CHAPTER - 3
MATERIALS AND METHODS

3.1 Pharmacognostic Study of Plants Fruit

3.1.1 Plant materials

❖ **Karonda:** *Carissa carandas*, L. (Family: Apocynaceae)

The fruits of this plant were collected in the month of August from local market of Gorakhpur, UP, India and authenticated by Dr. Tarique Husain from the NBRI, Lucknow, UP, India. A voucher specimen No-95395 was retained in the laboratory for further reference.

❖ **Amara:** *Spondias mangifera*, Willd. (Family: Anacardiaceae)

The fruits of this plant were collected in the month of November from the campus of Dibrugarh University, Assam and identified by Dr. Prof. Muhibul Islam from the Department of Life Sciences, Dibrugarh University, Assam. A voucher specimen No-DU/PS/HRB-02/2009 was retained in the laboratory for further reference.

❖ **Jangali baigun:** *Solanum torvum*, SW. (Family: Solanaceae)

The fruits of this plant were collected in the month of June from the campus of Dibrugarh University, Assam and identified by Dr. Prof. Muhibul Islam from the Department of Life Sciences, Dibrugarh University, Assam. A voucher specimen No-DU/PS/HRB-03/2009 was retained in the laboratory for further reference.

Pharmacognostic study was focused on the structural feature of the fruit and fruit powder of *Carissa carandas*, *Spondias mangifera* and *Solanum torvum* including macro and microscopic features, physicochemical parameters,
extractive value determination, fluorescence analysis of the fruit powder, preliminary phytochemical screening and TLC fingerprint analysis. These characteristics would be useful in identification and differentiation of these fruits from their substitutes and adulterants. For the standardization of the plant materials following parameters have been determined.

- Morphological study of the fruits
- Microscopical study of the fruits
- Histochemical analysis
- Tests for extraneous material
- Physicochemical parameters
  - Moisture content
  - Loss on drying
  - Ash value
  - Crude fibre content
- Extractive value
- Fluorescence analysis of the fruit powder
- Preliminary phytochemical screening and qualitative chemical test
- TLC fingerprint analysis

3.1.2 Morphology and microscopy

Fresh fruits were used for the examination of macroscopic and microscopic characters whereas the dried fruit powder was used for determination of physicochemical parameters. Microscopic sections were cut by free hand sectioning [1]. Microscopical examination and histochemical colour reactions of powdered fruit was carried out with Ruthenium red for mucilage, weak iodine solution for starch, Millon’s reagent for protein, Dragendorff’s
reagent for the alkaloidal substance, Conc. NaOH for flavonoids, aqueous ferric chloride for the phenolic compounds [2].

3.1.3 Tests for extraneous materials

A small portion was sprinkled and examine at a time with a good light and against a white back ground into a standard pepper sieve. The sieve was shaken moderately back and forth 10 times. The siftings were accumulated and small berries of pepper that pass through the pepper sieve were removed. The siftings were again weighed nearest to 0.1 g and the percentage of extraneous/foreign matter by sifting was calculated. In a composite sample two 100 g aliquots (A and C) were removed from opposite quarters after coning and quartering. They were hand pick for any sticks, stones, stems, foreign seeds and other extraneous matter. The pickings were weighed nearest to 0.1 g and the percentage of extraneous/foreign matter was calculated [1].

Calculation:

\[
\% \text{ of extraneous / foreign matter by sifting (Es)} = \frac{\text{Wt of sifting (g)}}{\text{Wt of sub sample (g)}} \times 100
\]

\[
\% \text{ of extraneous / foreign matter by hand picking (Ep)} = \frac{A \text{ (g)} + C \text{ (g)}}{2}
\]

\[
\% \text{ of extraneous / foreign matter in the sample} = \text{Es} + \text{Ep}
\]

The average % of extraneous matter was calculated into the sample to an accuracy of 0.0% by wt.
3.1.4 Physicochemical parameters

Quantitative analysis, viz. Loss on drying, total ash, acid insoluble ash, water soluble ash, crude fibre content and extractive value were assayed according to standard Indian Pharmacopoeia methods [3].

