RESEARCH ARTICLE

Development of two stable green nanoformulations with potent anticancer properties

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ABSTRACT

The most common cause of cancer death among women is breast cancer thus, developing new drugs is crucial. Essential oils (EO)s with a wide range of bioactivities, such as anticancer effects, have provided a valuable source for this purpose. In this study, components of two medicinally important EOs were identified using GC-MS analysis. Moreover, antioxidant effects, as well as anti-cancer properties, were evaluated.

The EOs formulated into nanoemulsion using the spontaneous emulsification approach, separately. Comprehensive stability tests were performed to select the optimum nanoemulsions of each EO. Anticancer effect of the most stable nanoemulsion of *Zataria multiflora* with a particle size of 43 ± 4 nm (PDI 0.4 ± 0.2 and SPAN 0.6 ± 0.1) was significantly better non-formulated form against four human breast cancer cell lines, MCF-7, MDA-MB-175, MDA-MB-231, and MDA-MB-468. Interestingly, the anticancer effect of *Artemisia dracunculus* nanoemulsion (16 ± 4 nm, 0.2 ± 0.1, and 0.4 ± 0.1) was also significantly improved in compassion to the non-formulated EO. Considering the results, prepared nanoemulsions could be used as supplementary drugs or food additives.

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INTRODUCTION

The most frequent cancer among women is breast cancer. Despite many attempts to developing or optimizing chemotropic agents, it is responsible for around 500.000 death annually [1]. Common side effects of using chemotrophic drugs such as hair loss, anemia, and toxic influence on other organs, encouraged researchers to find new medicines. Recently, anticancer investigation of herbal substrates (e.g., extractions and essential oils (EO)s) is common around the world [2].

EOs defined as natural liquid oils that secreted as secondary metabolites from different parts of aromatic plants such as rhizome, stem, bark, and fruit [3]. Hydrodistillation using the Clevenger type apparatus introduced as the most common approach for the extraction of EOs. Besides, EOs shows other bioactivities such as larvicidal action [4], antibacterial, and antifungal effects [5]. EOs with antioxidant activity can prevent oxidant-mediated phenomena by scavenging of free radicals [6]. Alzheimer's, Mellitus diabetes, development of cancer, and atherosclerosis are common chronic diseases, which related to free radicals [7, 8].

In this study, the ingredients of the two medicinally important EOs, including *Artemisia dracunculus* (ADEO) and *Zataria multiflora* (ZMEO), were identified using GC-MS analysis. Also, their antioxidant activities were investigated. Furthermore, for the first time, anticancer activities of ZMEO and ADEO was investigated on four human breast cancer lines, MCF-7, MDA-MB-175,

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MDA-MB-231, and MDA-MB-468. Additionally, we tried to improve their effectiveness by formulating stable nanoemulsions.

MATERIAL AND METHOD

Materials

Human breast cancer cell lines including MCF-7 (ATCC HTB-22), MDA-MB-175 (ATCC: HTB-25), MDA-MB-231 (ATCC: HTB-26) and MDA-MB-468 (ATCC: HTB-132) were provided by Pasteur Institute of Iran. Powders of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and tablets of phosphate-buffered saline (PBS) were bought from Sigma-Aldrich (USA). Dimethyl Sulfoxide (DMSO), Penicillin-Streptomycin, RPMI, and Trypsin were purchased from Shellmax (China). Fetal bovine serum (FBS) was bought from Gibco (USA). Zardband Pharmaceuticals Co (Iran) provided ADEO and ZMEO. Noted, the EOs were extracted from leaves of the dried plants.

GC-MS analysis

Components of the EOs were identified by GC-MS analysis, as described in our previous study [9].

Investigation antioxidant activities of the EOs

The DPPH assay was used for the investigation of the antioxidant activities of ZMEO and ADEO as follows. EOs were dissolved in ethanol, separately, for making defined concentration in a range of 81920 - 5 $\mu g.mL^{-1}$. The standard solution of DPPH (MW: 394.32 $g.mole^{-1}$) with a concentration of 0.3 mM was also prepared using the same solvent.

For the preparation of the reaction mixture, 40 and 160 µL of the prepared serial dilutions of EOs (separately) and DPPH standard solutions were added to each well. The treated plates were incubated 30 min away from light for completing the reaction. The absorbance (A) of wells was read at 517 nm using a plate reader machin (Model: Synergy HTX Multi-Mode Reader, USA). The antioxidant activity at different concentrations was calculated using equation 1. The test was repeated three times, and in each replicate, 12 wells considered as control groups, filled with 40 and 160 µL of ethanol and DPPH standard solution.

$$Antioxidant\ activity(\%) = (A\,control - A\,sample / A\,control) \times 100 \quad (1)$$

Preparation of nanoemulsions

Some of the components of EOs are volatile; the spontaneous emulsification method was used for the preparation of nanoemulsions. In this approach, no need to use of mechanically or sonically energies [10]. Defined amounts of ADEO (3.2 µL) or ZMEO (0.8 µL) and tween 20 were mixed 10 min (500 rpm, room temperature) to form a homogenous solution. Then, the PBS solution was added dropwise to the mixture up to the desired volume of 5000 µL. The prepared mixture was stirred for another 30 min at 1500 rpm.

Size analyses

The particle sizes (PS), polydispersity index (PDI), and particle size distribution (SPAN) of prepared emulsions were determined using a nanoparticle size analyzer apparatus (SZ-100 series, HORIBA Scientific, Japan). PDI was calculated using $(\sigma / d)^2$, which σ is the standard deviation of the mean diameter of nanoparticles, and d is the mean of the diameter of nanoparticles. Moreover, SPAN was determined using (D90-D10)/D50, Dn: percentile of particles that have a diameter lower than those values. Nanoformulations with PS <200 nm, PDI <0.7, and SPAN <1 were selected for further investigation, i.e., stability tests.

Stability tests

The stability of the selected nanoemulsions was investigated using short- and long-time tests. The samples that remained stable at each stage, chosen for the next stability test; un-stable samples were discarded from further investigation. Finally, stable nanoemulsions were chosen for anticancer research.

