Egypt. J. Chem. **Vol. 66**, No. 2 pp. 111 - 120 (2023)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Comparison of the Antimicrobial and Antioxidant Activities of Tea Tree (*Melaleuca alternifolia*) Oil and its Main Component Terpinen-4-ol with their Nanoemulsions

Mai M. Badr[a]; Nehad E. M. Taktak[a]; Mohamed E. I. Badawy[b,*]

[a] *Department of Environmental Health, High Institute of Public Health, 165 El-Horreya Avenue, El-Ibrahimia, Alexandria University, Alexandria, Egypt*

[b] *Department of Pesticide Chemistry and Technology, Laboratory of Pesticide Residues Analysis, Faculty of Agriculture, Aflatoun St., 21545 El-Shatby, Alexandria University, Alexandria, Egypt*

Abstract

An investigation of nanoemulsions of tea tree (*Melaleuca alternifolia*) oil and its main constituent terpinen-4-ol was carried out compared to non-formulated products for their antimicrobial and antioxidant properties. To prepare nanoemulsions from coarse oil-in-water (O/W) emulsions, the products were added to aqueous solutions containing 10% Tween 80 and stirred continuously under ultrasonication. Interestingly, the tea tree oil nanoemulsion produced droplets with a size of 70.67 nm, while the terpinen-4-ol nanoemulsion produced droplets with a size of 176.00 nm. The nanoemulsions showed high negative zeta potentials (-22.30 and -23.50 mV for oil and terpinen-4-ol, respectively. Compared with tea tree oil and terpinen-4-ol nanoemulsions as antioxidants, terpinen-4-ol nanoemulsion had the highest radical scavenging activity against DPPH with the lowest EC_{50} value (253.65 mg/L). Furthermore, terpinen-4-ol nanoemulsion exhibited the highest antibacterial activity (MIC = 600 and 475 mg/L against *Salmonella typhimurium* and *Staphylococcus aureus*, respectively). Tee tree oil nanoemulsion, on the other hand, had the strongest inhibition effect against *Candida albicans* (EC_{50} = 74.86 mg/L). Furthermore, a nanoemulsion of terpinen-4-ol exhibited high antifungal activity (EC_{50} = 253.13 and 312.53 mg/L against *Aspergillus flavus* and *A. niger*, respectively). Despite this, tea tree oil nanoemulsion was the most effective against fungal spores (EC_{50} = 484.61 and 291.99 mg/L against spores of *A. flavus* and *A. niger*, respectively). The information in this study will lead to a potential breakthrough for eco-friendly antimicrobials and antioxidants that contain tea tree oil and terpinen-4-ol.

Keywords: Tea tree oil; Terpinen-4-ol; Nanoemulsions; Antioxidant activity; Antimicrobial activity.

1. Introduction

The increase in microbial resistance to many drugs and the absence of new products in the market resulted in an increased need for alternative strategies to deal with infections caused by drug-resistant microbes [1]. Given public health, the medicinal properties of some plants have received significant attention due to their low toxicity, pharmacological activities, and economic viability [2, 3]. Several studies have focused on the benefits of phytochemicals extracted from plants (essential oils, EOs) and their impact on human health [4, 5]. Plant EOs and extracts exhibit an antioxidant or antimicrobial effect. Among these compounds of natural origin, tea tree oil is the volatile EO primarily derived from the Australian native plant *Melaleuca alternifolia* [6]. It consists of about 100 different compounds, and the major components are terpenes and sesquiterpenes, such as terpinen-4-ol, α- and γ-terpinene, 1,8-cineole, and terpinolene [7-10]. Concentrations of the key constituents of this EO can differ significantly between various preparations, affecting their antimicrobial activities. In previous studies, tea tree oil was used as a therapeutic agent compared to the antiseptic carbolic acid or phenol to evaluate their antimicrobial activity. Tea tree oil has clinical applications, particularly for removing methicillin-resistant *Staphylococcus aureus* or hand

*Corresponding author e-mail: mohamed.badawy@alexu.edu.eg
Receive Date: 06 April 2022, Revise Date: 15 May 2022, Accept Date: 31 May 2022
DOI: 10.21608/EJCHEM.2022.131758.5808

sanitizer to prevent infection with Gram-positive and Gram-negative pathogens [11]. In addition, recent data indicated the broad spectrum of the activity of this oil that includes antibacterial, antifungal, antiviral, and antiprotozoal. In terms of biological activity, antimicrobial activity is mainly related to terpinen-4-ol, a significant component of this oil [6]. As for the mechanism of antimicrobial action, tea tree oil and its components can preferentially split hydrocarbons into cell membranes and disturb their vital functions [12, 13].

EOs may lose their biological activity because of significant volatility or decomposition of their components, resulting in a limitation of their applications [14]. Therefore, developing newer antimicrobials from EOs using promising approaches such as nanotechnology enhances their availability and biological activity in drug delivery [15]. Nanoemulsions (NE) are dispersion composed of two immiscible liquids, which are characterized by homogeneity, dynamic stability, and composition as oil in water (O/W) or water in oil (W/O). This dispersion is stabilized by surfactants that form an interfacial film with droplet sizes ranging between 10 and 500 nm. NEs contribute to their increased spread in areas where microorganisms grow and multiply. In this form, EOs exhibit physical stability, low volatility, protection from environmental reactions (e.g., light, oxygen, moisture, and pH), promotion of vital activity, and reduced toxicity [16]. Moreover, tiny NE droplets of EOs close to the cell membrane surface will improve the accessibility to microbial cells and enable the disruption of cell membranes by altering the integrity of the phospholipid bilayer or by interfering with the embedded the phospholipid bilayer active transport proteins. Previous studies showed that nanoemulsions maintain the stability and efficacy of *M. alternifolia* extracts and suggest that the nanostructures of nanoemulsions may potentially treat microbial infections [17, 18].