3.1.4.1 Moisture content

The liquid part or constituent inherent in sample is the moisture content. The calculation for percentage moisture content (% MC) of fresh sample is mentioned as under

\[
% \text{ MC} = \frac{(\text{Fresh mass} - \text{Dry mass})}{\text{Fresh mass}} \times 100
\]

3.1.4.2 Loss on drying

Determination of water and volatile matter in crude drug is very essential for standardization of product. It determines the presence of excess of water in crude drug that encourages microbial and fungal growth, attack by insects and mites leading to deterioration of the drug. Therefore a set of limit for water content in plant material is essential. It should also be noted here that the phytochemicals present in the interior of the cell undergoes enormous changes through loss of water, oxidation-reduction and hydrolysis. Drying can be carried out by heating the drug to 100-105 °C to constant weight.

2-5g of air dried fruit powder was accurately weighed and placed in a previously dried and tarred petridish. The sample was dried in oven at 100-105 °C for 1 hr, till two consecutive weighing does not differ by more than 5mg. The loss of weight was calculated in mg/g of air dried material. For material containing considerable amount of volatile materials, drying may be
accomplished by spreading the weighed material over glass plate and placing in desiccators over phosphorus pentoxide R under atmospheric pressure or reduced pressure and at room temperature [3].

3.1.4.3 Ash value

Faulty collection, incorrect storage or substitution of artificially manufactured substance leads to substandard quality. Thus to prove acceptability as genuine drug determination of ash value is most important. It is determined by four different methods which measure total ash, acid insoluble ash, water-soluble ash and sulphated ash. Limits for ash content will be in a constant range for every plant material.

➤ Total ash

Total ash determines quantity of inorganic materials such as carbonate, silicates, oxalates and phosphates present in the crude drug. 2-4 g of accurately weighed air dried sample was taken in a previously ignited and tarred silica crucible. The crucible was placed in a Muffle furnace; the temperature was gradually increased up to 500-600 °C and ignited until white ash indicating the absence of carbon is formed. The furnace was cooled up to 100-200 °C; the crucible was removed, kept in a desiccator for further cooling and weighed. After weighing the content, the total ash was calculated [3].

➤ Acid-insoluble ash

‘Acid-insoluble ash is mostly non-physiological ash comprised of sand, grit and siliceous earthly material that come by adhering to plant surface. The powdered sample is frequently found to be adulterated with brick, sand or grit powder that can be easily evaluated by this method. In a crucible containing total ash, 25ml of 2M HCl was added and boiled for 5 minutes. The insoluble
matter was collected on an ash less filter paper and washed by hot distilled water until filtrate was neutral. The filter paper containing insoluble matter was transferred to the original crucible. It was ignited up to a constant weight and the content of acid-insoluble ash was calculated [3].

- **Water soluble ash**
  Substitution of exhausted drug devoid of any medicinally active constituents may be evaluated by this method. In crucible containing total ash, 25 ml of distilled water was added and boiled for 5 minutes. The insoluble matter was collected on an ash less filter paper and washed with 25 ml of boiling distilled water. It was then transferred to the original crucible and ignited to a constant weight. The weight of this residue subtracted from the weight of total ash is the water soluble ash [3].

- **Sulphated ash**
  The sulphated ash determines the quantity of basic radicals in ash reactive to sulphuric acid. 1gm of drug was placed in pre ignited tarred crucible. The drug was ignited to char and 1ml of sulphuric acid was added to residue and heated gently until the white fumes were no longer evolved. The crucible was ignited up to 800 ± 25 °C until white particles appeared. After cooling the crucible few drops of sulphuric acid was added and heated to ignition cooled and taken weight. This operation was repeated until two successive weights not differ by more than 5 mg [3].

- **Crude fibre content**
  Some resistant part of the plant cannot be dissolved in water and boiling mineral acid or alkali. ‘This procedure determines the presence of adulterant containing sclarenchyma or other resistant woody tissue, kernels etc. It denotes
the measurement of the content of cellulose lignin and cork cells in the plant tissue. It must also be noted that it is not an absolute value and that the crude fibre in the ideal case is identical with cellulose and it also depends on the degree to which the material has been ground up [4]. About 2-4 g of accurately weighed drug was refluxed with ether. The extract was boiled in 200 ml of 1.25 % sulphuric acid for 30 minutes under reflux. The mixture was then filtered through a harden filter and the residue was washed with boiling distilled water until free of acid. The residue was further refluxed in 200 ml of 1.25 % NaOH for 30 minutes. The mixture was filtered through a tarred filter paper and washed with boiling distilled water until filtrate became neutral. It was dried at 110 °C to a constant weight and then incinerated to a constant weight again. The difference between the weights of the dried residue represents the weight of crude fibre [3].

