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Evaluation of chemically characterized *Citrus sinensis* L. essential oil as botanical fungitoxicant against fungal deterioration of stored mustard oilseeds

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The study deals with the bioactive efficacy of *Citrus sinensis* L. essential oil (CSEO) against some storage fungi contaminating stored oilseeds of mustard (*Brassica compestris* L.). The average pH and percent moisture content of collected stored oilseeds of mustard ranged from 5.36 to 5.43 and 9.22 to 10.03%, respectively. Stored oilseeds of mustard were found associated with various storage moulds. During mycological screening of oilseeds, a total of 642 fungal isolates was recovered from three different stored samples. The percent occurrence frequency of sample 3 was found to be the highest (37.85%), whereas sample 1 exhibited the lowest (29.12%). The highest cumulative percent relative density was recorded in *Cladosporium* sp. (21.65%), followed by *Aspergillus niger* (18.06%) and *Aspergillus flavus* (11.37%), while the lowest relative density was found in *Aspergillus nidulans* (1.40%), followed by 1.86% in both *Aspergillus candidus* and *Aspergillus terreus*. The minimum inhibitory concentration (MIC) of CSEO against Aspergillus flavus was recorded at 100 µg/ml. CSEO also exhibited broad-spectrum fungitoxicity and was also comparable to the synthetic fungicide diphenylamine as well as having significant antioxidant activity (IC50 22.82). The chemotype of CSEO was determined by GC/GC-MS analysis, which showed 26 constituents. DL-Limonene was found to be the major component (90.44%), followed by linalyl acetate (2.80%) and β-myrcene (1.71%), whereas other compounds were in traces. The prospects of exploitation of CSEO as an acceptable plant-based additive in qualitative as well as quantitative control of biodeterioration of stored oilseeds have been discussed.

Keywords: *Citrus sinensis*, essential oil, antifungal, *Aspergillus flavus*, DL-Limonene, *Brassica compestris*, oilseeds.

1. Introduction

Oilseeds are among the most important agricultural commodities worldwide, serving as a primary source of edible oils, proteins, nutraceuticals, and industrial raw materials. However, during storage, these oilseeds are highly vulnerable to microbial deterioration, particularly by fungal species such as *Aspergillus*, *Penicillium*, *Fusarium, Cladosporium, Rhizopus, Mucor*, etc.[1,2]. Fungal colonization not only reduces the nutritional and germinative quality of oilseeds but also poses severe health hazards through the production of several mycotoxins[3]. These mycotoxins are known to be carcinogenic, hepatotoxic, and immunosuppressive, thereby representing a serious threat to food safety and global trade[4,5]. Conventional strategies to manage fungal spoilage primarily rely on synthetic fungicides and chemical preservatives.

Despite their effectiveness, the long-term and indiscriminate use of such chemicals is under increasing criticism due to their persistence in the environment, contribution to fungicide resistance, hazardous residues in food and adverse impacts on human health[6]. Consequently, there is a growing need for safer, eco-friendly, and sustainable alternatives to protect stored oilseeds from fungal deterioration.

Essential oils (EOs), volatile secondary metabolites obtained from aromatic plants, have attracted significant attention as promising natural preservatives. They are generally recognized as safe (GRAS), biodegradable, and exhibit broad-spectrum antimicrobial and antifungal properties[7]. The bioactivity of EOs is largely attributed to their chemically diverse constituents, including monoterpenes, sesquiterpenes, alcohols, aldehydes, and phenolic compounds[8]. Several aromatic plant EOs rich in bioactive compounds such as limonene, linalool, citral, ocimine, thymol, menthol, eugenol etc. have been reported to possess potent antifungal, antioxidant, and insecticidal activities[9,10].

The literature on the antifungal activity of plant Eos focusing on their application in stored oilseeds remain relatively limited. Furthermore, reports comprehensively correlate the chemical characterization of EOs with its preservative efficacy under simulated storage conditions[10,11]. Such studies are essential to establish scientific evidence supporting the integration of EOs into sustainable storage practices. The present study was therefore designed to evaluate the chemically characterized *C. sinensis* L. essential oil (CSEO) for its antifungal efficacy against common fungi associated with stored oilseeds for development as a "green preservative" in postharvest management of oilseeds.

2. Materials and Methods

2.1. *Collection of oilseed samples*

Three different yellow mustard seed samples (500g) of about 4-6 months of storage were procured from the local retailers of Gorakhpur, India. The oilseeds were collected in sterilized polythene bags to avoid further contamination and stored at 5°C for further analysis[12].

