CHAPTER 4

Materials and Methods
# Chapter 4: Materials and Methods

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4. MATERIALS AND METHODS

The present study was initiated with baseline survey through Ethno-pharmacological approach in Biligirirangana Hills, a religious mountain located in Chamarajanagar district of Karnataka state belongs to southern India. The successive analytical studies comprising, physico-chemical, phyto-chemical, antimicrobial & antioxidant properties, wound healing activities and consequently anticancer, antiscake venom activities respectively were executed with selected ethno-medicinal plant drugs and TMF drug formula at both *in vitro* followed by *in vivo* during the period of January, 2011 to November, 2014.

4.1. The Study Area and Ethno-pharmacological survey

The study area concentrates in and around of Biligirirangana hill tracts which come under reserved forest category located at the border region of Karnataka state. The uninterrupted hummocks are located along the line of Western Ghats by the side of the eastern periphery which is the distinctive hot-spot that supports wide range of flora and fauna accomplished naturally irrespective of the season.

![Geo-graphical location showing the Study Area Biligirirangana Hills (B.R. Hills), Chamarajanagara district of Karnataka, India](image)

(A: India; B: Karnataka State; C: Chamarajanagara district and D: Biligirirangana Hills)
The study area biogeographically is a unique sanctuary and it is positioned at 11° and 12° N followed by the ridges of the mounds which is travelled in the north-south direction. It is a projection of the Western Ghats passing in the direction of north-east and congregates the splintered hills of the Eastern Ghats at 78° E (Fig.4.1). This expansion of Western Ghats exclusively constitutes a live bridge between both Eastern and Western Ghats and the sanctuary is located almost in the central point of this bridge.446-448

4.1.1. Observation on Tribes and their Culture

In the baseline survey, it was observed that, the tribal communities are the major indigenous ethnic group of BR Hills-a study area. The Soliga Tribe lives in the different podus surrounded by forests, decisively, the tribe, ‘Soliga’ means ‘people of the bamboo’, a name based on their belief that their ancestors originated from the bamboo. Therefore, they live in podus or settlements of 10 to 50 thatched huts. Since time immemorial, soligas have a rich and deep traditional and indigenous knowledge about therapeutic uses of ethno-medicinal plant drugs which transmits verbally from one generation to the subsequent of their own community. During the survey, it was requested to the targeted community for contributing their knowledge and practices about different aspects of practicing of herbal medicine, drug formulation, mode and duration of treatment against different ailments/diseases starting form cold, cough, fever and to serious diseases like, cancer, wound healing, diabetes, snake bite and respiratory disorders in order to help the other people who are living main domein of the society.

4.1.2 Base-line survey and collection of Ethno-medicinal plants

The ethno-pharmacological survey in the study area mainly aims to identify and ethno-medicinal practices by the tribal and local people living in the surrounding area was recorded. The interviews and inquiries were also conducted individually and in group during the base line surveys. Interviews were chosen after seeking the consent from each Tribal Medicine Men (TMM).

The informations on ethno-medicinal plants comprising, type of plants with taxonomical details, family, genus, species and availability. In addition, the drug preparation parameters like, part used, processing of plant materials, dosage and duration of the treatments along with drug administration against the ailment is
concerned were documented systematically during interaction with the traditional healers.

4.1.3. Local Tribal/Traditional healers

The interaction was conducted with local traditional healers belongs to the study area B.R Hills followed by adjacent villages during October, 2010 to April, 2011. The traditional healers were staying in the region since several years and their main occupation was beekeeping, honey culture, basket making, pickles preparation and cultivation of some crop plants to a certain extent. Additionally, the traditional or tribal healers had rich traditional knowledge (TK) on ethno-medicinal plants, their practices against different ailments. They were interrogated periodically to acquire the necessary informations on traditional medicine knowledge it was found that, the transformation of TK from elders of the tribal community to the next generation which is restricted to the their own community. The very basis on prosperity of TK on ethno-medicinal plants was due to their string mind-set, cultural beliefs and incessant observations. Thus, the TK on ethno-medicinal plants has to be protected by this approach which is at verge of extinction in the modern days.

4.1.4. Interview with Tribal / Traditional healers

The ethno-medicinal plants data was documented by employing the standard methods through interrogation with traditional/tribal healers. The informants were classified according to their seniority, experience, knowledge and ability of therapeutic practices against wide range of diseases. Nearly, 25 informants including both male and female were interviewed with structured questionnaire which comprises their identity, medicinal knowledge, expectancy of practices, specific ailments, mode of drug preparations, treatment protocols and contact details were systematically documented (Fig. 5.3A-F).

The ethno-medicinal plant drugs emphasizing on wound and associated ailments was recorded. As a whole, significant information on ten plant species falls under ten genera of ten different families were documented relating to wound and associated ailments. The components of ethno-medicinal plants used in the drug formulations such as leaves, root, stem, bark, special modifications like, rhizome, tuber etc. were documented. Amongst, leaves and roots followed by whole plants were predominantly used to accomplish the drug.
4.1.5. Collection and Taxo-navigation of Ethno-medicinal Plant drugs

The outcome of the survey and the details comprising of ethnomedicinal plants, practices, parts used, mode of preparations of drug and their active formula etc. were documented and the plant drugs were collected for further critical analysis. Regarding plants, the Voucher specimens for *Andrographis serphyllifolia*, *Dioscorea hispida*, *Glycosmis mauritiana*, *Nothapodytes nimmoniana* Blume and *Rauwolfia densiflora* were collected and identified appropriately by consulting a floral expert. The species identified and the same have been deposited in the Bhoomigeetha Institute of Research & Development (BIRD), Tumkur in collaboration with Dept. of Engineering Chemistry, Akshaya Institute of Technology, Tumkur, Karnataka respectively. The different parts of five ethno-medicinal plant drugs, *A. serphyllifolia* (leaves), *D. hispida* (tubers); *G. mauritiana* (leaves); *N. nimmoniana* (leaves) and *R. densiflora* (whole plant) were collected from different tracts/regions of B.R. Hills of Chamaraja Nagar districts of Karnataka (Fig. 5.2A-H). During collection, the ethno-medicinal plants were subjected for taxonomical classification which was recognized with the help of Standard Flora and acknowledged with authentication as per the procedure. The baseline informations of selected ethno-medicinal plants are documented. The collected plant drug materials were subjected for shade drying till plant materials competent for grinding. The plant materials were then ground well into fine powder using suitable mechanical blender and transferred the material into appropriate containers and made airtight with correct labeling for further study.

