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Thyme Oil Nanoemulsion: Antifungal Activity Against *Sclerotinia sclerotiorum* and Phytotoxicity on Fennel Plant

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ABSTRACT

In the current study, thyme oil nanoemulsion was prepared for controlling of *Sclerotinia sclerotiorum*. Gas chromatography of thyme oil was performed and exhibited these bioactive components thymol, 1,8-cineole, α-terpinene and ρ-cymene as primary components. The nanoemulsion particles of thyme essential oil was evaluated using DLS. The results indicated that the PS after 30 min of sonication was about 36.65 nm, and Transmission electron microscopy analysis showed a spherical shape. Laboratory investigations showed that nanoemulsions at different concentrations (1000-5000 ppm) inhibited the growth of *Sclerotinia sclerotiorum* fungus. At a concentration of 3000 ppm, the nanoemulsion completely inhibited fungal growth. Treating fennel seeds with TEON, TEOE and TEOS had little effect on germination inhibition, with nanoemulsion being the most effective in promoting germination, while nanoemulsion was the best in terms of improving seed germination. As a result of nanoemulsion treatment, damping-off rates were significantly reduced, and plant growth was improved.
Keywords: Fennel, Thyme, Sclerotinia, Emulsion, Suspension.

INTRODUCTION

Fennel (*Foeniculum vulgare*) is an important medicinal herb in Egypt. The fennel plant is a fascinating herb. It has feathery green leaves, a bulbous base, and produces aromatic seeds. People often use different parts of the plant in various culinary applications. The bulb can be sliced and added to salads or roasted, while the seeds are commonly used as a spice in cooking and baking. Plus, the entire plant has a mild, licorice-like flavor that can elevate a dish. White rot diseases in fennel, which is caused by *Sclerotinia sclerotiorum* causing losses incurred in the growth and yield of plants (Hilal *et al.*, 2007 & Choi *et al.*, 2016). *S. sclerotiorum* is one of the most common and successful plant pathogens. Sclerotinia sclerotiorum, commonly known as white rot, is a notorious fungal pathogen that can affect various crops, including fennel. Patches of dead plant components increase and consolidate during the spring, causing significant stand losses (Bolton *et al.*, 2006). Fungicides are effective at controlling rot diseases; however, they have a severe impact on human health and the environment. Long-term intensive use of fungicides in open fields has resulted in the formation of fungicide-resistant populations. In general, the widespread use of fungicides has numerous detrimental environmental consequences that cannot be overlooked (Barnet and

Hollomon, 1998; Hilal *et al.*, 2007; Feng and Zheng, 2007). As a result, various attempts have been made to use natural fungicidal alternatives to control plant diseases. The term "essential" doesn't imply they are vital for life but rather indicates they contain the essence or characteristic fragrance of the plant. Essential oils are used for various purposes, such as aromatherapy, perfumery, skincare, and holistic health practices, due to their aromatic and potentially therapeutic qualities. Each essential oil has a unique chemical composition, giving it specific properties and potential benefits. Thyme oil is derived from the thyme plant (*Thymus vulgaris*) through steam distillation of its leaves and flowering tops. It's a versatile essential oil with a rich, herbaceous, and slightly medicinal aroma. In this respect, oil of thyme has been shown to exhibit antifungal properties against certain fungi (Zengin and Baysal, 2015; Attia *et al.*, 2023). Thyme oil emulsions are stable particles formed by dispersing immiscible or poorly mixed liquids together (Purwanti *et al.*, 2015). Nanoemulsions of thyme oil are interesting formulations that enhance the stability, solubility, and bioavailability of the essential oil. Nanoemulsions involve breaking down the oil into tiny droplets, usually in the nanometer range, which makes it easier to incorporate into various products. Thyme oil itself is known for its antimicrobial, antioxidant, and anti-inflammatory properties. When formulated as a nanoemulsion, it can have improved dispersibility and may be used in different applications such as food and pharmaceuticals. The nanoemulsion technology can enhance the efficacy of thyme oil and enable its use in more diverse products due to its improved stability and shelf life. This could be particularly beneficial in fields like food preservation, where thyme oil's antimicrobial properties could be leveraged. Particle size is what distinguishes oil emulsion from nanoemulsion; when oil particles decrease, emulsion stability increases significantly (Anton and Vandamme, 2011; Hassanin *et al.*, 2017). Thanks to their better active ingredient distribution and reduced environmental drift, recently developed nanomaterials (1–100 nm) hold the promise of safer and more effective fungicide administration (Gogoi *et al.*, 2009; Hassanin *et al.*, 2017; Hammad and Hassanin 2022). The main components of thyme oil that are responsible for its antifungal activity against Fusarium, *Sclerotinia*, *Aspergillus*, *Alternaria*, *Penicillium*, *Rhizopus sp., and Cladosporium* are p-cymene, thymol and 1,8-cineole (Šegvic Klaric *et al.*, 2007; Hassanin *et al.*, 2017; Hammad and Hassanin 2022). In this study, Thyme oil was chosen as a natural biocide for the synthesis of emulsion, nanoemulsion, and suspension in water using a non-ionic surfactant. *In vitro* and *in vivo* antifungal activities of thyme oil (emusion, nanoemulsion, and suspension) were investigated.

