

Antifertility Activity of Stems of *Plumbago zeylanica* Linn. in Female Albino Rats

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ABSTRACT

In the present study, *Plumbago zeylanica* Linn. (Plumbaginaceae), one of the folk medicinal plants commonly used as antifertility agent was evaluated for its antifertility effect. Five successive solvent extracts, petroleum ether, chloroform, acetone, ethanol and water extracts, of the stems of *P. zeylanica* were studied on estrous cycle at two dose levels, 200 and 400 mg/kg respectively. Among these, only the acetone extract was found to be most effective in interrupting the normal estrous cycle of the rats ($p < 0.05$) ($p < 0.01$) ($p < 0.001$). The rats exhibited prolonged diestrous stage of the estrous cycle with consequent temporary inhibition of ovulation. The anovulatory activity was reversible on withdrawal of the extract. The effective acetone extract was further studied on estrogenic functionality in rats. The extract showed significant estrogenic and antiestrogenic activity ($p < 0.05$) ($p < 0.01$) ($p < 0.001$) compared to control. Histological studies of the uteri were carried out to confirm the estrogenic activity. The results indicated the antifertility activity of *Plumbago zeylanica* stem extract in female Wistar rats.

Keywords: *Plumbago zeylanica*, anovulatory, estrous cycle, estrogenic activity

Population control is of immense importance for individual and national welfare. Seek for the oral contraceptive agent so as to control human fertility is as old as recorded history. Even though an extensive variety of synthetic contraceptive agents are available, these cannot be used constantly due to their severe side effects [1] and for this reason an approach was pursued to identify new antifertility agents from the natural sources. Numerous indigenous drugs are been explained in folkloric Indian medicine for the management of various reproduction-related purposes. Many plant preparations are accounted to have antifertility regulation property and only a few have been tested for such effects.

Plumbago zeylanica L. (Plumbaginaceae), commonly known as Chitrak [2], grows wild and abundantly in India. The aerial parts of the plant are reported to be used in various ailments like rheumatic pain, scabies, skin diseases, wounds, inflammations [3], while the roots are reported to have antioxidant [4], CNS stimulant [5], antimicrobial [6], antiplasmodial [7], wound healing [8], hypolipidaemic, and antiatherosclerotic [9] activities. The active constituents reported in this plant are plumbagin [10], hydroxy-1, 4-naphthaquinone, sitosterol glycoside, fatty alcohol and tannins [11].

Throughout the literature survey it was found that the plumbagin present in roots of this plant is responsible for its antifertility and uterine activity [12]. In our phytochemical study it was found that the stems also contain plumbagin. In this aspect we aimed to evaluate the antifertility effect of *P. zeylanica* stems. If the drug was proven to be effective, then the uprooting of this plant for abortifacient activity could be avoided.

MATERIALS AND METHODS

Plant Material

Stems of *P. zeylanica* were collected from Kanyakumari district, T.N and positively identified by Dr. H. S. Chatree, Botanist, Govt. Arts and Science College, Mandsaur, M.P. Voucher specimen (P/006/2006/BRNCOP) was deposited in the herbarium of Department of Pharmacognosy, BRNCP, Mandsaur for future reference.

Preparation of Extracts

The stems of the plant were shade dried and powdered. The powdered material was extracted using petroleum ether (60-80°) for 72 h and successively

extracted with chloroform, acetone, ethanol and water for 72 h each in soxhlet apparatus. The extracts were evaporated under reduced pressure to obtain solid masses and their percentage yield was found to be 1.23, 1.26, 4.67, 3.65 and 19.23% respectively.

Phytochemical Screening

In order to determine the presence of alkaloids, glycosides, flavones, tannins, terpenes, sterols, saponins, fats and sugars, a preliminary phytochemical study (colour reactions) with plant extracts was performed [13].

Estimation of Plumbagin in Various Extracts

The naphthaquinone identified in the petroleum ether, chloroform and acetone extracts was further confirmed by HPTLC technique [14]. Standard solution (1 mg/ml) was prepared by dissolving 10 mg of plumbagin (National Chemicals, Baroda, India) in 10 ml of petroleum ether and 10 mg/ml concentration of sample solutions were prepared by dissolving 100 mg of extracts in 10 ml of the respective solvents. Camag HPTLC system (Switzerland) equipped with a sample applicator Linomat IV, twin trough liner development chamber, Camag Scanner III combined with integration software CATS4.06 (Switzerland) and precoated aluminium silica gel F₂₅₄ plate (Merck) were used for the study.