2.2. *Moisture content and pH determination*

To determine the moisture content**,** a representative portion (≈ 50 g) of the mustard seed samples were weighed accurately and dried in a hot air oven at 100 ± 2 °C for 24 hours until a constant weight is obtained. The percentage of moisture is then calculated using the formula:

$$\text{Moisture content (\%)} = (W_1\text{-}W_2 \,/\, W_1) \times 100$$

Where W_1 is the initial weight and W_2 is the final weight after drying.

For pH determination**,** 10 g of each grounded mustard seeds were mixed separately with 100 ml of distilled water (1:10 w/v ratio), shaken for 30 minutes, and allowed to stand for 1 hour. The supernatant suspension is then filtered, and the pH is measured using a calibrated digital pH meter at room temperature[13].

2.3. *Mycobiota analysis of collected mustard seed samples*

Ten grams of each finely ground mustard seed samples were suspended separately in 90ml sterile 0.85% saline solution in an Erlenmeyer flask (250 ml) and homogenized on an electric shaker with a constant speed (120 rpm) for 15 min. Three-fold serial dilutions were prepared separately for each oilseed sample[13]. To inoculate the Petri dishes with 10 ml of freshly prepared potato dextrose agar (PDA) medium, 0.5 ml of the dilution (10^{-3}) was utilized followed by incubation for seven days at 27±2°C. On the third day of incubation, the colony counting process began. Each morphologically distinct mold colony was subcultured on PDA and identified[14,15].

2.4. *Detection of aflatoxigenic potential of isolated Aspergillus flavus*

Ten isolates of *A. flavus* from each mustard samples were randomly selected and tested for their aflatoxigenic potency using SMKY (Sucrose, 200.0 g; Magnesium sulphate, 0.5 g; Potassium nitrate, 0.3 g; Yeast extract, 7.0 g; Distilled water, 1000 ml; pH, 5.6±0.2) as broth nutrient medium[16]. One ml spore suspension (≈10^6 spores ml^{-1}) of each *A. flavus* isolate in 0.1% Tween-80 was inoculated aseptically

to 50 ml SMKY medium and incubated at 27±2 °C for 10 days. After incubation, the content of each flask was filtered (Whatman no. 1). Filtrate of each flask was separately extracted with 40 ml of chloroform in a separating funnel. The chloroform extract was separated and dehydrated with anhydrous sodium sulphate and evaporated till dryness on water bath at 70 °C. The residue left after evaporation was re-dissolved in 1 ml of chloroform and 100 µl of it was spotted on TLC plate (20×20 cm^2 of silica gel). The plate was then developed in Toluene:Isoamylalcohol: Methanol (90:32:2;v/v/v) solvent system[17,18]. The intensity of AFB_1 was observed in an ultraviolet fluorescence analysis cabinet at an excitation wavelength of 360 nm[19]. For quantitative estimation, blue spots of AFB_1 on TLC were scraped out and dissolved in 5 ml cold methanol and centrifuged at 3000 xg for 5 min. Optical density of supernatant was recorded at 360 nm and the amount of AFB_1 was calculated[18].

$$\text{Aflatoxin } B_1 \text{ content } (\mu gL^{-1}) = \frac{D \times M}{E \times l} \times 1000$$

Where, D-absorbance; M-molecular weight of AFB_1 (312); E-molar extinction coefficient of AFB_1 (21,800); *l*-path length (1 cm cell was used).

2.5. *Extraction of Citrus sinensis peel essential oil*

Peels of *Citrus sinensis* (L.) Osbeck fruits were collected from juice shops in Gorakhpur for the extraction of essential oil. Peels (500 g) were thoroughly washed with distilled water and subjected to Clevenger's hydrodistillation apparatus for three hours. The hydrophobic volatile fraction, *i.e.*, *C. sinensis* peel EO (CSEO) was separated followed by dehydration using sodium sulphate and stored in dark clean glass vial at 4-5 °C[18].

