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Original Article

TLC, HPTLC FINGERPRINTING AND ACUTE ORAL TOXICITY EVALUATION OF *HABB-E-AZARAQI*: A NUX-VOMICA-BASED TRADITIONAL UNANI FORMULATION

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ABSTRACT

Background and Objective: Nux-vomica based traditional Unani formulation, *Habb-e-Azaraqi* (HAZ) is an important drug used by Unani physicians since several decades. It possesses *Muqawwi-i-A'sab* (nervine tonic), *Muharrik-i-A'sab* (nervine stimulant) properties and is an effective treatment option for diseases like *Laqwa* (facial palsy), *Falij* (paralysis), *Niqris* (gout) and *Waja'al-Mafasil* (arthritis) etc. The aim of the study is to access and provide information of HAZ for its TLC, HPTLC Fingerprinting defining its clear qualitative perspective and acute oral toxicity evaluation for its safety assessment which was not done earlier, thus contributing in the field of research.

Materials and Methods: The chief ingredient, nux-vomica was detoxified as per method mentioned in Unani Pharmacopeia before its use in formulation. TLC and HPTLC was developed under four detection system i.e., UV 366nm, UV 254nm, exposure to iodine vapours and after derivatization with anisaldehyde sulphuric acid. Acute toxicity studies were performed as per OECD Guidelines 425 at a limit dose of 2000 mg/kg. Observations were done for signs of toxicity, body weight, and feed consumption at regular intervals followed by haematological and biochemistry evaluation.

Results: The generated data proved the authenticity and established the TLC and HPTLC profile of the formulation. Acute toxicity revealed no significant differences in HAZ-treated animals with respect to body weight gain, feed consumption, haematology, clinical biochemistry evaluation. No significant gross pathological observation was noticed in necropsy.

Conclusion: Data of the present study is substantial and scientific proof of HAZ in terms of standardization and toxicity study that can be utilize in future research activities.

Keywords: *Habb-e-Azaraqi*, Unani, TLC, HPTLC, Acute toxicity.

1. INTRODUCTION

Unani system of medicine is a holistic traditional system of medicine widely practiced across the globe. The importance of this system was validated upon its acceptance by WHO in the year 1976. This comprehensive system meticulously offers curative, promotive, preventive and rehabilitative treatment options. The System fits within the definition of traditional medicine as described by the World Health Organization (WHO), which states that traditional medicine is "the sum total of the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness'' (WHO, 2000).

Habb-e-Azaraqi has been in frequent use since several decades and is a potent tablet dosage form compound Unani formulation, cited in several classical texts and Pharmacopeia's like *Qarabadeen-e-Azam* (Khan, 1996), *Qarabadeen-e-Majeedi* (Latif, 1986) and National Formulary of Unani Medicine (Anonymous, 1981). Endowed with properties such as *Muqawwi-i-A'sab* (nervine tonic), *Muharrik-i-A'sab* (nervine stimulant), *Musakkin* (analgesic), *Muhallil* (resolvent), it is indicated in disorders like *Falij* (paralysis), *Laqwa* (facial palsy or Bell's palsy), *Niqris* (gout) and *Waja'al-Mafasil* (arthritis) (Anonymous, 2006). The formulation has four ingredients namely *Azaraqi mudabbar* (detoxified *Strychnos nux-vomica* L.), *Filfil siyah* (*Piper nigrum* L.), *Filfil daraz* (*Piper longum*

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L.) and *Araq-i-Ajwayin* (Distillate of *Trachyspermum ammi* L.) (Kabeeruddin, 2008). The chief ingredient of the formulation is *Azaraqi/ Kuchla* (*Strychnos nux-vomica* L.) which is considered to be toxic in nature, having strychnine and brucine as major alkaloids and was used originally as a nervine tonic and to treat rheumatic pain (Ghani, 2011). Being toxic in nature, the use of *Azaraqi* in formulation as an ingredient is endorsed only after its detoxification (*tadbeer*) which results in sharp decrease in content of toxic metabolites (*Anwar et al.,* 2015). The traditional procedure for detoxification is mentioned in Unani Pharmacopeia and detoxified nux-vomica is known as *Azaraqi mudabbar* (detoxified *Strychnos nux-vomica* L.). Review of the classical literature revealed that without detoxification of nux-vomica and over dosage of HAZ result in tachycardia, hyperthermia, hypertension, dyspnoea, weakness of limbs, myalgia, tonic– clonic convulsions and even death (Tariq, 2004).

