

Original Research Article

Preparation and Characterization of Nanoliposomal Beta-Cryptoxanthin and its Effect on Proliferation and Apoptosis in Human Leukemia Cell Line K562

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Abstract

Purpose: To prepare beta-cryptoxanthin-loaded nanoliposomes and evaluate their anti-proliferative activity in leukemia K562 cell line, compared to free beta-cryptoxanthin.

Methods: Beta-cryptoxanthin-loaded nanoliposomes were prepared by extrusion method. Morphological characterization of the nanoliposomes was performed by cryo-transmission electron microscopy (cryo-TEM). The anti-proliferation effect of beta-cryptoxanthin (BC) in free and liposomal forms on K562 cell line was studied using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay. Apoptotic activity, following treatment with beta-cryptoxanthin in the free and liposomal forms, was detected using flow cytometry.

Results: Entrapment efficiency of beta-cryptoxanthin was 86.3 % ± 1.0. Cryo-TEM analysis revealed that the nanoliposomes have spherical shapes. In all conditions, beta-cryptoxanthin-loaded nanoliposomes exhibited greater anti-proliferative activity than the free beta-cryptoxanthin ($p < 0.001$). Furthermore, in the presence of beta-cryptoxanthin-loaded nanoliposomes, the proportion of apoptotic cells was higher for free beta-cryptoxanthin ($p < 0.001$).

Conclusion: The data obtained indicate that beta-cryptoxanthin, especially in the liposomal form, inhibits the growth of K562 cells and may therefore provide a basis for the development of leukemia therapies.

Keywords: Beta-cryptoxanthin, Nanoliposome, Anti-proliferative, Apoptosis, Flow cytometry, Leukemia

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INTRODUCTION

Beta-cryptoxanthin (Fig 1) is one of the six major carotenoids routinely present in some yellow or orange fruits and vegetables, such as pumpkin, orange, sweet potatoes, corn and peas [1]. Like other carotenoids, beta-cryptoxanthin can help prevent free radical damage to biomolecules, as well as play an important role in the treatment of certain cancers [2]. It is documented that beta-cryptoxanthin has an inhibitory effect on different

types of cancer cells including lung, bladder, breast, and colon cancer cells [3,4]. However, the insolubility of the beta-cryptoxanthin in water has restricted its use in biomedical research [5].

Some studies have shown that encapsulation of plant-derived compounds in nanocarriers markedly compensated their water insolubility and altered their pharmacokinetics and effectiveness [6]. Liposomes are spherical and colloidal vesicles that range from a few

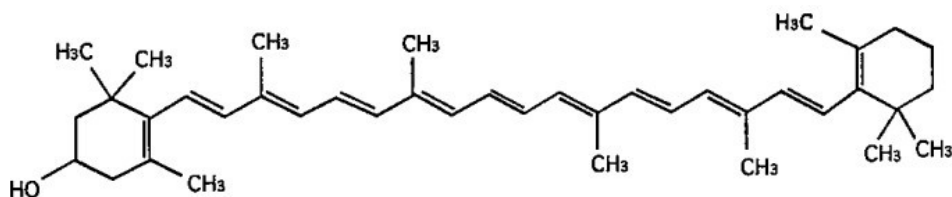


Figure 1: Chemical structure of beta-cryptoxanthin

nanometers to several micrometers in diameter [7]. These carriers are composed of natural phospholipids and other lipids, such as cholesterol, and can be used as a vehicle for the administration of drugs and nutrients [8,9]. To date, the anti-proliferative activity of beta-cryptoxanthin in the free and nanoliposomal forms against K562 cell line have not yet been studied. The aim of this study was to prepare beta-cryptoxanthin-loaded nanoliposomes and evaluate their *in vitro* anti-proliferation activity against the K562 cell line.

EXPERIMENTAL

Materials

Fetal bovine serum (FBS), RPMI-1640, penicillin, streptomycin, and trypan blue were obtained from Gibco BRL (Gaithersburg, MD, USA). Beta-cryptoxanthin (purity $\geq 97\%$), Hoechst 33342, soy lecithin and cholesterol were purchased from Sigma (St Louis, MO, USA). Ethanol, acetonitrile and diethylamine were obtained from Merck (Darmstadt, Germany).

