Research Article

Proximate Composition and Aflatoxin B1 Contamination of *Onosma hispida* Aerial Parts Powder from District Malakand, Pakistan

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Abstract | Medicinal plants are widely used as household remedies in Pakistan; however, information on the nutritional composition and mycotoxin contamination of locally used plant materials remains limited. This study evaluated the proximate composition and aflatoxin B_1 (AFB_1) concentration of powder prepared from the aerial parts of *Onosma hispida* Wall. ex G. Don collected from a single site in District Malakand, Khyber Pakhtunkhwa, Pakistan. Moisture, dry matter, crude protein, crude fat, crude fiber, total ash, and carbohydrate by difference were determined in triplicate according to the AOAC Official Methods of Analysis, 22nd edition. Energy value was calculated using Atwater conversion factors, while AFB_1 was quantified using a competitive enzyme-linked immunosorbent assay. The powder contained 5.30 ± 0.15% moisture, 60.88 ± 0.45% carbohydrate, 16.20 ± 0.35% crude fiber, 11.22 ± 0.25% crude protein, 2.10 ± 0.08% crude fat, and 4.30 ± 0.12% total ash, with an estimated energy value of 307.30 kcal/100 g. The AFB_1 concentration was 27.4 µg/kg. For comparison, this concentration was approximately 5.5-fold higher than the European Union maximum level of 5 µg/kg established for specified dried spices. Because hepatotoxic pyrrolizidine alkaloids have previously been reported in *O. hispida*, their exclusion from the present analysis represents an additional safety limitation. As the findings were obtained from a single collection site and batch, they should be regarded as preliminary baseline data. The results highlight the need for broader sampling, routine mycotoxin surveillance, improved post-harvest handling, and appropriate quality-control standards for medicinal plant products in Pakistan.

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Introduction

Traditional medicine remains an important component of healthcare worldwide (Guerrero *et al*., 1999; Ahmad *et al*., 2023). The World Health Organization reported that 170 of its 194 Member States use some form of traditional medicine, with use ranging from 40–90% of the population in many reporting countries (FDA, 2020; WHO, 2025). In Pakistan, medicinal plants are commonly prepared and consumed as powders, decoctions, infusions, and raw dried materials, particularly in rural communities where traditional medicinal knowledge remains deeply embedded in local healthcare practices (IARC, 2012; Ahmad *et al*., 2023). This reliance on medicinal plants is especially evident in Khyber Pakhtunkhwa, a region characterized by considerable plant diversity and a rich tradition of ethnomedicinal use (Wiedenfeld and Edgar, 2011; Karakaya *et al*., 2024). However, inadequate harvesting, drying, processing, storage, and transportation practices may compromise the quality and safety of medicinal plant materials by promoting fungal growth and mycotoxin contamination (Ashiq *et al*., 2014; Ałtyn and Twarużek, 2020; Jeyaraj *et al*., 2022). Consequently, two important aspects require simultaneous investigation: the proximate composition of the plant material, which provides information on its basic nutritional constituents, and the presence of hazardous mycotoxins that may pose risks to consumers (Niness *et al*., 1992; Zhang *et al*., 2018).

Onosma hispida Wall. Ex G. Don (Boraginaceae) is a perennial herb native from Afghanistan to the western Himalayan region, including Pakistan (Stadlmayr *et al*., 2011; Wiedenfeld and Edgar, 2011). In parts of Khyber Pakhtunkhwa, the plant is reportedly known by local names such as Ratanjot and Sra Zaila. Different parts of the plant have traditionally been used to manage bronchitis, wounds, eye disorders, abdominal complaints, and skin conditions. Its roots contain red naphthoquinone pigments, particularly alkannin- and shikonin-related compounds, which have traditionally been used as natural colorants and in medicinal preparations (Rajapara and Shah, 2021; Ahmad *et al*., 2022). In vitro investigations have also shown that extracts of *O. hispida* exert cytotoxic effects against human bone cancer cell lines, MG-63 and Saos-2, and breast cancer cell lines, BT-20 and MCF-7 (WHO, 2025; Marin *et al*., 2013). More broadly, phytochemical and pharmacological studies of the genus *Onosma* have reported antioxidant, antimicrobial, enzyme-inhibitory, and other biological activities associated with naphthoquinones and phenolic constituents (Rajapara and Shah, 2021; Jabbar et al., 2022). Nevertheless, information concerning the proximate composition of powdered aerial parts of *O. hispida* remains limited (Vishwakarma and Dubey, 2011; Stadlmayr *et al*., 2011).

An additional safety concern is the occurrence of pyrrolizidine alkaloids (PAs) in members of the Boraginaceae (Sheela *et al*., 2004; Sher *et al*., 2019). Several genera within this family, including *Onosma*, *Symphytum*, *Echium*, and *Heliotropium*, are known to produce PAs, some of which may cause hepatotoxicity and exhibit genotoxic and carcinogenic properties (El-Shazly *et al*., 1998; Wiedenfeld and Edgar, 2011; EFSA Contam Panel, 2023). Ahmad *et al*. (2003) qualitatively identified heliotrine, lycopsamine, and echimidine in the leaves of *O. hispida* collected from Pakistan (Selvaraj *et al*., 2023). Their detection demonstrates that the safety assessment of this species should not be restricted to microbial or post-harvest contaminants (Rajapara and Shah, 2021). Because PA concentrations were not determined in the present study, the potential exposure associated with consumption of the powdered material remains unknown and requires investigation through validated quantitative methods (Niness and Hollingsworth, 1992; Moretti *et al*., 2024).

