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Research Article

EXPLORING POST-COITAL ANTI-FERTILITY ACTIVITY WITH TOXICOLOGICAL AND HORMONAL PROFILING OF *SAPINDUS TRIFOLIATUS* LINN.

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Abstract: *Introduction:* Fruits of *Sapindus trifoliatus* (ST) Linn., are used as traditional medicine for birth control purpose. The present study is performed to evaluate its acclaimed post-coital pregnancy interception, along with associated toxicity profiles and to assess its effects on reproductive hormones. *Methodology:* The traditional formulation and saponin rich butanol extract of fruits of *Sapindus trifoliatus* Linn. are investigated on small animals for its acute and sub-acute toxicity study, contraceptive property, estrogenic and anti-estrogenic activities, gonadal and gonadotrophic hormones and lipid-carbohydrate profiling. *Results:* Toxicity studies reveal non-toxic nature of the extract at bioactive dose levels. The extract at dose of 20 mg/kg body weight inhibited fetal implantation (100%), as confirmed by laparotomy on 10th day of pregnancy. The extract also exhibits anti-estrogenic activity in presence of reference hormome and significant variations in gonadal and gonadotrophic hormones in serum. *Conclusions:* The present findings justify the use of soapnut fruits for pregnancy interception purpose, which may be due to antizygotic, blastocytotoxic or anti-implantation activity. Decreased levels of estrogen and progesterone in test animals may hinder tubal transport of blastocyst and make the uterus unreceptive for implantation. Changes in lipid and carbohydrate levels also confer Sapindus trifoliatus to be a promising anti-fertility agent with minimal risk factors
Key**words**: Anti-estrogenic, Anti-implantation, Gonadal and gonadotrophic hormones, Traditional medicine.

INTRODUCTION:

Sapindus trifoliatus (ST) Linn., a folk medicinal plant is commonly used as detergent, hence the name is soapnut tree. It is a medium-sized deciduous tree widely distributed throughout India. The pericarp is known for emetic, tonic, astringent and anti-helmintic properties and traditionally used in the treatment of asthma, colic, diarrhoea, paralysis of limbs and hemicranias [1]. Fruits of ST are traditionally used by rural people for contraception purposes [2]. ST has been reported for its antispermatogenic, antiandrogenic [3-5], antirheumatic [6], antinociceptive activities in various pain models [7], anti-inflammatory [8], hepatoprotective activities [9] as well as antidandruff components [10]. The pericarps of the fruits contain saponins (hederagenin group of glycosides) [11] and sugars [3, 12]. However, scientific profiles of the plant as effective antifertility agent along with its toxicological and hormonal evaluations are still fragmentary. Hence, the present study is taken up to validate acclaimed post-coital antifertility activity *in vivo* on animal models and explain possible mechanisms of its traditional use in birth control.

MATERIALS AND METHODS:

Plant collection and preparation of extract:

The dried fruits of *Sapindus trifoliatus* (ST) Linn, family Sapindaceae were collected from the local market and authenticated by submitting specimen (Ref. No. BSI/CNH/AD/Tech./2009 dated 06/07/2009) to M.S.Mondal, Additional Director, Botanical Survey of India, Kolkata.

Ethnomedicinal formulation (EF) of the pericarp of fruits was prepared according to traditional approach [2]. Fruits are crushed to remove the seeds, and the dried pericarp is grounded to rough powder (#30) with mortar and pestle. After size reduction, the crushed pericarp is vortexed in water by spinix and fed to the test animals at selected dose with gastric gavage tube.

While for the extract preparation, the crushed pericarp was exhaustively extracted with aqueous alcoholic mixture (1:1) and fractionated with *n*-butanol. The *n*-butanol fractions were pooled and concentrated with rotary evaporator (Eyela) to give brown viscous mass (extractive value 20.2% w/w). The butanol extract (BE) was reconstituted at required dose levels with water, and administered to test animals for bioactivity.

Dose selection was accomplished corresponding to dosage used in traditional practice [2], as well as considering the equivalence of animal dose to human dose [13]. Additional adjustments of dose were made based on biological response. Finally a dose level of 30 and 20 mg/kg body weight (bw) for EF and BE respectively were selected.

