Chapter 4

Experimental Work
4. EXPERIMENTAL WORK/ MATERIAL AND METHODS:

4.1. Collection of Plant Material:

The plant materials were collected from Pune and Thane District of Maharastra. The fresh unripe fruits of *Momordica cherantia* and seeds of *Eugenia jambolana* were collected from Pune distract in the month of September. Fresh steam bark of *Gmelina arborea* was collected from mahape forest area from Thane district. All the plant materials were primarily identified with help of local people and medicinal plant expert from Yerala Medical College, Navi Mumbai.

The plant material was then air dried at room temperature. The dried plant material was grounded into a fine powder. The powdered materials were used to prepare the aqueous extract.

4.2. Botanical Authentication:

The Seeds of *Eugenia jambolana*, fruits of *Momordica charantia* and stem bark of the *Gmelina arborea* were authenticated from Maharastra Association for the Cultivation of Science, Agharkar Research Institute, Pune (Maharastra). For future reference a voucher specimens were prepared and deposited in the Department of Pharmacology, SSPSPTM, NMIMS University, Mumbai. The authenticity of all the plant materials were reconfirmed with the help of technicians from Phyto concentrates Pvt. Ltd.

4.3. Preparation of Aqueous Extract

The extraction was performed under GMP compliant facility of Phyto Concentrates Pvt. Ltd because it was planned to use the same extract for clinical trials. The materials were sent for crushing, grinding and mixing after sufficient drying. Water (Ph 7.5) was used as solvent for extraction and the ratio of solvent and plant material were kept as 8:2 making is finally as eight part of water and two part plant material. The plant materials were diluted with solvent and extraction were performed by percolation method and steam distillation method after sufficient grinding and mixing. The recovered concentrate was subjected to distillation and tripping to obtain aqueous extract. The recovered solvent was recycled two times for complete extraction and to obtain additional quantity of extract. The final extracts were dried at 40°C.
and sifted through 40 size mesh to obtain uniform size aqueous extract. The yield obtained for plant materials were 28 % for EJ, 22 % for MC and 30 % for GA respectively.

4.4 Morphological studies: (Kay, 1938; Wallis, 1953):

The morphological/organoleptic assessments were done for selected plant material i.e. EJ seeds, MC fruit and GA bark. The various organoleptic characters like, size, shape, fracture, colour, odour and taste.

Powdered plant materials were evaluated for colour, odour, taste and texture. Presence or absence of glistening specks (calcium oxalate crystals) on examination with lens. THE effect of addition of a small quantity of water to the powder, the effect of shaking the powder with water in a test tube, the effect of pressing the little powder between the filter paper.


A. Extractive values:
Extractive values determine amount of active constituents present in given plant material in given solvent. Extracts were prepared with various solvents by standard methods (WHO). Percentage of dry extract was calculated in terms of air-dried stem powder.

Water soluble extractive values –
Accurately weigh (5 gm) powdered drug in the glass stoppered conical flask, macerate with 25 ml of distilled water for 6 hours with frequent shaking, then allow to stand for 18 hours. After completion of 18 hours filter the contents of flask and transfer the filtrate in tarred flat bottom porcelain dish. Then evaporated the filtrate to dryness on water bath and dry at 105°C for 6 hours cool in desiccator for 30 min, weigh and calculated the content of extractable matter in milligrams per gram of air dried material.

Alcohol / Chloroform / Petroleum ether soluble extractive values –
Accurately weigh 5 gm of powdered drug and place in the glass stoppered conical flask and Macerate with 25 ml of ethanol (95%) /Chloroform/ Petroleum ether for 6 hours with frequent shaking, mixture allowed to stand for 18 hours. After completion of 18 hours, filter rapidly taking care not to lose any solvent. Transfer the filtrate in tarred flat bottom porcelain dish. Evaporated the filtrate to dryness on water bath, dry at 105°C for 6 hours and cool in
desiccator for 30 min, weighed and calculated the content of extractable matter in milligrams per gram of air dried material.

**B. Loss on Drying:**
Loss on drying is the loss in weight in percent w/w resulting from loss of water and volatile matter of any kind that can be driven off under specific conditions. 5 gm of air dried drug reduce to powder and place in a crucible of silica. Originally the crucible should be cleaned and dried and weight of empty dried crucible should be taken. Spread the powder in a thin uniform layer. Then place the crucible in the oven at 105°C. Dry the powder for 2 hours and cool in a desiccator to room temperature and note the weight of the cooled crucible plus powder.

**C. Moisture content:**
Excess moisture in the sample suggests not only that the purchaser could be paying high price for unwanted water but also that the drug has been incorrectly prepared or subsequent to preparation has been incorrectly stored. Excess moisture can result in breakdown of important constituent by enzymatic activity and may encourage growth of yeast and fungi during storage. The result in either case will be eventual rejection of drug as an unsuitable material.

**D. Ash values** (Anonymous, 1996)
Ash values are indicative to some extent of care taken in collection and preparation of drug for market and of foreign matter content of natural drug. The purpose of ash preparation is to remove all traces of organic material interfering in an analysis of inorganic elements. Total ash, Acid insoluble ash and Water soluble ash of powdered material were obtained by reported methods (WHO).

**a. Total ash:**
This method is designed to measure total amount of material remaining after ignition. It includes both physiological ash and non physiological ash. The physiological ash is derived from plant tissue itself and non-physiological ash is residue of extraneous matter (e.g. Sand and soil) adhering to plant surface. Two gram of accurately weighed air dried powder was taken in tarred platinum crucible. Spread the drug material in fine even layer at bottom of the platinum crucible. This platinum crucible with drug material was kept in muffle furnace for ignition at high temperature. Temperature of furnace increased gradually up to 450°C. the
material was kept at this temperature for 6 hours till complete ignition of drug occurred, that is till complete white colored ash was obtained, intermittent weighing was also done and heating continued till constant weight of crucible. Crucible was then taken out from furnace, cooled and weighed.

The total ash was calculated by subtracting the weight of crucible with ash of drug after ignition from weight of crucible with drug powder before ignition. Percentage of total ash was calculated with reference to air dried drug.

b. Acid insoluble ash:
The acid insoluble ash value has been undertaken to remove variations caused by calcium oxalate. The ash obtained in the total ash method was taken and boiled with 25 ml of 2N Hydrochloric acid for 5 min. Insoluble matter was collected on ash-less filter paper and washed with hot water. The material was further ignited and weighed. Percentage of acid insoluble ash was calculated with reference to air dried material.

c. Water soluble ash:
Total ash value also varies from wide range; therefore, water soluble ash value is quite reliable parameter to judge adulteration. The ash obtained from total ash was taken, boiled with 25 ml water for 5 min. All insoluble matter was collected on ash less filter paper, washed with hot water and ignited for 15 min at the temperature not exceeding 450°C.

The percentage of water soluble ash was calculated by subtracting weight of insoluble matter from weight of total ash. The difference between weights represents water soluble ash. Percentage of water soluble ash was calculated with reference to air dried drug.

Elemental analysis of obtained ash has been performed to confirm the inorganic contents of ash, as per the Indian Pharmacopoeia, 1996.

E. Microbial Limit Tests:

Medicinal plant materials normally carry a great number of bacteria and moulds, often originating in soil. While a large range of bacteria and fungi form the naturally occurring microflora of herbs, aerobic spore-forming bacteria frequently predominate. Current practices of harvesting, handling and production may cause additional contamination and microbial growth. The determination of Escherichia coli and moulds may indicate the quality of production and harvesting practices. Dilute, suspend or emulsify the material being examined using a suitable method and eliminate any antimicrobial properties by dilution, neutralization or filtration. Water-soluble materials: 10 g or 10 ml of test extracts were dissolved or diluted,
in lactose broth medium which is proven to have no antimicrobial activity under the
conditions of the test, the volume was adjusted to 100 ml with the same medium. The pH of
the suspension was adjusted to about 7. Determination of microbial limits was performed
according to the manual “Quality control of Medicinal Plants” prescribed by World Health

4.6. Preliminary Phyto-chemical analysis:
The test extracts were tested for various chemical constituents with the help of following
different chemical tests (Kokate et al 2006).

