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Unveiling the cellular and molecular mode of action of *Melaleuca cajuputi* Powell. essential oil against aflatoxigenic strains of *Aspergillus flavus* isolated from stored maize samples

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ABSTRACT

This study aimed to reveal the bio-efficacy of *Melaleuca cajuputi* essential oil (McEO) against aflatoxigenic fungi and lipid peroxidation causing deterioration of stored maize samples. Three different toxigenic strains of *Aspergillus flavus*, namely AF-LHP-M2, AF-LHP-SP2, and AF-LHP-VS8 were investigated. Gas chromatography-mass spectrometry (GC-MS) analysis of EO revealed the presence of α-pinene (49.24%) as major compound. Investigation on efficacy showed that McEO exhibited remarkable inhibitory activity against growth and AFB_1 production by AF-LHP-M2 (2.0 and 1.4 μL mL^{-1}, respectively), AF-LHP-SP2 (1.2 and 1.0 μL mL^{-1}, respectively), and AF-LHP-VS8 (0.8 μL mL^{-1}) ($p < 0.05$). The McEO inhibited fungal growth via inhibition of ergosterol biosynthesis, cellular constituents' leakage, and damage of mitochondrial membrane potential, while AFB_1 production by inhibition of intracellular methylglyoxal. Further, molecular docking study was carried out to unveil the binding affinities of major compounds with the target protein Nor-1 (primarily catalyze an important step in AFB_1 biosynthesis), and the results revealed good correlation with the experimental findings. In addition, McEO showed significantly ($p < 0.05$) higher $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activity with IC_{50} values 3.16 and 4.29 μL mL^{-1}, respectively. Interestingly, McEO inhibited AFB_1 production, and malondialdehyde content in fumigated maize samples without significantly ($p < 0.05$) changing their sensory attributes, ascertaining its efficacy in food system with high safety profile (LD_{50} = 1800 mg kg^{-1} body weight) on mice model. The overall results proved McEO's potential as natural food preservative of stored food products.

1. Introduction

The majority of the maize (*Zea mays* L.) produced worldwide are susceptible to qualitative deterioration caused by fungal attack, mycotoxins production, and lipid peroxidation during storage, which do not only lessen their shelf-life but also lead to unpleasant taste (Chaudhari et al., 2021). Amongst different food borne contaminants, *Aspergillus flavus*, which is associated with the production of aflatoxin B_1 (AFB_1) is of alarming concern, owing to its hepatotoxic, mutagenic, teratogenic, immunosuppressive, neurotoxic, nephrotoxic, and estrogenic properties (Manso et al., 2014; Yogendrarajah et al., 2016). In addition, AFB_1 has been reported to induce toxicity via epigenetic modifications, including DNA methylation, histone protein modification, and non-coding RNAs regulations (Dai et al., 2017). Because of its extreme toxicities, many countries have set very strict guidelines to restrict or keep AFB_1 and total AFs concentration very low in the stored food commodities.

Generally, the elimination and degradation of this ubiquitous contaminant is achieved by application of chemical preservatives; however, in the past few years, their excessive use has begun to be questioned because many of them have deleterious effects, including residual toxicity to non-target organisms (aquatic and terrestrial organisms), emergence of resistant fungal strains, and ecotoxicity due to non-biodegradable nature, hence there is a growing interest for some novel alternatives (Ali et al., 2017; Chaudhari et al., 2019). In recent years, essential oils (EOs) have been explored as promising substitute of the chemical preservatives, owing to their biodegradable, non-residual, and non-toxic nature with strong antimicrobial, antimycotoxigenic, and antioxidant properties as well as high consumers safety (not directly

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edible, but shows high median lethal dose in mammalian system) (Chaudhari et al., 2021; Majeed et al., 2015). These EOs have an added effect of maintaining the overall quality (including organoleptic, nutritional, and functional) of the treated food items, since they are highly volatile and do not persist in the foods after application (Pérez-Alfonso et al., 2012).

Melaleuca cajuputi Powell. (Family: Myrtaceae), commonly called as 'cajeput' is a perennial aromatic tree, well known for the production of EO, which hold quite a good potential for the treatment of influenza, cough, abdominal flatulence, internal disorder, intestinal problems, and insect bite effects (Noor et al., 2020; Septiana, Yuliana, Bachtiar, Putri, et al., 2020). In addition, the EO has been reported to exhibit promising antibacterial, antifungal, and antioxidant activities (Siddique et al., 2020). United States Food and Drug Administration (US-FDA) has also approved it for use in foods (Septiana, Yuliana, Bachtiar, & Wijaya, 2020). However, to the best of our knowledge, there are no comprehensive studies in the literature showing the food preservative potential of this EO against qualitative losses caused by aflatoxigenic fungi and lipid peroxidation.

Thus, the main objective of this research was to analyze the chemical compositions of *M. cajuputi* EO (McEO) and to investigate its *in vitro* as well as *in situ* antifungal efficacies against three different toxigenic strains of *A. flavus* (AF-LHP-M2, AF-LHP-SP2, and AF-LHP-VS8) isolated from different varieties of stored maize, with special emphasis on cellular, biochemical, and molecular (binding interactions of the EO's major components with the putative target protein) levels. The outcome of this study may provide significant inferences for the development and commercialization of new antifungal food preservatives based on plant EOs.

2. Materials and methods

2.1. Materials

The major chemicals viz., dimethyl sulfoxide (DMSO), methylglyoxal (MG), 1,2-diaminobenzene, perchloric acid, Tween 20, Tween 80, silica gel-G, ethanol, methanol, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and ascorbic acid were supplied by Hi-Media laboratories, Mumbai, India. The culture media viz., potato, dextrose, and agar (PDA), and sucrose, $MgSO_4{\cdot}7H_2O$, KNO_3, and yeast extract (SMKY) were purchased from Sisco Research Laboratories (SRL), Mumbai, India.

2.2. Fungal strains

For this study, three different toxigenic strains of the *A. flavus*, namely AF-LHP-M2, AF-LHP-SP2, and AF-LHP-VS8, respectively isolated from Maukyrwat, Sohru Pnah II, and Varun suma variety of maize were selected as test fungi. The cultures were maintained in Tween 80 (0.1%) in the form of spore suspension (density = 10^3 spores mL^{-1}) at 4 °C.

2.3. Extraction and characterization of McEO

The EO was extracted from the mature leaves of *M. cajuputi* via hydro-distillation using a Clevenger's apparatus (Council of Europe, 1997). The voucher specimen (Melaleuca. 2021/1) was deposited in the herbarium of the Botany Department (Banaras Hindu University, Varanasi). For extraction, the mature leaves (500 g) of *M. cajuputi* were suspended in distilled water and subjected to a 5-L round bottom flask connected to the Clevenger's hydro-distillation apparatus. Temperature, time, and power were controlled by the operating system (power regulator and thermocouple) of the hydro-distillation apparatus. Next, the samples were heated to a fixed temperature of 75 °C for 3 h. The obtained oil was dried over anhydrous sodium sulfate, and stored in a dark amber glass vial at 4 °C until analysis.

Quantification of main components in the McEO was performed by means of gas chromatography-mass spectrometer (GC-MS) coupled with flame-ionization detector (FID). GC was performed on a TRACE 1300 series GC (Agilent Technologies) equipped with a TG-5 column (30 m length × 0.25 mm diameter × 0.25 μm thickness). During analysis, initial oven temperature was maintained at 60 °C for 2 min and then increased to 250 °C with a heating rate of 5 °C/min. Injector and detector temperatures were 220 °C and 250 °C, respectively. The EO was diluted with hexane (10 μL in 1 mL of hexane). Injection mode was pulsed with a split ratio equivalent to 1/50 with an injection volume 2 μL. Helium was used as a carrier gas at a flow rate of 1 mL min^{-1}. The column effluents were directly transferred into MS recorded with ionization energy of 70 eV. The components were identified by comparing their retention indices (RI) and mass fragmentation pattern with those available in the literature (Adams, 2007). The RI values of different components were calculated by using the retention times (RT) of a homologous series of n-alkanes (C_9–C_{33}) running in parallel with McEO under similar conditions.

