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ANTIFUNGAL POTENTIAL OF SOME PLANTS ON MYCELIAL GROWTH AND AFLATOXIN B1 BIOSYNTHESIS OF ASPERGILLUS FLAVUS

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Abstract

In this study, antifungal activities of 15 plant species were examined in vitro against toxicogenic Aspergillus flavus. Among the tested plants, Origanum vulgare powders showed the highest antifungal activity. O. vulgare powders inhibited mycelial growth of A. flavus by 100%. Our study continued with O. vulgare powders, which showed the highest in vitro antifungal activity against A. flavus. Minimum inhibitory concentration (MIC) value of O. vulgare aqueous extract was 250 µg/mL. The antifungal activity of O. vulgare on A. flavus mycelium growth in artificially infected sunflower, wheat, maize and rice grains was tested in vivo. These grains, artificially infected with A. flavus, were stored in test tubes containing O. vulgare extracts (2, 4, 8, 16 and 32 mg/mL) for 5 days (25oC). After the storage period, these grains were transferred to Petri dishes containing Potato Dextrose Agar (PDA). After the 5th day of incubation (25oC), O. vulgare extracts were unable to completely inhibit the mycelial growth of A. flavus. However, inhibition of mycelial growth of A. flavus was observed. Especially in infected sunflower grains, the inhibition of mycelial growth of A. flavus was significant ($p<0.05$) at all tested concentrations of O. vulgare extracts. Scanning electron microscope (SEM) analysis determined that aqueous extracts of O. vulgare made lysis, collapse, flattening and degenerative changes of cells with wrinkled cell surfaces on hyphal morphology. HPLC results showed that 8 mg/mL aqueous extract of O. vulgare inhibited A. flavus production of aflatoxin B1 by 100%. We can emphasize that aqueous extracts of O. vulgare have great potential against A. flavus, a producer of aflatoxin B1.

Keywords: *Aspergillus flavus*, *Origanum vulgare*, aflatoxin B1, antifungal, plant extract

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Introduction

Fungal growth and increase in the risk of mycotoxin production can be caused by improper handling, packaging, drying and transportation along with poor harvesting practices (Vila Donat, 2018). Contamination with mycotoxins is a major food safety problem for grains and other agricultural products. Each year, aflatoxins, which are among many dangerous mycotoxins, contaminate a significant percentage of the grain crops of the world.

During food crop production, the emergence of mycotoxins, as well as yield losses due to fungal infections, pose a critical health risk (Campos-Avelar et al., 2021). Mycotoxin as a term first came into usage in the 1960's in England during the investigations done on the turkeys that died after feeding on peanut meal. This toxic molecule was later identified as aflatoxin B1 (McCormick, 2013).

Aflatoxins (AFs) are one of the most important mycotoxins naturally occurring in agricultural products. AFs are coumarin difurano, consisting of two furans and a coumarin ring (Alkadi, 2021). AFs are classified as group 1 carcinogens in humans. As an AF, aflatoxin B1 (AFB1) is produced by *A. flavus* Link ex, which contaminates grains and other foods (Alshannaq et al., 2018).

Foods contaminated with this toxigenic fungi and the presence of aflatoxins are a major problem attracting worldwide attention due to their harmful effects on human and animal health and their importance in international food trade (Jallow et al., 2021). Synthetic fungicides are used worldwide to control fungi capable of producing mycotoxins that cause food spoilage. However, there is a growing worldwide consumer trend to reduce fungicides in the food chain due to the development of fungicide-resistant pathogens, their carcinogenic and teratogenic properties, and their potential to cause residue toxicity. This has led to the search for different approaches that are environmentally and economically friendly. In these studies seeking alternatives to synthetic fungicides, it was aimed to determine high-efficiency, low-residue and non-toxic biocontrol methods (Habschied et al., 2021; Kongolo Kalemba et. al., 2024).

It has been stated that as an alternative in preventing fungal growth and mycotoxin formation, usage of plant extracts, spices and their components may supply the desired result (*Dikhoba et al., 2019;* Ponzilacqua et al., 2019; Kavitha et al., 2020). Many spices and some plant extracts together with their essential oils have been proven to be effective antifungal against toxigenic fungi.

