3 Materials and Methods

3.1 Plant Material

*Elaeagnus indica* Servett. (Plate 1 and 2)

3.1.1 Taxonomy

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Plantae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class</td>
<td>Spermatopsida</td>
</tr>
<tr>
<td>Order</td>
<td>Rosales</td>
</tr>
<tr>
<td>Family</td>
<td>Elaeagnaceae</td>
</tr>
<tr>
<td>Genus</td>
<td>Elaeagnus</td>
</tr>
<tr>
<td>Specific epithet</td>
<td>indica - Servett.</td>
</tr>
<tr>
<td>Botanical name</td>
<td><em>Elaeagnus indica</em> Servett.</td>
</tr>
</tbody>
</table>

3.1.2 Habitat

In Tamil Nadu, *Elaeagnus indica* is prominently available in Kolli hills of Namakkal district and Pacchaimalai hills of Tiruchirappalli district, Tamil Nadu, India.

**Description**

- A large, branched, usually scendent shrub, often running over trees.
- Leaves variable, broadly elliptiv or elliptic-lanceolate, obtuse or acuminate apex, upper surface pale green, clothed with small whitish seals.
- Lower surface silvery white.
- Flowers usually many in cluster. Perianth covered with silvery scales. Fruit nearly 2cm long. The flowers are small, with a four-lobed calyx and no petals; they are often fragrant.
- Fruits are edible. The fruit is a fleshy drupe containing a single seed; it is edible in many species.
- *Elaeagnus* plants are deciduous or evergreen shrubs or small trees.
- The alternate leaves and the shoots are usually covered with tiny silvery to brownish scales, giving the plants a whitish to grey-brown colour from a distance (Matthew, 1995).
Elaeagnus indica Servattaz தௌன் வீழ்ச்சி 1 flower; 2 perianth, split open; 3-5 stamens; 6 scales; 7 hairs of perianth; 8 pistil; 9 & 10 ovary, l.s. & t.s.; 11 twig.
Plate 2: *Elaeagnus indica* Servett.

(a): Habitat of *Elaeagnus indica*  
(b): Flower of *E. indica*  
(c): Leaf of *E. indica*
3.2 Identification and Authentication of *E. indica*

In present study, *E. indica* plant are collected from places in Kolli Hills, Namakkal District, Tamil Nadu, identified with the help of Flora of Karnataka and authenticated by Rabinet Herbarium, St. Joseph’s College, Tiruchirappalli, Tamil Nadu

3.3 Pharmacognostic Methods

3.3.1 Organoleptic evaluation of *E. indica* (Sanjib and Kamaruz, 2009)

The sensory nature of dry leaves powder of selected plant was analysed by keeping a small quantity in a petri dish on a white background. The organoleptic characters such as appearance, colour, odour and taste of the powder were observed.

3.3.2 Fluorescence Analysis of *E. indica* (Chase and Pratt, 1949)

Fluorescence of the leaves powder of selected plant was observed under day and UV light (254nm) using various solvent extracts as well as treating with acids and alkaline solutions of the drug. The powder was treated with neutral solvents like hexane, benzene, chloroform, ethyl acetate, alcohol, acetone and acids like 1N Hydrochloric acid, 50% Sulphuric acid and alkaline solutions like aqueous and alcoholic 1N NaOH.

3.4 Physicochemical properties of *E. indica* (Anonymous, 2001)

3.4.1 Determination of Foreign Matter

100g of selected plant material was weighed and separated out in a thin layer. The foreign matter was detected by inspection with the unaided eye. It was separated, weighed and calculated the percentage of foreign matter present.

3.4.2 Determination of Moisture Content (Loss on Drying)

10g of leaves of selected plant was weighed (without preliminary drying) in a tarred evaporating dish. Care was taken so that no appreciable amount of moisture is lost during preparation. The weighed leaves were placed in the tarred evaporating dish, dried at 105°C for 5h, and weighed. The drying and weighing were continued at one-hour interval until difference between two successive weighing almost correspond and difference was not more than 0.25%. When Constant weight is reached, the material was cooled for 30m in a desiccator. The percentage of moisture content was calculated.
3.4.3 Determination of Total Ash

2g of accurately weighed leaves powder of selected plant was incinerated in a silica dish at temperature not exceeding 600°C until free from carbon and weighed. Calculate the percentage of ash with reference to the air-dried drug.

3.4.3.1 Determination of Acid Insoluble Ash

The ash obtained from total ash for 5m with 25ml of dilute hydrochloric acid was boiled. Then, collected insoluble matter in an ash less filter paper was washed with hot water and ignited to constant weight. The percentage of ash with reference to the air-dried drug was calculated.

3.4.3.2 Determination of Water Soluble Ash

The ash obtained from total ash for 5m with 25ml of distilled water was boiled. Then, collected the insoluble matter in an ash less filter paper was washed with hot water and ignited for 15m at a temperature not exceeding 600°C the weight of the insoluble matter subtracted from the weight of the ash, the difference in weight represents the water soluble ash. The percentage of water-soluble ash with reference to the air-dried drug was calculated.

3.4.4 Determination of extractive values

Suitable quantity of air dried, crushed leaves powder was weighed and extracted with various solvents in the order of increasing polarity by using Soxhlet extraction apparatus (for 6h). Then, the extract was filtered into a tarred evaporating dish and evaporated the solvents on a water bath. The residue dried at 105°C to get a constant weight. The percentage of extractive values with reference to the air-dried drug, for various solvents was calculated.

3.4.4.1 Determination of Alcohol Soluble Extractive Value

5g of the air-dried drug was macerated with 100ml of alcohol in a closed flask for 24h and frequently shaken during first 6h and allowed to stand for 18h. Evaporate the filtrate to dryness in a tarred flat bottomed china dish and dried at 105°C until constant weight is obtained. The percentage of alcohol soluble extractive value with reference to the air-dried drug was calculated.
3.4.4.2 Determination of Water Soluble Extractive Value

5g of the air-dried drug macerated with 100ml of water in a closed flask for 24h frequently shaken during first 6h and allowed to stand for 18h. Rapidly filtered and evaporated 25ml of filtrate to dryness in tarred flat-bottomed china dish and dried at 105°C until constant weight is obtained. The percentage of water-soluble extractive value was calculated with reference to the air-dried drug.

3.5 Phytochemical Screening

3.5.1 Extraction of plant materials- Cold extraction method

Nearly 500g of shade dried, coarsely powdered material was subsequently extracted with sufficient volume of various organic solvents in the order of increasing polarity and various extracts were subjected to preliminary phytochemical screening. All chemicals and solvents used for different studies were of analytical grade.

3.5.2 Behaviour of drug powder with various chemical reagents (Brindha et al., 1991)

Behaviour of drug powder were observed as per standard textual procedures

3.5.3 Preliminary Phytochemical Screening of various extracts (Harborne, 1984).

Preliminary phytochemical screening of various extracts was carried out as per the standard textual procedure.

Test for Saponins

The substance was shaken well with water.

Test for Tannins

The substance was mixed with basic lead acetate solution.

Test for Steroids (Liebermann burchard test)

The sample was taken in a test tube added few drops of glacial acetic acid, acetic anhydride and 1ml of concentrated Sulphuric acid was added along the sides of the test tube.

Test for Terpenoids (Salkowshi test)

The substance was warmed with tin and thionyl chloride.
Test for Flavonoids (Shinado’s test)

To the substance added alcohol, few magnesium turnings and few drops of concentrated hydrochloric acid and boiled for 5m.

Test for Coumarin

A small quantity of substance was mixed with few drops of 10% sodium hydroxide.

