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Development, Characterization, and Biological Evaluation of Clove Essential Oil Microemulsions

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Abstract

Clove essential oils (CEOs) are widely studied because of their biological potential; however, their applications are limited because of their water immiscibility. Microemulsions (MEs) can protect, deliver, and enhance the biological activities of CEOs, including their antioxidant and cytotoxic activities. In this research, the effects of ethanol as a cosurfactant and the polysorbate 80:cosurfactant mixture (S_{mix} = 1:0, 9:1, 7:1, 5:1, 3:1, and 1:1) on the formation of CEO-MEs were evaluated via a pseudo-ternary phase diagram. After 35 days, all the systems produced clear, monodisperse, and thermodynamically stable MEs, characterized by average sizes below 25.6 nm and low polydispersity index values (<0.21). The S_{mix} dose–response experiments without CEO revealed that the S_{mix} ratios of 1:1 and 3:1 resulted in the lowest cytotoxicity to HT-29 (colorectal adenocarcinoma) cells. The antioxidant capacity of the CEO-ME was greater than that of the CEO. Finally, the CEO-MEs enhanced the in vitro cytotoxic activity of the CEO against Caco-2, HT-29, HeLa, PC-3, and A549 cancer cells. These findings provide valuable information for the development of low-energy clove essential oil MEs for potential incorporation into functional foods and pharmaceutical products.

Keywords: pseudo-ternary phase diagram; antioxidants; cytotoxic activity; microemulsion; eugenol; *Syzygium aromaticum* L.

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1. Introduction

Breast, prostate, lung, colon, and cervical cancers are among the most prevalent types of cancers, accounting for approximately 44% of new cancer cases worldwide, and accounting for an estimated 47% of all cancer-related deaths [1]; however, an overall decrease in cancer mortality of 34% has been observed since 1991 due to changes in habits, early detection of cancer, and improved treatment. Despite advances in surgery, radiation therapy, and systemic therapies, including chemotherapy, hormonal treatments, and targeted biological therapies, the high costs and deleterious side effects of these treatments in patients are common problems. For example, some of the most common symptoms include systemic upsets (fatigue, swelling); gastrointestinal and mucosal effects (nausea, vomiting, mouth sores); dermatologic changes (hair loss, changes in skin color); hematologic and infectious effects (increased risk of infection, easy bruising or bleeding); neurologic effects; renal and

hepatic toxicity (kidney damage, liver damage); and cardiovascular effects (heart damage). Potential long-term effects include permanent nerve damage and an increased risk of developing other cancers [2,3]. Three main categories of lipid-based drug delivery systems have been extensively tested in cancer treatment: (1) vesicular systems; (2) lipid particle systems; and (3) emulsions, including nanoemulsions and microemulsions (MEs) [4].

The use and application of essential oils (EOs) against cancer is a very promising field because they exhibit a wide range of bioactive effects, including cytotoxic, antioxidant, antimutagenic, antiproliferative, and antimetastatic effects on cancer cells through multiple pathways and mechanisms of action. However, attempts to have in vivo/in vitro anticancer applications, with the specificity of cellular target and drug delivery systems, are largely insufficient [5,6]. Formulation strategies such as the use of nano-sized novel drug delivery systems, including emulsions, niosomes, and liposomes, have been used to increase the stability, efficacy, and bioactivity of EOs in several cancer cell lines [5–8]. Emulsions are manufactured through high- or low-energy processes, from which the kinetic stability, oil concentration, surface area, droplet size, optical properties, bioaccessibility, and bioavailability of the active compounds vary significantly. Low-energy processes involve the spontaneous self-assembly of surfactants, cosurfactants, oil, and water, in which a thermodynamically stable, low viscosity, and isotropic small-sized emulsion is produced, commonly named "microemulsion (ME)" [9]. The physical characteristics of the MEs are comprehensively explored by phase diagrams generated from varying ratios of oil, surfactant(s)/cosurfactant(s), and water, in which different structures, including biphasic, emulsion, and MEs, can be produced [10–12]. Surfactants and cosurfactants play essential roles in the formation of MEs, increasing their stability by reducing the interfacial surface tension, thereby compensating for the scattering entropy and making the system thermodynamically stable [10,11,13,14]. Ethanol is among the most popular cosurfactants used to stabilize pharmaceutical, cosmetic, and food emulsions because of its ability to blend with surfactants, reduce the surface tension, and increase the elasticity of the interfacial layer [10].

Hence, interest in exploring natural bioactive agents, such as essential oils, including *Allium sativum* L., *Citrus aurantium* L., *Citrus bergamia* Risso and Poit., *Citrus medica* L., *Curcuma longa* L., *Cymbopogon citratus* (DC.) Stapf., *Cymbopogon citratus* (DC.) Stapf., *Lippia graveolens* Kunth, *Ocimum basilicum* L., *Origanum vulgare* L., *Rosmarinus officinalis* L., *Salvia officinalis* L., *Thymus vulgaris* L., and *Syzygium aromaticum* L. have increased because of their potential in cancer treatment and their beneficial health properties [2,15]. *Syzygium aromaticum* L. is a fragrant blossom of the *Myrtaceae* family that grows in subtropical regions. Its flowers contain approximately 15 to 20% essential oils known as clove essential oils (CEOs), which, depending on the botanical origin and method of extraction, contain more than 30 volatile compounds. Eugenol is the major volatile compound in CEOs (50–90%), followed by β-caryophyllene and α-humulene [15,16]. CEO has been widely used for its antimicrobial properties, insecticidal effects, analgesic qualities, and anesthetic purposes, and more recently for its potential cytotoxic activities against various cancer cell lines [11,15–19].

