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ANTIFERTILITY EFFECT OF AQUEOUS EXTRACT OF *INDIGOFERA TRIFOLIATA* LEAVES ON REPRODUCTIVE ABILITIES OF FEMALE ALBINO RATS

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ABSTRACT: The present work deals with antifertility effect of the aqueous extract of *Indigofera trifoliata* leaves in female albino rats. Pregnant rats weighing 120 to 200 gm were randomized into 4 groups. From day 11 upto day 15 of pregnancy, rats were laprotomised on 10th day of pregnancy and live fetuses were observed in both the horns of the uterus. Rats in group 1 (control) were orally administered, with 0.5 ml of distilled water once daily while those in group 2 to 4 (experimental groups) were administered 100, 200 and 400 mg/kg body weight doses of aqueous extract of *I. trifoliata* leaves respectively. Then the animals were allowed to go full term. The effect of aqueous extract of *I. trifoliata* leaves on estrogenic and antiestrogenic activity and estrous cycle was observed to confirm the antifertility activity. The aqueous extract of *I. trifoliata* leaves exhibited significant antifertility activity (28.50 to 92.30%). It was found that the extract significantly reduced the number of live fetuses, whereas the resorption index and post implantation losses increased significantly. The % of abortion was found to be highest (92.30%) with 500 mg/kg dose of aqueous extract of *I. trifoliata* leaves. In ovariectomized immature young rats, the extract showed significant estrogenic effect (vaginal opening, vaginal cornification and increased uterine weight) and also prolonged the estrous cycle.

INTRODUCTION: Numerous herbs have been reportedly used historically by women to aid child delivery, stimulate menstrual flow or reduce fertility [1, 2]. Modern scientific studies in experimental animals have reproductive system [3-5]. Herbal contraceptive offer alternative for women who have problems with or lack access to modern contraceptive options particularly women living in the rural areas in developing nations with very high population like India, Africa and Bangladesh [6].

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Studying the potency and toxicity of local plants that are reputed for birth control in the folklore medicine of these countries may generate greater confidence in and wider acceptable of herbal contraceptive.

One plant that featured prominently from our ethanobotanical survey on herbal contraceptive is *I. trifoliata* which is used claimed to be used as "wash the uterus" by the tribal's of Dhamangaon region (20° 46′ N and to 78° 8′ E) of Amravati district of Maharashtra state of India.

Indigofera trifoliata L (Hindi: Diwali) belongs to family Fabaceae, which is a shrub widely distributed in various parts of India. The genus *I.* comprises around 700 species that are distributed geographically in tropical regions.

It is usually an erect plant about 70 cm tall, the leaves are trifoliolate, the lower surface particularly are densely covered with 2- armed or branched hairs; small circular glands are also present . Flowers are present in cluster on the leaf axils and are in red about 3.5 mm long, densely covered on the back with hairs and glands.

The pod is square in cross-section; length varies from 8-18 mm and contains 4-8 seeds. The stem is used as an antitumor, anti-inflammation, antiviral and anti-mycobacterial [7]. Leaves and flower are used in skin cancer, leprosy, cough and swelling of abdomen [8]. Leaves exclusively are used as abortifacient [9]. The plant has very specific use in psoriasis [10]. Root is chewed as a remedy for toothache [9].

The present work was undertaken to validate scientifically the antifertility role of *I. trifoliata* leaves as acclaimed by the traditional tribal user of Dhamangaon region of Amravati district, Maharashtra. But to the best of our knowledge, there is no information in the open scientific literature that has substantiated or refuted the abortifacient claims of *I. trifoliata* leaves in the folklore medicine.

MATERIALS AND METHODS:

Collection of Plant Material: The leaves *I. trifoliata* plant were collected from Dhamangaon region of Amravati district of during the flowering period of September to December, identified and authenticated by experts from Botanical Survey of India, Pune (Accession No. DD- 3).

Procurement and Rearing of Experimental Animal: Healthy wistar strain female albino rats of about two month old and weighing 150- 250 g were procured from Sudhakarrao Naik Institute of Pharmacy, Pusad (Maharashtra).

The rats were housed in polypropylene cages and maintained under environmentally controlled room provided with a 12:12 hr light and dark cycle approximately at 25 °C. They were fed on pellets (Trimurti Lab Feeds, Nagpur) and tap water *ad libitum.* The rats were allowed to acclimatize to laboratory environment for 15 days before experimentation.

