APPENDICES

Appendix 1

Qualitative analysis of phytochemicals

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

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 presenceof alkaloids.

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 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.

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 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 indicatesthe presence of saponins.

Detection of steroids

Salkowski's test: To 2 ml of extract added 2 ml of chloroform and concentrated sulphuricacid and

shaken well. Chloroform layer appeared red and acid layer showed greenishyellow 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 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.

Appendix 2

Determination of Moisture content

The moisture content was determined by AOAC (1999). Dry the empty dish and lid in the oven at 105°C for 3 h and transfer to desiccators to cool. Weighed the empty dish and lid. Individually, weighed about the 3gm of samples and spread the sample to uniformly and placed the dish with samples in the oven. Dried for 3 h at 105°C. After drying, transfer the dish with partially covered lid to the desiccator to cool. Reweighed the dish and its dried sample.

Moisture (%) =(W1 – W2)/W1 x100 where, W1= weight (g) of sample before drying W2=weight (g) of sample after drying

Appendix 3

Estimation of protein by Lowry's method

Protein content was estimated according to the method of Lowry *et al* (1951).

Principle

The blue colour developed by the reduction of the phosphomolybdic- phosphotungstic components in the Folin –ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartrate are measured in the Lowry‟s method.

Reagents

i) Solution A: 2.00 gm of sodium carbonate was dissolved in 100.00 ml of 0.1N NaOH.
ii) Solution B: 500.00 mg of copper sulphate was dissolved in 100.00 ml of 1% sodium potassium tartarate solution.
iii) Solution C: 50.00 ml of solution A was mixed with 1 ml of solution – B.
iv) Folin – phenol reagent : 1.0 ml of Folin – phenol reagent was mixed with 1.0 ml of double distilled water.

i) Protein Solution (Stock Standard)

Weigh accurately 50mg of bovine serum albumin (**fraction V**) and dissolve in distilled water and make up to 50ml in a standard flask.

ii) Working Standard Solution:

Dilute 10ml of the stock solution to 50ml with distilled water in a standard flask.

1.0ml of this solution contains 200µg protein.

Procedure

i) Pipette out 0.2, 0.4, 0.6, 0.8 and 1.0ml of the working standard into a series of test tubes.
ii) Pipette out 0.1 ml and 0.2 ml of the sample extract in two other test tubes.
iii) Make up the volume to 1.0 ml in all the test tubes. A tube with 1.0ml of water serves as the blank.
iv) Add 5.0 ml of reagent C to each tube including the blank. Mix well and allowed to standing for 10mins.
v) Then add 0.5 ml of reagent D, Mix well and incubate at room temperature in the dark for

30min, blue colour is developed.

Take the reading at 660nm.Draw a standard graph and calculate the amount of protein in the sample.

Appendix 4

Estimation of total carbohydrate

The total carbohydrate content was estimated by the method of Hedge and Hofreiter, 1962.

Principle

Carbohydrate is first hydrolysed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxmethyl furfural. This compound forms with anthrone a green coloured product with absorption maximum at 630 nm.

Reagents

1. Glucose stock standard: 100 mg of glucose was dissolved in 100 ml of water in a standard flask.
2. Working standard: 10 ml of the stock was diluted to 100 ml. 1.0 ml of this solution contains 100μg of glucose.
3. Anthrone reagent: 0.2% anthrone was dissolved in ice cold concentrated sulphuric acid. Prepared fresh before use
4. 2.5 N HCl.

Procedure

100mg of the sample into a boiling tube, hydrolysed by keeping it in a boiling water bath for three hours with 5.0 ml of 2.5 N HCl and cooled to room temperature. Neutralized it with solid sodium carbonate until the effervescence ceases made up the volume to 100 ml and centrifuged, collected the supernatant and take 0.2 to 1.0 ml for analysis. Prepare the standards by taking 0.2-1.0 ml of the working standards. 1.0 ml of water serves as a blank made up the volume to 1.0 ml in all the tubes with distilled water, then added 4.0 ml of anthrone reagent, heated for eight minutes in a boiling water bath, cooled rapidly and read the green to dark green colour at 630 nm.