MEs offer several advantages to EOs, including enhanced solubilization of lipophilic compounds, a reduction in unpleasant odors and tastes, and increased protection of encapsulated substances from hydrolysis or oxidation. Similarity, they can improve the biological activity of EOs by increasing their permeability and solubility [10,13,14,20,21]. For example, Kiromah et al. [22] reported that an ME generated with a polysorbate-80:PEG 400 ratio of 2:1 and an oil:S_{mix} ratio of 1:9 improved the stability, solubility, and effectiveness of clove essential oil through a system with a droplet size of 17.69 nm. Li et al. [23] reported that compared with pure *C. citratus* oil essential oil, the encapsulated oil in an ME showed

greater antiproliferative activity against HepG2 liver cancer cells, probably because the small size of the MEs increased their ability to permeate the cell membrane.

To our knowledge, there is little information on the development of MEs with clove oil and their cytotoxic effects on cancer cells. The scope of this research work was (1) to elucidate the impact of polysorbate 80 and ethanol mixtures on the formation of clove essential oil MEs using ternary diagrams, (2) to evaluate the impact of temperature under physiological conditions on the stability of formulated MEs, and (3) to determine their antioxidant capacity and cytotoxic activity against different cancer cell lines. Owing to the above-mentioned factors, there is growing research interest in determining whether MEs promote an increase in the bioactivity of essential oils for pharmaceutical and food applications.

2. Materials and Methods

2.1. Materials

Clove essential oil (CEO, No. W232300), polysorbate 80 (No. P1754), 2,2-diphenyl-1-picrylhydrazyl (DPPH, No. D9132), Folin reagent (No. F9252), Trolox (238813), Dulbecco's Modified Eagle's Medium (DMEM, D7777), 3–(4,5–dimethyl–2–thiazolyl)–2,5–diphenyl–2H–tetrazolium bromide (MTT, 298–93–1), and dimethyl sulfoxide (DMSO, 276855) were obtained from Sigma-Aldrich (Toluca, State of Mexico, Mexico). Absolute ethanol and methanol were purchased from "EMYR" (Guadalajara, Jalisco, Mexico). Deionized distilled water was used in all experiments. The cell lines used included two human colorectal adenocarcinoma cell lines, Caco-2 (ATCC® HTB-37, American Type Culture Collection, Rockville, MD, USA) and HT-29 (ATCC® HTB-38, American Type Culture Collection, Rockville, MD, USA); a human cervical adenocarcinoma cell line, HeLa (ATCC® CCL-2, American Type Culture Collection, Rockville, MD, USA); a human prostatic adenocarcinoma cell line, PC-3 (ATCC® CRL-1435, American Type Culture Collection, Rockville, MD, USA); a human lung adenocarcinoma cell line, A549 (ATCC® CCL-185, American Type Culture Collection, Rockville, MD, USA) and a non-cancerous human embryonic kidney cell line, HEK-293 (ATCC® CRL-1573, American Type Culture Collection, Rockville, MD, USA).

2.2. Methods

2.2.1. Microemulsion Formation

Pseudo-ternary phase diagrams were generated by mixing the CEO, water, and surfactant/cosurfactant mixture as needed to produce a pseudo-ternary diagram expressed in weight (*w*/*w* %) [13,24]. First, the S_{mix} dispersions were produced by mixing polysorbate 80 and ethanol at different mass ratios of 1:0, 9:1, 7:1, 5:1, 3:1, and 1:1 under continuous agitation at 200 rpm. Second, the oily phase was produced by mixing the CEO with S_{mix} at mass ratios. Finally, distilled water was added dropwise to the oily phase up to 90% (*w*/*w*) under continuous agitation at 200 rpm at 20 ± 0.5 °C. The samples were mixed for 2 min after the addition of water. A visual inspection was performed after each addition of water. The MEs were identified when a translucent and easily fluid appearance appeared. The samples were identified as emulsions when they were milky or cloudy liquids, and biphasic when they were immiscible two-phase systems.

2.2.2. Physiological Temperature Stability

The MEs with CEO (1%), water (90%), and different S_{mix} (9% of 1:9, 3:7, 5:5, 7:3, and 9:1) were stored in closed jars for 35 days at 37 °C (ICH 110 L, Memmert, Schwabach, Germany). The MEs were diluted (1 to 100) with distilled water prior to analysis. The mean droplet size (DS) and polydispersity index (PDI) of the MEs were measured in triplicate using a Zetasizer Nano-ZS90 (Malvern Instruments, Malvern, UK) and a plastic cuvette [7,8].

