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Research Article

The Effect of Thylakoid Membranes of Spinach Extract Supplementation on Atherogenic, Glycemic, and Anthropometric Indices and Renal Function in Obese PCOS Women under a Hypo-Caloric Diet: A Randomized, Double-Blind, Controlled Trial

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Background. Hyperandrogenism is a common disorder in women with polycystic ovary syndrome (PCOS) that can cause changes in body fat distribution and the amount of visceral adipose tissue. Visceral adiposity impairs insulin action, leading to insulin resistance (IR), cardiovascular disease, and renal disorders due to obesity and insulin resistance. Dietary thylakoids reduce visceral fat mass by suppressing appetite and regulating body weight. The present trial aimed to evaluate the fat distribution and renal function after thylakoid membranes of spinach supplementation along with a hypo-caloric diet. *Methods*. Forty-four obese women with PCOS participated in this randomized, double-blind, placebo-controlled clinical trial for 12 weeks and were allocated to receive 5 gr of thylakoid membranes of spinach extract combined with hypo-caloric diet or 5 gr placebo along with a hypo-caloric diet. Novel atherogenic and anthropometric indices including the atherogenic index of plasma (AIP), Castelli risk index I (CRI-I), Castelli risk index II (CRI-II), TyG-BMI (TyG-BMI), metabolic score for insulin resistance (METS-IR), abdominal volume index (AVI), body adiposity index (BAI), a body shape index (ABSI), and serum urea, creatinine, and total protein were assessed at the baseline and end of the intervention period. *Results*. Thylakoid membranes of spinach supplementation along with a calorie restriction diet showed a significant decrease in the AIP, CRI-I, II, TyG-BMI, and METS-IR ($P < 0.05$). AVI, BAI, and ABSI were found to reduce in the thylakoid and placebo groups ($P < 0.05$). However, the changes in serum urea, creatinine, and total protein did not show significant differences between the intervention and placebo groups. *Conclusion*. After 12-week supplementation with thylakoid membranes extracted from spinach, improvements in the value of atherogenic indices and insulin resistance surrogate markers were observed, while this intervention did not make a significant difference in the serum levels of renal function factors. This trial is registered with IRCT20140907019082N9.

1. Introduction

Polycystic ovary syndrome (PCOS) is a heterogeneous endocrine disorder that affects 5–10% of women during their reproductive age [1]. PCOS is defined as chronic anovulation (often shown as oligo—or amenorrhea), metabolic abnormalities (insulin resistance (IR), hyperinsulinemia, obesity, and acanthosis nigricans), and hyperandrogenism [2]. PCOS is a heterogeneous disease that is not only associated with long-term metabolic abnormalities such as IR but also associated with endothelial dysfunction, dyslipidemia, and systemic inflammation which exposes individuals to a higher risk of cardiovascular disease (CVD) [3] and kidney diseases due to obesity and IR, which have multiple links with kidney diseases [4]. Comorbidities of PCOS include traditional CVD risk factors such as obesity, impaired glucose tolerance (IGT), type 2 diabetes mellitus (T2DM), dyslipidemia, and hypertension [5, 6].

Women with PCOS who have hypertension are very susceptible to kidney disorders [7]. Furthermore, high levels of androgens in PCOS individuals contribute to renal impairment by increasing blood pressure and affecting renal function [8] as well as alterations in body fat distribution [9]. Previous research has found an association between androgen levels and visceral adipose tissue (VAT) and the amount of abdominal fat [10–12]. According to studies on fat compartmentalization in PCOS, women with PCOS show a predisposition to abdominal fat distribution when anthropometric measurements are performed [13]. It has been discovered that visceral obesity causes metabolic and endocrine abnormalities that are critical in the pathogenesis of PCOS [14–16]. Visceral obesity also affects the action of insulin, resulting in IR and compensatory systemic hyperinsulinism and hyperandrogenism development [17, 18], as well as being associated with elevated adipocytokine production, proinflammatory activity, a reduction in insulin sensitivity, a greater risk of developing T2DM, high triglyceride (TG), low-HDL cholesterol, dyslipidemia, atherosclerosis, hypertension, and an increased mortality rate [17]. Body mass index (BMI), waist circumference (WC), and waist-to-height ratio (WHTR) can all be used to evaluate central obesity in PCOS patients, although some studies have indicated that these indices provide limited data on fat distribution. Consequently, more appropriate anthropometric indices that incorporate body shape with disease prediction in order to assess central obesity are required; novel anthropometric indices can represent central obesity more accurately than traditional anthropometric indices [19]. Abdominal volume index (AVI) is a reliable anthropometric measure for estimating obesity that is strongly related to IGT and T2DM [20] and predicting metabolic abnormalities [21]. Body adiposity index (BAI) is a valuable measurement for predicting glucose metabolism problems [22]. A body shape index (ABSI) was found to be more closely related to the risk of T2DM than BMI [23]. Therefore, it is critical to quantitatively study body fat deposition changes in PCOS through novel anthropometric indices to evaluate the fat distribution and investigate the visceral adiposity independent of the overall obesity of women with PCOS.

Thylakoids are chloroplast inner photosynthesis membrane structures that could be isolated from green plants such as spinach [24]. Thylakoids are made up of protein-bound pigments like chlorophyll, beta-carotene, lutein, and zeaxanthin, as well as antioxidants including carotenoids and vitamin E [25, 26].

Dietary thylakoid membranes bind to pancreatic lipase and colipase, reducing fat digestion in the intestine, decreasing food consumption, as well as diminishing visceral fat mass [27]. In animal studies [28, 29], when the diet was supplemented with spinach extract containing thylakoids, a significant reduction in body weight and body fat percentage was observed. Montelius et al. [30] demonstrated that while green plant membranes were added to the daily diet for three months, they significantly decreased body weight, total cholesterol, and low-density lipoprotein cholesterol levels in overweight women compared to a control group. Meanwhile, thylakoids were shown to promote body weight loss significantly in addition to anthropometric indices including BMI, WC, WHR, and FM, which significantly decreased in 12 weeks of intervention [31, 32].

