Research Journal of Pharmacognosy (RJP) 9(2), 2022: 1–7
Received: 21 Sep 2021
Accepted: 15 Dec 2021
Published online: 21 Dec 2021
DOI: 10.22127/RJP.2021.305913.1792

Original article

Design and Optimization of PLGA-Based *Tribulus terrestris* Loaded Nanoparticles

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Abstract

Background and Objectives: Novel drug delivery systems improve bioavailability of standardized plant extracts which enables them to cross the biological membranes. Biodegradable polymeric nanoparticle systems are an approach to circumvent problems in drug delivery. *Tribulus terrestris* growing in subtropical areas has exhibited some biological and pharmacological activities; it contains compounds like flavonoids and steroids. To improve bioavailability of active compounds of the plant, its extract was subjected to prepare nanoparticles. **Methods**: Aqueous ethanol 80% extract of the whole plant was used for preparation of encapsulated nanoparticles using poly DL-lactic-co-glycolic acid (PLGA) polymer. Mean particle size, polydispersity, drug loading and encapsulation efficiency of the nanoparticles systems were evaluated in various ratios of *T. terrestris* extract. **Results**: All the applied concentrations of the extract provided particles in nano-scale size (163-214 nm). By increasing the extract ratio encapsulation efficacy also increased ranging between 40.3-78.5%. Above 50% of the loaded extract released in the first 3 h and it continued for 10 days. **Conclusion**: the plant extract has been successfully encapsulated into PLGA polymer. The quantification of encapsulation efficiency and in vitro release also showed that application of the plant in pharmaceutical field can be improved using nanoparticles.

Keywords: nano delivery system; nanoparticle; plant extract; polymers; *Tribulus terrestris*

Citation: Manayi A, Bagheri N, Dinarvand R, Esfandyari-Manesh M, Khanavi M. Design and optimization of PLGA-based *Tribulus terrestris* loaded nanoparticles. Res J Pharmacogn. 2022; 9(2): 1–7.

Introduction

Reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical and hydrogen peroxide are regularly generated endogenously during the cellular metabolic processes. Over production of ROS can further cause harmful effects on cell homeostasis, structures, and functions which has been implicated in the pathogenesis of a large list of chronic diseases such as cancer, atherosclerosis, cardio vascular disease, diabetes, rheumatoid arthritis, cataract, neurodegenerative disorders, liver and prostate disorders and aging [1,2]. In many studies, different types of medicinal plants have been characterized for their antioxidant potential in vitro and in vivo [3-7]. Natural antioxidant mechanisms have been found to be protective against ROS-mediated injury to cell and tissues. Therefore, the dietary supplements of

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antioxidants such as flavonoids for the treatment of diseases associated with oxidative stress have been prompted. Further, extensive research is being carried out on the use of medicinal plants, plant extracts or plant-derived pure chemicals to develop better therapeutics, and most of them have been proven to be pharmacologically active [8-10].

Tribulus terrestris L. is a flowering annual creeping herb belonging to the family Zygophyllaceae. It is found to be growing in subtropical regions around the world such as Asia, Southern Europe, Africa and Australia. It can thrive even in desert climates and poor soil [11]. The plant has been used in folk medicine as diuretic, tonic, aphrodisiac, analgesic, astringent, and stomachic-lithotripter. It has also been applied in treatment of colicky pain, hypertension, and hypercholesterolemia. The antioxidant, cytotoxic, antibacterial and antifungal activities of the plant have been demonstrated in many studies [12]. Twenty-five flavonoids' glycosides were isolated from *T. terrestris* extract which include common flavonols such as kaempferol, isorhamnetin and quercetin. Among those flavonoids quercetin is a well-known poly phenolic biomolecule that exhibits wide spectrum of pharmacological properties such as anti-tumor, anti-inflammatory, antioxidant and hepatoprotective activities. Extract of the plant also contains some steroidal saponins which have been reported to possess anticancer activity with other phytochemicals like alkaloids, and tannins [11,13].

