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HERBAL ANTI-FERTILITY POTENTIAL OF SHOREA ROBUSTA AND FICUS INFECTORIA: A RODENT-BASED INVESTIGATION

Sweta Kasna[1], Pougang Golmei[2], Devesh Tewari[3] Gaurav K Jain*[4]

[1,2]*Department of Pharmacology, Delhi Pharmaceutical Sciences and Research University, New Delhi-17, India.*
[3]*Department of Pharmacognosy and Phytochemistry, School of Pharmaceutical Sciences, Delhi Pharmaceutical Sciences and Research University, New Delhi, India*
[4]*Department of Pharmaceutics, Delhi Pharmaceutical Sciences and Research University, New Delhi-17, India*

***Corresponding Author:** Dr. Gaurav K Jain*
Department of Pharmaceutics,Delhi Pharmaceutical Sciences and Research University
Mehrauli – Badarpur Road,Pusph Vihar, New Delhi-17, India
Phone no: 98111 27909
Email ID: gkjdpsru@gmail.com

Abstract

Objectives: To evaluate the anti-fertility effects of hydroalcoholic bark extract (HBE) of S. robusta and F. infectoria in female rodents, focusing on implantation, ovulation, uterus, and hormonal alterations.

Study design: Female rats and mice were treated with HBE (400 mg/kg), and anti-implantation, anti-ovulatory, and estrogenic/anti-estrogenic effects were evaluated through biochemical, hormonal, and histopathological analyses.

Results: Flavonoids, glycosides, tannins, and alkaloids were detected during phytochemical analysis of the extracts. Both extracts significantly reduced estrogen and progesterone levels and altered ovarian and uterine morphology. S. robusta showed stronger anti-implantation activity (16.67%) and anti-fertility effect (23%). F. infectoria displayed 32% anti-fertility activity primarily via post-implantation loss. Further, histological and biochemical analyses also confirmed these findings.

Conclusion: The results suggest that these extracts possess promising multi-mechanistic anti-fertility activity and can serve as leads for developing herbal oral contraceptives.

Implications: S. robusta and F. infectoria can be used as promising oral contraceptive agents. This study can serve as a base for future research in utilizing the bark of S. robusta and F. infectoria as alternative oral contraceptive agents. This development has the potential to cater to a new era of anti-fertility medicine, providing innovative and beneficial healthcare solutions. To further enhance accessibility, additional research efforts must be undertaken to commercialize the HBE of S. robusta and F. infectoria as alternative contraceptive agents.

Keywords: Anti-fertility; Contraceptive; Estrogenic activity; Fertilization; Herbal medicine; Population

1. Introduction

The global population has reached approximately 8 billion, with India contributing approximately 1.4 billion [1], [2]. Swift population growth in developing nations, particularly India, poses a significant and potentially life-threatening challenge. Henceforth, it is imperative to limit the population growth [3]. Anti-fertility agents, also known as oral contraceptives, are designed to control pregnancies by preventing fertilization, ovulation, implantation, or inducing abortion, thereby affecting various stages of the reproductive process [4]. Over an extended time, researchers have explored multiple methods for controlling fertility, including hormonal, chemical, and immunological approaches [5]. However, chemical approaches, while effective, have diverse side effects such as obesity, cholelithiasis, gastric issues,

breast and cervical carcinoma, asthma, and thromboembolism [6]. Therefore, people seek alternative medicines with fewer or no side effects for anti-fertility action. As plants have been a natural source of medicinal compounds since ancient times [7], the exploration of fertility-regulating drugs derived from plants holds great significance.

Considering these issues, the present investigation deals with the evaluation of anti-fertility activity of ethno-medicinal plants: Shorea robusta Gaertn. and Ficus infectoria Roxb.

Indian ethnomedicine plant Shorea robusta Gaertn. (Dipterocarpaceae), known as Sal, it is widely used in Ayurveda and Unani medicine for diverse ailments. In an Indigenous system, the different parts of S. robusta, like bark, flower, resins, are known to possess pharmacological action to treat diarrheal diseases, diabetes mellitus, and bacterial infections, etc. [8] [9]. Analytical studies revealed that the plant contained tannin, triterpenoids, volatile matter, and phenolic content, which are the bioactive phytochemicals [10]. S. robusta is now being investigated for anti-fertility activity based on research that uses composite root extract including S. robusta for inhibiting implantation, hindering oocyte development [11], [12], leucorrhoea, menorrhagia, emmenagogue [13], and prevention for hypermenorrhea [14], [15]. F. infectoria (syn. Ficus virens Aiton, Ficus lacor Buch.-Ham.) is commonly known as pilkhan, belonging to the group of strangling figs. F. infectoria is a medium-sized tree that grows along the streamside in subtropical China, tropical south, and south-east Asia. In the traditional medicine system, many parts of F. infectoria, such as bark, latex, leaves, and fruits, are used in the treatment of blood diseases, apoplexy, vertigo, delirium, pain, rheumatism, diabetes, and also as antioxidants. Phenolic compounds are the main constituents of F. infectoria leaves and stem bark, according to phytochemical analyses of the plant. Furthermore, a thorough review of the literature revealed that tannins are found in various parts of this plant [16]. F. infectoria is evaluated for anti-fertility activity based on research mentioned that F. infectoria is a constituent of panchvalkala, a traditional Ayurvedic preparation (mentioned in Charak Samhita and Bhavaprakasha Nighantu) is being used for the treatment of female gynecological problems such as leucorrhea, endometriosis, menorrhea, menstrual disorders and vaginal disorders[17] [18] [19]. Bark extract of F. infectoria also contains bergapten and bergaptol, which are known to show anti-fertility activity [20] [21]. Although S. robusta and F. infectoria are used in composite traditional medicine, their direct anti-fertility effects in rodents through comprehensive pharmacological evaluation remain unexplored. This study was intended to investigate the anti-fertility effect of HBE of S. robusta and F. infectoria using female rodents for the first time.

