they appear bright against dark background. Magnifications of the figures are indicated by the scale-bars. Descriptive terms of the anatomical features are as given in the standard books (Esau, 1964).

Preparation of powder

The stem and leaf of B. nervosa (BNS and BNL) were cut into small fragments and shade dried until the fracture was uniform and smooth. The dried plant materials were powdered separately by using a blender and sieved to get uniform particles by using sieve No. 60. The final uniform powder of stem and leaf were used for various experimental studies.

Physicochemical characteristics

The percentage of loss of weight on drying, total ash, acid insoluble ash, water soluble ash, sulphated ash and extractive values in various solvents were obtained by employing standard method of analysis described in Pharmacopoeia of India (Anonymous, 1996).
**Determination of moisture content**

A known quantity of fresh parts of stem and leaf of *B. nervosa* were weighed separately and allowed to dry under shade until a constant weight was obtained. The percentage of loss of weight on drying was calculated.

**Determination of total ash**

Three gram of the powdered drugs (stem and leaf) 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 acid insoluble ash**

The ash obtained as described in the determination of total ash was boiled with 25 ml of 2N Hydrochloric acid for 5 minutes. The insoluble ash was collected on an ashless filter paper and washed with hot water. The insoluble ash was transferred to 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.

**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 allowed to cool in a desiccator. One gram of sample was weighed, transferred to the crucible and reweighed the crucible and the contents accurately. It was ignited gently at first, until the substance was thoroughly charred. It was cooled, moistened with 1 ml of sulphuric acid, heated gently until the white fumes were no longer evolved and ignited at 800°C ± 25°C until all black particles disappeared. The ignition was done in a place protected from air currents. The crucible was cooled, a few drops of sulphuric acid was added and heated. Again it was ignited as before and allowed to cool and weighed. This was repeated until two successive weighing did not differ by more than 0.5 mg.

**Extractive values**

The extractive values of the stem and leaf of *B. nervosa* in various solvents (petroleum ether, benzene, chloroform, ethyl acetate, methanol, ethanol and water) were determined by employing the methods of analysis described in Pharmacopoeia of India (Anonymous, 1996).

About 5 g of air dried sample was taken in a stoppered flask. 100 ml of the respective solvent were added, shaken well and allowed to stand for 24 hrs with occasional shaking. Then the content was filtered. 50 ml of the filtrate were pipette
out into a clean, previously weighed china dish and evaporated on a water bath. Finally it was dried at 105°C, cooled and weighed. The percentage of solvent soluble extractive with reference to the air-dried sample was calculated.

**Fluorescence analysis**

The drug powders were treated with acids like 1N HCl, conc. HCl, 50% H$_2$SO$_4$, conc. H$_2$SO$_4$, 50% HNO$_3$, conc. HNO$_3$, acetic acid and conc. HNO$_3$+NH$_3$; alkaline solutions like aqueous sodium hydroxide, 40% NaOH + 10% lead acetate and alcoholic sodium hydroxide; solvents like acetone, benzene, chloroform, petroleum ether, methanol and ethanol; other chemical reagents like ferric chloride and ammonia. They were subjected to fluorescence analysis in daylight and in short UV light (254 nm) and long UV light (365 nm). The fluorescence analysis was carried out as per the standard procedures (Chase and Pratt, 1949).

**Phytochemical analysis**

**Preparation of extract**

100 g of the coarse powder of stem and leaf of *B. nervosa* was extracted successively with 250 ml of petroleum ether, benzene, ethyl acetate, methanol and ethanol in a Soxhlet apparatus for 24 hrs. All the extracts were filtered through Whatman No. 41 filter paper separately and all the extracts were concentrated in a rotary evaporator. All the concentrated extracts were subjected to qualitative tests for the identification of various phytochemical constituents and used for *in vitro* antioxidant activity. The methanol and ethanol extracts were subjected to quantitative test for the phenols, flavonoids and tannins.
Qualitative phytochemical analysis

The qualitative phytochemical test for steroids, triterpenoids, reducing sugars, alkaloids, phenolic compounds, flavonoids, saponins, tannins, anthraquinones, xanthoproteins, glycosides, quinones, catechins and coumarin were carried out on the concentrated extracts using the standard procedures to identify the constituents as described by Harborne (1973), Brinda et al. (1981) and Trease and Evans (1986). Table 1 explains the various phytochemical tests performed.

Table: 1 Preliminary phytochemical screening

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Experiments</th>
<th>Observation</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Test solution + a few drops of CHCl$_3$ + 3 - 4 drops of acetic anhydride and one drop of conc. H$_2$SO$_4$</td>
<td>Purple colour changing to blue colour</td>
<td>Presence of steroids</td>
</tr>
<tr>
<td>2.</td>
<td>Test solution + piece of tin + 3 drops of thionyl chloride</td>
<td>Violet or purple colour</td>
<td>Presence of triterpenoids</td>
</tr>
<tr>
<td>3.</td>
<td>Test solution + 2ml of Fehling’s reagent and 3 ml of water and boil</td>
<td>Red or orange colour precipitate</td>
<td>Presence of reducing sugar</td>
</tr>
<tr>
<td>4.</td>
<td>Test solution taken with 2N HCl. Aqueous layer formed, decanted and to which are added one or two drops of Mayer’s reagent</td>
<td>White turbidity or precipitate</td>
<td>Presence of alkaloids</td>
</tr>
<tr>
<td>5.</td>
<td>Test solution in alcohol + one drop of neutral ferric chloride</td>
<td>Intense colour</td>
<td>Presence of phenolic compounds</td>
</tr>
<tr>
<td>6.</td>
<td>Test solution in alcohol + a bit of magnesium and one or two drops of conc. HCl</td>
<td>Red or orange colour</td>
<td>Presence of flavonoids</td>
</tr>
<tr>
<td>7.</td>
<td>Test solution + H$_2$O and shaken well</td>
<td>Foamy leather</td>
<td>Presence of saponins</td>
</tr>
<tr>
<td>No.</td>
<td>Reaction Description</td>
<td>Observation</td>
<td>Conclusion</td>
</tr>
<tr>
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<td>----------------------------------------------------------------</td>
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</tr>
<tr>
<td>8.</td>
<td>Test solution + H₂O + Lead acetate solution</td>
<td>White precipitate</td>
<td>Presence of tannins</td>
</tr>
<tr>
<td>9.</td>
<td>Test solution + Magnesium + acetate solution</td>
<td>Pink colour</td>
<td>Presence of Anthraquinones</td>
</tr>
<tr>
<td>10.</td>
<td>To the test solution + a few drops of conc. nitric acid + few ml of ammonia were added</td>
<td>Appearance of a red precipitate</td>
<td>Presence of xanthoprotein.</td>
</tr>
<tr>
<td>11.</td>
<td>The extract was mixed with a little anthrone on a watch glass + one drop of conc. sulphuric acid was added and made into a paste and warmed gently over the water bath.</td>
<td>Dark green colouration</td>
<td>Presence of glycosides.</td>
</tr>
<tr>
<td>12.</td>
<td>Test solution + a few drops of conc. sulphuric acid or aqueous sodium hydroxide solution</td>
<td>Colour formation</td>
<td>Presence of quinone compound.</td>
</tr>
<tr>
<td>13.</td>
<td>Test solution + a few drops of Ehrlich reagent and concentrate hydrochloric acid were added.</td>
<td>Appearance of pink colour</td>
<td>Presence of catechin.</td>
</tr>
<tr>
<td>14.</td>
<td>Test solution + a few drops of alcoholic sodium hydroxide were added.</td>
<td>Appearance of yellow colour</td>
<td>Presence of coumarin.</td>
</tr>
</tbody>
</table>

