4.1 Introduction

Pharmacology is the science of drugs. It contributes to the development of drugs, the understanding of their mechanisms of action and the description of their conditions of use. It also deals with the assessment of their efficiency and their safety. The study of medicinal plants and their products emphasize pharmaceutical use, and this can be based on pharmacological action.

The scientific study of traditional plant medicines can be considered as a major part of ethnopharmacology. Ethanopharmacology can be defined as ‘the scientific study of materials used by ethnic and cultural groups as medicine’ and in most instances this is synonymous with the study of traditional medicines.

It should also be commemorate that the active molecules isolated from traditional medicinal plants might not only provide valuable drugs but are also valuable as ‘lead molecules’ which might be modified chemically, or serve as a template for the design of synthetic molecules incorporating the pharmacophore responsible for the activity.

The term ‘drug discovery’ is generally used to refer to the isolation of molecules with activity. Several recent surveys have shown that using ethnopharmacology as a basis of selecting species for screening results in a significant increase in the discovery of novel active compounds.
The discovery process is composed of several stages. The first stage must be the reported use of a naturally-occurring material for some purpose which can be related to a medical use. Consideration of the ethnic practices associated with it is important in deciding possible bases of the reputed activity. If there is an indication of a genuine effect, then the material needs to be identified and characterized according to scientific nomenclature. It can then be collected for experimental studies, usually comprising some tests for relevant biological activity linked with isolation and structure determination of any chemicals present which might be responsible.

The ‘active’ compounds are usually discovered by several cycles of fractionation of the extract linked with testing for activity of each fraction, until pure compounds are isolated from the active fractions, a process known as bioassay-guided fractionation. These compounds, once their activity is proven and their molecular structure ascertained, serve as the leads for development of clinically-useful products.

The mostly reliable type of information arises from in-depth studies carried out by field workers, living in the particular community of a particular ethnic group, on the use of the local plants and other materials. Before such knowledge can be investigated scientifically, the information provided will often need clarification and translation into scientific terms.

In vivo animal models of disease states are the next best approach but expensive. For biological activity, therefore utilize in vitro animal tissue, cultured cells, cloned receptors or enzymes systems. A large number of tests have been developed in recent years and they offer the opportunity to carry out large numbers of tests using small amounts of materials in a short time and are, therefore, well suited to bioassay-guided fractionation.
4.2 Acute oral toxicity study

Acute oral toxicity study\cite{51}

The procedure was followed by using OECD guidelines (Organization of Economic Cooperation and Development) 423 (Acute Toxic Method). The acute toxic class method is a stepwise procedure with 3 animals of a single sex per step. Depending on the mortality and/or moribund status of the animals, on the average 2-4 steps may be necessary to allow judgment on the acute toxicity of the test substance (Fig:2). This procedure results in the use of a minimal number of animals while allowing for acceptable data based scientific conclusion. The method uses defined doses (5, 50, 300, 2000 mg/kg body weight) and the results allow a substance to be ranked and classified according to the Globally Harmonized System (GHS) for the classification of chemicals which cause acute toxicity.

Experimental procedure

Male wistar rats weighing 150-200 g were used for the study. The starting dose level of polyherbal formulation was 2000 mg/kg body weight p.o. As most of the crude extracts possess LD$_{50}$ value more value more than 2000 mg/kg p.o., the starting dose used was 2000 mg/kg p.o. Dose volume was administered 0.1 ml/10 gm body weight to the rat which were fasted over night with water \textit{ad libitum}. Food was with held for a further 3-4 hours after administration of ethanolic extracts of bark and leaf and observed for signs of toxicity. Body weight of the rats before and after termination were noted and any changes in skin and fur, eyes and mucous membranes and also respiratory, circulatory, autonomic and central nervous system and somatomotor activity an behavior pattern were observed, and
also sign of tremors, convulsion, salivation, diarrhoea, lethargy, sleep and coma were noted. The onset of toxicity and signs of toxicity also noted.
4.3 Invitro antioxidant activity of bark and leaf extracts of *Thespesia populnea*

