INTRODUCTION
Elevating human population throughout the world more particularly in developing and undeveloped parts have negative effects on the life fortifying the system on earth. The possibility of an efficacious check on human fertility may soon be realized through biological means. Fertility regulation, comprising contraception and management of infertility forms a paramount component of reproductive health [1]. Though, considerable progress has been made in the development of highly efficacious, acceptable and reversible methods of contraception among females, progress and possibilities on males are still slow and circumscribed. With recent progress towards a better understanding of male reproductive physiology there is a need to develop incipient contraceptive modalities for male. Several potential approaches for induction of infertility have been investigated over a long period, including hormonal, chemical and immunological approach, though the safety of their prolonged exposure is controversial [2]. In the present scenario, it is an alarming time to think some alternatives. Hence, an approach to identify new antifertility agents can be made with the search for contraceptive activity on androgenic as well as biochemical sensors also. The present experiment was carried out to select the minimum dose of a hydro-methanol extract of seed of *C. cyminum* having maximum contraceptive efficacy in male rats.

MATERIALS AND METHODS

**Chemicals/material**
Testosterone, Dehydroepiandrosterone (DHEA), Nicotinamide Adenine Dinucleotide (NAD), Pyrogallol were used of analytical grade obtained from Hi Media, Mumbai, India, or Sigma, St. Louis, MO, USA. Kits for the ELISA and various enzyme assays were obtained from Lilac Medicare Pvt. Ltd, Mumbai, India and Crest Bio systems, Goa, India. All other analytical grade reagents were locally purchased.

**Preparation of the plant extract**
Fresh seeds of *C. cyminum* was collected from the local market of Midnapore town and authenticated by the Department of Botany and Forestry, Vidyasagar University, Midnapore-721102, West Bengal, India and the voucher specimen have been deposited in the Herbarium of the same Department. The seeds were washed under tap water and dried in an incubator thoroughly at 37°C for 2 days and grinded to fine powder utilizing blender. Then 50 g of powder was suspended in 250 ml of hydro-methanol (2:3 v/v) solvent mixture and kept in an incubator at 37°C. After 48 hrs, the extract was filtered and the filtrate was evaporated under reduced pressure utilizing rotary evaporator (HAHN-SHIN HS-2000NS, Korea) at 40°C for consummate abstruction of methanol and the resulting plain aqueous extract was lyophilised and preserved in refrigerator at 4°C until use for the experiment.

**As a pilot work,** we have studied the different solvent extracts (aqueous, methanol, ethanol, hydro-methanol (2:3) hydro-ethanol (2:3)) of *C. cyminum* to determine their male contraceptive efficacy. Here, the hydro-methanol extract showed an outstanding male contraceptive activity on androgenic as well as biochemical sensors also. The present experiment was carried out to select the minimum dose of a hydro-methanol extract of seed of *C. cyminum* having maximum contraceptive efficacy in male rats.
Experimental animals

Twenty four Wistar strains male albino rats for three months of age and weighing about 120 ± 10g were used for the present study. Animals were housed in cages at an ambient temperature of 25 ± 2°C under 12 hr: 12 hr light-dark cycles and were kept for 15 days for acclimation prior to experimentation. They were provided with standard rat chow diet and water ad libitum. The Institutional Animal Ethical Committee (IAEC) approved the study and all the instructions given by our IAEC were followed throughout the experimentation.

Experimental design

After 15 days of acclimation of twenty four rats, body weight of each was measured and they were divided into four groups, each group contained six animals. The daily dose of the hydro-methanolic extract was prepared by suspending the extract in olive oil and administered to each animal through oral route by gavage in the morning (at 8.00 AM). The duration of the experiment was 28 days. The treatment schedule of each group was as follows

Group I (Vehicle-treated control): Rats of this group received 0.5 ml of olive oil/100g body weight once a day.

Group II (Hydro-methanol extract treated group at the dose of 30 mg): Rats of this group were treated with the hydro-methanol extract at the dose of 30 mg/0.5 ml olive oil/100g body weight once a day.

Group III (Hydro-methanol extract treated group at the dose of 60 mg): Animals of this group were treated with the hydro-methanol extract at the dose of 60 mg/0.5 ml olive oil/100g body weight once a day.

Group IV (Hydro-methanol extract treated group at the dose of 120 mg): Rats were treated with hydro-methanol extract at the dose of 120 mg/0.5 ml olive oil/100g body weight/day.