A. Alkaloids:-
 a) Dragendorff’s test: - Alkaloids give reddish brown precipitate with Dragendorff’s reagent
(Potassium bismuth iodide solution).
 b) Mayer’s test: - Alkaloids give cream color precipitate with Mayer’s reagent (Potassium
mercuric iodide solution).
 c) Wagner’s test: - Alkaloids give reddish brown precipitate with Wagner’s reagent (Iodine-
potassium iodide solution).
 d) Hager’s test: - Alkaloids give yellow precipitate with Hager’s reagent (Saturated solution of
picric acid).
 e) Tannic acid test: - Alkaloids give buff colour precipitate with tannic acid solution.
f) Picrolonic acid test: - Alkaloids give yellow colour precipitate picrolonic acid.

B. Amino acids :-
 a) Millon’s test: - To the test solution add about 2ml of Millions reagent white precipitate
indicates presence of amino acids.
 b) Ninhydrine test: - To the test solution add Ninhydrine solution, boil, violet colour indicates
presence of amino acid.

C. Carbohydrates:-
 a) Molisch’s test: -To the test solution add few drops of alcoholic α-naphthol, then add few
drops of concentrated sulphuric acid through sides of test tube, purple to violet colour ring
appears at the junction.
 b) Barfoed’s test: -1ml of test solution is heated with 1ml of Barfoed’s reagent on water bath,
if red cupric oxide is formed, monosaccharide is present. Disaccharides on prolong heating
(about 10 min.) may also cause reduction, owing to partial hydrolysis to monosaccharides.
c) Selivanoff’s test (Test for ketones): -To the test solution add crystals of resorcinol and equal volume of concentrated hydrochloric acid and heat on a water bath, rose colour is produced. (eg. Fructose, honey)

d) Test for pentoses: -To the test solution add equal volume of hydrochloric acid containing a small amount of phloroglucinol and heat, red colour is produced.

D. Flavonoids: -
a) Shinoda test: - To the extract add few magnesium turnings and concentrated hydrochloric acid dropwise, pink scarlet, crimson red or occasionally green to blue colour appears after few minutes.
b) Alkaline reagent test: - To the extract add few drops of sodium hydroxide solution, intense yellow colour is formed which turns to colourless on addition of few drops of dilute acid indicate presence of flavonoids.
c) Zinc hydrochloride test: - To the test extract add a mixture of zinc dust and conc. Hydrochloric acid. It gives red colour after few minutes.

E. Glycosides: -
I. General test: -
Test a: Extract 200mg of drug with 5ml of dilute sulphuric acid by warming on a waterbath. Filter it and then neutralize the acid extract with 5% solution of sodium hydroxide. Add 0.1ml of Fehling’s solution A and B until it becomes alkaline (test with pH paper) and heat on a waterbath for 2minutes. Note the quantity of red precipitate formed and compare with that of formed in Test B.
Test b: Extract 200mg of the drug using 5ml of water instead of sulphuric acid. After boiling add equal amount of water as used for sodium hydroxide in the above test. Add 0.1ml Fehling’s A and B until alkaline (test with pH paper) and heat on water bath for 2minutes. Note the quantity of red precipitate formed. Compare the quantity of precipitate formed in Test B with that of formed in Test A. If the precipitate in Test A is greater than in Test B then Glycoside may be present. Since Test B represents the amount of free reducing sugar already present in the crude drug, whereas Test A represents free reducing sugar plus those related on acid hydrolysis of any glycoside in the crude drug.
II. Chemical tests for specific glycosides: -

a. Anthraquinone glycosides: -
   i) Borntrager’s test: - Boil the test extract with 1 ml of sulphuric acid in a test tube for minutes. Filter while hot. Cool the filtrate and shake with equal volume of dichloromethane or chloroform. Separate the lower layer of dichloromethane or chloroform and shake it with half of its volume of dilute ammonia. A rose pink to red colour is produced in the ammoniacal layer.
   ii) Modified Borntrager’s test: - Boil 200 mg of the test extract with 2 ml of dilute sulphuric acid. Treat it with 2 ml of 5% aqueous ferric chloride solution (freshly prepared) for 5 minutes, shake it with equal volume of chloroform and continue the test as above. As some plants contain anthracene aglycone in a reduced form, if ferric chloride is used during the extraction, oxidation to anthraquinones takes place, which shows response to Borntrager’s test.
   iii) Hydrolyzed ether extract with methanolic magnesium acetate gives pink colour in daylight and greenish orange colour under ultraviolet (UV) light.
   iv) Test for hydroxyanthraquinones: - Treat the sample with potassium hydroxide solution red colour is produced.

b. Cardiac glycosides:-
   i) Kedde’s test: - Extract 200 mg of drug with chloroform, evaporate to dryness. Add one drop of 90% alcohol and 2 drops of 2% 3, 5-dinitro benzoic acid in 90% alcohol. Make alkaline with 20% sodium hydroxide solution, purple colour is produced. The colour reaction with 3, 5-dinitro benzoic acid depends on the presence of β- unsaturated lactones in the aglycone.
   ii) Keller-killiani test (Test for deoxy sugars): - Extract the 200 mg drug with chloroform and evaporate it to dryness. Add 0.4 ml of glacial acetic acid containing trace amount of ferric chloride. Transfer to a small test tube; add carefully 0.5 ml of concentrated sulphuric acid by the side of the test tube. Acetic acid layer shows blue colour.
   iii) Raymond’s test: - Treat the test solution with hot methanolic alkali, violet colour is produced.
   iv) Legal’s test: - Treat the test solution with pyridine and add alkaline sodium nitroprusside solution, blood red colour appears.
   v) Baljet’s test: - Treat the test solution with picric acid or sodium picrate, orange colour is formed.
c. **Coumarin glycosides:**
Place a small amount of sample in test tube and cover the test tube with a filter paper moistened with dilute sodium hydroxide solution. Place the covered test tube on water bath for several minutes. Remove the paper and expose it to ultraviolet (UV) light, the paper shows green fluorescence.

d. **Cyanogenetic glycosides:**
a) Place 200 mg of drug powder in a conical flask and moisten with few drops of water.
(There should be no free liquid at the bottom of the flask as the test will not work because the hydrogen cyanide produced will dissolve in the water rather than come off as a gas to react with the paper). Moisten a piece of picric acid paper with 5% aqueous sodium carbonate solution and suspended by means of cork in the neck of the flask. Warm gently at about 37°C. Observe the change in colour. Hydrogen cyanide is liberated from cyanogenetic glycosides by the enzyme activity and reacts with sodium picrate to form the reddish purple sodium isopurpurate.
b) Prepare solution of Guaiacum resin in absolute alcohol and allow it to dry on paper. Treat it with copper sulphate solution. The paper turns blue due to prussic acid with HCN.

e. **Saponine glycosides:**

i) Froth formation test: -Place 2ml solution of drug in water in a test tube, shake well, stable froth (foam) is formed.

ii) Haemolysis test: - Add 0.2 ml of extract to 0.2ml of blood in normal saline and mix well. Centrifuge and note the red supernatant. Compare with control tube containing 0.2 ml of blood in normal saline.

f. **Tannins (Phenolic compounds):**
a) Ferric chloride test: - Treat the extract with ferric chloride solution, blue colour appears if hydrolysable tannins are present and green colour appears if condensed tannins are present.
b) Phenazone test: - Add about 0.5 gm of sodium acid phosphate to 5ml of aqueous extract. Warm it and filter. To the filtrate add 2% phenazone solution, bulky precipitate is formed which is often coloured.
c) Gelatin test: - To the test solution add 1% gelatin solution containing 10% sodium chloride. Precipitate is formed.
G. Proteins:-

a) Biuret test: - To the test extract add Biuret reagent (2ml), violet colour indicates presence of proteins.
b) Hydrolysis test: - Hydrolyze the test extract with hydrochloric acid or sulphuric acid. Then carry out the Ninhydrine test for amino acids.
c) Test with trichloroacetic acid: - To the test extract add trichloroacetic acid, precipitate is formed.
d) Xanthoproteic test: - To the (5ml) of test solution, add 1ml of concentrated nitric acid and boil, yellow precipitate is formed. After cooling it, add 40% sodium hydroxide solution, orange colour is formed.