2.2.3. Antioxidant Activity Assays

DPPH Radical Capture Assay

DPPH reagent (4 mg) was dissolved in 100 mL of ethanol to prepare the DPPH solution. Then, 20 µL of each sample and 200 µL of the DPPH solution were added to a 96-well plate. The plate was incubated in the dark at 20 °C for 30 min. Following the incubation, the absorbance was measured with a spectrophotometer (Multiskan GO microplate, Thermo Fisher Scientific Inc., Waltham, MA, USA) at λ_{max} = 595 nm to quantify the residual DPPH radical. The CEO was diluted in ethanol to concentrations ranging from 0.015 to 0.0955 µg/mL, while the CEO–ME was diluted to concentrations from 0.04 to 2.44 µg/mL. The concentration was determined by the concentration of free oil or the amount of oil in the ME. Trolox was used as a standard and was evaluated at various concentrations ranging from 0.01 to 0.06 µg/mL The IC_{50} values (inhibitory concentration at 50%) were determined from their dose–response curves using linear regression ($y = 0.0057x + 0.0658$, $R^2 = 0.9973$). The antioxidant activity, expressed as Trolox equivalent antioxidant capacity (TEAC), was calculated with the following equation [8]:

$$\text{TEAC} = \frac{\text{IC}_{50} \text{ of Trolox } (\mu\text{g/L})}{\text{IC}_{50} \text{ of sample } (\mu\text{g/L})} \quad (1)$$

Total Phenolic Content (TPC) Determination

The TPC of the samples was quantified using a spectrophotometer method based on the Folin–Ciocalteu reagent. Briefly, 1 mL of the sample was diluted in 1 mL of 80% ethanol, and then, a 1:1 dilution with distilled water was made. From this diluted solution, 50 µL was added in triplicate to individual wells, followed by the addition of 50 µL of 1 N Folin–Ciocalteu reagent. The mixture was allowed to react for 5 min. Afterward, 100 µL of 7.5% Na_2CO_3 solution was added, and the mixture was homogenized, and incubated for 2 h at 20 °C. The absorbance was measured at λ_{max} = 765 nm. The samples were evaluated in triplicate for each determination, and the mean absorbance data were obtained. A standard curve was prepared using gallic acid (0.01–0.06 mg/mL). The results are expressed as mg of gallic acid equivalents per milliliter of sample (mg GA/mL of sample) [8].

2.2.4. Cytotoxic Activity Assay

The cytotoxic activity of CEO and its MEs was assessed against different cancer cell lines, including two colorectal adenocarcinoma cell lines (Caco-2 and HT-29), one human cervical adenocarcinoma cell line (HeLa), one human prostatic adenocarcinoma cell line (PC-3), and one human lung adenocarcinoma cell line (A549). A human embryonic kidney cell line (HEK-293) was used as a non-cancerous control (ATCC, Rockville, MD, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and a 1% penicillin–streptomycin–neomycin antibiotic mixture. All the cell lines were incubated under standard conditions (5% CO_2 and 95% air at 37 °C) (Heracell™ VIOS 160i CO_2, Thermo Fisher Scientific Inc., Waltham, MA, USA) [7]. Cytotoxicity assays were performed using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method. Approximately 1×10^4 cells/well were seeded in 200 µL of DMEM in 96-well plates and incubated for 24 h under standard conditions (5% CO_2 and 37 °C). The medium was replaced with 100 µL of the treatments (0–400 µg/mL), and the cells were incubated again. The treatment concentrations were standardized based on the CEO content within the MEs. CEO was dissolved in DMSO and treated with water. Untreated cells were used as negative controls [8]. After incubation, 20 µL of MTT solution (0.5 mg/mL in PBS) was added to each well and incubated for 4 h at 37 °C. The MTT dissolved in the medium was then carefully removed, and the insoluble formazan precipitates at the bottom of the wells were solubilized by addition of 100 µL of DMSO. The

absorbance was measured at 570 nm (reference wavelength at 690 nm) using a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Cell viability is expressed as a percentage with untreated cells representing 100% viability, according to the following equation:

$$\text{Cell viability}(\%) = \frac{\text{Sample}_{\text{abs}}}{\text{Control}_{\text{abs}}} \times 100\% \quad (2)$$

The median inhibitory concentration (IC_{50}) of each treatment was determined in accordance with the dose–response curve. IC_{50} was calculated with a non-linear fit log(inhibitor) vs. normalized response–variable slope in GraphPad Prism 5.01 software for Windows (GraphPad Software, Inc., San Diego, CA, USA).

2.3. Statistical Analysis

All assays were performed in triplicate, and the data are expressed as the mean ± standard deviation. One-way analysis of variance (ANOVA) was performed using Statgraphics Centurion XV 15.2.06 statistical software (StatPoint Technologies, Inc., Warrenton, VA, USA). Significant differences among treatments were identified using Tukey's test, with a significance level set at ($p < 0.05$).

3. Results and Discussion

3.1. Phase Diagrams

The triangular phase diagrams obtained at different S_{mix} ratios by the titration method from mixing CEO, water, and S_{mix} are shown in Figure 1. The formation of different structures was influenced by the change in oil solubility and its affinity for the surfactant and the S_{mix}. The phase diagrams show the formation of biphasic, emulsion, and ME regions, with the ME area increasing as S_{mix} decreases. A larger ME area (%) was observed for S_{mix} 1:1 (34.6%), whereas the other S_{mix} showed similar ME areas (approximately 25%). These results show that compared with the other formulations, the S_{mix} 1:1 formulation was more effective for the formation of MEs. Different authors have reported that the use of a cosurfactant decreases surface tension and aqueous phase polarity, improving the poor fluidity of the oil–water interface film generated when only one emulsifier is used, facilitating the formation of MEs [14,21,25].

