METHOD AND MATERIALS

*The present study "**In-depth Study of the Antioxidant Profile of Aegle Marmelos (L.) Correa and Its Impact on Type II Diabetes Mellitus Subjects"** was undertaken to fulfil the following objectives:*

SPECIFIC OBJECTIVES

1. To study in-depth antioxidant profile, proximate composition, trace elements and heavy metals in the cultivated ('Gomayasi') and wild variety of *Aegle Marmelos (L.) Correa* leaves.
2. To conduct knowledge, practice and use (KPU) of *Aegle Marmelos (L.) Correa* leaves with practitioners of Ayurveda and Naturopathy.
3. To assess the impact of *Aegle Marmelos (L.) Correa* (Bael) leaf juice supplementation on blood sugar levels, lipid profile, liver and kidney functions of type II diabetes subjects.

Based on the objectives, the methodology has been discussed under the following 3 phases

Phase I- In-depth antioxidant profile, proximate composition, trace elements and heavy metals in the cultivated ('*Gomayasi*') and wild variety of *Aegle Marmelos (L.) Correa* leaves

Phase II- Knowledge, practice and use (KPU) of *Aegle Marmelos (L.) Correa* leaves with practitioners of Ayurveda and Naturopathy.

Phase III- Impact of *Aegle Marmelos (L.) Correa* (Bael) leaf juice supplementation on blood sugar levels, lipid profile, liver and kidney functions of type II diabetes subjects.

PHASE I

To Estimate Proximate Composition Of Aegle Marmelos (L.) Correa Leaf

Sample Selection and identification

Wild variety (traditional and commonly available) was procured from five different *Aegle Marmelos (L.) Correa* trees from the identified zone (21004'49.7"N 70035'10.6"E) of Gir forest, Talala, Dist Gir Somnath, Gujarat, India.

Cultivated variety ('Gomayashi') was procured from ICAR-Central Horticultural Experiment Station, (CIAH) Godhra- Vadodara Highway, Vejalpur, Panchmahals, (Gujarat) India.

FIG 3.1: GLIMPSES OF THE SELECTION OF CULTIVATED VARIETY "GOMAYASI" AND WILD VARIETY OF *AEGLE MARMELOS (L.) CORREA* LEAF

Fresh leaves were thoroughly washed to remove unwanted material and dirt and then dried under shade. They were then dipped in 1% $HgCl_2$ solution for a minute and then dried on a filter paper. They were then identified and authenticated from The Department of Botany, The Maharaja Sayajirao University of Baroda, Vadodara. A voucher specimen of the leaves (FN/VG/I & II) was prepared and preserved in the department of herbarium.

Preparation of the plant material for testing

The fresh good quality 500 grams of leaves from each of 5 trees were plucked and mixed together. Total 2.5 kg of leaves were washed, cleaned and dried under the shade for 2-3 days until brittle. Dried leaves were then powdered to pass through 40 mesh size. The leaves were then stored in air tight plastic container for further use. Five hundred grams of leaves from both the varieties were taken for further analysis.

Aegle Marmelos (L.) Correa leaves were analyzed chemically for proximate composition- total ash method (Hot Oven) and available moisture (A.O.A.C., 2000), mineral content (X-ray Fluorescence), available carbohydrates (Dubois et al.,1956), crude fiber (FIBER THERM), protein (Lowry et al.,1951), fat content (soxhlet method) and heavy metal analysis (Atomic Absorption Spectroscopy).

Estimation

Ash

Two to four grams of the sample was weighed accurately in a previously ignited and tarred silica dish. The material was spread evenly and ignited in a muffle furnace at 600^0C until it was white, indicating the absence of carbon. The dish was cooled in a desiccator and weighed. Total ash was calculated when two consecutive weights were same. The percentage of the total ash of the air-dried material was calculated.

Calculation

$$\text{Total ash (\%)} = \frac{\text{Weight of ash}}{\text{Weight of the sample taken}} \times 100$$

Moisture

Moisture content of leaves were determined by drying the weighed sample of leaf at 105^0C in hot air oven for 5 hours and the loss of weight was expressed as moisture content (A.O.A.C., 2000). Five gram leaf sample from each variety was taken in pre-weighed Petri plates and calculate the moisture by the following formula:

$$\text{Moisture (\%)} = \frac{\text{(Fresh weight- Dry weight)}}{\text{Fresh weight}} \times 100$$

Total carbohydrate

The leaf (1 g) was homogenized in 2N hydrochloric acid using mortar and pestle and volume was made to 20 ml. The content was refluxed for one hour on boiling water bath at 70˚C. Supernatant was collected and residue was re-extracted twice with 2N HCl. All supernatant were pooled and final volume was made to 50 ml. The extract was used for the estimation of total carbohydrates. Total carbohydrates were estimated by following the method suggested by Dubois *et al.*, (1956). Aliquot 0.5 ml was taken and volume was made to 3 ml with the distilled water followed by 0.5 ml distilled phenol and mixed thoroughly. To this, 5.0 ml concentrated sulphuric acid was carefully added at the side of the tube. After the mixing thoroughly the tubes were kept for 30 minutes at room temperature for colour development. The absorbance was measured at 490 nm.

$$\text{Carbohydrates (\%)} = \text{Graph factor} \times \frac{\text{Sample reading}}{\text{weight of sample}} \times \frac{\text{Total volume}}{\text{volume taken}} \times 10^{-4}$$

Protein

Protein content was determined by the method developed by Lowry et al. 1951. The leaf sample of 1 gm was weighed and homogenized in five ml 0.1 N NaOH and filtered through Whatman No.1 filter paper. The sample extracts 0.2 ml was taken and made to 3.0 ml volume with distilled water. Five ml of alkaline copper solution (50 ml 2% Na2CO3 in 0.1 N NaOH + 1 ml 0.5 % CuSO4 in 10 % Sodium Potassium tartrate) was added. The content was allowed to stand for 10 minutes at

room temperature followed by addition of 0.5 ml solution Folin Ciocalteu reagent (1:1 v/v). The content was kept for 30 minutes at room temperature and the absorbance was measured at 750 nm. The protein content was calculated using bovine serum albumin as standard range from 50 – 300 µg.

