RESEARCH PAPER

Encapsulation of eucalyptus essential oil in chitosan nanoparticles and its effect on MDA-MB-231 cells

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ABSTRACT

Objective(s): Encapsulation of essential oil in polymeric nanoparticles (NPs) increases their retention and improves their efficacy. Here, eucalyptus essential oil (EEO) encapsulate in the chitosan (CS) NPs increases its retention, and enhances the anticancer effect of EEO.

Materials and Methods: The effects of pH, chitosan sodium tripolyphosphate ratio, and chitosan concentration on the size and charge NPs were evaluated. The success of EEO encapsulation was confirmed by FT-IR, UV–Vis spectroscopy, and GC techniques. The toxic effect of free EEO and CS-EEO NPs was investigated in MDA-MB-231 breast cancer cells and fibroblast normal cells.

Results: The optimized obtained EEO -loaded chitosan nanoparticles (CS-EEO NPs) were spherical with an average diameter of 86 nm, a polydispersity index below 0.4, and positive zeta potential (+14.25 mV) as confirmed. Increasing the concentration and pH of the chitosan solution and decreasing the chitosan/sodium tripolyphosphate ratio, the size of NPs decreased. Loading capacity (LC) and encapsulation efficiency (EE) of EEO in the NPs were about 45% and 32–76%, respectively. The chitosan nanoparticles exhibited a biphasic release profile with the release of 87% of the EEO in the first 5 h, followed by a sustained release for the next 43 h.

Conclusion: The free EEO was more toxic for MDA-MB-231 cells than fibroblast cells; however, CS-EEO NPs were non-toxic for fibroblast cells and more toxic for MDA-MB-231 cells compared to free EEO. Therefore, the CS-EEO NPs illustrate smart behavior in killing cancerous cells and will be suggested for breast cancer drug delivery.

Keywords: *Chitosan nanoparticles, Eucalyptus essential oil, Encapsulation, MDA-MB-231 cells*

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INTRODUCTION

The common methods of cancer treatment are chemotherapy, radiation therapy, and surgery. The lack of distinction between cancer and normal cells in chemotherapy causes serious side effects and damage to other tissues in chemotherapy. Low effects in large tumors and damage to surrounding healthy tissue are the problems in radiotherapy. Tumor recurrence in surgery due to incomplete removal of a lump causes attention to the new methods of cancer therapy [1]. Nanoparticle drug delivery represents strategies to overcome the limitations of current cancer therapies. NPs containing anticancer drugs improve the drug circulatory h(alf-life, enhances the drug accumulation in solid tumor by NP targeting, and provide the sustained release of drugs [2].

Breast cancer with 450000 death annually, is the leads to mortality in women. A high amount of side effects of chemotherapy or anticancer drugs, especially systemic toxicity, causes the

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change approach to the use of natural medical components with lower side effects. Natural compounds with higher tolerability and lower side effect are suggested for future cancer treatment in the scientific community [3].

Essential oils (EOs) as a secondary metabolite of plants have an anticancer effect in the six strategies, including; i) antioxidant activity, ii) antimutagenic action, iii) antiproliferative effect, iv) enhancement of immune functions and surveillance, v) enzyme induction and enhancing detoxification, vi) modulation of multidrug resistance [4].

Eucalyptus (*Myrtaceae*) is a native Australian tree, found in different areas of the world and its leaves are the main source of EOs containing terpenoids, phenolics, flavonoids and alkaloids. The antitumor activity of EEOs is due to the presence of compounds such as phenolic (epigallocatechin-3-gallate, apigenin, myricetin, naringenin, quercetin and gallic acid) and terpenoids (monoterpenes, sesquiterpenes, diterpenoids, and triterpenoids) [5].

Despite the significant medical advantages of EOs, their biological properties are limited due to high volatility, photosensitivity and degradability when exposed to temperature and light, reducing their bioavailability. However, the hydrophobicity of EOs prevents their application in oral usage. Recently nanoparticles are suggested for encapsulation to protect the EOs against evaporation and oxidation, increase EOs stability in front of harsh environmental conditions and enhance their water-solubility [6].

Chitosan (CS) is a second highly abundant natural polymer in the earth which is produced by the deacetylation of chitin. Chitin extract from crustaceans such as insects, crabs, shrimp, lobsters and some kinds of algae and fungi. Recently, CS NPs represented as an active biomaterial for the encapsulation of drugs, proteins, genes and other bioactive molecules. CS has a good ability to carry lipophilic compounds such as EOs [7]. On the other hand, CS has toxic characteristics against tumors which make it a potential biopolymer for carrying the anticancer drugs [8]. Therefore, encapsulating the EOs in CS NPs claims to enhance the anticancer effect of essential oil and shows the synergistic effect on cancer cells' apoptosis in the *in vitro* condition.

