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Role of *Euphorbia thymifolia* L. ethanolic root extract in treating female reproductive dysfunction in rats

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Abstract
Euphorbia thymifolia root is having the protective effect against female reproductive dysfunctions. This study is to evaluate the antioxidant activity of ethanolic extract of *Euphorbia thymifolia* root in treating stress induced female reproductive dysfunctions. Forced swimming stress (15min/day for 28 days) and restraint stress (3h/day for 28 days) were the methods employed to induce female reproductive dysfunction in rats. Ethanolic extract of *Euphorbia thymifolia* root was given to rats in two doses, 100 mg/kg and 200 mg/kg for 28 days along with induction of stress and its effectiveness was assessed by observing changes in SOD, catalase and lipid peroxidation of uterus and ovary. The results were analyzed by using one-way ANOVA followed by Dunnett's test. *Euphorbia thymifolia* root extract showed a significant antioxidant activity which is evident by increase in the levels of SOD and catalase, decrease in the levels of lipid peroxidation confirming the antioxidant effect which was found to be dose dependent. The antioxidant activity may be due to the presence of various phytochemical constituents like alkaloids, flavonoids and other constituents present in the *Euphorbia thymifolia* root.
Keywords: *Euphorbia thymifolia* L. root, Forced swimming stress, Restraint stress, Antioxidant activity.

1. Introduction
Infertility is defined as the inability to conceive after trying for at least one year. Infertility is a raising problem in today's society, influencing around 15% of couples globally. The event of infertility has moved ahead to expanding velocity and may impact 11% of couples of conceptive age.[1] As per World Health Organization, 2–10% and 10–25% of couples worldwide are unable to conceive due to primary and secondary infertility causes respectively. Among these couples, causative components are found in about 30-40% in females and 10–30% in males. In 15-30% of cases, both partners have detectable abnormalities[2] and in some cases without any cause.

There are so many confounding factors that can cause or continue to infertility. The major causes of female infertility are due to ovarian dysfunction, tubal obstruction, polycystic ovarian syndrome, endometriosis, stress and other unexplained factors.[3] Reproductive functions are suppressed under various stress conditions which includes infection, malnutrition, lifestyle factors, restraint, strenuous exercise, surgical trauma, heat, cold, noise exposures and environmental pollution.[4] Prolonged or chronic stress causes anovulation which results in infertility due to suppression of gonadotrophic hormones and oxidative stress (OS).[5]

Reactive oxygen species (ROS) can modulate cellular functions, and OS can impair the intracellular milieu, resulting in diseased cells or endangered cell survival. Reproductive cells and tissues remain stable when free radical production and the scavenging antioxidants remain in balance. The role of ROS in various diseases of the female reproductive tract has been discussed. ROS can affect a variety of physiological functions in the reproductive tract, and excessive levels can result in precipitous pathologies affecting female reproduction. The oxidant status can influence early embryo development by modifying the key transcription factors, hence modifying gene expression.

Under normal conditions, antioxidants act to oppose ROS production, scavenge existing free radicals, and promote the repair of ROS-induced damage to cell structures. Non-enzymatic antioxidants include vitamin C, vitamin E, selenium, zinc, beta carotene, carotene, taurine, hypotaurine, cysteamine, and glutathione. Enzymatic antioxidants include

SOD, Catalase, and GSH-Px, glutaredoxin and glutathione reductase. The degree of antioxidant defense present is often expressed as total antioxidant capacity (TAC). A disruption in the delicate balance between antioxidants and pro-oxidant molecules can result in OS. OS arises when the generation of reactive oxygen species and other radical species overrides the scavenging capacity by antioxidants, either due to the excessive production of ROS or an inadequate availability of antioxidants. Thus, oral antioxidant supplementation may serve to prevent and alleviate OS and its contribution to the pathogenesis of obstetrical disease such as preeclampsia and recurrent pregnancy loss and gynecological disorders such as polycystic ovarian syndrome (PCOS) and endometriosis.[6]

