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ORIGINAL PAPER

Antifungal Activity of Curly Parsley Essential Oil and Its Nanoemulsion Against *Alternaria solani* and *Rhizoctonia solani* Plant Pathogens

Gomaa, F.H.*[1] and Gomaa, S.E.[2]

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ABSTRACT

Curly Parsley (*Petroselinum crispum*) seeds were planted for the purpose of essential oil extraction from dried plants by hydro-distillation. GC-MS analysis of parsley essential oil (PEO) showed that *Parsley*-apiol (40.42%) and myristicin (34.77%) were the major components. PEO and its nanoemulsion (PEO-NE) were tested for their antifungal activities against *Alternaria solani*, the causal agent of tomato early blight, and *Rhizoctonia solani*, the causal agent of faba bean root rot. Isolated fungi were verified by ITS1and ITS4 primers. Their partial region was deposited in the GenBank (NCBI) under OL305707 and OL305708 accession numbers for *A. solani* and *R. solani*, respectively. *In vitro* treatments with PEO and PEO-NE showed a significant difference in the growth of both fungi under study when compared with their negative and positive controls. PEO highest effect was recorded at 3000 mg/L while PEO-NE was recorded at 1500mg/L without significant difference from the commercial fungicide (positive control). Pathogenicity test was confirmed by evaluating the antifungal activity by detached leaf assay on tomato and radical assay on faba bean for *A. solani* and *R. solani*, respectively. This study reflects the possibility of replacing harmful fungicides with natural products, such as PEO and its nanoemulsion, against *A. solani* and *R. solani*. Eco-friendly products, e.g., natural extracts, are a must now, to be used to decrease the negative impact towards general health and pollution from using commercial chemicals.

Keywords: *Alternaria solani*, *Rhizoctonia solani*, Essential Oils, Parsley, Nanoemulsion.

***Correspondence: Gomaa, F.H.**
E-mail: fayzahassan2003@yahoo.com
Fayza H. Gomaa
https://orcid.org/0000-0002-7332-0326
1- Seed Pathology Research Department, Plant Pathology Research Institute, Agricultural Research Center, 12619, Giza, Egypt.
Sara E. Gomaa
https://orcid.org/0000-0001-5484-2789
2- Medicinal and Aromatic Plants Research Department, Horticulture Research Institute, Agriculture Research Center, 12619, Giza, Egypt.

INTRODUCTION

Bio-ecological studies are becoming increasingly popular as a way to find novel alternatives to synthetic fungicides, as extensive fungicidal applications in open field, and thus consequently, raise pathogen resistance against those fungicides (Hassanin *et al.*, 2017). Plant extracts and essential oils became one of the most prevalent approaches against pathogenic organisms (Nazzaro *et al.*, 2017 and Grande-Tovar *et al.*, 2018). Natural substances utilization, as essential oils (EOs), is one of the distinguished alternatives known to lower toxicity for human beings and towards the environment (Hammer *et al.*, 2003). EOs are volatile substances extracted from plants, which can be used as a raw material in many industries, including pharmaceutical products, food and agriculture goods (Butnariu and Sarac., 2018). Similarly, EO derivatives are well documented to have antimicrobial effect against many microorganisms, such as viruses, bacteria and fungi (Pepeljnjak *et al.*, 2003 and Akthar *et al.*, 2014). EO derivatives as, lipophilic monoterpens (thymol, carvacrol, linalool, citral, geraniol, and 1,8- cineole) are used as antimicrobial compounds, and they are used in food preservation and as an antiseptics and disinfectants (Pauli, 2001 and Burt, 2004). One of the most promising EOs is parsley essential oil (PEO). Earlier, PEO was used as anti-microbial agent against different bacterial and fungal infections (Gutierrez *et al.*, 2008 and Teixeira *et al.*, 2013). PEO contains mainly α-pinene, myristicin and apiol (Khalil *et al.*, 2015), which give the EO its anti-inflammatory and antimicrobial characteristics (Salehi *et al.*, 2019 and Badr *et al.*, 2020). Parsley [*Petroselinum crispum*] is a bright green herb recognized with its flavor, aroma, medicinal and cosmetic use as well as being used in food and medicine industry (Gutierrez *et al.*, 2008).

In Agriculture, nanotechnology applications revealed a promising potential as in nano-fertilizers and/or nano-pesticides in crop production and protection (El-Wakil, 2020 and Usman *et al.*, 2020). Furthermore, it is considered as a new generation of ecofriendly pesticides for plant disease management) (Worrall *et al.*, 2018). There are three different types of lipids nano-particle carriers, namely,

solid lipid nanoparticles, liposomes and nanoemulsion (NE). Acceptable NE droplet sizes should range between 20 to 200 nm (Hassanin *et al.,* 2017 and Echeverría and de Albuquerque, 2019). NE particle size of 100 nm have recently created and provided the promise for a new era of more effective and secure insecticide, fungicide, herbicide as well as improved manure administration through better-quality active ingredient absorption and less environmental pollution (Gogoi *et al.,* 2009).

According to Anon. (2020), Egypt is considered the fifth country worldwide in tomato production, with 6.8 million MT productivity in 2019, compared to 8.6 million MT productivity in 2012. Tomato Early blight disease is considered the highest causal agent of tomato yield loss and reduced fruit quality (≈80%) in warm and humid regions caused by *Alternaria solani* (Ashour *et al.,* 2012 and Pandey *et al.,* 2014). Early symptoms are recognized as a brown spot-on leaves, fruits, and stems, it's mode of action is to reduce the photosynthetic surface and cause the leaf lower part to fall (Meenakshi *et al.,* 2014).

