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Evaluation of Ethyl Acetate, Chloroform and Toluene Fractions of *Thevetia peruviana* (Pers.) K. Schum Methanolic Leaf Extract for Uterotonic Activity

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Abstract: *Thevetia peruviana* (Pers.) K. Schum (Apocynacae) leaves have a reputation of abortifacient activity. We investigated traditional claim and found that methanolic leaf extract produce antifertility activity by lowering the progesterone level in rat model. Aim of the present study was to find out the chemical constituent (s) responsible for antifertility activity of methanolic leaf extract of *Thevetia peruviana* (Pers.). The ethyl acetate, chloroform and toluene fractions of methanolic extract of *T. peruviana* leaves freed from cardiac glycosides [TPL-Me-G] were selected for phytochemical investigation and *in-vitro* uterotonic activity. The methanolic extract of *T. peruviana* leaves (1.103g) was fractionated with toluene (100ml, n=20); chloroform (100ml, n=20); and ethyl acetate (100ml, n=20) in the successive order. These fractions were examined for phytoconstituents and evaluated for *in-vitro* uterotonic activity. The toluene fraction (TPL-T) was found to have triterpenes, flavonoids and phytosterols. Quercetin (0.8904%) is present in TPL-T. The chloroform fraction (TPL-Ch) was found to contain flavonoids, triterpenes and phytosterols. Presence of alkaloids and flavonoids (quercetin 0.1606%) were observed in ethyl acetate fraction (TPL-Et-Ac). In contrast to TPL-Et-Ac, the TPL-T and TPL-Ch induced dose dependent uterine contraction in the isolated estrogenized rat uterus model. Highest uterotonic activity was found with TPL-Me-G which has kaempferol as phyto-constituent additionally. The *in-vitro* uterotonic activity is not influenced by quercetin and primary contributor is kaempferol though some unknown phytoconstituent/s also contributes to uterotonic activity and synergizes the action of kaempferol too. So, further research is needed to identify other contributory unknown phytoconstituent/s for antifertility activity of methanolic leaf extract of *Thevetia peruviana* (Pers.).

Keywords: *Thevetia peruviana* (Pers.) Leaves, Methanolic Extract, Fractionation, Kaempferol, Unknown Phytoconstituent/s, Uterotonic Activity

1. Introduction

To meet the ever increasing need of safe and economic antifertility agents, plants with ethno-pharmacological or ethno-botanical reputation are now focused [1], Exhaustive literature survey revealed that *Thevetia peruviana* (Pers.) K. Schum (Apocyanaceae) is one of the 577 plants used traditionally for regulation of female fertility [2]. *T. peruviana* is a rich source of secondary metabolite like flavonoids and terpenoids [3-5]. Different parts of *T. peruviana* plant (fruits, leaves, seeds) have been found to possess cardiac glycosides such as thevetin A, thevetin B, neriifolin, peruvoside, thevetoxin and ruvoside etc [5] Researches on *T. peruviana* proved that it exhibits several pharmacological effect in life threatening diseases like cancer, AIDS as well as some common patho-physiological conditions like inflammation, pain, microbial and fungal infections.

Recently, *T. peruviana* had been explored for its male antifertility potential. *T. peruviana* stem bark methanolic extract causes significant decrease in spermatogenic elements and the weight of reproductive organs in male rats [6].

In our previous study, we have found that cardiac glycoside free methanolic extract of *T. peruviana* leaves (Quercetin 0.0326% and Kaempferol 0.138%) exhibited significant ($p<0.001$) antifertility activity by decreasing the serum progesterone level in female rat model [7].

In the present study, we aimed to identify the exact chemical constituent responsible for antifertility activity of methanolic extract of *T. peruviana* leaves (freed from cardiac glycosides). For the same, methanolic extract of *T. peruviana* leaves was fractionated with toluene, chloroform and ethyl acetate successively. The fractions of methanolic extract were selected for phytochemical investigation and evaluated of uterotonic activity.