4.2. MATERIALS

**Ethnomedicinal plant drugs**

The different parts of five ethno-medicinal plant drugs, *A. serphyllifolia* (leaves), *D. hispida* (tubers); *G. mauritiana* (leaves); *N. nimmoniana* (leaves) and *R. densiflora* (whole plant) were collected from different tracts/regions of B.R. Hills of Chamaraja Nagar districts of Karnataka (Fig. 5.2A-H).

**Instruments**

The instruments such as, Electric blender, Microscope (Olympus), Muffle furnace (Meta lab, Scientific industries, Mumbai), Centrifuge (REMI R-4C & 8RC Centrifuge Machine Bengaluru), Soxhlets Apparatus (Multiple Units), Flash Evaporator, Shaker
incubator, Laminar Air Flow (Meditech, Chennai), Spectrophotometer (UV/Visible, Elico Limited, Hyderabad) etc. were employed in the study.

**Chemicals**

In the study, the standard chemicals of analytical grade were used namely, Picric acid, α-naphthol, Benedict’s reagent, 5% Ferric chloride, 1% Gelatin, 10% sodium hydroxide, Alcohol, Biuret’s reagent, Ninhydrine reagent, Lead acetate, NaOH, Conc.H₂SO₄, copper sulphate, dimethylsulfoxide (DMSO), di-sodium hydrogen orthophosphate (Na₂HPO₄), ethylene diamine tetra-acetic acid (EDTA), Folin and Ciocalteu’s phenol (FC) reagent, ferric chloride, hydrogen peroxide (H₂O₂), Hydroxylamine hydrochloride, sodium dodecyl sulphate (SDS), magnesium sulphate (MgSO₄), potassium chloride (KCl), magnesium chloride (MgCl₂), sodium hydroxide (NaOH), sodium chloride (NaCl), sodium dihydrogen-ortho-phosphate (NaH₂PO₄), trichloroacetic acid (TCA), and Tris-HCl were obtained from Sisco Research Lab., (Mumbai, India). Additionally, other specific solvents and reagents of analytical grade were used in the studies, which were procured from authorized S.D. Fine chemicals Pvt. Ltd., India. The media like, agar, cholesterol, hypochlorite solution, proteose peptone and yeast extract were procured from Hi-Media (Mumbai, India).

**4.3. METHODOLOGY**

The preliminary experiments were conducted at Dept. of Biotechnology & Engineering, CSRF, SIET, Tumkur followed by Applied Chemistry laboratory, Akshaya Institute of Technology, Tumkur Visvesvaraya Technological University, Belagavi (Karnataka) in association with Bhoonigeetha Institute of Research and Development (BIRD), Tumkur. The fractionation and analysis studies were conducted at ‘Azyme Technologies’ and Raghavendra Biotechnologies, Bengaluru for completion of specific technical analysis. The plant materials were well dried in such a way that, all the water molecules evaporated from the sample was monitored and further, the dried plant materials were subjected for grinding using mechanical blender for further use.

**4.3.1. Validation of Ethno-Medicinal Plant materials and Tribal Medicine Formulation**

Ethno-medicinal plant materials and Tribal Medicine Formulations (TMF) were obtained from the Traditional Healers (Fig. 5.2A-H and 5.3A-J) during interactions
and then the samples were scientifically validated based on its physical characteristics in association with an authorized Ayurvedic practitioner, Nisarga Ayurveda Research Foundation, Sakaleshpur, Hassan district (India). The standard protocols were identified and the methodology was employed in the present study based on the descriptions.

4.3.2. Preparation and Processing of Ethno-medicinal Plant drug materials (EMP)

The collected plant materials were subjected for separating different desirable parts like leaves, stem, root/tubers from the main plants or whole plant parts. The selected ethno-medicinal plant drug materials such as leaves, stem, root/tubers were dried under shade for 20 days to ensure the active constituents were free from decomposition and also care was taken to avoid any photochemical degradation. The selected plant parts were powdered using suitable electric blender. The powder was stored in an airtight container and kept in a cool, dark and dry place until analysis commenced following the standard procedures.

4.3.2.1. Extraction of Ethno-medicinal Plant drug materials (EMP) via Successive solvent extraction

The powdered sample (170g) was extracted by maceration method using soxhlet apparatus with different solvents which comprises, from polar to less polar systems such as water, ethanol and methanol and petroleum ether respectively. The macerated extract was subjected for centrifugation at 5000rpm for 15 min and the supernatant was taken-out for the surplus solvent evaporation and the crude extract was taken for further analysis. The macerated extracts were then placed in shaker incubator for 24 h and later subjected to filtration using Whatmann No.1 filter paper.

The test solvents were confirmed based on the yield and feasibility of the solvents during extraction processes and then, organic solvents were removed, firstly by means of a water bath and then in an oven, yielding the extracted compound. The concentrate was designated as crude ethanolic extract of EMP. The extracts of ethno-medicinal plant drugs (EMP) were used to carry out the both phyto-chemical and pharmacological evaluation.
4.3.2.2. Water extraction of Crude EMP Samples

The crude extracts of EMP drugs were prepared using Soxhlet extraction method. The powdered plant material (20g) was uniformly packed into a thimble of Soxhlet unit for extraction. The pre-processed herbal material was subjected for heating with the help of hot plate through constant stirring at 30-40° C for 20 minutes. Then, the water extract was taken out and pass through a Whatmann No.1 filter paper and the filtrate was subjected to dryness. Then, the dried extract attained from filtrate was maintained in the refrigerator at 4°C for further phyto-chemical analysis.

4.3.3. Tribal Medicine Formulation (TMF)

Medicine Formulation is a mixture of the tribal medicinal components of various parts of plants that are used to treat various abnormalities. The parts used for the mixture can be leaves, roots, stem, tubers, twigs, fruits, seeds, flowers and whole plants. The formulation is usually prepared by mixing the components in various amounts and pasting it using cold or warm water. It can be directly applied on to the exterior parts of the body or given for the intake depending on the abnormality being treated. These formulations are the indigenous knowledge of Tribal people in India.

4.3.3.1. Preparation and Processing of Tribal Medicine Formulation (TMF)

The biochemical activity of Tribal Medicine Formulation (TMF) will not be known to the tribals, but their action will be known because of the practice, since many years. The components react with each other and show the suitable activity on the patient. The TMF constituent was subjected for devastating into tiny pieces in the mortar with the help of pestle and then ground well into powder using a suitable electric grinder. The powdered TMF sample comprising of ethno-medicinal plants was then subjected for extractions (aqueous and organic solvents) and finally, the extracted samples were subjected for preliminary phyto-chemical analyses.

