MATERIALS AND METHODS
Chapter – I

In Vitro Study Design
III. MATERIALS AND METHODS

The details of materials (sample selected, enzymes, standard kits, special chemicals) and the protocols adopted to carry out the research work are provided in this section.

I. In Vitro Study Design

a. Design Plant Material

*Ziziphus jujuba Mill.* fruits were purchased from a local grocery store in Birjand city located on the northeastern of Iran, which is one of the most famous city to cultivate Iranian Jujuba fruits.

b. Chemical/Enzymes

- Butylated hydroxyl toulene (BHT) was procured from Qualigens Fine Chemicals, Mumbai, India.
- Pepsin, Pancreatin, α – amylase (porcine origin), 2, 2-Diphenyl-1-picrylhydrazl (DPPH) and Thiobarbituric acid (TBA) were procured from Himedia laboratories Pvt. Ltd, Vadhani Ind. Est., LBS marg, Mumbai, India.
- Ascorbic acid and 5, 5’-Dithio (bis) nitro benzoic acid (DTNB) were procured from sigma chemicals Bangalore, India.
- Tris (hydroxymethyl) amionomethane was procured from SD Fine Chemicals, Mumbai, India.
- Total cholesterol, Triglycerides, Glucose, urea, Uric acid and Creatinine diagnostic kits were procured from Synergy Bio Company, Chennai, India.
- LDL cholesterol, HDL cholesterol, ALT, AST, Total protein and HbA1C diagnostic kits were procured from Erba Mannheim Company, Mumbai, India.
- Reduced glutathione (GSH) was procured from Sisco Research Lab. Pvt. Ltd. Mumbai, India.

All chemicals, reagents and solvents used in the present study were of analytical grade and obtained from reputed companies.
Processing of *Ziziphus jujuba* Mill.

3.1 Milling

Fruits were washed and separated into peels, pulps and seeds, and then the pulp and peel cut into slices. The small cut slices and the seeds were dried in a hot air oven (\(\leq 50^\circ\text{C}\)) for 48 h. The dried samples were ground to a fine powder. The peel and seed powder were sieved through a 60-mesh and all three samples stored in an airtight container at 4\(^\circ\text{C}\) until used.

3.2 Preparation of extracts and capsule contents

3.2.1 Methanolic and aqueous extracts

Plant materials were extracted with methanol (MtOH), cold (CA) and hot (HA) aqueous separately. To each of the powdered samples (20g), 100 ml of the solvents (Methanol or distilled water) were added and allowed to shake for 6 h on a mechanical shaker. For hot water extraction, the samples were extracted using hot water (70\(^\circ\text{C}\)) on a hot plate with occasional shaking for 45 minutes. Each of these preparations was centrifuged for 10 minutes at 3000 rpm then the slurry was collected and evaporated to dryness in a steady air current in a previously weighed crucible.

3.2.2 Pulp and Seed combination (PS)

To prepare a combination of pulp and seed (PS), the washed semi fresh fruits were soaked in water for 18 hours followed by heating for 45 minutes at <90\(^\circ\text{C}\). The pulp was passed through a 60-mesh sieve and the filtrate was concentrated on the hot water bath until it reduced to around 33\% moisture. Finally, the concentrated pulp extract and seed powder (56\% and 44\% respectively) was mixed well and dried in an oven (<30\(^\circ\text{C}\)). This standardized extract was made into 500 mg capsules which were used for the clinical trial study and stored in an airtight container at 4\(^\circ\text{C}\) until used (Figure 9).

3.2.3 Whole fruit powder

Whole semi dried *Ziziphus jujuba* Mill. were supplied in bulk and after washing and drying the entire parts of jujuba fruits including peel, pulp and seed were ground into a fine powder. Capsules were prepared from the powder at a local pharmacy (Figure 10).
c. The processed samples were analysed for the following:

I. Physicochemical parameters

i. Proximate composition
ii. Minerals
iii. Phytochemical screening
iv. Antinutrients
v. Antioxidant components
vi. Antioxidant activity
vii. Fiber

d. The procedures followed for analysing the mentioned parameters are as follows:

I. Physicochemical parameters

1. **Proximate composition**: Standard methods of AOAC [326] were used to determine Moisture (AOAC – 925.10), fat (AOAC – 2003.05) by soxhlet extraction and ash (AOAC – 923.03) by combustion. Protein (AOAC – 960.52) content (N × 6.25) was determined by micro Kjeldahl method.

