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Research Article

EFFECT OF *SARGASSUM ILICIFOLIUM* ON OVOGENESIS IN POLYCYSTIC OVARY SYNDROME-INDUCED RATS

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ABSTRACT

Objective: This study was conducted to evaluate the effect of *Sargassum ilicifolium* on testosterone-induced polycystic ovary syndrome (PCOS) in rats.

Methods: Female Wistar albino rats were divided into 6 groups consisting of 6 rats each. PCOS was induced in all the animals except normal group on the administration of testosterone (2 mg/100 g) for 21 days daily. After PCOS induction, the standard group rats were treated with clomiphene citrate (CC) (1 mg/kg. p.o.). The test groups were treated with 100, 200, and 400 mg/kg of ethanolic extract of *S. ilicifolium* orally, and normal animals were administered 2% carboxymethyl cellulose (2 ml/kg), respectively, for 21 days during induction and treatment period various parameters such as estrus cycle, histopathology, hormonal, and biochemical assays have been evaluated. Further to confirm the enhanced fertility rate, the rats were mated, and the litter size was observed.

Results: The results revealed the normalcy in estrus cycle, hormonal, biochemical, and histopathological parameters. The litter size was increased significantly compared to control.

Conclusion: Overall, from the obtained results, it is determined that the *S. ilicifolium* exhibited a good antiandrogenic effect by reducing the testosterone levels in PCOS-induced conditions.

Keywords: Clomiphene citrate, Infertility, Polycystic ovarian syndrome, *Sargassum ilicifolium*, Testosterone.

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INTRODUCTION

Infertility is considered as an individual problem, but the impact on women may vary from society to society depending on the culture [1]. Universally, every year 60-80 million couples suffer from infertility where India alone contributes probably between 15 and 20 million [2], in which half of the infertility cases are mainly due to the ovulatory dysfunction [3,4]. Polycystic ovary syndrome (PCOS) is one of the most important causes of ovulation disorders [5,6] with common characteristics such as irregular menstrual cycle, anovulation, hirsutism, amenorrhea and infertility [4,7]. It is generally accepted that hyperandrogenemia is the most important cause for PCOS [8,9].

Seaweeds are used widely to prepare meals as a supplement to the daily ration of the cattle, poultry, and other farm animals. Because seaweed meal enhances fertility, birth rate, general health of animals and improves yolk color in eggs [10]. Iodine has an ability to reduce ovarian cysts by lowering the amount of estrone and estradiol and promotes the amount of anticarcinogenic Estriol. Hence, there is a balance in three forms of estrogen in favor of cancer prevention [11,12]. Iodine is also effective in treating endometriosis, ovarian cysts, and premenstrual syndrome during the treatment of fibrocystic breast disease [12,13]. Considering these properties, the present study was carried to evaluate ovogenic effect of *Sargassum ilicifolium* on PCOS-induced infertility in rodents.

METHODS

Collection, authentication, and extraction

About 3 Kg of fresh marine brown algae *S. ilicifolium* (Turner) C. Agardh was collected from Mandapam coast, Rameswaram, and authenticated by botanist Professor P Jeyaraman, Director, Plant Anatomy Research Centre, West Tambaram, Chennai. (Ref..No. PARC/2016/3287). *S. ilicifolium* was shade dried, coarsely powdered, and subjected for Soxhlet extraction using 95% v/v of ethanol. The obtained ethanolic extract of *S. ilicifolium* (EESI) was preserved in an air tight container and used for further studies.

Drugs and chemicals

All reagents and chemicals were purchased from SD Fine Chemicals, Worli, Mumbai, India. Standard drug clomiphene citrate (CC) (Svizera Health Care, Division of Maneesh Pharmaceutical Ltd, Govandi, Mumbai, India) and testosterone (Sun Pharma Laboratories Ltd, Halol, Gujarat, India) were purchased from local Pharmacy, Bangalore.