The ME area depends strongly on the type and concentration of the cosurfactant used. For example, Kiromah et al. [22] reported that S_{mix} (1:2, 1:1, and 2:1) strongly impacted interfacial tension reduction, an important parameter to produce CEO-MEs stabilized with polysorbate-80 and polyethylene glycol 400. They reported that if the amount of cosurfactant increased beyond that of the surfactant, the ME area would have decreased; as a result, the emulsification process was less efficient. Gandhi et al. [26] reported that the largest ME area was obtained with a polysorbate 80: isopropyl alcohol S_{mix} ratio of 3:1.

Table 1 shows the average droplet size distribution of the initial formed CEO-MEs as well as after storage for 35 days at 37 °C. At the beginning of the experiment, the average droplet sizes of the CEO-MEs were less than 15.3 ± 0.2 nm, with PDIs less than 0.21 ± 0.02, indicating that they were monodisperse. A small and significant change in the average droplet size was observed between samples with different S_{mix} values. For example, Lam et al. [27] reported that the average sizes and PDIs of CEO-MEs were lower when a 3:1 S_{mix} (Z_{ave} = 36.3 nm and PDI = 0.17) was used than those observed when a 2:1 S_{mix} (Z_{ave} = 44.3 nm and PDI = 0.49) was used. The PDI values describes the variation in the droplet size distribution in a DLS measurement. Typically, PDI values below 0.2 indicate a monodisperse droplet size distribution, but the small fluctuation in the PDI values over time is also related to the stability of colloidal systems, i.e., the lower the PDI,

the higher the stability of the ME. Gandhi et al. [26] reported that for CEO-MEs produced with polysorbate 80: isopropyl alcohol at S_{mix} of 3:1, the increase in concentration S_{mix} and the decrease in water content reduce the Z_{ave} size of globules.

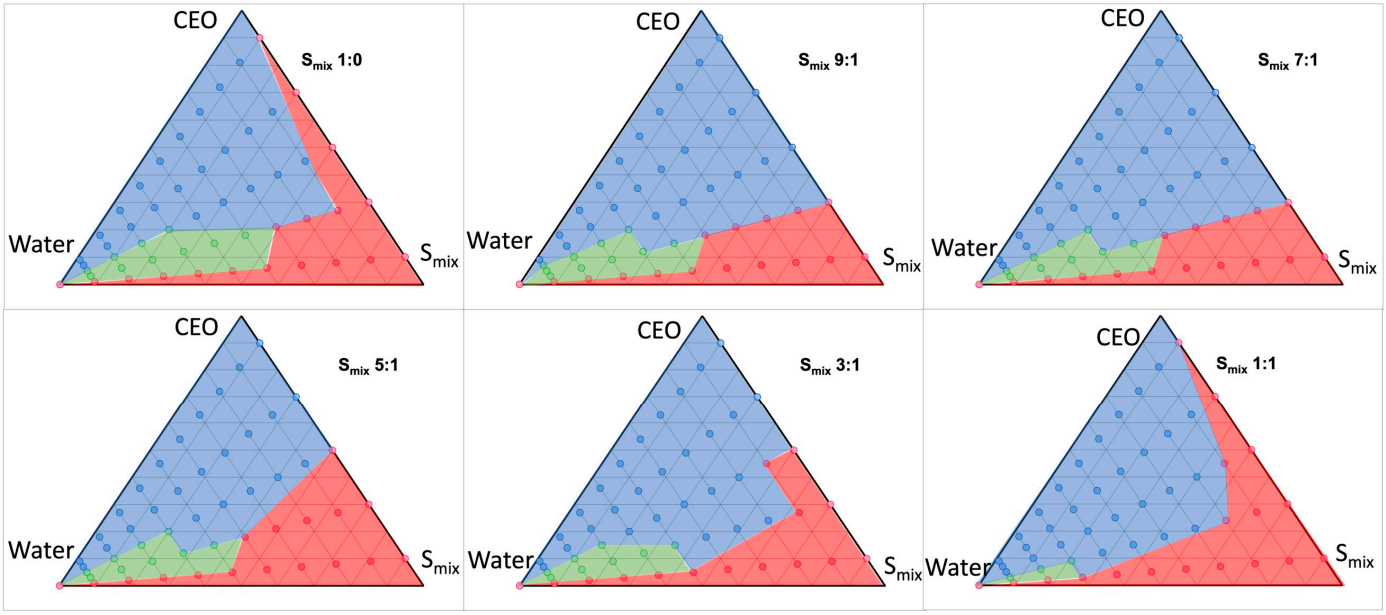


Figure 1. Pseudo-ternary phase diagrams constructed using clove essential oil, a surfactant–cosurfactant mixture (S_{mix}), and water. Illustrations show the biphasic (blue), emulsion (green), and microemulsion (red) regions.

Table 1. Average particle size and PDI of the clove essential oil MEs.

S_{mix}	Initial		Stored for 35 Days at 37 °C	
	Z-Ave (nm)	PDI	Z-Ave (nm)	PDI
1:0	10.2 ± 0.1^{aA}	0.08 ± 0.02^{aA}	12.2 ± 0.1^{aB}	0.20 ± 0.00^{bcB}
9:1	10.0 ± 0.0^{aA}	0.07 ± 0.01^{aA}	12.3 ± 0.1^{aB}	0.21 ± 0.00^{cB}
7:1	10.9 ± 0.1^{bA}	0.09 ± 0.02^{aA}	13.7 ± 0.1^{bB}	0.19 ± 0.00^{bB}
5:1	9.9 ± 0.1^{aA}	0.08 ± 0.01^{aA}	13.2 ± 0.1^{cB}	0.19 ± 0.01^{bB}
3:1	11.4 ± 0.1^{cA}	0.09 ± 0.01^{aA}	18.9 ± 0.1^{dB}	0.19 ± 0.00^{bB}
1:1	15.3 ± 0.2^{dA}	0.21 ± 0.02^{bA}	25.6 ± 0.3^{eB}	0.25 ± 0.00^{aB}

S_{mix}: polysorbate 80:ethanol; different lowercase letters indicate differences among treatments at the same time and different uppercase letters indicate differences among storage times within the same treatment ($p < 0.05$).

