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Original Article

β-cyclocitral Extracted from *Galium mite* (medicinal herb) and the Evaluation of its Effect in MCF-7 Cell Lines

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ABSTRACT

Carotenoids play important role as antioxidant and induction of gene expression changes lead to plants acclimation to environmental stresses. Betacyclocitral (BCC) derived from *Galium mite* Boiss. & Hohen. in the process of β-carotene oxidation has recently proved that has a new function in breast cancer treatment. In this project we used drug loaded delivery released system (DDRS) strategy, coupled with super paramagnetite nanoparticles (SNPs) based on graphene quantum dots (GQDs) synthesis to deliver drugs to tumor sites. The validation of synthesized SNPs was implemented with several techniques such as Fourier-transform infrared (FT-IR) spectroscopy, X-ray diffraction (XRD), scanning electron microscope (SEM) and transmission electron microscope (TEM). MCF-7 cell lines were treated with GQD, β-cyclocitral and GQD@β-cyclocitral (each with concentration of 3.93, 7.87, 15.75, 31.25, 62.5, 125, 250, 500, and 1000 µg/ml) in MTT assay in 24 and 48 hours respectively. IC50% was calculated in 24 h. Results indicated that β-cyclocitral had prominent effects in breast cancer cell lines controlling.

INTRODUCTION

Breast cancer is the most common malignancy in women and is a heterogeneous disease at the molecular level. Over the past 10 to 15 years, treatment concepts have evolved to account for this heterogeneity, with an emphasis on more biologic therapies and de-escalation of treatment to reduce adverse effects of treatment. Despite the inherent molecular heterogeneity, which is a driving principle of modern therapies, some features such as the influence of local tumor burden or metastatic patterns are shared and influence treatment. Primary breast cancer, meaning cancer that is present in the breast or has spread only to the lymph nodes in the armpit, is considered curable. Improvements in multimodality treatment have led to an increased chance of cure in 70-80% of patients. In contrast, advanced (metastatic) disease cannot be cured using available treatment options. However, advanced breast cancer is a curable disease whose main treatment goals are to prolong survival and control symptoms with low toxicity associated with treatment to maintain or improve quality of life [1]. Early detection of breast cancer is still a challenge for health professionals. According to the recommendations of the World Health Organization (WHO) on the National Cancer Control Program [2], assessing the importance of cancer, for example: incidence, prevalence and mortality, is the first step in this process. It is very important to know the important mechanisms involved in the development of cancer in order to advance therapeutic methods for the treatment of neoplasm. Apoptosis induction is one of the important characteristics of cytotoxic antitumor agents. It has been shown that a series of natural compounds, including plants, induce apoptotic pathways that are inhibited in cancer cells. The ability to induce apoptosis in cancer cells and stop the proliferation of these cells is the subject of many immunopharmacology researches. Among the main causes of cancers, we can mention the influence of environmental factors in causing

mutations and genetic changes responsible for the occurrence of malignancies [3]. Chemotherapy, radiotherapy and surgery can be mentioned among the treatment solutions of this malignancy. However, the mortality rate in the affected people who are treated is high, which indicates the ineffectiveness of these treatment methods [4]. Natural products, especially plants, have a high potential for making medicinal compounds. Many anticancer drugs that have been synthesized, including taxanes, vinca alkaloids, podophyllotoxins, and camptothecins, etc., are derived from plant compounds and are used to treat various metastatic and non-metastatic cancers [5]. A number of antitumor drugs have been extracted from plants [6-9]. Vinblastine, vincristine, etoposide and taxol are herbal compounds extracted from plants that have been approved for use as anticancer drugs [10]. A number of plants have inhibitory properties against tumor multicellularity in leukemia cell line K562. These plants include *G. mite*, *Ferula angulata*, *Stachys abtusicrena*, *Cirsium bracteosum* and *Echinophora cinera*. Among the species, *G. mite* is traditionally used as a sedative, invigorating and appetizing agent [11].

