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Cytotoxic / antioxidant activity and sensorial acceptance of yerba-mate development by oxidation process

Alice Teresa Valduga*, Itamar Luís Gonçalves, Ana Cláudia Piovezan Borges, Albanin Aparecida Mielniczki-Pereira and Ana Paula Picolo

*Universidade Regional Integrada do Alto Uruguai e das Missões, Erechim, Rua Sete de Setembro, 1621, 99700-000, Erechim, Rio Grande do Sul, Brazil. *Author for correspondence. E-mail: valice@uricer.edu.br*

ABSTRACT. Yerba-mate (*Ilex paraguariensis* St. Hil.) is a native species of South America, and its dried leaves are consumed mainly as a beverage. Its phytochemical profile includes phenolic compounds consisting of secondary metabolites with antioxidant activities. Current research optimizes conditions of yerba-mate leaves oxidation, evaluates the sensory acceptance and assesses the *in vivo* antioxidant activity of the oxidized product. The variables incubation chamber humidity and age of leaves were optimized within the yerba-mate leaves oxidative process by a 2^2 factorial design. The sensory acceptance and the antioxidant activity of yerba-mate extracts, oxidized by the yeast *Saccharomyces cerevisiae* exposed to hydrogen peroxide, were assessed. The variables chamber humidity and age of leaves affected the colorimetric coordinates L* and b* during the oxidative process, and validated empirical models. The minimum concentration of beverage-type yerba-mate extract which was able to enhance the cell survival of *S. cerevisiae* was 150 $\mu g \cdot mL^{-1}$, whilst rate for oxidized product reached 1,200 $\mu g \cdot mL^{-1}$. Extracts from the two processing forms increased the cell survival in *S. cerevisiae* exposed to 5 mM hydrogen peroxide. The sensory acceptance of oxidized product did not differ significantly when compared to control. The antioxidant activity of yerba-mate extracts should be exploited for the development of new products.

Keywords: oxidized yerba-mate, antioxidant activity, organoleptic proprieties.

Atividade citotóxica / antioxidante e aceitação sensorial de erva-mate desenvolvida por processo de oxidação

RESUMO. A erva-mate (*Ilex paraguariensis* St. Hil.) é uma espécie nativa da América do Sul, que tem como principal forma de consumo o chimarrão. Apresenta em seu perfil fitoquímico compostos fenólicos, metabólitos secundários com atividade antioxidante, o que representa uma condição capaz de estimular o potencial a ser explorado com sua utilização no desenvolvimento de novos produtos. Este trabalho teve por objetivo otimizar as condições de oxidação de folhas de erva-mate, avaliar a aceitação sensorial e a atividade antioxidante *in vivo* do produto oxidado. Foram otimizadas as variáveis umidade da câmara de incubação e idade da folha no processo oxidativo de folhas de erva-mate, através de um planejamento fatorial 2^2. A aceitação sensorial, e a ação antioxidante de extratos de erva-mate tipo chimarrão e oxidada na levedura *Saccharomyces cerevisae* exposta ao peróxido de hidrogênio foram avaliados. As variáveis idade da folha e umidade da câmara exerceram efeito sobre as coordenadas colorimétricas L* e b* durante o processo oxidativo, validando modelos empíricos. A concentração mínima do extrato de erva-mate tipo chimarrão capaz de melhorar a sobrevivência celular em *S. cerevisiae* foi 150 $\mu g \cdot mL^{-1}$, e para o produto oxidado este valor correspondeu a 1.200 $\mu g \cdot mL^{-1}$. Extratos obtidos a partir das duas formas de processamento foram capazes produzir aumento de sobrevivência de *S. cerevisiae* exposta ao peróxido de hidrogênio 5 mM. A aceitação do produto oxidado não se diferiu significativamente em relação ao controle. A atividade antioxidante dos extratos de erva-mate consiste em um potencial a ser explorado no desenvolvimento de novos produtos.

