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ORIGINAL PAPER

Effectiveness of Nano-emulsions and Essential Oil of Fennel and their Major Components against *Botrytis cinerea*

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ABSTRACT
This investigation aimed to identify the fungal pathogen responsible for gray mold in immature cucumber fruits and evaluate the influences of fennel essential oil (FEO) and nano-emulsion (FEO-NE) on *Botrytis cinerea* Pers: Fr., the causal agent of gray mold. *Botrytis cinerea* was identified as the fungal isolate through molecular characterizations obtained from sequencing of Internal Transcribed Space (ITS). The nucleotide sequence of fungi was registered in Gene Bank under accession number PP758474. Ultra-sonication based nano-emulsions containing fennel essential oil can act as antifungal agents, specifically for the production of plant-derived emulsions. Therefore, the aims of this study were to evaluate the efficacy of fennel nano-emulsion (FEO-NE) and essential oil (FEO) on the mycelial linear growth of *B. cinerea* at concentrations of 500, 250, 125, 65, and 30 µg ml^{-1}, compared to essential oil. The FEO-NE was the most effective in reducing the mycelial linear growth of *B. cinerea* isolate at a concentration of 30 µg ml^{-1}. The reduction rate in mycelial linear growth of fungi using FEO-NE was 13.87% and at the same concentration, FEO was 1.85% compared to the control treatment. *In vitro* the results were consistent with the *in vivo* results, showing that FEO-NE was better than FEO in terms of disease severity on fruits, with a ratio of 0.55 for FEO-NE and 0.83 for FEO compared to the control treatment. The essential oil of fennel was identified using Gas Chromatography-Mass Spectrometry (GC–MS); the main compounds identified were Fenchone (8.31%), Benzene (35.22%), Estragole (4.86%), Anethole (37.85%), 2-pentanone (25.21%), 11-Tricosene (26.58%), 2-Pentanone (5.76%), and Octadecanal (38.26%). Plentiful other compounds were also identified in trace quantities. Fennel nano-emulsion is a more effective option when applied as a foliar spray for controlling gray mold on cucumber fruits under greenhouses conditions without phytotoxicity to growing cucumber plants and be able to use as an environmentally friendly bio-cide.

Keywords: Fennel plants, cucumber, gray mold, environmentally friendly, GC-MS.

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1. INTRODUCTION

Cucumber is one of the most important vegetable crops grown in Egypt, which belongs to the family Cucurbitaceae. It is considered that cucumbers originated from South Asia, specifically India, but are currently grown all over the world and throughout the whole year (Ben-Shalom *et al*., 2003). Many diseases severely affect cucumber,causing significant production losses and reduced quality and yield (Khatab *et al*., 2016). Cucumber fruits are a valuable food that is rich in vitamins, antioxidants, minerals, and medical products. *B. cinerea* is one of the most epidemic fungal diseases causing significant fruits losses during the growth of cucumber plants in winter, causing gray mold in greenhouses (Kumar *et al*., 2010). Gray mold symptoms range from yellowish to dark brown discoloration, significantly affecting fruits yield quantity and quality (Soliman *et al*., 2015 and Ziedan *et al*., 2022). Fungal identification is often achieved through Internal Transcribed Spacer (ITS) ribosomal RNA (rRNA) sequencing, commonly used in amplicon sequencing methods. The ITS1

