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Effects of green tea, yerba mate and rooibos tea on C6 Astroglial cells

Trabalho de Conclusão de Curso

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Abstract

Ethnopharmacological relevance: Camellia sinensis, *Aspalathus linearis* and *Ilex paraguariensis* are distributed throughout several continents, such as China, Africa and South America, where they are widely consumed as teas and are known for their antioxidant potential. Several studies discuss green tea properties and its high antioxidant capability, however, it becomes necessary to investigate the effect of infusions of other traditionally used species that are widely consumed as teas.
Aim of study: To analyze the phenolic content and cytotoxicity of yerba mate (*I. paraguariensis*), green tea (*C. sinensis*) and rooibos (*A. linearis*) infusions which possibly will advance the understanding and treatment of a number of neurodegenerative diseases
Materials and methods: The effects of aqueous infusions of *C. sinensis* (CS), *A. linearis* (AL) and *I. paraguariensis* (IP) had their polyphenolic content evaluated by Folin–Ciocalteu test and expressed as gallic acid equivalents (GAE%). Cellular viability of the C6 glial cells exposed to infusions at different concentrations (10-500 μg/mL) for 1 h or 24 h, were evaluated by measuring MTT reduction.
Results: The total polyphenolic content observed in CS is higher than in the IP and AL (19. 5; 17.1 and 16.9 GAE%, respectively), AL having a lower polyphenolic content. There was no significant differences observed regarding cell viability in the 1 h treatment. In the 24 h treatment, it was verified that AL and CS significantly decreased C6 viability only at a higher concentration, starting from 450 and 500 μg/mL, respectively. CS also showed a significant increase in C6 viability when compared to control, at 250 μg/mL. On the other hand, IP significantly decreased cell viability at low concentrations (starting from 200 μg/mL).
Conclusion: This study has shown that various compounds present in the aqueous extracts of CS and AL, with emphasis on the polyphenols, might have beneficial effects and should be further evaluated for the development of a health-promoting functional beverage for the prevention or treatment of neurodegenerative diseases.

Keywords: Infusion, *Camellia sinensis*, *Aspalathus linearis*, *Ilex paraguariensis*, Cytotoxicity, Phenolic content

1. Introduction

Dementia can be defined as a progressive and chronic dysfunction of the cortical and subcortical brain activity, resulting in a complex cognitive decrease (Ritchie & Lovestone, 2002). The most common types of dementia are: Alzheimer's disease (AD), frontoparietal dementia and vascular dementia (Shagam, 2009), the first one being the most common with 50-70% of the cases, affecting around 40% of the individuals above 85 years old (Ferri *et al.*, 2005). AD can be considered one of the major threats to human health especially with an aging population (Blennow *et al.,* 2006; Cummings, 2004).

Clinically, AD is characterized by an irreversible and progressive cognitive decline with significant deficits in the ability of the individuals to form new memories and recall recent events. However, even before the appearance of the cognitive deficit it is possible to observe an increase in the production of reactive oxygen and nitrogen species (RONS), decrease in the quantity of reduced glutathione and glucose consumption (Sharma & Gupta, 2002; Duelli *et al.*, 1994).

Given that the medicines currently used to treat the disease have a single target and their effects are limited, such as the acetylcholinesterase inhibitors (AChE) (Maggini *et al.*, 2006), the search for potential therapeutic multi-target agents that enable to reach several targets of the disease at once is of paramount importance (Zhang, 2005). In this sense, medicinal plants are considered a potential new source for multipotent agents for neurodegenerative diseases such as AD, due to their complex and diverse chemical composition, lower toxicity and higher multi-target activity (Ke *et al.*, 2016). Accordingly, herbal drug extracts exerting multi-potent effect may have their efficacy explained due to a possible synergistic mechanism among the multiple compounds present in comparison to a single isolated product, which can make phytopreparations pharmacologically and therapeutically superior (Wagner & Ulrich-Merzenich, 2009). Moreover, the study of medicinal plants and their active principles have proven to be a useful tool in the identification of new and potential drugs relevant for the treatment of cognitive disorders such as AD.

