Antifertility Activity of Ethanolic and Aqueous Extracts of *Tabernaemontana divaricate* R.Br. ex Roem. & Schult on Female Wistar Rats: Rising Approaches of Herbal Contraception

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Abstract

The practice of traditional medicine for the control of fertility in most parts of India is based on the uses of plant medicines for many years. T*abernaemontana divaricata* (T. *divaricata*) from Apocynaceae family offers the traditional folklore medicinal benefits such as an anti-epileptic, antifertility, anti-inflammatory, anti-mania, brain tonic, and anti-oxidant. The aim of the present study was to evaluate the antifertility effect of aqueous and ethanolic extract of T. *divaricata* roots in female Wistar rats. The anti-fertility activity of the extracts was evaluated using five experimental animal models: 1) Estrogenic activity was carried out in immature female rats using ethinyl estradiol as standard. The evaluation parameters include changes in uterine weight and histopathology of uterus. 2) Anti-implantation and early abortifacient activity was performed in female Wistar rats. The number of implants and resorbtions were compared to vehicle control.3) Anti-ovulatory activity 4) Study of estrous cycle. 5) Study of reproductive hormones. In estrogenic activity, the ethanolic and aqueous extracts were offered significant estrogen-like activity at 400 mg/kg, p.o. by increasing the uterine weight compared to vehicle control group. In Anti-implantation and early abortifacient activity study, ethanolic (400 mg/kg, p.o.) showed significant effect and it was evident by decrease in the number of implants and increase in the number of resorbtions compared to vehicle control group. In anti-ovulatory activity the ethanolic extract of *T. divaricata* at 150 mg/kg and 300 mg/kg had no effect on ovarian weight or cholesterol levels as compared to the vehicle-treated group. In estrous cycle with a significant increase in diestrum phase length and estrus stage extension at the higher dose (450 mg/kg body weight/day). In reproductive hormones study's cytological, hormonal, and reproductive screening, the ethanolic extract of *T. divaricata* may have an antifertility effect. The above results revealed the potential, reversible female antifertility effect of ethanolic extract of *T. divaricata* root.

Keywords: T*abernaemontana divaricata*, Estrogenic activity, Anti-fertility effect, Anti-implantation activity, Abortifacient effect, Anti-ovulatory activity

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Introduction:

This century search for antifertility agents is continue to tackle the problem of population explosion that may lead too economic and health impact on the family in particular and the society in general especially in developing countries like Ethiopia where the population growth is very high [1]. The population of India is multiplying day by day at an alarming rate and has crossed on1.5 billion. Fertility regulation has therefore become the major concern of the people of all walks of life. Fertility control is an issue of global and national public health concern. There is a global need to support individuals in family planning due to the increasing growth rate of the world's population with its negative impact on the environment, economic growth, and poverty reduction in underdeveloped countries. About 90% of the world's contraceptive users are women. Although considerable progress has been made in the development of highly effective, acceptable, and reversible methods of contraception in females, progress and possibilities on males are still slow and limited. Aware of this responsibility, health organizations and pharmaceutical companies continue to financially support or actively pursue research toward new contraceptive approaches [2]. Current methods of contraception result in an unacceptable rate of unintended pregnancies and many side effects also. In recent years, plants are practice over synthetic contraceptive drug because plants are easily available, economic and devoid of harmful and no side effects [3]. Already several scientific papers have been published related to fertility control from medicinal plants. *T. divaricata* is one of such plants that has been advocated as a traditional medicine for family planning. Chewing of seven flowers of the plant, daily for two months after menses, with water in the morning will check the pregnancy for a year and may be continued for longer period for prolonging its effect [4, 5]. However, there is a paucity of scientific evidence for its usage as a anti-fertility agent, Henceforth the present study was undertaken to evaluate the anti-fertility effect of root extracts of *T. divaricata* using different experimental models.

