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Evaluation of Safety Parameters of Ayurvedic Antimicrobial Formulation for Treatment of Vaginitis during Acute and Chronic Oral Toxicity and Vaginal Irritation Studies in Animal Models

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Abstract— Introduction: *This in vivo preclinical toxicity study has been done to standardize a new vaginal herbal formulation consisting of dried parts of the stem barks of Azadirachta indica A. Juss. and Saraca asoca Roxb. who find mention in many Ayurvedic medicine texts for treatment of vaginitis. Both Acute and chronic toxicities of its hydro alcoholic (30:70) extract have been evaluated using the oral as well as vaginal route of administration through haematological 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 hydro-alcoholic extract of the research drug was administered orally at the level of 300, 500, 1000, 1500 & 2000 mg/kg to different groups and animals were observed for the appearance of toxic symptoms up to 24 hours.*

During chronic toxicity study, three doses of hydro-alcoholic extract, namely the therapeutic dose 500 mg/kg, 2500 mg/kg (5 times the therapeutic dose) and 5000 mg/kg body weight (10 times the therapeutic dose) were prescribed orally for 90 days to the three experimental groups each having 6 female Wister rats to assess the toxic effect or mortality on daily observation basis. Toxic symptoms including muscle spasm, loss of righting reflex, tremors, behavioral changes, locomotion, convulsions, and mortality were noted along with body weight changes, food and water intake. The animals were sacrificed on the 90th day, and their haematological 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 irritation 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 behavioural changes up to the dose of 2000 mg/kg and no mortality was reported up to 24 hours.*

During the chronic toxicity testing, no animal mortality was observed and no significant toxic effects were observed over a prolonged period. At the same time, the haematological 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 haematological 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*

Keywords— *Ayurvedic Formulation, Acute toxicity, Chronic toxicity, vaginal irritation test, Hematological biochemical & Hormones parameters, Histopathological studies*.

I. INTRODUCTION

Medicinal plants have been used as a source of traditional medicine in almost all cultures from time immemorial to treat acute and chronic diseases. The World Health Organization estimated that perhaps eighty percent of the world's inhabitants rely chiefly on traditional medicines, and therefore it approved the use of herbal products for national policies and emphasized upon drug regulatory measures to strengthen research and evaluation of the safety and efficacy of herbal products. The toxicity assessment of plants with proven therapeutic use is of utmost importance and hence toxicological studies such as acute, subacute and chronic toxic analysis in animal models are imperative to predict the safety associated before the use of medical products [1].

The research Ayurvedic vaginal herbal formulation used during the study was prepared by adding equal amounts of the dried parts of the stem bark of *Azadirachta indica A. Juss.* and *Saraca asoca Roxb.* since these two plants have been used since ancient times in the Ayurvedic system of medicine and elaborated in ancient texts such as Charak Samhita (Chikitsa Sthanam) as an astringent, anti-inflammatory & haemostatic and useful for arresting excessive abnormal vaginal discharge [2-4]. This is a new herbal vaginal formulation which has not been evaluated in the form of vaginal tablets till now although it is likely to exhibit sustained and significant antimicrobial

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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 [5].

Nimba (*Azadirachta indica A. Juss.*), the versatile medicinal plant belonging to Meliaceae family, is the unique source of various types of compounds having diverse chemical structure. Every part of the tree has been used as traditional medicine for against various human ailments from antiquity. The medicinal utilities have been described especially for leaf, fruit and bark. Nimba oil and the stem bark and leaf extracts have been therapeutically used in Ayurveda to control leprosy, intestinal helminthiasis, respiratory disorders, blood morbidity, biliary afflictions, itching, various skin diseases, burning sensations and phthisis and also as a general health promoter. Its fruits and seeds are the source of Nimba oil. The isoprenoids include diterpenoids and triterpenoids containing protomeliacins, limonoids, azadirone and its derivatives, gedunin and its derivatives, vilasinin type of compounds and Csecomeliacins such as nimbin, salanin and azadirachtin. The non-isoprenoids include proteins (amino acids) and carbohydrates (polysaccharides), sulphurous compounds, polyphenolics such as flavonoids and their glycosides, dihydrochalcone, coumarin and tannins, aliphatic compounds, etc. It is typically grown in tropical and semi-tropical regions. While Azadirachtin has exhibited pesticide and anti-feedant activities, Nimbidin, a major crude bitter principle extracted from the oil of seed kernels of this plant is having several biological activities. All parts of Nimba tree are used as anthelmintic, anti-fungal, anti-diabetic, antibacterial, antiviral, contraceptive and sedative [6,7].