The science of nanotechnology has helped diagnose and treat diseases through the use of nanoparticles and the development of targeted drug delivery methods. Magnetic nanoparticles have a high potential in the field of diagnosis and treatment of diseases, especially cancer. The use of these nanoparticles as contrast-enhancing agents in the conventional magnetic resonance imaging method and also as nanocarriers in modern drug delivery systems has been the focus of researchers in recent years [12-14]. Targeted delivery of chemotherapy agents to cancer cells with the help of magnetic nanoparticles has been studied in vivo and in vitro and has left valuable results. These particles have also been used in the treatment of cancer by heat treatment and in the transfer of nucleic acids, plasmids and siRNA into cells [12,15]. Diagnosing the disease in the early stages is very important for its recovery and treatment methods. Currently, the treatment methods used in the diagnosis of cancer are usually based on the changes in cells and tissues, which in turn can be done through the clinical tests of the doctor and conventional imaging methods. However, scientists are trying to detect cancer when the first molecular changes occur. Nowadays, iron oxide nanoparticles are the only magnetic nanomaterials that have been used in clinical medicine as a contrast agent in magnetic resonance imaging and as a carrier in targeted drug delivery [16]. Experiments conducted over several years on iron oxide nanoparticles show that these particles do not have any immediate or long-term toxic effects in the body, but the presence of some nanoparticles with nanocarriers enhances the effect and performanceon cancer cells. In this way, these nanoparticles strengthen the effect of nanocarriers through various mechanisms, such as increasing oxidative stress and proper drug accumulation in the cell [17]. Targeting of magnetic nanoparticles, unlike other nanocarriers, is done by using these nanoparticles to be affected by a magnetic field. The magnetic field causes the nano-drug to be transported to the tumor site [18]. The existing methods for the synthesis of graphene quantum dots can be generally divided into top-down and bottom-up processes [19]. As bottom-up methods, the synthesis of graphene quantum dots requires complex reaction steps and specific organic materials, which make it difficult to optimize the conditions. Therefore, it is preferable to use the top-down method that is, cutting large blocks of carbon material into small pieces. The raw materials required for this method are abundant carbon materials that are cheap and easy to obtain, and the method is relatively simple and easy to synthesize [20]. Hydrothermal method is a simple and fast method to prepare graphene quantum dots [21]. The principle is to break the bonds between carbon materials to form graphene quantum dots through high temperature and high pressure [22].

This method has many advantages such as low cost, high quantum efficiency, no need for dialysis and purification, simple experimental setup, etc. The prepared graphene quantum dots were environmentally friendly and exhibited water solubility to show their promising applications in the field of biomedical and bioelectronics devices [20]. B-cyclocitral is a derivative of carotenoids and is obtained by oxidation of β-carotene. B-cyclocitral is one of the reactive and electrophilic species and is biologically active and can induce changes in gene expression, which itself leads to plant adaptation to stress conditions [23].

MATERIALS AND METHODS

Graphene Quantum dot Synthesis

Fe3o4 colloid with 50 ml water (15-20 nm), β-cyclocitral, glucose and folic acid were purchased from Sigma-Aldrich chemical Co. All chemical compounds used directly without further purification. 1gr of Fe3O4 colloid nanoparticles in 50 ml was submerged under ultrasound for 15 minutes. Then 20 mg of glucose and 20 mg of folic acid were added to the solution containing magnetic nanoparticles and this mixture was autoclaved at 120°C for 6 h, in the reactor according to the method it was subjected to hydrothermal synthesis. The modified Fe3O4 graphene quantum dots (GQDs) were separated by magnetism and washed with deionized water and ethanol and dried in vacuum. In this study, glucose was used as carbon precursor as well as folic acid.

Conjugating GQD with β-cyclocitral

Graphene quantum dots was physically connected in deionized water in a shaker ratio of 1:25 with β-cyclocitral for 24 hours at a speed of 130 rpm, at -5 °C, magnet was separated, and it was washed with deionized water and ethanol and dried in vacuum.

