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RESEARCH ARTICLE

Preparation, characterization and evaluation of Ginkgo biloba solid lipid nanoparticles

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ABSTRACT

Objective(s): In this work, Ginkgo biloba extract (GBE) loaded solid lipid nanoparticles (SLNs) were synthesized via high pressure homogenization method and their physicochemical properties, as well as cytotoxicity and antibacterial activities were evaluated.

Methods: Ginkgo biloba extract SLNs (GBE-SLNs) were prepared using high pressure homogenization method. The morphology and size of SLNs were evaluated by scanning electron microscopy (SEM) and dynamic light scattering (DLS) techniques. The drug release of SLNs was also investigated using synthetic dialysis membrane. The antibacterial activity of nanoparticles was tested against both gram negative and gram positive bacteria strains. The probability of having toxicity of SLNs was studied on the rabbits.

Results: The spherical structure of GBE-SLNs was confirmed by SEM images. The mean particle size of the obtained SLNs was ranging from 104 to 621 nm for different formulations using DLS technique. An in-vitro study of synthesized SLNs illustrated that the percentage of ginkgo biloba released from the solid lipid nanoparticles was 85% of loaded GBE after 72 hours. There was no report of significant skin toxicity via in-vivo studies.

Conclusions: According to the above results, SLNs loaded with ginkgo extract showed acceptable particle size and shape, suitable loading of active substance and sustained release profile as well as appropriate antimicrobial effects without any significant skin toxicity.

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INTRODUCTION

The use of herbal supplements has increased unbelievable over the past 50 years due to the low side effects [1]. Ginkgo biloba is one of the most commonly herbal supplements were used in the world originating from China [2]. In medicine there are many reported for the clinical uses of dry extracts of Ginkgo biloba leaves, known as Egb761 [3] such as Alzheimer's disease or senile dementia [4], cardiovascular disease [5], cerebral vascular insufficiency and impaired cerebral

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performance [6], premenstrual syndrome [7], antidepressant-induced sexual dysfunction [8], vascular diseases [9], liver fibrosis [10], macular degeneration [11], tinnitus [12], Vertigo or equilibrium disorders [13], memory [14], cancer [15] and etc,. Ginkgo contains a number of biologically active components for its defense against insects, bacteria, and fungi [16].

Commercial extracts of the ginkgo leaves are enriched water-acetone or water-ethanol extracts of the ginkgo leaves. An extract from the leaves of Ginkgo biloba, was developed in the 1960s [17] and designated as EGb 761, which is an acetone-water (60:40) extract from the dried leaves [18]. The main bioactive constituents are terpene trilactones and flavonoid glycosides which are responsible for the pharmacological activities of the standardized leaf extract [19]. Other important constituents found in ginkgo include biflavonoids and traces of alkylphenols, such as ginkgolic acids [20].

Novel drug delivery systems (NDDS) can be an effective way to transfer active ingredients of commonly and widely used herbal plants such as Ginkgo biloba into the target sites [21]. Application of NDDS may improve efficacy and decreasing the side effects of various active ingredients of plants. Lipid-based drug delivery systems have been explored in different studies and have shown great potential in targeted drug delivery [22]. Solid lipid nanoparticles as a new colloidal drug carrier have many advantages such as drug delivery to specific sites and control drug release rate [23]. These colloidal systems have many significant benefits, such as good biocompatibility, tolerability, and ease of scale-up [24]. SLNs have been studied as a drug delivery system for the controlling of drug release and improving lasting effects in previous studies[25]. These carriers showed ability of increasing of antibacterial efficacy of some chemical and natural compounds too [26].

The aim of this work was development of solid lipid nanoparticles (SLNs) to be loaded with Ginkgo biloba extract and to be used as novel drug delivery system to enhance healing of wounds. Wound healing is a complex process with many potential factors that cause delay healing like bacterial and fungal [27]. Using safe and effective antimicrobial compounds could accelerate the wound healing process.

The structural properties of GBE- SLNs were studied by SEM, DLS techniques. Zeta potential was measured. Antimicrobial activity of SLNs was evaluated against two gram negative bacteria strains and one gram positive.