4.3.3.2. Extraction of Tribal Medicine Formulation (TMF)

The powdered sample (50g) of the Tribal Medicine Formulation (TMF) was subjected for successive solvents extraction with increasing order of polarity from petroleum ether to ethanol as well as methanol finally; the crude extraction was achieved with water. These organic solvents were specified based on the dissolving efficiency and recovery of the ethno-medicinal plant drugs amongst the organic
solvents used in the study. Meanwhile, the extracts were kept for evaporation to dryness and the dried extracts were subjected to various chemical tests in order to detect the presence of different phyto-constituents.

4.4. Physico-chemical analysis of EMP and TMF

The aqueous extract of the Ethno-medicinal plant drugs and tribal medicinal formulation were subjected to analyze various physico-chemical characteristic features based on WHO guidelines. The extracts from individual ethno-medicinal plant (EMP) drugs and the pre-processed Tribal Medicine Formulation (TMF) were prepared through maceration technique. The samples were exercised further for the physico-chemical analysis to distinguish qualitative parameters such as ash value, water soluble ash value, acid insoluble value, foreign organic matter, moisture content etc. respectively. The evaluation of Pharmaconostical status in plant material through Physico-chemical analysis was done using the standard protocols.

4.4.1. Determination of Ash Contents

The residual material obtained after blowing up of processed material of ethno-medicinal plants was evaluated for the parameters like, total ash content, water soluble ash and acid insoluble ash respectively. Accordingly, the total ash content was measured that the left over substance after the process of ignition; here, the ash obtained explicitly from plant tissue is designated as physiological ash whereas, non-physiological ash content was also generated from extraneous matter derived either from sand or soil which is irrelevant and stick on to the plant material. Similarly, the residual matter attained following boiling of the total ash with dilute hydrochloric acid followed by igniting the left over insoluble substance. In this, the totality of both sand and siliceous ground matter and the difference in the total ash content and the residue generated after treatment of total ash content with water was assessed as water soluble ash.

Total Ash

The material was weighed (about 2-4g) and subjected for ignition using silica crucible. The material was made wider as thin film and ignited steadily by increasing the temperature between 500-600°C till the material becomes white which is free from carbon. Subsequently, the material was allowed to cool with desiccators and weight of the material was recorded. If the ash free from carbon is not achieved, the crucible
was allowed to cool and moisturized by adding 2ml of water or else add saturated ammonium nitrate. Further, the sample was dried on a water bath followed by hot plate and then subjected for ignition to get steady weight. Later, the processed material was cooled in desiccators for 30 minutes weighed accurately and finally, the obtained ash content was calculated as per the standard formula.

\[
\% \text{Ash} = \left( \frac{M_{\text{ash}}}{M_{\text{dry}}} \right) \times 100
\]

**Acid-insoluble Ash**

25 ml of hydrochloric acid was added to the crucible containing total ash covered with watch glass. The sample was then subjected for boiling for 5 minutes after that the watch glass was rinsed with hot water (5 ml) and the same was transferred to another crucible.

The leftover and insoluble substance was maintained on filter paper followed by washing with hot water to get filtrate explicitly with neutral condition. The filter paper restrain with insoluble material was transferred to the crucible which was used in the beginning stage. Then, the sample was subjected for drying on hotplate followed by ignition to get a constant weight. Subsequently, the residue was cooled in desiccators for 30 minutes and weighed accurately. At the end, the substance of the acid insoluble ash was calculated using standard formula.

\[
\% \text{Ash} = \left( \frac{M_{\text{ash}}}{M_{\text{dry}}} \right) \times 100
\]

**Water-soluble Ash**

25 ml of water was added to the crucible containing total ash and boiled for 5 minutes. Then, the matter which was left as unsolvable was collected in a sintered glass crucible otherwise the material was also collected on ash less filter paper. Later, it was subjected for washing with hot water followed by ignition for 15 minutes in crucible at not more than 450°C. Further, the weight of sample was deducted in mg from the total ash content. At the end, the water soluble ash content was calculated.

\[
\% \text{Ash} = \left( \frac{M_{\text{ash}}}{M_{\text{dry}}} \right) \times 100
\]

4.4.2. **Proximate analysis**

4.4.2.1. **Determination of Foreign matter**

The plant drug materials were critically examined for any possible contamination by means of microorganisms or else with insects and animal excreta followed by check
up for any foreign matter, anomalous odor, discoloration leads to deterioration of the plant components etc. The foreign matter will be poisonous and dangerous to the actual status of the plant components therefore, the material should be cleaned before use. Meanwhile these unusual foreign matters should be analyzed through microscope in the plant materials such as soil, sand, dust particles and other inorganic matter should be removed before its usage in the subsequent analysis.

**Foreign organic matter**

10g of sample was taken and the organic matter in the sample was removed by hand picking. Then the collected organic matter was weighed. Finally, the % of foreign organic matter was determined.

\[
\text{% Foreign Organic matter} = \left( \frac{\text{Organic matter weight}}{\text{Total weight}} \right) \times 100
\]

**Sample size**

The foreign matters adheres to the plant components are basically uneven and heterogeneous. Therefore, in the selected ethno-medicinal plant materials where, a critical test was applied to assess the complexity of foreign matter which might be intimately similar to the plant material in appearance. The pooled sample of the collected plant materials were taken for analysis using physico-chemical or else by microscopy methods explicitly. The amount of the foreign matter was calculated based on the portion of the total quantity of foreign matters which was fails to take action in the test from the portions. Accordingly, the plant material was weighed, taking into account of the quantity indicated above as per the specifications mentioned in the test procedures which is based on the plant material is concerned. The material was widen as thin film and made into different groups by visual examination through magnifying lens of necessary resolution. The sample was sieved by an appropriate no. 250 sieve and the part of the sorted foreign matter was weighed (0.05 g) accurately. At the end, the matter in each group was calculated according to the standard formula.

**4.4.2.2. Determination of Moisture content**

The plant materials were dried properly in order to evaluate the water content (in %) in the dried samples of the ethno-medicinal plants. After weighing the sample of plant materials which was recorded as wet weight of the sample and the wet was removed by drying the sample to a steady state at not more than 239º F (115ºC). The sample was allowed to cool using the suitable drying equipment. After cooling, the plant
sample was again and documented as dry weight of the sample. Finally, the moisture content was subjected for calculation using numerical equation is as follows.

