This study comprised of four phases.

Phase I: Formative Research

Assessment of socio-demographic profile and menopause related scale in middle age women from free living population of Vadodara.

Phase II: Analytical Research

Identification and Quantification of different phytoestrogenic components in Fenugreek seeds, Flaxseeds, Pomegranate seeds and Yam.

Phase III: Experimental research

Impact of Pomegranate and Yam powder supplementation on MRS (Menopause Rating Scale), Serum Gonadotropin hormones and serum Estradiol in perimenopausal women – A supplementation trial.

Phase IV: Development and distribution of a guidebook on Menopause and its management to study subjects.

3.1 Experimental Design

Phase I: Formative Research

Cross-sectional study design was used to study the prevalence of menopauserelated symptoms experienced by middle age women and the differences in their experiences with respect to pre-, peri- and postmenopausal women.

Vadodara district was selected to enroll the women aged between 30-60 years from free living population (n=1000). Urban area of Vadodara district was divided in to five zones for screening; east, west, north, south and central. An equal number of women (n=200/zone) were enrolled from each zone; an institutional based approach was carried out through ICDS, so that we could get a mix population including all ethnic groups who represent the lower to middle income group population.

Sample size:

Considering prevalence of menopause as 10.4 % (based on previous study conducted by Nair et al., 2007- 08), with a 95 % confidence limit, and 20 % relative precision of estimate, the sample size required was calculated as follows:

$$\text{Formula for sample calculation} = \frac{(z)^2 \times (1 - p)}{(p) \times (e)^2}$$

$$\text{Sample size (N)} = \frac{(1.96)^2 \times (1 - 0.10)}{(0.10) \times (0.2)^2} = 860$$

Therefore, the sample size was set as N=1000.

Inclusion Criteria:

- Women aged between 30-60
- Who gave their consent

Exclusion Criteria:

- Pregnant and lactating women
- With history of any chronic diseases and hysterectomy

Data Collection:

Baseline data collection was obtained on the following:

- Socio demographic details: age, religion, educational status, profession, number of children, number of pregnancies etc – Using a predesigned and pretested questionnaire.
- Clinical History: Self reported clinical information for thyroid disorder, blood pressure, any other diseases, any medications; etc. was availed.
- Knowledge and Perception: women's knowledge and perception about menopause and related symptoms, hormone replacement therapy and associated risks, consequences of menopause with

thyroid and its symptoms, menopause and bone health - Using a predesigned and pretested questionnaire was carried out.

- Menopause related symptoms: were rated using a Menopause Rating Scale (MRS) which was obtained from the Professor Heinemann from Center of Epidemiology and Health Studies, Berlin, Germany.

Ethical Clearance and Approval:

This phase was approved by the Institutional Medical Ethical committee (Approval Number: F.C.Sc. / FND/ ME/ 65).

The following approvals were obtained for the study.

a. For anganwadi approach, the permission was taken from ICDS Office, Vadodara.
b. Prior Consent letter from women for participation

