Pharmacogn. Res., 2024; 16(1):98-109.
https://www.phcogres.com

Original Article

Quality Control and Acute Toxicity Study of *Habb-i-Hayat* (Unani Formulation) in Albino Wistar Rats

Ifra Qayoom[1], Athar Parvez Ansari[2,*], Ansari Huzaifa[3], Mohd Musaib Bhat[4], Bazilah Majeed Reshi[5], Pankaj Goswami[6], Noman Anwar[2]

[1]Department of Unani Pharmacy, Kashmir Tibbia College, Hospital and Research Center, University of Kashmir, Srinagar, Jammu and Kashmir, INDIA.
[2]Department of Clinical and Pharmacy Research, Regional Research Institute of Unani Medicine, (NABH & NABL Accredited), Central Council for Research in Unani Medicine, Ministry of Ayush, Govt. of INDIA, Chennai, Tamil Nadu, INDIA.
[3]Department of Unani Pharmacology, Mohammadia Tibbiya College and Assayer Hospital, Maharashtra University of Health Sciences, Nashik, Maharashtra, INDIA.
[4]ICMR INDIAB, Department of Clinical Research, Sher-i-Kashmir Institute of Medical Sciences, Srinagar, Jammu and Kashmir, INDIA.
[5]Department of Unani Pharmacology, Regional Research Institute of Unani Medicine, (NABH Accredited), Central Council for Research in Unani Medicine, Ministry of Ayush, Govt. of India, Srinagar, Jammu and Kashmir, INDIA.
[6]Division of Veterinary Pathology, Sher-e-Kashmir University of Agriculture Sciences and Technology Jammu, R. S. Pura, Jammu, INDIA.

ABSTRACT

Background: Several reports acknowledge that herbal products produce toxicities due to their poor quality. Hence, the worldwide promotion of traditional medicines is not at par due to a lack of quality control and safety aspects. *Habb-i-Hayat*, a Unani formulation, is used for the treatment of various bodily ailments. **Aim:** The present study was aimed at evaluating the quality control and acute toxicity of *Habb-i-Hayat*. **Materials and Methods:** The standardization and quality control study of *Habb-i-Hayat*, including organoleptic characters, physicochemical properties, qualitative phytochemical evaluation, microbial load, aflatoxins and heavy metals analysis, and HPTLC, were carried out. The acute toxicity study of the same was carried out at three dose levels following OECD guidelines 423. **Results:** The quality control findings of *Habb-i-Hayat*, including by weight variation, hardness, friability, moisture content, ash values, swelling index, etc., were found within the normal limits. The TBC and TFC were found within the permissible limits. The heavy metal analysis report showed that the heavy metals, including lead, cadmium, arsenic, and mercury, were present within the permissible limits. The number of peaks, R_f values, and area under the curve found in the HPTLC study may be used for identification and quality of *Habb-i-Hayat*. The toxicity data of *Habb-i-Hayat* revealed that it produces some serious toxic control effects including death at higher doses. The LD_{50} of *Habb-i-Hayat* was found to be 2500 mg/kg b. w. for rats. **Conclusion:** The standardization and quality control reports of *Habb-i-Hayat* have suggested that the preparation, drying, and storage of *Habb-i-Hayat* followed standard operating procedures. Moreover, the findings of standardization and quality control of *Habb-i-Hayat* may be referred in future. The acute toxicity profile of *Habb-i-Hayat* has confirmed that this important Unani formulation may be safely used at therapeutic dose levels.

Keywords: *Habb-i-Hayat*, Unani formulation, Standardization, Quality control, Acute toxicity.

Correspondence:
Dr. Athar Parvez Ansari
Department of Clinical and Pharmacy Research, Regional Research Institute of Unani Medicine, No. 1, West Madha Church Road, Royapuram, Chennai, Tamil Nadu, INDIA.
Email: aatharparvez@gmail.com
ORCID Id: 0000-0002-5755-3525

Received: 21-07-2023;
Revised: 14-08-2023;
Accepted: 20-09-2023.

INTRODUCTION

In the past few decades, the global interest in herbal drugs has increased[1] due to their great value in medicine, nutraceuticals, and cosmetic industries.[2] It has been estimated that around 80% world's population depends on herbal medicine for their health-related problems.[3] Henceforth, the demand for the recognition of plant-based herbal products has increased throughout the world.[4] Similarly, their increased global demand has eventually boosted the international trade and world market of the herbal industry, further raising the need for ensuring the safety and quality of herbal drugs.[5] Herbal drugs are considered relatively safe if they are judiciously used by expert health workers. Nonetheless, this common notion is not true when drugs are misidentified, adulterated, or contaminated, which may cause potential harmful effects on the body.[6] Moreover, it has been reported that several adverse effects produced by herbal products are due to their poor quality. The quality of herbal products is affected either by external or internal factors. External factors include wrong identification, substitution, adulteration, spoilage, contamination with heavy metals, microbes, pesticides, residues,

DOI: 10.5530/pres.16.1.12

etc., whereas internal factors are complexity and diversity in the chemical composition of herbal drugs.[5] Quality control of herbal products is a tough task[7] that is aimed at confirming their quality, purity, efficacy, and safety.[5] In order to achieve adequate reciprocity within the quality of raw substances, in-process materials, and finished products, it is necessary to establish definite and tactful quality control methods through an amalgamation of old and new analytical methods.[2] Moreover, concern has been raised about appropriate analytical tools for reliable identification, standardization, and detection of adulterants in herbal products, especially plant materials, due to their diverse environmental factors.[8] On these grounds, the WHO has provided guidelines to assess the quality of herbal drugs.[9] At present, the worldwide growth and promotion of traditional medicine are not at par due to a lack of quality control, safety evaluation, chemoprofiling, and efficient regulatory norms for herbal products.[5]

The complex mixture of herbal products is an exciting characteristic since most of their pharmacological activities are thought to be attributed to the synergistic actions among various components of such products. On the other hand, the meticulous safety and efficacy evaluation of such complex products is hampered because of their actions on multiple target organs.[7] The adverse effects may be caused either by an inherent property of the herbal drugs or by toxicities caused by contaminants. Thus, the toxicity study plays a pivotal role in the acceptance and promotion of traditional systems of medicine.[3] *Habb-i-Hayat*, a Unani formulation, is used as concoctive, laxative, and purgative[10-12] to evacuate the morbid humours from the body[11,12] in cases of several systemic diseases. This formulation is prepared with five plant-derived ingredients such as, *Sana* (*Cassia angustifolia* Vahl.), *Post-i-Halela Zard* (*Terminalia chebula* Retz. peel), *Halela Siyah* (*Terminalia chebula* Retz.), *Zanjbeel* (*Zingiber offininale* Rosc.), and *Maveez Munaqqa* (*Vitis vinifera* Linn.).[10-12] The present study was aimed at evaluating the quality control and acute toxicity of *Habb-i-Hayat* since no data is available so far on these studies.

MATERIALS AND METHODS

Source of data collection

The data were collected from Regional Research Institute of Unani Medicine, Srinagar, Jammu and Kashmir, Regional Research Institute of Unani Medicine, Chennai, Tamil Nadu, Sher-i-Kashmir University of Agricultural Sciences and Technology (SKUAST), Srinagar, Jammu and Kashmir, and Vowcare Products, SIDCO Lassipura, Pulwama, Jammu and Kashmir.

Collection and authentication of individual ingredients of *Habb-i-Hayat*

All the ingredients of *Habb-i-Hayat* were procured from an authorized drug supplier in Srinagar, Jammu and Kashmir, and authenticated by Dr. Akhtar H Malik, Centre of Biodiversity and Taxonomy (CBT), Dept. of Botany, University of Kashmir, Jammu and Kashmir, vide reference numbers 3164-KASH, 3165-KASH, 3166-KASH, 3167-KASH, and 3168-KASH.

