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Article

Nanosizing of Lavender, Basil, and Clove Essential Oils into Microemulsions for Enhanced Antioxidant Potential and Antibacterial and Antibiofilm Activities

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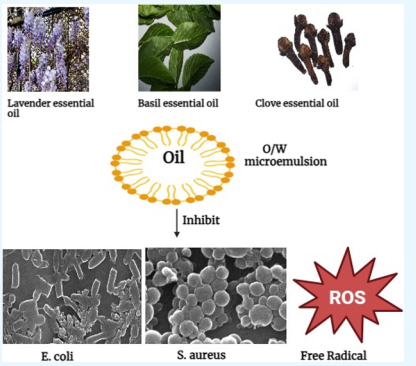
ABSTRACT: Plant essential oils (EOs) possess significant bioactivities (antibacterial and antioxidant) and can be substituted for potentially harmful synthetic preservatives in the food industry. However, limited water solubility, bioavailability, volatility, and stability limit their use. Therefore, the goal of this research was nanosizing lavender essential oil (LEO), basil essential oil (BEO), and clove essential oil (CEO) in a microemulsion (ME) to improve their physicochemical attributes and bioefficacy. Tween 80 and Transcutol P were utilized for construction of pseudoternary phase diagrams. It was observed that the concentration of EOs had a great impact on the physicochemical and biological properties of MEs. A spherical droplet of MEs with a diameter of less than 20 nm with a narrower size distribution (polydispersity index (PDI) = 0.10−0.27) and a ζ potential of −0.27 to −9.03 was observed. ME formulations were also evaluated for viscosity, conductivity, and the refractive index. Moreover, the impact of delivery systems on the antibacterial property of EOs was assessed by determining the zone of inhibition and minimum inhibitory concentration against two distinct pathogen classes (*S. aureus* and *E. coli*). Crystal violet assay was used to measure the growth and development of biofilms. According to bioefficacy assays, ME demonstrated more efficient antibacterial activity against microorganisms at concentrations lower than pure EOs. CEO ME had superior activity against*S. aureus* and *E. coli*. Similarly, dose-dependent antioxidant capacity was noted for MEs. Consequently, nanosized EO formulations with improved physicochemical properties and enhanced bioactivities can be employed in the food processing sector as a preservation agent.



1. INTRODUCTION

Food quality is vital to human health, and this issue has received a lot of interest in recent times. One of the greatest food quality challenges is the advent of food poisoning outbreaks associated with foodborne pathogens. Food infected with bacterial microbes, according to the literature, poses a major risk to humans. Urinary tract infection, cholecystitis, and bacteraemia can all be caused by *E. coli* existing in the human gut. *S. aureus* causes gastroenteritis, toxic shock syndrome, cardiomyopathy, and postoperative skin infections.[1] Herbal medicines have long been used to enhance the taste of foods owing to their evident sensory qualities and good preservative properties. Essential oils (EOs) are plant derivatives that have a strong flavor as well as antioxidant and antibacterial effects.[2] USFDA has licensed cinnamon oil, eugenol (a major component of clove bud oil), lavender oil, basil oil, and thyme oil as generally recognized as safe food additives. In recent years, EOs have received considerable attention for their usage as functional ingredients to boost food safety and quality. Furthermore, the primary use of EOs in the nutritional industry is restricted due to poor aqueous solubility and volatility of the phytochemicals.[3]

Lavender (*Lavandula angustifolia*) is a member of the *Lamiaceae* family shrub. It has become popular in the US, Australia, and southern Europe. LEO has some bioactivities including radical scavenging, anti-inflammatory, antibacterial, and analgesic.[4] LEO is used in food processing as an antibacterial as well as a flavoring component to protect food items against foodborne microbes.[5] Nonetheless, air, light, heat, and humidity all have an influence on LEO's stability in a natural environment.[6] Additionally, owing to their poor stability, EOs pose challenges in terms of handling and distribution.[7] Basil (*Osimum sanctum L.*), a member of the plant family *Lamiaceae*, is frequently utilized in cooking. The physicochemical characteristics of BEO vary depending on the plant origin, features of leaf and flowers, and active compounds, allowing it to provide a variety of main components. BEO is customarily utilized as a flavor enhancer in foods, dental and oral consumer items, and in perfumes.[8] Furthermore, it is extensively used in chemotherapeutic agents due to its numerous characteristics that are scientifically

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approved, including antinociceptive, antioxidant, antispasmodic, pesticide, and antibacterial properties. Clove oil (CEO) is a volatile oil extracted by hydrodistillation from the flower buds of *Syzygium aromaticum*, a plant in the *Myrtaceae* family. CEO is primarily composed of eugenol, as well as methyl salicylate, caryophyllene, pinene, humulene, vanillin, and eugenyl acetate.[9] It possesses several pharmacological activities, including antimicrobial, antibiofilm, antioxidant, antiviral, antiparasitic, cytotoxic, and insect-repelling characteristics.[10]

Although EOs possess numerous health functionalities, these are susceptible to oxidation as well as thermal and photodegradation.[7] In addition, due to their high volatilization and limited solubility in water, they are challenging to use in nutrition, cosmetics, and pharmaceutical industries. Furthermore, owing to the existence of interfering and interacting dietary ingredients (lipids and proteins) in the food system, the antibacterial activity of EOs would be frequently compromised. As a result, in order to ensure effectiveness of EOs, it is necessary to look for strategies that improve its stability. Nanotechnology has been investigated in order to preserve EOs from deterioration and evaporation to ensure their stability and therapeutic efficacy and to uniformly distribute the bioactive compound all through the product.[11] It also attempts to manage drug release, reduce adverse effects, decrease component interaction, increase pharmacological activity, and improve physical and chemical properties as well as improve food quality and safety. Nanoemulsions (NEs) and microemulsions (MEs) are types of colloidal dispersions that may be utilized to integrate EOs in water, and nanosized oil droplets can help food items retain their appearance. One such intriguing property has encouraged research on NEs.[12] MEs are clear, thermodynamically stable, spontaneously formed isotropic liquid mixtures of oil, surfactant, and water. However, NEs are kinetically stable dispersion systems with a size range of 100−500 nm.[13] On the other hand, NEs (thermodynamically unstable) can be destabilized by different mechanisms, causing changes in droplet size during storage, as well as potential phase separation.[14] Moreover, NE production often requires a significant amount of energy. Unlike NEs, MEs (thermodynamically stable) can be synthesized by spontaneously self-assembling water, oil, a surfactant, and a cosurfactant. The dispersed phase has a size of less than 100 nm, which correlates with ME's transparent appearance.[15] ME can be easily prepared using little external energy. As a result, ME provides the advantage of low production cost and shelf stability. MEs have been examined for their ability to dissolve hydrophobic substances in culinary, pharmaceutical, cosmetic, and oil recovery applications.[16] Sieniawska et al. designed a water-dilutable ME of citronella, mint, and eucalyptus oils. MEs improved the ingredient solubility of EOs and reduced volatile evaporation from a culture medium.[14]

The objective of this work was the utilization of the ME approach as a nanosizing strategy to enhance the stability and efficacy of LEO, BEO, and CEO against foodborne pathogens in order to identify potential candidates for food preservation application. The EO-loaded systems were characterized for physicochemical attributes and biological performance, such as antioxidative and antibacterial properties.

2. MATERIALS AND METHODS

2.1. Materials. The LEOs and CEOs were purchased from Go Natural Pakistan. BEO was procured from Aroma Farmacy, Karachi, Pakistan. Tween 80 and Transcutol P were supplied by DaeJung Chemical & Metals Co Ltd. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma-Aldrich. The bacterial cultures used in this study, *E. coli* (DH5α) and *S. aureus* (ATCC 12598), were purchased from the American Type Culture (Manassas, VA). Agar medium and nutrient broth were obtained from Lab Allay. Furthermore, analytical-grade compounds and reagents were employed in this research.