H. Steroids and Triterpenoids: -

a) Libermann-Burchard test: - Treat the extract with few drops of acetic anhydride, boil and cool. Then add concentrated sulphuric acid from the side of the test tube, brown ring is formed at the junction two layers and upper layer turns green which shows presence of steroids and formation of deep red colour indicates presence of triterpenoids.
b) Salkowski test: - Treat the extract with few drops of concentrated sulphuric acid red colour at lower layer indicates presence of steroids and formation of yellow coloured lower layer indicates presence of tri-terpenoids.
c) Sulfur powder test: - Add small amount of sulfur powder to the test solution, it sinks at the bottom.

I. Thin layer Chromatography:

- Sample concentration: 20 mg/ml in Methanol (Test Extracts)
- Application volume: 20 μl
- Stationary phase: Silicagel 60 F254
- Solvent systems:
  - *Eugenia Jambolana*: Toluene: Ethyl acetate (90:10)
  - *Momordica cherantia* Chloroform: Methanol (90:10)
  - *Gmelina arborea*: Chloroform: Methanol (80:20)
  - Saturated chamber for 30 minute and approx run distance 8 cm
  - Saturated chamber (30 min), aprox. run distant 8 cm
• Detection Methods:
  - U. V. Light (366nm) / Fluorescent Zones
  - Exposure to Iodine Vapour
  - For MC - On Spraying With 5% Methanolic Phosphomolybdic acid Reagent & Heating at 105°C for 10 Minutes
  - For EJ- On Spraying With Vanillin-Sulphuric acid Reagent & Heating at 105°C for 10 Minutes

4.7. Quantitative Estimation of Test Extracts:

Estimation of Total Phenolic Content (Sadashivam and Manickam, 1997):

Phenols, the aromatic compounds with hydroxyl groups are widespread in plant kingdom. They occur in plant parts. Phenols are said to offer resistance to diseases and pests in plants. Phenols include array of compounds like tannins, flavonols, etc. Total phenol estimation can be carried out with the Folin-Ciocalteau reagent.

**Principle**

Phenols react with phosphomolybdic acid in Folin-Ciocalteau reagent in alkaline medium and produce blue coloured complex (molybdenum blue).

**Materials**

- 80% Ethanol
- Folin-Ciocalteau reagent
- 20% Sodium carbonate solution
- Gallic acid (as a standard): Stock solution –1mg/ml.

**Preparation of test solution**

0.5 gm of test extract was mixed with 10-time volume of 80% ethanol and mixed. The homogenate was centrifuged at 10,000 rpm for 20min. The supernatant was saved. The residue was re-extracted with 5-times the volume of 80% ethanol, centrifuged and pooled the supernatants. The supernatant was evaporated to dryness. The residue was dissolved in 10ml of distilled water. 0.5ml of the above solution was pipetted out in a test tube.

**Procedure**

1. 0.2-1ml of the above stock solution was pipetted out in test tubes.
2. The volume was made upto 3ml with distilled water.
3. 0.5ml of Folin-Ciocalteau reagent was added to test tubes containing standard solutions and test solution.
4. After 3min, 2ml of 20% Sodium carbonate solution was added to test tubes containing standard solutions and test solution.
5. The solutions were mixed thoroughly and absorbance was measured at 765nm against a reagent blank after an hour.

**Estimation of Total Saponins:**
Take 5gm. test extract and add 25ml. 90% v/v alcohol and reflux it for half an hour. Repeat this process three times and combine all three extracts and distill off the solvent. Treat the soft extract left over after distillation of alcohol, with 25c.c. petroleum ether 60-80 by refluxing for half an hour. Cool and remove the solvent by decantation. Now treat the same soft extract similarly with 25c.c. chloroform and 25c.c. ethyl acetate and pour off the solvents after cooling. Keep the soft extract in the same flask. Dissolve the soft extract after three extractions cited above in 25c.c. methanol, filter & concentrate the methanolic extract to 5c.c. Add methanolic extract drop by drop with constant stirring to 25c.c. acetone to precipitate Glycosides. The precipitate is filter, collects and dried to a constant weight at 105 °C.

**Estimation of Total Alkaloid (Bitter):**
Reflux 3gm. Extract with 50cc of alcohol on water bath for half hour, Filter. Repeat the above process twice or till bitterness is observed in residue. Evaporate the alcohol under vacuum from the filtrate and take up residue repeatedly with 25, 15, and 15ml of hot water. Shake the above aqueous extract repeatedly with 25, 20 15 and 15 mil of ethyl acetate, collect the ethyl acetate, evaporate dry and weigh and calculate the % of Bitters.
4.8: Preclinical Safety Studies:

The acute and repeated dose toxicity screening of aqueous extract of stem bark was carried out on albino mice and wistar rats to predict safety data related use of test extracts in animal as an antidiabetic medicine. The protocols for toxicological study were approved by institutional ethical committee, which follows guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), complies with international norms of INSA. Acute Oral Toxicity was performed as per OECD Guidelines (423), with slight modification.

4.8.1 Acute Toxicity Study: *Testing of Chemicals, Section 4-Health Effect, Part 423, (OECD), 2001*

**Experimental Animals:** Female Albino mice of either sex obtained from Haffkine Institute, Mumbai were used for the acute toxicity study. Animals were maintained in the Animal House, under standard conditions (temperature 25°C±2°C, relative humidity 75%±5%, and 12-h light-dark cycle). During the experiments animals were provided with standard rodent pellet diet (Amrut feed) water *ad libitum*. The study was conducted after obtaining prior approval from the institutional Ethical Committee in accordance (IAEC) with the National Institute of Health “Guide for the Care and Use of Laboratory Animals” (NIH publication no. 86-23, 1985).

**Experimental Method:**

Fifty Female albino mice were randomly divided into ten groups as mentioned in the table-4.1, each containing five animals (Weight: 25±5 g, age: 6–8 weeks). The aqueous extract’s were administered orally at doses of 300, 2000, and 5000 mg/kg of body weight (OECD, 423). Distilled water was administered to control group. The general behaviour of the mice were continuously monitored for 1 h after dosing, periodically during the first 24 h with special attention given during the first 4 h (Hilaly et al., 2004), and daily thereafter, for a total of 14 days. Cage side functional observation batteries (FOBs) such as convulsion, vomiting, diarrhea, paralysis, breathing difficulties, bleeding, irritations, and abnormal posture, were also observed.
Changes in the normal activity of mice and their body weights were monitored and the time at which signs of toxicity or death appeared recorded. Signs of toxicity and mortality were observed daily for 14 days, with food and water intake ad libitum. During the study, food consumption was evaluated at on daily basis. Body weights of the animals were also recorded regularly. All surviving animals were euthanized with diethyl ether at day 14 and various organs like the liver, lungs, heart, spleen and kidneys were removed, weighed and carefully examined macroscopically for any abnormal, pathological signs of toxicity.