Another important leguminous crop in Egypt, is faba bean, with productivity reaching about 140 thousand tons during 2018 (Anon, 2020). This productivity is affected by many pathogens, mostly *Rhizoctonia* spp. according to (Shahda *et al.*, 2011). One hundred and thirty-one isolates of *Rhizoctonia* spp., were registered from different fields in Delta region, in Egypt, causing root rot and stem canker on faba bean plants (Helmy *et al.,* 2015).

One of which is *R. solani* having a wide range of hosts causing pre and post emergence seedlings damping-off and discoloration, necrosis of seeds and reduction in seed size (ElWakil *et al.,* 2009). *R. solani* might be associated with other pathogens such as *F. solani*, and *Macrophomina phaseolina.* This fungal association was reported to attack faba bean and soybean roots and stem base causing losses in seed germination and yield (Abdel-Kader *et al.,* 2011 and Atwa, 2018).

The purpose of this study was to investigate the antimicrobial effect of parsley essential oil (PEO) and its nanoemulsion (PEO-NE), then, determine the optimal concentration to be used against two destructive pathogens namely *A. solani* and *R. solani*. It was hypothesized that, PEO and PEO-NE might be needed to reduce *A. solani* and *R. solani* growth and thus preventing their infection, or at least reducing their severity. The chemical composition of (PEO) was determined by GC-MS.

MATERIALS AND METHODS

Plant Materials:

Parsley seeds were kindly provided by Enza Zaden Vegetable Breeding Co., Netherlands. Seeds were sown at the El-Sabaheya Horticulture Research Station, Alexandria governorate, in October 2020, and normal agricultural practices were performed according to Moustafa and Abdelwahab (2016).

Parsley Essential Oil Extraction:

Parsley Essential Oil (PEO) extraction took place from air dried plants by hydrodistillation. It was performed for two successive hours using a Clevenger-type apparatus as recommended by El-Massry *et al.* (2008) and Gomaa and Gomaa (2021). After extraction, PEO was dried using anhydrous sodium sulfate to be prepared for GC-MS analysis (Abd El-Kareem *et al.,* 2016 and Farouk *et al.,* 2018).

Gas Chromatography-Mass Spectrophotometry (GC-MS) Analysis:

GC-MS was performed in the Research Laboratories of Forensics and Toxicology Department, Faculty of Medicine, Zagazig University, as recommended by Abd El-Kareem *et al.* (2016). Chemical composition of PEO was determined using Trace GC-ISQ mass spectrometer (ThermoScientific, TX, USA) with a direct capillary internal column TG-5MS (30 m length, 0.25 mm diameter and packed with 0.25 µm film thickness). Sample volume was 1µl, using helium as a carrier gas at flow rate of 1ml/min. Column temperature was adjusted at 50°C and elevated every min 5°C until 250°C, and hold for 2 minutes, then increased to 300°C by 30°C every minute then hold again for 2 minutes. Injector temperature was kept at 270°C while MS transfer line temperature was kept at 260°C. Helium, as a carrier gas, was used in constant flow rate of 1 ml/min. Solvent was delayed 4 min, and then diluted samples of 1 µl were injected using the auto sampler coupled with GC in the split mode. Mass spectra were determined at 70 eV ionization voltages over the range of m/z 50-650 in full scan mode. The ion source temperature was set to 200°C. EO components were recognized by comparing them to their relative retention times on NIST 05 mass spectral database (NIST/EPA/NIH mass spectral library version 2.0d) as recommended by Mohamed *et al.* (2020).

Preparation of Nanoemulsion and Droplet Size Measurements:

Parsley Nanoemulsion (PEO-NE) preparation was performed at the Central Laboratory, Faculty of Pharmacy, Alexandria

University, Egypt. One ml of PEO and 0.5 ml of Tween 20 were added slowly under gentle stirring until homogenization. Then, volume was completed to 10 ml by distilled water with gentle stirring for 30 min. to get a homogeneous mixture. This blend was then, sonicated using Ultrasonicator (Germany) at 700 w for 30 min. Twenty-five µl of the PEO-NE were diluted with 1 ml of distillated water. Then droplet size estimation was performed using a dynamic light scattering analyses (Zetasizer, UK) at 25°C. Nano-particles size was expressed as the mean average of three impartial batches of the nanoemulsion (Hassanin *et al.,* 2017).

PEO-NE characterization was performed by Transmission electron microscopy (TEM) at the Electronic Microscope Unit - Faculty of Science, Alexandria University, Egypt. Twenty µl of diluted samples were placed on a film-covered 200-mesh copper specimen grid for 10 min. then, fluid leftovers were removed using filter paper. Stained grid with one drop of 3% phosphor tungstic acid was then left to dry for 3 minutes (Saloko *et al.,* 2013). At the end, the grid was tested below the TEM microscope (JOEL JEM-1400 Flash Electron Microscope-USA).