In this study, tea tree oil and its main component, terpinen-4-ol, nanoemulsions were prepared and compared with non-formulated products for stability, antioxidant activity, and antimicrobial properties. The physicochemical characteristics, including phenolics and flavonoids, were also evaluated. In addition, the Gram-negative (*Salmonella typhimurium*) and Gram-positive (*Staphylococcus aureus*) bacteria, fungi of *Aspergillus niger* and *Aspergillus flavus*, and yeast *Candida albicans* were tested for antimicrobial inhibition activity of the products. The antimicrobial activity was conducted to understand better the ability of the formulated and non-formulated products to inhibit microbial growth. This study represents the first time tea tree EO and terpinen-4-ol combined to form nanoemulsions for antimicrobial use.

2. Materials and methods

2.1. Materials

Tea tree (*Melaleuca alternifolia*) oil was purchased from Harraz Planta Medical Group (Cairo, Egypt). Terpinen-4-ol (95%), Folin-Ciocalteu phenol reagent, tween 80, dimethyl sulphoxide (DMSO), 2,2-diphenyl-1-picrylhydrazyl (DPPH, ≥96%), gallic acid (≥98%), and α-tocopherol (≥96%) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Antibiotic ceftriaxone (99%) was obtained from Pharco Pharmaceuticals Inc. (Alexandria, Egypt). A reference fungicide carbendazim (98%) was provided friendly by Shoura chemicals Co. (Cairo-Alexandria Desert Road, Egypt). All media, including nutrient agar (NA), nutrient broth (NB), potato dextrose agar (PDA), potato dextrose broth (PDB), Sabouraud dextrose agar, and Sabouraud dextrose broth (SDB) were purchased from Oxoid Ltd (Basingstoke, Hampshire, UK). All other chemicals were reagent grade.

2.2. Microorganisms

Salmonella typhimurium ATCC 1402 and *Staphylococcus aureus* ATCC 6538, Gram-negative and Gram-positive bacteria, respectively, were obtained from the Microbiology Laboratory, Department of Dairy Science, Faculty of Agriculture, Alexandria University, Egypt. A yeast strain of *Candida albicans* (ATCC 90028) was obtained from the Department of Microbiology, Faculty of Medicine, Alexandria University, Egypt. Isolates of fungal species, including *Aspergillus flavus* and *Aspergillus niger*, were obtained from the Microbiology Laboratory, High Institute of Public Health, Alexandria University, Egypt.

2.3. GC/MS analysis of the essential oil

GC/MS analysis was carried out according to the optimization method by Badr et al. [15] using a TRACE 1300 GC/MS (Thermo Scientific). Under the same conditions, using a homologous series of alkanes (C_8-C_{24}), the compounds were identified by comparing mass spectral patterns and the linear

retention indices with authentic references and NIST/Wiley databases (MS libraries) [19].

2.4. Preparation and characterization of nanoemulsions

Oil-in-water (O/W) nanoemulsions of tea tree oil and terpinen-4-ol were prepared using the high-energy ultra-sonication method [15] with minor modifications. It has been determined that a 10% mixture of EO and 10% tween 80 as a surfactant is the best combination for tea tree EO formulation. Nevertheless, the terpinen-4-ol nanoemulsion was prepared with 2% (v/v) of the active ingredient dissolved in DMSO (3.75%), which acted as an oil phase that dropped into the polar phase 90.50% distilled water. The emulsions formulated were then directed to ultrasonic emulsification under optimal conditions described before [15]. A dynamic light scattering instrument (Zetasizer Nano ZS, UK) was used to measure the droplet size, polydispersity index (PDI), and Zeta potential. Transmission electron microscopy (TEM, JEOL JEM-1400 Plus, Japan) was used to demonstrate the surface morphology. Rotary Myr VR 3000 digital viscometer was used to measure the absolute viscosity (μ) with an L4 spindle at 200 rpm and 25±1°C without further dilution. The pH values were measured by a digital pH meter (Mi 151 Martini Instruments, UK). Stability tests were conducted under different conditions [15].

2.5. Determination of phenolic and flavonoid contents

The total phenolic content of the EO and its nanoemulsion was determined using Folin-Ciocalteu phenol reagent [20]. The absorbance of the formed color was measured at 765 nm, and the total phenolic content expressed as mg gallic acid equivalent/g oil was obtained from the standard calibration curve of gallic acid. Total flavonoids were determined using the aluminium chloride colorimetric method [21]. The color-formed was measured at 510 nm against the blank, and the results were expressed as mg rutin equivalent/g oil.

2.6. Antioxidant activity assay

The antioxidant activity was evaluated by DPPH scavenging assay [22] in comparison with α-tocopherol as a reference with some modifications. Briefly, different concentrations (1-2500 mg/mL) of each product were added to 1 mL of 0.01 mM DPPH methanol solution. A range of 1-25 μg/mL α-tocopherol in DMSO was tested as a standard. The mixture was shaken vigorously and allowed to stand in the dark at 37°C for 15 min. Absorbance was recorded at 517 nm against blank. The scavenging activity was calculated using the following equation: DPPH scavenging activity (%) = $[(A_c-A_t)/A_c]\times100$. Where A_c is the absorbance of the control after 15 min and A_t is the absorbance of each treatment after 15 min. The EC_{50} in μg/mL (effective concentration required to inhibit DPPH radical formation by 50%) was calculated [23].

2.7. In vitro antimicrobial investigation

2.7.1. Antibacterial assay

The antibacterial activity was performed using the NA dilution method [24] compared to ceftriaxone as a standard drug. The pure oil and terpinen-4-ol were dissolved in DMSO. However, the nanoemulsions were used as stock without further dilution. The products were mixed with NA medium immediately at a temperature of 40-45°C. The mixture was poured into the Petri dishes to solidify. Parallel negative and positive controls were maintained with distilled water mixed with NA medium without the tested formulations. After solidifying the medium, one loopful of microorganism suspensions (≈ 6 μL) was spotted on the surface of the NA medium in the form of ten spots per plate. The minimum inhibitory concentration (MIC) was determined after 24 h of incubation in each case.

