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Research Article

Toxicity Studies on *Kushta Khabasul Hadeed* (calcined iron rust) prepared by Classical and Muffle Furnace Methods

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Abstract

Background: Though a number of studies has been carried out in terms of safety of *Kushta* but concerns have been raised about the presence of heavy metals in Unani formulations. Classical method of preparation of *Kushta Khabasul Hadeed* (KKH) and other *Kushta* preparation in Unani system of medicine has been found to eliminate all such issues from the end product. However, there is a need to provide scientific evidence to establish the impact of the procedure, hence; the present study was designed to evaluate the acute and sub-acute toxicity study of KKH prepared by Classical and Muffle furnace methods.

Objective: The investigators in this study made an attempt to ascertain the safety profile of *Kushta Khabasul Hadeed* (KKH) prepared by classical and muffle furnace methods.

Materials and methods: Both of the samples of KKH i.e. *Kushta Khabasul Hadeed* (KKHCM) and *Kushta Khabasul Hadeed* (KKHMF) prepared by Classical and Muffle Furnace Methods respectively were evaluated for acute and sub-acute toxicity by OECD guidelines 423 and 407 respectively. The starting dose in the acute test was 50 mg/kg and in sub-acute test, it was 134mg, 200 mg and 400 mg/kg b.w. Effect of both of the test samples were assessed for ponderal changes, food and water intake, relative organs weights, hematological, biochemical and histopathological features of various organs.

Results: Both of the samples did not produce any sign of toxicity in acute toxicity study up to the dose level of 2000mg/kg b.w. In sub-acute toxicity study no mortality in any of the test groups was observed. KKHCM at different doses showed a slight decrease in food intake in all the test groups but significant difference ($P < 0.05$) was observed in the test group B as compared to plain control. However, no alteration in food intake was noted in Satellite group. Repeated dose administration of KKHMF at different dose levels lead to a significant decrease in food intake in the test group A ($P < 0.01$) While in the test groups B, C and satellite, the decrease was not significant in comparison to plain control.

Conclusion: KKHMF was found to be relatively toxic than KKHCM at different dose levels. The study demonstrated that both the Classical and Muffle furnace methods of preparation of *Kushta* are useful for preparing KKH and also safe at the dose prescribed in Unani literature. However, the *Kushta* prepared by the classical method was found to be safer than the muffle furnace method.

Keywords: Classical and Muffle furnace methods, Toxicity, *Kushta*, Mortality, Metals and minerals

1. INTRODUCTION

The traditional system of medicine including Unani system advocates therapeutic uses of mineral and metallic preparations in many diseases since century in clinical practices. Minerals have formed as an important part of the Egyptian and Mesopotamian *Materia medica* and acted as an active component in many effective remedies¹. The medicinal uses of metallic and mineral origin drugs, methods of their purification and pharmacological properties have been mentioned by many eminent scholars in their books^{2,3}.

Knowing the possibility of toxic effects, Unani physicians emphasized on a set of exclusive pharmaceutical procedures such as *Tadbire-Adviah* (purification and/or detoxification) and *Taklees* (incineration and/or calcination) that convert the metals and minerals into *Kushta* (calx or calcined powder)^{2,3}. They do not use these preparations directly in a raw form but convert either into their carbonates or oxides by the process of roasting (*Ihraq*) or calcination (*Taklees*) so as to make them compatible and the product so obtained is known as *Mukallas* or *Kushta* (calcined)². *Kushta* is known as Calx in English, *Bhasma* in Ayurveda and *Parpam* in Siddha⁴. *Kushtan* in Persian means "to kill". *Kushta(s)* are prepared to

make the drug easy to use, remove the harmful and disadvantageous materials from it, minimize the dose and increase the potency of the drug ². *Kushta(s)* are unique preparations/dosage forms which are safely being practiced in Unani system of medicine and other traditional system of medicine (Ayurveda and Siddha system of medicine) without any noticeable side effects that can be considered as evidence to their safety but no objective confirmable data exist to support such claims. Preclinical studies of Unani and Ayurveda drugs provide scientific basis for their traditional use and to prove that they are safe and efficacious ⁵.

Kushta Khabasul Hadeed (KKH) (Calcined iron rust) is one of the Unani herbo-metallic preparations and used in the treatment of *Faqr-ud-Dam* (anemia), *Sayalan al-Raham* (leucorrhoea), *Istihaza* (menorrhagoa), *Sue-Hazm* (dyspepsia), *Zeeqan nafs* (asthma), *Niqris* (gout) and *Yarqan asfar* (jaundice) ⁶⁻⁹.

Iron based drugs are widely used in modern medicine as haematinics. These drugs are known to induce adverse drug reactions including gastro intestinal symptoms such as nausea, vomiting, epigastric pain, eructation, pyrosis, borborygami, colic pain, flatulence, constipation, black faeces and diarrhoea ¹⁰. Though a wide therapeutic utility of KKH has been mentioned in Unani literature, it is reported to be harmful if it is not processed or not purified properly as per the classical methods ⁶. Many eminent Unani scholars have also written in their books that KKH is *Muzir* (toxic) to the lungs ^{2, 3}. Previously conducted clinical studies reported about the safety of *Khabasul Hadeed* powder and *Majoon Khabasul Hadeed* ^{11, 12}. Though a number of studies have been carried out regarding the safety of *Kushta(s)*, concerns have been raised about the presence of heavy metals in Unani formulations ¹³. Classical method of preparation of KKH and other *Kushta* preparation in Unani system of medicine is found to eliminate all such issues from the final product. However, there is a need to provide scientific basis to establish the impact of this procedure, hence, the present study was designed to evaluate the acute and sub-acute toxicity study of KKH prepared by classical and Muffle furnace methods tuned to temperature variations as that in KKH in Wistar rats according to OECD Guideline 423 and 407 for comparing the preparations by these two methods.

2. MATERIALS AND METHODS

2.1. Drugs and chemicals

The study was carried out in National Institute of Unani Medicine (NIUM); Bengaluru and Indian Institute of Science (IISc); Bengaluru. *Khabasul Hadeed* was procured from apothecary shop in Bengaluru and *Haleela*, *Baleela*, *Amla* and *Kakronda booti* were procured from the pharmacy of NIUM, Bengaluru. All the drugs except *Khabasul Hadeed* were identified and authenticated by the experts of the Institute of Trans-Disciplinary Health Sciences and Technology (FRLHT); Bengaluru and assigned the authentication number viz. *Terminalia chebula* Retz. (*Haleela*), 5087 *Terminalia bellerica* (Gaertn.) Roxb (*Baleela*), 5088 *Phyllanthus emblica* L. (*Amla*), 5089 *Aloe vera* (L.) Burm. f. (*Elva*) 5090 and *Blumea balsamifera* (Linnaeus) Candolle (*Kakronda booti*) 5091. All the samples of the test drug were submitted to NIUM museum/Herbarium with voucher specimen no. 59/IA/Res/2019 for future reference. *Khabasul Hadeed* (iron rust) was identified by the Regional Ore Dressing Laboratory, Bengaluru, the Indian Bureau of Mines (IBM), Ministry of Mines, Government of India (No.K-23011/4/Chem /2018-19/Analys /Bng/OD, Lab Ref. no.CB-52). All bio-chemical and hematological reagents used in

the study were of analytical grade.

