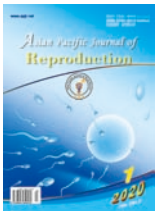

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Estrogenic activity of hydroalcoholic extract of *Clitoria ternatea* Linn. leaves on rats

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ABSTRACT

Objective: To assess the estrogenic activity of hydroalcoholic extract of *Clitoria (C.) ternatea* leaves in female Wistar rats.

Methods: Hydroalcoholic extract of *C. ternatea* leaves prepared by using cold maceration method was tested for estrogenic activity. An acute toxicity study was conducted to estimate the safe dose using OECD 423 guidelines. For estrogenic activity, ovariectomized female rats were divided into four groups, with 6 rats in each group. The control and standard groups were administered with 1% carboxymethyl cellulose orally and estradiol valerate at 1 μg/rat/day subcutaneously, respectively. The third group was administered with hydroalcoholic extract of *C. ternatea* at the dose 500 mg/kg body weight orally and the fourth group was administered with hydroalcoholic extract of *C. ternatea* at the dose 500 mg/kg body weight orally along with estradiol valerate at dose 1 μg/rat/day subcutaneously. All treatments lasted for 7 consecutive days and estrogenic activity was assessed by observing vaginal cornfication. On day 8, all animals were sacrificed and uterine horns were dissected out. Utrine weight was measured and blood serum was further processed for the estimation of biochemical parameters like cholesterol, total proteins, alkaline phosphatase and estrogen by autoanylser. Histological studies of uterus were also carried out.

Results: Acute toxicity studies indicated the hydroalcoholic extract of *C. ternatea* leave was found to be safe up to the dose level of 2 000 mg/kg. Oral administration of *C. ternatea* extract at the dose 500 mg/kg body weight and and estradiol valerate (1 μg/rat/day) caused morphological changes *i.e.* increase in uterine weight, vaginal opening and cornification of cells; biochemical changes *i.e.* increase in cholesterol, total protein, alkaline phosphatase and estrogen contents; histological changes *i.e.* increase in uterine diameter, thickness and height of endometrium. Simultaneous administration of *C. ternatea* extract with estradiol valerate showed a synergistic effect. Histological investigations further confirmed the strong estrogenic nature of *C. ternatea* extract.

Conclusions: *C. ternatea* extract (500 mg/kg) showed a significant estrogenic activity which is also supported by biochemical and histological studies. So, on the basis of these findings, it can be concluded that *C. ternatea* can be used as an alternative to synthetic oral contraceptives.

KEYWORDS: Estrogenic activity; *Clitoria ternatea*; Estradiol valerate; Ovariectomized rats

1. Introduction

The population boom has produced a grave hindrance in the commercial development, resources availability and all-around human progress in emerging nations[1]. According to the latest world population prospects, India population subsumed 17.74% of the total world population[2]. Current pandemic population growth demands an immediate improvement of new potential contraceptives[3]. Investigations and reports of numerous years have emphasized the unmet demand for reliable, affordable, and acceptable contraceptives to evade unwanted pregnancies.

The search for the oral herbal contraceptives is as old as recorded history[4]. Although various synthetic contraceptives are available in the market, they are in limited use due to their adverse side effects. Thus, there is a demand for herbal medicines, which have the least side effects[5].

India has an enormous wealth of medicinal plants due to climate diversity, and *Clitoria (C.) ternatea* Linn. (Fabaceae) is one of the plants originated from the humid and lowland tropics with alluring and charming flowers available in two varieties; blue & white[6]. It is commonly known as "Aparajita". It has many pharmacological activities like antioxidant, anticancer, anti-diabetic, insecticidal, anti-inflammatory, anti-microbial and many other pharmacological activities[7]. In the traditional (Asian) Indian systems of medicine and Ayurveda pharmacopeia, the roots, seeds, and leaves of *C. ternatea* has also been used as a brain stimulant and is believed to promote memory and intelligence[8]. The lead phytoconstituents reported are tannins, flavonoids, flavonol glycosides, anthraquinone, cardiac glycosides, volatile oils, saponins, and steroids. Specifically, the leaves are the rich source of phenolic compounds like 3-monoglucoside, 3-rutinoside, neohisperidoside, aparajitin, beta-

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sitosterol, kaempferol 3-O-rhamnosyl, and essential oils[9].

