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Potential of essential oils for protection of Couscous against *Aspergillus flavus* and aflatoxin B1 contamination

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ABSTRACT

Our study was aimed to evaluate antifungal, antiaflatoxin, and antioxidant potential of *Mentha pulegium, Myrtus communis,* and *Mentha piperita* essential oils (EOs). The EOs showed efficacy as a couscous preservative. A total of 387 fungal species were isolated from Couscous samples, with *Aspergillus flavus* BN (20) which is identified as the highest aflatoxin producer. The minimum inhibitory concentration (MIC) of *M. pulegium* and *M. communis* EOs against the toxigenic strain of *A. flavus* BN (20) determined to be 4.00 μL/mL, whereas the MIC of *M. piperita* was 3.50 μL/mL. The EOs inhibited the aflatoxin B_1 production at lower concentrations. The EOs showed fungitoxicity against wide-ranging of fungi and high antioxidant activity. The IC_{50} value of the oils ranged between 3.27 and 4.31 μL/mL. EOs may be able to contribute preservation of food commodities against quantitative and qualitative losses. During fumigation assays in Algerian traditional food system (Couscous), the three EOs showed protection of Couscous from *A. flavus* contamination indicating their efficacy as sustainable fumigant in food systems.

1. Introduction

The frequency of aflatoxin contamination of crops worldwide shows that the strategies presently used are not enough to ensure the safety of stored foods and that it is appropriate to develop others, in addition to or as a substitute for those that already exist (Upadhyay et al., 2018). Within this scope, methodologies based on the use of natural compounds, generally recognized as not harmful to the environment as well as to human and animal, seem interesting (Prakash et al., 2012; Kedia et al., 2014a; Kedia & Dubey, 2015). The health impact of these compounds has justified the implementation of consumer protection measures by the establishment of maximum tolerable standards in certain food categories. However, the existence of these standards also has important economic repercussions. By limiting trade from certain areas where the contamination is prevalent and greatly reducing the economic value of certain production in the event of contamination. It therefore seems imperative to develop means of control to prevent the contamination of food by these toxic compounds and/or to restrict their toxic effects (Belasli et al., 2020).

Pesticides and fungicides have been extensively used to prevent development of fungal agents in stored foods. However, because of their own toxicity, their use is subject to certain limitations (Cabral et al., 2013). Biological control is also an option possibility of struggle. However, none of these strategies seems, on its own, capable of solving the problem of contamination of raw materials by aflatoxins as evidenced by the many surveys that show levels of contamination sometimes high,

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especially in regions where the climate is friendly for fungal species development. There is a growing concern in identifying natural compounds able to limit the growth and the biosynthesis of mycotoxins. These compounds could be used to combat fungal contamination (Abd El-Aziz et al., 2015; Ben Miri et al., 2018).

This research aimed at screening of fungi responsible for biodeterioration of the stored couscous and detection of toxigenic strains of *Aspergillus flavus* and investigate the antifungal, anti-aflatoxin, and antioxidant properties of *Mentha pulegium, Myrtus communis,* and *Mentha piperita* essential oils (EOs) to evaluate their efficacy as plant based fumigants in Couscous stored in plastic containers so as to investigate the feasibility of their recommendation.

2. Materials and methods

2.1. Chemicals and equipments

The chemicals and solvents viz. Dimethyl sulfoxide (DMSO), chloroform, methanol, toluene, isoamyl alcohol, tween-80, sodium sulfate and constituents of media preparations vis. Potato Dextrose Agar (PDA) medium (Potato, 200 g; Dextrose, 20 g; Agar, 15 g and distilled water 1000 ml), Malt Extract Agar (M.E.A) (Malt extract, 20g; Peptone, 1g; Glucose, 20g; Agar, 20g; Distilled water, 1000 mL), Czapek concentrate ($NaNO_3$, 30 g; KCl, 5g; $MgSO_4.7H_2O$, 5g; $FeSO_4.7H_2O$, 0.1g; Distilled water, 1000 mL),Glycerol Nitrate Agar (G25N) (K_2HPO_4, 0.75g; Czapek concentrate, 7.5 mL; Yeast extract, 3.7g; Glycerol, 250g; Agar, 12g; Distilled water 750 mL), *Aspergillus flavus* and *parasiticus* agar (AFPA) (Peptone, 10g; Yeast extract, 20g; Ferric ammonium citrate, 0.5g; Chloramphenicol, 100 mg; Agar, 15g; Dichloran, 2 mg; Distilled water, 1000 mL), Dichloran Rose Bengale Chloramphenicol Agar (DRBC) (Glucose,10 g; Peptone, 5 g; KH_2PO_4, 1g; $MgSO_4$, $7H_2O$, 0.5 g; Rose Bengale, 25 mg; Dichloran, 2 mg; Chloramphenicol,100 mg; Agar, 15 g; Distilled water, 1000 mL) were purchased from Aldrich Sigma (France). 2,2-Diphenyl-1-picrylhydrazyl radical and Butylated Hydroxytoluene (BHT) were from Aldrich Sigma (France). The major equipments used were Electronic pH meter (7110 SET 2), Light microscope (Motic: BA210, China), Centrifuge (JOUAN E76), UV Transilluminator (CN-6, VILBER LOURMAY, France), Spectrophotometer UV–Visible (6705 UV/Vis, JENWAY).