Material and method

Plant materials

The roots of *T. divaricata* were collected from local area of Bhopal (M.P.) respectively. The sample was identified by senior Botanist Dr. Saba Naaz, Professor of department of Botany, Safia College of Arts and Science, peer gate Bhopal. A herbarium of plants was submitted to the specimen library of Safia College of Arts and Science, peer gate Bhopal and The specimen voucher no. of *T. divaricata* is **256//Saif/Sci/Clg/Bpl,**

Chemical reagents

All the chemicals used in this study were obtained from Hi Media Laboratories Pvt. Ltd. (Mumbai, India), Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India).All the chemicals and solvent used in this study were of analytical grade.

Defatting of plant material

Powdered plants material of *T. divaricata* was shade dried at room temperature. The shade dried plants material was coarsely powdered and subjected to extraction with petroleum ether using soxhlation method. The extraction was continued till the defatting of the material had taken place.

Extraction by soxhlation process

Defatted powdered of *T. divaricata* were exhaustively extracted with aqueous and ethanol solvent using soxhlation method. The extract was evaporated above their boiling points. The dried crude concentrated extract was weighed to calculate the extractive yield then transferred to glass vials (6 ×2 cm) and stored in a refrigerator (4°C), till used for analysis[6].

Animals used

This study included albino rats (Wister strain), immature female rats (Wister strain) measuring 40-60gms and albino mice weighing 20-25gms of either sex and laboratory developed virgin female Swiss albino mice aged 85-100 days weighing between 22-25 g, demonstrating consistent estrous cycle. Under laboratory circumstances, the animals were acclimatised for 10 days. They were housed in polypropylene cages and kept at a constant temperature of 27°C, relative humidity of 65°C, and a 12 hour light/dark cycle. Rodent pellet diet (Gold Mohur Lipton India Ltd.) and ad libitum water were provided to the animals. The Institutional Animal Ethical Committee gave its approval for the research to be conducted on animals (IAEC). The registration number for the Institutional Animal Ethical Committee is (Reg. No. 1824/PO/RcBi/S/15/CPCSEA), and the animal experiment proposal number is IAEC- PBRI/IAEC/PN-21028.

Acute oral toxicity

The Acute oral toxicity studies were carried out as per the guidelines of Organization for Economic Co-operation and Development (OECD- 423), Ministry of Social Justice and Empowerment, Government of India [7, 8].

Antifertility study

Antifertility activity of plant extracts was evaluated with the help of estrogenic activity, anti- implantation activity, abortifacient effect, anti-ovulatory activity, study of estrous cycle was also performed, which further supported by the hormonal analysis [9].

Estrogenic Activity in Immature Female Rats

Immature female rats of Wister strain 21–23 days old and weighing 40–60 g were used. They were divided into six groups of six animals each. The various groups were treated as follows: Group I: control (saline solution) p.o.

Group II: reference standard (ethinyl estradiol0.02mg/kg, p.o.),
Group III: ethanolic root extract of *TD* (200mg/kg,p.o.),
Group IV: ethanolic root extract of *TD* (400mg/kg, p.o.),
Group V: aqueous root extract of *TD* (200mg/kg, p.o.),
Group VI: aqueous root extract of *TD* (400mg/kg, p.o.).

The therapy was administered for six days, 24 hours following the last treatment; all of the animals were decapitated, and the uteri were taken out, cleansed of sticky tissue, blotted on filter paper, and promptly weighed on a sensitive balance. The tissues were dehydrated in alcohol and embedded in paraffin after being fixed in Bouin's fixative for 24 hours. For histological observations, the paraffin blocks were sectioned at 6 and stained with hematoxylineosin solution (H and E Stain) [10, 11].

Anti-Implantation Activity

Female rats in the proestrus period were housed in a 2:1 ratio with male rats with confirmed fertility. The next morning, the dense aggregates of spermatozoa in their vaginal smear were separated from their male partners. For the experiment, only rats with typical estrous cycles were chosen. female rats were checked for signs of copulation. Animals with

The animals were placed into six groups, each with six animals [12]. The following treatment was given to the respective groups:

Group I: control (saline solution) p.o.
Group II: reference standard (ethinyl estradiol0.02mg/kg, p.o.),
Group III: ethanolic root extract of *TD* (200mg/kg,p.o.),
Group IV: ethanolic root extract of *TD* (400mg/kg, p.o.),
Group V: aqueous root extract of *TD* (200mg/kg, p.o.),
Group VI: aqueous root extract of *TD* (400mg/kg, p.o.).

