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ANTIFERTILITY INVESTIGATIONS ON THE ETHANOL EXTRACT OF *EUGENIA SINGAMPATTIANA* BEDD LEAF IN MALE ALBINO RATS

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ABSTRACT: Antifertility effect of ethanol extract of leaf of *Eugenia singampattiana* was observed in male albino rats. The relative weight of the testes and epididymis were decreased. The epididymal sperm count, motility and sperm abnormality were reduced significantly in treated rats. There was an increase in serum urea, creatinine and the activity of liver marker enzyme (SGOT) levels of drug treated rats. The results of the hormonal assay showed increased serum levels of FSH and estrogen but decreased levels of LH and testosterone when compared to control. The results of the present study concluded that, ethanol extract of leaf of *E. singampattiana* inhibited sperm concentration, motility and testosterone which might result in a male fertility.

INTRODUCTION: The World Health Organization (WHO) has constituted a population control programme which includes studies on drugs used in traditional medical practices. Medicinal plant products have a long history of indigenous use in India as well as in other countries [1]. Phytotherapy has a very long tradition, although proper scientific explanation is relatively new. In our country as well as in the world, there are several medicinal plants associated with antifertility properties [2, 3]. Fertility regulation with plants or plant preparations has been reported in the ancient literature of indigenous systems of medicine.

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A large number of plant species with antifertility effects have been screened in China and India, since last 50 years and were subsequently fortified by national and international agencies[4, 5]. However, the search for an orally active, safe and effective plant preparation or its compound is yet to be fulfilled for fertility regulation as many of them have incomplete inhibition of fertility or side effects.

E. singampattiana Bedd belong to the family Myrtaceae. It is commonly known as "Kattukorandi" by *Kanikkar* tribals of Agasthiarmalai Biosphere Reserve, Western Ghats, Tamil Nadu, India. The paste prepared from the leaf of *E. singampattiana* is given to treat asthma and giddiness. Paste prepared from equal quantity of leaves and flowers is consumed by *Kanikkar* tribals to cure body pain and throat pain. Paste prepared from equal quantity of leaves, flowers and tender fruits are consumed by the *Kanikkars* to

relief from leg sores and rheumatism. Paste prepared from equal quantity of stems, leaves and flowers is consumed with palm sugar to get relief from gastric complaints [6]. *E. singampattiana* leaf extracts were reported for the biological activities such as antitumor, antidiabetic, anti-hyper-lipidaemic and *in vitro* antioxidant activity [7, 8, 9, 10]. In view of the above medicinal properties, the present study was designed to investigate the antifertility activity of ethanol extract of leaf of *E. singampattiana* on male albino rats.

MATERIALS AND METHODS:

Plant Material: The leaves of *Eugenia singampattiana* Bedd were freshly collected from the well grown healthy plants inhabiting the natural forests of Karaiyar, Agasthiarmalai Biosphere Reserve, Western Ghats, Tamil Nadu. The plant were identified and authenticated in Botanical Survey of India, Southern Circle, Coimbatore, Tamil Nadu, India. A voucher specimen was deposited in Ethnopharmacology Unit, Research Department of Botany, V.O. Chidambaram College, Tuticorin, Tamil Nadu.

Preparation of plant extract for phytochemical screening and antifertility studies: The *E. singampattiana* leaves were shade dried at room temperature and the dried leaves were powdered in a Wiley mill. Hundred grams of powdered *E. singampattiana* leaves was packed in a Soxhlet apparatus and extracted with ethanol. The extracts were subjected to qualitative test for the identification of various phytochemical constituents as per the standard procedures [11, 12, 13]. The ethanol extracts were concentrated in a rotary evaporator. The concentrated ethanol extract were used for antifertility studies.

Animals: Normal healthy male Wistar albino rats (180-240g) were used for the present investigation. Animals were housed under standard environmental conditions at temperature (25±2^{o}C) and light and Dark (12:12h). Rats were fed with standard pellet diet (Goldmohur brand, MS Hindustan Lever Ltd., Mumbai, India) and water *ad libitum*.

