Electronic Journal of Biotechnology 68 (2024) 20–30

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

journal homepage: www.elsevier.com/locate/ejbt

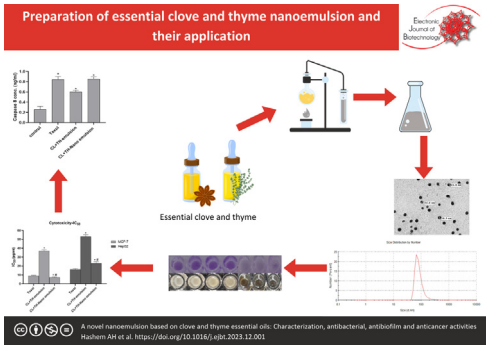
Research article

A novel nanoemulsion based on clove and thyme essential oils: Characterization, antibacterial, antibiofilm and anticancer activities ☆

Amr H. Hashem [a], Ahmed S. Doghish [b,c,*], Ahmed Ismail [c], Mahmoud M.H. Hassanin [d], Mohammad K. Okla [e], Ibrahim A. Saleh [f], Hamada AbdElgawad [g], Amr M. Shehabeldine [a,*]

[a] Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Nasr City, Cairo 11884, Egypt
[b] Department of Biochemistry, Faculty of Pharmacy, Badr University in Cairo, Badr, Cairo 11829, Egypt
[c] Biochemistry and Molecular Biology Department, Faculty of Pharmacy (Boys), Al-Azhar University, Nasr City, Cairo 11231, Egypt
[d] Ornamental, Medicinal and Aromatic Plant Disease Department, Plant Pathology Research Institute, Agricultural Research Center, Giza 12619, Egypt
[e] Botany and Microbiology Department, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia
[f] Faculty of Science, Zarqa University, Zarqa 13110, Jordan
[g] Integrated Molecular Plant Physiology Research, Department of Biology, University of Antwerp, 2020 Antwerp, Belgium

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 24 September 2023
Accepted 13 December 2023
Available online 3 January 2024

Keywords:
Antibacterial activity
Antibiofilm activity
Anticancer activity
Biofilm inhibition
Biomedical applications
Clove oil
DLS (Dynamic Light Scattering)
Essential oils
Nanoemulsion

ABSTRACT

Background: Essential oil nanoemulsions have received much attention in the last period due to their ability to fight microbes and cancers. In the current study, clove and thyme essential oils CL+TH-emulsion and CL+TH-nanoemulsion were prepared through an eco-friendly method. The prepared CL+TH-nanoemulsion was characterized using DLS and TEM analyses.

Results: Results revealed that CL+TH-nanoemulsion droplets were spherical in shape and nanoform in size (68.6 nm) with PDI 0.281. MIC concentrations of CL+TH-nanoemulsion against tested bacteria were found to be between 6.25 and 25 mg/mL. After being exposed to MICs of CL+TH-emulsion and CL+TH-nanoemulsion, which additionally prompted 1.43 log and 3.12 log declines, accordingly, as opposed to untreated (Control), the number of cells grown in the generated biofilms decreased. Furthermore, CL+TH-nanoemulsion exhibited anticancer activity more than CL+TH-emulsion toward HepG2 and MCF-7. Also, the effect of CL+TH-nanoemulsion is more effective and significantly cytotoxic than taxol on MCF-7. Besides, both prepared emulsions increased the rate of apoptosis and decreased the cell viability % of MCF-7 by increasing the activity of caspases 8 and 9. Moreover, CL+TH-nano emulsion decreased the activity of VEGFR-2 in MCF-7 in a more pronounced manner than CL+TH-emulsion and taxol.

☆ Audio abstract available in Supplementary material.

Peer review under responsibility of Pontificia Universidad Católica de Valparaíso

* Corresponding authors.
E-mail addresses: ahmed_doghish@azhar.edu.eg (A.S. Doghish), dramrshehab@azhar.edu.eg (A.M. Shehabeldine).

https://doi.org/10.1016/j.ejbt.2023.12.001

TEM (Transmission Electron Microscopy)
Thyme oil

Conclusions: The prepared CL+TH-nanoemulsion had antibacterial, and antibiofilm as well as anticancer properties, which can be used in different biomedical applications after extensive studies *in vivo*.
How to cite: Hashem AH, Doghish AS, Ismail A, et al. A novel nanoemulsion based on clove and thyme essential oils: Characterization, antibacterial, antibiofilm and anticancer activities. Electron J Biotechnol 2024;68. https://doi.org/10.1016/j.ejbt.2023.12.001.

1. Introduction

Antimicrobial resistance has grown and evolved as a result of widespread antibiotic use [1]. Additionally, when microbes constitute biofilms, their susceptibility to antimicrobial drugs rises. As the most common type of living existence, biofilm is an arrangement made up of surface-attached pathogens that are made visible through the exterior polymer scaffold [2,3]. Arguably the biggest health problem of the twenty-first century is the rise of antimicrobial resistance (AMR), which poses an obstacle to the efficient fight against and cure of an expanding array of illnesses brought on by microbes, viruses, fungi, and parasites that are resistant to the popular drugs used for managing them. It typically develops in water-based solutions where multiple microorganisms and solid objects are present. A major advantage of such biofilms being formed for microorganisms is a rise in antimicrobial medication rebellion when compared with the occasional position [4]. Following cardiovascular and infectious diseases as the major causes of death worldwide is cancer. Due to its high incidence rate, cancer is the second primary cause of death worldwide and has become a global health concern in the twenty-first century. Annually, approximately 15 million death cases are due to the persistence of malignant cells, and the number of cases is steadily rising [5]. Currently, there are many chemotherapeutic drugs for treating cancers that cause serious side effects on most human organs [6]. Therefore, it is crucial to prepare new chemotherapeutic agents that are safer and more effective.

Natural products like plant-derived compounds have a long history of usage as chemotherapeutic agents and overcome resistances [7,8,9]. Essential oils are among the most significant organic ingredients because of their biological nature and medicinal qualities, making the application of renewable substances in the manufacturing process of pharmacologic items extremely interesting [10]. Eugenol, a component of clove essential oil that is derived from *Eugenia caryophyllata* stem, leaves, and buds and may possess antimicrobial potential, is present in the oil. However, it is not frequently used as an oil in water nanoemulsion [11]. Thymol is an aromatic component, and these active compounds demonstrated significant antibacterial and antifungal activity. They are effective against a wide range of bacteria, including medically relevant diseases [12]. The most volatile component of the thyme essential oil is thymol [13]. Thymol is generally believed to be safe, and it has been used as an antibacterial and antioxidant compound [14]. However, thymol drawbacks, which restrict its utilization, include volatility, poor stability, and high hydrophobicity [15]. In contrast to regular fluids, protein-stabilized miniature emulsion demonstrated small particle droplets with improved stability and biocompatibility. Due to toxicological concerns regarding long-term use, nanoemulsion stabilizing requires a significant amount of a surfactant or emulsifying, which prevents its use in medical applications [16]. Herein, this study aimed to prepare a nanoemulsion based on both clove and thyme essential oils, and characterize this nanoemulsion using DLS and TEM analyses also, to evaluate its antimicrobial and antibiofilm nature against multidrug-resistant bacteria. Finally, to explore its anticancer activity via evaluating its effect on apoptosis-related markers like caspase-8 and caspase-9 and assessing its effect on the angiogenic factor receptor the vascular endothelial growth factor receptor 2 (VEGFR-2) using HepG2 and MCF-7 cancer cell lines.

2. Materials and methods

2.1. Materials

The taxol (paclitaxel) was obtained from Sigma Chemical in St. Louis, Missouri. The 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2 H-tetrazolium bromide (MTT) and the dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (Sigma, USA). The chemical and reagents required for cell lines including fetal bovine serum (FBS), phosphate buffer saline (PBS), Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin (Pen/Strep) solution, and trypsin-EDTA were obtained From Gibco (Gibco, TFS Inc., USA). The clove plant was obtained from the medicinal and aromatic plants department, Horticulture Research Institute, Agricultural Research Center, Giza, Egypt. The thyme plant was purchased from a local market of aromatic plants, Giza, Egypt.

