

Repeated Dose 90-Day Oral Toxicity Study of Kushta-e-Faulad: A Traditional Unani Hematinic Agent

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ABSTRACT

Background: Kushta is an ultra-fine powdered product in Unani medicine, created through calcination of animal, metals and mineral substances. Kushta-e-Faulad is a traditional compound Unani formulation to manage Su'al-Qinya (anemia) and Du'f al-Kabid (liver Dysfunction). Despite its traditional use, its safety profile has not yet validated through scientific studies. **Objectives:** The current investigation was aimed to evaluate 90-day oral toxicity of Kushta-e-Faulad, an Unani prescribed formulation commonly employed as hematinic. **Materials and Methods:** Kushta-e-Faulad safety was assessed in rats following OECD-408. The medication was given orally at 03 doses i.e. 6, 30, and 60 mg/kg bw-as an Aqueous suspension in 0.3% CMC. Animals were noticed for changes in body weight, feed consumption, toxicity signs, morbidity and mortality throughout the study. Hematology, clinical chemistry, level of electrolytes, gross pathology, comparative organ weight, and histological investigation were carried out at the conclusion of the study. **Results:** Increase in body weight, feed consumption, and clinical indicators of systemic toxicity did not significantly change throughout treatment with the tested medication. Haematological parameters did not differ significantly from those of the control group. At the end of the research, a gross necropsy showed that neither the drug-treated group nor the control animals had changed. Hematological values were similar to controls, and gross necropsy showed no changes in any group. It was discovered that the relative organ weight of the drug-treated and control groups was similar. When comparing drug-treated animals to those in the control group, no noticeable histological alterations were seen. Control and treatment groups showed similar relative organ weights, with no alterations in histology of various organs. **Conclusion:** The study confirmed that hematological and serum parameters remained unaffected, showing no significant changes in toxicity point of view. No significant difference observed in relative organ weight. Histological findings showed no significant changes. Therefore, based on findings Kushta-e-Faulad can be regarded as safe in rats at doses up to 60 mg/kg body weight.

Keywords: Hematinic, Kushta-e-Faulad, Unani, Traditional.

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INTRODUCTION

The acceptability of herbal formulations continuously expands across the world. Many Unani/Ayurveda products are regularly prescribed in countrywide medical. However, it is a common misconception that products of herbal origin carry no adverse effects (WHO 2004). Unani medicine is recognized for its highly effective therapies; however, these treatments need to be improved and evaluated according to modern regulatory standards for their

scientific validation (AYUSH 2013). Many people consume herbal medicine without professional guidance as a self-medication. Most of the herbal formulations lack scientific data in support of their safety claims. Hence, efforts should be made to elucidate the health benefits and risk assessment of such formulations (Riaz *et al.*, 2010). Kushta is the finely pulverized powder form of the Unani formulations, prepared by the calcinations of metal, mineral and animal drugs. Kushta-e-Faulad (KF) is a traditional unani multi-component formulation indicated for managing Su'al-Qinya (anemia with hypoproteinaemia), Du'f-i-Dimāgh (weakness of brain/ cerebraesthesia), Du'f al-Bāh (loss of libido) (NFUM 2006; NFUM 2008). Although this preparation is commonly utilized, its safety has not been established through research-based studies. Therefore, the investigators designed the study to evaluate 90-day repeated dose oral toxicity study in Sprague Dawley (SD) rats.



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MATERIALS AND METHODS

Experimental animals

SD rats of either sex (5-6 weeks) were sourced from the National Institute of Nutrition (NIN), Hyderabad, India. The designated female had not been pregnant and never give birth before. The polycarbonate cages were used for housing the animals for quarantine for 1 week prior to initiate the study. Animals were accommodated in polycarbonate cages for a 1-week quarantine prior to the start of the study. Animals were kept in a room maintained at 22°C±3°C, 30-70% relative humidity, under a 12:12-hr light/dark cycle. The study initiated after getting approval from Institutional Animals Ethics Committee vide protocol no. No. CRIUM/IAEC/2015/02/P01. Throughout the experiment, all recommended guidelines for laboratory animal care were strictly followed.

The standard diet for feeding purpose was acquired procured from National Institute of Nutrition, Hyderabad.