2.2. Method of detoxification of *Khabasul Hadeed*

Khabasul Hadeed was subjected to purification/detoxification procedure. *Khabasul Hadeed* powder was soaked overnight with vinegar (*Sirka desi*) and in the morning the vinegar was decanted and the remaining *Khabasul Hadeed* was used in the preparation of *Kushta Khabasul Hadeed* by both the methods i.e. classical and Muffle furnace ⁶.

2.3. Preparation of *Kushta Khabasul Hadeed*

2.3.1. Classical method

Detoxified *Khabasul Hadeed* was ground/ grinded by an electric grinder with the juice of *Triphala* (*Emblca officinalis*, *Terminalia bellerica* and *Terminalia chebula*) (250gm) as mentioned in the National Formulary of Unani medicine for about 12 hours. Thereafter, small cake (pellets) of same size and thickness (10-12 gm) was made. These cakes were dried well in the shade and put in earthen bowl and then covered by the process known as *Gil-e-Hikmat*, (which is nothing but application of specific semi solid materials all around and specially the junction point of the brim of bowels to make it air proof) thereafter the whole preparation was dried and named as *Boota* (earthen bowl). After this, a pit was dug in an open space which can accommodate 10 kg of cow-dung cakes (approximately 1.5 feet in length, 1.5 feet in width and 1.5 feet in depth), the *Boota* was placed in a pit and ignited with 10 kg of cow-dung cakes at the place secured from wind. The temperature was noted every five minutes with the help of pyrometer. After putting off the fire when *Boota* was cooled, it was removed from the pit, opened; all the ignited drugs were removed and again ground/grinded. Then the same process was repeated three times (teen *put*) to complete the preparation of *Kushta*. But in each *put* different herbs (*booti*) were used viz. in second *put* *Kakronda booti* and third *put* *Elva booti*. The temperature was recorded at every five minutes in each *put* and heat pattern was noted during the incineration process with the help of thermocouple, its sensor was placed near *Boota* in the pit. After completion of the process, *Boota* was cooled and removed from pit, opened and *Kushta Khabasul Hadeed* was powdered, sieved and preserved in an air tight glass container.

2.3.2. Muffle furnace method

The thermogram was prepared by recording temperature in classical method and utilized in this method. For the development of thermogram, an assembled pyrometer was used to record temperature at every five minutes till the *Kushta* is formed and cooled down. The temperature recorded in the classical method at various time intervals utilized by the thermogram was used for the gradual increase and decrease of temperature. The temperature pattern of thermogram was maintained for Muffle furnace when the *Kushta* was prepared in it. For this purpose, three samples were used to prepare *Kushta* in classical manner and temperatures were recorded after every five minutes from the start of the ignition till it cooled to normal temperature. The mean maximum temperature and duration for heat were calculated for the values of these samples which served as template for temperature variation used in Muffle furnace. Accordingly, the *Kushta* was prepared.

2.4. Animals

Wistar strain rats of both the sex, weighing 150-200g were used. The animals were maintained under ideal husbandry conditions viz. temperature (23±2°C),

relative humidity (50-60%) and exposed to 12-hour light and dark cycles. All the animals were exposed to the same environmental conditions and maintained on standard diet and drinking water *ad libitum*. The experimental protocol was approved by the IAEC, No: IAEC/6/14/IA/01 as per the guidelines of CPCSEA, India.

2.5. Dose fixation

The dose of the test drug samples was calculated by multiplying the human therapeutic dose as described in Unani literature; by conversion factor of seven¹⁴. The therapeutic dose of *Kushta Khabasul Hadeed* for human is 125 mg, mentioned in Unani literature. The dose i.e. 125 mg when converted by conversion factor was found to be 15mg/kg, since at this dose the drug is supposed to be not toxic; a starting dose of 50 mg/kg was selected for the acute toxicity study. Both the samples of *Kushta Khabasul Hadeed* were administered in the form of suspension in 1% of Carboxyl Methyl Cellulose (CMC) through gastric cannula.

2.6. Acute toxicity study

Young, healthy, nulliparous and non pregnant Wistar strain female rats were selected and acclimatized for seven days before the experiment. Suspension of both the test drugs i.e. KKHCM and KKHMFM were administered orally at starting dose of 50mg/kg body weight to overnight fasted female rats by following Organization for Economic Cooperation and Development (OECD) 423 guidelines¹⁵. The rats were observed closely for behavioral changes, signs and symptoms of toxicity and mortality for the first six hours and thereafter periodically up to 14 days. At this dose no sign of toxicity was observed, therefore, the next dose of 300 mg/kg was given to the additional three animals and further increased the dose to 2000 mg/kg. Observation was done as described in OECD 423 guidelines.

2.7. Sub acute toxicity study

Animals were divided into five groups namely; Plain control, Test group A, Test group B, Test group C and Satellite group, each consisting of 10 rats comprising five male and five female. The animals of each group were treated in the following manner¹⁶.

Experimental design

Control group:	1 ml distilled water/Wistar rat
Test group A: (Minimum dose)	1/15 th of maximum dose of acute toxicity study (134mg /kg b.w)
Test group B: (Intermediate dose)	1/10 th of maximum dose of acute toxicity study (200mg /kg b.w)
Test group C: (High dose)	1/5 th of maximum dose of acute toxicity study (400mg /kg b.w)
Satellite Test group: (High dose, 28 days + 14 days post- treatment)	1/5 th of maximum dose of acute toxicity study (400mg /kg b.w)

The suspensions of the test drugs were administered orally every day for 28 days¹⁷. Initial body weights of all the animals were recorded. General behavioral pattern was observed once a week by exposing each animal to open arena. On the 29th day, all the animals of each group except satellite group (43th day) were kept on fasting and in the morning overnight fasted rats were weighed again and anesthetized with theopentone sodium (50mg/kg b.w. ip). Blood was collected by cardiac puncture for hematological and bio-chemical analysis. Then, the rats were sacrificed; abdomen was opened through midline incision to record

the autopsy changes followed by dissecting out the important organs.