In this study, the effect of powders of 15 plant species on the mycelial growth of the food pathogen *A. flavus* was evaluated *in vitro*. The effect of *O. vulgare* aqueous extracts showing the highest inhibition on the hyphal structure of *A. flavus* and aflatoxin B1 production was examined. Its potential protective effect against artificially induced *A. flavus* infection in various cereals was tested.

Materials and methods

Fungal isolate, plant materials and extract preparation

Toxigenic *Aspergillus flavus* strain used in this study was obtained from the Arda Vocational School Mycology Culture Collection, Trakya University, Türkiye. The fungus conidia were inoculatedon Potato Dextrose Agar (PDA) plates and cultured at 25ºC. The 7 days old cultures were used for subsequent analysis.

The plant samples used in this research were assembled from different locations of the Yıldız Mountains to evaluate their antifungal properties against *A. flavus* (Kırklareli, Türkiye), identified, labeled and placed in the herbarium laboratory (Trakya University, Türkiye). After being cleaned and dried, they were ground with a laboratory type grinding mill (A11B, İKA). The prepared aqueous extracts were lyophilized and stored at -20ºC (Khadri et al., 2010).

In vitro effect of plant powders against mycelial growth of Aspergillus flavus

The suspensions obtained by adding plant powders (10 g) to 100 mL of PDA medium were autoclaved at 121ºC for 15 minutes. It was filtered through a 4-layer sterile cheese cloth before being poured into Petri dishes. 5mm agar discs taken from the active growth tip of seven-day-old *A. flavus* were used for inoculation. Incubation was carried out at 25ºC for 7 days. Mycelial growth inhibition was calculated using the following formula (Nduagu et al., 2008).

$$\text{MIR} = \frac{M_1 - M_2}{M_1 \times 100} \quad (1)$$

where MIR is the inhibitory rate (%) in colony diameter, M1 is the mycelia diameter of control and M2 is the diameter of *O. vulgare* aqueous extract treatment.

Minimum inhibitory concentration (MIC)

The MIC value was tested according to the CLSI (2002) standard procedure following the instructions for filamentous fungi (M38A). The MIC value of *O. vulgare* aqueous extract against *A. flavus* at concentrations from 0.98 to 1000 µg/mL was determined in RPMI-1640 (Sigma, St. Louis, MO, USA) buffered to pH 7.0 with MOPS. The inoculum suspension was adjusted to 10^4 CFU/mL. Inoculated 96-well microtiter plates were incubated at 25°C. The lowest concentration value at which *A. flavus* did not show visible growth was recorded as MIC.

Protection of aqueous extracts against grains infected with A. flavus

Various healthy cereal grains (sunflower, wheat, maise and rice) were dried at 40°C for 48 hours. The grains were packed into Erlenmeyers and autoclaved for 15 minutes at 121°C (Dambolena et al., 2010). Autoclaved grains were transferred to tubes containing 10^4 CFU/mL *A. flavus* spores and allowed to become infected for 1 minute. The infected grains were then placed in test tubes containing sterile aqueous plant extracts at concentrations of 2, 4, 8, 16, and 32 mg/mL. Controls consisted of tested cereal grains, *A. flavus* spores, and medium. The tubes were stored at 25ºC for 5 days. Afterwards, the cereal grains were transferred to Petri dishes containing PDA (Lima et al., 2019) and incubated for 5 days (25ºC). Radial mycelial growth was recorded daily.

Examination of A. flavus hyphal structure applied to O. vulgare aqueous extract

Mycelial agar disks were removed from the 7-day culture on the PDA plate with a cork borrer and placed in the center of another PDA plate. After 2 days of pre-incubation, *O. vulgare* aqueous extract (4MIC) was dripped and incubated at 25°C for 3 days (Soylu et al., 2010). *A. flavus* hyphal structures treated and untreated with *O. vulgare* aqueous extract were analyzed through Quanta FEG 250 scanning electron microscope (SEM).

Aflatoxin B1 analyses by HPLC

The potential effect of *O. vulgare* aqueous extracts on the aflatoxin B1 (AFB1) production of *A. flavus* was tested by HPLC (Agilent 1100). Different dilutions of the aqueous extracts (1, 2, 4, and 8 and mg/mL) were mixed with sterilized Potato Dextrose Broth (PDB) in tubes. Controls were prepared without plant extract. The experiments were repeated three times.

