EXPERIMENTAL PROCEDURE

3.0 EXPERIMENTAL PROCEDURE

The present study has been undertaken to investigate the antioxidant potential, free radical scavenging efficacy, cytotoxic and antibacterial Properties of leaves, flowers and bark of *Denolix elata*. The methodology adapted for the study is as follows:

3.1. Collection and Identification of plant samples

3.2. Preparation of plant extracts for analysis

3.3. Qualitative Screening of phytochemicals constituents.

3.4. Quantitative analysis of phytochemicals.

3.5. Determination of nutritive values in plant samples

3.6. Determination of Antioxidant status

3.6.1. Enzymic Antioxidants

3.6.2. Nonenzymic Antioxidants

3.7. Assessment of free radical scavenging effect

3.8. Biosafety Screening of plant samples

3.9. Evaluation of antibacterial activity

3.1. COLLECTION AND IDENTIFICATION OF PLANT SAMPLES

The fresh leaves, flowers and bark of the plant *Denolix elata* were composed from Coimbatore district, Tamilnadu. The plant was identified by their vernacular names and later taxonomical identification was made by the taxonomist of the Botanical survey of India, Southern circle, Tamilnadu Agricultural University, Coimbatore. Leaves, flowers and bark of the plant were cut into small pieces. Air dried in the shade, ground into coarse powder, stored in an airtight container and used for the assays.

3.2. PREPARATION OF PLANT EXTRACT FOR ANALYSIS

3.2.1. Extraction Procedure for Antioxidant Activity

The leaves, flowers and bark of *Denolix elata* were extracted with various buffers and the extracts were analyzed for the enzymic and nonenzymic antioxidants.

3.2.2. Extract Preparation for Free Radical Scavenging Activity

10g of each leaves, flower and bark of *Denolix elata* were shaken separately with petroleum ether, chloroform, methanol and water for 72 hours on an orbital shaker. Extracts were filtered using whatmann No.1 filter paper, the filtrate was concentrated to dryness and the residue was used for analysis.

3.2.3. Preparation of the Extract for the Determination of Antibacterial Activity and Cytotoxic Effect

10g of powdered plant samples of *Denolix elata* was subjected to soxhlet extraction with organic solvents with increasing order of polarity such as petroleum ether, chloroform, methanol and water. Each extract was kept in distillation unit to separate the solvents from samples and allowed to evaporate to dryness. Concentrated extracts were dissolved in DMSO and used for evaluation of antibacterial activity. Methanol extracts of samples were used for cytotoxic screening of plant samples.

3.3. QUALITATIVE SCREENING OF PHYTOCHEMICAL CONSTITUENTS

Phytochemical screening was performed using standard procedures. The presence or absence of alkaloids, flavonoids, saponins, phenols, glycosides (Raaman, 2006), tannins, reducing sugar (Iyengar, 1995), phytosterol, terpenoids (Siddiqui and Ali, 1997) and anthraquinones (Ayoola *et al.*, 2008) were detected in leaves, flowers and bark of *Denolix elata*. The procedure is given in Appendix I.

3.4. QUANTITATIVE ANALYSIS OF PHYTOCHEMICALS

3.4.1. Determination of Alkaloid

Alkaloid content was determined by the procedure as described by Harbone, 2005 and is elaborated in Appendix II.

3.4.2. Estimation of phenols

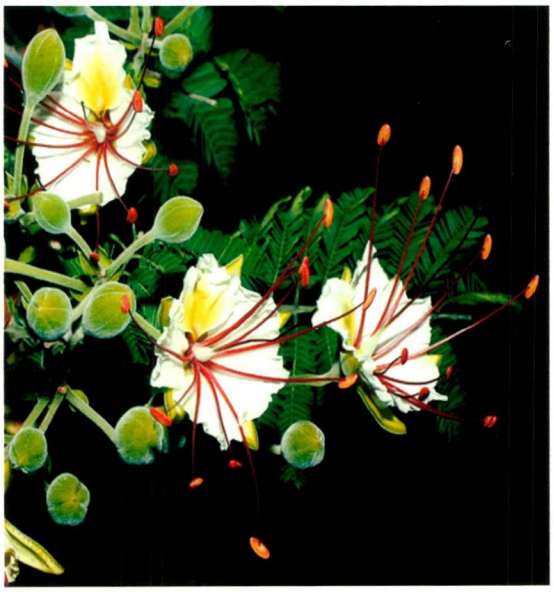
Phenolic content was estimated by the method of Malick and Singh (1980) which is explained in Appendix III.

