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Evaluation of antifertility potential of *Piper betle* (Petiole) on female wistar rats "rising approaches of herbal contraception"

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ABSTRACT

Piper betle (Petiole) is used of herbal methods for fertility regulation is widely accepted alternative for the synthetic drugs containing chemical having side effects. *Piper betle* (Petiole) is the plant having several medical properties but no reports were available on the antifertility activity. The aim of this study was to investigate the antifertility activity of extracts of *Piper betle* (Petiole) on female wistar rats at the doses 500 mg/kg b.wt./day for 30 days. Different parameters were studied in female wistar rats including effect of Reproductive outcome, Anti-implantation, Abortifacient and Estrogenic & Anti-estrogenic activity, were observed. *Piper betle* shown positive test for Alkaloids, Steroid, Flavonoids, Terpene, Carbohydrates and Tannin. The extract has anti-fertility effect the control rats showed good number of litters and treatment of animal with different extracts resulted a significant ($P < 0.05$, $P < 0.01$). Antifertility activity 51% and 37.2% was exhibited by Alcoholic extracts of *Piper betle* (Petiole) APB and Aqueous extracts of *Piper betle* (Petiole) WPB respectively. After 21 days of the extracts free period, the antifertility effect of the extracts was reversed. The extract treatment with APB, an increase in the percentage of resorption index indicates the failure in development of embryo. The mean percentage of anti-implantation and abortifacient were found to be highest for APB-38.45%, WPB 13.62, and APB-28.96%, WPB-12.75% respectively. The decrement in implantation caused by the extracts may be due to estrogenic or anti-estrogenic activity. However, along with standard APB exhibiting more potent estrogenic and less potent anti-estrogenic when compared with standard. Female antifertility agents should include acceptability, safety and efficacy during and after the treatment. The above results revealed the potential, reversible female antifertility effect of alcoholic extract *Piper betle* (Petiole).

1. 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. In recent years, plants are practice over synthetic contraceptive drug because plants are easily available, economic and devoid of harmful and no side effects [2].

Piper betle, Family: *Piperaceae* (commonly known in all over India as Paan) is a perennial herb that is grown in most part of India and it has been an important herb distributed throughout of world *Piper betle* are the most valued part of the plant, in the past were routinely used as a chewing agent to restrict unpleasant smell and they contain tannins, chavicol, phenyl, propane, sesquiterpene, cyneole, alkaloid, sugar and some essential oil and found various medicinal value, digestive, appetizer, aromatic, expectorant, stimulant, antibacterial, euphoria-inducing, antiprotozoan, carminative, anti-fungal and aphrodisiac etc. [3]. The leaves are also supposed to harden the gum, conserve the teeth and to prevent indigestion, bronchitis, constipation, congestion. However, scientific study of this plant in relation with the potentiality as effective antifertility agent is still fragmentary [4]. The present study was therefore carried out to evaluate the claimed antifertility effect of *Piper betle (Petiole)* using different aspects of reproductive physiology [5].

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2. Methods and materials

2.1. Collection of plant material

The plant specimens for the study were collected from the Satpura region of Madhya Pradesh, India, identified and authenticated by NISCAIR (National Institute of Science Communication and Information resources), New Delhi, A voucher specimen no. is (NISCAIR/RHMD/consult/2015/2859/52–1). Care was taken to select healthy fully grown plant and normal parts. The samples of different parts were cut suitably and removed from the plant and thoroughly washed with water to remove the adherent impurities and dried in sunlight [6].

2.2. Determination of physicochemical parameters

Physicochemical parameters of *Piper betle (*Petiole) were determined and reported as total ash, water-soluble ash, and acid-insoluble ash. Alcohol and water-soluble extractive values were determined to find out the amount of water and alcohol soluble components. The moisture content and pH was also determined [7].

