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Cypermethrin induced reproductive toxicity in male Wistar rats: Protective role of *Tribulus terrestris*

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Abstract

The present study was designed to investigate role of ethanolic extract of *Tribulus terrestris* (EETT) against α-cypermethrin induced reproductive toxicity in male Wistar rats. 24 male Wistar rats weighing about 250-300g were divided in four groups. Group-I was control. α-cypermethrin (3.38 mg kg^{-1}b.wt.) was given to group-II for 28 days. In Group-III, α-cypermethrin and EETT (100 mg kg^{-1}b.wt.) were administered in combination for 28 days. Rats in group-IV were given EETT for 28 days. At the end of the experiment, rats were sacrificed, testes and epididymis were removed and sperm characteristics, sex hormones and various biochemical parameters were studied. Decrease in weight of testes and epididymis, testicular sperm head count, sperm motility, live sperm count, serum testosterone (T), follicle stimulating hormone (FSH), leutinizing hormone (LH), catalase (CAT), superoxide dismutase (SOD), glutathione S transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx), total protein content and increase in sperm abnormalities and lipid peroxidation (LPO) level was observed in rats exposed to cypermethrin. In combination group-III, EETT treatment ameliorated α-cypermethrin induced damage. EETT treatment in group-IV increased testes and epididymis weight, sperm head counts, sperm motility, live sperm counts, testosterone, FSH, LH, GSH, CAT, SOD, GST, GR, GPx and total protein content. The study suggested that *Tribulus terrestris* plant possess reproductive system enhancement and antioxidant activity.

Key words

Cypermethrin, Reproductive toxicity, Sex hormones, *Tribulus terrestris*, Wistar rats

Introduction

Synthetic pyrethroids are commonly used pesticides/ insecticides in household applications for pest/ insect management. These pesticides are preferentially used because of their target oriented mechanism of action and rapid biodegradability. Cypermethrin is a synthetic pyrethroid commonly used in agriculture, veterinary and household insects/pests management (Solati *et al.*, 2010). Although considered to be safe for household applications, some studies indicated the adverse effects of cypermethrin on male reproductive system of laboratory animals (Mani *et al.,* 2002; Wang *et al.,* 2010). Recent studies reported, decrease in functional sperms as well as sex hormones in mice and rats on cypermethrin exposure (Solati *et al.,* 2010; Hu *et al.,* 2011). Since humans are under chronic exposure, cypermethrin induced damage to male reproductive system is not ruled out. Hence, household use of cypermethrin may be a contributing factor for human male infertility along with other emotional, occupational and environmental stress. The main cause of male infertility are Oligospermia (less number of sperm in the ejaculate of the male or less than 20 million sperm per milliter), Asthenospermia (reduction of spermatozoon motility), Teratozoospermia (high percentage of morphologically abnormal spermatozoa in ejaculates) and Azospermia (complete absence of sperm in the ejaculate). These causes accounts for 20-25% of the male infertility (Egozcus *et al.,* 2000; Hargreave, 2000). Exposure to environmental toxicants may be responsible for decrease in libido, impairment of reproductive system and male sterility. Many pesticides are now suspected of being endocrine disrupting chemicals (EDC) that lead to an increase in birth

defects, sexual abnormalities and reproductive failures (Lyons, 2000; Poongothai *et al.,* 2008).

A number of allopathic drugs such as sildenafil, cialis or levitra and synthetic testosterone have been developed to improve male sexual function impaired due to emotional, occupational and environmental stress but side effects associated with these drugs limit their utility. 'Vajikaran rasayana' are used in 'Ayurveda' for improving sexual function and enhancing male fertility. A number of medicinal plants listed as 'Vajikaran' are reported to possess aphrodisiac activity. *Tribulus terrestris* (Linn.) known as 'puncture wine' is a perennial creeping herb with a wide spread distribution in mediterranean, subtropical and desert climates around the globe. The plant has been used for treatment of diseases associated with urinary tract and reported to exhibit aphrodisiac activity (Adaikan *et al.* 2001). *T. terrestis* extracts are reported to improve semen quality, viability and motility of sperms, libido and active sexual behavior in animal models (Grigorova *et al.,* 2008). Protective effect of *T. terrestis* on cadmium-induced testicular damage in rats has been reported by Rajendra *et al.* (2011). The present study was carried out to evaluate the efficacy of *T. terrestris* against α-cypermethrin induced reproductive toxicity in male Wistar rats.

