APPENDICES

Appendix 1

Assay of alpha-amylase inhibitory activity

(Bernfeld, 1955)

Principle

Alpha-amylase inhibitory activity was assayed by quantifying the reducing sugar (maltose equivalent) liberated under assay conditions. The α-amylase inhibitory activity was expressed as the decrease in units of maltose liberated. A modification of the dinitrosalicyclic acid (DNS) method of Bernfeld (1955) was adopted for determining maltose content.

Reagents

1. Starch solution: Fresh solution (0.5% w/v) prepared in distilled water with slight warming.
2. Dinitrosalicylic acid reagent: One gram of dinitrosalicylic acid was dissolved in 20ml of 2N sodium hydroxide, 50 ml water and then 30g of potassium sodium tartarate was added and made up to 100ml with water.
3. Alpha- amylase enzyme solution: One mg/ml of stock solution was prepared and then 3ml of the enzyme solution was mixed with 2ml of phosphate buffer (pH-6.9).This solution was used for the assay.

Procedure

200 µl of the extract was allowed to react with 200 µl of porcine pancreatic α-amylase enzyme (Sigma-Aldrich 3176) and 100 µl of 200 mM phosphate buffer (pH-6.9). After 20 min of incubation, 500 µl of 0.5% starch was added. The same was performed for the control where 200 µl of enzyme was replaced by the buffer. After incubation for 5 minutes, 500 µl of dinitro salicylic acid was added to both the control and test. The tubes were kept in a boiling water bath for 10 minutes. The absorbance was recorded at 540 nm using a spectrophotometer and the percentage of α- amylase inhibition was calculated using the formula:

Inhibition (%) = 100 (Absorbance $_{Control}$ - Absorbance $_{Test}$ / Absorbance $_{Control}$)

Suitable reagent blank and inhibitor controls were also carried out and subtracted. Dose dependent variation in the α-amylase inhibition was measured using 0.2 - 1.0 mg/ml concentration of different solvent extracts of petroleum ether, chloroform, ethyl acetate,

ethanol, acetone and water of MCF, MCS, TGL and TGS. Acarbose was used as a positive control.

Appendix 2

Mechanism of alpha- amylase inhibition

(Dixon, 1953 and Cornish- Bowden, 1974)

Dixon plot

Different concentrations of extracts MCF, MCS, TGL and TGS (200-800 µg) were used for studying α-amylase inhibition as given by Dixon, (1953). 200 µl of porcine pancreatic α-amylase enzyme was allowed to react with various concentrations of inhibitor (200-800 µg) and 100 µl of 200 mM phosphate buffer (pH-6.9). After 20 min of incubation 500 µl four different concentrations of starch (0.25, 0.5, 0.75, and 1.0%) were added. The same was performed for the control where 200 µl of enzyme was replaced by the buffer. After incubation for 5 minutes, 500 µl of dinitro salicylic acid was added to both the control and test. The tubes were kept in a boiling water bath for 10 minutes. The absorbance was recorded at 540 nm using a spectrophotometer and the percentage of α- amylase inhibition was calculated using the formula

$$\text{Inhibition (\%)} = 100\ (\text{Absorbance}_{\text{Control}} - \text{Absorbance}_{\text{Test}} / \text{Absorbance}_{\text{Control}})$$

Test and control for enzyme at two different concentrations were also prepared. Read the absorbance at 560 nm. Absorbance values were extrapolated in a standard graph for maltose and the amount of maltose produced in presence of inhibitor was found. This was taken as the product concentration [V]. Calculated 1/ [V] values and plotted graph with concentration of [I] taken along X-axis and (1/ [V]) along Y-axis.

Parallel lines indicate uncompetitive inhibition; Lines intersecting in the space above the negative side of X-axis depict competitive inhibition; Lines intersecting on the negative side of X-axis, indicate non-competitive inhibition. From the nature of graph obtained, the mechanism of inhibition was studied. Since mixed and competitive mode of inhibition cannot be differentiated by Dixon Plot, Cornish- Bowden plot was performed to confirm the mode of inhibition.

Cornish- Bowden plot

The experiment was performed as mentioned in Dixon Plot. The difference here lies in plotting a graph with the concentration of inhibitor [I] taken along X-axis and substrate concentration divided by product concentration (S / [V]) along Y-axis. From the nature of graph obtained the mechanism of inhibition was studied as suggested by

Eisenthel and Cornish-Bowden (1974). Lines running parallel indicate competitive inhibition, intersection in the space above the negative side of X-axis indicates uncompetitive inhibition while intersection beneath the negative side of X-axis indicates mixed inhibition and intersection on the negative side of X-axis indicates non-competitive mode of inhibition.

Appendix 3

Determination of total antioxidant activity by ferric reducing antioxidant power (FRAP) assay

(Benzie and Strain, 1996)

Principle

FRAP method is based on the reduction of Fe^{3+}-tripyridyl-s-triazine (Fe^{3+} TPTZ) complex to ferrous form at low pH. This reduction is monitored by measuring the absorption change at 593 nm.

Reagents

1. Tripyridyltriazine (TPTZ) (10 mM)
2. Hydrochloric acid (40 mM)
3. Ferric chloride (20 mM)
4. Acetate buffer (300 mM, pH 3.6)

Procedure

The FRAP reagent contained 2.5 ml of a 10 mM TPTZ solution in 40 mM hydrochloric acid, 2.5 ml of 20 mM $FeCl_3.6H_2O$ and 25 ml of 300 mM acetate buffer (pH 3.6). It was freshly prepared and warmed at 37°C. 900µl FRAP reagent was mixed with 90µl water and 10µl of the MCF, MCS, TGL and TGS samples. The reaction mixture was incubated at 37°C for 30 minutes and the absorbance was measured at 593 nm.

Appendix 4

Estimation of DPPH radical scavenging activity

(Blois, 1958)

Principle

DPPH (2, 2'–diphenyl-2-picryl hydrazyl hydrate) is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diagnostic molecule. The reduction capacity of DPPH was determined by decrease in absorbance at 517 nm, which is induced by antioxidants.