2.2. Raw materials

Samples of high quality 100% medium semolina industrial dry couscous with the presence of gluten *viz.,* SIM (SIM GROUP), Safina (METIDJI GROUP), MAMA (SARL SOPI) and MEB (SARL MEB) (10 kg of each sample) were purchased from the local market in Rouiba, Algeria, in October 2021. *Mentha pulegium, Myrtus communis,* and *Mentha piperita* EOs and their GS/MS data were kindly supplied by Pr. Lamouri Saad (Algiers, Algeria). The EOs were produced using hydrodistillation and stored in sealed glass vials at room temperature. The density of the EOs, their refraction indexes, and major chemical volatile compounds are shown in Table 1.

2.3. Mycological analysis

The isolation of molds in the samples were carried out by the suspension-dilution technique and inoculation on agar medium. Ten (10) grams of each sample of Couscous were added to 90 mLof distillated water added to tween 80 and homogenized by stirring for 15 min. Serial dilution (10^{-2}, 10^{-3} and 10^{-4}) were made from the stock solution. One hundred (100) microliters of each dilution were subcultured on the surface of Petri dishes containing the DRBC medium and incubated at 28 °C for 5–7 days at darkness. Representative isolates from each sample were purified and then subcultured into test tubes containing PDA. After incubation for 7 days at 28 °C, the isolates were stored at 4 °C for subsequent analyzes. The identification of the main fungal genera and species was carried out on MEA, G25N, CYA and on AFPA (Pitt & Hocking, 2009).

2.4. Selection of the most toxigenic strain of A. flavus

A. flavus isolates of Couscous were screened for the production of AFB_1 according to Prakash et al. (2012). The isolates were cultured separately in 25 mL PDB broth for 10 days. The cultures were incubated at 28 ± 2 °C for 10 days. After incubation, the mycelia produced in the liquid cultures were removed by filtration and washing on Whatman No.1 filter paper. The weight of the mycelia was determined after incubation at 60 °C for 6 h then at 40 °C overnight. AFB_1 was extracted from the filtrate with 25 mL in a separatory flasks. After stirring, the

Table 1
Main physicochemical and compositional data for tested EOs.

EOs	Density (20 °C)	Refraction index (20 °C)	Volatile compounds	Percentage (%)	Formula	Classification	Total identifid (%)
M. pulegium	0.883–0.944	1.485–1.495	Pulegone	60.82	$C_{10}H_{16}O$	Monoterpene hydrocarbon	79.4
			5-methyl-2-(-1-methylethyl)-cycloxanone	14.27	$C10H_{18}O$	Oxygenated monoterpene	
			Bicyclol (4.1.0) heptane	2.54	C_7H_{12}	Monoterpene hydrocarbon	
			Limonene	1.77	C10H16	Monoterpene hydrocarbon	
M. communis	0.888	1.466	*α*-Pinene	46.7	$C_{10}H_{16}$	Monoterpene hydrocarbon	98.3
			1,8-Cineole	22.0	$C_{10}H_{18}O$	Oxygenated monoterpene	
			Myrtenyl acetate	14.0	$C_{12}H_{18}O_2$	Oxygenated monoterpene	
			Limonene	5.5	C10H16	Monoterpene hydrocarbon	
			Linalool	2.6	$C_{10}H_{18}O$	Oxygenated monoterpene	
			Geranyl acetate	2.50	C12H20O2	Oxygenated monoterpene	
			α-Terpineol	2.50	C10H18O	Oxygenated monoterpene	
			Terpenyl acetate	1.30	$C_{12}H_{22}O_2$	Oxygenated monoterpene	
			p-Cymene	1.20	C10H14	Monoterpene hydrocarbon	
M. piperita	0.870–0.950	1.450–1.464	Menthol	40.0	$C_{10}H_{20}O$	Oxygenated monoterpene	97.0
			1,8-Cineole	26.0	$C_{10}H_{18}O$	Oxygenated monoterpene	
			Menthone	25.0	$C_{10}H_{18}O$	Oxygenated monoterpene	
			Menthyl acetate	6.0	$C_{12}H_{22}O_2$	Oxygenated monoterpene	

Source: Technical data supplied by the producer.

mixture was left to stand overnight. The chloroform phase was recovered and evaporated to dryness at a temperature of 50 °C in a rotary evaporator. The residue was dissolved in 1 mL of chloroform. Using a microsyringe, 50 μL of the aflatoxin extract were spotted on Thin Layer Chromatograph (TLC) plate as spots in small portions. AFB_1 standard was spotted at the same time as the samples. The plates were then placed in a chromatographytank whose atmosphere had been previously saturated with Toluene/Iso-amyl alcohol/Methanol (90/32/2; v/v/v). After development, the plates were dried in a ventilated hood overnight to remove all traces of the solvent. The chromatograms were then observed under ultra-violet (UV) light to locate the spots exhibiting an absorbance at 365 nm. The fluorescent spots were scraped off, dissolved in 5 mL of methanol; the resulting solution was centrifuged at 3000 g for 5 min. The absorbance of the supernatant was performed in a UV–Visible spectrophotometerat 360 nm. The concentration of AFB_1 was calculated by the formula according to Singh et al. (2010):

$$[AFB_1] \text{ in } \mu g / mL = [(D \times M) / (E \times L)] \times 1000$$

Where, D: absorbance; M: molecular weight of aflatoxin (312 g/mol); E: molar extinction coefficient (21, 800 l/mol/cm); L: optical path length (1 cm cell).