**Quantitative estimation of phytochemicals**

**Estimation of total phenolics**

Total phenolic content was estimated using Folin-Ciocalteau reagent based assay as previously described (McDonald *et al.*, 2001) with little modification. To 1 ml of each extract (100 µg/ml), 5ml of Folin-Ciocalteau reagent (diluted ten-fold) and 4 ml (75 g/L) of Na₂CO₃ were added. The mixture was allowed to stand at
20°C for 30 min and the absorbance of the developed colour was recorded at 765 nm using UV-VIS spectrophotometer. 1mL aliquots of 20, 40, 60, 80, 100 µg/mL methanolic gallic acid solutions were used as standard for calibration curve. The absorbance of solution was compared with gallic acid calibration curve. The total phenolic content was expressed as gallic acid equivalents (GAE g/100 g dry weight of extract).

**Estimation of flavonoids**

Total flavonoid content was determined according to Eom *et al.* (2007). An aliquot of 0.5 ml of samples were mixed with 0.1 ml of 10% aluminium chloride and 0.1 ml of potassium acetate (1M). In this mixture, 4.3 ml of 80% methanol was added to make 5 ml volume. This mixture was vortexed and the absorbance was measured spectrophotometrically at 415 nm. The value of optical density was used to calculate the total flavonoid content present in the sample.

**Estimation of Tannins**

Total tannin content was quantified by Vanillin-HCl method of Burns (1971) using phloroglucinol as a standard at 500 nm with a spectrophotometer [ELICO UV-VIS Spectrophotometer SL 150]. The average values of triplicate estimations of all samples were expressed as g 100g⁻¹ on dry weight basis.

**HPTLC ANALYSIS**

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).

**Procedure**

**Extraction and Test solution preparation**

Five gram powdered samples of stem and leaf of the selected plant material were extracted separately with methanol in Soxhlet apparatus for 3 hrs. The content was cooled, filtered and concentrated using vacuum flash evaporator. The content was dissolved with 1 ml methanol and centrifuged at 3000 rpm for 5 minutes. The solution was used as test solution for HPTLC analysis.

**Sample loading**

2 µl of test solution and 2 µl of standard solution were loaded as 5 mm band length in the 4 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
(alkaloids, saponins, glycosides, steroids, terpenoids and flavonoids) and the plate was developed in the respective mobile phase up to 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, saponins, glycosides, steroids, terpenoids and flavonoids) and dried at 100°C in hot air oven. The plate was photo documented in visible light and UV 366 nm mode using Photo documentation (CAMAG REPROSTAR 3) chamber.

**Scanning**

Before derivatization, the plate was fixed in the scanner stage (CAMAG TLC SCANNER 3) and scanning was done at 254 nm. The peak table, peak display and peak densitogram were noted.

**Alkaloid profile**

**Sample code**

STD - Alkaloid standard as a reference compound

Sample A - Methanol extract of *B. nervosa* leaf

Sample B - Methanol extract of *B. nervosa* stem

**Analysis details**

**Mobile phase**: Ethyl acetate: Methanol: Water (10:1.35:1)
Spray reagent: Dragendorff’s reagent followed by 10% ethanolic sulphuric reagent

Detection

Orange yellow, brownish yellow coloured zone at visible light mode present in the standard and sample tracks were observed from the chromatogram after derivatization, which confirmed the presence of alkaloids in the given sample.

Flavonoid profile

Sample code

STD - Flavonoid standard as a reference marker
Sample A - Methanol extract of B. nervosa leaf
Sample B - Methanol extract of B. nervosa stem


Spray reagent: 1% Ethanolic chloride reagent

Detection:

Yellow, bluish yellow coloured fluorescent zone at UV 366 nm mode was present in the tracks. It was observed from the chromatogram after derivatization, which confirmed the presence of flavonoids in the given sample.

Glycoside profile

Sample code

STD - Glycoside standard as a reference marker
Sample A - Methanol extract of B. nervosa leaf
Sample B - Methanol extract of B. nervosa stem
Analysis Details

**Mobile phase**  : Ethyl acetate: Ethanol: Water (8:2:1.2)

**Spray reagent**  : Liebermann Burchard reagent was sprayed and dried at 100°C for 2 minutes.

**Detection**

Brownish violet coloured zones at visible light mode present in the standard and sample tracks were observed from the chromatogram after derivatization, which confirmed the presence of glycoside in the given sample.

**Saponin profile**

**Sample code**

- STD - Saponin standard as a reference marker
- Sample A - Methanol extract of *B. nervosa* leaf
- Sample B - Methanol extract of *B. nervosa* stem

Analysis Details

**Mobile phase**  : Chloroform: Glacial acetic acid: Methanol: Water (6.4:3.2:1.2:0.8)

**Spray reagent**  : Anisaldehyde sulphuric acid reagent was sprayed and dried at 100°C for 10 minutes.

**Detection**

Blue, yellow, green and violet coloured zones at visible light mode present in the track was observed from the chromatogram after derivatization, which confirmed the presence of saponins in the given sample.
Steroid profile

Sample code

STD - Steroid standard as a reference marker
Sample A - Methanol extract of *B. nervosa* leaf
Sample B - Methanol extract of *B. nervosa* stem

Analysis Details

Mobile phase : Toluene: Acetone (9:1)
Spray reagent : Anisaldehyde sulphuric acid reagent

Detection

Blue and violet coloured zones at visible light mode present in the standard and sample tracks were observed from the chromatogram after derivatization, which confirmed the presence of steroid in the given sample.

Terpenoid profile

Sample code

STD - Terpenoid standard as a reference marker
Sample A - Methanol extract of *B. nervosa* leaf
Sample B - Methanol extract of *B. nervosa* stem

Analysis Details

Mobile phase : n–Hexane : Ethylacetate (7.2:2.9)
Spray reagent : Anisaldehyde sulphuric acid reagent
Detection

Blue and violet coloured zones at visible light mode present in the standard and sample tracks were observed from the chromatogram after derivatization, which confirmed the presence of terpenoids in the given sample.

