RESEARCH ARTICLE

Assessment of oral toxicity and vaginal irritation test of novel antimicrobial mucoadhesive herbal vaginal tablet for the treatment of vaginitis

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Abstract

Introduction: The preclinical *in vivo* toxicity study aims to standardize new vaginal herbal formulation consisting of stem barks of *Ficus glomerata* Roxb. and *Symplocos racemosa* Roxb. which have been mentioned in Ayurvedic system of medicine as effective in the treatment of vaginitis. Acute and subacute toxicities of its aqueous extract have been evaluated using the oral route of administration through hematological and histopathological studies of the stomach, liver, and kidney. Vaginal irritation test has been also done to assess the redness, ulceration, bleeding, etc., during drug administration.**Materials and Methods:** Acute toxicity study was carried out on Swiss albino female mice weighing about 20–30 g following OECD guidelines. A single dose of aqueous extract of the research drug was administered orally at the level of 300, 600, 900, 1200, or 1500 mg/kg to different groups and animals were observed for the appearance of toxic symptoms up to 24 h. During subacute toxicity study, female albino mice divided into five groups of 6 animals were initially orally administered 500 mg/kg dose which was increased by 50 mg/kg every 3rd day to a maximum of 1200 mg/kg dose for a period of 4 weeks. After recording changes in body weight, food, and water intake and clinical signs, animals were sacrificed on the 30th day, and their hematological parameters and liver function test (LFT) were done. Histopathological studies were done on slides of dissected stomach, liver, and kidney and internal structures of cells, tissue, and mucous membrane, etc., were observed. Vaginal toxicity testing was performed on 18 female Wistar rats divided into 3 test groups, namely, control, drug-treated (500 mg/kg), and placebo-administered, using vaginal route of drug administration. Animals were necropsied 24 h after final vaginal dose and heart, kidney, liver, lung, ovary, pancreas, uterus, and vagina were excised, and histopathological examination of the internal structure of their cells, tissues, and glands was done. **Results and Discussions:** The acute toxicity tests performed on the research formulation revealed no signs of behavioral changes up to the dose of 1500 mg/kg and no mortality was reported up to 24 h. During the subacute toxicity (long-term toxicity) testing, no animal died when daily dose of the drug was gradually increased to 1200 mg/kg b.w. over 29 days, and no significant toxic effects were observed over a prolonged period. At the same time, the hematological studies, LFT and hormonal parameters of blood did not show any noticeable differences, and no significant damage of cells, tissues, epithelium lining, or other structures of stomach, liver, and kidney was observed during histopathological studies. The vaginal irritation (subacute toxicity) studies indicated that no differences were observed among treatment groups in respect of hematological parameters as well as weight and internal structures of all vital organs during the histopathological examination.**Conclusion:** The results clearly indicate the non-toxic attribute of research drug formulation on the basis of analysis of blood parameters and reproductive hormones using oral and intravaginal administration routes.

Key words: Herbal, mucoadhesive vaginal tablet, toxicity

INTRODUCTION

The vaginal microbiota is dominated by Gram-positive *Lactobacillus* bacteria, which maintain the acidic pH in the vagina (pH 4.5) by converting glycogen from exfoliated epithelial cells into lactic acid and protect it from pathogen invasion by the production of organic acid, bacteriocins, and hydrogen peroxide. The ecology of the vagina is influenced by factors

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such as the glycogen content of epithelial cells, glucose, pH, hormonal levels, trauma during sexual intercourse, birth-control methods, age, antimicrobial treatment, and delivery. The acidic environment of the vagina is a natural barrier to infection and irritation since it represses the growth of bad bacteria which prefer a less acidic environment. Therefore, as long as the good bacteria count is high and the vaginal pH is acidic, bad bacteria have a very slim chance of overgrowing, making the chance of infection low. However, the pH changes with age, stages of menstrual cycle, infections and sexual arousal while menstrual, cervical and uterine secretions and semen act as alkalizing agents and increase the pH.

Many herbal plants and their combinations in the nature of Ayurvedic drugs have been prescribed for oral administration and external application in the Ayurvedic text for the treatment of vaginitis or leucorrhoea. The aim of the present study was to standardize the new vaginal herbal formulation by mixing equal parts of stem barks of *Ficus glomerata* Roxb. and *Symplocos racemosa* Roxb. because both these plants have been used since ancient times in the Ayurvedic system of medicine and elaborated in ancient texts such as Charaka Samhita (Chikitsa Sthana) as an astringent, anti-inflammatory, and hemostatic and useful for arresting excessive abnormal vaginal discharge.[1-3] This is a new herbal formulation which has not been evaluated till now although it is likely to exhibit sustained and significant antimicrobial action due to the synergetic effect of the phenolic and flavonoidic compounds present in this research drug and the pharmacological properties of its constituent herbs.

