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Review Article

Evaluation of spermatotoxicity of *leonotis nepetifolia* in male albino rats

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ABSTRACT

The objective of the present study is to evaluate the potential spermatotoxic effect of ethanolic extract of *Leonotis nepetifolia* (EELN) in male albino rats. Ethanolic extract of *Leonotis nepetifolia* was given by gavage to rats in the *in vivo* test at a dose of 100,150 and 200mg/kg of bodyweight, along with normal controls and ther e by studying changes in sperm morphology consisting counts, motility and abnormalities of cauda epididymal sperm adapting light microscopy. Findings of this study revealed, the sperm concentration in the epididymis and sperm motility are decreased, whereas sperm abnormalities increased like sloughing of sperm neck, detached head, and coiling of end tail. In extract treated rats, the duration of sperm motility reduced with respect to the increased dose level. A triplet maner of reduction in the sperm counts and the viability, respectively were observed in EELN-200mg/kg body weight group. Sperm abnormality was increased by EELN in a dose-dependent manner was assessed by acridine Orange fluorescent staining and there was a highest elevation by the multiples of twenty with control in 200mg/kg group .This result indicates disruption of spermatogenic as well as androgenic compartment. The present study can be concluded the *Leonotis nepetifolia* extract suppress male fertility without altering the general metabolism.

Keywords: Acridine Orange fluorescent; Epididymis; *Leonotis nepetifolia*, Male contraception.

INTRODUCTION

The rapid increase of population has got an adverse effect on the international economy and as the increase is only limited to the developing countries, the problem becomes an acute on the fruits of improvement in the different sectors, which are being eroded by the growing population. Moreover, increasing number of births has got a deleterious effect of social and economic progress. Population explosion is alarming; it results in the exploitation of natural resources and affects the economic growth of a country. The control of population is the practice of curtailing the population raise, generally by reducing birth rate (Karthik YP et al 2012).

Mostly plant compounds are emerging into a therapeutic agent for various medicinal purposes. The developing countries particularly in villages still depend upon traditional plant based medicines due to its easy availability and low cost (Ogbuewu IP et al 2011). Many studies have been done on the male contraception. Some of the plants had spermicidal effects; others caused reduction in sperm counts and alter the mobility of the sperms. Some of the plants caused testicular change and altered hormone levels (Bhargava SK 1984).

Leonotis nepetifolia is an erect, branched herb belongs to family lamiaceae. It is commonly referred to as Lion's ear and Christmas candlestick (Imran S et al 2012). The plant is used in traditional medicine in therapy of bronchial asthma, diarrhea, fever, malaria and as an analgesic agent in menstrual Pains; also to treat common cold and to alleviate cough (Dhawan NG et al 2013). Preliminary phytochemical analysis revealed the presence of steroid, alkaloids, flavonoids, reducing sugar and tannins (Sarath Chandiran I et al 2015).

MATERIALS AND METHODS

Plant material

The whole plant of *Leonotis nepetifolia* was collected from Kalakadu, Thirunelveli district. Plant material was dried separately under shade and then powdered with a mechanical grinder to obtain a coarse powder, which were then subjected to extraction in a Soxhlet apparatus using ethanol. 50g of powered plant was extracted with 500mL of ethanol at 60^0C.Then extract was poured in petri plates and allowed to air dried (Bindu G and Sharddha NS 2011).

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Animals

Adult male Wistar rats were used for the study. Animals were housed under 12 h light/12 h dark cycle with controlled conditions and were fed by standard food and allowed water *ad libitum.* Food and water are given by animals are noted. Body weights of animals were also recorded on day 0 of the experiments and at end of the experiments.

Experimental design and treatment

Experimental animals

Male Wister albino rats were divided into 4 groups and their body weight ranging from 160g to 250g. Each groups containing 5 animals. Three groups are considered as the Treatment groups and one group is considered as the control group. Animals were administrated orally to the rat for 55 days by Plant extract of *L. nepetifolia* using oral gavage tube. The animals are divided into 4 groups based on their body weight and given by different doses, Group 1- Control, Group 2 – 100mg/kg (b/w), Group 3 – 150mg/kg (b/w), Group 4 – 200mg/kg (b/w). At the end of the treatment, animals were sacrificed by cervical dislocation and cauda epididymis, vas defenses were immediately dissect out. Sperm from cauda epididymis were released in phosphate buffer saline, followed by Sperm analysis was performed.

