



Comparative studies on chemical stability, antioxidant and antimicrobial activity from hot and cold hibiscus (*Hibiscus sabdariffa* L.) calyces tea infusions

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Abstract

Hibiscus calyx presents high content of bioactive compounds, such as anthocyanins and other phenolic compounds responsible for antioxidant activity. The present study investigated how the tea infusion temperature (hot and cold) can change the phytochemical profile, antimicrobial activity, and stability of hibiscus tea. The results indicated the same phytochemical profile for cold (infusion during 2 h/25 °C) and hot (7 min/75 °C) tea by using FTIR and UPLC-MS/MS analyses. Hibiscus tea, hot or cold, showed antimicrobial activity against strains of *Salmonella* Typhimurium, *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus*, with minimum inhibitory concentration (MIC), obtaining values between 62.5 and 125.0 mg/ml. The hot infusion was more efficient for the bioactive compounds extraction, being 47% higher for total anthocyanins. After 48 h at 5 °C, the anthocyanin content had an average decrease of 30% (cold tea) and 48% (hot tea). Tea cream content increased to 15% (cold tea) and 37% (hot tea) after 72 h storage. Regarding the color, it was observed that after 48 h storage there was a clearing of the teas, and the L* coordinate increased from 21.03 to 24.93, and 20.72 to 24.90 for cold and hot tea, respectively. Our study indicates that hibiscus tea provides more antioxidants when prepared in the hot condition (7 min at 75 °C), and soon after cooled to 5 °C. Finally, it is recommended that hibiscus tea be consumed up to 24 h from preparation even when kept refrigerated.

Keywords Hibiscus · Tea · Bioactive compounds · Anthocyanins · Tea cream

Introduction

Tea is among the three most consumed drinks in the world, along with cocoa and coffee. Tea is widely consumed as a daily drink in China and other countries. Its consumption is associated to a desire for healthier lifestyles and the attempt to reduce medication needs for the treatment of certain diseases [1]. Infusion or decoction of different plant parts are still the main extraction procedures for the preparation of herbal teas, currently used in the practice of traditional medicine [2].

Hibiscus sabdariffa L., also known as roselle, red sorrel or karkade, is an annual medicinal shrub of the genus *Hibiscus* in the family *Malvaceae*. This plant is native to Africa and grows in tropical and subtropical regions such as America and south Asia, being cultivated mainly for preparation of hot tea and cold drinks using the dry edible

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flowers. In addition to its consumption as a beverage and its uses in the food industry, hibiscus is also used in animal feed, cosmetics, nutraceuticals, and pharmaceuticals [3, 4].

Hibiscus calyces have in their composition fats, proteins, carbohydrates, acids, minerals, and bioactive compounds. Although the composition of this plant has been widely studied, a remarkable group of compounds is anthocyanins and phenolic compounds that attract much attention due to their beneficial effects on health [4].

The major bioactive compounds found in hibiscus calyces are the anthocyanins cyanidin 3-O-sambubioside and delphinidin 3-O-sambubioside which are responsible for the flowers' red color [5]. The phenolic compounds found in this plant include organic and phenolic acids, such as citric acid, hydroxycitric acid and caffeoylquinic acid. There are also flavonoids such as quercetin. Studies have confirmed the pharmacological properties of dry calyces' extracts, including action against hypertension, inflammation, liver disorders, diabetes, metabolic syndrome, among others [6].

A recent study evaluated different methods of preparing hibiscus tea by decoction, infusion, and cold maceration. The results revealed 33 metabolites, including sugars, flavonoids, anthocyanins, and organic phenolic and aliphatic acids. Cold maceration was effective in preserving anthocyanins, while infusion was more suitable for recovering organic acids. In addition, it was observed that all teas obtained by different extraction methods were able to inhibit the enzyme α -glucosidase [7].