2.4. Effect of McEO on growth and AFB_1 secretion by toxigenic strains of *A. flavus*

The effect of McEO on growth of toxigenic strains of *A. flavus* was recorded in term of minimum inhibitory concentration (MIC) according to the well known poisoned food technique (Singh et al., 2008). MIC was considered as the lowest concentration of EO that inhibited the visible growth of fungus on the PDA plate. The MIC was determined by the *in vitro* experiment measuring the growth of toxigenic strains of *A. flavus* in treatment sets against control. For this, briefly, requisite amounts of McEO (dissolved separately in 5% Tween 20) were separately added to the Petri plates containing 9.5 mL PDA medium to reach the final concentrations of 0.2–2 μL mL^{-1} for AF-LHP-M2, 0.2–1.2 μL mL^{-1} for AF-LHP-SP2, and 0.2–0.8 μL mL^{-1} for AF-LHP-VS8. Each plate was inoculated with 10 μL spore suspension of respective *A. flavus* strains (density = 10^3 spores mL^{-1}) along with controls (containing PDA without EO), sealed with parafilm to prevent EO volatilization, followed by incubation at 27 ± 2 °C. Parafilm sealing completely prevented hypoxia, hence, did not compromise the growth of *A. flavus* (Briard et al., 2016). After 7 days, the antifungal activity was evaluated by measuring the growth of fungus in treatments against control and inhibition rate (IR) was calculated using the following formula:

$$IR\ (\%) = (C_{FG} - T_{FG}/\ C_{FG}) \times 100$$

where,

C_{FG} = Fungal growth in control sets (cm)
T_{FG} = Fungal growth in treatment sets (cm)

The inhibitory effect of McEO on AFB_1 production was assessed in SMKY medium using our previously reported method (Chaudhari, Singh, Dwivedy, et al., 2020). Briefly, requisite amounts of McEO were separately added to the conical flasks containing SMKY medium to obtain the above-mentioned concentrations against respective *A. flavus* strains. Controls were prepared using SMKY without test EO. Then, each flask was inoculated with 25 μL spore suspension of test fungus (density = 10^3 spores mL^{-1}) and incubated in biological oxygen demand (BOD) incubator at 27 ± 2 °C for 10 days. Following incubation, the mycelia developed in the medium were filtered and dry weights were determined. During incubation, the conical flasks were sealed with cotton plugs, permitting the air to diffuse, resulting in normal growth of the test fungus. AFB_1 in the medium was extracted with 20 mL chloroform in a separating funnel and allowed to evaporate on water bath (70 °C). The residues left were re-suspended in 1 mL of methanol and 50 μL of each sample was spotted onto the silica gel-G thin layer chromatography (TLC) plates and developed in the mobile phase consisting of toluene:

isoamyl-alcohol: methanol (90:32:2 v/v/v). The amount of AFB_1 in the sample was calculated from the following equation:

$$AFB_1 \text{ amount } (\mu g\ mL^{-1}) = (\text{Absorbance of sample} \times \text{Molecular mass of } AFB_1) / (\text{Molar extinction coefficient} \times \text{Path length}) \times 1000$$

2.5. Antifungal mechanism of McEO

2.5.1. Effect on fungal plasma membrane integrity

The integrity of fungal plasma membrane was examined by determining the inhibition of ergosterol (Chaudhari, Singh, Singh, et al., 2020). Briefly, 25 μL spore suspension of each fungus (density = 10^3 spores mL^{-1}) was separately inoculated into the conical flasks containing SMKY medium amended with 0.2–2 μL mL^{-1} concentrations of McEO for AF-LHP-M2, 0.2–1.2 μL mL^{-1} for AF-LHP-SP2, and 0.2–0.8 μL mL^{-1} for AF-LHP-VS8. After 4-days of incubation at 27 ± 2 °C, the mycelia were extracted, mixed with 5 mL of 25% alcoholic KOH, vortexed, and incubated on water bath at 85 °C. The ergosterol was extracted from the sample by adding 2 mL of sterile distilled water and 5 mL of *n*-heptane. Following 2 min of vortexing, *n*-heptane layer was collected and analyzed by scanning between 230 and 300 nm using UV–visible spectrophotometer (Hitachi-2900, Shimadzu, Japan).

2.5.2. Effect on permeability of plasma membrane

The permeability of fungal plasma membrane was analyzed by measuring the efflux of cellular cations (Ca^{2+}, Mg^{2+} and K^+) and release of 260 (nucleic acids) and 280 nm (proteins) absorbing materials into the suspensions of McEO treated *A. flavus* cells as described previously by Das et al. (2019) with slight modifications. For ion leakage, 5-days old mycelium mass of each *A. flavus* strain was harvested, washed with distilled water, and suspended in 0.85% NaCl solution containing different concentrations of McEO (1/2MIC, MIC, and 2MIC). The fungal cultures without McEO were served as controls. The suspensions were then incubated at 27 ± 2 °C for overnight. The samples were centrifuged (13,000×*g* for 10 min) and respective ions were measured using Atomic Absorption Spectrophotometer (AAnalyst 800, PerkinElmer, USA).

For 260 and 280 nm absorbing materials, 5-days old cultured mycelial biomass of each test fungus was harvested by centrifugation (5000×*g*) for 10 min, washed thrice with phosphate buffer saline (PBS), and re-suspended in the same solution bearing 1/2MIC, MIC, and 2MIC concentration of McEO. After overnight incubation at 27 ± 2 °C, the mycelia were centrifuged and supernatants were subjected to UV–visible spectrophotometry (Hitachi-2900, Shimadzu, Japan) at 260 and 280 nm, respectively.

2.5.3. Effect on fungal mitochondrial membrane potential (MMp)

The effect of McEO on MMp was measured using fluorescent dye rhodamine (Rho123) according to Tian et al. (2012) with slight modifications. First, spore suspension of each fungus was adjusted to 10^6 cells mL^{-1} in PBS and then treated with different concentrations of McEO (1/2MIC, MIC, and 2MIC) for overnight. Samples without McEO were served as controls. Each sample was then centrifuged (5000×*g*), washed, dissolved in PBS, and stained with Rho123 at a final concentration of 1 μg mL^{-1}. After dark incubation for 30 min, the cells were centrifuged and fluorescence intensity of supernatants were measured at the excitation and emission wavelengths of 488 and 525 nm, respectively, using fluorescence spectrophotometer.

2.6. Antiaflatoxigenic mechanism of McEO

2.6.1. Effect on cellular methylglyoxal (MG) content

The antiaflatoxigenic mode of action of McEO was unravelled by determining the level of intracellular MG, which is one of the main precursors for up-regulating the biosynthesis of AFB_1 in the culture) according to our previously reported method (Upadhyay et al., 2018). Initially, an aliquot containing 25 μL spore suspension of AF-LHP-M2, AF-LHP-SP2, and AF-LHP-VS8 (density = 10^3 spores mL^{-1}) were inoculated into the conical flasks containing SMKY medium and different concentrations of McEO (0.2–2 μL mL^{-1}, 0.2–1.2 μL mL^{-1}, 0.2–0.8 μL mL^{-1}, respectively). The control sets were prepared without EO. After 7-days of incubation at 27 ± 2 °C, the tissue was extracted by addition of 3 mL of 0.5 M perchloric acid and incubated on ice bath for 15 min followed by centrifugation at 13000×*g* for 10 min. The supernatant was neutralized by drop-wise addition of saturated K_2CO_3 and centrifuged at 13000×*g* for 10 min. The supernatants were used for the estimation of MG in a total volume of 1 mL reaction mixture containing 250 μL 7.2 mM 1,2-diaminobenzene, 100 μL 5 M perchloric acid and 650 μL of resulting supernatant. Thereafter, absorbance of the sample was recorded at 341 nm using UV–visible spectrophotometer (Hitachi-2900, Shimadzu, Japan).

2.6.2. Homology modeling of Nor-1 gene product and its interaction with the major components of EO: Molecular docking

The molecular docking study was performed to interpret the molecular interaction of major bioactive components of McEO (viz., α-pinene, bornyl acetate, and camphor) with Nor-1 gene product. The docking procedure began with the preparation of the amino acid sequences of Nor-1 protein, which were obtained from UniProtKB database (http://www.uniprot.org) and submitted to the SWISS-MODEL (https://swissmodel.expasy.org). The quality of modeled protein was assessed through QMEAN Z-Scores and molprobity scores obtained from the results. The ligand structure of test compounds was downloaded directly from PubChem.

The structures were then prepared for molecular docking between the test compounds and Nor-1 protein using Molegro Virtual Docker 6.0.1. Before executing the docking process, protein and ligand structures were refined using protein preparation wizard and ligand preparation wizard, respectively. Ligand binding sites in protein was detected using auto detection mode and grid resolution was set to at 0.30 Å and number of runs at 30. Rest parameters were set to the default. The highest affinity-bound-ligands were predicted using MolDock Score.

