3. MATERIALS AND METHODS

3.1. BIOLOGY OF PLANT MATERIALS


- **Kingdom**: Plantae
- **Division**: Angiosperms
- **Class**: Eudicots
- **Order**: Asterids
- **Family**: Theaceae
- **Genus**: *Camellia*
- **Species**: *C. sinensis*

Tamil: Thacillai, English: Tea.

*Camellia sinensis* is native to mainland China, South and Southeast Asia, but it is today cultivated across the world in tropical and subtropical regions. It is an evergreen shrub or small tree that is usually trimmed to below 2 m (6.6 ft) when cultivated for its leaves. It has a strong taproot. The flowers are yellow-white, 2.5–4 cm (0.98–1.6 in) in diameter, with 7 to 8 petals. (Fig. 1).
Fig 1. Leaves of Camellia sinensis
3.1.2. Taxonomy of *Terminalia chebula* Retz.

Kingdom : Plantae
Division : Magnoliophyta
Class : Magnoliopsida
Order : Myrtales
Family : Combretaceae
Genus : *Terminalia*
Species : *T. chebula*

Tamil: Kadukkai. English: Black myrobalan.

It is a deciduous tree growing to 30-metre (98 ft) tall, with a trunk up to 1-metre (3 ft 3 in) in diameter. The leaves are alternate to subopposite in arrangement, oval, 7–8-centimetre (2.8–3.1 in) long and 4.5–10-centimetre (1.8–3.9 in) broad with a 1–3-centimetre (0.39–1.2 in) petiole. The fruit is drupe-like, 2–4.5-centimetre (0.79–1.8 in) long and 1.2–2.5-centimetre (0.47–0.98 in) broad, blackish, with five longitudinal ridges, (Fig. 2).
Fig. 2. Fruits of *Terminalia chebula*
3.2. PLANT MATERIAL

Healthy and young leaves (3-6 months old) of *Camellia sinensis* and fruits of *Terminalia chebula* were selected. They were collected from in and around Kolli hills and Ooty hills, Tamil Nadu, India and identified with help of the standard manuals such as "The Flora of the presidency of Madras" (Gamble, 1967) and Indian Medicinal plants (Kirtikar and Basu, 1994). The leaves were separated from stems, washed in clean water, and dried at room temperature. The shaded dried leaves were weighted and ground in a sterile mortar.

3.3. EXTRACTION OF PLANT MATERIALS (Harborne, 1984)

The leaves were cleaned and dried in shade for 7 days, then ground well to fine powder. About 500 g of dry powder was extracted with methanol (80%) at 70°C by continuous hot percolation using soxhlet apparatus. The extraction was continued for 24 hrs. The methanolic extract was then filtered and kept in hot air oven at 40°C for 24 hrs to evaporate the methanol from it. A dark brown residue was obtained. The residue was kept separately in air tight containers and stored in a deep freezer.

The leaves were cleaned and dried in shade for 7 days, then ground well to fine powder. About 500 g of dry powder was extracted with methanol (80%) at 70°C by continuous hot percolation using soxhlet apparatus. The extraction was continued for 24 hrs. The methanolic extract was then filtered and kept in hot air oven at 40°C for 24 hrs to evaporate the methanol from it. A dark brown residue was obtained. The residue was kept separately in air tight containers and stored in a deep freezer.

3.4. PHYTOCHEMICAL ANALYSIS

3.4.1. Qualitative Analysis

Phytochemical analysis of the plant extracts was undertaken using standard qualitative methods as described by various authors (Kapoor *et al.*, 1969; Odebiyi and Sofowora, 1990). The plant extracts were screened for the presence of biologically active compounds such as alkaloids, flavonoids, carbohydrates, phytosterols, proteins, phenolics, tannins and saponins.
3.4.1.1. Alkaloids (Salehi Surmaghi et al., 1992)

Dragendorff’s test (Kraut reagent – Potassium bismuth iodide)

8 g of Bi (NO₃)₃ 5 H₂O was dissolved in 20 ml of HNO₃ and 2.72 g of potassium iodide in 50 ml of distilled water separately. They were mixed and allowed to stand till KNO₃ got crystallized. The supernatant was decanted and made up to 100 ml with distilled water. The alkaloids were regenerated from the precipitate by treating with Na₂CO₃ followed by extraction of the liberated base with ether. To 0.5 ml of plant extract and 2 ml of HCl were added. Then 1 ml of reagent was added to this acidic medium. An orange red precipitate was produced immediately, which indicated the presence of alkaloids.

