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Effect of in vitro digestion of yerba mate (*Ilex paraguariensis* A. St. Hil.) extract on the cellular antioxidant activity, antiproliferative activity and cytotoxicity toward HepG2 cells

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ABSTRACT

The aim of this study was to evaluate the influence of the in vitro gastrointestinal digestion of yerba mate extract on the cellular antioxidant activity (CAA) and cytotoxic and antiproliferative action toward HepG2 cells. The total reducing substances, isolated phenolic acids, total flavonoids and total antioxidant activity of the yerba mate extract decreased significantly after in vitro digestion. The digested extract promoted lower CAA and inhibition of HepG2 cell proliferation when compared to the non-digested extract ($P < 0.05$). No significant difference was observed in the cytotoxicity assay. Dose–response correlations were found for both yerba mate extracts for CAA, antiproliferative activity and cytotoxicity. The simulated gastrointestinal digestion of yerba mate extract promoted a significant decrease in the phytochemical content, total antioxidant activity, CAA and inhibition of HepG2 cell proliferation. Nevertheless, digested yerba mate extract still has considerable CAA and antiproliferative activity, which merits further investigation in in vivo studies.

1. Introduction

Several biochemical mechanisms are involved in the genesis and development of cancer, including oxidative stress, which is induced by free radicals and causes DNA damage (Ames & Gold, 1991). It has been suggested that this potentially cancer-inducing oxidative damage, as well as cancer formation or progression, might be prevented or limited by dietary phytochemicals (Liu, 2013). Studies have demonstrated that dietary phytochemicals possess complementary and overlapping mechanisms of action for cancer prevention, including free radical scavenging and oxidative stress reduction, inhibition of cell proliferation, induction of cell differentiation, inhibition of oncogene expression, induction of tumor suppressor gene expression, regulation of cell cycle, and induction of apoptosis (Liu, 2004, 2013; Liu & Finley, 2005). In this context, the consumption of plant-based foods rich in bioactive phytochemicals is encouraged.

Yerba mate (*Ilex paraguariensis* A. St. Hil.), a plant species native to the subtropical region of South America, is an important plant food rich in antioxidant compounds, which may act against the development of cancer. Due to its increasing consumption, commercial availability and potential health benefits, yerba mate is becoming popular in North America and Europe (de Mejia, Song, Heck, & Ramirez-Mares, 2010). Various pharmacological properties of this plant have been attributed to the presence of phenolic compounds, mainly chlorogenic acid (de Morais et al., 2009). Previous in vitro and in vivo studies have

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led to reports on the antioxidant effects (Bassani, Nunes, & Granato, 2014; Boaventura et al., 2012; Boaventura, Di Pietro, et al., 2013; Boaventura, Murakami et al., 2013; Bravo, Goya, & Lecumberri, 2007), and the chemopreventive properties of yerba mate (de Mejia, Young, Ramirez-Mares, & Kobayashi, 2005; Ramirez-Mares, Chandra, & de Mejia, 2004). However, the antioxidant effect of yerba mate on cancer cells is still unknown. In addition, reports on the cytotoxic and antiproliferative action of yerba mate toward cancer cells are also scarce (Ramirez-Mares et al., 2004). Wolfe and Liu (2007) developed an assay to determine the cellular antioxidant activity (CAA) in relation to HepG2 human hepatocellular liver carcinoma cells, which is considered an alternative and more biologically relevant approach to measure the CAA of extracts. This is an assay for screening antioxidants in which the cellular uptake, distribution and efficiency of protection against peroxyl radicals under physiological conditions are considered (Wolfe & Liu, 2007). In this context, the measurement of the antioxidant activity of yerba mate extracts applied to cell cultures could provide an important tool for screening the potential bioactivity of this plant in relation to cancer.

It is well known that flavonoids and phenolic acids are highly metabolized after ingestion and gastrointestinal absorption, usually being transformed into plasma metabolites with lower antioxidant activity than the precursor molecules (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004). Although the bioavailability of yerba mate in plasma and tissues is still unclear, we hypothesized that the concentration of the bioactive compounds in yerba mate extract might change after gastrointestinal digestion, the first step of metabolism, modifying its biological effects in vivo. In this regard, in vitro methodologies that simulate the digestive processes in the gastrointestinal tract have been carried out as a simple and faster approach to in vivo trials (Miller, Schricker, Rasmussen, & Van Capen, 1981; Siracusa et al., 2011). The aim of the present study was to assess the CAA, cytotoxicity and antiproliferative activity toward HepG2 human liver cancer cells of a yerba mate extract submitted to in vitro simulated gastrointestinal digestion. The effect of the in vitro digestion of the yerba mate extract on its total reducing substances, total flavonoid content, and the total antioxidant activity of the extracts was also verified.