2.5. Determination of toxicity of EOs against A. flavus strain BN (20)

2.5.1. Antifungal activity

The activity of EOs on the mycelial growth of the test strain was evaluated using the direct contact method (José Velázquez-Nuñez et al., 2013). The EOs were incorporated into the agar medium so as to obtain the following concentrations: 2.00; 2.50; 3.00; 3.50; 4.00 μL/mL. The medium thus supplemented was poured into Petri dishes. An aliquot (10 μl) of the fungal suspension (≈1 × 10^6 spores/mL) were deposited in the center of the culture medium. The control was prepared without extract. Petri dishes were incubated at a temperature of 28 ± 2 °C for 7 days. Mycelial growth was followed by measuring the diameter along two straight lines perpendicular to the center. The percentage of inhibition (I %) was calculated according to the following equation (Shukla et al., 2012):

$$I\% = (D_{Control} – D_{Test} / D_{Control}) \times 100$$

D Control: Diameter of the control growth zone in mm;
D Test: Diameter of the test growth zone in mm.

2.5.2. Minimum inhibitory (MIC) and fungicidal (MFC) concentrations

The minimum inhibitory (MIC) and fungicidal (MFC) concentrations of each EO was determined using the liquid dilution method reported by Prakash et al. (2012). Ten (10) μL of the fungal suspension (≈1 × 106 spores/mL) were inoculated into test tubes containing 10 mL of the PDB liquid medium at different concentrations of EOs (0; 2.00; 2.50; 3.00; 3.50; 4.00 μL/mL). PDB tubes containing DMSO were used as control. The tubes were homogenized and incubated at 28 ± 2 °C for 7 days. After incubation, observation of a range allows access to the MIC, which corresponds to the lowest concentration capable of inhibiting the growth of the microorganism. Tubes which showed complete inhibition were subcultured into Petri dishes containing 10 mL of the PDA culture medium. When there is a resumption of mycelial growth, the concentration is called fungistatic (MFCs). Nevertheless, if there is no pursuance of growth of the mycelium because of persistent inhibition, then it is called as fungicide (MFCc).

2.5.3. Activity of EOs on spore germination

Spores from 7 day cultures of A. flavus BN (20), previously exposed to M. pulegium, M. communis, and M. piperita EOs (0; 2.00; 2.50; 3.00; 3.50; 4.00 μL/mL), were harvested with 5 mL of sterile distillated water with 0.1% (v/v) tween-80 by gently rubbing the mycelial surface with a L-shaped glass spreader. The same procedure was also applied for the control. Spore suspensions were inoculated into fresh PDA medium in depression slides and incubated (at 28 °C for 24 h). Each treatment was assessed by determining the extent of spore germination of 100 spores by checking their germ tube emergence. The percentage of spore germination was calculated by scoring the number of spores germinated (Aloui et al., 2014).

2.6. Evaluation of EOs as anti-aflatoxin B1

The concentrations changing from 2.00 to 4.00 μL/mL were prepared to by dissolving the necessary amounts of EOs. The medium was inoculated with the old culture of A. flavus BN (20) (≈1 × 106 spores/mL of seven days). The control contained no EO. AFB1 detection has been conducted by TLC according to the procedures described in the section of “Selection of the most toxigenic strain of Aspergillus flavus”.

2.7. Spectrum of fungitoxicity of EOs against some fungi isolated from Couscous

The MIC and MFC of EOs against A. niger, A. ochraceus, A. terreus, A. carbonarus, A. fumigatus, Fusarium sp., P. verrusosum, Altarnaria sp., Mucor, isolated from Couscous during mycological analysis, was evaluated in term of MIC and MFC by using PDB medium at concentrations from 2.00 to 4.00 μL/mL to record their fungitoxic spectrum.

2.8. Antioxidant activity

The antioxidant activities of M. pulegium, M. communis, and M. piperita EOs were evaluated by the DPPH• test according to the method of Blois (1958), with some modifications. Experimentally, 1 mL of the methanolic solution of DPPH• (0.004%) was added into the 1 mL of each EO at different concentrations. The vortexed mixture was left in the dark for 30 min and the absorbance was measured at 517 nm using a spectrophotometer against a blank without DPPH•. A blank for each concentration was prepared while replacing the volume of DPPH• with methanol. The antioxidant power of all EOs was compared to BHT. The percentage inhibition of the different samples was estimated according to the equation below:

$$I\% = [(A \text{ blank} - A \text{ sample}) / A \text{ blank}] \times 100$$

Where: A_{blank}: Absorbance of the control reaction containing all reagents except EO; A_{sample}: Absorbance of the sample containing a tested dose of EO.

The concentration of EOs required to obtain 50% of the reduced form of the $DPPH^•$ radical (IC_{50}) was also determined.

2.9. Antifungal activity of EOs vapors (application in Couscous)

The antifungal efficacy of EOs during storage of Couscous was estimated by storing 1 kg of each sample of Couscous, for 6 months at 28 ± 2 °C, separately in plastic boxes (total air volume, 2 L). Three milliliters (3 mL) of spore suspension (≈10^6 spores/mL) of *A. flavus* BN (20) was inoculated into the seed samples from each dish by uniform spraying using the micropipette. EOs at their MIC values were used to fumigate the couscous samples separately. Control samples were prepared in parallel (Prakash et al., 2012). After the storage period, the number of isolates of *A. flavus* BN (20) was determined by mycological analyzes by the previously mentioned serial dilution technique (Aziz et al., 1998).

The percent protection in the uninoculated and inoculated treatments was measured by following formula:

Percent protection = Dc − Dt/Dc × 100.where, Dc = percent occurrence of total fungi of control set and Dt = percent occurrence of total fungi in treatment set.