All experimental protocols were subjected to the scrutinization and approval of Institutional Animal Ethics Committee [registration number 1060/ac/07/ CPCSEA (IAEC/7/2009)].

Preparation of Extract: The leaves of *I. trifoliata* were collected, shade dried, powdered and subjected to soxhlet extraction with distilled water. The extract was evaporated to near dryness on a water bath, weighed and kept at 4 $^{\circ}$C in refrigerator until further use.

Phytochemical Screening: The presence of various plant constituents in the plant extract was determined by preliminary phytochemical screening as per Thimmaiah [11].

Acute Toxicity Study: Healthy female albino rats were starved for 3- 4 hrs and subjected to acute toxicity studies as per Organization of Economic Co-operation and Development (OECD) guidelines No: 423 [12]. They were divided into 4 groups of 6 animals each and kept singly in separate cages during the experiment. Group 1 represented the control group, which received 10 ml/kg of distilled water orally. Groups 2- 4 received suspension of aqueous extract of *I. trifoliata* leaf orally at the doses of 1000, 2000 and 4000 mg/kg daily for 7 days respectively. The rats were observed continuously for 2 hrs for behavioral, neurological and autonomic profile, and for next 24 and 72 hrs for any lethality or death.

Abortifacient Activity: The plant extracts were tested in female albino rats for abortifacient activity as per Khanna *et al* [13]. The female rats in proestrous phase were caged with males of proven fertility in the ratio of 2:1, in the evening and examined the following day for the evidence of copulation.

Rats exhibiting thick clump of spermatozoa in their vaginal smear were separated and that day was designated as day 1 of pregnancy. These rats were randomly distributed into 4 groups, 1 control group and 3 experimental groups of 6 animals each. On the day 10 of pregnancy animals were laprotomised under light ether anesthesia using sterile conditions. The two horns of uteri were examined to determine the implantation sites. Thereafter the abdominal wound was sutured in layers.

The extract to be tested were then fed to operated pregnant rats i.e. aqueous extract of *I. trifoliata* (leaves) at doses of 100, 200, 400 mg/kg body weight (one tenth of the highest tolerable dose) once daily by an intragastric (i. g.) soft rubber catheter from day 11 up to the 15th day of pregnancy. The animals were allowed to go full term. After delivery the pups were counted and the abortifacient activity of extract was evaluated.

Estrogenic and Anti-estrogenic Activity: The aqueous extract of *I. trifoliata* at 400 mg/kg was found to be most active amongst the three doses in the anti-fertility testing. Hence it was subjected to a detailed investigation for potential estrogenic and anti-estrogenic activity. The uterine weight and vaginal cornification method was employed for the estimation of estrogenic and anti-estrogenic activity [14, 15]. Immature ovariectimized female albino rats, 21-23 days old, weighing between 35-45 gm were used. The animals were divided into four groups, consisting of six rats each.

Group-I: Control, received 0.2 ml of distilled water orally.

Group-II: Treated, received 0.02 mg ethinyl estradiol/ kg/ rat per day in olive oil orally.

Group-III: Treated, received 400 mg aqueous extract of *I. trifoliata* (leaves)/ kg body weight in 0.2 ml of distilled water orally.

Group-IV: Treated, received 400 mg aqueous extract of *I. trifoliata* (leaves)/ kg body weight in 0.2 ml of distilled water orally +0.02 mg ethinyl estradiol / kg /rat per day in olive oil orally.

All the above treatments were given for 7 days. On the 8th day of experiment, the animal were sacrificed by decapitation and uteri dissected out and surrounding tissues removed .The uteri were blotted on filter papers and weighed quickly on a sensitive balance and fixed in Bouin's fluid for 24 hrs. The tissue were dehydrated and embedded in paraffin. The paraffin section were cut at 5 µm and stained with hematoxylin-eosin for histological observation. The diameter of the uteri and thickness of the endometrium were measured in 16 randomly selected sections using an ocular micrometer.

