Research Article

Antifertility activity of Thevetia peruviana (Pers.) K. Schum leaf in female Sprague-Dawley rat

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Abstract:

Objectives: Thevetia peruviana (Pers.) K. Schum. (Apocynaceae) is known to possess cardioactive glycoside such as thevetin A, thevetin B, neriifolin, peruvoside, thevetoxin, and ruvoside. Traditionally, T. peruviana leaves are used as abortifacient. The aim of the present study is to evaluate antifertility potential of T. peruviana leaves.

Subjects and Methods: Cardiac glycoside freed leaves of T. peruviana were extracted with methanol using maceration method. The dried cardiac glycoside-free methanolic extract of T. peruviana leaves (TPL-Me-G) was screened for phytoconstituents and evaluated for its effect on estrogen-primed female Sprague-Dawley rat uterus model. It was further studied for effects on the estrous cycle, implantation, and effect on estrogen and progesterone.

Statistical Analysis Used: Statistical analysis was done by ANOVA followed by Dunnett’s t-test.

Results: Alkaloids, flavonoids, essential oils, carbohydrates, and amino acids were found to be present in the glycoside-free extract. Thin-layer chromatography (TLC) in n-butanol: acetone: water (4:1:5) revealed the presence of quercetin and kaempferol. The presence of flavonoids (quercetin 0.0326% and kaempferol 0.138% on dry weight basis) was reconfirmed by high-performance TLC analysis. The extract was able to induce uterine contractions (EC50, 0.170 mg/ml) in a dose-dependent manner. Further investigation showed significant (P < 0.001) extension of estrous cycle and anti-implantation activity of the extract by reduction of the progesterone level.

Conclusions: Methanolic extract of T. peruviana leaves (TPL-Me-G) containing quercetin 0.0326% and kaempferol 0.138% possesses a significant (P < 0.001) antifertility potential by virtue of decreasing the progesterone level.

Key words: Abortifacient, anti-implantation, flavonoid, kaempferol, progesterone, quercetin, Thevetia peruviana

The total population of the world has reached 7 billion and it is assumed to reach 8 billion by 2025. The population of India is about 1.28 billion presently. Population explosion has caused serious problems in the economic growth and human development in developing countries such as India. At present, several methods of contraception are being used. The use of synthetic estrogen and progesterone is associated with severe side effects such as breast cancer, urinary tract infections, and cervical cancer. Traditionally used plants are now being focused on fertility regulation. Five hundred and seventy-seven plant species belonging to 122 families are traditionally used in fertility regulation in females. One of these plants is Thevetia peruviana (Pers.) K. Schum (Apocynaceae).

T. peruviana is a small evergreen plant of 2–6 m in height with a broad geographical and ecological distribution and its parts are used for medicinal purposes in Indian Systems of Medicine. Aqueous extract of T. peruviana leaves yielded 3.2% of a basic polysaccharide. The major fractions in the leaf were reported to be aucubin, ursolic acid, and cardenolides. The plant is also a source of terpenoids. Cardioactive glycoside such as thevetin A, thevetin B, neriifolin, peruvoside, thevetoxin, and ruvoside are found in T. peruviana.

Recently, T. peruviana has been explored for its male antifertility potential. The present study also explored the potential of T. peruviana leaf. Although the other plant parts (T. peruviana stem bark) were studied for antifertility activity, studies continued with raw extract only.

How to cite this article: Samanta J, Bhattacharya S, Rana AC. Antifertility activity of Thevetia peruviana (Pers.) K. Schum leaf in female Sprague-Dawley rat. Indian J Pharmacol 2016;48:669-74.
It is, therefore, aimed to evaluate antifertility effect of cardiac glycoside-free methanolic extract of *T. peruviana* leaves on female rat model and to identify the chemical constituents responsible for antifertility effect. Antifertility activity of the extract was studied using the models such as effect on estrogen-primed isolated rat uterus, effect on estrous cycle, on implantation along with the effect on serum estrogen, and progesterone level. Chemical constituents were identified by chemical tests, thin-layer chromatography (TLC), and high-performance thin-layer chromatography (HPTLC).

