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ASSESSMENT OF TOXICITY AND ANTIPROLIFERATIVE ACTIVITY OF *PUTRANJIVA ROXBURGHII* WALL.: A POTENTIAL THERAPEUTIC AGENT FOR THE TREATMENT OF ENDOMETRIOSIS

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Keywords:

Putranjiva roxburghii Wall, Leaf extract, Endometrial cells, Acute toxicity, Sub-acute toxicity

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ABSTRACT: The utility of herbal medicines incresed for curative purposes in the past few years. They serve as a curative alternative for a significant section of the worldwide community. Nonetheless, assessing the reliability of such facilities continues to be challenging in guaranteeing their safe use. Acute and subacute toxicity studies were conducted in a rat model. The cytotoxicity assay and AO/PI staining of HALEPR was carried out in an endometrial cell. Acute toxicity was evaluated in female Charles Foster rats over 14 days, revealing no mortality or severe adverse effects, suggesting a Lethal Dose 50 (LD50) above 2000 mg/kg. Subacute toxicity was assessed over 28 days with no significant changes ($P < 0.05$) in body weight, feed consumption, water intake, or biochemical parameters. Histological examination of organ tissues indicated no notable differences between treated and control groups. The antiproliferative activity of HALEPR was investigated using primary endometrial cell cultures. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay demonstrated a significant apoptotic effect with an IC50 of 41.46 µg/mL. This result was further confirmed by acridine orange/propidium iodide (AO/PI) staining, which showed clear signs of apoptosis in treated endometrial cells. In conclusion, HALEPR showed no obvious signs of toxicity in rats when dosed orally for up to 4 weeks and exhibited potent antiproliferative activity in endometrial cell cultures. These findings indicate that HALEPR could be considered a promising therapeutic agent for endometriosis treatment, with further studies needed to explore its potential clinical applications.

INTRODUCTION: Medicinal plants have been essential in treating different human diseases [1, 2]. These medicinal plants have diverse bioactive compounds, providing unlimited opportunities to discover novel drugs and herbal remedies [3, 4]. The use of plant-based products in traditional and modern societies as herbal remedies, crude drugs, or purified compounds has a long history [5, 6].

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Currently, herbal medicine is becoming famous worldwide, especially in developing countries where medicinal plants are available, accessible, and affordable. Even though these plants have shown the potential phytotherapeutic effects with high global demand, there are still concerns about their service and safety [7, 8].

Putranjiva roxburghii Wall. (*P. roxburghii* Wall) belongs to the family Putranjivaceae and is cultivated mainly in tropical regions of Asia [9]. It is known for its therapeutic properties and is grown all over India, abundantly in the Khammam forests of Andhra Pradesh [10, 11]. Traditionally, it has been used to treat health issues such as fever, cold, phlegm, skin ailments, acidity, and sterility [12].

Although the precise mechanism of action of this herb is still unknown in the female reproductive system, it is considered to provide nutritional support to the uterus, maintain endometrial health, normalise menstrual blood flow, and help prepare the uterus by improving the thickness of the endometrial lining for implantation. Furthermore, *P. roxburghii* Wall is regarded as a potential uterine tonic due to its ability to modulate ovarian insufficiency, alleviate anxiety, and restore hormonal balance [13, 14]. *P. roxburghii* Wall also has many biological activities, including the ability to reduce inflammation [15], lower blood sugar [16, 17], kill microbes [18], larvicidal [19], analgesic [20]. Lower body temperature [21], stop vomiting [22], anti-epileptic [17], calm the central nervous system and ease pain [23], anticancer [24], stop fleas [25] and antimicrobial [26, 27].

The toxicity effects of these compounds and the safety challenges encountered during the drug development process may account for this scenario. Therefore, it is essential to establish the safety of medicinal plants. Moreover, toxicity studies play a significant role in drug development, providing information on toxic doses and therapeutic indices of potential drugs [28, 29].

Despite these efficacy studies on various parts of *P. roxburghii* Wall, a cytogenic activity on Swiss albino mice bone marrow cell has been conducted for this medicinal plant [30]. As a result, there is no chromosomal aberration that occurred mice bone marrow cell that was treated with hydroalcoholic *P. roxburghii* Wall but there is no any report of organ related toxicity assessment [31]. The outcome of this study may fill the gap left by the previous studies on *P. roxburghii* Wall done by Awasthy *et al.,* 2000. This study aims to investigate the acute and subacute toxicity of HALEPR through biochemical and histological analyses of liver, kidney, uterus, and blood parameters in Charles Foster rats (*in-vivo*). Additionally, the antiproliferative effects of HALEPR on endometrial cells were evaluated to explore its potential as a therapeutic agent for endometrial health. Endometrial cells were selected for in vitro toxicity testing because they are a useful model for studying the impact of plant-based compounds on uterine health. Considering the reported therapeutic benefits of *P. roxburghii* Wall in supporting female reproductive health, particularly its role in maintaining endometrial health and regulating hormonal balance, evaluating its effects on endometrial cells could offer valuable insights into its potential as a uterine tonic [13].

MATERIAL AND METHODS:

Plant Material and Preparation of the Extract: The leaves of the *P. roxburghii* Wall plant were collected from the campus of Banaras Hindu University (BHU), Varanasi and gently washed with tap water to remove any dust particles before drying in the shade at room temperature. Sample identification and herbarium sheet (voucher specimen no. Putranjiva. 2022/01) were submitted to the laboratory of Prof. N. K. Dubey, Department of Botany, BHU. After drying (10 to 15 days), the leaf was pulverised in a grinder, and the powder was weighed to determine the yield percentage. The hydroalcoholic (70% ethanol and 30% water) extract was prepared following a standard protocol with minor modifications [30]. The semi solid extract was stored in an airtight container at 4°C.

