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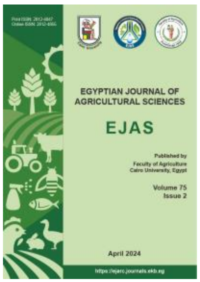

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Antifungal Activity of Thyme and Clove Essential Oil Nanoemulsions against Pothos Root Rot

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ABSTRACT

Fungi, including *Rhizoctonia solani* and *Sclerotium rolfsii,* pose a significant threat to agricultural productivity, causing substantial crop yield and quality losses. The excessive use of conventional fungicides raises concerns regarding their environmental impact and human health. This study explores the antifungal potential of thyme and clove oil nanoemulsions as a sustainable alternative. Nanoemulsions were prepared using high-energy ultrasonication and exhibited remarkable stability, with homogenous particle size distributions and low polydispersity index values. *In vitro* assays demonstrated complete fungal growth inhibition at 3000 ppm for both oils. Pot bioassays revealed significant antifungal activity, with thyme oil nanoemulsion reducing *R. solani* and *S. rolfsii* root rot by 75% and 50%, respectively. Clove oil nanoemulsion also effectively suppressed these pathogens by 41.67% and 25%, respectively. Thyme and clove oil nanoemulsions treatments significantly stimulated root growth under fungal challenge. In this regard, plants treated with thyme oil nanoemulsion showed remarkable increases in root length (4.61-fold and 11.18-fold), fresh weight (28.59-fold and 45.62-fold), and dry weight (48.29 and 48.43-fold) when exposed to *R. solani* and *S. rolfsii* infection, respectively, compared to untreated control. Clove oil nanoemulsion also significantly enhanced these root attributes, with remarkable increases in length (1.87-fold for *R. solani* and 4.12-fold for *S. rolfsii*), fresh weight (11.09-fold for *R. solani* and 21.31-fold for *S. rolfsii*), and dry weight (25.14-fold for *R. solani* and 16-fold for *S. rolfsii*). Molecular modeling studies supported the observed antifungal efficacy. In conclusion, thyme and clove essential oil nanoemulsions are promising candidates that act as natural fungicides for managing root rot diseases.

Keywords: *Essential oil; docking; Rhizoctonia solani; Sclerotium rolfsii; mode of action*

1. INTRODUCTION

Plant growth and development are threatened by various organisms, including fungi, bacteria, and viruses. Fungal diseases account for roughly 70–80% of all plant diseases (Peng *et al.*, 2021). Among these fungi, *Rhizoctonia solani* and *Sclerotium rolfsii,* persist as vegetative mycelium and/or sclerotia and cause uncountable numbers of diseases in cultivated plants (Elsharkawy *et al.*, 2022). Important crops threatened by *R. solani* and *S. rolfsii* include the tropical creeper golden pothos, *Epipremnum aureum* (Norman and Ali, 2018). This plant is grown commercially worldwide for ornamental purposes as a garden and houseplant.

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Increased use of fungicides in controlling plant diseases has led to several environmental issues, including the growth of resistant weed and pathogen populations, soil compaction and water pollution, which negatively influence agricultural sustainability and human health (Peng *et al.*, 2021). As a result, it is crucial to discover new fungicides that are mostly plant-derived, such as essential oils (EO), to control plant diseases (Zheng *et al.*, 2022).

Essential oils (EOs) are aromatic, plant-based, hydrophobic liquids containing many bioactive compounds (Masyita *et al*., 2022). Essential oils are currently used as green chemicals in the agricultural sector, and their application is extensively documented (Fierascu *et al.*, 2020). For example, thyme and lemongrass have great antifungal activity against *Alternaria linariae* (Saltos-Rezabala *et al.*, 2022). Furthermore, Mohammad *et al.* (2020) reported that thyme oil has a potent antifungal property against *Drechslera spicifera*, *Fusarium oxysporum* f. sp. *ciceris*, and *Macrophomina phaseolina*. Also, Zhang *et al.* (2022) reported that *Sabina chinensis* essential oil exhibited potent antifungal activity against *F. oxysporum* and *F. incarnatum*. However, a number of concerns about the high volatility, degradability, photosensitivity, destabilization, and wettability of essential oils restrict their use in the field (Song *et al.*, 2022). For this reason, some studies have looked into adding EOs to suitable delivery systems to increase their water solubility and antifungal effectiveness (Reis *et al.*, 2022).

Essential oil nanoemulsions are gaining popularity among delivery systems due to their advantages over EOs applied directly in bulk phase, including their superiority in terms of environmental friendliness, solubility, biodegradability, bioavailability, and physical stability. They can also increase EOs' antifungal activity (Perumal *et al*., 2021 and Perumal *et al*. 2022). Hence, plant oil-based nano-emulsion is one method for overcoming the essential oil disadvantage and improving product efficiency by incorporating nanotechnology into the formulation (Zaudin *et al.,* 2022).

Few studies are known for the antifungal activity of EO nano-emulsions, as they have not received as much attention as studies on bacteria. However, in the recent decades, particular studies demonstrated the antifungal effectiveness of EO nano-emulsions against some foodborne fungal strains (Maurya *et al.*, 2021). Also, some recent studies demonstrated that nanoemulsions like eugenol (Abd-Elsalam *et al.*, 2015), tea tree (de Souza Silveira Valente *et al.*, 2016), neem and citronella (Ali *et al.*, 2017) and peppermint (Pandey *et al*., 2020) have strong antifungal properties against different phytopathogenic fungi.

The mechanism by which essential oils fight fungi remains unclear (Huang *et al*., 2019). Scientists believe they directly damage the fungal cell wall (Mali *et al*., 2022), disrupt the flow of essential ions such as K+, H+, Ca2+, and Mg2+ (Mali *et al*., 2022), and inhibit the biosynthesis of fungal DNA, RNA, and proteins (Sun *et al*., 2022).

To determine the potential mechanism of action for the antimicrobial effects of essential oil components, numerous studies have undertaken molecular docking (Rawal *et al.,* 2019). In this approach, docking single or multiple small molecules to a receptor site is attempted to find putative ligands (Rawal *et al*., 2019). Some studies have shown that docking algorithms can find ligands and binding at a receptor site close to experimentally determined structures (Chen and Zhi, 2001). These algorithms are expected to be equally applicable to the identification of multiple proteins to which a small molecule can strongly or weakly bind (Chen and Zhi, 2001). Higher negative binding affinities meant that ligands could interact with the enzyme residues, inhibit them, and keep the complexes they had created with their target proteins (Ou-Ani *et al*., 2002).