2.2. Development of ME. The concentration ranges of components that can form MEs were calculated using a water titration method at 25 °C by a pseudoternary phase diagram.[17] Different oily phases (LEO, BEO, and CEO) were used to create three pseudoternary phase diagrams. The aqueous medium contains purified water and Tween 80 and Transcutol P as the surfactant and cosurfactant, respectively. The surfactant−cosurfactant mixture (S/CoS) was combined in a 1:1 (w/w) ratio for the first 12 h. Following that, the oily component was introduced to the S/CoS dispersion and blended in the following sequence: w/w ratio of 9:1 to 1:9. Finally, each combination was adjusted with distilled water (dropwise) while being magnetically stirred at room temperature. The mixes were examined physically, and the zones that created obvious mixtures are displayed on pseudoternary phase diagrams. Transparent systems were classified as MEs.

2.3. Measurement of Droplet Size, Polydispersity Index, and ζ Potential. Particle size and polydispersity index (PDI) of the ME were evaluated utilizing the dynamic light scattering (DLS) technique by a Malvern Zetasizer Nano ZS (Malvern Instruments, U.K.) at 25 °C. To avoid frequent scattering impacts, the MEs were diluted 1000-fold for each sample, and 1 mL of the sample was introduced to the measuring cell. ζ Potential, representing the oil droplet's surface potential, was calculated by monitoring the position and velocity of the particle motion in an electromagnetic field by a Zetasizer Nano ZEN3600 (Malvern Instruments, U.K.). All formulations were tested three times.

2.4. Evaluation of Conductivity, Viscosity, and Refractive Index. A conductivity meter (InoLab pH 730, Xylem, U.K.) was used to test the conductivity at 25 ± 0.2 °C. The conductometer was validated using a standard solution, KBr (84 μS/cm).[18] The conductivity test was done in triplicate.

The viscosities of the various MEs were measured using an NDJ-8S (Drawell International Technology Ltd., China), which was used with the spindle number 4 at 60 rpm The viscosity was measured at ambient temperature by taking the sample into a small sample holder.[19] All systems were tested in triplicates.

The refractive index was determined using an Abbe refractometer (Bausch and Lomb Optical Company) at room temperature. Single drops of formulation were dropped upon that glass surface, and the isotropic character was confirmed by putting a coverslip on it. A polarizing microscope was used to examine the slide under cross-polarized light, and the operation was repeated three times.[20]

2.5. Transmission Electron Microscopy (TEM). The geometry of the nanosystems was explored by utilizing TEM. At a voltage of 200 kV, the morphology of the produced particulates was studied using a JEM-2010 (JEOL, Tokyo, Japan) transmission electron microscope. Drop-casting of the appropriately diluted ME dispersion onto the carbon-coated copper grid was done, and before being loaded into the microscope, it was air-dried at ambient temperature. The high-

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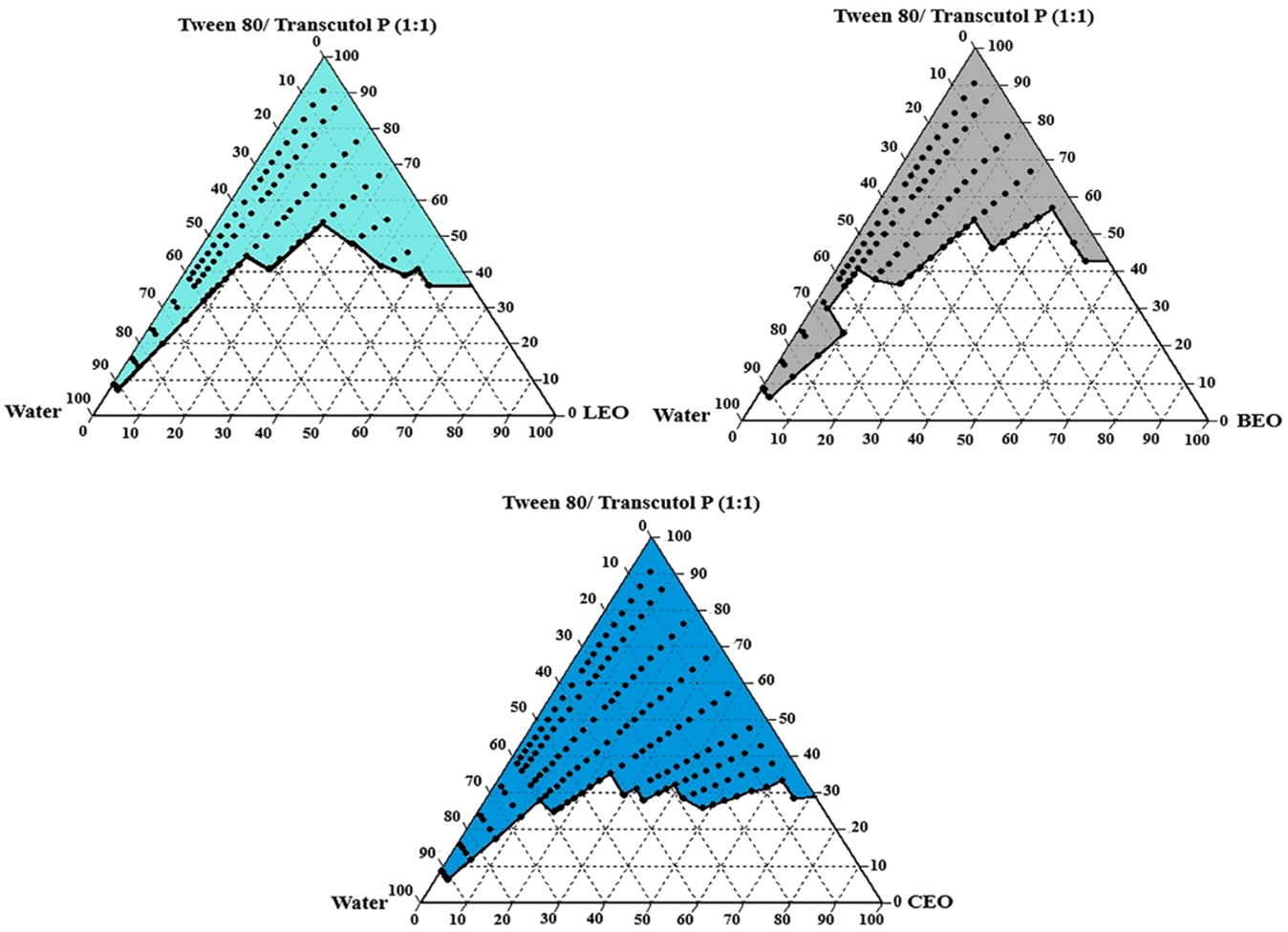

Figure 1. Pseudoternary phase diagrams for mapping the ME regions from LEO, BEO, and CEO as oils and a 1:1 mixture of Tween 80 and Transcutol P as a mixture of surfactant and cosurfactant, respectively.

resolution ME images were collected at 120,000× magnification.

2.6. Antibacterial Activities of Therapeutic Oils. *2.6.1. Microorganisms.* Bacteria are one of the major causes of food spoilage and various infections. Thus, one Gram-positive microbe (*S. aureus*) and one Gram-negative microbe (*E. coli*) were used to test the antibacterial activities of the ME. Strains were smeared onto agar plates and cultured at 37 °C for 24 h. From a Petri dish, a colony was isolated, and then, 0.5 mL of a bacterial solution formed by turbidity equivalent to 0.5 Mc Farland (1.5×10^8 CFU/mL), which was then adjusted using normal saline to the required bacterial concentration level (10^5−10^6 CFU/mL).

2.6.2. Inhibition Zone Measurement. The agar well diffusion method was utilized in order to determine the zones of inhibition of MEs. First, 10 mL of melted nutrient agar (Mueller-Hinton agar) was spread into sterilized plates and allowed to solidify. After the agar had cooled and solidified, 1 mL of the bacterial suspension was placed onto the agar plates. EOs and MEs were diluted in a 1:1 v/v solution of water and methanol, and 0.02 mL of the solution was dropped into the bore of the agar-containing plates. In order to observe differences in strain growth, culture dishes were positioned in the incubator for 24 h at 37 °C. Each experiment was repeated three times, and the average diameter of each inhibitory region was evaluated with the cross method.[21] All of this procedure was performed in a laminar flow hood.