Oxygen required in many metabolic reactions in the body particularly for the release of energy. In the process oxygen can be transformed under some condition into singlet oxygen hydroxyradical, superoxide anion and hydrogen peroxide. They are highly reactive and cause damage to biomolecules, cells and tissues. These free radicals have been implicated in the progress of several diseases such as cardiovascular disease, cancer, respiratory disease, diabetes and other degenerative disease\[^{52}\]. Many plants extract and phytochemicals especially the phenolic compounds such as flavonoids and tannins have been shown to have antioxidant free radical scavenging properties\[^{53}\].

Natural occurring polyphenolic compounds such as tannins, flavonoids are having good antioxidant activity. Several studies have been to access the antioxidant properties of natural products\[^{54}\]. Scientific information on chemical constituent and antioxidant property of various plants less widely used in the medicine is still rather scared. However relevant experimental work has not been exploited. Therefore our aim in this study was to evaluate the *in vitro* antioxidant activity of methanolic extract of bark and leaf of *Thespesia populnea* to determine the relation between its antioxidant properties and its use in traditional medicine.

Preparation of plant extract

Plant material:

The plant materials were powdered. The dried and powdered leaves and stem bark (each 50 gm) extracted with 500 ml of methanol by maceration process separately for 48 hour. The methanol was removed under vacuum and a solid mass were obtained.
Estimation of total phenolic content.\cite{55}

The phenolic content and the total tannin were estimated for bark and leaf powder separately by using the following method. Ten milligrams of standard gallic acid was dissolved in 100 ml distilled water in a volumetric flask (100 mg/ml of stock solution). From the above stock solution 0.5 to 2.5 ml of aliquots were pipette out into 25 ml volumetric flasks. Ten ml of distilled water and 1.5 ml of Folin Ciocalteu's reagent (diluted according to the label specification) were added to each of the above volumetric flasks. After 5 min, 4 ml of 20% sodium carbonate solution was added and the volume was made up to 25 ml with distilled water and incubated at room temperature for 30 min and the absorbance of the solution was recorded at 765 nm and a standard curve of absorbance verses concentration of gallic acid (50-250 µg) was plotted.

One gram of the powdered drug was extracted with 70% methanol (15 x 3 times), filtered, pooled and the volume was adjusted to 50 ml with 70% methanol in a volumetric flask. From the stock solution, suitable quantity of the extract was taken into a 25 ml volumetric flask and 10 ml of water and 1.5 ml of Folin Ciocalteu reagent were added to it. The mixture was kept for 5 min, and then 4 ml of 20% sodium carbonate solution was added and made up to 25 ml with double distilled water. The mixture was incubated at room temperature for 30 min and the absorbance was recorded at 765 nm in a spectrophotometer. Percentage of total phenolic contents was calculated from calibration curve of gallic acid (50-250 µg) plotted using the above procedure and total phenolic contents were expressed as % gallic acid.
Estimation of total tannins \cite{56}

Two grams of the powdered drug was extracted for 20 hr with petroleum ether. The residue was boiled for 2 hr with 300 ml of double distilled water. It was cooled, filtered with Whatman No. 1 filter paper and diluted to 500 ml with double distilled water. 25 ml of this infusion was pipetted into 2 liter porcelain dish to which 20 ml indigo solution and 750 ml double distilled water was added. This was titrated with standard KMnO\textsubscript{4} (0.1 N) solution by adding 1 ml at a time, until blue solution changed to green, after which a few drops were added at a time until solution turned golden yellow in color (A). Similarly, a mixture of 20 ml indigo solution and 750 ml of double distilled water was titrated (B). The percentage of total tannins was calculated using the formula, % Total tannins = \left[ \frac{(A-B) \times \text{Actual Normality of KMnO}_4 \text{ solution} \times 0.004157 \times 1000}{\text{Weight of drug sample taken} \times 0.1} \right]. Each ml of 0.1 N KMnO\textsubscript{4} \equiv 0.004157 \text{ g of total tannins.}

Preparation of the extraction for invitro antioxidant activity

Hundred milligram of dried methanolic extract was dissolved in 100 ml of methanol to make a stock solution of 1 mg/ml. Aliquots from this stock solution were further diluted with methanol as per the concentration required. Free radical scavenging activity of the methanol extract was tested in three \textit{in vitro} models.

