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Nanoformulation of Cedarwood Oil and Its Fungicidal Activity against *Fusarium solani* and *Alternaria tenuis*

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ABSTRACT

Plant pathogens are a major challenge to plant health, yield, and consequently food availability, food security, and human health. Therefore, newly natural or green fungicides in nanoformulation offer a well alternative to synthetic fungicides, with high activity and lower impact on humans and the ecosystem. This study aimed to prepare cedarwood oil nanoemulsions and evaluate their fungicidal activity against *F. solani* and *Alternaria tenuis*. In addition, acute oral toxicity on rats was studied. Nanoemulsion of cedarwood oil was prepared under different ratios and conditions of sonication time, tween 80, and Arabic gum. Four samples (A2, B2, C2, and D2) have high stability droplet size diameters of 129.9, 162.2, 124.4, and 224.2 nm. Samples with lower droplet sizes (A2 and C2) were used for the fungicidal activity study. These samples caused high inhibition in growth and the IC_{50} accounts for 3.57 % and 2.41 % against *F. solani* and 1.25% and 2.22% against *Alternaria tenuis*, respectively. These results showed that Cedarwood oil nanoemulsion of sample A2 (oil: tween at ration 1:1) and sample C2 (oil: Arabic gum: tween at ratio 1:1:1) with 10 minutes sonication time has high fungicidal activity against *F. solani* and *Alternaria tenuis*. There is no singes of toxicity or mortality in treated male rats. These results have important implications for the development of green nano fungicides as a safe and effective antifungal as an alternative to synthetic fungicides in green-integrated pest management programs.

Keywords: Fungicide, nanoemulsion, Cedarwood oil, Arabic gum, *F. solani* and *Alternaria tenuis,* rats.

1. Introduction

Plant health strategies are an effective approach to food safety and food security and human health. In these respect pests, pathogens, nutrition, climate change, and other factors are important issues. These factors affect plant and human health, produce yield losses and then influence economic status. Plant pathogens affect plant health, yield, and consequently food availability and food security (Rizzo *et al.,* 2021). Plant pathogens contain a wide variety of microorganisms including fungi that cause plant, fruit, and vegetable diseases, damage and then losses. Fungi pathogens cause damage and economic losses in the quantity and the quality of crops and their products in both the open fields and postharvest during transport and storage (Hahn, 2014; Mossa *et al.,* 2021; Nazarov *et al.,* 2020). The increase and widespread use of synthetic fungicides has led to adverse health effects on humans, the ecosystem, and develop fungi resistance (Bolognesi and Merlo, 2011; Hahn, 2014; Hassaan and El Nemr, 2020; Mesnage *et al.,* 2014; Mossa *et al.,* 2018b; Nicolopoulou-Stamati *et al.,* 2016). Therefore, there is a need for new green-natural fungicides from plant extracts, essential and fixed oils, and bio

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agents as new generation natural fungicides as alternatives for uses in green-integrated management strategies (Hou *et al.,* 2020; Mossa *et al.,* 2021, 2018b; Ogunnupebi *et al.,* 2020).

Natural fungicides, new compounds, and bio products have become much more requested because they have high effectiveness, no residues in food products, and relatively non-toxic to both human and the ecosystem (Aktar *et al.,* 2009; Santra and Banerjee, 2020). Newly, natural oils (essential or fixed), plant extracts, and their active components have received great attention for the development of commercial fungicide products based on laboratory and field studies. These fungicidal activities are based on the high content of secondary metabolites, active components of phenolic, flavonoid, steroid, tannin, alkaloid, and saponins compounds (Agyare *et al.,* 2013; Mossa *et al.,* 2021; Mujeeb *et al.,* 2014; Oloumi, 2014). Some researchers have stated the biological activity of some plant extracts and EOs against phytopathogenic fungi (Agyare *et al.,* 2013; Aktar *et al.,* 2009; Hou *et al.,* 2020; Mossa *et al.,* 2021; Mujeeb *et al.,* 2014; Ogunnupebi *et al.,* 2020; Oloumi, 2014; Rizzo *et al.,* 2021; Santra and Banerjee, 2020). In our previous study, clove, black seed, lemon, and orange oils were evaluated against *Galactomyces candidum*, *Alternaria tenuissima* and *Fusarium solani* (Mossa *et al.,* 2021). In our previous study, nanoemulsions of fixed oils of clove and black seed showed high fungicidal activity compared to normal oils and complete inhibition of mycelial growth after treatment with nanoemulsion was recorded (Mossa *et al.,* 2021). Cedarwood (*Cedrus atlantica*) essential oil and its active components have insecticidal, antifungal antiseptic, anti-inflammatory, antispasmodic, and diuretic (Zrira and Ghanmi, 2016) (Kačániová *et al.,* 2022). Zrira and Ghanmi (Zrira and Ghanmi, 2016) stated that the highest compounds in cedarwood oil were α-(E)-atlantone, β-himachalene,-8 Cedren-13-ol, α-himachalene, cedroxyde and deodarone. In GC-MS analysis also, the major components of the Cedar atlantica essential oil were γ-cadinene, (Z)-_-farnesene, viridiflorol and himachala-2,4-diene (Kačániová *et al.,* 2022).