Cell Culture and Viability

MTT Assay

MTT is a water-soluble tetrazolium salt and its activity is based on the mitochondrial succinate dehydrogenase enzyme of living cells. Living cells turn the yellow solution of MTT into purple insoluble formazan crystals. These crystals are dissolved by DMSO (dimethyl sulfoxide) and the percentage of living cells in each well can be obtained by reading the absorbance of the wells at 570 nm. So, the amount of formazan dye correlated to the number of MCF-7 living cells. The MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Scientific, USA) and supplemented with 10% fetal bovine serum (FBS, Thermo Scientific). The cells were incubated at 37 °C in a humidified atmosphere of 5% CO2 and 95% air, and the growth medium was changed twice a week. The cell seeding was performed by removing the medium and adding 2 mL of Trypsin (0.25%)-EDTA (0.53 mM) (Thermo Scientific) to the flask, then observing the cells under a microscope until the cell layer was dispersed. Finally, a 6-ml medium was added, and the cultures were incubated at 37 °C. The cytotoxicity of GQD@β-cyclocitral was measured using MTT assay. The MCF-7 were seeded at a density of 9.984×10^3 cells/well plates and incubated for 24h. GQD, β-cyclocitral and GQD@β-cyclocitral (each with concentration of 3.93, 7.87, 15.75, 31.25, 62.5, 125, 250, 500, and 1000µg/ml) were added to the culture media in the plate and untreated cells were used as controls. The cells were incubated in humidified 5% CO2 at 37°C for 24, and 48 h, respectively. At the end of the incubation time, 10µL MTT solution was added to each well plate. After 4 h of incubation, 50µL DMSO solution was added to each well plate. After 1h of incubation, the plates were shaken for 1 m, and the absorbance peak at 570 nm was measured and calculated with Eq.1 using a microtiter plate reader (Molecular Device, USA). After MTT assay LC50% was calculated using online softrware AATBioquest.

$$\text{Cell viability} = (mean\ test\ wells \div mean\ control\ wells) \times 100 \quad (1)$$

RESULTS AND DISCUSSION

The results of analysis of variance showed that there was a significant difference between the concentration treatments in terms of the amount of inhibition of MCF-7 cells ($P \leq 0.001$) as shown in table 1.

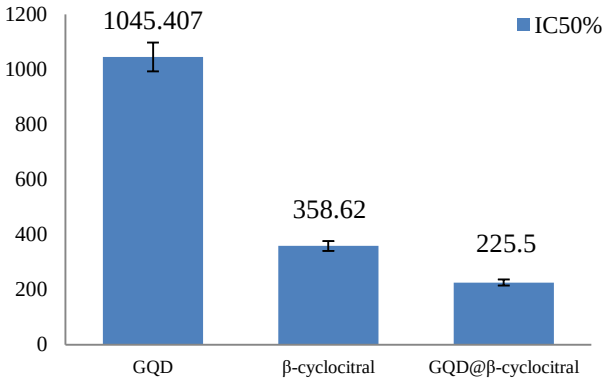

Fig. 4 IC50% of MCF-7 treated cell lines with GQD, β-cyclocitral and GQD@β-cyclocitral.

The study of the effect trend shows that the survival rate decreased with the increase in concentration and this trend was almost the same for all treatments. Examining the inhibition process at two time levels of 24 and 48 hours showed that the inhibition process was the same for the materials and the amount of inhibition increased with increasing treatment time. The amount of IC50% for each of the treatments was determined using AATBioquest online software. For 24 hours of treatment with

graphene quantum dot, β-cyclocitral and GQD@β-cyclocitral, the IC50% was calculated as 1045.407, 358.62 and 225.5µg/ml, respectively. Fig4. Previous studies demonstrated that the mechanism of action of anti-tumor agents can be due to two distinct processes of necrosisor apoptosis in the cells. Cell death by necrosis is a more passiveform of cell death that is characterized by organelle andcell swelling, loss of membrane integrity, rupture of theplasma membrane and cell lysis. Necrosis is often associated with extensive tissue damage and an intense inflammatoryresponse. Apoptosis, on the other hand, is an activeprocess that involves the activation of various cellsignaling cascades which results in characteristic morphologicaland biochemical changes such as chromatin condensation, DNA fragmentation, membrane blebbing, and cellshrinkage. The cell is eventually broken down into smaller membrane-bound vesicles termed apoptotic bodiesthat become engulfed by surrounding cells without initiatingan inflammatory response. Extracts isolated from *G. mite* represented anti-tumor activity via apoptosis induction process [11]. Many plant-derived substances are attractive sources for developing new anticancer agents. Among these natural products, monoterpenes are the primary components of plant essential oils and the effects of many medicinal herbs have been attributed to them. Monoterpenes, exhibit a variety of medicinal properties which had been investigated. They exhibit a very high anticancer potential [25]. B-cyclocitral has been shown to act as an inhibitor of enzymes involved in the biosynthesis of fatty acids, such as cyclooxygenase and lipoxygenase. Cyclooxygenase-2 (COX-2) is upregulated at an early stage in tumorigenesis and has been implicated as an important mediator of proliferation through the increased formation of bioactive arachidonic acid (AA) metabolites such as prostaglandin E2. Similarly, 5-lipoxoygenase-mediated AA metabolism also results in the formation of one-derived DNA adducts. The resulting Hε-DNA adducts are highly mutagenic in mammalian cell lines suggesting that these pathways could be (in part) responsible for the somatic mutations observed in tumorigenesis. As approximately 80% of cancers arise from somatic mutations, this provides an additional link between the upregulation of COX-2 and tumorigenesis (26). According to Javidnia *et.al* [2014], the illustration of the essential oils composition showed that β-cyclocitral is one of these compound extracted from *G. mite*. Therefore, the aim of this project was to target tumor site using synthesized superparamagnetite (Fe_3O_4) based upon GQDs strategy coupled with β-cyclocitral as apoptosis inducer. Results of this research project indicated that β-cyclocitral possesses potential property in apoptosis induction. So, β-cyclocitral can be used as apoptosis inducer in breast cancer cells.