### 3.1.5 Extractive value

Extractive value is the amount of extracts obtained by exhausting a given amount of crude drugs content with various solvents. Various polar and non-polar solvents are used to measure the diversity in chemical nature and properties of drug contents. Phytoconstituents like alkaloids, saponins, tannins, plant sugars, acids, mucilage and glycosides are hydrophilic in nature and easily comes into the cell fluid through the inner plasma membrane, tonoplast and protoplast of the living plant cell. While phytoconstituents like oils, balsams and resins are lipophilic in nature. They are formed in the plasma and gradually converted to ethereal oil which more or less completely fills the cell space. Hydrophilic and lipophilic constituents can be determined by successive extraction of crude powder in a sample [4].
3.1.5.1 Hot extraction

Hot extraction is one of the most crucial points of the analytical chain in an effort of achieving a complete recovery of targeted compound. In a 250 ml stoppered conical flask, 5.0 g of air-dried fruit powder was taken and 100 ml of water was added. The flask was shaken and allowed to stand for 1 hr. The material was refluxed for 1 hr and then cooled. The material was filtered through a dry filter with shaking. 25 ml of filtrate was placed in glass petridish and evaporated to dryness on water bath, followed by drying at 105 °C for 6 hrs in an oven. It was then cooled in a desiccator for 30 minutes and the weight was determined [5].

3.1.5.2 Cold maceration

5.0 gm of air-dried, accurately weighed fruit powder was placed in a 250 ml stoppered conical flask. Added 100 ml of solvent and shaking frequently for 6 hr then allowed to stand for 18 hr. Filtered the solvent and dried the 25 ml of filtrate in tarred glass petridish on water bath at 105 °C for 6 hr. Cooled it in desiccator and the weight was taken. Solvent like petroleum ether, chloroform, ethyl acetate, methanol and water were employed for the estimation of cold extractive value [5].

3.1.5.3 Successive extraction

About 30 gm of air dried powdered drug was extracted successively with Petroleum ether, Chloroform, Ethyl acetate, Methanol and finally with water. The fruit powder was macerated with above respective solvents for 24 hr in soxhlet apparatus and then extracted up to 6 hr with at least 6-8 siphoning. Each time before extracting with the next solvent, the powdered material was first dried in air and then in oven below 50 °C. Each extract was concentrated by
Materials and methods

3.1.5.4 Microwave-assisted extraction

Microwave energy is being used for extraction of phytoconstituents from plants as it is a good alternative to a conventional extraction. Microwave-assisted extraction method is useful for aqueous extraction of hard, dense and tough plants materials [5]. Microwave extraction method follows the same principal of extraction like maceration and percolation but the speed of breaking of plant cells and tissues is much more [6]. It requires shorter time with higher extraction rate and gives better products at lower costs. It reduces the processing time as well as decomposition or disintegration and oxidation of active constituents [7].

The dried fruits were crushed into small fragments. 25 g sample was taken in 1000ml conical flask and mixed with 500ml distilled water. The conical flask containing mixture was placed in microwave oven (LG Health Ware System MG – 605 AP, 900 Watts, 230 V, 50 Hz) at 650 W intensity for 45 minutes [7]. After irradiation in microwave oven the extract was cooled, filtered and the filtrate was evaporated under reduced pressure.

3.1.6 Fluorescence analysis

There are many chemical components in powdered drug that can be excited by electromagnetic radiation and as a consequence re-emit radiation. The measurement of fluorescence intensity permits the quantitative determination of many organic and inorganic species present in crude drugs. Almost all fluorescent chemical systems are complex organic compounds.
containing one or more aromatic functional groups and fluorescence in these compounds generally involves a $\pi-\pi^*$ transition. For visual fluorescence any exciting wavelength from 200-600µm may be used. It should be also noted that radiation below 300µm is injurious to the eyes and it contact with air to produce ozone and oxides of nitrogen, so adequate protection must be needed.

Fluorescence analysis of the powder sample was carried out by treating it with different chemical reagents in day light and UV light (254 nm and 365 nm). The dry powder was studied on glass slide whereas the different extracts were studied by adsorbing the extracts on Whatmann filter paper [8].