2.7. Antioxidant activity of McEO: In vitro

2.7.1. DPPH radical scavenging assay

The $DPPH^{\bullet}$ scavenging activity of McEO was performed following the procedure performed previously (Tomi et al., 2011) with minor modifications. Briefly, 0.004% solution of DPPH in methanol was prepared and different concentrations (2–12 μL mL^{-1}) of McEO were added to 2 mL DPPH solution. After 30 min of reaction under dark (color changed from purple to straw), absorbance of the sample was measured at 517 nm using UV–visible spectrophotometer (Hitachi-2900, Shimadzu, Japan). BHT, BHA, and ascorbic acid were used as positive control. The free radical scavenging (FRS) activity was calculated according to the following equation:

FRS activity (%) = [A $_{(blank)}$ − A $_{(sample)}$ / A $_{(blank)}$] × 100

where,

A $_{(blank)}$ = Absorbance of DPPH solution
A $_{(sample)}$ = Absorbance of DPPH solution containing McEO

2.7.2. ABTS radical scavenging assay

The FRS activity of the McEO against $ABTS^{\bullet+}$ was determined according to the standard protocol suggested by Re et al. (1999) with some modifications. Initially, $ABTS^{\bullet+}$ was produced by reacting 7 mM of ABTS solution with 2.45 mM potassium persulphate, and the mixture was placed in the dark at room temperature for 16 h. The solution was diluted with absolute ethanol to obtain the absorbance of 0.70 ± 0.02 at 734 nm. Then, different concentrations (2–12 μL mL^{-1}) of McEO were added to 2 mL of $ABTS^{\bullet+}$ solution and mixed thoroughly. BHT, BHA, and ascorbic acid were used as reference control. After 6 min of reaction, the absorbance of the sample was recorded at 734 nm using UV–visible spectrophotometer (Hitachi-2900, Shimadzu, Japan). The FRS activity was calculated similar to DPPH.

2.7.3. Determination of total phenolic content

The total phenolic content of McEO was assessed using Folin-Ciocalteu's (FC) reagent (Dwivedy et al., 2017). In brief, 100 μL McEO (mixed in DMSO) was added to a conical flask containing 23 mL of distilled water. To this sample, 0.5 mL FC reagent was added and incubated for 3 min. After thorough mixing, 2 mL of 2% Na_2CO_3 was added and allowed to stand for 1 h under dark condition. The absorbance of the sample was read at 760 nm using UV–visible spectrophotometer (Hitachi-2900, Shimadzu, Japan). The contents were presented as μg gallic acid equivalent per gram of EO (μg GAE g^{-1} EO).

2.8. Application of McEO in food system: In situ study

2.8.1. Experimental design

Herein, three different varieties of maize (Maukyrwat, Sohru pnah II, and Varun suma) were selected as model food commodity to demonstrate the *in situ* efficacy of McEO. These varieties were preferred because *A. flavus* strains namely AF-LHP-M2, AF-LHP-SP2, and AF-LHP-VS8 were isolated from these respective varieties. Samples were obtained from local market of Varanasi and Meghalaya, and then immediately transferred to the closed plastic containers (volume 0.5 L). It is crucial to understand the impact of interacting environmental factors such as water activity (a_w), temperature, pH, and moisture contents on growth and aflatoxins production by *A. flavus* in maize in order to predict the possible risk of AFB_1 contamination during storage (Garcia et al., 2012). However, among them, a_w is regarded as the main controlling factor, that influence both the rate of fungal growth and aflatoxin production (Magan & Aldred, 2007). The calculated a_w of the Maukyrwat, Sohru pnah II, and Varun suma were found to be 0.96, 0.97, and 0.90, respectively. Maize samples were divided into three different groups. In one group (inoculated treatment at MIC), maize samples (Maukyrwat, Sohru pnah II, and Varun suma) were fumigated with 2.0, 1.2, and 0.8 μL mL^{-1} concentration of McEO (impregnated in cotton swabs) and inoculated with 500 μL spore suspension (density = 10^3 spores mL^{-1}) of AF-LHP-M2, AF-LHP-SP2, and AF-LHP-VS8, respectively. In second group (inoculated treatment at 2MIC), maize samples were fumigated with 4.0, 2.4, and 1.6 μL mL^{-1} concentration of McEO and inoculated with 500 μL spore suspension (density = 10^3 spores mL^{-1}) of respective fungal strains. In third group, the samples were kept without McEO. All samples were then stored at 25 °C and observation for the presence of AFB_1 was made after three months of storage.

2.8.2. AFB_1 extraction and analysis

The AFB_1 extraction was performance according to the method of Upadhyay et al. (2021) with slight modifications. For this, approximately 10 g of the well milled representative samples were separately added to the conical flasks and mixed with 20 mL of methanol: double distilled water (8:10, v/v) with stirring on a mechanical shaker (300×*g*) for 30 min. Thereafter, the samples were centrifuged at 5000×*g* for 5 min and supernatants obtained were mixed with 300 μL of chloroform and 6 mL double distilled water containing 3% KBr. After 10 min of centrifugation at 5000×*g*, the settled phase were collected and evaporated on hot water bath. The residues obtained were suspended with 50 μL of HPLC grade methanol and injected to the high performance liquid chromatography (HPLC) system.

The AFB_1 in the sample was quantified using a Waters 515 HPLC instrument coupled with fluorescent detector set at the excitation and emission of 365 and 435 nm, respectively. The chromatographic separation was performed on a Nova Pack C18 column (4.6 mm × 25 cm × 5 μm) under isocratic condition at a flow rate of 1.2 mL min^{-1} using a mixture of methanol, acetonitrile, and Millipore water (17:19:64, v/v/v) and delivered at a flow rate of 1 mL min^{-1}. The limits of detection (LOD) and limit of quantification (LOQ) of AFB_1 were 0.5 and 1.5 ng mL^{-1}, respectively.

2.9. Efficacy of McEO against lipid peroxidation

The extent of lipid oxidation inhibition during storage was validated by measuring the level of thiobarbituric acid reactive substances (TBARS) as previously described with modifications (Mancini et al., 2017). This method is based on the spectrophotometric quantification of the pink coloured complex formed due to the reaction of malondialdehyde (MDA) with TBA. Briefly, 1 g powdered maize samples from the above control and treatments were separately homogenized for 5 min in 10 mL distilled water containing 4 mL 0.375% (w/v) TBA, 15% (w/v) trichloroacetic acid (TCA), and 2.5 mL 0.2 N of HCl. The solution was mixed and heated on a water bath at 80 °C for 30 min. Finally, after cooling at room temperature, the samples were centrifuged at 10,000×*g* for 10 min, and the absorbance was measured at 532 against a blank (consisting of the mixture of distilled water and TBA). Results were expressed as μg of MDA per gram of sample fresh weight (μg MDA $g^{-1}FW^{-1}$).

2.10. Oral acute toxicity assessment of McEO

In this study, a population of Swiss albino male mice (*Mus musculus* L., 3 months olds) were divided into 10 groups, each composed of 10 individuals. After being marked and weighed, the mice were transferred to polypropylene cages under controlled laboratory conditions and fasted overnight prior to the testing. The oral acute toxicity study was performed in accordance with Organization for Economic Cooperation and Development (OECD) guidelines (OECD, 2002 Test no. 423) class method at serial doses of 200–2000 mg kg^{-1} body weight diluted in Tween 80 (1%, v/v). The control groups consisted of 0.5% Tween 80 and distilled water. The assay was performed by the oral administration. Animals were monitored for 24 h before proceeding to the next dose. In addition, the animals were monitored for a week for possible long term lethal outcome. Finally, the numbers of dead mice were counted and median lethal dose (LD_{50} value, concentration required to kill 50% population of mice) was calculated by the probit analysis (Finney, 1971).

2.11. Organoleptic attributes analysis

The sensory acceptance of fumigated maize samples was performed by a panel of ten untrained assessors having experience in evaluating food quality following Stojanović-Radić et al. (2018). Maize samples from the *in situ* experiment were taken and prepared by roasting in a preheated oven (2 min at 100 °C). Tests were run on a 7-point hedonic scale (7 = like extremely, 1 = dislike extremely). Sensory evaluation were done for: color, texture, aroma, taste, and overall acceptance. Samples were served at room temperature, and marked with 3-digit

arbitrary codes.

2.12. Statistical analysis

Each experiment was conducted a minimum of three times, and each analysis was carried out in triplicate. The experimental data were subjected to one way analysis of variance (ANOVA), and significant differences between means were evaluated by Tukey's B multiple-range test (SPSS 16.0.). A p value < 0.05 was considered statistically significant.

3. Results

3.1. Extraction and characterization of McEO

The yield of EO obtained from *M. cajuputi* leaves via hydrodistillation was 4.5 mL kg^{-1} fresh weight. The EO was light yellow in color and exhibited strong aroma. The GC-MS analysis of EO revealed the identification of 10 principal components, making 85.83% composition of the oil (Table 1). The most frequent compounds were α-pinene (49.24%) followed by bornyl acetate (21.07%), and camphor (11.70). In addition, McEO also contained considerable amount of minor constituents such as 1,8-cineole (0.83%), p-cymene (0.77%), limonene (0.66%), fenchone (0.58%), camphene (0.41%), p-menthenol (0.37%), and α-terpineol (0.20%).

