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# Chitosan Nanoparticles Containing *Cinnamomum verum* J.Presl Essential Oil and Cinnamaldehyde: Preparation, Characterization and Anticancer Effects against Melanoma and Breast Cancer Cells

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## Abstract

Cancer is the second leading cause of death worldwide, and due to the emergence of resistance to synthetic drugs in different cancers, developing new green drugs have become crucial. In this study, chitosan nanoparticles containing *Cinnamomum verum* J.Presl essential oil and cinnamaldehyde (major ingredient) were first prepared. The obtained nanoparticles were then characterized using Dynamic Light Scattering (DLS), Transmission electron microscopy (TEM), and Attenuated Total Reflection-Fourier Transform InfraRed (ATR-FTIR). After that, anticancer effects of the as-prepared nanoparticles were investigated. $IC_{50}$ values of chitosan nanoparticles containing the essential oil were observed at 79 and 112 µg/mL against A-375 and MDA-MB-468 cells, respectively. These values for chitosan nanoparticles containing cinnamaldehyde were obtained at 135 and 166 µg/mL. The results of the current study indicated that chitosan nanoparticles containing *C. verum* essential oil can inhibit the growth of human melanoma (A-375) and breast cancer (MDA-MB-468) cells.

**Keywords:** Chitosan; Cinnamaldehyde; *Cinnamomum zeylanicum* Blume; Essential oils; Anticancer activity

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## Introduction

Cancer was the second cause of death after heart disease in 2019 [1]. Breast cancer is the most frequent cancer involving at least 2.1 million women annually [2]. Approximately 17% of all cancer deaths among women are caused by breast cancer; ~ 627000 women died only in 2018 [3]. The newly diagnosed female breast cancer increases in the developing world due to longer life expectancy, increased urbanization, and western lifestyles [4]. The modern lifestyle plays a critical role in emerging cancers such as breast cancer, oral cancer, and colorectal cancer due to oxidative stress [5,6]. The lowest prevalence rates are still found in most African countries; however, it increases day by day [7,8]. Melanoma is one of the high-risk forms of skin cancer, deriving from malignant alterations in the melanocyte cells. Moreover, around 75% of deaths from skin malignancies are caused by melanoma [9,10]. Around 100,000 new cases are detected in the US, and nearly 6850 estimated death cases were found in both genders in 2020 [11].

Chemotherapy, radiation therapy, surgery, hormonal therapy, and targeted therapy are accessible as general cancer treatment modalities. However, due to serious adverse effects in patients, herbal medicine and plant-derived phytocompounds have recently been introduced as alternative therapies [12,13]. Essential oils (EOs) with a wide range of bioactivities, such as antioxidant and anticancer activities, have received more attention due to their safety [14,15]. For instance, *Cinnamomum verum* J.Presl (cinnamon) -synonym: *Cinnamomum zeylanicum* Blume- has been widely used in traditional medicine for anticancer or antibacterial applications; e.g., its EO showed anti-tyrosinase activity [16]. The main ingredient of C. zeylanicum EO is cinnamaldehyde ($C_6H_5CH = CHCHO$ [17]) which has previously showed potent anti-tyrosinase and anti-melanogenic activities on α-MSH-stimulated B16 cells [18].

Despite the advantages of EOs, their efficacies should be improved as anticancer agents. The development of the EO-based nanoformulations is a promising approach which has great potential to overcome this limitation [19,20]. The small size of nanoparticles enables them to cross through small vessels, large transport amounts of agents, and probe inside the cells or organism [21-23]. Chitosan, as a biocompatible and biodegradable natural polymer, is one of the most common nanoparticles in developing new green drugs [24,25], and ionic gelation emulsification is common for preparing chitosan nanoparticles (CsNPs) containing EO [26]. Moreover, the bioadhesive property of CsNPs also causes better interaction with organisms, resulting in improved cargo efficacy [27,28]. Several research pieces on the preparation of various EOs or chemical molecules such as limonene, eugenol, and carvacrol have been reported. The results demonstrated those such nanoformulations increase their antimicrobial, antioxidant, and limonene properties [29-31].