2. Subjects and methods

2.1. Collection and Extract Preparation

Collection of *T. peruviana* leaves was done from vicinity of Panchkula (30.74°N, 76.80°E), Haryana, India in the year September'2011. Plant specimen was identified from Punjab University (Botany Department), Chandigarh, India by comparing with preserved specimen (specimen number PAN/5046) in the department. Air dried leaves were defatted with petroleum ether by continuous hot extraction method and defatted leaves were freed from cardiac glycoside by boiling leaves with 80% methanol: ethanol (8:2) for 3h at 45°C by the modified method of Oluwaniyi and Ibiyemi, 2007 [7, 8]. Treated leaves were extracted with methanol (solid to solvent ratio was 1:10) by cold maceration process at 27°C. The methanolic extract of *T. peruviana* leaves (pre-treated to remove cardiac glycoside) was designated as TPL-Me-G and dried to solid mass with rotary evaporator.

2.2. Fractionation and Chemical Characterization of Phyto-constituents

TPL-Me-G (1.103g) was fractioned with toluene (100ml, n=20); chloroform (100ml, n=20); and ethyl acetate (100ml, n=20) in the successive order and assigned as TPL-T, TPL-Ch and TPL-Et-Ac respectively. TPL-T, TPL-Ch and TPL-Et-Ac were subjected to chemical tests and TLC to find out the chemical component [9, 10].

Further these fractions of TPL-Me-G were subjected to HPTLC analysis. Desaga Densitometer CD60 and precoated (with silica gel GF254) HPTLC plate of dimension 100mm x100mm were employed for HPTLC analysis. TPL-T, TPL-Ch and TPL-Et-Ac were individually dissolved in methanol to prepare stock solution (50mg/ml). Standard solutions (0.5mg/ml) of two flavonoids namely kaempferol (KAMP) and quercetin (QUE) were also made in methanol. Standard and test solutions (10µl) were applied on precoated HPTLC sheet. The selected mobile phase was n-butanol: acetone: water (4:1:5). The TLC chamber (twin-trough) was sheathed with Whatman No. 1 filter paper and allowed to be impregnated with the vapors of n-butanol: acetone: water (4:1:5). Up to 80mm of the HPTLC plates were run over with the mobile phase. Then, plates were dried at 45°C after removal from the TLC chamber. Then dried plates were scanned at 300nm.

2.3. Animals

To perform uterotonic activity, estrogen primed rat uterus was required. Female virgin Sprague Dawley (SD) rats (50-60g body weight) were used to get estrogen primed rat uterus. The experimental animals were procured from National Institute of Pharmaceutical Education and Research (NIPER), Mohali, Punjab, India and experiments were performed in the animal house of Rayat Institute of Pharmacy, Ropar, Punjab, India. Total procedure related to animal handling and experimentation was conformed to Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSE) and with endorsement of Institutional Animal Ethics Committee. Institutional Animal Ethical Committee approved the research protocol vide ref. no. RIP/IAEC/2010-11/25 dated 26/7/11**.**

2.4. Uterotonic Activity

The effect of TPL-T, TPL-Ch and TPL-Et-Ac were evaluated on isolated estrogen-primed rat uterus [11, 12]. To procure the estrogen-primed uterus, the virgin female rats were injected (subcutaneously) with 17-β-estradiol benzoate (13.28 nM per animal) 24 hrs before the removal of uteri. 17-β-estradiol benzoate oil base injection (5mg/ml) was supplied by Macmillon Pharmaceutical Ltd., Amritsar, India. The rats (treated with17-β-estradiol benzoate) were sacrificed by decapitation and uteri were removed promptly. These estrogen primed uteri were removed of connective tissues and small strips (1cm long) were made. Each uterine strip was mounted in an organ bath. The capacity of the organ bath was20 ml and was filled with fresh De Jalon solution (mM). The composition of De Jalon solution (mM) was NaCl 153.85, KCl 5.64, $CaCl_2$ 0.648, $NaHCO_3$ 5.95 and glucose 2.78. The temperature of the organ bath unit was conserved at 37±0.5°Cand supply of air was continuous. A thirty minute time period was permitted as equilibrium period for each preparation. During this 30 minute, the wash up solution was changed three times, once in 10 minutes. Soon after the equilibration period, uterine cumulative contractile responses were observed after addition of oxytocin (0.05–1.50 µM). TPL-T, TPL-Ch and TPL-Et-Ac were added in a concentration range (0.05–1.60mg ml^{-1}) to evoke the same response. The uterine muscle contraction was noted after addition of every concentration of each extract. The log-concentration verses response curves were constructed using GraphPad Prism 6. An isotonic transducer connected to a single channel recorder was used to record the contractions. The transducer was calibrated to record changes in the tension generated on g versus mm displacement basis. The applied tension to the preparation was 0.5g.