Test for Quinones

The substance was mixed with few drops of concentrated sulphuric acid.

Test for Lignins

The substance was mixed with Alcoholic solution of Phloroglucinol and added few drops of concentrated Hydrochloric acid.

Test for Alkaloids

(1) To the substance few drops of acetic acid was added, followed by Dragendorff’s reagent and shaken well.

(2) The substance was mixed with little amount of dilute hydrochloric acid and Mayer’s reagent.

Test for Sugars

The substance was mixed with anthrone and 1 drop of concentrated Sulphuric acid and warmed gently.

Test for Proteins

The substance was mixed with saturated solution of picric acid
3.6 Quantitative Analysis of Phytochemical Constituents

3.6.1 Estimation of Total Alkaloids (Ferguson, 1956)

The alcoholic extract of plant sample was treated with 0.1N HCl and aqueous acidified layer thus obtained was partitioned with Chloroform in a separating funnel. The Chloroform layer was discarded and the aqueous layer was basified with Ammonium hydroxide to alkaline pH and partitioned with Chloroform in a separating funnel. The aqueous layer was discarded and the Chloroform layer was evaporated, the resultant content was treated as total alkaloid and confirmed for alkaloid with dragendorff’s reagent.

Following formula is used to calculate the total alkaloid percentage:

\[
\text{Alkaloid} \% = \frac{\text{Weight of Residue}}{\text{Total Weight}} \times 100
\]

3.6.2 Estimation of Total Flavonoids (Kadifkova-Panovska et al., 2005)

Aqueous plant extract was extracted with ethyl acetate. The extracts were dried over anhydrous sodium sulphate, filtered and concentrated under vacuum up to a concentration of 1g/ml of extract. They are further diluted with ethyl acetate to obtain 0.01g/ml solution and used in the experiments. About 10ml of the solution was transferred into a 25ml volumetric flask, 1ml of 2% aluminium chloride was added and the solution was filled to volume with methanol-acetic acid and was kept aside for 30m, the absorbance was measured at 390nm. A blank was also maintained. Luteolin was used to construct the calibration curve in the concentration range of 1 to 10µg/ml.

3.6.3 Estimation of Total Tannins (Polshettiwar et al., 2007)

Content of tannins in plant extract was determined by Follin-Denis method. Colorimetric estimation of tannins is based on the measurement of blue colour formed by the reduction of phosphotungstomolybdic acid by tannin like compound in alkaline medium. 1ml of plant extract and standard solution of tannic acid (10 – 50µg/ml) was made upto 7.5ml with distilled water. Then 0.5ml of Folin-Denis reagent and 1ml Na₂CO₃ solution were added. The volume was made up to 10ml with distilled water and absorbance was measured at 700nm. The total tannic acid content was expressed as mg of tannic acid equivalent per gram of extract.
3.6.4 **Estimation of Phenol (Malick and Singh, 1980)**

0.5 to 1gm of the sample was weighed and ground well with 10 times volume of 80% ethanol. The homogenate was centrifuged at 10,000rpm for 20m. The supernatant was collected and the residue was re-extracted with 5 times the volume of 80% Ethanol. Centrifuged and the supernatant was collected. It was then evaporated to dryness. The residue obtained was dissolved in 5ml of distilled water. Different aliquots (0.2 – 2.0ml) were pipetted out in test tubes. The volume was made upto 3ml with water. 0.5ml of Folin’s reagent was added to all the tubes after 3m and 2ml of 20% Sodium bicarbonate solution was added. The content was mixed thoroughly and the test tubes were placed in boiling water bath for 1m, cooled and the colour developed was measured at 650nm. The amount of total phenol was calculated using the standard graph.

3.6.5 **Estimation of Ascorbic Acid (Sathasivam and Manickam, 1996)**

500mg of the plant sample was weighed exactly and it was grinded with a mortar pestle with 10ml of 4% oxalic acid which was centrifuged for 10m. The supernatant was used for the estimation. 5ml of the working standard pipetted out into 100ml of conical flask. Then add 10ml of 4% oxalic acid and titrated against the dye (V₁ml). End point is the appearance of pink colour which persists for a few minutes. The amount of the dye consumed is equivalent to the amount of ascorbic acid. 5ml of the supernatant was pipetted out, 5ml of 4% oxalic acid was added and titrated against the dye (V₂ml). The ascorbic acid in the plant was expressed in the mg/100g of the plant sample.
3.7 In vitro Antioxidant Assay

There are numerous methods available to evaluate the antioxidant activity and in vitro antioxidant screening. However, total antioxidant activity of an antioxidant cannot be evaluated by using one or two methods, due to oxidative processes. Therefore, six methods were employed in present study in order to evaluate the total antioxidant activity. The most commonly used methods are DPPH free radical scavenging, ABTS free radical scavenging, ferric reducing power assay, superoxide anion radical scavenging, hydrogen peroxide scavenging and, hydroxyl radical scavenging activity.

3.7.1 DPPH Radical Scavenging Assay (Gyamfi and Aniya, 2002)

The free radical scavenging capacity of the aqueous extract of Elaeagnus indica was determined by using DPPH. DPPH solution (0.04% W/V) was prepared in 95% methanol. From stock solution 0.2-1.0 ml of solution were taken in five test tubes and serially dilute to concentration such as 20μg/ml to 100μg/ml respectively. Freshly prepared DPPH 0.5ml solution was incubated with test drug and after 10m, the absorbance was taken at 517nm using spectrophotometer. Ascorbic acid was used as reference.

Calculation

\[
\text{DPPH Scavenging activity (\%)} = \left(1 - \frac{A_{\text{Test}}}{A_{\text{Control}}}\right) \times 100
\]

The antioxidant activity of the leaf extracts was expressed as IC_{50} and compared with standard. The IC_{50} value was defined as the concentration of extract that inhibit the formation of DPPH radicals by 50%.

3.7.2 ABTS Radical Scavenging Assay (Re et al., 1999)

The basic principle underlying the ABTS decolorization assay is that ABTS on reaction with potassium persulfate (K_{2}S_{2}O_{8}) forms a greenish blue radical cation. Standard and sample antioxidants that are able to transfer an electron to ABTS radical scavenge the color of the solution proportionate to their amount. The extent of scavenging depends both upon the concentration of antioxidant and time duration for the reaction. ABTS assay is an excellent tool for determining the antioxidant activity of hydrogen-donating antioxidants and of chain-breaking antioxidants.
ABTS solution was freshly prepared by adding 5ml of a 4.9mM potassium persulphate solution to 5ml of a 14mM ABTS solution and the resulting solution was kept for 16h in dark at room temperature (25±1°C). This solution was diluted with methanol to yield an absorbance of 0.700±0.02 at 734nm and the same solution was used for the antioxidant assay. One milliliter of reaction mixture of standard and extracts comprised 950μL of ABTS solution and 50μL of the samples. This solution was vortexed for 10s and the absorbance was recorded at 734nm after 6m using a UV-visible spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) which was compared with the control ABTS solution. The calibration curve of vitamin C was prepared by plotting the percentage inhibition of vitamin C at various concentrations (5-100μg mL⁻¹). The percentage inhibition was calculated using the following formula:

\[
\text{Percentage inhibition of vitamin C and extracts } = \frac{A_0 - A_1}{A_0} \times 100
\]

where, Ao is the absorbance of the control and A1 is the absorbance of samples. The results were expressed as mg Vitamin C Equivalent Antioxidant Capacity (VCEAC) per gram extract.

