RESEARCH ARTICLE

Nanoliposomal formulation of Ecballium elaterium Extract: Cytotoxic Evaluation against Human Gastric Adenocarcinoma (AGS) Cell Line

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ABSTRACT

Objective(s): The aim of this study was to determine cytotoxic effect of nanoliposomal form of lyophilized aqueous extract of *Ecballium elaterium* fruit on gastric cell line (AGS) using cell viability tests.

Methods: An aqueous extract of the fruits of *Ecballium elaterium* was prepared. Nanoliposomal form was also prepared by thin-film hydration method and stability size was determined by SEM. The zeta potential and size characterized by Malvern zetasizer. Cytotoxic effect of the nanoliposomes encapsulated the extract on cell line was examined by MTT, Neutral Red and Frame methods.

Results: The size of nanoliposomes was 218.2 nm with proper dispersion (PDI=0.3). The morphology of the liposomes was suitable according to SEM image. The IC_{50} values indicated that the nanoliposomal form of extract was 2-3 times more active than extract alone. The average IC_{50} values for extract and nanoliposomal form of extract were 1±0.1 and 0.39±0.02 µg/ml, respectively.

Conclusions: The results from this study showed that the crude extract and nanoliposomal form extract of *Ecballium elaterium* have cytotoxicity effect on AGS cell line and these cells were significantly more susceptible to nanoliposomes encapsulated *Ecballium elaterium* extract than that of the extract itself.

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INTRODUCTION

Ecballium elaterium (L.) A. Rich (EE), which is also known as squirting cucumber, is the only species of the Ecballium genus. *Ecballium elaterium* is a wild medicinal plant which is found in Moghan (Ardabil province, Iran). EE fruit contains dark seeds and aqueous extract which has anti-inflammatory, anti-pyretic and analgesic effects and has been used by natives of Moghan for chronic sinusitis by nasal aspiration. Other traditional uses include the treatment of fever, cancer, liver cirrhosis, constipation, hypertension and rheumatic diseases [1]. *E.elaterium* contains triterpenoids (cocurbitacines), carbohydrates, gum, tannins and peptides. In addition, the fruit juice of EE and its triterpenoid constituent, cucurbitacin B [2] reported to have preventive and curative effects against CCl_4 (carbon tetra chloride) induced hepatotoxicity [2]. This plant contains elaterium which is a phenolic compound [3]. Liposomes are vesicles, having stable membranes composed of two layers, firstly were described by British hematologist Dr. Alec D Bangham FRs in 1961 [4] and because of their specific properties have

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become one of the most important drug carriers [5]. Nanoliposomes have gained broad spectrum application in medicine and biological researches due to similarity between their membrane with lipid bilayer of cells, encapsulation ability of hydrophobic and hydrophilic drugs, as well as gradual release of encapsulated drugs resulting in an enhanced efficacy [6]. It is known that nanoparticles have a high tendency to be trapped in the reticuloendothelial system, such as in spleen, liver and lungs [7].

On the other hand, it is known that size of liposome is highly related to its stability and trapped solute leakage. Many researches have been conducted in order to establish preparation methods of liposomes and to enhance their efficacy in chemotherapy [6]. Many studies have demonstrated that both hydrophilic and lipophilic chemicals can be encapsulated in liposomes because of their amphipathic characteristics. Encapsulation of plant or animal extracts in liposomes and their application for different purposes such as cancer therapy have been studied [8-10]. Although there are some researches about cytotoxicity of *Ecballium elaterium* extract [11], no study has been performed using liposomal entrapment. Therefore, in this study we investigated *in vitro* cytotoxicity activity of nanoliposomal form of lyophilized extract of *E. elaterium* on AGS cell line.

MATERIALS AND METHODS

Materials

Human Gasteric Adenocarcinoma cell line was provided from Pasteur institute of Iran (Tehran, Iran). Lecithin from sorbean type IV-sl-α-phosphatidyl chorine (pc) was obtained from sigma chemical co. Cholesterol and solven Folin – ciocaiteu phenolic were purchased from Merck co.

Plant material

At the end of summer, the fruits of *Ecballium elaterium* were collected from suburb area of Moghan, Ardabil province, Iran. In this season, seeds of the fruit collected when the seeds ripened.

