Nanomed Res J 8(3):301-310, Summer 2023

RESEARCH ARTICLE

Alginate nanoparticles containing *Rosmarinus officinalis* essential oil and α-pinene: cytotoxicity and effect on apoptotic-involved genes in human melanoma and breast cancer cell lines

Shekoufeh Hatami[1], Yaser Mansoori[2], Roghayeh Heiran[3], Atefeh Taheri[4], Abdolmajid Ghasemian[5], Mahmoud Osanloo[6*]

[1] Department of Biochemistry, School of Medicine, Fasa University of Medical Sciences, Fasa, Iran.

[2] Department of Medical Genetics, School of Medicine, Fasa University of Medical Sciences, Fasa, Iran

[3] Estahban Higher Education Center-Shiraz University, Estahban, Iran

[4] Department of Tissue Engineering and Regenerative Medicine, Faculty of Advanced Technologies in Medicine, Iran University of Medical Sciences, Tehran, Iran

[5] Noncommunicable Diseases Research Center, Fasa University of Medical Sciences, Fasa, Iran

[6] Department of Medical Nanotechnology, School of Advanced Technologies in Medicine, Fasa University of Medical Sciences, Fasa, Iran

ARTICLE INFO

Article History:
Received 06 Feb 2023
Accepted 18 Apr 2023
Published 01 May 2023

Keywords:
Cancer
Skin
Nanotechnology

ABSTRACT

Cancers are one of the major causes of death, and the development of new medicine using advanced technologies such as nanotechnology has thus received more attention. *Rosmarinus officinalis* as a common medicinal plant possess many biological effects, such as anticancer effects. This study investigated the chemical compositions of its essential oil using GC-MS analysis. Alpha-pinene (24.0%), eucalyptol (16.99%), verbenone (8.79%), L-borneol (7.79%), and camphor (6.87%) were the five major compounds. After that, alginate nanoparticles containing α-pinene (major compound) and *R. officinal*is essential oil were prepared with particle sizes of 137 ± 8 and 151 ± 7. Besides, successful loading of α-pinene or the essential oil in nanoparticles was confirmed using ATR-FTIR analysis. The efficacy (IC50) of alginate nanoparticles containing α-pinene against A-375 and MCF-7 were 582 (179-1886) µg/mL and 94 (47-187) µg/mL. These values for alginate nanoparticles containing *R. officinal*is essential oil were obtained as 807 (470-1383) µg/mL and 235 (168-328) µg/mL. Moreover, RT-PCR results show that the Bcl2 (anti-apoptotic gene) level in MCF-7 treated cells with alginate nanoparticles containing *R. officinal*is essential oil was higher than Bax (apoptotic gene); another cell death pathway rather than apoptosis was involved. Considering the proper efficacy of alginate nanoparticles containing the essential oil or α-pinene, especially against MCF-7 cells, they could be considered for further investigation in vivo.

How to cite this article
Hatami Sh., Mansoori Y., Heiran R., Taheri A., Ghasemian A., Osanloo M. Alginate nanoparticles containing Rosmarinus officinalis essential oil and α-pinene: cytotoxicity and effect on apoptotic-involved genes in human melanoma and breast cancer cell lines. Nanomed Res J, 2023; 8(3): 301-310. DOI: 10.22034/nmrj.2023.03.009

INTRODUCTION

Cancers were cause of about 10 million deaths only in 2020; it was 1 in six total death [1]. Breast cancer, with a 30% rate, is the most common cancer in women worldwide, and ~ 13% of cancers are related to skin cancer [2, 3]. Melanoma is the most aggressive and deadly skin cancer, accounting for 75% of skin cancer deaths [4, 5]. Moreover, melanoma is a new disease exacerbated by the migration of people with fair skin to more tropical regions and longer exposure to sunlight [5, 6].

The side effects of chemotherapy drugs and the emergence of resistance against them have drawn

* Corresponding Author Email: *m.osanloo@fums.ac.ir*

attention to developing new drugs, especially from herbal sources [7, 8]. However, in vitro models are still important in developing new drugs due to the strict rules of working with animals. MCF7 breast cancer cell line with high expression of hormone receptors (androgen, progesterone, and glucocorticoid) is the most selected model in research [9, 10]. Besides, they maintain characteristics similar to mammary epithelium; the cells forming domes and the epithelial-like cells grow in monolayers when grown in vitro [11, 12]. Furthermore, among 42 melanoma cell lines at the transcriptome level, A-375 cells are studied more due to aggressive behavior and low sensitivity to chemotherapy [13, 14].

Rosmarinus officinalis (rosemary) is widely used in headache treatment, physical and mental fatigue, inflammatory diseases, and poor blood circulation [15, 16]. The different biological properties of its essential oil (EO), such as anticancer, antibacterial, and antifungal activity, have also been reported [16, 17]. Moreover, α-pinene is the major component of rosemary EO, a well-known monoterpene with many biological effects such as anticancer effect, anti-inflammatory effect, and antioxidant effect [18]. Studies have shown terpenes are the most effective molecules against tumor growth, followed by triterpenoids [19]. However, EOs (e.g., rosemary EO) and their effective compounds (e.g., α-pinene) are hydrophobic and must be formulated to use them. Developing nanostructures containing EOs or natural compounds has recently received more attention as an approach to stability and efficacy improvements [20, 21]. Alginate is a hydrophilic biopolymer with proper biodegradability and biocompatibility features [22, 23]. Alginate nanoparticles have been widely used in pharmaceutical and biomedical researches such as antimicrobial products, tissue engineering, cancer treatment, and drug and gene delivery [22, 24]. To the authors' best knowledge, alginate nanoparticles containing rosemary EO and α-pinene were not reported; this study thus prepared them. After that, a comprehensive comparison was carried out on their cytotoxicity on human breast and melanoma cancer cell lines (MCF-7 and A-375). Besides their effects on apoptotic-involved genes, B-cell lymphoma protein 2 (Bcl_2) associated X (Bax) and Bcl2 were investigated using RT-PCR.

