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Evaluation of heavy metals, aflatoxins, pesticide residues and microbial load of Lactuca sativa L. (Tukhm-e-Kahu) used for Insomnia

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Abstract

Introduction and Background

Seeds of *Lactuca sativa* known as Tukhm-e-Kahu in Traditional medicine are highly valued for its therapeutic effect in diverse ailments including Insomnia. Despite its established use, it remains inadequately characterized regarding safety analysis as per WHO criterion. Its high fixed-oil content facilitates the retention of lipophilic contaminants and fungal proliferation, potentially compromising its chemical and microbiological safety. Comprehensive contaminant and safety assessment is therefore essential to establish its quality and ensure its safe therapeutic use.

Materials and methods

Authenticated, voucher-deposited *Lactuca sativa* L. seeds (Tukhm-e-Kahu) were subjected to a systematic assessment of chemical and microbiological quality. Arsenic, lead, cadmium and mercury were quantified by inductively coupled plasma–mass spectrometry (ICP-MS). Aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using matrix-matched calibration and an isotopically labelled internal standard. Pesticide residues were analysed by gas chromatography-tandem mass spectrometry (GC-MS/MS). Microbial load and specified pathogenic microorganisms were also assessed to evaluate the microbiological quality and safety of the crude drug.

Results

Lead, arsenic, cadmium, and mercury levels were below 5% of their respective permissible limits. All four aflatoxins were below the quantification limit of 1.0 µg/kg and no pesticide residues were detected at the reporting limit. The total aerobic microbial count and yeast and mould count were within the specified acceptable limits, and none of the specified pathogenic microorganisms were detected. All evaluated parameters complied with the applicable quality and safety limits specified in relevant WHO/API guidelines.

Conclusion

These comprehensive findings demonstrate that *Lactuca sativa* L. (Tukhm-e-Kahu) met the applicable quality and safety specifications across all parameters evaluated with no significant chemical or microbiological concerns identified. The findings provide product-specific safety evidence supporting the quality and suitability of Tukhm-e-Kahu for its intended therapeutic use.

1. Introduction

Unani System of Medicine (USM) is one of the oldest traditional systems that continues to be practised worldwide and utilizes the treasure from natural resources including plant, animal and mineral origin drugs. It has gained substantial global recognition in recent decades. Of the 194 WHO Member States, 170 have reported practising traditional medicine, and among the responding countries, 67% reported that 40–99% of their populations use traditional, complementary, or integrative medicine (WHO, 2024).

USM foundations lies in the Hippocratic (Buqrat) doctrine of the humours, which was further developed by Galen (Jalinoos) and subsequently organised into a coherent system of theory and practice by eminent Arab and Persian physicians during the medieval period, particularly Rhazes (Razi) and Avicenna (Ibn Sina) (Parveen et al., 2022). The system reached the Indian subcontinent in the eleventh century and has been practised there continuously ever since (Parveen et al., 2022).

This widespread use of USM underscores the need for rigorous standards of quality, safety, efficacy, and pharmacovigilance. The quality and safety of herbal medicine depends substantially on the quality of their raw materials, which may be influenced by cultivation, collection, drying, transportation, and storage conditions (WHO, 2007).

Lactuca sativa L. (*Tukhm-e-Kahu*) occupies a prominent place in USM. Classical authorities attribute to it refrigerant (*mubarrid*), sedative (*musakkin*), hypnotic (*munawwim*) and anaesthetic (*mukhaddir*) actions, rendering it especially suitable for disorders arising from excessive heat and dryness (Khan, 2018; Ghani, 2011; Ibn Baitar, 2000; Kabiruddin, 2000). Phytochemically, the seed yields approximately 30–40% fixed oil, composed predominantly of linoleic and oleic acids, together with proteins, phenolic compounds, flavonoids, phytosterols and tocopherols (Kim et al., 2022; Said et al., 1996). Experimental investigations have further demonstrated sedative, hypnotic, anxiolytic, antioxidant, neuroprotective and anti-inflammatory activities (Ahn et al., 2023; Malik et al., 2022; Kim et al., 2017; Ismail et al., 2015; Harsha et al., 2013; Yakoot et al., 2011), thereby lending preclinical support to its long-standing traditional use in Insomnia (*Sahar)*.

Insomnia is a prevalent sleep disorder characterised by persistent difficulty initiating or maintaining sleep, or early-morning awakening with an inability to return to sleep, occurring at least three nights per week despite adequate opportunity for sleep and resulting in clinically significant daytime impairment (AASM, 2023; APA, 2023; Jameson, 2018). Persistent insomnia is associated with an increased risk of cardiovascular, metabolic, neurological, and psychiatric disorders (Khara et al., 2024; Anderson et al., 2018; Brevik et al., 2017; Ylikoski, 2015; Gehrman et al., 2014). Globally, clinically relevant insomnia affects approximately 16.2% of adults, representing more than 850 million individuals, while 6–10% experience symptoms meeting the diagnostic criteria for insomnia disorder (Benjafield et al., 2025; Vgontzas et al., 2002). Its aetiology is multifactorial, involving psychological, physiological, biological, behavioural, and environmental factors. Although pharmacological therapies can provide symptomatic benefit, several hypnotic agents, particularly when used inappropriately or for prolonged periods, may be associated with adverse effects such as tolerance, dependence, psychomotor impairment, falls, and cognitive effects, particularly in older adults (Brandt et al., 2024). The substantial prevalence and health

burden of insomnia therefore support continued investigation of therapeutic approaches that are effective, safe, and supported by robust scientific evidence (Riemann et al., 2017; Morin et al., 2012; Edinger et al., 2005).

