3

Materials and Methods

The research methodology relevant to the study on "**Impact of Nutrition Interventions on Symptoms of Polycystic Ovarian Syndrome (PCOS) among Women of Reproductive Age (20-45 Years)"** is constituted in the following phases.

PHASE - I

Incidence of PCOS among the women in the Reproductive age groups (20-45 years)

A. Selection of the study area

B. Identification of subjects having PCOS using the validated screening tool

C. Formulation of research tools to conduct the study

PHASE – II

Mapping of subjects for Nutrition interventions

A. Conduct of the survey

B. Assessment of Nutritional status of the selected subjects

i) Checking Anthropometric measurements

ii) Estimation of Biochemical parameters

iii) Clinical examination and assessment

a) Assessment of Hirsutism

b) Assessment of Acne

c) Assessment of mental health status

d) Assessment of Physical Activity Level

e) Assessment of Menstrual irregularity

iv) Quantitative dietary Intake

PHASE - III

Formulation and Evaluation of Micro Nutrient rich supplement powder and Nutrition and Health Education modules on PCOS

A) Formulation and Evaluation of Micro Nutrient rich supplement powder

a) Selection of ingredients

b) Formulation of Nutritional Supplement

c) Sensory evaluation of the supplement

d) Estimation of nutrient content of the supplement.

e) Analysis of microbial count and cost effectiveness

f) Packing the nutritional supplement

B) Development and Validation of Nutrition Education modules on Nutritional status and polycystic ovarian syndrome

PHASE – IV

Effect of Nutrition interventions on improving the nutritional status and Symptoms of Polycystic Ovarian Syndrome (PCOS)

A) Selection and Grouping of PCOS participants for nutrition interventions

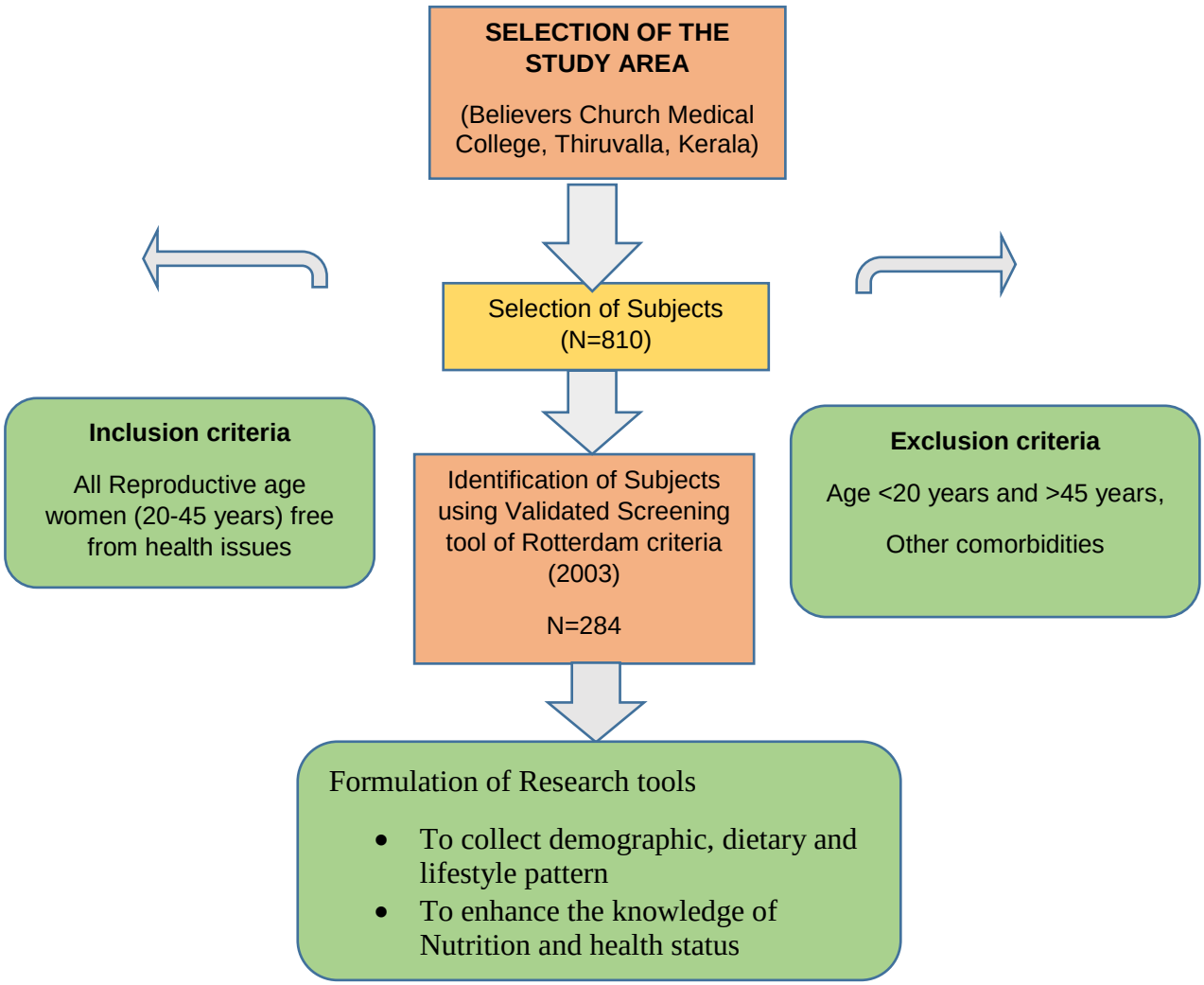
B) Effect of nutrition education and supplementation on nutritional status, biochemical and clinical symptoms among selected participants

PHASE – V

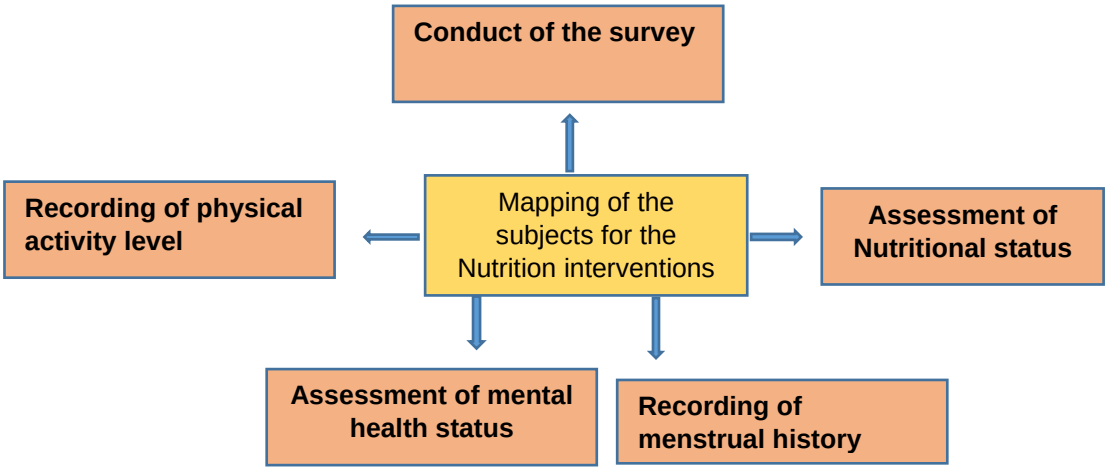
Statistical Analysis and Interpretation of data

