Original Research article

Threshold Dose Determination of Estrogenic Property using Morphometric Analysis of Mice Uterine Epithelial Proliferation and Study of Hepatic Toxicity of *Scoparia dulcis* L. Extract.

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Abstract: Ethnomedicinal plants have been the source of various therapeutic agents from time immemorial. *Scoparia dulcis* Linn., a commonly occurring plant in India is found to have potential in treatment of diabetes, kidney disorder and also found to possess abortifacients, antioxidant properties. This plant is also found to be used for treating menstrual disorder among women of Northeast India. The present study was designed to examine the threshold dose and estrogenic effect of crude plant extract of *Scoparia dulcis* in vivo. A suspension of methanolic crude extract was orally administered at doses of 250, 500 and 750 mg kg^{-1} body weight day^{-1} for two consecutive estrous cycles to albino mice. The threshold dose from the cyclic group was administered orally to Ovariectomized (OVX) mice for three consecutive days and was compared with OVX (Vehicle treated) and OVX-Estradiol-17β (E2) treated mice. The estrogenic effect of *Scoparia dulcis* on uterus was evaluated by performing uterotrophic histopathological examination in both cyclic and ovariectomized mouse models. Data were analyzed by ANOVA followed by Tukey's test ($p< 0.05$). Compared to cyclic control group, *Scoparia dulcis* extract treated groups exhibit pronounced increase in uterine epithelial proliferation. The uterine epithelium showed significant increase in thickness at the dose of 500mg/kg body weight/day and 750mg/kg body weight/day. The 250mg/kg body weight/day showed results which were insignificant when compared to the control group. The threshold dose was found to be 500mg/kg body weight/day. In the OVX group, the mice treated with the threshold dose (500mg/kg bodyweight/day) of *Scoparia dulcis* extract showed notable increase in the epithelial thickness in comparison to the OVX vehicle treated mice. The increase is similar to the OVX-E2 treated group (negative control). No toxicity in the hepatic tissues were seen in the studied doses of the extract. *Scoparia dulcis* treated group also exhibited increased vaginal cornification rate. Methanolic extract of *Scoparia dulcis* significantly increased the uterine epithelial proliferation and also the rate of showing the presence of efficient estrogenic compounds, which can be used as phytoestrogenic alternative to synthetic Estradiol-17β in near future.

Key words: Estrous cycle; endometrial epithelium; proliferation; *Scoparia dulcis*; uterotrophic assay

Introduction

The use of various herbs for the treatment of various diseases is in commonplace. *Scoparia dulcis* Linn. is a perennial shrub indigenous to tropical and subtropical regions of Asia, Africa and America (Wu *et al.,* 2012) and is abundant in North Eastern states of India (De, 2016). *Scoparia dulcis* Linn. is an important perennial ethnomedicinal herb, commonly known as Goatweed (English) (Jain *et al.*, 1989), Mithi patti (Hindi), Modhu-mehari (Assamese) belonging to the scrophulariaceae

family. It is widely distributed in tropical and subtropical regions (Zulfiker *et al.*, 2010). The plant is found to be used as treatment in various diseases like diabetes, pectoral problems, upper respiratory and skin disorders etc. in many countries (Mishra *et al.*, 2011). In India, the roots and leaves of the plants are used as a cure for toothache, blennorrhagia and stomach trouble (Kirtikar and Basu, 1935). Its use in menstrual disorder, dysmenorrhoea (painful menstruation) and as abortifacient (Technical Data Report for VASSOURINHA *Scoparia dulcis*, 2002), is well documented. Different chemical compounds including Scoparic acid A, Scoparic acid B, Scopadulcic acid A and B, Scopadulciol and Scopadulin has been isolated from *Scoparia dulcis* previously (Hayashi *et al.,* 1990; Hayashi *et al.,* 1991; Hayashi *et al.,* 1993). The Phytochemical investigation of *Scoparia dulcis* has revealed the presence of several alkaloids, diterpenoids, flavonoids, steroids, and triterpenoids (Mishra *et. al,* 2011). These compounds called phytoestrogens are very important as because these are phenolic compounds acting as agonist or antagonist to estrogen via estrogen receptors (Albertazzi and Purdie, 2002). Though use of *Scoparia dulcis* as the traditional treatment of menstrual disorders is documented (Lagachu *et al.,* 2013), but effects of the plant extract on reproductive regulation is yet to be evaluated. As documented in Technical Data Report for VASSOURINHA *Scoparia dulcis*, in Amazonia it is used as abortifacients and women of Tikuna Indian (a tribe of Brazil) drink the decoction of *Scoparia dulcis* for three days during menstruation as a contraceptive or abortifacients (Taylor, 2005).