Drug / Formulation

A fresh aqueous suspension of KF in 0.3% CMC (<2 mL/100 g bw) was prepared daily and administered orally at a volume of up to 2 mL/100 g bw. Control animals received only vehicle. To minimize variability, KF was given at the same time each day.

Dose Selection

Standard therapeutic dosage of KF according to Unani medicine is 60 mg/day. The animal dose equivalent to therapeutic dose for 60 mg human dose is 06 mg/kg bw/day. Hence, three dose levels were taken for the present study, viz. 06 (X), 30 (5X) and 60 (10X) mg/kg bw/day.

Drug administration

KF was prepared as 0.3% CMC aqueous suspension using mortar and pestle. Vehicle was administered orally via stainless steel gavage. Considering KF's therapeutic usage, investigation was carried over a period of 90 days.

Experimental design

The OECD test guideline-408 was followed in conducting the 90-day repeated dosage toxicity investigation (OECD 408). All the chosen *Sprague Dawley* rats, including male and female, were putted in 04 groups. Each group have been assigned 20 animals (10 males and 10 females):

Vehicle control treated with CMC water:

- KF 06 mg/kg bw/day (1X),
- KF 30 mg/kg bw/day (5X),
- KF 60 mg/kg bw/day (10X).

Throughout the experiment, animals were observed twice daily for any ailment or lethality. In addition, detailed evaluations such as clinical and functional parameters were assessed 1 hr after doing of vehicle or KF administration to monitor signs of toxicity. Data of body weights of all were collected each week. Every week the feed intake of animals was determined by measuring amount of feed provided to each cage and deducting the unused quantity the following day. At the end of the study, rats were fasted overnight with free access to water and then blood was collected from retro-orbital plexus for assessment of various hematological and biochemistry parameters by using using isoflurane anesthesia (EZ-Anesthesia-1339).

A fully automated analyzer (Swelab Autocounter-920E0+) was employed for measurement of hematology. While, serum parameters were determined using Erba-EM200. Serum electrolytes were analyzed with automated analyzer (Allcare-AC9801).

After study completion, necropsy was conducted, during which organs and tissues were grossly examined, dissected, and weighed. For histopathology, samples were stored in 10% neutral buffered formalin.

Statistical analysis

Data were reported as Mean±Standard error of mean. Statistical comparison between control and treated groups was performed by using One-Way ANOVA (GraphPad Prism version 5 Software). A *p* value of 0.05 was treated as cutoff for statistical significance.

RESULTS

The animals were monitored at different time time interval, during which neither behavioral nor physiological indicators of toxicity signs were detected. No either dosed animal or untreated animals of control group dies during the study. All animals survived within 90 days of the study in all drug-treated and control groups. The outline changes in body weight gain were predictable in the drug treated group in comparison with controls in both sexes (Figures 1A, 1B). Statistically non-significant alterations in feed consumption were noted in the drug treated group relative to the control group. (Figure 2A, 2B). Hematological assessments revealed no notable deviations from the control group, with all KF-treated groups maintaining values with in normal limits. (Table 1). Although the biochemical estimation showed a notable rise in total bilirubin in males in upper-dose treatment group (* *p*<0.05 vs. control) and ALP in the males treated with low dose (** *p*<0.01 vs. control), the values remained clearly falling within normal range and are unlikely to be of toxicological concern. ALT levels in males showed a significant (***) *p*<0.001 vs. control) upsurge in both lowest and middle-dose groups, as well as in the highest-dose group (* *p*<0.05 vs. control). However, effects on ALT were not dose-dependent and consistent and only observed in male rats and hence may be considered as toxicologically

