Chitosan nanoparticles containing limonene and limonene-rich essential oils: potential phytotherapy agents for the treatment of melanoma and breast cancers

Hiva Alipanah 1, Mojtaba Farjam 2,3, Elham Zarenehzad 2,3, Ghazaal Roozitalab 2 and Mahmoud Osanloo 4*

Abstract

Background: Melanoma and breast cancers are two common cancers worldwide. Due to the side effects of chemotherapy drugs and the occurring resistance against them, the development of green drugs has been received more attention.

Methods: The anticancer effects of three essential oils from the Citrus family and their identified major constituents (limonene) were first investigated against melanoma and breast cancer cell lines (A-375 and MDA-MB-468). By preparing chitosan nanoparticles containing them, an attempt was then made to improve their effectiveness.

Results: Chitosan nanoparticles containing Citrus sinensis and Citrus limon essential oils with IC50s of 0.03 and 0.124 μg/mL on A-375 cells, and 23.65 and 40.32 μg/mL on MDA-MB-468 showed distinct anticancer efficacies.

Conclusion: The prepared formulations could thus be considered as green anticancer agents in complementary medicine and therapies.

Keywords: Citrus, Triple negative breast neoplasms, Skin neoplasms, Phytochemicals, Cytostatic agents

Background

Breast cancer is one of the most common cancer, followed by prostate, lung, and colon cancers worldwide [1]. Breast cancer is the commonest life-threatening malignancy, causing 14% of all cancer-related fatalities among women [2, 3]. Skin cancer is another common and preventable carcinoma worldwide; its annually rising incidence has made it a pre- eminent public health threat [4]. Malignant melanoma is a type of skin cancer and responsible for the vast majority of skin cancer deaths; it begins with the abnormal proliferation of cells known as melanocytes [5]. Chemotherapy, surgery, and radiotherapy are common cancer treatments, but their nonspecific action and severe side effects are cancer treatment’s biggest obstacles [6, 7]. To bypass the side effects, developing new green drugs, especially essential oils (EO) s, has recently received more attention [8, 9].

Citrus aurantium (bitter orange) is a tree 4–6 m high, evergreen, hairless, with long blades of the Rutaceae (Citrus) family [10]. It possesses antispasmodic, anti-inflammatory, anti-flatulence, antihypertensive, and diuretic properties [11–13]. Citrus limon (L.) or lemon is another member of the Rutaceae family rich in vitamins, minerals, dietary fiber, and carotenoids [14]. Citrus sinensis (scientifically name for the orange) grows as a fruit-bearing shrub with green leaves and white flowers and is also a member of the Rutaceae family [15, 16].

EOs of the three mentioned specimens possesses
anticancer effects against different types of breast cancer cell lines. Their IC_{50}s against MCF-7 were reported at 82, 57, and 39 μg/mL, their IC_{50}s against MDA-MB-231 were reported at 74, 37, and 39 μg/mL, and their IC_{50}s against T47D were reported at > 300, 19, and 43 μg/mL. Moreover, limonene (1-methyl-4-(1-methylethenyl) cyclohexane) is a major constituent in the mentioned EOs; it is a colorless and aromatic liquid oil and acts as a potential chemotherapeutic monocyclic monoterpenone in nature [18–20]. Limonene has significant anticaner activity by inhibiting tumor initiation, growth and angiogenesis, and cancer cell apoptosis [21, 22]. For instance, the antiproliferative activity of limonene on BW5147 cells, colon, gastro, melanoma, mammary gland tumors has been confirmed [23, 24].

In this research, the anticancer effects of C. auran-tium, C. limon, and C. sinensis EOs (CAEO, CLEO, and CSEO) and limonene were first investigated against A-375 (human melanoma cancer cell line) and MDA-MB-468 (human breast cancer cell line). By preparing chito-san nanoparticles (ChiNPs) containing them, an attempt was made to improve their anticancer properties.

**Methods**

**Cells and reagents**

Pasteur Institute of Iran supplied the used cell lines, including breast cancer cell line MDA-MB-468 (ATCC HTB-132) and melanoma cell line A-375 (ATCC CRL-1619). Tetrazolium salt, 3-(4,5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazo-lium bromide (MTT), phosphate-buffered saline (PBS) tablets, Sodium-tripolyphosphate (TPP), chitosan low molecular weight, acetic acid, tween 20, and limonene were purchased from Sigma-Aldrich (USA). Penicillin streptomycin, trypsin, dimethyl sulfox-ide (DMSO), and Dulbecco’s Modified Eagle’s Media (DMEM) cell culture medium were purchased from Shellmax (China). Fetal bovine serum (FBS) was obtained from Gibco (USA). EOs were purchased from Iranian companies; CSEO Green Plants of Life Co. Ltd., CLEO Barij Essence Pharmaceutical Co., and CAEO Tabir Daru Co.