Furthermore, blank samples were prepared to evaluate the effect of the selected nanoemulsions constituents on the targeted cell lines. The blank samples were prepared using the described preparation process and the same ingredients; only no EOs were used.

Short-time

***Centrifugation*:** Selected nanoemulsions were centrifuged at 22000 g for 60 min at three temperatures, ± 4 and 25°C, separately. After that, samples visually checked for creaming or bi-phasic

***Heating and cooling process*:** Chosen nanoemulsions were placed at 4 and 45°C for six consecutive periods (48 h). They followed by checking for phase separation.

Freeze-thaw cycles: For performing this test, the selected nanoemulsions put down at ±25°C for four successive periods (48 h). Then, samples visually checked for creaming or phase separation.

Long-time

The samples were placed in two different conditions (4°C and ambient temperature) for six months. Then, samples were checked using DLS for investigating any significant changes in size parameters (PS, PDI, and SPAN).

Evaluation of anticancer activity

Preparation of serial dilutions

The EOs were dissolved in the PBS solution at a concentration of 5120 μg.mL^{-1}, separately. Then, serial dilutions were prepared with two-fold dilutions of the solution to make a concentration range of 5120 - 10 μg.mL^{-1}.

Cell culture

The cell lines were cultured in RPMI complete medium (containing FBS (10%) and Penicillin-Streptomycin (1%)) and incubated at 37°C, air (95%) and CO_2 (5%). After that, cells were seeded in 96-well plates, ~7000 cells/well. Then plates were incubated for another 24 h for attaching the cells to the plates and reached confluence 70-80%.

Followed by, the content of wells was discarded, and 150 μL of RPMI complete fresh medium was added to each well. After the addition of the prepared serial dilutions to the wells (50 μL/well), the concentration of each EO was fixed at 1280, 640, 320, 160, 80, 40, 20, 10, and 5 μg.mL^{-1}, respectively. Then treated plates were incubated for 48 h. The MTT assay was used for determining the anticancer activities of the EOs.

MTT assay

Briefly, MTT powder was dissolved (0.5 mg.mL^{-1}) in RPMI medium. Then, the content of the 48 h incubated plates were discarded, and 100 μL/well from MTT solution was added and were incubated for another 4 h. After that, 100 μL per well of DMSO was and mixed thoroughly to dissolve the dye crystals. Finally, the absorbance (A) of the wells was measured at 570 nm, using a plate reader (Synergy HTX Multi-Mode Reader, USA). The cell viability at each concentration was calculated by equation 2.

Furthermore, the anticancer effects of the selected nanoemulsions, as well as their ingredients, were also investigated using MTT assay as described.

$$Cell\,viability(\%) = (A\,sample / A\,control) \times 100 \quad (2)$$

Statistical Analyses

All the tests were performed in triplicates. Calculation of means, SD, and drawing charts were performed using Excel software (Version 2010, Microsoft Corporation, USA). The determination of 50% inhibitory concentration (IC50) of EOs was calculated by the CalcuSyn software (Free version, BIOSOFT, UK). For comparison of anticancer/oxidant activities of EOs or nanoemulsions together, one-way ANOVA or independent sample t-test with a confidence interval of 95% (CI 95%) were used. Those analyses were performed using SPSS software (Version 22, SPSS Inc, USA).

RESULTS AND DISCUSSION

Identified components in the EOs

Identified ingredients of ADEO (43 compounds) and ZMEO (37 compounds) are listed in Table 1. Estragole, cis-ocimene, β-ocimene, limonene, and 3-methoxycinnamaldehyde were identified as major constituents in ADEO with a portion of 67.62, 8.69, 7.57, 4.33, and 1.49% respectively. While Carvacrol (30.23%), thymol (25.20%), o-cymene (10.73%), γ.-terpinene (6.13%), and α-pinene (3.61%) were identified as major components of ZMEO.

Antioxidant properties of the EOs

Profiles of antioxidant activities of the EOs are illustrated in Fig. 1. The antioxidant effect was investigated in 15 different concentrations, 5 – 81920 μg.mL^{-1}. ADEO has no antioxidant properties, even at the highest concentration. While the antioxidant effect was improved (0 - ~ 85%) with an increasing level of ZMEO. The calculated IC50 for ZMEO was 4618 μg.mL^{-1}. Its lower and higher confidence limits were estimated as 2979 and 7158 μg.mL^{-1}, respectively.

As details show, no significant improvement was seen in the antioxidant activity of ZMEO at the three highest concentrations, including 20480, 40969, and 81920 μg.mL^{-1} (sig > 0.05, one-way ANOVA). This event was due to the final capacity of this substance as an antioxidant.

Reviewing the literature, ZMEO is more potent than many other EOs as an antioxidant agent; thus, it can be used to prevent oxidative-related diseases such as cancer. For instance, antioxidant activities

Table 1: Identified ingredients in ADEO (Left) and ZMEO (Right) using GC-MS analysis

