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Original Research

Nanoliposomal formulation of *Agrostemma githago* aqueous extract shows enhanced cytotoxic effect on gastric cancer cell line

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Abstract

Objective(s): The objective of this study was to determine the cytotoxic effects of nanoliposomal form of lyophilized aqueous extract of *Agrostemma githago* (*A. githago*) seeds on gastric cancer cell line (AGS) using cell viability tests.
Materials and Methods: Lyophilized aqueous extract of *A. githago* seeds was prepared. Liposomes were also prepared by thin-film hydration method and their stability and size were characterized by SEM. The size and zeta potential were determined by Malvern Zetasizer. Cytotoxic effects of nanoliposomes on gastric cancer cell line was determined using MTT, Neutral Red and Frame methods.
Results: The size of liposomes was around 171.5 nm with proper dispersion (PDI=0.268). The morphology of the liposomes was suitable according to SEM images. The IC_{50} values indicated that the nanoliposomal form of extract was 3-4 times more active than extract alone. Average IC_{50} values for extract and liposomal form of extract were 13.02 ± 0.95 and 4.43 ± 1.49 ug/ml, respectively.
Conclusion: This study showed that liposomal form of aqueous extract of *A. githago* seeds exerts cytotoxic effect at significantly lower concentrations than the extract itself.

Keywords: *Agrostemma githago*, Cytotoxicity, Gastric cancer, Nanoliposome

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Introduction

Agrostemma githago L. also known as Corn Cockle which is usually found as weed in grain fields is known to be toxic for both human and animals (1, 2). Its seed extract contains saponins, agrostin, githagin, githagenin and agrostemmic acid (3, 4). Studies elsewhere have exhibited that this plant has cytotoxic activity (5) related to a triterpenoid saponin, githagenin and a ribosome-inactivating protein (RIP), agrostin (6, 7). Agrostin is a type 1 RIP and combination with saponin augment its cytotoxic activity (5). This plant also contains flavonoids with cytotoxic (8-10) and antioxidant effects (11-13). Liposomes are vesicles, having stable membranes composed of two layers, firstly were described by British hematologist Dr. Alec D Bangham FRS in 1961 (14) and because of their specific properties have become one of the most important drug carriers (15). Nanoliposomes are nanoscale liposomes that are highly interesting pharmacologically because of their small size, high stability and therefore good tendency for application as drug carrier systems and have widely used as carrier agents in drug delivery systems for cancer chemotherapy (16-18). It is known that nanoparticles have a high tendency to be trapped in the reticuloendothelial system, such as spleen, liver and lung (19).

Using this phenomenon, nanoliposomes could be targeted toward tumor tissues as a strategy for cancer therapy (20).

On the other hand, it is known that the size of liposome is highly related to the stability and encapsulated solute leakage. The smaller the size, the more the stability and less the encapsulated solute might leak out (21). Numerous studies have demonstrated that both hydrophilic and lyophilic chemicals can be encapsulated on liposomes because of their amphipathic characteristics. Encapsulation of plant or animal extracts on liposomes and their application for different purposes such as cancer therapy have been studied.

It was shown that liposomes have suitable ability for entrapment and carrying not only the pure substances, but also the whole extract of a plant or animal (22-24).

A. githago is used as traditional medicine by Turkish people (25) and also people of north west of Iran for treatment of some diseases. Although there are some studies about cytotoxicity of this plant extract (5), no study has been performed using liposome encapsulated extract. This plant is widely grown in Ardabil province, Iran. Therefore, in this study we investigated in vitro cytotoxic activity of nanoliposomal form of lyophilized extract of *A. githago* on AGS cell line.

Materials and Methods

Materials

All solvents and chemicals were of analytical grade. Lecithin from soybean type IV-SL-α-phosphatidylcholine (PC) was obtained from Sigma Chemical Co. Cholesterol and Rutin were purchased from Merck and used without any further treatment.

Plant materials

A. githago seeds from suburb area of Ardabil province, North West of Iran were used for preparation of extract. Seeds were collected during the local harvesting season when the seeds were at their best condition and complete ripeness.

Preparation of A. githago lyophilized extract

Collected seeds were dried and grinded to make fine powder. Definite amount of powder was added into the distilled water and thoroughly mixed and homogenized using an ultrasonic homogenizer (UP200H, Hielscher, Germany) in maximum sonication level for complete cycle of 15 min. Subsequently the mixture was centrifuged and supernatant was collected and allowed to freeze at −80 ^{o}C. Afterward, the frozen extract lyophilized by Freeze dryer (ALPHA 2-plus, Martin Christ, Germany) and the residue stored at −20 °C for later use.