2.7.2. Candidacidal assay

The inhibitory effect against the yeast *C. albicans* was assessed according to the broth dilution method [15]. Portions from each stock solution of the tested products were mixed with the SDB medium in a total volume of 5 mL containing 10^3 cells/mL (0.5 mL of the stock). In an orbital shaking incubator (180 rpm), the tested tubes were incubated at 35°C for 24 h. For the positive control of the growth, the sterile medium was inoculated with the yeast (without the tested product). However, a negative control was the sterile medium and tested product without inoculum. After static incubation for 24 at 35°C, the turbidity of tubes was read at 540 nm using a drug-free medium as a blank. All determinations were performed in triplicate. The 24-h readings were used to calculate the yeast growth inhibition relative to the control, and the concentration that inhibited 50% of the yeast growth (EC_{50}) was determined by probit analysis [23].

2.7.3. Antifungal assay

The inhibition effect of the tested products on the mycelia growth of *A. flavus* and *A. niger* in the PDA medium was evaluated [15]. In addition, the sporicidal activity against the spores of both fungi was conducted in a PDB medium [25]. The products were examined on a scale of 25-2500 mg/L. In contrast, a carbendazim as a standard fungicide was assessed at 1-200 mg/L. A linear regression method determined the compound concentration that inhibited 50% of the mycelial growth or spore germination (EC_{50}) [23].

2.8. Statistical analysis

Statistical analysis was performed using the SPSS 25.0 software (SPSS, Chicago, IL, USA). The EC_{50} values for the antifungal and antioxidant assays were calculated according to the probit analysis [23]. Analysis of variance (ANOVA) of the other data was conducted and means property values were separated ($p \leq 0.05$) with Student-Newman-Keuls (SNK) test

3. Results and Discussion

3.1. Chemical constituents of the tea tree oil

The tea tree (*M. alternifolia*) oil contained 19 components representing 99.67% of the total oil (Table 1). The acceptable levels of mean components were α-pinene (3.36%), α-terpinolene (2.36%), limonene (21.35%), γ-terpinene (5.25%), α-phellandrene (2.46%), terpinen-4-ol (41.11%), α-terpineol (17.20%) and γ-terpineol (1.04%). The chemical structures of these main constituents are shown in Figure 1. Tea tree (*M. alternifolia*) oil comprises terpene hydrocarbons, mainly monoterpenes, sesquiterpenes, and associated alcohols. Terpinen-4-ol and α-terpineol were the main components of the oil and limonene as a hydrocarbon monoterpene. The results of this study are in line with an earlier study where the principal constituent of tea tree oil was determined to be terpinen-4-ol, which represents 39.4% of the total oil content [26].

3.2. Characterization of nanoemulsions

The results of the thermodynamic stability of nanoemulsions are presented in Table 2. The nanoemulsions of tea tree oil and terpinen-4-ol have viscosity values of 4.67 and 4.33 mPa.s, respectively. The pH values were 6.07 and 5.97 for the tea oil and terpinen-4-ol, respectively. The nanoemulsions of tea tree oil and terpinen-4-ol have droplet sizes of 70.67 and 176.00 nm, respectively (Table 2).

A PDI of 0.422 and 0.630 were found for the tea tree oil and the terpinen-4-ol nanoemulsions, respectively. There was strong electrostatic repulsion between the oil droplets in the aqueous phase of the prepared nanoemulsions, as demonstrated by zeta potential values (-22.30 and -23.50 mV) (Table 2).

Table 1: The main chemical constituents of tea tree EO isolated from *M. alternifolia*

Retention time (min)	Compound	Molecular formula	MW	Area (%)	Retention index (RI)
11.51	β-Ocimene	$C_{10}H_{16}$	136	0.49	1078
14.73	4-Carene	$C_{10}H_{16}$	136	0.42	1182
14.96	α-Pinene	$C_{10}H_{16}$	136	3.36	1189
15.28	α-Terpinolene	$C_{10}H_{16}$	136	2.36	1199
15.86	Limonene	$C_{10}H_{16}$	136	21.35	1218
17.09	γ-Terpinene	$C_{10}H_{16}$	136	5.25	1258
18.31	α-Phellandrene	$C_{10}H_{16}$	136	2.46	1297
19.75	2,7-Dimethylocta-2,6-dienol	$C_{10}H_{18}O$	154	0.63	1344
20.91	Artemiseole	$C_{10}H_{16}O$	152	0.58	1381
21.04	4-Caranol	$C_{10}H_{18}O$	154	0.25	1385
22.30	Terpinen-4-ol	$C_{10}H_{18}O$	154	41.11	1426
22.89	α-Terpineol	$C_{10}H_{18}O$	154	17.20	1445
23.06	γ-Terpineol	$C_{10}H_{18}O$	154	1.04	1450
30.17	β-Bisabolene	$C_{15}H_{24}$	204	0.74	1680
30.80	Elemene	$C_{15}H_{24}$	204	0.44	1700
32.09	6-(*p*-Tolyl)-2-methyl-2-heptenol	$C_{15}H_{22}O$	218	0.31	1742
32.61	α-Selinene	$C_{15}H_{24}$	204	0.52	1758
33.09	Mayurone	$C_{14}H_{20}O$	204	0.54	1774
33.37	Clovene	$C_{15}H_{24}$	204	0.62	1783
Total (%)				**99.67**	

Table 2: Characterizations and thermodynamic stability of tea tree EO and terpinen-4-ol nanoemulsions