The view of WHO on standardization and quality control of herbal drug clearly states that the process of physicochemical evaluation involving selection and handling of crude material, safety, efficacy and stability assessment of finished product needs to be properly scrutinized at every level (Folashade *et al.*,2012). Based on modern analytical techniques, *Habb-e-Azaraqi* needs attention in context of standardization for its scientific acceptability. Although, the formulation has been investigated for standardization constraints in the past such as total ash, acid insoluble ash, alcohol soluble and water-soluble matter but parameters like HPTLC Fingerprinting have not been evaluated earlier. In view of the immense benefits and significance of this formulation, the present study of HAZ was carried out to establish the standardization of HAZ complying with modern analytical techniques and its acute toxicity evaluation for its safety profile.

2. MATERIAL AND METHODS

2.1. Collection and authentication of ingredients of the formulation

All the ingredients of HAZ were procured from the pharmacy of National Research Institute of Unani Medicine for Skin Disorders, Hyderabad and herbal suppliers of Hyderabad, authenticated and identified by botanist and given voucher specimen numbers as SMPU/CRI-Hyd for *Azaraqi*-13552, *Filfil Daraz*-13553, *Filfil Siyah*-13554 and *Ajwayin* -13555. Calibrated analytical instruments were used and all the chemicals and solvents were of analytical grade.

2.2. Preparation of the formulation

HAZ was prepared at GMP certified pharmacy of National Research Institute of Unani Medicine for Skin Disorders (NRIUMSD), Hyderabad as per the composition of the formulation specified in National Formulary of Unani Medicine.

Table 1. Composition of *Habb-e-Azaraqi* (Anonymous, 1981).

S.no.	Name of drug	Quantity	Botanical name	Family	Part used
1.	*Azaraqi Mudabbar* (Detoxified)	20 gm	*Strychnos nux-vomica* L.	Loganiaceae	Seed
2.	*Filfil Siyah*	10gm	*Piper nigrum* L.	Piperaceae	Fruit
3.	*Filfil Daraz*	10gm	*Piper longum* L.	Piperaceae	Fruit
4.	*Araq-i- Ajwayin*	10gm	*Trachyspermum ammi* L.	Apiaceae	Seed distillate

2.3. Method of preparation

The ingredients of HAZ were cleansed separately for removal of any foreign material. *Azaraqi* was detoxified on the basis of traditional method mentioned in National Formulary of Unani Medicine for which it was buried in a pit of *Peeli Matti* (yellow clay), covered up with soil at which water was poured three times a day for 10 consecutive days in order to preserve the wetness of soil. On 11th day, *Azaraqi* was taken out and washed with normal water, the cotyledons were separated by peeling off the *testa* and the embryo part (*pitta*) was detached. It was again washed with hot water and draped in clean cloth. The clean cloth bag was than immersed in a container having milk and the milk was boiled till completely evaporated, taking care that the bag did not touch the bottom of the container. With this, the toxic metabolites of *Azaraqi* were separated in the milk and the bag containing *Azaraqi* was then removed, washed with normal water and now it is considered as *Azaraqi mudabbar* (detoxified *Strychnos nux-vomica* L.) (Anonymous, 2007).The other ingredients like *Filfil Siyah* and *Filfil Daraz* were powdered and filtered with sieve number 80 to obtain fine powder. The coarse powder of *Ajwayin*, soaked in purified water in the quantity 12 times of the drug for 24 hrs, was taken and transferred to the distillation plant along with the purified water to obtain *Araq-i-Ajwayin* (Seed distillate). The fine powder of *Filfil Siyah*, *Filfil Daraz*, *Azaraqi mudabbar* and *Araq-i-Ajwayin* were mixed uniformly to yield wet mass and dried at room temperature. The granules were taken to prepare HAZ tablets of 500 mg each by using automatic tablet making machine.