Calculation

A standard graph was drawn by taking the concentration of glucose on X axis and spectrophotometer reading on Y axis. From the graph the concentration of glucose in the sample was calculated.

Appendix 5

Estimation of lipid

Powdered dry sample (3 mg) was mixed into 10 ml solution of chloroform and methanol (in the ratio 1:2) and stirred with a glass rod. The resultant mixture was left over night and then centrifuged. After centrifugation, the clear supernatant was removed carefully into washed, dried and pre-weighed small bottles. These bottles were then put in an oven at 40-50°C to evaporate the solvent leaving the lipid fraction.

Appendix 6

Determination of Ash content

The Ash content was determined by AOAC (1999). Place the crucible and lid in the furnace at 550°C overnight to ensure that impurities on the surface of crucible are burned off. Then the crucible was cooled in the desiccator (30 min) and weighed the crucible and lid to 3 decimal places.Take 5g sample into the crucible. Heat over low bunsen flame with lid half covered. When fumes are no longer produced, placed crucible and lid in furnace. Heated at 550°C overnight. During heating, do not covered the lid. Placed the lid after complete heating to prevent loss of fluffy ash. Cool down in the desiccator. Weigh the ash with crucible and lid when the sample turns to gray. If not, returned the crucible and lid to the furnace for the further assaying. Ash (%) = weight of ash/weight of sample x 100

Appendix 7

Estimation of Red blood corpuscle (RBC) count

RBC count was made with a Neubauer crystalline counting chamber as described by Davidson and Henry (1969). The blood samples from the control fish and each group of treatment were collected in a vial containing 2% ethylene diamine tetra acetic acid (EDTA) as an anticoagulant. The blood was drawn up to 0.5 marks in RBC pipette and immediately the diluting fluid was drawn up to the mark 101 (thus the dilution is 1:200). The solution was mixed well by shaking gently. It was allowed to stand for 2 or 3 minutes. The counting chamber and cover glass were cleansed and the cover glass was placed over the ruled area. Again the solution was mixed gently and stem full of solution was expelled and a drop of fluid was allowed to flow under the cover slip holding the pipette at an angle of 40°, it was allowed to stand for 2 to 3 minutes to allow RBC to settle. Afterwards the ruled area of the counting chamber was focused under the microscope and the number of RBC‟s were counted in five small squares of the RBC column under high power and the number of RBC per cu.mm were

calculated accordingly.

Appendix 8

Estimation of White blood corpuscles (WBC) count

WBC count was made with a Neubauer crystalline counting chamber as described by Davidson and Henry (1969).The blood samples from the control fish and each group of treatment were collected in a vial containing 2% ethylene diamine tetra acetic acid (EDTA) as an anticoagulant. Blood is drawn from the vial into WBC pipette up to 0.5 marks and immediately the diluting fluid is drawn up to 11 marks. The solution is mixed thoroughly by shaking gently. It was allowed to stand for 2 or 3 minutes. The counting chamber and cover glass were cleansed and the cover glass was placed over the ruled area. Again the solution was mixed gently and stem full of solution was expelled and a drop of fluid was allowed to flow under the cover slip holding the pipette at an angle of 40°, it was allowed to stand for 2 to 3 minutes to allow RBC to settle. Afterwards the ruled area of the counting chamber was focused under the microscope and the number WBC were counted in bigger squares of the chamber. The WBC count was expressed in cu mm.

Appendix 9

Estimation of haemoglobin(Hb)

The haemoglobin concentration was estimated by Acid - haematin method (Sahli, 1962). N/10 hydrochloric acid was taken up to 20 marks in a graduated tube. Blood was collected directly from the eyeball up to 20 cu mm in the Hb pipette and the outer side was wiped out and this was transferred into the graduated tube containing N/10 hydrochloric acid. Pipette was rinsed two or three times with dilute hydrochloric acid. It was allowed to stand for 10 to 20 minutes after thorough mixing. Then N/10 HC1 was added drop by drop, mixing between each addition until the blood color matched with the standard color. And then the results were read from the scale on the graduated tube and the Hb concentration was expressed in grams percent.

