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Research Article

Eco-friendly preparation of thyme essential oil nano emulsion: Characterization, antifungal activity and resistance of *Fusarium* wilt disease of *Foeniculum vulgare*

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Abstract

Essential oil nanoemulsions have received much attention in the last period for controlling of fungal plant pathogens. In this study, thyme oil nanoemulsion (TONE) was successfully prepared from thyme oil which extracted from *Thymus vulgaris* (*T. vulgaris)*. The prepared TONE was characterized using DLS, Zeta potential, and TEM analyses. Results revealed that, TONE has spherical shape with size 32.7 nm. Moreover, results illustrated that TONE exhibited promising antifungal activity against *Fusarium oxysporum* (*F. oxysporum*) with minimum fungicidal concentration (MFC) 5 mg/ml. Additionally, TONE concentrations 1, 2, 3 and 4 mg/ml reduced the growth of *F. oxysporum* with percentages 7.78, 31.1, 52.2 and 67.8 % respectively. Disease index (DI) of *Fusarium* wilt reached the maximum rate by (85 %) in the *Foeniculum vulgare* (*F. vulgare*) plant infected with *F. oxysporum*. Application of TONE treatment on infected plants led to a decrease in DI to (17.5%) and an increase in the percentage of protection to (79.4%). Furthermore, DI was decrease to 42.5% with protection percentage 50% in the case of infected plant with TOE. Moreover, TOE, TONE played an important role in improving plant immunity by increasing phenol, proline, and antioxidant enzymes (POD&PPO) activities, as well as reducing oxidative stress by reducing (MDA & H_2O_2**)**. Results revealed that TONE led to significant increase in free proline in compared to TOE. We can conclude that TOE, TONE are considered eco-friendly safe strong inducers of *F. vulgare* plant immunity alternatives to difenoconazole against fusarial wilt disease to preserve plant, soil, and human health.

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Keywords: antioxidant enzymes; *Foeniculum vulgare*; *Fusarium oxysporum*; nanoemulsion; thyme oil; wilt disease

Introduction

Foeniculum vulgare Mill commonly called fennel has been used in traditional medicine for a wide range of ailments related to digestive, endocrine, reproductive, and respiratory systems. It is additionally used as a galactagogue agent by breastfeeding women (Badgujar *et al*., 2014). The ability of *F. vulgare* to display fungicidal, bactericidal, antioxidant, antithrombotic, and hepatic-protective effects has been well supported by many pharmacological studies (Rather *et al*., 2016). *Fusarium* fungus is ubiquitous since it can be found in all habitats including soil and water, the soil-born fungus prevalent in both conventional and organically grown soils are represented by *Fusarium* sp (Khalil *et al*., 2015; Shoayb *et al*., 2023). Wide range of plants, including *Solanum lycopersicum* (Attia *et al*., 2016), *Capsicum annuum* (Abdelaziz *et al*., 2022a), *Solanum melongena* (Abdelaziz *et al*., 2023b), *Cucumis sativus* (El-Batal *et al*., 2023) and *Foeniculum vulgare* (Shaker and Alhamadany, 2015), are thought to be susceptible to *Fusarium* wilt, which is a causing destruction effects.

Synthetic fungicides are the most effective method for combating a *Fusarium* wilt diseases. However, due to their continued use, unwanted pathogen resistance has emerged. In addition, their residues in soil and food are harmful to people and the environment (Farrag *et al*., 2017). Thus, alternative green, safe antifungal biofungicides are required (Hashem *et al*., 2022a). Nanoparticles are widely used as antimicrobial agents for combating pathogenic bacteria and fungi (Ali *et al*., 2022; Hasanin *et al.*, 2023; Hashem *et al*., 2022b; Hashem *et al*., 2022c; Lashin *et al*., 2023; Saied *et al*., 2022a; Saied *et al*., 2022b) Nanoemulsions have shown promising antimicrobial properties due to their tiny droplet size and larger surface area, which enhances their interactions with microorganisms (Hwang *et al*., 2013). They have been studied for their potential use as a natural alternative to traditional antimicrobial agents in various applications, including agriculture, food preservation, medical devices, and personal care products (Hashem *et al*., 2023). Several articles have reported the fungicidal effect of nanoemulsions against plant pathogens, including *Fusarium*, *Phytophthora*, and *Botrytis* (Javanmardi *et al*., 2023). *T. vulgaris* nanoemulsion exhibits antifungal activity against a variety of fungal plant diseases by suppress the growth of fungi that cause plant diseases such as *Fusarium oxysporum*, *Botrytis cinerea*, and *Penicillium expansum* (Hassanin *et al*., 2017a; Zhang *et al.,* 2022). Herein, this study aimed to prepare and characterize *T. vulgaris* essential oil nanoemulsion (TONE) through ecofriendly method. Also, to assess the antifungal activity of it toward *F. oxysporum* as well as control of *F. vulgare Fusarium* wilt *in vivo*.

Materials and Methods

Materials

Sodium hypochlorite, PDA, phosphotungstic acid, tween 80 were purchased from Sigma aldrich, Germany. F. vulgare seeds were obtained from ARC, Giza, Egypt.

Isolation and identification of pathogenic fungal isolate

Infected *F. vulgare* stem was washed repeatedly, then cut into 1 cm^2 and superficial sterilized by 1% NaClO for 2 min, splashed with sterilized water and dried by sterilized filter papers, Finally, rising fungi were purified using hyphal tip procedures after being first plated into potato dextrose agar (PDA) medium and cultured at 27 °C for 7 days (Abdelaziz *et al*., 2022b). Then identified morphologically (Crous *et al*., 2006; Huang *et al*., 2016; Phillips *et al*., 2013) but molecular identification was carried out method used by Khalil *et* *al*. (2021). The genomic DNA was isolated and purified using Quick-DNA Fungal Microprep Kit (Zymo research; D6007), and molecular identification was achieved by internal transcribed spacer (ITS) region. The primers sequences in this protocol were Forward ITS1-F (50 -TCCGTAGGTGAACCTGCGG-30) and Reverse ITS2-R (50 -TCCTCCGCTTATTGATATGC-30) according to Visagie *et al*. (2014). Gene JET PCR Purification Kit (Thermo K0701) was used for purification of PCR product according to the manufacturer's protocol. The resulting PCR products were sequenced by sequencing ready reaction kit (Applied Biosystems, Foster, CA, USA). Similar sequences via BLAST search database in the NCBI were compared with product sequence. Evolutionary study was directed in molecular evolutionary genetics analysis MEGA-x software (Kumar *et al*., 2018).