Hematological analysis was performed by using Horiba an automatic hematological analyzer (New Delhi Pvt. Ltd., India). ABX VET Pack reagent was used for hematological analysis. White blood cell (WBC), red blood cell (RBC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and platelets (PLT) count were measured from the blood samples. Bio-chemical parameters were carried out by using a fully automated STAR 20 Clinical Chemistry Analyzer (Rapid diagnostic Pvt. Ltd. Delhi). The studied parameters included serum glucose by GOD-POD, End point method, total cholesterol by Urease/GLDH method, urea by CHOD-POD, End point method, creatinine by Creatinine fixed time method, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) by Modified IFCC Kinetic method, albumin by BCG method, total protein by Biuret method, alkaline phosphatase by Kinetic UV test-optimized IFCC method, total bilirubin by TBIL-End point method and Sodium and Potassium by Colorimetric method.

All the important internal organs were carefully dissected namely; liver, kidney, heart, lungs, stomach, spleen, intestine, testis and prostate. After noting the signs of gross lesion and ponderal changes in liver, lungs and kidney, the organs were transferred to 10% formalin solution for preservation and then sent for histopathological study.

2.8. Statistical analysis

Data are presented as mean \pm SEM for ten rats in each experimental group. One-way analysis of variance (ANOVA) was used to compare the mean values of quantitative variables among the groups followed by Turkey-Kramer multiple comparison Test for unpaired data by using Graph Pad InStat [DATASET1.1SD] software to determine significant difference between groups at $P < 0.05$.

3. RESULTS

3.1. Acute toxicity study

The results of the acute toxicity study showed that both the samples of *Kushta Khabasul Hadeed* (KKHCM and KKHMFM) did not show any effect on behavioral and other parameters during the experiment period of 14 days. Also, both the samples of *Kushta* did not show any sign of toxicity and mortality when given orally up to 2000 mg/kg body weight.

3.2. Sub- acute toxicity study

After the sub-acute administration of KKHCM and KKHMFM at different dose levels, the body weight (From 0 to day-28th day) was decreased but not significant in all the test groups but in satellite group, the body weights were maintained as in plain control (Table 1).

KKHCM at different doses showed a slight decrease in food intake in all the test groups but significant difference ($P < 0.05$) was observed in the test group B in comparison to plain control. However, no alteration in food intake was noted in Satellite group (Table 2). The water intake was found increased significantly in the test group C ($P < 0.001$) and also increased in the test group B but not significant in comparison to plain control. On the other hand, slight decrease in water intake in the test groups A and satellite was observed in comparison to plain control (Table 2).

Repeated dose administration of KKHMF M at different dose levels lead to a significant decrease in food intake in the test group A ($P<0.01$) while in the test groups B, C and satellite, the decrease was not significant in comparison to plain control (Table 2). Water intake increased significantly in satellite group ($P<0.01$) while in the test group B, it was not significant in comparison to plain control. In test groups A and C, the water intake was found reduced but not significant in comparison to plain control (Table 2). No significant difference was observed in relative weight of liver, kidney, lungs, heart, stomach and spleen in all the test groups in comparison to plain control group (Table 3).

Effect of samples of KKHCM on hematological parameters was evaluated and all the treated groups were compared with plain control. No significant decrease in WBC, RBC and Hb in test groups B, C and satellite was observed and in the test group A, an increase was observed but non-significant. No significant decrease in HCT, MCH and MCHC values were observed in the test groups A, B, C and satellite. No significant increase in MCH value was noted in all the treated groups except the test group B in comparison to plain control. No significant difference was observed in platelets count in any of the test groups (Table 4). KKHMF M showed a significant reduction ($p<0.05$) in WBC in the test group A, and no significant decrease in the test group C and satellite group, except test group B, in comparison to plain control group was observed. Administration of the test drug at different dose levels lead to a decrease in RBC count and Hb concentration but non-significant in all the treated groups. A significant decrease in HCT value in the test groups A ($P<0.01$), B and C ($P<0.05$) was observed but in satellite group just decrease was observed but non significant. MCV value decreased in all the test groups but not significant, on the other hand, an increased MCH value was observed in all the treated groups but non-significant. Increased in MCHC value in the test groups A ($P<0.01$), C ($P<0.05$) and satellite ($P<0.05$) except test group B was observed in comparison to plain control. PLT count was found decreased in all the test groups but not significant (Table 4).

Among the 12 serum bio-chemical parameters, after treatment with test drug (KKHCM) the values of blood sugar, cholesterol, urea, creatinine, SGOT, SGPT, ALP and albumin were found altered and most of the parameters showed a slight increase in their values in comparison to plain control, however, no significant difference was noted. The total protein and bilirubin showed a significant increase in the test groups A and B ($p<0.05$) and test groups C and Satellite also showed a little increase in their values but not significant. After treatment the serum sodium was found significantly increased in the test groups B and Satellite ($P<0.01$) and highly significant increase in test group C ($P<0.001$) was observed in comparison to plain control. Serum potassium was decreased but not significant in test groups A, C and satellite while it was decreased significantly in the test group B ($P<0.05$) in comparison to the plain control (Table 5).

After treatment with test drug (KKHMF M), the values of glucose, total cholesterol, SGOT, albumin, alkaline phosphatase, sodium and potassium were found altered in comparison to plain control but not significant. Significant increase in serum urea level was observed in the test groups A ($P<0.01$), and B ($P<0.05$), and test groups C and satellite also showed an increase in urea level but statistically not significant. Creatinine was found significantly increased in the test groups B ($P<0.001$), C ($P<0.01$), and satellite ($P<0.01$) and in the test group A it was increased but non-significant in comparison to plain control group.

Repeated dose of KKHMF M at different dose levels lead to increased serum SGOT in the test groups A, C and satellite but not in the test group B in comparison to plain control. Slight increase in SGPT in all the test groups and significant increase in the test group A ($P<0.001$) was observed. After treatment the total protein was found to be significantly increased in the test groups A ($P<0.001$), B ($P<0.001$), C ($P<0.05$) and satellite ($P<0.05$) in comparison to plain control. After treatment the total bilirubin was found to be significantly increased in the test group B ($P<0.05$) and satellite group ($P<0.05$) while in the test groups A and C, it was increased but statistically not significant (Table 5).