### 3.7.3 Ferric Reducing Antioxidant Power (FRAP) Assay (Pulido et al., 2000)

The capability of the plant extracts to reduce ferric ions was evaluated through the FRAP assay (Benzie and Strain, 1996). An antioxidant capable of donating a single electron to the ferric-TPTZ (Fe(III)-TPTZ) complex would cause the reduction of this complex into the blue ferrous-TPTZ (Fe(II)-TPTZ) complex which absorbs strongly at 593nm. In the ferric reducing power assay (FRAP), the presence of antioxidants in the sample would result in the reduction of Fe³⁺ to Fe²⁺ by donating an electron. FRAP reagent (900μL), prepared freshly and incubated at 37°C, was mixed with 90μL of distilled water and 30μL of test sample or methanol (for the reagent blank). The test samples and reagent blank were incubated at 37°C for 30m in a water bath. The final dilution of the test sample in the reaction mixture was 1/34. The FRAP reagent was prepared by mixing 2.5mL of 20mM TPTZ in 40mM HCl, 2.5mL of 20mM FeCl₃, 6H₂O and 25mL of 0.3M acetate buffer (pH-3.6). At the end of incubation, the absorbance readings were taken immediately at 593nm against the reagent blank, using a spectrophotometer. Methanolic solutions of known Fe (II) concentration, ranging from 100 to 2000μM, (FeSO₄.7H₂O) were used for the preparation of the calibration curve. The parameter equivalent concentration was defined as the concentration of antioxidant having a
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ferric-TPTZ reducing ability equivalent to that of 1mM FeSO₄.7H₂O. Equivalent concentration was calculated as the concentration of antioxidant giving an absorbance increase in the FRAP assay equivalent to the theoretical absorbance value of a 1mM concentration of Fe (II) solution.

\[
\text{Ferric Reducing Power Assay} = \frac{A_{\text{Control}} - A_{\text{Test}}}{A_{\text{Control}}} \times 100
\]

3.7.4 Superoxide Radical Scavenging Activity (Halliwell and Gutteridge, 1985)

In cellular oxidation reactions, superoxide radical is normally formed first and its effect can be magnified because it produces other kinds of cell-damaging free radicals and oxidizing agents. Superoxide anion radical actively participate in the initiation of lipid oxidation. Oxidation of unsaturated fatty acids in biological membranes leads to formation and propagation of lipid radicals, uptake of oxygen, and rearrangement of the double bonds in unsaturated lipids and eventual destruction of membrane lipids, which produce breakdown products (Acharya et al., 2011). Superoxide is a reactive oxygen species (ROS), which can cause damage to the cells and DNA leading to various diseases. It was therefore proposed to measure the comparative interceptive ability of the antioxidant fractions to scavenge the super oxide radical. In this present study superoxide radicals were generated by auto-oxidation of hydroxylamine in presence of Nitro blue tetrazolium (NBT). Superoxide anions were generated using P:S/NADH system the superoxide anions are subsequently made to reduce nitro blue tetrazolumm, which yields a chromogenic product, which is measured at 560nm. Phenazine methosulphate:nicotinamides adenine dinucleotide (PMS:NADH) system was used for the generation of superoxide anion. About 1ml of nitro blue tetrazolium (156µM), 1ml NADH (468µM) 1N 100mM phosphate buffer of pH 7.8 and 0.1ml of different concentration of sample solution were mixed. The reaction started on the addition of 100µl phenazine methosulfate (60µM). Kept the reaction mixture at 25°C for 5m and absorbance of the mixture was measured at 560nm against blank samples. The percentage inhibition was determined by comparing the result of control and test samples.

\[
\% \text{ of Superoxide radical Scavenging activity} = \frac{A_{\text{Control}} - A_{\text{Test}}}{A_{\text{Control}}} \times 100
\]

Where \(A_{\text{Control}}\) was the absorbance of the control (blank, without extract) and \(A_{\text{Test}}\) was the absorbance with extract.
3.7.5  **H₂O₂ Radical Scavenging Activity (Ruch et al., 1989)**

A solution of H₂O₂ (40mM) was prepared in phosphate buffer (pH 7.4). The water extract at the 100µg/ml concentration in 3.4ml phosphate buffer were added to a H₂O₂ solution (0.6ml, 40mM). The absorbance value of the reaction mixture was recorded at 230nm. Blank solution was containing the phosphate buffer without H₂O₂. Scavenging of water extract and a standard compound was calculated as

\[
\% \text{ Scavenging of } H_2O_2 = \frac{A_{\text{Control}} - A_{\text{Test}}}{A_{\text{Control}}} \times 100
\]

Where \( A_{\text{Control}} \) is the absorbance of the control, and \( A_{\text{Test}} \) is the absorbance in the presence of the sample.

3.7.6  **Hydroxyl Radical Scavenging Activity (Klein et al., 1991)**

An aliquot of 10-40µg of different solvent extracts were added with 1mL of iron-EDTA solution (0.13% ferrous ammonium sulphate and 0.26 % EDTA), 0.5mL of EDTA solution (0.018%), and 1mL of DMSO (0.85 % V/V) in 0.1M phosphate buffer, pH 7.4. The reaction was initiated by adding 0.5mL of ascorbic acid (0.22 %) and incubated at 80-90°C for 15m in a water bath. After incubation the reaction was terminated by the addition of 1mL of ice cold TCA (17.5 % W/V). 3 mL of Nash reagent (75g of ammonium acetate, 3mL of glacial acetic acid and 2mL of acetyl acetone were mixed and raised to 1L with distilled water) was added and left at room temperature for 15m. The reaction mixture without sample was used as control. The intensity of color formed was measured spectrophotometrically at 412nm against reagent blank. The percentage of hydroxyl scavenging activity was calculated by the following formula,

\[
% \text{ HRSA} = 1 - \frac{\text{Difference in absorbance of sample}}{\text{Difference in absorbance of blank}} \times 100
\]

3.8  **Silver Nanoparticle Synthesise and Characterisation**

3.8.1  **Synthesise of Silver Nanoparticle (Harekrishna Bar et al., 2009)**

Optimization and synthesis of silver nanoparticles has done with different concentrations of leaf extracts (5, 10, 15 and 20ml) to different concentrations of Silver nitrate (0.5, 1.0, 1.5 and 2mM) solution at different range of boiling temperatures (40, 60, 80 and 100°C) for different incubation times (20, 40 and 60m). The colour change of the solution was checked periodically. The colour change of the leaf extract from yellow to dark
brown indicated the silver nanoparticles were synthesized from the leaves. Bioreduction of silver ions in the solution was monitored using Genesys 10 UV-VIS spectrophotometer. After cooling, the colored solution was centrifuged at 3000rpm for 5m. The colored supernatant was transferred to a new tube and centrifuged at 12000rpm for 10m. Silver nanoparticles were settled down as pellets. The colorless supernatant was discarded and the pellet was resuspended in sterile distilled water of same amount. The synthesised Silver Nanoparticles from Leaf powder of *E. indica* Servett. (*Ei*-AgNPs) can be used for further experiments.

### 3.8.2 Characterization of Silver Nanoparticles (Harekrishna Bar *et al.*, 2009)

#### 3.8.2.1 UV-Visible Spectroscopy

UV-Visible Spectroscopy is used for monitoring the signature of silver nanoparticles. As it is a powerful tool for the characterization of colloidal particles. Since they exhibit strong surface plasmon resonance absorption in the visible region and are highly sensitive to the surface modification.