No.	Compounds	%	tR[a]	RI[b]	Compounds	%	tR	RI
1	α-Thujene				α-Thujene	0.463	9.193	604
2	α-Pinene				α.-Pinene	3.616	9.483	613
3	Camphene				Camphene	0.199	10.036	625
4	Sabinene				β.-Pinene	0.587	11.248	646
5	β-Pinene				1-Octen-3-ol	0.093	11.569	704
6	β-Myrcene	0.233	8.979	605	3-Octanone	0.310	11.827	710
7	o-Cymene	0.410	10.448	662	β.-Myrcene	1.182	11.991	715
8	Limonene	4.338	10.727	673	3-Octanol	0.109	12.274	722
9	cis-Ocimene	8.691	11.321	696	α.-Phellandrene	0.172	12.502	727
10	β-Ocimene	7.577	11.899	712	3-Carene	0.058	12.757	734
11	γ-Terpinene	0.966	12.166	719	α.-Terpinene	1.443	13.083	742
12	α-Terpinolene	0.263	13.36	749	o-Cymene	10.731	13.584	755
13	Linalool	0.288	14.214	770	Limonene	0.915	13.688	757
14	Allocimene	0.438	15.853	809	1,8-Cineole	2.491	13.804	760
15	Estragole	67.623	19.176	876	β.-trans-Ocimene	0.043	14.607	780
16	Cuminic aldehyde	1.110	21.66	924	γ.-Terpinene	6.139	15.144	794
17	Carvone	0.048	21.698	925	cis-Sabinenehydrate	0.117	15.472	802
18	Anisaldehyde	0.124	21.947	930	α-Terpinolene	0.145	16.408	820
19	Geranial	0.118	22.365	938	Linalool	1.977	17.12	835
20	Nerol	0.025	22.69	944	Borneol	0.163	20.101	895
21	Bornyl acetate	0.517	22.929	948	4-Terpineol	0.936	20.617	905
22	α-Terpinene	0.095	24.827	984	β. Fenchyl alcohol	0.797	21.692	925
23	Cyclohexylmorpholine	0.080	25.265	992	2-isopropyl-5-methyl methoxybenzene	0.615	23.281	955
24	Eugenol	0.295	25.711	1001	Carvacrol methyl ether	1.329	23.705	963
25	α-Copaene	0.095	26.362	1013	l-Carvone	0.111	24.462	977
26	Cinnamic acid methyl ester	0.158	26.698	1019	trans-Anthole	0.215	25.594	998
27	β-Elemene	0.030	27.007	1025	Thymol	25.202	26.452	1015
28	Methyleugenol	0.768	27.687	1038	Carvacrol	30.238	27.05	1026
29	trans-Caryophyllene	0.159	28.119	1047	Thymyl acetate	1.180	28.69	1057
30	α-Bergamotene	0.041	28.742	1058	Carvacryl acetate	2.033	29.477	1072
31	α-Humulene	0.020	29.456	1072	trans-Caryophyllene	2.195	31.291	1107
32	trans-β-Farnesene	0.029	29.582	1074	γ.-Selinene	0.072	31.6	1113
33	2(3H)-Furanone, 5-hexyldihydro-	0.054	30.095	1084	Aromadendrene	1.198	32.066	1123
34	Acoradiene	0.255	30.314	1088	Aromadendrene	0.134	32.934	1140
35	Germacrene D	0.067	30.561	1093	Ledene	0.597	34.35	1168
36	cis-trans-α-Farnesene	0.114	31.131	1104	(+) spathulenol	0.820	37.648	1234
37	peri-Ethylenenaphthalene	0.238	31.242	1106	Caryophyllene oxide	1.364	37.823	1238
38	trans-trans-α-Farnesene	0.053	31.617	1114				
39	β-Sesquiphellandrene	0.123	32.246	1126				
40	3-Methoxycinnamaldehyde	1.491	34.245	1166				
41	Spathulenol	0.285	34.537	1172				
42	7-Methoxycoumarin	0.081	39.807	1278				
43	Nonadecane	0.044	45.615	1411				

[a]Retention Time and [b]Retention Index

(IC50) of EOs of *Allium rotundum* [11], *Piper nigrum* [12], *Achillea ageratum* [13], and *Eucalyptus gunnii* [14] were reported at 786000, 38770, 7490, and 7190 μg.mL^{-1}, respectively. It can be said that ZMEO has an acceptable antioxidant effect.

Anticancer activity of the EOs

In Fig. 2 effect of ADEO and ZMEO at different concentrations on four human breast cancer cell lines is demonstrated. Totally, by increasing the concentration of EOs, the viabilities of the cell lines were decreased. Interestingly viabilities of three cell lines, MCF-7, MDA-MB-175, and MDA-MB-468,

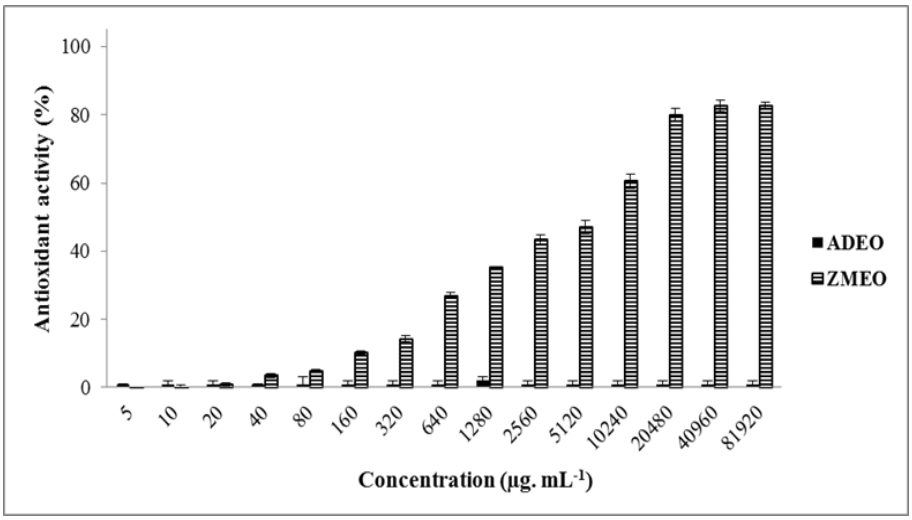
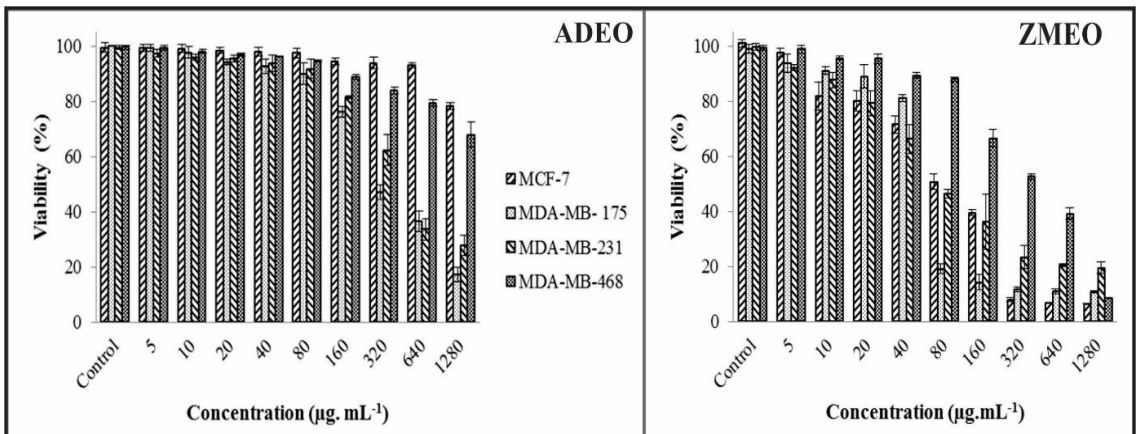


