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EFFECTS OF *ZIZIPHORA CLINOPODIOIDES* EXTRACT ON POLYCYSTIC OVARY SYNDROME, OBESITY, AND INSULIN RESISTANCE

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ABSTRACT

Background: Polycystic ovary syndrome (PCOS) is the most common hormonal disorder in women of reproductive age. The anti-inflammatory properties of the *Ziziphora clinopodioides* plant inhibit the NF-KB signal by suppressing the oxidative stress pathway in cells and preventing inflammation. Therefore, the present study aimed to investigate the effects of *Z. clinopodioides* extract on PCOS, obesity, and insulin resistance.
Methods: In this experimental study, 60 adult Wistar rats were divided into three control (n=12), PCOS (n12), and experimental groups(n=12). After inducing the syndrome for 60 days, the experimental groups were injected intraperitoneally with 100, 150, and 200 mg/kg body weight for 10 successive days. The animals were euthanized with chloroform and their ovaries and blood were collected for histomorphometric and hormonal studies and to determine the inflammatory index CRP levels. Data were analyzed using the one-way ANOVA at a significance level of $p < 0.05$.
Results: Changes in the weight of animals were observed in the control and PCOS groups (n = 12 per group). The body weight of the PCOS group rats increased significantly ($P < 0.001$) compared to the control group. A significant increase was recorded in the ovarian weight of the PCOS group compared to the control group (n = 12 per group). Comparison of ovarian weights in the PCOS group and the PCOS group treated with *Z. clinopodioides* extract (n = 12 per group) indicated a significant decrease in the PCOS group treated with *Z. clinopodioides* extract for 10 consecutive days compared to the PCOS group.
Conclusion: The anti-inflammatory effects of *Z. clinopodioides* extract can significantly reduce ovarian weight in the PCOS group.

Keywords: polycystic ovary syndrome, obesity, ovarian weight

INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most prevalent hormonal disorder in women of reproductive age. PCOS is the most common cause of hyperandrogenism and hirsutism. Hyperandrogenism and anovulation seen in PCOS may occur because of disorders in three important parts active in endocrinology: the hypothalamic-pituitary ovaries axis, and adipose tissue [1].This syndrome is a complex multifactorial complication with multiple factors. In response to LH stimulation, androgens are released from the ovarian theca cells. Cytochrome P450C17 triggers androgen production with the activity of 17-alpha hydroxylase, 17-20 lyase, and androstenedione production. Then, the androgenic steroid is converted to testosterone by 17β-hydroxysteroid dehydrogenase or to estrogen by the aromatase enzyme. *In vitro* and *in vivo* studies have shown that theca cells in polycystic ovaries are more active in converting the androgen precursor to testosterone. Since LH regulates androgenesis in theca cells, FSH regulates aromatase activity in granulosa cells [2,3].

Ziziphora clinopodioides belongs to the Lamiaceae family with thick bushes and a height of 20-50 cm. The leaves are small, opposite, more or less lanceolate, and without a petiole. It has small, full flowers in white, pink, and purple colors. The flowering stage of this plant occurs from July to September. *Z. clinopodioides* is a traditional medicinal plant widely used as a mild sedative, spasmolytic, and antibacterial agent. The leaves, aerial parts, and plant seeds are used in drug production. The seeds of this plant are used as an antipyretic. *Z. clinopodioides* plant has anti-inflammatory properties and inhibits the NF-KB signal and inflammation by inhibiting the oxidative stress pathway in cells[4,5].

Oxidative stress results from the imbalance between the production of free radicals and reactive oxygen species on the one hand and the antioxidant defense system on the other hand. In other words, antioxidant defense mechanisms are designed in aerobic biological systems to counteract free radicals and ROS to neutralize or minimize the harmful effects of these aggressive agents.