**Chemical composition of the EOs**

Ingredients of CSEO, CLEO, CAEO were analyzed by Gas Chromatography-Mass Spectrometry (GC–MS); the EOs were analyzed using a 6890 GC system coupled with a 5975 series of mass selective detectors (Agilent Technologies, USA). The separations were performed on HP-5MS silica fused column (length, 30 m; internal diameter, 0.25 mm; film thicknesses, 0.25 mm; stationary phase, 5% phenyl 95% methyl polysiloxane). The column temperature program started at 40 °C (fixed for 1 min), then raised with a rate of 3 °C · min^{-1 } to 250 °C, and was finally held for 60 min at this temperature. The temperature of the injection port and detector was fixed at 250 and 230 °C, respectively. Other operating conditions were as follows: carrier gas, helium (99.999%), split-flow 25 mL/min, septum purge 6 mL/min, and column flow rate 1 mL/min. Mass spectra were taken at full scan mode and 70 eV ionization energy and full scan mode. The scanned mass range was set at 50–550 m/z. The identification of ingredients was performed as described in our previous study, and the peak area normalization procedure was used for the quantitative determination of the compounds in the EOs [25].

**Preparation of chitosan nanoparticles**

Chitosan powder (0.25% w/v) was dissolved in a 1% acetic acid aqueous solution (4 h, 2000 rpm, ambient temperature). Preparation of chitosan nanoparticles containing limonene, CAEO, CLEO, and CSEO was obtained using a modified ionic gelation technique, as depicted in Fig. 1 [26]. Each EO or limonene (0.5% w/v) and tween 20 (0.5% w/v) was first mixed at 2000 rpm for 30 min at room temperature. The chitosan solution was then added and stirred for another 30 min. After that, an aqueous solution of TPP (0.15% w/v) was added 1 mL/h using the syringe pump, and the mixture was stirred for 40 min (2000 rpm) to stabilizing the nanoparticles. The prepared samples were abbreviated as CLChiNPs, CSChiNPs, CACHiNPs, and LimChiNPs. Moreover, the same methodology was used to prepare free chitosan nanoparticles (ChiNPs), but no EO or limonene was used.

**Characterization of the prepared nanoformulation**

**Size analysis**

Dynamic light scattering (DLS) technique was used to analyze all nanoformulations particle sizes (dynamic light scattering, scatter scope, K-ONE NANO. LTD, Korea). D50 was considered particle size, and particle size distribution (SPAN) was calculated as d90-d10/d50. D is diameter, and 10, 50, and 90 are percentile of particles with a smaller diameter than these specified diameters. Formulations with SPAN values less than 1 possess narrow particle size distributions [27].

**The attenuated Total reflection-Fourier transform infrared (ATR-FTIR) analysis**

ATR-FTIR has analyzed the loading of the EOs or limonene in the ChiNPs. Spectra of ChiNPs, limonene, lim-ChiNPs, CSEO, CSChiNPs, CLEO, CLChiNPs, CAEO, and CACHiNPs were recorded in 400–4000 cm^{-1}. The samples without preparation were subjected to the instrument (FTIR, Bruker Company, Model Tensor II, Germany).
Investigation of the anticancer activity

The anticancer activity of limonene, CAEO, CLEO, and CSEO (as non-formulated samples) also, Lim-ChiNPs, CAChiNPs, CLChiNPs, and CSChiNPs (as nanoformulations) were investigated using MTT assay. The EOs and limonene were dissolved (0.5% w/v) in a PBS solution containing 0.5% DMSO. The cell lines were cultured in 75 cm² culture flasks in DMEM medium supplemented with 10% of FBS and 1% of penicillin-streptomycin (P/S) and incubated at 37 °C air (95%) and CO₂ (5%). Cells (MDA-MB-468 and A-375) were separated by trypsin; they were then seeded (1 × 10⁴ cells per well) in 96 well plates and incubated overnight for attachment. The following day, the culture media was discarded, and a 75 μL complete fresh medium was added to each well. By adding an appropriate amount of the samples, concentrations were finally fixed at 1200, 600, 300, 150, and 75 μg/mL. The treated plates were incubated for 24 h at 37 °C with CO₂ 5%, and their content was then discarded, and wells were washed with 100 μL PBS to remove nanoformulations milky color. After that, 100 μL MTT reagent (0.5 mg.mL⁻¹) was added to each well and were incubated for another 4 h; created formazan crystals were then dissolved by adding DMSO (100 μL/well). The control group (six-well in each plate) was treated only with 25 μLPBS (0.5% DMSO) and 75 μL culture media. Finally, the absorbance (A) of the wells was monitored by an ELISA plate reader at 570 nm. The cell viability at each concentration was calculated using the following equation:
Cell viability (\%) = \frac{\text{Mean } A \text{ sample}}{\text{Mean } A \text{ control}} \times 100 \tag{1}

Results

Identified compounds in used EOs

Limonene was identified as the major compound in all three used EOs; it comprised 31.4% of CAEO, 61.8% of CLEO, and 71.3% of CSEO (see Table 1). Sabinene (15.6%), \(\gamma\)-terpinene (6.0%), linalool (5.6%), and nerolidol (5.1%) were the other four major compounds of CAEO. Alpha-pinene, sabinene, cis-limonene oxide, and trans-limonene oxide with a portion of 3.5, 17.0, 2.3, and 3.1% were the other four CLEO compounds. In SCEO, trans-p-2,8-Menthadien-1-ol, cis-limonene oxide, trans-limonene oxide, and trans-carveol were identified as the other four major compounds (5.0, 2.6, 2.3, and 2.9%).

