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CHROMATOGRAPHY (GC-MS) CHARACTERIZATION, ANTIOXIDANT AND ANTIFUNGAL ACTIVITIES OF *MELIA AZEDARACH* L. SEED OIL AND ITS APPLICATION TO INHIBIT THE AFLATOXINS BIOSYNTHESIS OF *ASPERGILLUS FLAVUS* AND *ASPERGILLUS PARASITICUS*

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Abstract *The current study was led to assess the secondary metabolites composition, antioxidant activity, antifungal potential, and anti-aflatoxigenic efficiency of Melia azedarach seed oil against aflatoxigenic moulds. The bioactive compounds screening exposed the occurrence of tocopherols (3.5 ± 0.1 mg/g), flavonoids (4 ± 0.2 mg CAE/g), and total phenolics (21 ± 0.5 mg GAE/g). The Gas Chromatographic-Mass Spectroscopic (GC-MS) analysis recognized 73.90% linoleic acid methyl ester as the chief fatty acid ingredient, followed through 8.99% and 6.78% of margaric acid methyl ester and oleic acid methyl ester, respectively. The antioxidant potential of seed oil was assessed by applying the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical quenching procedure. The seed oil displayed concentration-dependent antioxidant activities with 46.9mg/mL of IC_{50} value. The antifungal potential against Aspergillus parasiticus and Aspergillus flavus exhibited significant mould biomass inhibition at maximum oil dosage. The highest inhibition was determined at 500 mg/mL, ensuing 82% inhibition of Aspergillus parasiticus and 84% inhibition of Aspergillus flavus. The oil also efficiently inhibited the aflatoxins in both mould species in a dose-dependent way. The aflatoxin inhibition of G2, G1, B2, and B1 for Aspergillus parasiticus reached 96%, 86%, 94%, and 87%, respectively, while for Aspergillus flavus the aflatoxin inhibition reached 97% and 91% for B2 and B1, respectively, at 500mg/mL. These outcomes suggest that the seed oil of Melia azedarach exhibits significant free radical scavenging, anti-moulds, and anti-aflatoxigenic activities and can act as an efficient non-synthetic substance for inhibiting aflatoxigenic moulds and aflatoxin pollution.*

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Introduction

Aflatoxins (AFs) are poisonous compounds that might cause extreme threats to animals and human owing to which various cost-effective issues. Animal and poultry feed contamination with mycotoxins signifies a universal issue for agriculturalists. These poisons are produced from fungi whose growing on stored and living plants, which is nearly inevitable, especially under humid circumstances (Alim-un-Nisaa et al., 2012). They are most generally famous for producing chronic or acute liver ailments; they are also considered carcinogenic, teratogenic, mutagenic, hepatotoxic, and immunosuppressive. The four (4) main AFs are G2 (AFG2), G1(AFG1), B2 (AFB2), and B1 (AFB1), which are each illustrious through the color of their luminescence under UV-light as G for green and B for blue (Mohammedi and Atik, 2013).

Various approaches have been applied to control AFs pollution of vulnerable crops and plants (Mohammedi and Atik, 2013). The utilization of synthetic fungicides and pesticides has led to several health and environmental issues owing to their spermatotoxicity, hormonal imbalance, carcinogenicity, and residual toxicity (Mohammedi and Atik, 2013). There is a necessity to plan innovative and eco-friendly harmless techniques to minimize contamination of mycotoxigenic aspergilli and to control AFs production. Herbs are measured as an origin of beneficial phytochemicals (Javid et al., 2025). Herbal extracts are of countless values; subsequently, they are biologically vigorous substances that are simple to produce and are generally applied with an inordinate agreement of protection. Additionally, no worry for

their remnant possessions has been elevated, because they are biodegradable. Their motivating outcome on herb metabolism is obvious (Behiry et al., 2022). Herbs hold an extensive range of phytochemicals such as flavonoids, alkaloids, terpenoids, and tannins, which have well-known antifungal activities (Mohammedi and Atik, 2013).

Human beings have trusted in therapeutic herbs and their products from the ancient period all over the globe (Yousef et al., 2022). *Melia azedarach* Linn is a member of *Meliaceae* family and is generally known as the Chinaberry tree, and has been utilized as a traditional medicine for the treatment of various ailments in Pakistan and India. Numerous portions of the plant have been applied in different diseases like infections, kidney stones, leprosy, diabetes, and ulcers (Faiza et al., 2022). To date, many scientists around the world have focused their research on *Melia azedarach* (*M. azedarach*) for its promising properties of interest in agriculture and medicine. Also, *M. azedarach* exhibits a range of biological activities. Extracts of its fruits, seeds, and leaves have shown many properties (Lau et al., 2021), including pesticidal activity (Al-Rubae, 2009). The effectiveness of such extracts has been previously demonstrated against insects (Shadrach et al., 2018; Khaldi et al., 2022; Kumar et al., 2003), with antifeedant effects documented in numerous insects (Pavela and Benelli, 2016). The *M. azedarach* leaves extract against Dengue Vector Aedes aegypti (Ranchitha, et al., 2016); antifungal and anti-aflatoxigenic activities (Javid et al., 2025); Ripe fruits of *M. azedarach* antimicrobial activity against Gram-negative and Gram-positive bacteria (Abdelslam et al., 2020) and antifungal activities (Maria et al., 1999; Muhammad et al., 2017). However, the oil extracted from these tree parts displays several bioactivities against a wide range of insects and other organisms (Khaldi et al., 2022). Keeping in view the above facts, the current study was designed to determine the total phenolic, flavonoids, and tocopherol contents, Gas Chromatography-Mass Spectroscopy (GC-MS) analysis, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, antifungal, and anti-aflatoxigenic activities of *M. azedarach* seed Oil.

Material and Methods

Collection of Seeds

The *M. azedarach* Linn fruits were collected from the Medical Botanical Garden of Pakistan Council of Scientific and Industrial Research (PCSIR) Laboratories Complex, Peshawar, Khyber Pakhtunkhwa, Pakistan. The fruits were cleaned, and the stones were separated. The stones were wrecked manually to acquire seeds. The seeds were dried in a clean shade for seven days and stored in dark brown bottles for further analysis.

Extraction of Oil from Seeds

The seeds (200g) were crushed into minor fragments by applying a hammer and then ground into fine powder using a laboratory mill/grinder (Standard Model No.3 Wiley Mill USA). The powder was dried in an air-circulating Dehydrator (England) for one hour at 40°C. The oil was extracted from the seed powder using petroleum ether (boiling point 60-80°C), applying the Soxhlet Extractor equipment (Quickfit- England). The solvent was distilled off at 60 °C to obtain the crude seed oil.

