Appendices

APPENDIX - I

ESTIMATION OF CATALASE ACTIVITY

(Luck, 1974)

The enzyme catalase has a double function and it catalyses the following

a. It decomposes hydrogen peroxide to give water and oxygen.

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

b. It oxidizes H^+ donors, for example methanol, formic acid, phenol with the consumption of one mole of peroxide.

$$ROOH + AH_2 \xrightarrow{\text{Catalase}} H_2O + ROH + A$$

Principle

The UV light absorption of hydrogen peroxide solution can be easily measured between 230 and 250nm. On decomposition of hydrogen peroxide by catalase, the absorption decreases with time. The enzyme activity could be arrived at from this decrease. But this method is applicable only to enzyme solution, which do not absorb strongly at 230-250nm.

Reagents

1. Phosphate buffer 0.067M (pH 7.0) - Dissolved 3 .522g of KH_2PO_4 and 7.268g of Na_2HPO_4. 2 H_2O in distilled water and made up the volume to one litre.

2. Hydrogen peroxide - Phosphate buffer - Dissolved 0.16 ml of H_2O_2 (10% W/V) to 100 ml phosphate buffer, prepared fresh. The absorbance of the solution should be about 0.5 at 240nm with 1cm light path.

Procedure

Enzyme extract : The sample is homogenized in a prechilled mortar and pestle with M/150 phosphate buffer (assay buffer diluted 10 times) at 1 - 4°C and centrifuged. Stirred the sediment with cold phosphate buffer, allowed to stand in the cold with occasional shaking and then repeated the extraction once or

twice. The extraction should not take more than 24 hr. The combined supernatants were used for the assay. Used fresh extract for assay.

Assay

Read against a control cuvette 3ml of H_2O_2 containing the enzyme solution as in the phosphate buffer (M/15). Pipetted into the experimental cuvette 3ml of H_2O_2 phosphate buffer. Mixed in 0.01 – 0.04ml sample with the glass or plastic rod flattened at one end. Noted the time it required for a decrease in absorbance from 0.45 to 0.4. This value was used for calculations. If t' was more than 60 seconds, repeated the measurement with more concentrated solution of the sample.

Calculation

Calculated the concentration of H_2O_2 using the extinction coefficient 0.036μ mole/ml.

APPENDIX – II
ESTIMATION OF PEROXIDASE ACTIVITY
(Reddy *et al.*, 1995)

Principle

Peroxidase converts H_2O_2 to water and oxygen in the presence of hydrogen donor (pyrogallol or dianisidine) the oxidation of pyrogallol or dianisidine to colored product called purpurogalli is measured colorimetically.

$$\text{Pyrogallol} + H_2O_2 \longrightarrow \text{Oxidized pyrogallol} + H_2O$$

Reagents

1. Pyrogallol-0.05 M phosphate buffer (pH 6.5)
2. 1% H_2O_2

Enzyme extract

Macerated one gram of the sample with 5ml (W/V) 0.1M phosphate buffer (pH 6.5) in a homogenizer. Centrifuged the homogenate at 300g for

15 minutes. Used the supernatant as the enzyme source. All procedures were carried out at 0-5°C.

Procedure

Pipetted out 3ml of 0.05 M-pyrogallol solution and 0.5 to 0.1ml of enzyme extract in a test tube. Adjusted the spectrophotometer to read '0' at 400nm. Added 0.5ml of 1% H_2O_2 in the test cuvette. Recorded the change in the absorbance every 30 seconds up to 3 minutes.

Calculations

Change in absorbance/min	=	X
Weight of the plant material taken	=	300mg
Volume of the extract taken for the assay	=	0.02 ml
Change in absorbance	=	X
Change in absorbance for 1.5 ml extract	=	(X/0.02) x 1.50 – Y
(i.e) Peroxidase activity in 300 mg plant tissue	=	Y
Peroxidase activity / gram of plant tissue	=	Y x (1000/300) Units.

APPENDIX - III

ESTIMATION OF SUPEROXIDE DISMUTASE ACTIVITY

(Misra and Fridovich, 1972)

Principle

Superoxide dismutase uses the photochemical reduction of riboflavin as oxygen generating system and catalyses the inhibition of Nitro Blue Tetrozolium (NBT) reduction, the extent of which can be assayed spectrophotometrically.

