NATURAL RESOURCES WITH SWEETENER POWER: PHYTOCHEMISTRY AND ANTIOXIDANT CHARACTERISATION OF STEVIA REBAUDIANA (BERT.), SENSORIAL AND CENTESIMAL ANALYSES OF LEMON CAKE RECIPES WITH S. REBAUDIANA INCORPORATION

RECURSOS NATURAIS COM PODER EDULCORANTE: CARACTERIZAÇÃO FITOQUÍMICA E ANTIOXIDANTE DA STEVIA REBAUDIANA (BERT.), ANÁLISE SENSORIAL E CENTESIMAL DE RECEITAS DE BOLO DE LIMÃO COM INCORPORAÇÃO DE S. REBAUDIANA

RECURSOS NATURALES CON PODER DULCIFICANTE: CARACTERIZACIÓN FITOQUÍMICA Y ANTIOXIDANTE DE STEVIA REBAUDIANA (BERT.), ANÁLISIS SENSORIAL Y CENTESIMAL DE RECETAS DE PASTEL DE LIMÓN CON INCORPORACIÓN DE S. REBAUDIANA

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ABSTRACT

Stevia rebaudiana leaf extracts are calorie-free sweeteners of natural origin, derived from the Stevia rebaudiana plant known as a natural sweetener, which contains steviol glycosides and others bioactive compounds recognized by their biological properties. The present study was designed to evaluate the total phenolics (26.0 mg gallic acid/g) and total flavonoids contents (9.7 mg catechin/g) of a hydroalcoholic extract of Stevia rebaudiana dried leaves. A similar hydroalcoholic extract of commercial powder steviol sweetener was also evaluated, showing lower contents of bioactive compounds (11.9 mg/g and 5.1 mg/g, for total phenolics and flavonoids, respectively). The hydroalcoholic extract of dried Stevia rebaudiana leaves also showed high in vitro antioxidant activity, besides a positive correlation between total phenolic compounds and the DPPH and FRAP assays. Moreover, Stevia rebaudiana leaves have sensory and functional properties superior to those of many other high-potency sweeteners and is likely to become a major source of natural sweetener for the growing food market. Thus, four different lemon cakes formulations were studied (a traditional cake control recipe with sugar, two cakes with incorporation of Stevia rebaudiana fresh leaf and a cake with commercial powder steviol), using a sensory analysis covering 100 untrained consumers. Centesimal composition analyses of the four lemon cakes showed significant differences in fat, ashes, proteins and carbohydrates contents ($p<0.05$). Also, the raised energy value observed for the cake control was superior to the cake with Stevia rebaudiana leaves incorporation (309.8 Kcal/100 g, 268.0 Kcal/100 g,

respectively). Sensorial analysis results showed that Stevia rebaudiana leaves were accepted and, in the future, they can be a natural option to replace some or all the saccharose in cakes formulations.
Keywords: Stevia rebaudiana (Bert.) Bertoni; sweetener; bioactivity; antioxidant capacity; sensorial profile; nutritional analysis; lemon cake.

RESUMO

Os extratos de folhas de Stevia rebaudiana são edulcorantes não calóricos de origem natural, derivados da planta Stevia rebaudiana conhecida como adoçante natural, que contém glicosídeos de esteviol e outros compostos bioativos reconhecidos pelas suas propriedades terapêuticas. O presente estudo foi concebido para determinar o teor de fenólicos totais (26,0 mg de ácido gálico/g) e de flavonoides totais (9,7 mg catequina/g) de um extrato hidroalcoólico de folhas secas de Stevia rebaudiana. Foi também avaliado um extrato hidroalcoólico similar de esteviol em pó, edulcorante comercial, o qual apresentou menores teores de compostos bioativos (11,9 mg/g e 5,1 mg/g, para fenólicos e flavonoides totais, respetivamente). O extrato hidroalcoólico de folhas de Stevia rebaudiana também apresentou elevada atividade antioxidante in vitro, além de uma correlação positiva entre os compostos fenólicos totais e os ensaios DPPH e FRAP. As folhas de Stevia rebaudiana têm propriedades sensoriais e funcionais superiores às de muitos outros edulcorantes e provavelmente tornar-se-ão uma fonte natural importante para o crescente mercado de alimentos edulcorados. Nesse sentido, foram estudadas quatro formulações diferentes de bolo de limão (uma receita de controlo de bolo tradicional com sacarose, dois bolos com incorporação de folha fresca de Stevia rebaudiana e um bolo com steviol em pó comercial), posteriormente submetidos a uma análise sensorial por um painel de 100 provadores não treinados. A análise da composição centesimal dos quatro bolos de limão mostrou diferenças significativas a nível de humidade, teor total de gordura, cinzas, proteínas e hidratos de carbono ($p<0,05$). O valor energético do bolo controlo foi superior ao encontrado no bolo com incorporação de folhas de Stevia rebaudiana (309,8 Kcal/100 g; 268,0 Kcal/100 g, respetivamente). Os resultados da análise sensorial mostraram que as folhas da Stevia rebaudiana foram bem aceites e que, no futuro, podem vir a ser uma opção natural para substituir parte ou a totalidade da sacarose na composição dos bolos.
Palavras-chave: Stevia rebaudiana (Bert.) Bertoni; edulcorante; bioatividade; capacidade antioxidante; perfil sensorial; análise nutricional; bolo de limão.