Preparation of liposomes

The thin-film hydration method was used for preparation of nanoliposomes (26-28). Lecithin phospholipid (L-α-phosphatidyl-choline) was dissolved in chloroform to form the first solution. Cholesterol was also dissolved in chloroform to make the second solution. Tow solutions were combined and firmly mixed using a rotator in a final ratio of 4:1 (w/w), respectively. This mixture was evaporated in a rotary evaporator system (Heidolph Germany) connected with a vacuum system and was kept under the lipid transition temperature (T_C). Evaporation was performed under the atmosphere of nitrogen to prevent oxidation of phospholipids. This process was performed during 2 hours to remove whole solvents and the result was a thin film. The powder extract of plant was dissolved in distilled water and resultant solution was added to the film container. To improve the hydration process, glass beads (0.5 mm) were added and the mixture was stirred until the film was disappeared. For homogenizing of suspension and producing the nanoscale vesicles, obtained sample was sonicated for 15 minutes using ultrasonic homogenizer (UP200H, Hielischer, Germany). Homogenized suspension was taken under nitrogen atmosphere and was held under lipid transition temperature for 1 h. Then, product was centrifuged using a centrifuge (sigma 3- 30k, Germany) to yield a clear suspension of extract contained nanoliposomes and stored at 4°C until use.

Characterization of nanoliposomes

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

Determination of encapsulated amount of extract

A. githago extract contains some flavonoids that were used to determine the entrapment amount on nanoliposomes as an indicator for efficiency of whole extract entrapment.

UV–Vis absorption spectra on 415 nm at 25 $^{\circ}$C were measured using a spectrophotometer (PG-Instrument LTD, England) for determination of the total flavonoids loaded on nanoliposomes. Rutin was used as reference flavonoid (29).

The total entrapment amount of plant extract on nanoliposomes was calculated by following formula:

$$\text{Loaded Extract} = \frac{\text{flavoind}_{\text{total}} - \text{flavonoid}_{\text{remained}}}{\text{flavonoid}_{\text{total}}} \times 100$$

Cell culture

The human gastric cancer cell line, AGS, was provided from Pasteur Institute (Tehran, Iran). AGS cells were grown as monolayer culture at 37°C in RPMI-1640 medium (GIBCO, UK) containing 10% (v/v) fetal bovine serum (FBS; GIBCO, UK), pencillin (100 IU/ml) and streptomycin (100 µg/ml). Incubation was performed in a humidified atmosphere of 5% CO_2 in air until confluence. Cells were plated in 96-well plates at a density of 1×10^4 cells per well in 200 µl medium. After incubation for overnight, the medium was removed and cells were treated with a FBS-free medium supplemented with 10 mg/ml prepared extract-loaded nanoliposomes or nanoliposomes (without extract) and or extract (without nanoliposomes), by 1/4 serial dilutions in separate plates. Afterward, plates were incubated for 24 h.

Cytotoxicity studies by MTT assay

For performing the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide) assay. Four hours before completion of the incubation time, the medium of each well were replaced with 180 µl FBS-free medium and 20 µl of 2.5 mg/ml MTT (Merck, Germany). Finally, the medium were removed and 200 µl DMSO was added to each well. The plates were shaken gently for 10 min by a shaker and the absorbance was measured by a plate reader (Synergy HT, BioTek) at 570 nm. The wells without cells were treated as blank (30).

Neutral red assay

Neutral Red Uptake Assay was carried out as stated in our former study (31). Briefly, three hours before the end of the 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 ºC). Subsequently, fixative solution (150 µl) was added to each well and the absorbance was measured at 540 nm on the plate reader.

Protein content measurement

FRAME (Fund for the Replacement of Animals in Medical Experiments) method was used for the determination of protein content using the Comassie protein assay reagent as described previously (31). Briefly, after neutral red uptake assay, the

fixative solution was removed from each well and working stain solution (150 µl) was added to the wells and the plates were shaken for 20 min.

Then, the stain was removed and wash solution (250 µl) was added to each well. Wells were washed for two times and desorbing solution (150 µl, potassium acetate, 1M, in 70% ethanol) was placed into the wells. Plates were shaken on a shaker vigorously until a homogenous solution was observed. The absorbance was measured at 595 nm using the plate reader.

Statistical data analysis

Half Maximal Inhibitory Concentration (IC_{50}) values were calculated using Sigma Plot 11 software. Measurements were triplicate (n = 3 per experimental group) and differences among treatment groups were assessed by Student's t-test. Differences were considered significant at confidence limits of $p < 0.05$. Data values for the cell viability study are presented as mean ± standard deviation (SD).