2.10. Statistical analysis

All bioassays were given as means ± standard deviations and subjected to one-way ANOVA and Tukey's post hoc test ($p < 0.05$) using STATISTICA software (version 6).

3. Results and discussion

3.1. Mycological analysis and detection of aflatoxin producing potential

During mycological analysis, total of 387 fungal isolates were recovered belonging to 5 different genera and 10 species were recorded. The obtained results showed a relative dominance of the genus *Aspergillus* in the samples of couscous. *Aspergillus* is a ubiquitous fungus capable of colonizing various substrates and has a high capacity for sporulation (Tian et al., 2012). Current bibliographic data indicate that *Aspergillus* spp. are widespread in nature (Prakash et al., 2012, Kedia & Dubey, 2015) and especially in hot climate regions such as Algeria (Hocking & Pitt 2009). Thus, most investigations carried out in these arid regions have shown the predominance of fungal species of the genus *Aspergillus* in wheat and its derivatives. *A. flavus* was the most dominant fungus displaying the highest relative density (28.42%) and was detected in all the investigated samples (Table 2). Therefore, *A. flavus* was selected as test fungus to be used in further investigations. Moreover, *A. flavus* is common in subtropical and tropical areas and known to secrete highly toxic AFB_1. *A. flavus* BN (20) isolated from SIM sample was recorded to be the most toxigenic isolate which produced 386.422 μg/mL of AFB_1 and therefore selected as test fungus.

3.2. Antifungal and antiaflatoxigenic activity of EOs against aflatoxigenicA. flavus BN (20)

The effect of the different concentrations of *M. pulegium, M. communis,* and *M. piperita* EOs on mycelial growth of *A. flavus* BN (20) is presented in Fig. 1. EOs had a statistically significant effect on *A. flavus* BN (20) growth ($p < 0.05$). As presented, the inhibition of mycelial growth of the toxigenic *A. flavus* BN (20) was promoted by the increasing concentration of EOs. Compared with the control, a significant inhibition rate of *A. flavus* mycelium diameter was recorded at 3.50 μL/mL of *M. piperita* and *M. pulegium* EOs (84.40% and 87.93%, respectively) ($p < 0.05$), while, at 3.00 μL/mL of *M. communis* EO, the inhibition rate was about 90.50%. At the concentration of 4.00 μL/mL of *M. pulegium, M. communis,* and *M. piperita* EOs inhibited completely the mycelial growth.

The mechanism of action of EOs at the cellular level is still unclear elucidated. However, several potential cellular targets have been identified. Composed of several types of bioactive molecules, EOs can act simultaneously on several cellular targets (Kim et al., 2006; Kedia et al., 2014a; Kedia & Dubey, 2015). The hydrophobic nature of EO constituents facilitates their incorporation into plasma membranes and those of intracellular organelles (notably the mitochondria). These compounds can then act by altering the lipid composition membrane. They are often associated with a decrease in ergosterol levels (Kedia et al., 2014b; Prakash et al., 2015). The latter is a membrane lipid and a major

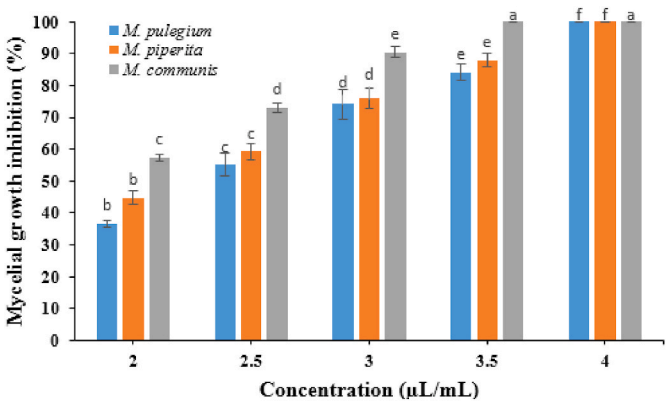

Fig. 1. Percentage inhibition of *A. flavus* BN (20) growth after 7 days of incubation. Values are means (n = 3) ±standard deviations.

constituent of the fungal cell membrane, essential to the integrity of the latter and to the fungal development. The destructuring of the cell wall can also take place by acting on polysaccharides membranes (galactose and galactosamine) (Tatsadjieu et al., 2010). These imbalances membranes lead to a leak of organelles and ions necessary for the survival of fungal cells. Kedia and Dubey (2015) thus observed morphological abnormalities related to rupture of the plasma membrane and leakage of Ca^{2+}, K^{+}, and Mg^{2+} ions from cells of *A. flavus* growing on chickpeas fumigated with *Mentha spicata* EO. Some EOs can also act at the level of membrane ATPase-H^{+}, essential for maintaining osmotic balance by regulating the concentration of ions intracellular. The study by Tatsadjieu et al. (2010) demonstrated the dose-dependent inhibition of the acidification of the medium extracellular by the EO of verbena in connection with the modulation of growth of *A. flavus*.

M. pulegium, M. communis, and *M. piperita* EOs showed pronounced antifungal efficacy, which can be attributed to 1,8-Cineole (Caputo et al., 2017; Dammaka et al., 2019), Linalool (Alouiet al., 2014), Limonene (Ben Miri, Djenane, & Ariño 2018), *p*-Cymene (Gusarovet al., 2009), α-Pinene (Nóbregaet al. 2021), Menthol (Mishra et al., 2013), Pulegone and Menthone (Boniet al., 2016; Piras et al., 2021) which have been reported to have a significant antifungal effect. Nevertheless, EOs consist of a broad diversity of chemical compounds, making it difficult to establish a direct link between one compound among others and antimicrobial activity. It would be obvious that even the minor components can play an important role in antifungal activities, and the inhibitory effects seemed to be related to their synergistic action (Djenane, Gómez, et al., 2019).