RESUMEN

Los extractos de hojas de Stevia rebaudiana son edulcorantes no calóricos de origen natural, derivados de la planta Stevia rebaudiana conocida como edulcorante natural, que contiene glucósidos de esteviol y otros compuestos bioactivos reconocidos por sus propiedades terapéuticas. El presente estudio fue concebido para evaluar el contenido de fenólicos totales (26,0 mg de ácido gálico/g) y de flavonoides totales (9,7 mg catequina/g) de un extracto hidroalcohólico de hojas secas de Stevia rebaudiana. También se evaluó un extracto hidroalcohólico similar de esteviol en polvo, edulcorante comercial, el cual presentó menores contenidos de compuestos bioactivos (11,9 mg/g y 5,1 mg/g, para fenólicos y flavonoides totales, respectivamente). El extracto hidroalcohólico de hojas de Stevia rebaudiana también presentó una elevada actividad antioxidante in vitro, además de una correlación positiva entre los compuestos fenólicos totales y los ensayos DPPH y FRAP. Las hojas de Stevia rebaudiana tienen propiedades sensoriales y funcionales superiores a las de muchos otros edulcorantes y probablemente se convertirán en una fuente natural importante para el creciente mercado de alimentos edulcorados. En este sentido, se estudiaron cuatro formulaciones diferentes de pastel de limón (una receta de control de pastel tradicional con sacarosa, dos pasteles con incorporación de hoja fresca de Stevia rebaudiana y un pastel con steviol en polvo comercial), utilizando un análisis sensorial cubriendo un panel de vidrio 100 probadores no entrenados. El análisis de la

composición centesimal de los cuatro pasteles de limón mostraron diferencias significativas con respecto a humedad, teor cantidad total de grasa, cenizas, proteínas e hidratos de carbono ($p<0,05$). El valor energético del pastel controlo fue superior al encontrado en el pastel con incorporación de hojas de Stevia rebaudiana (309,8 Kcal/100 g; 268,0 Kcal/100 g, respetivamente). Los resultados del análisis sensorial mostraron que las hojas de Stevia rebaudiana han sido bien aceptadas y que, en el futuro, podrán ser una elección natural para substituir parte o la totalidad de la sacarosa en la composición de los dos pasteles.

Palabras-clave: Stevia rebaudiana (Bert.) Bertoni; edulcorantes; bioactividad; actividad antioxidante; perfil sensorial; análisis nutricional; pastel de limón.

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INTRODUÇÃO

According to World Health Organization (WHO), the ingestion of foods with high amounts of sugar is directly related with the obesity and, consequently, with the development of diabetes *mellitus*. In recent years, the health authorities have alleged that the increase of the consumption of saccharose will be able to originate several nutritional problems and, consequently, in health. It exists a preoccupying increase of the prevalence of the obesity, diabetes *mellitus* type II and metabolic syndrome in children and adults in the whole world, being the developed countries the ones that have more cases of these illnesses (Sarodnik *et al.*, 2018; Arora *et al.*, 2010. Basu *et al.*, 2013; Brown *et al.*, 2011; Koning *et al.*, 2012; Mohd-Radzman *et al.*, 2013; Yang *et al.*, 2014). According to WHO, diabetes *mellitus* affects ~350 million people in the whole world and, it is expected that the global prevalence of diabetes *mellitus* type II will rise to 592 million in 2035. To minimize this ratio, it is recommended that the consumption of sugar added to foods does not exceed 10% of the total energy of the diet (Whiting *et al.*, 2011; Gelbert, 2014; Grembecka, 2015).

Thus, it is imperative that the consumer prevents the food products highest in sugar, having had in these last decades its substitution for intense sweeteners of low caloric value (Neacsu and Madar, 2014). However, the continuous alimentary additive ingestion has been associated with toxic, genotoxic and neoplastic effects (Demir *et al.*, 2010; Hoobs *et al.*, 2012; Saad *et al.*, 2014). So, food with synthetic sweeteners, such as saccharin, cyclamate, aspartame and acesulfame potassium must be prevented (Durán *et al.*, 2013; Thiyagarajan and Venkatachalam, 2012) For these reasons, the ingestion of natural sweeteners always must be preferential, since that the same one is in balanced dosages. Thus, a new concept of natural sweeteners, without nutritional value, as stevioside, extracted from *Stevia rebaudiana* leaves, appeared in recent years. The use of steviol glycosides as non-caloric sweeteners has proven to be beneficial for patients with diabetes *mellitus* type II, obesity, and metabolic syndrome (Panagiotou *et al.*, 2018). Additionally, Pol *et al*. (2007) refer that bioactive compounds present in *Stevia rebaudiana* don't have teratogenic, mutagenic or carcinogenic effects. Zhang *et al*. (2017) have studied the toxicity of ethanolic extract of *Stevia rebaudiana* Bertoni leaves through a battery of *in vitro* and *in vivo* tests and the results obtained demonstrated that this leaves does not possess adverse effects through oral administration, being safe to potential use in functional foods and nutritional supplements, beyond sweetener. According to European Food Safety Authority (EFSA), *Stevia rebaudiana* Bertoni could be used in a dosage of 4 mg/kg body weight by day (EFSA, 2010). Its sweetening capacity is directly related with the presence of steviol glycosides (stevioside, rebaudioside, dulcoside, rubuside and steviolbioside), that are concentrated in *Stevia rebaudiana* leaves and have a sweetening power 100 to 400 times superior to the one of saccharose (Surana *et al.*, 2006; Abdullateef *et al.*, 2015; Khalil *et al.*, 2014).

