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The design of propolis flavone microemulsion and its effect on enhancing the immunity and antioxidant activity in mice

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ABSTRACT

The objective of the present study was to formulate a microemulsion system for improving the activity of propolis flavone (PF). Pseudo-ternary phase diagrams were constructed to evaluate the existence area of PF microemulsion (PFM). The formulation was characterized by particle size, zeta potential, morphology and stability. The results showed that the optimal PFM formulation consists of 5.3% ethyl acetate, 14% RH-40, 7% ethanol and 73.7% water (w/w), with a solubility of PF up to 3.0 mg mL^{-1}. The immune-enhancing and antioxidant activity of PFM in vitro and in vivo were performed. The results showed that PFM could significantly promote the splenocyte proliferation and the secretion of IL-2 and IFN-γ in vitro. In vivo, PFM at high and medium doses was able to significantly increase the thymus and spleen indices, enhance splenocyte activity and improve the contents of IgG and IgM in serum, it could also improve the antioxidant activity, significantly increase the levels of superoxidase dismutase and glutathione peroxidase, and decrease the malondialdehyde levels compared with PF. These results indicated that microemulsion could be used as an effective formulation for enhancing the activity of PF. Therefore, microemulsion would be expected to exploit into a new-type preparation of PF.

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1. Introduction

The immune function is the fundamental defense mechanism of organism, and able to recognize and eliminate extraneous invaders, get rid of the senescent, injured, dead and degenerative cells of own organism, identify and handle the mutant and virus infected cells. If immunologic function is lower, it would not produce protective effect normally. In this case, the organism is easy to be infected by bacteria, virus, fungus, etc., and further suffer from a variety of diseases [1]. Diseases caused by negative environment (such as high temperature, radiation) and pathogen infection will lead to oxidative stress, which will easily induce digestive tract disease and result in a cascade of adverse consequences, and further affect the normal function of histocytes and immune cells, reduce the resistance to disease and even accelerate organism aging and death [2]. Therefore, it is of great practical significance to research immune-enhancement drugs and develop safe and efficient antioxidant product to protect the body from oxidative damage.

Natural product is a promising source for the discovery of new pharmaceuticals. In the last decades, several works dealing with propolis' composition and biological properties have been published, revealing the interest of researchers on this bee product and its potential for the development of new drugs as well [3,4]. Propolis is a complex mixture of plant-derived products gathered, modified and used by bees as a general purpose sealer, draught excluder and antibiotic in their hives. The internal and external use of propolis as a traditional medicine dates back to at least 2000 BC. It has been used in folk medicine due to its plenty of biological and pharmacological properties, such as anti-inflammatory, antibacterial, antiparasite, antifungal and antitumoral [5]. Moreover, its immunomodulatory and antioxidant effects were well known [6,7]. There are numerous studies confirm that propolis is able to activate immune system, scavenge free radicals, thereby protecting lipids and other compounds from being oxidized or destroyed during oxidative damage in mice and humans [8,9]. Propolis is rich in biochemical constituents, and more than 300 compounds have been

Abbreviations: PF, propolis flavone; PFM, propolis flavone microemulsion; RH-40, polyoxyethylene (40) hydrogenated castor oil; CTX, cytoxan; PHA, phytohemagglutinin; LPS, Lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; S, surfactant; CoS, cosurfactant; PDI, polydispersity index; TEM, transmission electron microscopy; BM, blank microemulsion; IFN-γ, interferon-γ; IL-2, interleukin-2; IgG, immunoglobulins G; IgM, immunoglobulins M; SOD, superoxidase dismutase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde.

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identified in it [10]. Flavone is one of the most important pharmacologically active constituents in propolis [11], and is thought to be responsible for many of its biological and pharmacological activities [12]. But due to its poor solubility in water, easy oxidation, low bioavailability and short half-life, its clinical use was limited [13].

Among the various drug delivery systems, microemulsion may be a better choice to solve these problems. Microemulsion has gained great attention as drug delivery systems in pharmaceutical research. Microemulsion is a thermodynamically stable transparent (or translucent), single optically isotropic liquid system of water, oil, co-surfactant and surfactants stabilized by an interfacial film of amphiphilic molecules with a droplet diameter usually within the range of 10–100 nm [14], and are considered the efficient carriers in the drug delivery system and effective vehicles of the solubilization of both hydrophilic and lipophilic drugs with ease of formulation, low viscosity, high solubilization capacity of lipophilic drugs and small particle size [15–17]. Furthermore, microemulsion can be envisaged as protecting medium for the entrapped of drugs from degradation, hydrolysis, and oxidation, and can also reduce toxicity, prolong release, and improve bioavailability of the drug as well [18].

In this study, oil in water (o/w) microemulsion system of propolis flavone (PF) was prepared. The physicochemical properties of PF microemulsion (PFM), such as particle size, zeta potential, morphology and stability, were investigated in detail. In addition, the pharmacological action of PFM on immunological activity in vitro, and on immune-enhancing and antioxidant activity in CTX-induced immunosuppressive mice were also evaluated. The main objective of this research was expected to manifest the possibility for microemulsion to increase solubility and improve the biological activity of PF.