The particle size of the prepared chitosan nanoparticles containing limonene or EOs

DLS analyses of the prepared nanoformulations are given in Fig. 2. CSChiNPs with a particle size of 156 ± 8 nm (SPAN 0.92) possess the smallest particles. CAChiNPs (173 ± 6 nm), CLChiNPs (181 ± 4 nm), and LimChiNPs (209 ± 13 nm) were situated in other ranks; their SPAN values were including 0.94, 0.90, 0.93, respectively.

Successful loading of limonene or EOs in chitosan nanoparticles

The ATR-FTIR spectrum of ChiNPs is depicted in Fig. 3A; the bond at about 1700 cm\(^{-1}\) can be related to carbonyl stretching of the pure chitosan’s secondary amide band and carbonyl group tween. The characteristic peak at 1094 cm\(^{-1}\) relates to symmetric and anti-symmetric stretching vibrations in the PO\(_2\) group, and 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\(^{-1}\) belong to anti-symmetric stretching vibrations of PO\(_2\) groups in TPP ions. These new peaks confirmed ionic crosslinks between protonated amino groups of chitosan and tripolyphosphate anionic groups.

Table 1

| RTa | Ri b | Compound                        | CAEO Area | %   | CLEO Area | %   | CSEO Area | %   |
|-----|------|---------------------------------|-----------|-----|-----------|-----|-----------|-----|
| 9.4 | 622.7| \(\alpha\)-pinene               | 58,484,827| 1.7 | 126,834,642| 3.5 | 37,874,128| 1.2 |
| 11.3| 694.5| sabinene                        | 542,668,432| 15.6| 623,674,861| 17.0| 34,246,642| 1.1 |
| 11.3| 696.9| \(\beta\)-pinene                 | 43,301,938| 1.2 | –          | –   | –         | –   |
| 12.0| 714.9| \(\beta\)-myrcene                | 108,784,770| 3.1 | –          | –   | –         | –   |
| 13.1| 742.0| \(\alpha\)-terpinene             | 59,177,141| 1.7 | –          | –   | –         | –   |
| 13.9| 762.4| limonene                        | 1,088,445,097| 31.4| 2,269,351,083| 61.8| 2,266,978,799| 71.3|
| 14.7| 781.9| \(\beta\)-ocimene Y              | 162,728,160| 4.7 | –          | –   | –         | –   |
| 15.1| 793.5| \(\gamma\)-terpinene            | 207,125,216| 6.0 | –          | –   | –         | –   |
| 17.2| 835.8| linalool                        | 192,637,034| 5.6 | –          | –   | –         | –   |
| 18.1| 853.7| cis-p-Menth-2,8-dienol          | –          | –   | –          | –   | 60,702,174| 1.9 |
| 18.6| 864.7| trans-p-2,8-Menthadien-1-ol      | –          | –   | –          | –   | 157,669,843| 5.0 |
| 18.8| 868.0| cis-limonene oxide              | –          | –   | 83,422,623| 2.3 | 82,287,580| 2.6 |
| 18.8| 869.1| trans-limonene oxide            | –          | –   | 113,204,736| 3.1 | 72,968,453| 2.3 |
| 20.6| 905.3| 4-terpineol                    | 66,692,764| 1.9 | –          | –   | –         | –   |
| 21.5| 921.1| nortricyclene                  | –          | –   | –          | –   | 31,832,189| 1.0 |
| 22.7| 943.8| trans-carveol                  | –          | –   | –          | –   | 92,456,195| 2.9 |
| 23.2| 953.7| cis-carveol                    | –          | –   | –          | –   | 55,583,810| 1.7 |
| 23.6| 960.1| cuminaldehyde                  | 128,003,231| 3.7 | –          | –   | –         | –   |
| 23.7| 963.5| carvone                        | –          | –   | –          | –   | 66,472,215| 2.1 |
| 25.8| 1002.3| 3-buten-1-ol, 4-chloro-2-methyl-1-phenyl| 41,752,965| 1.2 | –          | –   | –         | –   |
| 28.2| 1047.1| 1,2-yclochexanediol, 1-methyl-4-(1-methylethenyl)| –         | –   | 55,304,649| 1.5 | –         | –   |
| 37.1| 1223.5| nerolidol                     | 178,234,586| 5.1 | –          | –   | –         | –   |
| 42.9| 1345.6| farnesol                      | 34,776,356| 1.0 | –          | –   | –         | –   |

\(^{a}\) retention time, \(^{b}\) retention index
In the ATR-FTIR spectrum of limonene (Fig. 3B), the band at 3001 cm\(^{-1}\) relates to = C-H, and the bands at 2962, 2913, 2855, and 2855 cm\(^{-1}\) showed –CH stretching, the specific absorption at 1677 cm\(^{-1}\) corresponds to the stretching vibration of vinyl substituted C=C. The absorption band at 1644 cm\(^{-1}\) is assigned to the C=C vibration. The characteristic peaks at 989 and 890 cm\(^{-1}\) attributed to C-H bending absorption.