2.4. TLC (Thin layer chromatography) and HPTLC (High performance thin layer chromatography) evaluation

The chromatogram profile of HAZ was studied on three different batches and perceived under four detection systems viz., UV 366nm, UV 254nm, Iodine vapours and anisaldehyde sulphuric acid. The alcoholic extract of HAZ was used, for which 5 g of powdered HAZ was taken and reflux with 200 ml of alcohol using Soxhlet apparatus on a water bath for 30 min. The extract was then filtered and concentrated to 5 ml and this HAZ obtained was used for thin layer chromatography. The alcoholic extract was applied on TLC plate and was spotted on silica gel "G" plate and developed with Toluene: Ethyl Acetate: (8:2, v/v) as mobile phase. The R_f values of the spot were recorded through the software and calculated by the following formula-R_f Value=Distance travelled by spot/ Distance travelled by solvent.

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Fig. 1 Detoxification process and finished product (*Habb-e-Azaraqi*)

2.4.1. Method conditions
Make of Instrument: Desaga Sarstedt Gruppe (Germany),
Development Chamber: 20 X10 cm, Twin-trough chamber
Stationary phase: Pre coated silica gel 60 F254 Aluminium plates (Merck, KgaA, Germany)
Plate thickness: 0.2 mm
Plate size: 200 x 100 mm
Distance from starting: 20 mm
Distance from bottom: 10 mm
Volume applied: 5 µl
Band length: 10 mm
Distance between tracks: 20 mm
Development distance: 80 mm
Solvent used: HPLC grade
Extract storage vials: 5 ml glass vial
Mobile phase Used: Toluene: Ethyl acetate = 8: 2, v/v

2.4.2. Development of HPTLC technique
The developed TLC plates were dried completely and detected with UV Cabinet system for detection of spots at 366nm, 254nm and also under Iodine vapours and after derivatizing with Anisaldehyde sulphuric acid reagent. Moreover, it was scanned with Densitometer CD60 of DESAGA Sarstedt Gruppe system that exhibited typical densitogram in which peaks appeared for the corresponding spots being detected in the densitometer and the peak areas under the curve correspond to the concentration of the component in HAZ. The separation of the components in the formulation and its Rf values were recorded.

2.5 Acute toxicity evaluation
The study was approved by the Institutional Animals Ethics Committee (IAEC) meeting vide protocol no. CRIUM/IAEC/2017/01/P07, held at NRIUMSD, Hyderabad in the month of July 2017. CPCSEA guidelines of laboratory animal care was followed throughout the experiment (CPCSEA, 2010).

2.5.1. Experimental animals
Animals were procured from National Institute of Nutrition (NIN), Hyderabad and treated with standard feed pellets and water ad libitum, unless stated otherwise. Sprague Dawley rats (100 ± 20 g), females were acclimatized to the laboratory conditions of Animal House, NRIUMSD, Hyderabad, for one week before using them for experiment. The selected females were nulliparous and non-pregnant. Rats were housed in polycarbonate cages in the air-conditioned room maintained at the temperature of 22°C ± 3°C and relative humidity of 30-70%, with a 12:12 h light/dark illumination cycle (OECD, 1998).

2.5.2. Dose selection
A limit dose of 2,000 mg/kg bw per day was used for acute toxicity study (ICH, 2009). Aqueous carboxy methyl cellulose (CMC) 0.3% was used as vehicle.

2.5.3. Drug administration and duration of therapy
The drug was administered orally as suspension of HAZ in 0.3% CMC using mortar pestle and duration of therapy was 14 days.

2.5.4. Experimental design of acute toxicity study
The acute toxicity of HAZ was evaluated as per OECD Guidelines 425, following oral administration of a single dose and observation for 14 days. Overnight fasting of animals prior to dosing was done. Three female SD rats were used as per the limit test method by administering 2000 mg/kg bw orally to each animal one after another at an interval of 48 hrs. Firstly, one female rat was administered the dose using stainless steel feeding cannula and observations were done for its survival and consequently all the three animals were given the dose and observed for 14-day period (OECD, 2008). Observations for mortality and morbidity twice a day was done in all the experimental animals throughout the study period. A keen watch was kept on the day of dosing for first 1 hour till next 6 hours. Thorough clinical observations (i.e., functional observation parameters) were made periodically to detect signs of toxicity. Body weight of the animals was recorded prior to dosing and then once in a week. Average feed intake was recorded at weekly interval by weighing the amount of feed and leftovers on the next day. All animals were then subjected to gross necropsy at the culmination of study duration. Organs and tissues were examined macroscopically for toxic lesions and internal organs / tissues were isolated, trimmed and weighed. Organs / tissues were preserved in the neutral buffer formalin and data was noted.

