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Assessment of the Chemical Composition, Antifungal, Antiaflatoxigenic and Antioxidant Efficacy of *Hyptis suaveolens* (L.) Poit. Essential Oil for the Sustainable Management of Toxigenic Storage Fungus *Aspergillus flavus* and Aflatoxin Contamination

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ABSTRACT

The study deals with the bioactive efficacy of *Hyptis suaveolens* (L.) Poit. essential oil (HSEO), against an aflatoxigenic fungal isolate *Aspergillus flavus* DDUOS-06. The minimum inhibitory concentration (MIC) of HSEO against *A. flavus* DDUOS-06 was recorded at 25 µL/mL as well as completely checked the aflatoxin B1 (AFB1) production at 20 µL/mL. In addition, HSEO also exhibited strong antioxidant activity and its IC50 value was found as 36.7 µg/mL which was higher than ascorbic acid (15.8 µg/mL). The chemical composition of HSEO was determined by GC-MS analysis where, eucalyptol (51.16%) was found as major component followed by sabinene (11.72%), α-cedrene (6.59%), limonene (5.71%), β-pinene (3.91%) and β-Caryophyllene (3.48%). The multifunctional properties of HSEO i.e. antifungal, antiaflatoxigenic and antioxidant activity suggest that it can be a promising plant-based alternative of synthetic fungicides and chemical preservatives.

Keywords: *Hyptis suaveolens*; Essential Oil; Hydro–Distillation; Antifungal; *Aspergillus flavus*; MIC; Aflatoxin B1; SMKY; Antioxidant; GC-MS; Eucalyptol.

1.0.Introduction

Several agricultural products including cereals, pulses, oil seeds, spices, herbal raw materials, fruits, vegetables are extremely prone to post-harvest losses due to improper storage under hot and humid climatic conditions [1,2]. Such agricultural commodities are associated with a large number of storage fungi and mycotoxins produced by them during improper storage [3]. Such fungal deterioration significantly reduced their economic values as well as adversely affects customer's health by producing mycotoxins such as fumonisins and aflatoxins, which are strong carcinogens and immunosuppressive substances [4,5]. Despite the fact that conventional synthetic fungicides have been widely employed to control storage fungus, their use causes problems with food product residues, environmental contamination, hazards to human health, and the evolution of resistance fungal strains [6].

Alkaloids, phenolics, terpenoids, and other biologically active phytochemicals found in plants can be employed as safer alternatives to some common synthetic pesticides [7]. Essential oils (EOs) are volatile, complex blends of phenolic and terpenoid compounds that are derived from aromatic plants and have a variety of antibacterial qualities [8]. These EOs target the fungal cell membrane and break their lipid bilayer, causing cellular content leakage, mycelial growth suppression, and spore germination, according to a number of previous research [9]. It is now widely acknowledged that EOs considerably check various metabolic pathways for the synthesis of mycotoxins in various mycotoxigenic fungi [5,10].

Due to the widespread acceptance of plant-based products as safe, people are now depending on green consumerism. There are numerous EO-based formulations such as EcoSMART, Biorepel, Essentria IC3, Ecotrol etc. are used as botanical pesticides [11,12]. The chemical composition, antifungal, antiaflatoxigenic, and

antioxidant properties of *Hyptis suaveolens* essential oil (HSEO) were assessed in the current study. This work seeks to explore the feasibility to produce HSEO based botanical fungicide to decrease the fungal deterioration of stored agricultural products and prolong their shelf life by enhancing post-harvest management.

1.1. Study Objectives

The main objectives of the present study are:

1) To evaluate the antifungal efficacy of *Hyptis suaveolens* essential oil (HSEO) against aflatoxigenic fungus *Aspergillus flavus* by determining minimum inhibitory concentration (MIC).

2) To investigate the antiaflatoxigenic potential of HSEO by assessing its ability to inhibit aflatoxin B_1 production by toxigenic *A. flavus* under *in vitro* conditions.