2.6. *GC-MS analysis of CSEO*

The CSEO was analyzed through gas chromatography (Perkin Elmer Auto XL GC) equipped with a flame ionization detector. The GC conditions were as follows: column, EQUITY–5 (60m × 0.32mm × 0.25µm) fused silica capillary column; H_2 was the carrier gas; column Head pressure 10 psi; oven temperature program isotherm 2 min. at 70ºC, 3ºC/min. gradient to 250ºC, isotherm 10 min; injection temperature, 250ºC; detector temperature 280ºC. GC-MS analysis was also performed using Perkin Elmer Turbomass GC-MS. The GC conditions were as follows: Injection temperature, 250ºC; column temperature, isothermal at 70ºC for 2 min, then programmed to 250ºC at 37ºC/min and held at this temperature for 10 min; ion source temperature, 250ºC. Helium was used as the carrier gas. The effluent of the GC column was introduced directly into the source of MS. Spectra was obtained in the EI mode with 70ev ionization energy. The compounds were identified by comparison of their relative retention times and the mass spectra with those of authentic reference compounds shown in literature[20].

2.7. *Antifungal and antiaflatoxigenic activity of CSEO*

Minimum inhibitory concentration (MIC) and antiaflatoxigenic efficacy of CSEO was determined against most potent toxigenic isolate *A. flavus* DDUBC2-4 using SMKY broth medium. Different concentrations of the CSEO, *viz*., 20, 40, 60, 80 and 100µg/ml were prepared separately by dissolving their requisite amount in 0.5 ml 5% tween-20 and then mixing it with 49.5 ml of SMKY medium in 150 ml Erlenmeyer flask. The control sets were kept parallel to the treatment sets without CSEO. The flasks were inoculated aseptically with 1 ml spore suspension (≈10^6 spores/ml) of *A. flavus* DDUBC2-4 and incubated at 27±2 °C for 10 days. After incubation, mycelial biomass and aflatoxin B_1 content in broth medium of each flask was determined[17].

2.8. *Fungitoxic spectrum of CSEO*

The spectrum of fungitoxicity of the CSEO was determined at 100 µg/ml by the poisoned food technique using PDA against 11 isolated fungal species *viz*. *Alternaria* sp., *Aspergillus candidus*, *A. fumigatus*, *A. nidulans, A. niger*, *A. terreus*, *Bipolaris* sp., *Cladosporium* sp., *Curvularia lunata*, *Fusarium oxysporum* and *Penicillium* sp. from oilseeds during mycological analysis[13].

2.9. *Comparative efficacy of CSEO with some prevalent fungicides*

A few widely used synthetic fungicides, including benzimidazole (Benomyl), carbendazim 50%WP (Bavistin), diphenylamine (DPA), mencozeb (Dithane M-45), organo-mercurial dust (Agrosan GN) and Sulfur 80%WP (Wettasul-80) were compared to CSEO's fungitoxic effectiveness. 10, 50 and 100 mg/ml were the final concentrations that

were prepared by suspending the necessary amounts of the fungicides in 0.5 ml Tween-20 (5%) followed by 9.5 ml pre-sterilized melted PDA culture medium. Their MICs against toxigenic *A. flavus* DDUBC2-4 was ascertained by usual poisoned-food technique[16].

2.10. Antioxidant activity of CSEO

2.10.1. DPPH radical scavenging assay

To determine the antioxidant activity of CSEO, 50 µl (1:10 dilution in methanol) was applied on TLC plate and developed in ethyl acetate and methanol (1:1). The plate was sprayed with 0.2% DPPH (2,2-diphenyl-1-picrylhydrazyl) solution in methanol and left at room temperature for 30 min. Yellow spot developed due to bleaching of purple color of DPPH reagent, was recorded as positive antioxidant activity of CSEO[21].

2.10.2. Free radical scavenging activity

The magnitude that CSEO bleached the DPPH solution from purple to yellow was utilized to measure their ability to scavenge free radicals. Two-fold concentrations (1.0 to 64.0 µg/ml) of CSEO were prepared separately using 0.004% DPPH solution in methanol (5 ml) and incubated at room temperature for 30 min. Absorbance of the samples were recorded at 517 nm against a blank[21]. The free radical scavenging potential of CSEO was compared with positive control i.e. ascorbic acid.

Free radical scavenging activity (%) = $[(A_{blank} - A_{sample})/ A_{blank}] \times 100$

Where:

A_{blank} - absorbance of the blank (without any test compound); A_{sample} - absorbance of different tested samples

2.11. Statistical analysis

All experiments were conducted in triplicates, and data were expressed as mean ± standard error (SE). Statistical analysis was performed using SPSS software (SPSS 16.0; IBM, NY, USA). Differences between treatments were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. A *p*-value of less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. pH and moisture content