Z. clinopodioides extract possesses antibacterial activity against several types of Gram-positive and Gram-negative bacteria. The antibacterial activity of the extract may be attributed to the presence of thymol and polygon. Due to this property, this plant has beneficial effects on intestinal inflammation. A study on the molecular mechanism of the antibacterial activity of thymol indicates that it breaks down the cell wall[6,7].The essential oil of this plant shows an inhibitory effect against food bacteria and prevents food spoilage. According to these results, the main essential oil of *Z. clinopodioides* is used as a natural seasoning and to increase the shelf life of food products. The powdered leaves of this plant are used as food flavoring [8].

Obese people comprise at least 30% of PCOS cases, which can be seen up to 75% in some cases. More weight gain is usually seen in American women suffering from this syndrome than those in European countries. Hyperandrogenemia, insulin resistance, glucose intolerance, and impaired lipid metabolism lead to an increase in fat cells, particularly visceral fat, and an increase in waist size. Despite regular menstruation occurring in many obese women, the percentage of women with irregular menstruation increases with weight gain. Increases in androgens and LH as well as in the aromatization of androgens and estrogen production have been confirmed in obese girls. More than half of PCOS sufferers are obese, and weight loss can alleviate their androgen levels and hirsutism. Returned ovulation by weight loss has even been reported in some women with PCOS. Increased insulin, insulin resistance, and abnormal glucose tolerance tests are seen in obesity, especially when it is accompanied by PCOS. High levels of androgens along with increased insulin and elevated skin pigmentation in certain areas of the body, creating a condition called acanthosis nigricans, is indicative of insulin resistance, especially in women with non-insulin-dependent diabetes mellitus[9,10]. Demonstrations of this syndrome occur during puberty, and it is thought to be associated with weight gain during puberty [11].

PCOS patients typically present with insulin resistance and hyperinsulinemia, both of which play a role in ovarian steroidogenic dysfunction in PCOS. In PCOS, insulin impairs estrogen production in the ovary independent of gonadotropin secretion. Insulin receptors and IGF-1 are present in ovarian stromal cells. A specific disorder in the early stages of insulin receptor-mediated signaling has been observed in 50% of PCOS women. Obesity is the most common cause of insulin resistance and hyperinsulinemia. Despite the prevalence of obesity in PCOS, obesity alone does not explain this important relationship. Because hyperinsulinemia seems to play a role in ovulation with PCOS, treatment with insulin-sensitizing drugs may shift the endocrine balance on ovulation.

CRP was discovered in the sera of patients with inflammation by Tilley (1999)[12]. The biochemical structure of this protein is similar to pneumococcal polysaccharide, and since the polysaccharide is a carbohydrate, the first letter of carbohydrate, C, was chosen as the name of this protein. It combines with the membrane phospholipid of necrotic cells at the inflammation site and activates complement. It also combines with T-cells and prevents its activation by antigen, probably playing a role in immunization. With a molecular weight of about 105,000, this protein is secreted by adipocytes of the liver, and it is believed to be an alpha globulin [13].

The elucidation of the side effects and harmful consequences of chemical drugs has resulted in more attention to the return to herbal and natural drugs in recent years. A new attitude based on the study of medicinal plants and their physiological and pharmacological effects has been started during the past decades. Medicinal plants are also an important source of novel chemicals with strong therapeutic effects. In this regard, *Z. clinopodioides* (Lamiaceae) is an example of these plants. Based on previous studies, this research hypothesizes that the metabolic symptoms of PCOS can be reduced through the anti-inflammatory effects of *Z. clinopodioides* extract, along with the reduction of ovarian angiogenesis at the follicular and stromal level, as well as the appearance of corpora lutea, indicative of the ovary's normal activity in fertility. Given the therapeutic effects of *Z. clinopodioides* extract in the PCOS treatment, this study focused on the effects of this extract on PCOS, obesity, and insulin resistance.