3) To determine the antioxidant activity of HSEO using DPPH assay.

4) To determine the chemical composition of HSEO by GC-MS analysis.

5) To correlate the chemical composition of the essential oil with its biological activities.

6) To assess the potential application of HSEO as a natural and eco-friendly botanical preservative.

2.0. Literature Review

2.1. Fungitoxic potential in essential oils (EOs)

Higher plant EOs are gaining more attention as substitute of synthetic fungicides due to their broad spectrum fungitoxicity against a range of pathogens and eco-friendly nature. Numerous studies revealed that EO obtained from aromatic plants exhibited potent antifungal activity. These EOs are well known for their inhibitory activity against a number of fungal pathogens including *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp., *Alternaria* spp., *Rhizopus* spp. etc. [8,9,13-16]. In recent decade, there are several reports on inhibitory effect of several EOs against aflatoxin B_1 production by *A. flavus* [17-19].

2.2. Antioxidant Activity in essential oils

EOs are well known for their free radical scavenging ability, inhibition of lipid peroxidation and protection of biological system from oxidative damage. In earlier studies, several EOs showed remarkable antioxidant activity and some of them were comparable to synthetic antioxidants [19-21]. The antioxidant potential of EOs is mainly attributed to several bioactive components viz. thymol, eugenol, citral, limonene etc. [17,19,22,23].

3.0. Methodology

3.1. Test fungus

A toxigenic isolate *Aspergillus flavus* DDUOS-06, already available in Mycotoxin and Botanical Pesticide Laboratory, Department of Botany, Deen Dayal Upadhyaya Gorakhpur University (DDUGU), Gorakhpur was sub-cultured and used as test fungus.

3.2. Extraction of *Hyptis suaveolens* essential oil (HSEO)

Leaves of *Hyptis suaveolens* (L.) Poit. (Lamiaceae) were collected from the DDUGU Campus in the month of November 2025. The leaves (500 g) of the plant were carefully washed with tap water and introduced within Clevenger's hydro–distillation unit (3 hrs) for the extraction of essential oil (EO). Anhydrous sodium sulphate was used to dehydrate the extracted *H. suaveolens* essential oil (HSEO), which was then kept in a clean, dark glass vial at 4-6°C [17].

3.3. Antifungal activity of HSEO

Using the poisoned food method, the antifungal effectiveness of HSEO was tested against the toxic fungal isolate *A. flavus* DDUOS-06 [18]. Different concentrations of HSEO, viz. 5, 10, 15, 20, and 25 µL/mL, were created by dissolving their necessary amount in 0.5 mL of 10% tween-20, followed by 9.5 mL of melted potato dextrose agar (PDA) as nutrient medium, in order to determine the minimum inhibitory concentration (MIC). The control sets without HSEO were similarly kept parallel to the treatment sets. After solidification of nutrient media, each plate was aseptically inoculated with 5 mm diameter disc of test fungus *A. flavus* DDUOS-06 and cultured at 27±2°C for seven days. The diameter of fungal colony of treatment and control sets was measured and % mycelial inhibition was estimated [19].

Percent Mycelial Inhibition (%) = $\frac{C-T}{C} \times 100$

Where C = Mycelial growth diameter in control set, T = Mycelial growth diameter in HSEO treatment set.

3.4. Antiaflatoxigenic activity of HSEO

The capacity of the HSEO to prevent *A. flavus* DDUOS-06 from producing aflatoxin B_1 (AFB_1) was used to assess the antiaflatoxigenic activity of it. In Erlenmeyer flasks (150 mL), different concentrations of HSEO (5, 10, 15, 20, and 25 µL/mL) were prepared separately by dissolving the necessary amount in 0.5 mL 10% tween-20. This was followed by 49.5 mL of SMKY (sucrose 200 g; $MgSO_4.7H_2O$ 0.5 g; KNO_3 0.3 g; yeast extract 7.0 g; distilled water 1000 mL) broth medium, as well as control sets without HSEO. A spore suspension of *A. flavus* DDUOS-06 ($\approx 10^6$ spores/mL) was added to each flask containing SMKY broth, and the flasks were then incubated for ten days at 27±2°C. Mycelial biomass and AFB_1 production in each flask's broth medium were measured following incubation [24].