Anti ovulatory activity

Female rats were employed in this study, which were separated into three groups (n=6), fasted overnight, and given unlimited access to water. Female rats were given different doses of the test medication (150 and 300 mg/kg, p.o.) in different groups. Each rat's vaginal smear was inspected every day for 15 days, and those that showed three regular cycles were utilised. The drugs and vehicle were given orally every day for 15 days starting in the estrous period. Group I functioned as the control group, receiving simply the vehicle (1 percent gum acacia, p.o. daily). The ethanolic extract of J. Carcus was given to groups II and III at 150 and 300 mg/kg, respectively. Three regular estrous cycles were to be covered by the 15-day therapy. Each animal's vaginal smear was inspected every morning between 9 and 10 a.m. The rats in each group were anaesthetized and slaughtered on the 16th day, 24 hours following the previous treatment. Ovaries and uteri were dissected and weighed on a sensitive balance after being liberated of excess deposits. Each animal's ovary was processed for biochemical cholesterol analysis [13]. The following treatment was given to the respective groups:

Group I: vehicle (1% gum acacia, p.o. daily)
Group II: ethanolic root extract of *TD* (150 mg/kg, p.o.) Group III: ethanolic root extract of *TD* (300 mg/kg,p.o.),

Study of estrous cycle

The animals were placed into three groups, each of which included five animals. One group was the control group, and they were given the vehicle orally for 21 days. The other two groups were given a 250, 450 mg/kg animal body weight/day dosage of dry ethanolic extract of roots orally. The estrous cycle was investigated using a stained preparation of the animals' veginal smear. The extract was removed from the mice after 21 days of therapy, and the estrous cycle was investigated for another 21 days, i.e. the post-extract phase [14].

Study of reproductive outcome in mice

As previously stated, three groups of adult female mice (five animals/group) were chosen. For 8 days, two groups got root extract, while the control group received vehicle. The medication was continued for another 21 days after all of the experimental mice had mated with adult fertile partner mice. In all experimental groups, the number of litters was assessed after one gestation period. The litters were allowed to develop, and the growth of the extract-administered group's litters was compared to that of the control group's litters. The extract was given consistently for 21 days before being taken away from the trial. Animals were permitted to breed with male mice after 21 days of extract withdrawal. Following the completion of one gestation period, the number of litters was calculated [15].

Administration of test materials

Two experimental groups of albino mice were given the root extract orally at dosages of 250 mg/kg body weight/day and 450 mg/kg body weight/day. The dose for each group was established based on ethno medical applications of the herb for birth control and the human dose. The extract's hepatotoxicity was determined by performing liver function tests at regular intervals. Serum Glutamate Oxaloacetate Transaminase (SGOT) and Serum Glutamate Pyruvate Transaminase (SGPT) levels in mice given a dose of 250 mg/kg body weight/days did not alter significantly ($p > 0.05$). Initially, the other dose of 500 mg/kg body weight/days was chosen (which was double the first dose). However, following 10 days of extract treatment, the mice's SGOT and SGPT levels were found to be significantly higher. Since a result, a lower dosage of 450 mg/kg body weight/days was chosen, as it had no effect on transaminase activity during the research period.

Study of reproductive hormones

Blood samples were taken from the animal's caudal vein at all stages of the estrous cycle. ELISA micro well kits were used to assess the amounts of FSH, LH, prolactin, 17 estradiol, and 17 0H progesterone in the blood [16].

Statistical data

All values are expressed as mean ± SEM. Means were statistically analyzed by one-way analysis of variance (ANOVA) and values of $P < 0.05$ were considered statistically significant.