As alluded to above, *C. verum* EO, cinnamaldehyde, and chitosan as a green carrier could

be effectively applied for anticancer development. Thus, this study aims to prepare chitosan nanoparticles containing *C. verum* EO and cinnamaldehyde using ionic gelation emulsification. The anticancer effects of as-prepared nanoparticles were then investigated on A-375 and MDA-MB-468 cells.

## Materials and Methods

*Materials*

Breast cancer and melanoma cell lines, including MDA-MB-468 (ATCC HTB-132) and A-375 (ATCC CRL-1619), supplied by the Pasteur Institute of Iran. Tetrazolium salt, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazo-lium bromide (MTT), phosphate-buffered saline (PBS) tablets, tripolyphosphate (TPP), tween 20, chitosan low molecular weight (DD 75-85%), and cinnamaldehyde were purchased from Sigma-Aldrich (USA). Penicillin-streptomycin, trypsin, dimethyl sulfoxide (DMSO), and Dulbecco's Modified Eagle's Media (DMEM) cell culture medium were attained from Shellmax (China). Fetal bovine serum (FBS) and *C. verum* EO were acquired from Gibco (USA) and Zardband Pharmaceuticals Co. (Iran), respectively.

*Preparation of chitosan nanoparticles containing C. verum EO and cinnamaldehyde*

Constituents of *C. verum* EO were investigated using Gas Chromatography-Mass Spectrometry (GC-MS) analysis as detailed in our previous study; cinnamaldehyde with 62.04% was identified as the major compound [17]. In the current study, chitosan nanoparticles containing *C. verum* EO (CVCsNPs) and cinnamaldehyde (CinCsNPs) were prepared using the ionic gelation process as follows [32]. Chitosan powder (0.25% w/v) was first dissolved in an aqueous solution of acetic acid 1%. The obtained solution was then centrifuged for 30 min at 7000 rpm to precipitate potentially undissolved materials. After that, *C. verum* EO and cinnamaldehyde (0.5%) were dissolved (ambient temperature, 2000 rpm, and 10 min) in the chitosan solution containing 0.5% tween 20, individually. To transform chitosan to chitosan nanoparticles containing *C. verum* EO or cinnamaldehyde, the aqueous solution of TPP (0.15% w/w) was injected (1 mL/h) by a syringe pump. The obtained mixture was constantly stirred for 40 min to terminate the reaction and stabilization; the prepared solution was kept for characterization and anticancer investigations.

*Characterization of chitosan nanoparticles containing C. verum EO and cinnamaldehyde*

The particle size of CVCsNPs and CinCsNPs was examined at ambient temperature using a DLS type apparatus (Model of 9900, K-one Nano Ltd, Korea). Transmission electron microscopy (TEM, Philips EM208S 100KV, Max Res 0.2 nm, Netherlands) was employed to investigate the morphology of CVCsNPs and CinCsNPs. The samples were concisely diluted twice with distilled water; one drop was then positioned on a 200-mesh

carbon-coated copper grid and applied to the device. Attenuated Total Reflection-Fourier Transform Infrared (ATR-FTIR) was employed to approve EO or cinnamaldehyde loading in the CVCsNPs and CinCsNPs. For this purpose, *C. verum* EO, cinnamaldehyde, chitosan nanoparticles without cargo (CsNPs), and CVCsNPs and CinCsNPs were exposed to ATR-FTIR instruments without any preparation process (Tensor II, Bruker, Germany). The spectra were recorded in the wavelength of 400 – 4000 $cm^{-1}$.