**Subjects and Methods**

**Collection**

*T. peruviana* leaves were collected in September, 2011 from Panchkula (30.74°N, 76.80°E) India. The plant identification was done by Botany Department of Panjab University, Chandigarh, India. Our specimen was compared with their existing specimen (specimen number PAN/5046) on September 24, 2011. Leaves were dried at room temperature without exposure to sunlight, ground to a fine powder, passed through 80 mesh sizes, and preserved in an airtight container at room temperature for further studies. Air dried powdered leaves of *T. peruviana* (300 g) were used for extraction.

**Phytochemical Screenings**

Cardiac glycosides have been associated with toxicity\(^{[16,17]}\) and hence it was envisaged to remove glycosides from *T. peruviana* leaves. Leaves were defatted by boiling with petroleum ether for 2 h using Soxhlet apparatus. These defatted leaves were then exhaustively extracted from cardiac glycoside by boiling leaves with 80% methanol: ethanol (8:2) for 3 h at 45°C by the modified method of Oluwaniyi and Ibiyemi, 2007.\(^{[18]}\) The treated samples were collected after half an hour interval and each sample was analyzed for the presence or absence of glycoside using freshly prepared Baljet reagent (95 ml 1% picric acid and 5 ml 10% aqueous NaOH solution). Sample, collected after 3 h treatment, was found to be cardiac glycoside free (gave negative result with Baljet reagent). These cardiac glycoside free leaves were then extracted by cold maceration method at room temperature using methanol for 48 h. The extract was concentrated to dark brown, semi-solid mass. It was further dried using rotary evaporator and a dry solid residue (14.5% w/v) was produced. Glycoside-free methanolic extract of *T. peruviana* leaves (TPL-Me-G) was subjected to qualitative investigation for the presence or absence of alkaloids, glycosides, flavonoids, terpenoids, and phytosterols using chemical methods and TLC.\(^{[19,20]}\) Afterwards, HPTLC analysis was performed using Desaga Densitometer CD 60. A stock solution (50 mg/ml) of TPL-Me-G was prepared in methanol. Standard kaempferol (KAMP) and quercetin (QUE) solutions (0.5 mg/ml) were prepared in methanol. 10 μl of sample and standard was applied to HPTLC plate (100 mm × 100 mm) precoated with silica gel GF254. The solvent system used was n-butanol: acetone: water (4:1:5). The twin-trough TLC chamber was lined with Whatman filter paper no. 1 and allowed to saturate with vapors of the same solvent system. The solvent system was allowed to run up to 80% of the plate height. The plates were then taken out of the TLC chamber and dried at room temperature 45°C. Then, dried plates were scanned at 300 nm.

**Animals**

Adult, virgin female Sprague-Dawley (SD) rats (8-week-old and weighing between 150 and 200 g body weights) were used in the present study. Female SD rats (200–250 g body weight) were also used for acute toxicity studies. All the animals used for this experiment were bred in the animal house of the National Institute of Pharmaceutical Education and Research, Mohali, Punjab, India. The animals were housed in polypropylene cages and maintained in an environmentally controlled room provided with a 12:12 h light and dark cycle for each 24 h period at a temperature of 25°C. Animals were provided with sufficient quantity of food pellets and potable water. Animal studies were performed in the animal house of Rayat Institute of Pharmacy, Ropar, Punjab, India, with due permission from the Institutional Animal Ethics Committee (approval no. is RIP/IAEC/2010-11/25 dated 26/7/11).

**Drug Preparation for In vivo Experimentation**

Carboxymethyl cellulose suspension (CMC; 1% w/v) was prepared in distilled water. Previously, characterized TPL-Me-G extract was reconstituted in CMC suspension to get the concentration of 1000 mg/ml and 200 mg/ml. The suspension was administrated to experimental animals by the use of an intragastric tube.