Ashoka (*Saraca asoca* Roxb.) of Leguminosae family has been very widely used in Indian system from times immemorial for the treatment of uterine, genital and other reproductive disorders in women, ailments of urogenital tract, fever and pain, etc. Charak has mentioned its properties in the ancient Ayurvedic text book Charak Samhita under Vedanasthapan (analgesic, antipyretic and anti-inflammatory) category. It is an evergreen tree found almost throughout India up to an altitude of 750 m. This plant is medium sized and about 30 feet high. Leaves are compound paripinnate, leaflets 4-6 pairs, oblong to lanceolate, glabrous, acute and short petiole. Stem bark is channeled, outer surface is rough with warty protuberances, brown in colour and inner surface of the bark is smooth, reddish brown. Flowers are orange or orange-yellow, eventually turning vermillion, fragment in dense axillary corymbs. Fruits (Pods) are flat. Linear- oblong, leathery, 10- 25 cm. long, seeds are 4-8 in numbers, 1.5 inches long ellipsoid-oblong, compressed. Flowering is in spring and fruiting in autumn seasons. The stem bark of this plant in the Indian system of medicine has been used for its bitter, astringent, sweet, refrigerant, anthelmintic, styptic, stomachic, febrifuge, demulcent properties. It has stimulating effect on endometrium and ovarian tissue.

It is useful in dyspepsia, fever, biliousness, burning sensation, colic dysentery, internal bleeding, hemorrhoids, ulcers, menorrhagia, uterine fibroid and leucorrhoea. Flowers are also used in the burning sensation, hemorrhagic dysentery, and inflammation. Seeds are used as diuretic and in vesical calculi diseases. The stem bark of this tree is rich in tannins, flavonoids, steroids, volatile oil, glycosides, and various steroidal glycosides, an essential oil, haematoxillin, a keto-sterol, crystalline glycosidal constituents, a saponin and an organic iron compound, leucocynadin, quercetin. Leaves contain various carbohydrates, tannins, gallic acid and egallic acid. Flowers are rich in saracasin, saracadin, waxy substances, proteins, carbohydrates and steroids. Seeds of this plant contain various fatty acids like oleic, linoleic, palmitic and stearic acid. The pharmacological activities of stem bark are uterogenic, antibacterial, oxytocic, anti-tumor, anti-inflammatory, analgesic, anticancer, anti-progestational, etc. In females it is very commonly used to regularize hormones and menstrual cycles to improves the strength and stamina in young females having menstrual irregularities such as dysmenorrhea and leucorrhea [8].

The main aim of this preclinical evaluation of research formulation *in vivo* toxicity study in animal models was to validate a safe, effective and standardized herbal hydro alcoholic (30:70) formulation for oral route of administration as well as in the form of locally applicable vaginal tablet in the vaginal route of administration for the treatment of abnormal and excessive vaginal discharge. The objective is to arrest the excessive abnormal vaginal fluid secretion through a systemic approach using oral route of administration and also external application of the same research drug extract in different drug delivery form as a mucoadhesive vaginal tablet. Therefore, both the acute and chronic toxicities of hydro-alcoholic extract of research drug 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. During the studies, the herbal formulation provided the best physical parameters such as hardness, pH value, swelling index, and bio-adhesive tension which are essential for maintaining the vaginal flora [9,10].

II. Materials 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 chronic oral toxicity analysis and vaginal irritation test study of the hydro-alcoholic extract of the research formulation were done using female rodents in the animal house of the 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 after getting approval from the Institutional Animal Ethical Committee (IAEC) vide certificate no. SVP/PG/401(A) 2014 dated 27.3.2014.

A. Collection & identification of plant materials

The stem barks of Saraca asoca Roxb. And Azadirachta indica A. Juss. were purchased from the crude drug supplier of

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Katwa Chowrasta, Burdwan district and plant samples were authenticated as per Ref. no. BSI/Pharma/SD/Tech./2017 by the Botanical Survey of India, Howrah, India. Authenticated specimens were deposited bearing numbers IPGAE&R /Dravyaguna /M. Gupta /09 & IPGAE&R /Dravyaguna /M. Gupta / 10 in the herbarium museum of the department of Dravyaguna at I.P.G.A.E.&R., Kolkata for future reference.

B. Chemicals used for preparation of tablets

The pharmacognostical and chemical analysis of the research formulation were performed following the protocols of drug standardization mentioned in the Ayurvedic Pharmacopoeia of India (2001) [11].

Chemical reagents such as Toluene, Formic acid, Acetonitrile, Gallic acid, Phosphoric acid, Acetic acid, Vanillin, Resorcinol and HPLC grade water were procured from M/s Merck Specialities Pvt. Ltd and Chloroform, Ethyl Acetate, Ascorbic acid, Acetyl Salicylic acid, Catechol, Ellagic acid and Benzoic acid were purchased from M/s Nice Chemicals Pvt. Ltd. Di-calcium phosphate, Gum acacia, Lactose monohydrate, Sodium carboxymethyl cellulose, Sodium starch glycolate, Starch (maize), Ferric Chloride (FeCl3), 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 [12,13].

C. Continuous extraction of research formulation

The stem barks of both the plants were washed, air-dried and pre-heated in oven before being powdered in a grinding machine to 40# mesh particle size and stored in an airtight container. Powdered dried barks of the plants were mixed in an equal ratio and this coarse powder was sequentially extracted with petroleum ether (60°C – 80°C), chloroform, acetone, ethanol and water using Soxhlet apparatus. The hydro-alcoholic extract was also prepared using the Soxhlet apparatus in the ratio of 70:30. These extracts were filtered using a Buckner funnel and Whatman-1 filter paper at room temperature and concentrated at reduced temperature and pressure using rotary evaporator. All obtained extracts were stored in refrigerator below 8°C for subsequent experiments [14]. During this study, the hydro-alcoholic extract was standardized by using different types of instruments to assess the presence of chemical compounds which could be responsible for the antimicrobial and anti-inflammatory pharmacological activities required for curing the excessive abnormal vaginal discharge.