The collected mustard seed samples exhibited slight variation in their physical appearance, moisture content, and pH values, reflecting differences in storage duration and environmental conditions. The pH of crushed oilseed suspensions varied from 5.36±0.04 to 5.43±0.07 (Table 1), showing a slightly acidic nature favorable for fungal colonization. Moisture content of the samples ranged from 9.22±0.34% in sample 2 to 10.03±0.21% in sample 3 (Table 1), indicating lesser storage moisture for oilseeds but sufficient to support fungal growth under prolonged storage. Slightly acidic pH and higher moisture content create favorable conditions for fungal proliferation and mycotoxin production in stored oilseeds, particularly under hot and humid climates. Moisture levels above 8–10% raise seed water activity, promoting the growth of storage fungi such as *Aspergillus*, *Penicillium*, *Fusarium* species etc.[22,23,24]. These storage fungi flourish at slightly lower pH, reduce seed defense activity and enhance fungal enzyme activity, facilitating colonization[1,25]. High temperature and humidity accelerate respiration and lipid peroxidation, further deteriorating seed quality and encouraging mycotoxin synthesis—especially aflatoxins and ochratoxins—by *Aspergillus flavus* and *A. ochraceus*[26,27]. Thus, maintaining optimal moisture content (<7%) and neutral pH during storage is essential to suppress fungal and mycotoxin contamination in oilseeds in tropical conditions.

Table 1: pH and moisture content (%) of collected stored mustard seeds

Mustard seed samples	pH	Moisture content (%)
Sample 1	5.36±0.04[a]	9.56±0.34[a]
Sample 2	5.43±0.07[a]	9.22±0.36[a]
Sample 3	5.41±0.09[a]	10.03±0.21[a]

Values are mean (n = 3) ± SE; P < 0.05. The means followed by same letter in the same column are not significantly different according to One-Way ANOVA and Tukey's multiple comparison tests

3.2. Mycological analysis mustard oilseeds

Mycological examination of the collected mustard oilseeds revealed the occurrence of diverse fungal flora. Sample 1 showed the lowest occurrence frequency (29.12%) while highest (37.85%) in sample 3 (Table 2). The variation in occurrence frequency among samples, suggests differences in storage conditions such as moisture, temperature, and aeration that influenced fungal colonization. A total of 12 identified fungal species belonging to six genera were consistently isolated on Potato Dextrose Agar (PDA). The mycological analysis reflects the susceptibility of oilseeds to colonization by a wide range of storage fungi, particularly under suboptimal storage environments. Collectively, the highest relative density was shown by *Cladosporium* sp. (21.65%) whereas, lowest in *Aspergillus nidulans* (1.40%). The predominance of *Cladosporium* sp. is consistent with its role as a common airborne and surface contaminant thriving under moderate humidity[22,28,29]. The genus *Aspergillus* with six species was dominant during analysis and occupied 41.40% of total fungal isolates. Such dominance by *Aspergillus* indicates their adaptability to oil-rich substrates and warm, humid conditions typically prevailing during storage in tropical climates[25]. The presence of *A. nidulans* with a minimal relative density (1.40%) suggests that not all *Aspergillus* species are equally competitive in the given ecological niche. A total of 9.65% fungal isolates recovered during study were unidentified (Table 2) and point to potential novel or less-characterized fungal species that may require molecular identification for confirmation.

Table 2: Mycobiota analysis of collected stored mustard seed samples

Isolated Fungi	Mustard Sample 1	Mustard Sample 2	Mustard Sample 3	Total isolates	Relative density (%)
Alternaria sp.	7	6	6	19	**2.95**
Aspergillus candidus	4	2	6	12	**1.86**
Aspergillus flavus	19	24	30	73	**11.37**
Aspergillus fumigatus	14	11	19	44	**6.85**
Aspergillus niger	32	41	43	116	**18.06**
Aspergillus terreus	3	6	3	12	**1.86**
Aspergillus nidulans	2	2	5	9	**1.40**
Bipolaris sp.	9	12	15	36	**5.60**
Cladosporium sp.	39	43	57	139	**21.65**
Culvularialunata	9	11	12	32	**4.98**
Fusarium sp.	13	19	17	49	**7.63**
Penicillium sp.	14	10	15	39	**6.07**
Unidentified	22	25	15	62	**9.65**
Mucorales*	4	2	2		
Total isolates	**187**	**212**	**243**	**642**	
Occurrence frequency (%)	**29.12**	**33.02**	**37.85**		

