

5. SUMMARY AND CONCLUSION

Women are an integral part of society and are the nurturers in every country. Women's health is a complex and multifaceted area of study of the whole body. Number of factors like age, reproduction, health care, disease and deficiencies affect women directly or indirectly. Women's health focuses on biological differences between men and women, such as reproductive organs, body structure, early learning, hormonal changes, genetic factors, and brain chemistry. With a global prevalence of 6–28 percent, polycystic ovarian syndrome (PCOS) is the most popular endocrinopathy in women of reproductive age. Indeed, PCOS is a condition with significant decrease in serum antioxidant and vitamin levels and these women are in an increased risk of oxidative status.

Antioxidant supplementation has been shown to improve insulin sensitivity and other health threatening conditions in women with PCOS. Antioxidants and vitamins are shown to help with PCOS management in research. World Health Organization recommends a daily intake of more than 400g of vegetables per person protects against diet related chronic diseases. Apart from green leafy vegetables which are rich in nutrients and micronutrients, they are also recognized important for their non nutritive health promoting compounds such as antioxidants and phytochemicals.

The greens of the beet (*Beta vulgaris* L.) are a great source of functional compounds (i.e. polyphenols and betalains). They are one of the healthiest greens recommended in the diet for their low fat, no cholesterol but health benefiting vitamins, minerals and antioxidants. Antioxidants and polyphenols, are preferred as protective ingredients in pharmaceutical, food industries as food additives, preservatives and dietary supplements. Hence the present study was undertaken to assess management of oxidative stress in PCOS induced rats by administration of *Beta vulgaris* L. leaf aqueous extract.

The specific objective of the study were to

- Determine the total antioxidant, total phenol and total flavonoid in the selected underutilized vegetable greens powder
- Select the greens which scores the highest in these parameters such as total antioxidant, total phenol, total flavonoid for administration in PCOS induced rats.
- Screen for the presence of secondary metabolites, flavonoids, phytochemicals, nutrients and vitamin A, C and E in the underutilized vegetable greens powder that is selected for administration in PCOS induced rats.
- Assess the effect of selected *Beta vulgaris* L. greens powder on the antioxidant status in PCOS induced female rats
- Evaluate the effect the administered *Beta vulgaris* L. powder on PCOS induced female rats.

The study was carried out in five phases. In Phase I, carrot, radish and beetroot plants was collected from the natural habitat from Melvin's Hills Biodynamic farms, an organic farm in Kothagiri, Nilgiris District, Tamil Nadu. The plants was selected for research purpose and duly certified by a taxonomist to confirm its authenticity. The greens of Carrot, Radish and Beetroot were separated from the stalk, washed thoroughly in running water. The greens were blanched for 10-15 seconds at 80°C. Shade dried until complete removal of moisture. The dried vegetable greens were grinded into powder and then sieved in 1mm sieve to get a fine powder. The powder was packed individually in an air tight container for further analysis.

In Phase II, the vegetable greens powder were analyzed for total antioxidant, total phenol, and total flavonoid and the best scored greens powder were selected for administration in PCOS induced female rats.

In Phase III, *Beta vulgaris* L. leaf powder that scored high in antioxidant was selected for administration in PCOS induced female rats. Qualitative estimation for phytochemicals were carried out in aqueous, ethanol and methanol extracts of *Beta vulgaris* L. leaf with standard procedures.

Alkaloids, flavonoids, amino acids and proteins, phenols, tannins, cardiac glycosides, fats and oils (fixed), terpenoids, saponins, sterols and oxalates were

estimated qualitatively. Ethanol extract of *Beta vulgaris* L. leaf was examined for its antioxidant activity by DPPH method, Reducing Assay and Hydrogen Peroxide Assay. Identification of secondary metabolites in *Beta vulgaris* L. leaf was done by GCMS. HPLC was used for screening flavonoids, vitamin A and E in *Beta vulgaris* L. leaf powder. Estimation of moisture, ash, protein, fat, carbohydrate, fibre, energy, iron and calcium using standard procedure was done.