Table 1: Effect of KKHCM and KKHMF M on body weight

Groups	Weight gain (From 0 th day-28 th day) (Mean \pm SEM)	
	KKHCM	KKHMF M
Plain control (1ml D.W/Rat)	60 \pm 13.36	60 \pm 13.36
Test group A (134mg/kg b.w)	46.8 \pm 10.46	40.4 \pm 9.56
Test group B (200mg/kg b.w)	47.4 \pm 11.74	44.4 \pm 8.57
Test group C (400mg/kg b.w)	41.2 \pm 11.41	45.4 \pm 8.94
Satellite group (400 mg/kg b.w)	65.1 \pm 11.75	65 \pm 12.51

N=10 in each group, Test used: ANOVA test followed by Turkey-Kramer multiple comparison test.

Table 2: Effect of KKHCM and KKHMF M on Food and Water intake of animals

Groups	Food intake /Day/Rat (Mean \pm SEM)		Water intake /Day/Rat (Mean \pm SEM)	
	KKHCM	KKHMF M	KKHCM	KKHMF M
Plain control (1ml D.W/Rat)	14.06 \pm 0.15	14.06 \pm 0.1	23.76 \pm 0.52	23.76 \pm 0.52
Test group A (134 mg/kg b.w)	13.48 \pm 0.1814	12.93 \pm 0.21**	22.38 \pm 0.56	22.54 \pm 0.58
Test group B (200 mg/kg b.w)	13.28 \pm 0.14*	13.63 \pm 0.26	24.51 \pm 0.42	24.76 \pm 0.45
Test group C (400 mg/kg b.w)	13.75 \pm 0.20	13.26 \pm 0.22	27.49 \pm 0.68***	23.56 \pm 0.36
Satellite (400 mg/kg b.w)	14.59 \pm 0.21	13.65 \pm 0.16	21.82 \pm 0.59	26.09 \pm 0.47**

N=10 in each group, Test used: ANOVA test followed by Turkey-Kramer multiple comparison test, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, . Plain control vs Test groups

Table 3: Effect of KKHCM and KKHMF on relative organs weight

Groups	Relative Organ Weight in Wistar Rats (Mean ±SEM)											
	Liver (g/100g)		Kidney (g/00g)		Lungs (g/100g)		Heart (g/100g)		Stomach (g/100g)		Spleen (g/100g)	
	KKHCM	KKHMF	KKHCM	KKHMF	KKHCM	KKHMF	KKHCM	KKHMF	KKHCM	KKHMF	KKHCM	KKHMF
Plain control (1ml D.W/Rat)	4.01±0.13	4.01±0.13	0.38±0.01	0.38±0.01	1.07±0.05	1.07±0.05	0.36±0.01	0.36±0.01	0.66±0.04	0.66±0.04	0.40±0.03	0.40±0.03
Test group A (134mg/kg b.w)	4.05±0.08	3.92±0.17	0.39±0.02	0.36±0.02	1.02±0.06	0.96±0.04	0.39±0.02	0.35±0.01	0.74±0.06	0.58±0.02	0.42±0.04	0.30±0.03
Test group B (200 mg/kg b.w)	4.24 ± 0.22	3.94±0.10	0.43±0.03	0.37±0.01	1.08±0.08	0.97±0.06	0.35±0.01	0.35±0.01	0.58±0.03	0.56±0.02	0.35±0.01	0.41±0.04
Test group C (400 mg/kg b.w)	3.59±0.13	4.04±0.09	0.39±0.01	0.40±0.02	0.97±0.10	1.12±0.10	0.36±0.02	0.38±0.01	0.53±0.03	0.63±0.03	0.30±0.02	0.38±0.02
Satellite group (400 mg/kg b.w)	3.75±0.12	3.66±0.08	0.36±0.01	0.41±0.04	0.93±0.06	0.85±0.08	0.36±0.01	0.40±0.06	0.58±0.03	0.54±0.04	0.34±0.04	0.29±0.02

N=10 in each group, Test used: ANOVA test followed by Turkey-Kramer multiple comparison test, * Plain control vs Test groups

Table 4: Effect of KKHCM and KKHMF on Haematological Parameters

Haematological Parameter	Plain control (1ml D.W / Rat)	Test group A (134 mg/kg b.w)		Test group B (200 mg/kg b.w)		Test group C (400 mg/kg b.w)		Satellite group (400 mg/kg b.w)	
		KKHCM	KKHMF	KKHCM	KKHMF	KKHCM	KKHMF	KKHCM	KKHMF
WBC (10 ³ /mm ³)	12.96±0.82	14.74±1.30	6.64±1.668*	11.5±1.78	14.72±1.440	9.76±1.43	9.4±1.840	9.65±1.26	11.58±1.584
RBC (10 ⁶ /mm ³)	6.49±0.09	7.05±0.22	6.06±0.33	6.36±0.38	5.38±0.44	5.79±0.46	5.48±0.47	6.36±0.26	5.79±0.26
Hb (g/dl)	14.06±0.34	14.37±0.66	13.65±0.80	11.71±0.72	11.52±0.74	12.48±0.89	12.07±0.92	14±0.47	12.71±0.63
HCT (%)	39.61±0.91	38.66±1.18	27.58±3.57**	34.93±2.42	29.14±2.15*	32.4±2.59	27.95±3.00*	34.66±1.29	32.22±1.59
MCV (µm ³)	55.8±0.83	55±0.60	54.6±0.87	54.5±0.91	55.3±0.79	53.8±2.37	55.55±0.5031	55.6±1.07	55.3±0.4726
MCH (pg)	21.46±0.68	21.53±1.31	22.62±0.77	18.63±0.83	22.11±1.00	21.87±0.44	22.15±0.73	22.11±0.45	21.95±0.18
MCHC (g/dl)	34.05±1.04	39.26±2.56	41.5±1.96**	34.26±1.50	39.9±1.41	42.59±3.74	40.03±1.35*	38.76±0.63	39.69±0.26*
PLT (10 ³ /mm ³)	505.1±30.42	590.1±38.05	368.9±59.46	502.7±49.60	425.7±58.34	478±63.25	388.22±50.37	516.4±45.60	420.7±57.22

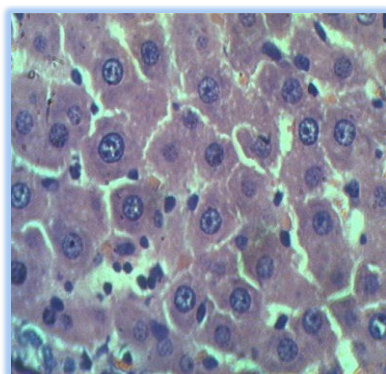
Data presented as mean± SEM, N=10 in each group, Test used: ANOVA test followed by Turkey-Kramer multiple comparison test, * P<0.05, ** P<0.01, * Plain control vs Test groups