Phytochemicals from traditional medicinal plants, like steroids[10], flavonoids (flavones, flavanones, and isoflavones)[11], alkaloids[12] and phenolic compounds[13] have already been reported in the literature for their antifertility activity. Plants such as *Michelia champaca*[14], *Jatropha gossypifolia*[15], *Aloe barbadensis*[16], *Derri*[17], *Tragia involucrata*[18], *Amaranthus spinous*[19], *Drosera burmanni*[20], *Achyranthus aspera*[21], *Cassia occidentalis*[22], *etc*. have been used as oral contraceptives since ages. So, the present study was undertaken to established the ethnomedicinal antifiertilty claim of plant by assessing estrogenic activity of hydroalcoholic extract of *C. ternatea* Linn. leaves on rats.

2. Materials and methods

2.1. Animals

Thirty-three female Wistar rats (100-120 g; 45-60 days old) were retrieved from the animal house, Lala Lajpat Rai University of Veterinary and Animal Sciences (Reg. No 1669/GO/abc/12/CPCSEA), Hisar, Haryana, India. The animals were kept in an institutional animal house for 7 days for acclimation. Female rats were bestowed in polypropylene cages with rice husk as bedding material and maintained at (27±2) ℃, relative humidity (65±10)% under a 12-hour light/dark cycle. The animals were bolstered with rat pellet diet and water *ad libitum*.

2.2. Procurement and identification of plant material

Fresh disease-free mature leaves of *C. ternatea* Linn. (1 kg) were gathered from the garden of the Sthaneshwar temple, in the region of Kurukshetra, Haryana, India amid the flowering season. Authentification of the plant was done by Prof. (Dr.) B.D. Vashistha, Department of Botany, Kurukshetra University, Kurukshetra, India (Voucher specimen No. KUK/Bot/IPS-37). The specimen of the mentioned plant was protected in the herbarium of the Department of Botany, Kurukshetra University, Kurukshetra for future reference.

2.3. Preparation of extract

The leaves of *C. ternatea* were cleaned in running tap water to remove adhering impurities and dried under shade. Then the leaves were pulverized into coarse powder by a mechanical grinder, passed through sieve No. 40 and stored in an impermeable container at room temperature. The extraction of 600 g powdered leaves of *C. ternatea* was carried out by cold maceration method with hydro-alcohol (10 : 90). The extract was concentrated to semi-solid mass by using rotary evaporator under reduced pressure and then lyophilized. The crude yield of the lyophilized extract was 11.8% w/w. The extract was stored at 4 ℃ in a refrigerator for further evaluation.

2.4. Phytochemical screening

Phytoconstituents were identified in the hydroalcoholic extract of *C. ternatea* leaves by using the established chemical methodology to establish its identity and purity[23,24].

2.5. Acute toxicity study

The acute toxicity study of hydroalcoholic extract of *C. ternatea* was determined in 9 female Wistar rats, under standard conditions. Toxicity study was executed as per the OECD Guidelines 423. Overnight fasting of animals was done before the experimentation. The test substance at a single dose (2 000 mg/kg) for the limit test was administered by oral gavage. The limit test was performed by the previous pilot experimentation, which indicated that the extracts were likely to be non-toxic up to the regulatory limit dose[19].

