MATERIALS AND METHODS

Collection of plant material

_Vaccinium leschenaultii_ Wight was collected during blooming season (January 2012) from Sholas of the Nilgiri Hills, Western Ghats, Southern India, Tamil Nadu (Plate-1). The plant was identified and authenticated by a plant taxonomist.

Description of the selected plant


Habit: Small trees up to 7 m tall.

Trunk\bark: Bark brownish, irregularly scaly; blaze yellowish.

Branchlets: Young branchlets angular to subterete, pubescent.

Leaves: Leaves simple, alternate, spiral; petiole 0.5-1 cm long, planoconvex in cross section, pubescent when young; lamina 4.5-6.5 x 2.5-3 cm, ovate, elliptic or elliptic-ovate, sometimes suborbicular, apex acute or acuminate, base attenuate to acute or obtuse, margin serrate, glabrous, coriaceous, glands on the midrib beneath; midrib slightly raised or flat above; secondary nerves 4-6 pairs; tertiary nerves broadly reticulate, not prominent.

Flowers: Inflorescence axillary and terminal, racemose; flowers urceolate, pink or rarely whitish; petals glabrous; stamens with awns; pedicel ca. 0.3 cm long.

Fruit& seed: Berry, smooth, globose; seeds many.

Ecology: In high elevation evergreen forests between 1600 and 2000 m.

Distribution: India in the Western Ghats- South Sahyadri.

Status: Endemic to sholas of the Nilgiri Hills, Western Ghats, Southern India.
Phytochemical study

250 g of collected *V. leschenaultia* whole samples (except fruits) were washed 2-3 times with water followed by distilled water and shade dried. All the dried parts were pulverized by mechanical grinder (willy mill) to get the powder through 100 mesh sieve and then stored in a refrigerator. The shade dried powdered plant material was defatted first with petroleum ether which also removed the gum and gum-resins and then the residue was re-extracted in methanol using a soxhlet apparatus. In the extracts were concentrated in a rotary evaporator to yield 5 gm of a syrupy residue. The petroleum ether, ethyl acetate, ethanol and aqueous residues were used for the qualitative study.

Qualitative identification Tests

Phytochemical analysis of each *V. leschenaultii* extract was carried out to identify the constituents using standard phytochemical methods as described by Trease and Evans (1989), Harborne (1973). All tests were carried out to find out the presence or absence of the active phytochemical constituents using the following procedures.

**Test for Alkaloids**

2 ml of extract was taken and mixed with few drops of 1% HCl. To 1 ml of this mixture, 6 drops of Mayer’s reagent, Wagner’s reagent and Dragendroff reagent were added. Within few minutes yellow-creamish precipitate colour in Mayer’s reagent and brownish red precipitate in Wagner’s reagents and orange precipitate in Dragendroff’s reagent were appeared.

**Test for flavonoids**

**Shinoda test:**

1 ml of extract was taken and three pieces of magnesium (chips) was added followed by a few drops of concentrated hydrochloric acid. The appearance of an orange, pink or red to purple colour indicates the presence of flavonoids.

**Sulphuric acid test:**

1 ml of extract was dissolved in few drops of concentrated sulfuric acid and the colour change was observed.
Ferric chloride test:

1 ml of extract was added with two drops of freshly prepared ferric chloride solution. Green, blue or violet colour formations indicate the presence of phenolic hydroxyl group.

Sodium hydroxide test:

2 ml of extract was dissolve in 2 ml of 10% aqueous sodium hydroxide solution and filtered to give yellow colour, a change in colour from yellow to colourless on addition of dilute HCl indicate the presence of flavonoids.

Test for terpenoids:

Salkowski test

Five ml of extract was mixed with 2 ml of chloroform, and 3 ml of concentrated sulphuric acid was carefully added to form a layer. A reddish brown colouration indicates the presence of terpenoids.

Test for Glycosides

Borntrager’s test

3 ml of extract was mixed with 3 ml of chloroform and shaken, chloroform layer was separated and 3 ml of 10% ammonia solution was added to it. Pink colour indicates the presence of glycosides.

Test for cardiac glycosides:

Keller-Killani test

Five ml of extract was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml of concentrated sulphuric acid. A brown ring of the interface indicates the presence of cardiac glycosides.

Test for Phenols:

1 ml of extract was added with 1 ml of alcohol and then few drops of neutral ferric chloride solution were added. The test result was observed.
Test for Sterols

Salkowaski test

2 ml of extract was treated with 2 ml of chloroform and 2 ml of concentrated sulphuric acid was added from the side of the test tube. Test tube was shaken for few minutes. The development of red color in chloroform layer indicated the presence of sterols.

Liebermann – Burchard Test

1 ml of extract was taken in a test tube and each 1 ml of chloroform and concentrated sulphuric acid was added. A blue color exhibited by chloroform layer and green fluorescence by the acid layer suggests the presence of sterols.

Test for Saponin

Foam test

1 ml of extract solution was diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes. Development of stable foam suggests the presence of saponins.

Test for Catachol:

1 ml of extract was taken in a test tube and added 1 ml of Erlich’s reagent followed by few drops of concentrated HCL. The result was observed.

Test for Free anthraquinones:

The 1 ml of extract was shaken with 10 ml of benzene and the content was filtered. 5 ml of 10% ammonia solution was added to the filtrate and the mixture was shaken. Appearance of a pink, red or violet colour in the ammoniacal layer (lower phase) indicates the presence of free anthraquinone.

Braemer’s test for tannin:

5 ml of extract was allowed to react with 1 ml of 5% ferric chloride solution. If dark green or deep blue color is obtained. It indicates the presence of tannin.
Test for Phlobatannins

Five ml of distilled water was added to 5ml of extract and boiled with 5ml of 1% HCl for 2min. Visible reaction was obtained. This indicates the presence of phlobatannins.

Test for Resin

Five ml of copper sulphate was added to 5ml of extract. The resultant solution was shaken vigorously and allowed to separate. A green colored precipitate indicating the presence of resin noticed, while the same mixed with ethanol gave a light blue coloration.