Characterization Techniques

Functional Groups of SNPs GQDs@β-cyclocitral

The typical FT-IR spectra of SNPs GQD@β-cyclocitral were shown in Fig 1. The presence of peak stretching vibrations around 437 cm^{-1} and 589 cm^{-1} is characteristic of Fe-O stretching vibrations of Fe3O4 nanoparticles. The change of the C═O band from 1712 cm^{-1} to1672 cm^{-1} was most likely due to the coordination of iron with the surface of GQDs and β-cyclocitral. In addition, the OH stretching vibration peak shifted from 3347 to 3100 cm^{-1}, indicating that the Fe-O chemical bonds were coordinated with the OH bonds. The bands appearing at 1167, 2850 and 2922 cm^{-1} are attributed to C-O and C-H stretching vibration peaks.

Crystalline Morphology of SNPs GQD@β-cyclocitral

Fig.2 represents XRD patterns of SNPs GQD@b-cyclocitral. Diffraction peaks around 2θ equal to 18.38, 30.20, 35.54, 43.16, 53.50, 57.02, and 62.77 degrees are attributed to (111), (220), (311), (400), (440), and (511). Fe3O4 plates confirm the formation of a spinal structure according to JCPDS card number 0629-19. It can be seen that the presence of GQDs and β-cyclocitral have no special effect on the structure of Fe3O4 nanoparticles. The average crystal size of Fe3O4 in Fe3O4@GQDs, calculated by the Debay-Scherrer equation, is about 31.1 nm.

Nanoparticle Size Confirmation Test

Cellular Exosome Extraction and Dynamic Light Scattering

After applying the treatments as well as untreated cells (control); the cells were centrifuged at 4000 rpm for 2 minutes.

Table 1 The results analysis of Variance (Means square) for the concentrations in terms of MCF-7 Cell inhibition.

S.o.V	df	Mean Squares (MS)		
		GQD	β-cyclositral	GQD@β-cyclocitral
Treatment (concentrations)	9	1453.25 **	154.21 **	712.5 **
Error	10	181.55	11.5	149.11
CV%		4.56	7.98	11.21