<table>
<thead>
<tr>
<th>Group No</th>
<th>Test Extract</th>
<th>Doses studies mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Aq. Extract of EJ seed + Aq. Extract of MC Fruit + Aq. Extract of GA bark (1:1:1)</td>
<td>300, 2000, 5000</td>
</tr>
<tr>
<td>B</td>
<td>Aq. Extract of EJ bark + Aq. Extract of MC Fruit + Aq. Extract of GA bark (1:1:1)</td>
<td>300, 2000, 5000</td>
</tr>
<tr>
<td>C</td>
<td>Aq. Extract of EJ seed + Aq. Extract of MC Fruit (1:1)</td>
<td>300, 2000, 5000</td>
</tr>
<tr>
<td>D</td>
<td>Normal Control</td>
<td>Dist. Water</td>
</tr>
</tbody>
</table>

4.8.2 Repeated dose toxicity:

**Animals:**

Wistar albino rats of either sex obtained from Bharat Serum, Thane were used for repeated dose toxicity study. All animals were maintained in an air-conditioned room at 25°C ± 2°C, with a relative humidity of 75% ± 5%, a 12-h light/dark cycle. A basal diet (Amrut feeds, Maharashtra, India) and tap water were provided ad libitum. Male and female rats were assigned to each dose group by stratified random sampling based on body weight. The animals were kept under laboratory conditions for an acclimatization period of 7 days before carrying out the experiments.

**Experimental Method:**

Fifty albino rats were used for study and randomly assigned into ten groups containing 5 animals in each group as mentioned in Table 4.2. Body weight (BW): 150-200 g; age: 6-8
weeks old). Treatment groups were administered with aqueous extract orally by gavage once a day for 28 days. A group of animals, serving as control, received normal saline and other groups received aqueous extract at doses of 500, 1000 and 2000 mg/kg BW respectively.

All animals were observed for morbidity and mortality, twice daily. Different Signs noted include, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (e.g. lacrimation, pupil size, unusual respiratory pattern). Changes in posture and response to handling as well as the presence of clonic or tonic movements, stereotypies (e.g. excessive grooming, repetitive circling) or bizarre behaviour (e.g. walking backwards) were also recorded. At the end of study surviving animals were fasted overnight, and anesthetized for isolation of vital organs for Histopathological observations. Blood were collected from the right ventricle for biochemical analysis. Body weight, water and food intake were measured on daily basis.

<table>
<thead>
<tr>
<th>Group No</th>
<th>Test Extract</th>
<th>Doses studies mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Aq. Extract of EJ seed + Aq. Extract of MC Fruit + Aq. Extract of GA bark (1:1:1)</td>
<td>500, 1000, 2000</td>
</tr>
<tr>
<td>C</td>
<td>Aq. Extract of EJ seed + Aq. Extract of MC Fruit (1:1)</td>
<td>500, 1000, 2000</td>
</tr>
<tr>
<td>D</td>
<td>Normal Control</td>
<td>Dist. Water</td>
</tr>
</tbody>
</table>

Hematology: Haematological analysis was performed using automatic hematological analyzed (Sysmex, Japan) at baseline and end of the study. The heparinised blood was used for a hematological estimation which included red blood cell count (RBC), hemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH),
mean corpuscular hemoglobin concentration (MCHC), platelets (PLT), white blood cell count (WBC) and white blood cell differential count.

**Biochemical parameters:** All the major biochemical parameters (like Blood glucose, creatinine (CRE), blood urea nitrogen (BUN), alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase (AST) total bilirubin (Bil), total protein (PRO), albumin (ALB)) were analyzed at the baseline and end of the study using autoanalyzer (Erba Chem 7, Germany)

**Gross Necropsy:** All animals in the study were subjected to a full, detailed gross necropsy which included careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. The liver, lungs, kidneys, adrenals, gonads, spleen, heart and brain of all animals were removed and their wet weights were taken immediately after dissection to avoid drying.

**Histopathology:** Liver, kidney, pancreas, heart, were fixed immediately in 10% formalin for routine Histopathological examination. Histopathological evaluations were performed by at Haffkine Institute.

### 4.9 Preclinical Efficacy Study:

#### 4.9.1 Antihyperglycemic Effect of Test Extracts and its Combinations in Alloxan Induced Diabetic rats

After confirming the safety of the test extracts in animal model, the antidiabetic potential of the test extracts were evaluated in Alloxan induced diabetes rat model. A study protocol for anti-hyperglycaemic activity of aqueous extracts of *Eugenia jambolana*, *Momordica charantia* and *Gmelina arboria* in alloxan induced diabetes in Wistar Rats was approved by Institutional Animal Ethics Committee. The study was planned with broad objective for elimination of less effective combination and confirm the efficacy at selected dose in animals. Considering future clinical trial work 48 rats were divided in eight different groups. The 500 mg/kg body weight dose was chosen as the dose for study on the basis earlier pilot study and literature review.

**i. Alloxan induced hyperglycemia:**

Hyperglycemia and glycosuria occur after administration of alloxan in several species (Brunschwig et al, 1943; Baily and Baily, 1943; Tasaka et al, 1988). Investigators found that
Alloxan has a selective destructive effect on the β-cells of islet of Langerhans of the pancreas. However, this effect varied with species and the dose (Richard and Robert, 1983). Alloxan (2,4,5,6-tetraoxypyrimidine; 5,6-dioxyuracil) was first described by Brugnatelli in 1818. Wohler and Liebig used the name “alloxan” and described its synthesis by uric acid oxidation. The diabetogenic properties of this drug were reported many years later by Dunn et al., (1943), who studied the effect of its administration in rabbits and reported a specific necrosis of pancreatic islets. Since then, alloxan diabetes has been commonly utilized as an animal model of insulin dependent diabetes mellitus (IDDM).

Alloxan exerts its diabetogenic action when it is administered parenterally: intravenously, intraperitoneally or subcutaneously. The dose of alloxan required for inducing diabetes depends on the animal species, route of administration and nutritional status. Human islets are considerably more resistant to alloxan than those of the rat and mouse (Eizirik et al. 1994). Fasted animals are more susceptible to alloxan (Szkudelski et al. 1998), whereas increased blood glucose provides partial protection (Bansal et al. 1980, Szkudelski et al. 1998). The mechanism of alloxan action has been intensively studied, predominantly in vitro, and is now characterized quite well. Using isolated islets and perfused rat pancreas (Kliber et al. 1996) it was demonstrated that alloxan evokes a sudden rise in insulin secretion in the presence or absence of glucose. This phenomenon appeared just after alloxan treatment and was not observed after repetitive exposure of islets to this diabetogenic agent. The sudden rise in blood insulin concentration was also observed in vivo just after alloxan injection to rats (Szkudelski et al. 1998). Alloxan-induced insulin release is, however, of short duration and is followed by complete suppression of the islet response to glucose, even when high concentrations (16.6 M) of this sugar were used (Kliber et al. 1996). Alloxan is a hydrophilic and unstable substance. Its half-life at neutral pH and 37 °C is about 1.5 min and is longer at lower temperatures (Lenzen and Munday 1991). On the other hand, when a diabetogenic dose is used, the time of alloxan decomposition is sufficient to allow it to reach the pancreas in amounts that are deleterious. The action of alloxan in the pancreas is preceded by its rapid uptake by the B cells. Rapid uptake by insulin-secreting cells has been proposed to be one of the important features determining alloxan diabetogenicity. Another aspect concerns the formation of reactive oxygen species. A similar uptake of alloxan also takes place in the liver. However, the liver and other tissues are more resistant to reactive oxygen species in comparison to pancreatic B cells and this resistance protects them against alloxan toxicity (Malaisse et al. 1982, Tiedge et al. 1997). The formation of reactive oxygen species is
preceded by alloxan reduction. In B cells of the pancreas its reduction occurs in the presence of different reducing agents. Since alloxan exhibits a high affinity to the SH-containing cellular compounds, reduced glutathione (GSH), cysteine and protein-bound sulphydryl groups (including SH containing enzymes) are very susceptible to its action (Lenzen and Munday 1991). However, other reducing agents such as ascorbate may also participate in this reduction (Zhang et al. 1992). Lenzen et al. (1988) proposed that one of the SH-containing compounds essential for proper glucose-induced insulin secretion is glucokinase, being very vulnerable to alloxan. Alloxan reacts with two -SH groups in the sugar binding side of glucokinase resulting in the formation of the disulfide bond and inactivation of the enzyme.

