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Original Article

Physicochemical Characterization and Clinical Evaluation of Final Formulation of Shallomin Liposomal Gel on Cold Sore

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Abstract

Acyclovir is used locally or systemically to treat cold sore and totally have no side effect. However, this antiviral drug is not suitable for some people. The main aim of this study was to prepare and characterize shallomin liposomal hydrogel formulation and evaluation and comparison of dermal efficacy of this new formulation with acyclovir ointment on cold sores. Thin-Film Hydration was used to prepare liposome consisting of lecithin and cholesterol (molar ratio: 1:1). Morphology, size analysis and liposome encapsulation efficiency were determined. For preparation of hydrogel, Hydroxyethyl cellulose (HEC) was used. *In vitro* Skin permeation assay through abdominal region skin of male wistar rat was also determined. After preparing liposomal shallomin 1% in hydrogel a randomized controlled trial was performed on three groups (15 students in each groups) who showed cold sore within 24 hours. Liposomal shallomin gel, acyclovir and placebo was used every 6 hours for the first, second and third groups, respectively. The size of liposomal shallomin particles were 139±31.8 nm whereas polydispersity index values were 0.219 ± 0.01. The TEM images showed that the shape of particles was spherical and any aggregation or fusion were not seen. Furthermore, the particle size diameter was < 150 nm. The encapsulation capacity with the liposomes was approximately calculated 82.7%. The cold sore and tingling was disappeared in liposomal shallomin gel treated group within 0-12 hours in 12 cases (80%) and within 12-24 hours in 2 cases. In the acyclovir treated group, skin was cleared from cold sore in 8 cases (53%) within 24-48 hours after using drug. Regarding the obtained results, the liposomal shallomin gel caused a significant improvement in the removing cold sores within 12 hours related to acyclovir (P=0.001). Because mucoidal properties of hydrogel can facilitate adhesion between the shallomin and skin membrane, which extend keeping of shallomin at the site of administration and enhancing drug permeation so, liposomal shallomin gel is more effective treatment than shallomin extract and acyclovir with fewer side effects for cold sores treatment.

Keywords: Cold sore, Acyclovir, Shallomin, Moosir, Liposome, Hydrogel

Introduction

Herpes simplex virus (HSV) type 1 (HSV-1) can transmit via direct exposure to contaminated aerosols or droplets through kissing, sharing toothbrushes or eating utensils in the presence of active cutaneous blister. At the moment, several antiviral drug such as acyclovir and its derivatives (penciclovir, famciclovir, and valacyclovir) are used to treat herpes infection, which can easily reduce severity of sign and spread of lesions. Recently, there have been

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some reports of rising resistant strains against antiviral drugs especially in patients with herpes- HIV coinfection [1]. Although acyclovir is generally well-tolerated, in some researches this antiviral drug has not been recommended in pregnancy [2] and has been reported to be associated with severe nephrotoxicity [3]. Given these facts, effort for discovering new sources of antiviral agents appears to be necessary. One of the most novels strategies for developing new antiviral drugs is the use of antiviral peptides and alternative medicine drugs such as Echinacea, L-Lysine, Zn and aloevera and bee products [4].

The *Allium hirtifolium* Boiss. (Persian shallot, also named shallomin) belongs to Alliaceae family and is one of the important eatable alliums in Iran [5]. Our previous research showed antibacterial activity of shallomin against gram positive and gram negative bacteria with a minimum inhibitory concentration (MIC) ranging from 5 to 40 µg/mL [6]. Antifungal activity of shallomin against yeast and fungus (the MIC value rang: 0.15 to 20 µg/mL) [7], as well as anti-protozoal activity (8-11) were previously revealed. Also, beneficial effects of shallomin on many disorders from colds and flu to cancer and inflammatory diseases should not be ignored (12-17). Antiviral effect of 5% shallomin alcoholic solution within the first 24 hours after appearance of blisters can prevent the progression of cold sores and reduce the period of ulceration [18]. The purpose of this study was to investigate different formulations of liposomal shallomin gel to find out one having the highest efficacy in treatment of cold sore.

Material and Methods

Ethical Consideration

The double-blind, randomized study was approved by the Ethics Committee of the Iranian Registry of Clinical Trials (IRCT ID: IRCT20200819048457N1).

