3. METHODOLOGY

The methodology for the research entitled ***"In vivo* Effects of *Beta vulgaris* L. Leaf Extract on Polycystic Ovarian Syndrome Induced Adult Rats"** is carried out through the following phases:

Phase I: Selection and Preparation of Underutilized Green Leafy Vegetable Powder

A. Selection of area for plant collection
B. Selection of underutilized green leafy vegetable of carrot, radish and beetroot
C. Collection of underutilized green leafy vegetable of carrot, radish and beetroot
D. Preparation of underutilized green leafy vegetable powder

Phase II: Determination of Total Antioxidant, Total Phenol and Total Flavonoid in the Selected Underutilized Green Leafy Vegetable Powder

A. Determination of Total Antioxidant in the selected underutilized green leafy vegetable powder
B. Determination of Total Phenol in the selected underutilized green leafy vegetable powder
C. Determination of Total Flavonoid in the selected underutilized green leafy vegetable powder
D. Justification for selection of *Beta vulgaris* L. leaf extract for further study

Phase III: Analysis of Phytochemical Constituents, Antioxidant Activity, Nutrients and Secondary Metabolites of *Beta vulgaris* L. Leaf Powder

A. Screening for pesticide in the selected *Beta vulgaris* L. leaf powder
B. Screening for phytochemicals in the selected *Beta vulgaris* L. leaf powder
C. Determination of antioxidant activity in the selected *Beta vulgaris* L. leaf extract
D. Estimation of nutrients in the selected *Beta vulgaris* L. leaf powder
E. Estimation of flavonoids in the selected *Beta vulgaris* L. leaf extract
F. GCMS screening of secondary metabolites in the selected *Beta vulgaris* L. leaf extract
G. HPLC screening of Vitamin A and E in the selected *Beta vulgaris* L. leaf extract

Phase IV: Acute Toxicity study of *Beta vulgaris* L. Leaf Aqueous Extract on Adult Female Rats

A. Acute toxicity study of *Beta vulgaris* L. leaf aqueous extract on adult female rats
B. General observation of *in vivo* acute toxicity study of *Beta vulgaris* L. leaf aqueous extract on adult female rats
C. Observation of Gross Behavior of *in vivo* acute toxicity study of *Beta vulgaris* L. leaf aqueous extract on adult female rats

Phase V:*In-vivo* effects of *Beta vulgaris* L. leaf aqueous extract of Polycystic Ovarian Syndrome (PCOS) in Adult Female Rats

A. *In vivo* study of the effects of *Beta vulgaris* L. leaf aqueous extract on PCOS in adult female rats
B. Monitoring the fluctuation's in the rat's estrous cycle
C. Determination of the effects of *Beta vulgaris* L. leaf aqueous extract on PCOS adult female rats in association with physical parameters
D. Determination of the effects of *Beta vulgaris* L. leaf aqueous extract on PCOS adult female rats in association with biochemical parameters
E. Determination of the effects of *Beta vulgaris* L. leaf aqueous extract on PCOS adult female rats in association with reproductive hormones
F. Determination of the effects of *Beta vulgaris* L. leaf aqueous extract on PCOS adult female rats in association with antioxidant status
G. Determination of histopathology of *Beta vulgaris* L. leaf aqueous extract on PCOS adult female rats
H. Consolidation and statistical analysis of the data

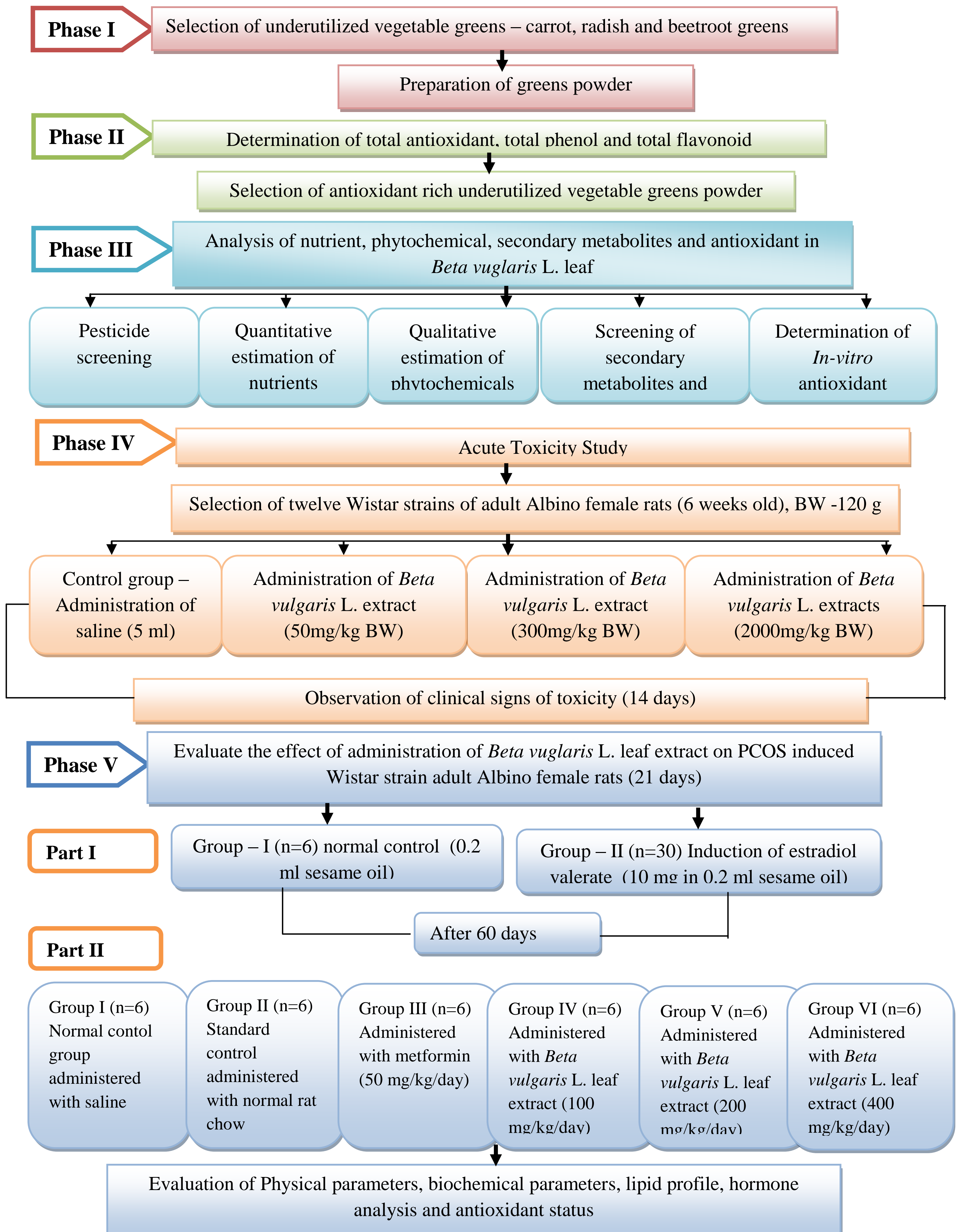


Figure 2: Research Design

Phase I: Selection and Preparation of Underutilized Green Leafy Vegetable Powder

A. Selection of area for plant collection

Traditional or underutilized leafy vegetables are grown in wild and cultivated. By habit, tradition, and custom, they are eaten as nutritional accompaniments to staples, either raw (fresh), cooked, or dried (Sivakumar *et al.,* 2020). Carrot, Radish and Beetroot are popularly grown in different parts of Nilgiris District, especially in Ooty, Coonoor and Kothagiri, which is a hilly area. Nilgiris District, the area selected for the collection of leaves of carrot, radish and beetroot are shown in Figure 2. The investigator, who is a native of the same district, collected the plant from an organic farm for further taxonomical certification. The collected plant from the natural habitat, Kothagiri, Nilgiris District was authenticated by Dr. Arulanandam, Botanist, The Rapinat Herbarium, St. Joseph's College, Tiruchirappalli, Tamil Nadu, India, and houses the herbarium specimens (Appendix I).

