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Acute And Subacute Toxicity Studies Of Picroside-Ii From *Picrorhiza Kurroa Rhizhome* Extracts In Wistar Rats

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Abstract:
The present study aimed to evaluate the acute and sub-acute oral toxicity profile of Picroside II (PK-II), a major bioactive constituent of Picrorhiza kurroa, in Wistar rats. Acute & sub-acute oral toxicity was conducted according to OECD guideline. Rats were orally administered PK-II at doses of 5, 50, 300, and 2000 mg/kg for the acute study and 1000 mg/kg daily for 28 consecutive days in the sub-acute study. Animals were monitored for clinical signs, mortality, body weight changes, hematological and biochemical parameters, and histopathological alterations. No mortality or clinical signs of toxicity were observed in any group. Hematological and biochemical profiles showed no significant deviations from controls. Histopathological evaluation revealed no treatment-related abnormalities in vital organs. The oral LD_{50} of PK-II was estimated to be greater than 2000 mg/kg, indicating a wide margin of safety. The findings confirm that PK-II is non-toxic under the tested conditions, supporting its potential use as a safe therapeutic candidate.
Keywords: Picroside II, Picrorhiza kurroa, Acute toxicity, Sub-acute toxicity, Wistar rats, OECD guidelines

Introduction:

Natural compounds derived from medicinal plants have long been recognized for their therapeutic potential and favorable safety profiles. *Picrorhiza kurroa* Royle ex Benth. (family: Plantaginaceae), commonly known as “Kutki,” is widely used in Ayurvedic medicine for the treatment of liver ailments. Its pharmacological properties are mainly attributed to its iridoid glycosides, Picroside I and Picroside II

(PK-II). Among these, Picroside II is recognized as a potent hepatoprotective agent, known to protect the liver against chemical and drug-induced toxicity.

Experimental studies have shown that PK-II mitigates liver injury by enhancing antioxidant defense mechanisms, inhibiting lipid peroxidation, and modulating inflammatory pathways such as NF-κB and MAPK. In addition to hepatoprotection, PK-II also exhibits antioxidant, anti-inflammatory, neuroprotective, and cardioprotective effects, further supporting its pharmacological value.

However, despite its extensive traditional use and promising pharmacological data, limited information is available regarding its toxicological safety. Therefore, the present study was designed to evaluate the acute and sub-acute oral toxicity of Picroside II in Wistar rats following OECD guidelines. The findings aim to establish its safety profile and support its future therapeutic applications as a natural hepatoprotective compound.

Material and Methodology:

Animals and Dosing:

Male and female healthy Wistar rats, with a minimum body weight of 125-130 grams, were procured from Crystal Biological Solutions Pune. The animals were housed under controlled environmental conditions, maintaining a temperature of 26 ± 2°C, relative humidity of 50 ± 5%, and a 12-hour light/dark cycle, in accordance with OECD guidelines. After a period of acclimatization lasting seven days, the animals were included in the study.

Test Material:

PK-II was used as the test compound. It was freshly dissolved in distilled water for oral administration.

Acute Toxicity Studies:

The single-dose acute oral toxicity study was evaluated following the recommendations by OECD Guidelines (423). Acute toxicity studies were carried out in Wistar rats, weighing 125–130 g each one, using a single dose, which administered orally. The general behaviour of mice and signs of toxicity were observed continuously for 1 h after the oral treatment and then intermittently for 4 h and thereafter over a period of 24 h. The mice were further observed once a day up to 14 days for following treatment for behavioural changes and signs of toxicity and/or death and the latency of death [6-7].

Experimental Design:

A total of 25 male rats were assigned to various treatment groups, each receiving a single dose of the test material according to the following experimental design (table 1).

Table 1. Experimental design for acute toxicity studies

Sr. No.	No. of rats	Treatment	Dose (p.o)
1	5	Control	Vehicle (d.w.)
2	5	PK-II	5 mg/ kg
3	5	PK-II	50 mg/ kg
4	5	PK-II	300 mg/kg
5	5	PK-II	2000 mg/kg

The test materials were dissolved in distilled water for oral administration to the rats. The animals were observed for mortality over a period of 21 days.