Reagents

1. DPPH (2, 2'–diphenyl-2-picryl hydrazyl hydrate 0.1 mM in methanol)
2. Methanol

Procedure

The sample extracts (MCF, MCS, TGL and TGS) at various concentrations (200 – 1000µg) were taken and the volume was adjusted to 100 µl with methanol. 5 ml of 0.1 mM methanolic solution of DPPH was added and allowed to stand for 20 min at 27°C. The absorbance of the sample was measured at 517 nm. Ascorbic acid was used as a standard. Percentage radical scavenging activity of the sample was calculated as follows:

% DPPH radical scavenging activity = (control OD-sample OD / control OD) × 100

The sample concentration providing 50% inhibition (IC_{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

Appendix 5

Determination of nitric oxide radical scavenging activity (Sreejayan and Rao, 1997)

Principle

Aqueous solution of sodium nitroprusside spontaneously generates nitric oxide (NO) at physiological pH, which interacts with O_2 to produce nitrite ions, which is measured at 546nm.

Reagents

1. Sodium nitroprusside (10mM)
2. Phosphate buffered saline (pH 7.4)
3. Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H_3PO_4)

Procedure

Three ml of 10mM sodium nitroprusside in 0.2 M phosphate buffered saline (pH 7.4) was mixed with different concentrations (200 - 1000µg) of MCF, MCS, TGL and TGS solvent extracts and incubated at room temperature for 150 min. After incubation time, 0.5 ml of Griess reagent was added. The absorbance of the chromophore formed was read at 546 nm. Ascorbic acid was used as a standard. Percentage radical scavenging activity of the sample was calculated as follows:

% NO radical scavenging activity = (control OD-sample OD / control OD) x 100

The sample concentration providing 50% inhibition (IC_{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

Appendix 6

Estimation of hydroxyl radical scavenging activity

(Klein *et al*., 1991)

Principle

Hydroxyl radicals were generated from ferrous ammonium sulphate and EDTA. This was detected by their ability to react with ascorbic acid to produce yellow colour complex which was measured at 412 nm.

Reagents

1. Ethylenediaminetetraacetic acid (EDTA)
2. Ferrous ammonium sulphate (0.13%)
3. Dimethyl sulfoxide (DMSO- 0.85% v/v in 0.1 M phosphate buffer, pH 7.4)
4. Ascorbic acid (0.22%)
5. Trichloroacetic acid (17.5% w/v)
6. Nash reagent (75.0g of ammonium acetate, 3 ml of glacial acetic acid, and 2 ml of acetyl acetone were mixed and raised to 1 L with distilled water)

Procedure

Different concentrations of the MCF, MCS, TGL and TGS extracts (200 - 1000µg) were added with 1ml of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 ml of EDTA solution (0.018%) and 1ml of DMSO. The reaction was initiated by adding 0.5 ml of ascorbic acid and incubated at 80-90°C for 15 min in a water bath. After incubation, the reaction was terminated by the addition of 1ml of ice-cold TCA. Three millilitres of Nash reagent was added and left at room temperature for 15 min. The intensity of the colour formed was measured spectroscopically at 412 nm against reagent blank. Quercetin was used as the standard. The % hydroxyl radical scavenging activity was calculated as follows:

% Hydroxyl radical scavenging activity = (control OD-sample OD / control OD) × 100

The sample concentration providing 50% inhibition (IC_{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

Appendix 7

Estimation of superoxide radical scavenging activity

(Beauchamp and Fridovich, 1971)

Principle

The assay was based on the capacity of the sample to inhibit farmazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system.

Reagents

1. Ethylenediaminetetraacetic acid (EDTA) (12 mM)
2. Riboflavin
3. Nitrotetrazolium blue chloride (NBT)
4. Sodium phosphate buffer (50 mM pH 7.6),

Procedure

Each 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.6), 20 mg riboflavin, 12 mM EDTA, 0.1 mg NBT and various concentrations (200 - 1000µg) of MCF, MCS, TGL and TGS extracts. Reaction was started by illuminating the reaction mixture with sample extract for 90 seconds. Immediately after illumination, the absorbance was measured at 590 nm. The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes with reaction mixture kept in dark served as blank. Ascorbic acid was used as the standard. The percentage inhibition of superoxide anion generation was calculated as:

% Superoxide radical scavenging activity = (control OD-sample OD / control OD) x 100

The sample concentration providing 50% inhibition (IC_{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

Appendix 8

Estimation of free radical scavenging activity on ABTS

(Re *et al*., 1999)

Principle

The test was based on the relative activity of antioxidants to quench the radical cation ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid). ABTS decolorisation assay involves the generation of the $ABTS^{+}$chromophore by the oxidation of ABTS with ammonium per sulphate. It is applicable for both hydrophilic and lipophilic compounds. The scavenging activity of the plant extract on ABTS radical cation was measured at 734 nm.

Reagents

1. 2, 2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid (ABTS) (7 mM)
2. Potassium persulfate (2.4 mM)
3. Ethanol (1:89 v/v)

Procedure

ABTS was produced by reacting 7 mM ABTS aqueous solution with 2.4 mM potassium persulfate in the dark for 12–16 h at room temperature. Prior to assay, this solution was diluted in ethanol (1:89 v/v) and equilibrated at 30^0 C to give an absorbance at 734 nm of 0.700 ± 0.02. The stock solution of the sample extracts were diluted such that after introduction of 10 µl aliquots into the assay, they produced between 20% and 80% inhibition of the blank absorbance. After the addition of 1 ml of diluted ABTS solution to 10 µl of sample MCF, MCS, TGL and TGS (200-1000 µg/ml), absorbance was measured at 734 nm at exactly 30 min after the initial mixing. Samples were analyzed in triplicate. Ascorbic acid is used as the standard. Percentage radical scavenging activity of the sample was calculated as follows:

% ABTS radical scavenging activity = (control OD-sample OD / control OD) × 100

The sample concentration providing 50% inhibition (IC_{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

Appendix 9

Determination of inhibition of lipid peroxidation

(Ohkawa *et al*., 1979)

Principle

The tissue malondialdehyde (MDA) was allowed to react with thiobarbituric acid (TBA). The MDA-TBA adduct formed during the reaction in acidic medium was extracted to the organic layer and the absorbance was measured at 532 nm.