Due to the EO's lipophilic nature, they have much weakness for the widespread use and application due to their physical and chemical properties. These properties are high volatility, insolubility in water, stability, and low residual activity (Hammoud *et al.,* 2022; Mossa *et al.,* 2021). Therefore, nanotechnology by developing new nanoformulations such as nanoemulsion is an important strategy for increasing the activity, uses, and application of EOs in pest management. Several researchers studied nanoformulation of essential oils such as nanoemulsion. In these formulations droplets with diameters, ranging from 5 to 100 nm were developmental in a stable colloidal system (Krishnamoorthy *et al.,* 2021; Mossa *et al.,* 2021; Mou *et al.,* 2008). Compared to the normal emulsion, the nanoemulsion has different properties including high physical stability, bioavailability, and low turbidity, which correlated with droplets size (Hassan *et al.,* 2021; Mohafrash *et al.,* 2020; Mossa *et al.,* 2021; Mou *et al.,* 2008). Therefore, the nanometric size of the nanoemulsion droplet increases the biological activity of EO because of increasing their bioavailability; improves the diffusion due to the subcellular size, and then enhances the activity (Krishnamoorthy *et al.,* 2021; Maurya *et al.,* 2021; Mossa *et al.,* 2021),

Therefore, the aims of the current work were undertaken to development new nanoemulsion of Cedarwood EO and to evaluate its antifungal activities against *F. solani* and *Alternaria tenuis*. Toxicity using Microtox® and rats as acute toxicity study of nanoemulsions were performed.

2. Materials and Methods

2.1. Chemicals and reagents

Cedarwood oil was obtained from Sigma-Aldrich, co., 3050 Spruce Street, Saint Louis, MO 63103 USA. Other substances were reagent-grade compounds.

2.2. Synthesize of Cedarwood oil nanoemulsion

Cedarwood oil, tween 80, Arabic gum, and distilled water was used for the preparation of nanoemulsion. Nanoemulsion of Cedarwood oil EO at a concentration of 5% w/w was prepared using different ratios of tween 80, Arabic gum (1:0:1, 1:0:2, 1:1:1, and 1:2:1). EO, AG, a tween at selected ratios were mixed and added to distilled water under stirring at 400 rpm for 30 minutes. The emulsion was sonicated at 5 and 10 minutes using a probe Sonicator (Ultrasonics, Sonics & Materials, INC. 53 Church Hill Rd. Newtown, CT, USA) at 20 kHz and a power output of 750 W and by resting an ice reservoir on the sample, the potential energy was neutralized. It was investigated how stable nanoemulsions are and how droplet sizes vary.

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2.3. Nanoemulsion characterization

2.3.1. Physicochemical studies

Nanoemulsion stability and physicochemical properties were studied. Nanoemulsion was subjected to different stress as cited in our previous study (Mossa *et al.,* 2018a). In the current study, four samples (A2, B2, C2 and D2) have high stability and droplet size distribution and fungicidal activity were performed on these nanoemulsions.

2.3.2. Droplet size distribution

Droplet size distribution of Cedarwood oil nanoemulsions samples (A2, B2, C2, and D2) were performed by dynamic light scattering (DLS) instrument (PSS, Santa Barbara, CA, USA) at 23 °C, HeNe laser with line 632-nm and angle 90° as the incident light. After 10 minutes of sonication, samples A2, B2, C2, and D2 have high stability with the lowest droplet size diameter of 129.9, 162.2, 124.4, and 224.2 nm. Samples with the lowest droplet size 124.4 and 129.9 nm (A2 and C2) was used for transmission electron microscopy (TEM)) and fungicidal activity studies.

2.3.3. Transmission electron microscopy (TEM)

For morphological analysis of Cedarwood oil nanoemulsions, JEOL Tokyo, Japan's model JEM-1230 TEM was utilized. Deionized water was used to dilute a drop of Cedarwood oil nanoemulsions before they were transferred to a copper grid and covered with carbon. Then stained for one minute with a phosphotungstic acid solution (2%, pH = 6.7). The replica was raised to dry at 30 °C room temperature after which the picture was seen using a TEM at 80 kV accelerating voltages.

2.4. Antifungal activity

2.4.1. Fungal culture

Fungal isolates *F. solani*, and *Alternaria tenuis* were isolated from the rhizosphere of soybean plants showing root rot symptoms. Diseased plants were collected from field of soybean cultivation located at El-Nobaria district, NRC of Egypt during 2021. These fungal were identified in the laboratory and further confirmed by the Department of Plant Pathology, NRC of Cairo, Egypt. Potato dextrose agar medium (PDA) was used for maintaining the isolated fungal at 25± 2℃ for 7 days. Growing fungi were further identified (Barnett *et al.,* 1972; Domsch *et al.,* 2008; Martin and Gilman, 1957). The isolates were used in this study for antifungal activity assay and were kept on PDA slants and at 27 ± 2˚C.