Phenols, Flavonoids and Tocopherol Determination in Seed Oil

The total phenolic quantity (phenolic compound) was measured through the Folin-Ciocalteu reagent procedure. The reaction blend was gained through a methanol solution (0.1mL) mixing with oil (1mg/mL dose) or standard solution (0.1mL) of Gallic acid (100, 80, 60,40 and 20mg/L), distilled H_2O (7.9mL), sodium carbonate 20% (1.5mL), and Folin -Ciocalteu Assay (0.5mL). The blind probe consisted of H_2O (distilled) in place of 0.1mL of the sample examined. After incubation (2 hrs), the optical density was calculated at 765nm (wavelength) using a Hitachi U-2900 UV-Vis Spectrophotometer (Tokyo- Japan) (Javid et al., 2025). Total phenolic compounds content in the seed oil was measured as g of gallic acid equivalents (GAE) per g of the sample. The flavonoids were measured rendering to the $AlCl_3$ colorimetric amount was utilized. The optical density was measured using a UV-Visible spectrophotometer at a wavelength of 510nm. The mean of flavonoid content was reported as mg catechin per gram of the dose (mg CAE/g). The tocopherol content was calculated as aliquots (40, 35, 30, 25, 20, 15, and 10 ppm) of tocopherol in the C_2H_5OH were diluted to a volumetric flask, and the volume was adjusted with ethanol (8mL). The 1.0 ml of 2,2´-dipyridyl reagent and each of the samples was poured into a volumetric flask (10mL) and blended. The ferric chloride reagent (0.1mL) was poured into a volumetric flask (10mL), and the mixture was agitated for ten seconds. The optical density of the sample was calculated at a wavelength (520nm) against a blank (ethanol), and then, after that, the standard graph was drawn (Mukhan and Deen, 2019).

GC-MS analysis of *Melia azedarach* L. Seed Oil

The GC-MS analysis of the seed oil was carried out using QP 2010 Plus Shimadzu-Tokyo (Japan) GC-MS equipment under computer control at 70 eV. Before GC-MS screening, the seed oil was arranged by altering the fatty acids into their matching fatty acid methyl ester (FAMEs), which enhances separation and volatility during chromatographic assay. The seed oil 100mg was dissolved in n-hexane (2mL) in a clean test tube. Afterward, add 0.5N methanolic sodium hydroxide (2 mL), and the blend was warmed for ten minutes at 60°C to saponify the oil. Then, cool the mixture, add the reagent boron trifluoride–methanol (2 mL), and the blend was re-warmed for 10min at 60°C for fatty acids methylation. When the reaction was completed, the blend was permitted to cool, and 2–3 mL of distilled H_2O. The fatty acid methyl esters were isolated with C_6H_{14}, and the organic layer

(upper) was collected. The oil extracted was dried by adding Na_2SO_4 (anhydrous) to eliminate the traces of H_2O. Then, the sample was filtered by a syringe filter system (0.45 µm). Lastly, the sample (1 µL) was injected by applying a micro syringe into the GC–MS system for the oil ingredients analysis, and the scanning time was 43 minutes. As the ingredients were parted, they released from the column and came into a sensor that was capable of generating an electric indicator whenever an ingredient was spotted. The greater the quantity of oil, the greater the indicator (peak) achieved, which was then treated through a computer. The period from which the injection was started (initial period) to when released is denoted as the Retention time (RT). Whereas the equipment was run, a graph from the signal generated through a computer known as a chromatogram. Every peak in the chromatogram denoted the indicator formed when a component was released from the Gas chromatography column into the sensor. The Y-axis calculated the strength of the indicator to enumerate the compound in the oil injected. As every molecule released from the Gas chromatographic column, they came into an electron ionization (mass spectroscopy) sensor, where they were bombarded with an electron stream producing them to break down separately into parts. The pieces achieved were actually charged ions with a specific mass. The Mass/Charge (m/z) proportion gained was calibrated from the obtained graph, which was known as the Mass spectrum graph, which is the molecule fingerprint. The Gas Chromatography condition during analysis was column oven temperature (80°C), injection temperature (250°C), injection mode (splitless), sampling time (1min), pressure (82.7kPa), total flow (40.0mL/min), column flow (1.22mL/min), purge flow 7mL/min, oven temperature program raised to 220°C, ion source temperature 280°C, interface temperature 280°C, solvent cut time 4min, Mass Spectroscopy conditions were start time (1min), end time (43min), ACQ mode (scane), start m/z (40) and end m/z (800). The Helium (He) gas was applied as a carrier as well as a releaser.

Identification of compounds

The identification of the compounds was accomplished through the matching of their mass spectra fragmentation and retention indices patterns with those stored in the computer library. The library of NIST08.LIB, WILEY8.LIB source was applied for comparing the recognized compounds from the seed oil.

DPPH radical scavenging assay

In methanol, the DPPH was dissolved to produce the 200µM solution; the seed oil 10µL in methanol, was added to the methanol DPPH solution (175µL), while methanol itself was used as a blank control. Various dosages (250, 125, 62.5, 31.25, 15.625, and 7.8 mg/mL) of seed oil were tested. Subsequently, blending, the reduction in optical density was measured through a UV-Visible Spectrophotometer after twenty minutes at 515nm. The real absorption reduction induction through the seed oil was measured by subtracting that from the blank. The ascorbic acid was applied as a positive control. The DPPH radical scavenging potential of the seed oil and vitamin C was measured as the inhibition % (Javid et al., 2022).

DPPH Radical Activity (%Inhibition) = [(A0 − A1)/A0] × 100%

Where: Optical density of the DPPH itself (A0); Optical density of treated samples (A1).

Antifungal Activities of Seed Oil

Inoculum Preparation

Aspergillus parasiticus (A. parasiticus) and *Aspergillus flavus* (*A. flavus*) were grown on Potato Dextrose Agar (PDA) slopes for seven to ten days at 25°C. The spores were collected in autoclaved distilled H_2O through applying an inoculation loop (sterilized) and mild stirring; to avoid the wetting of the spores, a drop of Tween 80 (0.1%) was added. The suspension was agitated shortly and afterward filtered by sterilized cloth or cotton wool into centrifuge tubes. The suspension of spores was washed through centrifugation in distilled H_2O, and the concentration of the spores was standardized at 10^6 cells per mL through applying an enhanced Neubauer Hemocytometer ART No. 1280 Marienfeld, Germany with suitable dilutions (Cuero et al., 1987).