Though no experimental validation is done on its fertility or abortifacients efficacy, this study can help us understand the effect of these steroidogenic compounds on the uterus. Moreover, not much study is done on its estrogenic properties as well as experimental validation of the plant on female reproduction. It is hypothesized that extract of *Scoparia dulcis* contains compound(s) possessing estrogenic properties which stimulate cellular proliferation in the uterine epithelium.

Estrogen plays diverse role in various reproductive as well as non reproductive tissues. Estrogen is known to induce responses in various reproductive tissues like the reproductive tract, mammary tissues and pituitary and also some non-reproductive targets such as bone formation and cardiovascular health (Hewitt & Korach, 2002). Nowadays, use of phytoestrogens as an alternative choice for estrogen replacement therapy is gaining popularity. The biological potencies of phytoestrogens vary greatly and have a stronger binding affinity to ER-β than ER-α. (Muthyala *et al.,* 2004, Mersereau *et al.,* 2008, Paruthiyil *et al.,* 2009). The majority of the compounds are nonsteroidal structures and vastly less potent than synthetic estrogens (10^{-2} to 10^{-5}) (Kuiper *et al.,* 1997, Kuiper *et al.,* 1998,)

Vaginal cytology assay in laboratory animals is used widely to determine the estrogenic activity of the synthetic estrogens (Wuttke *et al.,* 2003), xenoestrogens (Stroheker *et al.,* 2003) and phytoestrogens (Wang *et al.,* 2003). As reported by Montes and Luque, after administration of estrogen, the vaginal epithelium of rat becomes thicker with a larger number of cellular layers. There is transition from fixed basal cells through the maturation process, leading to superficial cornified cells as observed in smear (Montes and Luque, 1988).Uterotrophic assay of measurement of the thickness of endometrium is also a widely used technique to determine the estrogenic property of various synthetic estrogens (Granberg *et al.,* 2002), plant extracts (Vasudeva and Sharma, 2007) The endometrial thickness is found to increase in the presence of estrogenic compounds in the extracts (Sharangouda and Patil, 2008)

In the present investigation, crude extract of the aerial part of *Scoparia dulcis* has been tested in vivo for steroidogenic property. Different doses have been tested in cyclic mice for determination of threshold dose for uterine epithelium proliferation. Oral administration of the threshold dose was given to ovariectomized (OVX) mice to study the effects in and absence of native steroids and also compared to synthetic estrogen (Estradiol-17 β).

Materials and methods

Collection and Preparation of plant extract

Scoparia dulcis Linn. plant (Fig. 1) was collected from parts of Arunachal Pradesh and Assam. The stem and leaves were

Fig. 1. The plant *Scoparia dulcis* Linn.

cleaned and shade dried. The dried plant was chopped into small pieces then ground to make 60 mesh powders. The powder was immersed in methanol for a period of 72 hours at room temperature (25±2°C) for cold extraction and subsequently filtered. The filtered solution was allowed to dry at room temperature for removal of methanol. The semi solid methanolic extract was administered to the experimental animal.

Experimental animal

Adult cyclic female albino mice and ovariectomized (OVX) mice (body weight 25 ± 3 g) were used for the present in-vivo investigation to study the effect of methanolic crude extract of *Scoparia dulcis.* Animals were kept in the Central animal facility of Rajiv Gandhi University under uniform husbandry conditions and natural light and temperature. Cyclic mice were divided into four groups and OVX mice were divided into four groups each containing 5 female mice. Mice were fed with routine diet (Bengal gram, corn) and water *ad libitum*. The estrous cycle of the female mice was continuously monitored by following the method of *Montes & Luque, 1988*.

