Appendices

Appendix – 1
Estimation of superoxide dismutase activity in plant and liver tissue (Misra and Fridovich, 1972)

Principle

Superoxide dismutase uses the photochemical reduction of riboflavin as oxygen generating systems and catalyses the inhibition of Nitro Blue Tetrozolium (NBT) reduction, the extent of which can be assayed spectrophotometrically at 600nm.

Reagents

1. Potassium phosphate buffer (500 mM, pH 7.8)

2. Methionine (450 M)

3. Riboflavin (53 mM)

4. Nitro Blue Tetrazolium (NBT) (840 M)

5. Potassium cyanide (200 M)

Procedure

0.5g of sample (plant tissue and liver tissue) were ground separately with 3.0 ml of potassium phosphate buffer. The homogenates were centrifuged at 2000 rpm for 10 minutes and the supernatants were used for the assay. The incubation medium contained, in a final volume of 3.0 ml, 50 mM potassium phosphate buffer (pH 7.8), 45 M methionine, 5.3 mM riboflavin, 84 M NBT and 20 M potassium cyanide. The amount of homogenate added to this medium was kept below one unit of enzyme to ensure sufficient accuracy. The tubes were placed in an aluminium foil-lined box maintained at 25°C and equipped with 15W fluorescent lamps. After exposure to light for 10 minutes, the reduced NBT was measured spectrophoto-metrically at 600nm. The maximum reduction was observed in the absence of the enzyme. One unit of enzyme activity was defined as the amount of enzyme giving a 50% inhibition of the reduction of NBT. The values were calculated as units/mg protein.

Appendix – 2
Estimation of catalase activity in plant and liver tissue
(Luck, 1974)

Principle

The UV light absorption of hydrogen peroxide can be easily measured between 230 – 250 nm. On decomposition of hydrogen peroxide by catalase, the absorption decreases with time. The enzyme activity can be estimated by this decrease in absorption.

Reagents

1. Phosphate buffer : 0.067 M (pH 7.0)

2. Hydrogen peroxide in phosphate buffer (2mM)

Procedure

A 20% homogenate of the sample(plant tissue and liver tissue) was prepared in phosphate buffer (0.067M, pH 7.0) and the homogenate was employed for the assay. The samples were read against a control without homogenate, but containing the H_2O_2-phosphate buffer. To the experimental cuvette, 3 ml of H_2O_2-phosphate buffer was added, followed by the rapid addition of 40µl enzyme extract and mixed thoroughly. The time interval required for a decrease in absorbance by 0.05 units was recorded at 240nm. The enzyme solution containing H_2O_2-free 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 – 3
Estimation of peroxidase activity in plant tissue
(Reddy *et al.*, 1995)

Principle

Peroxidase catalyses the conversion of H_2O_2 to H_2O and O_2, in the presence of the hydrogen donor pyrogallol. The oxidation of pyrogallol to a coloured product called purpurogalli can be measured spectrophotometrically at 430nm with the specified time interval. The intensity of the product is proportional to the activity of the enzyme.

Reagents

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

Procedure

The plant samples were prepared as 20% homogenate in 0.1M phosphate buffer (pH 6.5) and used for the assay. Pyrogallol solution (3.0 ml) and enzyme extract (0.1 ml) were pipetted out into a cuvette. The spectrophotometer was adjusted to read zero at 30nm followed by the addition of 0.5 ml of 1% H_2O_2 and mixed. The change in absorbance was recorded every 30 seconds up to 3 minutes. One unit of peroxidase activity is defined as the change in absorbance per minute at 430nm.

Appendix – 4
Estimation of glutathione S-transferase activity in plant tissue (Habig *et al*., 1974)

Principle

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

Reagents

1. Glutathione (1mM)
2. 1-chloro-2,4-dinitrobenzene (CDNB) (1mM in ethanol)
3. Phosphate buffer (0.1M, pH 6.5)

Procedure

The samples (0.5g) were homogenized with 5.0ml of phosphate buffer. The homogenates were centrifuged at 5000rpm for 10 minutes and the supernatants were used for the assay. The activity of the enzyme was determined by observing the change in absorbance at 340nm. The reaction mixture contained 0.1ml of GSH, 0.1ml of CDNB and phosphate buffer in a total volume of 2.9ml. The reaction was initiated by the addition of 0.1ml of the enzyme extract. The readings were recorded every 15 seconds at 340nm against distilled water blank for a minimum of three minutes in a spectrophotometer (Genesys 10-S, USA). The assay mixture without the extract served as the control to monitor nonspecific binding of the substrates. GST activity was calculated using the extinction co-efficient of the product formed (9.6mM−1cm−1) and was expressed as nmoles of CDNB conjugated/minute.

Appendix – 5

Estimation of polyphenol oxidase activity in plant tissue

(Esterbauer *et al*., 1977)

Principle

Phenol oxidases are copper proteins of wide occurrence in nature, which catalyse the aerobic oxidation of phenolic substrates to quinones, which are autooxidized to dark brown pigments generally known as melanins, which can be estimated spectrophotometrically at 495nm.

Reagents

1. Tris HCl (50 mM, pH 7.2)
2. Sorbitol (0.4 M)
3. NaCl (10 mM)
4. Catechol (0.01 M) in phosphate buffer (0.1 M, pH 6.5)

Procedure

0.5g of powdered material of both leaf and pod was homogenized in about 20ml medium containing 50mM Tris HCl, pH 7.2, 0.4M sorbitol and 10 mM NaCl. The homogenate was centrifuged at 2000rpm for 10 minutes and the supernatant was used for the assay. The assay mixture contained 2.5ml of 0.1M phosphate buffer and 0.3ml of catechol solution (0.01M). The spectrophotometer was set at 495nm. The enzyme extract (0.2ml) was added to the same cuvette and the change in absorbance was recorded every 30 seconds up to 5 minutes. One unit of either catechol oxidase or laccase is defined as the amount of enzyme that transforms 1 µmole of dihydrophenol to 1 µmole of quinine per minute under the assay conditions. The activity of PPO was calculated using the formula,

Enzyme unit = K x (A/min)

where,

K for catechol oxidase = 0.272

K for laccase = 0.242

Appendix – 6

Estimation of ascorbic acid in plant and liver tissue

(Roe and Kuether, 1953)

Principle

Ascorbate is converted to dehydroascorbate by treatment with activated charcoal or bromine. Dehydroascorbic acid then reacts with 2,4- dinitrophenyl hydrazine to form osazones, which dissolves in sulphuric acid to give an orange coloured solution. The coloured product can be measured spectrophotometrically at 540nm.

Reagents

1. Trichloroacetic acid (4%)
2. Sulphuric acid (9N)
3. 2,4-dinitrophenylhydrazine reagent (2% in 9N sulphuric acid)
4. Thiourea solution (10%)
5. Sulphuric acid (85%)
6. Standard ascorbate solution: 10mg ascorbate in 100ml of 4% TCA.