MATERIALS AND METHODS

Materials

Ginkgo Biloba Terpene lactones mix (Ginkgolide A, Ginkgolide B, Ginkgolide C and Bilobalide, Ginkgo Biloba Flavone glycoside (Quercetin, Kampferol and isorhamnetin) as working standard, dialysis membrane as well as mannitol were purchased from Sigma Aldrich, Germany. Ginkgo biloba dried extract was provided by Iran darouk pharmaceutical co. Acetone, Tween80, ethanol, methanol, Acetonitril, Phosphoric Acid, Muller-Hilton agar and all materials were used to prepare phosphate buffer solution 7.4 were purchased from Merck, Germany.

Preparation of GBE- SLNS

GBE- SLNs were prepared by high pressure homogenization method. Briefly, 500 mg cholesterol as lipid matrix was added to the mixture of ethanol/acetone in ratio of 50/50 (%v/v) and the mixture heated at 50-60˚C under homogenization by homogenizer (MTOPS, SR30, Germany) at 500 round per minute (rpm). The desired amount of GBE is weighed and dispersed in 75ml deionized water containing 1% (w/v) Tween 80 as surfactant.

The hot oily phase added drop wise to aqueous phase and homogenized at 11000-13000 rpm for 10 minutes. Then the mixture was sonicated for 2 minutes using ultrasonic system (LIRARE, ARSONIC60, Italy). After sonication, prepared mixture was centrifuged at 3000 rpm for 5 minutes to separate probable aggregates.

To achieve optimum condition, SLNs were prepared by changing different experimental variables including GBE amount, solvents volume and homogenization time as well as centrifuge conditions according to Table 1.

Freeze-drying of SLN

The selected particles (S7) were lyophilized to prolong the shelf life of GBE loaded SLNs. Lyophilization of particles was done using 5% (w/v) mannitol as cryoprotectant to limit the risk of particle aggregation. GBE-SLNs emulsion was frozen at -40˚C for 24 hours and then lyophilization was done.

Particle size, zeta potential and polydispersity index were evaluated after freeze- drying again to ensure no significant size enlargement was happen.

Table 1. Preparation of GBE-SLNS with different experimental conditions.

Formulation name	Acetone/ Ethanol amount	GBE amount (mg)	Homogenization/ sonication condition	Centrifuge (time/temperature)
S1	8/8 (ml)	250 (mg)	11000-13000 (rpm) 2 min sonication	-
S2	8/8 (ml)	250 (mg)	11000-13000 (rpm) 2 min sonication	10 min in -4°C
S3	10/10 (ml)	175 (mg)	11000-13000 (rpm) 2 min sonication	-
S4	10/10 (ml)	175 (mg)	11000-13000 (rpm) 2 min sonication	10 min in -4°C
S7	10/10 (ml)	175 (mg)	11000-13000 (rpm) 10 min sonication	-
S7 freeze-dried	10/10 (ml)	175 (mg)	11000-13000 (rpm) 10 min sonication	-
S8	10/10 (ml)	175 (mg)	11000-13000 (rpm) 10 min sonication	45 min in -4°C

Characterization of GBE-SLNs

The size, zeta potential and polydispersity index (PdI) of GBE-SLNs were analyzed by dynamic light scattering using a Quidix, scatteroscpe 1, South Korea. The surface morphology of the SLNs was characterized using scanning electron microscopy (Hitachi model S4160).

Drug loading efficienc

In order to determination of drug loading efficiency (LE %), the samples were centrifuged at 26000 rpm for 45 minutes at -4˚C and the supernatant was collected. The concentration of GBE in supernatant was determined using HPLC by indirect method. LE% was calculated using indirect method by the following equation:

$$LE\% = \frac{drug_{total} - drug_{supernatant}}{drug_{total}} \times 100 \qquad (1)$$

Drug release study

Release study was performed by using dialysis sack method by Do405 dialysis tubing 23×15mm (Sigma, Germany). First 5ml of prepared formulation was placed in a dialysis membrane (10-12KD) and immersed in 100ml of phosphate buffer solution (pH 7.4) containing 2% (w/v) tween 80. Then 2 ml of samples around the dialysis sack were withdrawn in fixed time intervals and 2ml phosphate buffer containing 2% (w/v) tween 80 was replaced immediately after withdrawing to ensure that the sustained release profile is not due to membrane. Drug concentration was measured and analyzed by using pre-column derivatization HPLC technique.