Experimental animals and grouping

Around 6 weeks old healthy female Wistar albino rats (150-200 g) were selected for the study. They were well maintained under the standard hygienic conditions with temperature (22±2°C), room humidity (60±10%) with 12 hrs light and 12 hrs dark cycle, and provided with standard pellets and water *ad libidum*. Animals were quarantined for 5 days before the start of the experiment. The study was conducted on 36 healthy adult female rats after the approval of the Institutional Animal Ethical Committee (Approval no: XVI/MSRFPH/M-005/20-01-2016).

Initially, a group of animals was induced PCOS with testosterone for 21 days after PCOS induction, animals were randomly divided into 5 different groups consisting of six animals for drug treatment as shown in Table 1, and the separate untreated group was maintained as normal control.

Induction of PCOS

After quarantine, the rats were administered with testosterone at the concentration of 2 mg/100 g.b.wt. orally for 21 days. During the induction period of 21 days, all the rats were observed daily for estrus cycle in all the groups. On 22nd day, blood sample was collected through retro-orbital plexus under mild anesthesia from all the group of animals to estimate hormonal and biochemical parameters to ensure the induction of PCOS [14].

Evaluation of ovogenic activity

The EESI suspended in 2% carboxymethyl cellulose (CMC) solution to achieve 100 mg/ml stock solution, and animals of Groups 4 (EESI-I), 5 (EESI-II), and 6 (EESI-III) were treated with EESI 100, 200, and 400 mg/kg, respectively, for 21 days after the PCOS induction. Whereas Group 1 received 2% CMC and Group 3 received 1 mg/kg of CC only. Estrus cycle was observed daily for all the groups, and blood samples were collected on 43rd day to estimate various hormone and biochemical parameters. At the end of the treatment period, two animals from each group were sacrificed, and vital organs (uterus and ovary) were subjected for histopathological studies as shown in Fig. 1. Remaining treated active animals were allowed to mate with mature male rats, and the litter size was considered as an additional parameter for enhanced proven fertility.

Hormonal and biochemical assays

After PCOS induction followed by treatment period, hormones such as follicle stimulating hormone (FSH), luteinizing hormone (LH), testosterone, estrogen, and progesterone were estimated in blood samples. The biochemical parameters such as glucose, protein, alkaline phosphatase (ALP), and cholesterol were determined using commercially available diagnostic kits (HiMedia Laboratories Pvt. Ltd., Mumbai, India).

Statistical analysis

The results were expressed as Mean±standard error of mean. One-way analysis of variance followed by Tukey-Kramer multiple comparison test was done using Graph Pad Prism, Version-5-A computer software program for statistical analysis. $p<0.05$ was considered significant.

RESULTS

Extraction and preliminary phytochemical screening

The yield of pasty EESI was 1.35% and the preliminary phytochemical analysis revealed the presence of carbohydrates, fixed oil, fat, saponin, phenolic compounds, and tannins.

Estrus cycle

Estrus cycle was examined by vaginal smear method, one of the easiest ways to detect PCOS in rats. In the PCOS condition, the incidence of estrus phase and its duration is very low due to excess amount of testosterone existence, the main cause for the development of PCOS [15].

Normally estrus cycle of rats is in sequential order such as estrus, metaestrous, diestrous, and proestrous phase, respectively as shown in Fig. 2. However, testosterone-treated rats showed irregularity in its phases because of the physiological disturbance due to polycystic ovary condition. Most of the testosterone-treated rats showed persistent days of diestrous phase during the 21 days of treatment when compared to normal rats as shown in Fig. 3.

The estrus cycle was restored to regular in all the drug treated PCOS animals. Diestrous phase was reduced significantly, and estrus phase was extended in terms of days in the treated group when compared to control as shown in Fig. 4. Thus *S. ilicifolium* has potential effect by reverting the reproductive cycle toward normal in PCOS rats.