2.2. Methods

2.2.1. Preparation of nanoemulsion

Clove and thyme essential oils were extracted using the steam distillation method [17]. Dried and ground clove flower buds or thyme leaves (50 g) were put in a steam flask individually. The steam distillation lasted 6 h. The recovered condensate was distilled again using n-hexane as the solvent. To prepare CL+TH-nanoemulsion, 75 ml sterilized distilled water + 10 ml clove essential oil + 10 ml thyme essential oil and 5 ml of non-ionic surfactant Tween 80 were added slowly with gentle stirring until a homogeneous mixture formed. Subsequently, the mixture was agitated utilizing a homogenizer for 10 min. The combination underwent sonication using an Ultrasonicator (BANDELIN SONOPULS HD 2200, Germany) for 30 min at a power level of 350 W. The particle size of a nanoemulsion containing 10% essential oil was measured using a Hydrodynamic light scattering analyzer (DLS) after a 90-d storage period at room temperature (27°C) to check the stability. The essential oil emulsion was prepared as previously described, without the use of sonication [18].

2.2.2. Dynamic light scattering analysis (DLS)

Measurement of droplet size of a mixture of clove and thyme essential oil nanoemulsion was performed by DLS. This analysis was carried out according to the method used by Ghotbi et al. [19].

2.2.3. Transmission electron microscopy (TEM)

A volume of twenty microliters of the diluted blend of clove and thyme essential oil nanoemulsion was carefully deposited onto a film-coated copper specimen grid with a mesh size of 200 for 10 min. Subsequently, any excess fluid was removed using filter paper. The grid was subsequently subjected to staining using a single droplet of 3% phosphotungstic acid, followed by a drying period

of 3 min. The grid that had been coated was subjected to a drying process and subsequently analyzed using a transmission electron microscope (TEM) model Tecnai G20, Super twin, double tilt, manufactured by FEI in The Netherlands. The microscope was operated at an acceleration voltage of 200 kV [19].

2.2.4. Antibacterial activity

Using the disc diffusion approach, the antibacterial activity of clove CL+TH-emulsion and CL+TH-nanoemulsion was assessed [20]. *Bacillus cereus* ATTC 11778, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 35218, and *Klebsiella oxytoca* ATCC 51983 were employed for antibacterial screening. A total of 100 µL of every strain was applied to Mueller-Hinton agar (MHA) plates at a McFarland turbidity of 0.5. Both CL+TH-emulsion and CL+TH-nanoemulsion in 50 µL portions were aseptically put onto sterilized 6-mm filter sheets and placed in the center of the plates. The plates were then incubated at 37°C for 24 h. After the incubation, the radius of the inhibitory zone was measured using a measurement device [20]. The low detrimental levels of CL+TH-emulsion and CL+TH-nanoemulsion were established using the micro broth dilution technique. Different concentrations of CL +TH-emulsion and CL+TH-nanoemulsion (100, 50, 25, 12.5, 6.25, 3.125, 1.56, and 0.781 mg/mL) were incubated on plates overnight at 37°C. After 24 h of incubation, the difference in optical densities of each sample was compared, and MIC values were computed. The minimum bactericidal concentration (MBC) was also determined by sub-culturing investigated organisms, particularly those with wells that followed those MIC levels, onto plates with MHA at 37°C for 24 h. Then, it was found that MBC is the nano-formulated form that, when used at the lowest dose possible, prevents the growth of dangerous bacteria [21].

2.2.5. Evaluation of antibiofilm activity

Static biofilm models were used to conduct biofilm studies. Using the crystal violet staining method, the effect of the tested material CL+TH-nanoemulsion on the development of biofilms in *S. aureus* ATCC® 25923TM was evaluated [22]. The prepared substance was diluted onto 96-well plates as previously stated; six concentrations were diluted starting at 0.5xMIC. The plates were incubated for 48 h at 37°C under aerobic conditions. The wells were rinsed twice with sterile water before being stained for 15 min with 0.1 mL of 0.4% crystal violet after discarding the liquid combination. The dye that was associated with the biofilm was then solubilized by adding ethanol (95%), after which samples were washed twice with distilled water. At 540 nm, the separated dye absorbance was quantitatively quantified. The percentage of biofilm inhibition was estimated using **[Equation 1]** [23]:

$$\frac{OD\,growth\,control\ -\ OD\,sample}{OD\,growth\,control} \times 100 \quad (1)$$

where *OD*: optical density.

2.2.6. Anticancer activity

2.2.6.1. Cell lines. Both MCF-7 (breast cancer cell line) and HepG2 (liver cancer cell line) were obtained from ATCC (Manassas, USA) and grown in DMEM (Invitrogen) and supplied with 10% fetal bovine serum (FBS) and 1% pen/strep solution (Gibco, TFS Inc., USA) and incubated at 37°C with 5% carbon dioxide.

2.2.6.2. Cell viability assay. To examine the cytotoxic activity of CL +TH-emulsion and CL+TH-nanoemulsion on both cancer cell lines MCF-7 and HepG2, the MTT assay was used [8,9,24]. Briefly, in 96-well plates, the cells were planted in 96-well plates at a density of 1.2×10^4 cells/well for 24 h. After 24 h, the media were replaced with media containing CL+TH-emulsion or CL+TH-nanoemulsion at various concentrations for 48 h. After 48 h, the media were removed and wells were treated with 100 µL of MTT solution (5 mg/ml in PBS) and incubated at 37°C for 4 h [25]. The formed formazan crystals were solubilized with 100 µL of DMSO added to each well for 10 min at 37°C, and the developed violet color intensity was measured by a Microplate reader (Epoc-2 C microplate reader, Bio Tek, USA) at 570 nm and were used to calculate the IC_{50} [26].

2.2.6.3. Assessment of caspase-8 and caspase-9 activities and VEGFR-2. The activity of Caspase-8 and Caspase-9, in addition to VEGFR-2 level, were assessed by ELISA using the following kits human, EIA-4863, human, EIA-4860 (DRG International Inc., USA), and Catalogue #: OKAG02083 (AVIVA system biology, USA) respectively and according to manufacturer direction.

2.2.7. Statistical analysis

GraphPad Prism 8.0 (2019, San Diego, CA, USA) was used for data analysis. All results were represented as means ± SD. ANOVA followed by Tukey's multiple comparisons tests was used to assess the significant differences between groups, and $P < 0.05$ was considered significant.

3. Results and discussion

3.1. Characterization of CL+TH-nanoemulsion

3.1.1. DLS

The droplet size of the mixture of clove and thyme essential oils nanoemulsion was determined as shown in Fig. 1. Results illustrated that the mixture of clove and thyme essential oil nanoemulsion droplets was in nano size (68.6 nm). The polydispersity index (PDI) for particles was good (0.281). Tween 80 was employed as a surfactant due to its elevated hydrophilic-lipophilic balance (HLB) value, which promotes the formation of oil-in-water emulsions. The fast adsorption of Tween 80 onto the surface of emulsion droplets renders it more efficient in lowering droplet size compared to polymeric surfactants [27]. Dai et al. [28] conducted an evaluation on the production of nanoemulsion with reduced droplet size through the use of non-ionic surfactants containing double bonds in their nonpolar chain. Hashem et al. [18] succeeded in the preparation of CL-nanoemulsion where mean droplets were 91.3 nm and PDI was 0.448. In a study conducted by Krishnamoorthy et al. [29], a nanoemulsion of cleome viscosa oil was prepared, where results observed that the size of the droplets and their retention in the nanoemulsion at ambient temperature exhibited considerable variation. Specifically, the droplet diameters ranged from 10 to 19 nm, 23 to 24 nm, and 163 to 63 nm for oil: surfactant ratios of 1:3, 1:2, and 1:1 (v/v), respectively. Enayatifard et al. [30] demonstrated that the nanoemulsion of oregano displayed a low polydispersity index (PDI) value of 0.11, with a mean droplet size of 72.26 nm. The mean hydrodynamic diameter exhibited a proportional increase with the rise in the concentration of the added essential oil. This relationship can be attributed to the corresponding increase in the internal volume of the nanoparticle, which is occupied by the essential oil. The observed expansion in internal volume is indicative of a corresponding augmentation in the concentration of the essential oil within the particle. This phenomenon may also be ascribed to potential alterations in the organic viscosity and physicochemical characteristics resulting from the discharge of solvent into water.