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3. RESULTS

3.1 TLC and HPTLC outcomes

The TLC studies of alcoholic extract of HAZ with Toluene: Ethyl Acetate: (8:2, v/v) as mobile phase in three different batches detected under four detection system are as follows-

• Under UV 366nm- The chromatogram profile showed eight major spots at R_f values 0.14 (blue), 0.28 (blue), 0.35 (blue), 0.42 (yellow), 0.50 (light blue), 0.64 (light blue), 0.71(light blue),0.85 (light blue).

• Under UV 254nm- The chromatogram profile showed three spots at R_f values 0.35, 0.42, 0.57 (All black).

• Under Iodine vapours- HAZ showed one spots at R_f values 0.32 (brown).

• Under anisaldehyde sulphuric acid and heating at 105°C- HAZ showed three spots at R_f values 0.47 (dark blue),0.50 (light blue), 0.57(light blue)

The TLC plate under densitometer scanning revealed the typical densitogram having corresponding peaks for the separated components. The corresponding R_f values by the HPTLC software was represented graphically.

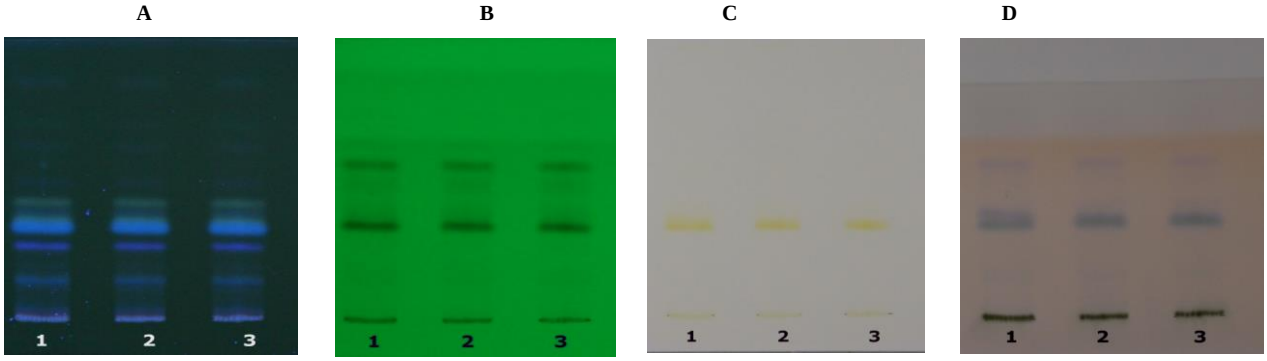


Fig. 2TLC of Alcoholic Extract of HAZ indicating A (at UV 366nm), B (at UV 254nm), C (Under Iodine vapours) and D (Under anisaldehyde sulphuric acid).