After completion of the experimental schedule, all the animals were sacrificed by light ether anesthesia after taking the body weight. Blood was collected using heparinized syringe and the plasma was isolated and kept in -20°C for testosterone assay. Reproductive organs i.e. the testes, epididymis, seminal vesicles were dissected out. Fat and connective tissues were abstracted from the surface of the organs and weights of these organs were recorded. Liver, kidney and left testis were kept in -20°C for enzymatic method by an ELISA reader [13]. 10 µl of each standard or sample was dispensed into felicitous well followed by integration of 100 µl of enzyme conjugate containing horse-radish peroxidase (HRP) and mixed. The strips were incubated for 60 min at 37°C. The reaction solution was decanted forcefully from all the wells followed by three washings. 100 µl of tetramethyl benzidine (TMB) substrate containing chromogen was included and after the scheduled time the reaction was stopped by addition of stop solution supplied in the kit. The absorbance of standards and samples were read against the blank at 450 nm.

Biochemical estimation of testicular cholesterol

Testicular cholesterol was estimated utilizing the kit following the supplied protocol[14].

Estimation of testicular Δ5, 3β-hydroxysteroid dehydrogenase (Δ5, 3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) activities

Testicular key androgenic Δ5, 3β-HSD and 17β-HSD enzyme activities were quantified spectrophotometrically utilizing the testicular homogenate following standard laboratory protocol[15].

Biochemical estimation of catalase, peroxidase, superoxide dismutase (SOD) and glutathione-s-transferase (GST) activities

The activities of catalase, peroxidase, SOD and GST in testis, epididymis and sperm pellet were performed biochemically following standard protocol [16].

Estimation of end products of lipid peroxidation (CD and MDA)

For quantification of end products of lipid peroxidation, i.e., conjugated diene (CD) and malondialdehyde (MDA), the sample tissue was homogenised at the tissue concentration of 50 mg/ml in 0.1 M of ice-cold phosphate buffer (pH 7.4), and the homogenates were centrifuged at 10000g at 4°C for 5 min. Supernatant was used for the spectrophotometrical estimation of the CD and MDA following the standard laboratory method[17].

Glutamate oxaloacetate transaminase (GOT) and Glutamate pyruvate transaminase (GPT) activities of liver and kidney

For the assessment of metabolic toxicity, GOT and GPT activities of liver and kidney were estimated [18].

Histological study

Testes were embedded in paraflin block, sectioned at 5 µm thickness and stained with hematoxylin-eosin. The prepared slides were observed under high power objective in a Trinocular microscope, which was handled with a computer. A photograph of a particular field was taken. Seminiferous tubular diameter (STD) was measured with the DeWinter Calipro-3.0 Softwares. Quantitative analysis of gametogenesis was carried out at the stage VII of the seminiferous epithelial cell cycle according to the method of Leblond and Clermont [19].

Statistical analysis

Data were expressed in mean ± SEM. For statistical analysis of data, Analysis of Variance (ANOVA) followed by multiple comparison two tail t-test was employed and p<0.05 was considered significant [20].

RESULTS

Body and organ weights

Throughout the experiment, there was no alteration in body weight in hydro-methanol extract treated groups when compared to the control group. Relative weights of testis, seminal vesicle and epididymis were significantly (p<0.05) decreased in all the treated groups in respect to the control.

From the comparative analysis of the relative weights of these sex organs, a significant (p<0.05) decrease in these indices was noted after treatment at a dose of 60 mg or 120 mg/100gbody weight in respect to the 30 mg/100g body weight treated group (table-1). The relative kidney and liver weights were not significantly change than the control (table 1).
Compared to the control as well as to the low dose experimental group, p<0.05.

Oral administration of the hydro-methanol extract at different doses exhibited a significant (p<0.05) decreased testicular Δ5, 3β-HSD and 17β-HSD activities of treated rats compared with the control.

Values are mean ± S. E. M. (n=6), (ANOVA followed by multiple comparison two tail t-test). *Indicates significance difference compared to the control, **Compared to the control as well as to the low dose experimental group p<0.05.

Table 1: Effect of hydro-methanolic extract of seed of C. cyanimum at different doses on body weight and relative organ weights in mature male albino rat

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight (g)</th>
<th>Relative organ weights (g/100g body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>Control</td>
<td>121.12±2.60</td>
<td>142.12±2.47</td>
</tr>
<tr>
<td>C. cyanimum treated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(30 mg/100g)</td>
<td>128.75±3.75</td>
<td>150.37±2.22</td>
</tr>
<tr>
<td>(60 mg/100g)</td>
<td>124.37±2.27</td>
<td>147.50±2.23</td>
</tr>
<tr>
<td>(120 mg/100g)</td>
<td>126.87±2.88</td>
<td>146.25±2.59</td>
</tr>
</tbody>
</table>

Values are mean ± S. E. M. (n=6), (ANOVA followed by multiple comparison two tail t-test), *Indicates significance difference compared to the control; **Compared to the control as well as to the low dose experimental group p<0.05.