Wagner’s reagent (Iodine-Potassium iodide solution)

1.2 g of iodine and 2.0 g of potassium iodide were dissolved in 5 ml of H₂SO₄ and the solution was diluted to 100 ml. 10 ml of plant extract was acidified by adding 1.5% HCl and a few drops of Wagner’s reagent. The formation of a yellowish brown precipitate confirmed the presence of alkaloids.

Meyer’s reagent (Potassium mercuric iodide)

1.36 g of mercuric chloride was dissolved in 60 ml of distilled water and 5 g of potassium iodide in 10 ml of water separately. The two solutions were mixed and diluted to 100 ml with distilled water. A few drops of the reagent were added to 1 ml of the plant extract. The formation of a pale precipitate showed the presence of alkaloids.

3.4.1.2. Flavonoids (Somolenski et al., 1972)

In a test tube containing 0.5 ml of plant extract, 5-10 drops of diluted HCl and a small pinch of zinc or magnesium were added, and the solution was boiled for a few minutes. In the presence of flavonoids, a reddish pink or dirty brown colour was produced.

3.4.1.3. Carbohydrates

Fehling’s test (Kokate, 1994)

Solution A: 34.65 g of copper sulphate was dissolved and made up to 500 ml with distilled water.
Solution B: 125 g of potassium hydroxide and 173 g of Rochelle’s salt (sodium potassium tartrate) and made up to 500 ml of distilled water.
The solutions ‘A’ and ‘B’ were added in to the test samples. The contents were boiled for a few minutes. The formation of a red or brick red precipitate indicated the presence of carbohydrates.

**Benedict’s test**

173 g of sodium citrate and 100 g of sodium carbonate were dissolved in 500 ml of distilled water. 17.3 g of copper sulphate dissolved in 100 ml of distilled water was added to the above solution. To 0.5 ml of plant extract, 5 ml of Benedict’s reagent was added and boiled for 5 min. The formation of a bluish green colour showed the presence of carbohydrates.

**3.4.1.4. Proteins**

**Millon’s test (Walsh and Farrel, 1961)**

One part of mercury was digested with 2 parts of concentrated HNO₃ and the resulting solution was diluted with 2 volumes of water. To a small quantity of plant extract, 5-6 drops of Millon’s reagent was added. A white precipitate which turned red on heat indicated the presence of proteins.

**3.4.1.5. Phenols (Malick and Singh, 1980)**

One ml of plant extract, 2 ml of distilled water followed by a few drops of 10% aqueous FeCl₃ solution were added. Formation of a blue or green precipitate indicated the presence of phenols.

**Lead acetate test**

One ml of plant extract was diluted to 5 ml with distilled water and then a few drops of 1% aqueous solution of lead acetate was added. Appearance of yellow precipitate indicated the presence of phenols.

**Libermann’s test**

A small amount of plant extract was dissolved in 0.5 ml of 20% sulphuric acid solution followed by the addition of a few drops of aqueous sodium nitrate solution. A red colour was obtained on dilution and it turned blue when made alkaline with aqueous sodium hydroxide solution, which indicated the presence of phenol.
3.4.1.6. Saponins (Malick and Singh, 1980)

In a test tube containing about 5 ml of plant extract, a drop of sodium bicarbonate solution was added. The mixture was shaken vigorously and kept for 3 min. Formation of a honey comb like froth showed the presence of saponins.

3.4.1.7. Tannins (Segelman et al., 1969)

Ferric chloride test

Two ml of plant extract, a few drops of 5 % aqueous FeCl₃ solution was added. A bluish black colour was formed, which then disappeared and addition of few ml of dilute H₂SO₄ formed yellowish brown precipitate.