MATERIALS AND METHODS

Materials

Chitosan, acetic acid, hydrochloric acid, tripolyphosphate (TPP), dichloromethane, sodium hydroxide, sodium sulfate, hexane (HPLC grade), ethanol (96%), acetone, sodium chloride were obtained from Merck Company (Darmstadt, Germany). DMEM cell culture media, trypan blue, trypsin, dimethyl sulphoxide (DMSO) and sodium bicarbonate were obtained from Sigma-Aldrich (USA). Also, fetal bovine serum and antibiotic PeneSerb from Gibco (USA).

Essential oil extraction

The eucalyptus leaves (*E. Microtheca*) were obtained from Iranian Research Organization Science and Technology (IROST) campus trees and dried in the shadow. The dried leaves were mild and screened. The extraction process is done in the Soxehalet device by the water vapor distillation method. The extracted EEO was kept in a sealed vial under the 4 °C until use.

Chitosan NP synthesis

Chitosan (CS) NPs were prepared based on ionic gelation in which electrostatic interaction between the amine group of CS and negative charge of poly-anion in the TPP is driving force. For optimization of NPs quality four variable factors, CS concentration, pH of CS solution, pH of TPP solution, and CS/TPP ratio were investigated. The CS concentration in 1, 1.25, 2, 3, 5 mg/ml in 1.75 % (v/v) acetic acid was prepared and the pH set in 3.5, 4 , 4.5 and 5. The TPP solution was prepared in 1, 2.5 and 5 mg/ml in PBS with pH 4.5 and 5. The TPP solution was slowly added to the CS solution under the stirring condition (homogenizer with 7000 rpm and room temperature) with a ratio of 1:3 and 1:3.5 of TPP/CS solution. The solution was mixed for 2 hr and then centrifuged at 13500 rpm for 20 min. The NPs collected at the button of the tube sonicate for 7 min to spread homogenously and freeze-dried or stayed in the refrigerator (4 °C) for future analysis.

Chitosan-essential oil NP synthesis

The CS solution was prepared based on the 2.3 section. The EEO in 0.1, 0.2 and 0.3g separately dissolved in 4 ml dichloromethane and slowly added to the aqueous solution CS under homogenizing with 13000 rpm in 10 min while the adding process took place in the ice bath. The TPP solution (insulin syringe) in different concentrations at room temperature and speed of 0.5 ml/min was added to the emulsion gradually to complete the CS-EEO NPs formation. The purification, ultra-sonification and retention of prepared NPs were done similar to section 2.3.

NPs size and morphology determination

The particle diameter and size distribution were measured by Dynamic light scattering (DLS) using the Zetasizer (Malvern, UK). Before measurement, the sample was diluted with distilled water to prevent particle aggregation due to multiple laser dispersion. The surface morphology of NPs investigated using scanning electron microscopy (SEM) (TESCAN, Czech Republic). The samples were diluted with double distilled water, dispersed on the aluminum foil and dried in the oven. The dried sample was coated with gold in the sputtering machine and the images were obtained on 15 kV accelerating voltage.

Fourier Transfer Infrared (FTIR) spectroscopy

FTIR spectroscopy (Thermo, WPA, Germany) for the determination of chemical characteristics of NPs was used. Samples containing CS, EO and CS-EEO NPs were analyzed in KBr disks (1% w/w) in the wavelength range 4000-450 cm^{-1}.

In vitro loading and release

For determination of encapsulation efficiency (EE%) and loading capacity (LC%) of EO in CS NPs, the 10 mg of CS-EEO NPs poured in 2 molar HCl for 30 min and 70 °C temperature. After cooling, the 2 ml 96% ethanol was added to the solution and mixed. The resultant mixture was centrifuged at 9000 rpm for 5 min to separate the whole of the encapsulated EEO from the CS NPs. The separated EEO on the supernatant was measured by UV-Visible spectroscopy at 218 nm. The EE% and LC% are determined by the following formula:

$$EE\% = \frac{Total\ amount\ of\ encapsulated\ EO}{Initial\ amount\ of\ EO} \times 100$$

$$LC\% = \frac{Total\ amount\ of\ encapsulated\ EO}{The\ total\ weight\ of\ NPs\ containing\ the\ EO} \times 100$$

The EEO release test was carried out in release media containing 60% buffer phosphate (pH=7.4) and 40% ethanol. 20 mg of NPs poured into 5 ml release media and put into the 37 °C shaker incubator. In the special time interval, the aliquots were withdrawn for concentration determination and replaced with the same amount of fresh-release media. The cumulative drug release profile was determined by the following formula:

$$\text{Cumulative drug release } \sum_{t=0}^{t} \frac{Mt}{M0} \times 100$$

The Mt is the cumulative amount of released EEO in special time points.

The M0 is the initial weight of encapsulated EEO in CS NPs.

Gas chromatography (GC)

For confirmation of EEO loading in the CS NPs the GC analysis was utilized. The GC instrument Yong ling 6500 model (Agilent, UK), was equipped with an FID detector and a capillary column of TBR-5 was used. The length of the column was 30 m and its internal diameter was 0.53 mm which the static phase layer diameter was determined at 3 µm. The column temperature setting starts from 50 oC and stays 1 min in it. The gradient of temperature increase based on special rate including 50-110 °C (15 °C/min), 110- 150 °C (5 °C/min), 150-250 oC (3 °C/min). The injector and detector temperature was 250 °C and nitrogen gas with 99.99% purity and flow rate of 4 mm/min was used as a carrier gas.