insignificant in the absence of derangement of other associated liver biomarkers such as AST, ALP and total bilirubin. Total protein (** $p < 0.01$ vs. control) and albumin (* $p < 0.05$ vs. control) levels were significantly elevated in the low and mid-dose male groups. KF treated female group at mid-dose was marginally increase in creatinine (* $p < 0.05$) but remained under normal physiological limits. Further, a significant decrease in BUN in the female group treated with Low KF-dose (* $p < 0.05$ vs. control) and in males of highest dose (** $p < 0.001$ vs. control) was observed. The alteration in lipid profile signifies an increase in the cholesterol level in the highest dose female group (** $p < 0.01$ vs. control) and a lowered value of triglycerides in the low and middle-dose groups of females (** $p < 0.01$ vs. control). These alterations are not dose-dependent and values within the physiological limit. Even though certain alterations in the biochemical profile noticed, but the results are not clinically and toxicologically significant as values maintained within physiological limits and showing no clear dose-related pattern (Table 2). Serum electrolytes, including calcium, potassium and chloride were within the normal range in the control and experiment groups, except for sodium which was lowered in female groups of low and mid-dose (** $p < 0.001$ vs. control, alteration in sodium level in all male groups (** $p < 0.001$ vs. control); reduction of calcium in female groups treated with KF mid and low dose (** $p < 0.001$ vs. control). Decrease in calcium in male group at all tested dose levels (** $p < 0.001$ for low, mid-dose and * $p < 0.05$ for high dose vs. control). Considering the lack of dose-dependent alterations in the electrolyte levels, and the fact that values are within physiological limit, such electrolyte changes may be considered toxicologically insignificant (Table 3). Additionally, non-significant differences were documented in relative organ weight of all KF dose received groups and control group (Figures 3A and 3B).

Histopathology

Histological examinations of the brain, heart, spleen, kidneys, trachea, sternum, bone marrow, pancreas, small intestine, stomach, ovaries/uterus and testes of KF high dose group (60 mg/kg bw) and control group revealed no changes of toxicological significance. Whereas, liver and lungs of High dose KF group

showed histologically significant changes. Both control and KF group showed varying grades of chronic interstitial pneumonitis, as the observed changes occurred in both control and KF treated groups, they are regarded as non-significant. The representative histological images of different organs of KF (60 mg/kg bw) and control animals are shown in Figure 4. Majority of animals in both control and high dose KF group revealed normal architecture. Microvacuolation of high grade was observed in 20% of cases in the liver of the KF (60 mg/kg bw) group.

KF group receiving dose of (30 mg/kg), 35% of animals showed normal hepatic histologic architecture. 45% of animals showed mild microvacuolation (5-33%) while moderate (33-66%) and severe (>66%) microvacuolation was seen in 10% of animals of mid-dose KF group.

Microvacuolation of varying degrees noticed in the livers of mid-and high-dose KF-treated groups may be associated with administration of KF.

DISCUSSION

Su'al-Qinya (anemia with hypoproteinaemia), Du'f-i-Dimāgh (weakness of brain/ cerebraesthesia), Du'f al-Bāh (loss of libido), and Du'f al-Kabid (hepatic insufficiency) can all be treated with Kushta-e-Faulad (KF), a multi-component Unani pharmacopoeial preparation. Despite the fact that impulsive and unmonitored use of various herbal formulations may result in hepatotoxicity, renal damage, reproductive toxicity, and carcinogenesis on long-term usage, herbal formulations are commonly acknowledged in the medical system. Safety data must be produced and publicized for available herbal formulations without jeopardizing patient safety, according to the widely accepted notion of "Evidence Based Medicine (Moreira *et al.*, 2014). Patient safety is the main goal of therapeutic intervention. Therefore, a ninety-day oral toxicity study in SD rats was used in the current investigation to assess the safety of KF.

The current study's findings offer comprehensive details on KF's safety profile following ninety days of repeated oral dosing. In toxicological research, rodent's growth is often assessed by

Table 1: Effect of KF on Haematological Parameters in Rats.

Sex		Female (n=10)				Male (n=10)			
Dose (mg/kg bw/day)		Control (0.3% CMC)	KF (06 mg/kg bw)	KF (30 mg/kg bw)	KF (60 mg/kg bw)	Control (0.3%CMC)	KF (06 mg/kg bw)	KF (30 mg/kg bw)	KF (60 mg/kg bw)
Hb	gm%	16.80±0.277	17.09±0.244	16.95±0.247	16.96±0.329	17.06±0.382	16.93±0.225	17.25±0.155	17.54±0.196
RBC	Million/mm ³	8.28±0.108	8.47±0.061	8.50±0.085	8.31±0.086	8.99±0.156	8.72±0.044	8.84±0.067	8.84±0.128
HCT	%	44.22±0.396	45.13±0.415	45.71±0.388	44.19±0.510	45.46±0.655	45.00±0.406	45.45±0.32	46.37±0.517
WBC	/ mm ³	6360±270.9	7900±493.5	7840±481.2	7150±438.7	7780±309.0	9100±603.5	7620±554.3	8550±511.0
PLT	Lakhs/mm ³	4.34±0.224	4.94±0.130	4.52±0.308	4.12±0.144	4.52±0.193	5.12±0.144	4.46±0.200	4.59±0.145

(Values presented as Mean±SEM; n=10/ sex; One-Way ANOVA)

Table 2: Effect of KF on Blood Biochemical Parameters in Rats.