Reagents

1. Phosphate buffer saline (pH 7.4)
2. Ferrous sulphate (0.07M)
3. 20% acetic acid (pH 3.5)
4. Thiobarbituric acid (0.8% TBA in 1% SDS)
5. Butanol

Procedure

Goat liver was washed thoroughly in cold phosphate buffer saline (pH 7.4) and homogenized to give a 10% homogenate. The homogenate was filtered and centrifuged at 10000 rpm for 10 min and the supernatant was used to carry out the assay. To 0.5 ml of 10% homogenate, 0.5 ml of the sample MCF, MCS, TGL and TGS (50 -250µg) was added. To this, 0.05 ml of 0.07M ferrous sulphate was added and incubated at room temperature for 30 min. To the incubated solution, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% TBA (in 1% SDS) were added. The tubes were incubated at 100°C for 1 hr and cooled to room temperature. About 5 ml of butanol was added and centrifuged at 3000 rpm for 10 min. The upper layer was used to read the absorbance at 532 nm.
The percentage inhibition was calculated as follows:

$$\% \text{ inhibition} = [(\text{Control OD} - \text{Sample OD})/ \text{Control OD}] \times 100$$

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC_{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

Appendix 10
Determination of proteins
(Lowry *et al*., 1951)

Principle

This method is a combination of both Folin-ciocalteau and Biuret reaction which involves two step reactions. In the first step protein binds with copper in alkaline medium and reduces it to Cu^{++}. In the second step Cu^{++} formed catalyses the oxidation reaction of aromatic amino acid by reducing phosphomolybdotungstate to heteropolymolybdenum that leads to the formation of blue colour which is measured at 640 nm.

Reagents

1. Alkaline copper reagent
2. Solution A (2 % w/v of sodium carbonate in 0.1 N sodium hydroxide)
3. Solution B (0.5 % w/v copper sulphate in 1 % sodium potassium tartarate) 50 ml of solution A was mixed with 1 ml of solution B just before use.
4. Folin's phenol reagent commercial reagent (1:2 dilutions)
5. Bovine Serum Albumin (BSA).

Procedure

To 0.1 ml of the serum sample, 0.9 ml of water, 4.5 ml of alkaline copper sulphate reagent were added and allowed to stand in the room temperature for 10 min. To this 0.5

ml of Folin's reagent was added. After 20 min, the blue colour developed was measured at 640 nm. The level of protein present was expressed as mg/dl.

Appendix 11

Estimation of glycogen

(Seifter *et al*., 1950)

Principle

Sulphuric acid in the anthrone reagent hydrolyses the glycogen into glucose and then dehydrates it into furfurals. This compound reacts with anthrone to produce a green colour which is read at 620 nm.

Reagents

1. Potassium hydroxide (60%)
2. Potassium hydroxide (30%)
3. Ethanol
4. Anthrone reagent (0.2 g of anthrone was dissolved in 95 % ice cold sulfuric acid)

Procedure

The sample was homogenized by adding 0.5 ml of 60% potassium hydroxide and 1 ml of 30% potassium hydroxide, both prepared in water. The mixture was incubated in a boiling water bath for 30 minutes. 4 ml of ethanol was added to the homogenate and it was kept in a refrigerator for 24 hours and then centrifuged at 3000 rpm for 20 minutes. The pellet was resuspended in 1 ml of distilled water and from this 0.25 ml was taken and mixed with 1.75 ml of anthrone reagent and kept in boiling water bath for 15 minutes. The colour developed was read at 620 nm.

Appendix 12

Estimation of total cholesterol

(Allian *et al*., 1974)

Principle

Cholesterol esterase hydrolyses the esters. Subsequent enzymatic oxidation by cholesterol oxidase leads to the formation of hydrogen peroxide. This is converted into colored quinonimine in a reaction with 4- aminoantipyrine and phenol, catalyzed by peroxidase and can be read at 505 nm.

Reagents

1. PIPES [Piperazine-N,N′-bis(2-ethanesulfonic acid)] buffer,(pH 6.7- 50 mmol /l)
2. Phenol (24 mmol/l)

3. Sodium cholate (0.5 mmol/L)
4. 4- aminoantipyrene (0.5 mmol/l)
5. Cholesterol esterase (180 U/L)
6. Cholesterol oxidase (200 U/L)
7. Peroxidase (1000 U/L)
8. Cholesterol standard solution (200 mg/dl)

Procedure

Ten microlitre of serum and cholesterol standard was mixed with 1000 µl of reagent, incubated for 5 min at 37 °C and read at 505 nm.

Cholesterol content in mg/dl = $A_{sample} / A_{standard}$ x Concentration of standard

Appendix 13

Estimation of triglycerides

(Schettler and Nussel, 1975)

Principle

Determination of triglycerides (TG) alters splitting with lipoprotein lipase. Indicator is qiuinoneimine which is generated from 4-aminoantipyrine and 4-chlorophenol by hydrogen peroxidase under the catalytic action of peroxidase**.**

Reagents

1. Pipes buffer (50 mmol/l, pH 7.0)
2. p-chlorophenol (5.3 mmol/l)
3. Potassium ferrocyanate (10 mmol/l)
4. Magnesium salt (17 mmol/l)
5. 4- aminoantipyrine (0.9 mmol/l)
6. ATP (3.15 mmol/l)
7. Lipoprotein lipase (1800 U/L)
8. Glycerol kinase (450 U/L)
9. Glycerol-3- phosphate oxidase (3500 U/L)
10. Peroxidase (450 U/L)
11. Triglyceride standard solution (200 mg/dl)

Procedure

Ten microlitre of serum/Triglyceride standard was mixed with 1000 µl of reagent, incubated for 5 min at 37 °C and read at 505 nm. Triglycerides was expressed in mg/dl and calculated by the formula

$A_{sample} / A_{standard}$ x Concentration of standard

Appendix 14

Estimation of HDL cholesterol

(Grove, 1979)

Principle

Chylomicrons, VLDL and LDL are precipitated by adding phosphotungstic acid and magnesium ions to the sample. Centrifugation leaves only the HDL in the supernatant. The cholesterol content in it is determined enzymatically.