Palavras-chave: erva-mate oxidada, atividade antioxidante, propriedades organolépticas.

Introduction

Yerba-mate is a native species of South America which is cultivated in Brazil, Argentina and Paraguay (

Burris, Harte, Davidson, Stewart, & Zivanovic, 2012). There are several studies on the biological effects produced by metabolites present in the yerba-mate phytochemical composition (Dartora et al., 2013; Pimentel et al., 2013; Bravo et al., 2014).

Phenolic compounds, saponins, methylxanthines, sugars and vitamins occurs in yerba-mate (Jacques,

Santos, Dariva, Oliveira, & Caramão, 2007; Heck & de Mejia, 2007; Burris et al., 2012; Hao et al., 2013). In fact, phenolic compounds show antioxidant activity *in vitro* (Nenadis & Tsimidou, 2002) and *in vivo* (Su, Wang, & Liu, 2009).

Different methodologies have been developed to measure *in vitro* and *in vivo* antioxidant activity (Alam, Bristi, & Rafiquzzaman, 2013). The first modality involves assays whose execution is simple and fast, but may not produce results capable of extrapolating the physiological conditions (Soares, Andreazza, & Salvador, 2005). In fact, assays using the eukaryotic yeast *Saccharomyces cerevisiae* have been employed to evaluate the antioxidant activity of flavonoids and vegetable extracts (Soares et al., 2005; Guarienti, Bertolin, & Costa, 2010; Peinado et al., 2013).

The phytochemical profile of yerba-mate and its effects on physiological systems is a condition capable of stimulating its use in the development of food product (Godoy et al., 2013). The oxidation of leaves of the *I. paraguariensis* is one of the alternatives to insert yerba-mate in food, similar to the processing of *Camellia sinensis* in black tea. Since black tea is the most consumed beverage worldwide, after water (Namita, Mukesh, & Vijay, 2012), the processing of yerba-mate leaves is a way of inserting antioxidants in the diet of a population that does not usually consume yerba-mate. In this context, this work aimed to develop a functional food from the oxidation of yerba-mate leaves.

Material and methods

Samples

Yerba-mate leaves were collected from a homogeneous crop, located in the municipality of Barão de Cotegipe, Rio Grande do Sul State, Brazil, 27º37'15 S and 52º22'47 W, at an altitude of 765 m.

Optimization of oxidation conditions

Oxidation conditions were established in a 2^2 factorial design by which the effects of the variables (i) humidity of chamber, and (ii) age of leaves, were evaluated on the values of L★, a★ and b★ colorimetric coordinates (Table 1).

Table 1. Values of central composite design 2^2 for the development of the oxidation process of mate leaves.

Variables		-1	0	1
Relative humidity (%)	x_1	80	90	99
Age of leaves (months)	x_2	1	6.5	12

The leaves were dehydrated during 2 hours in an air-circulation oven, at 30ºC. They were then rolled by hand (placed in a cotton cloth and pressed) at room temperature (25ºC) for 5 min. (Figure 1) and placed in a climatic chamber at 26°C and relative humidity as defined by factorial design for 3 hours. Thereafter, the leaves were dried in a fixed-bed dryer with hot air at 70ºC for 120 min. Dried leaves were crushed in a blender and sorted in a 2.36 mm sieve.

During the oxidative process, the color was evaluated at 0, 3, 6, 12, 24 and 48 hours with a colorimeter (Minolta CR 400), with CIELAB system scales, where L★ is the luminosity ranging between 0 (black) to 100 (white), and the parameters a★ and b★ (-a = green, +a = red; -b = blue, +b = yellow). Further, conditions for 80% humidity in the chamber and 12 months for the age of leaves were employed for sensory analysis and for the evaluation of *in vivo* antioxidant activity.