*Investigation anticancer activity of CVCsNPs and CinCsNPs*

The anticancer activity of CsNPs, CVCsNPs and, CinCsNPs was examined using the MTT assay. The cell lines were flourished in 75 cm2 culture flasks in DMEM medium, comprising FBS (10%) and Penicillin-streptomycin (1%) with 5% (v/v) CO2 at 37 °C. Cells (MDA-MB-468 and A-375) were isolated by trypsin and seeded in 96-well plates, and incubated for 24 h to reach a confluence of 70-80%. Then, the culture media was eliminated, and a 75 µL complete fresh medium was added to each well. By adding appropriate amounts of CVCsNPs and CinCsNPs following the standard procedure, concentrations were fixed at 1200, 600, 300, 150, and 75 µg/mL, and the plates were then incubated for 24 h. Next, the plate content was removed, and wells were washed with PBS for eliminating nano-formulations creamy color. Afterward, 100 µL MTT solution (0.5 mg/mL) and 100 µL/well DMSO was added to each well to dissolve the created formazan crystals after 4 h of incubation. Absorbance was tested in each well at 570 nm by ELISA Plate Reader. Cell viabilities at different concentrations were confirmed by equation 1. Six wells were investigated as the control group in each plate and filled out with 100 µL DMEM.

**Equation 1.**

Cell viability (%) = (Sample absrobance )/(Control absorbance) ×100

*Statistical analyses*

All the experiments were performed in triplicate, and the achieved results were described as mean ± SD. CalcuSyn software (Free version, BIOSOFT, UK) was applied to computing $IC_{50}$ values of the specimens with lower and upper assurance boundaries. Based on anticancer activities of specimens with proper control, independent sample t-tests with at least a confidence interval of 95% (SPSS software v. 22, USA) was employed.

## Results and Discussion

*CVCsNPs and CinCsNPs sizes and morphologies*

DLS and TEM analyses of the prepared nanoformulations are shown in figures 1 and 2. The hydrodynamic diameters of CVCsNPs and CinCsNPs were 111 ± 7, 161 ± 6, and 218 ± 11 nm, respectively. Nanoparticles without cargo (CsNPs) were smaller than CVCsNPs and CinCsNPs; it could be said that the size

of nanoparticles has increased with the loading of the EO or cinnamaldehyde. Besides, nanoparticles containing the EO were smaller than those containing cinnamaldehyde. As all parameters during the preparation of nanoparticles were the same, the smaller size of CVCsNPs is related to EO composition [33]. Preparation methods (i.e., ion gelation) rely on opposite charges, the concentration of initial materials, and pH; therefore, the presence of different constituents in *C. verum* EO affects nanoparticle size [34,35]. TEM images of CsNPs, CVCsNPs, and CinCsNP found that the shape of the particles was spherical, and their size was fairly uniform.

Some reports on the preparation of CVCsNPs and CinCsNPs were previously published; however, their anticancer effects on the examined cells were not yet reported. For instance, CinCsNPs (size range, 80-150 nm) with antibacterial effect on *Staphylococcus aureus* and *Escherichia coli* were introduced [36]. Furthermore, such nanoparticles with 65.1 nm diameter were tested against *E. coli* and *Listeria monocytogenes* [37]. In another report, *C. verum* EO was first encapsulated into CsNPs with a 100–190 nm size. The obtained nanoparticles were then used as cucumbers coating; improved their microbiological and physicochemical quality [38]. Another research, antibacterial effects CVCsNPs with a particle size of 100 – 200 nm against *E. coli, Erwinia carotovora*, and *Pseudomonas fluorescens* were investigated [39].