This study examined the GC-MS/MS analysis of thyme and clove oils as well as the *in vitro* and *in vivo* antifungal effects of their emulsions and nano-emulsions (NEs) phase against *R. solani* and *S. rolfsii* isolated from the rotten roots of the pothos plant. The effects of the identified compounds on several important fungal proteins were examined using molecular docking. The antifungal activity of thyme and clove essential oil-based nano emulsions and their in-silico mode of action against *R. solani* and *S. rolfsii* on pothos plants has been reported for the first time.

2. MATERIALS AND METHODS

2.1. Fungal pathogens culturing

Rhizoctonia solani and *Sclerotium rolfsii* were isolated from the rotten roots of the pothos plant from our previous work (Attia *et al*., 2020). In separate 500 mL flasks with 75 g autoclaved, sterilized barley, 25 g cleaned sand, and 100 mL water, the fungi were cultivated. The inoculated flasks were incubated at 25 °C for two weeks. Clay pots with a diameter of 30 cm that had been formalin-disinfected and were filled with peat moss, clay, and washed sand (1:1:1 v/v) were infected with 3% (w/w) of each pathogen separately. In the week before transplantation, the infected soil was irrigated two to three times (Reyad *et al.,* 2022).

2.2. Thyme and clove essential oils source

Both cold-pressed clove oil (SaEO) and thyme oil (TvEO) were obtained from the Natural Oil Extraction Unit, National Research Center, Giza, Egypt. Tween 80 was purchased from El Nasr Pharmaceutical Chemicals Co., Giza, Egypt.

2.3. GC-MS/MS analysis of clove and thyme essential oils

GC-MS/MS analysis of clove oil and thyme oil was performed at the Cairo University Research Park (CURP), Faculty of Agriculture, Cairo University, Giza, Egypt to identify their major compounds on an Agilent Triple Quad 7000 Series Chromatograph connected to a mass spectrometer (GC-MS/MS) with Elite-5MS (5% diphenyl, 95% dimethyl polysiloxane) in a capillary column (30 m× 0.25 µm ID × 0.25 µm df). Analyses were performed with helium as the carrier gas at a flow rate of 1.0 mL/min and a split ratio of 30:1 with the following temperature program: 110° C for 2 minutes, then 10 ° C/min to 200 °C, then 5 °C/min to 280° C/min, and hold for 9 minutes. The injector and ion-source temperatures were maintained at 250 °C and 200 °C, respectively. Mass spectra were obtained by electron ionization (EI) at 70 eV, using a spectral range of m/z 40–450. A sample was injected into a volume of 1.0 µL. The Wiley spectral library collection and the NSIT library database were used to identify the chemical components of the essential oils (Ezhilan and Neelamegam, 2012).

2.4. Thyme and clove oil emulsions and nano-emulsion preparations

Thyme oil and clove oil were utilized in all emulsion and nano-emulsion (NE) compositions at a concentration of 10% v/v (Marchese *et al.,* 2017). The emulsion was developed by combining the EO and surfactant (Tween 80) in a 2:1 (v/v) ratio before adding them to water. The mixture was divided into two parts. The first part (an oil-based nano-emulsion phase) was sonicated using an ultrasonic homogenizer from Bandelin Sonopuls HD 2200, Germany, with an output power of 700 W. The second part (the oil-based emulsion phase) was not sonicated. Sonicator treated portion formed strong disruption forces that decreased the stubbly emulsion's droplet diameter. An ice reservoir was placed on the sample during the 30-minute sonication-assisted emulsification process to reduce potential energy (Krishnamoorthy *et al*., 2021).

2.5. Characterization of clove and thyme essential oil nanoemulsions

Transmission electron microscopy characterization of thyme and clove oil nanoemulsions was studied by Ebrahim (2021). The nanoemulsions particle size distribution and polydispersity index (PDI) were calculated at the Nanotechnology Laboratory, Regional Center for Food and Feed (ARC, Giza, Egypt) according to Ghotbi *et al*. (2014).

2.6. Antifungal properties of essential oils *in vitro*

Using the poisoned food method, the effects of thyme oil emulsion (TvEO-e), thyme oil nano-emulsion (TvEO-ne), clove oil emulsion (SeEO-e), and clove oil nano-emulsion (SeEO-ne) at 1000, 2000, and 3000 ppm against *R. solani* and *S. rolfsii* were determined. One hundred mL of autoclaved potato dextrose agar (PDA), was mixed with a certain amount of stock solution to produce the required concentrations. A mycelial disk (0.4 cm in diameter) was cut from the margin of each fungus and placed centrally in each dish after the medium had solidified, then incubated at 25 °C. As soon as the plates in any treatment are filled with fungus mycelium, radial growth (mm) is measured. Three replicates were used for each treatment.

2.7. Pot experiments

In all pot experiments, 30-cm-diam. pots filled with soil, sand, and clay (2:1:1) were used for planting. Formalin-disinfested soil was infested with *R. solani* and *S. rolfsii,* each alone at a rate of 3% (w/w). Three replicates were used for each treatment. Before planting, the bases of pothos cuttings were individually soaked in each oil formulation (3000 ppm) and Carbendazim-fungicide (2g/L) for 30 minutes. Four treated cuttings were planted in a pot that was previously infested with the tested fungi. Basal stem parts of cuttings soaked in water were used as control.

Percentages of infection with root rot and basal stem root rot, root length, and fresh and dry weights were measured 60 days after planting.

2.8. Docking study

The major natural compounds (thymol, o-cymene, ɣ-terpinene, carvacrol, Eugenol, Caryophyllene, Eugenyl acetate, and α-Humulene) found in thyme and clove oils were docked with two of the most important enzymes (pectate lyase and cellobiose dehydrogenase) that were expressed mainly in *R. solani* and *S. rolfsii*, respectively by using Molecular Operating Environment (MOE) Software. The targeted proteins modeled and obtained from Uniprot (https://www.uniprot.org).

2.8.1. Preparation of targeted proteins

Protein structures were obtained from Uni Prot and the protein data bank; the complex water molecules were removed. Then, Quick preparation was done, missing amino acids were added, and errors in valence atoms were fixed. The energy of protein peptides was reduced by using CHARMM force fields. The protein essential Amino acids are selected and prepared for screening.