This research formulation contains the plant *F. glomerata* Roxb. or Cluster Fig which belongs to the Moraceae family. It is a moderate sized spreading lactiferous tree without much prominent aerial roots found throughout India whose fruits are eaten by villagers. Its leaves are dark green, ovate or elliptical while the fruits contain 2–5 cm diameter subglobose and smooth receptacles. The fruits are orange and dull reddish when ripe and having a pleasant smell. The stem bark is 0.5–1.8 cm thick, grayish green in color and having an uneven soft surface.[2,3] On rubbing it, white papery flakes come out from the outer surface; the inner surface is light brown, fracture fibrous, and mucilaginous taste. The stem bark, fruits, leaves, and latex of this plant have been used since ancient times as mentioned in the Ayurvedic textbook for treatment of dysentery, diarrhea, toothache, stomach-ache, vaginal disorders, menorrhagia, hemoptysis, diabetes, piles, and glandular swelling, etc. The roots of the plant are used in dysentery, pectoral complications, and diabetes, and also applied in inflammatory glandular enlargement, mumps, and hydrophobia. The latex is externally applied on wounds to decrease inflammation, pain, and edema and to promote healing. The phytochemical compounds isolated from the stem bark are leucocyanidin-3-o-B-glucopyranoside, leucopelargonidin 3-O-a- L-rhamnopyranoside, B- sitosterol, stigmasterol, tetracyclic triterpene - gluanol acetate and tiglic acid. The reported pharmacological properties of the different plant parts are hypoglycemic, antiulcer, antioxidant, wound-healing, anti-inflammatory, anti-diarrheal, antibacterial, antifungal, antipyretic, and antidiuretic.[4]

S. racemosa Roxb. known as Lodhra belonging to the Symplocaceae family is found distributed throughout North Eastern India up to 2,500 ft. elevation. It is a small evergreen tree with stem up to 6 m in height and 15 cm in diameter. Its stem bark is useful in bowel complaints such as diarrhea and dysentery, in dropsy, eye disease, liver complaints, wound healing, excessive vaginal discharge, menstrual problems, fevers, ulcers, and scorpion sting. The bark is often employed in the preparation of plasters and is reported to promote maturation or resolution of boils, stagnant tumors, and other malignant growths. A decoction of the bark or wood is used as gargle for giving firmness to spongy and bleeding gums and relaxed uvula. The phytochemical investigation of the n-butanol soluble fraction of the bark of stem of *S. racemosa* Roxb. yielded two phenolic glycosides of salirepin series, namely, symplocuronic acid and sympocemoside while salirepin has also been isolated from this plant.[5] The alcohol extract of stem bark indicated the presence of carbohydrates, glycosides, saponins, terpenoids, and alkaloids while its ether extract indicated the presence of glycosides, phytosterol, and steroids. The prominent pharmacological activities of its stem bark are antibacterial, anti-inflammatory, antiulcer, anti-tumor, antimicrobial, and antioxidant.[6]

To achieve the desired therapeutic effect, a good vaginal delivery system for curing vaginitis needs to reside at the site of infection for a prolonged period. Hence, there is need to develop an effective drug delivery system that should prolong the contact of the drug with the vaginal mucosal surface. Mucoadhesive drug delivery has been a topic of interest in the design of drug delivery systems to lengthen the residence time of the dosage form at the site of application or absorption and to facilitate intimate contact of the formulation with the underlying absorption surface so as to improve and enhance the bioavailability of the drug. Mucoadhesive controlled drug delivery systems are beneficial since they give a controlled drug release over a period of time and can also be utilized for localizing the drug to a specific site in the body. Mucoadhesive substances could also be used as therapeutic agents in their own right, to coat and protect and soothe the injured tissues (gastric ulcers or lesions of the oral mucosa) or as lubricants (in the oral cavity, eye, and vagina). Mucoadhesion is a complex process involving wetting, adsorption, and interpenetration of polymer chains. Thus, vaginal tablets appear to be useful dosage forms as they are easy to apply, portable and provide effective local absorption. During previous studies, the herbal formulation F-XII provided the best physical parameters such as hardness, pH value, swelling index, and bio-adhesive tension which are essential for maintaining the vaginal flora. The results indicate that this formulation will provide sustained slow releasing of anti-vaginitis and anti-leucorrhea drug delivery system in the form of an effective mucoadhesive vaginal

tablet.[7] This tablet consists of 500 mg active research drug mentioned above along with Carbopol 934P (15 mg), HPMC K4M (90 mg), microcrystalline cellulose (175 mg), lactose monohydrate (100 mg), talc (10 mg), and magnesium stearate (10 mg).[8-11]

The aim of this present preclinical *in vivo* toxicity study is to provide a safe and standardized herbal drug either for oral route of administration or in the form of vaginal tablet for treatment of vaginitis. The objective is primarily to arrest the excessive abnormal vaginal fluids through a systematic approach and also external application of the same research drug in different drug delivery forms. Therefore, both the acute and subacute toxicities of aqueous extract of research formulation have been evaluated using the oral route of administration to find out the dose-dependent therapeutic and toxic effect of this drug through hematological and histopathological studies of the stomach, liver, and kidney. The vaginal irritation test of tablet of the same research formulation has been also done to assess the redness, ulceration, bleeding, etc., of the vaginal mucous membrane during drug administration.