Some epidemiological studies have shown that the high consumption of specific foods, such as green tea (*Camellia sinensis* L. (Kuntze) (source of epigallocatechin-3-gallate, EGCG), wine (resveratrol), olive oil (omega 3) and "curry" (curcumin) are inversely associated with AD incidence (Kuriyama *et al.*, 2006; Ng *et al.*, 2006; Scarmeas *et al.*, 2006; Truelsen *et al.*, 2002). Green tea , a traditional beverage widely consumed around the world, is rich in polyphenols, such as ellagic acid, flavonoid, flavanones and proanthocyanidins, saponins and triterpenes. It also contains gallic acid, catechins, xanthines and caffeine (Liang *et al.*, 2001). Among the biologically active components present on green tea, catechins can be considered the most important ones, being EGCG the most studied. It is known that EGCG has a potential effect against AD, such as inhibiting the aggregation of Aβ (Porat *et al.*, 2006), attenuating the generation of Aβ through the activation of α-secretase (Obregon *et al.*, 2006; Rezai-Zadeh *et al.*, 2005), β-secretase inhibition (Jeon *et al.*, 2003) and reduction in the expression of the amyloid protein precursor (Reznichenko *et al.*, 2006), it also inhibits monoamine oxidase (MAO) (Mazzio *et al.*, 1998).

In addition to EGCG, green tea also has other catechins such as (-)-epicatechin (EC) and (-)-epicatechin-3-gallate (ECG), which also have an anti AD multipotent action, such as inhibitory activity of Aβ aggregation (Porat *et al.*, 2006), ROS capture and metallic ions chelation (Rice-Evans *et al*., 1996).

Several studies discuss green tea properties and its antioxidant capability, however, it becomes necessary to investigate the effect of infusions of other vegetable species that are widely used popularly, as the interest on them, both wide public and academic community, have been growing. For this reason, It is believed that both yerba mate (Gugliucci & Stahl, 1995) and rooibos tea (Ito *et al.*, 1991; Komatsu *et al*., 1994; Yoshikawa *et al.*, 1990) may also be beneficial to health.

Mate is an infusion of Yerba Mate (*Ilex paraguariensis* A. St.-Hil.), similar to tea, traditionally consumed among various countries of Latin America such as Argentina, Uruguay and south of Brazil (Small & Catling, 2001). It is known to contain phenolic compounds such as caffeic acid, caffeoylquinic acid, chlorogenic acid , feruloylquinic acid, quercetin, tannins , rutin , caffeine and theobromine (Burris *et al*., 2012). Furthermore, the consumption of flavonoids has been associated with the prevention of several age-related chronic diseases (Weisburger, 2002; McKay and Blumberg, 2002).

Additionally, among the medicinal properties that were already investigated *in vitro* and *in vivo* for *I. paraguariensis* extracts, are the stimulation of the central nervous system (Gosmann *et al.*, 1989), antioxidant (Miranda et al., 2008) and protective effect against induced DNA damage (Miranda et al., 2008). Moreover, it is known that quercetin is capable of reducing the peroxide levels and cellular death in glioma C6 cells treated with H_2O_2 and chemical anoxia (Chen *et al*., 2006). Quercetin can also inhibit the process of formation of free radicals in three different stages: in the beginning of the interaction with peroxide ions, in the hydroxyl radicals formation (iron ions chelation) and in the lipidic peroxidation (reaction with lipid peroxyl radicals) (Afanas'ev *et al.*, 1989). Overall, a higher antioxidant activity has been observed in infusions of *I. paraguariensis* when compared to green tea and black tea (Chaves and Maiocchi, 2002).