MATERIALS AND METHODS

For the experiments, 60 Wistar rats were assigned as the test group with an approximate weight of 170 ± 20 g. The animals were kept under standard conditions, *viz*. 24 °C, 12 hours of light and 12 hours of dark photoperiod, and free access to food and water. All rats were examined in terms of estrus cycles by preparing vaginal smears for 20 days, and those with four regular estrus cycles were selected and grouped into two large control and estradiol valerate (EV)-induced groups. Rats of the EV group received 2 mg of EV subcutaneously in a single phase during the estrous phase of their cycles. The sham group was injected with the same amount of sesame oil. After 60 days, PCOS induction was confirmed in the EV group compared to the control group with histological examinations. Then, EV rats were intraperitoneally injected with 100, 150, and 200 mg/kg body weight of ziziphora extract for 10 successive days. The saline was injected into the sham group corresponding to this group. After ziziphora extract treatment for 10 consecutive days, ovarian samples were isolated to determine the improvement of polycystic ovaries using histomorphometric and histomorphological analyses with hematoxylin and eosin staining.

Rats selected for PCOS induction should demonstrate at least two regular and consecutive estrous cycles. This event was examined using the vaginal smear test, Papanicolaou Smear Procedure or Shorr, S Staining Procedure [14].

PCOS was induced in rats through subcutaneous EV injection into rats selected according to the mentioned criteria. To this aim, the injection site was first sterilized with 70% alcohol and

the animal's skin was stretched until turn it into a triangular form. Then, 2 mg per animal weight of EV dissolved in sesame oil as a suitable solvent was injected into the skin using a 23 insulin syringe aligned with the body level. The animal was injected slowly due to the high viscosity of sesame oil. The control group rats were injected with the same amount of EV-free sesame oil. The control and PCOS groups were subjected to vaginal smear examinations for 60 days after the injection of sesame oil and EV dissolved in sesame oil to monitor estrous cycle irregularity and the occurrence of the persistent vaginal cornification phase, which is a symptom of ovarian follicular cysts [15].

To confirm PCOS induction, ovaries were removed from three euthanized rats and then analyzed with histomorphometric and histomorphological techniques after molding, sectioning, and staining. After confirming PCOS induction, the rats were divided into two treatment and control groups. The former group received ziziphora extract dissolved in saline at 100,150 and 200 mg/kg of animal weight for 10 consecutive days. The control group rats were injected with the same volume of saline without ziziphora extract. To inject ziziphora extract intraperitoneally, the skin of the animal in the abdominal area was sterilized with 70% alcohol, and the needle of the insulin syringe was inserted into the peritoneal area. For intraperitoneal injection, the animal can also be held upside down so that the head is lower than the body and the viscera are pushed toward the head. The needle of the syringe is then inserted between the groin and the abdomen in the sterilized injection site. Aspiration is done before injection to ensure no insertion to the viscera.

The ovary samples were fixed in Bowen's fixative for 22 h and then dehydrated using alcohol solutions with ascending grades of 20-100% each for 2 h. The samples were cleared with toluene for 2 h and then molded with paraffin. Paraffin molds were cut with a microtome 7 with a thickness and stained serially with hematoxylin/eosin. Finally, the number of follicles in different developmental stages and the thickness of follicular layers were examined through light microscopy. Data were analyzed statistically using INSTAT software and the one-way ANOVA parametric test to determine possible significant differences in the length and weight of the fetuses in the groups. The corresponding graphs were drawn with EXCEL software.

RESULTS

Previous studies suggest that obesity is a symptom of PCOS and is seen in almost 75% of patients with this syndrome. Thus, the observed metabolic changes were compared in the rats of the control and test groups by weighing. In addition to body weight gain in the test group, increased fat was observed macroscopically in the abdominal area compared to the control group.