MATERALS AND METHODS

The chemical and experimental studies were carried out in the laboratory of the Department of Dravyaguna (Medicinal Plant Pharmacology) at the Institute of Post Graduate Ayurvedic Education and Research, Kolkata. The acute and subacute toxicity and vaginal irritation test studies of the aqueous extract of the research formulation were done on female rodents after getting approval vide certificate no. SVP/PG/401(A) 2014 dated 27.3.2014 from the Institutional Animal Ethical Committee (IAEC) in the animal house of Institute of Post Graduate Ayurvedic Education and Research, Kolkata (registration number 1180/ac/08/CPSEA dated 27.03.2008 of CPCSEA), according to the guidelines of CPCSEA.

Plant Materials

The stem barks of *S. racemosa* Roxb. and *F. glomerata* Roxb. were purchased from crude drug supplier of Katwa Chowrasta, Burdwan district for the preparation of herbal vaginal tablet and the plant samples were authenticated by the Research Officer, Botanical Survey of India, Howrah, India (Ref. No. BSI/CNH/SF/Tech./2016).

Chemicals used for Preparation of Tablets

Di-calcium phosphate, Gum acacia, Lactose monohydrate, Sodium carboxymethyl cellulose, Sodium starch glycolate, Starch (maize), Ferric Chloride ($FeCl_3$), Magnesium stearate (IP grade), Microcrystalline cellulose (IP grade), Talc (IP grade), Folin–Ciocalteu's reagent, sodium carbonate, and sulfuric acid were obtained from M/s Merck Specialties Pvt. Ltd., Mumbai. Carbopol 934P and Hydroxy-propyl-methyl-cellulose K4M were purchased from reputed company M/s HiMedia Laboratories Pvt. Ltd while Citric acid monohydrate was procured from M/s B.D. Pharmaceutical works Pvt. Ltd and Sodium bi-carbonate (IP grade) from M/s Indian Drug House.

Preparation of Extracts

The stem barks of *S. racemosa* Roxb. and *F. glomerata* Roxb. were taken in equal quantity by weight, washed, sun-dried, and crushed to particle size of 40 mesh. This coarse powder was sequentially extracted with petroleum ether (60°C–80°C), chloroform, acetone, ethanol, and water using Soxhlet apparatus. These extracts were filtered using a Buckner funnel and Whatman No. 1 filter paper at room temperature and concentrated at reduced temperature and pressure using rotary evaporator. All obtained extracts were stored in refrigerator below 10°C for subsequent experiments. The aqueous extract of the research formulation was used in the study.[12,13]

Preparation of Mucoadhesive Vaginal Tablets

Bioadhesive vaginal herbal tablets (F-XII) were prepared using the dry compression technique of tablet preparation. These tablets consist of 500 mg active research drug mentioned above along with Carbopol 934P (15 mg), HPMC K4M (90 mg), Microcrystalline cellulose (175 mg), Lactose monohydrate (100 mg), Talc (10 mg), and Magnesium Stearate (10 mg). The polymers Carbopol and Hydroxy-Propyl-Methyl Cellulose were used as excipients, while talc and magnesium stearate were added as glidant and lubricant, respectively. Microcrystalline Cellulose and Lactose monohydrate were used as diluents. The binder hydroxypropyl methylcellulose was used to form sustained-release matrix with the polymer carbopol, which swells to form hydrogel-like matrices through which drug molecules could be released at a controlled rate. All the ingredients were thereafter passed through #44 mesh sieve and finally the mixture was compressed into tablet-form using single punch tablet compression machine.[14,15]

Experimental Animals

Swiss albino mice of female sex, weighing about 20–30 g, and Wistar female rats, weighing about 120–130 g, were used for different *in vivo* evaluation. All animals were procured from M/s Saha Enterprises of West Bengal, a CPCSEA registered breeder, Kolkata (Regd No. 1828/PO/BT/S/15/CPCSEA) and housed under standard environmental conditions with fixed 12 h light/dark cycles and a temperature of approximately 25°C in the animal house of Institute of Post Graduate Ayurvedic Education and Research, Kolkata. The animals were kept in standard polypropylene cages and provided