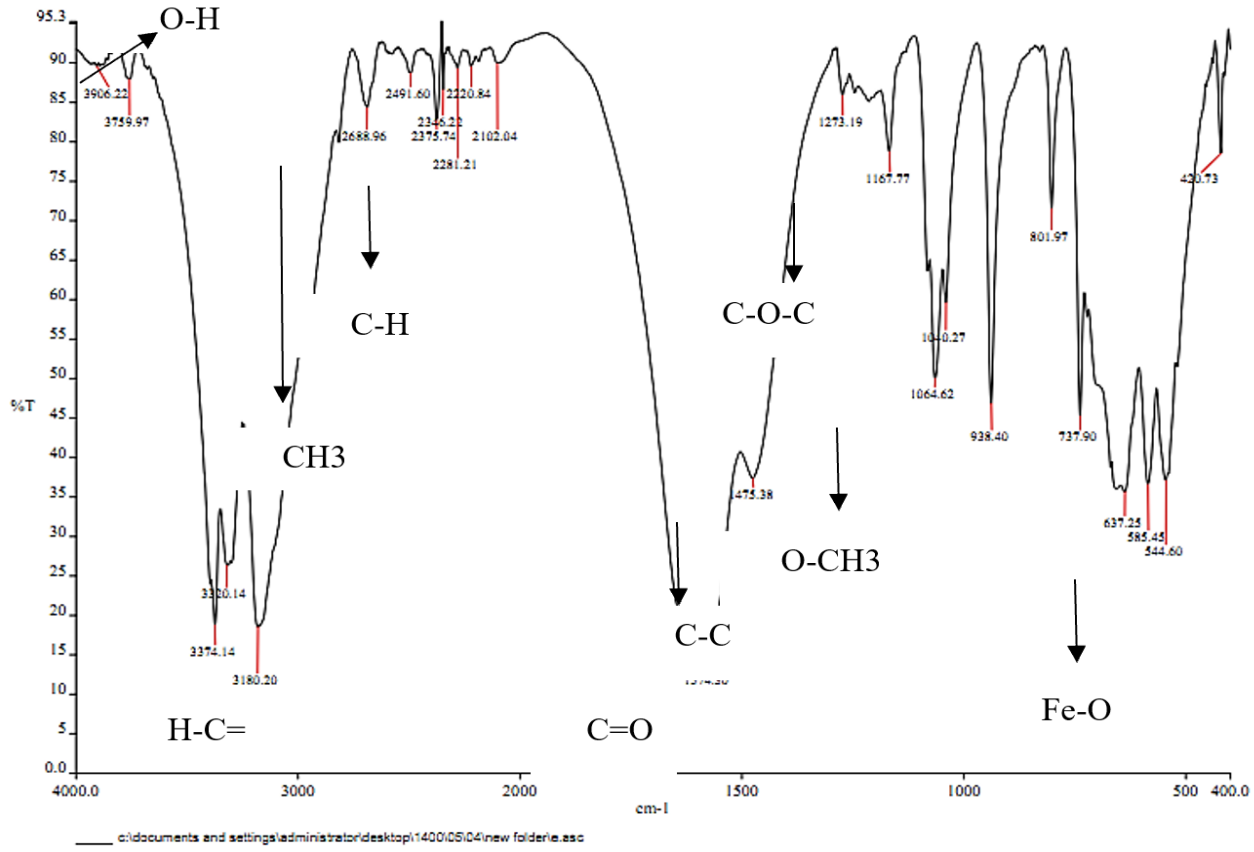

Fig. 1 FT-IR spectra of SNPsGQD@β-cyclocitral.