Monitoring of estrous cycle

Estrous cycle was monitored by cell identification in the vaginal smear every day 8:00hrs-9:00hrs after the onset of treatment regime. Briefly; Distilled water was taken in the tip of the glass dropper and flushed into vagina. The collected fluid was placed on the glass slides and spread to make a smear. Three clean glass slides were used for each female albino mice. The vaginal fluid on the glass slides was allowed to dry and a drop of methanol was put on dried vaginal smear. The slides were stained with Giemsa stain for 5 minutes and then washed in distilled water for removal of the excess stain. The slides were observed and photographed using Leica DM 5000B microscope. Adult female mice exhibiting at least three consecutive normal estrous cycles were considered for the present study. Different stages of estrous cycle of cyclic mice are given in figures (Fig. 2).

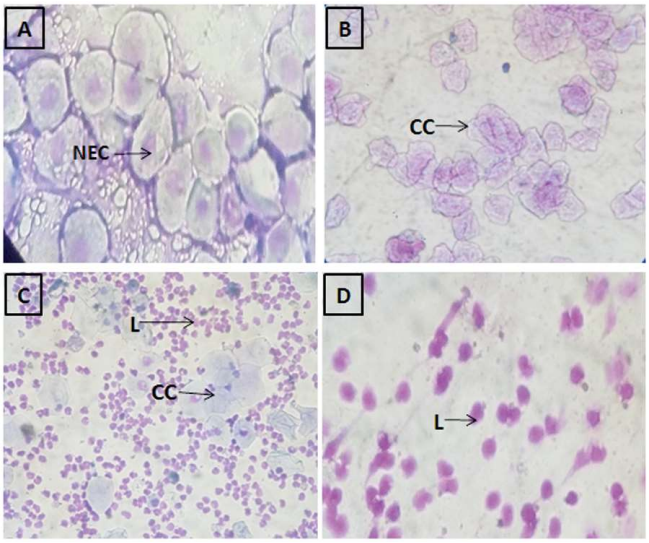

Fig. 2. Photomicrographs of different stages of estrous cycle of mice showing normal cell types during all phases of the cycle.
A. Proestrus, B. Estrus, C. Metestrus, D. Diestrus.
NEC-Nucleated Epithelial cells, CC- Cornified Cells, L-Leucocytes

Ovariectomy

Ovariectomy of the adult cyclic mice weighing 25 ± 3 grams was done following standard method (Hogan *et al.,* 1986). The mice were anaesthesized under mild diethyl ether. Briefly, the furs of the dorso-lateral on either side of the vertebral column and immediately behind the last rib were removed with a fine scissors.

A small dorso-lateral incision approximately 1cm in length was made through the skin and muscle of the back of the mice making the ovary and fat pad visible. The fat pad with attached ovary and oviduct was grasped with fine forceps and the ovary was removed with fine scissors. The incision was immediately stitched with catgut suture. Neosporin was then applied over the wound for healing. The ovariectomized (OVX) mice were kept under intensive care for three weeks to recover and were considered for the experiments.

Administration of extract and sample collection

The different cyclic female groups (n=5) were given three different doses of the extract. The methanolic extract was suspended in 100µl distilled water to prepare the final doses for oral administration. The control females (G-I) received the vehicle in similar manner. The mice were treated at the dose of 250 mg/kg body weight/day (G-II), 500 mg/kg body weight/day (G-III), 750 mg/kg body weight/day (G-IV), for 8 consecutive days (2 consecutive estrous cycles). Both control and extract treated cyclic females were sacrificed on next day following the administration of the extract in each group respectively during 8:00 to 9:00 hrs to collect the uterine horns and liver.

Three different groups of OVX females (n=5 in each group) were treated with vehicle (distilled water) (G-V), threshold dose of crude extract (500 mg/kg body weight/day) (G-VI), sesame oil (vehicle for estradiol-17 β) (G-VII) and estradiol-17 β (E2) (G-VIII) separately. Oral administration of the crude extract was done for three consecutive days during morning hours (8.00-9.00 hrs). Estradiol-17 β (Fulvestrant) was injected subcutaneously at the dose of 100ng/100µl of sesame oil for 3 consecutive days to the OVX females. Control OVX females received the 100µl sesame oil (vehicle) subcutaneously for three consecutive days. Females of each group were sacrificed on next day (day 4) of the last treatment during morning hours (8:00 - 9:00 hrs) for collection of uterine horn and liver.