3.1.2. TEM

TEM analysis was carried out for CL+TH-nanoemulsion to determine the size and form of the droplets. In the TEM images, the dro-

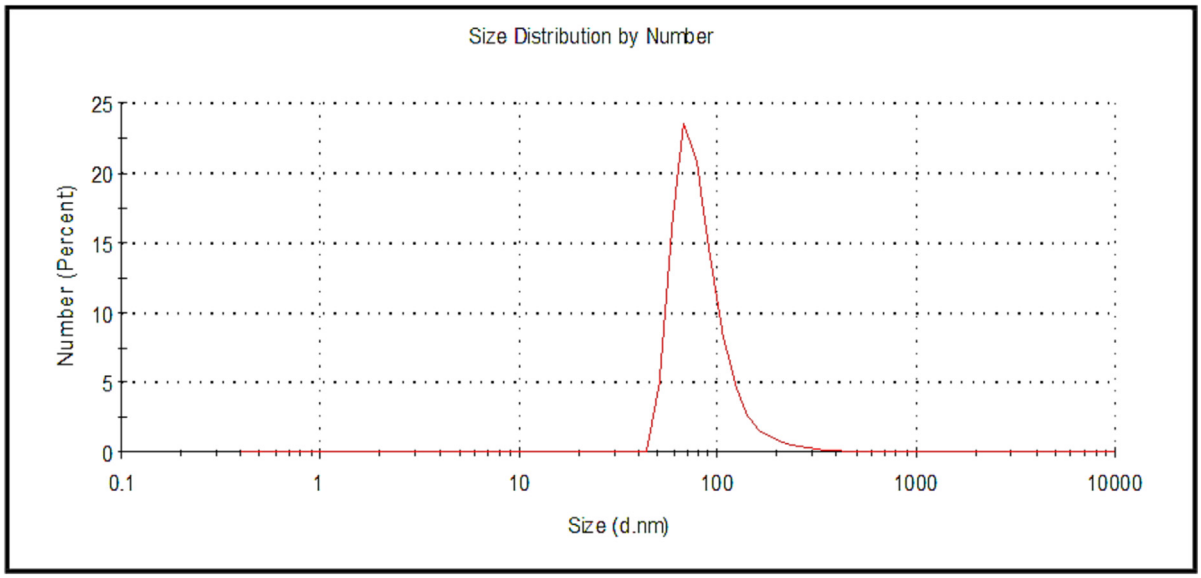


Fig. 1. DLS of CL+TH-nanoemulsion.

plets within the nanoemulsion exhibited a dark appearance. The transmission electron microscopy (TEM) micrograph revealed that the nanoemulsion of the essential oil had a spherical morphology and displayed monodispersion. The size of CL+TH-nanoemulsion droplets ranged from 25.6 to 41.0 nm, as depicted in Fig. 2. The obtained results were in agreement with Shehabeldine et al. [3] who found that TEM micrograph showed that CL-nanoemulsion droplets were approximately 225.8 nm with a spherical shape. Hassanin et al. [31] observed a reduction in the particle size of thyme essential oil nanoemulsion through the use of the ultrasonication method, resulting in an average size of approximately 34.6 nm. The decrease in droplet size can be attributed to the effectiveness and capabilities of the surfactants employed. Additionally, the act of stirring contributes to the reduction in droplet size within an oil-in-water emulsion. Hammad and Hasanin [32] observed that the nanoemulsions of Spearmint and Thyme had a spherical form, with either mono- or di-dispersed characteristics. Furthermore, the size of the Spearmint nanoemulsions ranged from 5.91 to 9.77, while the Thyme nanoemulsions ranged from 25.4 to 32.9. Abd-Elsalam and Khokhlov [33] demonstrated that

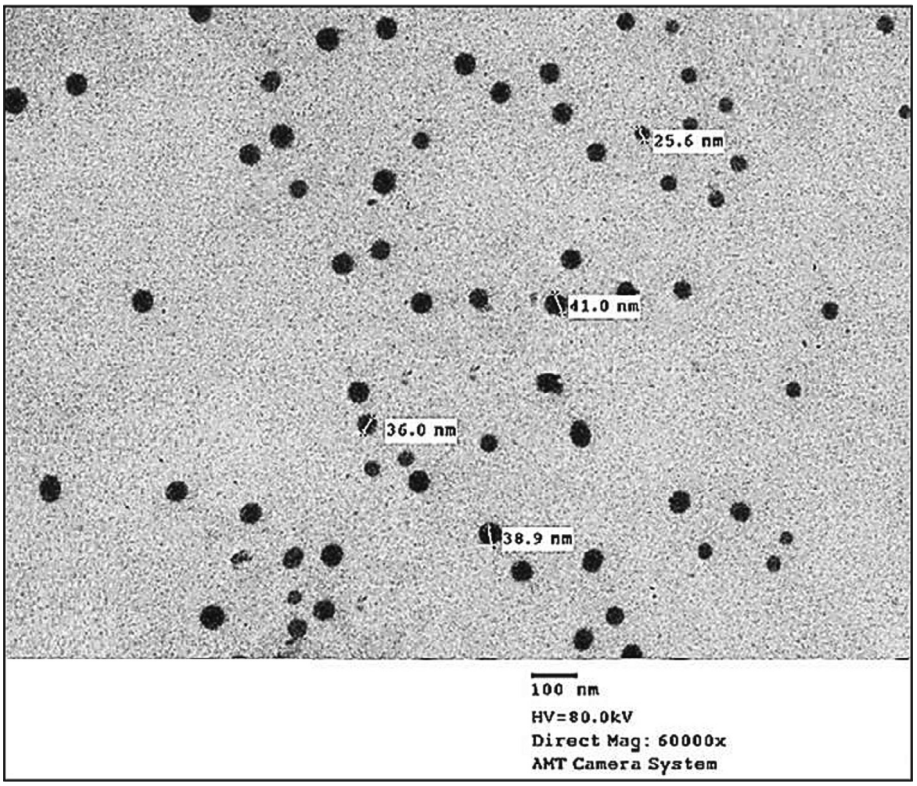


Fig. 2. Transmission electron microscopic image of a mixture of clove and thyme essential oil nanoemulsion prepared by ultrasonication method for 40 min. (size ranging from 25.6 to 41.0 nm).

the transmission electron microscopy (TEM) analysis revealed the spherical morphology of the nanoemulsion containing eugenol oil. The observed size of the nanoemulsion particles fell between the range of 50–110 nm.

3.2. The in vitro antimicrobial activity

Disc diffusion technique and micro broth dilution test were used to determine preliminary levels of CL+TH-emulsion and CL +TH-nanoemulsion against *Bacillus cereus* ATTC 11778, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 35218 and *Klebsiella oxytoca* ATCC 51983 as shown in Fig. 3. All of the species investigated were selected for their exceptional resistance to various medications. Results revealed that CL+TH-nanoemulsion suppressed *Klebsiella oxytoca*, *Escherichia coli*, *Bacillus cereus,* and *Staphylococcus aureus* with boundaries of inhibition of 23, 22, 21, and 19 mm, respectively (Table 1). The MIC concentrations of CL +TH-nanoemulsion toward *E. coli, K. oxytoca, S. aureus,* and *B. cereus* were 6.25 and 25 & 12.5 mg/L correspondingly in Table 1. CL+TH-nanoemulsion possessed lower MIC values as well as higher inhibitory activity compared to CL+TH-emulsion. This could possibly be explained by the fact that the particles have been shrunk to the nanoscale, increasing their surface area and favoring their movement through the studied pathogens' cell walls and consequent antimicrobial properties [34,35]. Additionally, nanoemulsion improves the stability of encapsulated oil by reducing the process of combustion and disintegration relative to their free equivalents, favoring their antibacterial action [35]. It ought to be emphasized that DMSO, which was utilized to dissolve free clove oil and thyme at a concentration of 2%, was not hazardous to the cells of bacteria (data not shown). The antibacterial qualities of thyme and clove oil may be connected to their ability to alter the lipid portion of bacterial walls [36,37]. The phenolic hydroxyl on the phenolic ring of thyme and clove oil increases its affinity for water and enables it to dissolve in antimicrobial membranes without causing harm to them [38]. As a result, thyme increases membrane penetration while lowering an interfacial state of equilibrium which causes interior substance loss.