2. Need of the Study

Despite significant therapeutic potential, medicinal plant materials may be exposed to a range of contaminants during cultivation, processing, and storage. Potentially toxic elements such as arsenic, lead, cadmium, and mercury may enter medicinal plants through contaminated soil, irrigation water, atmospheric deposition, and industrial sources (Jomova et al., 2025; Validandi et al., 2025; Asiminicesei et al., 2024). Medicinal seeds having oil and mucilage or water content particularly are susceptible to fungal contamination and aflatoxin production, particularly under improper storage conditions, while pesticide residues may persist following agricultural application and post-harvest handling (Jiang et al., 2026; Wang et al., 2022; IARC, 2012). Inadequate drying and storage may additionally promote microbial proliferation and contamination with pathogenic or opportunistic microorganisms (Shekhar et al., 2024; Opuni et al., 2023). Consequently, pharmacopoeial and international standards prescribe permissible limits and quality-control requirements for these contaminants in medicinal plant materials (Islam, 2026; Khan et al., 2025; WHO, 2007).

Despite the established traditional use and reported pharmacological potential of *Tukhm-e-Kahu*, comprehensive data on its quality and safety with respect to potentially heavy metals, aflatoxins, pesticide residues and microbial contamination remain limited. To address this research gap, the present study systematically evaluates the quality and safety of *Lactuca sativa* L. seeds (*Tukhm-e-Kahu*) against established permissible limits by quantifying potentially toxic elements using inductively coupled plasma mass spectrometry (ICP-MS), aflatoxins using liquid chromatography-tandem mass spectrometry (LC-MS/MS), and pesticide residues using gas chromatography-tandem mass spectrometry (GC-MS/MS). In addition, total bacterial and fungal counts are determined and the samples are also screened for specific pathogens like *Escherichia coli, Salmonella* spp, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

3 Materials and methods

3.1 Study Design

The present work was designed as a laboratory-based analytical study intended to establish the contaminant safety profile of the single Unani drug Tukhm-e-Kahu, the seed of *Lactuca sativa* L. (Asteraceae). Four contaminant categories recommended by the World Health Organization (2007) and specified in the pharmacopoeial standards for ASU (Ayurvedic, Siddha and Unani) drugs were investigated, namely toxic heavy metals, aflatoxins, pesticide residues and microbial load. Sample collection, authentication and preparation of the powder were carried out in the Department of Ilmul Advia, Faculty of Unani Medicine, Aligarh Muslim University (AMU), Aligarh, India, while the instrumental

determinations and microbiological assays were performed at AGSS Analytical and Research Lab. The results obtained were benchmarked against the permissible limits prescribed by the Unani and Ayurvedic Pharmacopoeia of India and the World Health Organization.

3.2 Instrumentation

Heavy metals were determined on an Agilent 7700 inductively coupled plasma mass spectrometer (ICP-MS) coupled to a closed-vessel microwave digestion system. Aflatoxins were quantified on an Agilent 1290 Infinity II liquid chromatograph coupled to an Agilent 6470 triple-quadrupole mass spectrometer fitted with an Agilent Jet Stream electrospray ionisation source. Pesticide residues were screened on an Agilent 7000D triple-quadrupole gas chromatograph-mass spectrometer (GC-MS/MS). The ancillary equipment comprised a temperature-controlled incubator (Amaze AI-216, India), a hot-air oven (NSW-143, India), an analytical balance (Ultima Smart Ti10), a vortex mixer (Bio Vortex V1, Taurus Scientific), a refrigerated centrifuge (REMI CM-08 Plus, India) and a laminar air-flow cabinet (ThermoFisher Scientific, India). All sample preparation, extraction and analytical operations were carried out in borosilicate glassware (Borosil Limited, India) which was acid-washed in 10% nitric acid overnight and rinsed with Milli-Q water before use in trace-metal work.

3.3 Chemicals, reagents and media

Heavy metal analysis

Certified aqueous calibration standards of lead, cadmium and arsenic (1000 µg/mL) and of mercury (10 µg/mL) were procured from Charlau Chemie, Spain. Ultra-pure nitric acid and hydrochloric acid used for digestion and for the preparation of calibrants were supplied by Merck, India. Ultrapure water (18.2 MΩ·cm) was drawn from a Milli-Q purification unit (Millipore, Bedford, USA).

Aflatoxin analysis

Analytical standards of aflatoxins B_1, B_2, G_1 and G_2 were obtained from Sigma-Aldrich (St. Louis, USA). Acetonitrile and LC-grade methanol were purchased from Merck (Darmstadt, Germany). Anhydrous magnesium sulphate, sodium chloride and the dispersive solid-phase extraction sorbents used for clean-up were of analytical grade.

Microbiological assessment

Soyabean-casein digest agar (M290-500G), Sabouraud dextrose agar MH063-500G), MacConkey agar (M008S-500G), Mannitol salt agar (MH118-500G), cetrimide agar (M1742-100G), lactose broth (M1003-500G), buffered sodium chloride-peptone solution of pH 7.0 (MM1275-500G), *Salmonella* agar and polysorbate-20 R (TC287M-100 ml) were obtained from HiMedia Laboratories, Mumbai, India. Sterile Petri plates, micropipettes and other routine consumables were used as and when required.

All reagents and standards were of analytical or LC-MS grade, and fresh batches were procured so that each was used well within its stated shelf life.

3.4 Plant material

3.4.1 Procurement

Seeds of *Lactuca sativa* L. (Tukhm-e-Kahu) were procured after taking Official permission from the Head of the Department in September 2025 under Batch No. 192/DTC from MSME Lab Dawakhana Tibbiya College, Aligarh Muslim University, Aligarh, India which manufacture the Traditional formulations on a global scale as per the National guidelines.

A single batch of 1 Kg was obtained so that the entire analytical programme was carried out on homogeneous material.

3.4.2 Authentication and voucher deposition

The plant material was authenticated in the Pharmacognosy Laboratory, Department of Ilmul Advia, Faculty of Unani Medicine, AMU, Aligarh by Dr Sumbul Rehman through macroscopic, microscopic and organoleptic examination and comparison with authentic reference material and the standard pharmacognostic descriptions given in The Unani Pharmacopoeia of India. A voucher specimen (Voucher No. SC-490/26) was deposited in the *Ibn-e-Baitar* Museum of the same department for future reference and verification. A representative photograph of the authenticated seed is presented in Fig. 1.

