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Efficacy of *Kleinia odora* Essential Oil on *Aspergillus flavus* Link Ex Fries Growth and Aflatoxin B1 Production

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Aflatoxin contamination in food products continues to be one of the most important food safety concerns worldwide. *Aspergillus flavus* and *A. parasiticus* are the primary producers of aflatoxins. Among the different aflatoxin types aflatoxin B1 (AFB1) is the most harmful foodborne mycotoxin. In this study, essential oil (EO) was extracted from *Kleinia odora* and its effect on *A. flavus* growth and AFB1 production was evaluated under *in vitro* condition. *K. odora* EO (KOEO) effectively suppressed *A. flavus* growth which resulted in a 43.1% reduction in mycelial weight over the control at a dose of 100 µL/50 mL of medium. The KOEO suppressed the toxin production by *A. flavus* in a dose-dependent manner and suppressed AFB1 production up to 93.7% at the concentration of 100 µL/50 mL as determined by liquid chromatography-mass spectrometry analysis. The chemical analysis of KOEO by gas chromatography-mass spectrometry showed the presence of ledol (21.0%), caryophyllene (12.0%), gamma-curcumene (10.2%), 4(10)-thujene (9.9%) and D-limonene (7.6) as major constituents. These compounds have been demonstrated to have a wide range of biological properties including antimicrobial activity. These results suggest that KOEO has huge potential to be used as a bio-preservative to suppress *A. flavus* growth and to minimize aflatoxin contamination in food products.

Keywords: Mycotoxin; *Aspergillus flavus*; plant products; medicinal herb; antifungal activity

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Aspergillus flavus Link ex Fries and *A. parasiticus* Speare belonging to *Aspergillus* section *Flavi* are ubiquitous fungi that colonize a wide variety of agricultural commodities including corn, peanut, tree nuts, and chillies under favourable conditions [1]. The temperature and humidity in storage, and the moisture content of the substrate determine the growth of *Aspergillus* spp. [2, 3]. During their growth, toxigenic strains of *A. flavus* and *A. parasiticus* produce toxins known as "aflatoxins" as secondary metabolites secreted via exocytosis. Aflatoxins are low-molecular-weight compounds and have carcinogenic, mutagenic, teratogenic and immunosuppressive properties. More than 20 different aflatoxin types and derivatives have been characterized; among them aflatoxin B1 (AFB1; $C_{17}H_{12}O_6$; MW 312.063), aflatoxin B2 (AFB2; $C_{17}H_{14}O_6$; MW 314.079), aflatoxin G1 (AFG1; $C_{17}H_{12}O_7$; MW 328.27) and aflatoxin G2 (AFG2; $C_{17}H_{14}O_7$; MW 330.29) which are often found in agricultural commodities [4,5]. On the basis of their chemical nature, aflatoxins are grouped into difurocoumarocyclopentenones (aflatoxin B-types and their derivatives) and difurocoumaro-lactones (aflatoxin G-types and their derivatives). The
alphabetical letters (B or G) indicate whether the fluorescence is blue (B) or green (G) when exposed to UV light, while the numbers (1 or 2) indicate its chromatographic mobility [6]. *A. flavus* produces B-type aflatoxins (AFB1 and AFB2), whereas, *A. parasiticus* produces both B- and G-type aflatoxins (AFB1, AFB2, AFG1 and AFG2) [7, 8]. Among them, AFB1 is recognized as one of the most potent liver carcinogens to humans. In humans, AFB1-exo-8,9-epoxide, which is formed by the activity of cytochrome P450 enzymes on AFB1, binds to DNA and protein molecules and induces mutation, and causes oxidative damage to various macromolecules. By virtue of its toxicity, AFB1 has been categorized into Group 1 human carcinogens [9].