The weight of the sample with moisture on adding water (A). The dried sample to a steady weight at a temperature not more than 239°F (115°C). The cooled sample was weighed again and recorded as the dry weight (B). The moisture content was reported to the nearest tenth of 1%.

\[ \%W = \left(\frac{A - B}{B}\right) \times 100 \]

Where, 
W – weight of the moisture in the given sample in %
A – Wet sample weight in g
B – Dry sample weight g

4.5. Evaluation of Plant drugs for Phyto-chemical analysis

4.5.1. Phyto-chemical analysis of EMP and TMF

The different extracts of Ethno-medicinal plant (EMP) drugs and Tribal Medicine Formulation (TMF) were subjected for the qualitative analysis of preliminary phytochemicals towards detection of carbohydrates, proteins, oils and fats followed by the active secondary metabolites such as alkaloids, flavonoids, terpenoids, steroids, tannins, saponins and total phenols etc. using standard procedures 453, 455, 456, 243, 457, 199, 458, 459, 460.

4.5.2. Materials for preliminary phyto-chemical screening

The chemicals used for the screening phyto-chemicals were of standard analytical grade, they are; Picric acid, α-naphthol, Benedict’s reagent, 5% Ferric chloride, 1% Gelatin, 10% sodium hydroxide, Alcohol, Biuret’s reagent, Ninhydrine reagent, Lead acetate, NaOH, Conc.H₂SO₄.

4.5.3. Test for Carbohydrates

The following are the qualitative tests conducted for Carbohydrates.

Molisch’s test

The test was performed using the filtrate which was subjected for treating with α-naphthol solution followed by adding of few drops of concentrated H₂SO₄ by the side of the test tube. The development of ring like structure with reddish violet color at the junction of two layers confirms the presence carbohydrate.
Iodine test

The filtrates were treated with few drops (2-3) of iodine solution and then, the intense blue color was formed which indicates the presence of polysaccharide, the condition of no color apart from iodine solution confirms the presence of mono or disaccharide and the subsequent formation of red color shows the presence of dextrin.

Benedict’s test

The test was performed using Benedict’s reagent which was treated with filtrate and mixed thoroughly. Then, it was subjected for boiling for two minutes which leads to formation of reddish precipitate and confirms the presence of reducing sugar.

Barfoed’s test

The test was performed using barfoed’s reagent which was treated with filtrate and subjected for boiling on a water bath for 3 minutes. After cooling, phosphomolybdic acid was added drop by drop till attaining the clear solution. Later, the intense deep blue color was formed which confirms the presence of monosaccharide similarly; the light blue colored solution was showed the presence of disaccharide.

4.5.4. Test for protein

Biuret test

This test was performed using 5% NaOH and CuSO4 solution which was treated with filtrate and formation of violet color that confirms the presence of protein.

Ninhydrin test

This was performed using Ninhydrin reagent which was treated with filtrate and boiled. The formation blue or purple color confirms the presence of amino acids.

Iso-electric precipitation

The test was performed by treating filtrate with few drops of bromocresol green indicator solution and mixed well. Then 1% acetic acid is added drop by drop until green color is obtained. The curdy white precipitate was formed which showed the presence of phosphor-protein. Subsequently, if no curdy white precipitate that, confirms the presence of glycoprotein.
Xanthoprotein test
The test was performed by treating the filtrate with few drops of conc. HNO₃, heated to boiling and cooled. 40% NaOH is added. Formation of yellow color in acid medium turns to orange color in alkaline medium indicates the presence of tyrosine and tryptophan.

4.5.5. Test for alkaloids
Wagner’s test
This was performed by treating the filtrate with iodine solution in potassium iodide which was designated as Wagner’s reagent. The development of brown precipitate confirms the presence of alkaloids.

Hager’s test
This was performed by treating the filtrate with saturated solution of picric acid which was designated as Hager’s reagent. The development of yellow precipitate confirms the presence of alkaloids

4.5.6. Detection of fixed oils and fats
Spot test
This test was performed by pushing little amount of petroleum ether and benzene extracts between two filter papers and the development of oil stains above the filter paper confirms the presence of fixed oil.

4.5.7. Detection of saponins
Foam test
This test was performed by taking aqueous extracts along with 1ml of alcohol and diluted separately using distilled water. Then, the mixture was shaken well in a graduated cylinder for 15 minutes. The development of froth which is above the surface confirms the presence of saponins.

4.5.8. Test for tannins
This test was performed by taking filtrate that was treated with few drops of 0.1% ferric chloride and development of brownish green or blue black color confirms the presence of tannins.

4.5.9. Test for phenols
The filtrates were treated with 10% ethanolic ferric chloride and the formation of dark blue or else blue green that confirms the presence of phenols.
4.5.10. Flavonoids test

The filtrates were treated with 5ml of diluted ammonia solution at the ratio 1:1 and Conc. H$_2$SO$_4$ will be added. The development of yellow color that confirms the presence of flavonoids and color disappears on standing.

4.5.11. Terpenoids test

The filtrates (5ml) were treated with 3ml of Conc. H$_2$SO$_4$ which was added by the side of the test tubes. The development of reddish brown color confirms the presence of terpenoids.

4.6. Assay for Total Phenolic content

The total phenolic content was determined in the drug formulation by Folin–Ciocalteu method $^{379}$ with slight modifications. About 1g of drug sample was added to 2.5ml of ethanol and subjected for centrifugation at 2°C for 10min. the supernatant was evaporated to dryness and redisolved in 10ml water. Further, 200µl of sample was added to 100µl diluted (1:10) Folin–Ciocalteu reagent and equilibrated for few min. Then, 800µl of 2.5 % aqueous Na$_2$CO$_3$ was added and the mixture was allowed to stand for 60min at room temperature with intermittent shaking. Finally, the absorbance of the blue colour solution was measured at 765nm on UV-visible spectrophotometer. Gallic acid (50mg %) was used as standard. The absorbance of solution was compared with gallic acid calibration curve. The total phenolic content was expressed as gallic acid equivalents (GAE mg/g dry weight of sample) and the values were presented as mean ±SD of triplicate analysis.

4.7. Assay for Cytotoxicity of TMF Extract

The cytotoxic assay was conducted to evaluate the probable action on brine shrimp lethality. The whole test was conducted as per the standard procedure $^{461,327, 462}$.

4.8. Antimicrobial activity $^{463,464}$

The different extracts of ethno-medicinal plant drugs were subjected for antimicrobial evaluation using agar well diffusion method followed by Minimum inhibitory concentration (MIC). Similarly, the extracts were tested for antifungal activity by using modified micro dilution technique. Therefore, the minimum inhibitory bacterial concentrations (MBC) and minimum inhibitory fungal concentrations (MFC) were systematically performed through a serial dilution technique with 96-well micro-titer plates$^{465}$. 
Collection of pathogenic microbes from healthcare centre

Dilution of cultures

Preparation of nutrient agar media.

Pouring the media into Petri plates.