Results and Discussion

Photon correlation spectroscopy

As it was shown in figure 1, zeta potential of prepared liposomes was -53 mV. Zeta potential value greater than 30 mV (negative or positive) is related with in vitro stability due to the electric repulsion between particles (32) and longer half-life of vesicles in intravascular system (16). Also, it was shown that negative value of zeta potential has a positive correlation with increased entrapment of water soluble pharmaceuticals, which is maximal at – 30 mv (33). The average size of liposomes was 171.5 nm (figure 2) which was considered nanoscale based on IUPAC definition (34). PDI value of prepared liposomes was 0.268 (figure 2). It has been stated elsewhere that PDI values less than 0.3 is indicative of a good monodispersity of vesicles (32).

Scanning electron microscopy (SEM)

Scanning electron micrographs of prepared liposomes exhibited the size of liposomes that are in nanoscale and this is in agreement with the results of Zetasizer. Also the morphology and size homogeneity of liposomes were in a range compatible with PDI value.

Entrapment efficiency

The loaded amount of extract in liposomes was 66%.

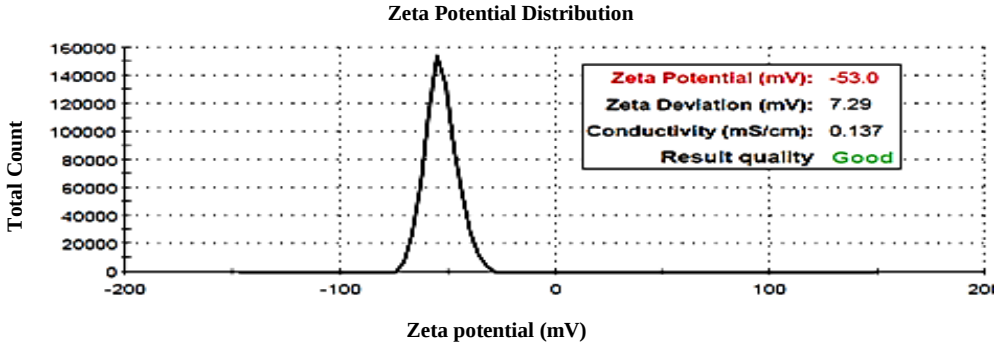

Figure 1. Zeta potential of encapsulated liposomes.

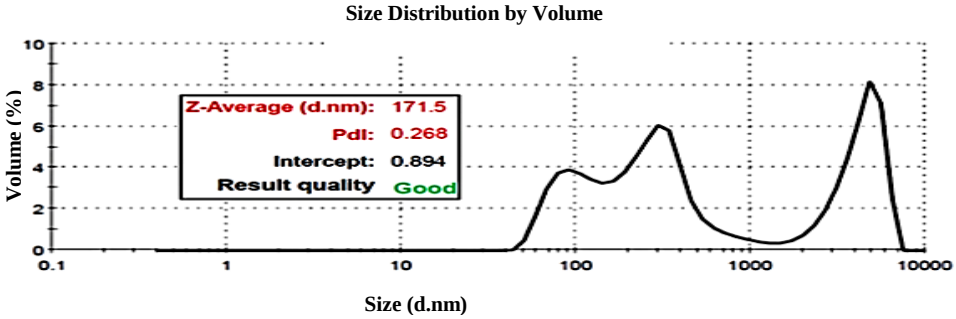

Figure 2. Size and PDI of encapsulated liposomes.

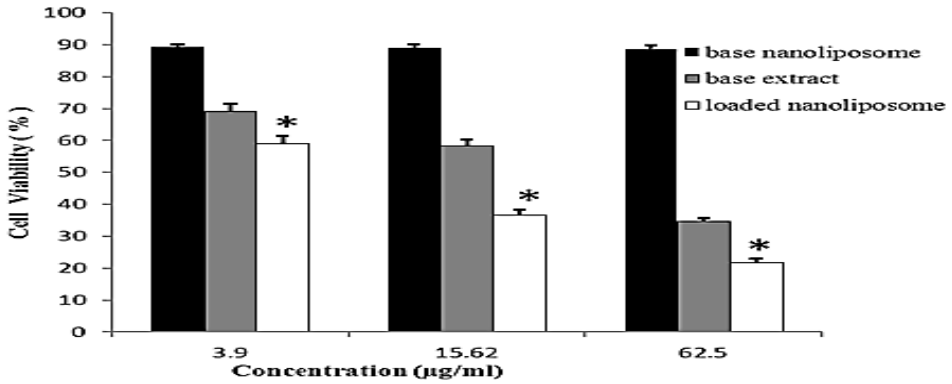

Figure 3. MTT assay of cytotoxic activity of nanoliposomal extract of *A. githago* on AGS cell line after 24 h compared to base extract. Data are presented as mean ± SD. (n=3). (* $p < 0.0001$ vs. base extract).