Aflatoxins are highly stable molecules in nature. Raters and Matissek [10] while studying the effect of heat on AFB1 reported 70% degradation of AFB1 at 150°C and 100% degradation at 180°C. Therefore, removal/degradation of aflatoxin from food products is extremely difficult by usual cooking

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processes. Some plant products [11-15] and microorganisms [16-18] have been reported to reduce aflatoxigenic
fungal infection and colonization on agricultural commodities, and aflatoxin production. For example, Safari et al. [12] showed that *Trachyspermum ammi*, *Peganum harmala*, and *Heracleum persicum* plant extracts totally prevented AFB1 production by *A. flavus*, without inducing significant changes in its growth. Further, the plant extracts down-regulated the expression of *aflR*, *aflM*, and *aflP* genes which are involved in the AFB1 biosynthesis. Several authors reported the antifungal properties of essential oils extracted from plants such as *Anethum graveolens* [19], *Melaleuca cajeputi* [20], *Pimenta dioica* [21], *Thymus eriocalyx* [22], *Cymbopogon citratus* [23], and *Zataria multiflora* [24, 25] against *A. flavus.* In addition, essential oils derived from *Heliotropium bacciferum*, *Ocimum dhofarense* and *Zataria multiflora* have been shown to detoxify AFB1 [13]. In the course of a search for biological detoxifying agents of aflatoxin B1, we found that the aqueous extract of *Kleinia odora* (Forssk.) DC. (Asteraceae) was capable of degrading AFB1 after incubation at 37°C for 72 h (Velazhahan et al. un-published). *K. odora*, a flowering medicinal herb with a succulent stem, is commonly distributed in Oman. This plant is reported to contain triterpenoids [26, 27] and essential oil [28]. Antimicrobial [28], antiprotozoal [26] and anti-inflammatory [29] activities of *K. odora* have been documented. In this study, the EO was extracted from *K. odora* and its effect on the growth and AFB1 production by a toxigenic strain of *A. flavus* was evaluated.

EXPERIMENTAL

Plant Material

The medicinal plant *Kleinia odora* (Forssk.) DC (Asteraceae) (Accession number 201000077) was collected from the plant nursery at the Oman Botanic Garden (OBG), Muscat, Sultanate of Oman, and stored at 4°C before use.

Aspergillus Flavus

Aflatoxin B1-producing strain of *Aspergillus flavus* STR10 (GenBank accession number OL437469) isolated from the diseased strawberry fruit [30] was obtained from the Department of Plant Sciences (Sultan Qaboos University) and used in this study. The fungal culture was maintained on potato dextrose agar (PDA; Oxoid, UK) slants at 4 °C.

Essential Oil Extraction

The fresh aerial parts of *K. odora* (1 kg) were washed thoroughly in running tap water and transferred to a glass reactor and 5.5 l of distilled water was added and the EO was extracted by using a microwave-assisted solvent extraction system (ETHOS X; Milestone Inc., Shelton, CT, USA) **[**13**]**. The EO was stored in opaque screw-capped glass vials at -20 °C until required.

Chemical Analysis of *K. Odora* Essential Oil

The chemical constituents of *K. odora* EO were analyzed by gas chromatography-mass spectrometry as described by Al-Harrasi et al. [13] using a Shimadzu GC-2010 Plus gas chromatograph, lined with a GCMS- QP2010 ULTRA MS (Shimadzu Corporation, Kyoto, Japan). The GC-MS system was equipped with a Rtx-5MS capillary column (30 m × 0.25 mm; 0.25 µm). The mass spectrum libraries viz., NIST 2011 Version 2.3 and Wiley 9th edition were used to identify the compounds. The chemical constituents of the EO were confirmed using Kovat's indices (KI).

Antifungal Activity of *K. Odora* Essential Oil

The antifungal activity of *K. odora* EO against *A. flavus* was tested using the standard poisoned food technique [31]. Fifty mL of SMKY liquid media (120 g sucrose, 0.3 g $MgSO_4$, 0.18 KNO_3, 4.2 g yeast to 600 mL of distilled water) in 250 conical flasks was amended with different concentrations of KOEO (10, 50,100 µL) diluted into 500 µL of ethanol. The liquid medium added with 500 µL of ethanol served as control. To prepare spore suspensions, 10 mL of sterile distilled water was added to a 7-day-old *A. flavus* culture grown on a 90 mm diameter PDA plate, and the spores were harvested by gently scraping the surface using a cell scraper. The concentration of the spores was determined using a hemocytometer and 500 µL of the spore suspension (6×10^7 spores mL^{-1}) was added to each flask and the inoculated flasks were kept at room temperature (25±2 °C). After 10 days of incubation, the contents of the flasks were filtered using a pre-weighed Whatman No.1 filter paper, and the filter paper containing the fungus was dried in an oven at 100 °C. The dry weight of the fungus was recorded when the weight was constant. All tests were performed in triplicate.