Preparation of beta-cryptoxanthin-loaded nanoliposomes

Beta-cryptoxanthin-loaded nanoliposomes were prepared by extrusion method, as described previously [10]. In brief, soy lecithin and cholesterol (4:1 molar ratio) were dissolved in chloroform and then dried to a lipid film with a rotary evaporator (Brinkman) under vacuum and nitrogen flow at 30 °C. Subsequently, the dried lipids were dispersed by agitation in 1 mL of beta-cryptoxanthin solution (100 $\mu\text{g}/\text{mL}$) and sonicated at 4 °C in ultrasonic bath (Braun-sonic 2000). Finally, beta-cryptoxanthin-loaded nanoliposomes were obtained by extruding of liposomal suspension through a polycarbonate membrane with 100 nm-sized pores 10 times, and separating the excess free beta-cryptoxanthin and larger lipid aggregation by ultracentrifugation (100,000 g, 30 min). The control nanoliposomes were prepared similarly, but PBS (pH 7.4) was used instead of the beta-cryptoxanthin solution.

Characterization of nanoliposomes

The content of the beta-cryptoxanthin in the nanoliposomes was determined by HPLC method [11] following dissolution in 0.1 % Triton X-100. To determination of beta-cryptoxanthin, 20 μL of the nanoliposomal lysate was injected into the HPLC column. In the HPLC analysis, a C 18 column (4.5 \times 150 mm, 5 μm , Phenomenex, Torrance, USA) and diode array UV detector was used. The mobile phase was an equivolume solution of ethanol and acetonitrile containing 0.1 mL of diethylamine per liter of solvent at a flow rate of 0.9 mL/min. Subsequently, the loading (L) of beta-cryptoxanthin was calculated as in Eq 1.

$$L (\%) = \{(W_n V) / (V W_i)\} 100 \dots \dots \dots (1)$$

where W_n is the amount of beta-cryptoxanthin in nanoliposomes, V the total sample volume tested and W_i the initial amount of beta-cryptoxanthin used in preparing the nanoliposomal formulation.

The mean particle size, zeta-potential, size distribution, and polydispersity index of the nanoliposomes were evaluated using a Malvern zetasizer (Malvern instrument) apparatus, as reported previously [8]. The analysis of the size and structure of the beta-cryptoxanthin-loaded nanoliposomes were carried out by cryo-TEM, as described previously [12]. Briefly, diluted nanoliposome samples were placed on a copper grid at room temperature and blotted with blotting paper. The sample was transferred to a grid holder and vitrified in liquid nitrogen. Thereafter, digital imaging was performed at 200 kV and size analysis was carried out with the aid of an image analysis software (NIH, Bethesda, MD, USA).

In vitro release study

The beta-cryptoxanthin released from the nanoliposomes was measured by the reported method [13]. Briefly, a cellulose membrane (molecular weight cut-off of 8000 kDa) was mounted between the donor and receptor compartments. The donor medium consisted of 1 mL of nanoliposomal sample and the receptor medium consisted of 10 mL of citrate-phosphate buffer (0.1 M, pH 7.4). During the dialysis, the

temperature was kept at 37 °C. At pre-determined time intervals, between 2 to 12 h, the amount of the released beta-cryptoxanthin was analyzed using HPLC as described above.

Cell culture

The K562 cell line (human CML, NCBI-C122) was purchased from Pasteur Institute of Iran (Tehran, Iran). The cells were grown in RPMI-1640 medium supplemented with 10 % (vol/vol) heat inactivated FBS along with penicillin (100 units/mL) and streptomycin (100 µg/mL) and were maintained at 37 °C in a humidified incubator 5 % CO₂. Beta-cryptoxanthin was dissolved in DMSO to obtain a 1 mg/mL stock solution. All subsequent dilutions were made in the RPMI medium.