Sex		Female (n=10)				Male (n=10)			
		Control (0.3% CMC)	KF (06 mg/kg bw)	KF (30 mg/kg bw)	KF (60 mg/kg bw)	Control (0.3%CMC)	KF (06 mg/kg bw)	KF (30 mg/kg bw)	KF (60 mg/kg bw)
AST	IU/L	139.2±6.037	134.4±5.167	135.7±7.699	135.2±8.000	144.2±6.005	111.3±7.216	130.4±14.93	142.5±4.334
ALT	IU/L	74.60±6.447	70.40±3.284	84.60±6.917	90.60±8.856	72.70±3.556	143.9±7.427***	139.3±2.868***	95.50±4.559*
Bilirubin Total	mg/dL	0.15±0.0076	0.14±0.012	0.15±0.0067	0.19±0.013	0.15±0.010	0.15±0.0070	0.16±0.0064	0.18±0.0059*
Alkaline Phosphatase	IU/L	82.40±2.993	94.80±3.797	86.30±3.239	86.70±5.447	110.2±9.926	164.4±14.88**	141.3±10.46	124.2±5.601
Total Protein	g/dL	6.97±0.0633	7.08±0.0854	7.09±0.0277	7.08±0.0629	6.90±0.044	7.15±0.076**	7.15±0.040**	6.91±0.031
Albumin	g/dL	4.090±0.067	4.210±0.054	4.270±0.049	4.260±0.056	3.92±0.090	4.20±0.044*	4.18±0.051*	4.00±0.039
Blood Urea Nitrogen	mg/dL	21.05±0.667	17.70±0.866*	20.51±0.922	20.85±0.907	21.51±0.605	18.80±0.613	20.24±0.944	16.71±0.746***
Creatinine	mg/dL	0.90±0.021	0.95±0.016	0.99±0.017**	0.90±0.009	0.90±0.021	0.89±0.017	0.87±0.015	0.90±0.014
Glucose	mg/dL	127.6±2.414	125.9±7.369	132.8±4.786	105.5±8.632	134.9±3.689	111.3±7.216	130.4±14.93	111.4±2.459
Cholesterol	mg/dL	106.0±5.746	122.7±3.211	124.1±6.366	134.6±3.494**	99.90±4.806	93.90±4.954	98.30±3.235	101.4±5.034
Triglycerides	mg/dL	80.20±5.356	54.60±2.876**	55.70±3.612**	69.91±6.019	64.10±3.271	73.80±5.118	77.70±6.429	68.30±3.276

(Values presented as Mean±SEM; n=10/ sex; one-way ANOVA; *=*p*<0.05, **=*p*<0.01, ***=*p*<0.001 vs. control)

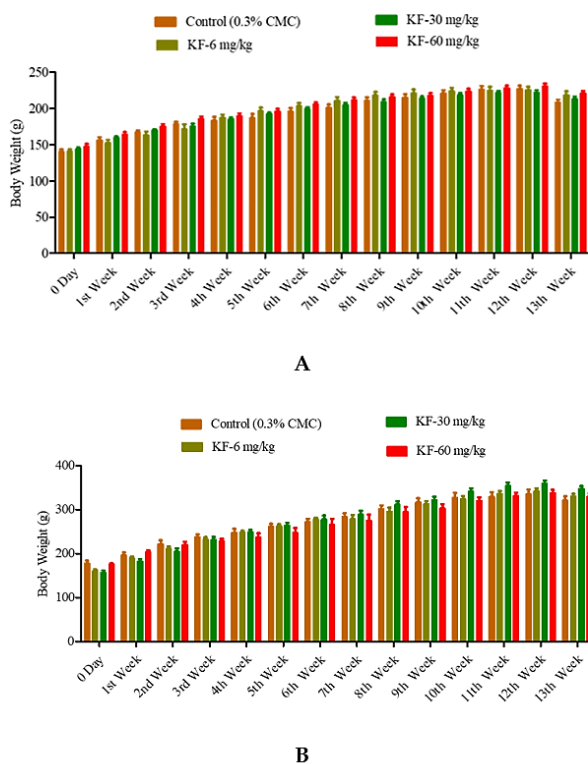


Figure 1: A: Effect of KF on Body Weight in Female Rats. B: Effect of KF on Body Weight in Male Rats.

