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Antioxidant properties, anti-hepatocellular carcinoma activity and hepatotoxicity of artichoke, milk thistle and borututu

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ABSTRACT

Cynara scolymus (artichoke), *Silybum marianum* (milk thistle) and *Cochlospermum angolensis* (borututu) are three plants widely used regarding hepatoprotective effects but to the best of our knowledge no anti-hepatocellular carcinoma activity has been studied in the most consumed forms: infusions and dietary supplements. Herein, antioxidant properties, anti-hepatocellular carcinoma activity and toxicity of infusions and dietary supplements of the mentioned plants were evaluated and compared. All the samples revealed antioxidant properties with EC_{50} values lower than the daily recommended dose, but infusions showed higher biological activity than dietary supplements. Borututu infusion gave the highest antioxidant activity ($EC_{50} \leq 170$ μg/mL), and also revealed anti-hepatocellular carcinoma activity ($GI_{50} = 146$ μg/mL) without toxicity in non-tumour liver cells ($GI_{50} > 400$ μg/mL). Artichoke infusion also presented antitumour activity ($GI_{50} = 52$ μg/mL) but with toxicity for normal cells at a higher concentration ($GI_{50} = 72$ μg/mL). The antioxidant and antitumour properties were positively correlated with phenolics and flavonoids content. Overall, among the three studied species, borututu infusion proved to be the most complete sample regarding antioxidant and anti-hepatocellular carcinoma activity.

1. Introduction

Artichoke (*Cynara scolymus* L.) is an herbaceous perennial plant native to the Mediterranean Basin that belongs to the Asteraceae family (Bianco, 2005). Consumed raw, boiled, steamed or fried, this plant is today widely cultivated all over the world for its large fleshy immature inflorescences, called heads or capitula, with edible leaves (bracts) and receptacle. Artichoke is known since ancient times as a tasty plant that can be used in soups, stews and salads, being perceived as a nutritious and healthy vegetable (Lattanzio et al., 2009) due to its antioxidant and hepatoprotective effects (Gebhardt and Fausel, 1997; Zapolska-Downar et al., 2002; Jimènez-Escrig et al., 2003; Wang et al., 2003; Falleh et al., 2008; Kukić et al., 2008; Gouveia and Castilho, 2012). This plant contains very little fat and high levels of minerals, vitamin C, fibre, inulin, polyphenols hydoxycinnamates and flavones, but most of its activity could be related to the polyphenolic fraction, mainly composed of mono- and dicaffeoylquinic acids and flavonoids (Schütz et al., 2004; Falleh et al., 2008; Lutz et al., 2011; Pandino et al., 2011; Gouveia and Castilho, 2012).

Milk thistle (*Silybum marianum* (L.) Gaertn) is also a member of Asteraceae family. It is an annual to biennial herbaceous plant, native to the Mediterranean countries with reported effects against hepatotoxicity and acute and chronic liver diseases, due to its main pharmacological active ingredient silymarin (standard mixture of flavonoligans) (Giese, 2001; Zuber et al., 2002; Doehmer et al., 2011). Beyond the hepatoprotective activity, this flavonoid complex also has antioxidant properties which seem to be related to liver damage prevention (Nencini et al., 2007; Shaker and Mahmoud Mnaa, 2010). Silibinin, the major active constituent of silymarin, has been reported as inhibitor of tumour growth in hepatocarcinoma cell (Brandon-Warner et al., 2010) and animal (Bousserouel et al., 2012) models.

Borututu (*Cochlospermum angolensis* Welw.) is a widespread tree in Angola that belongs to the Cochlospermaceae family. Its bark infusion is used in the traditional medicine of Angola for the treatment of hepatic diseases and for the prophylaxis of malaria (Poppendieck, 1981; Presber et al., 1991; Silva et al., 2011). Phenolic composition of borututu hydromethanolic and aqueous extracts were recently characterized and revealed to be rich in methyl ellagic acid and ellagic acid, respectively (Ferreres et al., 2013). The infusion of its dry roots also showed high 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity (Costa et al., 2012).

Despite the described hepatoprotective effects of the three mentioned plants and a few reports on their anti-hepatocellular

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carcinoma activity (Miccadei et al., 2008; Brandon-Warner et al., 2010), studies with the most consumed forms (infusions and dietary supplements) are scarce. Hepatocellular carcinoma is a major health problem with more than 660,000 new cases per year worldwide, being a rapid fatal disease with a life expectancy of about 6 months from the time of diagnostics; it has the third highest mortality rate among all cancers (Jemal et al., 2011). In the present work, infusions and dietary supplements of artichoke, milk thistle and borututu were prepared and studied for antioxidant properties, anti-hepatocellular carcinoma activity and hepatotoxicity in non-tumour liver primary cells. Furthermore, all these properties were correlated with the content in bioactive compounds.