Cell proliferation assay

In this test, K562 cell proliferation was evaluated using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay as described previously [14]. Briefly, 5×10^3 cells were incubated in 96 well plates in the presence of different concentrations of beta-cryptoxanthin in the free and nanoliposomal forms (0, 10, 50 and 100 µg/mL) for 24, 48, and 72 h in a final volume of 200 µL. At the end of the treatment period, 20 µL aliquots of MTT solution (5 mg/mL in PBS) was added to each well, and the plates were incubated at 37 °C. Subsequently, the purple-blue MTT formazan precipitate was dissolved in 200 µl of DMSO and the optical density read at 570 nm. Three replicates of each concentration of beta-cryptoxanthin in the free and nanoliposomal forms were performed.

Nuclear staining with Hoechst 33342

The K562 cells were incubated with beta-cryptoxanthin in the free and encapsulated forms (0, 10, 50 and 100 µg/mL) for 72 h and washed with PBS buffer. After fixing in freshly prepared ice-cold paraformaldehyde (0.1 %) for 10 min, the K562 cells were then stained with Hoechst 33342 (50 µg/mL) for 1 min and its morphologic

changes were observed under the fluorescent microscope. Subsequently, the percentage of apoptotic cells was determined. This experiment was done in triplicate.

Flow cytometric analysis

The K562 cells were treated with varying concentrations of beta-cryptoxanthin in the free and nanoliposomal forms (10, 50 and 100 µg/mL) in complete medium for 72 h. After treatment, the cells were collected and the quantitative apoptotic death assay was done using Annexin V and PI staining following the manufacturer's protocol. Subsequently, the stained cells were analyzed by flow cytometer using FACS (BD, San Diego, CA, USA).

Statistical analysis

All data were expressed as means \pm standard deviation (SD). The analysis of variance was performed to determine the significance level among the tested groups using IBM Statistics SPSS software version 19, and $p < 0.05$ was considered statistically significant.

RESULTS

The results showed that the percentage of beta-cryptoxanthin entrapment efficacy was $86.3 \% \pm 1$. Table 1 shows mean particle size, zeta-potential, and polydispersity index of empty and beta-cryptoxanthin-loaded nanoliposomes. According to previous studies, the size homogeneity of empty and loaded nanoliposomes suggested that beta-cryptoxanthin was entrapped into nanoliposomes [9,10]. Furthermore, the polydispersity index of the nanoliposomes revealed that the prepared nanoliposomes have appropriate stability in aqueous dispersion [12].

Cryo-TEM analysis showed that the nanoliposomes have a fine spherical shape and homogeneity (Fig 2).

Table 1: Particle size, zeta-potential and polydispersity index of empty and beta-cryptoxanthin-loaded nanoliposomes

Formulation	Mean particle size (nm)	Zeta-potential (mV)	Polydispersity index
Empty nanoliposomes	93.5 \pm 0.25	-1.12 \pm 0.10	0.18 \pm 0.06
Beta-cryptoxanthin-loaded nanoliposomes	96.2 \pm 0.18	-1.44 \pm 0.21	0.18 \pm 0.04

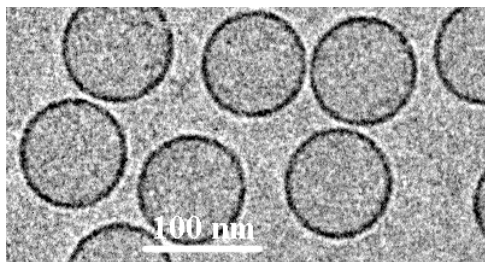


Figure 2: Cryo-transmission electron micrographs of the nanoliposomes loaded with beta-cryptoxanthin

The released amount of beta-cryptoxanthin at 37 °C from nanoliposomes was plotted as a function of time (Fig 3). Beta-cryptoxanthin recovery after 12 h was 84.1 % ± 1.8. The data suggest that the prepared nanoliposomes would be stable at the body temperature.