Materials and Methods

Adult male Wistar rats (3-4 months old, weighing about of 250-300g) obtained from animal research division, Indian Institute of Toxicology Research, CSIR, Lucknow (India) were used for this study. The rats were maintained in the animal house of Bundelkhand University, Jhansi under controlled conditions (25±2°C, 12 hr light/12 hr darkness, humidity 50–55%) with free access to pelleted food and tap water.

Technical grade α-cypermethrin (97%) used in this study was procured from Gharda Chemicals, Mumbai, India. Rat specific ELISA kits for estimation of serum testosterone (T), leutinzing hormone (LH) and follicle stimulating hormone (FSH) were purchased from *Labor Diagnostic Nord Gmbh* Company (Germany). All other chemical used were of high purity.

Aerial parts (with mature fruits) of *Tribulus terrestris* were collected from the local hills of Bundelkhand University in April- May, 2010. The plant material was identified with the help of experts in Department of Botany, Bundelkhand University and a voucher specimen no. BU/BMS/2010/03 was preserved in Department of Biomedical Sciences, Bundelkhand University. The plant material was shade dried, grinded to obtain coarse powder and macerated in 70% ethanol for 24 hrs. The extract was filtered, centrifuged at 3000rpm for 15 min and concentrated under vacuum to remove ethanol and further dried using freeze dryer to obtain ethanolic extract of *Tribulus terrestris* (EETT).

24 rats were divided in 4 groups of six rats each. Group I was taken as normal control. α-cypermethrin (3.38mg kg bw^{-1}) was administered to Group II. α-cypermethrin (3.38mg kg bw^{-1}) and EETT (100mg/kgbw) were co-administered to Group III. Group IV was treated with EETT (100mg kg bw^{-1}). α-cypermethrin and EETT were dissolved in DMSO administered orally by gavaging for 28 days. The animal experiments were carried out as per Institutional Animal Ethical Committee Guidelines (BU/Pharma/IAEC/10/028). At the end of the experiment, rats were sacrificed; both the testes and epididymis were removed and weighted. One testis was used for sperm head counts and other testis was used for estimation of lipid peroxidation, enzymatic and non-enzymatic antioxidants and total protein content. Epididymis was used for sperm motility and sperm morphology study. Blood samples were taken from the ventricle of heart and serum was separated for various reproductive hormones.

Sperm head counts were performed using hemocyto meter as described by Choi *et al.* (2008) with necessary modifications. The testes were removed, weighed and tunica albuginea (outer covering) was removed. The testes was minced and homogenized in 0.9% NaCl and 0.05% triton X solution for 2 min at 8000 rpm. 10-15 µl of homogenate was placed on hemocytometer. After 5 min sperm heads were counted in RBC chamber at 40X magnification.

A segment from distal end of epididymis was removed and minced in 2 ml of Dulbecco's phosphate buffer saline maintained at 36-38°C. Minced cauda was placed in a water bath to disperse the sperm for 1-5 min and gently mixed using pasture pipette. Mixture (5-10µl) was loaded in hemocytometer chamber and the numbers of non-motile sperms were counted in WBC counting area. Hemocytometer was kept at 40–50°C for 1 min (to kill the sperms) and then total number of dead sperms were counted (Williams, 1993).

A segment of epididymis was minced in 1 ml of 0.9% saline with the help of razor and 1ml of 10% neutral buffer saline was added. The suspension was diluted with water to suitable volume for performing the assay. 1-2ml of eosin (1%) was added to 20ml of above suspension and incubated at room temperature for 1 hr. One drop of suspension was taken on slide and a smear was prepared. The slides were viewed under light microscope at 40X magnification for evaluation of head and tail abnormalities. A total of 200 sperms were examined on each slide and head and tail abnormalities were expressed as percentage (Turk *et al.,* 2007).

Serum T, LH and FSH hormones were assayed by

Labor diagnostics Nord Gmbh (Germany) ELISA kit methods.