In addition to naturally occurring phytochemicals, medicinal plant materials may acquire mycotoxins through fungal contamination during harvesting, drying, transportation, and storage (Liu and Wu, 2010). Aflatoxin B_1 (AFB_1), principally produced by toxigenic strains of *Aspergillus flavus* and *A. parasiticus*, is a potent genotoxic carcinogen associated particularly with hepatocellular carcinoma (Amini *et al*., 2022; Liu and Wu, 2010). Warm temperatures, elevated moisture, inadequate drying, and unsuitable storage conditions can facilitate fungal growth and aflatoxin production in plant-derived commodities (Cotty and Jaime-Garcia, 2007; Ałtyn and Twarużek, 2020). Surveys conducted in different regions have detected AFB_1 and other aflatoxins in herbal materials, although their prevalence and concentration vary considerably according to the plant material, geographic origin, processing method, and storage conditions (Jeyaraj *et al*., 2022; Selvaraj *et al*., 2023; Hu *et al*., 2024).

Commission Regulation (EU) 2023/915 establishes a

maximum AFB_1 level of 5 µg/kg for certain specified dried spices (Marin *et al*., 2013). Although this limit is not directly established for *O. hispida* powder, it may provide a relevant comparative food-safety benchmark when interpreting the present findings (European Commission, 2023). Pakistan currently requires more comprehensive surveillance data and commodity-specific quality standards for medicinal plant materials intended for oral use. Such monitoring is particularly important for powdered products because visual examination alone cannot reliably establish the absence of aflatoxins (Khan *et al*., 2024). Despite increasing interest in the pharmacological properties of *O. hispida*, its proximate composition and possible aflatoxin contamination have generally been investigated as separate issues (Karakaya *et al*., 2024). To the best of our knowledge, no previous study has jointly characterized the proximate composition and AFB_1 concentration of *O. hispida* aerial-parts powder collected from Pakistan (Jeyaraj *et al*., 2022). The present study therefore provides preliminary baseline data integrating nutritional characterization with mycotoxin safety screening for a single batch collected from District Malakand, Khyber Pakhtunkhwa (Iheanacho and Udebuani, 2009).

Accordingly, this study aimed to: (i) determine the moisture, dry matter, crude protein, crude fat, crude fiber, total ash, carbohydrate content, and estimated energy value of *O. hispida* aerial-parts powder; (ii) quantify AFB_1 in the same material using a commercial competitive enzyme-linked immunosorbent assay; and (iii) interpret the findings using relevant international food-safety benchmarks and in the context of post-harvest handling and medicinal plant quality control in Pakistan. Competitive ELISA was selected as an accessible and cost-effective screening technique, while confirmatory chromatographic analysis, such as liquid chromatography tandem mass spectrometry, is recommended for future regulatory or multi-batch investigations.

Materials and Methods

Study area

The study was conducted in Matkani, Batkhela, District Malakand, Khyber Pakhtunkhwa, Pakistan (34.6686°N, 72.0603°E; elevation 716 m) (Hu *et al*., 2024). The area has a temperate climate with distinct seasonal variation, moderate spring rainfall, and relatively dry summer conditions (Guerrero Flores *et al*., 1999).

Plant collection

O. hispida was collected during the flowering stage from March to April 2024. Aerial parts, including leaves, stems, and flowers, were collected manually from multiple individuals distributed across the study site to reduce variation associated with a single plant and to improve the representativeness of the composite sample (Frankel, 2005).

Taxonomic identification and voucher deposition

The identity of *O. hispida* was confirmed by a qualified botanist through morphological comparison with authenticated herbarium specimens and published taxonomic keys for the family Boraginaceae (FAO/WHO, 2002). A voucher specimen was deposited in the Herbarium of the Institute of Plant Sciences, University of Malakand, under voucher number UOM-ONH-2024-001. Collection and voucher details are summarized in Table 1.