Anti-aflatoxigenic Potential of *K. Odora* Essential Oil

AFB1 content in the culture filtrate of *A. flavus* was analyzed by liquid chromatography-mass spectrometry. In a 1.5 mL microcentrifuge tube, 500 µL of culture filtrate was taken and mixed with 500 µL of chloroform by vortexing. The tubes were centrifuged at 14000 rpm at 5 °C for 2 min. The chloroform fraction was collected in a new microcentrifuge tube and kept in a water bath at 60 °C until completely dry. The residue was dissolved in methanol and analyzed using an Agilent 6460 Triple Quadrupole liquid chromatography/mass spectrometry system (Agilent Technologies Inc., CA, USA). Chromato- graphic separations were performed with Symmetry C8 5 µm, 3 mm × 150 mm column

(Waters) following the MS parameters described by Al-Owaisi et al. [32]. Data acquisition and processing were carried out using the Agilent MassHunter workstation and Agilent Mass- Hunter qualitative analysis software, respectively.

Statistical Analysis

The data obtained for *A. flavus* mycelial dry weight and AFB1 content were subjected to analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) at the 5% level was used for comparison of mean values (SAS v8; SAS Institute, NC, USA).

RESULTS AND DISCUSSION

The essential oil was extracted from the aerial parts of *K. odora* by microwave extraction method and its yield was 0.03%. The KOEO significantly inhibited the growth of *A. flavus* at the concentrations of 50 and 100 µL/50 mL of liquid medium and recorded 10.6 and 43.1% reduction in mycelial weight over control, respectively (Table 1). The antifungal activity of EOs from other plant species against *A. flavus* has been reported [19,21,33,34]. Rahimi et al. [25] observed that *Zataria multiflora* essential oil effectively inhibited the growth of *A. flavus*, with a minimum inhibitory concentration (MIC) of 100 ppm and minimum fungicidal concentration (MFC) of 400 ppm. Thanaboripat et al. [20] reported that *Melaleuca cajeputi* EO could completely inhibit the growth of *A. flavus* at a concentration of 25% (v/v) under in vitro conditions. Matasyoh et al. [23] documented the antifungal properties of *Cymbopogon citratus* EO against *A. flavus*, and they observed a MIC of 118 mg/ml. The lipophilic properties of EOs enable them to penetrate the cell walls of susceptible fungi, leading to membrane damage, leakage of cellular macromolecules and cytoplasm coagulation [35,36]. According to the findings of Gandomi et al. [24], the essential oil of *Z. multiflora* caused significant alterations in both morphology and structure of *A. flavus*. These changes included the loss of turgidity, cytoplasm vacuolization, and hyphae deformation. Tatsadjieu et al. [37] demonstrated that *Lippia rugosa* essential oil inhibited the growth of *A. flavus* and membrane H^+-ATPase enzyme activity. Tian et al. [19] reported the antifungal activity of EO extracted from the seeds of *Anethum graveolens* on *A. flavus*. The disruption of plasma membrane permeability and accumulation of reactive oxygen species (ROS) in *A. flavus* due to mitochondrial dysfunction were reported as the mechanisms of antifungal action of *A. graveolens* EO on *A. flavus* [19]. Chaudhari et al. [21] demonstrated that *Pimenta dioica* EO caused a reduction in methylglyoxal and ergosterol content of *A. flavus* plasma membrane and enhanced leakage of cellular ions. The antifungal activity of KOEO against *A. flavus* could be attributed to the fungitoxic phyto-constituents that may be present in the essential oil.

It is evident from this study that KOEO suppressed the toxin production by *A. flavus* in a dose-dependent manner and inhibited AFB1 production up to 93.7% at 100 µL/50 mL as determined by LC-MS analysis (Table 1). Restuccia et al. [38] observed that EOs extracted from *Citrus limon*, *C. bergamia*, and *C. aurantium* effectively impeded the growth of *A. flavus* and reduced AFB1 synthesis. Rammanee and Hongpattarakere [39] reported that EOs obtained from *C. hystrix* and *C. aurantifolia* exhibited inhibitory effects on the growth of *A. flavus* and *A. parasiticus*, resulting in a significant decrease in aflatoxin production. Specifically, *C. aurantifolia* EO, when used at a concentration of 2.25 mg/ml, completely inhibited the growth of *A. flavus* as well as its aflatoxin production. Furthermore, Gorran et al. [11] documented that EOs of *Satureja khozistanica* and *Thymus daenensis* as well as their ethanol extracts suppressed *A. flavus* growth and AFB1 production. The reduction in AFB1 concentration in the liquid cultures could be attributed to the decrease in *A. flavus* growth, potentially due to the effect of KOEO.

