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A New Iced Tea Base Herbal Beverage with *Spergularia rubra* Extract: Metabolic Profile Stability and In Vitro Enzyme Inhibition

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ABSTRACT: Beverages are an ideal format to offer nutrients, specific health-promoting functionality, and desirable sensory attributes to consumers. Taking into account previous research on *Spergularia rubra* (L.) J. Presl & C. Presl, the aim of this work was to describe the chemistry and biochemistry associated with the production of a new iced tea base herbal beverage containing a hydroethanolic extract of this species, including both naturally occurring and added components. Phenolic compounds of *S. rubra* hydroethanolic extract and of the iced tea base herbal beverage were determined by HPLC-DAD. Thirty compounds, comprising nonacylated *C*-glycosyl flavones, *C*-glycosyl flavones acylated with aromatic acids, and *C*-glycosyl flavones acylated with aliphatic acids, were identified, being essentially represented by apigenin derivatives. Organic acids of both samples were determined by HPLC-UV, malic acid being the major one. A strong inhibition of α-glucosidase, acetylcholinesterase, and butyrylcholinesterase was observed. Furthermore, the influence of the pH of the digestive tube on the chemical composition of both extract and iced tea base herbal beverage and, consequently, on their biological activity, was assessed. In a general way, pH variation significantly decreased ($p < 0.05$) the metabolites content and enzymes inhibitory capacity. Nevertheless, the beverage enriched with *S. rubra* extract represents a valuable addition to consumer's health and nutrition, once the loss of activity is lower than the one verified for the base iced tea. Thus, the results suggest that the ingestion of this beverage could be of potential interest for several chronic disorders, particularly Alzheimer's disease.

KEYWORDS: *iced tea base herbal beverage, Spergularia rubra, metabolic composition, antidiabetic, cholinesterases inhibition*

INTRODUCTION

The use of natural substances is generally preferred over synthetic ones as they are usually seen as safe. In this field, plants play an important role in the daily human intake of bioactive compounds because they are important sources of several compounds known to have biological properties, such as phenolic compounds, sterols, alkaloids, and carotenoids.[1] In fact, the increasing demand for natural ingredients, improving health and appearance, is also attracting beverages, being the fastest growing segment on the functional market.[2−7]

The aerial parts of *Spergularia rubra* (L.) J. Presl & C. Presl (Caryophyllaceae) are widely consumed as an infusion due to their biological properties.[8] Some studies were performed to characterize the presence in this species of metabolites known by their capacity to inhibit α-glucosidase and cholinesterases,[9,10] as well as by their free radicals scavenging properties.[9−12] Among the bioactive compounds are found phytoecdysteroids,[13,14] di-*C*-glycosyl flavones,[15,16] and *C*-glycosyl flavones, including acylated derivatives.[10,17] Thus, the development of an iced tea base herbal beverage able to control worldwide diseases, such as diabetes mellitus and Alzheimer's disease (AD), may be extremely important to the current society.

Diabetes mellitus is a metabolic disorder responsible for abnormal glucose levels in the blood, which is, most of the time, associated with obesity problems. α-Glucosidase is the enzyme responsible for the breakdown of α-glycosidic bonds of complex carbohydrates, resulting in less complex carbohydrates that can be absorbed. Thus, α-glucosidase inhibitors could retard the absorption of dietary carbohydrates, suppressing postprandial hyperglycaemia.[18]

AD is the most common cause of dementia in the elderly, being characterized by degeneration of cholinergic neurons in specific areas of the brain associated with higher intellectual functions, memory, and consciousness.[19] Its treatment is based on the cholinergic hypothesis that relates the cognitive decline observed with the loss of the neurotransmitter acetylcholine in the synaptic cleft. So, cholinesterases (acetyl- or butyrylcholinesterase) inhibitors could increase the levels of acetylcholine.[20,21]

Thus, the aim of this study was to develop an iced tea base herbal beverage containing an extract of *S. rubra* in order to be used in diabetes mellitus control and in the prevention of AD. Phenolic compounds and organic acids of both *S. rubra* hydroethanolic extract and iced tea base herbal beverage were

