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Nanoemulsion of *Thymus capitatus* and *Origanum vulgare* essential oil: stability, antimicrobial and cytotoxic properties

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ABSTRACT: This research focuses on the assessment of cytotoxic and antimicrobial properties of *Thymus capitatus* essential oil (TEO) and *Origanum vulgare* essential oil (OEO), before and after encapsulation in nanoemulsions, prepared utilizing high-pressure homogenization. These plants, indigenous to northern Albania and Mediterranean regions, produce EO with notable biological and cytotoxic activities. However, volatility, poor solubility, and chemical instability limit their practical application. Incorporating these oils into NE aims to enhance their stability and biological activity. The antimicrobial efficacy of the EO exhibited variability against different strains, showing particular effectiveness against *E. coli* ATCC 25922, *S. aureus* ATCC 29213, and *C. albicans* ATCC 10231, with no effect was observed against *P. aeruginosa* ATCC 27853. The EO-loaded NE exhibited enhanced cytotoxicity against MCF7, DU 145, and HT-29 cancer cell lines compared to the free oil. Encapsulation was found to augment the bioactivity of these volatile oils, with TEO-NE demonstrating superior cytotoxic effects than OEO-NE. Following encapsulation, OEO exhibited superior antimicrobial efficacy relative to TEO against *S. aureus*, *E. coli*, and *C. albicans*. Our results suggest that NE may enhance the cytotoxic and antimicrobial potential of the EOs, in different manner among EO used. The encapsulation of TEO and OEO in NE shows promising therapeutic potential, although further studies are required. However, these conclusions are drawn from in vitro analyses, underscoring the need for subsequent in vivo studies to ascertain this innovative clinical safety and efficacy.

KEYWORDS: *Thymus capitatus*; *Origanum vulgare*; nanoemulsion; MTT cell viability assay; antimicrobial activity.

1. INTRODUCTION

Nanoemulsions (NEs) are colloidal mixtures of oil and water, stabilized by surfactants or co-surfactants, featuring droplet sizes from 10 to several hundred nanometers [1–4].

As nanosystems, NEs are known for their biocompatible properties, from their preparation using completely natural ingredients, making them suitable and acceptable for human consumption. Renowned for their safe, biodegradable characteristics, NEs effectively transport biomolecules. They facilitate straightforward administration and effectively deliver anticancer agents to tumor cells for targeted therapy. Additionally, NE enhances tissue and cell absorption, provides protection against oxidation and hydrolysis, facilitates precise distribution, improves bioavailability, and can encapsulate a variety of biomolecules/complexes such as essential oils (EOs), vitamins, and drugs [2,3,5].

Despite their vast potential, NEs still need to be underutilized in medicine and pharmaceuticals, though ideally suited for use in the pharmaceutical, cosmetic, and food industries [6].

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Regarding preparation, NEs can be created using low-energy methods (such as spontaneous nanoemulsification, phase inversion temperature, and phase inversion composition) and high-energy methods (including high-pressure homogenization, microfluidization, and sonification). The low-energy approach involves mixing at specific temperatures and requires minimal energy, yielding droplet sizes of diverse sizes based on phase composition, temperature, pH, and mixing speed. Conversely, high-energy methods like high-pressure homogenization are preferred for their efficiency and ability to avoid toxic substances, with relatively short preparation time. Critical factors influencing the NEs size include pressure changes, number of homogenizer passes, and surfactant concentration [3,4,6,7].

With their superior efficacy in nanoencapsulation, NEs are exceptional for precisely releasing bioactive molecules/complexes. Moreover, it is crucial to assess their physicochemical and biological characteristics, and their stability [7].

The *Lamiaceae* family, known for its diverse medicinal/aromatic plants, is well-known for its EOs, which exhibit a range of biological activities, including cytotoxic activity on cancer cell lines, antioxidant activity, antibacterial activity, and more. Typically, medicinal plants are regarded as safe and exhibit lower toxicity than synthetic drugs, highlighting their therapeutic appeal. Specifically, genera such as *Thymus* (*T. capitatus*), natively found in the Mediterranean regions, and *Origanum* (*O. vulgare*), with a native range extending from Europe to Asia, have attracted scientific interest due to their therapeutic properties, prompting a heightened focus on EO as preventive or adjunct therapies for various cancers and their applications in the cosmetic and food industries [6,8]. In addition, a wide range of antibacterial activity was demonstrated for oregano and thyme [9,10].

Essential oils from species like *Thymus capitatus* (TEO) and *Origanum vulgare* (OEO) are known for their cytotoxic, antioxidant, and antimicrobial properties. These EOs are complex mixtures containing a significant number of bioactive components, which are mainly dependent on plant source and extraction method. This complexity contributes to their comprehensive therapeutic effects [6,7,11].

Research has confirmed TEO's cytotoxicity against multiple cancer cell lines, with thymol and carvacrol being vital bioactive compounds. Similarly, OEO displays a broad spectrum of biological activities, ranging from cytotoxic, antioxidant, anti-inflammatory, antifungal, and antibacterial activity, with carvacrol being a key active component. The effectiveness of these EOs is due to their active ingredients and the potential synergistic interactions with other minor/trace constituents [11–13].

The phytochemical composition of EOs is profoundly influenced by factors such as plant species, growth conditions, geographical areas, harvest periods, and climatic conditions. As several studies have emphasized, this underlines the importance of conducting research tailored to specific EOs rather than adopting a generalized approach across different types [6,14–16].

Despite their advantages, EOs show high volatility, low bioavailability, and stability issues, limiting their use in medicine and pharmaceuticals. Encapsulation in NEs offers a solution, effectively addressing these limitations [6].

Nanoemulsions demonstrate outstanding efficacy in targeting malignant cells and enabling the controlled release of complexes, improving selectivity towards malignant cells while reducing toxicity to normal cells, thus enhancing therapeutic outcomes.

Additionally, the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) have recently approved numerous nanosystem-based delivery methods for cancer treatment, underscoring their increasing importance in oncology [1,2,17]. Moreover, it was stated that employing NEs will not contribute to developing resistant strains and could address the bacterial resistance brought on by the improper and extensive use of antibiotics [18].

This trend indicates an increasing interest in exploring herbal products as therapeutic agents to integrate them as substitutes or complementary treatments alongside current synthetic agents in cancer therapy [7,19].

Additionally, the lack of new antibiotics entering the market has sparked a growing interest in developing natural-based antimicrobials, particularly those integrated into nanosystems that can potentially overcome the antibiotic resistance of the strains precisely [20–22].

The synergy of natural compounds and NE technology promises to significantly advance cancer treatment, offering a novel, less toxic, and potentially more effective solution. The successful development and characterization of these NEs could open new pathways for research and therapy [7,23].

This study aimed to develop NEs containing TEO or OEO to preserve and amplify their biological activities (cytotoxicity against various cancer types, antioxidant, and antibacterial activities) and enhance stability as a strategic approach for a successful therapeutic intervention.