3.3. Assessment of the antibiofilm activity of CL+TH-emulsion and CL +TH-nanoemulsion against S. aureus biofilms

The presence of biofilm colonies in healthcare facilities poses a threat to health. The settlement of planktonic organism bacteria eventually develops into a multispecies biofilm, which results in widespread pharmaceutical malfunctions. A common crystal violet test was used to quantify biofilm inhibition, and the findings were presented as a percentage. After 24 h, 8.11 log CFU cm^2 of adherent *S. aureus* cells were counted. Fig. 4 displays the outcomes of colonization being exposed to the CL+TH-nanoemulsion solutions and CL+TH-emulsion at 1 MIC and 2 MIC concentrations. The number of cultured cells in the produced biofilms decreased after exposure to MIC of free CL+TH-emulsion, which also caused 1.43 log decreases when compared with untreated (Control). Biofilm population decreased occurred with 3.12 log decreased after exposure to MIC of CL+TH-nanoemulsion. It has been established that the antibiofilm action of CL+TH-emulsion and CL+TH-nanoemulsion was dose-dependent ($P > 0.05$), as more dramatic decreases were observed at higher concentrations at 2MIC values. The fact that there were no distinctions among the CL+TH-emulsion at 2 MIC values and CL+TH-nanoemulsion at MIC suggests that CL+TH-nanoemulsion promoted the antibiofilm properties exhibited by the CL+TH-emulsion against *S. aureus* biofilms even when using lower concentrations.

The optimum regimens were exposure to 2 MIC of the CL+TH-nanoemulsion, which both eliminated biofilms to a 99.99% level with no discernible distinction ($P > 0.05$) between them. The nano-sized pills presumably allowed for excellent Clove and Thymol distribution throughout the biofilm architecture. As the dimension of particles reduces, it is also known that the amount of surface coverage expands, allowing for more interaction with microbes [39]. This is in agreement with previous research by Miladi et al. [40], 48-h-old *S. typhimurium* bacterial biofilms were reduced by 46.3% and 60.6% after being exposed to 2 MIC and 4 MIC carvacrol for 1 h.

3.4. Cytotoxic effect of clove oil+Thym (CL+TH) emulsion and CL+TH-nanoemulsion against HepG2 and MCF-7 cell lines

Drug carriers, such as nanoemulsions, are used to transport pharmaceuticals and phytochemicals into cells. They improve the antibacterial, antioxidant, and anticancer properties of their components. Cancer therapy strategies have been drastically altered thanks to the usage of nanoemulsions, which have proven to be a safe, biocompatible, and effective drug delivery method [41]. The cytotoxic effect of CL+TH-emulsion and CL+TH-nanoemulsion on HepG2 and MCF-7 cell lines was examined by MTT (Fig. 5). The IC_{50} values of both CL+TH-emulsion and CL+TH-nanoemulsion are lower in MCF-7 than HepG2, indicating the most effective cytotoxic impact. Besides, CL+TH-nanoemulsion is more cytotoxic than CL+TH-emulsion on both cell lines. Also, the effect of CL+TH-nanoemulsion is more effective and significantly cytotoxic than taxol on MCF-7.

Taxol, also known as paclitaxel, is a chemotherapy medication widely used to treat a wide variety of cancers. Taxol is one of the most widely used antitumoral drugs, having been used to treat over a million patients since its antitumoral action was discovered. Taxol was the first microtubule-targeting medication identified in the literature, and its principal method of action is the disruption of microtubule dynamics, which leads to mitotic arrest and cell death. However, apoptosis also has been found to follow secondary pathways, such as the increase of reactive oxygen species (ROS) and the activation of genes and proteins related to endoplasmic reticulum (ER) stress. However, more research is needed to confirm if the endoplasmic reticulum stress is caused by gene dysregulation brought on by p53 activation. On the other hand, there is a theory that mitochondrial impairment can be caused by an excessive accumulation of calcium ions (Ca2+) due to the release of Ca2+ from the endoplasmic reticulum (ER). The cumulative effect of these factors is an increase in ROS production [42].

In agreement with the study by Tawfik et al. [43], Teiama 3, we found that the nanoemulsion on MCF-7 exhibited lower IC50 values than the emulsion form did, demonstrating that the anticancer dosage of a thalidomide analog was significantly lowered from micromolar efficiency to nanomolar efficiency using the nanoemulsion formula. Our results are consistent with those of another study which found a dose-dependent antiproliferative effect of essential oil and two nanoemulsions against hepatocellular carcinoma cells. Its cell-inhibiting action was enhanced in a nanoemulsion.

3.5. Effect of CL+TH-emulsion, and CL+TH-nanoemulsion on caspase 8 and caspase 9 activities

The effect of CL+TH-emulsion, and CL+TH-nano emulsion on the apoptosis markers caspase-8 and caspase-9 are illustrated in Fig. 6

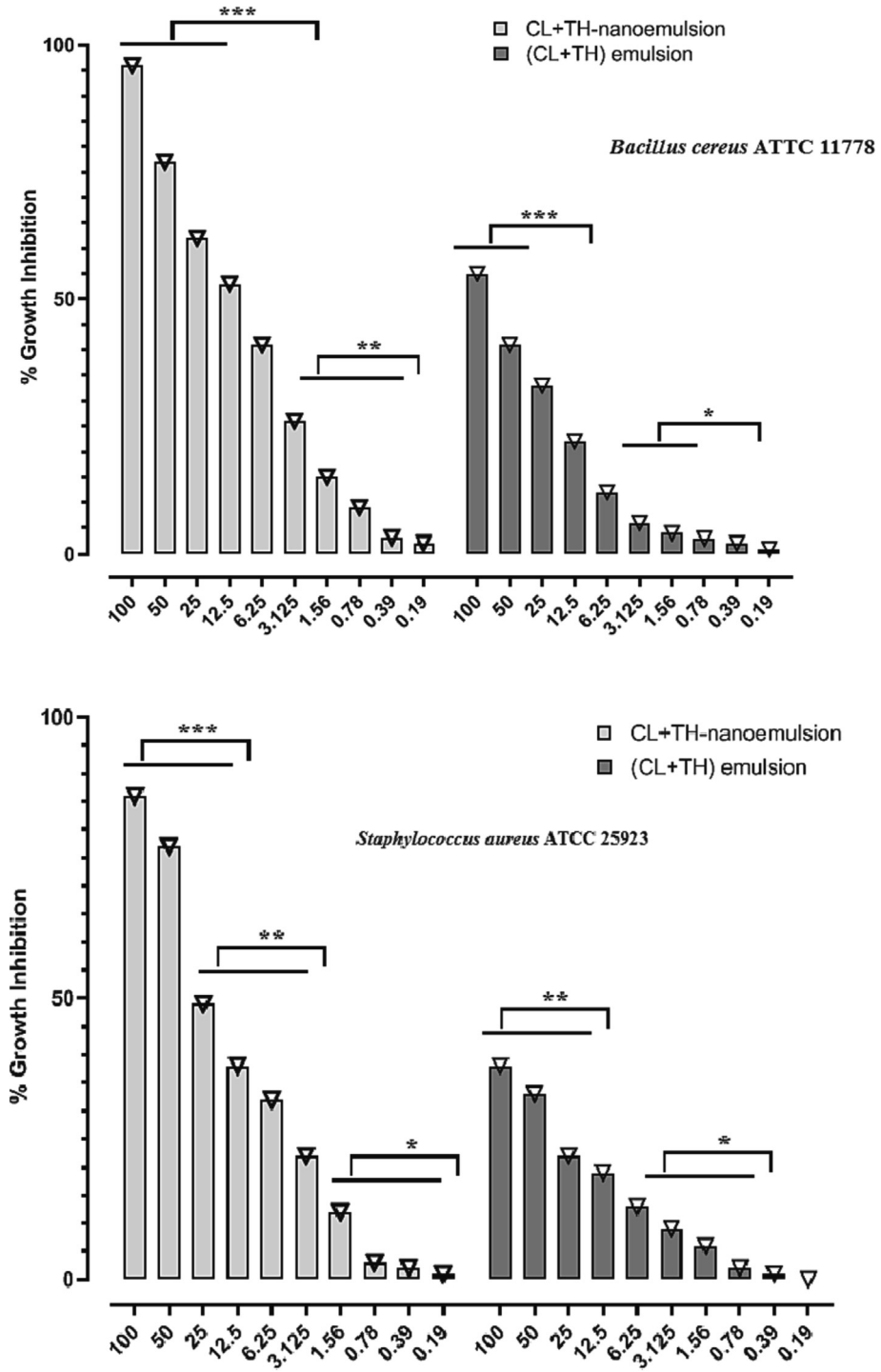


Fig. 3. CL+TH-emulsion and CL+TH-nanoemulsion limit the proliferation of tested bacteria in a concentration-dependent manner using a micro-broth dilution assay. One-way ANOVA was used for statistical analysis, and Tukey's test was used to evaluate mean differences; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