Acute Toxicity Studies: Acute oral toxicity study was performed as per OECD-423 guidelines (acute toxic class method); albino rats of either sex selected by random sampling were used for acute toxicity study [14]. The animals were kept fasting for overnight and provided only with water, after which the extracts were administered orally at 5mg/kg body weight by gastric intubations and observed for 14 days. If mortality was observed in two out of three animals, then the dose administered was assigned as toxic dose. If mortality was observed in one animal then the same dose was repeated again to confirm the toxic dose. If mortality was not observed, the procedure was repeated for higher doses such as 50, 100 and 2000 mg/kg body weight.

Experimental Design: The male rats were divided into four groups consisting of 5 animals.

Group I: Rats received normal saline daily for 21days, orally (Normal control).

Group II: Rats received ethanol leaf extract of *E. singampattiana* at the dose of 150mg/kg body weight daily for 21 days.

Group III: Rats received ethanol leaf extract of *E. singampattiana*, at the dose of 300mg/kg body weight daily for 21 days.

After 24 hours of last treatment, the final weight was recorded and the animals were sacrificed by decapitation. Blood was collected; Sera were separated by centrifugation at 3000g for 10 minutes and stored at 20^{o}C until used for various biochemical assays. Then testes, epididymis, vas deferens, seminal vesicle and ventral prostrate were dissected out, trimmed off extraneous and weighed accurately on torsion balance. The organ weights were expressed in terms of mg/100g body weight.

Sperm count: Epididymal fluid (for sperm count) was collected from caput and caudal segments separately and diluted with Sorenson's buffer (pH 7.2). The separated fluid was taken for sperm count. Sperm count was carried out by using Neubauer's haemocytometer as described by Zaneveld and Pelakoski [15].

Sperm motility and abnormality: After anaesthetizing the rats, the caudal epididymis was then dissected. An incision (about 1mm) was made in the caudal epididymis and drops of sperm fluid were squeezed onto the microscope slide and 2 drops of normal saline were added to mobilize the sperm cells. Epididymal sperm motility was then assessed by calculating motile spermatozoa per unit area.

Morphology (abnormality) was evaluated on sperm from the caudal epididymis. The total morphological abnormalities were observed as described by Linde *et al* [16].

Serum biochemical analysis: Serum protein [17] and serum albumins were determined by quantitative colorimetic method by using bromocresol green. The total protein minus albumin gives the globulin, urea [18], creatinine [19], serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate transaminase (SGOT) was measured spectrophotometrically by using the method of Reitman and Frankel [20]. Serum alkaline phosphatase (ALP) was measured by the method of King and Armstrong [21].

Hormonal Assay: Blood was removed from the rats by intracardiac method. Blood was centrifuged at 3000 rpm to separate the serum for the measurement of testosterone, Luteinizing hormone (LH), estrogen and follicle stimulating hormone (FSH). The quantitative determination of hormones was done by using Enzyme Immunoassay Method (EIA). The EIA kit was obtained from Immunometrics (London, UK).

Statistical Analysis: Data were expressed as Mean ± SEM. Student's t test was used for statistical comparison.

RESULTS:

Preliminary phytochemical screening and acute toxicity studies: Phytochemical screening of ethanol extract of leaf of *E. singampattiana* revealed the presence of alkaloid, catechin, coumarin, tannin, phenol, saponin, steroid, flavonoid, glycoside and xanthoprotein. In the acute toxicity study, ethanol extract of *E. singampattiana* leaf did not show any toxicity effect upto the dose of 2000 mg/kg body weight.

Body and Reproductive organ weight: The ethanol extract of leaf of *E. singampattiana* at different concentration were treated on male Wistar albino rats for antifertility activity.

The administration of ethanol extract of leaf of *E. singampattiana* to rats slightly decreased the body weight (Table 1) and on the libido treated rats; whereas weight of testes and the accessory organs were decreased significantly (p<0.05) (**Table 1**). Among the accessory sex organs, a significant weight reduction was seen in the caput and caudal epididymal segment.

The weight reduction was dose-dependent i.e. high dose (300 mg/kg body weight) treated group (Group III) drastically reduced followed by less in low dose group (Group II) (150 mg/kg body weight). Slight changes were observed in vas deferens, seminal vesicle and prostrate.