2. Materials and methods

2.1. Samples and samples preparation

2.1.1. Samples

C. scolymus L. (artichoke), *S. marianum* (L.) Gaertn (milk thistle) and *C. angolensis* Welw. (borututu) are plants widely used for their antioxidant and hepatoprotective properties. For the present study, artichoke, milk thistle and borututu were obtained from an herbalist shop in Bragança (Portugal), as dry material for infusions preparation (leaves, plant and bark, respectively; the taxonomical identification of plant species mentioned in the labels was confirmed) and dietary supplements (pills based-on plant and roots, in the case of borututu). All the samples were prepared according to the label and submitted to an evaluation of bioactive compounds (phenolics and flavonoids), antioxidant activity, anti-hepatocellular carcinoma activity and hepatotoxicity.

2.1.2. Infusions preparation

A dry weight of each material (20 g of artichoke and 8.5 g of milk thistle corresponding to the recommended 4 spoons; and 10 g of borututu) was added to 1 L or 0.5 L (in the case of borututu) of boiling distilled water, left to stand at room temperature for 10 min, filtered under reduced pressure, frozen, lyophilized and redissolved in distilled water at a final concentration of 10 mg/mL. The following dilutions were used in the biological assays: 10 mg/mL–1.22 μg/mL for antioxidant activity assays; 400, 100, 25, 6.25 and 1.56 μg/mL for hepatotoxicity assays.

2.1.3. Dietary supplements preparation

Three pills of each sample (1500 mg) were dissolved in 100 mL of distilled water (final concentration 15 mg/mL). Several dilutions of each sample were prepared to perform further assays. The following dilutions were used in the biological assays: 15–0.23 mg/mL for antioxidant activity assays; 400, 100, 25, 6.25 and 1.56 μg/mL for hepatotoxicity assays.

2.2. Standards and reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, USA). Gallic acid, catechin, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), ellipticine, phosphate buffered saline (PBS), acetic acid, sulforhodamine B (SRB), trichloroacetic acid (TCA) and Tris were purchased from Sigma (St. Louis, USA). Foetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin–EDTA (ethylenediaminetetraacetic acid), nonessential amino acids solution (2 mM), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively) and DMEM (Dulbecco's modified Eagle medium) were from Hyclone (Logan, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Quantification of total bioactive compounds

Total phenolics were estimated by Folin–Ciocalteu colorimetric assay according to procedures previously described (Batista et al., 2011) and the results were expressed as mg of gallic acid equivalents (GAE) per g of sample (lyophilized infusion or pill).

Total flavonoids were determined by a colorimetric assay using aluminium trichloride, following procedures previously reported (Batista et al., 2011); the results were expressed as mg of (+)-catechin equivalents (CE) per g of sample (lyophilized infusion or pill).

2.4. Evaluation of antioxidant activity

The antioxidant properties were evaluated by four different tests as there is no universal method that can measure the antioxidant capacity of all samples accurately and quantitatively: DPPH radical-scavenging activity, reducing power, inhibition of β-carotene bleaching and inhibition of lipid peroxidation using TBARS (thiobarbituric acid reactive substances) in brain homogenates (Rafael et al., 2011). The results were expressed in EC_{50} values (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay). Trolox was used as positive control.

2.4.1. DPPH radical-scavenging activity

This methodology was performed using an ELX800 microplate Reader (Bio-Tek Instruments, Inc; Winooski, USA). The reaction mixture on 96 well plate consisted in the sample solutions (30 μL) and methanolic solution (270 μL) containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to stand for 30 min in the dark, and the absorption was measured at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation: $\%RSA = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample, and A_{DPPH} is the absorbance of the DPPH solution.

2.4.2. Reducing power

The sample solutions (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and potassium ferricyanide (1% (w/v), 0.5 mL). The mixture was incubated at 50 °C for 20 min, and trichloroacetic acid (10% (w/v), 0.5 mL) was added. The mixture (0.8 mL) was poured in the 48 wells plate, the same with deionized water (0.8 mL) and ferric chloride (0.1% (w/v), 0.16 mL), and the absorbance was measured at 690 nm in the Microplate Reader mentioned above.

2.4.3. Inhibition of β-carotene bleaching

A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two millilitres of this solution were pipetted into a round-bottom flask. The chloroform was removed at 40 °C under vacuum and linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into test tubes containing sample solutions (0.2 mL). The tubes were shaken and incubated at 50 °C in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm. β-Carotene bleaching inhibition was measured by the formula: (β-carotene absorbance after 2 h/initial absorbance) × 100.

2.4.4. TBARS assay

Porcine (*Sus scrofa*) brains were obtained from official slaughtered animals, dissected, and homogenized with Polytron in an ice cold Tris–HCl buffer (20 mM, pH 7.4) to produce a 1:2 (w/v) brain tissue homogenate which was centrifuged at 3000 × *g* for 10 min.