RESEARCH DESIGN

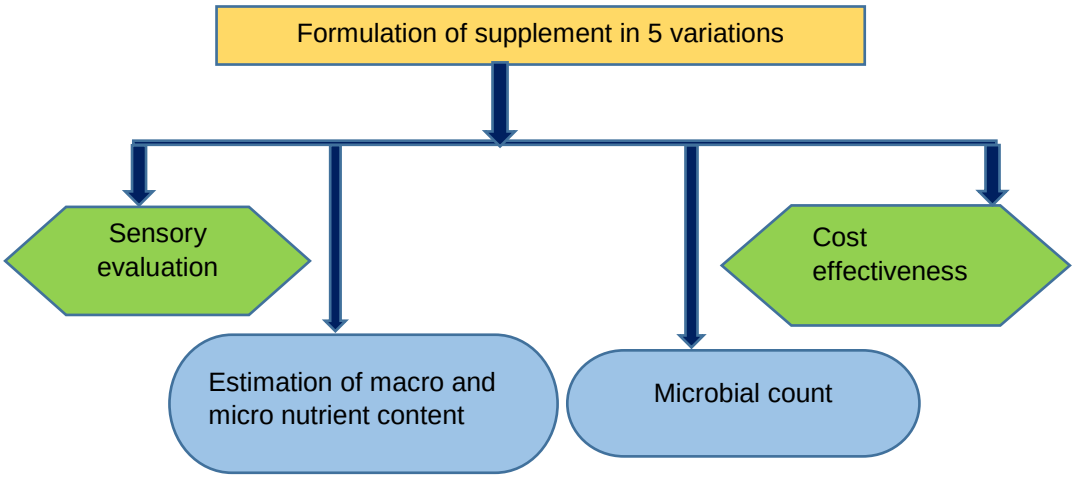
PHASE I: Incidence of PCOS among the women in the Reproductive age groups (20-45 years)



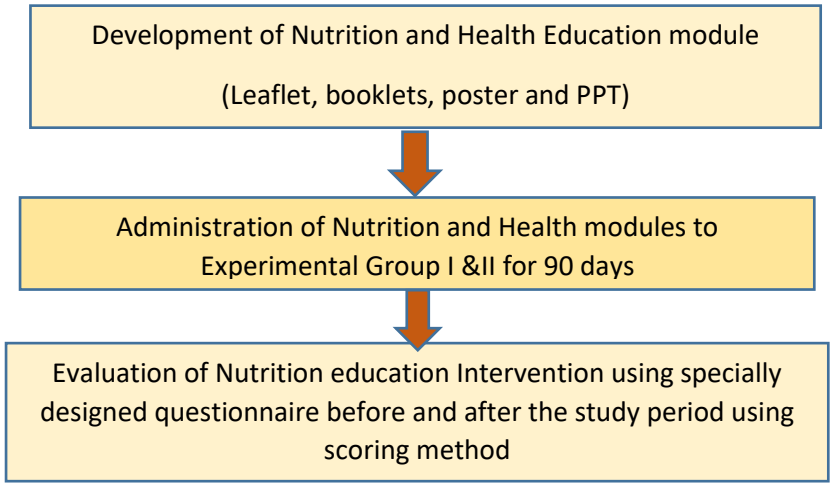
Phase II Mapping of subjects for nutrition intervention



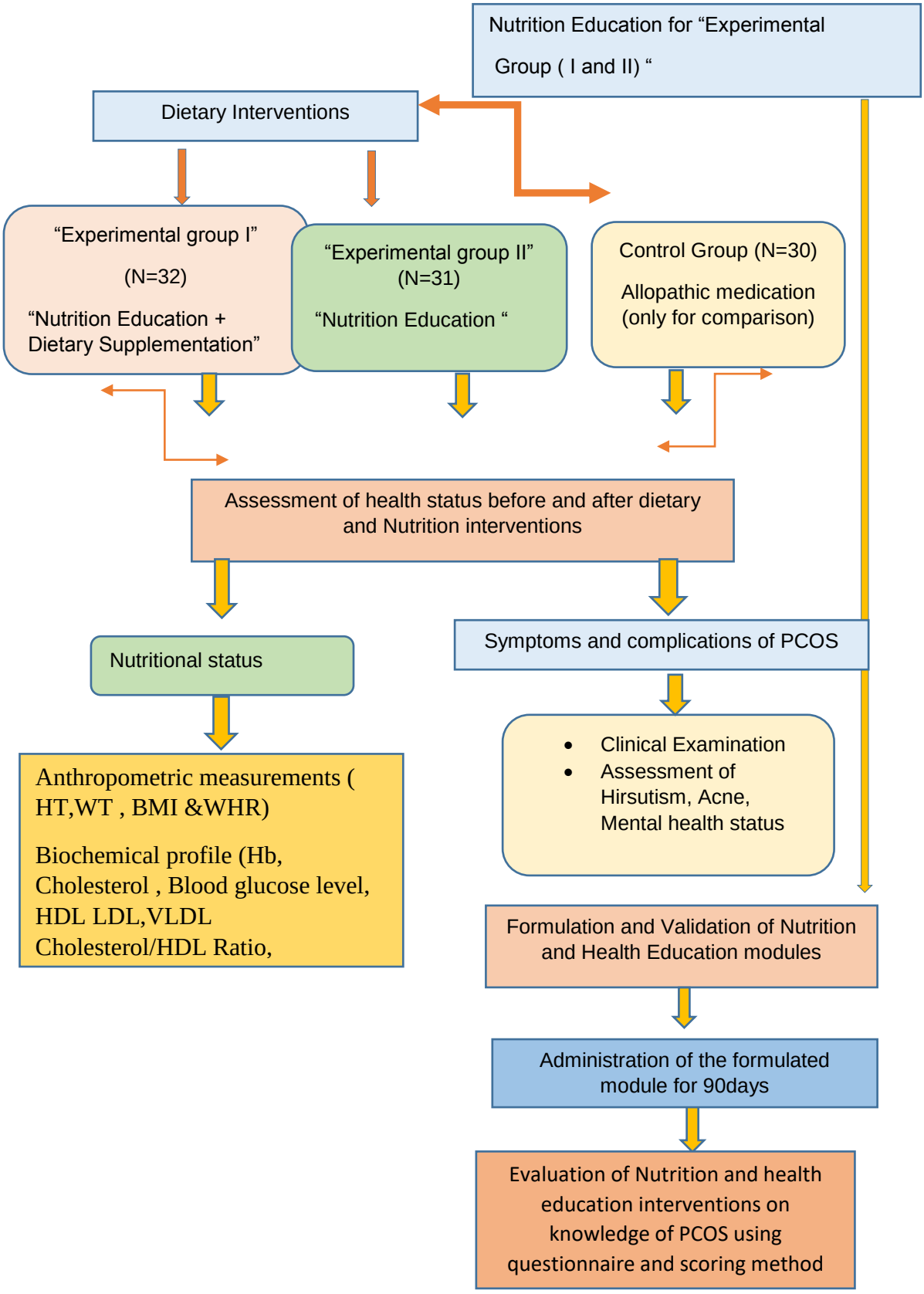
PHASE III (A) Formulation and Evaluation of Supplement – (Micro Nutrient rich Supplement powder)



PHASE III (B) Development and Validation of Nutrition and Health Education modules



Phase – IV Nutrition Interventions on Nutritional status and Clinical Symptoms of PCOS



PHASE – I : Incidence of PCOS among the women in the Reproductive age groups (20-45 Years)

A. Selection of the study area

The present study was performed in the Gynaecology and Dietetics Outpatient Clinic of Believers Church Medical College, which is a health care institution of Believers Church located in Thiruvalla, Kerala, India. The selected Medical College is attached to 743 beds multispecialty hospital.