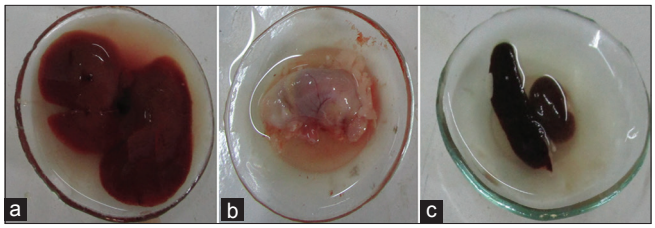

Figure 1: Photographs of dissected organs. (a) Liver, (b) stomach (c) kidney

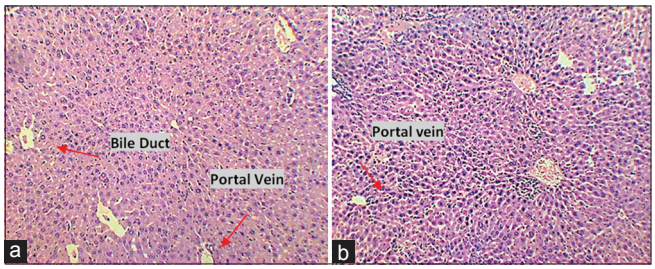

Figure 2: (a and b) Light micrographs of rat liver showing normal structure in control Group A and research drug-treated Group B of animals under a microscope (Olympus C×41) at ×10 magnification. No changes are noticed in the internal tissue structures such as hepatic cells, portal vein and bile duct and no infiltration or degeneration of cells is found in drug-treated liver relative to the control group

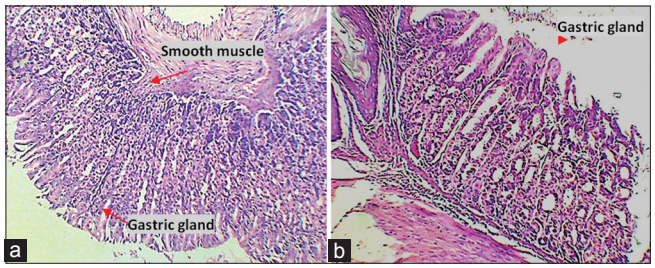

Figure 3: (a and b): Light micrographs of rat stomach showing normal structure in control Group A and research drug-treated Group B of animals under a microscope (Olympus C×41) at ×10 magnification. No changes are noticed in the internal tissue structures such as mucosa, submucosa, gastric glands and epithelium, and no infiltration of cells is found in drug-treated stomach relative to the control group

with food (standard pellet diet) and water *ad libitum*. These animals were acclimatized for a period of 14 days before performing any experiments. All experimental protocols were approved by the IAEC.

Animal Experimentation

Three types of toxicities of research drug formulation were analyzed to find out the lethal dose, therapeutic dose, and maximum tolerated dose using the acute and subacute toxicity tests using oral route of administration and vaginal irritation tests using intravaginal administration.

Acute toxicity (single dose) test

Acute toxicity study was carried out on healthy Swiss albino female mice following OECD guidelines. Animals were selected by random sampling technique and divided into 5 groups of 6 animals each after pilot study. A single dose of aqueous extract of the research drug formulation was administered orally to different group animals at the level of 300, 600, 900, 1200, or 1500 mg/kg body weight, respectively. All the animals were observed for the appearance of toxic symptoms including muscle spasm, loss of righting reflex, tremors, behavioral changes, locomotion, convulsions, and mortality for 1, 2, 4, 8, and 24 h. Long-term supervision was continued for a period of 14 days for observing any occurrence of toxic symptoms and mortality. Daily and weekly body weight changes, food and water intake and clinical signs were recorded on a regular basis.[16-18]

Subacute toxicity study (daily dose)

During this test the animals are dosed daily, starting at around expected therapeutic level and increasing stepwise every 2–3 days until toxic signs are observed. Hematological and biochemical monitoring is carried out and blood level of the compound checked to ensure its absorption. The animals are maintained at the maximum tolerated dose for a period of 2–3 weeks to allow development of any pathological changes, and then killed and subjected to full pathological and histological examinations. The purpose of this test is to determine the maximum tolerated dose, and to indicate the nature of toxic reactions so that suitable chronic toxicity studies can be designed to fully evaluate the toxic potential of the compound.

Daily doses of drug aqueous extract were administered to adult female albino mice that were divided into five groups of 6 animals each to determine the maximum tolerated dose, and to evaluate the toxic potential of the compound. During this test, the extract was orally administered by gavage starting at the expected therapeutic doses of 500 mg/kg b.w. which was selected on the basis of results obtained during acute toxicity study considering no mortality and no adverse symptomatic changes after administration of drug aqueous extract. This selected dose was increased by 50 mg/kg every 3rd day until toxic signs were observed. Thereafter, the animals were maintained at the maximum tolerated dose of 1200 mg/kg for a period of 4 weeks to allow development of any pathological or clinical changes. The changes in body weight, food, and water intake, and clinical signs were recorded on a regular basis until the animals were sacrificed on the 30th day. Animals were anesthetized and blood was obtained from hepatic portal vein at room temperature. Blood plasma was separated and stored at 4°C until the hematological parameters and liver function test (LFT) study were done. Hematological analysis was done using Sysmex BX-3010 analyzer, and biochemical test was carried out using Mindray BC3200, Labindia Healthcare ultima3, Access2, Beckman Coulter analyzer and blood level of the compound was checked to ensure its absorption.[16-18]

Histopathological study

The histopathological slides of dissected stomach, liver, and kidney organs were prepared by using the microtome, stained and the internal structure of cells, tissue, and mucous membrane, etc., were observed. Fresh portions of the lateral lobes of liver, stomach, and kidney were rapidly dissected out from sacrificed rat, fixed in neutral buffered formalin (10%), dehydrated with different grades of ethanol (70, 80, 90, 95, and 100%) and followed by clearing the samples in 2 changes of solute. Samples were then impregnated with two changes of molten paraffin wax and embedded into a block. One micron thickness of sections was then cut using microtome instrument. Paraffin sections (4-5 μm) were stained with hematoxylin and eosin and examined under Olympus C X 41 compound microscope.