PLATE NO.I

PARTS OF *Denolix elata*

LEAF

FLOWER



BARK

3.4.3. Estimation of reducing sugars

Determination of reducing sugar by Nelson-Somogyi method (1952) is stated in Appendix IV.

3.4.4. Estimation of tannins

Tannin content was estimated by the method of Robert, 1971 and the procedure is given in Appendix V.

3.4.5. Estimation of chlorophyll

Chlorophyll content of the plant samples was determined by the procedure as described by Aron, 1949, and is given in Appendix VI.

3.5. DETERMINATION OF NUTRITIVE VALUES OF THE PLANT SAMPLES

Nutrient analysis was done in leaves, flowers and bark of *Denolix elata* using parameters such as Carbohydrates and Proteins.

3.5.1. Estimation of Carbohydrates

The Carbohydrate content of the plant samples was analysed by the method of Hedge and Hofreiter (1962) as explained in Appendix VII.

3.5.2. Estimation of Proteins

The protein content was determined according to Lowry *et al.*, 1951 as stated in Appendix VIII.

3.6. DETERMINATION OF ANTIOXIDANT STATUS

3.6.1. Determination of the Activity of Enzymic Antioxidants

The leaves, flowers and bark of *Denolix elata* were analysed for enzymic antioxidants such as catalase, glutathione peroxidase, glutathione s-transferase, polyphenols oxidase, peroxidase and superoxide dismutase.

3.6.1.1. Estimation of catalase

Catalase was estimated by the method as described by Luck (1971) and is given in Appendix IX. The enzyme catalyses the decomposition of hydrogen peroxide, which is measured by decrease in absorbance at 240nm.

3.6.1.2. Estimation of glutathione peroxidase

Glutathione peroxidase catalyses the reduction of hydroperoxides by utilizing GSH. Determination of glutathione peroxidase activity is carried out according to the method of Rotruck *et al.*, 1973 as elaborated in Appendix X.

3.6.1.3. Estimation of glutathione s-tranferase

The enzyme was assayed by the method of Beutler *et al.*, 1984. Glutathione s-transeferase catalyses the formation of conjugates of GSH and 1-chloro-2,3-dinitrobenzene, which is read at 340nm and the procedure is explained in Appendix XI.

3.6.1.4. Estimation of peroxidase

Peroxidases catalyze the oxidation of a wide variety of organic and inorganic compounds (Matos, 2010). The activity of peroxidase of the plant sample was determined by the method of Reddy *et al.*, 1995 as described in Appendix XII.

3.6.1.5. Estimation of polyphenoloxidase

Polyphenol oxidase (PPO) is a copper-containing enzyme and it has multicatalytic functions such as the hydroxylation of monophenols to o-diphenols (cresolate activity) and the oxidation of o-diphenols to o-quinones (cathecolase activity) (Li *et al.*, 2010). The polyphenols oxidase activity of the sample was estimated by the method of Esterbauer *et al.*, 1977 as stated in Appendix XIII.

3.6.1.6. Estimation of superoxide dismutase

SOD catalyses the dismutation of superoxide radicals. Measurement of SOD activity was based on the adrenaline that transforms spontaneously to adrenochrome in the presence of air at p^H 10.2 (Misra and Fridovich, 1972). The procedure is explained in Appendix XIV.

3.6.2. Determination of the Levels of Nonenzymic Antioxidants

The levels of the non enzymic antioxidants such as Carotenoids, Vitamin C, Vitamin E, Flavonoids, Polyphenols and Reduced glutathione were assessed in the leaves, flowers and bark of *Denolix elata*.

3.6.2.1. Extraction and Estimation of Carotenoids

Carotenoids were analysed by the method of Zakaria *et al.*, 1979 and the details are mentioned in Appendix XV.