2.5. Test for antifungal activity

2.5.1. Agar well diffusion method

Using an agar well diffusion method and a sterile 6.0 mm cork borer, cedarwood oil and the nanoemulsions (A2, C2) were tested for their ability to inhibit the growth of fungi (Lira-De León *et al.,*2014). For the inoculation of fungi on PDA plates, old cultures of *F. solani* and *Alternaria tenuis* produced on a PDA medium were employed after 72 hours. Molten PDA was added to an aliquot (0.2 ml) of inoculums, which was then placed into a petri plate. After solidification, the proper wells on an agar plate were produced, 500 µl of various concentrations were homogenized using an ultrasonic cleaner, and deep blocks were filled in. The observation of antifungal activity was carried out by maintaining an incubation period of 4-5 days at 27±2˚C, by evaluating the zone of inhibition of fungal growth surrounding the well. The experiment was run in triplicates, and the zone of inhibition was quantified in millimeters.

2.5.2. Toxicity study on male rats

According to OECD 423 recommendations, a study on the acute oral toxicity of cedarwood oil nanoemulsion on male rats weighing 100 ± 5 g was conducted in the Animal Breeding House (ABH) of the National Research Centre, Dokki, Giza, Egypt (OECD, 2001). Under typical laboratory circumstances (12-hour light/dark cycle, 23 1.5 °C, 65.0% RH), the experiment was carried out. The NRC Local Ethical Review Committee approved the treatment and utilization of laboratory animals in ABH. The experiment was carried out in compliance with the National Research Council's Guide for the Care and Use of Laboratory Animals, which sets forth standards for animal protection and welfare (National Research Council., 2010).

There are five male rats in each of the four groups of male rats. Group I served as the control and groups II, III, and IV got either regular Cedarwood oil or their respective nanoemulsions (samples A2 and C2). One milliliter of either the nanoemulsions (samples A2 and C2) or the conventional emulsion (5% concentration) of cedarwood oil was ingested by each rat (Mossa *et al.,* 2021). Based on average human weights, a dose is equivalent to 600 ml of nanoemulsion per person and 25 g of cedarwood oil in nanoemulsions (60 kg). Over the course of 14 days, toxicity and mortality signs were noted every day.

2.6. Statistical analysis

Utilizing the Statistical Package for Social Sciences, data were statistically examined (SPSS 26.0 for Windows). The concentration (IC50) that inhibits growth by 50% was determined using a probit analysis.

3. Results

In the current study, Cedarwood oil was used for the preparation of nanoemulsion at a concentration of 5%. The nanoemulsion formulated was performed using polysorbate 80 (Tween 80) and Arabic gum at different ratios of 1:0:1; 1:0:2; 1:1:1; and 1:2:1 as an organic phase and distilled water as an aqueous phase (Table 1). All emulsions were prepared under the same conditions of dropping and steering at 400 rpm for 30 minutes at room temperature. The samples were sonicated under cooling for 5 and 10 minutes (Table 2). Eight nanoemulsion samples were formulated and have the following codes A1, A2, B1, B2, C1, C2, D1 and D2. Four nanoemulsion samples (A2, B2, C2 and D2) at 10 minutes as sonication times have high stability after subjected to stability studies. These samples have lower droplet size diameters of 129.9, 162.2, 124.4 and 224.2 nm, respectively (Table 2 and Figures 1 and 2). The morphological investigation of Cedarwood oil nanoemulsions was studied by transmission electron microscopy and confirmed the droplet size, the spherical shape, and good dispersion of droplets nanoemulsion (Figure 3).

Table 1: Samples cods and ratio of oil, Arabic gum, tween 80 and deionized water (w/w) used for nanoemulsion formulations.

Cod	Contain	Oil: arabic gum: Tween 80 ratio (w/w)	Formulation (g/100 g)			
			Oil	Gum	Tween 80	D.W
A	**Cedarwood + Tween**	1:0:1	5	0	5	90
B	**Cedarwood + Tween**	1:0:2	5	0	10	85
C	**Cedarwood + Arabic gum + Tween**	1:1:1	5	5	5	85
D	**Cedarwood + Arabic gum + Tween**	1:2:1	5	10	5	80

D.W = distilled water

Table 2: Stability and physiochemical studies of nanoemulsions and their droplets size.

Sample Code	Sonication time (min)	Centrifugation	Freeze–Thaw cycle	Heating–Cooling cycle	Result	Droplet size (nm)	Variance (P.I.)
A1	5	-	+	-	x	-	-
A2	10	+	+	+	√	129.9	0.438
B1	5	-	-	-	x	-	-
B2	10	+	+	+	√	162.2	0.466
C1	5	-	+	+	x	-	-
C2	10	+	+	+	√	124.4	0.411
D1	5	-	-	-	x	-	-
D2	10	+	+	+	√	224.2	0.260