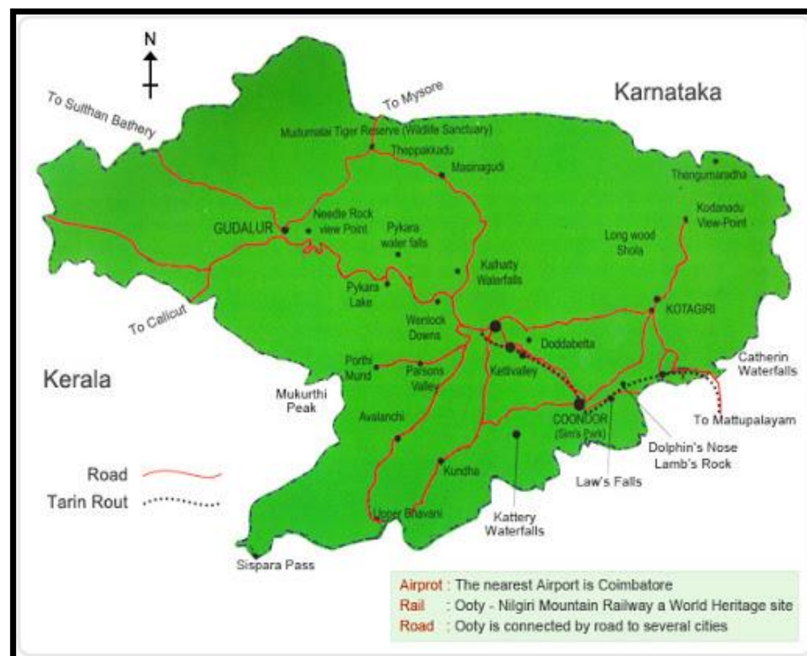


Figure 3: Selection of Area for Plant Collection

B. Selection of underutilized green leafy vegetable of carrot, radish and beetroot

Green Vegetables (GLVs) are an excellent component of phytonutrients and are an essential component of a healthy diet (Li*et al.*,2021). The greens of popularly consumed vegetables namely carrot, radish and beetroot are not utilized despite of its easy availability at a very low cost and dense in nutrients. These vegetable greens are neglected or discarded and used as fodder for animals. Majority of human population are ignorant about the nutrient content of these greens. These greens are underexploited during post harvest due to inadequate scientific knowledge of its nutritional potential. Based on the above mentioned criteria and reviewing on the waste profile these underutilized vegetable greens were selected for the study.Criteria for selection of Underutilized Green Leafy Vegetable is presented in Figure 4.

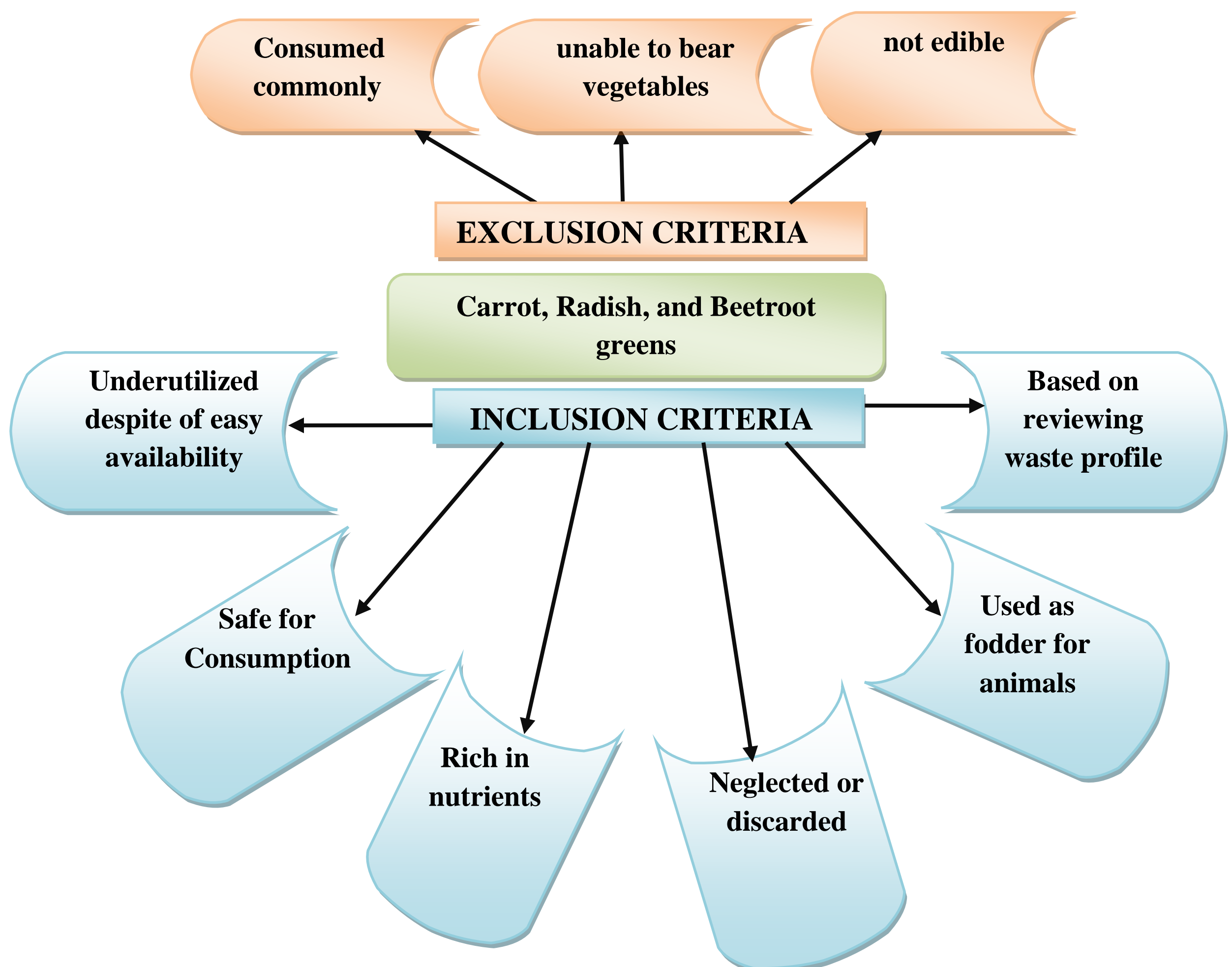


Figure 4: Criteria for Selection of Underutilized Green Leafy Vegetable

C.Collection of underutilized green leafy vegetable of carrot, radish and beetroot

