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Essential oil of *Eucalyptus citriodora*: Physio-Chemical analysis, Formulation with Hand Sanitizer Gel and Antibacterial Activity

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Abstract

Background: The essential oil-bearing plants are extensively being used in traditional systems of medicine due to the occurrence of the diversity of phytochemical constituents. The emerging crisis of developing resistance to conventional drugs has increased public health awareness and reliance on natural compounds as safer alternatives.

Methods: The essential oil extracted from *Eucalyptus citriodora* (Hook.) leaves was characterized for physicochemical attributes, formulated with hand sanitizer gel, tested for organoleptic parameters, and antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis*.

Results: *E. citriodora* essential oil (EEO) had a camphorous scent, and dark yellow coloration, while exhibiting 0.60% yield (v/w, 97% pure), 0.94 density, 1.47 refractive index, 11.10 viscosity, 0.92 specific gravity, 0.0-9.98° optical rotation, 11.20 acid number, 50.60 ester number, which satisfy the standards specified by ISO (The International Organization for Standardization). The chromatographic analysis of oil identified eucalyptol as the most abundant compound (80.08%) followed by α-terpinyl acetate, isopinocarveol, and globulol as the moderately abundant compounds (4.46-4.81%), while viridiflorol and terpinen-4-ol as less abundant compounds (3.06 and 2.69%, respectively). Formulated hand sanitizer with EEO exhibited physical and microbiological properties that were comparable with the market products. It also had a pleasant scent, was compatible with the skin, was easy to apply, and is acceptable to the users.

Conclusion: The current study clearly shows that EEO could be utilized as a potential ingredient in alcohol-based gel hand sanitizer formulation for giving a pleasant smell, acceptable physical appearance and microbial quality parameters.

Introduction

The Eucalyptus genus of the Myrtaceae family is one of the largest genera of around 900 species in the family [1], which has been harvested and traded for oil, gum, pulp, timber, and medicines around the world [2]. *Eucalyptus citriodora* Hook. also famous as lemon-scented eucalyptus is an evergreen tree that exhibits rapid growth and productivity, therefore commonly cultivated as an ornamental tree species. The essential oil extracted from its foliage hold a significant position in the food, perfumery, agriculture, and pharmaceutical industry due to its antibacterial, antifungal, antiseptic, and pest management properties [3,4,5]. Therefore, essential oil from Eucalyptus species has been categorized among the 18 most commonly traded oil [6]. Organic compounds are the main components of the essential oil, and the *E. citriodora* essential oil (EEO) is generally comprised of citronellol, citronellyl acetate, and β-caryophllene [5, 7]. The yield, quality, and chemical profile of EEO generally vary according to genetic factors and growing location. Moreover, management implemented after the harvest and the method of oil extraction also contribute to oil characteristics [8]. EEO yield of 0.6 to 5.9% [9] and abundance (60-80%) of oxygenated monoterpenes (e.g. citronellol and citronellal) have been noticed from the dried leaves [10].

The value of EEO for medicinal purposes is based essentially on the key active component of eucalyptol (1,8-cineole) [11]. Therefore, EEO can be infused into hand sanitizer formulations as a potential substitute for synthetic compounds, because of the resistance that has been increasingly developed by pathogenic microorganisms. Warnke *et al.* [12] found the considerable antibacterial potential of Eucalyptus, tea, and lemongrass essential oils against multi-resistant hospital-acquired infections caused by *Enterococcus* spp., *Staphylococcus aureus*, *Escherichia coli, Klebsiella pneumoniae*. Dinwiddie *et al.* [13] reported that the addition of tea extract and lemongrass essential oil enhanced the effectiveness of sanitizers against microbes and also offers the additional benefit of being free from toxic ingredients, which might be quite beneficial for the health and environment as well. Thus, EEO in hand sanitizer formulation can be both beneficial and sustainable without compromising efficacy or safety. Therefore, in the present investigation, EEO was extracted, characterized for physicochemical composition, and formulated in hand sanitizer. The antibacterial potential, physical, and organoleptic values of EEO-formulated hand sanitizer were also evaluated.

Methods

Extraction of *E. citriodora* essential oil (EEO)

Fresh leaves (5 kg) of *E. citriodora* were collected in the morning (7 a.m.) in the School of Chemistry, University of the Punjab (1° 30' 15" N and 74° 18' 23" E.) Lahore, Pakistan in April 2021 (high/low: 34/21 °C). The specimen was assigned to voucher no RP-2. The leaves were washed carefully to get rid of contaminants and dust particles. Essential oils from fresh leaves were obtained by hydrodistillation in a Clevenger apparatus for one hour. The hydrodistilled fraction, containing water and essential oil, was collected and dried with anhydrous Na_2SO_4 [14]. The oil samples were stored in sealed vials at 4 °C for further analysis.