Procedure

1g the sample(plant tissue and liver tissue) was taken and homogenized with 4% TCA to extract the ascorbate and the final volume was made up to 10ml with 4% TCA. The supernatant obtained after centrifugation at 2000 rpm for 10 minutes was treated with a pinch of activated charcoal, shaken well and kept for 10 minutes. Centrifugation was repeated once again to remove the charcoal residue. The volumes of the clear supernatants obtained were noted. Two different aliquots of the supernatant were taken for the assay.

(0.5ml and 1.0 ml). The assay volumes were made up to 2.0 ml with 4% TCA. A range of 0.2 to 1.0ml of the working standard solution containing 20-100g of ascorbate respectively were pipetted into clean dry test tubes, the volumes of which were also made up to 2.0 ml with 4% TCA. DNPH reagent (0.5ml) was added to all the tubes, followed by two drops of 10% thiourea solution. The osazones formed after incubation at 37°C for 3 hours, were dissolved in 2.5ml of 85% H2SO4, in cold conditions, to avoid an appreciable rise in temperature. To the blank alone, DNPH reagent and thiourea were

added after the addition of H2SO4. After incubation for 30 minutes at room temperature, the samples were read at 540 nm and the levels of ascorbic acid in the samples were determined using the standard graph constructed on an electronic calculator set to the linear regression mode and expressed as mg ascorbate /g leaf.

Appendix-7
Estimation of α-tocopherol in plant and liver tissue
(Rosenberg, 1992)

Principle

The estimation of tocopherols can be done using Emmerie-Engel reaction, based on the reduction of ferric to ferrous ions by tocopherols, which forms a red colour with 2, 2′-dipyridyl. Tocopherols and carotenes are first extracted with xylene and read at 460nm to measure carotenes. A correction is made for this after adding ferric chloride and read at 520nm.

Reagents

1. Absolute alcohol
2. Xylene
3. 2,2′-dipyridyl (1.2g in 1 litre of n-propanol)
4. Ferric chloride (1.2g in one litre of ethanol stored in brown bottle)
5. Standard solution of D,L-tocopherol, 10mg/L in absolute alcohol

(91mg of Alpha-tocopherol is equivalent to 100mg of tocopherol acetate)

6. Sulphuric acid (0.1N)

Procedure

2.5g of the sample(plant tissue and liver tissue) were homogenized in a small volume of 0.1N sulphuric acid and the volume was finally made up to 50 ml by adding 0.1N sulphuric acid slowly, without shaking and the contents were allowed to stand overnight. The contents of the flask were shaken vigorously on the next day and filtered through Whatman No.1 filter paper. Aliquots of the filtrate were used for the estimation of tocopherol. The plant extract, standard and water of 1.5ml were pipetted out into three centrifuge tubes namely test, standard and blank respectively. To all the tubes, 1.5ml each of ethanol and xylene were added, stoppered, mixed well and centrifuged.

After centrifugation, the xylene layer was transferred into another tube, taking care not to include any ethanol or protein. To 1.0 ml of xylene layer, 1.0ml of 2,2′-dipyridyl reagent was added, stoppered and mixed. This reaction mixture was taken in the spectrophotometric cuvettes and the extinctions of the test and the standard were read against the blank at 460nm. Then, in turn, beginning with the blank, 0.33ml of ferric chloride solution was added, mixed well and after exactly 15 minutes, the test and the standard were read against the blank at 520nm. The levels of tocopherol were calculated using the formula

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

Appendix – 8
Estimation of flavonoids in plant tissue
(Cameron *et al*., 1943)

Principle

Flavonoids react with vanillin reagent to produce a colored product which can be measured spectrophotometrically at 340nm.

Reagents

1. Vanillin reagent (1% in 70% sulphuric acid)

2. Catechin standard (110µg/ml)

Procedure

The plant samples (0.5g) were extracted first with methanol: water mixture (2:1) and secondly with the same mixture in the ratio 1:1. The extracts were shaken well and allowed to stand overnight, pooled the supernatants and measured the volume. This was concentrated and then used for the assay. An aliquot of the extract was pipetted out and evaporated to dryness. Vanillin reagent (4.0) ml was added and the tubes were heated for 15minutes in a boiling water bath. Varying concentrations of the standard were also treated in the same manner. The optical density was read at 340nm. The standard curve was constructed and the concentration of flavonoids was calculated. The values are expressed as mg flavonoids/g sample.

Appendix - 9
Estimation of polyphenols in plant tissue
(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. Diluted Folin - Ciocalteau reagent (1:10 dilution)
2. 20% Sodium carbonate
3. Ethanol
4. Stock solution:

   100 mg of catechol was made up with 100ml distilled water
5. Working standard:

   10ml of stock standard was diluted to 100ml. 1.0ml of this contains 100µg of catechol.

Procedure

1g of sample was homogenized using 20ml of 80% ethanol. The homogenate was centrifuged at 10,000rpm for 20 minutes. 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 (50ml) and 2.0ml was taken for the experiment. A working standard of 0.5 – 2.5ml catechol solution corresponding to 50 - 250µg of catechol were pipetted out into a series of test tubes. The volume was made upto 2.5ml with water. To all the tubes added 0.5ml of diluted Folin – ciocalteau reagent. After 3 minutes, added 2.0ml of 20% Na_2CO_3 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 sample was estimated and expressed as mg of polyphenols per g of the sample.

Appendix – 10
Estimation of reduced glutathione in plant and liver tissue
(Moron *et al.*, 1979)

Principle

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

Reagents

1. Phosphate buffer (0.2M, pH 8.0)
2. DTNB (0.6mM in 0.2M phosphate buffer)
3. TCA (5% and 25%)
4. Standard GSH (10mg/ml in 5% TCA)

Procedure

A 20% homogenate was obtained by homogenizing 0.5g of the sample(plant tissue and liver tissue) in 2.5 ml of 5% TCA. The homogenate was immediately acidified by adding 125 of 25% TCA to prevent aerial oxidation of glutathione. The precipitated protein was centrifuged at 1000rpm for 10 minutes. The homogenate was cooled on ice and 0.1ml of the supernatant was taken for the estimation. The supernatant was made up to 1 ml with 0.2M sodium phosphate buffer (pH 8.0). Two ml of freshly prepared DTNB solution was added to the tubes and the intensity of the yellow colour formed was read at 412 nm in a spectrophotometer after 10 minutes. A standard curve of GSH was prepared using concentrations ranging from 2-10 nano moles of GSH in an electronic calculator set to the linear regression mode and the values of the samples were read off it. The values are expressed as nmoles of GSH /g tissue.

Appendix – 11
Determination of Total antioxidant activity by Phosphomolybdenum method
(Prieto *et al*., 1999)

Principle

Antioxidants present in the sample reduce the Mo(VI) and Mo(V) which then react with the phosphate group of sodium phosphate to form a green coloured Mo(V)-

Phosphate complex (Phosphomolybdenum complex) in an acidic medium. This complex is then spectrophotometrically measured at 695nm. The reaction is highly time dependent.

Reagents

1. Phosphomolybdenum reagent

Dissolve 28mM(40mg) of sodium phosphate and 4mM ammonium molybdate(49mg) in 10ml of 0.6M sulphuric acid.

2. Sulphuric acid(0.6M)

Dissolve 170µl of sulphuric acid in 9.83ml of distilled water.