Díaz–Zepeda et al. [13] reported MEs of 11–18 nm and 18–21 nm prepared with polysorbate 20 and polysorbate 80/mono-propylene glycol, respectively; as the ratio of S_{mix} increased, more oil phase was incorporated, allowing easy dispersion and construction of a flexible film with the core of the oil droplets. Manzoor et al. [11] reported the formation of MEs with different essential oils, including lavender, basil and clove essential oils using polysorbate 80 and ethylene glycol ethyl ether at S_{mix} = 1:1. The authors reported that the MEs of the different essential oils had Z_{ave} values lower than 17 nm.

All the MEs significantly increased in average particle size after storage at 37 °C for 35 days (Table 1), with more pronounced changes observed in the systems prepared with S_{mix} ratios of 1:1 and 3:1. However, these variations did not result in visible phase separation or loss of the translucent appearance of the samples, suggesting that the nanoformulations preserved their stability under the evaluated conditions. Nevertheless, these results should be interpreted as evidence of short-term stability at 37 °C. Since the stability of MEs may be influenced by temperature, additional studies at room temperature, under refrigerated

conditions, and under accelerated stability testing are needed to determine their shelf-storage stability.

3.2. Cytotoxicity of the Surfactant–Cosurfactant Mixture Ratio (Smix)

Non-ionic surfactants are considered non-toxic and low-irritating materials. However, recent reports have shown that surfactants such as polysorbate 20, polysorbate 80, or their components cause cellular lysis through the dissolution of the cellular membrane [21,28,29]. Therefore, the appropriate S_{mix} to produce the MEs should be selected to reduce the potential cytotoxic effect. The effects of S_{mix} (polysorbate 80:ethanol mixing ratio essential oil free) on the viability of HT-29 cancer cells are shown in Figure 2. An increase in the ethanol concentration in the S_{mix} reduces the cytotoxicity in cells because of a relative reduction in polysorbate 80. Polysorbate 80 alone (corresponding to S_{mix} 1:0) had the greatest cytotoxic effect at concentrations above 500 µg/mL. The S_{mix} 9:1, 7:1, and 5:1 had cytotoxic effects at concentrations greater than 1500 µg/mL, whereas S_{mix} 3:1 and 1:1 and ethanol did not have cytotoxic effects at concentrations of 3500 µg/mL.

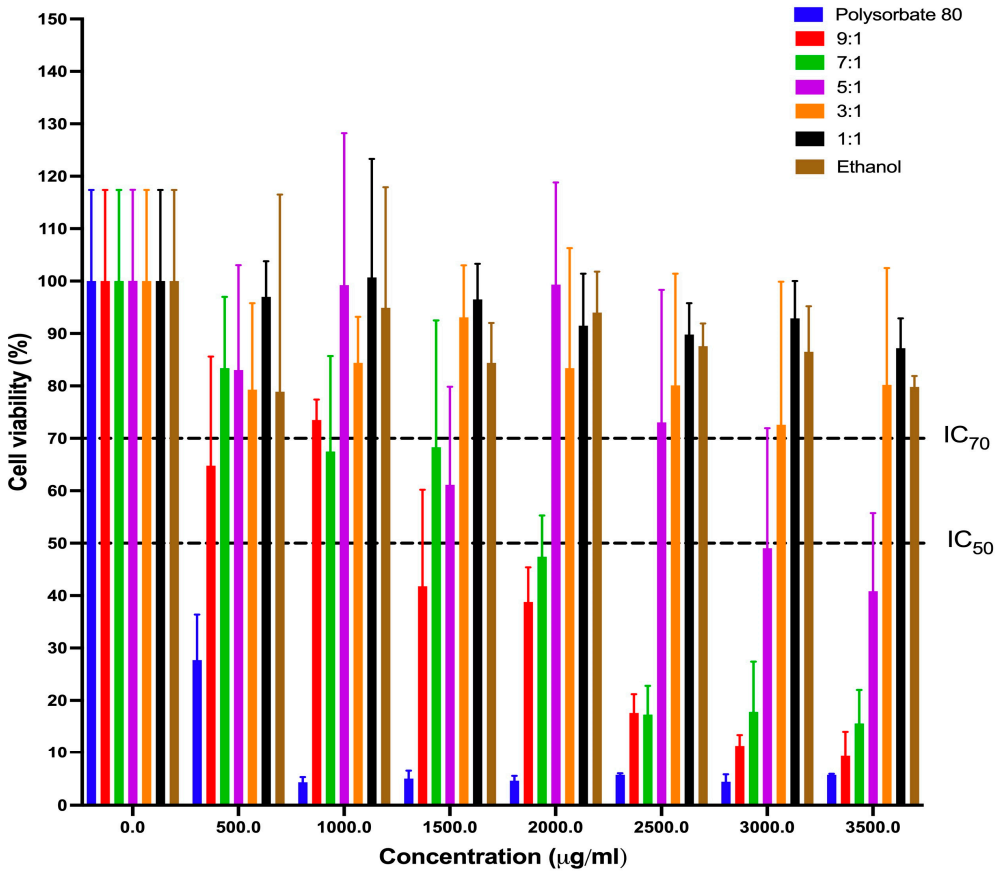


Figure 2. Effect of S_{mix} (polysorbate 80:ethanol) on the viability of HT-29 cells, as determined by MTT assays.