Moreover, several clinical studies related antioxidant, antimicrobial, antifungal, antineoplastic, anti-inflammatory and hepatoprotective properties of this vegetal species (Potocnjak *et al.*, 2017; Gupta *et al.*, 2013; Jayaraman *et al.*, 2008; Shivanna *et al.*, 2013; Silva *et al.*, 2008; Gaweł-Bęben *et al.*, 2015; Rao *et al.*, 2014).

The chemical compounds present in *Stevia rebaudiana* leaves assume a basic role in the promotion of the health, having recognized biological properties. Megeji *et al*. (2005) referred that *Stevia rebaudiana* leaf extracts have been used in the treatment of diabetes, helping in the control of the

serum glucose levels. Since dried leaves are edible, characterized for containing vitamins (C and E), minerals (calcium, zinc, potassium, magnesium, sodium and fluorine) and bioactive compounds, such as phenolics, flavonoids, carotenoids and tannins (Ciulua *et al*., 2017; Gawel-Beben *et al*., 2015; Goyal *et al*., 2010), we may conclude that the ingestion of these leaves may be advantageous, not only for its sweetening power, but also for their diversity in biological activity. Muanda *et al*. (2011) had described that quercetin, its aglycone and protocatechuic acid, are the predominant compounds obtained from a *Stevia rebaudiana* leaves aqueous extract. More recently, Gaweł-Bęben *et al*. (2015) have added other important compounds, nominated ferulic acid, chlorogenic acid, catechin, caffeic acid, rosmarinic acid, among others. Ruiz *et al*. (2014) and Abou-Arab *et al*. (2010) have also described considerable contents of chlorophyll a and chlorophyll b in leaf extracts of *Stevia rebaudiana*. Pande and Khetmalas (2011) have described other secondary metabolites, such as alkaloids, saponins and anthraquinones.

The sweet taste is a human desire that dates back for many centuries ago (Teixeira *et al*., 2011). So, the sweeteners used in detriment of the common sugar must supply qualities and identical sensations to the ones of saccharose when present in foods, qualities that are directly related with the reologic and organoleptic properties of the same ones (Bartoshuk, 1991). The artificial sweeteners are ingredients frequently integrant in alimentary diet, since provide intense sweet flavor, with significantly low caloric value (Benton, 2005). For moreover, the authorization of the alimentary additives use in the foodstuffs has been object of a standardisation criteria of the European Union, whose food free circulation in all the countries members has a guideline for a bigger protection of the health of the final consumers (Commission of the European Communities, 2006/0145). For these reasons, the artificial sweeteners can substitute sugar, partial or totally, promoting a superior sweetening power to the one of saccharose (Cagnasso *et al*., 2007).

The consumption of this plant has come to increase in the whole world, being *Stevia rebaudiana* leaf and stevioside (powder) used by food industry, in the production of foods and drinks (Obreiter and Roseno, 2017; Lemus-Mondaca *et al*., 2012). However, the use of *Stevia rebaudiana* in substitution of sugar can have some inconveniences. Rodríguez *et al* (2016) have evaluated the risk of fungal spoilage when *Stevia rebaudiana* was used as sweetener in cakes and breads. Their study suggested that commercial *S. rebaudiana* sugar substitute products alone may not be effective at controlling growth of spoilage fungi in bakery products. Stevioside is usually perceived with a significant bitter aftertaste (Chranioti *et al*., 2016). Mielby *et al.* (2016) have evaluated the sensorial profile of fruit beverages sweetened with *Stevia rebaudiana*. They have concluded that the sensorial characteristics of fruit drinks were affected by *Stevia rebaudiana* taste, but the addition of lime flavor masked the side effect of the aftertaste caused by *S. rebaudiana*. To mask off-flavours of *Stevia rebaudiana*, particularly for sweetener products, it is common to use an encapsulation technique. Chranioti *et al.* (2016) have concluded that the application of spray drying with maltodextrin and inulin as encapsulating agents reduced the bitter aftertaste of steviol glycosides.

For all the above-mentioned reasons, with this study, besides the evaluation of phytochemical and antioxidant properties of *Stevia rebaudiana* leaves, it was intended to study the nutritional composition of four different lemon cake formulations, with incorporation of *S. rebaudiana*, having also been carried through sensorial analysis to inquire the receptivity of the *Stevia rebaudiana* use as sweetener substitute of the sugar by the population.