2.6.3. Broth Microdilution Assay. The MICs of MEs were also evaluated following the CLSI guidelines (National Committee for Clinical Laboratory Standards). First, broth microdilution techniques were applied to nutrient broth. In detail, the bacterial isolate was grown on plates containing nutrient agar and then put in an incubator at 37 °C for 12 h and subcultured once more. Then, from the second batch, 2−3 bacterial colonies were separated into individual tubes. Each was introduced into 10 mL of a sterile nutritional medium and incubated at 37 °C in an incubator (shaking) for 8−12 h. The bacteria were supposed to be in the log phase after the incubation. Next, 100 μL of broth was added to sterile 96-well microplates. Starting with the first well, 100 μL of each stock sample was pipetted and 2-fold serially diluted from then on. Next, 100 μL of bacterial inoculum was adjusted to a 10^6 CFU/ml concentration and added in all wells except for the first well (functioned as a control well). In order to prevent dehydration of bacteria, the plate was wrapped with parafilm loosely, and the bacteria were incubated for 24 h at 37 °C; findings were assessed using a physical examination. The control well was first checked to verify the presence of visible growth (the medium had become cloudy due to microbial growth) and the lack of microbial development in the other

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wells (the medium remained clear, transparent).[22] These samples were prepared in triplicates.

2.7. Antibiofilm Activity. A microplate biofilm assay was used to determine the impact of the EOs and the ME at doses 1, 1/2, and 1/4 MIC on the bacterial strains' capacity to produce biofilms. Each well of the microplate received 100 μL of each formulation. 100 μL of MHB was used as the negative control. The final volume of each well was 200 μL after pipetting the 100 μL of bacteria culture (10^6 CFU/mL) in each well. To enable the cells to adhere to the surface, the plates were loosely wrapped with parafilm and incubated at 37 °C for 8 h with no shaking. The contents of each well were taken out after incubation. To get rid of nonadherent cells and cells that were loosely affixed, the wells were cleaned three times with sterile distilled water. After air-drying, the wells were stained with crystal violet (1%). After 15 min, the wells were cleaned of any remaining discoloration. By adding 150 μL of ethanol, the wells were destained. From this, 100 μL was taken and put in a new plate in order to measure the absorbance at OD_{590} nm by using an ELISA microplate reader (LABsystems, multiskan MS, Finland).[22]

$$\text{percentage inhibition} = OD_{\text{negative control}} - OD_{\text{experimental}}/OD_{\text{negative}}$$

2.8. Antioxidant Study. The formulation's free radical scavenging activity was determined by using 1,1-diphenyl-2-picryl-hydrazil (DPPH). In brief, a 0.2 mM DPPH methanolic mixture was formed, and from this solution, 1 mL was combined with 1 mL of a methanolic dilution of formulation at various concentrations. After vigorous shaking, the mixed solution was set aside for 30 min. A spectrophotometer was used to determine the absorbance at 517 nm. The smaller the absorbance of the reaction solution, the stronger the antioxidant effect.[23] The concentration of DPPH radicals was determined by following the equation below

$$\%\ \text{inhibition} = (A_0 - A_1)/A_0 \times 100$$

where A_0 represents control reaction absorbance and A_1 represents sample absorbance.

2.9. Statistical Analysis. GraphPad prism 5.0 software (GraphPad Software, INC.) was used for statistical analysis, and the data collected was investigated by employing one-way ANOVA, with a post hoc Tukey test. At a P value <0.05, all of the tests employed in this study were judged significant.

3. RESULTS AND DISCUSSION

3.1. Pseudoternary Phase Diagram. The mapping of ME regions was carried out through the ternary phase diagram. The stability can be expressed by the ME area in the diagram. An ME is formed when the oil−water contact has a very low degree of interfacial tension, and the intermediate surface is retained extremely elastic and fluid-like, resulting in a spontaneous emulsion. Normally, it is accomplished by the cautious and precise selection of S/CoS, as well as their relative percentage. In this work, the surfactant/cosurfactant (Tween 80/Transcutol P) ratio was held constant at 1:1. Figure 1 depicts the phase diagram where LEO, BEO, and CEO were used as an oil phase. The proportion of oil phase had a significant influence on size distribution, which may be attributed to ME droplet expansion. Furthermore, the higher the proportion of oil content, the greater the possibility of loading of hydrophobic payloads in the oily phase. The following was the order of the ME regions: CEO ME > LEO ME > BEO ME. Eugenol, the most abundant phenolic ingredient in CEO (89%), has a surfactant-like structure and a modest molecular volume (0.26 nm^3). As a result, this could adhere to the surfactant layer junction, generating a combined emulsifier−eugenol layer. This can improve the surfactant efficiency by increasing the effective chain volume of the surfactant. Furthermore, the molecule's hydrophobic tail (unsaturated) may aid in emulsifying the interfacial membrane and increasing its curvature to generate ME. Therefore, this cosurfactant behavior was the principal reason for the ME region of CEO.[24] Furthermore, additional elements of CEO, like eugenol acetate (6.9%), might be attributed to the adsorption on the surface with eugenol, resulting in reduced interfacial tension and therefore enhancing the ME production.[25]

Linalool, a terpenic alcohol with a surfactant-like structure, is found in LEO and, like eugenol, may offer greater interfacial tension, which could improve the interfacial curvature, resulting in enhanced LEO solubilization. As a result, it is possible that the narrower solubilization area in LEO compared to CEO is due to the lower proportion of linalool (35%) in LEO relative to eugenol.[26] Eugenol in BEO (41.5%) is less than CEO and gives less ME region as compared to CEO (Table 1).[27]

Table 1. % w/w Composition of ME, where L1−L4 Include LEO, B1−B4 Include BEO, and C1−C4 Include CEO

formulation	oils	surf/co-surf mix	water
L1	10	45	45
L2	10	50	40
L3	15	50	35
L4	20	50	30
B1	10	45	45
B2	15	50	35
B3	20	50	30
B4	25	55	20
C1	10	40	50
C2	15	45	40
C3	20	45	35
C4	25	45	30

3.2. Droplet Size, PDI, and ζ Potential. Particle diameter is an essential property to characterize ME, and the primary analysis indicated that the acquired MEs having a modest globule size were stable. Actually, droplet diameters of all of the MEs were below 20 nm, and the distributions of globule size that were relatively narrow confirmed the stability of MEs. The globule size could affect the interaction of the ME with the surrounding environment, such as microbes, food, and/or humans.

As shown in Figure 2, the diameter of LEO containing MEs (L1−L4) ranged from 11.65 ± 0.62 nm to 14.82 ± 0.91 nm, BEO MEs (B1−B4) presented a size from 12.99 ± 0.85 to 17.44 ± 1.1 nm, while CEO MEs (C1−C4) were of 11.85 ± 0.74 to 15.46 ± 0.85 nm, indicating that the MEs presented a comparable size. On increasing the oil concentration from 10 to 25% w/w, an increment in the globule diameter was observed.[28] The inclusion of a cosurfactant (Transcutol P) and the significant percentage of the S/COS mix might be related to a substantial drop in surface tension, resulting in globule size

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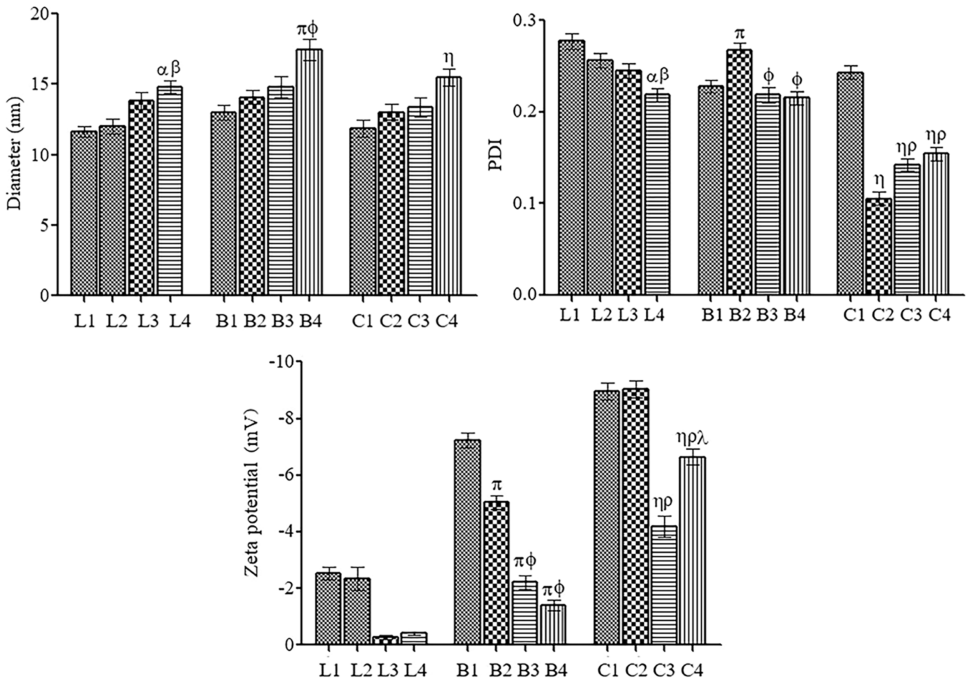