Nanoemulsion	Thermodynamic stability				Characterizations				
	Room temperature (25°C)	Centrifugation (5000 rpm)	Freezing cycle (-4°C)	Heating cycle (40°C)	μ (mPa.s) ± SE	pH ± SE	Droplet size (nm) ± SE	PDI ± SE	Zeta potential (mV)
Tea tree EO	√	√	√	√	4.93±0.67	6.07±0.03	70.67±3.22	0.422±0.05	-22.30
Terpinen-4-ol	√	√	√	√	4.33±0.33	5.97±0.03	176.00±3.21	0.630±0.08	-23.50

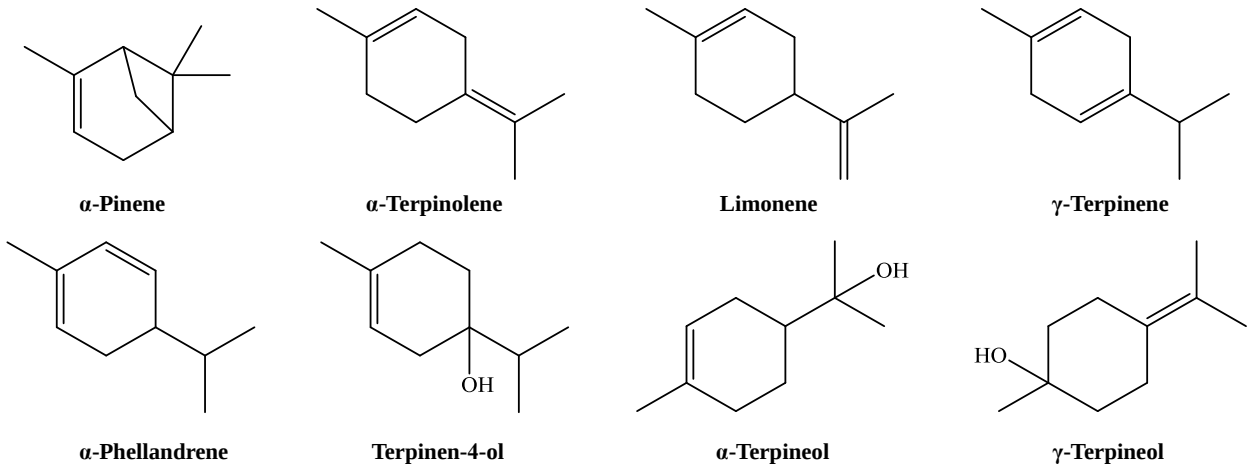

Figure 1: The chemical structures of the main constituents of tea tree essential EO based on GC/MS analysis.

A PDI of 0.422 and 0.630 were found for the tea tree oil and the terpinen-4-ol nanoemulsions, respectively. There was strong electrostatic repulsion between the oil droplets in the aqueous phase of the prepared nanoemulsions, as demonstrated by zeta potential values (-22.30 and -23.50 mV) (Table 2). TEM investigated the morphology of the nanoemulsions of tea tree oil and terpinen-4-ol (Figure 2). Microscopic images revealed that both nanoemulsions had a spherical shape.

By comparing EO and terpinen-4-ol nanoemulsions, neither creaming nor phase separation occurred in any formulation after two months, confirming the stability and efficacy. The nanoemulsions were stable over eight months, agreeing with previous thermal stability studies [27]. As long as the pH values are neutral, they can be used in various fields [15]. Emulsions with larger droplet sizes are more effective growth inhibitors and inactivating cells than smaller ones. This finding could be related to increased antimicrobial sequestration at emulsion interfaces and decreased solubilization of more than tween 80 micelles [28]. PDI characterizes the consistency of droplet size in the nanoemulsion. If a PDI is lower than 0.3, a sample can be classified as monodisperse, which signifies the uniformity of the globule size distribution [29]. Droplet stability is sometimes determined by Zeta potential, meaning that higher positive values than +30 mV and negative values than -30 mV indicate perfect stability [30].

3.3. Total phenolic and flavonoid contents

Figure 3A shows that tea tree oil and its nanoemulsion have total phenolic contents of 0.62 and 0.64 mg gallic acid equivalent/g oil, respectively. The overall flavonoid content was 0.10 and 0.08 mg rutin equivalents/g for tea tree oil and oil for nanoemulsion, respectively. (Figure 3B). The phenolic and flavonoid contents availability varied between crude oil and the nanoemulsion. The phenolic content of EO depends on several factors, including oil variety, environmental conditions, ripening, and genetic factors. A similar finding was obtained in which tea oil showed a low level of flavonoids [31].

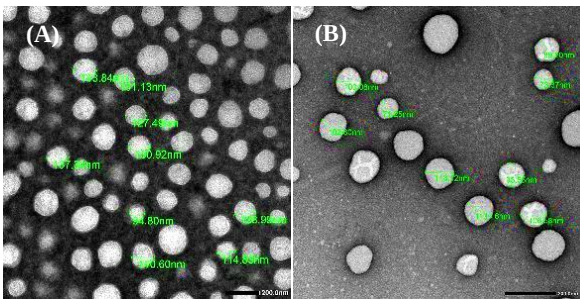

Figure 2: The TEM image of the nanoemulsions of tea tree essential EO (A) and terpinen-4-ol (B). The TEM was performed on a JEOL JEM-1400 Plus operating at an acceleration voltage of 80 kV with 20 µm aperture and a magnification of 25000x.

3.4. Antioxidant activity

Table 3 shows the EC_{50} of tea tree oil, terpinen-4-ol, nanoemulsions, and α-tocopherol with 95% confidence limits and other statistical parameters. By comparing tea tree oil with its monoterpene, terpinen-4-ol was the strongest DPPH radical scavenging activity with the lowest EC_{50} value (480.56 mg/L), while the tea tree oil was less active (2323.80 mg/L). As a result of the nanoemulsions, terpinen-4-ol nanoemulsion had an even higher antioxidant effect with the lowest EC_{50} value (253.65) than tea tree oil (EC_{50} = 1952.82 mg/L).

The antioxidant capacity of the prepared nanoemulsions was higher than the crude products. EOs are complex mixtures of mono-, di-, and sesquiterpenes, oxygenated terpenes, and phenols.