D. Preliminary phytochemical screening

The research extracts obtained from Soxhlet apparatus were subjected to preliminary phytochemical testing to detect the presence of different chemical group of compounds such as saponins, tannins, alkaloids, flavonoids, glycosides, carbohydrates, oils and fats, proteins and amino acids following the standard methods [15].

E. Preparation of mucoadhesive vaginal tablets

The various types of bio-adhesive vaginal herbal tablets were prepared using the dry compression technique of tablet preparation. During this study, 6 types of muco-adhesive herbal vaginal formulations were prepared for testing. All these formulations were prepared by mixing the same amount of the active research drug extract powder (500 mg-550mg) with different amounts of excipients, binders and developers. At last only Formulation (F- V_{NA} (iv)) was considered as a suitable vaginal herbal tablet on the basis of high muco-adhesive strength, swelling index and maximum sustained releasing pattern required for an effective muco-adhesive vaginal drug delivery system.

The final selected formulation of vaginal tablet F-V_{NA} (iv) having total weight 990 mg which consists of 550 mg active research drug mentioned above along with Carbopol 934P (26 mg), HPMC K4M (100 mg), Microcrystalline cellulose (170 mg), Lactose monohydrate (120 mg), Talc (12 mg), and Magnesium Stearate (12 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 [16, 17].

F. Experimental Animals

Experiment animals like Swiss albino mice of female sex, weighing about 20–30 g, and Wistar female rats, weighing about 130–140 g, were used for different *in vivo* evaluation. All the animals were procured from M/s Saha Enterprises, Kolkata, a CPCSEA registered breeder (Reg. No. 1828/PO/BT/S/15/CPCSEA) and housed under standard environmental conditions with fixed 12-hour 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 with standard diet and water. All experimental protocols were approved by the IAEC (Memo No. SVP/PG/401(A) 2014 dated 27.3.2014).

G. Acute toxicity study

Acute toxicity studies are commonly used to determine LD_{50} of drug or chemicals [19]. The acute study provides a guideline for selecting doses for the sub-acute and chronic low dose study, which may be clinically more relevant**.** The main objective of acute toxicity studies is to identify a single dose causing major adverse effects or life threatening toxicity, which often involves an estimation of the minimum dose causing lethality. Acute toxicity studies in animals are

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considered necessary for any pharmaceutical intended for human use.

Acute toxicity study was carried out on healthy Swiss albino female mice following OECD 425 guidelines. Animals were selected by random sampling technique and divided into 5 groups of 6 animals each after pilot study. A single dose of hydro-alcoholic extract of the research drug formulation was administered orally to different group animals at the level of 300, 500, 1000, 1500 &2000 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 hours. 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 [18-20].

H. Chronic toxicity study

Chronic effects are those produced by prolonged exposure of three months resulting from the cumulative tendencies of the toxicant. During this experiment, three doses are used – the therapeutic dose 500 mg/kg, 2500 mg/kg (5 times the therapeutic dose) and 5000 mg/kg body weight (10 times the therapeutic dose) of hydro-alcoholic extract of research formulation. These dosages were prescribed orally for 90 days to the three experimental groups each having 6 female Wister rats to assess the toxic effect or mortality. All the animals were observed on daily and weekly basis for the body weight changes, food and water intake and clinical signs, toxic symptoms and mortality were recorded for appearance of toxic symptoms including muscle spasm, loss of righting reflex, tremors, behavioral changes, locomotion, convulsions, and mortality up to 90 days in this study. Detailed physical observations were made twice daily for morbidity/ mortality.

The animals were maintained at the maximum tolerated dose for a period of three months to allow development of any behavioral, food and water intake, motor, sensory and other neurological activities, pathological changes, and then scarified by using the deep anesthesia for keeping in the chloroform chamber and subjected to full hematological, biochemical, pathological and histological examinations of the blood and stomach, liver and kidney organs. 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.

a. Hematological and Histopathological study

Animals were anesthetized and blood was obtained from cardiac puncture at room temperature. Blood plasma was separated and stored at 4°C until the hematological parameters and liver function test (LFT) study were done. 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. 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 [18-20].

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.

I. Vaginal Irritation test

It is important to identify and evaluate new antimicrobial herbal agents that can be used vaginally in effective doses without inactivating lactobacilli or causing overt vaginal irritation or other toxicity. Many of the new chemical vaginal antimicrobial compounds receiving attention during the past decade are cytotoxic, such cytotoxic agents are also likely to cause vaginal irritation and to inactivate the normal vaginal flora when used frequently at clinically effective doses [21].

Vaginal toxicity testing was performed on female wistar female rats using a procedure modified from that of Gad and Chengelis [22]. A total of 18 mature female rats were equally divided into three test groups of 6 animals each. Group A: Negative control, Group B: Positive control (containing only polymer excipients used for vaginal tablet and no active drug ingredient) & Group C: Research group (Research drug formulation in the form of vaginal tablet having ingredient 550 mg along with excipient) were treated accordingly using the vaginal cannula for 14 consecutive days.

