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Antimicrobial effects of essential oils of *Artemisia annua, Mentha longifolia,* and *Vitex agnus-castus* and their nanoemulsions against pathogenic microbes causing cattle mastitis

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Abstract
Cattle mastitis is one of the most common problems affecting the global economy. The main objectives of this study were: (i) assessing the antimicrobial activities of essential oils (EOs) from *Artemisia annua*, *Mentha longifolia*, and *Vitex agnus-castus*, and their nanoemulsions, against mastitis-associated pathogens, and (ii) identifying the chemical components of these EOs. Eucalyptol (15.34%), artemisia ketone (9.59%), and endo-borneol (8.24%) were found to be the main components of *A. annua* EO; pulegone (37.37%), isomenthone (32.03%), and eucalyptol (20.61%) were the major constituents of *M. longifolia* EO; cuminic aldehyde (66.15%) and 2-caren-10-al (6.65%) were the main compounds in *V. agnus-castus* EO. The EOs were assessed against the growth of the bacteria *Citrobacter diversus*, *Proteus vulgaris*, *Escherichia coli*, and *Staphylococcus aureus* as well as the fungi *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida krusei*, and *Candida albicans*. We found that the three EOs were most potent against *P. vulgaris*, *S. aureus*, and *E. coli*,with a significant activity against the other strains as well. The EOs also showed significant antifungal activities against all the five *Candida* strains, with *V. agnus-cactus* EO being the most potent. The EO nanoemulsions were found to be more active than the pure oils against all the microbes tested. Our results clearly demonstrate the potential of these EOs and their nanoemulsions as antimicrobial agents.
Keywords: Antimicrobial, *Artemisia annua, Mentha longifolia, Vitex agnus-castus*, essential oil, nanoemulsions, mastitis-associated microbes**..**

1. Introduction

Cattle mastitis is one of the most common problems affecting the dairy industry and economy, especially in developing countries. It involves inflammation of the mammary gland tissue, which causes the milk yield and quality to decrease [1,2]. Depending on the inflammation, mastitis can be chronic, sub-clinical, or clinical. Microbes, including bacteria and fungi, are the main etiological agents of this condition [1].

Besides vaccination, antibiotics represent a major treatment strategy and can be administered as intravenous or intramuscular injections, as well as intramammary infusions [3,4]. However, the wide use of commercial antibiotics to treat cattle, besides inducing allergies and causing immunodeficiency, has led to the emergence of non-responsive, drug-resistant microbes [5,6]. Hence, many studies have shifted their focus to natural products, investigating their potential to inhibit or control the pathogens isolated from the milk of mastitic cattle [7-11].

The essential oils (EOs) and extracts of numerous medicinal and aromatic herbs have been shown to exhibit significant antimicrobial and antioxidant activities [12-17. However, very few of them have been tested against the microbes isolated from the milk of mastitic cattle [11].

Artemisia annua L. (Asteraceae), *Mentha longifolia*, and *Vitex agnus-castus* (Lamiaceae) are common, important medicinal plants worldwide, possessing unique metabolites with documented uses in the treatment of several diseases [17-19]. Many studies have demonstrated the antimicrobial activities of enriched EOs from these plants [20- 23,12-17]. The antimicrobial efficiency of these EOs can be enhanced by using nanoemulsion formulations that increase their dispersibility and intervene with microbial cell membranes [24].

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The aim of this study was to evaluate the inhibitory activities of EOs from *A. annua*, *M. longifolia*, and *V. agnus-castus* and their nanoemulsions against some pathogenic microbes responsible for cattle mastitis, such as *Citrobacter diversus* (ATTC 13315), *Proteus vulgaris* (ATTC 13315), *Escherichia coli* (ATCC 35218), *Staphylococcus aureus* (ATCC 25923), *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, and *Candida krusei*, and *Candida albicans* (ATCC 10231).

Experimental section

Plant materials

A. annua and *M. longifolia* were collected from Adlya farm in the El-Sharkia Governorate, 80 km east of Cairo, Egypt, in April and May 2021. They were authenticated by Prof. Dr. M.A. Gibali, senior botanist at Orman Botanic Garden, Giza, Egypt. Two specimens of these two plants were deposited in the Orman Botanic Garden herbarium with specimen codes OB-MD-202xTY-1966 and OB-TV-202xTY-1985. *V. agnus-castus* plants were collected before the flowering period from populations growing in the wild in Saint Catherine, South Sinai, Egypt. The plants were identified by Prof. Ibrahim El-Garf, Department of Botany, Faculty of Science, Cairo University, Cairo, Egypt, and voucher specimens were deposited in the herbarium of the National Research Centre, Cairo (*Vitex agnus-castus* no. AH-1101)

EO extraction and chemical characterization by gas chromatography/mass spectrometry (GC/MS) analysis

The EOs of the air-dried, powdered aerial parts of *A. annua*, *M. longifolia*, and *V. agnus-castus* were extracted separately via the hydro-distillation of 150 g of each plant sample for 3 h on the Clevenger apparatus. This extraction was repeated thrice and the obtained oils were stored in glass vials at 4°C for further GC/MS analysis. The GC/MS analysis and the chemical characterization of the EOs were performed using the same protocol and experimental conditions described in recent studies [25,26].