Antifungal Activity Evaluation of Seed Oil (by determining mycelial growth)

To calculate the inhibitory effects of the seed oil on *A. parasiticus* and *A. flavus*, take 20 mL Potato Dextrose Broth (PDB) in a 50-mL conical flask and add numerous seed oil concentrations (500, 250, 125, 62.5, 31.25, and 15.625 mg/mL), and in each concentration, inoculate spore suspension (1.0×10^6 conidia per mL). These flasks were kept in a shaking incubator with 180 RPM with a rotational radius of 26 mm at 29°C; after five days of incubation, the mycelia were filtered using a filter paper and dried until a constant weight at 70°C, and the dry mycelia weight was calculated. The whole procedure was carried out aseptically in a Laminar Air Flow Cabinet (Streamline Laboratory Products, Maxwell Road—Singapore). The conical flasks, PDB, and mould but without seed oil were used as a control (negative), while the standard fungicide Benlate (25 µg/ml) was applied as a positive control. The supernatant fluid was reserved for AFs measurements (Yaping et al., 2019).

Aflatoxin Analysis

All chemicals, solvents, and reagents were LC grade, provided through authentic suppliers in Pakistan of Merck (Darmstadt, Germany) and Sigma (St. Louis, MO, USA). The water (LC-grade) was received through filtering deionized H_2O by a filter (0.45µ) with a Waters Millipore (Milford, MA, USA) system. The gases were removed from water and solvents through an ultrasonic bath (Model EIA CP104, Italy) for twenty minutes. The standard stock solution of

AFs (G2, G1, B2, and B1) of dosages 1 µg/mL each was made through dilution in benzene/ acetonitrile (98:2; v/v). The stock solution was kept in a cooled incubator (Gallenkamp—England) at 4°C, enfolded in Al foil, owing to the fact that AFs slowly degraded under ultraviolet (UV) light. The LC system consisted of a Hitachi-Japan model L-2000 equipped with a fluorescence detector L-2458 (Macao, Japan), an auto injector L-2200, and two pumps L-2130. The guard column, Intersil OD-3, 5 µm (GL Sciences Inc. Tokyo, Japan) was kept between the autoinjector and the separative column, Intersil ODS-3 (25cm x 4.6 m I.D., 5 µm, GL science, Tokyo-Japan). The photodetector reactor, UVE (LC Tech-Germany), for post-column derivatization of AFs was equipped between the fluorescence detector and the separative column.

Extraction procedure

Briefly, from each flask, take 25mL of sample and add one gram of NaCl and mix with methanol/water (80:20) of 40mL and n-hexane or cyclohexane (20mL) for three minutes. Subsequently, the isolation of the 2 phases, n-hexane or cyclohexane, was removed. Extracts were sieved using a Whatman filter paper # 4.

Immunoaffinity column clean-up

A 10 mL aliquot was diluted with 60mL of Phosphate buffer saline (PBS) of pH 7.4. An immune affinity column (IAC AlfaTest® Vicam-USA) was conditioned with PBS (10mL) buffer through calm syringe pressure at a 5 mL/min flow rate. Next, the blend of the filtrate diluted extract (70mL) was applied to the IAC column (1 to 2 drops per second), subsequent through a washing with double-distilled H_2O (20mL), and afterward dried with air. The AFs were then gradually released from IAC with methanol (2mL) into a glass vial.

LC analysis

The mobile phase consisted of acetonitrile/methanol/water (8:27:65, v/v/v). The gases were removed from the mobile phase through sonication. The Intersil ODS-3 (25 cm x 4.6 m I.D., 5 µm, GL science, Tokyo, Japan) column was linked as LC column. The column was sustained with a flow rate of 0.8mL/min at 40 ºC. The AFs were sensed at the emission and excitation of 450nm and 365 nm, respectively. While 20 µL was the injection volume. The measurement of AFs was grounded on the matching of RT with the genuine AFs standard. The quantification was carried out directly by HPLC software applying outside standard calibration curves arranged from recognized doses of AFs standards. The calibration curve was prepared through plotting peak area against doses, observing good linearity ($R^2 > 0.99$). The quantity of AFs in the sample was automatically measured through the equipment software. The inhibition % of AFs production in blended samples compared to the untreated was measured by applying the following formula:

% Inhibition $=C-T/C \times 100$

where C signifies AFs quantity in the untreated sample and T signifies AFs quantity in the treated sample.

Statistical analysis

All results handled and examined with SPSS 17.0 statistical software (IBM, Armonk, NY) and Microsoft Office Excel 2010 (Microsoft, Redmond, WA) and SPSS 17.0 statistical software (IBM, Armonk, NY). Outcomes were analyzed for statistical significance with one-way analysis of variance (ANOVA). The differences were measured as statistically significant at $P < 0.05$. The investigational outcomes were articulated as means ± standard errors of the means.

Results and Discussion

Results

The phytochemical substances of *M. azedarach* seed oil are displayed in Table 1. The results exposed that the oil exhibited a considerable quantity of bioactive components, comprising tocopherols, flavonoids, and total phenolics. The total phenolic quantified was noted as 21 ± 0.5 mg GAE/g, representing that the seed oil is wealthy origin of phenolic compounds. Flavonoid composition was relatively inferior, with a quantity of 4 ± 0.2 mg CAE/g. In addition, tocopherol quantity was recorded to be 3.5 ± 0.1 mg/g, signifying the occurrence of antioxidant-active Vitamin (E) ingredients in the seed oil. Generally, the phytochemical screening documented that the seed oil has significant free radical scavenging related secondary metabolites, which might contribute to its biological properties.

The GC-MS analysis of the *M. azedarch* seed oil is presented in Table 2. The GC-MS screening isolated twelve (12) fatty acid methyl esters (FAMEs) in the seed oil sample. Among the identified constituents, linoleic acid methyl ester was the main compound, showing 73.90% of the total seed oil constituent. The oleic acid methyl ester and margaric acid methyl ester were also present in sufficient quantity, accounting for 6.78% and 8.99%, respectively. The stearic acid methyl ester, octadecenoic acid methyl ester, and eicosadienoic acid methyl ester were detected at 5.09%, 1.31%, and 2.17%, respectively. Slight compositions comprised myristoleic acid methyl ester, palmitic acid methyl ester, arachidic acid methyl ester, and octadecanoic acid methyl ester were detected in a quantity of 0.10%, 0.20%, 0.40% and 1.07% respectively, while lauric acid methyl ester and hexanoic acid methyl ester were determined in trace quantities of 0.02% each. The findings revealed that the seed oil is chiefly comprised of unsaturated fatty acids, predominantly linoleic acid derivatives, representing its potential biological and nutritional significance.