All animals were weighed on the 1st day of dosing and every 7th day thereafter. Detailed physical observations were made twice daily for morbidity/ mortality. Approximately 4 hours after dosing, observations were also made for vaginal bleeding and discharges, appearance, behavior, and other pharmacologic signs & symptoms. The animals were necropsied 24 hours after the final vaginal dose on 14th day and the heart, kidney, liver, lung, ovary, pancreas, uterus, and vagina were excised and weighed. Histopathological slides of ovary, 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] The FSH, LT, TSH and progesterone and estrogen hormones were also estimated using the Elisa test method.

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J. 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.

III. RESULTS

A. Acute Toxicity

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

B. Chronic Toxicity Study

The chronic toxicity test was performed following the method detailed by Ghosh [18]. Observations of experimental animals during this period indicated no significant changes in normal motor and behavioral activities up to 500 mg/kg dose while some muscle spasm and spasticity was observed at 2500 and 5000 mg/kg dose after 70 and 84 days although no mortality was observed during and up to 15 days after completion of the experiment.

The observations relating to the hematological parameters have been highlighted in table 2. 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 chronic toxicity tests.

a. Histopathological studies during chronic toxicity test

During this study, almost all the tissues of the stomach, liver and kidney were noticed to be intact or unaffected as shown in figures 1-9. No appreciable or significant degeneration or changes were observed. Only minor damage of hepatocytes was found during the study of the T.S. of liver at the highest dose (figure 6).

TABLE 1. Clinical signs and symptoms observed in different groups during acute toxicity study

Clinical sign	**300 mg/kg Dose**	**500 mg/kg Dose**	**1000 mg/kg Dose**	**1500 mg/kg Dose**	**2000 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					

TABLE 2. Hematological and biochemical analysis of different groups during chronic toxicity tests (n=6)

Hematological & Biochemical Parameters	**Control Group**	**Research drug 500 mg/kg**	**Research drug 2500 mg/kg**	**Research drug 5000 mg/kg**
Total Bilirubin (mg/dl)	0.29±0.04	0.25±0.07	0.62±0.02	0.77±0.05
SGPT (IU/L)	37.00±2.84	39.00±8.52	88.30±5.02	65.70±7.43
SGOT (IU/L)	49.20±3.3	47.00±5.90	121.00±4.90	125.00±3.40
Total Protein (g/dl)	6.21±0.15	7.86±0.21	6.24±0.50	6.35±3.50
Alkaline Phosphatase (IU/L)	366.83±4.76	355.36±4.90	350.82±4.50	410.45±8.50
Haemoglobin (gm %)	12.80±0.62	13.40±0.13	10.60±0.13	14.00±0.50
Total Count				
Erythrocytic (10^6/cu.mm.)	4.20±0.20	5.60±0.05	5.45±0.08	6.55±0.54
Leucocytic (10^3/cu.mm.)	8.40±0.16	7.90±0.30	7.60±0.05	9.50±0.75
Differential Leucocyte Count				
Neutrophil (%)	27.00±2.83	28.80±7.99	23.80±7.50	50.80±7.50
Lymphocyte (%)	26.00±6.88	38.50±4.50	43.50±5.00	48.50±5.00
Monocyte (%)	2.50±0.97	2.96±0.05	2.60±0.05	2.60±0.05
Eosinophil (%)	3.00±0.75	2.40±0.74	4.40±0.30	4.40±0.30
Basophil (%)	0.00	0.00	0.00	0.00
Erythrocyte Sedimentation Rate (mm)	5.46±0.27	7.50±5.87	6.70±1.08	8.67±3.02