HPTLC ANALYSIS

ALKALOID PROFILE

Samples given

Sample A – Methanol extract of *Vaccinium leschenaultii* sample

STD – Alkaloid standard as reference marker

Procedure

Extraction & test solution preparation

The given *V. leschenaultii* dried plant material (5gm) was extracted with methanol in soxhlet apparatus for 3hrs. It was cooled, filtered the content and concentrated using vacuum flash evaporator. The residue was dissolved in 1ml methanol and centrifuged at 3000rpm for 5min. This solution was used as test solution for HPTLC analysis.

Sample application

2µl of *V. leschenaultii* test solution and 3µl of standard solution were separately loaded as 5mm band length in 4 x 10 silica gel 60F254, TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument.

Spot development

The sample 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 (alkaloid) 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 win CATS 1.3.4 version.

**ANALYSIS DETAILS**

**Mobile phase**

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

**Spray reagent**

Dragendorff’s reagent followed by 10% ethanolic sulphuric acid reagent.

**Detection**

Yellow, Orange-yellow and yellowish brown coloured zone at visible light mode present in the given standard and sample track observed in the chromatogram after derivatization, which confirmed the presence of alkaloid / nitrogen containing compound in the given standard and may be in the sample.

**FLAVONOID PROFILE**

**Samples given**

Sample A – Methanol extract plant sample (*Vaccinium leschenaultii*)

STD – Flavonoid standard as reference marker
Procedure

Extraction & Test solution preparation

The given dried plant material (5gm) was extracted with methanol in soxhlet apparatus for 3hrs. Cooled, filtered the content and concentrated using vacuum flash evaporator. Dissolved the content with 1ml methanol and centrifuged at 3000rpm for 5min. This solution was used as test solution for HPTLC analysis.

Sample application

2μl of test solution and 2μl of standard solution was 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 (Flavonoid) 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 (Flavonoid) 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 win CATS 1.3.4 version.
ANALYSIS DETAILS

Mobile phase

Ethyl acetate-Butanone-Formic acid-Water (5 : 3 : 1 : 1)

Spray reagent

1% Ethanolic Aluminium chloride reagent.

Detection

Yellow, Yellowish blue coloured fluorescent zone at UV 366nm mode were present in the tracks, it was observed from the chromatogram after derivatization, which confirmed the Presence of flavonoid / phenol carboxylic acid in the given standard and may be in the sample.

PHENOLIC PROFILE

Samples given

Sample A – Methanol extract plant sample (*Vaccinium leschenaultii*)

STD – Phenolic standard as reference marker

Procedure

Extraction & Test solution preparation

The given dried Plant material (5gm) was extracted with Methanol in soxhlet apparatus for 3hrs. Cooled, filtered the content and concentrated using Vacuum flash evaporator. Dissolved the content with 1ml Methanol and centrifuged at 3000rpm for 5min. This solution was used as test solution for HPTLC analysis.

Sample application

2µl of test solution and 3µl of standard solution was loaded as 5mm band length in the 4 x 10 Silica gel 60F \(_{254}\) TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument.
Spot development

The sample loaded plate was kept in TLC twin trough developing chamber (after saturated with Solvent vapor) with respective mobile phase (Phenolic) 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 (Phenolic) and dried at 100°C in Hot air oven. The plate was photo-documentated 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 win CATS 1.3.4 version.

ANALYSIS DETAILS

Mobile phase

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

Spray reagent

20% Sodium carbonate reagent followed by Folin Cio-calteu reagent.

Detection

Blue, Brown coloured zone at visible light mode present in the given standard and sample track observed in the chromatogram after derivatization, which confirmed the presence of phenolics in the given standard and may be in the sample.
STEROID PROFILE

Samples given

Sample A – Methanol extract plant sample (*Vaccinium leschenaultii*)

STD – Steroid standard as reference marker

Procedure

Extraction & Test solution preparation

The given dried Plant material (5gm) was extracted with Methanol in soxhlet apparatus for 3hrs. Cooled, filtered the content and concentrated using Vacuum flash evaporator. Dissolved the content with 1ml Methanol and centrifuged at 3000rpm for 5min. This solution was used as test solution for HPTLC analysis.

Sample application

2µl of test solution and 3µl of standard solution was loaded as 5mm band length in the 4 x 10 Silica gel 60F$_{254}$ TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument.

Spot development

The sample loaded plate was kept in TLC twin trough developing chamber (after saturated with Solvent vapor) with respective mobile phase (Steroid) 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 (Steroid) 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

After derivatization, the plate was fixed in scanner stage (CAMAG TLC SCANNER 3) and scanning was done at UV 366nm. The Peak table, Peak display and Peak densitogram were noted. The software used was winCATS 1.3.4 version.

ANALYSIS DETAILS

Mobile phase

Toluene-Acetone (9 : 1)

Spray reagent

Anisaldehyde sulphuric acid reagent.

Detection

Blue, Violet coloured zone at Visible light mode present in the given standard and sample track observed in the chromatogram after derivatization, which confirmed the presence of Steroid in the given standard and may be in the sample.

HPLC ANALYSIS

For sample extraction, 5 g of blueberries, in three replicated each, was extracted by grinding the sample 1 min at 20,000 rpm in a blender (Ultra-Turrax Miccra D-9 KT Digitronic, Germany) with 10 ml of acidified methanol (85:15 v/v, MeOH:HCl). The homogenate was centrifuged at 3500 rpm for 10 min. The extract was separated and the residual tissue was re-extracted until the extraction solvents became colorless (the total solvent volume was between 100-250 ml). After adding 10 ml of the same solvent mixture, the extraction was carried out under stirring. The filtrates were combined in a total extract, which was dried by vacuum rotary evaporator at 40 °C. Prior to each analysis, the dry residues were redisolved in 10 ml of methanol; the samples were centrifuged at 5000 rpm and filtered through 0.45 μm nylon filter (Millipore).

