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Research Article
Comparison Between Effects of Free and Niosomal Formulations of *Artemisia annua* L. (Asteraceae) on Chronic Myelogenous Leukemia (K562) Cell Line

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Abstract

Objective: *Artemisia annua* L. (Asteraceae) (artemisinin) has special antitumor activity against melanoma, breast, ovarian, prostate, central nervous system and renal cancer cell lines. The present study was conducted to compare effects of free and niosomal formulation of artemisinin on Chronic Myelogenous Leukemia (CML) using K562 cell line. **Materials and Methods:** Different concentrations of artemisinin were prepared using spectrophotometer in 195 nm wavelength. Two different methods of reverse phase evaporation and ether injection were adopted to prepare the niosomes formulations. Encapsulation, *in vitro* release, *in vitro* cytotoxicity determination and the K562 cells viability rates were assessed. **Results:** The percentage of encapsulation for niosome formulation obtained with reverse phase evaporation and ether injection methods were 87.27±0.93 and 74.9±4.2, respectively. The rate of the artemisinin release across the dialysis membrane was significantly slower for artemisinin-loaded niosomes than that of the free artemisinin ($p<0.05$). The percentage of K562 cells apoptosis in free artemisinin was significantly higher than that of the niosomal formulations ($p<0.05$). The niosomal formulations showed significantly more influence than that of the free artemisinin ($p<0.05$). **Conclusion:** The use of niosomal formulation of artemisinin could effectively improve its therapeutic index, release rate and apoptosis influence.

Key words: Artemisinin, drug delivery system, niosome, encapsulation, CML (K562) cell line

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Artemisia annua L. (Asteraceae) has been used throughout the ages to treat various ailments, specifically those related to the treatment and prevention of fevers which we now relate to malarial infections[1]. In ancient China, symptoms relating to *Plasmodium falciparum* infections were effectively treated with the *Artemisia* plant[2]. Because of its strong cytotoxic effects, it was reported that artemisinin has special antitumor activity against melanoma, breast, ovarian, prostate, central nervous system and renal cancer cell lines[3,4].

The structure of artemisinin includes lactone peroxide group. The adjacency of peroxides to high concentrated iron (like cancer cells) leads to release active oxygen species as well as to make the molecule instable. Due to wide receptor area against iron in cancerous cells, these cells would be degraded and result in iron deficiency[5,6]. It has been proved that artemisinin derivatives show anti-cancer effects on P388 cells (mice blood cancer) and A549 (human lung cancer)[7].

Besides the promising clinical results, the utility of artemisinin is limited in the biological systems due to its toxicity and very low solubility both in aqueous media and oils. Artemisinin has an initial burst effect and high peak plasma concentration but it metabolizes quickly *in vivo*[8]. It is unstable and mostly by the opening of the lactone ring is easily degraded[9]. Recently, it has been reported that its encapsulation into conventional liposomes is a reasonable method to prolong the circulating time of artemisinin in blood plasma and to enhance its half-life[7]. A drug delivery system has been introduced using Span 80, cholesterol and FU-5 (2:5:2 molar ratio), which is used mostly in the treatment of skin cancer[10,11].

Niosomes, non-ionic surfactant vesicles are now widely studied as an alternative to liposomes, because they alleviate the disadvantages associated with liposomes like chemical instability, variable purity of phospholipids and high cost[12,13]. Niosomes can be prepared by the same procedure as of liposomes. Most methods require large amount of organic solvent that are toxic to human and environments and have multistep. Most preparation methods even without using organic solvents such as heating method and polyol dilution method there are problems of using high temperature that is not suitable for heat labile substances. Thus preparation of niosomes using safer components and easier methods that have the least toxic effect on normal cells have always been considered[14,15].

Designing a suitable dose of medicine with increasing its influence in anticipated zone, decreasing drugs effects on non-target tissues for holding a stable state in blood and extending the effective time in target tissue are the main goals in drug delivery system[16]. Niosomes have some advantages over the other drug delivery systems like liposomes, including simpler preparation methods, low cost, structural flexibility and lower toxicity due to their non-ionic form. Moreover, niosomes are bio-consistent, biodegradable and non-immunogenic, while they can be prepared in a size range of 10-100 nm. Niosomes may trap wider range of drugs including hydrophilic and lipophilic ones[10].

