Thin layer chromatographic fraction of root extract of *Polygonum hydropiper* induces vaginal epithelial cell maturation in adult ovariectomized albino rat

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Summary

Dry root powder of *Polygonum hydropiper* is traditionally used as an antifertility agent by women of north east India. The effect of crude root extract of *P. hydropiper* on reproductive performance of female albino rat was reported earlier from this laboratory. In the present investigation, attempt was made to find estrogenic effect, if any, of thin layer chromatographic fraction of the crude extract. To achieve the goal, methanolic crude extract was fractionated in thin layer chromatography (TLC). Estradiol-17βwas used as the reference compound to identify the estrogenic compound in the crude root extract. The effect of TLC fraction was studied on the vaginal epithelial cell maturation in adult ovariectomized (OVX) rats and uterine histology of OVX and ovary-intact females. The TLC fraction was administered (*sc*) to adult OVX females at a dose of 5mg/kg/ day for 18 days. Estradiol -17β was injected as reference compound. Vaginal smears were stained with Giemsa's to observe the cell types. Rats were sacrificed by cervical dislocation at the end of the treatment and the uterine epithelium was subjected to histological analysis. The TLC fraction treated OVX females showed characteristic epithelial cell types (parabasal, intermediate, superficial and cornified) in the smear. Histological evidence revealed increased proliferation of the endometrial surface epithelium in the TLC fraction treated females. The results reveal that TLC fraction of root extract of *Polygonum hydropiper* contains compound(s) which has functional similarities with E2 and ovarian estrogen in female albino rats.

Keywords: Vaginal cell maturation, *Polygonum hydropiper*, TLC fraction, parabasal cell, cornified cell.

Introduction

Polygonum hydropiper is a weed growing in wild during spring and summer seasons in India and many other countries . In Europe, this plant is reported to be in use for correction of menstrual irregularities in women (Blatter et al., 1998). Taking leads from the Indian folk medicine this plant has been found to possess antifertility property (Garg et al., 1978; Satyavati, 1984). In the Chinese medicines this plant is used for its antiovulatory and pregnancy termination properties (Xiao and Gong, 1991). The leaf of this herb is reported to contain flavonoids having antioxidant property (Peng et al., 2003). Assam, situated in the sub - Himalayan region and one of the north eastern provinces of India, harbors numerous ethnic groups from time immemorial. These native ethnic groups have their own traditional medicines. The "Mishing", one such ethnic tribal group living on the banks of mighty Brahmaputra River, practices traditional herbal medicines for control of reproduction. These folk women consume dry root of *P. hydropiper* to terminate pregnancy. Earlier investigations from this laboratory showed that a crude root extract (CRE) of *P. hydropiper* mimics the ovarian estrogen (Hazarika and Sarma, 2006a) and induces follicular recruitment and endometrial hyperplasia in female albino rat (Hazarika and Sarma, 2006b). These findings led to the speculation that the estrogenic property exhibited by the methanolic crude root extract (CRE) of *P. hydropiper* is due to the presence of active steroidogenic compound(s). If such is the case, the chromatographic fraction of the root extract should induce epithelial cell maturation in the vagina and the uterine endometrium, since proliferation and differentiation of uterine squamous epithelium and vaginal squamous cell is under hormonal control. The ovarian estrogen is responsible for the growth and development of uterine endometrium and vaginal squamous cell. These epithelial cells respond to supra-physiological estradiol and show

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increase in cytoplasmic area with nuclear shrinkage (Chretien et al., 1998). Administration of estradiol -17β brings about vaginal epithelial cell growth, division and cornification in a stratified manner (Rao et al., 1997). Similar effect is also observed in the vaginal epithelial cell of the phytoestrogen treated females (Kayisli et al., 2002). The vaginal epithelium undergoes significant and cyclic changes under the influence of phytoestrogen that interferes with the reproductive physiology of both animals and human being (Montes and Luque, 1988). Therefore, the present study has been designed to examine the effect of a chromatographic fraction of root extract of *P. hydropiper* on the vaginal epithelial cells, with special reference to induction of cornification, in the absence of ovarian steroids *in situ*.

Material and Methods

Plant collection and extraction

Polygonum hydropiper growing wild in Assam was properly identified and collected during the month of April and May. Roots of the plants were cleaned and shade-dried. The dried roots were chopped into small pieces and finely powdered. The powder was soaked in methanol (in a ratio of 2 g powder in 25 ml methanol) for 72 hr and then filtered. The filtrate was evaporated to dryness using vacuum evaporator and stored at -20^0 C until use. Totally 5.5 g of dry crude extract was obtained from 85 g of dry root powder.