Preparation of nanoemulsion

The steam distillation method was used for extraction of essential oil according to method used by (Ratri *et al*., 2020). Dried and ground thyme leaves (50 g) were put in a steam flask. The steam distillation lasted 6 h. The recovered condensate was distilled again using n-hexane as the solvent. By evaporating the n-hexane, thyme oil was produced. To prepare TONE, 5 ml of tween 80 was added slowly to 20 ml of thyme oil with gently stirring for 40 min, and then completed to 100 ml with distilled water. An ultra-sonication was used to sonicate the mixture for 40 min at 350 W. Before sonication, the essential oil emulsion was made as previously indicated.

DLS and Zeta potential

At room temperature, the Zeta Nano ZS (Malvern Instruments, UK) was used to measure the size of the essential oil nanoemulsion droplets using a dynamic light scattering analysis. Before testing, 30 µl of nanoemulsion was watery diluted with 3000 µl at 25 °C. The mean of the Z-average of three separate batches of the nanoemulsion was used to express particle size information, the nanoemulsions droplet size and polydisparity index (PDI) were examined. The surface charge of each formulation was determined by measuring the zeta potential of the nanoparticles at 25°C (Honary and Zahir, 2013).

Transmission electron microscopy (TEM)

To carry out TEM, 20 microliters of diluted sample were placed on a film-coated 200-mesh copper specimen grid for 10 min, and the excess fluid was eliminated using filter paper. The grid was then stained with 1 drop of 3% phosphotungstic acid and allowed to dry for 3 min. The coated grid was dried and examined under the TEM microscope (Tecnai G20, Super twin, double tilt, FEI, Hillsboro, OR, USA), operating at 200 kV (Saloko *et al*., 2013).

Antifungal activity of TONE using radial growth method

Linear growth method was used for assessment of antifungal activity of TONE toward *F. oxysporum* according to the approach employed by Joshi, De Britto, Joshi *et al.* (2019) at various concentrations (1, 2, 3, 4 and 5 mg/ml) with minor modifications. Inhibition % of *F. oxysporum* was calculated as the following:

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

A= Linear growth of control.

B= Linear growth of treated fungus.

In vivo experiment (greenhouse experiment)

Seedlings of *F. vulgare* (20 days old) per pot (30 cm in diameter) containing a mixture of sand and clay (1:3 W/W) with a total weight of 4 kg were sowed to produce seedlings of the *F. vulgare* plant (soil infection with the pathogen at a rate of 1% w/w). Ten replicas of each treatment were delivered along with the pots. The managements were organized as follows: T1-healthy control, T2-infected control, T3-infected plants treated with TOE at conc. 5 ml/l water, T4-infected plants treated TONE) at conc. 5 ml\l water, and T5-infected plants treated with Difenoconazole 25% in Emulsifiable concentrate form. Daify Core manufactured by Sinochem Ningbo Chemicals Co., Ltd. – China) at the rate (2 ml/l).

Inoculum of pathogen and inducers preparation

Pathogenicity was made with the pathogenic fungus by method involves blending an electrical blender for 2 min with the contents of five pure *F. oxysporum* culture Petri dishes and 1000 ml of purified water (Abdelaziz *et al*., 2021). *F. vulgare* plants were applied with treatments after one week of planting (20 ml per plant once every week, after 45 days following sowing, biochemical signals from plant samples were analysed, and the progression of the disease was measured, with the purpose of evaluating plant resistance.

Disease index and protection

After 45 days of infection, disease symptoms were noted, and disease severity and protection % were evaluated using five score categories as follows: 0 (no symptoms), 1 (mild yellowing of lower leaves), 2 (moderate yellowing of the plant), 3 (wilting of the plant), and 4 (severe stunting and destruction of the plant). The five-grade scale was used to calculate the disease index (DI), which was determined as follows: DI= (1n1+ 2n2+ 3n3+ 4n4)100/4nt. Where Nt represents the total number of plants tested and n1–n4 the number of plants in each of the classes mentioned. Protection% = A-B/A X100% Where A is the PDI in the infected control plants and B is the PDI in the treated infected plants (Attia *et al*., 2016).

Metabolic indicators for F. vulgare plant resistance

Free proline was estimated by the method of Bates *et al*. (1973) as follow, a total of 5 g of dried shoots were subjected to extraction using 10 ml of sulfosalicylic acid solution (3% concentration). Subsequently, 2 ml of the resulting extract were combined with 2 ml of ninhydrin acid and 2 ml of glacial acetic acid. This mixture was then subjected to boiling conditions for a duration of one hour, after which the reaction was promptly halted by the addition of ice. Finally, 4 mL of toluene was added to the mixture, and assayed at 520 nm. plant phenolics (PPc) estimated by the method of Dai *et al.* (1993) , 1 g of dried Fennel leaves were extracted in 10 mL of 80% ethanol. The filtrate was subsequently replenished to a volume of 50 mL using a solution consisting of 80% ethanol. After that, a total of 0.5 mL of the filtrate was thoroughly combined with an equal volume of Folin's reagent, followed by agitation for a duration of 3 min. Subsequently, 3 mL of distilled water and 1 mL of a saturated sodium carbonate solution were added to the mixture. The resulting solution was then subjected to detection at a wavelength of 725 nm. The procedure of Hu *et a*l. (2004) was used to assayed the MDA content in fresh Fennel leaves. Fresh Fennel leaves also were established for hydrogen peroxide H_2O_2 content (Mukherjee and Choudhuri, 1983). Accepted method of Srivastava (1987) was used to determine POD. The activity of PPO enzyme was estimated by the technique of Matta (1969).