Green Leafy Vegetable (GLVs) are good source of nutrients and reported to contain vitamins, minerals and significant amount of pigments. Although vegetables, such as carrot, radish, and beetroot have been characterized for their high carotenoid content, GLVs still remain the richest source of carotenoids compared to roots, tubers and other vegetables (Koley*et al.*, 2014). For the purpose of the experimental study the underutilized vegetable greens of carrot, radish and beetroot were collected fresh in lot from Melvin's Hills Biodynamic farm, an organic farm in Kothagiri, Nilgiris District, Tamil Nadu. The collected plants are shown in (Plate 1). In organic farming, Navathaniya seeds are sown and watered periodically for three months for the plants to grow. Then the land is ploughed to remove the grown plants, to make the land free from chemicals. Materials like vermicompost, cowdung manure, neema cake, biopesticides like trycodema, metarizam, pseudomonas, pesslomyices, humic plus, baveria are mixed together and spread to finally form a rice bed. Simultaneously the seeds and seedlings undergo seed treatment, the seeds are soaked in cow pot pit solution and panjakavya to eradicate chemicals and dried for later use. The seeds are then sown for cultivation. To protect the leaves from insects, the vegetables are periodically sprayed with panjakvya, jeevamritham solution and neem oil. From the production of 100 kg of carrot, radish and beetroot vegetables approximately 20 kg of greens are wasted, which can be used for consumption and helps to reduce the waste index.

Carrot plant

Radish plant

Beetroot plant

Plate 1: Collection of Green Leafy Vegetables

D. Preparation of underutilized green leafy vegetable powder

Green Leafy vegetables have health benefits for consumers, due to the presence of vitamins, minerals, protein, fiber, antioxidant, carbohydrates etc., (Ioannou, 2013). The powder form dried green leafy vegetables were mostly used, which reduced the amount of space needed for storage and made them easier to handle (Satwase*et al.,* 2013). Vegetables of carrot, radish and beet root along with the greens were purchased in bulk from organic farm, the greens were separated from the stalk and weighed, the initial weight were noted. The greens were washed thoroughly under running tap water for the removal of adhering dust and impurities. The greens were weighed and blanched for 10-15 seconds at 80ºC. Blanching greens helps to reduce quality loss over time. The inactivation of enzymes preserve color, flavor, and nutritional value (Geerkens*et al*., 2015). After blanching, the greens were spread on the parchment paper and drained excess water for three hours at room temperature. The dried greens were weighed and noted. The dried greens were crushed in grinder to get powder form and then sieved in 1mm sieve to get fine powder, the final weight of the greens were noted. The greens were packed individually in High-density polyethylene (HDPE) air tight container for further study. Processing of the underutilized greens are shown in Plate 3.

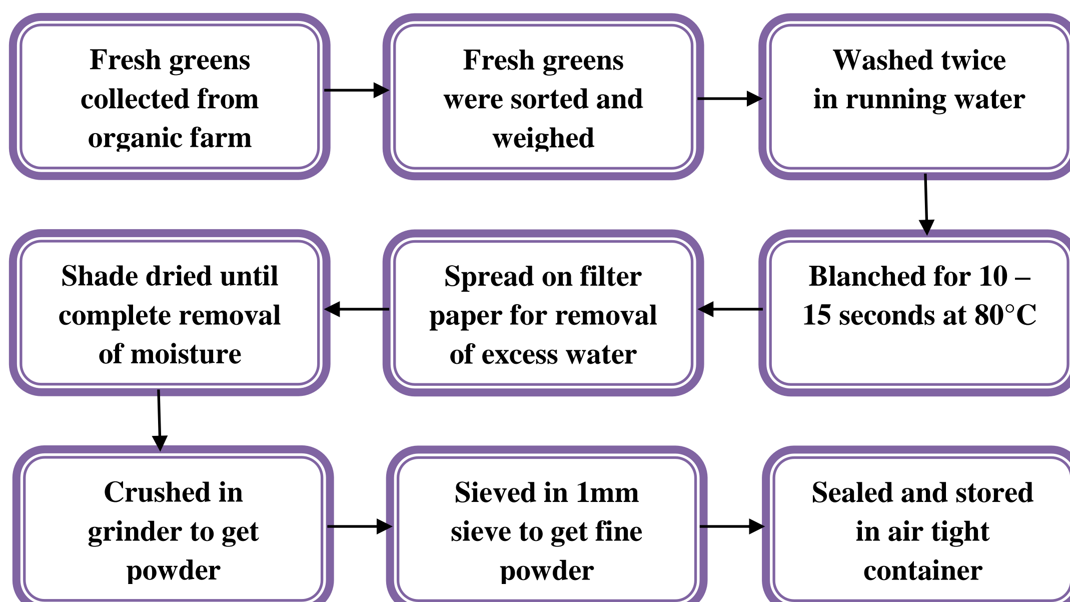
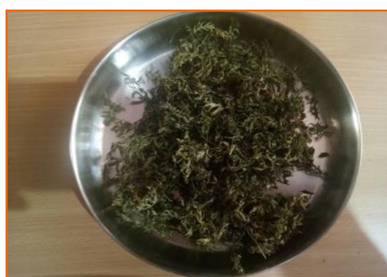


Figure 5: Preparation of Underutilized Vegetable Greens Powder

Fresh Carrot Greens

Fresh Radish Greens

Fresh Beetroot Greens



Dried Carrot Greens

Dried Radish Greens

Dried Beetroot Greens

Carrot Greens Powder

Radish Greens Powder

Beetroot Greens Powder

Plate 2: Processing of Underutilized Vegetable Greens

Phase II: Determination of Total Antioxidant, Total Phenol and Total Flavonoid in the Selected Underutilized Green Leafy Vegetable Powder

A. Determination of Total Antioxidant in the selected underutilized green leafy vegetable powder

Antioxidants are substances that can protect the body mainly due to their ability to remove the reactive oxygen species (ROS). Antioxidant-rich foods lessen the risk of a variety of chronic degenerative diseases(Boeing *et al*., 2012).

When antioxidants and other phytochemicals are added to food, they prevent peroxidation and the formation of oxidation products, conserving the quality of the food and trying to extend its shelf life (Barden and Decker, 2016).Antioxidants are a

type of molecule that can help to mitigate the harmful effects of free radicals formed in polycystic ovarian syndrome (Sulaiman *et al*., 2018). For the purpose of studying the antioxidant capacity, the greens powder of carrot, radish and beetroot were analyzed for quantification of total antioxidant, total phenol and total flavonoid using standard assay described by Sadasivam and Manickam (1996).The experimental study was carried out at Food Chemistry Laboratory, Jamal Mohamed College (Autonomous), Tiruchirappalli, India.