In sample preparation, 10 ml of CS-EEO NPs solution was added to 4 ml of 4N HCl at 60 °C and mixed. Then 1 ml of pure hexane was added to the solution and mixed. At the end, the resultant solution was centrifuged at 9000 rpm for 5 min and the supernatant containing the hexane and EEO was injected into the GC. Also, hexane and EEO solution in hexane separately were injected into the GC instrument for comparison with release EEO.

In vitro cell culture

Human breast cancer cell line, MDA-MB-231(pasture Institute, Iran), and human fibroblast cells were cultured in DMEM media containing penicillin, streptomycin and FBS. Both types of cells were propagated in a 95% humidified, 5 % CO_2 incubator at 37 °C. After 24 h incubation, the cells were washed with PBS, then trypsin-EDTA was added to detach the cells from the culture flask. An equal amount of fresh media was added to each flask and pipetting was performed to ensure uniform suspension of cells. The cell counting by trypan blue on the hemocytometer counting chamber ensures the suitable amounts of cells (1×10^4) for MTT assay.

In vitro cell toxicity

The EEO, CS NPs and CS-EEO NPs were assayed for cell toxicity. Cytotoxicity measurement was based on the viability of cells in 96-well plate.

Different concentration of each sample was added to seeded cells in wells. After 24 h incubation, the medium was removed and washed with PBS. MTT solution (100 µl) was added to each well and incubated for 4 hours in CO_2 incubator at 37 °C. Then, MTT solution was removed and DMSO was added to dissolve insoluble formazan crystals. Finally, the optical absorption in 570 nm wavelength was measured with ELIZA reader.

Statistical analysis

The results from three replication with mean ± standard deviation (SD) was reported. All data were analyzed using Minitab 16 software. Analysis of variance was examined with ANOVA method and significant differences were evaluated by Duncan's multiple range examination (at $P \leq 0.05$).

RESULTS AND DISCUSSION

Size and PDI of NPs

The CS NPs prepared by the ionic gelation method has been prepared with 4 parameters which affects the NPs size and dispersity. The CS concentration, pH of CS solution, pH of TPP solution and the ratio of CS/TPP. Firstly, the different pH of CS (3.5, 4 and 5) exerted. The size and polydispersity index (PDI) of CS NPs was obtained by dynamic light scattering (DLS) listed in Table 1.

Table 1 shows that the smallest CS NPs with smaller PDI will be obtained by pH 5 of CS and TPP solution, CS and TPP concentration, 1 mg/ml and their ratio should be 3:1. Increasing the pH of CS solution, decrease the viscosity, solubility, ionic interaction, which leads to a weaker network in the early stage and smaller particle formation [9]. In the lower pH, the protonation of amine groups, increases the repulsion between the CS molecules. Also, the protonation of TPP molecules in this condition, decreases its negative charge, so it could not forms a strong bonding with CS [10].

The effect of TPP pH in CS NP synthesis was investigated in 3 pH of 4, 4.5 and 5. The result is reported in Table 2.

According to Table 2, the smallest NPs related to the number 6 formulation with a mean diameter of 166 nm and PDI of 0.199.

The effect of CS concentration on CS NPs synthesis in 1, 1.25, 2.5, 3 and 5 mg/ml was investigated. The importance of CS concentration in Table 3 was considered as a variable parameter.

Table 1. The effect of CS solution pH on the size of CS NPs

Number	CS pH	CS Conc mg/ml	TPP pH	TPP Conc mg/ml	CS:TPP	Mean diameter (nm)	PDI
1	3.5	1	4.5	1	3:1	259	0.227
2	4	1	4.5	1	3:1	223	0.241
3	5	1	4.5	1	3:1	204	0.257
4	3.5	1	5	1	3:1	258	0.210
5	5	1	5	1	3:1	166	0.199
6	3.5	2.5	4.5	2.5	3:1	523	0.347
7	5	2.5	4.5	2.5	3:1	239	0.244
8	3.5	2.5	4.5	2.5	3.5:1	473	0.305
9	5	2.5	4.5	2.5	3.5:1	319	0.3

Table 2. The effect of TPP pH on the size and PDI of CS NPs

Number	CS pH	CS Conc mg/ml	TPP pH	TPP Conc mg/ml	CS:TPP	Mean diameter (nm)	PDI
1	3.5	1	4.5	1	3.5:1	299	0.296
2	3.5	1	5	1	3.5:1	275	0.275
3	5	1	4.5	1	3.5:1	224	0.268
4	5	1	5	1	3.5:1	255	0.243
5	5	1	4	1	3:1	301	0.217
6	5	1	4.5	1	3:1	204	0.257
7	5	1	5	1	3:1	166	0.199
8	5	2.5	4.5	2.5	3.5:1	319	0.300
9	5	2.5	5	2.5	3.5:1	354	0.304
10	3.5	2.5	4.5	2.5	3.5:1	523	0.347
11	3.5	2.5	5	2.5	3.5:1	663	0.354