2.6. Animal grouping

Twenty-four mature female rats of Wistar strain (100-120 g; 45-60 days old) were used for the estrogenic activity. They were bilaterally ovariectomized under light anesthesia. The uterine horns were exteriorized by laparotomy incision and ovaries were excised. After one week, the ovariectomized rats were divided into 4 groups, with 6 rats in each group. Group 1 (the control group) was administered with 1% carboxymethyl cellulose *p.o.*; Group 2 (the standard group) received standard drug *i.e.* estradiol valerate in arachis oil injected subcutaneously (1 μg/rat/day); Group 3 received 500 mg/kg body weight (b.w.) hydroalcoholic extract of *C. ternatea* in carboxymethyl cellulose suspension *p.o.*; Group 4 simultaneously received both hydroalcoholic extract of *C. ternatea* (500 mg/kg/b.w.) *p.o.* and estradiol valerate (1 μg/rat/day) subcutaneously. All treatments lasted for 7 consecutive days.

2.7. Estrogenic activity

Estrogenic activity was assessed by observing vaginal opening and cornification. At the assigned time period, *i.e.* the 8th day, all animals were sacrificed under light anesthesia; uterine horns were quickly dissected out; liberated from adhering fat, blotted, weighed quickly on a sensitive balance and uterine weight was measured. The uterine tissues were preserved in 10% formalin solution, dehydrated in alcohol and embedded in paraffin.

2.8. Biochemical parameters

Blood (1 mL) was collected by the retro-orbital puncture method before sacrificing the animals. Blood was centrifuged for 10 min at 10 000 rpm, and the serum was separated from the blood for the estimation of biochemical parameters like estrogen level, alkaline phosphates, total proteins, and cholesterolby Merck kit autoanalyser[25].

2.9. Histological findings

For histological studies, paraffin blocks were sectioned and stained with a hematoxylin-eosin solution (H & E stain). The stained slides were examined microscopically (400×) to determine the diameter

of the uterus, the thickness of the endometrium, and the height of endometrial epithelium in 10 randomly selected sections by using a calibrated ocular micrometer.

2.10. Statistical analysis

All data were expressed as mean±standard deviation (mean±SD). Means were statistically analyzed by one-way analysis of variance followed by Dunnett's test using Graphpad InStat software version 7.05[26]. $P<0.05$ was considered significant.

2.11. Ethical approval

Animal ethical clearance was accessed from Institutional Animal Ethical Committee (Protocol No. IPS/IAEC/2017/294), performing the experimental work for contraceptive activity.

3. Results

3.1. Phytochemical analysis

Preliminary phytochemical analysis of the hydroalcoholic extract revealed the presence of saponins, steroids, alkaloids, flavonol glycosides, flavonoids, carbohydrates, volatile oils, and tannins which releaved the presence of same phytoconstituents as reported in the literature.

3.2. Acute toxicity study

The dose was considered to be safe up to 2 000 mg/kg, orally. At 2 000 mg/kg, no morbidity and mortality were detected in the animals. Hence, 1/5th of this dose, *i.e.* (500 mg/kg) was used for the antifertility activity.

3.3. Estrogenic activity

Oral administration of hydroalcoholic extract of *C. ternatea* at 500 mg/kg b.w. caused a significant increase in the uterine weight *versus* the control group ($P<0.01$). The uterotropic changes, like the diameter of the uterus ($P<0.01$), height and thickness of endometrium ($P<0.01$) were significantly increased when compared with the control rats (Table 1 and 2).

The uteri of group 3 animals were inflated and full of fluid resembling the proestrous/estrous uterus. These animals also showed vaginal opening. Examination of vaginal smears of treated rats revealed predominantly cornified and nucleated epithelial cells. However, their number was less than that in estradiol valerate treated rats. Simultaneous administration of estradiol valerate and hydroalcoholic extract of *C. ternatea* caused a significant increase in the uterine weight, uterine diameter (*versus* the control; $P<0.01$), cornification and keratinization of cells in the vaginal smear. Synergistic effect with estradiol valerate further corroborated its estrogenic activity. It appeared that the hydroalcoholic extract had estrogenic activity at the dose of 500 mg/kg.

3.4. Biochemical parameters

The present findings also supported that hydroalcoholic extract of *C. ternatea* behaved like an estrogenic substance as it increased the protein and chloestrol content in the serum ($P<0.05$) (Table 3). Further, its combined administration with estradiol valerate acted in a synergistic way and these findings corroborated that protein and cholesterol content was estrogen motivated.