Sample preparation

Aerial parts, such as leaves, stems, and flowers, were collected by hand from approximately 15–20 individual plants spread across about 500 m^2 at the collection site (Ashiq *et al*., 2014). This method aimed to reduce variability from single plants and enhance the representativeness of the pooled sample (El-Shazly *et al*., 1998). Sub-samples from each plant were combined into one composite batch before drying and analysis (Amini *et al*., 2022). The freshly harvested plant material was carefully checked at the collection point, and any visibly diseased, insect-damaged, or mechanically injured parts were discarded, as physical damage is a primary entry point for toxigenic fungi like Aspergillus flavus (Hell *et al*., 2008). The remaining material was transported to the lab in clean paper bags, washed thoroughly underrunning tap water to remove surface soil and debris,

Table 1: *Collecting data and voucher information for O. hispida*

Botanical name	Plant habit	Part used	Collection site	Latitude	Longitude	Voucher No.	Collection date
Onosma hispida Wall. ex G. Don	Herb	Aerial parts	Matkani (Batkhela), District Malakand, KPK, Pakistan.	34.6686°N	72.0603°E	UOM-ONH -2024-001	March–April 2024

then given a final rinse with distilled water (Ałtyn and Twarużek, 2020). The material was cut into uniform segments approximately 2–3 cm long and dried in the shade at room temperature (25 ± 3°C) for about three weeks, following post-harvest practices common in regional medicinal plant preparation (Ashiq *et al*., 2014). Once dried, the material was ground into a coarse, homogeneous powder using a Romer Series II® laboratory mill and stored in clean, airtight, amber glass containers at 4°C, shielded from direct light, until analysis (Cotty and Jaime-Garcia, 2007).

Proximate composition analysis

Proximate composition was analyzed in triplicate following the official methods of analysis of AOAC International (AOAC International, 2023, 22nd Edition), with results expressed as mean ± standard deviation on a fresh-weight basis unless otherwise noted. Moisture content was measured by drying in a forced-air oven at 105°C until constant weight (AOAC Method 925.10) (Ahmad *et al*., 2023). Dry matter was calculated as the difference: Dry matter (%) = 100 – Moisture (%). Total ash was determined by incineration at 600°C for four hours (AOAC Method 923.03). Crude fat was extracted using Soxhlet extraction with anhydrous hexane for six hours (AOAC Method 920.39). Crude protein was measured by the micro-Kjeldahl method (AOAC Method 920.87) using the Jones nitrogen-to-protein conversion factor of 6.25, which is the standard default for mixed plant matrices (FAO/WHO, 2002). This factor does not differentiate true protein nitrogen from non-protein nitrogen compounds such as pyrrolizidine alkaloids and free amino acids present in Boraginaceae; hence, the reported value may slightly overestimate true protein content (WHO/FAO, 2003).

Crude fiber was determined using sequential acid-alkali digestion (AOAC Method 962.09). It is acknowledged that this method underestimates total dietary fiber because of incomplete recovery of hemicellulose and pectin; the values reported here specifically reflect crude fiber and should not be equated with total dietary fiber (Niness and Hollingsworth, 1992). Total carbohydrate was estimated by difference: Carbohydrate (%) = 100 – (% moisture + % crude protein + % crude fat + % crude fiber + % total ash) (Iheanacho and Udebuani, 2009). Energy value was calculated using the standard Atwater general conversion factors recommended by FAO (2003) for plant-based food materials: Energy (kcal/100 g) = (4 × % protein) + (9 × % fat) + (4 × % carbohydrate) (Ahmad *et al*., 2003). All proximate fractions used in this calculation- protein, fat, and carbohydrate by difference- were determined and expressed on a fresh-weight basis; the energy value is therefore also reported on a fresh-weight basis throughout (Aziz *et al*., 2026). Crude fiber is excluded from the Atwater energy calculation, and its prior subtraction in the carbohydrate-by-difference formula ensures this exclusion is consistently applied (FAO, 2003).

Aflatoxin B1 quantification by competitive ELISA

AFB1 was measured using the AgraQuant® Aflatoxin B1 2/50 ELISA kit (Romer Labs, Tulln, Austria), a competitive enzyme-linked immunosorbent assay with a validated quantitative range of 2–50 µg/kg and a kit-stated detection limit (LOD) of 0.5 µg/kg (Amini *et al*., 2022).

For sample extraction, 5 g of ground powder was mixed with 25 mL of 70% aqueous methanol and shaken vigorously for three minutes (Irshad *et al*., 2025). The extract was filtered through Whatman No. 1 filter paper by gravity, and the clear filtrate was kept for analysis. For the competitive ELISA procedure, 200 µL of enzyme conjugate was added to the dilution wells, followed by 100 µL of either the calibration standard or the sample extract (Ullah *et al*., 2023). The mixture was transferred to antibody-coated microplate wells and incubated at room temperature for 10 minutes. Wells were washed five times with deionized water and blotted dry on absorbent paper (Khan *et al*., 2024). Then, 100 µL of substrate solution (3,3',5,5'-tetramethylbenzidine; TMB) was added to each well and incubated for another 10 minutes (Manan *et al*., 2025). Finally, 100 µL of stop solution (1 N sulfuric acid) was added, and absorbance was measured at 450 nm using a BioTek ELx800 microplate reader (Khan *et al*., 2025).