Moreover, spinach extract, the richest source of nitrate, has been shown to have cardioprotective benefits by increasing postprandial plasma nitrate and nitrite concentrations [33] and lowering blood pressure. Furthermore, it could have antihypertensive actions by regulating body weight and insulin resistance [34, 35]. Based on observational research, hypertension has long been considered a risk factor for kidney function impairment [36, 37]. Furthermore, extensive epidemiological studies have shown that obesity, particularly visceral obesity, plays a critical role in the development of chronic renal disease [38]. Therefore, we hypothesize that thylakoid supplements along with a low-calorie diet can have beneficial effects on the distribution of visceral fat, in addition to improving glycemic, lipid, and anthropometric indices and renal function in obese PCOS women.

2. Methods

2.1. Trial Design and Participants. The present study was a randomized, double-blind, placebo-controlled clinical trial, conducted in the northwest of Iran, Tabriz city. A random sample of individuals with obesity and diagnosed PCOS were recruited from Sheykholrayis Polyclinic and the

gynecology and infertility clinics of AL Zahra Hospital (Tabriz, Iran). PCOS was diagnosed based on the Rotterdam criteria, which was diagnosed with two of the following three criteria: hyperandrogenism, ovulation disorder, and polycystic ovary. Eligibility criteria required individuals aged 20–45 years old to have received only oral contraceptive pills (OCPs) as routine medical therapy if there was no evidence of thyroid disorders, adrenal cortex dysfunction, or hyperprolactinemia. The exclusion criteria of subjects were as follows: menopause, or pregnancy, or lactation; being a smoker or a passive smoker; any other endocrine disorder; or cardiovascular disease; taking supplements or drugs affecting appetite and weight, insulin sensitivity, or ovulation induction; having any special diet before starting the study and/or undergoing special physical activity training. Forty-eight obese PCOS patients were recruited for this study, and the study's aim was explained to all participants before data gathering. All patients provided written informed consent after receiving a thorough description of the research protocols.

The current trial was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the research vice chancellor of Tabriz University of Medical Sciences, Tabriz, Iran (IRCT registration number: IRCT20140907019082N9 and ethics code: IR.TBZMED.REC.1401.892).

2.2. Study Protocol. Using random assignment software (RAS) and the randomized block method, an allocation sequence was generated, and participants were randomized into two groups with equal sample sizes (1 : 1). To perform allocation concealment, sequentially numbered opaque sealed envelopes are used. The envelopes are opened sequentially at the beginning of the intervention in the order of entry, and the grouping of participants is determined according to the envelope containing the allocation plan inside it. The personnel involved in the fieldwork and participants were kept concealed regarding the randomization during the whole study. The researchers and patients were kept concealed about the randomization and distribution until the final analyses were performed.

2.3. Interventions. The eligible individuals were randomly recruited to receive 5 gr/day thylakoid extract powder or 5 g/day placebo (raw corn starch powder) for 12 weeks along with a low-calorie diet (500 kilocalories less than the estimated daily energy requirement for each participant) prescribed by an expert dietitian for both groups. This trial was conducted under double-blind conditions; the intervention, randomization, and allocation were administered by an independent person who was not involved in any clinical procedures of the study; both study personnel and participants were blinded regarding the allocation and treatment until the end of the analysis. To ensure the blinding process, placebo sachets (corn starch) were colored with an edible green color. Both placebo and thylakoid powders were flavored with kiwi fruit so that the two types of sachets were completely identical in all visual, olfactory, and textural aspects. The participants were instructed to dissolve the sachet of powder content in a glass of water and consume it 30 minutes before lunch. Adherence to the recommended hypo-caloric diet was assessed using dietary intake records for 3 days (2 weekdays and 1 weekend) at the beginning of the study, the sixth week, and at the end of the study.

2.4. Outcomes. The primary study outcome was to evaluate and compare the value of novel glycemic, atherogenicity, and anthropometric indices among PCOS patients at the baseline and end of 12 weeks of thylakoid supplementation along with a low-calorie diet in two groups: intervention and placebo. Secondary outcome measures were the comparison of renal function markers, including urea, creatinine, albumin, and total protein, in two groups at the beginning and end of the study. For routine blood measurement and any anthropometric measurements, the participants were present between 8:00 and 10:00 am, following the fasting state in the designated place in the presence of staff who were professional healthcare providers. Detailed measurements of each index and biochemical factor are given below.

2.5. Anthropometric and Body Composition Measurements. The height and weight of the participants were measured using a wall-mounted stadiometer and a Seca scale (Seca co., Hamburg, Germany) to the nearest 0.5 cm and 0.1 kg, respectively. Waist circumference was measured at the midpoint between the lower costal margin and the iliac crest using a tape measure to the nearest 0.1 cm. Hip circumference was measured over the widest part of the buttocks and was recorded to the nearest 0.1 cm. Body composition was assessed using the Tanita MC-780 SMA (Amsterdam, the Netherlands), a validated instrument for bioelectrical impedance analysis (BIA).

2.6. Atherogenicity, Insulin Resistance, and Anthropometric Indices. The formulas that were implemented to calculate the indices measured in the present study are indicated below [19, 39, 40], and the optimal cut off values were also obtained from previous studies [41–46]:

$$
\begin{aligned}
\mathrm{AIP} &= \mathrm{Log10}\left(\frac{\text{triglycerides (mmol L)}}{\text{HDL} - \text{C (mmol L)}}\right),\\
\mathrm{CRI{-}I} &= \frac{\text{total cholesterol (mmol L)}}{\text{HDL} - \text{C (mmol L)}},\\
\mathrm{CRI{-}II} &= \frac{\text{LDL} - \text{C (mmol L)}}{\text{HDL} - \text{C (mmol L)}},\\
\mathrm{METS{-}IR} &= \mathrm{Ln}\left(2 \times \text{fasting glucose (mg/dL)} + \text{fasting triglycerides (mg/dL)} \times \left(\frac{\text{BMI (kg/}m2)}{\text{Ln (HDL} - \text{C (mg dl))}}\right)\right),\\
\mathrm{TyG{-}BMI} &= \left(\mathrm{Ln}\left(\frac{\text{fasting triglycerides (mg/dL)} \times (\text{fasting glucose (mg/dL)})}{2}\right)\right) \times \mathrm{BMI}\left(\frac{\mathrm{kg}}{m2}\right),\\
\mathrm{AVI} &= \frac{\left(2 \times (\text{waist})^2 + 0.7 \times (\text{waist} - \text{hip})^2\right)}{1000},\\
\mathrm{BAI} &= \left(\frac{\text{hip}}{\text{height}}\sqrt{\text{height}}\right) - 18,\\
\mathrm{ABSI} &= \frac{\mathrm{WC}}{\left(\mathrm{BMI}^{2/3}\text{height}^{1/2}\right)}.
\end{aligned}
\tag{1}
$$

2.7. Laboratory Analysis. At the study baseline and after the intervention period, venous blood samples (approximately 10 ml) were obtained following a minimum of 12 h overnight fasting and resting in bed during the early follicular phase of the menstrual cycle (days 2–5). The collected blood samples were immediately centrifuged, and the serum samples were separated from the whole blood and frozen at −80 °C until the time of measurement. Serum FBG (glucose oxidase, phenol, 4-amino antipyrine peroxidase method, Pars Azmoon, Cat No: 1500017, Karaj, Iran. Intra- and interassay coefficients of variation (CV) were 1.63 and 2.2, respectively), TG (glycerol-3-phosphate oxidase phenol 4-aminoantipyrine peroxidase method, Pars Azmoon, Cat No: 1500032, Karaj, Iran; Intra- and interassay CVs were 1.7 and 1.58, respectively), TC (cholesterol oxidase Phenol 4-aminoantipyrine peroxidase method, Pars Azmoon, Cat No: 1500010, Karaj, Iran; Intra- and interassay CVs were 1.6 and 2, respectively), and HDL-C analyzed are through enzymatic methods using the colorimetric technique by commercial kits (Man company, Cat No: 613066, Iran. Intra- and interassay CVs were 1.16 and 0.98) by an auto-analyzer (Hitachi-917, Tokyo, Japan). Also, the Friedewald equation was used to calculate LDL-C [47]. The serum insulin level was measured by using an ELISA kit (Insulin kit, Monobind, Lake Forest, Cat No: 2425300, USA. Intra- and interassay CVs were <5.6% both). Blood creatinine (Man company, Cat No.: 613027, Iran. Intra- and interassay CVs were <3.5% both), urea (Man company, Cat No: 613020, Iran. Intra- and interassay CVs were 1.1 and 1.4, respectively), albumin (Man company, Cat No: 613040, Iran Intra- and interassay CVs were 2 and 1.8, respectively), and total protein (Bioassay Technology Laboratory, Shanghai Korean Biotech, Shanghai City, Cat No: E1149Hu, China. Intra- and interassay coefficients of variation (CV) were 1.5 and 2.6, respectively) were measured using the commercial kits. Hormonal profiles including LH, FSH, and total testosterone were determined using ELISA kits (Bioassay Technology Laboratory, Shanghai Korean Biotech, Shanghai City, China. Cat No: E1037Hu, E1001Hu, and E1036H, respectively. Intra-and interassay CVs were less than 5.0% for these measurements), according to the manufacturer's instructions.

2.8. Statistical Analysis. Sample size calculation was performed using sample size software (PASS; NCSS, LLC, US, version 15), taking into consideration the mean ± standard deviation results for FBG from the study conducted by Montelius et al. [48] with a 95% confidence interval and a power of 90% in two-sided tests. Considering the drop-out rate of 15%, the final sample size of 24 participants within each group (48 in total) was estimated. All analyses were done using SPSS version 23 (SPSS Inc., Chicago, IL, USA). Results with P values below 0.05 were considered statistically significant. The Kolmogorov–Smirnov test was performed to examine the normality of the data distribution. The distribution of data was presented as the mean ± SD for quantitative data with normal distribution, and frequency (%) for qualitative data. The independent t-test was used to compare qualitative data between the two groups at the baseline and after the intervention. Qualitative data were compared with the chi-square test. A comparison of the two groups after

12 weeks of intervention of the study was performed by the analysis of covariance (ANCOVA) after adjusting for the baseline values and potential confounders including baseline values of each variable, age, and physical activity and total calorie intake. Also, one-way ANCOVA was used to estimate the effect size by taking the difference in the means of the two groups and dividing it by the pooled standard deviation. Independent *t*-tests are used for reporting the mean difference (MD) with a 95% confidence interval (CI) for between-group differences.

3. Result

Forty-eight eligible women diagnosed with PCOS with obesity who matched the selection criteria were recruited for this study and randomized to placebo and intervention groups ($n = 24$ in each group). Three participants from the thylakoid group (due to in vitro fertilization therapy and loss of follow-up) and one subject from the placebo group (due to travel) fell out of the research. Consequently, forty-four participants remained during the 12-week intervention period (intervention ($n = 21$) and placebo ($n = 23$)) (Figure 1). During the study, no adverse effects or signs were recorded after the participants consumed thylakoid powder, so no interim analysis and stopping instructions were done in the process of the present study.