Results and discussion

Compared with motor control, ethanolic treatment (200 and 400 mg / kg, p.o.) and aqueous extract (200 and 400 mg / kg, p.o.) resulted in a significant increase in uterine weight in a dose- dependent manner. At 400 mg / kg p.o., the estrogenic action of liquid excretion was equivalent to that of the normal reference of ethynil estradiol (0.02 mg / kg p.o.). In addition, ethanolic extraction has higher estrogenic activity

than the reference standard ethynil estradiol at 400 mg / kg. The extract significantly increased uterine weights, and the results were also linked and confirmed by histological studies. Ethanol extraction (400 mg / kg, p.o.) resulted in a significant increase in luminal epithelium length, loose and edemators stroma, and regenerative uterine glands, while fluid excretion (400 mg / kg, p.o.) resulted in a moderate increase in luminal height of the epithelium, loose stroma and edemators, and regenerated uterine glands. Histological architecture confirmed the results obtained by the removal of the uterine weight of immature female rats. Histopathological investigations were performed to determine what changes occurred as a result of the prescribed treatment. Normal uterine anatomy may be seen on the part of the control group. It means more epithelial cells with less secretion. Ethanolic discharge treatment has resulted in an increase in the length of the luminal epithelium, a loose and edematous stroma, and uterine glands activated in the treated groups Table 1 & Figure 1-6. Anti- implantation activity is measured on day 10 of pregnancy as a percentage decrease in the number of implantations in the uterus, and early abortifacient activity is measured on day 18 as the number of resorbed implants divided by the total number of implants. By lowering the number of implantation sites and displaying significant resorption of existing implants, the ethanolic and aqueous extracts revealed significant and dependent anti-implantation and early abortifacient effect in comparison to the vehicle control. At 400 mg/kg p.o., the ethanolic extract was found to have 74.27 percent anti-fertility activity, which was shown to be more potent than the aqueous extract, which had 46.78 percent anti-fertility activity. The findings are shown in Table 2. The ethanolic extract of *T. divaricata* at 150mg/kg and 300mg/kg had no effect on ovarian weight or

cholesterol levels as compared to the vehicle-treated group Table 3. According to the results of the current study's cytological, hormonal, and reproductive screening, the ethanolic extract of T. divaricata may have an antifertility effect Table 4. Mice were administered extract dosages of 250 and 450 mg/kg body weight/day for 21 days, resulting in a delayed estrous cycle with a significant increase in diestrus phase length (Table 5) and estrus stage extension at the higher dose (450 mg/kg body weight/day).

Table 1: Effect of *T. divaricata* extracts on uterine weight of immature female rats

S. No.	Group	Extracts / Drug	Dose (mg/kg)	Uterine weight (mg)
1	I	Control (vehicle)	--	215.15 ± 22.79
2	II	Ethynilestradiol (Standard)	0.02	285.12 ± 24.50*
3	III	Ethanolic Extract	200	324.15 ± 08.88***
4	IV	Ethanolic Extract	400	354.57 ± 11.32**
5	V	Aqueous Extract	200	283.14 ± 24.50*
6	VI	Aqueous Extract	400	293.5 ± 11.30*

Values are mean±SEM *(n=6).* *P < 0.05, **P < 0.01, ***P < 0.001 as compare to control group.

Table 2: Effect of *T. divaricata* root extracts on anti-implantation and early abortifacient

activity in rats ($x \pm s$, $n = 6$)

S. No.	Treatment/dose	% Anti-implantation activity	% Early Abortifacient activity	% Anti-fertility activity
1	Vehicle control	0	0	0
2	Ethanolic extract 200 mg/kg, p.o.	25.45 ± 0.58	2.86 ± 0.69**	27.48 ± 0.65
3	Ethanolic extract 400 mg/kg, p.o.	44.97 ± 0.40	5.72 ± 2.53***	75.28 ± 0.29
4	Aqueous extract 200 mg/kg, p.o.	28.42 ± 0.39	3.06 ± 0.43**	34.31 ± 0.17
5	Aqueous extract 400 mg/kg, p.o.	64.71 ± 0.44	10.09 ± 4.15***	47.79 ± 0.29