In Phase IV, Acute toxicity study was conducted to determine dosage of supplementation for animal experiment and its toxicity. Twelve Wistar Albino female rats, of 8 weeks old with mean body weight (BW) 120 grams were selected for acute toxicity study. The study was conducted as per OECD 423 guidelines. Animals were assigned into four groups, group I served as normal control administered with saline, group II served as experimental group 1 administered with low dose 150 mg/kg bw, group III with 300 mg/kg bw and group IV with 2000 mg/kg bw of *Beta vulgaris* L. leaf aqueous extract. The animals were monitored for any kind of toxicity symptoms for 14 days.

In Phase V, the animal study was approved by the Institutional animal ethical committee of Srimad Andavan Arts and Science College (Autonomous), Tiruchirappalli. The ethical number is SAC/IAEC/BC/2020/CP-004. Thirty six Wistar Albino adult female rats with mean body weight (BW) 120 g was selected for the study. After one week of acclimatization, 6-7 week old female rats (n=36) were divided into two groups of normal control and PCO rats. Group I served as normal control group, administered with saline. Group II, III, IV, V and VI served as experimental groups, injected intraperitoneal (i.p) of 10 mg of estradiol valerate in 5 doses at 10 days interval to induce PCO.

After confirmation of PCOS, rats were grouped into six as group I (normal control) which received only normal rat chow, group II served as PCOS induced standard control group administered with saline, group III served as standard control group which received Metformin (50mg/kg bw), group IV served as treatment group which received *Beta vulgaris* L. leaf extract of 100 mg/kg bw, group V served as treatment group which received *Beta vulgaris* L. leaf extract of 200 mg/kg bw, and group VI served as treatment group which received *Beta vulgaris* L. leaf extract of 400 mg/kg bw.

For analyzing the physical parameters i.e., body weight, liver weight, ovary weight and biochemical parameters i.e., blood glucose, lipid profile – total cholesterol, triglyceride, high density lipoprotein, low density lipoprotein, hormonal analysis i.e., luteinizing hormone, follicle stimulating hormone, estrogen, antioxidant assay i.e., super oxide dismutase, catalase, glutathione stimulating hormone, and lipid peroxidation (malondialdehyde) concentration and histopathology analysis of the ovary.

One way ANOVA with post hoc test (tukey's test) was carried out to interpret intra group variation of all biomarkers which had been tested before and after the experiments. P value <0.01 and <0.05 were accepted as statistically significant values.

Salient findings of the study are given as follows

- ❖ Determination of total antioxidants using DPPH assay revealed that the three greens powder namely *Daucus carota* L. ($24.70 \pm 0.73\%$), *Raphanus sativus* L. ($23.07 \pm 0.30\%$) and *Beta vulgaris* L. ($42.06 \pm 0.94\%$) had effective scavenging. *Beta vulgaris* L. greens powder showed highest radical scavenging activity.
- ❖ Total Phenols using Folin – Ciocolteu method (F-C) method analyzed in the vegetable greens powder revealed a higher phenolic content in *Raphanus sativus* L. (390.66 ± 12.80 mg), *Beta vulgaris* L. (346.60 ± 1.30 mg), and *Daucus carota* L. (118.82 ± 0.6 mg) per 100g, thus confirming the presence of phenols. Phenols were found to be superior in *Raphanus sativus* L. and *Beta vulgaris* L. greens powder.
- ❖ Total flavonoids analysed using spectrophotometric assay in the vegetable greens powder revealed *Daucus carota* L. (845.01 ± 13.07 mg), *Raphanus sativus* L. (381.8 ± 7.03 mg), and *Beta vulgaris* L. (2145 ± 21.06 mg) per 100 g, thus confirming the presence of polyphenol flavonoids.
- ❖ *Beta vulgaris*. L. leaf powder scored significantly higher total antioxidant (42.06 ± 0.94 %) and total flavonoids (2145 ± 21.06 mg). Though *Beta vulgaris*. L. powder had lesser amount of total phenolic content (346.60 ± 1.30 mg) when compared with *Raphanus stivus* L. (390.66 ± 12.80 mg). But on comparing total antioxidants, total phenols and total flavonoids parameters *Beta vulgaris* L.

leaf powder scored high in total antioxidants and flavonoids. Hence, *Beta vulgaris* L. greens powder was selected for the future analysis.