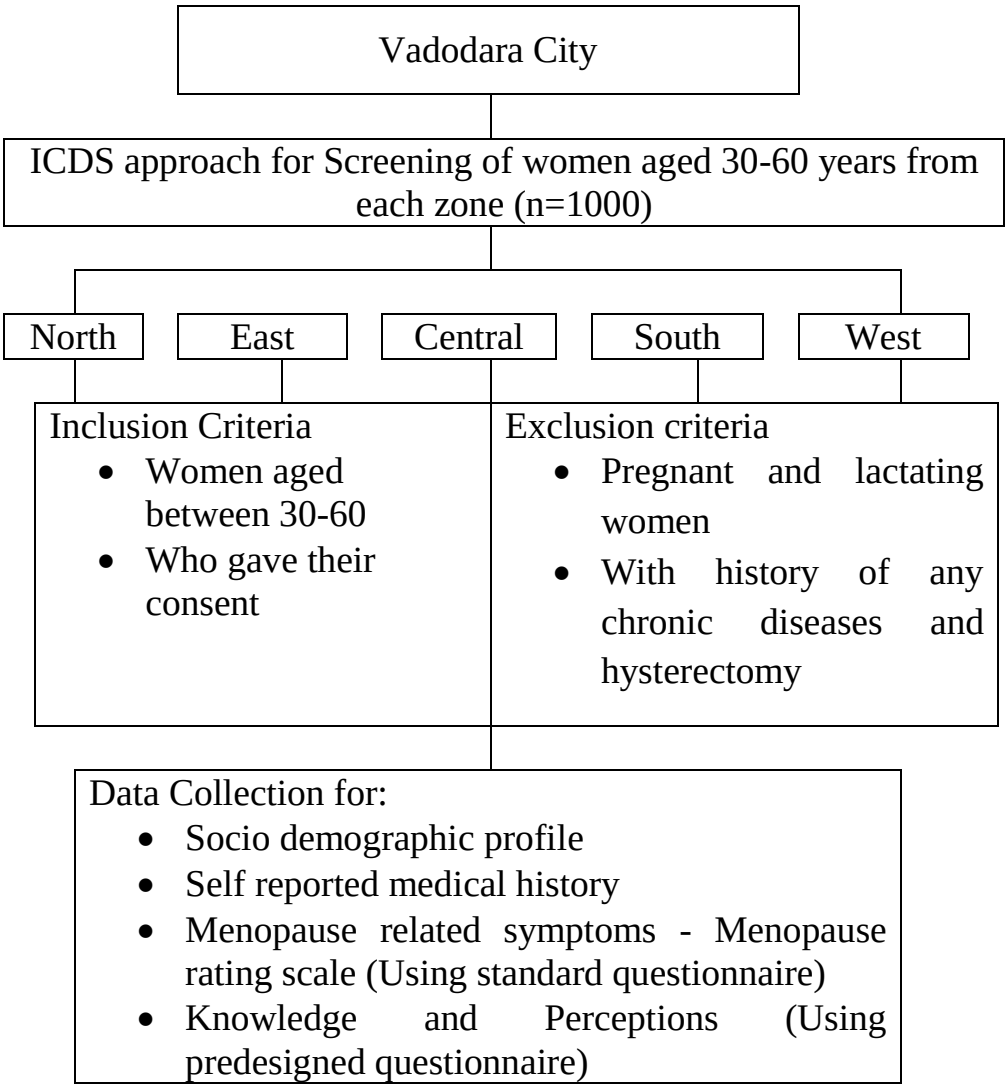


Figure 3.1: Experimental design for Phase I

Phase II: Analytical Research

An HPLC analysis was used for the identification and quantification of phytoetsrogens present in *Trigonella foenum-graecum* seeds (common name – Fenugeek, local name - Methi), *Amorphophallus paeoniifolius* (common name – Elephant foot yam, local name – Suran), *Punica granatur seeds* (Common name – Pomegranate, local name – Dadam), and Flaxseeds.

The raw samples for all four foods were procured from the local market of Vadodara.

Sample preparation was carried out as follows.

1) *Trigonella foenum-graecum* seeds (common name – Fenugeek, local name - Methi) and
2) *Linum usitatisimum* L seeds (Common name – Flaxseeds, local name – Alsi)

Dry seeds of above twofoods were dried in oven for 2 hours at 50C, to ensure complete dryness of seeds. After that, the seeds were made into fine powder using a mixer, this powder was then employed further for HPLC analysis.

3) *Punica granatur seeds* (Common name – Pomegranate, local name – Dadam)

Fresh pomegranate were peel off and seeds were collected. These collected seeds placed in an oven at 50^{o}C for 4 hours and then allowed to cool to room temperature. The same procedure was repeated until all the water content gets evaporated and seeds dries completely. These completely dried seeds were converted into fine powder using a mixer, this powder then employed further for HPLC analysis.

4) *Amorphophallus paeoniifolius* (common name – Elephant yam, local name – Suran)

Fresh elephant foot yam was washed thoroughly with water to avoid interference of dust particles and other contaminated materials. The outer skin layer of the stem was removed and cut down into small pieces (as thin as possible) of more or less equal dimensions, this helps to speed up the drying procedure. The cut pieces were placed in an oven at 50^{o}C for 4 hours and then allowed to cool to room temperature. Same procedure was repeated until all the water content gets evaporated and yam pieces dry completely. These completely dried pieces were made into fine powder using a mixer, this powder was then employed further for HPLC analysis. HPLC analysis of these powder forms of foods were carried out to identify and quantify the different phytoestrogens present.

Standards:

The standards for Isoflavone - Daidzein and Gensitein, Liganans - Secoisolariciresinol and Matairesinol and Comestans - Coumestrol were used to standardize the method for separation and quantification of the same in all four food components.