2.8.2. Preparation of natural metabolites

Chem-Bio Draw Ultra17.0 was used to create 2D structures of the tested compounds, which were then stored in SDF file format. Using MOE 2019 software, the saved file was opened, the ligands were protonated, and energy was reduced by using a 0.1 RMSD kcal/mol MMFF94 force field. Following that, the reduced structures were kept for molecular docking (Salih *et al.*, 2022).

2.8.3. Docking process for molecules

Utilizing docking algorithms, molecular docking was performed. The ligands were permitted to be flexible, while the targeted pocket was kept rigid. Each molecule was given twenty different opportunities to interact with the protein during the refining process. Following that, Discovery Studio 2019 Client software produced 3D orientations by recording the docking scores (affinity interaction energy) of the poses that best matched the active sites (Salih *et al.,* 2022).

2.9. Statistical analysis

The results of the agar dilution experiment and the disease incidence determined by the *in vivo* assessment were both subjected to ANOVA analysis. Fisher's Least Significant Difference (LSD) test was used to compare mean diameters when a significant F value was found (P≤0.05). MSTAT software was used to perform the statistical analysis.

3. RESULTS

3.1. GC-MS/MS analysis of thyme and clove essential oils

Data presented in Tables 1 and 2 showed the GC-MS/MS analysis of the tested oils. Fifteen ingredients were found in thyme oil, accounting for 99.98% of the total composition. In general, any chemical compounds that is calculated to make up more than 10% of the total oil is considered a major chemical compound. The most abundant constituent was thymol (40.66%), followed by p-cymene (16.19%) and ɣ-terpinene (14.45%) (Table 1 and Fig. 1). On the other hand, three components were identified in clove oil, accounting for 100% of the total composition (Table 2). Eugenol (65.67%) was the major ingredient, followed by benzyl alcohol (32.37%) (Table 2 and Fig. 2).

Table (1): Composition of thyme essential oil according to the GC-MS/MS analysis.

Peak no.	RT	Compound name	Area sum%
1	7.588	Alpha-phellandrene	1.01
2	7.920	1,1-Dicyclopropyl-2-methyl-1-pentene	0.79
3	9.996	beta-Terpinene	2.67
4	10.526	beta-Pinene	1.44
5	12.731	P-cymene	16.19
6	12.820	Eucalyptol	0.71
7	14.263	gamma-Terpinene	14.45
8	16.051	Linalool	3.96
9	19.093	Terpinen-4-ol	5.04
10	19.761	Estragole	5.9
11	20.691	Thymol methyl ether	1.23
12	24.593	Thymol	40.66
13	24.714	Carvacrol	3.14
14	25.654	Eugenol	0.50
15	27.461	Caryophyllene	2.29

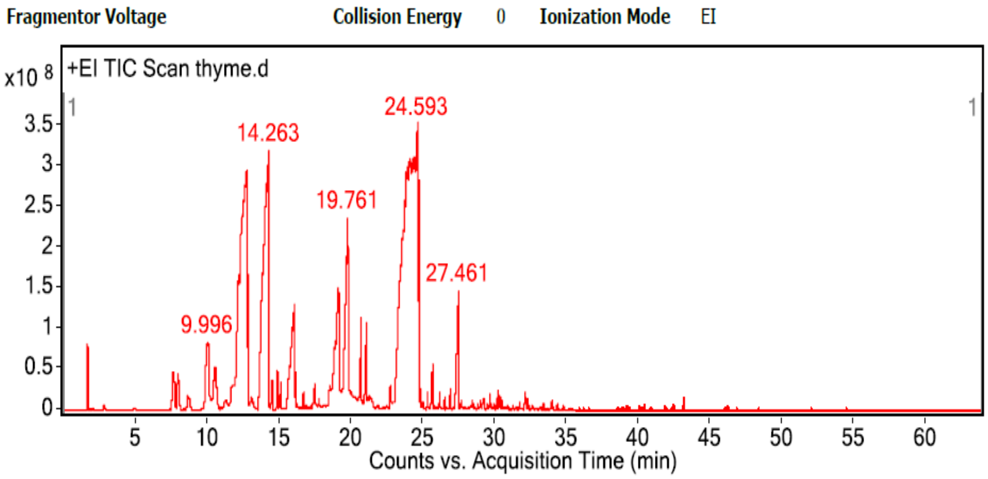


Fig. (1): GC-MS/MS analysis of thyme essential oil.

Table (2): Composition clove essential oil according to the GC-MS/MS analysis.

Peak no.	RT	Compound name	Area sum%
1	16.108	Benzyl alcohol	32.37
2	25.060	Phenol,2-methoxy-4-(1-propenyl)-	1.96
3	26.753	Eugenol	65.67

3.2 Droplet size measurement

Dynamic light scattering (DLS) technology was used to measure the droplet size and dispersion of the thyme and clove oil nano-emulsions. As shown in Fig. 3, the polydispersity index (PI) of thyme and clove oil nanoemulsions were 0.436 and 0.227, respectively, which showed that they are uniform. In addition, the average diameters of both formulations (droplet size) were 94.82 and 73.76 nm, respectively.