Sub-acute (4-week) toxicity studies:

Animal Selection and Dosing:

For the sub-acute toxicity investigation, a group of male and female healthy wistar rats, each weighing a minimum of 125 grams, were chosen as the study subjects. The rats were acclimatized under regulated environmental conditions, maintaining a temperature of 26 ± 2°C, relative humidity of 50 ± 5%, and a 12-hour light/dark cycle, conforming to the established OECD guidelines. Following a seven-day acclimatization period, the rats were included in the study [8].

Experimental Design:

A meticulous experimental design was employed for this study, aiming to assess the potential sub-acute toxicity of PK-II. The rats were allocated to different treatment groups, taking into consideration both sex and dosing, as follows (table 2).

Table 2. Experimental Design for sub-acute toxicity studies

Sr. No.	Sex	No. of rats	Treatment	Dose (p.o)
1	M	5	Control	Vehicle (d.w.)
2	F	5	Control	Vehicle (d.w.)
3	M	5	PK-II	1000 mg/ kg
4	F	5	PK-II	1000 mg/kg

Test materials were dissolved in distilled water for oral administration. Animals were observed for morality for 21 days.

Control Group:

- Male: 5 rats received the vehicle (distilled water) orally.
- Female: 5 rats received the vehicle (distilled water) orally.

PK-II Group:

- Male: 5 rats received 1000 mg/kg of PK-II orally.
- Female: 5 rats received 1000 mg/kg of PK-II orally.

The administration of PK-II was carried out once daily, within the time frame of 9:00 to 11:00 am, and was continued for a duration of 28 consecutive days.

Observations and Measurements:

Throughout the study period, the body weights of the rats were recorded on a weekly basis, every 7 days, until the completion of the 28-day duration. At the end of the study, the rats were humanely sacrificed, and both blood samples and major organs were collected for further analysis [9].

Blood and Serum Analysis:

The collected blood/serum samples were subjected to comprehensive hematological and biochemical analyses. Parameters including red blood cell count (RBC), white blood cell count (WBC), differential leucocyte count, platelet count, blood clotting time/potential, hemoglobin (Hb), platelet (Plt), glucose,

triglyceride (TG), cholesterol, bilirubin, alanine aminotransferase (ALT: SGPT), aspartate aminotransferase (AST: SGOT), urea, uric acid (UA), and creatinine were meticulously measured.

Effect of test materials on hematological parameters:

Blood Collection and Preparation:

On the 21st day of the study, approximately 1 ml of blood was carefully drawn from the tail vein of each rat. The collected blood was treated with EDTA as an anticoagulant for the purpose of hematological investigations. Furthermore, serum samples were obtained for subsequent biochemical analyses [10].

Hematological Investigations:

A comprehensive array of hematological parameters was meticulously investigated using manual techniques. These investigations encompassed essential blood parameters that provide insights into the overall health of the animals. The following hematological parameters were assessed:

- Red Blood Cell Count (RBC): The number of red blood cells per unit volume of blood was manually determined using an improved Neubauer counting chamber.
- White Blood Cell Count (WBC): The quantification of white blood cells in the blood samples was carried out manually using the Neubauer counting chamber.
- Platelet Count (PLT): The platelet count was manually performed on a thin blood film stained with Leishman stain.
- Differential Count (DC): The distribution of different types of white blood cells in the blood sample was assessed manually using a stained blood film.
- Hemoglobin Concentration ([Hb]): The concentration of hemoglobin in the blood was determined using Shali's method, which was chosen based on the laboratory's available facilities. This method involves a colorimetric approach.
- Packed Cell Volume (PCV) or Hematocrit: The micro-hematocrit method utilizing capillary tubes was employed to measure the packed cell volume, which represents the proportion of blood occupied by red blood cells.

Calculation of Derived Parameters:

From the obtained values of RBC, PCV, and [Hb], additional hematological parameters were calculated using established formulae, such as Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), and Mean Corpuscular Hemoglobin Concentration (MCHC).

Effect of the Extract on Biochemical Parameters:

Biochemical Investigations:

Biochemical analyses were carried out using POINTE reagent kits on a semi-automated chemistry analyzer (POINTE-180, Scientific Inc., USA). A range of essential biochemical parameters, crucial for assessing organ function and overall health, were meticulously evaluated. These parameters encompassed measurements related to blood glucose levels, lipid profile (including total cholesterol, high-density lipoprotein (HDL), and triglycerides (TG)), renal function (serum creatinine (Scr) and blood urea nitrogen (BUN)), and liver function (alkaline phosphatase (AP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) enzyme levels). Moreover, low-density lipoprotein (LDL) was calculated using formulae provided by the reagent kit based on lipid profile measurements [11].