Statistical analysis

The results were subjected to a one-way ANOVA, while mean differences were determined using the least significant difference with the help of Co State software.

Results

Isolation and identification of F. oxysporum

Isolation of pathogenic fungi from *F. vulgare* plants is an important step in studying the plant-fungal interactions and developing strategies for managing fungal diseases in plants. Fungal isolate AMMA2 was isolated from *F. vulgare*. AMMA2 isolate appeared pale violet to white in surface color, pale violet to brown in reverse color, growth diameter 25-45 mm (Figure 1 A & B). Macroconidia are tiny and slightly curved (Figure 1C). A BLAST search on NCBI revealed that the fungal isolate AMMA2 was recognized as *Fusarium oxysporum* with a similarity of 94.8%. Additionally, the gene bank's accession number for this sequence is OQ341165.

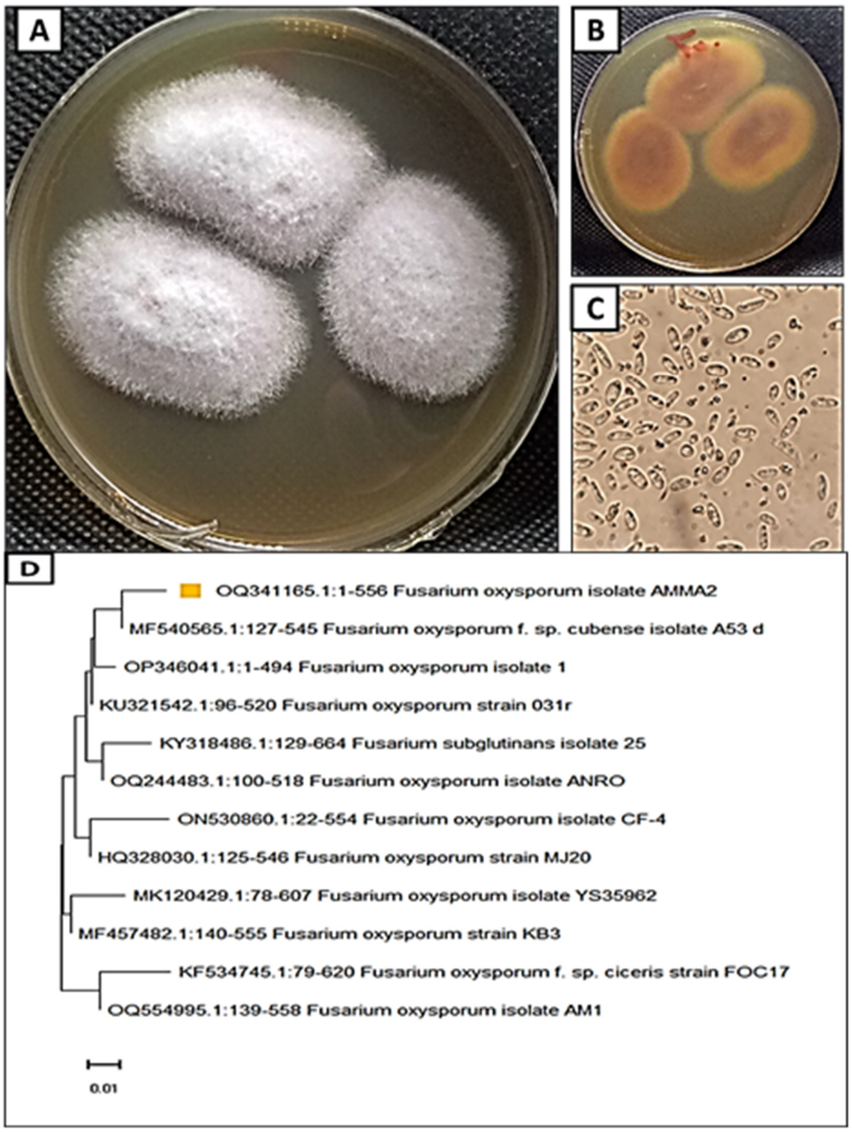

Figure 1. Morphological and molecular identification of *F. oxysporum* (A-D): A) Surface colonies on PDA; B) Reverse colonies; C) Conidia; D) The phylogenetic tree

Preparation and characterization of TONE

T. vulgaris essential oil was extracted from *T. vulgaris L.* using ecofriendly method. The extracted oil was used for preparation of TONE where color changes to white. To confirm formation of TONE, DLS, zeta potential and TEM analyses were carried out. DLS and Zeta potential of TONE was completed as depicted in Figure 2 A & B. TONE droplets was 32.7 nm in size, and Poly dispersity index for particles was 0.244. Moreover, results illustrated that, zeta potential of TONE had negative charge (-30.4 mV).

T. vulgaris essential oil nanoemulsion was characterized using transmission electron microscopy to determine the true size and shape of the droplets. TEM results illustrated that TONE appeared spherical in shape and monodispersed. Additionally, the size of TONE droplets was in the range of 32.3 – 53.4 nm (Figure 3).