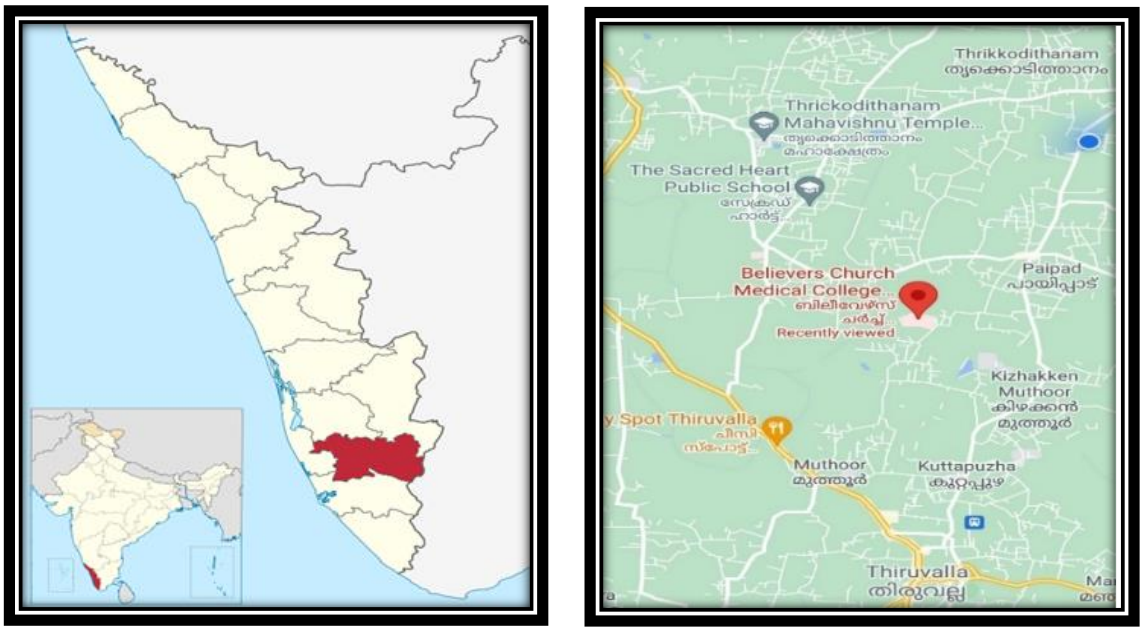


Figure- 7 Hospital area map

This medical college hospital was selected for the study on the basis of easy accessibility, availability of adequate number of subjects and the co-operation to carry out the present study effectively. The regular health check-up of the subjects was carried out between20-45 year age group and the presence of Polycystic Ovary syndrome was screened using a validated tool Rotterdam criteria (2003). The screened positive subjects were considered for the present study.

B. Identification of Subjects having PCOS

Ovarian abnormality is the primary cause of the disorder of Polycystic Ovarian Syndrome (PCOS) or Stein-Leventhal syndrome. Obesity and various other ecological factors responsible for development of specific symptoms (Wołczyński, 2012). Nearly 28 percent of women of Reproductive age suffers from Polycystic Ovarian Syndrome as per the latest studies done in India (Chatterjee 2020). Study done in South India among medical students confirmed eight percent of university students were confirmed the cases of PCOS (Joseph 2016).In a clinical study carried out by Vijayan (2013) revealed

that in Central Travancore, Kerala,15 percent of University students were confirmed the presence of PCOS .In the present study, reproductive age women of 20-45 age group consulted in the Gynaecologist and Dietician between "January 2019 and June 2021" were subjected to screening using the validated screening tool Rotterdam criteria (2003)

Table II

Selection of subjects for the study based on Rotterdam 2003 criteria

To confirm PCOS, the subject should have any two of the three features
1.Clinical features of Oligomenorrhea Irregular menstrual cycle - Absence of Menstruation for more than 35 days – 182 days, Amenorrhea (Absence of menstruation for more than 182 days)
2. Ultra sound scan with at least 12 follicles of 2-9mm in diameter with a pearl like appearance arranged in the ovarian stroma, Ovarian volume >10mm^3
3.Clinical or biochemical evidence of hyperandrogenism (Hirsutism, acne, androgenic alopecia or elevated serum androgen)

In order to find out the prevalence of PCOS among the reproductive age women a quick screening of symptoms of PCOS was carried out among 810 women consulted the gynaecologist from January 2019 to July 2021 this included not only PCOS but women of Reproductive age group.The presence of two criteria from clinical, Hormonal and Ovarian cyst on Ultra sound scan was considered as the diagnostic criteria. Based on the Rotterdam criteria (2003).Using the formula below mentioned, the adequate sample size was calculated as 268.

$$N = \frac{Z^2 \, P(1-P)}{d^2} = \frac{(1.96)^2 \times 0.225 \times 0.775}{(0.05)^2} = 268$$

[Where N= sample size; Z= Normal deviate at a level of significance of z=1.96to 5 % level of significance P= Prevalence of PCOS 22.5 percent (as per studies by Mohammad Ashraf Ganie *et .al,;*2019),d =margin of error , taken as 5 present .

The sample size of 268 was for finding the prevalence of PCOS ,for studying the socioeconomic profile, nutritional status , lifestyle pattern of the population as mentioned in the first, second and third objective of our study. Considering the drop out of 6 per cent total sample was calculated as 284 .After studying the Nutrient intake pattern of the population, the lacunae in the micronutrient intake was identified and micronutrient rich supplement was designed and standardization of formula was initiated. The selected 284 subjects were given orientation programme highlighted the protocol,

purposes and procedures to be followed for the study. Before the conduct of the study, an effective communication was created with the study subjects and also with their supportive family through proper counselling and interactive sessions in order to motivate them and to extend their complete support and cooperation for the effective outcome of the research study. The selected PCOS subjects were informed about the purpose of the study. They were also educated about the nutritional significance and health benefits of nutrient dense health mix to be used for dietary supplementation and the importance of nutrition education and nutrition assessment for evaluating the health status of them. They were also informed about the types of interventions such as medical nutritional care and support, suitable diet plan, the need for data collection, including biochemical values both before and after the three months of dietary intervention. The selected subjects and their family members were informed about the pathophysiology and causes for development of the syndrome and procedures to be carried out during the study for minimising the consequences of PCOS and also prevention of PCOS for promotion and preservation of optimum health status. All women were intimated about the study and collected the written consent from them. The selected women in the incidence study were included for anthropometric measurements, physical and clinical examination for hyperandrogenism such as acne, hirsutism, and menstrual history to diagnose PCOS as per the protocol of Rotterdam Criteria (2003) enclosed in the **Annexure I**.

For the nutrition interventions, 93 subjects were identified from 284 subjects on the basis of their willingness, cooperation and nutritional as well as health status. Table III points out the inclusion and exclusion criteria considered for inclusion of subjects for current research study.

TABLE III

Inclusion and Exclusion Criteria for selection of subjects

Inclusion criteria	*Exclusion Criteria*
Adult Women having irregular menstruation and fulfilment of Rotterdam criteria	Normal adult women, pregnant and lactating mothers
Women between the age of 20 and 45 years and giving written consent	Age >45 years, <20 years Having comorbidities
Clinical or Biochemical examinations and to willing to participate and co-operation to undergo	Not willing to participate in the clinical examination or biochemical estimation and non-co-operative
PCO morphology on ultrasound	Not willing to undergo ultrasound examination

C. Formulation of Research Tools to conduct the study.

Formulation of Research tools in accordance with the objectives completed initially. The tools were used to collect the data which are observable and measurable for further analysis and interpretation of data. Data collection was done using interview schedule and also by using specially designed questionnaire among the selected subjects of reproductive age 20-45 years. In the interview schedule, Demographic details including educational level, occupational status, income per month and food pattern were also included. In Anthropometric measurements Height, weight, Body Mass Index (BMI) and Waist Hip Ratio (WHR), body fat visceral fat, Triceps skin fold thickness were assessed.Clinical examination consisted of menstrual history, hyperandrogenism such as acne, hirsutism and so on and were executed to collect data.

Questionnaire schedule **(Annexure II)** was formulated incorporating closed ended and open-ended questions to obtain relevant specific data from the selected subjects. The comprehensive questionnaire consisted of socio demographic, dietary and lifestyle history, medical history reproductive history and food frequency questionnaire and was collected with one to one meeting and form were filled in questionnaire schedule manually and also by "*google docs"* forms. Before the questionnaire was administered for the study the validity and effectiveness of tool was evaluated .The specially designed tool was administered to a micro sample of 20 and based on the inputs of the respondents necessary changes were carried out to make it effective and relevant for the further study. An interview schedule was used to collect subjective information of the selected subjects. The study received the approval from "Clinical Trial Registry of India (CTRI) "**CTRI/2021/09/036850)",** Ethical clearance from the Institutional Human Ethical Committee (IHEC) of Avinashilingam Institute for Home Science and Higher Education foe Women (**AUW/IHEC-1920/FSN/FHP-01)"** and also approval from "Institutional Ethical Clearance Committee of Believers Church Medical College Hospital with approval number **IEC/2020/02/126** .