Shallomin Extract Preparation

Fresh Shallomin bulbs were collected at spring season from Zagros Mountain at Shiraz city, south of Iran. After confirmation by Medicinal Plant Research Center of Ahvaz Jundishapur University of Medical Sciences, five hundred grams of shallomin bulbs were washed and crushed into very small pieces using electrical grinder. Finally, 500 ml distilled water was added to crushed shallomin for 24 hour followed by filtering using Wattman No. 1. Fifty percent (50%) of ethyl acetate/water (v/v) mixture was added to the filtrated solution for another 24 hours. Thereafter, the aqueous extract was collected and the ethyl acetate fraction was separated using solvent. The ethyl acetate fraction was evaporated under a hood. The precipitate was collected and stored at 4 ℃ before use.

Liposome Synthesis using Thin-Film Hydration method

The thin film method was used to prepare liposomes. Briefly soya lecithin and cholesterol (molar ratio: 1:1) were liquated in chloroform-methanol (2:1) and shallomin was added, then the mixture was allowed to evaporate in a rotary evaporator. The thin film formed in the round-bottomed flask was hydrated with phosphate buffer and, then, the suspension was vortexed for 30 min. The liposomal suspension was centrifuged at 20000 rpm for 30 min. The supernatant was analyzed at 296 nm using spectrophotometer.

Determination of Liposome Encapsulation Efficiency

The percentage of the encapsulated liposome was determined after lysis of the prepared liposomes with absolute alcohol and sonication for 20 minutes [19]. The concentration of shallomin in absolute alcohol was spectrophotometrically determined at 265 nm using a UV-visible spectrophotometer (model UV-1700 (E); Schimadzu, Kyoto, Japan) in triplicate. Efficiency of encapsulation was considered as entrapment percentage and calculated through the following relationship [20]

Encapsulation efficiency % =Total drug−free drug/Total drug ×100.

Morphology and Size Analysis of Liposomes

The physical morphology of shallomin liposomes composed of lecithin and cholesterol in the 1:1 molar ratio were analyzed using a transmission electron microscope (TEM) (EM 10; Zeiss, Oberkochen, Germany). A drop of shallomin liposome was spilled on a 300-mesh collodion copper grid on paraffin and incubated for 10 min to adhere the shallomin liposome to collodion grids. The remaining non-adherent solution was removed, and treatment with 50 µl of 2% aqueous solution of uranyl acetate was performed for 3 min. Prior to size measurement with TEM, the remaining solution was washed and the grids were air-dried.

The mean particle size was determined by laser diffraction particle size analysis (Malvern Instruments Ltd., Malvern, UK). Furthermore, the size and polydispersity index (PI) of liposomes were measured by Zetasizer Nano ZS (ZEN 3600, Malvern Instruments Ltd., Malvern, Worcestershire, UK) as previously described (21-22).

Liposomal Shallomin Gel Preparation

Hydroxyethyl cellulose (HEC) was used as a key ingredient for making gel. Five gram (5 gr) of HEC was added to 100 ml deionized water containing 0.2% methylparaben and propylparaben (3:1) as preservative agents and mixed by stirrer (250 rpm) until appearing a transparent gel (15 min). Finally, the triethanol amin (10µl) was added to maintain the transparency of the gel. Three formulations including liposomal shallomin in

hydrogel, shallomin extract in hydrogel and the hydrogel base as control were developed.

Skin Permeation Assay

Skin permeation assessment was performed using Keshary-Chien Franz diffusion cells (Rama Scientific works, Delhi, India). Briefly, abdominal region skin of male wistar rat after removing hairs was washed by distilled water and then adhering fat was removed with isopropyl alcohol. The cleaned skin was placed between donor and receptor phase in diffusion cells so that the stratum corneum of skin was in connection with receptor phase. The receptor compartments were filled with 5 grams of each formulation (including shallomin plain, shallomin in hydrogel, liposomal shallomin in hydrogel and hydrogel plain) and diffusion cells were placed in a water bath at 37°C. To create homogeneity in donor phase fluid was stirred at a speed of 200 rpm. For a period of 24 hours at different and regular time intervals (0, 0.5, 1, 2, 3, 4, 5, and 24 h) 3 ml of aliquot was collected from the sampling arm from the donor comportment and replaced immediately with an equal volume of ethanol equilibrated at 37°C. The absorption rate of the samples was measured at λ max 254 nm using UV spectrophotometer (Beckman DU640 UV/Vis spectrophotometer, USA). The cumulative amounts of every formulation permeability in $\mu g/cm^2$ and the percentage of cumulative amounts at time intervals after 6h were calculated.