True protein (%)= Graph factor× $\frac{\text{Sample reading}}{\text{weight of sample}} \times \frac{\text{Total volume}}{\text{volume taken}} \times 10^{-4}$

Fat

Fat content was determined by the soxhlet method. Three to four grams of sample was taken in a thimble and it was placed in soxhlet apparatus fitted with a condenser. 100 ml of Petroleum ether (B.P. 40-600^0 C) in the round bottom flask and boiled for 4 hours. The extract was taken in pre-weighed conical flask. The conical flask was kept in a water bath to evaporate the petroleum ether. The traces of petroleum ether were removed in vacuum pump. The weight of fat was taken to constant weight.

Calculation

Fat content (%)= $\frac{\text{Weight of petroleum ether extract}}{\text{Weight of the sample taken}} \times 100$

Fiber

Fiber extraction was done by Fiber

The Fiber Extraction and Fiber content calculation

Weighed sample of 1 gram was taken as W1 (1gm). After washing in fibertherm it was dried at 105^o C for 3-4 h. After drying in oven cool in desiccators for 30 min it was transferred to crucible (weight of crucible - W0). Dried sample in crucible was taken as W2. It was kept into muffle furnace at 600^{0}C for 30 min and cooled to room temp. in desiccators. Then weight of crucible along with ash, was assumed to be W3.

Fiber (%) = $\frac{(W2-W0) - (W3-W0)}{W1} \times 100$

Note: wash with Acid H_2SO_4 0.313 and then NaOH 0.255N.

To analyze the mineral content in Aegle Marmelos (L.) Correa leaf by X-Ray Fluorescence

The X-ray Fluorescence is one of the most reliable and accurate, consistent and non-destructive method for analysis of main and trace elements using a single pressed pellet.

Sample Preparation

Aegle Marmelos (L.) Correa leaves were sun dried to evaporate water content from it, after then it was grinded in mixture and with the help of pallete maker, pallets of leaf sample were prepared and were used for further elemental analysis in X-ray Fluorescence instrument.

Instrument

Bench-top Energy Dispersive X-ray Fluorescence (EDXRF) of make Rigaku elemental analyzer with element range Na to U having Pd anode X ray Tube with high performance SDD detector with the use of NEX CG software.

To analyze trace metals in Aegle Marmelos (L.) Correa leaf

Digestion of the Sample

Leaf samples were digested in 1:2 Sulphuric acid and Nitric acid (30gm sample in 30ml acid solution).

Elemental analysis

It was done by (AAS) Atomic Absorption Spectrophotometer (Thermo scientific). The digested contents were transferred to acid washed bottles and made up to 100ml with double distilled water and subjected to various metal analysis in Atomic Absorption Spectrophotometer (Thermo scientific).

Instrument

The trace metals in the digested samples were determined by Atomic Absorption Spectrophotometer with either air-acetylene flame, a standard mercury analyzer (ECIL) was used for Mercury estimation by cold vapour Atomic Absorption Spectrophotometer following the AOAC method. The results were expressed in ppm.

To estimate the Total Antioxidant Capacity of Aegle Marmelos (L.) Correa leaf (FRAP/DPPH)

Ferric Reducing Antioxidant Potential (FRAP) -Benzie and Strain (1996)

a. Introduction

The FRAP assay is quick and simple to perform, and the reaction is reproducible and linearly related to the molar concentration of the antioxidant(s) present. It was originally developed as an assay for serum samples only. However, with its various advantages of ease of use etc.; it is very well adapted to botanical samples also.

b. Principle

This method is based on the reduction of a Ferric Tripyridal Triazine (TPTZ) complex to its ferrous, coloured form in the presence of antioxidant.

The FRAP assay directly measures antioxidant with a reduction potential below the reduction potential of the Fe^{3+} Fe^{2+} couple (Halvorsen et al, 2002). The FRAP method uses antioxidants as reductants in a redox-linked colorimetric reaction.

c. Instruments

Spectrophotometer and water bath

DPPH Method (Brand Williams et al, 1995)

a. Introduction

1,1-diphenyl-2-picrylhydrazyl radical scavenging (DPPH) is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The methodology involves reaction of specific compounds or extracts with DPPH in methanol solution. In the presence of hydrogen donors, DPPH is reduced and a free radical is formed from the scavenger. The reaction of DPPH is monitored by the decrease of the absorbance of its radical at 517nm, but upon reduction by an antioxidant, the absorption disappears (Williams, Cuvelier and Berset, 1995). Thus through this method we measure the radical scavenging property of the antioxidant.

b. Principle

This method is based on the ability of the antioxidants to scavenge DPPH cation radical.

c. Instruments

Spectrophotometer and water bath

To estimate total polyphenol content and individual phenols in Aegle Marmelos (L.) Correa leaf

Total phenol (Singleton and Rossi, 1965)

a) Principle

Folin-Ciocalteu (FC) colorimetry is based on a chemical reduction of the reagent, a mixture of tungsten and molybdenum oxides. The products of the metal oxide reduction have a blue colour that exhibits a broad light absorption with a maximum at 765nm. The intensity of light absorption at that wavelength is proportional to the concentration of phenols (Waterhouse, 2002).

b) Instruments

Spectrophotometer and water bath

c) Chemicals

All the chemicals used were of analytical grade from Merck and Hi-Media.

The details of the methods and tools used are given in annexure and Table 3.1

Individual phenols

For estimation of polyphenol composition, HPLC analysis was selected. The liquid chromatographic system consisted of a Shimadzu LC-6A model (Shimadzu, Tokyo, Japan) fitted with a Waters i-Bondapack (Water Corp., Milford, MA): Dionex-Acclaim C 18 column (100×2.1×2.2M) and an SCL-6A system controller.

The injection system used was a 20µl sample loop. Detection was done by a UV visible spectrophotometer SPD- 6AV. Elution was carried out at a flow rate of 1ml/min.

Mobile Phase

1% Acetic acid in water: Water: Me OH (1:4:5)

Specifications:

Column-(100mm×2.1mm×2.2M) column

Solvent- 1% Acetic acid in water: water: Methanol (1:4:5)

Flow rate- 1.0ml/min

Injection volume- 20µL

The HPLC analysis is the benchmark for studying polyphenol composition. This analysis was done at Food Testing Lab division of Junagadh Agriculture University, Junagadh.