MATERIALS AND METHODS

Materials

The calcium dichloride (C5670), alginate powder (w201502), and α-pinene (147524; 98% purity), MTT, and PBS tablets were purchased from Sigma-Aldrich (USA). A-375 and MCF-7 cell lines (ATCC = CRL-1619 and HTB-22) were purchesed from the Pasteur Institute (Iran). DMSO, DMEM cell medium culture, Penicillin-streptomycin, and Trypsin-EDTA were ordered from Shellmax (China). FBS was bought from Gibco (USA). Rosemary EO was obtained from Tabib Daru Pharmaceutical Company (Iran); they have dedicated medicinal plant cultivation farms. Trizol RNA extraction and cDNA synthesis kits were purchased from Yekta Tajhiz Company (Iran). Real Q plus 2x master mix Green high ROX kit was purchased from Ampliqon (Denmark). The designed primers were ordered from Pishgam Biotech (Iran).

Chemical composition of rosemary EO using GC-MS analysis

The chemical composition of rosemary EO was investigated using a 6890 gas chromatography system coupled with a 5973 network mass selective detector (Agilent Technologies, USA). For the separation of compounds, HP-5MS silica fused column (60 m length, 0.25 mm i.d, and film thickness was 0.25 μM) was used as described in our previous report [25].

Preparation and characterizations of alginate nanoparticles

A defined amounts of α-pinene and rosemary EO (0.16% w/v) and tween 20 as emulsifiers (0.2% w/v) were mixed for 3 min at 2000 rpm. Next, 2.5 mL Alginate aqueous solution 0.25% w/v was drop wisely added and mixed for another 3 min. After that, 2.5 mL calcium dichloride 0.1% w/v was added and stirred for 40 min to complete the cross-linking process. Besides, free alginate nanoparticles was prepared in the same approach, without α-pinene and rosemary EO. The prepared nanoparticles were named Alg-pinene, Alg-rosemary, and Alg-free; they were used for further characterization and biological investigations.

A dynamic light scattering (DLS) device (K-one Ltd. Korea) measured the mean size of nanoparticles. D90 – D10 D50 calculated the nanoparticle size distribution (SPAN). D is diameter, and 10, 50, and 90 are percentages of nanoparticles with smaller sizes than these specified points. Particle size < 200 nm and SPAN < 1 are optimal size characteristics [26].

ATR-FTIR (Attenuated Total Reflection Fourier Transform Infrared) analysis was used for the confirmation of the loading of α-pinene and rosemary EO in nanoparticles. For this purpose, the spectra α-pinene, rosemary EO, Alg-free, Alg-pinene, and Alg-rosemary were recorded at 400-4000 cm^{-1}. The samples were subjected to the spectrometer device (Tensor II, Bruker Company, Germany) without sample modifications.

Investigation of cytotoxicity of nanoparticles on A-375 and MCF-7 cells

The cytotoxicity of Alg-Pinene and Alg-rosemary against A-375 and MCF-7 was investigated using MTT assay. Briefly, the cultured cells in a complete DMEM medium (i.e., enriched with 1% penicillin and streptomycin and 10% FBS) were first seeded (10000 cells/well) in 96 well plates, incubated overnight for attachment, and reached 80% confluence. Liquid contents were then replaced with 50 µL fresh complete DMEM medium and treated with 50 µL of Alg-Pinene and Alg-rosemary with concentrations of 50, 100, 200, 400, and 800 µg/mL. Six well/plates were considered control and negative control groups; 50 µL of DMEM and Alg-free were added. After 24 h incubation, liquid contents were replaced with 100 µL of MTT solution (0.5 mg/mL) dissolved in DMEM. After 4 h incubation, formazan crystals were dissolved by adding DMSO (100 µL/well). The absorbance of all wells was read at 570 nm using a plate reader (SYNERGY, HTX, multi-mode reader, BioTek Instruments, USA). The viability of cells at each concentration was calculated using Equation 1. The test was repeated three times (n=3), and the results are expressed as mean ± SD.

Cell viability = (Absorbance sample / Absorbance control) × 100 (1)

Investigation of effects of nanoparticles on apoptotic genes

The cells were seeded in 6 well plates overnight, as described above. Alg-Pinene and Alg-rosemary were added to wells (400 µg/mL) and incubated for 24 h. Total RNAs were extracted from cells using the Trizol RNA extraction Kit. After quantification of extracted RNA using Nano-drop (SYNERGY, HTX, multi-mode reader, BioTek Instruments, USA), cDNA was synthesized according to the manufacturer's protocols. The RT-PCR reaction was performed to analyze Bax and Bcl2 apoptotic genes and the expression of β-actin (a housekeeping gene). Ten µL of master mix, 1 µL of the mixture of forward and reverse primers, 1 µL of the synthesized cDNA, and 8 µL of DEPC water were injected into a microtube and inserted into an RT-PCR device (Step One Plus Real-Time PCR machine, AB Applied Biosystems, Germany). Equation $2^{-\Delta\Delta CT}$ was used for normalizations; the results were expressed as fold change compared with untreated cells. The test was repeated three times (n=3), and the results are expressed as mean ± SD.

The sequence of used primers is as follows. Bax forward: 5 CCC GAG AGG TCT TTT TCC GAG 3, Bax reverse: 5 CCA GCC CAT GAT GGT TCT GAT. BcL2 forward: 5 CGG TTC AGG TAC TCA GTC ATCC 3, BcL2 reverse: 5 GGT GGG GTC ATG TGT GTGG 3. β-actin forward: 5 CCTGCTTGCTGATCCACATCT 3, β-actin reverse: 5 TTCCTCCTGAGCGCAAGTAC 3.

RESULTS

Identified compounds in rosemary EO

Twenty-seven identified compounds in rosemary EO are given in Table 1. Alpha-pinene (24.0%), eucalyptol (16.99%), verbenone (8.79%), L-borneol (7.79%), and camphor (6.87%) were the five major compounds.

Physicochemical properties of nanoparticles

DLS profiles of Alg-pinene (137 ± 8 nm) and Alg-rosemary EO (151 ± 7 nm) are shown in Fig. 1. SPAN values were obtained as 0.96 and 0.97, so their narrow particle size distributions were confirmed.