Cultures are inoculated over the Petri-plates containing media (Spread plate method)

Immersed paper discs in different organic solvent extracts separately and placed on the inoculums

Incubated for 24 h at 35°C for bacteria and room temperature for fungi

Fig.4.2. Flow chart for the analysis of Antimicrobial property

4.8.1. Preparation of Nutrient Agar Media

Weigh Peptone: 5g (a semi-digested protein), Beef extract: 3g (source of C, N, vitamins and inorganic salts), NaCl: 5g, Agar 6g, Distilled water: 500ml

Dissolve the weighed amount of peptone, beef extract, NaCl and agar in 250ml of distilled water with constant stirring and then make it up to 500ml with distilled water.

4.8.2. Preparation of Potato Dextrose Agar (PDA) Media

The constituents comprising of peeled potato tubers: 20g, Dextrose: 5g, Agar: 6g, Distilled water: 250ml prepared respectively. The weighed amount of potato tubers was washed peeled and sliced materials were taken in the beaker containing 100ml distilled water. These potato tubers are boiled for 30 minutes. The boiled potato tubers were filtered through muslin cloth/cheese cloth, squeezing out all liquid and weighed the amount of dextrose was added to the filtrate. Then, distilled water (100 ml) was taken in a separate beaker and subjected for heating at 56 °C and the amounts of agar was dissolved bit by bit to it then, add the potato extract (filtrate) to it and make up the volume to 250 ml by distilled water.

4.8.3. Development of Microbial Inoculum

The pathogenic microorganisms (bacteria and fungi) were collected from authorized healthcare center, Siddhartha Medical College, Tumkur. The procured
microorganisms were maintained on nutrient agar slants through the process of sub cultures. The bacteria species namely *Salmonella typhi*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* was used as a model organism. Similarly, the fungal species namely *Aspergillus niger* and *Aspergillus flavus* were spread over the Petri plates containing nutrient agar media. The antibacterial activity was evaluated by disc diffusion method using different extracts of ethno-medicinal plants and subjected for incubation at 35°C for 24 h for bacteria whereas, for fungi the room temperature condition was adopted.

4.9. Antioxidant Activities

The different extracts of ethno-medicinal plant drugs and tribal medicine formulation was subjected for the evaluation of antioxidant potentials using DPPH radical scavenging followed by ABTS methods respectively. Additionally, the antioxidant enzymes like, Superoxide dismutase and catalase were also determined as per the standard protocols.

4.9.1. DPPH Free Radical Scavenging Assay

The DPPH (1, 1-Diphenyl-2-Picrylhydrazyl) free radical scavenging assay was performed for the extracts of ethno-medicinal plant drugs as per the standard procedure. Initially, a solution of 0.007w/v DPPH in 95% methanol was prepared and different extracts of plant drugs were evaluated for antioxidant potentials with the variable test sample (50-300μg/ml) concentrations. About 3ml of different extracts of both ethno-medicinal plant drugs and tribal medicine formulations were mixed with 1 ml of DPPH solution in dark conditions. The strong oxidizing agent called ascorbic acid was used as standard. Similarly, the DPPH solution (1ml) was mixed with standard solution of ascorbic acid that was prepared at different concentrations in dark conditions. Then, the solutions both standard ascorbic acid and extracts of ethno-medicinal plant drugs were subjected for incubation for 30minutes. Later, the absorbance was measured at 517nm using UV Spectrophotometer. In addition, the extract or else solvent were considered as blank and the percent inhibition free radical scavenging activity in the sample was calculated using the standard formula is as follows.

\[
\text{DPPH radical scavenging activity (\%) = } \frac{\text{Control OD} - \text{Sample OD} \times 100}{\text{Control OD}}
\]
4.9.2. Total Antioxidant activity (FRAP assay)

The FRAP assay was performed to appraise the total antioxidant activities in the extracts of ethno-medicinal plant drugs by employing standard method. The stock solution was prepared with 300 mM acetate buffer (3.1 g C₂H₃NaO₂-3H₂O and 16 ml C₂H₄O₂) at pH 3.6 and 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) in the solution containing 40 mM HCl, and 20 mM FeCl₃·6H₂O. Subsequently, the working solution was prepared freshly by combination of 25 ml acetate buffer, 2.5 ml TPTZ, and 2.5 ml FeCl₃·6H₂O and the solution was maintained at 37°C prior to use. The extracts of plant drugs (150μl) were subjected for reaction with FRAP solution (2.85ml) for 30minutes in the dark conditions. The complex (ferrous tripyridyl-triazine complex) of colored product was read at 593nm and the linear standard curve was obtained ranging between 200 and 1000 μM FeSO₄. Finally, the result was represented in μM Fe (II)/g of dry mass which were compared with standards 467,562.

4.9.3. Assay for Total reducing ability

The aqueous extract of tribal medicine formulation was subjected to estimate total reduction capabilities using standard method. The reaction mixture was incubated 37°C and the absorbance was read at 700nm using UV spectrophotometer. The expression in the reaction mixture with increasing potential was measured as superior reducing potentiality 469.

4.9.4. ABTS radical scavenging assay

The ABTS assay (2, 2-azinobis 3-ethylbenzothiazoline-6-sulfonic acid) was performed by preparing a stock solution which is consisting of 7 mM ABTS solution and 2.45 mM potassium persulfate solution at equal proportion. This was subjected for incubation at room temperature for 12h during dark condition that was further yielded a dark colored solution which contains radicals of ABTS. Subsequently, to perform each assay, the fresh working solution was prepared by mixing stock solution with methanol (50%) and the initial absorbance was 0.700 (± 0.02) at 745 nm at 30°C temperature. Further, the extracts of ethno-medicinal plant drugs and tribal medicine formulation were used at variable concentrations (50-3000μg/ml). Then, these concentrations were subjected for reaction with known volume (3 ml) of ABTS solution and the absorbances were taken at 734 nm. Meanwhile, the ascorbic acid was taken as positive control. Finally, the radical scavenging activity was assessed based
on the percent activity of ABTS and calculated the value as per the standard formula

\[ \text{ABTS radical scavenging activity (\%)} = \frac{\text{Control OD} - \text{Sample OD} \times 100}{\text{Control OD}} \]

