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Formulation and characterization of an apigenin-phospholipid phytosome (APLC) for improved solubility, in vivo bioavailability, and antioxidant potential

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ABSTRACT

The apigenin-phospholipid phytosome (APLC) was developed to improve the aqueous solubility, dissolution, *in vivo* bioavailability, and antioxidant activity of apigenin. The APLC synthesis was guided by a full factorial design strategy, incorporating specific formulation and process variables to deliver an optimized product. The design-optimized formulation was assayed for aqueous solubility, *in vitro* dissolution, pharmacokinetics, and antioxidant activity. The pharmacological evaluation was carried out by assessing its effects on carbon tetrachloride-induced elevation of liver function marker enzymes in a rat model. The antioxidant activity was assessed by studying its effects on the liver antioxidant marker enzymes. The developed model was validated using the design-optimized levels of formulation and process variables. The physical-chemical characterization confirmed the formation of phytosomes. The optimized formulation demonstrated over 36-fold higher aqueous solubility of apigenin, compared to that of pure apigenin. The formulation also exhibited a significantly higher rate and extent of apigenin release in dissolution studies. The pharmacokinetic analysis revealed a significant enhancement in the oral bioavailability of apigenin from the prepared formulation, compared to pure apigenin. The liver function tests indicated that the prepared phytosome showed a significantly improved restoration of all carbon tetrachloride-elevated rat liver function marker enzymes. The prepared formulation also exhibited antioxidant potential by significantly increasing the levels of glutathione, superoxide dismutase, catalase, and decreasing the levels of lipid peroxidase. The study shows that phospholipid-based phytosome is a promising and viable strategy for improving the delivery of apigenin and similar phytoconstituents with low aqueous solubility.

1. Introduction

Apigenin is a hydrophobic, polyphenolic flavonoid compound commonly found in fruits, vegetables, tea, chamomile, wheat sprouts etc. (Shukla and Gupta, 2010). Apigenin-containing preparations are marketed as dietary and herbal supplements (Peterson and Dwyer, 1998). It has been demonstrated to possess anti-oxidant (Prince Vijeya Singh et al., 2004; Stanojević et al., 2009), anti-microbial (Taleb-Contini et al., 2003), anti-inflammatory (Funakoshi-Tago et al., 2011; Rithidech et al., 2012), anti-proliferative (Johnson and Gonzalez de Mejia, 2013), anti-viral (Shibata et al., 2014), antidiabetic (Choi et al., 2014), and tumor inhibitory activities (Choudhury et al., 2013; Ruela-de-Sousa et al., 2010).

The clinical potential of apigenin is stifled by its poor aqueous solubility, rapid metabolism, and low oral bioavailability. Apigenin has an aqueous solubility of <2.16 μg/mL, and rated as a Class II drug, based on the Biopharmaceutics Classification System (BCS) (Zhang et al., 2012). After oral administration in rats, apigenin undergoes rapid metabolism to key metabolites, such as *p*-hydroxy phenyl propionic acid, *p*-hydroxy cinnamic acid, and *p*-hydroxy benzoic acid (Griffiths and Smith, 1972). Furthermore, it demonstrated only 51% bioavailability after oral administration of *Flos chrysanthemi* extract (100 mg/kg) in rats (Chen et al., 2012). Thus, it is necessary to overcome these limitations using novel formulation strategies to improve apigenin delivery.

Several formulation strategies and techniques e.g. liposomes (Arsić et al., 2011), nanocrystal gel formulations (Al Shaal et al., 2011), and self-micro emulsifying drug delivery systems (SMEDDS) (Zhao et al., 2013) have been investigated to improve the delivery of apigenin. Munyendo et al. reported that the formulation of D-α-tocopherol acid and polyethylene glycol 1000 succinate (TPGS) stabilized the mixed micelles of apigenin and phospholipids in their evaluation anticancer properties

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(Munyendo et al., 2013). Karthivashan et al. prepared 'flavonosomes' (phytosomes loaded with multiple flavonoids) using phosphatidylcholine as a carrier, and evaluated their in vitro pharmacokinetics and toxicity (Karthivashan et al., 2016). Shen et al. evaluated a novel topical delivery system for apigenin by using soy lecithin-based ethosomes (Shen et al., 2014). The authors demonstrated a superior skin targeting with the prepared ethosomes. Additionally, apigenin-loaded ethosomes also showed a significant reduction of cyclooxygenase-2 levels in mouse skin inflammation induced by ultraviolet B (UVB) light. Pawlikowska-Pawlega et al. studied the interaction of apigenin within liposomes formed with dipalmitoylphosphatidylcholine (DPPC) using FTIR spectroscopy, ^{1}H NMR, and EPR techniques, and also investigated its chemopreventive activity via changing the fluidity of tumor cell membranes (Pawlikowska-Pawlega et al., 2013).

While these formulation methods were shown to improve the solubility, dissolution, or in vitro activity of apigenin, a systematic evaluation of the bioavailability and in vivo pharmacokinetics of apigenin is missing in literature; this is particularly true for oral administration of apigenin, or novel formulations of apigenin.