The spectrum of α-pinene EO (Fig. 2A) shows different bands related to C-H, C=C, CH_3, and CH_2 vibrations. The characteristic absorption bands between 3024-3834 cm^{-1} are attributed to =C-H and C-H stretching vibrations. Besides, the bands at 1658, 1469-1364, and 786 cm^{-1} might be assigned to C=C stretching, CH_2 and CH_3 bending, as well as out-of-plan (oop) C=C bending modes, respectively.

The ATR-FTIR spectrum of rosemary EO showed some characteristic key bands (Fig. 2 B). The band around 3446 cm^{-1} is attributed to hydroxy groups of rosemary EO, indicating Terpineol as one of the main components of rosemary EO. The characteristic bands around 2950-2834 cm^{-1} are assigned to =C-H and C-H stretching modes, and the ones at 1743, 1716, 1675, and 1618 cm^{-1} might be attributed to C=O and C=C stretching modes. The relatively strong bands around 1446 and 1364

Table 1. Identified compounds in rosemary EO using GC-MS analysis

Number	Retention time	Compound	%	KI [A]	Type
1	10.84	tricyclene	0.38	924	SH [B]
2	11.44	α-pinene	24.00	936	MH [C]
3	12.28	camphene	6.49	953	MH
4	13.71	B-pinene	1.06	982	MH
5	14.29	myrcene	1.86	993	MH
6	16.28	o-cymene	2.96	1033	SH
7	16.45	limonene	4.31	1036	MH
8	16.65	eucalyptol	16.99	1039	MO [D]
9	20.19	linalool	2.64	1108	MO
10	21.41	chrysanthenone	0.26	1133	MO
11	22.40	trans-pinocarveol	0.26	1152	MO
12	22.82	camphor	6.87	1161	MO
13	23.44	trans-pinocamphone	0.60	1173	MO
14	24.07	l-borneol	7.79	1186	MO
15	24.27	isopinocamphone	0.74	1190	MO
16	24.37	terpinene-4-ol	1.05	1192	MO
17	24.89	p-cymen-4-ol	0.20	1203	MO
18	25.19	α-terpineol	2.82	1209	MO
19	25.90	l-verbenone	8.79	1224	MO
20	27.63	geraniol	1.81	1261	MO
21	29.16	trans-bornyl acetate	2.45	1293	MO
22	31.98	piperitenone	0.17	1356	MO
23	33.30	geranyl acetate	0.15	1386	MO
24	34.59	methyleugenol	0.21	1416	other
25	35.10	caryophyllene	1.06	1428	SH
26	36.65	humulene	0.20	1465	SH
27	41.96	caryophyllene oxide	0.91	1597	SO [E]
		Total identified	97.01		

A: Kovats retention Index, B: Sesquiterpene Hydrocarbons, C: Monoterpene Hydrocarbons, D: Oxygenated Monoterpenes, E: Oxygenated Sesquiterpenes

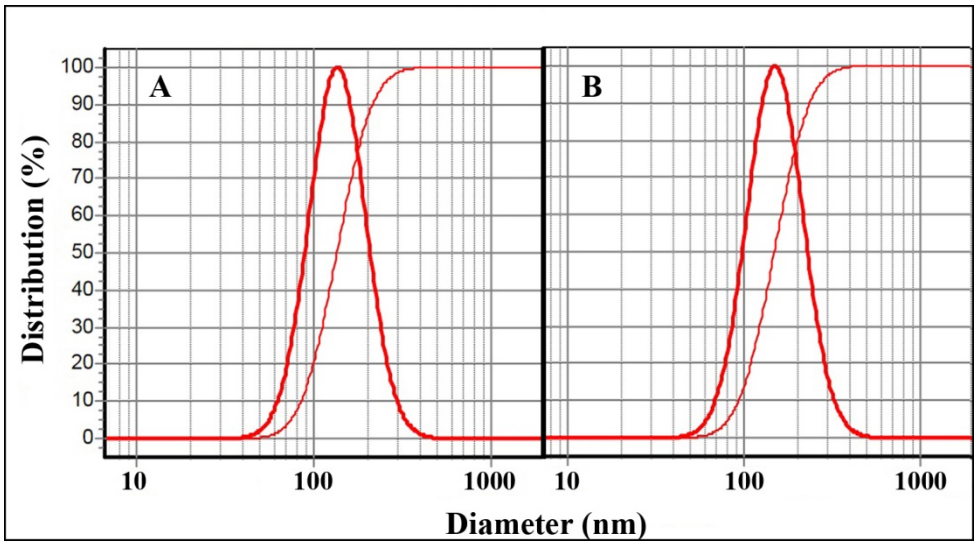

Fig. 1. DLS profiles of A) alginate nanoparticles containing α-pinene (Alg-pinene) with a mean particle size of 137 ± 8 nm and B) alginate nanoparticles containing rosemary EO (Alg-rosemary) with a mean particle size of 151 ± 7 nm