TABLE 1: EFFECT OF ETHANOL EXTRACT OF *EUGENIA SINGAMPATTIANA* LEAVES ON THE REPRODUCTIVE ORGAN WEIGHT OF ADULT MALE ALBINO RATS

Treatment Groups	Body wt (gm)		Testis (gm)	Epididymis (mg)		VD (mg)	SV (mg)	Prostrate (mg)
	Before	After		Caput	Cauda			
Group-I	225.15±10.8	205.41±11.6	2.431±0.75	206.32±4.2	294.22±11.5	118.52±9.4	296.24±11.3	184.34±6.3
Group-II	229.54±12.6	221.32±11.6	1.452±0.76*	153.56±3.4*	208.52±4.4*	98.53±2.3	279.51±13.1	179.34±7.6
Group -III	219.61±16.2	198.54±10.6	1.398±0.84*	142.24±8.52	201.24±5.61*	83.54±1.98	284.61±12.3	169.54±5.9

Each Value is SEM of 5 animals * p < 0.05 Control vs Treated

Sperm count and sperm motility: Sperm motility and sperm density in caudal epididymis, significantly decreased **(Table 2)** and the reduction was severe in higher dose treated group (Group III) followed by moderate and low dose groups (Group II) and the same trend was seen in the caput epididymal sperm density when compared to control (Group I).

Sperm abnormality: Sperm abnormality in caput and caudal region was drastically affected by ethanol extract of *Eugenia singampattiana* leaves ($p < 0.05$). Among the two dose treatment groups, high dose group have shown significant and drastic abnormality in the sperm morphology, further tail region of the sperm in all the treated groups much affected than the head region (**table 2**).

TABLE 2: EFFECT OF ETHANOL EXTRACT OF *EUGENIA SINGAMPATTIANA* LEAVES ON THE SPERM CONCENTRATION AND MOTILITY IN THE EPIDIDYMIS OF ADULT MALE ALBINO RATS

Treatment Groups	Sperm Concentration (Counts x 10^6 mil)		Sperm Motility (FMI) @ (cauda)	Sperm Abnormality #	
	Caput	Cauda		Head (%)	Tail (%)
Group-I	404.11±28.5	466.32±21.5	154.5±19.2	4.19±0.31	9.51±1.2
Group-II	389.4±14.3	314.59±12.3	129.54±6.2	81.5±9.4**	91.31±1.24**
Group -III	349.54±11.5*	298.54±11.5*	104.5±9.3*	85.4±10.2**	93.54±6.4**

Each Value is SEM of 5 animals * $p < 0.05$ ** $p<0.01$ Control vs Treated

Serum biochemical profile: Serum protein, albumin, globulin, glucose, urea and creatinine and the activity of liver marker enzymes (SGPT, SGPT and ALP) levels of control and treated rats were depicted in **Table 3**. Increase in urea, creatinine and liver marker enzymes were noted in the entire drug treated groups when compared to control group.

TABLE 3: EFFECTS OF ETHANOL EXTRACT OF *EUGENIA SINGAMPATTIANA* LEAVES ON FEW SERUM BIOCHEMICAL PROFILES OF ADULT MALE ALBINO RATS.

Parameters	Treatment Groups		
	Group I	Group II	Group III
Protein (gm/dl)	8.51± 0.7	7.61±0.71	7.02±0.62
Albumin (gm/dl)	4.50 ± 0.65	4.50±0.83	4.10±0.55
Globulin(gm/dl)	4.01±0.05	3.11±0.12	2.92±0.07
Urea(mg/dl)	13.54 ± 2.4	36.36±2.6*	30.51±1.4
Creatinine(mg/dl)	0.72±0.4	1.62±0.54	1.81±0.63
SGOT (U/L)	11.4±0.92	13.56±1.06	15.34±0.81*
SGPT(U/L)	16.5±0.84	18.49±0.64	15.41±0.91
ALP(U/L)	134.51±4.51	118.53±0.91	126.21±0.66

Each Value is SEM of 5 animals * $p < 0.05$, Control vs Treated

Reproductive hormone file: *Eugenia singampattiana* leaves (150 and 300 mg/kg body weight) repeated treatment daily.

