I. COLLECTION OF PLANT MATERIALS

The three different species of the genus *Morus* viz. *M. alba* (IC573059), *M. serrata* (EC493838), and *M. laevigata* (IC313789) were collected from Mulberry Germplasm Center, Central silk board, Govt. of India, Hosur, Tamil Nadu, India. Leaves were subjected for shade drying consequently to extraction procedures and the extracts were used for the phytochemical and pharmacological activities.

II. EXTRACTION OF PLANT MATERIALS

Fresh leaves of the plant (2kg) were collected, washed and dried under shade separately. Later, it was milled into coarse powder by mechanical grinder, sieved and subjected to extraction procedures. The dried powder was successively extracted with petroleum ether, chloroform and methanol in batches of 200g each in the Soxhlet extractor (50 cycles for each batch). After each extraction the marc was air-dried. The solvent was removed under reduced pressure using rotary vacuum evaporator and the extracts were stored in a desiccator. All these extracts were used for phytochemical investigations and pharmacological screening for various activities.

III. PHYTOCHEMICAL ANALYSIS

A. QUALITATIVE PHYTOCHEMICAL INVESTIGATION

The preliminary qualitative phytochemical studies were performed for testing the different chemical groups present in petroleum ether, chloroform and methanol extracts of three different plants (Trease and Evans, 1978; Kokate *et al.* 1990; Khandelwal, 2006).

Requirements

**Chemicals:** Distilled water, ethanol, chloroform, gelatin, sulfuric acid, hydrochloric acid, sodium carbonate, sodium chloride, sodium hydroxide, sodium nitroprusside, sodium picrate, sulphuric acid, lead acetate solution,
MATERIALS AND METHODS

Mayer’s reagent, Millon’s reagent, magnesium ribbon, neutral ferric chloride solution etc. (sd fine-chem Ltd, Mumbai / Himedia laboratories, Mumbai).

Glasswares: Beakers, conical flasks, glass rods, test tubes etc.

Apparatus: Burner, test tube holder etc.

1. Test for steroids
   i. Salkowski reaction
      
      To 2ml of extract add 2ml chloroform and 2ml conc. H$_2$SO$_4$. Shake well. Chloroform layer appears red and acid layer shows greenish yellow fluorescence.

   ii. Liebermann-Burchardt test
      
      To the chloroform solution (2mg in 1ml) added a few drops of acetic anhydride and mixed well. To this was added 1ml of concentrated sulfuric acid through the sides of the test tube; appearance of first red, then blue and finally green color indicated the presence of sterols.

   iii. Sulphur test
      
      Sulphur when added to the test solution, it sinks to the bottom indicating the presence of sterols.

2. Tests for triterpenes
   i. Salkowski test
      
      A few drops of concentrated sulphuric acid was added to the chloroform solution (2mg in 1ml), shaken and allowed to stand; the appearance of golden yellow color indicated the presence of triterpenes.

   ii. Liebermann–Burchardt’s test
      
      A few drops of acetic anhydride was added to the chloroform solution (2mg in 1ml) and mixed well. To this was added 1ml of concentrated sulphuric acid through the sides of the test tube; the appearance of red ring indicated the presence of triterpenes.
Test for alkaloids

The various fractions (10mg each) were basified with ammonia and extracted with chloroform. The chloroform solution was acidified with dilute hydrochloric acid. The acid layer was used for testing alkaloids.

i. *Wagner’s test (Solution of iodine in potassium iodide)*

The acid layer was treated with a few drops of Wagner’s reagent; the appearance of reddish brown precipitate indicated the presence of alkaloids.

ii. *Mayer’s test (Potassium mercuric iodide solution)*

The acid layer was treated with a few drops of Mayer’s reagent; the appearance of creamy white precipitate indicated the presence of alkaloids.

3. Test for phenolic compounds and flavonoids

i. *Ferric chloride test*

A few drops of neutral ferric chloride solution was added to a little quantity of extract; the formation of blackish green color indicated the presence of phenolic nucleus.

ii. *Shinoda test*

A few fragments of magnesium ribbon and 1ml of concentrated hydrochloric acid were added to the alcoholic solution of extract; the appearance of magenta color after few minutes indicated the presence of flavonoids.

iii. *Lead acetate test*

To small quantity of the extract solution add lead acetate solution and observation was done for yellow colored precipitation.

iv. *Alkaline reagent test*

Addition of increasing amount of sodium hydroxide to the extract solution shows yellow coloration, which decolorizes after the addition of acid.
4. Test for tannins

   To 2-3ml of the extract, add few drops of following reagents;
   
   i. *Ferric chloride test*

   A few drops of 1% neutral ferric chloride solution was added to the extract; the formation of blue, green or brownish green color indicated the presence of tannins.

   ii. *Gelatin test*

   The extracts were treated with 1% solution of gelatin containing 10% sodium chloride; the formation of white precipitate indicated the presence of tannins.

5. Test for glycosides

   i. *Baljet test*

   The test solution when treated with sodium picrate gives yellow to orange color.

   ii. *Keller-killiani test*

   To 2ml extract add glacial acetic acid, one drop 5% FeCl₃ and conc. H₂SO₄. Reddish brown color appears at junction of two liquid layers and upper layer appears bluish green.

6. Test for saponins

   *Foam test*

   In a test tube containing about 5ml of the extract, a drop of sodium bicarbonate solution was added. The test tube was shaken vigorously and left for 3 minutes. Formation of honeycomb like froth indicated the presence of saponins.
B. ISOLATION AND CHARACTERIZATION OF THE BIOACTIVE CONSTITUENTS

1. COLUMN CHROMATOGRAPHY
   i. Standardization of mobile phase
      Column chromatography is one of the most useful methods for the separation and purification of both solids and liquids when carrying out small-scale experiments. Column chromatography is widely used to isolate the component of complex mixture (preparative chromatography). Column chromatography can also be used to determine the quantity of different compounds in the mixture (analytical chromatography). It is a solid-liquid separation technique which has a solid stationary phase and a liquid mobile phase. The theory of column chromatography is analogous to that of thin layer chromatography. Various stationary phases as well as mobile phases are used to separate compounds either according to polarity (normal and reverse phase, silica gel, sephadex) or size of the compounds (Toyopearl-size-exclusion chromatography) and solvents alone or in different combinations.

      In the present research work, in order to select the best mobile phase for eluting the individual fraction, 5μL of a 100mg/ml solution was spotted on TLC and run with different combination of solvents with increasing polarity to get single spot. The active compounds can be detected using many methods viz. UV-active compounds were detected using UV chamber and non UV visible compounds were detected by iodine chamber. In this way the solvent system that exhibited the most favorable separation of compounds was chosen.

   ii. Column packing
      Glass column with 45cm long with diameter of 2.5cm was used for separation of constituents from the active extracts. Small plug of cotton was placed at the bottom of the column and approximately 40gms of silica gel (Merck, 60-120 mesh size) activated for 24h at 80°C in an oven. The slurry was prepared using chloroform and was transferred into the column using a long stirring glass rod covering \( \frac{3}{4} \)th of the column. It was then filled with eluting
solvent. Conical flask was placed under the column and allowed the solvent to flow freely for the rest of the experiment.

### iii. Loading the extract to the column

The eluting solvent was drained to approximately 1cm above the top of the silica bed. Using a long pipette, extract was transferred to the top of the silica bed making sure not to squirt the mixture onto the inner sides of the column. Later, mixture was allowed to adsorb on the top of the silica gel before adding more elution solvent. Whatmann filter paper was cut to the size of column diameter and inserted at top end of the stationary phase to prevent disruption during addition of solvent. Once addition of solvent to the column was started, the solvent level was maintained not to go below the top of the silica bed. The elutent, instead of rising by capillary action up a thin layer, flows down through the column filled with the adsorbent. Just as in TLC, there is an equilibrium established between the solute adsorbed on the silica gel and the eluting solvent flowing down through the column.