3.5. Antioxidant activity of MSEO

Using a spectrophotometer, the degree to which the purple-colored DPPH (2,2-diphenyl-1-picrylhydrazyl) solution turned yellow was used to measure the antioxidant activity of HSEO. The HSEO was produced in methanol at various two-fold doses (1.0 to 128.0 µg/mL). 2.0 mL of 0.004% (w/v) DPPH solution in methanol was mixed with 1 mL of HSEO solution in methanol, and the mixture was allowed to sit in the dark at room temperature for 30 minutes. After incubation, the absorbance was recorded against a blank at 517 nm using spectrophotometer. Scavenging of DPPH free radical with reduction in absorbance of the HSEO was taken as a measure of their antioxidant activity following Sharififar et al. [25]. HSEO's ability to scavenge free radicals was also contrasted with that of the artificial antioxidant ascorbic acid. Percent free radical scavenging activity was estimated using the following formula.

Percent Inhibition (%) = ($A_{blank} - A_{sample}$ / A_{blank}) x 100

Where, A_{blank} is the absorbance of the control (without test compound), and A_{sample} is the absorbance of the test compound.

3.6. Chemical composition of HSEO

The chemical characterization of HSEO by GC-MS analysis was performed at Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, Uttar Pradesh. The HSEO was subjected to gas chromatography (Perkin Elmer Auto XL GC) with a flame ionization detector. The GC conditions were as follows: column: EQUITY-5 (60m × 0.32mm × 0.25µm) fused silica capillary column; carrier gas: H2; column head pressure: 10 psi; oven temperature program isotherm: 2 minutes at 70ºC; gradient: 3ºC/min to 250ºC; isotherm: 10 minutes; injection temperature: 250ºC; detector temperature: 280ºC. Additionally, GC-MS analysis was performed using Perkin Elmer Turbomass GC-MS. The effluent from the GC column was directly fed to the MS source. Spectra were obtained in the EI mode with ionization energy of 70 eV. The compounds were identified by comparing their mass spectra and relative retention times to those of authentic reference compounds that had been published in the literature [26].

3.7. Statistical analysis

The experiments were performed in triplicate and data expressed as Mean±SD. One way ANOVA ($P < 0.05$) and Tukey's multiple range tests were analysed using SPSS (version 16.0).

4.0. Results and Discussions

The present study investigates the bioactive effectiveness of *H. suaveolens* essential oil (HSEO) against a toxigenic storage fungus *Aspergillus flavus* DDUOS-06. During study, HSEO exhibited significant antifungal efficacy and its minimum inhibitory concentration (MIC) against *A. flavus* DDUOS-06 was recorded at 25 µL/mL, demonstrating significant fungitoxic efficacy (Table 1; Figure 1). Similarly, antifungal activity of HSEO against *Aspergillus flavus* and other moulds like *Penicillium*, *Fusarium* and *Rhizopus* species have been reported by earlier workers [27-29]. The MIC of HSEO against *A. flavus* DDUOS-06 was found lower than previous reports of Moreira et al. [28], where it ranged between 40-80 µL/mL. The HSEO also showed potent antiaflatoxigenic activity and completely inhibited the AFB_1 production by *A. flavus* DDUOS-06 at 20 µL/mL concentration (Table 2, Figure 2). The literatures are almost silent on antiaflatoxigenic activity of HSEO. But, EOs of some other plants such as lemongrass, thyme, mint, holy basil, all spice, clove, cinnamon etc. exhibited strong antiaflatoxigenic activity [13,24,30-32].