Serum testosterone level: The ethanol extract for 21 days caused a significant decrease in the serum level of testosterone in male rats. The level of testosterone decrease was dose related **(Table 4).**

Serum luteinizing hormone (LH) level: Repeated treatment of male rats with *Eugenia singampattiana* leaves extracts for 21 days caused a dose related decrease in the serum level of LH (Table 4). The level of decrease was statistically significant ($p < 0.05$).

Serum estrogen level: The ethanol extracts of *Eugenia singampattiana* leaves (150 and 300 mg / kg body weight) caused an increase in the serum level of estrogen in male rats. Doses of 150 and 300 mg / kg body weight administered daily for 21 days caused a sharp rise in the serum level of estrogen (Table 4) whereas the highest dose of 300 mg / kg body weight induced gradual increase.

Serum follicle stimulating hormone (FSH) level: Pretreatment with ethanol extracts of *Eugenia singampattiana* leaves for 21days caused an increase in the serum level of FSH in male rats compared to control (Table 4). The increase in the serum level of FSH in male rats was statistically significant when treated with *E. singampattiana* ($p<0.01$).

TABLE 4: EFFECT OF ETHANOL EXTRACT OF *EUGENIA SINGAMPATTIANA* LEAVES ON SEX HORMONES LEVELS AND PITUITARY GONADOTROPHINS IN MALE ALBINO RATS

Treatment Groups	Parameters			
	Testosterone (ng/ml)	LH/ICSH (µIu/ml)	Estrogen (pg/ml)	FSH (µIu/ml)
Group I	2.23±0.92	1.54±0.34	19.24±0.34	0.99±0.05
Group II	1.78±0.54	1.05±0.24*	29.54±0.74	3.36±0.39*
Group - III	1.01±0.49**	0.52±0.33	31.33±0.94*	3.98±0.17*

Each Value is SEM of 5 animals * $p < 0.05$, ** $p<0.01$ Control vs Treated

DISCUSSION: Studies on the effects of plant products on male reproductive system and fertility are comparatively few and far fetched. From a public health perspective, the head for contraception has never been greater. The administration of ethanol extracts of *E. singampattiana leaf* to rats did not cause any significant change in the body weight and on the libido of treated rats, whereas, weights of testes and other accessory sex organs were decreased significantly during the experiment.

Among the accessory sex organs, a significant weight reduction was seen in the testes, caudal epididymal segments. Weight reduction was more significant in ethanol leaf extract of *E. singampattiana* treated rats (Group II and Group III) when compared Group I (Normal) animals. Reduction in the weight of testis and other accessory sex organs might be due to low level of androgen, which was not enough to maintain the weight of gonads and accessories [22]. It is known that the accessory sex organs viz., epididymis and vas deferens are androgen dependent target organs and manifest differential sensibility to androgens for maintenance of their structure and function. It is also known that, any change in circulating androgens would affect the internal microenvironment of epididymis and thereby lead to alteration in sperm motility and metabolism [23].

In the present study, *E. singampattiana* ethanol extract treated rats decreased the sperm motility and sperm density in caudal and caput epididymal segments (Table 2). Drastic effect on the nature of the normal sperms in the caput and caudal region was observed in *E. singampattiana* treated rats. Further tail region of the sperm in all the treated groups (Group II and III) were much affected than the head regions (Table 2). The development of normal mature sperm is the key to optimum male fertility. The production of the sperm cells (spermatozoa) and testosterone in the testis are mainly regulated by the follicle stimulating hormone (FSH) and Luteinizing hormone (LH), which are released from the anterior pituitary [24]. FSH stimulates spermatogenesis in the sertoli cells, while LH stimulates the production of testosterone in leydig cells of the testis [25]. Many studies on the testis of rat treated with plant extracts has also demonstrated that the inhibitory activity on the proliferation of spermatogonia in mammals [26, 27, 28]. Spermatogenesis is therefore, a complicated process, covering proliferation of the spermatogonia, long-lasting process of the tissue meiosis and numerous changes in the spermatids during their pre-formation [29, 30]. The result of the present study suggests that ethanol extract of *E. singampattiana* may affect the normal function of the sertoli and leydig cells on continuous oral administration for fourteen days.