After 7 days of incubation at 25^{o}C, 2 mL of the culture medium was taken and mixed with an equal volume of ethyl acetate and vortexed twice for 3 minutes. After waiting for 30 minutes, it was centrifuged at 10,000 rpm for 4 minutes. The supernatant was transferred to a new tube. Ethyl acetate was dried at 40^{o}C and evaporated. Dry extracts were rediluted with 100µL of methanol (Safari et al., 2020).

Aflatoxin B1 was determined using HPLC-fluorescence detector (FLD) and immunoaffinity column according to the modified AOAC 991.31 method (AOAC, 2005). 100uL of the dry extracts diluted with methanol was injected into the HPLC. During HPLC analysis, the composition of the mobile phase was formed from acetonitrile + methanol + water + 65% HNO_3 (200: 300: 600 + 350 µL 65% HNO_3 and 0.12 g KBr) and the flow rate of 1 mL/min was maintained throughout the analysis.

Statistical analysis

For the analysis of data, STATISTICA software was used to do ANOVA (analysis of variance) along with Mann-Whitney U tests. Using the statistical package program "SPSS for Windows, version 15.0", values lower than $p<0.05$ were considered significant for this evaluation.

Results and discussion

Selection of the plant with the highest inhibitory effect on the mycelial growth of A. flavus

Studies on obtaining "naturally derived" antimicrobial and antifungal agents have increased in the food industry. The reasons for this can be listed as the negative effects of synthetic chemicals and the increasing demand of consumers for natural, green and preservative-free foods (Almeida et al., 2022).

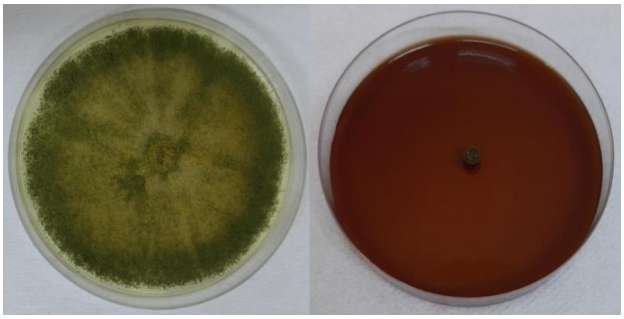
Since *A. flavus* is an airborne fungus, the conidia it produces in abundance spread over a wide area and infect grains and foods. Control of *A. flavus* and AFs in storage is more difficult than other pathogens (Gong et al., 2019). In this study, which was carried out to find a naturally obtained agent that could be an alternative to synthetic

chemicals for the control of toxigenic *A. flavus*, 15 plants were evaluated for their antifungal activities. The response of *A. flavus* to the powder (10% v/w) of the 15 tested plants was in the form of inhibition of mycelial development. This inhibition varies between 3.28 to 100% (Table 1). *O. vulgare*, belonging to the Lamiaceae family, inhibited the mycelial growth of *A. flavus* by 100% (Figure 1).

Table 1. *In vitro* effect of some plant powders on mycelial growth of *A. flavus* (10% v/w).

Family	Species	Inhibition (%)
Lamiceae	*Origanum vulgare* L.	100^{a}
Caprifoliaceae	*Sambucus ebulus* L.	50.38^{b}
Lamiaceae	*Salvia verticillate* L.	45.95^{bc}
Asteraceae	*Eupatorium cannabinum* L.	36.09^{d}
Lamiceae	*Marribium peregrinum* L.	27.09^{e}
Lamiceae	*Clinopodium vulgare* L.	20.58^{f}
Lamiceae	*Sideritis montana* L.	17.87^{fg}
Papaveraceae	*Chelidonium majus* L.	17.42^{fg}
Lamiaceae	M*elissa officinalis* L.	17.16^{fg}
Asteraceae	*Tanacetum parthenium* L.	16.07^{fg}
Asteraceae	*Lactuca serriola* L.	14.94^{gh}
Asteraceae	*Matricaria chamomilla* L.	14.65^{gh}
Asteraceae	*Artemisia absinthium* L.	7.74^{i}
Asteraceae	*Cirsium arvense* L.	5.19^{i}
Lamiaceae	*Mentha pulegium* L.	3.28^{i}

*Values are the average of three replicates. Values following the same letters did not show a significant difference ($p < 0.05$) according to the Mann-Whitney U Test.