Table 1. Antifungal activity of HSEO against *A. flavus* DDUOS-06 by poisoned food technique.

Concentration(µL/mL)	Colony diameter (cm)	Percent inhibition (%)
Control	4.39 ± 0.33^{e}	0.00
5	2.16 ± 0.53^{d}	50.79
10	0.73 ± 0.21^{c}	83.37

15	0.38±0.14[b]	91.34
20	0.17±0.06[ab]	96.13
25	0.00±0.00[a]	100.00

Values are mean (n = 3) ± SD; 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.

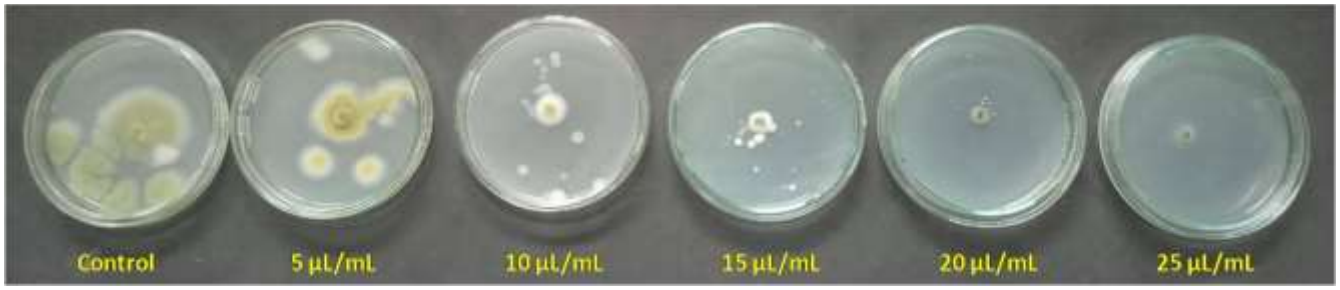

Figure 1. Inhibition of fungal growth with increasing HSEO concentration against *A. flavus* DDUOS-06.

The antifungal action of EOs may be related to the presence of physiologically active volatile molecules such linalool, eugenol, methyl eugenol, cineole, camphor and terpinenes [33,34]. These substances are known to cause fungal cell death by disrupting the integrity of the fungal cell wall and membrane, interfering with mitochondrial respiration, preventing spore germination, and altering enzymes activity [35,36]. Several EO components also inhibited ergosterol biosynthesis, an important constituent of fungal cell membrane, thereby membrane stability and its permeability are adversely affected [18,37]. EOs usually involves multiple biochemical and molecular targets to inhibit aflatoxin biosynthesis. Lipophilic constituents of EOs alter the cellular metabolism usually by membrane disruption and oxidative stress as well as regulate the expression of structural genes involved in aflatoxin biosynthesis [37]. Previous studies by Kumar et al. [33], Tripathi and Dubey [38], and Mishra et al. [39] have also emphasized the effectiveness of EOs as environmentally acceptable substitutes for synthetic fungicides in controlling storage fungus and preventing mycotoxin contamination.

Table 2. Antiaflatoxigenic potency of HSEO against *A. flavus* DDUOS-06

Concentration (µL/mL)	**AFB1 content (µg/L)**
0	457.98±75.08[c]
5	133.58±28.81[b]
10	22.90±11.45[a]
15	15.27±6.61[a]
20	0.00±0.00[a]
25	0.00±0.00[a]

Values are mean (n = 3) ± SD; 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.

The HSEO exhibited a significant antioxidant activity using DPPH assay and its IC_{50} value was recorded as 36.7 µg/mL, higher than ascorbic acid i.e. 15.8 µg/mL (Figure 3). The antioxidant potential of EOs is mainly involved

their ability to neutralize reactive oxygen species by releasing hydrogen atoms or electrons by which they prevent oxidative damage of biomolecules [40]. Such antioxidant potential of EOs may be associated with the presence of bioactive constituents such as terpenoids and phenolic compounds, well recognized for their radical-scavenging properties [41]. The variability in antioxidant potential in different EOs depends upon their chemical composition, plant genotype, age of plant, plant parts and mode of antioxidant assay [42].