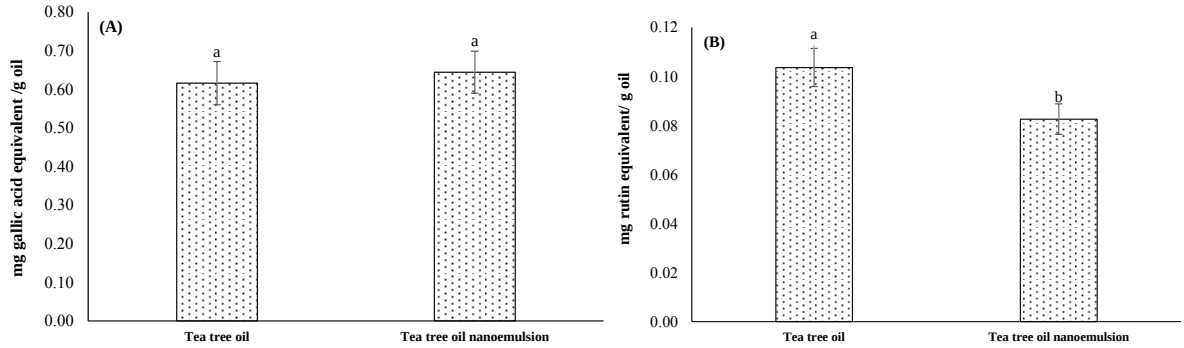

Figure 3: The total phenolic content (A) and total flavonoid content (B) in tea tree EO and its nanoemulsion. The data are mean ± SE ($n = 3$). The columns in the figure that are headed with the different letters are significantly different ($P \leq 0.05$) according to the Student-Newman-Keuls (SNK) test.

Table 3: The antioxidant activity of tea tree EO, terpinen-4-ol, and their nanoemulsions using DPPH reagent and the antibacterial activity against *S. typhimurium* ATCC1402 and *S. aureus* ATCC 6538

Product	EC_{50} [a] (mg/L)	MIC [b] (mg/L)	
		S. typhimurium	***S. aureus***
Tea tree oil	2323.80	2100	1650
Terpinen-4-ol	480.56	750	600
Tea tree oil nanoemulsion	1952.82	750	650
Terpinen-4-ol nanoemulsion	253.65	600	475
α-Tocopherol	9.16	-	-
Ceftriaxone	-	22	15

[a] The effective concentration value for a compound that required to inhibit 50% of the color reagent at specified test duration. [b] MIC: Minimum inhibitory concentration.

The high antioxidant activity of EOs can often be explained by the presence of different chemical substances in their composition, including phenols, alkaloids, flavonoids, tannins, glycosides, and other compounds [15, 32]. It is well documented that phenols can trap the chain-carrying lipid peroxyl radicals responsible for lipid oxidation by trapping them [33]. Furthermore, monoterpene hydrocarbons, particularly those with activated methylene groups like terpinene-4-ol and alpha and beta terpinene, have been found to be better antioxidants than sesquiterpenes. The present study shows that terpinen-4-ol (a phenolic monoterpene) had the highest percentage in tea tree oil. Therefore, it is critical to study the relationship between terpenic compounds and antioxidant activity in tea tree oil to provide a better understanding of its biofunctional benefits. Furthermore, the increased awareness of the antioxidant activity of tea tree oil may have a positive effect on the value of the product.

3.5. Antibacterial activity

The antibacterial activity against Gram-negative *S. typhimurium* ATCC1402 and Gram-positive *S. aureus* (ATCC 6538) are presented in Table 3 as MIC values in mg/L. Among the non-emulsified products and nanoemulsions, terpinen-4-ol was more active (MIC = 750 and 600 mg/L against *S. typhimurium* and 600 and 475 mg/L against *S. aureus*, respectively) than the tea tree oil (MIC = 2100 and750 mg/L against *S. typhimurium*, and 1650 and 650 mg/L against and *S. aureus*, respectively).

The results presented that the products exhibited high antibacterial action and terpinen-4-ol nanoemulsion was the most active against *S. typhimurium* and *S. aureus*. Some researchers stated that Gram-negative bacteria are more sensitive to the antibacterial activity of EOs [34]. Conversely, most researchers have concluded that Gram-positive bacteria are more susceptible to EOs than Gram-negative bacteria [35]. This finding may be due to the different structures of the cell walls of Gram-positive and Gram-negative bacteria. [36]. Tea tree oil and its nanoemulsion have an inhibitory effect due to several active components, the most prominent of which is terpinen-4-ol, which has antibacterial properties by disrupting the structural and functional integrity of bacterial membrane. According to the type and biochemical reactions inside the bacterial cell, different compounds are likely to reveal different mechanisms of action against microbes [35, 37]. EO's phenolic structure might explain its high activity against the tested microbial strains. Alcoholic monoterpenes play as either protein denaturation agents, solvents, or dehydrating agents [38]. In general, hydrocarbons are capable of partitioning into

the cell and cytoplasmic membrane of microorganisms and disrupt their vital functions, which may result in leakage of ions such as potassium, and the inhibition of respiration [39].

3.6. Candidacidal effect

The candidacidal effect of the tea tree oil, terpinen-4-ol, and their nanoemulsions against *C. albicans* are presented in Table 4, related to carbendazim as a standard. The results are shown as EC_{50} values in mg/L and their statistical parameters. Terpinen-4-ol was a significantly more toxic compound with lower EC_{50} (236.98 mg/L) than tea tree oil (EC_{50} = 482.47 mg/L). However, the tree oil nanoemulsion exhibited a higher inhibitory effect with a lower EC_{50} value (74.86 mg/L) than terpinen-4-ol nanoemulsion (EC_{50} = 152.27 mg/L). Based on the activity data of the tested products, nanoemulsions were superior active against *C. albicans* compared to the pure forms. However, the standard compound remained the most effective (EC_{50} = 10.12 mg/L). The high sensitivity of *C. albicans* to tea tree oil is connected mainly to terpinen-4-ol, the main bioactive component present in tea tree oil [6]. The findings of this study are similar to those of other investigations in which tea tree oil was utilized to boost the efficacy of fluconazole against *C. albicans* [40, 41].