2.3. Successive solvent extraction

The method is based on the extraction of active constituents present in the drug, using various solvents ranging from non-polar to polar. The solvents used were petroleum ether, ethanol and water. Crude drug was subjected to Soxhlet extraction with 1.5 litters of each solvent depending on their polarity. Each time before extraction with next solvents the marc was air-dried [6]. All the extracts were concentrated by distilling the solvent at low temperature [8]. They were then weighed and percentages of different extractive values were calculated with respect to air-dried substance [9].

2.4. Phytochemical screening

Identification of the chemical constituents was carried out on the powdered *Piper betle* (Petiole) and on the Petroleum Ether Extract (P.P.E.), Ethanol Extract (E.E.), and Aqueous extracts (W.E.) using chemical methods [10].

2.5. Animals

Anti-fertility test was performed on adult female wistar rats weighing between 180 and 200 g and Mice. They were housed in polypropylene cages and fed with standard chow diet and water ad labium [11]. The institutional ethical committee for animal cares and use approved all experimental procedures. The animals were exposed to alternate cycle of 12 h of darkness and light each. Before each test, the animals were fasted for at least 12 h [12]. The experimental protocols were subjected to the securitization of the Institutional Animal Ethics Committee and were cleared by the same (1587/PO/Re/S/11/CCSEA).

2.6. 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 [13,14].

2.7. Antifertility study

Antifertility activity of plant extracts was evaluated with the help of reproductive outcome, anti-implantation, abortifacient, estrogenic and anti-estrogenic study was also performed, which further supported by the hormonal analysis [15].

2.7.1. Reproductive outcome in rats

Five groups of mature female rats (five rat/group) were selected for received extracts for 8 days and control group received vehicle for the same period. All the experimental rats were then allowed to mate with mature fertile male rats and the treatment continued for 21 days. The number of litters was determined after the completion of one gestation period in all-experimental groups [16]. The litters were allowed to grow and the growth of litters produced from the extract-administered group was compared with those of control group. The reversibility of anti-fertility effect of the extracts was also studied in the treated groups. For this study, the extracts were administered continuously for 21 days and then the extract was withdrawn. After 21 days of extracts withdrawal, animals were allowed to mate with male rats. The number of litters was determined after the completion of one gestation period [17].

2.7.2. Anti-implantation study

Proven fertile female wistar rats, weighing between 150 and 200 g were selected and left overnight with male of proven fertile in the ratio of 3:1. The extracts were administered orally to separated group rats at the dose level of 500 mg/kg from day 1 to day 7 of pregnancy. Control animal received the vehicle (CMC 0.5%). The animals were then laparotomised on day 10 of the pregnancy under excess dose of thiopentone sodium and uteri were examined to determine the number of implantation sites [17].

2.7.3. Abortifacient study

Female rats at first day of pregnancy were divided into three groups, consisting of six animals in each group. The animals were laparotomised under light ether anaesthesia and semi-sterile conditions on tenth day of pregnancy. Both horns of the uterus were observed for the number of implants. The rats were sutured and allowed to recover. The first group served as control and received vehicle only (Tween-80, 1%) and group second and third received suspension of extract at a dose of 500 mg/kg body weight in 1% Tween-80, respectively, from day 10–18 of pregnancy. During the experiment, animals were observed for vaginal bleeding. On 21st day, animals were laparotomised under light ether anaesthesia and observed for number of litters and percentage of resorption compared with initial number of implantation observed on 10th day of pregnancy [18].

2.7.4. Estrogenic and antiestrogenic study

Colony breed immature ovariectomised female rats (21–23 days) weighing between 25 and 30 gm. were used. They were divided into experimental and control groups, consisting of six animals each group. The extracts were suspended in 0.5% CMC and administered orally for 7 days at the dose level of 500 mg/kg body weight. Ethinyl estradiol (Unicure Remedies Pvt. Ltd., Baroda, India) in olive oil 1 μg/rat per day was injected subcutaneously for 7 days in another group to induce estrous. CMC 0.5% was administered orally to the control animals. The extract at the dose level of 500 mg/kg was also administered orally along with ethinyl estradiol in olive oil at 1 μg/rat per day subcutaneously to different groups of rat for the same period [17].