Fig. 1: Antioxidant activities of ADEO and ZMEO



Parameter	MCF-7	MDA-MB-175	MDA-MB-231	MDA-MB-468	MCF-7	MDA-MB-175	MDA-MB-231	MDA-MB-468
IC50	5679	307	527	4504	76	70	104	302
LCL-UCL	3317-9722	218-433	345-805	2176-9323	54-106	43-114	80-134	208-438

Fig. 2: Anticancer activities of ADEO and ZMEO (IC50; Inhibitortry concentration 50%, LCL and UCL; Lower and Upper Confidence Limits)

reduced to ~ 8% after 48 h treatment with ZMEO with the concentration of 1280 µg.mL^{-1}.

As can be seen, ADEO 160 µg.mL^{-1} was the lowest concentration, whose effect is significantly better than the previous point (80 µg.mL^{-1}). Also, at this point, the viabilities of the cell lines were closed together (76-94%) in comparison with the higher concentrations. Therefore, this point was selected to investigate the effect of the formulating of ADEO to nanoemulsion. Besides, the concentration of 40 µg.mL-1 was also selected for comparison of the anticancer effect of ZMEO, and its nanoemulsion dosage form.

Observed IC50s (with lower and upper confidence limits) of ADEO and ZMEO on examined cell lines are also given in Fig. 2. As details show, the effectiveness of ZMEO on all targeted cell lines was significantly better than ADEO (Independent sample t-test, sig < 0.05).

Furthermore, comparing the observed IC50s of ADEO on cell lines, a significant difference was observed. For example, obtained IC50 for MDA-

Table 2: Ingredients and size analyses of the prepared emulsions of ADEO and ZMEO

No.	Ingredients (µL)				Size analyses		
	ADEO	ZMEO	Tween 20	PBS	Mean ± SD	PDI ± SD	SPAN ± SD
1	3.2	-	1.6	4995.2	14 ± 5	12.4 ± 2	0.1 ± 0.4
2	3.2	-	3.2	4993.6	14 ± 7	10.0 ± 5.1	1.2 ± 0.8
3	3.2	-	4.8	4992	3967 ± 8	11.9 ± 3.0	1.3 ± 0.7
4	3.2	-	6.4	4990.4	429 ± 5	3.0 ± 2.3	0.9 ± 0.4
5	3.2	-	8	4988.8	2 ± 1	6.8 ± 1.6	1.8 ± 1.1
6	3.2	-	9.6	4987.2	15 ± 6	0.9 ± 0.4	1.7 ± 0.1
7	3.2	-	11.2	4985.6	16 ± 4	0.2 ± 0.1	0.4 ± 0.1
8	3.2	-	12.8	4984	106 ± 4	1.1 ± 0.7	1.4 ± 0.7
9	3.2	-	14.4	4982.4	14 ± 2	0.5 ± 0.1	0.4 ± 0.1
10	3.2	-	16	4980.8		Not available	
1	-	0.8	0.4	4998.8	12 ± 3	8.1 ± 3.2	0.6 ± 0.1
2	-	0.8	0.8	4998.4	160 ± 10	0.5 ± 0.1	0.4 ± 0.2
3	-	0.8	1.2	4998.0	26 ± 4	2.9 ± 0.9	0.3 ± 0.1
4	-	0.8	1.6	4997.6	110 ± 26	5.7 ± 0.6	2.1 ± 1
5	-	0.8	2	4997.2	43 ± 4	0.4 ± 0.2	0.6 ± 0.1
6	-	0.8	2.4	4996.8	15 ± 7	3.9 ± 1.6	1.1 ± 0.9
7	-	0.8	2.8	4996.4	2 ± 2	12.3 ± 0.5	1.6 ± 0.5
8	-	0.8	3.2	4996.0	11 ± 2	0.6 ± 9	0.9 ± 0.1
9	-	0.8	3.6	4995.6		Not available	
10	-	0.8	4	4995.2		Not available	

MB-175 or MDA-MB-231 was significantly lower than MCF-7 and MDA-MB-468 (Independent sample t-test, sig < 0.05). While no significant difference was seen between the IC50s of MDA-MB-175 and MDA-MB-23, also MCF-7 and MDA-MB-468 (Independent sample t-test, sig < 0.05). Noted that, observed IC50 of ZMEO against MDA-MB-468 significantly higher than the other three cell lines (one-way ANOVA, sig < 0.05); confirmed it is more resistant than other cell lines to this EO.

Interestingly, no document was found on the anticancer activities of ZMEO, and ADEO on targeted cell lines. As well as, no report was found on the investigation of the anticancer activity of any EO against MDA-MB-175. Besides, just two reports were found on MDA-MB-468. IC50 of EOs of *Kelussia odoratissima*, *Peristrophe bicalyculata*, and *Borreria verticillata* were observed at 85.00, 66.6, and 20.4 µg.mL^{-1}, respectively [15, 16].

Furthermore, obtained IC50 (µg.mL^{-1}) of ZMEO (76.08) against MCF-7 in this study, was better than many other EOs. For example, *Lippia citriodora* (89.00) [17], *Ocimum basilicum* (170.00) [18], *Mentha spicata* (284.00) [18], and *Rosmarinus officinalis* (253.00) [19].