Morphological and Molecular Identification of Fungal Isolates:

Two fungal cultures were isolated from infected tomato plants at El- Sabaheya Research Station, Plant Pathology Research Institute, Alexandria Agricultural Research Center. Pure cultures were kept at 4°C on PDA for further studies. Isolates were identified as *A. solani* and *R. solani* according to their morphological features (Ellis, 1976) and molecular level using conserved ribosomal internal transcribed spacer (ITS) region (White *et al.,* 1990 and Moore *et al.,* 2011). From pure cultures of the isolated fungi, DNA was extracted by a repaid mini-preparation procedure (Edel *et al.,* 2001). PCR amplification was performed for *Alternaria solani* and *Rhizoctonia solani* in a final volume 50 µl as; 25 µL (2x PCR mix -Gene Direx), 1 µL of DNA template, 0.2 µM of each primer. PCR amplifications were conducted in Minicycler (PTC-150 Minicycler) with an initial determination at 95°C for 1 min followed by 35 cycles (30 Sec. at 94°C, 2 min. at 55°C and 1 min. at 72°C) and a final extension time of 10 min. at 72°C. (Gomaa, 2021). PCR amplified products were purified and sequenced using forward primer of the DNA facility of Sigma Laboratories, Egypt. Sequences obtained from the two isolates were confirmed by applying Basic Local Alignment Search Tool (BLAST search) on National Center for Biotechnology Information (NCBI). Then sequences were deposited in GenBank for accession numbers. Pathogenicity tests were then confirmed using the detached leaf assay for *A. solani* (Meenakshi *et al.,* 2014) and the radical assay for *R. solani* (Basbagci *et al.,* 2019).

Evaluation of Antifungal Activity of PEO and PEO-NE:

A) *In vitro* Evaluation on the Fungal Growth:

Media preparation was performed by dissolving 1 ml of PEO and PEO-NE in 25 ml of dimethyl sulfoxide (DMSO 99.99%), separately, to be added to the warm PDA to obtain the required concentrations (12.5, 25, 50, 100, 200, 375, 750, 1500, 3000 mg/L). After media solidification, it was inoculated with 5mm disk of *Alternaria solani* and *Rhizoctonia solani*. DMSO was used as a negative control while, the commercial fungicide (Meta, Laxyl-M - Azoxystrobin 39.1%), was used as a positive control. Plates were incubated at 25 ± 2°C for 10 days. Colony diameter means (mm) of 3 replicates were recorded and consequently standard error was calculated (Raj *et al.,* 2017).

B) Detached Leaf Assay for *A. solani*:

Detached leaf assay (tomato leaves) were modified from Meenakshi *et al.* (2014). Tomato leaves (Castle Rock cultivar) were surface sterilized with 70% ethanol followed by 1% Clorox, then washed with sterile distilled water and dried with sterilized tissue paper in petri dish. Tomato detached leaves, were kept fresh by being placed on a moist filter paper. To avoid leaf drying, all petioles were covered with sterile moist cotton and plates were contentiously moistened with sterile distilled water every 48 hours. As for control, leaves were treated by 2 ml of distilled water mixed with DMSO (25:1). Leaves were sprayed by 2 ml of the best concentration recorded previously *in-vitro* of PEO and PEO-NE. *Alternaria solani* was incubated on potato dextrose agar (PDA) at 25°C with 20 seconds UV exposure for sporulation induction (Yadav *et al.,* 2015) for seven days. *A. solani* discs, made with sterile corck borer (3mm), were placed on the leaf axial side center, and incubated in room temperature. Experiment was performed in four replicates. Lesion diameter (mm) was measured at the seventh day. Treatments effect (efficacy %) was calculated according to the following equation by Wang *et al.,* (2020):

$$\textbf{Efficacy (\%)} = \frac{\textbf{Infected lesion diameter - treatment lesion diameter}}{\textbf{Infected lesion diameter}} \times 100$$

C) Radical Assay for *R. solani*:

In vitro radial assay on faba bean seeds (Baldy cultivar) was used to determine the minimal inhibition effect of different PEO and PEO-NE concentrations on soaked seeds for 1 hour (Basbagci *et al.*, 2019). Mycelial discs (3 mm) of *R. solani* were taken from pre-grown fungal culture and placed in a Petri dish center containing water agar (2%) and incubated at 25±2°C for 48 hours. Faba bean seeds were surface sterilized by Clorox (1%), rinsed with sterilized distilled water and then surface dried. Seven seeds were placed around the fungus disc, where control disk was a sterile PDA disc placed at the middle of the seven seeds. Then, plates were incubated at 25±2°C for 10 days. Four replicates (plates) were used for each treatment. Disease severity measurement based on the scale from 0-5 as; 0=healthy, 1=1-10% infection of hypocotyls, 2=10≤30% infection of hypocotyls, 3=30≤50% infection of hypocotyls, 4=50≤80% infection of hypocotyls and 5= plant dead. Disease severity was calculated according to the Townsend–Heuberger formula (Maharjan *et al*., 2015):

$$\textbf{Disease severity (\%)} = \frac{\textbf{Sum of scorings of different ratings}}{\textbf{No. of samples} \times \textbf{highest rating}} \times 100$$

Statistical analysis:

All experiments were designed using the randomized complete design (RCD). Data were statistically analyzed using SAS System for Microsoft windows SAS 9.4.2016 using the least significant difference (L.S.D).

RESULTS AND DISCUSSION

Parsley seeds were planted for the purpose of essential oil extraction by hydrodistillation. Parsley essential oil (PEO) and its nanoemulsion (PEO-NE) were tested for their antifungal activity against *Alternaria solani* and *Rhizoctonia solani*.

Extraction from dried parsley plants produced a whitish yellow color extract with 0.45 ml/ 100g dw, which came in agreement with Moustafa and Abdelwahab (2016).

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis:

PEO was analyzed by GC-MS as explained earlier by Abd El-Kareem *et al.*, (2016) and Mohamed *et al.* (2020). Results showed that *Parsley*-apiol and myristicin were the most dominant components of PEO with 40.42 and 34.77%, respectively, followed by iso-elemicin (13.05%), then myrtenol (8.15%) and ϑ-terpinene (1.15) as shown in Figure (1) and Table (1). Results agreed with the previous studies recorded by Chatzopoulou and Katsiotis, (1995), Farouk *et al.* (2018) and Pineda *et al.* (2018) who stated that, *Parsley*-apiol and myristicin were the major components of PEO.