**Acute Toxicity Studies of Glycoside-free Methanolic Extract of *Thevetia peruviana* Leaves**

Three groups (1a, 1b, and 1c) of female SD rats (*n* = 5) were used for acute toxicity studies as per OECD Guidelines, 425 (limit test at 2000 mg/kg, Organization for Economic Co-operation and Development guideline for testing of chemicals, 2001). All animals were fasted overnight before dosing. All five animals of each group received 2000 mg/kg TPL-Me-G orally. The treated animals were kept under observation for 14 days for mortality and general behavior. Individual body weights of animals were determined before the administration of TPL-Me-G (2000 mg/kg) and checked daily until day 14. Treated animals were observed for 14 days after treatment for any changes in skin, fur, mucous membranes, and eyes and for tremors, convulsions, salivation, diarrhea, lethargy, sleep, and coma also.

**Uterotonic Activity of Characterized Glycoside-free Methanolic Extract of *Thevetia peruviana* Leaves**

Effect of TPL-Me-G was evaluated on isolated estrogen-primed rat uterus.\(^{[21]}\) To obtain the estrogen-primed uterus, virgin female rats were subcutaneously injected with 17-β-estradiol benzoate (13.28 nM per animal) and sacrificed 24 h later by decapitation. After prompt removal, uteri were cleaned of the connective tissues and small strips were prepared. Each uterine strip (1 cm long) was mounted in an organ bath of 20 ml capacity containing fresh De Jalon solution (mM): NaCl 153.85, KCl 5.64, CaCl\(_2\) 1.07, NaHCO\(_3\) 25.1, MgCl\(_2\) 1.07, and glucose 2.78. The length of the strip was kept constant in all the experiment. Organ bath unit was maintained at 37°C ± 0.5°C and continuously aerated. The preparation was allowed to equilibrate for 30 min during which the bathing solution was changed every 10 min to replenish the exhausted constituents. After 30 min of equilibration period, uterine cumulative contractile responses were elicited by adding oxytocin (0.05-1.50 μM), TPL-Me-G (0.05–1.60 mg/ml) to the bath containing the isolated uterus. After administration of each concentration, uterine muscle contractions were
recorded and log concentration versus response curve was plotted. The contractions were recorded by means of an isotonic transducer connected to a single channel recorder which was calibrated to record change in the tension generated on g versus mm displacement basis. The isotonic transducer is suitable for isotonic contraction in isolated tissue and small displacement in physiological preparation with preadjusted load. The applied tension to the preparation was 0.5 g.

**Effect of glycoside-free Methanolic Extract of *Thevetia peruviana* Leaves on the Estrous Cycle**

To study the effects of TPL-Me-G on the estrous cycle, three groups (2a, 2b, and 2c) of female SD rats (n = 6) were employed. The vaginal smear method was followed for studying the estrous cycle.[22,23] Vaginal smear (collected through suction pipette using normal saline) from each animal was placed on individual glass slide and examined under a microscope every morning at 9:00 a.m. for 21 days. Determination of the proportion of leukocytes, epithelial cells, and cornified cells present in the vaginal smear was the criteria for determination of different phase. The control group (2a) received the vehicle orally for 21 days. The TPL-Me-G-treated groups (2b and 2c) received 250 mg/kg and 500 mg/kg of the crude extract, respectively orally for 21 days, and the same parameters were determined.

**Effect of Glycoside-free Methanolic Extract of *Thevetia peruviana* Leaves on Implantation**

The effect of TPL-Me-G on implantation was studied by taking three groups (3a, 3b, and 3c) of matured female rats (n = 6).[24,25] All the experimental female rats (on estrous) were placed in the cages by proven male breeders in the proportion of 2:1. On the next day, the vaginal smear of each female rat was checked for the presence of sperm. Positive sperm plug was used as evidence that animal had mated and was considered as the 1st day of pregnancy and images of the sperm-positive vaginal smear were taken by high resolution camera. Control group (3a) received the vehicle orally for 8 days from 1st (D1) to 8th (D8) day of pregnancy. The other two groups (3b and 3c) received 250 mg/kg and 500 mg/kg of the crude extract orally, respectively/day for 8 days, from D1 to D8, respectively. On the 12th day, all the rats of control and treated group were anesthetized with ketamine-xylazine anesthesia and then sacrificed and number of implants of each rat was counted.