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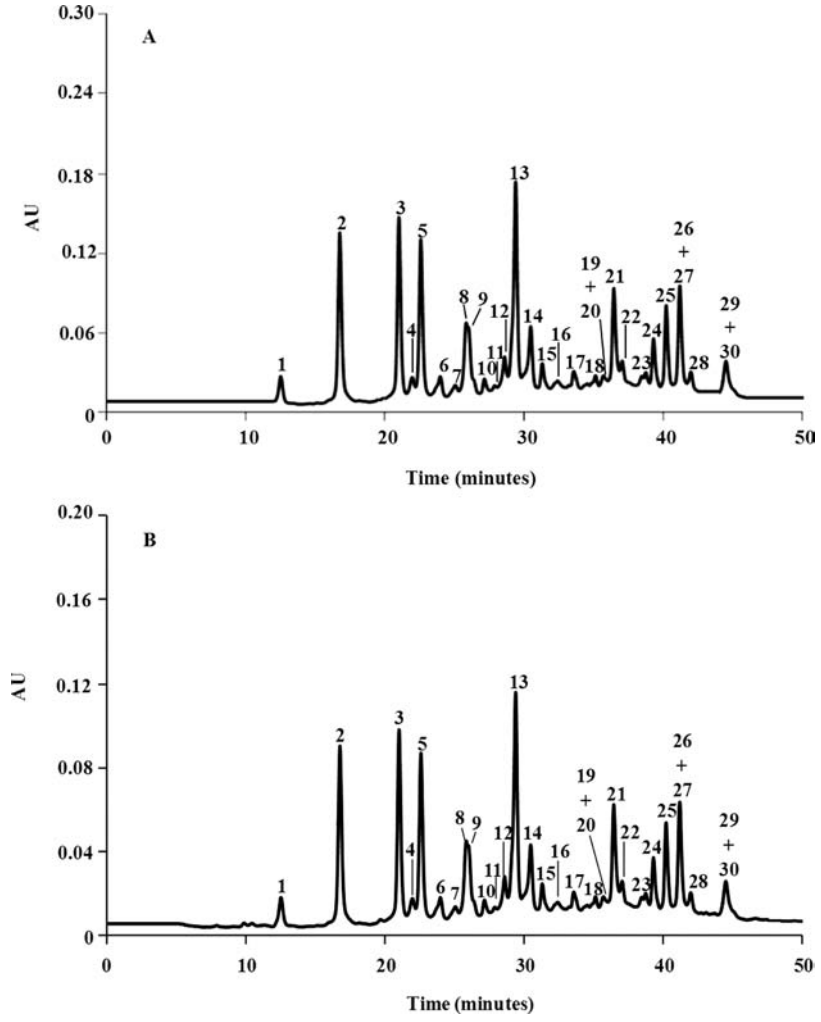

Figure 1. HPLC-DAD chromatogram of phenolic compounds present in *S. rubra* hydroethanolic extract (A) and in iced tea base herbal beverage (B). Detection at 350 nm. **1**, 7-*O*-glucosyl-6,8-di-*C*-glucosyl luteolin; **2**, 6,8-di-*C*-glucosyl luteolin; **3**, 6,8-di-*C*-glucosyl apigenin; **4**, 6,8-di-*C*-arabinosyl luteolin; **5**, 6,8-di-*C*-glucosyl chrysoeriol; **6**, 6-*C*-(6″-acetyl)glucosyl-8-*C*-glucosyl luteolin; **7**, 6-*C*-(6″-malonyl)glucosyl-8-*C*-glucosyl luteolin; **8**, 6-*C*-glucosyl-8-*C*-arabinosyl apigenin; **9**, 6-*C*-glucosyl-8-*C*-(4‴-malonyl)glucosyl luteolin; **10**, 6-*C*-arabinosyl-8-*C*-glucosyl chrysoeriol; **11**, 6-*C*-glucosyl-8-*C*-arabinosyl apigenin; **12**, 6-*C*-(4″-malonyl)glucosyl-8-*C*-glucosyl apigenin; **13**, 7,2″-di-*O*-glucosyl-6-*C*-arabinosyl apigenin; **14**, 7-*O*-glucosyl-6-*C*-arabinosyl-8-*C*-(6‴-malonyl)arabinosyl chrysoeriol; **15**, 6-*C*-(2″-feruloyl)glucosyl-8-*C*-glucosyl apigenin; **16**, 7-*O*-glucosyl-6-*C*-(2″-malonyl)-arabinosyl-8-*C*-arabinosyl chrysoeriol; **17**, 7-*O*-glucosyl-6-*C*-glucosyl-8-*C*-(2‴-sinapoyl)glucosyl luteolin; **18**, 6-*C*-glucosyl-8-*C*-(2‴-sinapoyl)glucosyl luteolin; **19**, 2″-*O*-glucosyl-6-*C*-arabinosyl apigenin; **20**, 6-*C*-glucosyl-8-*C*-(2‴-*p*-coumaroyl)glucosyl luteolin; **21**, 6-*C*-glucosyl-8-*C*-(2‴-feruloyl)-glucosyl luteolin; **22**, 6-*C*-glucosyl-8-*C*-(2‴-dihydroferuloyl)glucosyl luteolin; **23**, 6-*C*-glucosyl-8-*C*-(2‴-sinapoyl)glucosyl apigenin; **24**, 6-*C*-glucosyl-8-*C*-(2‴-sinapoyl)glucosyl chrysoeriol; **25**, 6-*C*-glucosyl-8-*C*-(2‴-feruloyl)glucosyl apigenin; **26**, 6-*C*-glucosyl-8-*C*-(2‴feruloyl)glucosyl chrysoeriol; **27**, 6-*C*-glucosyl-8-*C*-(2‴-dihydroferuloyl)glucosyl apigenin; **28**, 6-*C*-glucosyl-8-*C*-(2‴-dihydroferuloyl)glucosyl chrysoeriol; **29**, 6,8-di-*C*-(6″malonyl, feruloyl)glucosyl chrysoeriol; **30**, 6,8-di-*C*-(6‴malonyl, feruloyl)glucosyl apigenin.

determined and the potential against α-glucosidase and cholinesterases was assessed. Attending to the fact that pH may affect the stability of some components[22,23] and, consequently, their activity, the possible influence of the pH variations along the digestive tube was evaluated.