#### 3.8.2.2 Fourier Transform Infrared Spectroscopy (FTIR)

The dried silver nanoparticles were subjected to FTIR analysis by Potassium Bromide pellet (FTIR grade) method in 1: 100 ratios and spectrum was recorded in Nicolet Impact 400 FT-IR Spectrophotometer using diffuse reflectance mode.

#### 3.8.2.3 High Resolution Transmission Electron Microscopy (HR - TEM)

TEM is a method of producing images of a sample by illuminating the sample with electronic radiation (under vacuum), and detecting them using Philips CM12 Transmission Electron Microscope. Electrons in the electron gun were accelerated across a potential difference of the order of 100,000V between the cathode and anode. The condenser lens was used to focus the electron beam emerging from the electron gun onto the specimen to permit optimal illuminating conditions for visualizing and recording the image. The specimen image generated by the objective lens was subsequently magnified in one or two more magnification stages by the intermediate and projector lens and projected onto a photographic plate.
3.9 Studies on Anticancer Screening

3.9.1 In vitro Cytotoxicity Studies

3.9.1.1 Trypan blue dye exclusion method (Sheeja et al., 1997)

Trypan blue dye exclusion test is used to determine the number of viable cells present in a cell suspension. It acts as an exclusion test, not penetrating live cells with intact membrane structures (Demirci et al., 2001). It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, Eosin, or propidium, whereas dead cells do not. In this test, a cell suspension is simply mixed with dye and then visually examined to determine whether cells take up or exclude dye. The protocol presented here, a viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm (Shapiro, 1988). Short-term in-vitro cytotoxicity was assessed using Ehrlich Ascites Carcinoma cell lines cells by incubating different concentrations of the ethanolic extracts of the selected plant drugs at 37°C for 3h. The tumor cells were aspirated from peritoneal cavity of tumor bearing mice using an insulin syringe and transferred to a test tube containing isotonic saline. The cells were then washed in normal saline and cell number was determined using a Haemocytometer and adjusted at 10×10^6 cells/ml. For the cytotoxicity assay, different concentrations of the extracts (25-500µg/ml) were added to each tubes and the final volume was adjusted to one ml with normal saline. Control tubes were kept with the saline, tumor cells and without the drugs. All the tubes were incubated at 37°C for 3h. After incubation 0.1ml of 0.4% trypan blue dye in isotonic saline was added to each tube and the number of viable (unstained) and dead (stained) cells were counted using haemocytometer.

\[
\text{% of Dead cells} = \frac{\text{Total Cells Counted} - \text{Total Viable Cells}}{\text{Total Cells Counted}} \times 100
\]

3.9.1.2 MTT ASSAY (Francis and Rita, 1986)

MTT assay is based on the ability of viable cells with active mitochondrial to produce succinate dehydrogenate enzyme which cleave the tetrazolium rings of MTT (Mosmann, 1983) where the optical density (OD) obtained was proportional to the number of healthy viable cells.
Materials and Methods

Cell lines and Culture medium

HT-29 (Human, Colon carcinoma) cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO₂ at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25cm culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

Preparation of Test Solutions

For Cytotoxicity studies, each weighed test samples were separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1mg/ml concentration and sterilized by filtration. Serial dilutions were prepared from this for carrying out cytotoxic studies.

Procedure

The monolayer cell culture was trypsinized and the cell count was adjusted to 1X10⁶ cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO₂ atmosphere, and microscopic examination was carried out and observations were noted every 24h interval. After 72h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3h at 37°C in 5% CO₂ atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540nm. The percentage of growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC₅₀) values is generated from the dose-response curves for each cell line.

\[
\% \text{ of Growth Inhibition} = \frac{\text{Mean OD of Individual test group}}{\text{Mean OD of Control group}} \times 100
\]
3.9.2  *In vivo* Studies

3.9.2.1 Experimental design

Animals were divided into six groups of six mice each. The experimental design given below has been followed for both the ethanol extract of *E. indica* (*EiL-Et*) and green mediated silver Nanoparticles (*EiL-AgNPs*) selected for the study.

3.9.2.2 Experimental Design for Toxicity Studies

Adult Swiss albino mice, weighing about 25-30g (n=6) were used as experimental model. Swiss albino mice were divided into eleven groups. Group I orally administered with water. Group II to Group VI orally treated with *EiL-Et* (600, 1200, 1800, 2400 and 3000mg/Kg BW) and Group VII to Group XI orally treated with *EiL-AgNPs* (600, 1200, 1800, 2400 and 3000μg/Kg BW) for 14 consecutive days. On the 15th day, the animals were sacrificed and blood, samples organs such as liver and kidney collected for further analysis.

Group-I - Normal untreated mice  
Group-II – Normal mice Treated with 600 mg/Kg BW of *EiL-Et*.  
Group-III - Normal mice treated with 1200 mg/Kg BW of *EiL-Et*.  
Group-VI - Normal mice treated with 1800 mg/Kg BW of *EiL-Et*.  
Group-V - Normal mice treated with 2400 mg/Kg BW of *EiL-Et*.  
Group-VI - Normal mice treated with 3000 mg/Kg BW of *EiL-Et*.  
Group-VII - Normal mice treated with 600 μg/Kg BW of *EiL-AgNPs*.  
Group-VIII - Normal mice treated with 1200 μg/Kg BW of *EiL-AgNPs*.  
Group-IX - Normal mice treated with 1800 μg/Kg BW of *EiL-AgNPs*.  
Group-X - Normal mice treated with 2400 μg/Kg BW of *EiL-AgNPs*.  
Group-XI - Normal mice treated with 3000 μg/Kg BW of *EiL-AgNPs*.  

The plant extract administered orally for 14 days at five different dose levels of 600, 1200, 1800, 2400 and 3000 mg/kg BW to Group II, III, IV, V and VI respectively. Group VII, VII, IX, X and XI treated with different dosages (600, 1200, 1800, 2400 and 3000 μg/Kg BW) of green mediated silver naonoparticles from *E. indica*. 
At the end of the experimental period the animals were sacrificed by cervical decapitation. Blood was collected and used for hematological and biochemical estimations. Liver and kidney were dissected out and washed in ice-cold saline and processed for histopathological studies. Liver tissues were homogenized, in 0.1M phosphate buffer, pH 7.4 and used for analyzing various antioxidant and biochemical parameters.

3.9.2.3 Experimental animals

Healthy adult Swiss Albino male mice, weighing 25-35g were obtained from Kovai Medical Research Centre and Hospital, Kovai. The animals were allowed to acclimatize under laboratory conditions for a period of 5 days prior to the experiment. Animals were housed in standard polypropylene cages. Six animals were housed per cage, so as to provide them with sufficient space, and to avoid unnecessary morbidity and mortality. Animals were maintained under standard condition of 12: 12h light/ dark cycle and an ambient temperature at 23 ± 2°C with 65 ± 5 % humidity. Animals were fed with standard rat chow pellet obtained from Sai Durga Foods and Feeds, Bangalore, India and water ad libitum. LD\textsubscript{10} was considered for screening in vivo anticancer potential of E. indica. All the studies were conducted according to the ethical guidelines of CPCSEA after obtaining necessary clearance from the committee.