3.7. Antifungal effect

3.7.1. On mycelial growth

The results revealed that terpinen-4-ol has higher toxicity with lower EC_{50} values (575.17 and 594.23 mg/L against *A. flavus* and *A. niger*, respectively) than the tea tree oil (EC_{50} = 1169.25 and 1333.81 mg/L against *A. flavus* and *A. niger*, respectively) (Table 4). Based on the results of mycelial inhibition by the nanoemulsions of both tested products, terpinen-4-ol nanoemulsion presented high toxic action (EC_{50} = 253.13 and 312.53 mg/L against *A. flavus* and *A. niger*, respectively) compared to tea tree oil (EC_{50} = 690.14 and 732.03 mg/L against *A. flavus* and *A. niger*, respectively).

Tea tree oil also mediates its antifungal action in a similar way of the antibacterial action. In addition, plasma and mitochondrial membranes of fungal species are also thought to be negatively affected by inhibition of glucose-induced medium acidification by tea tree oil, which involves inhibition of membrane ATPase responsible for the expulsion of protons. The correlation between the chemical components of the tested oil and their antifungal activity could determine its effective toxicity against fungi [42]. Terzi et al. reported that terpinen-4-ol was the most effective compound for reducing mycelial growth of *Pyrenophora graminea*, *Fusarium graminearum* and *F. culmorum compared to* γ-terpinene and 1,8-cineole [43]. The authors found that the MIC_{50} values of terpinen-4-ol were 480, 390, and 490 mg/L for *F. culmorum*, *F. graminearum* and *P. graminea*, respectively. The present study results are also in agreement with that reported in previous research [44] that indicated terpinen-4-ol was the best active compound against all the tested microorganisms compared to 1,8-cineole, ρ-cymene, linalool, α-terpinene, γ-terpinene, α-terpineol, and terpinolene. In another study, filamentous fungi *A. flavus* and *A. niger* were entirely inhibited by tea tree oil for 24 h, and the MICs for other several dermatophytes and filamentous fungi ranged from 40 to 2500 mg/L [45].

3.7.2. On spore germination

Tea tree oil and nanoemulsion exhibited significant inhibitory effect (EC_{50} = 792.42 and 484.61 mg/L) against spores of *A. flavus* and (733.58 and 291.99 mg/L) and *A. niger*, respectively (Table 4). While, terpinen-4-ol and its nanoemulsion were less active (EC_{50} = 2694.82 and1222.63 mg/L, respectively) against spores of *A. flavus* and (2030.77 and 537.57 mg/L, respectively) *A. niger*. Huang et al. found that 50% of *A. niger* and 40% of *Rhizopus stolonifer* spores were inactivated over 60 min through a filter coated with tea tree oil [46]. The current findings show that the conidia of *Aspergillus spp*. are less sensitive to tea tree EO and terpinen-4-ol in both forms (crude and nanoemulsion) than yeast cells. This finding could be attributed to the thickness and density of the conidia wall, which results in less exposure to the antifungal agent [47].

Table 4: The inhibitory effect of tea tree EO, terpinen-4-ol, and their nanoemulsions against yeast *C. albicans* (ATCC 90028), mycelial growth and spore germination of *A. flavus* and *A. niger* compared to carbendazim as a standard

Product	EC_{50}[a] (mg/L)				
	C. albicans	Mycelial growth		Spore germination	
		A. flavus	*A. niger*	*A. flavus*	*A. niger*
Tea tree oil	482.47	1169.25	1333.81	792.42	733.58
Terpinen-4-ol	236.98	575.17	594.23	2694.82	2030.77

Product	EC_{50}[a] (mg/L)				
Tea tree oil nanoemulsion	74.86	690.14	732.09	484.61	291.99
Terpinen-4-ol nanoemulsion	152.27	253.13	312.53	1222.63	537.57
Carbendazim	10.12	20.45	46.08	26.63	19.75

[a] The concentration value for a compound that required inhibiting 50% of yeast, and the mycelia spore of tested fungi a specified test duration.

4. Conclusion

Tea tree oil, its main constituent terpinen-4-ol, and their nanoemulsions were tested for their antioxidant, and antimicrobial potency against bacteria, fungi, and yeast. Alkenes and alcohols were the predominant components of tea tree oil, with the significant individual component (terpinen-4-ol) accounting for approximately 41.11% of the total EO. The nanoemulsions were prepared by the high-energy homogenization method using ultrasonication. The nanoemulsions have droplet sizes of 70.67 and 176.00 nm for tea tree oil and terpinen-4-ol, respectively. The nanoemulsions were found to be more active than the unformulated products. Nanoemulsions were expected to provide the best results due to their stability and high penetration through the target cells. It is hoped that these natural products will provide a step forward in the search for novel antioxidant and antimicrobial agents, at a time when new drugs that have no or minimum side effects are urgently needed. However, it is necessary to conduct further studies to clarify various limitations regarding their mammalian safety, antimicrobial mode of action, and appropriate formulations for use as biorational antimicrobial agents.

5. Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

6. Competing interests

The authors confirm that this article content has no conflict of interest.

7. Funding

None.

8. Acknowledgement

The authors would like to thanks the staff members of the Microbiology Laboratory, Department of Dairy Science, Faculty of Agriculture, Alexandria University, Egypt for support the work by two food pathogenic bacteria. The authors would like to thanks the staff members of the Microbiology Department Faculty of Medicine, Alexandria University, Egypt for support the work by a yeast strain of *Candida albicans* (ATCC 90028).