### iv. Monitoring the column with TLC

The eluted fraction was monitored by comparative TLC of extract and column eluted fraction continuously. TLC was carried out on glass slides coated with 0.25mm silica (Merck, Silica gel for TLC). Using capillary tube, approximately 5μl column eluted fraction was loaded on silica gel 3mm above from the bottom. Fractions with similar TLC pattern were pooled together and concentrated at reduced pressure and temperature. The concentrated components were further dried in vacuum desiccator. Completely dried components were weighed to calculate the total mass extracted and the extracted compound was numbered tentatively with a prefix AD.

### v. Isolation and characterization of constituent from *Morus laevigata* methanol fraction

The methanol fraction of *M. laevigata* was chromatographed over silica gel (Merck, 60-120 mesh size) column, eluted with 100% ethyl acetate. The collected elutent from column chromatography was monitored by TLC,
fractions with similar TLC patterns were combined to yield single spot and labeled as AD5. The latter was kept for the evaporation of solvent and condensed compound was stored using micro centrifuge tubes in a desiccator. The isolated compound, AD5 was used for structure prediction based on IR, $^1$H-NMR and Mass Spectral analyses.

vi. **Isolation and characterization of constituent from *Morus laevigata* petroleum ether fraction**

The petroleum ether fraction of *M. laevigata* was chromatographed over silica gel (Merck, 60-120 mesh size) column, eluted with a mixture of petroleum ether and ethyl acetate (4:6 petroleum ether and ethyl acetate). The collected eluent from column chromatography was monitored by TLC, fractions with similar TLC patterns were combined to yield single spot and labeled as AD6. The latter was kept for the evaporation of solvent and condensed compound was stored using micro centrifuge tubes in a desiccator. The structure of the isolate was determined using IR, $^1$H-NMR and Mass Spectral analyses.

vii. **Isolation and characterization of constituent from *Morus alba* methanol fraction**

The methanol fraction of *M. alba* was chromatographed over silica gel (Merck, 60-120 mesh size) column, eluted with a mixture of methanol and ethyl acetate (6:4 methanol and ethyl acetate) which yielded AD11 and further with a mixture of ethyl acetate and petroleum ether (7:3 ethyl acetate and petroleum ether) which yielded AD13 respectively. The collected elutents from column chromatography were monitored by TLC, fractions with similar TLC patterns were combined. The latter was kept for the evaporation of solvent and condensed compound was stored using micro centrifuge tubes in a desiccator. The structure of the isolate was determined using IR, $^1$H-NMR and Mass Spectral analyses.
2. Characterization of the isolated constituent

The characterizations of the isolated compounds are done using infrared (IR), mass (LCMS) and proton nuclear magnetic resonance (\(^1\)H NMR) spectroscopy at Indian Institute of Sciences, Bangalore. All the spectral data were incorporated in the result section, so as to deduce the structure of the compound.

IV. SCREENING FOR PHARMACOLOGICAL ACTIVITIES

1. Animals

Albino mice weighing 20-25g were used for acute toxicity and for the various pharmacological screening studies except anti-inflammatory and wound healing activities which involved Albino Wistar male rats weighing 150-200g. The animals were procured from Shyamanur Shivashankarappa Institute of Medical Sciences and Research Centre (SSIMS & RC), Davanagere. They were maintained in the animal house of National College of Pharmacy, Shivamogga for experimental purpose. The animals were maintained under controlled conditions of temperature (23 ± 2°C), humidity (50 ± 5%) and 12h light-dark cycles. All the animals were acclimatized for seven days before the study. The animals were randomized into experimental and control groups and housed individually in sanitized polypropylene cages containing sterile paddy husk as bedding. They had free access to standard pellets (Pranav Agro Industries Pvt. Ltd.) as basal diet and water ad libitum. Animals were habituated to laboratory conditions for 48 hours prior to experimental protocol to minimize if any of non-specific stress. All the studies conducted was approved by the Institutional Animal Ethical Committee (IAEC) of National College of Pharmacy (Registration Number NCP/IAEC/CL/40/12/2011-12 dated 05-01-2012), Shivamogga, Karnataka, according to prescribed guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.
2. **Acute toxicity studies**

The acute toxicity studies were carried out as per stair case method (Ghosh, 1984). Animals weighing 20-25g were used for the study and were divided into groups of 6 animals each. The test extracts were administered intra-peritoneally as a suspension in Tween 80 to the different groups in increasing dose levels of 1000, 2000, 3000, 4000 and 5000 mg/kg body weight. The animals were then observed continuously for 3h for general behavioral, neurological, autonomic profiles and then every 30 minutes for next 3h and finally for death after 24h (OECD, 2001). Accordingly the LD\(_{50}\) of all the extracts calculated and the therapeutic dose was selected between 1/10\(^{th}\) and 1/5\(^{th}\) of this dose for the evaluation of pharmacological activities. 1% Tween-80 was used as the vehicle to prepare various fractions and administered intra-peritoneally.

3. **Statistical analysis**

All the experiments were carried out in triplicates. The observations are reported as mean ± standard error (SE). Differences between group’s means were assessed by one-way analysis of variance (ANOVA). The results obtained were compared with the control group. \(P\) value <0.01 was considered statistically significant. Correlation and regression analysis was done to ascertain the relationship between qualitative and quantitative antioxidant activities. All the statistical analysis was done using ez-ANOVA V.0.97 and SPSS statistics V.20 software.

A. **EVALUATION OF IN VITRO ANTIOXIDANT ACTIVITY**

1. **Quantitative antioxidant activities**

Phenolic and flavonoid compounds which are the secondary metabolites in plants are one of the most widely occurring groups of phytochemicals. They have biological and pharmacological properties especially exhibiting antimicrobial activity, antiviral, antimutagenic, anticarcinogenic, anti-inflammatory and cytotoxic activities (Prior *et al.* 2005). It has been recognized that phenolic compounds are a class of antioxidant compounds which act as
free radical terminators. The compounds such as flavonoids, which contain conjugated ring structures and hydroxyl functional groups, have the potential to function as antioxidants by scavenging or stabilizing free radicals involved in oxidative processes through hydrogenation or complex formation with oxidizing species that are much stronger than those of vitamins C and E (Amic et al. 2003).

i. Estimation of total phenolic content

Folin-Ciocalteau (FC) colorimetric method is based on a chemical reduction of the reagent, a mixture of tungsten and molybdenum oxides. The products of the metal oxide reduction have a blue color that exhibits a broad light absorption with a maximum at 725 nm. In the present investigation, Folin-Ciocalteau (FC) colorimetric method is employed for the quantitative estimation of total phenolic content present in different solvent extract of three mulberry species *viz.* *Morus alba*, *Morus serrata* and *Morus laevigata*.

Reagents

- Folin-Ciocalteu reagent
- Sodium carbonate solution
- Catechol (standard)

Procedure

The total phenolic content was estimated according to the method of Makkar *et al*. 1993. The aliquots of the extract was taken in a test tube and made up to the volume of 1 ml with distilled water. Then 0.5ml of Folin-Ciocalteu reagent (1:1 with water) and 2.5ml of sodium carbonate solution (20%) were added. After mixing, solution was incubated at 90°C for one minute and the absorbance was recorded at 725 nm against the reagent blank. Using catechol, a standard curve was prepared. Using the standard curve, the total phenolic content was calculated and expressed as catechol equivalent in µg/mg of extract.
ii. Estimation of total flavonoid content

In the present investigation aluminum chloride colorimetric method is employed for the quantitative estimation of flavonoids present in different solvent extract of three mulberry species *Morus alba*, *Morus serrata* and *Morus laevigata*. The principle of aluminum chloride colorimetric method is that aluminum chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols.