Characterization of Parsley Essential Oil Nanoemulsion (PEO-NE):

PEO-NE was prepared from PEO. At the day of preparation, PEO-NE droplet diameter (Z-Ave) and polydispersity index (PDI) were measured as described earlier by Hassanin *et al.* (2017). Figure (2) shows that droplet width is around 71.58nm. Mean NE droplet diameter was measured to be 125.1 nm with poly dispersity index (PDI) of 0.185.

PEO-NE characterization by Transmission Electron Microscopy (TEM) shows spherical and moderately mono-shape particles and they appeared white in color. Particles size ranged between 83.16 and 94.61 nm (Fig. 3, a and b).

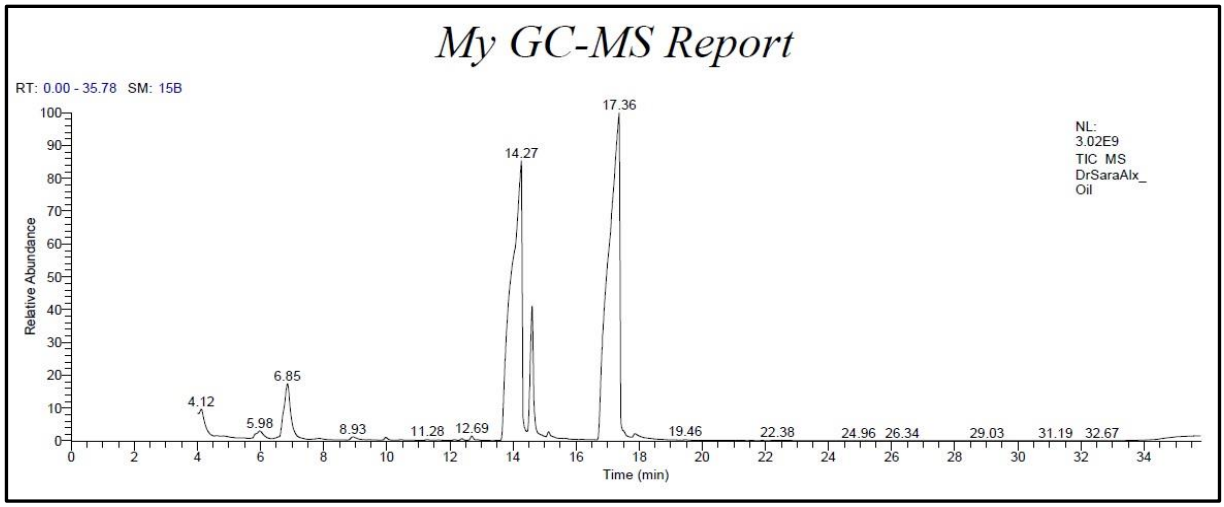

Figure (1): GC-MS spectrum of PEO with x-axis showing the retention time (min) and y-axis shows the relative abundance area.

Table (1): Chemical Composition (%) of PEO as inspected by Gas Chromatography Mass Spectrophotometry (GC-MS).

	Compound name	Retention time	Area %	Molecular formula	Library*	Cas number
1	ɑ-Terpinene	4.14	1.15	$C_{10}H_{16}$	R	99-85-4
2	β-Pinene	5.97	0.9	$C_{10}H_{16}$	W	127-91 -3
3	Myrtenol	6.85	8.15	$C_{10}H_{14}O$	M	564-94 -3
4	Estragole	8.93	0.08	$C_{10}H_{12}O$	W	140-67-0
5	Limonene	9.96	0.05	$C_{10}H_{16}$	R	138-86-3
6	Myristicin	13.85	34.77	$C_{11}H_{12}O_3$	R	607-91 -0
8	Iso-elemicin	14.61	13.05	$C_{12}H_{16}O_3$	M	5273-8 5-8
9	Epicubebol	15.13	0.62	$C_{15}H_{26}O$	R	38230-6 0-3
10	*Parsley*-Apiol	17.37	40.42	$C_{12}H_{14}O_4$	W	523-80 -8

*Library: (R = Replib, W = WileyRegi, M = Mainlib).