2. Materials and methods

2.1 Collection and authentication

Bark of S. robusta and F. infectoria was collected from the Khari Baoli market of Delhi, India. The samples were identified at CSIR-National Institute of Science Communication and Policy Research (NIScPR) on 25 March 2022 with an Authentication no NIScPR/RHMDC/Consult/2022/4049-50-3 and NIScPR/RHMDC/Consult/2022/4049-50-1, respectively, New Delhi, India

2.2 Extract Preparation and preparation of the extract suspension.

The HBE was prepared using the maceration technique after soaking the bark for 24 hours in distilled water/methanol (50/50) solvent. Then, the mixture was filtered using the #1 Whatman filter paper. The filtered mixture was thickened in the rotary evaporator; it was placed under the biological hood to allow the remaining solvent to evaporate. The dried extract was kept in the refrigerator in a sterile, closed container that protected it from light [22]. The dose of the extracts was selected based on previous studies[23] [24]. Carboxymethyl cellulose (0.5%, CMC solution) was used to prepare HBE suspension of S. robusta (400 mg/kg) and F. infectoria (400 mg/kg).

2.3 Preliminary phytochemical screening of bark extracts

The extract from various plant parts usually contains bioactive phytochemicals, which belong to various classes of phytochemicals, such as alkaloids, tannins, flavonoids, glycosides, etc. [25] [26] [27] [28]. These classes of phytochemicals were identified in the bark extract of S. robusta and F. infectoria using various preliminary phytochemical screening tests. A pinch of extracted powder of bark of S. robusta and F. infectoria was taken in a test tube and added with a few drops of chemical reagents such as strong acids, strong bases, and other reagents. The characteristic colours produced by the reactions were observed and recorded as per the standard methods. The bioactive compounds, such as alkaloids, carbohydrates, flavonoids, glycosides, phenols, saponins, tannins, terpenoids, etc., were screened by performing different tests for each phytochemical to ascertain the presence (Evans, 1989; Sofowara, 1993; Harborne, 1998) [29]. The

following preliminary phytochemical screening tests were conducted in this study (Table 1) [30].

2.4 Animals

Adult male and female albino Wistar rats (150–200 g) and immature female Swiss albino mice, approximately 3 weeks old (10-12 g), were procured from the animal house of DPSRU, New Delhi. Animals were housed in a controlled standard environment with unrestricted water access and ad libitum access to a pelleted diet. The animals were habituated for 7 days before experimental use. All animal investigations were executed as per the recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the approved protocol (IAEC/2019/II-R-12) of the Institutional Animal Ethical Committee (Registration No. 215/GO/ReBi/S/2000/CPCSEA) DPSRU, New Delhi.

2.5 Anti-fertility screening

The anti-implantation, anti-ovulatory, and estrogenic/anti-estrogenic studies, along with the hormonal analyses, were performed to assess the anti-fertility activity of bark extracts

2.5.1 Anti-implantation activity

Evaluation of anti-implantation activity was performed on 13 pregnant female rats in three subgroups (control = 3, treatment 1 = 5, treatment 2 = 5, where treatment 1 was HBE of S. robusta [31] and treatment 2 was HBE of F. infectoria) [32]. Briefly, adult fertile female rats were housed with mature male rats at a ratio of 3:1. Further, vaginal smears of the female rats were inspected daily in the morning to assess their estrous cycle (annexure 1 and Figure S1, supplementary material) and the presence of spermatozoa using Giemsa staining. The appearance of sperm in the vaginal smear for the first time was considered day 1 of pregnancy. Female rats were single-housed after the onset of pregnancy. The rats were orally administered 1.0 ml of normal saline, S. robusta extract (400 mg/kg) [23], and F. infectoria extract (400 mg/kg) [24], [33] in the control, treatment 1, and treatment 2 groups, respectively, for 7 consecutive days. On the 10th day of pregnancy, the animals were laparotomized to determine the implant count in the uterine horns and the number of corpora lutea in the ovaries [34]. Rats were allowed to complete their gestation period (usually 21-23 days), and the number of litters delivered was recorded [35]. The pre-implantation and post-implantation loss percentages and anti-fertility activity were calculated using the following formulas [36]:

1) Pre-implantation loss:
$$\text{No of corpora lutea on the 10th day} - \text{No of implant on 10th day}$$
2) Post-implantation loss:
$$\text{No of implants on the 10th day} - \text{No of litters delivered}$$
3) Percentage of Pre-implantation loss:
$$\frac{\text{no of corpora lutea} - \text{no of implants}}{\text{no of corpora lutea}} \times 100$$
4) Percentage of Post-implantation loss:
$$\frac{\text{no of implants} - \text{no of litters}}{\text{no of implants}} \times 100$$
5) Percentage of Anti-implantation activity [37]:
$$\frac{\text{no of implants of control} - \text{no of implants of treatment}}{\text{no of implants of control}} \times 100$$
6) Percentage of anti-fertility activity:
$$\frac{\text{no of corpora lutea} - \text{no of litters}}{\text{no of corpora lutea}}$$