3. Stock standard Ascorbic acid

Dissolve 50mg of ascorbic acid in a 50ml of standard flask and make up to the mark with 4% oxalic acid(1ml = 1mg).

4. working standard ascorbic acid

Dissolve 5ml of ascorbic acid in a 50ml of standard flask and make up to the mark with distilled water(1ml = 100µg)

5. Oxalic acid(4%)

Dissolve 4g of oxalic acid in 100ml of distilled water.

Procedure

1. The tubes containing 0.2ml of extract (100-1000µg/ml) is mixed with 1.8ml of distilled water, 2ml of phosphomolybdenum reagent solution.

2. Incubate it at 95°C for 90 minutes.

3. The mixture is cooled to room temperature and the absorbance is measured at 695nm against a reagent blank.

4. The antioxidant capacity is expressed as ascorbic acid equivalent(AAE).

Calculation

OD of test X Concentration of standard (µg) X made up volume (sample volume)
OD of standard

Appendix – 12

Preliminary phytochemical analysis in plant extracts

Preliminary phytochemical analysis was performed using methods of Harborne, (1984), Kokate *et al*., 1990, Siddiqui and Ali, (1997) and Basset *et al*., (1985).

Detection of Carbohydrates

A small quantity of various extracts of leaf was dissolved separately in 4ml of distilled water and filtered. The filtrate was subjected to the following tests to detect the presence of carbohydrates.

Molisch's Test

The filtrate was treated with 2-3 drops of 1% alcoholic alpha naphthol and 2ml of concentrated sulphuric acid was added along the sides of the test tube. Appearance of brown ring at the junction of two liquids shows the presence of carbohydrates.

Fehling's Test(Red sugar)

The filtrate was treated with each 1ml of Fehling's solution A and B and heated on a water bath. A reddish precipitate was obtained shows the presence of carbohydrates.

Detection of glycosides

Keller-Killani Test(Cardiac glycoside)

5ml of extract was treated with 1ml of glacial acetic acid containing 1 drop of ferric chloride solution .This was underplayed with 1ml of concentrated sulphuric acid. A brown ring of the interface indicates the presence of deoxysugar characteristic of cardenolides.

Modified Borntrager's Test

Extracts were hydrolysed with dil. HCl, and then treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonical layer indicates the presence of anthranol glycosides.

Detection of proteins and aminoacids

Xanthoproteic Test:

The extracts were treated with few drops of conc. Nitric acid. Formation of yellow colour indicates the presence of proteins.

Ninhydrin Test:

To the extract, 0.25% w/v ninhydrin reagent was added and boiled for few minutes. Formation of blue colour indicates the presence of amino acid.

Detection of alkaloids

Extracts were dissolved individually in dilute Hydrochloric acid and filtered.

Mayer's Test:

Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow coloured precipitate indicates the presence of alkaloids.

Wagner's Test:

Filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids.

Dragendroff's Test:

Filtrates were treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). Formation of red precipitate indicates the presence of alkaloids.

Detection of Flavonoids

Alkaline reagent Test

Small quantities of various extracts were dissolved separately in aqueous sodium hydroxide. Apperance of yellow color indicates the presence of flavonoids.

Sulphuric acid Test

To small portion of each extract concentrated sulphuric acid was added. Yellow orange color was obtained shows the presence of flavonoids.

Shinoda's Test

Small quantities of the extract were dissolved in alcohol. To that piece of magnesium followed by concentrated hydrochloric acid was added drop wise and heated. Appearance of magenta color shows the presence of flavonoids.

Detection of phenols

Ferric Chloride Test:

Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

Test for Tannins

5ml of the extract and a few drops of 1% lead acetate were added. A yellow precipitate was formed, indicates the presence of tannins.

Detection of Terpinoids and Steroids

Four milligrams of extract was treated with 0.5 ml of acetic anhydride and 0.5 ml of chloroform. Then concentrated solution of sulphuric acid was added slowly and red violet color was observed for terpenoid and green bluish color for steroids.

Detection of Diterpenes

Copper acetate test

Extracts were dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green colour indicates the presence of diterpenes.

Detection of Triterpinoids

10mg of the extract was dissolved in 1ml of chloroform; 1ml of anhydride was added following the addition of 2ml of Conc.H_2SO_4. formation of reddish violet colour indicates the presence of triterpinoids.

Detection of phytosterols

Small quantities of various extracts were dissolved in 5 ml of chloroform separately. Then this chloroform solution was subjected to the following test to detect the presence of phytosterols.

Salkowski test:

To 1 ml of above prepared chloroform solution, few drops of concentrated sulphuric acid was added. Brown color produced shows the presence of phytosterols.

Libermann Burchard test:

The above prepared chloroform solution was treated with a few drops of concentrated sulphuric acid followed by few drops of diluted acetic acid , 3ml of acetic anhydride. A blush green color appeared indicates the presence of phytosterols.

Detection of saponins

Froth Test:

Extracts were diluted with distilled water to 20ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

Appendix - 13
Estimation of total alkaloids in plant extracts
Singh *et al*., (2004)

Reagents

Standard : Colchicine

0.025M$Fecl_3$

0.05M of 1,10- phenanthroline in ethanol

Procedure

The reaction mixture contained 1ml plant extract, 1ml of 0.025M FeCl3 in 0.5M HCl and 1ml of 0.05M of 1,10- phenanthroline in ethanol. The mixture was incubated for 30 minutes in hot water bath with maintained temperature of 70 ± 20C. The absorbance of red coloured complex was measured at 510nm against reagent blank. Alkaloid contents were estimated and it was calculated with the help of standard curve of colchicines (0.1mg/mL, 10mg dissolved in 10ml ethanol and diluted to 100mL with distilled water). The values were expressed as mg/g of dry weight.

Appendix – 14
Estimation of total flavonoids in plant extracts
(Zhishen *et al.*, 1999)

Principle

Flavonoids are present in extract formed, a charge transfer complex with several heavy metals to give a characteristic colour. In this reaction, the high electro-positive nature of aluminium(Al^{3+}, Aluminium chloride) attracts the atomic nuclei of the aromatic rings in the flavonoids through the μ-electrons and creates a charge-transfer resonance hybrid. This hybrid is highly stable in the aqueous medium, which then interacted with the sodium nitrite in an alkaline medium to form a pink coloured complex that is spectrophotometrically measured at λ 510nm.

Reagents

1. Stock standard Rutin

Dissolve 100mg of rutin in 100ml of 80%ethanol (1ml=1mg)

2. Working standard solution

Dissolve 10ml of the stock solution in 100ml of 80% ethanol (1ml=100µg).

3. Sodium Nitrite (5%)

Dissolve 5g of sodium nitrite in 100ml of distilled water.

4. AlCl3.6H2O (10%)

Dissolve 10g of aluminium chloride in 100ml of distilled water.

5. NaOH (1M)

Dissolve 4g of NaOH in 100ml of distilled water.