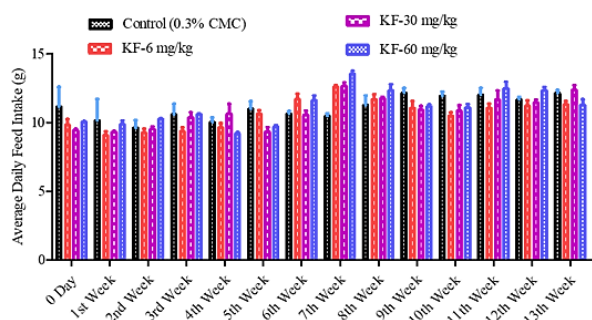
gathering information on body weight and feed intake (Hoffman *et al.*, 2002). Body weight gain and feed consumption did not significantly change as a result of treatment in this study. Over the duration of 90-day trial period, neither the control nor treatment groups had any deaths, nor there does no notable drug-related intoxication effect on the animals' behavior or clinical observations.

The main tissue hierarchical system is the hematopoietic system, in which adult cells with a finite lifespan are replaced by new ones. It is among the most vulnerable target organs for animal toxicity testing. (Bailey *et al.*, 2004). The data collected upon completion of trial showed no clinically significant observations with reference to HCT, PLT, RBC, Hb and WBC Count and in

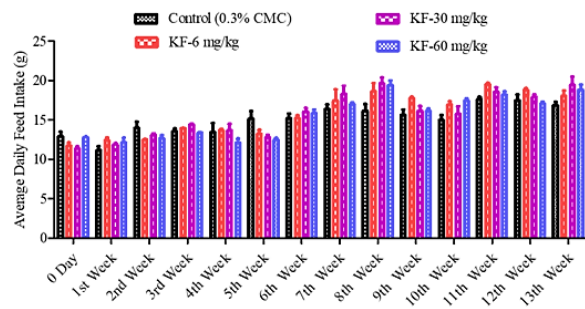
Table 3: Effect of KF on Serum Electrolytes in Rats.

Sex		Female (n=10)				Male (n=10)			
Dose (mg/kg bw/day)		Control (0.3% CMC)	KF (06 mg/kg bw)	KF (30 mg/kg bw)	KF (60 mg/kg bw)	Control (0.3% CMC)	KF (06 mg/kg bw)	KF (30 mg/kg bw)	KF (60 mg/kg bw)
Sodium	mmol/L	140.1±0.604	134.1±1.329***	133.1±0.912***	137.2±0.671	137.7±0.44	141.1±0.314***	142.2±0.33***	132.9±0.98***
Potassium	mmol/L	4.3±0.090	4.08±0.081	4.02±0.092	4.30±0.140	4.75±0.096	4.67±0.071	4.51±0.070	4.66±0.092
Chloride	mmol/L	104.6±0.884	102.7±0.300	103.5±0.428	103.6±0.859	100.1±0.849	103.4±0.426	104.1±0.504*	101.2±1.788
Calcium	mmol/L	3.24±0.168	2.23±0.066***	2.230±0.049***	3.18±0.085	3.640±0.219	2.300±0.066***	2.220±0.062***	2.960±0.200*

(Values presented as Mean±SEM; n=10/ sex; one-way ANOVA; * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ vs. control)



A



B

Figure 2: A: Effect of KF on Feed Intake in Female Rats. B: Effect of KF on Feed Intake in Male Rats.

treatment groups as compared to control, which indicates that KF does not have any toxic effects on the hematopoietic system.

