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RESEARCH

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# Cytotoxic effects of chitosan nanoparticles containing *Zataria multiflora* essential oil against human breast and melanoma cells

Hiva Alipanah[1], Fatemeh Yarian[2], Fatemeh Rasti[3], Mojdeh Safari[4], Shekoufeh Hatami[5] and Mahmoud Osanloo[6*]

## Abstract

**Background:** Breast cancer is the most common cancer among women, and melanoma incidence increases worldwide. The emergence of drug resistance and side effects of chemotherapy drugs has led to a great deal of attention being paid to the development of natural medicines, especially using essential oil. The preparation of essential oil-based nanoformulation has thus recently received more attention.

**Results:** In this study, chitosan nanoparticles (ChiNPs) containing *Zataria multiflora* essential oil with a particle size of 177 ± 10 nm, a narrow particle size distribution (SPAN 0.96), and a cubic-like shape were first prepared. $IC_{50}$ values of the prepared nanoformulation against human melanoma (A-375) and breast cancer cell lines (MCF-7 and MDA-MB-468) were obtained as 32 (12–84), 46 (32–67), and 105 (85–131) µg/mL. Besides, an electrospun polycaprolactone–polyethylene oxide scaffold was prepared as a dressing after treatment with the nanoformulation. Fourier transform infrared analysis confirmed the scaffold's preparation as well as successful loading of the essential oil in chitosan nanoparticles. Furthermore, the scaffold did not show a cytotoxic effect on A-375, MCF-7, and MDA-MB-468, and its surface was hydrophobic as the water contact angle with the surface was 136.5°.

**Conclusions:** The prepared prototype with natural ingredients and high efficacy could be considered for further consideration in vivo study or complementary medicine.

**Keywords:** Skin cancer, Electrospun nanofibers, Neoplasm, Natural anticancer

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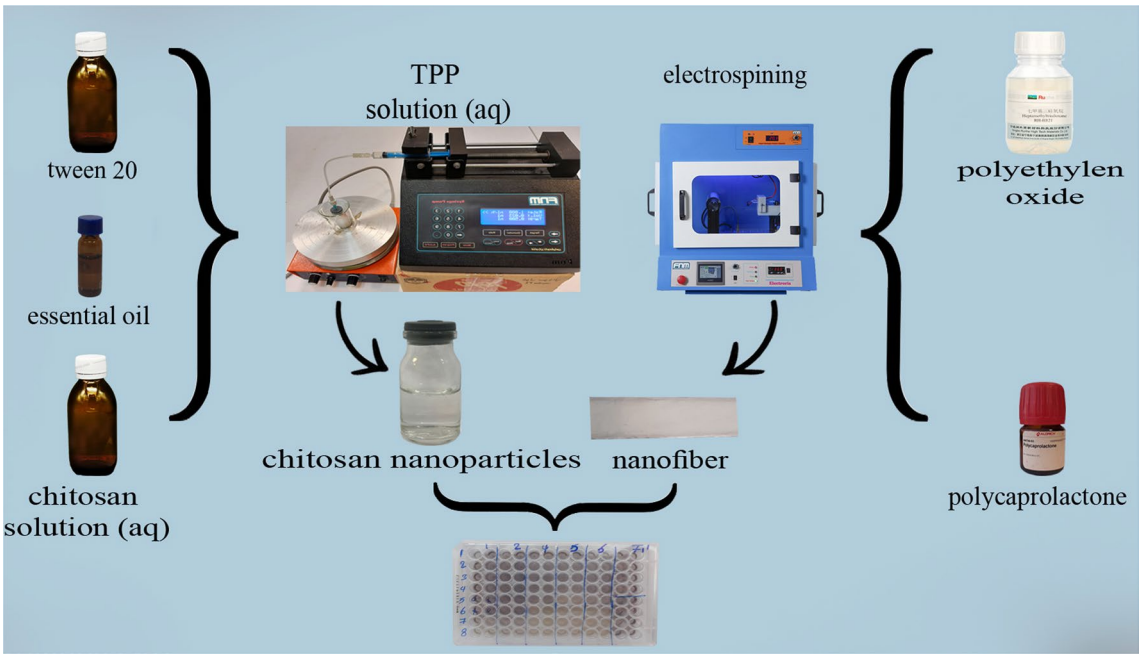
**Graphical abstract**




## 1 Background

After cardiovascular disease, cancers with approximately 17% of the global deaths are major health challenges worldwide. In addition, cancer imposes many onerous burdens, including emotional, physical, and financial encumbrance, on humankind societies [1]. Although the cancer rate was decreased by 3.1% in men yearly, it has a steady state in women (from 2009 to 2012) [2]. Melanoma (cancer of melanocytes) and breast cancer are two of the most dreadful cancers in the world. Breast cancer is the most predominant cancer in Asian nations [3]. As the incidence rate of melanoma has recently increased worldwide, an urgent consideration is required to reduce its morbidity and mortality [4, 5].