Histological studies and analysis

Uterine horns and liver were removed immediately and fixed in bouin's solution for 72hrs, dehydrated, cleared in xylene and embedded in paraffin. Histological sections were obtained by cutting the block in a rotary microtome and stretched on poly-L-Lysine (Sigma Cat.No. P8920) coated slides. Tissue sections were stained using standard Eosin-Hematoxylin stain method (Culling, 1974) and mounted with DPX. Photomicrographs were taken using LeicaDM5000B and all the measurements were done using Leica software LAS V4.4. Representative figures showed the uterine histological photomicrographs of mice treated of different groups.

Endometrial proliferation assay

The endometrial proliferation was studied by measuring the height/thickness of endometrial layer in control as well as each treated groups to study the estrogenic effects of the plant extract. The representative figures along with the mean value ± S.E.M of measurements are presented. Statistical analysis was done using the GraphPad Prism 8.0.ANOVA (post hoc Tukey test) was performed to analyze the level of significance of values among each groups.

Results

Cyclic mice

Administration of extract induced cell proliferation in the endometrial epithelium and glandular epithelium, evidenced by increase in the endometrial thickness. Administration of crude extract to adult cyclic females showed gradual increase of rate of proliferation of uterine epithelium. In the cyclic control uterus, the endometrial epithelium is intact with the uniform layer of epithelial cells without any significant proliferation (Fig. 3A, 3B). Oral administration of extract at 250 mg/kg body weight/day, for two estrous cycles (8 consecutive days) in the cyclic mice results in increase in the thickness of the uterine endometrial lining (Fig. 4A, 4B).But, the values of the thickness measured showed no significant ($P<0.05$) difference between the control (GI) and the 250 mg/ kg body weight/day treated group (GII). Increase in number of glands was seen in the 250 mg/kg body weight/day treated group (GII). The stroma is intact as seen in the control group of females (Fig. 3A, 3B).

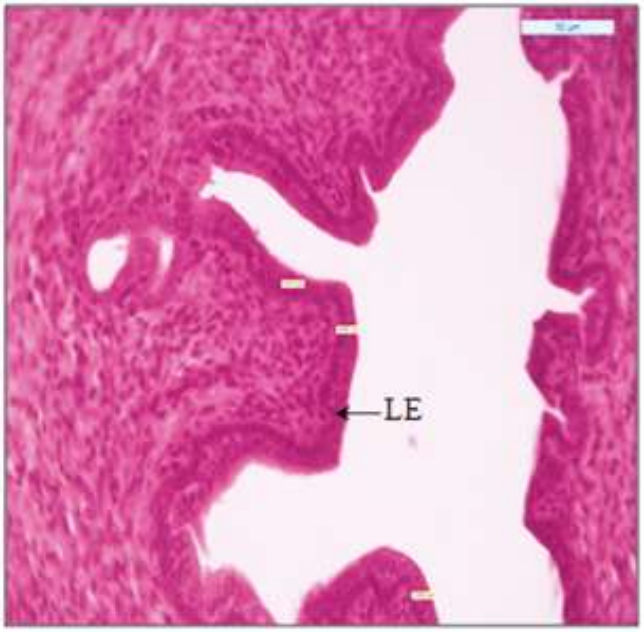

Fig. 3. Photomicrographs of uterus of control (Vehicle treated) mice uterus (cyclic mice) stained with Eosin-Hematoxylin stain showing the normal luminal epithelium and measurements of its height. LE-Luminal epithelium.

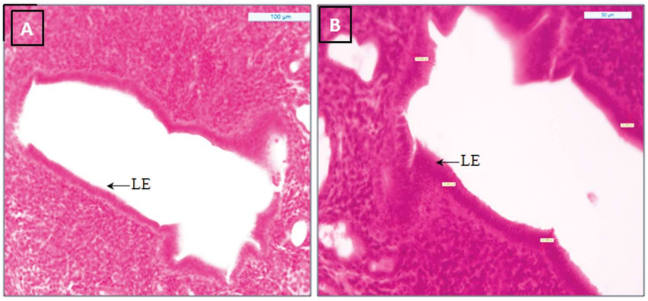

Fig. 4. Photomicrographs of uterus of extract treated (250mg/kg body wt./ day) mice uterus (cyclic mice) stained with Eosin-Hematoxylin showing the luminal epithelium and measurements of its height. LE-Luminal epithelium.