Many studies have investigated plants extracts and phytochemicals which have not provided excellent bio-activity in vitro with less or no in vivo actions due to improper molecular size or poor absorption and consequently poor bioavailability. Novel drug delivery systems can help to increase absorption profile of standardized plant extracts which enables them to cross the biological membrane, resulting in enhanced bioavailability [14,15].Thus, we can provide more amounts of active constituents at the site of action with similar or lower doses of the conventional plant extracts which can cause prolonged therapeutic action in oral or topical administration. One way to circumvent these problems are to entrap these molecules into biodegradable polymeric nanoparticles. Poly DL-lactic-co-glycolic acid (PLGA) has been widely used in drug delivery due to its excellent biocompatibility, biodegradability and mechanical strength. PLGA is hydrolytically degraded into nontoxic oligomers [16]. The preset study aimed to encapsulate *T. terresteris* into PLGA polymer to improve the bioavailability of the extract.

Materials and Methods

Ethical considerations

This study was approved by the Ethics Committee of the Tehran University of Medical Sciences, Tehran, Iran (ethics committee reference number: IR.TUMS.REC. 98/d/230/6715).

Chemicals

All the reagents sodium di-hydrogen phosphate (NaH_2PO_4), phosphoric acid (H_3PO_4), acetonitrile (HPLC grade), dichloromethane (DCM), acetone (ACE), polyvinyl alcohol (PVA), Tween-80, PLGA (LA/GA ratio 50:50, Mw 50000 and 100000), sodium dodecyl sulfate (SDS), analysis bag and the other reagents were of analytical grade and purchased from Merck, Germany.

Plant material

Tribulus terrestris was collected at the end of November from lands around the Qom-Arak highway 10 km away from Arak in Markazi province, Iran. Herbarium specimen of *T. terrestris* (7134-TEH) is preserved at the Herbarium of Department of Pharmacognosy, Tehran University of Medical Sciences, Iran. Dried and powdered whole plant including fruits, stems plus leaves and roots (100 g) were extracted with aqueous ethanol 80% in a percolation apparatus (3×24 h) and the extracts were dried in vacuo by a Heidolph model Laborta 4001 rotary evaporator at 40 °C.

Preparation of extract loaded nanoparticles

Extract-loaded nanoparticles were prepared using a modified simultaneous double-emulsion (water-in oil-in water) solvent evaporation/diffusion method [17]. Briefly, 200 mg of PLGA 502H was dissolved in 10 mL organic mixture of DCM and ACE (8:2) containing tween-80 (5%, v/v) as an emulsifier. The plant extract was dissolved in 2 mL of ethanol and water (8:2), and emulsified in the polymer solution through homogenization for 2 min with 50% amplitude. The primary

water/oil emulsion was further added to 40 mL of external water, polyvinyl alcohol (PVA) 0.5% with homogenization (2 min) to achieve the stable double emulsion (w/o/w). The resulting emulsion was dropped gradually into 60 mL of aqueous solution, PVA 0.3 % as surfactant, under steady stirring to solidify nanoparticles. The residual organic solvents were evaporated under negative pressure and the nanoparticles suspending in emulsion were collected by ultracentrifugation at 17000 rpm and washed with distilled water three times. Finally, the products were dried by lyophilization and stored at 4 °C.

Scanning electron microscope (SEM)

Extract-loaded nanoparticles were characterized by SEM. The particle samples were mounted on an aluminum stub using double-sided carbon adhesive tape. The solution was slowly evaporated at room temperature. The completely dried samples were coated with gold by sputter coating unit at 10 pa vacuums for 10 s. The image was captured on SEM mode at desired magnification.

Differential scanning calorimetry (DSC)

A DSC thermal analysis was employed to investigate the physical status of *T. terrestris* inside the nanoparticles. In the DSC measurement, approximately 8 mg of each sample (*T. terrestris* extract, PLGA and extract/PLGA nanoparticles) were respectively heated from 25-300 °C under a nitrogen atmosphere with the heating rate of 10 °C/min and the DSC thermal curves of those samples were then obtained.

In vitro release profile

The rate of the extract release from the nanoparticles was evaluated in the SDS 1% [18]. Briefly, 10 mg of dried extract-loaded nanoparticles were suspended in 5 mL distilled water in the dialysis bag and the samples were then placed in a thermostatic water bath at 37 °C with continuous agitation at 100 rpm. At various preset intervals, 25 mL SDS was withdrawn and replaced with 25 mL of fresh medium. The amount of the extract released was measured by the HPLC. The percentage (%) of released *T. terrestris* was calculated using the formula:

$$\frac{\text{released amount of } T.\ terrestris \text{ at time t}}{\text{total amount of quercetin entrapped in nanoparticles}} \times 100$$