On the 8th day of the experiment, all the animals were sacrificed by decapitation under light ether anaesthesia and the uteri were dissected out, surrounding tissues removed, blotted on filter paper and weighed quickly on balance sensitive to 0.0001 g [16,17]. A portion of the uterine tissues and adrenal glands from the control and treated animals were fixed in Bouin's fluid for 24 h, dehydrated in alcohol and then embedded in paraffin. The paraffin blocks were sectioned at 6 mm intervals and stained with haematoxylin-eosin for histological examinations [19].

2.8. Hormonal analysis

Blood (2 ml) was drawn by retro-orbital puncture and was immediately transferred into EDTA coated vacationer. The samples were

mixed gently and were left for more than half an hour at room temperature and finally centrifuged at 3000 rpm for 15 min. Serum was separated and assayed for FSH, LH, 17β- estradiol and prolactin and 17-OH progesterone using enzyme linked immunoassay (EIA) technique. Elisa reader (BIORAD 680 Microplate Reader) [20,21].

2.9. 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. [22]

3. Result and discussion

3.1. Physicochemical parameters

Physicochemical parameters of *Piper betle* petiole were determined. In physicochemical parameter, total ash was approximately two times and five times more than acid insoluble ash and water soluble ash respectively [21]. Ethanol soluble extractive was approximately two times higher than water soluble extractive. Moisture content was less than 2.2% and pH was 6.4. (Table 1)

3.2. Preliminary phytochemical investigation

A number of phytoconstituent from natural sources have been proved efficacy to prevent the pregnancy. Many scientific reports were published for antifertility activity of Flavonoids [23], Glycosides, Alkaloids [24]. Phytochemical investigation of *Piper betle* showed (Table 2) the preliminary phytochemical study of *Piper betle* Petiole showed that Alkaloid, Glycoside, Tannin, Flavonoid, was present Petroleum Ether and alcoholic extract. Whereas, Alkaloid, Terpene, Tannin, carbohydrate were present in Alcoholic extracts. Alkaloid, Terpene, carbohydrate and Tannins were present in aqueous extracts. The successive solvent extraction with petroleum ether, alcohol and water gave 17.5%, 21.45% and 26.56% practical yield.

3.3. Acute oral toxicity

Acute toxicity studies were carried out to evaluate toxicity and to determine the minimum lethal dose of the drug extracts, using Swiss albino rats. No clinical signs were evident in any animal during treatment period. (Clinical observations include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behaviour pattern, tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma). No mortality as well as any clinical sign of toxicity has been observed at a dose level of 2000 mg/kg indicating that all the extracts comes under category 5 and hence, LD50 cut-off was found to be 2000 mg/kg body weight. Hence, one-five of this dose, i.e. up to 500 mg/kg body weight, was used for antifertility investigations. Heamatological and biochemical parameters were also performed before and after treatment

and no significant changes were observed [24,25].

Table 1
Various physicochemical parameters.

Physicochemical parameter	Value % w/w* Mean ± SD.
Total Ash	20.75 ± 0.5% w/w
Acid insoluble ash	10.46 ± 0.1% w/w
Water soluble ash	4.1 ± 0.5% w/w
Water soluble extract	16.2 ± 0.3% w/w
Ethyl alcohol soluble extract	18.8 ± 0.3% w/w
Moisture content	2.2%
pH	6.4

w/w* weight/weight.
Value (%) Mean ± SD.

Table 2
Preliminary phytochemical study of *Piper betle* (Petiole).