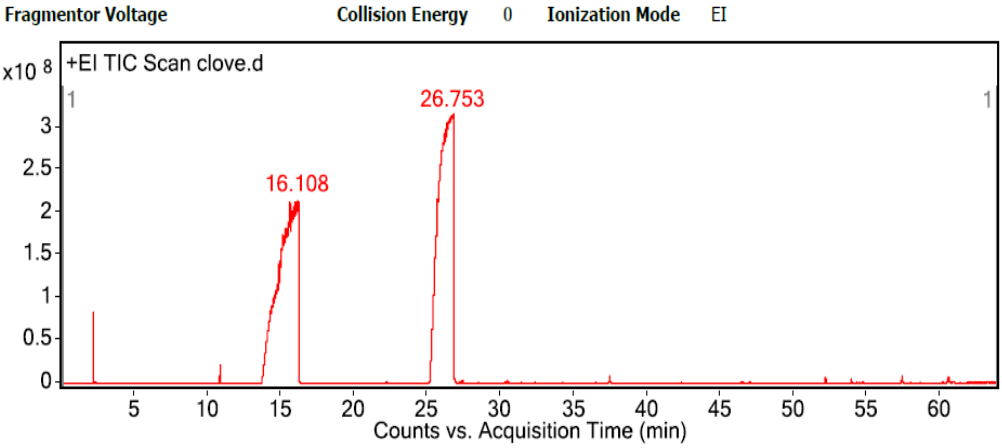
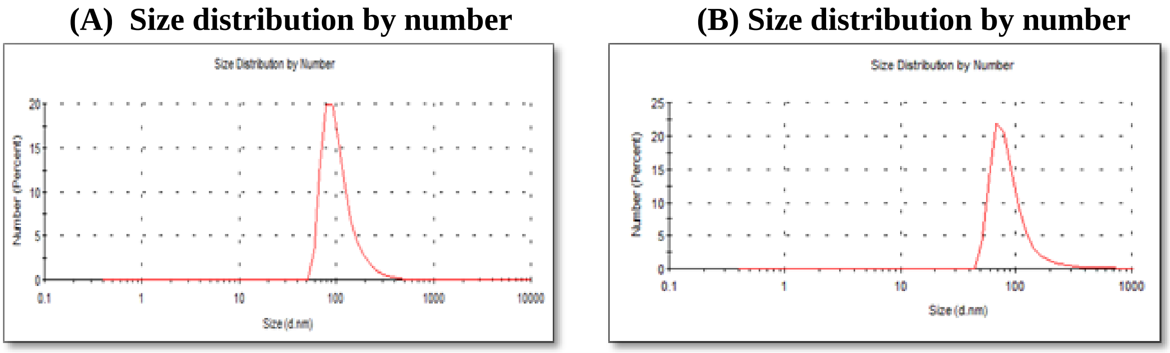


Fig. (2): GC-MS/MS analysis of clove essential oil.


(A) Size distribution by number

(B) Size distribution by number

Fig. (3): Particle size and distribution of thyme (A) and clove (B) oil nano-emulsion obtained by DLS analysis.

3.3 Antifungal properties of the tested essential oils *in vitro*

In vitro antifungal activity of thyme oil emulsion (TvEO-e), thyme oil nano-emulsion (TvEO-ne), clove oil emulsion (SeEO-e), and clove oil nano-emulsion (SeEO-ne) on radial growth of *R. solani* and *S. rolfsii* at 1000, 2000, and 3000 ppm was assessed (Figs 4 and 5). All treatments showed antifungal activity based on these radial growth values. Thyme oil nano-emulsion (TvEO-ne) at 3000 ppm completely inhibits both fungi. Also, SeEO-ne significantly reduced the growth of R. solani and S. rolfsii by 86.11% and 88.83%, respectively compared with the control. In general, essential oils nano-emulsions were more effective than their emulsion phase in reducing the growth of both fungi (Figs. 4 and 5).

3.4. Pot experiments

Rhizoctonia solani and *S. rolfsii* drastically reduced the root length, fresh weight, and dry weight of pothos plants. On the contrary, essential oil-based nano-emulsion and carbendazim-fungicide treatments significantly ($P \leq 0.05$) reduced disease incidence and attenuated the deleterious impact of the two pathogens on these parameters (Fig. 6). Thyme oil nanoemulsion was more effective than clove oil nanoemulsion in reducing the incidence of Rhizoctonia root rot and Sclerotium root rot, as shown in Fig. (6). In this regard, thyme oil nanoemulsion gave the lowest root rot incidence percentages (25 and 50%, respectively), while clove oil nanoemulsion gave the highest values (58.33% and 75%, respectively).

On the other hand, thyme oil-based nano-emulsion was more effective than clove oil-based nano-emulsion in improving the tested plant attributes (root length, fresh weight, and dry weight) as presented in Fig. (6), where thyme oil nano-emulsion significantly ($P \leq 0.05$) improved the root length (8.3 and 8.5 cm), root fresh weight (6.29 and 5.93 g/plant), and root dry weight (3.38 and 3.39 g/plant) of pothos plants under *R. solani* and *S. rolfsii* infection, respectively. Meanwhile, the corresponding values for clove oil nano-emulsion were 3.36 and 3.13 cm for root length, 2.44 and 2.77 g/plant for root fresh weight, and 1.76 and 1.12 g/plant for root dry weight, respectively.

Carbendazim-fungicide showed the best results. It showed the lowest values of disease incidence (8.33% and 16.67%) and exhibited the highest significant improvement in the root length (9.3 and 8.76 cm), root fresh weight (8.67 and 8.29 g/plant), and root dry weight (4.44 and 4.63 g/plant) of pothos plants under infection by *R. solani* and *S. rolfsii*, respectively (Fig. 6).

3.5.1 Pectate lyase

Thymol formed one Pi-Alkyl interaction with Cys174 and additionally interacted with Lys147 and Asp123 by two hydrogen bonds (Fig. 7 a, b). Carvacrol interacted with Lys147 and Asp123 by two hydrogen bonds and with His105 by pi-alkyl interactions (Fig. 7 c, d). Eugenol interacted with