Vaginal dermal toxicity test

Vaginal toxicity testing was performed on female Wistar rats using a procedure modified from that of Gad and Chengelis.[19] 18 mature female rats were equally divided into three test groups of 6 animals each. Group A consisted of untreated control animals while the other two groups were treated vaginally using the vaginal cannula for 14 consecutive days by administration with research drug formulation at the dose of 500 mg/kg bw (Group B) and by placebo containing only polymer excipients and no active drug ingredients (Group C). All animals were weighed on the 1st day of dosing and every 7th day thereafter. Detailed physical observations were made twice daily for moribundity/mortality. Approximately 4 h after dosing, observations were also made for vaginal bleeding and discharges, appearance, behavior, and pharmacologic signs. The animals were necropsied 24 h after the final vaginal dose, and the heart, kidney, liver, lung, ovary, pancreas, uterus, and vagina were excised and weighed. Histopathological slides of uterus and vagina were prepared to find out the internal structure of their cells, tissues, and glands. The hematological measurements made included hematocrit, hemoglobin concentration, erythrocyte count, total and differential leukocyte count, platelet count, serum glucose, protein, bilirubin, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alanine aminotransferase (ALT), and creatinine.[20-23]

Statistical Analysis

The data were statistically analyzed using one-way ANOVA followed by Dunnett's *t*-test for individual comparison of groups with control. Results were expressed as a mean ± standard deviation. $P < 0.05$ was used to indicate statistical significance.

RESULT

Acute Toxicity

The results obtained during acute toxicity tests are detailed in Table 1.

Subacute Toxicity Study

The subacute toxicity test was performed following the method detailed by Ghosh.[16] The observations have been detailed in Table 2 while the hematological parameters have been highlighted in Table 3. The hematological, LFT and biochemical parameters in case of the research drug showed almost similar results when compared with the values of the control group which suggests almost no toxic effect during the subacute toxicity tests.

Histopathological Study During Subacute Toxicity Test

During this study, almost all the tissues of the liver and stomach were noticed to be intact or unaffected as shown in Figures 1-3. No appreciable or significant degeneration or changes were observed. Only a slight degeneration was found in the renal cells and glomerular capsules during the study of the T.S. of kidney [Figure 4].

Vaginal Irritation Test

The results obtained during the hematological studies are detailed in Table 4. During the study LFT, erythrocyte sedimentation rate (ESR), total count (TC), differential count (DC), and hormonal levels were observed after sacrificing the animals. In the control group, the estrogen level was 7.87 ± 0.61 whereas it was 7.13 ± 0.1 and 7.23 ± 0.18 ng/ml, respectively, in research formulation treated and placebo-treated groups. Similarly, the progesterone level was 2.89 ± 0.26 in the control group while it was 2.73 ± 0.12 and 2.71 ± 0.11 ng/ml, respectively, in the formulation-treated and placebo-treated groups. The follicle-stimulating hormone (FSH) level was 0.20 ± 0.03, 0.05 ± 0.021, and 0.05 ± 0.07 ng/ml, respectively, in the control, drug-treated and placebo-treated groups. LFT and other blood parameters were also observed to be very similar in the other two groups when compared to the control group. Overall, almost all the studied parameters did not show any substantial or significant variation across the three groups.

Histopathological Study of Organs During Vaginal Irritation Test

The histopathological examination of the transverse sections of various vital organs was performed during the vaginal irritation test using Olympus C ×41 microscope, and the findings are shown in Figures 5 and 6. There were no noticeable signs of uterine degeneration or vaginal inflammation (ulceration, edema, and redness) in the representative control, research drug or placebo exposed groups.