Figure 2. Average globule size, PDI, and ζ potential for MEs of LEO (L1−L4), BEO (B1−B4), and CEO (C1−C4). α indicates $P < 0.05$ vs L1; β shows $P < 0.05$ vs L2; π indicates $P < 0.05$ vs B1; ϕ indicates $P < 0.05$ vs B2; η indicates $P < 0.05$ vs C1; ρ represents $P < 0.05$ vs C2; λ indicates $P < 0.05$ vs C3. Values are the mean ± SD.

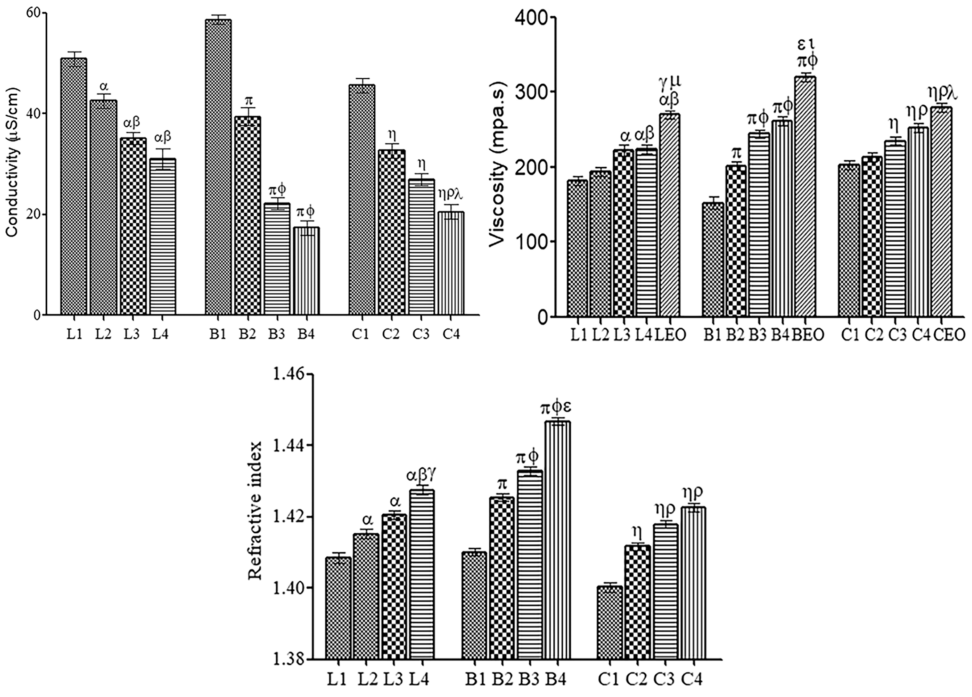

Figure 3. Conductivity, viscosity, and refractive indices for MEs of LEO (L1−L4), BEO (B1−B4), and CEO (C1−C4). α indicates $P < 0.05$ vs L1; β shows $P < 0.05$ vs L2; π indicates $P < 0.05$ vs B1; ϕ indicates $P < 0.05$ vs B2; and η indicates $P < 0.05$ vs C1; ρ represents $P < 0.05$ vs C2; λ indicates $P < 0.05$ vs C3. Values are the mean ± SD.

reduction. Furthermore, the reduced oil fraction in the formulation could be associated with a considerable reduction in particle size.[29] The prepared MEs displayed PDIs of around 0.10 ± 0.01 to 0.27 ± 0.02, indicating a narrow droplet size dispersion. PDI values smaller than 0.3 indicate low polydispersity, which would be consistent with the intended ME properties.[30]

The formation of droplets and the stability of the system were closely interrelated through interactions between different molecules in the sample, such as electrostatic contact. The results of ζ potential can be used to assess the sample's molecular interactions, which can then be utilized to estimate sample stability.[31] In this research, ζ potential values ranged from −0.27 ± 0.01 to −9.03 ± 0.13. This negative charge

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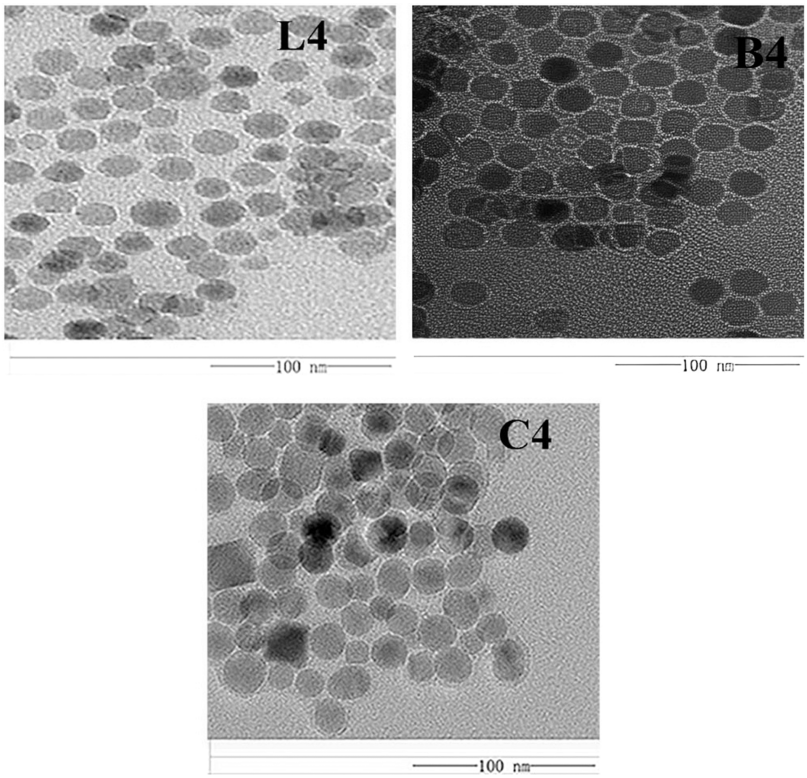

Figure 4. TEM images for the selected MEs of the LEO (L4), BEO (B4), and CEO (C4).

might be owing to the presence of fatty acids, or it could be indicative of nonionic surfactants and cosurfactants, which normally give a negative charge to molecules on which they would be bound.[32] This finding demonstrates that all of the MEs have sufficient charge and mobility that can reduce the particle aggregation. Hence, aggregation or coprecipitation of the system in the physiological system and during its shelf life is unlikely, as evidenced by the small globule size and low PDI. less than 0.5.[24]

3.3. Evaluation of Conductivity, Viscosity, and Refractive Index. MEs are characterized as oil in water (o/w), water in oil (w/o), or bicontinuous in theory, based on the oil/water phase ratio. As the water/oil phase ratio exceeds 1, an o/w ME is formulated, whereas when the oil/water phase ratio is greater than 1, a w/o ME is generated. When the water and oil phases are equivalent, a bicontinuous ME develops. Electrical conductivity is a commonly used criterion for determining the ME type. A w/o ME is defined as one with an electrical conductivity of less than 1 μS/cm, whereas a bicontinuous ME is considered as one with an electrical conductivity of 1−10 μS/cm. An o/w ME is specified as a system having a conductivity higher than 10 μS/cm.[33]