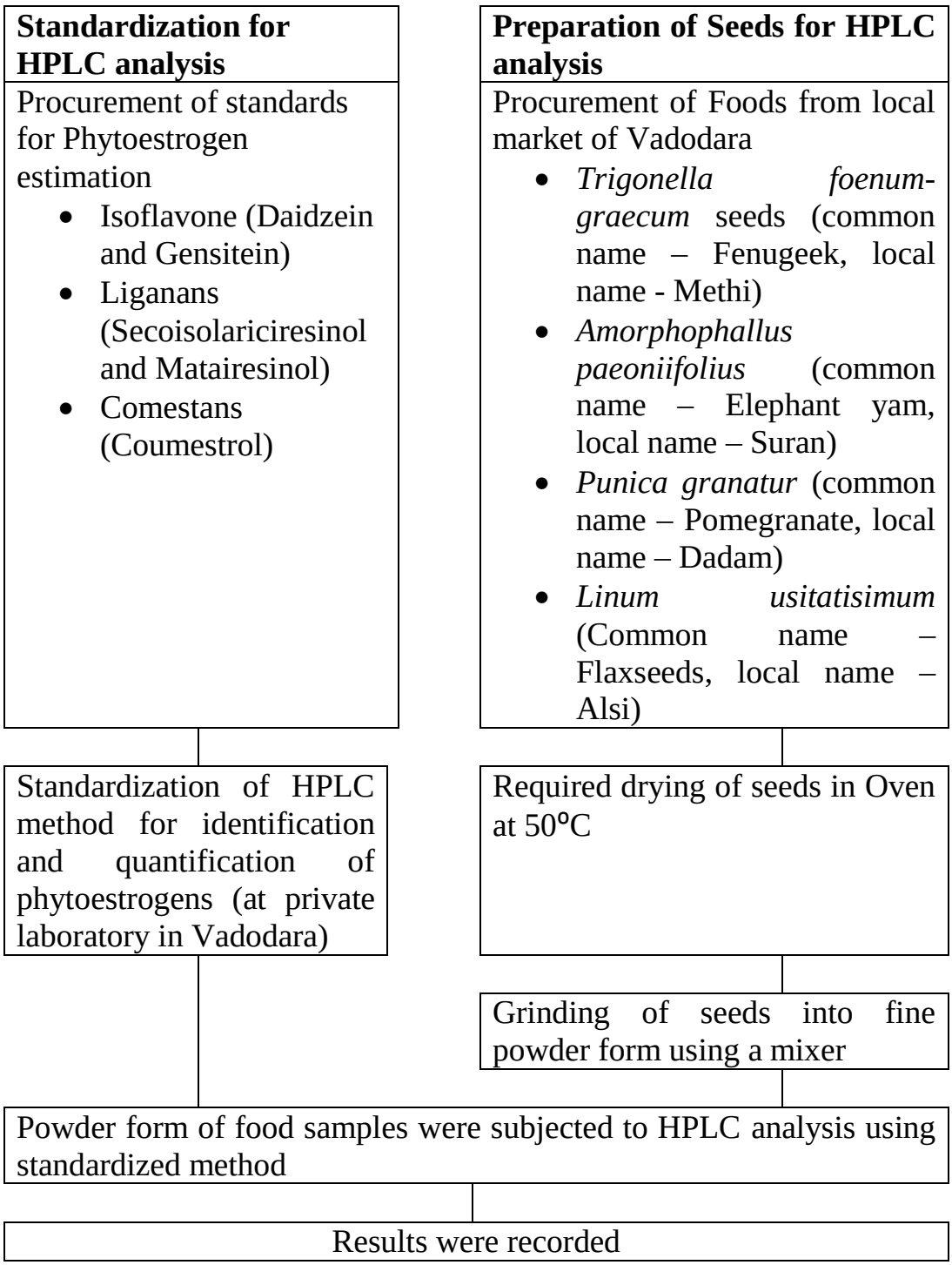


Figure 3.2: Experimental design for Phase II

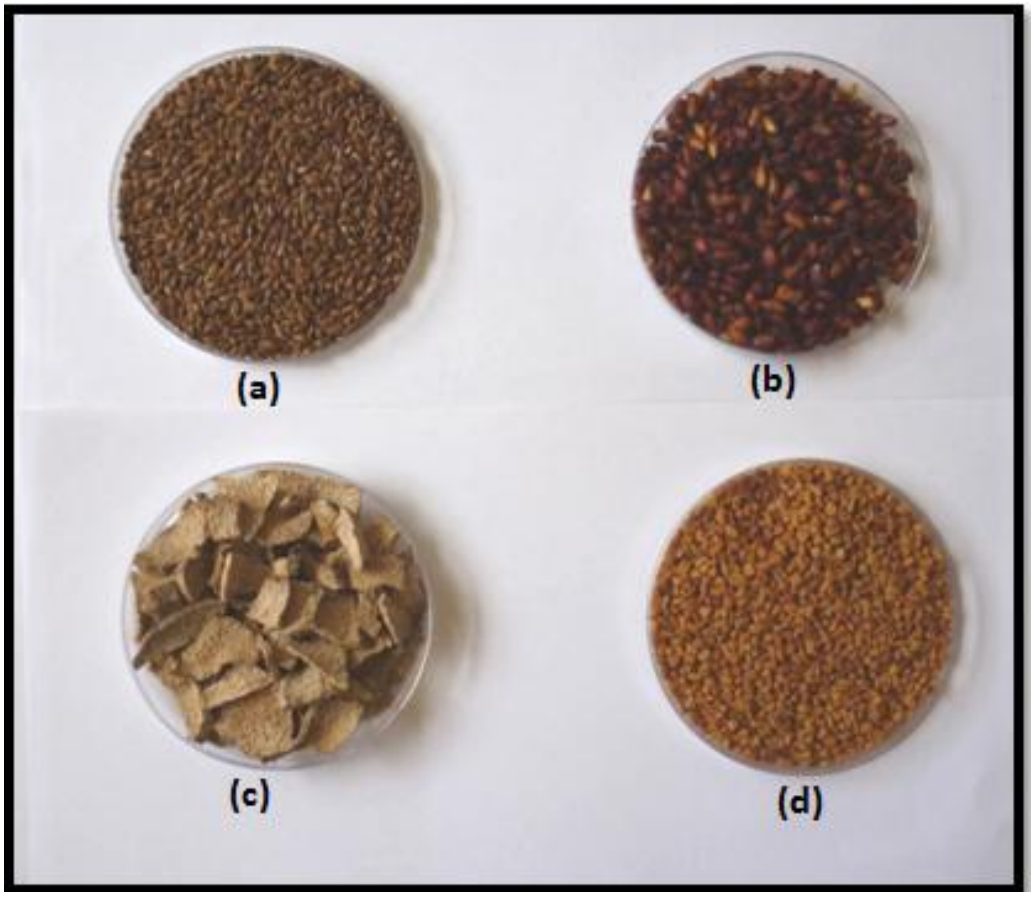


Figure 3.3: Oven dried seeds of

(a) *Linum usitatisimum* (Common name – Flaxseeds, local name – Alsi)

(b) *Punica granatur* (common name – Pomegranate, local name – Dadam)

(c) *Amorphophallus paeoniifolius* (common name – Elephant foot yam, local name – Suran)

(d) *Trigonella foenum-graecum* seeds (common name – Fenugeek, local name - Methi)

Phase III: Experimental research

An experimental-supplementation study was planned to observe the impact of consumption of phytoestrogen rich foods on MRS, serum gonadotropin levels and serum estradiol level in peri menopausal women. The participants were the perimenopausal women from phase I (n=36) and the women in perimenopause referred by gynecologists (n=114). The purpose of study, study design and related health benefits/risk if any, the type of food being supplemented etc and any cost or any kind of compensation was not provided to women for being part of the study - these were well explained to the subjects. Subjects were enrolled based on the inclusion and exclusion criteriawere enrolled (n=150, considering 20% attrition rate) and informed written consent was availed. The subjects were free to abscond from the study in between.

Inclusion Criteria:

- Women who are in peri menopause stage
- Women suffering from irregular menses since one year
- Women willing to participate

Exclusion Criteria:

- Pregnant and lactating women
- Women undergo any kind of surgery in last 6 months
- Women on any medication or HRT (Hormone replacement Therapy)
- Post menopausal women
- Women have any chronic disease

The home visit approach was used for the supplementation and data collection. The women were divided randomly into three groups;fifty in each using alternate numbers by blinding was assigned by a 3rd person.