In the spectrum of LimChiNPs (Fig. 3C), the characteristic peak at 2925 m\(^{-1}\) is showed C-H stretching in chitosan and C-H of limonene. A strong characteristic peak showed at 1278 cm\(^{-1}\) belong to C-N stretching indicates the complex formation via electrostatic interaction between NH\(^3\) + groups of chitosan within phosphoric groups of TPP. The band around 1580 cm\(^{-1}\) can be assigned to C-N stretching vibration and refers to the amide group because of the NH\(_2\) bending vibration. The band at 1017 cm\(^{-1}\) related to C-O in chitosan and the shape and position of the peaks proved that limonene was successfully encapsulated ChiNPs.

ATR-FTIR spectrum of CAEO is depicted in Fig. 3D; the broad peak at 3436 cm\(^{-1}\) attributes to NH, the bands at 2961, 2923, 2872 cm\(^{-1}\) displayed –CH cm\(^{-1}\), and the peak at 1706 cm\(^{-1}\) corresponding to the stretching vibration of carbonyl C=O. Absorption bands at 1644 and 1437 cm\(^{-1}\) are assigned to the aromatic ring C=C skeleton vibration of an aromatic substance. The peak at 1022 cm\(^{-1}\) is characteristic of a stretching vibration of C-N. The peak at 957 cm\(^{-1}\) is attributed to C-H bending absorption, and the strong peak at 758 cm\(^{-1}\) is assigned to benzene rings C-H vibration absorption.

From Fig. 3E, some of the specific peaks of CAEO disappeared when it was encapsulated in chitosan, i.e., CAChiNPs. A strong new peak showed at 1281 cm\(^{-1}\) belong to the C-N stretch indicating the complex formation via electrostatic interaction between NH\(^3\) + groups of chitosan within phosphoric groups of TPP. Also, the band around 1547 cm\(^{-1}\) can be assigned to C-N stretching vibration and refers to the amide group because of the NH\(_2\) bending vibration.

In ATR-FTIR spectra of CLEO, Fig. 3F, the bands at 3399 cm\(^{-1}\) related to OH stretching vibration, the bands at 3072 cm\(^{-1}\) related to = C-H. The bands at 2963, 2918, 2855, and 2834 cm\(^{-1}\) showed –CH stretching vibration,
and the characteristic bands $1677\text{ cm}^{-1}$ corresponding to $\text{C=O}$, the peak at around $1105\text{ cm}^{-1}$ related to $\text{C-O}$ bending vibration.

In ATR-FTIR of CLChiNPs (Fig. 3G), the new strong peak at around $1280\text{ cm}^{-1}$ relates to $\text{C-N}$ stretching was attributed to the electrostatic interaction between chitosan and TPP. Also, the sharp peak around $1545\text{ cm}^{-1}$ attributed to $\text{C-N}$ stretching vibration related to the amide group because of the $\text{NH}_2$ bending vibration. All other characteristic peaks appear in the spectra of CLEO and ChiNPs; it is confirmed that CLEO was successfully encapsulated in ChNPs.

In the spectrum of CSEO (Fig. 3H), the bond at about $3412\text{ cm}^{-1}$ can be related to $\text{OH}$ stretching vibrations, and the peaks at around $2964$, $2918$, $2885\text{ cm}^{-1}$ are related to $\text{-C-H}$. The vibrational bands around $1729\text{ cm}^{-1}$ related to $\text{C=O}$, and the band around $1677$ attribute $\text{C-O}$ stretching vibrations. The main peaks around $1110$, $1115$, and $1309\text{ cm}^{-1}$ are related to $\text{C-O}$. The peaks at $885\text{ cm}^{-1}$ attributed to $\text{C-H}$ bending absorption.

After CSEO was encapsulated, the spectrum showed a strong peak at $1280\text{ cm}^{-1}$ belongs to the $\text{C-N}$ stretch, indicating the complex formation via electrostatic interaction between chitosan and TPP (see Fig. 3I). The
strong band around 1547 cm$^{-1}$ can be assigned to C-N stretching vibration and refers to the amide group because of the NH$_2$ bending vibration. The band at 1096 cm$^{-1}$ due to the phosphoric acid root and the protonation of amino cross-linking effect.