The total antioxidant content of underutilized vegetable greens powders was determined using the DPPH assay.To determine the antioxidant activity of purified phenolics and organic plant extracts, the DPPH assay is widely used (Geng *et al*., 2015). According to Celik *et al*., (2010) and Gulcin (2009), spectrophotometric methods for the determination of antioxidant capacity in plant extracts are commonly used due to the simple, rapid, sensitive, and repeatable procedures involved in assays based on the use of 1-diphenyl-2-picryl-hydrazyl (DPPH) and 2,2'-azino-bis (3-ethyl-benzthiazoline-6-sulfonic acid) radical. By extracting in 80 percent methanol and then filtering with filter paper, the capacity of the underutilised vegetable greens extract to scavenge the stable 2, 2'-diphenyl-2-picrylhydrazyl (DPPH) free radical was determined. The free radical scavenging capacity of the underutilized vegetable greens extract was then determined.According to Sultan *et al*., (2009) and Ahmad *etal*., (2011), antioxidants extracted from plant material are frequently extracted using the solvent extraction method. The choice of solvent has been shown to have a significant impact on antioxidant extraction concentration.

Six millilitre of DPPH solution was added to 0.1 ml of freshly prepared underutilised vegetable greens powder extraction. The test tubes were stored in dark room at 35°C for one hour. The optimal density of the sample and the blank was measured spectrophotometrically at 517 nanometer. The readings were recorded in triplicates. Butylated hydroxytoluene was used as the standard. The procedure is presentedin (Appendix II).

B. Determination of Total Phenol in the selected underutilized green leafy vegetable powder

De Gaulejac *et al.,* (1999) opines that antioxidant compounds are usually in phenolic form and are most abundant in plant structures, therefore, in order to

determine the antioxidant capacity of the plant extracts it is very important to determine the quantity of phenolic compounds. Singleton *et al*.,(1999) suggests that Folin-Ciocalteu method (F-C) has been found to be the best method, for quantification measurement of phenolic compounds in plant components and plant extracts because it is simple and reproducible.

Plant extract polyphenol react with specific redox reagents (Folin-Ciocalteu reagent) to form a blue complex, which can be quantified by visible-light spectrophotometry, according to Blainski *et al*., (2013) and Schofield *et al*., (2001).Based on the reviewing of the significance of phenolic compound and the method used for measuring total phenol, Folin-Ciocalteu method was used to estimate the total phenolic compound present in the underutilized greens powder using ethanol as solvent. However, there is no universal extraction method suitable for extraction of all plant phenolic compound. Shi *et al.,* (2005) presumes that for extraction of polyphenol, ethanol is a good solvent and is considered safe for human consumption.

Fifty grams of carrot, radish, and beetroot greens powder were extracted using 100 ml of ethanol in soxhlet extractor for 24 hours and then the extracts were concentrated in a rotary flash evaporator. The extracts were preserved for future analysis. To 0.5 ml of prepared ethanol greens extract of carrot, radish, and beetroot powder were mixed with 8 ml of distilled water. Then 0.5 ml of Folin’s Ciocalteau reagent was added and kept for incubation for 10 minutes at 40°C. Later, 1 ml of sodium carbonate solution was added and the tubes were kept in dark under incubation for 1 hour.

The absorbance were read in triplicates using UV spectrophotometer at 660 nm against blank reagent and the results were calculated as gallic acid equivalents (GAE) (mg/100g) of sample against gallic acid used as standard (Appendix III). According to (Hu *et al*., 2010), gallic acid is commonly used as a comparison standard and values are measured in milligrams of gallic acid equivalent per kilogram or litre of extract among the samples.

C. Determination of Total Flavonoid in the selected underutilized vegetable greens powder

Flavonoids, a major class of polyphenols, widely present in the plant kingdom, represents a large group of plant secondary or specialized metabolites (PSMs) which are either produced naturally or induced by environmental stresses (Agati *et al.*, 2020). Hence flavonoids are considered as a major polyphenolic compound found naturally in plants. Flavonoids were quantified using Aluminium Chloride reagents ($AlCl_3$) (Bahorun *et al.,* 1996).

According to Pekal and Pyrzynska (2014), spectrophotometric assay based on aluminium complex formation is one of the most commonly used procedures for determining total flavonoid, as the content of these compounds is considered an important parameter for evaluating medicinal plant samples.Hence Aluminum Chloride method was used to determine the total flavonoid content in the selected underutilized vegetable greens powder. One gram of each carrot, radish and beetroot greens powder were soaked overnight in water. The mixture was then distilled using Whatman No.1 filter paper. To the tubes, 1.5 ml of the greens extract 3.5ml of distilled water and 0.3 ml of 5% sodium nitrate was added. During the 6th minute, 2 ml of 1M sodium hydroxide was added to the mixture. Immediately, the contents of the mixture were diluted with addition of 2.4 ml of distilled water and mixed thoroughly. Immediately a blank was prepared and the absorbance of the mixture was determined at 510 nanometre versus the blank. For quantification of total flavonoids quercetin was used as the standard. The readings were noted in triplicates (Appendix IV).

D. Justification for selection of *Beta vulgaris* L. leaf extract for further study

It was statistically analysed to identify the best-scoring underutilised vegetable greens powder for further study, with the results based on the significant difference in values for total antioxidant, total phenol, and total flavonoid.

PhaseIII: Analysis of Phytochemical Constituents, Antioxidant Activity, Nutrients and Secondary Metabolites of *Beta vulgaris* L.Leaf Powder

A. Screening for pesticide in the selected *Beta vulgaris* L. leaf powder

Tang *et al.* (2021) predicted that more than 70% of worldwide agricultural land was at danger to the environment of pesticide residues in the agricultural end products, which becomes a burden when consumed.Pesticides have been linked to a wide range of human health problems, from short-term effects like nausea and headaches to long-term effects like reproductive harm, cancer, and endocrine disruption.Pesticide residue refers to the pesticides or metabolic products of the pesticides that may remain in food grains, vegetables and fruits after they are applied to crops (Bankar *et al.*, 2012). The reason for choosing organically grown carrot, radish and beetroot vegetable greens for the experimental study was because of the injudicious use of pesticides on the locally grown vegetables with a special reference to carrot, radish and beetroot vegetables, where the greens are more exposed to pesticides since the greens cover the vegetables as they are grown under the ground. The *Beta vulgaris* L. greens powder chosen for supplementation was screened for the presence of pesticide residues using QuEChERs experimental procedure as described by Paramasivam, (2015) after seeing the noxious effect of pesticide residues (Appendix V).

B. Screening for Phytochemicals in the selected *Beta vulgaris*.L leaf powder

1. Preparation of Ethanol Extract

The ethanol extract were obtained by weighing 20 grams of the pulverized *Beta vulgaris* L. greens powder and soaked in 100ml of the 60% ethanol. The combination was allowed to settle for 24 hours at room temperature. The extracts were then filtered using Whatman no.1 filter paper. The filtrates were collected and stored for further analysis.

2. Preparation of Methanol Extract

The methanol extract were obtained by weighing 20 grams of the pulverized *Beta vulgaris* L. greens powder and soaked in 100ml of the 60% methanol. The combination was allowed to settle for 24 hours at room temperature. The extracts

were then filtered using Whatman no.1 filter paper. The filtrates were collected and stored for further analysis.

3. Preparation of Aqueous Extract

The aqueous extract was prepared by weighing out 20 grams of *Beta vulgaris* leaves powder and soaked in 100 ml of cold water in a conical flask , stirring vigorously with a glass rod for proper extraction. The combination was allowed to settle for 24 hours at room temperature. The extracts were then filtered using Whatman no.1 filter paper. The filtrates were collected and stored for further analysis.