Blood cancer or leukemia is a developmental and malign disease of body hematopoietic tissue. This disease is caused by incomplete proliferation and development of white blood cells and their progenitors in blood and bone marrow[17]. It is one of the four most common cancers among children[18]. The leukemic cells influence the other blood cells like red cells and platelets produced in bone marrow[19]. The chronic myeloid leukemia is one of the well-known types of blood cancer which is created due to displacement of "*Iba*" gene and "*bcr*" gene located on chromosome number 22 in stem cells[20]. The cell strain K562 is used as a model to study CML. The aim to treat CML is to retain the patient in chronic phase and to help prevent the disease to proceed to the next phases as well as to reduce the toxicity of common medications. Through some reactions, the oxygen free radicals including super oxides, hydroxyls and peroxyls are continuously produced in the body. Oxidative degradation caused by these molecules activity lead to chronic diseases including cancer[20].

The present study was conducted to compare effects of free and niosomal formulation of *Artemisia annua* L. (Asteraceae) on CML using K562 cell line.

MATERIALS AND METHODS

Preparation of standard concentrations of artemisinin: Different concentrations of artemisinin (Fluka, Sigma-Aldrich St., Louis, MO, USA) (0.312-0.5 mg mL^{-1}) were prepared using spectrophotometer XS Eave Power in 195 nm wavelength. Then, the standardization curve was drawn to determine unknown concentrations of medicine obtained by releasing pharm program.

Preparation of niosomes: Two different methods of reverse phase evaporation and ether injection were adopted to prepare the niosomes formulations. Surfactant, cholesterol and different concentrations of the artemisinin were utilized. In reverse phase evaporation method diverse portions of surfactants span 60, span 20, cholesterol and medicine were dissolved in ethanol and then solvent phase was separated by rotary evaporator (50°C, 90 rpm). The remaining agar was

hydrated by phosphate buffer and magnetic stirrer. To homogenize particles, the suspension was sonicated (35 W power, 5 min plus 992). In ether injection method, after dissolving different portions of span 60, span 20, cholesterol and drug in di-ethyl-ether, the resulted solution was dripped into saline buffer (10 mM, pH 7.4) on a magnetic stirrer (50°C, 300 rpm)[21-23].

Assessment of encapsulation rate: The encapsulation rate was assessed indirectly using a centrifuge (17000 g, 60 min and 4°C). A certain amount of niosome formulation was centrifuged and obtained sediment was washed two times by Phosphate Buffered Saline (PBS). Then, free artemisinin in supernatant was measured and encapsulation yield was calculated as follows:

$$\text{Encapsulation yield} = \frac{\text{Amount of trapped drug}}{\text{Total amount of drug}} \times 100$$

***In vitro* release study:** The *in vitro* release studies of artemisinin from vesicles were performed using dynamical diffusion via dialysis membrane. Two milliliter of all formulations were poured into a dialysis tube and then the tube was located in 25 mL PBS (10 mM, pH 7.4) and stirred with a magnetic stirrer (37°C, 32 h 300 rpm). Five milliliter aliquots of dialysate were taken at predetermined time and replaced immediately with the same volume of fresh PBS and withdrawn samples were assayed spectrophotometrically at 195 nm. The release pattern of niosomal formulations and free solution of artemisinin were compared to each other.

Determination of *in vitro* cytotoxicity: The cytotoxicity of niosomal formulations of artemisinin was measured by MTT[(3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl-tetrazoliumbromide blue-indicator dye]-based assay and compared to free artemisinin in CML (K562) cell lines[24]. The K562 cells were seeded at dilution of 2×104 well^{-1} in RPMI supplemented with 10% FBS, 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin, at 37°C in a humid atmosphere containing 5% CO_2 for 24 h . After that, the medium replaced with fresh medium containing the formulations at different concentrations. After 72 h, all media were then removed and 100 µL of MTT solution (0.5 mg mL^{-1} in RPMI) were added to the wells. The cells were incubated for 3 h. The MTT was removed and 100 µL of DMSO was added to dissolve the formazan crystals. The optical density at 492 nm was read using a BioTek ELISA reader. Untreated cells and doxorubicin (0.1 mM) were used as controls. All experiments were carried out for 3 times and in triplicate[25].