3.1.7 TLC fingerprint profile

For TLC fingerprint of *C. carandas* fruits, chloroform, ethyl acetate and methanolic extracts were analyzed. The mobile phase was made up of solvent system comprising toluene: acetone: formic acid in a ratio of 10: 1: 0.1. Anisaldehyde-sulphuric acid reagent was used as a detecting agent and the $R_f$ values were compared with standard drug lupeol dissolved in methanol [9].

For TLC fingerprint of *S. mangifera* water and methanolic extract of the fruits was analyzed. The mobile phase was made up of solvent system constituting Methanol: Ethyl acetate: Acetic acid: Formic acid in a ratio of (40: 20: 2: 2). Anisaldehyde-sulphuric acid reagent was used as a detecting agent. The $R_f$ values are compared with standard drug Gallic acid and colors are recorded [9].

For the TLC fingerprint of *S. torvum* fruits, different extractives of were analyzed. The mobile phase was made up of solvent system containing Chloroform: Methanol: Formic acid in ratio of 60: 40: 2. Anisaldehyde-sulphuric acid reagent was used as a detecting agent. The $R_f$ values are compared with standard drug Lupeol and Rutin [9].
3.2 Phytochemical Screening

Phytochemistry is the study of phytochemicals which are secondary metabolites found in plants. Phytochemical analysis mainly applies to the quality control of traditional medicine or herbal medicine to determine the various chemical components such as saponins, alkaloids, volatile oils, flavonoids and anthraquinones. In IR the spectra presented, transmission is plotted against wave-length. Absorption bands in the infra-red region studied here result from inter-atomic vibrations, whose frequencies are related to the strength of the atomic bonds involved. It should therefore be possible to assign certain absorption bands to particular atomic groups or linkages.

The NMR experiment makes the direct observation of atoms possible. The purified isolated sample was placed in an inert solvent [deuteron chloroform (CDCl₃), deuterium oxide (D₂O), carbon tetrachloride (CCl₄) or deuterated dimethyl sulphoxide (DMSO)] and the solution was placed between the poles of a powerful magnet. The different chemical shifts of the proton according to their molecular environments within the molecule were measured in the NMR apparatus relative to a standard, usually tetramethyl silane (TMS). European pharmacopoeia mostly uses NMR spectroscopy for the identification of drugs and reagents. UV, IR, NMR and Mass spectroscopy constitutes a method of value for the analysis of the chemical structure of newly synthesized and natural products isolated from plants.

3.2.1 Chemical and reagents

Silica gel (Qualigens, 60–120 mesh, Mumbai, India) was used for column chromatography. Silica gel G (Qualigens) was used for analytical TLC. Spots were visualized by exposure to iodine vapors, UV radiation and by spraying
anisaldehyde sulphuric acid reagents. The solvents for isolation were obtained from Merck Mumbai, India.

### 3.2.2 Preliminary phytochemical screening

Different extractives of the plant fruits obtained from the successive extraction were subjected to preliminary phytochemical investigation for the presence of various phytoconstituents. The presence or absence of different phytoconstituents viz. alkaloids, reducing sugar, glycosides, flavonoids, terpenoids, steroids, tannins and phenolic compounds were detected [10].

### 3.2.3 Extraction and isolation of constituent from C. carandas

The dried fruit powder (500 g) was defatted with petroleum ether and extracted with (95%) ethanol by using Soxhlet apparatus. The ethanolic extract was then concentrated and dried to yield 26.43% w/w. The ethanolic extract (40 g) was submitted to Si-gel CC eluting petroleum ether, chloroform and methanol to afford 41 fractions.

The compound was isolated from fractions 11-17 (Chloroform: Methanol 90:05) and subjected to TLC using the solvent system Chloroform: Methanol (90:10) which showed a single spot at Rf value of 0.81 detected by spraying with anisaldehyde sulphuric acid. This fraction was collected and dried to get 700 mg of amorphous brownish white powder with a melting point of 130-132 °C which was coded as Cc-01[11].

### 3.2.4 Extraction and isolation of constituent from S. mangifera

The air dried fruits of S. mangifera were made into a coarse powder. The dried fruit powder (1 kg) was defatted with petroleum ether and then extracted with (95%) ethanol by using Soxhlet apparatus. The extract was then concentrated and dried to give reddish brown mass. The yield of the extract was
Materials and methods

176 g. ‘It was subjected to column chromatography on silica gel (60-120) using solvents of varying polarities starting from petroleum ether, chloroform and methanol to yield several sub fractions (61 fractions). The compound was isolated from fractions 18-23 (methanol 40% in 60% chloroform) and subjected to TLC using the solvent system methanol: chloroform in the ratio 9:1 which showed a single spot at R_f value of 0.62 detected by spraying anisaldehyde sulphuric acid. This fraction was collected and dried to get 2.42 g of chocolate colour solid residue which was coded as Sm-01 [11].