4. Qualitative estimation of phytochemicals in the selected *Beta vulgaris* L. leaf extract

Phytochemical screening for *Beta vulgaris* L. leaf extract selected for supplementation was carried out as per standard procedures (Appendix VI). Alkaloids, steroids (Liebermann-Burchard test) and terpenoids (Salkowski test) were estimated qualitatively according to the procedure given by Majob *et al.,* (2010).Glycosides (reducing sugars – Fehling's test) and Anthraquinone (Borntrager's test) was done following the procedures given by Sofowora, (1993) and Usman *et al*., (2009). Quinones, saponins (foam test), tannins (Braymer's test), Cardiac glycosides (Keller Kelliani's test) and flavonoids (alkaline reagent test) were estimated qualitatively as per procedure given by Ugochukwu *et al*., (2013). Phenols and Coumarins (ferric chloride test) were determined with procedure by Mazimba *et al*., (2015) (Plate 3).

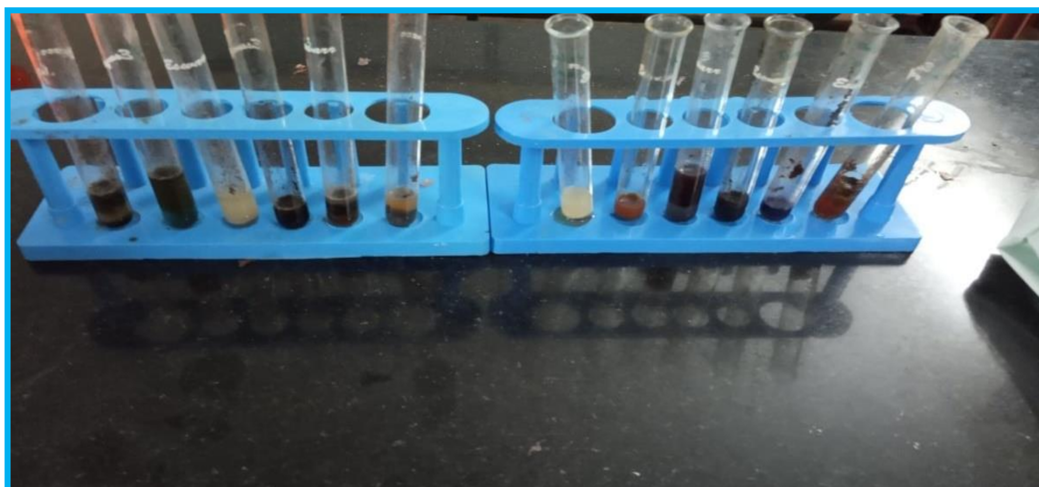


Plate 3: Qualitative Estimation of Phytochemicals in Beta vulgaris L. Leaf Extract

C. Determination of antioxidant activity in the selected *Beta vulgaris* L.leaf extract

(i) 1-Diphenyl 2-picryl Hydrazyl (DPPH) Assay

To determine the antioxidant activity of ethanolic extracts of *Beta vulgaris* L. leaf powderDPPH (1-Diphenyl 2-picryl hydrazyl) method were used. The free radical scavenging activity of 1-Diphenyl 2-picryl hydrazyl is used to determine the antioxidant activity of ethanol extracts (DPPH). The antioxidant activity of *Beta vulgaris* L. leaf extract was compared to that of ascorbic acid based on the scavenging effect of the 2,2-diphenyl-1-picryl hydrazyl (DPPH) free radical (a standard antioxidant) as described by Braca *et al*., (2001). Percentage of absorption of the extracts (%) were plotted against log concentration and IC_{50} (Concentration for 50% inhibition) values were deliberated with linear regression analysis.

(ii) Hydrogen Peroxide Scavenging Capacity

Ruch *et al.,* (1989) method was used to determine the hydrogen peroxide trapping capacity of *Beta vulgaris* L. leaf extract. Hydrogen peroxide solution (40 mM) was prepared in phosphate buffer (pH 7.4). The extract(100 µg/mL) in distilled water was added to the hydrogen peroxide solution. The absorbance of hydrogen peroxide at 230 nm was determined with hydrogen peroxide-free phosphate buffer. The percentage of hydrogen peroxide collection of *Beta vulgaris* L. leaf extract was calculated by the formula % recovered [H2O2] = [(Ac - As) / Ac] x 100 (Appendix VII).

(iii) Reducing Power Assay

The reducing power of an ethanolic extract of *Beta vulgaris* L. leaf was determined using the Oyaizu (1986) method.Substances with a reduction potential combine with potassium ferricynaide to form potassium ferrocynaide, which then combines with ferric chloride to form a ferric-ferrous complex with a 700 nm absorption maximum. As the reduction of ferric to ferrous ion increases, the absorbance increases, indicating that the ethanolic leaf extract of *Beta vulgaris* L. has reducing ability (Appendix VIII).

D. Estimation of nutrients in the selected *Beta vulgaris* L. leaf powder

Standard procedures were used to determine the moisture, ash, protein, fat, carbohydrate, dietary fibre, energy, iron, and calcium content of the *Beta vulgaris* L. leaf powder.Total moisture content, total ash content, fat, protein of the *Beta vulgaris* L. powder was determined according to AOAC (2005), anddietary fiber was estimate according to ASTA Method No 7.0. Estimation of calcium was determined by titration with potassium permanganate method. Estimation of Carbohydrate was done as described by Gayol *et al.,* (2010), where the content of the available carbohydrate was determined by subtracting from 100 the sum of the values (per 100g) of moisture, protein, fat, ash and crude fiber using the below formula.

Estimation of Carbohydrate=100-[% protein+% lipid+% ash+% moisture].

The energy content was calculated by multiplying the percent of crude protein, crude fat, and carbohydrate by 4, 9, and 4 factors, respectively. Iron was determined using an Atomic Absorption Spectrophotometer, as described in AOAC (1999), and calcium was determined using Indian Standard methods for calcium volume measurement using the EDTA (Ethylene Diamine Tetra Acetic Acid) titration method, as described in AOAC (1999) (Plate 4).

Plate 4: Estimation of Minerals in Beta vulgaris L. Leaf Powder

(i) **Estimation of vitamin C by titration method**.

Ascorbic acid (AA), an water-soluble primary health nutrient commonly known as vitamin C plays an important role in humans. Vitamin C acts as a lipid

peroxidation chain-breaking agent and protects against cell death, superoxide anion, and hydroxyl radicals.To estimate Vitamin C in *Beta vulgaris* L.leaf powder, titration method described by Sadasivam and Manickam (1996) was followed as shown in Plate 5.The readings were read in triplicates (Appendix IX).

Plate 5: Estimation of Vitamins in Beta vulgaris L. Leaf Powder

B. Estimation of Flavonoids in the selected *Beta vulgaris* L. leaf extract

Largest group of plant phenols are flavonoids. Chic *et al.,* (2014) opines that flavonoids are said to have anti-inflammatory properties, anticancer and diuretic properties.The selected *Beta vulgaris*L.leaf powder was further screened for the presence of flavonoid compounds using HPLC analysis. Kupiec, (2004) suggested that HPLC is a type of liquid chromatography that is now widely used to separate and quantify compounds that have been dissolved in solution in order to determine the amount of a specific compound.HPLC screening for flavonoids in *Beta vulgaris* L.leaf powder was performed according to experimental procedure by Samee *et al.,* (2007) (Appendix X).