and Fig. 7 respectively. The data revealed that CL+TH-nano emulsion treatment on MCF-7 enhanced both the activity of caspase 8 and caspase 9 significantly and in a similar manner as Taxol. In contrast, the effect of CL+TH-emulsion treatment on MCF-7 cells is less effective in enhancing the activity of both caspase 8 and caspase 9 than CL+TH-nano emulsion and Taxol. These data indicated that increasing the activity of caspases 8 and 9 led to an increase in the rate of apoptosis and a decrease in the cell viability %. These results are in accordance with previous work which indicated that Caspase-8 and -9 are both activated by extrinsic and intrinsic apoptotic stimuli [44].

3.6. Effect of CL+TH-emulsion, and CL+TH-Nano emulsion on VEGFR-2

The effect of Taxol, CL+TH-emulsion, and CL+TH-Nano emulsion on VEGFR-2 was illustrated in Fig. 8. The data revealed that the treatment of MCF-7 cells with Taxol, CL+TH-emulsion, and CL +TH-Nano emulsion significantly decreased VEGFR-2 than control. Besides, the effect is more pronounced in CL+TH-Nano emulsion treated cells than Taxol, CL+TH-emulsion but not significant. These data indicated that the treatment of the MCF-7 cancer cells decreased the angiogenesis via decreasing the VEGFR-2 and these data were in accordance with previous work [45,46].

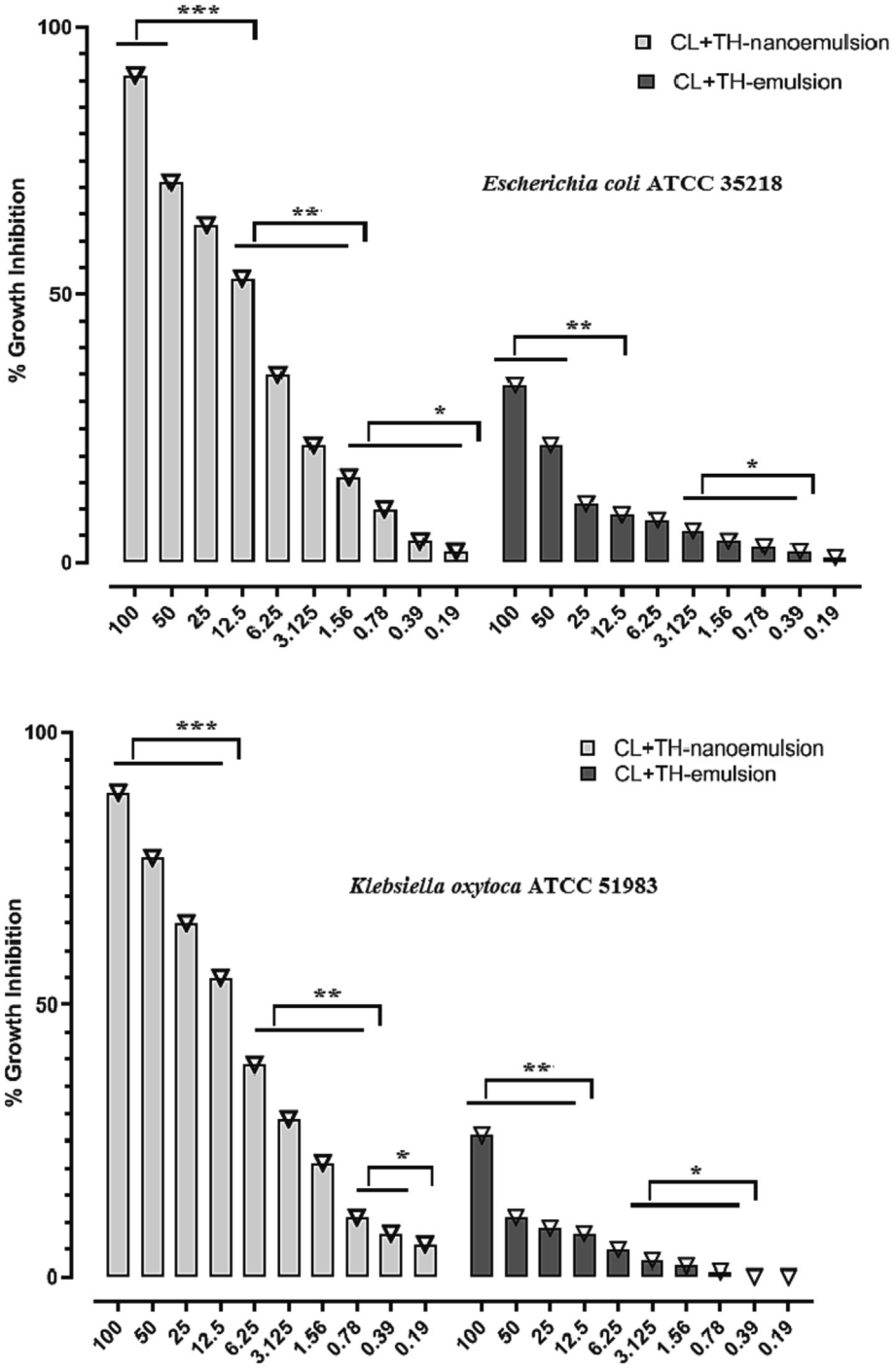


Fig. 3 (*continued*)

Table 1
Inhibition zones and MIC of CL+TH-emulsion and CL+TH-nanoemulsion.

	K. oxytoca		*E. coli*		*B. subtilis*		*S. aureus*	
	IZ/mm	**MIC (mg/mL)**	**IZ/mm**	**MIC (mg/mL)**	**IZ/mm**	**MIC (mg/mL)**	**IZ/mm**	**MIC**
CL+TH-emulsion	9.2 ± 3.1	ND	10 ± 1.9	ND	13 ± 2.8	100	8.9 ± 1.3	ND
CL+TH-nanoemulsion	23 ± 2.9	6.25	22 ± 3.1	6.25	21 ± 2.9	12.5	19 ± 2.1	25
Ciprofloxacin	15 ± 3.5	50	13 ± 2.6	100	21 ± 2.2	50	21 ± 3.1	50

