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Reproductive Toxicity in Male Wistar Rats Caused by Kaempferol Derivative Isolated from *Lysimachia ramosa*

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Abstract: **Plants have major role not only in maintaining the continuity of the environment but also in close association with human beings in day-to-day life. People use many plants as their food as well as to cure many of their health issues. But some plants are also there in nature which proves their effectiveness in dose dependent manner. In today's world, both toxic chemicals in the environment and phyto-products are affecting man's reproductive machinery by producing unhealthy sperms. Taking phyto-products into consideration, some are very effective in ameliorating the reproductive health of males and some act as toxic to the reproductive system. *Lysimachia ramosa* is a plant which is consumed as vegetable (in boiled condition) against worm infection. Kaempferol derivative is isolated from leaves of plant which is the active anthelmintic component in the plant. Before considering this component as potential candidate for health care, its adverse effects, particularly of antifertility effect, if any, should be investigated. Therefore this study has been undertaken to check effects of kaempferol derivative on its male consumer's reproductive system, taking wistar rats as animal model. The experiment was performed following OECD 407 guideline. 250 mg, 500 mg and 1000 mg kaempferol derivative per kg b.w. of animals were selected for the experimental animals and dosed continuously for 28 days. On 29th day, animals were sacrificed, testes along with cauda-epididymis were isolated and tested for gonado-somatic indices, sperm count, motility, viability and morphology abnormality of sperms. Seminiferous tubules where sperms are generated were also studied with the help of haematoxylin-eosin staining technique and scanning electron microscopy. Results show that at 250 mg per kg b.w. of the component, it exerts beneficiary effect to the reproductive system, however, at highest dose i.e. 1000 mg per kg b.w. the component affects the reproductive health in a negative way. Therefore the active component is considered to be safe for future study below 500 mg/kg b.w.**

Index Terms: **kaempferol derivative, *Lysimachia ramosa*, sperm, testes, wistar rats**

I. NTRODUCTION

Infertility is one of the major health issues of reproductively active couples with expanding frequency rates in males (Radford et al., 1999; Oyeyipo et al., 2014). It is reported that sperm quality in males has started degrading since 20th century (Jorgensen et al., 2012). The cause cannot be predicted accurately but might be due to the presence of various toxic chemicals in the surrounding environment including natural and industrial products (Dindayal, 2004). Natural phyto-products are of main concern here as they are the most useful substances in under-developed countries where they don't have required exposure to synthetic drugs to cure their illnesses (Satrija et al., 2001; Mali and Mehta, 2008). These natural herbal products are not only have good effects on public health but also some of them are poisonous to various vital organs including reproductive system of the body (Gaillard and Pepsin, 1999). In these circumstances, it becomes critical to evaluate the impact of plants used by the poor as remedies for other illnesses on sperm production. In this case, *Lysimachia ramosa* (common name: *Ka iarai*), of the family Primulaceae, has been selected for our study as the fluid extract of its leaves is being broadly utilized by Jaintia tribes to cure their intestinal helminth diseases (Challam et al., 2009). Dey and Roy (2020) observed that n-butanol fraction of the crude leaf extract was the most effective anthelmintic fraction of *L. ramosa* as it alter the glycogen content and many other tegumental enzymes of the helminths. The plant has been found to have different natural products such as alkaloid, tannin, saponins, phlobatannins. Recently, Dey et al. (2021) have isolated kaempferol derivative from the active n-butanol fraction of the crude plant extract and revealed its anthelmintic property. Upon treating the helminthes (*Raillietina echinobothrida*) with higher dose of the kaempferol derivative (40µg/ml and 80 µg/ml), it was found that the parasites started to be immobile within 3 hours of treatment and death occurs at 12 hours. However, literatures show that numerous phytochemicals of this type can cause toxic effects on their consumers if consumed at high doses over a long period of time (Adeneye et al., 2006). Moreover, this plant and its active anthelmintic principle have no scripts that support their benevolent property, if any, for the sperm-producing ability of their male consumers. Therefore, this experiment has been

attempted to assess the impacts of the anthelmintic kaempferol derivative isolated from the plant *Lysimachia ramosa* on typical sperm morphology and different characteristics of testicles.

II. MATERIALS AND METHODOLOGY

A. Collection of plant material and isolation of kaempferol derivative

Leaves of the plant *Lysimachia ramosa* were collected from distinctive parts of East Khasi hills of Meghalaya and air dried. Leaves were at that point crushed into fine powder and prepared for methanolic crude extract. N-butanol fraction was obtained from the methanolic extract using fragmentary refining strategy. Later, from the n-butanol fraction, the kaempferol derivative was obtained through column chromatography as described earlier (Dey and Roy, 2018; Dey et al., 2021).