Table 1: Clinical signs and symptoms observed in different dose groups during acute toxicity study

Clinical sign	300 mg/kg Dose	600 mg/kg Dose	900 mg/kg Dose	1200 mg/kg Dose	1500 mg/kg Dose
Motor activity↑	-	-	+	-	-
Motor activity↓	-	-	-	+	+
Clonic convulsions	-	-	-	-	-
Muscle spasm	-	-	-	-	-
Spasticity	-	-	-	-	-
Loss of righting reflex	-	-	-	-	-
Tremors	-	-	-	-	-
Sedation	-	-	+	+	+
Lacrimation	-	-	-	-	-
Diarrhea	-	-	-	-	-
Salivation Viscid Watery	-	-	-	-	+
Respiration Depression Stimulation	-	-	+	+	+
Hypnosis	-	-	-	+	+
Anesthesia	-	-	-	-	-
Drowsiness	-	-	-	+	+
Irritation	-	-	-	-	-

+ →Mild positive result - → Negative result

DISCUSSION

During acute toxicity tests, the test animals showed no significant toxic symptoms such as sedation, convulsion, diarrhea, and irritation and no signs of behavioral changes up to the dose of 1500 mg/kg of the research drug. No mortality was reported up to 24 h and even later during the subsequent 14 days at this dose. The therapeutic dose for the subsequent experiments was selected as 600 mg/kg on the basis of symptoms and mortality because some physical changes are observed in some animals at a higher dose up to 1500 mg/kg; however, these symptoms subsided within 1–2 h and no mortality occurred. After conducting acute toxicity study, the subacute toxicity of the combination drug was studied as the best results were observed in the phytochemical and chemical analysis of the combination drug.

The results of subacute toxicity (long-term toxicity) of the aqueous extract of research drug showed that no animal died when the daily dose of drug was gradually increased up to 1200 mg/kg b.w. over 29 days. There were no significant toxic effects observed up to a long time at the highest research drug concentration. While some symptoms were observed temporarily from the dose of 650 mg/kg to the maximum dose of 1200 mg/kg, these symptoms subsided within an hour during this study. The hematological studies, LFT and hormonal parameters of blood did not show any noticeable increase or decrease when compared with the corresponding values in case of the control group. No damage of cells, tissues, epithelium lining or other structures of stomach, liver, and kidney were observed during histopathological studies which indicate no toxic effect of the drug. Based on these results and observations, the safe dose of research drug using oral administration has been estimated as 500 mg/kg b.w. for further formulation studies.

During the vaginal subacute toxicity (irritation) studies performed on rats, the LFT, Hb, ESR, TC, DC, and hormonal level in the blood samples were analyzed after sacrificing the experimental animals and compared with the control group. Parameters such as total Bilirubin, SGPT, SGOT, total protein, and ALT of blood were also found to be in the normal range and quite similar to the values for the control group. The hormonal parameters FSH, thyroid stimulating hormone, luteinizing hormone, progesterone, and estrogen level were also observed in the three groups and noticed to be within the normal range. The estrogen level was found to be 7.87 ± 0.61 in the control group whereas it was 7.13 ± 0.1 and 7.23 ± 0.18ng/ml in the drug-treated and placebo-treated groups, respectively. Similarly, the progesterone level was 2.89 ± 0.26, 2.73 ± 0.12, and 2.71 ± 0.11ng/ml, and FSH level was 0.20 ± 0.03, 0.05 ± 0.021, and 0.05 ± 0.07 ng/ml, respectively, in the control, formulation treated and placebo-treated groups. During this study, no noticeable differences in mean body weight were observed among the three animal

Table 2: Clinical symptoms observed at different drug doses during subacute toxicity study

Dose and (study day)	500 (1st Day)	550 (3rd Day)	600 (5th Day)	650 (7th Day)	700 (9th Day)	750 (11th Day)	800 (13th Day)	850 (15th Day)	900 (17th Day)	950 (19th Day)	1000 (21st Day)	1050 (23rd Day)	1100 (25th Day)	1150 (27th Day)	1200 (29th Day)
Clinical sign															
Motor Activity↑	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Motor Activity↓	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
Clonic convulsions	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Muscle spasm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Spasticity	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Loss of righting reflex	-	-	-	-	-	-	-	-	-	-	-		-	-	-
Tremors	-	-	-	-	-	-	-	-	-	-		-	-	-	-
Sedation	-	-	-	-	-	-	-	-	-	-	-	–	-	-	-
Lacrimation	-	-	-	-	-	-	-	-					+	+	+
Diarrhea	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Salivation		-	-	-	-	-		-	-	-	-	-	+	+	+
Viscid							-		-	–					
Watery	-							-			-	+	+	+	+
Respiration Depression	-	-	–	–	–										
Stimulation						-	-	-	-	-	-	-	-	-	-
Hypnosis	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
Anesthesia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Drowsiness	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
Irritation	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+

+: → Mild positive result, – → Negative result

Table 3: Hematological and biochemical parameters observed during subacute toxicity study