Experimental Design:

A total of 20 rats (10 males and 10 females) were encompassed in this investigation, categorized as per the experimental design delineated below. The test materials, including the PK-II at a dose of 1000 mg/kg, were orally administered once daily between 9:00 to 11:00 am for a period of 28 consecutive days.

Histopathology of Vital Organs:

Histopathological Analysis:

Histopathological assessments of vital organs were conducted to scrutinize the potential impact of the test materials on tissue morphology and structural integrity. These evaluations were essential to ascertain whether the administration of the test materials at the specified doses led to any discernible abnormalities or pathological alterations within the examined organs. The histopathological examinations were performed using rotary microscopes, specifically the Leica RM 2125RT and Nikhon E600 [12].

Data Analysis:

The data obtained from the histopathological examinations were meticulously analyzed to deduce any notable changes in tissue structure and morphology. The findings were presented as the mean values accompanied by their corresponding standard errors of the mean (S.E.M). Additionally, a crucial parameter, the mean red blood cell (RBC) survival rate, was calculated. Furthermore, dose-response curves were constructed to evaluate the concentration that elicited a 50% response (CC50). The data was then subjected to graphing and interpretation using Microsoft Excel, which facilitated a comprehensive understanding of the impact of the test materials on the examined organs. The comprehensive histopathological analysis of the vital organs provided valuable insights into any potential structural changes or anomalies induced by the test materials, aiding in the determination of their safety profiles [13-14].

Results & Discussion:

Acute toxicity studies of PK-II in Rats:

The acute toxicity test using the Up and down method at an oral limit dose of 5, 50, 300 and 2000 mg/kg of the test material caused no death in the rats. No lethal effects were noted throughout the short and long-term observation period. No toxicity signs were observed in the animals throughout the 21 days' study period. Therefore, the test material may be safe at these doses and the oral LD50 considered greater than 2000 mg/kg in rats and mice.

The assessment of acute toxicity was conducted using the Up-and-Down method, with oral limit doses of 5, 50, 300, and 2000 mg/kg of the test material administered to the rats. Notably, no deaths were recorded among the rats subjected to these doses. Throughout both the short-term and long-term observation periods, there were no instances of lethal effects. Additionally, no signs of toxicity were observed in the animals over the entire 21-day study duration.

Consequently, it can be inferred that the test material, PK-II, is safe at the aforementioned doses. Furthermore, the oral LD50 (median lethal dose) is considered to be greater than 2000 mg/kg in both rats and mice, as there were no instances of toxicity-related deaths or adverse effects observed. This finding underscores the safety of the test material at the doses administered during the acute toxicity study.

Sub-acute Toxicity Studies of PK-II in Rats:

During the entire course of the 28-day treatment period, all the treated rats, irrespective of their gender and the administered doses (control and PK-II 1000 mg/kg), exhibited remarkable survival rates. An interesting observation emerged as the furs of the rats treated at various doses, including PK-II 1000 mg/kg, displayed a smoother texture compared to the control group. This intriguing alteration in fur texture might point towards potential physiological responses induced by the test material. Importantly, the treated rats did not manifest any discernible signs of toxicity, indicating the absence of overt adverse effects or observable toxic reactions in the PK-II-treated rats when compared to the control group.

Effect of the PK-II on Body Weight Gain of Rats:

The administration of the test material, PK-II, to both male and female rats did not result in any detectable impact on the body weight of the animals. This suggests that PK-II did not exert any significant influence on the weight gain patterns in rats when compared to the control group. The consistent body weight gain across the treated and control groups underscores the apparent lack of detrimental physiological effects on growth and development.

Effect of PK-II on Relative Organ Weight:

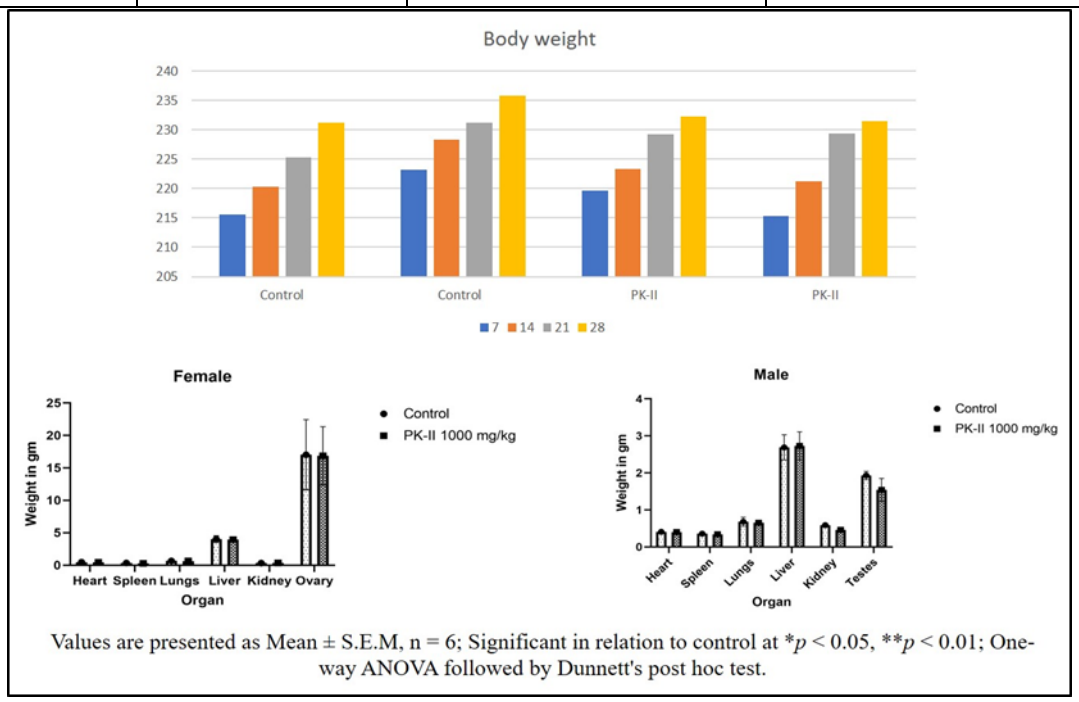
The impact of the test material, PK-II, on relative organ weight was further evaluated by assessing the relative organ weights of various organs in both female and male Wistar rats treated for 28 consecutive days. The results, detailed in figure 1 & Table 3, reveal that the administration of PK-II at a dose of 1000 mg/kg did not lead to any substantial alterations in the relative weights of the heart, spleen, lungs, liver, kidneys, and reproductive organs (ovaries in females and testes in males) in comparison to the control group. The close similarity in relative organ weights suggests the absence of any apparent organ-specific toxicity induced by PK-II administration.

These detailed findings underscore the favorable safety profile of PK-II during the sub-acute toxicity study, as evidenced by the absence of mortality, the absence of observable toxicity signs, minimal impact on body weight, and lack of significant alterations in organ weights.

Table 3. Effect of PK-II on relative organ weight in female and male Wistar rats treated for 28 consecutive days.

Animal	Organ	Control	PK-II 1000 mg/kg
Female	Heart	0.47 ± 0.05	0.44 ± 0.04
	Spleen	0.34 ± 0.03	0.28 ± 0.09
	Lungs	0.65 ± 0.07	0.64 ± 0.10
	Liver	4.02 ± 0.51	3.95 ± 0.25
	Kidney	0.33 ± 0.05	0.35 ± 0.05
	Ovary	17.02 ± 5.4	16.84 ± 4.5
	Testes	-	-
Male	Heart	0.41 ± 0.03	0.40 ± 0.05
	Spleen	0.36 ± 0.05	0.34 ± 0.04

Animal	Organ	Control	PK-II 1000 mg/kg
	Lungs	0.68 ± 0.12	0.65 ± 0.05
	Liver	2.69 ± 0.34	2.73 ± 0.38
	Kidney	0.59 ± 0.03	0.46 ± 0.07
	Ovary	-	-
	Testes	1.93 ± 0.11	1.54 ± 0.31



Values are presented as Mean ± S.E.M, n = 6; Significant in relation to control at *$p < 0.05$, **$p < 0.01$; One-way ANOVA followed by Dunnett's post hoc test.

Fig. 1. Effect of PK-II on relative organ weight in female and male Wistar rats treated for 28 consecutive days

Effect of PK-II on hematological parameters:

Upon analyzing the hematological parameters, it was discerned that there were no statistically significant differences observed when compared to the control group (fig.2 & table 4.). This indicates that the administration of the test materials did not provoke any noteworthy alterations in the assessed hematological parameters. The stability and consistency of these parameters in the treated group further corroborate the lack of substantial hematological impact induced by the test materials, reinforcing their overall safety profile.