3. Results

3.1. Fractionation and Chemical Characterization of Phyto-constituents

TPL-Me-G (1.103 gm) was fractionated with toluene (100ml, n=20); chloroform (100ml, n=20); and ethyl acetate (100ml, n=20). The extract was dissolved in toluene was 0.353 gm, in chloroform 0.155gm and in ethyl acetate 0.075gm. Phytochemical investigation revealed that the toluene and chloroform fraction contain triterpenes, alkaloids, flavonoids and phytosterols whereas ethyl acetate fraction contain only flavonoids [Table 1]. TLC of these entire fractions had been performed using different solvent medium to identify phytosterol and essential oil, flavonoid and alkaloids. TPL-T and TPL-Ch has shown the presence of phytosterols, flavonoids and alkaloids respectively, where as ethyl acetate fraction has shown the presence of only flavonoids. The presences of flavonoids (kaempferol and quercetin) were confirmed in TLC studies using solvent system n-butanol: acetone: water (4:1:5). The presences of these flavonoids were reconfirmed by HPTLC in the same solvent. Quercetin and kaempferol in the TPL-Me-G were quantified to be 0.0326 and 0.138 percent respectively [Table 2]. Presence of quercetin in TPL-T (0.8904%) and TPL-Et-Ac (0.1606%) was confirmed [Table 2]. These phytochemically characterized extracts were then used for further studies.

Table 1. *Phytochemical investigation of different fractions of TPL-Me-G.*

Test for active constituents	Results		
	TPL-T	TPL-Ch	TPL-Et-Ac
Triterpenes	+	+	–
Saponine	–	–	–
Alkaloids	+	+	–
Tannins	–	–	–
Flavonoids	+	+	+
Aminoacids	–	–	–
Sugar	–	–	–
Phytosterol	+	+	–
Triterpenes	+	+	–
Glycoside	–	–	–

*(+) sign indicates that identification tests gave positive result,
*(-) sign indicates that identification tests gave negative result

Table 2. *HPTLC of TPL-T, TPL-Ch and TPL-ET-Ac in Butanol: Acetone: Water (4:1:5).*

Sample	R_f	Height	Area	Assigned substance
TPL-T	0.01	124.51	90.5	Unknown
	0.33	63.47	100.37	Unknown
	0.82	612.94	4452.52	Quercetin
TPL-Ch	0.07	145.48	391.84	Unknown
	0.42	65.18	231.43	Unknown
	0.52	206.06	932.86	Unknown
	0.6	257.11	1151.46	Unknown
	0.69	320.45	2836.54	Unknown
TPL-Et-Ac	0.84	381.12	1894.7	Quercetin
QUE	0.84	1498.49	11787.62	Quercetin
KAMP	0.26	52.1	135.14	Unknown
	0.87	1144.6	3539.04	Kaempferol

3.2. Uterotonic Activity of Fractions of Thevetia Peruviana Leaves

TPL-Ch (EC_{50}, 0.525 mg ml^{-1}), TPL-T (EC_{50}, 0.930 mg ml^{-1}) and TPL-Et-Ac (EC_{50}, 6 mg ml^{-1}) as well as oxytocin (EC_{50}, 0.02 nM) evoked concentration-dependent contractions of the uterus. The response of TPL-Ch, TPL-T and TPL-Et-Ac were represented in Figure 1. TPL-Ch, TPL-T and TPL-Et-Ac uterotonic activity in descending order respectively. TPL-Et-Ac was observed with mild or almost no uterotonic activity.