Reagents

1. 50mM potassium phosphate buffer, pH 7.8
2. 45μM Methionine

3. 5.3μM Riboflavin
4. 84μM Nitro Blue Tetrozolium (NBT)
5. 20mM Potassium cyanide

Procedure

The incubation medium contained a final volume of 3ml, 50mM potassium phosphate buffer (pH 7.8), 45μM methionine, 5.3μM riboflavin, 84μM NBT and 20mM potassium cyanide. The tubes were placed in an aluminum Foil-lined box maintained at 25°C and equipped with 15W fluorescent lamps. Reduced NBT was measured spectrophotomerically at 600nm after exposure to light for 10 minutes. The maximum reduction was evaluated in the absence of the amount of enzyme giving 50% inhibition of the reduction of NBT.

APPENDIX – IV
ESTIMATION OF POLYPHENOL OXIDASE ACTIVITY
(Esterbauer *et al.*, 1977)

Principle

Polyphenol oxidases are copper proteins of wide occurrence in nature, which catalases the aerobic oxidation of certain phenolic substrate to quinines, which are auto oxidized to dark brown pigments generally known as melanins. The polyphenol oxidases (PPO) comprise catechol oxidase and laccase. One unit of either catechol oxidase or laccase in defined as the amount of enzymes that transforms 1μmol of quinone per minute under the assay conditions.

Reagents

1. 50mM Tris-HCL(Ph 7.2)
2. 0.4M Sorbitol
3. 10mM Sodium chloride
4. 0.1M Phosphate buffer (pH 6.5)
5. 0.01M Catechol solution

Preparation of enzyme extract

Ground about 5g of the plant tissue and made upto 20ml with the medium containing 50mM Tris-HCl (pH 7.2), 0.4M sorbitol and 10mM NaCl. Centrifuged the homogenate at 2000rpm for 10minutes and used the supernatant for the assay.

Procedure

Added 2.5ml of 0.2M phosphate buffer (pH 6.5), 0.3ml of catechol solution (0.01M) into the cuvette and set the spectophotometer at 495nm. Now added the enzyme extract (0.2ml) and started recording the change in absorbance for every 30seconds upto 5 minutes.

Enzyme units in the test = K(Δ/min)
K for catechol oxidase = 0.272
K for laccase = 0.242

APPENDIX – V

ESTIMATION OF GLUTATHIONE-S-TRANSFERASE ACTIVITY

(Habig *et al.*, 1974)

The enzyme activity was assayed by its ability to conjugate GSH and CDNB, the extent of conjugation causing a proportionate change in the absorbance at 340nm.

Reagents

1. 1mM - Chloro 2, 4-dinitrobenzene (CDNB) in ethanol
2. 1mM - Glutathione
3. 0.1 M -Phosphate buffer
4. 50mM -Tris-HCl buffer pH (7.2)

Procedure

The assay was done at 5°C under condition giving activities linear with respects to incubation time and protein concentration for at least 3 minutes. The enzyme activity was determined by monitoring the change in absorbance

at 340nm in a spectrophotometer. 0.1 ml of both substrates (GSH and CDNB was taken in 0.1M phosphate buffer (pH 6.5) at room temperature to make a volume of 2.9 ml. The reaction was started by the addition of 0.1 ml of sample to this mixture; the readings were recorded against distilled water blank for a minimum of three minutes. The complete assay mixture without the sample served as the control to monitor non-specific binding of the substrate. Care was taken to ensure that final concentration of ethanol in the mixture was always less than 4%.

Calculation

GST activity was calculated using the extinction coefficient of the product formed and the values have been expressed as nano moles and CDNB conjugated/minutes/g sample.