**Reagents**

- 5% NaNO$_2$
- 10% AlCl$_3$
- 1 M NaOH
- Catechol (standard)

**Procedure**

Total flavonoid contents of all the extracts were determined by the method of Zhishen (1999) and expressed as catechol equivalent in µg/mg of extract. An aliquot (1ml) of extracts or standard solution of catechol (20, 40, 60, 80 and 100 mg/ml) was added with 0.3 ml of 5% NaNO$_2$, 0.3 ml of 10% AlCl$_3$. The mixture was incubated for 5 min at room temperature then it was added with 2 ml NaOH. The total volume was made up to 10 ml by adding distilled water. The solution was mixed well and the absorbance was measured at 510 nm. Using the standard curve, the total flavonoid content was calculated.

iii. Determination of total antioxidant capacity

Total antioxidant capacity by phosphomolybdenum method assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of green phosphate/Mo (V) complex at acidic pH. The phosphomolybdenum method is quantitative since the total antioxidant activity is expressed as the number of equivalents of ascorbic acid (Prieto *et al.* 1999).
Reagents

- 0.6 M H$_2$SO$_4$
- 28mM Sodium Phosphate
- 4mM Ammonium Molybdate mixture
- Ascorbic acid (standard)

Procedure

The total antioxidant capacity was measured by spectrophotometric method of Prieto et al. 1999. At different concentration ranges, extracts were prepared in their respective solvents and mixed with 1ml of reagent solution (0.6M H$_2$SO$_4$, 28mM sodium phosphate and 4mM ammonium molybdate mixture). The tubes were incubated for 90 min at 95°C. The mixture was cooled to room temperature and the absorbance was read at 695 nm against blank. Ascorbic acid equivalents were calculated using standard graph of ascorbic acid.

2. Qualitative antioxidant activities

Various antioxidant activity methods have been used to monitor and compare the antioxidant activity of foods and natural products. In recent years, oxygen radical absorbance capacity assays and enhanced chemiluminescence assays have been used to evaluate antioxidant activity of foods, serum and other biological fluids. These methods are based on the direct interaction with reactive molecules or on their reactivity with metal ions and the effects are monitored by chemical measurements. Examples are determination of peroxyl radical scavenging, the ORAC assay, total antioxidant scavenging activity, the DPPH test or the FRAP method. In addition to these methods, chemical approaches have been developed which allow the detection of radical specific DNA-modifications in vitro (Chen et al. 2010).

i. DPPH radical scavenging

Free radical scavenging activity is determined using 1,1-diphenyl-2-picryl hydrazyl radical (DPPH), which is a stable free radical having purple
color. When free radical scavengers are added, DPPH is reduced and its color is changed to yellow, based on the efficacy of antioxidants. A 100µM solution of DPPH in methanol is added to the drug solution and the absorbance is read at 517 nm after 10 min. The change in absorbance with respect to the control (containing DPPH only without sample, expressed as 100% free radicals) is calculated as percentage scavenging.

Reagents

2,2-Diphenyl-2-picryl hydrazyl solution was prepared by dissolving 19mg in 50ml of methanol and then the total volume was made up to 500ml with methanol in a volumetric flask.

Procedure

DPPH free radical scavenging assay was measured using DPPH free radical test, by employing the method of Wong et al. 2006. The different concentrations of each of the extracts were prepared in methanol and were added to 3ml of 0.1mM methanolic solution of DPPH. The tubes were shaken vigorously and allowed to stand for 30 min at room temperature in dark. Changes in absorbance of samples were measured at 517 nm and methanol was read as blank. Free radical scavenging activity was expressed as inhibition percentage and was calculated using the following formula and the results are expressed as EC<sub>50</sub>, which is the amount of antioxidants necessary to decrease the initial concentration by 50%. Ascorbic acid was used as the standard.

\[
\% \text{ of Inhibition} = \frac{(A_0 - A_1)}{A_0} \times 100
\]

Where, \( A_0 \) = absorbance of the control (without test samples)
\( A_1 \) = absorbance of test samples.

ii. Nitric oxide radical scavenging

Nitric oxide is generated from sodium nitroprusside, which at physiological pH (7.4) liberates nitric acid. This nitric acid gets converted to nitrous acid and further forms nitrite ions on contact with air. The nitrite ions
diazotize with sulphanilic acid and couple with naphthylethylenediamine (Griess reagent), producing pink color which can be measured at 546 nm.

Reagents

- 10 mM Sodium nitroprusside
- Sulfanilic acid reagent
- 0.1% Naphthylethylenediaminedihydrochloride
- Saline phosphate buffer (pH 7.4)

Procedure

Nitric oxide radical scavenging activity was determined according to the method of Garrat, 1964. Nitric oxide (NO) was generated from sodium nitroprusside (SNP) and was measured by the Gries reagent. SNP (10mM) in phosphate buffer saline (PBS) was mixed with different concentration of extract (100-1000µg/ml) dissolved in ethanol and incubated at 25°C for 180 minutes. The samples from the above were reacted with Griess reagent (1% sulphanilamide, 0.1% naphthyl ethylene di-amine dichloride and 3% phosphoric acid). The absorbance of the chromophores formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthyl ethylene di-amine dichloride was read at 546 nm and referred to the absorbance of ascorbic acid, used as a positive control treated in the same way with Griess reagent. Nitric oxide radical scavenging activity was expressed as inhibition percentage and was calculated using the following formula and the results are expressed as EC50.

\[
\% \text{ of Inhibition} = \frac{(A_0 - A_1)}{A_0} \times 100
\]

Where, \(A_0\) = absorbance of the control (without test samples)
\(A_1\) = absorbance of test samples

iii. Hydroxyl radical scavenging

The scavenging activity for hydroxyl radical was measured by Fenton reaction as described by Halliwell et al. 1987. Biologically, the hydroxyl radical is widely believed to be generated when hydrogen peroxide reacts with Fe(II) (Fenton reaction). Antioxidants in food (such as vitamin C) may act as
pro-oxidants by reducing Fe(III) to Fe(II) and make the HO\(^-\) generation catalytic. In fact, ascorbic acid has been used in combination with catalytic Fe(II) and excess H\(_2\)O\(_2\) to generate a constant flux of HO\(^-\) radicals.

**Reagents**

- 1mM FeCl\(_3\)
- 1 mM 1,10-phenanthroline
- 0.2 M phosphate buffer pH 7.8
- 0.17M H\(_2\)O\(_2\)

**Procedure**

The hydroxyl radicals were generated in reaction mixture containing 60\(\mu\)l of 1.0mM FeCl\(_3\), 90\(\mu\)l of 1mM 1,10-phenanthroline, 2.4ml of 0.2M phosphate buffer (pH 7.8), 150\(\mu\)l of 0.17M H\(_2\)O\(_2\) and 1ml of extract at various concentrations. Addition of H\(_2\)O\(_2\) initiated the reaction. After incubation at room temperature for 5 minutes, the absorbance of the mixture was measured at 560 nm. The hydroxyl radicals scavenging activity was calculated according to the following equation and the results are expressed as EC\(_{50}\).

\[
\text{\% of Inhibition} = \frac{(A_0 - A_1)}{A_0} \times 100
\]

Where, \(A_0\) = absorbance of the control (without test samples)

\(A_1\) = absorbance of test samples.

iv. **Iron chelating**

The chelation of ferrous ions by extracts was estimated by method of Dinis et al. 1994. Iron and other transition metals (copper, chromium, cobalt, vanadium, cadmium, arsenic, and nickel) promote oxidation by acting as catalysts of free radical reactions. These red-ox active transition metals transfer single electrons during changes in oxidation states. Chelation of metals by certain compounds decreases their pro-oxidant effect by reducing their red-ox potentials and stabilizing the oxidized form of the metal (Reische et al. 2008). The basic principle of this test is based on the capacity to decolorize the iron-ferrozine complex. Ferrozine is a substance which can quickly act on iron to
form a colored complex. Ferrozime can act on divalent iron to form stable magenta complex-soluble in water. After 10 minutes at room temperature, the absorbance of the complex was measured at 562 nm spectrophotometrically.