4.10. Thin Layer chromatography (TLC)

The separation of diversified mixtures present in the plant drugs was demonstrated using thin layer chromatography which was carried out on a readymade TLC sheet of required grade and the TLC chamber was filled with two different solvents \textit{i.e.,} as a stationary phase and the mobile phase was contain toluene, chloroform and ethanol at the ratio of 5:5:2. Further, markings at the edges were done on the TLC sheet by leaving 1.5 cm and the different extracts were spotted on the TLC sheet with equal distances. After spotting of extracts the sheet was kept in TLC chamber immediately and the plates were left for half an hour. The mobile phase moves upwards along with the extracts which were spotted on the stationary phase and the compounds in the extracts gets separated based on their affinity. The fluorescence bands were observed at 254nm (short wavelength) in UV light followed by 366 nm (long wavelength) using UV light in the respective plates. After the run, plates were dried and sprayed with NP/PEG reagents were used to detect the bands on the TLC plates and the observation of chromatograms was done under long wavelength UV followed by visible light. The movement of the active compound was expressed by its retention factor (Rf), values were calculated for different samples\textsuperscript{470,471}.

\[ \text{Rf} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent front TLC plates}} \]

4.11. Evaluation of TMF drug for Wound healing

4.11.1. Ointment preparation for topical application

As per the standard procedure, the ointment was prepared with extracts (alcohol free) of Tribal Medicine Formulation (TMF) for the topical application. The ointment of 10\% and 15\% (w/w) in association with extract using soft white paraffin base was accomplished.

\textit{Experimental Animals}

The experiment consisting of animal model, albino rats (\textit{Rattus novergicus}) of both sexes, weighing about 400–500mg/kg each, were used for the study (Reg No. CSRF/
IAEC /2013/022). The animals were dwelling inside the polypropylene cages and standard conditions comprising of 12/12 h light - dark cycle at 25 ± 3 °C along with relative humidity between 35–60% were maintained. The animals were then monitored regularly by feeding with standard chow and water. The experimentation was implemented according to the ethical paradigm relating to handling of animals.

4.11.2. Acute Toxicity Studies

The experiment was performed by categorising the albino rats into 6 groups (n = 6 per cage) based on normal and good health of both sexes which were then subjected for fasting for overnight. Further, the animals were fed with increasing the doses (1, 2, 4, and 8 g/kg body wt.) of extracts of tribal medicine formulation. The animals administered orally with crude extract of TMF and ethanol extracts in doses of up to 8 g/kg body wt. did not produce any apparent symptom of toxicity or else mortality in animals even after 14 days administration.

4.12. Wound models

In the experiment, the animals were back shaved after subjecting them for anesthetization using Pentobarbitone as per the specification in wound models corresponding with different doses of 400 and 800 mg/kg body weight.472,473

4.12.1. Excision wound model

The animals after shaved at predetermined dorsal area were subjected for operation as per the clinical procedure. The skin was excised in a circular shape of complete thickness (500 mm²) using a distinct marker. The animals with excision type of wound were undressed and maintained separately in the open environment which facilitate monitoring and evaluation of wound contraction through re-epithelialisation process. The extracts comprising of 10 and 15% w/w of TMF drug was applied regularly to the wound area along standard drug, 0.2% w/w nitrofurazone ointment. The wound was completely healed on regular application of the drug which facilitate the promising changes occurred during the different phases of wound healing. The area of wound contraction on continuous days of treatment was calculated in terms of rate of wound contraction. Finally, the wound contraction achieved with respect days of treatment was considered as period of re-epithelialisation.472
**Measurement of Wound area**

The planimetry method comprising of tracing the wound margin using a graph paper on alternative days regular was employed to assess the developmental changes in the wound area. The measurement of wound contraction (in mm²) on graph paper was represented. The percent wound contraction was calculated using the following formula

\[
\text{% Wound Contraction} = \frac{\text{Healed Area}}{\text{Total Area}} \times 100
\]

**Method adopted**

In the animal experimentation with respect to wound healing, both excision and incision wound models were employed with topical application of the ointments prepared with different extracts of plant drug. The animals (albino rats) were categorized into following groups:

- **Group- I**: Served as control without local application of any ointment.
- **Group- II**: Treated with Nitrofurazone ointment (0.2% w/w) & served as standard control.
- **Group- III**: Tribal Medicine formulation (TMF) 400mg/kg body weight.
- **Group- IV**: Tribal Medicine formulation (TMF) 800mg/kg body weight.
- **Group- V**: Drug formulation in ethanol extract based on body weight -400mg/kg.
- **Group-VI**: Drug formulation in ethanol extract based on body weight- 800mg/kg.

The clinical experiment comprising of six animals in each category was maintained and the treatments were initiated on the day of operation as per the clinical protocol. The treatment of wound (2nd, 4th, 8th, 10th, 12th, 14th, 16th and 18th days) was incessantly monitored till the wound contraction (on 20th day) was achieved completely. Finally, the wound contraction was calculated based on the area of each animal with the help of Planimeter.

**4.12.2. Incision wounds**

The selected animals were shaven after anaesthetization and subjected for para-vertebral incisions (2-6cm). Subsequently, the wounds were bunged up with intermittent sutures of about 1 cm area separately and on the 7th day, the sutures were
taken away. Later, the animals were transferred to different cages for critical observation in which, wound recovery, breaking strength was measured in all the animals after 10th day of wounding.

In the clinical experiment, the animals were categorized into different groups consisting of six animals in each group. After 10th day of observation, the animals were subjected for dissected and the tensile strength was determined. After that, the sutures were quietly removed from both wound area cautiously. Further, with the help of knife the wound strips of same width were incised and at a predetermined distance two blades were placed. Consequently, a pair of steel clips was used to fix the either sides of each strip, wherein one clip was allowed lynching on a stand and on other side, polyethylene bottle was made to fill the water steadily till the strip of the wound broken down at the wound location. Finally, the breaking of wound with respect to the pressure exerted by the amount of water used was recorded and the tensile strength (g) was calculated in the wound.

4.12.2.1. Dead-space wounds

In this clinical test, the animals (albino rats) were subjected for formation of wound at the lumbar region of their dorsal surface by instilling two polypropylene tubes comprising of 0.5×2.5 cm each focussing one on both sides. Later, the granuloma tissues were formed at implanted tubes was excised vigilantly after the 10th day of wounding. On the other side, at a particular temperature (64 °C), the granuloma tissue in one tube was explicitly maintained and this was then subjected for evaluation of antioxidant enzymes. Similarly, other set of granuloma tissue was subjected for the determination of tensile strength after that; these were kept for drying in hot air oven at 60°C for 24 h and calculated the dry weight299,474. In addition, the analysis of hydroxyproline content in acid hydrolysate of the dry tissue was also evaluated

4.12.2.2. Biochemical Attributes

In the continued part of the clinical experiment, the granuloma tissue was excised from the dead space wound model which was then subjected for homogenization with phosphate buffered saline at pH 7.0 and the same was centrifuged in cold conditions. The antioxidant enzymes specifically, superoxide dismutase and the catalase were estimated in the supernatant through spectrophotometric method475-477.
4.12.2.3. Histopathological studies

Subsequently, the histo-pathological assessment was made through the sections of granuloma tissues with two imperative stains (Van Gieson and Masson Trichrome) wherein, the set down status of collagen prototype was critically examined\textsuperscript{478, 479}.