These results were consistent with those reported by Sun et al. [29], who reported an IC_{50} between 55 and 150 µg/mL for polysorbate 80 or its constituents. Surfactants damage the cell membrane and mitochondrial enzymes in a concentration-dependent manner. The effect of polysorbates on membrane integrity results in the solubilization of membrane components. Therefore, their use has a potential risk, and the optimal ratio of pharmaceutical excipient components and their toxicity need to be further investigated [28,29]. Based on the idea of reducing the surfactant concentration, S_{mix} = 1:1 polysorbate 80-ethanol was selected for the following sections.

3.3. Antioxidant Activity

Phenolic compounds found in essential oils are recognized for their ability to provide hydrogen to free radicals, which is strongly associated with their antioxidant characteristics. Table 2 presents the TPC and antioxidant activity of CEO and CEO-ME.

Table 2. Antioxidant activity of selected MEs.

	DPPH (IC_{50} μg CEO/mL)	**TEAC (mg of Trolox/mL)**	**TPC (mg of GA/mL)**
CEO	0.82 ± 0.05 [a]	88.44 ± 5.03 [a]	542.25 ± 26.89 [a]
CEO-ME	0.09 ± 0.05 [b]	1027 ± 586 [b]	547.33 ± 1.61 [a]

DPPH (IC_{50})—concentration of clove essential oil required to inhibit 50% of DPPH radicals; TEAC—Trolox equivalent antioxidant capacity; TPC—total phenolic content. The values are presented as the mean ± standard deviation. Within each column, means followed by different lowercase letters indicate significant differences among treatments ($p < 0.05$), whereas means sharing at least one lowercase letter is not significantly different.

The antioxidant capacity of the CEO was assessed by determining its capacity to donate hydrogen or eliminate radicals, leading to the transformation of DPPH• into the non-radical DPPH-H. The CEO showed an IC_{50} value of 0.82 μg/mL, which was nine times greater than that observed for the CEO-ME (containing 0.09 μg CEO/mL). Comparatively, the Trolox standard IC_{50} value was 72.56 μg/mL. However, despite similar concentrations of TPCs, the increase in antioxidant capacity persists. Manzoor et al. [11] reported an increase in the antioxidant activity of CEOs encapsulated in MEs, which was mainly attributed to greater surface area and reduced particle size. Several factors can influence the antioxidant capacity of emulsions, such as the size, type, and concentration of the oil phase and surfactant, as well as the pH, ionic composition, interfacial properties, location, and polarity of antioxidants within the oil phase. Various studies have reported that there is enhanced protection against antioxidants in emulsions with a high surfactant content [11,30–32]. These findings were also documented by Kiralan et al. [33], who studied the effect of surfactant concentration (polysorbate 20 at 0.1%, 0.5%, and 1%) on antioxidant activity, attributing it to the increased solubility of the oil phase with higher surfactant concentrations. Antioxidant compounds are more effective when located at emulsion droplet interfaces; this aligns with results from Villeneuve et al. [31], who noted that higher surfactant concentrations increase the interface density, allowing antioxidants to position themselves within it, thereby making free radical penetration more difficult and significantly enhancing antioxidant protection. The antioxidant effect is influenced not only by the structure and concentration of surfactants but also by their synergistic interactions [30,31,33]. However, this behavior should be interpreted in relation to the specific composition of the MEs system evaluated in this study, since it may not be applicable to all non-ionic surfactants regardless of their chemical structure. In addition, both the CEO components and the surfactant phase may be susceptible to oxidative changes, which could influence the overall antioxidant response of the formulation.

3.4. Cytotoxic Activity

Unlike the previous assay (Section 3.2), which sought to ensure the biocompatibility of the S_{mix}, the cytotoxic activity of the MEs on five cancer cell lines was evaluated. The antiproliferative effects of CEO-ME and CEO were evaluated with MTT assays against two colorectal adenocarcinoma cell lines (Caco-2 and HT-29), one human cervical adenocarcinoma cell line (HeLa), one human prostatic adenocarcinoma cell line (PC-3), and one human lung adenocarcinoma cell line (A549). The human embryonic kidney cell line (HEK-293) was used as a non-cancerous control cell line. The results are shown in Figure 3. CEO-MEs showed cytotoxic activity on all the evaluated cell lines.