** *P* <6 0.01, ****P* < 0.001 *vs* vehicle control

Table 3: Effect of the ethanolic extract of *T. divaricata* on ovarian weight and cholesterol level

S. No.	Treatment and dose (mg/kg p.o.)	Ovarian weight in mg/100g body weight	Cholesterol level in ovary (mg/50mg)
1	Gum acacia (1 %, 1 ml/kg)	39.46±0.61	0.39±0.06
2	Ethanolic extract (150)	41.55±0.84*	0.45±0.10
3	Ethanolic extract (300)	38.25±1.33	0.44±0.09**

*P<0.05, **P<0.01 compared with vehicle treated control group

Table 4: Hormone levels in various groups of animals during the study

S. No.	Different hormones	Groups	Stages of estrous cycle			
			Proestrus	**Estrus**	**Metestrus**	**Diestrus**
1	LH mlU/ml	Control	9.64 ± 0.21	4.48 ± 0.13	0.78 ± 0.23	0.87 ± 0.12
2		250 mg/kg bw/d	7.01 ± 0.21	3.38 ± 0.65	0.79 ± 0.12	1.08 ± 0.42
3		450 mg/kg bw/d	6.02 ± 0.41	2.43 ± 0.15	0.68 ± 0.18	0.48 ± 0.34
4	FSH mlU/ml	Control	7.18 ± 0.62	8.3 ± 0.43	3.37 ± 0.24	4.35 ± 0.57
5		250 mg/kg bw/d	8.38 ± 0.28	10.31 ± 0.25	3.05 ± 0.12	6.12 ± 0.24
6		450 mg/kg bw/d	5.86 ± 0.34	4.42 ± 0.44	3.24 ± 0.56	2.64 ± 0.34
7	Prolactin ng/ml	Control	30.60 ± 0.41	24.44 ± 0.53	12.10 ± 0.76	15.30 ± 0.15
8		250 mg/kg bw/d	34.22 ± 0.15	27.35 ± 0.52	10.18 ± 0.59	19.48 ± 0.25
9		450 mg/kg bw/d	43.56 ± 0.23	26.28 ± 0.66	10.47 ± 0.56	19.37 ± 0.75
10	Estradiol pg/ml	Control	806.42 ± 0.53	712.04 ± 0.44	274.00 ± 0.16	283.54 ± 0.45
11		250 mg/kg bw/d	667.21 ± 0.57	461.55 ± 0.15	264.19 ± 0.23	265.19 ± 0.87
12		450 mg/kg bw/d	944.55 ± 0.23	512.35 ± 0.66	215.08 ± 0.34	200.52 ± 0,62
13	17 OH Progesterone ng/ml	Control	10.55 ± 0.23	12.80 ± 0.48	16.61 ± 0.25	21.84 ± 0.68
14		450 mg/kg bw/d	11.52 ± 0.21	13.15 ± 0.75	15.47 ± 0.56	23.12 ± 0.53

N = 6 data are Mean±SEM

Table 5: Effect of ethanolic extract of *T. divaricata* on the estrous cycle of mice for 21 days and number of litters produced in different groups of mice

S. No.	Groups	Duration of estrous cycle (days)	Duration of different phages of estrous cycle (days)				No. of litters
			Proestrus (days)	Estrus (days)	Metestrus (days)	Diestrus (days)	
1	Group 1: Control	4.63 ± 0.14	0.97 ± 0.07	0.99 ± 0.19	0.87 ± 0.15	1.98 ± 0.21	7.9 ± 0.12
2	Group 2: 250 mg/kg bw/d	4.87 ± 0.52	0.56 ± 0.13	1.25 ± 0.23	0.63 ± 0.25	3.72 ± 0.45	3.8 ± 0.34
3	Group 3: Post-treatment of 250 mg/kg bw/d	4.62 ± 0.42	0.86 ± 0.56	1.23 ± 0.23	0.86 ± 0.13	1.85 ± 0.21	7.4 ± 0.42
4	Group 4: 450 mg/kg bw/d	6.21 ± 0.52	0.49 ± 0.32	178 ± 0.12	0.49 ± 0.56	3.50 ± 0.46	2.5 ± 0.23
5	Group 5: Post-treatment of 450 mg/kg bw/d	5.00 ± 0.54	0.47 ± 0.23	1.35 ± 0.48	0.78 ± 0.16	2.38 ± 0.25	5.9 ± 0.38