The seed oil of *M. azedarach* antioxidant activity (DPPH radical scavenging assay) is presented in Table 3. The seed oil documented concentration-dependent antioxidant activity, with inhibition rising from 20 ± 0.1% at 7.8 mg/mL to 90 ± 0.5% at 250

mg/mL. Similarly, ascorbic acid showed powerful free radical scavenging potential, creating 40 ± 0.2% hang-up at 7.8 µg/mL and attaining 98 ± 0.5% hang-up at 125 µg/mL. The IC_{90} and IC_{50} values of the seed oil were measured as 250mg/mL and 46.9mg/mL, respectively. In contrast, the ascorbic acid observed distinctly inferior IC_{90} and IC_{50} values of 62.5 µg/mL and 11.7 µg/mL, respectively, representing maximum antioxidant potential as compared to the seed oil of Chinaberry tree. Inclusive, the results verified that the seed oil exhibits significant free radical scavenging property, though inferior as compared with the reference antioxidant ascorbic acid. The antifungal activity (biomass inhibition) of M. azedarach seed oil against *A. flavus* and *A. parasiticus* is displayed in Table 4. The oil exposed a noteworthy dose-dependent inhibition of mould biomass in both mould species. The control flask (untreated), the mycelium of *A. parasiticus* and *A. flavus* were measured as 980 ± 0.2 mg and 950 ± 0.4 mg, respectively. At the lowermost experimental dose (15.625mg/mL), the oil contributed only minor inhibition, minimizing mould development via 6% in *A. parasiticus* and 5% in *A. flavus*. Though raising the dose gradually boosted antifungal activities. The inhibition gotten 44% and 47% against *A. parasiticus* and *A. flavus*, respectively, at 125mg/mL. The highest inhibition of the oil was documented at 500mg/mL, where mould inhibition touched 82% for *A. parasiticus* and 64% for *A. flavus*. Similarly, mould biomass was minimized to 180 ± 0.3 mg and 150 ± 0.2 mg for *A. parasiticus* and *A. flavus*, respectively. Generally, the findings documented that the seed oil holds significant fungal biomass reduction potential, predominantly against *A. flavus*, with activity rising in a concentration-dependent way.

The seed oil of M. azedarach inhibitory effect on AFs production via *A. flavus* and *A. parasiticus* in PDB is presented in Table 5. The findings documented a dose-dependent decrease in AFs biosynthesis with raising dosage of the seed oil. In the treatment (control), the *A. flavus* formed 60 ± 0.2 µg/kg of AFs (B2) and 290 ± 0.5 µg/kg of AFs (B1). Upon dealing with seed oil, AFs biosynthesis minimized gradually. At the maximum dosage (500mg/mL), AFs B2 and B1 quantities were distinctly minimized to 2 ± 0.1 µg/kg and 27 ± 0.4 µg/kg, respectively. Likewise, *A. parasiticus* formed an extensive quantity of AFs in the untreated group, including G2 (50 ± 0.7 µg/kg), G1 (270 ± 0.4 µg/kg), B2 (70 ± 0.1 µg/kg), and B1 (300 ± 0.5 µg/kg). Excrement with seed oil considerably inhibited AFs production in a concentration-dependent way. The AFs quantity was decreased to 2 ± 0.1 µg/kg (G2), 38 ± 0.5 µg/kg (G1), 4 ± 0.1 µg/kg (B2), and 40 ± 0.3 µg/kg (B1) at 500mg/mL. Generally, the outcomes documented that the seed oil efficiently suppressed AFs production in both mould species, with the maximum dosage observing the highest inhibition of AFs biosynthesis. The decrease in AFs buildup recommends the powerful anti-aflatoxigenic activity of the seed oil.

The inhibition % of AFs biosynthesis via *M. azedarach* seed oil against *A. flavus* and *A. parasiticus* is presented in Fig.1. The outcomes recorded that AFs inhibition augmented gradually with raising dosage of the seed oil, showing a powerful concentration-dependent anti-aflatoxigenic potential. The inhibition of AFs (B2) increased from 17% to 97%, while AFs (B1) inhibition increased from 7% to 91% at 15.625 mg/mL to 500 mg/mL via *A. flavus*. Likewise, in *A. parasiticus*, the AFs B1 and B2 suppression rises from 5% and 14% at the minimum dosage to 87% and 94%, respectively, at a concentration of 500mg/mL. The biosynthesis of AFs (G-type) through *A. parasiticus* was also distinctly inhibited through the oil (seed) treatment. The AFs (G1) suppression rose from 4% to 86% at 15.625 mg/mL and 500mg/mL, respectively, while AFs (G2) observed the maximum vulnerability, with suppression increasing from 40% to 96% across the experimental doses. Among all noticed AFs, B2 and G2 were the most susceptible to the suppression action of the seed oil. The maximum dose (500mg/mL) synthesized suppression against all AFs kinds, verifying the effective anti-aflatoxigenic property of the oil (seed) against both mould species.

Table 1. Selected Phytochemicals Contents in Seed Oil of *Melia azedarach*.

Parameters	Results
Total phenolics (mg GAE/g)	21±0.5
Flavonoids content (mg CAE/g)	04±0.2
Tocopherol content (mg/g)	3.5±0.1

Results are mean of three replicates ± standard error. Mean difference is significant at 0.05 level (p-value <0.05).

Table 2. Quantitative Composition of *Melia azedarach* Seed Oil.