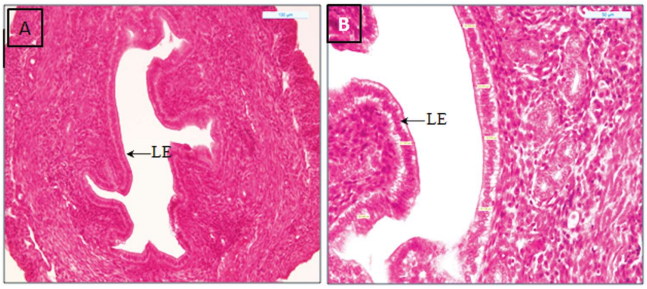

Fig. 5. Photomicrographs of extract treated (500mg/kg body wt./day) mice uterus (cyclic mice) stained with Eosin-Hematoxylin showing the proliferated luminal epithelium and measurements of its height. LE-Luminal epithelium.

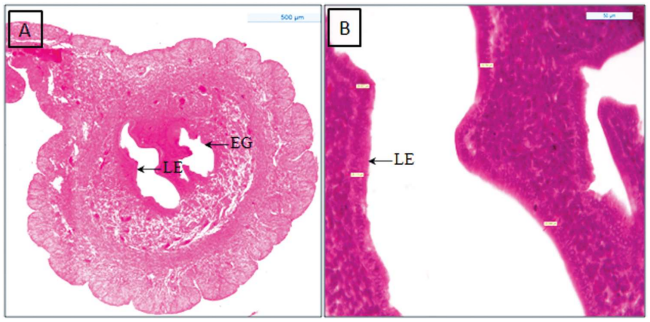

Fig. 6. Photomicrographs of extract treated (750mg/kg body wt./day) mice uterus (cyclic mice) stained with Eosin-Hematoxylin showing extensive proliferation of luminal epithelium and measurements of its height. LE-Luminal epithelium. EG-Endometrial gland.

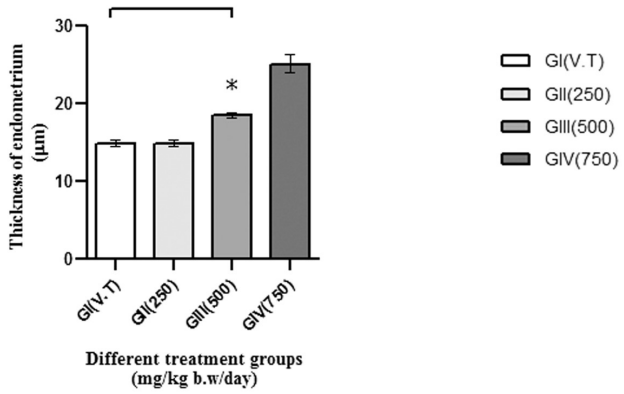

Fig. 7. Thickness of endometrium in each treated group. Data are presented as Mean ± SEM. Endometrial proliferation is measured taking Vehicle treated group as the baseline ($P<0.05$).V.T=Vehicle treated,

Uterus of 500 mg/kg body weight/day treated cyclic mice (GIII) showed uterine endometrial proliferation. Thickness of endometrial layer following treatment of crude plant extract significantly increased ($P < 0.5$) in GIII in comparison to the 250 mg/kg body weight/day treated group (GII) female mice (Fig. 5A). The cell layers in the endometrial lining have increased showing elongated cells (Fig. 5B). The increase in numbers of glands is evident from the Figures (Fig. 5A). The stroma consists of loose tissues (Fig. 5B).

Uterus of 750 mg/kg body weight/day treated cyclic mice (G-IV) showed uterine endometrial hyperplasia. Uterine lumen showed extensive proliferation leading to the formation of two lumens (virtual) (Fig. 6A). Increase in thickness of endometrial

layer following treatment of crude plant extract was seen ($P < 0.5$) in comparison to the 250 mg/kg body weight/day treated group (GII) female mice and 500 mg/kg body weight/day (G-III). In some region of endometrial lining the cellular layers even increased to multiple layers (Fig. 6B). The numbers of glands also increased extensively as evidenced in the figures (Fig. 6B). The Myometrium is intact with no any changes in all the treated groups.