Procedure

1. Pipette out 0.5, 1.0, 1.5, 2.0, and 2.5 ml(concentration varying from 50 to 250µg) of the standard solution into a series of test tubes.
2. Pipette out 0.1ml of sample in the test tubes.
3. To all the test tubes, including the blank, add distilled water to makeup to 2.5ml.
4. To all the test tubes add 75µl of 5% $NaNO_2$ and incubate at room temperature for 5 mminutes.
5. Add 150µl of 10% $AlCl_3$ and incubate at room temperature for 6minutes.
6. Then add 0.5ml of 1M NaOH, mix well and the pink coloured substance formed is spectrophotometrically measured at λ510 nm.
7. Draw a graph by plotting the concenteration of rutin along the X-axis and the optical density reading along the Y-axis. The values were expressed as mg/g of dry weight.

Appendix - 15
Estimation of total phenols in plant extracts
(Eberhardt *et al*., 2000)

Principle

The hydroxyl (-OH) groups of phenolic compounds reduce the phosphomolybdic acid to molybdenum blue in the presence of an alkaline medium(present in Folin's reagent). The blue coloured complex was then spectrophotometrically measured at λ 760 nm.

Regents

Stock standard solution

Dissolve 100mg of gallic acid in 100ml of distilled water(1ml=1µg).

Working standard solution

Maku up 10ml of the stock to 50ml with distilled water (1ml=200µg).

Folin-Ciocalteu reagent

Mix the Folin's reagent with distilled water in the ratio of 1:1. This reagent should be prepared freshly.

Sodium carbonate(7%)

Procedure

1. Pipette out 0.1, 0.2, 0.3, 0.4,and 0.5ml(concenteration varying from 2o to 1ooµg) of the working standard solution into a series of test tubes.
2. Pipette out 0.125ml of the unknowm sample into a test tubes.
3. To all the tubes, including the blank, add distilled water to make up to 3.5ml.
4. To all the test tubes, add 0.125ml of Folin's reagent.
5. Incubate the test tubes at room temperature for 6 minutes.
6. Add 1.25ml of 7% sodium carbonate in all the test tubes.
7. Incubate the test tubes at room temperature for 9ominutes.
8. Note the absorbance spectrophotometrically at λ 760nm.
9. Draw the graph by plotting the concenteration of total phenolics along the X-axis and the optical density reading along Y-axis.
10. From the satandard curve on the graph, calculate the unknown sample concenteration. The values were expressed as mg/g of dry weight.

Appendix - 16

Estimation of total tannins in plant extracts (Shanmugam *et al*., 2010)

Principle

Phenols reduce potassium ferricyanide to produce ferrous ions. These ferrous ions in turn react with ferric chloride in the presence of dilute Hcl to form a Prussian blue coloured complex, which can be measured spectrophotometrically at λ700 nm.

Reagents

Stock standard solution

Dissolve 50mg of tannic acid in 50ml of distilled water (1ml=1mg).

Working standard solution

5ml of the stock standard was made up to 50ml with distilled water(1ml=100µg).

$FeCl_3$ (0.02M) in 0.1 M Hcl

Pipette out 8.3 ml of concenterated Hcl in one litre standard flask and make up to the mark with distilled water. Then add 3.24 g of anhydrous $FeCl_3$ and mix well.

Potassium Ferric cyanide(0.008M)

Dissolve 3.38 g of potassium ferricyanide in one litre of distilled water. A yellow coloured solution.

Procedure

Pipette out 0.1, 0.2, 0.3, 0.4 and 0.5 ml (comcenteration varying from 10 to 50µg) of the working standard solution into a series of test tubes. Pipette out 0.1ml of sample into a test tube.

To all the test tubes, including the blank, add distilled water to 4. make up to 7ml. To all the test tubes, add 1ml of potassium ferricyanide and 1ml of $FeCl_3$. Mix well. Measure the absorbance spectrophotometrically at λ 700 nm. Draw the graph by plotting the concenteration of tannin along the X-axis and the optical density reading along Y-axis. From the standard curve of the graph, calculate the unknown sample concenteration. The values were expressed as mg/g of dry weight**.**

Appendix - 17
Estimation of total steroids in plant extracts
(Sabir *et al*., 2003)

Reagents

- Libermann Burchard Reagent (Acetic Anhydrate and Sulfuric acid)
  0.5ml Sulfuric acid dissolved 10ml of a Acetic Anhydrates and kept in ice.
- Standard: 10mg Cholesterol dissolved in 10ml of Chloroform

Procedure

1. Pipetted out 0.5, 1, 1.5, 2, and 2.5 ml of Standards into a series of test tubes and 0.2ml of the sample extract into another test tube.

2. Added 2ml of Libermann Burchard reagent to all the tubes.
3. Made up equal volume in all tubes with Chloroform.
4. Covered with carbon paper and Incubated at room temperature in dark for 30 minutes. A green colour was developed.
5. Read the absorbance at 640 nm. The values were expressed as mg/g of dry weight

Appendix – 18

Determination of DPPH radical scavenging activity
Mensor *et al*., (2001)

Principle

DPPH (2,2-diphenyl-2-picryl hydrazyl), a stable free radical, when acted upon by an antioxidant, is converted into diphenyl-picryl hydrazine with a colour change from deep violet to light yellow colour. This can be quantified spectrophotometrically at 518 nm to indicate the extent of DPPH Scavenging activity by the plant extracts.

Reagents

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

Procedure

The different solvent extracts of leaf and pod parts of *Cassia senna* (25µl) and 0.48 ml of methanol were added to 0.5 ml of methanolic solution of DPPH. The mixture was allowed to react at room temperature for 30 minutes. Methanol alone served as blank and DPPH in methanol, without the plant extracts, served as positive control. After 30 minutes of incubation, the discolourisation of the Purple colour was measured at 518nm. The radical scavenging activity was Calculated as follows.

$$\% \text{ of scavenging activity} = \frac{A_{518}\text{Control} - A_{518}\text{Sample}}{A_{518}\text{Control}} \times 100$$

Appendix – 19

Determination of ABTS radical scavenging activity
(Shirwaikaret *et al*., 2006)

Principle

ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) radical cation decolourization assay was ABTS is a chromogen, which changes into a coloured mono-

cation radical form(ABTS+) in the presence of oxidative agent and the ABTS+ has an absorption peak at 750nm. Antioxidants will reduce ABTS+ into its colourless form and the extent of decolourisation corresponds to the percent reduction of ABTS+.

Reagents

1. Ethanol
2. ABTS solution(7mM ABTS with 2.45mM ammonium persulfate)

(The solution was incubated at room temperature for 12-16hours before use.)

Procedure

The six different extracts (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,

$$\% \text{ of scavenging activity} = \frac{A \text{ Control} - A_{518} \text{Sample}}{A \text{ Control}} \times 100$$

Appendix – 20

Determination of hydroxyl radical scavenging activity (Apak *et al*., 2007)

Principle

Oxidative attack of hydroxyl radicals generated from such a Fenton reaction on deoxyribose produces Malondialdehyde (MDA) and similar substances that are reactive towards Thiobarbituric Acid (TBA) upon heating to yield a pink-coloured TBA-MDA adducts which can be calorimetrically absorbed at 532nm.