Effect on Estrous Cycle: The aqueous extract of *I. trifoliata* at 400 mg/kg was found to be most active amongst the three doses in the anti-fertility testing. Hence it was subjected to a detailed investigation for study of estrous cycle. The studies were conducted on adult female rats (150- 200 gm) for 30 days. To study the estrous cycle pattern, animal showing regularity in the normal cycle were separated and chosen for further studies. Those animals showing normal estrus cycle were divided in 2 groups of 6 animals each;

Group I- control, received distilled water (Vehicle)

Group II- treated, received aqueous extract of *I. trifoliata* at dose of 400 mg/kg body weight.

Vaginal smear using saline solution were taken twice daily during the entire treatment period, observation of the vaginal opening and the cell type obtained in a vaginal smear was also done. The duration of estrous cycle together with that of various phases was determined [16, 17].

Statistical Analysis: All the data are expressed as mean ± S.E. Statistical analysis was done by Student's t-test and one way ANOVA [18].

RESULTS AND DISCUSSION: Preliminary phytochemical screening of *I. trifoliata* leaves revealed the presence of alkaloid, steroids, flavanoids, phenolics compound, saponins, and tannins respectively. The flavonoid isolated from *Striga lutea* and *Striga orabanchioides* possessed strong estrogenic and abortifacient properties [19, 20].

Flavonoids isolated from *Butea monosperma* (Lam) have been reported to possess antifertility activity [21]. Sex hormones being steroidal compounds, the plant sterols were suspected to be responsible for the antifertility effects of the leaves of *I. trifoliata* [22].

Alkaloids like constituent were reported to be possibly responsible for the suppressant effect on the uterine normal contraction and the high anti-implantation activity exhibited by the aqueous extract of *Graptophyllum pictum* [23]. These alkaloids, steroids, flavonoids, saponins present in *I. trifoliata* extract might be responsible for its contraceptive activity.

Clinical toxicity symptoms such as respiratory distress, salivation, weight loss and change in appearance of hair as well as maternal mortality were not observed at any period of the experiment. Similarly no mortality and changes in the behavioural, neurological and autonomic profile were observed in treated groups. The highest dose 4000 mg/kg body weight was used for abortifacient activity. This suggested that short term use for this purpose is apparently safe. Similar finding was also observed by Tajuddin *et al* [24], while working on ethanolic extract of *Myristica fragrans* and Zade *et al*, [25] on *Moringa oleifera* in female rats.

The leaves of *I. trifoliata* have been in use by the tribals of Dhamangaon region of Amravati district as a means of abortifacient even without recourse to the scientific validity of the claim. Hence this study was carried out to validate scientifically this tribal claim. The oral administration of aqueous extract of *I. trifoliata* leaf at the doses of 100, 200 and 400 mg/kg body weight produced a dose dependent adverse effect on fertility index and number of implantation in the uterine horns of the female rats by virtue of an increase in the percentage of the post-implantation embryonic loss.

All the experimental extract when evaluated for their abortifacient activity, were found to exhibit pregnancy interceptive activity. Administration of 400 mg/kg body weight of the aqueous extract resulted in 92.30% abortion, while at 100 and 200 mg/ kg body weight of the aqueous extract resulted in 28.50% and 44.45% abortion (**Table 1**).

This was evident from decrease in the percentage of live fetuses. The percent resorption index increased from zero in the control animals to 92.30% in 400 mg/kg body weight aqueous extract treated animals. Our result were corroborated with the findings of ethanolic extract of root powder of *Cassia occidentalis, Derris brevipes* variety *Brevipes* and *Justica simplex* which showed 100% abortifacient activity at 600 mg/kg body weight [14]. Alcoholic extract of *Plumeria rubra* at a dose of 200 mg/kg resulted in 100% abortifacient effect in female albino rats [26]. Similar finding were reported by Yakubu *et al* [27], using *Senna alata* leaves and in antifertility activity of methanolic extract of three varieties of *Ricinus communis* Linn [22]. The litter born to the experimental animal did not show any morphological defects hence, it can be stated that the treatment does not exhibit any teratogenic effect.