The baseline characteristics of participants are shown in Table 1. No significant differences were observed between the two groups regarding the age (P, 0.81), body composition including fat mass and fat-free mass (P, 0.84, and 0.42, respectively), energy intake (P, 0.70), fasting blood glucose (P, 0.08), and lipid profiles such as TG and TC (P, 0.77, and 0.62, respectively). Regarding the status of sex hormones, no significant difference in the testosterone level (P, 0.12) and the LH/FSH ratio (P, 0.31) was observed between the two groups at baseline. The results obtained from the preliminary analysis of atherogenic indices are presented in Table 2. Despite a significant reduction in the atherogenic index of plasma (P_{adjusted}, <0.001; mean change, −0.02), Castelli risk index I (P_{adjusted}, 0.002; mean change, −0.12), and II (P_{adjusted}, 0.003; mean change, −0.07) in the thylakoid group, no significant differences were observed in the AIP, CRI-I, and II indices in the case of controls ($P_{\text{crudeoradjusted}} > 0.05$). TyG-BMI and METS-IR decreased in both the thylakoid and placebo groups ($P < 0.001$). Following our results in anthropometric indices, a significant reduction in AVI (P for both groups <0.001), BAI (P for both groups <0.001), and ABSI ($P_{\text{intervention}}$, 0.029 and $P_{\text{control}} < 0.001$) was found in the thylakoid and placebo groups ($P < 0.05$). Table 3 shows renal function markers among the two groups of study; no significant differences were found in serum urea, creatinine, and total protein between the baseline and end of the study ($P > 0.05$). There was a significant increase in creatinine levels in the intervention group (P, 0.021), which lost its significance after adjusting for confounding factors (P_{adjusted}, 0.694). The serum albumin was observed to increase in the thylakoid and placebo groups (mean change for intervention and placebo group, 2.14 and 1.41, respectively; $P < 0.001$), but it disappeared after adjustment for potential confounders both in the intervention and placebo groups ($P = 0.955$ and 0.768, respectively). Table 4 shows no statistical difference in the mean difference (MD) of AIP (MD, −0.03; 95% CI = −0.06, 0.01; $P = 0.21$), CRI I (MD, −0.22; 95% CI = −0.5, 0.12; $P = 0.19$), CRI II (MD, −0.18; 95% CI = 0.45,0.11; $P = 0.22$), TyG-BMI (MD, −4.32; 95% CI = −17.84, 9.20; $P = 0.52$), METS-IR (MD, −1.28); 95% CI = −3.76, 1.19; $P = 0.29$), BAI (MD, 0.19; 95% CI = −1.13, 1.51; $P = 0.77$), ABSI (MD, <0.001; 95% CI = −0.00, 0.00, 1.51; $P = 0.12$), urea (MD, −2.28; 95% CI = −8.62, 4.06; $P = 0.47$), and albumin (MD, −0.57; 95% CI = −1.18, 0.03; $P = 0.06$ between the thylakoid and placebo groups after 12 weeks of treatment. However, between-group comparison of the effect of 12-week interventions revealed a significant difference in the MD of BMI (MD, −1.64; 95% CI = −0.22, −3.07; $P = 0.02$), AVI (MD, −1.58; 95% CI = −2.58, −0.56; $P < 0.01$), and total protein (MD, −1.78; 95% CI = −2.97, −0.59; $P < 0.01$). There was a high estimated effect size of thylakoid supplementation on the BMI ($\eta^2 = 0.93$), TyG-BMI ($\eta^2 = 0.13$), AVI ($\eta^2 = 0.54$), and total protein ($\eta^2 = 0.17$). Likewise, a low estimated effect size of intervention on the METS-IR ($\eta^2 = 0.01$), urea, and creatinine (η^2 for both <0.01) is observed in Table 4.

4. Discussion

In the present study, forty-four obese women with PCOS treated with 5 gr thylakoid combined with a hypo-caloric diet showed improved atherogenicity, glycemic, and anthropometric indices, but no significant differences in renal function along the treatment period.

To the best of our knowledge, this trial was the first clinical trial that investigated the effects of thylakoid-rich spinach extract supplementation along with a caloric restriction on novel atherogenic, glycemic, and anthropometric indices in addition to renal function. Supplementation of obese PCOS patients with 5 gr/day thylakoid along with a restricted diet resulted in significant reductions in BMI, AIP, CRI-I, and CRI-II compared to placebo. Meanwhile, other indices including TyG-BMI, METS-IR, AVI, BAI, and ABSI, significantly decreased at the end of the 12-week study in both the thylakoid and placebo groups. Likewise, no meaningful impact was observed on renal function factors including urea, creatinine, albumin, and total protein in any of the intervention and placebo groups.

These results are similar to previous reports where thylakoid supplementation decreased body weight [29, 30, 48–50], glycemic parameters [25, 48, 50], and serum lipid profile [48, 50] which are explained in detail below. A randomized, placebo-controlled study with a twelve-week intervention on 53 healthy nonsmoking females, with a BMI range between 25 and 33 revealed that thylakoids reduced body weight, and total and LDL cholesterol levels [29]. In the previous project with the same population, we reported that supplementation with 5 g/day spinach-derived thylakoid along with a hypo-calorie diet improved traditional anthropometric indices such as BMI and WHR, as well as weight and FM that provides limited information about fat