Table 1
for Drug details

Unani Name	Botanical Name	Family	Part Used	Voucher No.
Tukhm-e-Kahu	*Lactuca sativa* L.	Asteraceae	Seed	SC – 490/26

3.4.3 Preparation of the powder

Prior to pulverization, the seeds were spread out and freed of chaff, husk, dust, immature and damaged seeds, and other extraneous matter by hand picking. The cleaned seeds were dried in a hot-air oven maintained at a temperature not exceeding 40°C until a constant weight was obtained, so that the fixed oil of the seed was not subjected to thermal stress. Drying was followed by pulverization in an electric grinder at the Murakkabat Laboratory, Department of Ilmul Advia, AMU and the powder so obtained was passed through sieve no. 80 to obtain a uniform particle size. The sieved powder was transferred to an airtight amber container and stored at ambient temperature, protected from light and moisture, until analysis. All subsequent determinations were performed on this single homogenised powder.

3.5 Determination of heavy metals

3.5.1 Instrumental conditions

Lead, cadmium, arsenic and mercury were determined by ICP-MS on an Agilent 7850 instrument, adopting with minor modification the procedure described for Unani formulations by Razi et al. (2026).

The instrument was configured with a micro-mist nebuliser, a quartz spray chamber, a quartz torch with nickel sampling and skimmer cones, and a mass spectrometric detector. Digested samples were delivered to the plasma through an auto-sampler. Acquisitions were made in helium collision mode with an ORS4 octapole collision/reaction cell, which suppresses the polyatomic interferences generated by the carbon-rich matrix, and the instrument was tuned before each run under the optimised auto-tune conditions of MassHunter 4.4 software.

3.5.2 Preparation of calibration standards

Working calibrants were prepared from the certified reference solutions on the day of analysis. For lead, cadmium and arsenic, 1 mL of the respective 1000 µg/mL stock was transferred accurately to a 100 mL volumetric flask and made up to the mark with 5% nitric acid in Milli-Q water. Mercury was treated separately: 1 mL of the 10 µg/mL stock was diluted to 100 mL with a mixture of 3% nitric acid and 2% hydrochloric acid in Milli-Q water, the chloride being included to stabilise the element in solution. These intermediate solutions were stored under refrigeration, protected from light, and served for the construction of the calibration curves. Calibration series were obtained by further dilution of aliquots with 5% nitric acid in Milli-Q water, covering 0.1–100 µg/L for lead, cadmium and arsenic and 0.1–10 µg/L for mercury. Linearity was accepted where the correlation coefficient of the calibration curve was not less than (0.999).

2.5.3 Sample digestion

Sample processing comprised homogenisation, acid digestion and dilution. A quantity of 0.50 ± 0.01 g of the dried, homogenised seed powder was weighed accurately into a PTFE vessel, 10.0 mL of concentrated ultra-pure nitric acid was added and the vessel was sealed. Because the seed carries a high proportion of fixed oil, the charged vessels were allowed to stand for 15 min before the programme was initiated, so that the initial exothermic reaction subsided. Digestion was carried out in a microwave digester at a constant power of 1000 W in three ramped stages: to 110°C over 20 min with a hold of 5 min; to 145°C over a further 10 min with a hold of 5 min; and finally, to 190°C over 10 min with a hold of 10 min. The vessels were cooled to room temperature and vented, and the digests were made up to 50 mL with Milli-Q water, filtered through a [0.45 µm] membrane and diluted hundred-fold prior to aspiration. Digests were examined visually and were accepted for analysis only when clear and free of particulate residue.

3.6 Determination of microbial load

The microbiological quality of the seed powder was assessed at AGSS Analytical Laboratory following the standard protocol recommended for herbal materials by the World Health Organization (2007) and the corresponding pharmacopoeial provisions. All interventions were carried out aseptically in a laminar air-flow cabinet, media were prepared as per the manufacturer's instructions and sterilised by autoclaving at 121°C for 15 min and plane controls were incubated alongside the test plates in every run. Determinations were made in triplicate and the mean values were recorded.

3.6.1 Pre-treatment of the test drug

The sample was first diluted to a level at which any inhibitory activity of the drug itself was neutralised, the mode of dispersion being selected according to the physical nature of the material. Buffered sodium chloride-peptone solution of pH 7.0 (MM1275-500G, HiMedia Laboratories, Mumbai, India) was used as the diluent. Being an oily, water-insoluble powder, Tukhm-e-Kahu was dispersed by the procedure prescribed for non-water-soluble substances: 10 g of the powder was suspended in lactose broth (M1003-500G, HiMedia Laboratories), a medium shown to possess no antibacterial activity under the test conditions, with the addition of a suitable solution of polysorbate 20 R as the wetting and inactivating agent. The pH of the suspension was adjusted to approximately 7.0 and the volume was made up to 100 mL with the same medium. Method suitability was established beforehand by inoculating separate portions of the suspension with viable cultures of *Escherichia coli*, *Salmonella* spp., *Pseudomonas aeruginosa* and *Staphylococcus aureus* and confirming that the test material did not inhibit their recovery under the conditions employed.

3.6.2 Total aerobic bacterial and fungal counts

The suspension was shaken vigorously so that the microorganisms were evenly dispersed. For the stock, 5 mL of the suspension was mixed with 95 mL of sterile distilled water and subjected to tenfold serial dilution, 1 mL of the stock being transferred aseptically into 9 mL of sterile distilled water. Bacterial and coliform counts were obtained by plating the dilutions with about 15 mL of liquefied soybean-casein digest agar, while for fungal counts 1 mL of the suspension was streaked on Sabouraud dextrose agar plates. The bacterial plates were incubated at 30–35°C for 48–72 h and the fungal plates at 20–25°C for 5 days. After incubation, the colonies were counted and the total aerobic bacterial count and the total yeast and mould count were calculated with reference to the dilution factor and expressed as colony-forming units per gram of the drug.