Testis was homogenized in 10 % (w/v) ice cold buffer (0.1 M phosphate buffer, pH 7.4+150mM KCl). A part of this homogenate was used for lipid peroxidation and GSH estimations and other part was centrifuged at 9000 rpm for 20 min to obtain supernatant which was used for SOD, CAT, GPx, GR, GST and protein estimations.

Lipid peroxidation was estimated by the method of Okhawa *et al.* (1979). 1ml homogenate was incubated at 37ºC for 10 min 1 ml of 10% (w/v) chilled trichloroacetic acid (TCA) was added to it and centrifuged at 2500 rpm for 15 min at room temperature. 1 ml of 0.67% thiobarbituric acid (TBA) was added to 1 ml of supernatant and kept in boiling water bath for 10-15 min. The tubes were cooled under tap water, followed by addition of 1ml of distilled water. Absorbance was recorded at 530 nm and the results were expressed as n moles MDA $hr^{-1}g^{-1}$ tissue. GSH was estimated by the method described by Ellman (1959). 1 ml of 10% crude homogenate was mixed with 1 ml of 5% TCA (w/v), the mixture was allowed to stand for 30 min. and centrifuged at 2500 rpm for 15 min. 0.5 ml of supernatant was taken and 2.5 ml of 5' 5' -dithionitrobenzoic acid (DTNB) was added, mixed thoroughly and absorbance was recorded at 412 nm. The results were expressed as µmole g^{-1} tissue.

Superoxide dismutase activity (SOD) was estimated by the method of Kakkar *et al.* (1984). 650 µl of sodium pyrophosphate buffer, 50 µl phenazine methasulphate (PMS), 150 µl of nitroblue tetrazolium chloride (NBT) and 100 µl NADPH were added to 50 µl of supernatent. The mixture was vortexed thoroughly, incubated for 90 sec and 500µl glacial acetic acid was added to stop the reaction. 2.0 ml of n-butanol was added to the mixture, vortexed thoroughly and kept at room temperature for 10 minutes. Absorbance was measured at 560 nm and the results were expressed as µmoles min^{-1} mg^{-1} $protein^{-1}$. Catalase activity (CAT) was estimated by the method of Sinha (1972). 1ml of phosphate buffer and 0.4 ml water was added to 0.1 ml of supernatent. Reaction was started by adding 0.5 ml H_2O_2 and mixture was incubated at 37^{0}C for 1 min. Reaction was stopped by adding 2ml of dichromate : acetic acid reagent and kept at boiling water bath for 15 min. The mixture was cooled and absorbance was read at 570 nm. CAT activity was calculated in terms of µmoles min^{-1} mg^{-1} protein. GPx was estimated by the method of Rotruck *et al.* (1973). 0.4 ml Tris HCl buffer, 0.2 ml GSH, 0.1 ml water and 0.2 ml H_2O_2 were added to 0.1ml of supernatent. The mixture was incubated at 37º C for 15 min. and 0.5 ml TCA (10%) was added. The mixture was centrifuged at 2000 rpm for 15 min., 0.5 ml of supernatant was taken and 2 ml di-sodium hydrogen phosphate buffer and 0.5 ml Ellman's reagent were added and the absorbance was read at 420 nm. The results were expressed as nmoles min^{-1} mg^{-1} protein. GST was estimated as per method of Habig *et al.*(1974). The reaction mixture consisting of 1.425 ml phosphate buffer (0.1 M, pH 6.5) 1.475 ml GSH (1.0 mM), 20µl 1-chloro-2,4-Dinitrobenzene (CDNB,1mM) and 60 µl water were added to 20µl of supernatent to give 3.0 ml of reaction mixture. Absorbance was recorded at 340nm and the GST activity was calculated as µmoles CDNB conjugate formed min^{-1} mg^{-1} protein using molar extinction coefficient of $9.6x10^3M^{-1}$ cm^{-1}. GR was estimated by the method of Carlberg and Mannervik (1985). 2.5 ml buffer, 0.2 ml NADPH, 0.2 ml GSSG and 0.1 ml 'supernatent' were mixed and allowed to stand for 30 sec. Absorbance was recorded at 340 nm for 3 min at 30 sec intervals. GR activity was calculated in terms of n moles min^{-1} mg^{-1} protein.