Preparation of EO nanoemulsions of *A. annua, M. longifolia,* and *V. agnus-castus*

Tween 80, a non-ionic surfactant derived from sorbitan esters, was used to make nanoemulsions from the EOs of *A. annua*, *M. longifolia*, and *V. agnus-castus*. As the organic phase, Tween 80 was added to the EO (1:1, w/w). With vigorous stirring at 25°C, the organic phase mixture was slowly added in droplets to distilled water (aqueous phase). The instrument Ultrasonic (Sonics & Materials, Inc., 53 Church Hill Rd., Newtown, CT, USA) was used to sonicate the produced emulsion at 20°C for 15 min at a high frequency of 20 kHz with a power output of 750 W. [26, 27].

Characterization of EO nanoemulsions using high-resolution transmission electron microscopy (TEM)

The nanoemulsions were characterized using high-resolution TEM (Model JEOL-JEM-2100, Japan) to determine the size and shape of droplets. A drop of the nanoemulsion was placed on a carbonated copper grid, negatively stained with 1% phosphotungstic acid, and allowed to dry at room temperature for 2 min using a Whatman filter paper before examination [28].

Droplet size and zeta potential analysis

At 23°C, a dynamic light scattering apparatus (PSS, Santa Barbara, CA, USA) was used to evaluate the average size, size distribution, and zeta potential utilizing the 632.8 nm line of a helium–neon laser as the incident light with an angle of 90° and the zeta potential with an external angle of 18.9°. (Fernandes et al., 2014; Sugumar et al., 2014)[29-30].

Antimicrobial assays using EOs and their nanoemulsions

Bacterial strains

The bacterial strains used in this study were: *Citrobacter diversus* (ATTC 13315), *Proteus vulgaris* (ATTC 13315), *Staphylococcus aureus* (ATTC 25923), and *Eschericia coli* (ATTC 35218). They were obtained from the culture collection of the Department of Microbiology and Immunology, National Research Centre, Cairo, Egypt. In addition, two resistant strains of *S. aureus* and *E. coli* were used.

Fungal strains

Four *Candida* species – *C. glabrata*, *C. albicans*, *C. tropicalis*, and *C. krusei* – were identified by polymerase chain reaction and used in this study [31].

Agar disc diffusion method

Strains were prepared by transferring a loopful of bacterial culture into nutrient broth (Oxoid, England) or a loopful of fungal culture into Sabouraud dextrose broth (Oxoid, England). A sterile cotton swab was dipped into the 0.5 McFarland standard microbial inoculum (1.5 x 10^8 CFU/mL suspension), and streaked over Mueller–Hinton agar (bacteria) or Sabouraud dextrose agar plates (fungi) (Oxoid, England). Sterilized 6 mm blank discs were loaded with EOs diluted with dimethyl sulfoxide (DMSO) (bacteria) or 10% Tween 80 in distilled water (fungi). The impregnated discs were applied on the inoculated agar plates using sterile forceps. Standard antibiotic discs (30 µg vancomycin for *S. aureus*, 5 µg ciprofloxacin for other bacteria, and fluconazole for *Candida*) were used as positive controls while distilled water and DMSO-loaded discs were used as negative controls. The plates were inverted and incubated for 24 h at 37°C (bacteria) or 48 h at 28°C (fungi). The diameter of inhibition zone was measured in millimetres with a ruler for assessing the antimicrobial activity [32,33]**.** The results with three replicates were analyzed by one-way analysis of variance (version 6.311; CoHort Software, Monterey, CA, USA).

Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC)

The EOs which showed inhibition zones in the agar disc diffusion method were tested to determine their MIC values by broth microdilution method**.** A 100 µg/mL stock solution of the EO was prepared in sterile Muller–Hinton broth (MHB, Oxoid, England) containing 10% Tween 20 (bacteria) or in sterile distilled water containing 10% Tween 80 (fungi). The stock solution was serial-diluted in 100 µL sterile MHB (bacteria) or Sabouraud dextrose broth (fungi) in 96-well microtiter plates. Then, 1 µL of microbial suspension adjusted to 0.5 McFarland was added to each well. A well containing broth with the inoculum was the positive growth control while one containing broth without the inoculum was the negative control. After incubation, the lowest concentration of the EO showing no visible growth was recorded as the MIC value [34,35]. MBC and MFC were determined by sub-culturing of MIC wells. The lowest concentrations of the EO which showed no microbial growth were recorded as the MBC and MFC values [34,36].

