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Phytochemical investigation of alkaloid fractions of *Fumaria vaillantii* and *F. asepala* and their antifungal influence on gene expression pattern in *Aspergillus* species

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Abstract: The genus Fumaria is known for its strong medicinal effects. This research aimed to assess the phytochemical profile in various alkaloid fractions from *Fumaria vaillantii* Loisel and *Fumaria asepala* Boiss. grown in the Saveh region of the central province of Iran and their antifungal activity against *Aspergillus flavus* and *Aspergillus niger* was investigated in laboratory conditions. Also, the types and amount of alkaloid compounds and the effect of compounds on the expression of key genes of aflatoxin biosynthesis were conducted in order to investigate the ability of alkaloids of two *Fumaria* species to prepare environmentally friendly disinfectants. The total alkaloid contents were extracted using the soxhlet apparatus and then fractioned to perform GC-MS analysis. The effect of the extract of two species of Fumaria on the activity of *Aspergillus flavus* and *Aspergillus niger* were evaluated considering time factors (24 h, 48 h, and 72 h) and two concentrations (50 and 100 mg/mL). The total alkaloids of the leaf and fruit of *F. vaillantii* respectively contain (3.48 and 3.32 mgg^{-1}DW,) and *F. asupala* (2.38 and 2.26 mgg^{-1}DW, respectively). Fractions 1 and 4 of leaf and fruit showed the highest total alkaloid content, which was then mixed for GC-MS analysis; isoquinoline alkaloids, including protopine and sanguinarine; parfumine and allocryptopine accounted for the highest percentage of alkaloids in both species. *F. vaillantii* had a higher content of these alkaloids. The antifungal activity of fractions 1 and 4 reduced radial growth in both *Aspergillus* species. The alkaloid fractions at both species caused downregulation of aflatoxin B_1 biosynthesis key genes (*aflR*, *aflD,* and *aflM*) in *A. flavus* and fumonisin B_2 key genes (*fum8* and *fum6*) in *A. niger*. The obtained data shows that alkaloid fractions extracted from Fumaria species, *F. vaillantii*, can be useful in the preparation of nature-friendly disinfectants for postharvest disinfection of fruits and vegetables.

Key words: Alkaloid fractions, *Aspergillus flavus*, *Fumaria* sp., antifungal activity, aflatoxin

Abbreviation: GC-MS: Gas chromatography-mass spectrometry; DW: Dry weight

1. Introduction

The genus *Fumaria* (Papaveraceae family) includes 60 herbaceous species (Pérez-Gutiérrez et al., 2012) with a wide distribution range from the west of Asia to the Mediterranean and west of Europe (Europaea, 1993). To date, 8 species of *Fumaria* have been identified in the flora of Iran, which all belong to the microsepala section (Lidén, 1986; Mozaffarian, 2006; Wendelbo, 1974), mostly favor agricultural fields and orchards in the west and north of the country, often as a weed. Morphologically, they have lush narrow shoots and thin leaves, white or reddish-white flowers, and small drupe-like fruits (Araii et al., 2011).

Significant medicinal benefits have been reported in various species in traditional medicine, especially as a remedy for hepatobiliary disorders, cough, eczema treatment, and skin diseases (Orhan et al., 2012). Besides, the phytochemical potential of extracts from *Fumaria* species have known to have antifungal (Moghtader, 2013), antibacterial (Khamtache-Abderrahim et al., 2016), and antiinflammatory (Bribi et al., 2016) effects. Isoquinoline alkaloids are often cited as responsible for these activities, among which protopine is the most prevalent (Vrancheva et al., 2012). However, a high level of polyphenols and flavonoids (Vrancheva et al., 2014) has also been reported, which confer antioxidant (Ivanov et al., 2014) diuretic and antiprotozoal (Orhan et al., 2015) potentials. While the literature on phytochemical diversity and potential of *Fumaria* species is rich, particularly on *F. apreolata* L. (Maiza-Benabdesselam et al., 2007; Noureddine et al., 2013), *Fumaria parviflora* Lam. (Rizvi et al., 2017), *Fumaria officinalis* L. (Suau et al., 2002), *Fumaria indica* Hausskn. (Rathi et al., 2008; Singh et al., 2013), and to some extent, *Fumaria vaillantii* Loisel (Soušek et al., 1999;

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Srivastava and Choudhary, 2014), reports on species like *Fumaria asepala* Boiss. are scarce.

On the other hand, despite the well-known antifungal effects of isoquinoline alkaloids and the extent of *Fumaria* genus as a rich source, so far, published reports on the antifungal study of essential oils or aqueous and alcoholic extracts from this genus are just a few (Khan et al., 2014b; Moghtader, 2013; Orhan et al., 2007; Sarma et al., 1999). Among agriculturally important fungi *Aspergillus* species, *A. lavus,* and *A. niger* are the most commonly found in agricultural products ranging from vegetables to industrial crops (e.g., maize, peanut, and cottonseed) till harvest and postharvest. The contamination of food products with these species leads to spoilage and degrades quantity and quality (Caceres et al., 2020; Kumar et al., 2017; Torres et al., 2014). *A. flavus* is a saprophytic fungus that produces aflatoxins, lethal and carcinogenic substances (Liang et al., 2015). *A. niger* is known as black mold, responsible for several diseases in agro-products without a significant specificity, and damages a wide range of fruits and vegetables (Mogensen et al., 2010). Similar to *A. flavus*, this species also produces carcinogenic compounds as fumonisins, which are polyketide-derived mycotoxins that give rise to cancer and neural complications in animals (Desjardins, 2006); similar disorders have been reported in human populations where maize contaminated with *A. niger* is the main part of their diet (Carballo et al., 2021).