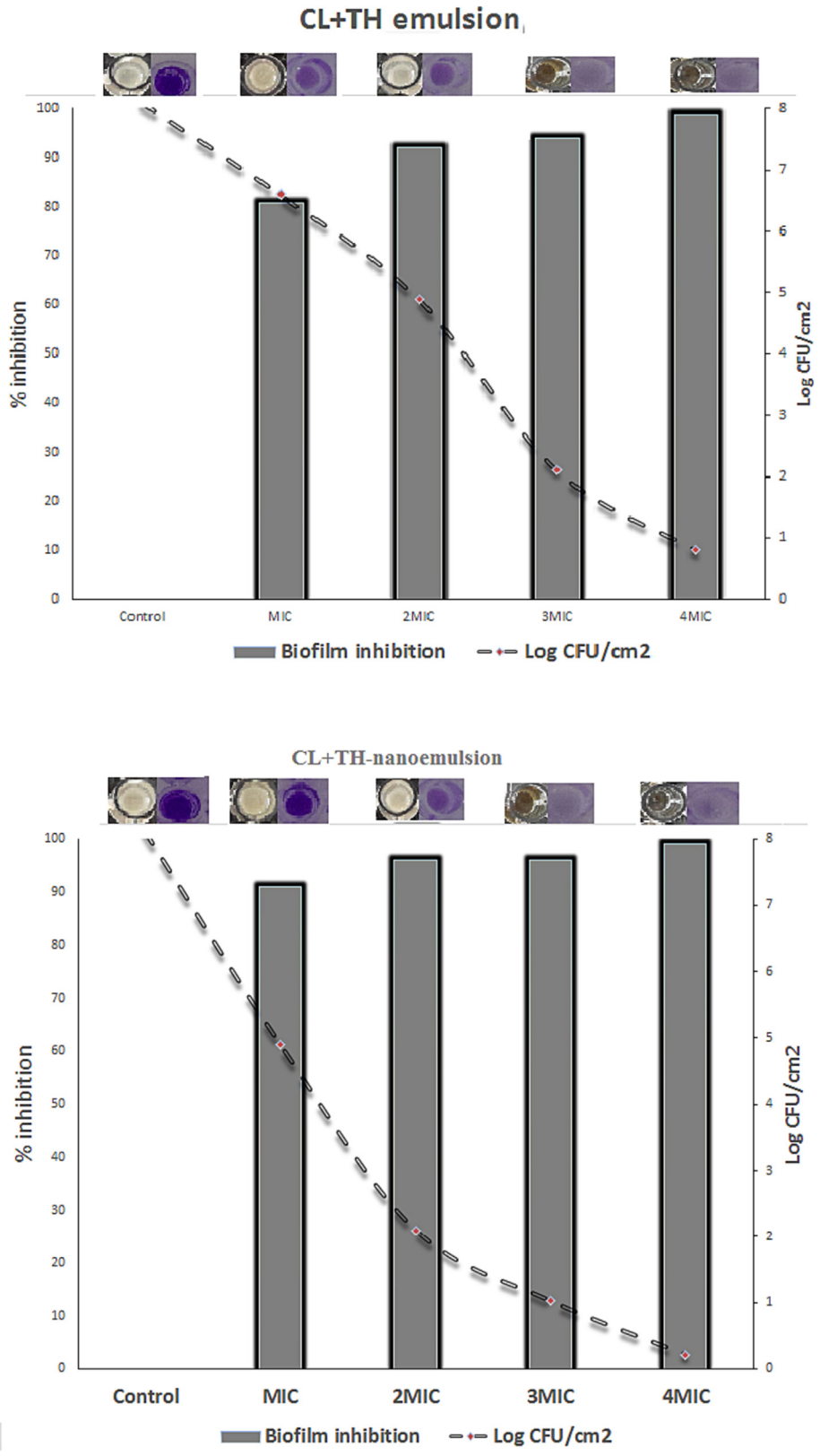


Fig. 4. The reduction in the growth of *S. auerus* and biofilm inhibition by CL+TH emulsion and CL+TH emulsion nanoemulsion at various concentrations.

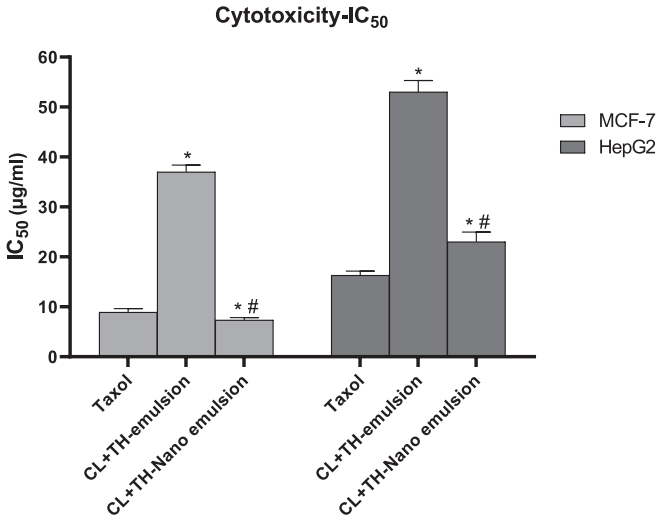


Fig. 5. Cytotoxic effect of Taxol, CL+TH-emulsion, and CL+TH-Nano emulsion against MCF-7, and HepG2 cell lines. Data are represented as mean ± SD of three independent experiments. *Significant *P*-value from Taxol group at $P < 0.001$, # significant *P*-value from CL-emulsion group at $P < 0.001$.

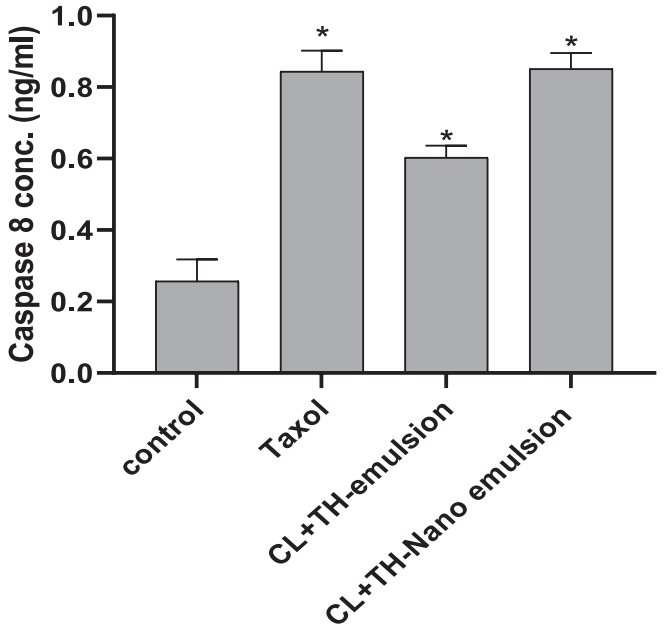


Fig. 6. Effects of Taxol, CL+TH-emulsion, and CL+TH-Nano emulsion on caspase-8 in MCF-7 cells compared to Taxol. Data are represented as mean ± SD, *: significant from the control group at *P*-value < 0.0001.

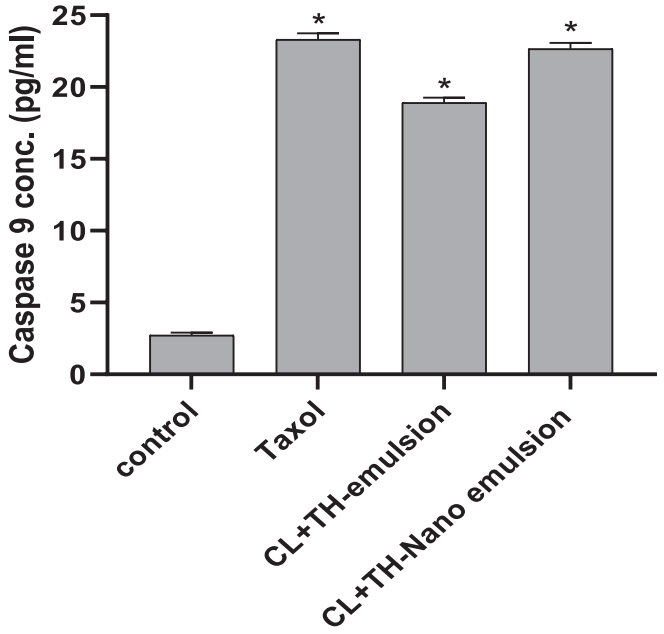


Fig. 7. Effects of Taxol, CL+TH-emulsion, and CL+TH-Nano emulsion on caspase-9 in MCF-7 cells compared to Taxol. Data are represented as mean ± SD, *: significant from the control group at *P*-value < 0.0001.