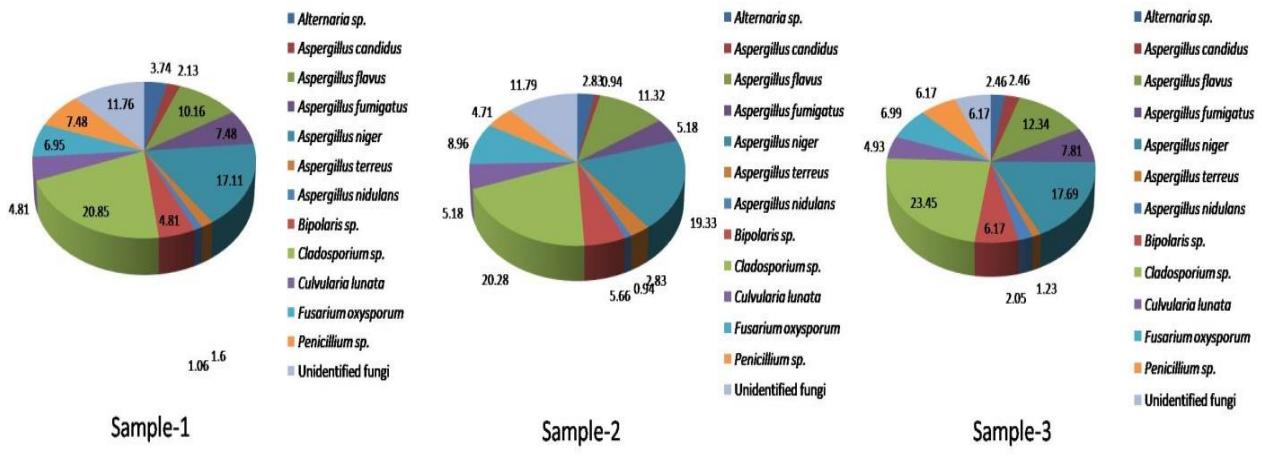

Figure 1. Mycobiota analysis of stored mustard oilseeds

3.3. Detection of aflatoxigenic isolates of A. flavus

Ten isolates of *Aspergillus flavus* were randomly screened for aflatoxin production from each oilseed sample using TLC method revealed a significant toxigenic potential among the fungal populations associated with stored mustard oilseeds. Out of 10 isolates, sample 1, 2 and 3 exhibited 4 (40%), 3 (33.33%) and 5 (50%) toxigenic isolates respectively with intense blue fluorescence under UV light at 365 nm (Table 3). The highest aflatoxin B_1 production (1511.34 µg/L) was reported from isolate *A. flavus* DDUBC2-4 (Table 3) highlights its superior toxigenic capacity and selected as test fungus for further study. The predominance of *A. flavus* as both a frequent and toxigenic species emphasizes its ecological fitness and preference for lipid-rich substrates like mustard seeds, particularly under warm and humid storage conditions conducive to aflatoxin biosynthesis[3,24]. Similar findings have been reported by earlier workers[30,31,32], where, *A. flavus* was identified as a major contaminant in oilseeds with significant aflatoxin production potential.

Table 3: Toxigenicity of *Aspergillus flavus* isolated from selected mustard oil seeds

Mustard sample 1		**Mustard sample 2**		**Mustard sample 3**	
Fungal isolates	AFB_1 (µgL^{-1})	Fungal isolates	AFB_1 (µgL^{-1})	Fungal isolates	AFB_1 (µgL^{-1})
A. flavus DDUBC1-1	-	*A. flavus* DDUBC2-1	1036.18	*A. flavus* DDUBC3-1	-
A. flavus DDUBC1-2	-	*A. flavus* DDUBC2-2	-	*A. flavus* DDUBC3-2	-
A. flavus DDUBC1-3	40.07	*A. flavus* DDUBC2-3	-	*A. flavus* DDUBC3-3	1248.00
A. flavus DDUBC1-4	1162.13	***A. flavus* DDUBC2-4***	**1511.34**	*A. flavus* DDUBC3-4	538.13
A. flavus DDUBC1-5	-	*A. flavus* DDUBC2-5	-	*A. flavus* DDUBC3-5	-
A. flavus DDUBC1-6	-	*A. flavus* DDUBC2-6	1139.23	*A. flavus* DDUBC3-6	612.55
A. flavus DDUBC1-7	618.28	*A. flavus* DDUBC2-7	-	*A. flavus* DDUBC3-7	-
A. flavus DDUBC1-8	1013.28	*A. flavus* DDUBC2-8	-	*A. flavus* DDUBC3-8	417.91
A. flavus DDUBC1-9	-	*A. flavus* DDUBC2-9	-	*A. flavus* DDUBC3-9	641.17
A. flavus DDUBC1-10	-	*A. flavus* DDUBC2-10	-	*A. flavus* DDUBC3-10	-