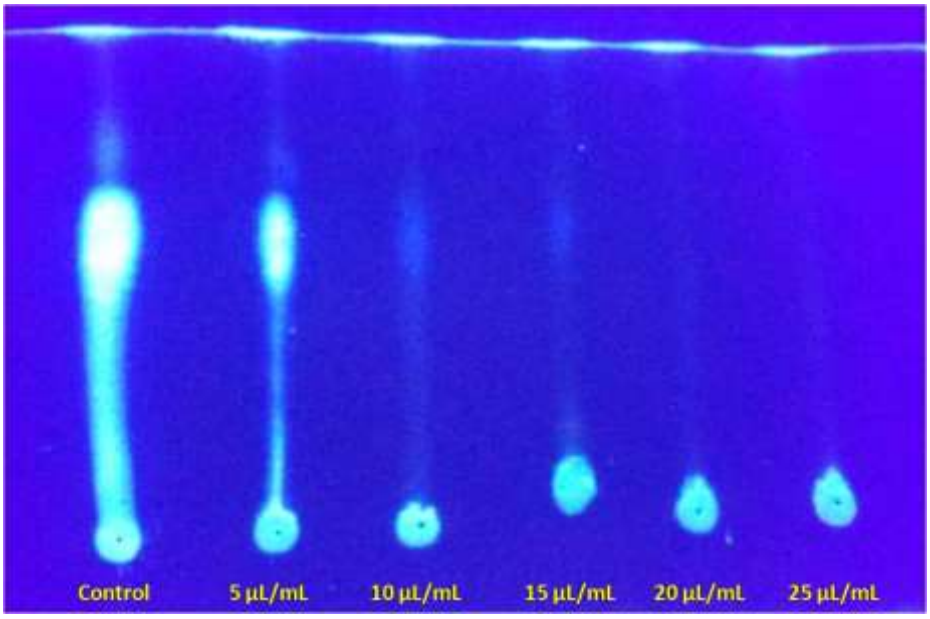

Figure 2. Reduction in intensity of fluorescent blue spot of AFB_1 (at 365 nm) with increasing HSEO concentration.

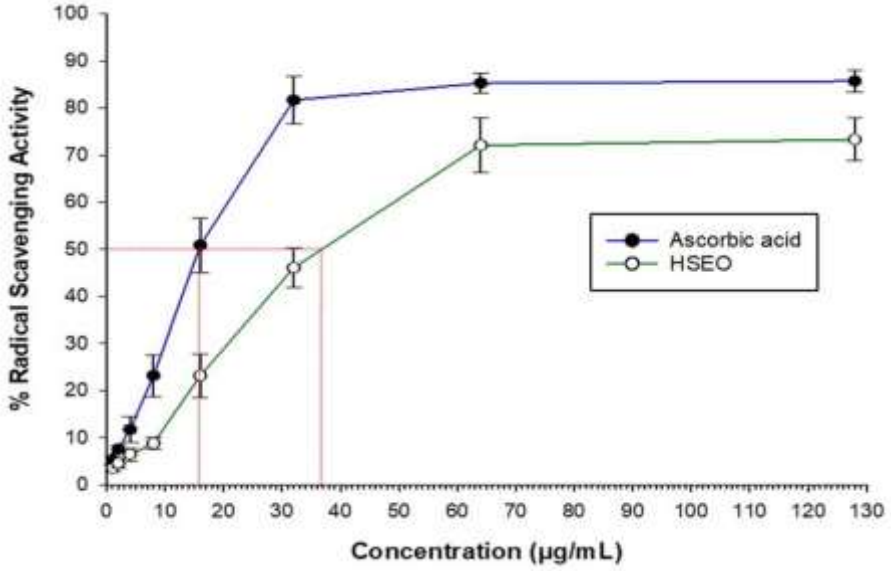

Figure 3. Comparative Free radical scavenging activity of HSEO with ascorbic acid using DPPH assay.