We aim to develop and characterize TEO-NE and OEO-NE, prepared via high-pressure homogenization, to enhance their cytotoxic efficacy against three cancer cell lines (MCF7, DU 145, and HT-29) and antimicrobial activity against Gram-negative bacteria (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853), Gram-positive bacteria (*Staphylococcus aureus* ATCC 29213), and fungus (*Candida albicans* ATCC 10231). In addition, this is the first study to evaluate the antimicrobial properties of nanoemulsified TEO and OEO isolated from northern Albanian wild-grown plants.

Our research contributes to understanding key factors necessary for the formulation of NEs that increase the biological efficacy of TEO and OEO, potentially broadening their application in the pharmaceutical, food, and cosmetic fields.

2. RESULTS AND DISCUSSION

2.1. Essential Oil Composition

The analysis Through GC/FID/MS, the composition of TEO and OEO were analyzed according to our previous work and the results are shown in Figure 1 and Table 1 [24,25]. As stated in previous publications of our working group, the phytochemical composition of EOs varies depending on several conditions such as growth conditions, and extraction methods [24,25]. Thymol was identified as the primary component of TEO. Meanwhile, carvacrol was shown to be the dominant component in OEO. The data are consistent with the data reported in our presentations and those from the literature [24,25].

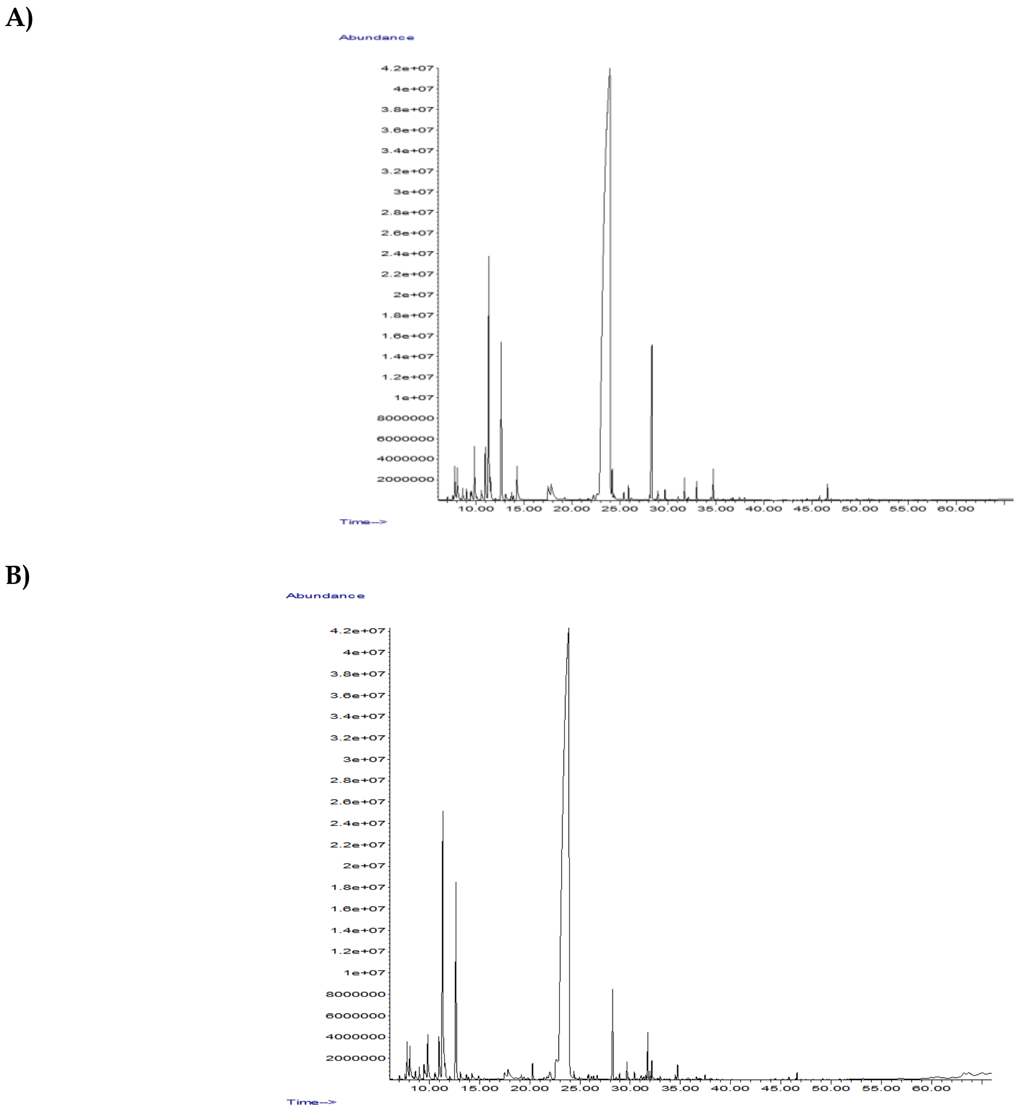


Figure 1. GC/FID/MS chromatogram of A) *T. capitatus* and B) *O. vulgare essential oil*

2.2. Size, PDI, Zeta Potential and Rheology Properties

For each EO, NE formulations were prepared, with subsequent evaluations conducted on the droplet size (DS), polydispersity index (PDI), and zeta potential (ZP) of blank, TEO-NE, and OEO-NE samples. The results are summarized in Table 2. The blank NE exhibited a DS of 114 ± 1.72 nm. Encapsulation of TEO reduced the DS significantly to 96 ± 0.32 nm ($p<0.001$), while OEO encapsulation led to a DS decrease to 89 ± 0.22 nm ($p<0.001$), compared to the blank NE. Our findings align with similar studies, showing DS remains stable after encapsulating the EOs. This observation could be attributed to the EOs potentially influencing the membrane dynamics of the nanosystems [21,26–30]. The differences between TEO-NE and OEO-NE DS may be due to different EO compositions. The effect of the oil phase composition on the EO-NE DS distribution has been reported [7,31].

Regarding uniformity, the blank NE demonstrated a PDI value of 0.19 ± 0.01, indicating moderate dispersion. However, upon encapsulating TEO and OEO, the PDI values significantly improved, dropping to 0.10 ± 0.01 for TEO-NE and 0.09 ± 0.01 for OEO-NE ($p<0.001$), which signifies the formation of more monodisperse nanosystems. Our results align with established literature, posing that a PDI of approximately 0.30 or lower indicates a monodisperse system, an essential criterion for developing efficient, stable, and safe nanosystems [5,32,33].

Table 1. Chemical composition of A) *T. capitatus* and B) *O. vulgare* essential oil.