Table 1. Effect of hydroalcoholic extract of *Clitoria ternatea* on estrogenic activity in ovariectomized rats.

Groups	Group 1	Group 2	Group 3	Group 4
Uterine weight	83.33±0.04	218.00±0.01**	140.33±0.07**#	368.66±0.06**#
Vaginal cornification	Nil	+ + +	+ to + +	+ + +

Values are expressed as mean±SD; n=6 in each group. **$P<0.01$ as compared to the control group. #$P<0.01$ when compared to the standard group. + = Nucleated cells; ++ = Nucleated and cornified cells; +++ = Cornified cells. Group 1 (the control group) is treated with 1% carboxymethyl cellulose *p.o.*; Group 2 (the standard group) is treated with standard drug estradiol valerate (1 μg/rat/day) *s.c.*; Group 3 is treated with hydroalcoholic extract of *Clitoria ternatea* 500 mg/kg *p.o.*; Group 4 is treated with hydroalcoholic extract of *Clitoria ternatea* 500 mg/kg *p.o.* + estradiol valerate 1 μg/rat per day *s.c.*

Table 2. Histological changes after treatment with hydroalcoholic extract of *Clitoria ternatea*.

Groups	Group 1	Group 2	Group 3	Group 4
Diameter of uterus (μm)	303.56±30.90	575.35±13.70**	364.96±12.00**#	601.93±10.36**#
Height of endometrium (μm)	47.24±2.91	120.87±3.59**	62.87±2.45**	202.42±1.81**#
Thickness of endometrium (μm)	8.27±0.54	23.20±2.00**	14.30±1.22**	31.16±0.71**#

Values are expressed as mean±SD; n=6 in each group. **$P<0.01$ as compared to Group 1 (the control group); #$P<0.01$ when compared to Group 2 (the standard group).

Table 3. Biochemical changes after treatment with hydroalcoholic extract of *Clitoria ternatea*.

Groups	Group 1	Group 2	Group 3	Group 4
Estrogen (pg/mL)	0.27±0.07	8.73±0.09**	3.10±0.13**#	11.21± 0.29**#
Cholesterol (mg/dL)	52.57±4.60	78.26±4.90**	59.23±2.21*	79.22±5.36**#
Total protein (mg/dL)	6.18±0.28	9.83±0.41**	6.99±0.56*	11.21±0.29**#
Alkaline phosphatase (U/L)	9.61±0.22	20.90±0.59**	16.88±0.19**#	22.04±0.78**#

Values are expressed as mean±SD; n=6 in each group. *$P<0.05$ and **$P<0.01$ when compared to Group 1 (the control group); #$P<0.01$ when compared to Group 2 (the standard group).