Results and Discussion

The EOs of the three plants *A. annua*, *M. longifolia*, and *V. agnus-castus* were extracted and chemically characterized via GC/MS. The chemical components of the three plants are listed in **Table 1**. The nanoemulsions of these EOs were prepared and characterized by TEM (Figure 1) followed by droplet size and zeta potential analysis (Figure 2). Finally, the three EOs and their nanoemulsions were tested against some pathogenic microbes associated with bovine and cattle mastitis.

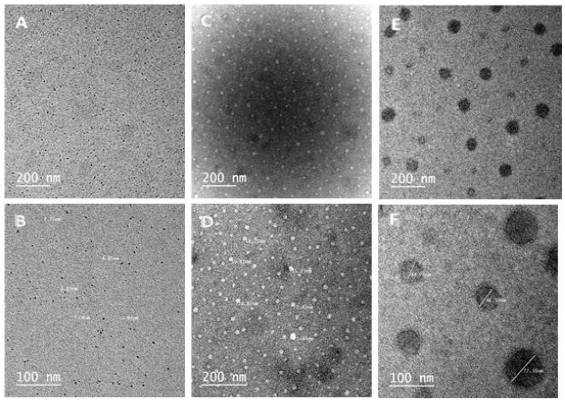

Figure 1: High-resolution transmission electron micrographs of EO nanoemulsions of (**A, B**) *Artemisia annua*, (**C, D**) *Mentha longifolia*, and (**E, F**) *Vitex agnus-castus*.

Chemical profiling of *A. annua, M. longifolia,* and *V. agnus-castus* EOs

The GC/MS analysis of the *A. annua* EO revealed 32 compounds representing 99.19% of the overall oil mass, as summarized in **Table 1**. From all the identified compounds, eucalyptol (15.34%), artemisia ketone (9.59%), endo-borneol (8.24%), 1,5,9,9-tetramethyl-2-methylene-spiro[3.5]non-5-ene (6.16%), o-cymene (5.33%), germacrene D (5.25%), *Z*-α-santalol (5.09%), camphor (4.27%), and *trans*-caryophyllene (4.01%) were the main constituents. All these components have already been reported as the main constituents of *A. annua* EO around the world [37-41,17].

We identified 23 compounds from *M. longifolia* EO, accounting for 99.78% of the overall oil mass (**Table 1**). The preponderance of terpenoids in our study, especially the monoterpenes, was in complete agreement with previous findings (Farzaei et al., 2017). Pulegone (37.37%), isomenthone (32.03%), and eucalyptol (20.61%) were the main components, which has already been reported in *M. longifolia* samples collected from different countries[42-47, 12]. The GC/MS chemical profiling of *V. agnus-castus* EO led to the identification of 28 compounds constituting 99.46% of the overall EO mass (**Table 1**). Monoterpenes were the main components, similar to previously reported data (Kustrak et al., 1994; Habbab et al., 2016; Benmeddour et al., 2019). Cuminic aldehyde (66.15%) and 2-caren-10-al (6.65%) were identified as the principal constituents of the EO, which was specific to our study since they were not reported in the previous ones[48-50].

TEM analysis of EO nanoemulsions

The EO nanoemulsions were prepared and the high-resolution TEM analysis revealed that all of them were of critical nano-size (less than 100 nm). *A. annua* EO nanoemulsions (**Figure 1A–B**) contained spherical particles with a size ranging from 2.75–4.35 nm, whereas the sizes of the spherical particles present in *M. longifolia* and *V. agnus-castus* EO nanoemulsions ranged from 16.23–20.82 nm and 44.23–77.35 nm, respectively (**Figures 1C–F**).

Droplet size and zeta potential analysis

The size distribution of the *V. agnus-castus* nanoemulsion droplets was estimated. The mean droplet diameter was 437.8 ± 0.037 nm, with 25% of the distribution smaller than 377.4 nm, 50% smaller than 429.6 nm, 75% smaller than 489.0 nm, 80% smaller than 504.9 nm, 90% smaller than 549.4, and 99% of the distribution smaller than 671.5 nm (Figure 2B). In a previous study, the mean droplet diameter of *Araucaria heterophylla* EO nanoemulsions was estimated to be 106 ± 0.655 nm, with 25% of the distribution smaller than 62.3 nm and 75% smaller than 153.5 nm[26]. The concentration of Tween 80 and the duration of sonication were both essential factors in the creation of nanoemulsions [51]. The zeta potential of an EO in suspension is a physical parameter that can be exploited to improve emulsion formulations and forecast long-term stability. The surface potential of nanoemulsion droplets is related to the zeta value. EO nanoemulsions with zeta potentials greater than +30 mV or less than -30 mV are deemed stable [52]. The mean zeta potential of the *V. agnus-castus* nanoemulsion was -30.12 mV, indicating that it was stable (Figure 2A). A previous study showed that *Araucaria bidiwillii* EO nanoemulsion had a mean

diameter of 106 nm and an average zeta potential of -1.84 mV [27].