Inhibition of superoxide radicals by riboflavin photoreduction method\cite{57}

Superoxide scavenging activity of \textit{Thespesia populnea} was determined by the NBT (Nitroblue tetrazolium) reduction method. The reaction mixture contained EDTA (0.1 M) containing 0.0015% NaCN, riboflavin (0.12 \mu M), NBT (1.5 \mu M), various concentration of the extract and phosphate buffer (M /15 M pH 7.8) in a final volume of 3 ml. The tubes were uniformly illuminated under an incandescent lamp for 15 minutes and the optical density was measured at 530 nm before and after illumination. The percentage inhibition of superoxide generation
was evaluated by comparing the absorbance values of the control and experimental tubes.

**Inhibition of lipid peroxide formation by Fe$^{2+}$/ ascorbate system**

The reaction mixture contained rat liver homogenate (0.1 ml, 25% w/v) in Tris.HCl buffer (20 µM, pH 7.0), KCl (150 µM), Ferrous ammonium sulphate (0.8 µM) and ascorbic acid (0.3 µM) in a final volume of 0.5 ml and was incubated for 1 hr at 37°C. The lipid peroxide formation was measured. The incubated reaction mixture (0.4 ml) was treated with SDS (0.2 ml, 8%), thiobarbituric acid (1.5 ml, 20%, pH 3.5). The total volume was then made up to 4 ml by adding distilled water and kept in a water bath maintained at 100°C for 1 hr. After cooling 1 ml of distilled water and 5 ml of mixture of n-butanol with pyridine (15:1 v/v) were added and shaken vigorously. The absorbance of the organic layer was measured at 560 nm after centrifugation. The percentage inhibition of lipid peroxide formation was determined by comparing the results of the drug treated and non treated samples.

**Hydroxyl radical scavenging activity**

Hydroxyl radical scavenging was measured by studying the competition between deoxyribose and the extract for hydroxyl radicals generated for the Fe$^{3+}$/ ascorbate/ EDTA/ H$_2$O$_2$ system. The hydroxyl radicals attack deoxyribose, which eventually results in TBARS formation. The reaction mixture contained deoxyribose (2-8 µM), FeCl$_3$ (0.1 µM), EDTA (0.1 µM), ascorbate (0.1 µM), potassium hydrogen phosphate-potassium hydroxide buffer (20 µM, pH 7.4) and various concentrations of the drug in a final volume of 1 ml. The reaction mixture was incubated for 1 hr at 37°C. Deoxyribose degradation was measured on TBARS and percentage inhibition was calculated.
4.4 Antidiabetic activity of *Thespesia Populnea* bark and leaf extract against streptozotocin induced diabetic rats

Diabetic mellitus (DM) is the condition arising due to abnormal metabolism of carbohydrate, proteins and fats. It is caused by insulin deficiency, often combined with insulin resistance \[^{60}\]. This disorder occur world wide and its occurrence is increasing quickly in most of the countries. Various complications develop as a consequence of the metabolic derangement in diabetes \[^{61}\]. The treatment of DM is based on parenteral insulin and oral anti-diabetic drugs. Oral hypoglycemic agents, currently used have serious side effect hence there is a need to search a newer anti-diabetic agents that having high therapeutic efficacy with minimum side effect \[^{62}\]. This may be fulfilled by treating DM with traditional medicine using anti-diabetic agents from medicinal plants.