X = failed; √ = passed

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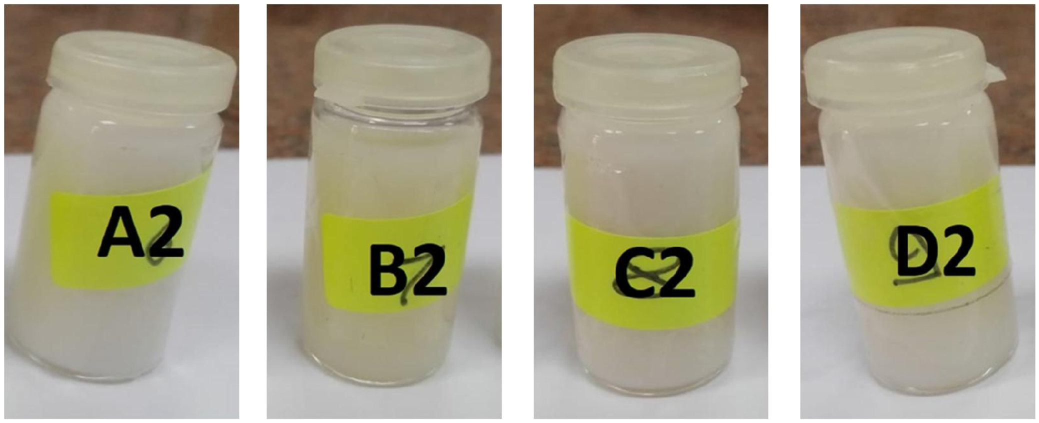
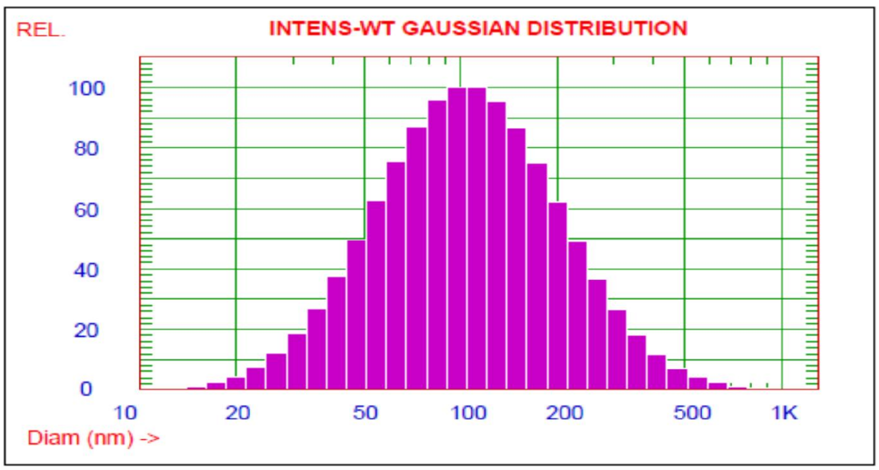
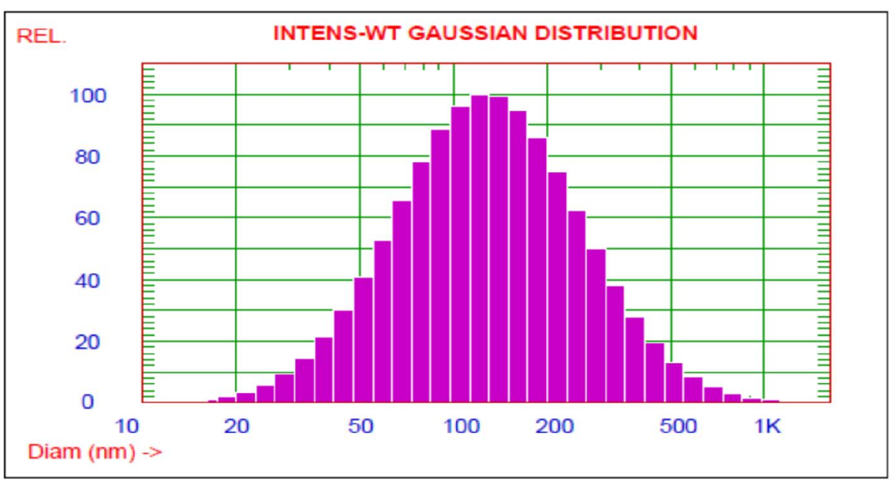


A2 B2 C2 D2

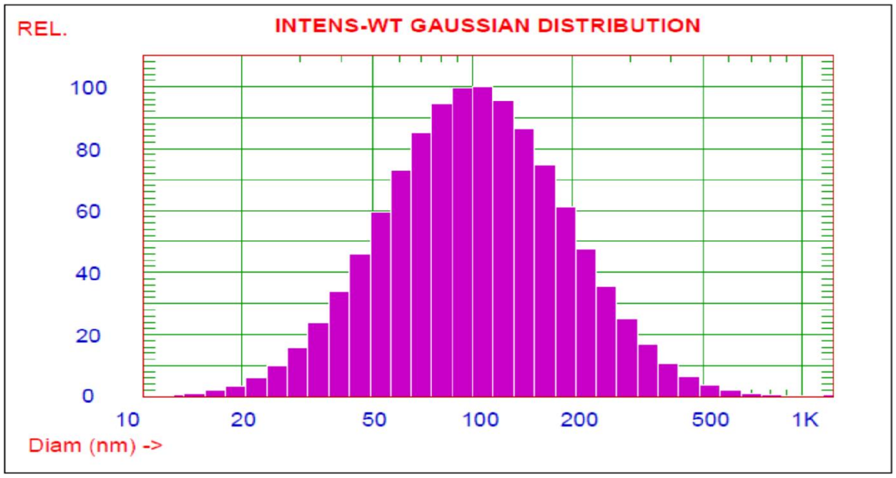
Fig. 1: Nanoemulsion of Cedarwood essential oil with Arabic gum and Tween 80 at different ratios.