Reagents

- 2 mM FeCl$_2$ solution
- 5 mM Ferrozine
- De-ionized water

Procedure

To 0.5ml of different concentrations of the extracts, 1.6ml of de-ionized water and 0.05ml of 2mM FeCl$_2$ was added. After 30 seconds, 0.1ml of 5mM ferrozine was added. Ferrozine reacted with divalent iron to form stable magenta complex species that were very soluble in water. After 10 minutes at room temperature, the absorbance of the Fe$^{2+}$-Ferrozine complex was measured at 562 nm. EDTA was used as a standard metal chelating agent (Lei et al. 2010). The chelating activity of the extract for Fe$^{2+}$ was calculated using the following formula and the results are expressed as EC$_{50}$.

\[
\% \text{ of Inhibition} = \frac{(A_0 - A_1)}{A_0} \times 100
\]

Where, $A_0$ = absorbance of the control (without test samples)

$A_1$ = absorbance of test samples.

v. Reducing power assay

The reducing power of the extracts was evaluated according to Oyaizu, 1986. The reducing capacity of a compound Fe$^{3+}$/ferricyanide complex to the ferrous form may serve as a significant indicator of its antioxidant capacity. The existence of reductones are the key of the reducing power, which exhibit their antioxidant activities through the action of breaking the free radical chain by donating a hydrogen atom. The reduction of the Fe$^{3+}$/ferricyanide complex to the ferrous form occurs due to the presence of reductants in the solution. Absorbance of Fe$^{3+}$ can be observed by measuring the O.D. values at 700 nm.
Reagents

- 0.2 M phosphate buffer pH 6.6
- 1% potassium hexacyanoferrate
- 10% trichloroacetic acid
- 1% ferric chloride solution

Procedure

Different amounts of extracts were mixed with 2.5ml of 0.2M phosphate buffer (pH 6.6) and 2.5ml of 1% K₃Fe(CN)₆. This mixture was incubated at 50°C for 20 min. 2.5ml of 10% TCA was added to the blend and centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5ml) was assorted with distilled water (2.5ml) and FeCl₃ (0.5ml, 0.1%) and the absorbance was measured at 700 nm. Increase in absorbance of the reaction mixture indicates increased reducing power. EC₅₀ value (µg of extract/ml) is the effective concentration at which the absorbance was 0.5 for reducing power. Ascorbic acid was used as standard.

3. Correlation and regression analysis for antioxidant activities

Correlation and regression analysis was done to ascertain the relationship between qualitative and quantitative activities. For bivariate analysis, correlation coefficient (r) was calculated using dot plot method for different qualitative estimations, viz. reducing power assay, DPPH radical scavenging activity, hydroxyl radical scavenging activity, nitric oxide radical scavenging activity and iron chelating activity versus different quantitative estimations viz. total phenolics, total flavonoids and total antioxidants (Mau et al. 2006). Regression lines were plotted for EC₅₀ values of all the extracts from different qualitative activities against equivalence of respective extract from different quantitative estimations (Li et al. 2009).
B. ANTICANCER ACTIVITY OF THE PLANT EXTRACTS

The anticancer effect of the methanol leaf extracts of three mulberry species viz. *Morus alba, Morus serrata* and *Morus laevigata* were screened under non-tumorigenic and tumorigenic model systems.

1. *In vitro* anticancer activity

Cytotoxicity of sample on tumor cells was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Mosmann, 1983) for methanolic extracts of three species of *Morus* viz. *Morus alba, Morus serrata* and *Morus laevigata*.

i. Maintenance of cell culture

The cells were cultured using Dulbecco’s Modified Eagle Medium (DMEM) media with 10% FBS. Once the cells in the culture dish (T25 flask) reaches confluence, the monolayer cells were detached and single cell suspensions were made using trypsin-ethylene diamine tetra acetic acid (EDTA). Trypan blue was added to check the viability of the cells and hemocytometer was used to do the cell counting. $1 \times 10^6$ cells/ per well/100μl was seeded in a 96-well plate. Cells were allowed to attach in the well plate for 24 hr at 37°C, 5% CO$_2$, 95% relative humidity.

The cells were treated with different concentrations of the test samples after 24 hours of incubation. The required final drug concentrations of 50, 100, 200, 400, 800μg/ml from the stock of 1mg/ml were obtained by serial dilution and the final volume was made up to 200μl/well using media. After addition of the drug, the plates were incubated for an additional 48 hours at 37°C. The medium without samples served as control and triplicate was maintained for all concentrations. Since the samples were dissolved in DMSO to make 1mg/ml stock. The DMSO controls of 50, 100, 200, 400, 800μg/ml concentration was maintained in triplicates.
ii. Cytotoxicity assay

After 48h incubation, cell viability was determined by adding tetrazolium salt (Sigma) as cytotoxicity indicator. 20μl from MTT stock of 5mg/ml (PBS was used to make the stock) was mixed with 80μl of media to give a final concentration of 1mg/ml was added to each well and incubated at 37°C for 4h. The medium with MTT was discarded and 100μl of DMSO per well was added to solubilize the formazan crystals. The absorbance was measured using micro plate reader at 590 nm. Tetrazolium salts are cleaved to formazan dye by cellular enzyme mitochondrial succinate dehydrogenase present only in the viable cells. The % of cell inhibition was determined using following formula.

\[
\% \text{ of cell Inhibition} = 100 - \frac{\text{Abs (sample)}}{\text{Abs (control)}} \times 100
\]

2. In vivo anticancer activity

In vivo anticancer activity was performed using EAT cell lines in mice for the methanolic extracts of three species of Morus viz. Morus alba, Morus serrata and Morus laevigata.

i. Ehrlich ascites tumor cell lines

Ehrlich ascites tumor (EAT) is referred to as an undifferentiated carcinoma which was of mammary origin, and is originally hyper diploid, has high transplanatble capability, no-regression, rapid proliferation, shorter life span, 100% malignancy and also does not have tumor specific transplantation antigen (TSTA) (Ozaslan et al. 2013). EAT cells were obtained from Dept of Radiotoxicology, Manipal Life Sciences, Manipal, India. They were maintained by weekly intraperitoneal inoculation of 10^6 cells/mouse.

ii. Experimental design

Male Swiss albino mice were divided into 5 groups (n=6). All the groups were injected with EAT cells (0.2 ml of 2×10^6 cells/mouse) intra-peritoneally except the control group. This was taken as day zero, from the first day to every alternative day. The drugs were administered intra-peritoneally for
14 days and after the administration of last dose followed by 18hr fasting, 3 mice from each group were sacrificed for the study of antitumor activity. The remaining animals in each of the groups were kept to check the mean survival time (MST) and percent increase in life span of the tumor bearing hosts (Khanam et al.1997; Gupta et al. 2004; Sachin Hiradev et al. 2010).

Further, anticancer effect of Morus species were assayed by observation of change with respect of body weight, ascitic tumor volume, packed cell volume and viable cell count (Nicol and Prasad, 2006). The experimental design is shown below (Table 3.1).

Table 3.1: Experimental design for in vivo anticancer activity

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Treatment</th>
<th>Groups</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>I</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Morus alba methanol extract</td>
<td>II</td>
<td>200mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III</td>
<td>300mg/kg</td>
</tr>
<tr>
<td>3</td>
<td>Morus serrata methanol extract</td>
<td>IV</td>
<td>200mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V</td>
<td>300mg/kg</td>
</tr>
<tr>
<td>4</td>
<td>Morus laevigata methanol extract</td>
<td>VI</td>
<td>200mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VII</td>
<td>300mg/kg</td>
</tr>
</tbody>
</table>

iii. Tumor transplantation

Ehrlich’s Ascites Tumor was maintained by serial transplantation from tumor bearing Swiss Albino mice. Ascitic fluid was drawn out from tumor bearing mice at the log phase (day 7-8 of tumor bearing) of the tumor cells. The tumor cell number was adjusted to $2 \times 10^6$ tumor cells/ml. Sample showing more than 90% viability was used for transplantation. Each animal received 0.2ml of tumor cell suspension containing $2 \times 10^6$ cells/ml intra-peritoneally (Nicol and Prasad, 2006).
iv. **Tumor cell volume and packed cell volume**

The mice were dissected to collect ascitic fluid from peritoneal cavity and centrifuged to determine packed cell volume at 1000 rpm for 5 min (Sivakumar *et al.* 2005). The transplantable murine tumor was carefully collected to measure the tumor volume.

v. **Viable cell count**

Viable cell counting of the ascitic cell was done by staining with tryphan blue (0.4% in normal saline), dye exclusion test and count was determined in a neubauer counting chamber. The cells that did not take up the dye were viable and those that took the stain were not viable (Nicol and Prasad, 2006).

vi. **Mean survival time (MST) and percent increase in life span (ILS)**

The effect of *Morus* species on tumor growth was observed by MST and % ILS. MST of each group continuing 3 mice was monitored by recording the mortality daily for 6 weeks and % ILS was calculated by using following equation (Sivakumar *et al.* 2005; Nicol and Prasad, 2006).