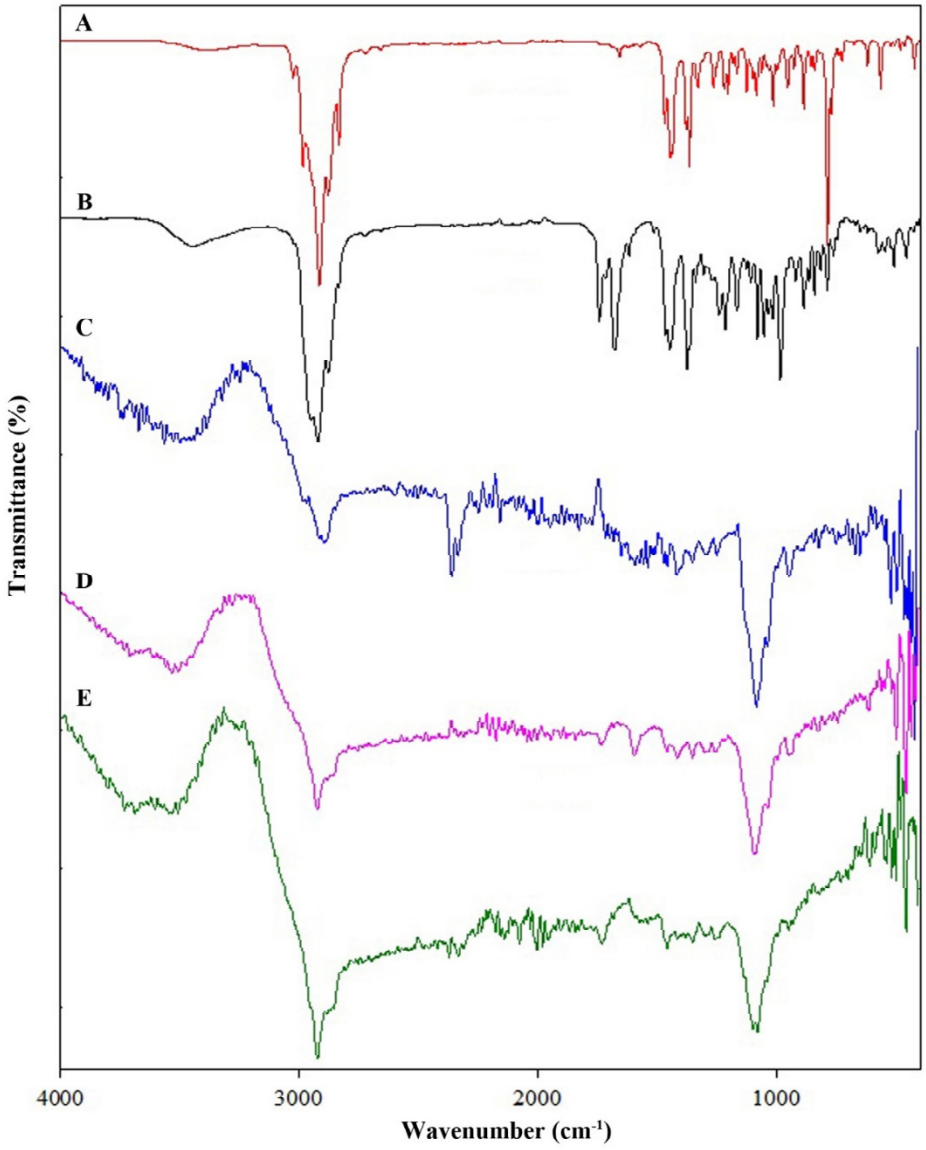

Fig. 2. ATR-FTIR spectra of A) α- pinene, B) rosemary EO, C) Alg-free, D) Alg-pinene, E) Alg-rosemary

cm^{-1} belong to bending modes of CH2 and CH3 and C=C vibration modes. The presence of C-O bonds was confirmed by observing bands around 1266-1053 cm^{-1}. Moreover, the C-H oop bending modes appeared at 984-784 cm^{-1}. These bands indicate that 1,8-cineole and α- pinene are the main components of rosemary EO.

The ATR-FTIR spectrum of the Alg-free formed from the tween 20, alginate sodium, and $CaCl_2$ has been shown in Fig. 2C. The broad band around 3450 cm^{-1} ranged corresponding to hydroxy stretching and hydrogen bonding. The various bands around 2992-2870, 1604, 1417, and 1089 cm^{-1} belong to the C-H stretching, asymmetric and symmetric COO^- vibrations, and deformation of C-OH and C-O-C vibrations, respectively.

By incorporating α- pinene into Alg-free, the O-H vibrations were observed as a broad band around 3526 cm-1, and those bands related to C-H stretching vibrations appeared around 2923-2842 cm^{-1}. Compared with Alg-free spectra, some changes were observed around 3705-2842 cm^{-1}, suggesting some interaction between Alg-free and α-pinene. The characteristic absorption bands of

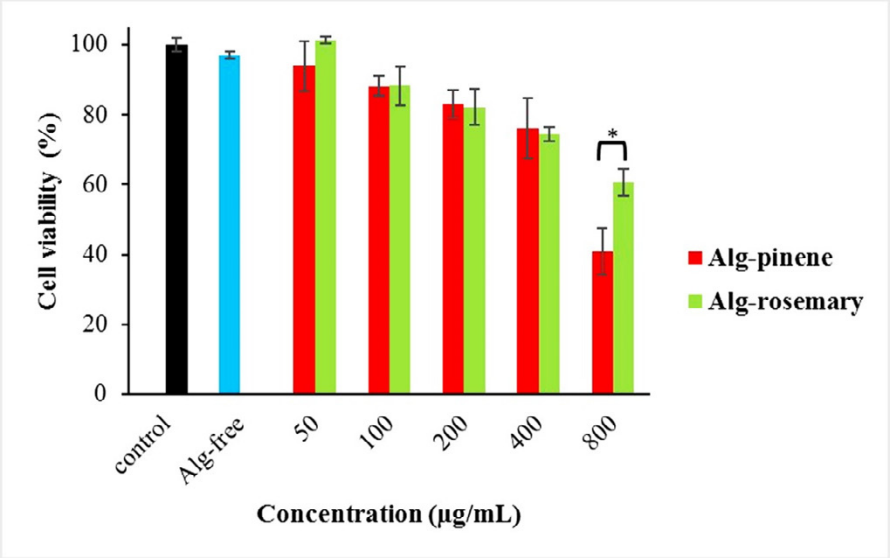

Fig. 3. Viability of A-375 cells after 24 hours of exposure to alginate nanoparticles containing α-pinene and rosemary EO (Alg-pinene and Alg-rosemary). *: $P < 0.05$

Alg-free and α-pinene are seen at 1735, 1604 cm^{-1}, 1464, 1417, and 1342 cm^{-1}, corresponding to C=O and C=C stretching and CH_2 CH_3 bending modes. The relatively strong band around 1098 cm^{-1} relates to C-O stretching and C-OH deformation (Fig. 2D).

The spectrum of Alg-free after rosemary EO incorporation was similar to Alg-free slight difference in wave number and the width of the peaks. Two broad bands at 3685 and 3526 cm^{-1} are assigned to OH groups of both rosemary EO and Alg-free, as well as hydrogen bonding. After treatment with rosemary EO, the C-H stretching bands shift and overlap, creating a relatively strong band at 2935 and 2850 cm^{-1}. Different bands between 1735-1248 cm^{-1} are attributed to C=O and C=C stretching, CH_2 and CH_3 bending, and C-O stretching modes, while the strong band at 1079 cm^{-1} is assigned to C-O vibrations, and the weak one at 938 cm^{-1} might be assigned to C-H oop bending modes. These changes could prove that rosemary EO is loaded into Alg-free (Fig. 2E).