Test for constituent	*Piper betle* (Petiole)		
	P.E.E	A.E.	W.E.
Alkaloid	+ve	+ve	+ve
Steroid	−ve	+ve	−ve
Terpene	−ve	+ve	+ve
Flavonoid	+ve	+ve	−ve
Glycoside	+ve	+ve	−ve
Sugars	+ve	+ve	+ve
Saponins	−ve	+ve	−ve
Tannin	+ve	+ve	+ve
Carbohydrate	−ve	+ve	+ve
Colour and Consistency	Dark green	Dark green	Dark brown
Yield	17.8%	21.45%	26.56%
Code	PPB	APB	WPB

3.4. Antifertility study

3.4.1. Reproductive outcome study

(Table 3) shows the effect of different extracts on the fertility of female rats. The control rats showed good number of litters. Treatment of animal with different extracts resulted a significant ($P < 0.05$, $P < 0.01$). A significant antifertility activity (62.2%) was exhibited by APB.

It was also found that the litters of the extract treated rats did not show any physical deformity [24]. All litters grew up to normal adult stage, which indicates that the extracts do not have teratogenic effect and the absence of teratogenic effect of extracts at a given dose justifies the safety of the plant. The present observations agree with Salhad who reported the reversible antifertility effect of *Ricinus communis* (castor beans) on female rabbits [17] and also supported by Endalk who reported the same effect of the methanolic root extract of *Rumex steudelii* on female rats [26].

After 21 days of the extracts free period, the antifertility effect of the extracts was reversed for all animals. An increase in the number of litters observed in all the post treatment groups may indicate the reversible antifertility effect of all extracts. These observations correlate the findings of Ganguly and Gebrie who reported the reversible antifertility effect with similar observations on the treatment with methanolic extract of *Cissampelos pareira* leaves in mice and methanolic root extract of *Rumex steudelii* in rats respectively [27,28]. The animal groups gave 7.89 ± 0.05 litters at an average. This showed that there was no statistically significant change from the control group (10.00 ± 0.03). Hence, the sequence of extracts from more potent to less potent was

3.4.2. Anti-implantation and abortifacient activities

Postcoital antifertility study showed the anti-implantation activity in the treated animals. Treated animals delivered litters which, was significantly less than control (Table 4). The extract treatments with

Table 3
Effect of Extracts on Reproductive Outcome.

Group	Estrogenic cycle	Fertility	Litters present
Control	Regular	100% + ve	10.00 ± 0.03[a]
APB	Irregular	62.2% - ve	8.20 ± 0.05[a]
W.D-APB	Regular	54.6% + ve	7.16 ± 0.08
W.D-WPB	Regular	45.5% + ve	6.20 ± 0.02[a]

[a] Values are expressed as mean ± S.D., P values a = $P < 0.001$ when compared with normal control.

Table 4
Effect of Extracts on Anti-Implantation Activity.

Treatment (Dose)	Anti-Implantation Activity		
	No. of implants	No. of litters	Mean % Anti-implantation
Control	7.23 ± 0.52[a]	7.50 ± 0.65[a]	Nil
APB	5.23 ± 0.52[a]	3.50 ± 0.55 [a]	39.45 [a]
WPB	6.10 ± 0.10 [b]	5.32 ± 0.12 [b]	38.10 [b]

Values are expressed as mean ± S.D.
P values a = P < 0.05, b = P < 0.01, c = P < 0.001 when compared with normal control.

Table 5
Effect of extracts on abortifacient activity.

Treatment (Dose)	Abortifacient activity		
	No. of implants	No. of litters	% resorption
Control	7.42 ± 0.62[a]	7.52 ± 0.30[a]	5.45 [a]
APB	5.62 ± 0.53 [b]	4.43 ± 0.41 [b]	28.12[b]
WPB	5.42 ± 0.47 [b]	4.33 ± 0.30 [b]	26.22

Values are expressed as mean ± S.D.
P values a = P < 0.05, b = P < 0.01, c = P < 0.001 when compared with normal control.

Table 6
Effect of Extracts on Estrogenic and Anti-Estrogenic Study.