region of the rRNA cistron is a frequently used DNA marker for recognizing fungal species (Ziedan *et al*., 2018b, 2022). Fennel (*Foeniculum vulgare* L.) essential oils (EOs) exhibit diverse biological functions and have been utilized as anti-microbials and antifungals (Seow *et al*., 2013). Fennel is considered one of the most significant medicinal and aromatic plants, with its ripe seeds and oils used in many food products such as bread, warm drinks, pastries, and cheese, as well as in the manufacture of cosmetics and medicinal products (Piccaglia and Marotti, 2001; Mohammad and Amene, 2011 and Mohamed *et al*., 2022). The frequent use of chemical fungal pesticides severely harms human health, leading us to develop environmentally clean substitutes to perform the same function as chemical pesticides and reduce the adverse impact on the environment and humans (Dihazi *et al*., 2011and Mohammadi and Aminifard, 2013). Fennel plants are valuable sources of secondary metabolites, containing powerful properties that can fight against various types of harmful fungi and potentially trigger natural defense mechanisms (Pusztahelyi *et al*., 2015). These natural substances are environment-tally friendly, promote sustainable methods for controlling diseases and have a low risk of harming non-target organisms. According to Roby *et al*. (2013), fennel essential oils were found to have varying levels of impact on the growth of microorganisms. According to Bakkali *et al*. (2008) and Calo *et al.* (2015), EOs often comprise 20–60 different compounds, including flavonoids, alcohol, terpenoids, alkaloids, polyphenols, aldehydes, fat-soluble pigments, and esters. A stable colloidal system of nano-metric droplets with diameters ranging from 5 to 100 nm is referred to as a nano-emulsion (Mou *et al*., 2008). Because of the droplets' sub-cellular size and increased bio-availability due to their nan-ometric size, EO-NE skillfully penetrates bacteria (Ferreira *et al*., 2010). Hence, the objective of the present study is to prepare EO-NE to analyze its anti-pathogenic efficacy and biological activity, due to the hazardous effects of fungicides on edible plant parts, environmental pollution, and resistant fungal strains (Bakkali *et al*., 2008; Kadoglidou *et al*., 2020 and Ziedan *et al*., 2020, 2022).

2. Materials and Methods

The present study is a collaborative effort between El-Sbaheya Horticultural Research Station (SHRS), Horticulture Research Institute (HRI), and Seed Pathology Department, Plant Pathology Research Institute, Agricultural Research Center (ARC), Alexandria, Egypt.

2.1. Molecular characterization of genomic DNA from fungal isolate:

One fungal isolate of *Botrytis* sp., was obtained from the Seed Pathology Laboratory, Plant Pathology Research Institute, Agricultural Research Center (ARC), Alexandria, Egypt. *Botrytis* isolate was grown on potato dextrose agar (PDA) medium to identify according to the morphological and cultural characteristics, and to extract genomic DNA from the isolate, a quick micro preparation process was used (Mohamed and Gomaa, 2019 and Shakam *et al*., 2022). The ITS DNA region of this isolate was amplified via PCR using universal primers. One isolate's amplified ITS1-5.8s and ITS2 regions (500–700 bp) of selected *Botrytis* sp., was sent for sequencing (Macrogen, Scientific Services Company, Korea) (Kumar *et al*., 2016). The sequences were contrasted with GenBank sequences (http://www.ncbi.nlm.nih.gov) using a BLAST search on the National Center for Biotechnology Information (NCBI). The sequences used in the present study were submitted to GenBank.

2.2. Plant material:

The fennel cultivar used in this study was kindly provided by the Vegetables,

Aromatic and Medicinal Plant Breeding Research Dept., Horticultural Research Institute (HRI). Fennel seeds were sown at the El-Sbaheya Hort. Res. Stat., Alexandria and usual agricultural carried out until obtaining fully ripened seeds.

2.3. Extraction of essential oil:

Fennel essential oil (FEO) was extracted from fennel seeds by hydro-distillation using a Clevenger-type apparatus. The extracted oil was dried over anhydrous sodium sulfate and stored at 4°C until used in tests and analyses, according to Bettaieb *et al*. (2011).