2.5.2 Estrogenic/Anti-estrogenic activity

For estrogenic/anti-estrogenic activity, immature female mice (approximately 3 weeks old and 10-12 g) were subdivided into six groups: (A) control (n=3), (B) standard (estradiol, 0.5 µg/kg, subcutaneous) (n=5), (C) S. robusta extract (n=5), (D) S. robusta + standard (n=5), (E) F. infectoria (n=5), and (F) F. infectoria + standard (n=5). The rats in these groups received 1.0 ml of normal saline, estradiol, S. robusta extract, estradiol + S. robusta extract, F. infectoria extract, and estradiol + F. infectoria extract, respectively, for 4 consecutive days. Animals were euthanized 24 h after treatment completion. The uterus was then collected, pressed, and weighed. The mean uterine weight of each group was compared with the control and standard to determine the percentage reduction in uterine weight and stored in 10% formalin for histopathological evaluation of estrogenic/anti-estrogenic activity using hematoxylin and eosin (H&E) staining [38], [39].

2.5.3 Anti-ovulatory activity

Non-pregnant female rats were used for evaluating anti-ovulatory activity, which were subdivided into groups: control (n=3), treatment 1 (n=5), and treatment 2 (n=5). The rats were orally administered 1.0 ml of normal saline, HBE of S. robusta (400 mg/kg), and HBE of F. infectoria (400 mg/kg) in the control, treatment 1, and treatment 2 groups, respectively, for three consecutive days. Further, cupric acetate (4 mg/kg) was injected intravenously to induce ovulation after 30 minutes of the last dose administration. For investigating ovulation, rats were sacrificed after 18-24 hours of cupric acetate administration, and ovaries were harvested and stored in 10% formalin for

histopathological evaluation of anti-ovulatory activity using hematoxylin and eosin (H&E) staining [40], [41].

2.6 Hormonal assay

Blood samples were collected under light anesthesia through the retro-orbital plexus in centrifugation tubes for serum collection. The blood samples were incubated at 37˚C for 45 min and centrifuged at 1500 rpm for 15 min at 4˚C to collect serum. Blood serum was used to measure the levels of progesterone and estrogen using the progesterone and estradiol ELISA kit (Elabscience) [42].

2.7 Tissue cholesterol estimation

One ovary from each rat was used for the estimation of tissue cholesterol levels (mg/dl of tissue) following the protocol of Shrestha et al. [43]. The weight of the ovary was estimated and then homogenized to acquire a uniform suspension in cold saline (i.e., 1 mg tissue in 20 µL of saline). Cholesterol level was assessed using the manual ELISA kit procured from Aspen Laboratory Pvt. Ltd., Delhi.

2.8 Histopathological investigation

The isolated ovaries and uteri were fixed in 10% formalin and dehydrated in alcohol. Transverse sections of the fixed ovary and uterus of five-micrometer diameter were prepared and mounted on glass slides and then stained with hematoxylin and eosin using a standard protocol [44]. Slides were observed under a trinocular microscope with LED and camera (by Radical Scientific, model no-RxLr-5) using Pro-CAM software at 20X magnification to investigate morphological changes in the ovaries and uterus.

2.9 Statistical Analysis

All experimental data are conveyed as mean ± SEM. The data from all groups were analysed using one-way and two-way ANOVA of Graph Pad Prism 8 software. The standard for statistical significance was set at $p < 0.05$.[45]

3. Results

3.1 Percentage yield of the extracts.

The percentage yield of the prepared bark extract of S. robusta and F. infectoria was found to be 13% and 11%, respectively. These bark extracts were screened for the presence of bioactive phytochemicals and used for evaluating their anti-fertility activity in rodents.

3.2 Preliminary phytochemical screening of bark extracts

The HBE of S. robusta and F. infectoria was investigated for the presence of different classes of phytochemicals using preliminary screening tests. The list of experiments performed and classes of phytochemicals found were compiled in Table 1. Many research has already been conducted, which has shown that these classes of phytochemicals, such as alkaloids, tannins, flavonoids, glycosides, etc., have potential as effective antifertility agents [25] [26] [27] [28]

Preliminary test	Class of phytochemical	S. robusta	F. infectoria
Lead acetate, Potassium dichromate, and Ferric chloride tests	Tannins	+	+
Foam test	Saponins	-	+
Sodium hydroxide and conc. Sulphuric acid tests	Flavonoids	+	+
Borntrager's and Keller-Killiani tests	Glycosides	+	+
Dragendorff's, Hager's, and Wagner's tests	Alkaloids	+	+
Fehling's, Benedict's, and Barfoed's tests	Carbohydrates	+	+
Biuret, Ninhydrin's, and Millon's tests	Proteins	-	-

+ Present; - Absent

Table 1: Preliminary phytochemical screening of HBE of S. robusta and F. infectoria

3.3 Anti-fertility activity

3.3.1 Anti-implantation activity

The effect of HBE of S. robusta and F. infectoria was investigated for the presence and count of the corpora lutea and implant. As shown in Table 2, there was no significant change in the corpora lutea count in the ovaries of the treatment 1 and 2 groups. However, a considerable loss in the number of implants in treatment 1 (10±0.84) ($p<0.01$) (Figure 1) was observed as compared to the normal control (12±0.71).