\[
\text{MST} = \frac{\text{Day of first death} + \text{Day of last death}}{2}
\]

&

\[
\text{ILS} = \frac{\text{MST of treated group}}{\text{MST of control group}} (-1) \times 100
\]

C. **ANALGESIC ACTIVITY**

Pain is an unpleasant sensation which informs structural and functional changes in the body and acts as a warning signal against disturbances in the body. Even though pain is an unpleasant sensation, is mainly a protective mechanism for the body (Kanodia and Das, 2008). It is a consequence of complex neurochemical processes in the central and peripheral nervous systems (Mary *et al.* 1997). In the present investigation, the analgesic activity of leaf extracts of three mulberry species *viz.* *Morus alba*, *Morus serrata* and *Morus laevigata* were evaluated by tail immersion test.
Tail immersion Test

The basal reaction time to radiant heat source was taken by placing the tip of the tail on the radiant heat source (Kulkarni, 1987). The animals were screened for the sensitivity test by immersing the tail of the mice gently in hot water maintained at 55°C. The time the animal’s tail spent in the water before reacting to the pain is recorded with a stop watch. The animal immersing the tail from hot water within 5 seconds was selected for the study. The drugs were administered @ 200mg/kg & 400mg/kg intra-peritoneally. After administration of the drugs, the reaction time was measured (in seconds) after 60 minutes. The selected mice were then divided into twenty groups of six mice each and the experimental design is shown in Table 3.2.

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Extract</th>
<th>Group</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>I</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Aspirin</td>
<td>II</td>
<td>100mg/kg</td>
</tr>
<tr>
<td>3</td>
<td>Morus alba Petroleum ether</td>
<td>III</td>
<td>200mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IV</td>
<td>400mg/kg</td>
</tr>
<tr>
<td>4</td>
<td>Morus alba Chloroform</td>
<td>V</td>
<td>200mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VI</td>
<td>400mg/kg</td>
</tr>
<tr>
<td>5</td>
<td>Morus alba Methanol</td>
<td>VII</td>
<td>200mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VIII</td>
<td>400mg/kg</td>
</tr>
<tr>
<td>6</td>
<td>Morus serrata Petroleum ether</td>
<td>IX</td>
<td>200mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>X</td>
<td>400mg/kg</td>
</tr>
<tr>
<td>7</td>
<td>Morus serrata Chloroform</td>
<td>XI</td>
<td>200mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>XII</td>
<td>400mg/kg</td>
</tr>
<tr>
<td>8</td>
<td>Morus serrata Methanol</td>
<td>XIII</td>
<td>200mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>XIV</td>
<td>400mg/kg</td>
</tr>
<tr>
<td>9</td>
<td>Morus laevigata Petroleum ether</td>
<td>XV</td>
<td>200mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>XVI</td>
<td>400mg/kg</td>
</tr>
<tr>
<td>10</td>
<td>Morus laevigata Chloroform</td>
<td>XVII</td>
<td>200mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>XVIII</td>
<td>400mg/kg</td>
</tr>
<tr>
<td>11</td>
<td>Morus laevigata Methanol</td>
<td>XIX</td>
<td>200mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>XX</td>
<td>400mg/kg</td>
</tr>
</tbody>
</table>
D. ANTI-INFLAMMATORY ACTIVITY

Inflammation is a normal, protective response to tissue injury caused by physical trauma, noxious chemicals, or microbial agents and is the body's effort to inactivate or destroy invading organisms, remove irritants, and set the stage for tissue repair (Mary et al. 1997). Upon interaction of foreign pathogens with innate immune cells like macrophage or monocytes, inflammatory immune response is triggered off. A series of pro-inflammatory mediators, specialized cytokines, prostaglandins, chemokines are produced as a result in a way to amplify the inflammatory response (Beg, 2002).

Carrageenan-induced paw edema

The method used was similar to that described by Winter et al. 1962. The albino rats of either sex were divided into twenty groups of six animals each. The initial paw volume was measured plethysmographically before carrageenan injection. Acute inflammation was induced in all groups by injecting 0.1 ml of 1% carrageenan into the sub-plantar region of the right hind paw of rats. The right paw served as a reference to non-inflamed paw for comparison. The relative increase in paw volume was measured in control, standard and treated group at 60, 120, 180 and 240 min after carrageenan injection. The percentage increase in the paw volume over the initial reading was calculated. This increase in the paw volume in the animals treated with the standard drug and the different doses of the crude extracts were compared with increase in paw volume of untreated control animals. Mean decrease in the paw volume was measured. The experimental design is shown below (Table 3.3).

The percentage inhibition of paw edema is calculated using the following formula,

\[
\text{Percentage inhibition of paw edema} = (1 - \frac{V_t}{V_c}) \times 100
\]

Where \(V_c\) = average increase in paw volume (average inflammation) of the control group of rats at a given time; \(V_t\) = average inflammation of the drug treated (ie. plant extracts) rats at the same time.
Table 3.3: Experimental design for in vivo anti-inflammatory activity

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Extract</th>
<th>Group</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control animals received vehicle</td>
<td>I</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Indomethacin</td>
<td>II</td>
<td>50mg/kg</td>
</tr>
<tr>
<td>3</td>
<td><em>Morus alba</em> Petroleum ether</td>
<td>III</td>
<td>200mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IV</td>
<td>400mg/kg</td>
</tr>
<tr>
<td>4</td>
<td><em>Morus alba</em> Chloroform</td>
<td>V</td>
<td>200mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VI</td>
<td>400mg/kg</td>
</tr>
<tr>
<td>5</td>
<td><em>Morus alba</em> Methanol</td>
<td>VII</td>
<td>200mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VIII</td>
<td>400mg/kg</td>
</tr>
<tr>
<td>6</td>
<td><em>Morus serrata</em> Petroleum ether</td>
<td>IX</td>
<td>200mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>X</td>
<td>400mg/kg</td>
</tr>
<tr>
<td>7</td>
<td><em>Morus serrata</em> Chloroform</td>
<td>XI</td>
<td>200mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>XII</td>
<td>400mg/kg</td>
</tr>
<tr>
<td>8</td>
<td><em>Morus serrata</em> Methanol</td>
<td>XIII</td>
<td>200mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>XIV</td>
<td>400mg/kg</td>
</tr>
<tr>
<td>9</td>
<td><em>Morus laevigata</em> Petroleum ether</td>
<td>XV</td>
<td>200mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>XVI</td>
<td>400mg/kg</td>
</tr>
<tr>
<td>10</td>
<td><em>Morus laevigata</em> Chloroform</td>
<td>XVII</td>
<td>200mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>XVIII</td>
<td>400mg/kg</td>
</tr>
<tr>
<td>11</td>
<td><em>Morus laevigata</em> Methanol</td>
<td>XIX</td>
<td>200mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>XX</td>
<td>400mg/kg</td>
</tr>
</tbody>
</table>

E. WOUND HEALING ACTIVITY

Wound healing disorders present a serious clinical problem and are likely to increase since they are associated with diseases such as diabetes, hypertension, and obesity. Additionally, increasing life expectancies will cause more people to face such disorders and further aggravate this medical problem. Thus, several animal models have been established to serve as an experimental basis to determine molecular and cellular mechanisms underlying and controlling an undisturbed healing process. The wound healing model *viz.* excision, incision and dead space wound models are extensively used to determine the healing property of natural and chemical products. The excision wounding method represents an animal model that provides access to investigate complex tissue movements associated with repair such as hemorrhage, granulation tissue formation, re-epithelialization and angiogenic
processes. Measurement of wound strength (incision wound model) and granulation tissue (produced in dead space wound model) provides highly quantifiable estimates of the efficacy of the aggregate healing process. Determination of various individual components of the phases of healing can provide important insights about events operative during repair. In the present investigation, wound healing property was assessed by incision and excision wound models in three leaf extracts of *Morus alba*, *Morus serrata* and *Morus laevigata*.