MIC and MFC methods were used to evaluate the fungistatic and fungicidal properties of EOs against foodborne fungi. The MIC of *M. pulegium* and *M. piperita* was 4.00 μL/mL, while that of *M. communis* was 3.50 μL/mL, respectively against *A. flavus* BN (20). These results are consistent with some earlier reports on EOs viz. *Cinnamomum cassia*, *Litsea cubeba*, *Cymbopogon martini* and *Thymus mongolicus* (Wang et al., 2018), *Cinnamomum jensenianum* (Tian et al., 2012), which displayed similar MIC values against other fungal strains. MIC is important for

Table 2
Mycological screening of Couscous.

Couscous	Fungal species										
	Ao	*An*	*Af*	*At*	*Ac*	*Afu*	*Fm*	*P v*	*Alt*	*Mucor*	Total colonies
SIM	25	10	26	11	12	8	5	5	1	1	104
SAFINA	23	13	32	8	12	9	6	3	1	–	107
MAM	16	8	21	13	10	8	–	–	–	–	76
MEB	18	12	31	12	8	6	9	4	–	–	100
Total colonies of particular fungus	82	43	110	44	42	31	20	12	2	1	387
% Relative density	21.18	11.11	28.42	11.36	10.85	8.01	5.16	3.10	0.51	0.25	

Af: *Aspergillus flavus*; An: *A. niger*; Ao: *A. ochraceus*; At: *A. terreus*; Ac: *A. carbonarus*; Afu: *A. fumigatus*; Fm: *Fusarium* sp.,Pv: *Penicilium verrusosum*; Alt: *Altarnaria* sp., *Mucor*.

measuring the antifungal activity of plant extracts. Since a lower MIC value indicates that a lower dose is required to control the growth of foodborne fungi, extracts with a lower MIC are more effective antifungal agents. The MFCs were relatively higher than MICs, showing that the toxicity was fungistatic.

The effects of *M. pulegium, M. communis,* and *M. piperita* EOs on the spore germination percentage are presented in Fig. 2 A and B. The EOs significantly inhibited the spore germination of *A. flavus* BN (20) in a dose dependent manner ($p < 0.05$). When the *M. piperita* and *M. pulegium* EOs concentrations were between 2.00 and 3.50 μL/mL, the reduction in spore germination ranged from 55.83% to 91.66%, and from 47.33% to 92.16%, respectively, while the reduction in spore germination of *M. communis* EO ranged from 66.00% to 94.5% at concentrations between 2.00 and 3.00 μL/mL. According to Tian et al. (2011), *Cicuta virosa* L. EO greatly inhibited the spore germination in *A. flavus*at the concentration of 4 μL/mL. Similar results were also presented by Bajpai and Kang (2010), Tian et al. (2012), Hu et al. (2019). The effects of EOs on spores may be due to the denaturation of enzymes that are responsible for spore germination or interference with amino acids involved in germination. The activity of EO components on the perception/-transmission of signals participating in the transition from vegetative growth to reproductive development could also be responsible for the inhibition of spore germination (Negeri et al., 2014). Similarly, Songsamoe et al. (2016) also reported that limonene could affect some enzyme functions involved in spore germination by extending the lag phase during spore germination after UV-C radiation.

Regarding mycelia dry weight (MDW) and AFB_1 production, all the three checked AFB_1 production by the toxigenic strain *A. flavus* BN (20) at concentrations lower than their MICs for fungal growth inhibition (Fig. 3 A and B). The MDW was significantly lower in comparison to the control ($p < 0.05$). *M. pulegium* and *M. piperita* EOs caused complete inhibition of MDW of *A. flavus* BN (20) up to 3.50 μL/mL; while *M. communis* EO caused complete inhibition of MDW at 3.00 μL/mL, whereasAFB_1 complete inhibition was recorded at these concentrations, respectively ($p < 0.05$), emphasizing two different mechanisms essential for the inhibition of fungal growth and AFB1 production as highlighted by Kedia and Dubey (2015) & Kohiyama et al. (2015). Previously, many studies reported that the AFB_1 inhibition by EOs cannot be completely attributed to reduced fungal growth, since EOs might interact with certain steps of the metabolic pathways of *A. flavus*, which control AFB_1 biosynthesis (Mishra et al., 2012; Prakash et al., 2012; Shukla et al., 2012; Tian et al., 2012; Ben Miri et al., 2018). Lately, the research by Hu et al. (2019) revealed that the level of transcription of some aflatoxin biosynthesis genes in *A. flavus*treated with EO upregulated the expression as an antiaflatoxigenic mechanism. Oliveira et al. (2020) depicted that thyme EO could have an inhibition role on growth and AFB_1 production in *A. flavus* by gene suppression of fungal secondary metabolism, through the cell death by apoptotic mechanisms. Nazareth et al. (2020) and Wang et al. (2019) showed that the gene transcriptomes involved in the synthesis of AFB_1 have been amended by certain plant biomolecules. The AFB_1 biosynthesis genes have been suggested as the possible site of molecular action of the bioactive molecules contained in