For the consumers and food industries, is very important to know the tea shelf life in different extraction conditions. There are few studies about the investigation of infusion methods, as well as the stability of hibiscus tea when stored for later consumption, so this can justify the innovative factor of this paper. A recent research evaluated storage stability of jabuticaba peel tea in relation to its antioxidant capacity. Storage of tea at refrigerated conditions (4 and 10 °C) for up to 72 h did not promote major changes in color, anthocyanin content and antioxidant capacity [8]. In this context, the present study investigated how the tea infusion temperature (hot and cold) can change the phytochemical profile, antimicrobial activity, and storage stability of hibiscus tea.

Materials and methods

Chemicals and reagents

6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), DPPH (2,2-diphenyl-1-picrylhydrazyl), Folin reagent, Calcium gallate, ABTS (2,2'-AZINO-BIS (3-ethylbenzo-thiazoline-6-sulfonic acid)) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Methanol

(chromatography grade) was purchased from Vetec Ltda. (São Paulo, São Paulo State, Brazil). All other reagents used were of analytical grade.

Tea preparation

Hibiscus calyces *in natura* were purchased from a producer in the city of Marialva, Paraná-BR (23° 29' 8" N, 51° 47' 34" E). The calyces were cleaned (sodium hypochlorite solution 200 ppm for 15 min) and dried in an oven at 65 °C for 22 h [9]. Cold tea infusions were prepared using 0.5 g of hibiscus in 20 ml of water at room temperature (20–25 °C) and allowed to stand for 2 h. Hot tea was prepared using 0.5 g of hibiscus in 20 ml of water at 75 °C and left to infuse for 7 min [10]. The infusions were then filtered through cloth filters and analyzed.

Tea characterization

Phytochemical profile by UPLC-MS/MS

UPLC-MS/MS was used to identify the bioactive compounds in cold and hot teas. The teas were injected into a UPLC Acquity UPLC® Bridged Ethane Hybrid (BEH) system coupled to the Xevo TQD™ triple quadrupole (Milford, MA, USA) mass spectrometer, equipped with a Waters Zspray™ electrospray ionization (ESI) source (Milford, MA, USA). The mass spectrometer was operated in positive and negative mode using the following conditions: 3.5 mbar collision gas pressure, 500 °C gas desolvation temperature, 800 l h⁻¹ desolvation gas flow and 3 kV capillary voltage. The analyzed molecules were separated in an Acquity UPLC® bridged ethane hybrid (BEH) C18 column (50 mm × 2, 1 mm, 1, 7 μm), maintained at 40 °C and injection volume of 4 μl. The mobile phase was composed of water, which was acidified with 0.1% formic acid (A) and methanol (B). The gradient developed for separation was 0.22 ml min⁻¹, with 87A:13B from 0 to 3 min, 35A: 65B from 3 to 7.2 min, 0A:100B from 7.2 to 8.3 min, after that the mobile phase returns to the initial conditions generating a total chromatographic run of 12 min.

FTIR-ATR analysis

To perform the attenuated total reflectance (ATR) analysis, an infrared spectrometer with Fourier transform (model Vertex 70v, Bruker, Germany) was used coupled to the attenuated total reflectance accessory (model Platinum, Bruker, Germany). The sample was positioned on the diamond crystal, maintaining contact with it throughout the measurement time. Each spectrum was an average of 128 scans, with a spectral resolution of 4 cm⁻¹. The spectral range of measurement was 4000 to 400 cm⁻¹.

Assessment of antibacterial activity

The minimum inhibitory concentration (MIC) of cold and hot teas were determined by the broth microdilution method in 96-well microplates (TTP, Trasadingen, Switzerland) according to the Clinical and Laboratory Standards Institute [11]. Tea concentrations ranging from 250 to 0.49 mg/ml were tested. Bacterial cultures were grown in Mueller–Hinton broth (MHB; Difco, Le Pont-de-Claix, France) at 37 °C for 6 h, standardized on the McFarland 0.5 scale and diluted to obtain 5×10^6 colony forming units (UFC)/ml. A 10 μ l aliquot of the standardized bacterial suspension was inoculated into each well and after 24 h of incubation at 35 °C, MIC was defined as the lowest concentration of tea that inhibited bacterial growth by visual reading. To determine the minimum bactericidal concentration (MBC), 20 μ l were removed from each well in which bacterial growth was not observed and inoculated in culture media specific to each bacterium. The plates were incubated for 24 h at 35 °C and MBC was defined as the lowest concentration at which bacterial growth did not occur.