1. MATERIALS AND METHODS

1.1. CHEMICALS

Chemical reagents sodium carbonate, gallic acid, catechin, sodium nitrite, 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) solution, sodium acetate, glacial acetic acid, iron(III) chloride and iron(II) sulfate were purchased from Sigma Chemical Co. (St. Louis, USA), absolute ethanol from Fisher Chemical (Loughborough, England), Folin-Ciocalteau reagent from Merck (Darmstadt, Germany), aluminium chloride from Panreac (Barcelona, Spain); sodium hydroxide from VWR International (Leuven, Belgium).

1.2. MATERIALS

The *Stevia rebaudiana* plants were supplied by "Cantinho das Aromáticas" (Vila Nova de Gaia, Portugal), harvested at December in 2015. For determination of phenolic and flavonoids content and antioxidant activity *S. rebaudiana* leaves were dried in an oven at 45ºC during 3 days. The *Stevia rebaudiana* powder was obtained in commercial store "Celeiro" (Vila Nova de Gaia, Portugal). The other ingredients used in cake formulations were purchased in a local market (Continente, Vila Nova de Gaia, Portugal).

1.3. *IN VITRO* ANTIOXIDANT ACTIVITY

1.3.1. Hydroalcoholic extracts preparation

Dried *Stevia rebaudiana* leaves (0.5 g) were submitted to solid-liquid extraction with 50 mL of ethanol:water (1:1) at 40 °C for 60 minutes, according to Costa *et al.* (2014). The same procedure was followed with commercial *Stevia rebaudiana* powder. Extracts were filtered through Whatman no. 1 filter paper, kept at -25 °C and lyophilized. These hydroalcoholic extracts, concentrated to 0.1 mg/L, were prepared in triplicate and used for quantification of total phenolics, total flavonoids and antioxidant activity assays.

1.3.2. Total phenolics

Total phenolic content of *S. rebaudiana* leaves extracts were determined according to Vinha *et al.* (2015). Briefly, 500 µL of each extract were mixed with 2.5 mL of the Folin-Ciocalteau reagent (1:10) and 2.5 mL of a sodium carbonate solution (7.5%, m/v). The mixture was first incubated at 45 ºC, during 15 min, followed by 30 minutes incubation at room temperature (~ 20 ºC) before absorbance readings at 765 nm in a microplate reading Synergy HT (Bio Tek Instruments, Synergy HT GENS5, EUA). Total phenolic contents were calculated from a calibration curve prepared with

gallic acid (0-100 mg/L; r = 0.9978) and results expressed as mg of gallic acid equivalents (GAE)/g of sample.

1.3.3. Total flavonoids

The total flavonoid contents of *Stevia rebaudiana* leaves hydroalcoholic extracts were determined according to Rodrigues *et al.* (2013). In a test tube, 1 mL of extract was mixed with 4 mL of distilled water and 300 µL of 5% $NaNO_2$. After 5 minutes, 300 µL of 10% $AlCl_3$ were added, allowing reacting for 1 minute. After this time, 2 mL of 1 M NaOH and 2.4 mL of distilled water were added. Solutions were homogenized and the absorption was measured at 510 nm in a microplate reading Synergy HT (Bio Tek Instruments, Synergy HT GENS5, EUA). Total flavonoid contents were calculated from a calibration curve prepared with catechin (0-450 mg/L; r = 0.9983) and results expressed as mg of catechin equivalents (CE)/g of sample.

1.3.4. DPPH• free radical scavenging assay

DPPH• (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method is an antioxidant assay based on electron-transfer that produces a violet solution in ethanol. This analysis was performed according to Rodrigues *et al.* (2013) with minor modifications. The reaction mixture was prepared directly on a 96 well plate and consisted of a solution of different sample concentrations (14 µL) and an ethanolic solution (186 µL) containing DPPH• radicals (6×10^{-5} mol L^{-1}) in each well. The reduction of the DPPH• radical was determined by measuring the absorption at 517 nm, during 60 minutes until stabilization of the reaction. 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 when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH• solution.

1.3.5. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out according to Costa *et al*. (2014). The method is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}-TPTZ). An aliquot (90 µL) of an extract was added to 270 µL of distilled water and 2.7 mL of the FRAP reagent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10 mM TPTZ solution and 1 part of 20 mM $FeCl_3 \cdot 6H_2O$ solution) and the reaction mixture was incubated at 37 °C. The increase in absorbance at 595 nm was measured after 30 minutes. Solutions of known Fe(II) concentrations ($FeSO_4 \cdot 7H_2O$) were used for calibration. A calibration curve was prepared with ferrous sulphate (linearity range: 0-2000 mg/ L, r = 0.9953), and the results were expressed as mmol of ferrous sulphate equivalents/g of sample material.

1.4. MACRONUTRIENTS ANALYSIS

Macronutrients were analyzed following the Association of Official Analytical Chemists methods. Moisture content was determined using an infrared moisture determination balance (Scaltec® modelo SMO 01, Scaltec Instruments, Alemanha). The ash content was determined by incinerating the sample in a muffle furnace (Thermolyne 48000, F48010- 26, Electrothermal Engineering Ltd, Essex, Reino Unido) at 550 °C (AOAC 920.153). The protein content ($N \times 6.25$) was determined using the Kjeldahl procedure (AOAC 928.08, 2012). Total fat was determined by Soxhlet extraction with petroleum ether (AOAC 991.36, 2012). The total carbohydrate content was determined by difference. Energy was calculated according to the general Atwater factors (Atwater and Benedict, 1902): Energy (kcal) = 4 × (g protein) + 3.75 × (g carbohydrate) + 9 × (g fat). The results are expressed as kcal/100 g of dry mass.

