Experimental Procedure

3. EXPERIMENTAL PROCEDURE

The methodology followed for the "Evaluation of Antioxidant potential, Antimicrobial activity and Antidiabetic effect of *Syzygium cumini* and *Momordica charantia*" is as follows:

3.1 COLLECTION OF PLANT SAMPLE

The plants namely *Syzygium cumini* and *Momordica charantia* were collected for the present investigation. Fresh leaves and seeds of *Syzygium cumini* and *Momordica charantia* were collected, washed and air dried in the shade at room temperature for seven days. The dried samples were separately powdered and used for further investigation.

3.2 PREPARATION OF HERBAL MIXTURE

The powdered leaf sample of the two different plants were mixed in equal amounts to prepare the herbal seed mixture and in the same way herbal leaf mixture was prepared and analyzed for the antioxidant potential and antimicrobial activity.

3.3 PREPARATION OF PLANT EXTRACT

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

The herbal mixture and the powder obtained from various plant parts namely leaf and seed were extracted with respective buffers to analyze the enzymic and non-enzymic antioxidants present in them.

(b) Preparation of the extract for determining antimicrobial efficacy

5g of each sample was extracted with alcohol 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 in water bath at 40°- 60°C. The residue was dissolved in dimethyl sulphoxide (DMSO) which was used for screening antimicrobial activity.

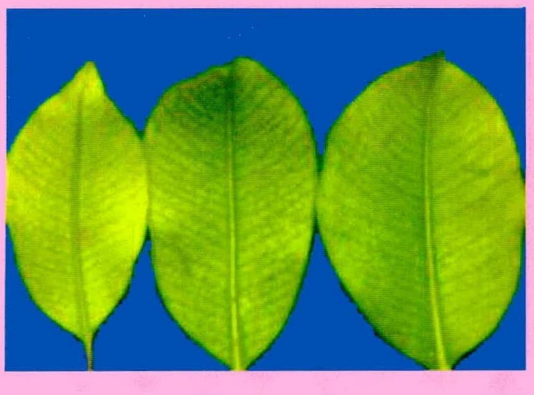
PLATE - 1
FRESH AND POWDERED SEED SAMPLES

Syzygium cumini

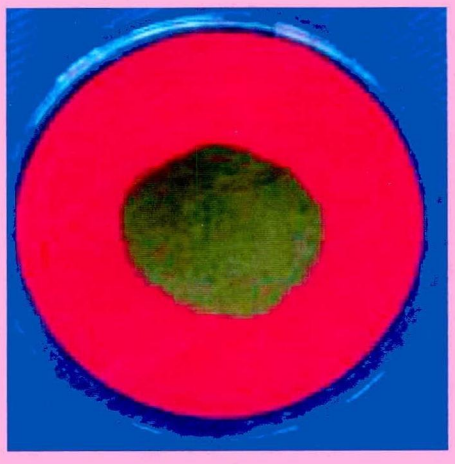
Momordica charantia

Seed Mix

PLATE 2
FRESH AND POWDERED LEAF SAMPLES



Syzygium cumini



Momordica charantia

Leaf Mix

3.4 DETERMINATION OF ANTIOXIDANT STATUS

Antioxidants play an important role to protect the human body against damage by reactive oxygen species. An antioxidant is a molecule stable enough to donate an electron to a rampaging free radical and neutralize it, thus reducing its capacity to damage (Shivaprasad *et al.*, 2008).

3.4.1 Enzymic antioxidants

The major ROS scavenging mechanisms include enzymatic system, which consists of Superoxide Dismutases (SOD), Catalase (CAT), Peroxidase (POD), Polyphenol oxidase (PPO), Glutathione-S-Transferase (GST), Glutathione peroxidase (GSH-Px) and Glutathione reductase (GR) (Yin *et al.*, 2008).

3.4.1.1 Estimation of catalase (CAT)

Catalases are enzymes that catalyze the conversion of hydrogen peroxide to water and oxygen, using either an iron or manganese cofactor (Chelikani *et al.*, 2007). The activity of Catalase was determined by the method of Luck, (1974) which is detailed in Appendix I.

3.4.1.2 Estimation of peroxidase (POD)

Peroxidase (POD) catalyzes the reduction of hydrogen peroxide, organic hydroperoxides as well as peroxynitrite (Rhee *et al.*, 2005). Peroxidase activity was estimated by the method of Reddy *et al.* (1995) as described in Appendix II.

3.4.1.3 Estimation of Superoxide Dismutases (SOD)

Superoxide dismutases are a class of closely related enzyme that catalyzes the breakdown of the superoxide anion into oxygen and hydrogen peroxide (Johnson and Giulivi, 2005). The plant sample SOD activity was estimated by the method described by Misra and Fridovich (1972) and is explained in Appendix III.

3.4.1.4 Estimation of Polyphenol oxidase (PPO)

Polyphenol oxidase (PPO) is frequently reported as a latent enzyme, which can be activated *in vitro* by a number of different factors and treatments such as detergents (Wititsuwannakul *et al.*, 2002). The method described by Esterbouver *et al.* (1977) was followed to determine the activity of polyphenol oxidase and the procedure is given in Appendix IV.

3.4.1.5 Estimation of Glutathione-S-Transferase (GST)

Glutathione-S-Transferases (GST) are class of glutathione dependent antioxidant enzymes that show activity with lipid peroxides (Sharma *et al.*, 2004). The activity of Glutathione-S-Transferase (GST) was determined by the method proposed by Habig *et al.* (1974) and the procedure is described in Appendix V.