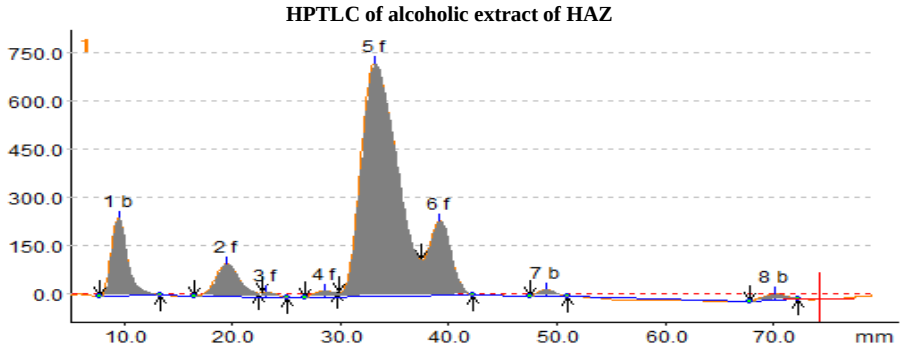


Fig.3 Densitogram of alcoholic extract of *Habb-e-Azaraqi* at UV 366nm

Table 2. Peak list of alcoholic extract of *Habb-e-Azaraqi* at UV 366nm

Peak no	Y-Pos	Area	Area %	Height	R_f value
1	9.50	409.60	9.98	239.99	0.01
2	19.4	259.00	6.31	101.42	0.14
3	23.1	22.22	0.54	15.45	0.20
4	28.5	37.41	0.91	18.64	0.27
5	33.1	2745.32	66.92	719.33	0.33
6	39.1	551.65	13.45	230.19	0.42
7	49.0	32.81	0.80	18.84	0.56
8	70.2	44.49	1.08	17.70	0.85

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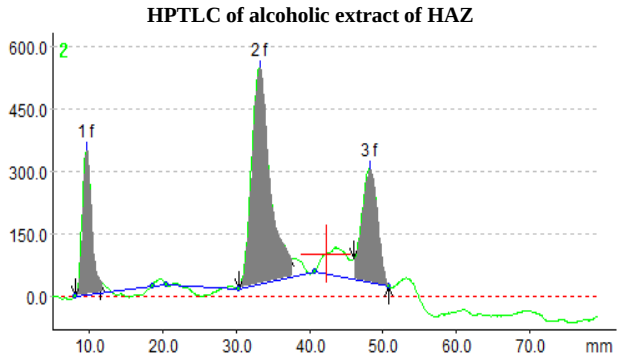


Fig. 4 Densitogram of alcoholic extract of *Habb-e-Azaraqi* at UV 254nm

Table 3. Peak list of alcoholic extract of *Habb-e-Azaraqi* at UV 254nm

Peak no	Y-Pos	Area	Area %	Height	R_f value
1	9.6	493.88	18.79	340.57	0.01
2	33.2	1472.21	56.00	508.65	0.34
3	48.2	662.94	25.22	268.83	0.54

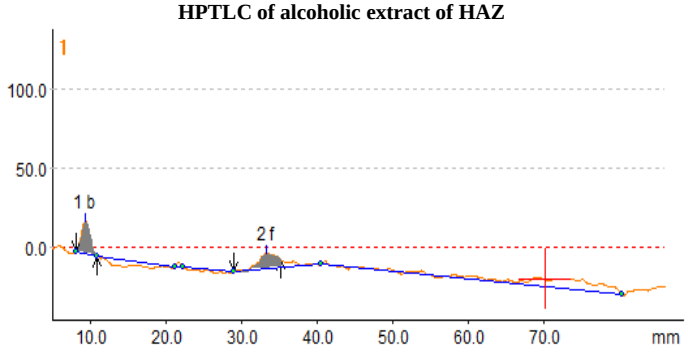


Fig. 5 Densitogram of alcoholic extract of *Habb-e-Azaraqi* Upon exposure to Iodine vapour

Peak no	Y-Pos	Area	Area %	Height	R_f value
1	9.4	27.20	50.22	20.13	0.01
2	33.4	26.96	49.78	9.47	0.34

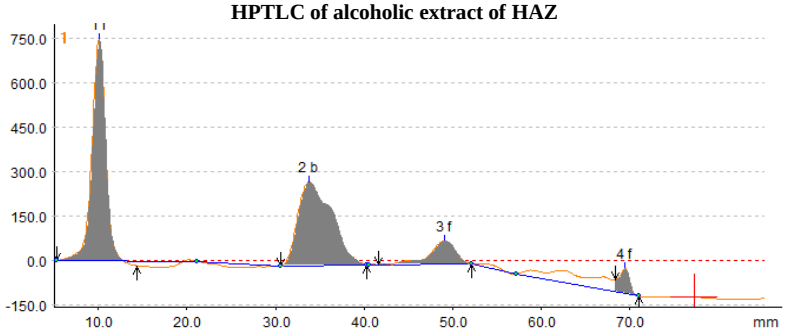


Fig. 6 Densitogram of alcoholic extract of *Habb-e-Azaraqi* upon derivatized withanisaldehyde sulphuric acid

Table 5. Peak list of alcoholic extract of *Habb-e-Azaraqi* upon derivatized with anisaldehyde sulphuric acid at 580nm.