Lead acetate test

In a test tube containing about 5 ml of plant extract, a few drops of 1 % solution of lead acetate was added. Formation of yellow or red precipitate indicated the presence of tannins.

3.4.1.8. Phytosterols (Malick and Singh, 1980)

About 0.5 ml of test solution was mixed with minimum quantity of chloroform and the 3-4 drops of acetic acid and one drop of concentrated H₂SO₄ were added. Formation of a deep blue or green colour showed the presence of steroids.

3.4.1.9. Terpenoids

5 ml of each extract was mixed in 2 ml of chloroform. 3 ml of concentrated H₂SO₄ was then added to form a layer. A reddish brown precipitate colouration at the interface formed indicated the presence of terpenoids.

3.4.1.10. phlobatannins:

Deposition of a red precipitate when an aqueous extract of each plant sample was boiled with 1 % aqueous hydrochloric acid was taken as evidence for the phlobatannins.

3.4.2. GC-MS analysis (Ivanova et al., 2002)

30 g powdered sample of Psidium guajava and Azadirachta indica were soaked and dissolved in 75 ml of methanol for 24 hrs. Then the filtrates were collected by evaporated under liquid nitrogen. The GC-MS analysis was carried out using a Clarus 500 Perkin Elmer (Auto
System XL) GasChromatograph equipped and coupled to a mass detector Turbo mass gold – Perking ElmerTurbomas 5.2 spectrometer with an Elite-1 (100% Dimethyl ploy siloxane), 300 m x 0.25 mm x 1μm df capillary column. The instrument was set to an initial temperature of 110°C, and maintained at this temperature for 2 min. At the end of this period, the oven temperature was raised up 280°C, at the rate of an increase of 5°C/min, and maintained for 9 min. Injection port temperature was ensured as 250°C and Helium flow rate as 1 ml/min. The ionization voltage was 70 eV. The samples were injected in split mode as 10:1. Mass Spectral scan range was set at 45-450 (m/z). The chemical constituents were identified by GC-MS. The fragmentation patterns of mass spectra were compared with those stored in the spectrometer database using National Institute of Standards and Technology Mass Spectral database (NIST-MS). The percentage of each component was calculated from relative peak area of each component in the chromatogram.

3.5. HEPATOPROTECTIVITY ACTIVITY

Albino rats of either sex weighed about 150-220 g were divided into six groups of six each. The rats were used after an acclimatization period of 7 days to the laboratory environment. They were provided with food and water ad libitum.

3.5.1. Experimental conditions

The animals were divided in to 7 groups of 6 animals each. The animals from Group I which served as control received vehicle for 7 days. Group II-VII received 1ml/kg/day p.o of CCl4 for all 7 days. The standard drug Silymarin (100mg/kg p.o.) was administered to Group III animals for 7 days. Group IV & V received methanolic extract of T. chebula in the dose for 7 days respectively. Group VI & VII received methanolic extract of C. sinensis in the dose for 7 days respectively. The CCl4, Silymarin & the extracts were administered concomitantly to the respective group of animals. On 7th day, blood was collected through retro orbital vein and serum was separated by centrifugation at 2500 rpm for 10 minutes.
3.5.2. Estimation of Serum Glutamate Oxaloacetate Transaminase (SGOT) (Reitman and Frankel, 1957) Reagents

1. Substrate solution
   L-aspartate, 100 M mole/L
   L-oxalglutarate, 100 M mole/L

2. Colour reagent
   2, 4 Dinitrophenyl hydrazine, 5 M mole/L

3. Alkaline reagent
   Sodium hydroxide, 0.4 N.

4. Standard
   Sodium pyruvate, 1601.11/L

Procedure
From the serum, 0.1 ml was mixed with 0.5 ml of substrate solution and incubated for 60 min at 37°C. After the incubation time, 0.5 ml of colour reagent 2,4-dinitrophenyl hydrazine (5 M mole) was added and further incubated for 20 mm at 37°C. After the incubation, the reaction was stopped by adding 3 ml of alkaline reagent (Sodium hydroxide, 0.4N) and the colour intensity was read at 505nm. Sodium pyruvate was used as a standard. The SGOT activity was expressed as IU/L.