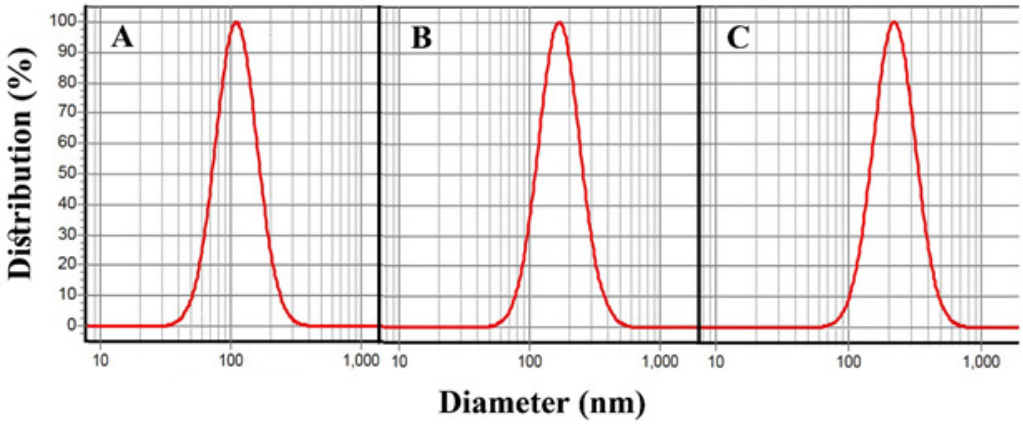


**Figure 1.** The hydrodynamic diameter of A) chitosan nanoparticles 111 ± 7 nm, B) chitosan nanoparticles containing *C. verum* EO 161 ± 6, and C) chitosan nanoparticles containing cinnamaldehyde 218 ± 11 nm

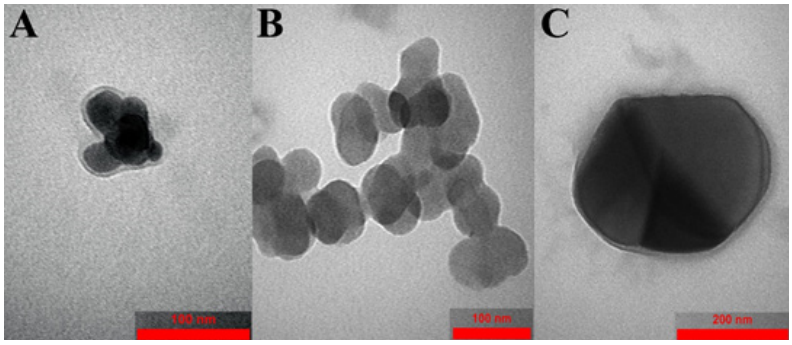


**Figure 2.** TEM images of A) chitosan nanoparticles, B) chitosan nanoparticles containing *C. verum* EO, and C) chitosan nanoparticles containing cinnamaldehyde

*Confirming successful loading of C. verum EO and cinnamaldehyde in chitosan nanoparticles*

ATR-FTIR spectra of samples, including CsNPs, cinnamaldehyde, CinCsNPs, *C. verum* EO, and CVCsNPs, are shown in Figures 3A-E. As detailed in figure 3A, 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 $cm^{-1}$ related 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 $cm^{-1}$ belong to anti-symmetric stretching vibrations of $PO_2$ groups in TPP ions. This new peak showed the formation of ionic crosslinks between protonated amino groups of chitosan and TPP anionic groups.

ATR-FTIR of cinnamaldehyde (Figure 3B) showed the bands at 3060 and 3028 $cm^{-1}$ attributed to =C-H, the bands 2815 and 2741 $cm^{-1}$ assigned to C-H of aldehyde, the peak at 1723 $cm^{-1}$, indicating to the aldehyde, and the main and sharp peaks at 1671, 1624 $cm^{-1}$ corresponding to the stretching vibration of an aldehyde carbonyl C=O.

In figure 3C, the bands at 1596 and 1449 $cm^{-1}$ can be related to the C=C skeleton vibration of an aromatic substance. Besides, two characteristic bands at 1279 and 1121 $cm^{-1}$ can be related to anti-symmetric stretching vibrations of $PO_2$ groups in TPP ions. These peaks showed the formation of ionic interaction between amine groups of chitosan and TPP anionic groups [40]. All the characteristic peaks of cinnamaldehyde appear in the spectra of CinCsNPs at the approximately same wavenumber. The results indicate that cinnamaldehyde is encapsulated into the CsNPs.