Figure 3 shows the results of ME having LEO 10−20% w/w had electrical conductivity ranging from 50.87 ± 2.45 to 31.02 ± 2.03 μS/cm, while MEs containing BEO and CEO represent conductivity ranging from 58.66 ± 2.25 to 17.4 ± 1.94 μS/cm and from 45.61 ± 1.65 to 20.5 ± 1.32 μS/cm, respectively. According to the aforementioned classification, this study concluded that all of the formulations were oil-in-water MEs.[34] Formulations with 10% w/w oil and 50% water had high conductivity, whereas MEs having 25% w/w oil content and 20% water possessed low conductivity. These results indicated that electrical conductivity is directly related to water content in the formulation, as a higher water concentration results in the increment of electrical conductivity.[35]

Viscosity of ME containing three distinct EOs is illustrated by Figure 3. In terms of mean particle size, substantial variation in viscosity was observed, particularly based on the type of EO. The individual LEO had a viscosity of 270 mPa·s, and MEs ranged from 181 to 224 mPa.s for 10−20% oil content. Viscosities of BEO and its MEs were 320 and 152−261 mPa.s, respectively. CEO having a viscosity of 280 mPa·s and its MEs presented 203−252 mPa.s. By enhancement of the percentage of EOs in ME, an increase in the viscosity was noted. The percentage of surfactant also seemed to be correlated to viscosity; this might be due to water molecules becoming trapped in the surfactant's cross-linking networks. On the basis of these findings, it could be possible to suggest that the ME's decreased viscosity correlates to the smaller diameter. In this regard, it has been observed that the average diameter of a NE is proportional to the oily phase's viscosity.[36] A decrease in viscosity causes the emulsifier to flow more quickly and easily, resulting in smaller particles. The resistance of oil particles to breakage is boosted by their comparatively high viscosity. Conversely, when these oil particles are sheared, they begin to spin along their axis, resulting in bigger droplets. It was also observed that reducing the percentage of water in the system from 50 to 20% promotes the increase in viscosity of the ME.[37] The smaller droplet size and lower viscosities of the EOs in the formulation contribute to the overall lower viscosity of the ME, which also indicates the existence of one phase in the designed formulations as reported elsewhere.[38]

Refractive index can also give information about the microstructures of ME. Hashem et al. revealed that refractive indices in the range of 1.39−1.42 denote the presence of monophasic isotropic systems.[39] This region of refractive index explains that MEs are optically clear (transparent) in

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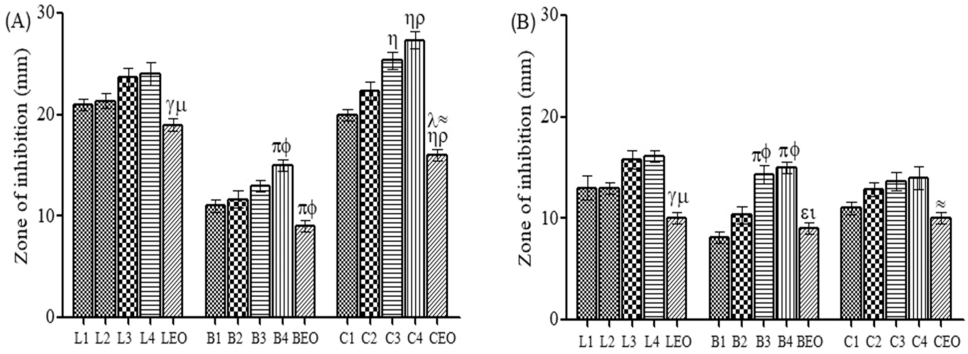

Figure 5. Antibacterial activity of LEO, BEO, CEO, and their respective MEs against (A) *S. aureus* and (B) *E. coli*. γ indicates $P < 0.05$ vs L3; μ indicates $P < 0.05$ vs L4; π depicts $P < 0.05$ vs B1; ϕ indicates $P < 0.05$ vs B2; η indicates $P < 0.05$ vs C1 and ρ represents $P < 0.05$ vs C2; λ indicates $P < 0.05$ vs C3; $\approx$ indicates $P < 0.05$ vs C4. Values are the mean ± SD.

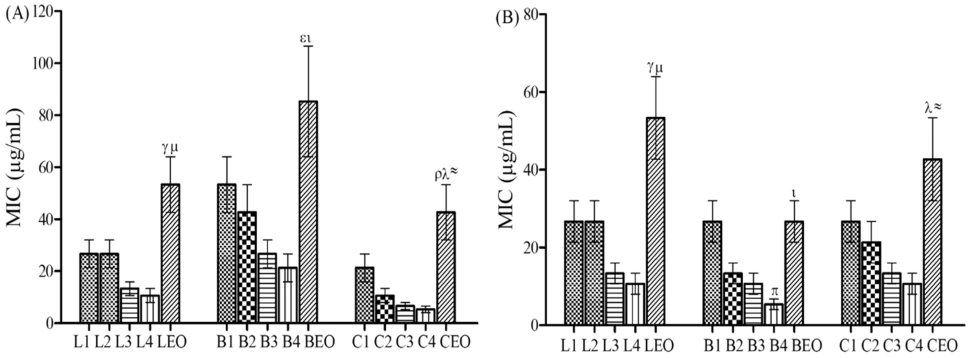

Figure 6. MIC of LEO, BEO, CEO, and their respective MEs against (A) *S. aureus* and (B) *E. coli*. γ indicates $P < 0.05$ vs L3; μ represents $P < 0.05$ vs L4; π depicts $P < 0.05$ vs B1; ε represents $P < 0.05$ vs B3; ι illustrates $P < 0.05$ vs B4; ρ represents $P < 0.05$ vs C2; λ indicates $P < 0.05$ vs C3; $\approx$ indicates $P < 0.05$ vs C4. Values are the mean ± SD.

appearance.[40] As the concentration of oil increased, the refractive index increased slightly. Basheer et al. discovered that the refractive index of w/o MEs is greater than that of bicontinuous and o/w MEs. This is due to surfactants having a greater refractive index than water as an exterior pseudo-phase.[41] This discovery was supported by the findings of our ME. As the water percentage in MEs increased from 20 to 50%, the refractive index values dropped from 1.43 to 1.41. Our findings align well with the visual observations of transparency that showed that monophasic systems were obtained.

3.4. Transmission Electron Microscopy (TEM). TEM images of the specified MEs L4, B4, and C4 verified the uniform spherical-shaped particles with a size smaller than 20 nm, as shown in Figure 4. These results were consistent with the dynamic light scattering results obtained using the DLS method. Globules were found to be uniformly dispersed, indicating good stability of the MEs.

3.5. Antibacterial Activity and MIC. Antibacterial activity of EOs before and after encapsulation into MEs was evaluated against two pathogenic bacterial strains. Terpenes, alcohols, aldehydes, and other volatile components have been linked to bactericidal activity in plant EOs, and hence, variations in volatile component's concentration alter their antimicrobial characteristics.[31] As shown in Figures 5 and 6, nanosizing of the EOs increased their antibacterial potential toward S. aureus and *E. coli*. The zone of inhibition of LEO for *S. aureus* was 19 ± 1 mm, and by encapsulating it in an ME system, the inhibition zone increased up to 24 ± 2 mm. The range of inhibition zone of L1−L4 was 21 ± 1 to 24 ± 2 mm. The zone of inhibition of BEO against *S. aureus* was 9 ± 1, whereas for its MEs (B1−B4), it was 11 ± 1.5 to 15 ± 2 mm, respectively. The inhibition zones of CEO and C1−C4 against *S. aureus* were 16 ± 1 and 20 ± 2 to 27 ± 2.5 mm, respectively. Against *E. coli*, inhibition zones of LEO and L1−L4 were 10 ± 1.2 mm and 13 ± 1.2 to 16.1 ± 1.5 mm, respectively; for BEO and B1−B4, they were 9 ± 1 mm and 8.1 ± 1.1 to 15 ± 2.5 mm, respectively; and for CEO and C1−C4, they were 10 ± 1 and 11 ± 1.1 to 14 ± 1.8 mm, respectively. Increase in activity is most likely due to the nanosized formulations' higher surface area, as well as the ME feature that allows for more active ingredient diffusion through the membrane of the micro-organism. It is considered that the hydrophilic surface of the nanostructure permeates the cellular membrane via hydrophilic transport networks with the help of proteins in Gram-negative bacteria (*E. coli*), whereas nanosized particles transport the chemicals right into the site of action against Gram-positive bacteria.[42]