2.4. Nano-emulsion preparation:

Fennel nano-emulsion (FEO-NE) was extracted according to the protocol described in previous research (Gomaa and Gomaa, 2022) at City of Scientific Research and Technological applications, New Borg El-Arab City, Alexandria governorate. Fennel essential oil nano-emulsion (FEO-NE) was prepared on the day of preparation, and its droplet diameter (ZAve) and polydispersity index (PDI) were determined in accordance with previous instructions from Hassanin *et al*. (2017). Through Transmission Electron Microscopy (TEM), the characterization of FEO-NE was accomplished at the Faculty of Science, Alexandria University, Egypt. 20 µl of diluted samples were held for 10 minutes in a 200-mesh film-wrapped copper specimen grid. The last step was removing any leftover fluids using filter paper. The grid was stained for three minutes after being exposed to a drop of 3% phosphotungstic acid (Saloko *et al*., 2013). Lastly, a TEM microscope (JOEL JEM-1400 Flash Electron Microscope USA) was used to investigate the grid.

2.5. Antifungal activity *in vitro*

Fennel essential oil nano-emulsion (FEO-NE) and FEO were tested *in vitro* for their anti-fungal activity against *Botrytis* sp., The FEO and FEO-NE were prepared a series of two fold dilutions ranging from 30 to 500 µg ml^{-1} by diluting the oil in 10% di-methyl sulfoxide (DMSO) with the addition of a few drops of Tween-80 (Mansour *et al*., 2020; Mohamed *et al*. 2020a, 2020b and Shakam *et al*., 2022) were compared to referenced fungicide viz., Topsin-M 70 WP (thiophanate methyl) was also tested at the recommended dosage (gm/L) for antifungal activity using the poisoned food technique (Gupta *et al*., 2015). The anti-fungal activity of the FEO and FEO-NE were assessed against *Botrytis* sp., isolate by linear growth. The isolate was cultivated on PDA medium, then a cork-borer was used to extract a 6-mm culture disk from actively grown cultures, which was then placed in the center of Petri dishes with different concentrations. The Petri dishes were left in a 28°C incubator for 7 days (Kottearachchi *et al*., 2012; Mohamed *et al.* 2022). PDA Petri dishes without FEO-NE or FEO served as control, each treatment was repeated three times. The linear growth of fungal mycelia was measured when the pathogen in the control plates completely covered the surface. Fungal growth inhibition (FGI %) of the tested fungi was calculated with the following formula according to Ziedan *et al*., 2018a, Mohamed *et al*., 2020a and Hassan *et al*., 2021):

Fungal growth inhibition % (FGI %) =

[(Growth in control - Growth in treatment) / Growth in control] × 100.

2.6. GC–MS analysis of essential oils:

The phytochemicals profile of the extracted EOs from fennel was determined using the Trace GC Ultra-ISQ mass spectrometer (Thermo Scientific, Austin, TX, USA). GC–MS analysis was extracted as per protocol described in previous research (Abd-ElSalam *et al*. 2015; Okla *et al*. 2019; Abd-Elkader *et al*. 2021 and Ali *et al*. 2021).

2.7. Antifungal activity *in vivo*

The experiment was carried out in December 2023 at El-Sbaheya Horticulture Research Station in Alexandria, Egypt, to examine the antifungal activity of FEO-NE and FEO on

the infection by *Botrytis* sp., of cucumber cv., Prince under greenhouse conditions. The artificial inoculation with conidial suspension with 10^8 spores ml^{-1} of *Botrytis* sp. was done (El-Hefny *et al*., 2023 and Hassanin *et al*., 2018). Cucumber plants, 35 days old, were sprayed with the suspension and incubated for 24 hours. Each experimental plot was divided of cucumber cv. Prince by *Botrytis* sp. into 3 replicates and sprayed 2 times, at 7 and 14 days. The plants were sprayed using 500 ml for each treatment of FEO-NE and FEO at the concentration 500 µg ml^{-1}, while the commercial fungicide Topsin-M 70 WP was sprayed at the recommended dose (one gm/L). This experiment was carried out in a randomized complete block design and comprised of four treatments:

1- Control treatment.
2- Fennel essential oil (FEO).
3- Fennel nano-emulsion (FEO-NE).
4- Treatment with the commercial fungicide Topsin-M 70 WP.