Acid hydrolysis of anthocyanin. 2 mL extract was mixed with 2 ml of 2 M HCl in a screw-cap test tube. The mix was capped and hydrolyzed for 1 h at 90°C, then cooled in an ice bath.
The phytochemicals alkaloids, flavonoids, phenol and sterols of \textit{V L determination by HPLC} methods. Analyses were performed on a Shimadzu HPLC system equipped with a binary pump delivery system LC-20 AT (Prominence), a degasser DGU-20 A3 (Prominence), diode-array SPDM20 A UV–VIS detector (DAD) and a Luna Phenomenex C-18 column (5μm, 25 cm x 4.6 mm). The mobile phase consisted in: solvent A -formic acid (4.5%) in bidistilled water and solvent Bacetonitrile. The gradient elution system was: 10% B, 0-9 min; 12% B, 9-17 min; 25% B 17- 30 min; 90% B, 30-50 min; 10% B, 50-55 min. The flow rate was 0.8 ml/min and the analyses were performed at 35 °C. The chromatograms were monitored at 520 nm.

\textit{In vitro Antioxidant activity}

\textbf{Total antioxidant capacity} (Mitsuda \textit{et al.}, 1996).

1 ml of the various extract of \textit{V. leschenaultii} of different concentrations were treated with 1ml of reagent solution (0.6M sulphuric acid, 28 mM sodium phosphate and 4mM ammonium molybdate) in eppendrof tube. Capped tubes were incubated in thermal block at 95°C for 90 min. After cooling to room temperature, the absorbance was measured at 695nm against blank.

\textit{Scavenging of superoxide radical by riboflavin-NBT-system} (Fontana \textit{et al.}, 2001)

\textbf{Principle}

The method involves generation of superoxide radical of riboflavin and its detection by nitrite formation from hydroxylamine hydrochloride. The nitrite reacts with sulphanilic acid to produce a diazonium compound, which subsequently reacts with naphtylamine to produce a red azo compound whose absorbance is measured at 543 nm.

\textbf{Reagents}

0. 50 mM phosphate buffer, pH7.4
1. 20 mM L-Methionine
2. 1% (v/v) Triton X-100
3. 10 mM hydroxylamine hydrochloride
4. 50 μM EDTA
5. 50 µM Riboflavin

6. Griess reagent: 1 % sulphanilamide, 2% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride.

Procedure

1.4 ml aliquot of the reaction mixture was Pipetted in a test tube. 100 µl of the sample was added followed by preincubation at 37°C for 5 min. 80 µl of riboflavin was added and the tubes were exposed for 10 min. to 200 W Philips fluorescent lamps. The control tube contained equal amount of buffer instead of sample. The sample and its respective control were run together. At the end of the exposure time, 0.1 ml of Greiss reagent was added to each tube and the absorbance of the colour formed was measured at 543 nm. One unit of enzyme activity was defined as the amount of SOD capable of inhibiting 50% of nitrite formation under assay condition.

**Diphenyl-1-picryl hydrazyl (DPPH) method** (Hwang et al., 2001)

The antioxidant activity of the extract of *Vaccinium leschenaultii* was assessed on the basis of radical scavenging effect of the stable DPPH free radical.

**Chemicals and reagents**

1. DPPH solution: 22 mg of DPPH (2, 2-diphenyl-1-picryl hydrazyl) was accurately weighed and dissolved in methanol. The volume was made up to 100ml. From this stock solution, 18 ml was taken and diluted to 100ml using methanol to obtain 100 µM DPPH solutions.

2. DMSO, distilled.

3. Methanol, distilled.

**Preparation of test solutions**

105 mg of *Vaccinium leschenaultii* extract was dissolved in 5.0 ml of DMSO to get 21 mg/ml solutions. This solution was serially diluted with DMSO to obtain lower dilutions.
Preparation of standard solution

105 mg of ascorbic acid was weighed separately and dissolved in 5.0 ml DMSO to get 21 mg/ml solution. This was serially diluted with DMSO to get lower dilutions.

Procedure

The assay was carried out in a 96 well micro titer plate (Tarson’s products, Kolkata). To 200 µl of DPPH solution, 10 µl of each of the test sample or the standard solutions was added separately in microtitre plates. The final concentration of the test and standard solution used were 400, 200, 100, 50, 25, 12.5, 6.25 and 3.12 µg/ml. The plates were incubated at 37º C for 30 min. and absorbance of each solution was measured at 490 nm using microtitre plate reader (ELISA, Biorad Laboratories Inc., California, USA, Model 550) against the corresponding test and standard blanks. The remaining DPPH was calculated. IC₅₀ is the concentration of the sample required to scavenge 50% DPPH free radicals. IC₅₀ value was calculated for the test solutions and also compared with the IC₅₀ value of standard.

Anti-inflammatory activity

The whole plant unadultered powdered materials of Vaccinium leschenaultii was successively extracted with ethanol, methanol and chloroform in a soxhlet apparatus and concentrated to dryness. These extracts were made free from any solvent by distillation. All the extracts were subjected for acute and sub-acute toxicity studies and LD50 doses were determined for the pharmacological activity. These extracts were used as an emulsion in 5% suspension with gum acacia and administered orally at the dose of 100and 200 mg/kg b.wt. The animals were grouped in cage in an air conditioned room at the temperature of 22±1ºC with 12 hour light and dark cycle. The animals were maintained with pellet diet and water adlibitum. They were further segregated in to various groups. This experiment was performed according to ethical guidelines for the investigation of experimental pain in conscious animals (659/02/a/CPCSEA). Stranded Intra Gastric Catheter tube (IGC) was used for oral drug administration.

Carrageenan-induced paw oedema in albino rats (Winter and Poster, 1957)

Animals were divided into 5 groups comprising five animals in each group. In all groups acute inflammation was produced by sub plantar injection of 0.1 ml freshly prepared
1% suspension of carrageenan in normal saline in the right hind paw of the rats and paw volume was measured plethysommetrically at 0 to 180 mins after carrageenan injection. All the animals were premedicated with indomethacin (10mg/kg b.wt.) orally two hour before infection. Mean increase in paw volume was measured and percentage was calculated for all the extracts. All the extracts were subjected for acute toxicity studies and 1/10th of the LD 50 dose was selected for pharmacological activity. Percentage inhibition of paw volume was calculated by the following formula

\[
\% \text{ inhibition} = \frac{V_c - V_t}{V_c} \times 100
\]

Where

Vt- means increase in paw volume in rats treated with test compounds
Vc- means increase in paw volume in control group of rats.

**Statistical analysis**

The mean paw volume was expressed in terms of mean ± SEM and evaluated for statistical significance by ANOVA followed by Dunnett’s test, P<0.05 was considered by statistically significant.