**Sperm count, motility and viability**

Sperm count, sperm motility and sperm viability were decreased significantly (p<0.05) after the treatment of hydro-methanol extract at the dose of 30 mg/100g body weight group in respect to the control. A significant (p<0.05) low values were noted in the above sensors after the treatment of hydro-methanol extract at the dose of 60 mg or 120/100g body weight group in respect to 30 mg dose treated group. No significant difference in the levels of the said parameters was noted between the groups treated with 60 mg and 120 mg/100g body weight (table-2).

**Testicular cholesterol level**

The testicular cholesterol level was elevated significantly (p<0.05) in all the treated groups in comparison with the control in a dose dependent manner. The result showed a significant (p<0.05) increased in this parameter when animals were treated at a dose of 60 mg/100g body weight or 120 mg/100g body weight in comparison to 30 mg/100g body weight, though the level of testicular cholesterol did not differ significantly when comparison was made between 60 mg and 120 mg/100g body weight dose treated groups (table-2).

**Seminal vesicular fructose**

A significant (p<0.05) reduction in seminal vesicular fructose level was noted in all the treated groups in comparison with the control. The level of this parameter was decreased significantly (p<0.05) in 60 mg or 120 mg/100g body weight treated group in respect to 30 mg/100g body weight treated group. No significant difference was noted in the level of this parameter when comparison was made between the groups treated with 60 mg and 120 mg/100g body weight (table-2).

**Testicular testosterone**

Plasma level of testosterone was decreased significantly (p<0.05) after treatment of a hydro-methanol extract of C. cyanimum at a dose of 60 mg or 120 mg/100g body weight in respect of the 30 mg/100g body weight treated group. After treatment with 30 mg/100g body weight, plasma level of testosterone was also diminished significantly (p<0.05) in comparison with the control. But no significant difference in the plasma level of this parameter was noted between 60 mg and 120 mg/100g body weight treated groups (table-2).

Table 2: Comparative analysis of sperm count, sperm motility, viability, testicular cholesterol, seminal vesicular fructose and plasma testosterone after treatment with hydro-methanolic extract of C. cyanimum at different doses in mature male albino rat

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total sperm count (millions/ml)</th>
<th>Sperm motility (%)</th>
<th>Sperm viability (%)</th>
<th>Testicular cholesterol (mg/gm of tissue)</th>
<th>Seminal vesicular fructose (µM/gm)</th>
<th>Plasma Testosterone ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24.87±1.45</td>
<td>81.00±1.79</td>
<td>87.62±2.32</td>
<td>28.35±1.06</td>
<td>0.098±0.010</td>
<td>15.96±0.63</td>
</tr>
<tr>
<td>C. cyanimum treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(30 mg/100g)</td>
<td>19.62±1.17*</td>
<td>64.75±1.14*</td>
<td>67.87±1.48*</td>
<td>33.35±1.37*</td>
<td>0.063±0.008*</td>
<td>12.92±0.92</td>
</tr>
<tr>
<td>(60 mg/100g)</td>
<td>14.37±0.88**</td>
<td>57.07±1.28**</td>
<td>60.12±1.71**</td>
<td>42.27±1.05**</td>
<td>0.029±0.003**</td>
<td>8.07±0.61**</td>
</tr>
<tr>
<td>(120 mg/100g)</td>
<td>13.50±0.86**</td>
<td>55.50±1.34**</td>
<td>57.37±1.45**</td>
<td>43.29±1.45**</td>
<td>0.026±0.003**</td>
<td>7.30±0.63**</td>
</tr>
</tbody>
</table>

Values are mean ± S. E. M. (n=6), (ANOVA followed by multiple comparison two tail t-test). *Indicates significance difference compared to the control, **Compared to the control as well as to the low dose experimental group, p<0.05.

**Testicular Δ5, 3β-HSD and 17β-HSD activities**

Oral administration of the hydro-methanol extract at different doses exhibited a significant (p<0.05) decrease in testicular Δ5, 3β-HSD and 17β-HSD activities of treated rats compared with the control.

