Appendices

APPENDIX- I

ESTIMATION OF CATALASE ACTIVITY

(Luck, 1974)

Catalase activity was assayed spectrophotometrically by the method proposed by Luck (1974).

PRINCIPLE

The UV light absorption of hydrogen peroxide can be easily measured between 230 and 250nm. On decomposition by catalase, the absorption of H_2O_2 decreases with time and is proportion to the activity of the enzyme. The enzyme activity can be arrived at from this decrease.

REAGENTS

1. Phosphate buffer 0.067M (PH 7.0)
2. Hydrogen peroxide (30% in phosphate buffer)

PROCEDURE

PREPARATION OF THE ENZYME EXTRACT

0.5g of pulp was homogenized in a prechilled mortar and pestle with 7.5ml phosphate buffer. The pulp debris was removed by centrifugation (1- 4c) and the supernatant obtained was used for the assay of catalase. Use fresh extracts for assay.

ASSAY

H_2O_2 –phosphate buffer (2.9ml) was pipette out into a quartz cuvette. The enzyme extract (0.1ml) was rapidly added and mixed thoroughly. The time required for a decrease in absorbance by 0.05 units was recorded. The H_2O_2– phosphate buffer served as control. One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240nm by 0.05 units.

APPENDIX – II

ESTIMATION OF PEROXIDASE ACTIVITY

(Reddy *et al.*, 1995)

Peroxidase activity was assayed by the method of Reddy et al. (1995) in the ripe and unripe pulp of the fruits.

PRINCIPLE

In the presence of the hydrogen donor pyrogallol, peroxidase converts H_2O_2 to water and oxygen. The oxidation of pyrogallol to the coloured product purpurogalli can be quantified spectrophotometrically at 430nm. The formation of the product is proportional to the activity of the enzyme and can be used as a measure of the same.

REAGENTS

1. Pyrogallol (0.05M in 0.1 M phosphate buffer, pH 6.5)
2. H_2O_2 (1% in 0.1% phosphate buffer, pH 6.5)
3. Phosphate buffer (0.1M, pH 6.5)

PROCEDURE

PREPARATION OF THE ENZYME EXTRACT

0.5g of the pulp sample with 2.5ml (w/v) 0.1M phosphate buffer (pH 6.5) was homogenizer. The homogenate was centrifuged at 3000rpm for 15 minutes. The supernatant was used as the enzyme source. All procedures were carried out at 0-5°C.

ASSAY

Pyrogallol solution (2.4ml) and enzyme extract (0.1ml) were pipette out into an experimental cuvette. The spectrophotometer was adjusted to read zero at 430nm. To the experimental cuvette, 0.5ml of 1% H_2O_2 was added and

the change in absorbance was recorded for every 30 seconds up to 3 minutes. One unit of peroxidase activity is defined as the change in absorbance per minute at 430nm.

APPENDIX – III

ESTIMATION OF SUPEROXIDE DISMUTASE ACTIVITY

(Kakkar *et al.*, 1984)

Super oxide dismutase activity was determined by the method proposed by Kakkar *et al.* (1984).

PRINCIPLE

The assay of SOD is based on the inhibition of formation of NADH-phenazine methosulphate- nitroblue tetrozolium formazon, the extent of which can be assayed spectrophotometrically at 560nm.

REAGENTS

1. Sodium pyrophosphate buffer (0.025M, pH 8.3)
2. Phenazine methosulphate (PMS) (186μM)
3. Nitroblue tetrazolium (NBT) (300μM)
4. NADH (700 μM)
5. Glacial acetic acid
6. n –butanol

PROCEDURE

PREPARATION OF THE ENZYME EXTRACT

0.5g of the fruit pulp was grounded in 7.5ml of sodium phosphate buffer, centrifuge at 3000rpm for 15 min and the supernatant was used for the assay.