Table 4. Effect of PK-II on hematological parameters

Animal	Parameters	Control	PK-II 1000 mg/kg
	WBC (x103/ul)	7.59 ± 1.34	8.13 ± 1.12
	LY (x103/ul)	3.29 ± 1.02	3.48 ± 1.06
	MO (x103/ul)	1.28 ± 0.42	1.34 ± 0.21

Female	GR (x103/ul)	3.71 ± 1.29	3.74 ± 0.41
	LY (%)	43.7 ± 10.57	39.28 ± 5.61
	MO (%)	12.59 ± 2.04	12.87 ± 1.89
	GR (%)	41.49 ± 10.05	45.37 ± 5.32
	RBC (x106/ul)	7.01 ± 0.59	7.09 ± 0.62
	Hgb (g/dl)	14.25 ± 1.25	16.52± 1.12
	HCT (%)	45.64 ± 4.26	47.56 ± 3.54
	MCV (fl)	70.84 ± 0.35	66.54 ± 0.94
	MCH (pg)	22.35 ± 0.38	24.26 ± 0.22
	MCHC (g/dl)	30.26 ± 0.31	34.25 ± 0.63
	RDW (%)	15.65 ± 0.32	17.25 ± 0.58
	PLT (x103/ul)	562.26 ± 45.26	563.25 ± 71.25
	PCT (%)	0.48 ± 0.05	0.48 ± 0.08
	MPV(fl)	10.25 ± 0.65	9.84 ± 0.24
	PDW (fl)	15.24 ± 1.06	14.25 ± 1.26
Male	WBC (x103/ul)	9.65 ± 2.21	10.32 ± 4.25
	LY (x103/ul)	8.54 ± 1.34	10.25 ± 3.26
	MO (x103/ul)	0.31 ± 0.14	0.48 ± 0.21
	GR (x103/ul)	4.21 ± 0.84	4.26 ± 1.25
	LY (%)	90.58 ± 5.34	84.85 ± 6.28
	MO (%)	2.84 ± 0.19	6.54 ± 2.35
	GR (%)	42.65 ± 6.8	45.68 ± 4.25
	RBC (x106/ul)	8.6 ± 0.15	8.65 ± 0.42
	Hgb (g/dl)	15.64 ± 0.84	16.52 ± 0.49
	HCT (%)	45.64 ± 2.76	43.25 ± 3.65
	MCV (fl)	36.25 ± 2.35	40.26 ± 2.31
	MCH (pg)	20.25 ± 0.19	19.25 ± 1.35
	MCHC (g/dl)	34.37 ± 0.27	35.26 ± 0.21
	RDW (%)	18.41 ± 0.21	19.43 ± 0.30
	PLT (x103/ul)	605 ± 39.51	634.74 ± 31.30
	PCT (%)	0.48 ± 0.03	0.49 ± 0.04
	MPV(fl)	8.19 ± 1.07	7.15 ± 2.01
	PDW (fl)	16.84 ± 2.08	19.56 ± 3.58

Values are presented as Mean ± S.E.M, n = 6; Significant in relation to control at *$p < 0.05$, One-way ANOVA followed by Dunnett's post hoc test.