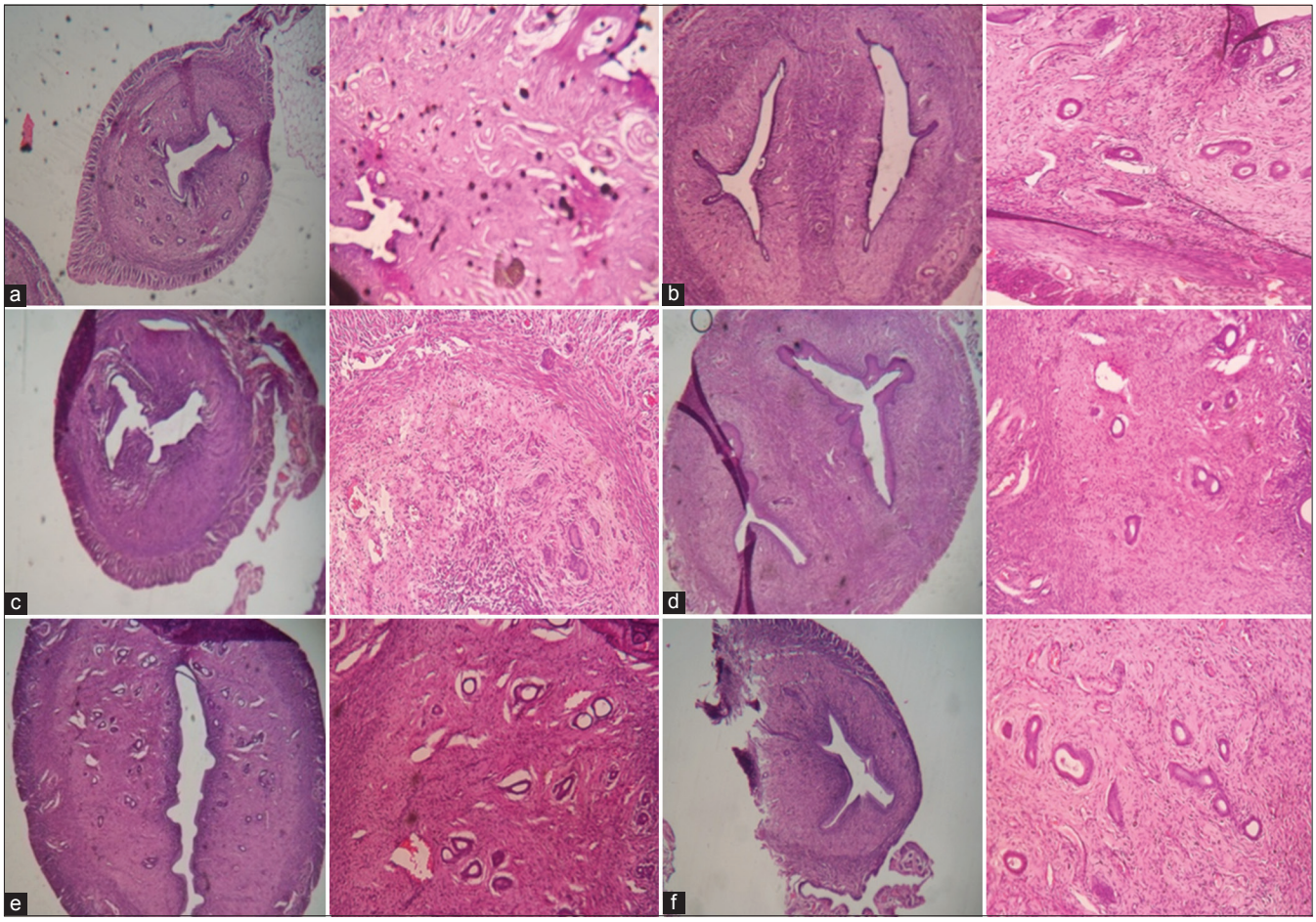

Fig. 1: Photomicrograph of ovary of female rats. (a) Normal group (40 and ×100), (b) control group (40 and ×100), (c) standard group (40 and ×100), (d) ethanolic extract of *Sargassum ilicifolium* I group (40 and ×100), (e) ethanolic extract of *Sargassum ilicifolium* II group (40 and ×100), (f) ethanolic extract of *Sargassum ilicifolium* III group (40 and ×100)

Hormonal parameters

In the present investigation, the PCOS induction after testosterone administration was confirmed by decreased level of FSH, LH, estrogen, progesterone, and elevation of testosterone concentrations.