The cytotoxicity of nanoparticles on A-375 and MCF-7 cells

As shown in Fig. 3, the Alg-free did not significantly affect A-375 cells compared to the control group ($P > 0.05$). The cytotoxicity effects of Alg-pinene and Alg-rosemary were dose-dependent. Besides, cell viability after treatment with Alg-pinene (800 µg/mL) was significantly lower ($P = 0.011$) than cells treated with Alg-rosemary (800 µg/mL). However, at concentrations of 400, 200, 100, and 50 µg/mL, no significant difference ($P > 0.05$) was observed between cell viability after treatment with the samples. Moreover, the efficacy of Alg-pinene with IC50 582 µg/mL (179-1886) showed more potency than Alg- rosemary with IC50 807 µg/mL (470-1383; however, this difference was not significant ($P > 0.05$).

Effects of Alg-pinene and Alg-rosemary on MCF-7 cell viability are shown in Fig. 4. There is no significant difference was observed between cell viability in control group cells (untreated) and Alg-free treated cells ($P > 0.05$). The efficacy of Alg-pinene in reducing cell viability in concentrations of 50 ($P = 0.002$), 100 ($P < 0.001$), and 200 ($P < 0.001$) µg/mL was significantly more potent than Alg-rosemary. However, at concentrations of 400 ($P = 0.003$) and 800 ($P < 0.001$) µg/mL, the efficacy of Alg-rosemary was significantly more potent than Alg-pinene. Interestingly, 94% of MCF-7 cell viability was decreased after treatment with 800 µg/mL Alg-rosemary. Furthermore, no significant difference ($P > 0.05$) was between IC50 values of Alg-pinene and Alg-rosemary; 94 µg/mL (47-187) and 235 µg/mL (168-328).

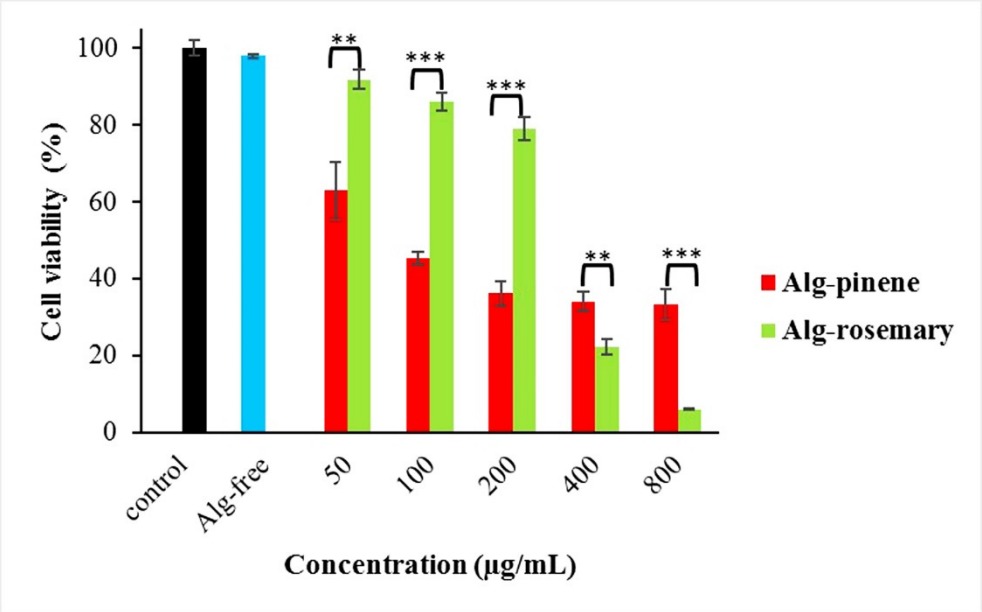

Fig. 4. Viability of MCF-7 cells after 24 hours of exposure to alginate nanoparticles containing α-pinene and rosemary EO (Alg-pinene and Alg-rosemary). **: $P < 0.01$ and ***: $P < 0.001$

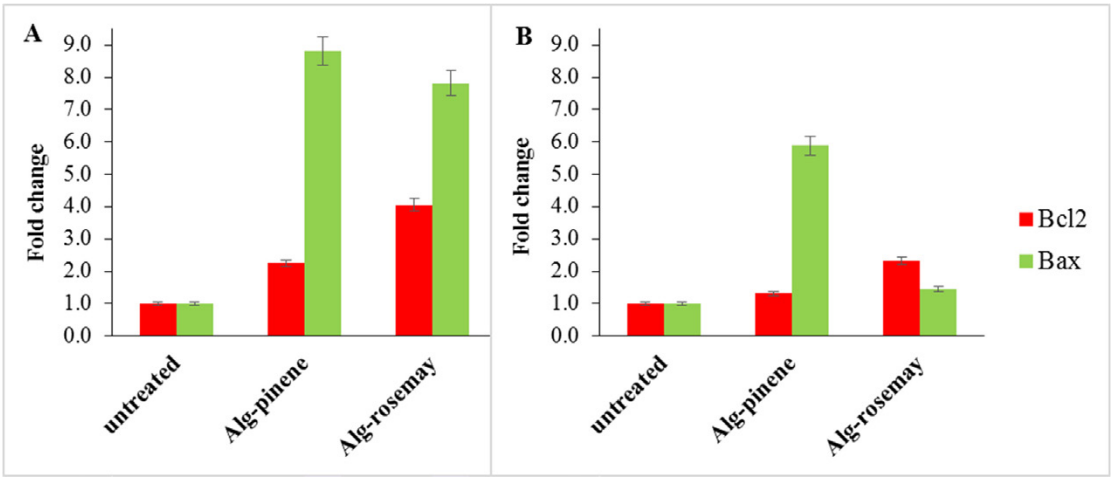

Fig. 5. Effects of 400 µg/mL of alginate nanoparticles containing α-pinene and rosemary EO (24 hours exposure) on apoptotic involved genes in A) A-375 and B) MCF7 cells

Effects of nanoparticles on apoptotic genes

The effects of Alg-pinene and Alg-rosemary on Bcl2 and Bax genes expression compared with untreated in A-375 cells is depicted in Fig. 5A. Bax/Bcl-2 ratio in both treated cells is more than 1, so their effects could be confirmed concerning the apoptotic pathway. Besides, the samples' effects on MCF-7 cells are depicted in Fig. 5B. Again, Bax/Bcl-2 ratio in Alg-pinene-treated cells is more than 1. However, this ratio in Alg-rosemary-treated cells is less than 1; it seems another cell death pathway rather than apoptosis was involved.