Experimental Animals: Approximately 6 to 7-week-old female Charles Foster rats weighing 100–110 g were purchased from Banaras Hindu University's Institute of Medical Science's animal laboratory in Varanasi. The Organisation for Economic Cooperation and Development (OECD) guidelines 407 [30] and 423 [32] were followed for assessing toxic effect. The animals were housed in sterile paddy material and plastic cages filled with husks and fed a pellet diet. The rats were acclimatized to a specific room temperature, relative humidity, and a 12-hour light/dark cycle. All procedures were approved by the Institutional Animal Ethical Committee (IAEC), BHU (permission reference No. BHU/DoZ/IAEC/2021-2022/030).

Acute Oral Toxicity: The acute toxicity assay was carried out in accordance with the Organization for Economic Cooperation and Development (OECD) guideline 423 using a single dose of 2000 mg/kg body weight. Two groups were selected: Group I (control) received normal saline, while Group II was administered the extract (2000 mg/kg) via gavage and monitored for 14 days. Behavioral and physiological activities were observed at 2, 6, 12, and 24 hours post-administration on day 1, and periodically thereafter. Body weight was recorded weekly. On day 14,

animals were euthanized by cervical dislocation under deep anesthesia induced by an intraperitoneal overdose of pentobarbital (200 mg/kg). Blood and vital organs (kidney, liver, and uterus) were collected for histological, hematological, and biochemical analyses.

Subacute Toxicity: The sub-acute toxicity study followed OECD Guideline 407. Four groups (*n* = 3) were established: one control group (distilled water) and three test groups treated with HALEPR at doses of 100, 200, and 400 mg/kg body weight for 28 days. Animals were monitored daily for general health and toxicity, with body weight recorded at 0, 7, 14, 21, and 28 days. At the end of the study, animals were fasted overnight before blood collection via the retro-orbital plexus. Blood samples were divided into EDTA and non-EDTA tubes. Haematological parameters were analyzed from the anti-coagulated blood, while serum for biochemical analysis was obtained by centrifugation at 3000 rpm for 10 minutes at 4 °C. The liver, kidneys, and uterus were excised, weighed, and examined histologically.

Histological and Biochemical Parameters: At the conclusion of the acute (14th day) and sub-acute (28th day) toxicity studies, all rats were fasted overnight, weighed, and euthanized by cervical dislocation. Blood samples were collected via retro-orbital plexus puncture for haematological (RBC, Hb, MCV, MCH, MCHC, platelet count, WBC, lymphocytes, monocytes, basophils, neutrophils) and biochemical (ALP, ALT, serum urea, serum glucose, cholesterol) analyses [33]. These parameters were assessed to evaluate changes in hematological and biochemical profiles during acute and sub-acute toxicity studies.

Histological Examination: At the end of the acute and sub-acute toxicity studies, the liver, kidneys, and uterus were excised, and their relative organ weights were calculated. Tissues were fixed in 10% neutral buffered formalin and stained with hematoxylin and eosin using standard protocols. Stained sections were examined microscopically for cell injury or morphological changes.

Cell Culture and Viability Measurement

MTT Assay: Human endometrial tissue was obtained from an endometriosis patient in the Department of Gynecology at Sir Sunder Lal Hospital, BHU. After cutting the tissue up, it was grown in DMEM media that had 10% (v/v) heat-inactivated FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL) added to it. All cell cultures were grown in a humidified incubator (Eppendorf Cellxpert, C170 India) at 37 °C in 5% CO_2.

Cell viability was measured by monitoring the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO, USA) dye, following a method slightly modified [35]. Approximately 7500 cells/well were seeded in 96-well plates and incubated overnight for adhesion. The media were replaced with serum-free media containing various concentrations of HALEPR and incubated for 24 hours. Subsequently, 20 µL of 5 mg/mL MTT reagent was added, followed by incubation for 4 hours in the dark at 37 °C. Formed crystals were dissolved with DMSO, and absorbance was measured at 570 nm using a microplate reader. Cellular viability was calculated using MS Excel, with appropriate controls.

AO/PI Staining for Cell Morphological Analysis: A cell death study was performed using Acridine orange (AO)/propidium iodide (PI) double staining [36]. Endometrial cells were treated with various concentrations of leaf extract (0, 0.2, 0.4, 0.5, 0.8, 1.0, mg) for 24 hours in 24-well plates. Following treatment, cells were washed with PBS (1X) and stained with AO/PI (100 µg/mL each in PBS). Stained cells were visualized and imaged using an inverted phase-contrast fluorescence microscope (Nikon Eclips T*s*2).

Statistical Analysis: The data were presented as mean values with standard deviation (±SD) and analysed using student T- test using Excel program and ANOVA (analysis of variance) followed by post hoc Tukey HSD test. Differences were considered statistically significant at a level of $p < 05$.