**Drug formulation**

The drugs were prepared for each of the extracts for topical administration. 5g of each extract was mixed with 45g of white petroleum jelly to get 10% (w/w) concentration and were applied topically in the form of ointments (Varma and Giri, 2013). 0.2% (w/w) Nitrofurazone was used as a standard reference. All the formulations were prepared freshly before the commencement of each experiment.

Eleven groups of animals containing six each were used for each of the excision and incision wound models. The animals of group I was considered as the control and received 5% sodium alginate ointment base and group II animals received 0.2% (w/w) Nitrofurazone. The experimental design is shown below (Table 3.4).
### Table 3.4: Experimental design for *in vivo* wound healing activity

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Treatment</th>
<th>Groups</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>I</td>
<td>5% sodium alginate</td>
</tr>
<tr>
<td>2</td>
<td>Nitrofurazone</td>
<td>II</td>
<td>0.2% (w/w)</td>
</tr>
<tr>
<td>3</td>
<td><em>Morus alba</em> Petroleum ether</td>
<td>III</td>
<td>5g of each extract + 45g of white petroleum jelly</td>
</tr>
<tr>
<td>4</td>
<td><em>Morus alba</em> Chloroform</td>
<td>IV</td>
<td>5g of each extract + 45g of white petroleum jelly</td>
</tr>
<tr>
<td>5</td>
<td><em>Morus alba</em> Methanol</td>
<td>V</td>
<td>5g of each extract + 45g of white petroleum jelly</td>
</tr>
<tr>
<td>6</td>
<td><em>Morus serrata</em> Petroleum ether</td>
<td>VI</td>
<td>5g of each extract + 45g of white petroleum jelly</td>
</tr>
<tr>
<td>7</td>
<td><em>Morus serrata</em> Chloroform</td>
<td>VII</td>
<td>5g of each extract + 45g of white petroleum jelly</td>
</tr>
<tr>
<td>8</td>
<td><em>Morus serrata</em> Methanol</td>
<td>VIII</td>
<td>5g of each extract + 45g of white petroleum jelly</td>
</tr>
<tr>
<td>9</td>
<td><em>Morus laevigata</em> Petroleum ether</td>
<td>IX</td>
<td>5g of each extract + 45g of white petroleum jelly</td>
</tr>
<tr>
<td>10</td>
<td><em>Morus laevigata</em> Chloroform</td>
<td>X</td>
<td>5g of each extract + 45g of white petroleum jelly</td>
</tr>
<tr>
<td>11</td>
<td><em>Morus laevigata</em> Methanol</td>
<td>XI</td>
<td>5g of each extract + 45g of white petroleum jelly</td>
</tr>
</tbody>
</table>

1. **Excision wound**

The rats were inflicted with excision wounds as described by Morton and Malone (1972) under light ether anesthesia. In this model, the skin of the impressed area was excised to full thickness on the dorsal thoracic region of the rats to obtain a wound area of about 500 sq mm. The ointment gel was topically applied daily till the complete epithelialization starting from the day of operation. The parameters studied were wound closure percentage and time of complete epithelialization. The wounds were traced on 1 mm² graph paper on the days of 4, 8, 12 and 16 and thereafter on alternate days until healing were complete.

2. **Incision wound**

In incision wound model (Ehrlich and Hunt, 1969), 6cm long para vertebral incisions were made through full thickness of the skin on either side
of the vertebral column of the rat. Care was taken to see that incision was at least 1 cm lateral to vertebral column. The wounds were closed with interrupted sutures of 1 cm apart. The animals were caged individually. The sutures were removed on 8\textsuperscript{th} post wounding day. The skin breaking strength of the wounds was measured on 10\textsuperscript{th} post wound day by using tensiometer by the method of Lee and Tong (1968) and the results were recorded and subjected to statistical analysis.

F. CNS DEPRESSANT ACTIVITY

CNS depressants sometimes referred to as sedatives and tranquilizers, are substances that can slow normal brain function. Because of this property, some CNS depressants are useful in the treatment of anxiety and sleep disorders. Among the medications that are commonly prescribed for these purposes are Barbiturates, Benzodiazepines and Alprazolam. While CNS stimulants increase alertness, attention, and energy, which are accompanied by increases in blood pressure, heart rate, and respiration which include norepinephrine, ephrin and dopamine. Stimulants increase the levels of these chemicals in the brain and body. In addition, the increase in dopamine is associated with a sense of euphoria that can accompany the use of stimulants. In the present investigation, locomotor activity was employed to determine the CNS depressant activity.

Locomotor activity

The locomotor activity was studied in petroleum ether, chloroform and methanol extracts at two doses of 200 and 400 mg/kg and evaluated by using photoactometer. Mice were placed individually in photoactometer and counts were taken for the duration of 5 min. The basal reaction time was noted before and 1 hour after the administration of treatment (Kulkarni, 1999). The animals were divided into XX groups of six mice each and the experimental design is shown below (Table 3.5).
G. ANTHELMINTIC ACTIVITY

Helminth infections are prevalent in people all over the world, but most common in the tropical and subtropical regions. Considerable research has shown that some plants have antiparasitic effects. For example, plants that contain condensed tannins, a class of phenolic secondary metabolites, have these effects (Jalalpure et al. 2007). Search for anthelmintic factor in plants therefore remains a potential area of investigation. In view of this leaf extracts of *M. alba*, *M. serrata* and *M. laevigata* were screened for anthelmintic activity by using *Pheretima posthuma* (Indian Earthworm) due to its anatomical and physiological resemblance with the intestinal round worms, parasites of human beings (Vidyarthi, 1967; Thorn, 1977; Vigar, 1984).
Drug formulation: The sequential extracts of the plant, were prepared in Tween 80 solution so as to obtain five dosage forms at the concentration of 20, 40, 60, 80 and 100mg/ml. 20mg/ml Albendazole was used as standard and 20% tween-80 served as control.

Organisms used
All the experiments were carried out on Indian adult earthworms (Pheretima posthuma) collected from Earthworm Rearing Center, Dummalli, Shimoga (Karnataka).

Procedure
The anthelmintic assay was carried out as per the method described by Ghosh et al. (2003). Six worms of nearly equal size were placed in petri-plates containing 15ml of solution of either standard drug or test extracts solutions at room temperature. 20% Tween 80 used as control sample was also tested. Thus a total of five groups (20, 40, 60, 80 and 100mg) in each extract along with one negative control (20% Tween 80) and one positive control (Albendazole 20mg/ml) were subjected to the evaluation of anthelmintic property. Observations were made for the time taken to paralyze, and death of individual worms. Paralysis was said to occur when no movement of any sort could be observed except the worms were shaken vigorously. Time for death of worms were recorded after ascertaining that the worms neither moved when shaken vigorously nor when dipped in warm water at 50°C (Chopra et al.1956).

H. ANTIMICROBIAL ACTIVITY
The antibiotics are used to treat microbial infections caused by bacteria, fungi or protozoa. Most exert a highly selective toxic action upon their target microbial cells but have little or no toxicity towards mammalian cells. They can therefore be administered at concentrations sufficient to kill infecting organisms (or at least inhibit their growth) without damaging mammalian cells. Study of the mechanism of action of the antibiotics reveals the basis of their selective toxicity.
Microorganisms used

Pure culture of bacteria used for this study were *Pseudomonas aeruginosa* (NCIM 2945), *Staphylococcus aureus* (NCIM 5022), *Proteus vulgaris* (NCIM 2027), *Bacillus subtilis* (NCIM 2920), *Salmonella typhi* (NCIM 2501), *Shigella flexneri* (NCIM 4924), and two fungal species *Candida albicans* (NCIM 3103) and *Aspergillus niger* (NCIM 789). These organisms were obtained from National College of Industrial Microorganisms (NCIM), Pune, India.

**Drug formulation:** The different extracts of *M. alba*, *M. serrata* and *M. laevigata* were dissolved in DMSO to obtain 20, 40, 60 and 80mg/ml of extracts and standards were prepared at a concentration of 10mg/ml.

**Procedure**

**Preparation of nutrient agar media**

Peptone (5g), Beef extract (3g), were weighed accurately and dissolved in 500ml distilled water taken in conical flask. The pH was adjusted to 7.0. Agar (20g) was weighed and dissolved in 500ml of distilled water taken in another flask. Then the content of previous flask was added to agar. The flask was cotton plugged and the media was sterilized by autoclaving at 15lbs. pressure at 121°C for 15-20 min.