Leaf processing to obtain mate-type yerba-mate

Some leaves were submitted to a mate-type yerba-mate processing, which involved the following steps: (i) roasting at 180ºC for 5 minutes at 20 rpm in a prototype laboratory equipment, previously described (Valduga, Finzer, & Mosele, 2003); (ii) drying in an air-circulation oven at 70ºC, for about 12 hours (moisture < 3%); (iii) trituration in a knife mill, followed by homogenization in a Tyler-series sieve with mesh 16 (< 2.36 mm). Figure 2 describes the yerba-mate processing forms employed.

Figure 1. Oxidation of yerba-mate leaves. (a) previously dehydrated leaves; (b) material in a wrung cloth; and (c) the leaves after the rolling process.

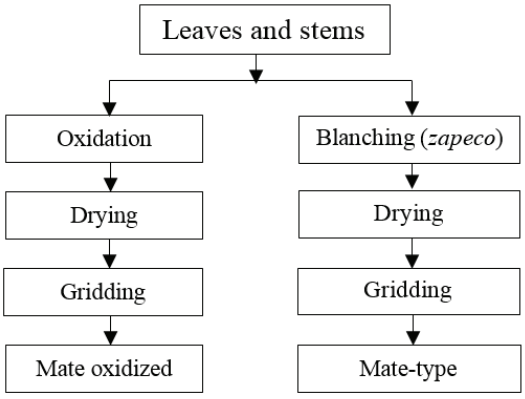

Figure 2. Flowchart describing the processing forms to obtain mate-type and oxidized yerba-mate.

Obtaining extracts

Approximately 200 g of each sample (mate-type processed yerba-mate and oxidized yerba-mate) were submitted to aqueous extraction (1,000 mL) in a reflux for 2 hours, with three replications. After the extraction, the solid was filtered and the extracts were concentrated and lyophilized. The lyophilized extracts (10 g) were solubilized in distilled water/ethanol, (3/1 v/v) and stored at -20ºC for 6 hours for the precipitation of insoluble compounds. Samples were centrifuged (8,500 rpm, 20 min., 4ºC) and supernatant was concentrated under reduced pressure and lyophilized (Dartora et al., 2011).

Assessment of cytotoxic and antioxidant activity

As a preliminary step, the cytotoxic potential of oxidized and processed yerba-mate was evaluated through the survival rate of *S. cerevisiae* cells exposed to extract concentrations of 0, 150, 600 and 1,200 $\mu g \cdot mL^{-1}$. For *in vivo* antioxidant activity measurements, the same assay was performed with hydrogen peroxide 5 mM.

The *S. cerevisiae* yeast was pre-inoculated from an isolated colony in YEL liquid medium (1% yeast extract, 2% peptone, 2% glucose). The cells were incubated in phosphate-buffered saline (PBS; 3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl, pH 7.4) for 90 minutes at 30°C, at 150 rpm. Cells were diluted and plated on solid YEPD (1% yeast extract, 2% peptone, 2% glucose, 2% agar) and kept in an oven at 30ºC for 3 days so that the surviving colonies could be counted.

Sensory analysis

Aqueous infusions of mate-type and oxidized yerba-mate (8 $g \cdot L^{-1}$) were prepared. The assays were performed with 40 semi-trained male and female tasters, aged between 18 and 30 years, by applying a seven-point graphic hedonic scale whose extremes corresponded to extremely dislike (1) to extremely like (7). The samples were served in plastic cups, with a random 3-digit code, concomitantly with a cracker and water for palate cleansing between samples.

Statistical analysis

The survival percentage of *S. cerevisiae* cells submitted to different concentrations of yerba-mate extracts and scores, attributed to the samples in sensory test, were compared by variance analysis followed by *Tukey*'s test at 5% significance level, with software GraphPad Prism 6.0.

Results

Effect of variables on color

Table 2 represents L⋆ values at the start and after 6h of exposure to conditions defined by factorial design. Responses at 3, 12 and 24h are not represented since they were not affected by the studied variables. The analysis of variance yielded a calculated F (6.63) that was higher than the tabulated F (3.0), thus validating an empirical model ($p < 0.05$) for color parameter L⋆, as represented in Figure 3-A. Younger leaves showed a low brightness index when submitted to oxidation. The lowest lightness was obtained at 90% humidity and leaf age of 6.5 months. Response was significantly affected only by the quadratic term of the variable age of the leaf (Figure 3-B).