ASSAY

To assay mixture contained in a volume of 3.0ml, 1.2ml of sodium pyrophosphate buffer, 0.1ml of PMS, 0.3ml of NBT, 0.2ml of enzyme preparation and 1.0ml of water. NADH (0.2ml) was added to start the reaction. The assay mixture was incubated at 30°C for 90 seconds and the reaction was stopped by the addition of 1.0ml of glacial acetic acid. n-butanol (4ml) was added to the above mixture, allowed to stand for 10 minutes and then centrifuged at 2000g for 5 minutes. The intensity of the chromogen in the butanol layer was measured at 560nm against butanol as blank. The system devoid of enzyme served as control. One unit of enzyme activity is defined as the amount of enzyme causing a 5% reduction in NBT oxidation/ minute.

APPENDIX –IV

ESTIMATION OF GLUTATHIONE REDUCTASE ACTIVITY

(David and Richard 1983)

Glutathione reductase activity was determined by the method of David and Richard (1983).

PRINCIPLE

The enzyme glutathione reductase involves the conversion of oxidized glutathione to its reduced form by using NADPH as a substrate. The amount of NADPH utilized is a direct measure of enzyme activity.

PROCEDURE

PREPARATION OF THE ENZYME EXTRACT

0.5g of the grounded fruit pulp were homogenized in 7.5ml of buffer, homogenate spun at 3000rpm for 15 min and the supernatant was used for the assay.

ASSAY

The reaction mixture contained in a final volume of 3.0ml, EDTA (0.1ml), sodium azide (0.1ml), oxidize glutathione (0.1ml), enzyme source (0.1ml) and water. The reaction mixture was incubated for 3 minutes, after which NADPH (0.1ml) was added to the reaction mixture. The absorbance was recorded at an interval of 15 seconds for 3 minutes at 340nm. The assay mixture free from oxidized glutathione served as control. One unit of glutathione reductase is defined as the μmoles of NADPH oxidized/ minutes.

APPENDIX –V

ESTIMATION OF REDUCED GLUTATHIONE

(Moron *et al.*, 1979)

The amount of reduced glutathione present in the leaf sample was estimated by the method proposed by Moron *et al.* (1979).

PRINCIPLE

Reduced glutathione (GSH) was measured by its reaction with 5, 5'-dithio- 2 -nitrobenzoic acid (DTNB) (Ellman's reaction) to give a yellow coloured compound that absorbs at 412nm.

REAGENTS

1. TCA (5%)

2. TCA (25%)

3. Sodium phosphate buffer (0.2M, pH 8.0)

4. DTNB (0.6M in 0.2M sodium phosphate buffer

PROCEDURE

PREPARATION OF THE PULP EXTRACT

0.5g of the sample was homogenized in 5%TCA to give a 20% homogenate. The precipitated protein was centrifuged at 3000rpm for 15 minutes. The homogenate was cooled on ice and 0.1ml of supernatant was taken for the estimation of GSH.

ESTIMATION

The volume of different aliquots (0.2 to 1.0ml) was made up to 1ml with phosphate buffer. Freshly prepared DTNB (2ml) was added to the tubes and the intensity of the yellow colour was read at 412nm in a spectrophotometer after10 minutes. A standard curve of GSH was prepared using concentrations ranging from 2 to 10moles of GSH. The concentration of GSH in the samples was calculated from this and the results were expressed as nmoles GSH/g fruit pulp.

APPENDIX –VI

ESTIMATION OF ASCORBIC ACID

(Roe and Kuether, 1943)

The levels of ascorbic acid in *Citrullus colocynthis* fruit were quantified spectrometrically by the method of Roe and Kuether (1943).

PRINCIPLE

Activated carcoal treatment converts ascorbate to dehydroascorbate,which reacts with 2,4- dinitrophenyl hydrazine to form

osazone. Osazone dissolves in H_2SO_4 to give an orange coloured solution, whose absorbance can be measured spectrophotometrically at 540nm.

REAGENT

1. Trichloro acetic acid (TCA) (4%)
2. Sulphuric acid (H_2SO_4) (9N)
3. 2,4- dinitrophenyl hydrazine (DNPH) (2% in 9N H_2SO_4)
4. Thiourea (10%)
5. H_2SO_4 (85%)
6. Standard ascorbate solution: 10mg ascorbic acid in 100ml of 4% TCA.