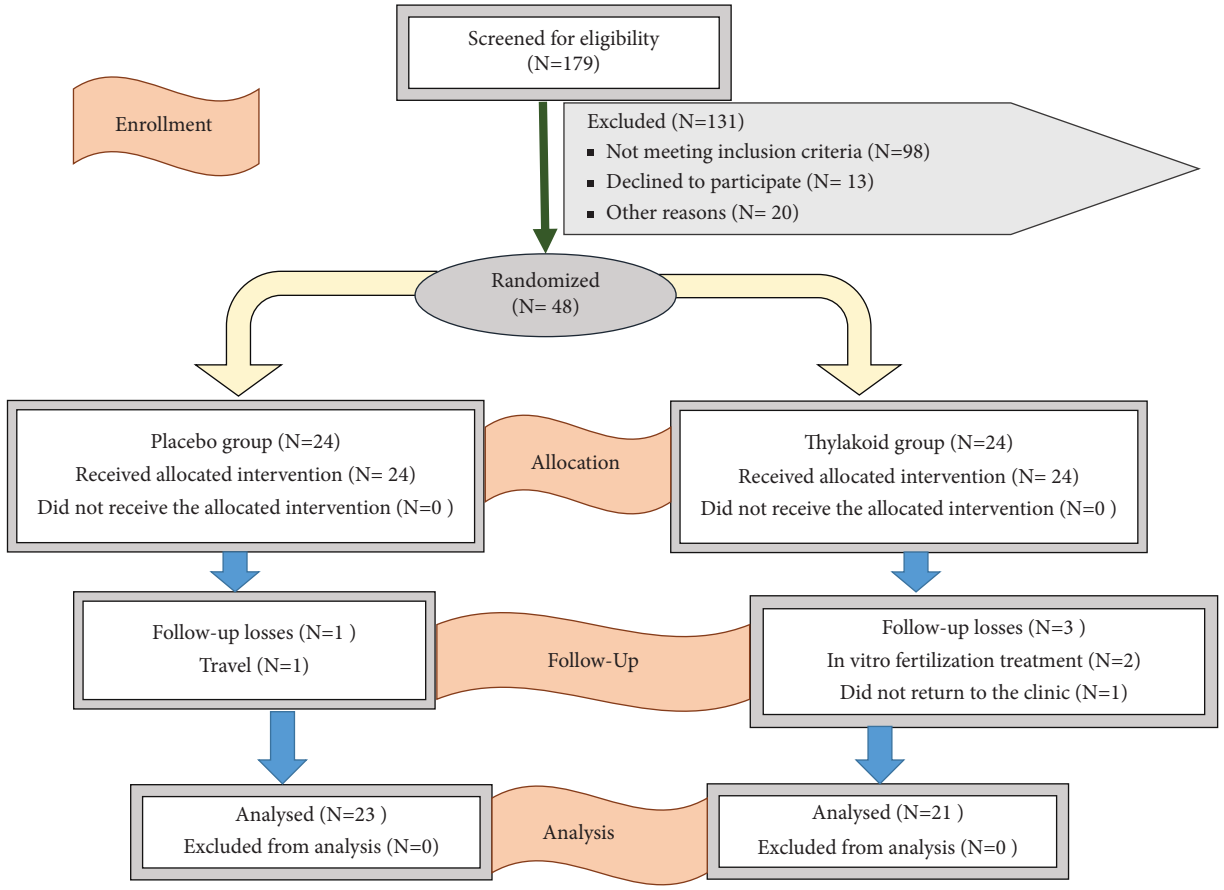

Figure 1: Study flow diagram.

Table 1: General demographic characteristics of study participants.

Variables		Normal range	Thylakoid group ($n = 21$)	Placebo group ($n = 23$)	P^*
Age (y)		—	31.86 (2.35)	32.04 (2.83)	0.81
Blood pressure (mmHg)	Systolic	<120	125.95 (6.64)	125.47 (6.79)	0.82
	Diastolic	<80	92.01 (3.66)	91.81 (3.07)	0.84
Weight (kg)		—	89.21 (6.50)	88.14 (7.27)	0.61
BMI (kg/m^2)		18.5–24.9	35.13 (2.16)	35.31 (2.77)	0.80
WC (cm)		<80	108.09 (3.89)	108.18 (4.22)	0.94
FM (kg)		—	32.40 (3.46)	32.17 (3.90)	0.84
FFM (kg)		—	56.79 (3.21)	55.96 (3.53)	0.42
Energy (Kcal)		—	2346.3 (399.96)	2297.8 (446.26)	0.70
FBS (mg/dl)		70–99	93.09 (5.39)	96.56 (7.44)	0.08
Insulin (μU/mL)		5–15	17.97 (2.45)	18.63 (2.33)	0.37
TG (mg/dl)		<100	145.57 (14.86)	144.09 (18.23)	0.77
TC (mg/dl)		<200	193.52 (8.54)	192.30 (7.45)	0.62
Total testosterone (nmol/L)		0.5–2.4	0.65 (0.06)	0.69 (0.08)	0.12
LH/FSH		1-2	1.80 (0.19)	1.74 (0.22)	0.31

BMI, body mass index; WC, waist circumference; WHR, waist-to-hip ratio; FM, fat mass; FFM, fat -free mass; FBS, fasting blood sugar; TC, total cholesterol; TG, triglyceride; LH, luteinizing hormone; FSH, follicle stimulating hormone. All values are presented as the mean (SD). $^*P$ based on independent sample t-test.

distribution [31]. Considering the need to investigate more appropriate and novel anthropometric indices that combine body shape and disease prediction ability to measure fat distribution, in this study, our investigation showed that thylakoids could also have a beneficial effect on novel indices associated with central obesity and visceral fat distribution.

Table 2: Within-group comparison of the effect of 12 week interventions on values of glycemic, atherogenicity, and anthropometric indices.

Variables	Optimal cut-off	Thylakoid group ($n = 21$)				Placebo group ($n = 23$)			
		Baseline	After 12 weeks	P^*	P^{**}	Baseline	After 12 weeks	P^*	P^{**}
BMI (kg/m^2)	24.5	35.13 (2.16)	32.38 (2.08)	**<0.001**	**<0.001**	35.31 (2.77)	34.03 (2.60)	**<0.001**	**<0.001**
Atherogenic index of plasma	0.66	0.599 (0.066)	0.578 (0.057)	0.063	**<0.001**	0.608 (0.078)	0.603 (0.072)	0.778	0.194
Castelli risk index I	5.17	5.343 (0.651)	5.223 (0.466)	0.362	**0.002**	5.510 (0.794)	5.444 (0.639)	0.769	0.499
Castelli risk index II	3.27	3.539 (0.556)	3.459 (0.384)	0.482	**0.003**	3.686 (0.691)	3.631 (0.535)	0.776	0.463
TyG-BMI	222.9	309.671 (19.947)	297.523 (18.145)	**<0.001**	**<0.001**	312.271 (27.639)	301.843 (25.853)	**<0.001**	**<0.001**
Metabolic score for insulin resistance	31.1	56.734 (3.850)	54.448 (3.474)	**<0.001**	**<0.001**	57.828 (5.791)	55.735 (4.548)	0.004	**<0.001**
Abdominal volume index	17.52	23.456 (1.672)	20.337 (1.757)	**<0.001**	**<0.001**	23.485 (1.816)	21.913 (1.558)	**<0.001**	**<0.001**
Body adiposity index (%)	35.28	40.234 (2.045)	39.538 (1.855)	**<0.001**	**<0.001**	40.419 (2.626)	39.347 (2.468)	**<0.001**	**<0.001**
A body shape index ($m^{11/6} \cdot kg^{-2/3}$)	0.07	0.079 (0.001)	0.078 (0.002)	**0.002**	**0.029**	0.080 (0.001)	0.079 (0.001)	**0.001**	**<0.001**

TyG-BMI, triglyceride glucose-body mass index. P^* values derived from unadjusted ANCOVA P^{**} values derived from ANCOVA after adjustment for confounders (baseline values, age and physical activity, and total calorie intake). The bolded values present a significant threshold.