From the obtained results, FSH, LH, estrogen, and progesterone hormone level showed a significant reduction ($p<0.001$) in their levels when compared to normal as shown in Table 2. Moreover, there was a significant increase ($p<0.001$) in testosterone hormone level. Many studies have revealed that elevation in testosterone and downfall of FSH is commonly found in PCOS condition. The similar results were obtained in the present research work. After the PCOS induction, the testosterone level was high when compared to the normal, meanwhile the other hormone levels were decreased.

Table 1: Treatment plan

Groups	Group and treatment
1	Normal control (2% carboxymethyl cellulose 2 ml/kg p.o)
2	Disease control (testosterone alone 2 mg/100gp.o)
3	Standard (testosterone 2 mg/100g+ clomiphene citrate1 mg/kg p.o)
4	EESI-I low dose (testosterone 2 mg/100 g+ EESI 100 mg/kg p.o)
5	EESI-II median dose (testosterone 2 mg/100 g+ EESI 200 mg/kg p.o)
6	EESI-III high dose (testosterone 2 mg/100 g+ EESI 400 mg/kg p.o)

EESI: Ethanolic extract of *Sargassum ilicifolium*

Table 2: Influence of testosterone on hormonal parameters in untreated healthy rats

Parameters	Normal control	Disease control (2 mg/100 g testosterone only)
FSH (IU/L)	8.17±0.12	3.37±0.15###
LH (IU/L)	4.09±0.16	2.59±0.12###
Testosterone (nmol/l)	8.19±0.10	28.74±0.11###
Estrogen (pg/ml)	58.31±0.15	27.35±0.26###
Progesterone (ng/ml)	7.57±0.14	2.11±0.15###

###p<0.001 compared to normal. Values are expressed as mean±SEM, n=6. FSH: Follicle stimulating hormone, LH: Luteinizing hormone, SEM: Standard error of mean

±The hormonal parameters such as FSH, LH, estrogen, and progesterone levels in standard and EESI treated groups showed a significant increase ($p<0.001$) in their levels, and there is also a significant decrease ($p<0.001$) in testosterone levels as shown in Table 3. The effect of *S. ilicifolium* treatment with high dose was found to be comparable with the standard drug CC.

Biochemical parameters

Since PCOS is associated with obesity, type 2 diabetes and other disorders [16]. Hence, the biochemical parameters such as glucose, protein, ALP, and cholesterol also considered as valid parameters in this study.

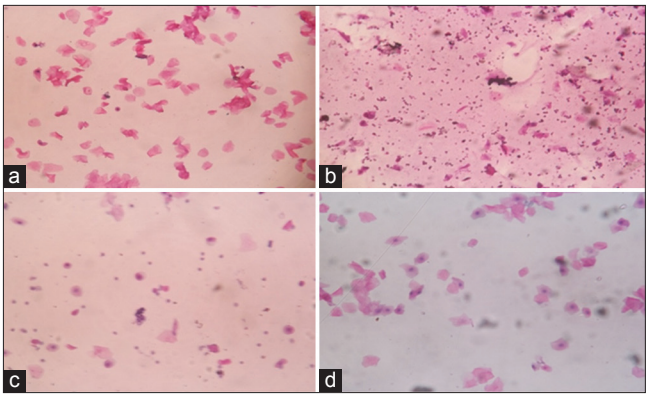

Fig. 2: Photographs of vaginal smear for confirmation of reproductive phases. (a) Estrus phase, (b) metaestrous phase, (c) diestrous phase, (d) proestrous phase

