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Encapsulation of a citrus by-product extract: Development, characterization and stability studies of a nutraceutical with antioxidant and metalloproteinases inhibitory activity

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ABSTRACT

Spray-dried aqueous extract from citrus by-product ("pastazzo", ExO) was studied to evaluate the polyphenols content, the antioxidant properties and the inhibition of metalloproteinases over-expressed in dysmetabolic disease. During *shelf life* studies, ExO changes polyphenols content and decreases the antioxidant properties. Furthermore, Citrus flavonoids are unstable in gastric environment and ExO showed an incomplete *in vitro* dissolution rate in simulated biological fluids, probably due to their low solubility. For these reasons, formulation studies have been performed to develop spray-dried gastro-resistant microsystems for oral administration. Our studies revealed that all the prepared gastroresistant microsystems preserved the polyphenols content, prolonged the *shelf-life* and the antioxidant efficiency of ExO and maintaining its inhibitory effects on metalloproteinases.

1. Introduction

Citrus sinensis (L. Osbeck, var., Tarocco, Moro, and Sanguinello) is the most widespread and commercialized citrus specie in Sicily. It is an important source of flavonoids and anthocyanins, secondary metabolites of plants also known as nutraceuticals or functional compounds, recognized for their antioxidant (Rapisarda et al., 1997) and radical scavenging activities (Escobedo-Avellaneda, Gutierrez-Uribea, Valdez-Fragosoa, Torres, & Welti-Chanesa, 2014). They also have been shown a protective role against metabolic diseases such as obesity and lipid disorders (Mayumi Nakajima, Alves Macedo, & Alves Macedo, 2014) modulating the activity against metalloproteinases overexpress in these pathologies (Bahadoran, Mirmiran, & Azizi, 2013).

Citrus fruits are principally consumed by humans as fresh fruit or processed juice. After juice extraction from the fruit, a residual called "pastazzo" comprised of peel, pulp, rag and seeds still remains. Every year amount of 600 tons of "pastazzo" are produced in Sicily, creating a series of issues related to the cost of the production process and placement of these residual materials (Calabretta & Intrigliolo, 2007). In part, these problems could be overcome by allocating large amounts of this product in commercial sectors different from the traditional food industry such as the nutraceutical, so to obtain products with high added value. In particular, the *Citrus sinensis* has aroused great interest because of its potential beneficial effects on human health, such as anti-diabetic and, hypocholesterolemic activities (Titta et al., 2009). Unfortunately, the absorption from the gastro-intestinal tract of flavanones predominant in *Citrus* species is slow and irregular, as a result of their very slight solubility in water, slow dissolution rate and instability in the gastric environment *in vivo* (Lauro, De Simone, Sansone, Iannelli, & Aquino, 2007; Sansone et al. 2009; Sansone, Picerno, et al., 2011).

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Therefore, to obtain a nutraceutical with high added value reusing a waste product of food industry, the purpose of the present research is the design and development of a "dietary supplement" of spray-dried aqueous extract (ExO) from "pastazzo" actives on metabolic disorders such as the dyslipidemic pathologies. In order to confirm the high value added, we evaluated the polyphenol components and the antioxidant properties (ORAC assay). The amount of inhibition of extracellular enzymes overexpresses in presence of high levels of cholesterol and triglycerides such as metalloproteinase MMP-2 and -9, (Derosa et al., 2007) was also studied. Furthermore, to improve the oral bioavailability of unformulated ExO, the dietary supplement was formulated as spray-dried gastro-resistant microsystems.

In the literature only few works are reported concerning spray-dried gastroresistant microsystems of pure citrus polyphenols (Lauro, Maggi, Conte, De Simone, & Aquino, 2005; Lauro et al., 2007; Sansone et al., 2009; Sansone, Picerno, et al., 2011); in addition, no information is given about these type of microsystems containing herbal extract and their ORAC and MMPs inhibitory activity.

The spray drying technique was chosen because of its capacity to decrease the water content and activity, ensuring a great microbiological stability of products in food industry, avoiding the risk of chemical and/or biological degradations, reducing the storage and transport costs (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007).

Among the most suitable coating materials for this application, we selected cellulose acetate phthalate (CAP), a polymer with a pH-dependent solubility used to protect the extract in the gastric environment (Sansone et al., 2009; Sansone, Picerno, et al., 2011). Tween 60 and Tween 80 (Ibrahim, El-Faham, Mohammed, & El-Eraky, 2011; Mingzhong, Ning, & Ke, 2013) were tested to enhance the dissolution rate in the intestinal medium. The influence of different parameters such as the polymer concentration and the drug/polymer ratio on particle yield, distribution and morphology were investigated. Solid state, dissolution properties and *shelf life* of extract loaded microparticles were also studied.