Procurement of chemicals and reagents

Eosin, DPX mountant, formaldehyde, acetone, benzene, and paraffin were procured from Merck Life Science Pvt. Ltd., Godrej One, 8th floor, Pirojshanagar, Eastern Express Highway, Vikhroli (East), Mumbai, INDIA (400079). Haematoxylin was procured from HiMedia Laboratories Pvt. Ltd., Registered office 23, Vadhani Industrial Estate, LBS Marg, Mumbai, INDIA (400086). Ethanol absolute was purchased from Changshu Hongsheng Fine chemical Co. Ltd. No. 8, Haifeng Road, New Material Industrial Park, Changshu city, Jiangsu province, and Thiopentone sodium was procured from Pfizer Ltd., Bandra East, Mumbai (400051).

Composition of *Habb-i-Hayat*

The composition of *Habb-i-Hayat* (100 mg) was as follows: *Sana* (*Cassia angustifolia* Vahl.) (44mg), *Post-i-Halela Zard* (*Terminalia chebula* Retz.) (22mg), *Halela Siyah* (*Terminalia chebula* Retz.) (22 mg), *Zanjbeel* (*Zingiber officinale*Rosc.) (12 mg), and *Maveez Munaqqa* (*Vitis vinifera* L.) (q. s.).[10]

Preparation of *Habb-i-Hayat*

Habb-i-Hayat in the form of pills was prepared according to the method described in the National Formulary of Unani Medicine (NFUM), Part-III.[10] All the ingredients except *Maveez Munaqqa* were powdered and sieved using 100 No. of mesh size. *Maveez Munaqqa* was boiled into a sufficient amount of water for a short duration, and the powdered drug was mixed in it to make a *lubdi* (mass). Thereafter, the *lubdi* was rolled into small sticks, and a pill of 500 mg was prepared.

Physicochemical standardization and quality control study

The physicochemical standardization and quality control study of *Habb-i-Hayat* including organoleptic characters,[13] weight variation,[14] hardness, friability, pH, loss of weight on drying, ash values, alcohol and water-soluble matter,[15] foreign matter, swelling index, foaming index, microbial load, aflatoxins,[16] heavy metals,[17] florescence analysis,[18] High-Performance Thin Layer Chromatography (HPTLC), and preliminary phytochemical evaluation[15] were carried out by following standard methods and procedures.

Acute toxicity study

The acute toxicity study of *Habb-i-Hayat* was carried out following the Organization for Economic Cooperation and Development by (OECD) guidelines 423.[19] The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of RRIUM, Srinagar, Jammu and Kashmir, vide reference number 927/ GO/ Re/ S/ 2006/ CPCSEA.

Experimental animals

The acute toxicity study was carried out on healthy female albino Wistar rats with 150-200 g of body weight. The rats were procured from IIIM, Jammu, Jammu and Kashmir, INDIA. Before the commencement of the experiment, the rats were acclimatized in the animal house of RRIUM, Srinagar. They were housed in the propylene cages under standard laboratory conditions at 22°C±3°C and 12 hr light and dark cycles as per CCSEA guidelines. During the whole study and housing period, standard food pellets and water *ad libitum* were given to the rats.

Experimental design

A total of 12 female albino Wistar rats were randomly divided into four groups three in each.

Group I (Plain Control): This group received 1 ml of distilled water *per os* daily for the whole study period.

Group II (Toxicant Group A): This group received a single dose of *Habb-i-Hayat* at 300 mg/kg b. w. dissolved in distilled water by oral route.

Group III (Toxicant Group B): When no signs of toxicity or mortality were found in group II for 48 hr after administration of the test drug, a single dose of *Habb-i-Hayat* at 2000 mg/kg b. w. dissolved in distilled water, was administered to this group by oral route.

Group IV (Toxicant group C): When no signs of toxicity or mortality were found in group III for 48 hr after administration of the test drug, a single dose of *Habb-i-Hayat* at 5000 mg/ kg b. w. dissolved in distilled water was given to this group by oral route.

Physical observations

All the rats were observed after dosing at least once during the first 30 min, periodically during the first 24 hr, with special attention given for the first 4 hr and daily thereafter for a period of 14 days. The observations include mortality, changes in the skin, fur, eyes, mucous membrane, behaviour pattern, tremors, convulsions, salivation, diarrhoea, lethargy, sleep, and coma. The body weight of each rat was recorded shortly before administration of the test drug and weekly thereafter. The food intake of each group was also measured daily. The relative organ-body weight was recorded at the end of the experiment.[19]

Histopathological examination of liver, kidney and heart

At the end of the study, all the rats were sacrificed by administering Thiopentone sodium (50 mg/kg b. w.) intraperitoneally[20] after an overnight fast. The liver, kidney, and heart of each rat were collected for gross and microscopic histopathological examinations.

Statistical analysis

The recorded data was compiled and entered in a spread sheet, then exported to the data editor GraphPad Instat and GraphPad Prism software versions 8.4.2. The continuous variables are expressed as Mean ± Standard Error of Mean (SEM). A one-way ANOVA was employed for the analysis of various parameters among different groups. A repeated measure of ANOVA was also employed in cases where observations of parameters were recorded more than twice. All these parametric tests were employed subject to the condition that the continuous data under consideration passed the normality test. In order to analyze the possible pair-wise significance between the groups, Dunnett's comparison test was applied. The p value <0.5 was considered statistically significant.

RESULTS

Physicochemical standardization and quality control

Organoleptic characters

The findings of the organoleptic characters of each ingredient of *Habb-i-Hayat* and its whole preparation were found to be same as mentioned in Unani classical literature.

Weight variation, Hardness and Friability

The average weight of 20 randomly selected pills of *Habb-i-Hayat* was found to be 338.7 mg. The deviation of individual pill weight from the average weight of 20 pills was found to be within the percentage limit of 5% of the mean weight. The hardness and friability of *Habb-i-Hayat* were found to be 78.4 Newton and 0.045%, respectively.

Loss of weight on drying at 105°C and pH

The loss of weight on drying of *Habb-i-Hayat* at 105°C was found to be 3.0%. The pH of *Habb-i-Hayat* in 1% and 10% aqueous solutions was found to be 4.9 and 4.6, respectively.

Ash value

The total ash value, acid-insoluble ash value, and water-soluble ash value of *Habb-i-Hayat* were found to be 3.0, 2.0, and 2.5%, respectively.

Water and alcohol soluble matter

The water-soluble matter of *Habb-i-Hayat* tested through hot and cold extraction methods was found to be 15.4 and 18.2%, respectively, while the alcohol-soluble matter of *Habb-i-Hayat* tested through hot and cold extraction methods was found to be 24.6% and 17.8%, respectively.

Foreign matter

The test drug was found to be free from any contamination, including insects, moulds, animal excreta, soil, and other adulterants. In addition, no abnormal odour, discoloration, sign

of deterioration, or innocuous matter were found in the sample. After analysis, the amount of foreign matter in the sample was calculated as 0.0016%.

Swelling index and Foaming index

No swelling index and foaming index were found in the test drug.

Table 1: **Microbial load of *Habb-i-Hayat***

Parameters	Results	Permissible limits as per WHO (2007)
Total Bacterial Count (TBC)	3×10^2 cfu/g	10^5 cfu/g
Total Fungal Count (TFC)	1×10^1 cfu/g	10^3 cfu/g
Enterobacteriaceae	Absent	10^3
Salmonella spp.	Absent	10
Escherichia coli	Absent	Absent
Staphylococcus aureus	Absent	Absent
Pseudomonas aeruginosa	Absent	Absent

Fluorescence analysis

The fluorescence analysis of the powder of *Habb-i-Hayat* carried out separately by treating it with various chemical reagents, showed different colours under daytime and ultraviolet lights.

Microbial load and Aflatoxins

The microbial analysis of *Habb-i-Hayat* showed that TBC and TFC were found to be$3x10^2$cfu/g and $1x10^1$ cfu/g, respectively**.** The microorganisms, including *Salmonella* spp., *Enterobacteriaceae, Escherichia coli, Pseudomonas aeruginosa*, and *Staphylococcus aureus*, were found to be absent in the test sample (Table 1). The aflatoxins analysis of the test drug showed that the aflatoxins included AFB1, AFB2, AFG1, and AFG2, were estimated as 0.3 ppb.