2. Materials and methods

2.1. Materials

PF was kindly provided by Institute of Traditional Chinese Veterinary Medicine (Nanjing Agricultural University). Polyoxyethylene (40) hydrogenated castor oil (RH-40) was purchased from BASF (Berlin, Germany). Cytoxan (CTX) was purchased from Alading Co. Ltd. Lymphocytes separation medium was manufactured by Tianjin Haoyang Biological Co. Ltd. RPMI-1640 (GIBCO) with the supplement of 100 IU mL^{-1} benzylpenicillin, 100 IU mL^{-1} streptomycin and 10% fetal bovine serum was used for washing and re-suspending cells, diluting mitogen and culturing the cells. Phytohemagglutinin (PHA, Sigma), as a T-cell mitogen, was dissolved into 0.1 mg mL^{-1} with RPMI-1640. Lipopolysaccharide (LPS, Sigma), as a B-cell mitogen, was dissolved into 0.05 mg mL^{-1} with RPMI-1640. Hanks' solution was used for diluting blood. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, American Co.) was dissolved into 5 mg mL^{-1} with calcium and magnesium-free phosphate-buffered saline (PBS, pH 7.2). These reagents were filtered through a 0.22 μm millipore membrane filter. Dimethyl sulfoxide was produced by Zhengxing Chemical Co. Ltd. (Suzhou, China). Ethanol and ethyl acetate were analytical grade and supplied from Kermel Chemical Co. Ltd. (Tianjin, China). All other chemicals are analytical grade.

2.2. Preparation of propolis flavone microemulsion

2.2.1. Solubility study

The solubility of PF in various menstruums was determined by adding excess amount of PF into 1 mL of each vehicle in a centrifugal tube, followed by mixing in a shaking incubator at 25 °C for 3 days. The samples were centrifuged to remove the excess drug. After filtered by a 0.45 μm filter, the PF in the supernatant was diluted with ethanol and measured by rutin method [19]. The menstruums were selected with a better solubility to PF. Based on the results, ethyl acetate, RH-40 and ethanol showed better solubility for PF than others. Therefore, ethyl acetate, RH-40, ethanol, deionized water were selected as the oily phase, surfactant, cosurfactant and aqueous phase respectively.

2.2.2. Construction of pseudo-ternary diagrams

In order to find out the ratio of components for the existence area of microemulsion, pseudo-ternary phase diagrams were constructed using water titration method at ambient temperature. Surfactant and cosurfactant (S&CoS) were blended into each tube at specific weight ratios as 3:1, 2:1 and 1:1, and then were mixed adequately for 1 min to make the surfactant mixture. The ratios of oil phase to the mixture of surfactant and co-surfactant were changed from 0.5:9.5 to 9.5:0.5 (w/w). The mixture of oil and S&CoS was titrated dropwise with water under gentile magnetic stirring. After being equilibrated, the mixtures were assessed as microemulsions when they were low viscosity and clear appearance. The critical points between microemulsion region and other phase regions were determined when the appearance of the system changed from clear to turbid and turbid to clear respectively. Based on these diagrams, appropriate ratio of S&CoS was selected for the preparation of PFM.

2.2.3. Preparation of PFM

PFM were prepared at desired component ratios. PF was added to the mixtures of oil, surfactant and cosurfactant with a certain ratios. Then water was added to the mixture dropwise and stirring for 24 h at 25 °C. Then the solution was filtered by 0.45 μm membrane.

2.3. Characterization of propolis flavone microemulsion

2.3.1. Particle size and zeta potential

The particle size, zeta potential and its distribution (characterized by polydispersity index, PDI) of PFM were determined by using a Malvern laser particle size analyzer (Nano-ZS, Malvern, UK), after equilibration for 2 min. The temperature used was 25 °C. Particle size data were reported as the intensity-weighted distribution.

2.3.2. Morphology detection by TEM

The morphology of PFM was examined by transmission electron microscopy (TEM) (H-600 II, Hitachi, Japan). One drop of diluted samples (50 μL) was stained by 2% phosphotungstic acid and placed on a copper grid coated with carbon film followed by drying at 25 °C before examination under the TEM.

2.3.3. Stability of PFM

Two batches of PFM were produced (0 and 1 in Table 1). The physicochemical stability of PFM was assessed at 4 °C, 25 °C and 40 °C for 3 months. At various time points, the stability parameters, including appearance, particle size, PDI, zeta potential, pH and the content of PF were determined as a function of storage time.

2.4. The design of experiment in vivo

2.4.1. Splenocytes culture

The mouse spleens were aseptically removed from the sacrificed mice by using scissors and forceps in 0.1 M cold PBS, gently homogenized, and passed through a 40 μm nylon cell strainer to obtain single-cell suspensions. Erythrocytes were lysed with red cell lysis buffer (0.5 M ammonium chloride, 10 mM potassium bicarbonate and 0.1 mM disodium ethylene diamine tetraacetic acid, pH 7.2)

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