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Group I – 500mg/kg	Group II– 2500mg/kg	Group III– 5000mg/kg
KIDNEY	**KIDNEY**	**KIDNEY**
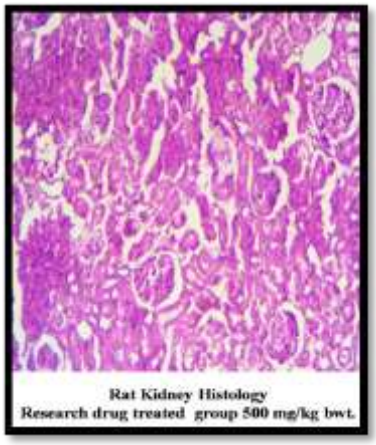	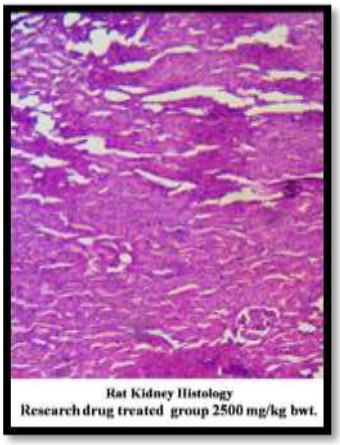	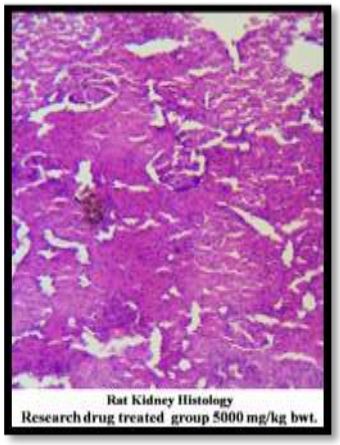
Fig.1	Fig.2	Fig.3
Fig. 1, Fig. 2 & Fig. 3 - TS microscopy of rat kidney showing normal structure in group I- 500mg/kg, group II- 2500mg/kg and group III- 5000mg/kg research drug treated animals under under microscope (Olympus CH20i) at 10X magnification. The internal tissue structures like nephritic cells, Bowman's capsule, both proximal and distal convoluted tubules, no infiltration of the cells and intact epithelium show that the kidney of rat is unchanged in drug treated groups relative to the control section.		
LIVER	**LIVER**	**LIVER**
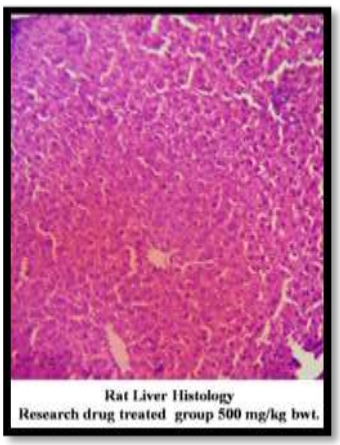	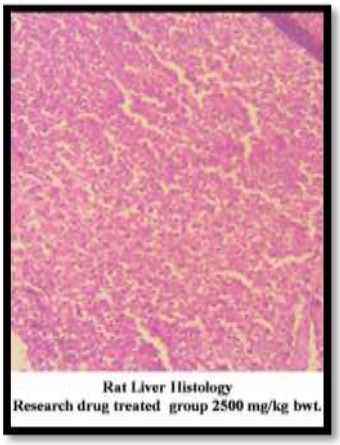	
Fig.4	Fig.5	Fig.6
Fig. 4, Fig. 5 & Fig. 6 - TS microscopy of rat liver showing normal structure in group I- 500 mg/kg and group II- 2500 mg/kg and minor damage of hepatocytes 7 other structures in group III- 5000 mg/kg research drug treated animals under microscope (Olympus CH20i) at 10X magnification.		
STOMACH	**STOMACH**	**STOMACH**
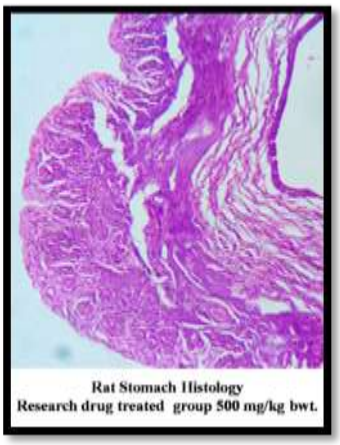	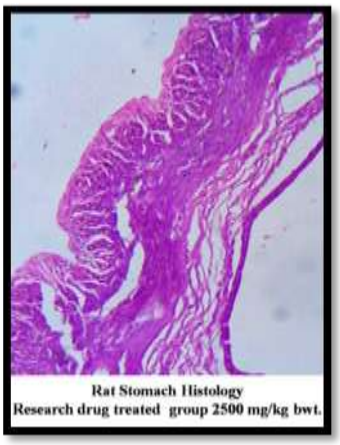	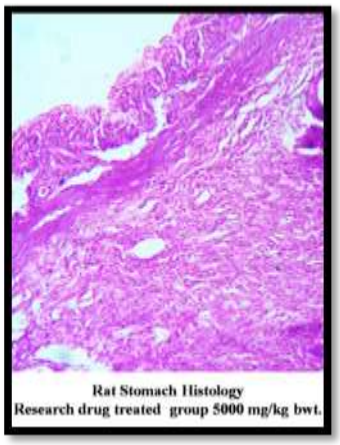
Fig.7	Fig.8	Fig.9
Fig. 7, Fig. 8 & Fig. 9 - TS microscopy of rat stomach showing internal tissue structures like mucosa, sub-mucosa and epithelium in group I- 500 mg/kg, group II- 2500 mg/kg and group III- 5000 mg/kg research drug treated group of animals under microscope (Olympus CH20i) at 10X magnification		

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The results of chronic toxicity (long term toxicity) of the hydro-alcoholic extract of Research drug showed no damage of cells, tissue & epithelium lining and other structures of stomach, liver and kidney during histopathological studies indicating no toxic effect and safety of drug up to 500 mg/kg dose. Therefore, the 500 mg/kg body wt dose of research drug was selected for further pharmacological studies. There was no mortality found after 90 days of the prescription of oral administration of the research drug in all groups but the some physical, behavioral changes were observed in some animal of group at higher doses group II- 2500 mg/kg and group III- 5000 mg/kg during this study.