APPENDIX – VI

ESTIMATION OF GLUTATHIONE PEROXIDASE ACTIVITY

(Rotruck *et al.*, 1973)

A known amount of enzyme preparation was used to react with H_2O_2 in the presence of GSH for a specified time period. Then the remaining GSH was measured by the method of Ellman.

$$2GSH + H_2O_2 \xrightarrow{\text{Se -GPx}} GSSG + 2H_2O_2$$

Reagents

1. 0.4M Tris buffer
2. 10mM Sodium azide
3. 10% TCA
4. 0.4mM EDTA
5. 10mM Hydrogen peroxide
6. 2mM Glutathione

Procedure

To 2ml of Tris buffer, 0.2ml of EDTA, 0.1ml of sodium azide and 0.5ml of plant extract were added. 0.2ml of glutathione followed by 0.1ml of hydrogen peroxide were added to the mixture, mixed well and incubated at 37°C for 10 minutes along with a tube containing all the reagents except sample. After 10 minutes the reaction was arrested by the addition of 0.5 ml of 10% TCA centrifuged and the supernatant was assayed for glutathione by the method of Ellman.

The activities are expressed as μg GSH consumed /minute/mg protein.

APPENDIX – VII

ESTIMATION OF GLUTATHIONE REDUCTASE

(David and Richard, 1983)

Principle

Glutathione reductase catalyses the conversion of oxidized glutathione to reduced glutathione employing NADPH as a substrate. The amount of NAPDH utilized is a direct measure of enzyme activity.

Reagents

1. 0.12M phosphate buffer, pH 7.2
2. 15mM EDTA
3. 10mM sodium azide
4. 6.3 mM oxidized glutathione
5. 9.6 mM NADPH

Procedure

20% aqueous extract was prepared in 0.12 M phosphate buffer pH 7.2 was used as the source of enzyme. The assay system contained 1 ml of 0.12M potassium phosphate buffer, 0.1 ml of 15mM EDTA, 0.1ml of 10mM sodium azide, 0.1 ml 0f 6.3mM oxidized glutathione and 0.1ml of enzyme source and

water in the final volume of 2 ml. Kept for 3 minutes. The 0.1 ml of NADPH was added. The absorbance at 340nm was recorded at an interval of 15 seconds for 2 to 3 minutes. For each series of measurement controls were done that contained water instead of oxidized glutathione. The enzyme activity was expressed as mille moles of NADPH oxidized/minutes/ g sample

APPENDIX – VIII

ESTIMATION OF ASCORBIC ACID

(Roe and Kuether, 1953)

Principle

Ascorbate is converted to dehydroascorbate by treatment with activated charcoal and bromine. Dehydroascorbic acid then reacts with 2, 4-dinitrophenyl hydrazine to form osazones, which dissolves in sulpuric acid to give an orange coloured solution whose absorbance can be measured spectrophotomerically at 540nm.

Reagents

1. 4%TCA
2. 9 N H_2SO_4
3. 2% 2, 4- dinitrophenyl hydrazine: dissolved 2g of DNPH in 100ml of 9N H_2SO_4
4. 10% thiourea
5. 80% sulpuric acid
6. Stock standard solution: Dissolved 100mg of ascorbic acid in 100ml 4%TCA
7. Working standard: diluted 10ml of the stock solution to 100ml with 4%TCA

Procedure

About 1g of the sample was homogenized in 4% TCA up to 10ml. Centrifuged at 2000rpm for 10 minutes. To the supernatant obtained, a pinch of activated charcoal was added, shaken well and kept for 10minutes. Centrifuged once again and removed the charcoal residue. The volume of the clear

supernatants was noted. 0.5 and 1.0 ml aliquots of this supernatant were taken for the assay. The assay volumes were made up 2.0 ml with 4%TCA. 0.2 to 1.0 ml of the working standard solution containing 20-100μg of ascorbate respectively were pipetted out into clean dry test tube, the volume of which were also made up to 2.0 ml with 4% TCA. Added 0.5 ml of DNPH reagent to all the test tubes, followed by 2drops of 10% thiourea solution. Incubated at 37°C for 3 hours. The osazones formed were dissolved in 2.5 ml of 85% sulphuric acid, in cold, drop by drop, with no appreciable rise in temperature. To the blank alone , DNPH reagent and thiourea were added after the addition of H_2SO_4. The tubes were incubated for 30 minutes at room temperature, and the absorbance was read spectrophotometrically at 540nm. Calculated the content of ascorbic acid in the sample using the standard graph.