C. GCMS Screening of secondary metabolites in the selected *Beta vulgaris* L. leaf extract

According to De-Fatima *et al*., (2006), secondary metabolites produced by plants are biologically active. In general, secondary metabolites are important sources because of their properties and structural diversity. Thus, the active compounds present in the *Beta vulgaris* L. leaf extract were identified using Gas Chromatography Mass Spectrometry (GCMS).

(i) Sample preparation

To 10 gm of *Beta vulgaris* L.leaf powder 80 ml of ethanol was added to soak and left overnight and then filtered through Whatmann filter paper No.41 along with 2gm sodium sulfate to remove the sediments and traces of water in the filtrate. Before filtering, the filter paper was wetted with ethanol, and the filtrate was then concentrated to 1ml by flushing nitrogen gas into the solution. Both polar and non-polar phytocomponents were present in the extract.The chemical composition of 1 ml were investigated through Gas Chromatography Mass Spectrometry (GC-MS). The National Institute of Standards and Technology (NIST) database, which contains over 1,62,000 patterns, was used to interpret the mass spectrum GC-MS. Peak area normalisation was used to quantify the compounds (Appendix XI).

D. HPLC screening of vitamin A and E in the selected *Beta vulgaris* L. leaf extract

Van den Berg *et al*., (2000) identified beta-carotene as the most well-known carotenoid in plant foods, and it is well known that provitamin A carotenoids contribute the most to vitamin A intake, accounting for up to 82 percent of total vitamin A intake. Carotene is a generally safe and effective source of vitamin A in both conventional foods and vitamin supplements, according to the Council for Responsible Nutrition (Dickinson *et al*., 2014). High Performance Liquid Chromatography (HPLC) was used to screen beta-carotene in beetroot greens powder, according to Khalil and Varananis (1996) analytical work. (Appendix XII).

Because only plants can synthesise vitamin E, it is regarded as a critical dietary nutrient for both humans and animals. Trang (2013) points out that many HPLC methods for vitamin analysis have been developed and reported over the last three decades. The current trend is to develop simple and easy-to-transfer multivitamin methods. To determine the presence of fat soluble vitamins, vitamin A, and vitamin E in the *Beta vulgaris* L. leaf powder "High Performance Liquid Chromatography (HPLC)" was used.For screening fat soluble vitamins A and E in *Beta vulgaris* L. leaf powder the procedure was determined referring Qian and Sheng (1998) experimental procedure (Appendix XIII).

Phase IV: Acute Toxicity Study of *Beta vulgaris* L. Leaf Aqueous Extracton Adult Female Rats

The ethical clearance for conducting animal experiment was obtained from Institutional Animal Ethical Committee (IAEC Certificate number – SAC/IAEC/BC/2020/CP-004 dated 12.02.2020) (Appendix XIV).Study was conducted at animal laboratory of Srimad Andavan Arts and Science College (Autonomous), Affiliated to Bharathidasan University, Tiruchirappalli and supervision of Experiment of Animals (CPCSEA) according to the guide for the care and use of laboratory animals.

A. Acute Toxicity Study of *Beta vulgaris* L. leaf aqueous extract on adult female rats

The researcher intended to carry out an acute toxicity study in order to determine the maximum safe dose of *Beta vulgaris* L. leaf aqueousextract for supplementation in experimental animals. The study was to be carried out in accordance with OECD's 423 guidelines. Acute oral toxicity is defined as toxic effects that occur within 24 hours of taking a single or multiple doses of any substance through the oral route (OECD, 2001).

(i) Selection of animal for acute toxicity study

Twelve Wistar strain Adult Albino female rats (six weeks old with mean BW of 120 g) were selected to conduct acute toxicity study. For acute toxicity study, the preferred rodent species according to OECD (2000) is female rats.According to Lipnick *et al.,* (1995), studies of conventional LD50 tests revealed that there is a slight difference in sensitivity between male and female species; in those cases, differences are detected, and female animals are usually more sensitive than male animals.

(ii) Housing and feeding of the selected animals for acute toxicity study

Twelve Wistar strain Adult Albino female rats selected for the conduct of acute toxicity study was housed in polypropylene cages. Throughout the study period, the temperature was kept at 22±3°C and the light/dark cycle was repeated every 12 hours.Humidity of the laboratory was maintained at 40-60 percent. The experimental animals were provided with commercially available normal rat chow containing moisture 5.3%, crude protein 20.1%, crude fibre 4.2%, calcium 1.1%,

phosphorus 0.8%, total ash 8.2% and water activity 0.54 aw (Tetrogon Chemie Pvt. Ltd) with water *ad libitum.* Animals were chosen at random and labelled for identification. Rats were kept in cages for one week prior to dosing to acclimate to laboratory conditions.

(iii) Preparation of *Beta vulgaris* L.leaf aqueous extract for acute toxicity study

Aqueous extracts of *Beta vulgaris* L.leaf were administered to the animals.

(iv) Grouping of animals for acute toxicity study

Grouping of animals for acute toxicity study is presented in Table I.

Table I

Grouping of Animals for Acute Toxicity Study

(N=6)

Groups (n=3/group)	**Administration of *Beta vulgaris*L. leaf extract**
Control group	Administration of saline 5ml
Experimental group I	Administration of *Beta vulgaris* L. leaf aqueousextract 50mg/kg/bw
Experimental group II	Administration of *Beta vulgaris* L. leaf aqueousextract 300 mg/kg/bw
Experimental group III	Administration of *Beta vulgaris* L.leaf aqueousextract 2000 mg/kg/bw

(v) Administration of *Beta vulgaris* L. leaf aqueous extract to the selected animals

Doses varying from 50 mg , 300 mg and 2000 mg//kg body weight (bw) of aqueous extract of *Beta vulgaris* L.leaf extract were administered to the animals by oral gavage tube. Distilled water used as vehicle for administration. Animals were fasted overnight and weighed to determine body weight prior to dosage administration. Following the administration of leaf aqueousextract, food was withheld for 3-4 hours.

B. General observation of *in vivo* acute toxicity study of *Beta vulgaris* L. leafaqueousextract on adult female rats

(i) Measurement of body weight of the animals

Before beginning drug administration, on the 7th day, and on the 14th day, the body weight (bw) of the animals in all the groups was noted by weighing them in a weighing balance.

C. Observation of Gross behavior of *in vivo* acute toxicity study of *Beta vulgaris* L. leaf aqueous extract on adult female rats

Following dosage administration, the animals were carefully observed for the first 30 minutes and occasionally for the first 24 hours, with special attention given to gross behaviour such as Autonomous Nervous System (ANS) and Central Nervous System (CNS) activity during the first four hours (CNS). When compared to the control group that were fed standard rat chow and water *ad libitum*, the animals were monitored for clinical signs and symptoms of acute toxicity and/or mortality, abnormalities, food and water consumption, and changes in body weight for 14 days.