2. **Mineral Content**: Mineral analysis was carried out on samples digested with hydrochloric acid. Total iron (AOAC – 944.02) was analysed by colorimetric method using αα bipyridyyl method [326]. Total phosphorus was analysed colorimetrically using Taussky & Shorr method [327]. Total calcium, magnesium, manganese, potassium, sodium, zinc and copper was analysed by flame atomic absorption spectroscopy (Spectr AA 400; Varian, Mulgrave, Australia).

3. **Fiber**: Total dietary fiber (TDF) was measured as the sum of water-insoluble and water-soluble fractions, based on digestion of sample (1g) with enzymes, as described by Asp et al [328].

4. **Phytochemical screening**: The powder of samples was subjected to a thorough phytochemical screening to detect the presence of the following secondary metabolites: alkaloids, tannins, phenols, saponins, flavonoids, reducing sugar, triterpenoids and steroids (Figure 7).
Sequential extraction of phytochemicals

- 10 g of powdered sample
- About 150 ml of solvents for extraction
  - Petroleum ether
  - Chloroform
  - Acetone
  - Methanol
  - Water
- Mechanical shaking for 24 hrs
- Concentrate the extract in rotary vacuum under reduced pressure

FIG. 7 Flow diagram for sequential extraction of phytochemicals

4.1 Alkaloids: Various solvent extracts (corresponding to 2.5 g dried sample) were evaporated to dryness and the residue was heated on a boiling water bath with 5 ml of HCl (2 N). After cooling, the mixture was filtered and the filtrate, divided into two equal portions. One portion was treated with a few drops of Mayor’s reagent and the other with equal amounts of Wagner’s reagent [329].

The samples were then observed for the presence of turbidity or precipitation.

Slight opaqueness - (+)
Definite opaqueness - (+ +)
Heavy precipitation or flocculation - (+ + +)

Reagents used:

a. Wagner’s reagent: Iodine (2 g) and potassium iodide (6 g) were dissolved in 100 ml of distilled water.

b. Mayor’s reagent: HgCl2 (1.358 g) was dissolved in 60 ml distilled water, the solution obtained was mixed with 10 ml distilled water containing 5 g KI and the volume adjusted to 100 ml with distilled water.
4.2 **Tannins:** The extract (corresponding to 1 g of dried sample) was evaporated to dryness and the residue was extracted with 10 ml of hot NaCl solution (0.9%). The extract was filtered and divided into 3 equal portions. A sodium chloride solution was added to the first portion, gelatine solution (1%) to the second one and the gelatine-salt reagent to the third portion. Precipitation with the latter reagent or with both the second and third reagents is indicative of presence of tannins. Positive tests were confirmed by the addition of FeCl₃ solution to the sample that should result in a characteristic blue, blue-black, green or blue green colour (phenolic compounds) [329].

4.3 **Saponins:** About 1 g of dried sample was extracted with 20 ml boiling distilled water. The extract was cooled and then filtered. The filtrate was shaken vigorously to froth and then allowed to stand for 15-20 min [330]. The samples were classified for saponins content as following:

- No froth - Negative
- Froth < 1 cm - Weakly positive
- Froth 1.2 cm - Positive
- Froth > 2 cm - strongly positive

4.4 **Flavonoids:** The methanolic extract (5 ml, corresponding to 1 g of dried sample) was treated with few drops of concentrated HCl and magnesium turnings (0.5 g). The presence of flavonoids is indicative, if pink or magenta-red colour develops within 3 min [329].

4.5 **Reducing sugar:** About 0.5 g of dried sample was extracted with 10 ml boiling distilled water. After cooling, it was filtered and heated with 1 ml of H₂SO₄ solution (0.1 M) in a boiling water bath for 5 min. The extract was then cooled, mixed with few drops of NaOH (1 N) and Fehling’s reagent, and heated in a boiling water bath. The orange precipitate is indicative of presence of reducing sugars [331].