Tea stability evaluation

Cold and hot teas were evaluated for pH, tea cream, total phenolic compounds (TPC), total anthocyanins (TA), antioxidant (DPPH and ABTS), instrumental color and microbiological evaluation at times 0, 24, 48 and 72 h for temperature storage condition of 5 °C.

The pH was determined by direct measurement in a digital potentiometer (PG2000). The content of total phenolic compounds (TPC) was carried out using the Folin-Ciocalteu method. The absorbance was measured at 725 nm in a spectrophotometer (UVmini—1240 Shimadzu) and the results are expressed in mg of gallic acid equivalent (GAE) g^{-1} of tea [12].

The content of total anthocyanins (TA) was determined by the single pH method (pH 1) [13]. Total anthocyanins were calculated from Eq. 1 and expressed in mg equivalent of cyanidin-3-glucoside (C3G) $0.100 g^{-1}$ tea.

$$\text{Total anthocyanins} = \frac{A_{535nm} \times \text{dilution factor}}{98.2} \quad (1)$$

For determination of the reduction of the DPPH stable radical (2,2-diphenyl-1-picrylhydrazil), absorbance was measured at 515 nm in a spectrophotometer (UVmini—1240 Shimadzu) and the antioxidant activity was calculated from the standard Trolox curve ($R^2 = 0.99$) and expressed in μ M Trolox (TE) g^{-1} of tea [14]. To determine ABTS \cdot^+ (2,2'-AZINO-BIS

(3-ethylbenzo-thiazoline-6-sulfonic acid) elimination capacity) absorbance was measured at 734 nm in a spectrophotometer (UVmini—1240 Shimadzu) and antioxidant activity was calculated from the standard Trolox curve ($R^2 = 0.99$) and expressed in μ M Trolox (TE) g^{-1} of tea [14].

Tea cream content was determined after cold and hot teas were kept for 16 h under refrigeration (5 °C). After this time, the sample was centrifuged at 5600 rpm for 20 min to remove insoluble solids and determine soluble solids in the supernatant. The difference between solids in the sample and in the supernatant indicates how much tea cream was formed during cooling [15].

The determination of parameters for color analysis of cold and hot teas was made using a colorimeter (Minolta, model CR400, Konica Minolta Sensing, Inc., Japan) with the determination in CIE $L^*a^*b^*$ mode. Since the coordinate L^* varies from 0 (completely black) to 100 (completely white), the chromaticity coordinate a^* can assume values from -80 (green) to $+100$ (red) and the chromaticity coordinate b^* varies from -50 (blue) to $+70$ (yellow).

For the microbiological analysis Petri film plates were used (3 M Company, St. Paul, MN, USA) for the determination of mesophilic aerobic bacteria according to [16], microbiological method 990.12; total coliforms and *Escherichia coli*, microbiological method 991.14.

Statistical analysis

The analyzes were performed in triplicate and evaluated statistically, using the Analysis of Variance (ANOVA), compared by the Tukey test with a significance level of 5% ($p \leq 0.05$) using the Sisvar 5.6 statistic program.

Results and discussion

Tea characterization

Phytochemical profile by UPLC-MS/MS

Bioactive compounds present in cold and hot hibiscus tea were identified by the UPLC-MS/MS method. The profile of the compounds identified (Table 1) was the same for both preparation methods, with the following being identified: cyanidin (m/z 287.1), delphinidin (m/z 303.1), 4-caffeoylquinic acid (m/z 353.0), quercetin hexoxide (m/z 463.0), quercetin (m/z 595.0) and myricetin (m/z 611.0). These compounds have already been identified in previous studies in aqueous and ethanolic hibiscus extract [3, 5, 9]. A study on white tea evaluated eight samples and compared hot and cold infusions, concluding that the samples phytochemical profile was the same, with differences in antioxidant activity only [10].