B. Experimental animals

The experiment was carried out on male wistar rats following animal ethical committee's rule. Approval to conduct experiments on animals was obtained from Institutional Ethical Committee, North-Eastern Hill University. The animals were put up in cages within the consistent temperature of 22-25 °C. Legitimate nourishment and water as per necessity was given to the animals. All the rats were acclimated to the environment 14 days prior to the starting of the experiments.

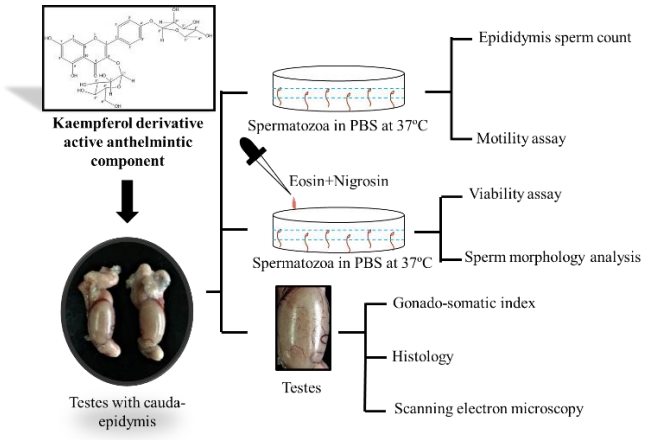

Fig. 1: Diagrammatic work-flow representation of obtaining sperm and testicular tissue to investigate effects of kaempferol derivative isolated from *L. ramosa* on reproductive system of male wistar rats.

C. Gonado-somatic index, epididymis sperm count and motility analysis

All the animals were clustered into four groups. They were control group, groups treated with 250 mg, 500 mg and 1000 mg kaempferol derivative/kg bw of rats following OECD guideline 407. After giving treatment persistently for 28 days, on 29th day the animals were anaesthesised and testes with cauda-epididymis were obtained. Once evacuated, the epididymis are smeared on sterile filter paper to expel blood and is afterward incised carefully. Spermatozoa are pressed out, recuperated and submerged into PBS at 37°C to permit sperm cells to diffuse (Naghdi et al., 2016). The sperm concentration of the cauda epididymis was determined using the standard hemocytometer and the motility of the sperm was analyzed under the microscope (Leica DM 1000) and reported as mean motile sperm according to the WHO method. (WHO, 1999). Gonado-somatic index were measured by following formula (Ali, 2001):

Gonado-somatic index of an organ= (weight of the testes/weight of the body)x100%

D. Viability and sperm morphology analysis

After sperm collection, the samples were stained with eosin-nigrosin (WHO, 2010). Eosin penetrates into the dead sperms and thus stains the non-viable sperms, whereas nigrosin brings contrast. Smears were prepared on glass slides. A total of 500 sperm from each rat were examined and individually classified as normal or abnormal. Morphological abnormalities of sperms were divided into head and tail defects. Sperm mid-piece anomalies were included as part of the sperm tail assessment. Normal and abnormal sperm rates were calculated. (Adamkovicova et al., 2016).

E. Histological analysis of testes

For histological study, testes were poised and perpetuated in 10% formalin solution. Next through microtome, tissue sections were received on clean slides which are then rolled through different alcoholic grades of different strength, stained with haematoxylin-eosin and finally studied under compound microscope (Bancroft and Stevens, 1996).

F. Scanning electron microscopic study of testes

For investigation on alterations of the surface structures of testes, samples were fixed in 2% Gluteraldehyde followed by air-drying in TMS (Reville and Cotter, 1991). After coating with gold, tissues were observed in JOEL JSM 6360 at 25 kV.

G. Statistical analysis

The results were communicated as mean±standard error of mean. Statistical analyses were performed utilizing students's t-test. Data were considered statistically significant at $p \leq 0.05$ when values of treatment groups were compared to the values of control group.

III. RESULTS

A. Gonado-somatic index, epididymis sperm count and motility analysis

Gonado-somatic index was seen to be decreased significantly when male rats were treated with 1000 mg of kaempferol derivative per kg b.w. of rats. Similarly sperm count was also observed to be decreased at the highest dose treatment groups. Motility percentage and rate of viable

sperms were also noticed to be significantly decreased on treating with the highest dose but after treatment with lower dosages, rates of motile sperms and viable sperms were counted with significant rise.