MATERIALS AND METHODS

Biochemical analysis of thyme oil using Gas Chromatography:

The biochemical constituents of the emulsion of thyme oil tested was determined using Gas Chromatography (GC) technique, as described by Elhalawany *et al.*, 2019. In this method, GC was performed using a Ds Chrome 6200 Gas Chromatograph. The machine was composed of a flame ionization detector; a 5% phenyl polysillphenylene - siloxane (0.25 mm IDo × 30 m × 0.25 μm film thickness), and Column: BPX-5. The temperature program ranged from 70 to 200 °C at an average of 10 °C/ min., while the used temperature for the detector was 280 °C. Nitrogen flowed at the rate of 30 ml/min., hydrogen at 30 ml/ min. and air at 300 ml/min.

Preparation of emulsions and suspension of thyme oil:

The following is how thyme essential oil nanoemulsion (TEON) is made: Five milliliters of Tween 80, a nonionic surfactant, and ten milliliters of thyme oil were carefully combined until a homogeneous mixture was achieved. After that, 85 ml water was added to the mixture to help it spread and absorb all of the thyme oil, making the final volume 100 ml. After that, a magnetic stirrer was used to agitate the mixture for 30 minutes. An ultrasonicator

(Bande-lin SONOPULS HD 2200, Germany) was used to sonicate the mixture for 15 and 60 minutes at 350 W after it had been divided into two halves. Using DLS, the particle size of 10% TEON for each quantity was determined following 30 d of storage at 27 °C. Thyme emulsion oil essential (TEOE) was created without sonication, whereas thyme essential oil suspension (TEOS) was prepared as follows: 10 ml of oil and five ml of Tween 80 were carefully mixed together until a homogenous mixture formed. Then, 85 ml water was added to make the final mixture 100 ml.

Droplet size of nanoemulsion:

The size of TEON droplets was measured at room temperature utilizing DLS investigations with the Zeta Nano ZS instrument from Malvern Instruments, UK. Prior to testing, 30µl of the nanoemulsion was mixed with 3 ml of water at a temperature of 25 °C. The particle size (PS) data were presented as the average of the Z-averages of three distinct batches of nanoemulsions.

Transmission electron microscopy (TEM):

Twenty microliters of diluted samples were applied onto a film-coated 200-mesh copper specimen grid and left for 10 minutes before being taken off using filter paper. Subsequently, a single droplet of 3% phosphotungstic acid was applied to the grid, which was then allowed to air dry for a duration of three minutes. Following the drying process, the grid that had been coated was examined using TEM microscope model Tecnai G20, Super twin, double tilt, manufactured by FEI in The Netherlands. The samples were analyzed at an operational voltage of 200 kv.

Effect of TEON, TEOE and TEOS on growth of *S. sclerotiorum*:

Method of the paper disc published by Šegvic Klaric *et al.,* (2007) was used to investigate the efficacy of TEON, TEOE and TEOS in inhibiting fungal growth. Three PDA medium-containing plates were infected with *S. sclerotiorum* discs (5 mm in diameter). The Petri dishes were inoculated with microorganisms and small circular pieces of filter paper (5 mm in diameter) were soaked with different concentrations (0, 1000, 3000, 5000 parts per million) of the volatile oil solutions. These paper discs were then positioned in the middle of the covers of the plates and kept in a controlled environment at a temperature of 18 °C for incubation. The formula proposed by Topps and Wain (1957) was used to calculate the inhibition of fungal growth. This was determined when the fungal growth of the control plates completely covered the plates. as follows:

$$\% \text{ Inhibition} = \frac{A - B}{A} \times 100$$

A= The linear growth of control.
B= The linear growth of treated fungus.