Table 1 Bioactive compounds identification

Identification trial	TR (min)	m/z	Fragments	Cone (v)	Collision energy (v)	Mode	Samples	
							Cold tea	Hot tea
Cyanidin	2.65	287.1	121.0	70	45	ES+	+	+
			137.1	70	45			
			213.0	70	45			
Delphinidin	1.53	303.1	152.9	80	50	ES+	+	+
			229.0	80	45			
			257.0	80	40			
4-Caffeoylquinic Acid	0.97	353.0	173.0	30	20	ES-	+	+
Quercetin Hexoxide	4.01	463.0	301.0	30	20	ES-	+	+
Quercetin	1.61	595.0	300.0	30	20	ES-	+	+
			463.0	30	20			
Myricetin	3.24	611.0	316.0	30	25	ES-	+	+

FTIR-ATR analysis

FTIR-ATR spectroscopy was used to identify the functional groups based on the peak values in the IR present in the samples of cold and hot teas. It was observed that the spectra for teas are similar (Fig. 1), indicating that both samples have the same compound profile after infusion, as confirmed by UPLC-MS/MS analysis (Item 3.1.1). The broadband in the

3360 cm^{-1} region corresponds to the stretching of the O–H intermolecular bond in the hydroxyl function group, indicating the presence of phenolic compounds in hibiscus teas [17, 18]. The band at 1606 cm^{-1} corresponds to the stretching of the C–N–H bond of imine groups, the band at 1265 cm^{-1} to the stretching of the C–N link of amine groups and the band at 1400 cm^{-1} to the stretching of the imine groups. The bond C=N indicates the presence of proteins and/or amino