C. Vaginal Irritation Test

The results obtained during the hematological studies are detailed in table 3. 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 negative control group, the estrogen level was 4.54 ± 1.61 whereas it was 5.00 ± 1.02 and 5.89 ± 0.34 ng/ ml respectively in the research formulation treated group and the placebo-treated group. Similarly, the progesterone level was 7.28 ± 0.44 in the negative control group while it was 4.30 ± 0.24 and 8.40 ± 0.02 ng/ml in the research formulation treated group and the placebo-treated groups respectively. The follicle-stimulating hormone (FSH) level was 0.19 ± 0.14, 0.23 ± 0.21, and 0.10 ± 0.31 ng/ml respectively in the negative control, research formulation treated group and the placebo-treated group. 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.

TABLE 3. Hematological parameters during vaginal irritation test toxicity study (n=6)

Treatment Group →	Group A	Group B	Group C
Total Bilirubin (mg/dl)	0.19±0.13	0.17±0.09	0.21±0.05
SGPT (IU/L)	31.00±1.69	21.80±1.18	23.00±0.20
SGOT (IU/L)	39.32±2.13	43.47±2.53	41.50±1.80
Total Protein (g/dl)	8.63±0.18	6.3±0.98	7.28±0.22
ALP (U/L)	98.50±2.31	108.00±1.21	103.00±1.20
Haemoglobin (gm %)	11.65±1.76	12.00±1.55	12.60±0.80
Erythrocytic (10^6/cu.mm.)	6.20±0.76	5.25±1.04	5.15±0.79
Leucocytic (10^3/cu.mm.)	5.69±1.84	4.29±0 .54	5.10±0.24
Neutrophil (%)	22.56±1.05	20.59±1.84	19.00±1.43
Lymphocyte (%)	25.90±4.41	25.90±4.41	23.50±0.41
Monocyte (%)	3.00±2.07	2.40±1.07	3.30±0.72
Eosinophil (%)	2.00±0.60	2.00±1.50	2.00±1.40
Basophil (%)	0.00	0.00	0.00
Erythrocyte Sedimentation Rate (mm)	4.46±1.22	5.06±1.40	5.1±0.12
FSH (mIU/ml)	0.19±0.14	0.23±0.21	0.10±0.31
TSH (mcU/ml)	0.36±0.53	0.18±1.32	0.34±0.64
LH (mIU/ml)	0.02±0.11	0.32±1.14	0.06±0.65
Progesterone (ng/ml)	7.28±0.44	4.30±0.24	8.40±0.02
Prolactin (ng/ml)	0.17±2.26	0.09±0.06	0.22±1.63
Estrogen (ng/ml)	4.54±1.06	5.00±1.02	5.89±0.34

a. Histopathological Study of Organs During Vaginal Irritation Test

Light micrographs of the rat uterus after 14 days of daily intravaginal treatment in case of the control group A, Research drug-treated in the form of vaginal tablet formulation with excipients in 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. Similarly, there were no visible signs of vaginal or ovarian inflammation (ulceration, edema, or leukocyte infiltration) in all the groups as shown in figures 10 - 12.

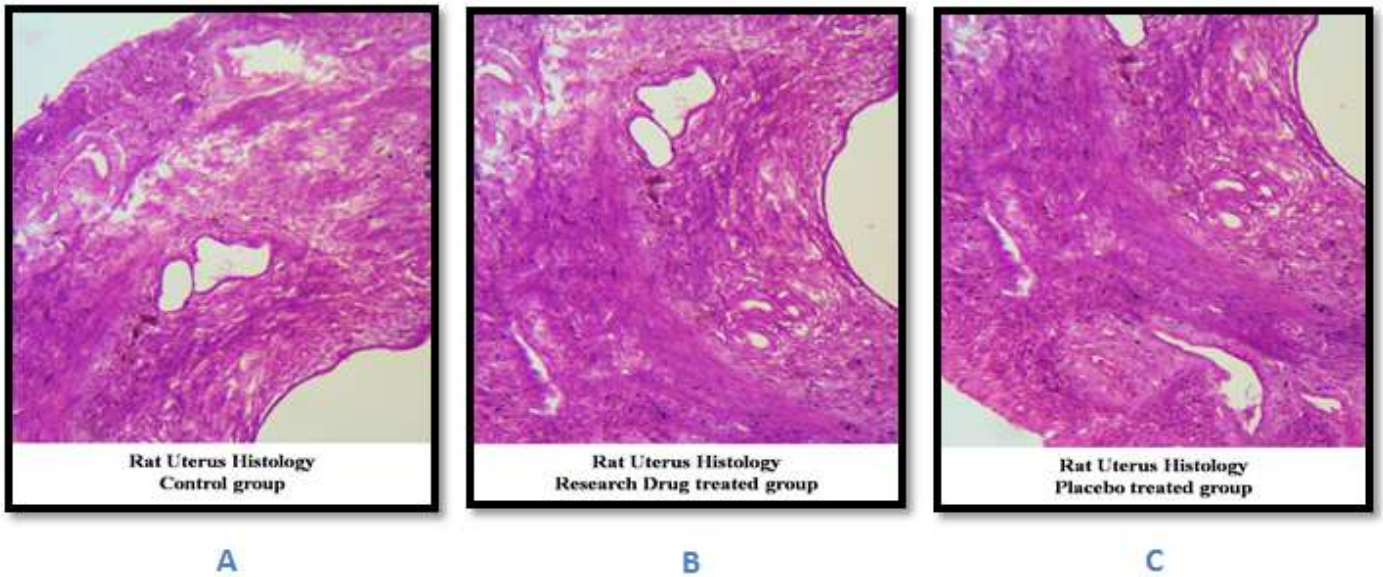

Fig. 10. Hematotoxyline and eosin stained section of rat uterus under Olympus CH20I microscope at ×10 magnification showing internal tissue structures such as mucosa, submucosa & uterine glands in Groups A, B & C.