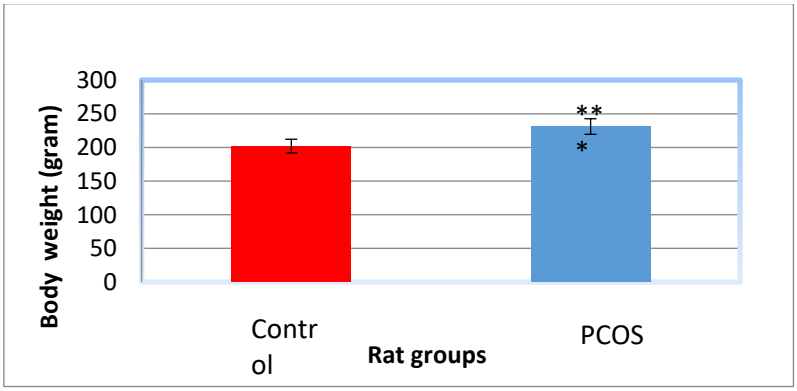

Figure 1. Changes in the weight of animals in the control and PCOS groups (n = 12 per group). The body weight of the PCOS group increased significantly compared to the control group (Mean ± SEM, P < 0.001).

The body weight decreased significantly in the rats treated with ziziphora extract compared to the control group. Accordingly, the injection of ziziphora extract for 10 days led to significant metabolic changes in rats. A surgery after 10 days revealed only a small amount of abdominal fat in the abdominal area of the control animals.

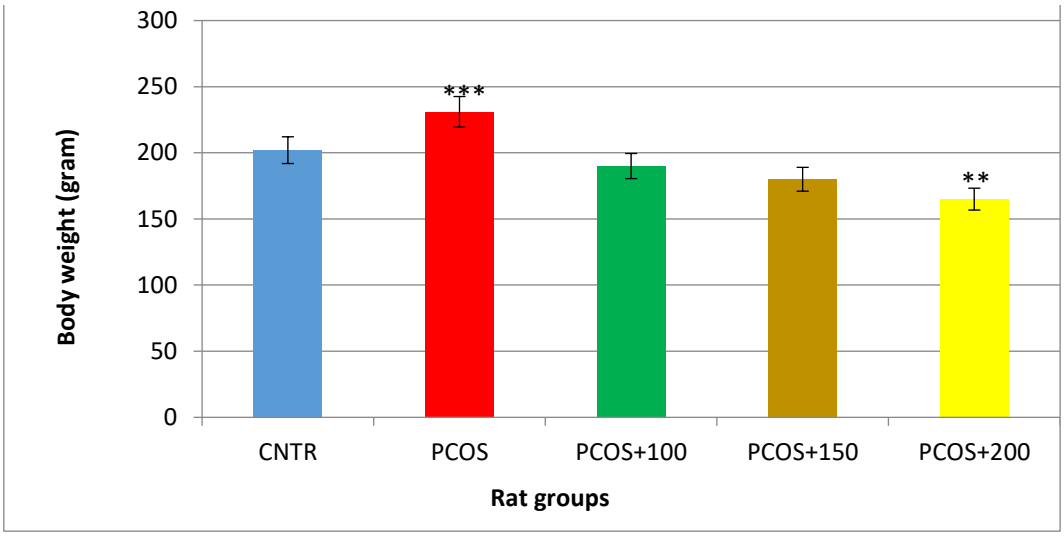

Figure 2. Changes in the body weight of animals in the control group, PCOS group, and group of rats treated with ziziphora extract (n = 12 per group). The body weight increased significantly in PCOS rats ($P < 0.001$) compared to the control group. The body weight decreased significantly with the intraperitoneal injection of ziziphora extract for 10 days (Mean ± SEM, $P < 0.01$). PCOS + 100, PCOS + 150, and PCOS+ 200 indicate the PCOS groups treated with 100, 150, and 200 mg of *Z. clinopodioides* extract, respectively.

Blood was sampled from rats euthanized with chloroform, and the skin of the abdominal area and peritoneum was incised to remove the ovaries and separate the extra fat and oviduct tubes. During this time, serums containing physiological serum were used to prevent tissue desiccation. The ovaries were weighed with a sensitive scale and a significant increase was observed in the weight of ovaries in the test group compared to the control rats. Morphological examinations showed increases in follicular fluid and stroma in polycystic ovaries, elevating ovarian weight in the samples of the test group. Accordingly, it was expected to observe the expansion of the ovarian stroma region in the prepared tissue sections, which was confirmed in this research.