S#	Name of Compounds	Area	Retention Time	m/z	Concentration %
1	Hexanoic acid, methyl ester	1254	3.058	87.00	0.02
2	Lauric acid, methyl ester	2748	8.550	87.00	0.02
3	Myristoleic acid, methyl ester	10460	10.995	87.00	0.10
4	Palmitic acid, methyl ester	1720993	14.679	87.00	0.20
5	Margaric acid, methyl ester	14254	16.976	87.00	8.99
6	Stearic acid, methyl ester	752369	19.697	87.00	5.09
7	Oleic acid, methyl ester	481780	20.254	97.00	6.78
8	Octadecenoic acid, methyl ester	57967	20.422	97.00	1.31
9	Linoleic acid, methyl ester	3342543	21.945	95.00	73.90

10	Octadecanoic acid, methyl ester	20890	22.113	95.00	1.07
11	Arachidic acid, methyl ester	26694	27.297	87.00	0.40
12	Eicosadienoic acid, methyl ester	35076	29.741	95.00	2.17

Table 3. Comparative antioxidant activity, IC_{50} and IC_{90} values of oil extract and Vitamin C determined by DPPH assay

Oil Extract (mg/mL)	% Inhibition	Vitamin C (µg/mL)	% Inhibition
7.8	20 ± 0.1	7.8	40 ± 0.2
15.625	30 ± 0.4	15.625	60 ± 0.4
31.25	40 ± 0.2	31.25	80 ± 0.1
62.5	60 ± 0.1	62.5	90 ± 0.3
125	80 ± 0.3	125	98 ± 0.5
250	90 ± 0.5	—	—
IC_{50}	46.9 mg/mL	IC_{50}	11.7 µg/mL
IC_{90}	250 mg/mL	IC_{90}	62.5 µg/mL

Results are presented as mean ± standard deviation (n = 3). Mean difference is significant at 0.05 level (p-value <0.05).

Table 4. Antifungal activities seed oil of *Melia azedarach*

Oil Concentration (mg/mL)	*Aspergillus flavus*		*Aspergillus parasiticus*	
	Biomass (mg)	**Inhibition (%)**	**Biomass (mg)**	**Inhibition (%)**
15.625	900±0.5	5	920±0.8	6
31.25	700±0.3	26	730±0.5	25
62.5	600±0.1	37	650±0.4	34
125	500±0.6	47	550±0.6	44
250	400±0.4	58	430±0.2	56
500	150±0.2	84	180±0.3	82
Control	950±0.4	0	980±0.2	0
Benlate (25 µg/ml)	100±0.1	89	130±0.1	87

Values are mean of three replicates n=3) ± standard error. Mean difference is significant at 0.05 level (p-value <0.05).

Table 5. AFs Biosynthesis (µg/kg) in PDB using *Melia azedarach* Seed Oil.

Oil/Control Dose (mg/mL)	*Aspergillus flavus*		*Aspergillus parasiticus*			
	B1	**B2**	**B1**	**B2**	**G1**	**G2**
15.625	270±0.8	50±0.5	285±0.6	60±0.2	260±0.5	30±0.5
31.25	250±0.5	40±0.4	265±0.1	50±0.5	240±0.7	20±0.4
62.5	220±0.5	33±0.1	240±0.2	40±0.3	210±0.6	13±0.2
125	170±0.2	20±0.5	150±0.1	25±0.5	165±0.2	8±0.5
250	90±0.5	8±0.2	100±0.5	12±0.3	85±0.8	4±0.1
500	27±0.4	2±0.1	40±0.3	4±0.1	38±0.5	2±0.1
Control	290±0.5	60±0.2	300±0.5	70±0.1	270±0.4	50±0.7

Values are presented as mean ± standard deviation (n = 3). Mean difference is significant at 0.05 level *(p-value <0.05).*

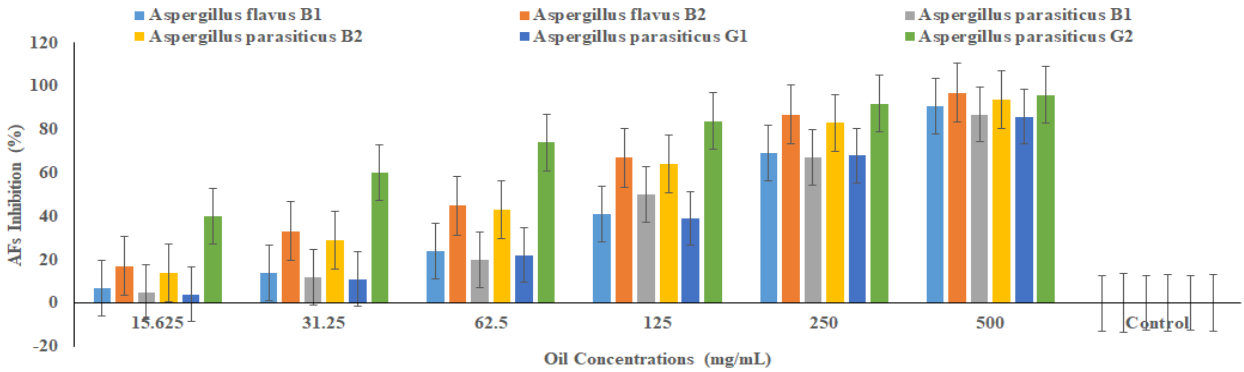

Figure 1. AFs Inhibition (%) Using *Melia azedarach* Seed Oil.

Discussion

Phytochemicals Contents

The secondary metabolites screening of various portions of the Chinaberry tree has documented the occurrence of a broad degree of bioactive compounds, such as flavonoids, tannins, alkaloids, phenols, cyanogenic glycosides, phenolic glycosides, cardioactive glycosides, glycosides, anthraquinones, and saponins (Muhammad et al., 2017). Likewise, the leaves extract of the Chinaberry was documented to hold cardiac glycosides, tannins, flavonoids, saponins, and alkaloids, representing that the species

is a rich source of phytochemicals with noteworthy biological properties (Shumirai et al., 2022). Earlier secondary metabolites assessments additionally exposed the incidence of various ingredients in various plant parts, such as five flavonoids, three anthraquinones, terpenoids, ninety-six limonoids, and seven carboxylic acids (Kumar et al., 2003). These phyto-compounds are recognized for their varied physiological and pharmacological activities, such as digestive stimulant, antimutagenic, anticarcinogenic, hypolipidemic, antioxidant, antifungal, antibacterial, and anti-inflammatory activities (Muhammad et al., 2017). The flavonoids and phenolic compounds are measured as the chief givers to the antifungal and antioxidant potential of herbal extracts. The occurrence of these constituents, together with other small compounds, has been linked with boosted suppression of mould development and pollution (Alharthi et al., 2021). In the phenolic extracts of Chinaberry tree seed oil from two various localities, the total phenolic compounds spectrum from 26.5 ± 0.4 to 28.7 ± 0.3 mg GAE/g, while defatted seed cake methanol extracts observed quantities ranging from 16.8 ± 0.3 to 18.5 ± 0.4 mg GAE/g (Mukhan and Deen, 2019). In another finding, the leaves' aqueous extracts displayed flavonoids and total phenolic contents of 12.7 ± 0.2 mg QE/g DW and 67.5 ± 0.4 mg GAE/g DW
(Javid et al., 2025). These results indicate that various extraction methods and plant parts meaningfully affect the recapture of phenolic substances. Tannins, which relate to the category of polyphenol substances, are documented as powerful free radical scavengers and antioxidants. They have also been documented to affect protein digestibility, net metabolizable energy, feed efficiency, growth rate, and feed intake in experimental animals (Shumirai et al., 2022). The flavonoids are among the most significant naturally occurring phenolics due to their capacity to give up hydrogen atoms or electrons willingly, thus counteracting reactive oxygen species (ROS). Furthermore, flavonoids behave as antioxidant agents and metal chelators, contributing importantly to the defensive properties of botanical extracts against oxidative stress (Mukhan and Deen, 2019). The antifungal activities displayed in various extracts of plants have also been associated with their phenolic compounds, as documented for seed extracts of mandarin against poisonous mould strains (Alharthi et al., 2021).