* Fungal isolate ***A. flavus* DDUBC2-4** from Mustard sample 2exhibited the aflatoxin B1 producing potential

3.4. Chemical characterization of CSEO

EO extracted from *C. sinensis* peels via hydro-distillation yielded 2.4±0.2% (v/w) of very light greenish-yellow aromatic oil indicating efficient recovery of volatile compounds typical of *Citrus* species. Gas Chromatography–Mass Spectrometry (GC–MS) profiling of CSEO revealed the presence of 26phytochemical constituents, accounting for 98.96% of the total composition. DL-Limonene (90.66%) was found as dominant compound followed by linalyl acetate (2.80%) and β-myrcene (1.71%) while, rest other constituents were in traces (Table 4). The predominance of DL-limonene aligns with previous reports describing it as the major monoterpene hydrocarbon responsible for the characteristic citrus aroma and potent antioxidant, antimicrobial, and antifungal activities of CSEO[33,34,35]. The presence of minor constituents such as linalyl acetate and β-myrcene further contributes to the EO's bioactivity and fragrance profile[36]. The dominance of oxygenated monoterpenes and terpenoids suggests that the EO may possess significant biological potential, particularly as a natural preservative or antifungal agent[34].

Table 4: Chemical composition of CSEO

Sr No.	**Retention time (RT)**	**Retention index (RI)**	**Compounds**	**Percentage (%)**
1	9.52	931	α-Pinene	0.34
2	10.87	962	Sabinene	0.37
3	11.05	968	β-Pinene	0.03
4	11.17	972	Methyl heptenone	0.01
5	11.24	992	Octanal	0.42
6	11.35	1020	β-Myrcene	1.71

7	12.05	1028	α-Phyllandrene	0.03
8	12.27	1035	β-Ocimene	0.23
9	12.50	1046	α-Terpinene	0.04
10	**13.20**	**1057**	**DL-Limonene**	**90.66**
11	13.65	1062	Cis-Ocimene	0.03
12	14.55	1068	Caprylic alcohol	0.05
13	15.52	1078	α-Terpinolene	0.18
14	15.78	1128	Nonanal	0.05
15	15.85	1137	Cosmene	0.03
16	15.94	1236	Linalyl acetate	2.80
17	22.80	1253	DL-Carvone	0.07
18	22.52	1267	Z-Citral	0.09
19	17.65	1306	Myrtenyl acetate	0.05
20	18.35	1438	t-Sabinine hydrate	0.37
21	19.67	1484	Decanal	0.02
22	20.77	1574	Isopulegol	0.26
23	21.95	1717	Geranyl formate	0.62
24	23.87	1737	β-citronellol	0.21
25	24.12	1739	6-isopropenyl-3-methyl-2-Cyclohexene-1-one	0.26
26	24.22	1777	Perillaldehyde	0.03
			Total	**98.96**

3.5. Antifungal and antiaflatoxigenic efficacy of CSEO

CSEO showed potent fungitoxicity against *A. flavus* DDUBC2-4 and its minimum inhibitory concentration (MIC) was recorded at 100 mgmL^{-1}. In addition, CSEO was also found efficient to inhibit the AFB_1 production by *A. flavus* DDUBC2-4 and completely checked at 60 mgmL^{-1} (Table 5). A direct relation was found between fungal growth and AFB_1 production i.e. decreases in mycelial biomass resulted in low AFB_1 production and vice versa.

Table 5: Antifungal and antiaflatoxigenic activity of CSEO against *A. flavus* DDUBC2-4

Concentration (mg ml^{-1})	Mycelial Biomass (g)	Aflatoxin B_1 content (µg L^{-1})
Control	0.503±0.026^{c}	1483.670 ± 78.914^{c}
20	0.130±0.014^{b}	305.320 ± 53.431^{b}
40	0.050±0.009^{a}	111.633 ± 22.358^{ab}
60	0.030±0.006^{a}	0.000±0.000^{a}
80	0.021±0.006^{a}	0.000±0.000^{a}
100	0.000±0.000^{a}	0.000±0.000^{a}

Values are mean (n = 3) ± SE; P < 0.05. The means followed by same letter in the same column are not significantly different according to One Way ANOVA and Tukey's comparison tests