**ii. Experimental Animals:**
Healthy Wistar rats (body weight (150-200gm) procured from Bharat Serum, Thane were used for this study. The animals were fed on a pellet diet (Amrut feed, India). Rats were devoid of any disease, at difference stages during the time of the experiment.

**iii. Experimental Induction of Diabetes in Rats**
Diabetes was induced in overnight fasted rats of Wistar strain by a single i.p. injection of alloxan, at a dose of 90 mg/kg body weight (in normal saline). The dose of alloxan was 90 mg/kg-body weight. The same volume of acetate buffer was given to each control rat. Animals were provided with 10% glucose solution after injection of alloxan. This decreased the mortality of the animals. Control was provided with normal tap water. Animals with blood glucose level more than 250 mg/dl were selected and used for further study.

**IV. Experimental Design:**
Five groups of Alloxan inducted diabetic rats (n = 6) were administered orally with aqueous extract (500 mg/kg BW) and a standard drug i.e glibenclamide (600 µg/kg BW) was administered to group F. Two groups of rats (n = 6) served as normal and diabetic controls. The treatment duration for all the groups was 28 days. The different test groups were number as A to H. The details of the combination and doses are mentioned in table 4.3. Plasma blood glucose levels and body weight (BW) were determined at 0 (D 0), day 7 (D7), day 14 (D14), day 21 (D21)) and day 28 (D27) after administration of test samples. Also to assess acute effect, the plasma glucose levels were determined at 30, 60, 120, 240 and 360 min after the administration of the test samples. On all the days the effect food and water intake of each group was monitored. Blood samples were collected from the retro-orbital plexus of the rats.
under light ether anesthesia. Plasma was separated by centrifugation (5 min, 5000 rpm). Plasma was stored at 20 °C until assayed. The plasma was used for the estimation of glucose, albumin, total proteins, blood urea nitrogen (BUN) creatinine, cholesterol, HDL, triglycerides, AST, ALT, ALP. All the analysis were done by using commercially available diagnostic kits (Erba diagnostics, Germany).

**Statistical Analysis:**

All data were expressed as mean ± S.E.M. The effects of test extracts on BW, feed intake, liquid intake and blood glucose levels were determined using one-way analysis of variance test. P values less than 0.05 were considered significant. The differences among experimental and control groups were determined using the statistical program prism 4.0. Comparisons among different groups were performed by analysis of variance using ANOVA test. Significant difference between control and experimental groups were assessed by student’s t-test. All data are expressed as mean ± standard error of mean (S.E.M.); P-values less than 0.05 was considered to be significant.

<table>
<thead>
<tr>
<th>Table- 4.3: Animal Groups and doses of test extract for Antihyperglycemic effect of test extract study.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group</strong></td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>D</td>
</tr>
<tr>
<td>E</td>
</tr>
<tr>
<td>F</td>
</tr>
<tr>
<td>G</td>
</tr>
<tr>
<td>H</td>
</tr>
</tbody>
</table>
4.8.2: Study of Insulin Potentiating Effect of Test Extract in Alloxan induced Diabetic Rats:

After confirming the efficacy of test extract in Alloxan diabetic rat model the test extracts were assed for insulin potentiating effect. The study was planned considering that in clinical trial we may get few patients who are taking insulin for the treatment of diabetes and the results of this study will help to justify the protocol for clinical trial with same intention. The objective of the study was to evaluate insulin potentiating effect of aqueous extract of *Momordica charantia*, *Eugenia jambolana* and *Gmelina arboria* in alloxan induced hyperglycaemic rats at a dose of 500 mg/kg body weight.

Diabetes was induced in overnight fasted rats of Wistar strain by a single i.p. injection of alloxan, at a dose of 95 mg/kg body weight (in normal saline). Each animal with a plasma glucose concentration level above 250 mg/dl was considered to be diabetic. The combinations of three and two extracts were selected for analysis. After confirming stable hyperglycemia on day 4, 0.5 IU of insulin was given intravenously and blood sugar were determined at 30, 60, 90, 120, 180 and 240 minutes. The same procedure was repeated after test extract administration at a dose of 500 mg/kg body weight.

The 24 rats were divided in following four groups containing 6 rats in each group.

- Group 1: Insulin + (EJ+MC+GA) 1:1:1 orally
- Group 2: Insulin + (EJ+MC) 1:1 orally
- Group 3: Insulin + vehicle (orally)
- Group 4: Vehicle Control

The SEM of obtained bold glucose readings at different time point were compared for analyzing reduction in blood glucose level between different groups.
4.10 Formulation Used For Clinical Studies:

The effective dose from preclinical study was 500 mg/kg and the calculation for the HED (Human Effective Dose) was made using Guidance for Industry and Reviewers Estimating the Safe Starting Dose in Clinical Trials, U.S. Department of Health and Human Services Food and Drug Administration 2002. After calculation the effective dose for human use was coming to more than 3000 mg per day and however this dose is not practically possible for human use considering the patient compliance. The literature search done for available herbal formulations in the market suggested the competitive dose of 500 mg/day two times a day (BID). So the 500 mg/day BID was considered as final does for all clinical trials in present work.

Considering the suitability and compatibility capsule was selected as dosage form for clinical studies. The dry powdered extract of EJ and MC were taken for future clinical trials. The capsule feeling capsule filling and packaging of extracts were done at GMP premises of Manbro Pharmaceuticals Ltd. Mumbai.

Upon performing pilot formulation studies the size 0 capsules were selected for final formulation. The empty capsules were obtained from Associate Capsule by Manbro Pharmaceuticals and the same capsules were used filling extract and placebo on hand filling capsule machine. The fill weight of capsules were 500 mg. The extracts of EJ and MC were filled and packed separately. The dried extracts were filled individually in suitable capsule size. The fill weight of extracts of EJ and MC were 500 mg per capsule. The filled capsules were subjected to in-process quality checks. The capsules were filled in HDPE container in controlled humidity and temperature. Similarly placebo capsule were filled with Maize Starch and packed. The filled capsules were assessed for all the in-house quality control tests like appearance, weight variation, defect of capsule shell, disintegration time as per Manbro Pharmaceuticals SOP.

Four thousand each of aqueous extract and placebo capsules were filled and packed in air tight container. The batch numbers assigned were as below:

I. Aq. Extract of Momordica Charantia Fruit:

**BATCH:** KARELA/PC1/JUN09

Mfg Date: June 2009
Exp Date: May 2011
Quantity: 4000 Capsules (500 mg each)

II. Aq. Extract of Eugenia jambolana Seeds:

**BATCH:** JAMB/PC1/JUN09
- Mfg Date: June 2009
- Exp Date: May 2011
- Quantity: 4000 Capsules (500 mg each)

III. Placebo:

**BATCH:** PLACEBO/MB/DEC09
- Mfg Date: June 2009
- Exp Date: May 2011
- Quantity: 2000 Capsules (500 mg each)

Label used on Container:

<table>
<thead>
<tr>
<th>Protocol: CT/EJMC/01</th>
</tr>
</thead>
<tbody>
<tr>
<td>For Clinical Trial Use Only</td>
</tr>
<tr>
<td>Batch #: JAMB/PC1/JUN 09, KARELA/PC1/JUN09, PLACEBO/MB/DEC/09</td>
</tr>
<tr>
<td>Mfg. Date: June 2009</td>
</tr>
<tr>
<td>Randomization Number: _ _</td>
</tr>
<tr>
<td>Dispensing Date: ___ /<em><strong>/</strong></em></td>
</tr>
<tr>
<td>Investigator Name: Dr.</td>
</tr>
<tr>
<td>Contact Number:</td>
</tr>
<tr>
<td>Storage: Store at Room temperature:</td>
</tr>
<tr>
<td>Direction for use: Refer patient instruction</td>
</tr>
</tbody>
</table>
4.11 CLINICAL STUDIES:

4.11.1 Human Exploratory study in patient with NIDDM and already on Oral Hypoglycaemic Agents: **CTRI Reg #: CTRI/2010/091/000424:**

After confirming safety and efficacy of individual and combined extracts in preclinical models the clinical study was carried out in the patients with NIDDM and already receiving treatment with OHAs. The study protocol and all other documents were approved by the Independent Ethics Committee before study initiation. After getting EC approval the study was registered in Clinical Trial Registry of India (CTRI/2010/091/000424). The study was published on CTRI website http://ctri.nic.in before enrolment of first patient. The study protocol and all the relevant documents were approved by Independent Hunan Research Ethics Committee (IHREC). The IHREC was working as per Good Clinical Practice Guidelines issued by Central Drug Standard Control Organization and Ethical Guidelines for Biomedical Research on Human Subjects, issues by Indian Council of Medical research (ICMR). The EC approval and composition are attached as appendix II.