**Preparation of Fehling’s reagent:** CuSO₄, hydrated (7 g) was dissolved in 100 ml distilled water (Fehling 1). Sodium-potassium tartrate (35 g) and NaOH (10 g) were dissolved in 100 ml of distilled water (Fehling 2). Equal portion of these two solutions were mixed to obtain Fehling’s reagent.
4.6 Triterpenoid/Steroids: The Lieburmann-Burchard test was used to detect the presence of steroids and triterpenoids in samples. Methanolic extract (2 drops) was mixed with equal volume of acetic anhydride, and then 2 drops of concentrated sulphuric acid was added from the sides of the test tubes. Development of red colour is indicative of triterpenoids, while a green colour in the upper layer is indicative of steroids [331].

1. Antinutrients

5.1 Tannins: Tannin content in the samples was measured by the AOAC method [332].

5.2 Saponins: Saponins content were estimated according to the method of Makkar et al [333].

2. Antioxidant components:

6.1 Ascorbic acid: Ascorbic acid was estimated by a visual titrimetric method using 2, 6-dichlorophenol indopenol dye on reaction with ascorbic acid which is blue in alkaline solution and red in acid solution and turns colourless when reduced by ascorbic acid [334].

6.2 Flavonoids: The total flavonoids content of the samples were determined according to Miliauskass et al (2004) using quercetin as a reference compound. One ml of plant extract in methanol (10g/l) was mixed with 1ml if aluminum trichloride in ethanol (20g/l) and diluted with ethanol to 25ml. The mixture was incubated at 20°C and the absorbance was measured at 415 nm. The blank was prepared by diluting 1 ml plant extract and 1 drop acetic acid to 25 ml with ethanol. The flavonoids in the samples were calculated using the following formula.

\[
X = \frac{(A \times m_o \times 10)}{(A_o \times m)}
\]

Where: X– Flavonoid content, mg/g plant extract in quercetin Equivalents.
A– Absorption of Sample
A_o – Absorption of standard quercetin solution
m – Weight of Sample
m_o – Weight of quercetin in the solution
6.3 Total polyphenols: Total phenolic content of each extract was determined by the Folin–Ciocalteu micro-method [335]. Extract solution (20 µl) was mixed with 1.58 ml distilled water and 100 µl of Folin–Ciocalteu reagent, followed by addition of 300 µl of Na2CO3 solution (20%) after 1 min and before 8 min. Subsequently, the mixture was incubated in a shaking incubator at 40°C for 30 min and its absorbance was measured at 760 nm. Gallic acid was used as a standard for calibration curve. The phenolic content was expressed as gallic acid equivalents using the following linear equation based on the calibration curve:

\[ A = 0.980C + 9.925 \times 10^{-3}, \quad R^2 = 0.9996 \]

Where:  
A - Absorbance  
C - Concentration as gallic acid equivalents (µg/ml).

6.4 Glutathione: Reduced glutathione (GSH) was estimated based on the reaction of 5, 5′-Dithio (bis) nitro benzoic acid (DTNB) with compounds containing sulphydryl groups [336]. Dried sample (1g) was homogenized in 8 ml Tris-buffer (0.2 M, PH 8.2) and filtered. To 1 ml filtrate, 2 ml of Tris-buffer (0.4 M, PH 8.2) and 0.1 ml of DTNB reagent were added. The absorbance was read within 2 min at 412 nm. GSH in unknown sample was estimated based on glutathione standard curve.

3. Antioxidant activity:

3.1 DPPH radical scavenging activity: The ability of the methanolic and aqueous extracts to scavenge free radicals was determined against a very stable free radical DPPH determined spectrometrically Blois [337]. Aliquot of the sample extract at different concentrations were added to 1mM methanolic solution of DPPH. The mixture was vortexed vigorously and left for 30 min at room temperature in the dark. The absorbance was measured at 517 nm and activity was expressed as percentage DPPH scavenging relative to control using the following equation:

\[ \text{DPPH scavenging activity (\%)} = \left( \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \right) \times 100 \]

3.2 Reducing power assay: The ability of methanolic and aqueous extracts to reduce iron (III) to iron (II) was assessed by the method of Yildrim et al (2001). The dried extract (125-1000µg) in 1ml of the corresponding solvent was mixed with 2.5ml of phosphate buffer (0.2 M, pH 6.6) and 2.5ml of potassium ferricyanide (K₃Fe
(CN)$_6$; 10 g l$^{-1}$), and then the mixture was incubated at 50$^{\circ}$C for 30 min. After incubation, 2.5ml of trichloroacetic acid (100 g l$^{-1}$) was added and the mixture was centrifuged at 1650 rpm for 10 min. Finally, 2.5ml of the supernatant solution were mixed with 2.5ml of distilled water and 0.5ml of FeCl$_3$ (1g l$^{-1}$) and the absorbance was measured at 700 nm. High absorbance indicates high reducing power.