**Sabouraud’s dextrose agar medium**

Peptone (10g), dextrose (40g), were weighed accurately and dissolved in 500ml distilled water taken in conical flask. Agar (15g) was weighed and dissolved in 500ml of distilled water taken in another flask. Then the content of previous flask was added to agar. The flask was cotton plugged and the media was sterilized by autoclaving at 15lbs. pressure at 121°C for 15-20 min.

**Preparation and standardization of stock cultures**

The cultures were sub-cultured in nutrient agar medium for bacterial cultures and Sabouraud’s dextrose agar medium for fungal culture plates. They were stored in slants as stock cultures. For the studies, stock culture was
prepared by inoculating each culture from slants to flask in sterile nutrient broth media and incubated at 37°C for 24 hours for bacterial test whereas for fungal screening, sterile Sabouraud’s dextrose broth media was used and incubated at 28°C for 48 hours.

The *in vitro* antimicrobial activity of all the extracts at different concentrations (20, 40, 60 and 80mg/ml) were studied by agar well diffusion method (Thorn *et al.* 1977; Vidyarthi, 1977), against five bacterial species and two fungal species.

1. **Screening of antibacterial activity**

   The screening was initiated by inoculating the test bacteria on to nutrient broth under incubation temperature of 37°C for 24hrs. From the nutrient broth, lawn of each test organism was made with the help of sterile cotton swabs on nutrient agar plates. Well of 0.5cm in diameter was punched on the center of the plate with the help of sterile cork borer. The well was filled with varied concentrations of extracts. Plates were incubated for 24h at 37°C. The plates were observed for clear zone formation around the well. Antibacterial activities were determined by measuring the zone of inhibition as per standard procedure (Vigar, 1984) and expressed in millimeter. Results were evaluated by comparing the zone of inhibition shown by the extracts *vs.* streptomycin. The experiment was carried out in triplicate and the mean values were tabulated.

2. **Screening of antifungal activity**

   The antifungal activities of different solvent extracts of the three plant species were assessed by the cup plate method (Pelczar, 1993) using two selected fungal strains *viz.* *Candida albicans* and *Aspergillus niger*. Culture of each test organism was inoculated with the help of sterile cotton swabs on Sabouraud’s agar plates. After inoculation four bores were made at equal distance by using sterile steel cork borer. In to these cups different concentrations of standard drug and the test extracts along with control (DMSO) were introduced. After introduction of standard drug and extracts,
these plates were placed in a refrigerator at 8-10° C for two hours for proper diffusion of the drugs. After 2 hours of cold incubation, the petriplates were transferred to incubator and maintained at 25± 2° C for 24-36 hours. After the incubation period, the plates were observed for zone of inhibition and the diameter of zones was measured by using standard procedure. Results were evaluated by comparing the zone of inhibition shown by the extracts with fluconazole. The experiment was carried out in triplicate and the mean values were expressed in millimeter.

3. Estimation of Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC) for the isolated compounds

The in vitro antimicrobial activity of drugs is usually assessed by determining of the MIC and MBC. The minimum bactericidal concentration (MBC) is the lowest concentration of an antibacterial agent required to kill a particular bacterium. It can be determined from broth dilution minimum inhibitory concentration (MIC) tests by sub-culturing to agar plates that do not contain the test agent.

In order to measure the MIC values, micro-broth dilution method was used (Abu-Shanab et al. 2006). The preparations of the extracted compounds were diluted in Mueller–Hinton broth medium to obtain various concentrations of the stock using serial dilution method (800, 400, 200, 100, 50, 25, 12.5, 6.25, 3.125 and 1.56µg/ml) and was assayed against the test organisms viz. Staphylococcus aureus (gm +ve) and Pseudomonas aeruginosa (gm -ve). The minimum inhibitory concentration was defined as the lowest concentration able to inhibit any visible bacterial growth. Equal volume of the test compound concentrations and Mueller–Hinton broth were mixed in micro-tubes to make up to a final volume on 2ml. 0.5 ml of McFarland standard of the organism suspension was added to each tube (Shahidi, 1995). The tubes were incubated at 37° C for 24 h. Two control tubes were maintained for each test batch. These
include tube containing test compounds without inoculum and the tube containing the growth medium and inoculum.

The minimum bactericidal concentration (MBC) was determined by subculturing the test dilution on Mueller–Hinton agar and further incubated for 24h. The highest dilution that yielded no single bacterial colony was taken as the minimum bactericidal concentration (Akinyemi et al. 2005).

I. **IN SILICO SCREENING FOR TOXICOLOGY AND PHARMACOLOGY OF ISOLATED COMPOUNDS**

The term ‘in silico’ is a modern word usually used to mean experimentation performed by computer and is related to the more commonly known biological terms in vivo and in vitro. In silico biology refers to computational models of biology. In silico biology draws from the vast amounts of biological information available, and applies sophisticated algorithms or simulations to advance scientific understandings. The results of these simulations can then tested experimentally or serve as a guide for future physical experimentation.

The term in silico toxicology generally refers to a computational experiment, mathematical calculation, or scientific analysis of substances and organization of substance related data through a computer based analysis. In silico toxicology studies are the non-testing methods which include models for predicting the absorption, distribution, metabolism and elimination (ADME) characteristics of chemicals in biological system (Mostrag and Worth, 2010).

**Tools and servers used**

Different bioinformatics and chemi-informatics software were used to study the pharmacological and toxicological parameters of isolated compounds in silico. Below are the details of the servers (Table 3.6) and software (Table 3.7) used for in silico studies.
Table 3.6: List of online servers used in *in silico* studies

<table>
<thead>
<tr>
<th>Sl No</th>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>ChemSpider</td>
<td>ChemSpider providing fast access to over 32 million structures, properties and associated information of known compounds.</td>
</tr>
<tr>
<td>3</td>
<td>Drug Bank</td>
<td>The DrugBank database is a unique bioinformatics and cheminformatics resource that combines detailed drug (<em>i.e.</em> chemical, pharmacological and pharmaceutical) data with comprehensive drug target (<em>i.e.</em> sequence, structure, and pathway) information.</td>
</tr>
<tr>
<td>4</td>
<td>Molsoft</td>
<td>Molsoft molecular properties predictor calculates an overall drug-likeness score using Molsoft’s chemical fingerprints.</td>
</tr>
<tr>
<td>5</td>
<td>admetSAR</td>
<td>AdmetSAR provides the manually curetted data for diverse chemicals associated with known Absorption, Distribution, Metabolism, Excretion and Toxicity profiles.</td>
</tr>
<tr>
<td>6</td>
<td>Molinspiration</td>
<td>Molinspiration calculates important molecular properties needed in QSAR, molecular modelling and drug design and high quality molecule depiction.</td>
</tr>
<tr>
<td>7</td>
<td>Protein Data Bank</td>
<td>The Protein Data Bank (PDB) archive is the single worldwide repository of information about the 3D structures of large biological molecules, including proteins and nucleic acids</td>
</tr>
<tr>
<td>8</td>
<td>Ligplot</td>
<td>Automatically generates schematic diagrams of protein-ligand interactions for a given PDB file.</td>
</tr>
</tbody>
</table>
Table 3.7: List of software used in *in silico* studies

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ChemDraw</td>
<td>chemical structure drawing program</td>
</tr>
<tr>
<td>2</td>
<td>ChemSketch</td>
<td>2D structure cleaning and geometry optimization</td>
</tr>
<tr>
<td>3</td>
<td>Open Babel</td>
<td>Construction of 3D coordinates for ligands</td>
</tr>
<tr>
<td>4</td>
<td>AutoDock Tools</td>
<td>Molecular Interaction studies</td>
</tr>
</tbody>
</table>

1. *IN SILICO TOXICOLOGY STUDIES*

   i. **Preparation of ligands**

   Structure of the drug molecules were drawn in ChemDraw Ultra 6.0 assigned with proper 2D orientation (ChemOffice package). 3-D geometrical optimization was done using Chemsketch v12.01 (ACD/labs). Openbabel, a standalone tool was used to obtain 3D coordinates for all the drug candidates. Ligand molecules are assessed for the ADME-Toxicity profile using their SMILES notations (Simplified molecular-input line-entry system) generated from ChemSketch software (Table 3.8).

