3.0 EXPERIMENTAL PROCEDURE

The methodology followed for the "Evaluation of Antioxidant properties, Cytotoxic effect and Antibacterial activity of *Mukia maderaspatana* (L.)" is as follows:

3.1 COLLECTION AND IDENTIFICATION OF PLANT SAMPLE

The plant sample *Mukia maderaspatana (L.)* (Family: Cucurbitaceae) was collected from Coimbatore region of Western Ghats, Tamilnadu. The plant material was further taxonomically identified by the Botanical survey of India, Southern Regional centre, Tamil nadu Agricultural University, Coimbatore, India. Fresh leaves and stems of *Mukia maderaspatana (L.)* were collected, washed and air dried under shade at room temperature for three days. The dried parts of the plant were then separately powdered using a mechanical grinder and stored in an air tight container and used for further investigation.

3.2 PREPARATION OF PLANT EXTRACT

(a) Preparation of the extract for analysis of antioxidant levels

The powder obtained from various plant parts namely leaf and stem were extracted with respective buffers to analyze the enzymatic and nonenzymatic antioxidant levels in the plant parts.

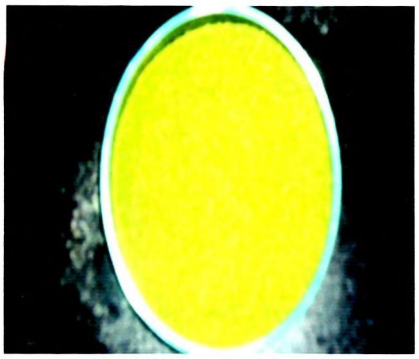
(b) Preparation of the extract for determining the free radical scavenging activity

Five grams of each sample was extracted with petroleum ether (40 - 60° C), ethanol, ethyl acetate and water separately by keeping in a mechanical shaker for 48 hours. It was then filtered through Whatmann No. 1 filter paper and the crude extracts obtained were evaporated at 50° C. The residue obtained was dissolved in dimethyl sulphoxide (DMSO) which was used for determining the free radical scavenging activity.

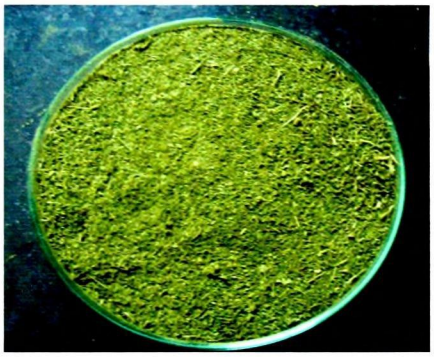
PLATE I

Mukia maderaspatana L.

LEAF POWDER



STEM POWDER



(c) Preparation of the extract for evaluating antibacterial activity and cytotoxicity test

Ten grams of each plant sample was extracted with petroleum ether (40 - 60° C), ethanol, ethyl acetate and water by Soxhlet apparatus for 24 hours. The crude extracts obtained were evaporated by distillation unit. The residue was dissolved in their respective solvents and used for screening antibacterial activity. The ethanolic extract alone is used for cytotoxicity test.

3.3 PHYTOCHEMICAL ANALYSIS

Variety of herbs and plants contain different phytochemicals with bioactive compounds that are valuable for therapeutics. It may help in protection against chronic diseases (Babu *et al.*, 2009).

3.3.1 PRELIMINARY QUALITATIVE PHYTOCHEMICAL ANALYSIS

Important constituents of plants such as alkaloids, flavonoids, saponins, phenols, glycosides (Raaman, 2006), anthroquinones (Ayoola et al, 2008), tannins, reducing sugars (Iyengar, 1995) terpenoid and phytosteroid (Siddiqui and Ali, 1997) were screened by the methods as previously described and is in Appendix – I.

3.3.2 DETERMINATION OF PHYTOCHEMICAL CONTENT

3.3.2.1 Alkaloids

Determination of alkaloids was carried out by the method of Harbone, (2005) and is explained in Appendix – II.

3.3.2.2 Total phenols

Estimation of total phenols was analyzed by the procedure explained by Malick and Singh, (1980) and is described in Appendix – III.