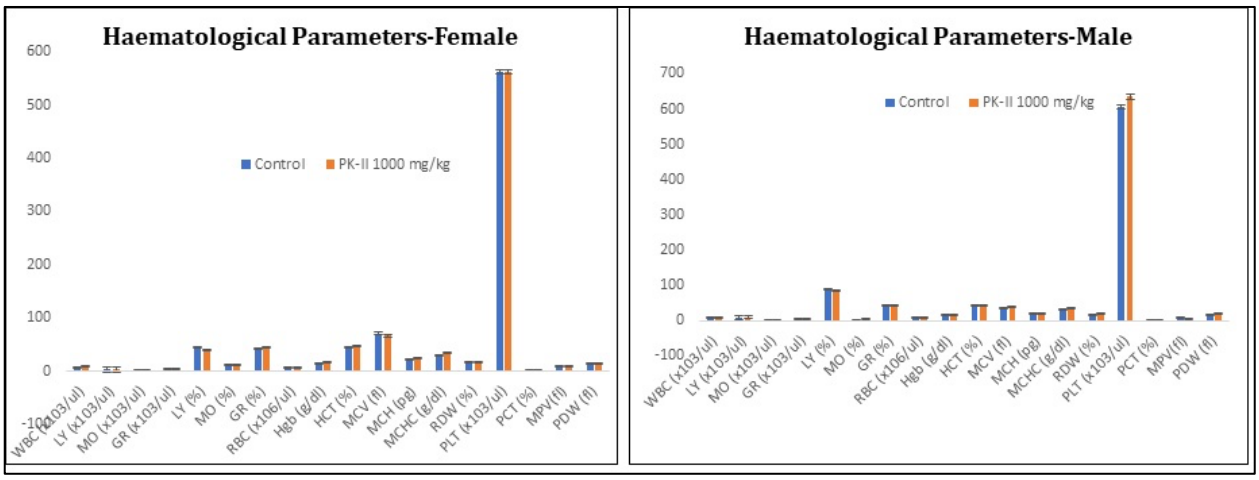


Fig. 2. Effect of PK-II on hematological parameters

Effect of the PK-II on biochemical parameters:

The findings of the biochemical investigations underscored that the administration of the test materials, particularly at a dose of 1000 mg/kg, did not induce any statistically significant alterations in the assessed parameters when compared to the control group (fig.3 & table 5). Both male and female rats exhibited no significant deviations in the studied biochemical markers during the 28-day treatment duration. Additionally, no noteworthy discrepancies in these parameters were observed in comparison to the control group. This further affirms the absence of adverse biochemical effects due to the administration of the test materials at the specified dose, underlining their potential safety for the subjects.

Table 5. Effect of PK-II on biochemical parameters.

Animals	Parameters	Control	PK-II 1000 mg/kg
Female	U (mmol/l)	6.38 ± 0.93	5.98 ± 0.41
	Cr (mg/dl)	0.8 ± 0.00	0.8 ± 0.01
	Na+ (mmol)	142 ± 3.6	144 ± 5.62
	K+ (mmol)	5.21 ± 0.42	4.97 ± 0.31
	Cl− (mmol)	98.52 ± 3.62	95.25 ± 2.36
	HCO3- (mmol)	22.36 ± 0.32	26.35 ± 2.05
	Total Protein (g/dl)	7.10 ± 1.02	7.42 ± 0.21
	Albumin (g/dl)	3.26 ± 0.07	3.64 ± 0.19
	AST (U/L)	184.62 ± 6.32	179.85 ± 10.29
	ALT (U/L)	56.34 ± 11.25	52.26 ± 2.32
	T. Bilirubin (mg/dl)	0.65 ± 0.10	0.72 ± 0.29
	D. Bilirubin (mg/dl)	0.16 ± 0.04	0.19 ± 0.03
	U (mmol/l)	4.12 ± 0.62	4.13 ± 0.56
	Cr (mg/dl)	0.95 ± 0.26	0.89 ± 0.15
	Na+ (mmol)	143.25 ± 0.85	149.55 ± 1.34
	K+ (mmol)	5.28 ± 0.04	5.63 ± 0.16

Male	Cl− (mmol)	105.64 ± 0.84	112.25 ± 1.35
	HCO3- (mmol)	26.54 ± 1.84	27.56 ± 0.86
	Total Protein (g/dl)	6.21 ± 0.14	6.34 ± 0.16
	Albumin (g/dl)	4.01 ± 0.21	4.21 ± 0.38
	AST (U/L)	256.21 ± 5.49	245.25 ± 13.64
	ALT (U/L)	70.15 ± 18.21	67.25 ± 4.37
	T. Bilirubin (mg/dl)	0.38 ± 0.04	0.41 ± 0.03
	D. Bilirubin (mg/dl)	0.13 ± 0.06	0.15 ± 0.04

Data presented as Mean ± S.E.M. *$p < 0.05$; U= Urea, Cr= Creatinine, aspartate aminotransferase (AST), alanine amino transferase (ALT), T. = Total, C. = Conjugated, D. = Direct.