RESULTS:

Acute Toxicity: According to the OECD 423 guidelines, acute toxicity refers to a single dose of any compound or plant extract tested for 14 days. There was no mortality in rats in the first four hours of intense observation or after 24 hours. There is

also no evidence of a lethal effect after 14 days of extract administration. The fur and skin colour appeared to have typical morphological characteristics. Salivation, diarrhoea, lethargy, unusual behaviour, or altered respiration were absent **Table 1.**

TABLE 1: IMPACT OF ORAL ADMINISTRATION OF HALEPR ON BEHAVIORAL RESPONSES OF RAT DURING ACUTE TOXICITY STUDIES

Parameters	Control	HALEPR (2000 mg/kg)
Temperature	Normal	Normal
Alteration in skin	Not reported	Not reported
Eye color change	Not reported	Not reported
Food consumption	Normal	Normal
General physique	Normal	Normal
Diarrhea	Not reported	Not reported
Coma	Not reported	Not reported
Drowsiness	Not reported	Not reported
Breathing difficulty	Not reported	Not reported
Sedation	Not reported	Not reported
Tremor	Not reported	Not reported
Death	Alive	Alive

Subacute Toxicity:

Effect on Body Weight, Feed and Water Consumption: There was no significant difference in the mean body weight of rats treated with 100, 200, and 400 mg/kg of HALEPR compared to the control group. However, at the end of 28 days of exposure, there was a slight reduction in body weight at a dose of 400 mg/kg compared to the control **Fig. 1A.** Over 28 days, feed intake rates of rats exposed to various doses of HALEPR had no significant impact, in the treatment groups compared to the control **Fig. 1B.** Water consumption in treated animals also remained unchanged compared with control animals (data not shown).

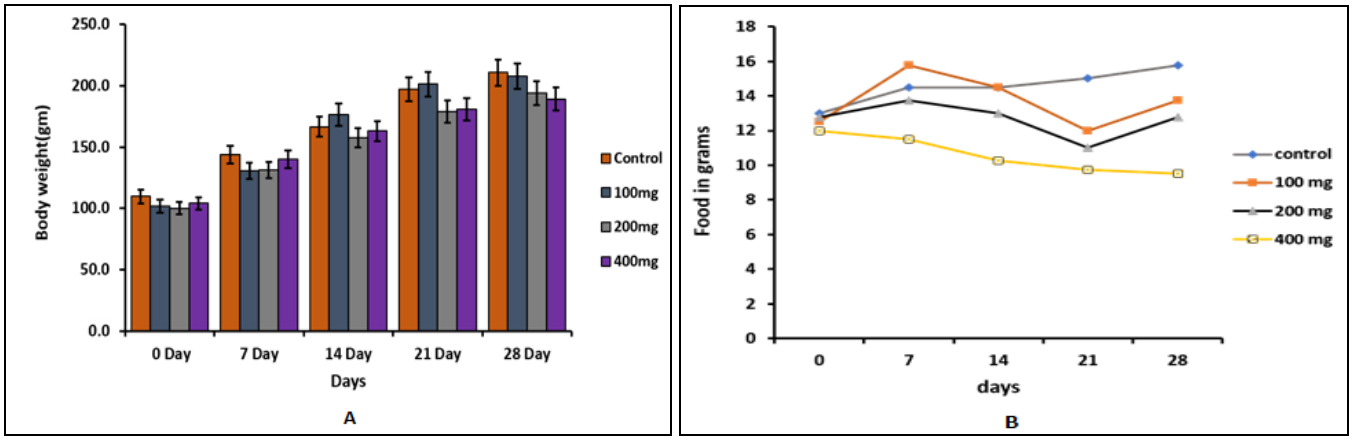

FIG. 1: SHOWS CHANGES IN BODY WEIGHT AND FEED CONSUMPTION IN RATS. FOR 28 DAYS, THE CHANGE IN BODY WEIGHT WITH HALEPR TREATMENT AT CONTROL 100 mg/kg, 200mg/kg AND 400 mg/kg BW/DAY AND CONTROL (FIG. 1A). THE CHANGES IN FEED CONSUMPTION IN RATS DURING 28 DAYS WITH HALEPR TREATMENT AT 100 mg/kg, 200 mg/kg AND 400 mg/kg BW/DAY AND CONTROL (FIG. 1B)

Effect on Relative Organ Weight: Prior to the autopsy, all animals fasted for the night. Then, the rats were euthanized by cervical dislocation, and their vital organs (kidney, spleen, uterus, and liver) were excised and examined microscopically for lesions or abnormal signs. Next, the dissected body parts were weighed after being placed on blotting paper for a few minutes to evaluate their weights (relative organ weight). The relative organ weight was calculated as a ratio of organ weight divided by body weight at the euthanized date, which is then multiplied by 100.There was no significant change in the absolute organ weight of the left and right kidneys, spleen, liver, and uterus in rats treated with a 28-day oral dose of 100, 200, and 400 mg/kg of HALEPR **Fig. 2.**