3.3.2.3 Tannins

Estimation of tannins by Vanillin- hydrochloride method was determined by the procedure proposed by Robert, (1971) and is given in Appendix – IV.

3.3.3.4 Reducing sugar

Determination of reducing sugar by Nelson-Somogyi method was explained by Somogyi M., (1952) and is described in Appendix – V.

3.3.3.5 Chlorophyll

Chlorophyll content was estimated by the method of Arnon, (1949) and is in Appendix- VI.

3.4 DETERMINATION OF NUTRITIVE VALUE OF THE PLANT

Primary plant metabolism synthesizes essential compounds which are present in all plant species. These are like natural laboratories with a great source of biosynthesized chemical compounds such as proteins, carbohydrates, phenols and fats which are chief components of animal nutrition (Neha and Rekha, 2010).

3.4.1 ESTIMATION OF CARBOHYDRATES

The total carbohydrate content of the plant parts was determined by the method described by Hedge and Hofreiter (1962) and is given in Appendix – VII.

3.4.2 ESTIMATION OF PROTEIN

The total protein content of the plant sample was estimated by Lowry *et al.*, (1951) method and the procedure is given in Appendix – VIII.

3.5 DETERMINATION OF FREE RADICAL SCAVENGING ACTIVITY

Reactive oxygen species (ROS), which consists of free radicals such as hydroxyl (OH^-), superoxide (O_2^-), nitric oxide (NO), peroxyl (RO_2^-), lipid peroxyl (LOO^-) radicals and non-free radical species such as hydrogen peroxide (H_2O_2), singlet oxygen, ozone (O_3), lipid peroxidation (LOOH) are different forms of activated oxygen (Palash *et al.*, 2009).

3.5.1 DPPH RADICAL SCAVENGING ACTIVITY

The 1,1-diphenyl 1-2-picryl hydrazyl (DPPH) scavenging activity was determined by the method described by Mensor *et al.*, (2001) and the procedure is given in Appendix- IX

3.5.2 ABTS RADICAL SCAVENGING ACIVITY

The procedure proposed by Shirwaiker *et al.*, (2006) was followed for determining 2,2-azino-bis (3-ethyl benzothiazoline-6-sulfonic acid) ammonium salt (ABTS) scavenging effect and the method is explained in Appendix X.

3.5.3 HYDROGEN PEROXIDE SCAVENGING ACTIVITY

Appendix- XI explains the method for analysis of hydrogen peroxide scavenging activity proposed by Ruch *et al.*, (1989).

3.5.4 FERROUS ION CHELATING ACTIVITY

Ferrous ion chelating activity was determined by the method explained by Carter, (1971) and is in Appendix – XII

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3.5.5 DETERMINATION OF INHIBITION OF SUPEROXIDE GENERATION

Inhibition of superoxide generation was determined by the method described by Mccord and Fridovich, (1968) and the procedure is given in Appendix-XIII.

3.5.6 DETRMINATION OF INHIBITION OF NITRIC OXIDE GENERATION

The method of Green and Hill (1984) was followed for the determination of inhibition of nitric oxide generation and is explained in Appendix – XIV.

3.5.6 DETERMINATION OF INHIBITION OF HYDROXYL RADICAL GENERATION

Inhibition of hydroxyl radical generation was done by the method proposed by Elizabeth and Rao, (1990) and is in Appendix- XV.

3.5.8 DETERMINATION INHIBITION OF LIPID PEROXIDATION IN GOAT LIVER HOMOGENATE

Inhibition of lipid peroxidation in goat liver homogenate was analyzed by the method of Okhawa *et al.*, (1979) and is explained in Appendix- XVI.

3.6 DETERMINATION OF ANTIOXIDANT POTENTIAL

Antioxidants are vital substances which possess the ability to protect the human body from damage by free radical-induced oxidative strees (Khalil *et al.*, 2007). The antioxidative system includes both enzymatic and non enzymatic systems.

3.6.1 ENZYMATIC ANTIOXIDANTS

The enzymatic antioxidants which consists of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione– s- transferase (GST), peroxidase (POX) and polyphenol oxidase (PPO), which limit the cellular concentration of free radicals and prevent excessive oxidative damage (Patil *et al.*, 2007).