3.2. Effect of McEO on growth and AFB_1 secretion by toxigenic strains of *A. flavus*

The efficacy of McEO on growth and AFB_1 biosynthesis by toxigenic stains of *A. flavus* are presented in Fig. 1A–C. As can be seen in the figures, there were significant ($p < 0.05$) efficacy against fungal strains at tested concentrations comparable to the controls. The McEO caused complete inhibition of mycelial growth of AF-LHP-M2, AF-LHP-SP2, and AF-LHP-VS8 with MIC value of 2.0, 1.2, and 0.8 μL mL^{-1}, respectively (Fig. 1A–C). Additionally, McEO achieved complete suppression of AFB_1 secretion by respective fungal strains at 1.4, 1.0, and 0.8 μL mL^{-1} concentrations, respectively (Fig. 1A–C).

3.3. Antifungal mechanism of McEO

The results of ergosterol inhibition in plasma membrane of toxigenic strains of *A. flavus* is shown in Fig. 1D. Results revealed a significant ($p < 0.05$) decline in ergosterol contents with the increasing concentrations of McEO. The reduction percentage were 7.06–100% for AF-LHP-M2, 25.34–100% for AF-LHP-SP2, and 24.49–100% for AF-LHP-VS8 when the concentrations of McEO increased from 0.2 to 1.6 μL mL^{-1}, 0.2–1.0 μL mL^{-1}, and 0.2–0.8 μL mL^{-1}, respectively (Fig. 1D). Further, a significant enhancement ($p < 0.05$) in the leakage of Ca^{2+}, Mg^{2+}, and K^{+} as well as 260 and 280 nm absorbing materials was observed (Fig. 2A–F). The fungal strains treated with 2MIC concentrations showed greater leakage of cellular materials than treated at MIC doses. The results of disruption of MMp in *A. flavus* cells exposed to 1/2MIC, MIC and 2MIC concentrations of McEO is presented in Fig. 3A. A dose-dependent degradation of fluorescence intensity of Rho123 dye was noted with the increasing concentrations of McEO.

Table 1
Chemical composition of *M. cajuputi* EO analyzed through GC-MS analysis.

Peak. no.	Identified compounds	Area (%)	RT	RI calculated	KI (Adams, 2007)
1	**α-pinene**	**49.24**	**8.25**	**964**	939
2	Camphene	0.41	9.78	1005	954
3	p-cymene	0.77	12.49	1068	1024
4	Limonene	0.66	12.69	1073	1029
5	1,8-cineole	0.83	12.78	1082	1031
6	Fenchone	0.58	15.56	1137	1086
7	**Camphor**	**11.70**	**18.51**	**1202**	1146
8	α-terpineol	0.20	20.99	1257	1188
9	p-menthenol	0.37	23.28	1308	1177
10	**Bornyl acetate**	**21.07**	**25.13**	**1351**	1285
	Total	85.83%			

Compounds are listed in order of their elution from a MS column.
Compounds in bolds represent the major components.
RT = Retention times; RI = Retention indices; KI = Kovats indices; Area (%) = Percentage of compounds.

3.4. Antiaflatoxigenic mechanism of McEO

The antiaflatoxigenic mode of action of test EO was determined by measuring the level of cellular MG. There were a good linear relationship between the inhibition of MG contents with the values 695.55–8.56 μM g^{-1} FW^{-1}, 550.02–29.97 μM g^{-1} FW^{-1}, and 698.80–50.02 μM g^{-1} FW^{-1} exposed to 0.2–2.0 μL mL^{-1} concentrations against AF-LHP-M2, 0.2–1.2 μL mL^{-1} against AF-LHP-SP2, and 0.2–0.8 μL mL^{-1} concentrations of McEO against AF-LHP-VS8 were observed (Fig. 3B).

To reveal the molecular mechanism behind the inhibitory role of McEO on AFB_1 biosynthesis, molecular docking between the test compounds and Nor-1 protein was performed. The 3D structure of Nor-1 protein obtained through homology modeling was found suitable for the molecular docking and revealed 88.82% of amino acid residues in the favored region. The results revealed that the test compounds strongly interacted with the amino acid residues of the target protein Nor-1. The binding affinity was calculated in terms of MolDock score, which was found to be −37.414, −58.178, and −38.628 for α-pinene, bornyl acetate, and camphor, respectively (Fig. 4).

3.5. Antioxidant activity of McEO: In vitro

The antioxidant activity of the McEO at different concentrations against DPPH and ABTS radicals are presented in Fig. 5A and B, with BHT, BHA, and ascorbic acid as positive control. McEO showed very strong FRS activity with IC_{50} value 4.29 and 3.16 μL mL^{-1} against $DPPH^{\bullet}$ and $ABTS^{\bullet+}$, respectively. These values were much higher than that of the BHT (IC_{50} = 6.6 and 5.13 μL mL^{-1}, respectively), BHA (IC_{50} = 5.12 and 3.91 μL mL^{-1}, respectively), and ascorbic acid (IC_{50} = 4.31 and 3.52 μL mL^{-1}, respectively). The total phenolic content of McEO was found to be 11.23 μg GAE g^{-1} EO.

3.6. Application of McEO in food system: In situ study

From HPLC results, it was found that the maize samples fumigated with McEO had significant differences ($p < 0.05$) in the AFB_1 contamination rate. Control sample presented the highest level of AFB_1 contamination (131.58 μg kg^{-1}). However, the samples (Maukyrwat, Sohru pnah II, and Varun suma) fumigated with McEO at MIC concentrations showed relatively lower AFB_1 content (4.021, 7.967, and 3.754 μg kg^{-1}, respectively), which was non-detectable in 2MIC fumigated maize samples.

3.7. Efficacy of McEO against lipid peroxidation

The effect of different doses of McEO (MIC and 2MIC) on TBARS values is shown in Fig. 6A. The MDA contents significantly decreased ($p < 0.05$) in all McEO fumigated maize samples as compared to controls. MDA content in control set was 917.2 μM g^{-1} FW^{-1}. After fumigation with MIC and 2MIC doses, MDA contents were decreased in all tested varieties of the maize (Maukyrwat, Sohru pnah II, and Varun suma). Highest MDA inhibition occurred in the maize treated with 2MIC doses.

3.8. Oral acute toxicity assessment of McEO

There were no significant changes in the behaviour of the treated

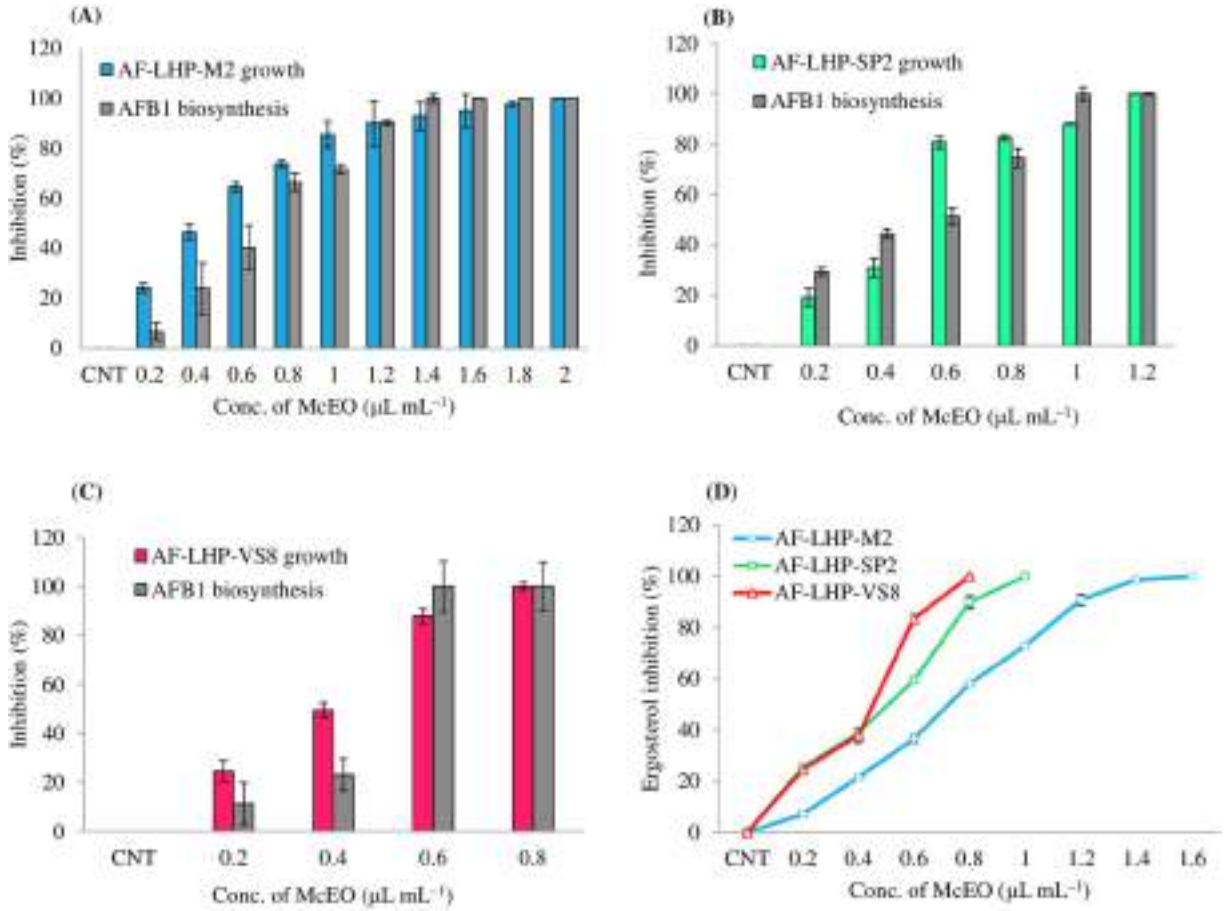

Fig. 1. Effect of varying concentrations of McEO on growth and AFB_1 production of toxigenic strains of *A. flavus*: (A) AF-LHP-M2, (B) AF-LHP-SP2, (C) AF-LHP-VS8, and (D) plasma membrane integrity (ergosterol contents). Error bars indicate the mean ± standard error.