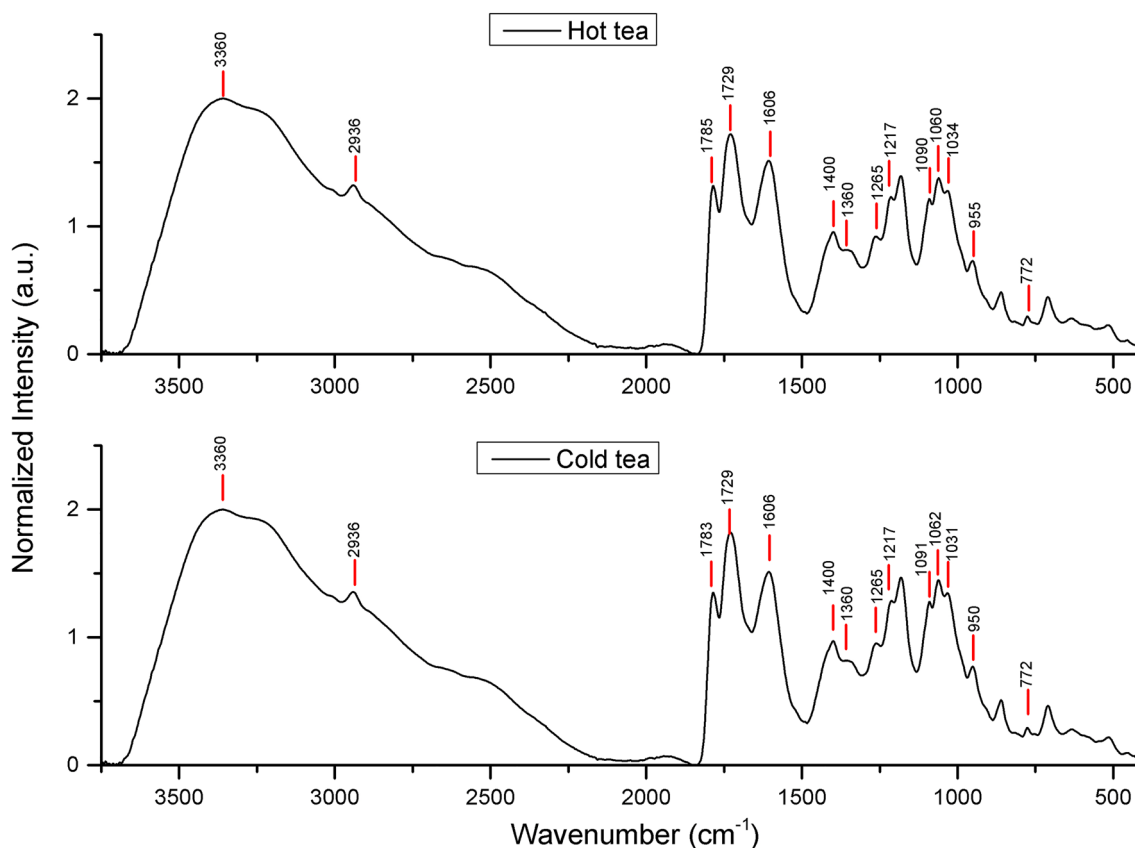


Fig. 1 Fourier infrared spectra of cold and hot hibiscus tea (normalized at 1400 cm^{-1})

acids [18, 19]. The 1783 cm^{-1} band is attributed to the C=O stretch, the band at 1217 cm^{-1} corresponds to the stretch of the group C–H, the band 955 cm^{-1} is assigned to the stretch C=C [17, 19].

Assessment of antibacterial activity

The MIC and MBC of hot and cold hibiscus tea against the *Salmonella* Typhimurium, *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* are shown in Table 2. Cold tea showed better efficiency in the inhibition of *P. aeruginosa*, with MIC of 62.5 mg/ml, and for the bacteria *Salmonella* Typhimurium, *E. coli* and *S. aureus*, obtained a minimum inhibitory concentration of 125 mg/ml. For bactericidal (MBC), cold hibiscus tea was more effective against the species of pathogenic bacteria *Salmonella* Typhimurium and *P. aeruginosa* at a concentration of 125.0 mg/ml for both strains.

A recent study evaluated the antimicrobial activity of ethanolic extract of 25 different varieties of hibiscus from Mexico against *Escherichia coli*, *Salmonella enteritidis*, *Staphylococcus aureus*, and *Micrococcus luteus*, concluding that

the most potent extracts against the microorganisms studied were the ones with the greatest amount phenolic compounds. Another study evaluated the antimicrobial activity of hibiscus methanolic extract and a MIC was found between 25 and 50 mg/ml against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Vibrio cholera* strains concluding that the extract can be used in many applications with antimicrobial intentions [18]. Thus, the antibacterial activity hibiscus tea can be attributed to the specific phenolic composition and possible synergistic effects with other compounds. Plant polyphenols act as antimicrobials, usually by disrupting bacterial cell membranes and reducing bacterial growth or multiplication [4].

Evaluation of tea stability

Regarding the pH of cold and hot tea (Table 3), there was a significant difference between the samples in Time 0 with a pH of 2.39 for cold tea and 2.28 for hot tea. After 24 h, the pH increased to an average of 2.61, keeping those values for 72 h of storage, corroborating a previous study that evaluated different Beninese Roselle (*Hibiscus sabdariffa* L.) beverage preparation procedures, and had pH ranging from 2.79 to 2.95 [20].

Cold and hot hibiscus tea showed initial levels of tea cream of 11.26 and 20.45%, respectively (Table 3). After 72 h of storage (Fig. 2), tea cream content increased by 15.14% for cold tea and 37.14% for hot tea. It was observed that the levels of tea cream for hot tea were significantly higher, probably due to the higher content of phenolic compounds. The formation of tea cream consists of solids precipitating from the solution, from the complexation of phenolic compounds with other tea constituents, such as

Table 2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of hot and cold hibiscus tea