**Effects of Glycoside-free Methanolic Extract of *Thevetia peruviana* Leaves on Reproductive Hormones**

Effects of TPL-Me-G on reproductive hormones (serum estrogen and progesterone) were studied with three groups of matured female rats (n = 6).[26] Animals were marked as control, TPL1, and TPL2 (TPL-Me-G-treated group). The control group received vehicle from D1 to D21. TPL1 and TPL2 groups received TPL-Me-G at dose 250 mg/kg, 500 mg/kg, respectively from D1 to D21.

Blood samples were collected from each animal by retroorbital puncture (under mild anesthesia) on 12th day (D12), 19th (D19), and 21st (D21) day of pregnancy. Parturitions occur on 23rd day (D23). Serum 17-β estradiol and progesterone concentration were measured by the methods of Enzyme-linked immunosorbent assay (ELISA) using rat kit (96 well DRG estradiol ELISA kit, Ref-EIA-2693; 96 well DRG progesterone ELISA kit, Ref-1561) and ELISA micro plate reader (Model: Mios Mini, Brand: Merck).

**Statistical Analysis**

Data were expressed as mean ± standard error of mean. The data were statistically analyzed using statistical packages (GraphPad InStat, California), and statistical analysis was done by ANOVA followed by Dunnett’s t-test. The significance level considered was P < 0.05, P < 0.01, P < 0.001 compared with control.

**Results**

**Phytochemical Screening of Glycoside-free Methanolic Extract of *Thevetia peruviana* Leaves**

Phytochemical investigations of the glycoside-free TPL-Me-G using chemical tests [Table 1] indicated that it contained alkaloids, flavonoids, and essential oils. TLC in n-butanol: water: acetone (4:5:1) showed the presence of kaempferol and quercetin. The presence of these flavonoids was reconfirmed by HPTLC in the same solvent [Table 2]. Quercetin and kaempferol in the extract were quantified to be 0.0326% and 0.138%, respectively. This phytochemically characterized extract was then used for further studies.

**Acute Toxicity Study of Characterized Glycoside-free Methanolic Extract of *Thevetia peruviana* Leaves**

All five rats of each group were alive after treatment with TPL-Me-G orally at a dose of 2000 mg/kg. No mortality and behavioral changes were observed in the treated groups up to 2000 mg/kg. No significant changes were observed in skin, fur, eyes, and mucous membranes, and no tremors, convulsions, salivation, diarrhea, lethargy, sleep, and coma were found in

| Sample     | R² | Height | Area | Assigned substance |
|------------|----|--------|------|-------------------|
| TPL-Me-G   | 0.04 | 87.33  | 98.79 | Unknown           |
| 0.09 | 676.55 | 1772.49 | Unknown |
| 0.58 | 98.52 | 296.21 | Unknown |
| 0.78 | 51.37 | 126.99 | Unknown |
| 0.84 | 128.66 | 365.12 | QUE |
| 0.87 | 176.37 | 455.12 | KAMP |
| QUE       | 0.84 | 1498.49 | 11,787.62 | QUE |
| KAMP      | 0.26 | 52.1 | 135.14 | Unknown |
| 0.87 | 1144.6 | 3539.04 | KAMP |

TPL-Me-G=Glycoside-free methanolic extract of *Thevetia peruviana* leaves, KAMP=Kaempferol, QUE=Quercetin

**Table 1: Phytochemical investigation of glycoside-free methanolic extract of *Thevetia peruviana* leaves**

| Test for active constituents | TPL-Me-G |
|-----------------------------|----------|
| Triterpenes                 | +        |
| Saponin                     | –        |
| Alkaloids                   | +        |
| Flavonoids                  | –        |
| Glycosides                  | +        |
| Tannins                     | +        |
| Amino acids                 | +        |
| Sugar                       | +        |
| Phytosterol                 | +        |

+: Identification tests gave positive result, -: Identification tests gave negative result. TPL-Me-G=Glycoside-free methanolic extract of *Thevetia peruviana* leaves.
treated animals. There was no significant change in body weight in TPL-Me-G (2000 mg/kg) treated rats. All these observations help us to conclude that TPL-Me-G up to dose 2000 mg/kg has no acute toxicity in respiratory, circulatory, autonomic, and central nervous systems. 500 mg/kg (1/4th of the safe dose) and 250 mg/kg (1/8th of the safe dose) of extract were selected as doses to be used safely in the antifertility experiments.