Representative figures of histological photomicrographs of vehicle treated control and extract treated ovary intact females are presented in Fig. 3 to Fig. 6. and the graph showing the increase in the thickness of endometrium at different doses is presented in Fig. 7.

OVX mice

The ovariectomized control mice (GV) treated with the vehicle (distilled water) did not show any proliferated endometrial lining (Fig. 8A, 8B). The OVX-extract treated with 500 mg/kg body weight/day females (GVI) showed high degree of proliferation of the endometrial lining (Fig. 9A) .The stroma showed presence of loose tissues in some area alongwith enlargement in the size of glands (Fig. 9B). The effects seen in this group of female mice treated with the threshold dose of the extract showed changes that were similar to that of the OVX-E2 treated group (GVII)(Fig.11A,11B).There was significant($P < 0.5$) increase in the values of the thickness of the endometrial lining in the extract treated group in comparison to the control group. No changes in the myometrium is seen all the OVX groups.

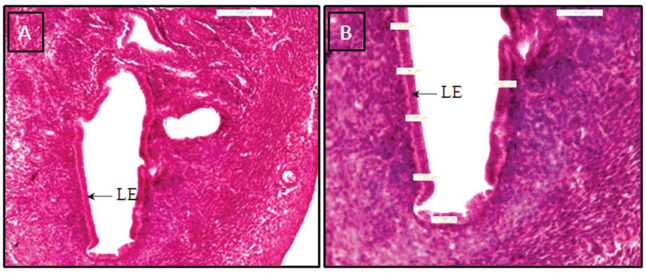

Fig. 8. Photomicrographs of Ovariectomized (OVX)-vehicle treated mice uterus stained with Eosin-Hematoxylin showing the luminal epithelium and measurements of its height. LE-Luminal epithelium.

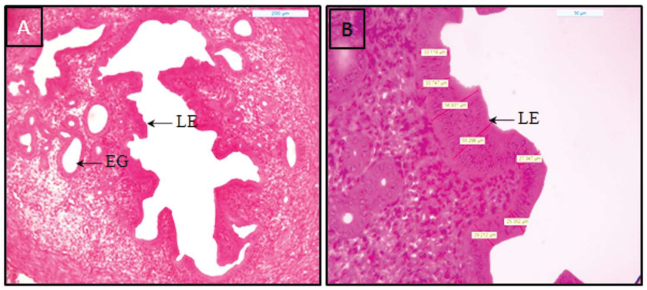

Fig. 9. Photomicrographs of Ovariectomized (OVX)-extract treated (500mg/kgbodywt./day) mice uterus stained with Eosin-Hematoxylin showing the luminal epithelium and measurements of its height. LE-Luminal epithelium.EG-Endometrial gland.

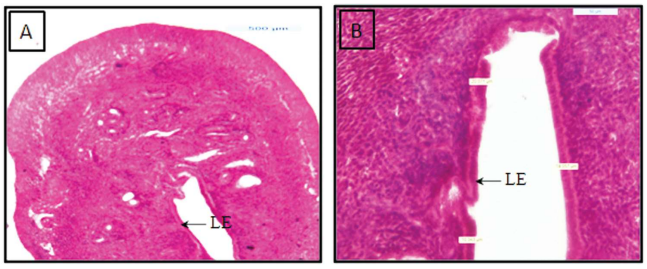

Fig.10. Photomicrographs of Ovariectomized (OVX)-SOT (Sesame oil treated) mice uterus stained with Eosin-Hematoxylin showing the luminal epithelium and measurements of its height. LE-Luminal epithelium.

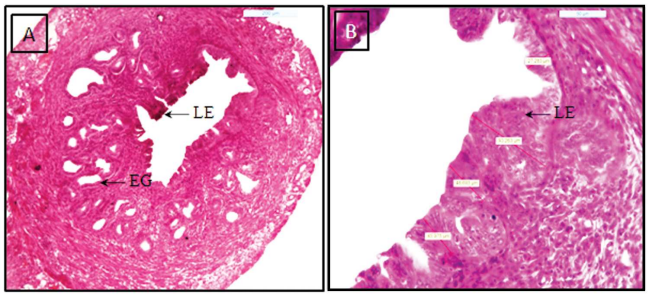

Fig. 11. Photomicrographs of Ovariectomized (OVX)-Estradiol-17β (E2) treated mice uterus stained with Eosin-Hematoxylin showing the proliferated luminal epithelium and measurements of its height. LE-Luminal epithelium.EG-Endometrial gland.