A decrease in implant count (11.4±0.51) in treatment 2 was also observed, but the change was not statistically significant (Figure 1). The anti-implantation activity of the HBE of S. robusta and F. infectoria was 16.67% and 5%, respectively, evaluated using formula 4 (Table 2). A significant loss in the number of litters delivered was also observed in treatment 1 (9.2±1.02) and treatment 2

(8.8±0.73) compared to the control (11.2±0.66). Both treatments yielded significant results ($p < 0.01$) (Figure 1).

Groups/ Treatment	No. of corpora lutea (Mean ±SEM)	No. of implantation sites (Mean ±SEM)	Anti-implantation activity%	No. of litters born (Mean± SEM)
Control, Normal saline	12.8±0.54	12±0.71	0	11.2±0.66
Treatment 1 S. robusta, 400mg/kg	12±0.71	10±0.84**	16.67%	9.2±1.02**
Treatment 2 F. infectoria, 400 mg/kg	13.2±0.92	11.4±0.51	5%	8.8±0.73***

Values are expressed in mean ± SEM. Significant difference was observed at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****). SEM = Standard error of mean

Table 2: Changes in different parameters of implantation on intervention with treatment 1 and treatment 2 when administered orally for 1-7 days post-coital in female Wistar rats. Treatment 1 = HBE of S. robusta and treatment 2 = HBE of F. infectoria.

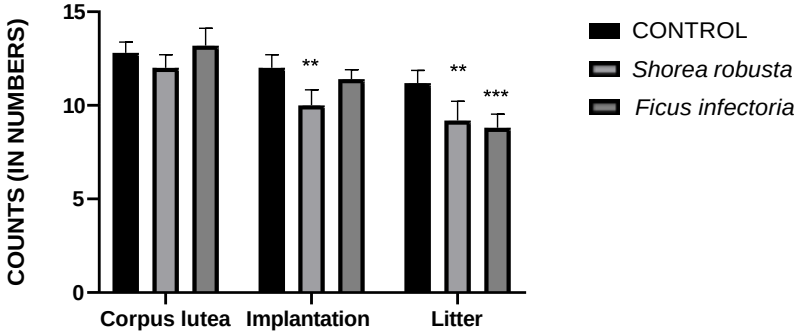

Figure 1: Graph showing changes in corpora lutea, implants, and litter counts in female Wistar rats after 7 days of oral administration of HBE of S. robusta and F. infectoria. Significant difference was observed at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

3.3.2 Pre-implantation effect

The effect of HBE of S. robusta and F. infectoria on the pre-implantation effect was investigated by evaluating the change in the number of CL and implants using formula 3. There was a reduction in the number of CLs and implants (pre-implantation determinants), which increased pre-implantation loss (16.60±3.67%, $p<0.01$) (Figure 2) in the treatment 1 group compared to the control (6.19±4.30%). Similarly, a reduction in the number of CLs and implants was also observed in the treatment 2 group, where there was an increase in pre-implantation loss (12.62±4.4%), but the change was not statistically significant (Table 3).

3.3.3 Post-implantation effect

After comparing the change in the number of implants and litters using formula 4, it was observed that there was a reduction in post-implantation determining parameters, which increased post-implantation loss in treatment 1 (8.72±2.23%) (Figure 2) and treatment 2 (22.07±7.45%, $p<0.0001$) groups compared to the control (6.63±2.34). However, the increase in the post-implantation loss was not statistically significant in the treatment 1 group.

3.3.4 Anti-fertility effect

The anti-fertility activity of HBE of S. robusta and F. infectoria was evaluated using formula 5. The anti-fertility percentage was increased in the treatment 1 and 2 groups as compared to the control (12.25±5.73%) with rates of 23±4.93% ($p< 0.01$) and 32.05±7.44% ($p< 0.0001$), respectively.

Groups/Treatment	Mean Percent Pre-implantation loss ± SEM	Mean Percent Post-implantation loss ± SEM	Mean Percent Anti-fertility ± SEM
Control (Normal saline, 3ml/kg)	6.19±4.30	6.63±2.34	12.25±5.73
Treatment 1 (HBE of S. robusta, 400 mg/kg)	16.60±3.67 **	8.72±2.23	23±4.93 **
Treatment 2 (HBE of F. infectoria, 400 mg/kg)	12.62±4.4	22.07±7.45****	32.05±7.44 ****

Values are expressed in mean ± SEM. Significant difference was observed at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****). SEM = Standard error of mean

Table 3: Effect of HBE of S. robusta and F. infectoria on different parameters of anti-fertility activity.