Viability of the K562 cells: The K562 cells were stained with acridine orange and propidium iodide (Sigma-Aldrich). Survival rate was quantitatively assessed using fluorescent microscope in 5 per high power fields ($\times400$)[26].

Statistical analysis: Statistical tests were performed with the SPSS software (IBM, SPSS, Statistics 19). Results are expressed as Mean±Standard Deviation. Statistically significant difference was determined using the Student's t-test and analysis of variance (ANOVA). A value of $p<0.05$ was considered to be statistically significant.

RESULTS

Assessment of encapsulation rate: The encapsulation percentage is an important factor in drug delivery systems. This is especially true for expensive drugs. This rate for all formulations of the artemisininis shown in Fig. 1. The percentage of encapsulation for noisome formulation obtained with reverse phase evaporation and ether injection methods were 87.27±0.93 and 74.9±4.2, respectively.

***In vitro* release study:** The release studies of niosomal formulations of artemisinin were performed using dialysis tube containing 2 mL of artemisinin-loaded niosomes dispersion placed into a flask containing 100 mL PBS. Collected samples in predetermined times, were analyzed by spectrophotometer at 195 nm. The rate of the artemisinin release across the dialysis membrane was significantly slower for artemisinin-loaded niosomes than that of the free artemisinin ($p<0.05$). The in vitro release rate values are shown in Fig. 2.

Determination of *in vitro* cytotoxicity: The *in vitro* cytotoxicity assays are widely used to screen the cytotoxic

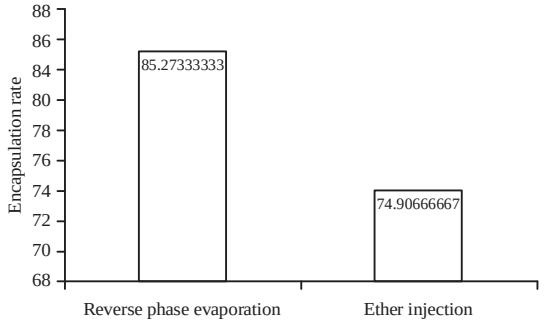

Fig. 1: Percentage of encapsulation of niosomal formulations of artemisinin prepared with both reverse phase evaporation and ether injection methods

activity of a compound. The CML (K562) cell lines were evaluated for cytotoxicity assay using MTT test. Based on the findings of the present study, the niosomal formulations showed significantly more influence than that of the free artemisinin ($p<0.05$) (Fig. 3).

Viability of the K562 cells: The findings of the present study showed that the percentage of K562 cells apoptosis in free artemisinin was significantly higher than that of the niosomal formulations ($p<0.05$) (Fig. 4, 5).

DISCUSSION

The membrane structure and layer number of vesicles are very important for the size, stability and application of the vesicles. Tween 60 has a large hydrophilic head group that is not soluble in water and form order bilayer aggregation by hydrogen binding and hydrophilic action, however, cannot form vesicles alone. Span 60 contains long hydrophobic group and is hardly soluble in water, however can interaction with artemisinin and form vesicles alone because of long alkyl chain. The balance between hydrophilic and hydrophobic moiety of mixed surfactants are important factors for high entrapment efficiency[27].

Increase the amount of span 60 leads to high entrapment efficiency and stability of the vesicles. This is due to its stronger hydrophobicity of Span 60 that can cause more rigidity in vesicle wall. In the present study the niosomal formulation of artemisinin was successfully prepared by a simple method and characterization of the niosomal

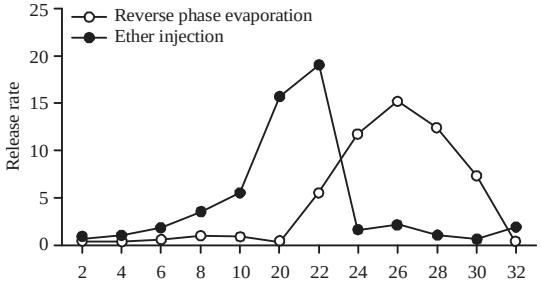

Fig. 2: Line graph shows comparison of drug release rate between free artemisinin and niosomal formulations over 32 h using dialysis tube method. The rate of the artemisinin release across the dialysis membrane was significantly slower for artemisinin-loaded niosomes than that of the free artemisinin ($p<0.05$)