Clinical Evaluation

The double-blind, randomized study was approved by the Iranian Registry of Clinical Trials. A total of 45 participants after obtaining the informed consent were recruited in each arm of intervention and during the study were examined in department of microbiology or dermatology of Ahvaz Jundishapur University of Medical Sciences. Participants were male and female students of mentioned university who were examined by dermatologist and cold sore had been diagnosed for them. All the volunteers were shown cold sores within 24 hours before checkup. Participants were randomly divided to three groups each included 15 students. Two groups were randomly chosen as treatment groups (first group were exposed to treatment with acyclovir and second group treated with liposomal shallomin gel) and third group was selected as control group (treated with liposomal gel whiteout shallomin). The subjects in both treatment groups were asked to use the administered medicine on the cold sore every hour until the tingling sensation and sores healed. Vehicle (only liposomal gel) was prepared in similar looking containers and was administered for the control group and asked them to apply it hourly on sores. Dermatologist examined the sores in both treatment groups during 12, 24, 48 and 72 hours after initiation of intervention until cold sores healed.

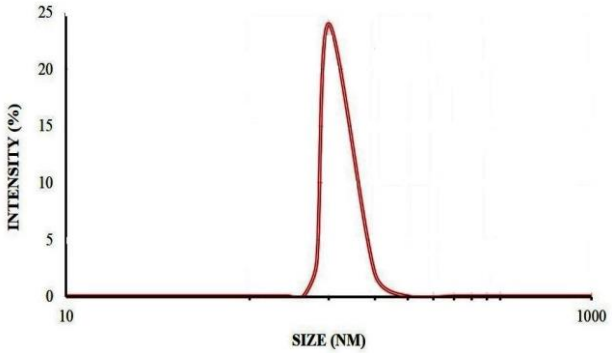

Fig. 1 Particle size distribution of shallomin liposomal gel

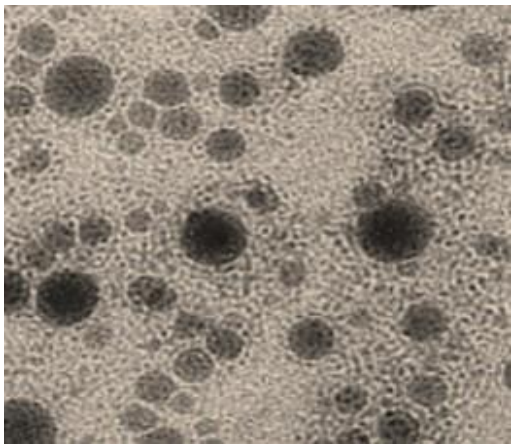

Fig. 2 TEM image of shallomin liposomal

Statistical Analysis

To determine the level of significance ($p < 0.05$), statistical analyses were carried out using Chi-square and Fisher's exact test followed by SPSS software version 22.0 (IBM Corporation, Armonk, NY, USA).

Results

As shown in figure 1, the size of liposomal shallomin particles were 139±31.8 nm whereas polydispersity index values were 0.219±0.01. The TEM images showed that the shape of particles was spherical and any aggregation or fusion were not seen. Furthermore, the particle size diameter was < 150 nm (Fig. 2). Besides the shape and size of particles, the surface charge of them is another the most important feature for their biological activities. Zeta potential values of liposome particles were approximately -44.2 mV whereas the surface charge of liposome contained shallomin extract were more increased than liposome, -59.6 mV, which the reason of this negative charge increasing could be caused by the shallomin interaction with lipid bilayers (Fig. 3).