Reagents

1. HDL cholesterol reagent
2. Phosphotungstate (14 mmol/l)
3. Magnesium chloride (1 mmol/l)
4. HDL cholesterol standard (50 mg/dl)

Procedure

Three hundred micro litre of serum was mixed with 300 µl of HDL reagent, allowed to stand for 10 min at room temperature, mixed again and centrifuged for 10 min at 4000 rpm. After centrifugation the clear supernatant was separated from the precipitate within 1hr and HDL was determined using cholesterol reagent. 50 µl of supernatant/ standard was mixed with 1000 µl of cholesterol reagent, incubated for 5 min at 37 ° C and read at 500nm.

HDL cholesterol concentration (mg/dl) = A_{sample} / $A_{standard}$ x Concentration of standard x sample dilution factor

Appendix 15

Determination of the activity of superoxide dismutase

(Kakkar *et al*., 1984)

Principle

The assay of superoxide dismutase is based on the inhibition of the formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazon. The colour formed at the end of the reaction can be extracted into butanol and measured at 560 nm.

Reagents

1. Sodium pyrophosphate buffer (0.025M, pH 8.3)
2. Phenazine methosulphate (PMS 186µM)
3. Nitroblue tetrazolium (NBT 300µM)
4. NADH (780µM)
5. Glacial acetic acid
6. n-butanol
7. Potassium phosphate buffer (50mM pH 6.4)

Procedure

The assay mixture contained 1.2ml of sodium pyrophosphate buffer, 0.1ml of PMS, 0.3 ml of NBT, 0.2 ml of the liver homogenate preparation and water in a total volume of 2.8 ml. The reaction was initiated by the addition of 0.2ml of NADH. The mixture was incubated at 30°C for 90 seconds and arrested by the addition of 1.0ml of glacial acetic acid. The reaction mixture was then shaken with 4.0ml of n-butanol, allowed to stand for 10 minutes and centrifuged. The intensity of the chromogen in the butanol layer was measured at 560 nm in a spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme that gave 50% inhibition of NBT reduction in one minute.

Appendix 16

Determination of the activity of catalase

(Luck, 1974)

Principle

The UV absorption of hydrogen peroxide can be measured at 240nm, whose absorbance decreases when degraded by the enzyme catalase. From the decrease in absorbance, the enzyme activity can be calculated.

Reagents

1. Phosphate buffer(0.067M, pH 7.0)
2. Hydrogen peroxide (2mM in phosphate buffer)

Procedure

Liver homogenate was prepared in phosphate buffer. The homogenate was centrifuged and the supernatant was used for the enzyme assay. H_2O_2-phosphate buffer (3.0ml) was taken in an experimental cuvette, followed by the rapid addition of 40µl of enzyme extract and mixed thoroughly. The time required for a decrease in absorbance by 0.05 units was recorded at 240nm in a spectrophotometer. 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 17

Determination of the activity of glutathione peroxidase

(Rotruck *et al.,* 1973)

Principle

The assay is based on the principle that reduction of hydrogen peroxide (H_2O_2), by glutathione peroxidase through simultaneous oxidation of reduced glutathione (GSH) to form oxidized glutathione (GSSG)

Reagents

1. Phosphate buffer (0.32 M pH 7.0)
2. Ethylene diamine tetra acetic acid (EDTA 0.8 mM)
3. Sodium azide (10mM)
4. Reduced glutathione (3mM)
5. Hydrogen peroxide (2.5 mM)
6. Trichloroacetic acid (TCA 10%)
7. Disodium hydrogen phosphate (0.3 M)
8. 5, 5-dithio-bis-(2-nitrobenzoic acid) (DTNB) solution (40 mg of DTNB in 100ml of 1% sodium citrate)
9. Reduced glutathione

Procedure

To 0.1 ml of the tissue homogenate, 0.2 ml of EDTA, sodium azide and hydrogen peroxide were added and mixed. Then 0.4 ml of phosphate buffer was added and allowed to incubate at room temperature. The reaction was arrested by addition of 0.5 ml of TCA. The reaction mixture was centrifuged at 2000 rpm and supernatant was collected. To 0.5 ml of supernatant 4 ml of disodium hydrogen phosphate and 0.5ml of DTNB were added. The blank contained only DTNB and phosphate buffer while standard glutathione was treated with DTNB reagent. The absorbance of standard and liver tissue homogenate samples were measured at 412 nm. The enzyme activities were expressed as µg of GSH consumed /min /mg liver protein.

Appendix 18

Determination of vitamin C

(Roe and Kuether, 1953)

Principle

Ascorbate is converted to dehydroascorbate by treatment with activated charcoal or bromine. Dehydroascorbic acid then reacts with 2,4 dinitro phenyl hydrazine to form osazones, which dissolves in sulphuric acid to give an orange colored solution whose absorbance can be measured spectrophotometrically at 540nm.