There are several organs in biological systems which release certain enzymes after injury, like the elevation of ALT and AST levels in hepatic injury or during hepatocellular necrosis. Liver dysfunction is also associated with elevation of total bilirubin content (Testa *et al.*, 1989). In the current research notable significant changes were seen in liver enzymes, but the values stayed within the normal physiological limit and effects were not noticed in a dose-dependent manner in both sexes. Therefore, they may be considered toxicologically non-significant. Serum creatinine and urea are the primary biomarkers for kidney damage and a rise in levels of both is generally associated with renal impairment (Testa *et al.*, 1989). The values of creatinine in KF-treated groups (except in mid-dose females; though value remained within normal physiological limits) were comparable to

the control. The BUN was consistently lowered in KF-treatment group upon comparison to the control group. Hence, KF may be considered nontoxic to renal functions. Electrolytes were found comparable in normal and KF-treated rats. There were some changes in sodium and calcium. However, considering the lack of dose-dependent alterations, such electrolyte changes may be considered toxicologically insignificant. The investigation of organ weight in preclinical studies is an important end point for the determination of the potential toxic effects of treatment (Alimba *et al.*, 2012). The relative organ weights did not differ significantly between the treatment group and the control group.

Both humans and animals that are exposed to different harmful chemicals have higher amounts of oxidants and oxidative DNA damage, which results in cellular damage and tissue necrosis. Histopathological analysis aids in assessing any organ damage at the cellular level brought on by a chemical's harmful effects

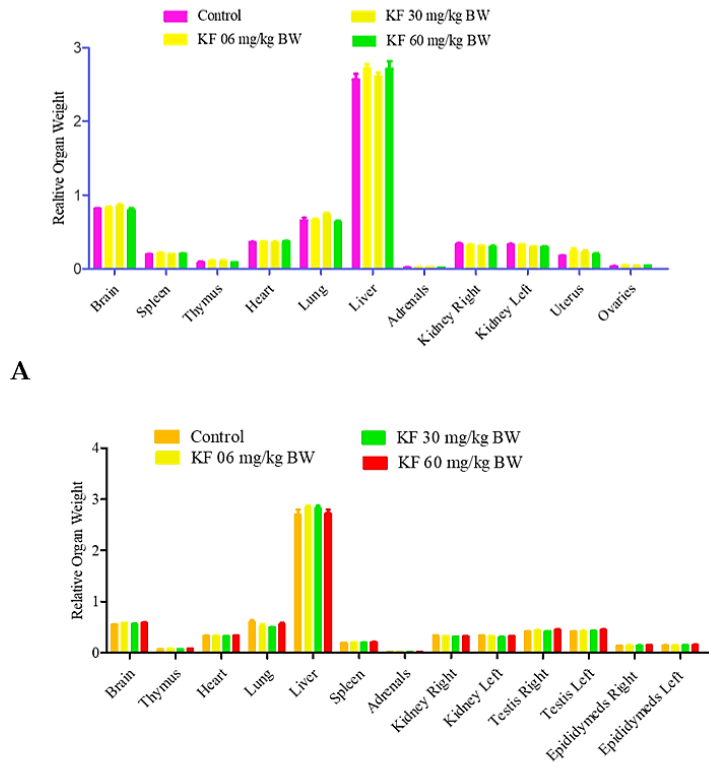


Figure 3: A. Effect of KF on Relative Organ Weight in Female Rats. B. Effect of KF on Relative Organ Weight in Male Rats.