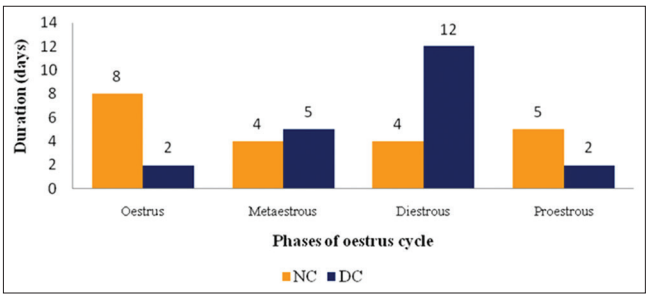

Fig. 3: Influence of testosterone on estrus cycle in untreated healthy rats. NC: Normal control, DC: Disease control

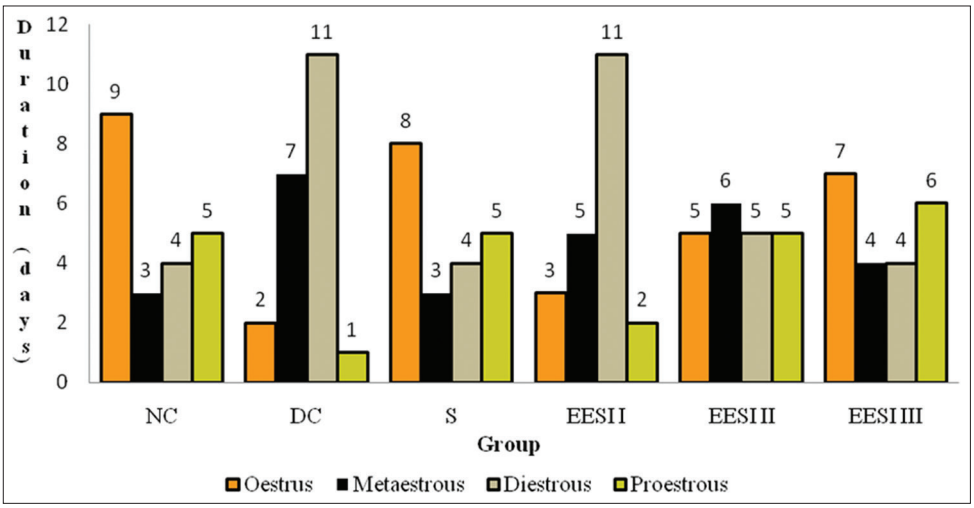

Fig. 4: Influence of ethanolic extract of *Sargassum ilicifolium* on estrus cycle in testosterone-induced polycystic ovary syndrome rats. NC: Normal control, DC: Disease control, S: Standard and ethanolic extract of *Sargassum ilicifolium*-test groups

From the obtained results, biochemical parameters such as glucose and cholesterol levels showed a significant increase ($p<0.001$) in their levels in different groups when compared to normal as shown in Table 4. The elevation of these parameters could be PCOS associated with type 2 diabetes and obesity. However, ALP is found to be non-significant and protein to be less significant in all the groups.

No significant alterations found in ALP and protein. However, glucose and cholesterol were found to be reduced significantly ($p<0.001$) in treated groups compared to PCOS control shown in Table 5.

DISCUSSION

Seaweeds are one of the commercially important marine living resources. They contain several bioactive substances and are known as the excellent sources of iodine [17]. For many decades, seaweeds were the primary sources of iodine for medicinal purpose. The total iodine content in *Sargassum* species was found to be 30 µg/g [18].

Normally in PCOS, increase in testosterone may be due to multifactorial issues such as insulin resistance, obesity, LH dysregulation, and also by thecal hyperplasia [19] Increase in testosterone level is responsible for anovulation by direct effect on the ovary. Due to the entry of androgens into the granulosa layer of pre-antral follicles, androgen-induced follicular atresia is thought to occur, where they bind to the cell receptors and cause cell death.