PHASE –II : Mapping of subjects for nutrition intervention

A) Conduct of the survey

The survey was carried out to collect the data related to demographic profile, age, educational, marital status, income levels, family history, medical and reproductive profile including disease history such as diabetes mellitus hypertension and so on in any other family members , menstrual irregularities , hirsutism and alopecia in male family member were recorded .Dietary pattern, Food frequency pattern **(annexure III),** 24 hour recall ,food consumption details of selected participants (N= 284)was collected using validated

questionnaire after getting approval from the Ethical study committee of Avinashilingam Institute for Home science and Higher education for women, Coimbatore and Ethical clearance committee of the Hospital. The socio-economic profile of the selected subjects was analysed using Kuppuswamy Socioeconomic Scale (Saleem, 2020) and is given in **annexure IV**. Before conducting the survey, a good rapport was created with the selected study subjects. The purposes, objectives and benefit of the study was clearly explained to the selected subjects and written and oral consent were obtained to conduct the study effectively and systematically. The investigator collected the reliable and correct data using the specially designed interview schedule and the observations cum interactions and exchange of their views from the selected subjects.

B Nutritional Status assessment of the selected Subjects

i) Anthropometric measurements

Assessment of nutritional status of the selected subjects included anthropometric, biochemical, dietary, medical profile and clinical examination .In the present study, the nutritional status of the selected subjects was evaluated by ABCD Techniques Anthropometric measurements such as weight, height, BMI, WHR, body fat, and Triceps Skin Fold (TSF) were recorded using standard procedures. Height was measured using a portable stadiometer (Model HM01) to nearby the head was held straight and erect and arms hanging at the sides in a natural manner. A head piece (scale) was lowered, touching the hair and top of the head and height was measured to the nearest 0.1 cm. The process was repeated three times to ensure consistency in the reading obtained. Weight was measured with an accuracy of 10 g using digital weighing scales (SAMSO)**.** BMI was evaluated by calculating the ratio between weight in kilograms to square of height in meters**.** According to Asian Classification, the BMI was classified and mentioned in Table III

Table IV Grading of malnutrition based on BMI

Criteria	BMI range
Severely underweight	<16.5kg/m²
Underweight -	<18.5 kg/m²
Normal weight	≥18.5 - 22.9 kg/m²
Overweight	≥23 - 24.9 kg/m²
Obesity	≥ 25 kg/m²
Obesity grade I	25 - 29.9 kg/m²
Obesity grade II	30-39.9 kg/m²
Obesity grade III	≥40 kg/m²

Ref: Asia Pacific Classification of BMI, WHO Expert Consultation. (2004).

Waist measurements were taken with fibre glass tape at thinnest point between the last rib and pelvic crest, soon after exhalation. Waist circumference (WC) of the selected subjects of 284 subjects were measured at the centre point between the lower border of the ribcage and iliac crest and Hip circumference (HP). The largest circumference between the waist and knees was measured using a measuring tape. Widest part was measured for Hip circumference in inches. Waist to hip ratio was tabulated (Lohman,*et. al.,*1988). Waist Hip Ratio (WHR) was computed as the ratio of waist Circumference(cm) to hip circumference (cm) and used to assess abdominal obesity.As per the guidelines (ICMR, 2019), the cut-offs for WHR is 0.80 for Indian woman. Reproductive age women who were having ≥0.80 were considered as abdominally obese. A Digital Body composition analyser, Eagle EEF 2001A was used for measuring the total body fat. Triceps Skin Fold thickness was measured using Baseline – USA 60Mm slim guide skin fold calliper, 9”x0.5”x6” and recorded for further analysis.

Ia) .Body composition Analysis

A digital body composition Analyser , Eagle EEF 2001A was used for measuring body fat , body water and visceral fat .Body fat scales employ a method called bioelectrical impedance analysis (BIA) to calculate the relative percentages of different body tissues and substances. Bioelectrical Impedance analysis (BIA) sends a mild electrical impulse throughout the body.A safe, mild, and imperceptible electrical current is passed through the body when stepping on the scale. It travels from one foot to one leg, across the pelvis, and down the other leg,measuring resistance in accordance with the quantity of fat.The scale's sensors will then compute the body fat and other pertinent data in accordance with that.The different materials and tissues exhibit different levels of impedance, or resistance, to the impulse. Following that, the electrical resistance value is combined with information about an individual's age, height, and gender using a mathematical formula that the scales apply. People typically supply this information via their cell phones or other electronic devices. The mathematical formula is then used to estimate the proportional amounts of bone, muscle, water, and total fat.Fat provides more resistance than either muscle or water. Therefore, calculations of a higher proportion of body fat usually result from higher resistance levels.

ii) Biochemical estimation

In the initial stage of data collection random blood glucose levels (RBG) and serum lipid profile, serum testosterone, and haemoglobin was carried out in PCOS subjects to diagnose defects in sugar tolerance ,high lipid levels in blood and associated risk factors .The normal values considered were Random blood glucose less than 140mg/dl, total cholesterol less than 200mg/dl, High Density Lipoprotein less than

50mg/dl, Low Density Lipoprotein less than 130mg/dl, Triglycerides less than 150mg/dl and Very Low Density Lipoprotein less than 50mg/dl. Moreover, BP was also measured (>/= 130/85mmhg) was considered increased levels and to be reported as hypertension. As a part of the immune assay, total testosterone levels were estimated .The National Cholesterol Education Panel ATP, III Diagnostic criteria (2001) revised in (2004) was used to select subjects with metabolic syndrome, the definition was , Waist Circumference above 102cm, Plasma Triglyceride above 150mg /dl, Serum HDL Cholesterol below 40mg/dl, BP levels above 130/85mm Hg, Fasting plasma glucose above 100mg/dl .

iii) Clinical examination

Clinical history including the use of hormonal drugs including Oral Contraceptive Pills (OCP), antihypertensive medications, and mediations for lowering blood glucose and lipid levels in the blood. Menstruation history was collected (irregular menstrual cycle, hyper androgenic features, and family history of ovarian cyst, infertility, clinical examination).The selected subjects were screened for clinical signs of androgen excess mainly manifested as dermatological changes. Modified Ferriman-Gallwey (FG) score (Ferriman and Gallwey, 1983) was used to assess hirsutism, Level of severity of Acne was assessed using Global Acne Grading System (Ramli, R.2012), Mental health status using Perceived Stress Scale (Cohen, and Williamson, 1988) and physical activity Scoring using Godin Leisure Scale (Godin,2011) and Ultrasonography was also used to detect PCOS among the selected 284 subjects

a) Assessment of Hirsutism

Modified Ferriman-Gallwey (FG) score defined the clinical feature hirsutism and hyper androgenism **.(annexure V)**.It is the standard scoring system used for evaluation and quantification of hirsutism by including nine androgen dependent sites (Hatch et. al, 1981) and (Ferriman and Purdie,1983). The nine body areas hair growth was examined and evaluated the score .The body parts examined were, upper lip, chin, chest, upper and lower abdomen, thighs, upper and lower back and upper arm. The hair growth was rated from 0 (no growth of terminal hair) to 4 (extensive hair growth) in each of the nine locations. A score >/= 8 was indicative of hyperandrogenism. In each of the nine location the hair growth was assessed from zero to four, where zero indicates no terminal hair growth and four indicates extensive growth of hair. Hyperandrogenism is designated with a total score of greater than or equal to eight.

b) Assessment of Acne

Acne lesions were counted by Global Acne Grading System **(annexure VI)** was used where the numbers of open and closed comedones, papules, pustules and nodules were counted. Based on the location, the scores are given. Different type of lesions were allotted a severity score. According to the lesion type the score was given .Different types of lesions were assigned scores (lesion score) similarly the score was assigned to each location . By multiplying types of lesions / lesion severity score by its the particular location score (the factor score) total acne score can be tabulated by adding the scores assigned to each location.

c) Assessment of mental health status

The perception of stress was assessed by Perceived Stress Scale (PSS) **(annexureVII)**, the most extensively handled psychological instrument by the psychologists for assessing the stress perception of individuals. Stress scoring is a method of quantifying the of the degree stress a person is suffering from. It measures to which situations in one's life accounted as stressful. The questions in the PSS revealed the feelings and thoughts during the last month of the study period.

d) Physical Activity Level

Randeva *et al.,* (2012) showed that physical activity such as brisk walking, reduces Waist Hip Ratio, and homocysteine levels as an indicators of insulin resistance, cardiovascular risk respectively in overweight PCOS women.Godin Leisure Time questionnaire **(annexure VIII**),asked about the frequency and intensity of different kinds of exercise more than 15 minutes in seven-day period, which includes 13 types of vigorous intensity activities (e.g., long distance bicycling, jogging or running, vigorous swimming),10 types of moderate intensity activities (fast walking, baseball, dancing) and eight types of light exercises (yoga, easy walking). Physical activity scores were calculated by multiplying the scores of regularity by intensity of each activity and adding the scores of all the activities. Score less than 14 units indicated insufficiently active in physical activity level and the physical activity level of the participants were carefully recorded for statistical analysis

e) Assessment of Menstrual irregularity

The selected 284 subjects were asked about the menstruation history, frequency of menstrual flow and regularity of cycles. Secondary amenorrhea was encountered as a problem in menstrual cycle, where absence of periods for at least three of the previous cycle intervals or for more than six months. Menstrual intervals shorter than 20days or greater than 35 days to six months was considered as oligomenorrhea and data related to menstrual cycle were gathered from the selected 284 subjects.