3.5.3. Estimation of Serum Glutamate Pyruvate Transaminase (SGPT) (Reitman and Frankel, 1957)

Reagents
1. Substrate solution
   L-alanine, 200 M mole/L
   L-oxalglutarate, 100 M mole/L

2. Colour reagent
   2, 4 Dinitrophenyl hydrazine, 5 M mole/L
3. Alkaline reagent
   Sodium hydroxide, 0.4 N.

4. Standard
   Sodium pyruvate, 160 μl/L

Procedure
   From the serum 0.1 ml was mixed with 0.5 ml of substrate solution and incubated for 60
   min at 37°C. After the incubation time, 0.5 ml of colour reagent 2,4-dinitrophenyl hydrazine (5
   M mole) was added and further incubated for 20 min at 37°C. After the incubation, the reaction
   was stopped by adding 3 ml of alkaline reagent (Sodium hydroxide, 0.4 N) and the colour
   intensity was read at 505 nm. Sodium pyruvate was used as a standard. The SGPT activity was
   expressed as IU/L.

3.5.4. Estimation of lipid peroxidation (LPO) (Nichan and Samuelson, 1968)

Reagents
   1. Trichloro acetic acid (TCA) — Tertiary butyl alcohol (TBA) — Hydrochloric acid (HCl)
      reagent (1%).
   2. Standard malondialdehyde solution.

   To prepare 3 M solution of standard, 16 ml of 1, 1', 3, 3' tetramethoxy propane was
   dissolved and made upto 100 ml with distilled water.

Procedure
   From the serum, 0.5 ml was mixed with 2 ml of 1% TCA-TBA-HCl reagent and mixed
   thoroughly. The solution was heated for 15 mm in a boiling water bath. After cooling, the
   precipitate appeared was removed by centrifugation at 1000 rpm for 10 mm. The absorbance of
   the supernatant was read at 520 nm against reagent blank. Malondialdehyde was used as
   standard. The level of LPO was expressed as moles/L.

41
3.5.5 Estimation of plasma TBARS

Lipid peroxidation was estimated as evidenced by the formation of thiobarbituric acid reactive substances (TBARS). TBARS in plasma were assayed by the method of Nichars And Samuelsen (1968).

Plasma was deproteinised with phosphotungstic acid and the precipitate was treated with thiobarbituric acid at 90°C for 1 hour. The pink colour formed gives a measure of the thiobarbituric acid reactive substances (TBARS) which was read at 530nm.

Reagents

1. 0.083 N sulphuric acid
2. 10% phosphotungstic acid
3. Thiobarbituric acid (TBA): 670mg was dissolved in 100ml water. To this, 100ml of glacial acetic acid was added.
4. Standard malondialdehyde stock solution: 1, 1', 3, 3'-tetramethoxy propane (184µg/ml).

Procedure

To 0.5ml of plasma, 4.0ml of 0.083N sulphuric acid was added. To this mixture, 0.5ml of 10% phosphotungstic acid was added and mixed. After standing at room temperature for 5 minutes, the mixture was centrifuged at 3000g for 10 minutes. The supernatant was discarded. The sediment was mixed with 2.0ml of sulphuric acid and 0.3ml of 10% phosphotungstic acid. The mixture was shaken well and centrifuged at 3000g for 10 minutes. The sediment was suspended in 4.0ml of distilled water and then 1.0ml of TBA reagent was added. The reaction mixture was heated at 95°C for 60 minutes. After cooling, 5.0ml of n-butanol was added and the mixture was shaken vigorously and centrifuged at 3000g for 15 minutes. The colour extracted in the butanol layer was read at 530nm. Tubes containing standard malondialdehyde 1 to 5 nmoles were treated in a similar manner along with a blank containing 4.0 ml of distilled water.