TABLE 1: EFFECT OF AQUEOUS EXTRACT OF *INDIGOFERA TRIFOLIATA* (LEAVES) ON FERTILITY OF RATS WHEN FED ORALLY FROM DAY 11 TO 15 OF PREGNANCY

Treatment groups (dose, mg/kg body wt)		No. of foetus individual rats	No. of rats delivered	No. of resorption in	No. of resorption	Abortifacient activity (%)
Control	Group- I Vehicle	8,8,9,8,6,6	6(8,8,9,8,6,6)	0,0,0,0,0,0	0	Nil
Aqueous extract of *I. trifoliata*	Group- II 100	9,3,9,6,13,8	6(7,2,7,3,11,6)	2,1,2,3,2,2	2±0.25***	28.50
	Group- III 200	6,10, 9,11,7,9	6(3,8,5,7,2,5)	3,2,4,4,5,4	3.66±0.42***	44.45
	Group- IV 400	9,13,8,10,7,11	6(0,1,0,0,0,0)	9,12,8,10,7,11	9.5±0.76***	92.30

Values are mean ± SE from 6 animals in each group, P values: *<0.05, **<0.01, ***<0.001, When compared between group, ns= non-significant

In the estrogenic and anti-estrogenic study the effect of aqueous extract of *I. trifoliata* revealed that under control group none of the rats could show vaginal opening during the period of study. The aqueous extract at the dose of 400 mg/kg when administered orally for 7 days, all the animals showed vaginal cornification and also increased the uterine weight (P<0.001) of immature rats significantly when compared with control. **Table 2** revealed the effect of aqueous extract of leaves of *I. trifoliata* when administered conjointly with ethinyl estradiol caused significant increase in the uterine weight (P< 0.01) when compared with control, but the extent of the uterotropic response was less than that produced by ethinyl estradiol alone (P<0.001). The number of cornified cells in the vaginal smears were considerably higher in aqueous extract treated group (+ to ++) than those of the control (0 to +), but notably less than ethinyl estradiol treated rats (+++) (**Table 2**). The test drug significantly increases the diameter of the uterus and thickness of the endometrium (P< 0.01; P<0.001) when compared to control group, but notably less than ethinyl estradiol (P<0.01) treated rats.

TABLE 2: ESTROGENIC AND ANTI-ESTROGENIC POTENTIALS OF THE AQUEOUS EXTRACT OF *INDIGOFERA TRIFOLIATA* LEAVES IN RATS

Groups	Treatment dose (mg/ kg body wt.)	Uterine weight (mg/ 100 gm body wt.)	Vaginal status	Vaginal cornification
I	Control (distilled water)	72.83±2.28	Not opened	0 to +
II	Ethinyl estradiol (0.02mg/kg)	179±2.97***	Opened	+++
III	Aqueous extract of *I. trifoliata* (400 mg/kg)	97±2.68*** [c]	Opened	+ to ++
IV	Aqueous extract of *I. trifoliata* (400 mg/kg) + Ethinyl estradiol (0.02mg/kg)	119±1.53** [c]	Opened	+++

Values are mean ± SE from 6 animals in each group, P values: *<0.05, **<0.01, ***<0.001, When compared with control, P values: [a] <0.05, [b] < 0.01, [c] <0.001, when compared with ethinyl estradiol group, ns= non-significant**.** + -nucleated epithelial cells, ++ -nucleated and cornified cells, +++ -cornified cells.

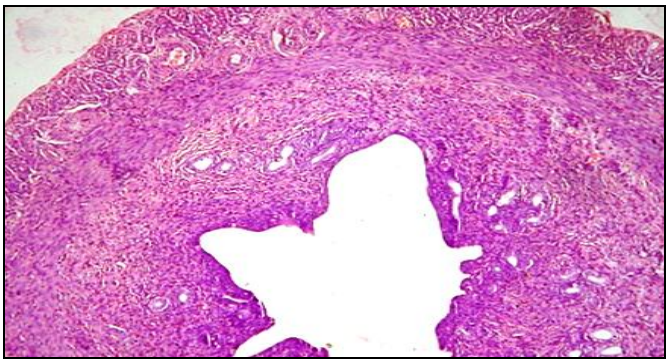
The aqueous extract of leaves of *I. trifoliata* when administered conjointly with ethinyl estradiol caused significant increase in the diameter of the uterus (P< 0.001) and thickness of endometrium (P<0.001), when compared with control (**Table 3**).