N = 6 data are Mean±SEM Group 2 and group 4 compared with group 1: **= highly significant ($p \leq 0.001$)

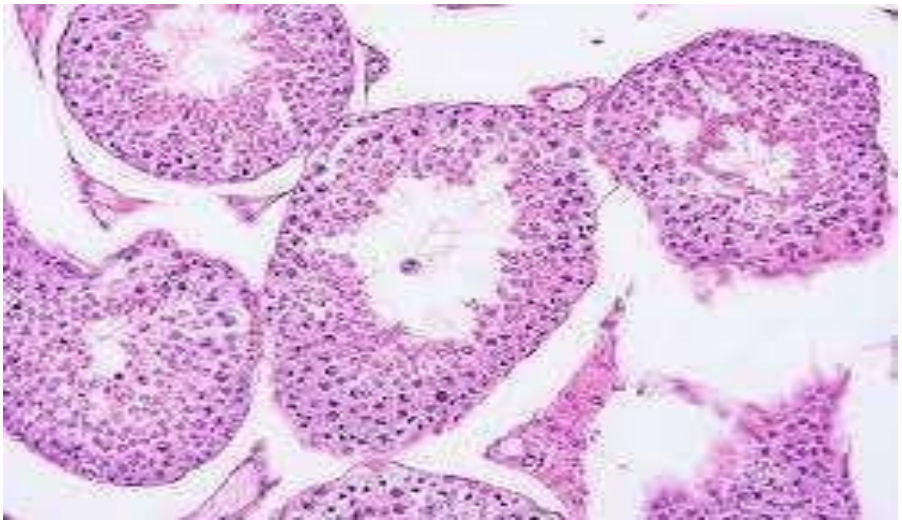

Figure 1: Photomicrograph showing section of uterus indicating surface epithelium with no secretory activity (Control group) HE 300×

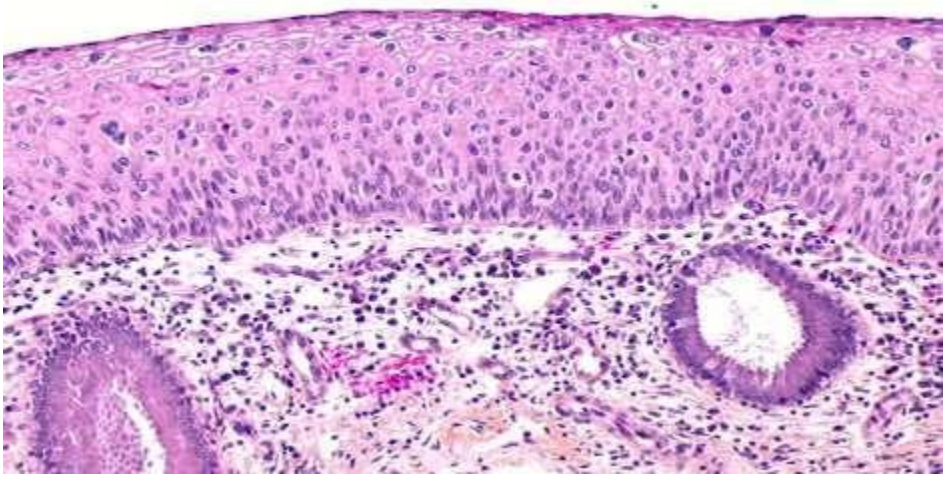

Figure2: Photomicrograph showing section of uterus indicating increasing height of luminal epithelium (Ethinyl Estradiol) HE 300×