Peak no	**Y-Pos**	**Area**	**Area %**	**Height**	**R_f value**
1	10.1	1312.51	44.68	735.30	0.02
2	33.8	1236.95	42.10	278.56	0.34
3	49.1	272.76	9.28	78.52	0.56
4	69.4	115.68	3.94	84.48	0.84

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3.2. Acute toxicity study outcomes

- Mortality- There were no deaths either on the day of treatment or throughout the 14-day post treatment observation period.
- Clinical Observations- No signs of systemic toxicity were observed throughout the observation period.
- Body weight- No loss of body weight was noticed in any of the rats and probable body weight gain were accredited.
- Feed and water consumption- Feed and water intake was found to be within limits.
- Laboratory results- Biochemistry and haematological data showed all parameters within normal limits.
- Necropsy- All the three animals survived until the scheduled necropsy on Day 15. No abnormalities were noted and all organs of rats proved to be free of HAZ treatment related gross pathological changes.

Table 6. Summary of Clinical Observations

Animal ID	Sex	HAZ Dose (mg/kg bw)	Clinical Observations
F1	Female	2000	No treatment related toxic sign & symptoms or mortality was observed.
F2	Female	2000	No treatment related toxic sign & symptoms or mortality was observed.
F3	Female	2000	No treatment related toxic sign & symptoms or mortality was observed.

Table 7. Body Weight data of HAZ treated rats

Day of study period	Sex	HAZ Dose (mg/kg bw)	Weight in grams		
			F1	F2	F3
Day 1	Female	2000	181.3	190.8	186.3
Day 7	Female	2000	192.3	201.5	195.2
Day 14	Female	2000	218.1	234.3	222.4
Mean ± S.D			197.23 ± 15.42	208.8 ± 18.41	201.3 ± 15.35

Table 8. Feed Consumption data of HAZ treated rats

Day of study period	Sex	HAZ Dose (mg/kg bw)	Weight in grams		
			F1	F2	F3
Day 1	Female	2000	14.6	20.2	17.3
Day 7	Female	2000	21	22.8	20.9
Day 14	Female	2000	16.2	17.2	16.8
Mean ± S.D			17.26 ± 2.71	20.06 ± 5.23	18.33 ± 1.82

Table 9. Summary of Necropsy Findings

Animal ID	Sex	HAZ Dose (mg/kg bw)	Necropsy Findings
F1	Female	2000	No noteworthy finding was observed in the animal, which was sacrificed on day 15th after dosing
F2	Female	2000	No noteworthy finding was observed in the animal, which was sacrificed on day 15th after dosing
F3	Female	2000	No noteworthy finding was observed in the animal, which was sacrificed on day 15th after dosing

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Table 10. Summary of Hematology of HAZ treated rats

Parameters	F1	F2	F3
Hb (gm%)	11.8	16.2	16.6
RBC (million/mm^3)	6.0	8.4	7.8
WBC (million/ mm^3)	7200	5600	3400
Platelet (lakhs/ mm^3)	4.4	7.0	7.2
HCT (%)	42	40	42
Neutrophil (%)	02	02	02
Lymphocyte (%)	96	94	93
Eosinophil (%)	02	02	03
Monocyte (%)	01	02	02

Table 11. Summary of Hematology of HAZ treated rats

Parameters	F1	F2	F3
Glucose (fasting) (mg/dl)	68	115	74
AST (IU/L)	216	159	158
ALT (IU/L)	60	42	53
Bilirubin (mg/dL)	0.34	0.29	0.34
ALP (IU/L)	101	99	79
Total Protein (g/dL)	6.6	5.9	6.3
Albumin (g/dL)	5.0	4.9	5.6
Globulin (g/dL)	1.6	1.0	0.7
BUN (mg/dL)	21.9	21.9	22.8
Creatinine (mg/dL)	0.7	0.8	0.7
Uric acid mg/dL)	2.1	2.2	2.3
Sodium ions (mmol/L)	141	140	141
Potassium ions (mmol/L)	4.3	3.7	3.6
Chloride ions (mmol/L)	106	105	106
Calcium ions (mmol/L)	1.8	1.8	1.7

DISCUSSION

The traditional medicines are often questioned for their paucity of information and scientific credibility and the solution can be pharmacological evidence of safety and efficacy, development of standardized dosage forms and quality control studies. The formula and method of preparation of HAZ for the present study was selected from

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National Formulary of Unani medicine and the work for the present study was aimed into two parts i.e., TLC and HPTLC profile and acute toxicity evaluation of HAZ. From the present study, it is evident that HAZ is well standardized for its qualitative aspect and can be used as safe therapy in individuals as proved by toxicity study.