GC-MS Analysis

GC-MS analysis of ethanol extracts of stem and leaf of *B. nervosa* were performed using a GC Clarus 500 Perkin-Elmer system comprising a AOC - 20i autosampler and gas chromatograph interfaced to a mass spectrometer (GC-MS) equipped with a Elite-1, fused silica capillary column (330 mm × 0.25 mm ID × 1µm df, composed of 100% Dimethyl polysiloxane). For GC-MS detection, an electron ionization system with ionizing energy of 70 eV was used. Helium gas (99.999%) was used as the carrier gas at constant flow rate of 1ml/minute and an injection volume of 0.5 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 minutes), with an increase of 10°C/minute, to 200°C, then 5°C/minute to 280°C, ending with a 9 minutes isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 seconds and fragments from 40 to 550 Da. Total GC running time was 36 minutes. The relative percentage 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 of GC-MS was conducted using the database of National Institute of Standard and Technology (NIST) having more
than 62,000 patterns. The spectrum of the unknown components 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.

**FT-IR analysis**

A little powder of plant specimen was mixed with KBr salt using a mortar and pestle and compressed into a thin pellet. Infrared spectra were recorded as KBr pellets on a Thermoscientific Nicot iS5 iD1 transmission, between 4000 - 400 cm$^{-1}$ (Kar eru et al., 2008).

**Pharmacological studies**

**In vitro antioxidant activity**

**DPPH radical scavenging activity**

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-diphenyl-2-picryl-hydrazyl (DPPH) according to the previously reported method (Blois, 1958). Briefly, 0.1 mM solution of DPPH in methanol was prepared and 1 ml of this solution was added to 3 ml of the solution of all extracts at different concentrations (50, 100, 200, 400 and 800 µg/ml). The mixtures were shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance was measured at 517 nm using a UV-VIS spectrophotometer (Genesys
Ascorbic acid was used as the reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated by using the following formula.

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

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

**Hydroxyl radical scavenging activity**

The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell *et al.* (1987). Stock solutions of EDTA (1 mM), FeCl\(_3\) (10 mM), Ascorbic Acid (1 mM), H\(_2\)O\(_2\) (10 mM) and Deoxyribose (10 mM) were prepared in distilled deionized water.

The assay was performed by adding 0.1 ml EDTA, 0.01 ml of FeCl\(_3\), 0.1 ml H\(_2\)O\(_2\), 0.36 ml of deoxyribose, 1.0 ml of the extract of different concentration (50, 100, 200, 400 & 800 µg/ml) dissolved in distilled water, 0.33 ml of phosphate buffer (50 mM, pH 7.9), 0.1 ml of ascorbic acid in sequence. The mixture was then incubated at 37°C for 1 hour. 1.0 ml of the incubated mixture was mixed with 1.0 ml of 10% TCA and 1.0 ml of 0.5% TBA (in 0.025 M NaOH containing 0.025% BHA) to develop the pink chromogen measured at 532 nm. The hydroxyl radical scavenging activity of the extract is reported as % inhibition of deoxyribose. The degradation is calculated by using the following equation

\[
\text{Hydroxyl radical scavenging activity} = \frac{(A_0 - A_1)}{A_0} \times 100
\]
Where, $A_0$ is the absorbance of the control and $A_1$ is the absorbance of the test samples and reference. All the tests were performed in triplicates and the results were averaged.

**Superoxide radical scavenging activity**

The superoxide anion scavenging activity was measured as described by Srinivasan *et al.* (2007). The superoxide anion radicals were generated in 3.0 ml of Tris - HCl buffer (16 mM, pH 8.0) containing 0.5 ml of NBT (0.3 mM), 0.5 ml NADH (0.936 mM) solution, 1.0 ml extract of different concentrations (50, 100, 200, 400 & 800 µg/ml) and 0.5 ml Tris - HCl buffer (16 mM, pH 8.0). The reaction was started by adding 0.5 ml PMS solution (0.12 mM) to the mixture, incubated at 25°C for 5 min and the absorbance was measured at 560 nm against a blank sample, ascorbic acid. The percentage inhibition was calculated by using the following equation

$$\text{Superoxide radical scavenging activity} = \frac{(A_0 - A_1)}{A_0} \times 100$$

Where, $A_0$ is the absorbance of the control and $A_1$ is the absorbance of the test samples and reference. All the tests were performed in triplicates and the results were averaged.

**Antioxidant Activity by Radical Cation (ABTS\(^+\))**

ABTS assay was based on the slightly modified method of Huang *et al.* (2011). ABTS radical cation (ABTS\(^+\)) was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12-16 hrs before use. The ABTS\(^+\) Solution were diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. After addition of 100 µL of
sample or trolox standard to 3.9 ml of diluted ABTS⁺ solution, absorbance was measured at 734 nm by Genesys 10S UV-VIS (Thermo scientific) exactly after 6 minutes. Results were expressed as trolox equivalent antioxidant capacity (TEAC).

\[
\text{ABTS radical cation activity} = \frac{(A_0 - A_1)}{A_0} \times 100
\]

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

**Reducing Power**

The reducing power of the extract was determined by the method of Kumar and Hemalatha (2011). 1.0 ml of solution containing 50, 100, 200, 400 & 800 µg/ml of extract was mixed with sodium phosphate buffer (5.0 ml, 0.2 M, pH 6.6) and potassium ferricyanide (5.0 ml, 1.0%). The mixture was incubated at 50°C for 20 minutes. Then 5ml of 10% trichloroacetic acid was added and centrifuged at 980 g (10 minutes at 5°C) in a refrigerated centrifuge. The upper layer of the solution (5.0 ml) was diluted with 5.0 ml of distilled water and ferric chloride and absorbance read at 700 nm. The experiment was performed thrice and results were averaged.

**Acute toxicity study**

Acute oral toxicity study was performed as per OECD - 423 guidelines (acute toxic class method). Wistar albino rats / Swiss albino mice (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 intubations and observed for 14 days. If mortality was observed in two out of six 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 ….. 2000 mg/kg body weight (data not shown). All the experimental protocol was approved by the Institutional Animal Ethics Committee.

**Anticancer activity**

**Experimental Animals**

Male adult Swiss albino mice (20-25 g) 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 (12:12 hrs light/dark cycle) and temperature (25±2°C) with standard laboratory diet (Sai Durga feeds and Foods, Bangalore) and water *ad libitum*. The experiments were conducted according to the rules and regulations of Institutional Animal Ethics Committee, Government of India.