TABLE 3.1 : METHODS AND TOOLS USED FOR PHASE I ANALYSIS OF AEGLE MARMELOS (L.) CORREA LEAF

PARAMETERS	METHODS/TOOLS
Proximate composition of *Aegle Marmelos (L.) Correa* Leaf	
Ash	Hot oven
Moisture	A.O.A.C., 2000
Total Carbohydrate	Dubois M. et al., 1956
Protein	Lowry O.W. et al., 1951
Fats	Soxhlet Method
Crude Fiber	Fully automated Fiber Therm Method
Elemental Analysis	
Minerals	X-ray Fluorescence
Heavy Metal Analysis	Atomic Absorption Spectroscopy
Antioxidant Capacity	
Ferric Reducing Antioxidant Power (FRAP)	Benzie & Strain, 1996
1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging (DPPH)	Brand and William et al., 1995
Total Polyphenol	Folin Ciocalteu Assay
Polyphenol composition	HPLC : Column/Solvent/Flow rate/Injection volume (250mm×4.6mm×5µm) column/ 1% Acetic acid in water: water: Methanol (1:4:5) /1.0ml/min./ 20µL

The proximate analysis, mineral & antioxidant activity was carried out in Food Testing Lab of the Bio-chemical department of Junagadh Agriculture University while heavy metal content of the *Aegle Marmelos (L.) Correa* leaf was done in Central Institute of Fishing Technology (CIFT), Veraval, Dist Gir Somnath.

FIG 3.2: EVALUATION OF ANTIOXIDANT PROPERTY OF *AEGLE MARMELOS (L.) CORREA* LEAF

PHASE II

Desk review on various commercial formulations of Aegle Marmelos (L.) Correa used in health and disease specially diabetes

Pub Med and other web sources

Desk reviews on on various commercial formulations of *Aegle Marmelos (L.) Correa* used in health and disease specially diabetes was extracted using internet with the help of various key words like Medicinal plant *Aegle Marmelos (L.) Correa*, diabetes, Ayurveda, Naturopathy, commercial formulations, poly herbal formulations, traditional healers, folk lore medicine, herbal formulations, Ayurvedic Pharmacopeia of India (API), etc. Based on information available on internet, various colleges and agricultural universities were identified where the detailed information on *Aegle Marmelos (L.) Correa* plant and its therapeutic role in Ayurveda and Naturopathy was extracted through personal visits. The places visited were

1. Junagadh Agricultural University (JAU),
2. Sardar Patel Agricultural University
3. Anand Aromatic Plant Research Station
4. Jamnagar Ayurveda College Jamnagar
5. Bael Vejalpur Research Station CSIR Panchmahal Godhra;

Knowledge, practice and use of Aegle Marmelos (L.) Correa with practitioners of Ayurveda and Naturopathy

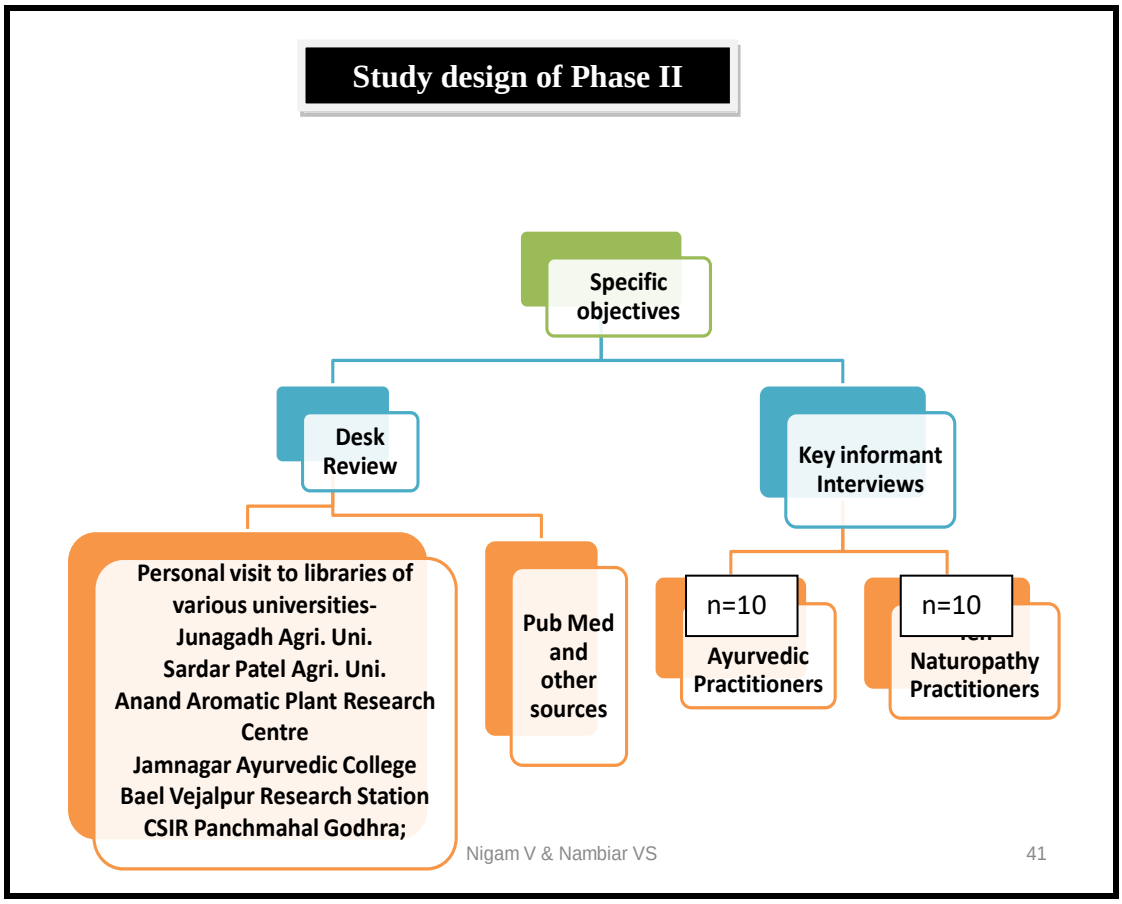
Sample selection:

Based on the desk review, purposive selection of Ayurveda (n=10) and Naturopathy (n=10) practitioners was done using snow ball sampling technique from Vadodara and adjoining areas based on their oral consent to share data.