Treatment group	Control	Aqueous extract
Total bilirubin (mg/dl)	0.27±0.04	0.25±0.05
SGPT (IU/L)	33.00±2.84	37±8.52
SGOT (IU/L)	44.00±4.34	45±11.90
Total protein (g/dl)	7.90±0.15	6.90±0.51
ALT (U/L)	90.00±4.76	70.50±7.23
Hemoglobin (g %)	13.80±0.62	13.84±0.13
Total count		
Erythrocytic (10^6/cu.mm.)	5.90±0.22	5.92±0.08
Leukocytic (10^3/cu.mm.)	5.40±0.16	5.50±0.30
Differential leukocytic count		
Neutrophil (%)	20.00±2.83	32.00±7.99
Lymphocyte (%)	27.00±6.88	35.50±6.75
Monocyte (%)	7.00±0.97	4.96±0.16
Eosinophil (%)	2.00±0.60	3.00±0.74 (P=0.03)
Basophil (%)	0.00	0.00
Erythrocyte sedimentation rate (mm)	5.46±0.27	7.50±5.87 (P=0.009)

SGPT: Serum glutamic pyruvic transaminase, SGOT: Serum glutamic oxaloacetic transaminase, ALT: Alanine aminotransferase

Table 4: Hematological, biochemical and hormonal analysis of different groups of animals

Treatment Group →	Control	Research formulation extract	Placebo
Total Bilirubin (mg/dl)	0.27±0.04	0.26±0.05	0.20±0.10
SGPT (IU/L)	34.00±2.81	28.00±1.2	29.00±0.12
SGOT (IU/L)	43.00±4.32	40.00±1.8	42.00±2.3
Total Protein (g/dl)	7.90±0.15	7.40±0.12	7.10±0.10
ALT (U/L)	107.00±4.26	103.00±1.22	100.00±1.12
Hemoglobin (g %)	13.80±0.62	11.60±0.80	11.12±0.40
Total Count			
Erythrocytic (10^6/cu. mm)	5.00±0.20	4.20±0.70	4.50±0.80
Leukocytic (10^3/cu. mm)	4.80±0.14	4.10±0.24	4.60±0.11
Differential Leukocytic Count			
Neutrophil (%)	20.00±2.83	19.00±1.43	18.90±2.23
Lymphocyte (%)	27.00±6.81	23.00±0.41	26.00±0.41
Monocyte (%)	7.00±0.97	1.00±0.72	2.00±0.51
Eosinophil (%)	2.00±0.60	2.0±0.40	2.0±0.20
Basophil (%)	0.00	0.0	0.0
Erythrocyte sedimentation rate (mm)	5.46±0.22	5.1±0.12	4.9±0.11
FSH (mIU/ml)	0.20±0.03	0.05±0.02	0.05±0.07
TSH (mcU/ml)	0.90±0.02	0.88±0.05	0.80±0.05
LH (mIU/ml)	0.21±0.07	0.6±0.03	0.5±0.06
Progesterone (ng/ml)	2.89±0.26	2.73±0.12	2.71±0.11
Estrogen (ng/ml)	7.87±0.61	7.13±0.10	7.23±0.18

SGPT: Serum glutamic pyruvic transaminase, SGOT: Serum glutamic oxaloacetic transaminase, ALT: Alanine aminotransferase, FSH: Follicle-stimulating hormone, LH: Luteinizing hormone, TSH: Thyroid stimulating hormone

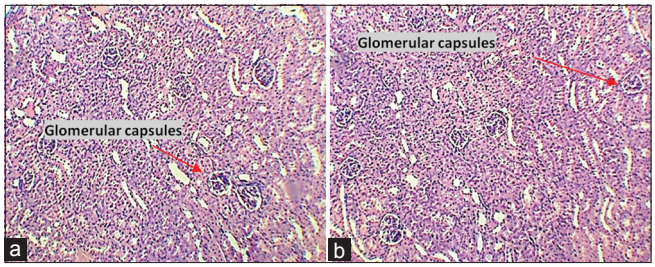

Figure 4: (a and b) Light micrographs of rat kidney showing normal structure in control Group A and research drug-treated Group B of animals under microscope (Olympus C×41) at ×10 magnification. No changes are noticed in the internal tissue structures such as nephrotic cells, Bowman's capsular, both proximal and distal convoluted tubules and epithelium, and no infiltration of cells is found in drug-treated kidney relative to the control group