**Tumour cells**

Echrislh Ascites Carcinoma (EAC) cells were obtained under the courtesy of Department of Biochemistry, Adayar Cancer Institute, Chennai, India. The freshly drawn ascitic fluid was taken in a haematocrit (micro) tube and diluted upto 1000 times in phosphate buffer solution pH (6.8). Then a drop of the diluted cell suspension was placed on the Neubauer counting chamber and the cells in 64 small squares were counted. An aliquot of 1ml (1 × 10⁶ cells) of the diluted solution was injected intraperitoneally to mice belonging to age group of 5 to 6 weeks and weight (20 to 25 g)
Experimental protocol

Healthy mature male Swiss albino mice were divided into seven groups of six each. All the groups except group I, were injected with EAC cells \((1 \times 10^6 \text{ cells/mouse})\) sub-cutaneously at the abdomen region to cause mammary tumor. This was taken as day 0. Group I served as normal saline control \((1 \text{ ml/kg, p.o.})\) and group II served as EAC bearing control. On day 1, the ethanol stem extract of *B. nervosa* at a dose of 150 mg/kg and 300 mg/kg each to the Group III and IV and leaf extracts at a dose of 150 mg/kg and 300 mg/kg each to the Group V and VI were administered orally and continued for 9 consecutive days respectively. Group VII served as tumour induced animals administered with vincristine \((80 \text{ mg/kg body weight})\) for 9 consecutive days. On day 10, body weight of the animal was noted. One set of the animals were sacrificed, 24 hrs after the last dose of the drug and the weight of the vital organs such as spleen, thymus, liver, kidney and lungs were recorded. They were expressed as relative organ weight \((\text{g}/100\text{g body weight})\). The rest of the animals were kept with food and water *ad libitum* to study the parameters like mean survival time, increase in life span, packed cell volume, viable cell count and non viable cell count (Gothoskar and Ranadive, 1971; Mazumdar *et al.*, 1997).

**Determination of Mean Survival Time**

Animals will be inoculated with EAC cells \((1 \times 10^6 \text{ cells/mouse})\) on day ‘0’ and the mean survival time (MST) of each group will be noted.

\[
\text{Mean survival} = \frac{\text{Day of 1st death + Day of last death}}{2}
\]
Percentage of increase life span (% ILS)

The effect of ethanol extract of *B. nervosa* on 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 \( T \) = Mean survival time of treated group

Where \( C \) = Mean survival time of control group

**Determination of Packed Cell 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 was determined by centrifuging at 10000 rpm for five minutes.

**Estimation of Viable and Non viable Cell Count**

The cells were then stained with trypan blue (0.4% in normal saline) dye. 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.

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

**Hematological Studies**

Hemoglobin content (Hb), red blood cell count (RBC) 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.
**Sloid Tumor Volume**

Tumor was induced by injecting EAC cells \((1 \times 10^6\text{cells/mouse})\) subcutaneously to the right hind limb of the animals for six groups. The radii of the tumor were measured using Vernier Calipers at 5 days intervals for one month starting with 15\(^{th}\) day. The volume of the tumor was calculated using the formula

\[ V = \frac{4}{3} \pi r_1^2 r_2 \]

Where, ’r1’ and ‘r’ represent the major and minor diameter respectively. This was compared with tumor control (Group I).

**Antidiabetic activity**

**Experimental induction of diabetes in rats**

Three months old male Wistar albino rats weighing 180 - 240 g were obtained from the animal house of the laboratory of Agricultural University, Trissur, Kerala. All animals were kept in an environmentally controlled room with 12 hrs light/12 hrs dark cycle. The animals had free access to water and standard rat diet. The rats were injected with alloxan monohydrate dissolved in sterile normal saline at a dose of 150 and 300 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 hrs. The rats were then kept for the next 24 hrs 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. Diabetic was induced in rats two weeks before starting the treatment. The rats were divided into the following seven groups after the induction of diabetics. Each group consists of 6 rats.

Group - I : Rats received normal saline daily for 14 days, orally by using an intragastric catheter tube (IGC) and served as normal control.

Group - II : Diabetic rats received normal saline daily for 14 days, orally by using an IGC, at a dose of 2.5 ml/kg body weight and served as diabetic induced control.

Group – III : Diabetic rats received stem extract of *B. nervosa* at the dose of 150 mg/kg body weight daily for 14 days, orally by using an IGC.

Group –IV : Diabetic rats received stem extract of *B. nervosa* at the dose of 300 mg/kg body weight daily for 14 days, orally by using an IGC.

Group – V : Diabetic rats received leaf extract of *B. nervosa* at the dose of 150 mg/kg body weight daily for 14 days, orally by using an IGC.

Group –VI : Diabetic rats received leaf extract of *B. nervosa* at the dose of 300 mg/kg body weight daily for 14 days, orally by using an IGC.

Group – VII : Diabetic rats received glibenclamide (600 µg/kg body weight) for 14 days, orally by using an IGC.
The plant drug treatments were given between 9.30 to 10.00 hrs 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 rpm for 10 minutes and stored at -20°C until used for enzyme and biochemical assays.

**Estimation of Glucose (Sasaki et al., 1972)**

**Principle**

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

**Reagents**

1. Ortho toluidine boric acid reagent: This reagent consists of 2.5 g of thiourea and 2.4 g of boric acid in 100 ml of a mixture of water, acetic acid and ortho toluidine (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. It was mixed well and centrifuged. 0.5 ml of the supernatant was taken. To this, 2.0 ml of ortho toluidine reagent was added and heated in a boiling water bath for 15 minutes 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. The samples and control 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 conjugates are then washed off with wash buffer. The amount of bound peroxidase is proportional to the concentration of the insulin present in the sample. The concentration of insulin in the samples is determined by the intensity of the colour developed upon the addition of the substrate and chromogen.

Assay procedure

- The designed number of coated wells in the holder was secured and the data sheet with sample identification was marked.
- 25 µl of serum sample, control and reference were dispensed into the assigned wells.
- 100 µl of enzyme conjugate was dispensed into each well and mixed for five seconds.
- It was incubated for 30 minutes at 25°C.
- Incubation mixture was removed and the wells were rinsed well with washing buffer for five times.
100 µl of solution A and then 100 µl of solution B were dispensed into each well.

It was incubated for 15 minutes at room temperature.

The reaction was stopped by adding 50 µl of 1N sulphuric acid or 2N HCl to each well and the OD was read at 450 nm with a micro well reader.