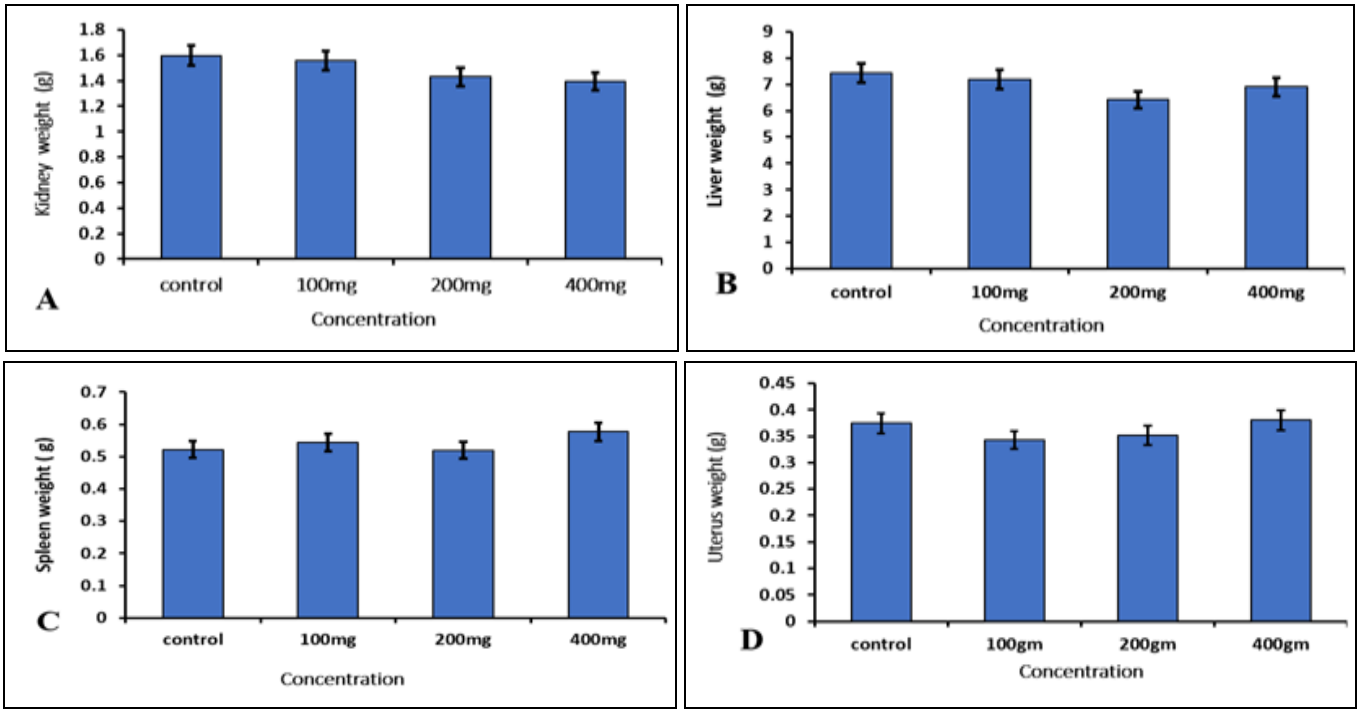

FIG. 2: RELATIVE ORGAN WEIGHTS OF CHARLES FOSTER RATS AFTER 28 DAYS OF REPEATED EXPOSURE TO HALEPR. THE RELATIVE ORGAN WEIGHTS OF THE LIVER (A), SPLEEN (B), KIDNEY (C), AND UTERUS (D) WERE MEASURED AND COMPARED BETWEEN THE CONTROL GROUP AND GROUPS RECEIVING DIFFERENT DOSES OF HALEPR (100, 200, AND 400 mg/kg). NO STATISTICALLY SIGNIFICANT CHANGES (P > 0.05) WERE OBSERVED IN THE RELATIVE WEIGHTS OF THE LIVER, SPLEEN, OR KIDNEY ACROSS ALL DOSE GROUPS COMPARED TO THE CONTROL. HOWEVER, THE UTERUS SHOWED SLIGHT VARIATIONS IN WEIGHT AT HIGHER DOSES (200 AND 400 mg/kg), ALTHOUGH THESE CHANGES WERE NOT STATISTICALLY SIGNIFICANT. ERROR BARS REPRESENT THE STANDARD ERROR OF THE MEAN (SEM)

Hematological Parameters: The **Table 2** represents hematological parameters of acute and subacute toxicity. Results of hematological reveals that no significant differences in blood cell counts were observed on the 28th day in the treated group, except for PLT levels in rats, which also showed a considerable increase in the acute toxicity study with the control group. All relative blood parameters, such as WBC, Hb, RBC, MCH, and MCV, were not statistically different between the HALEPR treated group and the control group in acute and sub-acute toxicity studies **Table 2.** The statistical findings were most prominent in the low-dose group. These statistical shifts remained within the bounds of historical control. Further investigation is necessary to uncover profound explanations for the differences.

TABLE 2: HEMATOLOGICAL PARAMETERS OF RATS FOLLOWING ACUTE AND SUBACUTE TOXICITY TESTING OF HALEPR. ACUTE TOXICITY WAS ASSESSED AFTER A SINGLE ADMINISTRATION OF 2000 mg/kg of THE EXTRACT, WHILE SUBACUTE TOXICITY WAS EVALUATED OVER 28 DAYS AT DOSES OF 100 mg/kg, 200 MG/KG, AND 400 mg/kg

Parameters	Acute toxicity		Sub-acute toxicity			
	Control	2000mg HALEPR	Control	100mg HALEPR	200mg HALEPR	400mg HALEPR
WBC (×10^3/µl)	4.97±0.22	5.93±0.09	4.08±1.32	4.9±0.41	4.45±0.63	5±0.42
RBC (×10^6/µl)	7.05±0.26	6.95±0.14	6.5±0.42	8.32±0.67	7.61±0.16	7.22±0.31
MCH (pg)	17.04±0.60	18.4±1.55	16.85±0.70	17.75±0.56	17±0.35	17.65±0.48
PLT (×10^3/µl)	350±7.53	437.5±6.67*	332±11.31	405.5±10.60*	420±14.14*	461±15.55*
Hb (g/dl)	13.02±0.53	12.26±0.48	12.85±0.64	13.05±0.21	12.95±0.07	13.02±0.42
MCV (fl)	47.10±0.60	49.38±1.24	46.30±0.70	46.30±0.56	47.75±0.35	48.60±0.84
MCHC (g/dl)	35.4±0.16	35.80±0.42	36.8±0.28	37.45±3.60	36.15±0.49	36.35±0.49

RBC - Red Blood Cells; WBC - White Blood Cells; Hb - Hemoglobin, PLT-Platelet. Values are expressed as means± SD, *p value less than 0.05, (p< 0.05) significant value.