Table 2. Matrix of the 2^2 experimental design (real and coded values), with response color parameter L⋆ at 0 and 6h of oxidation.

Run	Factors		Color L⋆	
	X_1	X_2	0h	6h
1	-1 (80)	1 (12)	19.28	21.88
2	1 (99)	1 (12)	19.91	20.66
3	0 (90)	1 (12)	14.31	19.10
4	-1 (80)	-1 (1)	17.04	20.05
5	1 (99)	-1 (1)	18.90	19.70
6	0 (90)	-1 (1)	17.98	18.00
7	1 (99)	0 (6.5)	20.46	18.04
8	-1 (80)	0 (6.5)	16.43	18.24
9	0 (90)	0 (6.5)	15.10	18.21
10	0 (90)	0 (6.5)	17.98	18.01
11	0 (90)	0 (6.5)	18.76	18.80

The color parameter b⋆ was significantly influenced by the studied factors, which suggested a tendency for yellowing in the samples at 3h (Table 3). The analysis of variance yielded a 0.80 correlation coefficient, and the calculated F (9.31), which was higher than tabulated F (3.0), resulted in the validation of an empirical coded model, as Figure 4-A shows. Increase in relative humidity produced an increase in the color

parameter b* only for the young leaves, possibly due to the loss of chlorophyll in the sample. However, when the mature leaves (12 months) were subjected to a different incubation humidity (80%), the effect of color degradation was less intense. Except for the quadratic term of the humidity of chamber variable, all the terms significantly affected the response (Figure 4-B). The studied factors did not statistically ($p < 0.05$) influence color parameter a*.

Antioxidant and cytotoxic effects on *S. cerevisiae*

Cell concentration that produced the highest survival percentage in preliminary assays was $1{\cdot}10^7$ cells·mL^{-1}. This condition was employed in all tests. Extracts of mate-type and oxidized yerba-mate showed no cytotoxic effects on *S. cerevisiae* cells since they did not decreased the survival percentage of cells exposed to different extract concentrations. Increase in survival was statistically significant at all tested mate-type concentrations (Figure 5-A), whereas this result was observed only with 1.200 μg·mL^{-1} with oxidized yerba-mate (Figure 5-B).

Table 3. Matrix of the 2^2 experimental design (real and coded values) with the responses color parameter b* at 0 and 3h of oxidation.

Run	Factors		Color b*	
	X_1	X_2	0h	3h
1	-1 (80)	1 (12)	8.06	8.92
2	1 (99)	1 (12)	7.24	8.29
3	0 (90)	1 (12)	9.12	8.30
4	-1 (80)	-1 (1)	7.81	8.14
5	1 (99)	-1 (1)	8.28	10.08
6	0 (90)	-1 (1)	7.86	9.37
7	1 (99)	0 (6.5)	9.20	10.22
8	-1 (80)	0 (6.5)	6.87	8.92
9	0 (90)	0 (6.5)	8.16	9.83
10	0 (90)	0 (6.5)	7.86	9.37
11	0 (90)	0 (6.5)	8.35	9.24