The side effects of synthetic or semisynthetic anticancer drugs, including a remarkable decrease in white blood cell count, loss of immunity, bone marrow depression, severe physical weakness, and alopecia, were given serious concern [6]. Therefore, natural products, especially essential oils (EOs), have received special attention in developing new anticancer drugs with less harmful effects [7]. However, since EOs are hydrophobic, to enhance their performance in laboratory and animal research, the preparation of EO-loaded nanostructures (e.g., nanofibers, nanoparticles, and lipid nanocarriers) has received more attention [8, 9]. For instance, as a natural biocompatible and biodegradable polymer, ChiNPs have been widely employed in drug delivery research. For example, ChiNPs containing *Torreya grandis* EO with a particle size of 349.6 nm offered a more potent antibacterial agent than non-formulated EO [10]. In another research, ChiNPs (30–80 nm) containing *Carum copticum* EO showed a better antioxidant effect than the bulk EO [11].

Our previous studies investigated the cytotoxicity of some EOs against A-375 melanoma cells and MCF-7 and MDA-MB-468 human breast cancer cells. For instance, the $IC_{50}$ value of *Myrtus communis* EO against A-375 was 580.8 µg/mL [12]. Besides, *Mentha spicata* and *Tanacetum balsamita* EOs $IC_{50}$ values' against A-375 were 1136 and 1312 µg/mL. On the other hand, their efficacy against MDA-MB-468 cells was 1067 and 2323 µg/mL [13]. $IC_{50}$ values of their major ingredients, i.e., carvone, were obtained as 3657 and 6038 µg/mL against A-375 and MDA-MB-468 cells [13]. Moreover, $IC_{50}$ values of clove EO against A-375 and MDA-MB-468 were 545 and 243 µg/mL [14]. Besides, $IC_{50}$ values of *Anethum graveolens*, *Citrus limon*, and *Zingiber officinale* EOs against MCF-7 were 1908, 201, and > 500 µg/mL; their efficacy on MDA-MB-468 cells were 403, 210, and 775 µg/mL [15]. However, the efficacy of *Zataria multiflora* Bioss. EO was more potent than the mentioned EO; its $IC_{50}$ values against A-375, MCF-7, and MDA-MB-468 were obtained

as 59, 76, and 302 µg/mL [8, 16]. Therefore, this EO was selected for further investigation in the current study. ChiNPs containing *Z. Multiflora* EO were thus first investigated, and their efficacy was then investigated on melanoma and breast cancer cells (A-375, MCF-7, and MDA-MB-468). Besides, a polycaprolactone–polyethylene oxide electrospun scaffold was proposed as dressing after topical treatment with the nanoparticles.

## 2 Methods

### 2.1 Materials

The cell lines were purchased from the Pasteur Institute of Iran; A-375 (ATCC CRL-1619), MCF-7 (ATCC HTB-22), and MDA-MB-468 (ATCC HTB-132). Polycaprolactone (PCL 80.000 Da), polyethylene oxide (PEO 20.000 Da), acetic acid, phosphate-buffered saline tablets, tween 20, sodium-tri-polyphosphate (TPP), chitosan low molecular weight, and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazo-lium bromide (MTT) were purchased from Sigma-Aldrich (USA). Fetal bovine serum was purchased from Gibco (USA). Dulbecco's Modified Eagle's Media (DMEM) cell culture medium, trypsin, penicillin–streptomycin, and dimethyl sulfoxide were obtained from a Chinese company, Shellmax.

### 2.2 Preparation of chitosan nanoparticles containing *Z. multiflora* EO

Chitosan powder (0.25% w/v) was dissolved in a 1% acetic acid aqueous solution (4 h, 2000 rpm, ambient temperature). In order to prepare ChiNPs containing *Z. multiflora* EO, a modified ionic gelation technique was employed [17]. In the first step, the EO (0.5% w/v) and tween 20 (0.25% w/v) were mixed at room temperature for 3 min while the rotation speed was 2000 rpm (Fig. 1). In the next 30 min, the chitosan solution was added and stirred. Then, a syringe pump was employed to add 1 mL/h TPP (0.15% w/v) aqueous solution. The mixture was stirred for 40 min (2000 rpm) to stabilize the ChiNPs containing *Z. multiflora* EO. The same methodology was used for preparing free chitosan nanoparticles; only no EO was used.