Infertility, like any disease, is simply a sign that something is not right inside the body and must be fixed. The body can reverse infertility naturally if given the correct resources. Currently, female infertilities are treated by natural plants, drugs, surgical procedures in addition to dietary and life style changes. Treatment of infertility with drugs and surgical procedures may lead to complications like multiple pregnancy, twins, ectopic pregnancy, stress, ovarian hyperstimulation syndrome, ovarian cancer, birth defects etc. Although significant advances have been made in treatment of reproductive disorders, there are serious limitations in existing therapies because of cost, utilization and toxicity. Medications from natural sources (medicinal plants) are attractive therapeutic alternatives and supplements to existing therapy and have not really been explored in depth.[6]

Euphorbia thymifolia also Known as *Chamaecyse thymifolia*, Dudhi, Dugdhikaa, Naagaarjuni and Swaaduparni.[7] This plant is reported to have antiviral[8], antibacterial, antioxidant[8]-[11], anti-inflammatory[12] and hepatoprotective[12] activities. Roots of *Euphorbia thymifolia* are known to show female fertility improving properties[7], but not reported scientifically. So the current study was undertaken to evaluate the antioxidant activity of ethanolic extract of *Euphorbia thymifolia* root in rat models against experimentally induced stress models.

2. Materials and Methods

2.1 Collection of Plant material

Euphorbia thymifolia fresh roots were collected from Tirupati, Andhra Pradesh, identified and authenticated by Dr. K. Madhava chetty, Asst. Professor, Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India.

2.2 Preparation of ethanolic extract

The roots of *Euphorbia thymifolia* L. was chopped and dried under shade at room temperature and submitted for extraction to Green Chem Herbal Extracts and Formulations, Bangalore, India. The ethanolic extract and COA were obtained from Dr. Rajendran, Green Chem Herbal Extracts and Formulations with Batch no: ETE/RD/01.

2.3 Experimental Animals

Experimental study was carried out using adult female Wistar albino rats weighing between 175-200g. Animals were housed in a group of 6 in polyethylene cages under standard housing conditions of 12-12h light and dark cycle, temperature 22±2°C and humidity 50±10% with standard feed pellet and free access to water *ad libitum*. Standard hygiene conditions were maintained. Experiment was conducted with strict compliance to ethical principles and guidelines formulated by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and performed in accordance with the Institutional Animal Ethics Committee (IAEC/NCP/66/11) of Nargund college of Pharmacy, Bangalore.

2.4 Dose selection based on Acute oral toxicity study

Two doses of ethanolic extract 100 mg/kg and 200 mg/kg of *Euphorbia thymifolia* L. root were selected as per the acute oral toxicity study performed in accordance with Office of Prevention, Pesticides and Toxic Substances (OPPTS) guidelines following the Up and Down procedure.[13] The ethanolic extract of *Euphorbia thymifolia* L. root found safe up to 5000 mg/kg body weight.

2.5 Forced swimming stress (FSS) model

Animals with regular estrous cycle were selected and divided into four groups, six animals in each group. The forced swimming stress was induced to all the rats by placing them individually in acrylic plastic pool (60 cm in height x 30 cm in diameter) filled with water up to a depth of 50cm for 15 min/ day for 28 days at ambient room temperature.[14]

Group I: Vehicle control - distilled water, orally (5 mL/kg b.w) for 28 days.

Group II: Forced swimming stress (15 min/day) for 28 days.

Group III and IV: Rats were treated with ethanolic extract of *Euphorbia thymifolia* root (EEET) (100 mg/kg and 200 mg/kg b.w, per oral), continued for 28 days along with induction of stress. Animals were subjected to forced swimming stress for 15 min/day after half an hour of administration of the extract.

After the last stress session on 28th day all animals were sacrificed by cervical dislocation. Ovaries, uteri were

isolated and placed in KCl (10%w/v) solution and homogenated using a homogenizer and centrifuged at 4000rpm for 10min. The supernatant was separated and estimated for SOD, catalase and lipid peroxidation.

2.6 Restraint stress (RS) model:

The animals with regular estrous cycle were selected & divided into four groups, six animals in each group. The restraint stress was induced to all the rats by placing them individually inside the plastic cylindrical restrainers (21cm in length x 6cm in diameter) with ventilated sliding doors at ambient temperature.[14]-[16]

Group I: Vehicle control - distilled water, orally (5 mL/kg b.w) for 28 days.