1.5. SENSORIAL ANALYSIS

In order to evaluate the acceptability of *Stevia rebaudiana* powder and leaves as future substitutes of saccharose, four lemon cakes were prepared, under identical conditions (Table 1), with different sweeteners rate: cake A – control (100% saccharose), cake B –steviol powder and saccharose (20 g: 50 g), cake C - *Stevia rebaudiana* fresh leaves and saccharose (20 g: 50 g), and cake D – 100% *Stevia rebaudiana* leaves. Cakes were presented on plastic plates and the sensorial analysis was performed under white light and at approximately 20ºC.

Table 1. Formulations of the four lemon cakes.

INGREDIENTS	QUANTITY			
	CAKE A	CAKE B	CAKE C	CAKE D
STEVIA REBAUDIANA LEAVES	-	-	20 G	20 G
STEVIOL POWDER	-	20 G	-	-
1 LEMON	PEEL	PEEL	PEEL	PEEL
SUGAR	250 G	50 G	50 G	-
EGGS	3	3	3	3
NATURAL YOGHURT	1	1	1	1
MARGARINE	75 G	75 G	75 G	75 G
VANILLA SUGAR	15 G	15 G	15 G	15 G
WHEAT FLOUR	200 G	200 G	200 G	200 G
BAKING POWDER	10 G	10 G	10 G	10 G

A sensory analysis was performed, using 100 untrained consumers, with ages between 10 and 83 years of both genders, using a nine-point hedonic scale (1= extremely bad to 9 = extremely good) regarding six parameters: appearance, aroma, flavor, texture, sweetness and color.

1.7. DATA ANALYSIS

Data regarding centesimal analysis (moisture, ashes, fat, proteins and carbohydrates, all variables in %) were presented as average ± standard deviation of three measurements for each sample. For each variable, the comparison between the four cake recipes was performed using the one-way ANOVA. Upon finding significant differences, the cake (or cakes) significantly different for each variable were detected using a least significant differences (LSD) post-hoc test.

Data regarding the sensorial analysis was ordinal, therefore median and inter-quartil ranges were calculated. Parameters median scores comparison of the four cake recipes were performed using the Friedman's test, and upon finding significant differences, those were identified using paired comparisons with Bonferroni adjusted p-values.

Data analysis were performed using IBM© SPSS© statistics vs23.0 (IBM© Corporation, Chicago, USA), and the level of significance was set to 0.05 for all inference situations.

2. RESULTS AND DISCUSSION

2.1. TOTAL PHENOLICS AND TOTAL FLAVONOIDS CONTENT

The analysis of the results of total phenols quantification allowed verifying that the leaf presented higher content of these compounds, more than the double of the one of the commercial powder (26.0 mg GAE/g and 11.9 mg GAE/g,). These results were expected, because the technological processes necessary to the attainment of the commercial stevioside promote the loss of phytochemicals compounds.

Comparatively with other published works, the results are concordant with other identical studies. Milani *et al*. (2016) reported a content of 22.81 mg/g in total phenols for water leaf extracts, using Folin Ciocalteu reagent, while Tadhani *et al*. (2007), through HCl in methanol extracts, reported 25.18 mg/g. Shukla *et al.* have obtained 56.73 mg/g (2012) and 61.50 mg/g (2009) for leaf water and ethanolic extracts, respectively. Barroso *et al*. (2016) refers a higher value, 151 mg/g relative to a methanol:H_2O extract of dry leaf, following a HPLC analysis with a diode array detector. Periche *et al.* (2015b) have compared conventional extraction method with extraction by means of ultrasound technique and microwave energy of dried *Stevia rebaudiana* leaves. They have verified that the first method allowed the highest efficiency. These authors have obtained 93.41 mg gallic acid/g for phenols when conventional extraction was carried out with water at 90ºC, but at 50ºC and after 20 minutes the value decrease to 80 mg gallic acid/g *S. rebaudiana*. The same authors (2015 b) reported 44.4 mg gallic acid/g *S. rebaudiana* fresh leaves and 76.8 mg gallic acid/g leaves dried at 180ºC, concluded in this study that the most suitable drying method was hot air at 180ºC, since it substantially increased the total phenolics content. Tavarini and Angelini (2013) have obtained total phenols values in the range between a minimum of 37.26 mg gallic acid/g of *Stevia rebaudiana* and a maximum of 78.24 mg gallic acid/g of *S. rebaudiana*, depending on the harvest time, using methanolic extracts of the leaves dried at 40ºC. The difference between these values shows the

importance of the choice of the extractor solvent and the quantification method, being the leaves drying method and the harvest time also important (Perez-Jiménez and Saura-Calixto, 2006).