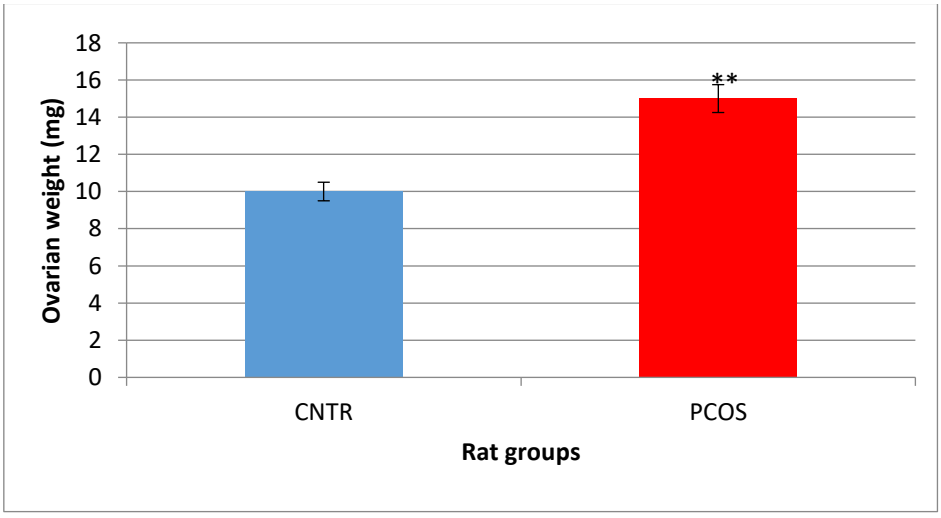

Figure 3. Comparison of ovarian weight in the control and PCOS groups (n = 12 per group). Ovarian weight rose significantly in the PCOS group compared to the control animals ($P < 0.001$).

Treatment of the control group animals with ziziphora extract led to reductions both in the follicular fluid and the stromal size. A significant decrease was observed by weighing and comparing the ovaries of the two groups ($P < 0.01$). Precise examinations of the tissue sections revealed decreases in the density of follicular fluid and stroma.

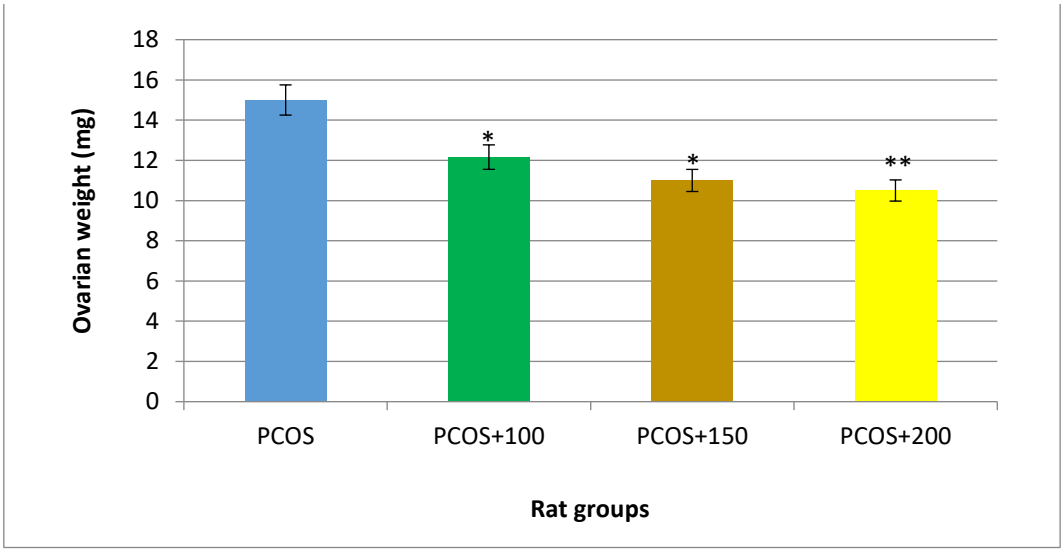

Figure 4. Comparison of ovarian weights in the PCOS group and the PCOS group treated with *Z. clinopodioides* extract (n = 12 per group). The ovarian weight significantly decreased in the PCOS group treated with *Z. clinopodioides* extract for 10 consecutive days compared to the PCOS group (**P < 0.01).