9. References

[1] Murugaiyan J.; Kumar P. A.; Rao G. S.; Iskandar K.; Hawser S.; Hays J. P.; Mohsen Y.; Adukkadukkam S.; Awuah W. A.; Jose R. A. M. Progress in alternative strategies to combat antimicrobial resistance: Focus on antibiotics. Antibiotics. 2022; 11:200-237.

[2] Jamshidi-Kia F.; Lorigooini Z.; Amini-Khoei H. Medicinal plants: Past history and future perspective. Journal of Herbmed Pharmacology. 2018; 7.

[3] Jamshidi-Kia F.; Lorigooini Z.; Amini-Khoei H. Medicinal plants: Past history and future perspective. Journal of Herbmed Pharmacology. 2018; 7:1-7.

[4] Sharma D. R.; Kumar S.; Kumar V.; Thakur A. Comprehensive review on nutraceutical significance of phytochemicals as functional food ingredients for human health management. Journal of Pharmacognosy and Phytochemistry. 2019; 8:385-395.

[5] Zhao Y.; Hu X.; Zuo X.; Wang M. Chemopreventive effects of some popular phytochemicals on human colon cancer: A review. Food and Function. 2018; 9:4548-4568.

[6] Carson C. F.; Hammer K. A.; Riley T. V. *Melaleuca alternifolia* (tea tree) oil: a review of antimicrobial and other medicinal properties. Clinical Microbiology Reviews. 2006; 19:50-62.

[7] Hammer K. A.; Carson C. F.; Riley T. V. Effects of *Melaleuca alternifolia* (tea tree) essential oil and the major monoterpene component terpinen-4-ol on the development of single-and multistep antibiotic resistance and antimicrobial susceptibility. Antimicrobial Agents and Chemotherapy. 2012; 56:909-915.

[8] Yasin M.; Younis A.; Javed T.; Akram A.; Ahsan M.; Shabbir R.; Ali M. M.; Tahir A.; El-Ballat E. M.; Sheteiwy M. S. River tea tree oil: Composition, antimicrobial and antioxidant activities, and potential applications in agriculture. Plants. 2021; 10:2105-2120.

[9] Hart P. H.; Brand C.; Carson C. F.; Riley T. V.; Prager R. H.; Finlay-Jones J. J. Terpinen-4-ol, the main component of the essential oil of *Melaleuca alternifolia* (tea tree oil), suppresses inflammatory mediator production by activated

human monocytes. Inflammation Research. 2000; 49:619-626.
[10] Alirezalu K.; Pateiro M.; Yaghoubi M.; Alirezalu A.; Peighambardoust S. H.; Lorenzo J. M. Phytochemical constituents, advanced extraction technologies and techno-functional properties of selected Mediterranean plants for use in meat products. A comprehensive review. Trends in Food Science and Technology. 2020; 100:292-306.
[11] May J.; Chan C. H.; King A.; Williams L.; French G. L. Time–kill studies of tea tree oils on clinical isolates. Journal of Antimicrobial Chemotherapy. 2000; 45:639-643.
[12] Sikkema J.; de Bont J. A.; Poolman B. Mechanisms of membrane toxicity of hydrocarbons. Microbiological Reviews. 1995; 59:201-222.
[13] Li X.; Shen D.; Zang Q.; Qiu Y.; Yang X. Chemical components and antimicrobial activities of tea tree hydrosol and their correlation with tea tree oil. Natural Product Communications. 2021; 16:1-7.
[14] Al-Maqtari Q. A.; Rehman A.; Mahdi A. A.; Al-Ansi W.; Wei M.; Yanyu Z.; Phyo H. M.; Galeboe O.; Yao W. Application of essential oils as preservatives in food systems: challenges and future prospectives–a review. Phytochemistry Reviews. 2021:1-38.
[15] Badr M. M.; Badawy M. E. I.; Taktak N. E. M. Characterization, antimicrobial activity, and antioxidant activity of the nanoemulsions of *Lavandula spica* essential oil and its main monoterpenes. Journal of Drug Delivery Science and Technology. 2021; 65:102732.
[16] Cimino C.; Maurel O. M.; Musumeci T.; Bonaccorso A.; Drago F.; Souto E. M. B.; Pignatello R.; Carbone C. Essential oils: pharmaceutical applications and encapsulation strategies into lipid-based delivery systems. Pharmaceutics. 2021; 13:327-363.
[17] Battisti M. A.; Caon T.; de Campos A. M. A Short review on the antimicrobial micro-and nanoparticles loaded with *Melaleuca alternifolia* essential oil. Journal of Drug Delivery Science and Technology. 2020:102283.
[18] Mallika L.; Thanigaivel S. Comparison of antimicrobial and antipathogenic efficacy of tea tree oil nanoemulsion against gram negative *Pseudomonas aeruginosa* infection in *Cyprinus carpio*. ECS Transactions. 2022; 107:14027.
[19] Davies N. W. Gas chromatographic retention indices of monoterpenes and sesquiterpenes on methyl silicon and Carbowax 20M phases. Journal of Chromatography A. 1990; 503:1-24.
[20] Cicco N.; Lanorte M. T.; Paraggio M.; Viggiano M.; Lattanzio V. A reproducible, rapid and inexpensive Folin–Ciocalteu micro-method in determining phenolics of plant methanol extracts. Microchemical Journal. 2009; 91:107-110.
[21] Chang C.-C.; Yang M.-H.; Wen H.-M.; Chern J.-C. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. Journal of Food and Drug Analysis. 2002; 10.
[22] Sharma O. P.; Bhat T. K. DPPH antioxidant assay revisited. Food Chemistry. 2009; 113:1202-1205.
[23] Finney D. J. Probit analysis. 3d Ed1971.
[24] EUCAST. Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by agar dilution. EUCAST Definitive Document E. Def 3.1. Clinical Microbiology and Infection. 2000; 6:509-515.
[25] Das K.; Tiwari R. K. S.; Shrivastava D. K. Techniques for evaluation of medicinal plant products as antimicrobial agents: current methods and future trends. Journal of Medicinal Plants Research. 2010; 4:104-111.
[26] Flores F. C.; Ribeiro R. F.; Ourique A. F.; Rolim C. M. B.; Silva C. d. B. d.; Pohlmann A. R.; Beck R. C. R.; Guterres S. S. Nanostructured systems containing an essential oil: protection against volatilization. Química Nova. 2011; 34:968-972.
[27] Biju S. S.; Ahuja A.; Khar R. K.; Chaudhry R. Formulation and evaluation of an effective pH balanced topical antimicrobial product containing tea tree oil. Die Pharmazie-An International Journal of Pharmaceutical Sciences. 2005; 60:208-211.
[28] Terjung N.; Löffler M.; Gibis M.; Hinrichs J.; Weiss J. Influence of droplet size on the efficacy of oil-in-water emulsions loaded with phenolic antimicrobials. Food & Function. 2012; 3:290-301.
[29] Danaei M.; Dehghankhold M.; Ataei S.; Hasanzadeh Davarani F.; Javanmard R.; Dokhani A.; Khorasani S.; Mozafari M. R. Impact of particle size and polydispersity index on the clinical applications of lipidic nanocarrier systems. Pharmaceutics. 2018; 10:57.
[30] Honary S.; Zahir F. Effect of zeta potential on the properties of nano-drug delivery systems-a review (Part 1). Tropical Journal of Pharmaceutical Research. 2013; 12:255-264.
[31] Ksouri R.; Megdiche W.; Falleh H.; Trabelsi N.; Boulaaba M.; Smaoui A.; Abdelly C. Influence of biological, environmental and technical factors on phenolic content and antioxidant activities of Tunisian halophytes. Comptes Rendus Biologies. 2008; 331:865-873.
[32] Amorati R.; Foti M. C. Mode of antioxidant action of essential oils. In: Hashemi S. M. B.;