MATERIALS AND METHODS

Plant Material. The dried aerial parts of *S. rubra* were from a medicinal plants distributor (Morais e Costa & Ca. Lda, Portugal), as before.[10] The plant material was powdered (mean particle size lower than 910 μm). A voucher specimen was deposited at Laboratório de Farmacognosia, Faculdade de Farmácia, Universidade do Porto (SRA-AO-112012).

Standards and Reagents. Reference compounds were purchased from various suppliers: luteolin-3′,7-di-*O*-glucoside, isovitexin-7-*O*-glucoside, and chrysoeriol were from Extrasynthèse (Genay, France), and oxalic, citric, malic, aconitic, shikimic, and fumaric acids were obtained from Sigma (St. Louis, MO, USA). Acetylcholinesterase (AChE) from electric eel (type VI-s, lyophilized powder), acetylthiocholine iodide (ATCI), butyrylcholinesterase (BuChE) from equine serum (lyophilized powder), *S*-butyrylthiocholine chloride (BTCC), α-glucosidase (type I from baker's yeast), and 4-nitrophenyl α-D-glucopyranoside (PNP-G) were purchased from Sigma (St. Louis, MO, USA). Acetic acid, methanol, and ethanol were obtained from Merck (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Extracts Preparation. Aerial parts of *S. rubra* were extracted with ethanol:water (1:1) mixture (1 g/100 mL) as follows: 0.5 h of sonication, 2 h of stirring (200 rpm) maceration at room temperature, plus 0.5 h of sonication. The resulting extract was filtered over a filtration funnel, ethanol was evaporated under reduced pressure, and then the water was lyophilized in a Labconco Freezone 4.5 apparatus

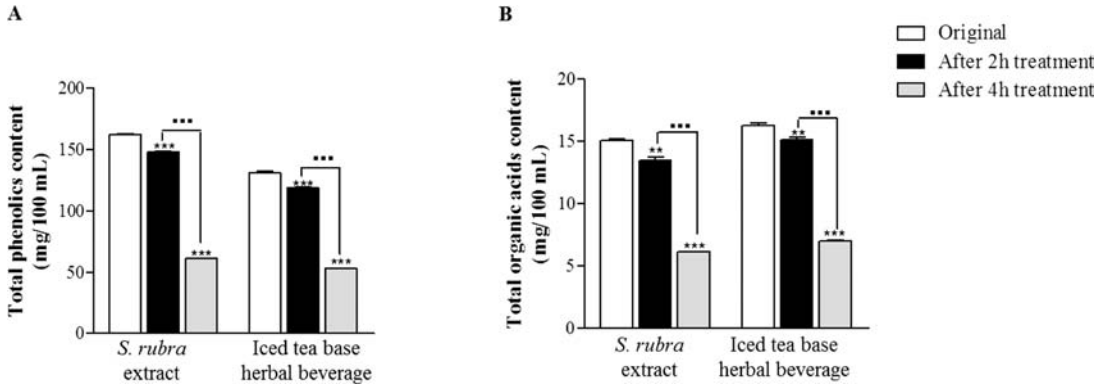

Figure 2. Phenolic compounds (A) and organic acids (B) contents (mg/100 mL) in *S. rubra* extract and in the iced tea base herbal beverage. ** $p < 0.01$, *** $p < 0.001$ relative to original sample; ■■■ $p < 0.001$ among pH variations.

(Kansas City, MO, US). The lyophilized extracts were kept in a desiccator, in the dark, until analysis.

Formulation of the Iced Tea Base Herbal Beverage. *S. rubra* lyophilized extract (66 mg/mL) was incorporated in several beverages commercialized by UNICER in Portugal. Beverages included juices (only when the juice was identified as being a brand containing ≥50% of real fruit juice), noncola carbonated beverages, flavored and nonflavored waters (including carbonated ones), and soft tea drink (iced tea).

A panel composed of six people (professors, university students, and laboratory personnel) was engaged in sensorial evaluation of different mixtures, relative to the original beverages, concerning color, odor, and flavor. Tests were performed using tulip glasses containing 30 mL of each test mixture, in an adequate room (without sensory odors) at 25 °C.

Effects of pH Variation along the Digestive Tube. The iced tea base herbal beverage and *S. rubra* hydroethanolic extract were subsequently submitted to stomach pH conditions (1.99 with Tris-HCl) for 2 h, followed by intestine pH conditions (8.00 with phosphate buffer) for another 2 h, in the dark at 37 °C with stirring at 150 rpm. Aliquots of the initial iced tea base herbal beverage and *S. rubra* hydroethanolic extract and of the two samples after the 2 and 4 h treatments were taken for analyses.

