Experimental Procedure

3.0 EXPERIMENTAL PROCEDURE

The present study was undertaken to compare the levels of some known enzymic and non-enzymic antioxidants in the unripe fruits and the ripe fruits of *Citrullus colocynthis* (bitter apple). The methodology adopted for the work entitled '**A comparative study on the antioxidant properties of unripe and ripe fruits of *Citrullus colocynthis* (Linn.)**' is given below.

3.1 COLLECTION OF FRUITS

3.2 PREPARATION OF THE EXTRACT

3.3 DETERMINATION OF ANTIOXIDANT LEVELS

3.3.1 ENZYMIC ASSAYS

3.3.1.1 Estimation of catalase

3.3.1.2 Estimation of peroxidase

3.3.1.3 Estimation of super dismutase

3.3.1.4 Estimation of glutathione reductase

3.3.2 NON- ENZYMIC ASSAYS

3.3.2.1 Estimation of reduced glutathione

3.3.2.2 Estimation of ascorbic acid (vitamin c)

3.3.2.3 Estimation of total carotenoids and lycopene

3.3.2.4 Estimation of tocopherol (vitamin e)

3.4 FREE RADICAL SCAVENGING ACTIVITY

3.4.1 DPPH scavenging activity

3.4.2 Inhibition of *in vitro* hydrogen peroxide scavenging

3.4.3 ABTS scavenging activity

3.4.4 Hydroxyl radical scavenging

3.4.5 Inhibition of *in vitro* superoxide generation

3.1 COLLECTION OF FRUITS

The *Citrullus colocynthis* (bitter apple) fruit was collected from a local market at Coimbatore. The fruits obtained were wiped thoroughly with a wet cloth to remove the adhering dirt particles. The pulps of the fruits were scraped out separately according to the level of ripening and the pulps were shade- dried for 5 days. The well dried fruit pulps were then grounded into a fine powder and sieved out. The required amount of powder was taken for the preparation of the extract for the determination of various parameters.

Plate1 and Plate II show the unripe and the ripe fruits of *Citrullus colocynthis.*

3.2 PREPARATION OF THE EXTRACT

5 grams of the dried powered fruit pulp (unripe and ripe) was taken and centrifuged with distilled water. The supernatant was carefully separated out using a pipette and used for enzymic and non- enzymic antioxidant analysis.

Ten grams of each sample was individually packed in a Whatmann No.1 filter paper and extracted with distilled water, methanol and petroleum ether. Extraction was done by using Soxhlet according to the increasing polarity of the solvents, starting with petroleum ether, methanol and distilled water. The Soxhlet was run from 6- 10 back suctions. The residue was then

dissolved in the respective solvents. These crude extracts were stored in a refrigerator and screened for free radical scavenging activity.

3.3 DETERMINATION OF ANTIOXIDANT LEVELS

Measuring *in vivo* antioxidant status is important in understanding the role of oxidative events in the initiation and progression of numerous diseases, including cancer, atherosclerosis and diabetes. *In vivo* antioxidant status can be assessed by measuring individual plasma or tissue levels of antioxidants such as vitamin C, vitamin E and carotenoids. However, the task is more difficult for numerous other compounds, including flavonoids and polyphenol-like compounds that may influence *in vivo* antioxidant status (Prior, 2010).

3.3.1 ENZYMIC ASSAYS

The activity of antioxidants depends on complex factors including the nature of the antioxidants, the conditions of oxidation, the properties of the oxidizing substrate and the stage of oxidation (Kancheva, 2009).

3.3.1.1 Estimation of Catalase

The catalase activity was estimated by the Method of Luck (1974) and the detailed procedure is given in Appendix – I.

3.3.1.2 Estimation of Peroxidase

Peroxidase activity was determined by the Method of Reddy *et al.* (1995) as described in Appendix – II.

3.3.1.3 Estimation of Superoxide Dismutase

Assay for SOD was done by the Method of Kakkar *et al.,* (1984). The procedure is given in Appendix –III.

PLATE I

UNRIPE FRUIT OF *Citrullus colocynthis*

PLATE II

RIPE FRUIT OF *Citrullus colocynthis*

3.3.1.4 Estimation of Glutathione Reductase

Glutathione reductase activity was determined by the Method proposed by David and Richard (1983) and the procedure is described in Appendix- IV.

3.3.2 NON- ENZYMIC ASSAYS

Non enzymic antioxidant activity is represented by a series of antioxidant molecules that the plant uses against reactive oxygen species formation (Patil *et al.*, 2008).

3.3.2.1 Estimation of Reduced Glutathione

Reduced glutathione was estimated by the Method of Moron *et al.* (1979) and the procedure is explained in Appendix – V

3.3.2.2 Estimation of Ascorbic Acid (Vitamin C)

Ascorbic acid is a free radical scavenger. Ascorbic Acid was estimated by coupling with DNPH using the Method described by Roe and Keuther, (1943) as outlined in Appendix-VI.

3.3.2.3 Estimation of Total Carotenoids and Lycopene

The total carotenoids and lycopene were estimated by the Method of Zakaria *et al.*, (1979) and given in Appendix- VII.

3.3.2.4 Estimation of Tocopherol (Vitamin E)

Tocopherol was estimated by the Method of Rosenberg (1992) and the detailed procedure is given in Appendix – VIII.

3.4 FREE RADICAL SCAVENGING ACTIVITY

3.4.1 DPPH scavenging activity

DPPH scavenging activity was measured by the Method of Mensor *et al.*, (2001) and the details are explained in Appendix – IX.

3.4.2 Inhibition of *in vitro* hydrogen peroxide scavenging

Hydrogen peroxide scavenging activity was measured by the Method of Ruch *et al.*, (1989). The procedure is explained in Appendix - X.

3.4.3 ABTS scavenging activity

ABTS scavenging activity was measured by the Method of Shirwaikar *et al.*, (2006). The procedure is explained in Appendix – XI.

3.4.4 Hydroxyl radical scavenging

Hydroxyl radical scavenging was measured by the Method Elizabeth and Rao (1990). The procedure is given in Appendix – XII.

3.4.5 Inhibition of *in vitro* superoxide generation

Inhibition of superoxide generation was measured by the Method of Winterbourn *et al.,* (1975). The procedure is given in Appendix - XIII.

3.5 STATISTICAL ANALYSIS

The data obtained were analyzed by two-way analysis of variance (ANOVA) for free radical scavenging and t- test for the enzymic and non- enzymic antioxidants.

Using the above methods, the findings of the present study are explained and interpreted in the following chapter.