4.13. Evaluation of ethno-medicinal plant drug formula for anti-cancer property using Cancer cell lines

The anticancer analysis was done in the tribal medicinal formulation as per the standard procedure explained by the authors\textsuperscript{480, 481, 563}.

4.13.1. Ethno-medicinal plant Drug dilutions

Dilution is a process of reducing the concentration by adding of a solution such as water. The crude extracts were diluted according to the requirements. The extracts were syringe flittered to avoid contamination prior to use.

4.13.2. Drug addition Procedure

1. After 24 h of incubation of the cells, the spent media was discarded from all the wells.
2. The 2\textsuperscript{nd} and 3\textsuperscript{rd} column was added with media and vehicle (10\% distilled water made in media) respectively. The first and second column was taken as control for evaluating the extracts.
3. Add 100µl of diluted drugs to each well in the order of the lowest dilution to the highest dilution.
4. The plate was incubated for 24 h in a CO\textsubscript{2} incubator.

4.13.3. Plating of HeLa cell lines

Procedure

1. The flask in which the cells have reached the confluent stage was selected. The cells were then washed twice with the saline after discarding the media. The saline should be discarded properly from the flask as saline inhibits the action of trypsin.
2. Then, 500µl of trypsin-EDTA was added to the flask and wash the inner surface properly with trypsin. Wait for 1-5 minutes until the cells get detached from the surface.
3. After the addition of fresh complete media (2 ml) and collected this complete solution in a sterile centrifuge tube and centrifuge at 1500rpm for 10 minutes.

4. The supernatant was disposed and the total cell count was done after adding 1ml of fresh media.

5. Then, 0.1 to 0.2 million cells were pipetted out and made up the volume up to 6ml.

6. Subsequently, 10µl of cell suspension was transferred to each well of the 96 well plates. The left over cell suspension was subjected for cryopreservation.

7. The plate was kept in 5%CO₂ incubator for 24h.

4.13.4. Trypan Blue assay

Trypan blue is an imperative stain applied to examine the dead tissues or the cells which takes blue colour selectively. This assay is used to determine the dead cell count as well as the living cell count. The living cells will have an intact membrane which does not allow the dye to pass since the cells are very selective in compounds. The dead cell does not process an intact membrane and takes up the stain.

Procedure
1. After 24h of addition of the drug, the spent media and drug was discarded.
2. Then, 50µl of trypan blue was added to each of the wells from which media and drug has been discarded. The plate is kept undisturbed for 1 minute.
3. The trypan blue was then discarded from the wells properly with the help of pipette.
4. The plate was observed under the microscope.
5. The same procedure after 48 h of addition of the drug was repeated.

4.13.5. Hoechst stain assay

This assay was done to check that the cell death has occurred due to apoptosis (the cell death due to destruction in the actual functions of membrane followed by cells that in turn leads to inflammation) or not.

Procedure
1. This assay was performed after 48h of addition of the drug.
2. Then, 100µl of Hoechst dye was put into the wells and subjected for incubation for 10 minutes in dark conditions.
3. The fluorescent microscope was switched on and waited for 10 minutes to allow the microscope to warm and emit the fluorescent light.

4. The plate under the microscope was observed and note down the observations.

4.13.6. MTT assay

The MTT assay was performed to reduce the yellow composite called 3-(4,4-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) under the influence of succinate dehydrogenase enzyme in the complex-II at the electron transport chain that occurs in Mitochondria. The turn down of MTT can occur only physiologically active cells so, the activity will be determined based on the viability status of the cells. The whole reaction comprises, the MTT which go into the cell subsequently to the mitochondria wherein, MTT was reduced to form an insoluble Formosan product which appears in dark purple color. Meanwhile, the cells are solubilized through DMSO solvent thus, the released Formosan product was assessed through spectrophotometric method $^{564}$.

Procedure

1. After 24 /48 h of addition of the drug this colorimetric assay was performed.
2. Then, 20µl of MTT reagent was added to the wells already containing the media as well as the drug.
3. The plate was incubated in the incubator for 3h.
4. After 3 h the MTT reagent was discarded along with the media and the drug and adds 100µl of DMSO to stop the reaction of MTT.
5. The plate was kept for incubation for 1 hr.
6. After incubation, the suspension was pipetted out from each well into the plate reader.
7. The measurement was taken on a plate reader using 550nM as analytical wavelength and 630nM as wavelength of standard range.
8. The data was recorded and columns were tabulated.


4.14.1. Requirements

The chemicals used for the phyto-chemical analysis were of standard analytical grade namely, Picric acid, α- naphthol, Benedict’s reagent 5%Ferric chloride, % gelatin,
10% Sodium hydroxide, Alcohol, Biuret’s reagent, Ninhydrine reagent, Lead acetate, NaOH, Conc.H₂SO₄. For Thin layer Chromatography, TLC sheet, TLC chamber, Micro pipettes, 1% Ninhydrine solution and solvents used are n-propanol and water.

4.14.2. Methodology

Buffer extraction method is used to extract the proteins (salting in).

4.14.2.1. Protein Precipitation using Ammonium Sulfate

The protein will be solubilized when the salts (<0.15 M) are added increasingly which is referred as salting-out. Correspondingly, the solubility factor will be decreased considerably at higher concentrations of salt which leads to precipitation. The protein is finely suitable for gel filtration where, buffer can play a lead role in exchange for ammonium sulfate⁴⁸².

4.14.2.2. Separation of proteins by Gel-filtration chromatography (GFC)

The proteins will be separated exclusively with respect to variability in molecular dimension and the permeability or else porous matrix which are the deciding factors in accessing the proteins based on size. Generally, the larger molecules have lesser accessibility similarly, the smaller molecules have larger accessibility. Subsequently, the column will be packed with matrix along with test sample then, the separation process will be influenced by aqueous buffer that is passed in the course of column which is referred as mobile phase. The protein molecules will be cramped in the volume of the column and the beads will be sweep via column by means of mobile phase. Therefore, the sample will be eluted on the basis of category of size that will be in decreasing pattern. Finally, the eluted samples will be analyzed through spectrophotometric method which further facilitates the qualitative measurement of proteins using SDS-Polyacrylamide gel Electrophoresis.
4.15. Source of Venom

The snake venom type, *Naja naja* which is generally called common cobra was procured from the authorized sources, Irula Snake Catchers Industrial Society Ltd., Chennai, (Tamilnadu), India. The snake venom was subjected for lyophilization and maintained with control conditions that specifies at 2 to 8°C for further study.