### 2.3 Preparation of PCL–PEO scaffold

PCL granules and PEO powder (10%:4% w/v) were dissolved in hexafluoroisopropanol (overnight/ 2000 rpm/ room temperature). The solution was poured into a 10 mL syringe connected to a blunted needle (23 G) and was situated in a syringe pump of the electrospinning machine (Fanavaran nano-meghyas, Iran). Instrumental factors were optimized for preparing the beadles nanofibers with the nano-sized diameter; 0.7 mL/h the injection rate, 20 kV applied DC voltage between needle and collector, and 120 mm distance between needle and collector. In order to separate the formed nanofibrous scaffold, the cylindrical collector (diameter 7.5 cm) was covered using a thin layer of aluminum foil. The collector was rotated during the preparation of scaffolds (110 rpm).

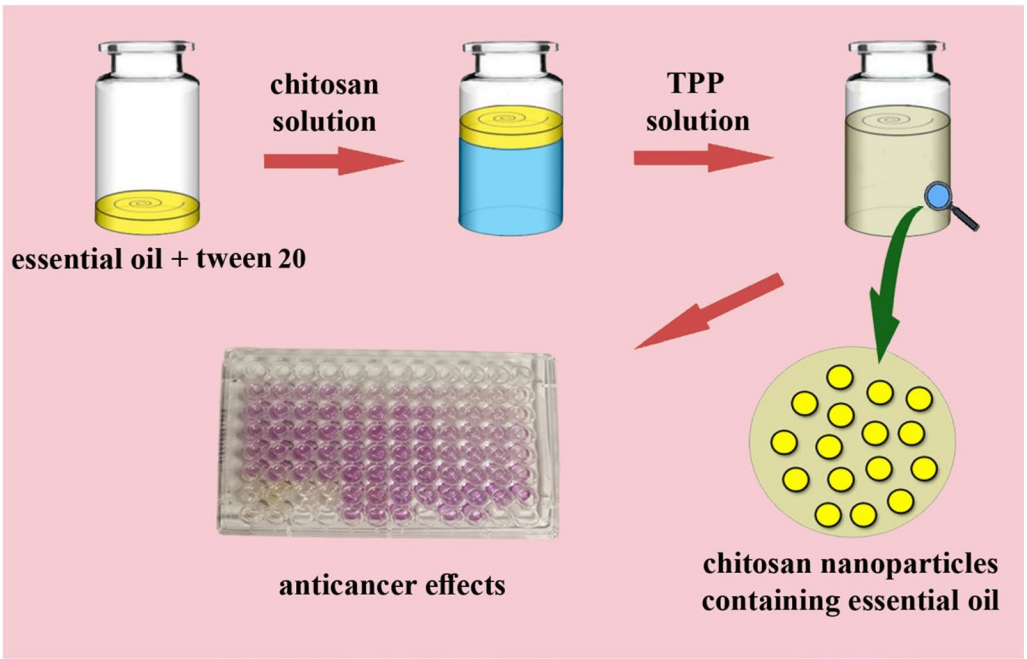



**Fig. 1** Preparation of chitosan nanoparticles containing *Z. Multiflora* EO

### 2.4 Characterizations of the prepared nanostructures

The dynamic light scattering (DLS) technique (K-ONE NANO. LTD, Korea) analyzed the particle size of ChiNPs containing *Z. multiflora* EO. D50 and d90-d10/d50 were considered particle size and size distribution (SPAN). D is the diameter, and 10, 50, and 90 show the percentile of particles with a smaller diameter than these specified diameters. Transmission electron microscopy (LEO 906E Zeiss, Germany) confirmed the particle size of ChiNPs containing *Z. multiflora* EO and determined their morphology. The sample was first concisely diluted using distilled water twice, and one drop was then located on a 200-mesh carbon-coated copper grid and applied to the device. ChiNPs nanoparticles and preparation of PCL–PEO scaffold were confirmed using Fourier transform infrared analysis (Bruker Company, Model Tensor II, Germany). The spectra of ChiNPs containing *Z. multiflora* EO, the scaffold, and their ingredients were recorded in 400–4000/cm.

Scanning electron microscopy was used to investigate the morphology and size of the PCL–PEO scaffold (Scanning Electron Microscopy, Vega 3, TESCAN, Czech Republic). The scaffolds were punched and coated with gold vapor (sputtering coater, Q150R-ES, Quorum Technologies, UK) before subjecting to the scanning electron microscopy instrument. Besides, the wettability of the prepared scaffold was evaluated by determining the contact angle of deionized water with its surface. A five µL volume of deionized water was injected into the surface of the scaffold, and the contact angle was measured.