Methods

Key informant interviews were conducted in the form of open ended questions based on checklist related to dosage and formulations of *Aegle Marmelos (L.) Correa* in various diseases including diabetes (annexure 1). Questions pertaining to procurement of leaves, medicinal properties of this plant, its role in treatment of various diseases particularly diabetes, importance of leaf parts in various diseases, various forms of leaf supplements such as decoction, powder etc, most acceptable form, dose and duration of treatment given to patients and its acceptability were asked to them after their consent with prior appointment on one to one basis. The dosage and form of supplementation for clinical trial was based on the results of phase II.

FIG.3.3: STUDY DESIGN OF PHASE II



PHASE III

Selection of the dosage of Aegle Marmelos (L.) Correa fresh leaf juice (Bael) for supplementation in clinical trial

Based on the results of the KPU of Ayurveda and Naturopathy practitioners, fresh *Aegle Marmelos (L.) Correa* leaf juice (AMLJ or bael juice), of varying amounts of leaves (10, 20, 30g in 100 ml water), was extracted in an electrical mixture, strained and subjected to sensory evaluation to a panel of semi trained judges.

Sensory evaluation

A panel of 30 semi trained panel members (Type II diabetic subjects, age range 42-69 years, 11 F ; 19 M) who volunteered to be a part of this study evaluated the Bael juice using a 9-point Hedonic rating test (Annexure 2). The panellists were asked to rank coded samples of 100ml juice prepared with different samples (sample A-10g leaves; sample B-20g leaves and sample C -30g leaves) for the degree of liking, on a 9-point scale which ranged from 'like extremely' to 'dislike extremely'.