Table I: Effect of kaempferol derivative isolated from *L. ramosa* on gonado-somatic indices (GSI), sperm count, motility and viability of male wistar rats. Values are communicated as mean±SEM. *P-value is significant at p≤0.05 when values of treatment groups were compared to the values of control group.

	GSI (%)	Sperm Count (mill sperm/ml)	Motility (%)	Viability (%)
Control	1.33±0.09	48.50±0.87	49.81±0.41	69.49±0.36
250 mg/kg	1.38±0.07	49.97±0.41	51.16±0.28*	70.58±0.12*
500 mg/kg	1.22±0.05	47.77±0.43	48.39±0.41	68.80±0.04
1000 mg/kg	1.08±0.06*	46.40±0.32*	47.89±0.06*	68.32±0.10*

B. Sperm morphology analysis

Fig. 2(A) showed a stained dead sperm and unstained living sperm on the basis of their viability. From morphological point of view, a normal sperm has a perfect hook shaped head and a long tail with middle piece in between them. Different types of abnormalities were observed in all the groups such as two tailed sperm, thin narrow head, acephalic sperm, paired sperms, pin-head sperm, bent neck sperm, coil tailed sperm and detached head. All kinds of abnormalities had been categorized into broadly two types-abnormal head and abnormal tail (Table II). In the rats exposed to higher doses of the phytochemical, a significant decrease in normal morphology was observed along with a significant increase in abnormal sperm count.

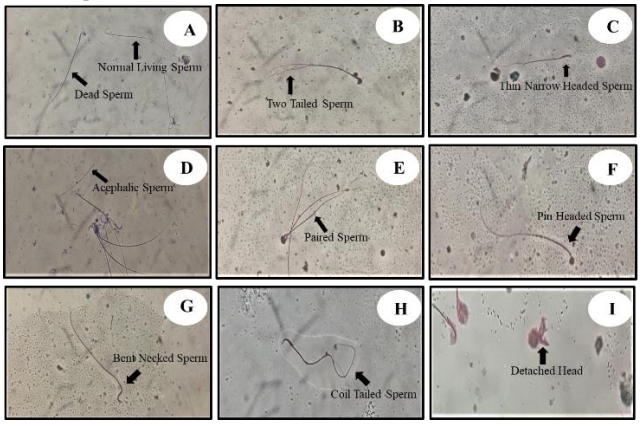

Fig. 2: Microphotographs illustrating morphologically normal sperm and various deformed sperms stained with eosin-nigrosin. A. Normal living sperm and dead sperm; B. Two tailed sperm; C. Thin narrow headed sperm; D. Acephalic sperm; E. Paired sperm; F. Pin headed sperm; G. Bent necked sperm; H. Coil tailed sperm; I. Detached head (Magnification: 40X objective).

Table II: Effect of kaempferol derivative isolated from *L. ramosa* on sperm morphology of wistar rats. Values are communicated as mean±SEM. *P-value is significant at p≤0.05 when values of treatment groups were compared to the values of control group.

	Normal morphology (%)	Abnormal head (%)	Abnormal tail (%)
Control	97.47±0.29	1.87±0.41	0.66±0.13
250 mg/kg	97.66±0.41	1.47±0.41	0.87±0.07
500 mg/kg	96.83±0.15	2.07±0.12	1.07±0.33
1000 mg/kg	96.27±0.18*	2.80±0.15*	0.93±0.15

A. Histological analysis of testes

Histological observations on control testes demonstrated normal epithelium resting on a basement membrane and surrounded a lumen where spermatozoa were released. Spermatogonia were located in the basal compartment of membrane. These cells were appeared round shaped and had prominent nuclei. Sertoli cells with their characteristic oval shaped nuclei were also visible. Interstitial or leydig cells were found to be located in the connective tissue surrounding the seminiferous tubules. Myoid cells were also seen to be in its natural position covering the tubules (Fig. 3A). When rats were treated with 250 mg kaempferol derivative per kg b.w. of animals, no structural or cellular anomalies were observed (Fig. 3B). All the cell types were in normal condition. Upon exposure of rats to 500 mg of the component per kg b.w., both alterations and distortions were observed in germinal epithelium in the form of disturbed arrangement of major cells in few seminiferous tubules of the rat population (Fig. 3C). Noticeable changes appeared in the rat group which got exposed to 1000 mg of the isolated component per kg b.w. of animals. The seminiferous tubule was irregular in shape. The normal organization of the germinal epithelium was reduced, and the cells of the germinal epithelium were abnormally arranged, and partial depletion of spermatogonic cells was observed in comparison with the control group (Fig. 3D). In some affected tubules, the spermatogonic cells were only the major cell type seen.