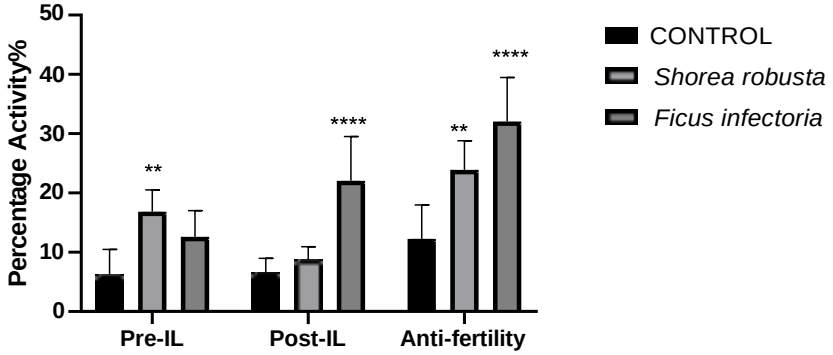

Figure 2: Graph showing pre- and post-implantation loss along with the anti-fertility activity of HBE of S. robusta (Treatment 1) and F. infectoria (Treatment 2). Significant difference was observed at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

3.3.5 Estrogenic/Anti-estrogenic Activity

The effect of HBE of S. robusta and F. infectoria on estrogenic/anti-estrogenic supported change in uterine growth was also investigated. The uterine weight was significantly increased in mice injected with estradiol for 4 days. This clearly represents the role of estrogen in reproductive development. Uterine weight was found to be increased significantly compared to the control in all groups where treatments were administered for 4 days (Table 4). However, it was observed that there was a significant decrease in uterine weight in mice fed with HBE of F. infectoria either alone ($P < 0.001$) or in combination with estradiol injection ($P < 0.001$) compared to mice injected with estradiol alone (Figure 3). These results clearly indicate the estrogenic and anti-estrogenic activity of HBE of S. robusta and F. infectoria, respectively, which was further supported by histopathological findings (Figure 4).

Group	Extracts/treatment	Dose	Uterine weight (mg)	% change in uterine weight (control)	% change in uterine weight (standard)
A	Control	1 ml distilled water	18.4±3.049	-	-44.91
B	Standard (estradiol)	0.5µg/kg, s.c	33.4±5.409****	+ 81.52%	-
C	S. robusta extract	400mg/kg, p.o	26±2.168*	+ 22.15%	-22.75
D	S. robusta extract + estradiol	400mg/kg, p.o + 0.5µg/kg, s.c	31.6±3.124****	+ 71.74%	-5.39
E	F. infectoria extract	400 mg/kg, p.o	22±3.701	+ 19.57%	-34.13
F	F. infectoria extract + estradiol	400mg/kg, p.o + 0.5µg/kg, s.c	20.8±3.555	+ 11.96%	-37.72

Values are expressed in mean ± SEM. Significant difference was observed at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****). SEM = Standard error of mean. +, - Represents % increase and decrease in uterine weight.

Table 4: Showing change in uterine weight after 4 days of intervention with HBE of S. robusta, F. infectoria, and estradiol.

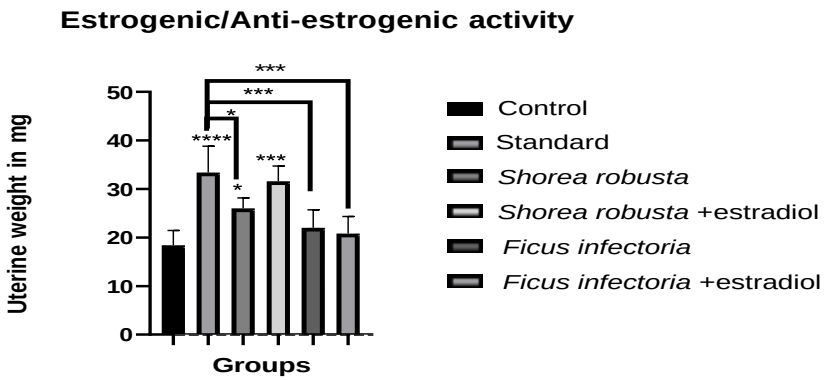

Figure 3: Graph showing a change in uterine weight as a function of estrogenic/anti-estrogenic activity of HBE of S. robusta and F. infectoria ($p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

3.5.6 Histopathological changes in uterine morphology

The uterus of immature female control mice consisted of a slender lumen, few glands, differentiated prominent layers of the uterine wall, visible columnar epithelial cells, and prominently visible blood vessels (Figure 4A). These morphological features indicate the proliferative phase of the endometrium. After dosing with estradiol for 4 days, the uterine lumens became tortuous with numerous prominent glands and blood vessels, an undifferentiated endometrial layer, and degeneration in the perimetrium, myometrium, and epithelial cells, indicating a prominent secretory phase with increased uterine weight (Figure 4B). However, treatment with the bark extract of S. robusta resulted in a tortuous lumen with an increased number of prominent glands with irregular shapes, along with thickening of the endometrium, differentiated endometrial layer, perimetrium, myometrium, and epithelial cells, along with visibly extended and enlarged blood vessels, indicating an initial secretory phase (Figure 4C). The mice administered with combined a dose of estradiol and the bark extract of S. robusta showed numerous uterine glands with a thickened, undifferentiated uterine wall, epithelial cell layers with signs of degeneration in the widened layers of the endometrium, prominently visible blood vessels, and a small uterine lumen. These changes indicate a prominent secretory phase and may be the reason for increased uterine weight (Figure 4D). Similarly, F. infectoria bark extract-treated female mice showed no hypertrophy, lumen dilation, and differentiated layers of uterine tissue. In addition, some signs of degeneration in differentiated uterine walls, epithelial cells, and blood vessels, and fewer uterine gland counts were also observed. Moreover, the endometrium appeared slightly thickened and differentiated, indicating that it is not the secretory phase (Figure 4E). Combined treatment with bark extract of F. infectoria and estradiol led to scattered uterine glands, necrosis in epithelium layers, few blood vessels, and widened lumen, and also showed perimetrium, myometrium, and endometrial degeneration (Figure 4F).