Animals:

Colony-bred adult male (350-400 g), female (140-220 g) and immature female (40-60 g) Wistar rats, and adult female Swiss mice (20-35 g), maintained in air-conditioned surroundings (25 ± 2^0C) and provided with 12 h alternate light and dark cycle for each 24 h period with regular husbandry conditions, were used for the experiments. The animals were housed in polypropylene cages with husk beds and fed on normal pellet diets and reverse osmosis ozonized drinking water (Silicon Food & Beverages, Kolkata). The animal experimentations were carried out on prior approval of Institutional Animal Ethics Committee (Reg. No. 506/01/a/CPCSEA).

Acute & sub-acute toxicity experiment:
BE was administered i.p. to albino mice at doses 50, 100, 200, 500 and 1000 mg/kg bw. Each group consisted of five animals of either sex. Mortality and behavioural changes were observed daily for one week. The LD_{50} value was calculated by graphical method [14, 15].
Sub-acute toxicity was estimated with control (vehicle) and single dose of BE (20 mg/kg bw). Each group (albino mice) were treated with vehicle and BE, i.p. one injection per day for 14 consecutive days. The animals were observed at weekly intervals for different parameters, like body weight, food and water consumption, spontaneous movement and posture in both treated and control groups. Changes in gait and response to handling as well as the presence of clonic or tonic movements, stereo types (e.g., excessive grooming, repetitive circling) or bizarre behaviour (e.g., self-mutilation, walking backwards), and mortality were also recorded on weekly basis [16]. On the fifteenth day, blood was drawn by cardiac puncture for haematological examination of TC (Total Leucocyte count), DC (Differential Count WBC), RBC count, Hb (hemoglobin), platelet count, PCV (packed cell volume; Hematocrit value), and biochemical examination of urea, creatinine, SGPT/ALT, SGOT/AST and alkaline phosphatase. The animals were sacrificed by cervical dislocation, and organ tissues of liver and kidney were surgically removed for histopathological studies. The organs were carefully examined macroscopically for any abnormal, pathological signs of toxicity. The organ weights were checked and then fixed in 10% formalin solution. The tissues were embedded in paraffin blocks, sliced and placed onto glass slides. After histological (hematoxylin-eosin) staining, the slides were examined under microscope (B1 series, Motic, Xiamen, China).

Evaluation of estrous cycle and post-coital pregnancy interceptive activity:
The evaluation of estrous cycle [17] of female rats was performed to screen the normal cycling females. Vaginal smears of the animals, collected in small quantity of distilled water on microscope slides, were observed under low power (10X) of microscope for prominent diestrous, proestrous or estrous phases. The animals exhibiting cyclical repetition of diestrous to proestrous to estrous phases were selected for study. Variations in estrous cycle were monitored for a period of 12 days in two groups of normal cycling rats (control and BE (20 mg/kg bw)).
The female rats in proestrous phase were kept for mating with male of proven fertility in the ratio of 2:1. Day 1 of the pregnancy was confirmed by the presence of spermatozoa in the vaginal smear. Mated female rats were isolated, randomized into various treatment groups. The test agent and distilled water (vehicle) were administered orally during first 7 days of post-coitum. On the 10th day of pregnancy, laparotomy was performed under ether anesthesia and the uterine horns were inspected for number of implantation sites. The rats were allowed to recover and deliver after full term [18]. Experimental outcomes of pregnancy inhibition (PI) were expressed as % AI and % AF, which describe percentage of anti implantation and anti-fertility activity respectively, in number of animals (N) along with number of corpus lutea (n_{Cl}), implantation sites (n_I) and litters born (n_L) for control and test groups.

Estrogenic and anti-estrogenic activity:
BE (20 mg/kg bw) was further assessed for estrogenic and anti-estrogenic activities [19, 20]. 17β-Ethinyl estradiol (0.03 mg/kg bw) was used as standard. For determination of estrogenic activity [21], immature female rats were treated orally with the test agent or the vehicle for 3 days once daily. Autopsy of the rats were done 24 h after the last treatment to observe uterine fresh weight, premature opening of vagina and the extent of vaginal cornification. The histological studies on isolated and fixed uteri and ovaries were carried out.