3.6.1.1 ESTIMATION OF SUPEROXIDE DISMUTASE (SOD)

Superoxide dismutase activity was estimated by the method described by Misra and Fridovich (1972) and is explained in Appendix-XVII.

3.6.1.2 ESTIMATION OF CATALASE (CAT)

The activity of catalase was determined by the method of Luck, (1974) which is described in Appendix – XVIII.

3.6.1.3 ESTIMATION OF PEROXIDASE (POX)

The activity of peroxidase was determined by the method described by Reddy *et al.*, (1995) and the procedure is explained in Appendix- XIX.

3.6.1.4 ESTIMATION OF GLUTATHIONE PEROXIDASE (GPx)

The method for estimation of glutathione peroxidase was followed which was proposed by Rotruck *et al.*, (1973) and explained in Appendix- XX.

3.6.1.5 ESTIMATION OF GLUTATHIONE-S-TRANSFERASE (GST)

The method of Beutler (1984) was followed to determine the glutathione-s-transferase and the procedure is explained in Appendix- XXI

3.6.1.6 ESTIMATION OF POLYPHENOL OXIDASE (PPO)

The method of Esterbauer *et al.*, (1977) was followed to estimate the polyphenol oxidase activity which is elaborated in Appendix- XXII.

3.6.2 NON ENZYMATIC ANTIOXIDANTS

Non enzymatic antioxidants like reduced glutathione (GSH), α-tocopherol (vitamin E), ascorbic acid (vitamin C), carotenoids etc., oppose the toxic actions of lipid peroxides and oxygen radicals and limit the amount of lipid peroxide formation (Sadanand *et al.*, 2008).

3.6.2.1 ESTIMATION OF ASCORBIC ACID

The procedure of Roe and Kuether, (1953) was followed to determine the ascorbic acid content as explained in Appendix- XXIII.

3.6.2.2 ESTIMATION OF α-TOCOPHEROL

Appendix-XXIV explains the Emmeric-Engel method by Rosenberg (1992) followed for the estimation of α-tocopherol.

3.6.2.3 ESTIMATION OF TOTAL CAROTENOIDS

Carotenoids content was analyzed by the method of Zakaria *et al.*, (1979) as described in Appendix-XXV.

3.6.2.4 ESTIMATION OF REDUCED GLUTATHIONE

The level of reduced glutathione was estimated by the method proposed by Moron *et al.*, (1979) as described in Appendix-XXVI.

3.6.2.5 ESTIMATION OF POLYPHENOLS

The content of polyphenols was determined by the method explained by Malick and Singh, (1980) and is given in Appendix-XXVII.

3.6.2.6 ESTIMATION OF TOTAL FLAVONOIDS

The procedure in Appendix- XXVIII explained by Zhishen *et al.*, (1999) was followed for determination of flavonoids.

3.7 CYTOTOXIC EFFECT OF THE PLANT

Brine shrimp lethality test (BST) was used to predict the cytotoxic activity in the plant exracts (Zakaria *et al.*, 2007). Brine shrimp lethality bioassay was done by Meyer *et al.*, (1982) method with minor modifications and given in Appendix- XIX.

3.8 DETERMINATION OF ANTIBACERIAL ACTIVITY

3.8.1 BACTERIAL STRAINS

Microorganisms used in the determination of antibacterial activities of different plant extracts were *Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumoniae, Escherichia coli, Shigella dysentriae.* All bacterial strains were obtained from the Department of Biochemistry, Biotechnology, Avinashilingam Deemed University, Coimbatore. Different bacterial strains were maintained on nutrient agar and subcultures were freshly prepared before use.

3.8.2 ANTIBACTERIAL SCREENING TEST

The bacterial test was performed using agar-well diffusion assay (NCCLS, 1997) with different plant extracts and explained in Appendix-XXX.

3.9 STATISTICAL ANALYSIS

All data were expressed as mean±S.D. The results were subjected to statistical evaluation with Student's t-test for phytochemical content and antioxidant activities in leaves and stem of *Mukia maderaspatana (*L.*).* LC_{50} values for the samples were determined for the Brine shrimp lethality assay using Probit analysis (Finney, 1971).