Determination of drug loading and encapsulation efficiency

The content of extract in nanoparticles was analyzed by HPLC with indirect method. The supernatant was collected and injected into HPLC. Compounds were separated on a 250 mm×4.6 mm i.d., 5-µm particle, C_{18} column (Teknokroma, Spain) with 35:65 (v/v) acetonitrile-pH 2.4 phosphate buffer (35% acetonitrile in 0.025 M NaH_2PO_4) as the mobile phase at a flow rate of 1.2 mL/min. The concentration of extract was calculated from a standard curve, prepared by using the calibration curve of quercetin (0.2-0.8 µg/mL). Drug loading and encapsulation efficiency were calculated as follows [19]:

$$\text{Loading\%} = \frac{\text{weight of loaded drug in the nanoparticles}}{\text{weight of prepared nanoparticles}} \times 100$$

$$\text{Encapsulation efficiency\%} = \frac{\text{actual drug loading}}{\text{theoretical drug loading}} \times 100$$

Statistical analysis

All data were expressed as means (standard deviation, SD), and analyzed with one-way analysis of variance (ANOVA). Schaffer's test was used to calculate statistical significance by SPSS software. Values of $p<0.05$ and 0.001 were considered statistically significant.

Results and Discussion

The extract of *T. terrestris* was successfully incorporated in nanoparticles using PLGA polymer. The mean particle size and polydispersity index (PDI) of the extract loaded nanoparticles were in range of 163-214 nm and 0.122-0.201, respectively. The effect of extract concentration was evaluated on the nanoparticles characteristics. The results stated that increasing portion of the extract can cause bigger size of particles with no considerable increase in PDI. For example, a small particle size (163 nm) with a small PDI (0.122) was obtained when the ratio of extract was in the lowest amount. An increase in the amount of the extract resulted in better encapsulation efficiency, which was measured by HPLC method.

The encapsulation efficiency of *T. terrestris* loaded nanoparticles was 40.3 to 78.5 and the drug loading was 0.80 to 6.10 %, as obtained

from different ratios of the extract (Table 1). Encapsulation efficiency at the concentration of 16 mg of extract and 200 mg PLGA 502H, would be the best compared to other investigated concentrations. The morphological characteristics such as particle size and shapes were determined by zetasizer, DSC graphs and SEM pictures. Figure 1 (a, b, and c) show the surface morphology of nanoparticles prepared with 16 mg of the extract. The nanoparticles were of regular spherical shape with smooth surfaces and without any aggregation or adhesion.

Table 1. Mean particle size, polydispersity, drug loading and encapsulation efficiency of the nanoparticles systems with various ratios of *Tribulus terrestris* extract (mg). All determinations were performed in triplicate and values were expressed as mean (SD), n=3

Amount of used extract (mg)	Mean particle size (nm)	Polydispersity index (PDI)	Drug loading (%)	Encapsulation efficiency (%)
4	163 (4.93)	0.122 (0.016)	0.80	40.3 (5.1)
8	173 (8.34)	0.192 (0.058)	2.70	69.9 (2.8)
12	169 (14.73)	0.149 (0.063)	4.17	72.2 (5.9)
16	214 (38.87)	0.201 (0.111)	6.10	78.5 (5.8)

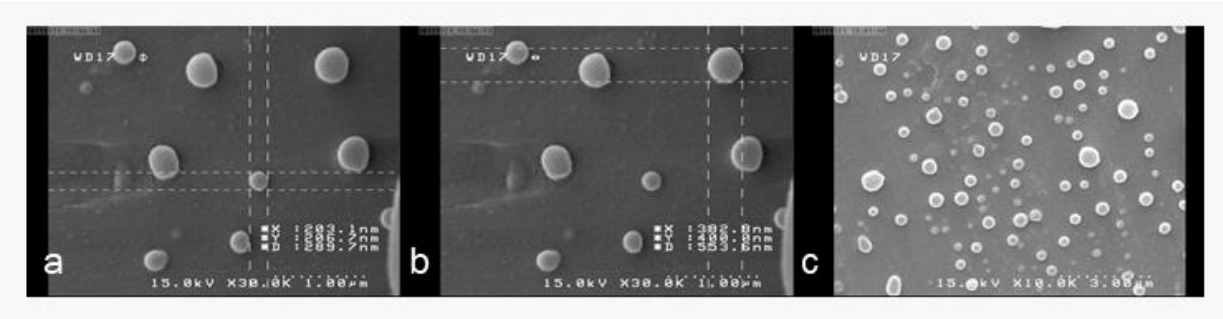

Figure 1. SEM images show the surface morphology of nanoparticles prepared with 16 mg *Tribulus terrestris* extract-loaded polymeric nanoparticles. a: nanoparticles at 203 nm particle size in 30000 magnification; b: nanoparticles at 382 nm particle size in 30000 magnification; c: uniform size distribution in 10000 magnification