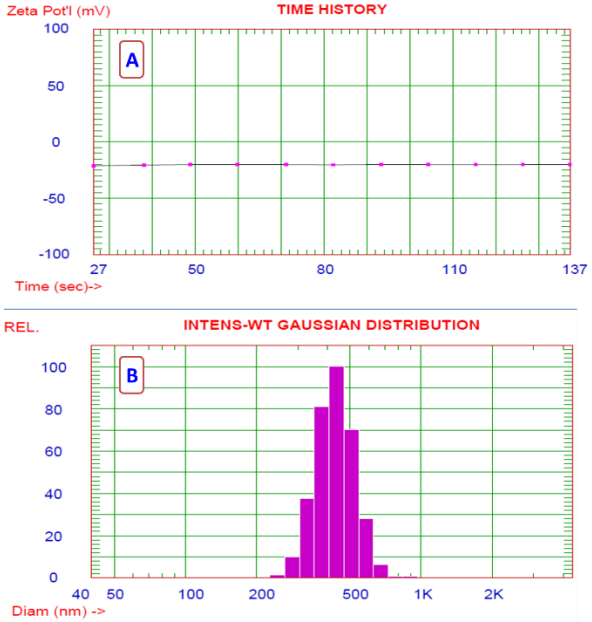

Figure 2. A) Zeta potential analysis and B) particle size distribution of *V. agnus-castus* EO nanoemulsion (the most active one from the three tested nanoemulsions).

Antimicrobial activities of EOs and their nanoemulsions

Artemisia annua

The antimicrobial assay results, including the inhibition zones (mm), MIC, MBC, and MFC (µg mL^{-1}), of *A. annua* EO and its nanoemulsion are summarized in Table 2. All the data displayed significant effects of the EO and its nanoemulsion compared with the two reference antibiotics vancomycin and ciprofloxacin.

The *A. annua* EO exhibited potent antibacterial effects against *P. vulgaris*, *S. aureus*, and *E. coli* with MIC and MBC values of 0.19 and 0.39 µg mL^{-1}, respectively, for all the strains. Similarly, the growth of these three strains was strongly inhibited by the nanoemulsion, with MIC and MBC values of 0.39 and 0.78 µg mL^{-1}, respectively, for all the strains.

The *A. annua* EO moderately inhibited the growth of *C. diversus* and resistant (res.) *E. coli*, with MIC and MBC values of (12.5 and 25.0) and (25.0 and 50.0) µg mL^{-1}, respectively. The nanoemulsion was more active against these two strains than the EO itself, with MIC and MBC values of (6.25 and 12.5) and (6.25 and 10.0) µg mL^{-1}, respectively.

The *A. annua* EO weakly inhibited the growth of res. *S. aureus*, with the same MIC and MBC value of 100.0 µg mL^{-1}. The nanoemulsion significantly increased the potency of the EO, with MIC and MBC values of 12.5 and 25.0 µg mL^{-1}, respectively.

The results described in Table 2 also demonstrate the significant antifungal activities of *A. annua* EO and its nanoemulsion compared with the reference antifungal fluconazole. The EO exerted potent antifungal effects against all the tested *Candida* strains: it was more active against *C. albicans*, *C. tropicalis*, and *C. krusei* with MIC values of 0.19, 0.78, and 1.56 µg mL^{-1} and MFC values of 0.78, 1.56, and 3.13 µg mL^{-1}, respectively. The weakest inhibition was observed against *C. albicans* (ATCC 10231) and *C. glabrata* with MIC values of 3.13 µg mL^{-1} for both strains, and MFC values of 3.13 and 25.0 µg mL^{-1}, respectively.

The nanoemulsion strongly enhanced the inhibitory activity of the EO against all the five strains: *C. glabrata* (MIC and MFC, 0.78 and 1.56 µg mL^{-1}), *C. albicans* (0.19 and 0.39 µg mL^{-1}), *C. tropicalis*, *C. krusei*, and *C. albicans* (ATCC 10231) (0.39 and 0.78 µg mL^{-1}).