3.9.2.4 Ehrlich Ascites Carcinoma (EAC)

Since the first description of the Ehrlich ascites tumor in 1932 by Loewenthal and Jahn the ascites tumor has been used comparatively and frequently by investigators for chemotherapeutic studies. The Ehrlich ascites tumor is a useful tool for testing the activity of chemicals and besides the ascites tumor provides an easy challenge to chemotherapeutic agents. Ascites tumor was obtained from the Ehrlich mouse carcinoma, which originated as a tumor of the mammary gland. Intraperitoneal injection of the tumor emulsion produces ascites. Solid tumors were obtained by subcutaneous injection of fresh ascetic fluid containing cancer cells, and ascites tumors were induced by intraperitoneal injection (Kanemasusugiura, 1953).
3.9.2.5 Maintenance of EAC cells (Gothoskar and Ranadive, 1971)

Ehrlich Ascites Carcinoma cells were obtained through the courtesy of Amla Cancer Research Centre, Thrissur and were maintained by weekly intraperitoneal inoculation of 1X10^6 cells/mouse.

3.9.2.6 Experimental Design for Anticancer Studies

Group-I - Normal untreated rats (Control)
Group-II – EAC cell line (1X10^6 cell mouse) (Tumor Control)
Group-III - EAC cell line (1X10^6 cells) treated with 100mg /kg BW of $E_iL$-Et.
Group-IV - EAC cell line (1X10^6 cells) treated with 200mg /kg BW of $E_iL$-Et.
Group-V - EAC cell line (1X10^6 cells) treated with 300mg /kg BW of $E_iL$-Et.
Group-VI - EAC cell line (1X10^6 cells) treated with 100 $\mu$g /kg BW of of $E_iL$-AgNPs.
Group-VII - EAC cell line (1X10^6 cells) treated with 200 $\mu$g /kg BW of $E_iL$-AgNPs.
Group-VIII - EAC cell line (1X10^6 cells) treated with 300 $\mu$g /kg BW of $E_iL$-AgNPs.
Group IX - EAC cell line (1X10^6 cells) treated with standard drug 5- Fluorouracil (20mg/kg. BW).

Treatment with the plant extract started after 24h of EAC inoculation. The $E_iL$-Et administered orally for 14 days at three different dose levels of 100,200 and 300 mg/kg BW to Group III, IV, and V respectively. The $E_iL$-AgNPs administered orally for 14 days at three different dose levels of 100,200 and 300 $\mu$g/kg BW to Group III, IV, and V respectively.

At the end of the experimental period, the Ascites fluid was collected and the animals were sacrificed by cervical decapitation. Blood was collected and used for hematological and biochemical estimations. Liver was dissected out and washed in ice-cold saline and processed for histopathological studies. Liver tissues were homogenized, in 0.1M phosphate buffer, pH 7.4 and used for analyzing various antioxidant and biochemical parameters.
3.9.2.7 Survival Time (Majumdar et al., 1997)

The percentage of increase in life span was calculated as follows.

\[
\text{Increased Life Span (\%)} = \left( \frac{\text{Mean survival time of treated group}}{\text{Mean survival time of control group}} - 1 \right) \times 100
\]

\[
\text{Mean Survival Time} = \frac{\text{Day of first death} + \text{Day of last death}}{2}
\]

3.9.2.8 Body Weight of the Animals

The body weight of the experimental animals was measured by using the rough table top balance. The body weight of the animals was weighed before the introduction of EAC cell lines and during the treatment with plant extracts at an interval of 5 days till the end of the experimental period.

3.9.2.9 Tumor Growth Response (Ramnath et al., 2002)

**Tumor volume**

The mice were dissected and the ascitic fluid was collected from the peritoneal cavity. The volume was measured using a graduated centrifuge tube and packed cell volume was determined by centrifuging at 1000rpm for 5m.

**Tumor cell count**

The ascitic fluid was taken in a WBC pipette and diluted 100 times. Then a drop of the diluted cell suspension was placed on the Neubaur counting chamber and the numbers of cells in the 64 small squares were counted.

**Viable/non-viable tumor 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 treated as viable and those that took up the stain were treated as nonviable. Thus viable and nonviable cells were counted.

\[
\text{Cell Count} = \frac{\text{No. of Cells} \times \text{Dilution}}{\text{Area} \times \text{Thickness of liquid film}}
\]
3.9.2.10  **Determination of Haemoglobin**

Hemoglobin content was estimated by the method of Wintrobe *et al.*, (1961). The Sahli’s pipette was filled upto 20 mark with 0.1N hydrochloric acid. Then 20μl of blood was added and mixed well. The mixture was allowed to stand at room temperature for 10m. The mixture was diluted with distilled water until the colour matches with the standard which was given in the sahil’s hemoglobinometer. The level of hemoglobin was expressed as g(%).

3.9.2.11  **Determination of Red Blood Cells Count**

RBC counts were determined by the method of Armour *et al.*, (1965). 20μl of blood was taken in the RBC pipette. It was diluted upto the mark with RBC diluting fluid. The dilution of blood was 200 times. The Hemocytometer was cleaned with cotton and the counting chambers were observed under microscope. Then the counting chamber was filled with diluted blood and placed on stage. The number of Red blood cells were counted and recorded. The result was expressed as millions of cells/mm.

3.9.2.12  **Determination of White Blood Cells Count**

WBC counts were determined by the method of Wintrobe *et al.*, (1961). 20μl of blood was taken in the WBC pipette. It was diluted upto the mark with WBC diluting fluid. The dilution of blood was 20 times. The Hemocytometer was cleaned with cotton and the counting chambers were observed under microscope. Then the counting chamber was filled with diluted blood and placed on the stage of the microscope. The number of white blood cells were counted and recorded. The counted white cells were calculated and the result was expressed as cells/mm.

3.9.2.13  **Estimation of Blood Glucose (Folin and Wu, 1919)**

0.1ml of the plasma was added to 3.4ml of water, 0.2ml of 10% sodium tungstate and 0.2ml of 2/3N sulphuric acid in order to precipitate the protein. Mixed well and centrifuged. To 1ml of the filtrate added 2ml of alkaline copper sulphate solution and placed in a boiling water bath for 8m, cooled and added 2ml of phosphomolybdic acid. Various concentrations of standard solution was taken and was made up to 1ml with water and added 2ml of alkaline copper sulphate solution and placed in a boiling water bath for 8m, cooled and added 2ml of phosphomolybdic acid. A Blank was also maintained. The blue colour, developed was read at 620nm. The amount of glucose was estimated using a standard graph. The glucose content was expressed as mg/dl.
3.9.2.14 Estimation of Protein

Serum protein content was estimated by the method of Lowry et al., (1951). 0.1ml of diluted serum (0.1ml of serum diluted to 20ml) was made up to 1.0ml with water and 4.5ml of alkaline copper reagent was added to all the tubes including blank, containing 1.0ml water and standards containing aliquots of working standard BSA (0.1 to 0.5ml) and made up to 1ml with water. The tubes were incubated for 10m at room temperature. 0.5ml of Folin’s Phenol reagent was added to all the tubes and incubated for 20m at room temperature. The blue colour developed was read at 640nm. From the absorbance the amount of protein was calculated using a standard graph. The protein content was expressed as g/dl.

3.9.2.15 Estimation of Serum Urea

Urea was estimated by the Diacetyl monoxime method (Natelson et al., 1951). To 0.1ml of blood 3.3ml of distilled water, 0.3ml of 10% sodium tungstate and 3.3ml of 2/3 N H₂SO₄ were added and centrifuged for few minutes at 3000rpm. To 2ml of the supernatant 2ml of distilled water, 0.4ml of DAM reagent and 1.6ml sulphuric and phosphoric acid reagent were added. Various concentrations of standards (20-100µg/ml) were added with 0.4ml of DAM and 1.6ml of sulphuric and phosphoric acid reagent. The tubes were incubated in a boiling water bath for 20m. The pink colour developed was read at 480nm. The amount of urea was calculated using a standard graph. The amount of urea was expressed as mg/dl.