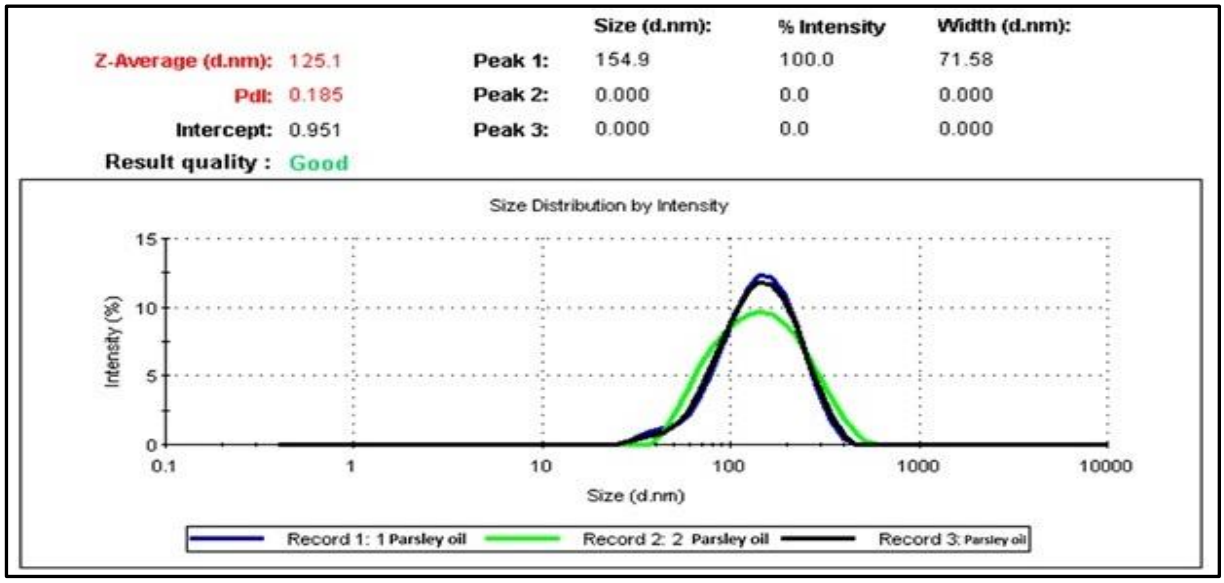

Figure (2): PEO-NE particle size prepared by ultra-sonication for 30 min.

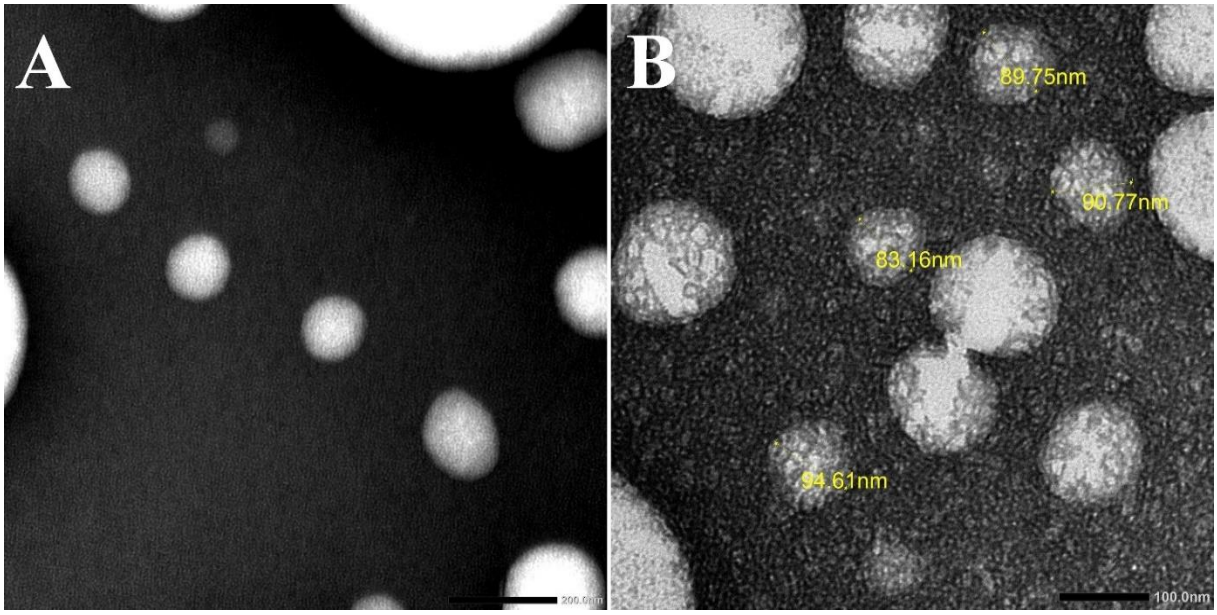

Figure (3): Transmission Electron Microscope (TEM) characterization of PEO-NE using ultra-sonication method for 30 min., with two magnifying powers; (a) 200 nm and (b) 100 nm.

Morphological and Molecular Identification of Fungal Isolates:

Isolated fungi were identified and verified according to their cultural properties and microscopy characterization as mentioned before by Ellis (1976) and confirmed using the universal primers ITS1 and ITS4 which were used to amplify a single stranded band of approximately 200–400 kbp. Partial amplified ITS region for the two fungal isolates were accepted, deposited and released on 30 October 2021 at the GenBank (NCBI, National Center Biotechnology Information) under accession numbers (OL305707 and OL305708) as *A. solani* and *R. solani*, respectively. Sequences analysis revealed that, *A. solani* isolate was 99.78% homologues as compared to another isolate from China (accession number: ON790493), while *R. solani* isolate showed 99.01% homologues when compared to an isolate from Turkey (accession number: MT397135) on NCBI database.

Evaluation of Antifungal Activity of PEO and PEO-NE:

A) *In vitro* Evaluation on the Fungal Growth:

The antifungal effect of PEO and PEO-NE was tested *in vitro* against *Alternaria solani* and *Rhizoctonia solani*. Tested concentrations were compared with the commercial fungicide (Meta, Laxyl-M - Azoxystrobin 39.1%), as a positive control. Results concerning growth inhibition are presented in Table (2) expressed in colony diameter mean (mm). Both isolated fungi growth diameter measurements indicated a significant difference in growth ranges between PEO and PEO-NE treatments with their positive and negative controls. Also, they both showed the highest effect on growth when treated with PEO at 3000 mg/L while PEO-NE treatments showed the highest effect at a lower concentration (1500mg/L) with no significant difference from the commercial fungicide (positive control). The concentration which gave the best inhibition was indicated at 1500 mg/L and 750 mg/L, as 27 and 35 mm for *A. solani* and 11 and 27 for *R. solani*, respectively. PEO-NE showed a significant difference with *R. solani* from the first concentration (12.5 mg/L) when compared with the negative control, while PEO showed the significant effect at the fifth concentration (200 mg/L). Commonly, it was noted that PEO-NE treatments showed better effect on fungal growth as it reduced the growth at earlier concentrations as shown in Figures (4 and 5). Efficiency of PEO was confirmed by Burt (2004) who reported that PEO showed anti-fungicidal activities at low concentration against *Pseudomonas funiculosum* and *Trichoderma viride;* detected mostly in food contamination. PEO affect fungi by reducing cell wall degrading enzymes activity (Khaledi *et al.*, 2015). Moreover, PEO-NE was more effective as its smaller particle size led to additional proficiency as supported by Abd-ElSalam and Khokhlov (2015), Hassanin *et al.* (2017), Hassanin *et al*. (2018), Shahbazi, (2019) and Abdullahi *et al*. (2020). PEO-NE small particle size makes it significantly stable against aggregation, coalescence, and creaming (Anton and Vandamme, 2011 and Moradi and Barati, 2019). Early studies stated that, nanoemulsion (NE) state of EOs is selected over oil suspensions because of their better solubilization capacity and thermodynamic stability (Anton and Vandamme, 2011 and Abd-ElSalam and Khokhlov, 2015).

Table (2): Means of *A. solani* and *R. solani* colony growth (mm) in the presence of PEO and PEO-NE nine different concentrations with the –ve and +ve controls.

Concentration's (mg/L)	*A. solani*		*R. solani*	
	PEO	PEO-NE	PEO	PEO-NE
DMSO (-ve control)	70 ± 0.000 a*	70.0 ± 0.000 a	90 ± 0.000 a	90 ± 0.000 a
12.5	67 ± 0.153 b	60.0 ± 0.000 b	90 ± 0.000 a	62 ± 0.519 b
25	66 ± 0.100 b	57.0 ± 0.346 b	90 ± 0.000 a	57 ± 0.757 b
50	66 ± 0.058 b	54.0 ± 0.361 bc	90 ± 0.000 a	48 ± 0.265 c
100	65 ± 0.058 b	48.0 ± 0.513 cd	90 ± 0.000 a	33 ± 0.289 d
200	62 ± 0.058 c	46.7 ± 0.577 d	73 ± 0.144 b	32 ± 0.643 d
375	48 ± 0.115 d	45.7 ± 0.289 e	70 ± 0.500 b	28 ± 0.577 d
750	45 ± 0.058 e	35.0 ± 0.866 f	45 ± 0.500 c	27 ± 0.058 d
1500	27 ± 0.252 f	0.0 ± 0.000 g	11 ± 0.404 d	0 ± 0.000 e
3000	0 ± 0.000 g	0.0 ± 0.000 g	0 ± 0.000 e	0 ± 0.000 e
Fungicide (+ve control)	0 ± 0.000 g	0.0 ± 0.000 g	0 ± 0.000 e	0 ± 0.000 e

* Means of the fungal colony diameter in three samples ± standard deviation (SD). Means having letter in common do not significantly differ using L.S.D., p= 0.05 level of significance.

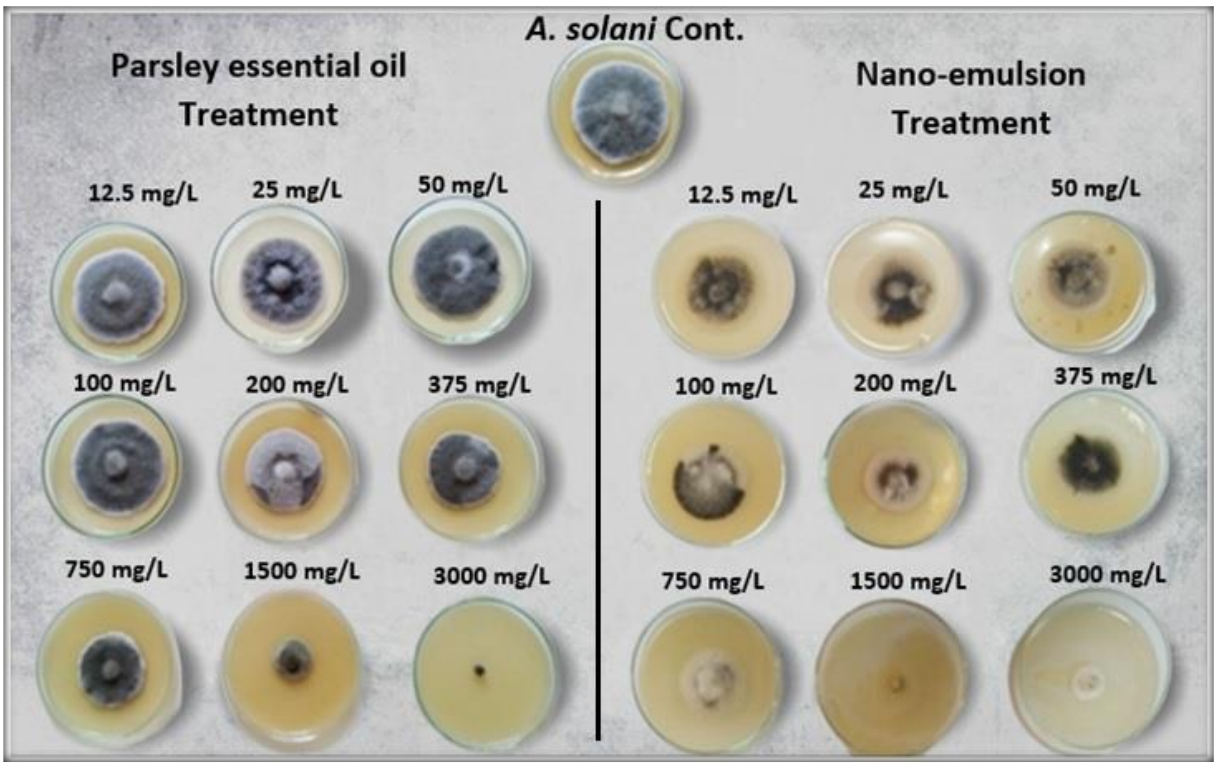

Figure (4): ***In vitro, A. solani*** **growth on PDA with different concentrations of PEO and PEO-NE.**