	MIC (mg/ml)		MBC (mg/ml)	
	Cold Tea	Hot tea	Cold tea	Hot tea
<i>Salmonella typhimurium</i>	125.0	125.0	125.0	250.0
<i>Pseudomonas aeruginosa</i>	62.5	125.0	125.0	250.0
<i>Escherichia coli</i>	125.0	125.0	250.0	250.0
<i>Staphylococcus aureus</i>	125.0	125.0	250.0	250.0

Table 3 Storage stability for cold and hot tea

	pH	TPC	TA	DPPH	ABTS	L	a	b	Tea cream (%)
Time 0									
Cold tea	2.39 ^a ±0.02	4.35 ^b ±0.08	34.96 ^b ±3.11	212.13 ^b ±0.22	60.08 ^b ±0.13	21.03 ^a ±0.01	6.66 ^b ±0.01	3.63 ^b ±0.12	11.26 ^b ±0.57
Hot tea	2.28 ^b ±0.00	4.55 ^a ±0.01	66.02 ^a ±0.78	219.34 ^a ±0.13	85.15 ^a ±0.13	20.72 ^b ±0.01	7.95 ^a ±0.02	4.23 ^a ±0.06	20.45 ^a ±1.01
Time 24 h									
Cold tea	2.61 ^a ±0.01	4.33 ^b ±0.05	33.44 ^b ±2.12	211.40 ^a ±3.09	60.52 ^b ±2.00	21.08 ^a ±0.02	6.68 ^b ±0.02	3.63 ^b ±0.06	12.5 ^b ±0.00
Hot tea	2.63 ^a ±0.01	4.44 ^a ±0.04	65.85 ^a ±1.79	214.93 ^a ±2.05	85.68 ^a ±0.96	20.73 ^b ±0.02	7.96 ^a ±0.03	4.17 ^a ±0.06	25.64 ^a ±1.00
Time 48 h									
Cold tea	2.61 ^a ±0.01	4.36 ^a ±0.09	24.44 ^b ±0.51	203.16 ^b ±0.84	45.68 ^b ±3.15	24.93 ^b ±0.00	8.02 ^b ±0.01	3.00 ^b ±0.00	15.55 ^b ±1.05
Hot tea	2.61 ^a ±0.01	4.41 ^a ±0.01	34.62 ^a ±0.51	213.46 ^a ±0.22	71.81 ^a ±3.95	24.98 ^a ±0.01	8.85 ^a ±0.01	3.57 ^a ±0.06	31.31 ^a ±1.09
Time 72 h									
Cold tea	2.67 ^a ±0.00	3.99 ^a ±0.24	22.74 ^b ±0.77	196.02 ^b ±0.22	45.10 ^b ±0.94	24.97 ^a ±0.03	8.32 ^b ±0.02	3.10 ^b ±0.1	15.14 ^b ±1.03
Hot tea	2.65 ^b ±0.00	3.74 ^a ±0.06	33.77 ^a ±1.06	207.57 ^a ±0.92	70.57 ^a ±4.25	24.93 ^a ±0.01	8.47 ^a ±0.01	3.40 ^a ±0.1	37.14 ^b ±1.00

The data are expressed as means ± standard deviations. Mean values denoted by a different letter along a column are significantly different at $p \leq 0.05$. TPC-mg of gallic acid equivalent (GAE) g^{-1} of tea; TA-mg cyanidin-3-glucoside equivalent (C3G). 100 g^{-1} tea; DPPH- expressed in μM Trolox (TE) g^{-1} of tea; ABTS- μM Trolox (TE) g^{-1} of tea