2. Materials and methods

2.1. Chemicals

Methanol (MeOH), hydrochloric acid (HCl), acetic acid, acetone, phosphate-buffered saline (PBS), sodium carbonate, and potassium phosphate were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). Ethanol (EtOH, anhydrous, 100%), sodium borohydride ($NaBH_4$), chloranil, vanillin, butylated hydroxytoluene (BHT), bile extract (from porcine), pepsin (from porcine stomach mucosa), pancreatin (from porcine pancreas), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), fluorescein disodium salt, 6-hydroxy-2,5,7,8-tetramethylchoman-2-carboxylic acid (Trolox), Folin–Ciocalteu reagent, quercetin dehydrate, ethyl acetate, 1-butanol, 5-*O*-caffeoylquinic acid, 3,5-dicaffeoylquinic acid, 3,4-dihydroxybenzoic acid and *p*-coumaric acid were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Tetrahydrofuran (THF), aluminum chloride ($AlCl_3 \cdot 6H_2O$) and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Pittsburgh, PA). 2,2-Azobis(2-amidinopropane) (ABAP) was purchased from Wako Chemicals USA, Inc. (Richmond, VA). Gallic acid was purchased from ICN Biomedical Inc. (Costa Mesa, CA). HepG2 human liver cancer cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Williams' Medium E (WME) and Hanks' Balanced Salt Solution (HBSS) were purchased from Gibco Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA). All chemicals used in this study were of analytical grade.

2.2. Yerba mate extract preparation

Leaves of yerba mate (*I. paraguariensis* A. St. Hil.) were harvested in Catanduvas-SC/Brazil, in June 2012. The leaves were oven-dried in a forced-air oven (FABBE, 171, São Paulo-SP, Brazil) for 24 h at 45 °C and ground in a knife mill (Marconi, MA-580, Piracicaba-SP, Brazil) before the extraction of phytochemicals.

Phytochemicals from yerba mate leaves were extracted according to a modified previously reported method (Wolfe & Liu, 2003). Briefly, 10 g of dried and milled yerba mate leaves were homogenized with 50 mL of 80% chilled acetone in a Virtis High Speed Homogenizer (VirTis Co., Gardiner, NY) for 10 min. The mixture was vacuum filtered through a no. 1 Whatman filter paper. Solvent in the filtrate was evaporated using a rotary evaporator at 45 °C until the weight of the evaporated filtrate was <10% of the weight of the original filtrate. The samples were then resuspended with water to give a final volume of 25 mL and stored at −40 °C until use. All extractions were performed in triplicate.

2.3. Gastrointestinal in vitro digestion

The in vitro digestion was conducted applying the method described by Miller et al. (1981) and modified by Liu, Glahn, and Liu (2004). Briefly, 2.25 mL of yerba mate extract, in triplicate, was mixed with saline (140 mM NaCl, 5 mM KCl, and 150 μM BHT) to give a final volume of 9 mL and acidified to pH 2.0 with 0.1 M/1 M HCl. The sample was then mixed with 0.25 mL of pepsin solution (0.2 g of pepsin in 5 mL of 0.1 M HCl) and incubated in a shaking water bath at 37 °C for 1 h. After gastric digestion, the pH of the digestate was increased to 6.9 with 0.1 M/1 M $NaHCO_3$. Further intestinal digestion was performed with the addition of 1.25 mL of pancreatin–bile solution (0.45 g of bile extract and 0.075 g of pancreatin in 37.5 mL of 0.1 M $NaHCO_3$) and incubated in a shaking water bath at 37 °C for 2 h. The total digestate was adjusted to give a final volume of 12 mL with saline. The digestate was stored at −70 °C until analysis.

2.4. Determination of total reducing substances

The total reducing substances of yerba mate samples were determined using the Folin–Ciocalteu colorimetric method described by Singleton, Orthofer, and Lamuela-Raventos (1999) with modifications (Wolfe & Liu, 2007; Wolfe et al., 2008). Briefly, yerba mate aqueous extracts were reacted with Folin–Ciocalteu reagent and then neutralized with sodium carbonate. After 90 min, the absorbance of the resulting solution was measured at 760 nm (Molecular Devices, Sunnyvale, CA). Gallic acid was used as the standard and the results were expressed as g% of gallic acid equivalents (GAE) per 100 g of sample. Data were reported as the mean ± SD for at least three replicates.

2.5. Determination of total flavonoids content

The total flavonoids content of the yerba mate extract was determined using the sodium borohydride/chloranil-based assay (He, Liu, & Liu, 2008). Briefly, a volume of 4 mL of extract was added to a test tube, the solvent was evaporated under nitrogen gas and the sample reconstituted in 1 mL of tetrahydrofuran/ethanol (THF/EtOH, 1:1, v/v). Catechin standards (0.1–10.0 mM) were freshly prepared before use in 1 mL of THF/EtOH (1:1, v/v). Next, 1 mL of 50 mM $NaBH_4$ solution and 0.5 mL of 74.6 mM $AlCl_3$ solution were added into each test tube with 1 mL of sample solution or 1 mL of catechin standard solution. The test tubes were shaken in an orbital shaker at room temperature for 30 min. An additional 0.5 mL of 50.0 mM $NaBH_4$ solution was added to each test tube and shaking was applied for a further 30 min at room temperature. After this period, 2.0 mL of chilled 0.8 M acetic acid solution was added to each test tube and the solutions were kept in the dark for 15 min after thorough mixing. One milliliter of 20.0 mM chloranil was added to each tube, which was kept at 95 °C