GC-MS analysis of EEO

A sample of 1 µL is injected into GC-MS (Shimadzu GC-9A) equipped with a capillary column (SPB-5) maintained with a flame ionization detector at 220 °C. Carrier gas (N2:1.0 mL min-1) was adjusted at the initial temperature of 50 °C for an initial 5 min, followed by an increase in temperature (5 °C min-1) up to 235 °C and finally sustained for 5 min. A column (HP-5 with dimensions: 25 m × 0.22 mm and 0.25 µm df) was used for the complete analyses of the fraction. The observed mass chromatogram generated was interpreted.

Quality parameter analysis of EEO

Quality properties of EEO like density, viscosity, specific gravity, optical rotation, acid number, ester number, consistency, appearance, odor, color, odor, and purity were analyzed according to the prescribed methods.

Preparation of hand sanitizer gel

Hand sanitizer was formulated following the protocol described earlier with slight modifications [15]. Briefly, formulated hand sanitizer (FHS) was prepared by dissolving and stirring carbopol (0.25 g) in water (30 mL) at 60 rpm for 30 minutes at 80 °C (Mixture I). Afterward, a mixture of triethanolamine (0.2 mL), propylparaben (0.2 g), and glycerin (1 mL) was prepared in 50 mL of 70% alcohol (Mixture II). Finally, a homogeneous gel of FHS was obtained after mixing mixture II with the mixture I. For the preparation of FHS-EEO, drops of EEO (0.5% v/v) were added with constant stirring to the FHS. The remainder of each formula was completed with distilled water. Control formulation was also prepared without adding EEO.

Product characterization

The hand sanitizers (FHS and FHS-EEO) were examined for pH, density, and viscosity through prescribed methods. Organoleptic tests were carried out visually by observing the homogeneity, color, smell, and clarity of the hand sanitizer [15]. Consumers' response to the formulated hand sanitizer was assessed by observing sensory tests (color, smell, viscosity, spreadability, and clarity) involving 20 untrained respondents. The respondents rated the product by scoring 1 (strongly disliked) to 5 (strongly liked) after applying the sample to their palms.

Antimicrobial assays

The antimicrobial activity of EEO, FHS-EEO, and FHS was studied against gram-positive bacteria viz., *Escherichia coli* (FCBP-WB-0005), *Staphylococcus aureus* (FCBP-WB-0005), and *Bacillus subtilis* (FCBP-WB-0005) using the agar diffusion method [16]. The bacterial cultures used were sub-cultured and maintained on LBA (Luria-Bertani agar).

For the antimicrobial assay, overnight nutrient agar-derived cultures were used for the preparation of inoculum suspension. EEO (5 mg/mL) was dissolved in dimethylsulfoxide (DMSO). About 300 µL of a colloidal suspension of the bacterium was spread on the fresh LBA. Wells of 6 mm diameter were punched in the agar medium with the help of a sterile cork-borer and filled with 100 µL of the EEO. Inoculated plates were incubated overnight at 37 °C for 24 hours. The antibacterial activity was determined by measuring the diameter of the zone of inhibition in millimeters (mm) around each well. The results are represented as the mean of three replicates. Controls using DMSO and water were adequately prepared. The antimicrobial activity of EEO, FHS, and FHS-EEO was compared with two commercially available hand sanitizers.

Results

The physical and chemical properties of *E. citriodora* essential oils (EEO) extracted from the fresh leaves through the hydro-distillation method are depicted in Table 1. The EEO exhibited 0.94 g/mL density, 1.47 refractive index, 11.10 mPa viscosity, 0.92 specific gravity, 0-0.98 degree optical rotation, 11.20 acid number, and 50.60 ester number. The EEO was 97% pure, dark yellow liquid having thin consistency with fruity odor and purity.