Thin layer chromatography

The crude methanol extract was fractionated by thin layer chromatography (TLC). Separation of the fractions was done on TLC plates, 20 x 20 cm, coated with silica gel 60 having 60μ pore size (MERCK, Germany, Cat No. 1.05641). A solvent mixture of n-butanol : acetic Acid : water, in a ratio of 100 : 10 : 10 (v/v/v), was used in the glass chamber to run the chromatogram. Estradiol -17β (Lancaster, Germany, Cat No.LO3801, F.W. 272.39) was run as the reference compound along with the crude extract (Fig.1). Iodine was used as developing agent. The separated fractions, which showed similar Rf value as Estradiol -17β (E2), were scrapped out. The fractions from 100 lanes were collected in 10 ml of absolute ethanol and thoroughly mixed to dissolve the active compound. The ethanol-silica gel mixture was filtered to separate the compound dissolved in ethanol and the filtrate was dried in a vacuum evaporator at room temperature. A semisolid mass of dark brown material was obtained and stored at -20^0 C until use for administration to the female rats. The eluted material was subjected to the Liebermann-Burchard test to confirm the presence of steroid (Nath et al., 1946; Cook, 1961).

Experimental design

Adult female albino rats were overiectomized (OVX) following standard method (Hogan et al., 1986). Briefly, normal cycling rats were subjected to ketamine hydrochloride anesthesia, and dorsolateral incisions, approximately 1 cm long, were made on both sides to expose the ovaries. The ovaries were removed and single cat gut sutures were made bilaterally. The rats were allowed to recover for three weeks period before beginning the treatments. The OVX females were divided into three groups consisting of three adult females each. The chromatographic fraction was administered through subcutaneous route to one of the groups. E2 was administered to the second group, while the third group was treated with the vehicle (sesame oil) and formed the control. The TLC fraction was administered to a group of three ovary-intact females. Here, ovary-intact females treated with the vehicle formed the control.

*Administration of TLC fraction and Estradiol-17*β

The eluted TLC fraction was suspended in sesame oil at a concentration of 2mg / ml and kept overnight. Thus, a fine suspension of the TLC fraction in sesame oil was obtained. The preparation was administered (*sc*) in a dose of 5mg/kg body weight/day for 18 days between 7.00 – 9.00hr to one group of OVX as well as ovary-intact females. Injection was given on both right and left of the nape and inner thigh region, alternately. Estradiol-17β was administered at a concentration of 0.1μg/ml sesame oil (2.5μg/kg bodyweight/day) for 4 days. The control OVX animals were injected with the vehicle (sesame oil) in a dose of 0.3 ml / rat/day for 18 days.

Analysis of vaginal smear

The vaginal smears of all rats were studied during the entire treatment period. The smears were prepared on microscope slides and allowed to dry. The cells in the dry smears were fixed in methanol and stained with Giemsa's. The slides were washed in distilled water, dehydrated in graded ethanol, mounted in DPX mountant and observed in the microscope. Quantitative analysis of the cells in the smear was made to determine the degree of maturation of the epithelial cells. Total 1000 cells were counted at random from each slide, and the percentage of each of the cell types, *viz*., parabasal, intermediate, superficial and cornified, were calculated. On the basis of percentage of the cell types, the slides were graded. Grade 1 represents 0-25% each type

of cells in each slide, Grade 2 represents 25-50% each type of cells in each slide, Grade 3 represents 50-75% each type of cells in each slide and Grade 4 represents 75-100% each type of cells.

Histological analysis of uterus

The rats were sacrificed by cervical dislocation at completion of the treatments. Thus, the TLC fraction treated OVX and ovary-intact females and the vehicle-treated OVX females were sacrificed on day 19, while the E2-treated OVX females were sacrificed on day 5. The rats in the other group of ovary-intact females were sacrificed at the estrus phase. The uterine horns were collected and fixed in 10% formaldehyde. Tissues were processed for routine histological analysis adopting hematoxylin-eosin staining for observation in the microscope.

Statistical analysis

The data on the four cytological grades (grades 1-4) were statistically analyzed to determine the significance of difference of each cell type among the experimental groups. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS), version 10.0. One way ANOVA and post-hoc multiple comparison test, when necessary, were performed by LSD to find the significance of the difference between the groups. The data are presented as mean of grade ± standard error (SE). The statistical significance was defined as $P < 0.05$ for all tests.