A **B**

Figure 1. A. Control; **B**. Effect of *O. vulgare* powders (10% v/w) on mycelial growth of *A. flavus* in Petri dishes.

Shireen et al. (2015) and Ullah et al. (2023) reported that *Aloe vera* and *Citrus limon* extracts inhibited the mycelial growth of *A. flavus* by 51% and 46.5%, respectively. The different inhibition percentages shown by these plant species on the mycelial growth of *A. flavus* may be proportionally related to the biological compounds they contain, which are responsible for various biological activities (Mongalo et al., 2019).

It has been stated that plants can be effective on fungal growth thanks to the secondary metabolites they contain such as alkaloids, flavonoids, terpenoids, and phenolics (Zhao et al., 2022). The antifungal activity of phenols is closely connected with their hydroxyl group, which is bonded directly to a benzene ring (Tian et al., 2022). Many modes of action of phenolic compounds have been proposed to inhibit the development of pathogenic agents. Due to these effects, the enzymatic processes of the pathogenic agent in energy production are disrupted, the permeability barrier of the cell membrane is damaged or weakened, and the physicochemical structure of the cell changes or cytoplasmic content is affected (Kauffmann and Castro, 2023). Considering this situation and similar studies (Alkadi, 2021; Yousef et al., 2022; Bhat et al., 2024), we can say that plants showing mycelial growth inhibition have potential antifungal activity. In order to determine its protective effect against *A. flavus* infections and its effect on aflatoxin B1 biosynthesis, subsequent studies were continued with *O. vulgare*, which showed the highest inhibitory effect (100%) on mycelial growth.

Minimum Inhibitory Concentration (MIC)

The study to determine MIC of antifungal agents is very important as it helps to estimate the effectiveness of these agent in controlling fungal growth (Verma et al., 2021). The MIC value of *O. vulgare* against *A. flavus* was 250 µg/mL. When we compare it with the results of similar studies conducted with extracts of some plant samples against *A. flavus*, we can say that the MIC value for *O. vulgare* extracts is low.

The MIC's of some crude extracts of *Chamaerops humilis* L. against the toxigenic strain of *A. flavus* have been reported to vary between 3.00 mg/mL and 3.50 mg/mL (Boudjaber et al., 2023). The MIC values of *Avicennia marina*'s ethanolic and *Citrus aurantium*'s aqueous extracts against *A. flavus* has been found as 16 mg/mL and 130 mg/mL, respectively (Alizadeh Behbahani et al., 2014; Ullah et al., 2023).

Possible protective activity of O. vulgare aqueous extracts against A. flavus mycelial growth in various cereal grain contamination model

It was determined that the grains transferred to Petri dishes at the end of the storage period (5 days) in tubes containing different concentrations of *O. vulgare* could not completely prevent the mycelial growth of *A. flavus*, but there was a regression in mycelial growth. Especially in sunflower, the protection of *O. vulgare* was effective during the incubation period at all tested concentrations. This effect was significant at concentrations from 2 to 32 mg/mL (Figure 2, $p<0.05$). Efficacy was observed at concentrations of 16 and 32 mg/mL in infected maize and 32 mg/mL in wheat grains on days 2 to 4 of the incubation period (Figures 3, 4; $p<0.05$). *O. vulgare* extracts

had significant protective potential in rice at all concentrations on the 2nd day and at 16 and 32 mg/mL concentrations on the 3rd and 4th days (Figure 5; $p<0.05$). We can state that the antifungal effect of *O. vulgare* against *A. flavus* in *in vivo* studies is not as effective as in *in vitro* studies.