Chemical Composition. *HPLC-DAD Conditions for Phenolic Compounds Analysis.* Phenolic compounds were analyzed as before.[10] Compounds were identified by comparing their retention times and UV−vis spectra with those of flavonoids already reported by us for hydromethanolic extract of *S. rubra*.[17] Quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. Because there is no standard of the *C*-glycosyl flavones commercially available, luteolin derivatives were quantified as luteolin-3′,7-di-*O*-glucoside, apigenin derivatives as isovitexin-7-*O*-glucoside, and chrysoeriol derivatives as chrysoeriol. In the cases of coelution, the UV spectra allowed us to notice which was the most abundant derivative. Thus, 6-*C*-glucosyl-8-*C*-arabinosyl apigenin and 6-*C*-glucosyl-8-*C*-(4′″-malonyl)glucosyl luteolin (compounds **8** + **9**) were quantified together as luteolin-3′,7-di-*O*-glucoside, while the pairs 2″-*O*-glucosyl-6-*C*-arabinosyl apigenin plus 6-*C*-glucosyl-8-*C*-(2′″-*p*-coumaroyl)glucosyl luteolin (compounds **19** + **20**), 6-*C*-glucosyl-8-*C*-(2′″-feruloyl)glucosyl chrysoeriol plus 6-*C*-glucosyl-8-*C*-(2′″-dihydroferuloyl)glucosyl apigenin (compounds **26** + **27**), and 6,8-di-*C*-(6″malonyl, feruloyl)glucosyl chrysoeriol plus 6,8-di-*C*-(6′″malonyl, feruloyl)glucosyl apigenin (compounds **29** + **30**) were quantified as isovitexin-7-*O*-glucoside.

HPLC-UV Conditions for Organic Acids Analysis. Compounds were determined as before.[10] Quantification was achieved by the absorbance recorded in the chromatograms relative to external standards.

In Vitro Enzymes Inhibition. Spectrophotometric microassays were performed in a Multiskan Ascent plate reader (Thermo Electron Corporation), using 96-well plates.

α-Glucosidase. The evaluation was based on the reaction with PNP-G, as previously reported.[10] The absorbance was measured at 400 nm, and three independent assays were performed in triplicate.

Cholinesterases. The determination was based on Ellman's method, as previously described.[10] The absorbance was measured at 405 nm, and the rates of reactions were calculated by Ascent Software version 2.6 (Thermo Labsystems Oy). Three independent assays were performed in triplicate.

Statistical Analysis. Data were analyzed using GraphPad Prism software (version 5.02 for Windows). One-way analysis of variance (ANOVA), using the Turkey's multiple comparison test, was carried out on data obtained from triplicate determinations of each sample. The level of significance was set at *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

■ RESULTS AND DISCUSSION

Selection of the Iced Tea Base Herbal Beverage. The hydroethanolic extract of *S. rubra* was added to different beverages, including waters, juices, noncola carbonated beverages, and iced tea. As the *S. rubra* hydroethanolic extract has a dark-brown color, its incorporation in the noncola carbonated beverages and waters caused important changes in the color of the original beverages, which acquired a brown color and may not be enjoyed by consumers. So, these beverages were immediately excluded. The only two beverages in which original color was not affected by the addition of *S. rubra* extract were the juice and the iced tea. So, the mixtures prepared with these beverages were given to the panel, which evaluated them just in terms of pleasant and tasty or not. We chose iced tea as the base of the new beverage once its flavor and odor were less affected than the juice, the resulting mixture being considered to be pleasant and tasty.

Chemical Characterization. *Phenolic Compounds.* Phenolic compounds are generally known by their favorable effects in human health, mainly due to their antioxidant, anti-inflammatory, and anticarcinogenic properties. Thus, the addition of these metabolites to foods and beverages will continue to be of great interest to the food industry.[24] Thirty phenolic compounds, comprising nonacylated flavones and *C*-glycosyl flavones acylated with aromatic or aliphatic acids, were identified by HPLC-DAD in the hydroethanolic extract of *S. rubra* and in the iced tea base herbal beverage (Figure 1). No phenolic compound was detected in the original beverage (iced tea). In a general way, the qualitative composition of the hydroethanolic extract revealed to be similar to that of the hydromethanolic one previously described.[10,17]

Table 1. Phenolic Compounds in the Original *S. rubra* Hydroethanolic Extract and Iced Tea Base Herbal Beverage and Subjected to pH Variation (mg/100 mL)[a]