Effect of TEON, TEOE and TEOS on seed germination:

A total of 300 fennel seeds were immersed in oil formulations (TEON, TEOE, & TEOS) at concentrations of 0, 1000, 3000, and 5000 ppm for a duration of 15 minutes. Subsequently, the treated seeds were placed in Petri dishes with moistened blotters, with each dish accommodating 100 seeds. The dishes were subsequently placed in a highly regulated environment (27 °C) and incubated for a period of 15 days, following alternating cycles of 12 hours of light and 12 hours of darkness. Ultimately, the germination ratio for each treatment was calculated as follows:

$$\% \text{ Germination} = \frac{\text{Germinated seeds number}}{\text{Total numbers of tested seeds}} \text{x } 100$$

Greenhouse studies

The greenhouse investigations utilized pots with a diameter of 25 cm, which were filled with soil consisting of a 1:1 ratio of sand to clay (by weight). Formalin-treated soils were contaminated with *S. sclerotiorum* at a rate of 3% of the soil's weight, with three replicates. Antifungal activity of TEON (3000 ppm), TEOE & TEOS (5000 ppm), and fungicide: Switch 62.5% WG (a-Cyprodinil b-Fludioxonil) (2 g/l) suspension was applied. Prior to being planted in the contaminated soil, the seeds were immersed in the emulsions and fungicide that were previously created for a duration of 20 minutes. As a control, some seeds were soaked in water only. Each treatment and control group consisted of three pots. Sixty seeds of fennel (20 seeds per pot) were distributed to each treatment. Damping-off percentages were observed after 15 and 45 days for both pre-emergence and post-emergence. Plant height and root length of healthy surviving plants were measured 90 days following seeding.

RESULTS AND DISCUSSION

Thyme oil composition:

Using GC technique, the results in Table (1) appeared that the major components of thyme oil were thymol (27.945 %); 1.8-cineole (16.34 %), ρ-cymene (15.744 %) and α-terpinene (10.983 %). On the other hand, little amounts of β-caryophyllene (1.628%) and α-pinene (1.356 %) were detected. These findings are somewhat in according with those of Grigore *et al.*, 2010, who performed a quantitative analysis of thyme essential oil using GC and a qualitative analysis HPTLC. The results reported that essential oil of thyme contained thymol and p- cymene in high quantities.

Table 1. Chemical composition of thyme oil detected using GC.

Component	Area %
α-terpinene	10.983
α-pinene	1.356
Broneol	4.179
Camphene	2.187
Myrcene	4.682
Thymol	27.945
ρ-cymene	15.744
Limonene	5.729
1.8-cineole	16.34
γ-terpineol	4.184
β-caryophyllene	1.628
Sabinene	3.122
Total	98.079

Effect of sonication on droplet of oil:

The influence of sonication on size of TEON was investigated. (Fig.1). After 30 days of storage at room temperature, it demonstrated the stability of TEON generated by ultrasonication for 30 minutes at 350 W. Sonication has a significant impact on size of droplet. The observed increase in sonication duration can be attributed to an elevation in shear pressures exerted on the droplets, resulting in enhanced droplet fragmentation. It is recommended that the droplet size decreases as the sonication time increases. Figure (1) represents a PS created by ultrasonication for 30 minutes. It was small (about 36.65 nm) and had a high polydispersity index (PDI) of 244. Surfactant ability and performance may impact droplet size reduction. Stirring has been found to decrease the size of droplets in an oil-in-water emulsion (Sajjadi et al., 2002). In the presence of double bonds in the nonpolar chain of nonionic surfactants, Dai *et al.* (1997) examined the creation of nano-emulsions with smaller sizes of droplet. The findings confirmed recent findings (Shahavi *et al.*, 2015; Doghish *et al.*, 2023).