Reagents

1. Trichloroacetic acid (TCA 4%)
2. 2,4-dinitrophenyl hydrazine (Dissolved 2g of DNPH in 100ml of 9N H_2SO_4)
3. Thiourea (10%)
4. Sulphuric acid 85%
5. Stock standard solution: Dissolved 100mg of ascorbic acid in 100ml 4% TCA

6. Working standard: Diluted 1.0ml of the stock solution to 100ml with 4% TCA.

Procedure

One gram of liver tissue was ground and homogenised 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 10 minutes. Centrifuged once again and removed the charcoal residue. The volumes of the clear supernatants were noted. 0.5 and 1ml aliquots of this supernatant were taken for the assay. The assay volume was made up to 2ml with 4% TCA. 0.2 to 1.0ml of the working standard solution containing 20 – 100 µg of ascorbic acid respectively were pipetted out into clean dry test tubes, the volumes of which were also made up to 2.0ml with 4% TCA. Added 0.5 ml of DNPH reagents to all the test tubes, followed by 2 drop of 10% thiourea solution. Incubated at 37^0C for 3 hours. The osazones formed were dissolved in 2.5ml 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 sulphuric acid. The tubes were incubated for 30 minutes at room temperature and the absorbance was read spectrophotometrically at 540nm.

Appendix 19

Determination of vitamin E

(Rosenberg, 1992)

Principle

Tocopherols can be estimated using Emmerie-Engel reaction, which is based on the reduction of ferric to ferrous ions by tocopherols, which forms a red colour with 2, 2'-dipyridyl. Tocopherols and carotenes were 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 - tocopherol is equivalent to 100 mg of tocopherol acetate).
6. Sulphuric acid (0.1N)

Procedure

A small volume of 0.1N sulphuric acid was used for homogenizing 2.5g of tissue sample and the volume was finally made up to 50 ml by adding 0.1N sulphuric acid slowly, without shaking and 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. Into 3 stoppered centrifuge tubes (test, standard and blank) 1.5ml of the test sample, standard and water respectively were pipetted out. 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 stoppered tube, taking care not to include any ethanol or protein. To 1.0 ml of xylene layer, 1.0 ml of 2, 2'-dipyridyl reagent was added to each tube, stoppered and mixed. This mixture was taken in the colorimetric cuvettes and the extinctions of the test and the standard were read against the blank at 460nm. Then, in turn, beginning with the blank, 0.33 ml of ferric chloride solution was added, mixed well and after exactly 15 mins, the test and the standard were read against the blank at 520nm. The results are expressed as µg tocopherol/g of sample. The level of tocopherol in the sample was calculated using the formula:

$$\text{Tocopherol (µg)} = \frac{\text{Reading at 520nm} - \text{Reading at 450nm}}{\text{Reading of standard at 520nm}} \times 0.29 \times 15$$

Appendix 20

Estimation of reduced glutathione

(Moron *et al.,* 1979)

Principle

Reduced glutathione (GSH) is measured by its reaction with DTNB (5,5'- dithio-2-nitro benzoic acid). DTNB is a disulfide compound which is reduced by sulphydryl groups present in GSH. This reduction leads to the formation of yellow colour and is measured at 412 nm.

Reagents

1. Sodium phosphate buffer (0.2M pH 8.0)
2. 5,5'- dithio-2-nitro benzoic acid (0.6 mM DTNB in 0.2 M phosphate buffer)
3. Trichloroacetic acid (TCA 5%)
4. Trichloroacetic acid (25%)
5. Standard GSH solution (M.W. 307.33) (Dissolved 10 mg of GSH in 100ml of 5% TCA)

Procedure

A 20% homogenate was obtained by homogenizing 0.5g of sample in 2.5 ml of 5% TCA. To precipitate the protein, 125 µl of 25% TCA was added to 0.5 ml of tissue homogenate. The precipitated protein was centrifuged at 1000rpm for 10 mins. The homogenate was cooled on ice and 0.1 ml 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). 2.0 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 min against a reagent blank. The values are expressed as n moles of GSH /g of tissue.

Appendix 21

Determination of lipid peroxidation

(Ohkawa *et al*., 1979)

Principle

Malondialdehyde (MDA) formed from the breakdown of polyunsaturated fatty acids serves as a convenient index for the determination of the extent of peroxidation reaction. MDA, a product of lipid peroxidation reacts with TBA (Thiobarbituric acid) to give a pink coloured product having absorption maxima at 535nm.

Reagents

1. Trichloroacetic acid (10%TCA)
2. Thiobarbituric acid (0.1M TBA)
3. Phosphate buffer (0.12M, pH 7.2)

Procedure

A 20% liver homogenate was prepared in phosphate buffer (pH 7.2). To 0.5ml of the homogenate, 1.0ml of TCA and 1.0ml of TBA were added and mixed thoroughly. The mixture was heated in a boiling water bath for 20 minutes. The tubes were centrifuged at 1000g for 10 minutes and the absorbance was read at 535nm in a spectrophotometer against a blank containing all the reagents except the homogenate. The concentration was expressed as n moles of MDA per mg of protein.

Appendix 22

Estimation of glucose -6-phosphatase activity

Koide and Oda (1959)

Principle

Glucose-6-phosphatase was assayed according to the method of Koide and Oda (1959) based on the hydrolysis of inorganic phosphorus (Pi) from glucose-6-phosphate by the sample containing enzyme glucose-6-phosphatase.

Glucose-6-phosphate +H_2O ⟶ glucose + Pi

Reagents

1. Citrate buffer (0.1M pH 6.5)
2. Substrate (Glucose-6-phosphate 0.01M in distilled water)
3. Ammonium molybdate solution (2.5%)
4. ANSA (1-amino-2-naphthol-4-sulfonic acid)
5. Trichloroacetic acid (10% TCA)

Procedure

The incubation mixture in a total volume of 1 ml contained 0.3 ml of buffer, 0.5 ml of substrate and 0.2ml of enzyme solution. Incubation was carried out at 37^oC for 60min. The reaction was terminated by the addition of 1ml of 10% TCA solution. The suspension was centrifuged and the phosphorus content in the supernatant was estimated by the method given by Thomas (1998). In acid medium the phosphate reacts with ammonium molybdate to form yellow molybdate complex which was measured at 340nm. One ml of the reagent (0.4mmol/L ammonium molybdate, 210 mmol/L of H_2SO_4) was treated with 10µl standard / 10µl supernatant, mixed and incubated for 5 min at 20-25^oC and measured the absorbance of standard and reagent blank within 60 minutes. The enzyme activity is expressed as n moles of Pi liberated/min/mg protein.