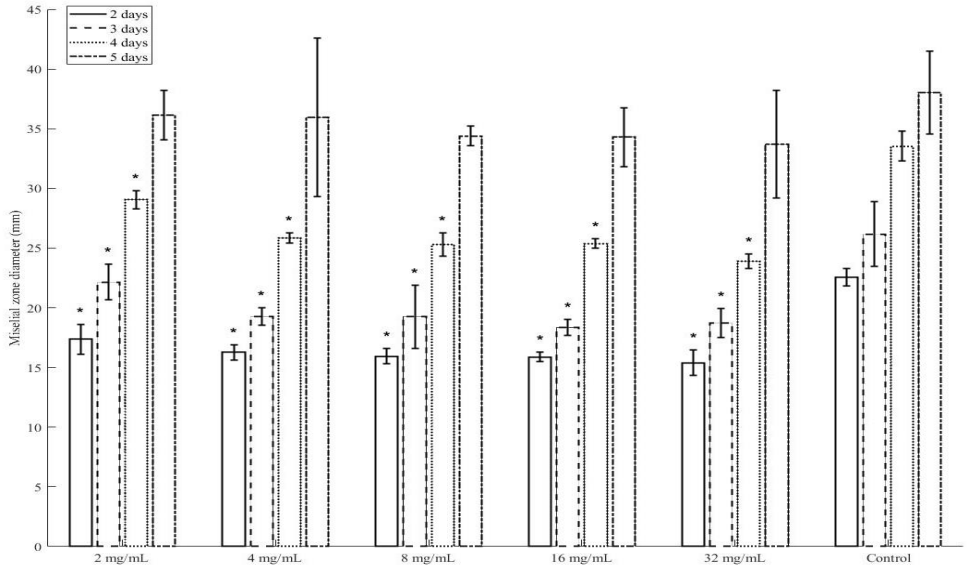


Figure 2. Protective effect of *O. vulgare* aqueous extracts against *A. flavus* infection in sunflower. *p<0.05.

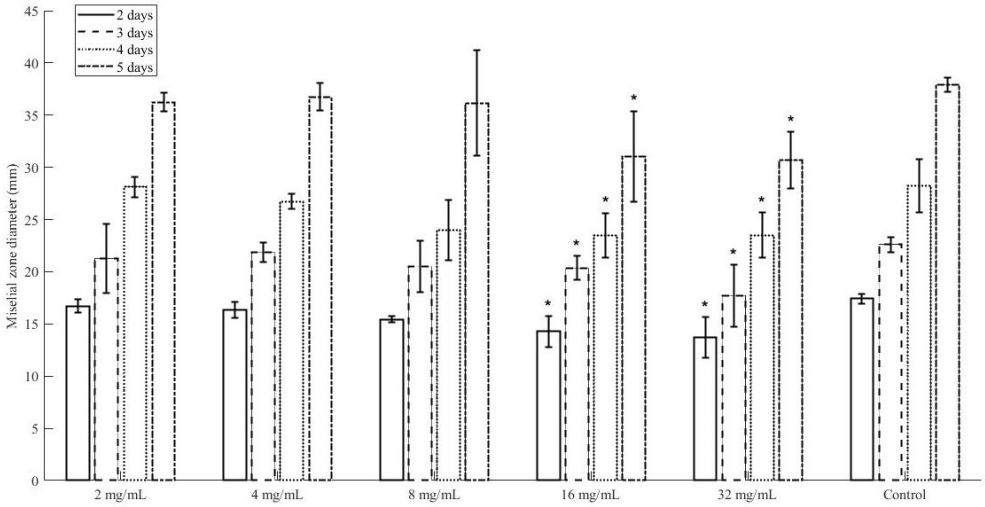


Figure 3. Protective effect of *O. vulgare* aqueous extracts against *A. flavus* infection in maize. *p<0.05.