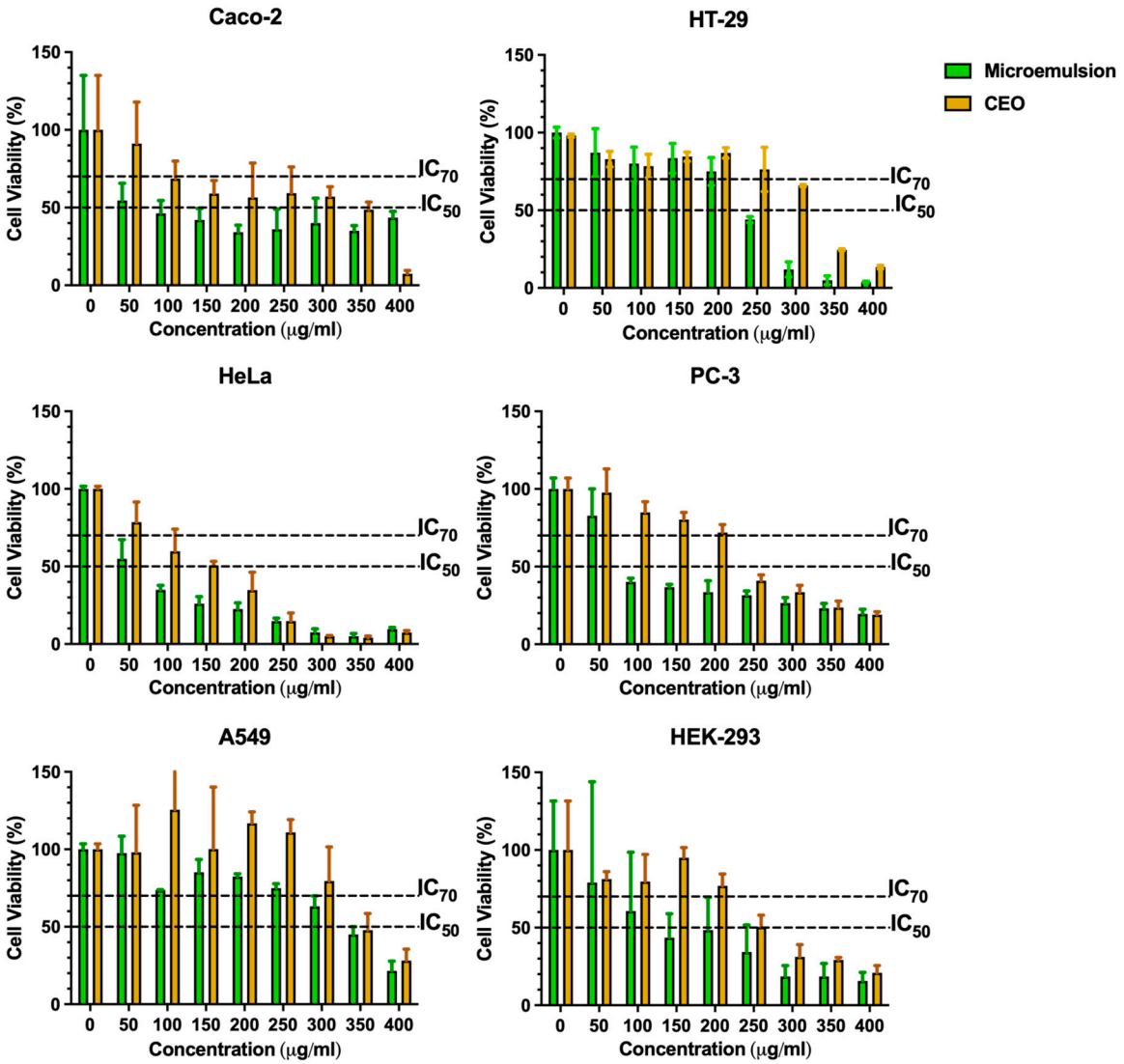


Figure 3. Effect of treatment on cell viability after 24 h of incubation, as determined by MTT assays. The green bars represent CEO-MEs while the yellow bars represent free CEO.

Compared with free clove essential oil, the CEO-MEs showed greater cytotoxic activity for all the cell lines, showing cytotoxic effects below 50, 50, 100, 100, 250, and 300 μg/mL for the Caco-2, HeLa, HEK-293, PC-3, HT-29, and A549 cell lines, respectively.

Table 3 shows the IC_{50} values for all the cell lines obtained for CEO-MEs and clove essential oil. The results from the MTT assay revealed a reduction in cellular viability upon treatment with MEs, as opposed to those treated with pure oil. The chemotherapeutic properties of CEO are ascribed to its copious phytochemical constituents, encompassing an array of mono- and sesquiterpene compounds, with eugenol being the predominant component. Various studies have corroborated the potent, dose-proportional cytotoxic effects of CEO across diverse human carcinoma cell lines, such as hepatic (HepG2), mammary (MCF-7), prostate (PC3), colonic (HCT116), and pulmonary (A549) cell lines [7,8].

CEO mechanisms against cancer cells include apoptosis promotion, inhibition of metastasis, and suppression of proteins involved in inflammation processes. The CEO promotes apoptosis in cancer cells through alterations in mitochondrial activity, preventing them from generating energy correctly during respiration. Munteanu et al. [34] reported that CEO and eugenol inhibited the active respiration of the HT-29, A341, and NCI-H460 cancer cell lines, which resulted in their death. Likewise, cancer cells need ROS for homeostasis, but an excess of ROS can negatively impact their development. The CEO and

specifically eugenol act as antioxidant and prooxidant components against cancer cell lines. This mechanism has been demonstrated through the death evaluation of several cancer cell lines, such as HCT-15, HT-29, A431, RPMI-7951, and NCI-H460, confirming the dual effect of this essential oil on ROS modulation [16,35].

Table 3. IC_{50} (μg/mL) values of clove essential oil and MEs against different types of human cell lines.