Biochemical Parameters: The biochemical parameters (lipid profile, hepatic, and kidney function test) of treated animals in acute and sub-acute studies with HALEPR did not produce any significant changes compared with the control group.

Evaluating Liver Parameters: Table 3 displays the results of toxicological studies on liver function tests. The administration of 100, 200, and 400 mg/kg of HALEPR in a sub-acute toxicity study showed an insignificant difference from the control group. The exposure of 2000 mg/kg BW in acute study was also insignificant except for aspartate aminotransferase (AST), which is significantly increased compared to the control group ($P< 0.05$).

However, the levels of alkaline phosphatase (ALP), alanine aminotransferase (ALT), total protein (TP), and total bilirubin (TB) were higher when compared to control.

TABLE 3: EFFECTS OF DIFFERENT CONCENTRATIONS OF HALEPR ON LIVER PARAMETERS IN RAT SERUM DURING ACUTE AND SUBACUTE TOXICITY STUDIES. BIOCHEMICAL PARAMETERS MEASURED FOLLOWING ACUTE AND SUBACUTE TOXICITY TESTING IN CONTROL AND TREATED GROUPS. ACUTE TOXICITY WAS ASSESSED AFTER ADMINISTERING A SINGLE 2000 mg/kg DOSE OF THE TEST SUBSTANCE, WHILE SUBACUTE TOXICITY WAS EVALUATED OVER 28 DAYS AT DOSES OF 100 mg/kg, 200 mg/kg, AND 400 mg/kg

Biochemical parameters	Acute toxicity		Sub-acute toxicity			
	Control	2000mg HALEPR	Control	100mg HALEPR	200mg HALEPR	400mg HALEPR
Aspartate Aminotransferase (AST, U/L)	125.15 ± 3.61	213.76 ± 2.63*	127.15±2.61	127.15±2.61	134.9±2.91	138.2±2.54
Alanine aminotransferase (ALT, U/L)	49.05 ± 5.2	59.92 ± 2.29	48.05±5.2	48.05±5.2	57.2±2.54	61.25±5.30
Alkaline Phosphatase (AP U/L)	267.65 ± 5.77	274 ± 2.48	264.65±6.57	264.65±6.57	275.85±6.71	238.8±3.11
Total bilirubin (TB, mg/dL)	0.60 ± 0.14	0.7 ± 0.003	0.50±0.01	0.50±0.42	0.60±0.03	1.61±0.11
Total protein (TP, g/dL)	5.61 ± 0.05	8.67 ± 0.91	5.21±0.26	5.21±0.04	5.30±0.04	5.08±0.03

Values are expressed as means± SD, *p-value less than 0.05, ($p< 0.05$) significant value.

Effect on Kidney Function Test: The administration of HALEPR in rats for 14 days (acute) as well as 28 days (subacute) showed no significant change in the renal parameters **Table 4.** The levels of urea, creatinine, uric acid, glucose, serum Ca^{++}, and BUN were analysed.

TABLE 4: EFFECTS OF DIFFERENT CONCENTRATIONS OFF SERUM ON RENAL PARAMETERS IN RAT SERUM DURING ACUTE AND SUBACUTE TOXICITY STUDIES. BIOCHEMICAL PARAMETERS MEASURED AFTER ACUTE AND SUBACUTE TOXICITY TESTING IN CONTROL AND TREATED GROUPS. ACUTE TOXICITY WAS ASSESSED USING A 2000 mg/kg DOSE OF THE HALEPR, WHILE SUBACUTE TOXICITY WAS EVALUATED AFTER 28 DAYS OF ADMINISTRATION AT DOSES OF 100 mg/kg, 200 mg/kg, AND 400 mg/kg

Biochemical parameters	Acute toxicity		Sub-acute toxicity			
	Control	2000mg HALEPR	Control	100mg HALEPR	200mg HALEPR	400mg HALEPR
Urea (mg/dL)	32.05 ± 0.31	23.24±0.77*	30.05±0.21	33.10±0.42	27.25±0.35	28.95±0.07
Creatinine (mg/dL)	0.54 ± 0.042	0.60±0.078	0.44±0.057	0.47±0.004	0.52±0.040	0.41±0.025
Uric Acid (mg/dL)	3.05 ± 0.029	2.845±0.23	2.72±0.049	0.63±0.099*	1.48±0.106	0.97±0.008*
Sugar (mg/dL)	97.05 ± 2.19	144.88±3.42*	96.05±2.19	113.05±2.75	98.70±1.13	92±2.12
Serum Ca++ (mg/dL)	7.98 ± 0.71	6.80±0.134	8.03±0.81	11.36±0.20*	11.64±0.10	10.49±0.36
Blood Urea Nitrogen (BUN, mg/dL)	15.14 ± 0.36	11.43±1.52	14.54±0.46	15.1±0.29	12.28±0.45	13.04±0.55

BUN- Blood Urea Nitrogen, Values are expressed as means± SD. *p-value less than 0.05, ($p< 0.05$) significant value.