<table>
<thead>
<tr>
<th>Protocol No.</th>
<th>CT/EJMC/001</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protocol title:</td>
<td>A multicentric prospective randomized, single blind study, to evaluate the efficacy and safety of <em>Eugenia jambolana</em> and <em>Momordica charantia</em> following multiple dose administration in subjects diagnosed with NIDDM</td>
</tr>
<tr>
<td>Type of trial:</td>
<td>Prospective, Randomized, Single blind</td>
</tr>
</tbody>
</table>
| Investigational Product: | Group A: Metformin + Aqueous Extract of *Eugenia jambolana* (seed) and *Momordica charantia* (Fruit)  
Group B: Metformin+ Placebo 500 mg |
| Doses to be studied: | Aq. Extract of *Eugenia Jambolana*: 500 mg/day  
Aq. Extract *Momordica charantia*: 500 mg/day |
| Dosage forms: | Capsule |
| Route: | Oral |
Primary objective: To evaluate the clinical efficacy of EJ and MC aqueous extract’s as adjunct with other OHAs in the management of NIDDM.

Secondary objective: To evaluate the safety of EJ and MC extract as adjunct with other OHAs in the management of NIDDM.

Total sample size: 30 patients

Trial population (summary): Subjects diagnosed NIDDM at least 6 month prior to screening

Dosing regimen: 2 capsule of 500 mg (one of EJ and one of MC) / placebo

Safety parameters: Number of Adverse Events
Patient compliance to therapy

Efficacy parameters: Change in FBS Level
Change in PPBS Level
Changes in symptoms related to disease

Treatment Duration 90 days + follow-up visit at 150 day

4.11.1.1 Study Design:

The present study is a multicentric, prospective, randomized, single blind study. Is was planned to enroll thirty eligible subjects randomly dividing in to two groups First group subject will receive the test drug in addition to their routine Antidiabetic medicines and second group will receive the placebo in addition to their routine Antidiabetic medicines. There will be three month treatment period and 2 month follow-up period
4.11.1.2 Trial Methodology:

a. Patient Population:

Thirty patients already diagnosed with NIDDM consuming OHAs were randomized from three sites during the course of the study. The selected patients were having NIDDM at least six month prior to study enrollment and taking OHAs like Metformin and/or one of the sulphonylurea. Sulphonylurea were stopped from the time randomization for those patients who were taking sulphonylurea along with the Metformin. The rescreening patients after were screened for study after obtaining voluntary written informed consent. All the patients were informed about plan and purpose of the study. The attempt was made to select mild and moderate diabetic patients and severe diabetic patients were excluded from the study.
Table 4.4: Site wise patient distribution

<table>
<thead>
<tr>
<th>Site Number</th>
<th>Number of Patients Screened</th>
<th>Number of patient Randomized</th>
<th>Number of patient discontinued</th>
<th>Number of Patients Completed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22</td>
<td>13</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>17</td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>9</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td>32*</td>
<td>2</td>
<td>30</td>
</tr>
</tbody>
</table>

* As per protocol ver. 1.0 it was planned to randomize 30 patients but 32 patients were randomized due to discontinuation of two patients. Two patients were early terminated due to protocol noncompliance at day 15 visit

b. Subject Eligibility:

Inclusion criteria:

Male and female subjects who are diagnosed with Diabetes at least prior to six month from screening and are already on the treatment of OHAs and meet all of the following criteria were randomized into the study:

1. Patients who are willing to give signed informed consent.
2. Male or female subjects at least 18 years of age and no more than 70 years of age
3. Women of childbearing potential, or men of reproductive potential, must be using adequate (in the investigator’s opinion) birth control measures (e.g. abstinence, oral contraceptives, intrauterine device, barrier method with spermicide, or surgical sterilisation) during the study. Female subjects of childbearing potential must test negative for pregnancy prior to enrolling in the study. Post-menopausal (cessation of menses for more than 2 years) women are eligible for this study.
4. Patient having fasting plasma glucose level above 110 mg/dl and/or postprandial plasma glucose level in between 140 to 300 mg/dl
5. Patient known to have diabetes at least 6 month prior to screening
Exclusion criteria

Subjects who meet any of the following criteria were excluded from this study:

1. Body weight >98 kg.
2. Pregnant, nursing, or planning a pregnancy (both men and women) within 9 months of enrolment.
3. Screening laboratory tests:
   - Haemoglobin ≤ 8.0 gm/dl
   - White blood cells ≤ 3.0 x103 cells/µl
   - Neutrophils ≤ 1.5 x 103 cells/µl
   - Platelets ≤100 x 103 cells/µl
   - Serum transaminase level (AST and ALT) ≥ 2 times upper limit of normal (ULN)
   - Serum creatinine ≥ 0.15 mmol/l
4. Subjects with a history of any clinically significant adverse reaction to study medication, including serious allergic reactions.
5. Subjects with a history of, presence of, or at high risk of serious infection including:
   - History of active TB, or positive contact history with a subject with active TB within the past 3 months.
   - A serious infection during the 3 months prior study entry (hospitalised or received IV antibiotics for an infection).
   - Chronic or recurrent infectious disease.
   - Systemic fungal infections
   - Subjects known, or suspected, to be infected with HIV, hepatitis B, or hepatitis C.
6. Subjects with evidence of severe, progressive, or uncontrolled renal, hepatic, haematological, gastrointestinal, endocrine, pulmonary, cardiac, neurologic, psychiatric, or cerebral disease.
7. Concurrent CHF, including medically controlled, asymptomatic CHF or ECG findings suggestive of CHF.
8. Subjects previously enrolled in this study, currently participating in another investigational study or treated with any investigational drug within the previous 3 months.
9. Any other clinically significant disease or disorder or factors such as substance abuse which in the opinion of the investigator make the subject ineligible for participation in this study.

10. Patients with concomitant severe illness necessitating other medications, patients with severe hypertension, history of severe unstable angina, myocardial infarction, CVAs, renal failure, and those patients
c. Study Flowchart:

Figure 4.2: Study Flowchart CT/EJMC/01
4.11.1.3: Study Specific Procedures:

Informed Consent Procedure:

All patients considered eligible were invited to enroll in the study. Voluntary written informed consent was obtained from each patient before any study-related activity is performed that alters the management of the patient or may affect the individual patient in any other way. The investigator or co-investigator had explained the nature, purpose, and risks of the study to the patient. The patient was given sufficient time to consider the implications prior to deciding whether to participate. Informed consent was recorded by signing the consent form by investigator and patient and a copy was given to the patient. A copy of English informed consent is attached as appendix.

Demography:

Demographic details like date of birth, sex, race, height and weight were recorded at screening visits for all the subjects.

Medical/Surgical history:

Diabetes related medical/surgical history and the subject’s other medical/surgical history were recorded at screening from all the subjects.

Vital signs:

Vital signs were recorded at each visit by the investigator which include body temperature, pulse and systolic and diastolic blood pressure and body weight. Wherever possible, vital signs were recorded in the seated position after the subject has rested for at least five minutes.

Physical examination:

The general physical examination of the subject was performed at all visits according to commonly accepted medical standards and the results recorded on the source documents. The physical examination include:

- General appearance
- Head, including detailed examination of the face, scalp, eyes and ears
- Nose, throat, neck
- Respiratory system
- Central and peripheral nervous system
- Cardiovascular system
- Gastrointestinal system, including mouth
- Musculoskeletal system
- Skin
- Lymph node palpation (head, neck, axillary)

**ECG recording:**

A 12 lead ECG was performed for all the subject in the supine position at the screening assessment and at day 90/Early Termination visits.