Several studies demonstrated that *Stevia rebaudiana* presents pharmacological and therapeutic applications, which are related with phytochemicals contents. Wölwer-Rieck (2012) referred that *S. rebaudiana* extract contains more than 100 important compounds, such as proteins, sterebins, flavonoids and phenolics, which can be used in foods, incorporating nutritional, medicinal and functional value. Face to the importance of these compounds, in this study total flavonoids had also been quantified and expressed in CE/g of sample.

Relatively to total flavonoids content, the dry leaves of *Stevia rebaudiana* had presented higher values (9.7 mg CE/g) comparatively with the results obtained to the sample of stevioside in powder (commercial product) (5.1 mg CE/g).

As in the total phenols, the results attained in this study had been superior to the values reported by Milani *et al.* (2016), which has described 123.27 mg/g for aqueous extract of dried *Stevia rebaudiana* leaf. However, Barroso *et al.* (2016) obtained 22.7 mg/g of total flavonoids in a methanol: water extract of the leaf. Tadhani *et al.* (2007), in turn, relate a value of 21.73 mg/g, gotten for a HCl in methanol extract. Kim *et al*. (2011) have reported a value of 15.64 mg/g of quercetin for leaf aqueous extract, using a method similar to ours. Periche *et al.* (2015a, 2015b) obtained 52.92 - 69.18 mg catechin/g for conventional extraction of dried leaves with water at 100ºC, decreasing the value for lower temperatures. In another paper (2015 b) these authors have reported 2.5 mg catechin/g *Stevia rebaudiana* fresh leaves and 45.1 mg catechin/g *S. rebaudiana* dried leaves.

Flavonoids are the predominant chemical compounds in the vegetable kingdom, depending their content on edaphoclimatic conditions, type of botanical species and experimental analytical conditions used. Anthocyanins are flavonoids that are widely distributed in the nature and are responsible for the majority of the tonalities of the plants, being common its presence and, consequently, the increase of the concentration of flavonoids in the vegetal species (Panche *et al*., 2016; Dewick, 2002). However, according to Ozturk *et al*. (2015), the dry *Stevia rebaudiana* plants present high contents of chlorophyll (a and b) and, when compared with the quantities of these pigments in fruits and vegetables, the amount of chlorophyll of *S. rebaudiana* is significantly bigger. These pigments are directly related with the inhibition of the synthesis of anthocyanins and, consequently, with the increase of the flavonoids content. Nonetheless, the presence of chlorophylls in *Stevia rebaudiana* leaves enhance its use, in detriment of the commercial steviol powder, since the plant can be used as coloration agent in some industrial applications.

2.2. DPPH RADICAL SCAVENGING ASSAY

Radical $DPPH^{\bullet}$ is frequently used for the determination of primary antioxidant activity. The activity of elimination of this free radical has been studied in pure antioxidant compounds, nominated in extracts of plants and fruits, as well as in alimentary products (Wong *et al.*, 2006). In this study the antioxidant activity was express in percentage of RSA.

Relatively to the antioxidant activity obtained in the hydroalcoholic extracts studied, the leaf presented higher values (35%). The commercial powder sweetener only inhibited 18% of the radical. Previous studies have demonstrated the significant antioxidant activity of *Stevia rebaudiana* leaf face to radical DPPH (Gaweł-Bęben *et al.*, 2015; Goyal *et al.*, 2010; Rao *et al.*, 2014). Although it has an agreement with the values reported in other identical studies, Milani *et al.* (2016) had gotten 39% of inhibition and Tadhani *et al*. (2007) have reported 39.86% for water extracts and 33.17% for methanolic extracts. Yildiz-Ozturk *et al.* (2015) have obtained higher values for DPPH inhibition: 91.39 % and 92.40%, using microwave–assisted and ultrasonically assisted ethanol extractions, respectively. The same authors, in 2014, using subcritical water extraction, have reported 92.50% (Yildiz-Ozturk *et al.*, 2014). Tavarini and Angelini (2013) reported 86.52 – 89.75% for DPPH inhibition with methanolic extracts of *Stevia rebaudiana* leaves harvested at different time.

2.3. FERRIC REDUCING ANTIOXIDANT POWER (FRAP)

FRAP method supplies indications about the antioxidant potential of vegetal extract. To determine the total antioxidant activity *in vitro* is necessary to perform other analytical methods, such as $DPPH^{\bullet}$, ABTS, acid linoleic/β-carotene system, among others, to determine specific antioxidant substances present in each extract. FRAP method is the more reproducibly technique and the one that has high correlation with ascorbic acid and phenolic compounds contents (Grozeva *et al*., 2015). The capacity of *Stevia rebaudiana* leaves and steviol extracts to reduce the iron ions was determined using the FRAP assay. This assay measures the capacity of reduction ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) in the presence of antioxidant substances

The values obtained for hydroalcoholic extracts had varied between 0.30 (commercial steviol powder) and 1.05 mmol/g (leaf), showing a superior antioxidant profile of the dried leaves. In an identical study, Álvarez-Robles *et al.* (2016) obtained a value of 1.00 mmol/g in water extracts of *Stevia rebaudiana* leaves. Grozeva *et al*. (2015) have reported a much lower value (0.10 mmol/g) for the same sample, using an ethanolic extract. Tavarini and Angelini (2013) have obtained FRAP values between 0.296 mmol Fe^{2+}/g and 0.813 mmol Fe^{2+}/g depending on the harvest time, using methanolic extracts of the leaves dried at 40ºC. These differences could be related with the extracting capacity of the solvent used and the harvest time of the *Stevia rebaudiana* leaf.