Analysis of hormones and lipid- carbohydrate profiles:
The study includes estimation of serum gonadal and gonadotropic hormones by EIA and CLIA estimations for gonadal and gonadotrophic hormones in ELISA reader, Eldex 3.8 (Lilac) and CLI analyzer, ALFA PRIME (Lilac) respectively. Serum lipid-carbohydrate profiles were estimated in a UV-2550, UV-visible spectrophotometer, Shimadzu. Assay procedures were followed as specified by manufacturers of standard EIA, CLIA or enzymatic assay kits. Blood was drawn from orbital plexus of adult female rats before and after 8 days of administration of the extract (BE, 20 mg/kg bw), and the serum was used for analysis. LDL and VLDL-cholesterol contents in serum were estimated by the Friedewald equations [22].
[LDL-Cholesterol] = [Total Cholesterol] - [HDL-Cholesterol] - [VLDL-Cholesterol]
[VLDL-Cholesterol] = [TG]/5

Statistical analysis:
The experimental data were expressed as mean±SEM and statistically analyzed using Student's t-test and analyses of variance (ANOVA) in one-way between control and test values. Statistical analysis of hormonal and lipid-carbohydrate profiles between control and test values were done using pair wise Student's t-test. The results were judged significant, if $p < 0.05$.

Qualitative analysis:
Qualitative analysis of EF was performed to search for saponins, sterols and triterpenoids, reducing sugars, tannins, alcohols, essential oils, glycosides, alkaloids and flavonoids [23, 24].

RESULTS:
Acute & sub-acute toxicity:
The mortality of mice on dose dependent manner (Suppl. Table 1), obtained from acute toxicity study, were plotted to get the LD_{50} value (Suppl. Figure 1). LD_{50} value was determined by finding the dose corresponding to probit 5. No behavioural changes were observed in the live animals. The effective dose (20 mg/kg) used for bioactivity studies is less than $1/10^{th}$ of the lethal dose (240 mg/kg).
In case of sub-acute toxicity study, gross necropsy findings of the animals did not show any adverse effects in any organ. No significant differences in organ weights (liver and kidney) were present (Suppl. Table 2) in the treated group

compared to the control group. No lethality was found during the treatment period. No gross histo-pathological changes were observed in the treated group as compared to control (Figure 1A and B). The biochemical and hematological parameters (Suppl. Table 2) also did not show any significant changes in control and treated groups. Therefore it is ascertained that the BE is safe at 20 mg/kg dose in small animals.

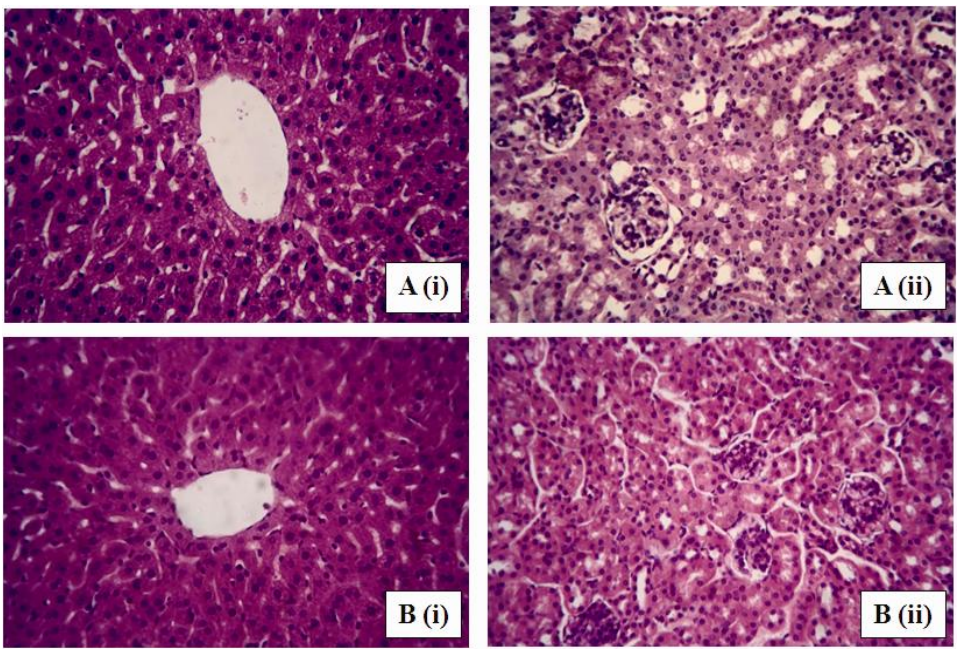


Figure 1: Histopathology study of Control (A) and treated (B) groups respectively [i) Liver and ii) Kidney].