Heavy metals

The heavy metals including lead, cadmium, arsenic and mercury were found to be 2.1157 mg/ L, 0.0147 mg/ L, 1.9522 µg/L and 0.9825 µg/L, respectively, in *Habb-i-Hayat* (Table 2).

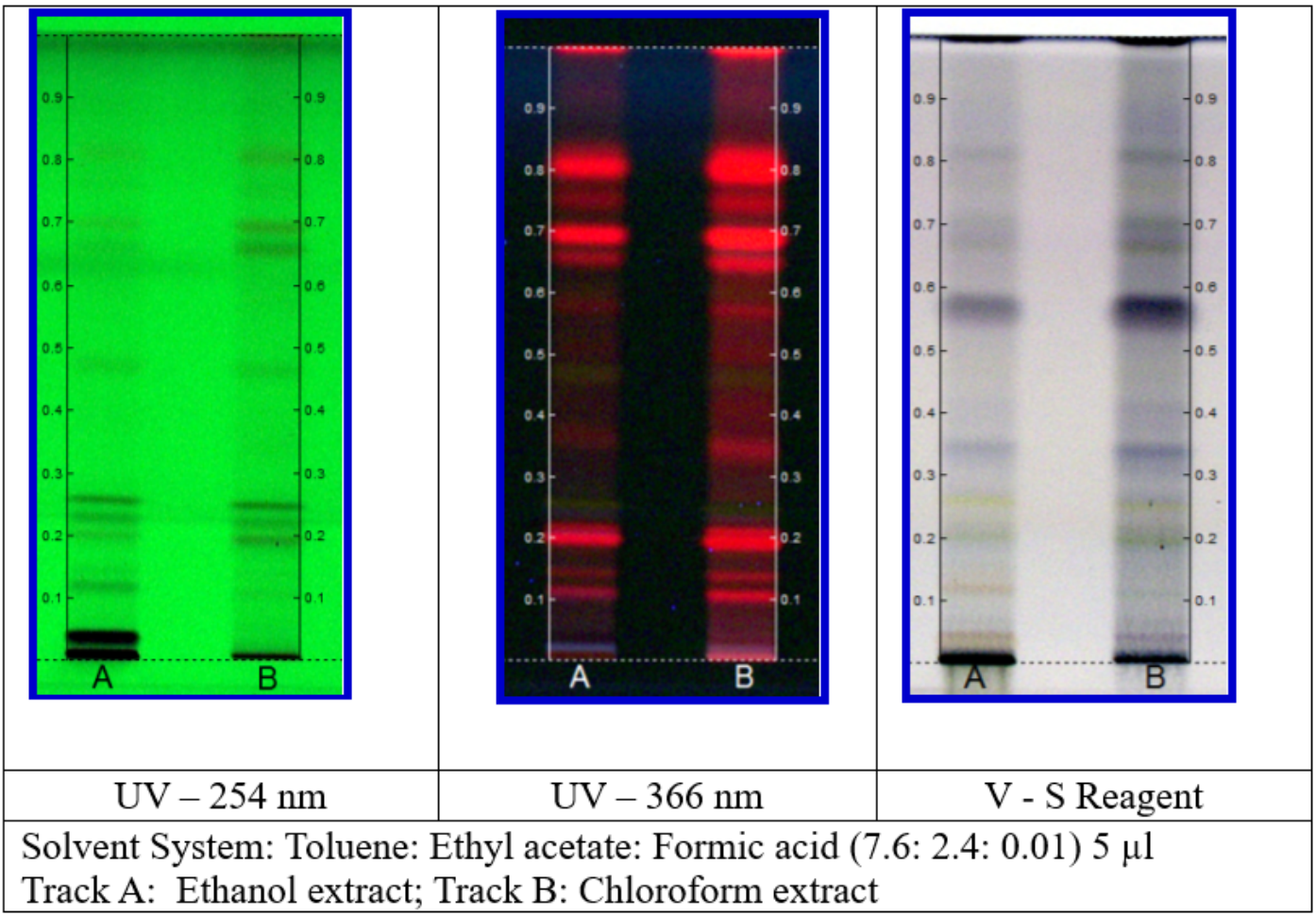

Figure 1: Chromatograms of ethanolic and chloroform extracts of *Habb-i-Hayat*.

Preliminary phytochemical screening

Various phytoconstituents, including alkaloids, phenols, tannins, anthraquinones glycosides, flavonoids, coumarins, carbohydrates, proteins, and steroids, were present in the ethanolic extract of *Habb-i-Hayat*.

HPTLC analysis of ethanolic and chloroform extracts of *Habb-i-Hayat*

In the present study, HPTLC analysis was performed on ethanolic and chloroform extracts of *Habb-i-Hayat* (Tracks A and B), and both chromatograms after scanning at 254 nm and 366 nm, followed by derivatization with vanillin sulphuric acid (V.S.) expressed, diverse phytochemical profile (Figure 1).

HPTLC of ethanolic extract

The emerged TLC plate of the ethanolic extract was scanned at 254 nm, which has shown 15 spots (Track A), with major spots at R_f 0.01, 0.20, and 0.24 (Green) (Table 3; Figures 1 and 2), and 13 peaks at 366 nm with important peaks at R_f values of 0.16, 0.20,0.24 (Fluorescent Red), and 0.68 (Red) (Table 4; Figures 1 and 3), and derivatization with vanillin-sulphuric acid reagent followed by heating at 110°C for about 5 min and observe under visible light, the plate shows major spots at R_f 0.81, 0.68 (Light grey), 0.58, 0.34 (Grey), 0.25 (Light yellow), 0.20 (Light grey), and 0.12 (Light brown).

Table 2: Heavy metal analysis of *Habb-i-Hayat*

Heavy Metals	Results	Permissible limits as per ASU Pharmacopoeias
Lead	2.1157 mg/L	10 ppm
Cadmium	0.0147 mg/L	0.3 ppm
Arsenic	1.9522 µg/L	3.0 ppm
Mercury	0.9825 µg/L	3.0 ppm

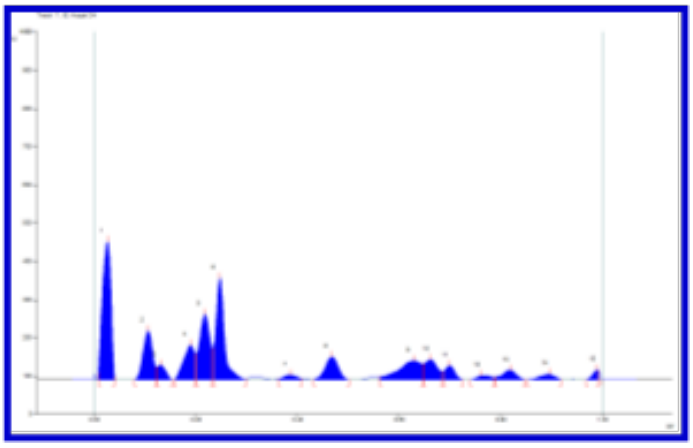

Figure 2: HPTLC finger print of *Habb-i-Hayat* of ethanol extract at 254 nm (Absorbance mode).