Appendix 23

Estimation of fructose 1, 6-diphosphatase activity

(Gancedo and Gancedo, 1971)

Principle

The enzyme, fructose-1-6-diphosphatase catalyzes the conversion of fructose-1-6-diphosphate to fructose-6-phosphate and Pi. The phosphorus content was estimated by the method of Fiske and Subbarow.

Fructose-1, 6-diphosphate + H_2O ⟶ fructose-6-phosphate + Pi.

Reagents

1. Tris-HCl buffer (0.1M pH 7.0)
2. Substrate (0.05M fructose-1-6-diphosphate solution 21.4mg/1.0ml)
3. Magnesium chloride (0.1M)
4. Potassium chloride (0.1M)
5. Ethylene diamine tetra acetic acid (0.001M EDTA)
6. TCA (10%)

7. Ammonium molybdate solution (2.5%)
8. ANSA (195ml of 15% sodium bisulphate was added to 0.5g of amino naphthol sulphonic acid followed by 5.0ml of 20% sodium sulphate)

Procedure

The assay medium in a final volume of 2ml contained 1.2ml of buffer, 0.1ml of substrate solution, 0.25ml of Magnesium chloride, 0.1 ml of Potassium chloride solution, 0.25ml of EDTA solution and 0.1ml of enzyme. The incubation was carried out at 35^{o}C for 15min. The reaction was terminated by the addition of 1ml of TCA. The suspension was centrifuged and the phosphorus content of the supernatant was estimated by the method of Fiske and Subbarow (1925). Protein was determined by the method of Lowry *et al*. (1951). The enzyme activity is expressed as n moles of Pi liberated/min/mg protein.

Appendix 24

Estimation of glucose -6-phosphate dehydrogenase activity (Ellis and Kirkman, 1961)

Principle

The enzyme assay involves measurement of increase in absorbance when NADP reduces to NADPH. The reaction takes place when electrons are transferred from Glucose 6-phosphate to NADP catalyzed by Glucose 6-phosphate dehydrogenase.

Reagents

1. Tris HCl buffer (0.05 M, pH 7.5)
2. Magnesium chloride (0.1 M)
3. $NADP^{+}$ (0.1 M)
4. 2,6-Dichlorophenol indophenol (0.01 % in distilled water) (freshly prepared)
5. Phenazine methosulphate (0.005% in distilled water) (freshly prepared)
6. Substrate (0.02 M glucose 6-phosphate solution)

Procedure

The incubation mixture in a total volume of 5.5 ml contained 1.0 ml of tris buffer, 0.1 ml of magnesium chloride, 0.1 ml of NADP+, 0.5 ml of phenazine methosulphate, 0.4 ml of the dye solution and the requisite amount of the enzyme extract. The mixture was allowed to stand at room temperature for 10 min to permit the oxidation of endogenous materials. The reaction was initiated by the addition of 0.5 ml of glucose 6-phosphate. The absorbance was read at 640 nm against water blank: at one minute intervals for 3.5 min in a UV spectrophotometer. The activity of the enzyme was calculated in units by multiplying

the change in OD/min by the factor 6/17.6, which is the molar extinction co-efficient of the reduced enzyme activity.

Change in OD/min x molar extinction co-efficient x temperature correction factor (Tf). Tf at 37°C is 0.76 and molar extinction co-efficient of NADPH is 6/17.6. The activity of enzyme was expressed as ml U/mg of protein for tissue.

Appendix 25

Histopathological analysis of pancreas

(Culling, 1979)

The rats were sacrificed by cervical dislocation and an autopsy was carried out to obtain pancreas of the rats. The steps followed in the histopathological analysis were,

1. Tissue samples were taken and preserved in 10 % formalin solution for a minimum of one hour.
2. Dehydration of the fixed tissue was done by giving three changes of acetone (each 100ml).
3. Cleaning of tissue from acetone was done by three changes of xylene (each 500 ml) in a total duration of three hours.
4. Incubation of processed tissue in melted paraffin was done by two changes for 3-4 hours in an incubator maintained at 58 - 60°C.
5. Embedding of the tissue in paraffin wax was done by immersing the tissue in molten paraffin and then cooling it to harden the paraffin.
6. Sections of the paraffin embedded tissue were done using a microtome adjusted to 1-3 µ thickness.
7. The paraffin sections were carefully taken on glass slides.
8. The sections were then cleaned by immersing in xylene.
9. The sections were stained with hematoxylin and aqueous eosin (1 %) and screened to evaluate the morphology and cellular composition.

Appendix 26

Qualitative analysis of phytochemicals

***(*Trease and Evans 1989; Tiwari *et al.,* 2011)**

Detection of flavonoids

Lead acetate Test: To 0.5 ml of extract, few drops of lead acetate solution was added. Formation of yellow colour precipitate indicates the presence of flavonoids.

Shinoda test: To dry powder or extract, 5 ml of 95% ethanol, few drops of concentrated HCl and 0.5 g of copper turnings are added. Appearance of pink colour indicates the presence of flavonoids.

Detection of phenols

Ferric Chloride Test: To 2 ml of extract, 3-4 drops of ferric chloride solution was added. Formation of bluish black colour indicates the presence of phenols

Detection of tannins

Lead acetate test: To 5 ml of extract, 1 ml of 10% lead acetate solution was added. Formation of yellow precipitate indicates the presence of tannins.

Ferric chloride test: To 5ml of extract, 6.1 ml of ferric chloride solution was added. Formation of greenish black precipitate indicates the presence of tannins.