TBARS level was expressed as nmol/mL plasma.
3.5.6. ESTIMATION OF ENZYMATIC ANTIOXIDANTS

3.5.6.1. Estimation of superoxide dismutase [SOD]

Superoxide dismutase activity was assayed by the method of Kakkar et al., (1984).

The assay is based on the inhibition of the formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazan. The reaction is initiated by the addition of NADH. After incubation for 90 seconds, adding glacial acetic acid stops the reaction. The color developed at the end of the reaction is extracted into n-butanol layer and measured at 520nm.

Reagents

1. Sodium pyrophosphate buffer, 0.052 M, pH 8.3.
2. Absolute ethanol
3. Chloroform
4. n-butanol
5. Phenazine methosulphate (PMS), 186 μmol
6. Nitroblue tetrazolium (NBT), 300 μmol
7. Reduced nicotinamide adenine dinucleotide (NADH), 780 μmol

Procedure

0.5 ml of the plasma or hemolysate or tissue homogenate was diluted to 1.0 ml with ice-cold water, followed by 2.5 ml ethanol and 1.5 ml chloroform (chilled reagents). This mixture was shaken for 60 seconds at 4°C and then centrifuged. The enzyme activity in the supernatant was determined as follows.

The assay mixture contained 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of PMS and 0.3 ml of NBT and appropriately diluted enzyme preparation in a total volume of 3 ml. The reaction was started by the addition of 0.2ml NADH. After incubation at 30°C for 90 seconds, the reaction was stopped by the addition of 1ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4 ml n-butanol. The mixture was allowed to stand for 10 minutes, centrifuged and the butanol layer was separated. The color intensity of the chromogen in butanol layer was measured in a colorimeter at 520 nm. A system devoid of enzyme served as control. One unit of enzyme activity is defined as the enzyme concentration, which gives 50% inhibition of NBT reduction in one minute under assay conditions.
Superoxide dismutase activity was expressed as U/ml for plasma, U/mg Hb for erythrocyte lysate and U/mg protein for tissues (U-The amount of enzyme required to inhibit 50% nitroblue tetrazolium (NBT) reduction).

3.5.6.2 Estimation of catalase [CAT]

The activity of catalase was assayed by the method of Sinha (1972).

Dichromate in acetic acid was reduced to chromic acetate, when heated in the presence of hydrogen peroxide with the formation of perchromic acid as an unstable intermediate. The chromic acetate formed was measured at 590 nm. Catalase was allowed to split H₂O₂ for different periods of time. The reaction was stopped at different time intervals by the addition of dichromate-acetic acid mixture and the remaining H₂O₂ was determined by measuring chromic acetate colorimetrically after heating the reaction mixture.

Reagents

1. Phosphate buffer, 0.01 M, pH 7.0
2. Hydrogen peroxide, 0.2 M
3. Potassium dichromate, 5%
4. Dichromate-acetic acid reagent: Potassium dichromate and glacial acetic acid were mixed in the ratio of 1:3. From this 1 ml was diluted again with 4 ml of acetic acid.
5. Standard hydrogen peroxide, 0.2 Mm.

Procedure

To 0.9 ml phosphate buffer, 0.1 ml plasma or hemolysate or tissue homogenate and 0.4 ml H₂O₂ were added. The reaction was arrested after 15, 30, 45 and 60 seconds by adding 2.0 ml of dichromate-acetic acid mixture. The tubes were kept in a boiling water bath for 10 minutes, cooled and the color developed was read at 530 nm. Standards in the concentration range of 20-100 μmoles were processed as for the test.

The activity of catalase was expressed as U/ml for plasma, U/mg Hb for erythrocytes and U/mg protein for tissues (U-μ moles of H₂O₂ utilized/sec.).
3.5.6.3.**Estimation of glutathione peroxidase (GPx)**

The activity of glutathione peroxidase was determined by the method of Rotruck *et al.*, (1973) with modifications.