Table 3. The effect of chitosan concentration on size and PDI of CS NPs

Number	CS pH	CS Conc mg/ml	CS:TPP	Mean diameter (nm)	PDI
1	3.5	1	3.5:1	275	0.275
2	3.5	1.25	3.5:1	260	0.339
3	3.5	2.5	3.5:1	663	0.354
4	3.5	5	3.5:1	415	0.385
5	3.5	1	3:1	258	0.210
6	3.5	2.5	3:1	523	0.347
7	3.5	3	3:1	411	0.328
8	5	1	3:1	204	0.257
9	5	2.5	3:1	239	0.244
10	5	1	3.5:1	255	0.243
11	5	2.5	3.5:1	354	0.304

The result shows with increasing the CS concentration, the viscosity of the solution will increase and restrict the dispersion of the liquid phase, so the bigger particle will be obtained. In the low concentration of CS, the repulsion between amine groups forms the extended structure [11]. The presence of vast steric in the CS molecules allows TPP for dispersing and binding between the CS chains and forms the small NPs. In a higher concentration of CS, the steric hindrance causes weak and uneven penetration of TPP molecules between CS chains, so higher NPs with different size ranges will be formed [12].

The importance of CS/TPP ratio was the fourth factor that assayed. Table 4 compares the CS/TPP ratio in addition to other factors.

The CS:TPP ratio is an effective parameter in CS NP synthesis because with increasing the TPP (decrease of CS:TPP ratio), the binding between the CS chain and TPP will increase, the high amount of water involved in the gelation process, thereupon the smaller particle will be formed [13].

Optimization of CS NPs synthesis condition based on changing the four parameters showed that 1 mg/ml of CS and TPP concentration, pH 5 for CS and TPP, 3:1 ratio of CS:TPP, gain the best result with 166 nm diameter NPs with PDI 0.199, in which the zeta potential of NPs was +38 mV. The gained zeta potential was admissible for creating the stable suspension through electrostatic repulsion [14].

Microscopic examination

The imaging by SEM from the smallest NPs obtained by DLS showed that the real size of NPs was 72-75 nm. The reason for such difference is that the DLS determines the hydrodynamic diameter of NP, the mean diffusion of nanoparticles within the fluid which is higher than the real NP's diameter due to weakly attached liquid molecules around the NP [15].

The morphology of CS NPs was spherical with

Table 4. Effect of CS: TPP ratio on size and PDI of CS NPs

Number	CS:TPP ratio	CS Conc mg/ml	CS pH	TPP Conc mg/ml	TPP pH	Mean diameter (nm)	PDI
1	**3:1**	1	3.5	1	5	258	0.210
2	**3.5:1**	1	3.5	1	5	275	0.275
3	**3:1**	1	5	1	5	166	0.199
4	**3.5:1**	1	5	1	5	255	0.243
5	**2.5:1**	1	5	1	5	198	0.278
6	**3:1**	1	5	1	4.5	204	0.257
7	**3.5:1**	1	5	1	4.5	224	0.268
8	**3:1**	2.5	5	2.5	4.5	239	0.244
9	**3.5:1**	2.5	5	2.5	4.5	319	0.3
10	**3:1**	2.5	3.5	2.5	4.5	523	0.347
11	**3.5:1**	2.5	3.5	2.5	4.5	473	0.305

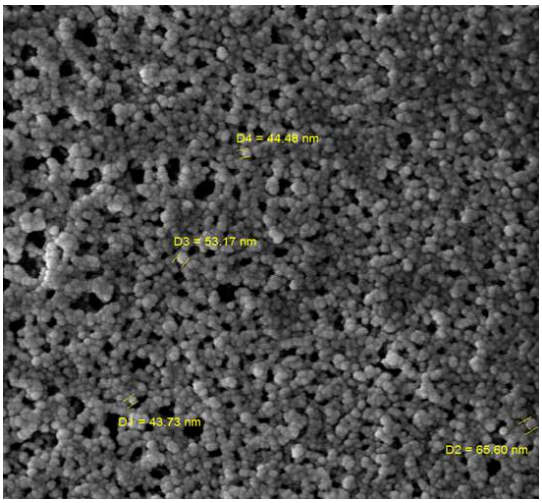

Fig.1. SEM image of chitosan nanoparticles

relatively smooth edges have shown in Fig. 1. The real size of NPs, lower than 100 nm has the advantage of transporting from the blood vessels and pores in the epithelial cells.