Semalty et al. reviewed the potential benefits of pharmacosomes, novel lipid-based drug delivery systems, in improving the biopharmaceutical properties of several drugs (Semalty et al., 2009). Previous studies have demonstrated that an appropriate carrier system like phospholipids can improve the aqueous solubility, permeability, and overall bioavailability of such compounds. The studies with *Centella asiatica* (Saoji et al., 2016), matrine (Ruan et al., 2010), oxymatrine (Yue et al., 2010), curcumin (Zhang et al., 2013), catechin (Semalty et al., 2012), chrysophanol (Singh et al., 2012), naringenin (Semalty et al., 2014) have shown that molecular aggregation with phospholipids increased their oral bioavailability by improving the aqueous solubility, permeability, and other properties of these phytochemicals. Semalty et al. discussed the usefulness of supramolecular phospholipids–polyphenolics adducts (PHYTOSOME®) in improving the solubility and permeability of low water-soluble polyphenolic compounds across a broad pharmacological spectrum (Semalty et al., 2010). In a recent review, Semalty A. provided a comprehensive analysis of cyclodextrin-, and phospholipid-based aggregates in the enhancement of solubility, dissolution, and overall bioavailability of Biopharmaceutical Classification System (BCS) Class II and Class IV drugs (Semalty, 2014).

The present work aims to formulate, optimize, characterize an apigenin–phospholipid phytosome (APLC), and evaluate the optimized formulation for both enhanced aqueous solubility and oral bioavailability of apigenin in a rat model. Additionally, a preliminary pharmacological analysis was carried out to evaluate the in vivo antioxidant potential after oral administration of APLC in rats.

2. Materials and methods

2.1. Materials

Apigenin was obtained from Shandong Northwest Manufacturing Co., China. Phospholipon® 90H was obtained from Lipoid GmbH, Ludwigshafen, Germany. 1, 4 – dioxane, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's Reagent), and 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p*,*p*'-disulfonic acid monosodium salt hydrate (FerroZine™ Iron Reagent) were obtained from Loba Chemie Pvt. Ltd., Mumbai, India. *n*-hexane, *n*-octanol, thiobarbituric acid, and trichloroacetic acid were purchased from Sigma-Aldrich Corporation, St. Louis, MO. All other chemicals used were of analytical grade.

2.2. Formulation of apigenin-phospholipid phytosome

The APLC was prepared using different molar ratios of (1:1, 1:2 and 1:3) based on earlier reported techniques (Bhattacharyya et al., 2014; Maiti et al., 2007). Briefly, accurately weighed amounts of apigenin and phospholipid were placed into a 100 mL round bottom flask, and dissolved in 20 mL of a mixture of 1, 4–dioxane:methanol (14:6). The reaction temperature of the reflux was controlled at 40 °C, 50 °C, or 60 °C using a water bath for 2 h. The flask contents were then concentrated to obtain a dry residue. This dried material was dissolved in a 50 mL mixture of chloroform: methanol (45:5). The solution was then concentrated to 2–3 mL and poured into 100 mL of *n*-hexane. The resulting APLC was precipitated and filtered. Subsequently, the phytosome was dried completely under vacuum at 40 °C. The dried APLC (yield ~90% *w*/w) was transferred to a light-protected (amber) glass vial purged with nitrogen, and stored at room temperature.

2.3. The extent of Apigenin incorporation in the prepared APLC (% yield)

An accurately weighed amount of APLC (equivalent to 10 mg of apigenin) was added to 5 mL of chloroform. The formed phytosome and the unreacted phospholipid dissolves in chloroform, but the free (non-aggregated) apigenin remains practically insoluble in chloroform (Tan et al., 2012). The dispersion was filtered and the free or non–aggregated apigenin was separated as a precipitate. This non-aggregated apigenin was dissolved in methanol and assayed using a UV–visible spectrophotometer (Model: V-630, JASCO International Co. Ltd., Tokyo, Japan) at 335 nm for apigenin.

2.4. Full-factorial design (3^2)

A full factorial design was adopted to study the overall influence of specific independent variables, viz., drug: phospholipids ratio (X_1, w:w), and temperature (X_2, °C) on the extent of apigenin incorporation (% yield). The two independent variables (X_1 and X_2) were selected at three levels, coded as −1 (low), 0 (middle), and +1 (higher), resulting in a 3^2 factorial design of nine possible combinations. The extent of apigenin incorporation (% yield) was defined as the dependent variable. The experimental trials were performed using all nine possible combinations of the selected variables. The mathematical model containing coefficient effects, interactions, and polynomial terms was analyzed to assess the response using the following equation:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_{12} + b_4X_{22} + b_5X_1X_2$$

where *Y* is the dependent variable, *b* is the coefficient of the independent variable *X*. The main effects (X_1 and X_2) represent the possible aggregate effect of both factors as they change independently from their low to high level. The interaction term (X_1X_2) shows how the response changes when two factors are simultaneously changed. The polynomial terms (X_{12} and X_{22}) describes the non-linearity. The design levels and the real values of the independent variables are shown in Table 1. The composition of experimental trials along with obtained yield (%) values are shown in the Table 2.

2.5. Physicochemical characterization

2.5.1. Particle size analysis and zeta potential

Photon Cross-Correlation Spectroscopy (PCCS) with dynamic light scattering was used to analyze the particle size distribution of the prepared APLC. Briefly, about 5 mg of APLC powder was dispersed in 10 mL

Table 1
Coded levels and "Real" values for each factor studied.

Variables	Levels		
	−1	0	+1
Independent	Real values		
Apigenin: Phospholipid ratio (X_1, w:w)	1:1	1:2	1:3
Reaction temperature (X_2, °C)	40	50	60
Dependent			
Extent of apigenin incorporation or Yield (Y, % *w*/w)			