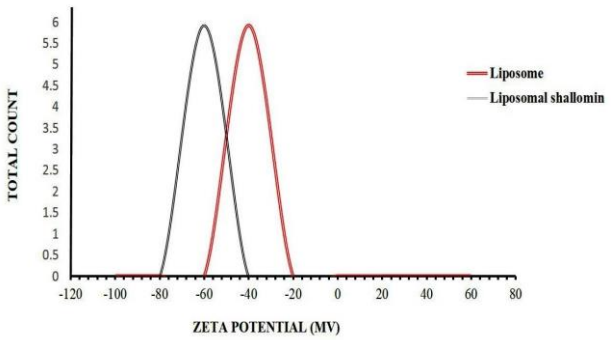

Fig. 3 Surface charge of shallomin liposomal

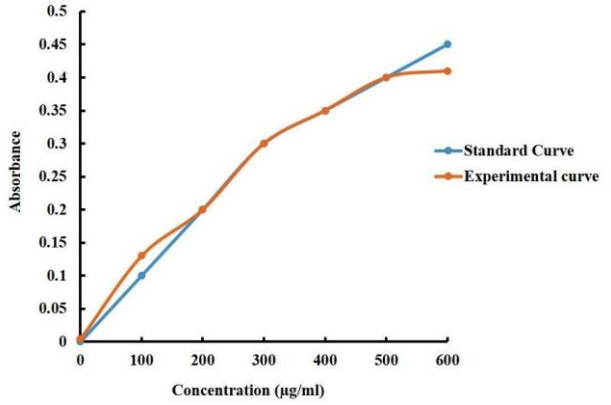

Fig. 4 The absorbance level at various concentrations ofshallomin at 296nm.

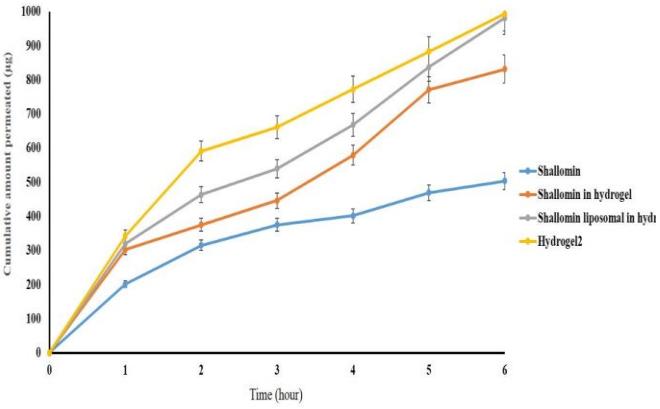

Fig. 5 Skin permeation ability of shallomin in different formulations

Evaluation of Liposome Size and Encapsulation Efficacy

The absorbance level at various concentrations of shallomin was measured by a UV-visible spectrophotometer at 296 nm. The standard curve of the shallomin was drawn by plotting absorbance versus the concentration (Fig. 4). Based on this graph, the absorbance is no linear at concentration between 10 to 200 µg/ml and 500–600 µg/ml. The concentration of the free drug in the supernatant was measured by this curve. So that, the amount of encapsulation of liposomes entrapping shallomin was easily calculated. The amount of encapsulation was determined as the mass ratio of the shallomin encapsulated in liposomes and obtained ratio was applied in the liposome preparation.

The total drug applied in the preparation of liposomes was 1 ml of shallomin (1M). So that by using the mentioned formula at method section, the encapsulation efficiency of the lecithin and cholesterol (molar ratio: 1:1) liposomes was found to be 82.7%. These results demonstrated that the prepared liposome with the high encapsulation have the potential to be delivered more efficiently to skin.

In vitro Permeation Study

Skin permeation ability of shallomin in different formulations was shown in figure 5. The cumulative amounts of shallomin permeated after 6 hours were 993, 981, 831, and 503 µg from hydrogel extract, liposomal shallomin in hydrogel, shallomin in hydrogel, and shallomin extract, respectively.

The cumulative amounts of the liposomal shallomin in hydrogel (1%, w/w, 981 µg/cm^2) was exhibited greater than shallomin in hydrogel (1%, w/w; 831 µg/cm^2), this may be explained by the more solubility and facility of release of liposomal shallomin in hydrogel.