FIG. 3.4 : STEPS FOR SENSORY EVALUATION

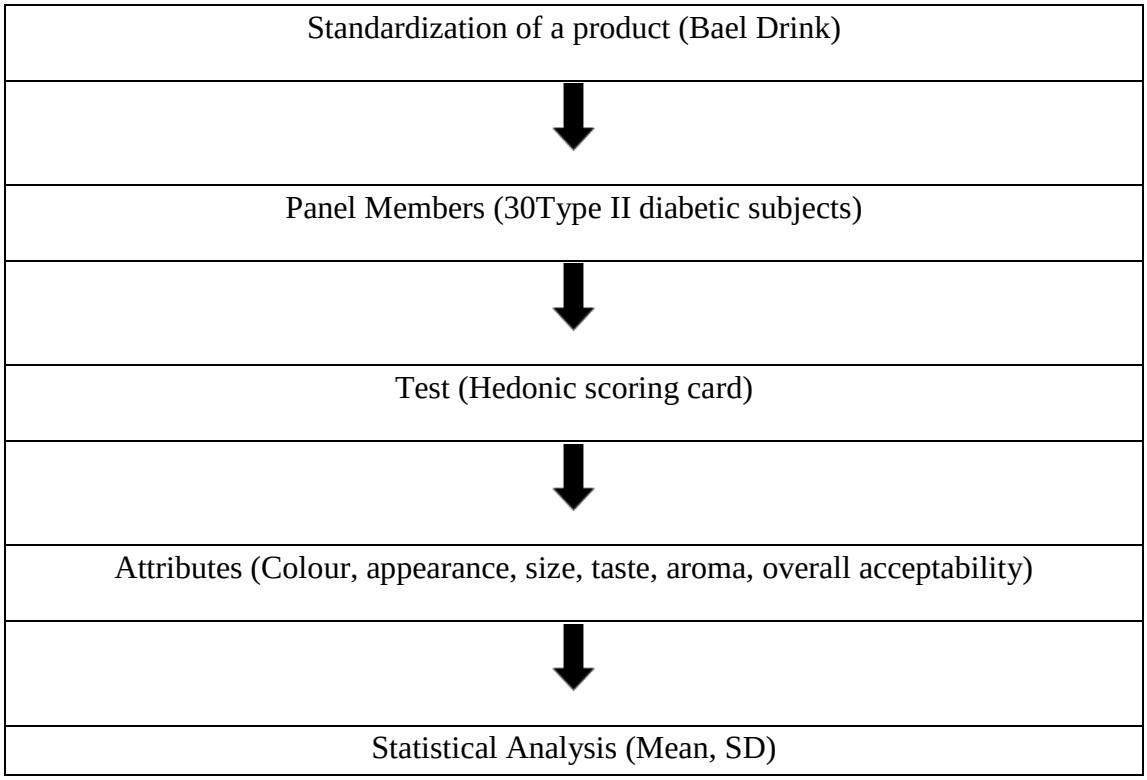


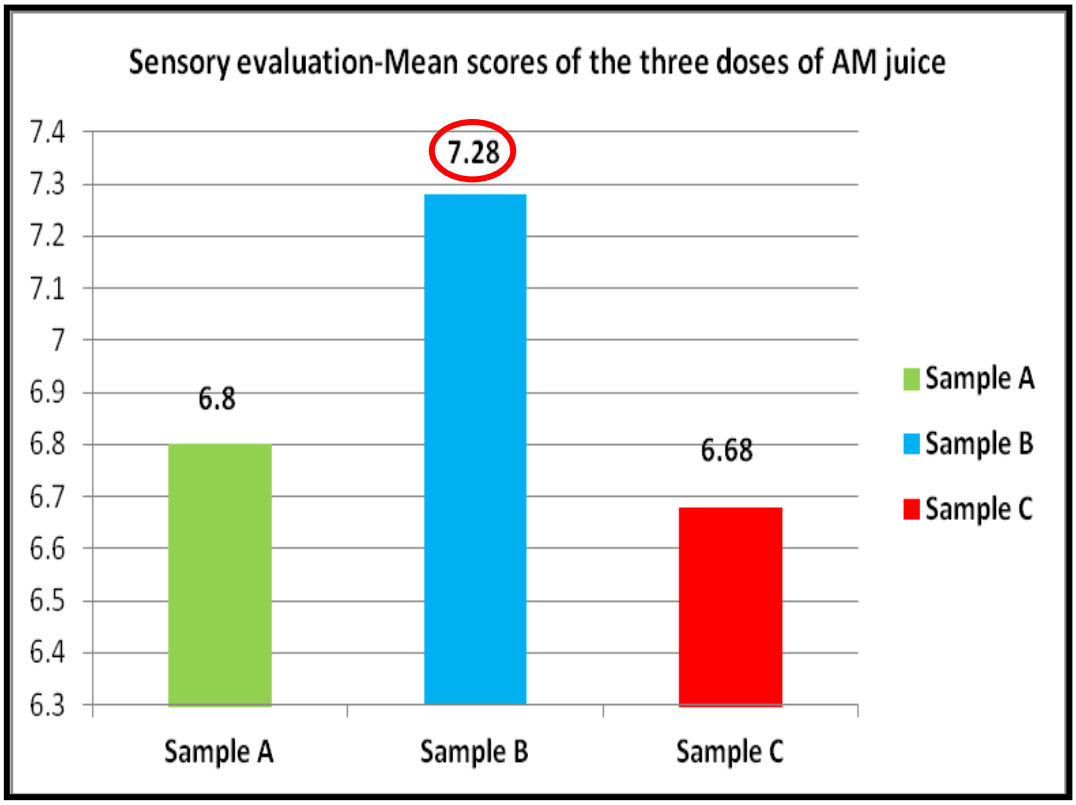
FIG 3.5: SENSORY EVALUATION-THREE DOSES OF FRESH *AEGLE MARMELOS (L.) CORREA* LEAF JUICE (10G LEAVES- SAMPLE A), (20G LEAVES- SAMPLE B) (30G LEAVES -SAMPLE C)

FIG 3.6: PANELISTS OF SENSORY EVALUATION

TABLE 3.2 : RESULTS OF THE SENSORY EVALUATION OF AEGLE MARMELOS (L.) CORREA LEAF JUICE (AMLJ)

No. of Subjects (n=30)	Sample A (10g)	Sample B (20g)	Sample C (30g)
Mean Score	6.8	7.28	6.68
Range	3-9	3-9	2-9
Std. Deviation	1.25	1.51	2.13
Coefficient of Variation CV	0.18	0.21	0.31

FIG 3.7: RESULTS OF SENSORY EVALUATION OF DIFFERENT DOSES OF *AEGLE MARMELOS (L.) CORREA* LEAF JUICE (AMLJ)



Based on results of Sensory evaluation sample B (prepared using 20g *Aegle Marmelos (L.) Correa* leaf juice) was selected for supplementation to the experimental group for clinical trial in the next phase.

TABLE 3.3 : NUTRIENT CONTENT OF 20G AEGLE MARMELOS (L.) CORREA LEAF JUICE

Nutrient	%
Ash	0.48
Protein	0.57
Carbohydrates	0.77
Fat	0.38
Fibre	1.5

Study Area for the clinical trial

Veraval city in Gir Somnath district of Gujarat state in India was selected purposively for sampling and clinical trials to study the impact of *Aegle Marmelos (L.) Correa* leaf juice supplementation on blood sugar level of type II diabetic subjects.

FIG 3.8: MAP INDICATING LOCATION OF GIR SOMNATH DISTRICT IN GUJARAT

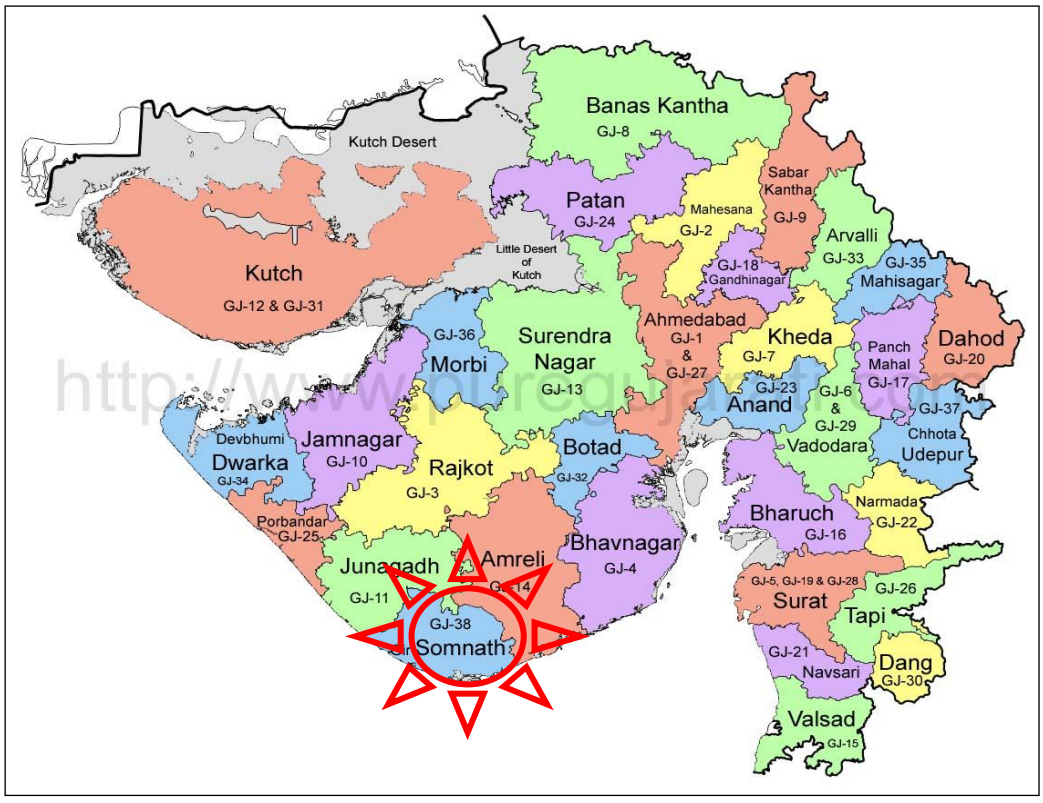


FIG 3.9 AND 3.10: CLINICAL TRIAL- STUDY VENUE: SPIRITUAL CENTRE OF VERAVAL CITY, BHALKA, DIST GIR SOMNATH, GUJARAT, INDIA