Table 3: Within-group comparison of the effect of 12 -week interventions on renal function markers.

Variables	Normal ranges	Thylakoid group ($n = 21$)				Placebo group ($n = 23$)			
		Baseline	After 12 weeks	P^*	P^{**}	Baseline	After 12 weeks	P^*	P^{**}
Urea (mg/dl)	15–40	31.39 (8.08)	34.01 (9.4)	0.236	0.521	39.32 (9.9)	36.29 (11.35)	0.317	0.881
Creatinine (mg/dl)	0.5–1.1	0.86 (0.21)	1.07 (0.25)	**0.021**	0.694	1.05 (0.14)	0.97 (0.27)	0.267	0.267
Albumin (g/dl)	3.4–5.4	3.38 (0.64)	5.53 (1.26)	**<0.001**	0.955	4.69 (1.37)	6.10 (0.67)	**<0.001**	0.768
Total protein (g/dL)	6.0–8.3	6.53 (1.23)	6.70 (2.09)	0.798	0.941	6.10 (1.47)	8.48 (1.77)	<0.001	0.635

P^* values derived from unadjusted ANCOVA. P^{**} values derived from ANCOVA after adjustment for confounders (baseline values, age, physical activity, total calorie intake, and changes in weight and body mass index during the intervention period). The bolded values present a significant threshold.

Moreover, in PCOS-induced rats, 8 weeks of thylakoid supplementation at a dose of 6 mg chlorophyll/gr food intake daily by oral gavage for 8 weeks significantly improved the FBS, luteinizing hormone, insulin resistance, and body weight [50]. In addition, thylakoid supplementation along with a high-fat diet for 14 days resulted in increased fecal fat excretion that led to decreased body weight, fat mass, and liver fat accumulation in 30 mice [51]. Köhnke et al. [29] found that a high-fat meal in the combination of thylakoids and pesto resulted in lower levels of ghrelin, serum-free fatty acids, and insulin in normal-weight healthy participants. A double-blind, placebo-controlled, randomized crossover-designed study that was conducted among sixty overweight or obese males and females indicated that supplementation with 5 g of thylakoids could increase satiety as well as a greater increase in the postprandial plasma glucose response compared to the placebo group [25]. Stenblom et al. [52] concluded that the dietary addition of thylakoids to a carbohydrate-rich breakfast resulted in a significant increase in CCK levels postprandially and prevented postprandial hypoglycemia in 20 healthy women.

These observed positive effects of thylakoid extracts on anthropometric indices, glucose homeostasis, and lipid profile could be explained by probable mechanisms, such as lipase/colipase inhibition which stimulates the compensatory release of lipase/colipase and as a mechanism to increase enterostatin which is an appetite suppressant peptide [53]. Thylakoid membranes also delay lipolysis and fat digestion by inhibiting pancreatic lipase/colipase activity that improves the lipid profile [54]. Another possible mechanism is that after consuming a thylakoid-rich meal, cholecystokinin (CCK) secretion increases and postprandial circulating levels of GLP-1 elevate [30], CCK and GLP- 1 can increase insulin secretion, and thylakoids may exert incretin-like effects in this way [53].

The atherogenic index of plasma (AIP) and Castelli indices are used as markers of plasma atherogenicity and are more accurate predictors of cardiovascular risk, especially among individuals with IR [55–57]. In Nawrocka-Rutkowska et al.'s study, the AIP and Castelli index values were higher in the PCOS group with IR compared to the control group [57], and the increased value indicators of atherogenicity indicate that IR has a unique, unfavorable influence on the progression of atherosclerosis. Also, Zhu et al. found a strong association of AIP values with obesity [55]. In the current study, beneficial effect of thylakoid on atherogenicity indices such as AIP, CRI-I, and II can be related to weight loss and central obesity reduction, as well as insulin sensitivity improvement and insulin resistance attenuation following thylakoid supplementation [25, 30, 31, 58]. Furthermore, TyG-BMI, a combination of the TyG index and BMI, has a good performance in the evaluation of IR, and it has also been introduced as a simple and effective index for the evaluation of IR among Chinese women with PCOS [59]. TyG-BMI has also been suggested as a clinical indicator that predicts the occurrence of metabolic syndrome, dysglycemia, and visceral adiposity in women with PCOS [60]. In a two-year prospective cohort study, Bello-Chavolla et al. [61] confirmed that the METS-IR index is a novel score for assessing cardiometabolic risk in healthy and high-risk individuals, as well as a promising tool for screening for insulin sensitivity. Based on the results of the current study, 5 g/day of spinach-derived thylakoid among obese women with PCOS resulted in significant reductions in IR-related indices including TyG-BMI and METS-IR that were observed in the placebo group participants that were under calorie restriction too, so it can be interfered that weight loss is the main core of improvement in TyG-BMI and METS-IR indices. Our results also show that thylakoid membranes supplementation in combination with a hypo-caloric diet significantly affects anthropometric indices including abdominal volume index (AVI), body adiposity index (BAI), and a body shape index (ABSI) that are associated with visceral obesity and body fat distribution [22, 62–64]. Women with PCOS have a similar amount of total and trunk fat as controls but have a larger amount of visceral fat, which is defined by increased thickness of the abdomen fat deposit, particularly in the intraperitoneal area [65]. Visceral adipose tissue (VAT) has fewer insulin receptors located on the cell surface, lower insulin receptor substrate protein-1 expression, and lower insulin receptor affinity. As a result, VAT has less insulin sensitivity and has decreased glucose absorption and utilization [66]. In addition, free fatty acid from VAT lipolysis disrupts the insulin signaling system, reduces liver and skeletal muscle sensitivity to insulin, limits glucose uptake and oxidation, and exacerbates glucose regulation abnormalities [67] (Figure 2).