	CEO-MEs	CEO
	IC_{50} (μg/mL)	
Caco-2	23.0 ± 6.5 [b]	233.9 ± 67.6 [a]
HT-29	226.5 ± 14.9 [c]	306.7 ± 11.8 [b]
HeLa	59.6 ± 16.6 [c]	124.4 ± 14.4 [b]
PC-3	107.1 ± 27.2 [c]	239.5 ± 12.6 [b]
A549	324.1 ± 10.6 [b]	351.1 ± 21.6 [b]
HEK-293	43.6 ± 29.0 [b]	255.6 ± 26.1 [a]

The values are presented as the mean ± standard deviation. Within each column, means followed by different lowercase letters indicate significant differences among treatments ($p < 0.05$), whereas means sharing at least one lowercase letter are not significantly different.

CEO inhibition of metastasis is carried out through the downregulation of the expression of genes responsible to produce metalloproteinases. These proteins are used by cancer cells to degrade the extracellular matrix, which generally limits cancer cell migration or spread, as they act as physical and chemical barriers [36]. Baharara et al. [37] reported that eugenol downregulated the mRNA expression of matrix metalloproteinase-9 (*MMP-9*) and paxillin in MCF-7 cancer cells. Both proteins are essential for metastasis, demonstrating the previously mentioned effect. Similarly, Abdullah et al. [38] reported that eugenol reduced the expression of the *MMP-2* and *MMP-9* genes in MDA-MB-231 and SK-BR-3 breast cancer cell lines. As all these proteins (*MMP-2*, *MMP-9*, and paxillin) are essential for metastasis, the effects of CEO, but mainly eugenol, on the metastasis of different cancer cell lines can be widely demonstrated.

The antioxidant activity of CEO is the main mechanism through which it prevents inflammation processes. However, some studies have shown the specific activity of eugenol (CEO main component) against pro-inflammatory cytokines. Wang et al. [39] reported that eugenol inhibited, in fibroblast-like synovial cells, the effect of tumor necrosis factor alpha (TNF-α) on prostaglandin–endoperoxide synthase 2 (COX-2) expression. COX-2 is a protein involved in inflammatory processes, whose expression can be regulated by TNF-α. Furthermore, eugenol reduced the expression of proteins associated with inflammatory processes generated by NF-κB.

CEO-ME formation could have improved cell uptake, increased the surface area, solubility, and stability of clove essential oil, and caused a decrease in the IC_{50} compared with those of its free form [7,8,17,18,24]. In the Caco-2 cancer cell line, CEO-MEs had the greatest cytotoxic activity. The cellular sensitivity to CEO-ME treatments was as follows: Caco-2 > HEK-293 > HeLa > PC3 > HT-29 > A549. For the CEOs, the order was as follows: HeLa > Caco-2 > PC3 > HEK-293 > HT-29 > A549. Although cytotoxic effects were evident in the evaluated cell lines, the results should be interpreted with caution, as the non-cancerous HEK-293 cell line was also sensitive to the treatments. In a scenario of potential application of CEO-MEs, Caco-2 cells would be a suitable target given their sensitivity. However, for the other cancer cell lines, the efficacy and safety of the treatments could be compromised by their effects on healthy cells, overcoming the detrimental effects on the benefit achieved. Nirmala et al. [24] reported a CEO-ME IC_{50} value of 0.7 μL/mL for the HTh-7 thyroid cancer cell line, using a S_{mix} 1:4 polysorbate 20- polysorbate 80. However, no cytotoxic effects were detected in the HEK-293 cell line. These results also indicate that the cytotoxic

activity of CEO-MEs is based on late-stage apoptosis and early necrosis. MEs increase the solubility, penetration, absorption, bioavailability, and protection against drug oxidation and hydrolysis, facilitating the induction of cell death [7,8,17,24]. The cytotoxic activity of clove essential oil has been reported to be caused by its antioxidant, antimetastatic, apoptotic, or necrotic effects, as well as its ability to induce cell cycle arrest [15–17,19,24].

4. Conclusions

The clove essential oil microemulsion was obtained through the development of a pseudo-ternary phase diagram. The results indicated that the use of ethanol as a cosurfactant enables the generation of nanometric-sized droplets. Proper cosurfactant selection, along with the mixing ratio between the surfactant and cosurfactant, facilitates the adequate solubilization of clove essential oil, as well as the generation of a thermodynamically stable microemulsion. Additionally, the inclusion of a cosurfactant may decrease the potential cytotoxic characteristics of non-ionic surfactants. The microemulsion was able to maintain the phenolic content while increasing the antioxidant capacity compared to the free essential oil. The anticancer cytotoxicity of the microemulsions varied depending on the evaluated cell line, with the Caco-2 and HeLa lines exhibiting greater sensitivity than the PC3, HT29, and A549 lines did. These findings provide valuable insights for developing easily formulated clove essential oil microemulsions for functional food applications.

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Abbreviations

The following abbreviations are used in this manuscript:

CEO	Clove essential oil
EO	Essential oil
S_{mix}	surfactant:cosurfactant mixture
ME	Microemulsion
CEO-ME	Clove essential oil microemulsion
DMSO	Dimethyl sulfoxide
MTT	3–(4,5–dimethyl–2–thiazolyl)–2,5–diphenyl–2H–tetrazolium bromide
DMEM	Dulbecco's Modified Eagle's Medium
TPC	Total phenolic content

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