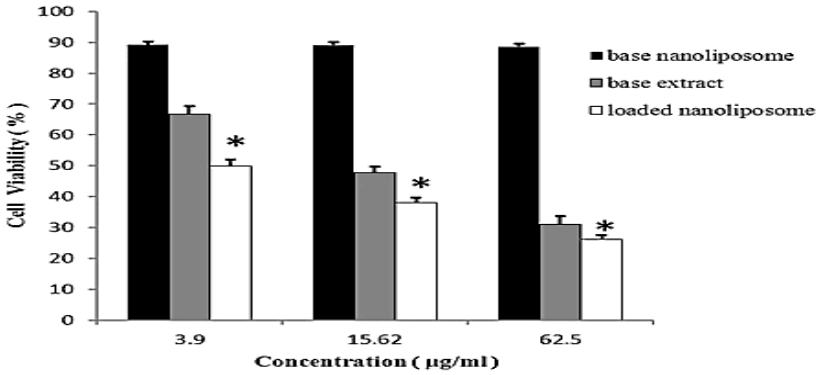

Figure 4. Neutral red assay of cytotoxic activity of nanoliposomal extract of A. githago on AGS cell line after 24 h compared to base extract. Data are presented as mean ± SD (n=3). (* $p < 0.0001$ vs. base extract).

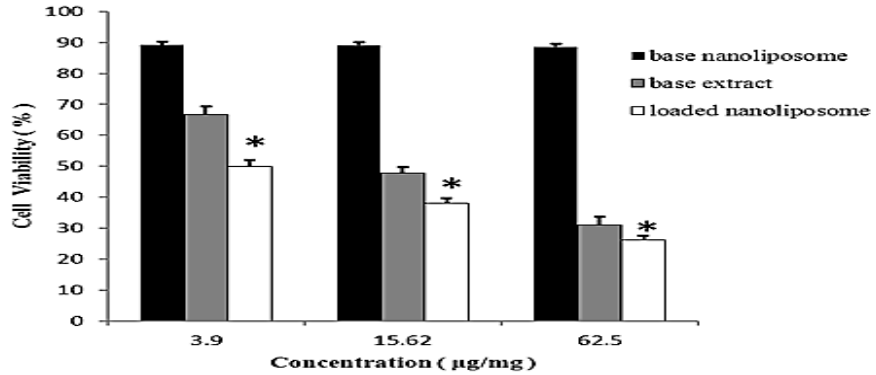

Figure 5. Frame assay of cytotoxic activity of nanoliposomal extract of *A. githago* on AGS cell line after 24 h compared to extract. Data are presented as mean ± SD (n=3). (* p < 0.0002 vs. base extract).

This value is assumed satisfactory when compared to previously published data on liposomal drug delivery systems of compounds from herbal origin (35).

Cytotoxic study

The results of viability studies are shown in figures 3-5. The results demonstrate that encapsulated *A. githago* extract shows significant cytotoxic effect in comparison with the base extract. It could be concluded that the cytotoxic effect of liposomal form of *A. githago* extract on AGS cell line was 3 to 4 times more than the free extract. During the experiments the liposomes without extract were used as control.Table 1 shows the IC50 values calculated from MTT, neutral red and frame methods after 24 h incubation.

By comparing the IC50 values for extratct and encapsulated extract, it seems that the prepared nanoliposomes are efficient for delivering the active ingredients of the extract to AGS cell line. The two most important compounds of *A. githago* that have cytotoxic effect are agrostin and agrostemma saponin 1 (githagenin). It is known that when these two compounds are used in combination, the toxicity effect enhances by 10,000 fold over that of each (5). The role of saponin is to facilitate the penetration of the non-permeable agrostin through the cell membrane (36). From this point of view, it could be postulated that liposomal encapsulation boosts saponin ability in enhancing the agrostin entrance into the target cells.

Table 1. IC_{50} values of base and nanoliposomal extract of *A. githago* 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)	13.26 ± 1.31	15.29 ± 0.94	10.53 ± 0.61	13.02 ± 0.95
Loaded nanoliposomes	4.3 ± 0.64*	5.6 ± 0.59**	3.41 ± 0.79***	4.43 ± 1.49

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

On the other hand, investigations 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 (37).

Conclusion

Nanoliposomal form of lyophilized extract of *A. githago* was prepared and characterized by photon correlation spectroscopy and scanning electron microscopy. Results showed that the liposomes were in nanosized scale with good stability and dispersion. Cytotoxic effect of liposomes on AGS cell line which was determined by MTT, Neutral Red and Frame assays was significantly more than the extract.

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