**II. In Vivo Study Design:** Different parts of jujuba such as peel, pulp and seed were used in the preparation of supplement (capsule). The efficacy of a various forms of supplement was evaluated in subjects with dyslipidemia. Upon review of the current literature only one clinical trial were reported concerning the hypolipidemic effects of *Ziziphus jujuba* Mill. fruit in the human. Thus, the present study was conducted to investigate the effect of aqueous and fresh products of *Ziziphus jujuba* fruit based on folk medicine process on serum lipid profile. Therefore, the antihyperlipidemic activity of *Ziziphus jujuba* Mill. was experimentally born out but it has to be standardized for common use.

**Screening and Selection of Subjects:**

In the present research clinical studies were undertaken to evaluate the lipid lowering ability of *Ziziphus jujuba* Mill. on dyslipidemic subjects who were willing to participate in the study. The subjects were recruited from multi-center (Vikram clinic, Diabetes clinic and Mediwave Research Hospital) and University health centers in Mysore city, India with the help of the attending physicians. Details of the subjects, recruited for the study from various centers is presented in Table 12.

**Table. 12 Subjects Selected from Different Centers in Mysore city**

<table>
<thead>
<tr>
<th>No</th>
<th>Clinic</th>
<th>No of subjects screened</th>
<th>No of subjects selected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vikram clinic</td>
<td>87</td>
<td>65</td>
</tr>
<tr>
<td>2</td>
<td>Diabetes clinic</td>
<td>68</td>
<td>55</td>
</tr>
<tr>
<td>3</td>
<td>Mediwave Research Hospital</td>
<td>80</td>
<td>49</td>
</tr>
<tr>
<td>4</td>
<td>University health center (Maharaja’s College)</td>
<td>51</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>University health center (Manasagangotri)</td>
<td>19</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>305</strong></td>
<td><strong>217</strong></td>
</tr>
</tbody>
</table>
Dyslipidemia was confirmed according to Asian Indian population [205] by screening for total cholesterol or LDL-cholesterol and triglyceride, type 2 diabetes mellitus were confirmed by physician and based on use of antihyperglycemic medication(s). Each subject had 6 ml of blood drawn from an anterior cubital vein after a minimum 12-hour fast. All blood tests were performed at a single certified laboratory. Informed written consent was obtained from all subjects prior to participation, and the University ethics committee approved the study (IHEC–UOM No. 25/PhD/ 2008-09. 11.12.2008).

Inclusion criteria

- Subjects with type 2 diabetes mellitus and dyslipidemia (with cholesterol ≥160 mg/dl or triglyceride ≥150 mg/dl or LDL ≥100 mg/dl) without any complications
- Subjects with good general health except for having dyslipidemia (with cholesterol ≥160 mg/dl or triglyceride >150 mg/dl or LDL ≥100 mg/dl)

Exclusion criteria

- Subjects with a history of hypothyroidism
- Subjects with any acute renal, liver, heart disease
- Subject suffering from type 1 diabetes mellitus
- Pregnant and/or lactating women

This section of the study was conducted in 2 phases as described below.

1. PHASE 1:

1.1 **Study design:** A randomized, cross sectional study was conducted to study the nutritional and biochemical status of subjects with dyslipidemia.

1.2 **Subjects selection:** From among 305 subjects, a total of 217 subjects (M=129, F=88), aged between 25-69 years met the inclusion criteria (Table 12).

1.3 **Data collection:**

Their dietary, biochemical and anthropometry status were assessed. Questionnaires were used to elicit information on the following aspects:
a) Demography – Gender, Age, Education, Marital status, Occupation and Religion.
b) Socio economic status (SES) – Family type, Order of birth and Income.
c) Family history – Diabetes, CVD, Hyperlipidemia, Obesity and Hypertension.
d) Disease history
e) Anthropometric measurement – Height (cm), Weight (kg), Waist and Hip circumference (cm), Mid arm circumference (cm) and Skin fold measurement.
f) Body composition – Total body fat, Visceral fat and BMR.
g) Medication
h) Blood parameters – Total cholesterol or LDL and triglyceride.
i) Health habits – Physical activity, Smoking and Drinking.
j) Dietary pattern and Food habits – Food frequency