The observed inverse relationship between fungal biomass and AFB_1 production supports earlier findings that toxin biosynthesis is growth dependent and can be significantly reduced by disrupting cellular and metabolic processes[25]. One of the most widely recognized mechanisms is disruption of fungal cell membrane and cell wall integrity. Lipophilic EO components such as limonene, thymol, carvacrol, and citral penetrate the lipid bilayer, increasing membrane permeability, causing leakage of vital cellular contents (ions, proteins, nucleic acids), and leading to cell lysis[37,38]. Inhibition of ergosterol biosynthesis, a key structural sterol in fungal membranes, also weakens cell structure and disrupts membrane bound enzyme activity[39]. EOs also interfere with mitochondrial function and energy metabolism, reducing ATP synthesis and impairing fungal growth to inhibit respiratory chain enzymes, leading to oxidative stress and accumulation of reactive oxygen species[39]. The inhibition of mycotoxin biosynthetic pathways is another critical mechanism, certain EO constituents downregulate aflatoxin biosynthetic genes, thereby reducing toxin formation even at sub-inhibitory concentrations[40,41].

3.6. Fungitoxic spectrum of CSEO

CSEO exhibited broad fungitoxic spectrum against some other storage fungi. It completely checked the proliferation of all the tested fungal species at 100 mgml^{-1} (MIC against *A. flavus* DDUBC2-4) except *Alternaria* sp. (86.89±0.92%), *Bipolaris* sp. (83.70±0.94%), *Cladosporium* sp. (74.67±6.58%), *Curvularia lunata* (85.42±0.74%) and *Fusarium oxysporum* (88.70±1.01%) whereas, at 200 mg mL^{-1} (2×MIC against *A. flavus* DDUBC2-4) could not completely inhibit *Alternaria* sp. (94.85±2.59%), *Cladosporium* sp. (89.34±1.07%) and *Curvularia lunata* (94.44±2.78%) (Table 6). The broad-spectrum fungitoxic activity of CSEO against various storage fungi suggests that its bioactive constituents, particularly DL-limonene and linalyl acetate, may interfere with membrane integrity and enzyme systems necessary for fungal growth[8,33]. The partial inhibition of some fungal species even at higher concentrations (2×MIC) reflects variability in fungal susceptibility, likely due to differences in cell wall composition and metabolic adaptation. CSEO as fungitoxicant was evaluated to be less efficacious compared to tested synthetic fungitoxicants except wettasul-80 (> 100 mg mL^{-1}) while MIC of Diphenylamine (100 mg mL^{-1}) against *A. flavus* DDUBC2-4 was found comparable to CSEO (Table 7), highlighting its potential as a natural alternative for controlling fungal contamination and aflatoxin production in stored oilseeds. Overall, the results support the promising role of CSEO as an eco-friendly fungitoxicant with significant antitoxigenic potential.

Table 6: Fungitoxic spectrum of CSEO against some storage fungi

Test Fungi	Percent inhibition	
	MIC (100 mgmL^{-1})	2×MIC (200 mgmL^{-1})
Alternaria sp.	86.89 ± 0.92^{b}	94.85 ± 2.59^{ab}
Aspergillus candidus	100.00 ± 0.00^{c}	100.00 ± 0.00^{b}
Aspergillus fumigatus	100.00 ± 0.00^{c}	100.00 ± 0.00^{b}
Aspergillus nidulans	100.00 ± 0.00^{c}	100.00 ± 0.00^{b}
Aspergillus niger	100.00 ± 0.00^{c}	100.00 ± 0.00^{b}
Aspergillus terreus	100.00 ± 0.00^{c}	100.00 ± 0.00^{b}
*Bipolaris*sp.	83.70 ± 0.94^{ab}	100.00 ± 0.00^{b}
Cladosporium sp.	74.67 ± 6.58^{a}	89.34 ± 1.07^{a}
Curvularialunata	85.42 ± 0.74^{ab}	94.44 ± 2.78^{ab}
Fusarium oxysporium	88.70 ± 1.01^{b}	100.00 ± 0.00^{b}
Penicillium sp.	100.00 ± 0.00^{c}	100.00 ± 0.00^{b}

Values are mean (n = 3) ± SE; P < 0.05. The means followed by same letter in the same column are not significantly different according to One Way ANOVA and Tukey's comparison tests

Table-7: Comparative fungitoxicity of CSEO with some prevalent synthetic fungicide