3.6.3 Tests for specified pathogens

The pre-treated and neutralised sample was further examined for the absence of specified pathogens. Enrichment was carried out in lactose broth at 30–35°C for 24–48 h, after which portions of the enriched culture were subcultured on Salmonella agar for *Salmonella* spp, MacConkey agar for *Escherichia coli*, Mannitol salt agar for *Staphylococcus aureus* and Cetrimide agar for *Pseudomonas aeruginosa*. The plates were incubated at 30–35°C for 24–48 h and examined for characteristic colonial morphology, suspect colonies being confirmed by (Gram staining and biochemical identification). The sample was regarded as complying where no growth characteristic of the respective organism was observed in 1 g of the material.

3.7 Determination of aflatoxins

Aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2) were determined by Agilent 6475 liquid chromatography-tandem mass spectrometry (LC-MS/MS) after extraction by a QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) procedure (Anastassiades et al., 2003), modified from Sirhan et al.

(2013) and Ouakhssase et al. (2019, 2023). QuEChERS extraction coupled with LC-MS/MS is now the preferred approach for mycotoxins in medicinal plants and herbal products, offering multi-analyte capability and low detection limits in complex botanical matrices (Huang et al., 2025; Kshirsagar et al., 2026), a matter of importance given the documented occurrence of aflatoxins in medicinal plants worldwide (Wang et al., 2024). Because aflatoxins are photolabile, all solutions were handled in amber glassware and exposure to daylight was minimised throughout the analysis.

3.7.1 Preparation of standard and working solutions

An intermediate mixed standard solution was prepared by diluting 125 µL each of the AFB_1 and AFG_1 standards and 450 µL each of the AFB_2 and AFG_2 standards to 5 mL with acetonitrile-water (50:50, v/v). The resulting solution contained 0.050 µg/mL each of AFB_1 and AFG_1, 0.047 µg/mL of AFB_2 and 0.045 µg/mL of AFG_2. The individual standards and the intermediate mixed solution were stored in amber flasks at -18°C until use.

3.7.2 Calibration

Aliquots of 5, 10, 15, 20 and 25 µL of the intermediate mixed solution were each diluted to 1 mL to give five calibration levels: 0.25, 0.50, 0.75, 1.00 and 1.25 ng/mL for AFB_1 and AFG_1; 0.235, 0.470, 0.705, 0.940 and 1.175 ng/mL for AFB_2; and 0.225, 0.450, 0.675, 0.900 and 1.125 ng/mL for AFG_2. In view of the lipid-rich nature of *L. sativa* seed, the calibration levels were prepared in blank *L. sativa* seed extract previously confirmed to be free of aflatoxins, and quantification was carried out against these matrix-matched standards so as to compensate for ionisation suppression by co-extracted material.

3.7.3 Sample preparation

Seeds of *L. sativa* were ground in a laboratory mill and passed through a 80 No. mesh sieve, and the powder was homogenised and stored in airtight amber containers at 4°C until analysis. One gram of the powdered seed was weighed into a 50 mL polypropylene centrifuge tube and extracted with 3 mL of methanol- acetonitrile (60:40, v/v) by vortexing for 1 min. Anhydrous magnesium sulphate (1.32 g) and sodium chloride (0.25 g) were added and the tube was shaken vigorously for a further 1 min (Ouakhssase et al., 2019, 2023). After centrifugation at 4000 rpm for 5 min, 0.5 mL of the upper organic layer was cleaned up by dispersive solid-phase extraction (d-SPE) with C18 sorbent (50 mg per mL of extract, the amount being increased to account for the high lipid content of the seed) and anhydrous magnesium sulphate (150 mg per mL of extract) (Lehotay et al., 2005), vortexed for 30 s and centrifuged again under the same conditions. The supernatant was filtered through a 0.2 µm regenerated cellulose syringe filter and injected directly into the LC-MS/MS system.

3.7.4 Mobile phase and gradient elution

Three mobile-phase combinations were evaluated during method development, eluent A being water in each case and eluent B being acetonitrile, methanol, or methanol containing 5 mM ammonium acetate. On the basis of peak shape, sensitivity and repeatability, methanol containing 5 mM ammonium acetate

was adopted as eluent B for all subsequent analyses. The proportion of eluent B was increased linearly from 10% to 100% over 4 min, held at 100% for 2 min and returned to 10% for 3 min of column re-equilibration, giving a total run time of 9 min.

3.7.5 Chromatographic and mass spectrometric conditions

Analyses were performed on an Agilent 1290 Infinity II UHPLC system (flexible pump, vial sampler and multicolumn thermostat) coupled to an Agilent 6475 triple-quadrupole mass spectrometer equipped with an Agilent Jet Stream electrospray ionisation (AJS-ESI) source (Agilent Technologies, Santa Clara, CA, USA). Separation was achieved on a ZORBAX Eclipse Plus C18 RRHD column (50 mm × 3.0 mm i.d., 1.8 µm; Agilent Technologies) maintained at 40°C, with an injection volume of 5.0 µL and a flow rate of 0.6 mL/min. The source was operated in positive ionisation mode with a drying gas temperature of 300°C, drying gas flow of 8 L/min, sheath gas temperature of 400°C, sheath gas flow of 12 L/min, nebuliser pressure of 45 psi, capillary voltage of 3500 V and nozzle voltage of 500 V. Nitrogen from a generator served as nebuliser, drying and sheath gas and high-purity nitrogen (99.999%) as the collision gas. Data were acquired in multiple reaction monitoring (MRM) mode with two transitions per analyte, the more abundant being used for quantification and the second for confirmation. Data acquisition and processing were performed with MassHunter software.