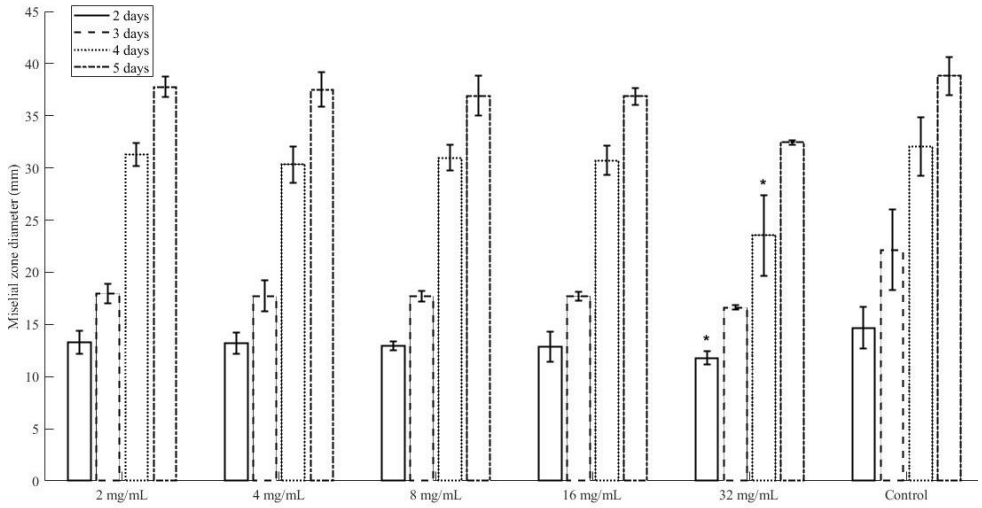


Figure 4. Protective effect of *O. vulgare* aqueous extracts against *A. flavus* infection in wheat. *p<0.05.

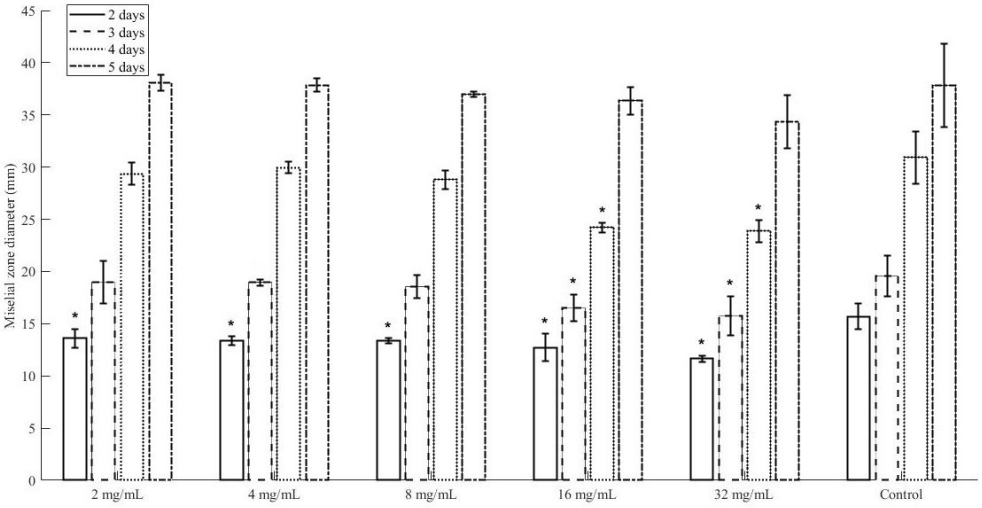


Figure 5. Protective effect of *O. vulgare* aqueous extracts against *A. flavus* infection in rice. *p<0.05.

Lima et al. (2019) reported in their study on the inhibitory activity of thymol and carvacrol in maize grains infected with *A. flavus* that the mycelial development of the fungus could not be prevented. It has been stated that the protective effect only occurs in the tubes when in contact with maize grains. We can say that the *in vivo* activity was lower due to the relationship between the compounds found in the aqueous extracts of *O. vulgare* and the fiber, mineral, phenolic compound, protein

and vitamin levels of the tested grains. At the same time, we can explain the difference in the activity of the aqueous extract of *O. vulgare* in the tested grains by the fact that the interactions between the natural compounds of plant origin and the components of the grains can cause an antagonistic effect.

Effect of O. vulgare aqueous extracts on the hyphal morphology of A. flavus

SEM scanning of A. flavus hyphae after treatment with O. vulgare aqueous extract unequivocally revealed the antifungal activity of O. vulgare. While healthy hyphen morphology of A. flavus had a linear, regular and homogenous cell wall (Figure 6), the aqueous extracts of O. vulgare had caused hyphae structure flattening, collapse, lysis and wrinkling in A. flavus (Figure 7). These results obtained by SEM analysis prove that O. vulgare aqueous extracts have phytotoxic properties against A. flavus. The antifungal activity of O. vulgare aqueous extracts damaged the rigidity and integrity of the A. flavus cell wall.