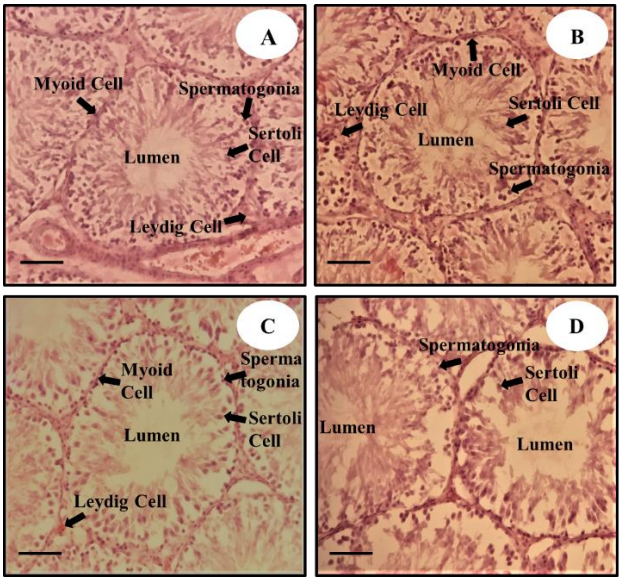

Fig. 3: Photographs of testes (A-D), stained with hematoxylin-eosin, showing seminiferous tubules which has a lumen of the tubule, epithelium composed of spermatogonia (primitive male germ cells), sertoli cells (nourish the spermatocytes), basement with myoid cells (responsible for contraction of seminiferous tubules). All the seminiferous tubules are connected with connective tissue rich in Leydig cells (produce male hormones). A: A normal seminiferous tubule. B: Seminiferous tubule of rat's testes treated with 250 mg kaempferol derivative per kg b.w. C: Rat's seminiferous tubule exposed to 500 mg kaempferol derivative. D: Seminiferous tubule after treatment with 1000 mg of the isolated component from *L. ramosa* (Scale bar: 200 µm).

A. Scanning electron microscopic study of testes

Scanning electron micrograph of control rat showed that the outer surface of the seminiferous tubule was covered with single layer of flat myoid cells, the nuclei of which appeared as small bulges. The myoid cells were arranged in a continuous monolayer (Fig 4A). Germ cells layers were visible. The tubular lumen is filled with flagella of spermatozoa. When treatments of rats were performed with 250 mg of kaempferol derivative per kg b.w., all cellular architectures were observed to be normal as compared to control (Fig 4B). Scanning electron micrograph of rat's seminiferous tubule on exposure to 500 mg component per kg b.w. showed narrow tubule having irregular outlines with exfoliated germ cells in the lumen (Fig 4C). Rats treated with 1000 mg of the phytochemical per kg b.w., showed many cavities and depletion of germinal epithelial layers with decreased spermatozoa in the lumen of seminiferous tubule along with detachment of the epithelium from the basal myoid cell layer (Fig 4D).

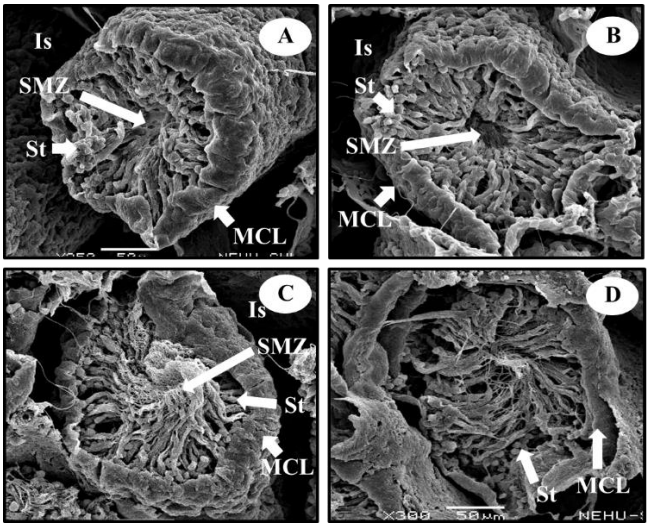

Fig. 4: Scanning electron micrographs (A-D) of rat's testes showing myoid cell layer (MCL), spermatid (St), spermatozoa (SMZ) and interstitial space (Is). A: seminiferous tubule of a normal rat testes, B: seminiferous tubule of rat treated with 250 mg kaempferol derivative per kg b.w. of rats. C: 500 mg per kg b.w. treated rat showing seminiferous tubule D: seminiferous tubule after treatment with 1000 mg of kaempferol derivative per kg b.w. of rat (Scale bar: 50µm)