Chemical analysis of NPs

The FTIR spectroscopy of CS NPs, EEO and CS-EEO NPs was shown in Fig. 2. In the CS NPs spectrum type I amide group emerged in 1638 cm^{-1} and type II amide in 1557 cm^{-1}. The C-O-C group shows a sharp peak in 1086 cm^{-1} and a C-H peak exit in 2922 cm^{-1}. The NH and OH emerge on 3429 cm^{-1}. The EEO spectrum illustrates the significant peaks 3466 cm^{-1} and 2926 cm^{-1} which are related to OH and CH groups in EEO, respectively. The 1464 cm^{-1} shows the presence of CH_2 and CH_3 groups, 1375 cm^{-1} is related to CH_3CO groups. Also, the peaks in the 1214 cm^{-1}, 1079 cm^{-1} regions related to C-O-C in 985 cm^{-1} and 887 cm^{-1} show the CH_2 group. Both CS NPs and EEO peaks emerged in the CS-EEO NPs peak which identifies the binding between CS and EEO molecules.

Loading and release analysis by UV-Visible and GC

The entrapment efficiency and loading capacity of EEO in CS NPs were obtained based on formulas

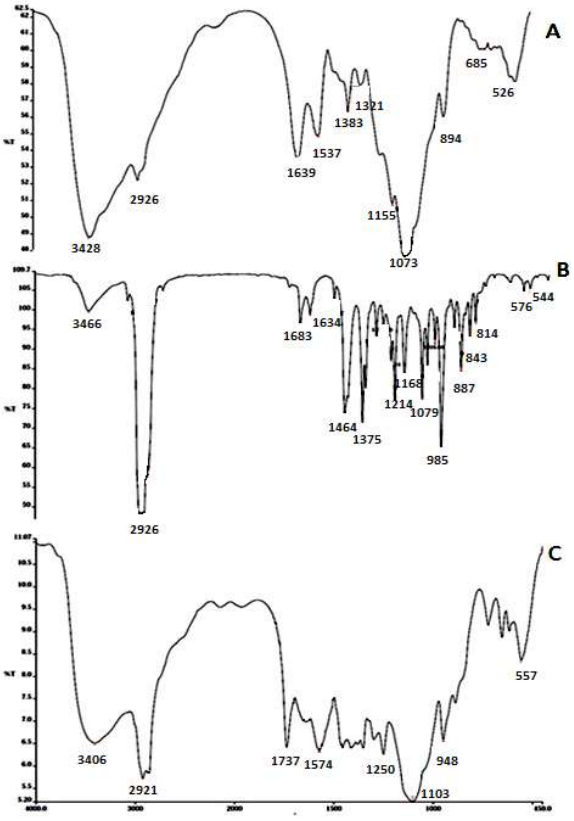

Fig. 2. FTIR analysis of Chitosan (A), EEO (B) and chitosan-EEO (C)

1 and 2. The data come in Table 5 based on the initial concentrations of 0.2 and 0.4 g of EEO and CS:EEO ratios of 1:0.5 and 1:0.25.

Data from the Table 5 indicates that increase in the initial concentration of EEO, the EE decrease, but the LC will increase. This result was adapted to Hosseini and et al. which is the oregano essential oil encapsulated in CS NPs [16]. The oregano EEO in 4 concentrations of 0.04, 0.08, 0.16 and 0.32 g. The results showed with increasing the EEO concentration, the LC increased from 1.3 to 2.12; however, the EE decreased from 24.72 to 5.45. An increase in EE is due to the saturation of CS NPs with EEO, but LC is related to the initial content of EEO.

The release rate of EEO from the CS NPs was investigated a shaker incubator 37 °C and

Table 5. The EE and LC percent of EEO in CS NPs

CS:EEO ratio	Initial amount of EEO (g)	Absorption wavelength (218 nm)	EE (%)	LC (%)
1:0.5	0.2	0.526	75.8	45.4
1:0.25	0.4	0.544	32.1	46.1

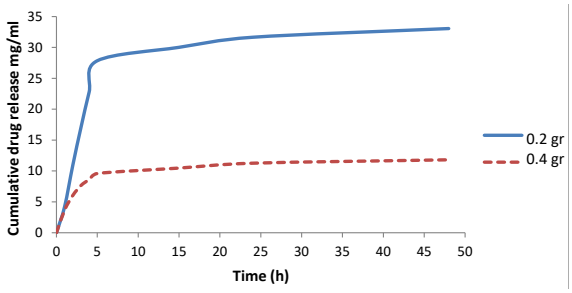

Fig. 3. The release profile of EEO from chitosan nanoparticles in 2 concentrations

shaking speed of 150 rpm at 48 h. The absorption wavelength of EEO at each time was gained by UV-Visible spectroscopy at 218 nm. The cumulative drug release EEO with an initial amount of 0.2g and 0.4 g was reported in Fig 3.

The cumulated released EEO from the CS NPs with an initial amount of 0.2 g was 33.06 mg/ml and the percent of cumulative release was 21.8 %; however, this amount with 0.4 g of initial EEO was 11.812 mg/ml and 9.19 %, respectively.

For confirming the loading of EEO on CS NPs, the GC analysis was performed [17]. Here, hexane is used as an extraction solvent that can separate the organic phase (EEO) from the CS NPs (aqueous phase). Two samples including pure hexane and EEO were injected into GC before injection of CS-EEO NPs. Fig. 4 shows the hexane, EEO and EEO extracted from CS-EEO NPs chromatogram, respectively.