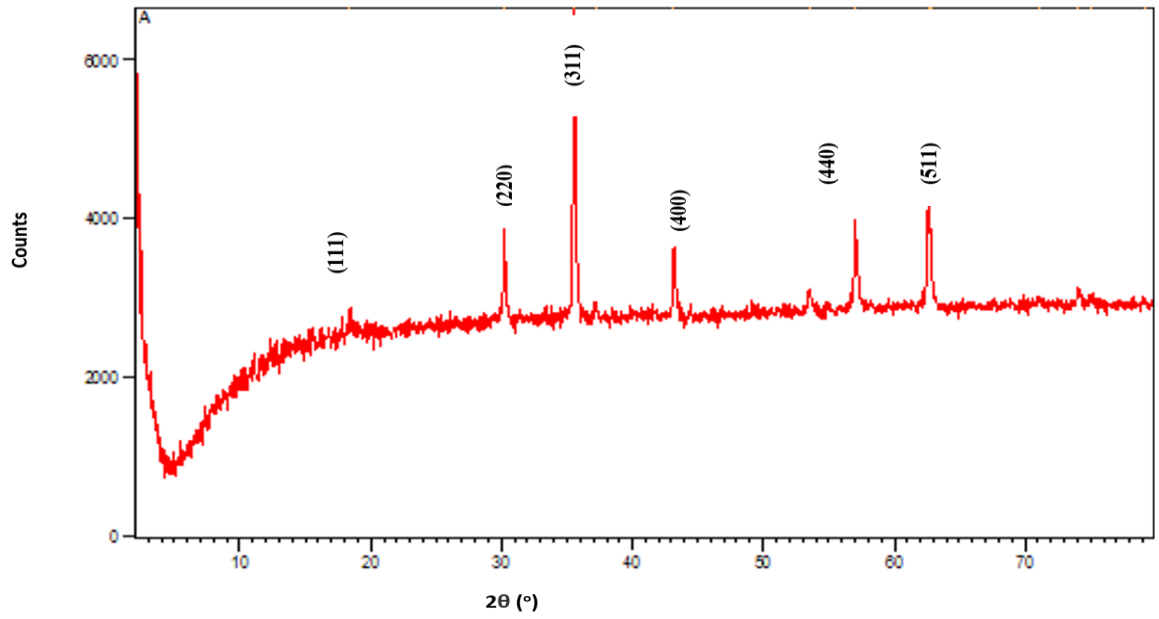

Fig. 2 XRD pattern of SNPs GQD@β-cyclocitral

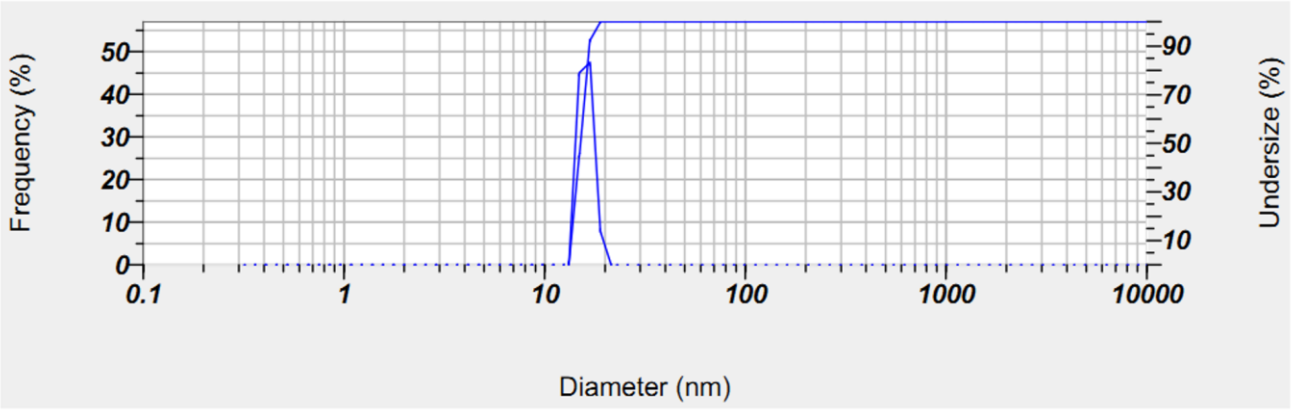

Fig. 3 Dynamic light scattering pattern of extracted exosome of MCF-7 treated cell lines.

Then cell sediment was formed. The supernatant of the cells was removed and centrifuged at 5000 rpm, 8000 rpm, and 14000 rpm at -4 °C and the supernatant was removed at all stages. Cells were centrifuged again at 40,000 RPM for 5 minutes at -4 °C. After the previous centrifugation for 5 minutes, the supernatant was centrifuged at 60000 rpm. Finally, centrifugation was performed at 80,000 rpm for 15 minutes and cell sediment was removed, DLS test and zeta potential test were performed for the extracted exosomes and the final result confirmed the extraction of exosomes. After treating MCF-7 cells at IC50% concentration level of graphene quantum dot (GQD), β-cyclocitral and GQD@β-cyclocitral, exosome was extracted from the treated cells. The size of the extracted exosome was confirmed using D.L.S (Dynamic Light Scattering) technology. The size of exosome extracted in different treatments varied between 10 and 100 nm. (Fig. 3).

CONCLUSION

Tremendous endeavors have being conducted in breast cancers treatment. In this pathway tumor site targeting, drug loaded deliveryreleased system (DDRS) and enhanced permeability and retention time (EPR) strategies play an important role in cancer treatent. Nanocarriers coupled with secondary metabolites derived from herbal medicine exhibited apoptosis induction property in cancer cells. This project conducted in order to produce manipulated nanocarrier with β-cyclocitral as addictive substances for breast cancer treatment. Confirmation tests including FT-IR, XRD, SEM and TEM implemented. According to results, β-cyclocitral can be used as apoptosis inducer in breast cancer treatment.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

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