3.2.5 Experimental procedure for characterization of isolated constituents

The chemical constituents isolated from the fruits of Carissa carandas and Spondias mangifera was established on the basis of chemical tests and spectroscopic evidences such as UV, FTIR, 13C NMR, 1H NMR and MASS spectroscopy. UV spectra scanned in chloroform and methanol on Lambda Bio 20 Spectrophotometer Shimadzu-U Singapore. IR spectra were recorded in KBr pellets on a Win IR FTS 135 instrument (Biorad. USA). 1H NMR (300 MHz) and 13C NMR (75 MHz) spectra were recorded on a Brucker Spectrometer (Brucker, USA) in CDCl3 with TMS as internal standard. The MS were measured in DART dried Helium was used for ionization mode with a JEOL-AcuTOF JMS-T100LC. Melting points were determined on a Perfit melting point apparatus. Silica gel (Qualigens, 60-120 mesh, Mumbai, India) was used for column chromatography. Silica gel G (Qualigens) was used for analytical TLC. Spots were visualized by exposure to iodine vapors, UV Lamp 254 nm and by spraying with anisaldehyde sulphuric acid reagents.
3.3 Screening for adaptogenic activity

Different parameters of adaptogenic activity have been investigated in vivo by using healthy albino mice (20-25 g) of either sex.

- Safety profile studies for determination of lethal dose and effective dose.
- Anoxia stress tolerance test.
- Swimming endurance test and post-swimming motor function test
- Immunological related studies (Cyclophosphamide induced immunosuppression test) and estimation of immunological parameters like haemoglobin, total RBCs and total WBCs counts.

3.3.1 Preparation of extract

The size of fruits was reduced by cutting with clean and sharp knife and dried in shade. The air dried fruit of *C. carandas*, *S. mangifera* and *S. torvum* were made into coarse powder and 200 gm of fruit powder was defatted with petroleum ether (40–60 °C). The marc was dried and further extracted with 95% ethanol. The ethanolic extract was evaporated under reduced pressure and vacuum dried. The yield of ethanolic extract of fruit *C. carandas* (EEFCC), *S. mangifera* (EEFSM) and *S. torvum* (EEFST) were 24.12, 9.4 and 7.8% w/w.

3.3.2 Experimental animals

Healthy adult Swiss albino mice of either sex weighing between 20-25 g were procured from Central Drug Research Institute Lucknow, India. They were maintained in clean, sterile, polypropylene cages and fed with commercial pellet and water *ad libitum*. After randomization into various groups, the mice were quarantined for the period of a week for environmental and trainer handling acclimatization before initiation of experiments like Anoxia stress tolerance test.
Materials and methods

and swimming endurance test and Cyclophosphamide induced immunosuppression activity. The Experimental protocol was approved by the Institutional Ethical Committee (Approval no- 1213/ac/08/CPCSEA/IU) following the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) which complies with International norms of INSA (Indian National Science Academy).

3.3.3 Administration of drugs

The ethanolic extract of fruits *C. carandas* (EEFCC), *S. mangifera* (EEFSM) and *S. torvum* (EEFST) and isolated constituents Cc-01 and Sm-01 was used in all the models. The extract and constituents were formulated into an emulsion using gum acacia (2%) to obtain the desired dose on body weight basis (mg/kg) of the animal and administered to animal daily by oral gavage using a ball ended feeding needle. Drugs were prepared fresh daily before administration. Control groups were administered with 0.5 ml only acacia emulsion orally. Geriforte tablets (Himalaya drugs) 50 mg/kg was used as a standard adaptogenic drug which is a multi-constituent ayurvedic drug with 35 herbal and natural constituents like *Withania somnifera*, *Asparagus racemosus*, *Glycyrrhiza glabra*, *Centella asiatic*, *Terminalia chebula*, *Piper longum*, *Shilajit*, etc.