2.8. Disease assessment

The small infected or healthy cucumber fruits were, randomly, collected and evaluated 15 days after foliar spraying as percentage of diseased fruits and disease severity using the symptoms scale that was given by Ziedan *et al*. (2018a, b) as following in Table (1). The diseased fruits equation was as follows:

Diseased fruits (%) = N / T ×100.

Where;

N: number of diseased fruits, T: total number of fruits.

Table 1: Disease severity scale of gray mold disease on cucumber fruits

Scale	Percentage of fruit infected by Gray mold	Symptom
0	0	No symptoms of infection
1	50	Yellowish
2	100	Yellowish
3	50	gray mold
4	100	gray mold

2. 9. Statistical analysis:

Data were analyzed using the appropriate methods of statistical analysis of variance (ANOVA), as described by Snedecor and Cochran (1989). A statistical test was administered to compare the treatment means using the L.S.D. test procedure at a probability level of $p \leq 0.05$.

3. Results

3.1. Pathogenic fungus identification:

One fungal isolate of *Botrytis* sp. was obtained from Seed Pathology Laboratory, Plant Pathol. Res. Inst. Agric. Res. Cen. (ARC), Alexandria, Egypt. The isolate was also identified as *Botrytis cinerea* Pers. by ITS sequence analysis, and the nucleotide sequence was submitted to GenBank under the accession number PP758474. The sequence analysis revealed a 99% identity with *B. cinerea* isolate, confirming the fungal pathogen.

3.2. Characterization of essential oil and nano-emulsion:

The essential oil extracted from fennel seeds was light yellow, with a pleasant aroma and a yield of 1.9% (v/w). The GC-MS analysis identified 11 major compounds in fennel essential oil (FEO), with Anethole (37.85%) being the most abundant, followed by Octadecanal (38.26%), Fenchone (8.31%), Benzene (35.22%), Estragole (4.86%), and 2-Pentanone (5.76%), as shown in Table (2).

3.3. Effect of FEO and FEO-NE *in vitro* on growth of *B. cinerea:*

Fennel essential oil nano-emulsion was prepared on the day of preparation. Particle sizes ranged from 54.76 to 92.00 nm, (Fig. 1, A and B). FEO-NE particles

that were characterized by Transmission Electron Microscopy (TEM) were white and looked to be spherical with a relatively mono-shape. The droplet width in Fig (2) was approximately 16.75 nm. The Z-average of FEO-NE droplet diameter was 1665 nm, and the polydispersity index (PDI) was 1.000. The growth of *B. cinerea* was evaluated on agar medium amended with FEO and FEO-NE at concentrations 500, 250, 125, 65 and 30 µg ml^{-1} of fennel (Fig. 3).

Data presented in Table (3) indicated that fennel essential oil nano-emulsion displayed significant anti-fungal activity against the growth of *B. cinerea* compared to fennel essential oil (FEO). The FEO-NE was most effective in inhibiting the linear growth of *B. cinerea* isolate. At the concentration of 30 µg/ml, FEO-NE reduced the mycelial growth by 13.87%, while FEO reduced it by 1.85% compared to the control treatment but both FEO-NE and FEO were able to completely eliminate fungal growth at the concentration of 500 µg ml^{-1}.

Table 2. Chemical composition (%) of fennel essential oil by using GC-MS

Compound name	Percentage in oil (%)
Fenchone	8.31*
Camphor	0.82
Estragole	4.86
Benzene	35.42
Fenchyl acetate	1.93
Anethole	37.85
Caryophyllene	2.95
Humulene	1.13
Caryophyllene oxide	2.06
Baimuxinal	0.75
2-pentanone	25.21
benzaldehyde-4-methoxy	2.06
2-Pentanone	5.76
Octadecanal	38.26
11-Tricosene	26.58
Sabinene	9.54

*Values are relative percentage (RSI: Reverse Standard index- SI: Standard Index).