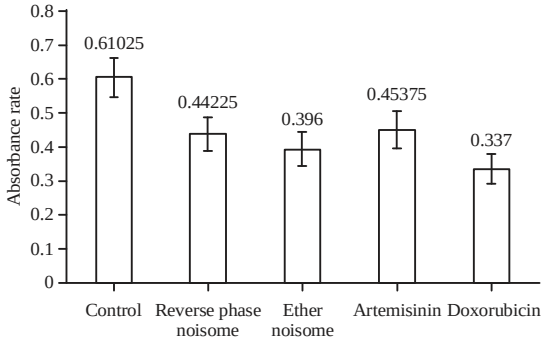

Fig. 3: Bar graph shows cytotoxic activity of niosomal formulations of artemisinin against MCL (K562) cell lines in both reverse phase evaporation and ether injection methods. Doxorubicin was used as control. The percentage of K562 cells apoptosis in free artemisinin was significantly higher than that of the niosomal formulations ($p<0.05$)

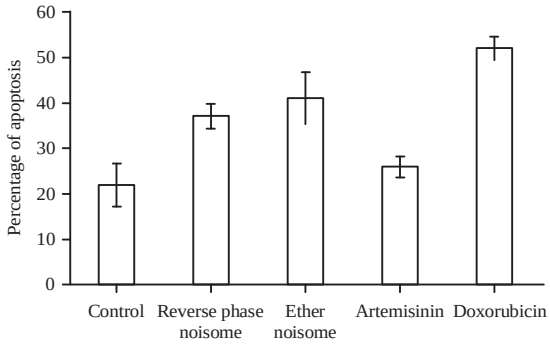

Fig. 4: Rate of apoptosis among K562 cells after treating with artemizin and encapsulated artemizin with two preparation methods. The niosomal formulations showed significantly more influence than that of the free artemisinin ($p<0.05$)

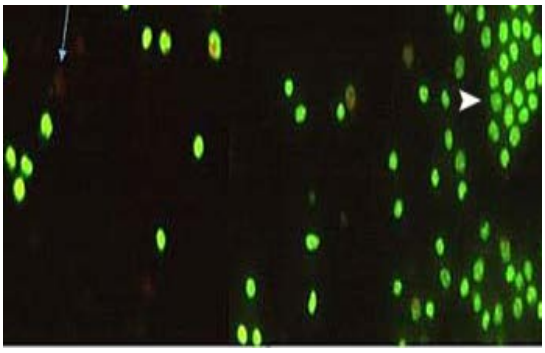

Fig. 5: Photomicrograph shows K562 cells stained with acridine orange and propidium iodide using fluorescent microscope. Apoptotic cells are stained brown (arrow) and normal cells are stained bright green (arrowhead) (×400)

formulation indicated that this vesicular carrier could be used as a potential delivery system.

Because of smaller size, higher stability and stealth mode of niosomes they have long blood circulation half-life in the body[28]. The particle size was decreased with increase in span 60 content, which was due to the critical packing parameter of span 60. Because of its strong cytotoxic activity, it was reported that artemisinin bears potential antitumor activity against many cancers[29]. For evaluation of antitumor activity of the ART-loaded niosomes, its cytotoxicity was evaluated on a model of cancer cell lines of CML (K562). The CML is defined as a clonal myelo proliferative disorder, which is derived from oncogenic conversion of hematopoietic stem cells[30]. The philadelphia (Ph) chromosome which functions because of areciprocal translocation fusing the 5′ sequence of the *bcr* gene with the upstream exon 2 sequence of the c-abl proto-oncogene on chromosome 22 is detected in fundamentally all cases of CML. The fusion production, named BCR-ABL is a protein (210 kDa) that it is larger than the normal Abl protein (160 kDa) and in which the tyrosine kinaseis constitutively active[31]. The chimeric BCR-ABL oncoprotein, which has tyrosine kinase activity and involves nuclear import and export signals is the main molecular signal of CML. The IC_{50} is an index to express the effectiveness of a compound in inhibiting biological or biochemical function. It is the half maximal (50%) inhibitory concentration of a substance[32]. This index is obtained by MTT assay. The MTT results indicated that artemisinin-loaded niosomes bear more antitumor activity (lower IC_{50} values) on the cancer cell lines compared to the free form.