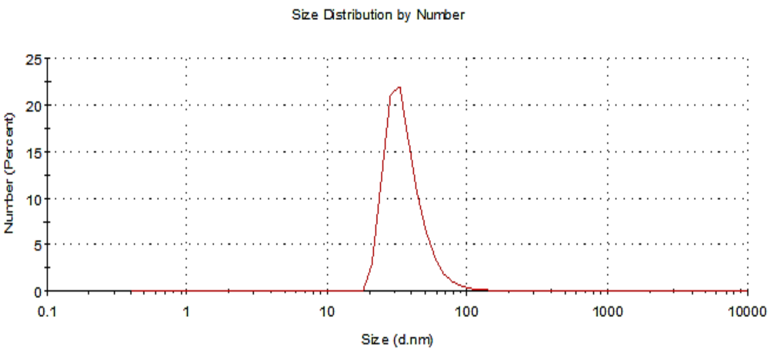

Fig. 1. Effect of sonication on particle size of TEON prepared by sonicator for 30 min. (peak at 36.65 nm) & (PDI= 244).

TEM analysis:

The real shape and size of the TEON are revealed by TEM analysis; droplets of nanoemulsion appear black. The TEON was round in form and relatively homogeneous, with dimensions ranging from 29.1 nm to 51.3 nm (Fig.2). Size of droplet correlated well with the results of DLS droplet size study (Abd-Elsalam and Khokhlov, 2015; Doghish *et al.*, 2023).

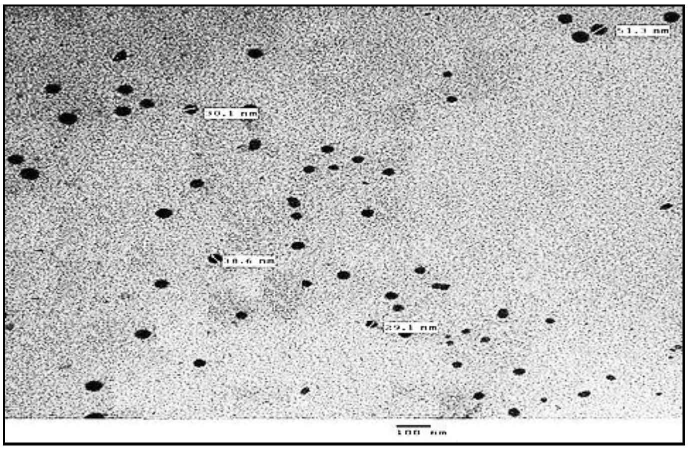

Fig.2. The result of sonication on the TEM of TEON generated by sonicator for 30 minutes (with sizes ranging from 29.1 nm to 51.3 nm).

Effect of thyme oil on *S. sclerotiorum* growth:

According to the data in Table 2, TEON was associated with a decrease in fungal growth where fully stopped. At a concentration of 3000 ppm, mycelial growth occurs. In contrast, TEOE was always associated with a reduction in growth. The diameter of fungal growth, however, was 9.0 (control), 2.3, 1.7 and 0.5 cm at concentrations of 0 (control), 1000, 3000 and 5000 ppm, respectively. Also, decreased the fungal growth with TEOS, 9.9, 3.1, 2.2 and 0.9 at the concentrations of 0 (control), 1000, 3000 and 5000 ppm, respectively. The size of the nano particles is what distinguishes it from emulsion. The stability of an emulsion much improves when the size of the oil particles is reduced (Anton and Vandamme, 2011). Nanoparticles (100 nm) are being developed and offer the possibility to administer fungicides, insecticides, fertilizers and herbicides more efficiently and safely due to greater active component delivery and reduced environmental drift (Gogoi *et al.*, 2009). Zedan *et al.*, 1994 reported on the antifungal harmful effects of many oils, including the one under investigation. According to Šegvic Klaric *et al.*, 2007, thymol, 1,8-cineole, and p-cymene discovered in thyme oil are responsible for its antifungal effect. However, the activity of thyme oil can be ascribed to its ability to penetrate the cell wall made of chitin. This penetration destroys the lipoprotein cytoplasmic membrane, which in turn allows the cytoplasm to escape (Zambonelli *et al.*, 1996).

Table 2. Effect of different concentrations of TEON, TEOE & TEOS on linear growth of *S. sclerotiorum*.