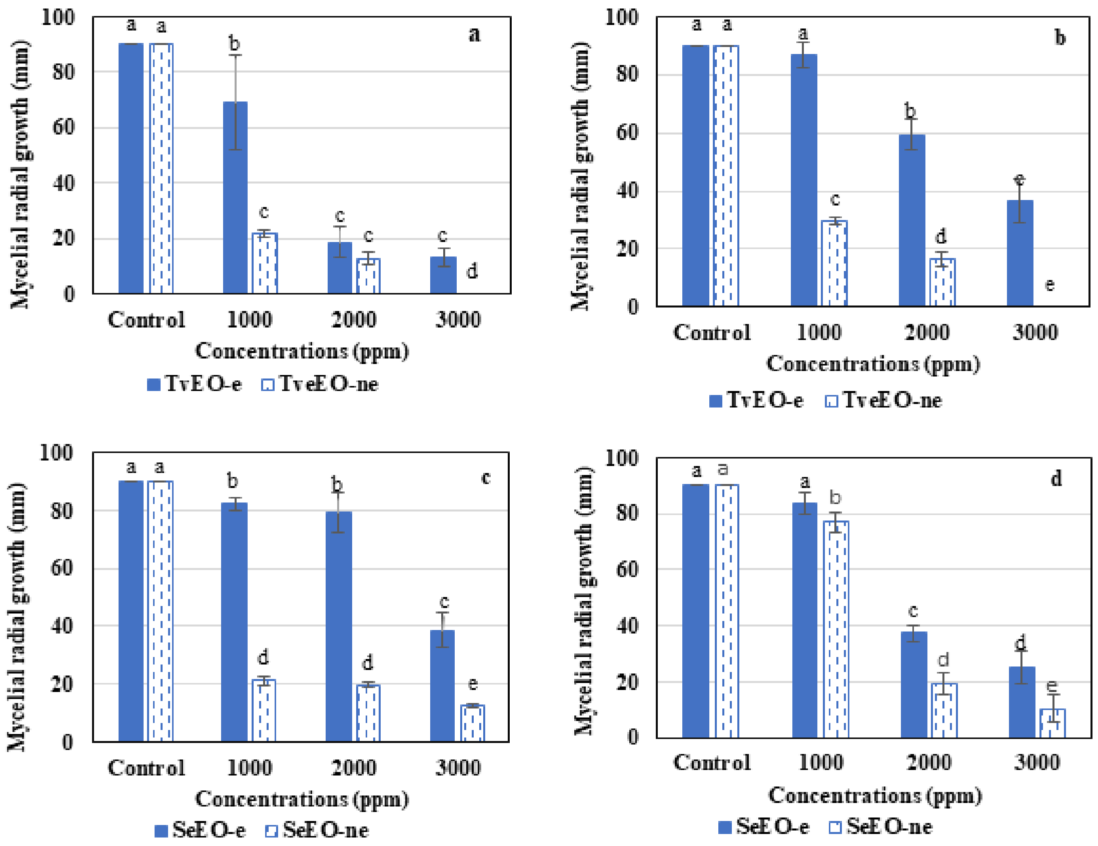


Fig. (4): Mycelial radial growth of *Rhizoctonia solani* (a,c) and *Sclerotium rolfsii* (b,d) with different concentrations of TvEO-e: thyme oil emulsion, TvEO-ne: thyme oil nanoemulsion, SeEO-e: clove oil emulsion and SeEO-ne: clove oil nanoemulsion. Columns with different letters indicate significant differences according to Duncan's test ($p < 0.05$), The vertical bar indicates standard error.

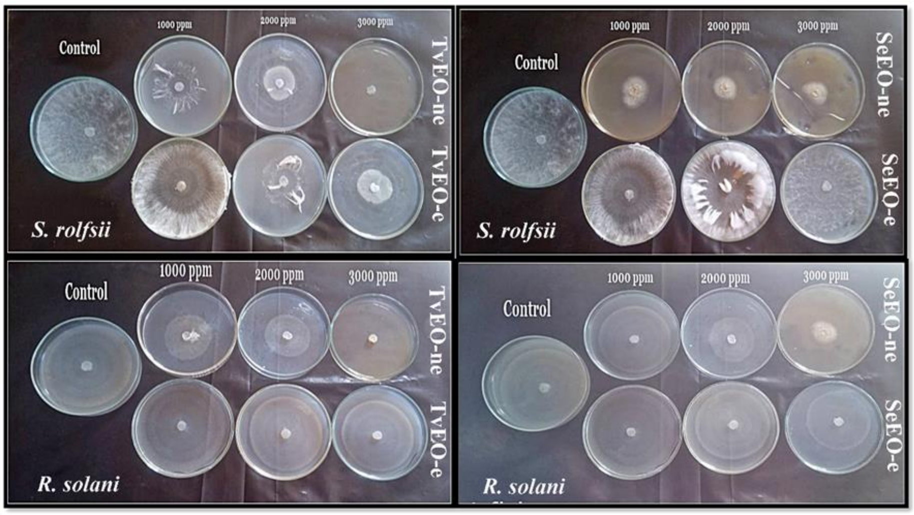


Fig (5): Mycelial radial growth of *Rhizoctonia solani* and *Sclerotium rolfsii* with different concentrations of TvEO-e: thyme oil emulsion, TvEO-ne: thyme oil nanoemulsion, SeEO-e: clove oil emulsion and SeEO-ne: clove oil nanoemulsion.

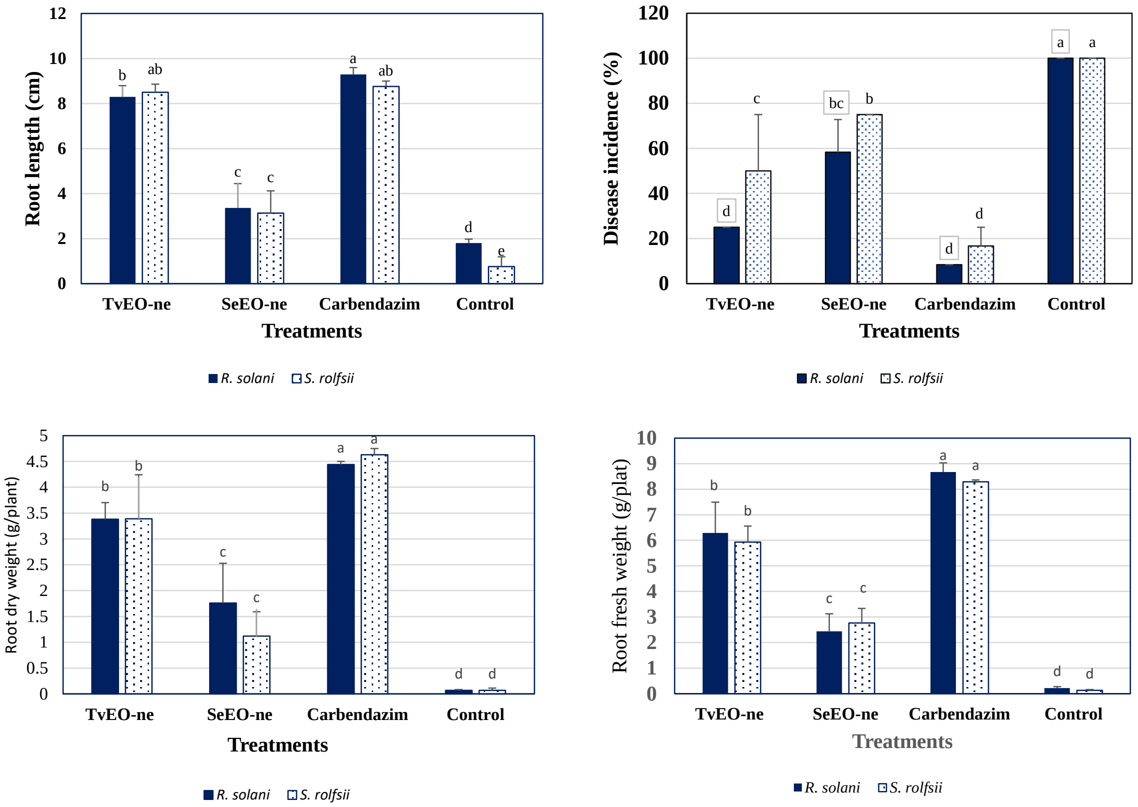


Fig. (6): Effect of TvEO-ne: thyme oil nanoemulsions, SeEO-ne: clove oil nanoemulsions, Carbendazim on disease incidence, root length, root fresh weight and root dry weight. Columns with different letters indicate significant differences according to Duncan's test ($p < 0.05$), The vertical bar indicates standard error.