- ❖ Ethanol, methanol and aqueous extracted *Beta vulgaris* L. greens powder contains phytochemicals like alkaloids, flavonoids, amino acids, proteins, sterols, cardiac glycosides, tannins, phenols, saponins and oxalate. The total alkaloids, total saponin, total oxalate, total tannins of *Beta vulgaris* L. greens powder were 68.8 ± 0.08 , 20.91 ± 0.01 , 3.60 and 7.76 mg per 100g respectively.
- ❖ DPPH is one of the free radicals widely used for testing preliminary radical scavenging activity of leaf extract. Scavenging of DPPH radical is related to the inhibition of lipid peroxidation. *Beta vulgaris* L. greens powder showed the maximum inhibitory activity in the aqueous extract at 80mg/ml. The percentage of inhibition was from 30, 40, 50, 60, 70 at 56, 65, 70, 76, 80 mg/mL for *Beta vulgaris* L. and the IC_{50} value for *Beta vulgaris* L. was 60 mg/ml while for standard it was found to be 8.5 mg/ml.
- ❖ *Beta vulgaris* L. greens powder contains ash ($1.4 \pm 0.01\%$), moisture ($9.13 \pm 0.8\%$), energy (21.14 ± 0.04 kcal), carbohydrate (1.030 ± 0.09 g), protein (2.4 ± 0.03 g), total fat (0.79 ± 0.03 g), dietary fibre (2.93 ± 0.03 g), iron (2.54 ± 0.02 mg), calcium (52.50 ± 0.73 mg) and vitamin C (382 ± 4.68 mg) per 100 g respectively.
- ❖ HPLC analysis of *Beta vulgaris* L. greens powder revealed presence of important natural flavonoid compounds with high peak percentage of ellagic acid (RT 4.145), gallic acid (RT 5.321) and catechin (RT 7.155).
- ❖ GCMS screening on secondary metabolites in *Beta vulgaris* L. greens powder identified fifteen different compounds that showed a peak area of 73.1% tetraoxane followed by Decane, 4-Hepten-2-one, 5-ethyl-3,3,4-trimethyl, Phthalic acid, Hexadecanoic acid, Phytol and 17-Pentatriacontene that are considered to possess antioxidant, hepatoprotective and antimicrobial activities.
- ❖ HPLC screening of Vitamin A (Beta carotene) was $3.5 \mu\text{G}/100\text{g}$ and vitamin E was below detection level.

- ❖ Aqueous extracts of *Beta vulgaris* L. greens ranging from 50 mg/kg body weight (low dose) to 2000 mg /kg body weight (high dose) was administered to female rats and observed for 14 days. None of the rats in experimental study showed toxicity signs in their general behavior. Central nervous system stimulant activities such as stub tail, irritability and hyperactivity exhibited immediately after supplementation of high dose of 2000 mg/kg BW of *Beta vulgaris* L. extract got subsided after 3-4 hours.
- ❖ Central Nervous System (CNS) depression activities namely hypoactivity, passivity, relaxation, necrosis and ataxia were absent in the control and experimental groups after supplementation of *Beta vulgaris* L. leaf aqueous extract. The Autonomous Nervous System (ANS) activities such as urination and salivation after supplementation of high dose of *Beta vulgaris* L. aqueous extract of 2000 mg/kg body got diminished after 3-4 hours.
- ❖ A normal estrous cycle of 4-5 days with all four phases in sequential order was observed in the control group whereas estrous cycle in the PCOS induced standard control groups was irregular and long. PCOS induced metformin standard control Group III and *Beta vulgaris* L. leaf extract treated groups IV, V and VI showed restoration of estrous cycle.
- ❖ All the groups except group II (PCOS induced standard group) showed increase in mean body weight. Among the treatment groups mean body weight in treatment group IV 143.33 ± 2.9 g and group V 152.83 ± 2.6 g, showed similar weight gain compared with Group III (standard group) treated with metformin 156.17 ± 3.2 g. Treatment group IV, V and VI with *Beta vulgaris* L. leaf extract showed significant ($p < 0.05$) increase in body weight compared to PCOS induced standard group II.
- ❖ High Blood Glucose level noted in PCOS induced group II were 156.67 mg/dl due to insulin resistance is considered to be a central link in the pathogenesis of PCOS. In treatment groups, group VI treated with 400 mg of *Beta vulgaris* L. greens powder showed blood glucose 116.50 ± 3.62 mg/dl and group V treated with 200 mg of *Beta vulgaris* L. greens powder showed 125.17 ± 1.72 mg/dl. Similar result were obtained in PCOS induced standard control group III (103.83 ± 4.12 mg/dl) treated with metformin.