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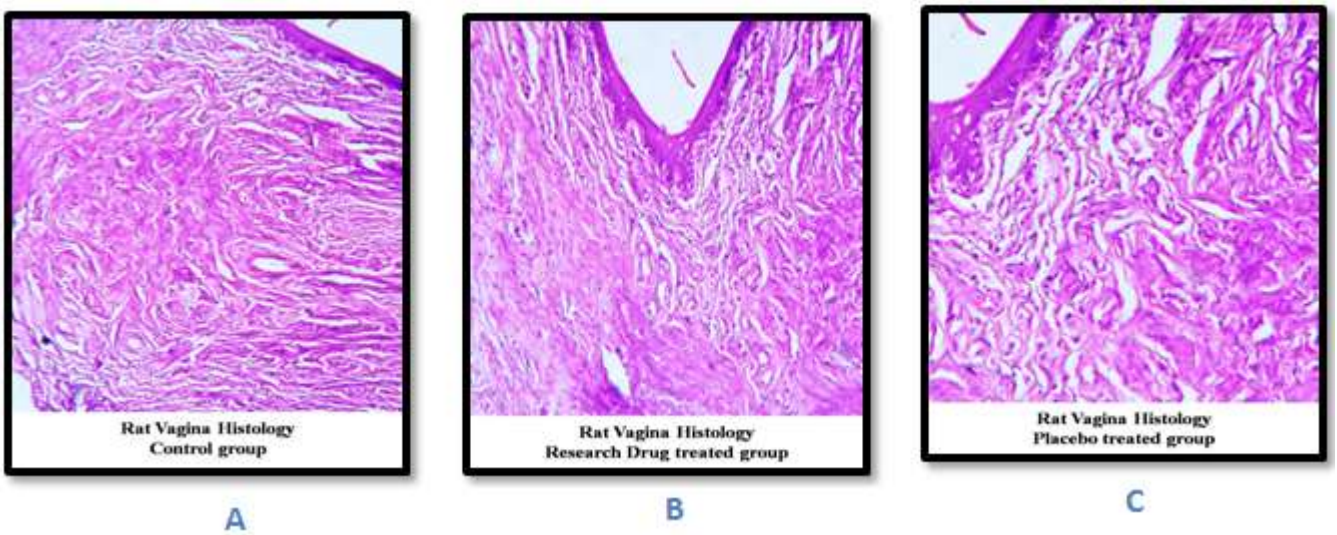

Fig. 11. Hematotoxyline and eosin stained section of rat vagina under Olympus CH20I microscope at ×10 magnification showing internal tissue structures such as mucosa, submucosa and epithelium in Groups A, B & C

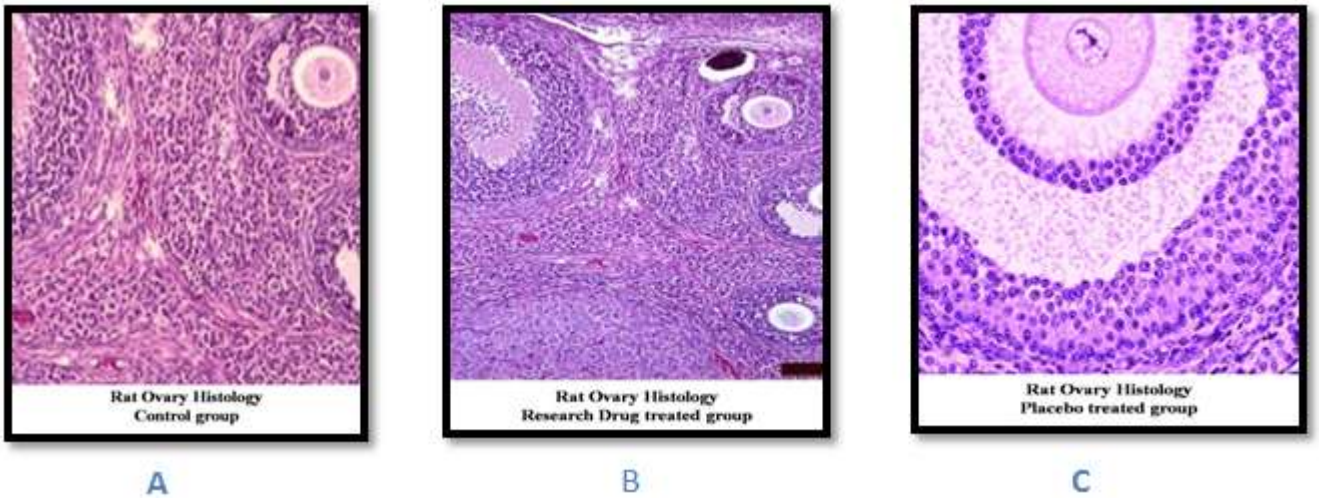

Fig. 12. Light micrographs of the Hematotoxyline and eosin stained section of rat ovary under Olympus CH20I microscope at ×10 magnification showing internal tissue structures such as mucosa, submucosa, ovarian glands and intact epithelium of the ovary of different groups