Plate 1 Recording of Anthropometric measurements

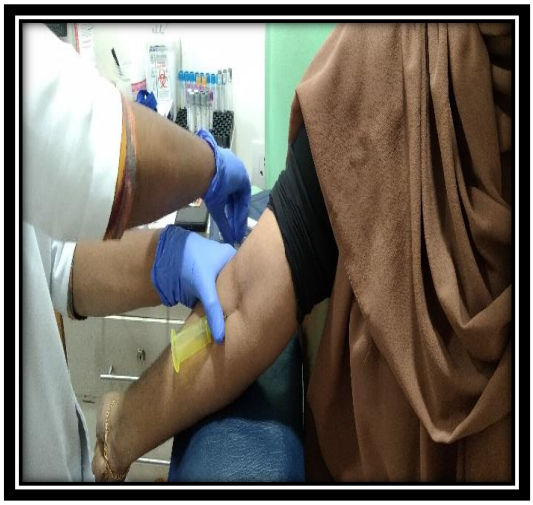


Plate 2 Collection of blood sample

Plate 3 Clinical Examination

Plate 4 Documentation of Dietary Intake

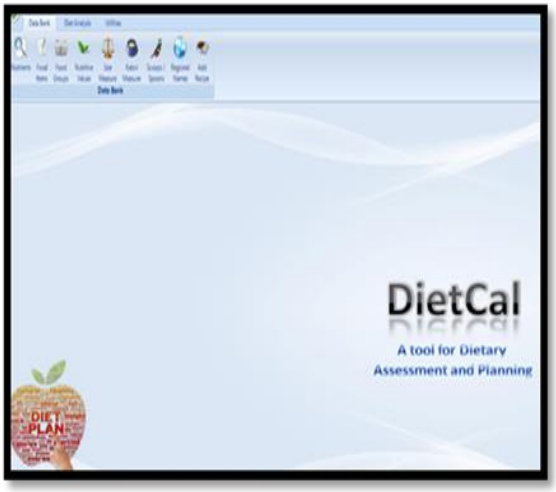


Plate 5 Diet Cal dietary software to calculate nutrients

iv) Quantitative Dietary intake

Quantity consumption of food items and meals were collected from the selected 284 subjects using two methods: Assessment of quantitative dietary intake was performed by “one-day food record from the last 24 hour dietary recall. Collected in formation’s and the interview included: meals timing, ingredients, quantity, and preparation method of every meal. The sizes of consumed portions were determined according to the Album of photographs of food products and dishes of IFCT (Indian Food Composition Table, NIN 2019) and also by showing the household measuring cups and spoons. A dietary software DIET CAL was used to calculate the calorie, protein, fat, carbohydrate, vitamins, minerals, and dietary fibre. The obtained results were compared to the standard values suggested by ICMR RDA (2020). A questionnaire consisting of 15

food groups were included in the Food frequency questionnaire" .It was used to find out the consumption of different food groups. It contained 15 food types and questions to get information on consumption pattern to categorise the selected subjects into regular, occasional and rare consumers.

PHASE – III : Formulation and evaluation of Micro Nutrient rich Supplement Powder and nutrition education modules on PCOS

A. Formulation and evaluation of Micro Nutrient rich Supplement powder

A long term remedy for degenerative diseases focuses on intervening balanced diet to affected population. It imparts a curative role in management of any lifestyle disease. Nutrition supplementation with suitable nutrition education intervention is one of the effective methods to achieve the nutritional requirements of an individual. Nutritional supplements were made using the commonly available oilseeds, nuts and grains available in the market, which were low cost, seasonal and easy to prepare. All these factors taken into consideration for preparing a micronutrient dense supplement which is predicted to improve the reproductive issues and also alleviating biochemical, clinical , psychological, and metabolic issues of the patient .

a) Selection of Ingredients

Micronutrients such as calcium, phosphorus , vitamin B complex , Vitamin E, Omega 3 fatty acid , iron, selenium, zinc, magnesium and fibre was rich in the nutritional supplement, and was prepared using millets and oil seeds available in the market. Table-V and Figure 2 indicate the details of the food components used in the preparation of nutritional supplements in different variations

Table V Ingredients used in the Nutritional supplement powder

Ingredients	Botanical name	Specific nutrient	Action
Sprouted Finger millet 	*Eleusine coracana*	Iron and Calcium	• Involved oocyte maturation, • Successive follicular development. • Helps insulin signalling pathway
Sprouted Pearl millet	*Pennisetum glaucum*	Vitamin A, Selenium, Magnesium, MUFA	• Controls glucose metabolism • Regulates insulin

Ingredients	Botanical name	Specific nutrient	Action
			metabolism • Decrease testosterone level
Flax seed 	*Linum usitatissiumum*	Omega-3fatty acids, Zinc , Potassium Selenium , Phosphorus	• Increase IGF 1, • Increase oocyte production • Reduces insulin resistance • Selenium can reduce dyslipidaemia and Insulin resistance
Pumpkin seed 	*Cucurbita pepo*	Zinc, Potassium, Iron Phosphorus, Magnesium, MUFA, PUFA	• Reduces dyslipidaemia • Reduce Insulin resistance • Decreases testosterone level
Sunflower seed 	*Helianthus annuus*	Zinc , Potassium MUFA, PUFA	• Decrease testosterone level
Sesame seed 	*Seasmum indicum*	Rich in EFA , Iron and Calcium	• Improve blood Iron and Calcium levels, • Improve insulin sensitivity
Brown sugar 	*Sucrose*	Simple sugar , Iron	• Add flavour and taste • Improves Iron