Considering that obesity, especially visceral obesity, is a risk factor for chronic kidney disease [68, 69], obesity, independent of metabolic problems, contributes to renal dysfunction and structural damage, and so the preventive effect of weight loss on CKD is relatively evident [68]. In overweight or obese diabetic individuals, a lifestyle intervention that included calorie restriction and increased physical activity reduced the incidence of CKD by 30% when compared to controls who received an education

Table 4: Between-group comparison of the effect of 12-week interventions on values of glycemic, atherogenicity, anthropometric indices and renal function markers.

Variables	Periods	Thylakoid group ($n = 21$)	Placebo group ($n = 23$)	MD (95% CI) between groups*	P value	Effect size
BMI (kg/m^2)	Baseline	35.13 (2.16)	35.31 (2.77)	−0.18 (−1.71, 1.34)	0.80	
	End	32.38 (2.08)	34.03 (2.60)	−1.64 (−0.22, −3.07)	0.02	0.93
	MD (95% CI) within groups**	−2.75 (−2.66, −2.82)	−1.27 (−1.15, −1.40)			
Atherogenic index of plasma	Baseline	0.59 (0.06)	0.60 (0.07)	−0.01 (−0.5, 0.03)	0.68	
	End	0.57 (0.05)	0.60 (0.07)	−0.03 (−0.06,0.01)	0.21	**0.03**
	MD (95% CI) within groups	−0.02 (−0.04, 0.00)	−0.00 (−0.04, 0.03)			
Castelli risk index I	Baseline	5.34 (0.65)	5.51 (0.79)	−0.17 (−0.61, 0.27)	0.45	
	End	5.22 (0.46)	5.44 (0.63)	−0.22 (−0.5, 0.12)	0.19	**0.03**
	MD (95% CI) within groups	−0.12 (−0.3,0.14)	−0.06 (−0.52, 0.39)			
Castelli risk index II	Baseline	3.53 (0.55)	3.68 (0.69)	−0.15 (−0.53, 0.23)	0.44	
	End	3.45 (0.38)	3.63 (0.53)	−0.18 (−0.45,0.11)	0.22	**0.03**
	MD (95% CI) within groups	−0.07 (−0.031, 0.15)	−0.05 (−0.45, 0.34)			
TyG-BMI	Baseline	309.67 (19.94)	312.27 (27.63)	−2.59 (−17.39, 12.19)	0.72	
	End	297.52 (18.14)	301.84 (25.85)	−4.32 (−17.84, 9.20)	0.52	0.13
	MD (95% CI) within groups	−12.14 (−13.70, −10.58)	−10.42 (−11.64, −9.20)			
Metabolic score for insulin resistance	Baseline	56.73 (3.85)	57.82 (5.79)	−1.09 (−4.11, 1.92)	0.46	
	End	54.44 (3.47)	55.73 (4.54)	−1.28 (−3.76, 1.19)	0.29	**0.01**
	MD (95% CI) within groups	−2.28 (−3.15, −1.41)	−2.09 (−3.45, −0.72)			
Abdominal volume index	Baseline	23.45 (1.67)	23.48 (1.81)	−0.03 (−1.09, 1.03)	0.95	
	End	20.33 (1.75)	21.91 (1.55)	−1.58 (−2.58, −0.56)	**<0.01**	0.54
	MD (95% CI) within groups	−3.11 (−3.53, −2.70)	−1.57 (−1.83, −1.30)			
Body adiposity index (%)	Baseline	40.23 (2.04)	40.41 (2.62)	−0.18 (−1.62, 1.25)	0.79	
	End	39.53 (1.85)	39.34 (2.46)	0.19 (−1.13, 1.51)	0.77	0.08
	MD (95% CI) within groups	−0.6 (−0.99, −0.39)	−1.07 (−1.33, −0.80)			
A body shape index ($m^{11/6}.kg^{-2/3}$)	Baseline	0.07 (0.00)	0.08 (0.00)	−0.01 (−0.00, 0.00)	0.64	
	End	0.07 (0.00)	0.07 (0.00)	−0.00 (−0.00, 0.00)	0.12	0.06
	MD (95% CI) within groups	−0.00 (−0.00, −0.00)	−0.00 (−0.00, −0.00)			
Urea (mg/dl)	Baseline	31.39 (8.08)	39.32 (9.9)	−7.92 (−13.47, −2.37)	**<0.01**	
	End	34.01 (9.40)	36.29 (11.35)	−2.28 (−8.62, 4.06)	0.47	**<0.01**
	MD (95% CI) within groups	2.62 (−1.84, 7.09)	−3.02 (−9.14, 3.09)			
Creatinine (mg/dl)	Baseline	0.86 (0.21)	1.05 (0.14)	−0.18 (−0.29, −0.07)	**0.02**	
	End	1.07 (0.25)	0.97 (0.27)	0.09 (−0.07, 0.25)	0.26	**<0.01**
	MD (95% CI) within groups	0.20 (0.03, 0.37)	−0.07 (−0.20, 0.06)			
Albumin (g/dl)	Baseline	3.38 (0.64)	4.69 (1.37)	−1.30 (−1.96, −0.63)	0.03	
	End	5.53 (1.26)	6.10 (0.67)	−0.57 (−1.18, 0.03)	0.06	0.04
	MD (95% CI) within groups	2.14 (1.48, 2.80)	1.41 (0.78, 2.03)			
Total protein (g/dL)	Baseline	6.53 (1.23)	6.10 (1.47)	0.43 (−0.41, 1.27)	0.31	
	End	6.70 (2.09)	8.48 (1.77)	−1.78 (−2.97, −0.59)	**<0.01**	0.17
	MD (95% CI) within groups	0.17 (−0.98, 1.26)	2.41 (1.29, 3.53)			

Data are presented as the mean (SD). TyG-BMI, triglyceride glucose-body mass index; SD, standard deviation; MD, mean difference; CI, confidence interval. *Independent t-tests are used at the baseline of the present study for between-group comparison, represented as the mean difference (95% CI). The bolded values present a significant threshold.