A five-point calibration series was prepared using AFB1 standards at 0 (zero reference), 2, 5, 20, and 50 µg/kg supplied by the kit manufacturer (Manan *et al*., 2025). Bound-to-maximum absorbance ratios (B/B_0) were calculated by dividing each standard or sample absorbance by the zero-standard absorbance (B_0) (Khan *et al*., 2026). The logit-log transformation [$\text{Logit}(B/B_0) = \log_{10}(B/B_0 / (1 - B/B_0))$ plotted against $\log_{10}$(concentration)] was applied to linearize

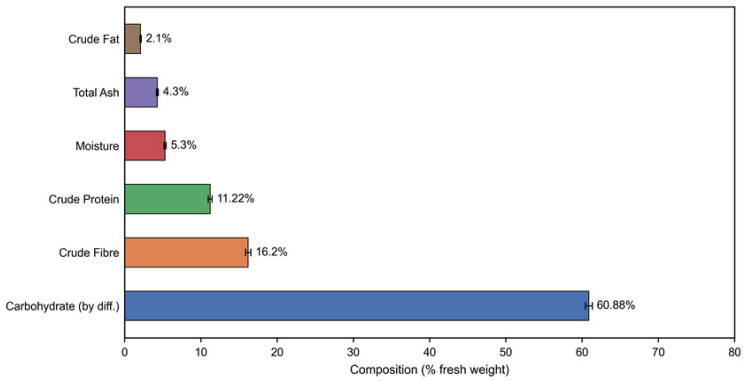

Figure 1: *Proximate composition of O. hispida aerial parts powder from District Malakand, Pakistan. Values expressed as percentage (%) on a fresh-weight basis. n = 3; error bars represent standard deviation.*

the calibration response, and the 50% inhibition concentration (IC_{50}) was derived from the resulting linear regression equation (Naz *et al.*, 2026). The final sample AFB1 concentration was determined by interpolation using Romer Labs polynomial regression software and expressed in (µg/kg) after applying the gravimetric extraction dilution factor of 5 (5 g sample extracted in 25 mL methanol) (Saifullah *et al.*, 2025). No independent matrix spike recovery experiment was performed specifically using O. hispida powder; instead, "kit-reported recovery data of 70–103% in grain and feed matrices (Amini *et al.*, 2022)" provide partial assurance of performance. No independent recovery data for herb powder matrices are available for this kit (Amini *et al.*, 2022), and true recovery in this specific matrix remains uncharacterized (Shakir *et al.*, 2023). This is an acknowledged limitation; confirmatory HPLC-FLD or LC-MS/MS quantification is recommended before any regulatory enforcement or clinical risk conclusions are drawn from these results (European Commission, 2006; Zhang *et al.*, 2018). All calibration standards and the sample extract were analyzed in duplicate wells on the same microplate, and the mean absorbance of each duplicate pair was used for all calculations (Shakir *et al.*, 2025).

Statistical analysis

All proximate analyses were performed in triplicate, and results are shown as the mean ± standard deviation. ELISA absorbance readings were taken, and the average value was used to determine concentration from the calibration curve. Descriptive statistics and percentage energy contributions were calculated using Microsoft Excel 365, while graphical data were generated with GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). Since the main focus was on the compositional characterization of a single plant material rather than testing hypotheses, inferential statistical analyses were not conducted (Iheanacho and Udebuani, 2009; Saha *et al.*, 2015).

Table 2: *Proximate composition and energy value of O. hispida aerial parts powder (n = 3; values expressed as mean ± SD)*

Parameter	Value	Energy contribution (%)
Moisture	5.30 ± 0.15%	—
Dry matter	94.70 ± 0.15%	—
Crude protein	11.22 ± 0.25%	14.6
Crude fat	2.10 ± 0.08%	6.2
Crude fiber	16.20 ± 0.35%	—
Total ash	4.30 ± 0.12%	—
Carbohydrate (by difference)	60.88 ± 0.45%	79.2
Energy value	307.30 kcal/100 g	100

Energy calculated using standard FAO Atwater general conversion factors: protein = 4 kcal/g; fat = 9 kcal/g; carbohydrate = 4 kcal/g (FAO, 2003).

Results

Proximate composition and energy value

The proximate composition of *O. hispida* aerial parts powder is shown in Table 2 and visualized in Figure 1. Standard deviations across all triplicate measurements were ≤ 0.45 percentage points. Moisture content was 5.30 ± 0.15%, resulting in a dry matter fraction of 94.70 ± 0.15%. Among the macronutrient fractions, carbohydrate by difference was the dominant component at 60.88 ± 0.45%, followed by crude fiber at 16.20 ± 0.35% and crude protein at 11.22 ± 0.25%. Crude fat was 2.10 ± 0.08%, and total ash was 4.30 ± 0.12%. Using standard Atwater general conversion

factors protein: 4 kcal/g; fat: 9 kcal/g; carbohydrate: 4 kcal/g (FAO, 2003), the calculated energy value was 307.30 kcal per 100 g. As shown in Figure 2, carbohydrate contributed the largest portion of total energy at 79.2% (243.52 kcal), followed by protein at 14.6% (44.88 kcal) and fat at 6.2% (18.90 kcal).