Representative figures of histological photomicrographs of OVX vehicle treated control and extract treated OVX females are presented in Fig. 7 to Fig. 11. and the graph showing the increase in the thickness of endometrium at different doses is presented in Fig. 12.

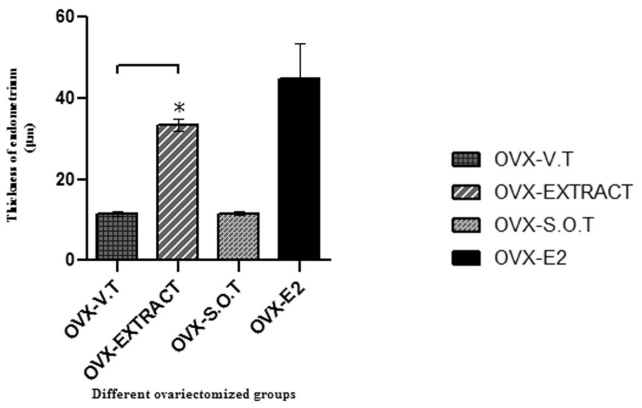

Fig. 12. Thickness of endometrium in each OVX group. Data are presented as Mean ± SEM. Endometrial proliferation is measured taking OVX-Vehicle treated group as the baseline ($P<0.05$). OVX-VT=(GV), OVX-EXTRACT=GVI, OVX-SOT=GVII, OVX-E2=GVIII. VT-Vehicle treated, SOT-Sesame oil treated, E2-Estradiol-17 β

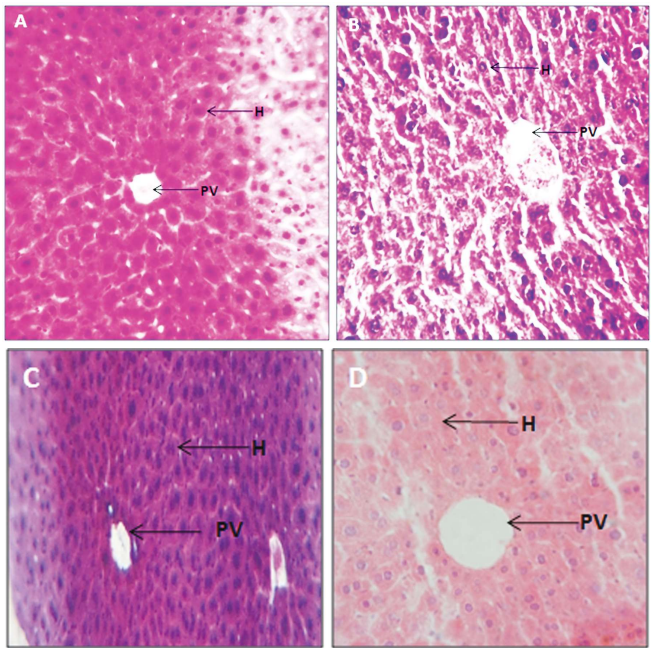

Fig. 13. Photomicrographs of T.S. of liver of mice, A-Control and B-Extract treated, C-OVX control and D-OVX extract treated. The histological sections shows no structural changes. H-Hepatocytes, PV-Portal Vein.

Liver histology

The effect of the extract of *Scoparia dulcis* on hepatic tissues was studied in both presence (ovary intact) and absence (OVX) of ovary of the adult female mice. The histological structures of control ovary intact female (Fig. 13A) showed compact hepatic lobules with the portal vein and hepatocytes. These structures were observed in both the controls of ovary intact as well as the OVX female mice (Fig. 13A, 13C).

Oral administration of the extract did not show any prominent effects on the histological structure of liver in either ovary intact (Fig. 13B) or OVX females. Compact structures of hepatic lobules were observed in liver of OVX females too as seen in the photomicrographs (Fig. 13D). However infiltration of neutrophils in the extract treated ovary intact mice liver was observed (Fig. 13A).