Group II: Restraint stress (3h/day) for 28days.

Group III and IV: Rats were treated with ethanolic extract of *Euphorbia thymifolia* root (EEET) (100 mg/kg and 200 mg/kg b.w, p.o), continued for 28 days along with induction of stress. Animals were subjected to restraint stress for 3h/day after half an hour of administration of the extract.

After the last stress session on 28th day all animals were sacrificed by cervical dislocation. Ovaries, uteri were isolated and placed in KCl (10%w/v) solution and homogenated using a homogenizer and centrifuged at 4000rpm for 10min. The supernatant was separated and estimated for SOD, catalase and lipid peroxidation.

3. Results

Table 1: Effect of ethanolic root extract of *Euphorbia thymifolia* on antioxidant enzyme levels-forced swimming stress model

Groups	SOD	CATALASE	LIPID PEROXIDATION
	Uterus (SOD units/mg of protein)	**Uterus** (CAT Units/mg of protein)	**Uterus** MDA (nanomol/mg of protein)
Vehicle control	1.009±0.031	363.4 ±7.72	247.7±5.35
FSS	0.3097±0.018^{***a}	132.3 ±5.16^{***a}	349.4±3.29^{***a}
FSS + EEET (100mg/kg)	0.5833±0.015^{***b}	277.9 ±5.21^{***b}	278.1±3.83^{***b}
FSS + EEET (200mg/kg)	0.8318±0.022^{***b}	319.3 ±6.08^{***b}	250.7±3.37^{***b}

Values are expressed as Mean ± SEM. Data were analyzed by one way ANOVA followed by Dunnett's *t* test. Number of animals in each group n = 6, ^{a} comparison made with vehicle control group, ^{b} comparison made with Forced swimming stress group. *** P<0.001.

Table 2: Effect of ethanolic root extract of *Euphorbia thymifolia* on antioxidant enzyme levels-forced swimming stress model

Groups	SOD	CATALASE	LIPID PEROXIDATION
	Ovary (SOD units/mg of protein)	**Ovary** (CAT Units/mg of protein)	**Ovary** MDA (nanomol/mg of protein)
Vehicle control	1.104±0.043	255.2±4.84	103.8±2.79
FSS	0.3595±0.016^{***a}	119.3±5.61^{***a}	210.8±3.38^{***a}
FSS + EEET (100mg/kg)	0.5926±0.018^{***b}	213.4±3.06^{***b}	149.1±2.77^{***b}
FSS + EEET (200mg/kg)	0.8630±0.021^{***b}	218.5±8.91^{***b}	113.7±1.95^{***b}

Values are expressed as Mean ± SEM. Data were analyzed by one way ANOVA followed by Dunnett's *t* test. Number of animals in each group n = 6, ^{a} comparison made with vehicle control group, ^{b} comparison made with Forced swimming stress group. *** P<0.001.

Forced swimming stress group showed significant decrease in the enzyme activity of superoxide dismutase and catalase, where as a significant increase in lipid peroxidation activity in the uterus as well as ovary when compared with vehicle control group. Groups that received ethanolic extract of *Euphorbia thymifolia* root 100mg/kg b.w and 200mg/kg b.w for 28 days along with forced swimming stress showed significant increase in the enzyme activity of superoxide dismutase, catalase and significant decrease in lipid peroxidation in the uterus as well as ovary when compared with stress treated group.

Table 3: Effect of ethanolic root extract of *Euphorbia thymifolia* on antioxidant enzyme levels-restraint stress model

Groups	SOD	CATALASE	LIPID PEROXIDATION
	Uterus (SOD units/mg of protein)	**Uterus** (CAT Units/mg of protein)	**Uterus** MDA (nanomol/mg of protein)
Vehicle control	1.009±0.031	363.4±7.72	247.7±5.35
RS	0.3008±0.019^{***a}	137.3±3.96^{***a}	359.8±3.90^{***a}
RS + EEET (100mg/kg)	0.573±0.018^{***b}	318.3±5.51^{***b}	270.9±3.73^{***b}
RS + EEET (200mg/kg)	0.8176±0.019^{***b}	349.2±4.69^{***b}	254.7±2.88^{***b}

Values are expressed as Mean ± SEM. Data were analyzed by one way ANOVA followed by Dunnett's *t* test. Number of animals in each group n = 6, ^{a} comparison made with vehicle control group, ^{b} comparison made with Restraint stress group. *** P<0.001.