**Antidiabetic Activity**

**Experimental induction of diabetes in rats**

When administrating a foreign chemical substance to a biological system, various types of interactions could occur and a series of dose-dependent results may be occurred. These responses are desired and useful, however a number of other effects may be disadvantageous. These effects on the biological systems are harmful or beneficial. The types of toxicity studies that are carried out by several pharmaceutical industries for a new drug are acute, subacute and chronic toxicity.

Acute toxicity involves LD50, the dose which has proved to be lethal to 50% to the tested group of animals. Determination of acute oral toxicity is usually an initial screening step in the assessment and evaluation of the toxic characteristics of all compounds.
Three month old male Wistar Albino rats weighing 180-240g were obtained from the small animal breeding center of Kerala Agricultural University, Trissur, Kerala. All animals were kept in an environmentally controlled room with a 12h light/12h dark cycle. The animals had free access to water and standard rat diet. The rats were injected Streptozotocin dissolved in sterile normal saline at a dose of 200 mg/kg body weight, intraperitoneally. Since STZ is capable of producing fatal hypoglycaemia as a result of massive pancreatic insulin release, rats were treated with 20 % glucose solution intraperitoneally after 6h. The rats were then kept for the next 24h on 5 % glucose solution bottles in their cages to prevent hypoglycaemia (Dhandapani et al., 2002). After a fortnight, rats with marked hyperglycaemia were selected and used for the study.

Experimental design

In the experiment, a total of 25 rats (20 diabetic surviving rats, 5 normal rats) were used. Diabetes was induced in rats 2 weeks before starting the treatment. The rats were divided into six groups, each group comprised of 5 rats. Group I (untreated normal rats), Group II streptozotocin induced diabetic rats, Group III (treated diabetic given ethanolic extract of *V. leschenaultii* at dose of 100 mg/kg b.wt. daily using an intragastric gavage), Group IV (treated diabetic given ethanolic extract of *V. leschenaultii* at dose of 200 mg/kg b.wt. daily using an intragastric gavage), Group V (treated diabetic given ethanolic extract of *V. leschenaultii* at dose of 400 mg/kg b.wt. daily using an intragastric gavage), Group VI (glibenclamide treated rats), given ethanolic extract of *V. leschenaultii* at dose of 600 mg/kg b.wt. daily using an intragastric gavage). The animals were carefully monitored every day and weighed every week (2 weeks). No sign of toxicity was noticed on the behaviour and general health of the animals when exposed to extract. Animals described as fasted were deprived of food for at least 12 h but allowed free access to drinking water. Blood samples were drawn at end of study. Blood glucose estimation, body weight, food and water intake measurement were done on 14th day of the study. On day 14, rats were sacrificed by cervical dislocation under ether anesthesia. Blood was collected from overnight fasted rats, allowed to clot and centrifuged at 3000 rpm for 15 minutes. Serum samples were separated and used for biochemical analysis. The samples were stored at -80º C, till further use.

**Estimation of Insulin** (Anderson et al., 1993)
The UBI MAGIWEL TM INSULIN QUANTITATIVE is solid phase enzyme-linked immunosorbent assay (ELISA).

**Principle**

UBI MAGIWEL TM Insulin is a solid phase enzyme-linked immunosorbent assay (ELISA). The wells are coated with monoclonal antibody with higher activity for insulin. When the samples, and controls are incubated in the wells with enzyme conjugate, which is another antibodies linked to horse radish peroxidase to form a sandwich complex bound to the well. Unbound conjugate are then washed off with wash buffer. The amount of bounded peroxidase is proportional to the concentration of the insulin present in the sample. The addition of the substrate and chromogen, the intensity of the color developed is proportional to the concentration of insulin in the samples.

**Assay procedure**

- Secured the designed number of coated wells in the holder. Marked data sheet with sample identification.
- Dispensed 25 μl of serum sample, control and reference into the assigned wells.
- Dispensed 100 μl of enzyme conjugate into each well and mixed for 5 secs.
- Incubated for 30 min. at 25º C.
- Removed incubation mixture and rinsed the wells five times with washing buffer.
- Dispensed 100 μl of solution A and then 100 μl of solution B in to each well.
- Incubated for 15 min. at room temperature.
- Stop reaction by adding 50 μl of 1N sulphuric acid or 2N HCl to each well and read O.D at 450 nm with a micro well reader.

**Estimation of Glucose** (Sasaki *et al.*, 1972)

**Principle**

Ortho toludine reacts with glucose in hot acetic acid solution to produce blue color, which is measured at 630nm.

**Reagents**
1. Ortho toludine boric acid reagent: This reagent consists of 2.5g of thiourea and 2.4g of boric acid in 100ml of a mixture of water, acetic acid and ortho toludine (distilled) in the ratio of 10:75:15.

2. Standard glucose: 100mg of glucose in 0.1% benzoic acid. 10ml of the above solution was diluted to 100ml to give 100μg of glucose per ml.

Procedure

To 0.2 ml of serum added to 0.8ml of 10% TCA mixed well and centrifuged. 0.5 ml of the supernatant was taken. To this 2.0 ml of ortho toludine reagent was added and heated in a boiling water bath for 15min. along with standard solution containing 20-100 μg of glucose. The blue colour developed was read at 640nm. The result was expressed as mg/dl in serum.

Estimation of Urea (Varley, 1976)

Principle

Diacetyl monoxime in the presence of acid, hydrolysis to produce the unstable compound diacetyl. This reacts with urea to produce a yellow diazone derivative. The color of this product becomes pink by addition of thiosemicarbazide which is measured colorimetrically at 520nm.

Reagents

- TCA, 10%
- Stock Diacetylmonoxime, 25g/L
- Stock Thiosemicarbazide 2.5g/L
- Acid ferric chloride solution: Added 1.0 ml sulphuric acid to 100 ml of ferric chloride solution containing 50 g/L in water.
- Acid reagent: Added 10ml of ortho phosphoric acid, 80 ml sulphuric acid and 10 ml acid ferric chloride solution to 1 litre of water and mixed.
- Color reagent: To 300 ml acid reagent added 200 ml water, 10 ml stock diacetylmonoxime and 2.5 ml thiosemicarbazide.
- Stock urea standard: 5,10,15,20,30,40, and 50 mmol/L (30, 60, 90, 120, 180, 240 and 300 mg/100 ml).