And finally, the third medicinal infusion studied was Rooibos (*Aspalathus linearis* (Brum. f.) R. Dahlgren), an endemic plant of South Africa (Dahlgren, 1968), which has a high content of flavonoids and isoflavonoids (specially pterocarpans), known for their anti-inflammatory, anticlastogenic and antioxidant capability (Selvam *et al*., 2004; Njamen *et al*., 2003; Maurich *et al*., 2004; Erasto *et al.*, 2004; Miyase *et al*., 1999). Rooibos tea has small quantities of (+)-catechins (Marais, 1996), being aspalathin, a dihydrochalcone, one of the main and most important compounds found in the unfermented rooibos tea, however, most of it is oxidised during fermentation (Joubert, 1996) being transformed to flavanones and unknown polymeric substances (Marais, 1996). Aspalathin has several therapeutic properties such as antioxidant, anti-inflammatory and anti-carcinogenic effects, hepatoprotective and phytoestrogenic (Joubert & De Beer, 2011). Moreover, it has antispasmodic effects, modulates the immune system, has anti-proliferative effects, vasodilatory effects, anti-aging properties (Joubert *et al.*, 2008). These effects are attributed to phenolic composition and associated with antioxidant activity (Niwa & Miyachi, 1986).

Considering the importance of the study of multifunctional agents in the search for adjuvant therapies that assist in the prevention or reversal of neurodegenerative damage caused by phosphorylation imbalance in the brain, the objective of this project was to analyze the antioxidant role of yerba mate, green tea and rooibos infusions which possibly will advance the understanding and treatment of a number of neurodegenerative diseases, not only AD.

2. Methods and materials

2.1. Plant materials and Chemicals

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO,USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle medium (DMEM) and other materials for cell culture were purchased from Gibco BRL (Carlbad, CA, USA). A sample of organic green tea (*Camellia sinensis* L. (Kuntze) (Chás Campo Verde Ltda.) was

purchased at a local supermarket. Fermented rooibos tea (*Aspalathus linearis* (Brum. f.) R. Dahlgren) was purchased at a speciality tea shop (Tea Shop, Porto Alegre - RS) and organic maté (*Ilex paraguariensis* A. St.-Hil.) (Madrugada Alimentos, Venancio Aires - RS) was purchased at local supermarket. All other chemicals were purchased from common commercial suppliers.

2.2. C6 Astroglial Cell Culture

The C6 astroglial cell line was obtained from the American Type Culture Collection (Rockville, MA, USA) and was cultured according to a previously described procedure. The cells were seeded in asks and cultured in DMEM (pH 7.4) containing 5% FBS, 0.1% amphotericin B and 0.032% gentamicin. Cells were maintained at a temperature of 37ºC in an atmosphere of 5% CO_2/95% air. At log phase, cells were detached from the culture asks using 0.05% trypsin/ethylene-diaminetetracetic acid (EDTA) and seeded (56103 cells/cm^2) in 96-well plates.

2.3. Preparation of aqueous tea infusions

Tea extracts were prepared by pouring boiling distilled water onto the dry leaves in a proportion of 1:60 (w/v), since a typical cup of brewed tea is made using 1-2 g of tea leaves in 100 mL of hot water (Lambert & Elias, 2010), the leaves were statically macerated for 30 min (von Gadow *et al*., 1997) Subsequently, the extracts were filtered in qualitative paper and brought down to room temperature. Aliquots of the extracts were kept frozen (-18°C) until they were lyophilized and kept in a desiccator until further use.

2.4. Total phenolic content of the extracts

The total phenolic content of the infusions were determined by the classic colorimetric assay using Folin–Ciocalteu reagent in 96-well microplate. The assay was performed as described (Zhang et al., 2006) with some modifications. Briefly, the absorbance of the solution was measured after incubation in the dark at room temperature during 90 min, at a wavelength of 750 nm in a Spectramax i3 spectrophotometer. The results are expressed as gallic acid equivalents.

2.5. *Cellular viability: MTT Assay*

Cells were seeded into 96-well plates for 24 h, followed by treatment with different concentrations of each extract (10μg/mL-500μg/mL) for a further 24 h, or 1 h, respectively. During all treatments, the cells were maintained at 37 ºC in an atmosphere of 5% CO_2/95% air. Cells were incubated with 50 mg/mL MTT for 30 min, at 37 ºC in 5% CO_2/95% air. Subsequently, the medium was removed and the MTT crystals were dissolved in dimethylsulfoxide (DMSO). Absorbance values were measured at 560 nm and 650 nm. The results are expressed as percentages relative to control conditions (Bobermin et al., 2012).