### 2.5 Investigation of the anticancer activity

The MTT assay was used to investigate the anticancer activity of ChiNPs containing *Z. multiflora* EO. The cell lines were cultured in 25 $cm^2$ culture flasks using DMEM medium cell culture (supplemented with 10% and 1% of fetal bovine serum and penicillin–streptomycin) and incubated at 37 °C in an air/$CO_2$ mixture (95:5%). First, the cells (A-375, MDA-MB-468, and MCF-7) were separated by trypsin; then, they were seeded ($1 \times 10^4$ cells per well) in 96 well plates and incubated overnight for attachment. The next day, the culture media was discarded, and a 75 µL complete fresh medium was added to each well. Finally, concentrations were fixed at 1200, 600, 300, 150, and 75 µg/mL by adding appropriate amounts of ChiNPs containing *Z. multiflora* EO. Moreover, a piece (0.5 cm) of PCL–PEO scaffold was got into other wells to investigate their cytotoxicity.

The treated plates were incubated for 24 h at 37 °C with $CO_2$ 5%. Then, their content was discarded, and wells were washed with 100 µL phosphate-buffered saline to remove the nanoformulations' milky color and non-degraded scaffold. In the next step, 100 µL MTT reagent (0.5 mg $mL^{-1}$) was added to each well and incubated for another 4 h; dimethyl sulfoxide then dissolved created formazan crystals (100 µL/well). Finally, by an ELISA plate reader, the absorbance of the wells was measured at 570 nm; the cell viability at each concentration was calculated using the following equation; (mean absorbance sample/mean absorbance control) × 100. Noteworthy, the control group (six-well/plate) was not treated.

## 3 Results

### 3.1 Characterization of chitosan nanoparticles containing *Z. multiflora* EO

DLS diagram of ChiNPs containing *Z. multiflora* EO with particle sizes of 177 ± 10 nm is depicted in Fig. 2A. The SPAN value is less than 1 (0.96) [18], so its narrow particle size distribution was confirmed. In addition, the presence of a peak sharp in nanoformulations is also indicated narrow particle size distribution. Finally, the transmission electron microscopy verified the morphology of ChiNPs containing *Z. multiflora* EO; it revealed a cubic shape (Fig. 2B).

### 3.2 Loading of *Z. multiflora* EO in chitosan nanoparticles

Fourier transform infrared analysis is popular optical spectroscopy for identifying the molecular structure and possible interactions between the main components of polymeric nanoparticles or scaffolds [19]. Spectra of free chitosan nanoparticles, *Z. multiflora* EO, ChiNPs containing *Z. multiflora* EO, as well as PCL, PEO, and PEO–PCL scaffold spectra, are shown in Fig. 3A.

In the spectrum of free chitosan nanoparticles, the strong bond at about 1700 $cm^{-1}$ can correspond to carbonyl stretching of the secondary amide band of the pure chitosan and carbonyl group in tween. The characteristic peak at 1094 $cm^{-1}$ relates to symmetric and anti-symmetric stretching vibrations in the $PO_2$ group. The strong band at 1020 $cm^{-1}$ belongs to symmetric and anti-symmetric stretching vibrations in the $PO_3$ group. After the crosslinking process, two bands at 1280 and 1152 $cm^{-1}$ belonging to anti-symmetric stretching vibrations of $PO_2$ groups in TPP ions appeared. This new peak showed the formation of ionic crosslinks between protonated amino groups of chitosan and tripolyphosphate anionic groups [20]. The *Z. multiflora* EO spectrum showed broadband between 3200 and 3600 $cm^{-1}$, characteristic of the hydroxyl functional group, and a band at 3019 $cm^{-1}$, attributed to the stretching vibration of =C–H groups from olefins. In addition, peaks at 2959, 2925, and 2869 $cm^{-1}$ relate to –CH's stretching vibrations and the absorption peak around 1737 $cm^{-1}$ relates to C=O. The absorption peaks around 1619 and 1420 $cm^{-1}$ are attributed to