Sr. No.	Property	Mean values ± S.D.
1	Oil yield	0.60 % (v/w, fresh weight basis)
2	Density	0.94 ± 0.08
3	Refractive index (25°C)	1.47 ± 0.11
4	Viscosity (25°C)	11.10 ± 0.15
5	Specific gravity (25°C)	0.92 ± 0.04
6	Optical rotation	0.0-9.98 degrees
7	Acid number	11.20 ± 0.02
8	Ester number	50.60 ± 0.02
9	Consistency	Thin
10	Appearance	Liquid
11	Color	Dark yellow
12	Odor	Minty, camphorous scent
13	Purity	97%

Table 1: Physical and chemical properties of the *Eucalyptus citriodora* essential oil

The GC chromatogram of EEO is presented in Fig. 1, and the detail of isolated chemical constituents are depicted in Table 2. On the percentage occurrence of the individual compound, the 5 compounds representing 100% of the total content were grouped as most, moderately, and less abundant. Accordingly, the most abundant compound was eucalyptol, which occupied the maximum peak area of 80.08%. However, 3 other compounds namely α-terpinyl acetate, isopinocarveol, and globulol were among the moderately abundant compounds (4.46-4.81%), while viridiflorol and terpinen-4-ol were reported as less abundant compounds (3.06 and 2.69%, respectively).

Compounds	Molecular Formula	Molecular weight (g/mol)	Retention time (min)	Peak area (%)
Eucalyptol	$C_{10}H_{18}O$	154	3.94	80.48
3-(R)-alpha-terpinyl acetate	$C_{12}H_{20}O_2$	196	5.86	4.81
Bicyclo[3.1.1]heptan-3-ol, 6,6-dimethyl-2-methylene-, [1S-(1α,3α,5α)]- or Isopinocarveol	$C_{10}H_{16}O$	152	5.22	4.46
Globulol	$C_{15}H_{26}O$	222	10.86	4.46
1H-Cycloprop[e]azulen-4-ol, decahydro-1,1,4,7-tetramethyl-, [1aR-(1aα,4β,4aβ,7α,7aβ,7bα)]- or Viridiflorol	$C_{15}H_{26}O$	222	10.86	3.06
Terpinen-4-ol	$C_{10}H_{18}O$	154	5.693	2.69

Table 2: Bioactive compounds identified from *Eucalyptus citriodora* essential oil through GC-MS analysis

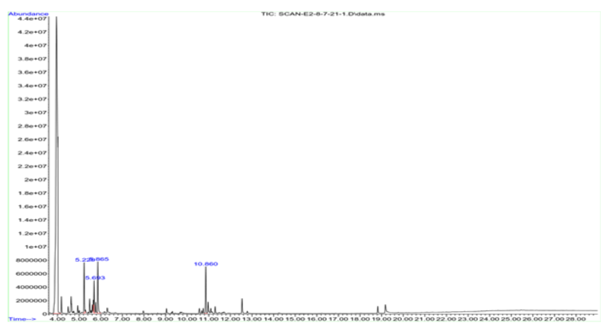

Figure 1: Chromatogram of essential oil from leaves of *Eucalyptus citriodora* through GC-MS analysis

A hand sanitizer was formulated according to the prescribed formula, and it was also incorporated with EEO. The organoleptic characteristics of the FHS-EEO and EEO were also compared with the available market products (Table 3). It was revealed that FHS-EEO had comparable color (pale yellow), odor (sweat, EEO distinctive odor), consistency (clear gel, light to spread), pH (5.41), viscosity (9834 cP), and density (0.90 g/mL) with the FHS and market products.

Antimicrobial assays confirmed the potential of all treatments to inhibit the growth of *E. coli*, *S. aureus*, and *B. subtilis* (Table 4). Formulated hand sanitizer with EEO (FHS-EEO) showed more antimicrobial potential (90-97%) as compared to FHS without EEO (70-74%) or EEO alone (50-55%). The acceptability test study for a formulated gel with EEO was performed on 20 volunteers, and the results are shown in Table 5. The

Sr. No.	Property	FHS Mean values ± S.D.	FHS-EEO Mean values ± S.D.	Market hand sanitizer 1 Mean values ± S.D.	Market hand sanitizer 2 Mean values ± S.D.
1	Color	Transparent	Dull yellow	Transparent	Transparent
2	Odor	Alcoholic	Characteristic of EEO	Alcoholic	Alcoholic
3	Consistency	Clear gel	Clear gel	Clear gel	Clear gel
4	pH	4.80 ± 0.24	5.41 ± 0.21	4.92 ± 0.35	4.67 ± 0.13
5	Viscosity (cP)	9910 ± 0.19	11041 ± 0.14	9159 ± 0.34	9457± 0.14
6	Density (g/mL)	0.90 ± 0.31	0.93± 0.07	0.93± 0.15	0.98 ± 0.11

Table 3: Physical properties of the formulated hand sanitizer (FHS) and FHS-*Eucalyptus citriodora* essential oil (FHS-EEO).