The phytochemicals extraction efficiency mainly depends upon the solvent polarity as applied. Polar solvents are commonly more efficient in breaking the cell walls of plants and discharging intracellular ingredients such as anthocyanins, polyphenols, and phenols (Muhammad et al., 2017). Therefore, crude extract achieved with polar solvents frequently shows greater potential due to the maximum dose of bioactive compounds. This thought is reinforced through earlier findings documenting that crude botanical extracts can be reported to have powerful antifungal properties due to the process of extraction, which concentrates bioactive compounds (Yousef et al., 2022). Like results were documented by Dahham et al. (2010), who displayed potential antifungal activities in methanol extracts of pomegranate and attributed this potential to the occurrence of tannins, flavonoids, and phenols. The *M. azedarach* seed oil flavonoid content in phenolic extracts ranged from 3.5 ± 0.3 to 3.5 ± 0.4 mg CAE/g, whereas defatted seed cake methanol extracts possessed 2.9 ± 0.0 to 4.2 ± 0.1 mg CAE/g (Mukhan and Deen, 2019). Differences in flavonoid quantity among samples obtained from various localities can be attributed to variations in ecosystem stress, climate conditions, soil composition, and other environmental aspects affecting botanical metabolism.

Tocopherols are non-synthetic occurring antioxidants broadly dispersed in vegetable oils and show a vital function in defensive fats against oxidative deterioration during stowage. These constituents act as quenchers of free radicals and also function as a significant source of Vitamin E in the human diet (Mukhan and Deen, 2019). The phenolic extracts of Chinaberry tree seed oil from Palwal and Hisar, the tocopherol content ranged from 3.4 ± 0.2 to 4.3 ± 0.2 mg/g, while defatted seed cake methanol extracts confined pointedly maximum degree ranging from 23.1 ± 0.2 to 26.5 ± 0.4 mg/g (Mukhan and Deen, 2019). The relatively maximum tocopherol quantity in seed cake extracts can be linked with variances in agroclimatic circumstances between cultivation areas and extraction efficiency. The *M. azedarach* seed on a dry basis after pressing contains about 4–6% oil. The oil of the seed is considered to have a maximum amount of unsaturated fatty acids, predominantly linoleic acid (65.95%), tracked by oleic acid, palmitic acid, and stearic acid with concentrations of 18.71%, 9.31%, and 3.08%, respectively (Gu et al., 1995). The oil also possessed various terpene ingredients in its non-oil ingredients, which contribute to its biological properties. Both field and laboratory studies have documented auspicious pesticidal activities of the oilseed emulsion, proposing its latent utilization as a non-synthetic bioactive compound (Gu et al., 1995).

GC-MS Analysis

Linoleic acid, a vital forerunner for the production of trihydroxy oxylipins in flora, has been documented for its substantial antifungal activities (Behiry et al., 2022). Oxylipins originated from linoleic acid act an important function in plants' protective system against mould pathogens. Earlier findings reported that both allylphenols and linolenic acid controlled the mycelium development of *Rhizoctonia solani* and *Pythium ultimum* through 74% and 65%, respectively, at 1000 µM concentration. Additionally, these constituents meaningfully minimized mould biomass synthesis and showed suppression potential against various other plant pathogenic moulds (Behiry et al., 2022). These results propose that unsaturated fatty

acids and their byproducts contribute considerably to the antifungal defense efficiency of flora extracts. The GC-MS analysis of fruit extracts of hexane and fatty acid methyl esters (FAMEs) of Chinaberry tree exposed the incidence of 13 ingredients, representing about 86.84% of the total extract component. The main compounds quantified 9.8%, 16.1%, and 18.8% of methyl linoleate, methyl linolenate, and methyl palmitate, respectively (Khaldi et al., 2022). The maximum concentration of fatty acid esters, chiefly unsaturated fatty acids, might contribute to the biological properties of the extracts, such as insecticidal, antifungal, and anti-aflatoxigenic activities.

The secondary metabolites assessment of ethanol leaves extract of *M. azedirach* additional confirmed the incidence of flavonoids, chlorogenic acid, phenols, tannins, saponins, steroidal glycosides, carbohydrates, and alkaloids (Ranchitha et al., 2016). These phytochemicals are recognized to constrain insect development and feeding and exhibit ovicidal as well as pesticidal properties. Furthermore, various terpenoids extracted from Meliaceae family members have been documented to display latent insecticidal activities. Likewise, GC-MS assay carried out in other research isolated 27 constituents in *M. azedarach* fruit ethanol extract, with the extract being predominantly rich in flavonoids, tannins, and other polyphenol components (Abdelslam et al., 2020). These results jointly designate that the biological property of the flora is linked with the synergistic properties of manifold secondary metabolite compounds. The palmitic and its derivatives (ethyl and methyl esters) have been documented to hold a wide-spectrum antimicrobial property against numerous human and plant pathogens. These comprise plant-pathogenic fungi like *Fusarium oxysporum* and *Alternaria solani* as well as clinically significant fungi species such as Aspergillus species, *Candida tropicalis*, and *Candida albicans* (Egbo et al., 2024). Investigational studies have also revealed that palmitic acid, whether alone or in combination with oleic acid, inhibits biofilm construction and minimizes cell survivability in *Candida glabrata*, thereby verifying its anti-inflammatory and antifungal activities (Mulatu et al., 2022).