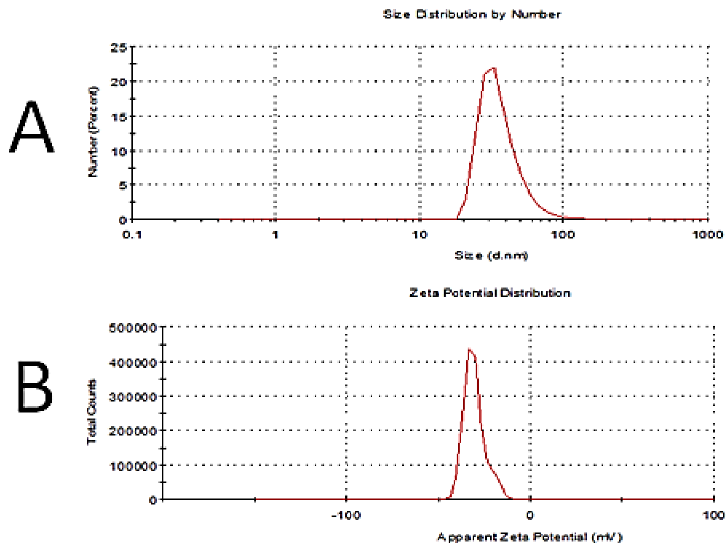

Figure 2. DLS (A) and Zeta potential (B) of TONE

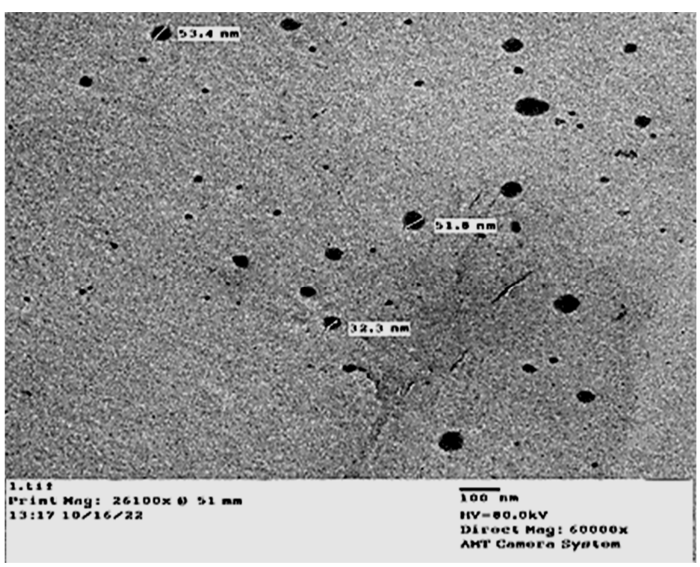

Figure 3. TEM of the prepared TONE

In-vitro antifungal activity of TONE using linear growth method

In the current study, antifungal activity of TONE toward *F. oxysporum* using linear growth was evaluated (Figure 4). Results illustrated that the increase in TONE concentration led to a decrease in linear growth of *F. oxysporum* as shown in Figure 4A. Moreover, the low concentration of TONE at 1 mg/ml led to mild decrease in linear growth where was 89 mm, but in higher concentrations at 3 and 4 mg/ml led to severe decreasing in linear growth where were 43 and 29 mm (Figure 4B). Furthermore, growth reduction percentages of *F. oxysporum* was calculated using linear growth of control and treated fungus. As illustrated in Figure 4B, results revealed that MFC of TONE was 5 mg/ml where reduction of growth was 100%. On the other hand, TONE concentration 1, 2, 3 and 4 mg/ml reduced the growth of *F. oxysporum* with percentages 7.78, 31.1, 52.2 and 67.8% respectively.

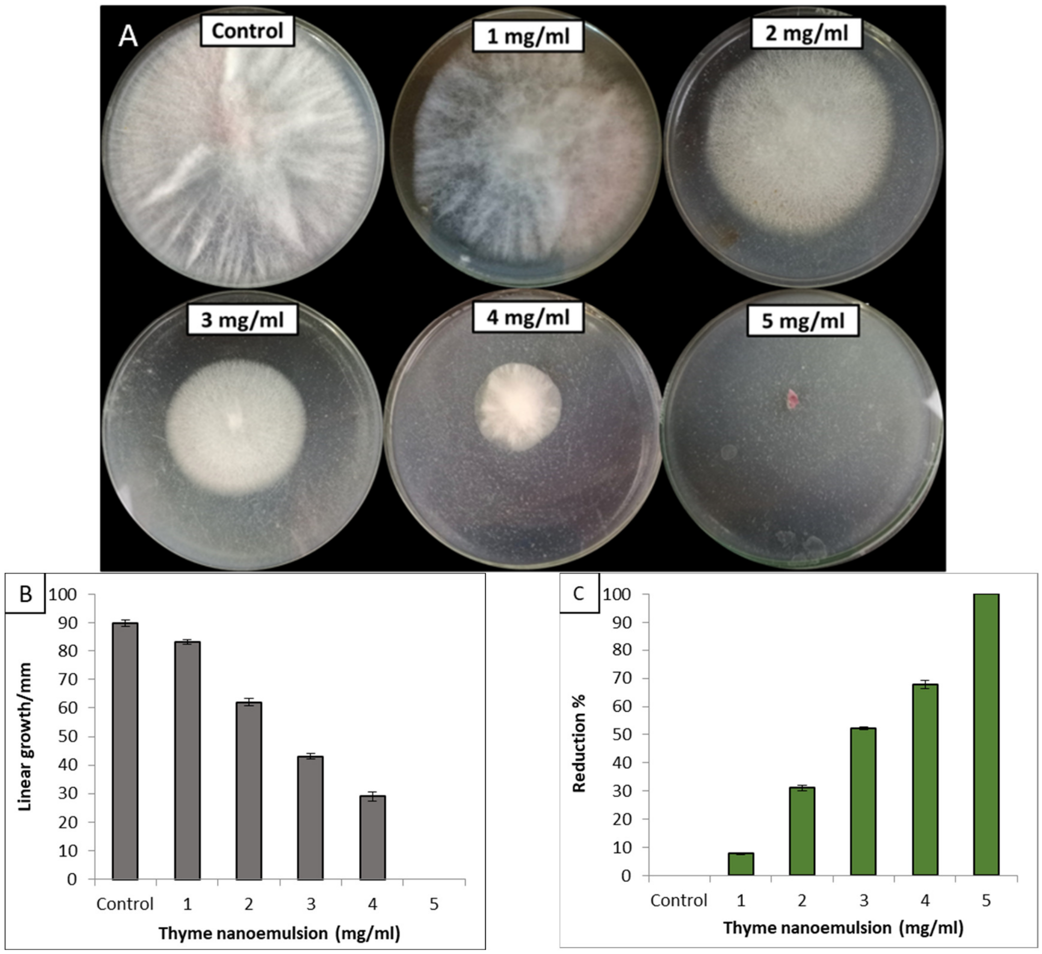

Figure 4. Effect of TONE concentrations on linear growth (A&B) and reduction % of *F. oxysporum*.