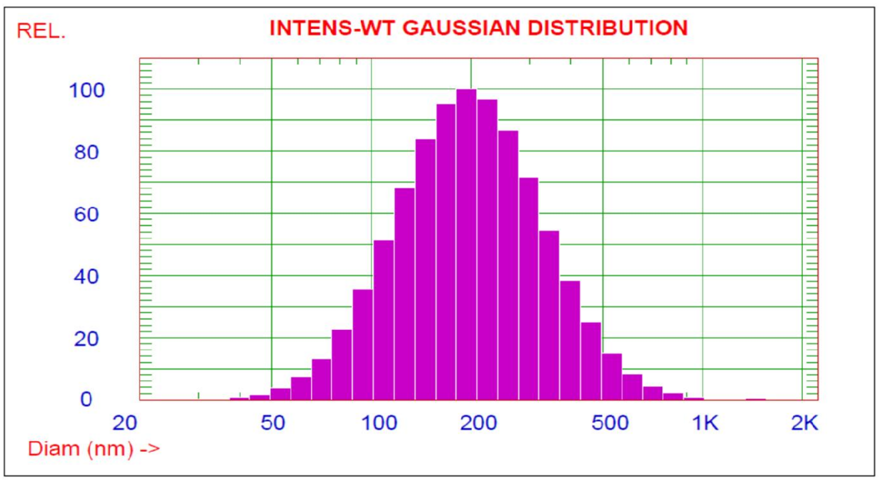
Cedarwood : Tween 80 (1:0:1) (129.9 nm)



Cedarwood : Arabic gum : Tween 80 (1:0:2) (162.2 nm)

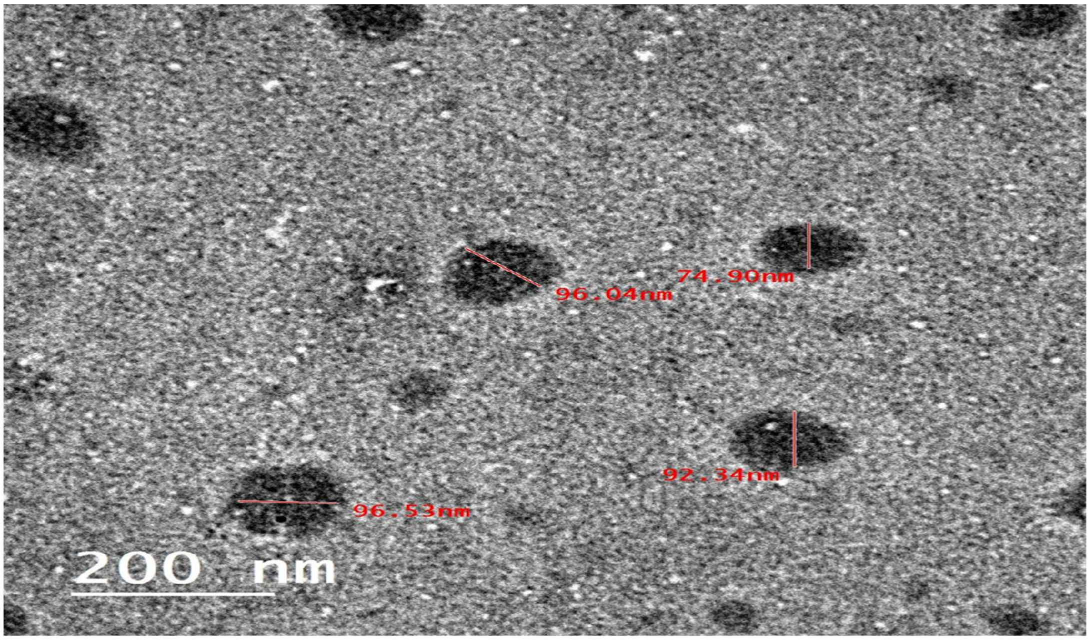


Cedarwood : Arabic gum : Tween 80 (1:1: 1) (124.4 nm)