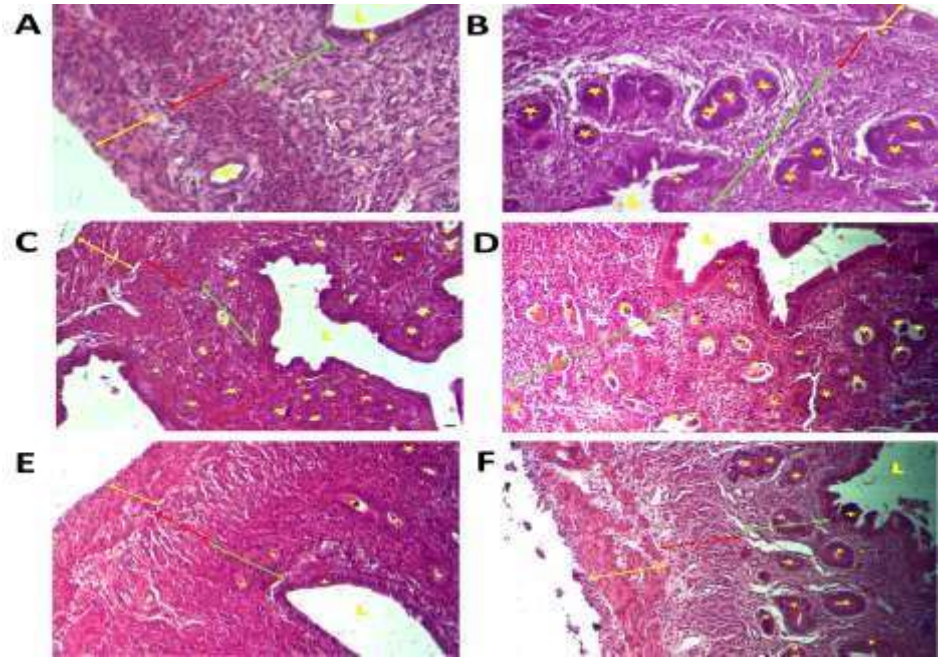

Figure 4: Histological morphology of the uterus of immature female mice of 3 weeks old without any treatment that is Control (A) and under-treatment of estradiol (B), S. robusta (C), S. robusta + estradiol (D), F. infectoria (E), and F. infectoria + estradiol (F). Abbreviation and marking: L (lumen), green double-sided arrow (endometrium layer), red double-sided arrow (myometrium layers), yellow double-sided arrow (perimetrium layers), yellow plus sign (epithelium layers), yellow star (uterine glands). (H&E, 200x, 50 µm)

3.5.7 Histopathological investigation of ovary to evaluate anti-ovulatory activity

Histopathological investigation showed that the ovarian tissues were covered by a solitary layer of modified mesothelium and represented the surface epithelium. Also, primordial follicles (PF) contained germ cells exclusively, whereas maturing Graafian follicles (GF) had a nucleus (N), granulosa cells (G), theca interna (TI), theca externa (TE), and corpora lutea (CL) (Figure 5A), which

represent induction of ovulation by cupric acetate in control. The transverse sections of the ovary in treatment 1 (Figure 5B) showed an absence of oocytes in the secondary follicles (SF); however, atretic follicles (AF), zona pellucida, granulosa cells (G), theca externa (TE), and theca interna (TI) were visible and can be easily differentiated. In addition, vacuolization and degeneration of interstitial cells were observed, and the secondary follicle (S) was distorted, with a large antrum. Transverse sections from the ovaries of rats in treatment 2 (Figure 5C) exhibited interstitial cells and numerous follicles with clearly distinguishable theca Interna (TI) and theca Externa (TE). Additionally, some follicles displayed slightly distorted nuclei along the antrum (A), and atretic follicles (AF) were observed. This result clearly indicated the inhibition and disruption of ovulation by the HBE of S. robusta and F. infectoria, which was clearly shown in the histopathological images. (Figure 5).

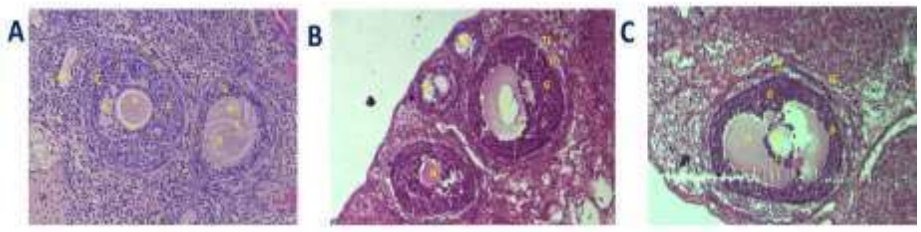

Figure 5: Histological picture showing hematoxylin and eosin-stained transverse sections of rat ovary. A (control), B (treatment 1, S. robusta), C (treatment 2, F. infectoria), A = Antrum, AF = Atretic Follicle, B = Basement Membrane, C = Corona Radiata, CL = Corpora Lutea, G = Granulosa Cells, N = Nucleus Representing Oocyte, S = Secondary Follicle, P= Primary Follicle TI = Theca Interna, TE = Theca Externa, Z = Zona Pellucida. (200x, 50 µm).