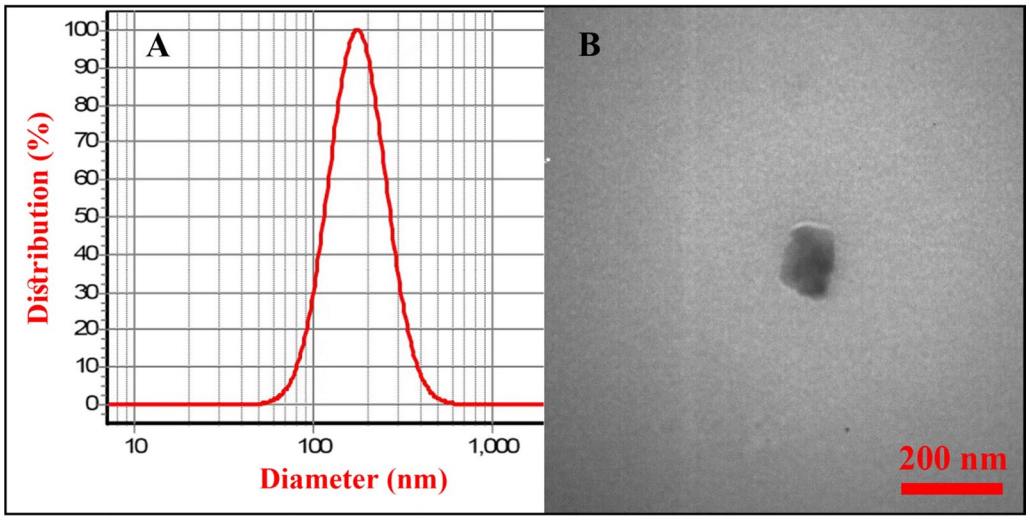



**Fig. 2** **A** DLS diagram of ChiNPs containing *Z. multiflora* EO and **B** transmission electron microscopy image of them

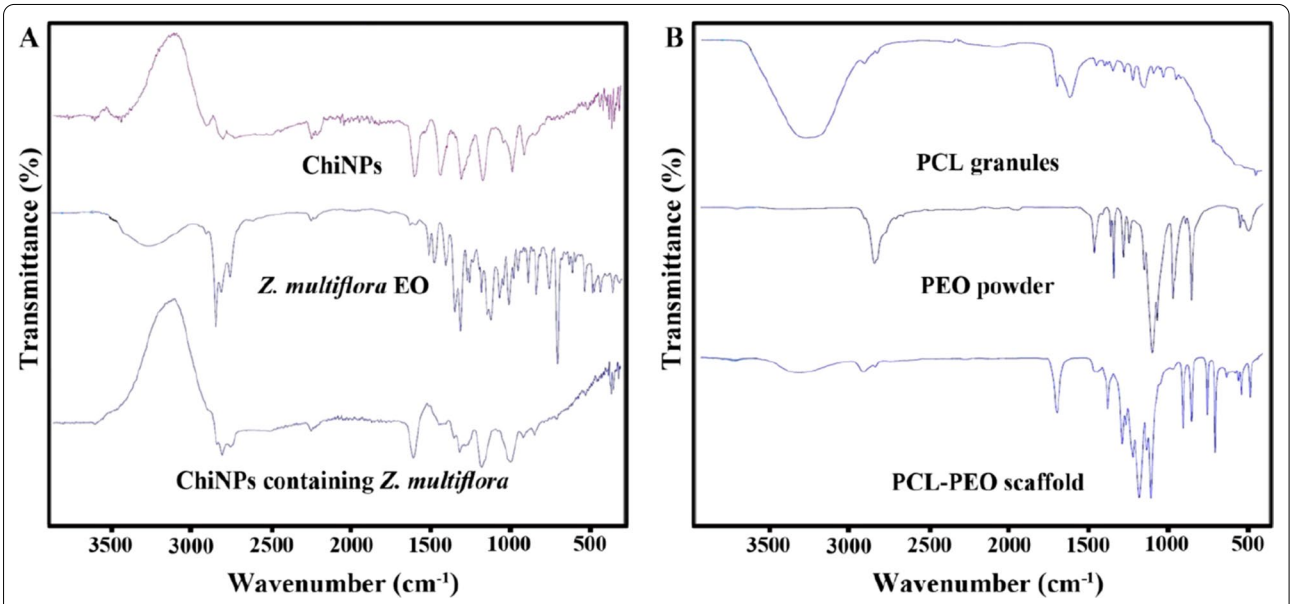



**Fig. 3** Fourier transform infrared spectra: **A** free chitosan nanoparticles (ChiNPs), *Z. multiflora* EO, ChiNPs containing *Z. multiflora* EO and **B** polycaprolactone (PCL), polyethylene oxide (PEO), and PCL–PEO scaffold

C=C, peaks at 1222 and 1175 $cm^{-1}$ relate to (C–O–C) bonds, and the ones at 809 $cm^{-1}$ are attributed to angular deformations of $CH_2$ groups. In spectra of ChiNPs containing *Z. multiflora* EO, two bands at 1280 and 1098 $cm^{-1}$ belong to the linkage between phosphoric groups of TPP and the ammonium group of chitosan. This new peak showed the formation of ionic crosslinks between protonated amino groups of chitosan and TPP anionic groups. The other characteristic peaks are similar to *Z. multiflora* EO.