A known amount of enzyme preparation was allowed to react with H₂O₂ in the presence of GSH for a specified time period. The GSH content remaining after the reaction was measured by the method of Beutler and Kelley (1963).

\[ 2 \text{GSH} + \text{H}_2\text{O}_2 \xrightarrow{\text{GPx}} \text{GSSG} + 2 \text{H}_2\text{O} \]

**Reagents**

1. Phosphate buffer, 0.4 M, pH 7.0
2. Sodium azide solution, 10 mM
3. 10 % Trichloroacetic acid (TCA),
4. 0.4mM Ethylene diamine tetra acetic acid (EDTA),
5. 0.2 mM H₂O₂
6. 2 mM Glutathione (GSH).

**Procedure**

The reaction mixture in a total volume of 1ml-contained 0.2ml of phosphate buffer, 0.2ml EDTA, 0.1ml of sodium azide and 0.5ml of the enzyme preparation (tissue homogenate/plasma/erythrocyte lysate). 0.2ml of glutathione and 0.1ml of H₂O₂ were added, to this mixture and incubated at 37°C for 10 minutes. The reaction was arrested by the addition of 0.5 ml of 10 % TCA. The tubes were centrifuged and the supernatant was assayed for GSH by the method of Beutler and Kelle. A blank was treated similarly to which 0.2ml of the enzyme was added after the incubation.

3.5.6.4.**Estimation of glutathione** (Rotruck *et al.*, 1973)

**Reagents**

1. Trichloroacetic acid, 8 %.
2. Disodiumhydrogen phosphate, 0.3 M.
3. Ellman's reagent: Prepared by dissolving 19.8 mg of 5, 5' dithionitro benzoic acid in 100ml of sodium citrate (1%).
Stock standard

Prepared by dissolving 100 mg of reduced glutathione in 100 ml of distilled water.

Working standard

Prepared by diluting 10 ml of stock into 100 ml with distilled water (10 times diluted).

Procedure

From the serum, 0.5 ml was pipetted out into a test tube, to which 2 ml of 8% TCA was added and centrifuged. To 1 ml of supernatant, 1 ml of Ellman's reagent and 3 ml of 0.3 M disodium hydrogen phosphate were added. The yellow colour developed was read at 412 nm against reagent blank. Glutathione was used as standard. The amount of glutathione was expressed as moles/L.

3.5.7. Estimation of Non-Enzymatic Antioxidants

3.5.7.1. Estimation of Vitamin E

Vitamin E was estimated in plasma and erythrocyte membrane by the method of Desai (1984) based on the classical Emmerie Engle reaction.

This method involves reduction of ferric ions to ferrous ions by the tocopherol and the formation of a pink colored complex with bathophenanthroline orthophosphoric acid. Absorbance of the stable chromophore is measured at 536nm.

Reagents

1. Redistilled ethanol.
3. Bathophenanthroline reagent: 0.2% solution of 4,7-dipyridyl-1-10-phenanthroline in purified absolute ethanol.
4. 0.01 M Ferric chloride in absolute ethanol.
5. 0.001 M Orthophosphoric acid in absolute ethanol.
6. Standard solution: 1g/100ml α-tocopherol in absolute ethanol.
Procedure

To 0.2ml of the sample (plasma/erythrocyte membrane) taken in a glass stoppered centrifuged tube, 1.8ml of redistilled ethanol was added and thoroughly mixed. 3.0ml of petroleum ether was then added and tubes were shaken rapidly in a mechanical shaker for 3 minutes. The tubes were centrifuged and 2.0ml of the ether layer transferred to fresh tubes and evaporated to dryness. To the lipid residue, carefully redissolved in 3.0ml of absolute ethanol, 0.2ml of 0.2% baphenanthroline reagent was added and mixed. The tubes were protected from exposure to direct light and the assay was carried out rapidly from this point. 0.2ml of ferric chloride reagent was added and mixed in a vortex mixture. After one minute, 0.2ml of orthophosphoric acid reagent was added and shaken well. Tubes containing standard α-tocopherol, 2-10µg were treated in a similar manner along with a blank containing 3.0ml of ethanol.

The α-tocopherol values were expressed as mg/dl for plasma and µg/mg protein for erythrocyte membranes.