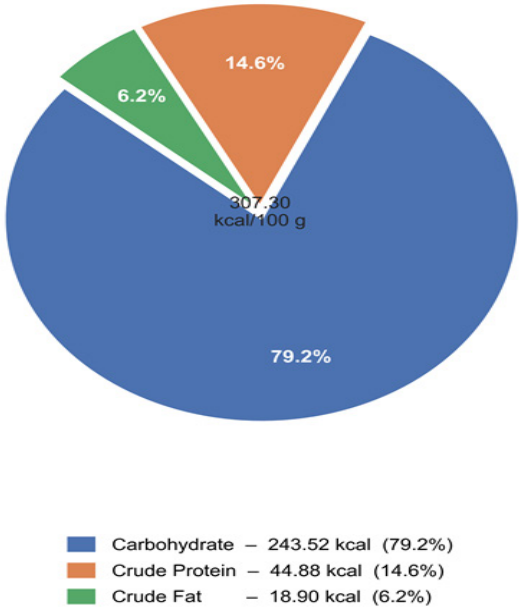

Figure 2: *Macronutrient contributions to the total energy value of O. hispida aerial parts powder. Total energy: 307.30 kcal/100 g, calculated using standard FAO Atwater general conversion factors (protein: 4 kcal/g; fat: 9 kcal/g; carbohydrate: 4 kcal/g) (FAO, 2003).*

Aflatoxin B1 detection and quantification

AFB1 quantification was performed using the AgraQuant® Aflatoxin B1 2/50 competitive ELISA kit. The calibration data, including calculated B/B_0 ratios and logit-transformed values, are presented in Table 3. The B/B_0 ratio, defined as each standard or sample absorbance divided by the zero-standard absorbance (B_0 = 1.611 at 450 nm), decreased from 0.786 at 2 µg/kg to 0.084 at 50 µg/kg. The logit-log calibration relationship [$\text{Logit}(B/B_0) = \log_{10}(B/B_0 / (1 - B/B_0))$ plotted against $\log_{10}(\text{concentration})$] yielded a linear regression equation of Logit = −1.100 × Log(C) + 0.778, with R^2 = 0.970. The Romer Labs polynomial regression applied to raw absorbance-concentration data yielded a polynomial fit with R^2 = 0.9966, which was used for final sample-concentration interpolation. The IC_{50} was 5.10 µg/kg (Figure 3).

Table 3: *ELISA calibration data for AFB1 quantification using AgraQuant® Aflatoxin B1 2/50 kit*

Standard (µg/kg)	Absorbance (450 nm)	B/B_0	Log_{10} (Conc.)	Logit B/B_0
0 (B_0 reference)	1.611	1.000	—	—
2	1.267	0.786	0.301	+0.566
5	0.666	0.413	0.699	−0.152
20	0.286	0.178	1.301	−0.666
50	0.136	0.084	1.699	−1.035

Based on the sample absorbance of 0.789 at 450 nm,

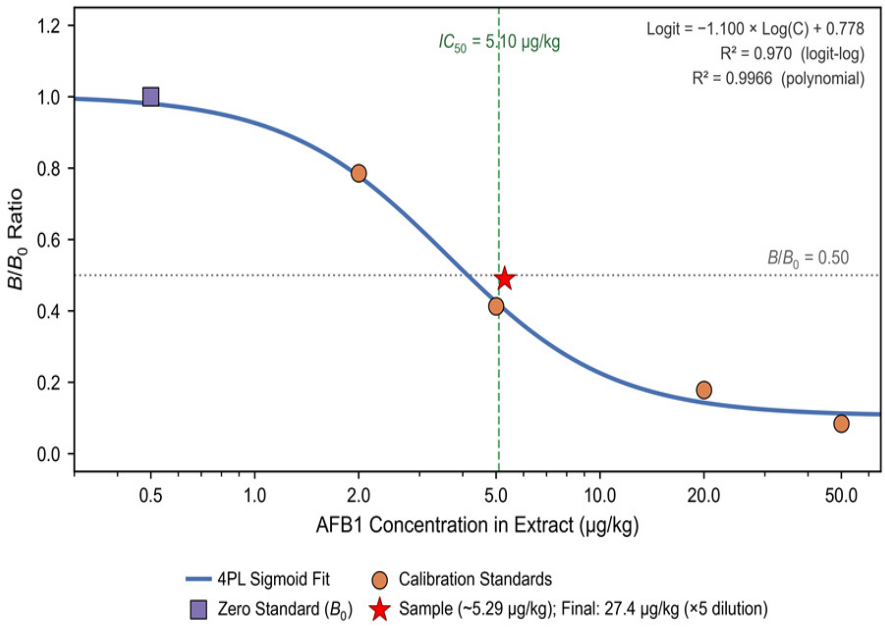

Figure 3: *ELISA calibration curve for AFB1 quantification using AgraQuant® Aflatoxin B1 2/50 kit. Standards: 0, 2, 5, 20, and 50 µg/kg. Logit-log linear regression: Logit = −1.100 × Log(C) + 0.778; R^2 = 0.970. Polynomial regression R^2 = 0.9966 (used for final sample quantification). IC_{50} = 5.10 µg/kg.*