The fruit extract of the plant has already shown a significant hypoglycemic effect. Hence in present study, the ethanolic extract of *Thespesia populnea* bark and leaf was investigated for hypoglycemic effect in steptozotocin induced diabetic rats and to compare this effect with glibenclamide, a standard hypoglycemic agent and also measure the lipid peroxide, superoxide dismutase and catalase enzyme level in the liver and kidney of the animal.
Animal

Wistar rats (150-200 gm) were purchased from King Institute, Chennai for experimental study. They were acclimated to animal house condition feed with commercial pellets Rats chon (Hindustan Lever Ltd, Bangalore, India) and had free access to water. The experimental protocol was approved by the IAEC (Institute Animal Ethical Committee) of CPCSEA (Committee for the Purpose of Control and Supervision of Experiment on Animal).

Anti-diabetic activity

Experimental Induction of diabetes

After fasting for 18 hr 40 rats were injected by intraperitoneally with a single dose of 50 mg/kg streptozotocin after dissolving it in freshly prepared ice cold citrate buffer (pH 4.5). After the injection they had free access to feed and water and were given 5% glucose solution to drink over night to counter the hypoglycemic shock. The development of diabetes was confirmed after 48 hr of the streptozotocin injection. The animal having fasting blood glucose levels more than 200 mg/dl were selected for the experimentation. Out of 40 animals 3 were died before grouping and one was omitted from the study because of mild hyperglycemic. Remaining 36 diabetic animals were divided into 6 groups each having 6 rats.
Experimental protocol:

Group I – streptozotocin induced diabetic animals received 1% SCMC 5 ml/kg p.o. for 15 days.

Group II – streptozotocin induced diabetic animals received *Thespesia populnea* leaf extract (TPLE) extract 200 mg/kg p.o. for 15 days.

Group III – streptozotocin induced diabetic animals received *Thespesia populnea* leaf extract (TPLE) extract 400 mg/kg p.o. for 15 days.

Group IV – streptozotocin induced diabetic animals received *Thespesia populnea* bark extract (TPBE) extract 200 mg/kg p.o. for 15 days.

Group V – streptozotocin induced diabetic animals received *Thespesia populnea* bark extract (TPBE) extract 400 mg/kg p.o. for 15 days.

Group VI – streptozotocin induced diabetic animals received the standard drug glibenclamide 1.25 mg/kg p.o. for 15 days.

All the group of animals received the treatment for 15 days. Blood samples were collected one hr after the drug administration and the day 5\(^{th}\), 10\(^{th}\), 15\(^{th}\) to determine the blood glucose level by electronic glucometer \(^{[64]}\). The organ like liver and kidney were removed on 15\(^{th}\) day and 10% tissue homogenate were prepared with 0.025 M Tris-Hcl buffer (pH 7.5). After centrifugation at 10,000 rpm for 10 minutes, the clear supernatant was used to measure thiobarbituric acid reactive substances (TBARS) and hydroperoxides.
For the estimation of non-enzymic and enzymic antioxidants, tissue was minced and homogenized (10% w/v) in 0.1 M phosphate buffer (pH 7.0) and centrifuged for 10 minutes and the resultant supernatant was used for estimation such as catalase, superoxide dismutase and glutathione-S-transferase \(^{[65]}\).

Statistically analysis:

The statistical analysis was carried out by using one-way ANOVA (Analysis of Variance) followed by Dunnet’s ‘t’ test. P values < 0.05 were considered as significant.
4.5 Diuretic activity of bark and leaf extract of *Thespesia populnea*

Diuretic activity[^66]

Albino rats of either sex weighing 150 to 200 gm were divided into six groups of six animals each. The animals were fasted for 24 hr and water was given *ad libitum* during fasting. Then the drug administered as by the following protocol. All the extracts were suspended with 1% SCMC for administration to the animal by p.o.