3.5.7.2. Estimation of Vitamin C

The level of plasma vitamin C was determined by the method of Omaye et al., (1979).

Ascorbic acid is oxidized by copper to form dehydro ascorbic acid and diketoglututaric acid. These products when treated with 2,4-dinitrophenyl hydrazine (DNPH) form the derivatives bis-2,4-dinitrophenylhydrazone which undergoes rearrangement to form a product with an absorption maximum at 520nm. Thiourea provides a mild reducing medium that helps to prevent interference from non-ascorbic acid chromogens.

Reagents

1. 2,4-dinitrophenylhydrazine – thiourea - copper sulphate reagent (DTC): 0.4g thiourea, 0.05g copper sulphate and 3.0g of DNPH in 100ml of 9 N H₂SO₄.
2. 10% TCA
3. 65% H₂SO₄
4. Standard solution: 10mg/100ml of 5% TCA.
Procedure

1.0 ml of the sample was mixed thoroughly with 1.0 ml of ice cold 10% TCA and centrifuged for 20 minutes at 3500g. To 0.5 ml of the supernatant, 0.1 ml of DTC reagent was added and mixed well. The tubes were incubated at 37°C for three hours. 0.75 ml of ice cold 65% sulphuric acid was added and the tubes were allowed to stand at room temperature for an additional 30 minutes. A set of standards containing 10 - 50 µg of ascorbic acid was processed similarly along with a blank containing 0.5 ml of 10% TCA. The colour developed was read at 520 nm.

Vitamin C values were expressed as mg/dl for plasma.

3.6. HISTOLOGICAL ASSAY

On the 28th day, pancreatic tissues were taken from animals which were fasted overnight under ether anaesthesia. The whole pancreas from each animal was removed after killing the animals, was placed in 10% formulation solution and immediately processed by the paraffin technique section of 5 µm thickness were cut and stained by haematoxylin and Eosin (H and E) for histological examination. The photomicrographs of histological studies are taken.

3.7. ANTI-INFLAMMATORY ACTIVITY (Winter et al., 1962)

3.7.1. Carrageenan Induced Hind Paw Edema

Albino rats of either sex weighed about 150-200 g were divided into six groups of six animals each. The dosage of the drugs administered to the different groups was as follows. All the drugs were administered orally.

3.7.2. Experimental conditions

Seven groups of five animals each were used. Paw swelling was induced by sub-plantar injection of 0.1 ml 1% sterile carrageenan in saline into the right hind paw. The methanolic extracts of T. chebula and C. sinensis at dose of 100 and 200 mg/kg were administered orally 60 min before carrageenan injection. Aspirin (10 mg/kg) was used as reference drug. Control group received the vehicle only (10 ml/kg). The inflammation was quantified by measuring the volume displaced by the paw, using a plethysmometer at time 0, 1, 2, 3, and 4 h after carrageenan injection. The difference between the left and the right paw volumes (indicating the degree of
inflammation) was determined and the percent inhibition of edema was calculated in comparison to the control animals.

\[
\text{Percentage inhibition} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100
\]

*Increase in paw volume in 3rd hour

3.8. ANTIBACTERIAL ACTIVITY

3.8.1. Micro organisms

Bacterial culture of *Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella aerogenes* and, *Pseudomonas aeruginosa* were clinical isolates of patients isolated from clinical patients at dental clinics in and around Thanjavur and Chennai, Tamil Nadu, India.

3.8.2. Determination of antimicrobial activity

Culture supernatants with fractions and crude extract of the plants were used in the disc-diffusion method separately. Bacterial cultures were swabbed on the surface of the sabouraud agar plates and discs (Whatman No.1 filter paper with 9 mm diameter) impregnated with the 50 µl of each plant sample was place on the surface individually. To compare the anti-bacterial activities, Ampicillin (20 µg/disc) used as standard antibiotic and negative control, a blank disc impregnated with solvent followed by drying was used. The plates (triplicates) were incubated 28°C for 72 h. The antimicrobial potency of the test samples was measured by determining the diameter of the zones of inhibition in millimeter.