2.6. Statistical analysis

Data are presented as mean ± S.E.M. Each experiment was performed in triplicate from two independent cultures. The data were subjected to one-way analysis of variance (ANOVA) followed by the Dunnett's test. Values of $P< 0.05$ were considered significant. All analyses were performed using the GraphPad Prism 6 software.

3. Results

3.1. Total phenolic content

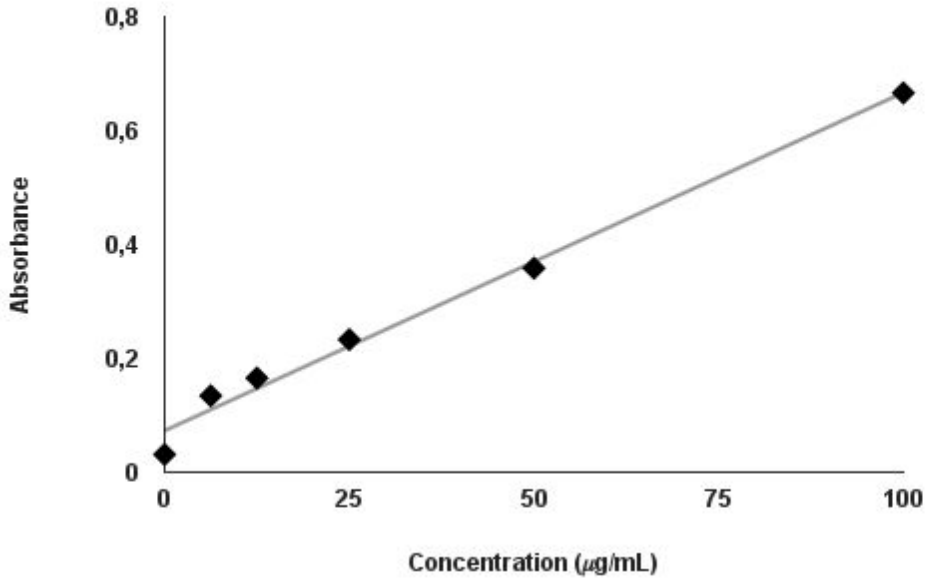

Fig. 1: Absorbance curve versus concentrations for the gallic acid. The curve is described by the equation $y=0.059x-0.0729$ ($R^2=0.988$).

Table 1: Polyphenol content of aqueous extracts of *A. linearis*, *C. sinensis* and *I. paraguariensis* in gallic acid equivalent (%).

Species	**GAE%**
Aspalathus linearis	16,9
Camellia sinensis	19,5
Ilex paraguariensis	17,1

The Folin–Ciocalteu test is an established method to give a rough estimate of the total polyphenolic content. The calibration curve showed good linearity between gallic acid concentration and absorbance. The calibration curve of standard gallic acid solutions is shown in Figure 1. The polyphenol content of aqueous CS, AL and IP extracts can be observed in Table 1. Concentrations observed were multiplied by the dilution factor of the original sample. Therefore, by the Folin–Ciocalteu test, it is possible to observe that the total polyphenolic content in CS is higher than in the IP and AL, the last one having a lower polyphenolic content.

3.2. MTT assay

Cellular viability of the C6 glial cells exposed to rooibos (AL), green tea (CS) and yerba mate (IP) extracts (10-500 μg/mL) for 1 h or 24 h, were evaluated by measuring MTT reduction. As shown in Figure 2A, in the 1 h treatment there were no significant differences observed, however it is possible to observe that IP slightly decreases C6 viability at 500 μg/mL (Fig. 2A). In the 24 h treatment, it was verified that AL and CS significantly decreased C6 viability only at a higher concentration, starting from 450 and 500 μg/mL, respectively (Fig. 2B), when compared to their respective control groups. CS also showed a significant increase in C6 viability when compared to control, at 100 μg/mL (Fig. 2B). On the other hand, IP significantly decreased cell viability at low concentrations (starting from 200 μg/mL), when compared to control, Fig. 2B.