Group	Treatment (Dose)	Uterine weight (mg/100 g body weight; mean ± S.D)	Vaginal cornification
1	Control	70.24 ± 5.35[a]	NIL
2	Ethinyl Estradiol (1 μg/rat per day)	335.40 ± 7.56[a]	+ + +
3	APB (500 mg/kg)	102.65 ± 7.43[c]	+ to + +
4	Ethinyl estradiol (1 μg/rat per day) + APB (500 mg/kg)	410.67 ± 9.09 [a]	+ + +
5	Ethinyl estradiol (1 μg/rat per day) + WPB (500 mg/kg)	408.58 ± 8.27	+ + +

Values are expressed as mean ± S.D.
P values a = P < 0.05, b = P < 0.01, c = P < 0.001 when compared with normal control + : Nucleated Epithelial Cells, + +: Nucleated Epithelial Cells and Cornified Cells, + + +: Cornified cells.

APB, significantly (P < 0.001) reduced the number of litters born (Table 5). This indicates the abortifacient nature of extracts. An increase in the resorption index (%) by the extract is an indication of failure in the development of the embryo [29]. Such occurrence of fetal resorption suggests that interruption of pregnancy also occurred after implantation [30]. These observations indicate the pregnancy terminating potential of the extract. Embryonal resorption could be due to modifications of uterine lining function or maternal toxicity which consequently may increase early resorption and late fetal death [31,32]. Hence, the present investigation clearly reveals that the extracts are effective before and after the implantation occurs [33].

Both these activities were calculated on the basis of number of implants and number of litters. The mean percentage of anti-implantation and percentage of resorption (abortifacient) were found to be highest for APB - 39.45%, whereas in the case of percentage of resorption; APB-28.12%, these results indicated that all the extracts inhibited the conversion or development of implants into litters. The decrement in implantation caused by the extracts may be due to estrogenic or anti-estrogenic activity [34].

3.4.3. Estrogenic and anti-estrogenic study

Antifertility activity of all the extracts were finally evaluated with the help of estrogenic and anti-estrogenic activity associated with hormonal level and histological parameter like uterine weight, diameter of uterus, thickness of endometrium and height of endometrium epithelium [27]. The stages of estrous cycle and its duration were determined as described by Makonnen [28]. The detail data has given in (Table 6) and (Table 7). A highly significant value show increase in the uterine weight (410.67 ± 09.09[a]) mg/100 g b.wt. and uterine contents was observed in estrogen treated group (P < 0.001) (Table 6). However, co-administration of Ethinyl Estradiol and extract caused a highly significant (P < 0.001) decrease in uterine weight [(102.65 ± 7.43[b]) mg/100 g b.wt.] when compared to estrogen treated group [27,28]

The uterotrophic changes such as diameter of the uterus, thickness of endometrium and height of endometrial epithelium were also insignificantly changed (versus control) (Table 7) A highly significant increase in the uterine weight [(821.45 ± 6.25 [a]) mg/100 g b.wt.] and uterine contents was observed in estrogen treated group (P < 0.001). However, co administration of ethinyl estradiol and extract caused a highly significant (P < 0.001) decrease in uterine weight (515.15 ± 8.66[c]).

3.5. Hormonal analysis

Sex hormones were assayed based on their roles in maintaining pregnancy, since a failing pregnancy could be correlated to the levels of these hormones in the body fluids [13]. The reduction in the concentration of FSH is an indication of disturbance of estrus cycle and ovulation [27]. LH is required for continued development and normal function of corpora lutea. The significant reduction in the level of serum LH could be associated with the physiological process of luteolysis preceding parturition [13]. It could possibly be attributed to pregnancy failure resulting from a luteal phase that is not being maintained [28]. The reduced level of hormone may also be due to inactivation of lutenization of ovarian follicles, which could be responsible for the reduction in the concentration of serum progesterone in this study. Elevated level of progesterone during pregnancy plays a key role in maintaining the conditions and is an important factor in the implantation process.

Table 7
Histological changes in the uterus and endometrium after treatment with extracts.