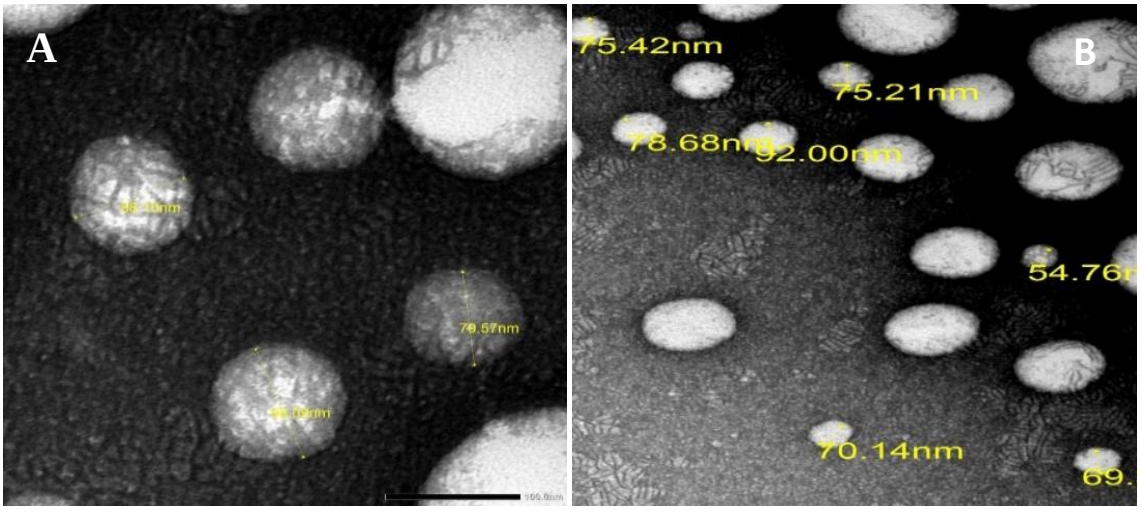

Fig. 1: Transmission Electron Microscope (TEM) characterization of FEO-NE using ultrasonication method for 30 min., with two magnifying powers; (A) 100 nm and (B) 200 nm.

3.4. Effect of FEO and FEO-NE *in vivo*:

Under greenhouse conditions, FEO-NE was more effective than FEO in reducing gray mold disease severity on cucumber fruits. FEO-NE showed that disease severity ratio was 0.55 compared to 0.83 at FEO, while the commercial fungicide Topsin-M 70 WP was 1.063 and in the control was 3.763 (Table 4).