HPTC of chloroform extract

The refined TLC plate of chloroform extract (Track B), has manifested 16 spots at 254 nm with major spots at R_f 0.14, 0.20, 0.23, and 0.63 (Light Green) (Table 5; Figures 1 and 4) and 12 peaks at 366 nm with significant peaks at R_f values of 0.15 (Red),

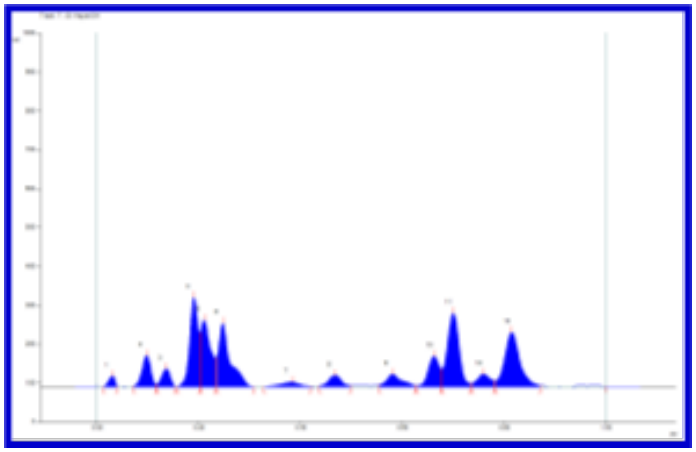

Figure 3: HPTLC finger print of *Habb-i-Hayat* of ethanol extract at 366 nm (Absorbance mode).

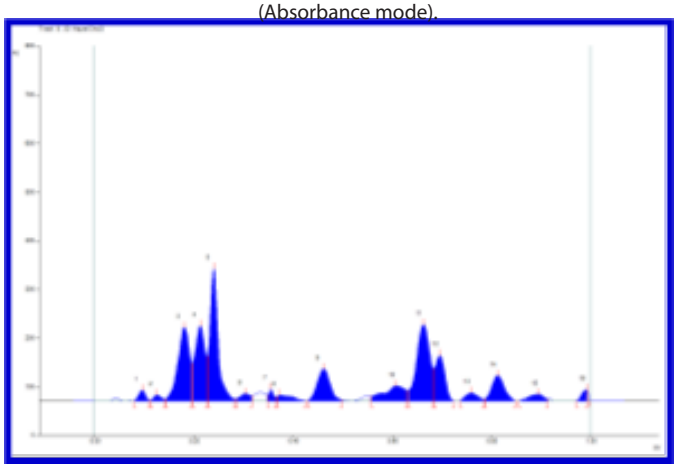

Figure 4: HPTLC finger print of *Habb-i-Hayat* of chloroform extract at 254 nm (Absorbance mode).

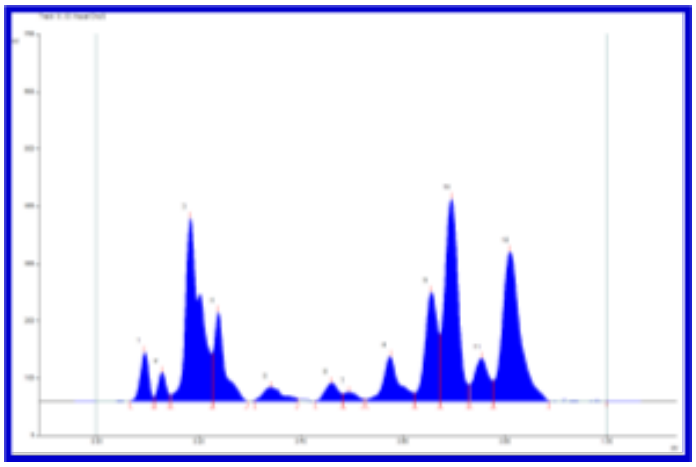

Figure 5: HPTLC finger print of *Habb-i-Hayat* of chloroform extract at 366 nm (Absorbance mode).

Table 3: R_f values of ***Habb-i-Hayat*** of ethanol extract at 254 nm (Absorbance mode).

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.01 R_f	18.6 AU	0.03 R_f	362.9 AU	26.88%	0.04 R_f	7.4 AU	5034.1AU	22.46%
2	0.08 R_f	0.1 AU	0.11 R_f	128.6 AU	9.53%	0.12 R_f	32.4 AU	1986.4AU	8.86%
3	0.13 R_f	32.5 AU	0.13 R_f	39.0 AU	2.89%	0.16 R_f	0.0 AU	535.4 AU	2.39%
4	0.16 R_f	0.1 AU	0.19 R_f	90.9 AU	6.74%	0.20 R_f	67.7 AU	1640.7AU	7.32%
5	0.20 R_f	68.4 AU	0.22 R_f	171.7 AU	12.72%	0.23 R_f	80.5 AU	2913.9AU	13.00%
6	0.24 R_f	82.9 AU	0.25 R_f	266.5 AU	19.74%	0.30 R_f	1.0 AU	3688.9AU	16.46%
7	0.36 R_f	1.4 AU	0.39 R_f	13.2 AU	0.97%	0.41 R_f	3.3 AU	275.9 AU	1.23%
8	0.43 R_f	0.3 AU	0.47 R_f	60.0 AU	4.45%	0.50 R_f	0.1 AU	1296.0AU	5.78%
9	0.56 R_f	7.4 AU	0.63 R_f	50.1 AU	3.71%	0.65 R_f	41.5 AU	1874.0AU	8.36%
10	0.65 R_f	41.9 AU	0.66 R_f	52.2 AU	3.87%	0.69 R_f	21.2 AU	1126.1AU	5.02%
11	0.69 R_f	21.8 AU	0.70 R_f	36.9 AU	2.73%	0.73 R_f	0.1 AU	595.0 AU	2.65%
12	0.74 R_f	0.0 AU	0.76 R_f	11.5 AU	0.85%	0.79 R_f	6.0 AU	257.9 AU	1.15%
13	0.79 R_f	6.0 AU	0.82 R_f	25.5 AU	1.89%	0.85 R_f	0.1 AU	588.6 AU	2.63%
14	0.85 R_f	0.1 AU	0.90 R_f	14.8 AU	1.10%	0.92 R_f	0.3 AU	355.1 AU	1.58%
15	0.97 R_f	0.1 AU	0.99 R_f	26.0 AU	1.93%	0.99 R_f	24.4 AU	246.3 AU	1.10%

Table 4: R_f values of ***Habb-i-Hayat*** of ethanol extract at 366 nm (Absorbance mode).

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.01 R_f	0.1 AU	0.03 R_f	28.3 AU	2.29%	0.04 R_f	0.7 AU	301.2 AU	1.33%
2	0.07 R_f	1.5 AU	0.10 R_f	80.7 AU	6.51%	0.12 R_f	6.5 AU	1185.7 AU	5.22%
3	0.12 R_f	6.9 AU	0.14 R_f	46.7 AU	3.78%	0.16 R_f	0.5 AU	675.7 AU	2.98%
4	0.16 R_f	0.2 AU	0.19 R_f	230.3 AU	18.60%	0.20 R_f	39.0 AU	3154.3 AU	13.89%
5	0.20 R_f	139.8 AU	0.21 R_f	172.1 AU	13.90%	0.23 R_f	74.1 AU	2849.8AU	12.55%
6	0.24 R_f	76.1 AU	0.25 R_f	164.2 AU	13.26%	0.31 R_f	0.1 AU	3194.5 AU	14.07%
7	0.33 R_f	0.2 AU	0.38 R_f	12.2 AU	0.98%	0.42 R_f	0.8 AU	396.2 AU	1.74%
8	0.44 R_f	2.0 AU	0.47 R_f	29.4 AU	2.37%	0.50 R_f	3.6 AU	657.2 AU	2.89%
9	0.56 R_f	5.1 AU	0.58 R_f	32.1 AU	2.60%	0.63 R_f	3.1 AU	836.4 AU	3.68%
10	0.63 R_f	3.2 AU	0.66 R_f	78.9 AU	6.37%	0.68 R_f	45.5 AU	1474.9 AU	6.49%
11	0.68 R_f	46.2 AU	0.70 R_f	189.8 AU	15.33%	0.74 R_f	8.6 AU	3693.5 AU	16.26%
12	0.74 R_f	8.7 AU	0.76 R_f	33.1 AU	2.67%	0.78 R_f	15.4 AU	742.8 AU	3.27%
13	0.78 R_f	15.4 AU	0.82 R_f	140.4 AU	11.34%	0.87 R_f	3.3 AU	3546.5 AU	15.62%

0.63, 0.68 and 0.78 (Fluorescent red) (Table 6; Figures 1 and 5), and derivatized in vanillin-sulphuric acid reagent, followed by heating at 110°C about 5 min and observing under visible light, the plate shows major spots at R_f 0.80, 0.70, 0.68 (Light grey), 0.58 (Grey), 0.34 (Light grey), 0.26 (Light yellow), 0.20, and 0.11 (Light green). The denistometric analysis of both the extracts under UV 254 nm, 366 nm, and fluorescence mode was also carried out and is depicted in Figures 6-9.