The chemical constituents of HSEO were analyzed by GC-MS to determine the presence of major and minor bioactive compounds associated with their biological activities.The GC-MS analysis of HSEO showed 37 considerable peaks comprising 98.09 % identified compounds. HSEO exhibited eucalyptol (51.16%) as major constituent followed by sabinene (11.72%), α-cedrene (6.59%), limonene (5.71%), β-pinene (3.91%) and β-Caryophyllene (3.48%). Several common bioactive constituents like camphene, α-pinene, myrcene, α-terpinene,

methyleugenol, α-thujeneetc, were found in very low quantity or in traces (Table 3; Figure 4). The composition showed resemblance with earlier reports where 1,8-cineole (eucalyptol) was reported as major of HSEO [28,43]. Whereas, variation in major components is also reported in earlier findings as sabinene [44-46], β-Caryophyllene [47] etc. The variation in EO composition depends on plant age, plant part, geographic distribution and method of extraction [48,49]. The biological activity of EO not only depends on major compounds but also regulated by interaction among major and minor components [50]. The variation in chemical composition significantly influences a range of biological activity of EOs [51].

Table 3. Chemical composition of HSEO determined by GC-MS analysis.

Retention Time (RT)	**Compounds**	**Percentage (%)**
3.11	Camphene	0.05
3.69	Sabinene	11.72
3.75	β-Pinene	3.91
4.16	β-Myrcene	0.38
4.53	α-Thujene	0.49
4.72	α-Pinene	0.34
4.94	α-Terpinolene	0.76
5.21	o-Cymene	1.17
5.32	β-Caryophyllene	3.48
5.42	Eucalyptol	51.16
6.04	α-Terpinene	1.24
6.23	Isodihydrocarvone	0.57
6.58	α-Cedrene	6.59
6.72	Dihydrocarvone	0.42
6.96	endo-Borneol	0.32
7.07	α-Phellandrene	0.18
7.31	Isopinocampheol	0.19
7.36	Cubenol	0.31
7.62	Borneol	0.11
7.74	4-Terpinenol	1.62
7.84	m-Cymenol	0.29
7.89	Carenol	0.24
8.07	Methyleugenol	0.23
9.29	δ-Cadinene	0.05
9.82	Limonene	5.71
9.91	α-Bergamotene	0.98
10.03	β-Farnesene	0.07
10.06	Humulene	0.68

10.24	Germacrene D	0.16
10.34	β-Cyclogermacrane	0.97
10.91	Caryophyllene oxide	0.72
13.24	Isopimarol	1.03
13.28	Falcarinol	0.09
13.43	Pulegone	1.12
13.49	Cedrol	0.26
13.57	Abietadiene	0.32
14.62	Manool	0.16