TABLE 3: HISTOLOGICAL CHANGES IN THE UTERUS AND ENDOMETRIUM AFTER TREATMENT WITH THE AQUEOUS EXTRACT OF *INDIGOFERA TRIFOLIATA* LEAVES IN RATS

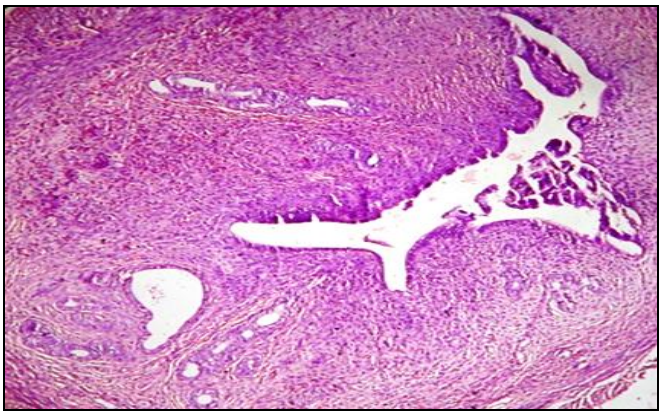
Groups	Treatment dose (mg/kg body weight)	Diameter of uterus (µm)	Thickness of endometrium (µm)
I	Control (distilled water)	292.00±7.27	131.70±3.63
II	Ethinyl estradiol (0.02mg/kg)	514.29±6.62**	345.50±5.67*
III	Aqueous extract of *I. trifoliata* (400 mg/kg)	415.16±5.34*** [c]	294.8±8.29** [b]
IV	Aqueous extract of *I. trifoliata* (400 mg/kg) + Ethinyl estradiol (0.02mg/kg)	501.3±3.13*** [b]	308±2.65*** [c]

Values are mean ± SE from 6 animals in each group, P values: *<0.05, **<0.01, ***<0.001, When compared with control, P values: [a] <0.05, [b] < 0.01, [c] <0.001, when compared with ethinyl estradiol group, ns= non-significant**.**

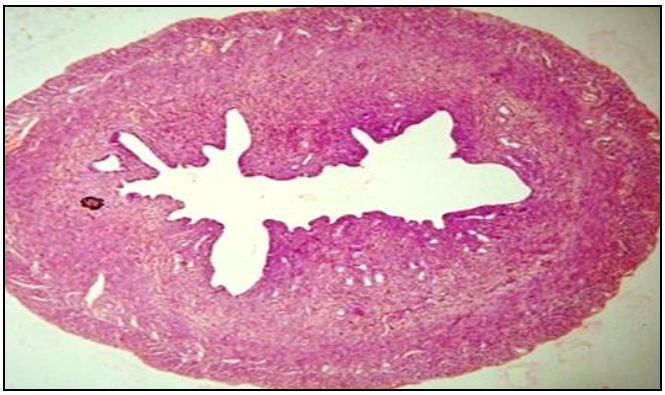
In the present study the histological evidences of the uterus treated with aqueous extract of *I. trifoliata* leaves clearly supports an unfavourable uterine milieus, showing obliterated lumen with loose stroma, increased height of luminal epithelium and stimulated uterine gland in respective extracts (**Fig. 1**), therefore from the present finding it can safely be said that the extract possess estrogenic activity.



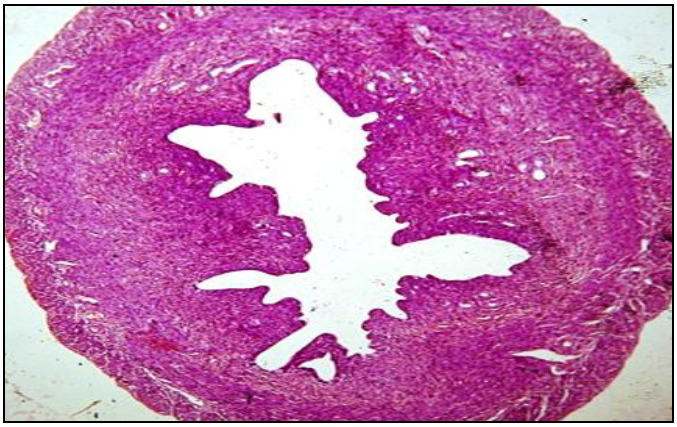
T. S. OF UTERUS OF IMMATURE OVARI ECTOMIZED RAT TREATED WITH ETHINYL ESTRADIOL



T. S. OF IMMATURE OVARIECTOMIZED CONTROL RAT UTERUS



T.S. OF UTERUS OF OVARIECTOMIZED RAT TREATED WITH 400 mg/kg b. w. AQUEOUS EXTRACT OF *I. TRIFOLIATA* LEAVES



T.S. OF UTERUS OF OVARIECTOMIZED RAT TREATED WITH ETHANYL ESTRADIOL + 400 mg/kg b. w. AQUEOUS EXTRACT OF *I. TRIFOLIATA* LEAVES