5 µl of (1 mg/ml) standard plumbagin and 5 µl of (10 mg/ml) sample solutions (extracts) were applied as 6 mm band width from about 1 cm of the edge of HPTLC plate using Camag Linomat IV applicator. The solvent system was chloroform-ethyl acetate-hexane-acetic acid (10:5:5:0.3). The chromatogram was developed and scanned at 366 nm using TLC scanner.

Animals

Healthy Animals obtained from Calcutta fishing agency, Indore, with no prior drug treatment were used for the present studies. Female albino rats (Wistar strain weighing 150-200 g) were used for anovulatory activity and immature female rats (Wistar strain) 21-23 days old were used for estrogenic activity. The animals were housed in standard environmental conditions of temperature (21 ± 2°C), humidity (55 ± 10%) and a 12 h light-dark cycle. Rats were supplied with standard pellet diet and water *ad libitum*. The animals were acclimatized to laboratory hygienic conditions for 10 days before starting the experiment. Animal study was performed in Division of Pharmacology, B R Nahata College of Pharmacy, Mandsaur with due permission from Institutional Animal Ethics Committee (Reg No.-918/ac/05/CPCSEA).

Acute Toxicity Studies

The acute toxicity test of the extracts was determined according to the Organization for Economic Co-operation and development (OECD) guidelines No. 420. Female Wistar rats (150–180 g) were used for this

study. After the sighting study, starting dose of 2000 mg/kg (p.o.) of the test samples were given to various extract groups containing 5 animals in each groups. The treated animals were monitored for 14 days for mortality and various responses like behavioural, neurological and autonomic responses. No death was observed till the end of the study. The test samples were found to be safe up to the dose of 2000 mg/kg and from the results 200 and 400 mg/kg dose levels were selected for further experimentation [15].

Antifertility Activity

Anovulatory Activity: Experiments was carried out in female Wistar rats weighing (150-200 g) [16]. Vaginal smear of each rat was examined daily morning between 9-10 A.M for 15 days to select the animals showing regular cycles (4–5 d). The selected rats were divided into 11 groups of six animals each. The extracts were administered orally for 5 days to cover one regular estrous cycle. Group I received vehicle (1% Tween 80, p.o. daily) and served as control. Groups II to XI received petroleum ether, chloroform, acetone, ethanol and aqueous extracts of *P. zeylanica* stems at 200 mg/kg and 400 mg/kg body weight. Vaginal smear from each animal was observed every morning between 9-10 A.M for 5 days of treatment and during the following 15 days in the morning.

Estrogenic and Antiestrogenic Activity: The extract, which showed anovulatory activity, was further studied for the estrogenic and antiestrogenic activity [17]. Immature female Wistar strain rats, 21–23 days old, weighing between 35-45 g, was divided into 6 groups (n = 6). The first group served as control and received only vehicle (1% Tween 80). The second group received ethinyl estradiol (standard) (Rajesh Chemicals, Indore, India) in distilled water using Tween-80, (1%) at a dose of 0.02 mg/kg body weight [18]. The third and fourth groups received acetone extract of *P. zeylanica* at two dose levels, 200 and 400 mg/kg body weight respectively. The groups, five and the six received ethinyl estradiol in addition to a test dose of acetone extract of the plant at the same dose. All the above treatments were given for 3 days (p.o.). On the fourth day, the rats were sacrificed by decapitation, the uteri were dissected out and surrounding tissues were removed. The uteri were blotted on filter papers and weighed quickly on a sensitive balance and fixed in Bouin's solution for 24 h. The paraffin embedded tissues were cut at 6 µm and stained with haematoxylin-eosin solution for histological observations.

Statistical Analysis

The data was statistically analyzed and expressed as mean ± SEM. Statistical analysis of the variance between control and experimental values was done by student's *t*-test.

Table 1. Preliminary Phytochemical Studies on Various Extract of *P. zeylanica*

Plant extracts	Constituents
Pz-P	Fats, steroids and napthaquinone
Pz-C	Steroids and napthaquinone
Pz-A	Tannins, flavonoids, triterpenoids and napthaquinone
Pz-E	Carbohydrates, glycosides, tannins, flavonoids and saponins
Pz-W	Carbohydrates, glycosides, tannins, flavonoids and saponins

Pz- *Plumbago zeylanica*, P-petroleum ether extract, C-chloroform extract, A-acetone extract, E-ethanolic extract, W-aqueous extract.