2.4. MACRONUTRIENTS ANALYSIS

The centesimal analysis was carried through in the four cakes studied, allowing to find significant differences between them. The results are presented in Table 2.

Table 2. Results of the centesimal analysis of studied cakes. All the values are express in percentage (%).

Samples	Moisture	Ashes	Fat	Proteins	Carbohydrates
Cake A	20.75±1.39^{c}	1.63±0.17^{c}	7.71±0.42^{c}	12.31±0.32^{b}	58.32±0.78^{a}
Cake B	29.47±1.18^{b}	2.03±0.08^{b}	10.32±0.02^{b}	10.98±0.13^{c}	47.22±1.61^{b}
Cake C	33.78±3.62^{a}	2.13±0.07^{b}	10.55±0.27^{b}	11.19±0.30^{c}	40.78±0.72^{c}
Cake D	34.45±4.21^{a}	2.47±0.08^{a}	12.63±0.67^{a}	15.72±0.16^{a}	32.75±3.91^{d}

*The results are presented on average ± standard deviation of three repetitions for each sample. $^{a, b, c, d}$ Different lower-case letters in each column suggest significant differences between the samples when compared with each other ($p<0.05$) according to the LSD post-hoc test.

Results presented significant differences in all the studied parameters. Cake A showed higher sugars content (~58%) while cake D constituted only by *Stevia rebaudiana* leaves, presented the lowest value (~33%). Curiously, cake D also presented the highest value of total protein (15.72%), total fat (12.63%), ashes (2.47%) and moisture (34.45%), and regarding moisture this formulation did not differ significantly from cake C. These results are easily explained. Moisture is directly related with the water percentage presented in fresh leaves of *S. rebaudiana*. On the other hand, the ashes represent the remaining inorganic residue after incineration of the organic substance presented in the sample. Since *Stevia rebaudiana* leaf is a natural raw material, not subject to industrial refinement processes, the mineral content becomes significantly superior in the formulations where the leaves are incorporated (cake C and cake D). Relatively to the total protein content, it was observed that cake D (only with *S. rebaudiana* leaves) exhibited the highest content with a significant difference when compared with the values obtained in the other cakes. This result can be based by the increase of amino acids and proteins that the leaves contain: 15 amino acids had been already identified in *Stevia rebaudiana* leaves, some essentials (glutamate, aspartate, lysine, serine, isoleucine, alanine, proline, tyrosine, arginine, histamine, phenylalanine, leucine, valine, tryptophan and glycine) (Abou-Arab *et al*., 2010; Li *et al*., 2011; Rafiq *et al*., 2007). The fat was also manifestly superior in cake D, however, this could be associated to the liposoluble compounds content present in *Stevia rebaudiana* leaves, such as essential oils, chlorophylls and carotenoids. The presence of fatty acids and cerosides could also interfere with the quantitative superiority founded in cake D. However, the main objective of this work focuses in the sugars content and, for the presented data, it was verified that the reduction of the saccharose content in the formulations and the substitution by *Stevia rebaudiana* allow to diminish considerably the total carbohydrate content (cake A > cake B > cake C > cake D), what is directly related with the caloric value of each cake.

Table 3. Results of the energy values of studied cakes. All the values are express in kcal/100 g of dry mass.

Samples	Energy
Cake A	309.8
Cake B	287.2
Cake C	277.3
Cake D	268.0

By the analysis of table 3, it is possible to observe that the caloric value per 100 g of each cake decreases as the sugar is substituted by *Stevia rebaudiana*, since saccharose has a superior caloric value that *S. rebaudiana*. This decrease also occurs with the exchange of commercial powder steviol sweetener for *Stevia rebaudiana* leaf.

2.5. SENSORIAL ANALYSES

Visually appearance of cakes with *Stevia rebaudiana* leaves is clearly different, since chlorophyll "dyes" the cake with a green tonality, total different of the habitual standard of the pastry shop cake batters. Since leaves modify the rheological characteristics of the batter, different sensorial parameters had been studied, namely, the appearance, the aroma, the flavor, the texture, the sweetness and the color (Figure 1).

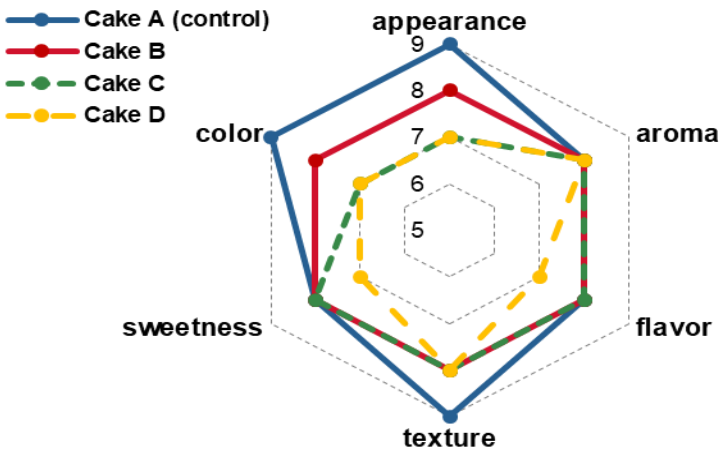

Figure 1 – Spyder plot of the comparison of median classification attributed to the studied sensory attributes of the four cakes (9="extremely good", 5="Nor good, nor bad").