Reagents

1. Phosphate Buffer (0.2M) pH (7.0)
2. 2-deoxyribose (10mM)
3. EDTA (20mM)
4. Ferrous Chloride (20mM)
5. H_2O_2 (10mM)
6. TCA (2%)
7. Thiobarbituric Acid (TBA) (1%)

Procedure

1. To a test tube add 1.3ml of phosphate buffer (pH-7.0), 0.5 ml of 10mM 2-deoxy-D-ribose, 0.25ml of 20mM Na_2-EDTA, 0.25ml of 20mM $FeCl_2$ solution,, 0.1ml of sample solution (100-1000µg/ml), 1.9ml distilled water and 0.5ml of 10mM H_2O_2 rapidly in this order.
2. The mixture in a total volume of 10ml is incubated for 4hours at 37°C in a waterbath.
3. At the end of the period, the reaction is arrested by adding 2.5ml of 2.8% TCA.
4. To this add 2.5ml of 1%TBA and keep the reaction mixture at 100°C in a boiling waterbath for 10minutes.
5. Cool the mixture under running tap water and record the absorbance at 520nm.
6. Express the hydroxyl radical scavenging ability in percentage
   [1-(test sample absorbance/blank sample absorbance)]×100%
7. Mannitol is used as a positive control.

Appendix – 21
Determination of inhibition of superoxide radical generation (Winterbourn *et al*, 1975)

Principle:

This assay is based on the inhibition of the production of nitroblue tetrazolium (NBT) Formosan of the superoxide ion by the plant extracts and is measured spectrophotometrically at 530nm.

Reagents:

1. EDTA (0.1M containing 1.5mg NaCl / 100ml)
2. 1.5mM NBT
3. 0.12mM riboflavin
4. 0.067M phosphate buffer (pH – 7.8)
5. Dimethyl sulphoxide (DMSO)

Procedure:

The reaction mixture consisted of 2.63ml phosphate buffer, 20µl plant extract, 200µl EDTA, 100µl NBT, and 50µl riboflavin .The control tubes were also set up where DMSO was added instead of sample. All the tubes were vortexed and measured the initial optical density at 560nm. After that, these tubes were placed in an area where they

received uniform illumination for 30 minutes. Again the optical density was measured at 560nm. The difference in optical density before and after illumination is the generation of superoxide by the test sample and calculated by comparing with the optical density of the control.

(% inhibition) = O.D after illumination – O.D (Initial)/ Control O.D

Appendix – 22

Determination of inhibition of nitric oxide radical generation

(Green and Hill, 1984)

Principle:

Aqueous solution of sodium nitroprusside spontaneously generates nitric oxide (NO) at physiological pH, which interacts with oxygen to produce nitrite ions, which is measured spectrophotometrically at 546nm.

Reagents:

1. 100mM sodium nitroprusside
2. Phosphate buffered saline (PBS) (pH – 7.4)
3. Griess reagent:

   Sulphanilamide – 1%

   Orthophosphoric acid (H_3PO_4) – 2%

   N – (1-napthyl) – ethylene diamine hydrochloride – 0.1%

Procedure:

The reaction mixture (3ml) containing 2ml of sodium nitroprusside, 0.5ml of phosphate buffered saline and 0.5ml of extracted sample was incubated at 25˚C for 150 minutes. Control without test compound was kept in an identical manner. After incubation, 0.5ml of griess reagent was added. Incubate for 30 minutes. The absorbance of the chromophore formed was read at 546nm and the percentage inhibition was calculated by the following formula

$\%\ \text{inhibition} = A_e / A_c \times 100$

Where,

A_c = Absorbance of control

A_e = Absorbance in the presence of plant extract

Appendix – 23

Determination of hydrogen peroxide scavenging activity
Ruch *et al*., (1989).

Principle

The UV light absorption of hydrogen peroxide can be easily measured at 230nm. On scavenging of hydrogen peroxide by the plant extracts, the absorption decreases at this wavelength, which property can be utilized to quantify their H2O2 scavenging ability.

Reagents

1. Phosphate buffer (40mM, pH 7.4)
2. H2O2 in phosphate buffer (40mM)

Procedure

The ability of the plant extracts to scavenge hydrogen peroxide was determined according to the method of Ruch et al (1989). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Extracts (100 µg/mL) in distilled water were added to a hydrogen peroxide solution (0.6 mL, 40mM). Absorbance of hydrogen peroxide at 230 nm was determined 10 minutes later against a blank solution containing the phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging of both *C. monogyna* extracts and standard compounds were calculated.

% Scavenged $[H_2O_2]$ = [(AC – AS)/AC] x 100

Where AC is the absorbance of the control and AS is the absorbance in the presence of the sample.

Appendix – 24

HPTLC analysis for alkaloid and flavonoid profiles

Reagents

Mobile phase and spraying reagent for alkaloids

Ethyl acetate-Methanol-Water (10:1.35:1)

Dragendorff's reagent followed by 10% Ethanolic sulphuric acid reagent.

Mobile phase and spraying reagent for flavonoids

Toluene-Acetone-Formic acid (4.5 : 4.5 : 1)

Folin Cio-Calteu reagent

Procedure

Test solution preparation

The given plant samples each 25mg were weighed accurately in an electronic balance (Afcoset) dissolved in 312.5µl of respective solvents and centrifuged at 3000rpm for 5min. These solutions were used as test solutions for HPTLC analysis.

Sample application

2 µl of test solutions and 2 µl of standard solution were loaded as 5mm band length in the 3 x 10 Silica gel $60F_{254}$ TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument.

Spot development

The samples loaded plate was kept in TLC twin trough developing chamber (after saturated with Solvent vapor) with respective mobile phase (Alkaloid) and the plate was developed in the respective mobile phase up to 90mm.

Photo-documentation

The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in Photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at Visible light, UV 254nm and UV366nm.

Derivatization

The developed plate was sprayed with respective spray reagent and dried at 100°C in Hot air oven. The plate was photo-documented in Visible light and UV 366nm mode using Photo-documentation (CAMAG REPROSTAR 3) chamber.

Scanning

Before derivatization, the plate was fixed in scanner stage (CAMAG TLC SCANNER 3) and scanning was done at UV 254nm. The Peak table, Peak display and Peak densitogram were noted. The software used was winCATS 1.3.4 version.

Detection

Coloured zones at Visible mode and UV366nm were observed from the chromatogram befor and after derivatization, to confirm the presence of alkaloids and flavonoids of leaf extracts of *C. senna*.

Appendix – 25

HPLC analysis for alkaloid and flavonoid profiles

Identification of selected phytoconstituents was carried out in the ethanolic extracts namely DEE and SEE of *C. senna* leaves using a Shimadzu HPLC system with RP C18 column, temperature control module and UV detector.