animal groups and no evident signs of toxicity as well as mortality were noted following administration of McEO at the dose levels of 200–1600 mg kg^{-1} body weight. The symptoms of toxicity was observed at higher doses, mice having taken McEO >1600 mg kg^{-1} body weight showing symptoms such as torpor, nose and eyelid bleeding within 24 h of administration. All animals died at 2000 mg kg^{-1} body weight. The LD_{50} in mice was found to be 1800 mg kg^{-1} body weight. These results demonstrate that the mice appeared to well tolerate the McEO at doses between 200 and 1600 mg kg^{-1} body weight. The toxicity was sub-acute when the dose exceeds from 1600 to 1800 mg kg^{-1} body weight, and acute at the dose of up to 2000 mg kg^{-1} body weight, indicating its dose-dependent effects. As far as the current study is concerned, the no observed adverse effect level (NOAEL) of the McEO can be established at the dose of 1600 mg kg^{-1} per day, since at this dose, no sign of toxicity as well as mortality of any mice was observed.

3.9. Organoleptic attributes analysis

The results of the organoleptic evaluation of maize samples fumigated with MIC and 2MIC concentrations of McEO are plotted in Fig. 6B–D, where the sensory scores of different maize samples were shown. The results obtained indicated that McEO fumigated maize samples had significantly higher ($p < 0.05$) scores as compared to control samples. In contrast, maize fumigated with MIC and 2MIC concentrations of McEO showed significant improvement ($p < 0.05$) in the sensory scores for almost all the tested parameters except aroma, which achieved lower scores for all maize varieties at 2MIC.

4. Discussion

It is generally evident that the biological activity of EO is depending upon their chemical composition and the percentage of these compounds may be influenced by environmental factors (Kedia, Prakash, Mishra, & Dubey, 2014). Therefore, the identification of actual chemical composition of EO is crucial before recommending its use as food preservative and to assure consumers safety. The chemical composition of McEO recorded in the present investigation showed slight variation from the previously recorded chemical profiles of the same EO obtained from Indonesia by Septiana, Yuliana, Bachtiar, Putri, et al. (2020). The authors reported 1,8-cineole, α-terpineol, caryophyllene, α-pinene, and γ-terpinene as the major components. However, there were some differences in the per cent composition. This might have resulted from the differences in climatic condition, geographical location, plant part used, genetic variations, and methods of extraction (Chaudhari, Singh, Das, et al., 2020; Teerarak & Laosinwattana, 2019).

The present results on antifungal activity showed that McEO exhibited potent inhibitory activity against tested toxigenic strains of *A. flavus*, the main deteriorating strains found in stored maize samples. These results are in consistent with those reported by Boukaew et al. (2017), who noted the effect of capsicum, cinnamon, clove, and vatica EO treatment on growth inhibition of ten isolates of *A. flavus*. In an attempt to investigate the efficacy of EO as natural fungicides, Hu et al. (2021) also examined the inhibitory effect of *Perilla frutescens* EO on toxigenic *A. flavus* strain. The authors reported that test EO had a pronounced antifungal activity at MIC value of 0.4 μL mL^{-1}. More importantly, in the present investigation, MIC values of 2.0, 1.2, and 0.8 μL mL^{-1} were reported because complete inhibition of visual growth of

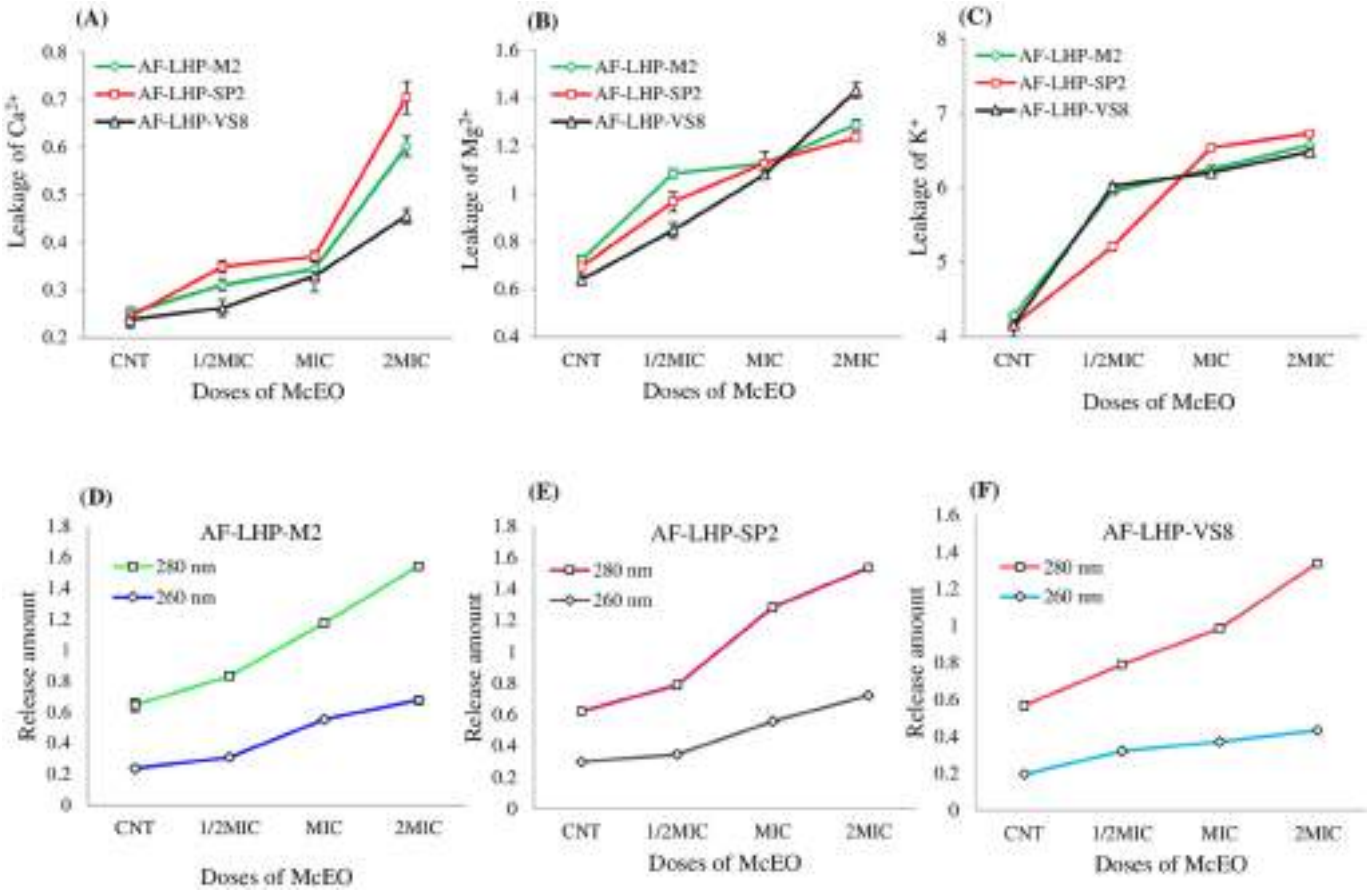

Fig. 2. Effect of varying concentrations (1/2MIC, MIC, and 2MIC) of McEO on plasma membrane permeability of toxigenic strains of *A. flavus*: (A–C) Leakage of cellular ions, and (D–F) Release of 260 (nucleic acids) and 280 nm (proteins) absorbing materials. Error bars indicate the mean ± standard error.