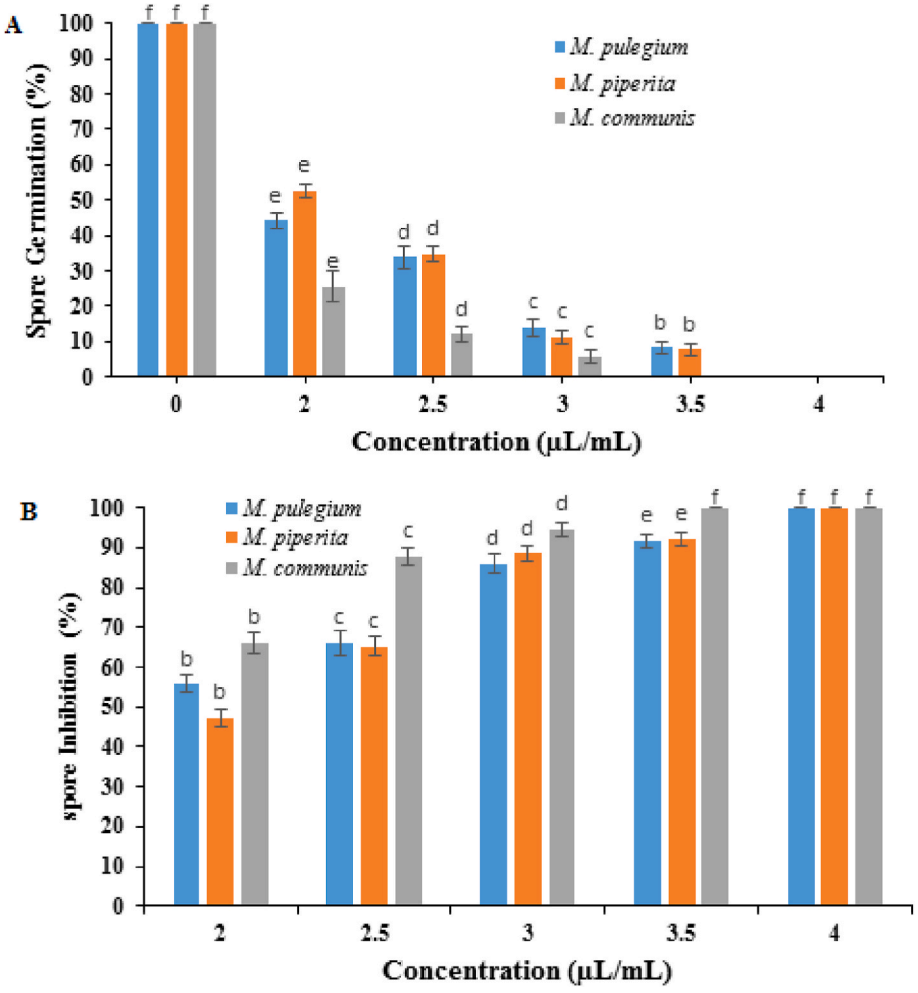

Fig. 2. Effects of *M. pulegium, M. communis,* and *M. piperita* EOs on spore germination of *A. flavus* BN (20). (a) Effect of different concentrations of EOs on spore germination of the tested fungus. (b) Effect of different concentrations of EOs on spore inhibition of the tested fungus. Values are means (n = 6) ±standard deviations.