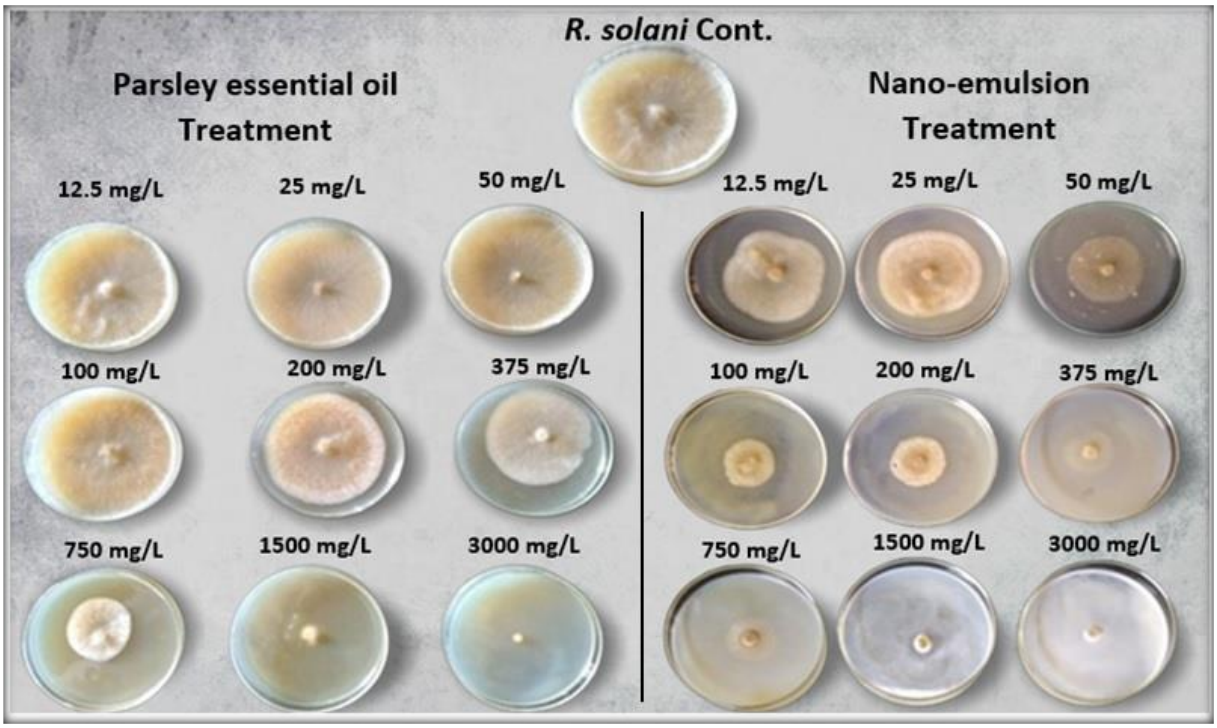

Figure (5): ***In vitro, R. solani*** **growth on PDA with different concentrations of PEO and PEO-NE.**

B) Detached Leaf Assay for *A. solani*:

Tomato leaves were inoculated with *A. solani* discs and lesion areas of the infected leaves were measured (mm) and efficacy (%) was calculated according to Wang *et al.* (2020). Infected leaves with *A. solani* turned yellow to brown and others became completely brown. Lesion area means for infected leaves recorded about 27.5 mm, whereas leaves treated with PEO (3000mg/L) showed lesion with 7.25 mm diameter and PEO-NE treatment (1500mg/L) was approximately 4.75 mm. Treatment with PEO (3000mg/L) and PEO-NE (1500mg/L) showed efficiency up to 73.6% and 82.7%, respectively, compared to infected leaves as shown in Table (3) and Figure (6). Results of efficacy showed that PEO-NE treatment was more effective than PEO by almost 10%.

Table (3): Means of lesion area (mm) on tomato detached leaves treated with PEO and PEO-NE against *A. solani* infection.

Treatment	*Mean of lesion area (mm)	Efficacy (%)
Control	0.00 ± 0.000 d	-
Infected (untreated)	27.50 ± 0.289 a	-
PEO (3000mg/L)	7.25 ± 0.263 b	73.6
PEO-NE (1500mg/L)	4.75 ± 0.050 c	82.7

* Means of the fungal colony diameter in four samples ± standard deviation (SD). Means having letter in common do not significantly differ using L.S.D. at p= 0.05 level of significance.