APPENDIX – IX

ESTIMATION OF α-TOCOPHEROL

(Emmerie-Engel method, 1938 as described by Rosenberg, 1992)

Principle

Tocopherol can be estimated using Emmerie-Engle reaction which 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. Xylene
3. 2, 2'-dipyridyl
4. Standard solution: Dissolved 10mg/10 ml of α-tocopherol in absolute alcohol 91mg of α-tocopherol is equivalent to 100mg of tocopherol acetate.

Extraction

The sample was homogenized with water in a blender. Weighed accurately, 2.5g of the homogenized sample into a conical flask. Added 50 ml of 0.1N H_2SO_4 slowly without shaking. Stoppered and allowed to stand overnight. The next day contents of the flask were shaken vigorously and filtered through whatman No.1 filter paper, discarding the initial 10-15ml of filtrate. Aliquots of the filtrate were used for the estimation.

Procedure

Into 3 stoppered centrifuge tubes (test, standard and blank), pipetted out 1.5 ml of extract, 1.5 ml of standard, 1.5 ml of water respectively. To the test and blank added 1.5 ml of ethanol and to the standard, added 1.5 ml of water. Added 1.5 ml xylene to all the test tubes, stoppered, mixed well and centrifuged. Transferred 1.0 ml of xylene layer into another stoppered tube, taking care not to include any other ethanol or protein. Added 1.0 ml 0f 2, 2'-dipyridyl reagent to each tube, stoppered and mixed. Pipetted out 1.5 ml of the mixture into colorimeter cuvettes and read the extinction of the test and standard against the blank at 460nm. Then in turn beginning with the blank, added 0.33 ml of ferric chloride solution. The amount of vitamin E can be calculated using the formula,

$$\text{Amount of tocopherols in } \mu g = \frac{\text{Reading at 520nm - Reading at 460nm}}{\text{Reading of standard at 520nm x 0.29 x 15}}$$

APPENDIX – X

ESTIMATION OF REDUCED GLUTATHIONE

(Moron *et al.*, 1979)

Principle

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

Reagents

1. DTNB
2. 5% TCA
3. 0.2M Sodium phosphate buffer

Procedure

1g of the sample was homogenized in 5% TCA to give a 20% homogenate. The precipitated protein was centrifuged at 1000rpm for 10 minutes. The homogenate was cooled on ice and 0.1 ml of supernatant was taken for the estimation. The volume of the aliquot was made up to 1.0 ml with 0.2M Sodium phosphate buffer (pH 8.0), 2 ml of freshly prepared DTNB solution (0.6mM) in 0.2M phosphate buffer (pH 8.0), was added to the tubes and intensity of the yellow colour formed was read at 412nm in a spectrophotometer after 10minutes. A standard curve of GSH was prepared using concentration ranging from 2 to 10nmoles of GSH IN 5% TCA.

APPENDIX – XI

ESTIMATION OF POLYPHENOLS

(Malick and Singh, 1980)

Principle

Phenols react with phosphomolybdic acid in Folin-ciocalteau reagent in alkaline medium and produce blue coloured complex (molybdenum blue), which is read in a spectrometer at 650nm.

Reagents

1. 80% Ethanol
2. Diluted Folin-ciocalteau reagent
3. 20% Sodium carbonate
4. Stock standard: 100mg of catechol is made up with 100ml distilled water
5. Working standard: 10ml of stock standard was diluted to 100ml. 1.0ml of this contains 100 µg of catechol.

Procedure

1gm of sample was homogenized using 20 ml of 80% ethanol. The homogenate was centrifuged at 10,000 rpm for 20minutes. The supernatant was saved. The residue was reextracted with 10ml of 80% ethanol, centrifuged and collected the supernatant and evaporated to dryness. The residue was dissolved in a known volume of distilled water (50 ml) and 2.0 ml was taken for the experiment. A working standard of 0.5-2.5 ml catechol solution corresponding to 50-250 µg of catechol were pipetted out into a series of test tubes. The volume was made up to 2.5 ml with water. To all the tubes added 0.5 ml of diluted Folin-ciocalteau reagent. After 3minutes, added 2.0 ml of 20% Na2CO3 solution to each tube and mixed thoroughly. The tubes were placed in a boiling water bath for exactly one minute. Cooled and measured at 650nm against a reagent blank. Constructed a standard graph by plotting the concentration of catechol on X-axis and absorbance on Y-axis. From the graph, the amount of polyphenols present in the samples was estimated and expressed as mg of polyphenols per g of the sample.