Table 6 describes the retention time (RT) of the sample (EEO release from the CS-EEO NPs) and EEO retention in the chromatogram peaks. By comparing the peaks area in two chromatograms, the amount of loading was obtained.

Based on the Table 6, some of the EEO components with different percentage of loading

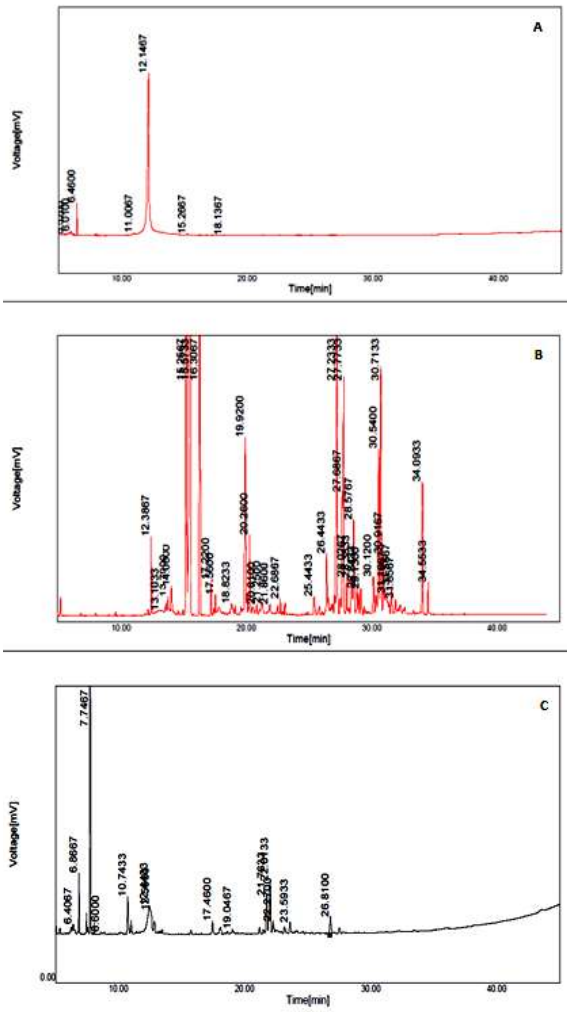

Fig. 4. GC chromatogram of hexane (A), EEO (B) and EEO extracted from the CS-EEO NPs (C)

from 14 % to 92 % is available. However, some ingredient of EEO was not loaded in CS NPs.

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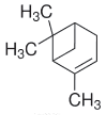
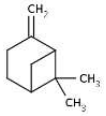
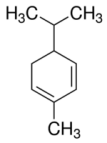
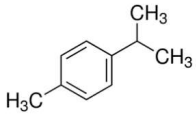
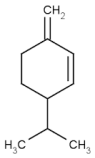
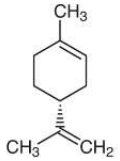
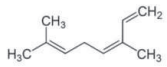
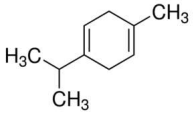
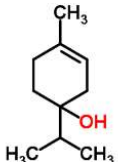
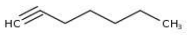
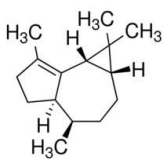
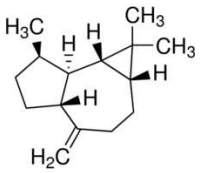
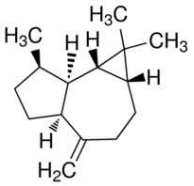
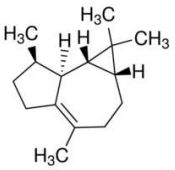
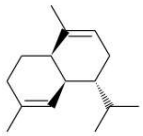
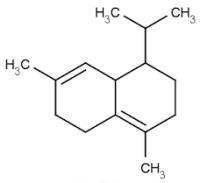
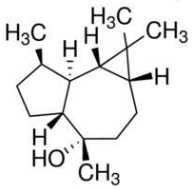
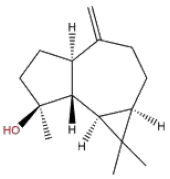
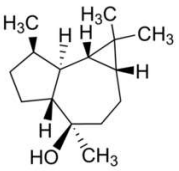
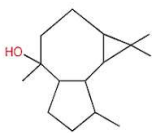
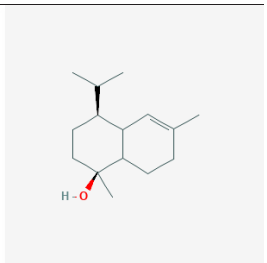
Table 6. The percent of every EEO ingredient loading into the CS-EEO NPs

Peak name	RT_{sample}	RT_{EO}	Loading amount=sample area/standard area	Loading %
A	12.4433	12.3867	0.7952	79%
B	12.5600	13.1033	0.8087	80%
C	17.4600	17.2200	0.5506	55%
D	19.0467	18.8233	0.32337	32%
E	21.7633	21.2500	0.9246	92%
F	22.2700	22.6768	0.3315	33%
G	23.59	25.4433	0.5234	52%
H	26.8100	26.4433	0.1477	14%