A)

Peak No.	**RT**^{a} **(min)**	**KIL**^{b}	**Compound**	%^{c}
1	7.79	924	β-pinene	0.19
2	8.07	932	α-terpinene	1.09
3	8.63	946	camphene	0.24
4	9.57	974	γ-terpinene	3.18
5	9.85	988	myrcene	1.1
6	10.56	1002	α-phellandrene	0.27
7	10.97	1014	α-thujene	0.64
8	11.35	1020	p-cymene	6.19
9	12.6	1054	thymol	74.5
10	13.1	1065	cis-sabinene hydrate	0.17
11	13.7	1086	terpinolene	0.13
12	14.28	1095	linalool	0.9
13	17.5	1165	borneol	0.66
14	17.85	1174	terpinen-4-ol	1.05
15	19.25	1186	α-terpineol	1.05
16	22.23	1239	carvone	0.21
17	23.93	1289	α-pinene	0.69
18	24.38	1356	eugenol	0.16
19	25.88	1370	carvacrol acetate	0.12
20	28.31	1417	trans-caryophyllene	4.02
21	29.67	1452	α-humulene	0.18
22	31.72	1505	β-bisabolene	0.33
23	32.99	1506	α-(Z)-bisabolene	0.28
24	34.72	1582	caryophyllene oxide	0.57
			Total:	**97.92**

B)

Peak No.	RT[a] (min)	KIL[b]	Compound	%[c]
1	8.067	932	α-pinene	0.82
2	8.633	946	camphene	0.21
3	9.852	988	myrcene	0.99
4	10.567	1002	α-phellandrene	0.22
5	10.968	1014	α-terpinene	0.97
6	11.351	1020	p-cymene	7.29
7	12.667	1054	γ-terpinene	4.31
8	13.108	1065	cis-sabinene hydrate	0.24
9	13.703	1086	terpinolene	0.10
10	14.252	1095	linalol	0.24
11	17.502	1165	borneol	0.28
12	17.851	1174	terpinen-4-ol	0.58
13	19.161	1186	α-terpineol	0.10
14	20.283	1232	thymol, ethyl ether	0.31
15	22.022	1233	pulegone	0.43
16	22.652	1289	thymol	1.29
17	23.882	1298	carvacrol	71.41
19	26.714	1387	β-burbonene	0.11
20	28.254	1417	trans-caryophyllene	1.69
21	28.952	1439	aromadendrene	0.14
22	29.678	1452	α-humulene	0.29
23	30.417	1478	γ-murrolene	0.16
24	31.750	1505	β-bisabolene	0.77
25	31.973	1513	γ-cadinene	0.14
26	32.156	1522	δ-cadinene	0.30
			Total:	**93.39**

a RT (min) = Retention time
b KIL = Kovat's retention index
c The percentages of compounds were obtained by FID peak-area normalization. The percentage composition of the oil was computed by the normalization method from the GC peak areas, calculated as the mean of three samples without correction factors.
The methodology and phytochemical composition presented in the above tables are the same as reported in our previous work [24,25].

The ZP for blank NE was measured at - 7.51 ± 0.50 mV. Upon encapsulation of TEO and OEO into NE, the ZP significantly shifted to - 36.63 ± 2.51 mV and - 36.67 ± 1.87 mV, respectively ($p<0.001$). Similar studies highlighted the interactions between EOs with the NE membrane, which modify the surface charge. These data imply that the vesicle membrane composition and the preparation method significantly impact the DS, PDI, ZP, and EE% [7,34]. The viscosities of OEO and TEO NEs were shown to be 1.2629 ± 0.005 mPa.s and 1.2670 ± 0.004 mPa.s for TEO-NE and OEO-NE, respectively (Table 2), with no significant differences ($p>0.05$). The results were similar to the reported data, with some minor differences, which can be related to the nature of EO, EE% of EO-NE, and the preparation method [35].

Table 2. Characteristics of blank and *T. capitatus* and *O. vulgare* essential oil-loaded nanoemulsions regarding mean droplet size, polydispersity index (PDI), zeta potential (ZP), and encapsulation efficiency (EE%).

Nanoemulsion batches[a]	Mean size(nm)	PDI[b]	ZP[c] (mV)	Viscosity (mPa.s)	EE%[d]
Blank NE	114 ± 1.72	0.19 ± 0.01	- 7.51 ± 0.50	1.10 ± 0.006	
T. capitatus EO-loaded NE	96 ± 0.32	0.10 ± 0.01	- 36.63 ± 2.51	1.2629 ± 0.005	85.89 ± 3.13
O. vulgare EO-loaded NE	89 ± 0.22	0.09 ± 0.01	- 36.67 ± 1.87	1.2670 ± 0.004	80.59 ± 2.69

[a] NE: nanoemulsion, EO: essential oil
[b] PDI: polydispersity index
[c] ZP: zeta potential
[d] EE%: encapsulation efficiency

Data are expressed as a mean value ± standard deviation (*n* = 3)

2.3. Evaluation of the Encapsulation Efficiency

A high encapsulation efficiency (EE%) is crucial for a nanosystem's efficacy. It ensures optimal delivery of bioactive molecules with minimal carrier volume. The variations in EE% are dictated by the composition and concentration of the EO and the nanosystem [36].

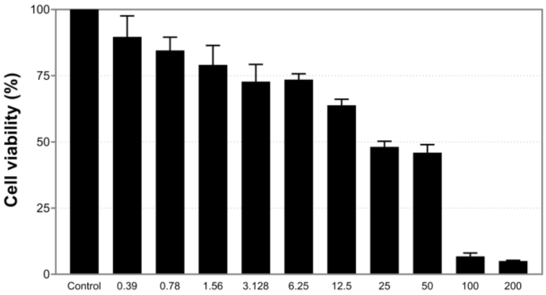
The study findings highlight NEs' exceptional capability in encapsulating TEO and OEO, with EE% of 85.89 ± 3.13% for TEO-NE and 80.59 ± 2.69% for OEO-NE, respectively, with no significant changes ($p>0.05$) as detailed in Table 2. The findings align with earlier research, indicating that high-pressure homogenization methods yield stable NEs with elevated EE values [35,37]. In contrast, some previous studies have pointed to lower EE% rates, suggesting that EE% is subject to change based on the EOs composition, concentration, the characteristics of the NE itself, and the preparation techniques [35,37–40].

The encapsulated substance's nature and interaction with the NE membrane are crucial in determining EE%. Differences in membrane permeability contribute to the observed variability in EE% among different EOs presented in the literature. Additionally, the interaction between NE membrane components and the EOs hydroxyl groups has been found to enhance EE%. In addition, the reduced EE% observed in nanosystems containing particular EOs may be due to the NE membrane's saturation level with the EOs components [5,11,16].

2.4. Cytotoxic potential

Unencapsulated TEO/OEO, TEO-NE, and OEO-NE cytotoxic activities were evaluated and compared *in vitro* using the MCF7, DU 145, and HT-29 cancer cell lines (breast, prostate, and colorectal cancer, respectively). The viability of the cells was determined using the MTT Cell Viability Assay, and the results are presented in Figures 2–4.