### Uterotonic Activity of Glycoside-free Methanolic Extract of *Thevetia peruviana* Leaves

TPL-Me-G (EC<sub>50</sub> 0.170 mg/ml) and oxytocin (EC<sub>50</sub> 0.02 nM) evoked concentration-dependent contractions of the uterus. The response of oxytocin is represented in Figure 1 and the response of TPL-Me-G is represented in Figure 2. These results are comparable with our previous study where we had reported the uterotonic activity of *Cissampelos pareira* stem methanol extract and its different fractions.\[23\]

### Effect of glycoside-free Methanolic Extract of *Thevetia peruviana* Leaves on the Estrous Cycle

Female SD rats that were used in the study all were in regular estrous cycle (4 days estrous cycle comprises proestrus phase, estrus phase, metestrus phase, and diestrus phase). The effects of TPL-Me-G on the estrous cycle of female rats are shown in Table 3. Treatment of rat with TPL-Me-G for 21 days caused a significantly (P < 0.001) prolonged estrous cycle in rats of group 2b (250 mg/kg) and group 2c (500 mg/kg) as indicated in Table 3. A significant increase (P < 0.001) in the length of the diestrus phase and decrease in the duration of proestrus and metestrus stage in treated group than those of the control animals had been tabulated in Table 3.

### Effect of glycoside-free Methanolic Extract of *Thevetia peruviana* Leaves on Implantation

The animals bearing sperm-positive vaginal smear were treated with TPL-Me-G. The decrease in the mean number of implants in extract treated group 3b (250 mg/kg) and 3c (500 mg/kg) had been shown in Table 4.

### Table 3: Effects of glycoside-free methanolic extract of *Thevetia peruviana* leaves (250 and 500 mg/kg) for 21 days) on the estrous cycle of rat

| Phases (days)      | Control (Group 2a) | Extract, 250 mg/kg (Group 2b) | Extract, 500 mg/kg (Group 2c) |
|--------------------|--------------------|--------------------------------|--------------------------------|
| Estrous cycle      | 4.18±0.087         | 6.11±0.16                      | 7.29±0.24                      |
| Proestrus phase    | 0.96±0.033         | 0.49±0.05***                   | 0.45±0.054***                  |
| Estrus phase       | 0.99±0.035         | 0.95±0.03***                   | 0.88±0.036***                  |
| Metaestrus phase   | 0.98±0.014         | 0.66±0.06***                   | 0.57±0.062***                  |
| Diestrus phase     | 1.25±0.017         | 4.01±0.045***                  | 5.39±0.15***                   |

***P<0.001 (significant)

### Table 4: Effect of glycoside-free methanolic extract of *Thevetia peruviana* leaves (250 and 500 mg/kg for 8 days from 1<sup>st</sup> day of pregnancy) on implantation of rat

| Groups    | Number of implants |
|-----------|--------------------|
| 3a (control) | 9.3±0.30           |
| 3b (250 mg/kg) | 3.8±0.19***     |
| 3c (500 mg/kg) | 2.9±0.37***     |

***P<0.001 (significant)

### Effects of Glycoside-free Methanolic Extract of *Thevetia peruviana* Leaves on Reproductive Hormones

In control group, the estradiol level increased throughout gestation. Estradiol levels for TPL1 and TPL2 group also increased. The mean estradiol level of all treated group (TPL1 and TPL2) was higher than the control group’s value at D<sub>19</sub>. The marked rise in estradiol was observed in control group from D<sub>19</sub> to D<sub>21</sub> (day 21) which was not observed in TPL1 and TPL2 (Table 5). The progesterone level was increased in D<sub>19</sub> from D<sub>21</sub> for control group and then declined in D<sub>21</sub>. The progesterone level was found to be less in TPL1 and TPL2-treated groups on D<sub>21</sub>. On D<sub>21</sub>, progesterone level was tended to be lower values in extract treated groups [Table 5].