3.7.6 Method performance

Calibration was linear over 0.225–1.25 ng/mL with correlation coefficients (r) > 0.995 for all four aflatoxins. Mean recoveries were 70–110%, with relative standard deviations below 20%. The LOD and LOQ were 0.3 and 1.0 µg/kg, respectively, for each aflatoxin, below the limits for herbal drugs of 2 µg/kg for aflatoxin B1 and 4 µg/kg for the sum of aflatoxins B1, B2, G1 and G2 (EDQM, 2022), and below the limits prescribed for Ayurvedic, Siddha and Unani drugs (Anonymous, 2016). A procedural blank was included in every batch. An aflatoxin was considered identified only when its retention time agreed with that of the matrix-matched standard within ± 0.1 min and its qualifier-to-quantifier ion ratio was within ± 30% of that of the standard.

3.8 Estimation of pesticide residues

3.8.1 Sample preparation

Pesticide residues were extracted by an ethyl acetate-based procedure (Pihlström et al., 2007) combined with d-SPE clean-up (Anastassiades et al., 2003), an approach widely applied to multiresidue GC-MS/MS analysis of herbal medicines (Ji et al., 2023). Two grams of powdered *L. sativa* seed were moistened with 5 mL of water and allowed to hydrate for 15 min, since dry commodities require hydration for efficient partitioning of polar residues (Anastassiades et al., 2003). The sample was then extracted with 5 mL of ethyl acetate by vortexing for 2 min and centrifuged at 10,000 rpm for 2 min. To reduce the load of seed lipids, the organic layer was held at -20°C for 2 h and centrifuged again while cold, which precipitated the bulk of the triglycerides. A 1 mL aliquot of the supernatant was then cleaned up with 50 mg of C18 sorbent and 150 mg of anhydrous magnesium sulphate to remove the remaining co-extracted

lipids (Lehotay et al., 2005), vortexed for 30 s and centrifuged again. The cleaned supernatant was filtered through a 0.22 µm PTFE syringe filter into an autosampler vial for analysis.

3.8.2 Chromatographic and mass spectrometric conditions

Analyses were performed on an Agilent 7890B gas chromatograph coupled to an Agilent 7000D triple-quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). Separation was carried out on an HP-5ms Ultra Inert capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; Agilent Technologies) with helium (99.999%) as carrier gas at a constant flow of 1.2 mL/min. One microlitre of extract was injected in splitless mode with the inlet held at 280°C. The oven temperature was initially held at 70°C for 2 min, raised at 25°C/min to 150°C and then at 8°C/min to 300°C, where it was held for 5 min (total run time ≈ 29 min). The transfer line, ion source and quadrupole temperatures were 280, 280 and 150°C, respectively. Ionisation was by electron ionisation (EI) at 70 eV, and detection was in dynamic multiple reaction monitoring (dMRM) mode with nitrogen (1.5 mL/min) as collision gas and helium (2.25 mL/min) as quench gas, after a solvent delay of 3 min. Two transitions were acquired for each analyte, one for quantification and one for confirmation.

3.8.3 Scope of the screen and method performance

Thirty-four (34) pesticides residue from the organochlorine, organophosphate, pyrethroid and nitrogen-containing groups corresponding to the residues specified for Ayurvedic, Siddha and Unani drugs in the Ayurvedic Pharmacopoeia of India (Anonymous, 2016) were screened. Quantification was performed against matrix-matched calibration standards prepared in blank seed extract. A residue was reported as detected only when its retention time (± 0.1 min) and qualifier-to-quantifier ion ratio (± 30%) matched those of the matrix-matched standard. Method performance was assessed in accordance with the criteria for pesticide residues in herbal drugs (EDQM, 2022; WHO, 2011). Recoveries determined in triplicate at 0.05 mg/kg ranged from 70 to 110%, with relative standard deviations below 20%, within the pharmacopoeial requirements of 70–110% recovery and RSD ≤ 20%. Residues below the limit of quantification of 0.01 mg/kg were reported as < LOQ.

3.9 Statistical analysis

All determinations were carried out in triplicates and the results are expressed as mean ± standard deviation. Where all replicates fell below the limit of quantification, the result is reported as below limit of quantification and no standard deviation is given. Descriptive statistics were computed using GraphPad Prism 11.0.2 (92) (GraphPad Software, San Diego, USA).

3.10 Results

The safety of *Tukhm-e-Kahu* was assessed through its microbial load, heavy metal content, aflatoxins and pesticide residues, and each result was compared with the limits prescribed for Ayurvedic, Siddha and Unani (ASU) drugs in the Unani and Ayurvedic Pharmacopoeia of India (Anonymous, 2016) and with WHO recommendations for herbal materials (WHO, 2011). The analyses were carried out at AGSS Analytical and Research Lab Pvt. Ltd.

3.10.1 Microbial load

The total bacterial count of the seed powder was 5.0×10^4 cfu/g, half of the permissible limit of 1.0×10^5 cfu/g, and the total yeast and mould count was 1.4×10^2 cfu/g against a limit of 1.0×10^3 cfu/g (Table 2). The specified pathogens *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus* and *Pseudomonas aeruginosa* were absent. The drug therefore complied with the microbiological requirements for ASU drugs, although the bacterial count, at 50% of the limit, indicates that hygienic storage and handling of the seed should be maintained.

Table 2
Microbial load of *Tukhm-e-Kahu*

S. No.	Parameter	Result	Limit (API)
1	Total bacterial count (cfu/g)	5.0×10^4	1.0×10^5 (max.)
2	Total yeast and mould count (cfu/g)	1.4×10^2	1.0×10^3 (max.)
Specific pathogens			
1	*Escherichia coli* (per g)	Absent	Absent
2	*Salmonella* spp. (per g)	Absent	Absent
3	*Staphylococcus aureus* (per g)	Absent	Absent
4	*Pseudomonas aeruginosa* (per g)	Absent	Absent

cfu, colony-forming units; API, Ayurvedic Pharmacopoeia of India.

3.10.2 Heavy metals

Lead, mercury, arsenic and cadmium, determined by ICP-MS, were all below their limits of quantification in *Tukhm-e-Kahu* (Table 3). Since each LOQ was lower than the corresponding pharmacopoeial limit, the drug complied with the requirements for heavy metals, and no metallic contamination of the seed was detected.