All our results corroborated previously reported data on *A. annua* Eos (Cavar et al., 2012; Juteau et al., 2002; Massiha et al., 2013; Li et al., 2011; Bilia et al., 2014). The main compounds, such as eucalyptol and artemisia ketone, were shown to have very strong antimicrobial activities against multiple bacterial and fungal strains (Bilia et al., 2014). The abundance of endo-borneol, o-cymene, germacrene D, and the other compounds was associated with antimicrobial functions exerted by EOs of different plants [20-23].

Mentha longifolia

The antimicrobial activity results of *M. longifolia* EO and its nanoemulsion are listed in **Table 3**. The growth of *P. vulgaris*, *S. aureus*, and *E. coli* was strongly inhibited by *M. longifolia* EO with MIC and MBC values of 0.39 and 0.78 µg mL^{-1}, respectively, for all three strains. In contrast, the antimicrobial activity of the nanoemulsion was weaker than the pure oil with MICs and MBCs of (6.25 and 6.25 µg mL^{-1}), (0.78 and 1.56 µg mL^{-1}), and (6.25 and 6.25 µg mL^{-1}), respectively. Additionally, the EO exhibited strong inhibition of *C. diversus* and res. *S. aureus* growth at MICs and MBCs of (3.13 and 6.25 µg mL^{-1}) and (3.13 and 6.25 µg mL^{-1}), respectively. The nanoemulsion inhibited the growth of these pathogens to a similar extent with an MIC and MBC value of 6.25 µg mL^{-1} for both the strains. The *M. longifolia* EO showed the lowest inhibitory effect against res. *E. coli* with MIC and MBC values of 6.25 and 12.5 µg mL^{-1}; this effect was enhanced by the nanoemulsion, with an MIC and MBC value of 0.78 and 1.56 µg mL^{-1}, respectively.

The *M. longifolia* EO also potently inhibited the growth of two fungi – *C. glabrata* and *C. albicans* – with MICs and MFCs of (0.19 and 0.19 µg mL^{-1}) and (0.19 and 0.78 µg mL^{-1}), respectively. Though relatively weaker, the antifungal activity was considerable against *C. tropicalis* (MIC and MFC, 6.25 and 6.25 µg mL^{-1}), *C. krusei* (12.5 and 6.25 µg mL^{-1}), and *C. albicans* (ATCC 10231) (6.25 and 6.25 µg mL^{-1}). The nanoemulsion displayed relatively stronger inhibition of *C. glabrata*, *C. albicans*, *C. tropicalis*, *C. krusei*, and *C. albicans* (ATCC 10231)

with the same MIC and MFC values of 0.78, 3.13, 3.13, 3.13, and 1.56 µg mL^{-1}, respectively.

Our results on the antimicrobial properties of *M. longifolia* EO agree with previously described data [12]. The wide-spectrum antimicrobial potential of *M. longifolia* EO has been reported [44] (Gulluce et al., 2007) and its activity is largely contributed to by the main components, such as pulegone, isomenthone, and eucalyptol[44].

Vitex agnus-castus

V. agnus-castus EO showed the strongest inhibitory effects against *P. vulgaris* and *S. aureus* with the same MIC and MBC value of 3.13 µg mL^{-1} for both the strains. This EO also strongly inhibited the growth of *C. diversus*, res. *S. aureus*, and res. *E. coli* at the same MIC and MBC value of 6.25 µg mL^{-1} for all three strains. The inhibitory activity of the nanoemulsion against all the tested bacterial strains was stronger than the EO itself, with MIC and MBC values of 0.78 and 1.56 µg mL^{-1} for all strains.

V. agnus-castus EO also exerted potent antifungal activities against the growth of *C. tropicalis*, *C. krusei*, and *C. albicans* (ATCC 10231), with MIC and MFC values of 0.39 and 0.78 µg mL^{-1} for all strains. The inhibition of *C. glabrata* and *C. albicans* was considerably strong, with MIC and MFC values of 0.78 and 1.56 µg mL^{-1} for both the strains. The nanoemulsion showed stronger antifungal activity than the oil against all five fungal strains. The MIC and MFC values against *C. tropicalis*, *C. krusei*, and *C. albicans* (ATCC 10231) were 0.10 and 0.19 µg mL^{-1} for all three strains, while the MIC and MFC values against *C. glabrata* and *C. albicans* were 0.19 and 0.39 µg mL^{-1} for both. These findings are presented in Table 4.

In this study, the nanoemulsions of the three EOs showed more showed more potency than the pure EOs to inhibit microbial growth. The nanoemulsions of soybean and citral oils have been shown to exhibit significantly higher bactericidal effects than the oils themselves [53,54]. The enhanced activity displayed by the nanoemulsions might be due to increased cellular absorption [55], which reduces the resistance to mass transfer [56]. Additionally, the nanoemulsion particles might inhibit microbial growth via direct interaction with the microbes [57-59].