3.3.4 Safety profile study

Safety profile studies of fruit ethanolic extract were performed according to organization for economic cooperation and development (OECD) guidelines, received draft guidelines 425, received from CPCSEA, Ministry of social justice and empowerment, Government of India [12]. For safety profile studies total 20 mice fasted overnight were used. The mice were divided in four groups each
group consisting of 6 mice. One time single dose of 0.5, 1, 2, and 3g/kg of ethanolic vacuum dried extract was administered orally to each group of overnight fasted mice. After extract administration the animals were provided with food and water immediately and closely observed in their cages for any mortality and signs of severe toxic effects such as hypo-activity, piloerection, anorexia, salivation, diarrhoea, muscle cramping, convulsions, if any, for 24 h and further daily for next 14 days.

3.3.5 Anoxia stress tolerance

The animals were divided into 5 groups of 6 mice each (n= 6). The animal of the group-I served as control and received vehicle alone (0.5 ml/kg 2% gum acacia). Group-II and III were treated with (100 and 200 mg/kg/day) ethanolic extract of fruit C. carandas (EEFCC), S. mangifera (EEFSM) and S. torvum (EEFST). Group-IV was treated with (10 mg/kg/day) isolated constituents (Cc-01 and Sm-01) of respective plants fruit and group-V was treated with standard drug (Geriforte 50 mg/kg/day) for three weeks respectively. Each animal was placed in an air tight glass container (250 ml) and the time taken for the development of clonic convulsion was taken as the end point. The time duration from the entry of the animal into the hermetic vessel and the appearance of first convulsion was taken as time of anoxia tolerance. Different groups of animal were treated as follows for three weeks [13].

- (Control) Gum acacia (2%) 0.5 ml / kg/day (p.o.)
- (Test) extract 100 mg / kg / day (p.o.)
- (Test) extract 200 mg / kg/day (p.o.)
- (Test) isolated drug 10 mg / kg/day (p.o.)
- (Std.) Geriforte 50 mg / kg/day (p.o.)
3.3.6 Swimming endurance and post-swimming motor function test

The swimming test has been employed widely to evaluate the effect of various drugs for adaptogenic activity. The exhaustion in these models is dependent upon physiological and nutritional factors and environmental parameters such as ambient temperature. Mice were divided into 5 groups of 6 mice each (n=6). Mice of the group-I served as control and received vehicle alone (0.5ml/kg/day 2% gum acacia). Group-II and III were treated with (100 and 200 mg/kg/day) ethanolic extract of fruit *C. carandas* (EEFCC), *S. mangifera* (EEFSM) and *S. torvum* (EEFST). Group-IV was treated with (10 mg/kg/day) isolated constituents (Cc-01 and Sm-01) and group-V was treated with standard drug (Geriforte 50 mg/kg). All the drugs were given orally once a day for seven days. On seventh day one hour after drug administration all the mice were made to swim in a water tank (140 × 60 × 45 cm) maintained at room temperature (30 ± 2 °C) until they sank. This was recorded as the swimming time [14 & 15].

Mice were removed and allowed to recover and dry for about 5 min. They were subsequently tested for muscle coordination on a rota rod (Medicraft rota rod M. no.519/E-30) rotating at 15 rpm and the duration of stay on the rod was recorded [16].

3.3.7 Cyclophosphamide induced immunosuppression

In this experiment Cyclophosphamide (Cyp) was used as to suppress the immune activity of mice [17]. The Cyp was suspended in 2% gum acacia solution in distilled water. The solution was administered orally at a dose of 25 mg/kg b/w. Mice were divided into the 4 groups containing 6 mice each (n= 6). Group I (Control group) received 2% gum acacia while group II received Cyp 25mg/kg alone and group III Cyp 25mg/kg along with 100 mg/kg fruit extract (EEFCC, EEFSM and EEFST) and group IV received 10 mg/kg isolated drugs.
(Cc-01 and Sm-01) along with Cyp 25mg/kg. The treatment was continued for 16 days as following dosage schedule.

- Control Gum acacia (2%)/kg (p.o)
- Cyp 25 mg/kg (p.o)
- Cyp + Extract 25 mg +100 mg/kg (p.o)
- Cyp + isolated drug 25 mg +10 mg/kg (p.o)

At the end of the treatment schedule, mice were anaesthetized and blood sample was collected by cardiac puncture into tubes containing Ethylenediaminetetraacetic acid (EDTA) as an anti-coagulant [18]. The vital organs liver, kidney, spleen, and heart were carefully dissected out, cleaned of the adhering connective tissues, blotted and accurately weighed. The ratio of each organ to body weight was determined. The WBC count was done by Turke’s method [19], RBC by Hayem’s method [20] and Haemoglobin by Sahli’s method [21]. The body weight of animal was also recorded prior to Cyp treatment and every 4th day up to the period of 16 days.