Changes in estrous cycle:

Duration of estrous cycle in control female rats (5.5 ± 0.5 days) did not change significantly ($p<0.05$) upon administration of BE (5.8 ± 0.3 days).

Post-coital antifertility activity:

Post-coital pregnancy interception, expressed as % AI and % AF along with n_I, n_{CI} and n_L for control and test groups are represented in (Table 1). EF and BE both exhibited significant AI and AF activities at 30 and 20 mg/kg bw respectively, however BE showed 100% AI and AF, since no prominent implantation sites were observed in this group. Number of corpus luteum also decreased significantly in test animals. This might be due to the resorption of the implantation sites or due to abortion. However, no vaginal bleeding was observed. (Figure 2) illustrates implantation sites in uterine horns in control (A) and BE (B) groups, indicate that fruit extract of *Sapindus trifoliatus* has significant post-coital anti-fertility activity.

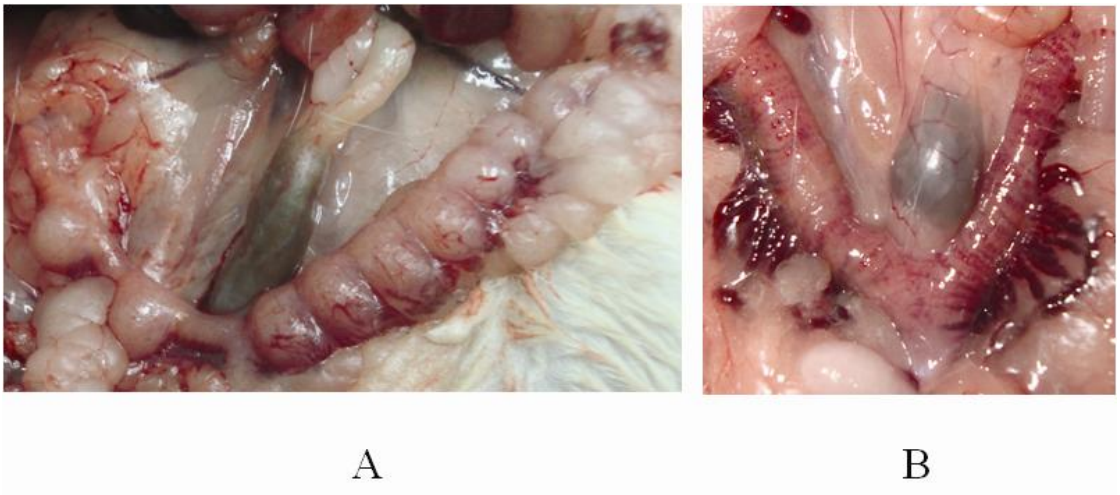


Figure 2: Implantation sites in uterine horns in control (A) and BE (B) groups.

Table 1: Estimation of Contraceptive Property of Extract of *Sapindus trifoliatus*.

Parameters	Control Vehicle	EF 30 mg/kg	BE 20 mg/kg	ANOVA	LSD	Ranked Group Means
N_p	8	8	8			
N_{ni}	0	6	8			
% AI	0	85.52	100			
% AF	0	87.5	100			
n_I	(11,8,7,7,12,10,8,6) 8.63±2.13	(0,0,6,0,0,0,4,0) 1.25±2.38[a]	(0,0,0,0,0,0,0,0) 0.0±0.0[a]	51.13[a]	1.04	(C) (EF) (BE)
n_{Cl}	(14,10,11,15,14,15,16,10) 13.13±2.42	(10,9,8,10,12,11,10,12) 10.25±1.39[a]	(10,7,12,9,8,11,10,8) 9.38±1.69[a]	8.71[b]	1.04	(C) (EF, BE)
n_L	(9,6,5,5,8,9,7,4) 6.63±1.92	(0,0,3,0,0,0,2,0) 0.63±1.19[a]	(0,0,0,0,0,0,0,0) 0.00±0.00[a]	62.88[a]	1.04	(C) (EF, BE)