### 3.3 Physicochemical characteristics of PCL–PEO scaffolds

The spectra of PCL and PEO and the PEO–PCL scaffold are shown in Fig. 3B. The main characteristic peaks of pure PEO at 2878 and 1466 $cm^{-1}$ are attributed to the asymmetric stretching and asymmetric bending of $CH_2$. The peaks at 1341 and 1359 $cm^{-1}$ are associated with the bending vibration of $-CH_2$. The triplet peaks at 1144, 1094, and 1059 $cm^{-1}$ are related to the C–O–C vibration and are also assigned to the existence of the crystalline PEO. The peaks at 960 $cm^{-1}$ and 841 $cm^{-1}$ are associated

with the $CH_2$ rocking vibrations of the methylene (–CH3) group [21, 22]. In the case of PCL, the prominent peak at 1722 $cm^{-1}$ is associated with the carbonyl (C=O) group. The characteristic peaks located at 2943 and 2865 $cm^{-1}$ are assigned to the stretching vibration of $–CH_2$. The FTIR spectrum of PCL also exhibited absorption bands at 1292 $cm^{-1}$ for C–O and C–C stretching and at 1164 and 1236 $cm^{-1}$ for COC symmetric and asymmetric stretching, respectively [23]. Importantly, the main characteristic bands of each compound (PEO and PCL) appeared in the FTIR spectrum of the PEO–PCL scaffold, thus suggesting that both PEO and PCL were present in the prepared scaffolds [24]. The PCL's C=O stretching vibration at 1722 $cm^{-1}$ gets shifted to 1704 $cm^{-1}$ in the PCL–PEO scaffold with lower intensity. The absorption peaks at 2943 and 2865 $cm^{-1}$ corresponding to stretching vibration of $–CH_2$ in PCL are decreased in intensity in the PCL–PEO scaffold. The shifting of the main peaks indicates the molecular interaction of PEO and PCL in the final scaffold [22, 24].

The wettability of solid surfaces is investigated with a water contact angle meter. If the measured contact angle is above 90 degrees, the solid has poor wetting and is called hydrophobic [25]. The water contact angle of the PCL–PEO scaffold was high as 136.5° (Fig. 4A). Besides, Fig. 4B shows randomly oriented and beadles PCL–PEO nanofibrous with a mean diameter of 246 ± 39 nm.

### 3.4 In vitro anticancer activity of chitosan nanoparticles containing *Z. multiflora* EO

Figure 5 indicates the dose-dependent effects of the ChiNPs containing *Z. multiflora* EO on all examined cell lines, including the A-375 melanoma cell line and two breast cancer cell lines, MCF-7 and MDA-MB-468. Interestingly, the viability of A-375 and MCF-7 more than 90% were reduced after treatment with ChiNPs containing *Z. multiflora* EO 600 and 1200 µg/mL. Besides, the viability of cells after treatment with free ChiNPs 10–15% was reduced. Moreover, the scaffold did not significantly affect three cell lines (data not shown).

Furthermore, $IC_{50}$ values of ChiNPs containing *Z. multiflora* EO against A-375, MCF-7, and MDA-MB-468 were obtained as 32 (12–84), 46 (32–67), and 105 (85–131) µg/mL, respectively.

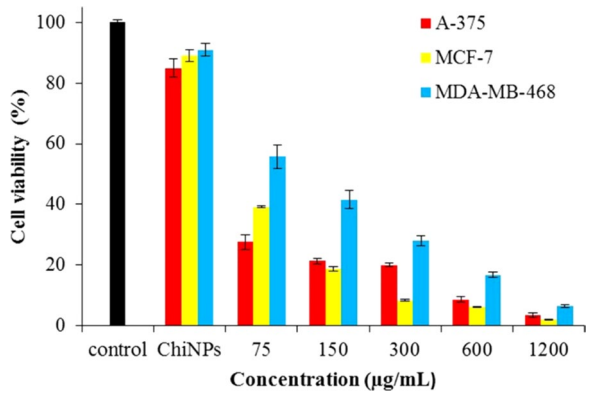



**Fig. 5** Anticancer effects of chitosan nanoparticles containing *Z. multiflora* EO against human melanoma (A-375) and breast cancer cell lines (MDA-MB-468 and MCF-7), $n = 3$