Table 4: Effect of ethanolic root extract of *Euphorbia thymifolia* on antioxidant enzyme levels-restraint stress model

Groups	SOD	CATALASE	LIPID PEROXIDATION
	Ovary (SOD units/mg of protein)	**Ovary** (CAT Units/mg of protein)	**Ovary** MDA (nanomol/mg of protein)
Vehicle control	1.104±0.043	255.2±4.845	103.8±2.79
RS	0.364±0.019^{***a}	129.4±6.42^{***a}	237.1±2.20^{***a}
RS + EEET (100mg/kg)	0.639±0.014^{***b}	201.9±6.15^{***b}	156.6±2.84^{***b}
RS + EEET (200mg/kg)	0.916±0.013^{***b}	224.8±6.17^{***b}	129.0±2.82^{***b}

Values are expressed as Mean ± SEM. Data were analyzed by one way ANOVA followed by Dunnett's *t* test. Number of animals in each group n = 6, [a] comparison made with vehicle control group, [b] comparison made with Restraint stress group. *** P<0.001.

Restraint stress group showed significant decrease in the enzyme activity of superoxide dismutase, catalase and significant increase in lipid peroxidation level in the uterus as well as ovary when compared with vehicle control group. Groups that received ethanolic extract of *Euphorbia thymifolia* root with 100mg/kg b.w and 200mg/kg b.w along with the restraint stress showed significant increase in the enzyme activity of superoxide dismutase, catalase and significant decrease in lipid peroxidation in the uterus as well as ovary when compared with stress treated group.

4. Discussion

Euphorbia thymifolia is well known in folk medicine and well recognized to have different activities towards health improvement. The plant consisting of different active ingredients and notably roots are known to have phytosterols, beta-sitosterol, brassicasterol, alkaloids and terpenes which are known to have protective effect in infertility.

Forced swimming stress (a moderate physical or metabolic stress) and Restraint stress (physical and psychological stress) are the stressor's which were known to induce female reproductive dysfunctions. These two methods were chosen to induce stress in rats.[15]

The estrous cycle in rats involves many histological, physiological, and morphological and biochemical changes within the ovary. During the estrous cycle the maturation and ovulation of preovulatory follicles takes place under the combined and balanced influence of ovarian and extra ovarian hormones. Imbalance in these hormones leads to irregularity in ovarian function and changes in the duration of estrous cycle.[17]

Oxidative stress is known to cause damage during oocyte maturation and ovulation. These ROS are very unstable and highly reactive. They become stable by acquiring electron from nucleic acids, proteins, carbohydrates and lipids there by cascade of chain reaction are initiated resulting in cellular damage and causes lipid peroxidation. SOD is the first antioxidant enzyme to deal with oxyradicals by accelerating the dismutation of superoxide (O_{2*}) to hydrogen peroxide (H_2O_2). Catalase is a peroxisomal haem protein that catalyses the removal of H_2O_2 formed during the reaction catalysed by SOD. Thus, they provide protective defense against reactive oxygen species.[18]

In ovary & uterus of stress treated rats there was a significant decrease in the SOD, catalase enzyme activities due to the excess generation of free radicals and increased lipid peroxidation. Oxidative stress results in cellular damage. Malondialdehyde a secondary product of lipid peroxidation is a major reactive aldehyde; higher levels can lead to peroxidation of biological membranes.

Euphorbia thymifolia treated groups showed significant increase in the SOD and catalase levels and significant decrease in the lipid peroxidation which may be due to the free radical scavenging properties of its active phyto-constituents.

5. Conclusion

The experimental studies carried out on ethanolic extract of *Euphorbia thymifolia* root showed antioxidant effect against stress induced female reproductive dysfunction. Further work regarding isolation of bioactive compounds responsible for this potent activity will provide more insight about the role of plant.

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