The fungal cell wall consists of a complex and dynamic structure of chitin, glycans and glycoproteins. It plays a role in the morphogenesis of fungal cells, protection of protoplasts from physical damage and osmotic stress, cell recognition in various interactions and drug resistance to antifungal agents, as well as exchange of nutrients and metabolic products (Bowman and Free, 2006). Chitin and glucan are mainly found in the inner layer of the fungal cell wall. Disruption of chitin biosynthesis causes disruption of the fungal cell wall, which leads to cell lysis and death (Pandey et al., 2013). At the same time, inhibition of glucan synthesis causes disruption of fungal cell wall formation and inhibition of fungal growth.

Phenolic substances interfered with the life cycle of the fungi, regardless of being in a combination or acting singly, by causing changes in structural component synthesis, weakening or damaging the permeability barrier of the cell membrane, altering the physiological conditions of the cells and acting as chelating agents (Nazzaro et al., 2013).

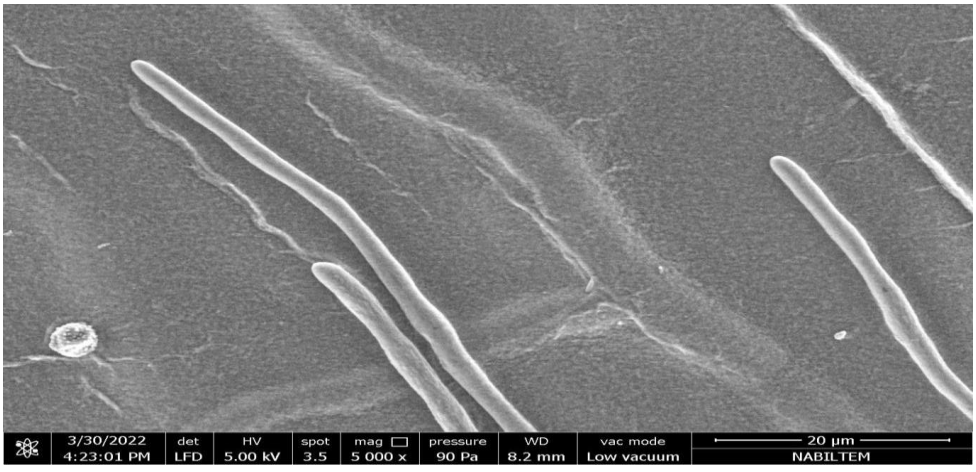


Figure 6. SEM image of *A. flavus* healthy hyphae.

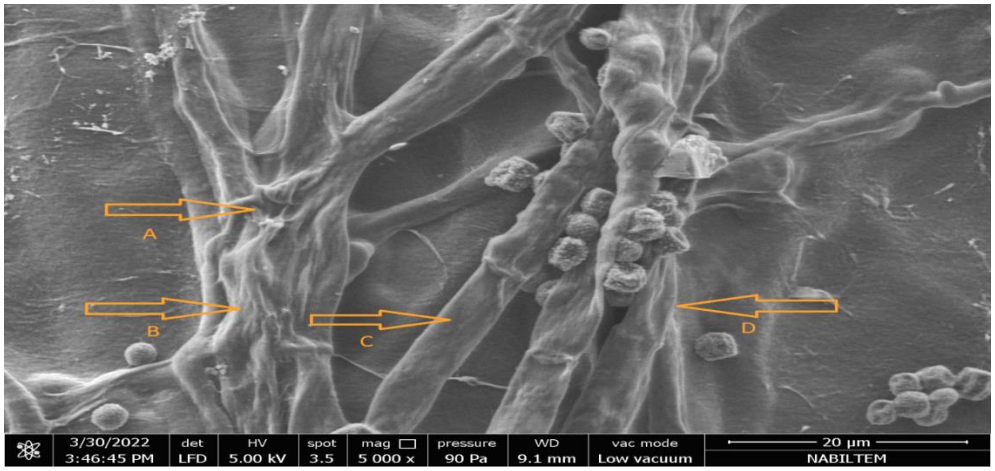


Figure 7. SEM image of *A. flavus* hyphae exposed to 4MIC *O. vulgare* aqueous extract. A; lysis, B; wrinkling, C; flattening, D;collapse.