To prevent the entrance of harmful aflatoxins and fumonisins into our food chain, numerous approaches have been introduced to inhibit the growth of *A. flavus* and *A. niger* growth on food products (Bennett and Klich 2003; Chang and others 2005; Amaike and others 2011). Of these, a more sustainable method to control fungal growth in postharvest through using natural products as an application of natural substances in postharvest of agro-products has frequently been reported (Liang et al., 2015; Youssef et al., 2021). Applying isoquinoline alkaloids such as sanguinarine and chelerythrine showed high antifungal activity against agriculturally important pathogens, including *Alternaria alternate*, *Curvularia lunata,* and *Valsa mali* (Yang et al., 2015), revealing the potential of these alkaloids as an alternative for synthetic antifungals. Zhao et al., 2019 similarly reported the mechanism of action of isoquinoline alkaloids against *Rhizoctonia solani* Kühn, *Botrytis cinerea*, *Fusarium graminearum* and *Mycosphaerlla melonis*. The main goal of the present study was to investigate the phytochemical profile of different alkaloid fractions of two species of *F. vaillantii* and *F. Asepala* using a gas chromatography-mass spectrophotometer (GC-MS) and assess the potency of fractions rich in alkaloids on the growth of *A. flavus* and *A. niger* and the gene expression pattern.

2. Materials and methods

2.1. Plant material

Different parts of F. vaillantii and F. asepala (root, shoots, leaves, and fruit) were collected from Saveh, Iran (35° 4'4.25"N, 50°23'23.54"E). A voucher specimen (No. 6563 TEH for F. vaillantii and No. 6564 TEH for F. asepala) was deposited at the Herbarium of Faculty of Agricultre at Islamic Azad University, Saveh Branch by Dr. Babak Delnavaz Hashemloian. The samples collected for extraction were dried under shade for 5 days, then kept in a zip-pack in the refrigerator at 4 °C until the performing of extraction.

2.2. Extraction and fractioning of alkaloids

Grounded materials form different organs (root, stem, leaf, and fruit) (10 g from each) were extracted with 200 mL of 96% ethanol for 4 h in a Soxhlet system following Suau et al., 2002 with slight modifications. Afterward, the extract was filtered using vacuum filtration (JP-40 ⎡ V model). Then hydrochloric acid (2.5%) was used to take out the residue, and by the addition of 150 mL of diethyl ether, the fats were removed. The pH of the solvent was then adjusted to 8 using concentrated ammonium hydroxide prior to adding 150 mL of dichloromethane. Extracts were dried over magnesium sulfate and were evaporated to dry the crude alkaloid extract under high pressure using a rotary instrument (EV 311 VAC, Lab Tech).

The alkaloids fractioned following the Marek et al., 2003 and Khamtache-Abderrahim et al., 2016 with some modification; fraction 1: The pH was adjusted to 8–10 using sodium carbonate. Then, ether was added to form two phases in a decanter. The ether phase was separated and dried by a rotary instrument. Fraction 2 (lipophilic alkaloid): The dried extract was solved in 250 mL of distilled water. The pH was adjusted to 3-4 by H_2SO_4. Then ether was added and poured into a decanter. Two phases were formed, and the ether phase was separated. The ether phase contained lipophilic alkaloid. Fraction 3 (nonpolar alkaloid): The pH was adjusted to 8–10 using sodium carbonate for nonpolar alkaloid extraction. Then, the ether was added to a decanter, and two phases were formed. Fraction 4 (high polar alkaloid): The pH was adjusted with H_2SO_4 and KI. Then, 200 mL chloroform was added, and the solvent was poured into a decanter.

2.3. Identification of alkaloids

Analyses were carried out using a Hewlett Packard (HP; Palo Alto, CA, USA) model 5988A MS coupled to an HP 5980 GC equipped with an HP-1 fused silica capillary column (12 m 0.2 mm i.d.; 0.33 m film thickness). The column temperature was initially held at 200 °C for 0.8 min, then increased at 10 °C/min to 250 °C, and then held at 250 °C for 24 min: the helium flow rate was 10 mL/min. The ion source of the MS was operated at 250 °C and the transfer line at 280 °C. Electron impact (EI) ionization

was carried out at 70 eV, and quantitative determination was based on the total ion current corrected for the detector response of each alkaloid. The mass range from 125 to 450 amu was scanned at a rate of 2.6 scans/s. For analysis, alkaloid fractions (10 mg) were dissolved in dichloromethane (10 mL), and 0.5–1.5 L aliquots were injected directly. Alkaloids were identified by directly comparing their MS and retention times (Rt) with those of authentic samples and with data from the NBS MS library.

2.4. Fungi culture preparation

Toxigenic fungi, *A. flavus* (code: 50041, ID: AR 111) and *A niger* (code 50101, ID: ATCC9142), were procured from Pasteur Institute of Iran, Pathogenic Fungi Culture Collection (PFCC). The fungi were cultured on Sabouraud dextrose agar (SDA) media (Oxioid CM 41) at 25 °C + 2 °C for 6 days and identified according to McClenny, 2005.

2.5. Alkaloid fractions × fungi species

For testing the antifungal activity of alkaloid fractions against *A. flavus* and *A. niger*, two concentrations (50 mg and l00 mg/mL) of each fraction were mixed separately with 50 mL of SDA media, then placed into sterile Petri dishes (11 cm in diameter) and remained until solidification. Mycelial disks (6 mm diameter) were removed from the growing margins of the tested fungi cultures (*A niger* and *A flavus*), transferred on the SDA agar media's surface, and then incubated at 25 °C + 2 °C for 6 days. Controls of *A. flavus* and *A*. niger without the plant extract were also involved in the experiment. Three replications for each concentration were carried out. The growth inhibition for each tested fungal species at different concentrations (50 and 100 mg/mL) was monitored after 24, 48, and 72 h by measuring the radial growth diameter and compare with the control (Özcan, 1998).

2.6. Gene expression analysis

2.6.1. RNA extraction and reverse transcription

The culture media was filtered to isolate fungi hyphae, and RNA extraction was performed using NORGEN© Fungi RNA Isolation Kit (BIOTEK CORP, ON, CA) according to the manufacturer's instructions. RNA samples were treated with DNA-free DNase. Samples were stored at –80 °C prior to further use. The purity and concentrations of RNA were determined by measuring the absorbance of samples at 260 and 280 nm using a NanoDrop 2000 (Gene Company Limited, Hong Kong, China). A Thermo Scientific kit (Thermo Fisher Scientific, Inc, USA) was used for reverse transcription to obtain cDNA according to the manufacturer's instructions. For negative control, similar reactions were performed in the absence of the enzyme.