   Table 3.8: SMILES generated from ChemSketch software

<table>
<thead>
<tr>
<th>Sl No</th>
<th>Ligand</th>
<th>SMILES</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AD 5</td>
<td>CC12CCC3C(C1CCC2O)CC=C4C3(CCC(C4)O)C</td>
</tr>
<tr>
<td>2</td>
<td>AD 6</td>
<td>CC(=CCC1=C(C=CC2=C1OC(=C2)C3=CC(=CC(=C3CC=C(C(C=C)O)O)O)O)C</td>
</tr>
<tr>
<td>3</td>
<td>AD11</td>
<td>CC1CCC2(CCC3(C(=CCC4C3(CCC5C4(CCC(C5(C(C)O)C)O)C)C)2C1C)C(=O)O)C</td>
</tr>
<tr>
<td>4</td>
<td>AD 13</td>
<td>C1=CC2=C(C=C1O)OC(=C2)C3=CC(=CC(=C3)O)O</td>
</tr>
</tbody>
</table>

   ii. **Primary screening of the drug molecule**

   The Lipinski rule-of-five also known as Pfizer's rule of five (RO5) was employed for the screening of competent drug molecules based on their molecular properties. Rule of five has become a computational approach for the estimation of solubility and permeability of new drug candidates. It is also a standard protocol to check the pharmacological and biological properties of the
ligand for virtual screening (VS) (Oprea, 2002). The rule describes the molecular properties important for a drug’s pharmacokinetics in the human body, including their absorption, distribution, metabolism and excretion (ADME) which makes sense for drug-likeness prediction and oral bioavailability (Lipinski et al. 2012).

iii. **In silico ADME-Toxicology studies**

The ADME-T properties of each drug molecule were calculated by submitting the competent drug candidates to the molinspiration, admetSAR and molsoft server which also provided the information about solubility, permeability, and drug-likeness of each drug molecule along with Pharmacokokinetic and pharmacodynamic properties like GPCR ligand, ion channel modulator, protease inhibitor, kinase inhibitor, enzyme inhibitor, nuclear receptor inhibitor, aqueous solubility, TPSA, blood brain barrier, human intestinal absorption, Caco-2 permeability, AMES toxicity, carcinogenicity and acute oral toxicity.

2. **IN SILICO PHARMACOLOGY**

Initial stages of structure based virtual screening involve the preparation of ligand and target molecules. The protein structural files were fetched out from PDB (www.rcsb.org/pdb). The structure of the active pocket was predicted using ligplot and the residues forming the active pocket were identified. Protein–ligand interactive visualization and analysis was carried out in Pymol viewer 1.5.4.

i. **Preparation of ligands**

Structure of the drug molecules prepared for *in silico* toxicity studies were utilized for structure based virtual screening studies. Openbabel, a standalone tool was used to obtain 3D coordinates for all the drug candidates prepared for *in silico* toxicity studies.
ii. Selection of macromolecule

Based on the evidences, molecular docking studies were carried out for the constructed ligand molecules with different protein molecules to identify the drug-target interactions (Table 3.9).

Table 3.9: Target molecules for in silico docking studies

<table>
<thead>
<tr>
<th>Target molecule</th>
<th>PDB ID</th>
<th>Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>P 38 Map Kinase</td>
<td>1OUK</td>
<td>Antitumor activity</td>
</tr>
<tr>
<td>γ-Aminobutyric acid receptor</td>
<td>4MS4</td>
<td>CNS Depressant activity</td>
</tr>
<tr>
<td>Cyclooxygenase-2</td>
<td>6COX</td>
<td>Anti-inflammatory activity</td>
</tr>
<tr>
<td>Glycogen synthase kinase 3-β</td>
<td>1Q5K</td>
<td>Wound healing activity</td>
</tr>
<tr>
<td>B-Tubulin</td>
<td>1OJ0</td>
<td>Anthelmintic activity</td>
</tr>
<tr>
<td>Glucosamine-6-phosphate Synthase</td>
<td>2VF5</td>
<td>Antibacterial activity</td>
</tr>
<tr>
<td>Cytochrome p450 14 Alpha-sterol Demethylase</td>
<td>1EA1</td>
<td>Antifungal activity</td>
</tr>
</tbody>
</table>

iii. Preparation of macromolecule

The selected protein molecules are edited by removing the hetero atoms like metal ions, water and ligand molecule. Later it was added with C-terminal oxygen, polar hydrogen and Gasteiger charges (Gasteiger and Marsili, 1980).

iv. Molecular docking

Automated docking was used to study the binding of different drug molecules to the active pocket of the macro molecule. A genetic algorithm method implemented in the AutoDock 4.2 was employed to study appropriate binding modes of the ligand in different conformations. For the ligand molecules, Gasteigere–Marsili partial charges were assigned and non-polar hydrogen was merged. All the torsions were allowed to rotate during docking. The grid map was set around the residues forming the active pocket, which was predicted using ligplot (Table 3.10). Grid file was generated using AutoGrid program (Table 3.11) and Lamarckian genetic algorithm and the pseudo-Solis and Wets methods were applied for energy minimization using default parameters. Docking energies were calculated using the following equation (Sanner et al. 1996).
Docking energy = A + B + C + D

Where

- A = Intermolecular energy,
- B = Total internal energy,
- C = Torsional free energy and
- D = Energy of the unbound system

Table 3.10: Amino acid binding pockets of different target molecules for in silico docking studies

<table>
<thead>
<tr>
<th>PDB ID</th>
<th>Amino acids forming active pocket</th>
</tr>
</thead>
<tbody>
<tr>
<td>4MS4</td>
<td>Trp 65, Cys 129, Gly 151, Ser 152, Ser 153, His 170, Tyr 250, Trp 278, Glu 349</td>
</tr>
<tr>
<td>6COX</td>
<td>Tyr 355, Leu 359, Arg 513, Ala 516, Phe 518, Met 522, Val 523, Gly 526, Ala 527, Ser 530, Val 349</td>
</tr>
<tr>
<td>1Q5K</td>
<td>Ile 62, Val 70, Ala 83, Leu 132, Asp 133, Tyr 134, Val 135, Pro 136, Arg 141, Leu 188</td>
</tr>
<tr>
<td>1OJ0</td>
<td>His 6, Ile 16, Phe 20, Gln 134, Thr 136, Phe 167, Glu 198, Phe 200, Met 233, Val 236, Leu 250, Leu 253</td>
</tr>
<tr>
<td>2VF5</td>
<td>Ala602, Val399, Ala400, Gly301, Thr302, Ser303, Cys300, Gln348, Ser349, Thr352, Ser347, Lys603</td>
</tr>
<tr>
<td>1EA1</td>
<td>Gln 72, Tyr 76, Phe 78, Arg 95, Arg 96, Leu 100, Ala 256, Thr 260, Thr 264, Gly 287, Pro 320, Leu 321, Leu 324, Arg 326, Pro 386, Phe 387, Gly 388, His 392, Arg 393, Val 395, Gly 396, Phe 399, Ala 400</td>
</tr>
</tbody>
</table>

Table 3.11: Grid box orientation of AutoGrid program

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>NPTS</th>
<th>Grid center</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>1OUK</td>
<td>60, 60, 48</td>
<td>44.583</td>
</tr>
<tr>
<td>4MS4</td>
<td>52, 46, 54</td>
<td>-23.172</td>
</tr>
<tr>
<td>6COX</td>
<td>56, 46, 56</td>
<td>21.748</td>
</tr>
<tr>
<td>1Q5K</td>
<td>60, 64, 58</td>
<td>18.468</td>
</tr>
<tr>
<td>1OJ0</td>
<td>58, 54, 46</td>
<td>8.412</td>
</tr>
<tr>
<td>2VF5</td>
<td>40, 50, 40</td>
<td>32.198</td>
</tr>
<tr>
<td>1EA1</td>
<td>78, 64, 74</td>
<td>-17.658</td>
</tr>
</tbody>
</table>

NPTS: Number of X-, Y- and Z- grid points