PROCEDURE

EXRACTION OF ASCORBIC ACID

About 0.5g of the sample was homogenized in 4% TCA up to 10ml, centrifuged at 3000rpm for 15 minutes. To the supernatant obtained, a pinch of activated charcoal was added, shaken well and kept for 10 minutes. The centrifugation was repeated to remove the charcoal residue. Aliquots (0.5ml) of the supernatants were used for the estimation.

ESTIMATION

Aliquots (0.2 to 1.0ml) of the working ascorbate were made up to 2.0ml with 4% TCA. DNPH reagent (0.5ml) was added to each tube, followed by two drop of thiourea solution and incubated at 37°C for 3 hours. The osazone crystals formed were dissolved by the addition of 85% H_2SO_4 (2.5ml) on ice to avoid local heat generation. The plant sample was treated in the way similar to that of ascorbate standard.

To the blank alone, DNPH reagent and thiourea were added after the addition of H_2SO_4. The absorbance was read at 540nm. The concentration of ascorbic acid in the samples was calculated from the standard curve constructed on an electronic calculator set to the linear regression mode and expressed as mg ascorbate/g fruit pulp.

APPENDIX- VII

ESTIMATION OF TOTAL CAROTENOIDS AND LYCOPENE

(Zakaria *et al.*, 1979)

The method proposed by Zakaria *et al.* (1979) was used for the estimation of total carotenoids and lycopene.

PRINCIPLE

The total carotenoids in the sample were extracted in petroleum ether. The total carotenoids were estimated in a UV/Visible spectrophotometer at 450nm.

REAGENTS

1. Petroleum ether (40 to 60°C)
2. Anhydrous sodium sulphate
3. Calcium carbonate
4. Alcoholic potassium hydroxide (KOH) (12%)

PROCEDURE

All the steps subsequent to the saponification were carried out in the dark to avoid photolysis of carotenoids. The leaves (5.0g) were grounded and saponified with 2.5ml of 12% ethanolic KOH in a water bath at 60°C for 30 minutes. The saponified extract was transferred into a separating funnel (packed with glass wool and calcium carbonate) containing 10- 15 ml of

petroleum ether, mixed and allowed to separate. The petroleum ether layer containing the carotenoid pigments was transferred into another separating funnel. The extraction was repeated until the aqueous phase was colourless. A small quantity of anhydrous sodium sulphate was added to the petroleum ether extract to remove excess moisture, if any. The final volume of the petroleum ether layer was noted and diluted (if needed) by a known dilution factor. The absorbance of the yellow colour was read at 450nm and 503nm using petroleum ether as blank. The amount of total carotenoids and lycopene was calculated using the formula,

$$\text{Amount of total carotenoids present} = \frac{P \times 4 \times V \times 100}{W} \text{ mg}$$

where,

P = Optical density of the sample

V = Volume of the sample

W = Weight of the sample

The total carotenoids are expressed as mg/g fruit pulp.

APPENDIX –VIII

ESTIMATION OF TOCOPHEROL

Rosenberg (1992)

The spectrophotometic method proposed by Rosenberg (1992) was adopted to estimation the level of tocopherol in *Citrullus colocynthis* fruit pulps.

PRINCIPLE

The Emmerie – Engel reaction is based on the reduction of ferric to ferrous ions by tocopherols, which then forms a red colour with 2, 2'-dipyridyl. Tocopherol and carotenes are first extracted with xylene and the

extraction read at 460nm to measure carotenes. A correlation is made for these after adding ferric chloride and reading at 520nm.

REAGENTS

1. Absolute alcohol
2. Xylen
3. 2, 2'- dipyridyl (1.2g/L in n-propanol)
4. Ferric chloride (1.2g/L in ethanol)
5. Standard solution of D,L- α tocopherol: 10mg/L in absolute alcohol (91 mg of α-tocopherol is equivalent to 100mg of tocopherol acetate).
6. Sulphuric acid (0.1N)

PROCEDURE

PREARATION OF PLANT EXTRACT

The grounded fruit pulp sample was homogenized in a blender. Weighed accurately 2.5g of the homogenized sample into a conical flask, 50ml of 0.1N sulphuric acid was added slowly without shaking. The content of the flask was allowed to stand overnight. The next day, the homogenate was shaken vigorously and filtered through Whatman No. 1 filter paper. Estimation of tocopherol was done using aliquots of the filtrate.