FTIR spectrum of *C. verum* EO is displayed in figure 3D. The broad peak at 3468 $cm^{-1}$ related to OH, the bands at 3061 and 3028 related to =C-H, the band at 2923 showed –CH, 2812, and the band at 2740 $cm^{-1}$ indicates C-H aldehyde. The peak at 1728 $cm^{-1}$ representing the aldehyde of saturated fat, and the peak at 1671 at 1624 $cm^{-1}$ corresponding to the stretching vibration of an aldehyde carbonyl C=O. These main and strong peaks are related to high cinnamaldehyde and aldehydes in the *C. verum* EO. The absorption band at 1573 $cm^{-1}$ is assigned to the aromatic ring C=C skeleton vibration of an aromatic substance. The peak at 1449 $cm^{-1}$ is very characteristic of an alcohol C-OH within the bending vibration absorption. The strong peak at 1120 $cm^{-1}$ and 1071 and 1004 $cm^{-1}$ is assigned to C-O's stretching vibrations and the C-OH deformation vibration. The peak at 970 $cm^{-1}$ is attributed to C-H bending absorption, and the strong peak at 745 $cm^{-1}$ is assigned to benzene rings C-H vibration absorption. The peak at 687 $cm^{-1}$ related to the vibration absorption of alkenes.

All the characteristic peaks that appear in the spectra of *C. verum* EO were also observed in CVCsNPs at the approximately same

wavenumber (Figure 3E). Two new peaks at about 1278 and 1124 $cm^{-1}$ related to ionic crosslinks formed between $NH_3^+$ groups of chitosan and TPP anionic groups [40]. The results confirmed that *C. verum* EO was encapsulated into the CsNPs.

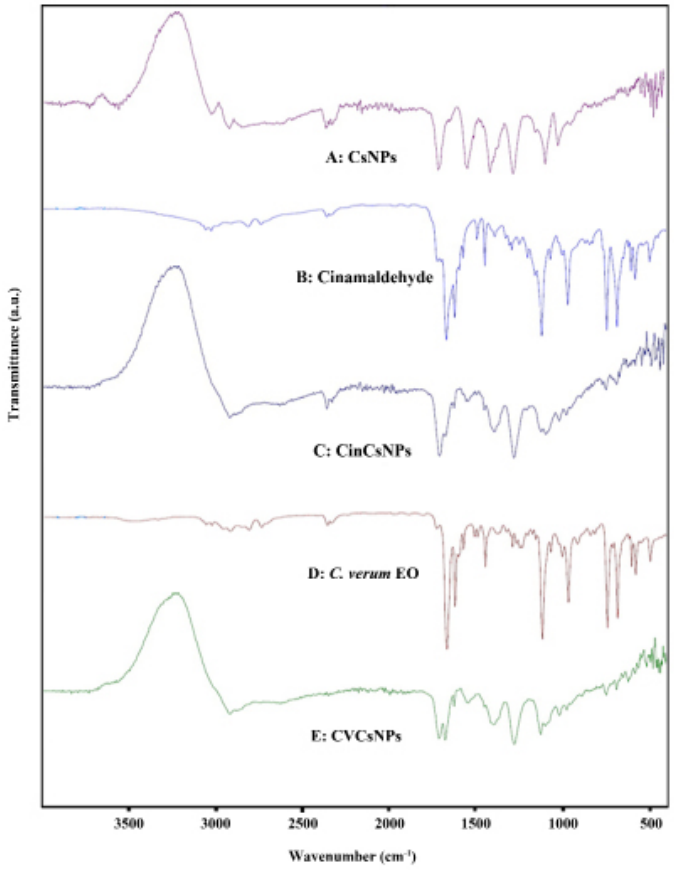


**Figure 3.** Confirmation successful loading of *C. verum* EO and cinnamaldehyde in chitosan nanoparticles; A) chitosan nanoparticles, B) cinnamaldehyde, C) chitosan nanoparticles containing cinnamaldehyde, D) *C. verum* EO, and E) chitosan nanoparticles containing *C. verum* EO

*Anticancer effects of CsNPs, CVCsNPs, and CinCsNPs*

Anticancer effects of CsNPs, CVCsNPs, and CinCsNPs against A-375 and MDA-MB-468 cells as human melanoma and breast cancer in vitro are depicted in Figure 4. The MDA-MB-468 is one of the common cell lines for investigating new drugs as it is triple-negative (without estrogen and progesterone receptors and expression of $HER_2$ [41]. On the other hand, the A375 cell line has been extensively applied for these dangerous forms of skin cancer as it shows low sensitivity to chemotropic drugs [42]. Thus, these cells were chosen for the current study.