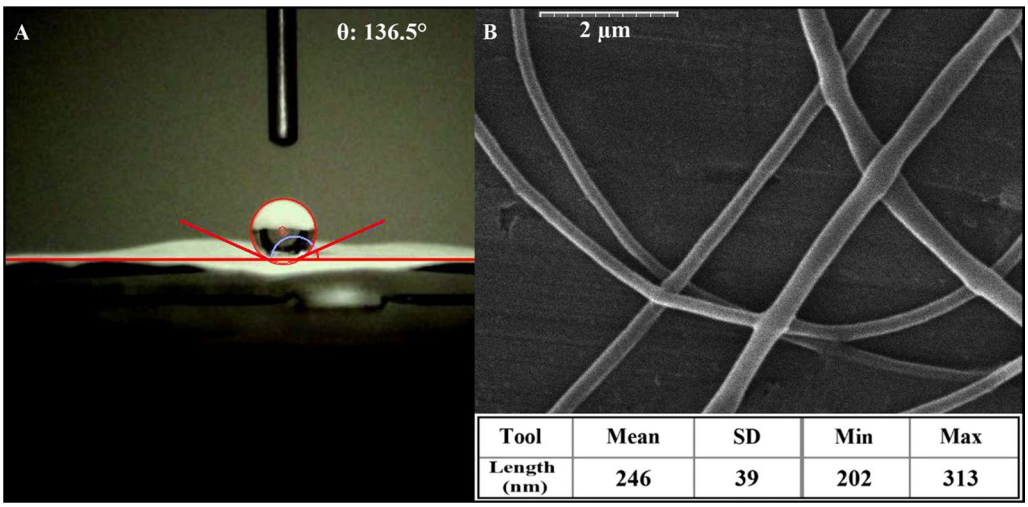



**Fig. 4** **A** Water contact angle with the surface of PCL–PEO scaffold and **B** its scanning electron microscopy image

## 4 Discussion

*Zataria multiflora* (*Lamiaceae* family) is one of the most common medicinal plants that grows in Pakistan, Afghanistan, and southern Iran [26]. Its EO possesses some biological properties, such as antibacterial, antioxidant, and anticancer effects [27, 28]. In this study, the anticancer effect of ChiNPs containing *Z. multiflora* EO on the viability of melanoma (A-375) and breast cancer (MCF-7 and MDA-MB-468) cells was examined. A-375 human melanoma cell maintains typical cutaneous melanoma characteristics and is a suitable in vitro model for studying human cancers' most aggressive, treatment-resistant, and chemo-resistant form [29, 30]. MCF-7 cell is a suitable in vitro model for investigating breast cancer pathogenesis and anticancer drugs and is the most studied ER-positive cell line globally [31, 32]. MDA-MB-468, as an ER-negative breast cancer cell line, is a target cell line for evaluation in vitro invasive and metastatic cancer models [33]. Since the 1970s, incidence rates for many cancers like lung cancer have decreased, but breast and skin cancers are the most commonly diagnosed cancers with high incidence and poor survival rates [34]. Therefore, further research and more effective chemotherapeutic agents are needed to achieve the best outcomes for treating these cancers.

Plant-derived substances originate about 50% of the clinically active chemotherapeutic agents [35, 36]. At least four classes of herbal anticancer agents are on the market today, vinca alkaloids (vincristine, vindesine, and vinblastine), epipodophyllotoxins (teniposide and etoposide), taxanes (docetaxel and paclitaxel), and the camptothecin derivatives (irinotecan and camptothecin) [37]. Moreover, many attempts have been made to exploit EOs as antioxidant, antimicrobial, and anticancer drugs. Nevertheless, besides low water solubility, EOs main compounds have low absorption, low efficiency, and low plasma membrane permeability, which has the limited clinical application of these herbal compounds [29, 38]. Therefore, the preparation of EO-based nanoformulation is a promising approach that increases cellular uptake, solubility, and biological and pharmacological activities. Nanoformulation could also reduce the dosage use, toxicity, and side effects and increase the noticeable efficacy of EOs [39]. For example, nanoformulated *Mentha piperita* EO, as noted by *Abedinpour* et al., increases noticeably cytotoxic and efficacy of *Mentha piperita* EO against human breast cancer cell lines [18]. It has also been indicated that lipid nanoparticles increased the release rate of EO compared to pure EO. Furthermore, *Poladi* et al. have shown that *Artemisia* EO inhibits cancer cell viability, which is more effective by nanoformulation [40]. Furthermore, emerging evidence suggests that ChiNPs can penetrate cancerous cells and induce DNA damage, and finally disrupt cancer cell growth and metabolism [41, 42]. ChiNPs containing EOs have thus been widely used to improve the EOs' therapeutic and pharmacological effects. For instance, *Soltani* et al. designed a cytotoxicity study against liver hepatocellular carcinoma cells by loading *Boswellia sacra* EO in ChiNPs with a particle size of 80.13 nm [43]. Another group proposed ChiNPs containing green tea EO with a mean particle size of $30.7 \pm 1.13$ nm as a natural drug delivery system to cancer against hepatocellular breast and colon carcinoma cells [44].