A)



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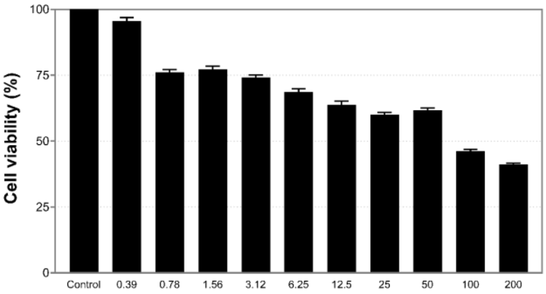

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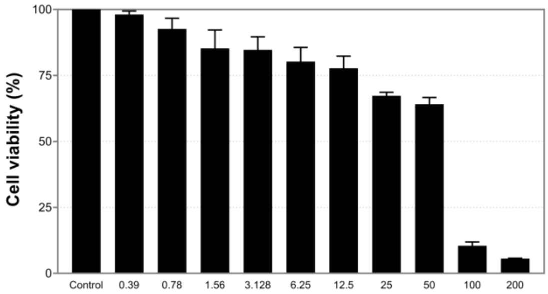

Figure 2. Viability of A) MCF7, B) DU 145, and C) HT-29 Cancer Cell Lines after treatment with different concentrations of *T. capitatus* essential oil (0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 200 µg/mL). Each bar represents means ± SD.

3A)

3B)

3C)

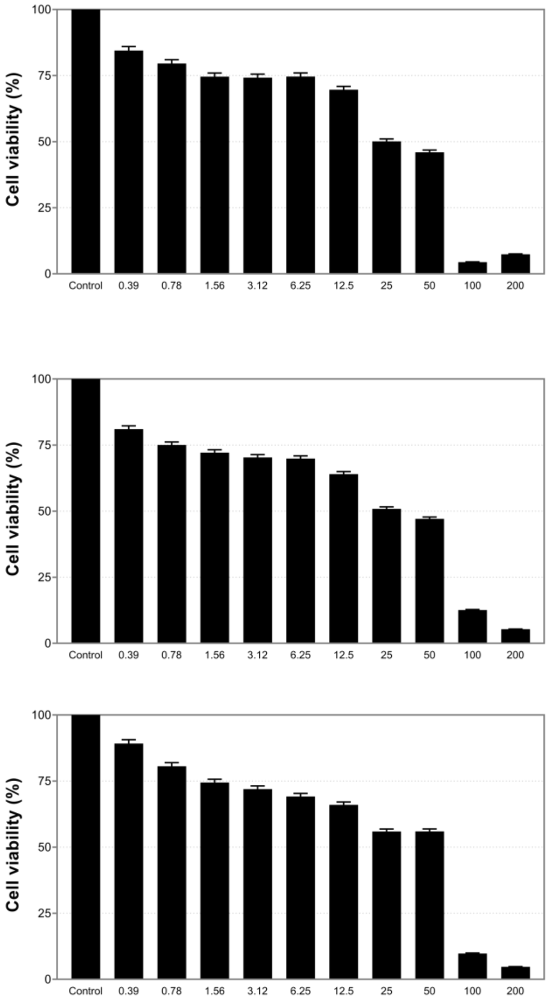

Figure 3. Viability of A) MCF7, B) DU 145, and C) HT-29 Cancer Cell Lines after treatment with different concentrations of *O. vulgare* essential oil (0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 200 µg/mL). Each bar represents means ± SD.

The cell viability of the tested cells with TEO was shown to be 47.90 ± 2.91% and 50.04 ± 5.41% in MCF7 and DU 145; the cell viability of HT-29 was reduced to 48.29 ± 3.99% (as previously reported by our studies) [25].

In the same cells, OEO showed the following results: 50.10 ± 8.88%, 48.87 ± 2.47%, and 45.94 ± 3.53% (Figure 3). No significant differences were observed between OEO and TEO in the same cells ($p>0.05$).

Our study results indicate that TEO and OEO play a significant role in *in vitro*, reducing the viability of malignant cells across several cell lines, including breast, prostate, and colorectal cancers.

Similar previous studies have shown that *Thymus* and *Origanum* plant EOs are cytotoxic to a variety of human cancer cells, including colon cancer cells (HCT-116 and CaCo-2), breast cancer cells (MCF-7), and lung cancer cells (A-549) [11,41,42]. According to previous findings, the cytotoxic activity of EOs is not conditionally directly related to the components with the highest concentrations. The phytochemical components of the EOs interact with cells at different concentrations. As a result, even trace components may play a role in the overall cytotoxic activity of EOs [11,41,42]. This leads to a synergistic effect that enhances the overall activity of the EO [6,43].

This synergistic effect is especially significant for the cancer-fighting abilities of two compounds, thymol, and carvacrol, which have been the subject of much research [11,44,45]. In contrast, as the literature reports, EOs containing thymol, carvacrol, *p*-cymene, and γ-terpinene as crucial components are non-toxic to cancer cell lines [11,15,20].

Furthermore, the significant components of TEO and OEO have been shown to possess less potent biological activity than the oil [3,11,12,46].