One group was supplemented with fine powder of elephant foot yam (Experimental Group 1 - EG1), one with fine powder of pomegranate seeds (Experimental group 2 – EG2) and control group (CG) was provided nutrition and physical health education for 45 days (reinforced at 7 days interval). The information and frequency of nutrition and physical health education intervention was similar in all study groups. The quantity of food being supplemented was determined based on the results of analytical results of phase II, which amounted to 50 mg of phytoestrogen.

Along with food supplementation and NHE, emphasis was given to laughing and stretching exercise to all women during home visits. Steps in laughing exercise were demonstrated by the researcher and a copy of the same was provided to all. The compliance of food supplements and their experiences during consumption were recorded. The withdrawal of blood was performed by the researcher, since she is trained personnel with DMLT degree.

Data Collection:

Baseline data collection was obtained on the following:

- Socio demographic details: age, religion, educational status, profession, number of children, number of pregnancies etc – Using a predesigned and pretested questionnaire.
- Clinical History: Self reported clinical information for thyroid disorder, blood pressure, any other diseases, any medications; last medical test etc. was availed.
- Anthropometric Measurements: Measurements for weight, height, waist circumference and hip circumference were carried out and BMI and WHR were calculated for all women.

- Knowledge and Perception: Menopause and related symptoms, Hormone replacement therapy and associated risks, consequences of menopause with thyroid and its symptoms, menopause and bone health - Using a predesigned and pretested questionnaire was carried out.
- Menopause related symptoms: were rated using a Menopause Rating Scale (MRS) which was obtained from the Professor Heinemann from Center of Epidemiology and Health Studies, Berlin, Germany.
- Biochemical estimation: This was carried out for the parameters – Hb, Serum estradiol, Serum Gonadotropins and thyroid hormones (T_3, T_4 and TSH)
- Food frequency: Consumption frequency of major phytoestrogen, iodine and iron rich foods were recorded.