PCOS + 100, PCOS + 150, and PCOS+ 200 indicate the PCOS groups treated with 100, 150, and 200 mg of *Z. clinopodioides* extract, respectively.

DISCUSSION

To confirm the idea of PCOS association its cause and effect relationship with inflammation, Spaczynsk et al. (1999) believe that insulin, IGF-1, and IGF-2 are among autocrine and paracrine regulators of Theca-interstitial in humans and rats and can stimulate the proliferation and steroidogenic activity of these cells. Abnormal function and morphology of these cells contribute to insulin resistance, hyperinsulinemia, increased IGF-1, and PCOS pathogenesis[16]. They examined the serum levels and growing expression of TNF-α in fatty tissue in humans and polycystic rodents and reported its role in regulating the normal activities of a healthy ovary. TNF-α is found in oocytes and healthy and atretic granulosa cells, as well as in a subgroup of single, cells called T-I, in this group of animals. It is also secreted locally by granulosa-lutein cells, macrophages, and ovarian lymphocytes and is involved in the formation of internal theca that requires cell proliferation and differentiation. It also mediates the transition from G0 to G1 in the cell cycle and the proliferation of T-I cells induced by insulin and IGF-1. TNF-α causes the proliferation of granulosa-luteal cells in humans and induces the apoptosis of granulosa cells in antral follicles of rats. Later, Kelly (2001) found that the serum CRP of PCOS women was significantly higher than that of healthy people, and their CRP levels rose with rising BMI in the examined samples[17]. It was concluded that the expression of cytokines e.g., IL6 and TNF-α derived from adipose tissue in people with high BMI played an important role in inflammation induction in these patients.

Other studies evidenced a direct and close relationship between systemic and local inflammation and PCOS. To prove this, a mechanism was proposed by Gonza´lez (2006), who carefully explained the expression of NF-KB and hyperglycemia levels in PCOS patients[18]. Accordingly, hyperglycemia increases ROS production from the blood mononuclear cells of

this group of patients. ROS induces oxidative stress in cells and increases TNF-α transcription and insulin resistance by activating the NF-κB pathway. TNF-α induces the phosphorylation of the insulin receptor serine substrate-1 and then inhibits tyrosine kinase activity. Insulin resistance resulting from NF-κB activity is among the causes of ovarian cysts because of changes in LH activity. As such, an increase in inflammation and the development of PCOS occurred by increasing the dietary glucose count.

Baravalle (2007) claimed that PCOS could be regarded as a metabolic disorder directly associated with Low-Grade Inflammatory. Classic LGI markers, such as CRP, IL-1β, TNF-α, MCP1, MIF, and MMP2, rise in PCOS[15].

In our study, the increased CRP levels in PCOS samples indicate the induction of metabolic manifestations of this syndrome, suggesting that EV could affect the angiogenesis pathway and increase the expression of VEGF, thereby raising the expression of inflammatory metabolites. Some of the syndrome induction methods indicate PCOS-associated metabolic abnormalities and cardiovascular factors common to humans. Repaci et al. proposed a model by DHT that reduced insulin sensitivity and visceral adiposity without changing plasma lipids. Prenatal rats that received free testosterone produced a model showing insulin resistance, adiposity, and increased plasma lipids[19].

Dyslipidemia is a disorder that includes increases in triglycerides, total cholesterol, and Postprandial Lipemia, which has been observed in 70% of women with PCOS according to recent reports. Rhesus monkeys and PL sheep show both metabolic and ovarian aspects of PCOS [20].Accordingly, it can be reported for the first time that estradiol has inflammatory effects similar to DHT to cause symptoms of metabolic disorder among the induction methods of this syndrome; however, this feature is not seen in some of the methods mentioned in the introduction.