		S. rubra hydroethanolic extract			iced tea base herbal beverage		
	phenolic compounds	O	2 h	4 h	O	2 h	4 h
1	7-*O*-glucosyl-6,8-di-*C*-glucosyl luteolin	1.52 a (0.00)	1.32 bbb, A (0.03)	0.53 ccc, BBB (0.01)	1.45 a (0.02)	1.25 bbb, A (0.01)	0.46 ccc, BBB (0.00)
2	6,8-di-C-glucosyl luteolin	11.75 a (0.08)	10.16 bb, A (0.00)	3.83 ccc, BBB (0.02)	9.50 a (0.16)	8.98 bb, A (0.04)	3.37 ccc, BBB (0.02)
3	6,8-di-C-glucosyl apigenin	25.81 a (0.24)	25.48 a, A (0.03)	10.69 bbb, BBB (0.12)	23.30 a (0.30)	23.10 a, A (0.09)	9.57 bbb, BBB (0.01)
4	6,8-di-C-arabinosyl luteolin	1.39 a (0.10)	1.25 a, A (0.04)	0.40 bbb, BBB (0.00)	1.19 a (0.10)	1.06 a, A (0.03)	0.33 bbb, BBB (0.00)
5	6,8-di-C-glucosyl chrysoeriol	8.32 a (0.05)	6.93 bbb, A (0.01)	2.97 ccc, BBB (0.03)	6.53 a (0.08)	6.40 b, A (0.03)	2.64 ccc, BBB (0.01)
6	6-C-(6″-acetyl)glucosyl-8-C-glucosyl luteolin	0.92 a (0.01)	0.66 bbb, A (0.00)	0.59 ccc, BBB (0.01)	0.79 a (0.02)	0.59 bbb, A (0.01)	0.46 ccc, BBB (0.00)
7	6-C-(6″-malonyl)glucosyl-8-C-glucosyl luteolin	0.86 a (0.01)	0.66 bbb, A (0.00)	0.20 ccc, BBB (0.00)	0.79 a (0.01)	0.53 bbb, A (0.02)	0.20 ccc, BB (0.00)
8 + 9	6-C-glucosyl-8-C-arabinosyl apigenin + 6-C-glucosyl-8-C-(4′″-malonyl)glucosyl luteolin	8.45 a (0.06)	7.26 bbb, A (0.01)	2.71 ccc, BBB (0.04)	6.01 a (0.06)	5.41 bbb, A (0.03)	2.31 ccc, BBB (0.01)
10	6-C-arabinosyl-8-C-glucosyl chrysoeriol	0.99 a (0.00)	0.86 bbb, A (0.01)	0.26 ccc, BBB (0.01)	0.73 a (0.01)	0.59 bbb, A (0.00)	0.20 ccc, BBB (0.00)
11	6-C-glucosyl-8-C-arabinosyl apigenin	0.53 a (0.10)	0.40 a, A (0.03)	0.13 bbb, BBB (0.00)	0.46 a (0.05)	0.33 bb, A (0.03)	0.13 ccc, BB (0.00)
12	6-C-(4″-malonyl)glucosyl-8-C-glucosyl apigenin	1.91 a (0.02)	1.58 bbb, A (0.03)	0.73 ccc, BBB (0.01)	0.79 a (0.03)	0.59 bbb, A (0.01)	0.20 ccc, BB (0.02)
13	7,2″-di-*O*-glucosyl-6-*C*-arabinosyl apigenin	41.91 a (0.24)	36.56 bbb, A (0.12)	14.32 ccc, BBB (0.12)	31.61 a (0.40)	30.23 bb, A (0.11)	12.94 ccc, BBB (0.04)
14	7-*O*-glucosyl-6-*C*-arabinosyl-8-*C*-(6′″-malonyl) arabinosyl chrysoeriol	3.89 a (0.03)	3.63 bb, A (0.02)	1.72 ccc, BBB (0.03)	3.50 a (0.07)	3.04 bbb, A (0.02)	1.45 ccc, BBB (0.00)
15	6-C-(2″-feruloyl)glucosyl-8-C-glucosyl apigenin	4.16 a (0.10)	3.96 a, A (0.10)	1.52 bbb, BBB (0.00)	4.03 a (0.10)	3.56 bbb, A (0.00)	1.32 ccc, BBB (0.02)
16	7-*O*-glucosyl-6-C-(2″-malonyl)arabinosyl-8-*C*-arabinosyl chrysoeriol	0.66 a (0.00)	0.59 bb, A (0.01)	0.20 ccc, BBB (0.00)	0.53 a (0.00)	0.26 bbb, A (0.00)	0.07 ccc, BBB (0.00)
17	7-*O*-glucosyl-6-*C*-glucosyl-8-C-(2′″-sinapoyl) glucosyl luteolin	0.99 a (0.00)	0.86 bbb, A (0.00)	0.46 ccc, BBB (0.00)	0.92 a (0.03)	0.73 bbb, A (0.00)	0.33 ccc, BB (0.00)
18	6-C-glucosyl-8-C-(2′″-sinapoyl)glucosyl luteolin	1.65 a (0.01)	1.52 bb, A (0.04)	0.40 ccc, BBB (0.00)	1.52 a (0.01)	1.39 bbb, A (0.01)	0.13 ccc, BBB (0.00)
19 + 20	2″-*O*-glucosyl-6-*C*-arabinosyl apigenin +6-*C*-glucosyl-8-*C*-(2′″-*p*-coumaroyl)glucosyl luteolin	1.19 a (0.01)	0.46 bbb, A (0.01)	0.26 ccc, B (0.01)	1.58 a (0.00)	0.99 bbb, A (0.01)	0.40 ccc, BBB (0.00)
21	6-C-glucosyl-8-C-(2′″-feruloyl)glucosyl luteolin	5.68 a (0.06)	5.54 a, A (0.10)	2.18 bbb, BBB (0.01)	4.36 a (0.05)	3.83 bbb, A (0.03)	1.78 ccc, BBB (0.00)
22	6-C-glucosyl-8-C-(2′″-dihydroferuloyl)glucosyl luteolin	1.91 a (0.10)	1.85 a, A (0.04)	0.53 bbb, BBB (0.00)	1.39 a (0.10)	1.25 a, A (0.05)	0.40 bbb, BBB (0.01)
23	6-C-glucosyl-8-C-(2′″-sinapoyl)glucosyl apigenin	1.58 a (0.03)	1.45 a, A (0.10)	0.59 bbb, BBB (0.00)	1.52 a (0.02)	0.92 bbb, A (0.01)	0.20 ccc, BBB (0.00)
24	6-C-glucosyl-8-C-(2′″-sinapoyl)glucosyl chrysoeriol	2.90 a (0.10)	2.77 a, A (0.05)	1.19 bbb, BBB (0.00)	2.71 a (0.20)	2.51 a, A (0.01)	0.79 bbb, BBB (0.01)
25	6-C-glucosyl-8-C-(2′″-feruloyl)glucosyl apigenin	11.81 a (0.30)	11.42 a, A (0.12)	4.69 bbb, BBB (0.00)	7.73 a (0.09)	5.28 bbb, A (0.01)	3.96 ccc, BBB (0.01)
26 + 27	6-C-glucosyl-8-C-(2′″feruloyl)glucosyl chrysoeriol + 6-C-glucosyl-8-C-(2′″-dihydroferuloyl)glucosyl apigenin	15.91 a (0.10)	15.77 a, A (0.04)	6.53 bbb, BBB (0.02)	12.87 a (0.13)	11.68 bbb, A (0.02)	6.53 ccc, BBB (0.01)
28	6-C-glucosyl-8-C-(2′″-dihydroferuloyl)glucosyl chrysoeriol	1.19 a (0.06)	1.12 a, A (0.02)	0.40 bbb, BBB (0.00)	1.12 a (0.01)	0.79 bbb, A (0.00)	0.20 ccc, BBB (0.00)
29 + 30	6,8-di-C-(6″malonyl, feruloyl)glucosyl chrysoeriol + 6,8-di-C-(6′″malonyl, feruloyl)glucosyl apigenin	4.29 a (0.20)	4.09 a, A (0.02)	3.17 bbb, BBB (0.03)	4.09 a (0.30)	3.63 a, A (0.20)	2.90 bb, BBB (0.01)