### Discussion

Preliminary phytochemical screening of the TPL-Me-G indicated the presence of tannins, reducing sugars, alkaloids, and flavonoids. The presence of flavonoids in TPL-Me-G could be responsible for the antifertility effect of *T. peruviana* leaves.\[28\] HPTLC data suggest that TPL-Me-G contains kaempferol, quercetin as chemical constituent [Table 2]. As TPL-Me-G was found to possess promising chemical constituents (flavonoids) and was not previously explored for antifertility activity; the present study was designed to observe the effect of TPL-Me-G on isolated estrogen-primed rat uterus, on estrous cycle, on implantation, and on reproductive hormones. The uterotonic activity of TPL-Me-G could be due to its estrogenic property or oxytocin-like property.\[29\] The extended duration of estrous cycle and diestrus phase have been observed with the TPL-Me-G could suggest the antifertility effect of *T. peruviana* leaves. The chance of the rats to be fertile becomes minimized due to prolongation of diestrus phase.\[30\] Treatment of rats with TPL-Me-G decreased the mean number of implants compared with control suggesting the antifertility effect of extract. Decrease in mean number of implants were found to be less in group treated with 250 mg/kg extract (Group 4b) than that of group treated with 500 mg/kg (Group 4c) suggests that the antifertility effect of the TPL-Me-G is dose dependent. High estrogen levels cause toxic effect to embryo and affect the implantation.\[31\] High estrogen level in TPL-Me-G-treated groups may be the cause for anti-implantation activity of it. Progesterone is one of the reproductive hormones that prepare the endometrium as a bed
Table 5: Effects of glycoside-free methanolic extract of Thevetia peruviana leaves on reproductive hormones

| Groups | Hormone     | 12<sup>th</sup> day | 19<sup>th</sup> day | 21<sup>st</sup> day |
|--------|-------------|---------------------|---------------------|---------------------|
| Control| Estradiol (pg/ml) | 22.44±0.41          | 34.43±1.12          | 47.39±1.60          |
|        | Progesterone (ng/ml) | 64.17±1.86          | 73.40±2.06          | 37.95±2.10          |
| TPL1   | Estradiol (pg/ml) | 29.69±1.3***        | 49.56±1.8***        | 38.83±0.8*          |
|        | Progesterone (ng/ml) | 45.33±1.04***       | 36.79±1.9***        | 30.79±1.2***        |
| TPL2   | Estradiol (pg/ml) | 34.69±1.3***        | 56.16±1.48***       | 43.83±0.7           |
|        | Progesterone (ng/ml) | 29.19±1.04***       | 23.75±2.1***        | 19.4±2.12***        |

***P<0.001 (significant), *P<0.05 (significant). TPL=Thevetia peruviana leaves

Conclusion

The present study revealed significant antifertility activity of cardiac glycoside-free methanolic extract of T. peruviana leaves. TPL-Me-G exhibits anti-implantation activity by virtue of increasing the estrogen and decreasing the progesterone levels. Kaempferol and quercetin present in TPL-Me-G may be responsible for the antifertility effect of the extract. Elaborate studies are required for establishing the exact phytoconstituents responsible for the antifertility effect of TPL-Me-G.

Acknowledgment

Authors are thankful to Dr. Richa Puri, Botany Department of Panjab University, Chandigarh, India for her help for identification of the plant. Authors are also thankful to Rayat and Bahra Institute of Pharmacy, Mohali, Punjab, India; International Testing Centre, Panchkula, Haryana, India, and Satyam Diagnostic Laboratory, Chandigarh, India, for providing necessary laboratory facilities.

Financial Support and Sponsorship

Nil.

Conflicts of Interest

There are no conflicts of interest.

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