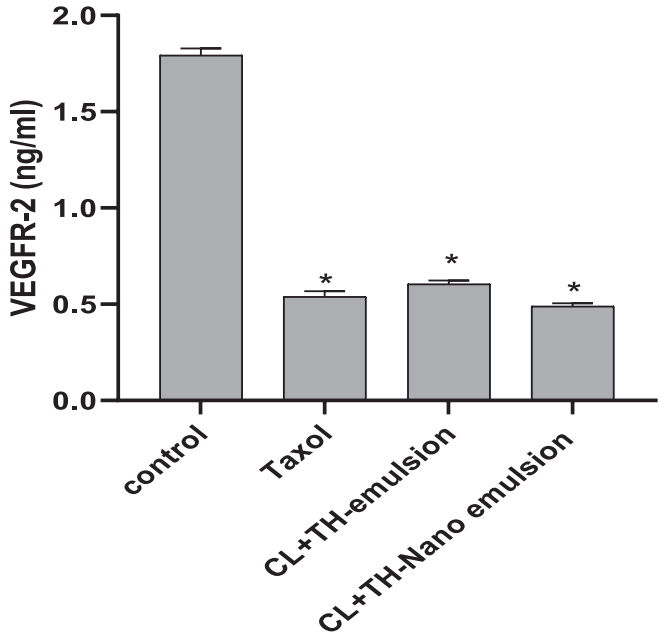


Fig. 8. Effects of Taxol, CL+TH-emulsion, and CL+TH-Nano emulsion on VEGFR-2 in MCF-7 cells. Data are represented as mean ± SD, *: significant from the control group at *P*-value < 0.0001.

4. Conclusions

In the current study, a novel nanoemulsion based on clove and thyme essential oils was successfully prepared. Characterization results revealed that the prepared nanoemulsion was in a nano-form with a spherical shape. Moreover, results illustrated that the prepared nanoemulsion exhibited promising antibacterial activity toward Gram-positive and Gram-negative bacteria where MICs were in the range of 6.25–25 mg/L. Furthermore, the prepared nanoemulsion shows antibiofilm activity against S. aureus where log CFU was significantly decreased. Moreover, the prepared nanoemulsion shows anticancer activity with activation of the apoptotic marker's caspase-8 and caspase-9. Also, the prepared nanoemulsion suppressed the activity of VEGFR2. Finally, the prepared clove and thyme oil nanoemulsion can be used in biomedical and pharmaceutical applications after *in vivo* studies

Author contributions

- Study conception and design: AH Hashem; AM Shehabeldine; AS Doghish; A Ismail; MMH Hassanin.
- Data collection: A Ismail; AH Hashem; AS Doghish; AM Shehabeldine.

- Analysis and interpretation of results: AH Hashem; AM Shehabeldine; AS Doghish; MMH Hassanin; A Ismail; MK Okla; IA Saleh; H AbdElgawad.
- Draft manuscript preparation: AH Hashem; AM Shehabeldine; A Ismail; AS Doghish.
- Revision of the results and approval of the final version of the manuscript: AH Hashem; AS Doghish; A Ismail; MK Okla; AM Shehabeldine; IA Saleh; H AbdElgawad.

Financial support

The authors extend their appreciation to the Researchers Supporting Project number (RSP2023R374) King Saud University, Riyadh, Saud Arabia.

Conflict of interest

There are no conflicts to declare.

Acknowledgment

The authors extend their appreciation to the Researchers Supporting Project number (RSP2023R374) King Saud University, Riyadh, Saud Arabia.

Supplementary material

https://doi.org/10.1016/j.ejbt.2023.12.001.

Data availability

Data available on request from the authors upon reasonable request.

References

[1] Gabaldón T. Nothing makes sense in drug resistance except in the light of evolution. Curr Opin Microbiol 2023;75:102350. https://doi.org/10.1016/j.mib.2023.102350. PMid: 37348192.
[2] Su Y, Yrastorza JT, Matis M, et al. Biofilms: Formation, research models, potential targets, and methods for prevention and treatment. Adv Sci 2022;9 (29):2203291. https://doi.org/10.1002/advs.202203291. PMid: 36031384.
[3] Shehabeldine AM, Doghish AS, El-Dakroury WA, et al. Antimicrobial, antibiofilm, and anticancer activities of *Syzygium aromaticum* essential oil nanoemulsion. Molecules 2023;28(15):5812. https://doi.org/10.3390/molecules28155812. PMid: 37570781.
[4] Sauer K, Stoodley P, Goeres DM, et al. The biofilm life cycle: Expanding the conceptual model of biofilm formation. Nat Rev Microbiol 2022;20 (10):608–20. https://doi.org/10.1038/s41579-022-00767-0. PMid: 35922483.
[5] Singh D, Vignat J, Lorenzoni V, et al. Global estimates of incidence and mortality of cervical cancer in 2020: A baseline analysis of the WHO Global Cervical Cancer Elimination Initiative. Lancet Glob Health 2023;11(2): e197–206. https://doi.org/10.1016/S2214-109X(22)00501-0. PMid: 36528031.
[6] Schirrmacher V. From chemotherapy to biological therapy: A review of novel concepts to reduce the side effects of systemic cancer treatment. Int J Oncol 2019;54(2):407–19. https://doi.org/10.3892/ijo.2018.4661. PMid: 30570109.
[7] Altwaijry N, El-Ghlban S, El Sayed IET, et al. *In vitro* and *in vivo* antitumor activity of indolo[2,3-b] quinolines, natural product analogs from neocryptolepine alkaloid. Molecules 2021;26(3):754. https://doi.org/10.3390/molecules26030754. PMid: 33535575.
[8] Ismail A, Doghish AS, Elsadek BEM, et al. Hydroxycitric acid potentiates the cytotoxic effect of tamoxifen in MCF-7 breast cancer cells through inhibition of ATP citrate lyase. Steroids 2020;160:108656. https://doi.org/10.1016/j.steroids.2020.108656. PMid: 32439410.
[9] Ismail A, Mokhlis HA, Sharaky M, et al. Hydroxycitric acid reverses tamoxifen resistance through inhibition of ATP citrate lyase. Pathol Res Pract 2022;240:. https://doi.org/10.1016/j.prp.2022.154211. PMid: 36401980154211.
[10] Guzmán E, Lucia A. Essential oils and their individual components in cosmetic products. Cosmetics 2021;8(4):114. https://doi.org/10.3390/cosmetics8040114.
[11] Khalil AA, ur Rahman U, Khan MR, et al. Essential oil eugenol: Sources, extraction techniques and nutraceutical perspectives. RSC Adv 2017;7 (52):32669–81. https://doi.org/10.1039/C7RA04803C.
[12] Gholami-Ahangaran M, Ahmadi-Dastgerdi A, Azizi S, et al. Thymol and carvacrol supplementation in poultry health and performance. Veterinary Medicine and Science 2022;8(1):267–88. https://doi.org/10.1002/vms3.663. PMid: 34761555.
[13] Juven BJ, Kanner J, Schved F, et al. Factors that interact with the antibacterial action of thyme essential oil and its active constituents. J Appl Bacteriol 1994;76(6):626–31. https://doi.org/10.1111/j.1365-2672.1994.tb01661.x. PMid: 8027009.
[14] Najafloo R, Behyari M, Imani R, et al. A mini-review of Thymol incorporated materials: Applications in antibacterial wound dressing. J Drug Delivery Sci Technol 2020;60:. https://doi.org/10.1016/j.jddst.2020.101904101904.
[15] Hajibonabi A, Yekani M, Sharifi S, et al. Antimicrobial activity of nanoformulations of carvacrol and thymol: New trend and applications. OpenNano 2023;13:. https://doi.org/10.1016/j.onano.2023.100170100170.
[16] Deghiedy NM, Elkenawy NM, Abd El-Rehim HA. Gamma radiation-assisted fabrication of bioactive-coated thyme nanoemulsion: A novel approach to improve stability, antimicrobial and antibiofilm efficacy. J Food Eng 2021;304:. https://doi.org/10.1016/j.jfoodeng.2021.110600110600.
[17] Ratri PJ, Ayurini M, Khumaini K, et al. Clove oil extraction by steam distillation and utilization of clove buds waste as potential candidate for eco-friendly packaging. Jurnal Bahan Alam Terbarukan 2020;9(1):47–54. https://doi.org/10.15294/jbat.v9i1.24935.
[18] Hashem AH, Abdelaziz AM, Hassamim MMH, et al. Potential impacts of clove essential oil nanoemulsion as bio fungicides against *Neoscytalidium* blight disease of *Carum carvi* L. Agronomy 2023;13(4):1114. https://doi.org/10.3390/agronomy13041114.
[19] Ghotbi RS, Khatibzadeh M, Kordbacheh S Preparation of neem seed oil nanoemulsion. In: Proceedings of the 5th International Conference on Nanotechnology: Fundamentals and Applications; 2014 August 11–13; Prague, Czech Republic; 2014. p. 11–13.
[20] Krishnamoorthy R, Athinarayanan J, Periasamy VS, et al. Antimicrobial activity of nanoemulsion on drug-resistant bacterial pathogens. Microb Pathog 2018;120:85–96. https://doi.org/10.1016/j.micpath.2018.04.035. PMid: 29684541.
[21] Bendary MM, Ibrahim D, Mosbah RA, et al. Thymol nanoemulsion: A new therapeutic option for extensively drug resistant foodborne pathogens. Antibiotics 2020;10(1):25. https://doi.org/10.3390/antibiotics10010025. PMid: 33396722.
[22] Kwasny SM, Opperman TJ. Static biofilm cultures of Gram-positive pathogens grown in a microtiter format used for anti-biofilm drug discovery. Current Protocols Pharmacology 2010;50(1):13A.8.1–13A.8.23. https://doi.org/10.1002/0471141755.ph13a08s50. PMid: 22294365.
[23] Chaieb K, Kouidhi B, Jrah H, et al. Antibacterial activity of Thymoquinone, an active principle of *Nigella sativa* and its potency to prevent bacterial biofilm formation. BMC Complement Altern Med 2011;11(1):29. https://doi.org/10.1186/1472-6882-11-29. PMid: 21489272.
[24] Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65(1–2):55–63. https://doi.org/10.1016/0022-1759(83)90303-4. PMid: 6606682.
[25] Farag MM, Ahmed MM, Abdallah NM, et al. The combined antibacterial and anticancer properties of nano Ce-containing Mg-phosphate ceramic. Life Sci 2020;257:117999. https://doi.org/10.1016/j.lfs.2020.117999. PMid: 32585244.
[26] Okba MM, Ezzat MI, Shehabeldine AM, et al. Eurycomanol and eurycomanone as potent inducers for cell-cycle arrest and apoptosis in small and large human lung cancer cell lines. Nat Prod Res 2023;37(11):1856–62. https://doi.org/10.1080/14786419.2022.2119387. PMid: 36054770.
[27] Marhamati M, Ranjbar G, Rezaie M, et al. Effects of emulsifiers on the physicochemical stability of Oil-in-water Nanoemulsions: A critical review. J Mol Liq 2021;340:. https://doi.org/10.1016/j.molliq.2021.117218117218.
[28] Dai L, Li W, Hou X. Effect of the molecular structure of mixed nonionic surfactants on the temperature of miniemulsion formation. Colloids Surf A Physicochem Eng Asp 1997;125(1):27–32. https://doi.org/10.1016/S0927-7757(96)03859-9.
[29] Krishnamoorthy R, Gassem MA, Athinarayanan J, et al. Antifungal activity of nanoemulsion from *Cleome viscosa* essential oil against food-borne pathogenic *Candida albicans*. Saudi Journal of Biological Sciences 2021;28(1):286–93. https://doi.org/10.1016/j.sjbs.2020.10.001. PMid: 33424308.
[30] Enayatifard R, Akbari J, Babaei A, et al. Anti-microbial potential of nanoemulsion form of essential oil obtained from aerial parts of *Origanum vulgare* L. as food additive. Adv Pharm Bull 2021;11(2):327. https://doi.org/10.34172/apb.2021.028. PMid: 33880355.
[31] Hassanin MM, Halawa AE, Ali AAA. Evaluation of the activity of thyme essential oil nanoemulsion against Sclerotinia rot of fennel. Egypt J Agric Res 2017;95(3):1037–50. https://doi.org/10.21608/ejar.2017.149559.
[32] Hammad EA, Hasanin MMH. Antagonistic effect of nanoemulsions of some essential oils against *Fusarium oxysporum* and root-knot nematode *Meloidogyne javanica* on coleus plants. Pak J Nematol 2022;40(1):35–48. https://doi.org/10.17582/journal.pjn/2022/40.1.35.48.