2.6.2. Real-time PCR analysis

A quantitative comparison of gene expression was performed using real-time PCR using ABI (Applied Biosystems step-one) machine. Following Liang et al., 2015, *A. flavus* 18S ribosomal RNA amplification was used as the internal control gene (Table 1). The sequences of the encoding genes *aflD*, *aflM*, and *aflR* in *A. flavus* were obtained from Liang et al., 2015 and *fum6* and *fum8* in *A. niger* from Palumbo et al., 2013. Three replicates were assigned for each reaction. The reaction mixture volume for each sample consisted of 10 µL of SYBR Green mixture, 3 µL of cDNA, 1.8 µL of each of the precursor-specific primers with a concentration of 10 µm, and 2.6 µL of sterile distilled water. The polymerase chain reaction was performed under the following conditions: 10 min at 95 °C and 35 repetitions: 15 s at 95 °C, 15 s at 60 °C, and 45 s at 60 °C for cDNA synthesis. The $2^{-\triangle\triangle C_T}$ method was used for the quantitative analysis of relative fold gene expression (Livak and Schmittgen, 2001)

2.7. Statistical analysis

All experiments were performed in triplicate. Data for each sample were statistically analyzed using SPSS software (Version 16.0, SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by Duncan's multiple comparisons was used for statistical significance analysis. Differences were considered to be significant at $p < 0.05$ and $p < 0.01$. Graphs were prepared using Graph pad prism 8.

Table 1. Primers used in this study for qt-PCR.

Gene	Forward primer(5′-3′)	Reverse primer(5′-3′)
Aspergillus flavus		
aflD	ATGCTCCCGTCCTACTGTTT	ATGTTGGTGATGGTGCTGAT
aflM	GAGCCAAAGTCGTGGTGAAC	GCCTGGATTGCGATAGCGTC
aflR	CCTTTCTCACTACTCGGGTTT	GCAGGTAATCAATAATGTCGG
18S rRNA	GCTCTTTTGGGTCTCGTAATTGG	CGCTATTGGAGCTGGAATTACC
Aspergillus niger		
fum6	GAAATGGGCGCGTCTTGGGGAA	CGCTCAACCGCTCTCCCGTTTT
fum8	CCGGGACTTGAAAGCATGGCGT	TGACAACCTCTCGTGTCGGGCA
b-tubulin	GGTAACCAAATCGGTGCTGCTTTC	ACCCTCAGTGTAGTGACCCTTGGC

3. Result

3.1. GC-MS analysis

The total alkaloid contents of the different organs of two species, *F. vaillantii* and *F. asepala* extracted using ethanol 96% in the Soxhlet system described by Suau et al., 2002 as shown in Table 2. Leaves and fruits in *F. vaillantii* with 3.48 and 3.32 mgg^{-1} DW had a higher total alkaloid content compared to *F. asepala* 2.38 and 2.26, respectively (Table 2). The majority of alkaloid content was observed in fractions 1 and 4 in both species (Table 3). The order of alkaloid content in organs was leaf>fruit>stem>root, wherein roots, no measurable amount of alkaloids were detected in fractions 2 and 3.

Considering the results presented in Table 3, alkaloids in fractions 1 and 4 for leaf and fruit were mixed and profiled as shown in Table 4. In *F. vaillantii,* protopine accounted for 20.67 % as a primary alkaloid detected, while in *F. asepala*, protopine was considerably lower with 9.71% . The other major alkaloids, sanguinarine had the second-highest percentage in *F. vaillantii* (18.70 %) and *F. asepala* (8.91 %). The content of parfumine showed a relatively significant interspecies difference as it was lower in *F. vaillantii* (6.91) compared to *F. asepala* (8.62). Two compounds sinactine and stylopine were not detected in *F. vaillantii* while in *F. asepala* accounted for 5.11% and 2.15%, respectively. Fumaricine also was not detected in *F. asepala* while it was 4.26% in *F. vaillantii.* The content of 9 compounds manifested a difference between the two species. However, the content of compounds found in *F. asepala* were notably lower (Table 4).

Table 2. Total alkaloids (mg g^{-1}DW) in various organs of *F. vaillantii* and *F. sepala.*

Organ	*F. vaillantii*	*F. asepala*
Root	0.21 ± 0.01*	0.10 ± 0.01
Stem	0.4 ± 0.02	0.26 ± 0.08
Leaf	3.48 ± 0.2	2.38 ± 0.12
Fruit	3.32 ± 0.21	2.26 ± 0.05

*Data are mean ± standard error (Mean±SE)

3.2. Antifungal activity

The inhibitory effect of alkaloid fractions extracted from *F. vaillantii* on two fungi species of *A. flavus* and *A. niger* was observed (Figure 1). In this multi-factor test, the radial growth of *A. flavus* was influenced by fractions as well

Table 3. Total alkaloids (mgg^{-1}DW) in various organs of different fractions in *F. vaillantii* and *F. asepala*

Species	Organ	F. 1	F. 2	F. 3	F. 4
	Root	0.12 ± 0.01*	-	-	0.09± 0.02
F. vaillantii	Stem	0.1 ± 0.03	0.08 ± 0.01	0.07 ± 0.01	0.15 ± 0.05
	Leaf	1.11 ± 0.17	0.51 ± 0.08	0.42 ± 0.01	1.44 ± 0.1
	Fruit	1.12 ± 0.12	0.42 ± 0.02	0.59 ± 0.06	1.19± 0.15
	Root	0.05 ± 0.01	-	-	0.05 ± 0.1
F. asepala	Stem	0.09 ± 0.01	0.04 ± 0.00	0.05 ± 0.01	0.08 ± 0.01
	Leaf	0.92 ± 0.14	0.14 ± 0.35	0.2 ± 0.18	1.12 ± 0.23
	Fruit	0.90 ± 0.31	0.17 ± 0.05	0.19 ± 0.04	1.0 ± 0.05

*Data are mean ± standard error (Mean±SE)

Table 4. The profile of a mixture of fraxion 1 and 4 from laef and fruite of *F. vaillantii* and *F. asepala* using GC-MS

No. of peak	Alkaloid	RT (min)	*F. vaillantii* (%)	*F. asepala* (%)
1	Adlumine	13.19	4.3±0.5*	2.03±0.13
4	Allocryptopine	49.59	5.73±0.91	7.83±0.84
4	Fumariline	21.50	4.09±0.24	3.19±0.35
4	Fumaricine	53.75	4.26±1.04	-
4	Parfumine	25.39	6.91±0.63	8.62±1.4
1	Protopine	1.54	20.67±3.14	9.71±0.82