The calculated R_f values, which represent different phytoconstituents present in the tested extracts, would be of significant help in the validation of unknown chemical constituents by correlating with the reference standard. Also, from the area percentage values pertaining to different chemical constituents, concentration can be determined. The evolved TLC plates for both types of extract, and the formulation under the given method displayed different natures of compounds (Polar and non-polar) with excellent resolution. These results may help to isolate and characterize the compounds in the future and identify the marker compounds with the help of spectroscopic techniques like NMR, MS, IR, etc.

Table 5: R_f values of ***Habb-i-Hayat*** of chloroform extract at 254 nm (Absorbance mode).

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.08 R_f	0.2 AU	0.10 R_f	22.0 AU	1.97%	0.11 R_f	1.2 AU	252.4 AU	1.26%
2	0.11 R_f	1.3 AU	0.13 R_f	12.2 AU	1.09%	0.14 R_f	3.1 AU	159.8 AU	0.80%
3	0.14 R_f	3.5 AU	0.18 R_f	151.5 AU	13.55%	0.20 R_f	77.1 AU	2989.5 AU	14.89%
4	0.20 R_f	78.1 AU	0.21 R_f	154.6 AU	13.82%	0.23 R_f	90.6 AU	2605.2 AU	12.98%
5	0.23 R_f	93.8 AU	0.24 R_f	272.6 AU	24.38%	0.28 R_f	3.8 AU	3706.6 AU	18.47%
6	0.29 R_f	3.8 AU	0.31 R_f	15.3 AU	1.37%	0.32 R_f	11.2 AU	263.1 AU	1.31%
7	0.35 R_f	13.0 AU	0.36 R_f	24.3 AU	2.17%	0.37 R_f	8.8 AU	200.8 AU	1.00%
8	0.37 R_f	8.8 AU	0.38 R_f	11.9 AU	1.06%	0.43 R_f	0.2 AU	309.3 AU	1.54%
9	0.43 R_f	0.3 AU	0.46 R_f	65.7 AU	5.88%	0.50 R_f	2.7 AU	1427.1AU	7.11%
10	0.56 R_f	9.3 AU	0.61 R_f	31.2 AU	2.79%	0.63 R_f	20.7 AU	1109.8 AU	5.53%
11	0.63 R_f	20.9 AU	0.66 R_f	157.1 AU	14.06%	0.68 R_f	65.6 AU	3519.7 AU	17.53%
12	0.69 R_f	66.5 AU	0.70 R_f	94.1 AU	8.42%	0.73 R_f	0.2 AU	1502.7AU	7.49%
13	0.74 R_f	0.4 AU	0.76 R_f	16.8 AU	1.50%	0.79 R_f	3.5AU	349.4 AU	1.74%
14	0.79 R_f	3.6 AU	0.81 R_f	51.9 AU	4.64%	0.85 R_f	0.2AU	1112.8 AU	5.54%
15	0.86 R_f	0.1 AU	0.90 R_f	13.8 AU	1231%	0.92 R_f	3.7 AU	349.2 AU	1.74%
16	0.97 R_f	0.3 AU	0.99 R_f	23.0 AU	2.06%	0.99 R_f	22.1 AU	215.2 AU	1.07%

Table 6: R_f values of ***Habb-i-Hayat*** of chloroform extract at 366 nm (Absorbance mode).

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.07 R_f	0.0 AU	0.10 R_f	85.4 AU	5.21%	0.11 R_f	6.6 AU	1151.1 AU	3.11%
2	0.12 R_f	7.3 AU	0.13 R_f	50.2 AU	3.07%	0.15 R_f	10.6 AU	653.8AU	1.77%
3	0.15 R_f	10.7 AU	0.19 R_f	319.2 AU	19.48%	0.23 R_f	821 AU	7786.5 AU	21.06%
4	0.23 R_f	83.6 AU	0.24 R_f	155.8 AU	9.51%	0.30 R_f	0.0 AU	2722.5AU	7.36%
5	0.31 R_f	0.9 AU	0.34 R_f	24.0 AU	1.46%	0.40 R_f	4.9AU	781.4 AU	2.11%
6	0.43 R_f	0.3 AU	0.46 R_f	31.6 AU	1.93%	0.48 R_f	12.4 AU	691.7 AU	1.87%
7	0.49 R_f	12.6 AU	0.50 R_f	15.5 AU	0.95%	0.52 R_f	3.9 AU	323.9 AU	0.88%
8	0.53 R_f	2.8 AU	0.58 R_f	77.7 AU	4.75%	0.63 R_f	14.2 AU	1996.6 AU	5.40%
9	0.63 R_f	14.5 AU	0.66 R_f	190.6 AU	11.64%	0.68 R_f	15.5 AU	3876.3 AU	10.48%
10	0.68 R_f	117.3 AU	0.70 R_f	352.7 AU	21.53%	0.73 R_f	28.3 AU	7645.0 AU	20.68%
11	0.73 R_f	28.5 AU	0.76 R_f	74.4 AU	4.54%	0.78 R_f	35.5 AU	1768.1 AU	4.78%
12	0.78 R_f	35.9 AU	0.81 R_f	261.1 AU	15.94%	0.89 R_f	0.4 AU	7573.1 AU	20.48%

Acute toxicity

Physical observations

The rats treated with the low dose level of *Habb-i-Hayat* (300 mg/kg b. w.) did not show any abnormal signs, while the rats receiving the drug at 2000 mg/kg b. w. had diarrhoea. One rat out of three treated with the test drug at 5000 mg/kg b. w. died 24 hr after administration of the dose. The rats belonging to this group exhibited some abnormal signs like cyanosis, hair loss, diarrhoea, piloerection, disturbance in respiration, sluggish reflexes, emaciation, lethargy, abnormal gait, edema, tremors, and vocalization.

LD_{50}

The LD_{50} of *Habb-i-Hayat* was calculated as 2500 mg/kg b. w. for rats. This calculation was done by following OECD guidelines 423.

Body weight

No significant gain or reduction in the body weight of rats belonging to groups I, II, and III was noted throughout the study

Table 7: Effects of *Habb-i-Hayat* on total body weight of rats.

Showing average body weight variation by rats among different groups										*p* Value
Groups	1st day			7th day			14th day			
	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM	
Group I	181.6	9.71	5.60	198.33	10.01	5.78	203.33	12.22	7.05	<0.01**
Group II	153.33	4.16	2.40	162	11.79	6.80	171.33	15.69	9.06	<0.01**
Group III	177	16.52	9.53	196	15.52	8.96	206.33	15.50	8.95	>0.05
Group IV	157	7.55	4.35	170	18.24	10.53	168.5	3.53	2.50	<0.01**
Test applied repeated measures of ANOVA.										

**p <0.01: highly significant [comparison between groups was made using one-way ANOVA followed by Dunnett's multiple comparison test; values are expressed as Mean ± SEM (n=3)].