In vivo experiment (greenhouse experiment)

Disease evaluation

As shown in the Table 1, the results showed that the symptoms of *Fusarium* wilt infection were very clear and DI reached the maximum rate by (85%) in infected *F. vulgare* with *Fusarium* only. On the other hand, the symptoms of *Fusarium* wilt infection were reduced in infected *F. vulgare* plants with all treatments. Also, it is expected to reduce the severity of the infection by using the fungicide (difenoconazole). The results showed that the application of the difenoconazole gave the lowest DI% to (15%). Interesting application of

TONE treatment on infected plants led to a reduction in DI to (17.5%) and an increase in the percentage of protection to (79.4%) then treatment with TOE on infected plants which led to a lessening in DI by (42.5%) and an increase in the percentage of protection to (50%).

Table 1. Effect of TOE and TONE and difenoconazole on disease index

Treatment	Disease symptoms classes					DI (disease index) (%)	Protection (%)
	0	1	2	3	4		
Control Infected	0	0	1	2	7	85	0
Infected + TOE	2	3	2	2	1	42.5	50
Infected + TONE	5	3	2	0	0	17.5	79.4
Infected + fungicide	6	2	2	0	0	15	82.3

Effect of TOE and TONE and difenoconazole on biochemical defense system F. vulgare plants

Free proline and phenol contents

The contents of free proline and total phenols in infected plants, infected plants treated with TOE, TONE and difenoconazole were determined (Figures 5 & 6). Results revealed that *F. oxysporum* caused a marked significant increase in free proline and phenol contents of the infected *F. vulgare* plants by 55.83% and 8.12 % compared with healthy control. Concerning the effect of TOE, TONE and difenconazole on the challenged *F. vulgare* plants with *F. oxysporum*, it was found that TONE show significant increase in free proline compared to TOE and difenoconazole. Regarding the effect of treatments (TOE, TONE and difenoconazole) on the challenged fennel plants with *F. oxysporum*, it was found that (TONE) show significant increase in total phenol by (47.4%) related to difenoconazole by (4.04%) and came next (TOE) by (1.15%), respectively (Figure 6).

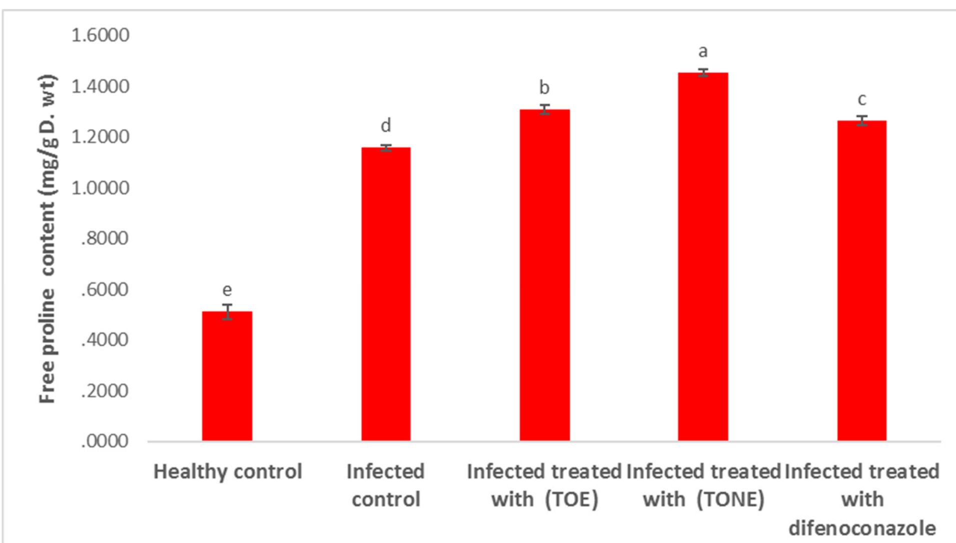

Figure 5. Effect of thyme oil emulsion (TOE), thyme oil nanoemulsion (TONE) and fungicide (Difenoconazole 25%) on free proline content of infected *F. vulgare* plants with *Fusarium* wilt
(Data represent mean± SD, n=3), letters revered to significant in statically analysis.

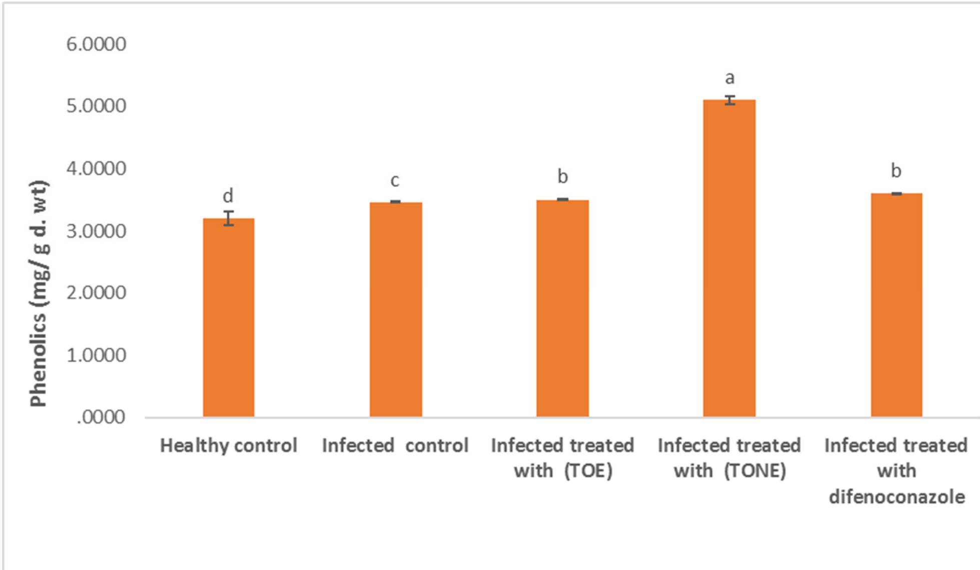

Figure 6. Effect of thyme oil emulsion (TOE), thyme oil nanoemulsion (TONE) and fungicide (Difenoconazole 25%) on phenolics content of infected *F. vulgare* plants with *Fusarium* wilt
(Data represent mean± SD, n=3), letters revered to significant in statically analysis.