Protein was estimated by the method of Lowry *et al.* (1951). Results were expressed as Mean ± SD. Data was subjected to one way analysis of variance (ANOVA). The treatment groups were compared with control group using Dunnett's test. All the statistics were carried out in GraphPad InStat Software Inc., v. 3.06, San Digeo, USA.

Results and Discussion

Sex organ weight : The safety claims of synthetic pyrethroids including cypermethrin have lead to their indiscriminate use in agricultural practices and household. In the present study, the weight of the testes and epididymis decreased as compared to the control. Significant ($P<0.01$) decrease in weight of epididymis and testes (31 and 8.8%) in group-II, non significant ($P>0.05$) increase (9 and 0.08%)

Table 1 : Effect of EETT and cypermethrin on reproductive parameters of Wistar rats

Groups	Testicular sperm head Count×10^6 g^{-1} tissue	Sperm morphology abnormality (%)	Sperm motility (%)	Live sperm count (%)
I	261.50±15.08	18.16±2.04	85.74±2.18	73.33±3.22
II	89.56±4.023^c	65.61±3.30 ^{c}	40.695±1.79 ^{c}	42.916±3.44 ^{c}
III	250.76±8.28 ^{a}	22.0± 2.60 ^{b}	78.58±6.93^b	69.583±3.0 ^{a}
IV	278.66±12.24^b	12.33±0.87 ^{c}	92.92±2.11^b	79.916±4.49^c

Values are mean of six replicates ± SD. c= $P<0.01$ b= $P<0.05$ a=$P>0.05$ as compared with control value. Group I- Control, Group II- Cypermethrin, Group III-Combination (cypermethrin + *EETT*), Group IV-EETT, EETT- Ethanolic extract of *Tribulus terrestris*

Table 2 : Effect of EETT and cypermethrin on sperm tail and head abnormalities

Tail morphology	G-I	G-II	G-III	G-IV
Headless tail (%)	2.5	15	5.0	1.5
Broken tail (%)	1	3	1.5	1
Coiled tail (%)	1	3	1	1.5
Bent tail (%)	2	4	2	1.5
Head morphology				
Excessive hook (%)	0	0.5	0	0
Amorphous hook (%)	0	0.5	0	0
Pin head (%)	0	1	0	0
Detached head (%)	2.5	5.5	1.5	1

in group-III and significant (P<0.05) increase (31 and 26.6%) in group-IV was observed as compared to group-I (Table 4). The decrease in organ weight may be due to decrease in level of serum T, FSH and LH observed in the study. Pyrethroids and other pesticides have exhibited antiandrogenic effect largely because of their accumulation in testes. Previous studies have shown decrease in sex hormone level on exposure of deltamethrin (Issam *et al.,* 2009). Accumulation of pesticides in testes induces oxidative stress leading to accelerated death of spermatogenic cells associated with sperm abnormalities (Sharma and Singh, 2010). Oxidative stress may also be responsible for decrease in testes and epididymis weight observed is this study.

Sperm characteristics : Sperm head count (67.75%), sperm motility (53.41%), live sperm count (41.48%) were significantly (P<0.01) decreased in group-II as compared to control. Group-III showed the non significant (P>0.05) decrease in sperm head count (4.10%) and live sperm count (5.11%) and significant (P<0.05) decrease in sperm motility (8.35%) whereas in group-IV these parameters increased significantly (P<0.05) with 6.65, 8.37 and 8.98% respectively (Table1). Significant increase (P<0.01) in sperm morphology abnormality (261.28 %) in group-II, significant (P<0.05) increase (18.66%) in group-III, significant (P<0.01) decrease (32.10%) in group-IV was observed as compared to group-I (Table 1).

Increase in headless tail (15%), broken tail (3%), coiled tail (3%), bent tail (4%), excessive hook (0.5%), amorphous hook (0.5%), pin head (1%), detached head (5.5%) was observed in group-II. In group-III and IV increase in headless tail (5.0, 1.5%), broken tail (1.5, 1%), coiled tail (1, 1.5%) and bent tail (2, 1.5%) while no change in head abnormalities except detached head (1.5, 1%) was observed (Table 2).