HPLC method

HPLC technique is used to separate, identify, and quantify GBE components in samples. In this study, a suitable method was developed to identify and quantify the two major components of GBE including Flavone glycoside (Quercetin, kampferol and isorhamnetin) and Terpene lacton (Ginkgolide A, Ginkgolide B, Ginkgolide C and Bilobalide). But all of the calculation of drug concentration for drug release and loading efficiency studies was considered by Area Under the Curve (AUC) of quercetin (one of the compounds of flavone glycoside). In brief C8 column was used, the mobile phase composition was: 35% methanol, 25% acetonitrile, 40% phosphate buffer solution, the flow rate was 1ml/min and the UV detector wave length was set on 270nm. The derivatization and HPLC analysis were validated for the GBE in concentrations between 5-2000 ppm. Limit of Detection (LOD) and Limit of Quantitation (LOQ) of designed method were 57.89561 and 192.9854 ppm respectively.

Antimicrobial studies

In order to compare the antibacterial activity of nanoparticles of GBE with that of free drug, the conventional broth macro-dilution tube method was used to determine minimum inhibitory concentration (MIC) and minimum bacteriostatic concentration (MBC). In this method, three bacterial strains including E.coli and and P.aueroginosa, as the gram negative pathogenic strains and S.aureus as gram positive strain were cultured 24-48 hours at 35-37˚C on the surface of Muller-Hinton agar

plates. Serial dilutions of stock solution to reach a concentration range of 0.5 to 32 mg/ml were prepared. The bacterial suspensions with density equivalent to 0.5 -32 mg/ml were transferred individually onto the surface of Muller-Hinton agar plates. Well with 8mm diameters were prepared aliquots of 100 µl of each 3 solutions, GBE SLN, free drug and Blank-SLN were delivered into the wells. After 24-48 hours incubation at 35-37˚C, the zones of inhibition around the well measured in mm using a caliper.

Cytotoxicity tests

The method used for conducting in vivo studies is the accepted standard described in OECD 404 Guideline for the Testing of Chemicals, Acute dermal irritation and corrosion. Two healthy female Albino rabbits were selected for this study. The method of randomization was based upon the random selection of numbers generated from a set of numbers without replacement. Approximately 24 hours before the test, fur was removed by closely clipping the dorsal area of the trunk of the animals. Care was taken to avoid abrading the skin, and only animals with healthy, intact skin should be used.

The test substance was applied to a small area (approximately 6 cm^2) of skin and covered with a gauze patch, which was held in this place with non-irritating tape.

Animals were observed individually at least three times during the first 4 hours after dosing, periodically during the first 24, 48, 72 hrs. Dermal reactions were graded and recorded according to the grades as below.

Scoring systems

Erythema and Eschar Formation

No erythema: 0, Very slight erythema (barely perceptible): 1, well defined erythema, 2, Moderate to severe erythema, 3, severe erythema (beef redness) to eschar formation preventing grading of erythema, 4 and Maximum possible: 4

Oedema Formation

No oedema, 0

Very slight oedema (barely perceptible): 1, Slight oedema (edges of area well defined by definite raising): 2, Moderate oedema (raised approximately 1 mm): 3, Severe oedema (raised more than 1 mm and extending beyond area of exposure), 4 and maximum possible: 4.

Skin dryness

No dryness: 0, Slight dryness: 1, Mild dryness: 2 and severe dryness: 3.

RESULTS AND DISCUSSION

Particle size analysis and Morphology study

In present study, DLS analysis of prepared SLNs is summarized in Table 2. PdI value of less than 0.5 was obtained for all SLNs. The PdI values of samples namely S1, S2, S3 and S7 after lyophilization were 0.509, 0.495, 0.198 and 0.233 respectively. Particle size of more than 93% and 97% of S2 particles were smaller than 111 nm and 152 nm respectively. It can be deduced from Table 2 that centrifugation has considerable effect on reducing particles size.

SEM micrographs of prepared SLNs have shown in Fig. 1.