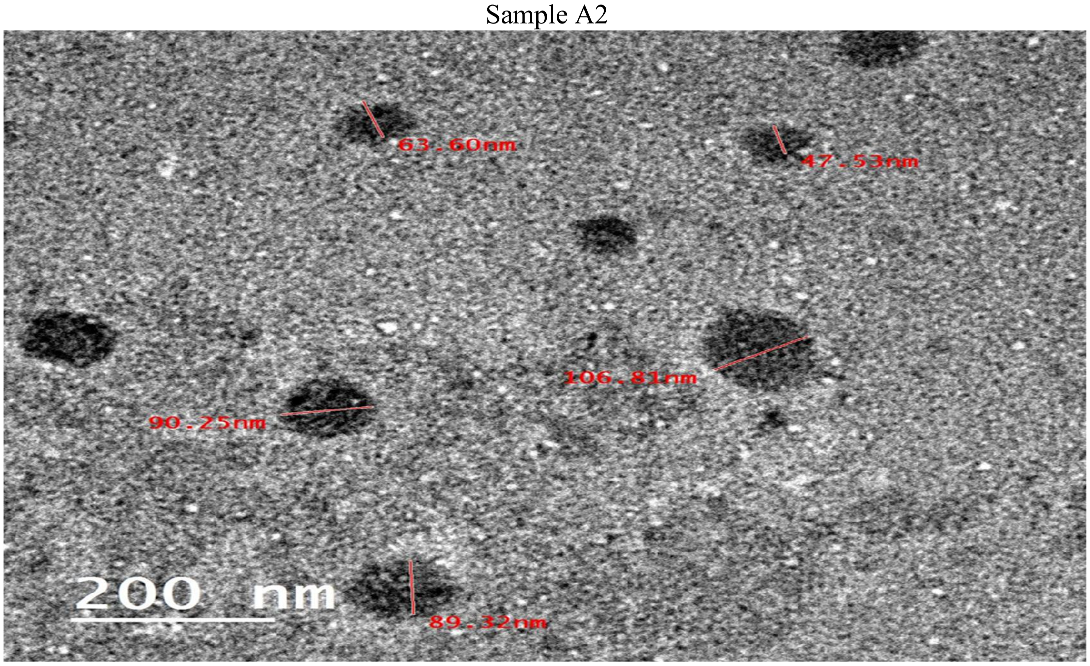


Cedarwood : Arabic gum : Tween 80 (1:2:1) (224.2 nm)

Fig. 2: Nanoemulsion droplet size distribution of samples A2, B2, C2 and D2.



Sample A2



Sample C2

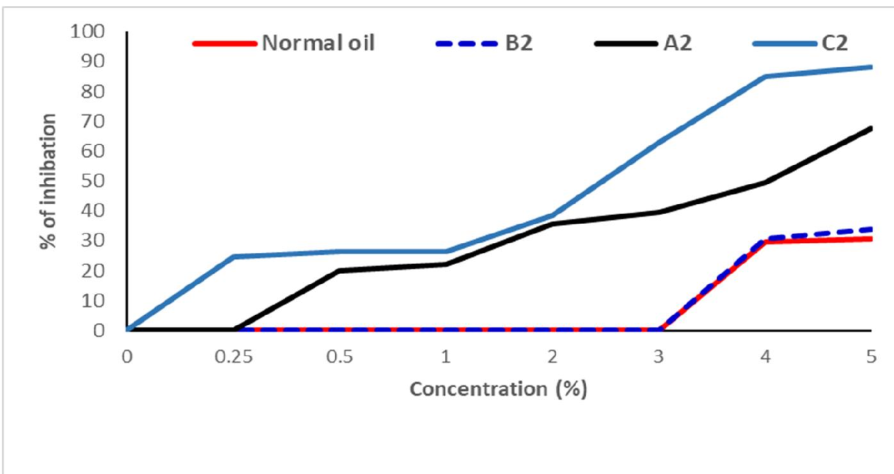
Fig. 3: Transmission electron microscopy (TEM) of Cedarwood normal oil nanoemulsions sample A2 and C2.

As shown in Table 3 and Figure 4, Cedarwood oil nanoemulsion samples A2 (129.9 nm), and sample C2 (124.4 nm) have high fungicidal activity and caused inhibition of mycelial linear growth of *F. solani*, and *Alternaria tenuis*. The concentration that caused 50% inhibition in growth (IC_{50}, concentration) accounts for 3.57 % and 2.41 % against *F. solani* and account for 1.25% and 2.22% against *Alternaria tenuis*, respectively (Table 3 and Figures 4) while normal oil has lower activity against both fungi at concentration 5% which account less than 50% inhibition. The current results demonstrated that Cedarwood oil nanoemulsion of sample A2 (oil: tween at ration 1:1: with 10 minutes sonication time) has high fungicidal activity against *F. solani* compared to sample C2, while sample 2 (oil: Arabic gum: tween at ratio 1:1:1 with 10 minutes sonication time) has high fungicidal activity against *Alternaria tenuis*. An acute oral toxicity study of Cedarwood oil nanoemulsions (samples A2 and C2) was performed on male rats. Results showed that both nanoemulsions did not show singes of toxicity or mortality in treated male rats. Nanoemulsions (5%) was administrated at 1 ml/rat orally as a single dose that corresponds to 25 g of Cedarwood oil in nanoemulsions / person and is equal to 600 ml nanoemulsion/persona if calculated based on average human weights (60 kg).