4.15.1. Partial purification of Snake venom protein by Gel filtration chromatography

The venom of snake, *Naja naja* sample was obtained from the authorized sources as per the procedure. The venom sample was purified using column chromatography loaded with the silica gel. A known amount (30mg) of *Naja naja* venom was dissolved in buffer and loaded to column of bed volume; 94.2 and fraction was eluted at the flow rate of 2ml per 5 minutes. The bed volume; 11/2 of protein samples was eluted for each tube the optical density was measured at 280nm.
4.15.2. Purification of sPLA$_2$ from *Naja naja* venom

sPLA$_2$ from *N. naja* snake venom was purified to homogeneity as described in the previous report$^{483}$. Briefly, *N. naja* venom (110 mg) was fractionated on CM-Sephadex C-25 column (1.4 x 120 cm) using phosphate buffer of different molarities (0.02 - 0.4) and pH (7.0 - 8.5). This was eluted into thirteen major fractions. Fraction NN-I-PLA$_2$ was chosen for further purification. The lyophilized fraction NN-I-PLA$_2$ (25 mg) was re-chromatographed on CM-Sephadex C-25 column (1.6 X 30 cm) which was pre-stabilized by way of instituting phosphate buffer (0.1 M) at pH 7.0. Correspondingly, the protein was eluted using phosphate buffers (0.1 M) at pH 7.0 followed by 0.15 M at pH 7.5. This was eluted into NN-I-PLA$_2$ (a) and NN-I-PLA$_2$ (b) fractions. These 2 fractions were dissolved separately in 0.1 M NaCl and loaded on to Sephadex G-50 column (1.4 X 92 cm) which was pre equilibrated with NaCl (0.1 M) and the same solvent will be eluted. The PLA$_2$ activity was assessed at both peaks which are of homogenous conditions. The homogeneity was checked by SDS-PAGE$^{484}$ and RP-HPLC (Fig.4.4).

4.15.3. Sodium Dodesyl Sulfate-Poly-Acrylamide Gel Electrophoresis (SDS-PAGE)

In the study, the standard protocol was employed to analyze the patterns of protein using SDS-PAGE$^{484}$. The homogeneity of purified sPLA$_2$ samples were checked using
12.5 % resolving gel. The gel was stained with 0.25 % Coomassie brilliant blue (R-250) and the wide range of proteins were envisaged as a result of staining.

4.15.3.1. Estimation of Protein

The TMF sample was subjected for Protein measurement by employing the standard method using BSA as protein standard\textsuperscript{385}. The aliquots of 0-1 ml standard BSA was taken in clean and dry test tubes, 5 ml Lowry’s reagent (98 ml of 4 % sodium carbonate + 1 ml of 2 % copper sulphate + 1 ml of 2 % sodium potassium tartarate) was added. Later, 0.5 ml of Folin Ciocalteu’s (FC) phenol reagent was added at the proportion of 1:1 (diluted with water) after 15 min and the same was kept for 30 min. Finally, the OD was taken at 660 nm.

4.15.3.2. Phospholipase A\textsubscript{2} Activity assay

The PLA\textsubscript{2} assay was conducted by employing the standard protocol prescribed in the previous report\textsuperscript{418} which involves the measurements of free fatty acids liberated by the enzyme catalyzed hydrolysis of egg- phosphotidyl choline. A set of 7 cleaned and dried vials were taken; to this, 50\mu l of phosphotidyl choline and 50-200ug of concentrated venom samples were added. The PLA\textsubscript{2} enzyme was calcium dependent thus, 20\mu l of calcium was added after that, Tris HCl buffer at pH 7.5 was also added ultimately the volume was made up to 1ml. Subsequently, petroleum ether (200ul) was added and subjected for incubation for 1 hour at 37\degree C. The reaction was then arrested (after incubation) by adding 500ul of doles mixture and 1000ul of petroleum ether furthermore, the mixture was subjected for centrifugation at 500xg for 5 min. Later, the supernatant (0.5 ml) was collected; to this 500ul of chloroform: petroleum ether was added along with; 500ul of cobalt nitrate reagent was also added. Further, the mixture was centrifuged at 1200xg for 10 min and the supernatant of about 0.5ml was collected. Finally, to this 1-nitroso 2-naphthol solution of about 750ul and 2000ul of methanol was added. Then, the OD for the sample was spectrophotometrically read at 540nm.

4.15.3.3. Anti-hemorrhagic Study

This analysis was conducted in order to find out the Snake venom activity through Myonecrosis and lung hemorrhage activity, the Mouse lung tissue and also the muscle tissue layer was collected and added with Saline buffer to one layer and the cell death did not occur. Then the other layer was added with Snake venom and the cells started dying by forming tumors. Then the other layer was added with the Snake venom sample
along with the TMF extract sample and there was decreased activity of the enzymes which were present in the venom and this showed the activity of TMF extract over venom in neutralizing the effect of the venom.

4.15.3.4. Anti-hemorrhagic Test

The matured and fertile eggs were procured from the authorized sources for conducting the experiment as per the standard protocol. The selected eggs were subjected for incubation at 38°C for 3-4 days. The eggs were splintered on 4th day and again incubated till 6th day of experiment. The filter paper, Whatman No.2 was used in the form of discs of about 2-3mm diameter and these discs were drenched with a standard hemorrhagic dosage (SHD) of snake venom, *Naja naja* of 3g/1.5l alone followed by, venom along with different concentrations of TMF drug respectively. Further, the discs were placed emphasizing on main bilateral vein of yolk sac membrane and kept for 3h in order to assess the hemorrhagic corona which was measured explicitly using a specific tool. Meanwhile, the buffered saline solution was used as control which was used for the preparations of venom long with TMF drug extract as well. Finally, anti- hemorrhagic readings were taken as Minimum Effective Neutralizing Dose (MEND).
4.16. Statistical Analysis

The animal experiments were statistically analyzed explicitly by means of one-way ANOVA as well as Dunnet’s multiple comparisons (Computer programme Excel Software, 1999). The results generated from all the parameters in response to the treatments with both EMP and TMF were compared with each other. The outcome of the statistical analysis (p <0.05) were considered to be statistically significant. The concentration producing 50% of the maximum response (LC_{50} or IC_{50}) was obtained by the best visual fit from the plot of the individual experiments. The concluding data were analyzed by employing SPSS Software.10 (Chicago, IL, USA).