ESTIMATION

The plant extract (1.5ml), standard (1.5ml) and water (1.5ml) were pipette out into three centrifuge tube namely test, standard and blank respectively. To all the tube, xylene (1.5ml) was added, stoppered, mixed well and centrifuged. The xylene layer (1.0ml) was taken and transferred to another set of stoppered tubes, 1.0ml of of 2, 2'- dipyridyl was added to each

and mixed. The reaction mixture (1.5ml) was taken in a spectrophotometric cuvette and the extinction of test and standard were read against the blank at 460nm. Ferric chloride solution (0.33ml) was added and after exactly 15 minutes, the absorbance of the red colour was read against blank at 520nm. Tha amount of tocopherol in the sample was calculated using the formula,

$$\text{Tocopherols } (\mu g) = \frac{\text{Reading at 520nm} - \text{Reading at 460nm}}{\text{Reading of standard at 520nm}} \times 0.29 \times 15$$

The results are expressed as µg tocopherol/g sample.

APPENDIX – IX

DPPH SCAVENGING ACTIVITY

(Mensor *et al.*, 2001)

The method proposed by Mensor *et al.* (2001) was adopted to test the DPPH scavenging ability of different solvent extracts of the fruit pulp.

PRINCIPLE

Antioxidants react with (DPPH - 1, 1-diphenyl-2-picryl hydrazyl) and converted into α,α' - diphenyl-β-picryl hydrazine by donating its OH group. This can be identified by the conversion of purple colour to yellow colour.

REAGENTS

1. DPPH (0.3mM in methanol)
2. Methanol

PROCEDURE

The different solvent extracts and crude aqueous extract (5µl) was added with 0.5ml of methanolic solution of DPPH and 0.495ml of methanol. The mixture was then allowed to stand at room temperature for 30minutes. DDPH-

methanol solution was used as positive control and methanol alone acted as blank. After incubation, the conversion of purple colour to yellow colour was read at 518nm in a spectrophotometer. The percent incubation was calculated using the following formula;

$$\text{Scavenging activity (\%)} = 100 - \frac{A(\text{Control}) - A(\text{Sample})}{A_{518}\,(\text{Control})} \times 100$$

APPENDIX – X

HYDROGEN PEROXIDE SCAVENGING ASSAY

(Ruch *et al.*, 1989)

The effect of the fruit pulp to scavenge H_2O_2 was determined by the method proposed by Ruch *et al.* (1989).

PRINCIPLE

H_2O_2 scavenging activity was measured in term of a decrease in the absorbance at 230nm spectrophotometrically.

REAGENTS

1. H_2O_2 (40mM in 0.1M phosphate buffer)
2. Phosphate buffer (0.1M, pH7.4)

PROCEDURE

The fruit extracts was diluted to a concentration of 10mg in 10µl. This extract (10µl corresponding to 10mg) was added to 0.6ml of H_2O_2 solution and the final volume was made up to 3ml with the same buffer. After 10 minutes, the absorbance value at 230nm of the reaction mixture were recorded

against a blank containing phosphate buffer without H_2O_2 for each sample. The percent inhibition was calculated using the formula,

$$\text{The \% of scavenging } H_2O_2 = = \frac{\text{A(Control)} - \text{A(Sample)}}{\text{A (Control)}} \times 100$$

APPENDIX – XI

ABTS RADICAL SCAVENGING ACTIVITY

(Shirwaikar *et al.*, 2006)

ABTS (2', 2'- azino- bis- 3- ethyl benzthiozline- 6- sulphonic acid) radical cation de- colourization assay according to the method proposed by Shirwaikar *et al.(*2006) was employed to assess the radical- scavenging effect of the fruit pulp of *Citrullus colocynthis*.

PRINCIPLE

ABTS is a chromogen, which changes into a coloured mono-cation radical ($ABTS^+$) in the presence of oxidative agent and the $ABTS^+$ has an absorption peak at 750nm. Antioxidant will reduce $ABTS^+$ into its colourless form and the extent of decolourization corresponds to the percent reduction of $ABTS^+$.