Discussion

The present investigation on the experimental validation of crude extract of aerial parts of *Scoparia dulcis* L. has been carried out on the basis of traditional knowledge of the use of the plant for treating various menstrual disorders among folk people of Assam and Arunachal Pradesh as stated earlier. Traditionally among the folk people, women with menstrual irregularities are treated with a decoction of the plant. As mentioned earlier, in several countries pregnant women are strictly not allowed to have the plant as it may cause abortion (Taylor, 2005). As there is no scientific evidence of the mechanism of the action of the extract of this plant, these research findings are the first of its kind to report the estrogenic property of the extract of *Scoparia dulcis*. It is seen in the result that the *Scoparia dulcis* exerts effects on the uterine tissues which is evident by the proliferation of the endometrial lining. It is observed that the rate of proliferation is higher in the treated groups in comparison to the control group in the ovary intact cyclic mice. Additionally the higher rate of proliferation is also seen in the ovariectomized (OVX) group treated with the crude extract when compared to the control group. This proliferation is statistically significant and nearer to that of the OVX-Estradiol 17 β treated group, which serves as the negative control for the experiment.

In the present investigation three doses were used for the treatment and the dose of 500mg/kg body wt/day is found to the minimum dose at which the extract showed the estrogenic

property, so this dose is considered as the threshold dose for treatment in the OVX group of females. As this dose showed the desired result in the absence of ovary too, so this dose is established as the dose of treatment for further studies.

Toxicity studies of the methanolic extract of *Scoparia dulcis* revealed that it to be non-toxic, as they found LD50 for the extract to be 3807mg/kg body weight (Abdulsalaam *et al.,* 2013). Moreover as no report of poisoning of humans has been reported till date, it is probably the narrative of its widespread use for treatment of numerous diseases worldwide. Different extracts of *Scoparia dulcis* is found to have potential hepatoprotective activity as it showed protective effect against CCl4 induced changes in serum glutamate oxaloacetate transaminase (SGOT), glutamate pyruvate transaminase (SGPT) and histoarchitecture of liver (Praveen *et al.,* 2009; Sahoo and Madhavan, 2009). As seen in present investigation the extract does not show any abnormal structural changes in the hepatic tissues of both ovary intact and OVX groups of treated female showing that it does not have any toxic effect on liver.

Estrogen is a steroid hormone which can promote the development of vagina and cervix, uterine epithelial hyperplasia, and keratinization. In simple endometrial hyperplasia without cytologic atypia there is an abundance of endometrial tissue, and the glands are enlarged (Colgan and McLachlin, 2008). Proliferation of the endometrium under the influence of estrogen is a prime phenomenon of a cyclic estrous cycle in mice and menstrual cycle in human females. This cyclicity ensures the proper implantation if fertilization takes place. Estradiol-17β (Fulvestrant) used as negative control in this study is the synthetic alternative of the estrogen present in our body. Morphometric uterotrophic assay of the uterine endometrial lining is one of the key tools to identify the estrogenic effect of any compounds/extract (Lemini *et al.*, 2004). It is evidenced by the result of this study that the methanolic extract of the aerial part of *Scoparia dulcis* possess estrogenic properties. The higher endometrial proliferation in the extract treated groups than the control group (untreated group) and lower but significantly nearer to the values of the Estradiol-17β treated group shows the presence of compound(s) that exerts this estrogenic effect. Such plant compounds with estrogenic properties are known as phytoestrogens. And this experiment validates the claims of presence of phytoestrogens in this plant as mentioned earlier.

As seen in the cyclic group, in the presence of the circulating estrogen the extract further enhanced the proliferation of the uterine endometrial lining showing hyperplasia in the uterus; it shows that the compound(s) might act as the estrogen agonistic. Moreover the proliferation showing nearer values to the estradiol-17β treated values makes us speculate that these compound(s) can be used a alternative to synthetic estradiol, though the scientific trials is yet to be done.

As stated in earlier studies the phytoestrogens are found to act via ER receptors ERα and ERβ. This claim can be substantiated by further *in-silico*/*in-vivo* investigations on this extract and its mechanism of action. Studies on its chemical constituents and their effect on the pathways of its action and there intermediates can be very much helpful to understand its effect on the female reproductive regulation. This can further help in putting this plant for clinical trials to formulate a non-steroidal estrogen/progestin alternative in near future.

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