2. PHASE 2: The effect of *Ziziphus jujuba Mill.* supplementation on blood lipid profile was evaluated. This phase of the study included two sections as described below:

2.1 Pilot Study: Since there is not enough clinical trials report on the effects of *Ziziphus jujuba Mill.* in dyslipidemic subjects, only one randomized, single blind pilot study was undertaken to confirm the hypolipidemic effect and to arrive at the ideal dosage. The dosage selected for the pilot study provided about 40 mg of saponin which is equivalent to 4 capsules per day. This is based on the literature suggesting saponins [338-344] in *Ziziphus jujuba Mill.*

Subject selection- From among the participants on the phase 1, five subjects met the following inclusion criteria which included:

- High cholesterol and high triglyceride
- Willingness to participate

Capsule size- 500 mg

Dosage- 4 capsule per day (2 capsules in the morning and 2 capsules at night) before meal
Study duration- 8 weeks

Data collection- A detailed assessment of the various parameters (as given below) was administered on this sub set of subjects at the baseline and at the end of the intervention.

a. Biochemical blood tests- lipid profile (Total cholesterol, LDL-C, HDL-C and triglyceride), Fasting blood sugar (FBS), HbA1C, liver function tests (ALT and AST), Renal function tests (urea, uric acid and creatinine), Total protein, Hb, Reduced glutathione (GSH) and Lipid peroxide (LPO) two times at the initial and at the end of 8 weeks of the study respectively.

b. Dietary pattern and Food habits- Food frequency and 24 hours dietary recall.

c. Blood Pressure (mmHg)- Systolic (SBP) and Diastolic (DBP) blood pressure with a sphygmomanometer and stereoscope after seating the subject for >5 min.

The subjects were briefed about the objectives of the study. Experimental capsules required for 4 weeks were packed separately (120 capsules per box) and provided to the subjects. After four weeks, each subject returned to the clinic to receive another box of the supplement capsules for the next 4 weeks while each subject was questioned about intervention and adverse events. All medication was permitted during the trial, except those that could affect serum lipids. The subjects were advised to follow their usual food habits and subjects were also checked for the blood pressure. They were allowed to ask any questions related to intervention, to contact the researcher throughout the study period.

2.2 Clinical trial: This study was aimed to develop for the first time new formulas for hypolipidemic effects of cooked and raw products of *Ziziphus jujuba* Mill. based on traditional medicine. After verification of hypolipidemic effects of the supplement in the pilot study trial, main studies were performed on the larger groups of the dyslipidemic subjects. From among of 217 subjects in the phase 1, sixty two subjects who expressed their willingness to co-operate were randomly divided into three groups for implementation of clinical trials. Seventeen of these subjects
dropped out before completing the intervention, leaving 45 subjects who completed the study. Reasons for leaving the study included hospitalization, being too busy, traveling and several participants gave no reason for dropping out. Before 10 days of starting blood lipid screening tests, all lipid lowering agents were stopped in four subjects who were under medication with the help of attending physician. Participants were randomly assigned to receive experimental or placebo of identical appearance (Fig. 9). Drug compliance was good as judged by participants during the period of the study. Two different supplements were used in the clinical trials. Based on the type of supplement administration, separate clinical trials were conducted, the details are as follows (Table 13):

Table 13. The Number of Groups and Study Design Characteristics

<table>
<thead>
<tr>
<th>No of Groups</th>
<th>Group-code</th>
<th>Study design</th>
<th>Blood Lipid Profile</th>
<th>Type of Supplement</th>
<th>No of Subjects</th>
<th>Duration (Weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NDM</td>
<td>Randomized, Single blind</td>
<td>↑Chol+↑TG</td>
<td>PS</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(HCHT)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>DM-1</td>
<td>Randomised, double-blind, placebo-controlled</td>
<td>↑Chol (HC)</td>
<td>WF</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>DM-2</td>
<td>Randomised, double-blind, placebo-controlled</td>
<td>↑Chol+↑TG (HCHT)</td>
<td>WF</td>
<td>16</td>
<td>8</td>
</tr>
</tbody>
</table>


The criteria for subjects selection (except high cholesterol and normal triglyceride subjects in second trial group), data collection, capsule size, supplement dosage and study duration procedure followed for these trials are similar to those followed in the pilot study (phase II).
2.2.1 Blood lipid lowering effect of the PS supplement: Capsules were prepared from the powder (Fig. 9) which has been described in the earlier chapter (page 58). The study plan is summarized as follows:

Study design- A randomized, single-blind trial

Subject Selection- From among the participants in the phase 1, fourteen subjects with high cholesterol and high triglyceride non-diabetic subjects (M=12, F=2), age 51.57±10.15 years (Group 1) met the inclusion criteria.