Appendix 10

Hematocrit (Packed Cell Volume)

Hematocrit was estimated by employing micro hematocrit (capillary) method by sodium heparinized micro hematocrit capillaries as described by Nelson and Morris (1989) using RM 12°C micro centrifuge and a micro hematocrit reader.

Principle

When the whole blood with anticoagulant was centrifuged at a constant speed, erythrocytes (RBC), which are heavier than white cells, platelets and plasma, are settled at the bottom.

This red cell column is called hematocrit or packed cell volume which was expressed as fraction of the whole blood (level of plasma).In microhematocrit method, the anticoagulated blood is centrifuged in a sealed capillary tube and with help of a special hematocrit reader the volume of packed red cells and percentage of the whole blood (level of plasma) are determined.

Procedure

Two sodium heparinized capillary microhematocrit tubes each having length about 7 cm long with a uniform bore of about 1mm were taken. Then the blood from control and experimental groups kept in their respective vials were filled up to 5 cm by capillary movement. The sucking end of each tubes were sealed with modeling clay and the filled tubes were placed in radial grooves of the microhematocrit centrifuge head with the sealed end away from the centre and speed at 10,000 rpm for 5 min, using RM 12°C micro centrifuge. The tubes were then taken and the length of the whole column including plasma and that of the red cell column alone was measured in a millimeter rule of the microhematocrit reader. The concentration of the red cells were taken as the hematocrit value which is expressed in percentage.

Appendix 11

Estimation of serum protein

Plasma protein estimation was done according to the method of Lowry *et al* (1951).

Principle

The blue colour developed by the reduction of the phosphomolybdic- phosphotungstic components in the Folin –ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartrate are measured in the Lowry‟s method.

Reagents

i) Solution A: 2.00 gm of sodium carbonate was dissolved in 100.00 ml of 0.1N NaOH.

ii) Solution B: 500.00 mg of copper sulphate was dissolved in 100.00 ml of 1% sodium potassium tartarate solution.

iii) Solution C: 50.00 ml of solution A was mixed with 1 ml of solution – B.

iv) Folin – phenol reagent : 1.0 ml of Folin – phenol reagent was mixed with 1.0 ml of double distilled water.

i) Protein Solution (Stock Standard)

Weigh accurately 50mg of bovine serum albumin (**fraction V**) and dissolve in distilled water and make up to 50ml in a standard flask.

ii) Working Standard Solution:

Dilute 10ml of the stock solution to 50ml with distilled water in a standard flask.

1.0ml of this solution contains 200µg protein.

Procedure

1. Pipette out 0.2, 0.4, 0.6, 0.8 and 1.0ml of the working standard into a series of test tubes.
2. Pipette out 0.1 ml and 0.2 ml of the sample extract in two other test tubes.
3. Make up the volume to 1.0 ml in all the test tubes. A tube with 1.0ml of water serves as the blank.
4. Add 5.0 ml of reagent C to each tube including the blank. Mix well and allowed to standing for 10mins.
5. Then add 0.5 ml of reagent D, Mix well and incubate at room temperature in the dark for 30min, blue colour is developed.
6. Take the reading at 660nm.Draw a standard graph and calculate the amount of protein in the sample.

Appendix 12

Estimation of serum glucose

Plasma glucose was estimated by O-Toluidine method (Cooper and McDaniel,
1970).

Principle

Glucose reacts with O-Toluidine in presence of acetic acid to form a green colour derivative which is measured at 630 nm by using UV Spectrophotometer.