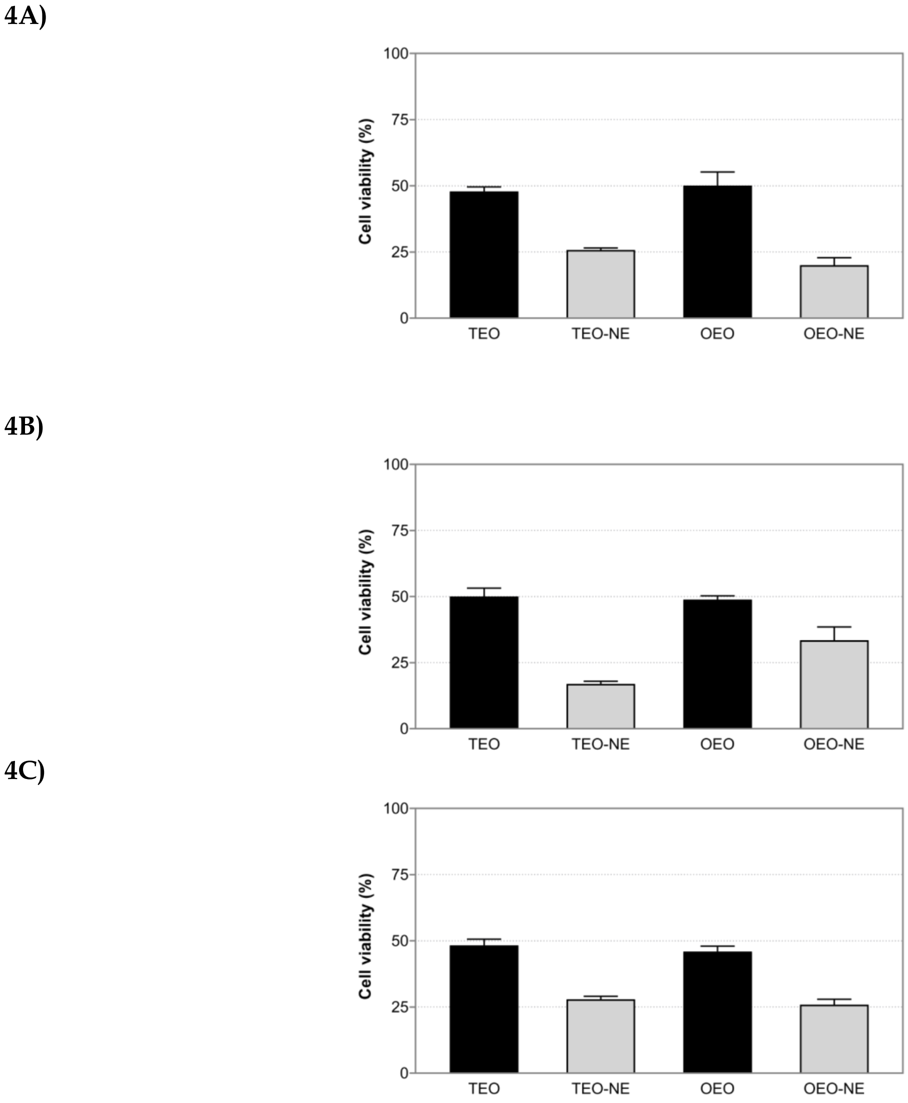

Figure 4. Cytotoxicity of Free *T. capitatus* and *O. vulgare* essential oil and nanoencapsulated essential oils on A) MCF7, B) DU 145, and C) HT-29 Cancer Cell lines. The charts show cell viability post-treatment with free essential oil and essential oil encapsulated in nanoemulsions, as assessed by the MTT Cell Viability Assay. Each bar represents ± SD (*n* = 3).

In addition, EOs, including TEO and OEO, have demonstrated cytotoxic effects via several mechanisms like apoptosis, cell cycle arrest, and the disruption of cell membranes. The specific nature of these mechanisms can vary based on the EOs composition and targeted cell type. The advantage of utilizing EOs lies in their diverse array of potentially bioactive compounds, though this diversity also necessitates thorough characterization for their effective use [15].

The findings of this study indicate that TEO and OEO exhibit cytotoxic properties against the three evaluated cell lines. Even though no significant differences were revealed in cell viability exposed to unencapsulated OEO and TEO, more substantial differences are observed after incorporation in NE. As shown in the figures, NEs proved to be capable of significantly improving the cytotoxic action of these EOs in different manners.

For the breast cancer cell line MCF7, treatment with TEO-NE and OEO-NE decreased cell viability to 25.79 ± 1.29% and 20.02 ± 4.89% ($p<0.001$), respectively. In the DU 145 prostate cancer cell line, a reduction in cell viability was observed to 16.96 ± 1.77% and 33.47 ± 8.70% for TEO-NE and OEO-NE, respectively, with the former showing statistical significance ($p<0.001$) and the latter showing significance ($p<0.05$) when compared to the non-encapsulated EOs. Similarly, for the HT-29 colorectal cancer cell line, cell viability was reduced to 27.94 ± 1.89% for TEO-NE and 25.90 ± 3.48% for OEO-NE, with significant reductions ($p<0.001$).

The results demonstrate that the EO-loaded NE outperformed free EO in cytotoxic activity *in vitro*. According to our knowledge, the evaluation and comparison of the cytotoxic activity of TEO-NE and OEO-NE on the MCF7, DU 145, and HT-29 cancer cell lines has not been studied so far. As shown in Figure 3, TEO-NE and OEO-NE were significantly more cytotoxic than the free EOs. Blank formulations did not show any activity.

These results align with previously reported studies, indicating improved EO activity after NE encapsulation [7,15]. The enhanced cytotoxic activity observed for TEO/OEO-NE may be explained by the increased ability of TEO/OEO to be transported efficiently inside the cell, compared to the non-encapsulated form of the EO. Several investigations have demonstrated the enhanced intracellular accumulation of a compound when encapsulated in NE [47,48]. Additionally, prolonged EO stability following NE encapsulation and the sustained presence of EO from NE through controlled release may explain the enhancement of EO NE's cytotoxic activity [7,48–51]. In all cases, TEO-NE and OEO-NE significantly improved cytotoxic activity ($p<0.001$). Research indicates that NE-containing EOs do not impact the viability of normal cell lines. Furthermore, the literature highlights the suitability of EOs as anticancer agents due to their lack of adverse effects on healthy cells [52,53].

2.5. Antioxidant potential

This research employed the DPPH radical scavenging assay to evaluate the antioxidant activities of both unencapsulated EOs and EO-loaded NEs. Figure 5 depicts the antioxidant activities represented as a percentage. According to our previous results, TEO and OEO demonstrated antioxidant activities of 47.28 ± 0.58% and 53.52 ± 4.27%, respectively [25]. No significant difference was shown between EOs.

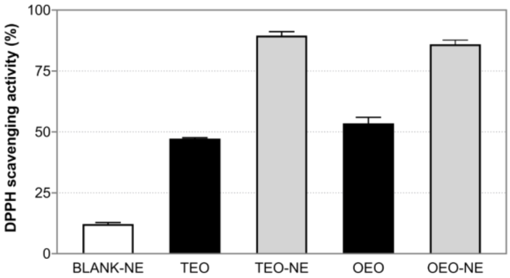

Figure 5. Antioxidant Activity of Free *T. capitatus* and *O. vulgare*, Blank nanoemulsions, and essential oil-loaded nanoemulsions. Each bar represents ± SD ($n = 3$).

Encapsulating TEO and OEO into NE significantly enhanced their antioxidant activities (Figure 5). TEO-NE were found to be the most effective, improving the antioxidant activity to 89.53 ± 2.87% ($p<0.001$), followed by OEO-NE: 86.00 ± 1.70% $p<0.001$). These findings are supported by data previously reported in the literature, confirming the improved antioxidant activity of EO nanosystems compared to free EOs [6,7,24,38]. Factors such as the EO's phytochemical composition, the NE's ZP, and DS critically influence the

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formation of the antioxidant-NE membrane complex and, thus, the antioxidant activity [6,7,48,54]. Because of their ability to form chemical complexes with free radicals, the compounds thymol, carvacrol, and *p*-cymene (as central components of TEO and OEO) have good antioxidant activity. Furthermore, other monoterpenes in the oil have been shown to have antioxidant activity, implying that this biological activity results from interactions among the oil's components [6,55,56].

2.6. Antimicrobial potential

The antimicrobial potencies of unencapsulated TEO/OEO, TEO-NE, and OEO-NE were evaluated both qualitatively and quantitatively against *Escherichia coli*, *S. aureus*, *P. aeruginosa*, and *C. albicans*. This was achieved by determining MIC and MBC values, defined by microdilution assay. The results obtained are presented in Table 3.

The MIC values of OEO were shown to be 0.25 mg/mL, 0.5 mg/mL, and 0.125mg/mL against *E. coli*, *S. aureus*, and *C. albicans*, respectively. Encapsulation in NE significantly increased the antimicrobial activity of OEO for all microorganisms tested with MIC values 0.0625, 0.25, and 0.0625, respectively.