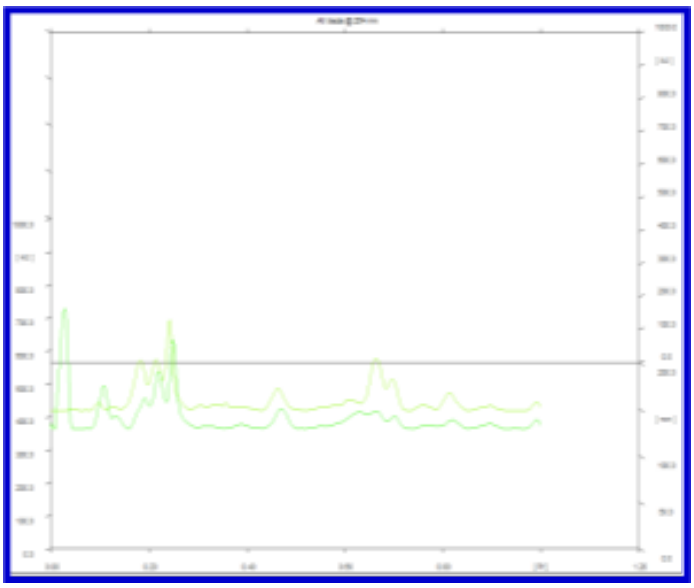

Figure 6: Densitometric chromatogram of ethanol and chloroform extract at 254 nm (Absorbance mode).

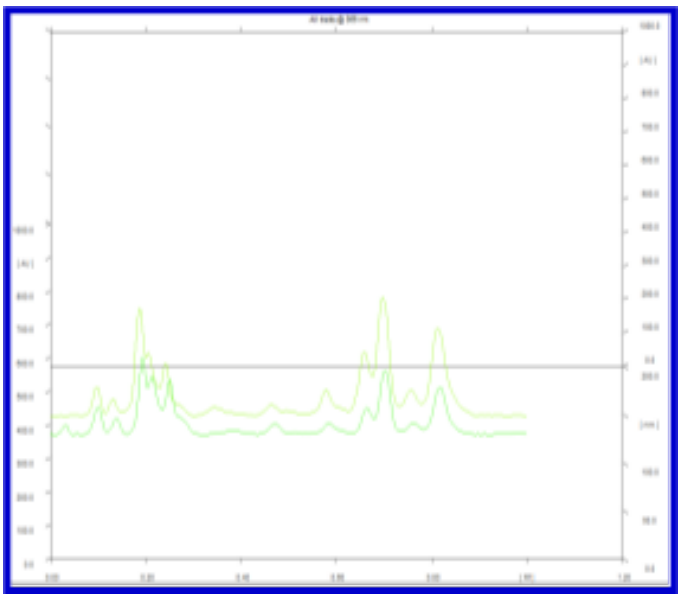

Figure 7: Densitometric chromatogram of ethanol and chloroform extract at 366 nm (Absorbance mode).

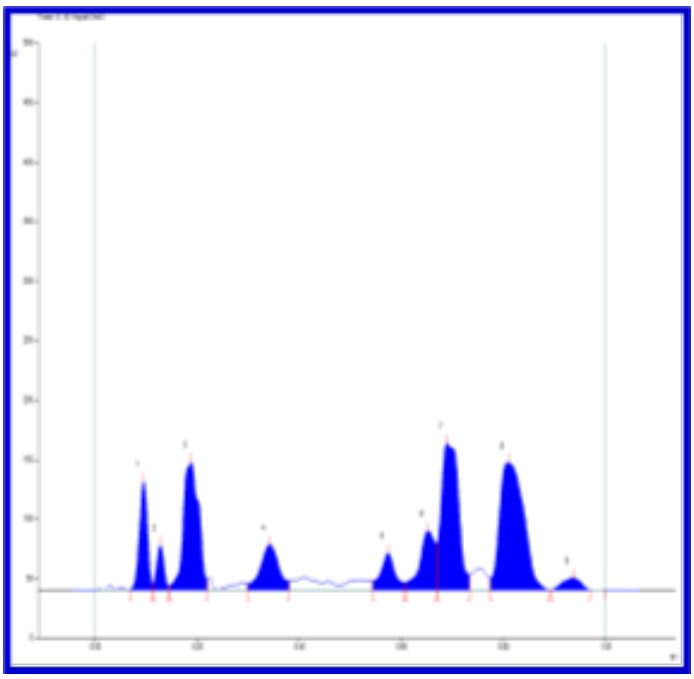

Figure 8: Densitometric chromatogram of ethanol and chloroform extract at 366 nm (Fluorescence mode).

period, whereas the body weight of rats belonging to group IV (p<0.01) was found to be slightly reduced at the end of the study as compared to the second week (Table 7).

Food intake

The mean value of the food intake for each group per day belonging to groups I, II, III, and IV was found to be 98.6±1.41, 95.6±1.61, 98.2±1.86, and 78.87±4.2, respectively.

Relative organ-body weight

The relative organ-body weight of the liver of rats belonging to groups I, II, III, and IV was found to be 3.40±0.11, 4.06±0.50, 3.77±0.42, and 3.92±0.89, respectively. The relative organ-body weight of the kidneys of rats belonging to groups I, II, III, and IV was recorded as 0.66±0.03, 0.68±0.02, 0.63±0.01 and 0.87±0.19, respectively. The relative organ-body weight of the heart of rats

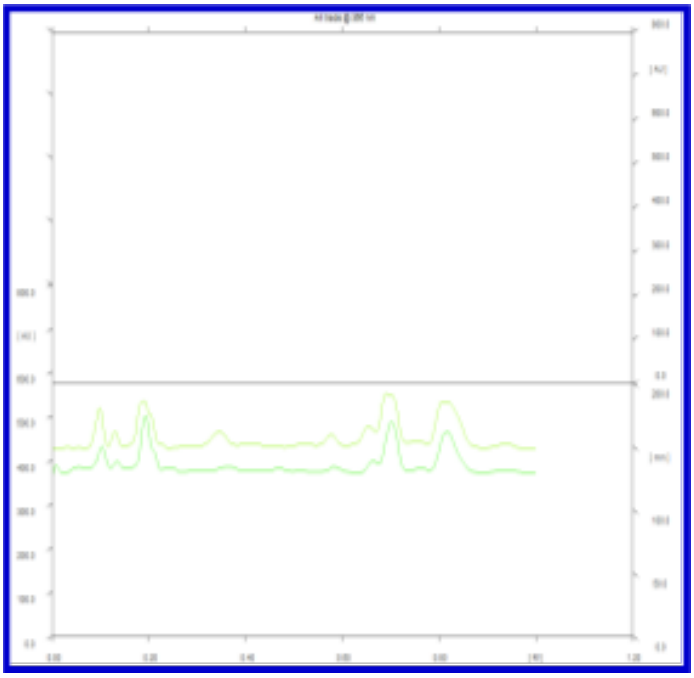

Figure 9: Densitometric chromatogram of ethanol and chloroform extract at 366 nm (Fluorescence mode).

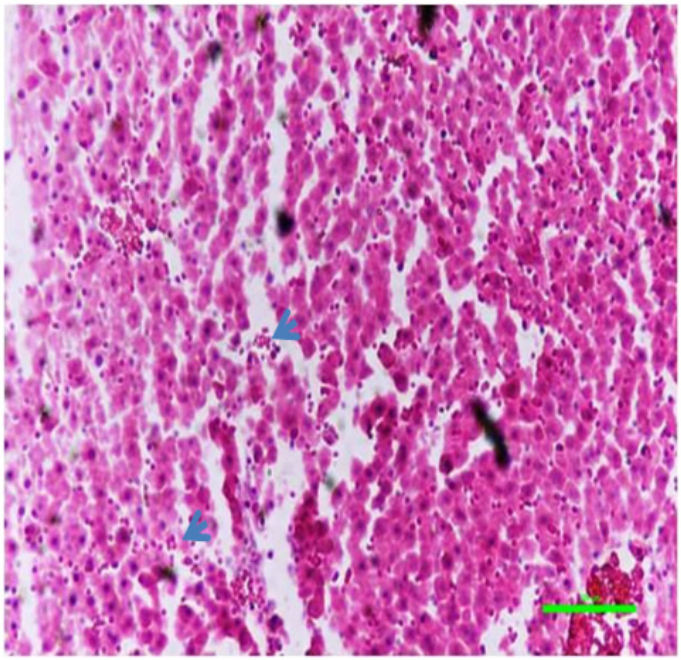

Figure 10: (Liver of Group III): Moderate haemorrhage and congestion in sinusoids, hypertrophy and hyperplasia in kupffer cells (arrow) along with necrotic changes (H & E 20X).

belonging to groups I, II, III, and IV was found to be 0.36±0.01, 0.35±0.01, 0.34±0.01, and 0.37±0.03, respectively.