The palmitic acid antifungal activities are arbitrated by manifold interconnected approaches. As a saturated long-chain fatty acid (C16:0), palmitic acid may mix into mould cell membranes, creating weakening of the fat bilayer and disturbance of membrane health. Such changes could damage diet conveyance systems and delay membrane-linked enzymatic jobs (Bashir et al., 20204; Prasath et al., 2020). Furthermore, palmitic acid has been documented to induce oxidative stress through inspiring the synthesis of ROS, predominantly in Candida parapsilosis. Raised ROS synthesis might injure organelles, proteins, and DNA, eventually compromising the mould cell's existence. Augmented compassion to palmitic acid has also been shown in mutants scarce in fatty acid desaturation pathways. Additionally, palmitic acid can suppress vital biosynthesis pathways of lipid, comprising sphingolipid and triacylglycerol production, which are critical for mould morphogenesis, membrane stability, and growth. Additional suggested approaches comprise the competitive hang-up of phospholipase A_2 (PLA_2), an enzyme linked to inflammatory signaling and phospholipid metabolism. While this suppression potential has been widely categorized in mammalian systems, like properties in mould might disturb phospholipid makeover procedures and contribute to antifungal activities (Guimaraes et al., 2022; Aparna et al., 2012). Oleic acid is an aliphatic carboxylic acid. It has anti-tumor, antibacterial, and antifungal properties. It has an additional degree of antibacterial potential (Padma et al., 2019). The occurrence of phenolic compounds, tocopherol fraction, and fatty acids was described to boost the antimicrobial properties of botanical extracts (Alharthi et al., 2021). Jointly, these results propose that fatty acids and their byproducts existing in Chinaberry tree seed oil might play a noteworthy role in the displayed antifungal properties.

Antioxidant Activities

The AFs are categorized as free radicals, which may inhibit the usual cell development and metabolic systems, leading to biological function disruption and cellular damage (Alharthi et al., 2021). The DPPH procedure is the most general assay applied for the measurement of the antioxidant activity in plant extracts, substances, or other biological materials. The DPPH is a steady N-centered free radicals which quenched efficiently in the presence of an antioxidant molecule, and at 517nm, powerful absorbance was observed (Faiza et al., 2022). The herb's phenolic compounds are electively secondary metabolites which are obviously aromatic and are extremely antioxidant in terms of their capability to control the active oxygen and free radicals. It is delightful that polyphenolic compounds contribute to the antioxidant properties of herbal items. Actually, phenols exhibit noteworthy free radical scavenging properties (Mukhan and Deen, 2019). The outcome of the study showed the occurrence of substances holding antioxidant property in the extract of ethanol, which have the capacity to quench DPPH free radical, then the primary purple dye of the solution transforms into yellow because of the synthesis of the diphenyl picryl hydrazine (Muhammad et al., 2017).

The occurrence of bioactive compounds that hold a free radical scavenging activity could have a vigorous purpose in inhibiting the existence of oxidative stress, which leads to delivering a defensive scheme against microbial contamination. The problem might be resolved via the non-synthetic free radical scavengers obtained from herbal extracts (Alharthi et al., 2021). The extracts' antioxidant activity will act as a dynamic function for the reduction of environmental

circumstances' impact on mould through growth, which subsequently reduces the oxidative stress on the cell and direct to reduce the production of mycotoxin (Abdel-Razek et al., 2021). The seeds of plants contents generally hold various compounds, which act in their protective scheme to shield the 2nd flora generation. Whereas botanical germination could be hurt by inappropriate environmental circumstances during development, the occurrence of these small ingredients may deliver defence throughout the initial phase of germination. Commonly, these inappropriate circumstances are connected to oxidative stress happening because of the occurrence of free radicals and ROS (Alharthi et al., 2021). The extracts of plants function as antioxidants to suppress AFs through scavenging free radicals and inhibiting their proliferation, changing them into minimal-poison components (Behiry et al., 2022). Antioxidant properties are attributed to the occurrence of flavonoids and total phenolic ingredients in the extracts (Muhammad et al., 2017). The extracts with maximum antioxidant activity were documented to inhibit AFs quantity in the development media of moulds (Abdel-Razek et al., 2021).

The antioxidant activities of the aqueous extract of Chinaberry tree displayed the maximum (61.30+3.55%) DPPH inhibitory activity, while the chloroform, methanol, and n-hexane were found 52.61+3.59%, 52.95+0.53%, and 51.16+1.70%, respectively. All extracts have been found to have above 50% inhibitory activities at 500µg/mL (Faiza et al., 2022). The Chinaberry tree leaves extracts at 100µg/mL, using petroleum ether, aqueous, and ethanol were found 64.76±0.06%, 68.87±0.09%, and 71.42±0.04% respectively (Mohammed et al., 2012). The free radical scavenging activity through DPPH assay of flower extracts (5mg/mL) of Chinaberry tree and vitamin C (0.5mmol/mL) disclosed Inhibition (%) of dichloromethane, ethyl acetate, methanol, n-butanol, ethanol, and ascorbic acid were 6.6, 8.2, 16.5, 18, 50, and 93.74, respectively (Muhammad et al., 2017). The secondary metabolites screening of the flower extract of the Chinaberry tree disclosed the occurrence of flavonoids and phenols. The extract of ethanol displayed the maximum inhibition % of free radical suppression, owing to which it is evident that Chinaberry tree ethanol extract might be applied as an antioxidant because of its antioxidant activity (Muhammad et al., 2017).

Antifungal Activities

Earlier research has reported substantial antifungal activity of Chinaberry tree extracts against a wide range of pathogenic moulds. The ethanol fruit extracts observed both fungicidal and fungistatic properties, where the minimum fungicidal concentration (MFC) and minimum inhibitory concentration (MIC) varied among mould species. The MFC and MIC values of *A. flavus* exhibited 500 and 300 mg/mL, respectively, whereas lower dosages were effective against *Candida albicans stains*, *Microsporum canis*, and *Fusarium moniliforme* (Maria et al., 1999). Similarly, ethanol, methanol, n-butanol, ethyl acetate, and dichloromethane flower extracts of Chinaberry tree were documented to suppress the development of *A. fumigatus*, *A. niger*, and *A. flavus* at various dosages ranging from 1 to 100 mg/mL (Muhammad et al., 2017). Various investigations have also emphasized the antifungal potential of extracts synthesized from various plant portions of the Chinaberry tree. The ethanol and hexane extracts gained from senescent leaves, seed kernels, and fruits showed fungistatic effects against *Sclerotinia sclerotiorum*, *Fusarium verticillioides*, *Fusarium solani*, *Fusarium oxysporum*, *Diaporthe phaseolorum* var. *meridionales*, and *A. flavus* (Carpinella et al., 2003). Likewise, ethanol leaves extracts observed controlling property against significant plant pathogens such as *Botrytis cinerea*, *Fusarium sambucinum*, *F. oxysporum* f. sp. *lycopersici*, *F. oxysporum* f. sp. *melonis*, *Fusarium solani*, and *Alternaria alternata* (Maroua et al., 2016). These former investigations sturdily support the anti-mould activity displayed in the current study.