Among the ethanol extract treated rats, Group III produced a significant reduction in total sperm count and viable sperms. This may be as a result of the ability of the extract at the given doses, to either interfere with spermatogenetic process in the seminiferous tubules, epididymal functions or activities of testosterone on hypothalamic release factor and anterior pituitary secretion of gonadotropins which may result in alteration of spermatogenesis [31, 32].

The presence of immature sperms was also observed in the experimental rats treated with 300mg/kg body weight of ethanol leaf extract could affect the maturation of the spermatozoan in the male rats, which might also be a contributory factor to the decrease in the mean total sperm count. The data generated in the present study, by and large, confirm to those already reported and studied with various plant extract [33, 34, 35].

The decrease in the caudal epididymal sperm counts in Group II and III when compared to Group I animals (Table 2) are clear indications that, *E. singampattiana* extract can affect one or more aspects of spermatogenesis as well as spermigenesis. Though a direct effect of *E. singampattiana* extract on the cellular mechanisms of spermatogenesis cannot be concluded, it is likely that the impairment of the hormonal mechanisms concerned with the regulation of spermatogenesis may be underlying cause.

The various other sperm abnormalities like sluggish motility, coiled tail and sperm immaturation are also due to *E. singampattiana* toxicity. The hitherto unreported abnormal sperm morphology, coiled tail and malformed head could be attributed to both testicular and epididymal effects of *E. singampattiana* extract. Coiling of the sperm tail is usually the product of abnormal axoneme and or outer dense fibril. The outcome of the present study affirms the male reproductive toxic effects of *E. singampattiana* when applied as therapeutic agent.

Since male reproductive toxicology and male contraception are two sides of the same coin, the negative consequence of *E. singampattiana* on the sperm may be taken as the advantage for futher study. By the treatment employed in the study, no toxic effect was produced in the liver and kidney, neither was it directly involved on the development of functioning of the male reproductive system nor in the reproductive organs.

The present study revealed a decrease in the serum level of testosterone. This observation was similar to the earlier findings of [36, 37, 38]. The reduction in the serum level of testosterone could probably be due to the decrease of serum levels of LH/ICSH observed in this investigation. Leydig cells secrete testosterone by the stimulatory effect of LH [38, 39, 40].

In males, reduction of testosterone level may impair spermatogenesis and cause male infertility. This study further observed a significant increase in the serum estrogen level of ethanol leaf extract of *E. singampattiana* treated rats. This increase might probably be due to the conversion of testosterone to estrogen [41, 42].

Treatment with the ethanol extracts of *E. singampattiana* leaf and bark (300mg/kg b.wt) was highly effective in producing reversible functional sterility. The drug treated male rats clearly indicated structural and functional alteration in testis, epididymis and seminal vesicle. Depletion of sperm count and sperm motility in the drug treated rats suggests alteration in sperm production in the testes and maturation in the epididymis. Changes in both sperm count and motility resulted in partial infertility within twenty one days. This resulted in abnormal sperm functions which ultimately gave rise to complete male sterility.

Among the plant based contraceptives, inhibition of male fertility after administration of natural substances has been related to decreased spermatozoa density [43]. For male contraception, it is not necessary to stop spermatogenesis, but it is enough to eliminate the fertilizing ability of the spermatozoa by causing changes in the morphology or in the function of sperm [44].

Saponins are important mainly because of their steroid structure. They are precursors for the hemisynthesis of birth control pills (with progesterone and estrogens) as well as similar hormones and corticosteroids [45]. Recently many laboratories are engaged in developing male contraceptives from plants [46]. Plant products as contraceptives will be more acceptable for economic reasons in terms of self reliance and the possible practicability for a male pill approach in countries where population pressure is high. Recently extensive efforts have been made to study the antifertility drugs from plants [47, 48, 49].

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