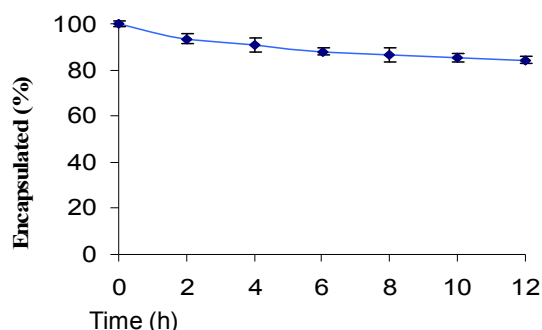


Figure 3: Recovery of beta-cryptoxanthin in nanoliposomes at 37 °C

The viability of the K562 cells after treatment with the free and nanoliposomal forms of beta-cryptoxanthin was examined by MTT assay. The data showed that cell proliferation was inhibited in K562 cells in a dose- and time-dependent manner (Fig 4). In all conditions, the beta-cryptoxanthin-loaded nanoliposomes were more effective than those of free beta-cryptoxanthin on the K562 cell proliferation ($p < 0.001$). As shown in Figure 4, the extent of inhibition increased significantly at 24 h with the lowest concentration of beta-cryptoxanthin in the free and encapsulated forms which was continued to rise at 48 and 72 h durations at their maximum concentration.

The Hoechst staining of the beta-cryptoxanthin-treated cells after 72 h showed characteristic apoptotic features such as nuclear fragmentation and chromatin condensation (Fig 5). In particular, in the presence of beta-cryptoxanthin-loaded nanoliposomes the percentage of the apoptotic cells was higher than those of free beta-cryptoxanthin ($p < 0.001$).

As shown in the representative FACS analysis scatter-grams, the treated cells with beta-cryptoxanthin, in particular in the loaded form, at 10, 50 and 100 µg/mL doses for 72 h showed a strong shift from the vital cells to the late and early apoptotic cells with a little change in the necrotic cell population (Fig 6).

DISCUSSION

The use of plant-derived materials for cancer therapy has been widely investigated [15,16]. The main problem associated with the application of such compounds is insufficient water solubility [17].

As an interesting approach to drug delivery research, the incorporation of plant-derived materials into nanoparticles could overcome this limitation [18]. It has been reported that encapsulation of plant-derived materials in the liposomes could increase their water solubility, bioavailability and effectiveness [19,20].

Beta-cryptoxanthin, a carotenoid pigment and phytochemical, is naturally present in many vegetables and fruits such as apricots, grapefruit, tangerines and rutabagas [1,2]. Evidence for beta-cryptoxanthin's benefit was strongest for anti-oxidative, anti-tumour, anti-inflammatory, and anti-arthritis effects [4,5]. In this study we found that beta-cryptoxanthin, in particular in the encapsulated form, significantly inhibited proliferation of K562 cells with a dose- as well as time-dependent manner. Our results showed that anti-proliferation of beta-cryptoxanthin-loaded nanoliposomes against K562 cells was higher than those of free beta-cryptoxanthin. Several hypotheses, including increased water suspensibility and penetration of plant-derived compounds into cells may explain the mechanism of enhanced anticancer efficacies of these nanovesicle formulations [15-20]. When nanocarriers such as nanoliposomes were used in vitro, they can interact with the cancer cell membranes, and therefore they can selectively deliver drugs to the cancer cells [19,20].

In this case, similar observations have been reported in some previous studies with other natural plant products such as curcumin [15], epigallocatechin-3-gallate [16], celastrol [17], tea polyphenol [18], berberine [19] and gossypol [20] in various cancer cell lines. Therefore, beta-cryptoxanthin and other effective plant-derived agents, in particular in loaded form, could be considered as a promising strategy for developing anticancer drugs.