DISCUSSIONS

Rosemary EO possess many pharmacological effects, such as anti-inflammatory, antioxidant, antimicrobial, hepatoprotective, antispasmodic, anticarcinogenic, and antitumorigenic

properties [27]. Also, α-pinene with wide range of pharmacological activities such as anti-inflammatory, antioxidant, and anticancer effects has known as an effective component of rosemary EO [18]. This study investigated the anticancer activity of Alg-pinene and Alg-rosemary on melanoma and breast cancer cells. The results showed that the efficacy of Alg-pinene was higher than Alg-rosemary, although this difference was not significant. Investigating whether the efficacy of nanoparticles containing EO is better or nanoparticles containing the main compounds is one of the hot research fields in the world, and there is still no definitive conclusion. For instance, the cytotoxic effects of chitosan nanoparticles containing *Cinnamomum verum* EO and its major compound, i.e., cinnamaldehyde against A-375 and MDA-MB-468 cell lines, were investigated. Nanoparticles containing the EO with IC50 values of 79 and 112 µg/mL were more potent than cinnamaldehyde nanoparticles (135 and 166 µg/mL)[28]. Another study investigated the cytotoxic effects of chitosan nanoparticles containing carvone and two carvone-reach EOs, including *Mentha spicata* and *Tanacetum balsamita,* against A-375 cells. Chitosan nanoparticles containing *T. balsamita* with IC50 value 85 µg/mL was more potent than nanoparticle containing -carvone (IC50 99 µg/mL) and –*M. spicata* EO (IC50 113 µg/mL)[29]. In another study, the efficacy of chitosan nanoparticles containing clove EO and the major component (eugenol) against A-375 cells was almost equal; 73 and 79 µg/mL. However, the efficacy of chitosan nanoparticles containing eugenol against MDA-MB-468 cells was more potent than those containing clove EO; IC50 values were 51 and 177 µg/mL [30]. In another study, the IC50 of chitosan nanoparticles containing limonene (30 µg/mL) against A-375 was reported to be more effective than *Citrus aurantium* EO (55 µg/mL). However, its efficacy was reported to be lower than that of another limonene-reach EO, i.e., *Citrus limon* with IC50 0.12 µg/mL [31]. These reports can be summarized as saying that EOs have selective effects on cells, which are independent of the effects of their main compound.

Furthermore, to clarify the anticancer mechanism of the alginate nanoparticles, the expression ratio of two apoptotic-involved genes, Bax and Bcl2, were investigated in the current study. Apoptosis is a biologically programmed cell death that is critical in homeostasis, development, and stress condition [32]. Macrophages feed these dead cells, and to keep homeostasis, other similar cells are generated [33]. The Bax and Bcl_2 genes function as promoters and inhibitors of apoptosis, respectively, controlling cell life or death. Bax and Bcl2 are prognostic markers in various cancers [34]. Inducing apoptosis in normal cells signals death; however, in cancer cells, this pathway is impaired [35]. In the current study, the level of Bax in Alg-pinene-treated A-375 and MCF-7 cells was more than Bcl2, which was the reason for apoptosis. Recent studies have demonstrated that α-pinene can exert its antitumor activity by inducing apoptosis or cell cycle arrest [36, 37]. However, the level of Bcl-2 in Alg-rosemary-treated MCF-7 cells was higher than Bax in the current study; the sample caused the apoptosis pathway to not be activated in this cell. Noted that, Bax/Bcl-2 ratio >1 is a significant criteeia in apoptosis patway.

Furthermore, the number of distinct energy-dependent processes, such as autophagy, apoptosis, and pyroptosis, or energy-independent processes, such as necrosis and oncosis, could influence cell death [38]. Literature suggested that treatment with rosemary EO in some cell lines increased the intracellular ROS (reactive oxygen species) that lead to necrosis cell death. Moreover, the morphological microscopy observation of rosemary EO-treated cells emphasizes necrosis rather than the apoptosis mechanism [39, 40]. In the current study, cell viability decreased after treatment with Alg-rosemary; however, the Bax/Bcl-2 ratio was less than 1, which is consistent with what is mentioned in the literature. However, more study is needed to understand the cell death pathway better.

CONCLUSION

The chemical composition results showed that α-pinene was the major compound of *R. officinalis* EO. Alginate nanoparticles containing α-pinene and rosemary EO were prepared, and their physicochemical properties were characterized using DLS and ATR-FTIR analyses. Their potency against human breast and melanoma cancer cells (MCF-7 and A-375) was compared; no significant differences were not observed. Their efficacy with IC50 values of 94 and 235 µg/mL against MCF-7 was promising. Therefore, they could be considered for further investigations to find the pathway of their effects.

ACKNOWLEDGMENTS

Fasa University of Medical Sciences supported this study, grant No. 400229. Moreover, it has been approved by the ethical committee of Fasa University of Medical Sciences, Fasa, Iran (IR.FUMS.REC.1401.021).

AUTHORS' CONTRIBUTIONS

ShH prepared nanoparticles, performed MTT tests, and drafted MS in contribution with MO. YM and AT performed RT-PCR. RH interpreted ATR-FTIR spectra. AGh revised MS. MO designed the study, analyzed data, and drafted the MS. All authors contributed to the drafting of the manuscript and approved the final version.