Results

Administration of the TLC fraction of crude root extract of *P. hydropiper* exerted effects on the vaginal epithelium of OVX females. Changes of the cell types were observed from day 4 onwards of the treatment. The effect of E2 began on the 2nd day of treatment, with the appearance of parabasal cells advancing to intermediate, superficial and finally a dominant group of cornified cells. As shown in the figure 2, four different cell types were observed following administration of the TLC fraction and estradiol-17β. Rounded parabasal and intermediate nucleated epithelial cells were observed in all treated (E2 and TLC fraction) groups. Nucleated epithelial (intermediate) cells, with a small nucleus, approaching cornification, were considered as the superficial cells. The superficial cells and cornified cells were observed in the estrous phase of ovary-intact females as well as E2 and TLC fraction administered ones. The cornified cells of TLC fraction treated females were elongated, aberrant and smaller in size (Fig. 2E) as compared to the ovary-intact estrus phase rats (Fig. 2D) and ovariectomized E2 treated rats (Fig. 2F). The control OVX females, treated with sesame oil, did not show any form of epithelial cell types in the smear during the experiment. The mean cytological scores of all groups are shown in the table1.

Histological study of the uterus revealed regression of the uterine endometrial epithelium in OVX control females (Fig. 3A). Administration of TLC fraction to the OVX females induced proliferation of endometrial epithelium (Fig. 3B). In the ovary-intact treated females, the endometrial epithelium showed higher degree of cellular proliferation (Fig. 3D) than the normal females in estrus phase (Fig. 3C). Administration of E2 to OVX females resulted in extensive proliferation of the endometrial tissues (Fig. 3E).

Statistical analysis (ANOVA) revealed no significant difference among the ovary intact, OVX-E2 treated and OVX- TLC fraction treated rats at 5% level of significance.

Discussion

The present study revealed effect of the TLC fraction of *P. hydropiper* root extract on the vaginal cell maturation of adult OVX female rats. The fraction induced epithelial cell proliferation and maturation in the vagina and uterine endometrial epithelium both in OVX and ovary-intact females. In ovary-intact females the TLC fraction increased the extent of proliferation of endometrial tissue. The objective of using E2 as reference drug was to study if the TLC fraction of root extract has estrogenic property. The cellular proliferation and maturation following TLC fraction treatment in OVX females is comparable to that in E2 treated OVX and ovary-intact females' vaginal epithelial cell types. However, the cornified cells, produced due to TLC fraction treatment, were aberrant morphologically. Thus, the results of the present investigation suggest estrogenic property of the compound present in the TLC fraction of *P. hydropiper* root but also with a property which may lead to the structural deformation of the cornified cells.

The estrogenic effects of phytoestrogens on vaginal cell proliferation and maturation have been reported both in animal models and human beings (Kurzer and Xu, 1997). Studies on animal models have revealed variable effects of phytoestrogens on the estrus cycle depending on the species and the phytoestrogen used (Whitten and Naftolin, 1998). The effect of the particular phytoestrogen would depend on the threshold dose and the duration of treatment. The red clover (*Trifolium prentense*) induced partial

Table 1. The mean values of grades of different cell types counted in groups of experimental females (ovary intact, OVX- E2 treated, OVX- TLC fraction treated and OVX control).

Group	Parabasal	Intermediate	Superficial	Cornified
Ovary Intact	1.20 ± 0.13	1.10 ± 1.0	1.60 ± 0.16	3.30 ± 0.48
OVX-E2 treated	1.16 ± 0.16	1.33 ± 0.21	2.33 ± 0.21	3.00 ± 0.63
OVX- TLC fraction treated	1.10 ± 1.00	1.20 ± 0.13	1.20 ± 0.13	3.20 ± 0.63
OVX-Control	00	00	00	00

Values are expressed as mean ± SE (Standard error of the mean)
The cell counts were based on evaluation of total 1000 cells from vaginal smear in each slide of the respective group of control and treated females. Number of each cell type was graded as 1 (0-25%), 2 (25-50%), 3 (50 – 75%) and 4 (above 75%). The cell types were compared among the groups (ovary intact, OVX-E2 treated and OVX-TLC fraction treated rats) at 5% level of significance. The data on cell types (parabasal, intermediate, superficial and cornified) was not significantly different among the first three groups.

cornification of vaginal cells at the two highest doses of 500mg/kg and 750mg/kg on treatment for 21 days (Burdette et al., 2002). Resveratrol (trans-3,5,4'-trihydroxystibene), an active phytoestrogen abundant in the grapes, produced a significant effect on the vaginal cell proliferation when administered to ovariectomized rats indicating that resveratrol does act as a potent partial estrogen agonist on vaginal cytology at a does of 5 mg / kg for 35 days (Hascalik et al., 2005). In the present investigation comparable results were produced when treated an optimal dose of 5mg / kg / day for 18 days.