As the concentration of therapeutic oil increased up to 25% w/w, the zone of inhibition also increased, as shown in Figure 5, whereas the MICs were inversely related to the oil content, as shown in Figure 6. Against *S. aureus*, the MICs of LEO and its MEs (L1 to L4) were 53.33 ± 3.5 μg/mL 26.66 ± 2.1 to 10.66 ± 2.9 μg/mL, respectively; MICs of BEO and B1−B4 were 85.33 ± 4.7 μg/mL and 53.33 ± 2.5 to 21.33 ± 2.4 μg/mL, respectively; and MICs for CEO and C1−C4 were 42.66 ± 2.9 μg/mL and 21.66 ± 2.4 to 5.33 ± 1.5 μg/mL, respectively. In the case of *E. coli*, the MICs of LEO and L1−L4 were 53.33 ± 2.4 μg/mL and 26.66 ± 1.6 to 10.66 ± 1.5 μg/mL, respectively; MICs for BEO and B1−B4 were 26.66 ±

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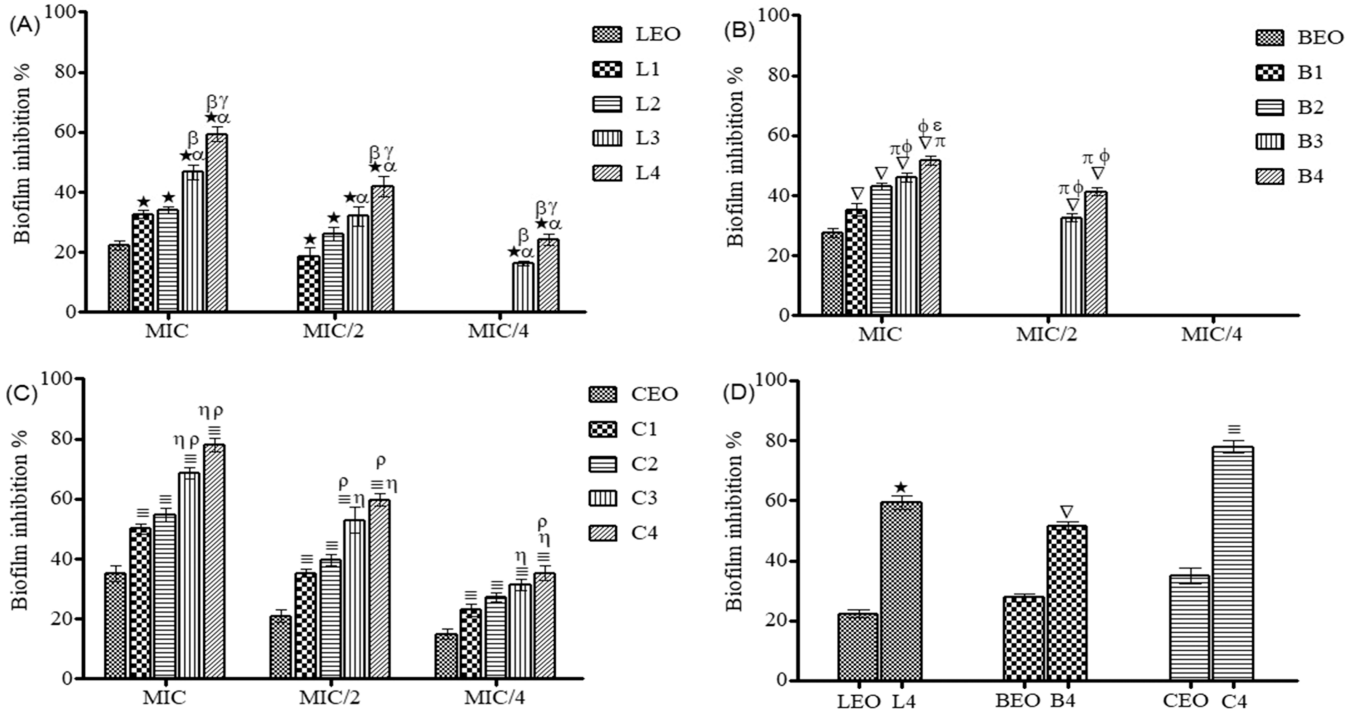

Figure 7. Antibiofilm activity of (A) LEO, ME, (B) BEO, ME, (C) CEO, ME, and (D) LEO vs L4, BEO vs B4 and CEO vs C4 against *S. aureus*. ★ indicates $P < 0.05$ vs LEO; α indicates $P < 0.05$ vs L1; β shows $P < 0.05$ vs L2; γ indicates $P < 0.05$ vs L3; Δindicates $P < 0.05$ vs BEO; π depicts $P < 0.05$ vs B1; ≡ represents $P < 0.05$ vs CEO; η indicates $P < 0.05$ vs C1; ρ represents $P < 0.05$ vs C2. Values are the mean ± SD.

2.6 μg/mL and 26.66 ± 3.2 to 5.33 ± 1 μg/mL, respectively; and MICs for CEO and C1−C4 were 42.66 ± 2.9 μg/mL and 26.66 ± 2.4 to 10.66 ± 1.5 μg/mL, respectively.

Terpinen-4-ol and linalool are among the antibacterial components found in LEO. Linalool is the most effective main ingredient that combats a variety of pathogens.[35] The prepared ME was of nanodroplet size that could directly fuse well with the microbes' cellular surface, and the surfactants utilized allow rupture of the exterior surface, leading to killing of the microorganisms. The hydrophobicity of CEO partitioned the phospholipids of the plasma membrane, rendering them extremely permeable by disrupting the structure. As a result, bacterial cells die due to substantial spillage from the cytoplasm or the outflow of essential chemicals and electrolytes. CEO has long been practiced to combat cariogenic and periodontopathogenic bacteria as an effective antimicrobial.[43]

According to the literature, an EO mixture of clove, cinnamon, and lavender was utilized to develop a formulation against *E. coli*. The stability of these compounds set in the NE system has demonstrated its effectiveness, as the antibacterial activity has been maintained and even improved over time.[44] Nanoformulations interacting with bacterial cell walls causes some internal components to deplete and leads to creating a disruption to the membrane of the bacterium. Active EO hydrophobic chemicals penetrated the bacterial cytoplasm, which led to substantial ion leakage, cell proteolysis, ATP depletion, and autolysis.[45] Porins exist in the external membrane structure of *E. coli* and permit the entrance of water absorbent compounds while serving as a barrier to lipophilic substances and limiting the chemical entry via size.[46] As a result, it is reasonable to predict that solubilizing lipophilic antibacterial substances in ME systems will increase the number of binding sites that can interface with porins.[47] Recent research publications have reported an increase in the antibacterial activity of NE, including EOs such as thyme oil and cinnamon, rosemary, oregano, etc.[48] Furthermore, the findings of this investigation revealed that the antibacterial activity of MEs was significantly more effective. In this regard, MEs are well-established antimicrobial delivery vehicles, and it is well-known that proper release of entrapped active substances from small particles improves the microbiological action of dispersed antibacterials.[49]

Because of their cell wall structure, microbes (Gram-positive) are substantially more susceptible to EOs. The Gram-positive strains' peptidoglycan cell wall permits hydrophobic compounds to enter the cell. Contrary to this, Gram-negative bacteria's lipopolysaccharide outer layer allows tiny hydrophilic molecules and is somewhat favorable for hydrophobic molecules. According to a study, ME encapsulating LEO preserved by starch has a unique antimicrobial activity for *S. aureus* and *E. coli*. Linalool L, found in LEO, has antimicrobial effects.[50] The ME encapsulating the EOs permeates the cellular membrane of the microbes more easily due to the tiny size of the particles. This permits the hydrophobic compounds in the EOs to cause harm to biological membranes by changing the stability of the phospholipid bilayer or interference with the bilayer's active carrier proteins. Changes in the porosity of the ruptured biological membrane allow nucleic acids, proteins, and potassium to seep out of the bacterial cell, causing the membrane to become unstable and inhibiting bacterial cell development. When EOs and ME are compared in terms of antibacterial action, it is clear that ME was significantly better. Smaller ME particulates may transport EOs to the interface of biomembranes, but pure EOs cannot easily interact with the cytoplasmic membrane due to their bigger size than ME. The terpenoid chemicals sabinene, myrcene, and transcaryophyl-