4.1. TLC and HPTLC profile

Drug standardization is the need of the hour to ensure the quality of the drug. Additionally, the fact that herbal drugs contain several ingredients, there should be quality control studies for the entire preparation as well as for every single drug present in that to ensure the quality of herbal medicines (Purohit *et al.,* 2004). Thin layer chromatography is a physical method of separation in which the components to be separated are distributed between stationary and mobile phases that embraces a distinct place in qualitative as well as quantitative analysis, thereby assuring the purity of a compound. It is based on the principle of adsorption chromatography or partition chromatography or combination of both depending on adsorbent and nature of solvents employed. If the drug is adulterated, there might be appearance of the other compounds present in adulterant, in turn may increase the number of spots. On the other hand, the exhausted or deteriorated drugs may lose the component and the number of spots appeared might be less when investigated. Thus, HPTLC is a modern analytical technique that reveal disparity in the number of spots in TLC plate in context of adulteration. In this respect, HAZ was standardized for regulating the therapeutic efficacy on the modern parameters such as TLC and HPTLC for global recognition and acceptance as a validated product. Modern scientific tool and technologies of standardization adopted in this study will serve as a noteworthy factor relating quality assurance which is a necessity nowadays. The systematic approach of this study will aid in laying down pharmacopeial standards and strengthening the outcome of quality products.

4.2. Acute toxicity study

Herbal drugs being efficacious are always not safe and needs proper analysis in terms of toxicity studies for potential adverse responses and interactions. For the safety profile of these drugs, animal models are used for understanding the safety hazards and the mechanism of a particular toxicity. The widespread therapeutic use of HAZ in Unani system of medicine without scrutiny of its toxicity profile was long due in the research field and therefore this study was taken up for acute toxicity study. Acute toxicity studies unveil the significant short term adverse/toxic effects with respect to behavioural signs of toxicity, body weight gain, feed intake, haematology parameters, biochemistry profile and gross necropsy. Laboratory investigations is done to find out the extent of the adverse effect on blood and is vital because changes in the haematological and biochemistry parameters have noteworthy correlation to human toxicity upon translation of preclinical statistics. OECD guideline 425 symbolizes the drug to be safe when LD-50 dose is 2000 mg/kg and above. The present study provides detailed evidence of acute toxicity profile of HAZ. As no mortality reported and there were no signs of toxicity detected with respect to gross examination, haematology and clinical chemistry profile, HAZ may be considered as non-lethal and the LD-50 is greater than 2000 mg/kg in rats. Moreover, based on the present results, HAZ with its extensive past of its clinical use in the Unani system of medicine is confirmed as non-toxic and safe formulation in the tested experimental conditions. With the study, the authors provided a substantial proof of the safety of HAZ and it may be considered as a suitable treatment option for use in therapeutic dose in humans.

CONCLUSION

Habb-e-Azaraqi, an effective Unani drug especially for nervine disorders was studied using existing modern technical standard and subjected to significant and scientific parameter of standardization i.e., TLC and HPTLC analysis that proved its identity and purity. The data can be used as standards for future evaluation and reference which in turn may help in the development of standard parameters of various other Unani formulations too. The traditional method of detoxification effectively lessens the toxicity of nux-vomica and therefore, detoxified and standardized HAZ was used for toxicity assessment. The acute toxicity study of HAZ, not performed earlier, presented no toxic sign and symptoms or mortality in any of the animals. Based on the study information, the oral LD50 of HAZ in the female sprague-dawley rats showed NOAEL (No Observed Adverse Effect Level) and this acute study toxicity confirmed the safe profile of HAZ for short term therapy. Further studies are warranted for future research endeavours to ensure probable challenges in standardization and safety studies.

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CONFLICT OF INTEREST

None

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