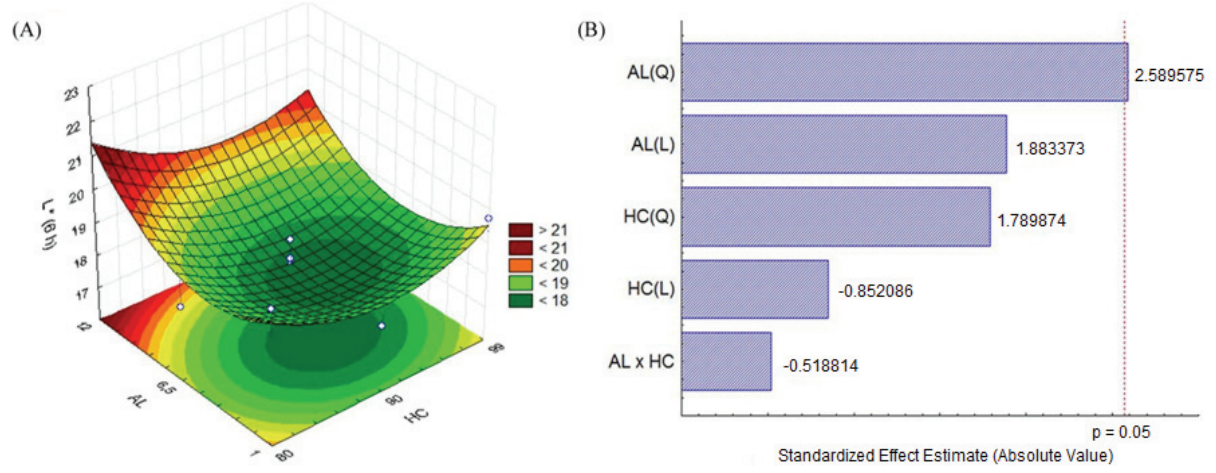

Figure 3. Effects of variable on colorimetric coordinate L* during the oxidative process at 6 h. (a) Response surface for color L* as a function of humidity of chamber (x) and age of leaves (y). (b) Pareto chart. AL: age of leaf; HC: humidity of chamber.

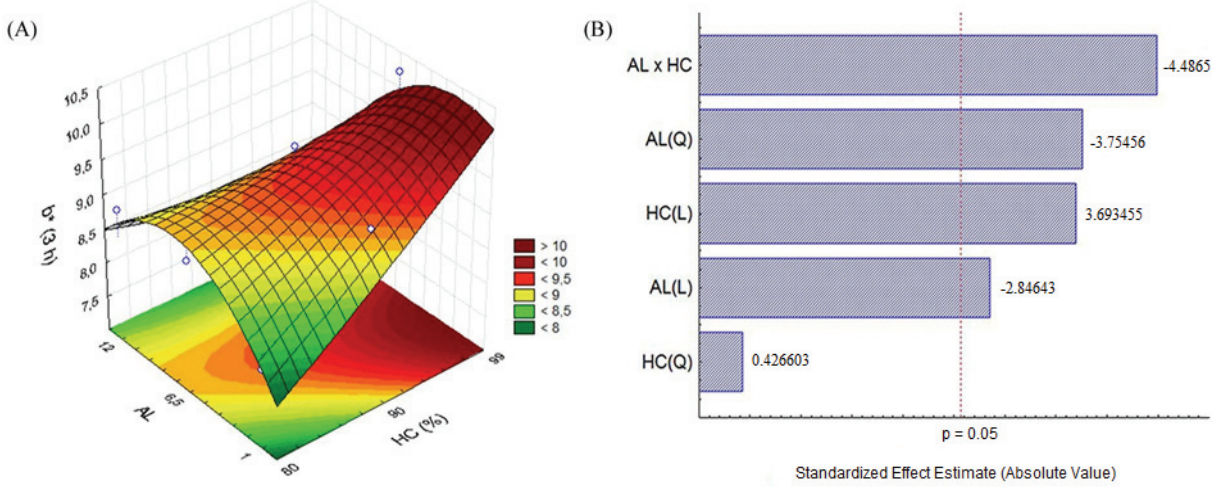

Figure 4. Effects of variable on the colorimetric coordinate b* during the oxidative process at 3h. (a) Response surface for color b* as a function of the humidity of chamber (x) and age of leaves (y). (b) Pareto chart. AL: age of leaf; HC: humidity of chamber.