There was gradual increase of the proliferated cells in the vaginal smear reaching a maximum level, similar to the cell types that appeared in the smear of estrous phase ovary-intact females, at the end of the period of treatment, suggesting duration-dependence of the effect. It is reveled that the TLC fraction of the *P. hydropiper* root extract has functional similarity with E2 and ovarian estrogen in regard to the epithelial cell proliferation in the female reproductive tract. At the same time, the cause underlying the aberrant structure of cornified cells in the TLC fraction treated female remains to be elucidated. Perhaps, the fraction possesses other properties in addition to estrogenic one. Several phytoestrogens have been reported to bring about considerable physiological effects in relation to women's reproductive health (Murkies et al., 1998) and in animal model system. *Pueraria mirifica*, a herb containing phytoestrogens, causes cornification of the vaginal epithelium of female albino rat. Oral administration of the plant in a dose of 100 and 1000mg/kg body weight produced cornification within 5 days of treatment (Malaivijitnond et al., 2004). Dietary intake of soy protein has been reported to increase the length of follicular phase, suppression of mid-cycle gonadotropin surge (Cassidy et al., 1994) and decrease in serum estradiol and luteal phase progesterone (Lu et al., 1996). In addition, studies on women revealed that certain phytoestrogens can produce mild estrogenic effect on vaginal cytology (Wilcox et al., 1990; Baird et al., 1995). Several such phytoestrogens are used for human welfare, particularly woman. For example, resveratrol has been reported to suppress breast cancer (Whitsett et al, 2006). The ability of phytoestrogens to alleviate menopausal symptoms and reduce breast cancer risk has surged extensive research towards evaluation of phytoestrogens for female reproductive health (Umland et al, 2002). The present investigation is yet another attempt in this direction and shows the presence of compound(s) functionally similar to ovarian estrogen and/or estradiol - 17β in the root of *P. hydropiper* that can induce cellular maturation in vaginal epithelium. The historical background and indigenous knowledge on the use of roots of this wild herb by women of north east India for their fertility control provides leads that the dry root powder of this herb can induce infertility in women. However, the mechanism of action remains to be understood. It is speculated that the active fraction would bind to the estrogen receptor and induce transcription of target gene(s) of vaginal epithelial cells, resulting in cell proliferation and cornification. The basis of deformation of cornified