Procedure

Both the ethanolic extracts namely DEE and SEE of *C. senna* leaves were dissolved in appropriate volume of HPLC grade methanol, filtered through a membrane filter and then injected into the HPLC instrument. The flow rate and injection volume were 1ml /min and 20µl respectively. The mobile phase used was acetonitrile : water . The mobile phase was also filtered through a 0.45 µm membrane filter. The total run time was 20minutes at 1200 psi. For HPLC chromaotogram of alkaloid profile using caffeine as standard, the detection wavelength for chromatographic peaks was 254nm and pH of mobile phase was 3.6. For HPLC chromaotogram of flavonoid profile using kaempferol as standard, the detection wavelength for chromatographic peaks was 282nm and pH of mobile phase was 7.3. For HPLC chromaotogram of flavonoid profile using quercetin as standard, the detection wavelength for chromatographic peaks was 215nm and pH of mobile phase was 3.5.

Appendix - 26

MTT assay in cell lines

(Scudiero *et al*., 1988)

Principle

The reaction between MTT and "mitochondrial dehydrogenase" of viable cells produce water-insoluble formazan salt which is solubilized and quantified with an ELISA plate reader. The absorbance directly correlates with the cell number.

Reagents

Trypsin-0.25%

FBS(Fetal bovine serum)

MTT

Dimethyl sulphoxide

Lysis buffer

RPMI

Procedure

Cells were cultured in 96 well plate and incubated for 24 hrs at CO_2 incubator for cell attachment. Then the media was replaced with a fresh growth medium along with increasing concentrations of plant extracts and incubated at 37˚C for 14 hrs at CO_2 incubator with 5% CO_2. Then the media was replaced with a fresh growth medium along with 20µl of 3(4,5-dimethyl thiazol-2-yl) 2,5 diphenyl tetrazolium bromide (MTT, sigma) MTT reagent was added to it. Again it was incubated for 4 hrs at 37˚C. After incubation purple precipitate was clearly visible under the inverted microscope then the grown medium was removed and 200ml of 0.1% 0.1N acidic isopropyl alcohol was added to the cells to dissolve the formazan crystals. Then the covered plates were kept in the dark at 18-24˚C per overnight. The samples were drawn every 2 hrs and observed the readings at 570nm. The absorbance was recorded at 570nm immediately after the development of purple color. The percentage growth inhibition was calculated using the following formula.

$$\% \text{ cell viability} = \frac{\text{OD of treated cells}}{\text{OD of Control cells}} \times 100$$

Appendix - 27

Estimation of Gamma glutamyl transferase(GGT) activity in serum (Persijn and van der Slik, 1978)

The activity of Gamma glutamyl transferase was assayed in the serum of mice using kits procured from Span Diagnostics Limited, Sachin, India.

Principle

GGT catalyzes the transfer of amino group between L-g-glutamyl-3-carboxy-4-nitroanilide and glycylglycine to form L-g-glutamyl glycylglycine and 5-amino-2-nitrobenzoate, which is measured as an increase in absorbance proportional to the GGT activity in the sample.

Reagents

1. Buffer
2. Substrate (L-γ-glutamyl-3-carboxy-4-nitroanilide)
3. Working reagent : The substrate tablet was dissolved in 2.2ml of buffer

Procedure

Working reagent (1.0ml) was incubated at assay temperature (37°C) for one minute and 0.1ml of sample was added. The contents were mixed well and the initial absorbance was read at 405nm in a spectrophotometer (Genesys 10-S, USA) after one minute and the absorbance reading was repeated after every 1, 2 and 3 minutes. The mean absorbance change per minute was calculated (DA/minute) and enzyme activity isexpressed as IU/L.

Appendix - 28

Estimation of nitric oxide level in serum
Green *et al*., (1982)

Reagents

Cadmium granules. small pieces (60–100 mg) of cadmium granules and stored them in H_2SO_4 (0.1 mol/L).

Glycine buffer. 1.5 gm of glycine was dissolved in about 90 ml of water, pH was adjusted to 9.7 with 2 mol /L of NaOH solution and the final volume was made up to 100 ml (200 mmol /L).

CuSO4 solution. A) 5 mmol /L in glycine buffer for Cortasand Wakid method. B) 200 mmol /L in glycine buffer for our Rapid Cadmiumcopper reduction method.

ZnSO4 solution. 75 mmol /L in analytical water.

NaOH solution. 55 mmol /L in analytical water.

Sulfanilamide. 1000 mg was dissolved in 100 ml of 3 mol /L HCl solution (58 mmol /L). This is stable for one year at room temperature.

N-Naphthylethylene diamine dihydrochloride. 20 mg was dissolved in 100 ml of water (0.8 mmol /L). Stored at 4–8°C.

Standards. 10, 25, 50, 75 and 100 mmol /L working standards were prepared by diluting stock of 1.0 mmol /L solution of

Procedure

Nitrite was estimated by Griess reaction. 600 mL of water/standards/serum filtrates were placed in glass tubes. The reaction was started by adding two granules of

Cu-coated cadmium. These were put on a shaker for 5 min. Addition of equal volume of glycine buffer is omitted

From the above tubes 500 mL of sample were placed into fresh glass tubes. To it 250 mL sulfanilamide solution were mixed in, followed by 250 mL NED solution. Tubes were incubated for 10 min at room temperature for a pink colour development and absorbance was read at 545 nm within 60 min.

Appendix - 29
Estimation of glutathione peroxidase activity in liver tissue
(Rotruck *et al.,* 1973)

Principle

A known amount of enzyme preparation was allowed to react with H_2O_2 in the presence of reduced glutathione (GSH) for a specified time period. Glutathione peroxidase converts GSH to oxidized glutathione. Then the remaining GSH was measured at 421 nm.

Reagents

1. 0.4 M Tris buffer, pH 7.0
2. 10 mM Sodium azide solution
3. 10 per cent Trichloroacetic acid
4. 0.4 mM EDTA
5. 20 mM Hydrogen peroxide solution
6. 2 mM Glutathione solution

Procedure

To 2ml of Tris buffer, 0.2ml of EDTA, 0.1ml of sodium azide and 0.5ml of tissue homogenate were added. 0.2ml of glutathione and 0.1ml of hydrogen peroxide were also added. The contents were mixed well and incubated at 37°C for 10 minutes along with a tube containing the reagents except tissue homogenate. After 10 minutes, the reaction was arrested by the addition of 0.5ml of 10 per cent TCA, centrifuged and the supernatant was assayed for glutathione by the method of Moron *et al.* (1979) as in Appendix –XIX. The activities were expressed as nmoles of GSH utilized/min/mg protein.

Appendix - 30

Assessment of lipid peroxidation in liver tissue (Nichans and Samuelson, 1968)

Principle

The pink chromogen formed by the reaction of 2-thiobarbituric acid with breakdownproducts of lipid peroxidation malondialdehyde and other thiobarbituric acid reactivesubstances (TBARS) in acidic solution was read at 535 nm.

Reagents

1. Tricholro acetic acid (TCA) – 15%
2. Hydrochloric acid (HCl) – 0.25N
3. Thiobarbituric acid (TBA) – 0.38% in hot distilled water
4. TCA-TBA-HCl reagent-solution: 1,2 and 3 were mixed freshly in the ratio of 1:1:1.
5. Stock standard – (MDA bis diethyl acetate) or (1,1,3,3-tetra methoxy propane) was made upto 100ml with double distilled water.
6. Working standard-50 n mol/ml in double distilled water.