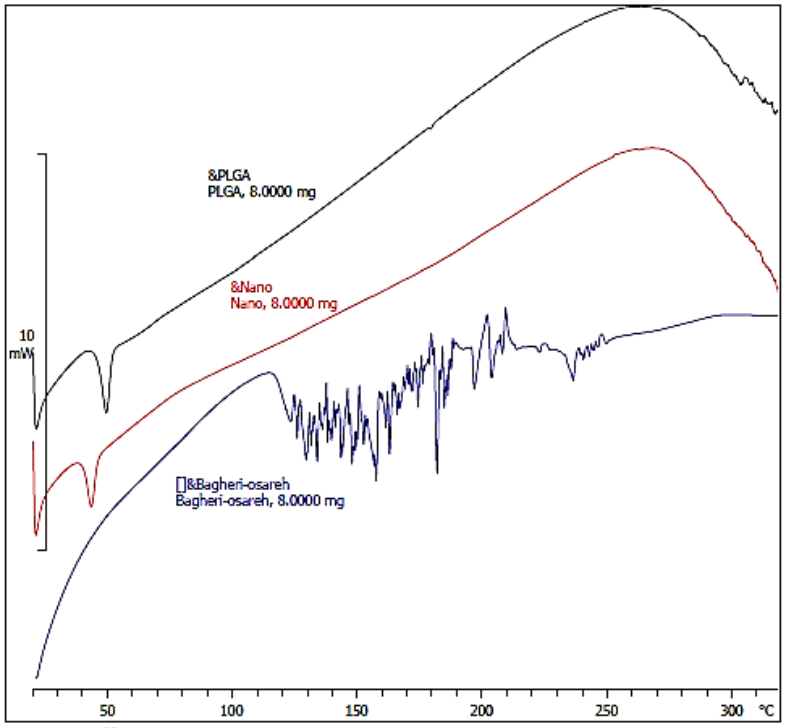

Figure 2. DSC thermograms of *Tribulus terrestris* (blue), *Tribulus terrestris* extract nanoparticles (red), PLGA polymer (black)

Differential scanning calorimetry gives us information about the physical status of the extract in the nanoparticles. DSC curves of the pure extract and of the *T. terrestris* loaded nanoparticles are displayed in figure 2. The extract showed several peaks at 120-250 °C that are probably related to its melting point (Figure 2, blue line). However, all these peaks completely disappeared in the lyophilized extract nanoparticles thermogram (Figure 2, red line). Moreover, the pure polymer materials all showed a phase transition that can correspond to amorphous solid material as the endothermal peaks appeared around 50 °C.

The release profiles of different ratios of extract from the loaded nanoparticles were analyzed through in vitro assay in SDS 1% using dialysis bag at 37 °C (Figure 3). The overall pattern of the release in all formulations was same and followed a biphasic release kinetic, an initial burst release in the first day followed by a constant release over 10 days. The results indicated a burst release over 50% within initial 3 h followed by sustained release. The half release of the extract was observed after 3 h and almost complete release was observed after 96 h. The results indicated that the high burst release was depended to the proportion of the plant's extract, as the proportion of the extract in the formulation increased, it resulted in higher burst release. The high proportion of the extract could result in a burst release because more extract can be adsorbed into the surface of nanoparticles.

The results of our study revealed that extract of *T. terrestris* can be loaded on PLGA based nanoparticles with encapsulation efficacy up to 75.8%. Considering PDI values, it can be stated that the free and loaded nanoparticles obtained by the method were homogeneous, and the employed method was reproducible and stable [20]. To analyze molecular dispersion of drug and polymer, DSC has been used to evaluate polymer-drug interactions [21-23]. Our results showed that the extract loaded polymeric nanoparticles had a lower glass transition temperature compared with the pure extract which can be due to the incorporation of the plant components. Changes in the location of the peaks and some newly formed signals suggested that there was an interaction between the plant extract and PLGA. Similar to the previous study of quercetin and catechin encapsulated into PLGA nanoparticles, our findings indicated that *T. terrestris* extract is dispersed in the polymeric matrix of PLGA nanoparticles [24]. As the previous investigations show, the common double emulsion techniques for encapsulating hydrophilic drugs into polymeric nanoparticles suffer from low encapsulation efficiency, which is caused by the drug rapidly penetrating from the inner aqueous phase to the external aqueous phase [25,26].