Reagent utilized

Reagent 1 : O-Toluidine colour reagent Reagent 2 : Glucose standard, 100 mg% **Procedure**

Eight test tubes were taken and marked as Blank (B), Control (C), Test (T1), Test (T2),Test (T3),Test

(T4),Test (T5), Test(T6) and Standard (S). To each test tube 5 ml of Reagent-1 (O-Toluidine colour reagent) was added. Then 0.1 ml of distilled water was added to the test tube marked B (Blank). Similarly, 0.1 ml of plasma from control and m treated fish was added to the respective tubes (Control and Test tubes). Then, 0.1 ml of Reagent -2 (Glucose standard) was added to the test tube marked as S (Standard). The contents in all the tubes were mixed well and heated in boiling water for 10 minutes. Then, the test tubes were cooled under running tap water for 5 minutes and the optical density of the test samples were measured at 630 nm within 30 minutes against blank using UV Spectrophotometer

Calculation

$$\text{Plasma glucose in mg/100 ml} = \frac{\text{O.D. of the test}}{\text{O.D. of the Standard}} \times 100$$

Appendix 13

Estimation of serum cholesterol

Serum cholesterol was estimated by the method of Zak (1977).

Principle

Cholesterol in glacial acetic acid gives a yellow-red colour with ferric chloride and a polar sulphuric acid. This reaction has been employed by Zak‟s to estimate the cholesterol in an unknown serum sample.

Reagents

1. **Stock ferric chloride reagent**: 840mg ferric chloride is weighed and dissolved in 100ml glacial acetic acid.
2. **Ferric chloride precipitating reagent**: Dilute 10ml stock ferric chloride in 100ml glacial acetic acid.
3. **Ferric chloride diluting reagent**: Dilute 8.5mL of stock using 100ml of glacial acetic acid.
4. **Standard cholesterol**: Dissolve 200mg of cholesterol in 10ml of ferric chloride precipitating reagent and made up to 100mL with glacial acetic acid.

Procedure

Eight test tubes were taken and marked as Blank (B), Control (C), Test (T1), Test (T2),Test (T3),Test (T4),Test (T5), Test(T6) and Standard (S). 0.10 ml of plasma from control and treated fish was taken in respective tubes (Control and Test tubes). Then 0.90 ml of distilled water was added. 1 ml of distilled water was taken in „Blank‟ tube.To all the test tubes add 4.9mL ferric chloride reagent. Mix well using

glass rod and centrifuge for 15minutes. From this, take 2.5mL filtrate and add 2.5mL of ferric chloride diluting reagent followed by 4mL concentrated sulphuric acid with thorough mixing. For standard curve, take various concentration of cholesterol and made up to 5mL using ferric chloride diluting reagent. Then add 4mL concentrated sulphuric acid to all tubes. Mix well and read the colour developments at 560nm. Plot a standard graph and calculate the amount of cholesterol present in the given sample.

Calculation

Serum cholesterol in g / 100 ml = O.D. of test / O.D. of standard x 100

Appendix 14

Composition and preparation of Muller Hinton culture medium

Muller Hinton Agar Medium (1 L) Composition

Beef extract – 2 grams

Acid hyrolysate of casein – 17.50 grams Starch – 1.50 grams

Agar – 17.00 grams Distilled water – 1000ml pH- 7.3

Preparation

The medium was prepared by dissolving 38g of the commercially available Muller Hinton Agar Medium (Hi Media) in 1000ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure 121° C for 15 minutes. The medium was cooled and poured into 100mm petri plates (25 – 30 ml/ plate).

Appendix 15

Composition and preparation of Nutrient broth Nutrient broth (1 L)

Composition

Peptone -5grams Beef extract - 3grams

Sodium chloride - 5grams Distilled water - 1000ml PH - 6.8

Preparation

One litre of nutrient broth was prepared by dissolving 13 g of commercially available nutrient broth medium (Hi Media) in 1000 ml of distilled water and boiled to dissolve the medium completely. The medium was dispensed as desired and sterilized by autoclaving at 15 lbs pressure at 121° C for 15 minutes.

Appendix 16

Composition and preparation of Nutrient agar medium Nutrient agar medium (1L)

Composition

Peptone -5grams Beef extract - 3grams

Sodium chloride - 5grams Agar agar - 15 grams Distilled water - 1000ml PH - 6.8

Preparation

The medium was prepared by dissolving 28 g of the commercially available Nutrient Agar Medium (Hi Media) in 1000ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure 121° C for 15 minutes. The autoclaved medium was cooled and poured into 100mm petri plates (25 – 30 ml/ plate) while still molten.