Table 4: *AFB1 quantification results for O. hispida powder*

Sample ID	Absorbance (450 nm)	B/B_0	Logit B/B_0	AFB1 — Extract (µg/kg)	Dilution factor	AFB1 — Final (µg/kg)
0 µg/kg Standard	1.611	1.000	N/A	—	—	—
2 µg/kg Standard	1.267	0.786	+0.566	~2.0*	—	—
5 µg/kg Standard	0.666	0.413	−0.152	~5.0*	—	—
20 µg/kg Standard	0.286	0.178	−0.666	~20.0*	—	—
50 µg/kg Standard	0.136	0.084	−1.035	~50.0*	—	—
O. hispida Sample	0.789	0.490	−0.018	~5.29	5	27.4

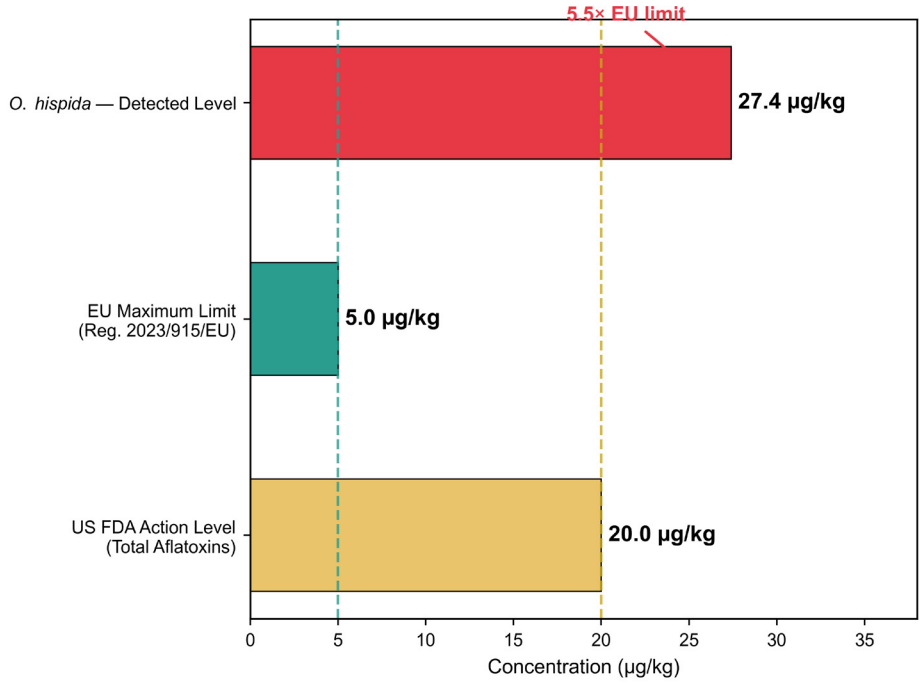

Figure 4: *Comparison of the AFB1 level detected in O. hispida aerial parts powder from District Malakand (27.4 µg/kg) against applicable international regulatory thresholds. EU: maximum limit of 5 µg/kg for dried herbs and spices (EU Regulation 2023/915/EU, European Commission, 2023). US FDA: action level of 20 µg/kg for total aflatoxins in human food (FDA, 2020). Note: Pakistan currently has no established national regulatory limit for AFB1 in medicinal herbal products. All values expressed as µg/kg.*

and the corresponding Logit B/B_0 was −0.018. Interpolation from the logit-log regression produced an extract concentration of approximately 5.29 µg/kg (Figure 4). Using the gravimetric extraction dilution factor of 5 (5 g sample extracted in 25 mL of 70% methanol), the final AFB1 concentration was 27.4 µg/kg according to Romer Labs polynomial regression software, which aligns with the logit-log estimate of 26.5 µg/kg (Table 4).

Back-calculated concentrations for calibration standards are shown for reference validation only. N/A = logit transformation undefined at B/B_0 = 1.000. Dilution factor = extraction volume (mL) ÷ sample mass (g) = 25 mL ÷ 5 g = 5. Final AFB1 concentration = extract concentration × dilution factor = 5.29 µg/kg × 5 = 26.45 µg/kg (logit-log regression); 27.4 µg/kg (Romer Labs polynomial regression software).

Discussion

Proximate composition and nutritional implications

This study provides the first proximate characterization of *Onosma hispida* aerial parts powder from District Malakand, Pakistan, a material consumed in repeated powder doses by local populations in the absence of any existing compositional baseline (Subhan *et al.*, 2025). The carbohydrate-dominant profile (60.88%), substantial crude fiber (16.20%), and moderate crude protein (11.22%) are consistent with the typical macronutrient composition of dried herbaceous aerial tissues, where structural polysaccharides

form the principal dry-mass fraction (Iheanacho and Udebuani, 2009). At realistic medicinal doses of 2–10 g per day, however, the absolute nutritional contribution is minimal, approximately 0.56 g of protein and 15.4 kcal per 5 g dose, and these findings are best interpreted as a compositional safety baseline rather than a nutritional claim (Ullah *et al*., 2018).