**Procedure**

To 0.2 ml of serum added 1.0 ml water and 1.0 ml of 10% TCA. Mixed well and centrifuged. 0.2 ml of the supernatant was taken and added 3.0 ml of color reagent. At the same time took 0.2 ml of water bath for 20 min. Cooled to room temperature and read the color developed at 520 nm within 15 min. The result was expressed as mg/dl in serum.

**Estimation of Creatinine** (Owen et al., 1954)

**Principle**

Creatinine forms a coloured complex with picrate in alkaline medium. The rate of formation of the complex is measured at 540 nm.

**Reagents**

- Picric acid: 8.02 g/L
- Sodium hydroxide: 12.8 g/L
- Standard creatinine: Dissolved 100 mg of creatinine in 100 ml with distilled water.
- Working standard: Diluted 2.0 ml of stock solution was diluted to 100 ml with distilled water. This contains 20 μg of creatinine/ml.
- Reagent mixture: Mixed one part by volume of diluted NaOH with one part by volume of picric acid at least 30 minutes before the assay.

**Procedure**

Pipetted out 0.2 ml of serum and 2.0 ml of the reagent mixture in to a cuvette. Simultaneously, a blank was set up with the reagent mixture and distilled water. Mixed well and the change in absorbance was measured after 30 seconds which was taken as A1 and exactly after 2 min., the absorbance was read as A2 at 490 nm. Sets of standards were also treated in the same manner. A1-A2 gives the change in absorbance, which was the measure of the creatinine present in the sample. The result was expressed as mg/dl in serum. The values are expressed as mg of creatinine/dl.
Estimation of Protein (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 tartarate are measured at 660nm.

Reagents

1. 1.2% Sodium carbonate 0.1N NaOH (Reagent A)
2. 0.5% Copper sulphate in 1% potassium sodium tartarate (Reagent B)
3. Alkaline copper reagent: Mixed 50ml of A and 1.0ml of B prior to use
4. Folin-ciocalteau reagent: Mixed 1 part of reagent with 2 part of water.
5. Stock standard: Weighed 50mg of bovine serum albumin and made up to 50ml in a standard flask with saline.
6. Working standard: Diluted 10ml of the stock to 50ml with distilled water. 1.0ml of this solution contains 200μg of protein.

Procedure

Pipetted out 0.2ml to 1.0ml working standard solution. 0.1ml of the sample was taken. The volume in all the tubes was made upto 1.0ml with distilled water. Added 5.0ml of alkaline copper reagent to each tube. Mixed well and allowed to stand for 10min. Then added 0.5ml of folin-ciocalteau reagent. Mixed well and incubated at room temperature for 30 min. A reagent blank was also prepared. After 30 minutes, the blue color developed was read at 660nm. The result was expressed as g/dl in serum and mg/g in tissue.

Estimation of Alkaline Phosphatase (King and Armstrong, 1934)

Principle

The method used was that of King and Armstrong in which disodium phenyl phosphate is hydrolysed with the liberation of phenol and inorganic phosphate. The liberated phenol is measured at 700 nm with Folin-Ciocalteau reagent.
Reagent

- Sodium carbonate-sodium bicarbonate buffer, 100mmol/L: Dissolved 6.36g anhydrous sodium carbonate and 3.36g sodium bicarbonate in water and made to a litre.
- Disodium phenyl phosphate, 100mmol/L: Dissolved 2.18g in water, heated to boil, cooled and made to a litre. Added 1.0ml of chloroform and stored in the refrigerator.
- Buffer substrate: Prepared by mixing equal volume of the above two solutions. This has a pH of 10.
- Sodium carbonate solution, 15%: Dissolved 15g of anhydrous sodium carbonate in 100ml of water.
- Standard phenol solution, 1 g/L: Dissolved 1g pure crystalline phenol in 100mmol/L HCl and made to a litre with the acid.
- Working standard solution: Added 100ml dilute phenol reagent to 5.0 ml of stock standard and diluted to 500ml with water. This contained 10μg phenol/ml.

Procedure

Pipetted 4.0 ml of the buffer substrate into a test tube and incubated at 37°C for 5 min. Added 0.2 ml of serum or tissue homogenate and incubated the exact for 15min. and immediately added 1.8ml of diluted phenol reagent. At the same time a control was set up containing 4.0 ml buffer substrate and 0.2ml sample to which 1.8ml phenol regent was added immediately. Mixed well and centrifuged. To 4.0ml of supernatant added 2.0ml of sodium carbonate. Took 4 .0ml of working standard solution and for blank taken 3.2 ml water and 0.8ml of phenol regent. Then added 2.0ml of sodium carbonate. Incubated all the tubes at 37°C for 15 min. The colour developed was read at 700 nm. The activity was expressed as units/L in serum and units/protein in tissue.

Estimation of Aspartate Transaminase (SGOT) (Reitman and Frankel, 1957)

Principle

The enzyme catalyses the following reaction:

\[
\text{L-Aspartate + } \alpha\text{-oxoglutarate} \xrightarrow{\text{Oxaloacetate + L-glutamate}}
\]
The oxaloacetate is measured by the reaction with 2,4-dinitrophenylhyrdrazine giving a brown coloured hydrazone after the addition of sodium hydroxide. The colour developed is read at 520 nm.

**Reagent**

- Phosphate buffer 0.1M, pH 7.5
- Solution A: 0.1M solution of monobasic sodium phosphate (13.9g/l).
- Solution B: 0.1 M solution of dibasic sodium phosphate 6.8 of Na$_2$PO$_4$.
- 7H$_2$O g/L. 16ml of A and 84 ml of B, diluted to a total of 200ml.
- Substrate: Dissolved 146 mg of α-Ketoglutarate and 13.3 g of aspartic acid in 1 N NaOH with constant stirring. Adjusted the pH to 7.4 and made up to 1000 ml with phosphate buffer.
- Standard pyruvate, 2 mmol/L: Dissolved 22 mg of sodium pyruvate in 100ml of phosphate buffer. 0.2ml of standard contained 0.4 μM of sodium pyruvate.
- Dinitrophenylhydrazine reagent, 1 mmol/L: 200 mg in 1 mol/L HCl.
- 0.4 N NaOH: Dissolved 16 g of NaOH in 1000 ml water.