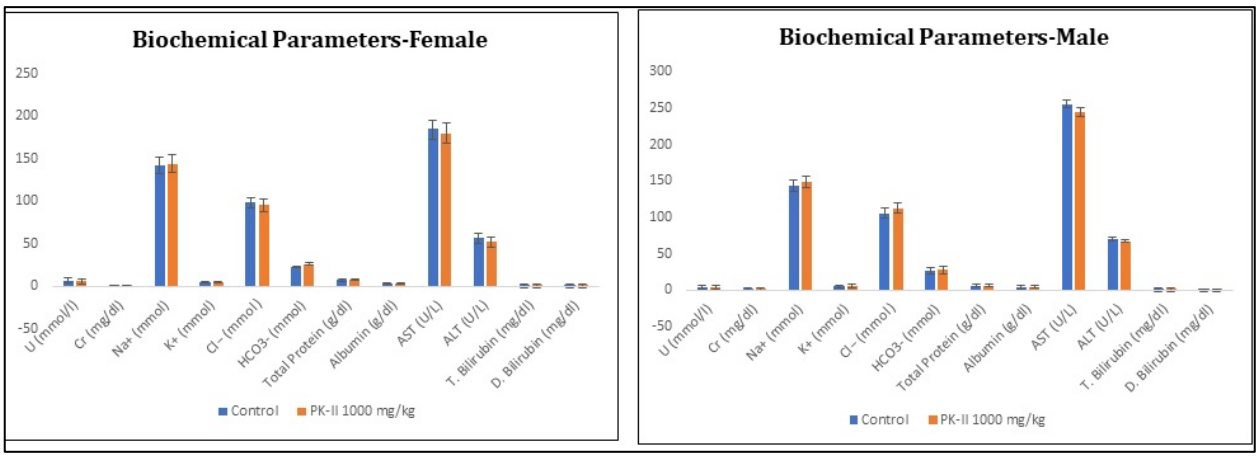


Fig. 3. Effect of PK-II on biochemical parameters

Histopathology studies of PK-II:

Histopathological examinations of specific internal organs were conducted to support the hematological and biochemical assessments in accordance with OECD guidelines 423 for the ongoing preclinical toxicity studies. Upon macroscopic examination, no morphological abnormalities were observed in any of the essential organs in the rats administered PK II compared to the untreated group (fig 4 & 5). Additionally, histological analysis revealed that the Liver, heart, kidney, spleen, brain, lung, pancreas, fat tissue, muscle, ovary and testis exhibited normal architectural structures consistent with those observed in the control group, showing no signs of histological abnormalities.