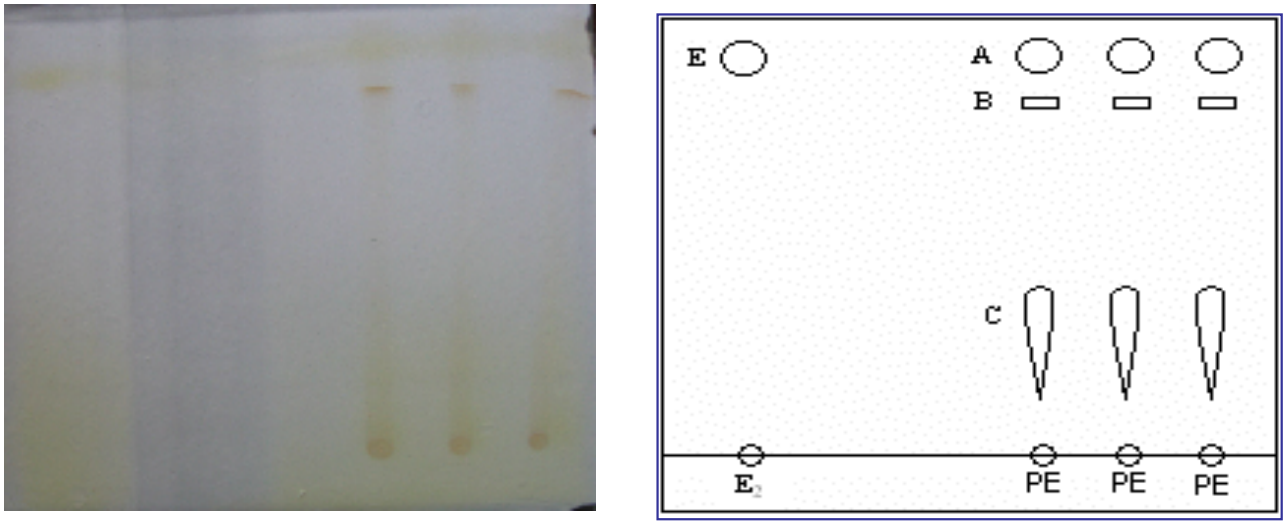

Fig. 1. The crude root extract (PE) of Polygonum hydropiper was separated in TLC plates using butanol/acetic acid as solvent. Three fractions (A, B, C) were observed in the TLC separation. Estradiol - 17β (E) was run as the reference to identify the compound in crude extract having steroidal nature. Fraction A having similar Rf value as estradiol - 17β was eluted and resuspended in methanol. The methanol was evaporated in vacuum evaporator and the extract was used for sc administration to female (OVX and ovary-intact) rats.

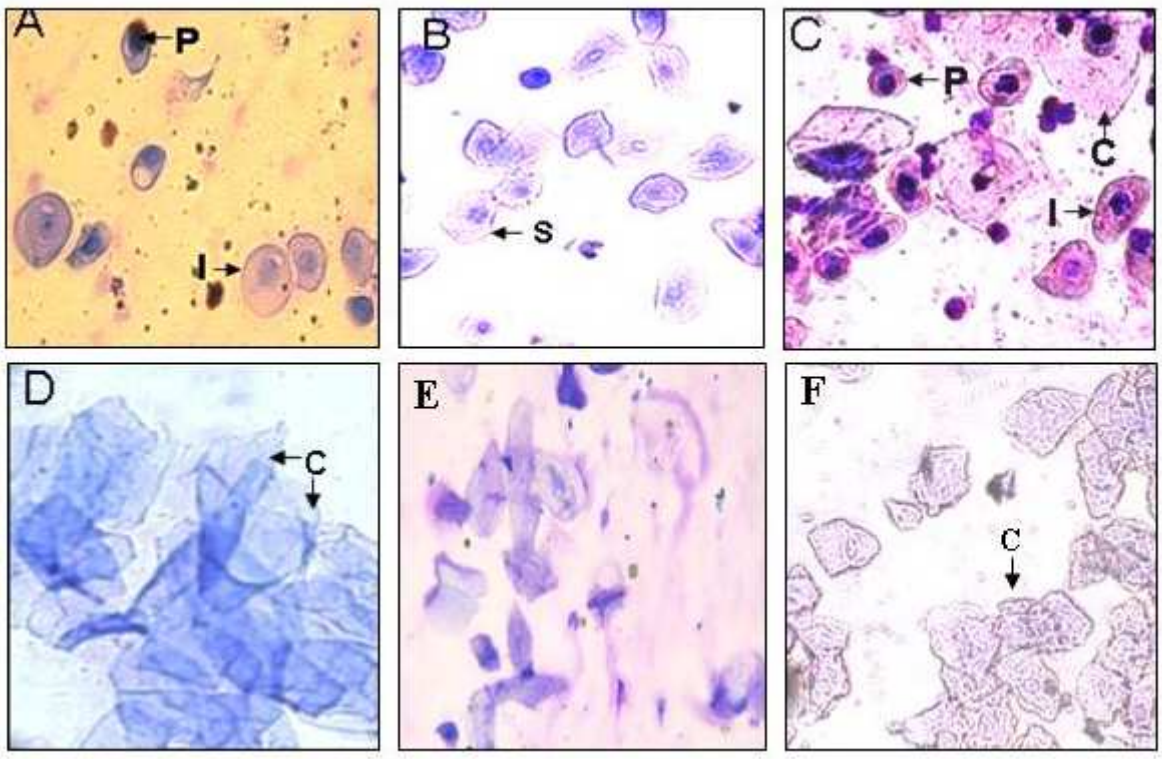

Fig. 2. Administration of TLC fraction of root extract stimulates the cell proliferation of ovariectomized female rat uterus producing parabasal (P) cells and intermediate (I) cells (Fig. 2A). The intermediate cells gradually convert into the superficial (S) cells (Fig. 2B) and cornified (C) cells (Fig. 2C) . The cornified cells of TLC fraction treated females (Fig. 2E) were elongated, smaller and deformed compared to the ovary intact estrus phase (Fig. 2D) and ovariectomized E2 treated females (Fig. 2F). (Original magnification x 40).