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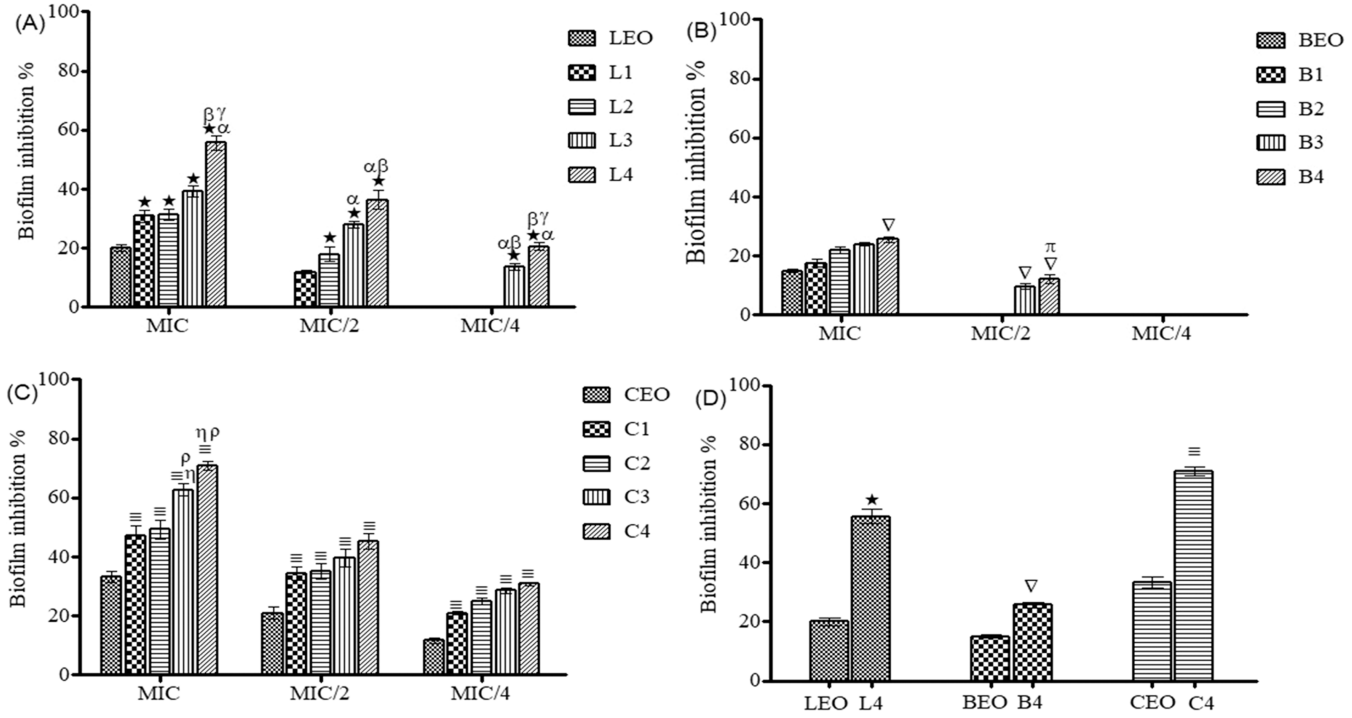

Figure 8. Antibiofilm activity of (A) LEO, ME, (B) BEO, ME, (C) CEO, ME, and (D) LEO vs L4, BEO vs B4 and CEO vs C4 against *E. coli*. ★ indicates $P < 0.05$ vs LEO; α indicates $P < 0.05$ vs L1; β shows $P < 0.05$ vs L2; γ indicates $P < 0.05$ vs L3; Δindicates $P < 0.05$ vs BEO; π depicts $P < 0.05$ vs B1; ϕ indicates $P < 0.05$ vs B2; ε represents $P < 0.05$ vs B3; ≡ represents $P < 0.05$ vs CEO; η indicates $P < 0.05$ vs C1; ρ represents $P < 0.05$ vs C2. Values are the mean ± SD.

lene, which are found in BEO, have a role in antibacterial action.[51]

The variation in the zone of inhibition diameter and MIC indicates that EOs and EOs-MEs inhibit *S. aureus* growth quite effectively compared to *E. coli*. Gram-negative bacteria's cell walls are more complicated than Gram-positive bacteria's.[52] The *E. coli* cell wall is made up of three layers: an exterior lipoprotein layer, an intermediary lipopolysaccharide layer, and an interior membrane of peptidoglycan, as well as a bilayer of an outer membrane (improved chemical resistance that enter and exit cells and cause toxicity). The hydrophobicity of EOs can destroy lipids in the bacterial membrane, disrupt membrane integrity, induce cell membrane leakage, and eventually limit bacterial development.[53] BEO was reported to possess the most effective antibacterial properties against *E. coli*, followed by *S. typhimurium* and *S. aureus*.[51] Overall, CEO MEs displayed a higher degree of killing of Gram-positive bacteria as well as Gram-negative bacteria.

3.6. Antibiofilm Activity. To assess the ability of EOs and ME to suppress the cell attachment at MIC, MIC/2, and MIC/4 value concentrations, the crystal violet assay was carried out. Figures 7 and 8 depict how three different EOs affected the cell's ability to form a biofilm. The results indicate that all of the formulations inhibit biofilm in a manner that CEO > LEO > BEO. The inhibitory effect of ME was much higher than that of the pure EOs. The increased percentage inhibition by ME as compared to EOs at a low concentration could be due to the specific characteristics of ME. First, the EO particles in the ME have a larger surface area and a small size that make interaction with the bacterial surface easier. Additionally, the concentration of the active component was presumably boosted by MEs' capacity to disperse EOs into the nutrient broth. As shown in Figure 7(A), at the MIC value, the percentage inhibition of LEO and LEO ME against *S. aureus* ranged from 22.3 to 59.3%, respectively. As the concentration was decreased to MIC/2 and MIC/4, the activity also reduced up to 18.6−44% and 0−24.3%, respectively. In the case of MIC/4, LEO, L1, and L2 were unable to inhibit the biofilm due to the very low concentration. In Figure 7(B), the activity of BEO and BEO ME was 27.6−51.7% at MIC. At MIC/2, the BEO, B1, and B2 did not show any activity. At MIC/4, there was no inhibition noted in any formulation. CEO and CEO ME (Figure 7(C)) showed a higher activity against *S. aureus* up to 35−78% at an MIC value. At MIC/4, 15−35% activity was noted. BEO exhibited less activity as compared to LEO and CEO. In the case of *E. coli*, 8Figure Figure 8(A) depicts that LEO and its ME have activity from 20 to 55.6% at MIC. At MIC/2, LEO showed zero activity, while L1−L4 inhibited 12−36% of the biofilm. L3 and L4 have 13.6 and 20.6% inhibitions, respectively, at MIC/4. BEO showed the least activity against *E. coli* as compared to LEO, CEO, and the activity against *S. aureus*. Figures 7(D) and 8(D) represent the comparison of EO activity with a higher concentration of formulations. CEO ME demonstrated the strongest antibiofilm activity with percentage inhibitions of 78% (MIC) and 71% (MIC) against *S. aureus* and *E. coli*, respectively. In the study by Sharma et al.,[54] CEO underwent an antiadhesion test utilizing a crystal violet assay to assess the reduction of cell attachment. They discovered that CEO had a variety of impacts on the formation and growth of biofilms, resulting in 75% inhibition in cell attachment against *E. coli*. Our findings are relevant to a study by Campana et al. that nanoencapsulation of EOs enhances the antimicrobial and antibiofilm activities.[55] The examined EOs and MEs had a dose-dependent inhibitory effect on the biofilm. According to a previous study, the antibiofilm activity is directly proportional to the concentration of the antibiofilm