ABBREVIATIONS

EO: Essential oil

ATR-FTIR: Attenuated Total Reflection- Fourier Transform InfraRed

RT-PCR: Reverse Transcription-Polymerase Chain Reaction

GC-MS: Gas Chromatography-Mass Spectrometry

DLS: Dynamic Light Scattering

Alg-pinene: alginate nanoparticles containing a-pinene

Alg-rosemary: alginate nanoparticles containing rosemary EO

Alg-free: alginate nanoparticles without cargo

CONFLICT OF INTEREST

There is no conflict of interest

REFERENCES

1. WHO. Cancer Ket Facts. Available at: https://www.who.int/news-room/fact-sheets/detail/cancer. Accessed Agust, 2022.
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: A Cancer Journal for Clinicians, 2018;68 (6):394-424.https://doi.org/10.3322/caac.21492
3. Toma A-O, Prodan M, Reddyreddy AR, Seclaman E, Crainiceanu Z, Bloanca V, Bratosin F, Dumitru C, Pilut CN, Alambaram S, Vasamsetti NG, Decean L, Pricop M. The Epidemiology of Malignant Melanoma during the First Two Years of the COVID-19 Pandemic: A Systematic Review. International Journal of Environmental Research and Public Health, 2023;20 (1):305.https://doi.org/10.3390/ijerph20010305
4. Domingues B, Lopes JM, Soares P, Pópulo H. Melanoma treatment in review. ImmunoTargets and therapy, 2018;7:35. https://doi.org/10.2147/ITT.S134842
5. Davis LE, Shalin SC, Tackett AJ. Current state of melanoma diagnosis and treatment. Cancer Biology & Therapy, 2019;20 (11):1366-1379.https://doi.org/10.1080/15384047.2019.1640032
6. Rebecca VW, Sondak VK, Smalley KS. A brief history of melanoma: from mummies to mutations. Melanoma Research, 2012;22 (2):114-122.https://doi.org/10.1097/CMR.0b013e328351fa4d
7. Nieto G, Huvaere K, Skibsted LH. Antioxidant activity of rosemary and thyme by-products and synergism with added antioxidant in a liposome system. European Food Research and Technology, 2011;233 (1):11-18.https://doi.org/10.1007/s00217-011-1486-9
8. Noorpisheh Ghadimi S, Sharifi N, Osanloo M. The leishmanicidal activity of essential oils: A systematic review. Journal of Herbmed Pharmacology, 2020;9 (4):300-308. https://doi.org/10.34172/jhp.2020.38
9. Comşa Ş, Cimpean AM, Raica M. The story of MCF-7 breast cancer cell line: 40 years of experience in research. Anticancer Research, 2015;35 (6):3147-3154.
10. Cavo M, Fato M, Peñuela L, Beltrame F, Raiteri R, Scaglione S. Microenvironment complexity and matrix stiffness regulate breast cancer cell activity in a 3D in vitro model. Scientific Reports, 2016;6 (1):1-13.https://doi.org/10.1038/srep35367
11. Wu S, Wang Y, Yuan Z, Wang S, Du H, Liu X, Wang Q, Zhu X. Human adiposederived mesenchymal stem cells promote breast cancer MCF7 cell epithelialmesenchymal transition by cross interacting with the TGFβ/Smad and PI3K/AKT signaling pathways. Molecular Medicine Reports, 2019;19 (1):177-186.https://doi.org/10.3892/mmr.2018.9664
12. Fayyad RJ, Ali ANM, Dwaish AS, Al-Abboodi AKA. Anticancer activity of Spirulina platensis methanolic extracts against L20B and MCF7 human cancer cell lines. Plant Arch, 2019;19 (1):1419-1426.
13. Massi D, Brusa D, Merelli B, Ciano M, Audrito V, Serra S, Buonincontri R, Baroni G, Nassini R, Minocci D. PD-L1 marks a subset of melanomas with a shorter overall survival and distinct genetic and morphological characteristics. Annals of Oncology, 2014;25 (12):2433-2442.https://doi.org/10.1093/annonc/mdu452
14. Vincent KM, Postovit L-M. Investigating the utility of human melanoma cell lines as tumour models. Oncotarget, 2017;8 (6):10498.https://doi.org/10.18632/oncotarget.14443
15. Yu M-H, Choi J-H, Chae I-G, Im H-G, Yang S-A, More K, Lee I-S, Lee J. Suppression of LPS-induced inflammatory activities by Rosmarinus officinalis L. Food Chemistry, 2013;136 (2):1047-1054.https://doi.org/10.1016/j.foodchem.2012.08.085
16. Nieto G, Ros G, Castillo J. Antioxidant and antimicrobial properties of rosemary (Rosmarinus officinalis, L.): A review. Medicines, 2018;5 (3):98.https://doi.org/10.3390/medicines5030098
17. Ngo SN, Williams DB, Head RJ. Rosemary and cancer prevention: preclinical perspectives. Critical Reviews in Food Science and Nutrition, 2011;51 (10):946-954.https://doi.org/10.1080/10408398.2010.490883
18. Park BB, An JY, Park SU. Recent studies on pinene and its biological and pharmacological activities. EXCLI journal, 2021;20:812-818.
19. Allegra A, Tonacci A, Pioggia G, Musolino C, Gangemi S. Anticancer activity of Rosmarinus officinalis L.: mechanisms of action and therapeutic potentials. Nutrients, 2020;12 (6):1739.https://doi.org/10.3390/nu12061739
20. Kelidari HR, Alipanah H, Roozitalab G, Ebrahimi M,