Phase V: *In vivo* effects of *Beta vulgaris* L. Leaf Aqueous Extract on Polycystic Ovarian Syndrome in Adult Female Rats

A. *In vivo* study of the effects of *Beta vulgaris* L. leaf aqueous extract on PCOS induced adult female rats

1. Selection of animals for experimental study of PCOS

Six weeks old female (Wistar strain Albino) rats (mean body weight 120 g) were obtained from the BIOGEN laboratory animal facility, Bangalore. Housing conditions were same as in the acute toxicity study. For acclimatization the animals were kept for one week.

As the vaginal changes reflect the presentation of PCOS, experimental animals were monitored by daily examination of vaginal smears. Only animals with two consecutive normal 4-5 day cycles were chosen for the experiment (Kafali *et al*., 2004 and Bravalle *et al*., 2006). At the beginning of the experiments, all animals had a regular cycle.

2. **Polycystic Ovarian Syndrome Induction (PCOS)**

Brower's (1996) experimental induction of Polycystic Ovarian Syndrome (PCOS) in rodents was made possible by intraperitoneal (i.p) injection of estradiol valerate (EV) in 6-week-old rats. In rodents, estradiol valerate is an effective drug for inducing PCOS. The rats stopped ovulating and developed symptoms similar to those seen in people with PCOS, such as large cystic follicles in the ovaries and low luteinizing hormone levels (Brower *et al*., 2019).

After one week of acclimatization, 6-7week-old rats (n=36) were divided into two groups of control and PCO rats. The control group received 0.2 ml sesame oil and the experimental group assigned to the PCO group received 5 doses of intraperitoneal (i.p) injection of 10 mg of estradiol valerate (EV), in 0.2 ml of sesame oil, to induce PCO (Brawer 1986). Administration was done between 0800 hours and 0900 hours daily. They were allowed free access to rat chow and water before and after their daily administration. Rats were weighed weekly from the initial day to till the termination of experiment (Plate 6). All the estradiol valerate treated rats were evaluated 60 days after the injection, when vaginal smear were tested, with irregular estrous cycle, increase in luteinizing hormone and decrease in follicle stimulating hormone.

Plate 6: Assessment of Body Weight of the Experimental Animals

3. Grouping of animals for supplementation study

Rats were randomly assigned into six groups which included one normal control, two standard control and three treatment groups with six rats in each group (Plate 7). Group I served as normal control which received standard rat chow and water *ad libitum* for 21 days. Group II served as PCOS induced standard control administered saline orally for 21 days. Group III served as PCOSstandard control administered with Metformin (50 mg/Kg/bw) orally for 21 days. Group IV served as PCOS treatment group I, administered with *Beta vulgaris* L. aqueousleaf extract (100 mg/kg/bw) (low dose). Group V served as PCOS treatment group II, administered with *Beta vulgaris* L. aqueous leaf extract (200 mg/kg/bw) (medium dose). Group VI served as PCOS treatment group III, administered*Beta vulgaris* L.aqueous leaf extract (400 mg/kg/bw) (high dose) as shown in Plate 8.

Table II

Grouping of Animals for Supplementation Study

<table>
<tr><td rowspan="3">Control Groups</td><td>Group I(N=6)</td><td>Normal Control Group
Received standard rat chow and water ad libitum</td></tr>
<tr><td>Group II(N=6)</td><td>PCOS induced Standard Control
Administration of saline orally for 21 days</td></tr>
<tr><td>Group III(N=6)</td><td>PCOS Standard Control administered with metformin
Administration of metformin (50 mg/kg/ bw) orally for 21 days</td></tr>
<tr><td rowspan="3">Treatment Groups</td><td>Group IV(N=6)</td><td>Treatment Group I
Administration of Beta vulgaris L. aqueousleaf extract (100mg/kg/bw) (low dose) orally for 21 days</td></tr>
<tr><td>Group V(N=6)</td><td>Treatment Group II
Administration of Beta vulgaris L. aqueousleaf extract (200mg/kg/bw) (medium dose) orally for 21 days</td></tr>
<tr><td>Group VI(N=6)</td><td>Treatment Group III
Administration of Beta vulgaris L. aqueous leaf extract (400mg/kg/bw) (high dose) orally for 21 days</td></tr>
</table>

GROUP I – Normal Control Group

GROUP II – PCOS Induced Standard Control

GROUP III – PCOS + Metformin (50 mg/kg/bw) Standard Control

GROUP IV –Treatment Group I: PCOS + *Beta vulgaris* L. leaf extract (100mg/kg/bw)

GROUP V - Treatment Group II: PCOS + *Beta vulgaris* L. leaf extract (200mg/kg/bw)

GROUP VI - Treatment Group III: PCOS + *Beta vulgaris* L. leaf extract (400mgkg/bw)

Plate 7: Grouping of Experimental Animals

Plate 8: Administration of Estradiol Valerate (intraperitoneal route) and Beta vulgaris L. leaf Aqueous Extract (Oral Route)

B. Monitoring the fluctuation's in estrous cyclicity of the selected rats

Microscopic analysis of the predominant cell type in vaginal smears obtained daily from day one to the end of the experiment determined the stage of cyclicity.Every morning between 8.00 a.m and 9.00 a.m vaginal secretion was collected. With 10 and 40x objective lenses, vaginal smears were examined microscopically for estrous cycle determination using the Hemacolor staining set (Merck KGaA, Germany).According to (Marcondes *et al*., 2002), a proestrus phase smear primarily consisted of unnucleated round cells, an estrous phase smear primarily consisted of anucleated cornified epithelial cells, a metestrus phase smear primarily consisted of leucocytes and a small number of large, non-granular, and anucleated cornified epithelial cells, and a diestrus phase smear constitued prominent polymorphonuclear leukocytes and few epithelial and cornified cells.

C. Determination of the effects of *Beta vulgaris* L. leaf aqueous extract on PCOS adult female rats in association with physical parameters

(i) Measurement of body weight of the animals

Individual body weight of rats in all groups of animals were recorded once in every week in the electronic weighing balance.

(ii) Measurement of ovary of the animals

Conditional dissection of the animals were done according to the standard operating procedure (SOP). The liver and ovary were cleaned of fat and weighed individually (Plate 9).