3.5.8 Evaluation of hormone level

A hormone-specific **ELISA kit experiment** on serum from non-pregnant female rats was performed to evaluate the change in the levels of estrogen and progesterone in rats in treatment 1 and treatment 2 groups compared to the control; however, the cholesterol level was evaluated in the ovary tissue. Since cholesterol serves as the precursor for steroidogenesis in ovarian endocrine tissues [46], [47], the notable increase in ovarian cholesterol levels (Figure 6A) in the treated groups suggests that cholesterol may not be utilized for steroidogenesis. Figure 6B and Figure 6C clearly show a significant drop in estrogen and progesterone levels in female rats in the treatment 1 and 2 groups. These results clearly indicate the inhibitory effect of the HBE of S. robusta and F. infectoria on the reproductive secretory system.

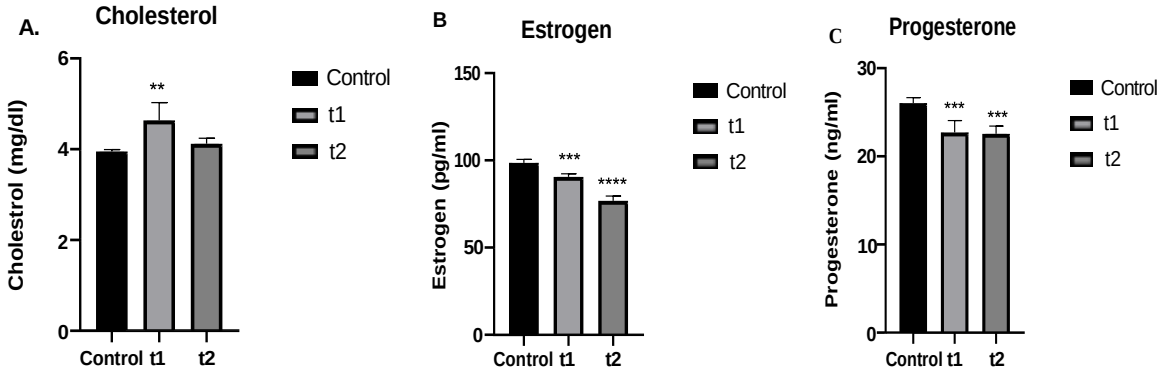

Figure 6: Graph showing change in cholesterol, estrogen, and progesterone levels to investigate the effect of HBE of S. robusta and F. infectoria.

4. Discussion

The screening of plants to evaluate the anti-fertility potential can be performed via (a) Investigation of plants that have a folkloric/traditional reputation as contraceptives. (b) Evaluation of plants that are known to contain constituents which theoretically affect the female cycle and thus produce anti-fertility effects, e.g., estrogenic sterols, isoflavones, and coumestanes, or those which have the potential to contract the uterus. (c) Random screening of plants for mass screening for fertility regulation [48].

Plants are the natural source of active pharmaceutical chemical compounds and have

been utilized in various female physiological conditions since ancient times. However, despite the extensive advantages of natural remedies, efforts have not been made to develop any clinically acceptable contraceptives from traditionally known medicinal plants.

A literature survey revealed that composite root extract, which includes Shorea robusta in mixed form, has been traditionally used by the folk women of the North East region of India through the oral route during the first seven days of a menstrual cycle to prevent conception [11]. Research also suggests that extracts containing Shorea robusta (Sal) root, especially in composite formulations, may have potential for inhibiting implantation and hindering oocyte development [11], [12]. Bark of F. infectoria was being used for the treatment of women with endometriosis-related problems, leucorrhea and vaginal ailments [18][17][19]. However, their direct anti-fertility effects through comprehensive pharmacological evaluation remain unexplored.

In the current study, the anti-fertility effect of HBE of S. robusta and F. infectoria was explored in three important steps of the reproductive processes (implantation, ovulation, and estrogenic modification of uterine wall morphology) in female rodents.

HBE of S. robusta and F. infectoria was found to contain alkaloids, flavonoids, glycosides, and tannins (Table 1). These tannins, alkaloids, flavonoids, and glycosides are also known to alter the various phases of female fertility [25], [26], [27], [28]. After phytochemical analysis, these HBE were investigated for their in vivo anti-fertility activity. In rodents, successful implantation of the fertilized ovum requires synchronized development of the embryo and endometrium, governed by a delicate balance of estrogen and progesterone. Early pregnancy is maintained by progesterone produced by the corpora lutea. The agents that prevent implantation of the embryos by interfering with pre- and post-implantation events of pregnancy are known as anti-implantation agents. In contrast, those disrupting early pregnancies are known to possess abortifacient activity. Emmenagogues are the agents that increase the menstrual flow in the non-gravid uterus and have been used extensively to induce abortion [52] [53].