Table 2. Effect of Treatment of Various Extracts of Stems on Estrous Cycle for 5 Days in Rat

Treatment	Dose (mg/kg)	Duration of cycle (Days)	Duration of different phases of estrous cycle (days)			
			Proestrous	Estrous	Metestrous	Diestrous
Control	--	4.32 ± 0.22	0.83 ± 0.17	0.83 ± 0.17	0.83 ± 0.31	1.83 ± 0.40
Pz. Pet.ether	200	4.98 ± 0.47	0.66 ± 0.42	0.83 ± 0.40	1.66 ± 0.61	1.83 ± 0.47
	400	4.32 ± 0.48	0.83 ± 0.44	1.16 ± 0.65	1.66 ± 0.33	1.66 ± 0.42
Pz. Chloroform	200	4.16 ± 0.33	0.66 ± 0.21	1.00 ± 0.36	1.00 ± 0.36	1.50 ± 0.50
	400	4.48 ± 0.44	0.66 ± 0.54	1.33 ± 0.42	1.16 ± 0.33	1.33 ± 0.49
Pz. Acetone	200	5.15 ± 0.31*	0.66 ± 0.30	0.50 ± 0.34	0.83 ± 0.42	3.16 ± 0.30*
	400	5.85 ± 0.34**	0.83 ± 0.54	0.33 ± 0.21*	0.33 ± 0.43	4.36 ± 0.21***#
Pz. Ethanol	200	3.99 ± 0.35	0.83 ± 0.36	0.35 ± 0.42	1.00 ± 0.25	1.83 ± 0.40
	400	4.48 ± 0.36	1.16 ± 0.21	0.83 ± 0.30	0.83 ± 0.54	1.66 ± 0.42
Pz. Aqueous	200	4.32 ± 0.52	0.66 ± 0.54	1.00 ± 0.36	0.66 ± 0.60	2.00 ± 0.59
	400	4.81 ± 0.49	0.66 ± 0.44	1.33 ± 0.49	1.16 ± 0.54	2.33 ± 0.49

Pz- *P. zeylanica*

Values are expressed in mean ± SEM, n=6, *P<0.05, **P<0.01, ***P<0.001 Vs Control #P<0.01 Vs Pz. Acetone 200 (Students 't' test)

RESULTS

Phytochemical Screening

The phytochemical screening of different extracts revealed the presence of various constituents as shown in table 1. The R_f value of the standard plumbagin and the plumbagin peak of the extracts were found to be 0.94 (Fig 1). The quantity of plumbagin was estimated by comparing the peak area of the standard with that of the extracts. The amount of plumbagin present in petroleum ether, chloroform and acetone extracts of stems was found to be 1.94% (Fig 2), 0.54% (Fig 3) and 2.65% (Fig 4) respectively.

Acute Toxicity Studies

No mortality and changes in the behavioural, neurological and autonomic responses were observed in the treatment groups up to 2000 mg/kg body weight. From the results 200 mg/kg and 400 mg/kg doses were chosen for further experimentation.

Effect of Extract on the Estrous Cycle of Rats

The present study indicated that the acetone extract of *P. zeylanica* stems were responsible for the antifertility effect. Rats showing routine estrous cycle (Fig 5) were used. Treatment of rats with acetone extract for 5 days prolonged the estrous cycle significantly ($p<0.05$) ($p<0.01$) ($p<0.001$) compared to

control as indicated in Table 2. The estrous cycle in rats treated with acetone extract reduced duration of estrous and metestrous phases and on the other hand it was also characterized by a prolongation of the diestrous phase. Among the two doses of acetone extract, significant difference was found only in the diestrous phase. The estrous cycle was found to be reversible on withdrawal of the treatment (Table 3). Apart from the acetone extract, remaining all the extracts were found inactive.

The effect of acetone extract of *P. zeylanica* stems on immature rats uteri are shown in Table 4. Oral administration of the extract at 200 and 400 mg/kg body weight caused significant increase in the uterine weight of immature rats ($p<0.05$) ($p<0.01$) ($p<0.001$) when compared to control. No significant difference in estrogenic and antiestrogenic activity was observed between the two doses of the acetone extracts. The height of the endometrial epithelium and thickness of the endometrium was significantly increased when compared to the control rats (Fig 6) (Fig 7). The epithelium of the endometrium consisted of spindle shaped cells with basal nuclei and the endometrial glands were dilated. The stroma consisted of loose and edematous fibroblast type cells with oedema (Fig 8). The control rats showed closed vagina, at the same time the treated rats showed an open vagina.