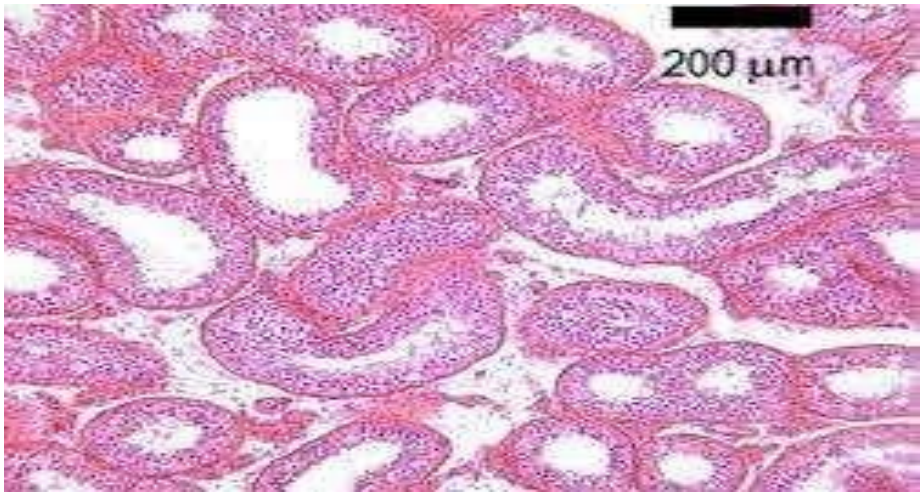

Figure3: Photomicrograph showing section of uterus indicating increase in height of luminal epithelium (EtTD-200 mgkg-1) HE 300×

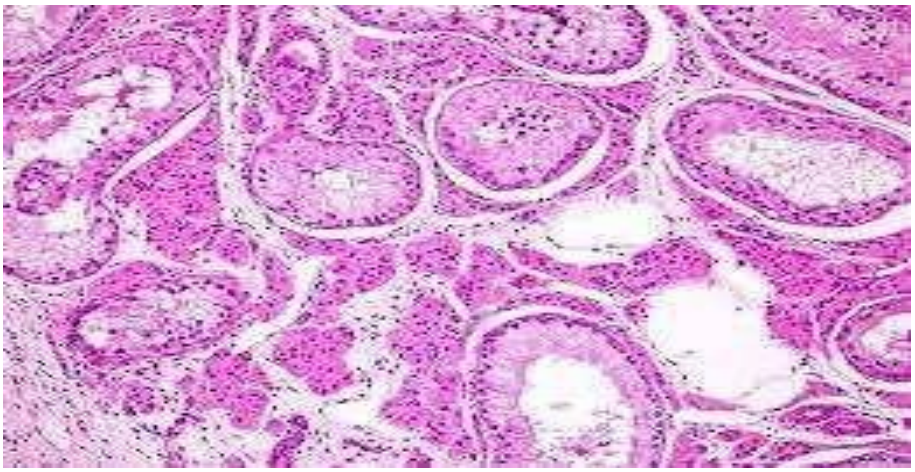

Figure 4: Photomicrograph showing section of uterus indicating increase in height of luminal epithelium, loose and edematous stroma with stimulated uterine glands (EtTD 400 mgkg^{-1}) HE 300×