In the present study, preclinical cancer models (MCF-7, MDA-MB-468, and A-375) investigated the anticancer effect of ChiNPs containing *Z. multiflora* EO with a particle size of $177 \pm 10$ nm. In vitro treatment of both ER-positive (MCF-7), negative breast cancer (MDA-MB-468), and melanoma (A-375) cells by ChiNPs containing *Z. multiflora* EO inhibited growth percentages in cancer cells. The obtained $IC_{50}$ value for ChiNPs containing *Z. multiflora* EO against A-375 melanoma cells ($IC_{50} = 32$ µg/mL) lower than ER-negative MDA-MB-468 cells ($IC_{50} = 105$ µg/mL). ER-positive MCF-7 breast cancer cells ($IC_{50} = 46$ µg/mL) was sensitive compare to the ER-negative MDA-MB-468 cells ($IC_{50} = 105$ µg/mL). Our results agreed with the findings of previous studies indicating that *Z. multiflora* EO has anticancer properties on breast, melanoma, and colon cancers and has the potential to be used in cancer treatment [45, 46]. The results demonstrated that A-375 melanoma cells are more sensitive toward ChiNPs containing *Z. multiflora* EO than ER-positive and –negative breast cancer. It can be concluded that ChiNPs containing *Z. multiflora* EO has a cell line-dependent anticancer activity. In agreement with these results, *Yerlikaya* et al. showed that chitosan could reduce cell proliferation in melanoma cell lines (A-375, SKMEL28, and RPMI7951) with different mechanisms and cells line-dependent manner [47]. It is important to note that, the potency of ChiNPs containing *Z. multiflora* EO in our study was more potent than non-formulated *Z. multiflora* EO in previous studies against these cells; A-375 ($IC_{50} = 59$ µg/mL), MCF-7 ($IC_{50} = 76$ µg/mL), and MDA-MB-468 ($IC_{50} = 302$ µg/mL) [8, 16]. The findings reported in this study and previous results give depth to our understanding of cell line-dependent anticancer activity of ChiNPs.

The wettability of biomaterials is one of the most important properties considered in tissue engineering for skin diseases such as metastatic melanoma [48, 49]. Pathogenic infections increase patient mortality because of a weakened immune system in cancer patients [50]. This study prepared a PCL–PEO scaffold to create a skin coating for melanoma patients after treatment with ChiNPs containing *Zataria multiflora*

EO. The scaffold could be used as a protective coating in melanoma patients for inhibiting the entry of environmental pathogens into the site, allowing air exchange [51, 52].

## 5 Conclusions

The findings of in vitro cancer models suggest that the ChiNPs containing *Z. multiflora* EO could act as a drug supplement to inhibit cancer cell proliferation (breast and melanoma cells). The comparative study showed that the anticancer effect of ChiNPs containing *Z. multiflora* EO was more pronounced in melanoma cells than in breast cancer cells. The PCL–PEO scaffold was also proposed as a skin coating after treatment with the nanoformulation. The prepared prototype could be considered for further investigations in in vivo studies.

### Abbreviations

ChiNPs: Chitosan nanoparticles; Eos: Essential oils; MTT: 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyltetrazo-lium bromide; PCL: Polycaprolactone; PEO: Polyethylene oxide; DLS: Dynamic light scattering; TPP: Sodium-tri-polyphosphate.

### Acknowledgements

Not applicable.

### Author contributions

HA performed MTT tests. FY wrote the introduction. FR prepared the scaffold. ShH reviewed the literature. MS interpreted ATR-FTIR spectra. MO designed the study, analyzed data, and prepared chitosan nanoparticles. All authors contributed to the drafting of the manuscript. All authors read and approved the final manuscript.

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### Availability of data and materials

All data are given in the current report.

## Declarations

### Ethics approval and consent to participate

This study has been approved by the ethical committee of Fasa University of Medical Sciences, Fasa, Iran. Besides, **t**his research did not involve human study; thus, no constant form was used.

### Consent for publication

Not applicable.

### Competing interests

Researchers have no conflict of interest in this study.

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