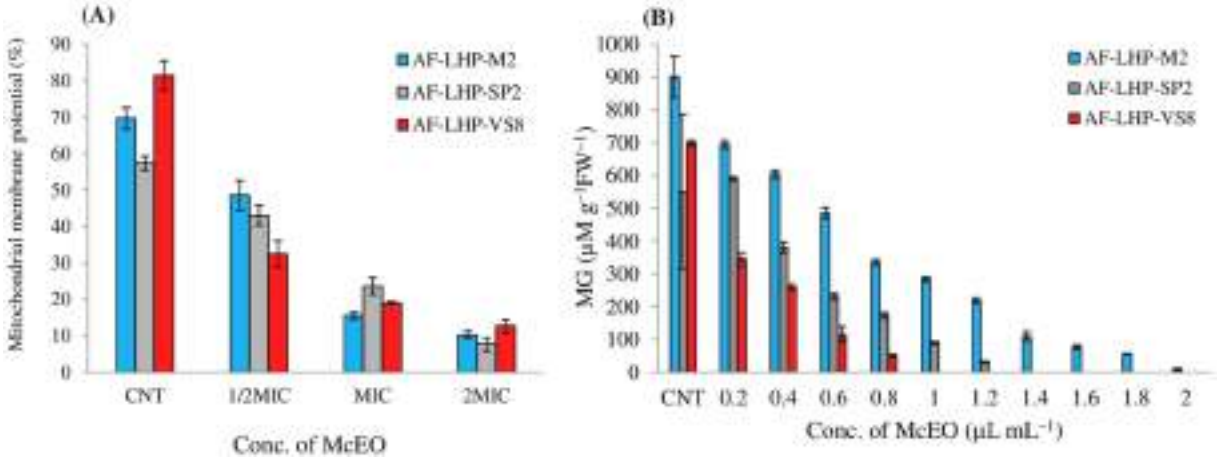

Fig. 3. Effect of different concentrations of McEO on (A) Mitochondrial membrane potential, and (B) Methylglyoxal biosynthesis of toxigenic strains of *A. flavus*. Error bars indicate the mean ± standard error.

toxigenic strains of *A. flavus* by McEO has been achieved at these concentrations. Reported inhibitory activity of McEO might be attributed to the presence of high contents of monoterpene compounds, including α-pinene, bornyl acetate, camphor etc (Deba et al., 2008; Kordali et al., 2005; Yong et al., 2021). While differences in the MICs relative to controls were attributed to the sensitivity of different fungal strains toward test EO or due to the variations in its chemical composition having different mode of action (Avanço et al., 2017). However, it has been also documented that the efficacy of EO is not restricted to a single or dominant compounds but is rather a synergism of both major and minor compounds present in the oil (Farzaneh et al., 2015). Further, the presence of monoterpenes in the McEO may lead to oxidative stress, damage the cell integrity, inhibition of the respiration process in the mitochondrial membrane, and decrease the virulence as well as growth of the fungi (Abdel-Aziz et al., 2019; Hua et al., 2014). To date, the capacity of the McEO to inhibit growth and AFB_1 biosynthesis by tested *A. flavus* strains remained unexplored. Nevertheless, the antifungal effect against other fungal and bacterial pathogens has been investigated

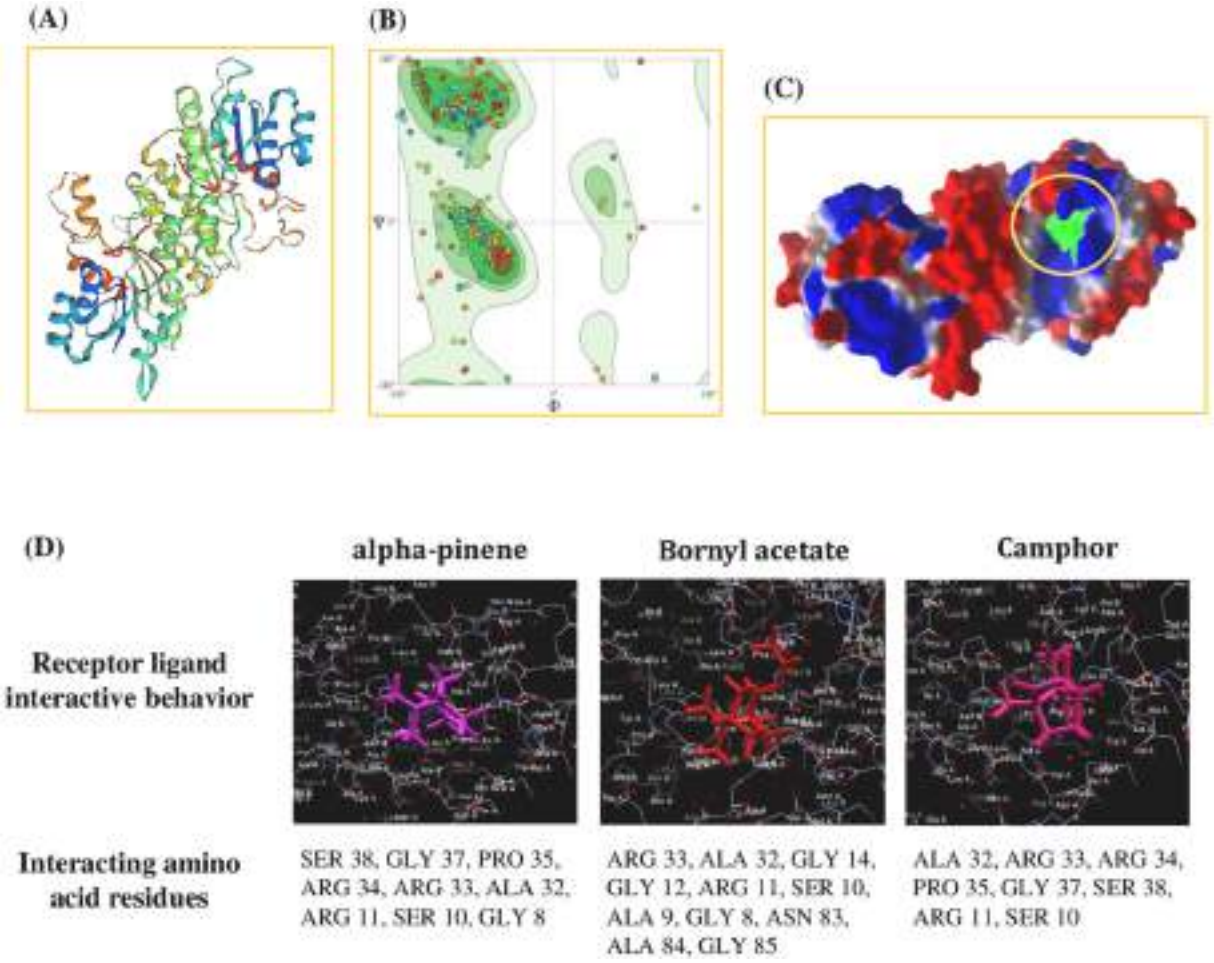

Fig. 4. Homology modeling of Nor-1 gene product and its interaction with the major components of McEO: (A) 3D model of Nor-1 protein obtained through homology modeling, (B) Ramachandran plot of the assessment of homology model, (C) Protein showing the catalytic cavity for docking, and (D) Interaction of different ligands with Nor-1 protein and interacting amino acid residues.

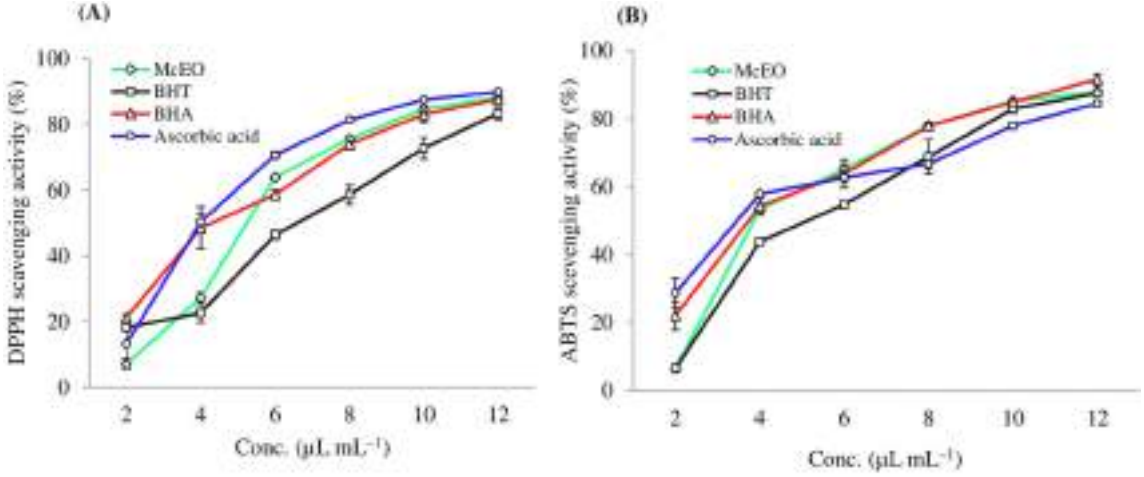

Fig. 5. Free radical scavenging activities of McEO determined through (A) DPPH, and (B) ABTS assay. Error bars indicate the mean ± standard error.