**Anticancer effects of CSEO, CLEO, CAEO, and limonene**

Figure 4 illustrates the cytotoxicity effects of limonene, CAEO, CLEO, and CSEO and their nanoformulated forms on A-375 cells; obtained IC$_{50}$s are listed in Table 2. Interestingly, the viability of A-375 cells treated with CLChiNPs, CSEO, and CSChiNPs at all examined concentrations (150, 300, 600, and 1200 μg/mL) was reduced < 20%. IC$_{50}$s of CLChiNPs, CSEO, and CSChiNPs were 0.124, 0.02, and 0.03 μg/mL. Besides, IC$_{50}$s of LimChiNPs and CAChiNPs were observed at 30.24 and 55.00; there was no significant difference together (p > 0.05). However, their potency was significantly more than their no-formulated forms, i.e., limonene and CAEO, with IC$_{50}$s of 246.05 and 10,564.00 μg/mL.

From Fig. 5, the cytotoxicity effects of limonene, CAEO, CLEO, and CSEO on MDA-MB-468 cells are depicted. Generally, the samples had less potency on the growth of this cell line than A-375 cells; viability of cells reduced to lower than 10% after treating with only LimChiNPs at a concentration of 1200 μg/mL. The best-obtained IC$_{50}$s were related to CSChiNPs (23.65 μg/mL) and CLChiNPs (40.32 μg/mL). Moreover, IC$_{50}$s of LimChiNPs and CAChiNPs (650.70 and 240.44 μg/mL) were significantly more potent than their non-formulated forms (2118.94 and 2037.53 μg/mL).

**Discussions**

Obtained IC$_{50}$ for CSEO (0.02 μg/mL) against A-375 in the current study was more potent than that reported in the literature, e.g., IC$_{50}$s of Ajuga chamaepity, Sideritis montana, and Eryngium campestre EOs were reported at 67.44, 34.89, and 1.57 [28–30]. Besides, the efficacy of the most potent non-formulated sample against MDA-MB-468 in the current study (i.e., CLEO 137 μg/mL) was assessed as moderate compared to those published in the literature; Kelussia odoratissima EO 85.00 μg/mL, Zataria multiflora EO 302 μg/mL, and Mentha piperita EO 2536 μg/mL [25, 31, 32]. Interestingly, no report was found on preparing chitosan nanoparticles containing CAEO, CLEO, CSEO, and limonene as anticancer agents on these cell lines in the literature.

![Anticancer effect of limonene and EOs of C. aurantium, C. limon, and C. sinensis (CAEO, CLEO, and CSEO) and chitosan nanoparticles contained them (LimChiNPs, CACHiNPs, CLChiNPs, and CSChiNPs) against A-375 cells. Data are presented as mean ± standard deviation (n = 3).](image-url)
chitosan microcapsules containing limonene with a particle size of 2–12 μm were reported [33]. Electrosprayed Alyssum homolocarpum seed gum nanoparticles containing limonene with a mean diameter of 65.68 ± 8.80 nm was reported in another research [34]. In a study, CAChiNPs with a particle size of 40 nm was proposed as mushroom food packaging; it significantly decelerated the rate of color change, weight loss, and firmness compared to fumigation with EO [35]. In another study, by incorporating CAEO in ChiNPs (20–60 nm), antioxidant and antimicrobial effects were improved [36]. Chitosan nanoemulsion containing CSEO as a green fruit juice preservative was also reported [37]. In another report, CLEO was encapsulated in chitosan/hicap with a mean particle size of 339.3 nm; it was concluded that due to the desirable physicochemical properties and thermal stability, this formulation could be used in medicine and food industries [38].

Furthermore, the preparation of nanoformulations to improve the anticancer effects of EOs has recently received much attention [39]. For instance, a study confirmed that by loading limonene into ChiNPs with a particle size of 339.5 nm, its antioxidant became more potent by improving its solubility [40]. CAEO nanoemulsion showed cytotoxicity against A549 cells with the IC_{50} value of 152 μg/mL [41]. Besides, isolated nanovesicles from C. lemon have been shown an inhibitory effect in a time-dependent manner on cancer cell proliferation in different tumor cell lines by inducing apoptotic cell processes [42]. It has also been proposed that loading limonene into solid lipid nanoparticles (one of the pharmaceutical drug delivery systems) can reduce growth percentages in cancer cells with a low toxic effect on the non-tumoral cell line [43]. In vitro cytotoxicity assay showed that D-limonene-loaded niosome had a noticeable anticancer effect than D-limonene against HepG2, MCF-7, and A549 cell lines [39].

In the current study, by preparing ChiNPs containing EOs/limonene, their anticancer effects were improved, e.g., IC_{50}s of limonene against A-375 and MDA-MB-468 were obtained at 246 and 2118 μg/mL, while IC_{50}s of its nanoformulated state (LimChiNPs) were observed at 30 and 650 μg/mL. CAEO did not show proper efficacy on both mentioned cell lines (IC_{50} 10,564 and 2037 μg/mL). However, after the preparation of ChiNPs containing CAEO, its IC_{50}s were reduced dramatically to 55 and 240 μg/mL. Moreover, only IC_{50} of CSEO (0.02 μg/mL) against A-375 cells was not improved after preparation of its nanoformulation (CSChiNPs: 0.03 μg/mL); further investigation is needed at low concentrations. However, as the hydrophobic nature of CSEO, for its practical administration in vivo or clinical trials, it should be formulated; thus, its nanoformulated form (CSChiNPs) was the best sample against A-375 cells. Interestingly, IC_{50}s of CSChiNPs and CLChiNPs (0.03 and 0.124 μg/mL) against A-375 were more potent than cisplatin with IC_{50} of 0.40–0.45 μg/mL [29, 44]. Their efficacies against MDA-MB-468 (23.65 and 40.32 μg/mL) were also comparable or more potent than cisplatin (IC_{50} 32.50 μg/mL) [45].