Artemisinin is a natural compound and has lesser side effects compared to chemical drugs. Other terpenoids and flavonoids present in this plant have also cytotoxic effects. Artemisinin easily is removed by macrophages, digested by lipases and metabolized by liver with decreased blood circulation half-life. It has been suggested that artemisinin 0.02% decreased the cancer rate in comparison with positive control group in mice affected by cancer[33]. Peroxides present in artemisinin release active oxygen species and receptors capture iron so, the cancerous cells are destroyed. Studies have suggested that artemisinin may defeat cancer cells by itself while induce no undesired effect on normal cells[34]. Our findings showed that higher encapsulation rate for niosome preparation was obtained by reverse phase evaporation method compared to ether injection method.

In recent years, it has increasingly been recognized that malignancy may not exclusively result from enhanced cell proliferation but also from decreased physiological cell death, i.e., apoptosis[35]. Apoptotic induction has been a new target for innovative mechanism-based drug discovery[33,34]. Chemoprevention, a relatively new strategy to prevent cancer, depends on the use of nontoxic chemical substances, to block, reverse or retard the process of carcinogenesis. Plant-based diet is regarded one of the potential chemopreventiveagents[35,36]. If a plant-derived extract induces apoptosis and has anti-proliferative and antioxidant effects, it might protect normal cells from the damage caused by reactive oxygen species while inducing apoptosis and inhibiting proliferation in tumor cells. The findings of the present study on apoptosis confirmed this.

Niosomes could be a promising and economical carrier in topical drug delivery[37]. They are osmotically active and stable against chemical degradation or oxidation, therefore, enhance the stability of the entrapped drug[38]. Their handling and storage of surfactants do not require any special conditions and can be used for oral, parenteral as well topical[39]. The surfactants are biodegradable, biocompatible and non-immunogenic that improve the therapeutic performance of the drug by protecting it from the biological environment and restricting effects to target cells, thereby reducing the clearance of the drug[10,40]. Furthermore, controlling composition, size, lamellarity, stability and surface charge of niosomes is feasible using the type of preparation method, surfactant, cholesterol content, surface charge additives and suspension concentration[41,42]. Niosomes have longer shelf life and are able to deliver medication at sustained or controlled pattern. Outcomes of a study revealed that doxorubicin loaded magneto-niosomesexhibited high stability, controlled release and 25% decrease of K562 cell line viability in 24 h compared to drug alone[43,44]. Using nanocarrierslike niosomesto deliver atremisin, low bioavailability, stability and solubility problems of the drug can be solved and the efficiency of it can be enhanced. As in one study, MTT assay showed better cytotoxic effect. Moreover, stability and release study indicated that the optimized formulation has the improved therapeutic index, stability and solubility[27,43]. The niosomal dispersions in an aqueous phase can beemulsified in a non-aqueous phase to control therelease rate of the drug and administer normal vesiclesin external non-aqueous phase[11]. However, few disadvantages have been reported for the niosomes including their physical instability, aggregation, fusion, leaking of entrapped drug and hydrolysis of encapsulated drugs with limiting the shelf life of the dispersion[45-47]. Moreover, since heat sterilization and membrance filtration are unsuitable for niosomes, much effort is needed for niosomes sterilization process[41].

Niosomal formulation of artemisinin could enhance drug therapeutic parameters and lower its dosage. Thus,

the side effects of medication are decreased and the treatment could be more affordable.

CONCLUSION

Our findings showed that preparation of noisome using a mixture of non-ionic surfactants and a simple method can provide stable and small vesicles in nano dimensions. The use of niosomal formulation of artemisinin could effectively improve its therapeutic index, release rate and apoptosis influence. These vesicles can be a suitable carrier for delivery of a very range of hydrophobic, hydrophilic or amphiphilic molecules.

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