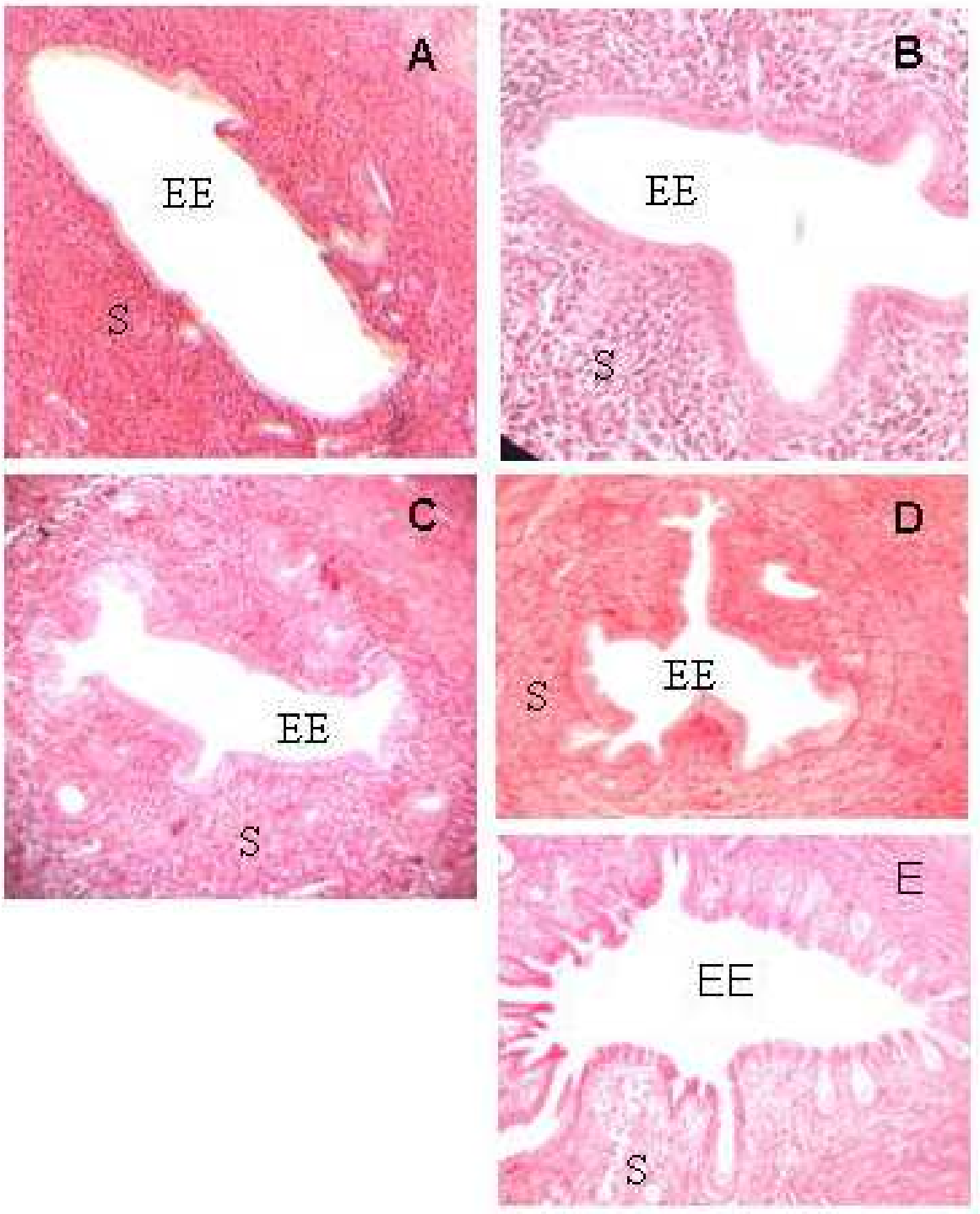

Fig. 3. Rat uterine lumen and the endometrial epithelium showed stimulation following treatment of TLC fraction of *Polygonum hydropiper.* Control OVX female uterus showed non-proliferated epithelium (A). TLC Fraction treatment to OVX female stimulated the proliferation of stromal cells as well as endometrial epithelium (B). Administration of fraction to ovary intact females showed proliferation of endometrial epithelium (D) in comparison to that of the control, ovary intact, rat uterus during estrus (C). Injection of estradiol-17β (sc) to OVX female stimulated the endometrial epithelium showing extensive proliferation (E). EE, endometrial epithelium; S, stroma. (Original magnification x 10).

cells remains to be addressed, which may throw light on mechanism of inducing infertility.

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