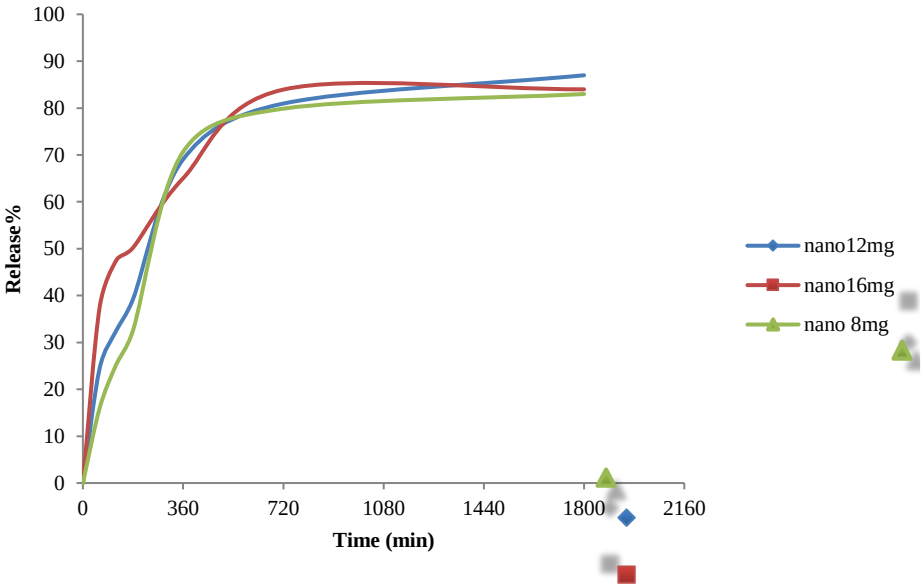

Figure 3. The effect of the *Tribulus terrestris* ratio on release profile of the nanoparticles

In order to improve the encapsulation of hydrophilic drugs, we used a modified double-emulsion solvent diffusion/evaporation technique, in which acetone was introduced into the emulsion process as a co-solvent. As our results indicate, more than half of the extract released in 3 h, rapid initial release is normally due to the tightly adsorbed extract on the PLGA nanoparticles. The adsorbed fraction of the extract was rapidly diffused into the surrounding liquid of the release medium. This is accounting for the rapid initial part of the release profile. At the second stage, the release of the extract was slower and sustained. This part of release is assigned to diffusion of the extract entrapped within the core of the PLGA nanoparticles.

The results indicated that the high burst release depends on the proportion of the plant's extract, as the proportion of extract increased in the formulation, more burst release happened. Higher proportion of the extract caused more burst release, because more amount of the extract can be adsorbed to the surface of the nanoparticles.

Conclusion

Polymeric nanoparticles using PLGA was prepared and applied to encapsulate, protect, and release the extract of *T. terrestris*. Therefore, the delivery system may be suitable to increase bioavailability of flavonoids or other phytochemicals available in the plant, which has to be evaluated in vivo. Generally, our results have important implications for the design and fabrication of polymeric nanoparticle delivery systems for bioactive compounds with beneficial properties for human health and wellness.

Acknowledgments

This study was supported by Tehran University of Medical Sciences (98/d/230/6715).

Author contributions

Azadeh Manayi was involved in analyzing and drafting the study; Nikoo Bagheri participated in the acquisition of data; Rasoul Dinarvand was involved in design of the study; Mehdi Esfandyari-Manesh took part in administrative technical or logistic support; Mahnaz Khanavi was involved in designing and conception of the study.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

ACE: acetone; DCM: dichloromethane; DSC: differential scanning calorimetry; PLGA: poly DL-lactic-co-glycolic acid; PVA: polyvinyl alcohol, ROS: reactive oxygen species; SEM: scanning electron microscope, SDS: sodium dodecyl sulfate