Fungicides	**MIC against *A. flavus* (mg ml^{-1})**
Benzimidazole **(**Benomyl)	20
Carbendazim 50%WP (Bavistin**)**	40
Diphenylamine (DPL)	100
Mencozeb (Dithane M-45)	40
Organo-mercurial dust (Agrosan GN)	20
Sulfur 80%WP (Wettasul-80)	> 100
CSEO	100

3.7. Antioxidant activity of CSEO

The antioxidant activity of CSEO was evaluated using standard *in vitro* DPPH assays. The results revealed that CSEO exhibited considerable free radical scavenging activity (IC_{50} 22.82 µg mL^{-1}) showed the comparatively lower antioxidant potential against synthetic antioxidant ascorbic acid (IC_{50} 8.64 µg mL^{-1}) as positive control (Figure 2). DL-limonene, the predominant monoterpene constituent of CSEO, and minor compounds like linalyl acetate and β-myrcene are largely responsible for its notable activity. These compounds work together to donate electrons and stabilize reactive oxygen species[33,42,43]. The moderate IC_{50} value suggests that CSEO could serve as a promising natural antioxidant source with potential applications in food preservation and pharmaceutical formulations, offering a safer and eco-friendly alternative to synthetic antioxidants[38].

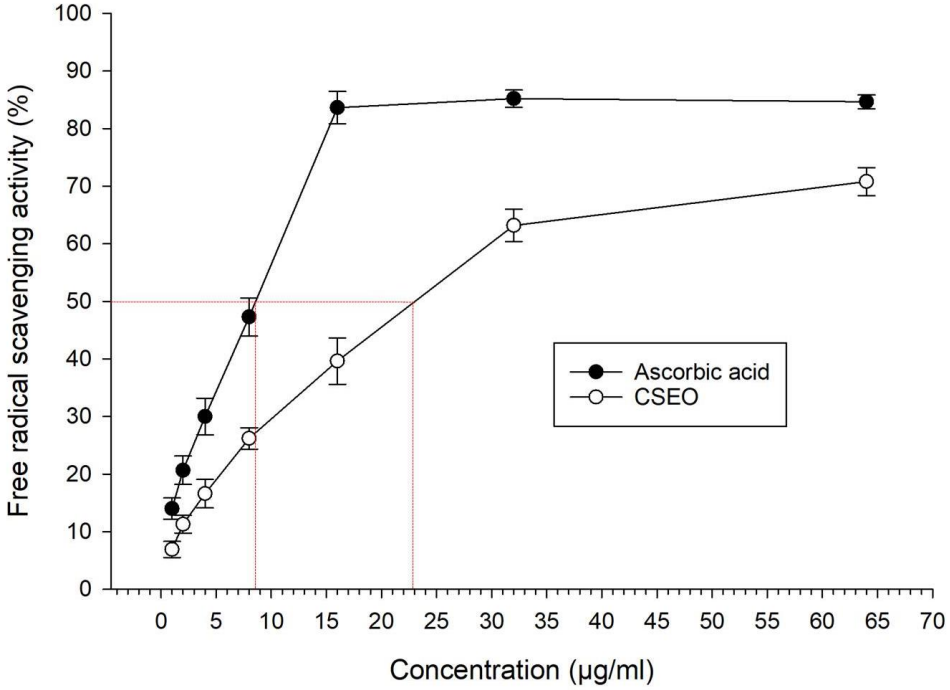

Figure 2. Comparative antioxidant activity of CSEO with ascorbic acid

4. Conclusion

The findings of this study provide a strong basis for the application of CSEO as a natural, multi-functional preservative for oilseeds and other stored commodities. CSEO demonstrated pronounced antifungal, antiaflatoxigenic, and antioxidant activities attributable to its high limonene content, can help minimize postharvest losses and aflatoxin contamination in storage systems. The EO's broad-spectrum fungitoxicity and efficacy comparable to commercial fungicides underscore its potential as an eco-friendly biopreservative for safe storage of mustard oilseeds. These findings support the integration of CSEO into botanical fungicide formulations as sustainable alternatives to synthetic chemicals in postharvest management systems. Future studies should focus on the microencapsulation or vapor-phase application of CSEO to enhance its stability and long-term efficacy under commercial storage conditions.

Author contributions

PP conducted the experiment and wrote the original draft of the paper. **SK** performed mycological analysis and fungal identification. **AK** conceptualized and supervised the whole experimental work as well as editing and corrections in the original draft. All the authors have read and agreed to the final version of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Funding

Nothing to declare.

Data Availability

Data supporting this study is available from the corresponding author upon reasonable request.

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