Preparation of lyophilized Ecballium elaterium

Fruits were washed and cut into small pieces and homogenized by silent crusher M (Heidolph, Germany) in distilled water. The subsequent mixture was further treated by ultrasonic homogenizer (up 200H, Hielscher, Germany) in maximum amplitude and complete cycle for 15min. Then the mixture was centrifuged and supernatant was collected allowed to freeze at -80 °C. Next day, the frozen extract was lyophilized using freezer dryer (ALPHA2-Plus, Martin Christ, Germany) and dried residue kept at -20 °C.

Preparation of liposomes

The thin-film hydration method was used for preparation of nanoliposomes [12-14]. Cholesterol and lecithin phospholipid (L-α-phosphatidyl-cholin) were dissolved in chloroform. A mixture of two solutions was combined completely by a rotator in a ratio of 4:1 (w/w), respectively. The solution was transferred to a rotary evaporator system (Heidolph Germany) related with a vacuum system and was stored under the lipid transition temperature (T_c). To prevent the oxidation of phospholipids, evaporation was carried out under the atmosphere of nitrogen for 2 hours until a thin film was obtained. Then, freeze-dried plant extract was dissolved in distilled water and added to the solution. For a faster dissolution, glass beads (0.5mn) were added and the mixture was stirred until the film was disappeared. The obtained product was sonicated by an ultrasonic homogenizer (up200H, Hielis cher, Germany) for 15 minutes. Homogenized suspension was taken under nitrogen atmosphere and kept under lipid transition temperature for 1 h. Then, the product was centrifuged (sigma 3-30k, Germany) to yield a clear suspension of nanoliposomes encapsulating the extract and held at 4 °C until next days.

Characterization of nanoliposomes

Zeta potential, hydrodynamic diameter (size) and polydispersity index (PDI) of nanoliposome determined by photon correlation spectroscopy using a Malvern ZetaSizer (Nano ZS nanoseries, Malvern Instruments, UK). Size, PDI, and zeta potential were obtained using the water as dispersant agent at 25 °C. The data were analyzed using the Malvern software. The morphology of the prepared liposomes was determined by Scanning Electron Microscopy, SEM (LEO 1430VP, Germany and UK).

Determination of encapsulation amount of extract

Ecballium elaterium has many component antioxidants in the fruits, especially cocurbitacines B and E which have phenolic structure that were used to determine the encapsulated amount on nanoliposomes as an indicator for efficiency of whole extract entrapment. For determination of the total phenolic loaded on nanoliposomes, UV-Vis absorption spectra at 415nm (25 °C) were measured by a spectrophotometer (PG-Instrument LTD,

England). Gallic acid was used to plot the calibration curve [15]. The total encapsulation amount of plant extract in the nanoliposomes was calculated using following formula:

$$\text{Loaded extract} = (\text{phenolic}_{\text{total}} - \text{phenolic}_{\text{remained}}) / \text{phenolic}_{\text{total}} \times 100$$

Cell culture

Human gastric adenocarcinoma (AGS) cells were cultured at 37 ºC in RPMI-1640 medium (GIBCO, UK) supplemented with 10% (v/v) fetal bovine serum (FBS; GIBCO, UK), Penicillin (100 IU/ml) and streptomycin (100µg/ml). The cells were provided from Pasteur Institute (Tehran, Iran). AGS cells were harvested and plated in 96-well flat bottom plates at a density of 1×10^4 cells per well in 200µl medium. After the attachment of cells, the medium was removed and cells were indicated with a FBS-free medium supplemented with 10 mg/ml prepared extract-loaded nanoliposomes or nanoliposomes (without extract) and/or extract (without nanoliposomes), by ¼ serial dilution for 24h in separate plates. The cytotoxicity of the above-mentioned formulations was determined using MTT, neutral red and frame assays.

MTT assay

Four hours prior to the completion of incubation time, the medium was removed and 180 µl of serum-free medium and 20 µl of 2.5 mg/ml MTT (Merck, Germany) were added to each well. Finally, the medium was removed and 200 µl of DMSO was added to each well. Plates were shaken for 10 min. The absorbance at 570 nm was measured by a plate reader (Synergy HT, Biotek). The wells without cells were considered as blank [16].

Neutral red assay

Briefly, three hours before the end of incubation period, the medium was aspirated from each well. Immediately, the working neutral red solution (0.05% of the neutral red stock in cell culture medium without serum) was added to each well and incubated for 3 hours. At the end of incubation time, the neutral red solution was removed and the wells were washed with warm PBS (37 ˚C). After that, 150 µl of a destain solution was added to the wells and was shaken for 20 min in order to remove the neutral red. The destain solution contained 50 ml distilled water, 49 ml ethanol and 1 ml glacial acetic acid. Finally, absorbance of the cells at 540 nm was measured [17].