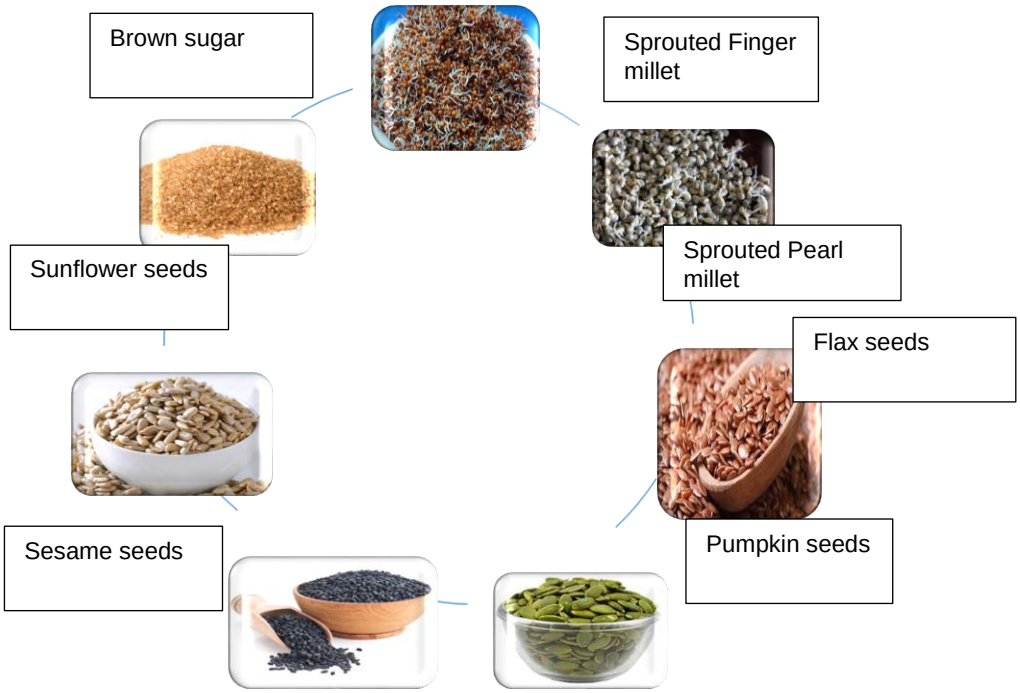


Figure 8 Ingredients of the nutritional supplement

i) Pre preparation of the ingredients

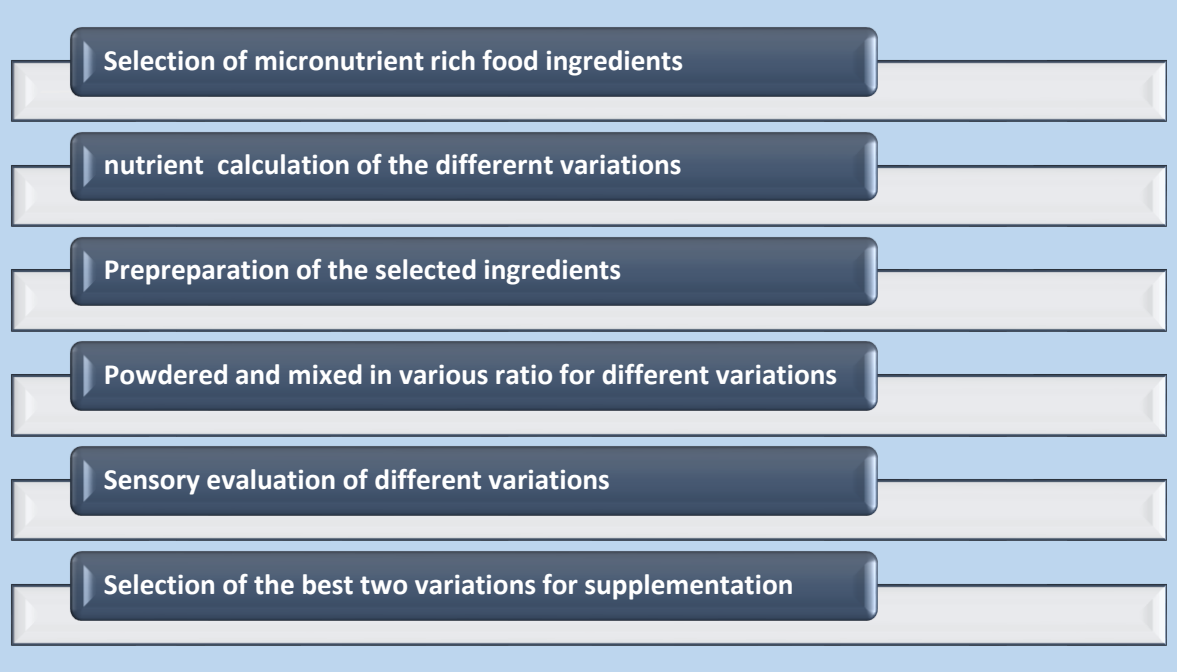
Finger millet and pearl millet were germinated by soaking in cold water for 24 hours. Then it was dried in a mechanical drier for 18 hours. Both the germinated ingredients were roasted for 3-6 minutes and powdered in a mixer grinder. The seeds were introduced in specified proportions after slight roasting for different variation. The ingredients were mixed in various combinations and ground into a powder for ready to consume form .Quantity of ingredients used in each of the variations is given in Table –VI.

Table VI Quantity of ingredients for the formulation of Supplement powder

Ingredients	VARIATIONS IN AMOUNTS (100g)				
	i.	ii.	iii.	iv.	v.
Sprouted, dried and roasted Pearl millet	15	15	15	15	10
Sprouted , dried and roasted Finger millet	10	15	10	15	5
Flax seed	20	20	15	10	20
Pumpkin seeds	20	20	15	15	20
Sesame seeds	15	10	20	20	20
Sunflower seeds	15	15	20	20	20
Brown Sugar	5	5	5	5	5
Total	100g	100g	100g	100g	100g

b) Formulation of Nutritional Supplement

The steps involved in the formulation of Nutritional supplement includes



Steps involved in the formulation of nutritional supplement

c) Sensory evaluation of the supplement

Sensory evaluation is the judging the overall acceptability of the food products in terms of taste, appearance, colour, flavour and mouth feel. In Sensory evaluation the following steps are adopted. In the first step of sensory evaluation the variations were prepared at different point of time and considered for sensory evaluation scoring. Sensory evaluation was repeated to avoid the biased results and remarks were registered systematically. Sensory evaluation for the five variations were executed thrice to have more reliable results. In Five variations, the variation which secured the highest acceptability scores were opted for further analysis in terms of standardisation of supplement and estimation of nutrient content.

i) Selection of panel members

In sensory panel, a group of assessors, were selected to participate in the sensory evaluation, twenty semiskilled nutrition personnel were selected on the basis of their health, co-operation, willingness and knowledge of sensory assessment and also has the ability to differentiate the various criteria considered for sensory evaluation

ii**. Sensory evaluation using hedonic scale**

A “Nine-point hedonic scale” was used for sensory evaluation in terms of appearance, colour, taste, flavours and texture and grades were given according to the

degree of acceptance by the selected taste panel members **(annexure-IX)**. Scores secured in the Sensory evaluation of the nutritional supplement is given in Table VII. Variation III secured the highest score for all sensory properties followed by variation I

Table VII Scores of the supplement in different variations m

Properties	Scores of Variations				
	Variation I	Variation II	Variation III	Variation IV	Variation V
Appearance	6.5	6	8.1	6.3	5.5
Taste	6.3	5.6	7.5	6.6	6.0
Colour	6.5	3.25	6.75	6.25	6.2
Flavour	6.6	4.3	7.3	6.75	6.2
Texture	6.5	2.8	8.1	5.7	5.5
Mean Score	6.48	4.38	7.55	5	5.8

d) Estimation of Nutrient content of the supplement

Five variations of the supplements were prepared and considered for sensory evaluation. The two variations which got the highest scores were chosen for nutrient analysis. The acceptability of the best two variations were tested after preparation of different types of porridges. Each of the variations were supplied as 30g as one feed .Variation I and III of supplement were used for the preparation of porridges and used for analysis of the nutrient content suggested by ICMR-NIN Laboratory Manual (2020).The selected two variations of nutritional supplement were considered for the nutrient analysis, using Standard methods the procedures are adopted in nutrient analysis. Table VIIII gives the procedures adopted in nutrient analysis. The details of the methods and procedure is provided in **Annexure X**. The developed Variation I was named as Formula I and Variation III was named as Formula II respectively.

Table VIII –Methods/Procedures adopted for Nutrient analysis

Nutrients	Procedures
Moisture (%)	ISI1623-2008 -Karl Fischer Titration
Protein(g)	IS5983(Part1)-2005, Ra2016-Kjeldahl Method
Fat (g)	IS1011-2002,Ra2013,Soxhlet Extraction method
Carbohydrate(g)	IS2234-1989,Ra2015

Total ash (%)	IS1155-1968,Ra2012
Energy Kcal	FAO manual ,2003
Calcium(mg)	IS13433(part), 1992
Crude fibre (g)	IS10226(Part1)-1982, Ra2005
Iron (mg)	IS14433:2007AnnexD
Vitamin C (mg)	FSSAI Manual2016
VitaminB3(mg)	CKL/ANL/HPLC/014
Vitamin B6(mg)	CKL/ANL/HPLC/014
VitaminB2(mg)	CKL/ANL/HPLC/014
VitaminB5 (mg)	CKL/ANL/HPLC/014
Chromium (mg)	CKL/ANL/FP/019
Zinc(mg)	CKL/ANL/FP/019
Magnesium(mg)	CKL/ANL/FP/019
Phosphorus(mg)	AOAC21st Edition vol III

e) Analysis of the Microbial count and cost effectiveness

i) Microbial count

The plate count of the developed supplement Formula 1 and Formula II was carried out in the microbiology laboratory using the procedure of Total Aerobic Microbial count (TAMC) IP (2018 (Volume -1) pg no -38, The reference range is0 to 10^4 cfu / g. The result of the score was tabulated. The microbial count gave valuable information on the shelf life quality and keeping quality of the supplement prepared without much processing. The shelf life of the formulas was analysed in the first month, second month and third month. After one month, plate count was observed 1.2X104 cfu/g for formula I. For formula II, after one month the plate count was noticed as 1.8x104 cfu/g. Preservatives were not used at any stage of the preparation of the supplement and the subjects were requested to store the supplement in suitable temperature preferably below 15 degree Celsius throughout the supplementation period of 90 days.