The administration of acetone extract aggravated a significant increase in the uterine wet weight signifying

Table 3. Effect of Post- Treatment of Various Extracts of Stem on Estrous Cycle for 15 Days in Rats; *Estrogenic and Antiestrogenic Activity*

Treatment	Dose (mg/kg)	Duration of cycle (Days)	Duration of different phases of estrous cycle (days)			
			Proestrous	Estrous	Metestrous	Diestrous
Control	---	4.32 ± 0.22	0.83 ± 0.17	0.83 ± 0.17	0.83 ± 0.31	1.83 ± 0.40
Pz. Pet.ether	200	4.82 ± 0.33	1.16 ± 0.17	1.00 ± 0.36	0.83 ± 0.41	1.83 ± 0.40
	400	4.48 ± 0.37	0.83 ± 0.30	0.83 ± 0.47	1.16 ± 0.40	1.66 ± 0.33
Pz. Chloroform	200	4.82 ± 0.43	1.16 ± 0.40	1.00 ± 0.33	1.00 ± 0.44	1.66 ± 0.55
	400	4.48 ± 0.37	0.66 ± 0.33	0.83 ± 0.40	1.16 ± 0.30	1.83 ± 0.47
Pz. Acetone	200	4.82 ± 0.52	0.83 ± 0.44	1.33 ± 0.66	0.66 ± 0.49	2.00 ± 0.51
	400	4.82 ± 0.42	1.00 ± 0.63	0.66 ± 0.33	0.83 ± 0.42	2.33 ± 0.21
Pz. Ethanol	200	3.82 ± 0.50	1.00 ± 0.44	0.66 ± 0.47	1.00 ± 0.36	1.16 ± 0.55
	400	4.15 ± 0.42	1.16 ± 0.40	1.16 ± 0.47	0.83 ± 0.50	1.00 ± 0.33
Pz. Aqueous	200	4.99 ± 0.39	1.00 ± 0.36	0.83 ± 0.40	1.16 ± 0.36	2.00 ± 0.44
	400	4.82 ± 0.35	0.66 ± 0.33	0.83 ± 0.31	1.00 ± 0.46	2.33 ± 0.33

Pz- *P. zeylanica*, Values are expressed in mean ± SEM, n=6, values are non significant Vs Control (Students 't' test)

Table 4. Estrogenic and Antiestrogenic Activity of Acetone Extract of *P. zeylanica* Stem

Groups	Dose (mg/kg body weight)	Uterine weight (mg/100 g body weight)
Control	(Tween-80, 1%)	45.50 ± 2.27
Ethinyl estradiol	0.02	142.00 ± 4.83 ^{***}
Pz. Acetone	200	60.17 ± 2.98 ^{**}
Pz. Acetone	400	66.19 ± 3.48 ^{***}
Ethinyl estradiol + Pz. Acetone	0.02 + 200	128.13 ± 3.70 ^{***†}
Ethinyl estradiol + Pz. Acetone	0.02 + 400	120.87 ± 3.97 ^{***†}

Pz- *P. zeylanica*

Values are expressed in mean ± SEM, n=6, ^{**}P<0.01, ^{***}P<0.001 Vs Control,

[†]P<0.05 Vs Ethinyl estradiol (Students 't' test)

the estrogenic activity but when given together with ethinyl estradiol it lowered the effect of estrogenic activity produced by ethinyl estradiol alone (Table 4).

DISCUSSION

The acetone extract of stems of *P. zeylanica* exhibited significant ($p<0.05$) ($p<0.01$) ($p<0.001$) antifertility activity when compared with control. The duration of estrous cycle in rats is normally 4-5 days. When observed using a microscope, the vaginal smear during a routine rat estrous cycle (Fig 5) shows three cell types and depending upon the presence and absence of these cell types and their relative proportion, the stages of estrous cycle of rats can be determined. Among the five extracts tested for anovulatory activity, only acetone extract produced a temporary and reversible modification on the estrous cycle. The prolongation in diestrous phase explains the remote possibility of the rats to get pregnant. The reversible nature of the antifertility activity of the extract is explained through the observation that there was no significant change in the diestrous and the estrous cycle after withdrawal of the extract from those of the control. As a result the extract provoked inhibition of the ovulation with consequent reduction of the cyclicity. Estrous cycle and the shift in different stages are mainly governed by the synthesis of ovarian estrogen which in turn, is controlled by the secretion of pituitary gonadotropins and hypothalamic-releasing factor [16].