**Laboratory Tests:**

Haematology, clinical chemistry and urinalysis were performed at screening and day 90 visit for all the randomized subjects at local lab designated at each site. The abnormal lab parameters were evaluated for their clinical significance by the respective clinical investigator.

**Pregnancy Test**

For female subjects of child bearing potential serum pregnancy test were performed at screening and at day 90 visit

4.11.1.4 **Allocation of Treatment and Randomization**

Subjects who satisfy the inclusion and exclusion criteria were randomized for investigational product. The patients were randomized on the basis of computer generated master randomization list and the patient was blinded for the study medication. All the subjects were randomly assigned to either group Test Extract (A) or Placebo (B).
4.11.5 Dietary Restrictions

There were no restrictions on non-alcoholic fluid or food intake during the study and all the subjects were requested to continue their routine diet plan. All the subject were advised for standard exercise in the morning.

4.11.1.6 Study Treatment Discontinuation Criteria:

Early Study Treatment Discontinuation

All subjects were assessed for baseline investigations. After obtaining baseline investigations and assigning treatment, the patients were enrolled in the study. Patients were requested to come for follow up as per protocol. After 10 days of proposed visit of default the patient was considered as dropout.

Permanent suspension of study treatment was considered in the following cases:

• Withdrawal of consent (voluntary withdrawal)

• Development of an adverse event or medical condition that in the opinion of the principal investigator necessitates permanent suspension of study treatment

4.11.1.7 Concomitant Treatment:

No concomitant Antidiabetic treatment was advised apart from Metformin. Other medications for other indication were allowed as per the discretion of the physician. Similarly regular exercise were advised

4.11.1.8 Investigational Product:

Identity and Formulation of Investigational Products

Investigational Product supplied in capsule formulation. Each capsule contains 500 mg of each extract. The Investigational Product supplied in capsule formulation. Each capsule contains 500 mg of each extract or placebo. One container was containing 30 capsules each of 500 mg.

Study drug dispensing:

At each visit, drugs were dispensed to patient to suffice for at least next 30 days.
Drug accountability procedure:

The drug accountability records were maintained in the IP dispensing and accountability logs. All the used and unused IP were accounted at the end of study.

4.11.1.9 Adverse Events:

An Adverse Event (AE) is defined as any untoward medical occurrence in a clinical investigation when a subject is administered a drug and does not necessarily have a causal relationship with this treatment. An AE can therefore, be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of an investigational product, whether or not related to the investigational product.

All adverse events reported or observed by patients were recorded with information about the severity, date of onset, duration and action taken regarding the study drug. Relation of adverse events to study medication was predefined as “Unrelated” (a reaction that does not follow a reasonable temporal sequence from the time of administration of the drug), “Possible” (follows a known response pattern to the suspected drug, but could have been produced by the patient’s clinical state or other modes of therapy administered to the patient), and “Probable” (follows a known response pattern to the suspected drug that could not be reasonably explained by the known characteristics of the patient’s clinical state).

Patients were allowed to voluntarily withdraw from the study, if they had experienced serious discomfort during the study or sustained serious clinical events requiring specific treatment. For patients withdrawing from the study, efforts were made to ascertain the reason for dropout. Non-compliance (defined as failure to take less than 80% of the medication) was not regarded as treatment failure, and reasons for non-compliance were noted.

A serious adverse event (SAE) is defined as “any experience that is fatal or life threatening, requires or prolongs inpatient hospitalization, results in persistent or significant disability/incapacity, or is a congenital anomaly or birth defect
4.11.2 Human Exploratory study in patient with newly diagnosed NIDDM:
CT/EJMC/002: CTRI Reg #CTRI/2010/091/001158:

After confirming safety and efficacy of test extracts in diabetic patients already on OHAs one more clinical study was carried on newly detected diabetic patients who have not received any treatment. Study protocol and all other documents were approved by the Independent Ethics Committee before study initiation. The study protocol and all the relevant documents were approved by Independent Hunan Research Ethics Committee (IHREC). The IHREC was working as per Good Clinical Practice Guidelines issued by Central Drug Standard Control Organization and Ethical Guidelines for Biomedical Research on Human Subjects, issues by Indian Council of Medical research (ICMR). The EC approval and composition are attached as appendix II. Also the study was registered on CTRI (CTRI/2010/091/001158). The patients diagnosed with diabetes within 6 month from screening and not on any OHAs were considered for this study provided they comply with all other requirement of protocol.

The brief details about the study design are as below:

<table>
<thead>
<tr>
<th>Protocol No/ID</th>
<th>CT/EJMC/002</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial title:</td>
<td>A randomized, single blind, study to investigate the efficacy and safety of <em>Eugenia Jambolana</em> and <em>Momordica Charantia</em> following multiple dose administration in subjects newly diagnosed with NIDDM</td>
</tr>
<tr>
<td>Type of trial:</td>
<td>Prospective, Randomized, Single blind,</td>
</tr>
<tr>
<td>Investigational Product:</td>
<td>Aqueous Extract of <em>Eugenia jambolana</em> (seed) and <em>Momordica charantia</em> (Fruit) and matching placebo</td>
</tr>
</tbody>
</table>
| Doses to be studied: | *Eugenia Jambolana*: 500 mg  
*Momordica charantia*: 500 mg |
| Dosage forms: | Capsule |
| Route: | Oral |
Trial centres: 2

Primary objective: To evaluate the clinical efficacy of EJ and MC extract as monotherapy in the management of NIDDM.

Secondary objective: To evaluate the safety of EJ and MC extract in the management of NIDDM.

Total sample size: 20 patients

Trial population: Subjects newly diagnosed with NIDDM according to WHO diabetes diagnosis criteria 1999

Dosing regimen: 2 capsule of 500 mg (one of EJ and one of MC) or 2 capsules of placebo/day

Safety parameters: Number of Adverse Events
Patient compliance to therapy

Efficacy parameters: Change in FBSL
Change in PPBSL
changes in the disease symptom score

4.11.2.1 Study Design:

The present study was a multicentric, prospective, randomized, single blind study. It was planned to enroll twenty eligible subjects randomly dividing in to two groups First group subject received the test drug and second group will receive the placebo. Due to use of placebo arm and recommendation from Ethics committee the study was limited to patients with mild diabetics only. The treatment duration was of three month and the follow-up period was for two month. However during follow-up period patients were allowed to take standard OHAs.
4.11.2.2 Subject Eligibility Criteria:

Adult patients with a confirmed diagnosis of NIDDM as per WHO diagnosis criteria. It is anticipated that up to 50 subjects will be screened to randomise 20 subjects.

**Inclusion criteria:**

Male and female subjects who meet all of the following criteria can be entered into the study:

1. Patients who are willing to give signed informed consent.
2. Male or female subjects at least 18 years of age and no more than 70 years of age
3. Women of childbearing potential, or men of reproductive potential, must be using adequate (in the investigator’s opinion) birth control measures (e.g. abstinence, oral contraceptives, intrauterine device, barrier method with spermicide, or surgical sterilisation) during the study. Female subjects of childbearing potential must test negative for pregnancy prior to enrolling in the study. Post-menopausal (cessation of menses for more than 2 years) women are eligible for this study.
4. Diagnosis of Diabetes as per WHO diagnosis criteria 1999 ((For newly diagnosed patients: FBSL >110 mg % and after 2 hours of consuming 75 grams of glucose: >180 mg %).