- ❖ Total cholesterol decreased in *Beta vulgaris*.L leaf extract treatment group IV, V and VI with 100 mg, 200mg and 400 mg were 375.83 ± 3.71 , 297.50 ± 3.61 and 196.33 ± 3.93 mg/dl respectively. Triglyceride levels decreased in *Beta vulgaris*.L leaf extract treatment group IV, V and VI were 190.00 ± 3.40 , 152.33 ± 3.01 , and 124.50 ± 2.66 mg/dl compared with PCOS induced metformin standard control group II were 264.83 ± 3.87 mg/dl. High Density Lipoprotein Cholesterol increased in *Beta vulgaris* L. leaf extract treatment group IV,V and VI were 37.50 ± 1.87 , 48.33 ± 2.16 , and 60.33 ± 1.63 mg/dl compared with PCOS induced metformin standard control group II were 28.66 ± 2.50 mg/dl. Low Density
- ❖ Lipoprotein Cholesterol decreased in *Beta vulgaris* L. leaf extract treatment group IV,V and VI were 73.31 ± 1.92 , 61.89 ± 0.22 and 57.97 ± 1.42 mg/dl compared with PCOS induced standard control group II 104.77 ± 2.67 mg/dl. Total cholesterol, triglycerides, and LDL-C significantly ($P < 0.01$) decreased and HDL-C significantly increased ($P < 0.01$) in the treatment groups compared with the PCOS standard control group.
- ❖ Administration of *Beta vulgaris* .L leaf extract in treatment group VI of 400 mg for 21 days decreased leutinizing hormone 148.17 ± 5.45 mIU/ml hormone level in treatment group VI compared with the PCOS induced metformin standard control group II 104.77 ± 2.67 mIU/ml.
- ❖ Follicle stimulating hormone levels in the PCOS induced control group II 9.05 ± 0.37 mIU/ml were significantly lower compared to the treatment group IV, V and VI 20.25 ± 0.17 , 25.16 ± 0.87 and 34.61 ± 1.20 mIU/ml. FSH level significantly increased in metformin treatment group III 36.96 ± 0.63 mIU/ml. Administration of *Beta vugaris*.L leaf extract in treatment groups V and VI showed significant increase ($p < 0.01$) in FSH hormone level.
- ❖ Estradiol levels in *Beta vulgaris* .L leaf extract treatment groups IV, V and VI 1.10 ± 0.24 , 1.01 ± 0.01 and 0.76 ± 0.01 pg/ml significantly decreased ($p < 0.01$) compared to the PCOS induced standard control group II (1.32 ± 0.01 pg/ml).
- ❖ Lipid peroxide level of treatment group IV, V and VI with *Beta vulgaris* L. were 99.00 ± 2.09 , 80.66 ± 3.26 and 60.00 ± 2.82 mM MDA/g respectively. Lipid peroxidation level significantly decreased ($p < 0.01$) in treatment groups

compared with PCOS induced metformin standard control group III 56.66 ± 3.26 mM MDA/g.