Table 5: Effect of KKHCM and KKHMF on biochemical parameters

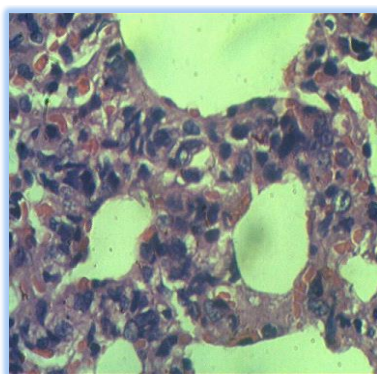
Biochemical Parameter	Plain control (1ml D.W/Rat)	Test group A (134mg/kg b.w)		Test group B (200 mg/kg b.w)		Test group C (400 mg/kg b.w)		Satellite group (400 mg/kg b.w)	
		KKHCM	KKHMF	KKHCM	KKHMF	KKHCM	KKHMF	KKHCM	KKHMF
Glucose (mg/dl)	122.74±7.59	114.31±13.66	94.64±15.05	154.43±12.15	122.42±11.69	154.02±21.33	107.27±7.74	168.80±25.42	100.39±17.99
T.Cholesterol(mg/dl)	107.26±21.49	132.29±3.14	194.83±14.31	136.29±3.09	103.38±18.73	137.41±4.65	138.05±29.60	120.14±2.26	101.89±25.53
Urea (mg/dl)	40.07±3.47	41.78±3.08	69.46±8.61**	34.99±4.67	66.86±4.60*	39.19±3.22	60.89±4.27	38.24±3.95	55.34±5.77
Creatinine (mg/dl)	1.30±0.15	2.35±0.17	2.02±0.27	1.88±0.24	2.75±0.21***	1.73±0.23	2.42±0.23**	3.65±1.26	2.46±0.08**
SGOT (IU/L)	113.95±8.13	112.131±6.77	122.76±7.11	140.63±14.04	83.05±12.37	87.33±16.54	123.67±12.96	109.03±10.24	123.07±15.15
SGPT (IU/L)	39.04±6.53	38.70±3.68	58.812±6.51***	50.41±3.10	45.26±2.62	44.78±2.15	51.35±3.50	56.45±7.38	55.23±5.75
Albumin (g/dl)	3.08±0.31	3.079±0.24	3.64±0.25	4.08±0.26	3.31±0.20	3.77±0.25	3.48±0.16	2.62±0.29	3.58±0.06
T. Protein (g/dl)	3.96±0.27	5.639±0.70*	6.23±0.30**	5.78±0.26*	6.51±0.37***	5.35±0.30	5.56±0.36*	4.70±0.35	5.38±0.34*
ALP (IU/L)	132.2±14.13	116.2±17.05	194.61±27.44	149.8±7.271	148.8±17.20	116±11.38	205.22±24.37	119.4±14.93	170±20.92
T. Bilirubin (mg/dl)	1.58±0.11	2.645±0.20*	2.21±0.191	2.562±0.3846*	2.28±0.182*	1.56±0.16	1.95±0.2002	2.06±0.21	2.29±0.15*
Sodium (mEq/L)	24.19±10.18	26.70±5.70	19.45±3.39	56.477±4.784**	51.01±9.86	68.10±3.60***	15.603±1.76	56.7±2.94**	31.54±10.13
Potassium (mEq/L)	7.45±0.65	4.81±0.93		4.075±0.5630*	7.34±0.79	5.31±0.55	6.72±0.63	7.21±0.72	6.57±0.76

Data presented as mean± SEM, N=10 in each group, Test used: ANOVA test followed by Turkey-Kramer multiple comparison test, * P<0.05, ** P<0.01, *** P<0.001, - Plain control vs Test groups

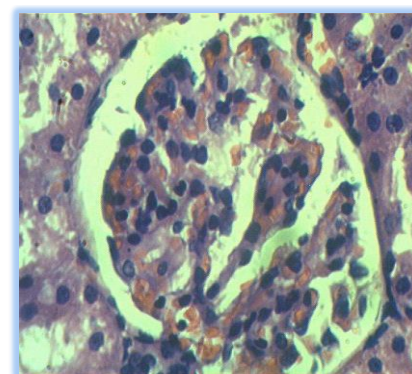
Plain control (Showing histopathology of liver, lungs and Kidney)



A



B



C

Figure 1: Plain control, A (Liver) showing normal Central vein, bile ducts were intact and functional in portal triad, B (Lung) normal bronchial lining epithelial cells with goblet cell activity and C (Kidney) showing normal Kidney reveal uniformly spread nephrons

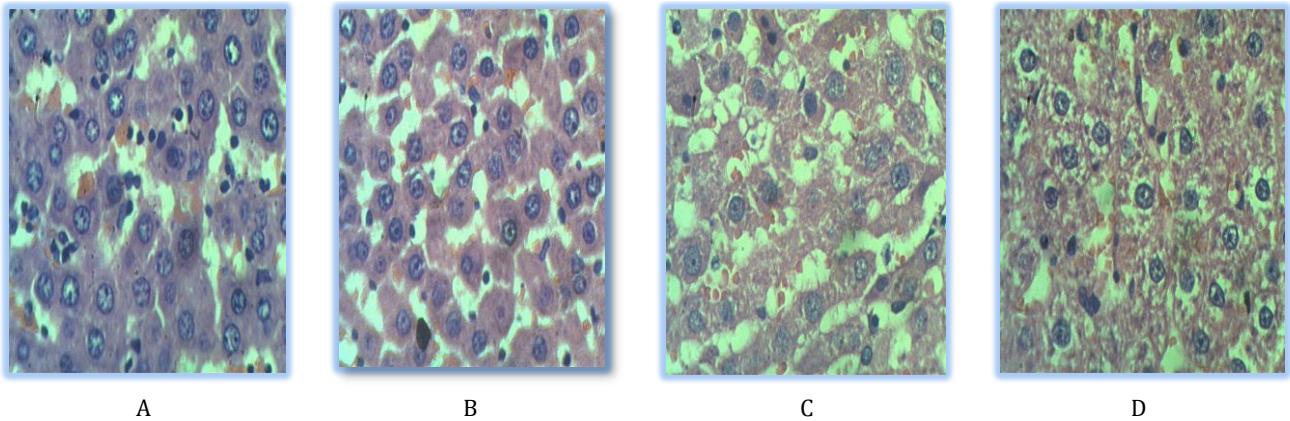
Effect of KKHCM on liver

Figure 2: **A:** (Test group A) showing Mild degenerative, circulatory and infiltrative changes, **B** (Test group B) showing mild degeneration noticed along with aggregation of mononuclear cells. **C** (Test group C) showing liver is found to be intact and normal and **D** (Satellite group) showing liver, structurally destroyed and the intactness of hepatocytes are bizarre.