Khaneghah A. M.; Sant'Ana A. S., editors. Essential Oils in Food Processing: Chemistry, Safety and Applications: John Wiley & Sons Ltd; 2017. p. 267-291.
[33] Zeb A. Applications of phenolic antioxidants. In: Zeb A., editor. Phenolic Antioxidants in Foods: Chemistry, Biochemistry and Analysis: Springer, Cham. ; 2021. p. 385-411.
[34] Zaika L. L. Spices and herbs: their antimicrobial activity and its determination 1. Journal of Food Safety. 1988; 9:97-118.
[35] Nazzaro F.; Fratianni F.; De Martino L.; Coppola R.; De Feo V. Effect of essential oils on pathogenic bacteria. Pharmaceuticals. 2013; 6:1451-1474.
[36] Muta T.; Parikh A.; Kathawala K.; Haidari H.; Song Y.; Thomas J.; Garg S. Quality-by-design approach for the development of nano-sized tea tree oil formulation-impregnated biocompatible gel with antimicrobial properties. Pharmaceutics. 2020; 12:1091.
[37] Álvarez-Martínez F. J.; Barrajón-Catalán E.; Herranz-López M.; Micol V. Antibacterial plant compounds, extracts and essential oils: An updated review on their effects and putative mechanisms of action. Phytomedicine. 2021; 90:153626.
[38] Guimarães A. C.; Meireles L. M.; Lemos M. F.; Guimarães M. C. C.; Endringer D. C.; Fronza M.; Scherer R. Antibacterial activity of terpenes and terpenoids present in essential oils. Molecules. 2019; 24:2471.
[39] Xu J.; Zhou F.; Ji B. P.; Pei R. S.; Xu N. The antibacterial mechanism of carvacrol and thymol against *Escherichia coli*. Letters in Applied Microbiology. 2008; 47:174-179.
[40] Mertas A.; Garbusińska A.; Szliszka E.; Jureczko A.; Kowalska M.; Król W. The influence of tea tree oil (*Melaleuca alternifolia*) on fluconazole activity against fluconazole-resistant *Candida albicans* strains. BioMed Research International. 2015; 2015.
[41] Francisconi R. S.; Huacho P. M. M.; Tonon C. C.; Bordini E. A. F.; Correia M. F.; Sardi J. d. C. O.; Spolidorio D. M. P. Antibiofilm efficacy of tea tree oil and of its main component terpinen-4-ol against *Candida albicans*. Brazilian Oral Research. 2020; 34:1-9.
[42] Kumar U.; Kumari P.; Sinha D.; Yadav M.; Singh D.; Singh B. K. Antimicrobial activity of essential oils against plant pathogenic fungi: A review. International Journal of Inclusive Development. 2020; 6:37-44.
[43] Terzi V.; Morcia C.; Faccioli P.; Vale G.; Tacconi G.; Malnati M. *In vitro* antifungal activity of the tea tree (*Melaleuca alternifolia*) essential oil and its major components against plant pathogens. Letters in Applied Microbiology. 2007; 44:613-618.
[44] Carson C. F.; Riley T. V. Antimicrobial activity of the major components of the essential oil of *Melaleuca alternifolia*. Journal of Applied Bacteriology. 1995; 78:264-269.
[45] Hammer K. A.; Carson C. F.; Riley T. V. In vitro activity of *Melaleuca alternifolia* (tea tree) oil against dermatophytes and other filamentous fungi. Journal of Antimicrobial Chemotherapy. 2002; 50:195-199.
[46] Huang R.; Pyankov O. V.; Yu B.; Agranovski I. E. Inactivation of fungal spores collected on fibrous filters by *Melaleuca alternifolia* (tea tree oil). Aerosol Science and Technology. 2010; 44:262-268.
[47] Deak E.; Wilson S. D.; White E.; Carr J. H.; Balajee S. A. *Aspergillus terreus* accessory conidia are unique in surface architecture, cell wall composition and germination kinetics. PLOS One. 2009; 4:e7673.