As the acetone extract showed anovulatory activity, it was further studied for its estrogenic and antiestrogenic activity. The extract also exhibited estrogenic activity as shown by the significant increase in diameter of uterus, uterine weight, thickness of the endometrial epithelium when compared to the control. It was also observed that the acetone extract suppressed the action of ethinyl estradiol when administered together. The extract showed a significant estrogen like activity when given alone but with ethinyl estradiol it exhibited slight antiestrogenic nature. This indicates that extract acted as competitive antagonist to the much potent ethinyl estradiol [18].

Preliminary phytochemical studies indicated the presence of tannins, flavonoids, triterpenoids and naphthaquinone in the acetone extract. According to the literatures flavonoids and plumbagin (naphthaquinone) are known to exhibit antifertility activity [1,18,19]. In our study also, the activity may be due to the presence of flavonoids and naphthaquinones. The petroleum ether and chloroform extracts too showed the presence of naphthaquinones, but the activity was not found. The possible reason may be this that the sufficient concentration or the particular active constituent (flavonoid or naphthaquinone) responsible for antifertility activity is not present in petroleum ether, chloroform, ethanol and aqueous extract or the activity of acetone extract may be due to the synergism produced by naphthaquinone along with flavonoid.

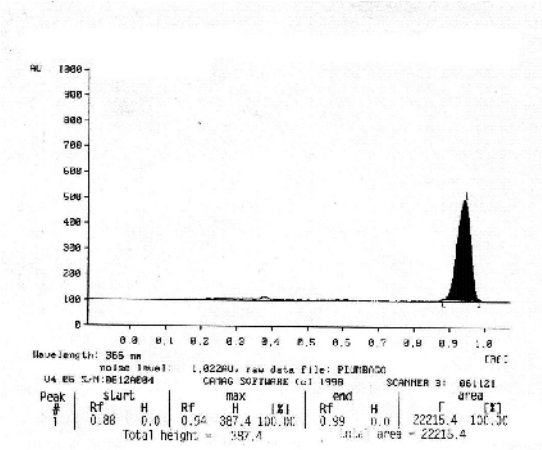


Fig 1. HPTLC of standard plumbagin

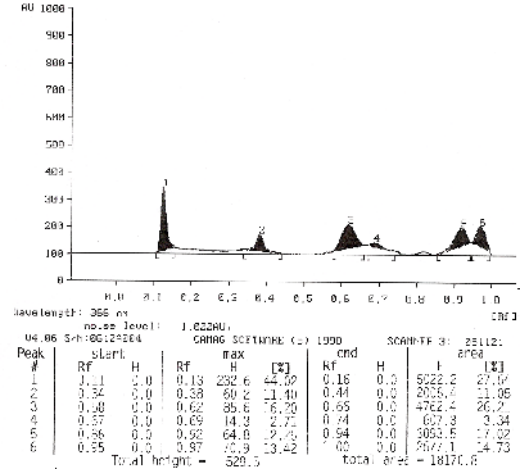


Fig 2. HPTLC of petroleum ether extract of *Plumbago zeylanica* stem.

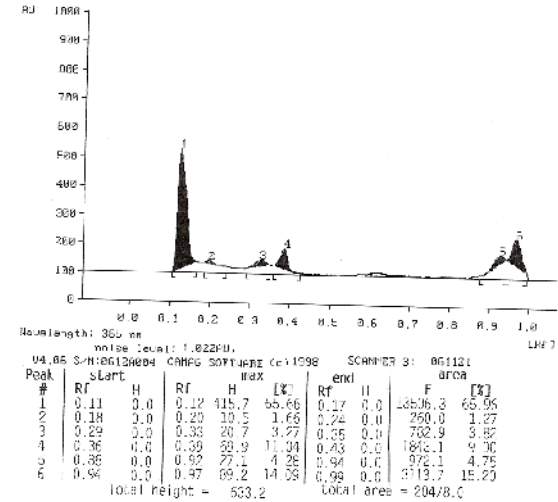


Fig 3. HPTLC of chloroform extract of *Plumbago zeylanica* stem.