Parameters in Semen Analysis

Dilution of Semen

The cauda epididymal duct on one side was exposed and incised. The Connective tissue capsule around the epididymis was teased out and the duct was coiled. The fluid was oozed out into the cavity block is quickly sucked into a capillary tube up to 0.05µl mark and transferred to a 2mL vial. It is diluted 5mL in phosphate buffer saline. After thorough mixing by blowing air throw blowpipe the sperm suspension is used for analysis, the *L. nepetifolia* treated was observed thorough sperm motility, sperm morphology and sperm count.

Epididymal Sperm Counts

Sperm counts were made according to Gopalakrishnan (Gopalakrishnan K et al 1994). The semen diluted was thoroughly mixed and drop of the dilute semen was transferred to an improved Neubauer counting chamber and a cover glass was overlaid. The counting chamber was observed under a research microscope at x400 magnification and sperm heads in the central square were counted. The central square has 25 large squares. The volume of each of the 25 squares is 0.1ml. The sperm counts were calculated using the following formula:

$$\frac{Number\ of\ sperm\ in\ 25\ squares}{10} \times 10 \times diluted\ factor \times 1000$$

Which gives the number of sperm in one ml.

Data on each group were used to calculate the mean and the standard deviation (Mean± SD)

Sperm Motility

A drop of dilute semen was transferred on to a cover glass. The cover glass was inverted over a cavity slide to obtain a hanging drop. The preparation was observed at regular intervals in such a way as to find the rate in µ/sec and duration, in mins, of the progressive motility of the sperm. The rate of motility was measured using a calibrated ocular grid. For each animal two separate hanging drop preparations were made and two independent observers assessed the mobility. The data from each animal were used to obtain the respective averages (Gopalakrishnan K et al 1994).

Acridine Orange and Ethidium Bromide (AO/EtBr) Staining of Sperm

In order to find the viability of sperm, fresh sperm were stained with acridine orange (AO) and ethidium bromide (EtBr). A fine suspension was made and stained with 25µl of AO-EtBr. One drop of stained suspension was placed on the clean slide and allowed to dry. Then cover slip is placed on the slide. The preparations were observed in the light microscope with the attachment of fluorescent. The images were captured in the camera. In all cases of counts of sperm with morphological abnormalities, randomly selected the sperm from each slide and were observed. This will assigned to the categories *viz.,* normal, head alone and tail break defect.

Statistical Analysis

All data for control and experimental groups were subjected to statistical evaluation, using analysis of variance (ANOVA) for significant differences between the controls and experimental groups at $p<0.05$. All data were recorded systematically in preformed data collection sheet. Statistical analysis was performed by using SPSS for windows version 16.0. 95% confidence limit was taken.

RESULTS

Sperm Parameters

Sperm count

The total sperm counts were significantly decreased in Group II, Group III and Group IV animals compared to that of control animals. In Group IV animals, the total sperm counts were highly decreased when compared to that of control rats. (Table 1)

Sperm motility

The motility of sperm was highly inhibited in Group II, Group III and Group IV animals (Table 1).

Sperm morphology

In the control rats of cauda epididymidal sperm retained the cytoplasmic droplet (CD) when subjected to

Table 1: Effect of EELN on Sperm count, Motility and Morphology

Groups	Treatment	Total sperm count		Sperm motility	Abnormal sperm morphology
		Head	Tail		
Group 1	Saline	97.34+3.78	93.24+3.09	98.76±6.32	3.98+0.23
Group 2	*L. nepetifolia* (100mg/kg)	74.61±10.31	71.57±9.76	74.43±9.78	32.65±7.65
Group 3	*L. nepetifolia* (150mg/kg)	51.23±12.79	55.12±11.47	56.51±14.78	39.07±6.54
Group 4	*L. nepetifolia* (200mg/kg)	35.17±7.15	33.78±7.09	34.23±7.64	55.34±7.02

Values represent the Mean ± SD of the observation made on five rats in each group. Statistical analysis one way analysis of variance (ANOVA) with hoc testing least significant difference.