[33] Abd-Elsalam KA, Khokhlov AR. Eugenol oil nanoemulsion: Antifungal activity against *Fusarium oxysporum* f. sp. *vasinfectum* and phytotoxicity on cottonseeds. Appl Nanosci 2015;5:255–65. https://doi.org/10.1007/s13204-014-0398-y.

[34] Barradas TN, de Holanda e Silva KG. Nanoemulsions of essential oils to improve solubility, stability and permeability: A review. Environ Chem Lett 2021;19(2):1153–71. https://doi.org/10.1007/s10311-020-01142-2.

[35] Low LE, Siva SP, Ho YK, et al. Recent advances of characterization techniques for the formation, physical properties and stability of Pickering emulsion. Adv Colloid Interface Sci 2020;277:102117. https://doi.org/10.1016/j.cis.2020.102117. PMid: 32035999.

[36] Chouhan S, Sharma K, Guleria S. Antimicrobial activity of some essential oils—present status and future perspectives. Medicines 2017;4(3):58. https://doi.org/10.3390/medicines4030058. PMid: 28930272.

[37] Chouhan S, Sharma K, Zha J, et al. Recent advances in the recombinant biosynthesis of polyphenols. Front Microbiol 2017;8:2259. https://doi.org/10.3389/fmicb.2017.02259. PMid: 29201020.

[38] Xu J, Zhou F, Ji BP, et al. The antibacterial mechanism of carvacrol and thymol against *Escherichia coli*. Lett Appl Microbiol 2008;47(3):174–9. https://doi.org/10.1111/j.1472-765X.2008.02407.x. PMid: 19552781.

[39] Hasheminejad N, Khodaiyan F. The effect of clove essential oil loaded chitosan nanoparticles on the shelf life and quality of pomegranate arils. Food Chem 2020;309:125520. https://doi.org/10.1016/j.foodchem.2019.125520. PMid: 31698187.

[40] Miladi H, Zmantar T, Kouidhi B, et al. Use of carvacrol, thymol, and eugenol for biofilm eradication and resistance modifying susceptibility of *Salmonella enterica* serovar *Typhimurium* strains to nalidixic acid. Microb Pathog 2017;104:56–63. https://doi.org/10.1016/j.micpath.2017.01.012. PMid: 28062292.

[41] Khan I, Bahuguna A, Kumar P, et al. *In vitro* and *in vivo* antitumor potential of carvacrol nanoemulsion against human lung adenocarcinoma A549 cells via mitochondrial mediated apoptosis. Sci Rep 2018;8:144. https://doi.org/10.1038/s41598-017-18644-9. PMid: 29317755.

[42] Gallego-Jara J, Lozano-Terol G, Sola-Martínez RA, et al. A compressive review about Taxol®: History and future challenges. Molecules 2020;25(24):5986. https://doi.org/10.3390/molecules25245986. PMid: 33348838.

[43] Tawfik NM, Teiama MS, Iskandar SS, et al. A novel nanoemulsion formula for an improved delivery of a Thalidomide analogue to triple-negative breast cancer; synthesis, formulation, characterization and molecular studies. Int J Nanomed 2023;2023(18):1219–43. https://doi.org/10.2147/IJN.S385166. PMid: 36937550.

[44] McComb S, Chan PK, Guinot A, et al. Efficient apoptosis requires feedback amplification of upstream apoptotic signals by effector caspase-3 or -7. Sci Adv 2019;5(7):eaau9433. https://doi.org/10.1126/sciadv.aau9433. PMid: 31392262.

[45] Falcon BL, Chintharlapalli S, Uhlik MT, et al. Antagonist antibodies to vascular endothelial growth factor receptor 2 (VEGFR-2) as anti-angiogenic agents. Pharmacol Ther 2016;164:204–25. https://doi.org/10.1016/j.pharmthera.2016.06.001. PMid: 27288725.

[46] Elkady H, Elwan A, El-Mahdy HA, et al. New benzoxazole derivatives as potential VEGFR-2 inhibitors and apoptosis inducers: Design, synthesis, anti-proliferative evaluation, flowcytometric analysis, and *in silico* studies. J Enzyme Inhib Med Chem 2022;37(1):403–16. https://doi.org/10.1080/14756366.2021.2015343. PMid: 34961427.