Post data Collection:

Post data collection was carried out for 145 women, 5 women were dropped out from the study.

The reasons:

EG1: n=2 (One got shifted from Vadodara city and one found with poor compliance)

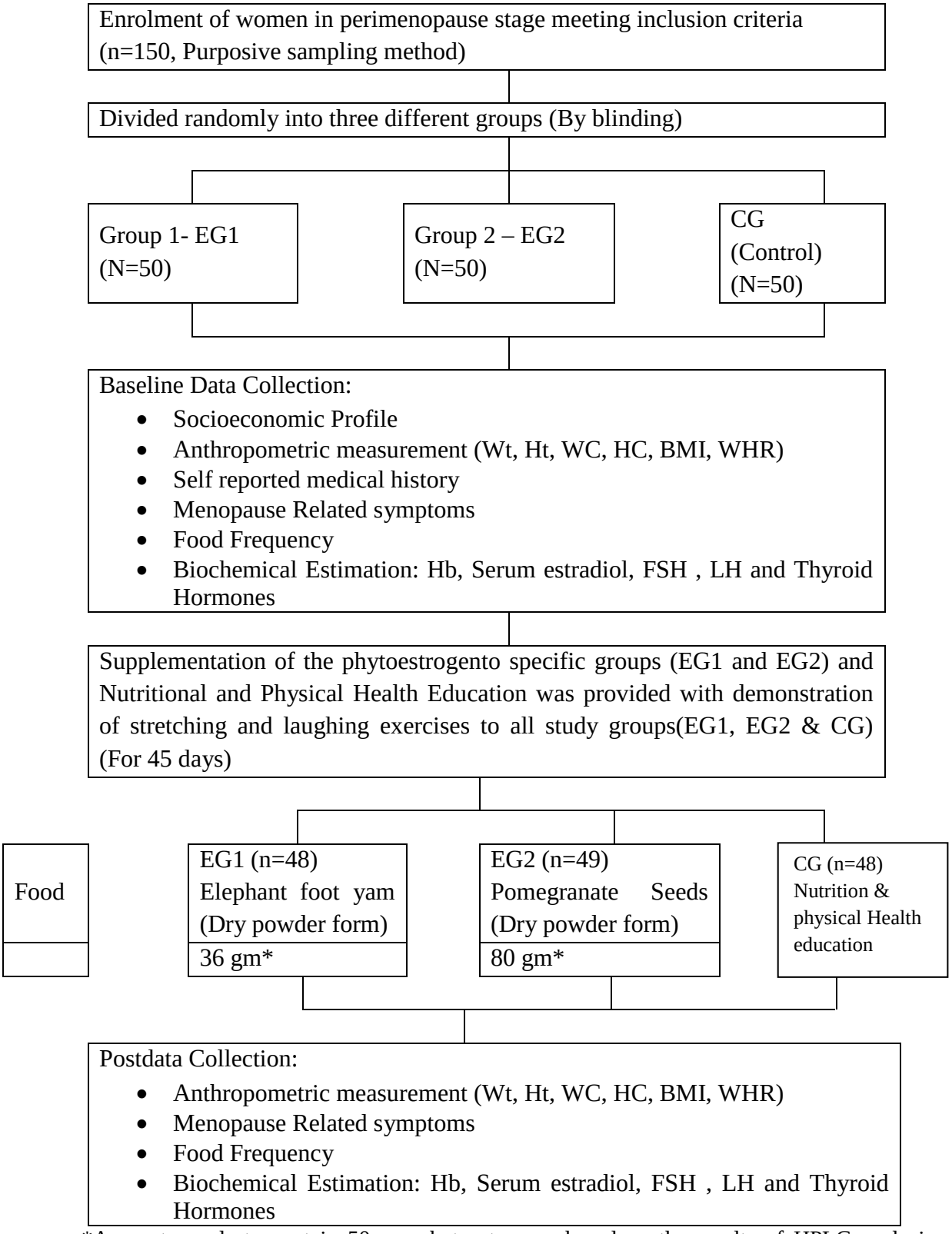
EG2: n=1 (Had malarial infection)

CG: n=2 (not allowed post blood withdrawal)

Data on anthropometric measurements, experiences of menopause related symptoms, biochemical estimations and food frequency were availed.

Ethical Clearance and Approval:

This phase was approved by the Institutional Medical Ethical committee (Approval Number: IECHR/2014/20).



*Amount equals to contain 50 mg phytoestrogens based on the results of HPLC analysis (Phase-2).