REAGENTS

1. Ethanol
2. ABTS solution (7 mM of ABTS with 2.45 mM ammonium persulpfate). The solution was incubated at room temperature for 12- 16 hours before use.

PROCEDURE

The six different extract (100µl each) were added to ABTS solution (300µl) and the final volume of each was made up to 1ml with ethanol. The absorbance was read at 745nm and the percentage inhibition was calculated using the formula,

$$\text{Inhibition (\%)} = = \frac{\text{A(Control)} - \text{A(Sample)}}{\text{A (Control)}} \times 100$$

APPENDIX – XII

HYDROXYL RADICAL SCAVENGING ACTIVITY

(Elizabeth and Rao, 1990)

The effect of *Citrullus colocynthis* fruit pulp extracts on oxidant-induced damage to deoxyribose *in vitro* was quantified as the amount of thiobarbituric acid reactive substances (TBARS) formed, according to the procedure explained by Elizabeth and Rao (1990).

PRINCIPLE

Hydroxyl radical are generated from a Fe^{2+} /ascorabate/ EDTA/H_2O_2 system, which attacks deoxyribose and eventually produces TBARS. The ability of the plant extracts to inhibit TBARS formation is measured spectrophotometrically at 532nm.

REAGENTS

1. Deoxyribose (2.8Mm)
2. $FeCl_3$ (1mM)
3. EDTA (1mM)

4. H_2O_2 (10mM)
5. Ascorbate (1mM)
6. KH_2PO_4- KOH buffer (20mM, pH 7.4)
7. Thiobarbituric acid (1%)
8. HCl (25%)

PROCEDURE

The reaction mixture contained deoxyribose (0.1ml), $FeCl_3$ (0.1ml), H_2O_2 (0.1ml), ascorabate (0.1ml), buffer (0.1ml) and 20µl of leaf extract which corresponded to 10mg concentration. The total volume was made up to 1ml with water. The tube were capped tightly and incubated in a water bath at 37°C for one hour. The reaction was terminated by the addition of TBA (0.5ml) and HCl (0.5ml). the tube were heated in a boiling water bath for 20 minutes for colour development. The intensity of the pink colour formed, as the indication of TBARS formation, was measured at 532nm. The percent TBARS produced for positive control (H_2O_2) was fixed as 100% and the relative percent TBARS was calculated for the extract treated groups.

APPENDIX – XIII

SUPEROXIDE SCAVENGING ACTIVITY

(Winterbourn *et al.*, 1975)

The efficiency of the fruit extracts to inhibit the in vitro generation of SO was studied by the method of Winterbourn *et al.* (1975).

PRINCIPLE

The extent of superoxide generation was studied on the basis of inhibition of the production of nitroblue tetrazolium formazone of the superoxide ion by the fruit extract and is measured spectrophotometrically a 560nm.

REAGENTS

1. ETDA (0.1M containing 1.5mg of sodium cyanide/ 10ml)
2. Nitroblue tetrazolium (NBT) (1.5mM)
3. Riboflavin (0.12mM)
4. Phosphate buffer (0.067M, pH 7.6)
5. Dimethly sulfoxide (DMSO)

PROCEDURE

The assay mixture contained 0,02ml of fruit (solvent and crude aquesous) extracts with 0.2ml of EDTA, 0.1ml of NBT, 0.05ml of riboflavin and 2.63ml of phosphate buffer. DMSO, instead of plant extract, was considered as control. All tubes were votexed and the initial absorbance was read at 560nm. The tubes were illuminated uniformly using a fluorescent lamp for 30 mminutes. Teh absorbance was read again at 560nm. The difference in optical dansity before and after illumination is the measure of superoxide generation an dthe percentage inhibition was calculated using the formula,

$$\% \text{ Superoxide Scavenged} = \frac{A(\text{Control}) - A(\text{Sample})}{A(\text{Control})} \times 100$$

Success is not final, failure is not fatal:

It is the courage to continue the counts

Wilson Churchill