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

**Principle**

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

**Reagents**

1. TCA, 10%
2. Stock: Diacetyl monoxime, 25 g/L
3. Stock: Thiosemicarbazide 2.5 g/L
4. Acid ferric chloride solution: 1.0 ml sulphuric acid was added to 100 ml of ferric chloride solution containing 50 g/L in water.
5. Acid reagent: 10 ml of ortho phosphoric acid, 80 ml sulphuric acid and 10 ml of ferric chloride solution were added to 1 litre of water and mixed well.
6. Color reagent: To 300 ml acid reagent, 200 ml water, 10 ml stock diacetyl monoxime and 2.5 ml thiosemicarbazide were added.
7. 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. It was mixed well and centrifuged. 0.2 ml of the supernatant was taken and 3.0 ml of colour reagent was added. The test tubes were kept in water bath for 20 minutes. It was cooled to room temperature and the colour developed was read at 520 nm within 15 minutes. 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**

1. Picric acid: 8.02 g/L
2. Sodium hydroxide: 12.8 g/L
3. Standard creatinine: 100 mg of creatinine was dissolved in 100 ml of distilled water.
4. Working standard: 2.0 ml of stock solution was diluted to 100 ml with distilled water. This contains 20 µg of creatinine / ml.
5. Reagent mixture: One part by volume of diluted NaOH and one part by volume of picric acid were mixed 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 prepared with the reagent mixture and distilled water. It was mixed well and the change in absorbance was measured after 30 seconds at 490 nm, which was taken as $A_1$ and exactly after 2 minutes, the absorbance was read and it was taken as $A_2$. Sets of standards were also treated in the same manner. $A_1 - A_2$ gave the change in absorbance, which was the measure of the creatinine present in the sample. The result was expressed as mg/dl in serum.

Estimation of Glycosylated Haemoglobin (HbA1c)

At the end of the experimental period, the 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 proposed by Karunanayake and Chandrasekharan (1985).

Estimation of Protein (Lowry et al., 1951)

Principle

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

Reagents

1. 1.2% Sodium carbonate in 0.1N NaOH (Reagent A).
2. 0.5% Copper sulphate in 1% potassium sodium tartarate (Reagent B).

3. Alkaline copper reagent: 50 ml of reagent A and 1.0 ml of regent B were mixed prior to use.

4. Folin-Ciocalteau reagent: One part of Folin’s phenol reagent was mixed with two parts of water (1:2).

5. Stock standard: 50 mg of bovine serum albumin was weighed and made up to 50 ml in a standard flask with saline.

6. Working standard: 10 ml of the stock was diluted to 50 ml with distilled water. 1.0 ml of this solution contained 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 up to 1.0 ml with distilled water. 5.0 ml of alkaline copper reagent was added to each tube, mixed well and allowed to stand for 10 minutes. Then 0.5 ml of Folin-Ciocalteau reagent was added, mixed well and incubated at room temperature for 30 minutes. 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 was 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 formed a coloured complex specifically with albumin. The intensity of the colour was measured at 620 nm, and the intensity of colour was directly proportional to the albumin concentration in the serum. The total protein minus the albumin gave the globulin (James *et al.*, 2007).
Estimation of Serum Glutamate Pyruvate Transaminase (SGPT or ALT)  
(Reitman and Frankel, 1957)

Principle

The enzyme catalyses the following reaction:

\[ \text{L-Alanine} + \alpha\text{-oxoglutarate} \rightarrow \text{Pyruvate} + \text{L-glutamate} \]

The pyruvate was 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

1. Phosphate buffer: 0.1M, pH 7.5
2. Substrate: 146 mg of \(\alpha\)-ketoglutarate and 17.8 g of L-alanine were dissolved in 1N NaOH with constant stirring. The pH was adjusted to 7.4 and made up to 1000 ml with phosphate buffer.
3. Standard pyruvate, 2 mmol: 22 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer. 0.2 ml of standard contains 0.4 \(\mu\)M of sodium pyruvate.
4. Dinitrophenyl hydrazine (DNPH) reagent (1 mM / L): 200 mg in 1 mol/L HCl.
5. 0.4 N NaOH: 16 g of NaOH was dissolved in 1000 ml water.

Procedure

0.2 ml of sample and 1.0 ml of the buffer substrate were incubated for 30 minutes 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 minutes. Then, 10 ml of 0.4 N 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 was 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.

**Reagent**

1. Phosphate buffer 0.1M, pH 7.5

   Solution A: 0.1M solution of monobasic sodium phosphate (13.9 g/L).

   Solution B: 0.1M solution of dibasic sodium phosphate (6.8 g/L of \( \text{Na}_2\text{PO}_4\cdot7\text{H}_2\text{O} \)) 16 ml of solution A and 84 ml of solution B were mixed and diluted to a total of 200 ml.

2. Substrate: 146 mg of \( \alpha\text{-Ketoglutarate} \) and 13.3 g of aspartic acid were dissolved in 1 N NaOH with constant stirring. The pH was adjusted to 7.4 and made up to 1000 ml with phosphate buffer.
3. Standard pyruvate (2 mol/L): 22 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer. 0.2 ml of standard contains 0.4 µM of sodium pyruvate.

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

5. 0.4 N NaOH: 16 g of NaOH was dissolved in 1000 ml water.

**Procedure**

0.2 ml of sample and 1.0 ml of the buffer substrate were incubated for 60 minutes 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 minutes. Then, 10 ml of 0.4 N 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**

In this method, disodium phenyl phosphate was hydrolysed with the liberation of phenol and inorganic phosphate. The liberated phenol was measured at 700 nm with Folin-Ciocalteau reagent.

**Reagent**

1. Sodium carbonate-sodium bicarbonate buffer, 100mM/L: 6.36 g of anhydrous sodium carbonate and 3.36 g of sodium bicarbonate were dissolved in water and made up to a litre.
2. Disodium phenyl phosphate solution, 100 mM/L: 2.18 g of Disodium phenyl phosphate was dissolved in water, heated to boiling, cooled and made up to a litre. 1.0 ml of chloroform was added and stored in a refrigerator.

3. Buffer substrate: It was prepared by mixing equal volumes of the above two solutions and the pH was adjusted to 10.

4. Folin-Ciocalteau reagent: 1.0 ml of reagent was mixed with 2.0 ml of water.

5. 15% Sodium carbonate solution: 15 g of anhydrous sodium carbonate was dissolved in 100 ml of water.

6. Standard phenol solution: 1 g of pure crystalline phenol was dissolved in 100 mmol/L HCl and made up to a litre with the acid.

7. Working standard solution: 100 ml of dilute phenol reagent was added to 5.0 ml of stock standard and diluted to 500 ml with water. This contains 10 µg of phenol/ml.