Osanloo M. Anticancer Effect of Solid-Lipid Nanoparticles Containing Mentha longifolia and Mentha pulegium Essential Oils: In Vitro Study on Human Melanoma and Breast Cancer Cell Lines. Biointerface Research in Applied Chemistry, 2021;12 (2):2128-2137.https://doi.org/10.33263/BRIAC122.21282137
21. Qasemi H, Fereidouni Z, Karimi J, Abdollahi A, Zarenezhad E, Rasti F, Osanloo M. Promising antibacterial effect of impregnated nanofiber mats with a green nanogel against clinical and standard strains of Pseudomonas aeruginosa and Staphylococcus aureus. J Drug Deliv Sci Technol, 2021;66:102844.https://doi.org/10.1016/j.jddst.2021.102844
22. Fernando IPS, Lee W, Han EJ, Ahn G. Alginate-based nanomaterials: Fabrication techniques, properties, and applications. Chemical Engineering Journal, 2020;391:123823.https://doi.org/10.1016/j.cej.2019.123823
23. Zeng Y, Xiang Y, Sheng R, Tomás H, Rodrigues J, Gu Z, Zhang H, Gong Q, Luo K. Polysaccharide-based nanomedicines for cancer immunotherapy: A review. Bioactive Materials, 2021;6 (10):3358-3382.https://doi.org/10.1016/j.bioactmat.2021.03.008
24. Hariyadi DM, Islam N. Current Status of Alginate in Drug Delivery. Adv Pharmacol Pharm Sci, 2020;2020:8886095. https://doi.org/10.1155/2020/8886095
25. Moemenbellah-Fard MD, Shahriari-Namadi M, Kelidari HR, Nejad ZB, Ghasemi H, Osanloo M. Chemical composition and repellent activity of nine medicinal essential oils against Anopheles stephensi, the main malaria vector. Int J Trop Insect Sci, 2021;41 (2):1325-1332.https://doi.org/10.1007/s42690-020-00325-2
26. Sanei-Dehkordi A, Moemenbellah-Fard MD, Saffari M, Zarenezhad E, Osanloo M. Nanoliposomes containing limonene and limonene-rich essential oils as novel larvicides against malaria and filariasis mosquito vectors. BMC Complementary Medicine and Therapies, 2022;22 (1):140.https://doi.org/10.1186/s12906-022-03624-y
27. Hamidpour R, Hamidpour S, Elias G. Rosmarinus officinalis (Rosemary): a novel therapeutic agent for antioxidant, antimicrobial, anticancer, antidiabetic, antidepressant, neuroprotective, anti-inflammatory, and anti-obesity treatment. Biomed J Sci Tech Res, 2017;1 (4):1-6.https://doi.org/10.26717/BJSTR.2017.01.000371
28. Kamyar K, Hiva A, Hadi B, Negar R, Mahmoud O. Chitosan Nanoparticles Containing Cinnamomum verum J.Presl Essential Oil and Cinnamaldehyde: Preparation, Characterization and Anticancer Effects against Melanoma and Breast Cancer Cells. Traditional and Integrative Medicine, 2022;7 (1).
29. Alipanah H, Rasti F, Zarenezhad E, Dehghan A, Sahebnazar B, Osanloo M. Comparison of Anticancer Effects of Carvone, Carvone-Rich Essential Oils, and Chitosan Nanoparticles Containing Each of Them. Biointerface Res Appl Chem, 2022;12:5716-5726.https://doi.org/10.33263/BRIAC124.57165726
30. Valizadeh A, Khaleghi AA, Alipanah H, Zarenezhad E, Osanloo M. Anticarcinogenic Effect of Chitosan Nanoparticles Containing Syzygium aromaticum Essential Oil or Eugenol Toward Breast and Skin Cancer Cell Lines. BioNanoScience, 2021.https://doi.org/10.1007/s12668-021-00880-z
31. Alipanah H, Farjam M, Zarenezhad E, Roozitalab G, Osanloo M. Chitosan nanoparticles containing limonene and limonene-rich essential oils: potential phytotherapy agents for the treatment of melanoma and breast cancers. BMC Complementary Medicine and Therapies, 2021;21 (1):1-10.https://doi.org/10.1186/s12906-021-03362-7
32. Rogers C, Erkes DA, Nardone A, Aplin AE, Fernandes-Alnemri T, Alnemri ES. Gasdermin pores permeabilize mitochondria to augment caspase-3 activation during apoptosis and inflammasome activation. Nature communications, 2019;10 (1):1-17.https://doi.org/10.1038/s41467-019-09397-2
33. Nagata S. Apoptosis and clearance of apoptotic cells. Annual Review of Immunology, 2018;36:489-517.https://doi.org/10.1146/annurev-immunol-042617-053010
34. Kulsoom B, Shamsi TS, Afsar NA, Memon Z, Ahmed N, Hasnain SN. Bax, Bcl-2, and Bax/Bcl-2 as prognostic markers in acute myeloid leukemia: are we ready for Bcl-2-directed therapy? Cancer Management and Research, 2018;10:403-416.https://doi.org/10.2147/CMAR.S154608
35. Pistritto G, Trisciuoglio D, Ceci C, Garufi A, D'Orazi G. Apoptosis as anticancer mechanism: function and dysfunction of its modulators and targeted therapeutic strategies. Aging, 2016;8 (4):603-619.https://doi.org/10.18632/aging.100934
36. Xu Q, Li M, Yang M, Yang J, Xie J, Lu X, Wang F, Chen W. α-pinene regulates miR-221 and induces G2/M phase cell cycle arrest in human hepatocellular carcinoma cells. Bioscience Reports, 2018;38 (6).https://doi.org/10.1042/BSR20180980
37. Matsuo AL, Figueiredo CR, Arruda DC, Pereira FV, Scutti JAB, Massaoka MH, Travassos LR, Sartorelli P, Lago JH. α-Pinene isolated from Schinus terebinthifolius Raddi (Anacardiaceae) induces apoptosis and confers antimetastatic protection in a melanoma model. Biochemical and Biophysical Research Communications, 2011;411 (2):449-454.https://doi.org/10.1016/j.bbrc.2011.06.176
38. D'Arcy MS. Cell death: a review of the major forms of apoptosis, necrosis and autophagy. Cell Biology International, 2019;43 (6):582-592.https://doi.org/10.1002/cbin.11137
39. Pérez-Sánchez A, Barrajón-Catalán E, Ruiz-Torres V, Agulló-Chazarra L, Herranz-López M, Valdés A, Cifuentes A, Micol V. Rosemary (Rosmarinus officinalis) extract causes ROS-induced necrotic cell death and inhibits tumor growth in vivo. Scientific Reports, 2019;9 (1):1-11.https://doi.org/10.1038/s41598-018-37173-7
40. Golstein P, Kroemer G. Cell death by necrosis: towards a molecular definition. Trends in Biochemical Sciences, 2007;32 (1):37-43.https://doi.org/10.1016/j.tibs.2006.11.001