The results are presented as mean ± standard deviation (s.d.). ANOVA represents one-way analysis of variance. LSD explains least significant difference (Critical difference at 5% level). Ranked group means explain means under different parentheses are statistically different at p<0.05. ^{Np} number of rats with sperm-positive smear. ^{Nni} number of rats having no implantation sites on day 10. ^{nI} implantation sites., ^{nCl} number of corpus lutea., ^{nL} litters born, $^{\%AI}$ and $^{\%AF}$ describe percentage of anti implantation and anti-fertility activity respectively, $^{a}p<0.05$ and $^{b}p>0.05$ in comparison to control (Student's *t*-test), $^{a}p<0.001$ and $^{b}p>0.001$: Significance levels of *F* values in ANOVA

Estrogenic and anti-estrogenic activity:

The histological sections of ovarian tissues (Figure 3A) did not exhibit any significant changes from normal conditions. The extract exhibited no significant changes (Figure 3B) in uterine wet weight ratio, vaginal opening or its cornification (Suppl. Table 3). However, simultaneous administration of reference hormone and BE reduced the extent of uterotropic effects produced by ethinyl estradiol alone. Therefore, it is revealed that BE shows a weak anti-estrogenic property in presence of ethinyl estradiol.

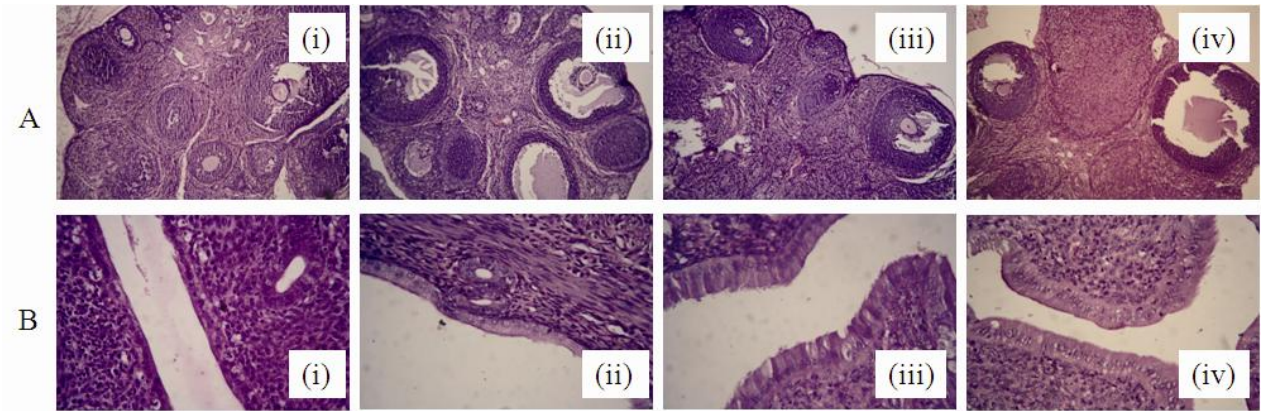


Figure 3: Histological sections of ovarian (A) and uterine tissues (B) of various treatment groups (i) Control, (ii) Test, (iii) Standard drug and (iv) Standard drug along with Test.

Analysis of hormones:

Percentage change of gonadal and gonadotrophic hormones, and lipid-carbohydrate profiles in blood-serum are depicted in (Figure 4) and also listed in (Suppl. Table 4), and showed that the changes are significant at 95% confidence level. Serum level of gonadal hormones decreased significantly after BE treatment in comparison to control, whereas gonadotrophic hormone levels were elevated. Hence, it is evident that reduced levels of hormones make the uterine environment hostile for implantation of fertilized ovum; however LH and FSH levels were increased due to feedback mechanism of low gonadal hormone levels on hypothalamus pituitary gonadal axis. Lipid profile after BE treatment showed that HDL level increased significantly, whereas TG and VLDL levels decreased in comparison to control group.