Procedure

The tissue homogenate was prepared in Tris-HCl buffer (pH 7.5) 1.0 ml of the tissue homogenate was treated with 2.0ml of TBA-TCA-HCl reagent and mixed thoroughly. The mixture was kept in boiling water bath for 15 minutes. After cooling, the tubes were centrifuged for 10 minutes and the supernatant was taken for measurement. The absorbance of chromophore was read at 535 nm against the reagent blank. The amount of TBARS was calculated using the extinction co-efficient 1.56 X 105 M-1cm-1. The results were expressed as nM of MDA / mg protein.

Appendix - 31

Estimation of aspartate transaminase(AST) and alanine transaminase(ALT) activities in serum (Bergemeyer *et al.*, 1978)

Principle

This assay is based on the principle that AST and ALT catalyse the transfer of amino group from L-aspartate/L-alanine to a-ketoglutarate to yield oxaloacetate/pyruvate respectively. Oxaloacetate/pyruvate can oxidise NADH to NAD+ in the presence of

malate dehydrogenase/lactate dehydrogenase. The decrease in absorbance at 340nm in a spectrophotometer (Genesys 10-S, USA) due to the oxidation of NADH is monitored kinetically and is proportional to AST/ALT activity.

Reagents

1. Reagent 1 – Buffer

Tris (80 mmol/l pH 7.8)

L-aspartate or L-alanine (240 mmol/L)

MDH ³ 600 U/L

LDH ³ 600 U/L

2. Reagent 2 – Substrate

2-oxoglutarate (12 mmol/L)

NADH (0.18 mmol/L)

3. Working reagent

Four parts of reagent 1 were mixed with one part of reagent 2.

Procedure

To 100µl of serum, 1000µl of working reagent was added. The tubes were mixed well and the absorbance was read after 60 seconds and the change in absorbance was measured for 2 minutes at 340nm in a spectrophotometer (Genesys 10-S, USA). AST/ALT activity is expressed as IU/L.

Appendix - 32

Estimation of alkaline phosphatase(ALP) activity in serum (Schlebusch *et al.*, 1974)

Principle

At alkaline pH, ALP catalyzes the hydrolysis of p-nitrophenyl phosphate to yellow coloured p-nitro phenolate and phosphate; the change in absorbance measured at 415nm is directly proportional to the enzyme activity.

Reagents

1. p-nitrophenyl phosphate (PNPP)

2. Buffer

The working reagent was prepared by mixing one vial of PNPP substrate with 5.0ml buffer.

Procedure

To 20µl of serum, 1.0ml of working reagent was mixed and after one minute, theincrease in absorbance was measured at 415nm in a spectrophotometer (Genesys 10-S, USA). The ALP activity is expressed as IU/L.

Appendix - 33
Estimation of serum cholesterol
(Allain *et al.*, 1974)

Principle

Cholesterol esters are hydrolysed to cholesterol and fatty acids. The cholesterol is oxidized by cholesterol oxidase to form cholesten-3-one and H2O2. This H2O2 oxidizes 4-amino antipyrene and phenol to a red coloured compound, which can be measured at505nm.

Reagents

1. Reagent 1

1. Buffer (50 mmol/L pH 6.7)
2. Cholesterol oxidase ³ 50 U/L
3. Cholesterol esterase ³ 100 U/L
4. Peroxidase ³ 3 IU/L
5. 4-amino antipyrine (0.4 mmol/L)

2. Reagent 2

Cholesterol standard (200 mg/dL)

Procedure

To 1.0ml of cholesterol reagent, 10µl of serum and standards were added separately. The contents were mixed well and incubated at 37°C for 10 minutes. The absorbance of the standard and sample was measured against reagent blank at 505nm within 60 minutes. The serum cholesterol is expressed as mg/dl.

Appendix - 34
Estimation of serum triglycerides
(Bucolo and David, 1973)

Principle

Triglycerides are hydrolysed by lipases to glycerol and free fatty acids. Glycerol is converted to H2O2 and dehydroxyacetone phosphate. H2O2 combines with 4-chlorophenol to form a pink coloured complex, whose absorbance is measured at 500nm.43

Reagents

1. Buffer Magnesium chloride 9.8 mmol/L

PIPES 100 mmol/L

Chloro-4-phenol 3.5 mmol/L

2. Enzymes Lipase ³ 1000 IU/L

POD ³ 1700 IU/L

Glycerol 3 phosphate oxidase ³ 3000 IU/L

Glycerol kinase ³ 600 IU/L

3. 4-amino-antipyrine (PAP) 0.5 mmol/L

4. Adenosine triphosphate Na (ATP) 1.3 mmol/L

5. Standard Glycerol 2.8 mmol/L

Triglycerides 200 mg/dL

Procedure

To three tubes, namely blank, calibrator and assay tubes, 300µl of buffer was added and 3µl of sample was added only to the assay tube. The contents were mixed vigorously and allowed to stand for 10 minutes at 37°C. The absorbance was recorded at 546nm in a spectrophotometer (Genesys 10-S, USA) against blank. The values are expressed as mg/dL.

Appendix - 35
Estimation of serum free fatty acids
(Falholt *et al.*, 1973)

Principle

Lipids are solubilized in the presence of phosphate buffer, and mixed with high density copper reagent (pH 8.1). The copper soap remaining in the upper organic layer is determined spectrophotometrically with diphenylcarbazide at 550nm.

Reagents

1. Phosphate buffer (33 moles/L, pH 6.4)
2. Extracting solvent (chloroform : hexane : methanol in the ratio 5:5:1)
3. Copper reagent (500nmoles/L each of CuSO4, triethanolamine, sodium hydroxide, sodium chloride, pH 8.1)
4. Standard palmitic acid (2mM/L)
5. Diphenyl carbazide

Procedure

To 0.1ml of serum, 1.0ml of phosphate buffer, 6.0ml of extracting solvent and 2.5ml of copper reagent were added and mixed well. The tubes were mixed vigorously for 90 seconds and left for 15 minutes. The contents were centrifuged and 3.0ml of the upper layer was transferred to another tube containing 0.5ml of diphenyl carbazide and mixed. Standard palmitic acid was treated with copper reagent and diphenyl carbazide. The absorbance was measured at 550nm in a spectrophotometer (Genesys 10-S, USA) against phosphate buffer as blank. The serum free fatty acids are expressed as mg/dL.

Appendix - 36
Extraction of lipids from liver tissue
(Folch *et al*., 1970)

Reagents

1. Chloroform : methanol- 2:1 (v/v)
2. Saline: 0.89 % solution

Procedure

A known volume of suspension was mixed with 10 ml of chloroform methanol mixture and homogenized. The homogenate was filtered through Whatmann filter paper(No.42) into a separating funnel . The filtrate was mixed with 0.2 ml of physiological saline and the mixture was kept overnight undisturbed. The lower phase containing the lipid was drained off into pre weighed beakers. The upper phase was re-extracted with more of chloroform-methanol mixture and the extracts were pooled and evapourated under vaccum at room temperature. The lipid extract were redissolved in 3ml of chloroform methanol (2:1) and aliquots were taken for the estimation of cholesterol and phospholipids.