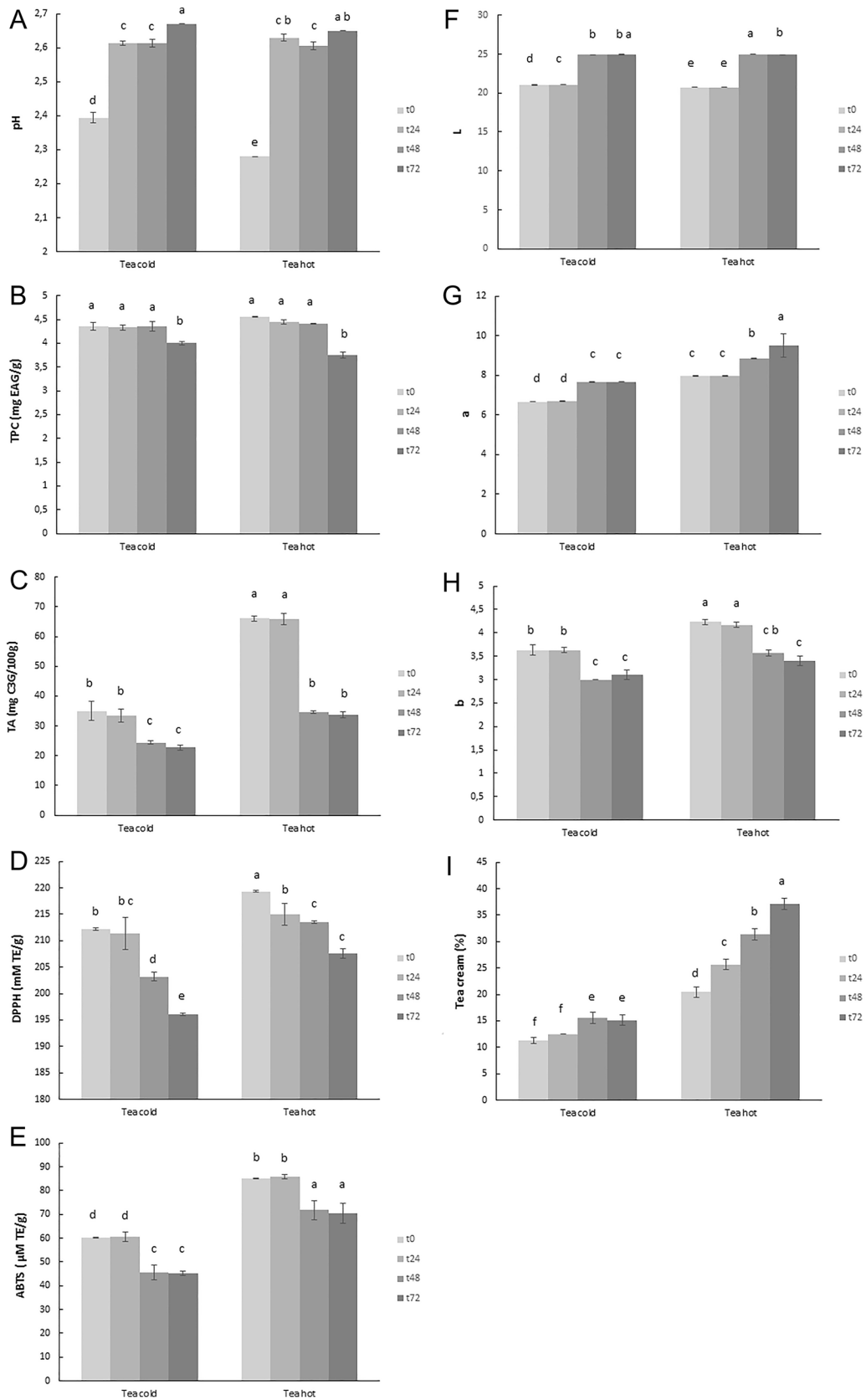


Fig. 2 Storage stability for cold and hot tea at different times. pH (a), Total phenolics (b), Total anthocyanins (c), Antioxidant activity: DPPH (d) and ABTS (e), Color: L (f), a (g) and b (h), and Tea cream (i). *Equal letters indicate no significant difference ($p < 0.05$) between samples and times

metal cations, proteins, lipids, and polysaccharides [21]. A previous study evaluated the stability of green tea extract and observed an increase from 30 to 45% in the content of tea cream after storage under refrigeration [22].