[a]Results are expressed as mean (standard deviation) of three assays; O, original; 2h, after being submitted to acid pH (2h); 4h, after being submitted to acid pH (2h) plus basic pH (2h); distinct lowercase and uppercase letters in the same line correspond to significant differences relatively to the corresponding original sample and among pH variation (2h and 4h), respectively (one letter, $p < 0.05$; two letters, $p < 0.01$; three letters, $p < 0.001$).

The content of the determined phenolic compounds in *S. rubra* extract and in the iced tea base herbal beverage corresponded to 162.17 and 131.02 mg/100 mL, respectively (Figure 2A). However, these amounts significantly decreased in both *S. rubra* extract and iced tea base herbal beverage, with pH variation mimicking the conditions along the digestive tube (Figure 2A). In a general way, a significant reduction ($p < 0.05$) of the content of each phenolic compound was observed after submission to both pH variations, the decrease being more evident after acid (2 h) followed by basic (2 h) variations (Table 1). This leads us to conclude that the pH affects the stability of the phenolic compounds, which is in agreement with previous works by other authors.[22,23] Furthermore, some compounds, namely compounds **3**, **4**, **11**, **15**, and **21**−**30**, were less affected by the acidic pH (2 h treatment) than the compounds **1**, **13**, **14**, **16**, **17**, **19**, and **20** (Table 1).

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Table 2. Organic Acids in the Original *S. rubra* Hydroethanolic Extract and Iced Tea Base Herbal Beverage and Subjected to pH Variation (mg/100 mL)[a]

		S. rubra hydroethanolic extract			iced tea base herbal beverage		
organic acids	RT (min)	O	2 h	4 h	O	2 h	4 h
oxalic	19.69	2.13 a (0.02)	1.76 bbb, A (0.05)	0.73 ccc, BBB (0.02)	nd	nd	nd
cis-aconitic	23.16	1.06 a (0.01)	0.99 bb, A (0.02)	0.63 ccc, BBB (0.01)	0.98 a (0.01)	0.93 b, A (0.01)	0.61 ccc, BBB (0.04)
citric	30.49	0.04 a (0.01)	0.03 a, A (0.00)	0.01 bb, BBB (0.00)	1.50 a (0.01)	1.47 bb, A (0.00)	0.75 ccc, BBB (0.00)
malic	37.11	10.08 a (0.01)	9.20 bb, A (0.39)	3.88 ccc, BBB (0.01)	11.87 a (0.23)	11.21 bb, A (0.20)	4.73 ccc, BBB (0.02)
trans-aconitic	44.25	1.29 a (0.12)	1.08 b, A (0.02)	0.70 ccc, BB (0.01)	1.26 a (0.01)	0.99 bb A (0.09)	0.65 ccc, BB (0.04)
shikimic	48.23	0.14 a (0.01)	0.08 bbb, A (0.00)	0.03 ccc, BBB (0.00)	0.12 a (0.00)	0.07 bbb, A (0.01)	0.03 ccc, BBB (0.00)
fumaric	61.74	0.32 a (0.01)	0.28 bb, A (0.00)	0.15 ccc, BBB (0.01)	0.54 a (0.00)	0.44 bbb, A (0.02)	0.24 ccc, BBB (0.01)

[a]Results are expressed as mean (standard deviation) of three assays; RT, retention time; O, original; 2h, after being submitted to acid pH (2h); 4h, after being submitted to acid pH (2h) plus basic pH (2h); nd, not detected; distinct lowercase and uppercase letters in the same line correspond to significant differences relatively to corresponding original sample and among pH variation (2h and 4h), respectively (one letter, $p < 0.05$; two letters, $p < 0.01$; three letters, $p < 0.001$).