3.4.1.6 Estimation of Glutathione peroxidase

Glutathione peroxidase is an enzyme containing four selenium cofactors that catalyzes the breakdown of hydrogen peroxide and organic hydroperoxides (www.wikipedia_glutathione peroxidase.htm). The method of Rotruck *et al.*, (1973) was followed to assess the activity of glutathione peroxidase and it is given in Appendix VI.

3.4.1.7 Estimation of Glutathione reductase (GR)

Glutathione reductase (GR) is important in maintaining high levels of reduced glutathione in cells (Demir *et al.*, 2006). Glutathione reductase activity was determined by the method proposed by David and Richard, (1983) which is elaborated in Appendix VII.

3.4.2 Non-enzymic antioxidants

The non-enzymic antioxidant defense system includes ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), β-Carotene and Vitamin A. There is a balance between both the activities and intracellular

levels of these antioxidants that are essential for the survival of organisms and their health (Vaisi-Raygani *et al.*, 2007).

3.4.2.1 Estimation of Ascorbic acid

Ascorbic acid or 'Vitamin C' is a monosaccharide antioxidant found in both animals and plants. Ascorbic acid is a reducing agent and thereby neutralizes reactive oxygen such as hydrogen peroxide (Padayatty *et al.*, 2003). Appendix XIII describes the detailed method followed for the estimation of ascorbic acid as proposed by Roe and Keuther, (1953).

3.4.2.2 Estimation of α-tocopherol

α-tocopherol is the most important lipid-soluble antioxidant, and that it protects membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction (Traber and Atkinson, 2007). Emmeric-Engel method given by Rosenberg, (1992) was followed for the estimation of α-tocopherol which is explained in Appendix IX.

3.4.2.3 Estimation of reduced Glutathione

Glutathione (GSH) metabolism is one of the most essential antioxidative defense mechanisms present in tissues and blood (Solak *et al.*, 2005). The content of reduced glutathione of the sample was determined by the method of Moron *et al.* (1979) as given in Appendix X.

3.4.2.4 Estimation of Polyphenol

Polyphenolic compounds in plant, exert anticarcinogenic, antimutagenic and cardioprotective effects linked to their free radical scavenging (Karou *et al.*, 2005). The procedure of Malick and Singh, (1980) was followed to estimate the total phenolic content as explained in Appendix XI.

3.4.2.5 Estimation of total Carotenoids and Lycopene

Lycopene belongs to a class of compounds known as the carotenoids. Carotenoids are protective against chronic diseases which are thought to be caused by damage from free radicals (Roy, 2005). Carotenoids and Lycopene

were analysed by the method of Zakeria *et al.* (1979) as described in Appendix XII.

3.4.3 Estimation of Proteins

Proteins are the most abundant organic molecules to be found in cells and form over 50% of their total dry mass (Taylor *et al.*, 2003). The total protein of samples was estimated by the method of Lowry *et al.* (1951) which is in Appendix XIII.

3.4.4 Estimation of Carbohydrate

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

3.4.5 Free radical scavenging effect of plant samples

3.4.5.1 Determination of *in vitro* lipid peroxidation

Lipid peroxidation refers to the oxidative degradation of lipids. It is the process whereby free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage (www.wikpedia.org/wiki/lipid peroxidation). Quantitative measurement of lipid peroxidation was performed by the method of Okhawa *et al.* (1979) as given in Appendix XV.

3.4.5.2 Determination of nitric oxide generation *in vitro*

Nitric oxide is a free radical generated by endothelial cells, macrophages, neurons etc. *In vitro* inhibition of nitric oxide radical is also a measure of antioxidant activity (Shivaprasad *et al.*, 2008). Appendix XVI explains the procedure given by Green *et al.* (1982) which was used to estimate the extent of nitric oxide inhibition by plant samples and crude extracts.

3.4.5.3 Determination of Superoxide production *in vitro*

Superoxide radical O^{o-} is a highly toxic species, which is generated by numerous biological and photochemical reactions (Govindarasan *et al.*, 2003). Inhibition of superoxide generation in plant samples was determined by the method of Winterbourn *et al.* (1975) as explained in Appendix XVII.

3.5 DETERMINATION OF ANTIBACTERIAL ACTIVITY

3.5.1 Antibiotic Sensitivity Test

The inocula were prepared from fresh overnight cultures in nutrient broth. Plates were prepared by pouring sterile nutrient agar into sterile petri dishes. Sterile swabs were dipped in the inocula and swabbed on the agar plate. Standard antibiotic disc were placed with care on the swabbed plates and kept for overnight incubation.

3.5.2 Antibacterial Sensitivity Test

The antibacterial activity of the crude alcoholic extract was determined by disc diffusion assy.

3.5.3 Agar Disc Diffusion Assay

Plates were prepared by pouring sterile nutrient agar into sterile petri dish previously autoclaved. The sterile cotton swab was dipped in the bacterial subculture in nutrient broth and then swabbed on agar plate. Sterile disc saturated with different extracts were placed on nutrient agar seeded with test organisms. Disc fed with DMSO served as control. The plates were incubated at 37°C and observed for zones of growth inhibition after 48 hours.

3.6. ANTIDIABETIC EFFECT

3.6.1 Preparation of seed powder

Seeds from the pulp of *Syzygium cumini* and *Momordica charantia* were removed and thoroughly washed with distilled water to remove the traces of pulp from the seeds. The seeds were dried in a hygienic place at room temperature and powdered. The powdered seed samples were used for the supplementation process.

3.6.2 Oral Supplementation

Forty volunteers were selected at random from diabetic centre operating in Puducherry. Twenty patients were supplemented with the seed powder of *Syzygium cumini* (3g/day) and the other twenty with *Momordica charantia* (3g/day) for forty days. The body weight of the volunteers were taken before and after supplementation. Glucose levels of the diabetic patients were measured by using Acucheck strips with the help of lab technicians before and after supplementation.