Appendix - 37

Estimation of liver cholesterol
(Zak *et al*., 1953)

Reagents

1. Stock ferric chloride solution:

   840 mg of ferric chloride in 100 ml of glacial acetic acid.

2. Ferric chloride precipitating reagent:

   10 ml of stock was diluted to 100 ml with acetic acid.

3. Ferric chloride diluting reagent:

   8.5 ml of stock was diluted to 100 ml with acetic acid.

4. Stock Standard solution

   200 mg of cholesterol is dissolved in 10 ml ferric chloride precipitating reagent and made up to 100 ml with glacial acetic acid.

5. Working standard solution:

   1 in 50 dilutions

Procedure

0.1 ml of lipid extract was added to 4.9 ml of ferric chloride precipitating reagent and mixed well and centrifuged. From this 2.5 ml of filtrate was taken. A cold ice bath was set and added 2.5 ml of diluting reagent and 4ml of concentrated sulphuric acid with thorough mixing various concentrations of working standard solution were taken and made up to 5ml with diluting reagent. Added 4 ml of sulphuric acid to all the tubes. A blank was also maintained. The colour developed was read at 560nm.

Appendix - 38

Estimation of liver triglycerides
(Foster and Dunn, 1973)

Reagents

1. Isopropanol
2. Aluminium oxide: neutral
3. Saponification reagent:

   5g of potassium hydroxide dissolved in 60 ml of distilled water and 440 ml of isopropanol was added to it.

4. Acetyl acetone Reagent:

   0.75 ml of acetyl acetone was added to 20 ml of isopropanol and mixed.

5. Sodium metaperiodate reagent:

   77 g of anhydrous ammonium acetate was dissolved in about 700 ml of distilled water; 60 ml of glacial acetic acid was added to it followed by 650 mg of sodium metaperiodate.The mixture was dissolved and diluted to one litre.

6. Stock Standard Solution:

1.0 g of tripalmitin was dissolved in 100 ml of isopropanol.

7. Working Standard Solution:

3.0 ml of stock standard was dissolved in 10 ml of isopropanol in a 10 ml volumetric flask. (300mg /100 ml).

Procedure

4.0 ml of isopropanol was added to 0.1 ml of lipid extract and mixed well, followed by 0.4 g of alumina and shaken well for 15 min. Centrifuged at 2000 rpm for 10 min and then 2.0 ml of the supernatant was transferred to appropriately labeled tubes. The tubes were placed in a water bath at 65° C for 15 min for saponification after adding 0.6 ml of the saponification reagent. After cooling 1.0 ml of Sodium metaperiodate was added followed by 0.5 ml o acetyl acetone reagent. After mixing, the tubes were kept in a water bath at 65° C for half and hour. The contents were cooled and read at 430 nm.

Appendix - 39

Estimation of liver phospholipids
(Fiske and Subbarow, 1925)

Reagents

1.Perchloric acid

2.Molybdic acid: 2.5% ammonium molybdate in 3N H_2SO_4.

3.ANSA

4.Phosphorus stock: 35.1 mg of KH_2PO_4 dissolved in 100ml of distilled water.

Procedure

0.1ml of lipid extract was digested with 0.2ml of perchloric acid over a sand bath. Digestion was continued till it was colorless. The liberated phosphorus was estimated.4.3ml of deionised water was added to the digested sample followed by 0.5ml of ammonium molybdate. After 10min 0.2ml of ANSA was added. Tubes were well shaken and kept aside for 20mins. Blue colour was read at 620nm.

Appendix - 40

Estimation of liver free fatty acids
(Falholt *et al*., 1973)

Reagents

1.extraction solvent: chloroform: methanol (5:1)

2.stock copper solution (500mM)

3.trietanolamine-1M

4.sodium hydoxide-1M

5. Copper reagent

6. diphenyl carbazide solution-1.5M in ethanol

7. Standard Palmitic acid

Procedure

0.1ml of lipid extract was evaporated to dryness. 0.1 ml of phosphate buffer, 6.0ml of extraction solvent and 2.5 ml of copper reagent were added. All the tubes were shaken vigorously, 200mg of activated silicic acid was added and left aside for 30 minutes. The tubes were centrifuged and 3ml of the copper layer was transferred to another tube containing 0.5 ml of diphenyl carbazide and mixed carefully. The absorbance was read at 550nm.

Appendix - 41
Histological analysis of liver tissue
(Culling, 1974)

The following steps were followed in the histological techniques

- Liver homogenates are preserved in 10 percent formalin solution for minimum 1 hour.
- Dehydration of liver homogenates was done by 3 changes of acetone (each 500ml).

- Cleaned the homogenate form acetone by 3 changes of xylene (each 500ml) for about 3hours.
- Incubated the processed tissue bits in paraffin wax- 2 changes for 3-4 hours in an
- incubator at 58-600C.
- Embedded the tissue bits in paraffin wax after incubation in melted paraffin.
- The sections were cut from autopsy bit embedded in wax (sections are 1-36m thick autopsy bit).
- Sections were taken on the glass slide.
- Sections on glass slide from wax by immersing in xylene.
- Sections were histochemically reacted with haemotoxylin and eosin staining to evaluate the morphology and cellular composition.

Appendix - 42

Gas Chromatography - Mass Spectrometry Analysis (Maciejewicz *et al.*, 2007)

Principle

As the name implies, it is actually two techniques that are combined to form a single method of analyzing mixtures of chemicals. Gas chromatography separates the components of a mixture and mass spectroscopy characterizes each of the components individually. By combining the two techniques, both qualitatively and quantitatively evaluate a solution containing a number of chemicals.

Procedure

Chromatographic analysis of derivatized extracts were performed with a GC-MS Hewlett Packard 6890 Series II instrument equipped with an on-line injection system and mass – selective detector Model HP5973A. A capillary column HP-J fused silica column (30m x 0.25mm, column thickness 0.25m (5 %)-biphenyl-(95%) dimethylsilioxane copolymer) was used. The carrier gas was at 1.21 ml/min constant flow. The compounds were separated by following method: isothermal at 100°C for 5 min, 250°C at 15°C/min and finally isothermal at 280°C for10 minutes. Mass spectra were obtained in electron impact ionization at 70eV. The injection volume was 1µl (split ratio 60:1). The identification of components was accomplished
using computer searches in commercial libraries.

Appendix - 43

Fourier Transform-Infrared Spectroscopy

(Mohd nasir *et al.*, 2006)

Principle

Fourier transform-infrared spectroscopy is very much helpful in examining the peak variation of amino groups and carboxylic groups. Some of the infrared radiation is absorbed by the sample and some of it is passed through (transmitted). The resulting spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of the sample, which corresponds to the frequencies of vibrations between the bonds of the atoms making up the material. Because each different material is a unique combination of atoms, no two compounds produce the exact same infrared spectrum. In addition, the size of the peaks in the spectrum is a direct indication of the amount of material present.

Procedure

Infrared spectroscopy of Shimadzu Corporation of model IR prestige 21 was used. A drop of each extract was applied on a sodium chloride cell to obtain a thin layer. The cell was mounted on the IR and scanned through the IR region.