(Harkenthal et al., 1999; Keereedach et al., 2020).

The use of EOs has always been of great interest to the researchers owing to their great potential to inhibit fungal growth and mycotoxin production. Even though, numerous records are available on the efficacy of EOs to control fungal and mycotoxin production (Chaudhari et al., 2021). Kedia, Prakash, Mishra, Chanotiya, and Dubey (2014) found *Mentha spicata* EO as one of the strongest inhibitor of *A. flavus* and AFB_1 production, better than the synthetic fungicides nystatin and wettasul-80. Likewise, Kiran et al. (2016) observed complete inhibition of *A. flavus* growth and AFB_1 production after treatment with *Cinnamomum zeylanicum* EO at 0.6 and 0.3 μL mL^{-1}, respectively. Based on findings of the present study along with supportive results of others, it

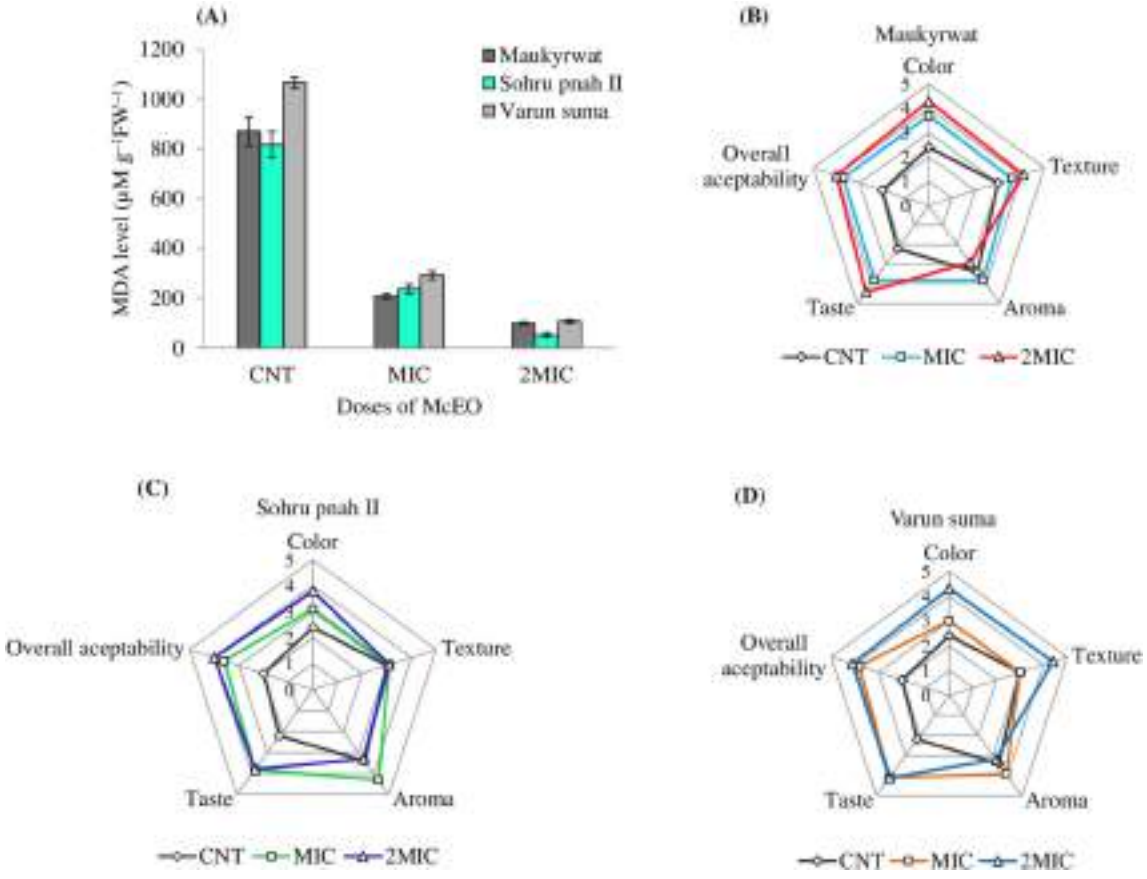

Fig. 6. Effect of varying concentrations of McEO on (A) Lipid peroxidation (TBARS value), and (B–D) Organoleptic qualities of fumigated maize samples.

may be concluded that McEO could be used for the development of effective substitute to the harmful synthetic preservatives to overcome fungal and mycotoxin contamination.

The plasma membrane provides a permeability barrier to the passage of cellular constituents, which are vital for maintaining the normal activity and metabolism of the fungal cells. Previous studies have demonstrated that plasma membrane of fungus is one of main antifungal targets of EOs. To investigate plasma membrane integrity, the ergosterol contents in McEO treated *A. flavus* strains were determined. Ergosterol is a unique and major sterol component of the fungal cell membrane, aid in regulating the structure, integrity, fluidity, and permeability of the membrane, and has also been regarded as important target site for most of the commercially available antifungal drugs such as azoles and polyenes (Siahmoshteh et al., 2018). Earlier studies suggest that even relatively minor changes in the cell membrane structure can lead to harmful effects on cell metabolism and cause cell death. A very significant inhibition of ergosterol biosynthesis was noted even at lower MIC values of McEO, indicating that this EO was effective in interdicting the plasma membrane integrity. The impairment of ergosterol biosynthesis by tested EO may be associated with the inhibition of lanosterol 14-α demethylase, a key enzyme involved in the conversion of lanosterol to ergosterol (Das et al., 2021). Similar trend for diminution of ergosterol biosynthesis in *A. flavus* by cuminaldehyde has been reported by Xu et al. (2021). The authors found 90% reduction in *Erg 25* gene expression, resulting in down-regulation of differentially expressed genes (DEGs) associated with impaired ergosterol biosynthesis. The inhibition of ergosterol biosynthesis may thus negatively affect the integrity and functions of some membrane associated proteins, resulting in permeability disorder, ultimately causing the leakage of important cellular constituents and cell death.

Cellular ions homoeostasis is an important factor for maintaining the energy status, membrane coupled soluble transport, and cellular turgor pressure (Cai et al., 2019). The release of cellular constituents could reflect changes in membrane permeability. The plasma membrane permeability was confirmed on the basis of leakage of the cellular ions and absorbance of 260 (nucleic acids) and 280 nm (proteins) absorbing materials from *A. flavus* cells exposed to McEO at 1/2MIC, MIC, and 2MIC concentrations. Our result showed that McEO caused dose-dependent enhancement of cellular ions and 260 and 280 nm absorbing materials leakage. This result is in corroboration with our previous findings, reporting enhancement of cellular cations and 260 and 280 nm absorbing leakage from *A. flavus* cells following exposure of nanoencapsulated allspice EO (Chaudhari et al., 2022). Excessive loss of these vital cellular constituents may hamper the fungal respiratory reactions, causing detrimental effect on hyphal growth and cellular metabolism. Therefore, even minor changes in permeability caused by EO fumigation could led to cytoplasmic accumulations of ions and irreversible damage to gross cellular metabolic activity (Tao et al., 2014). The results in this study suggests that the relative permeability of the fungal cell membrane increased with the increasing concentrations of McEO, causing leakage of important cellular constituents and leading to cell death.

MMp is a very sensitive indicator of the energy-coupling status of mitochondria. Rho123 is a cationic cell-permeant dye and extensively used to determine the MMp. In normal cells, Rho123 can selectively enter the mitochondrial matrix depending on membrane potential across inner membrane. The fluorescence intensity of the dye is enhanced when cell death occurs and the integrity of mitochondrial membrane is destroyed (Jouan et al., 2014; Pinto et al., 2013). Hence, changes in MMp can be determined by the change of fluorescence level. The fluorescence was increased when *A. flavus* strains were treated with 1/2MIC and MIC concentrations of McEO. When McEO concentration

reached 2MIC, the fluorescence reached maximum, indicating loss of MMp. Similar result was obtained for the EO of *Foeniculum vulgare*, suggesting antifungal mode of action through destruction of the mitochondrial membrane potential (Kumar et al., 2020).

To further understand the antiaflatoxigenic mode of action of McEO that led to AFB_1 inhibition, we investigated MG, which have been reported to induce AFB_1 biosynthesis in *A. flavus* cells. According to Chen et al. (2004), MG biosynthesis in *A. flavus* is essential for up-regulating the expression of AFB_1 biosynthesis gene (*Afl*R). The disruption or down-regulation of this gene resulted in loss of the ability to convert aflatoxin intermediates to AFB_1. Thus, it seems possible that McEO may interfere with the genes involved in AFB_1 biosynthesis, which subsequently resulted in the inhibition of AFB_1. Similar finding for the inhibition of MG biosynthesis by *Cistus ladanifer* EO in *A. flavus* cells has been reported by Upadhyay et al. (2018), who suggested that the inhibition of MG level in *A. flavus* culture might be the possible reason for down-regulation of *AflR* gene expression, and subsequently the AFB_1 biosynthesis. However, further research, especially on gene and protein expressions is needed to shed light on the possible mechanism concerned with the AFB_1 inhibition by McEO.