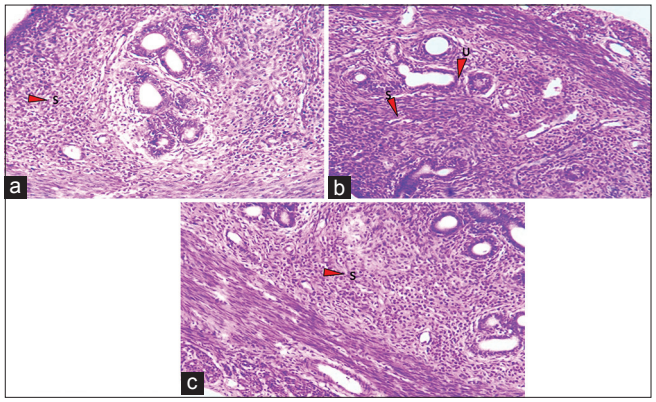

Figure 5: Hematotoxyline and eosin stained section of rat uterus. Light micrographs of the rat uterus after 14 days of daily intravaginal treatment with a control animal A, Research drug-treated Group B and placebo-treated Group C showed that the internal tissue structures such as mucosa, submucosa, uterine glands, and intact epithelium of the uterus of rat are unchanged relative to the control section. There were no visible signs of vaginal inflammation (ulceration, edema, or leukocyte infiltration) in all the groups. (a) Group A: T.S. of control rat uterus with normal histoarchitecture under a microscope (Olympus C×41) at ×10 magnification, showing internal tissue structures such as mucosa, submucosa, uterine glands, and intact epithelium of the vagina of rat (P). (b) Group B: T.S. of drug-treated rat uterus with normal histoarchitecture under a microscope (Olympus C×41) at ×10 magnification, showing internal tissue structures such as mucosa, submucosa, uterine glands, and intact epithelium of the vagina of rat (P), smooth muscles (S), and uterine glands (U). (c) Group C: T.S. of Placebo-treated rat uterus with normal histoarchitecture under a microscope (Olympus C×41) at ×10 magnification showing internal tissue structures such as mucosa, submucosa, uterine glands, and intact epithelium. The uterus of placebo-treated rat is unchanged relative to the control section where smooth muscles (S) and uterine glands (U) were shown

groups (control, placebo-treated, and research drug treated). Furthermore, no differences were observed among the treatment groups in respect of (1) hematological parameters, blood chemistry, and blood coagulation parameters (2)

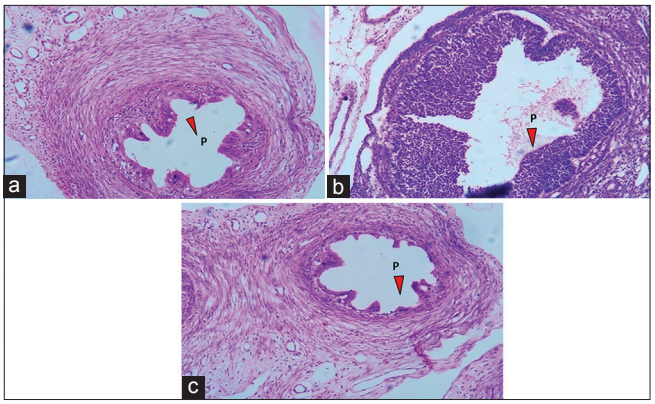

Figure 6: Hematotoxyline and eosin stained section of rat vagina. Light micrographs of the rat vagina after 14 days of daily intravaginal treatment with a control animal A, Research drug-treated Group B and placebo-treated Group C showed that the internal tissue structures such as mucosa, submucosa, uterine glands, and intact epithelium of the uterus of rat are unchanged relative to the control section. There were no visible signs of vaginal inflammation (ulceration, edema, or leukocyte infiltration) in all the groups. (a) Group A: T.S. of control rat vagina with normal histoarchitecture under a microscope (Olympus C×41) at ×10 magnification, showing internal tissue structures such as mucosa, submucosa, uterine glands, and intact epithelium of the vagina of rat (P). (b) Group B: T.S. of drug-treated rat uterus with normal histoarchitecture under a microscope (Olympus C×41) at ×10 magnification, showing internal tissue structures such as mucosa, submucosa, uterine glands, and intact epithelium of the vagina of rat (P), smooth muscles (S), and uterine glands (U). (c) Group C: T.S. of placebo-treated rat vagina with normal histoarchitecture under a microscope (Olympus C×41) at ×10 magnification showing internal tissue structures such as mucosa, submucosa, uterine glands, and intact epithelium. The vagina of placebo-treated rat is unchanged relative to the control section.

gross observations of the organs at necropsy, and (3) weight of organs. No histopathological alterations that could be attributed to either research drug or the placebo were observed in the tissues, and no degeneration of uterus, vagina or ovary was observed. Thus, neither the research drug nor the placebo had produced any systemic toxicity in the rats following the vaginal application during the study. The results of this analysis clearly indicate the non-toxic and safe attribute of the research drug formulation on the basis of the examined blood parameters and reproductive hormones using the intravaginal administration route.

CONCLUSION

The acute toxicity tests performed on the research formulation revealed no signs of behavioral changes up to the dose of 1500 mg/kg and no mortality was reported up to 24 h.

During the subacute toxicity (long-term toxicity) testing, no animal died when daily dose of the drug was gradually

increased to 1200 mg/kg b.w. over 29 days, and no significant toxic effects were observed over a prolonged period. At the same time, the hematological studies, LFT and hormonal parameters of blood did not show any noticeable differences, and no significant damage of cells, tissues, epithelium lining or other structures of stomach, liver, and kidney was observed during histopathological studies.

The vaginal dermal subacute toxicity studies indicated that no differences were observed among treatment groups in respect of hematological parameters as well as weight and internal structures of all vital organs during histopathological examination.

The results clearly indicate the non-toxic attribute of research drug formulation on the basis of analysis of blood parameters and reproductive hormones using oral and intravaginal administration routes.

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