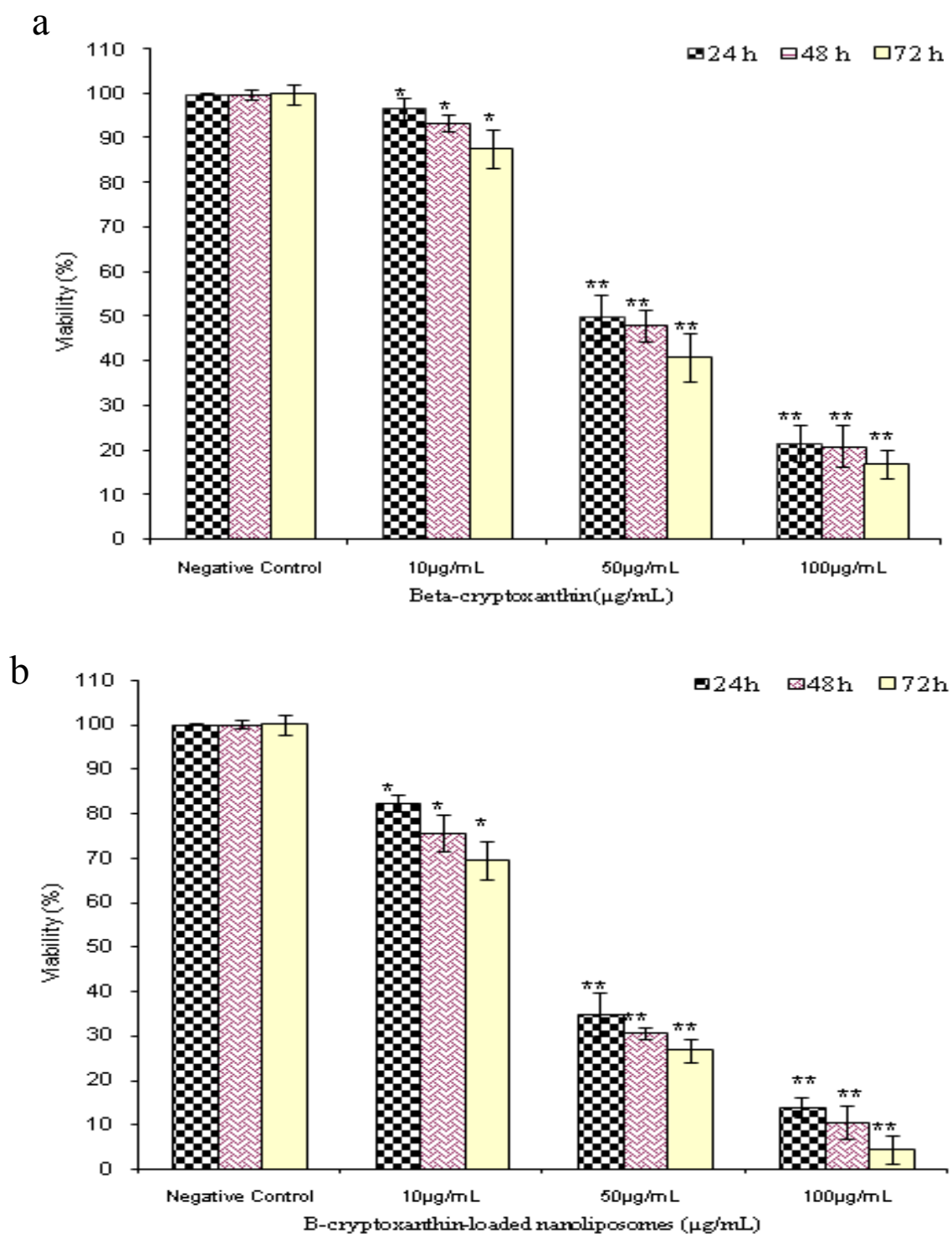


Figure 4: Dose- and time-dependent inhibition of K562 cell growth by beta-cryptoxanthin in the free (a) and nanoliposomal (b) forms. The cells were incubated with increasing concentration of beta-cryptoxanthin in the free and nanoliposomal forms and then the cell survival was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay. Data are expressed as mean \pm standard deviation ($n = 3$, * $p < 0.05$, ** $p < 0.001$)