However, its efficiency on MDA-MB-231 was worse than some of the reports. For instance, IC50 of *Hedychium spicatum* [20], *Blepharocalyx salicifolius* [21], *Teucrium yemense* [22], and *Decatropis bicolor* [23] were reported as 70.00, 46.00, 59.81, and 53.81 µg.mL^{-1}, respectively. Regarding the mentioned docs, ZMEO possesses acceptable potency as an anticancer agent.

Prepared emulsions

Ingredients of 10 prepared emulsions of each EOs and their size analyses are listed in Table 2. PS of the prepared emulsions of ADEO was in the range of 2 – 3967 nm. However, PS of all prepared ZMEO emulsion was lower than 200 nm (Expectancy No. 9 and 10). Noted that size analyses of sample 10 in ADEO and samples of 9 and 10 in

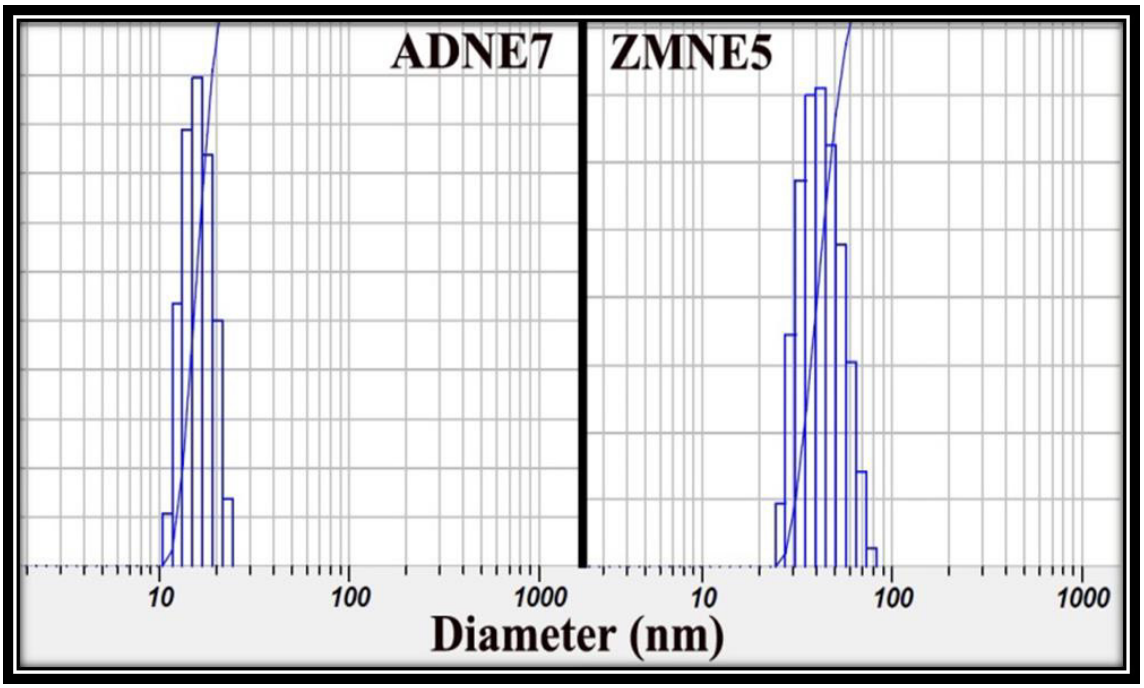


Fig. 3: DLS analysis of optimum nanoemulsions (PS, PDI, and SPAN): ADNE7 (16 ± 4 nm, 0.2 ± 0.1, and 0.4 ± 0.1) and ZMNE5 (43 ± 4 nm, 0.4 ± 0.2, and 0.6 ± 0.1)

ZMEO were not available; it is due to the limit of the range (0.3 – 8000 nm) for the measurement of the particles.

Furthermore, using distilled water as an aqueous phase in the preparation of nanoemulsions is common. However, in this study, the PBS solution was used as an aqueous phase for the prevention of cell damages caused by osmotic pressure change during MTT assays [24]. Moreover, for obtaining optimum nanoemulsion, using co-surfactant (one or more types of alcohol) is common [25]. Nevertheless, for a precise examination of the effect of formulating of EOs into nanoemulsions on anticancer activities, co-surfactant was not used.

For the preparation of optimum nanoformulations, the balance between ingredients is necessary [26]. Among the prepared emulsions of ADEP, only two samples (No. 7 and 9) had acceptable values (PS < 200 nm, PDI < 0.7, and SPAN < 1). They named ADNE7 and ADNE10 and selected for stability investigations

Besides, PDI and SPAN of only three samples of ZMEO (2, 5, and 8) were in the acceptable range (PDI < 0.7 and SPAN <1). They called ZMNE2, ZMNE5, and ZMNE8 and were selected for stability tests.

From the literature, the PS of the selected nanoemulsions is comparable with other reports on essential oil-based nanoemulsions. For instance, PS of nanoemulsions of *Thymus daenensis* and *Rosmarinus officinalis* EOs as antibacterial and larvicidal agents reported as 143 and 200 nm, respectively [27, 28], In another study, antibacterial, antibiofilm, and antioxidant, activities nanoemulsion *Citrus medica* EO were investigated; PS was determined at 73 nm [29].

Stability of the selected nanoemulsions

Stability in short-time tests

Centrifugation: Selected nanoemulsions (ADNE7, ADNE10, ZMNE2, ZMNE5, and ZMNE8) were centrifuged at 22000 g for 60 min at three temperatures (± 4 and 25°C), separately. Samples visually checked and reveal that a precipitant was created only in ZMNE2. Thus, it was discarded from further investigations.