In both *S. rubra* extract and iced tea base herbal beverage, apigenin derivatives dominated, corresponding to ca. 70% of the determined compounds (Table 1). The main compound in each analyzed matrix was 7,2″-di-*O*-glucosyl-6-*C*-arabinosyl apigenin (**13**) (Table 1), as observed before for *S. rubra* hydromethanolic extract.[10]

Organic Acids. Regarding the organic acids composition, seven compounds, namely oxalic, *cis*-aconitic, citric, malic, shikimic, *trans*-aconitic, and fumaric acids, were found in hydroethanolic extract of *S. rubra* (Table 2), revealing to be generally similar to the hydromethanolic one previously characterized.[10] On the other hand, in the iced tea base herbal beverage, oxalic acid was absent (Table 2).

The total organic acids content in *S. rubra* extract and in the iced tea base herbal beverage was 15.06 and 16.27 mg/100 mL, respectively (Figure 2B). Significant decreases were observed in the total organic acids content of samples subjected to the different pH conditions (Figure 2B). Furthermore, as it happened with the phenolic composition, the different pH conditions led to a significant reduction of the content of each compound, which was more pronounced after acid pH (2 h) and subsequent by basic pH (2 h) variations (Table 2).

Citric and malic acids (1.41 and 0.08 mg/100 mL, respectively) and trace amounts of fumaric acids were found in the iced tea. Unlike *S. rubra* hydromethanolic extract, which was predominantly composed by oxalic acid,[10] the hydroethanolic one and the iced tea base herbal beverage contain high amounts of malic acid, which corresponds to more than ca. 50% of the determined organic acids (Table 2). Malic acid is directly involved in Krebs' cycle and in the metabolism of carbohydrates.[25] Additionally, it is known by its antioxidant and antimicrobial properties. This metabolite is also used in food industry as acidifier, stabilizer, and as flavoring.[26−28]

Oxalic acid was the second major compound in *S. rubra* hydroethanolic extract (ca. 14%), which is not surprising once the Caryophyllaceae family is known by its high capacity to accumulate oxalate.[29] Nevertheless, its absence in the iced tea base herbal beverage was not expected (Table 2). This compound renders calcium (and sometimes other minerals like sodium, iron, zinc, aluminum) unavailable for nutritional absorption, thereby being considered an antinutrient.[30] In this context, perhaps the absence of oxalic acid in the iced tea base herbal beverage may be related with its capacity to form complexes with other metabolites of the original beverage.[31]

In *S. rubra* hydroethanolic extract, citric and fumaric acids were minor compounds (Table 2). These acids were found in higher amounts in the iced tea base herbal beverage than in *S. rubra* hydroethanolic extract (Table 2), which is not surprising because they were already present in the iced tea, as mentioned above. In fact, these compounds are commonly used in the food industry due to their antioxidant and antimicrobial properties.[26]

In Vitro Enzyme Inhibition vs Chemical Composition. *α-Glucosidase.* As referred above, the antidiabetic properties of *S. rubra* are already known.[8,10,32] Thus, the development of an iced tea base herbal beverage with *S. rubra* hydroethanolic extract could be useful to control the levels of blood glucose in diabetes mellitus patients. The α-glucosidase inhibitory activity of the beverage used as base (iced tea) was assessed, and a strong effect was observed (96%) (Figure 3). Thus, we tried to understand the high activity against α-glucosidase revealed only by the iced tea. As described above, citric acid was present in this beverage. This compound is known to inhibit this enzyme.[33] As such, we studied the influence of this organic acid on α-glucosidase activity and observed that at the concentration present in the iced tea, citric acid inhibited α-glucosidase to ca. 56% (data not shown). According to these results, it can be concluded that citric acid has a main role in the effect of the iced tea against this enzyme. Nevertheless, we cannot ignore the presence of other nondetermined constituents in the iced tea formulation which can contribute to this activity.

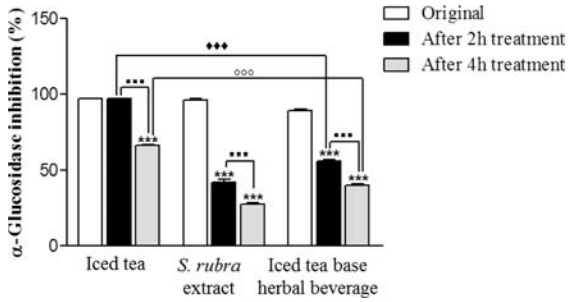

Figure 3. α-Glucosidase inhibitory activities of iced tea, *S. rubra* extract, and iced tea base herbal beverage. Results show mean ± SEM of three experiments performed in triplicate; *** $p < 0.001$ relative to original sample; ■■■ $p < 0.001$ among pH variations; ○○○ $p < 0.001$; ♦♦♦ $p < 0.001$ relative to iced tea.