**Conclusions**

Compounds of three EOs from the *Citrus* family, including *C. aurantium*, *C. limon*, and *C. sinensis*, were first identified using GC-MS analysis. Their anticancer effects were then investigated on human breast cancer and melanoma cell lines. After that, chitosan nanoparticles containing EOs and limonene (as their major ingredients)
were prepared for improving their efficacies. Chitosan nanoparticles containing \textit{Citrus sinensis} and \textit{Citrus limon} essential oils with IC$_{50}$s of 0.03 and 0.124 μg/mL on A-375 cells, and 23.65 and 40.32 μg/mL on MDA-MB-468 showed more potency than other samples. Thus two mentioned formulations could be considered as green anticancer agents in future studies.

**Abbreviations**

GC–MS: Chromatography–Mass Spectrometry; ATR–FTIR: Attenuated Total Reflection–Fourier Transform Infrared; EO: Essential Oil; CAEO: \textit{Citrus aurantium} essential oil; CLEO: \textit{Citrus limon} essential oil; CSEO: \textit{Citrus sinensis} essential oil; ChiNPs: Chitosan nanoparticles; CACHiNPs: Chitosan nanoparticles containing \textit{Citrus aurantium} essential oil; CLChiNPs: Chitosan nanoparticles containing \textit{Citrus limon} essential oil; CSChiNPs: Chitosan nanoparticles containing \textit{Citrus sinensis} essential oil

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Not applicable.

**Authors’ contributions**

HA performed MTT assays and was the main draver of the manuscript. MF was the data analyzer. EZ interpreted ATR-FTIR. GH reviewed the literature. MO designed the study and prepared nanoformulations. All authors contributed to drafting the manuscript and confirmed the final version. The author(s) read and approved the final manuscript.

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**Availability of data and materials**

All data generated or analyzed during this study are included in this published article.

**Declarations**

**Ethics approval and consent to participate**

The ethical committee has ethically approved this research; IR.FUMS.REC.1399.188. This research did not involve in vivo or human study, so consent to participate did not use.

**Consent for publication**

Not applicable.

**Competing interests**

None.

**Author details**

1Department of Physiology, School of Medicine, Fasa University of Medical Sciences, Fasa, Iran. 2Noncommunicable Diseases Research Center, Fasa University of Medical Sciences, Fasa, Iran. 3Clinical Research Development Unit, Valie-Asr Hospital, Fasa University of Medical Sciences, Fasa, Iran. 4Department of Medical Nanotechnology, School of Advanced Technologies in Medicine, Fasa University of Medical Sciences, Fasa, Iran. 5Department of Medical Nanotechnology, School of Advanced Technologies in Medicine, Fasa University of Medical Sciences, Fasa, Iran.