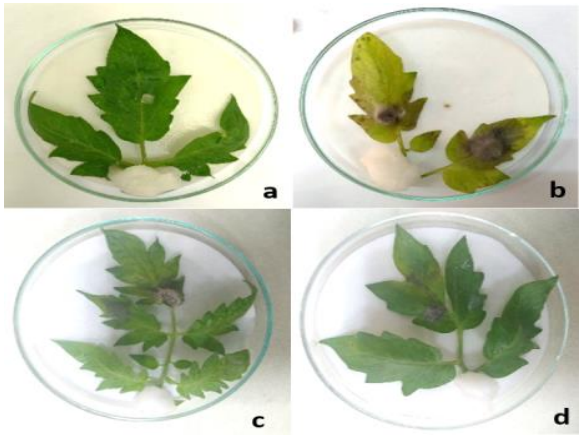

Figure (6): Effect of different treatments against *A. solani*

a: Control leaves; **b**: Infected leaves (untreated); **c**: Infected leaves sprayed with PEO (3000 mg/L); **d**: Infected leaves sprayed with PEO-NE (1500 mg/L)

C) Radical Assay on *R. Solani*:

Radical assay was performed on faba bean seeds to evaluate the effect of the best treatments against *R. solani*. Disease severity (D.S) was calculated according to Maharjan *et al*. (2015) and results in Table (4) show that, PEO-NE (1500mg/L) performed 20% better than PEO treatment. Both PEO-NE and PEO treatments reduced the fungal growth rate when compared to the control as illustrated in Figure (7). There was an obvious significant difference between PEO-NE and PEO treatments as PEO-NE efficacy was 22% higher than PEO treatments (Table 4).

Table (4): Radical assay for Faba Bean seeds treated with PEO and PEO-NE against *R. solani* infection.

Treatment	Disease severity (%)	Efficacy (%)
Control	0.00 ± 0.000 d	-
Infected	90.3 ± 4.509 a	-
PEO (3000mg/L)	39.7 ± 4.509 b	48.4
PEO-NE (1500mg/L)	21.3 ± 3.215 c	70.5

* Mean percentage of the fungal colony diameter in four replicates with 7 faba bean seeds per each ± standard deviation (SD). Means having letter in common do not significantly differ using L.S.D., at p= 0.05 level of significance.

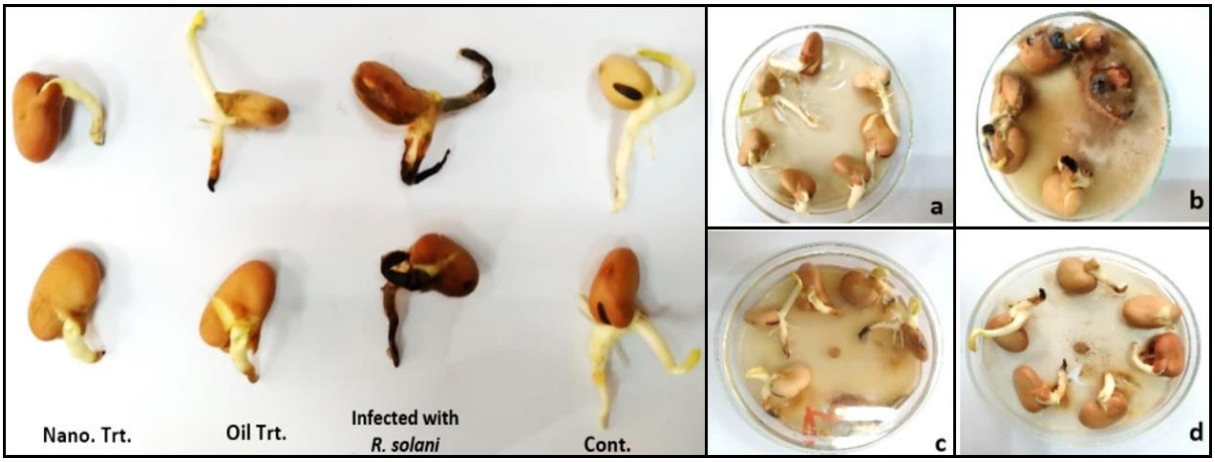

Figure (7): Effect of different treatments against *R. solani* after 12 days of inoculation.

a: Control seeds; **b**: Infected seeds (untreated); **c**: Infected seeds treated with PEO (3000mg/L); **d**: Infected seeds treated with PEO-NE treatment (1500mg/L)

The same trend of results was supported by Hammer *et al.* (2003), as they explained that, EOs bind with the cellular membrane and confer its permeability, which leads to cytoplasmic leaking and loss, then EOs make its way to be attached to special proteins capable of damaging the chromosomal replication process. It was suggested also that, terpenes in EOs can inhibit virulence encoding genes (Qiu *et al.* 2012). Myristicin antifungal capability has been reported by Meepagala *et al*. (2005) and Moreira-Valente *et al*. (2015) against *A. flavus* and *A. ochraceus* almost by 80% for both at very low concentrations of 3%. Although, *parsley*-apiole and myristicin are very similar in their chemical structure, as the difference is only

a methoxyl group; *parsley*-apiole is thought to be significantly active than myristicin which is suggested to be due to electronic and/or steric factors in phenylpropenes which is essential in the fungal growth inhibition (de Almeida *et al*., 2009 and Pineda *et al.*, 2018). Both components showed a significant antifungal effect against *C. acutatum* as compared to control. It was also indicated by Semeniuc *et al*. (2017) and Khalil *et al.* (2018) that PEO has an antibacterial effect when tested with Gram +ve and –ve bacteria.

CONCLUSION

The present study reflects the significant potential of introducing a natural alternative product in managing two fungal plant pathogens as well as reducing fungicides chemical usage, and accordingly obtains healthier and nontoxic plant products in addition to reducing the environmental chemical pollutants. Results clarified that parsley essential oil (PEO) has a significant effect as an antifungal agent. Nanoemulsion form showed higher effect on *A. solani* and *R. solani* growth than the PEO, due to its smaller particle size and accordingly its lipophilicity. The authors recommend more applicable studies concerning field experiments to be applied on a sustainable and wide range.

CONFLICTS OF INTEREST

The author(s) declare no conflict of interest

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