In such studies, anticancer effects of non-formulated samples (i.e., *C. verum* EO and cinnamaldehyde) compare with their nanoformulated states. Generally, EOs are dissolved in medium culture or PBS with/out adding 1% DMSO [43,44]. In the current study, cinnamaldehyde was not dissolved in such men-

tioned solvents. It needed to increase the content of DMSO to 10%; this amount of DMSO showed strong cytotoxicity; thus, only anticancer effects of the nanoformulations of cinnamaldehyde and *C. verum* were investigated. CsNPs was applied as a control for observing the possible effects of free CsNPs. CsNPs was found to possess a significantly reduction in viabilities of A-375 ($p = 0.007$) and MDA-MB-468 ($p = 0.010$) compared to control group (no treatment); their viabilities were observed at 85 and 89%. This cytotoxic effect is maybe related to its size and structure, which is discussed elsewhere [45].

CVCsNPs and CinCsNPs' effects on A-375 cells are shown in figure 4A; the cytotoxic effect increased by augmentation particle concentrations in all samples. Efficacy of CVCsNPs were significantly more potent than CinCsNPs at concentrations of 75 µg/mL ($p = 0.01$), 150 µg/mL ($p = 0.025$), and 1200 µg/mL ($p < 0.001$). However, their efficacies at concentrations of 300 and 600 µg/mL were not significantly different; $p = 0.11$ and $p = 0.066$. In figure 4B, the cytotoxic effects of CVCsNPs and CinCsNPs on MDA-MB-468 cells are depicted. CVCsNPs with lower IC50 value showed more potency than CinCsNPs at three concentrations, including 75 µg/mL ($p = 0.025$), 300 µg/mL ($p = 0.007$), and 600 µg/mL ($p < 0.001$). However, its effect at concentrations of 150 µg/mL ($p = 0.930$) and 1200 µg/mL ($p = 0.10$) was not significantly different compared with CinCsNPs. From table 1, although the obtained IC50 values for *C. verum* EO (79.12 and 112.35 µg/mL) against A-375 and MDA-MB-468 cells were lower than cinnamaldehyde (135.06 and 166.47 µg/mL), however, this differences are not significant ($p > 0.05$). Smaller particles possess higher kinetic energy and surface-to-volume ratio, may increasing their possible interaction with cells membrane [46].

Although anticancer effects of cinnamaldehyde and *C. verum* EO were reported in the literature [47-49]; however, CVCsNPs and CinCsNPs have not been yet evaluated against melanoma cell line A-375 and breast cancer cell line MDA-MB-468. In the literature, *C. verum* is considered an antiproliferative and anti-tumorigenic agent [50]. A study by Abd Wahab et al. reported that *C. verum* extracts had a cytotoxic effect on human breast cancer cell line (MCF-7) [47]. In addition, *C. verum* EO contains antioxidant compounds, and due to them, its anti-proliferative effects increase in cancer cells, such as HeLa cell (cervical cancer epithelium) and Raji cell (Burkitt lymphoma) lines [51]. It has also been reported that cinnamaldehyde could prevent oxidative damage and induce antiproliferative pathways in cancer cells, such as apoptosis [49,50].

Furthermore, *C. zeylanicum* EO and cinnamaldehyde anticancer effects are mediated via different apoptosis-related pathways. The anticancer properties of cinnamon and its components are presented under inhibition and suppression of NF-κB. Moreover, suppression of the TNF-α stimulated the expression of IL-8 and inhibitions of invasiveness,

tumor growth, and proliferation of melanoma cells [52,53]. Besides, in a previous study, an extract of *C. zeylanicum* was introduced as a model for hormone therapy of breast cancer [54]. However, no report was not found on investigating the mode of action of CVCsNPs and CinCsNPs; more investigation is thus required for better understanding their anticancer effects pathways.