Table 1. Inhibitory effect of *Kleinia odora* essential oil (KOEO) on the growth of *Aspergillus fla*vus and aflatoxin B1 production.

KOEO (µL/50 mL)	*A. flavus* growth (mycelial dry weight) (mg/50 mL)	% reduction over control	AFB1 production (ppb)	% reduction over control
10	1.89 ab	4.5	6149 b	28.2
50	1.77 b	10.6	3762 c	56.1
100	1.11 c	43.1	541 d	93.7
Untreated Control	1.98 a	-	8564 a	-

Data are mean of 3 replications
Values followed by the same alphabetical letter, do not differ significantly (DMRT; *P*=0.05)

GC-MS analysis of KOEO revealed the presence of ledol (21.0%), gamma-curcumene (10.2%), caryophyllene (12.0%), 4(10)-thujene (9.9%), and D-limonene (7.6) as major constituents (Table 2; Figure 1). Ledol ($C_{15}H_{26}O$; MW 222.37) is an anti-fungal, toxic sesquiterpenoid. It has been identified as a component of *Rhododendron tomentosum* [40] and *Ledum palustre* [41] essential oils. Gamma-curcumene ($C_{15}H_{24}$; MW 204.35) has been identified as a major component of *Helichrysum microphyllum* subsp. *tyrrhenicum* EO that showed antimicrobial activities [42]. The antimicrobial property of caryophyllene ($C_{15}H_{24;}$ MW 204.35), a sesquiterpene, isolated from *Aquilaria crassna* EO has been documented [43]. Franca et al. [44] reported that the EO of *Schinus terebinthifolius* seeds that showed antimicrobial activity contained 4(10)-thujene (syn: Sabinene; $C_{10}H_{16}$; MW 136.23), a bicyclic monoterpene, as a major component. Limonene ($C_{10}H_{16;}$ MW 136.23), a terpene, is commonly present in the essential oil of lemon and orange [45]. Antimicrobial activity of limonene against *Listeria monocytogenes* has been documented [46]. Al-Taweel et al. [28] previously reported (+)- epi-bicyclosesquiphellandrene, caryophyllene, α-caryophyllene, and α-pinene as the major constituents among the 32 components of *K. odora* EO identified by GC-MS analysis. The differences in the chemical composition of KOEO might be due to differences in their habitats, geographical locations, climatic conditions, and growth stage of the plant sample used for EO extraction [47, 48].

Table 2. Chemical constituents of *Kleinia odora* essential oil.

S. No.	Compound	Retention Time (min)	Area%	KI (NIST)	KI (calculated)
1	n-Hexanol	6.549	2.89	852	893
2	1R-.alpha.-Pinene	7.68	0.73	922	936
3	4(10)-Thujene	8.758	9.91	964	976
4	(-)-.beta.-Pinene	8.845	3.06	965	979
5	.beta.-Myrcene	9.209	4.12	979	992
6	.alpha.-Phellandrene	9.581	0.75	997	1006
7	D-Limonene	10.259	7.59	1018	1030
8	(+)-4-Carene	11.895	1.23	1018	1089
9	β-Elemen	19.654	1.10	1398	1369
10	Caryophyllene	20.376	12.09	1424	1421
11	alpha.-Caryophyllene	21.133	3.68	1456	1454
12	.gamma.-Curcumene	21.635	10.23	1472	1475
13	.alpha.-Curcumene	21.691	0.89	1472	1478
14	Germacrene D	21.748	2.20	1480	1480
15	.beta.-Eudesmene	21.882	2.77	1478	1486
16	o-Menth-8-ene, 4-isopropylidene-1-vinyl-	22.09	0.72	1492	1495
17	.beta.-Bisabolene	22.279	3.76	1500	1504
18	.beta.-Humulene	22.361	1.08	1454	1507
19	Unidentified	22.662	0.84		1521
20	Ledol	22.845	21.04	1597	1529
21	Unidentified	22.954	1.25		1534
22	Nerolidol 2	23.401	1.54	1564	1555
23	Caryophyllene oxide	24.002	4.26	1576	1582
24	.β-Selinenol	25.377	0.90	1627	1648
25	Guai-1(10)-en-11-ol	25.725	1.25	1651	1664