![Fig. 1: Comparative analysis of the activities of testicular Δ5 3β-HSD and 17β-HSD after treatment with hydro-methanolic extract of C. cyanimum at different doses in mature albino rats. Values are given as mean ± SEM, (n=6). Bars with significant difference have been indicated by * and ** (ANOVA followed by multiple comparison two tail t-test): *Compared to the control; **Compared to the control as well as low dose experimental group p<0.05.](image-url)
A significant (p<0.05) difference in these parameters was noted when comparison was made between 30 mg treated group and 60 mg or 120 mg/100g body weight treated groups though there was no significant difference in the activities of these parameters between the groups treated with 60 mg and 120 mg/100g body weight (fig. 1).

**Activities of catalase, peroxidase, superoxide dismutase and glutathione-s-transferase**

Activities of catalase, peroxidase, superoxide dismutase and glutathione-s-transferase were decreased significantly (p<0.05) in the testis, epididymis and sperm pellet in all of the hydro-methanol treated groups in respect to the control. The activities of all enzymes were significantly (p<0.05) decreased at the dose of 30mg/100g body weight extract treated group in respect to the control. But after the administration of 60 mg or 120 mg doses of the extract resulted a significant diminution in the activity of said enzymes in respect to 30mg dose treated group. There was no significant changes in the level of said enzymes between the dose of 60mg and 120mg/100g body weight of the extract treated group (fig 2).

**Levels of CD and MDA**

A significant (p<0.05) elevation in the levels of the CD and MDA in testis, epididymis and sperm pellet were noted in all the treated groups in comparison with the control. However, the levels of these parameters were elevated significantly (p<0.05) when the comparison was made between 30 mg and 60 mg or 120 mg/100g body weight treated groups (fig. 2). No significant difference was noted in the levels of these parameters when comparison was made between the groups treated with 60 mg and 120 mg/100g body weight (fig 2).

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**Table 3: Glutamate Oxaloacetate Transaminase (GOT) and Glutamate Pyruvate Transaminase (GPT) activities in liver and kidney after treatment with hydro-methanolic extract of C. cyminum at different doses in mature albino rat**

<table>
<thead>
<tr>
<th>Groups</th>
<th>GOT activity (unit/mg of tissue)</th>
<th>GPT (unit/mg of tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Kidney</td>
</tr>
<tr>
<td>Control</td>
<td>28.12±1.78</td>
<td>17.67±1.16</td>
</tr>
<tr>
<td><em>C. cyminum</em> treated (30 mg/100g)</td>
<td>23.56±1.66</td>
<td>20.00±1.50</td>
</tr>
<tr>
<td><em>C. cyminum</em> treated (60 mg/100g)</td>
<td>26.20±2.26</td>
<td>22.13±1.83</td>
</tr>
<tr>
<td><em>C. cyminum</em> treated (120 mg/100g)</td>
<td>25.32±1.94</td>
<td>19.54±1.41</td>
</tr>
</tbody>
</table>

Values are mean ± S. E. M. (n=6), (ANOVA followed by multiple comparison two tail t-test). No significant different between the groups, p>0.05
Toxicity assessment in liver and kidney

Activities of GOT and GPT are most consequential indicators of metabolic toxicity in the liver and kidney. These enzyme activities were not significantly affected by hydro-methanol extract of seed of C. cyminum among those treated groups (table-3).

Quantification of different generation of germ cells at stage VII

Quantitative study of germ cells at the stage VII of seminiferous epithelial cell cycle revealed that treatment with the extract in all of the said doses resulted a significant (p<0.05) decrease in the numbers of ASg, pLSc, mPSc and 7Sd in the extract treated groups in respect to the control in a dose dependent manner.

The numbers of these cells were decreased significantly (p<0.05) on increasing the dose from 30 mg to 60 mg/100g body weight. However, no significant variation in the number of these cells was noted between 60 mg and 120 mg/100g body weight treated groups (table-4).

Histological observation

Treatment with the extract established a significant (p<0.05) reduction in the seminiferous tubular diameter (STD) of the testes in all the treated groups as compared to the control. A significant (p<0.05) reduction in STD was noted after treatment at the dose of 60 mg or 120 mg /100g body weight in comparison to 30 mg/100g body weight treated group without any significant variation between 60 mg and 120 mg treated groups (fig. 3).