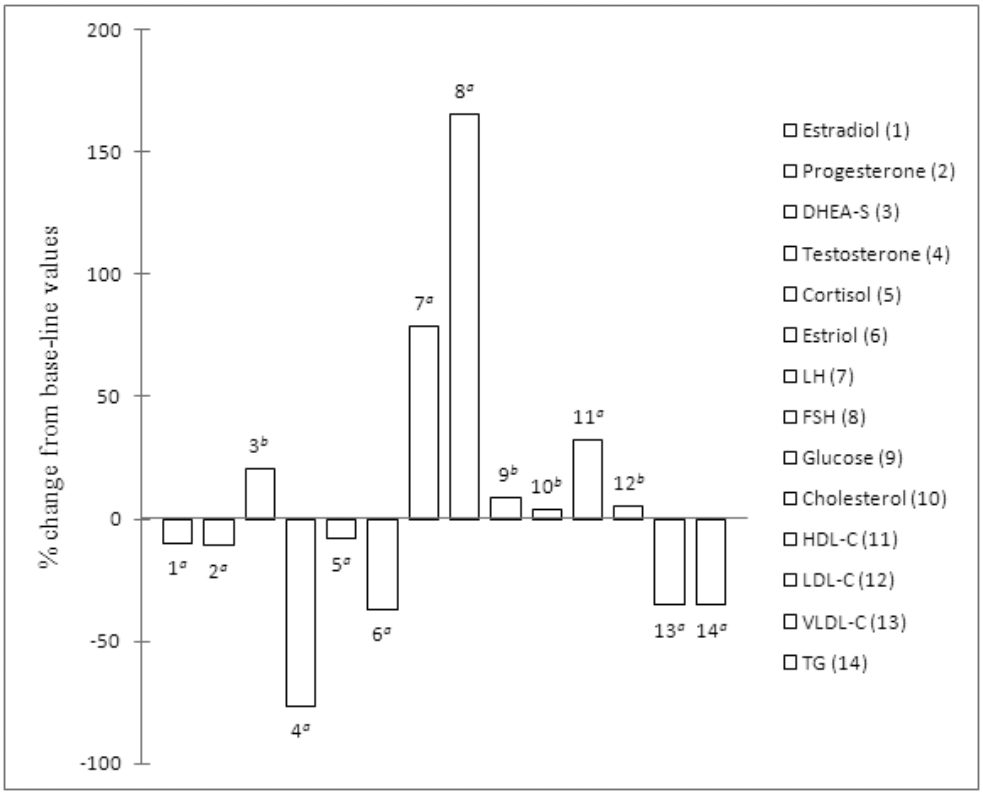


Figure 4: Percentage changes of serum gonadal and gonadotrophic hormones and lipid-carbohydrate parameters on treatment with BE (20 mg/kg), [a]p<0.05 and [b]p>0.05 (Pair wise Student's t-test).

Qualitative analysis:

The phytoconstituent profile of the extract showed the presence of significant content of saponins, and lesser extent of reducing sugars and essential oils (Suppl. Table 5).

DISCUSSION:

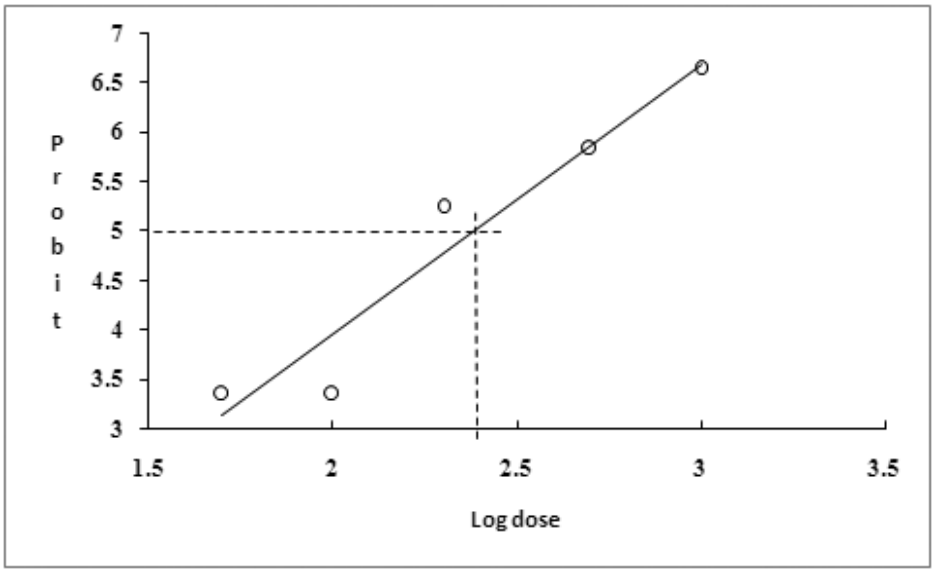
The LD_{50} value (240 mg/kg) of BE lays within the range of 50-500 mg/kg, which is considered as "moderately toxic" category [25]. However a detailed sub-acute toxicity evaluation of the effective dose showed the extract does not pose any threats to vital organs as well as hematological and biochemical parameters, and therefore considered to be safe at the bioactive dose level.

In the present study, the fruits of ST showed prominent anti-fertility activity which may be due to antizygotic, blastocytotoxic or anti-implantation activity [26] as indicated by lowering or complete loss of implantation sites compared to control group with no signs of implantation at all (i.e., 100% preimplantation loss) in case of BE. Saponins present in the fruit pericarps might have an essential role on pregnancy interception as observed from the fact that BE, rich in saponin, seems to be more effective than EF. Further investigations are required to support the finding regarding saponins. No teratogenic effects were observed either by gross visual examination or in the weight of litters born. After discontinuation of the treatment, the females were further mated, resulting in normal pregnancy and delivery, indicating reversible action of the extract.

Uterine wet weights, vaginal cornifications and histological examination of uterus implied that BE exhibits mild anti-estrogenic activity in presence of reference hormone. Changes in the level of gonadal and gonadotrophic hormones provided an insight into the post coital anti-fertility phenomenon. The steps of implantation are not yet completely understood though it is known that exact equilibrium between estrogen and progesterone hormones is needed for the process of tubal transport of rodent embryo and its apposition, adhesion and implantation [27]. Any disturbance in the hormonal milieu in the uterine environment leads to infertility. In presence of reduced levels of gonadal hormones, the blastocysts either fail to implant or maintain if implanted. Gonadotrophic hormone levels in turn elevates due to negative feedback mechanism. Lipid-carbohydrate profiles also implicate BE to be safe at bioactive level.

The present findings are comparable with the findings on *Hibiscus rosa-sinensis* leaf extract [28] and the extract showed 100% anti-implantation along with antiestrogenic potential in presence of estrogen leading to altered physiology of the endometrium, oxyradical and antioxidant status in the endometrium and failure of blastocyst implantation.

Suppl. Fig. 1 Determination of LD_{50} value of BE of *Sapindus trifoliatus* using a graphical method.



Suppl. Table 1 Acute Toxicity Studies and LD_{50} Calculation for BE of *Sapindus trifoliatus*

Sl No.	Dose (mg/kg bw)	Log dose	N	N_d	% Mortality	Corrected mortality (%)	Probit
1	50	1.70	5	0	0	5	3.36
2	100	2.00	5	0	0	5	3.36
3	200	2.30	5	3	60	60	5.25
4	500	2.70	5	4	80	80	5.84
5	1000	3.00	5	5	100	95	6.64
LD_{50} value of BE =240.00 mg/kg bw							

[N] number of test animals., [Nd] number of animals that died.