ii) Cost effectiveness

The ingredients used in the nutritional supplement were locally available, low cost and were rich in micronutrients. These ingredients were procured from the local market and cost of the formula I and formula II were calculated to know about the cost effectiveness.300g (1 packet) of formula I supplement costs Rs 132 and Formula II costs Rs 129 which was comparatively cost effective to commercial medical management formula. Variation I was named as Formula I and Variation III was named as Formula II.

f) Packing the Nutritional supplement

Considering the sensory evaluation score, cost effectiveness, nutritive value, Formula II was selected for the nutrition supplementation intervention .Weight of the Formula II was recorded using electronic food weighing balance. Each packet contained 300g of Formula II supplement powder ,prepared by including 30g Sprouted , dried and roasted Finger millet flour, 45g Sprouted, dried and roasted Pearl millet flour,45g powdered Flax seed, 45 g powdered pumpkin seeds , 60g powdered sunflower seeds , 60g powdered sesame seeds and 15g of powdered brown sugars measured and portions were mixed together and weighed again for confirming the measurement amount as 300g.each of the measured quantity supplement was packed in aluminium foil and 15g measurement spoon was put in each packet for easy usage of the powder. Packing was done in a standard aluminium foil packing, manually and was kept in air tight containers for storage. The colour of the formula I was light brown coarse powder, and formula II was brown colour coarse solid powder .The moisture, and pH of formula I was 4.35percent and 7.13 and formula II was 5.3percent and 7.21 respectively .Both the formulas were not completely soluble in water **.**Total ash content of Formula I was3.39 percent and formula II was 3.28 percent.

Plate 6 Packed Nutritional supplement for distribution

B. Development and Validation of nutrition education modules on nutritional status and polycystic ovarian syndrome.

a) Preparation of the nutrition and health education module

The Nutrition and health education modules were developed by including a brief introduction, pathophysiology, prevalence, aetiology, signs and symptoms, dietary, lifestyle and other interventions management by including pictures related to PCOS. Leaflets and PowerPoint presentation was developed on the initial stage of nutrition education. Nutrition programme was executed as one to one counselling method. Group discussion was carried out on the specified days, leaflets and booklets were also distributed to the selected participants to update their knowledge related to food, nutrition health, PCOS and its preventive measures.

Plate 7 Nutrition and Health education Intervention (using leaflets and power point presentation)

Plate 8 Progressive muscle relaxation techniques as part of nutrition education

Plate 9 Nutrition Education leaflet

Dietary and lifestyle modification , increased physical activity , management of body weight since obesity and insulin resistance are related with PCOS and stress management and recommended as preventive measures for women with PCOS .The investigator developed a Nutrition and health education module covering all the aspects with knowledge related to PCOS , using the relevant scientific literature with suitable evidences and pictures for preservation , suitable evidences and pictures in the

developed module , meaning , prevalence , causes , role of nutrients for preservation suitable dietary and lifestyle modification and other approaches including physical activities and stress management were highlighted to minimize the consequences and symptoms of PCOS .Hand books , power points and Nutrition education posters were developed in English and Regional language of Malayalam .Handbooks were distributed to the selected participants. In What's app group doubts were interacted with the investigator.

b) Conduct of the Nutrition and Health Education Intervention programme

Nutrition and Health education intervention with the distribution of handbook and PowerPoint presentation was given to the selected participants in the study groups (Experimental group I and Experimental group II) for three months. The content validity of the Nutrition and health education module was done by five subject experts in the area. Their suggestions were incorporated and module was modified accordingly **(annexure XII)** .The investigator discussed the diet plan, counselled with supportive aids once in a week and covered all the aspects within the study period of 90 days, with initial and final evaluation using the specially developed questionnaire. A specially designed short structured Questionnaire, content validated by the subject experts in the area was framed and used for evaluating the Nutrition knowledge of the participants. All the questions in the questionnaire were multiple choice types with one correct answer and has equal weightage of scores and carries one mark(annexureXI).Total scoring for the entire questionnaire was 20 .As first step of nutrition and health education, a specially framed questionnaire was administered in the experimental I and II groups . At the end of the nutrition and health education intervention programme, the same questionnaire was administered to evaluate the effect of nutrition and health education on the knowledge of the selected participants (annexureXI).Statistical analysis also revealed the effect of Nutrition and Health education on the significant improvement in the knowledge of the selected participants in the two study groups

PHASE – IV Effect of Nutrition interventions on nutritional status and symptoms of Polycystic Ovarian Syndrome.

Nutrition interventions including dietary supplementation, nutrition education, sessions on stress management and assessment of physical activity levels were totally meant for minimising the consequences and severity symptoms of PCOS.

A. Selection and grouping of PCOS Participants for nutrition interventions

Women who were diagnosed as PCOS using validated tool were considered for the supplementation study. Purposive random sampling technique was used to select 93

women participants for the intervention study .Among the recruited 93 participants 32 participants were randomly selected for the selected for nutrition interventions including dietary supplementation and nutrition education and 31 participants were recruited for Nutrition Education alone ,for the period of three months and the sample size calculation is explained below

Sample size: The formula suggested for clinical trials by considering type one error (α) of 0.05 and type two error (β) of 0.20 (power = 80%).The sample size for the Nutritional intervention part of the study was 25 participants in each group, that is the total of 75 participants .This was calculated based on the concentration of testosterone change as per previous study (Garg et al 2015), where the standard deviation was 0.11ng/ml , difference in mean was 0.09ng/ml and was based on the formula

$N=2(Z\alpha/2+Z\beta)^2$ (Standard Deviation ÷Difference in mean $)^2$ =24

Considering a drop out of 20 % the minimum sample size was calculated as 30 per each group that is 90 participants. But we were able to recruit 32 in Experimental group I , 31 in Experimental group II and 30 in Control group. So the power of the study is not compromised for getting valid results. Those subjects who were recommended Medications (n=30) were kept as control group.

All subjects identified as PCOS and those who fit into the Inclusion and Exclusion criteria has been be enrolled in the study from the Gynecology Department. The study was a Quasi Experimental study design, because those on medications continued in the control group of the trial. Those who were advised Dietary management were divided into two Supplement group and Education group. They have been randomized either to receive supplement +Nutrition education or Nutrition education alone Randomization has been carried out using Fish bowl technique, in which 32 slips were kept as Experimental group I and 32 slips were kept as Experimental group II , all 64 slips of papers were put into a fishbowl/ container and shuffled and each slip is randomly picked out one- by- one by the participants

1st group -Experimental group I: Multi-Seed Health mix will be given 30gram daily, along with Nutrition education ,standard calories diet plan , leaflet and module for a period of 3 months :32 participants

2nd group -Experimental group II: Participants were provided Nutrition education, standard calories diet plan , leaflet and module alone will be given for a period of 3 months : 31 articipants

3rd group – Control group : On medication mainly to improve insulin sensivity , pain killers whenever they face severe pain and was not taking continuously : 30 participants

Drop out : 1 in the Experimental group II within one week

A total number of 93 participants with PCOS were included in the study

Limitation of sampling

The hospital selected for the study was a tertiary care Centre .The catchment area of the study area consists of five Districts and our sample consists of mixture of subjects from all these Districts. Nevertheless, we have added line in the limitation that ours was a single Centre Trial

B. Effect of Nutrition interventions on nutritional status, biochemical and clinical symptoms of PCOS among the selected participants

Nutrient dense health mix supplement in the form of ready to consume powder (300g) in the airtight sealed container was distributed once in a week. The participants of Experimental group I was were called to assemble to receive the supplement. During the study period of three months they were instructed to consume the supplement, Formula II in the morning 15g and in the evening 15 g without sharing with others and also any wastage. The Outcome of Nutrition Education on nutritional status and symptoms of PCOS was assessed using the specially designed research tools. Anthropometric measurements, biochemical estimation, clinical examination and individual dietary intake by one day food record and 24-hour recall method were used to assess the nutritional status of the selected participants.