The decrease in testicular sperm head count was observed on cypermethrin exposure. The diminution in testicular sperm count by cypermethrin treatment in our study is in agreement with the findings of others, where cypermethrin treatment was associated with the inhibition of T, LH and FSH level (Assayed *et al.,* 2008; Joshi *et al.,* 2011; Liu *et al.,* 2010). Decrease in sperm motility, live sperm and increase in the number of the abnormal sperm may be due to enhanced ROS production by α-cypermethrin in the testes and epididymis as observed in this study. Pesticide induced ROS production is known to adversely affect sperm motility, live sperm and increased sperm abnormality (El-Demerdash *et al.,* 2004; Joshi *et al.,* 2011; Elbetieha *et al.*, 2001).

Reproductive hormones : Significant (P<0.01) decrease in serum T (38.41%), FSH (61.07%), LH (35.08%) in group-II, significant (P<0.05) increase in T (11.87%) , significant (P>0.05) increase in FSH (1.78%), and LH (0.925%) in group-III, while significant (P<0.01) increase in T (13.12%), FSH (41.96%) and LH (24.07%) was observed in group-IV as compared to group-I (Table 3). The decrease in the T, LH and FSH hormone levels may be either due to the direct effect of the α-cypermethrin on the androgen biosynthesis pathway in the testes or its effect on brain hypothalamus/ anterior pituitary gland may be indirectly affecting the testes and sexual function. Decrease in T, FSH and LH levels was also observed by Issam *et al.* (2009). Gauthaman *et al.* (2002) reported increase in serum level T by steroidal compound protodioscin from *Tribulus terresteris.*

Lipid peroxidation : The significant (P<0.01) increase in lipid peroxidation level (179.66%) in group-II, significant (P<0.05) increase (17.87%) in group-III whereas significant (P<0.01) decrease (62.94%) in group-IV was observed as compared to control (Table 4). In the present study, significant increase in the testicular LPO level was observed as compared to the control, possibly due to increase in the generation of the free radicals by cypermethrin accumulation in the testes causing damage to cell membrane and enzyme systems. Pesticide induced increase in lipid peroxidation has previously been reported by Giray *et al.* (2001), Vaithinathan *et al.* (2009), Sharma and Singh (2010).

Non-enzymatic antioxidant : GSH (71.09%) decreased significantly (P<0.01) in group-II. Group-III showed significant (P<0.05) decreased (1.49%) in GSH level, while significant (P<0.01) increase (56.31%) was observed in group-IV as compared to group-I (Table 4). GSH and GSH related enzymes are involved in detoxification and metabolism of reactive oxygen species as well as pesticides (Knapen *et al.*, 1999). In the present study, significant decrease in the GSH level was observed in the cypermethrin exposure group as compared to the control, possibly due to increased utilization of GSH for detoxification of cypermethrin induced free radicals (Rajindra *et al.,* 2009; Raina *et al.,* 2009).

Enzymatic antioxidants : SOD (66.87%), CAT (65.11%), GPx (29.8%), GR (86.40%), GST (72.03%) activities and total protein

Table 3 : Effect of EETT against cypermethrin induced reproductive hormone alterations in Wistar rats

Parameters	Group-I	Group-II	Group-III	Group-IV
Testosterone (ng ml^{-1})	2.940±0.047	1.811±0.136^{c}	2.591±0.295^{b}	3.326±0.205^{c}
FSH (mlU ml^{-1})	0.112±0.004	0.0436±0.013^{c}	0.114±0.005^{a}	0.159±0.036^{c}
LH (mlU ml^{-1})	0.108±0.004	0.070±0.008^{c}	0.109±0.013^{a}	0.134±0.023^{c}

Values are mean of six replicates ± SD. c=P<0.01, b= P<0.05 a=P>0.05, as compared with control value. Group I- Control, Group II- Cypermethrin, Group III-Combination (cypermethrin + *EETT*), Group IV-EETT, EETT- Ethanolic extract of *Tribulus terrestris.*

Table 4 : Effect of EETT and cypermethrin on Organ weight, Lipid peroxidation, Non-enzymatic antioxidants and Enzymatic antioxidants activity in rat testes