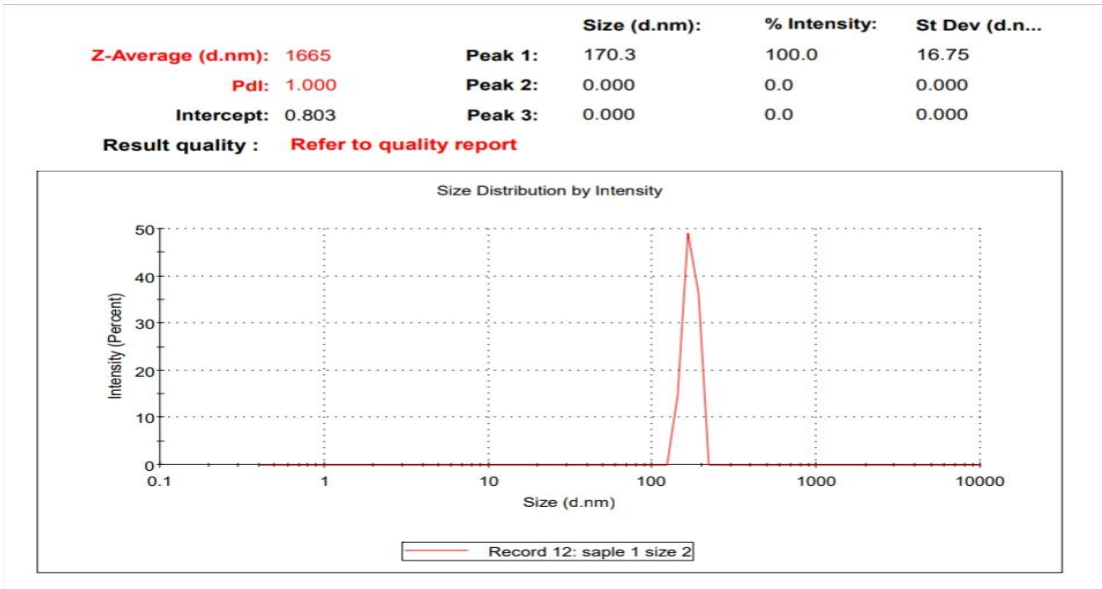

Fig 2: FEO-NE particle size was created using a 30-minute ultra-sonication process.

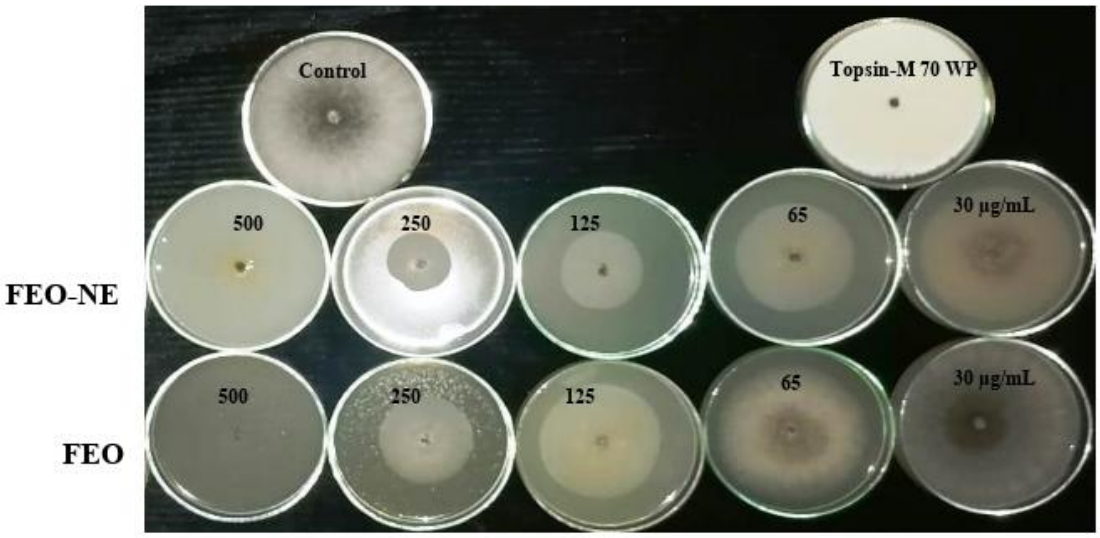

Fig. 3: Antifungal bioassay FEO and FEO-NE against the mycelial growth of *B. cinerea* at the concentrations of, 30, 65, 125, 250 and 500 µg/mL compared to the control treatment and commercial fungicide.

Table 3: Effect of FEO and FEO-NE on the Reduction (%) of *Botrytis cinerea* linear growth

Concentration (µg mL^{-1})	FEO	FEO-NE
DMSO (Control)	0.0^{h*}	0.0^{h}
30	1.85^{h}	13.87^{g}
65	10.74^{g}	33.332^{e}
125	20.17^{f}	50.37^{c}
250	42.2^{d}	64.07^{b}
500	100^{a}	100^{a}
Topsin-M 70 WP	96.67^{a}	96.67^{a}

*Values in each column followed by the same letter are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test. (LSD 0.05 = 5.145)

Table 4: Effect of FEO and FEO-NE on infection of cucumber fruits by gray mold under greenhouse conditions.