OEO was more effective than TEO for *S. aureus* and *C. albicans*, while no significant difference was observed for *E. coli*.

With MIC 0.25 mg/mL, free TEO exhibited better antimicrobial activity against C. albicans, followed by significantly increased activity after incorporation in NE with MIC 0.125 mg/mL. The MIC values of TEO for *E. coli* and *S. aureus* were shown to be 2.5 and 0.5 mg/mL, respectively, with significantly increased antibacterial activity after encapsulation in NE, with MIC values of 1.0 mg/mL and 0.125 mg/mL.

The chemical components of EOs determine their antibacterial action. Significant components of phenolic Carvacrol in OEO, thymol in TEO, and terpene hydrocarbons (γ-terpinene) seem to be associated with their antimicrobial action [57,58].

Table 3. Minimum inhibitory concentrations, minimum bactericidal concentrations, and minimum fungicidal concentrations (mg/mL) of nanoemulsions and non-emulsified *T. capitatus* and *O. vulgare* essential oils against microorganisms.

Strain		*O. vulgare* EO[a]	*O. vulgare* EO-loaded NE[b]	*T. capitatus* EO	*T. capitatus* EO-loaded NE
E. coli	MIC[c]	0.25	0.0625	0.5	0.125
	MBC[d]	0.5	0.25	1.0	0.5
S. aureus	MIC	0.5	0.25	2.5	1.0
	MBC	0.5	0.5	>2.5	1.0
P. aeruginosa	MIC	>2.5	>2.5	>2.5	>2.5
	MBC	>2.5	>2.5	>2.5	>2.5
C. albicans	MIC	0.125	0.0625	0.25	0.125
	MFC[e]	0.25	0.25	2.5	2.5

[a] EO: essential oil
[b] NE: nanoemulsion
[c] MIC: Minimum Inhibition Concentration
[d] MBC: Minimum Bactericidal Concentration
[e] MFC: Minimum Fungicidal Concentration
Blank NE did not show any antimicrobial activity

Thymol and carvacrol can break down the outer membrane of Gram-negative bacteria and appear to render the cell membrane permeable [59]. The second primary compound by percentage, *p*-cymene in both EOs analyzed in this study, was shown to lack antibacterial efficacy when used alone [35,58].

The research highlights the significance of each component in the biological activity of EOs [59]. Additionally, research has demonstrated that the antibacterial properties of EOs can be impacted by the synergism and antagonism between their main molecules and smaller components.

In the current investigation regarding the antimicrobial action of OEO and TEO, *C. albicans* was shown to be more sensitive than *S. aureus*, *P. aeruginosa*, and *E. coli*.

Similar to our findings, previously published data indicated that EOs (including thyme oil) were more efficient against *Candida albicans* than against *Salmonella* species, *E. coli*, *S. aureus*, and *P. aeruginosa* [60].

According to the study, OEO and TEO nanoemulsification increases antimicrobial activity. This can be attributed to the EO's homogenous distribution in the microbial broth medium, which allowed EO to overcome the microorganism under investigation effectively [61,62]. The previous investigations reported similar outcomes with EO nanoemulsion indicating better action against gram-positive and gram-negative bacteria than the unencapsulated EO [59]. The differences in antimicrobial activity observed among EOs tested and their NE are related to differences in the phytochemical composition of EO, physicochemical properties of the EO-NE, and the sensitivity of certain microorganisms [63]. It was demonstrated that the droplet size, content of EOs, formulation, and strains of microbes studied significantly impact the antimicrobial efficacy of essential oil-based NEs. Nanoencapsulated EO may withstand bacterial mutation and retain its bactericidal properties, limiting the emergence of resistant bacterial strains. It works by non-specifically disrupting the membranes of bacterial cells [5].

Emulsification of natural EOs is recognized to enhance their efficacy in combating resistant germs that pose a threat to the population. For instance, it has been demonstrated that the Copaíba resin oil and Copaíba resin oil NE are potential antifungals against *Paracoccidioides spp*. since they effectively reduce the growth of the fungus. Copaíba resin oil and Copaíba resin oil NE had a comparable and additive impact with a decrease in their MIC values when mixed with amphotericin B [64].

Previous studies stated that employing NEs will not contribute to the development of resistant strains and, therefore, could address the issue of bacterial resistance brought on by the improper and extensive use of antibiotics [18,65].

The significant antimicrobial effectiveness of NE systems may stem from various factors, such as their interaction with the microbial cell membrane facilitated by their large surface area and passive transport across the cell membrane. This interaction could involve the electrostatic contacts between the NE and the microbial cell wall, the stability of the components of EOs, the fusing of nanoemulsions with the cell membrane, or their prolonged release. Due to these mechanisms, the concentration of EOs or their components at the target site may rise [53,63,66].

2.7. Stability Studies

Understanding the storage stability of NE systems is crucial for evaluating their potential as therapeutic delivery systems, especially in clinical settings and large-scale manufacturing. As a result, changes in the NE physicochemical properties were measured after six months. The DS, ZP, and storage conditions have been shown to influence NE stability [67,68].

The DS, PDI, and ZP of TEO/OEO-NE before and after six months of storage at 25 °C were evaluated in this study. Table 4 shows that TEO-NE and OEO-NE remained stable regarding the DS. After six months, TEO-NE and OEO-NE demonstrated stable DS with significant increases to 167 ± 3.81 nm for TEO-NE and 105 ± 3.85 nm for OEO-NE ($p<0.01$). The PDI values rose to 0.79 ± 0.01 for TEO-NE and 0.72 ± 0.04 for OEO-NE ($p<0.001$), and ZP values showed maintained stability at -29.30 ± 0.11 mV (TEO-NE) and -26.78 ± 0.62 mV (OEO-NE) ($p<0.001$). The formulation process, specifically the component ratios, plays a crucial role in the overall stability of NEs [3,5,48]. The formulations remained stable until three months at 25°C with minimal difference in DS, PDI, and ZP. Previous studies reported similar findings, observing a slight increase in NE droplet size after 30 days of storage at 25°C using a high-pressure homogenizer [5,69]. Nonetheless [70], noticed a rise in NE size after 60 days of storage. Smaller droplet NEs often show less tendency to aggregate. The ZP is also essential for preserving NE stability and avoiding aggregation. This might account for research that has already been published, suggesting that, in some situations, nanosystems with smaller vesicles are less stable [33].

Furthermore, the literature review reveals that using medium-chain triglycerides (MCT), similar to our approach in NE preparation, can enhance the stability of NE encapsulating EOs [7].

Table 4. Characteristics of blank and *T. capitatus* and *O. vulgare* essential oil-loaded nanoemulsions regarding mean droplet size, PDI, and zeta potential.