Suppl. Table 2 Sub Acute Toxicity Profiles of Control and Test Groups

Bio parameters		Control	Test
Organ weight	Liver (mg)	951.06±13.49	968.63±19.88
	Kidney (mg)	171.25±14.40	170.14±17.63
Haematological parameters	TC (Total Leucocyte count) (10^3/mm^3)	8.4±0.52	10.69±0.44
	Neutrophils (%)	37±7.25	30±5.33
	Lymphocytes (%)	63±5.19	69±8.19
	Monocytes (%)	0	0
	RBC count (10^6/mm^3)	4.9±0.43	4.5±0.52
	Hb (hemoglobin) (g/dl)	14.0±0.57	13.5±0.52
	Platelet count (10^3/mm^3)	782±15.67	724±12.52
	PCV (packed cell volume; Hematocrit value)	42±0.53	39±0.81
Biochemical parameters	Urea (mg/dl)	36±3.44	38±3.19
	Creatinine (mg/dl)	0.35±0.02	0.32±0.02
	SGPT/ALT (IU/l)	80±13.83	77±14.63
	SGOT/AST (IU/l)	502.90±17.8	496±16.83
	Alkaline phosphatase (IU/l)	222±18.73	196±19.27

Suppl. Table 3 Estrogenic and Anti-Estrogenic Potencies

Treatment (dose, mg/kg body weight)	Uterine weight mg/100 g body weight	Vaginal opening	Vaginal cornification
Control	64.79±8.51	-	-
BE (20)	67.65±3.19	-	-
Ethinyl estradiol (0.03)	228.16±6.44*	+	+
Ethinyl estradiol (0.03) + BE (20)	163.62±8.83*, †	+	+

* $P < 0.001$: Significance relative to control., † $P < 0.001$: Ethinyl estradiol relative to Ethinyl estradiol + BE., + stands for positive and − stands for negative outcome.

Suppl. Table 4 Hormone, Glucose and Lipid Profiles of Blood on BE Treatment

Bio-parameters				Mean ± s.e.	Percentage change#
Serum gonadal and gonadotrophic hormone levels	**Estradiol**	**pg/ml**	***bt***	378.4±40.4	-9.7[a]
			at	341.7±42.5	
	Progesterone	**ng/ml**	***bt***	50.2±8.8	-10.9[a]
			at	44.7±8.4	
	DHEA-S	**µg/ml**	***bt***	1.3±0.3	20.7[b]
			at	1.5±0.3	
	Testosterone	**ng/ml**	***bt***	1.6±0.4	-76.2[a]
			at	0.4±0.2	
	Cortisol	**ng/ml**	***bt***	433.1±36.1	-8.1[a]
			at	398.2±40.3	
	Estriol	**ng/ml**	***bt***	363.2±31.3	-37.1[a]
			at	228.5±41.1	
	LH	**mIU/ml**	***bt***	0.4±0.1	79.0[a]
			at	0.7±0.2	
	FSH	**mIU/ml**	***bt***	0.4±0.1	165.6[a]
			at	1.0±0.3	
Serum carbohydrate levels	**Glucose**	**mg/dl**	***bt***	85.4±15.0	8.8[b]
			at	92.9±14.5	
Serum lipid levels	**Cholesterol**	**mg/dl**	***bt***	84.8±5.4	3.8[b]
			at	88.0±5.5	
	HDL-C	**mg/dl**	***bt***	6.2±0.8	32.2[a]
			at	8.2±1.3	
	LDL-C	**mg/dl**	***bt***	71.5±4.3	5.2[b]
			at	75.2±4.7	
	VLDL-C	**mg/dl**	***bt***	7.0±1.3	-35.1[a]
			at	4.6±1.0	
	TG	**mg/dl**	***bt***	35.2±6.6	-35.1[a]
			at	22.8±4.9	

* bt and at stand for before and after treatment., # changes are significant at [a]$p<0.05$ and [b]$p>0.05$

Suppl. Table 5 Phytoconstituent Profile of EF

Saponins	Sterols and triterpenoids	Reducing sugars	Tannins	Alcohols	Essential oils	Glycosides	Alkaloids	Flavonoids
+	-	+	-	-	+	-	-	-

+ and - indicate presence and absence respectively.

CONCLUSION:

The present study emphasises that the folklore formulation as well as the butanol extract of the fruits of *Sapindus trifoliatus* Linn, have prominent post-coital pregnancy interceptive activity in female rats. The observed effects could be mediated at several levels of the hypothalamic-pituitary-gonadal (HPG) axis due to altered levels of gonadotrophic hormones. Anti-implantation activity may also be due to altered gonadal hormone levels making uterine environment unreceptive.

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