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Preparation and characterization of liposomes containing methanol extract of aerial parts of *Platycladus orientalis* (L.) Franco

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Abstract

Objective: *Platycladus orientalis* or *Thuja orientalis* is a native plant of Iran different parts of which are used in the treatment of various diseases such as: gout, rheumatoid arthritis, common cold, cough, bronchitis, asthma, high blood pressure and hormonal disorders like hirsutism and baldness. Also, various organs of this species have been used as appetizer. The purpose of this study was to prepare and characterize liposomal formulations that contain methanol extract of aerial parts of *P. orientalis* for hirsutism treatment.

Material and Methods: Plant's leaves were dried in room temperature, and powdered by grinding. Then, methanol extract was prepared by maceration method. Liposomes containing mathanol extract were produced by two methods of fusion and solvent evaporation. To evaluate mathanol extract and encapsulation efficiency of liposomes, quercetin was chosen as standard. The amount of quercetin in samples was determined by high pressure liquid chromatography (HPLC) method.

Results: Mean size of liposomes prepared by solvent evaporation and fusion methods was 373 and 320 nm, respectively. According to the quercetin concentration, encapsulation efficiency of liposomes containing menthanol extract was 69.3±3.1% for solvent evaporation and 62.2±4.9% for fusion method.

Conclusion: In the current study, a suitable liposomal formulation was prepared. The pharmacological activity of these carriers should be evaluated in the future study.

Keywords: *Platycladus orientals*, Hirsutism, Liposomes, HPLC.

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Introduction

Platycladus orientalis (L.) Franco [*Thuja orientalis* L., *Biota orientalis* (L.) Endl., Oriental Arborvitae] is a monoecious and evergreen tree belongs to Cupressaceae family. *P. orientalis* is the only species of genus *Platycladus*. This coniferous shrub or tree has 5-12 m height, scale minute leaves, closely imbricate, of two types in opposite and decussate pairs, composed of an inner, median facial pair and an outer, lateral pair, adnate for much of their length with small, free, obtuse tips. Male cones are terminal and female cones are ovoid-pyriform, with 0-8 thick valvate scales. The apical pair of scales is sterile, only the central four scales are usually fully fertile. Seeds are under each scale and ovate, 3 mm thick, and are not winged. The plant is indigenous of Korea, Manchuria, north of China and Iran. Its Persian names are "Noosh", "Sarv-e-Khomrehi" and "Sarv-e-Tabari" (Kubitzki, 1991; Assadi, 1998; Sabeti, 1975).

P. orientalis has different medicinal uses and pharmacological activities. For example ethanol extract of *P. orientalis* inhibits the 5α-reductase activity. Its diterpenes and flavonoids can be used in androgenic alopecia, hirsutism, acne, etc (Takahashi et al.,1996; Takeda et al., 2000). It also has platelet activating factor (PAF) inhibitory effect related to pinusolid and pinusolidic acid (Yang et al., 1995a,1995b; Yang and Han, 1998), neuroprotective activity related to 90% methanol fraction of *Biota orientalis* against glutamate-induced neurotoxicity (Koo et al.,2002), cytotoxic activity by isolated deoxy podophyllotoxin (a lignane) from *P. orientalis* leaves against HeLa cells (Kosuge et al.,1985a), hemostatic activity of condenced tanines and quercitin (a glycosid flavonoid) of *P. orientalis* leaf (Kosuge et al., 1985b; Sun et al.,1987) and antimicrobial activity because of essential oils from twigs by hydrodistillation method and fruits by steam distillation method (Bagci and Digrak, 1996; Hassanzadeh et al., 2001).

Liposomes are colloidal, vesicular structures based on (phospho) lipid bilayers. In these structures, an aqueous core is surrounded by lipids arranged in a bilayer configuration. They can be as small as 20 nm and as large as several microns in diameter (Lasic, 1995; Crommelin and Storm, 2003; Crommelin et al., 2003; Barenholz, 2003; Felnerova et al., 2004; Torchilin, 2005). These vesicles are nontoxic, biodegradable and practically nonimmunogenic (Lasic, 1995, 1996; Felnerova et al., 2004). Because of liposome biocompatibility, they are suitable for every route of administration (Fielding, 1991). They are also used in cosmetics and topical formulations because of their collodial size, easily controllable surface and membrane properties and large carrying capacity (Chen et al., 2001). Improved penetration into tissues, especially in the case of dermally applied liposomal dosage forms was reported in several studies. Examples include anesthetics, corticosteroids, and insulin (Lasic, 1995). In the recent years, the topical delivery of liposomes has been used for different applications and in different disease models. After topical application of liposomal formulations, such formulations can significantly increase the rate and extent of drug absorption into epidermis (Torchilin, 2005; Crommelin and Storm, 2003; Crommelin et al., 2003; Barenholz, 2003; Lasic, 1995, 1996; Felnerova et al., 2004; Fielding, 1991). Current efforts in this area concentrate on optimization procedures and new compositions (Torchilin, 2005).