Effect of O. vulgare aqueous extracts on AFB1 production from A. flavus

Inhibition of AFB1 production by *A flavus* was related to the concentration of the extract tested. A decrease in AFB1 production from *A. flavus* was observed in all tests with increasing extract concentration. By HPLC analysis, it was determined that *O. vulgare* aqueous extracts at concentrations of 1, 2 and 4 mg/mL reduced AFB1 production, and 8 mg/mL aqueous *O. vulgare* extract inhibited AFB1 production of *A. flavus* by 100% (Table 2). We can state that *O. vulgare* aqueous extracts may have great potential in protecting foods from AFB1 producer *A. flavus*.

Table 2. HPLC analysis of the potential effect of *O. vulgare* aqueous extracts on the aflatoxin B1 production of *A. flavus.*

Extract concentration (mg/mL)	AFB1 (ppb)	Inhibition (%)
1	1.51±0.61	90.46
2	0.76±0.15	95.20
4	0.21±00.7	98.68
8	0	100
Control	15.82±8.3	

The antiaflatoxigenic effects of natural plant-derived compounds are not fully understood. Hypotheses for these effects include (a) modulating extracellular and intracellular factors affecting aflatoxin biosynthesis; (b) interrupting cell signaling upstream of the aflatoxin biosynthetic pathway; (c) inhibiting carbohydrate catabolism dependent on aflatoxin biosynthesis; (d) inhibiting specific genes or enzymes in the aflatoxin biosynthetic pathway (Chaudhari et al., 2019).

Mitochondria, which play critical roles in aflatoxin biosynthesis in *A. flavus*, are responsible for providing ATP, NADPH, and acetyl-CoA. Natural components derived from the plant can disrupt mitochondria of *A. flavus* and stop the formation of acetyl-CoA, which feeds the aflatoxin biosynthesis pathway. As a result, inhibition of aflatoxin biosynthesis may occur (Tian et al., 2022).

It has also been suggested that aflatoxin synthesis is affected by plant-based natural substances inhibiting some key enzymes responsible for anti-aflatoxigenic activity and suppressing the expression of aflatoxin biosynthetic genes (Moon et al., 2018).

The gene clusters responsible for aflatoxin biosynthesis are coordinately regulated and present as a single copy in the genome. Their expression is coordinated by two cluster-specific regulators; regulatory gene (*aflR)* and structural gene (*aflS*). In addition, the role of at least 27 enzymatic reactions in the aflatoxin synthesis process has been demonstrated (Safari et al., 2020). AFB1 biosynthesis in A. flavus is a complex, multistep process involving enzymes of metabolic pathways and intermediate pathways. Detailed studies are needed on the anti-aflatoxigenic mechanism of action of the *O. vulgare* aqueous extract tested in our study.

In the study conducted on the aflatoxin B1 production of *A. flavus* by *Thymus vulgaris* L., it was reported that complete inhibition occurred at a concentration of 150 mg/mL (Kohiyama et al., 2015). Boudjaber et al. (2023), evaluated the antiaflatoxinogenic effect of *Chamaerops humulis* hexane extract. They reported that this effect caused a decrease in AFB1 production of *A. flavus* between 43.47% and 94.88%. The inhibitory effect of *Quercus ilex* and *Ulmus sp.* extracts on the Aflatoxin B1 production of two mycotoxigenic strains of *A. flavus* was investigated by Boy et al., (2023). Both plant extracts were reported to reduce aflatoxin B1 production, but could not completely inhibit it even at a concentration of 250 mg/L. It has been stated that the phenolic compounds contained in plants may affect the aflatoxin production of *A. flavus* (Al Khoury et al., 2022). In our previous study (Balkan et al., 2017) in which the phenolic compounds of *O. vulgare* were determined, it was found that the carvacrol content was high. We believe that detailed studies are needed to determine the effect of carvacrol and other phenolic compounds contained in *O. vulgare* on aflatoxin production of *A. flavus*.

Conclusions

The results obtained from this study, which we think can help as a reference for future studies, show that aqueous extracts of *O. vulgare* significantly inhibited mycelial growth and AFB1 production of *A. flavus*. However, the antifungal effect of *O. vulgare* against *A. flavus* in *in vivo* studies was less effective compared to the *in vitro* studies. Degenerations in the hyphal structure of *A. flavus* were determined in SEM analysis. *O. vulgare* extracts can be evaluated as biofungicide that can be an alternative to synthetic fungicides against *A. flavus* contamination. We suggest that *O. vulgare* aqueous extracts may be suitable as antifungal and anti-AFB1 agents in food preservation.

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