The concentration of total anthocyanins (TA) and phenolic compounds (TP), showed a significant difference between all times, and the hot infusion was more efficient for the extraction of these compounds. In Time 0, it was observed that hot tea presented 47.0% anthocyanins and 4.4% more phenolic compounds than cold tea. Recent studies have found that higher temperatures generally increase the diffusion and solubility rates of solute in the solvent. However, the extraction temperature is limited, because exceedingly high temperatures are normally associated with the degradation of anthocyanins and phenolic compounds, requiring the determination of an optimal extraction temperature [23, 24].

The anthocyanin content remained constant up to 24 h of storage, with an average drop of 30% for cold tea and 48% for hot tea in 48 h and 72 h (Fig. 2). Therefore, the tea is better when prepared at home, consumed within 24 h, and kept under refrigeration. Recent studies show that cleavage of anthocyanins cyanidin and delphinidin occurs in hibiscus extract, thus forming degradation products such as protocatechuic acid and gallic acid. Other studies also identified phloroglucinaldehyde as a degradation product of anthocyanins [5, 25]. Anthocyanin loss has already been observed during the storage of jabuticaba peel tea, with loss of monomeric anthocyanins occurring after 72 h of storage under refrigeration [8].

To evaluate the antioxidant activity of cold and hot teas, two different methods were used (DPPH and ABTS) because antioxidant compounds can act by different radical mechanisms, resulting in different degrees of antioxidant capacity in different tests [26]. It was observed that the antioxidant activity of hot tea was significantly higher when compared to cold tea (Table 3). In addition, it was observed that after 48 h of tea storage (Fig. 2) there was a decrease in antioxidant activity, and after 72 h, this reduction was of 6.5% and 17% for the DPPH and ABTS methods, respectively. A previous study with jabuticaba peel tea also observed a reduction in antioxidant activity after 24 h of storage under refrigeration [8].

In relation to color, it was observed that the a^* coordinate is significantly higher for hot tea, being more pronounced for the hot tea after 72 h (Table 3). Measurement at Time 0 was 6.66 and 7.95 for cold and hot tea, respectively. This is

related to the red color of hibiscus calyces, rich in anthocyanins, as the hot infusion was more efficient for anthocyanin extraction it is justified that the hot tea showed higher values for the a^* coordinate [27]. Regarding the storage time, it was observed that after 48 h (Fig. 2) there was a clearing of the teas, and the L^* coordinate increased from 21.03 to 24.93 for cold tea and from 20.72 to 24.90 for hot tea. The change in tea color may be due to the loss of monomeric anthocyanins during storage, including several factors such as anthocyanins co-pigmentation with other phenolic compounds. A recent study evaluated thermal processing and subsequent storage of hibiscus extract observed clearing of the extracts with storage at 4 °C for 20 days [28].

In relation to microbiological control for all samples, at times 0, 24, 48 and 72 h, there was no growth (< 1 CFU/ml) of aerobic bacteria (standard count), and total and fecal coliforms (*E. coli*), being within the standards established by the Ministério da Saúde, Portaria nº 451, de 19 de Setembro de 1997, grupo IX: Products to be consumed after adding liquid, using heat, with tea included in this group [29].

Conclusion

Infusion temperature (hot or cold) did not influence the phytochemical profile identified by UPLC-MS/MS: cyaniding (m/z 287.1), delphinidin (m/z 303.1), 4-caffeoylquinic acid (m/z 353.0), quercetin hexoxide (m/z 463.0), quercetin (m/z 595.0) and myricetin (m/z 611.0). The teas showed anti-microbial activity for the strains of *Salmonella* Typhimurium, *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus*.

The hot infusion was more efficient for the extraction of anthocyanins and phenolic compounds, thus presenting a higher content of antioxidants. Regarding storage, it was observed that from 48 h tea cream formation increases, color changes, and there is a sharp decay in the concentration of anthocyanins, phenolics and antioxidant compounds. Thus, it is recommended that hibiscus tea be consumed up to 24 h from preparation even if kept refrigerated.

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Methodology. GSM: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Software; Writing-original draft; Writing-review & editing.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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