Furthermore, permeation of shallomin plain (503 µg/cm^2) was less than that of liposomal shallomin in hydrogel (981 µg/cm^2) and shallomin in hydrogel (831 µg/cm^2), this means that hydrogel increases permeation 2-fold appromixately higher than aqueous solution extract. Retional explanation for this phenomen might be that mucoidal properties of hydrogel can facilitate adhesion between the shallomin and skin membrane, which extend keeping of shallomin at the site of administration which can enhance drug permeation.

Clinical Evaluation

Participants were randomly divided to three groups each included 15 students.

Table 1 The results of administration of different formulations within 72h.

Formulations	Times (hour); n(%)			
	0-12 h	12-24 h	24-48 h	48- 72 h
Liposomal shallomin gel	12 (80)	2 (13)	1 (6)	-
Acyclovir	8 (53)	5 (33)	2 (13)	-
Placebo	-	-	11 (73)	4 (27)

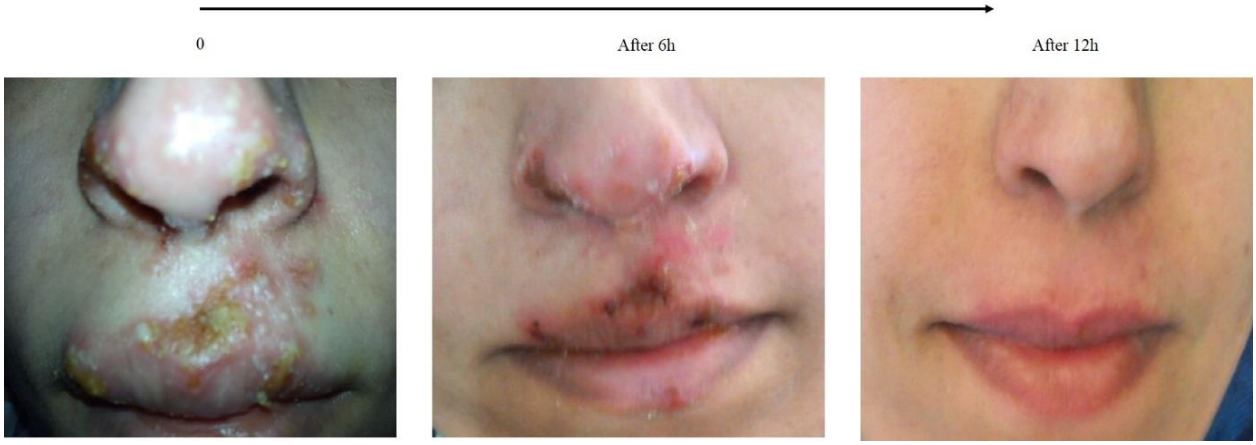

Fig. 6 The cold sore and tingling was disappeared in shallomin treated group within 12-24 hours

Two groups were randomly chosen as treatment groups (first group were exposed to treatment with acyclovir and second group treated with liposomal shallomin gel) and third group was selected as control group (treated with liposomal gel whiteout shallomin).

The results of administration of every formulation were shown in table 1. The cold sore and tingling was disappeared in second group within 0-12 hours in 12 cases (80%) and within 12-24 hours in 2 cases (Fig. 6). In the acyclovir treated group, skin was cleared from cold sore in 8 cases (53%) within 24-48 hours after using drug. The timetable for treating remaining cases were listed in table 1. In the placebo group, the cold sores were disappeared in all cases after 72 hours. Regarding the obtained results, the liposomal shallomin gel caused a significant improvement in the removing cold sores within 12 hours related to acyclovir ($p=0.001$). No complications, range from mild to severe, was reported while using the liposomal shallomin gel. There was not reported any mild or serious adverse event in the topical use of liposomal shallomin gel.