Figure 3.4: Experimental design for Phase III

Phase IV: Development and distribution of a guidebook on Menopause and its management.

The mid life women were provided with optional counseling regarding the menopause and its management. They should understand and make efforts to incorporate the necessary attributes with menopausal transition and use integrated approaches towards healthy lifestyle. With this view, a booklet with guidance on "Menopause-and Its Management" was developed in local language (Gujarati) by the researcher. This provides basic information on menopause and its transitions, long-term effects of estrogen loss, and side effects of HRT and use of available therapies to enhance health, steps of some basic exercise and stretching exercise.

Further the booklet includes updated information based on recent scientific evidences (Results of Phase II and III).

3.2Methods for assessment

3.2.1 Anthropometric Assessment

These include measurements weight, height, weight circumference, hip circumference, and waist to hip ratio which are the most common and important parameters for assessing the nutritional status of an individual. All measurements were taken by aresearcher complying standard protocols.

Weight measurements (kg)

Weight is the primary measure of body mass; it is simple and widely used assessment. It is a composite of all body constituents like water, fat, proteins, and minerals, bone etc and very sensitive to small changes in nutrition. Weight measurements were made using bathroom scale. It is a portable tool and can be conveniently used in the field. Subjects were asked to stand erect on the scale without touching anything, without leaning against, with no heavy clothing or footwear and looking straight ahead. The weights were taken twice to ensure accuracy. The scale was recalibrated to zero using standard weights before taking measurement of a new individual.

Height measurements (cm)

Height is a linear measurement of the body, a vertical distance measured from crown of head to bottom of feet (heels).

Height measurements were made using a flexible, non-stretchable fiberglass tape. Subjects were asked to stand erect without touching anything, with no footwear, heels touching the measuring wall (flat wall) and looking straight, ahead in the Frakfort plane. 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 Gluteus maximuswere ensured to be in contact with the measuring wall.In this position, a mark was made on the

wall indicating height of the subjects, which was then measured with the fiberglass tape. Height was taken twice to ensure accuracy and recorded to the nearest 0.1 cm.

Body mass index (BMI) (kg/m^2)

It indicates current nutritional status of an individual. BMI has been recommended as an indicator of choice for under nutrition and over nutrition. BMI was calculated as:

BMI = weight (kg)/height (m^2).

Table 3.1: Cut-offs for BMI

Classification	BMI (kg/m^2)
Underweight	<18.50
Normal	18.50-24.9
Overweight	25.00-29.99
Obese	≥30.00

(Source: WHO 2004)

Waist circumference (WC) (cm)

For the waist circumference,a subject was asked to stand with feet together and arms placed on either side. The measurement was done at the waist mid-way between the lowest rib and the iliac crest with thesubject standing at the end of gentle expiration with light clothing using a fiber glass tap wrapped around subjects. The measurement was recorded to the nearest o.1 cm.

Hip circumference (HC) (cm)

For the hip circumference, a subject was asked to stand with feet together and arms placed on either side.

Hip measurements were taken immediately after waist circumferences. The measurement was done at the maximum circumference over the buttocks with the subject standing with light clothing using a fiber glass tap wrapped around subjects. The measurement was recorded to the nearest 0.1 cm.

Waist to hip ratio (WHR)

Waist to hip ratio is an important tool that helps you determine your overall health risk. People with more weight around their waist are at greater risk of lifestyle related diseases such as heart disease and diabetes than those with weight around their hips. It is a simple and useful measure of fat distribution. WHR is the ration between waist circumference and hip circumference.

WHR = WC (cm) / HC (cm)

WHO - >/= 0.85 – abdominal obese

3.2.2 Assessment of menopause related symptoms

A semi structured pre tested questionnaire was used to inquire about the menopausal symptoms experienced by the women. The subjects were interviewed in the language comprehensible to them (Gujarati/Hindi/English). One to one interview pattern was followed. There were total 18 symptoms listed, of which 11 was mentioned for calculating Menopause rating scale (MRS) score.

Menopause rating Scale (MRS):

The MRS scale is a valuable tool for assessing health related quality of life of women in the menopausal transition and it is used worldwide.

MRS (Menopause Rating Scale) was obtained from the Professor Heinemann from Center of Epidemiology and Health Studies in Berlin.

The MRS includes 11 symptoms. Each of the eleven symptoms contained in the scale get 0 (no complaints) or up to 4 scoring points (very severe symptoms) depending on the severity of the complaints perceived by the women. The score increases point by point with increasing severity of symptoms in each of the 11 symptoms.

The MRS is divided into three subscales:

(a) Somatic – hot flushes, heart discomfort/palpitation, sleeping problems and muscle and joint problems;

(b) Psychological – depressive mood, irritability, anxiety and physical and mental exhaustion and

(c) urogenital – sexual problems, bladder problems and dryness of vagina.