FHS-EEO was well-tolerated, felt comfortable upon use (not sticky), had not produced skin irritation, it was liked by a majority of the user (≤ 80%) because of its pleasant smell.

Treatment	Percentage inhibition in bacterial growth		
	Escherichia coli	*Staphylococcus aureus*	*Bacillus subtilis*
Control (water)	0.00^{e}	0.00^{e}	0.00^{e}
Control (DMSO)	14.00^{d}	18.00^{d}	21.00^{d}
Formulated hand sanitizer without EEO	70.09^{b}	68.91^{b}	73.81^{b}
Formulated hand sanitizer with EEO	92.32^{a}	97.23^{a}	93.81^{a}
EEO	50.33^{c}	54.16^{c}	55.17^{c}
Market hand sanitizer 1	93.12^{a}	96.26^{a}	93.22^{a}
Market hand sanitizer 2	72.76^{b}	70.07^{b}	74.49^{b}

Table 4: Antimicrobial assays with *Eucalyptus citriodora* essential oil (EEO). Alphabets in each column show significant (p ≤ 0.05) differences as detrmined by the LSD-test.

Property	Color	Clarity	Aroma	Thickness	Sticky
Score 1	0	0	0	0	0
Score 2	0	0	0	0	0
Score 3	0	10	0	10	10
Score 4	20	20	10	10	10
Score 5	80	80	90	80	90

Table 5: Consumer response to formulated hand sanitizer with *Eucalyptus citriodora* essential oil (FHS-EEO).

Discussion

Among the species of Eucalyptus used for obtaining essential oils, *E. citriodora* is commonly used. In the present study, the essential oil extracted from the leaves of *E. citriodora* exhibited a yield of 0.60% (v/w), which was in accordance with the previous reports on the *Eucalyptus* spp. Accordingly, Boukhatem *et al.* [17] described 0.13-1.87% (v/w) yield of essential oil from different species of Eucalyptus from Algeria. About 1.2 to 3.0% (w/w) yield of essential oils for the different Eucalyptus species was also documented in Tunisia, while the highest yield was obtained from *E. cinerea* and *E. sideroxylon* (3.0%) [18]. The differences in the yield of essential oil were ascribed to the difference in the Eucalyptus species, leave age, harvest date, and distillation method [18-21]. It has been known that hydro-distillation products hold greater market value than products from different extraction methods mostly due to the procurement of a greater number of essential oil components [22].

The results on physical and chemical attributes i.e., density (0.94 g/mL), refractive index (1.47 at 25°C), viscosity (11.10 mPa at 25°C), specific gravity (0.92 at 25°C), optical rotation (0-0.98 degree), acid number (11.20), ester number (50.60), consistency (thin), appearance (liquid), color (dark yellow), odor (fruity) and purity (97%) of EEO were comparable with the earlier findings [17, 23, 24, 25]. For an instant, Boukhatem *et al.* [17] and Subramanian *et al.* [23] acknowledged similar values of refractive (1.46 to 1.48), specific gravity (0.918 to 0.919), and optical rotation (0 to 10 degrees) in the essential oil of *E. globulus*. Furthermore, their findings also related high values to the refractive index with less water content and richness of 1,8-cineole. Zrira *et al.* [24] and Dalal [25] also noticed EEO as a pale yellow liquid, which exhibited a camphoraceous odor, 0.92 g/mL density, and 1, 8-cineole as a major component. Zhou *et al.* [26] documented yield of essential oil is 0.7% (w/w, relative to the dry weight) with a color of light yellow and a persistent smell from *Eucalyptus grandis* × *E. urophylla* leaves essential oils.