Enrolment of Subjects

In this phase, based on a minimum sample of 30, required for clinical trial (Hogg and Tanis, 2008), 65 confirmed Type II diabetic subjects from the largest private hospital in Veraval city, dist Gir Somnath, Gujarat, India who gave their written consent for the study were selected and randomly divided into two groups, Experimental (E Gp) (N=30) and Control group (C Gp) (N=30). Out of 65 Subjects, 5 dropped out (1 from E Gp and 4 from C Gp), thus in final results data of 60 Type II diabetic subjects are presented (inclusion and exclusion criteria for selection is given in Table 3.4)

TABLE 3.4: INCLUSION AND EXCLUSION CRITERIA FOR THE SELECTION OF SUBJECT

Sr. No.	Inclusion	Exclusion
1	25 – 69 years of individuals	Individuals with renal and hepatic dysfunction
2	HbA1c values > 7%	Pregnant ladies
3	FBS 125-250mg/dl	FBS>250mg/dl
4	Willing to participate	Not willing to participate
5	On hypoglycaemic drugs or medication	Any other complications

Baseline Information or Pre Data

Baseline data were collected from both the groups regarding general information, medical history using pre-tested structured questionnaire, their health related quality of life (using Quality of life questionnaire- Diabetes Care Profile, self-administered modified questionnaire of The Michigan Diabetes Research and Training Centre), anthropometry (weight, height and BMI), dietary intake (24 hr dietary recall method and food frequency questionnaire). Their biochemical parameters (glycemic and lipid profile, serum creatinine, total proteins, glycosylated haemoglobin (HbA1c), highly sensitive-C reactive protein (HsCRP), serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and antioxidant capacity in the form of serum ferrous reducing antioxidant potential (serum FRAP) were estimated using the tools and techniques, the details of which are presented in Table 3.5.

TABLE 3.5 : DETAILS OF TOOLS AND TECHNIQUES USED FOR PHASE III

Parameters	**Method/Tools**
General Information	Semi-structured questionnaire
Medical history	Medical records
Diet history	24 hour recall (data was entered in Diet cal software and calculated) and Food Frequency
Quality of life	Diabetes Care Profile, self-administered modified questionnaire of The Michigan Diabetes Research and Training Center (MDRTC) (https://www.med.umich.edu/mdrtc/profs/documents/svi/dcp.pdf)
Anthropometric & Biophysical	
Height, weight, BMI	Standard methods**,** BMI-Asia Pacific Classification
SBP, DBP	Digital BP measuring instruments
Biochemical	
Serum anti-oxidant capacity	FRAP assay (Benzie and Strain, 1996) carried out at CFMRI Lab, Veraval, Gir Somnath, Gujarat
serum lipids	Erba Diagnostics, Manheim, Germany
Fasting Blood Glucose	DiaSys Diagnostics India Pvt. Ltd, Mumbai
HbA1c	Nyco card ™ (Alere)
Hs-CRP	SPINREACT, S.A.U., Spain
SGOT and SGPT	Piramal Diagnostics Ltd. Mumbai
Creatinine	Span Diagnostics Ltd. Surat, India
Total protein	Span Diagnostics Ltd. Surat, India

Enzymatic Kits

Supplementation of *Aegle Marmelos (L.) Correa* leaf juice (AMLJ)

Fresh leaves were plucked from the identified zone of Gir Somnath district in the evening, cleaned and kept for the preparation of juice to be supplemented to the Experimental group (E Grp) next morning. For this group (n=30), 3000ml (100ml/subject) fresh juice was prepared daily using 600g *Aegle Marmelos (L.) Correa* leaves (20g /18-20 leaves / per subject) using a kitchen mixer grinder, strained using strainer with single fine mesh, 7-Inch diameter.

FIG 3.11: GLIMPSES OF COLLECTION OF WILD VARIETY OF AEGLE MARMELOS (L.) CORREA LEAVES FOR PREPARATION OF JUICE

Administration of juice

Bael juice (100ml volume using measuring cup) was served to the subjects empty stomach in disposable glasses in the morning daily between 7:45 am to 8:15 am in a

large spiritual centre located in Bhalka area on the main road of Veraval city as this place was centrally located.

Compliance

The bael juice was given to all subjects in the presence of the investigator every day. In case of absence, an extension was made in the number of days, so that the total number of juice supplementation amounted to 60 days for all subjects (n=30) for the Experimental group.

Impact Analysis (Post Data)

Post data were collected after 60 days for alterations if any on the anthropometric measures, biophysical parameters, dietary information and bio-chemical parameters.

Baseline information

Semi structured questionnaire (Annexure 4 and 5) was filled in by the subjects and base line information was collected for the socio economic data and medical history.

Socio-economic data

General information was collected regarding age, education, family information, income level, occupation and lifestyle pattern using semi-structured questionnaire.

Medical and Family history

Information about onset of disease, medications and presence of any complications, if any were asked and confirmed with medical records. Family history and background history of diabetes were asked by recall method.

FIG 3.12: GLIMPSES OF SUPPLEMENTATION OF AEGLE MARMELOS (L.) CORREA LEAF JUICE DURING CLINICAL TRIAL

Dietary Information

For assessing the dietary intake, two methods were used.

24 hour dietary recall

A 24-hour recall method (24-HDRM) was used to assess the dietary intake of the subjects. Information on dietary intake was taken by recall of diet of the previous day (24 hour) with details of ingredients and amounts using standard cups and spoons. All data were entered in MS excel and average daily calorie intake and nutrient intake was calculated using Diet cal software and daily % RDA met were calculated for each subject.