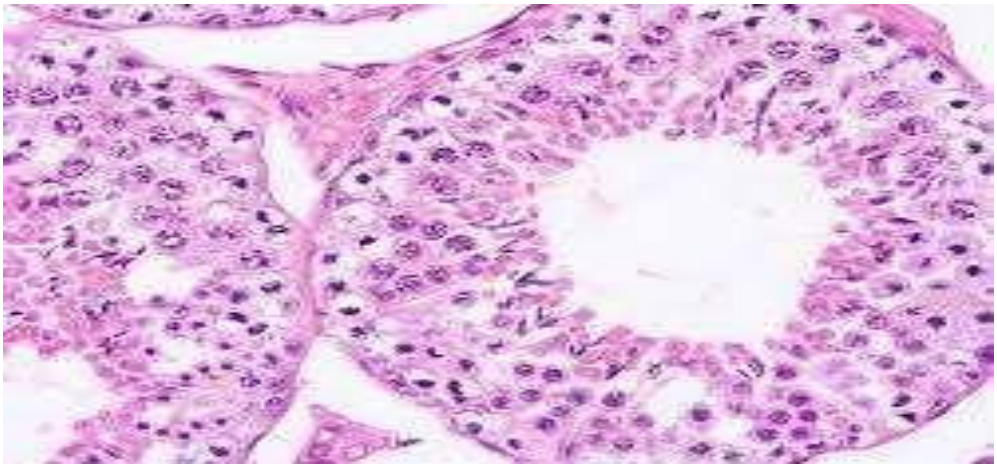

Figure 5: Photomicrograph showing section of uterus indicating moderate increase in height of luminal epithelium with moderate stimulation of uterine weight (AqTD 200 mgkg^{-1}) HE3 00×

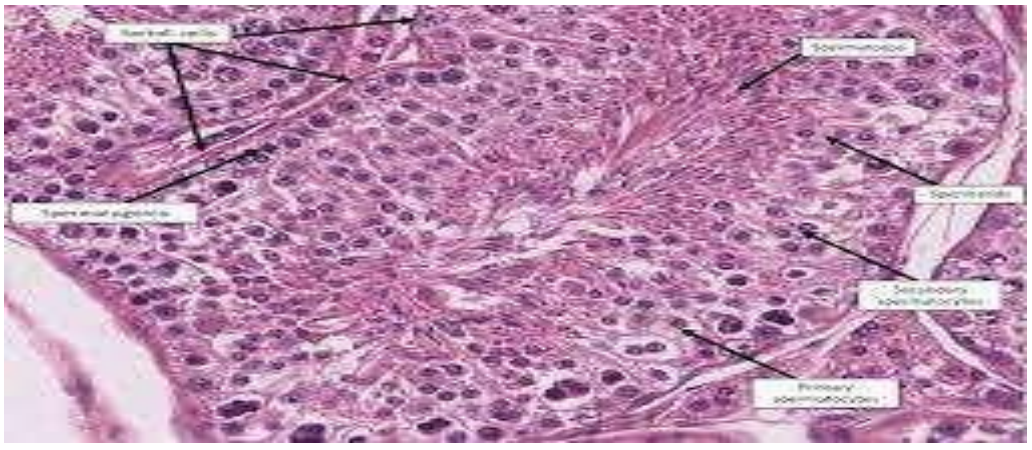

Figure 6: Photomicrograph showing section of uterus indicating moderate increase in height of luminal epithelium with stimulated uterine glands (AqTD 400 mgkg^{-1}) HE 300×

Conclusion

With these preliminary results we can conclude that the ethanolic and aqueous

extract of *T. divaricata* showed significant anti-fertility activity. At 400 mg / kg, ethanolic extraction has greater estrogenic activity than the reference standard ethynil estradiol. Abortion greatly increased uterine weights, and the results were correlated with and supported by histopathological findings. In terms of anti-implant function, ethanolic extraction of 400 mg / kg
p.o. showed 74.27 percent anti-fertile activity, making it more potent than liquid extraction. Since ethanolic extracts and aquoes were tested for anti-egg maturation, there was no difference in egg weight or cholesterol levels compared to the car-treated group. In the case of reproductive hormones, treatment with a higher dose resulted in a longer estrous cycle and a significant increase in the duration of the diestrus phase and an increase in the estrus stage. The results of the present study provide that evidence for the antifertility activity of *T. divaricata* as claimed in the traditional use. The flavonoids, alkaloids, and glycosides present in the extracts may be responsible for their activity.

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