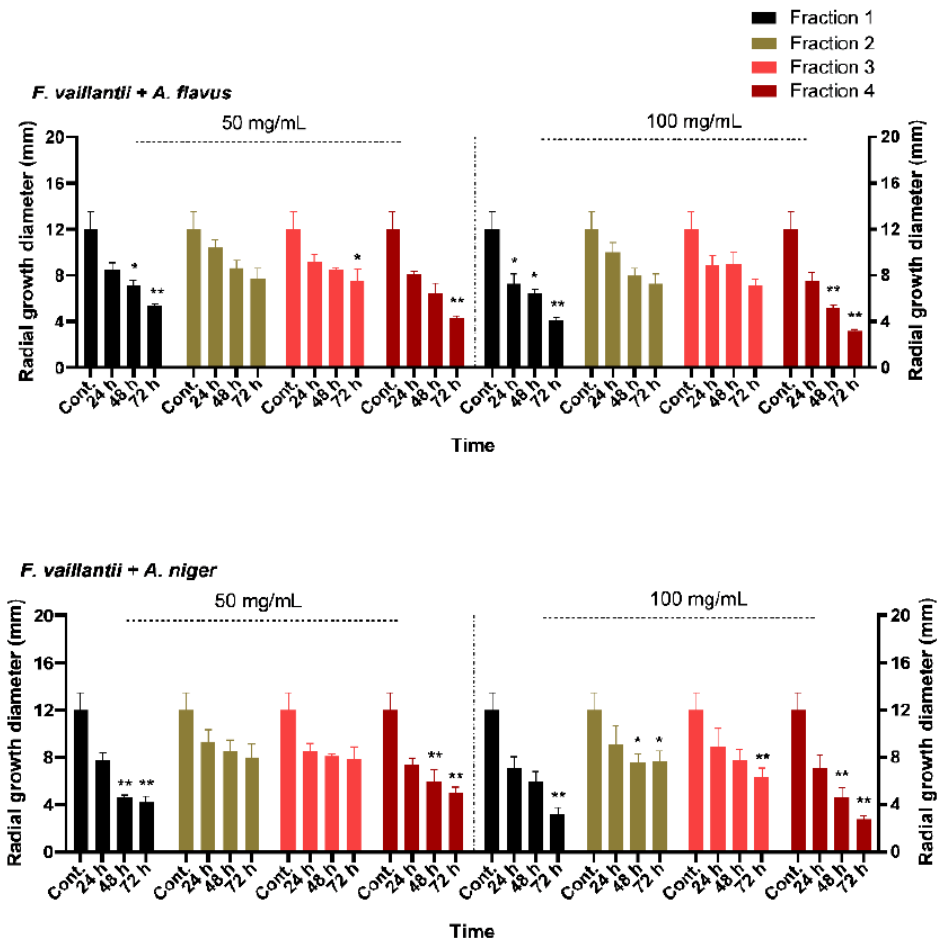

Figure 1. Inhibitory effect of *F. vaillantti* alkaloids fractions on the radial growth (mm) of *A. falvus* and *A. niger* after 24, 48 and 72 h incubation period under 50 and 100 mg/mL concentrations. ∗ Statistically significant when compared to control, $P < 0.05$; ∗∗ Statistically 6 significant when compared to control, $P < 0.01$. Data are mean ± standard error (Mean±SE)

as the time treatment; as the time increased from 24 h to 72 h, the fungi growth was inhibited further. The greatest decrease in radial growth was seen in samples treated with 100 mg/mL of fraction 4 at 72 h, and after that the decrease was noticeable at 48 h, when both treatments showed a significant difference at 1% level with the control. The intensity of reducing the radial growth of exposing *A. niger* to fraction was seemingly higher as fraction 4 with 50, and 100 mg/mL concentration under 72 h notably decreased with radial growth of 5.2 and 3.1 mm, respectively. Fractions 2 and 3 indicated a lower inhibitory effect, in general. However, 100 mg/mL concentrations of 2 and 3 in 72 h made a significant difference with control.

It seemed the fractions of *F. asepala* overall showed a lower antifungal effect since the radial growth was significantly higher (Figure 2). The radial growth of *A. flavus* was slightly higher when treated with different concentrations of *F. asepala* fractions. In fractions 1 and 4, still, the radial growth decremented considerably, at 72 h exposed to fraction 4 revealed a significant decrease compared to control. In *F. asepala* × *A. niger*, the effect of fractions increased where fractions 2 and 3 also had a significant reduction in radial growth under 100 mg/mL concentration and 48 h and 72 h.

3.3. Gene expression analysis

Alongside evaluating the antifungal effect of alkaloid fractions, the expression of key genes encoding aflatoxin biosynthesis in *A. flavus* was also analyzed. The expression levels of three genes involved in aflatoxin biosynthesis in *A. flavus* under the influence of *F. vaillantii* alkaloid fractions are shown in Figure 3. The relative expression of genes, *aflM*, *aflmR*, and *aflD*, in *A. flavus* in *F. vaillantii* showed severe downregulation at the first 24 h, however, expression increased with time intangibly. Amongst the three genes of *A. flavus* under the *F. vaillantii* alkaloid fractions, *aflM* seemed to have the lowest expression level