Test for terpenoids

Salkowski test: Five ml of plant extract was mixed in 2 ml of chloroform followed by the careful addition of 3 ml concentrated sulphuric acid. A layer of reddish brown colouration that is formed at the interface indicates the presence of terpenoids.

Detection of steroids

Salkowski's test: To 2 ml of extract added 2 ml of chloroform and concentrated sulphuric acid and shaken well. Chloroform layer appeared red and acid layer showed greenish yellow fluorescence in presence of steroids.

Libermann Burchard's test: Two ml of extract was treated with chloroform and filtered. The filtrate was treated with few drops of acetic anhydride, boiled and cooled. Concentrated sulphuric acid was added. Formation of brown ring at the junction indicates the presence of steroids.

Detection of saponins

Froth test: One ml of the extract was 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.

Detection of alkaloids: Extracts were dissolved individually in dilute hydrochloric acid and filtered.

Mayer's test: To 1 ml of filtrate, few drops of Mayer's reagent (Potassium Mercuric Iodide) was added. Formation of a yellow coloured precipitate indicates the presence of alkaloids.

Wagner's test: Ten ml of extract was treated with few drops of Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids.

Dragendroff's test: To 0.5 ml of filtrate 1 ml of Dragendroff's reagent (solution of potassium bismuth Iodide) was added. Formation of red precipitate indicates the presence of alkaloids.

Detection of glycosides

A small amount of the extract was dissolved in 1 ml of water and then aqueous 10% sodium hydroxide was added. Formation of yellow colour indicates the presence of glycosides.

Appendix 27

Estimation of total flavonoids

(Zhishen *et al*., 1999)

Principle

The basic principle of colorimetric method is that aluminium chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. Aluminium chloride also forms acid labile complexes with the ortho - dihydroxyl groups in the A- or B-ring of flavonoids resulting in pink colour and is measured at 510 nm.

Reagents

1. Sodium nitrite (5%)
2. Aluminium chloride (10%)
3. Sodium hydroxide (4%)

Procedure

0.5ml aliquot of appropriately (10mg/2ml) diluted sample solution was mixed with 2ml of distilled water and subsequently with 0.15ml of 5% $NaNO_2$ solution. After 6 min, 0.15 ml of 10% $AlCl_3$ solution was added and allowed to stand for 6 min, and then 2ml of 4% NaOH solution was added to the mixture. Immediately, water was added to bring the final volume to 5ml, and then the mixture was thoroughly mixed and allowed to stand for another 15min. Absorbance of the mixture was determined at 510 nm versus water blank. The analysis was performed and the results were expressed as rutin equivalent.

Appendix 28

Estimation of total phenols and tannins

(Siddhuraju and Becker, 2003; Siddhuraju and Manian, 2007)

Principle

Phenols react with phosphomolybdic acid in Folin- Ciocalteau reagent to produce a blue coloured complex in alkaline medium, which can be estimated spectrophotometrically at 725 nm.

Reagents

1. Folin-Ciocalteu phenol reagent (1:1 with water)
2. Sodium carbonate (20%)
3. Tannic acid
4. Polyvinyl polypyrrolidone (PVPP)

Procedure

The total phenolic content was determined according to the method described by Siddhuraju and Becker. Ten microlitre aliquots of the extracts (10mg/2ml) were taken in test tubes and made up to the volume of 1 ml with distilled water. Then 0.5 ml of Folin-Ciocalteu phenol reagent (1:1 with water) and 2.5 ml of sodium carbonate solution (20%) were added sequentially in each tube. Soon after vortexing the reaction mixture, the test tubes were placed in dark for 40 min and the absorbance was recorded at 725 nm against the reagent blank. The analysis was performed and the results were expressed as tannic acid equivalents.

Using the same extracts, the tannins were estimated after treatment with polyvinyl polypyrrolidone by the method of Siddhuraju and Manian. One hundred milligrams of PVPP was weighed into a 100×12 mm test tube and to this 1 ml distilled water and then 1 ml of the sample extracts were added. The content was vortexed and kept in the test tube at 4°C for 4h. Then the sample was centrifuged (3000 rpm for 10 min at room temperature) and the supernatant was collected. This supernatant has only simple phenolics other than tannins (the tannins would have been precipitated along with the PVPP). The phenolic content of the supernatant was measured as mentioned above and expressed as the content of non-tannin phenolics (tannic acid equivalents) on a dry matter basis. From the above results, the tannin content of the sample was calculated as follows:
Tannin (%) = Total phenolics (%) – Non-tannin phenolics (%)

Appendix 29
Estimation of total terpenoids
(Ferguson, 1956)

Principle

Total terpenoids were estimated by successful extraction of plant extracts with ethanol followed by petroleum ether since terpenoids are lipid soluble.

Reagents

1. Ethanol (95%)
2. Petroleum ether

Procedure

Two grams of MCS and TGS was taken and soaked in 50 ml of 95% ethanol for 24 hours. The extract was filtered and the filtrate was extracted with petroleum ether and concentrated to dryness. The dried ether extract was treated as total terpenoids.

Appendix 30
High Performance Thin Layer Chromatography (HPTLC) fingerprinting analysis for flavonoids and phenols
(Leena *et al*., 2014)

Sample preparation and application

The samples were prepared by dissolving 5 mg of MCS and TGS in 5ml of ethyl acetate of chromatographic grade and then filtered using Whatman filter paper No. 1. Prepared samples were applied on TLC aluminium sheets silica gel 60 F 254 (Merck) 10 µl each with band length of 5 mm using Linomat 5 sample applicator set at a speed of 200 nl /sec.

Development of chromatogram

The chromatograms were developed in twin trough glass chamber saturated with respective solvents for 20 minutes up to the distance of 70 mm.