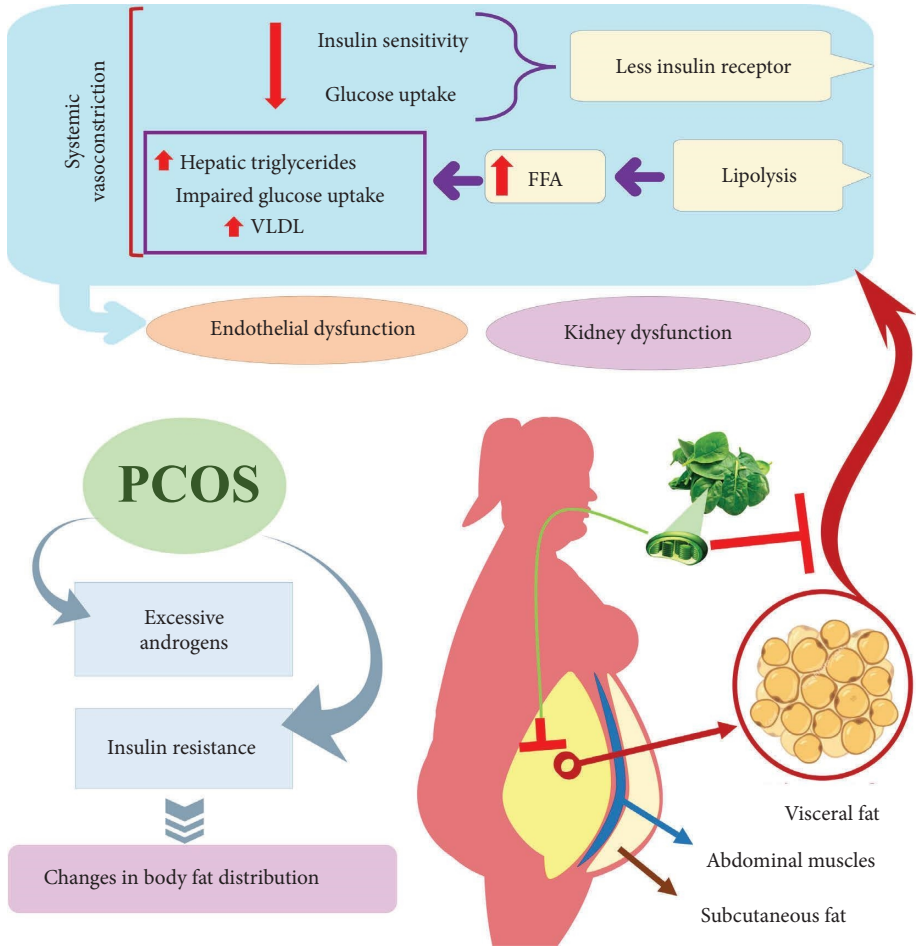

Figure 2: Hyperandrogenism and insulin resistance are the two main biochemical indicators of polycystic ovary syndrome, which causes disturbances in fat distribution and an increase in abdominal fat and visceral adipose tissue in women with this syndrome. Visceral obesity is associated with impairment of insulin sensitivity, increased free fatty acids as a result of lipolysis, high-triglyceride (TG)/, endothelial dysfunction, and kidney disorders. PCOS, polycystic ovary syndrome; FFA, free fatty acids; VLDL; very low-density lipoprotein.

intervention [70, 71]. However, the changes observed in renal function factors including urea, creatinine, albumin, and total protein did not differ significantly between the two groups in the present study.

Some of the limitations of the current study should also be addressed; first, our study was conducted among obese subjects with PCOS aged 20–45 years old, and therefore generalization of our results to other populations should be done with caution. Also, since the evaluation of kidney function in this study was done with blood biomarkers, it is better to find the effects of this intervention on kidney function with urinary markers in further studies. Also, due to prescribing a low-calorie diet to participants, it was uncertain whether they would follow the diet. In addition, another limitation of the present study is the relatively small sample size which reflects the need for further studies with a larger sample size to confirm the findings of this study. Although the most accepted and accurate atherogenic, glycemic, and anthropometric indices have been investigated in this study, one of the most challenging and controversial issues is the interpretation of the results, considering that the quality of the surrogate markers has not been definitively established and is controversial. Therefore, the results should be interpreted with caution until further studies confirm the association between each surrogate factor and cardiometabolic risk factors. Our study also has several strengths. To our knowledge, this is the first investigation in a sample of obese PCOS women in which the risk of visceral obesity and renal disorders is assessed based on the novel anthropometric indices and renal function biomarkers. Follow-up of patients in the form of visits once every two weeks and diet according to the condition of each patient, and adjusting the results for potential confounders that increase the reliability of the results are also other strengths of this study.

5. Conclusion

The results of this trial have led us to conclude that 5 gr per day of thylakoid membrane derived from spinach supplementation in 12 weeks modulates atherogenic and anthropometric indices including AIP, CRI-I and II, TyG-BMI, METS-IR, AVI, BAI, and ABSI of obese PCOS women. However, the changes in urea, creatinine, albumin, and total protein were not statistically meaningful.

Data Availability

The data of the current study are available upon a reasonable request to the corresponding author.

Additional Points

Protocol registration number in Iranian Registry of Clinical Trials. IRCT20140907019082N9.

Disclosure

This study was a part of Negin Nikrad's master's thesis [72].

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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