Protein content measurement

After the measurement of cell absorbance in neutral red assay, the medium was removed and wells were rinsed twice with warm PBS. Then, fixative solution was removed from each well and replaced with 150 µl added to the wells and plates were shaken for 20 min. Thereafter, the plates were rinsed with the washing solution (250 µl) as described above and finally, 150 µl of the desorbing solution (150 µl, potassium acetate, 1M, in 70% ethanol) was placed into the wells and shaken for 5 min at 150 rpm.

Finally, the absorbance was measured at 595nm using the plate reader.

Statistical data analysis

Concentration for 50% inhibition of cell growth (IC_{50}) value, was calculated using sigma plot 11 software and presented as mean±SD. Measurements were triplicate (n=3 per experimental group) and the statistical significance was evaluated by student's t-test. Differences were considered significant at confidence limits of $P<0.05$.

RESULTS AND DISCUSSION

Photon correlation spectroscopy

Zeta potential of prepared liposomes was -45.5 mV as it is shown in Table 1. Due to the electric

Table1. Zeta potential of nanoliposomes

System				
Temperature(°C):	24.9	Zeta Runs:		12
Count Rate (kcps):	2030.4	Measurement Position (mm):		2.00
Cell Description:	Clear disposable zeta c…	Attenuator:		6
Results				
		Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV):	-45.5	Peak 1:0.00	0.0	0.00
Zeta Deviation (mV):	0.00	Peak 2: 0.00	0.0	0.00
Conductivity (mS/cm):	15.8	Peak3 : 0.00	0.0	0.00
Result Quality	Good			

repulsion between particles [18], zeta potential values greater than 30 mV is related with in vitro stability and longer half-life of vesicles in intravascular system [19]. Also, it was shown that negative values of zeta potential has a positive correlation with increased entrapment of water soluble pharmaceuticals, which is maximal at -30 mV [20]. The average size of liposomes was 218.2 nm (Fig. 1) which was considered as nanoscale, based on IUPAC definition [21]. PDI value of prepared nanoliposomes was 0.3 (Fig. 1). It has been stated that PDI values less than 0.3 or corrected 0.3 is indicative of a good monodispersity of vesicles [18].

Scanning electron microscopy (SEM)

Scanning electron micrographs of prepared nanoliposome showed the size of nanoliposomes that are in nanoscale and this is in agreement with the conclusion of Zetasizer (Fig. 2). Also the morphology of nanoliposomes were in a rang compatible with PDI value.

Entrapment efficiency

The loaded amount of extract in nanoliposomes was 62.76%. This amount assumed satisfactory when compared to previously published data on liposomal drug delivery system for compounds with herbal origin [22].

Cytotoxicity study

The results of viability studies are shown in Fig. 3. The results demonstrate that entrapped *Ecballium elaterium* extract shows significant cytotoxic effect in comparison with the base extract. It could be resulted that the cytotoxic effect of liposomal form of *Ecballium elaterium* extract on AGS cell line was 2 to 3 times more than the free extract. The nanoliposomes without extract during the experiments were used as control. Table 2 shows the IC_{50} values calculated from MTT, neutral red and frame methods after incubation for 24h. By comparing the IC_{50} values for extract and encapsulated extract, it seems that the prepared nanoliposomes are efficient for delivering the active