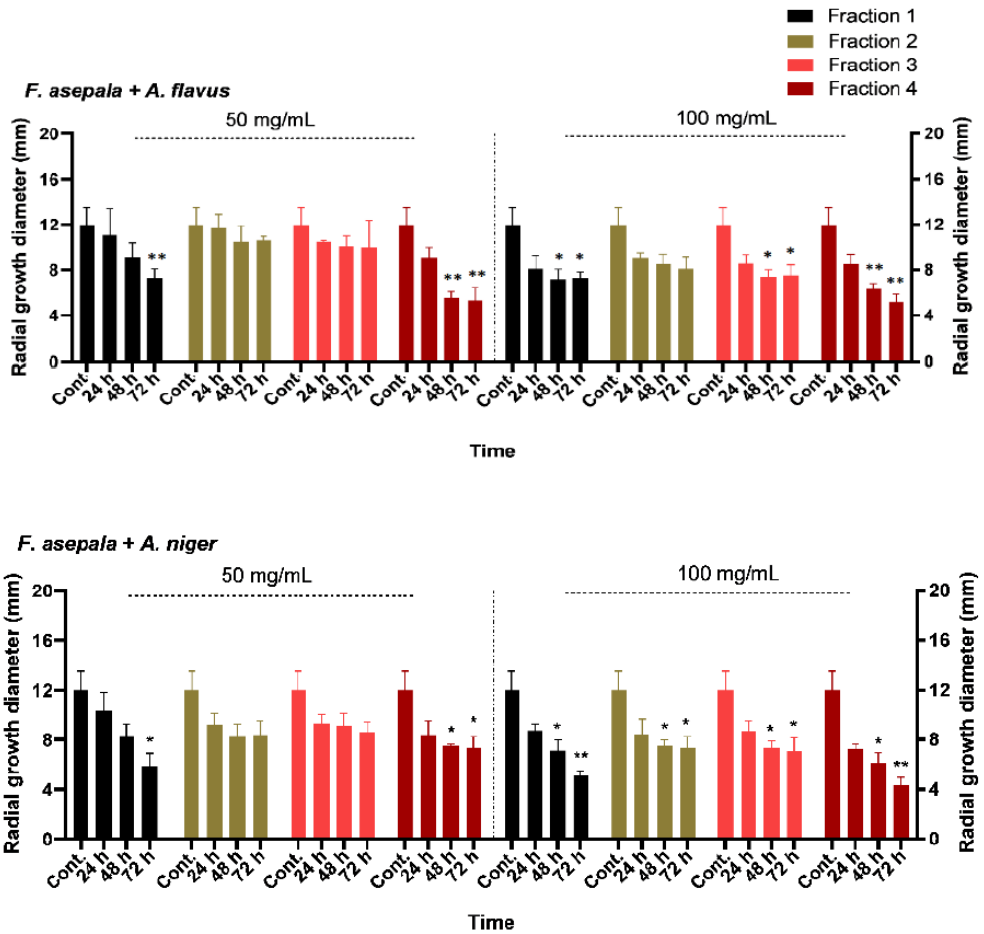

Figure 2. Inhibitory effect of *F. asepala* alkaloids fractions on the radial growth (mm) of *A. falvus* and *A. niger* after 24, 48 and 72 h incubation period under 50 and 100 mg/mL concentrations. ∗Statistically significant when compared to control, $P < 0.05$; ∗∗ Statistically significant when compared to control, $P < 0.01$. *Data are mean ± standard error (Mean±SE)

in general while *aflD* on the other hand, showed a higher relative expression (Figure 3). Downregulation of the *A. flavus* genes was also observed in treatment with *F. asepala* alkaloid fractions; however, by increasing in time from 24 h to 72 h the relative expression incremented but still showed a significant difference with control, particularly under 100 mg/mL concentration (Figure 4). Overall, *F. asepala* alkaloid fraction had a lower inhibitory effect on the expression of *aflM*, *aflmR*, and *aflD* compared with *F. vaillantii.* in this study, radial growth decreased over time while expression level was either steady or increased insignificantly. In the current study, both expression and fungi radial growth decreased with an increase of concentration from 50 to 100 mg/mL.

The expression of genes responsible for the biosynthesis of fumonisin in *A. niger* (fum8 and fum6) under the treatment of alkaloid fractions was analyzed with qtPCR. The alkaloid fractions 1 and 4 of *F. vaillanttii* managed to hamper the expression of *fum8* and *fum6* to the lowest levels at both 50 and 100 mg/mL concentrations (Figure 5). It is worth mentioning that in *A. flavus* treated with fraction 1, at the first 24 h, the expression of fum8 was zero (Figure 4). In 2 and 3 fractions, expression of fum8 and fum6 downregulated at 24 h to a significant level while notably increased at 48 h and 72 h. Interestingly, in fraction 4, the expression of fum8 downregulated at 72 h while the fum6 continued its upregulation. Similar to the effect of *F. asepala* alkaloids on gene expression in *A. flavus*, the expression of *fum8* and *fum6* genes in *A. niger* overall was higher under this species' alkaloid fractions (Figure 6). Fraction 1, at 50 mg/mL concentration, downregulated *fum8* and *fum6* at the first 24 h to a significant level, whereas at 48 h and 72 h after, their expression surged.

4. Discussion

The alkaloid contents reported for these two species were significantly lower than *F. officinalis* (6.3 mgg^{-1} DW)