Table 7. The 21 compounds of E.microtheca leave EO with more than 1% concentration

	Compound	Percent %	Molecular structure
1	α -pinene	6.752	
2	β - pinene	5.006	
3	α -phellandrene	16.487	
4	p- cymene	5.251	
5	β -phellandrene	2.194	
6	Limonene	1.503	
7	Cis-ocimene	1.655	
8	γ -terpinene	1.235	
9	4-terpineol	1.256	
10	1-methoxyhept-1-yne	1.809	
11	α –gurjunene	1.897	
12	Aromadendrene	12.773	
13	Alloarmadendrene	2.520	
14	Ledene	5.665	
15	α –amorphene	1.666	
16	δ -cadinene	2.663	
17	Epiglobulol	1.167	
18	Spathlenol	1.915	
19	Globulol	5.997	
20	Veridiflorol	1.243	

Continued Table 7.

21	Tau-muurolol	1.580	

of *E.microtheca* by GC-MS. They reported more than 100 compounds in the EO of *E.microtheca* leaves [18]. In Table 7, we bring 21 compounds of *E.microtheca* leaves EO, which have more than 1 percent concentration in the EEO ingredients. Since all compounds in EEO are nonpolar with almost similar structures, thereupon, the nonpolar-nonpolar interaction between EEO compounds and CS NPs in aqueous media are identical [19]. Therefore, the concentration of every EEO compound can be determining factor for CS adsorption. Adapting the GC result of Table 6 with the compounds in Table 7, the possible EO compounds in CS structure will be harvested. The α -pinene, β – pinene, α -phellandrene, p-cymene, Aromadendrene, Ledene, δ -cadinene and Globulol will be the possible compound in CS structure [18].

In vitro cell toxicity

Effect of EEO on human fibroblast and MDA-MB-231 cells

The concentrations including 0.5, 1, 5, 10, 15 and 20 mg/ml of EEO in 10% ethanol solution were investigate in human fibroblast cells. Fig. 5 shows the result after 24 h incubation. At the concentration lower than 1 mg/ml of EEO, 80% of the cells stayed alive, but at 20 mg/ml the availability of the cell was 23 %. The IC_{50} of EEO on fibroblast cells was 12.3 mg/ml. Different concentrations of EEO including 0.5, 1, 5, 10, 15, and 20 mg/ml in 10% ethanol solution affected MDA-MB-231 breast cancer cells. As illustrated in Fig. 5 after 24 h incubation, in the lower concentration of EEO (lower than 1 mg/ml) the availability of MDA-MB-231 cells was 76%; however, in the concentrations of 20 mg/ml, cell availability lowered to 15%. The analysis was carried out with Minitab 16 software and a one-directional analysis of variance (One Way ANOVA). The IC_{50} of EEO based on Excel software obtained 6.13 mg/ml. As it is clear, the sensitivity of MDA-MB-231 cells is higher than fibroblast cells against the EEO concentration.

Effect of CS NPs on the fibroblast and MDA-MB-231 cells

Different concentration of CS NPs including 0.5, 1, 5, 10, 15 and 20 mg/ml was affected on fibroblast and MDA-MB-231 cells. The MTT assay on both cells showed that CS NPs are not cytotoxic (Fig. 6).

Effect of CS-EEO NPs on fibroblast and MDA-MB-231 cells

The concentration of 0.5, 1, 5, 10, 15 and 20 mg/ml of CS-EEO NPs on water solvent affected fibroblast cells. The results show, in the

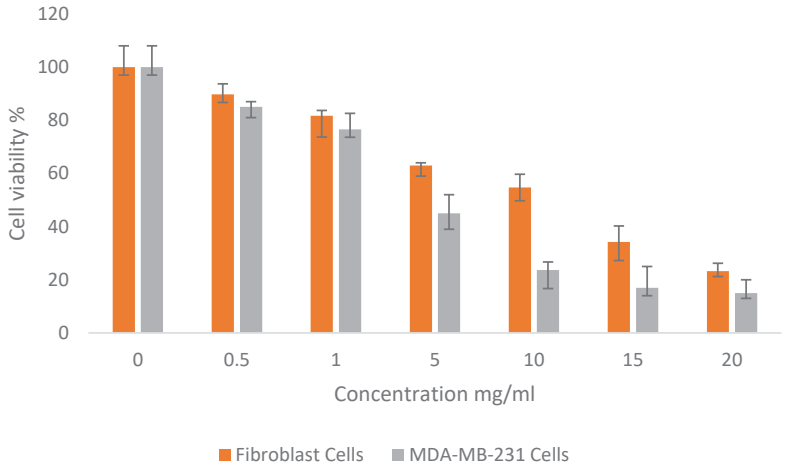


Fig. 5. The curve shows the effect of different concentrations of EEO on human fibroblast and MDA-MB-231 cells after 24 h incubation in 37 C. Results are expressed as mean ± SD ($P \leq 0.05$)