The antifungal activities of the Chinaberry tree might be linked with its secondary metabolites' ingredients, especially phenolic and fatty acid compounds. Previous GC-MS screening displayed the occurrence of linoleic, oleic, palmitoleic, palmitic, and stearic acids in the extracts. These fatty acids have formerly been documented for their antimicrobial properties (McGaw et al., 2002; Seidel and Taylor, 2004). Oleic acid has been recognized to display antimicrobial activities against human pathogens (Kabara et al., 1972), whereas oleic, linoleic, and linolenic acids were also reported to exhibit antifungal properties (Walters et al., 2004). Furthermore, oleic and linoleic acids, which originated from *Helichrysum pedunculatum* confirmed antibacterial potential and synergistic connections (Dilika et al., 2000). Palmitoleic acid has also been known as a strong antibiotic ingredient against decay microorganisms (Ouattara et al., 1997). The current findings are also coherent with outcomes reporting antifungal activity of Chinaberry tree leaves extracts against numerous mould pathogens, such as *Ascochyta rabiei* (Jabeen et al., 2011), *Sclerotinia sclerotiorum*, *F. verticillioides*, *F. solani*, *Fusarium oxysporum*, and *A. flavus* (Carpinella et al., 2003). Methanol leaves extracts showed inhibition zones of 13 mm against *Trichoderma* spp., 10 mm against *Sclerotium* spp., 10.6 mm against *Geotrichum* spp., 11 mm against *Rhizoctonia solani*, and 12.3 mm against *Fusarium oxysporum* at 200mg/mL (Neycee et al., 2012). In addition, the extracts of *M. azedarach* have been revealed to be effective against numerous pathogens (bacteria) such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus*, as well as mould species including *Rhizopus stolonifer*, *F. oxysporum*, *A. flavus*, and Aspergillus

niger (Sen and Batra, 2012). Likewise, antifungal activities were also documented for methanolic fruit-seed and flower extracts of Chinaberry tree (Neycee et al., 2012). Differences in antifungal properties documented in various findings can be attributed to variations in phytochemical composition of the plant material, extraction solvents, environmental conditions, and differences in geographical origin (Gottlieb et al., 2001; Szewczuk et al., 2003; Orhan et al., 2012). Preceding phytochemical findings on Tunisian Chinaberry tree leaves recommended that chlorogenic acids and connected phenolic ingredients might meaningfully contribute to the experimental antimicrobial activities (Akacha et al., 2016). Caffeic and chlorogenic acids have been documented to suppress conidial germination and mould growth of *Fusarium oxysporum* f. sp. *niveum* (Ling et al., 2013; Maroua et al., 2016). Additionally, protocatechuic acid has proved powerful antifungal properties against *Rhizoctonia solani* and *Botrytis cinerea*, representing its possibilities as a substitute for unnatural fungicides (Nguyen et al., 2015). The occurrence of linoleic acid might also be responsible for the antifungal activity of Chinaberry tree extracts (Abdelillah et al., 2013).

Anti-aflatoxigenic Activities of Seed Oil

The herb extract, especially non-traditional ones, possesses numerous active components and biomolecules. These substances have been documented to have the capability to minimize the injurious influence of toxic molecules that occur in the plant development atmosphere (Alharthi et al., 2021). Likewise, the seed oil extracted observed a noteworthy capacity to control the mycotoxin biosynthesis through their poisonous mould strains, among which hibiscus oils (Badr et al., 2019), pomegranate oil (Badr et al., 2020), black cumin seed (Abdel-Razek et al., 2018), and jojoba, jatropha (Badr et al., 2017). The nanoparticles (NPs) produced from pumpkin seed oil (PSO) and its seed oil exhibited anti-mould activities against *A. flavus*, as well as inhibited the AFs production. Concerning AFs, PSO and its NPs revealed a decrease in AFs to 66.6% and 32.2%, respectively (Shaimaa and Amira, 2025). The *Amomum subulatum* oil was known to be effective against *A. flavus*, total suppression of AFs (B1) was observed at 500µg/mL, and mycelium development at 750µg/mL (Samuel et al., 203). Furthermore, the effect of phenolic quantity was prolonged to minimize the quantity AFs excretion that may happen in the media from the mould development (Alharthi et al., 2021). Numerous previous investigations have examined the outcome of herbal bioactive compounds on AFs gene cluster of poisonous moulds and the AFs production pathways. For example, in the presence of essential oils, the AFs gene expression was shown to be downregulated. Additionally, the secondary metabolites, such as phenolic acids, have been observed to control gene expression in the AFs pathway. Moreover, the plants' phytochemicals are significant controllers of gene expression in poisonous moulds (Abdel-Razek et al., 2021).

Conclusion

The current study concluded that Chinaberry tree seed oil holds noteworthy secondary metabolites, free radical scavenging, fungicidal, and anti-aflatoxigenic activities. The oil was rich in tocopherols, flavonoids, phenolic compounds, and unsaturated fatty acids, especially linoleic acid methyl ester, which could contribute to its mentioned biological properties. The DPPH activity verified significant activity of the oil, though less than the reference antioxidant ascorbic acid. The seed oil possessed significant antifungal properties against *A. parasiticus* and *A. flavus* by meaningfully minimizing mould biomass in a dose-dependent way. Furthermore, the oil efficiently controlled AFs production, presenting powerful inhibition of AFs (B2, G1, B2 an B1) at maximum doses. Among the investigated moulds, *A. flavus* seemed extra vulnerable to the suppression effects of the seed oil. Generally, the outcomes propose that seed oil of the Chinaberry tree might act as a talented phyto origin of antifungal and antioxidant substances for the control of AFs moulds and AFs pollution in the feed and food system. Additional research is suggested to isolate the bioactive compounds on an industrial scale and investigate their field/in vivo utilization in feed/food protection and agricultural preservation.

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Statements and Declarations

Data Availability statement

All relevant data are within the manuscript file.

Author's Contribution Statement

MS, JA, MA, JA, and MY collected data and wrote manuscript equally. All authors have read the final manuscript and approve its submission.

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