The crude protein content of 11.22% is notable in comparison to related species. It exceeds the 4.35% reported for *Chenopodium murale* from Spain (Guerrero *et al*., 1999), surpasses the 3.2–8.7% range documented for underutilized green leafy vegetables from Assam, India (Saha *et al*., 2015), and falls within the upper range of 5.8–12.4% reported for wild edible herbs from Eastern Chhattisgarh (Vishwakarma and Dubey, 2011). The substantial crude fiber content of 16.20% may partly account for the traditional use of *O. hispida* as a laxative and digestive remedy, as dietary fiber is well established to promote colonic motility and beneficial shifts in gut microbiota composition (Barber *et al*., 2020). Crude fat was low at 2.10 ± 0.08%, consistent with most aerial plant tissues; the limited lipid content reduces the substrate available for lipid peroxidation and is associated with reduced oxidative rancidity risk during storage (Frankel, 2005). Total ash of 4.30% reflects a moderate inorganic mineral residue comparable to values reported for wild vegetables in South Asia (Sheela *et al*., 2004), though specific mineral identification and bioavailability assessment would require dedicated atomic absorption spectroscopy or ICP-MS analysis beyond the scope of this study (Ullah *et al*., 2019).

Pyrrolizidine alkaloid safety context

Any study assessing the safety of an ingested Onosma preparation must recognize the documented presence of pyrrolizidine alkaloids (PAs), an internal plant chemical hazard that is chemically and etiologically different from the external mycotoxin contamination described in Section 4.3. Sher *et al*. (2019) directly identified heliotrine, lycopsamine, and echimidine in O. hispida leaves collected from Pakistan using HPLC, marking the first confirmed report of PA contamination in this species from the study area (Aziz *et al*., 2026). EFSA has concluded that PAs are genotoxic carcinogens with no safe threshold for long-term exposure, that they undergo liver bioactivation to reactive pyrrole-DNA adducts, and that they are linked to veno-occlusive disease at low chronic doses (Ullah *et al*., 2023). Systematic PA monitoring using LC-MS/MS is now recommended for all Boraginaceae-based herbal products destined for commercial distribution (EFSA, 2023; Moretti *et al*., 2024). PA screening was outside the scope of this study. For regular consumers of O. hispida powder, AFB1 and PAs therefore pose two co-existing, mechanistically separate hepatotoxic risks, one external and mold-related, the other internal and biosynthetic, whose combined health impact remains unquantified and represents a key priority for future safety research on this material (Irshad *et al*., 2025).

Aflatoxin B1 contamination and safety implications

The detection of AFB1 at 27.4 µg/kg is the most directly actionable finding of this study (Ullah *et al*., 2025a). This level exceeds the EU maximum limit of 5 µg/kg for dried herbs and spices under Regulation 2023/915/EU by approximately 5.5-fold, placing this material outside compliance with EU standards when applied as an international benchmark (European Commission, 2023). It should be noted that Regulation 2023/915/EU technically governs products within the EU food supply chain; its application here is for comparative benchmarking only and does not imply formal legal non-compliance within Pakistan's domestic jurisdiction, as no equivalent national regulatory limit for AFB1 in medicinal herbal products currently exists in Pakistan (Khan *et al*., 2024). The detected level also surpasses the US FDA action level of 20 µg/kg; however, the FDA threshold applies to total aflatoxins in human food rather than AFB1 alone, making the EU framework the more directly applicable regulatory reference for this material type (FDA, 2020). To contextualise the practical health risk, an illustrative exposure estimate is instructive. The assumed dose of 5 g per day is based on the general FAO/WHO reference range of 2–10 g per administration for powdered medicinal plant preparations (FAO/WHO, 2002); no dose-specific data for *O. hispida* in KPK communities were identified in the available literature (Khan *et al*., 2025). This calculation assumes 100% bio accessibility of AFB1 from the matrix, a conservative assumption likely to overestimate rather than underestimate true consumer exposure. At a daily dose of 5 g and the detected level of 27.4 µg/kg, the estimated daily AFB1 intake = (5 g × 27.4 µg/kg) ÷ 1000 g/kg = 0.137 µg/day. As EFSA does not establish a tolerable daily intake (TDI) for genotoxic carcinogens such as AFB1, a Margin of Exposure (MOE) approach is applied (EFSA, 2023). Using the JECFA provisional

maximum tolerable daily intake (PMTDI) of 1 ng/kg body weight per day as a reference, a 5 g daily dose at the detected level would deliver approximately 2.3 ng/kg bw for a 60 kg adult, exceeding this benchmark by a factor of approximately 2.3 (Khan *et al*., 2026). This estimate does not account for cumulative dietary AFB1 exposure from other food sources, lower-body-weight consumers, or individuals using higher doses, and is presented as an illustrative calculation to anchor the safety signal rather than as a definitive risk assessment, given the single-sample design of this study (Ullah *et al*., 2025b).