The aim of the present study was to prepare and characterize liposomes containing methanol (MeOH) extract of *P. orientalis* leaves that contain flavonoids and diterpens which has 5α- reductase inhibitory effects and can be used in diseases like hirsutism and androgenic baldness.

Liposomes were prepared with two different methods: fusion and solvent evaporation. As several flavonoidic constituents of the leaves of *P. orientalis* such as rutin, quercitrin, quercetin, amentoflavone, aromadendrin, myricetin and hinokiflacone have been reported (The Dictionary of Chinese Traditional Medicine, 1992), we used quercetin as our standard material reference.

Materials and Methods

Materials

HPLC-grade methanol and acetonitrile were purchased from Caledon (Canada). Methyl paraben, propyl paraben and propylene glycol and sodium acetate (≥85%) were obtained from Merck (Germany). Quercetin was from Sigma (USA). Egg phosphatidylcholine and cholesterol were ordered from Avanti Polar Lipids (USA). All solvents were of analytical grade.

Plant

The leaves and branches of *P. orientalis* (L.) were collected from Soorkesh valley, Aliabad Katool, Golestan province North of Iran. This plant was identified by Mr. M.R. Joharchi from Ferdowsi University of Mashhad Herbarium (FUMH) where vouchers specimen has been deposited (No. is 37063).

Extraction

Total methanol extract was prepared by maceration technique, in four 24 hour cycles. For this purpose, dry and powdered leaves and branches (500 g) were extracted with 1.5 liter methanol and then were evaporated to dryness by rotary evaporator (Heidolph, Germany). The total methanol extract was deposited in 4°C until being analyzed. The identification of quercetin was accomplished by direct comparison with standard by HPLC assay.

HPLC method

Calibration

A stock solution consisting of quercetin (1 mg/ml) was prepared. 0.5, 1.0, 2, 4.0 and 8.0 ml of the stock solution were, respectively, adjusted with methanol into five 25 ml volumetric flasks for the calibration of standard curves. Standards contain 0.02, 0.04, 0.08, 0.16 and 0.32 mg/ml quercetin. Analysis was done by HPLC method using C18 column (250×4.6 mm) and phosphate buffer: acetonitrile (25:75) mobile phase in wavelength of 266 nm and with flow rate of 0.8 ml/min (Lu et al., 2006).

Validation

For inter-day validation, the lower and upper limits of standard concentrations (0.02 and 0.32 mg/ml) were analyzed by HPLC, 5 times per day and CV% was calculated. To validate HPLC data intra-daily, lower and upper limits of standards were injected in 5 days continuously.

Sample preparation

For analysing the amount of quercetin, 20 mg of methanol extract was solved in 1 ml methanol and centrifuged in 14000 rpm for 10 min. The supernatant was filtered through a 0.45 µm membrane then 20 µl of the filtrate was injected to HPLC and amount of quercetin was calculated according to the calibration curve.

Preparation of liposomes

In the present study, liposomes were prepared by two methods:

A) Solvent evaporation method: In this method, lipid phase containing egg phosphatidylcholine and cholesterol were dissolved in organic solvent (methanol: chloroform, 2:1 v/v). The organic solvent was removed by rotary evaporation to form a thin film of the lipid mixture in the inner wall of round bottom flask. Phosphate buffer saline (PBS) was added to the dried lipid film

as rehydration medium at temperature above the Tm. Liposomes were formed after 15 min vortexing. Liposomal formulation is summarized in Table 1 (Jaafari et al., 2005).

B) Fusion method: First, components of lipid phase (Table 1) and propylene glycol were kept at 60°C water bath to form uniform lipid phase. Then, herbal extract dissolved in suitable amount of acetone was added to lipid phase. To evaporate acetone, the mixture was kept at 60°C. Subsequently, the aqueous phase (phosphate buffer saline) was warmed up to 60°C and added to lipid phase. Liposomes were formed after 15 min vortexing (Jaafari et al., 2005).

Characterization of liposomes

Mean size of prepared liposomes was determined by Zetasizer (3000HSA, Malvern, UK) after suitable dilution. To determine the encapsulation efficiency of liposomes, certain amount of liposomes was dissolved in specific amount of organic solvent. The quantity of quercetin in samples was determined by HPLC method described above. The amount of entrapped quercetin was directly determined using the standard curve.

Results

We obtained 60 g total methanol extract out of 500 g *P. orientalis* (L) dry and powdered leaves and branches (12% w/w). It was colored in dark green. The HPLC method for quercetin quantitation was linear to the concentration of 0.02 to 0.32 mg/ml with R^2 of 0.998 (Figure 1). Quercetin's peak in HPLC graph was appeared after about 8.9 minute. The concentration of quercetin in methanolic extract was 0.0425 mg/ml. The results of inter-day validation showed CV of 1.7% and 1.8% for lower and upper limits of standard concentrations, respectively.