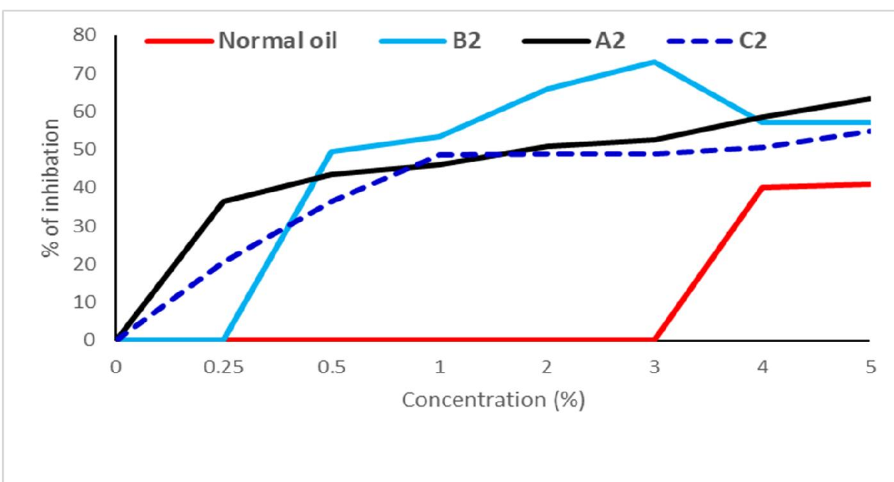
Table 3: Effect of different concentration s in PDA medium on the zone of inhibition of *F. solani* and *Alternaria tenuis* after 7 days incubation at 27±1°C

Formulation	Probit analysis of Mycelial growth								
	IC_{50} %	**Lower limit**	**Upper limit**	**LC_{90} %**	**Lower limit**	**Upper limit**	**Slop**	**Chi-square (df = 3)**	**P value**
				F. solani					
A2	3.57	2.88	4.78	42.05	21.98	126.43	1.29	9.38	0.052
C2	2.41	2.12	2.67	5.39	4.59	6.98	3.68	0.02	0.88
				Alternaria tenuis					
A2	1.25	0.63	2.73	31.68	23.38	99.15	0.53	0.23	0.63
C2	2.22	1.39	4.94	56.76	30.78	72.41	0.65	0.07	5.39

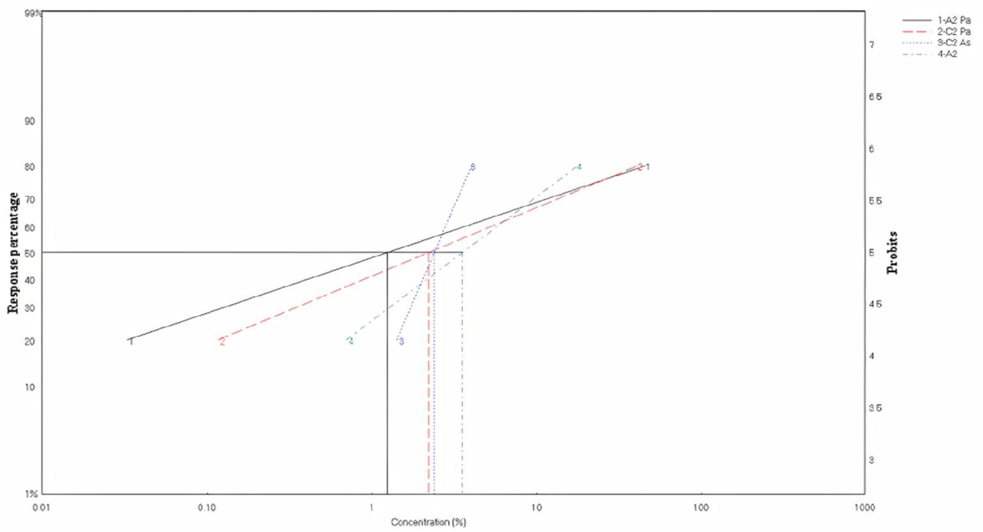
A2; Cedarwood: Arabic gum: Tween 80 (1:0:1); C2; Cedarwood: Arabic gum: Tween 80 (1:1:1) IC_{50}, concentration induced 50 % inhibition in mycelial growth, Probit analysis was completed using SPSS 26.0 for Windows.



A



B



C

Fig. 4: Effect of Cedarwood normal oil, Cedarwood nanoemulsion sample A2 (129.9 nm), sample B2 (162.9 nm), and sample C2 (124.4 nm) on mycelial linear growth of *F. solani* (A), *Alternaria tenuis* (B) and their probit analysis line, ldp lines (C)

4. Discussion

F. solani and *Alternaria tenuis* are fungus-caused plant diseases on fruits and vegetables including onions, peanuts, grapes, and apricots that is a common contaminant of food. *F. solani* has been observed in a broad range of habitats and causes the disease black mold (Gray *et al.,* 1999; Sabbir *et al.,* 2022). Due to the extensive use of synthetic fungicides in plant protection and their adverse health effects on humans and the ecosystem. This study was conducted to prepare and evaluate the nanoemulsion of the Cedarwood oil against *F. solani* and *Alternaria tenuis* fungi. Previous studies has been showed that Cedarwood oil *(Cedrus atlantica)* has high biological activity against fungi, bacteria, viruses and senile (Bouchra *et al.,* 2003; Hammer *et al.,* 1999; Lahlou, 2003; Loizzo *et al.,* 2008; Mossa and Mohafrash, 2022).