The contamination level detected here falls within the range documented in recent comparable international studies. Selvaraj *et al*. (2023) detected AFB1 in 80.6% of herbal supplement samples from Malaysia at levels up to 13.94 µg/kg, and Hu *et al*. (2024) reported AFB1 up to 32.13 µg/kg in Chinese medicinal herb samples. Within Pakistan, Khan *et al*. (2024) documented aflatoxin contamination in 30% of medicinal plant samples from Upper Dir and Upper Swat in KPK, with toxigenic *Aspergillus* species present in up to 90% of analysed material (Manan *et al*., 2025). The AFB1 level detected in *O. hispida* from Malakand is consistent with this regional contamination pattern and most likely reflects inadequate post-harvest management at one or more stages along the drying, storage, or transit chain (Ashiq *et al*., 2014; Altyn and Twarużek, 2020).

Aflatoxin formation needs enough substrate moisture, temperatures between 25°C and 40°C, and relative humidity above 70%, conditions that can occur during traditional shade-drying over three to four weeks under Malakand's spring conditions (Cotty and Jaime-Garcia, 2007; Shakir *et al*., 2023). Aflatoxins are chemically stable and resistant to heat; once they form, they are not inactivated by later drying or storage with lower moisture levels (Marin *et al*., 2013). A powder with only 5.30% moisture at the time of analysis can still contain a significant AFB1 load from earlier post-harvest phases (Ullah *et al*., 2025c; Saifullah *et al*., 2025). To effectively reduce risk, quick mechanical drying to below 10% moisture within 24–48 hours of harvest, moisture-barrier packaging, and cold dry storage are needed practices that are not currently standard in this region's informal medicinal plant trade (WHO, 2007; Hell *et al*., 2008; Naz *et al*., 2026).

Conclusions and Recommendations

This study provides preliminary baseline information on the proximate composition and aflatoxin B_1 (AFB_1) concentration of *O. hispida* aerial-parts powder collected from District Malakand, Khyber Pakhtunkhwa, Pakistan. The examined powder was characterized by a high carbohydrate-by-difference content of 60.88 ± 0.45%, a crude protein content of 11.22 ± 0.25%, and an estimated energy value of 307.30 kcal/100 g. However, at the relatively small quantities generally associated with medicinal use, its contribution to daily nutrient and energy intake would be limited. Competitive ELISA screening indicated an AFB_1 concentration of 27.4 µg/kg. This value was approximately 5.5-fold higher than the European Union maximum level of 5 µg/kg established for certain specified dried spices. Because this limit is not specifically applicable to *O. hispida* powder, it should be regarded only as a comparative food-safety benchmark. Moreover, the result was obtained from a single composite batch and was not confirmed by a chromatographic method; therefore, it should not be interpreted as evidence of species-wide contamination or formal regulatory non-compliance. The previously reported occurrence of potentially hepatotoxic pyrrolizidine alkaloids in *O. hispida* represents an additional safety concern that was not evaluated in the present study. Future investigations should include multiple independent samples collected from different locations and seasons, matrix-matched method validation, confirmatory AFB_1 analysis by HPLC-FLD or LC–MS/MS, and quantitative profiling of pyrrolizidine alkaloids and other relevant contaminants. Overall, the findings emphasize the importance of improved harvesting and drying practices, moisture-controlled storage, routine mycotoxin surveillance, comprehensive chemical safety assessment, and the development of appropriate quality-control standards for medicinal plant materials intended for oral use in Pakistan.

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Novelty statement

To the best of our knowledge, this is the first study to integrate proximate composition analysis with aflatoxin B_1 screening in *Onosma hispida* aerial-parts powder collected from District Malakand, Pakistan. The study provides preliminary baseline data on both the nutritional composition and mycotoxin safety of this medicinal plant material and highlights the need for broader multi-site sampling, confirmatory chromatographic analysis, pyrrolizidine alkaloid profiling, and improved quality-control measures for medicinal plant products intended for oral use.

Author Contributions

Amir Suhail: Conceptualization, Investigation, Data curation, and Writing original draft.
Kainat Ali: Investigation, Data collection, and laboratory analysis.
Gul Rahim: Sample collection, Methodology, data collection, and Supervision.
Shazia Ali: Laboratory analysis, Data organization, and Validation.
Sahar Nasim: Investigation, Literature review, and Manuscript preparation.
Sheema Rahman: Methodology, Data interpretation, and Technical guidance.
Ghani Subhan: Conceptualization, Supervision, and Writing review and editing.
Lubna Shakir: Literature review, Data presentation, and Manuscript editing.
Murad Ali: Data analysis, Visualization, and Validation.
Shakir Ullah: Conceptualization, Supervision, Writing review and editing, and Project administration. Amir Suhail and Ghani Subhan contributed equally to this work. All authors have read and approved the final manuscript.

Future work
Future studies should analyze multiple *O. hispida* samples collected from different locations and seasons. Aflatoxin B_1 results should be confirmed using HPLC-FLD or LC–MS/MS, together with matrix recovery studies. Quantitative profiling of pyrrolizidine alkaloids, other mycotoxins, minerals, and heavy metals is also recommended. Further research should evaluate the effects of drying, packaging, and storage conditions on the safety and quality of *O. hispida* powder.

Generative AI and AI-assisted technology statement
The authors declare that no generative AI and AI-assisted technology was used in the creation of this manuscript.

Conflict of interest
The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Data availability statement
The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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