Change in Lipid Parameters: Similar to the control group, the treated rats' lipid profiles (HDL, LDL, cholesterol, and VLDL) showed no significant variation at a graded dose concentration. However, there was a significant increase in the levels of total cholesterol (TC) and triglycerides (TG) at doses of 200 and 400 mg/kg BW **Table 5.**

TABLE 5: LIPID PARAMETERS IN RATS SERUM TREATED WITH DIFFERENT CONCENTRATIONS OF HALEPR DURING ACUTE AND SUBACUTE TOXICITY STUDIES. THE ACUTE TOXICITY GROUP WAS TREATED WITH 2000 mg/kg OF THE TEST SUBSTANCE, AND SUBACUTE TOXICITY GROUPS WERE ADMINISTERED 100 mg/kg, 200 mg/kg, AND 400 mg/kg DOSES OVER 28 DAYS. PARAMETERS ASSESSED INCLUDE. NO SIGNIFICANT VARIATION WAS SEEN IN THE LIPID PROFILE OF TREATED RATS WHEN COMPARED TO THE CONTROL GROUP. NONETHELESS, THE VALUES REMAINED WITHIN LABORATORY RANGES

Biochemical parameters	Acute toxicity		Sub-acute toxicity			
	Control	2000mg HALEPR	Control	100mg HALEPR	200mg HALEPR	400mg HALEPR
Total Cholesterol(TC, mg/dL)	83.86±1.08	85.71±1.71	84.3±1.69	76.45±6.85	93.35±3.74	96.35±2.33
High Density Lipid (HDL, mg/dL)	30.05±1.98	25.39±1.82	29.55±2.89	33.15±0.35	30.55±2.19	31.30±0.56
Low Density Lipid (LDL, mg/dL)	42.90±1.34	43.89±0.43	42.90±1.83	654.40±2.68	50.45±0.77	51.05±1.76
Triglycerides (TC, mg/dL)	57.80±2.08	69.63±1.93	57.80±1.69	61.5±5.65	68.10±4.80	75.70±5.23
Very Low-Density Lipoprotein (VLDL, mg/dL)	11.06±0.93	16.83±0.89	10.66±0.93	12.95±2.47	13.35±1.90	15.50±0.35

Values are expressed as means ± SD. *p-value less than 0.05, (P< 0.05) significant value.

Histological Examination: The histological sections were analysed of the liver, kidney, and uterus in control and treated groups with different doses (100, 200, and 400) of HALEPR on Charles foster rats. The liver, kidney, and uterus tissue sections showed no significant damage was recognised in the liver, kidney, and uterus after treatment with various concentrations of extract in sub-acute toxicity experiments. However, a slight modification was observed in the liver, kidney, and uterus tissue when exposed to higher concentrations (2000 mg/kg BW) in acute toxicity, as shown in **Fig. 3.**