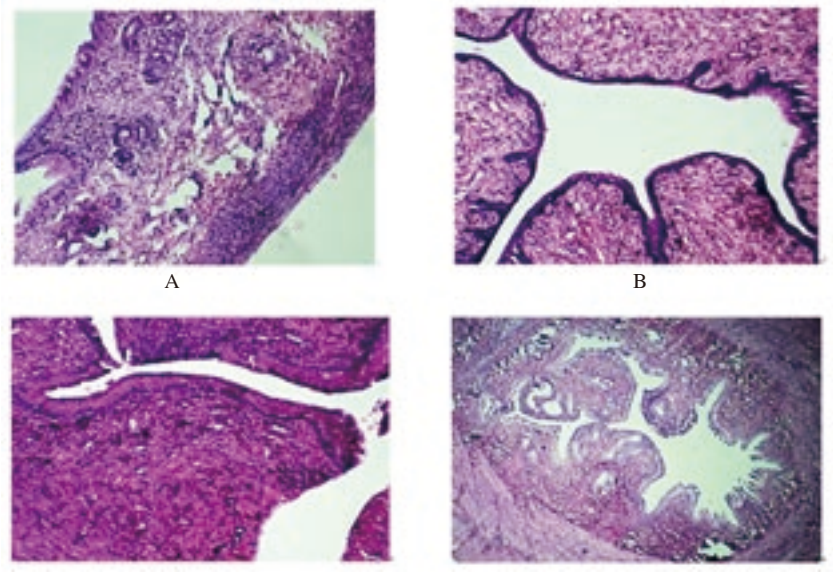

Figure 1. Photomicrograph showing a transverse section of uterus (H & E stain; 400×). A: Transverse section of the uterus of the control group, showing surface epithelium with no secretary activity; B: Transverse section of the uterus of estradiol valerate treated rats, indicating proliferation stage *i.e.* broad lumen and loose stroma and increasing height of luminal epithelium; C: Transverse section of the uterus of hydroalcoholic extract of *Clitoria ternatea* treated rats, indicating an increase in height of endometrium and loose and edematous stroma with stimulated uterine glands; D: Transverse section of the uterus of *Clitoria ternatea* extract+estradiol valerate treated rats, showing stratified columnar cells with prominent nuclei and stimulated uterine glands.

The hydroalcoholic extract of *C. ternatea* (500 mg/kg, *p.o.*) when administered alone or conjointly with estradiol valerate to mature ovariectomized rats significantly ($P<0.01$) increased the estrogen level, in comparison to the control, indicating the estrogenic nature of the extract (Table 3).

An increment in alkaline phosphatase was found after estrogen administration. After the oral administration of hydroalcoholic extract of *C. ternatea* (500 mg/kg), the activity of soluble phosphate increased ($P<0.01$) as shown in Table 3. Conjoint treatment of *C. ternatea* extract and estradiol valerate acted synergistically. These findings also provided strength to the estrogenic nature of the extract.

3.5. Histology findings

The hydroalcoholic extract of *C. ternatea* significantly increased the weight of uteri (Table 1), and results obtained were also correlated and supported by the histology findings as shown in Table 2 and Figure 1.

The hydroalcoholic extract of *C. ternatea* at the dose 500 mg/kg showed a significant increase in the number of uterine glands, enhanced glandular secretion, enlarged uterine lumen, and an increase in the height of luminal epithelium when compared to the control group, therefore showing the estrogenic nature (Figure 1C). Animals in the control group showed confined lumen, compact stroma, atrophic endometrium, inactive endometrial glands, basal nuclei, and less cytoplasm (Figure 1A). The uterus of estradiol valerate-treated animals showed obliterated lumen lined with stratified columnar epithelium, stimulated uterine glands with loose and edematous stroma, the thickness of endometrium and height of luminal epithelium were increased (Figure 1B). A synergistic effect was manifested by the group where hydroalcoholic extract of *C. ternatea* (500 mg/kg) conjointly administered with the standard drug, *i.e.* estradiol valerate reported dilated endometrial glands constituted by large cuboidal cells, distinct centralized and pronounced nucleoli (Figure 1D).

4. Discussion

A combination of estrogen and progesterone are responsible for maintaining the biochemical milieu of the female genital tract. Exogenous administration of estrogen to ovariectomized rats is known to change the uterine morphology *i.e.* increase in the uterine weight, distended/bulged uterus because of retention of the fluid, vaginal opening and increase in the biochemical parameters *viz.* increase in the cholesterol, total proteins, estradiol and alkaline phosphatase content of the uterus, hence making on receptive condition and changing the uterine milieu in the genital tract[27]. Besides, the presence of cornified cells in vaginal smear indicated the estrogenic activity. Based on this inherent virtue, the estrogenic and antiestrogenic nature of numerous prophylactic contraceptive agents has been evaluated.

The plant products having estrogenic nature were known to increase and that of antiestrogenic property tended to decrease the cholesterol, total proteins, alkaline phosphatase and estradiol level in ovariectomized rats. In general, protein content was directly proportional to the uterine weight. So, increment in protein content of reproductive system was responsible for the increase in uterine weight.

