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.
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.

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.

**Table 1: Treatment plan**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Group and treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal control (2% carboxymethyl cellulose 2 ml/kg p.o)</td>
</tr>
<tr>
<td>2</td>
<td>Disease control (testosterone alone 2 mg/100 gp.o)</td>
</tr>
<tr>
<td>3</td>
<td>Standard (testosterone 2 mg/100 g+ clomiphene citrate 1 mg/kg p.o)</td>
</tr>
<tr>
<td>4</td>
<td>EESI-I low dose (testosterone 2 mg/100 g + EESI 100 mg/kg p.o)</td>
</tr>
<tr>
<td>5</td>
<td>EESI-II medium dose (testosterone 2 mg/100 g + EESI 200 mg/kg p.o)</td>
</tr>
<tr>
<td>6</td>
<td>EESI-III high dose (testosterone 2 mg/100 g + EESI 400 mg/kg p.o)</td>
</tr>
</tbody>
</table>

EESI: Ethanolic extract of *Sargassum ilicifolium*

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

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

***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.

**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 pycnomatic 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 the reason for the complications in PCOS pregnant women. Similarly, a threefold higher prevalence of autoimmune thyroiditis (AIT) is the reason for the complications in PCOS pregnant women. Similarly, a threefold higher prevalence of autoimmune thyroiditis (AIT) is the reason for the complications in PCOS pregnant women. Similarly, a threefold higher prevalence of autoimmune thyroiditis (AIT) is the reason for the complications in PCOS pregnant women. Similarly, a threefold higher prevalence of autoimmune thyroiditis (AIT) is the reason for the complications in PCOS pregnant women. Similarly, a threefold higher prevalence of autoimmune thyroiditis (AIT) is the reason for the complications in PCOS pregnant women. Similarly, a threefold higher prevalence of autoimmune thyroiditis (AIT) is the reason for the complications in PCOS pregnant women. Similarly, a threefold higher prevalence of autoimmune thyroiditis (AIT) is the reason for the complications in PCOS pregnant women. Similarly, a threefold higher prevalence of autoimmune thyroiditis (AIT) is the reason for the complications in PCOS pregnant women. Similarly, a threefold higher prevalence of autoimmune thyroiditis (AIT) is the reason for the complications in PCOS pregnant women.

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 inAIT, 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 hyperinsulinemia in PCOS [14]. Around 50% of women with PCOS

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control (2% CMC)</th>
<th>Disease control (2 mg/100g Testosterone only)</th>
<th>Standard (1 mg/kg CC)</th>
<th>EESI I (100 mg/kg)</th>
<th>EESI II (200 mg/kg)</th>
<th>EESI III (400 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH (IU/L)</td>
<td>8.01±0.14</td>
<td>3.34±0.12***</td>
<td>7.82±0.19***</td>
<td>6.26±0.15***</td>
<td>6.81±0.12***</td>
<td>7.45±0.16***</td>
</tr>
<tr>
<td>LH (IU/L)</td>
<td>4.19±0.11</td>
<td>2.61±0.11***</td>
<td>3.78±0.09***</td>
<td>3.44±0.13***</td>
<td>3.60±0.08***</td>
<td>3.64±0.08***</td>
</tr>
<tr>
<td>Testosterone (nmol/L)</td>
<td>8.20±0.13</td>
<td>28.3±0.24***</td>
<td>11.4±0.17***</td>
<td>13.86±0.17***</td>
<td>13.31±0.14***</td>
<td>12.91±0.09***</td>
</tr>
<tr>
<td>Estrogen (pg/ml)</td>
<td>58.1±0.24</td>
<td>27.95±0.23***</td>
<td>54.9±0.12***</td>
<td>50.5±0.17***</td>
<td>53.19±0.51***</td>
<td>53.23±0.26***</td>
</tr>
<tr>
<td>Progesterone (ng/ml)</td>
<td>7.66±0.14</td>
<td>2.20±0.12***</td>
<td>7.12±0.15***</td>
<td>6.19±0.20***</td>
<td>6.35±0.23***</td>
<td>6.48±0.18***</td>
</tr>
</tbody>
</table>

**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: Ethanol extract of Sargassum ilicifolium, PCOS: Polycystic ovary syndrome, SEM: Standard error of mean

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control</th>
<th>Disease control (2 mg/100 g testosterone only)</th>
<th>Standard (1 mg/kg CC)</th>
<th>EESI I (100 mg/kg)</th>
<th>EESI II (200 mg/kg)</th>
<th>EESI III (400 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dl)</td>
<td>94.1±2.33</td>
<td>115.7±0.64***</td>
<td>94.1±2.33</td>
<td>115.7±0.64***</td>
<td>115.7±0.64***</td>
<td>115.7±0.64***</td>
</tr>
<tr>
<td>Protein (mg/dl)</td>
<td>7.70±0.12</td>
<td>6.25±0.09***</td>
<td>7.70±0.12</td>
<td>6.25±0.09***</td>
<td>6.25±0.09***</td>
<td>6.25±0.09***</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>53.5±4.52</td>
<td>51.7±0.78</td>
<td>53.5±4.52</td>
<td>51.7±0.78</td>
<td>51.7±0.78</td>
<td>51.7±0.78</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>60.9±8.28</td>
<td>98.7±0.56***</td>
<td>60.9±8.28</td>
<td>98.7±0.56***</td>
<td>98.7±0.56***</td>
<td>98.7±0.56***</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control (2% CMC)</th>
<th>Disease control (2 mg/100 g testosterone only)</th>
<th>Standard (1 mg/kg CC)</th>
<th>EESI I (100 mg/kg)</th>
<th>EESI II (200 mg/kg)</th>
<th>EESI III (400 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose mg/dl</td>
<td>94.49±0.42</td>
<td>117.5±1.27***</td>
<td>112.8±1.52</td>
<td>104.3±2.33***</td>
<td>100.3±1.57***</td>
<td>98.0±0.67***</td>
</tr>
<tr>
<td>Protein mg/dl</td>
<td>7.58±0.26</td>
<td>6.82±0.28</td>
<td>6.68±0.15</td>
<td>6.78±0.31</td>
<td>6.91±0.38</td>
<td>7.23±0.20</td>
</tr>
<tr>
<td>ALP IU/L</td>
<td>52.9±0.28</td>
<td>53.03±0.84</td>
<td>52.25±0.56</td>
<td>53.43±0.62</td>
<td>53.03±0.58</td>
<td>52.53±0.41</td>
</tr>
<tr>
<td>Cholesterol mg/dl</td>
<td>60.4±0.32</td>
<td>98.31±0.18***</td>
<td>98.14±0.53</td>
<td>61.43±0.39***</td>
<td>61.56±0.33**</td>
<td>61.09±0.29***</td>
</tr>
</tbody>
</table>

**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: Ethanol 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 [1-4]. 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. ilicifolium* 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.

**ACKNOWLEDGMENT**

Authors are grateful to Dr. V. Madhavan, Dean, Faculty of Pharmacy, M.S.RI.I.U.I.A.S., Bangalore, for providing all the facilities to carry out this research work.

**REFERENCES**