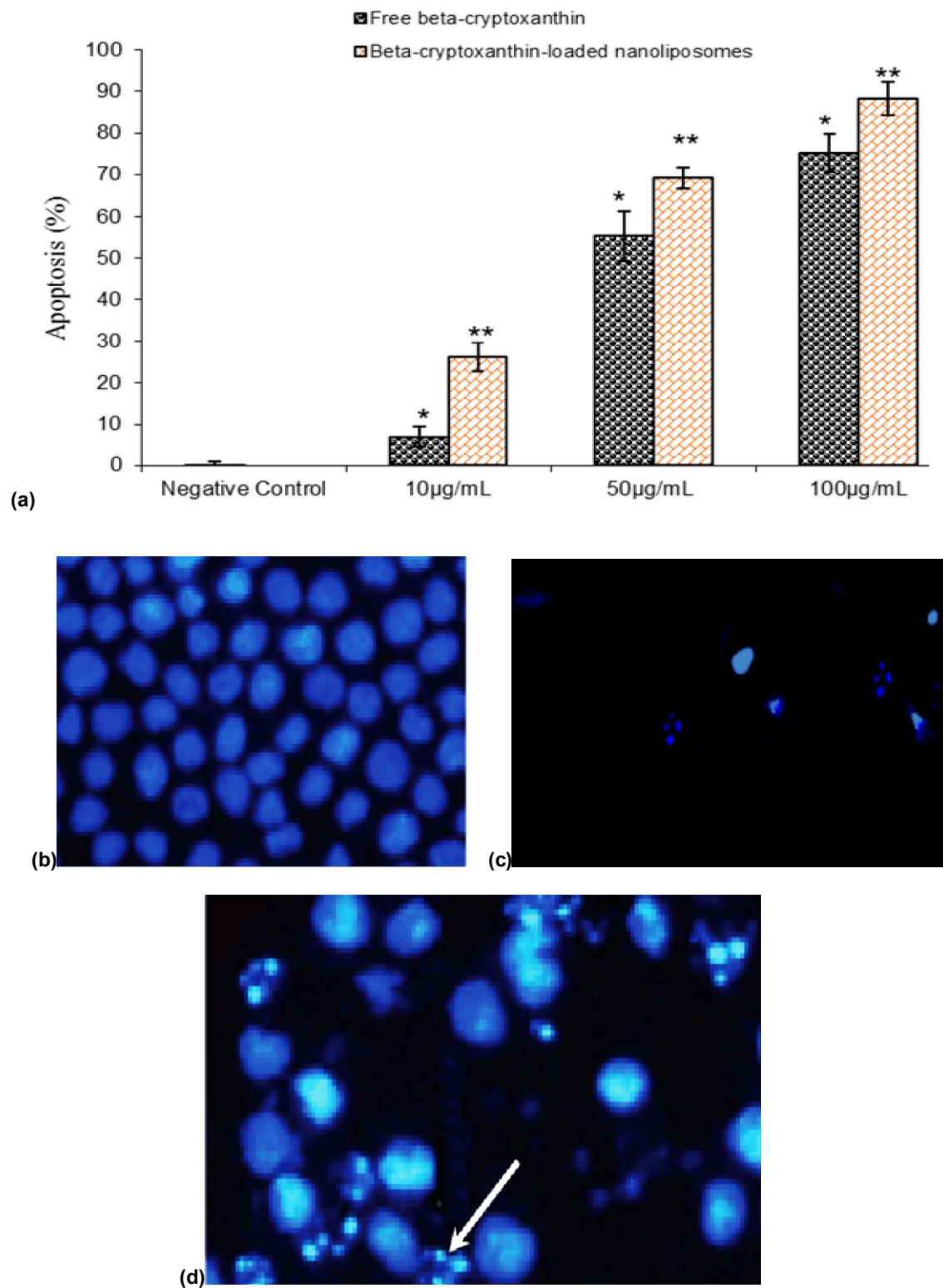


Figure 5: (a) Rate of apoptotic K562 cells after treatment with beta-cryptoxanthin in the free and nanoliposomal forms; (b) control cells; (c) fragmented or condensed nuclei indicative of apoptosis in the beta-cryptoxanthin-treated cells with free form; and (d) with nanoliposomal forms, as indicated with arrows. Data represent mean \pm standard deviation (n = 3, *p < 0.05, **p < 0.001)