Food frequency data

Frequency of consumption of selected foods- cereals, legumes, citrus and sweet fruits, green leafy vegetables and other vegetables was elicited. The FFQ method contains an inventory of foods in each category. It helped to validate the general dietary pattern. It was valuable information for assessing the nutritional profile of the subjects, they playing a role in risk factor for development of T2DM

Quality of life

The subjects were asked to fill up a Diabetes Care Profile, self-administered modified questionnaire of The Michigan Diabetes Research and Training Center (MDRTC) (https://www.med.umich.edu/mdrtc/profs/documents/svi/dcp.pdf) having four-sections assessing their health status scoring on a Likert scale ranging from 1 (excellent) to 5 (poor). Questions regarding how they felt during the past 4 weeks about their behaviour whether they were calm, downhearted and angry were scored on a Likert scale ranging from 1(all of the time) to 5 (none of the time). Their perception regarding how they felt during the last month was also elicited. Sub-section II assessed their understanding regarding various aspects related to self-care, medicine, diet and weight management scoring on a Likert scale ranging from 1 (poor) to 5 (excellent). Also various activities that they might be doing during a day were scored on a Likert scale ranging from 1 (Limited a lot) to 3 (Not Limited at all).

Anthropometric measurements

Anthropometry is the measurements of body dimensions to characterize skeletal and tissue development, and relationship between nutrient levels of the individuals. The anthropometric measurements included height, weight and Body Mass Index.

Weight

It is the most widely used and the simplest reproducible anthropometric measurement. It indicated the body mass and is composite of all body constituents like water, minerals, fats, protein, bone, etc (Robinson et al., 1986). A digital platform weighing scale to the nearest of 100g was used to measure weight. The subject was weighed in standard indoor clothing, bare feet and without leaning against or holding anything. The scale was 'zeroed' before taking any weight.

Height

It is a linear measurement made up of the sum of four components i.e. Legs, pelvis, Spine and Skull. Flexible non stretchable tape was used to measure the height of the subjects. A convenient flat wall was identified at the subject's place for the measurement of the heights. The subject was made to stand barefoot with the arms hanging freely by the side. Heels of the feet were placed together with the medial (inner) border of the feet at an angle of 60 degrees. The scapula and the buttock were ensured to be in contact with the measuring wall. The head was held straight with tragus of the ear and the lateral angle of the eye in a horizontal line. Height was recorded to the nearest of 0.1 cm after the subject inhaled fully and maintained the erect position without altering the load on the heels. In this position a mark was made on the wall and height was recorded with a measuring tape.

Body Mass Index (BMI)

The BMI is a convenient and valid measure of adiposity and found to be positively correlated with blood pressure and plasma lipid concentrations and therefore with the risk of cardiovascular diseases (Brown et al., 2000). The Asia Pacific Classification cut-offs are given in table 3.6. It is computed as, BMI (Kg/m^2) = Wt (Kg)/ Ht (m^2)

TABLE 3.6: CLASSIFICATION FOR OBESITY

CATEGORY	BMI
Underweight	< 18.5
Normal	18.5-22.9
Overweight	23-24.9
Obese grade I	25-26.9
Obese grade II	27-29.9
Obese grade III	≥ 30

(Source: Asia Pacific Classification for Obesity, 2004)

Biophysical Investigations

Body Fat

Body fat percentage is the amount of fat tissue in the body as percentage of total body weight. Due to stiffness in body composition, BMI is not necessarily an accurate indicator of body fat for e.g. individuals with greater than average muscle mass will have a higher BMI. Body fat is an accurate indicator to assess the health risk of CVD, hypertension and diabetes. Guidelines for body fat percentage are given in table. Body fat measurement was taken using Eagle EEF 2001A Digital Body Fat/Hydration Monitoring Scale.

FIG 3.13: GLIMPSES OF ANTHROPOMETRIC MEASUREMENTS OF THE SUBJECT

Blood Pressure

Blood pressure was measured using Omron HEM-7130 Automatic Blood Pressure Monitor with Comfit Cuff. It gives accurate and precise readings. It is recommended brand by the doctors. It gives accurate results with the touch of one button. It has the ability to review the last 14 readings.

Biochemical Analysis

Biochemical analysis included the following parameters such as glycemic profile (FBS, HbA1c, PPBS), lipid profile (TC, TG, LDL, HDL, VLDL), Serum antioxidant status (serum FRAP), Hs-CRP (Anti-Inflammatory Status), SGOT, SGPT (Liver function), serum creatinine and total protein (kidney function). After an overnight fast of 13 hours, fasting venous blood samples (5ml) were drawn by trained technician using disposable syringes and needles and blood was dispensed into respective vaccutainers. Serum was then separated for further biochemical analysis. The bio-chemical analysis was done using enzymatic kits which was out-sourced to largest laboratory of Veraval. The details of the methods and tools is given in (Annexure 6).

FIG 3.14: GLIMPSES OF LABORATORY TECHNICIAN DRAWING BLOOD FOR BIO-CHEMICAL ESTIMATION

Estimation of

Fasting Blood Sugar (FBS) and Post Prandial Blood Sugar (PPBS)-

Glucose is oxidized by glucose oxidase (GOD) to produce gluconate and hydrogen peroxide. The hydrogen peroxide is then oxidatively coupled with 4 amino-antipyrene (4-AAP) and phenol in the presence of peroxidase (POD) to yield a quinoeimine dye that is measured at 505nm. The absorbance at 505nm is proportional to concentration of glucose in the sample. FBS and PP2BS was estimated using enzymatic reference method (Trinder P., 1969) using enzymatic GOD-POD Method.

Glycated haemoglobin (HbA1c)

Method: Fully automated H.P.L.C> using TOSOH G8
Detection Technique: H.P.L.C

Principle

It works on the principle of high performance liquid chromatography (HPLC), which is considered the "Gold Standard" technology in the follow-up of the plasma glucose concentration of diabetic patients over time, via the measurement of HbA1c (glycated haemoglobin fraction).

Lipid Profile

Lipid profile is a panel of blood tests that serves as an initial broad medical screening tool for abnormalities in lipids such as cholesterol and triglycerides. The result of this test can determine approximate risks for cardiovascular diseases, certain forms of pancreatitis and other diseases.

Total Cholesterol (TC)

The total cholesterol was estimated using the CHOD POD method with the help of enzymatic kits. Cholesterol esters are enzymatically hydrolyzed by cholesterol esterase to free cholesterol to free fatty acids. This free cholesterol is then oxidized by cholesterol oxidase (CHOD) to cholest-4-ene-3-one & Hydrogen Peroxide (H202). The liberated H_20_2 then combines with phenol & 4-aminoantipyrine to produce

quinonimine, a red coloured complex in presence of peroxidase (POD). The colour of which is directly proportional to the quantity of cholesterol present in the serum. This is measured calorimetrically by automated chemistry analyzers at 500 nm.