MDA and H_2O_2

Data presented in Figure 6 revealed that, *F. oxysporum* cause a marked significant increase in MDA and H_2O_2 contents of the infected *F. vulgare* plants by 76.32 % and 93.19 % compared with healthy control. It was noticed in (Figure 7 A, B) that, those levels of MDA as well as H_2O_2 in *F. oxysporum* - infected plants were decreased due to (TOE, TONE and difenoconazole) treatments. Concerning the effect of (TOE, TONE and difenoconazole) on the challenged fennel plants with *F. oxysporum*, it was found that (TONE) show highly significant decline in (MDA and H_2O_2) by (21% and 44%) of related to (TOE) by (14.5% and 42%) and came next (difenoconazole) by (10.6% and 40.7%), respectively (Figure 7 A, B).

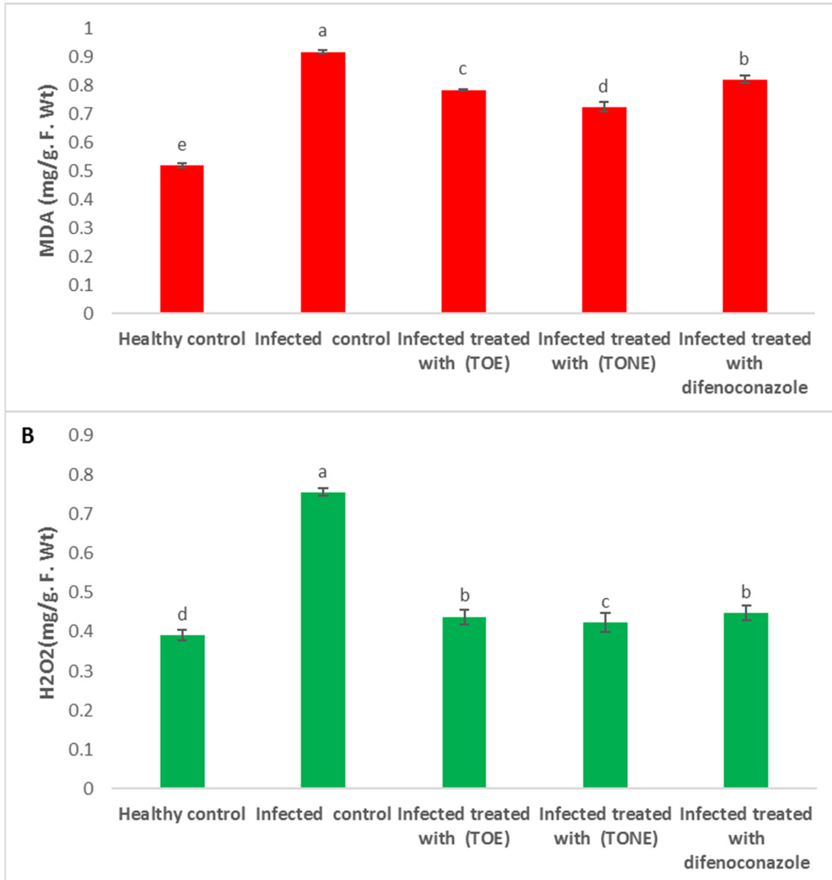

Figure 7. Effect of thyme oil emulsion (TOE), thyme oil nanoemulsion (TONE) and fungicide (Difenoconazole 25%) on A) MDA and B) H2O2 of infected *F. vulgare* plants with *Fusarium* wilt
(Data represent mean± SD, n=3), letters revered to significant in statically analysis.

Antioxidant enzymes activity

Figure 8 showed that, *F. oxysporum* cause a noticeable increase in peroxidase (POD) as well as PPO activities of the infected *F. vulgare* plants by (145.14 % and 68.70 %) compared with healthy control. As shown in Figure 8, the results showed that that, those activities of POD as well as PPO in *F. oxysporum* - infected plants were raised due to TOE, TONE and difenoconazole treatments. Concerning the effect of (TOE, TONE and difenoconazole) on the challenged fennel plants with *F. oxysporum*, it was found that (TONE) show highly significant increase in POD and PPO by (17% and 101%) related to (TOE) by (4.6% and 64.1%), respectively (Figure 8). On the other hand, application of difenoconazole on infected plants caused significant decrease in POD by 7.6% and significant increase in PPO by (28.5%) Consequently, we can opinion that TOE, TONE is a vital elicitors of plant resistance and a safe alternative to chemical fungicides. The results of the current study confirm that the application of TOE, TONE on infected plants induces the formation of antioxidants and increases the activity of enzymes that play an important role in mitigating the damage caused by infection, which indicates interest in these treatments as safe and effective alternatives in eliminating plant diseases.