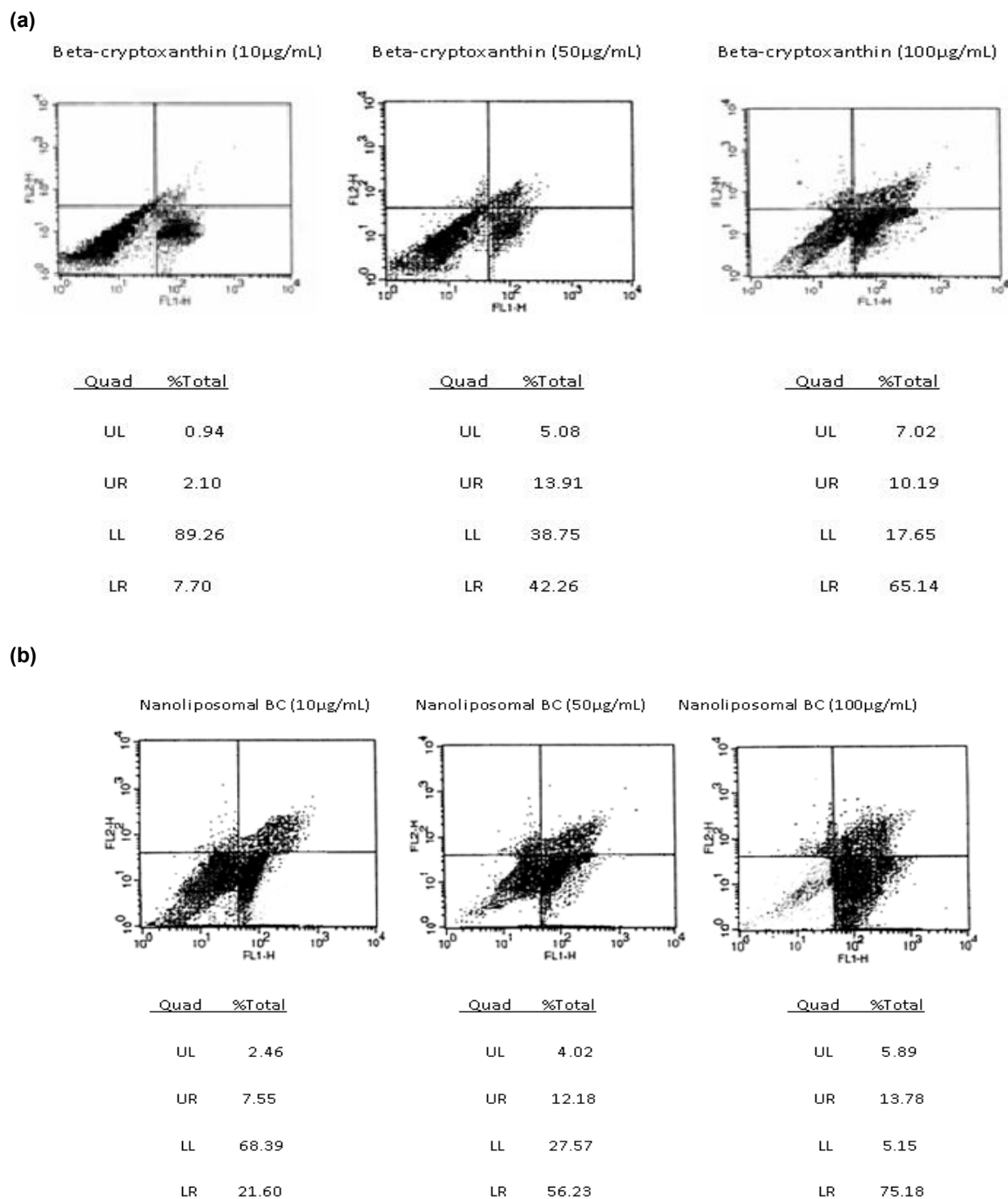


Figure 6: Representative FACS analysis of treated cells with 10, 50 and 100 µg/mL of beta-cryptoxanthin in the free (a), and loaded (b) forms after staining with AnnexinV/PI. Lower Left (LL): AnnexinV⁻PI⁻, Lower Right (LR): AnnexinV⁺PI⁻, Upper Right (UR): AnnexinV⁺PI⁺, Upper Left (UL): AnnexinV⁻PI⁺

CONCLUSION

Findings from this work demonstrate that nanoliposomal beta-cryptoxanthin, can be prepared by extrusion method. Beta-cryptoxanthin-loaded nanoliposomes have strong apoptosis induction effect in human leukemia cell line K562. Therefore, this novel formulation

provides a basis for the future development of leukemia therapies.

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