For the preparation of stable nanoformulation, a proper ratio between EO or drug to carrier (such as surfactant or polymer) is crucial [30]. Generally, the increasing proportion of carriers to cargo leads to forming a stable complex [31]. This un-stability of ZMNE2 was resulted from using an equal ratio

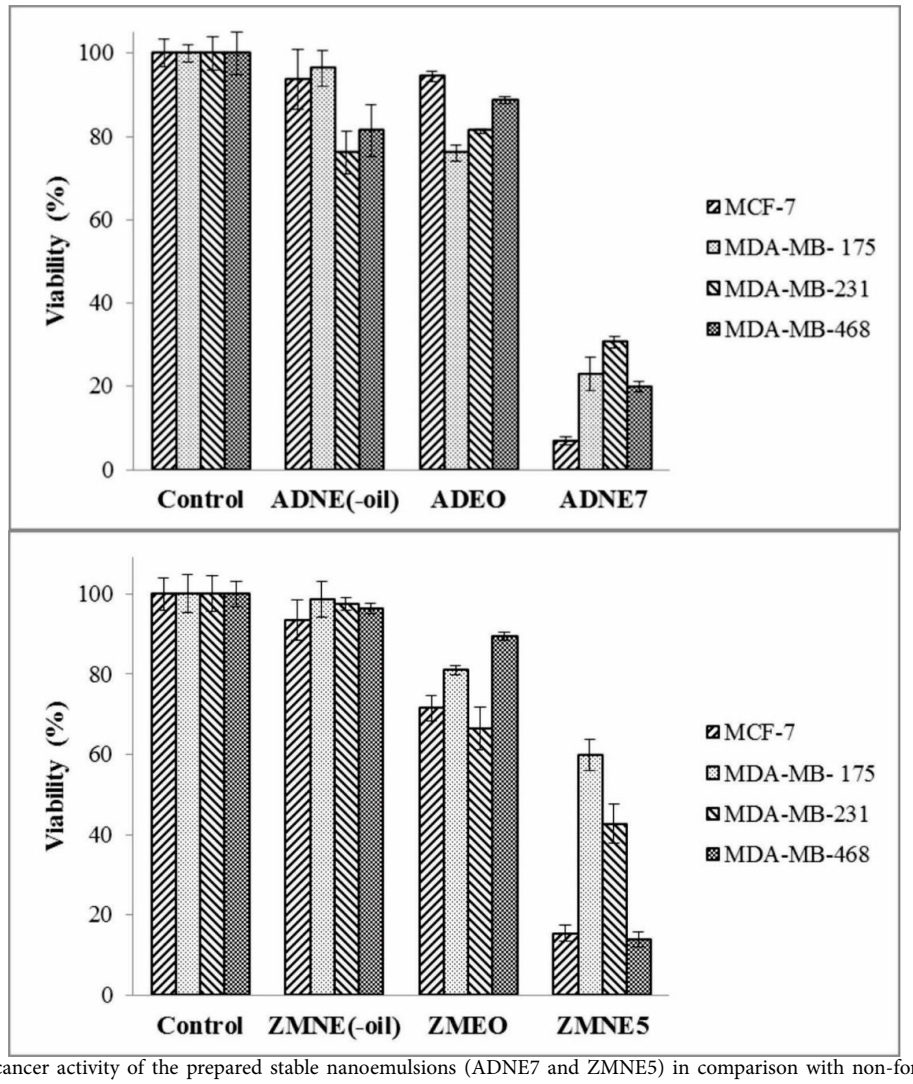


Fig. 4: Anticancer activity of the prepared stable nanoemulsions (ADNE7 and ZMNE5) in comparison with non-formulated EO (ADEO and ZMEO) and ingredients of nanoemulsion (ADNE(-oil) and ZMNE(-oil))

of ZMEO (cargo) and tween 20 (carrier); 0.8 µL in 5000 µL.

Heating and cooling process: The remained nanoemulsions were placed at a defined temperature (4 and 45°C) for six continuous 48 h periods. No changes (phase separation or creaming) were observed in nanoemulsions. Thus, all four examined nanoemulsions, including ADNE7, ADNE9, ZMNE5, and ZMNE8, were selected for further investigations.

Freeze-thaw cycles: the mentioned nanoemulsions were placed at ±25°C for four successive periods (48 h). Particulate matter was observed in ZMNE8 and ADNE9 after this test. So they were also excluded from further examinations. Among the five selected nanoemulsion, just ADNE7 and ZMNE5 passed the short-time stability tests and were chosen for a long-time stability test. Their DLS analysis is illustrated in Fig. 4.

As details are shown in Table 2, PS of ADNE7 and ADNE9 had no significant difference together, but PDI of ADNE9 was almost 2.5 times bigger than stable nanoemulsion (ADNE7). In addition, the PDI and SPAN of un-stable nanoemulsion (i.e., ZMNE8) were significantly bigger than ZMNE5 (stable sample). According to Ostwald's ripening phenomenon, larger particles can be grown by devouring smaller particles in critical condition like Freeze-thaw cycles. Finally, the disintegration in the formulation can be occurred, as was observed in the results of this study.

Stability of chosen nanoemulsions in long-time test

ADNE7 and ZMNE5 were stored at 4°C and room temperature for six months. After that, no creaming, phase separation, or sedimentation was observed. Also, no significant changes in PS, PDI, and SPAN were detected (data not reported). According to the results, both nanoemulsions of ADNE7 and ZMNE5 were entirely stable and selected for anticancer investigation.

Anticancer activity of the chosen nanoemulsion in comparison to non-formulated EOs

In Fig. 4, the anticancer activities of ADNE7 and ADEO at the concentration of 160 $\mu g.mL^{-1}$ were compared. Also, the anticancer effect of blank formulation, i.e., ADNE(-oil), was given. ADNE(-oil) had a significant effect on MDA-MB-231 and MDA-MB-468; their viabilities reduced to 76 and 81%, respectively. Besides, the viabilities of MCF-7, MDA-MB-175, MDA-MB-231, and MDA-MB-468 were reduced to 94, 76, 81, and 88%, respectively, after treatment with ADEO. These values were significantly decreased after treatment with ADNE7 (Independent sample t-test, sig < 0.05); MCF7 6%, MDA-MB-175 23%, MDA-MB-231 30%, and MDA-MB-468 19%.

Effect of ZMEO, ZMNE5, and ingredients of nanoemulsion (ZMNE(-oil)) on human breast cancer cell lines are also shown in Fig. 4. ZMNE(-oil) had no significant effect on the viabilities of the cell lines in comparison with the control group (Independent sample t-tets, sig > 0.05). Viability of all treated cell lines with ZMNE5 were significantly decreased in comparison with ZMEO: MCF-7 (15 < 71%), MDA-MB-175 (59 < 80%), MDA-MB-231 (42 < 66%), and MDA-MB-468 (13 < 89%) (Independent sample t-test, sig < 0.05).