IV. DISCUSSION

Herbal product is a type of alternative medicine made from plants and plant extracts. Herbal remedies have been in use for centuries and are the precursors of modern medicine to treat various health issues involving reproductive system related problems. In the present investigation, it has been noticed that the active anthelmintic component, kaempferol derivative, isolated from the plant *L. ramosa* has some effects on the male reproductive system of wistar rats in a dose dependent manner. While evaluating effect of any natural or synthetic drug on the male reproductive system, some criteria are chosen to analyze the results such as changes in gonad weight, sperm count, sperm motility etc. (Queiroz-Neto et al., 1997; Ban et al., 1995). In our study also, gonado-somatic index was noticed to be decreased significantly when treatment was done with 1000 mg of the isolated kaempferol derivative per kg b.w. At this treatment group of animals, the sperm count, motility as well as viability have also been found to be decreased significantly. Decrease in sperm motility and viability may be due to high concentration of isolated phytoproduct which is acting as spermatoxic agent on maturing or matured spermatozoa (Pacifici et al., 1995). Similar kind of results were obtained in the study of Auta and Hassan (2016) when they treated albino mice with the higher doses of aqueous wood-ash extract of *Azadirachta indica* (neem). In this investigation, it has also come to light that, although the higher doses caused undesirable effects on testes weight, concentration, motility and viabilty of sperms in the rats, lower doses has healthier results on these aspects of sperm study. On treating animals with 250 mg of the phyto-product per kg b.w. gonado-somatic index and sperm count increment have been noticed but significant increase in the percentage has taken place in motility and viability of sperms in animals of the lowest dose treated group. Like our observations, when rats were treated with lower concentrations of citrus fruits peel extracts similar kind of increments in the sperm parameters were observed in a separate study

done by Khaki et al. (2011). Sperm morphology study is another important criteria for evaluation of reproductive health of male animals (Parreault and Cancel, 2001). In the present study, the number of morphologically normal sperm cells decreased compared to the control when the animals were treated with 1000 mg kaempferol derivative per kg b.w. In this group of treated animals, head abnormalities were prominent. *Azadirachta indica* (neem) treated albino mice also showed similar kind of observations (Auta and Hassan, 2016). Abnormal sperm shape may be caused by protein abnormality, as sperm shape is partially maintained by structural protein. The significant increase of sperm head abnormality in highest dose exposed male rats may be due to damage to the pre-meiotic stages of spermatogenesis as it is known that during spermatogenesis DNA synthesis occurs only in pre-meiotic phase (Odeigah, 1997; Otubanjo and Mosuro, 2001). Histological observation through light microscope supported the above results as testicular tissue of rats treated with 1000 mg of the isolated natural product, showed deformities and distortions in the seminiferous tubule's epithelium having lesser number of spermatogonia. In the present study, light microscopic observation is also supported by scanning electron microscopy, which is considered as advanced tool to evaluate any kind of toxic effect exerted by any chemical on a tissue. In our findings, seminiferous tubule of rats exposed to lower dosages of the plant product did not show any remarkable structural effect on the tubules whereas treatment with highest dose i.e. 1000 mg of kaempferol derivative developed cavities and degenerations in the epithelium of the tubule where germ cells produce vital spermatozoa. Similar type of results were also observed by Kumari and Dutta (2014) where they treated mice with pan-masala, a commonly used herbal blend in Indian sub-continent, for long term. Lesser count of spermatogonia and spermatozoa in the highest dose exposed rats may be due to the disturbance in micro-environment of sertoli cells which are responsible for protein synthesis machinery essential for germ cell differentiation (Manivannan et al., 2009).

CONCLUSION

When animals are treated with higher doses of kaempferol derivative isolated from *Lysimachia ramosa*, which has been proven to be an anthelmintic component of the plant in the earlier studies, some undesirable effects have been observed in male reproductive system of wistar rats. But running alongside, it has also been noticed that at lower concentrations, preferably at 250 mg of the component per kg b.w. the isolated component shows some ameliorating impacts on the male reproductive system of wistar rats such as increase in the motility and viability of spermatozoa. Therefore, it can be concluded that the kaempferol derivative isolated from *L. ramosa* can be taken for further study or for trials for human safely at the dose below 500 mg/kg b.w.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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