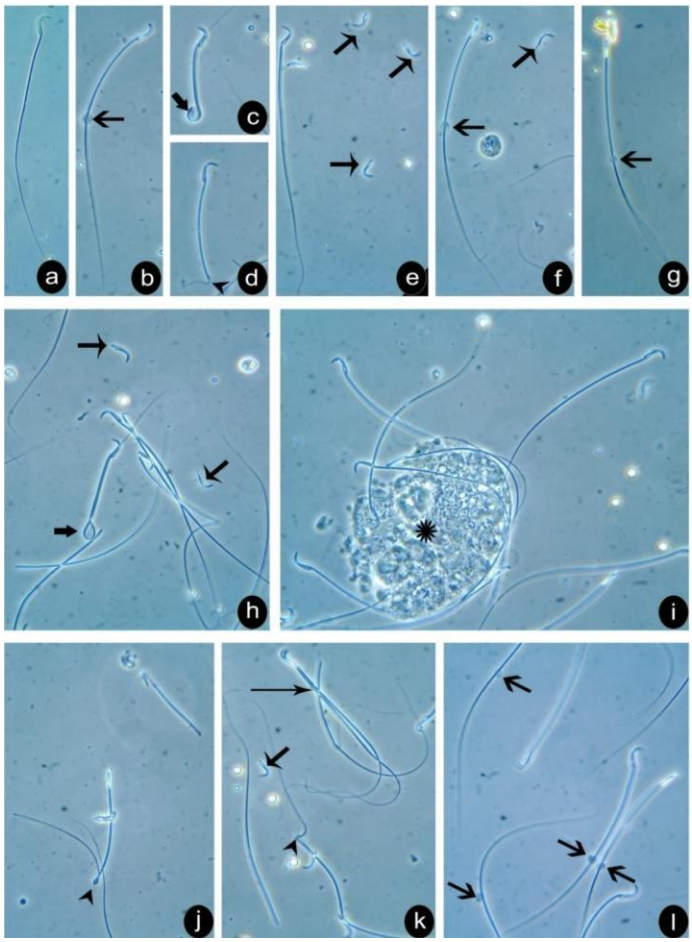

Figure 1: Phase contrast photomicrograph of Control and and L. nepetifolia ethanolic extrat treated rats.

a. Control; **b-l.** *L. nepetifolia* treated rats sperm showing various abnonormalities. Cytoplasmic droplet (**Pointed arrows**); Head alone (**arrow**); coiling of tail (**solid arrow and arrow heads**); immature germ cells (**asterisk**); fussion of sperms (**long arrow**).

slight hypo-osmotic stress. In *L. nepetifolia* extract treated rats the percentage of the sperm retaining the CD increased, and treated rats almost 50% all the sperm retained the CD (Fig 1). More than 50% of the sperm had abnormal morphologies of various kinds, which included broken head, DNA damage sperm, coil in tail region of two or more sperm etc., were observed in phase contrast microscope (Fig 1). The plant extract exerted a significant decrease epididymal sperm concentration and sperm progress motility. The live/dead sperm count was increased in Group II, Group III and Group IV animals when compared to Control animals.

Smear of Testis

Acridine orange-stained smears of testis of the both control and *L. nepetifolia* extract treated rats revealed spermatogenic cells, including sperm, to fluoresce green. There was hardly a cell than fluorescent red and highly increased bi- and multi- nucleated giant cells in the treated rats. A few round spermatids were double