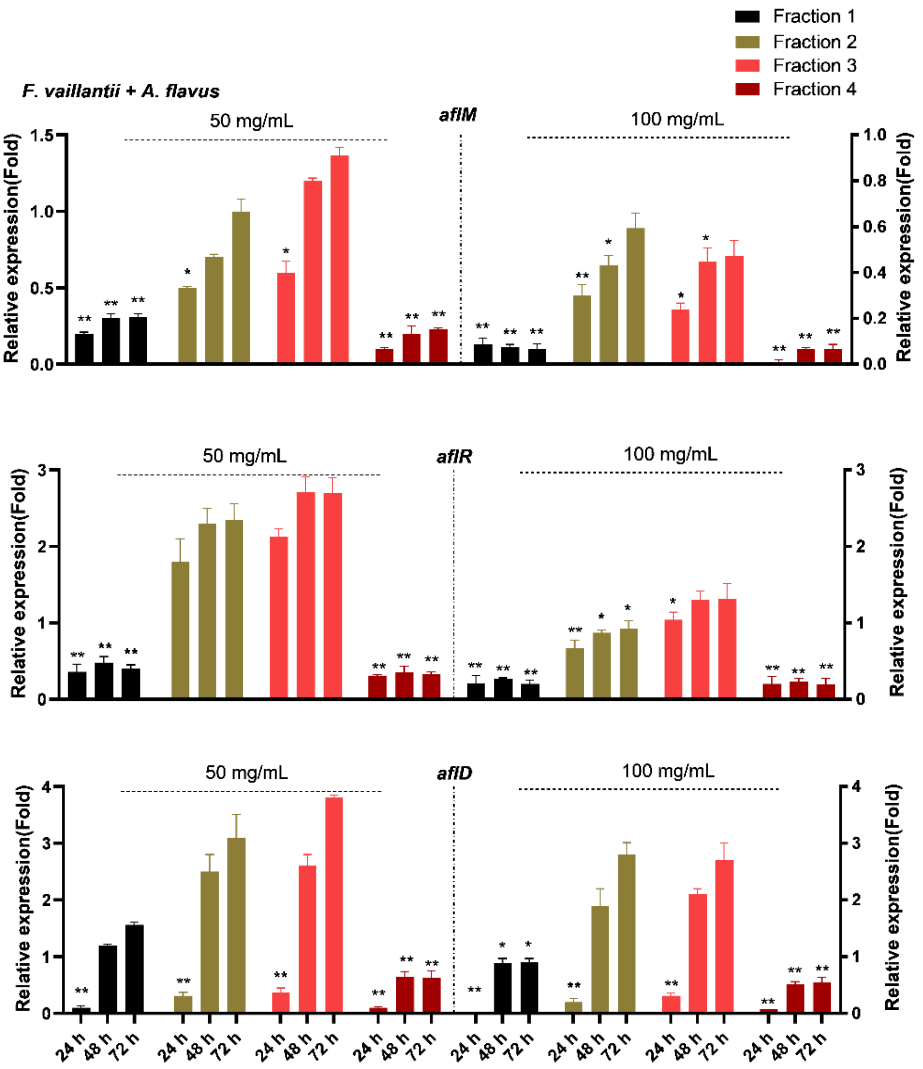

Figure 3. Effect of *F. vaillantii* extract fractions on the expression of *aflM, aflR* and *aflD* in *A. flavus* fungus. The relative expression of genes in *A. flavus* treated with 50 mg/mL extract (left-side bar chart and 100 mg/mL extract (right-side bar chart). ∗Statistically significant when compared to control, $P < 0.05$; ∗∗ Statistically significant when compared to control, $P < 0.01$. Data are mean ± standard error (Mean±SE)

reported by Khamtache-Abderrahim et al., 2016 from Turkey or the same species reported from Spain (5.4 mgg^{-1} DW) (Suau et al., 2002). The alkaloid content of *F. vaillantii,* previously reported by Soušek et al., 1999, was notably higher (17.44 mgg^{-1} DW) than the result of the current study. Alkaloids are often provided a defense for plants against pests and herbivores, which can explain the high content of alkaloids in the leaves and fruits of these two species. Protecting the reproductive organs is the prime importance for plant survival (Otani et al., 2005). Alkaloids are produced in roots and translocated to aerial parts, as reported in *Nicotiana tabacum* L. where leaf vacuoles are rich in alkaloids translocated from roots via xylem (Steppuhn et al., 2004). Therefore, a similar biosynthesis process involved in *Fumaria* may explain the order of alkaloid content observed in this study.

In consistent with our results, Soušek et al., 1999 found protopine to be one of the major isoquinoline alkaloids in *F. vaillantii* (21.1%). However, they used RP-HPLC (reversed-phase high-performance liquid chromatography) and identified 13 alkaloids, some of which, like fumarophycine was not detected in our study using GC-MS. Suau et al., 2002 reported that protopine was the primary alkaloid in 10 species of *Fumaria* (did not include the two species in this study). The second major alkaloid was stylopine, while in our research here, this compound was identified only in *F. asepala*. Sanguinarine, on the other hand, was the second main compound for both *F.vaillantii* and *F. asepala*, Şener