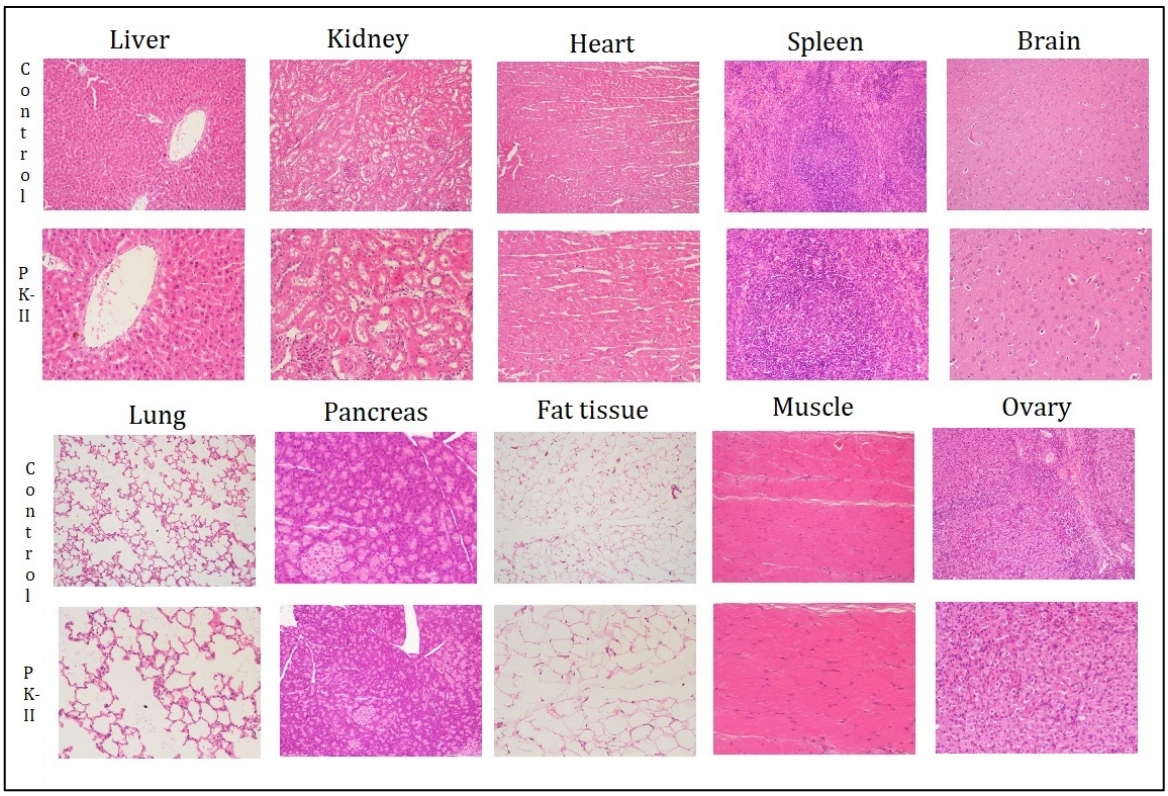


Fig. 4. Histopathology of Vital Organs of female

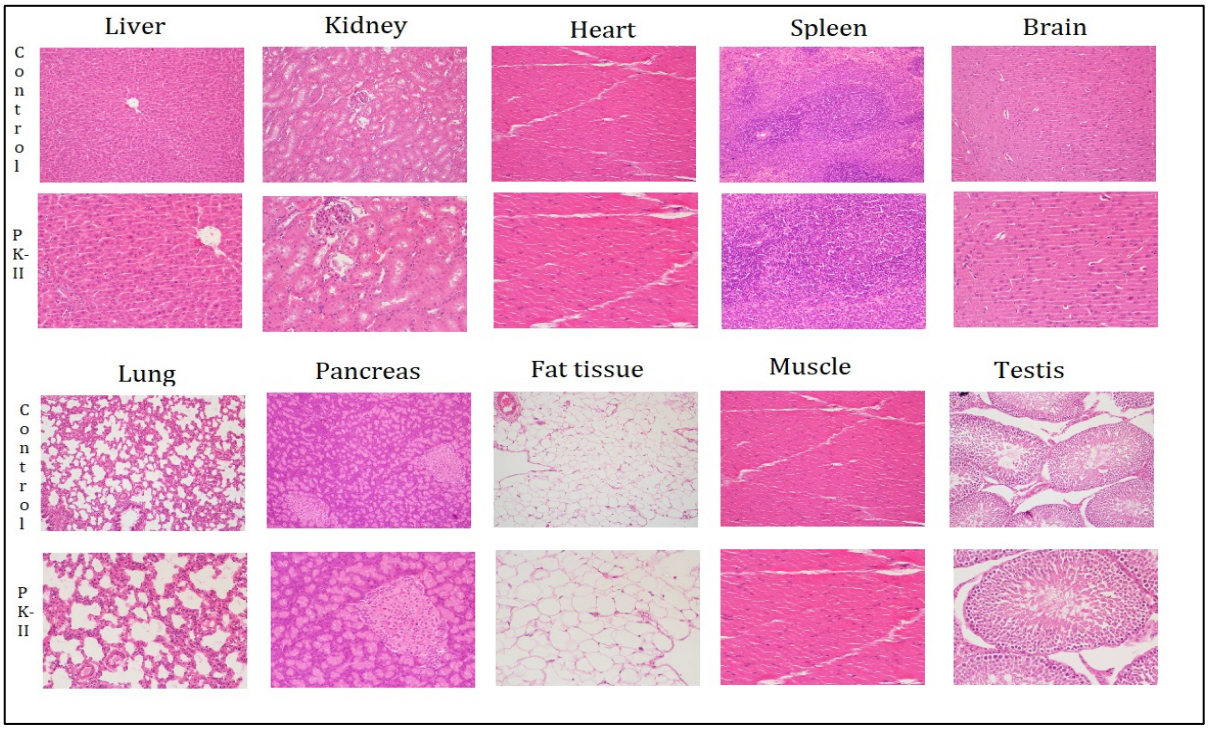


Fig. 5. Histopathology of Vital Organs of Male

Conclusion:

The present study demonstrates that PK-II is safe and well-tolerated in Wistar rats following both acute and sub-acute oral administration. No mortality, behavioral abnormalities, or significant alterations in hematological, biochemical, or histopathological parameters were observed, even at the highest tested dose of 2000 mg/kg. The findings indicate that PK-II possesses a wide safety margin and does not elicit systemic or organ-specific toxicity. These results confirm that PK-II possesses a favorable safety profile, supporting its further development as a safe bioactive compound for therapeutic use.

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