Since the EOs of these medicinal plants have potent antimicrobial effects, they could be used as natural biopreservatves and antimicrobial agents in various food products[60]. This study highlighted that *V. agnus-castus* EO showed a promising antifungal effect against *Candida*, so it can be used to preserve dairy products, especially cheese. Further, *in vivo* studies are needed to investigate the safety, efficacy, and appropriate dosage of these EOs. Future studies should also focus on identifying the active constituents responsible for these antimicrobial properties and their mode of action.

Table 1: GC/MS profiles of essential oils from *Artemisia annua*, *Mentha longifolia*, and *Vitex agnus-castus*

No	Rt[a]	KI[b]	Compound Name	MF[c]	Relative Concentration[d]		
					A. annua	*M.longfolia*	*V. agnus-cactus*
1	3.27	924	α-Thujene	$C_{10}H_{16}$	----	0.03±0.00	----
2	3.39	932	α-Pinene	$C_{10}H_{16}$	----	1.08±0.02	0.05±0.00
3	3.97	946	Camphene	$C_{10}H_{16}$	1.74±0.03	0.06±0.01	1.20±0.04
4	4.02	969	Sabinene	$C_{10}H_{16}$	----	0.86±0.02	----
5	4.12	974	β-Pinene	$C_{10}H_{16}$	----	1.88±0.05	0.58±0.03
6	4.26	988	α-Myrcene	$C_{10}H_{16}$	----	0.46±0.03	----
7	5.02	1026	o-Cymene	$C_{10}H_{14}$	5.33±0.21	----	----
8	5.37	1029	D-Limonene	$C_{10}H_{16}$	----	0.90±0.01	----
9	5.53	1031	Eucalyptol	$C_{10}H_{18}O$	15.34±0.37	20.60±0.46	0.04±0.00
10	6.01	1059	γ-Terpinene	$C_{10}H_{16}$	----	----	1.28±0.06
11	6.04	1062	Artemisia ketone	$C_{10}H_{16}O$	9.59±0.23	----	----
12	6.53	1096	Linalool	$C_{10}H_{18}O$	0.81±0.03	0.10±0.01	----
13	7.17	1098	*trans*-Sabinene hydrate	$C_{10}H_{18}O$	1.26±0.05	0.10±0.02	----
14	7.43	1124	Chrysanthenone	C15H24	----	0.05±0.00	----
15	7.58	1137	*trans*-Limonene oxide	$C_{10}H_{16}O$	----	1.36±0.04	0.05±0.01
16	7.72	1139	endo-Borneol	$C_{10}H_{18}O$	8.24±0.19	----	----
17	7.91	1142	*trans*-Sabinol	$C_{10}H_{16}O$	----	0.12±0.01	----
18	8.07	1144	*trans*-Verbenol	$C_{10}H_{16}O$	----	0.05±0.00	----
19	8.21	1146	Camphor	$C_{10}H_{16}O$	4.27±0.29	1.02±0.02	----