Scanning and detection of spots

The air dried plates were viewed in ultraviolet radiation. Spots were visible without derivatization at 254 and 366 nm wavelengths but best results were shown when TLC plates were sprayed with detection reagent. Scanning was performed by CAMAG HPTLC Densitometer (Scanner 3) in absorbance mode at both 254 and 366 nm. The Rf values and colour of the resolved bands were noted.

HPTLC profile for flavonoids

The plant samples MCS and TGS along with reference compounds like quercetin, rutin, kaempferol, catechin were applied as bands and placed in HPTLC twin trough glass chamber. The mobile phase used was chloroform: methanol: formic acid in the ratio of 8.8:1:0.2The plate was sprayed with 1% ethanol aluminium chloride reagent and heated at 120^{o} C for 5 min in hot air oven. The presence of flavonoids was confirmed by the appearance of fluorescence bands at UV 366 nm.

HPTLC profile for phenols

The plant samples MCS and TGS along with reference compounds like resorcinol catechol, gallic acid and hydroquinone are applied as bands and placed in HPTLC twin trough glass chamber. The mobile phase used was chloroform: ethyl acetate: formic acid in ratio 5:4:1. After development, the plate was sprayed with folin-ciocalteau reagent and heated at 120^{o} C for 5 min in hot air oven. The presence of phenolics was confirmed by the appearance of fluorescence bands at UV 366 nm

Appendix 31

Separation of active compounds by Thin Layer Chromatography (TLC)

(Roger *et al.,* 1987)

Thin Layer Chromatography (TLC) was performed on a pre-coated silica gel TLC plate to separate the compounds present in the plant crude extract.

Application of the Samples

A total of 5 µl (10 mg/ml) of sample was spotted at 1 cm from the bottom of silica gel plates using capillary tubes.

Development of Chromatogram

About 120 ml of the solvent mixture (Chloroform: Methanol- 95:5) of the corresponding ratio was added to the TLC tank. In order to saturate the chamber, a filter paper was wetted and was placed over the inner sides of the tank. The TLC plate was placed inside the chamber and tightly covered by means of the lid. When the solvent system reaches the top of the plate, it was removed and air dried. Finally the plate was sprayed uniformly with the spraying reagent and the spots were noted. The Rf values of the spots were calculated and the corresponding silica gel fraction was scraped.

Preparative Thin Layer Chromatography

For preparative TLC, the sample was applied as a streak, developed and then a portion of the TLC plate was sprayed to detect the sample. Then the corresponding region

of silica gel was scraped. The scraped silica gel was extracted by mixing with methanol and then centrifuged to get the clear filtrate. The filtrate evaporated was made up with water. This was used as the inhibitor source for the assay.

General screening of compounds

TLC plate was placed in Iodine chamber and left for 5 min. Iodine will visualize most of the compounds present on the plates. Appearance of brown colored spots indicates the presence of unsaturated compounds.

Appendix 32
Analysis of potent TLC fractions by Fourier transform Infra-Red (FT- IR) spectrophotometry
(Murugan *et al.,* 2014)

Fourier transform infrared spectrophotometer (FT-IR) is perhaps the most powerful tool for identifying the types of chemical bonds and functional groups present in the phytochemicals. The wavelength of light absorbed is salient feature of the chemical bond as can be seen in the annotated spectrum. By interpreting the infrared absorption spectrum, the chemical bonds in a compound can be determined.

Dried powder of MCS and TGS was used for FTIR analysis. 10 mg of the dried extract powder was encapsulated in 100 mg of KBr pellet, in order to prepare translucent sample discs. The powdered sample of each extract was loaded in FT-IR spectroscope (Shimadzu, Japan), with a scan range from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1}.

Appendix 33
Gas Chromatography Mass Spectroscopy (GCMS) analysis of potent TLC fractions
(Mercy *et al*., 2013)

GC-MS analysis was carried out on a GC clarus 500 Perkin Elmer system and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions. Column Elite-1 fused silica capillary column (30mm x 0.25mm ID x 1 µ Mdf, composed of 100% dimethyl poly siloxane), operating in electron impact mode at 70eV; Helium (99.999%) was used as carrier gas at a constant flow of 1ml /min and an injection volume of 1 ml was employed (split ratio of 10:1); Injector temperature 250 ^{0}C; Ion-source temperature 280^{0}C. The oven temperature was programmed from 110^{0}C (isothermal for 2 min) with an increase of 10^{0}C / min, to 200^{0}C then 5^{0}C / min, to 280^{0}C, ending with a 9 min Isothermal at 280^{0}C. Mass spectra were taken at 70eV; a scan

interval of 0.5 seconds and fragments from 45 to 450 Da. Total GC running time was 47 min for MCS and 38 min for TGS The relative percentage of each compound was calculated by comparing its average peak area to the total area. Software adopted to handle mass spectra and chromatograms was a Turbo Mass Ver 5.2.0.

Identification of compounds

Interpretation of mass spectrum GC-MS was conducted using the database of National Institute Standard and Technique (NIST) having more than 62,000 patterns. The spectrum of the unknown compound was compared with the spectrum of the known compounds stored in the NIST library. The name, molecular weight, structure of the compounds in the test material was ascertained.

Appendix 34

Molecular docking of selected compounds with human pancreatic alpha amylase and porcine pancreatic alpha amylase

In the first step, amino acid sequences of human pancreatic alpha-amylase and porcine pancreatic alpha-amylase enzyme were retrieved from Protein Data Bank website. Structures of selected compounds were obtained from Pubchem database. Molecular docking web server was used for docking study (http://www.dockingserver.com/web). Docking calculations were carried out using Docking Server. Gasteiger partial charges were added to the ligand atoms. Non-polar hydrogen atoms were merged, and rotatable bonds were defined. Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added with the aid of AutoDock tools. Docking simulations were performed using the Lamarckian genetic algorithm (LGA) and the Solis & Wets local search method. Initial position, orientation, and torsions of the ligand molecules were set randomly. All rotatable torsions were released during docking. Docking experiment was derived from 10 different runs that were set to terminate after a maximum of 250000 energy evaluations.