Asp123 by two hydrogen bonds and with Cys177 and Cys174 by pi-alkyl interactions (Fig. 7 e, f). Eugenyl acetate interacted with Glu236 by one hydrogen bond and with Cys174 and Glu75 by pi-sulfur and pi-lone pair interactions, respectively (Fig. 7 g, h). Thymol, p-cymene, ɣ-terpinene, carvacrol, eugenol, caryophyllene, eugenol acetate, and ά-humulene have binding energies of -6,65, -6.02, -5.70, -5.80, -7.38, -5.56, and -6.07 kcal/mol with pectate lyases (Table 3).

3.5.2 Cellobiose dehydrogenase (CDH)

Molecular docking results based on binding energy revealed that thymol, p-cymene, ɣ-terpinene, carvacrol, eugenol, caryophyllene, eugenol acetate, and ά-humulene have binding energies of -5.20, -5.28, -5.40, -5.59, -5.69, -5.52, -5.92, and -5.06 kcal/mol with CDH, as shown in Table (3). Thymol formed five pi-alkyl interactions with Val308, Phe294, Trp290, Phe282, and His689 and additionally interacted with Asp688 by one hydrogen bond (Fig. 8 a, b). o-cymene interacted with Phe278, Leu312, Gly310, and His689 by four Pi-Alkyl and Pi-Pi interactions (Fig. 8 c, d). ɣ-terpinene showed interaction with Phe278, Leu312, Pro773, and His689 by six Pi-Alkyl and Pi-Pi interactions (Fig. 8 e, f). Carvacrol interacted with Asn521 by one hydrogen bond and with Pro563, Arg586, Phe278 and Tyr609 by four Pi-Alkyl and Pi-Pi interactions (Fig. 8 g, h). Eugenol interacted with Leu312 by one hydrogen bond and with Asn309 by Pi-sigma interaction (Fig. 8 I, j). Caryophyllene showed interaction with Leu312, Tyr609, Phe278, His689, and Phe282 by six Pi-Alkyl interactions (Fig. 8 k, l). Eugenyl acetate interacted with Tyr604, Thr584, and Asn732 by five hydrogen bonds and interacted with Phe282,

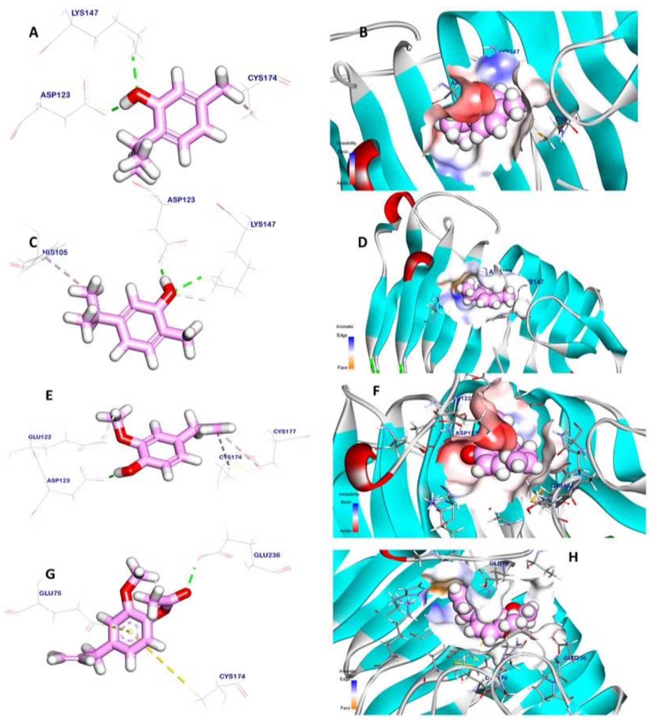


Fig. (7): Natural metabolites docked in pectate lyase, hydrogen bonds (green) and the pi interactions represented in purple lines with Surface Mapping showing *Natural metabolites* occupying the active pocket of pectate lyase.

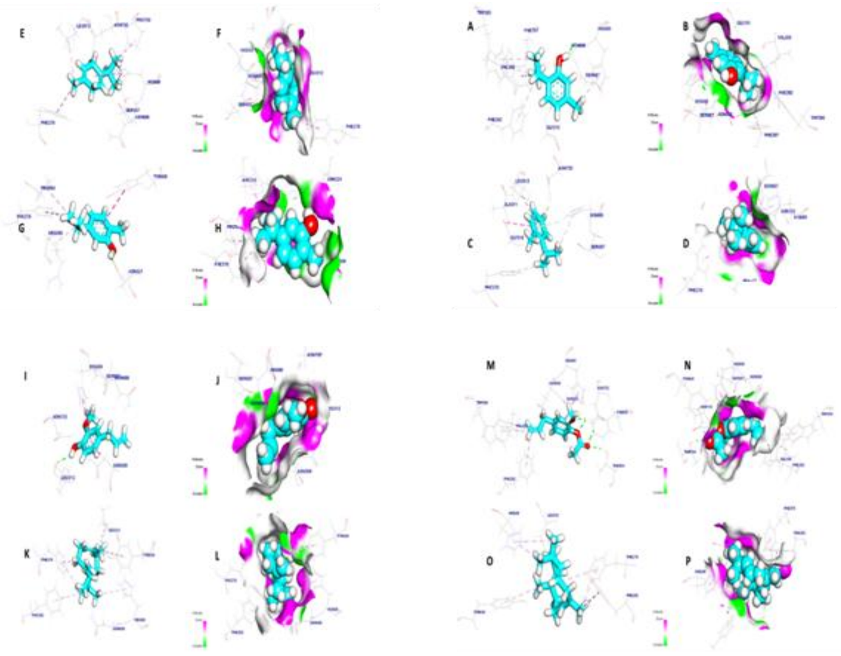


Fig. (8): Natural metabolites docked in cellobiose dehydrogenase, hydrogen bonds (green) and the pi interactions are represented in purple lines with Surface Mapping showing Natural metabolites occupying the active pocket of cellobiose dehydrogenase

Table (3): Docking scores kcal/mol and RMSD value of tested metabolites against targeted sites of the fungal proteins.