IV. Discussions

Plant based medicines play an important role in disease prevention and treatment through enhancement in inhibition of bacterial growth and modulation of genetic pathways. The therapeutic role of a number of plants in disease management is still being enthusiastically researched due to their lower side effects and affordable properties. In recent years, multiple drug resistance has developed due to indiscriminate use of synthetic drugs. This drives the need to screen medicinal plants for novel bioactive compounds as plant based drugs are biodegradable, safe and have fewer side effects. It is a largely accepted fact that numerous pharmacologically active drugs are derived from natural resources including medicinal plants and herbs. Therefore, different types of preparations based on plants or their constituents are very popular in many countries in disease management [23].

Toxicity refers to the toxic effect of a particular or a group of substance(s) on a cell or a particular organ (like kidney/ liver) or the whole organism. All substances are potentially toxic when administered exceeding a certain limit; similarly, many therapeutic medicines can be acutely toxic but are beneficial when used appropriately [9-10].

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, this herbal formulation provided good 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.

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 2000 mg/kg of the research drug. No mortality was reported up to 24 hours and even later during the subsequent 14 days at this dose. The therapeutic dose for the subsequent experiments was selected as 500 mg/kg on the basis of symptoms and mortality because some physical changes were observed in some animals at the higher dose of 1500 mg/kg & 2000 mg/kg; however, these symptoms subsided within 1–2 hours and no mortality occurred [24].

Organ weight changes have long been accepted as a sensitive indicator of chemically induced changes to organs and in toxicological experiments, comparison of organ weights between control and treated groups have conventionally been used to predict toxic effect of a test material since organ weight is an index of swelling, atrophy or hypertrophy. The heart, liver, kidneys, spleen and lungs are the primary organs affected by metabolic reactions caused by toxicants while liver is the major site of foreign compounds metabolism in the body. Body weight and condition of internal organs such as stomach, liver, kidney, heart spleen, thymus glands, etc. are simple and sensitive indices of toxicity after exposure to toxic substance. Therefore, the histopathological study of the stomach, liver & kidney were done in chronic oral toxicity and uterus, vagina and ovary organs were done in vaginal irritation test analysis.

The serum biochemical tests are frequently used in diagnosis of diseases of hearts, liver, kidney and cardiovascular system. They are also widely used in monitoring the response to exogenous toxic exposure [52]. When a herbal product is ingested, the body interacts with it in an attempt to get rid of any harmful toxins, especially if the body cannot convert the foreign substance into cellular components. These insults are commonly manifested by changes in enzyme levels and other cell components. The enzymes commonly involved are glutamate oxaloacetate transaminase (AST/GOT) glutamate pyruvate transaminase (ALT/GPT), alkaline phosphatase (ALP). Also component like urea and uric acid are vital diagnostic tools for toxicity [53]. Generally, liver cell damage is characterized by a rise in serum enzymes like AST, ALT, ALP, etc. [54]. The urea and creatinine are good indicators for renal function. If kidney function falls, the urea and creatinine levels will rise [25].

No significant toxic effects were observed up to a long time during the chronic toxicity studies up to the highest research drug concentration. While some symptoms were observed temporarily at the highest dose of the research drug, these symptoms subsided within an hour during this study and no animals died in the trial period. 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 [26-28].

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 as well as the hormonal parameters FSH, thyroid stimulating hormone, luteinizing hormone, progesterone and estrogen level were also observed. During this study, no noticeable differences in the mean body weight were observed among the three animal 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) 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 [29].

V. CONCLUSION

The new vaginal herbal formulation used during the study prepared using stem barks of *Azadirachta indica A. Juss.* and *Saraca asoca Roxb.* based on their Ayurvedic properties described in ancient texts such as an astringent, anti-inflammatory & efficacy in arresting excessive abnormal vaginal discharge has been evaluated on account of its exhibited pharmacological properties and the presence of phenolic and flavonoidic compounds in its constituent herbs [30].

The acute toxicity tests performed using the research formulation revealed no signs of behavioral changes up to the dose of 2000 mg/kg and no mortality was reported up to 24 hours.

During the chronic toxicity (long-term toxicity) testing, no animal died when high daily dose of the drug (5000 mg/kg bw.) was prescribed to the animal for 90 days. A little toxic

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effect upon their behavior and physical activities was observed over a certain period but these adverse temporary effects subsided gradually and the animals reverted to their normal activities in respect of food intake, urination, etc. 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 sub-acute 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 attributes of the research drug formulation on the basis of analysis of blood parameters, histopathological study of the organs and reproductive hormones using oral and intravaginal administration routes which validate its safety and efficacy for the treatment of vaginitis.

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