2. Material and methods

2.1. Materials

Spray-dried aqueous citrus by-product extract (ExO) was produced by ORTOGEL S.p.A. (Belpasso (CT), Sicily, Italy), cellulose acetate phthalate (CAP) was supplied by Eastman® Kodak (Kingsport, Tennessee, United States); Tween 60 (Tw60), Tween 80 (Tw80), Hesperidin (Hd), Narirutin (Nr), Kuromanin chloride (Cyanidine-3-glucoside, CG), Fluorescein (FL), AAPH (2,2′ azobis (2-methylpropionamide) dihydrochloride), 97%, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid) were purchased from Sigma–Aldrich S.r.l. (Milan, Italy). OmniMMP Fluorescent Substrate Mca-Pro-Leu-Gly-Leu- Dpa-Ala-Arg-NH_2, MMP-9 (refolded) (human) (recombinant) (Catalytic Domain) and MMP-2 (catalytic domain) (human) (recombinant) were purchased from Vinci-Biochem S.r.l. (Firenze, Italy). All the other chemicals used in the study were of analytical grade and were obtained commercially.

2.2. Qualitative and quantitative analyses of polyphenols content of *Citrus aurantium* spray-dried aqueous waste extract (ExO)

2.2.1. Qualitative analyses

The analyses were performed according to Picerno et al. (2011) with slight modifications. 1 mg of ExO was suspended in 1 mL of MeOH. The suspension was centrifuged at 3000 rpm for 5 min and then filtered with 0.45 μm filters. A part of supernatant was separated by HPLC (Agilent 1100 series system; Model G-1312 pump; Rheodyne Model G-1322A loop (20 μl); DAD G-1315 detector; 150 × 3.9 mm i.d. C18 μ-Bondapack column). The flow rate was 1.0 mL min−1. All constituents were expressed as μg/mL. ***Bioflavonoids.*** Mobile phase: water (solvent A), and methanol (solvent B). Elution gradient: 0 → 5 min, 15 → 30% B; 5 → 10 min, 30 → 35% B, 10 → 20 min, 35 → 50% B, 20 → 30 min, 50 → 75% B; 30 → 35 min, 75 → 95% B; 35 → 40 min, 100% B. DAD detector set at λ 283 nm. ***Anthocyanins.*** Mobile phase: water/acetonitrile/formic acid 87:3:10 (v/v/v) (System A), and water/acetonitrile/formic acid 40:50:10 (v/v/v) (System B). Linear gradient: 0 min, 6% B; 20 min, 20% B; 35 min, 40% B; 40 min, 60% B; 45 min, 90% B; 55 min, 6% B. (Hillebrand, Schwarz, & Winterhalterj, 2004). DAD detector set at λ 520 nm.

2.2.2. Quantitative analyses

2.2.2.1. Total polyphenol content. The polyphenol content was determined by UV/Vis spectrometry (1 mg of ExO raw was solubilized in 5 mL of water and analysed at λ 310 nm) and HPLC analyses (see below) according to Picerno et al. (2011) with slight modifications. Each analysis was made in triplicate. The total phenols were expressed as mg of Hd per 100 mg dry weight (mg bioflavonoids/100 mg extract dw, mean ± S.D. of three determinations).

2.2.2.2. Total monomeric anthocyanin (TMA) content. Total monomeric anthocyanin (TMA) content was determined using the pH-differential method (Lee, Durst, & Wrolstad, 2005) approved by AOAC (Official Method 2005.02). Two dilutions were performed on each sample. The first used potassium chloride (0.025 M) at pH 1.0 and the second was with sodium acetate (0.4 M) at pH 4.5. Absorbance was measured at 520 and 700 nm and the difference in absorbance and wavelengths was used to calculate anthocyanin content as CG as follows:

$$\left(A \times MW \times DF \times 10^3\right) \Big/ \varepsilon \times l$$

where $A = (A_{520nm} - A_{700nm})$ pH 1.0 $- (A_{520nm} - A_{700nm})$ pH 4.5; MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside (cyd-3-glu); DF = dilution factor established in D; l = pathlength in cm; ε: molar extinction coefficient, in 26,900 $L \times cm^{-1} \times mol^{-1}$ for CG; and 10^3 = factor for conversion from g to mg. The TMA content was reported as mg of anthocyanins per 100 mg dry weight (mg anthocyanins/100 mg dw, mean ± S.D. of three determinations).

2.2.3. ExO solubility

Solubility of the powders was determined in distilled water (2.6 g/L) and in simulated biological fluids, gastric fluid, pH 1.2 (2.5 g/L), and intestinal fluid, pH 7.5 (2.7 g/L) without enzymes (USP 37) by UV/Vis spectrometry at λ 310 nm and expressed as Hesperidin equivalents. Each analysis was made in triplicate.

2.3. Microparticles preparation

In preliminary experiments, 1% or 2% (w/v) of CAP was dissolved in simulated intestinal fluid (IF, pH 7.5, without enzymes, according to USP 37), then ExO in 3:1 polymer: extract weight ratios was suspended in the polymeric solutions under magnetic stirring (Sansone, Picerno, et al., 2011) to give ExO1 and ExO2 microsystems. In the second set of experiments, 0.1% w/v of Tween 60 or Tween 80 as enhancers of dissolution rate were suspended in 2% w/v CAP aqueous buffer feed solution with 3:1 polymer/extract ratio to obtain CAPExOTw60 microparticles. As control, CAP-free