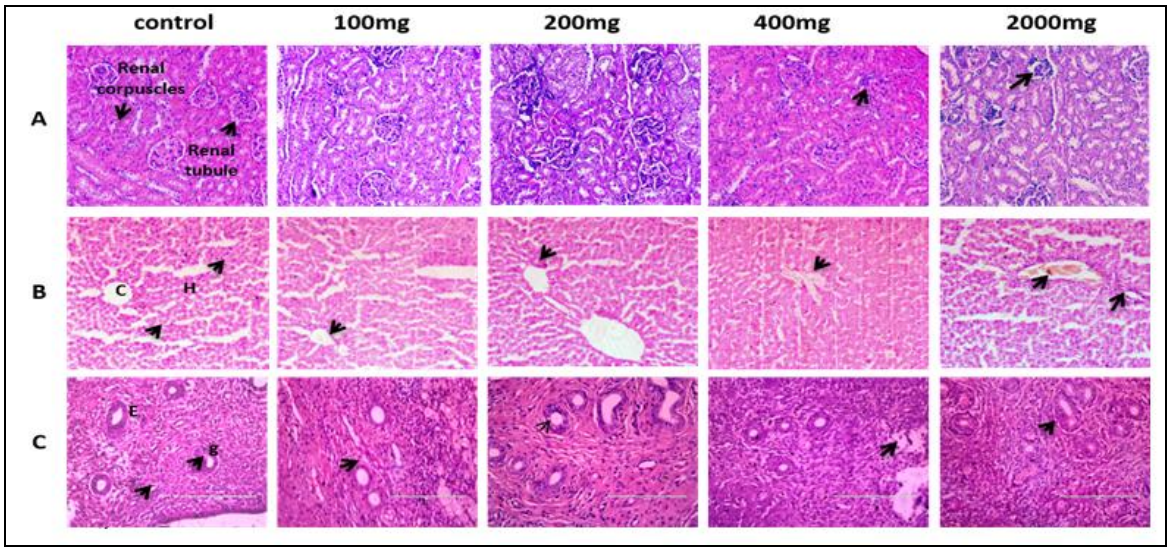

FIG. 3: THE EFFECT ON HISTOLOGICAL CHANGES BY TREATING HALEPR IN CHARLES FOSTER RAT MODEL. THE REPRESENTATIVE DIAGRAM SHOWS THE H&E STAINING ANALYSIS OF THE UTERUS (A), KIDNEY(B) AND LIVER (C). FIG 3A CONTROL SHOWING NORMAL HISTOLOGY OF UNTREATED RAT KIDNEY. THE EFFECT OF HALEPR TREATMENT AT 100 AND 200 MG/KG BW SHOWING NO SIGNIFICANT CHANGE WHILE THE TREATMENT GROUP WITH 400 AND 2000mg/kg BW SHOWING GLOMERULAR AND TUBULAR CONGESTION. HISTOLOGY OF THE UNTREATED RAT LIVER SHOWED A NORMAL CENTRAL CANAL AND PROPER ARRANGEMENT OF HEPATOCYTE CELLS (CONTROL AND 100mg AND 200mg/Kg TREATED GROUP), THE TREATED GROUP IN THE ACUTE STUDY (2000mg/Kg) SHOWED LITTLE CONGESTION AND VASCULARISATION IN THE CENTRAL CANAL AS WELL AS LOOSELY ARRANGED HEPATOCYTES CELL. IN THE CASE OF THE UTERUS, NO SIGNIFICANT CHANGE APPEARS IN THE TREATED GROUP AT 100, 200 AND 400mg/Kg. IN COMPARISON, AT 2000mg/Kg TREATED GROUP SHOWS MINOR VASCULARISATION AND SHRINKAGE OF ENDOMETRIAL GLANDS.(MAGNIFICATION: 20X)

Cell Viability Assay:

MTT Assay: The cell viability of primary endometrial cells was determined using MTT analysis to assess the anti-proliferation effects of HALEPR. **Fig. 4** shows the viability of primary endometrial cells treated with doses (0.1, 0.2, 0.4, 0.6, 0.8, and 1 mg/ml) of HALEPR for 24 h. The leaf extract inhibited the primary endometrial cells with an EC_{50} of 41.46µg.

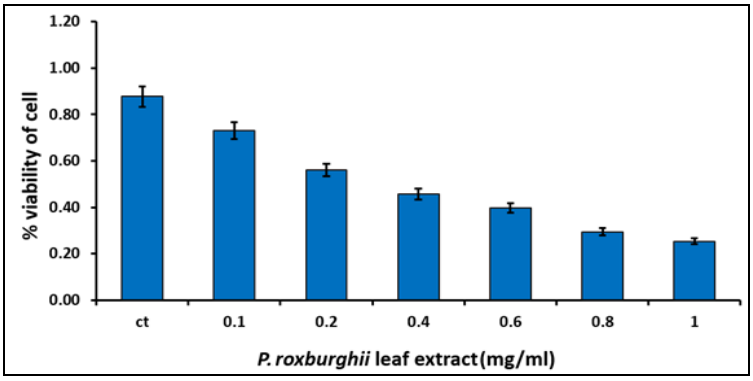

FIG. 4: ANTI-PROLIFERATIVE EFFECT OF HALEPR ON ENDOMETRIAL PRIMARY CELL CULTURE AT DIFFERENT CONCENTRATIONS (0.1 TO 1 mg/ml) FOR 24 H. THE EXPERIMENTS WERE CARRIED OUT IN TRIPLICATE, AND DATA ARE EXPRESSED AS MEAN ± SD VALUES

AO/PI Staining: As shown in **Fig. 5,** endometrial cells with exposer of different concentrations (100, 400, and 800 µg/ml) of HALEPR showed cellular apoptosis in primary cultured endometrial cells. Supplementation of an extract with 400µg induces apoptotic change by 50% in endometrial cells, as shown in the diagram. In comparison, exposure at 800µg increases the apoptosis rate by more than 50%, as evidenced by the high intensity of red fluorescence (PI staining). Disclosure at 100µg provides less apoptotic effect, as evidenced by the lower red fluorescence intensity of PI staining. Three independent experiments were conducted to confirm the results.