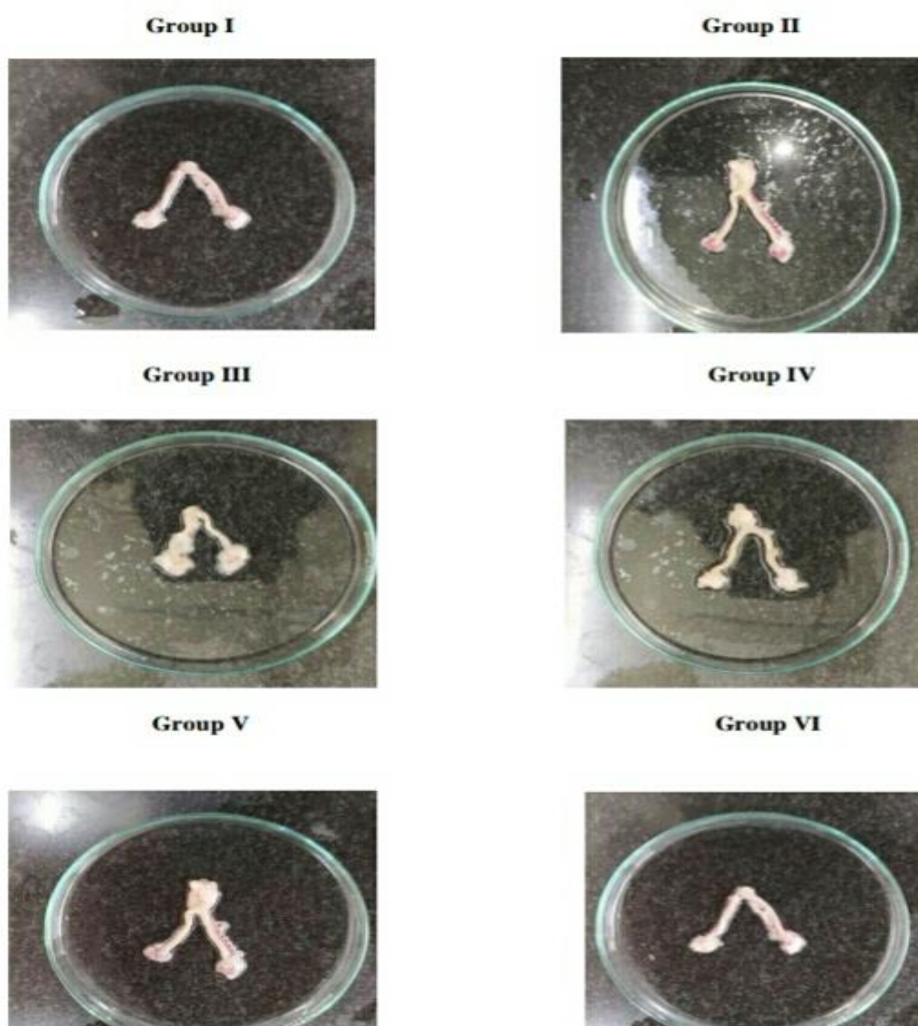


Plate 9: Assessment of Ovary Weight of Experimental Animals

D. Determination of the effects of *Beta vulgaris* L. leaf aqueous extract on PCOS adult female rats in association with biochemical parameters

Biological blood sample collection of the animals

Blood samples were collected from retro-orbital plexus using capillary tubes under mild anaesthesia with ether between 09.00 and 12.00 hours after the last dose of treatment and after an 18 hour fasting period. For further estimation of biomarkers, blood was collected in EDTA (anti-coagulant) containing fresh vials and serum was

separated in a cooling electric centrifuge (REMI cooling certrifuge-VCBF-1322) at 3000 rpm for 10 minutes.

(i) Estimation of blood glucose

Commercially available glucose kit was used to estimate fasting and postprandial blood glucose levels (FBS and PPBS), which was tested and standardised.

(ii) Estimation of lipid profile

The lipid profiles of rats in each group were analysed after 21 days of the study period. Henkel and Stoltz (1982) used the Enzymatic –GPO method to measure triglycerides, Trinder and Webster (1984) used the CHOD-PAP method to measure total cholesterol, Lopes-Virella *et al*., (1977) used the Precipitation method to measure HDL, and Friedewald equation to calculate LDL (Friedewald *et al*., 1972).

E. Determination of the effects of *Beta vulgaris* L. leaf extract on PCOS adult female rats in association with reproductive hormones

(i) Hormonal assay

For the hormonal assay, blood samples were centrifuged at 3000 rpm for 15 minutes with EDTA anticoagulant to separate the plasma. The plasma was kept at -20°C in the freezer. Leutinizing hormone, follicle stimulating hormone and estradiol serum concentrations were measured using a Rat/Mouse ELISA kit (Cosmo Bio Co.Ltd.Japan).

F. Determination of the effects of *Beta vulgaris* L. leaf aqueous extract on PCOS adult female rats in association with antioxidant status

(i) Determination of Lipid peroxidation by 2-thiobarbituric acid reactive substances (TBARS)

To each 1 ml of tissue homogenates and plasma samples, 8.1 percent sodium didecyl sulphate (SDS, 1 ml), 20% acetic acid (2 ml), and 0.75 percent TBA were added (1 ml). The mixture was centrifuged for 10 minutes at 14,000 rpm after boiling for 30 minutes. The absorbance of the malondiadehyde (MDA) –TBA adduct formed at 533 nm was colorimetrically measured using a specrophotometer (Bidlack and Tappel, 1973). The MDA values were calculated and expressed as TBARS values after a standard curve was prepared with tetramethoxypropane and TBA.

(ii) Superoxide dismutase (SOD)

Superoxide dismutase activity was determined by the ability of SOD to inhibit the auto-oxidation of epinephrine to adrenochrome at alkaline pH.First, 50mM sodium carbonate buffer was added to 25µL of the supernatant solution obtained from centrifuged liver homogenate. In a total volume of 2 ml buffer medium, add 0.1mM epinephrine. At 480 nm, absorption was measured. The activity of superoxide dismutase (SOD) was measured in units per milligram of protein. (Sun and Zigman, 1978).

(iii) Catalase (CAT)

In a total volume of 3.0 ml, the assay mixture contained 1.95 ml phosphate buffer (0.05 M, pH 7.0), 1.0 ml hydrogen peroxide (0.019 M), and 0.05 ml homogenate (10 percent w/v), with changes in absorbance measured at 240 nm. The catalase activity was measured in units per milligram of protein (Njomen, 2008).

(iv) Reduced Glutathione (GSH)

To 0.25 mL of 10% ovarian homogenate, an equal volume of ice cold 5% Trichloroacetic acid (TCA) was added. To remove the precipitate, centrifuge at 4000 rpm for 10 minutes.A 1ml aliquot of supernatant was thoroughly mixed with 0.25 ml of 0.2 M phosphate buffer, pH 8.0, and 0.5 ml of 5,5'-Dithio-bis 2-nitrobenzoic acid (DTNB). A spectrophotometer was used to measure the absorbance at 412 nm. The results were expressed in units per milligramme of protein. (Browne and Armstrong, 1998).

G. Determination of histopathology of *Beta vulgaris* L. leaf aqueous extract on PCOS adult female rats

(i) Histopthological Evaluationof *Beta vulgaris* L. leaf aqueous extract on PCOS adult female rats

Each rat's ovary tissues were collected after euthanasia. The tissues were then fixed in 10% neutral buffered formalin before being embedded in paraffin. The ovaries of the control and estradiol valerate treated rats were removed, cleaned of adherent fat and connective tissue, and fixed for at least 24 hours in 10% formaldehyde buffer. Longitudinally and serially sectioned with a microtome at 4 m and mounted on poly-lysine coated slides, deparaffinised with xylene, rehydrated and

stained with hematoxylin and cosin, dehydrated, cleared, and mounted on DPX under glass cover slips. As described by Kafali *et al*., (2004) the slides were then examined under a light microscope connected to a camera to capture images. The corpus luteum and preantral, antral, atretic, and cystic follicles were identified and described.

H. Consolidation and statistical analysis of the data

The data from the experimental study was statistically analysed using SPSS version 16 (Statistical Package for Social Sciences). All of the results of the experimental study were represented using descriptive statistics, which included the mean and standard deviation. The intragroup variations were interpreted using a one-way ANOVA with a post hoc test. To determine the significance, the values obtained from the animal model experiment were compared between the control and treatment groups. P values of <0.01 and <0.05 were considered statistically significant. The significance level was determined by comparing all five treatment groups with the control group.