2.2.2 Blood lipid lowering effect of the whole fruit supplement: Capsules were prepared from the whole fruit dried powder (Figure. 8) which has been described in the earlier section (page 58). For blinding reasons, two different boxes of experimental and placebo capsules were packed individually with identical presentation and identified as experimental and placebo with the code of ‘E’ and ‘P’ respectively on the label of the boxes (Figure. 9). The third person performed randomization and distributing of the capsule boxes. Researchers and study participants remained blind to capsule allocation throughout the study. The study plan is summarized as follows:

Study design- A randomized, double-blind, placebo-controlled and parallel-group trial

Subject Selection- From among the participants in the phase 1, two different groups of subjects (Groups 2 and 3) met the following inclusion criteria as follows:

- Fifteen high cholesterol and normal triglyceride diabetic subjects (M=12, F=3) age 58.66±7.37 years (Group 2).
- Sixteen high cholesterol and high triglyceride diabetic subjects (M=13, F=3), age 54.06±9.29 years (Group 3).
Screening Subjects

PHASE I

Screening Subjects
(n= 305)

Cross Sectional
(n= 217)

Dyslipidemia Subjects
DM & NDM
(n=50)

PHASE II

Pilot Study
(n=5)

Main Study
(n=45)

NDM
↑ Chol↑ TG
(HCHT)

DM-1
↑ Chol
(HC)

WF
(n=15)

WF
(n=16)

Dyslipidemia Subjects
DM & NDM
(n=50)

FIG. 8 Schematic representation of the Protocol for Recruiting of subjects for Two Phases of Clinical trials

Anthropometric Measurements:

The subjects were assessed for Height, Weight, Triceps skin fold (TSF), Mid Upper Arm Circumference (MUAC), Waist circumference (WC) and Hip circumference (HC). BMI was calculated using Height and Weight, Mid Upper Arm Muscle Circumference (MUAMC) was calculated using TSF, MUAC and Waist/Hip ratio was calculated using waist and hip circumference.

Body Fat Composition:

The total body fat (TBF), visceral fat (VF) and basal metabolic rate (BMR) of the subjects were analyzed using body fat analyzer (Omron) (Figure 10).

Biochemical Assessment:

Blood Collection:

Blood sample was drawn from each subject from the anterior cubital vein after an overnight minimum 12 hours after the last meal. Subjects were instructed not to rush or climb stairs before the blood sample. After taking few drops of blood for estimation of haemoglobin, blood samples were centrifuged at 3000 rpm for 15 minutes. After clotting for 20 minutes, serum was analyzed for total lipids (Total cholesterol, triglycerides, Low density lipoprotein- LDL-C, High density Lipoprotein- HDL-C), glucose, liver function tests (ALT and AST), renal function tests (urea, uric acid and creatinine), Hb and total protein. Further serum samples were frozen in micro tubes at -20°C for analyzing reduced glutathione (GSH) and lipid peroxide (LPO). All serum samples were tested for two times at the initial and at the end of 8 weeks of the study respectively.

Estimation of Lipids (mg/dl):

Serum lipids were measured using standard diagnostic kits.

- Serum cholesterol (Total Chol) was determined using enzymatic kits (Synegy Bio, Chennai), which is based on the cholesterol oxidase- phenol peroxide method.
- Serum triglyceride (TGL) was determined by using enzymatic glycerol- 3 phosphate peroxide method (Synegy Bio, Chennai).
• Serum Low density lipoprotein (LDL-C) was measured using a direct assay by using enzymatic kit (Synegy Bio, Chennai), which is based on the cholerterol oxidase and peroxidase method.

• Serum High density lipoprotein (HDL-C) was measured using a direct assay by using enzymatic kit (ERBA Mannheim, Germany), which is based on cholesterol oxidase and cholesterol esterase method.