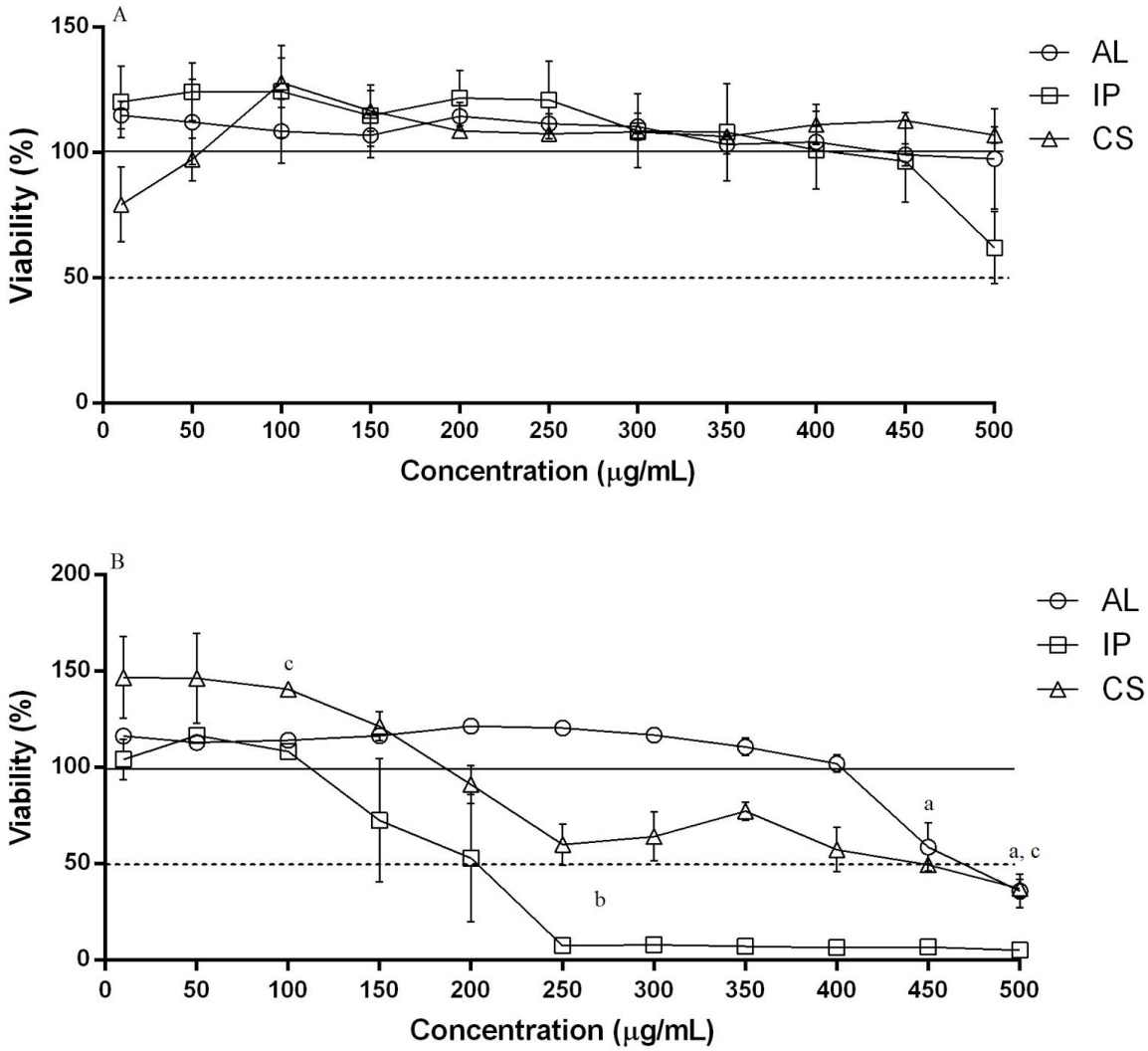

Fig. 2: (A) Cell viability curve of C6 cells when exposed to different concentrations (10-500 μg/mL) of aqueous extracts of rooibos (AL), green tea (CS) and yerba mate (IP), during 1 h treatment. (B) Cell viability curve of C6 cells when exposed to different concentrations (10-500 μg/mL) of aqueous extracts of rooibos (AL), green tea (CS) and yerba mate (IP), during a 24 h treatment. (a) AL significant difference, (b) IP significant difference, (c) CS significant difference, all when compared to their respective control.

4. Discussion

In the present study, the effects of aqueous infusions of CS, AL and IP had their phenolic content and cytotoxicity analysed. The main findings of this study are that CS and IP presented a high phenolic content when compared to AL. CS polyphenols content is in agreement with the values found in the literature, 21.02 to 14.32% GAE (Anesini et al., 2008). However, the AL and MT polyphenols content was lower than the the values found in the literature, those being 35,6% GAE and 3g/l, respectively (Bixby *et al*., 2005; von Gadow *et al*., 1997). In this sense, one has to bear in mind that the content in polyphenols can vary due to the proportion of drug material per mL of water used for each infusion which were, in the cited studies (1:20), higher than in our study (1:60).

Other important findings of the present study are that CS and AL infusions decreased cell viability only at high concentrations. IP, known for its potential anti-inflammatory (Montanha *et al.*, 1990) and antioxidant effects (Miranda et al., 2008), has decreased the cell viability starting at relatively low concentrations (200 μg/mL). These results are in agreement with the ones found by Ramirez-Mares *et al.* (2004), that showed IP extracts dominant cytotoxicity when compared to CS. Those results may be due to *I. paraguariensis* saponins content (352 µg/mL in the aqueous extract) (Gnoatto *et al.*, 2005), since cytotoxic effects can be related to several saponins due to their ability to stimulate apoptotic process in tumor cells, usually through its intrinsic pathway (Podolak *et al.