3.9.2.16 Estimation of Serum Uric Acid

Serum uric acid level was estimated by the method of Caraway and Seligson (1963). To 0.5ml of serum added 2.0ml of 10% sodium tungstate, 2.0ml of 2/3N sulphuric acid. Mixed well and centrifuged the contents. 3.0ml of filtrate was taken and added 0.6ml of sodium bicarbonate and 0.6ml of phosphotungstate reagent. A blank was taken consisting of 3ml of water and treated similarly. Various concentrations of standards were also added with 0.6ml sodium carbonate and 0.6ml of Phosphotungstate reagent. The tubes were kept in room temperature for 30m. The color developed was read at 700nm. The amount of uric acid was calculated using standard graph. The values were expressed as mg/dl.
3.9.2.17  **Estimation of Serum Bilirubin**

Serum bilirubin was estimated in serum by the method of Malloy and Evelyn, (1937). For the determination of total bilirubin 0.2ml of serum was taken and made up to 2ml with water. Then added 0.5ml of diazo reagent and 2.5ml of methanol. Various concentration of standards were made up to 1ml. To the blank added 2ml with water, 0.5ml of diazo blank and 2.5ml methanol. The colour developed was read at 540nm. From the absorbance the amount of bilirubin was calculated using a standard graph. The values were expressed as mg/dl.

3.9.2.18  **Estimation of Creatinine**

The colorimetric determination of Creatinine by the Jaffe reaction (Bonsnes and Taussky, 1945). The series of test tubes with various concentration of standard solution was taken and a volume was made up to 3ml with distilled water. For the test 2ml of serum was added to 2ml of sulphuric acid and 2ml of sodium tungstate and centrifuged. From that 3ml was taken. Then added 1ml of picric acid and 1ml of sodium hydroxide to all tubes are heated in a boiling water bath for 15m. The colour developed was read at 500nm. The values were expressed as mg/dl.

3.9.2.19  **Estimation of Liver Glycogen**

Hepatic glycogen was estimated by the method of Morales et al., (1973). A known amount of the liver tissue was subjected to alkali digestion in a boiling water bath for 20m after adding 5ml of 30% KOH. The tubes were cooled and 3ml of absolute ethanol and a drop of ammonium acetate were added. The tubes were then placed in a freezer overnight to precipitate glycogen. The precipitated glycogen was collected after centrifugation at 3000g for 10m. The precipitate was washed thrice with alcohol and dissolved in 3ml of water. Various concentrations of standard aliquotes were taken and made up to 1ml with water. 4ml of anthrone was added to the tubes kept in an ice bath, mixed and heated in a boiling water bath for 20m. The green colour developed was read at 640nm. The amount of glucose was calculated using standard graph. The factor 0.93 was used to convert glucose to glycogen. The values were expressed as mg/g tissue.
3.9.2.20 Extraction of Lipids

Tissue lipids were extracted by the method of Folch et al. (1970). A known volume of tissue suspension was mixed (2:1 V/V) with 10ml of chloroform methanol mixture and homogenized. The homogenate was filtered through Whatmann filter paper (No.42) into a separating funnel. The filtrate was mixed with 0.2ml of physiological saline (0.89%) and the mixture was kept overnight undisturbed. The lower phase containing the lipid extract was drained off into pre weighed beakers. The upper phase was re-extracted with more chloroform-methanol mixture and the lower phase was collected. The lipid extracts were re-dissolved in 1.0ml of chloroform- methanol mixture and aliquots were used for estimation of various lipid components such as Cholesterol, phospholipids, Triglycerides and Free fatty acids.

3.9.2.21 Estimation of Serum / Tissue Cholesterol

Cholesterol was estimated in serum and tissue by the method of Parekh and Jung (1970). To 0.1ml of the lipid extract (tissue / serum), 2.9ml of uranyl acetate reagent and 2ml of sulphuric acid –ferrous sulphate reagent were added and mixed well. Blank comprised of uranyl acetate reagent and 2ml of sulphuric acid –ferrous sulphate reagent. A calibration curve was prepared using the standard cholesterol. The optical density was measured after 20m at 560nm. From the absorbance the cholesterol content was calculated using a standard graph. The cholesterol content was expressed as mg/dl serum or mg/100g wet tissue.

3.9.2.22 Estimation of Serum / Tissue Triglycerides

Triglycerides were estimated in serum and tissue by the method of Foster and Dunn (1973). 4.0ml of isopropanol was added to 0.1ml of lipid extract (tissue / serum) and mixed well, followed by 0.4g of alumina and shaken well for 15m. Centrifuged at 2000rpm for 10m and then 2.0ml of the supernatant was transferred to appropriately labeled tubes. The tubes were placed in a water bath at 65° C for 15m for saponification after adding 0.6ml of the saponification reagent. After cooling, 1.0ml of Sodium metaperiodate was added followed by 0.5ml of acetyl acetone reagent. After mixing, the tubes were kept in a water bath at 65° C for half an hour. The contents were cooled and read at 430nm. From the absorbance the triglyceride content was calculated using a standard graph. The triglyceride content was expressed as mg/dl serum or mg/100g wet tissue.
3.9.2.23 Assay of Serum HDL Cholesterol

Serum HDL was estimated by the method of Friedewald et al., (1972). To 1.0ml of plasma, 0.18ml of heparin manganese chloride reagent was added and mixed. This was allowed to stand in an ice bath for 30m and then centrifuged in a refrigerated centrifuge at 2500g for 30m. The supernatant contained HDL fraction. Aliquots of the HDL supernatant were estimated for cholesterol, phospholipids and triacylglycerol. The HDL cholesterol content was expressed as mg/dl serum.

Aggregation of VLDL

Sodium dodecyl sulphate (SDS) – 10% in 0.15M NaOH (pH – 9)

1ml of plasma was added to 0.15ml of SDS solution. The contents were mixed well and incubated at 37°C for 2h. The contents were centrifuged in a refrigerated centrifuge at 10,000g for 30m. VLDL aggregated as a pellicle at the top. The supernatant was a mixture containing HDL and LDL fractions. The fractions of lipoproteins were assayed after heparin manganese chloride and SDS precipitation. The values were expressed in mg/dl plasma. After precipitation the cholesterol levels in supernatant was measured to get HDL cholesterol. SDS precipitated VLDL and the cholesterol content in the supernatant was measured for HDL cholesterol, LDL cholesterol and VLDL cholesterol.

\[
\text{LDL Cholesterol} = \frac{(\text{Total Serum Cholesterol}) - (\text{Total Serum TGL}) - (\text{HDL Cholesterol})}{5}
\]

\[
\text{VLDL Cholesterol} = \frac{(\text{Total Serum TGL})}{5}
\]

3.9.2.24 Estimation of Alanine Transaminase (ALT) [EC 2.6.1.2]

The serum ALT levels were assayed using the method of King (1965). The assay mixture containing 1ml of substrate (Alanine) and 0.2ml of serum was incubated for 1h at 37°C. Added 1ml of DNPH and kept at room temperature for 20m. Various concentrations of pyruvate was used as standard. Serum was added to the control tubes after the reaction was arrested by the addition of 1ml of DNPH. Added 5ml of 0.4N NaOH and the colour developed was read at 540nm. The Levels of ALT was expressed as U/L.
3.9.2.25  Assay of Aspartate Transaminase (AST) [EC 2.6.1.1]

The serum AST levels were assayed using the method of King (1965). The assay mixture containing 1ml of substrate (Aspartate) and 0.2ml of serum was incubated for 1h at 37ºC. Added 1ml of 0.2% DNPH in 1N HCl and kept at room temperature for 30m. Various concentrations of Pyruvate were used as standard. Serum was added to the control tubes after the reaction was arrested by the addition of 1ml of DNPH. Added 5ml of 0.4N NaOH and the colour developed was read at 540nm. The Levels of AST was expressed in U/L.