- ❖ PCOS induced control group II 3.65 ± 0.67 mg/g of estradiol valerate significantly decreased ($p < 0.01$) the superoxide dismutase activity in the ovary. *Beta vulgaris* L. leaf extract administered treatment group V significantly increased ($p < 0.01$) the superoxide dismutase activity in the ovaries in comparison to the PCOS induced metformin standard control group III 11.05 ± 1.05 mg/g in rats.
- ❖ Treatment with *Beta vulgaris* L. leaf extract of 100mg, 200mg and 400 mg in treatment groups IV, V and VI reversed the estradiol valerate effect as 12.50 ± 0.70 , 15.00 ± 0.53 and 21.40 ± 0.90 mg/g respectively evidenced by a significant increase ($p < 0.01$) in catalase activity, in the ovarian homogenate.
- ❖ Glutathione stimulating hormone levels were significantly decreased ($p < 0.01$) in PCOS induced group II (3.50 ± 0.78 mg/g) in comparison to normal control group I 17.41 ± 1.31 mg/g. *Beta vulgaris* L. leaf extract treatment group IV and V were 5.25 ± 1.21 and 10.66 ± 1.50 mg/g showed significance at ($p < 0.01$) and treatment group VI were 14.08 ± 1.16 mg/g showed significance at ($p < 0.05$) resulted in a significant increase in the Glutathione stimulating hormone levels in comparison to the PCOS induced metformin treatment group III 16.33 ± 1.16 mg/g.
- ❖ Low dose of *Beta vulgaris* L. leaf extract treatment group IV exhibited follicles larger in size and few corpora lutea. In treatment group V and VI cysts were absent and normal sized healthy follicles were found.

Conclusion

Based on the findings of the present study summarized above, it may be concluded that *Beta vulgaris* L. greens extract contain complex mixture of total phenols and flavonoid compounds that are antioxidants. Phytochemicals such as flavonoids, carotenoids, and other phenolic compounds were shown in studies to have high antioxidant activity and health benefits. This could account for high antioxidant activity of *Beta vulgaris* L. greens extract and thus considered as naturally occurring potential antioxidant source. Estradiol valerate was induced to PCOS in female Wistar rats. There are many hypotheses for the etiology of PCOS, but the exact initiating

event in the pathophysiology of the syndrome is not clear and is poorly understood. Since the study of pathophysiology or any manipulation for treatment of PCOS in human have logistic and ethical limitations; animal modeling is a good choice to improve the understanding of the difficult aspects of PCOS. Since the 1960s, many animal models such as rodents, sheep and nonhuman primates have been applied for this purpose. Using rat models provide an all-purpose tool for studying and understanding the comprehensive biologic mechanisms of PCOS. They have stable genetic background, shorter reproductive, estrous cycle and generation times. Besides, these models have shown same reproductive and metabolic conditions as human PCOS. Exposure to a single dose of estradiol valerate (EV) in adult rat can cause irregular cycles, lack of ovulation and polycystic ovaries with high number of atretic follicles and cysts. These changes in ovarian morphological features are similar to those of PCOS women. Treatment with metformin was used as standard, significantly metformin ameliorated hormones, lipid profiles, glucose and insulin as well as oxidative stress in PCOS condition. Treatment with *Beta vulgaris* L. leaf extract supplement significantly reversed the levels of hormones, lipid profile, glucose and oxidative stress as compared to PCOS induced group. Treatment with metformin and *Beta vulgaris* L. leaf extract effectively reversed the PCOS induced damage to ovaries.

Strength of the study

The supplementation of *Beta vulgaris* L. leaf extract in estradiol valerate induced rats improved antioxidant status, decreased oxidative damage, reverse reproductive hormone levels, improved lipid profile, improved glucose level and reversed the damage of the ovaries, therefore, the results of the study proves that a regular intake of red beet greens is a better way to improve oxidative damage and possibly contributes to reduced risk of developing chronic diseases.

Limitation of the study

Analysis of parameters related to reproductive hormones like estrogen level, progesterone level, testosterone level, Antioxidant assay like total antioxidant capacity, Myeloperoxidase (MPO) would have given more details on the effect of administration of *Beta vulgaris* L. leaf extract on PCOS.

Future Recommendations

1. Studies on bioavailability of bio-active components of Beta vulgaris L. leaf like betacyanin, betaxanthin and polyphenols can be isolated and used for incorporation in drug formulation for treatment of PCOS.
2. Identification of nutraceutical compounds of Beta vulgaris L. leaf will help in formulation of drugs for many chronic diseases.
3. Future studies on encapsulation of Beta vulgaris L. leaf powder would be helpful of human consumption.
4. As PCOS has complex pathophysiological characteristics, which have not yet been understood completely a number of clinical trials has to be carried out in the future for formulation of drug based on plant sources.