FIG. 1: HISTOPATHOLOGICAL CHANGE IN IMMATURE OVARIECTOMIZED UTERUS OF RAT WHEN TREATED WITH AQUEOUS EXTRACT OF *I. TRIFOLIATA* LEAVES (PHOTOMICROGRAPH AT A MAGNIFICATION OF 100X)

The aqueous extract of *I. trifoliata* leaves has shown estrogenic activity in immature rats which becomes responsible to cause abortifacient activity. It is expected that due to the estrogenic activity, the aqueous extract may disturb the normal estrogenic titre in the uterus in order to insult the egg to implant. The estrogenic activity of the extract may also affect the rate of ovum transport or may create non receptive uterine milieu. Thus, under the unfavourable circumstances a fertilized egg is unable to make a successful contact on the endometrium [28].

The high dose of estrogen improportionate to progesterone leads to resoption of foetuses [29, 30]. Our results also corroborating with the finding of Keshari *et al* [31]., reported that hexane extract of the seeds of *Nigella sativa* L. when treated orally possess estrogenic activity in immature rats. Similar finding was recorded by Dabhadkar and Zade [26], while working on abortifacient and estrogenic activity of *Plumeria rubra* pods on female rats.

The control rats exhibited regular estrous cycle and normal duration of each phases of the estrous cycle. Analysis of the estrous cycle revealed that oral administration of 400 mg/kg body weight of aqueous extract of *I. trifoliata* leaves produced an irregular pattern of cycling in all the treated rats. The length of the estrous cycle was significantly increased (Table 4).

TABLE 4: EFFECT ON ESTROUS CYCLE OF FEMALE ALBINO RATS AFTER THE ADMINISTRATION OF 400 mg/kg AQUEOUS EXTRACT OF *INDIGOFERA TRIFOLIATA* LEAVES

Phases (Days)	Group-II Control group	Group-II Aqueous extract of *I. trifoliata* 400 mg/kg	Vaginal opening/ cell type obtained in a vaginal smear
Proestrous phase	0.63±0.09	0.43±0.03**	25% to 40% / Epithelial cells only
Estrous phase	0.60±0.15	0.48±0.01*	Above 70% / Few cornified cells
Metaestrous phase	0.87±0.31	1.14±0.02***	50% to 70% / Cornified cells plus many leukocyte
Diestrous phase	2.37±0.13	4.19±0.18***	50% to 70% / Leukocytes plus epithelial cells
Complete Estrous cycle	4.47±0.68	6.24±0.90**	--

Values are mean ± SE from 6 animals in each group, P values: *<0.05, **<0.01, ***<0.001, When compared with control, ns= non-significant

The duration of the diestrous phase was significantly increased while the proestrous and estrous phases were decreased. This disruption of the estrous cycle may be due to the effect of this extract on the ovary which control ovarian functions and estrous cycle via ovarian and extra ovarian hormones [32, 33]. Cyclic changes in the vaginal smear observed in the estrous cycle gives a reasonable index of the ovarian activity and its hormonal synthesis of estrogen and progesterone. The levels of these hormones are controlled by hypothalamic releasing hormones and pituitary gonadotrophins [34]. A feedback mechanism also operates where the pituitary gonadotrophins secretion in turn is controlled by estrogen and progesterone. The cornification in the vaginal epithelial cells is mainly due to high levels of estrogens secreted by the ovarian matured follicles.

It is also known that exogenous administration of estrogen consistently stimulates the proliferation of the vaginal epithelium in adult spayed animals [35, 36]. Similar observation was recorded by Yadav and Agrawal [37], while working on *Nigella sativa* and Amah *et al* [38] on *Momoedica charantia* on rats.

CONCLUSION: The abortifacient activity lends support to the claims for its traditional usage of *Indigofera trifoliata* as an abortive medicine. Thus, this study may prove to be an effective and safe alternative remedy for contraception. Further studies to identify the bioactive principle of abortifacient and estrogenic activity of the extract are in progress.

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