PCOS association with the increase of adhesion molecules, such as SICAM-1, SVCAM-1, ET-1, and SE-Selectin, which are markers of endothelial dysfunction and are observed with hyperandrogenemia and insulin resistance, was reported in some other studies. This, therefore, reveals the role of hyperandrogenemia in triggering endothelial disorders. An *in vitro* study has shown that high levels of testosterone promote the development of atherosclerotic lesions through VCAM induction on endothelial cells through an NF-κB-dependent pathway, as well as a final induction of monocyte adhesions. Healthy endothelium is known to regulate vascular tonicity and inhibit many proatherogenic processes, such as the use of monocytes, the adhesion of platelets, the proliferation of smooth muscle cells, the oxidation of LDL, and the synthesis of inflammatory cytokines. Endothelial disorder is a change in endothelial function that leads to incomplete vasoconstriction and vasodilation, procoagulation, activation and adhesion of platelets, and antifibrillization, thereby increasing vascular strength and oxidative stress. Insulin resistance and hypo-ghrelinemia are conditions observed in PCOS that play a key role in the creation and persistence of endothelial dysfunction. These two complications mediate the expression and activation of iNOS [21,22].

Molecules involved in LGI are involved in the pathogenesis of hyperandrogenemia and the opposite also occurs: TNF-α produced in LGI induces the proliferation and steroidogenesis of single cells and enhances the activity of insulin and IGF-1 in a dose-dependent manner. Additionally, it reduces insulin resistance by reducing the activity of insulin tyrosine kinase receptors.

IL-6 can stimulate human adrenal cells and increase adrenal steroidogenesis, including androgens. Androgens indirectly cause the hypertrophy of adipocytes by influencing the expression of enzymes and proteins involved in the metabolism of carbohydrates and fats and are involved in oxidative stress and the differentiation of preadipocytes into mature adipocytes. Androgens also elevate lipolysis and the release of FFA levels [23,24].

A direct relationship between androgens and adipokines in endothelial dysfunction and LGI has been established in other studies. For example, visfatin is an adipokine with high mRNA levels in adipose tissues of PCOS women. In addition, visfatin is directly linked to LH levels, androstene diones, and free testosterone and is indirectly associated with SHBG. Adiponectin and leptin are two adipokines involved in systemic inflammation. With low blood levels in PCOS, adipokine is negatively associated with body weight, abdominal fat, and insulin resistance. The ratio of adiponectin to leptin is sometimes regarded as a marker to investigate inflammation [19,25].

In addition to glucose, the role of consumed fat and proteins in inflammation investigated elsewhere reveals that an acute inflammation in response to food promotes insulin resistance in PCOS women. On the other hand, oxidative stress reduction reduces inflammatory mediators in conditions of calorie restriction and two-day starvation in obese people. Abdominal fat elevation, migration of MNCs to adipose tissue, and macrophages from MNCs in the stromal vascular parts can increase TNF-α production and inflammation. According to Sathyapalan and Atkin (2010), free fatty acids increase in obesity that is a state of chronic systemic inflammation associated with increased serum levels of inflammatory cytokines and changes in the number and function of peripheral blood lymphocytes and account for primary ligands for TOll-like receptors[26]. These receptors are central regulators of the innate immune response, hence obesity-induced inflammation is considered an innate immune problem. Free fatty acids and TOll-like receptors act as a link between inflammation regulatory systems and obesity regulatory systems. At the molecular level, intracellular signaling pathways involved in glucose homeostasis and inflammation have many common signals. At the cellular level, adipocytes and macrophages are closely related being derived from common ancestral cells. Therefore, a parallel evolutionary cycle exists between metabolic systems and inflammation systems.

CONCLUSION

The results of the present study is consistent with the previous studies indicating that ziziphora clinopodioides extract reduce body weight and insuline resistance in the PCOS group.

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