The composite scores for each of the subscales are based on adding up the scores of each item of the respective subscales. (Appendix III)

The symptoms not mentioned in MRS but included for this study were irregular menses, swelling, weight fluctuation, hair loss, constipation, visual problem and nails cracking. The level of severity for these symptoms was also recorded.

3.3Methods for analysis

3.3.1 Biochemical Estimation

Hemoglobin estimation (Cyanmethemoglobin Method)

Principle for Hemoglobin estimation

When blood is mixed with Drabkin's reagent containing potassium cyanide and potassium ferricyanide, hemoglobin reacts with ferricyanide to form methhemoglobin which is converted to stable cyanmethemoglobin by the cyanide. The intensity of the color is proportional to hemoglobin concentration and it is compared with a known cyanmethemoglobin standard at 540 nm (green filter).

Reagent requirement: Drabkin's solution and Cyanmethemoglbin standard

Sample: Whole blood

Procedure for estimation of Hemoglobin:

- Pipette out exactly 5 ml of the Drabkin's reagent in a test tube.
- Add 0.02 ml of whole blood in to a pre-coded test tube having 5 ml of Drabkin's solution.
- Mixed the content thoroughly and wait for 5 minutes.
- Absorbance of standard was read by pipetting directly in a cuvette.
- Absorbance of sample was read at 540 nm by setting blank to 100% T.
- Calculate the Hb for samples using the formula:

Hemoglobin (g/dl)= (O.D. of test/O.D. of Standard) X 15

Hemoglobin cut-off used to define anemia

Normal - $\geq$ 12 gm/dl

Mild anemia – 11 to 11.9 g/dl

Moderate anemia – 8.0 to 10.9 g/dl

Severe anemia - <8 g/dl

Hormone Estimations (T_3, T_4, TSH, FSH, LH, Estradiol)

Blood samples collected were centrifuged, and serum samples were collected with appropriate tools & techniques and analyzed with ECL Assay principles.

ECL Assay Principles (Mathew BC, 2005)

Electrochemiluminescence (ECL) processes are known to occur with numerous molecules including compounds of ruthenium, osmium, rhenium or other elements. ECL is a process in which highly reactive species are generated from stable precursors at the surface of an electrode. These highly reactive species react with one another producing light. The development of ECL immunoassays is based on the use of a ruthenium chelate as the complex for the development of light. The chemiluminescent reactions that lead to the emission of light from the ruthenium complex are initiated electrically rather than chemically. This is achieved by applying a voltage to the immunological complexes (including the ruthenium complex) that are attached to Streptavidin – coated micro particles.

Test Principles

The following test principles were used for the estimation of analytes and antibodies in the samples:

1. Competitive principle

   This principle is applied to analytes of low molecular weight such FT_3, FT_4, Cortisol, Testosterone and Estradiol.

   The measurement is indirectly proportional to the sample concentration.

   High signal = low concentration

   Low signal = high concentration

2. Sandwich principle

   This principle is applied to high molecular weight antigens such as Thyroid stimulating hormone (TSH), Follicle stimulating hormone (FSH), Luteinizing hormone (LH).

   The measurement is directly proportional to the sample concentration.

   Low signal = low concentration

   High signal = high concentration

All the hormone level estimations were carried out at Thyrocare which is nationally accredited laboratory.

Table 3.2: Reference range for biochemical indicators

BIOCHEMICAL INDICATORS	REFERENCE RANGE
T_3	60-200ng/dl
T_4	4.5-12.0µg/dl
TSH	0.30-5.5µIU/ml
FSH	**(mIU/ml)**
Follicular phase	2.5-10.2
Mid –cycle peak	3.4-33.4
Luteal phase	1.5-9.1
Pregnant	<0.3
Perimenopause	>20
Postmenopausal	23.0-116.3
LH	
Follicular phase	1.9-12.5
Mid –cycle peak	8.7-76.3
Luteal phase	0.5-16.9
Pregnant	0.1-1.5
Perimenopause	>20
Postmenopausal	15.9-54.0

3.3.2 High Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is a separation technique that can be used for the analysis of organic molecules and ions. HPLC can be used to assess the purity and/or determine the content of many pharmaceutical substances.

Principle:

HPLC is based on mechanisms of adsorption, partition and ion exchange, depending on the type of stationary phase used. HPLC involves a solid stationary phase, normally packed inside a stainless-steel column, and a liquid mobile phase. Separation of the components of a solution results from the difference in the relative distribution ratios of the solutes between the two phases.

Materials

The solvents – water, acetonitrile and acetic acid used for HPLC and optical density readings were of analytical or HPLC grade, vacuum-filtered through a 0.45 µm filter. HPLC grade water, acetic acid and acetonitrile were purchased from Fisher Scientific Company.