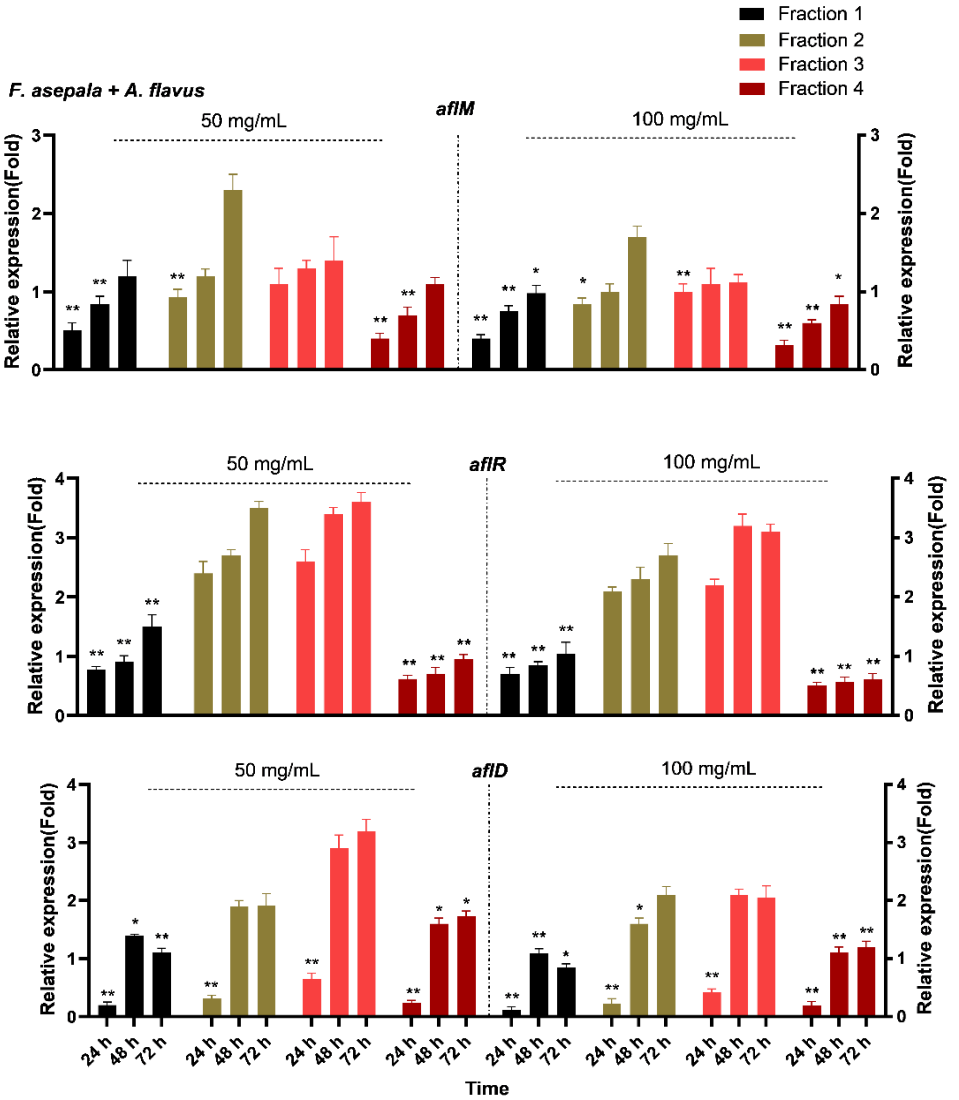

Figure 4. Effect of *F. asepala* extracts fractions on the expression of *aflM, aflR* and *aflD* in *A. niger.* The relative expression of genes in *A. niger* treated with 50 mg/mL extract (left- side bar chart and 100 mg/mL extract (right-side bar chart). ∗Statistically significant when compared to control, $P < 0.05$; ∗∗ Statistically significant when compared to control, $P < 0.01$. Data are mean ± standard error (Mean±SE)

et al., 1983 and Sener, 1985 similarly reported the presence of this important compound in aerial parts of these two species; however, (+)-Bicuculline also was reported, which was not detected in this study.

The antimicrobial activities for some of the isoquinoline alkaloids have long been known (Orhan et al., 2007; Yang et al., 2015). There is an ongoing attempt to limit the application of chemical fungicides in agronomy and horticulture (Liang et al., 2015), thus here the alkaloid fractions of F. vaillantii and F. asepala were evaluated for their antifungal potential against two of the most common and important fungi, A. flavus and A. niger. The application of individual isoquinoline alkaloids against pathogenic bacteria and fungi has been frequently reported revealing significant variation in their potency of effect; for instance, Orhan et al., 2007 evaluated 33 isoquinoline alkaloids from *Famaria* and *Corydalis* species for their antifungal and antiviral activity where mostly, protopine, in particular, had a significant effect on *Candida albicans* (4 µg/mL). Investigating the crude methanolic extract of *F. indica* on *A. flavus* revealed significant antifungal activity, however, in high concentrations (300–500 mg/mL) (Khan et al., 2014a). Similarly, Moghtader, 2013 observed the potent antifungal activity of *F. vaillantii* essential oil

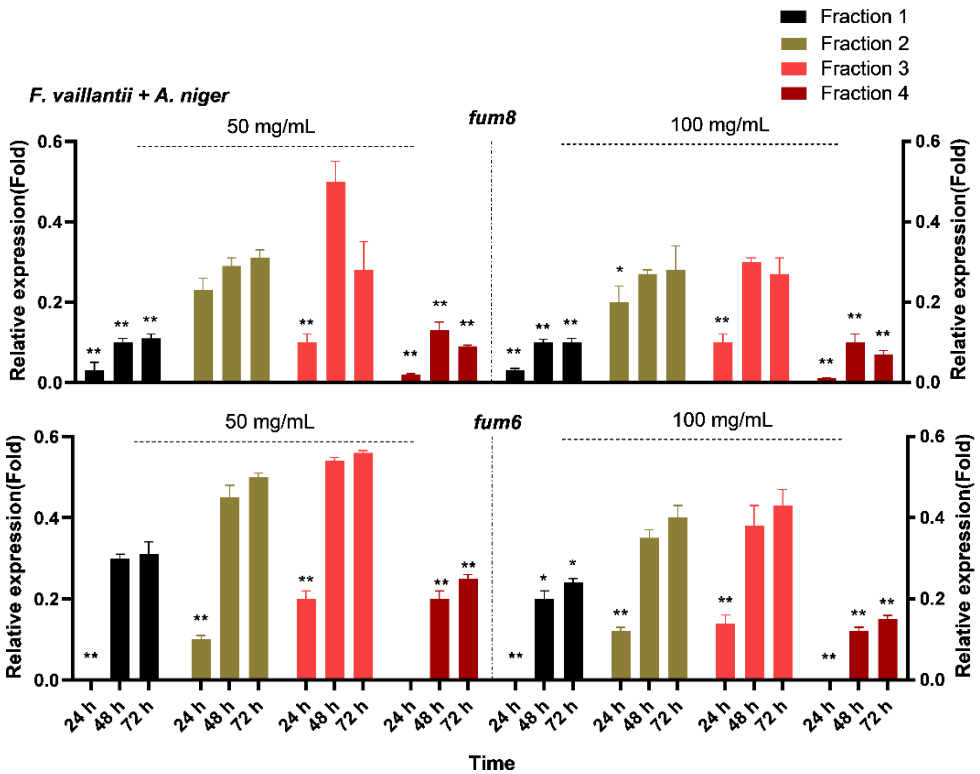

Figure 5. Effect of *F. vaillanttii* extract fractions on the expression of *fum8* and *fum6* in *A. niger.* The relative expression of genes in *A. niger* treated with 50 mg/mL extract (left-side bar chart and 100 mg/mL extract (right-side bar chart). ∗Statistically significant when compared to control, P < 0.05; ∗∗ Statistically significant when compared to control, P < 0.01. Data are mean ± standard error (Mean±SE)

rich in isoquinoline alkaloids on *A. flavus*. Then again, the synergistic interaction among the isoquinoline alkaloids presented from *F. vaillantii* and *F. asepala* may confer higher antifungal activity than the compounds individually (Zhang et al., 2020). The potent inhibitory effect of fractions 1 and 4 on fungal growth suggested a membrane-located target for their action (Khan et al., 2010). Sarma et al., 1999 studied the effect of berberine derivatives from *F. indica* on *Erysiphe cichoracearum* and *Fusarium udum* Butler and observed the inhibitory effect of the extract by preventing spore germination. The current study suggests the potential of alkaloid fractions of these two species of *Fumaria* in controlling damaging fungi species like *A. flavus* and *A. niger* under field and postharvest conditions.