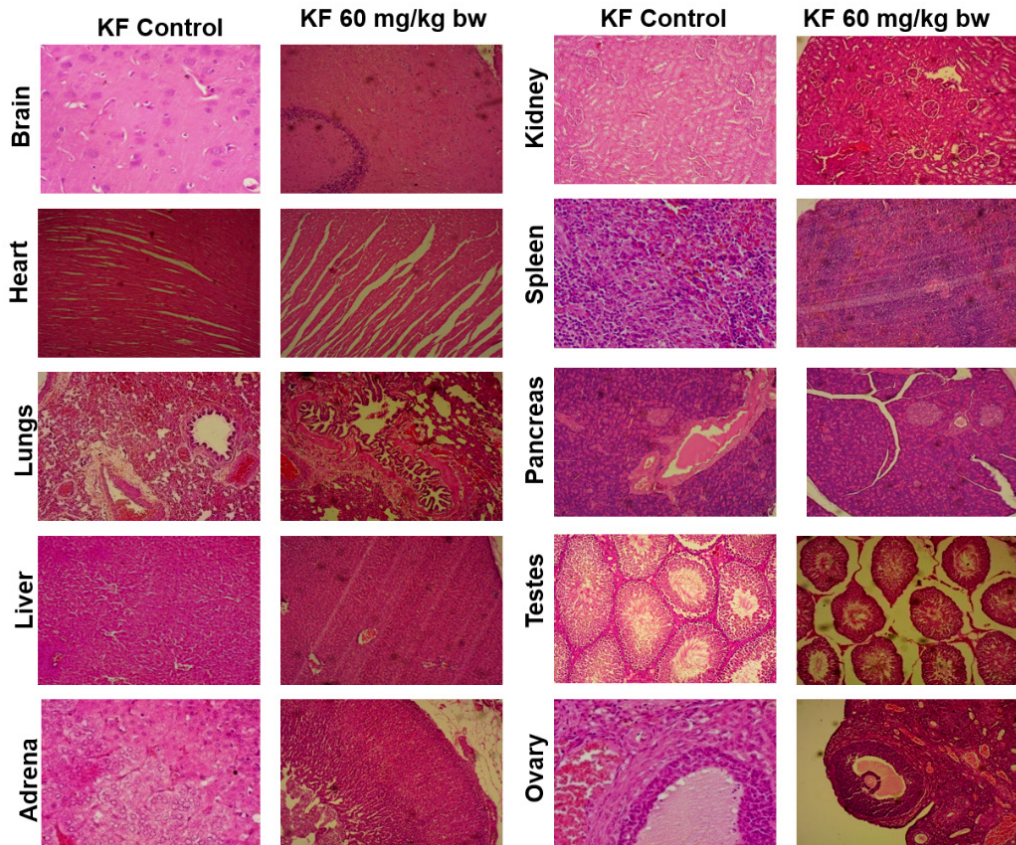


Figure 4: Histopathological sections of Control vs KF treated (60 mg/kg bw) rats.

(Auti *et al.*, 2019). Our study's findings imply that plasma influx into the cytoplasm caused vacuoles to develop in the liver. Hepatic sinusoidal congestion was linked to this alteration. As a result, the mid- and high-dose KF group's livers showed micro microvacuolation of different grades, which could be related to the medication used. However, despite notable differences from control animals, biochemical changes in ALP and AST levels did not corroborate histological findings since the changes in values happened within the normal physiological range.

CONCLUSION

Rats were used in a repeated dosage oral toxicity study for the duration of ninety-day administration of the polyherbal Unani formulation Kushta-e-Faulad (KF). Up to the tested dose level, no group experienced any fatality or morbidity. The KF-treated rats' body weight and feed intake increase which was appeared equivalent to those of the control group. Biochemical parameters showed some dose-independent changes, although the values stayed within the typical physiological range. They could therefore be regarded as toxicologically unimportant. Contrasting to the control group, the hematological results were normal. Organ weight (Relative) did not fluctuate significantly between the treatment group and the control group. The mid- and high-dose KF-treated group's histological results revealed varying degrees of micro-ovulation, which could be related to KF treatment. Thus, up to the highest tested dose level of 60 mg/kg bw in rats, KF may be deemed safe based on the results.

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ABBREVIATIONS

CMC: Carboxymethyl cellulose; **KF:** Kushta-e-Faulad; **OECD:** Organisation for Economic Co-operation and Development; **bw:** body weight; **SD:** *Sprague Dawley*; **NIN:** National Institute of Nutrition; **CPCSEA:** Committee for the purpose of control and supervision of experiments on animals; **Hb:** Hemoglobin; **RBC:** Red Blood Cells; **WBC:** White Blood Cells; **PLT:** Platelets; **HCT:** Hematocrit; **AST:** Aspartate Aminotransferase; **ALT:** Alanine Aminotransferase; **ALP:** Alkaline Phosphatase; **BUN:** Blood Urea Nitrogen; **SEM:** Standard Error of Mean; **ANOVA:** Analysis of Variance.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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AUTHORS CONTRIBUTIONS

Husain GM involved in constructing an idea or hypothesis for research and supervise the project. Husain GM also reviewed the article before submission not only for spelling and grammar but also for its intellectual content. Urooj M design the methodology to reach the conclusion and supervise the course of the project or the article and taking the responsibility. Urooj M also performed the statistical analysis and presented the results. Kodavath D involved in taking care of animal health and provide the technical support for histopathological analysis. Ahmad T provide the support for hematological and biochemical analysis of rat blood and serum samples. Munshi YI reviewed the article before submission.

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