Androgens cause deterioration of follicles by increasing the number of pycnotic granulosa cells and degenerating oocytes [20]. This could be the reason for the complications in PCOS pregnant women. Similarly, a threefold higher prevalence of autoimmune thyroiditis (AIT) is found in PCOS patients due to the imbalance of normal high estrogens and low progesterone levels; the so-called "unopposed estrogens" thought to be responsible for the apparent increase in the prevalence of AIT. High estrogen levels, which are implicated as enhancers of humoral immunity, while androgens and progesterone are thought to be protective as natural immune suppressors. Although androgens are known to protect from autoimmune disease, the mildly elevated androgens in PCOS do not appear to protect PCOS patients from the development of AIT. Hypothyroid conditions can result from a lack of iodine and supply of iodine can improve AIT [21]. Iodine also has an impact on glucose metabolism. It appears that iodine increases the sensitivity of the insulin receptor, which improves glucose control which favors the diabetic patients [22].

Many studies have revealed that increase in testosterone levels disturbs the gonadotropin-releasing hormone secretion which, in turn, affects the release of sex hormones such as progesterone and estrogen [23,24]. For the maintenance of implantation of blastocyst and pregnancy in humans and other mammals, the sex hormones such as progesterone and estrogen are the most important hormones [25,26]. Not only progesterone and estrogen even there is a decrease in concentration of FSH and LH. FSH is responsible for the development of eggs into mature follicles by acting on immature follicular cells of the ovary [27]. Furthermore, Luteinizing hormone is responsible for the secretion of progesterone and estrogen [28].

It is observed that iodine reduces serum cholesterol levels [29]. Iodine not only plays a major role in AIT, insulin resistance, and obesity but also helps in nourishing and strengthening the bone. PCOS is associated with an approximately seven-fold increased risk of type 2 diabetes mellitus or insulin resistance. Insulin resistance is mainly due to a post-binding defect in insulin receptor-mediated signal transduction and defects in insulin synthesis/secretion. In addition, decreased hepatic clearance and pancreatic sensitivity of insulin was also one of the causes for hyper insulinemia in PCOS [14]. Around 50% of women with PCOS

Table 3: Influence of EESI on hormonal parameters in testosterone-induced PCOS rats

Parameters	Normal control (2% CMC)	Disease ontrol (2 mg/100g Testosterone only)	Standard (1 mg/kg CC)	EESI I (100 mg/kg)	EESI II (200 mg/kg)	EESI III (400 mg/kg)
FSH (IU/L)	8.01±0.14	3.34±0.12$^{###}$	7.82±0.19***	6.26±0.15***	6.81±0.12***	7.45±0.16***
LH (IU/L)	4.19±0.11	2.61±0.11$^{###}$	3.78±0.09***	3.44±0.13***	3.60±0.08***	3.64±0.08***
Testosterone (nmol/l)	8.20±0.13	28.3±0.24$^{###}$	11.48±0.17***	13.86±0.17***	13.31±0.14***	12.91±0.09***
Estrogen (pg/ml)	58.13±0.24	27.95±0.23$^{###}$	54.99±0.12***	50.5±0.17***	53.19±0.51***	53.23±0.26***
Progesterone (ng/ml)	7.66±0.14	2.20±0.12$^{###}$	7.12±0.15***	6.19±0.20***	6.35±0.23***	6.48±0.18***

***p<0.001 verses control, $^{###}$p<0.001 verses normal. Values are expressed as mean±SEM, n=6. FSH: Follicle stimulating hormone, LH: Luteinizing hormone, CMC: Carboxymethyl cellulose, CC: Clomiphene citrate, EESI: Ethanolic extract of *Sargassum ilicifolium*, PCOS: Polycystic ovary syndrome, SEM: Standard error of mean

Table 4: Influence of testosterone on biochemical parameters in untreated healthy rats