Table 3
Heavy metal content of *Tukhm-e-Kahu*

S. No.	Heavy Metal	Result (mg/kg)	LOQ (mg/kg)	Limit (API, mg/kg)
1	Lead (Pb)	BLQ	2.50	10.0 (max.)
2	Mercury (Hg)	BLQ	0.05	1.00 (max.)
3	Arsenic (As)	BLQ	0.25	3.00 (max.)
4	Cadmium (Cd)	BLQ	0.05	0.30 (max.)

BLQ, below the limit of quantification; LOQ, limit of quantification. Method: AGSS/CHEM/SOP/ICPMS/13 (ICP-MS).

3.10.3 Aflatoxins

None of the four aflatoxins was detected above the LOQ of 1.0 µg/kg by LC-MS/MS. No chromatographic peak in the sample extract met the identification criteria of retention time (± 0.1 min) and quantifier-to-qualifier ion ratio (± 30%) against the matrix-matched standards. Because each aflatoxin was below 1.0 µg/kg, the sum of aflatoxins AFB_1, AFB_2, AFG_1 and AFG_2 was below 5.0 µg/kg, and the drug complied with the individual and total aflatoxin limits for ASU drugs (Anonymous, 2016). The absence of aflatoxins is notable because oil-bearing seeds are susceptible to aflatoxigenic fungi during storage (Wang et al., 2024), and it agrees with the low yeast and mould count recorded for the same material.

Table 4
Aflatoxin content of *Tukhm-e-Kahu*

S. No.	Aflatoxin	Result (µg/kg)	LOQ (µg/kg)	Limit (API, µg/kg)
1	Total Aflatoxins (B_1, B_2, G_1 and G_2	BLQ	1.0	≤ 5.0
2	Aflatoxin B_1	BLQ	1.0	≤ 2.0
3	Aflatoxin B_2	BLQ	1.0	≤ 2.0
4	Aflatoxin G_1	BLQ	1.0	≤ 2.0
5	Aflatoxin G_2	BLQ	1.0	≤ 2.0

BLQ, below the limit of quantification (1.0 µg/kg). Method: AGSS/CHEM/SOP/AFL/12 (LC-MS/MS).

3.10.4 Pesticide residues

Thirty-eight (34) pesticide residues were screened: 11 organochlorines, 15 organophosphates, 7 pyrethroids and pyrethrins, and 5 compounds of other classes (alachlor, bromopropylate, carbendazim, the dithiocarbamates and piperonyl butoxide). None was detected above its LOQ in *Tukhm-e-Kahu* (Table 5). The LOQs ranged from 0.01 to 0.10 mg/kg and, for most analytes, were five- to hundred-fold lower than the corresponding API limits, so that absence of these residues was demonstrated with an ample margin. For alachlor, aldrin with dieldrin, and heptachlor, the LOQ coincided with the API limit, and for chlordane and endrin it was close to the limit; for these compounds the results establish that the limit was not exceeded rather than that the residue was absent. For carbendazim, cyhalothrin, dicofol and fluvalinate, the API specifies no limit, and none of them was quantifiable. Overall, the seed complied with the pesticide residue requirements for ASU drugs, which is consistent with the use of drug-grade seed rather than treated sowing seed.

Table 5
Pesticide residues in *Tukhm-e-Kahu*

S. No.	Pesticide	Class	Result (mg/kg)	LOQ (mg/kg)	Limit (API, mg/kg)
1	Alachlor	Chloroacetanilide	BLQ	0.01	0.02 (max.)
2	Aldrin and dieldrin (sum)	OC	BLQ	0.01	0.05 (max.)
3	Azinphos-methyl	OP	BLQ	0.01	1.00 (max.)
4	Bromopropylate	Acaricide (benzilate)	BLQ	0.01	3.00 (max.)
5	Chlordane (sum of *cis*-, *trans*- and oxychlordane)	OC	BLQ	0.01	0.05 (max.)
6	Clofenvinphos	OP	BLQ	0.01	0.50 (max.)
7	Chlorpyrifos	OP	BLQ	0.01	0.20 (max.)
8	Chlorpyrifos-methyl	OP	BLQ	0.01	0.10 (max.)
9	Cypermethrin (and isomers)	PY	BLQ	0.01	1.00 (max.)
10	DDT (sum of *p,p′*-DDT, *p,p′*-DDE and *p,p′*-TDE)	OC	BLQ	0.01	1.00 (max.)
11	Deltamethrin	PY	BLQ	0.01	0.50 (max.)
12	Diazinon	OP	BLQ	0.01	0.50 (max.)
13	Dichlorvos	OP	BLQ	0.01	1.00 (max.)
14	Dithiocarbamates (as CS_2)	Dithiocarbamate	BLQ	0.01	2.00 (max.)
15	Endosulfan (sum of isomers and endosulfan sulphate)	OC	BLQ	0.01	3.00 (max.)
16	Endrin	OC	BLQ	0.01	0.05 (max.)
17	Ethion	OP	BLQ	0.01	2.00 (max.)

S. No.	Pesticide	Class	Result (mg/kg)	LOQ (mg/kg)	Limit (API, mg/kg)
18	Fenitrothion	OP	BLQ	0.01	0.50 (max.)
19	Fenvalerate	PY	BLQ	0.01	1.50 (max.)
20	Fonofos	OP	BLQ	0.01	0.05 (max.)
21	Heptachlor (sum of heptachlor and heptachlor epoxide)	OC	BLQ	0.01	0.05 (max.)
22	Hexachlorobenzene	OC	BLQ	0.01	0.10 (max.)
23	Hexachlorocyclohexane isomers (other than γ)	OC	BLQ	0.01	0.30 (max.)
24	Lindane (γ-hexachlorocyclohexane)	OC	BLQ	0.01	0.60 (max.)
25	Malathion	OP	BLQ	0.01	1.00 (max.)
26	Methidathion	OP	BLQ	0.01	0.20 (max.)
27	Parathion	OP	BLQ	0.01	0.50 (max.)
28	Parathion-methyl	OP	BLQ	0.01	0.20 (max.)
29	Permethrin	PY	BLQ	0.01	1.00 (max.)
30	Phosalone	OP	BLQ	0.01	0.50 (max.)
31	Piperonyl butoxide	Synergist	BLQ	0.01	3.00 (max.)
32	Primiphos-methyl	OP	BLQ	0.01	4.00 (max.)
33	Pyrethrins (sum of isomers)	PY (natural)	BLQ	0.01	3.00 (max.)
34	Quintozene (sum of quintozene, pentachloro aniline and methyl pentachlorophenyl sulphide)	OC	BLQ	0.01	1.00 (max.)