Antimicrobial study

Antimicrobial tests were performed on three bacterial strains including two gram-negative pathogenic strains such as E.coli and P.aueroginosa and S.aureus as gram-positive strain. The antimicrobial activity of the GBE-SLNs after preparation in first dispersion according to broth micro dilution method is shown.

Table 3 showed the antibacterial properties of GBE-SLNs against three bacterial strains. The GBE loaded SLNs show less MIC/MBC than Free-drug, it can be seen that SLNs of Ginkgo Biloba extract possessed high antimicrobial activity against all three strains of bacteria. It may be concluded that SLNs could effectively reduce the clinical dosage of drug and its side effects.

Table 2. The size of PdI, Particle size and Zeta potential in samples

Sample	PdI	Particle size (nm)	Zeta potential(mV)
S1	0.509	621	-12.6
S2	0.495	469	-19.4
S3	-	136.9	-
S4	-	104.8	-
S7	0.198	106	-18
S7 (freeze-dried)	0.223	119	-28

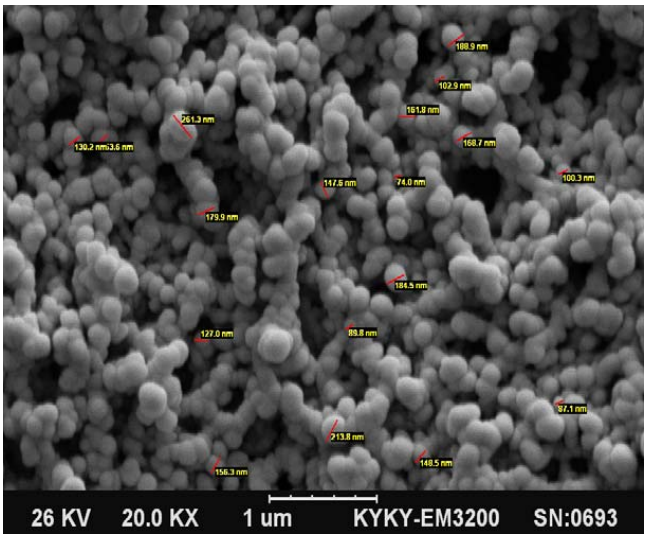

Fig. 1. SEM micrographs of sample S7

Table 3. MIC and MBC of GBE in comparison with GBE SLNs

Bacteria	Test	Result
P.aueroginosa	SLN/MBC	1/16
	SLN/MIC	1/32
	Blank/MBC	1/4
	Blank/MIC	1/4
	Free drug/MBC	Initial concentration
	Free drug/MIC	1/2
E.coli	SLN/MBC	1/4
	SLN/MIC	1/4
	Blank/MBC	Initial concentration
	Blank/MIC	1/2
	Free drug/MBC	Not effective
	Free drug/MIC	Not effective
S.aureus	SLN/MBC	1/2
	SLN/MIC	1/2
	Blank/MBC	Initial concentration
	Blank/MIC	Initial concentration
	Free drug/MBC	-
	Free drug/MIC	Initial concentration

Drug release and loading efficiency

By using Eq. (1), the calculated loading efficiency was calculated as 89/79%. The release profile of GBE loaded on SLNs showed a prolong manner. The results showed after 24, 48 and 72 hours 67%, 78% and 85% of loaded drug. Drug release studies revealed that the release profile of GBE loaded solid lipid nanoparticles was in a sustainable manner and no burst effect was observed Fig. 2.

In vivo studies

The GBE-SLNs didn't show any toxicity in short term from dermal application. In long term dermal after exposure erythema, edema and skin dryness were not observed in case group. The product didn't show any irritant effects and corrosive response in long term. The total score was 0 in this rabbit.

The Blank showed signs of toxicity and well defined irritant effects after 1 hour. In long term dermal exposure, erythema and skin dryness was observed in case group which didn't received in control group. The signs of toxicity were not recovered to normal appearance after the remove of the patch. The total score was 24 in this rabbit. The product showed moderate to severe irritant effects

with corrosive response in long term(24-48hrs) and sever irritant with corrosive effects after 72 hrs from dermal exposure. Dermal erythema and skin dryness were still observed after 5 days from initial application.