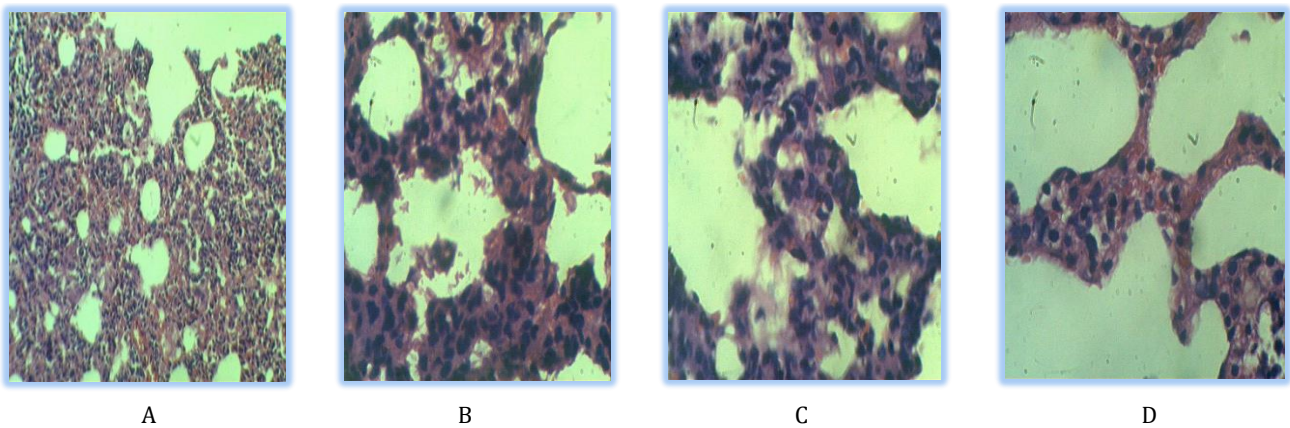
Effect of KKHCM on lungs

Figure 3: **A:** (Test group A) showing parenchyma thickened walls of alveoli, with cellular infiltrations, **B** (Test group B) showing mild degenerative noticed along with aggregation of mononuclear cells, **C** (Test group C) showing lung parenchyma pneumonia, and **D** (Satellite group) showing pneumonia

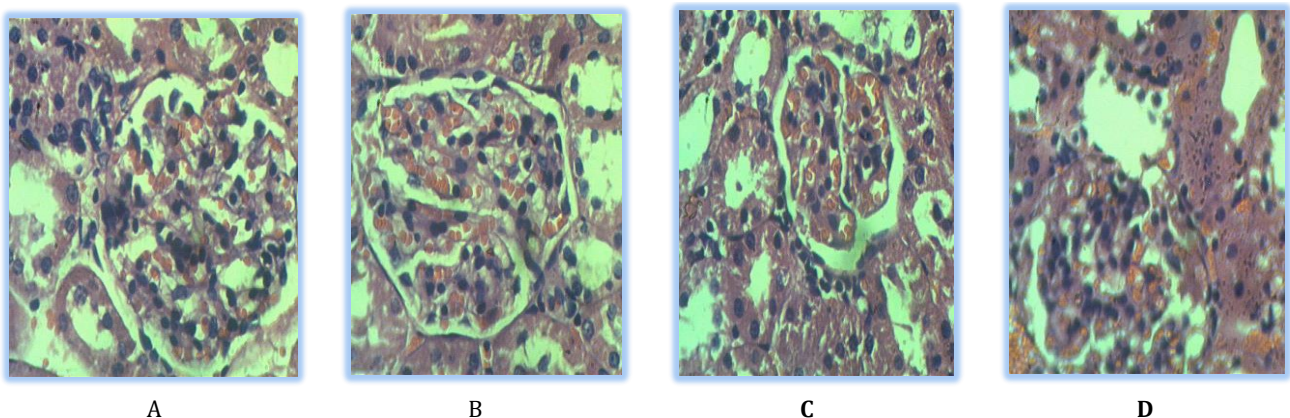
Effect of KKHCM on Kidney

Figure 4: **A:** (Test group A) showing Mild degenerative, circulatory and infiltrative changes, **B** (Test group B) showing mild degenerative noticed along with aggregation of mononuclear cells, **C** (Test group C) showing kidney revealing a moderate degenerative cytoplasm, and **D** (Satellite group) showing a moderate degenerative cytoplasm with mild congestion across the cortex

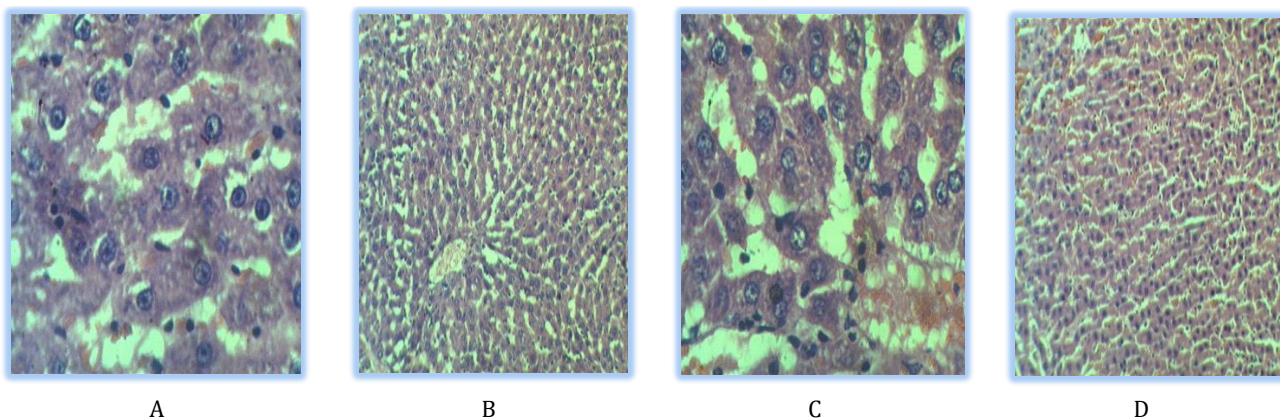
Effect of KKHMF on liver

Figure 5: **A:** (Test group A) showing liver are found to be intact, **B** (Test group B) showing liver are found to be intact, with few areas of hepatosis, **C** (Test group C) showing liver are found to be intact, with few areas of hepatosis I, and **D** (Satellite group) showing few areas of hepatosis and congestion