3.9.2.26  Estimation of Serum Alkaline Phosphatase [EC3.1.3.1]

The serum ALP levels were assayed using the method of King (1965). The reaction mixture containing 1.5ml carbonate buffer, 1ml Di sodium phenyl phosphate, 0.1ml Magnesium Chloride and 0.1ml of serum was incubated at 37ºC for 15m. The reaction was arrested by the addition of Folin’s Ciocalteau phenol reagent. Control tubes were also treated similarly but serum was added after the reaction was arrested with Folin’s phenol reagent and then added 1ml of Sodium Carbonate. Various concentrations of Phenol were used as standard. The colour developed was read after 10m at 640nm. The activity of ALP was expressed as U/L.

3.9.2.27  Assay of Gamma- Glutamyl Transferase [EC 2.3.2.2]

The serum GGT levels were assayed using the method of Rosalki and Rau (1972). The incubation mixture contained 0.5ml of substrate, 1ml of Tris HCl, 2.2ml of Glycyl glycine, 0.2ml of homogenate. The total volume was made upto 4ml with water. After incubation for 30m at 37ºC the samples were heated at 100ºC for 5m and centrifuged. The amount of p-nitroaniline in the supernatant was measured at 410nm. The Levels of GGT was expressed as U/L.

3.9.2.28  Assay of Lactate Dehydrogenase (LDH) (EC 1.1.1.27)

The enzyme level was assayed according to the method of King (1965). To a set of tubes, 1.0ml of the buffered substrate and 0.1ml serum were added and the tubes were incubated at 37ºC for 15m. After adding 0.2ml of NAD solution, the incubation was continued for another 15m. By adding 0.1ml of DNPH reagent the reaction was arrested, and the tubes were incubated for a further period of 15m at 37ºC. 0.1ml of serum was added to blank tubes after arresting the reaction with DNPH. 7.0ml of 0.4N sodium hydroxide solution was added and the color developed was measured at 420nm in a Systronics 119UV
Spectrophotometer. Suitable aliquots of the standards were also analyzed by the same procedure. The enzyme activity was expressed U/L.

### 3.9.2.29 Extraction of Nucleic Acids

The nucleic acids extraction was done as advocated by Schneider et al., (1957). Known amount of Liver tissue were homogenised in 5.0ml of ice-cold distilled water. 5.0ml of 5% TCA was added to the homogenate and this was kept in ice for 30m to allow complete precipitation of proteins and nucleic acids. The mixture was centrifuged and the precipitate obtained was washed thrice with ice cold 10% TCA. Then it was treated with 95% ethanol to remove lipids. The final precipitate was heated at 90°C for 15m with occasional shaking, which facilitated the quantitative separation of nucleic acids from protein. The supernatant after centrifugation was used for the estimation of DNA and RNA.

### 3.9.2.30 Estimation of Deoxy Ribonucleic Acid (DNA)

DNA was estimated as according to the method of Burton (1956). A known volume of the nucleic acid extract was made upto 3.0ml with 1N perchloric acid. This was mixed with 2.0ml of diphenylamine reagent. A reagent blank and standards were also carried out concurrently. This was kept in a boiling water bath for 10m and the blue colour developed was read at 640nm. The amount of DNA was calculated using the standard graph. The DNA level was expressed as mg/g wet tissue.

### 3.9.2.31 Estimation of Ribonucleic Acid (RNA)

RNA was estimated by the method of Rawal et al., (1977). Aliquots of nucleic acid extracts were made upto 2.0ml with 5% TCA. To this 3.0ml of orcinol-ferric chloride reagent was added and mixed well. The tubes were heated in a boiling water bath for 20m. Reagent blank and standards were also treated in the same way. The tubes were cooled and the colour developed was measured at 640nm. The amount of RNA was estimated using the standard graph. The RNA level was expressed as mg/g wet tissue.
3.9.2.32 Estimation of Protein Bound Hexose

Protein bound hexose was estimated by method of Niebes (1972). Neutralized sample was obtained by centrifuging blood at 5000rpm. Separated serum was precipitated with alcohol. The alcoholic precipitate was hydrolyzed with 2ml of 4N Hydrochloric acid at 100°C for 4h. The hydrolyzed material was neutralized with Sodium hydroxide. To 0.5ml of neutralized sample, 0.5ml of water and various concentrations of standards were added made upto 1ml with water. 2.0ml of Orcinol-Sulphuric acid reagent was added to all the tubes very slowly by keeping the tubes on ice. The tubes were heated at 80°C for 15m. The tubes were cooled in an ice-bath and colour intensity was read at 540nm. From the absorbance the amount of hexose calculated using standard graph. The amount of hexoses was expressed as mg/dl serum or mg/g of dry defatted tissue.

3.9.2.33 Estimation of Protein Bound Hexosamine

Protein bound hexosamine was estimated by the method of Wanger (1972). Neutralized sample was obtained by centrifuging blood at 5000rpm. Separated serum was precipitated with alcohol. The alcoholic precipitate was hydrolyzed with 2ml of 4N Hydrochloric acid at 100°C for 4h. The hydrolyzed material was neutralized with Sodium hydroxide. To 0.8ml of the neutralized sample, 0.6ml of acetyl acetone reagent was added. Blank contain 0.8ml of water. The tubes were heated in a boiling water bath for 30m. The tubes were cooled and 2.0ml of Ehrlich reagent was added. All the tubes were shaken well and colour developed was read at 540nm. The amount of hexosamine was calculated using a standard graph. The amount of hexosamine was expressed as mg/dl serum or mg/g of dry defatted tissue.

3.9.2.34 Estimation of Fucose

The Fucose was estimated by the method of Dishce and Hettles (1948). 0.1ml of serum as well as tissue homogenate was precipitated with ethanol. The precipitate was dissolved in 1ml of 0.1N sodium hydroxide. 1ml of water and various concentrations of fucose were treated as blank and standard. All the tubes were placed in an ice bath and added 4.5ml of cold sulphuric acid water reagent. Then the tubes were kept in a boiling water bath for 30m. 0.1ml of 3% cysteine hydrochloride was added. The tubes were incubated in dark at room temperature for 75m. The colour developed was read at 396nm. The amount of fucose
was calculated using a standard graph and expressed as mg/dl serum or mg/g of dry defatted tissue.

3.9.2.35 Estimation of Sialic Acid

The amount of Sialic acid was estimated by the method of Warren (1959). Alcoholic precipitate of serum and tissue homogenate was hydrolyzed with 2ml of 0.1N sulphuric acid for 1h at 90°C. The hydrolyzed sample was used for estimation of Sialic acid. To 0.2ml of hydrolyzed sample, 0.2ml of sodium metaperiodide reagent was added and kept at room temperature for 30m. Then 0.2ml of sodium arsenate was added and shaken well added 3.0ml of thiobarputric acid to all the tubes and was heated in a boiling water bath for 15m. The tubes were cooled and 5.0ml of acidified butanol was added to all tubes. The absorbance of pink butanolic phase after centrifugation was read at 540nm. The amount of Sialic acid was calculated using a standard graph. The amount of Sialic acid was expressed as mg/dl serum or mg/g of defatted tissue.