In the current study, Cedarwood oil *(Cedrus atlantica)* showed high fungicidal activity against *F. solani* and *Alternaria tenuis*. The fungicidal activity could be due to the presence of the secondary metabolites components such as phenolic, flavonoid, steroid, tannin, alkaloid, and saponins (Agyare *et al.,* 2013; Mossa *et al.,* 2021, 2018b; Mujeeb *et al.,* 2014; Oloumi, 2014). Cedarwood oil analysis showed α-(E)-atlantone, β-himachalene,-8 Cedren-13-ol, α-himachalene, cedroxyde and deodarone (Zrira and Ghanmi, 2016) and γ-cadinene , (Z)-_-farnesene, viridiflorol and himachala-2,4-diene (Kačániová *et al.,* 2022) were the major components. Although natural fungicides as a bioproducts have become much more requested (Aktar *et al.,* 2009; Santra and Banerjee, 2020), it has many limitations for widespread application. These limitations are volatility, insolubility in water, and shortage of life span (Hammoud *et al.,* 2022; Mohafrash *et al.,* 2020; Mossa *et al.,* 2021, 2018b).

Currently, new trends in plant protection are starting for using nanotechnology including nano-fungicides. This technology is estimated to play a significant role in the management of plant disease as an alternative to synthetic fungicides. These nano-fungicides have high fungicidal activity, lower toxicity to humans, and as eco-friendly (Hassan *et al.,* 2021; Krishnamoorthy *et al.,* 2021; Mossa and Mohafrash, 2022; Mossa *et al.,* 2021; Myc *et al.,* 2003). In the current study, nanoemulsion of Cedarwood oil was performed. Nanoemulsion with high stability and lower droplet size were obtained after 10 minutes of sonication. These nanoemulsions samples A2 and C2 have lower droplet size diameters of 129.9, and 124.4 nm, respectively. Sporting to these results the morphological studies of Cedarwood oil nanoemulsions by TEM showed spherical shape, and good dispersion of droplets nanoemulsion. This finding showed the correlation between Hydrophilic–lipophilic balance (HLB), lower droplet size and sonication time for nanoemulsion development. Other studies have reported the correlation between HLB of essential oil, and surfactant, sonication time, and droplet size (Campolo *et al.,* 2020; Ghosh *et al.,* 2013; Mohafrash *et al.,* 2020; Mossa and Mohafrash, 2022; Mossa *et al.,* 2021).

Fungicidal activity of Cedarwood oil nanoemulsion samples A2 (129.9 nm), and sample C2 (124.4 nm) showed high inhibition in mycelial linear growth of *F. solani*, and *Alternaria tenuis*. The IC_{50} accounts for 3.57 % and 2.41 % of *F. solani* and 1.25% and 2.22% of *Alternaria tenuis*, respectively. The current finding showed that nanoemulsion of sample A2 (oil: tween 80 at ration 1:1: with 10 minutes sonication time) has high fungicidal activity against *F. solani* while nanoemulsion sample 2 (oil: Arabic gum: tween at ratio 1:1:1 with 10 minutes sonication time) has high fungicidal activity against *Alternaria tenuis*. These results demonstrated that Arabic gum has increased the fungicidal activity of Cedarwood oil nanoemulsion. The increase in fungicidal activity of nanoemulsion compared to normal oil could be due to the changes in the nanoemulsion properties, which correlated to the droplet size. These properties including water solubility, physical stability, bioavailability, and low turbidity (Hassan *et al.,* 2021; Kesrevani and Sharma, 2016; Mohafrash *et al.,* 2020; Mossa *et al.,* 2021; Mou *et al.,* 2008). Changes in the properties increase the biological activity of EO via increased bioavailability; increases the distribution due to the subcellular size, and then enhance the fungicidal activity (Aqil *et al.,* 2013; Jaisamut *et al.,* 2021; Kapustová *et al.,* 2021; Krishnamoorthy *et al.,* 2021; Maurya *et al.,* 2021; Mossa *et al.,* 2021). The fungicidal activity of Arabic gum was reported by other studies (Atrash *et al.,* 2018; Dong and Wang, 2018).

The acute oral toxicity study of Cedarwood oil nanoemulsions showed no signs of toxicity or mortality in treated rats. These results suggest that Cedarwood oil nanoemulsions could be considered safe (at dose corresponds to 25 g of Cedarwood oil in nanoemulsions / person/ day) as alternatives to synthetic fungicides.

5. Conclusions

Cedarwood oil nanoemulsion was formulated and evaluated against *F. solani* and *Alternaria tenuis*. Cedarwood oil nanoemulsion of sample A2 (oil: tween at ration 1:1) and sample C2 (oil: Arabic gum: tween at ratio 1:1:1) with 10 minutes sonication time has high fungicidal activity against *F. solani* and *Alternaria tenuis*. An acute oral toxicity study showed no singes of toxicity or mortality in treated male rats. These results have important implications for the development of green nano fungicides as a safe and effective antifungal as an alternative to synthetic fungicides in green-integrated pest management programs.

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