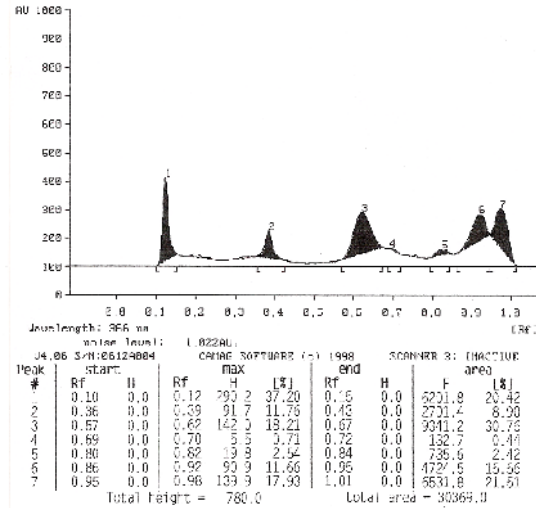


Fig 4. HPTLC of acetone extract of *Plumbago zeylanica* stem.

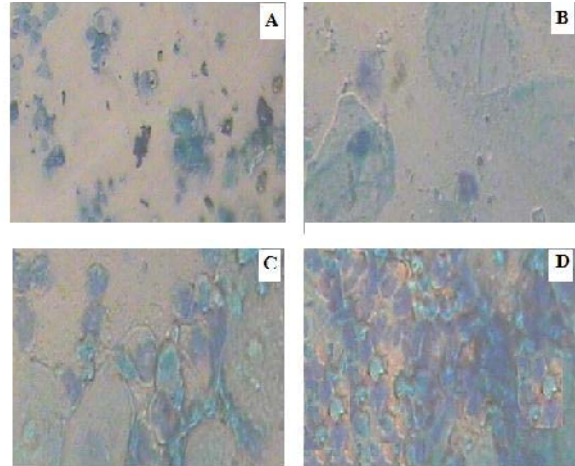


Fig 5. Vaginal smear of a rat during the estrous cycle (four days). Stained with 5% aqueous methylene blue for 10 minutes, as per the standard literatures. CXR camera x 100. Control: 1st day (A), 2nd day (B), 3rd day (C), 4th day (D).

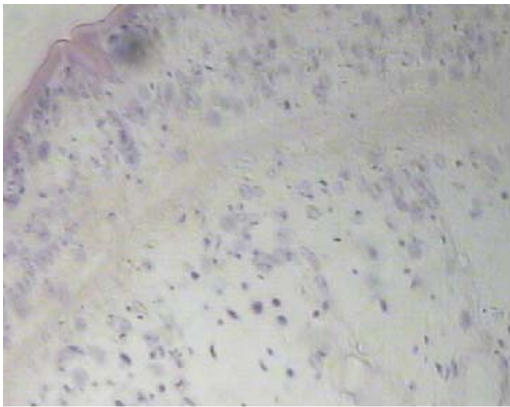


Fig 6. Photomicrograph of transverse section of the uterus (x 45) of control Wistar strain rat, stained with haematoxylin-eosin.

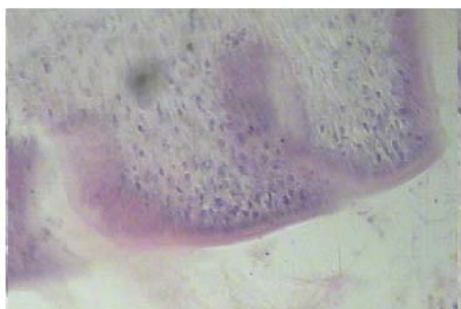


Fig 7. Photomicrograph of transverse section of the uterus (x 45) of a rat treated with ethinyl oestradiol (0.02 mg/kg p.o.), showing endometrial proliferation stained with haematoxylin-eosin.

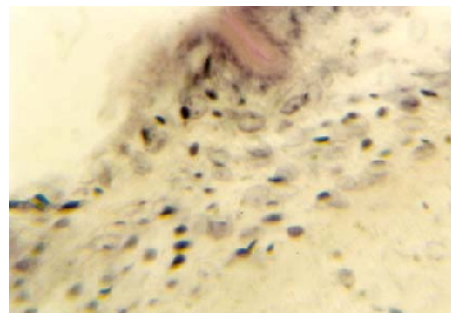


Fig 8. Photomicrograph of transverse section of the uterus (x 45) of a rat treated with the acetone extract (400 mg/kg p.o.), showing sloughing of the endometrial lining of epithelium stained with haematoxylin-eosin.

CONCLUSION

The results of the present study conclude that the acetone extract of *P. zeylanica* stems have significant antifertility activity and it could be used as an alternative medicine instead of roots. The extract of this plant can further be developed into a contraceptive and uprooting of this plant could be avoided.

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