As results show in Fig. 4, the viabilities of all cells after treatment with the prepared stable nanoemulsion were significantly decreased in comparison to their ingredients as well as non-formulated EOs. Nowadays, it has been accepted that the use of surfactants in the preparation of nanoformulation has a direct effect on enhancing permeability, stability, and efficacy of cargo [32, 33]. Moreover, the reduction of the size of the particles to nanoscale leads to higher contact with the cell surface and improvements of penetration [34]. Therefore, in this research, by formulating ADEO and ZMEO to nanoemulsions, significant reductions in cell viabilities were observed.

Furthermore, the main reasons for nanoformulating of the chemotherapy drugs are reducing side effects, improvement of efficacy, and prolonged circulation time in the bloodstream [35, 36]. While side effects of EOs are lower than industrial drugs. Interestingly, the effectiveness of some EOs is comparable to chemical drugs. For example, IC50s of *Eryngium campestre* on A375, MDA-MB231, and HCT-116 were reported as 1.57, 2.99, and 1.64 $\mu g.mL^{-1}$, respectively, While these values for Cisplatin were 0.41, 2.74, and 2.34 $\mu g.mL^{-1}$, respectively [37]. In this type of researches (i.e., developing green nano drugs), the focus of researchers is only on improving the effectiveness or better targeting.

Considering the mentioned comments, as well as the necessity for developing new anti-cancer drugs, it is anticipated that the nanoformulating of EO as anticancer drugs or supplementary medicine will be considered around the world in early ahead years.

CONCLUSION

Chemical composition and antioxidant properties of two medicinally important EOs were investigated. For the first time, anticancer activities of ZMEO and ADEO were investigated on four types of human breast cancer cell lines including, MCF-7, MDA-MB-175, MDA-MB-231, and MDA-MB-468. Comprehensive tests were performed for selecting optimum nanoformulations. Anticancer activities of the stable nanoemulsions were significantly better than no-formulated EOs. Thus could be used as supplementary anticancer drugs or food additives.

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CONFLICT OF INTEREST

There is no conflict of interest among the authors.

REFERENCES

1. Costa A, Kieffer Y, Scholer-Dahirel A, Pelon F, Bourachot B, Cardon M, Sirven P, Magagna I, Fuhrmann L, Bernard C. Fibroblast heterogeneity and immunosuppressive environment in human breast cancer. Cancer Cell, 2018;33 (3):463-479. e410.
2. de Sousa AC, Gattass CR, Alviano DS, Alviano CS, Blank AF, Alves PB. Melissa officinalisL. essential oil: antitumoral and antioxidant activities. Journal of Pharmacy and Pharmacology. 2004;56(5):677-81.