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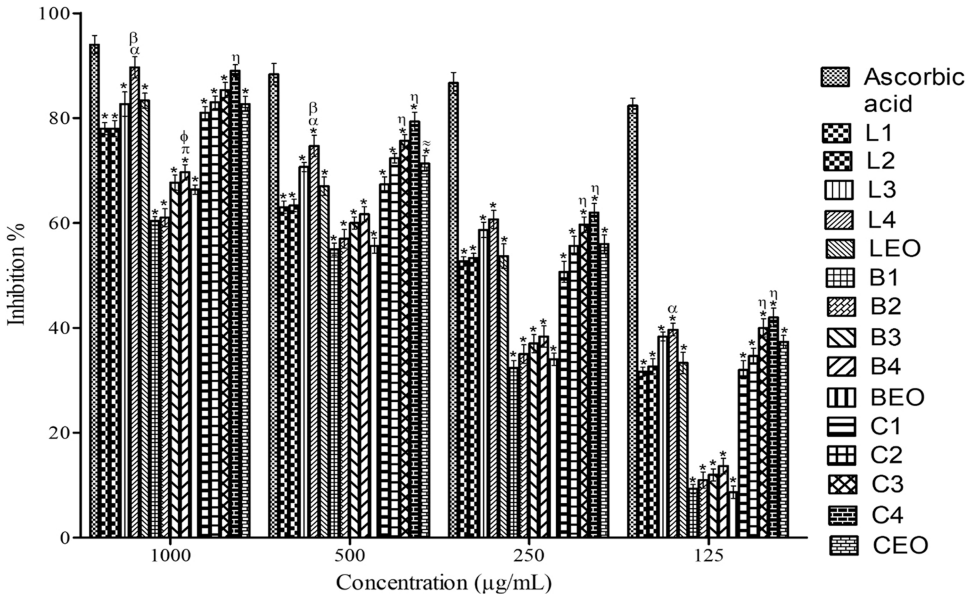

Figure 9. Antioxidant activities of LEO, BEO, CEO, their respective MEs, and ascorbic acid. * Illustrates $P < 0.05$ vs ascorbic acid; α indicates $P < 0.05$ vs L1; β shows $P < 0.05$ vs L2; π depicts $P < 0.05$ vs B1; ϕ indicates $P < 0.05$ vs B2; η indicates $P < 0.05$ vs C1 and $\approx$ shows $P < 0.05$ vs C4. Values are the mean ± SD.

agent.[56] Our results were in agreement with those of Millezi et al., indicating that by increasing the concentration of EOs, the activity against the *E. coli* biofilm gets stronger.[57] It had been demonstrated by Olszewska et al. that the main chemical component of CEO is eugenol, which showed the greatest activity by hampering the cell membrane, thus reducing the enzymatic reaction. As a result, it alters physical properties like the surface texture and organic matter of biofilms.[58] Numerous studies have been conducted on EO's antimicrobial activity and mechanisms.[59] Eugenol, a member of phenylpropenes, alters the cell membrane's composition and combats bacterial enzymes by using its free hydroxyl group.[60] Lou et al. explained that by encapsulating the EOs, the antioxidant, antimicrobial, and antibiofilm activity enhanced as compared to pure EOs.[61] Nanoencapsulation of EOs will encourage the use of EOs internationally.

3.7. Free Radical Scavenging Activity. DPPH free radical scavenging is an established method for antioxidants to reduce the oxidation process in a short period of time. In the DPPH test, antioxidants can reduce the stable free radical to the yellow-colored diphenyl-picrylhydrazine. The approach relies on the interaction of an alcohol-related DPPH carrier with a hydrogen-donating antioxidant to create the nonradical version of DPPH-H.[5] Ascorbic acid or vitamin C is used as the reference, and the relative values exhibited by the formulation components reflect a somewhat potent antioxidant activity of the formulation.

Figure 9 shows the bioactivity findings, which indicate that EOs have considerable antioxidant activity. Polyphenols or pigments like anthocyanins, volatiles like methyl chavicol and eugenol found in EOs, tannins like catechin, and flavonoids like quercetin, kaempferol, and myricetin are all responsible for this characteristic. The antioxidant activity tends to vary significantly due to variations in the quantity of these substances. The EOs inhibited the production of DPPH radicals according to the dose. Our results show that CEO (82.6 ± 1%) is a potent antioxidant agent followed by LEO and BEO. CEO is a natural substitute to eugenol in order to improve the antioxidant activity. According to some studies, BEO is a strong antioxidant since it contains eugenol and linalool.[62] However, in our comparison with CEO and LEO, BEO was found to be a weak agent. However, owing to the higher concentration of linalool present in LEO as compared with BEO, the activity of BEO was reduced. Additionally, CEO has 2−3 times higher content of eugenol as compared to BEO. The synergistic impact among phenolic contents of CEO at a low concentration can also explain its strongest radical scavenging agent. Radunz et al. also explained that eugenol and phenolic components present in CEO show the strongest antiradical activity.[63]

It can be observed from Figure 9 that the EOs encapsulated in ME were comparable to the reference (ascorbic acid). The scavenging abilities of LEO ME (89 ± 1.5%), BEO ME (69 ± 2.0%), and CEO ME (90 ± 1.5%) were compared with the ascorbic acid reference (97 ± 2%) in 1000 μg/mL, but as the concentration decreased, the activity also decreased. LEO (93.4 ± 1.55%) and limonene (93.1 ± 1.17%) were found to have a scavenging action.[64] These results make it possible to draw the conclusion that the antiradical activity of EOs has been enhanced by encapsulation in the ME system. ME facilitates the interaction of EOs with DPPH. The larger surface area and small particle size of oil droplets present in the ME system are the obvious reasons for the improved antioxidant activity. However, an additional aspect that should also be taken into consideration is the usage of a surfactant and a cosurfactant. Due to the increased number of carbons in Tweens, the EOs would be more easily maintained in ME.[14] The antioxidant properties of different solvent extracts produced using basil leaf and flower components were studied in another study. The percentages of ethanol, water, and acetone that scavenge DPPH radicals were 72.55, 50.98, and 37.91%, respectively.[65] When compared to published data, the CEO under research had a higher DPPH scavenging activity. For instance, at a dosage of 10000 $\mu g\,mL^{-1}$, 92.82% activity had been recorded.[63] In general, a concentration-dependent antioxidant phenomenon was observed for EOs and their

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corresponding MEs. MEs with a lower oil content had a slightly lower free radical scavenging capacity than their corresponding oils, but all of the MEs containing 25% EOs had a better antioxidant potential compared to their EOs.

The natural form of antioxidants is of vital importance since free radicals have harmful effects on food and the human body. According to research, lipid oxidation speeds up in food due to the presence of excessive free radicals that lower the quality of food. Our finding implies that in place of synthetic antioxidants, EOs should be considered.

4. CONCLUSIONS

In this study, we developed a microemulsion system to encapsulate EOs of lavender, basil, and clove, which were commonly utilized as flavoring agents as well as preservatives in foods. This approach produced miniscule and spherical droplets of EOs in the nanometer range with a significantly higher stability. Encapsulated EOs had better antimicrobial and radical scavenging activity toward foodborne microorganisms than pure EOs, indicating that nanosizing and stabilization of EOs improve their surface area, hydrophilicity, and biological activity, which broaden their applicability. Our findings suggest that these natural nanosystems might be a good substitute for chemical preservatives in the food processing industry for limiting the incidence of foodborne illnesses and assuring higher food safety through nanotechnology.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c05394.

Conductivity, viscosity, and refractive indices for MEs of the LEO (L1−L4), BEO (B1−B4), and CEO (C1−C4); antioxidant activities of LEO, BEO, CEO, their respective MEs, and ascorbic acid; zone of inhibition and MIC values of EOs and their respective MEs against *S. aureus* and *E. coli*; and visual appearance of MEs of the LEO (L1−L4), BEO (B1−B4), and CEO (C1−C4) (PDF)

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Notes

The authors declare no competing financial interest.

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