GC-MS profile of EEO-S showed the occurrence of eucalyptol, isopinocarveol, globulol, and terpinen-4-ol, along with other components. Most of the compounds belonging to monoterpene alcohol represented the main characterized class of the total oil content. Elaissi *et al.* [27] also documented oxygen-containing monoterpenoids as the key content in 20 different types of Eucalyptus oil in northern and northwestern Tunisia. Likewise, essential oils of different Eucalyptus species (*E. maideni; E. astringent*; *E. cinerea*; *E. leucoxylon*; *E. lehmani; E. sideroxylon* and *E. bicostata*) contained 1,8-cineole, α-pinene, and α-terpineol as major common compounds [18]. In many studies, eucalyptol (1,8-cineol) has been found as a major compound (44-84%) in the essential oil of Eucalyptus spp. and it is known to possess significant antioxidant and antimicrobial activity [18, 26, 28]. Moreover, the essential oil of eucalyptus exhibits 70% eucalyptol and is recommended for medicinal purposes, according to the European and British Pharmacopoeia [29]. However, in many other studies, citronellal was also reported as the major constituent in EEO [30, 31]. The disparity with currently reported results may be ascribed to the differences in the plant varieties and regions along with the associated factors (climate, nature of the soil, time of collection, mode of extraction, and the age of the tree). Another important monoterpene ester found in EEO was α-terpinyl acetate. It bears antimicrobial potential and has a sweet, herbaceous floral and lavender odor. Hence, α-terpinyl acetate is widely utilized as an important ingredient in air fresheners, laundry, dishwashing products deodorizers, soaps, shampoo, and lotions. Moreover, α-terpinyl acetate is used as a flavoring food additive in baked items, drinks, fruit ice creams, sugar candies, chewing gum, gelatin, and puddings [32].

Isopinocarveo [33] and viridiflorol [34] are well-known bioactive natural compounds, endowed with different medicinal properties including antiviral and radical scavenging potentials [35,36]. Over and above, globulol, isolated from the *E. globulus* has been recommended as an inexpensive and environmentally acceptable agrochemical in agriculture and pharmacy due to its strong antimicrobial potential. It was traditionally used in China to treat skin inflammation and infectious diseases [37]. GCMS profile of EEO confirmed the presence of chemical compounds of sweat odor, antimicrobial and antioxidant action, therefore, EEO could be a valid alternative as an additive in alcohol-based hand sanitizers on account of increasing consumer interest in natural products.

Formulated hand sanitizer (FHS-EEO) exhibited comparable attributes of pale yellow color, sweat odor, clear gel, light to spread with neutral pH, 9834 cP viscosity, and 0.90 g/mL density. Wijana *et al.* [15] also recognized similar properties of the alcoholic-based hand sanitizer formulated with citrus peel essential oil. The results also revealed that formulated hand sanitizer with EEO is safe for the skin as a pH range of 4.5 to 6.5 is recommended for a broad range of skin [38]. The density of the FHS-EEO was lower than water, which indicated easy application of the product on the skin. The viscosity (consistency) of the hand sanitizers was also in accordance with the specification standard (2381-16893 cP) which may show the ease with which the product can easily be poured and dripped on the palm [15]. The slightly higher viscosity values of FHS-EEO (11041 cP) among all hand sanitizer gels (>1000 cP) might be attributed to the presence of essential oil.

Antimicrobial assays confirmed the potential of all treatments to inhibit the growth of *E. coli*, *S. aureus,* and *B. subtilis*. The antimicrobial activity of all hand sanitizers seemed to be caused by alcohol, as alcohol-based hand sanitizers have been shown to kill gram-positive and negative bacteria by denaturing their protein and lipid membranes. Czerwinski *et al.* [39] and WHO [40] also recommended the utilization of alcohol-based hand sanitizers due to broad and rapid action against microbes with minimal risk of generating microbial resistance. EEO was also effective in performing an antimicrobial activity (50-55%), as indicated by the area of inhibition. High levels of eucalyptol in the EEO could be accountable for the antimicrobial potential. Similar investigations have been documented previously with essential oil of Eucalyptus species against *E. coli*, *Staphylococcus aureus, Enterococcus faecalis, Listeria ivanovii*, and *Bacillus cereus* [18]. However, other components may also contribute to the antimicrobial activity of EEO, which possibly caused cell lysis by disturbing microbial cell permeability. Bedsides, formulated hand sanitizer with EEO showed more antimicrobial potential (90-97%) as compared to hand sanitizer without EEO (70-74%) or EEO alone (50-55%). The antimicrobial activity of the formulated hand sanitizers was as good as available hand sanitizers in the market.

Essential oil from leaves of *E. citriodora* contained compounds that may have potential applications in food and pharmaceutical products. It can be a potential ingredient for alcohol-based hand sanitizer gel for improving the aroma, physical, and microbial quality parameters. The sensory test results revealed that the product was liked by the majority of the respondents (⩽ 70%). It is essential to ensure the shelf-life of the formulated hand sanitizers by evaluating their stability test.

Competing Interest

The authors declare that there is no conflict of interest.

Author Contributions

Shabnam Javed: Extraction of essential oil; Amna Shoaib: Wrote and edited manuscript, prepared all tables; Amina Bibi: Experiments; Shagufta Perveen: Formatted manuscript; Malik Fiaz Hussain Firdosi: GC-MS analysis

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