3. Sangwan N, Farooqi A, Shabih F, Sangwan R. Regulation of essential oil production in plants. Plant Growth Regul, 2001;34 (1):3-21.
4. Osanloo M, Mehdi Sedaghat M, Esmaeili F, Amani A. Larvicidal Activity of Essential Oil of Syzygium aromaticum (Clove) in Com-parison with Its Major Constituent, Eugenol, against Anopheles stephensi. Journal of Arthropod-Borne Diseases. 2019.
5. Baharfar R, Azimi R, Mohseni M. Antioxidant and antibacterial activity of flavonoid-, polyphenol- and anthocyanin-rich extracts from Thymus kotschyanus boiss & hohen aerial parts. Journal of Food Science and Technology. 2015;52(10):6777-83.
6. Venditti A, Frezza C, Bianco A, Serafini M, Cianfaglione K, Nagy DU, et al. Polar Constituents, Essential Oil and Antioxidant Activity of Marsh Woundwort (Stachys palustrisL.). Chemistry & Biodiversity. 2017;14(3):e1600401.
7. Dakah A, Zaid S, Suleiman M, Abbas S, Wink M. In vitro propagation of the medicinal plant Ziziphora tenuior L. and evaluation of its antioxidant activity. Saudi Journal of Biological Sciences. 2014;21(4):317-23.
8. Yoo KM, Lee CH, Lee H, Moon B, Lee CY. Relative antioxidant and cytoprotective activities of common herbs. Food Chemistry. 2008;106(3):929-36.
9. Osanloo M, Sereshti H, Sedaghat MM, Amani A. Nanoemulsion of Dill essential oil as a green and potent larvicide against Anopheles stephensi. Environmental Science and Pollution Research. 2017;25(7):6466-73.
10. Osanloo M, Abdollahi A, Valizadeh A, Abedinpour N. Antibacterial potential of essential oils of Zataria multiflora and Mentha piperita, micro- and nano-formulated forms. Iranian Journal of Microbiology. 2020.
11. Assadpour S, Nabavi SM, Nabavi SF, Dehpour AA, Ebrahimzadeh MA. In vitro antioxidant and antihemolytic effects of the essential oil and methanolic extract of Allium rotundum L. European Review for Medical and Pharmacological Sciences, 2016;20 (24):5210-5215.
12. Nikolić M, Stojković D, Glamočlija J, Ćirić A, Marković T, Smiljković M, et al. Could essential oils of green and black pepper be used as food preservatives? Journal of Food Science and Technology. 2015;52(10):6565-73.
13. Kasrati A, Alaoui Jamali C, Bekkouche K, Wohlmuth H, Leach D, Abbad A. Comparative evaluation of antioxidant and insecticidal properties of essential oils from five Moroccan aromatic herbs. Journal of Food Science and Technology. 2014;52(4):2312-9.
14. Bugarin D, Grbović S, Orčič D, Mitić-Ćulafić D, Knežević-Vukčević J, Mimica-Dukić N. Essential Oil of Eucalyptus Gunnii Hook. As a Novel Source of Antioxidant, Antimutagenic and Antibacterial Agents. Molecules. 2014;19(11):19007-20.
15. Momtazi AA, Askari-Khorasgani O, Abdollahi E, Sadeghi-Aliabadi H, Mortazaeinezhad F, Sahebkar A. Phytochemical Analysis and Cytotoxicity Evaluation of Kelussia odoratissima Mozaff. Journal of Acupuncture and Meridian Studies. 2017;10(3):180-6.
16. Ogunwande IA, Walker TM, Bansal A, Setzer WN, Essien EE. Essential Oil Constituents and Biological Activities of Peristrophe Bicalyculata and Borreria verticillata. Natural Product Communications. 2010;5(11):1934578X000501.
17. Fitsiou E, Mitropoulou G, Spyridopoulou K, Vamvakias M, Bardouki H, Galanis A, et al. Chemical Composition and Evaluation of the Biological Properties of the Essential Oil of the Dietary Phytochemical Lippia citriodora. Molecules. 2018;23(1):123.
18. Fitsiou E, Mitropoulou G, Spyridopoulou K, Tiptiri-Kourpeti A, Vamvakias M, Bardouki H, et al. Phytochemical Profile and Evaluation of the Biological Activities of Essential Oils Derived from the Greek Aromatic Plant Species Ocimum basilicum, Mentha spicata, Pimpinella anisum and Fortunella margarita. Molecules. 2016;21(8):1069.
19. Jardak M, Elloumi-Mseddi J, Aifa S, Mnif S. Chemical composition, anti-biofilm activity and potential cytotoxic effect on cancer cells of Rosmarinus officinalis L. essential oil from Tunisia. Lipids in Health and Disease. 2017;16(1).
20. Mishra T, Pal M, Meena S, Datta D, Dixit P, Kumar A, et al. Composition and in vitro cytotoxic activities of essential oil of Hedychium spicatum from different geographical regions of western Himalaya by principal components analysis. Natural Product Research. 2015;30(10):1224-7.
21. Furtado FB, Borges BC, Teixeira TL, Garces HG. Chemical Composition and Bioactivity of Essential Oil from Blepharocalyx salicifolius. Int J Mol Sci, 2018;19 (1).
22. Ali N, Chhetri B, Dosoky N, Shari K, Al-Fahad A, Wessjohann L, et al. Antimicrobial, Antioxidant, and Cytotoxic Activities of Ocimum forskolei and Teucrium yemense (Lamiaceae) Essential Oils. Medicines. 2017;4(2):17.
23. Estanislao Gómez CC, Aquino Carreño A, Pérez Ishiwara DG, San Martín Martínez E, Morales López J, Pérez Hernández N, et al. Decatropis bicolor (Zucc.) Radlk essential oil induces apoptosis of the MDA-MB-231 breast cancer cell line. BMC Complementary and Alternative Medicine. 2016;16(1).
24. Sleator RD, Hill C. Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence. FEMS Microbiology Reviews. 2002;26(1):49-71.
25. Najafi-Taher R, Ghaemi B, Amani A. Delivery of adapalene using a novel topical gel based on tea tree oil nano-emulsion: Permeation, antibacterial and safety assessments. European Journal of Pharmaceutical Sciences. 2018;120:142-51.
26. Osanloo M, Sedaghat MM, Sereshti H, Rahmani M, Saeedi Landi F, Amani A. Chitosan nanocapsules of tarragon essential oil with low cytotoxicity and long-lasting activity as a green nano-larvicide. Journal of Nanostructures, 2019;9 (4):723-735.
27. Moghimi R, Ghaderi L, Rafati H, Aliahmadi A, McClements DJ. Superior antibacterial activity of nanoemulsion of Thymus daenensis essential oil against E. coli. Food Chemistry. 2016;194:410-5.
28. Duarte JL, Amado JRR, Oliveira AEMFM, Cruz RAS, Ferreira AM, Souto RNP, et al. Evaluation of larvicidal activity of a nanoemulsion of Rosmarinus officinalis essential oil. Revista Brasileira de Farmacognosia. 2015;25(2):189-92.
29. Lou Z, Chen J, Yu F, Wang H, Kou X, Ma C, et al. The antioxidant, antibacterial, antibiofilm activity of essential oil from Citrus medica L. var. sarcodactylis and its nanoemulsion. LWT. 2017;80:371-7.
30. Haznedar S, Dortunç B. Preparation and in vitro evaluation of Eudragit microspheres containing acetazolamide. International Journal of Pharmaceutics. 2004;269(1):131-40.
31. Abyadeh M, Sadroddiny E, Ebrahimi A, Esmaeili F, Landi FS, Amani A. Electrosprayed chitosan nanoparticles: facile and efficient approach for bacterial transformation. International Nano Letters. 2017;7(4):291-5.
32. Jaiswal M, Dudhe R, Sharma PK. Nanoemulsion: an advanced

mode of drug delivery system. 3 Biotech. 2014;5(2):123-7.
33. Kakumanu S, Tagne JB, Wilson TA, Nicolosi RJ. A nanoemulsion formulation of dacarbazine reduces tumor size in a xenograft mouse epidermoid carcinoma model compared to dacarbazine suspension. Nanomedicine: Nanotechnology, Biology and Medicine. 2011;7(3):277-83.
34. Khan I, Bahuguna A, Kumar P, Bajpai VK, Kang SC. In vitro and in vivo antitumor potential of carvacrol nanoemulsion against human lung adenocarcinoma A549 cells via mitochondrial mediated apoptosis. Scientific Reports. 2018;8(1).
35. Li Z, Tan S, Li S, Shen Q, Wang K. Cancer drug delivery in the nano era: An overview and perspectives. Oncology Reports. 2017;38(2):611-24.
36. Yang K, Feng L, Liu Z. The advancing uses of nano-graphene in drug delivery. Expert Opinion on Drug Delivery. 2014;12(4):601-12.
37. Cianfaglione K, Blomme EE, Quassinti L, Bramucci M, Lupidi G, Dall'Acqua S, et al. Cytotoxic Essential Oils fromEryngium campestreandEryngium amethystinum (Apiaceae) Growing in Central Italy. Chemistry & Biodiversity. 2017;14(7):e1700096.