Triglycerides (TG)

Detection technology: Photometry

Serum triglycerides are measured by an enzymatic method using lipoprotein lipase, glycerol kinase, glycerol phosphate oxidase and peroxide enzymes. Glycerol relaeased from the hydrolysis of triglycerides by lipoprotein lipase is converted into glycerol-3-phosphate which is oxidised by glycerol phosphate oxidase to dihydroxyacetone phosphate and hydrogen peroxide. In the presence of peroxidise, hydrogen peroxidise oxidises phenolic chromatogen to a red coloured compound which is then measured colorimetrically on automated chemistry analyzers at 546 nm.

High Density Lipoprotein-C (HDL-C)

Separated serum was precipitated by adding precipitating reagent (Phosphotungstic acid and dextran sulfate-magnesium chloride) after centrifugation at 3000 rpm. The supernatant was estimated for HDL-C by using cholesterol reagent (Cholesterol esterase and cholesterol oxidase) as the catalase eliminates the VLDL cholesterol, LDL cholesterol and cylomicrons. The cholesterol ester is hydrolyzed by cholesterol esterase to cholesterol and fatty acid. The cholesterol is then oxidized to cholestenone and hydrogen peroxide by cholesterol oxidase. Hydrogen peroxide in presence of peroxidase reacts with 4-aminoantipyrine and HDAOS to produce a quinine pigment. The intensity of the dye produced is directly proportional to the HDL cholesterol concentration when measured at 600 nm.

Very Low Density Lipoprotein-C (VLDL-C)

VLDL-C was calculated using the Friedlewald's formula (1972) by diving triglycerides values by 5.

$$VLDL = TG/5$$

Low Density Lipoprotein-C (LDL-C)

The LDL-C values were calculated using the Friedlewald's formula (1972).

LDL-C in mg % TC - (HDL + TG/5)

Serum Creatinine-

It is used for the determination of Creatinine in human serum/ plasma and urine using Jaffe's method (Murray R.L., 1984)

Principle-

Creatinine present in serum or plasma directly reacts with alkaline picrate resulting in the formation of a red colour, the intensity of which is measured at 505nm/green filter. The rate of formation of this complex is measured by reading the change in absorbance at 505 nm in a selected interval of time and is proportional to the concentration of creatinine. The reaction time and the concentration of Picric Acid and Sodium Hydroxide have been optimised to avoid interference from ketoacids.

$$\text{Creatinine + Picric Acid} \xrightarrow{\text{Alkaline medium}} \text{Orange coloured complex}$$

Highly Sensitive C- Reactive Protein (HsCRP)

The HsCRP ELISA is intended for the quantitative determination of C-reactive protein (CRP) in human serum. Enhanced sensitivity measurements of CRP can be useful for the detection and evaluation of infection, tissue injury, inflammatory disorders and associated diseases.

Principle:

The HsCRP ELISA is based on the principle of a solid phase enzyme-linked immunosorbent assay. The assay system utilizes a unique monoclonal antibody directed against a distinct antigenic determinant on the CRP molecule. This mouse monoclonal anti-CRP antibody is used for solid phase immobilization (on the micro titer wells).

Instruments

Spectrophotometer

Alanine amino-transferase (ALT/GPT)

For the determination of Alanine Aminotransferase (ALT/GPT) in serum, optimized UV method is used (IFCC) (Thomas L., 1998).

Total Protein

Principle

The Peptide bonds of Proteins react with Cupric ions in alkaline solution to form a coloured chelate, the absorbance of which is measured at 578 nm. The Biuret Reagent contains Sodium-potassium Tartrate, which helps in maintaining solubility of this complex at alkaline p^H. The absorbance of final colour is proportional to the concentration of Total protein in the sample (Koller A., 1984).

Total Antioxidant Capacity (TAC)

Total antioxidant capacity of the subjects was measured using Ferric Reducing Antioxidant Potential (FRAP) assay (Benzie and Strain, 1996).

Principle

This method is based on the reduction of a ferric tripyridal triazine (TPTZ) complex to its ferrous, coloured form in the presence of antioxidant.

The FRAP assay directly measures antioxidant with a reduction potential below the reduction potential of the Fe3 + Fe3 + couple (Halvorsen et al., 2002). The FRAP method uses antioxidants as reductants in a redox-linked colorimetric reaction. Same method as given in 4.2.1 was used but instead of botanical sample, serum of the subjects was used for serum FRAP analysis. Serum drawn was stored at -20^0 C in deep freeze.

This test was carried out at Central Marine Fisheries Research Institute CFMRI, Veraval, Dist Gir Somnath under the guidance of senior scientist. The day of sample

collection was considered as 1st day and at the end of the supplementation period (60 days), analysis was repeated for the same parameters. The details of the study design is given in Fig 3.1.

Instruments

Spectrophotometer and water bath

FIG 3.15: GLIMPSES OF ANALYSIS OF SERUM ANTIOXIDANT (FRAP) ASSAY OF THE ENROLLED SUBJECTS

ETHICAL APPROVAL

The study was approved by Ethics Committee of Department of Foods and Nutrition, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat. The Ethical Clearance No. is IECHR/2013/20.

DATA MANAGEMENT AND STATISTICAL ANALYSIS

The data was entered in an excel spreadsheet and checked for its validity and then subjected to appropriate statistical analysis. Data analysis was performed using the Statistical Package for the Social Sciences (SPSS 17.0 version, SPSS Inc., Chicago, IL, USA). Descriptive statistics was used: mean comparison, standard deviation, percentages, paired t- test was performed to compare the mean values between pre and post data of the same group. Student t test was performed for the comparison between control and experimental group for various biochemical and anthropometric parameters. The significance levels were set at 5% by two sided tests and their statistical significance was ascertained using Microsoft Excel and computer programme package.

FIG 3.16: IMPACT OF BAEL JUICE SUPPLEMENTATION (STUDY DESIGN PHASE III)