5. Patient having Fasting Blood Glucose level above 110 mg/dl and/or postprandial plasma glucose level in between 140 to 250 mg/dl

**Exclusion criteria**

Subjects who meet any of the following criteria will be excluded from this study:

1. Body weight >98 kg.

2. Pregnant, nursing, or planning a pregnancy (both men and women) within 9 months of enrolment.

3. Patient already on OHAs

4. Screening laboratory tests:
   a. haemoglobin ≤ 8.0 gm/dl
   b. white blood cells ≤ 3.0 x103 cells/µl
   c. neutrophils ≤ 1.5 x 103 cells/µl
   d. platelets ≤100 x 103 cells/µl
   e. serum transaminase level (AST and ALT) 2 times upper limit of normal (ULN)
   f. serum creatinine ≥ 0.15 mmol/l

5. Subjects with a history of any clinically significant adverse reaction to study medication, including serious allergic reactions.

6. Subjects with a history of, presence of, or at high risk of serious infection including:
   - history of active TB, or positive Mantoux test or QuantiFERON Gold test or chest x-ray suggestive of active or healed TB or positive contact history with a subject with active TB within the past 3 months. If patients have a positive Mantoux test but a negative QuantiFERON Gold test, they may be enrolled.
   - a serious infection during the 3 months prior study entry (hospitalised or received IV antibiotics for an infection).
   - chronic or recurrent infectious disease.
   - systemic fungal infections
• opportunistic infection within 3 months prior to screening (refer to 1993 CDC Classification System for HIV Infection).
• subjects known, or suspected, to be infected with HIV, hepatitis B, or hepatitis C.

7. Concurrent CHF, including medically controlled, asymptomatic CHF or ECG findings suggestive of CHF.

8. Subjects receiving cytotoxic drugs including cyclophosphamide, cyclosporine, or alkylating agents within 6 months prior to first study dose.

9. Subjects previously enrolled in this study, currently participating in another investigational study or treated with any investigational drug within the previous 3 months or within 5 half-lives, whichever is greater, prior to first study dose.

10. Any other clinically significant disease or disorder or factors such as substance abuse which in the opinion of the investigator make the subject ineligible for participation in this study.

4.11.2.2. Study Visit Procedures:

I. Screening Visit (Day -10 to Day -1):

• Informed consent
• Urine pregnancy test (female subjects with child bearing potential only)
• Assessment of compliance with the inclusion and exclusion criteria
• Collect demographic information
• Obtain medical, surgical, smoking, alcohol and Diabetes history
• Evaluation of Symptoms of NIDDM
• Record subject diet and exercise habits
• Record all concomitant medications
• Height, Weight & Vital signs
• Physical examination
• ECG
• Laboratory tests for hematology, Biochemistry, Lipid profile, urine analysis
• Blood Glucose i.e Fasting and Postprandial

The following procedures will be conducted for each subject successfully screened.

• Randomize the subject as per randomization envelop and record the allocated treatment
• Inform the subject of the date of the Baseline (Day 0) visit

II. Baseline (Day 0):

Review inclusion/exclusion criteria to confirm subject eligibility

• Randomize subject
• Record vital signs
• Perform brief physical examination
• Record adverse events
• Record all concomitant medications used since screening
• Study drug dispensing
• Complete drug dispensing and accountability records
• Remind subject about their next study visit
• Blood Glucose i.e Fasting and Postprandial
• Record subject diet and exercise habits

III. Day 15 (+/- 2 days), 30(+/- 2 days) and 60 (+/- 2 days)

• Blood sample collection for lab test (Blood Glucose i.e Fasting and Postprandial)
• Record vitals
• Weight
• Evaluation of change symptoms of NIDDM
• Record concomitant medication
• Record change in diet and exercise habit
• Physical examination
• Study drug dispensing
• Complete drug dispensing and accountability records
• Adverse event recording
• Remind subject about their next visit

IV. Day 90 (+/- 2 days)/Early Termination

• Evaluation of Symptoms of NIDDM
• Measure body weight and calculate and record BMI
• Record subject diet and exercise habits
• Record concomitant medications.
• Record change in diet and exercise habit
• Weight & Vital signs
• Physical examination
• Urine pregnancy test (female subjects with child bearing potential only)
• Physical examination
• Laboratory tests for hematology, Biochemistry, Lipid profile, urine analysis
• Blood Glucose i.e Fasting and Postprandial
• Adverse event recording
• Remind subject about their next visit

V. Day 150 (+/- 2 days) Follow up

• Blood Glucose i.e Fasting and Postprandial
• Adverse event recording
• Evaluation of symptoms of NIDDM

4.11.2.3 Study Specific Procedures:

Informed Consent Procedure:

The Investigator explained each subject the nature of the treatment, its purpose, procedures, expected duration and the potential risks and benefits involved along. Additionally, the subjects were provided with a printed information sheet containing more detailed information and ample time was given to read and understand the document and to ask any questions they may have. The written informed consent were administered before performing any study related procedures.

Demography:

Demographic information of all the study subjects were captured in source in CRF which includes recording the date of birth, sex, race, height, and weight of the subject and calculation of Body Mass Index (BMI).
Medical/Surgical history:
A complete medical/surgical history were taken at the screening visit, and recorded in the source documents with particular emphasis on other disorders of relevance, recent and current medications, and allergies. Separate sections on the CRF are provided to record the diabetes related medical/surgical history and the subject’s other medical/surgical history.

Vital signs:
Vital signs include tympanic body temperature, pulse and systolic and diastolic blood pressure and body weight. Wherever possible, vital signs shall be recorded in the seated position after the subject has rested for at least five minutes. More frequent monitoring of vital signs may be implemented at the discretion of the Investigator.

Physical examination:
The general physical examination of the subject will be performed according to commonly accepted medical standards and the results recorded on the source documents. The physical examination will include:

- General appearance
- Head, including detailed examination of the face, scalp, eyes and ears
- Nose, throat, neck
- Respiratory system
- Central and peripheral nervous system
- Cardiovascular system
- Gastrointestinal system, including mouth
- Musculoskeletal system
- Skin
- Lymph node palpation (head, neck, axillary, inguinal)

Laboratory Tests:
Haematology, clinical chemistry and urinalysis will be performed as shown in the Study Schedule of Assessments. If a screening test result is abnormal and clinically significant, the subjects were excluded from the study.
**Pregnancy Test:**
For female subjects of child bearing potential only serum pregnancy test will be performed at screening and at day 90 visit

**Allocation of Treatment and Randomisation**
Subjects who satisfying the inclusion and exclusion criteria were allocated to treatment prior to the Baseline (Day 0) visit. Subjects were randomly assigned to either test extracts or placebo.

**Dietary Restrictions:**
There were no restrictions on non-alcoholic fluid or food intake during the study. Subjects were instructed to required to limit their alcohol intake to no more than the equivalent of 2 standard drinks per day for males and 1 standard drink per day for females (1 standard drink is defined as 10 grams of absolute alcohol or ethanol e.g. 330 ml beer, 100 ml table wine, 30 ml straight spirits equal 10 grams of absolute alcohol). In addition subjects will be requested to abstain from alcohol for the twelve (12) hours immediately prior to each safety blood draw

**4.11.2.4 Efficacy and Safety Parameters:**

**Efficacy parameters**
Efficacy parameters used in this study include

1. Change Fasting blood glucose level
2. Change in Postprandial Blood Glucose level
3. Change Weight
4. Symptomatic improvement in NIDDM: Polyuria, weakness/tiredness, Fatigue, Loss of appetite, Burning sensation in heart and s, thirst, dry mouth, blurring of vision

**Safety Parameters**
Safety parameters measured include treatment-emergent AEs (defined as those beginning on or after administration of Investigational Product or pre-existing conditions that worsened on or after Investigational Product administration), laboratory values and vital signs.
4.11.2.5 Investigation Product:

Investigational Product supplied in capsule formulation. Each capsule contains 500 mg of each extract/placebo. The detailed IP accountability records maintained by means of dispensing and inventory log. The logs were updated on ongoing basis.

4.11.2.5 Statistical Considerations:

The sample size of 20 subjects was considered for this study. The data entered in CRF were analyzed by using descriptive statistics and blood sugar level at screening and different visits were compared by one way ANOVA using t test.