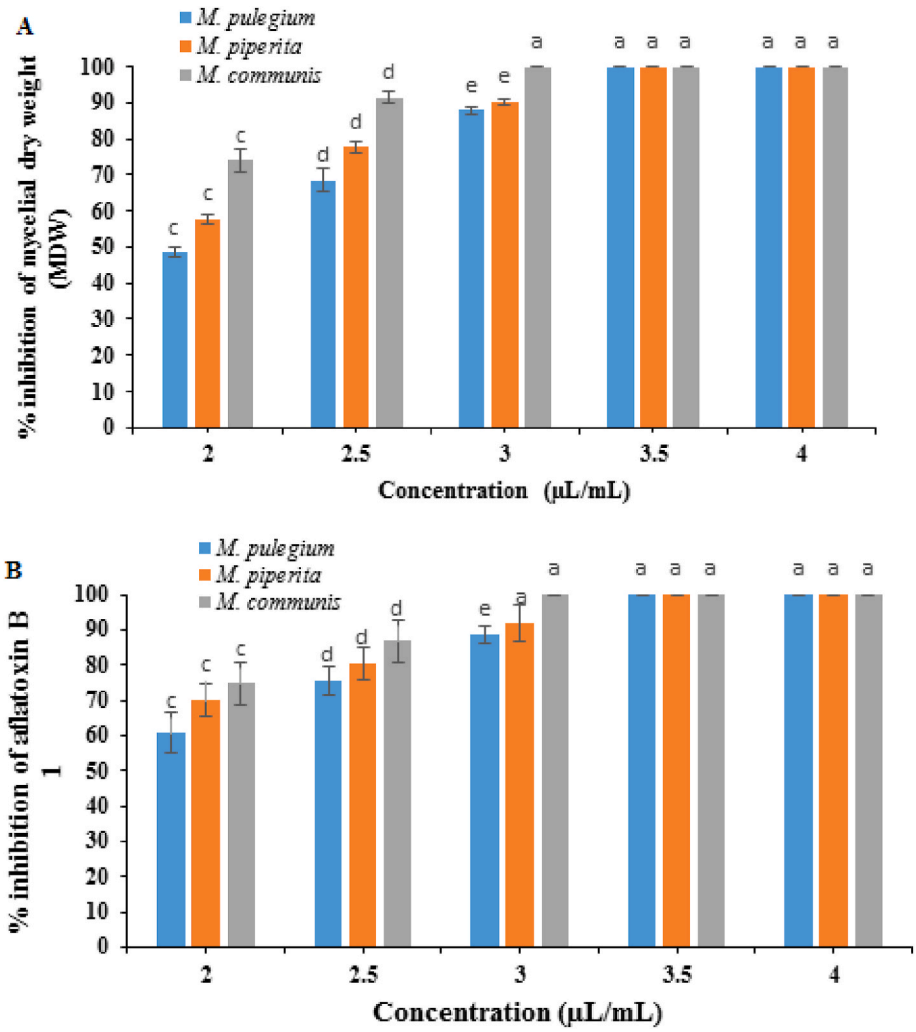

Fig. 3. Effect of *M. pulegium, M. communis,* and *M. piperita* EOs on mycelial biomass (A) and aflatoxin B_1 production (B) by *A. flavus* BN (20). Values are means (n = 3) ± standard deviations.

EOs (Kujur et al., 2019; Yuan et al., 2019).

3.3. Spectrum of toxicity of EOs

M. pulegium, M. communis, and *M. piperita* EOs exhibited wide spectrum of fungitoxicity by inhibiting the growth of 9 additional fungi (Table 3). The MIC varied from 2.00 to 3.83 µL/mL with respect to test fungi. The type of conidia formed as a protective mechanism for EOs toxicity might be effective on the variable sensitivity levels of the tested fungi to different concentrations of EOs. Furthermore, external factors such as stress from the use of fungicides and environmental factors (temperature, pH and humidity) have also been related with the formation of fungal conidia (Dikhoba et al., 2019).

3.4. Antioxidant activity of EOs

Food is damaged by free radicals during storage which leads to the formation of some toxic compounds. For example, oxidative stress during foods storage is a stimulator for *A. flavus* to produce higher amount of AFB_1 (Upadhyay et al., 2018). According to the antioxidant

Table 3
Fungitoxic spectrum of EOs against some fungi.

Fungal species	MIC (µL/mL)			CMF (µL/mL)		
	M.pulegium	*M.communis*	*M.piperita*	*M.pulegium*	*M.communis*	*M.piperita*
A. niger	2.16 ± 0.28^{a}	2.66 ± 0.28^{a}	3.00 ± 0.86^{a}	2.33 ± 0.28 ^{a}	2.66 ± 0.28 ^{a}	3.16 ± 0.57^{a}
A. ochraceus	2.50 ± 0.5 ^{a}	2.33 ± 0.28^{a}	2.83 ± 0.28^{a}	2.50 ± 0.50 ^{a}	2.83 ± 0.28 ^{a}	3.00 ± 0.50^{a}
A. terreus	2.00 ± 0.00 ^{a}	2.00 ± 0.00^{a}	2.33 ± 0.28^{a}	2.50 ± 0.50 ^{a}	2.33 ± 0.28 ^{a}	2.50 ± 0.00^{a}
A. carbonarus	3.66 ± 0.28^{b}	3.00 ± 0.50^{ab}	2.66 ± 0.28^{a}	3.83 ± 0.28 ^{a}	3.16 ± 0.57 ^{a}	2.83 ± 0.28^{a}
A. fumigatus	3.50 ± 0.00 ^{a}	3.16 ± 0.57 ^{a}	3.16 ± 0.57^{a}	3.66 ± 0.28 ^{a}	3.33 ± 0.28 ^{a}	3.50 ± 0.50^{a}
Fusariumsp	3.50 ± 0.00 ^{a}	3.00 ± 0.00^{b}	3.66 ± 0.28^{a}	3.83 ± 0.28 ^{a}	3.16 ± 0.28 ^{a}	3.83 ± 0.28^{a}
P.verrusosum	3.00 ± 0.50 ^{a}	3.50 ± 0.00 ^{a}	3.83 ± 0.28^{a}	3.66 ± 0.28 ^{a}	3.66 ± 0.28 ^{a}	3.83 ± 0.28^{a}
Altarnaria sp	3.83 ± 0.28 ^{a}	3.66 ± 0.28 ^{a}	3.50 ± 0.00^{a}	>4.00	4.00 ± 0.00 ^{a}	3.66 ± 0.28^{a}
Mucor	3.16 ± 0.28 ^{a}	2.66 ± 0.28 ^{a}	3.33 ± 0.28^{a}	3.83 ± 0.28 ^{a}	3.50 ± 0.00 ^{a}	3.50 ± 0.00^{a}

Values are means (n = 3) ± standard deviations.

activity results, which were detected by measuring the amount of DPPH• free radical revealed that *M. pulegium*, *M. communis*, and *M. piperita* EOs had quite high antioxidant activities and their IC_{50} values are summarized in Table 4.

The bioactive components of EO can act at the enzymatic level on mechanisms directly involved in the production of aflatoxins such as catabolism glucose and lipid peroxidation. Indeed, during development on a medium containing glucose, most of the fungi uses the latter to produce energy through mitochondrial respiration oxidative. Glucose entering the cell is metabolized to pyruvate in the cytoplasm and the latter is transformed into acetyl-CoA, at the level of the mitochondrial membrane (Strijbis & Distel, 2010). It has been demonstrated that the anti-toxigenic activity of the phenolic compounds, constituents, among others, of EO, is associated with an inhibition of the activity of MnSOD (or Sod2), a mitochondrial superoxide dismutase which has the role of detoxifying free radicals generated during this respiration (Kim et al., 2006). This phenomenon leads to the inhibition of the mitochondrial respiratory chain and, subsequently, the blocking the synthesis of acetyl-CoA, precursor of aflatoxin. This could therefore contribute to the observed *anti*-aflatoxinogenic effect of EOs (Prakash et al., 2012). Eugenol also inhibits the activity of enzymes involved in glucose catabolism (glucose- 6-phosphate dehydrogenase, as well as other cytoplasmic enzymes involved in lipid peroxidation, thereby reducing oxidative stress, a prerequisite for the aflatoxin production (Jayashree & Subramanyam, 2000).