*, 2010). Moreover, non apoptotic processes can also be involved in saponin cytotoxic activity, such as cell cycle arrestment, autophagic cell death stimulation, inhibition of metastasis and cytoskeleton disintegration (Podolak *et al.*, 2010). Therefore, the saponins present on the *I. paraguariensis* infusions might have affected C6 cell viability due to C6 astroglial cell line tumor-like characteristics, since the cell line derived from rats brain tumors induced by *N*,*N*'-nitrosometilurea (Benda *et al.*, 1968).

Furthermore, AL infusions cell viability decrease only at high concentrations, probably due to its antioxidant, anti-inflammatory and anti-carcinogenic effects (Joubert & De Beer, 2011). Such effects can be attributed to AL phenolic composition and also associated with its high antioxidant activity (Niwa & Miyachi, 1986). However, insufficient data are available on the dietary exposure to rooibos phenolic compounds (Joubert et al., 2009) and further studies are needed to enable the connection between potential health benefits of the AL to its phenolic content.

Finally, another important finding was the significant increase on C6 cell viability when exposed to CS at the concentration of 250 μg/mL. Those results may be due to the potential antioxidant capability of CS, which might be related to its polyphenols content (Cotelle, 2001), making green tea a promising candidate to therapies that assist in the prevention or reversal of neurodegenerative damage.

5. Conclusion

Neurodegenerative diseases are multifactorial, and strategies for treating these diseases need to include a variety of interventions directed at multiple targets. On the basis of the current results, we suggest that various compounds present in the aqueous extracts of CS and AL, with emphasis on the polyphenols, might have beneficial effects. Thus they be further

evaluated for the development of a health-promoting functional beverage for the prevention or treatment of neurodegenerative diseases in which oxidative stress and astrocytic cell death play a role. It remains to be elucidated why IP decreased cell viability and which chemical components could be the ones affecting cell viability.

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