3.3.8 Statistical Analysis

The data was represented as mean ± S.E.M. for six mice. One way analysis of variance (ANOVA) and Dunnett’s post hoc test were performed by using Graph Pad Prism 2.01 (Graph Pad Software Inc.). The data was expressed as the mean ± standard error of the means (SEM) and a value of P < 0.05 was considered as statistically significant.
3.4 Antioxidant Activity

Natural product resources provide excellent raw material for the discovery and development of novel oxidative stress defence and anti-ageing compounds. Phenolic, flavonoids, acids glycosides, glucopyranosides and the triterpenoids compounds in fruits and vegetables have been reported to exhibit a wide range of biological activity and this effect is mainly attributed to their anti-oxidant properties [22].

3.4.1 Drugs, chemicals and reagents

The chemicals for determinations of antioxidant activity Ferrous sulphate, ascorbic acid, trichloroacetic acid (TCA), potassium ferricyanide, aluminium chloride (AlCl₃), sodium nitrite (NaNO₂), sulphanilamide, phosphoric acid, naphthylendiamine dihydrochloride and rutin were obtained from Qualigens Fine Chemicals, Mumbai, India. 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma-Aldrich Co MO, USA. Folin-Ciocalteu reagent, sodium carbonate (Na₂CO₃) and Gallic acid were obtained from Titan Biotech Limited, Rico Industrial area Phase-III, Bhiwadi (Raj).

- Griess reagent
  1% Sulphanilamide, 2% phosphoric acid and 0.1% Naphthylene diamine.

- Phosphate buffer saline pH 7.4 (PBS)
  2.38 g of disodium hydrogen phosphate, 0.19 g of Potassium hydrogen phosphate and 8.0 g of sodium chloride were dissolved in sufficient water to produce 1000 ml and adjusted to pH 7.4.

- Sodium nitroprusside (5µ M)
  149 mg Sodium nitroprusside were dissolved in 100 ml PBS
Materials and methods

Chapter 3

- **Phosphate buffer**
  
  Phosphate buffer 0.2 mM, pH 6.6 (13.872 g of Potassium dihydrogen phosphate and 35.084 g of disodium hydrogen phosphate were dissolved in distilled water and finally made the volume to 1000 ml.)
  
  1% Potassium ferricyanide in distilled water
  
  10% Tri chloroacetic acid in distilled water
  
  0.1% Ferric chloride in distilled water

3.4.2 Determination of total phenolics content

Phenolic and flavonoid compounds in fruits and vegetables have been reported to exhibit a wide range of biological activity and this effect is mainly attributed to their antioxidant properties [23].

The total soluble phenolics in the different extractives of the fruits *C. carandas, S. mangifera* and *S. torvum* were determined by the Folin Ciocalteu reagent. ‘Aliquots (1ml) of the extractives (25, 50 and 100 mg/ml) in a 25 ml volumetric flask were diluted with distilled water (9 ml). Then about 1 ml of Folin Ciocalteu reagent was added and the contents of the flask were mixed thoroughly. After 5 min, 3 ml sodium carbonate was added and the volume was made upto 25 ml. A reagent blank using distilled water was prepared. The mixture was allowed to stand in the dark for 1 h with intermittent shaking and then the absorbance was measured at 760 nm [24]. All determinations were performed in triplicate. The total phenolic content was expressed as the gallic acid equivalent %w/w of the extract.

3.4.3 Determination of total flavonoid content

The content of total flavonoids in the different extractives of fruits *C. carandas, S. mangifera* and *S. torvum* were estimated by the aluminium chloride
colorimetric method [25]. Aliquots (1ml) of the extracts (25, 50 and 100 mg/ml) were added to a 10 ml volumetric flask containing 4 ml distilled water and mixed with 0.3 ml 5% sodium nitrite followed by 0.3 ml 10% aluminium chloride after 5 min. At the 6th minute, 2 ml 1M-NaOH was added and the volume was made up to 10 ml with distilled water. Then, the mixtures were allowed to stand at room temperature for 30 min with intermittent shaking and the absorbance was measured at 514 nm. The calibration curve was constructed by preparing rutin solutions of different concentrations (100, 200, 400, 800, 1200 and 1600 μg/ml) in distilled water. The total flavonoid content was expressed as the rutin equivalent %w/w of the extracts.