Standard Solutions

The standard chemicals of daidzein 98%, genistein 100%, secoisolariciresinol 95%, matairesinol 85%and coumestrol 97.5% were purchased from Sigma Aldrich. Co. (USA).

The standards for each phytoestrogen (daidzein, Gensitein, Secoisolariciresinol, matairesinol and coumestrol) were dissolved in 60% ethanol to prepare a stock solution of 1000 ppm. Working standard solutions were prepared by serial dilution of the stock standard.

Sample extraction

The dry seeds of all food sampleswere procured from local market of Vadodara (Gujarat, India). The seeds were ground into fine powder using a mixture and resulting sample was used for further analysis. The extraction was carried with the help of 60% ethanol. The resultant mixture was sonicated for 4-5 minutes and allowed to stand for 40 minutes. The supernatant was collected and filtered before injecting into HPLC system.

Alkaline Hydrolysis

For alkaline hydrolysis, 0.5 g powder was treated with 25 ml 2M NaOH, for 1 hour at room temperature, followed by acidification step with 40% H_2SO_4 to adjust for pH 3. The extraction was done with the same procedure as described earlier.

All the analysis was carried out in duplicates. The values were expressed in µg/g (ppm).

HPLC Analytical Conditions:

The details of the instrument used were Merck Hitachi HPLC (Lachroma model) L-7100 pump with UV-VIS L-7420 detector having D-7000 interface. The C_{18} column (Chrombudget-Bischoff Chromatography, A German Company) of 0.46x100 cm long having 5 micron was used for the separation. The mobile phases were composed of water, acetonitrile and acetic acid. The adjustable experimental variables were the conditions of gradient modes and mobile phase compositions. The mobile phase A consists of water/acetonitrile/acetic acid (94.9/5/0.1 v/v/v) and mobile phase B consisted of water/acetonitrile/acetic acid (5/94.9/0.1 v/v/v). The flow rate was kept at 1 ml/min, the injection volume was 20 µl with following step wise gradient over 25 minutes- where 100% of

mobile phase A ran for 15minutes; from 15 to 20 minutes where mobile phase changes from 0% to 70% and mobile phase A changes from 100% to 30%; and finally during last five minutes mobile phase A again changes to 100%.Phytoestrogens were detected using UV absorbance at 245 nm. Optimisation of HPLC conditions as a standard procedure was carried out prior to analysis.

(a) (b)

Figure 3.5: Instruments used for (a) Filtration of solvents (b) HPLC analysis

Figure 3.6: Process of HPLC analysis (Technical support by aLab technician in HPLC analysis)

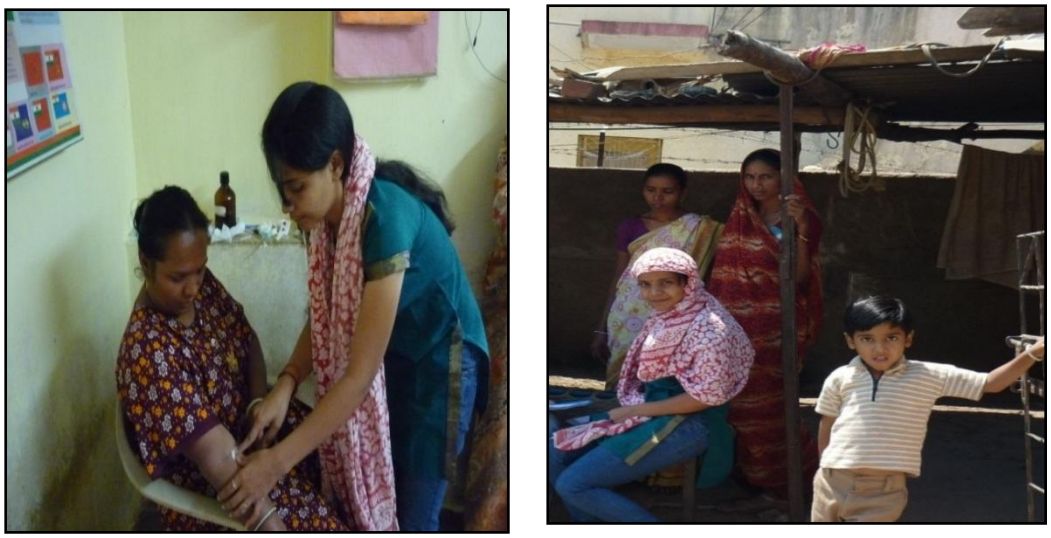


Figure 3.7: Home visits during data collection

Figure 3.8: A valuable supporters of the study – women participants

Figure 3.9: Nutrition and physical health education

Figure 3.10: Community outreach – distribution of booklet "Menopause and its management"