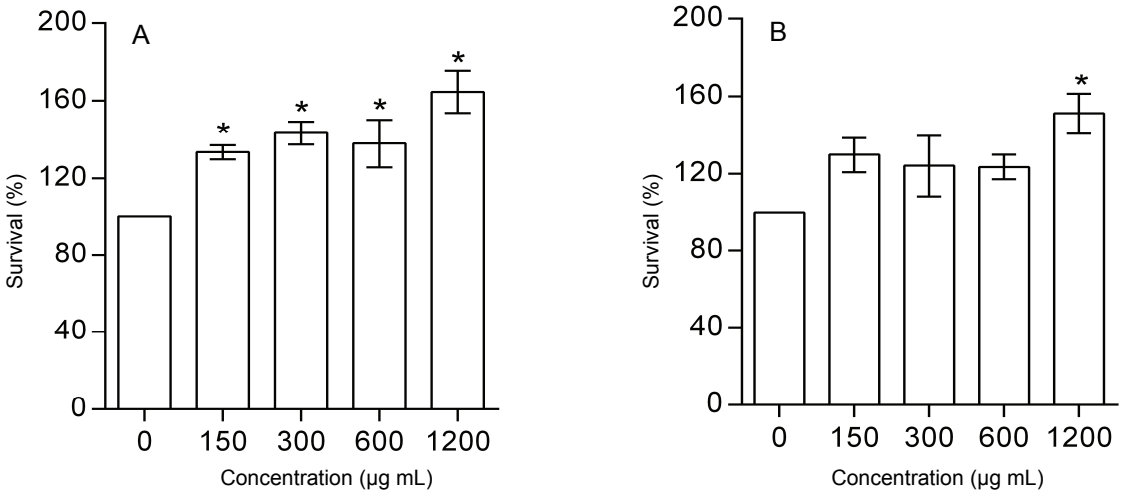

Figure 5. Effect of yerba-mate extracts on cell survival of *S. cerevisiae*. (a) Effect of mate-type yerba-mate extracts, and (b) effect of oxidized yerba-mate extracts. Results are expressed as mean ± standard error, (⋆) $p > 0.05$ compared with control according to analysis of variance followed by Tukey's test.

The mate-type yerba-mate extract (control) had a protective effect on the cells of *S. cerevisiae* against the oxidative stress induced by hydrogen peroxide at concentrations 150 and 600 $\mu g \cdot mL^{-1}$. However, the oxidized yerba-mate extract produced a significant protection only at 150 $\mu g \cdot mL^{-1}$ (Figure 6).

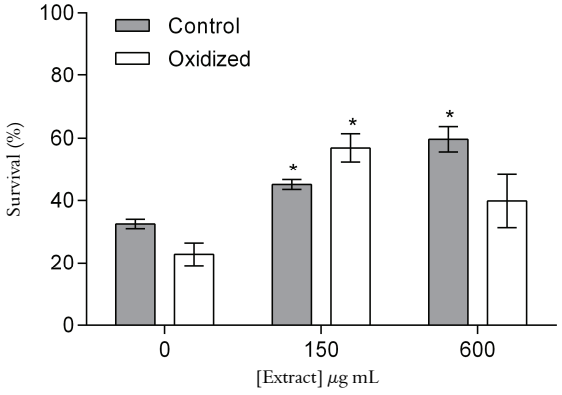

Figure 6. Effect of mate-type (control) and oxidized yerba-mate on survival of cells of *S. cerevisiae* exposed to 5 mM hydrogen peroxide. Results are expressed as mean ± standard error, (⋆) $p > 0.05$ compared to control according to analysis of variance followed by Tukey's test.

Sensory acceptance

A lower level of sensory acceptance was associated with black tea when compared to mate-type yerba-mate infusion ($p < 0.05$). Sensory acceptance of yerba-mate oxidized infusion was not statistically different when compared to mate-type yerba-mate (Figure 7-A). There was no difference in color and odor for the analyzed infusions (Figures 7-B and 7-C).