Observations of the first, second and third examined rabbits were described in Tables 4 and 5 respectively.

Discussion

In this study ginkgo biloba extract (GBE) loaded SLNs were prepared successfully via a high pressure homogenization method to enhance antimicrobial efficacy and sustaining release profile as well as prolonging application intervals in wound healing treatment in future. Many studies were carried out to establish antibacterial efficacy of GBE and enhancement of antibacterial efficacy of some antibiotics using GBE [28, 29]. Since some other studies demonstrated that nanotechnology could decrease MIC and MBC of some compounds [24], in this study the probability of antibacterial efficacy enhancement of GBE using nanotechnology was evaluated. The PdI value of prepared SLNs was obtained lower than 0.5 which is in an acceptable range in pharmaceutical field of science. The Spherical shape with size ranging from 104 to 621 nm was obtained according to SEM micrographs. Drug release studies revealed that the release profile of GBE loaded solid lipid nanoparticles was in a sustained manner and no initial rapid release was

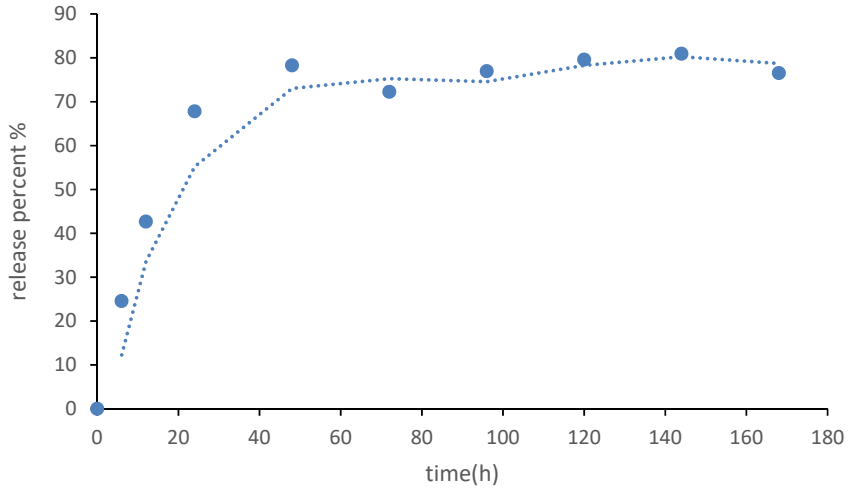

Fig. 2. GBE release profile

Table 4. Skin reactions of rabbit 1 after 3min, 1hrs, 4 hrs,24 hrs,48 hrs , 72 hrs,5 days and 14 days to GBE-SLN

Time	3 min	1 hr	4 hrs	24 hrs	48 hrs	72 hrs	5 days	14 days
Erythema	0	0	0	0	0	0	0	0
edema	0	0	0	0	0	0	0	0
Skin dryness	0	0	0	0	0	0	0	0
Total score:8	0	0	0	0	0	0	0	0
Weight of Animal	1563+148g							

Table 5. Skin reactions of rabbit 2 after 3 min, 1 hr, 4 hrs, 24 hrs,48 hrs , 72 hrs and 5 days to proposed Blank

Time	3 min	1 hr	4 hrs	24 hrs	48 hrs	72 hrs	5 days
Erythema	0	2	2	3	3	4	2
Edema	0	0	0	0	0	0	0
Skin dryness	0	0	0	1	2	3	2
Total score:7	0	2	2	4	5	7	4
Weight of Animal :1609±28g							

observed. The bacterial test has shown that GBE-SLNs have a bacteriostatic effect against all three studied bacterial strains in concentrations lower than free drug. In vivo results revealed that GBE-SLNs works without any skin toxicity. It seems that these SLNs could be good semi-finished products to be used in dosage forms for wounds healing in future.

CONCLUSION

It seems that using nanotechnology could improve the therapeutic effects of Ginkgo like antibacterial efficacy. Enhancement of antibacterial efficacy of a natural product could help to avoid using more toxic compounds in future. We are thinking about design of semi-solid preparations using mentioned GB nanoparticles in this study in near future.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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