Nanoemulsion batches[a]		**Mean size (nm) ± SD (*n* = 3)**	**PDI ± SD (*n* = 3)**[b]	**ZP (mV) ± SD (*n* = 3)**[c]
***T. capitatus* EO-loaded NE**	t0	96 ± 0.32	0.10 ± 0.01	- 36.63 ± 2.51
	t6	167 ± 3.81	0.79 ± 0.01	- 29.30 ± 0.11
***O. vulgare* EO-loaded NE**	t0	89 ± 0.22	0.09 ± 0.01	- 36.67 ± 1.87
	t6	105 ± 3.85	0.72 ± 0.04	- 26.78 ± 0.62

a NE: nanoemulsion, EO: essential oil
b PDI: polydispersity index
c ZP: zeta potential
Data are expressed as a mean value ± standard deviation (*n* = 3)

3. CONCLUSION

The research highlighted the effectiveness of TEO and OEO in reducing the viability of cancer cells (MCF7, DU 145, and HT-29). It emphasized the increased efficacy when these oils were encapsulated in NEs compared to their unencapsulated forms. The study indicated that encapsulation enhanced the bioactivity of these volatile oils, with TEO-NE exhibiting more powerful cytotoxic effects than OEO-NE, suggesting its higher potential as a cytotoxic agent against malignant cells. Moreover, after nanoencapsulation, OEO demonstrated superior antimicrobial effectiveness compared to TEO against pathogens such as *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. This finding has significant implications for applications in food preservation, pharmaceuticals, and cosmetics, where natural antimicrobial agents are desired. The study employed high-pressure homogenization, a two-step, solvent-free process, to prepare these NE. This ensured a safe and natural method that created stable NEs with high encapsulation efficiencies, low separation phases, and small droplet sizes. Both nanoemulsified systems maintained physicochemical stability over six months at 25°C. The research recommends further *in vitro* studies to validate these cytotoxic and antimicrobial effects and to define the pharmacological profiles of both the free and encapsulated forms of TEO and OEO. Such studies are crucial for establishing safe dosage guidelines and understanding the mechanisms of action, which are essential for their pharmaceutical use.

In conclusion, the study expands the knowledge base on TEO and OEO, demonstrating their cytotoxic and antimicrobial capacities and highlighting the advantages of NE encapsulation for increased efficacy. Compared to their free forms, the encapsulated oils enhanced cytotoxicity and antimicrobial activity, suggesting potential applications in the medicinal and food industries and providing new avenues for cytotoxic and antimicrobial strategies. Encapsulated TEO and OEO emerge as valuable subjects for ongoing anticancer and antimicrobial therapy research and development.

4. MATERIALS AND METHODS

4.1. Chemicals

Reagents including DPPH (2,2-diphenyl-1-picrylhydrazyl), hexane, cholesterol 95%, and HPLC grade absolute ethanol were procured from Sigma-Aldrich, Germany. Medium Chain Triglycerides (MCT) were obtained from Herba Chemosan Apotheker, Austria. Polysorbate (Tween 80) and Lecithin were acquired from Caelo and ROTH, respectively, in Germany.

4.2. Bacterial strains and human cell lines

For cell culture experiments, Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12, MTT (Methylthiazolyldiphenyl-tetrazolium bromide), and DMSO (Dimethyl sulfoxide) were procured from Sigma–Aldrich, Germany. The human cell lines (MCF 7 (ATCC® HTB-22™), HT 29 (ATCC® HTB-38™), and DU 145 (ATCC® HTB-81™), Gram-negative bacteria (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853), Gram-positive bacteria (*Staphylococcus aureus* ATCC 29213), and fungus (*Candida albicans* ATCC 10231) were procured from the American Type Culture Collection (ATCC®), Virginia, USA.

4.3. Plant material and essential oil extraction

The aerial parts of the plants TEO and OEO were specially used to extract essential oil when the plant material was collected in May from Albania's northern region. Voucher specimens of the plant material were placed at the University of Tirana Herbarium after Professor Skerdilaid Xhulaj (University of Tirana) identified the plant material. Plant samples were allowed to air dry at room temperature to extract EOs and then hydrodistillated using Clevenger-style apparatus. To obtain EO at a collection rate of 2-3 mL/min, 20 g of the plant material was hydro-distilled for three hours at 100 °C with 500 mL of distilled water. Following published instructions, the resulting EO was dried over anhydrous sodium sulfate (Sigma-Aldrich) and kept at 4 °C [71]. This extraction method is consistent with those previously reported by our research group [24,25].

4.4. GC-FID/MS analysis

The essential oil samples were analyzed on a Gas Chromatography system with Flame Ionization Detection and Mass Spectrometry provided with a Mass Quadrupole Detector (Agilent 7890A and Agilent 5975C, respectively) as detailed in our previous studies [24,25]. An GC column (HP-5ms), measuring 30 m × 0.25 mm with a film thickness of 0.25 µm, was employed. The GC-FID/MS settings were set as described below: the oven temperature was initially set to 60 °C, then increased by 3 °C/min to 240 °C, and further amplified to 280 °C at 10 °C/min. The injector and FID detector temperatures were 220 and 270 °C, respectively. Helium functioned as the transporter gas, flowing at 1 mL/min. Each sample was injected with a split ratio of 1:1 [24,25]. For mass spectroscopy, the settings were: a mass range of 50–550 Da, ion source temperature 230 °C, and ionization voltage 70 eV. To verify each constituent's identity, MS spectra were compared with those in the NIST and Wiley databases, as well as with spectra from the literature. A normalizing approach was used to quantify the GC-FID peak areas without applying correction factors [24,25,72].

4.5. Nanoemulsion preparation

An optimized NE preparation method was developed through collaboration with Prof. Dr. Zimmer's team at the University of Graz, Austria. This procedure involves separate preparation stages for the oil and water components. Initially, the oil phase was created by mixing 1.5 g of lecithin with 8.5 g of Medium Chain Triglycerides (MCT), totaling 10 g. Meanwhile, in the water phase, 1.5 g of Polysorbate 80 was dissolved in distilled water to make a total of 150 g. Each phase was heated to 75 °C with a thermoregulator and homogenized separately. NE were initially produced by homogenizing the mixture using a T25 Ultra–Turrax (IKA Works, USA) at 8,000 rpm for a set duration to ensure pre-homogenization. In the second step, the NE underwent eight cycles (8–9 minutes at 75 °C) of high-pressure homogenization at 800 bar using a GEA Niro Soavi NS1001L2K (GEA, Italy). After homogenisation, the EOs were encapsulated into the blank NE by being stirred for two hours with a magnetic stirrer. The final NE were stored at 4 °C for further testing alongside control groups comprising blank NEs without EOs.

4.6. Nanoemulsion Droplet size, PDI, and zeta potential measurements

The characteristics of TEO-NE and OEO-NE, such as droplet size (DS), polydispersity index (PDI), zeta potential (ZP), and encapsulation efficiency (EE%) were comprehensively examined. The DS, PDI, and ZP of blank and EO-loaded NE were determined using a Malvern Zetasizer Nano ZS (Malvern, UK).