3.6.2.2. Estimation of Ascorbic acid

Vitamin C acts as antioxidant by scavenging superoxides, hydroxyl radicals and various lipid peroxides (Patil *et al.*, 2008). Ascorbic acid was estimated by the method of Roe and Kuther 1953 and the procedure is given in Appendix XVI.

3.6.2.3. Estimation of α-Tocopherol

Tocopherol content was determined by Rosenberg 1992 as elaborated in Appendix XVII.

3.6.2.4. Estimation of Flavonoids

Flavonoids with some specific chemical structure can react with Al^{3+} and form a red complex which gives a maximum absorption at 510nm (He, 2009). The procedure in Appendix XVIII explained by Zhishen *et al.*, 1999 was followed for determination of flavonoids.

3.6.2.5. Estimation of Polyphenols

The procedure of Malick and Singh, 1980 was followed to estimate the polyphenols as stated in Appendix XIX.

3.6.2.6. Estimation of Reduced glutathione

GSH content was estimated according to the method of Moron *et al.*, 1979 as explained in Appendix XX.

3.7. ASSESMENT OF FREE RADICAL SCAVENGING POTENTIAL

Following assays were performed to assess the free radical scavenging effects of the various parts of the plant extracts.

3.7.1. ABTS radical scavenging assay

The actual procedure employed is presented in Appendix XXI as described by Shirwaiker *et al.*, 2006.

3.7.2. DPPH radical scavenging assay

The experimental details of DPPH (Mensor *et al.*, 2001) assay are documented in Appendix XXII.

3.7.3. H_2O_2 radical scavenging activity

The H_2O_2 scavenging ability of the extracts of the plant parts was determined according to the method of Ruch *et al.*, 1989. The procedure is explained in Appendix XXIII.

3.7.4. Hydroxyl radical scavenging activity

The procedure of Elizabethbeth and Rao, 1990 was followed to determine hydroxyl radical scavenging activity as explained in Appendix XXIV.

3.7.5. Ferrous iron chelating activity

Ferrous iron chelating capacity of the plant samples was determined by the method of Carter, (1971) as predicted in Appendix XXV.

3.7.6. Determination of Lipid peroxidation assay in plant homogenate

An *invitro* model of goat liver was used for the induction of lipid peroxidation with H_2O_2 as an oxidant. The procedure is explained in Appendix XXVI (Okhawa *et al.*, 1979).

3.7.7. Determination of Superoxide production *invitro*

The potential of plant sample to inhibit the generation of superoxide was determined by the method of Mccord and Fridovich, 1968 as elaborated in Appendix XXVII.

3.7.8. Determination of Nitricoxide production *invitro*

Appendix XXVIII explains the procedure given by Green *et al.*, 1982 to estimate the extent of nitric oxide inhibition by plant extracts.

check year

3.8. BIOSAFETY SCREENING OF PLANT EXTRACTS

Brine shrimp lethality bioassay was carried out to investigate the cytotoxicity of extracts of *Denolix elata*. The actual procedure is given in Appendix XXIX as explained by Zakaria *et al.*, 2007.

3.9. ASSESMENT OF ANTIBACTERIAL ACTIVITY

A rapid increase in the resistance of human bacterial pathogens during the recent three decades is caused by emergence of new mechanisms of microbial resisitance and a widespread application of antibiotics in medicinal treatment. Plant extracts are a very rich source of secondary metabolites with antibacterial action and their application provides an opportunity to effectively combact, among other things, antibiotic-resistant bacterial strains. It has been shown that, *invitro*, test compounds originating from plants possess antibacterial activity against wide spectrum of human bacterial pathogens (Krolicka *et al.*, 2009). In the present study, the antibacterial activity of extracts from *Denolix elata* was tested against bacterial isolates that are major cause of infections namely *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Staphylococus aureus* and *Shigella flexneri.* Antibacterial activity was carried out by agar well method elaborated in Appendix XXX as described by NCCLS (1997).

3.10. STATISTICAL ANALYSIS

Experimental results are expressed as means ± SD. All measurements were replicated three times. Analysis of the data was performed on the original data by one-way analysis of variance (ANOVA) by sigma stat. Differences at $P < 0.05$ were considered significant. The LC_{50} values were calculated for the Brine shrimp lethality assay using Probit analysis (Finney, 1971).