**Procedure**

0.2 ml of sample and 1.0 ml of the buffer substrate was incubated for 60 min. at 37°C. To the control tubes, enzyme was added after arresting the reaction with 1.0 ml of DNPH and the tubes were kept at room temperature for 20 min. Then 10 ml of 0.4 N NaOH was added. A set of standard pyruvate was also treated in a similar manner. The colour developed was read at 520 nm. The enzyme activity was expressed as units/L in serum and units/protein in tissue.

**Estimation of Alanine Transaminase (SGPT)** (Reitman and Frankel, 1957)

**Principle**

The enzyme catalyses the following reaction:

\[
\text{L-Alanine} + \alpha\text{-oxoglutarate} \quad \xrightarrow{} \quad \text{Pyruvate} + \text{L-glutamate}
\]
The oxaloacetate is measured by the reaction with 2, 4-dinitrophenylhydrazine giving a brown coloured hydrazone after the addition of sodium hydroxide. The colour developed is read at 520 nm.

**Reagents**

- Phosphate buffer: 0.1M, pH 7.5
- Substrate: Dissolved 146 mg of β-ketoglutarate and 17.8 g of L-alanine in 1N NaOH with constant stirring. Adjusted the pH to 7.4 and made up to 1000 ml with phosphate buffer.
- Standard pyruvate, 2 mM: Dissolved 22 mg of sodium pyruvate in 100 ml of phosphate buffer, 0.2 ml of standard contained 0.4 μM of sodium pyruvate.
- Dinitrophenyl hydrazine reagent, 1 mmol/L: 200 mg/L in 1 mol/L HCl.
- 0.4 N NaOH: Dissolved 16 g of NaOH in 1000 ml water.

**Procedure**

0.2 ml of sample and 1.0 ml of the buffer substrate were incubated for 30 min. at 37°C. To the control tubes, enzyme was added after arresting the reaction with 1.0 ml of DNPH and the tubes were kept at room temperature for 20 min. Then 10 ml of 0.4 N NaOH was added. A set of standard pyruvate was also treated in a similar manner. The colour developed was read at 520 nm. The enzyme activities were expressed as units/L in serum and units/protein in tissues.

**Estimation of Albumin and Globulin** (Wolfson et al., 1948)

**Principle**

The blue colour developed by the aminoacids tyrosine and tryptophan present in the protein by the biuret reaction of the protein with the alkaline cupric tartarate are measured at 555 nm.

**Reagents**

- Sulphate-sulphite solution: Weighed 208 gm of sodium sulphate (anhydrous) and 70 gm of sodium sulphite (anhydrous) and dissolved with stirring in about
900ml of distilled water, to which 2.0ml of concentrated sulphuric acid was added. Transferred to 1L volumetric flask and made up to the mark with distilled water. The pH as adjusted to 7.0.

- Stock biuret reagent: Dissolved 45gm of Rochelle salt in 400ml of 200mmol/L NaOH and added 15gm of copper sulphate and mixed. 5gmof potassium iodide was added and made up to a litre with 200mmol/L NaOH.
- Working biuret reagent: Diluted 20ml of stock reagent to 100ml with 200mmol/L NaOH
- Ether
- Stock standard solution: 100mg of bovine serum albumin was dissolved in 100ml of saline.
- Working standard: 1ml of stock was diluted to 5ml with distilled water. Therefore 1ml of this solution contains 200μg of protein.

**Procedure**

**Total protein**

Pipetted out 6ml of sulphate-sulphite solution in a test tube and added 0.4ml of serum and mixed well. From the mixture, 2.0ml were taken and to it 5.0ml of biuret reagent was added.

**Albumin**

Added about 3.0ml of ether to the rest of the serum-sulphate mixture and shaken 40 times, twice each second for 20 seconds. The tube was centrifuged for 5mins. After centrifuging, the tube was tilted and inserted a pipette into the clear solution below the globulin layer and pipette out 2.0ml. To this, 5.0ml of biuret reagent was added.

A set of standards were taken, to this 6.0ml of sulphate-sulphite solution was added and mixed well. To 2.0ml of this mixture, 5.0ml of biuret reagent was added. All the tubes were warmed at 37°C for 10mins. Allowed to cool for 5mins at room temperature and colour was read at 555nm. The difference between the amount of total protein and albumin gives the globulin. The values in the serum were expressed as gm/dl.
**Estimation of Total Cholesterol** (Parekh and Jung, 1970)

**Principle**

Cholesterol reacts with ferric chloride in the presence of concentrated sulphuric acid to give a pink color. The intensity of color developed is directly proportional to the amount of cholesterol present and is read at 540 nm in a colorimeter.

**Reagents**

- Stock ferric chloride: 840 mg of pure dry ferric chloride was weighed and dissolved in 100ml of glacial acetic acid.
- Ferric Chloride precipitation reagent: 10ml of stock ferric chloride reagent was taken in 10 ml of standard flask and made upto the mark with pure glacial acetic acid.
- Ferric chloride diluting reagent: 8.5 ml of stock ferric chloride is diluted to 100ml with pure glacial acid.
- Standard cholesterol solution: 100 mg of cholesterol was dissolved in 100 ml of glacial acetic acid.
- Working standard: 10 ml of stock was dissolved in 0.85 ml of stock ferric chloride reagent and made up to 100 ml with glacial acetic acid. The concentration of working standard is microgram/ml.

**Procedure**

To 0.1 ml of plant extract added 4.9 ml of ferric chloride precipitating reagent. Centrifuged and to 2.5ml of supernatant added 2.5 ml of ferric chloride diluting agent and 4.0ml of concentrated sulphuric acid. A blank was prepared simultaneously by taking 5.0 ml of diluting reagent and 4.0ml of concentrated sulphuric acid. A set of standards (0.5-2.5ml) were taken and made up to 5.0 ml with ferric chloride diluting reagent. Then added 4.0 ml of concentrated sulphuric acid. After 30 min., the intensity of color developed was read at 540 nm against a reagent blank. The amount of cholesterol in the sample is expressed as mg/dl.
Estimation of Triglycerides (Rice, 1970)

Principle

The glycerol moiety is oxidized to formaldehyde and the later condensed with ammonia and 2, 4-pentanedione (acetyl acetone) to produce 3,5-diacetyl 1,4-dihydrotoludine, which is yellow in color and has absorption at 450nm.