For the ZP measurements, after allowing a 2-minute equilibration period, one mL of each NE was placed into a folded capillary cell (Malvern, UK). The ZP was then measured using Smoluchowski's equation. All tests were conducted in triplicate at 25 °C, following a 2-minute equilibration time. The results were presented as means ± SD ($n = 3$).

4.7. Rheological properties

The rheological characteristics of the TEO-NE and OEO-NE were assessed using a rotary rheometer, the Physica MCR 301 (Anton Paar, Austria). The testing parameters were established using an Anton Paar CP50-1 cone plate, which included a 50 mm diameter, a cone truncation at 100 µm, and a 1° angle. For each

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test, 800 µl of the sample was carefully placed between the cone (Anton Paar CP50-1) and the plate, reducing the gap incrementally. The experiments were performed at a stable temperature of 25 °C, employing a shear rate (γ) that varied between 50 and 150 s^{-1}.

4.8. Determination of encapsulation efficiency

The method for determining the encapsulation efficiency was adapted from the literature, with slight modifications [73,74]. The NE were centrifuged for 30 minutes at 4,000 rpm. The collected supernatant (2 mL) was mixed with hexane (2 mL). The concentration of the encapsulated EOs were quantified using a UV 1800 UV-Vis spectrophotometer (Shimadzu, Japan), set to measure at a wavelength of 306 nm. The experiment was performed in triplicate. The concentration of EOs were determined against a standard curve, which was prepared using various concentrations of EOs diluted in hexane. The EE% was calculated as a percentage using the formula:

$$EE\% = \frac{(Total\ oil\ -\ Free\ oil)}{Total\ oil} \times 100$$

4.9. Determination of Cytotoxicity with an MTT Cell Viability Assay

The MCF7, DU 145, and HT-29 cell lines were grown in a 1:1 combination of HAM's Nutrient Medium Mixture F12 nutrient medium and Dulbecco's Modified Eagle Medium (DMEM), containing 10% FBS, 100 IU/mL penicillin, 10 mg/mL streptomycin, and 1% L-glutamine. The cultures were maintained in an incubator with 5% CO2. The cells were seeded into 96-well plates at a density of 7x103 cells per well (180 µL). The plates, after seeding, were then putted for incubation at 37 °C for 24 hours, with 5% CO2 [24,25].

The EOs were initially dissolved in DMSO to create a 100 mg/mL stock solution and then further diluted in a medium before being added to plates. Following the incubation period, 20 µl of the diluted sample was added to the cells in the wells at a concentration starting from 0.39 to 200 µg/mL, to calculate the IC50.

In a separate step, the cells were treated with 20 µL of EO-loaded NE. Following a 24-hour incubation, the plates were incubated for 4 hours at 37 °C with the addition of MTT solutions (20 µL/200 µL each well of a 5 mg/mL solution). Lastly, in each well 200 µL of DMSO was added. Using a microplate reader (Thermo Scientific, USA), the optical densities were determined at 492 nm. 100% cell viability was interpreted from the absorbance of untreated cells [24,25].

Based on the absorbance values, the percentage of cell viability and the IC50 values were calculated by using the following formula:

$$Cell\ Viability\ (\%) = \frac{Sample\ absorbance\ value}{Control\ absorbance\ value} \times 100$$

The control group without treatment was set to 100%, and the treatment results were reported as percentages in comparison to the control. The cytotoxicity assay was conducted in triplicate over different days, following methods previously described in the literature [24,25]. Results are expressed as the mean value ± SD ($n = 3$).

4.10. Determination of Antioxidant Activity (DPPH Radical Scavenging Assay)

The antioxidant capabilities of free-unloaded EOs and EO-loaded NE were assessed using a slightly modified version of a previously documented method [23,24]. In this procedure, 1 mL of either EO or EO loaded-NE (500 µg/mL) was combined with 2 mL of a 0.125 mM DPPH ethanolic solution. These combinations were then incubated at 25 °C in a dark environment for 10 min, followed by a centrifugation step at 15,000 rpm for 15 min. Post-centrifugation, the absorption of the supernatant was measured at a wavelength of 517 nm using a UV 1800 UV-Vis spectrophotometer (Shimadzu, Japan). All measurements were performed in triplicate, with results presented as mean values ± SD ($n = 3$). The percentage of DPPH scavenging activity was calculated using the formula:

$$DPPH\ Scavenging\ activity\ (\%) = \frac{Sample\ absorbance\ value}{Control\ absorbance\ value} \times 100$$

4.11. Antimicrobial Activity, Determination of MIC and MBC/MFC

The *in vitro* antibacterial and antifungal activity of OEO, TEO, and their NE was utilized using Gram-negative bacteria (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853), Gram-positive bacteria (*Staphylococcus aureus* ATCC 29213), and fungus (*Candida albicans* ATCC 10231). The MIC, MBC, and MFC of the TEO, OEO, TEO-NE, and OEO-NE against each microorganism were evaluated using the microdilution method in 96 well plates according to the Clinical and Laboratory Standards Institute (CLSI) [18,30,75]. Serial dilutions were applied to OEO, TEO, and respective NE (OEO-NE and TEO-NE) to rich final concentrations (2.5–0.0625 mg/mL) using sterile Mueller Hinton broth, and Mueller Hinton broth with 0.5% Tween 80, respectively for bacterial strains and RPMI 1640 medium buffered with MOPS at pH 7.0 (for Candida albicans). Each well was inoculated with microbial suspensions from 24h broth cultures and incubated at 37 °C for 24h. Wells containing microorganisms inoculum and respective dilution medium (Mueller Hinton broth; Mueller Hinton broth with 0.5% Tween 80; RPMI 1640 medium buffered with MOPS) were used as positive controls and wells containing respective dilution medium and empty NE were used as negative control. The plates were then incubated at 37 °C for 24 h. The OD was measured using a microplate reader at 595 nm. At the time of testing, agar plates were used to determine the bacterial numbers. Following the incubation period, the initial dilutions revealed that no bacterial growth was recorded as MICs. To ascertain MBCs and MFCs, the suspensions from wells that did not exhibit microbiological growth were cultured on respective Agar plates for the bacteria and yeast and incubated at 37 °C for 48 h. The lowest concentrations that did not result in colonies on the agar plates were known as the MBC and MFC values. Each experiment was performed in triplicates.

4.12. Stability Testing of Nanoemulsions

TEO- and OEO-loaded NE were evaluated for their physicochemical stability after a storage period of 6 months at 25 °C. Parameters such as droplet size (DS), polydispersity index (PDI), and zeta potential (ZP) were assessed during this period. The storage conditions were maintained using the Memmert Climate Chamber ICH110, set to a temperature of 25 ± 2 °C and a relative humidity of 60 ± 5% [25].

4.13. Statistical analysis

All experiments were repeated three times and expressed as a mean value ± SD (n = 3). Statistical analyses were performed using GraphPad Prism 3.0, specifically by using one-way ANOVA and Tukey cross-comparison tests for all study groups. Values of $p<0.05$ were considered significant.

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