Procedure

4 ml of the buffer substrate was pipetted into a test tube and incubated at 37°C for 5 minutes. 0.2 ml of serum or tissue homogenate was added to the test tube and incubated further for exact 15 minutes. The tube was removed and 1.8 ml of diluted phenol reagent was added immediately. 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. It was mixed well and centrifuged. To 4 ml of supernatant,
2 ml of sodium carbonate was added. The blank was prepared by mixing 4 ml of working standard solution, 3.2 ml of water and 0.8 ml of phenol reagent. 2 ml of sodium carbonate was then added and all the tubes were incubated at 37°C for 15 minutes. The colour developed 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**

1. Stock ferric chloride: 840 mg of pure dry ferric chloride was weighed and dissolved in 100 ml of glacial acetic acid.

2. Ferric chloride precipitation reagent: 10 ml of stock ferric chloride reagent was taken in 10 ml of standard flask and made up to the mark with pure glacial acetic acid.

3. Ferric chloride diluting reagent: 8.5 ml of stock ferric chloride was diluted to 100 ml with pure glacial acetic acid.

4. Standard cholesterol solution: 100 mg of cholesterol was dissolved in 100 ml of glacial acetic acid.
5. Working standard: 10 ml of stock was dissolved in 0.85 ml of ferric chloride precipitation reagent and made up to 100 ml with glacial acetic acid. The concentration of working standard is microgram/ml.

Procedure

To 0.1 ml of the serum, 4.9 ml of ferric chloride precipitating reagent was added and centrifuged. To 2.5 ml of supernatant, 2.5 ml of ferric chloride diluting reagent and 4.0 ml of concentrated sulphuric acid were added. 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 up to 5.0 ml with ferric chloride diluting reagent. Then, 4.0 ml of concentrated sulphuric acid was added. After 30 minutes, 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

1. Chloroform-methanol mixture (2:1).

2. Activated silicic acid: It was activated by washing silicic acid with 4 N or 2 N 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.

3. Saponification reagent: 5 g of KOH was dissolved in 60 ml of water and 40 ml of isopropanol was added.

4. Sodium-metaperiodate reagent: 77 g of anhydrous ammonium acetate was dissolved in 700 ml of water. To this, 60 ml acetic acid and 650 mg of sodium metaperiodate were added and diluted to 1000 ml with distilled water.

5. Acetyl acetone reagent: 0.75 ml of acetyl acetone was added to 20 ml of isopropanol and 80 ml of distilled water and it was mixed well.

6. Tripalmitin standard contains 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 and mixed well. Then, 400 mg of silicic acid was added, placed them in a mechanical shaker and centrifuged.

To 2.0 ml of the supernatant, 0.6 ml of saponification reagent was added and incubated at 60-70°C for 15 minutes. After cooling, 1 ml of sodium metaperiodate was added and mixed well. Then, 0.5 ml of acetyl acetone reagent was added and mixed again. The tubes were incubated at 50°C for 30 minutes. After cooling, the colour was read at 405 nm. Standard tripalmitin (20-100 µg) were taken in tubes and treated similarly. Triglycerides are expressed as mg/dl 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.

**Procedure**

(i) HDL - Cholesterol separation

The lavender coloured complex formed was mixed well, kept at room temperature for 10 minutes and then centrifuged at 2000 rpm for 15 minutes to obtain a clear supernatant. Then proceeded to step II.

(ii) HDL – cholesterol estimation

<table>
<thead>
<tr>
<th></th>
<th>Blank (B)</th>
<th>Standard (S)</th>
<th>Test (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reagent 1:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol reagent</td>
<td>3.0 ml</td>
<td>3.0 ml</td>
<td>3.0 ml</td>
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<tr>
<td><strong>Reagent 2:</strong></td>
<td></td>
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<tr>
<td>Working cholesterol</td>
<td>-</td>
<td>0.015 ml</td>
<td>-</td>
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<tr>
<td>Standard (200 mg%)</td>
<td></td>
<td>15 (µl)</td>
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<tr>
<td><strong>Supernatant from step – I</strong></td>
<td>-</td>
<td>-</td>
<td>0.12 ml</td>
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<td></td>
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<td>(120 µl)</td>
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</tbody>
</table>

The content in the tubes were mixed well and the tubes were kept immediately in the boiling water bath exactly for 90 seconds (1½ minutes). The tubes were cooled
immediately at room temperature under running tap water. The OD of Standard (S) and Test (T) against Blank (B) were read on a colorimeter with a yellow green filter or on a spectrophotometer at 560 nm.

**Determination of Low Density Lipoprotein - Cholesterol (LDL-C) and Very Low Density Lipoprotein - Cholesterol (VLDL-C)**

LDL - cholesterol and VLDL - cholesterol levels in serum were calculated as per the procedure proposed by Friedwald *et al.* (1972).

**Determination of Phospholipids:**

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**

1. 15% KCl
2. 1% Phosphoric acid
3. n-butanol
4. 0.6% thiobarbituric acid
5. 10 mM ferrous sulphate
6. 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 minutes in a boiling water bath and after addition of 4.0 ml of n-butanol vigorously vortexed and centrifuged at 2000 rpm for 20 minutes. 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 tetra ethoxy propane at concentration of 5.125, 10.25 and 20.5 nmol ml\(^{-1}\) or using an extinction coefficient of the chromophore 1.56 × 10\(^{-5}\) M\(^{-1}\) cm\(^{-1}\) and the results were expressed as nmoles of MDA formed / mg protein.

Estimation of Glutathione Peroxidase (GPx) and Reduced Glutathione (GSH)

GPx activity was measured by the method described by Rotruck \textit{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 \(\text{H}_2\text{O}_2\). The contents were incubated at 37°C for 10 minutes. The reaction was arrested by 0.4 ml of 10% TCA and centrifuged. The 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 Ellmans 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 naphtylamine to produce a red azo compound whose absorbance is measured at 543 nm.

Reagents

1. 50 mM phosphate buffer, pH 7.4
2. 20 mM L-Methionine
3. 1% (v/v) Triton X-100
4. 10 mM hydroxylamine hydrochloride
5. 50 µM EDTA
6. 50 µM Riboflavin
7. 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 minutes. 80 µl of riboflavin was added and the tubes were exposed for 10 minutes 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 Griess 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.

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

The method is based on the fact that, dichromate in acetic acid reduces to chromic acetate when heated in the presence of H\(_2\)O\(_2\) with the formation of perchloric acid as an unstable intermediate. The chromic acetate thus produced is measured colorimetrically at 610 nm. Since dichromate has no absorbance 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 is allowed to split H\(_2\)O\(_2\) for different periods of time. The reaction is stopped at specific time intervals by the addition of dichromate-acetic acid mixture and the remaining H\(_2\)O\(_2\) is determined by measuring chromic acetate colorimetrically after heating.