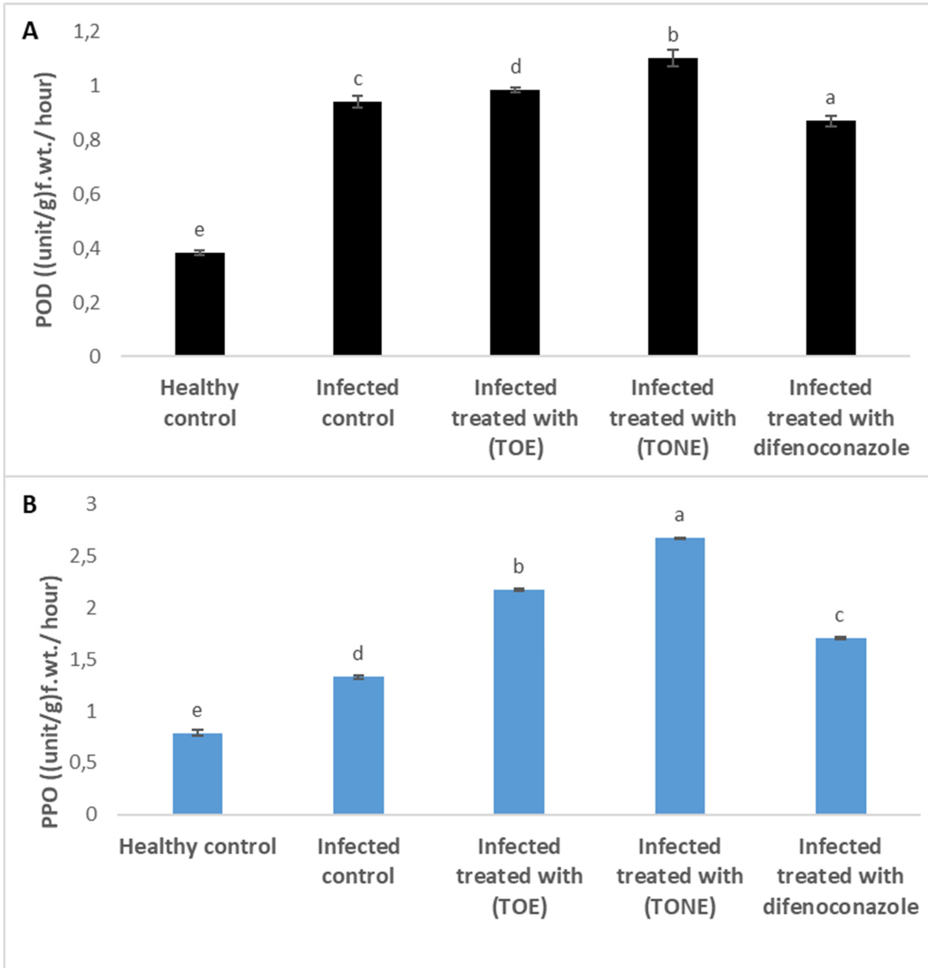

Figure 8. Effect of thyme oil emulsion (TOE), thyme oil nanoemulsion (TONE) and fungicide (Difenoconazole 25%) on A) POD and B) PPO of infected *F. vulgare* plants with *Fusarium* wilt
(Data represent mean± SD, n=3), letters revered to significant in statically analysis.

Discussion

Plant pathogens are becoming increasingly ferocious day by day, and it has become necessary to find safe and effective alternatives to control plant diseases. Isolation of pathogenic fungi from *F. vulgare* plants is an important step in studying the plant-fungal interactions and developing strategies for managing fungal diseases in plants. A BLAST search on NCBI revealed that the fungal isolate AMMA2 was recognized as *Fusarium oxysporum* with a similarity of 94.8%. Additionally, the gene bank's accession number for this sequence is OQ341165. Our results confirmed by Shaker and Alhamadany (2015) who isolated *F. oxysporum* from *F. vulgare* that causing wilting and yellowing, stunting on the plants. *T. vulgaris* essential oil was extracted from *T. vulgaris L.* using ecofriendly method.

TONE droplets was 32.7 nm in size, and PDI for particles was 0.244. The small size of the nanoemulsion is attributed to performance of surfactant with good stirring (Sajjadi *et al.*, 2002). Moreover, results illustrated that, zeta potential of TONE had negative charge (-30.4 mV). Hassanin *et al.* (2017b) prepared TONE for using as antifungal agent, and characterization illustrated that the size of TONE was 34.6 nm. Another study, TONE was prepared for controlling of *F. oxysporum*, the size of TONE was 48.1 nm (Hammad & Hasanin, 2022). Hassanin *et al.* (2017a) confirmed that TONE was in nanoform, where the size was less than 100 and stills stable at room temperature for 3 months of storage.

T. vulgaris essential oil nanoemulsion was characterized using transmission electron microscopy to determine the true size and shape of the droplets. In the present study TEM results illustrated that TONE appeared spherical in shape and monodispersed. Additionally, the size of TONE droplets was in the range of 32.3 - 53.4 nm. The size of the droplets matched up well with the data from the dynamic light scattering analysis of droplet size. Abd-Elsalam and Khokhlov (2015) prepared and characterized TONE where TEM analysis confirmed that TONE was spherical in shape and size was in range 50-110 nm. Furthermore, TONE was prepared in previous study where were spherical in shape, and the size was 34 80.8 nm Hassanin *et al.* (2017a). Moreover, Hassanin *et al.* (2017b) reported that the prepared TONE appeared spherical and shape, and its size was in range 26.6 - 45.3 nm according to TEM. Also, Hammad and Hasanin (2022) revealed that TONE was spherical in shape, and its size was in range 25.4 32.9 nm. In the current investigation, the antifungal TONE activity toward *F. oxysporum* using linear growth Results illustrated that the increase in TONE concentration led to a decrease in linear growth of *F. oxysporum* Moreover, the low concentration of TONE at 1 mg/ml led to mild decrease in linear growth where was 89 mm, but in higher concentrations at 3 and 4 mg/ml led to severe decreasing in linear growth where were 43 and 29 mm. Antifungal mechanism of thyme oil is attributed to their ability to penetrate chitin of cell wall, then damages the lipoprotein in cytoplasmic membrane leading to escape of cytoplasm (Zambonelli *et al*., 1996) In a previous study, TONE was successfully prepared and exhibited promising antifungal activity against *Sclerotinia sclerotiorum* (Hassanin *et al*., 2017b). Moazeni *et al.* (2021) studied the antifungal activity of TONE toward *Candida albicans, C. glabrata* and *Aspergillus fumigatus,* where found that TONE has promising antifungal activity toward selected fungal strains. Hassanin *et al.* (2017a) reported that, maximum inhibition of TONE against *F. oxysporum* isolated from geranium plant at 2 mg/ml.