Reagents

- Chloroform-methanol mixture (2:1)
- Activated silicic acid: It was activated by washing silicic acid with 4N or 2N HCl and then with water until the washings become natural. After drying, ether was added. Silicic acid was then dried at 60°C and activated at 100°C over night prior to use.
- 0.2 N H₂SO₄
- Saponification reagent: Dissolved 5g of KOH in 60ml water and added 40ml of isopropanol.
- Sodium-metaperiodate reagent: To 77g of anhydrous ammonium acetate in 700ml water, added 60ml acetic acid and 650mg of sodium metaperiodate. Dissolved and diluted in 1litre with distilled water.
- Acetyl acetone reagent: Added 0.75ml of acetyl acetone to 20ml of isopropanol and mixed well. Added 80ml of distilled water and mixed.
- Tripalmitin standard was containing 100μg/ml in chloroform.

Procedure

Take 0.1ml of the serum or dried lipid extract. Make up the volume to 4.0ml with isopropanol. Mixed well and added 400mg of silicic acid. Placed them in a mechanical shaker and centrifuged. To 2.0ml of the supernatant added 0.6 ml of saponification reagent and incubated at 60-70°C for 15min. After cooling added 1.0ml of sodium metaperiodate and mixed well. Then added 0.5ml of acetyl acetone reagent and mixed again. Incubated the tubes at 50°C for 30min. After cooling read the color at 405nm. Standard tripalmitin (20-100μg) were taken in tubes and treated similarly. Triglycerides are expressed as mg/100ml in serum.
**Estimation of HDL - Cholesterol** (Warnick et al., 1985)

**Principle**

Cholesterol reacts with hot solution of ferric per chlorate, ethyl acetate and sulphuric acid (Cholesterol reagent) and gives lavender colored complex which is measured at 560 nm. High density lipoproteins (HDL) are obtained in the supernatant after centrifugation. The cholesterol in the HDL fraction is also estimated by this method.

**Procedure**

(i) HDL – Cholesterol separation

Mixed well, kept at room temperature for 10 min. and then centrifuged at 2000 rpm for 15 min. to obtain a clear supernatant. Proceed to step II.

(ii) HDL – cholesterol estimation

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Blank (B)</th>
<th>Standard (S)</th>
<th>Test (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reagent 1</strong>: Cholesterol reagent</td>
<td>3.0 ml</td>
<td>3.0 ml</td>
<td>3.0 ml</td>
</tr>
<tr>
<td><strong>Reagent 2</strong>: Working cholesterol Standard, (200 mg%)</td>
<td>-</td>
<td>0.015 ml</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15 (μl)</td>
<td>0.12 ml</td>
</tr>
<tr>
<td>Supernatant from step – 1</td>
<td>-</td>
<td>-</td>
<td>(120 μl)</td>
</tr>
</tbody>
</table>

Mixed well and kept the tubes immediately in the boiling water bath exactly for 90 seconds (1 ½ minutes). Cooled them immediately to room temperature, under running tap water. Measured the O.D of standard (S) and Test (T) against Blank (B) on a colorimeter with a yellow green filter or on a spectrophotometer at 560 nm.
Determination of LDL Cholesterol

LDL cholesterol level in serum was calculated by Friedbald et al., (1972) formula.

Estimation of Lipid Peroxidation (Uchiyama and Mihara, 1978)

Principle

Malondialdehyde has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid to give a red colour, absorbing at 535 nm.

Reagents

- 15% KCl
- 1% Phosphoric acid
- n-butanol
- 0.6% thiobarbituric acid
- 10mM ferrous sulphate
- 0.2mM ascorbate

Procedure

Tissue slices were homogenized in ice cold 1.15% Kcl. 0.5 ml of aliquot of the homogenate was mixed with 3.0 ml of 1% phosphoric acid and 1.0 ml of 0.6% thiobarbituric acid. The mixture was heated for 45 min. in boiling water bath and after addition of 4.0 ml of n-butanol vigorously, vortexed and centrifuged at 2000 rpm for 20 min. the absorbance of the upper organic layer at 535 nm was measured in a spectrophotometer and compared with a standard of freshly prepared 1,1,3,3 tetraethoxy propane at concentration of 5.125,10.25 and 20.5 nmol ml$^{-1}$ or using an extinction coefficient of the chromophore $1.56 \times 10^{-5}$ M$^{-1}$ cm$^{-1}$ and the results were expressed as n moles of MDA formed / mg protein.

Estimation of Superoxide Dismutase (Das et al., 2000)

Principle

The method involves generation of superoxide radical of riboflavin and its detection by nitrite formation from hydroxylamine hydrochloride. The nitrite reacts with sulphanilic
acid to produce a diazoum compound, which subsequently reacts with naphtylamine to produce a red azo compound whose absorbance is measured at 543 nm.

**Reagents**

- 50 mM phosphate buffer, pH7.4
- 20 mM L-Methionine
- 1% (v/v) Triton X-100
- 10 mM hydroxylamine hydrochloride
- 50 μM EDTA
- 50 μM Riboflavin
- Griess reagent: 1% sulphanilamide, 2% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride.

**Procedure**

Pipetted 1.4 ml aliquot of the reaction mixture in a test tube. 100 μl of the sample was added followed by pre incubation at 37°C for 5 min. 80 μl of riboflavin was added and the tubes were exposed for 10 min. to 200 W Philips fluorescent lamps. The control tube contained equal amount of buffer instead of sample. The sample and its respective control were run together. At the end of the exposure time, 0.1 ml of Greiss reagent was added to each tube and the absorbance of the colour formed was measured at 543 nm. One unit of enzyme activity was defined as the amount of SOD capable of inhibiting 50% of nitrite formation under assay condition.