APPENDIX – XII

EXTRACTION AND ESTIMATION OF TOTAL CAROTENOIDS AND LYCOPENE

(Zakaria *et al.*, 1979)

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
2. Anhydrous sodium sulphate
3. Calcium carbonate
4. 12% alcoholic potassium hydroxide

Procedure

Weighed 5-10g of the sample. Saponified for about 30 minutes in a shaking water bath at 37°C after extracting the sample in 12%alcoholic KOH. Transferred the saponified extract into a separating funnel (packed with glass wool and $CaCO_3$ containing 10 to 15 ml of petroleum ether layer. Transferred the lower aqueous phase to another separating funnel, and the petroleum ether extract containing the carotenoid pigments to amber coloured bottle. Repeated the extraction of the aqueous phase. To the petroleum ether extract added a small quantity of anhydrous Na_2SO_4 to remove the turbidity. Noted the final volume of the petroleum ether extract and diluted if needed by a known dilution factor. The absorbance of the extract at 450nm and 503nm was noted in a spectrophotometer.

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

P = Optical Density of the sample V = Volume of the sample, W = Weight of the sample

APPENDIX - XIII

ESTIMATION OF PROTEIN

(Lowry *et al.*, 1951)

Principle

The amino acid tyrosine and trytophan present in the protein will react with the Folin-ciocalteau reagent. By the reduction of phosphomolybdic phosphotungstic components it will produce blue colour. Also the colour developed by the biuret reaction of the protein with the alkaline cupric tartarate is measured in Micro Lowry's method.

Reagents

1. Solution A: 1% copper sulphate
2. Solution B: 2% sodium potassium tartarate
3. Solution C: 2% sodium carbonate in 0.1N NaOH
4. Solution D: Mixed just before use, 1ml of solution A, 1ml of solution B and 100ml solution C.
5. Solution E: 1N Folin-ciocalteau reagent (stored protected from light)

Procedure

Pipetted out 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the working standard and known volume of the sample in duplicates to different tubes. Made up the volume to 1 ml with 0.1N NaOH. Added 3.0ml of solution D, followed by 0.3ml of solution E to each tube, mixed well and incubated for 3 min at 37^0C. Read the colour developed at 750 nm against a reagent blank.

APPENDIX - XIV

ESTIMATION OF CARBOHYDRATE

(Hedge and Hofreiter, 1962)

Principle

Carbohydrates are first hydrolyzed into simple sugar using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxyl furfural. This compound forms a green coloured product with anthrone which has an absorption maximum at 630nm.

Reagents

1. 2.5N HCl
2. Anthrone reagent; Dissolved 200mg anthrone in 100 ml 0f ice cold 95% H_2SO_4 (Prepared fresh before use)
3. Stock standard: Dissolved 100mg of glucose in 100 ml distilled water
4. Working standard: 10 ml of stock solution is made up to 100 ml of distilled water.

Procedure

Weighed 100mg of the sample in a boiling tube. Hydrolyzed by keeping it in a water bath for 3 hours with 5 ml of 2.5N HCl and cooled to room temperature. Neutralized it with solid sodium carbonate until the effervescence ceases. Made up the aliquots for analysis. Prepared the standards by making 0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the working standards. '0' served as the blank. Made up the volume to 1 ml in all the test tubes including the sample tubes by adding distilled water. Then added 4.0 ml of anthrone reagent. Heated for eight minutes in boiling water bath. Cooled rapidly and read the green to dark green colour at 630 nm.