Molecular docking have been used to decipher the interactions that occur between the ligands with the active site residues of important proteins involved in aflatoxin biosynthesis (Singh et al., 2021). In the present investigation, molecular docking simulations were used to evaluate the complementary interactions between α-pinene, bornyl acetate, and camphor with the Nor-1 binding site. Nor-1 is a 29-kDa protein containing short chain alcohol dehydrogenase motif in its amino acid chain and primarily involved in conversion of norsolorinic acid (NOR) into averantin (AVN), which is an important step in AFB_1 biosynthesis. More importantly, Nor-1 is synthesized in the cytoplasm and transported to the vacuoles of fungal hyphae, where it carried out the regulatory step of AFB_1 biosynthesis (Hong & Linz, 2009). Hence, Nor-1 was selected as the receptor protein for molecular docking.

The result clearly confirmed that the test compounds strongly interacted with the amino acid residues of the Nor-1 protein. This result is consistent with the previous study of Kumar et al. (2020), reporting molecular interaction of anethole with Nor-1 gene products during investigating the antiaflatoxigenic mode of action of *Foeniculum vulgare* EO. Das et al. (2020) also demonstrated strong interaction of thujanol, elemicin, and myristicin (components of *Myristica fragrans* EO) with other proteins (Ver-1 and Omt-A) involved in AFB_1 biosynthesis. Regarding this interactive structure dependent binding mechanism, we concluded that the antiaflatoxigenic mechanism of action of test EO could be attributed to the bindings of its major components (ligands) with amino acids present in the protein binding pocket of Nor-1.

Owing to the complex reactive facets of plant bioactives, the antioxidant activity of plant EO cannot be measured solely by a single method, but at least by two to ascertain authenticity (Chaudhari, Singh, Singh, et al., 2020; Ye et al., 2013). For this reason the FRS activity of McEO was determined by two spectrophotometric methods, ABTS and DPPH assays. Our results showed that the McEO exhibited moderately higher FRS capacity than that of synthetic antioxidants like BHT, BHA, and ascorbic acid. This fact might be explained by the dominance of monoterpene compounds viz., α-pinene, bornyl acetate, and camphor, whose antioxidant capacity has already been reported by others (Kim et al., 2013; Kordali et al., 2005; Shahriari et al., 2018; Yang et al., 2010).

Many studies have reported that the antioxidant activity of EO largely depends on the total phenolic contents. Therefore, besides measuring the antioxidant activity, the total phenolic content of McEO was also recorded. The results showed a strong linear correlation between the FRS activity and total phenolic content. More importantly, most of the reactions involved in AFB_1 biosynthesis are mediated by oxygenases enzymes (Manso et al., 2014). Hence, blocking the enzymatic activity of a biosynthetic enzyme, probably due to the high phenolic content (high antioxidant activity) of McEO may constitutes one of the possible mechanisms for the inhibition of AFB_1 biosynthesis. In addition, the high phenolic content in McEO may also lead to its higher antifungal activity (Bagamboula et al., 2004). According to these authors, the EO that contains more phenolic compounds could interact more rapidly with fungal cell membrane structures and functions, and hence, exhibited strongest antifungal properties. The results confirmed that the evaluated McEO could exhibit great potential for being applied as a good shelf-life enhancer of the stored food items.

Maize is one of the world's highest yielding crops and rich source of carbohydrates, protein, starch, and other micro/macronutrients, and serves as an excellent substrate for fungal proliferation, especially for aflatoxigenic fungi. Therefore, in order to reveal the preservative efficacy of McEO against AFB_1 contamination in food system, *in situ* study was performed on maize (var. Maukyrwat, Sohru pnah II, and Varun suma). The results of *in situ* investigation on maize in storage containers during three months of storage confirmed the strong efficacy of EO against AFB_1 contamination. Hu et al. (2017) similarly confirmed our results as they found that *Curcuma longa* EO at 4 μL mL^{-1} to had the remarkable effect on extending the shelf-life of treated maize. Our previous research also indicated that the fumigation of maize with *Origanum majorana* EO could significantly inhibit AFB_1 accumulation during storage (Chaudhari, Singh, Das, et al., 2020). Although, it is difficult to compare the efficacy of different treatments employed to control AFB_1 contamination; however, the level of control of AFB_1 achieved in this study is relevant, and might be possible to achieve complete protection of stored maize using McEO as a fumigant.

Lipid peroxidation is one of the major factors contributing to post-harvest losses of food commodities during storage, resulting in serious loss of flavor and nutritional value. In this study, the extent of lipid peroxidation was recorded by measuring the level of TBARS in maize samples treated with McEO. TBARS test is one of the most prevalent methods used to measure the second stage oxidation products, especially MDA (Papastergiadis et al., 2012). From the obtained results, it can be concluded that the test EO retarded the rate of lipid peroxidation in fumigated maize samples during storage. Inhibition of MDA content by McEO in stored maize samples might be associated with the presence of different bioactive components having prominent free radical scavenging activities (Youdim et al., 2002). Our result is in agreement with previous study of Amiri et al. (2019), who observed significant inhibition of lipid peroxidation in ground beef patties after treatment with *Zataria multiflora* EO fortified with cinnamaldehyde. Our results in this study suggested that adding McEO might prevent the production of MDA as a secondary lipid oxidation product in stored maize and other agricultural commodities during storage.

The calculated LD_{50} value of McEO was found more higher than some of the commonly used preservatives like pyrethrum (300–500 mg kg^{-1}), bavistin (1500 mg kg^{-1}), and formic acid (700 mg kg^{-1}) (Prakash et al., 2012). Moreover, the higher LD_{50} value of McEO as compared to different EOs and bioactive components such as *Artemisia dracunculus* EO (1250 mg kg^{-1}) thujone (870 mg kg^{-1}), pulegone (150 mg kg^{-1}), and carvacrol (910 mg kg^{-1}), suggesting mammalian non-toxicity and satisfying the acceptability of consumers for large scale application in food system (Maham et al., 2014; Moazeni et al., 2019). In light of these results, fumigating model food (maize) with the McEO may be advised to consumers to prevent from fungal and AFB_1 contamination during storage, as consumption of maize treated at this recommended dose of tested EO is likely to be safe for human.

The results of sensorial properties of treated maize samples showed that the maize samples without any treatment (controls) had the lowest scores for all the tested parameters viz., color, texture, aroma, taste, and overall acceptability. This is probably due to the oxidation of essential fatty acids (palmitic, linoleic, oleic and linolenic acid) during storage, and thus, decreased scores by the panelists. However, McEO treated maize samples showed significant improvement ($p < 0.05$) in the sensory scores for almost all the tested parameters except aroma, which achieved lower scores for all maize varieties at 2MIC. This may be due to

the interactions of EO with food matrix components such as carbohydrates, fats, and proteins, resulting in decrease of their organoleptic properties, and subsequently the consumer acceptance. This result is supported by the earlier results of Castro-Rosas et al. (2017) and Sharma et al. (2017), they also reported that the application of higher doses of EOs induced negative impact on sensory characteristics of treated food items. Nevertheless, nanoencapsulation of McEO in polymeric matrix could be an effective strategy to overcome this issue, since the application of encapsulated EO in food did not negatively alter its sensory attributes due to the controlled release properties when compared to the control samples over the assessed storage period (Hasheminejad & Khodaiyan, 2020).

5. Conclusion

The outcome of this study clearly indicated that the tested McEO exhibited potential antifungal and antiaflatoxigenic activity against three toxigenic strains of *A. flavus* in culture media and in maize grains without significantly altering their sensory acceptability. The antifungal modes of action of McEO was attributed to the disruption of plasma membrane integrity, permeability, and mitochondrial membrane potential, while antiaflatoxigenic action was ascribed to the inhibition of methylglyoxal biosynthesis and molecular functioning of the Nor-1 protein in fungal cell. The non-toxic nature of McEO on mice, ascertained its high safety profile on mammalian system. The findings of the study could support the utilization of this EO as a novel substitute of synthetic chemical preservatives for the protection of stored maize grains from fungal attack, AFB_1 contamination, and lipid peroxidation.

Compliance with ethical standards

The experiment related to animal study was in accordance with the ethical standards of the institution at which the study was conducted.

CRediT authorship contribution statement

Anand Kumar Chaudhari: Conceptualization, Writing – original draft, Methodology, Investigation, Funding acquisition. **Vipin Kumar Singh:** Methodology, Investigation. **Somenath Das:** Validation, Data curation. **Anupam Kujur:** Methodology, Software. **Deepika:** Formal analysis. **Nawal Kishore Dubey:** Writing – review & editing, Supervision, All authors have reviewed and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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