3.5. Antifungal efficacy of EOs vapors (application in Couscous)

The potential use of *M. pulegium, M. communis,* and *M. piperita* EOs as preservative depended on their protection percentage against the *A. flavus* BN (20) in EOs fumigated Couscous when compared to the untreated samples. The efficacy of *M. pulegium, M. communis,* and *M. piperita* EOs on contaminated couscous was presented in Table 5. The three EOs showed above 50% protection of Couscous from *A. flavus* BN (20) infestation contamination.

The hydrophilic nature of EO molecules will certainly require the implementation of optimization and formulation procedures in order to be able to apply them in food. Microencapsulation could make it possible to maintain the stability of the active molecules and their easy dispersion in the treated food, the release of the active principle only taking place in the event of accidental rewetting during storage (Bouzidi et al., 2019; Djenane, Gómez, et al., 2019; García-Díaz et al., 2019; Li et al., 2019). Moreover, the chemical nature of the identified components will be a key point in the choice of the coating material to maintain the quality. The incorporation of EOs on the coating can have an effect on the prolongation of its antimicrobial activity. Although the films can decrease the extent of dispersion in food products, EO can interact with the chemical nature of the film, react with the polymer and emulsifying agent and thus lead to better dispersion and the formation of a homogeneous coating. Additionally, EO compounds are gradually released onto the product surface over time, maintaining the appropriate concentration of antimicrobial components during the storage period. This mechanism allows the use of lower quantities compared to direct application (Avila-Sosa et al., 2012; Taoudiat et al., 2018). Recently, the incorporation of EOs or their main components in films for active packaging has an increasing trend to maintain antimicrobial properties and improve the shelf life of food products (Maisanaba et al., 2016). Active packaging has generated considerable interest to limit fungal growth and mycotoxin production in cereals and cereal products, fruits and dairy products are also being introduced (Hedayati et al., 2022). Additionally, research has confirmed that biopolymer films and coatings incorporating antimicrobial agents offer great potential to control fungi and mycotoxins and improve food quality and safety (Jafarzadeh et al., 2022).

Table 4
Antioxidant activity of EOs.

EOs	DPPH (IC_{50}) (μL/mL)
M. piperita	3.97 ± 0.05[a]
M. communis	3.27 ± 0.29[c]
M. pulegium	4.31 ± 0.05[a]
BHT	2.66 ± 0.04[b]

Values are means (n = 3) ±standard deviations.

Table 5
Protection of Couscous (%) treated with EOs after 6 months of storage.

EOs	Concentration (μL/mL)	The total number of isolates of *A. flavus* BN (20)		%protection
		Control	Traitements	
SIM				
M. pulegium	4.00	54[a]	25	53.70
M. communis	3.50	54[a]	17	68.51
M. piperita	4.00	54[a]	21	61.11
Safina				
M. pulegium	4.00	90[b]	27	70.00
M. communis	3.50	90[b]	20	77.77
M. piperita	4.00	90[b]	23	74.44
MAMA				
M. pulegium	4.00	40[c]	20	50.00
M. communis	3.50	40[c]	12	70.00
M. piperita	4.00	40[c]	15	62.50
MEB				
M. pulegium	4.00	72[d]	23	68.05
M. communis	3.50	72[d]	10	86.11
M. piperita	4.00	72[d]	14	80.55

[a] Control for all EOs treated sets of couscous SIM.
[b] Control for all EOs treated sets of couscous Safina.
[c] Control for all EOs treated sets of couscous MAMA.
[d] Control for all EOs treated sets of couscous MEB.

4. Conclusion

Given their potential to inhibit aflatoxin formation and fungal growth and their effectiveness as a fumigant in the food system to control fungal contamination, EOs have the potential to be used as herbal preservatives to improve the shelf life of food products during post-harvest processes. Furthermore, active packaging could represent an interesting prospect for the study.

Authorship contribution statement

Yamina Benmiri: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft. **Amina Benabdallah:** Writing original draft-review and editing. **Aldjia Taoudiat:** Formal analysis. **Djamel Djenane:** Writing-review and editing. **Mohamed Mahdid:** Conceptualization, Methodology. **ZeynepTacer-Caba:** Review and editing. **CansuTopkaya:** Review and editing. **Jesus Simal-Gandara:** Conceptualization, Visualization, Writing review and editing.

CRediT authorship contribution statement

Yamina Ben Miri: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Amina Benabdallah:** Writing – original draft, Writing – review & editing. **Aldjia Taoudiat:** Formal analysis. **Mohamed Mahdid:** Conceptualization, Methodology. **Djamel Djenane:** Writing – review & editing. **Zeynep Tacer-Caba:** Writing – review & editing. **Cansu Topkaya:** Writing – review & editing. **Jesus Simal-Gandara:** Conceptualization, Visualization, Writing – review & editing.

Declaration of competing interest

The authors report no conflict of interest.

Data availability

Data will be made available on request.

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