Discussion

Cold sore is a painful and common viral infection caused by the HSV-I or HSV-II. Both of these viruses can affect mouth or genital and easily spread from person to person by close contact such as kissing. Two-thirds of the population (aged 0–49 years) around the world have been affect by these viruses at least once in their life [23]. Acyclovir is used locally or systemically to treat cold sore and totally have no side effect. However, this antiviral drug is not suitable for some people such as people suffering from kidney and immunocompromised diseases (HIV), as well as pregnant women [24]. The extract of Allium hirtifolium Boiss, known as Persian shallot and also named shallomin, is a natural and traditional herbal medicine in Iran. Based on our previous study this medicine has not any *in vitro* and *in vivo* hematotoxicity, hepatotoxicity, and renal toxicity [18]. Furthermore, up to our knowledge, the antimicrobial activity of raw extract of shallomin against bacteria, viruses and fungi was previously evaluated. These studies revealed *in vitro* antibacterial activity of shallomin against methicillin-resistant *Staphylococcus aureus* (MRSA) [25], antiviral activity against HPV and HSV [18, 26].

and fungicidal activity against *Aspergillus*, *Penicillium*, and *Microsporum* [27]. In our previous clinical trial study, hourly topical application of 5% shallomin alcoholic solution for five to six hours caused a rapid clearance of the sores with no significant side effects and with a minimal crust or scab formation. In the present study, 45 participants with cold sores were divided into 3 groups (15 students in each group) and the effectiveness of extract was examined on patients who developed cold sore during last 24 hours. The results of the current study exhibited that the 1% liposomal shallomin in hydrogel led to disappearance of cold sores during 0-12 hours without any crust and scab, whereas our previous clinical trial study revealed that 5% shallomin alcoholic solution was able to remove cold sore and tingling during the same time period.

The greater effectiveness of the extract at lower concentrations may be due to the drug formulation. The results of *in vitro* permeation study of shallomin showed that the cumulative amounts of the liposomal shallomin in hydrogel were exhibited greater than shallomin in hydrogel, which may be explained by the more solubility of liposomal shallomin in hydrogel. In addition, permeation of shallomin plain was less than that of liposomal shallomin in hydrogel and shallomin in hydrogel, revealing that hydrogel enhances permeation more than aqueous solution. Retional explanation for this phenomen might be that mucoidal properties of hydrogel can facilitate adhesion between the shallomin and skin membrane, and consequently resulting in the increase of availability and permeation of shallomin at the site of administration. It seems that loading of shallomin in

liposome improves solubility and encapsulation. In addition, the mocoidal property of hydrogel leads to the increase of permeation and release of drug to skin.

The cold sore and tingling were removed during 0-12 hours in 80% of cases treated with liposomal shallomin gel, while in acyclovir-treated group, skin was cleared from cold sore in 53% of cases during the first 24-48 hours after the treatment. The reason for higher effectiveness of our new formulation compared to our older formulations, shallonin alcoholic solution and acyclovir, in previous study might be due to the use of hydrogel and liposome in the present formulation [18]. In agreement with results of our study, Kumar A, *et al*., have revealed that compared to acyclovir cream with a moderate effectiveness, microemulsion-based topical formulations exhibited greater skin permeability levels and consequent suppression of HSV-1 at the site of infection [28].

Although to improve the antiviral efficacy of our new formulation other characteristics such as stability and the pharmaceutical dosage form, which are important for better delivery to skin, should be investigated, this was not done in the present study. This study revealed that application of liposomal shallomin gel for treatment of cold sore reduces the healing time compared to shallomin alcoholic solution, resulting from enhancement of encapsulation of shallomin (1%) and its adhesion to skin at the site of infection. Besides, preparation of the natural shallomin extract in a liposome-encapsulated gel form simplify its topical usage.

The results of the present study showed that the safe and easy-to-use topical application of liposomal shallomin 1%, could be as effective as microemulsion-based acyclovir in the treatment of cold sore. The antiviral mechanisms of shallomin have not yet been well understood, and to find out more about these mechanisms, studies with more details are needed. The effectiveness of therapeutic properties of shallomin may be enhanced by increasing the concentration of liposome-encapsulated form of shallomin or its combination form with conventional antivirals, which still need to be investigated in more depth.

In conclusion, our results showed significant difference between the efficacy of liposomal shallomin gel and acyclovir in the treatment of cold sore. Moreover, self-application of this formulation was as easy as acyclovir ointment and shallomin extract and its side effect was reduced. In the present study, a new formulation of a natural extract was applied for the treatment of cold sore. However, more studies are required to explain the underlying mechanism of its action of this new formulation.

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Authors' Contribution: Mansour Amin proposed the original idea. All authors developed the protocol and abstracted and analyzed the data and wrote the manuscript.

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