20	8.56	1148	*p*-Menthone	$C_{10}H_{18}O$	----	0.77±0.02	----
21	8.59	1158	Isomenthone	$C_{10}H_{18}O$	----	32.03±0.61	----
22	8.76	1164	Pinocarvone	$C_{10}H_{14}O$	0.48±0.03	----	----
23	8.88	1177	4-Terpineol	$C_{10}H_{18}O$	1.62±0.04	----	0.25±0.03
24	9.35	1182	*cis*-Pinocarveol	$C_{10}H_{16}O$	----	----	0.08±0.01
25	9.49	1188	α-Terpineol	$C_{10}H_{18}O$	0.84±0.03	0.34±0.01	----
26	9.93	1199	*trans*-*p*-Menth-2-en-7-ol	$C_{10}H_{18}O$	----	----	0.05±0.00
27	10.45	1237	Pulegone	$C_{10}H_{16}O$	----	37.37±0.54	----
28	11.01	1239	Cuminic aldehyde	$C_{12}H_{20}O_2$	----	----	66.15±0.72
29	11.08	1252	Piperitone	$C_{10}H_{16}O$	----	0.21±0.02	----
30	11.23	1275	Phellandral	$C_{10}H_{16}O$	----	----	0.04±0.00
31	11.4	1285	Bornyl acetate	$C_{10}H_{14}O$	0.54±0.02	0.20±0.03	0.06±0.01
32	11.47	1297	2-Caren-10-al	$C_{10}H_{14}O$	----	----	6.65±0.17
33	11.75	1299	α-Terpinenyl acetate	$C_{12}H_{20}O_2$	----	----	0.27±0.03
34	12.44	1328	1,5,9,9-Tetramethyl-2-methylene-spiro[3.5]non-5-ene	$C_{15}H_{24}O$	6.16±0.26	----	----
35	12.86	1384	Styrene glycol	$C_8H_{10}O_2$	----	----	21.10±0.73
36	13.84	1373	α-Ylangene	$C_{15}H_{24}$	----	----	0.08±0.01
37	14.05	1374	α-Copaene	$C_{15}H_{24}$	0.65±0.01	----	0.09±0.02
38	14.46	1403	Methyleugenol	$C_{11}H_{14}O$	----	----	0.07±0.01
39	14.96	1419	*trans*-Caryophyllene	$C_{15}H_{24}$	4.01±0.12	----	0.17±0.01
40	15	1456	α-Patchoulene	$C_{15}H_{26}O$	0.53±0.03	----	----
41	15.14	1462	α-Famesene	$C_{15}H_{24}$	3.60±0.31	----	0.07±0.01
42	15.16	1480	*ar*-Curcumene	$C_{15}H_{22}$	----	----	0.07±0.00
43	15.33	1484	Germacrene D	$C_{15}H_{24}$	5.25±0.22	----	----
44	15.4	1493	Zingiberene	$C_{15}H_{24}$	----	----	0.06±0.00
45	15.45	1498	α-Selinene	$C_{15}H_{24}$	1.08±0.05	----	----
46	15.65	1500	Bicyclogermacrene	$C_{15}H_{24}$	0.57±0.03	----	----
47	15.88	1507	α-Bisabolene	$C_{15}H_{24}$	----	----	0.04±0.00
48	15.9	1509	α-Bulnesene	$C_{15}H_{24}$	0.69±0.02	----	----
49	16	1522	δ-Cadinene	$C_{15}H_{24}$	----	----	0.08±0.00
50	16.23	1522	β-Sesquiphellandrene	$C_{15}H_{24}$	----	----	0.08±0.01
51	16.49	1577	Spathulenol	$C_{15}H_{24}O$	1.62±0.09	0.10±0.02	0.19±0.02
52	16.88	1582	Caryophyllene oxide	$C_{15}H_{24}$	5.05±0.27	0.18±0.03	0.07±0.00
53	17.09	1590	Globulol	$C_{15}H_{24}O$	3.96±0.18	----	----
54	17.25	1591	*cis*-*Z*-α-Bisabolene epoxide	$C_{15}H_{26}O$	1.96±0.11	----	----
55	17.32	1594	Carotol	$C_{15}H_{26}O$	----	----	0.54±0.03
56	17.51	1601	Ledene oxide-(II)	$C_{15}H_{24}O$	1.44±0.04	----	----
57	17.71	1617	Longipinocarveol, trans-	$C_{15}H_{24}O$	2.12±0.06	----	----
58	17.97	1624	Farnesene epoxide, E-	$C_{15}H_{24}O$	0.97±0.02	----	----
59	18.24	1631	Ledene oxide-(I)	$C_{14}H_{22}$	1.24±0.05	----	----
60	18.34	1674	*Z*-α-Santalol	$C_{15}H_{24}O$	5.09±0.10	----	----
61	18.9	1685	α-Bisabolol	$C_{15}H_{26}O$	0.63±0.03	----	----
62	19.28	1770	α-bisabolol oxide A	$C_{15}H_{26}O_2$	2.51±0.13	----	----
Total identified					**99.19**	**99.87**	**99.46**

[a] Rt: retention time; [b] KI: Kovats retention index from literature; [c] MF: molecular formula, [d] Data shown as mean ± SD for n=3 replicates.

Table 2. Antimicrobial effects of *Artemisia annua* EO and its nanoemulsion

Strain	EO (2 µL/disc)			EO Nanoemulsion			Reference drug	
	[a] IZ mm	MIC	MBC/ MFC	[a] IZ mm	[b] MIC	[b] MBC/ MFC		
Antibacterial results							**VA 30 µg**	**CIP 5 µg**
C. diversus (ATCC 13315)	12.0±0.11	12.5	25.0	9.0±0.06	6.25	12.5	—	35
P. vulgaris (ATCC 13315)	15.0±0.16	0.19	0.39	10.0±0.11	0.39	0.78	—	30
S. aureus (ATCC 25923)	15.0±0.13	0.19	0.39	12.0±0.10	0.39	0.78	—	30
S. aureus (resistant)	10.0±0.09	100.0	100.0	8.0±0.09	12.5	25.0	15	—
E. coli (ATCC 35218)	20.0±0.18	0.19	0.39	14.0±0.15	0.39	0.78	Resis.	—
E. coli (resistant)	10.0±0.12	25.0	50.0	8.0±0.07	6.25	10.0	—	33
Antifungal results							**Fluconazole 25 mg**	
C.glabrata	8.0±0.10	3.13	25.0	8.0±0.11	0.78	1.56	21	
C. albicans	10.0±0.12	0.19	0.78	9.0±0.05	0.19	0.39	20	
C. tropicalis	10.0±0.15	0.78	1.56	9.0±0.09	0.39	0.78	18	
C. krusei	9.0±0.07	1.56	3.13	8.0±0.14	0.39	0.78	26	
C. albicans (ATCC 10231)	10.0±0.13	3.13	3.13	9.0±0.08	0.39	0.78	23	