Histopathological examination

Gross examination of the liver

The normal reddish-brown colour of the liver was observed in all the sacrificed rats. No significant changes were observed in the livers of rats during post-mortem examination.

Microscopic examination of the liver

The cut sections of the liver tissues of rats belonging to the plain control showed a normal hexagonal structure of hepatocytes with numerous hepatic lobules. The hepatic lobules appeared normal with a clear central vein. Mild congestion in the hepatic central vein was observed in group II, whereas prominent degenerative changes of hepatocytes were seen as evident with moderate haemorrhage and congestion in sinusoids, proliferation of kupffer cells, and necrotic changes in group III (Figure 10). Some of the sinusoids were over-infiltrated with erythrocytes. The liver specimens of rats belonging to group IV showed diffuse necrosis of the hepatic parenchyma and kupffer cell hyperplasia. Marked degenerative changes were seen with endothelial damage, haemorrhages, and necrotic heaptocytes in this group (Figure 11).

Gross examination of the kidneys

The normal bean-shaped, brown-coloured kidneys were observed in the rats of all groups on post-mortem examination. The cut surface of the kidneys did not reveal any gross abnormality and showed proportionate cortex and medulla.

Microscopic examination of the kidneys

The cut sections of the kidneys of rats belonging to the plain control showed normal glomerulus and tubular architecture in the cortex and medulla. The renal lesion in all treatment groups demonstrated a variable extent of renal tubular necrosis. The kidney specimens of rats belonging to group II showed acute tubular necrosis in the cortex with marked congestion in the medulla. In group III, there was loss of tubular structure, necrosis, and atrophy of the glomerulus. Markedly distorted tubules, loss of nephron, and desquamated epithelium were evident in the lumen of the kidney tubules in group IV (Figure 12).

Gross examination of the heart

There were no visible changes in the hearts of all the rats in the different groups.

Microscopic examination of the heart

The cardiac histology of the rats in plain control revealed single, oval, and centrally arranged nucleus and regularly arranged cardiac myofibres. The histoarchitecture of the hearts of rats belonging to groups II and III showed similar patterns; however, cytoplasmic vacuolation was evident in both groups. The hearts of rats from group IV showed disarrayed cardiac myofibre with marked haemorrhages and degenerative changes (Figure 13).

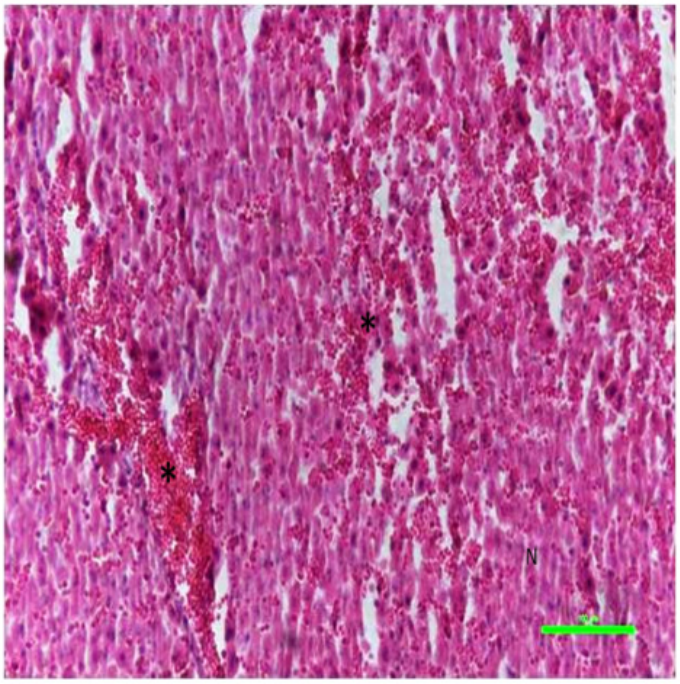

Figure 11: (Liver of Group IV): Necrosis (N) of hepatic parenchyma and kupffer cell hyperplasia and haemorrhages (H & E 20X).

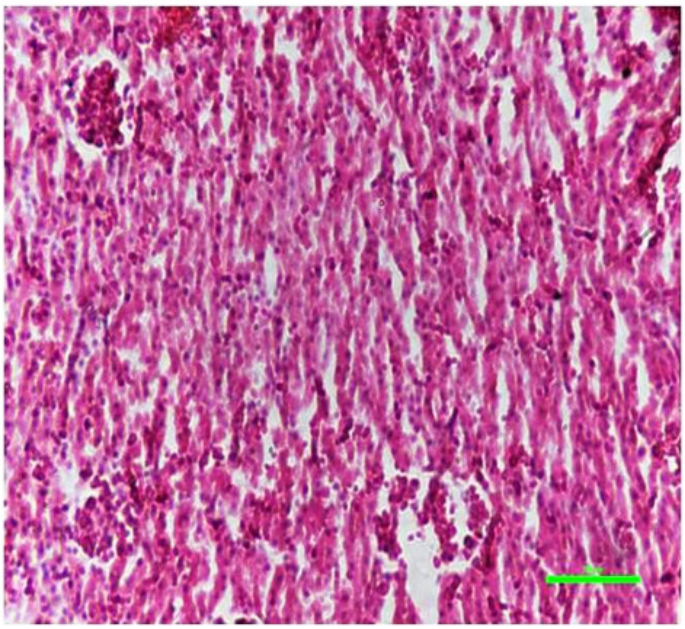

Figure 12: (Kidney of Group IV): Distorted tubules, haemorrhages and loss of nephron (H & E 20X).

DISCUSSION

Quality assurance is essential for ensuring the quality of drugs in all systems of medicine. Numerous multidisciplinary and analytical tactics like macroscopic, microscopic, physicochemical, phytochemical, chromatographic fingerprinting, safety, biological evaluations, etc. are employed in this context.[21] The present study was carried out to evaluate different physicochemical and quality control parameters of *Habb-i-Hayat* to ensure its quality

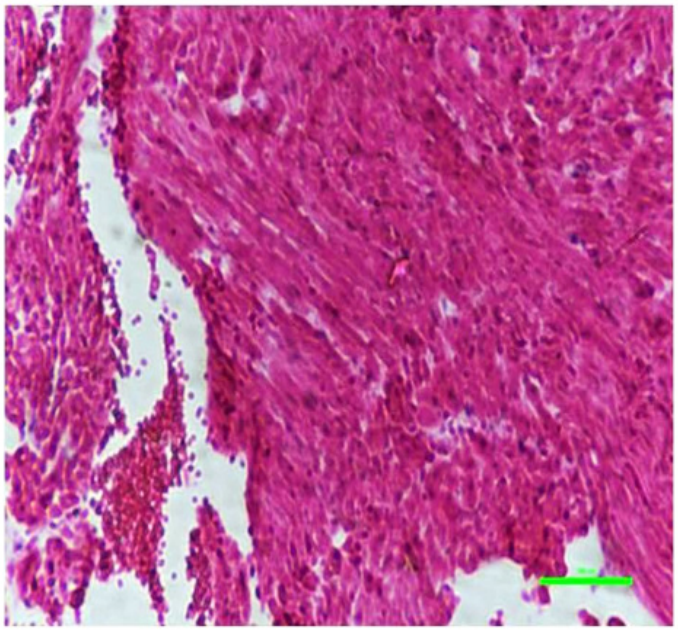

Figure 13: (Heart of Group IV): Distorted disarrayed cardiac myofibre with marked haemorrhage and degenerative changes (H & E 20X).