Targets screened	Tested compounds	RMSD value (Å)	Docking (Affinity) score (kcal/mol)
Pectate lyase	Thymol	0.93	-6.65
	o-Cymene	0.67	-6.02
	ɣ-terpinene	1.42	-5.70
	Carvacrol	1.25	-5.80
	Eugenol	0.86	-7.38
	Caryophyllene	1.078	-5.56
	Eugenyl acetate	1.44	-7.78
	α-humulene	1.07	-6.07
Cellobiose dehydrogenase	Thymol	1.39	-5.20
	o-Cymene	1.46	-5.28
	ɣ-terpinene	0.92	-5.40
	Carvacrol	1.12	-5.59
	Eugenol	1.81	-5.69
	Caryophyllene	0.48	-5.52
	Eugenyl acetate	1.48	-5.92
	α-humulene	1.02	-5.06

(Fig. 8 m, n). α-Humulene has seven pi-alkyl interactions with Phe202, Phe278, Tyr609, Leu312 and His689 (Fig. 8 o, p).

4. DISCUSSION

The novel achievement of the current studies is to develop an environmentally friendly approach that counteracts the negative effects of synthetic fungicides on humans and the environment as well as discover its antifungal activity by modeling. Natural plant products, especially essential oils, are a safe and powerful alternative to fungicides. Pathogenic fungi feed intracellularly and disrupting the normal metabolic balance of plant cells (Duan *et al.*, 2013). To invade plants, fungi secrete a range of phytotoxins and cell wall-degrading enzymes (Peng *et al.,* 2021). These cellular enzymes are suitable targets for drugs. In this study, we examined the effect of thyme and clove oil as emulsions and nano-emulsions on pothos root rot caused by *R. solani* and *S. rolfsii* and their modes of action by using molecular docking. Our results clearly showed that the oil-based nanoemulsions exhibited excellent antifungal activity against *R. solani* and *S. rolfsii* (Figs 4, 5 and 6). The effect of thyme oil and clove oil nanoemulsions in a 3000 ppm is considerably superior to that of thyme and clove oil emulsions with the same dose. These findings agree with previous studies that claimed that oil-based nanoemulsions have greater antimicrobial activity than essential oil emulsions in their original form (Badawy *et al.,* 2017 and Peng *et al.,* 2021). The high efficiency of the oil-based nanoemulsions is attributed to their small droplet size and larger surface area as presented in this study (Fig. 3). According to Miastkowska *et al*., (2020), the small droplet size and larger surface area of the oil-based nanoemulsions allow them to alter the delivery of essential oils to the fungal cell membrane and their interaction with the molecular sites on the cell membrane. These alterations enhance the antifungal activity of the oil-based nanoemulsions compared to essential oil emulsions in their original form. Additionally, the increased surface area of the nanoemulsions allows for better dispersion and coverage on surfaces, leading to more effective antimicrobial action.

Rhizoctonia solani and *S. rolfsii* drastically reduced root length, root fresh, and dry weight of pothos plant. In contrast, treatments with essential oil-based nanoemulsions and the fungicide carbendazim significantly reduced disease incidence ($P \leq 0.05$) and mitigated the negative effects of the two pathogens on these attributes (Fig. 6). These findings are consistent with those obtained by Moghaddam and Mehdizadeh (2020), who reported that thyme oil has potent antifungal activity against *Drechslera spicifera*, *Fusarium oxysporum* f. sp. *ciceris*, and *Macrophomina phaseolina*. Our observations also agree with the results reported by Zhang *et al.* (2022), who found that *Sabina chinensis* essential oil exhibited potent antifungal activity against *F. oxysporum* and *F. incarnatum*. The beneficial effects of the investigated oils on promoting vegetative development involving root length, root fresh weight, and root dry weight (Fig. 6) could be attributed to their direct impact on the tested pathogens by inhibiting their cell wall degrading enzymes (pectate lyase and cellobiose dehydrogenase), as indicated by the molecular docking results (Figs. 7 and 8 and Table 3). This inhibition prevented the degradation of the plant cell walls by these enzymes, thereby reducing the incidence of root rot caused by *R. solani* and *S. rolfsii* (Fig. 6), and contributing to improve vegetative development, better root growth, and overall enhanced plant performance.

Thyme and clove oils are promising antifungal plant metabolites, but their specific mechanism of action against *R. solani* and *S. rolfsii* is still poorly known. In fact, numerous studies on the antifungal mode of action of the essential oils clearly imply that the mechanism of action is related to ergosterol-binding and channel development, which causes destabilization of the fungal cell membrane, breakdown of the fungal cell membrane, and abnormalities in the mitochondrial structure (Badawy *et al*., 2019).

Enzymes that break down plant cell walls, known as plant cell-wall-degrading enzymes, are indicative of a necrotrophic lifestyle in fungi, which involves exploiting weaknesses in plant tissues to penetrate and infect them (De Silva *et al*., 2016 and Nazar Pour *et al*., 2022). Pectic enzymes are the first cell-wall-degrading enzymes secreted by pathogens when cultivated on isolated plant cell walls, as well as the first produced in diseased tissue (Martínez *et al.*, 1991; Niture *et al*., 2006 and Nazar Pour *et al*., 2022). Pectic enzymes modify the plant cell wall structure, exposing cell wall components to breakdown by other enzymes (Panda *et al*., 2004). Also, the cell wall degrading enzyme, cellobiose dehydrogenase plays an important role in necrotrophy (Razali *et al*., 2020). It acts as lignocellulose degradations (Ludwig and Haltrich, 2002). Due to the important role played by the pectin degrading enzyme like pectin lyase and lignocellulose degrading enzymes like cellobiose dehydrogenase we proceed these proteins for the molecular docking analysis.