Parameter	Group-I	Group-II	Group-III	Group-IV
Weight of testes (g)	1.24±0.027	1.13±0.030^{c}	1.22± 0.023^{a}	1.31±0.041^{c}
Weight of epididymis (g)	0.22±0.023	0.15±0.027^{c}	0.23± 0.023^{a}	0.28±0.013^{c}
LPO (nmole MDA $hr^{-1}g^{-1}$ tissue)	2.680±0.102	7.495±0.119^{c}	3.159±0.146 ^{b}	0.9935±0.064 ^{c}
GSH (µmole g^{-1} tissue)	0.467±0.011	0.135±0.002^{c}	0.460±0.057 ^{b}	0.730±0.005 ^{c}
CAT (µmole min^{-1} mg^{-1})	58.985±0.773	20.576±0.283^{c}	58.578±0.912 ^{b}	65.964±1.174 ^{b}
SOD (µmole min^{-1} mg^{-1} protein)	6.56±0.234	2.173 ±0.105^{c}	5.51±0.429 ^{b}	7.66±0.238 ^{b}
GPx (nmole min^{-1} mg^{-1} protein)	35.478±0.845	24.95±0.580 ^{c}	35.57±0.767 ^{a}	41.156±2.49 ^{b}
GR (nmole min^{-1} mg^{-1} protein)	2.346±0.132	0.319±0.007 ^{c}	2.151±0.071 ^{a}	6.154±0.128 ^{c}
GST (µmole min^{-1} mg^{-1} protein)	0.329±0.015	0.092±0.0050 ^{c}	0.321±0.007 ^{a}	1.136±0.0013 ^{c}
Protein (mg ml^{-1})	4.488±0.234	1.676±0.105 ^{c}	3.659±0.190 ^{a}	6.057±0.606 ^{b}

Values are mean of six replicates ± SD. c=P<0.01 b= P<0.05 a=P>0.05 as compared with control value. Group I- Control, Group II- Cypermethrin, Group III-Combination (cypermethrin + *EETT*), Group IV-EETT, EETT- Ethanolic extract of *Tribulus terrestris.*

(62.76%) was significantly (P<0.01) decreased in group-II as compared to group-I (Table 4). Significant (P<0.05) decrease in SOD (16%), CAT (0.69%) activities non significant (P>0.05) decrease in GPx (0.25%), GR (8.31%) and total protein(18.47%) while significant (P<0.01) decrease in GST(2.43%) was observed in group-III. There was significant (P<0.05) increase in CAT (11.83%), SOD (15.18%), GPx(16%) and total protein (11.83%) and significant (P<0.01) increase in GR(162%) and GST(245%) in group-IV as compared to group-I (Table 4). SOD is considered the first line of defense against deleterious effects of ROS in the cell by catalyzing dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen (Kanematsu and Asada, 1994). Decrease in testicular SOD activity was observed as compared to control. Excessive ROS may have inactivated SOD leading to further ROS accumulation and enhanced tissue damage reflected by testes weight loss and diminution in sperm characteristics. The present results are in accordance with Raina *et al.* (2009), Vaithinathan *et al.*(2009) and Yousef *et al.*(2003). Decrease in SOD activity is likely to accumulate hydrogen peroxide in testes. Accumulated H_2O_2 is known to inhibit CAT activity (Latchoumycandane and Mathur, 2002) Significant decrease in CAT activity was observed as compared with control group in this study.

Present study showed decrease in testicular GPx level which might be due to decrease in GSH level as the later is used as substrate for GPx. GR activity also decreased in cypermethrin exposure group as compared to control. Significant decrease in the GR may be activity due to cypermethrin induced damage to the tertiary structure of the enzyme (Latchoumycandane and Mathur, 2002).

Decrease in the GST activity was observed in the testes of exposed rats as compared to control. GST catalyzes the conjugation of the reduced glutathione to electrophiles and protects cellular components from oxidative damage (Hayes and Pulford, 1995). GST is known to bind strongly to hydrophobic compounds like pyrethroids. Previous studies reported decrease in GST level in rat testes on lindane exposure (Sharma and Singh, 2010). Since the activity of all antioxidant enzymes and level of GSH decreased on cypermethrin exposure, the findings were concomitant with decrease in total protein content in exposure group.

Cypermethrin induced alteration in fertility parameters, reproductive hormone and biochemical parameters were reverted to normal level by EETT treatment. The study concluded that EETT ameliorated cypermethrin induced testicular damage by reducing oxidative stress and by enhancing level of sex hormones. Further research is required to understand the mechanism of action of EETT or its purified molecule(s) on sex hormone biosynthesis.

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