APPENDIX - XV

EXTENT OF INHIBITION OF *INVITRO* LIPID PEROXIDATION

(Okhawa *et al.* , 1979)

An *in vitro* model of goat liver homogenate was used for induction of lipid peroxidation mediated by $FeSO_4$ as a pro-oxidant .Application of the relevant plant tissue extract in the medium was tried with an objective of assessing the extent of inhibition of *in vitro* lipid peroxidation by measurement of Thio Barbituric Acid Reactive Substances(TBARS) in the experimental mixtures. TBARS were measured spectrophotometrically at 535 nm.

Reagents

1. Tris buffered saline (TBS.pH,7.4)- 10mM Tris , 0.15M NaCl
2. Ferrous sulphate- Ferrous sulphate was prepared fresh in TBS and added at 10 μ moles final concentration in the assay medium.
3. 1% Thio Barbituric Acid 1g TBA in 100ml hot water or TBS.
4. 70% Alcohol.
5. Acetone.
6. 8.5% Goat liver homogenate prepared in TBS (cold).

Procedure

A 5% liver homogenate was prepared in TBS (cold). 50 µl of it was used in the assay.0.5 g of fresh plant tissue was weighed accurately and homogenized in 1ml of cold TBS. 50µl it was used in the assay. Ferrous sulphate at a concentration of 10µ moles was added in the assay medium to induce oxidation. The final volume in the test tubes were made up to 500µl with cold TBS.

Controls were prepared for each sample containing the respective plant extract (50µl) liver homogenate (50 µl) and TBS to make up final volume to 500µl. Pro-oxidant was not added to the control tubes. A blank containing 50µl of ferrous sulphate and 450µl of TBS was also prepared. An assay medium corresponding to 100% oxidation was prepared by adding all other constituents expect leaf extracts. The experimental medium corresponding to auto oxidation contained only the liver homogenate and TBS to make up final volume to 500µl .All the tubes were incubated at 37 °C for one hour. At the end of incubation period, 500µl pf 70 percent alcohol was added to all the tubes to stop the LPO reaction. 1ml of 1 per cent TBA was added to all the tubes and heated in a boiling water bath for 20 minutes. After cooling to room temperature, tubes were centrifuged. To the clear supernatants collected 500µl of acetone was added and measured the TBARS at 535nm in a spectrophotometer.

APPENDIX – XVI

DETERMINATION OF INHIBITION OF SUPEROXIDE PRODUCTION *IN VITRO*

(Winterbourn *et al* ., 1975)

The extent of superoxide generation was studied on the basis of inhibition in the production of superoxide ion by the plant sample, which was measured colorimetrically at 560nm.

Reagents

1. EDTA (0.1 M containing 1.5mg NaCN/100ml)
2. NBT (1.5mM)
3. 0.12mM Riboflavin
4. 0.067M phosphate buffer, pH 7.8

Procedure

The assay tubes contained test sample (20mg concentration) with 0.2 ml of EDTA , 0.1 ml NBT, 0.05 ml riboflavin and 2.55 ml of phosphate buffer. The control tubes were also set up without leaf extracts. The initial optical densities of the solutionswere recorded at 560nm. After that, these tubes were placed in an area where they received uniform illumination for 30minutes.

Again the optical density was measured at 560nm. The difference in optical density before and after illumination is the quantum of superoxide production and the percentage of inhibition by the test sample was calculated by comparing with the optical density of control.

APPENDIX – XVII

DETERMINATION OF INHIBITION OF NITRIC OXIDE GENERATION

(Green and Hill, 1984)

Aqueous solution of sodium nitroprusside spontaneously generates nitric oxide (NO) at physiological pH, which interacts with oxygen to produce nitrite ion, which is measured colorimetrically.

Reagents

1. Phosphate buffered saline
2. Sodium nitroprusside (100mM)
3. Griess reagent - (1% sulfanilamide, 2% H_3PO_4, 0.01% Naphthalene diamine dihydrochloride)

Procedure

3ml of reaction mixture containing sodium nitroprusside in PBS and extract was incubated at 25°C for 150 minutes. Controls were kept without test compound in an identical manner. After incubation, 0.5ml of Griess reagent was added. The absorbance of the chromopore formed was read at 546nm.

The percentage inhibition of nitric oxide generation was measured by comparing the absorbance values of control and those of test compounds.