The activity of the iced tea was not enhanced by the addition of *S. rubra* hydroethanolic extract (Figure 3). In fact, a decrease

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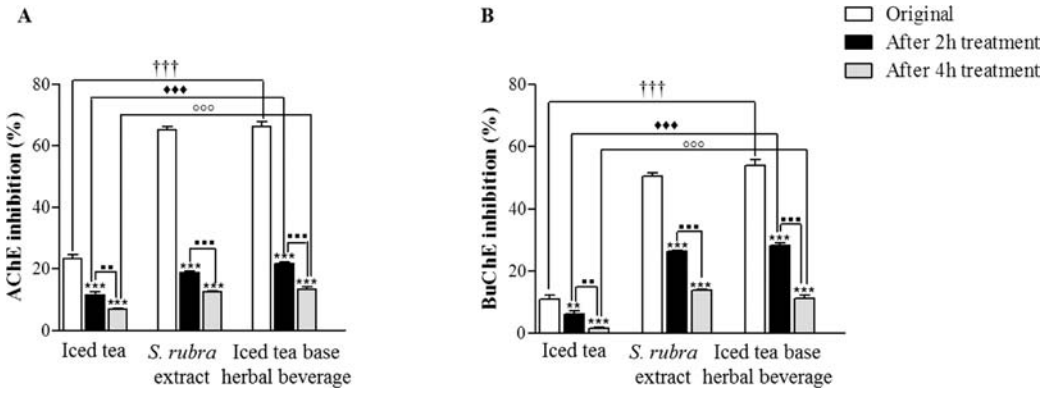

Figure 4. AChE (A) and BuChE (B) inhibitory activities of iced tea, *S. rubra* extract, and iced tea base herbal beverage. Results show mean ± SEM of three experiments performed in triplicate; ** $p < 0.01$, *** $p < 0.001$ relative to original sample; ■■ $p < 0.01$, ■■■ $p < 0.001$ among pH variations; ○○○ $p < 0.001$; ♦♦♦ $p < 0.001$, ††† $p < 0.001$ relative to iced tea.

of the α-glucosidase inhibitory activity of the iced tea base herbal beverage (89%) relatively to the original tea (96%) was verified (Figure 3). This fact may be correlated with the existence of possible antagonisms between the different metabolites present in *S. rubra* extract and in the base iced tea. Furthermore, the α-glucosidase inhibitory activity of the iced tea base herbal beverage and *S. rubra* hydroethanolic extract for the distinct pH variations decreased significantly (Figure 3), as it happened with the phenolics and organic acids contents (Figure 2).

The α-glucosidase inhibitory activity of the iced tea base herbal beverage can be, at least partially, related to the presence of organic acids, namely citric acid, but also with the apigenin and luteolin derivatives, which are known by their capacity to inhibit this enzyme.[34] Furthermore, in the major compounds of *S. rubra* hydroethanolic extract and iced tea base herbal beverage (apigenin derivatives), the presence of unsaturation between C-2 and C-3, in addition to the OH-groups in C-5, as well as the presence of an OH-group at C-4′, are molecular characteristics important for this inhibitory property.[34,35]

Cholinesterases. Following the previous observation of the capacity of *S. rubra* hydromethanolic extract to inhibit cholinesterases,[10] we also considered the development of an iced tea base herbal beverage with these properties to be used by AD patients. Unlike what happened against α-glucosidase, *S. rubra* hydroethanolic extract significantly improved the cholinesterases inhibitory properties of the iced tea (Figure 4). In fact, although there was a significant decrease of the *S. rubra* hydroethanolic extract and of the iced tea base herbal beverage activity under the pH conditions along the digestive tube (following what was seen with phenolics and organic acids contents), the addition of *S. rubra* extract increased two and four times the activity of the iced tea against AChE and BuChE, respectively (Figure 4A,B).

Attending to the chemical composition of *S. rubra* extract, the presence of *C*-glycosyl flavones may partially contribute to the anticholinesterase (AChE and BuChE) inhibitory activity, which was already verified for other plants.[9] In fact, as previously described by other authors,[9,36] the presence of a 4′-OCH_3 group in the B-ring and an *O*-glycosylation at C-7 are responsible for the high AChE inhibition of these compounds. For BuChE activity, the methoxy group at C-4′ seems to be not so relevant.[37] Thus, as there is an increase of the inhibitory activity of the iced tea base herbal beverage relative to the original one (Figure 4), in which no phenolic compound was determined, this activity can be partially explained by the presence of ca. 30% of *C*-glycosyl flavones, namely 7-*O*-glucosyl-6,8-di-*C*-glucosyl luteolin, 7,2″-di-*O*-glucosyl-6-*C*-arabinosyl apigenin, 7-*O*-glucosyl-6-arabinosyl-8-*C*-(6′″-malonyl)-arabinosyl chrysoeriol, 7-*O*-glucosyl-6-*C*-(2″-malonyl)-arabinosyl-8-*C*-arabinosyl chrysoeriol, and 7-*O*-glucosyl-6-*C*-glucosyl-8-*C*-(2′″-sinapoyl)glucosyl luteolin. As far as we know, there is no study describing the capacity of organic acids to inhibit both cholinesterases.

In conclusion, in this study a new designed iced tea base herbal beverage with *S. rubra* hydroethanolic extract was developed and its metabolic profile was described, revealing a wealth of phenolic compounds (nonacylated flavones and *C*-glycosyl flavones) and organic acids. Taking into account the minimum alterations observed concerning color, flavor, and odor relatively to the original beverage, the iced tea base herbal beverage may have good consumer acceptance. Furthermore, the new formulation offers some advantages to the consumers concerning the highest capacity to inhibit cholinesterases (AChE and BuChE), which may be partially related to the determined metabolites. Nevertheless, we cannot ignore the presence of other compounds not determined in this study and that can contribute for this activity. Therefore, the product proposed represents a value addition to the consumer's health, mainly against Alzheimer's disease, and is expected to have good market potential in an era of new herbal beverages.

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Notes

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