According to the molecular docking results, all tested compounds showed excellent affinity for interactions with pectate lyase and cellobiose dehydrogenase (Table 3), which interfered with their normal functioning, eventually leading to their inhibition. This inhibition prevented the degradation of the plant cell walls by these enzymes, thereby reducing the incidence of root rot and contributing to enhanced plant performance. The pectate lyase and cellobiose dehydrogenase were found to be interacting well with the tested essential oil constituents and the interaction was found to be stronger in case of Eugenyl acetate (-7.78 and -5.92 kcal/mol, respectively) and Eugenol (-7.38 and -5.69 kcal/mol, respectively) as compared to the other components. Thymol and Eugenol compounds demonstrate high binding affinity with these enzymes, with thymol showing the strongest interactions with pectate lyase and eugenol showing the strongest interactions with cellobiose dehydrogenase. In the case of pectate lyase, thymol forms five pi-alkyl interactions with different residues in the enzyme's active site, including Val308, Phe294, Trp290, Phe282, and His689. Additionally, it interacts with Asp688 by one hydrogen bond. These interactions play a pivotal role in stabilizing the thymol-pectate lyase complex. Furthermore, the formation of a hydrogen bond with Asp688 further strengthens the binding affinity, contributing to the stability of the complex. Similarly, in the case of cellobiose dehydrogenase, eugenol forms five pi-alkyl interactions with different residues in the enzyme's active site, including Phe278, Leu312, Gly310, Pro773, and His689. These interactions are critical in anchoring eugenol within the active site, potentially influencing the enzyme's catalytic activity or stability. These results highly correlate with the biological data, and the nanoemulsions of the total extract of targeted compounds showed excellent activity against *R. solani* and *S. rolfsii*. Thus, the docking studies conclude that the active sites of selected proteins (pectate lyase and cellobiose dehydrogenase) can act as key areas to inhibit the enzymatic activity of disease development and are vital for finding inhibitors for pathogenesis. The result shows that these proteins could be potential targets of the essential oils and further studies would be required to be confirmed. Therefore, thyme oil and clove oil are promising candidates that act as natural fungicides.

Conclusions

The present study has demonstrated the efficacy of thyme and clove oil-based nanoemulsions as potent antifungal agents against *R. solani* and *S. rolfsii* in controlling pothos root rot. The results revealed that nanoemulsions were more effective than traditional oil emulsions in reducing mycelial growth of both fungi. The effectiveness of the nanoemulsions was further confirmed by the significant reduction in root rot incidence and the mitigation of adverse effects on plant roots attributes. Molecular docking analysis revealed the strong affinity of main components in thyme and clove oils with pectate lyase and cellobiose dehydrogenase enzymes in the targeted pathogens. Overall, the study highlights the potential of thyme and clove oils as eco-friendly alternatives to synthetic fungicides, offering a sustainable solution for disease management in agriculture. In the future, further in-depth studies must be conducted to further elucidate the specific modes of action and optimize the use of these oils as effective antifungal agents in agricultural practices.

Authors' contributions

All authors contributed in conceptualization, methodology, software, validation, formal analysis investigation, resources, data curtain, writing the original draft preparation, writing, review, editing, supervision and funding acquisition. All authors have read and agreed to the published version of the manuscript.

Competing interests

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

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النشاط المضاد للفطريات للمستحلبات النانونية لزيت الزعتر والقرنفل ضد تعفن جذور البوتس

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ملخص

نظراً للمشاكل البيئية الناجمة عن استخدام المبيدات في مكافحة أمراض النبات، فإن البحث عن بدائل آمنة لتلك المبيدات أصبح أمراً ضرورياً، لذلك استهدفت هذه الدراسة البحث في تأثير استخدام المستحلبات الزيتية النانوية القائمة على الزيوت العطرية كبديل آمن للمبيدات الفطرية في مكافحة أمراض النبات. تركزت الدراسة حول البحث في النشاط المضاد لإثنين من الزيوت العطرية الطيارة هما زيت الزعتر وزيت القرنفل ضد فطرين مسببان لأعفان جذور نبات البوتس هما رايزوكتونيا سولاني و اسكليروثيوم رولفسياي. ولقد استخدمت الموجات الفوق صوتية عالية الطاقة لإنتاج مستحلبات الزيوت النانوية بعد مزجها بمادة التوين 80 بنسبة 2:1 (حجم / حجم). ولتقييم ثبات المستحلب تم دراسة توزيع حجم الجزيئات في المستحلب باستخدام تقنية تشتت الضوء الديناميكي (DSL). وأوضحت نتائج مؤشر التفاوت في الاحجام (PI) لمستحلبات الزعتر وزيت القرنفل النانوية انها تبلغ 0.436 و0.227 على التوالي، مما يدل على أنهما متماثلان. بالإضافة إلى ذلك، كان متوسط قطر كلا المستحلبين (حجم القطرات) هو 94.82 و73.76 نانومتر على التوالي. أما عن كفاءة تلك المستحلبات الزيتية ضد كل من الفطر رايزوكتونيا سولاني والفطر اسكليروثيوم رولفسياي فإن المعاملة باستخدام ثلاث تركيزات (1000 – 2000 – 3000 جزء في المليون) من زيت الزعتر وزيت القرنفل سواء في صورة مستحلب أو مستحلب نانوي أكدت النتائج أن الصورة النانوية هي الأكثر كفاءة في خفض النمو الميسليومي لكلا الفطرين وأن التثبيط الكامل لنمو كلا الفطرين نتج عن التركيز الأعلى (3000 جزء في المليون). وأكدت تجارب مكافحة المرض تحت ظروف العدوى الصناعية نتائج التجارب المعملية، حيث ساعدت تلك المستحضرات الزيتية النانوية على خفض نسبة الإصابة وتخفيف حدة الضرر الناتج عن تلك الفطريات على نمو النبات متمثلة في زيادة طول الجذر ووزنه الطازج والجاف. ولقد درست ميكانيكية فعل الزيوت العطرية محل الدراسة باستخدام النمذجة الجزيئية وأظهرت النتائج تفاعلاً قويا بين مكونات الزيت الفعالة وبروتينات الفطريات المسؤولة عن الإمراضية (انزيم البكتات لايز والديهيدروجينيز) .وجد أن التفاعل كان أقوى ما يمكن في حالة مركب الايوجينول وأسيتات الايوجينول مقارنة بالمكونات الأخرى للزيتين وترتبط هذه النتائج ارتباطًا وثيقًا بنتائج الدراسة البيولوجية.

المجلة المصرية للعلوم الزراعية – المجلد (75) العدد الثانى (أبريل 2024) 78-92.