Table 1. Ingredients of different liposomal formulations prepared by solvent evaporation and fusion methods.

Liposomal formulation	Ingredients	Percent	Liposomal formulation	Ingredients	Percent
prepared by solvent evaporation method	Egg phosphatidylcholine	15	prepared by fusion method	Egg Phosphatidylcholine	15
	Cholesterol	2		Cholesterol	2
	alpha-tocopherol	0.3		alpha-tocopherol	0.3
	methyl paraben	0.1		methyl paraben	0.1
	propyl paraben	0.02		propyl paraben	0.02
	propylene glycole	0		Propylene glycole	7
	methanolic extract	2		methanolic extract	2
	Aqueous phase	up to 100		Aqueous phase	up to 100

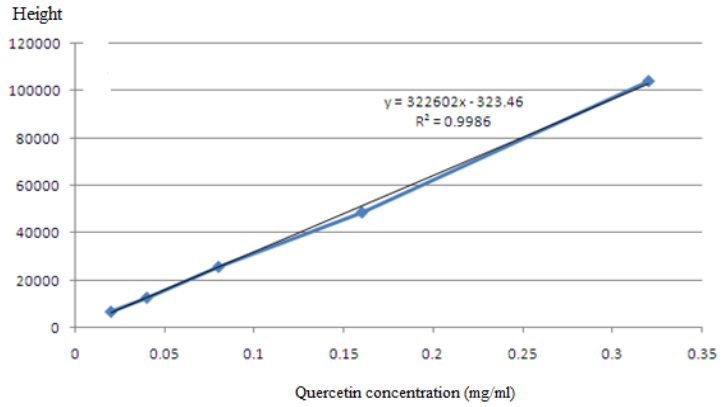

Figure 1. Quercetin standard curve. Standard concentrations are 0.02, 0.04, 0.08, 0.16 and 0.32 mg/ml.

Intra-day validation CVs for lower and upper concentrations were 2.9% and 2.2%, respectively.

The mean size of two liposomal formulations after preparation and two months storage in 4 °C are summarized in Table 2. The encapsulation efficiency of quercetin in liposomes containing methanolic extract was 69.3±3.1% in solvent evaporation method and 62.2±4.9% in fusion method.

Table 2. Mean size of liposomes containing methanol extract of *Platycladus orientalis* after preparation and two months after storage at 4 ◦C (mean±SD, n=3).

	Mean size (nm)	
	After preparation	Two months after preparation
Liposomes prepared by solvent evaporation method	373.0±8.47	409.8±12.1
Liposomes prepared by fusion method	319.6±13.3	677.5±18.0

Discussion

P. orientalis (L.) Franco has different diterpenoides and flavonoides which has 5α-reductase inhibitory effects and can be used in androgenic alopecia. We obtained 12% w/w methanol extract which is a quantitative amount. 127.5 mg quercetin can be achieved out of 500 g powder. Quercetin was used for standardizing methanol extract of this plant. We also prepared liposomes containing ethyl acetate and chloform extracts of *P. orientalis* but the results showed that the amount of quercetin in these extracts was lower than the limit of detection of analysis method. Application of other standards such as pinusolidic acid and amentoflavonne has also been suggested for these extracts.

Previous studies showed that the effect of liposomal drugs was increased topically and their systemic side effects were decreased. In the study of Mezei and *et al.,* liposomal formulation of triamcinolone delivered 4-times more drugs as compared to conventional forms while drug concentration in deep layers was less (Mezei, 1993). Similar studies have been carried out for other steroids such as hydrocortisone (Wohlrab and Lasch, 1987; Kim et al., 1998), triamcinolone (Jaafari et al., 2005) and betametasone (Korting et al., 1991).

When colloidal drug carriers are administered by other routes, such as subcutaneous or intramuscular injection or topical application, they are generally retained at the site of administration longer than the free drug (Barratt, 2000). When a liposome associated drug is applied to the skin, the amount penetrating into the superficial layers may be increased compared to the the free drug, while its passage to the systemic circulation may be reduced.

It has been shown that topical application of liposomes and niosomes containing finasteride could enhance drug concentration at the pilosebaceous unit more than that of hydroalcoholic solution (Tabbakhian et al., 2006). As the PSU is main target of component of *P. orientalis* extracts, administration of these components (diterpenoides and flavonoides) as a liposomal form can improve the therapeutic effect of the prepared extracts. On the other hand, reduction of liposome size may help increasing the penetration of diterpenoides and flavonoides to the PSU units.

Conclusion

In the current study, a suitable liposomal formulation was prepared. The pharmacological activity of these carriers should be evaluated in the future study.

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