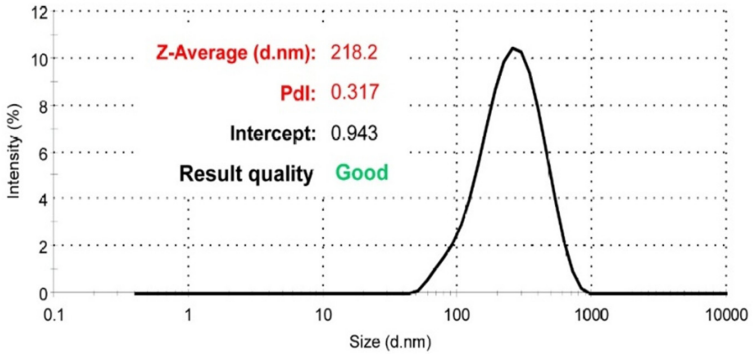

Fig.1. Size and PDI of extract-loaded liposomes

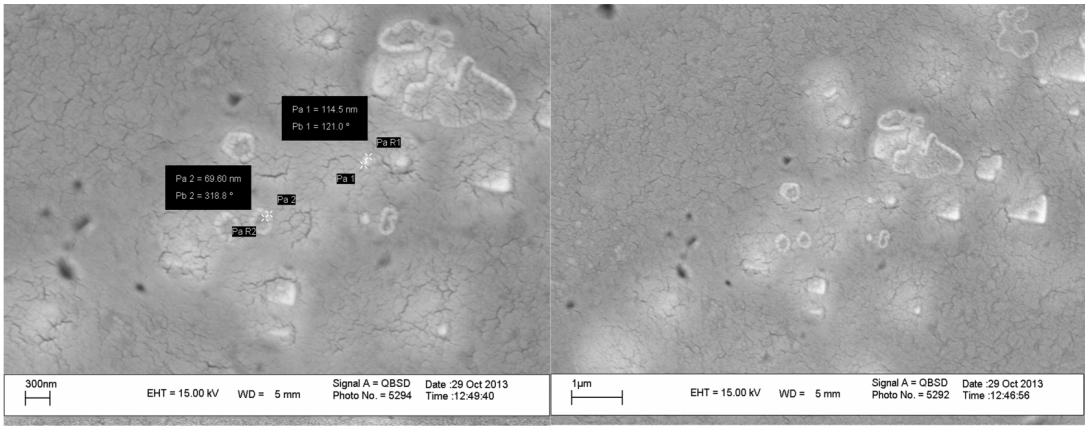

Fig 2. SEM images of prepared nanoliposome

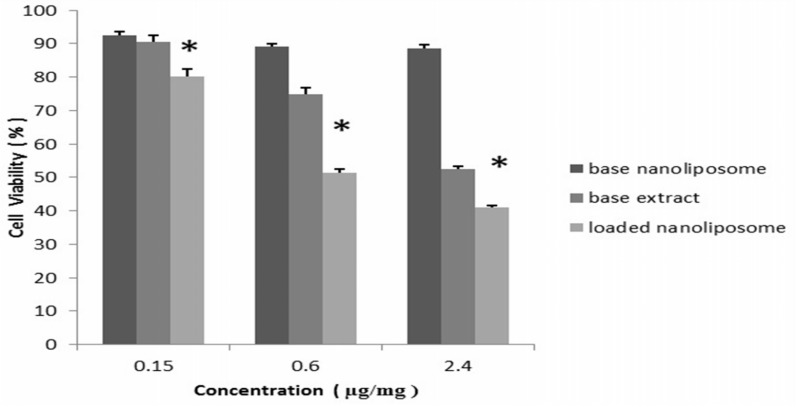

Fig. 3. MTT assay of nanoliposomal extract on AGS cell line after 24 h compared to base extract. Data are presented as mean ±SD. (n=3). (* p<0.0001 vs. base extract).

Table 2. IC50 values of base and nanoliposomal extract of E. elaterium on gastric cancer cell line after 24 h

Samples	MTT assay (µg/ml)	Neutral Red assay (µg/ml)	Frame assay (µg/ml)	Total IC_{50} (µg/ml)
Base extract (control)	0.98±0.08	1.08±0.15	0.94±0.10	1±0.11
Loaded nanoliposomes	0.39±0.006*	0.41±0.01**	0.38±0.01	0.39±0.02***

Data are presented as mean ± S.D. (n=3). *p <0.0002 vs. control **p<0.0003 vs. control ***p<0.0003 vs. control

ingredients of the extract to AGS cell line. The most important compounds of *Ecballium elaterium* that have cytotoxic effects are phenolic compounds. From this point of view, it could be postulated that liposomal encapsulation boosts phenol ability in enhancing the phenol entrance into the target cells. On the other hand, investigations have confirmed that encapsulation of bioactive ingredients in nanoliposomes may enhance their cellular uptake and offer some advantages such as high metabolic stability, high membrane permeability, improved bioavailability, and longer duration of action [23].

CONCLUSION

Nanoliposomal form of lyophilized extract of *Ecballium elaterium* was prepared and characterized by photon correlation spectroscopy and scanning electron microscopy. Results showed that the liposomes were in nano-sized scale with good stability and dispersion. Cytotoxic effects of extract-loaded liposomes on AGS cell line was determined by MTT, neutral red and frame assay and was shown to significantly be more than the extract alone.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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