Treatments	Linear growth (cm) at concentration of:			
	0 ppm	1000 ppm	3000 ppm	5000 ppm
(TEON)	9.0	0.8	0.0	0.0
(TEOE)	9.0	2.3	1.7	0.5
(TEOS)	9.0	3.1	2.2	0.9
L.S.D. at 5% for:	**Treatment (A)= 0.92**	**Concentrations (B)= 1.0**	**A × B= 1.3**	

Effect of TEON, TEOE, and TEOS on seed germination:

The results demonstrated the influence of TEON, TEOE, and TEOS on germination of fennel seeds when employed as a soaking treating for a specific period of time (Table 3). TEON treatment resulted in 100% germination of treated seeds at doses of 1000, 3000, and 5000 ppm. In the instance of TEOE, germination (%) was gradually enhanced by increasing concentration to achieve 50.0%, 66.7%, and 70.0% at the concentrations of 1000, 3000 and 5000 ppm, respectively. On other hands, the effect of TEOS on seeds germination of fennel was the least effective in germination increasing. The results of our study can be interpreted in terms of the various types of terpenoids found in plant oils and their potential impacts on seed germination. Terpenoids, particularly sesquiterpenes, are said to be a class of chemicals with varying biological activity. These compounds revealed a unique structure-activity relationship at low concentrations (Beekman *et al.*, 1997; Hassanin *et al.*, 2017).

Table 3. Effect of TEON, TEOE & TEOS on percentage of seeds germination.

Treatments	% Concentrations			
	0 ppm	1000 ppm	3000 ppm	5000 ppm
TEON	100	100	100	100
TEOE	100	50.0	66.7	70.0
TEOS	100	44.0	54.0	68.7
L.S.D. at 5% for:	Treatment (A)= 10.3	Concentrations (B)= 9.4	A × B= 11.03	

Greenhouse tests:

Based on the findings shown in Table (4), all of the treatments significantly reduced the ratios of pre- and post-emergence damping-off induced by Sclerotinia sp. compared to the control group. Additionally, the treatments noticeably improved plant survival. TEON exhibited the highest efficacy in reducing both pre- and post-emergence damping-off rates, with percentages of 8.3% and 13.3% respectively. As a consequence, it outperformed the control in terms of seedling survival (78.4%). All treatments examined enhanced plant growth parameters such as root length and plant height when compared to the control (untreated), according to the data (Table 4). There was substantial variation between these treatments and the controls. The choicer seed soaking treatment overall was TEON, which resulted in higher parameters (plant height: 30.0 cm & root length: 17.3 cm) than the other treatings, followed by TEOE and Switch fungicide. In comparison to the control, TEOS had the least influence on plant height (27.0 cm) and root length (15.5 cm). The small size of nano-emulsion particles may contribute to its antifungal efficacy (Hassanin *et al.*, 2017). The rise in plant growth metrics can be attributed to biochemical alterations in the stem base tissues, or it may be linked to their effectiveness in reducing or preventing disease infection and progression. This change leads to an increase in growth hormones, peroxidase enzyme activity, and phenol compounds. Hassanin (2013 & 2017), Hammad and Hassanin (2022), and Zedan *et al.* (2011) reported similar results on several crops grown in soil that was either naturally or intentionally contaminated .

Table 4. Effect of (TEON, TEOE and TEOS) and Switch on damping-off incidence % and growth of fennel plants under greenhouse conditions.

Treatments	Pre-emergence (%)	Post-emergence (%)	Survivals (%)	Plant height (cm)	Root length (cm)
(TEON)	8.3	13.3	78.4	30.0	17.3
(TEOE)	11.7	20.0	68.3	29.2	17.0
(TEOS)	23.3	26.7	50.0	27.0	15.5
Switch	18.3	20.0	61.7	28.3	16.4
Control	38.3	41.7	20.0	21.0	11.2
L.S.D. at 5%	13.4	10.3	19.7	6.5	6.1

Conclusions:

The present investigation evaluated efficacy of TEON, TEOE, & TEOS as antifungal and management Sclerotinia rot disease of fennel compared with fungicide (Switch 62.5% WG). TEON and TEOE were the more effective treatments tested since they gave significant decreases in disease. These results indicate TEON, TEOE possesses strong antifungal properties and also, the possibility of using as an alternative fungicide agent against Sclerotinia rot disease to fennel plants.

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