Further, the histological studies revealed that alkaline phosphatase by altering the cell permeability increased the absorption of nutrient material by the cell. The involvement of soluble phosphate was believed to be in the growth and secretory function of the tissue, lipid and carbohydrate metabolism, nucleic acid and an increase in cell permeability.

Therefore, an increase in activity of alkaline phosphatase in reproductive tract may lead to alteration of secretory functions by modifying the cell permeability, by changing the reproductive or uterine milieu which was directly involved in the implantation of eggs.

In the present investigations, oral administration of hydroalcoholic extract of *C. ternatea* at dose level 500 mg/kg to ovariectomized

mature rats showed the significant increase in uterine weight, cholesterol, total proteins, alkaline phosphatase, estradiol, vaginal opening, and presence of cornified cells in the vaginal smear compared to the control group. Conjoint administration of hydroalcoholic extract of *C. ternatea* with estradiol valerate showed the synergistic effect. Further, the histological studies also supported the estrogenic nature of the extract. It is confirmed from the significant increase in the diameter of the uterus, the height and thickness of the endometrium in the extract-treated groups as compared to the control group. The animals simultaneously treated with estradiol and hydroalcoholic extract of *C. ternatea* showed synergistic effect, *i.e*., additionally increased the height and thickness of luminal epithelium; stimulated and enlarged uterine glands, thus, suggesting the strong estrogenic nature of the extract.

Previous reports on antifertility activity also highlight the similar estrogenic effect on uterine histology after oral administration of various plant extracts[15,28,29]. Different phytoconstituents occurring in a variety of plants like steroids[10], β - sitosterol[30], flavonoids (flavones, flavanones, and isoflavones)[11], alkaloids[12] and phenolic compounds have well been known to possess antifertility activity[13]. Especially, the flavonoids (like apigenin, luteolin, and kaempferol) and isoflavones including coumestans and lignans fall under non-steroidal phytoestrogens responsible for causing infertility in animals. They mimic the biological effects of 17 β -estradiol by their capability to bind and activate the nuclear estrogen receptors. Flavonoids isolated from *Thevetia peruviana* and *Striga orobanchioides* possessed strong estrogenic and contraceptive properties[11,31].

C. ternatea, an ethnomedicinal plant, is used by the tribal people of the West Rarrh region of West Bengal as an oral contraceptive[32]. But no scientific claim was available for justifying the contribution of this plant in fertility control till date. Therefore, the present study was performed to investigate the antifertility effect of hydoalcoholic leaves extract of *C. ternatea* by estrogenic study including effect on biochemical and histological parameters. It is conspicuous that this plant has a variety of phytochemical constituents, which have a myriad of pharmacological activities. The present preliminary phytochemical findings on leaf extracts of *C. ternatea* revealed the presence of carbohydrates, steroids, flavonol glycosides, flavonoids, alkaloids, volatile oils and tannins in the hydroalcoholic extract. As discussed above, several of these compounds are known to exhibit antifertility activity; the similar effect of hydroalcoholic extract of *C. ternatea* might be due to the presence of such compounds.

In conclusion, based on the above perceptions *i.e*. morphological, biochemical and histological studies, it might be presumed that hydroalcoholic extract of *C. ternatea* leaves inferable from its intense estrogenic nature alters the biochemical and hormonal milieu in the conceptive tract, which further changes the natural phenomenon of the reproduction in the females. Hence, the antifertility activity of the hydroalcoholic extract of *C. ternatea* may be due to its inherent estrogenic activity and these research findings provide the basis for the antifertility activity of *C. ternatea* as claimed in the customary use. Further studies are going on in our laboratory to find out the active constituents and the exact mechanism of action involved in fertility regulation of this plant.

Conflict of interest statement

All authors declare that there is no conflict of interest.

Authors' contributions

Mandeep Kaur conducted experimental work of the study. Dr. Avtar Chand Rana and Dr. Sunil Kumar helped in analysis of experimental part, manuscript writing and monitoring work.

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