As shown in the current results showed that the symptoms of *Fusarium* wilt infection were very clear and DI reached the maximum rate by 85% in infected *F. vulgare* with *Fusarium* only. Also, it is expected to reduce the severity of the infection by using the fungicide (difenoconazole). The present results showed that the application of the difenoconazole gave the lowest DI% to (15%). Daify Core works as a sterol demethylation inhibitor, preventing the growth of the fungus by preventing the formation of ergosterol in cell membranes (Thakur *et al*., 2018). Interesting application of TONE treatment on infected plants led to a reduction in DI to (17.5%) and an increase in the percentage of protection to (79.4%) then treatment with TOE on infected plants which led to a lessening in DI by (42.5%) and an increase in the percentage of protection to 50 %. The first guide to governing the occurrence of resistance in plants against the pathogen is a Disease Index (Attia *et al*., 2022a; Roux *et al*., 2014). This study shows that applying TONE causes induction of *F. vulgare* resistance which reduced the disease severity percentage and provided a high protection against *Fusarium* (Das *et al*., 2021; Escobar *et al*., 2020; Gaber *et al.*, 2023). In this regard, TONE can inhibit the growth of the *Fusarium* mycelium directly through antibiosis (Sharma *et al*., 2023) ,which indicates the importance of TONE as a safe alternative to chemical fungicide against fungal infection.

Plants store proline for its direct function as an osmosis regulator to safeguard cells from free radical toxicity when there is biotic stress (Attia *et al.,* 2022b; Attia *et al.,* 2022c). Numerous studies have observed an increase in phenols in plants that have been affected by pathogens (Elbasuney *et al*., 2022). It was noticed in current results, that, those contents of free proline as well as total phenols in *F. oxysporum* - infected plants were increased due to (TOE, TONE and difenoconazole) treatments. Previous research has shown that TOE and TONE elicit systemic responses against plant pathogens (Pandey *et al*., 2018; Yadav *et al*., 2023). Also, our results appeared that (TONE) show significant increase in total phenol related to difenoconazole and TOE.

Antioxidants play a crucial function in protecting plant defense cells from damage and free radicals, in addition to helping to build cell walls (Bolouri-Moghaddam *et al*., 2010; Sharma *et al*., 2018).

Fungal infection causes oxidative stresses to occur within the cells, which are expressed by an increase in H_2O_2 and MDA (*Abdelaziz et al*., 2023a). Plant cells underwent serious biotic disorder as a result of oxidative stress, which also increased the levels of MDA and H_2O_2 in plant leaves (Abd Alhakim *et al*., 2022; Ye *et al.,* 2006). Our results appeared that, those levels of MDA as well as H_2O_2 in *F. oxysporum*-infected plants were decreased due to TOE, TONE and difenoconazole treatments. Concerning the effect of (TOE, TONE and difenoconazole) on the challenged fennel plants with *F. oxysporum*, it was found that TONE show highly significant decline in MDA and H_2O_2 of related to TOE and difenoconazole. Previous studies have documented that reducing MDA and H_2O_2 levels is a strong evidence for the recovery of infected plants from oxidative stress (Ciriolo *et al*., 1997; Munne-Bosch and Penuelas, 2003). Numerous studies arrived the induction of resistance with thyme oil nanoemulsion that increase enzymatic activity to reduce oxidative stress (Gill *et al*., 2016; Hassanin *et al.,* 2017a; Jiang *et al*., 2023). The stimulation of antioxidant enzyme activity is another way to defend the plant against various stress factors (Caverzan *et al*., 2016).

Infected plants show antioxidant enzyme activity has significantly increased to get rid of free radicals formed as a result of infection (Radwan *et al*., 2010). As shown in Figure 8, the results showed that that, those activities of POD and PPO in *F. oxysporum*-infected plants were raised due to TOE, TONE and difenoconazole treatments. Previous studies have documented that reducing POD and PPO levels is strong evidence for the recovery of infected plants from oxidative stress (Abdelaziz *et al*., 2022b; Attia *et al*., 2023). The maximum increase of POD and PPO activities expressed in response to the use of TONE. The activity of (POD and PPO) enzymes is one of the key control mechanisms for cellular protection against versus infection (Akladious *et al*., 2019; El-Fawy *et al*., 2021; Harb *et al*., 2010). Induction of these enzymes plays a vital role in cellular defense against oxidative stress (Contreras-Zentella *et al.*, 2022; El-Beltagi *et al*., 2010; Zulfiqar and Ashraf, 2022).

Conclusions

In the current study, *T. vulgaris* oil nanoemulsion was successfully prepared and characterized using different modern techniques. Characterization results confirmed that TONE was in nanoform with spherical shape. Furhtermore, results revealed that TONE has promising antifungal activity toward *F. oxysporum* in-vitro. The results of the current study confirm that the application of nanoemulisions on infected plants played an effective role in reducing the severity of infection, which resulted in an increase in the rate of protection against disease. Also, the use of *T. vulgaris* nanoemulisions had great results in inducing the formation of phenolic substances, proline and antioxidants, which play an important role in mitigating the damage caused by *F. oxysporium* infection, which indicates the interest in these treatments as safe and effective alternatives in eliminating *F. vulgare F. oxysporium* wilt.

Authors' Contributions

Data curation; M.S.A., A.M.A., A.H.H.,M.M.H.H., Formal analysis; M.S.A,A.M.A.,A.H.H.,M.M.H.H Funding acquisition; A.A.A.,S.A.M., M.S.A.,A.M.A., A.H.H., Investigation; M.S.A.,A.M.A., A.H.H.,M.M.H.H ., Methodology; M.S.A.,A.M.A., A.H.H.,M.M.H.H .,Project administration; A.A.A.,S.A.M., M.S.A.,A.M.A., A.H.H., Resources; M.S.A.,A.M.A., A.H.H.,M.M.H.H., Software; M.S.A.,A.M.A., A.H.H.,M.M.H.H., Supervision; M.S.A.,A.M.A., A.H.H.,M.M.H.H., Validation;

M.S.A.,A.M.A., A.H.H.,M.M.H.H., Visualization; M.S.A.,A.M.A., A.H.H.,M.M.H.H. A.A.A.,S.A.M., Writing - original draft; Writing - review and editing; M.S.A.,A.M.A., A.H.H.,All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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