Liang et al., 2015 reported increased expression with time in *A. flavus* treated with eugenol, cinnamaldehyde, and citral. In earlier studies, it has been observed that the expression of aflatoxin-encoding genes is affected by a considerably lower concentration of plant-based compounds. In contrast, a higher concentration is needed to inhibit the fungal growth; case in point, Yan et al. (2004) observed that cyclo produced by *Achromobacter xylosoxidans* impeded aflatoxin production in *Aspergiloius parasiticus* at 0.20 mg/mL. Still, the fungal growth inhibition happened at higher than 6 mg/mL. Assessing the expression of the genes unveiled that cyclo can repress *aflR*, which consequently the transcription level of structural genes such as *fas-1* (*aflB*), *pksA* (*aflC*), and *omtA* (*aflP*) inhibited, reducing the biosynthesis of aflatoxin. Also, the application of caffeic acid at 12 mmol/L reduced 95% of aflatoxin biosynthesis through down-regulating aflatoxin biosynthetic genes in *A. flavus*, with a more negligible effect on the fungal growth (Kim et al., 2008).

Here, the order of downregulation of genes was *aflM*> *aflmR*>*aflD* which to a large extent was consistent with the previous report replaced by Liang et al., 2015, where they found downregulation of *aflmR* responsible for the downregulation of other key genes involved. Similar results were obtained by Jahanshiri et al., 2012, who proposed the downregulation of *aflR* in *Aspergilous parasiticus* exposed to curcumin, which is responsible for the downregulation of other key genes including *aflT*, *aflD*, *aflM*, and *aflP*. The fumonisin biosynthetic gene cluster (*fum*), first identified in *Fusarium verticillioides* regulates the fumonisin production composed of 16 genes with

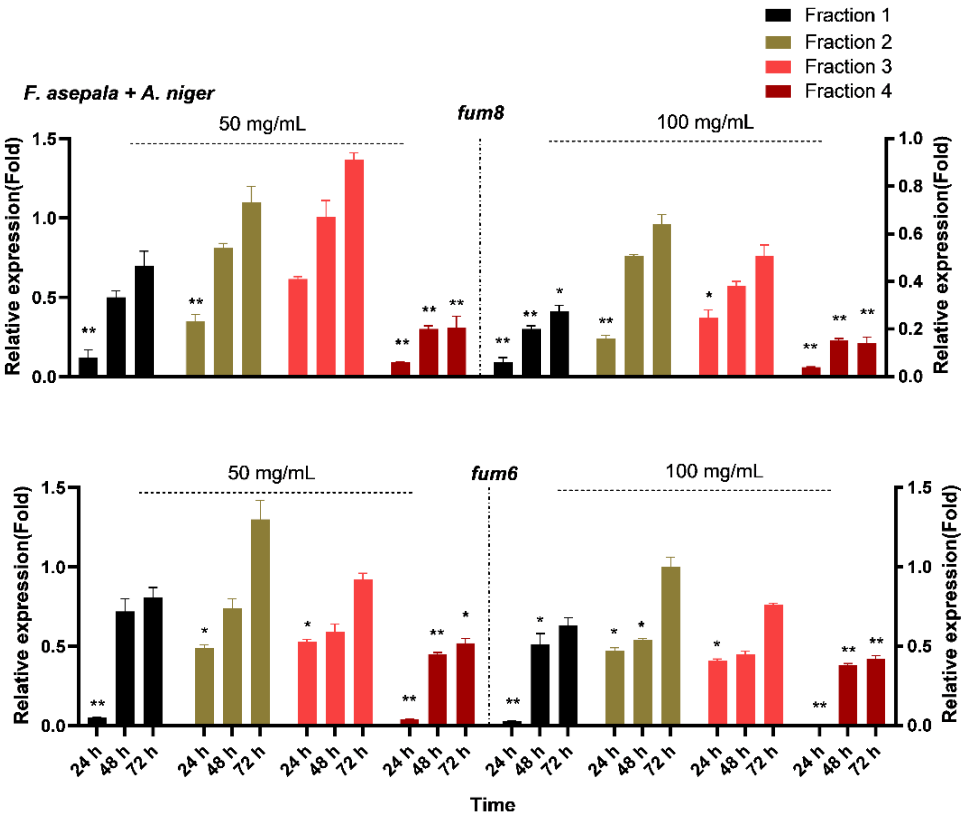

Figure 6. Effect of F. asepala extracts fractions on the expression of *fum8* and *fum6* in *A. niger* fungus. The relative expression of genes in *A. niger* treated with 50 mg/mL extract (left-side bar chart and 100 mg/mL extract (right-side bar chart). ∗Statistically significant when compared to control, $P < 0.05$; ∗∗ Statistically significant when compared to control, P 5 < 0.01. Data are mean ± standard error (Mean±SE)

encoding, regulatory, and protein transport roles (Proctor et al., 2013). The putative *fum* cluster in *A. niger* consists of at least 10 genes that are orthologous to *F. verticillioides* fumonisin biosynthetic genes. Among them, two are in our interest in this study, fum8 with predicted functions of encoding an α-oxoamine synthase responsible for the condensation of the linear polyketide with alanine has a focal role in the biosynthesis of fumonisin (Lazzaro et al., 2012; Medina et al., 2013), and *fum6* also that functions in cytochrome P450 monooxygenase (Palumbo et al., 2013). The *fum* genes are keys in the biosynthesis of mycotoxin fumonisin B_2, particularly *fum8,* as Susca et al., 2010 established a correlation between the production of fumonisin B_2 and the presence of *fum8* in grapes.

5. Conclusion

The current investigation comprehensively evidences the rich phytochemical profile of alkaloid fractions 1 and 4 of the *Annona squamosa* leaves and fruits, where the main alkaloid compounds were protopine, sanguinarine, parfumine, and allocryptopine, respectively (Shami, 2017). The alkaloid fractions revealed significant antifungal activity against *A. favus* accompanied by downregulating the expression of key biosynthetic genes for aflatoxin B_1, *aflR*, *aflD*, and *aflM*. Similarly, the downregulation of genes encoding fumonisin B_2, fum8, and fum6 in *A. niger* also resulted from exposure to alkaloid fractions. Although both species of fumaria showed strong potential but *F. vaillanttii* had a more substantial effect, indicating the potential of the alkaloid fractions of this species to be used as plant-based compounds replacing chemical fungicides during the postharvesting process of crops and vegetables. The result of this study is first-handed data that can be exploited for sustainability in the postharvest process of agricultural products. However, utilizing a broader range of alkaloid fractions and analyzing more key genes involved in the biosynthesis process of aflatoxin and fumonisin are highly recommended in future research endeavors.

Conflict of interest declaration

We have no conflict of interest to declare.

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