since no monograph on the standardization and quality control of *Habb-i-Hayat* has been published in the Unani Pharmacopoeia of INDIA (Part II). The findings of the organoleptic characters of each ingredient reveal that all the individual drugs of *Habb-i-Hayat* were authentic and of good quality, as mentioned in the Unani Pharmacopoeia of India, Part I, Volume I and IV.[22,23] The organoleptic characteristics of *Habb-i-Hayat* reveal that the pills were prepared as per the standard method described in the National Formulary of Unani Medicine.[10] The slight bitter taste of the pill is due to the presence of *Cassia angustifolia* and *Zingiber officinale*. The greyish-brown colour, characteristic odour, round shape, and hard and smooth texture of *Habb-i-Hayat* may be referred to as its standard morphological parameters in the future. The weight variation among the randomly selected 20 pills was found to be within the acceptable limit, which indicates that the pills prepared for the present study were uniform in size. The disintegration time and dissolution time of a tablet depend on its hardness. The disintegration time of a too-hard tablet is longer, which ultimately decreases its dissolution rate and absorption. Similarly, too-soft tablets are easily broken during packaging and transportation.[24] Thus, the hardness of an oral tablet should be 4 to 10 kg.[24,25] The hardness of *Habb-i-Hayat* was found to be 78.4 Newton (7.99 kg) through the EI Digital Tablet Hardness Tester (Model 3956), which is within the normal range as per the standards. It is pertinent to mention that the quality and quantity of the binder used in the preparation of any tablet or pill play an important role in its hardness.

In the present study, the syrup prepared with *Vitis vinifera* (10% of the total weight of the powder) was used as a binder. The friability of the pill is an essential parameter to know the strength of the pill or tablet. The friability of *Habb-i-Hayat* was found to

be within the normal range, i.e., 0.5-1%.[15] The moisture content refers to the presence of the total water content in any drug. A drug is more likely to develop microbial growth if its moisture content is raised, which eventually affects its efficacy.[15] The loss of weight on drying at 105°C of *Habb-i-Hayat* was found to be within the normal limit, which indicates the drug was of good quality. The determination of ash value that is an essential criteria to identify adulterants in herbs.[15] The higher ash value in a plant-derived drug indicates the presence of inorganic substances, which may be due to adulteration or impurities.[26] The total ash, acid-insoluble ash, and water-soluble ash values of *Habb-i-Hayat* were found to be 3.0%, 2.0%, and 2.5%, respectively. These are the ash values of the compound drug prepared by four plant-derived drugs in combination. There is no previous data available regarding the ash value of this compound formulation. Hence, the constants of different ash values obtained from the present study may be considered as references for future studies. The greater concentration of the water-soluble extractive indicates the presence of sugar, acids, and inorganic substances, while the higher concentration of the alcohol-soluble extractive attributes to the presence of glycosides, phenolic compounds, alkaloids, steroids, and flavonoids in the test drug. The greater concentration of both extractive values also acknowledged that the test drug was of good quality and was free from exhaustive materials and adulterations.[15] The pH of the 1% and 10% solutions of the test drug was found to be acidic in nature, which indicates its good absorption through the mucous membrane of the stomach.[27] The total bacterial count and total fungal count were found within the permissible limits in the test drug.[28] Specific microbes such as *Salmonella* spp., *Enterobacteriaceae, Escherichia coli, Pseudomonas aeruginosa*, and *Staphylococcus aureus* were found to be absent. The aflatoxins include AFB1, AFB2, AFG1, and AFG2, were estimated as 0.3 ppb in *Habb-i-Hayat*, which is within the permissible limit as per the Unani Pharmacopoeia.[28] The quality control report also showed that four important heavy metals including lead, cadmium, arsenic, and mercury were found within the permissible range in the test drug.[28] All these constants affirmed that the manufacturing, drying, and storage processes of *Habb-i-Hayat* strictly adhered to the standard operating procedures as mentioned in the Unani Pharmacopoeia. HPTLC fingerprinting is an important parameter for the authentication of herbal products. The number of peaks, R_f values, and area under the curve play a pivotal role in the identification, quality, and adulteration of herbal drugs.[15] First time, HPTLC fingerprinting of *Habb-i-Hayat* in ethanol and chloroform extracts has been carried out, which may be useful in the standardization and quality control of this preparation in the future.

In the present era, the use of herbal products has increased throughout the world, but their safety is a major concern. Some reports asserted that herbal materials produce toxicities due to their inherent properties or adulterants, which raised the necessity for toxicity studies of herbs.[29] The acute toxicity report of *Habb-i-Hayat* reveals that its higher dose level (5000 mg/kg) has produced some significant toxic effects, including death, reduced body weight, body hair loss, diarrhoea, sluggish reflexes, lethargy, abnormal gait, and tremors. The LD_{50} of *Habb-i-Hayat* was found to be 2500 mg/kg b. w. for rats. Marked degenerative changes and necrosis were observed in the hepatic cells of rats treated with the higher dose level of the test drug. Similarly, marked distorted renal tubules and loss of nephrons in the kidneys, marked hemorrhages, and degenerative changes in the myocardium were seen in the rats of the same group. These findings suggested that the higher dose level of *Habb-i-Hayat* accumulates and damages the liver and kidneys. The test drug at lower dose levels, such as 300 mg/kg and 2000 mg/kg didn't produce any adverse effects, which suggested that it is safe up to 2000 mg/kg b. w. in rats. Studies carried out on the safety evaluation of individual ingredients of *Habb-i-Hayat* supported the findings found in the present study. Some reports indicate that the chronic use of *Cassia angustifolia* at higher doses may produce hepatic toxicities.[30] A study has reported that the prolonged use of *Zingiber officinale* at higher doses produced bradycardia, hypotension, and degeneration in the myocardium in rats.[31]

The overall standardization and quality control results of *Habb-i-Hayat* have suggested that the individual ingredients of this formulation were of good quality and were free from adulterations. The physicochemical constants and other quality control parameters of *Habb-i-Hayat* may be used as standards for future studies. The acute toxicity reports of the present study have suggested that the test drug produces toxic effects only at higher dose levels, which further reveals its wide therapeutic window. Hence, it is recommended that it be safely used at a therapeutic dose level for treatment purposes.

CONCLUSION

The Unani system of medicine is lacking in standardization and safety evaluations of Unani formulations. The safety and quality control data developed in the present study may be referred to in the future for worldwide acceptance of the Unani system.

AUTHORS' CONTRIBUTION

Ifra Qayoom and Athar Parvez Ansari: Designed protocol, carried out the whole study, collected data and compiled the whole manuscript. **Pankaj Goswami:** Involved in histopathology of liver, kidney and heart specimens and gave interpretation of the results with histopathological changes. **Mohd Musaib Bhat, Bazilah Majeed Reshi and Noman Anwar:** Critically reviewed and edited the manuscript. **Huzaifa Ansari:** Assisted the pre-clinical trial.

ACKNOWLEDGEMENT

We are thankful to the Director General, Central Council for Research in Unani Medicine, New Delhi, Ministry of Ayush, Govt. of INDIA for providing all the facilities required in this study.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FINANCIAL SUPPORT

This work is part of the post graduate research programme of Regional Research Institute of Unani Medicine, Srinagar, CCRUM, New Delhi, Ministry of Ayush, Govt. of INDIA. All the required facilities for this study were provided by the Central Council for Research in Unani Medicine, New Delhi.

ABBREVIATIONS

CCSEA: Committee for Control and Supervision of Experiments on Animals; **HPTLC:** High-performance thin layer chromatography; **IAEC:** Institutional Animal Ethics Committee; **IIIM:** INDIAn Institute of Integrative Medicine; **NFUM:** National Formulary of Unani Medicine; **OECD:** Organization for Economic Cooperation and Development; **RRIUM:** Regional Research Institute of Unani Medicine; **TBC:** Total Bacterial Count; **TFC:** Total Fungal Count; **WHO:** World Health Organization.

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