**Estimation of blood glucose (mg/dl):**

Blood glucose was determined using the enzymatic glucose oxidase and peroxidase method (Dr Reddy’s Lab, Hyderabad).

**Estimation of ALT (U/L):**

Blood Alanin Amino Transferase was determined using the enzymatic alanin amino transferase and lactate dehydrogenase method (ERBA Mannheim, Germany).

**Estimation of AST (U/L):**

Blood Aspartate Amino Transferase was determined using the enzymatic aspartate amino transferase and malate dehydrogenase method (ERBA Mannheim, Germany).

**Estimation of urea (mg/dl):**

Blood urea was determined using the enzymatic urease and Glutamate dehydrogenase method (LiNEAR Chemicals S.L., Spain).

**Estimation of uric acid (mg/dl):**

Blood uric acid was determined using the enzymatic uricase and peroxidase method (LiNEAR Chemicals S.L., Spain).

**Estimation of creatinin (mg/dl):**

Blood creatinin was determined using the alkaline reacts with picrate ions method (LiNEAR Chemicals S.L., Spain).
Estimation of Hemoglobin (mg/dl):

Hemoglobin was measured by the cyanmethemoglobin method.

Estimation of total protein (g/L):

Blood total protein was determined using the Biuret method (Synegy Bio, Chennai).

Dietary Status:

The subjects were interviewed with the help of a questionnaire, for assessing the individual’s 24 hrs food intake by recall for two days at the initial and at the end of study. They were asked to recall the food consumed on the previous day. The food intake of the individuals was recorded using a set of household measures, relevant to Indian cuisine, models and serving bowls of various portion size, spoons, ladles and chapatti disk were used to determine the portion size of different menu items consumed by the subjects. Raw ingredients for the cooked items were derived by standardizing different menu items and preparatory methods.

Risk Assessment:

Framingham risk scoring [302] is an established method to predict an individual’s 10 year risk for coronary heart disease (CHD). The participants’ cardiovascular risk was assessed via a 2 step procedure. First the participants’ major risk factors for CVD include gender, age, total cholesterol, HDL cholesterol, systolic blood pressure, treatment for hypertension, and cigarette smoking are scored using the Framingham point scores. Secondly the points were added and compared against the 10 year risk % score sheet. This estimation is using a “paper-and-pencil” approach. Electronic calculators to determine 10-year risk developed by Boston University are available on the ATP III page of the NHLBI Web site (www.nhlbi.nih.gov/guidelines/cholesterol). The electronic calculators give a more precise value for 10-year risk because they use continuous variables as opposed to the discrete cutpoints used in the tables. Framingham scoring categories persons with multiple risk factors into those with 10 year risk for CHD of 20% (high risk), 10 to 20% (moderate risk) and 10% (low risk).
Computation:

The collected data was consolidated in terms of each subject and computed for whole group.

a) Anthropometric data was used for the calculation of following

- Body mass index (BMI)= weight (kg)/ height (mts)$^2$

- Mid upper arm muscle circumference (MUAMC)= MUAC- ($\pi$ x TSF/10)

Where TSF- Triceps skin fold,

MUAC- mid upper arm circumference (cm)

- Waist Hip Ratio (WHR)= waist (cm) / hip (cm)

b) Food consumed by each subject was converted to its raw weight i.e. breaking up the menu items into its basic raw ingredients and assigning them to different food groups like cereals, pulses, vegetable, milk and milk products, meats fish, eggs, fruits, fats and oils, sugar and jiggery [345]. Food group intake data was translated into energy and nutrient- macro nutrients (carbohydrate, protein, fat) and micro nutrients (calcium, iron, carotene, vit A, ascorbic acid, B complex vitamins- thiamine, riboflavin, niacin, pyridoxine, folic acid and vit B12) by using food composition tables [346].

Statistical Analysis:

Results were expressed as means ± standard deviation. Descriptive statistical analysis, one-way analysis of variance (ANOVA), scheffe multiple range test, student ‘t’ test, paired ‘t’ test, ANCOVA test and Pearson correlation coefficients were performed using SPSS for windows version 18 program. Excel have been used to generate graphs, tables etc.
FIG. 9 Processing of PS Capsule Content Preparations
FIG. 10 Processing of Whole Fruit Capsule Content Preparation

FIG. 11 Experimental & Placebo Capsules

FIG. 12 Body Composition Analyzer