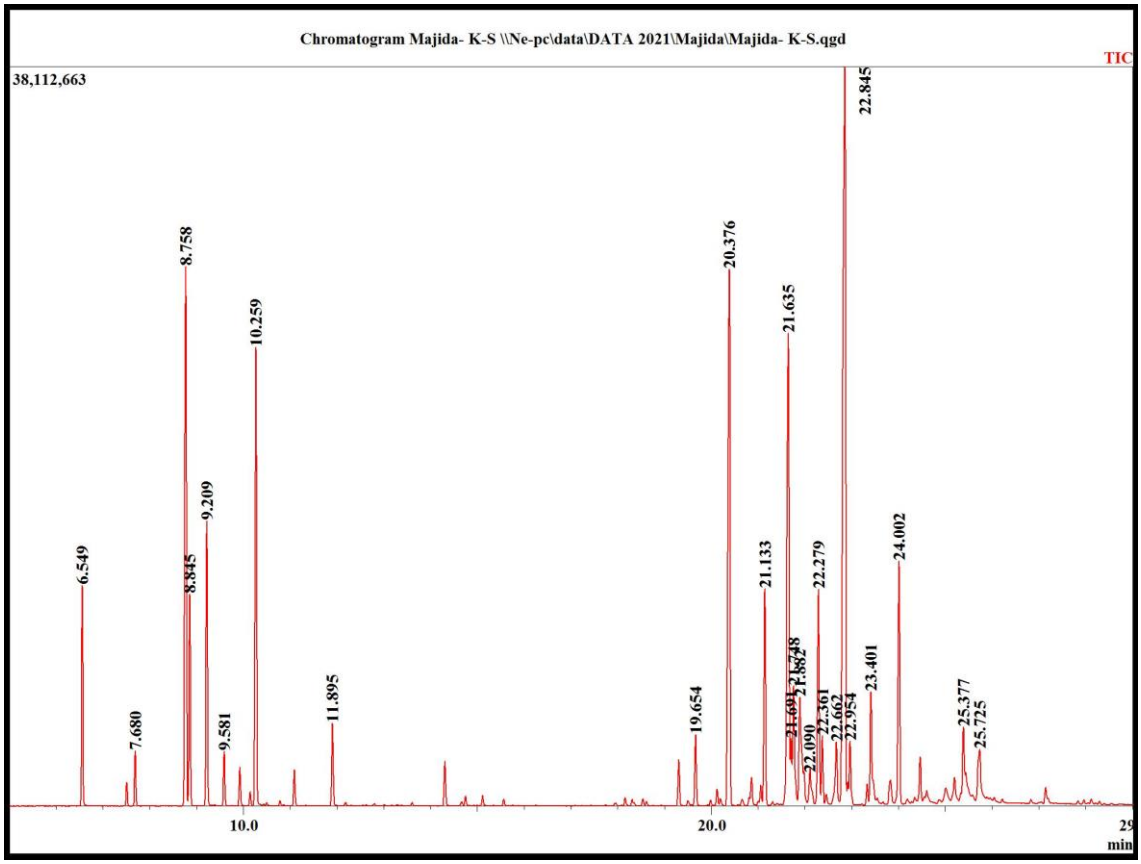

Figure 1. GC-MS chromatogram of *Kleinia odora* essential oil.

CONCLUSION

Our results showed that KOEO was capable of reducing the growth of *A. flavus* and production of AFB1 *in vitro.* The antifungal activity of KOEO against *A. flavus* was proportional to its concentration. These findings suggest that KOEO could be used as a food preservative and/or antimicrobial agent against *A. flavus* contamination. Further studies are needed to elucidate the mechanism of antifungal activity of KOEO. The efficacy of KOEO on the other foodborne mycotoxigenic molds should be determined.

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