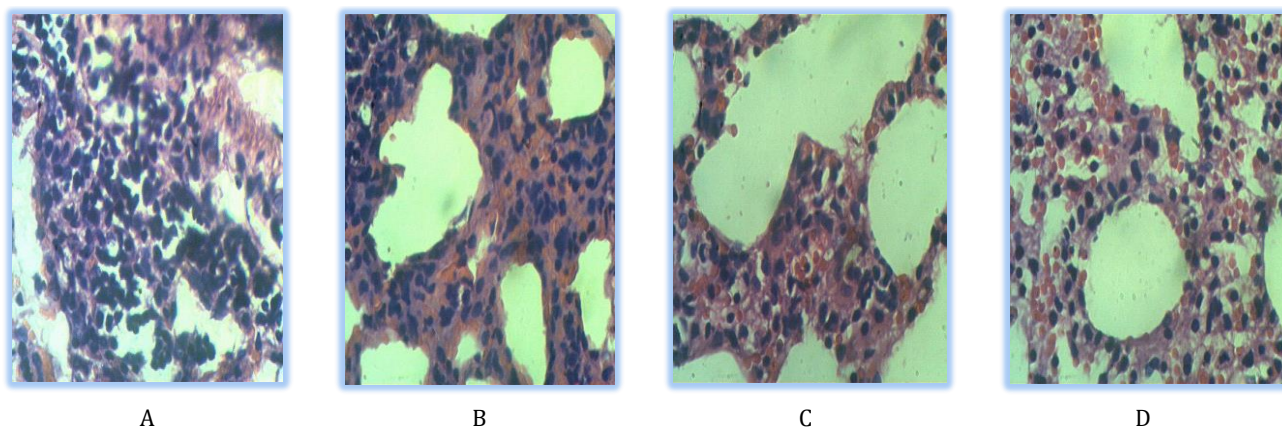
Effect of KKHMF o lungs

Figure 6: **A:** (Test group A) showing pneumonia, **B** (Test group B) showing pneumonia, **C** (Test group C) showing lung parenchyma pneumonia, and **D** (Satellite group) showing pneumonia

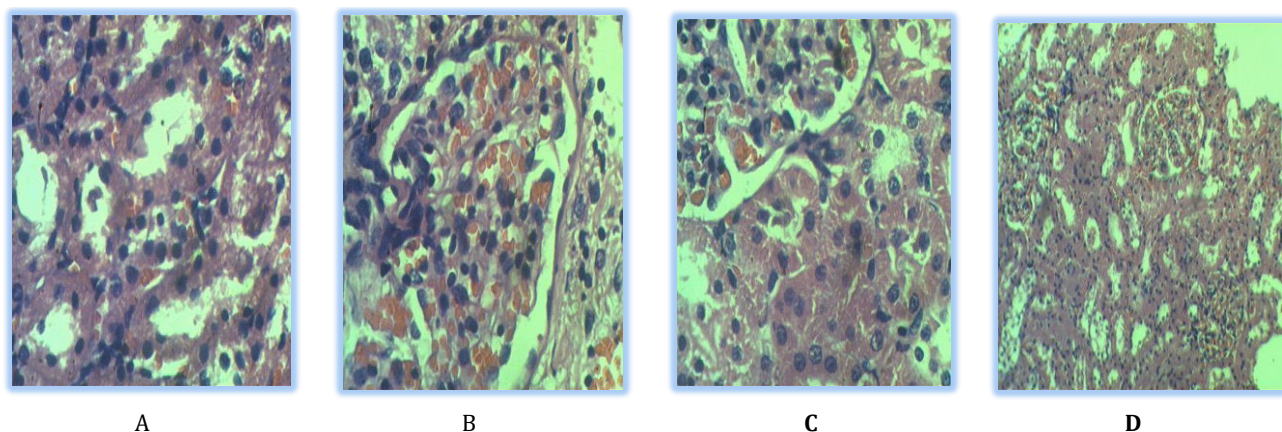
Effect of KKHMF on Kidney

Figure 7: **A:** (Test group A) showing moderate degenerative lesions and congestion, **B** (Test group B) Slide showing kidney, a moderate degenerative lesions and congestion, **C** (Test group C) showing moderate degenerative lesions severe congestion across parenchyma, and **D** (Satellite group) showing moderate degenerative lesions and severe congestion across parenchyma

4. DISCUSSION

Metals and minerals are extensively used in Unani formulations since time immemorial and became an important part of Unani therapeutics. To make them suitable for therapeutic use, Unani physicians emphasized on a set of exclusive classical pharmaceutical procedures.

In this study, both the samples of KKH (viz. KKHCM and KKHMF) did not exhibit any observable toxic effects during the entire duration of acute toxicity study at the dose level upto 2000 mg/kg and all female rats survived 14 days of observation which suggest that LD₅₀ value may be higher than 2000 mg/kg body weight by oral route. As per UN classification, any substance which has oral LD₅₀ or

more than 2000 mg/kg body weight is considered as low hazard potential and categorized as UN 6.1 PG III^{18,19}. Thus, as per the above criterion, both KKHCM and KKHMFM can be categorized as substances with low health hazard potential (Class 4 of GHS and UN 6.1 PG III)²⁰.

After multiple dose administration of KKHCM and KKHMFM at different dose levels, the animals were observed for different parameters. Body weight is an important indicator of the state of appetite and food intake. The toxic drugs disturb the metabolism of protein fat and carbohydrate and subsequently decrease the body weight. In the present study, both the samples of the test formulation did not show any significant alteration in the body weight (From 0 to day-28th day) in all the test groups in comparison to plain control which clearly indicate that test formulations did not disturb the metabolism (Table 1). However, food intake was found significantly decreased with KKHCM in the test group B ($P < 0.05$) and with KKHMFM ($P < 0.01$) in the test group A (Table 2). After treatment with KKHCM, the water intake was found significantly increased in the test group C ($P < 0.001$) and with KKHMFM in satellite group ($P < 0.01$) (Table 2). Normal food and water consumption of animals support the gain in the body weight of animals throughout the study period. Similar findings were also observed in this study as no significant reduction in the body weight was noted in any group of the animals²¹. Moreover no significant difference was observed in relative weight of any organ (viz. liver, kidney, lungs, heart, spleen and stomach) in KKHCM and KKHMFM treated groups which further support the safety of the test compounds (Table 3).