Table 4: Effect of hydro-methanol extract of seed of C. cyminum on the number of different generations of germ cells at stage VII of seminiferous epithelial cell cycle

<table>
<thead>
<tr>
<th>Groups</th>
<th>ASg (×10^6)</th>
<th>pLSc (×10^6)</th>
<th>mPSc (×10^6)</th>
<th>7Sd (×10^6)</th>
<th>STD (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.75±0.16</td>
<td>10.62±0.59</td>
<td>17.25±0.88</td>
<td>62.50±1.59</td>
<td>474.35±12.08</td>
</tr>
<tr>
<td>C. cyminum treated(30 mg/100g)</td>
<td>1.25±0.17*</td>
<td>14.87±0.71*</td>
<td>13.12±0.61*</td>
<td>54.37±2.02*</td>
<td>383.19±18.23*</td>
</tr>
<tr>
<td>C. cyminum treated(60 mg/100g)</td>
<td>0.75±0.16**</td>
<td>10.62±0.88**</td>
<td>10.12±0.78**</td>
<td>45.75±1.38**</td>
<td>296.37±12.60**</td>
</tr>
<tr>
<td>C. cyminum treated(120 mg/100g)</td>
<td>0.62±0.18**</td>
<td>9.87±0.93**</td>
<td>9.50±0.82**</td>
<td>42.75±2.19**</td>
<td>280.14±13.53**</td>
</tr>
</tbody>
</table>

Values are mean ± S. E. M. (n=6), (ANOVA followed by multiple comparison two tail t-test). *Indicates significance difference compared to the control, **Compared to the control as well as to the low dose experimental group p<0.05.

DISCUSSION

The present experiment provided a number of observations regarding the effect of hydro-methanol extract of C. cyminum on testicular function in mature albino rats. Treatment with the extract at the dose of 60mg is the most efficacious dose showed maximum efficacy in comparison with the doses of 30mg and 120mg. From the result it has been revealed that there was no significant alteration in somatic growth in treated rats compared to the control. This suggests that this seed extract has no general toxic effect on body growth. Weight reduction of the reproductive organs of treated rats clearly denoted that the extract caused structural and functional alteration in testis, epididymis and seminal vesicle and lowered the testosterone as these organs are androgen dependent [21].

The diminution in the activities of testicular Δ5, 3β-HSD and 17β-HSD, the key enzymes of androgenesis [22, 23] may be inhibited by low level of pituitary gonadotropins secretion [24-26]. This inhibition supported here by the low plasma testosterone level. This
view has been further supported here by the testicular cholesterol assessment which was elevated that may be due to inhibition in testicular androgenesis because cholesterol is the mother molecule for gonadal steroidogenesis [27]. This was further confirmed by the quantification of seminal fructose as fructose quantity in seminal plasma is regulated by testosterone [28].

The diminution in epididymal sperm count and the number of different generations of germ cells at the stage VII of seminiferous epithelial cell cycle in the seed extract treated rats may be due to a low plasma level of pituitary gonadotropins and testosterone, which are the major regulators of spermatogenesis [29-32]. Moreover, diminution in STD in seed extract treated rats also supported the low plasma level of testosterone as STD is one of the designators of plasma level of testosterone [33].

To find out whether the extract besides modulating the testicular testicular axis inhibits the testicular activity by inducing oxidative stress, several oxidative stress parameters were considered. Assessment of the activities of catalase, peroxidase, superoxide dismutase and glutathione-s-transferase and quantification of the CD and MDA levels in testis, epididymis and sperm pellet, which are the indicators of oxidative stress were changed significantly in a further oxidative stress generation. This result supports the fact that the extract may additionally affect male reproduction by developing oxidative stress [34] and it may have some direct spermiocidal effect on the germ cells. Oxidative stress additionally affects the sperm motility and sperm viability [35].

To determine whether the doses of the seed extract of C. cyminum that induce antigonal effect have any metabolic toxicity, we measured GOT, and GPT activities in the liver and kidney because these enzymes are the indicators of metabolic toxicity [36]. Because there was no significant alteration in the hepatosomatic and renosomatic indices, hence, it may be stated that the applied dose of the extract has no specific toxic effect on such metabolic organs. Specific enzyme assay for toxicity study indicated that there was no significant alteration in the GOT, and GPT activities in liver and kidney of extract-treated rats. Therefore, it may be claimed that the applied doses of the extract of C. cyminum have antigonal effect without any toxic effect on metabolic organs.

CONCLUSION

It may be concluded that hydro-methanolic extract of C. cyminum that induce antigonal activity in a dose dependent manner showing maximum contraceptive effect at a dose of 60 mg/100g body weight without causing any metabolic toxicity.

ACKNOWLEDGEMENT

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CONFLICT OF INTERESTS

The authors have declared that there is no conflict of interest.

REFERENCES