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**References**

1. SEER Cancer Statistics Review, 1975-2018, National Cancer Institute. [https://seer.cancer.gov/csr/1975_2018].
2. Sharma GN, Dave R, Sandhya I, Sharma P, Sharma K. Various types and management of breast cancer: an overview. J Adv Pharm Technol Res. 2010; 1(2):109–26.
3. McGuire A, Brown JA, Kerin MJ. Metastatic breast cancer: the potential of miRNA for diagnosis and treatment monitoring. Cancer Metastasis Rev. 2015;34(1):145–55. https://doi.org/10.1007/s10555-015-9551-7.
4. Gordon R. Skin cancer: an overview of epidemiology and risk factors. In: Semin Oncol Nurs. 2013: Elsevier; 2013: 160–169.
5. Uong A, Zon Li. Melanocytes in development and cancer. J Cell Physiol. 2010;222(1):38–41. https://doi.org/10.1002/jcp.21935.
6. Haider T, Pandey V, Banjare N, Gupta PN, Soni V. Drug resistance in cancer: mechanisms and tackling strategies. Pharmacol Rep. 2020;72(5):1125–51. https://doi.org/10.1007/s43440-020-00138-7.
7. Blowman K, Magalhães M, Lemos M, Cabral C, Pires I. Anticancer properties of essential oils and other natural products. Evid Based Complement Alternat Med. 2018;2018:1–12. https://doi.org/10.1155/2018/3149362.
8. Pavithra PS, Mehta A, Verma RS. Essential oils: from prevention to treatment of skin cancer. Drug Discov Today. 2019;24(2):644–55. https://doi.org/10.1016/j.drudis.2018.11.020.
9. Russo R, Corasaniti MT, Bagetta G, Morrone LA. Exploitation of cytotoxicity of some essential oils for translation in cancer therapy. Evid Based Complement Alternat Med. 2015;2015:397821.
10. Dobetsberger C, Buchbauer G. Actions of essential oils on the central nervous system: an updated review. Flavour Fragr J. 2011;26(5):300–16. https://doi.org/10.1002/ffj.2045.
11. Shen C-Y, Jiang J-G, Zhu W, Ou-Yang Q. Anti-inflammatory effect of essential oil from 
\textit{Citrus aurantium} L. var. Amara Engl. J Agric Food Chem. 2015;63(5):8586–94. https://doi.org/10.1021/acs.jafc.7b02586.
12. Suryawanshi JAS. An overview of \textit{Citrus aurantium} used in treatment of various diseases, African J Plant Sci. 2011;5(7):39–5.
13. Choi H-S, Song HJ, Ueda H, Sawamura M. Radical-scavenging activities of \textit{Citrus} essential oils and their components: detection using 1,1-diarylhydrazyl. J Agric Food Chem. 2000;48(9):4156–61. https://doi.org/10.1021/jf000227d.
14. González-Molina E, Domínguez-Perles R, Moreno D, García-Víquez C. Natural bioactive compounds of \textit{Citrus limon} for food and health. J Pharm Biomed Anal. 2010;51(3):527–45. https://doi.org/10.1016/j.jpba.2009.07.027.
15. Ling APK, Chia JY, Hussein S, Harun AR. Physiological responses of \textit{Citrus sinensis} to gamma irradiation. World Appl Sci J. 2008;5(1):12–9.
16. Kammoun AK, Ahyar AE, Gad HA. Comparative metabolic study of \textit{Citrus Sinensis} leaves cultivars based on GC-MS and their cytotoxic activity. J Pharm Biomed Anal. 2021;198:113991.
17. Najar B, Shortrede JE, Pestilli L, Buhagiar J. Chemical composition and in vitro cytotoxic screening of sixteen commercial essential oils on five Cancer cell lines. Chem Biodivers. 2020;17(13):e201900478.
18. Sun J. D-limonene: safety and clinical applications. Altern Med Rev. 2007;12(2):259–64.
19. Jia S-S, Xi G-P, Zhang M, Chen Y-B, Lei B, Dong X-S, et al. Induction of apoptosis by D-limonene is mediated by inactivation of Akt in LS174T human colon cancer cells. Oncol Rep. 2013;29(1):349–54. https://doi.org/10.3892/or.2012.2039.
20. Crimmina R, Lomeli-Rodriguez M, Cara PD, Lopez-Sanchez JA, Pagliaro M. Limonene: a versatile chemical of the bioeconomy. Chem Commun. 2014; 50(97):1528–56. https://doi.org/10.1039/C4CC0147K.
21. Araújo-Filho HGD, Dos Santos JV, Carvalho MT, Picot L, Frutieri-Araúna I, Groult H, Quintans-Júnior LJ, Quintans JS. Anticancer activity of limonene: A systematic review of target signaling pathways. Phytother Res. 2021;35:1–14. https://doi.org/10.1002/ptr.7125.
22. de Vasconcelos Cerqueira Braz J, de Carvalho FO, de Vasconcelos Cerqueira J. Comparative metabolic study of \textit{Citrus} leaves cultivars based on GC-MS and their cytotoxic activity. J Pharm Biomed Anal. 2010;51(2):327–45. https://doi.org/10.1016/j.jpba.2009.07.027.
23. Ling APK, Chia JY, Hussein S, Harun AR. Physiological responses of \textit{Citrus sinensis} to gamma irradiation. World Appl Sci J. 2008;5(1):12–9.
24. van de Velde I, van der Linden C, van den Broek W, Voest EE. Cytotoxic Effect on Human Breast Cancer Lines. BioNanoScience. 2021;11:892/or.2012.2093.
26. Osanloo M, Sedaghat M, Sereshki H, Rahmanian M, Saeedi Landi F, Amani A. Chitosan nanocapsules of tarragon essential oil with good cytotoxicity and long-lasting activity as a green nano-larvicidal. J Nanostruct. 2019;9(4):723–35.

27. Abdollahi A, Mizaei E, Armoogumar F, Moemenbella-Fard MD, Zarennejad E, Osanloo M. High Antioxidant Effect of Impregnated Nanolipid Mats with a Green Nanogel Against Major Human Pathogens. BioNanoScience. 2021; 11:549–58.

28. Vettori A, Frezza C, Maggi F, Lupidu G, Iafrate M, Quasimini L, et al. Secondary metabolites, glandular Trichomes and biological activity of Sidernits montana L. subsp. Montana from central Italy. Chem Biodivers. 2016;13(10):1380–90. https://doi.org/10.1002/cbdv.201600882.

29. Canfaglione K, Blomme EE, Quassinti L, Bramucci M, Lupidi G, Dall’Acqua S, et al. Phytochemistry, micromorphology and bioactivities of Ajuga chamaeptidis (L.) Schreb. (Lamiaceae, Ajugidae): two new harpagide derivatives and an unusual idyllic glycosides pattern. Fitoterapia. 2016;133:45–43. https://doi.org/10.1016/j.fitote.2016.06.009.