Participants on medical management was considered as the control group**.** Supplementation intervention was provided to experimental group I along with nutrition education and diet plan. Nutritional supplement of 30g daily for 90 days was prescribed and was given in the form of a ready to eat supplement powder, packed in airtight containers, distributed every two weeks. Daily intake of 2 tablespoon (30g) was suggested and was advised to continue for 90 days period. For Experimental group II, nutrition education and standard calorie diet plan was given. All the basic details were collected using the common questionnaire, food frequency questionnaire and Clinical assessment tools. For the present study, Nutritional anthropometry, biochemical estimation, clinical examination and individual dietary intake by 24 hour recall method were used for Nutritional status assessment of the selected participants. Scores on knowledge of PCOS were record for both the Experimental groups before and after the nutrition intervention for the period of 90 days (annexure XI)

Plate 10 Execution of Nutrition and Health Education /Counseling

a) Effect of nutrition interventions on nutritional status of the selected Participants

i. Anthropometric measurements

Anthropometric measurements like height, weight, BMI, Waist Circumference(WC) ,Hip Circumference(HC) , body fat and Triceps Skinfold Thickness(TSF),visceral fat and body water were recorded for the selected participants (N=93) in the study groups, before and after the nutrition intervention studies for a period of 90 days

ii. Biochemical estimation

Biochemical estimation of blood haemoglobin level, serum lipid profile, random blood sugar and total testosterone were conducted. Two ml of the blood sample was collected from selected 93 participants who were involved in the three study groups (Plate-2).

a) Haemoglobin estimation

Blood Haemoglobin level was estimated by cyan methaemoglobin method. Beckmann Coulter AU480 automated machine was used to estimate haemoglobin. Normal reference serum level in adult woman was 11-15g/dl

b) Random Blood Glucose estimation

Random blood glucose estimation was done based on Hexokinase method in automated Beckmann Caulter AU480/ Beckmann Coulter AU680 machines. The normal reference range was <140mg/dl

c) Lipid profile (Non fasting)

Cholesterol estimation was done by Cholesterol Oxidase/Peroxidase Enzymatic Method (CHOD-PAP) The normal reference range for adult females was 0-200mg ,

Triglycerides was estimated using Glycerol3-Phosphate/ Peroxidase Enzymatic Method (GPO-POD), the reference range of triglycerides was found to be 0-150mg/dl .High Density Lipoprotein levels were estimated by direct enzymatic method and reference range found to be 40-59mg/dl .LDL cholesterol in serum levels were estimated by Direct enzymatic method normal range is between 0-100mg/dl.The procedure was performed in automated Beckmann Coulter AU680 machine. Cholesterol to HDL ratio and VLDL estimated by calculation and reference ranges were 0-4.5 and 0-20mg/dl respectively

d) Total Testosterone

Testosterone levels were estimated using by chemiluminescence immunoassay (CLIA), based on the principle of competitive binding. The automated machine used for testosterone estimation was Beckmann Coulter DXH 800. The reference ranges were 11-0.59ng/ml

iii. Clinical examination

Clinical examination schedule consists of menstrual regularity, Acanthosis nigricans, skin health, metabolic profile such as body fat, insulin resistance and body fat percent were carried out using standard procedures. A modified Ferriman-Gallawey (FG) score for quantification of scores of "hirsutism in women using nine body areas (upper lip, chin, chest, upper and lower abdomen, thighs, upper and lower back and upper arm) "(Ferriman1983) .The severity rating was done for hair growth grading from zero to four (from no growth to extensive hair growth) A total score of >=8 was indicated hyperandrogenism .For acne standard screening tools Global Acne Grading system (Ramil *et al*., 2012), was used were used before and after intervention. Each type of acne will have a severity score and type of leision in specific location such as cheeks, forehead, nose, chin, neck and back will also have particular score by multiplying of each type of lesion score by its severity index score. The product value of each area can be summed up for getting total score for acne. A score of 1-18 was considered mild, 19-30 as moderate and 31-39 as severe and >39 as very severe

iv. Assessment of stress level

Perceived Stress Scale (PSS) was the standard instrument and broadly used psychological tool used for evaluating the stress level (Cohen, 1988). It is a gauge of how stressful a person perceives their life's circumstances to be. PSS is administered to note the stress level of the participants in the study groups at the beginning and end of the study period of 90 days. The feelings and thoughts of the participants during the last month will be considered for scoring.The score of 0-13 was considered as low stress, 14-26 as moderate stress and 27-40 as high stress and collected data were systematically recorded for further analysis.

v) Physical activity level

Physical activity level was assessed using Godin Leisure Time Scoring (Godin 2011) before and after the study period. During a one week period how many times on the average the participants taking part in physical activity (for more than 15 minutes) that was multiplied by the intensity score of the type of physical activity provided the total physical activity score If the score obtained is <14 less active, 14 to 23 is moderately active, 24 and above is very active and the same procedure has adopted for the assessment of physical activity level of the participants

Weekly Leisure Activity Score = (9 × Strenuous) + (5 × Moderate) + (3 × Light)

vi) Individual dietary intake

Each subjects were asked to complete a multiple food items food frequency intake questionnaire and a one day food record, 24 hour recall. Training on household measurements both using pictures and models representing various portion sizes and were provided with written materials containing an example of a complete dietary record. Diet cal software was used to calculate the nutrient intake of the selected participants to know about their actual intake of nutrients of the selected participants before and after the study period of 90 days. Individual dietary intake of micronutrients were calculated. Biochemical measurements of micronutrients were not included in the methods due to cost constraints

a) Assessment of Individual Dietary Diversity Score

The 24-hour recall, Food frequency questionnaire were used for the measurement of IDDS. Twelve food groups was used for measurement of Household Dietary Diversity Score indicator. Each food group was allotted a score of one (if consumed) or 0 (if not consumed). By this way in which from the 24-hour recall, the food groups taken was noted and summed up to get scores for each person (ranging from 1 to 12). The score will range from 1 to 12(1 will be the lowest and 12 will be the highest score)

HDDS (1-12) = Sum IDDS (A+B+C+D+E+F+G+H+I+J+K+L)

Average IDDS = Sum IDDS/ Total Number of Individuals

b) Nutrient Adequacy Ratio

Nutrient Adequacy Ratio was calculated as the ratio of the intake of nutrients of the participants to current recommended allowance of for a given nutrient for the age group. The ratio is calculated based on the ICMR Recommended Dietary Allowances (2020) and the current nutrient intake of the participants.

NAR =Actual Nutrient intake of a nutrient (per day) /Recommended Dietary allowance of the nutrient

PHASE – V Statistical Analysis and Interpretation of data

After the collection of data, it is mandatory to organize the data for systematic consolidation and also for obtaining the desired results. Interpretation was also carried out with statistical analysis to assure the results of the effect of different nutrition interventions on reducing or relieving the symptoms and consequences of PCOS. Descriptive statistics like percentage and mean were used to explore the demographic profile, dietary and lifestyle pattern, BMI and incidence of PCOS and knowledge level of participants. Inferential statistics like correlation and chi-square were applied to find out the association and differences between variables and effect of Nutrition Interventions on the symptoms of PCOS. Categorical variables were compared using the Mc Nemar's χ^2 test and Mc Nemar's odds ratio. Inferential statistics like correlation and chi-square were applied to find the association and differences between the variables. Paired t- test was used to check the effect of intervention on different symptoms reduction before and after intervention. Comparison of means performed using independent sample T- test. Within the group comparison was performed to determine the significance of within-group change using Paired *t*-test. Three-group comparison was performed by analysis of variance, adjusted for the baseline values. The effect size was analyzed by repeated measure analysis and proportion for agreement was analysed by Kappa of agreement. The rate of change of each variable over time and rate of change was also analysed. The data was analyzed using SPSS 23 version and SAS university edition