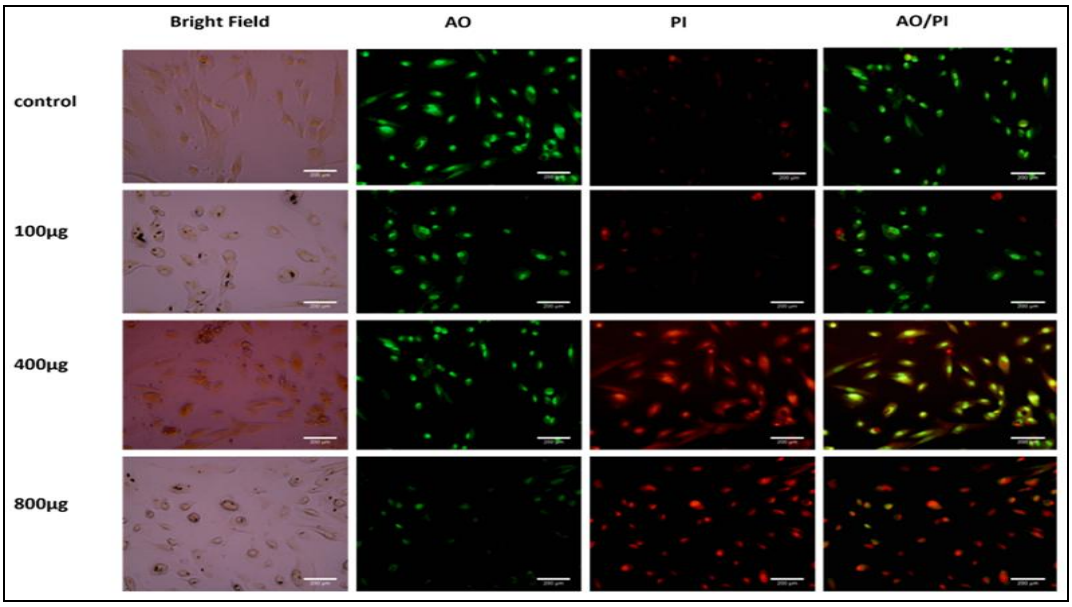

FIG. 5: REPRESENTATIVE PHOTOGRAPHS SHOW ACRIDINE ORANGE /PROPIDIUM IODIDE (AO/PI) STAINING FOR THE ANALYSIS OF CELL VIABILITY AFTER THE CONTROL AND DIFFERENT CONCENTRATIONS OF HALEPR TREATMENT IN ENDOMETRIAL PRIMARY CULTURED CELLS. HALEPR TREATMENTAT 100µg DOES NOT INDUCE SIGNIFICANT MORPHOLOGICAL APOPTOTIC CHANGES IN ENDOMETRIAL CELLS, AS EVIDENCED BY THE INCREASED INTENSITY OF AO STAINING AND THE REDUCED INTENSITY OF PI. SUPPLEMENTATION OF EXTRACT AT 400µg INDUCES APOPTOTIC CHANGE BY 50% OF ENDOMETRIAL CELLS, AS SHOWN IN THE DIAGRAM. WHEN ENDOMETRIAL CELLS ARE EXPOSED TO 800µg/ml LEAF EXTRACT, THE RATE OF APOPTOSIS INCREASES, AS EVIDENCED BY THE INCREASED INTENSITY OF PI STAINING. MAGNIFICATION: 20X

DISCUSSION: Medicinal plants have considerable potential due to their several bioactive compounds, which have shown therapeutic benefit in a variety of health disorders [1, 2]. Typically, researchers conduct acute, sub-acute, and chronic toxicity tests in experimental animals to conduct safety studies on herbal medicines [37].

This study comprehensively investigated the acute and sub-acute toxicity profiles of HALEPR in Charles Foster rats and explored its antiproliferative activity on primary endometrial cells. The findings provided insights into the non-toxic nature of HALEPR at therapeutic doses and its potential as a therapeutic agent for endometrial health.

In the acute toxicity investigation, a single dose of 2000 mg/kg HALEPR resulted in no deaths or apparent side effects. Body weight, feed consumption, and behavior were unaffected, indicating that HALEPR has an elevated tolerance of safety (OECD, 2001). Similarly, a sub-acute toxicity trial with dosages of 100, 200, and 400 mg/kg for 28 days found no substantial adverse effects, as demonstrated by stable hematological and biochemical profiles. This finding suggests that the extract has an impact on metabolic activity. Therefore, few side effects may occur after long-term leaf extract exposure, including loss of appetite and reduction in weight, when the dose is above 400 mg/kg [38]. However, this effect was not associated with significant changes in organ weight or histological structure.

Histological examinations confirmed the biochemical findings, revealing no major damage to liver, kidney, and uterine tissues. Minor structural changes noticed at the highest acute dose (2000 mg/kg) were most likely caused by physiological stress rather than inherent toxicity. The drop in urea and uric acid levels, notably at 2000 mg/kg in the acute phase and 400 mg/kg in the sub-acute study, shows that HALEPR may improve renal clearance or regulate nitrogen metabolism. This is consistent with prior research highlighting the importance of plant-derived polyphenols in regulating critical enzymes in the urea cycle and purine metabolism [39]. Furthermore, phytochemicals in HALEPR may block xanthine oxidase, lowering uric acid levels [40]. HALEPR's antiproliferative efficacy against primary endometrial cells suggests its therapeutic potential. The MTT experiment showed a dose-dependent reduction in cell viability, with an IC50 of 41.46 µg/ml. Morphological study with AO/PI staining revealed apoptotic alterations in treated cells, demonstrating that HALEPR causes programmed cell death. These findings are consistent with previous observations on flavonoids that have antiproliferative and proapoptotic effects on endometrial cells [41, 42]. This study fills a knowledge gap on the organ-specific toxicity and therapeutic potential of *P. roxburghii* Wall. The lack of considerable toxicity and the strong antiproliferative effects on endometrial cells underscore HALEPR's dual promise as a safe and effective treatment agent for endometrial health and fertility.

CONCLUSION: This study demonstrated the safety and therapeutic potential of the hydroalcoholic leaf extract of HALEPR. Acute toxicity studies confirmed a high safety margin, as HALEPR showed no mortality or significant toxicity at doses up to 2000 mg/kg. Sub-acute administration at doses of 100, 200, and 400 mg/kg revealed no adverse effects on hematological, biochemical, or histological parameters over 28 days. The reduction in urea and uric acid levels suggests nephroprotective properties, likely mediated by bioactive compounds that enhance renal function or inhibit xanthine oxidase. Additionally, HALEPR exhibited strong antiproliferative and apoptotic effects on primary endometrial cells, supporting its potential for managing endometrial health and fertility. Further studies are necessary to confirm these findings, explore long-term safety, and investigate clinical applications for endometrial and reproductive health.

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±Equal Contribution of both the Authors:

Author Contribution: JKC: conceptualization, methodology, Validation, investigation. SA:

Manuscript preparation and investigation DP: Manuscript preparation. AT: Supervision, Conceptualization, Validation, Reviewing the manuscript.

Declarations:

Ethical Approval The ethical permission for animal study was obtained by IAEC (Institutional Animal Care and Ethics Committee) with approval no. BHU/DoZ/IAEC/2021-2022/030 dated February 15, 2022.

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