BLQ, below the limit of quantification; LOQ, limit of quantification; NS, not specified; OC, organochlorine; OP, organophosphate; PY, pyrethroid. Method: AGSS/CHEM/SOP/PEST/15.

4. Discussion

Herbal medicines are used by a large part of the world's population, and their safety is often assumed because they are natural, although plant material can pick up contaminants from soil, water, agrochemicals and post-harvest handling (Ekor, 2014; WHO, 2011b). Stronger regulation and routine quality control have therefore been called for (Sileshi et al., 2025), and the WHO methods for medicinal plant materials (WHO, 2011a) and the national protocol for testing Ayurvedic, Siddha and Unani medicines (Lohar, 2007) provide the framework for such screening.

Lactuca sativa L. seed (*Tukhm-e-Kahu)* is taken for insomnia over weeks or months, so the contaminant burden of the raw seed matters. In this study the seed was examined for microbial load, heavy metals, aflatoxins and pesticide residues and every parameter was within the limits of the Ayurvedic Pharmacopoeia of India (API).

4.1 Microbial load

Microbial contamination of herbal material arises from soil, water, handling, drying and storage, and it is a recognised health concern for oral preparations (de Sousa Lima et al., 2020; Khurana et al., 2011).

In this study, the total bacterial count (5.0×10^4 cfu/g) was half of the permissible limit (1.0×10^5 cfu/g), and the yeast and mould count (1.4×10^2 cfu/g) was about one-seventh of its limit (1.0×10^3 cfu/g). *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus* and *Pseudomonas aeruginosa* were absent in 1 g. Plant materials are not sterile, so standards limit the microbial load and exclude specified pathogens (WHO, 2007). The seed was hand-cleaned, dried below 40°C to constant weight and stored airtight, since inadequate drying and storage are the main drivers of microbial growth (Shekhar et al., 2024; Opuni et al., 2023). The absence of *E. coli*, a faecal indicator, argues against contamination from manure, unclean water or poor handling. The sample pre-treatment neutralised the antimicrobial activity of the seed, so the negative results are not due to inhibition by the oily matrix. The low yeast and mould count is consistent with the absence of aflatoxins. Because the bacterial count was at 50% of the limit, dry storage and periodic re-testing are advisable. The methods were culture-based and do not detect non-culturable cells or bacterial toxins.

4.2 Heavy metals

Lead, cadmium, mercury and arsenic are the metals of greatest concern in herbal medicines because they have no physiological role, accumulate in the body and damage several organ systems. Lead is neurotoxic and affects the renal, haematological and cardiovascular systems (Debnath et al., 2019) and it impairs neurodevelopment in children (Desye et al., 2023). Cadmium is linked to kidney damage and to metabolic and vascular disease (Charkiewicz & Backstrand, 2023; Yang et al., 2025). The toxicity of

mercury depends on its chemical form (Kumar et al., 2022; Wu et al., 2024), and long-term exposure to inorganic arsenic causes skin lesions, cancers and cardiovascular disease (NIEHS, 2024; WHO, 2022). Plants take up these metals from soil, irrigation water, fertilisers and industrial emissions (Asiminicesei et al., 2023; Vinogradova et al., 2023), and herbal products with detectable lead, mercury or arsenic or with metals above permissible limits have been reported repeatedly (Validandi et al., 2025; Luo et al., 2021; Saper et al., 2004, 2008).

None of the four metals was quantifiable in *Tukhm-e-Kahu*. The LOQs of 2.50, 0.05, 0.25 and 0.05 mg/kg for lead, mercury, arsenic and cadmium were four, twenty, twelve and six times lower than the API limits of 10.0, 1.00, 3.00 and 0.30 mg/kg, so compliance was demonstrated for each metal. The margin was narrowest for lead, but a concentration just below 2.50 mg/kg would still be a quarter of the limit. They suggest that this lot of seed was not appreciably contaminated during cultivation, drying, storage or milling.

4.3 Aflatoxins

Aflatoxins are produced by *Aspergillus flavus* which forms the B-type toxins, and *A. parasiticus*, which forms both B and G types. Herbal medicines are prone to contamination when raw plant material is exposed to high moisture and temperature during harvest, drying and storage. Aflatoxins have been detected in herbal products from Nigeria and Uganda (Eniola et al., 2023; Onen et al., 2021) and their occurrence in medicinal plants, including oil-bearing seeds is documented worldwide (Wang et al., 2024). Aflatoxin B1 is the most potent. After activation to the 8,9-epoxide by hepatic cytochrome P450 enzymes (CYP1A2 and CYP3A4), it binds to guanine in DNA and causes the G→T mutation at codon 249 of *TP53* that is seen in aflatoxin-associated liver cancer and are classified as carcinogenic to humans (IARC, 2012; Milki & Abdeta, 2023). The WHO guidelines give limits of 5 µg/kg for aflatoxin B1 and 20 µg/kg for total aflatoxins (WHO, 2007), the European Pharmacopoeia gives 2 and 4 µg/kg (EDQM, 2022), and the API gives 2.0 µg/kg for each of the four aflatoxins and 5.0 µg/kg for their sum.