**Reagents**

1. 0.01 M Phosphate buffer, pH 7.0
2. 0.2 M Hydrogen peroxide
3. Stock dichromate/acetic acid solution: 5% potassium dichromate was mixed with glacial acetic acid (1:3 by volume).
4. Working dichromate/acetic acid solution: The stock solution was diluted in 1:5 ratio with water to make the working dichromate/acetic acid solution.
**Procedure**

The assay mixture contained 0.5 ml of H$_2$O$_2$, 10 ml of buffer and 0.4 ml of 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 H$_2$O$_2$ decomposed/minutes/mg protein.

**Methodology for Tissue antioxidant Preparation of tissue homogenate**

After the experimental regimen, the animals were sacrificed under mild chloroform anesthesia. Liver and kidney were excised and 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 minutes, 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 kidney**

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 minutes in a water bath and then cooled and centrifuged at 3500×g for 10 minutes at room temperature, the absorbance of clear supernatant was measured at 535 nm against a reference blank. The values were expressed as nanomole/mg protein.
Estimation of Superoxide Dismutase (SOD)

The activity of superoxide dismutase (SOD) was assayed as per the procedure designed by Kakkar et al. (1984) based on the oxidation of epinephrine adrenochrome transition by enzyme. 0.5 ml of tissue homogenate was diluted with 0.5 ml of distilled water. To this, 0.25 ml of chilled ethanol and 0.15 ml of chloroform was added. The mixture was shaken for 1 minute and centrifuged at 2000×g for 10 minutes. From this mixture, 0.5 ml was added to 1.5 ml of PBS buffer (pH 7.2). The reaction was initiated by adding 0.4 ml of epinephrine and change in optical density OD (min⁻¹) was measured at 470 nm. SOD activity was expressed as U/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)

Catalase (CAT) was estimated by the method designed by Sinha (1972). The reaction mixture, 1.5 ml contained 1.0 ml of 0.01 M phosphate buffer (pH 7.0), 0.1 ml of tissue homogenate and 0.4 ml of 2 M H₂O₂. The reaction was stopped by adding 2.0 ml of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid in 1:3 ratio). Then the absorbance was measured at 530 nm. The CAT activity was expressed as U/mg protein.

Estimation of Glutathione Peroxidase (GPx)

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 of 10 mM sodium azide, 0.2 ml tissue extract, 0.2 ml of reduced glutathione and 0.1 ml of 0.2 mM hydrogen peroxide.
The contents were incubated for 10 minutes at 37°C. 0.4 ml of 10% TCA was added to stop the reaction and centrifuged at 3200×g for 20 minutes. 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 U/mg protein.

**Estimation of Reduced Glutathione (GSH)**

Reduced glutathione (GSH) was measured by the method proposed by Ellman et al., (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 minutes, 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 U/mg protein.

**Hepatoprotective activity**

**Experimental animal**

Normal healthy adult male Wistar albino rats weighing about 180 - 240 g body weight were selected for this work. They were housed under standard environmental conditions at room temperature (25 ± 2°C) in a well-ventilated animal house with constant 12 hrs of darkness and 12 hrs of light schedule. The rats were fed with standard pellet diet (Goldmohar brand, Hindustan Lever Ltd., Mumbai, India) and water *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

A total of 35 rats were taken and were divided into seven groups of 5 rats each, of which, six groups contained CCl₄ hepatic toxicity induced rats and the remaining one group contained normal rats.

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

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

Group – III : Liver injured rats received stem extract of B. nervosa at the dose of 150 mg/kg body weight for 14 days by using an IGC.

Group – IV : Liver injured rats received stem extract of B. nervosa at the dose of 300 mg/kg body weight for 14 days by using an IGC.

Group – V : Liver injured rats received leaf extract of B. nervosa at the dose of 150 mg/kg body weight for 14 days by using an IGC.

Group – VI : Liver injured rats received leaf extract of B. nervosa at the dose of 300 mg/kg body weight for 14 days by using an IGC.
Group – VII: Liver injured rats received silymarin 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 hrs in the morning. After 24 hrs 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×g for 10 minutes and stored at -20°C until used for various biochemical assays.

Estimation of alkaline phosphate (ALP), serum glutamate oxalo transaminase (SGOT or AST) and serum glutamate pyruvate transaminase (SGPT or ALT), blood glucose and serum protein was done as per the procedure already given in the diabetic methodology.

Estimation of Total, Conjugated, Unconjugated Bilirubin and GGTP

Total bilirubin and conjugated bilirubin were determined as described by Balistrei and Shaw (1987). The concentration of unconjugated bilirubin was calculated as the difference between total and conjugated bilirubin concentrations. Gamma-glutamyl transferase (GGT) was estimated by the method of Szasz (1968).

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

Qualitative estimation of MDA formation was done by the method proposed by Okhawa et al. (1979) and it was done by determining the concentration of thiobarbituric acid reactive substance (TBARS) in 10% liver homogenate. Enzymatic antioxidants, superoxide dismutase (SOD) (Mishra and Fridovich, 1972), Catalase (Aebi, 1974), non enzymatic antioxidant glutathione peroxidase (GPx) (Paglia and
Valentine, 1967) and glutathione reductase (GRD) (Goldberg and Spooner, 1983) were also assayed in liver homogenate.

**Antifertility activity**

**Experimental animal**

Normal healthy adult male Wistar albino rats weighing about 180 - 240 g body weight were selected for this work. They were housed under standard environmental conditions at room temperature (25 ± 2°C) in a well-ventilated animal house with constant 12 hrs of darkness and 12 hrs of light schedule. The rats were fed with standard pellet diet (Goldmohar brand, Hindustan Lever Ltd., Mumbai, India) and water

*ad libitum.*

**Experimental design**

The animals were divided into five groups and each group was consisting of 5 animals.

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

**Group II** : Rats given stem extract of *B. nervosa* at the dose of 150 mg/kg body weight daily, orally for 14 days by using an IGC.

**Group III** : Rats given stem extract of *B. nervosa* at the dose of 300 mg/kg body weight daily, orally for 14 days by using an ICG.

**Group IV** : Rats given leaf extract of *B. nervosa* at the dose of 150 mg/kg body weight daily, orally for 14 days by using an ICG.
**Group V**: Rats given leaf extract of *B. nervosa* at the dose of 300 mg/kg body weight daily, orally for 14 days by using an ICG.

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 hrs in the morning. After 24 hrs 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×g for 10 minutes and stored at -20°C until used for various biochemical assays. Then testis, epididymis, vas deferens, seminal vesicle and ventral prostate were dissected out, trimmed off extraneous tissues and weighed accurately on a torsion balance. The weight of the organs was expressed in terms of mg/100 g body weight.

**Assessment of sperm motility**

Immediately after animals were sacrificed, caudal 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 ocularimeter and expressed as micrometre (μm) traversed per minute.