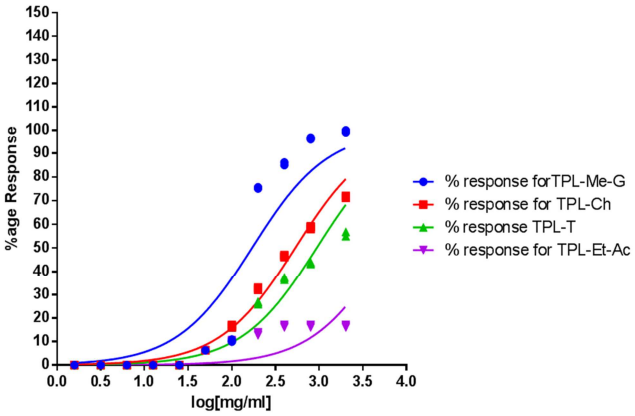

Figure 1. *Concentration vs response curve for uterotonic activity of TPL-Me-G, TPL-Ch, TPL-T and TPL-Et-Ac.*

4. Discussion

TPL-Ch (EC_{50}, 0.525 mg ml^{-1}) and TPL-T fraction (EC_{50}, 0.930 mg ml^{-1}) evoked contractions onthe isolated rat uterus which was directly proportional to concentration of extracts employed. TPL-Et-Ac (EC_{50}, 6 mg ml^{-1}) has very mild effect on uterine contraction. Watcho *et al* (2011) reported uterotonic activities of Ethanolic extracts of *Ficus asperifolia* fruits in rats with concentration depended contraction of the isolated estrogenized rat uterus which was matched with our observations [12]. Urugo *et al* (1998) demonstrated uterotonic properties of the methanol extract of *Monechma ciliatum* [13] and Veale *et al* (1999) reported contractile effects of *Agapanthus africanus* on the isolated rat uterus [14]. These observations and our previous study reports [7, 15] are also in conformation with the present study.

The presence of flavonoids as phyto- constituent was the governing factors for many anti-fertility agents [16]. TPL-Et-Ac contained quercetin (0.1606%) as flavonoid and TPL-Et-Ac was observed with a negligible uterotonic activity which proved that flavonoid particularly quercetin was not responsible for uterotonic effect. TPL-Ch was found to contain some unknown substances which were neither kaempferol nor quercetin but the fraction (TPL-Ch) was able to induce uterine contraction in isolated estrogenized rat uterus model to a significant extent. The toluene fraction (TPL-T) was found to have triterpenes, flavonoids and phytosterol. Quercetin (0.8904%) was present in the TPL-T but the same fraction was devoid of kaempferol. The TPL-T

was also able to induce uterine contraction in the isolated estrogenized rat uterus model in a dose dependant manner. The activity in this case was not linked to the presence of quercetin and kaempferol. It means the toluene fraction illicit the *in vitro* uterotonic activity independent of the flavonoids quercetin and kaempferol.

TPL-Me-G (EC_{50}, 0.170 mg ml^{-1}) [7] produced highest uterotonic activity in comparison with its fractions which may suggest that kaempferol (flavonoid) and these unknown substances produced synergistic action to exhibit antifertility activity.

5. Conclusion

The uterotonic activity in TPL-Ch exhibited due to the phyto-constituent other than quercetin and kaempferol. The *in vitro* uterotonic activity of TPL-T was illicited independent of kaempferol. These results emphasized the fact there are some phytoconstituent/s apart from kaempferol could be able to produce uterotonic activity. Negligible uterotonic effect of TPL-Et-Ac emphasized the fact that quercetin has no role for this activity. But TPL-Me-G itself produces higher uterotonic activity than its fraction which emphasizes the synergistic action of unknown phytoconstituent/s along with kaempferol.

The anti-fertility activity of *Thevetia peruviana* leaves is attributable to the presence of phyto-constituent yet to be identified and needs further investigation. Semisythetic modification of kaempferol and other chemical constituent/s may give us revolutionary anti-fertility agents

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