30. Ghanbariasad A, Osanloo M. Development of two stable green nanoformulations for preserving the postharvest quality of Agaricus bisporus. J Sci Food Agric. 2016;96(5):1016–25. https://doi.org/10.1002/jsfa.9036.

31. Momtazi AA, Askari-Khorasgani O, Abdollahi E, Sadeghi-Alaladi H, Mortazeinezhad F, Shokouhi A. Phytochemical analysis and cytotoxicity evaluation of Kelussia odoratissima Mozaff. J Acupunct Meridian Stud. 2017; 10(3):180–6. https://doi.org/10.1016/j.jams.2017.02.002.

32. Gharianihe A, Osanloo M. Development of two stable green nanofluids with potent anticancer properties. Nanomed Res J. 2020;5(3):224–44.

33. Souza JM, Caldas AL, Tohidi SD, Molina J, Souto AP, Fangueiro R, et al. Properties and controlled release of chitosan microencapsulated limonene oil. Rev Bras Farmacogn. 2014;24(6):691–8. https://doi.org/10.1590/1519-8495.2014.11.007.

34. Khoshakhlagh K, Kochocki A, Mehebbi M, Allafchian A. Development and characterization of electrosprayed Alyssum homolocarpum seed gum nanoparticles for encapsulation of d-limonene. J Colloid Interface Sci. 2017; 490:562–75. https://doi.org/10.1016/j.jcis.2016.11.067.

35. Karimirad R, Behnamian M, Dehzshtan S, Sonnenberg A. Chitosan nanoparticles-loaded Citrus aurantium essential oil: a novel delivery system for preserving the postharvest quality of Agaricus bisporus. J Sci Food Agric. 2018;98(13):5112–9. https://doi.org/10.1002/jsfa.9050.

36. Karimirad R, Behnamian M, Dehzshtan S. Bitter orange oil incorporated into chitosan nanoparticles preparation, characterization and their potential application on antioxidant and antimicrobial characteristics of white button mushroom. Food Hydrocoll. 2020;101:105387. https://doi.org/10.1016/j.foodhyd.2019.105387.

37. Benito R, Pagán E, Berdejo D, de Carvalho RJ, García-Embid S, Maggi F, et al. Chitosan nanoemulsions of cold-pressed orange essential oil to preserve fruit juices. Int J Food Microbiol. 2020;331:108786. https://doi.org/10.1016/j.ijfoodmicro.2020.108786.

38. Hasani S, Ogah SM, Ghorbani M. Nanoencapsulation of lemon essential oil in chitosan-Hicap system. Part 1: study on its physical and structural characteristics. Int J Biol Macromol. 2018;115:143–51. https://doi.org/10.1016/j.ijbiomac.2018.04.038.

39. Hajizadeh MR, Maleki H, Barani M, Fahmidehkari MA, Mahmoodi M, Torkzadeh-Mahani M. In vitro cytotoxicity assay of D-limonene niosomes: an efficient nano-carrier for enhancing solubility of plant-extracted agents. Res Pharm Sci. 2019;14(5):448–58. https://doi.org/10.14197/rps.2019.14.5.004.

40. Li PH, Lu WC. Effects of storage conditions on the physical stability of d-limonene nanoemulsion. Food Hydrocoll. 2016;65:218–24. https://doi.org/10.1016/j.foodhyd.2015.01.031.

41. Navaei Shoovan S, Shahraki F, Shafiei N, Karimi E, Osoueian E. Citrus aurantium L. bloom essential oil nanoemulsion: Synthesis, characterization, cytotoxicity, and its potential health impacts on mice. J Food Biochem. 2020;44(5):e13181.

42. Raimondo S, Naiselli F, Fontana S, Monteleone F, Dico AL, Salvia L, et al. Citrus Limon-derived nanovesicles inhibit cancer cell proliferation and suppress CML xenograft growth by inducing TRAIL-mediated cell death. Oncotarget. 2015;6(23):19514–27. https://doi.org/10.18632/oncotarget.4004.

43. Souza EB, Ziolinka S, Souza SB, Durazzo A, Lucarini M, Santini A, et al. (+)-Limonene 1, 2-epoxide-loaded slns: Evaluation of drug release, antioxidant activity, and cytotoxicity in a HaCaT cell line. Int J Mol Sci. 2020;21(4):1446.

44. Venditti A, Bianco A, Frezza C, Serafini M, Giacomello G, Giuliani C, et al. Secondary metabolites, glandular Trichomes and biological activity of Sidernits montana L. subsp. Montana from central Italy. Chem Biodivers. 2017;14:17.

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45. Gambini V, Tilio M, Maina EW, Andreani C, Bartolacci C, Wang J, et al. In vitro and in vivo studies of gold(I) azolate/phosphane complexes for the treatment of basal like breast cancer. Eur J Med Chem. 2018;155:418–27. https://doi.org/10.1016/j.ejmech.2018.06.002.