Treatment	Diseased fruits (%)	Disease severity on cucumber fruits (%)
Control	26.23^{a}	3.763^{a*}
FEO-NE	2.79^{b}	0.55^{c}
FEO	3.86^{b}	0.83^{bc}
Topsin-M 70 WP	5.57^{b}	1.063^{b}

* Values in each column followed by the same letter are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test. **LSD 0.05 = 0.2962**

Fig. 4: Effect of FEO and FEO-NE on cucumber fruits infection by gray mold under greenhouse conditions: no symptoms of infection (0), 50% yellowish (1), 100% yellowish (2), 50% gray mold (3) and 100% gray mold (4).

4. DISCUSSION

Botrytis cinerea is responsible for the epidemic distribution of cucumber fruit gray mold during winter season under plastic greenhouses, which significantly increases losses in developing cucumber fruits. Moreover, (An and Ma, 2005-2006; Al-Sadi *et al*. 2011; Elad *et al*. 2016;

Ziedan *et al*. 2018a, b; 2022). In the present study, *Botrytis* sp. isolate was identified DNA from the isolate was amplified with the internal transcribed spacer (ITS) primers. In addition, the (ITS) region was sequenced to study the diversity of the isolate of *Botrytis*. The sequence under comparison bears similarities to the data collected by Ziedan *et al*. (2018b). It was compared to those of *Botrytis* species available in the GenBank database (www.ncbi.nlm.nih.gov). Accordingly, the fungus was identified as *Botrytis cinerea* Pers.

Fennel essential oil and nano-emulsions were evaluated *in vitro* against the linear growth of isolated *B. cinerea* mycelia, with large amounts of crude and essential oil nano-emulsions, which is consistent with the results of Moustafa and Abd Elwahab 2016; Ziedan *et al*., 2022; Gomaa and Gomaa, 2022. Due to its numerous chemical components, EOs' biological activity against fungi, bacteria, and weeds is made up of various chemical, physical, and biological characteristics. The majority of EOs' effects on weeds and microbes come from their bio-active volatile components, which block the production of chitin and glucan cell wall building enzymes and increase plasma membrane permeability. Highly penetrating EOs break down chemical components, damage mitochondrial and cytoplasmic membranes, and cause death of cells in bacteria and fungi (Viuda-Martos *et al*. 2007; Hua *et al*., 2017; Lagrouh *et al*., 2017; Bouyaha *et al*., 2019). As a result, the EOs of oregano and fennel alter the morphology of fungal mycelia, produce cytoplasmic co-agulations and lysis, and alter *Sclerotinia sclerotiorum* forms sclerotia (Soylu *et al*., 2007) according to its morphological and cultural characters. Eugenol had a detrimental impact on the structure of the plasma membrane, the shape of the hyphae, the accumulation of cytoplasm, and the number of cell vacuoles, all of which significantly decreased the mycelial linear growth of the fungus *B. cinerea* (Wang *et al*., 2010). According to earlier explanations by Abd El-Kareem *et al*. (2016), Alam *et al*. (2019) and Mohamed *et al*. (2020a, b), FEO was examined using GC-MS. Results showed that the chemical composition of fennel was Fenchone (8.31%), Benzene (35.22%), Estragole (4.86%), Anethole (37.85%), 2-pentanone (25.21%), 11-Tricosene (26.58%), 2-Pentanone (5.76%) and Octadecanal (38.26%). Results are in agreement with the previous studies recorded by Alam *et al*. (2019) who stated that, 2-pentanone, fenchone and benzaldehyde-4-methoxywere the major compo-nents of FEO.

5. CONCLUSION

Results of the present study show that fennel essential oil nano-emulsion (FEO-NE) is more effective than Fennel essential oil (FEO) in inhibiting the growth of *B. cinerea* and reducing disease severity in cucumber fruits under greenhouse conditions. FEO-NE presents a promising, safe, and environmentally friendly alternative to chemical fungicides for controlling gray mold in greenhouse production cucumber. Further researches are recommended to refine the formulation and assess the long-term effects of FEO-NE application on plant health and productivity. Enzyme quantify-cation will be given attention in future research.

CONFLICTS OF INTEREST

The author (s) declare no conflict of interest.

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