3.9.2.36 Estimation of Lipid Peroxides

Lipid peroxide content was estimated by the method of Ohkawa et al., (1979). 0.1ml of tissue homogenate was mixed with 4ml of 0.85N H₂SO₄ and mixed gently. 0.5ml of 10% phosphotungstic acid was added and stirred well. The contents were centrifuged for 10m. The supernatant was discarded and the sediment mixed with 2.0ml of 0.85N H₂SO₄ and 0.3ml of 10% phosphotungstic acid. The mixture was centrifuged for 10m. The sediment was suspended in 4.0ml of distilled water and 1ml of TBA reagent. The tubes were kept in a boiling water bath for 1h. After cooling 5ml of butanol was added to each tube and the colour extracted in the butanol phase was read at 532nm. The lipid peroxide content was expressed as nanomoles of malondialdehyde formed/mg tissue.

3.9.2.37 Assay of Glutathione Peroxidase (GPx) (EC 1.11.1.9)

The assay of glutathione peroxidase was carried out using the method of Rotruck et al., (1973). The reaction mixture consisted of 0.2ml each of EDTA, sodium azide, H₂O₂, 0.4ml phosphate buffer and 0.1ml tissue (liver) homogenate. The mixture was incubated at 37°C at different time intervals. The reaction was arrested by adding of 0.5ml of TCA and the tubes were centrifuged at 2000rpm. To 0.5ml of supernatant, 4ml of disodium hydrogen phosphate and 0.5ml of DTNB were added and the colour developed was read at 420nm immediately. The levels were expressed as U/mg of protein.
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3.9.2.38 Assay of Reduced Glutathione

Reduced Glutathione was estimated using the method of Moron et al., (1979). One ml of tissue (Liver) homogenate was precipitated with 1ml of TCA and the precipitate was removed by centrifugation. To 5ml of the supernatant added 2ml of DTNB and the total volume was made up to 3ml with phosphate buffer. The absorbance was read at 412nm. The level of glutathione was expressed as nmol of mg/g of wet tissue.

3.9.2.39 Assay of Superoxide Dismutase (EC 1.15.1.1)

The assay of SOD was carried out using the method of Misra and Fridovich (1972). 0.1ml of tissue (Liver) homogenate was added to tubes containing 0.75ml ethanol and 0.15ml chloroform (chilled in ice) and centrifuged. To 0.5ml of supernatant added 0.5ml EDTA solution and 1ml of buffer. The reaction was initiated by the addition of 0.5ml of epinephrine and the increase in absorbance was measured at 480nm. The enzyme level was expressed as U/mg protein.

3.9.2.40 Assay of Catalase (EC 1.11.1.6)

Catalase was assayed by the method of Maehly and Chance (1954). 0.05ml of tissue (Liver) homogenate was added to 1.2ml of phosphate buffer. To this 1.0ml hydrogen peroxide was added to initiate the enzyme reaction. The decrease in the absorbance was measured at 620nm at 30seconds interval for 3m. The enzyme blank was run simultaneously with 1.0ml of distilled water instead of hydrogen peroxide. The level of catalase was expressed as U/mg of protein.

3.10 Histological Studies (Sujai, 1993)

Histopathology is the microscopic study of the tissues affected by disease. The technique deals with the preparation of tissues for microscopic examination. The various steps involved in the preparation of tissue for histological examination are (i) Fixation (ii) Dehydration (iii) Clearing (iv) Impregnation (v) Embedding (vi) Cutting and Sectioning (vii) Staining in H&E stains (Harris et al., 1987) (viii) Mounting in DPx (ix) observation under microscope.
3.11 Isolation and Prediction of Marker Compound (Vvedenskaya et al., 2004)

The isolation and purification of the bioactive components were achieved from 2g of ethanol extract of *E. indica* to a Sephadex LH-20 column. The ethanol extract (2g) was dissolved in a small amount of hexane for transfer to column chromatography system pre-packed with sephadex LH-20 (column size 100mm \( \times \) 45mm) on silica gel using solvents of increasing polarities starting from hexane, chloroform, ethylacetate, ethanol and isopropanal in different ratios to yield several fractions. The fractions were monitored by TLC on silica gel plates developed with the solvent system. The column was subsequently eluted with as follows as: Hexane (100%), Hexane –Chloroform (90:10 upto 10:90), Chloroform (100%), Chloroform–Ethylacetate (90:10 upto 10:90), Ethylacetate (100%), Ethylacetate –Isopropanol (90:10 upto 10:90), Isopraponal (100%).

The mixtures of decreasing polarities are yielding different fractions. The fractions were monitored by TLC on silica gel plates developed with the solvent system: Chlroform: Ethylacetate (90:10). The spots were detected using Iodine reagent and visualized under UV light at 254 and 365nm. The fractions eluted from Chloroform (100%) were collected. The fraction was further characterized by using FT-IR, NMR (\(^1\)H-NMR & \(^{13}\)C-NMR) and GC-MS studies.
3.12 Bioinformatic Studies

3.12.1 Docking

3.12.1.1 Protein preparation

AutoDock is a suite of automated docking tool. It is designed to predict how small molecules, such as substrates or drug candidates, bind to a receptor of known 3D structure (Scott et al., 1999). The protein add with Kollmann charges were assigned. Through which hydrogens were added, side chains were optimized for hydrogen bonding. The energy minimized protein was then saved in PDB format. Using MGLTools-1.4.6 nonpolar hydrogens were merged, AutoDock atom type AD4 and Gasteiger charges were assigned and finally saved in protein.pdbqt format (Morris et al., 1998).

3.12.1.2 Ligand preparation

Structure of ligands were drawn using ChemSketch, optimized with 3D-geometry and the two-dimensional structure of plant compound are converted into 3-D structure using the Babel format molecule converter (Guha et al., 2006) and saved in PDB format for AutoDock compatibility. MGLTools-1.4.6 (The Sripps Research Institute) was used to convert ligand.pdb files to ligand.pdbqt files.

3.12.1.3 Active site prediction

The active site of the protein is the binding site or usually a pocket at the surface of the protein that contains residues responsible for substrate specificity which often act as proton donors or acceptors. Identification and characterisation of binding site is the key step in structure based drug design. The binding site has been identified by computational and literature reports. The active site region of the protein is identified by Qsite (Alasdair et al., 2005). These servers analytically furnish the area and the volume at the probable active site of each pocket to envisage the binding site.

3.12.1.4 Docking protocol

Grid parameter files (protein.gpf) and docking parameter files (ligand.dpf) have written using MGLTools-1.4.6. Receptor grids were generated using 90x60x60 grid points in xyz with grid spacing of 0.375Å. Grid box was centered co crystallized ligand map types were generated using autogrid. Docking of macromolecule was performed using an empirical free energy function and Lamarckian Genetic Algorithm, with an initial population of 250
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randomly placed individuals, a maximum number of 106 energy evaluations, a mutation rate of 0.02, and a crossover rate of 0.80. One hundred independent docking runs were performed for each ligand. Results differing by 2.0Å in positional root-mean square deviation (RMSD) were clustered together and represented by the result with the most favorable free energy of binding. It is expressed in the units- kcal/mol.

3.13 Statistical Analysis

All the results were expressed as Mean±SE. The data were statistically analyzed by “t” test. One way analysis of ANOVA was carried out to compare the significant differences between the groups. The significant values at $P < 0.05$, $P < 0.01$, $P < 0.001$ were considered as significant, more significant and most significant respectively.

IC$_{50}$ values of In vitro antioxidant studies and cytotoxicity studies were determined by using GraphPad Prism 6.0 (Trail Version).