[a]Average ± SD (n=3) of the inhibition zone diameter (mm).
[b] MIC, MBC, and MFC values are expressed in µg mL^{-1},
VA: vancomycin, CIP: ciprofloxacin

Table 3. Antimicrobial effects of *Mentha longifolia* EO and its nanoemulsion

Strain	EO (2 µL/disc)			EO Nanoemulsion		
	IZ mm	MIC	MBC/ MFC	IZ mm	MIC	MBC/ MFC
Antibacterial results						
C. diversus (ATCC 13315)	15.0±0.18	3.13	6.25	8.0±0.12	6.25	6.25
P. vulgaris (ATCC 13315)	15.0±0.26	0.39	0.78	8.0±0.09	6.25	6.25
S. aureus (ATCC 25923)	15.0±0.20	0.39	0.78	9.0±0.16	0.78	1.56
S. aureus (resistant)	25.0±0.31	3.13	6.25	9.0±0.14	6.25	6.25
E. coli (ATCC 35218)	15.0±0.22	0.39	0.78	9 .0±0.11	6.25	6.25
E. coli (resistant)	20.0±0.28	6.25	12.5	8.0±0.13	0.78	1.56
Antifungal results						
C.glabrata	30.0±0.35	0.19	0.19	9.0±0.12	0.78	0.78
C. albicans	10.0±0.12	0.19	0.78	9.0±0.17	3.13	3.13
C. tropicalis	10.0±0.14	6.25	6.25	9.0±0.11	3.13	3.13
C. krusei	12.0±0.07	12.5	6.25	9 .0±0.08	3.13	3.13
C. albicans (ATCC 10231)	22.0±0.16	6.25	6.25	9.0±0.15	1.56	1.56

Table 4. Antimicrobial effects of *Vitex agnus-castus* EO and its nanoemulsion

Strain	EO (2 µL/disc)			EO Nanoemulsion		
	IZ mm	MIC	MBC/ MFC	IZ mm	MIC	MBC/ MFC
Antibacterial results						
C. diversus (ATCC 13315)	16.0±0.22	6.25	6.25	13.0±0.15	0.78	1.56
P. vulgaris (ATCC 13315)	15.0±0.31	3.13	3.13	11.0±0.18	0.78	1.56

S. aureus (ATCC 25923)	17.0±0.12	3.13	3.13	13.0±0.12	0.78	1.56
S. aureus (resistant)	20.0±0.36	6.25	6.25	14.0±0.19	0.78	1.56
E. coli (ATCC 35218)	20.0±0.42	6.25	6.25	16.0±0.21	0.78	1.56
E. coli (resistant)	14.0±0.19	6.25	6.25	10.0±0.11	0.78	1.56
Antifungal results						
C.glabrata	Full plate (10 cm)	0.78	1.56	50.0±0.30	0.19	0.39
C. albicans		0.78	1.56	45±0.25	0.19	0.39
C. tropicalis	27.0±0.31	0.39	0.78	18±0.15	0.10	0.19
C. krusei	55.0±0.39	0.39	0.78	35±0.27	0.10	0.19
C. albicans (ATCC 10231)	50.0±0.34	0.39	0.78	32±0.31	0.10	0.19

Data treatments

The antimicrobial assay results with three replicates were analyzed by one-way analysis of variance (version 6.311; CoHort Software, Monterey, CA, USA).

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Authors' contributions

Conceptualization: all authors; Formal analysis and microbial investigation: Tamer I.M. Ragab, Doaa D. Khalaf and Asmaa S. Mansour; Chemical investigation of plant extract and their EOs and Validation: Tamer I.M. Ragab, Abdelsamed I. Elshamy, and Abd El-Nasser G. El-Gendy; Writing—original draft preparation, review and editing: Doaa D. Khalaf, Tamer I.M. Ragab, Asmaa S. Mansour, Abdelsamed I. Elshamy, and Abd El-Nasser G. El-Gendy; All authors have read and agreed to the published version of the manuscript.

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