In *Tukhm-e-Kahu*, none of aflatoxins B1, B2, G1 and G2 was detected by LC-MS/MS, as no peak in the sample extract matched the matrix-matched standards for retention time (± 0.1 min) and quantifier-to-qualifier ion ratio (± 30%). The LOQ of 1.0 µg/kg was 50% of the API limit for each aflatoxin and 20% of the API limit for the total, and 20% and 5% of the WHO limits for aflatoxin B1 and the total respectively. Each toxin was therefore below 1.0 µg/kg and their sum below 4.0 µg/kg which satisfies the API, WHO and European Pharmacopoeia limits.

4.4 Pesticide residues

Pesticides applied to medicinal crops can remain as residues in the harvested plant material, so herbal medicines are screened for them. Organochlorines persist in the environment and accumulate in tissue and Khan et al. (2025) note that they stimulate the nervous system while organophosphates inhibit acetylcholinesterase. Residues have been reported in dry herbs, tea, herbs and the infusions made from them and Chinese herbal medicines (Caldeirao et al., 2023; Mello et al., 2023; Szternfeld et al., 2023; Wang et al., 2022). Multi-residue methods are used to monitor herbal decoctions and root-based herbal

medicines (Jeong et al., 2022; Yang et al., 2023), and pesticides banned in the European Union continue to appear in imported food (PAN Europe, 2025).

None of the 34 pesticides screened in Tukhm-e-Kahu (10 organochlorines, 15 organophosphates, 5 pyrethroids and pyrethrins, and 4 compounds of other classes) was detected by GC-MS/MS at an LOQ of 0.01 mg/kg. The seed therefore complied with the API limits for all 34 pesticides.

5. Limitations

In this study, a few limitations should be acknowledged. Only a single batch of Tukhm-e-Kahu from one supplier was analysed at one outsourced laboratory, so batch-to-batch variation could not be assessed. The findings describe this sample and cannot be extended to the drug as a whole, which is particularly relevant to aflatoxins because they are unevenly distributed within a lot. Only the raw powdered seed was tested, not the decoctions or compound formulations in which it is consumed. The metals were measured as total concentrations, without speciation, and the scope was restricted to four metals, four aflatoxins and 34 pesticides. Other mycotoxins, polar pesticides, fumigant residues and radioactivity, which are covered by the WHO guidelines for herbal materials (WHO, 2007) were not assessed. Microbial testing was culture-based and does not detect non-culturable cells or bacterial toxins. Finally, no dose-based exposure assessment was performed, so the results were compared with concentration limits and not with permitted daily intakes. Future studies should therefore include multiple batches from different sources and seasons, a wider analytical scope, and the finished Unani preparations containing the seed.

6. Future perspectives

Future work should analyse multiple batches from different sources and seasons and lower the LOQs for mercury and cadmium below their limits by reducing sample dilution. A dose-based exposure assessment using the classical dose of *Tukhm-e-Kahu* would translate concentrations into daily intake. The scope should also be widened to include other mycotoxins, polar pesticides and radioactivity and the finished Unani preparations containing the seed should be screened in the same way.

7. Conclusion

This study assessed the contaminant safety of *Tukhm-e-Kahu*, the seed of *Lactuca sativa* L., in the four categories recommended by the World Health Organization (2007) and compared each result with the limits for Ayurvedic, Siddha and Unani drugs (Anonymous, 2016). The total bacterial count (5.0×10^4 cfu/g) and the yeast and mould count (1.4×10^2 cfu/g) were within their limits, and *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus* and *Pseudomonas aeruginosa* were absent. Lead, mercury, arsenic and cadmium were below LOQs that were four to twenty times lower than their limits. No aflatoxin (B1, B2, G1, G2) was detected at an LOQ of 1.0 µg/kg, and none of the 34 pesticides screened, covering organochlorines, organophosphates, pyrethroids and other classes, exceeded its LOQ of 0.01

mg/kg. The margin of compliance was narrowest for alachlor and for aldrin with dieldrin, chlordane, endrin, fonofos and heptachlor, so for these the results show that the limit was not exceeded and not that the residue was absent.

Within these limits, the examined sample shows a favourable contaminant profile, consistent with drying and storage that restricted microbial and fungal growth. The findings support the continued use of *Tukhm-e-Kahu* in *Sahar* and provide a baseline for the quality control of Unani formulations containing it. Because they come from a single batch of raw seed, routine batch-wise testing against pharmacopoeial limits remains necessary to assure the safety of the drug over prolonged use.

Abbreviations

AD
Anno Domini
AFB_1, AFB_2, AFG_1 and AFG_2
Aflatoxins B1, B2, G1 and G2
API
Ayurvedic Pharmacopoeia of India
BLQ
Below the limit of quantification
Cfu
Colony-forming units
DNA
Deoxyribonucleic acid
GC-MS/MS
Gas chromatography-tandem mass spectrometry
gm
Gram
ICP-MS
Inductively coupled plasma-mass spectrometry
Kg
Kilogram
LC-MS/MS
Liquid chromatography-tandem mass spectrometry
LOQ
Limit of quantification
µg
Microgram
NS
Not specified
OC

Organochlorine
OP
Organophosphate
PTFE
Polytetrafluoroethylene
PY
Pyrethroid
USM
Unani System of Medicine
UPI
Unani Pharmacopoeia of India
UHPLC
Ultra-high-performance liquid chromatography
W.H.O
World Health Organisation

Declarations

Declaration of Competing Interest:

The authors declare no competing interest.

Ethics, Consent to Participate, and Consent to Publish declarations

Plant samples were obtained after the Official permission taken from Head of the Department of Ilmul Advia, Aligarh Muslim University, Aligarh to purchase from the MSME Lab named Dawakhana Tibbiya College, AMU, Aligarh INDIA which manufacture the Traditional formulations on a global scale as per the National guidelines.

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Author Contribution

SR: Conceptualization, Supervision, Investigation and Validation, Reviewing, Editing, NA: Data curation, Writing- original draft preparation, Methodology, AR: Project Administration, Visualization

Data Availability Statement:

All data generated or analyzed during this study is included in this article

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Figures



Figure 1

Sample of *Lactuca sativa*L. used in safety analysis

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