3.4.4 DPPH radical scavenging capacity

DPPH• is a stable free radical due to the delocalization of the spare electron on the whole molecule. Thus, DPPH• does not dimerize as happens with most free radicals. The delocalization on the DPPH• molecule determines the occurrence of a purple colour with an absorption band with a maximum around 520 nm. When DPPH• reacts with a hydrogen donor the reduced (molecular) form (DPPH) is generated accompanied by the disappearance of the violet colour. Therefore, the absorbance diminution depends linearly on the antioxidant concentration [26].

The radical scavenging activities of the ethanolic extracts of fruits C. carandas (EEFCC), S. mangifera (EEFSM) and S. torvum (EEFST) against DPPH were determined by UV spectrophotometer at 517 nm. An aliquot of (0.05, 0.5, 1.0 and 2.0 mg/ml) of extract was mixed in a test tube containing 3 ml of methanol and 0.5 ml of 1mM DPPH. Ascorbic acid was used as standard at concentrations of 0.025, 0.05, 0.5 and 1.0 mg/ml. A blank solution was prepared containing the same amount of methanol and DPPH. The reaction
mixture was incubated at 37 °C for 30 min [27]. The radical scavenging activity was calculated using the following equation.

\[
\% \text{ Scavenging} = \frac{Ac - As}{Ac} \times 100
\]

Ac: absorbance of control,
As: absorbance of sample

3.4.5 Nitric oxide anion scavenging activity

The procedure is based on the principal that sodium nitroprusside solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitric ion that can be estimated using Greiss reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthylenediamine dihydrochloride). Scavenger of nitric oxide competes with oxygen leading to red 

ute production of nitric ion.

For the experiment, an aliquot (1 ml) of different concentrations of (EEFCC), (EEFSM) and (EEFST) were dissolved in Phosphate buffer solutions (PBS) and added 1ml of sodium nitroprusside (10 mM) and incubated at room temperature for 150 min. The reaction without the extract sample but equivalent amount of methanol served as control. After incubation period, 0.5 ml of Greiss reagent was added. The absorbance of the chromophore formed was read at 546 nm [28]’. Ascorbic acid was used as positive control.

3.4.6 Reducing power

In the PFRAP (potassium ferricyanide reducing power) method, the absorbance increased can be correlated to the reducing ability of
antioxidants/antioxidant extracts. The compounds with antioxidant capacity react with potassium ferricyanide to form potassium ferrocyanide. The latter reacts with ferric trichloride yielding ferric ferrocyanide a blue coloured complex with a maximum absorbance at 700 nm. Increased absorbance of the reaction mixture indicates increased reducing power.

Different concentrations of (EEFCC), (EEFSM) and (EEFST) in 1 ml of distilled water were mixed with 2.5 ml phosphate buffer (0.2 mM, pH 6.6) and 1% of 2.5 ml potassium ferricyanide. The mixture was incubated at 50 °C for 30 min. Subsequently 2.5 ml of trichloroacetic acid (10%) was added to the mixture which was then centrifuged for 10 min at 3000 rpm. The upper layer of the solution (1.5 ml) was mixed with 1.5 ml distilled water and FeCl₃ (0.3 ml, 0.1%) and the absorbance was measured at 700 nm using UV spectrophotometer [29].

3.4.7 Effect on linoleic acid peroxidation

The peroxy radical scavenging activity was determined by ferric thiocyanate method using ascorbic acid as a standard. Different concentration of (EEFCC), (EEFSM) and (EEFST) were dissolve in 0.5 ml of distilled water and mixed 2.5 ml of 0.02 M linoleic acid emulsion in (0.04 M phosphate buffer pH 7) and 2 ml phosphate buffer (0.04 M pH 7) in a test tube and incubated in darkness as 37 °C. At intervals during incubation the amount of peroxide form was determine by reading the absorbance of red colour developed at 500 nm by addition of 0.1 ml 30% ammonium thiocyanate solution and 0.1 ml 20 mM ferrous chloride in 3.5 % HCl to the reaction mixture [30].
References