**Estimation of Catalase** (Sinha, 1972)

**Principle**

Catalase causes rapid decomposition of hydrogen peroxide to water.

\[
\text{Catalase} \\
2\text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \text{O}_2
\]
The method is based on the fact that dichromate in acetic acid reduces to chromic acetate when heated in the presence of H\textsubscript{2}O\textsubscript{2} with the formation of perchloric acid as an unstable intermediate. The chromic acetate thus produced is measured colorimetrically at 610 nm. Since dichromate has no absorbance in this region, the presence of the compound in the assay mixture does not interfere with the colorimetric determination of chromic acetate. The catalase preparation is allowed to split H\textsubscript{2}O\textsubscript{2} for different periods of time. The reaction is stopped at specific time intervals by the addition of dichromate/acetic acid mixture and the remaining H\textsubscript{2}O\textsubscript{2} is determined by measuring chromic acetate colorimetrically after heating the reaction.

**Reagents**

- M Phosphate buffer, pH 7.0 2.
- 0.2M Hydrogen peroxide
- M phosphate buffer, pH 7.02.
- 2.02M Hydrogen peroxide
- Stock dichromate/acetic acid solution: Mixed a 5% potassium dichromate with glacial acetic acid (1:3 by volume).
- Catalase
- Working dichromate/acetic acid solution: The stock was diluted to 1:5 with water to make the working dichromate/acetic acid solution.

**Procedure**

The assay mixture contained 0.5 ml of H\textsubscript{2}O\textsubscript{2}, 10 ml of buffer and 0.4 ml water. 0.2 ml of the enzyme was added to initiate the reaction. 2.0 ml of the dichromate/ acetic acid reagent was added after 0, 30, 60, 90 seconds of incubation. The enzyme was added to the control tube and read at 610 nm. The activity of catalase was expressed as μ mole of H\textsubscript{2}O\textsubscript{2} decomposed/min/mg protein.
Estimation of Glutathione Peroxidase (Beutler et al., 1984)

Reagents

- 0.4 M sodium phosphate buffer, pH 7.0
- 10 mM sodium azide
- 2.5 mM hydrogen peroxide
- 4 mM reduced glutathione
- 10% TCA
- 0.3 M phosphate solution
- 0.04% DTNB in 1% sodium citrate
- Reduced glutathione standard: 20 mg reduced glutathione was dissolved in 100 ml of water.

Procedure

0.4 ml of buffer, 0.1 ml of sodium azide, 0.2 ml of reduced glutathione, 0.1 ml of H₂O₂, 0.2 ml of enzyme and 1.0 of water were added to a final incubation volume of 2.0 ml. The tubes were incubated for 0, 30, 60, 90 seconds. The reaction was then terminated by the addition of 0.5 ml TCA. To determine the glutathione content, 2.0 ml of the supernatant was removed by centrifugation and added 3.0 ml disodium hydrogen phosphate solution and 1.0 of DTNB reagent. The colour developed was read at 412 nm. Standards in the range of 200-1000 μg were taken and treated in the similar manner. The activity was expressed in terms of μg of glutathione utilized/mg protein.

Microbial assay

Disc Diffusion Assay (Bauer et al., 1966)

The following microbial strains were used in the present study. They are Salmonella paratyphi G(-), Salmonella paratyphi-A G(-), Salmonella paratyphi-B G(-), Bacillus subtilis G(+), Escherichia coli G(-), Bacillus thuringiensis G(+), and Pseudomonas aeruginosa G(-) for bacteria and Aspergillus niger, Rhizopus oryzae, Aspergillus flavus, Cladosporium carrionii, Mucor sp., Pencillium notatum and Alternaria alternata for fungi. The bacterial
and fungal strains were procured from the Department of microbiology, Bharathidasan University, Thirichirapalli. Bacteria were maintained on nutrient agar slants, and fungi on Potato Dextrose agar slants at 4°C and subcultured monthly.

Two colonies of a 24-hour plate culture of each organism were transferred aseptically into 10 ml nutrient and potato dextrose broth in a test tube and mixed thoroughly using an electric shaker for uniform distribution. Petri dishes were plated with Nutrient agar and Potato Dextrose agar medium were prepared according to the manufacturer’s manual and allowed for 30 minutes to solidify. The test organisms were then spread on the surface of the media using a sterile swap stick. The different concentrations of the plant extracts were (10mg/ml) introduced on the disc (0.7 cm) and then allowed to dry. Then the disc was impregnated on the agar plates and tetracycline used as reference drug for the bacteria. The extracts of Vaccinium leschenaultii were tested against seven fungi and 10µg Nystatin used as the reference drug for the fungi. The plates were then incubated at 37º C for 24 h. Microbial growth was determined by measuring the diameter of zone of inhibition.

**Determination of Minimum Inhibitory Concentrations (MICs) of the extracts of Vaccinium leschenaultii**

MIC was determined by the standard method of Van Den Berghe and Vlietinck, 1991. Nutrient broth was prepared and sterilized using autoclave. One ml of the prepared broth was dispensed in to the test tubes numbered 1-8 using sterile syringe or needle. Then 1 ml of the extract (500µl/ml) was dispensed into the tubes numbered 1. Subsequently, from tube 1, serial dilution was carried out and 1 ml from tube 1 was transferred up to tube number 7 and 1 ml from the tube 7 was discarded. Tube 8 was control for sterility of the medium. An overnight culture (inoculums) of each of the test isolates was prepared in sterile nutrient broth. 10µl of the inoculums was transferred into each tube from tube 1 to tube 8. The final concentration of the plant extract in each of the test tubes numbered 1-7 after dilution 500µl, 250µl, 125µl, 62.5µl, 31.25µl, 15.62µl and 7.81µl/ml were incubated at 37°C for 24 h and examined for growth. The last tube in which growth failed to occur was the MIC tube. The tubes with the extract and broth were inoculated with a micro-organism suspension at a density of $10^5$ CFU per ml. The tubes were incubated at 37°C for 24 h and then observed for the Minimum Inhibitory Concentration (MIC). The growth of organisms was observed as
turbidity determined by a spectrophotometer (Elico SL177) at 620 nm. Control tubes without the tested extracts were assayed simultaneously. The minimum dilution of fractions completely inhibiting the growth of each organism was taken as the MIC.

**Statistical analysis:**

All the data were subjected to Duncans Multiple Range Test (DMRT) was done by using the SPSS version 2007 WINSAT software.