In general, cakes A and B were equally and significantly more appreciated regarding the parameters appearance, flavor, sweetness and color (half of the consumers classified all these parameters as "extremely good" or, at least "very good") ($p<0.001$). Cakes C and D were significantly less appreciated regarding all parameters (the worse median classification was "moderately good") ($p<0.001$). The incorporation of *Stevia rebaudiana* powder was more appreciated than *S. rebaudiana* leaves and the replacement of saccharose by *Stevia rebaudiana* proved to be well accepted for all consumers (Figure 2).

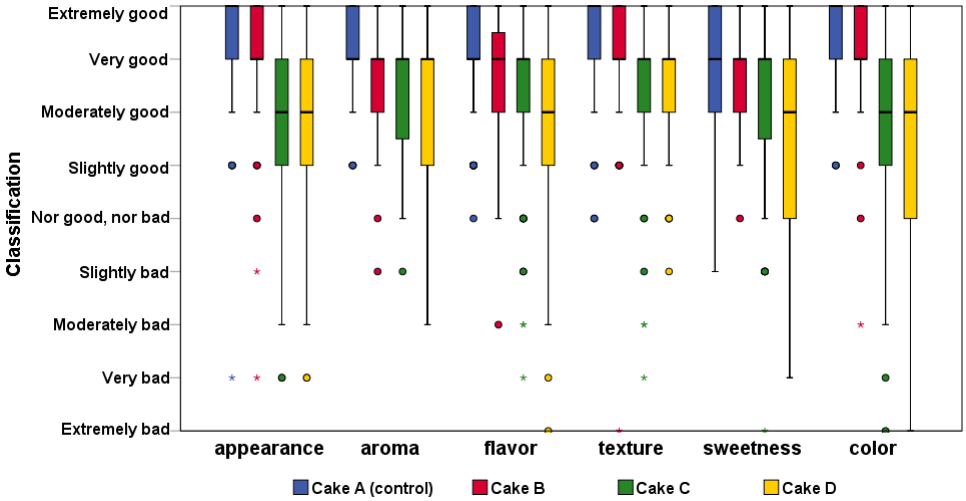

Figure 2 - Boxplot of the results obtained in the sensorial analysis of each cake.

Obreiter and Roseno (2017) have evaluated consumer acceptability of lemon pound cake when sugar is replaced by *Stevia rebaudiana* and sucralose, using a 10-point hedonic scale. The variables measured in this study include appearance, taste, texture, sweetness, aftertaste and overall acceptability. Generally, participants preferred the formulation sweetened with sugar within all aspects studied and preferred the appearance of the pound cake sweetened with *Stevia rebaudiana* when compared to the one sweetened with sucralose. Andersen *et al*. (2017) studied the acceptance of apple-cherry fruit drinks with different levels of beta-glucans and different sweeteners, sucrose or *Stevia rebaudiana* and concluded that the replacement of sucrose by *S. rebaudiana* did not affect the hedonic and post-ingestive sensations.

CONCLUSION

This work intended to value the plant *Stevia rebaudiana* (Bert.) Bertoni, aiming at a possible application in food industry and, eventually, as nutraceutic. The use of *Stevia rebaudiana* (Bert.) Bertoni leaf for the food industry has come to increase at world-wide level, trying to substitute saccharose for this plant. *Stevia rebaudiana* has the advantage of being exempt from calories and does not modify the levels of sugar in the blood at human metabolism, nor stimulate the insulin production, allowing concluding that its use/consumption can be recommended to all the population, in particular to the diabetic ones.

This plant has a high content of bioactive compounds, what is directly related with the antioxidant activity, recognized for the sequestering action of free radicals resulting from oxidative stress. These results suggest larger resource to its application, having as sustainable base the allegations of different organizations, being safe the use of *Stevia rebaudiana* leaves. Comparing *Stevia rebaudiana* leaves with steviol commercial powder, it is possible to conclude that, in all the evaluated parameters, leaves present higher contents of all the compounds than the powder.

The centesimal analysis of the four cakes showed that the substitution of sugar by *Stevia rebaudiana* increases moisture, ashes and fat content. However, total carbohydrate content decreases significantly and, consequently, the caloric value. On the other hand, cake sweetened only with *Stevia rebaudiana* leaves had the highest protein content comparatively with the values obtained in the other cakes.

The sensorial profile of *Stevia rebaudiana* sweetened cakes allows knowing how natural sweetener is perceived in combination with flavor and fibers. Sensorial analysis results showed that *S. rebaudiana leaves* can replace some or all the saccharose in cakes formulations without changing drastically the acceptability of the product.

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