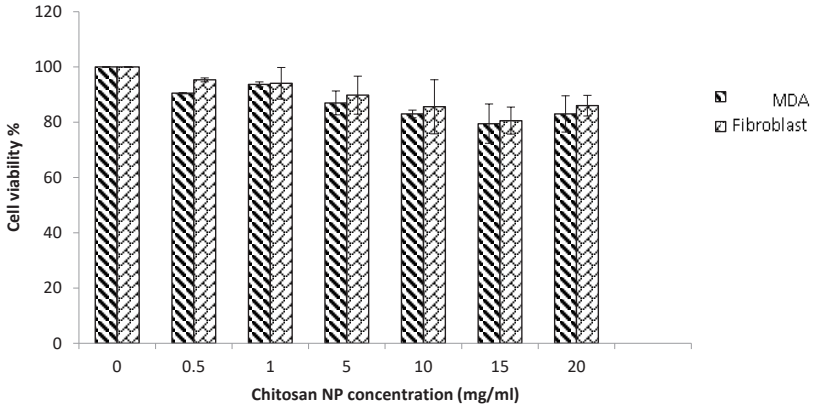


Fig. 6. The curve related to CS NPs effect on fibroblast and MDA-MB-231 cells after 24 hr of treatment. Results are expressed as mean ± SD ($P \leq 0.05$)

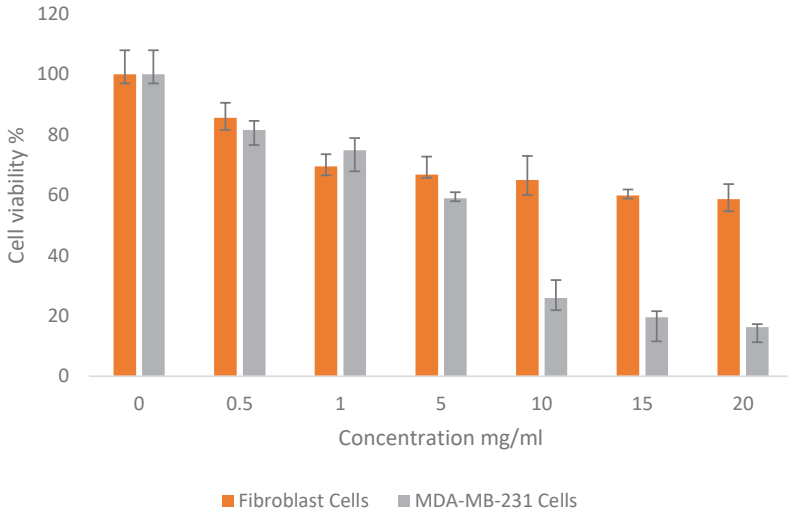


Fig. 7. The curve related to CS-EEO NPs effect on fibroblast and MDA-MB-231cells after 24 hr of treatment. Results are expressed as mean ± SD ($P \leq 0.05$)

concentration of 0.5 mg/ml and below 85% of the cells are alive. With increasing the concentration of CS-EEO NPs to 1 mg/ml the availability of cells decreased to 69%. According to this experiment, it can be stated that CS-EEO NPs are not toxic to fibroblast cells unless at a much higher concentration. Fig. 7. illustrates the result. The concentration including 0.5, 1, 5, 10, 15 and 20 mg/ml of CS-EEO NPs was affected on MDA-MB-231 cells. with the Excel software, the IC_{50} of the CS-EEO NPs calculated at 7.85 mg/ml. Based on the curve in Fig.7, in concentrations below 0.5 mg/ml, more than 80% of cells were alive. Increasing the concentration of CS-EEO NPs to 20 mg/ml 83.72 % of cells were killed. Fig. 7 shows the results.

The comparing the result of cell bioavailability between the EEO effect of MDA-MB-231 cells with CS-EEO NPs shows, the encapsulation of EEO on the CS NPs was no significant effect on the mortality of cancerous cells. Accordingly, CS-EEO NPs have a more lethal effect on MDA-MB-231 cells than fibroblast cells. So, encapsulation of EEO in CS NPs not only decreases the toxic effect of EEO for normal human fibroblast cells but also, increases the anticancer effect against the MDA-MB-231 cancerous cells.

CONCLUSION

Synthesis of chitosan NPs depends on various parameters such as pH of CS solution, pH of TPP solution, CS concentration and CS:TPP ratio that 1 mg/ml of CS and TPP concentration, pH 5 for CS and TPP, 3:1 ratio of CS:TPP, gain the best result with 166 nm diameter NPs with PDI 0.199,

in which the zeta potential of NPs was +38 mV. However, in CS-EEO NPs the average diameter of 86 nm, a PDI below 0.4 and zeta potential +14.25 mV were obtained by the same condition. The SC NPs not only protect the EEO from evaporation, but increase its anticancer effect against the MDA-MB-231 breast cancer cells. 0rthermore, the CS NPs decrease the toxic effect o f EEO on fibroblast normal cells.

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CONFLICTS OF INTEREST

There is no conflict of interest between the authors.

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