Treatment (Dose)	Diameter of uterus (μm ± S.D)	Thickness of endometrium (μm ± S.D)	Height of endometrial epithelium (μm ± S.D)
Control	330.54 ± 5.25 [a]	54.14 ± 2.12 [a]	17.4 ± 0.25 [a]
Ethinyl Estradiol (1 μg/rat per day)	821.45 ± 6.25 [a]	230.45 ± 15.15 [a]	65.52 ± 4.18 [a]
APB (500 mg/kg)	515.15 ± 8.66[c]	145.20 ± 4.59[c]	30.00 ± 1.43[b]
Ethinyl Estradiol (1 μg/rat per day) + APB (500 mg/kg)	725.50 ± 6.56[c]	278.73 ± 5.29[c]	58.42 ± 2.26[a]
Ethinyl Estradiol (1 μg/rat per day) + WPB (500 mg/kg)	745.05 ± 4.04[c]	279.12 ± 5.10[c]	54.12 ± 2.52[a]

Values are expressed as mean ± S.D.
P values a = P < 0.05, b = P < 0.01, c = P < 0.001 when compared with normal control.

Table 8
Hormonal Levels in Various Groups of Animals.

Treatment 500 mg/kg	LH	FSH	Prolactin	17β estradiol	17 OH Progesterone
Control	7.25 ± 2.42	8.64 ± 5.20	40.25 ± 6.10	745.12 ± 45.40	15.10 ± 1.10
APB	6.18 ± 02.44 [a]	6.14 ± 4.10 [a]	36.70 ± 3.25 [a]	714 ± 25.14 [a]	25.42 ± 1.10 [a]
WPB	6.24 ± 01.25 [a]	6.14 ± 4.10 [b]	38.10 ± 2.10 [a]	724 ± 14.02 [a]	22.14 ± 1.10 [a]

N = 5, Data representation as Mean ± SD.
P values a = P < 0.05, b = P < 0.01, c = P < 0.001 when compared with normal control.

Therefore, luteolysis and reduction in the blood levels of progesterone may contribute to abortion and anti-implantation activity of the all extracts. The findings of present study were agreed with previous studies which reported the effect of *Inula viscose* and *Bambusa vulgaris* leaf extract on implantation and abortion in rats and rabbits [13]. In this study, an increase in prolactin level was observed (Table 8). These findings were also supported by Ganguly, who reported that a combination of enhanced prolactin and suppressed LH secretion is due to prolongation of estrus cycle [27]. An imbalance in endogenous estrogen and progesterone levels could be responsible for Anti-implantation activity.

4. Conclusion

The present findings inferred that the gathering treated with the most noteworthy convergence of plant concentrate indicated great come about as that of the standard medication and was underpinned by histopathological investigations of the antifertility activity on albino rats. The result of our study clearly demonstrates that Extract of *Piper betle*. The control rats showed good number of litters and treatment of animal with different extracts resulted a significant (P < 0.05, P < 0.01). A significant antifertility activity (62.2%) was exhibited by APB. It was also found that the litters of the extract treated rats did not show any physical deformity. All litters grew up to normal adult stage, which indicates that the extracts do not have teratogenic effect and the absence of teratogenic effect of extracts at a given dose justifies the safety of the plant. After 21 days of the extracts free period, the antifertility effect of the extracts was reversed for all animals. An increase in the number of litters observed in all the post treatment groups may indicate the reversible antifertility effect of all extracts.

Anti-Estrogenic in nature at the dose of 500 mg/kg body weight as evident form the significance increases in the diameter of uterus, height of endometrial epithelium, and thickness of endometrium in extracted animal compared with control, while the animal treated with aqueous extract showed increase height of luminal epithelium with stimulated uterine glans. The extract did not exhibit any estrogenic activity. Proper equilibrium between estrogen and progesterone is essential for implantation, and any disturbance in the level of these hormones may affect the fertility.

The results of the present study provide that evidence for the antifertility activity of *Piper betle* as claimed in the traditional use. The flavonoids, Phytosterol, and Terpenoid present in the extracts may be responsible for their activity.

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Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbrep.2018.08.001.

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