The anti-implantation activity of HBE of S. robusta and F. infectoria was found to be 16.67% and 5%, respectively (Table 2). The results showed a significant mean percent pre-implantation loss ($p<0.01$) and anti-fertility activity ($p<0.01$) with HBE of S. robusta (Figure 2, Table 3), which may be due to inhibition of corpora lutea and the number of implants (Figure 1, Table 2). In contrast, HBE of F. infectoria showed a significant post-implantation ($p<0.0001$) and anti-fertility percentage ($p<0.0001$) (Figure 2, Table 3), which indicates F. infectoria may inhibit a number of implants and no of litters (Figure 1, Table 2). Hence, S. robusta possesses significant pre-plantation loss, abortifacient activity, anti-implantation activity, and anti-fertility, while F. infectoria only possesses significant post-implantation and anti-fertility

Further, the estrogenic/anti-estrogenic effect of HBE of S. robusta and F. infectoria and their combination with estradiol on uterine weight and their morphological change was also evaluated. Endometrial thickness is positively influenced by estradiol concentration[54]. The endometrial lining plays a major role in implantation. A thin endometrium, or uterine lining, can prevent successful implantation of an embryo due to insufficient support for the fertilized egg. A thin endometrium may not provide enough blood flow or nutrients for the embryo to grow and develop, leading to implantation failure or miscarriage. In contrast, excessive thick endometrial linings, especially over 14mm, can sometimes hinder successful implantation. So, for successful embryo implantation, an optimally thickened endometrium is generally beneficial for implantation. The ideal implantation thickness is generally considered to be 8 - 14mm. [55] [56]. Results indicate that groups B, C, and D (Table 4) show a significant percentage increase in uterine weight of mice that were found to be 81.54%, 22.15%, and 71.74%, respectively, indicating an estrogenic effect, endometrial hyperplasia, and uterotrophic action of HBE of S. robusta and estradiol. It may reveal that the anti-fertility of HBE of S. robusta is due to estrogenic activity.

Group E and F (Table 4) do not show a significant percentage increase in uterine weight of mice, i.e., 19.57% and 11.96%, respectively, compared to the control. However, when compared with the positive control (estradiol injected alone), groups E and F show a significant percentage decrease in uterine weight, i.e., 22.75% and 5.39% at $p<0.001$ (Figure 3), which indicates anti-estrogenic activity of F. infectoria. As, it did not show significant uterotrophic activity when administered alone, but together with estradiol, it inhibited the uterotrophic action of estrogen. Histopathological examination of the uterus of female mice treated with HBE of S. robusta revealed major morphological changes in uterine structure, like endometrial hyperplasia with dilated glands, as shown in Figure 4C. HBE of S. robusta with estradiol (Figure 4D) shows a synergetic effect

because of the secretory phase of uterine walls with numerous glands due to uterotrophic action, which may also contribute to the prevention of implantation into the endometrium. HBE of F. infectoria revealed no hypertrophy but shows differentiated layers of uterine tissue with some degeneration and few glands, as shown in Figure 4E. The group F that received HBE of F. infectoria with estradiol (Figure 4F) did not show secretory changes.

Furthermore, the anti-ovulatory effect was evaluated using histopathological and biochemical analyses. During the menstrual cycle, two phases of elevated estrogen concentrations can be distinguished. During the proliferative phase, the growing follicles produce increasing amounts of estradiol that peak at ovulation. Hence it can be said that, the estrogen level depends on the number of ovarian follicles. After ovulation, the corpora lutea continues to produce significant amounts of estrogens, in addition to progesterone [57]. Critical concentration of estradiol is needed to initiate the positive feedback for successful ovulation. Any disturbance in the concentration of estradiol during the phase leads to inhibition of ovulation. The anti-ovulatory action may be mediated through a lower level of estrogen.

The present biochemical findings also supported the anti-ovulatory nature of the HBE of Shorea robusta and Ficus infectoria, as it increased the cholesterol in the tissue, indicating it may not be utilized in steroidogenesis, which may also be the reason for the reduced concentration of estrogen and progesterone (Figure 6). Decline in the concentration of estradiol and progesterone may be responsible for the interruption in ovulation. Further, histopathological results on the ovaries in the extract-treated groups showed ovarian follicle degeneration, vacuolization of interstitial cells, which may lower estrogen and progesterone levels, confirming the disruption of ovulation (Figure 5). This indicates significant suppression of ovulation. These results clearly suggest that HBE of S. robusta and F. infectoria possess potent anti-fertility activity and inhibit various processes of reproduction, such as ovulation, uterine endometrial morphology, and implantation.

5. Conclusion

The hydroalcoholic bark extracts of Shorea robusta and Ficus infectoria exhibit multi-targeted anti-fertility effects, including ovulation suppression, anti-implantation activity, and hormonal modulation. These findings support their potential as herbal contraceptives, although further pharmacological and toxicological studies are warranted to facilitate their development into safe and effective oral contraceptive agents.

Declaration

Ethical approval and consent to participate

The study was approved by the Institutional Animal Ethical Committee (IAEC) Protocol no. (IAEC/2019/II-R-12) DPSRU, New Delhi.

Consent for publication

Not applicable

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The availability of data and materials

The data supporting the findings of this study will be made available upon reasonable request.

Competing interest

No competing interests.

Author Contributions

SK contributed to conceptualization, experiment, data analysis, and manuscript drafting. PG and SR helped with the in vivo study. DT contributed to manuscript writing, editing, and proofreading. GKJ perceived this idea, proofread it, and prepared the final manuscript.

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