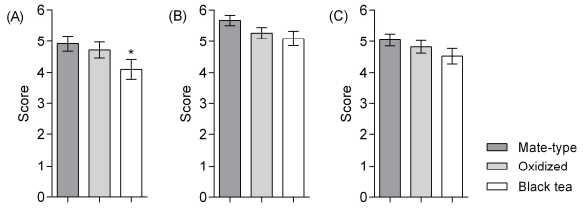

Figure 7. Scores attributed to infusions of yerba-mate mate-type, oxidized and black tea compared to flavor (a), color (b) and odor (c). (⋆) $p < 0.05$ compared to mate-type.

Discussion

Yerba-mate color is affected by processing. Approximately 80% of the chlorophyll content in yerba-mate leaves undergo degradation during the 'zapeco' step (Schmalko & Alzamora, 2001). UV light was utilized to produce vegetable pigments degradation in processed yerba-mate (Lewinski et al., 2015). Colorimetric parameter and plant pigment changes also occurred during the yerba-mate shelf-life. In a previous study, the parameters L⋆, a⋆ and b⋆ as well as chlorophylls and pheophytins showed changes among consecutive measurements. A strong positive correlation was reported between decreased levels of chlorophyll and a change in the a⋆ parameter to a value of zero (Cabral-Malheiros, Hecktheuer, Canto, & Balsamo, 2010). In results reported in current assay, lower L⋆ values were registered in younger leaves submitted to oxidation. Behavior could be due to more intense enzymatic activity in these leaves.

The differences in *S. cerevisiae* cell viability may be explained by the fact that yerba-mate contains multiple nutrients that may be used by yeast. Increase in cell survival was reported in the

two extracts, which may be due to the presence of carbohydrates in the extracts, when compared to control PBS without any carbohydrates. Different mono- and di-saccharides were identified in the two extracts (Dartora et al., 2011). Polysaccharides isolated from yerba-mate leaves revealed activities against murine sepsis (Dartora et al., 2013) and gastric lesion induced by ethanol (Maria-Ferreira et al., 2013). Several studies indicate that other metabolites are present in yerba-mate, such as methylxanthines, flavonoids, phenolic acids, saponins, vitamins, minerals and others (Dartora et al., 2011; Heck & de Mejia, 2007).

Yerba-mate extracts show a high level of phenolic acids and flavonoids. Rutin, followed by kaempferol, is the most abundant flavonoid in yerba-mate (Bojić, Simon Haas, Šarić, & Maleš, 2013). The role of these metabolites in health has been extensively studied during the last years. Yerba-mate extracts reduced the damage by DNA, lipid peroxidation and protein carbonylation in rats skin tissue induced by UVA and UVB radiation (Barg et al., 2014). The yerba-mate phytochemical profile may be greatly explored in the development of food products. Recently, yerba-mate extracts have been used in formulations of yogurt (Preci et al., 2011), gelatin (Berté, Izidoro, Dutra, & Hoffmann-Ribani, 2011) and cereal bars (Chiesa, Schlabitz, & Souza, 2012).

The yeast *S. cerevisiae* has been utilized as an *in vivo* model to assess antioxidant activity. Hydrogen peroxide, apomorphfine and paraquat were used to induce oxidative stress in *S. cerevisiae* cells (Guarienti et al., 2010; Soares et al., 2005). A recent literature review counted 19 *in vitro* and 10 *in vivo* assays to quantify antioxidant activity. The largest fraction of *in vivo* methods involved animals (Alam et al., 2013). The use of *S cerevisiae*, as a model for the evaluation of antioxidant activity, is a manner to obtain reproducible to physiological conditions, albeit without the use of animals.

Conclusion

The variables age of leaves and humidity of chamber affected colorimetric coordinates $L^\star$ and $b^\star$ during the oxidative process of yerba-mate leaves. Cytotoxic effects of oxidized and mate-type yerba-mate extracts on studied cell lineage have not been reported. In the two conditions, extracts were able to stimulate cell survival of *S. cerevisiae* exposed to hydrogen peroxide, whilst oxidized yerba-mate showed a good level of sensory acceptance.

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