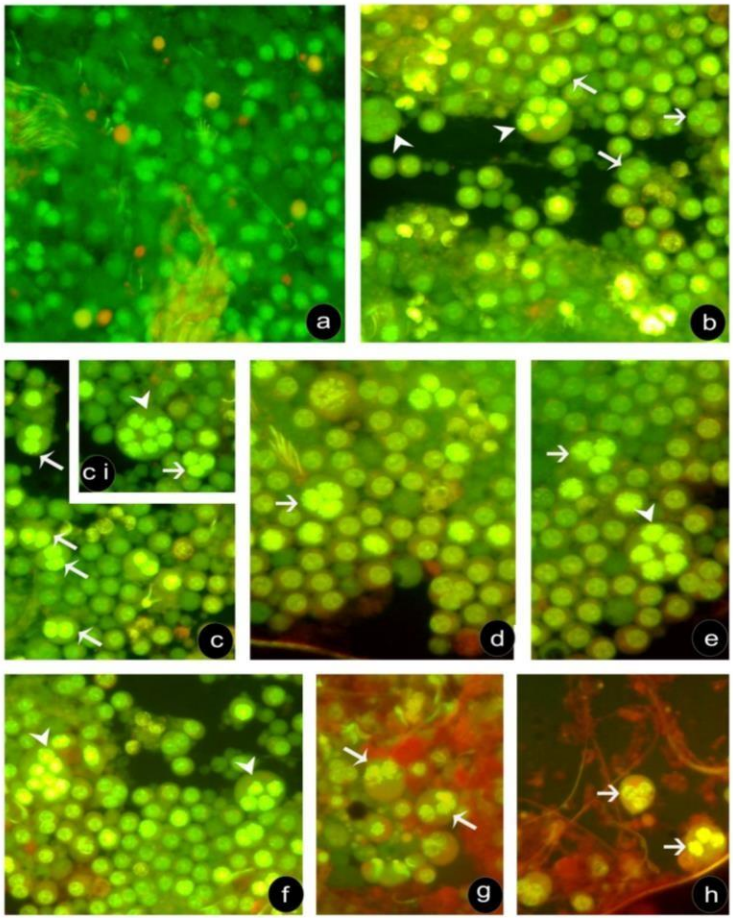

Figure 2: Agredin orange / Ethidium bromide (AO/EtBr) staine semier phomicrograph of Control and L. nepetifolia ethanolic extract treated rats testis showing various abnormalities.

a. Conrol; **b-h** treated rat`s testis smears. Bi-nucleate cells (**Pointed arrows**); multi nucleated gaint cells (**arrow heads and arrows**). Green flourecent inticats the live cells and red flourecent indicats the dead cells.

the size of normal round spermatids and contained two nuclei, either widely separated or closely held together (Fig.2). These observations indicated that the treatment of *L. nepetifolia* extract treated rats did not produce any perceptible damage to the DNA and viability of the cells.

DISCUSSION

The herbal medicines are being used up to 80% of the population in developing countries. Based on this widespread use, few scientific studies have been undertaken to ascertain the safety and efficacy of traditional remedies. The present study was undertaken to evaluate the male reproductive toxicity of the ethanolic extract of *Leonotis nepetifolia,* which is an herbal medicine.

L. nepetifolia did bring about adverse changes in the parameters of male reproduction, namely sperm counts, sperm motility, sperm morphology and sperm smear. While these changes are desirable endpoints towards testing for contraceptive development (Cooper TG and Yeung CH 2003), they also produced gross changes in the testis, affecting the cellular compartments concerned with spermatogenesis as well as androgen synthesis.

In *L. nepetifolia* treated rats, invariably, all the cauda epididymidal spermatozoa retained the cytoplasmic droplet CD; in other words, the spermatozoa failed to shed the CD even after arrival at the cauda epididymidis. During spermatogenesis, spermatozoa lose much of their cytoplasm as the residual body that is phagocytosed by Sertoli cells (de Kretser DM and Kerr JB 1994), (Bardin CW et al 1994). However, the spermatozoon leaves the testis with a small droplet of cytoplasm, the cytoplasmic droplet (CD). The organization of the CD and its displacement from the neck to the principal piece during transit of the spermatozoon towards the distal part of the epididymis have been reported (Akbarsha MA et al 2000), (Agnes VF and Akbarsha MA 2003). CD is the part of the sperm containing the largest portion of cytoplasm of the sperm during the epididymal transit (Cooper TG and Yeung CH 2003). Thus, *L. nepetifolia* treatment specifically targets the aspects of contribution of epididymis to sperm maturation, such that the flagellum is coiled in which alteration in the CD is the primary cause, and the swelling and coiling are the consequences.

Multinucleate giant cells arise due either to failure of cytokinesis (Russell LD et al 1987), (Zheng Y et al 2001) or opening up of the cytoplasmic bridges connecting the male germ cell clones (Ren HP and Russell LD 1991). The cytoplasmic bridges are narrow passageways of cytoplasmic continuity between the cells. These bridges are maintained by actin microfilaments (Weber JE et al 1985), (Russell LD et al 1987), (Zheng Y et al 2001). Disruptors of actin microfilaments of the cytoplasmic bridges, like cytochalasin D, carbendazim, etc., bring about depolymerization of actin microfilaments such as to affect the integrity of the bridges, resulting in their collapse and generation of symplasts, which are multinucleate giant cells (Hess RA 1990), (Akbarsha MA et al 2000), (Kadalmani B et al 2002). The uni- and multinucleate giant cells are abnormal products of spermatogenesis since the cells do not differentiate further. Such cells die through apoptosis, get discharged from the sertoli cells and their fragments arrive at the lumen of the seminiferous tubules and reach the ductus epididymidis through the efferent ductules (Nakai M et al 1995).This is precisely what was observed in the *L. nepetifolia* treated rats.

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