KKHCM after 28 days of administration showed no significant toxic effect on hematological parameters except slight reduction in Hb percentage in the test groups B and C. Other hematological parameters were also found not altered significantly in comparison to plain control (Table 4). On the other hand, KKHMFM showed some alteration in parameters such as WBC count reduced in the test group A ($P < 0.05$), slight reduction in Hb percentage in all the treated groups, significant decrease in HCT in the test groups A ($P < 0.01$), B and C ($P < 0.05$) and MCHC increased in all the treated groups significantly except in the test group B (Table 4).

KKHCM showed significant alteration in bio-chemical parameters. For example, total protein and bilirubin increased significantly in the test groups A and B ($P < 0.05$), sodium increased significantly in the test groups B, Satellite ($P < 0.01$) and C ($P < 0.001$) and a significant reduction in serum potassium level was observed in the test group B ($P < 0.05$) (Table 5). In case of KKHMFM treated animals, the level of urea increased significantly in the test groups A ($P < 0.01$) and B ($P < 0.05$). Urea contributes most of the body's non protein nitrogen. It is the major end product of protein catabolism synthesized in liver, released in blood and excreted by the kidneys. This is the chief indicator of renal and hepatic integrity. Elevated serum urea level may be due to pre renal, renal or post-renal etiology²². Creatinine was also found to be significantly increased in the test groups B ($P < 0.001$), C and satellite ($P < 0.01$). Serum creatinine is a product of creatine and phosphocreatine which are the important components of muscle. Creatinine is freely filtered and therefore the serum creatinine level depends on the glomerular filtration rate. Renal dysfunction diminishes the ability to filter creatinine and raises its serum level. The only condition that causes a significant increase in serum creatinine level is damage to a large number of nephrons²². Serum glutamic pyruvic transaminase (SGPT) is a liver enzyme that aids in

producing proteins. Besides liver, it is also found in other organs such as heart, muscle, brain and kidney. Injury to any of these tissues can cause an elevated blood level. It also helps in detecting hepatocellular necrosis but is considered as a less specific biomarker enzyme for hepatocellular injury as it can also signify abnormalities in heart, muscle, brain or kidney. A significant increases in SGPT in the test group A ($P < 0.001$) indicates the toxic effect of KKHMFM.

The estimation of total proteins in the body is helpful in differentiating between normal and altered liver functions. This is because the majority of the plasma proteins such as albumins and globulins are produced in the liver. Low total protein level is suggestive of kidney disorder or disorder where protein is not absorbed or digested properly. The total protein was found to be increased significantly in the test groups A, B, C and satellite when compared to plain control²² and the total bilirubin was found to be increased significantly in the test groups B and satellite in comparison to plain control when the repeated dose of KKHMFM was administered. (Table 5) The elevation of both the parameters (liver and kidney) is due to the toxic effect of KKHMFM.

After 28 days of treatment with KKHCM, the histological findings of liver revealed some degree of degenerative, circulatory and infiltrative changes in all the groups. Lungs histology showed a parenchyma with thickened walls of alveoli and cellular infiltrations, and mild degenerative change was noticed along with aggregation of mononuclear cells in the test groups A and B, and Test groups C and Satellite showed pneumonic lesion. Kidney showed Mild degenerative, circulatory and infiltrative changes in all the groups. On the other hand KKHMFM showed less toxic effect on liver as only negligible alteration in the histopathological features of liver was observed. However, relatively greater toxic effect was observed in lungs as pneumonic lesions were observed in all the groups. Kidney showed moderate degenerative lesions and congestion in all the groups.

It is important to mention here that after treatment with KKHMFM, all the treated groups showed pneumonic lesions of lungs parenchyma. Unani physicians have already written clearly in their classical books that *Kushta Khabasul Hadeed* is *Muzir* (Toxic) to lungs. The findings of this study confirm the claims made by the Unani physicians. After analysis of hematological and bio-chemical parameters, it was observed that KKHMFM has showed more toxic effect on liver and kidney in comparison to KKHCM. This finding is in agreement with Szalay *et al* (2012) [Fig.3, 6]²³.

Metals, while being made into *bhasma*, get chelated with organic molecules (ligands) present in the herbs which lead to better assimilation. Chelation therapies have been used to bring down the levels of toxic metals in patients. During pharmaceutical processing of *Lauha bhasma*, right from *sodhana* till *amrtikarana*, *Triphala* is used as an organic media to convert metal Iron (*Lauha*) into a herbomineral complex. *Triphala* mainly consists of tannins, gallic acid, ascorbic acid (Vitamin C) and phenolics. Ascorbic acid increases the bioavailability of iron by converting ferric to ferrous ion while phenolics can reduce the iron by binding to it. The presence of ascorbic acid or a lack of dietary tannins has been suggested to be contributing to clinical/pathological iron storage disease. Excess iron causes iron overloading in the body and can damage the liver, heart and pancreas and irritate the stomach and gut, causing constipation and diarrhoea. In other words, the various constituents of *Triphala* have antagonizing activity and thereby too much iron absorption is prevented²². Similar procedure has been adopted in this study. In the light of the

above explanation, the findings of the present study are justified.

There are various factors which probably may be responsible for the mild toxic effect of *Kushta*. For instance Unani physicians always advise to use *Kushta* at least after six months of its preparation so that its *Mizaj* can be stabilized. In this study the stabilization period was not maintained, it may be one of the reasons for its toxic effect. Secondly, *Kushta* is always used with some of vehicles such as butter, ghee, milk, honey etc. In this study, the CMC was used as a vehicle to suspend the required dose of *Kushta* in order to administer the whole dose of *Kushta* conveniently.

It was found that the classical method is superior to muffle furnace method in *Kushta* preparation as the particle size was found to be very small (14.12 nm) than those prepared by muffle furnace method (23.11 nm). SEM study also showed that the particles of hematite are agglomerated due to controlled and uniform heat in the muffle furnace method but in classical method the heat was not controlled and uniform as it was done in open space²⁴.

On the basis of the above findings, it can be concluded that good quality of *Kushta* can be prepared by classical method as more fine particles were observed in X-ray diffraction analysis. Shifting the process of preparing *Kushta* from classical method to technologically convenient Muffle furnace method needs more improvement in respect of monitoring the temperature gradients over the time.

5. CONCLUSION

The study has clearly demonstrated that both the methods of preparation of *Kushta* are useful for preparing KKH and safe at the prescribed dose as mentioned in Unani literature. However, the classical method has an advantage over the muffle furnace method. While administering orally both KKHCM and KKHMF up to 2000 mg/kg body weight did not produce any toxic effects. KKHCM at different doses showed a slight decrease in food intake in all the test groups and repeated dose administration of KKHMF at different dose levels lead to a significant decrease in food intake in the test group A.

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Conflict of interest

None

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