Parameters	Normal control	Disease control (2 mg/100 g testosterone only)
Glucose (mg/dl)	94.12±0.33	115.7±0.64$^{###}$
Protein (mg/dl)	7.70±0.12	6.25±0.09$^{###}$
ALP (IU/L)	53.54±0.32	51.7±0.78
Cholesterol (mg/dl)	60.98±0.28	98.72±0.36$^{###}$

$^{###}$p<0.001when compared to normal. Values are expressed as mean±SEM; n=6. ALP: Alkaline phosphatase, SEM: Standard error of mean

Table 5: Influence of EESI on biochemical parameters in testosterone-induced PCOS rats

Parameters	Normal control (2% CMC)	Disease control (2 mg/100 g testosterone only)	Standard (1 mg/kg CC)	EESI I (100 mg/kg)	EESI II (200 mg/kg)	EESI III (400 mg/kg)
Glucose mg/dl	94.49±0.42	117.5±1.27$^{###}$	112.8±1.52	104.3±2.23***	100.3±1.57***	98.05±0.67***
Protein mg/dl	7.58±0.26	6.82±0.28	6.68±0.15	6.78±0.31	6.91±0.38	7.23±0.20
ALP IU/L	52.9±0.28	53.03±0.84	52.25±0.56	53.43±0.62	53.03±0.58	52.53±0.41
Cholesterol mg/dl	60.41±0.32	98.31±0.18$^{###}$	98.14±0.53	61.43±0.39***	61.56±0.33***	61.09±0.29***

$^{###}$p<0.001 verses normal;***p<0.001 verses control. Values are expressed as mean±SEM, n=6. ALP: Alkaline phosphatase, CMC: Carboxymethyl cellulose, CC: Clomiphene citrate, EESI: Ethanolic extract of *Sargassum ilicifolium*, PCOS: Polycystic ovary syndrome, SEM: Standard error of mean

are overweight or obesity. Moreover, this obesity may further lead to insulin resistance and other metabolic syndrome [14]. Acute increase in LH triggers the growth of corpus luteum, ovulation, and also the release of progesterone in women [30]. Therefore, a significant increase in the level of FSH and LH elevates the level of estrogen and progesterone.

In the present study, the serum FSH, LH, estrogen, and progesterone concentration was found to be increased on the administration of EESI with decrease in concentration of testosterone levels. There was also a significant decrease in both glucose and cholesterol level. The estrus cycle was reverted to regular and normal. Microanatomical architecture of EESI treated rat ovary tissues was found to be normal. A significant increase in ovary weight, endometrium thickness, and litter size was observed when compared to disease control. The different doses of EESI treated animals showed normal granulosa layer, well-defined thecal layers, and existence of corpora lutea, suggesting that EESI restored regular estrus cycle. The ovarian cortex showed the presence of multiple follicles indicating normal oogenesis. An increase in fertility rate, litter size was observed in the treated group and comparable to standard drug CC treated group.

The ameliorative effect of *S. ilicefolium* is may be due to the presence of an excessive or adequate level of iodine which meets the daily requirement of women/pregnant women to enable the normal fertilization process.

CONCLUSION

The estrus cycle is restored to regular in the animals treated with EESI. Thus, it can be concluded that the test drug has a potential effect on PCOS bringing the reproductive cycle and other related complications to normal by reducing the risk of type 2 diabetes mellitus or insulin resistance that may be by diminishing the insulin resistance with the post-binding modifications in insulin receptor-mediated signal transduction. The EESI may enhance the hepatic clearance and pancreatic sensitivity of insulin. The findings of this study confirmed that EESI causes an elevation of serum concentration of FSH, LH, estrogen, progesterone, and decrease in testosterone levels. Similarly, EESI was more effective in reducing elevated glucose and cholesterol level. Hence, EESI showed remarkable antiandrogenic effect and helped in regulating normal ovulation and iodine content of EESI could act in regulating progesterone and estrogen for imbedding of the blastocyst. Increased concentration of iodine may be responsible for promoting fertility in PCOS rats. Therefore, this drug can be used clinically for the treatment of PCOS with appropriate dose levels.

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