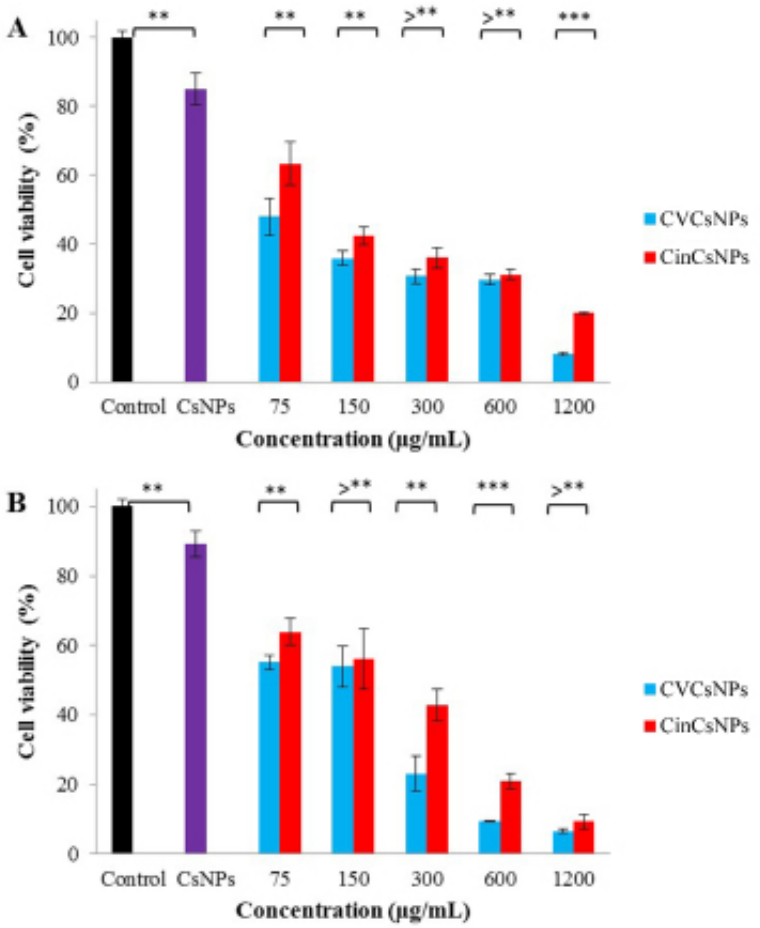


**Figure 4.** Cytotoxicity effect of chitosan nanoparticles (CsNPs) containing *C. verum* EO and cinnamaldehyde (CVCsNPs and CinNPs) on A-375 (A) and MDA-MB-468 (B) cells at different concentrations

**Table 1.** $IC_{50}$ values of chitosan nanoparticles containing *C. verum* EO and cinnamaldehyde on A-375 and MDA-MB-468 cells

| Sample (µg/mL) | A-375 | | | MDA-MB-468 | | |
|---|---|---|---|---|---|---|
| | LCL[c] | $IC_{50}$[d] | UCL[e] | LCL | $IC_{50}$ | UCL |
| CVCsNPs[a] | 30.98 | 79.12 | 202.07 | 75.34 | 112.35 | 167.54 |
| CinCsNPs[b] | 94.82 | 135.06 | 192.37 | 126.19 | 166.47 | 219.61 |

[a]Chitosan nanoparticles containing *C. verum* EO; [b]Chitosan nanoparticles containing cinnamaldehyde;

[c]Lower Confidence Limit (95%); [d]Inhibitory Concentration 50%; [e]Upper Confidence Limit (95%)

## Conclusion

Chitosan nanoparticles containing *C. verum* EO and cinnamaldehyde were first prepared and characterized. Their anticancer effects were then investigated on human melanoma and breast cancer cells. Their IC50 values against A-375 were obtained at 79 and 135 µg/mL, these values for MDA-MB-468 were observed at 112 and 166 µg/mL. Regarding the results, chitosan nanoparticles containing *C. verum* EO could be considered a green potent anticancer for further investigation in vivo study or complementary medicine.

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## Conflict of Interests

None.

## Acknowledgments

None.

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