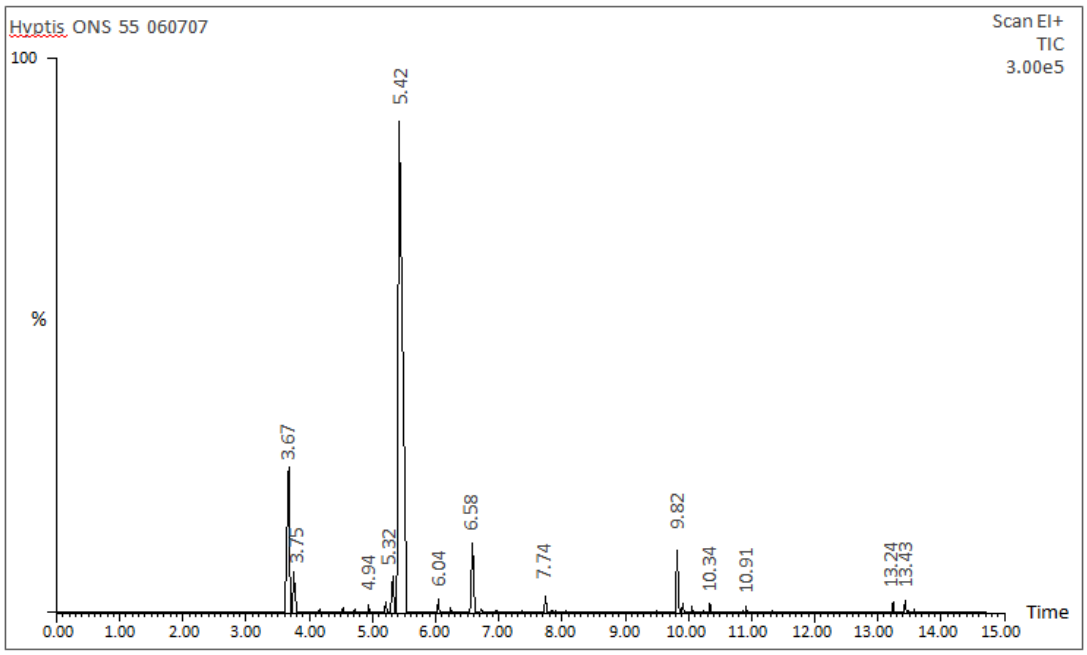

Figure 4. GC-MS chromatogram of HSEO showing peaks of some major compounds at their retention time.

5.0. Conclusion and Future Recommendations

The present study revealed that the investigated HSEO possesses a complex mixture of biologically active volatile components, responsible for its significant fungitoxic and antioxidant activity. GC-MS analysis of HSEO showed the presence of eucalyptol as major component which may interact with other minor components, responsible for its bioefficacy. HSEO exhibited a strong antifungal and antiaflatoxigenic activity against a toxigenic isolate *Aspergillus flavus* DDUOS-06. Furthermore, its potent antioxidant activity indicating its ability to scavenge free radicals. Such multifunctional properties of HSEO suggest that it can be a promising plant-based alternative of synthetic fungicides and chemical preservatives.

- The following recommendations are made for future research to further investigate the potential of HSEO as a natural plant-based preservative or pesticidal agent in light of the current investigation's findings:

- To validate the efficacy of the HSEO under real storage conditions using several agricultural products to determine its practical effectiveness against fungal deterioration and aflatoxin contamination.

- To investigate the spectrum of antimicrobial activity against a wider range of food-borne fungi, phytopathogenic fungi, spoilage microorganisms, and mycotoxin-producing species to broaden its potential applications.
- To isolate and characterize the major bioactive constituents of the HSEO and evaluate their individual and synergistic antifungal, antiaflatoxigenic, and antioxidant activities to identify the principal active compounds.
- To develop novel delivery systems to enhance the stability, solubility, controlled release, and efficacy of the essential oil.
- To elucidate the molecular mechanism of action of HSEO by investigating its effects on fungal cell components and the expression of genes involved in aflatoxin biosynthesis.
- To conduct comprehensive toxicological and safety evaluations to establish safe application limits of HSEO for food and pharmaceutical uses.

Declarations

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Competing Interests Statement

The authors declare that they have no competing interests related to this work.

Consent for Publication

The authors declare that they approved this study for publication.

Authors' Contributions

All the authors contributed in the Conception and Design of the work, Data collection, Drafting the article, and Critical revision of the article.

Availability of Data and Materials

Supplementary information is available from the authors upon reasonable request.

Institutional Review Board Statement

Not applicable in this study.

Ethical Approval

Not applicable in this study.

Informed consent

Not applicable in this study.

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