Experimental protocol:

- **Group I** – Administered with normal saline 10 ml/kg, p.o.
- **Group II** – Administered with aqueous extract of bark 400 mg/kg, p.o.
- **Group III** – Administered with ethanol extract of bark 400 mg/kg, p.o.
- **Group IV** – Administered with chloroform extract of bark 400 mg/kg, p.o.
- **Group V** – Administered with ethyl acetate extract of bark 400 mg/kg, p.o.
- **Group VI** – Administered with standard drug furosemide 2 mg/kg, p.o.

Immediately after the respective treatments the animals were placed in metabolic cages and urine was collected in the measuring cylinder up to 5 hr. The volume of urine, sodium, potassium and chloride ions[^67,68] were estimated in the urine for assessing diuretic activity.
Diuretic activity of leaf extract of *Thespesia populnea*

Diuretic activity[^66]

Albino rats of either sex weighing 150 to 200 gm were divided into six groups of six animals each. The animals were fasted for 24 hr and water was given *ad libitum* during fasting. Then the drug administered as by the following protocol. All the extracts were suspended with 1% SCMC for administration to the animal by p.o.

Experimental protocol:

- **Group I** – Administered with normal saline 10 ml/kg, p.o.
- **Group II** – Administered with aqueous extract of leaf 400 mg/kg, p.o.
- **Group III** – Administered with ethanol extract of leaf 400 mg/kg, p.o.
- **Group IV** – Administered with chloroform extract of leaf 400 mg/kg, p.o.
- **Group V** – Administered with ethyl acetate extract of leaf 400 mg/kg, p.o.
- **Group VI** – Administered with standard drug furosemide 2 mg/kg, p.o.

Immediately after the respective treatments the animals were placed in metabolic cages and urine was collected in the measuring cylinder up to 5 hr. The volume of urine, sodium, potassium and chloride ions[^67,68] were estimated in the urine for assessing diuretic activity.
4.6 Cytotoxic effect of ethanolic extracts of bark and leaf of *Thespesia populnea* on human cancer cell lines

**Extracts**

*Thespesia populnea* bark extract (TPBE)

*Thespesia populnea* leaf extract (TPLE)

**Cell lines used**

HT29-Colon cancer, U251-Glioblastoma and HepG2- Liver cancer

**MTT assay**[69]

MTT assay is laboratory test and standard colorimetric assay (an assay which measures changes in color) for measuring cellular proliferation (cell growth). It is used to determine cytotoxicity of potential medicinal agents and other toxic materials. Yellow MTT (3-(4, 5-Dimethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide) is reduced to purple formazan in the mitochondria of living cells. A solubilization solution (usually dimethyl sulfoxide) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at 562 nm by a spectrophotometer. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion is directly related to the number of viable (living) cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced by using the formula.

Percentage growth inhibition = 100 – \{(mean OD of test group / mean OD of control group) ×100\}
Procedure

0.1 ml of the U251, HT29 and HepG2 cell suspensions (containing $5 \times 10^5$ cells / 100 micro liter in DMSO such that the final concentration of DMSO in media is less than 1%) were added to the 96 well plates and kept in carbon dioxide incubator with 5% CO$_2$, at 37$^\circ$C for 72 hour. Blank contains only cell suspension and control wells contain 1% DMSO and cell suspension. After 72 hour, 20 microliter of MTT was added and kept in CO$_2$ incubator for 2 hour followed by 80 microliter of lysis buffer (15% Sodium Lauryl Sulphate in 1:1 Dimethyl formamide and water). The plates were covered with aluminum foil to protect it from light, and then the 96 well plates are kept in rotary shaker for 8 hour. After 8 hour, the 96 well plates were processed on ELISA reader for absorption at 562 nm. The readings were averaged and viability of the bark and leaf extracts were compared with DMSO control (Table 12). The mean GI$_{50}$ were found to be 57.33 mg/ml and 46.67 mg/ml for extracts respectively. From the results, we have concluded that various extracts of *Thespesia populnea* possess cytotoxic effect against human cancer cell lines$^{[70]}$. 