**Sperm count determination**

**Collection of epididymal fluid**

Epididymal fluid was collected from caput and cauda segments separately for sperm count, 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 of epididymal fluid was added to 1.9 ml of Sorenson’s buffer. The preparation was thoroughly mixed and one drop was added to both sides of a standard blood cell Neubauer haemocytometer. The number of spermatozoa in the appropriate square of the haemocytometer was counted under the microscope at 100 x. Both sides of the haemocytometer were counted and an average was taken.

**Calculation**

The sperm concentration refers to the number of spermatozoa per ml of 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, the spermatozoa that crossed the lines at the top and right hand sides were also 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 mm³ or 10⁻⁴ ml. The multiplication factor of corner square E is therefore 10⁻⁴ or 1,00,000. 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 would be obtained.

When the spermatozoa in small square E1, E2, E3, E4 and E5 were counted, the multiplication factor was 5 times greater than that 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 ×10^6/ml.

The basic formula that is applied to obtain the sperm concentrations is: Sperm concentration = No. of spermatozoa × multiplication factor × dilution factor.

**Sperm motility, viability and counts**

The rats were anesthetized with 25% urethane at a dose of 0.6 ml/100 g intraperitoneally. The caudal epididymis was then dissected. An incision (about 1 mm) was made in the caudal epididymis, drops of sperm fluid were squeezed on to 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 with 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 stem and leaf ethanol extracts of *B. nervosa* in the control males’ 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 estrons 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 foetuses and the number of resorption sites were recorded.

**Hormonal analysis**

The blood removed from the animals by intra-cardiac method was centrifuged at 2000 rpm (Revolutions per minute) to separate the serum for the measurement of Follicle Stimulating Hormone (FSH), Luteinizing Hormone (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) which 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 at 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-assay and inter-assay variations were 10.02%, 10.12%, 9.7% and 10.4% for Testosterone, FSH, LH and Estrogen respectively.
**Antiinflammatory activity**

**Acute toxicity study**

For toxicity studies, two different groups of six albino rats of both sexes were administered orally with the test substances, in the range of doses 100 - 3000 mg/kg and the mortality rates were observed after 72 hrs. The ethanol extracts of stem and leaf of *B. nervosa* showed no mortality at 3000 mg/kg. Therefore, 3000 mg/kg dose was considered as LD$_{50}$ cutoff dose (safe dose) and hence 1/20 (150 mg/kg) 1/10 (300 mg/kg) of LD$_{50}$ doses were the selected as safe doses.

**Animals**

Wistar albino adult rats of both sexes with 150 - 200 g body weight were used for present investigation. The animals were kept in individual cages under standard environmental conditions at temperature of 25 ± 2°C and a relative humidity of about 55%. The rats were fed with standard pellet diet (Goldmohar brand, Hindustan Lever Ltd., Mumbai, India) and water *ad libitum* and fasted for 16 hrs before the start of the experiment.

**Drugs (Synthetic antiinflammatory agents)**

Commercial name of the reference antiinflammatory drug used in our study is Indomethacin. It is chemically known as 1-(4-Chlorobenzoyl)-(-methoxy-2-methylindole-3-yl) acetic acid and was obtained from Pharmco Pharmaceuticals Company.
Chemical used for the induction of inflammation

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

Rats were divided into six groups and each group comprising of five rats.

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

**Group II**: Rats received carbon tetrachloride to induce paw oedema

**Group III**: Rats received stem extract of *B. nervosa* at the dose of 150 mg/kg body weight.

**Group IV**: Rats received stem extract of *B. nervosa* at the dose of 300 mg/kg body weight.

**Group V**: Rats received leaf extract of *B. nervosa* at the dose of 150 mg/kg body weight.

**Group VI**: Rats received leaf extract of *B. nervosa* at the dose of 300 mg/kg body weight.

**Group VII**: Rats received standard Indomethacin 10 mg/kg body weight.

Paw edema was induced by injecting 0.1 ml of 1% W/V carrageenan in physiological saline into the sub-plantar tissues of the left hind paw of each rat (Winter *et al.*, 1962). The ethanol extracts of stem and leaf of *B. nervosa* were administered orally 30 minutes prior to carrageenan administration. The paw volume was measured at intervals of 60, 120, 180 and 240 minutes by the mercury
displacement method using a plethysmograph. The inhibition percentage of paw volume in drug treated groups was compared with the carrageenan control group. Percentage inhibition was calculated using the formula:

\[
\text{Percentage inhibition} = \left[ \frac{(V_c - V_t)}{V_c} \right] \times 100
\]

Where, \(V_t\) represents the percentage difference in increased paw volume after the administration of test drugs to the rats and \(V_c\) represents the difference in increased volume in the control groups.

**Antibacterial studies**

**Collection of microorganisms**

Stock cultures of bacteria such as, *Bacillus thuringiensis*, *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus aureus* (Methicillin sensitive), *Enterococcus faecalis*, *Salmonella paratyphi-A*, *Salmonella paratyphi-B*, *Salmonella paratyphi*, *Proteus mirabilis*, *Escherichia coli*, *Escherichia coli* (ESBL), *Proteus vulgaris*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens* and *Mycobacterium smegmatis*, were obtained from Research Laboratory, University of Madras, Chennai, Tamil Nadu.

**Preparation of media**

The growth media employed in the present study included Nutrient agar and Nutrient broth. Nutrient agar is composed of

- **Beef extract**: 3.0 g
- **Peptone**: 5.0 g
- **Agar**: 15.0 g
Distilled water - 1000 ml

Nutrient broth is composed of

Beef extract - 3.0 g
Peptone - 5.0 g
Distilled water - 1000 ml

The medium was adjusted to pH 7.4 and sterilized by autoclaving at 15 lb pressure (121°C) for 15 minutes.

**Sub culturing of microorganisms**

The pure culture of microorganisms was maintained on nutrient agar slants by frequent sub culturing. The culture was stored at 4°C.

**Preparation of inoculum**

Each organism was recovered for testing by sub culturing on fresh media. A loopful inoculum of each bacterium was suspended in 5ml of nutrient broth and incubated overnight at 37°C. These overnight cultures were used as inoculum.

**Antimicrobial activity**

Antimicrobial activity was demonstrated by modification of the method described by Barry and Thornsberry, (1985). 0.1 ml of the diluted microbial culture was spread on sterile nutrient agar plate. The presoaked and dried discs of 6mm diameter of Whatman No.1 filter paper were then placed on the seeded plates and gently pressed down to ensure contact. At the same time standard antibiotic of Tetracycline was used as reference or positive control. Respective solvents without
plant extracts served as negative control. The plates were incubated at 37°C for 24 hrs. After the incubation period, the diameter of the inhibition zone around the plant extract saturated discs were measured and also compared with the diameter of inhibition zone of commercial standard antibiotic discs. The inhibition zone around the discs were measured and recorded as the difference in diameter between the disc (6 mm) and growth free zone.

**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).