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Effects of an herbal extract composed of white tea, roasted yerba mate and fermented rooibos on the antioxidant activity and sensory properties of popsicles manufactured with different protein sources

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Abstract

Popsicle-type edible ice cream is consumed worldwide for its sensory properties. However, its nutritional composition is limited to carbohydrates, sweeteners and synthetic flavors. In this work, the objective was to develop popsicles manufactured with different protein sources (rice protein, concentrated bovine milk whey protein and a mixture of both proteins) and added with an herbal lyophilized extract (LME) composed of white tea, fermented rooibos, and roasted yerba mate. Six formulations were produced and their proximate composition, physicochemical properties, sensory acceptability, total phenolic content, condensed tannins, and *in vitro* antioxidant activity determined. Popsicles added with LME showed a higher total phenolic content compared to the controls (without LME). The popsicles formulated with animal protein and LME showed the highest antioxidant activity as measured by the DPPH and FRAP assays. In relation to sensory analysis, the highest acceptance rates, 91 and 88%, were observed in formulations added with animal protein without and with LME, respectively. On the other hand, the vegan formulation added with LME had the lowest acceptance rate (69%). Overall, the addition of LME and concentrated bovine whey protein provides a viable option for the development of phenolic-rich protein-based popsicles.

Keywords: Technology application; Vegetarian diet; Dairy desserts; Food development; Popsicle.

1. Introduction

Ice creams are defined as frozen products obtained from an emulsion of fats and proteins; or a mixture of water and sugar. Other ingredients can be added as long as they do not mischaracterize the product (Brasil, 2005). The production of edible ice cream in Brazil has shown stability in recent years. According to the Brazilian

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Ice Cream Industry Association–ABIS, Brazil currently has roughly 8,000 ice cream producers. The sector generates about 75,000 direct and 200,000 indirect jobs, with annual revenues above US$ 2.5 billion (ABIS, 2020). Frozen desserts, such as popsicles, are popular around the world because of their sweet taste, versatility in terms of formulations and refreshing sensation.

Popsicles and conventional ice creams are marketed in various forms such as conventional ones, light, fat-free, and low sugar content among others (Granato et al., 2018). Recent studies encourage the addition of plant-based extracts, which are sources of bioactive compounds, in ice cream and other dairy products to replace synthetic preservatives and taste agents (Öztürk et al., 2018). Martins et al. (2018) evaluated the bioactivity, chemical and sensory characteristics of popsicles manufactured with bovine milk whey protein and concentrated watermelon juice. According to these authors, the popsicles were sensorially acceptable and presented antioxidant activity. In fact, a diversity of herbal extracts has been studied in relation to their bioactivity, such as antioxidative, anti-obesity, and anti-allergic effects, among others (Khan and Mukhtar, 2019).

The health outcomes of plant-based diets have been increasingly recognized for presenting several benefits in both the short and long term (Satija and Hu, 2018). For instance, Patel et al. (2017) reported that plant-based diets decrease some risk factors and mortality from cardiovascular diseases and should be a therapeutic goal in patient care. Ferdowsian and Barnard (2009) performed a biographical survey regarding the effects of plant-based diets had on plasma lipids. Results showed that individuals who chose to follow plant-based diets have significantly lower blood lipid concentrations compared with those who follow milk, egg and meat-based diets. Recently, Adeva-Andany et al. (2019) concluded that reduction in consumption of animal-based foods is beneficial in decreasing the risk of type 2 diabetes.

A recent study conducted by Santos et al. (2018) described the characterization of an herbal extract that is composed of 82.9% *Camellia sinensis* (while tea), 10.9% *Ilex paraguariensis* (roasted yerba mate) and 6.2% *Aspalathus linearis* (fermented rooibos). This mixture presented a total phenolic content of 1,200 mg/L and high antioxidant activity measured by different chemical and cell-based assays. The beverage showed antibacterial activity and antiproliferative effects against human cancer cell lines (Caco-2 and HepG2). However, there is no application of this mixture on the manufacture of ice creams or other related desserts. In addition, the lyophilized extract composed of 82.9% white tea, 10.9% roasted yerba mate and 6.2% fermented rooibos showed toxicological safety, antioxidant, anti-hyperglycemic, and antihypertensive potential using different *in vitro* methodologies (Santos et al., 2020). However, there is no technological application of this bioactive-rich extract in food models.

Natural extracts made of herbs, edible flowers and other sources of bioactive compounds should be further explored by the food industry, in particular, for the development of products with a lower content (or absence) of synthetic additives, such as flavorings, antioxidants and antimicrobial agents (Granato et al., 2018). Thus, considering the demand for natural products and the technological need to develop nutritionally balanced foods, this work is aimed at developing popsicles that are sources of proteins and natural antioxidants, without the addition of artificial sweeteners/flavors, and to evaluate their nutritional, sensory acceptance and antioxidant activity.

2. Materials and methods

2.1. Chemicals

Folin-Ciocalteau reagent, DPPH (2,2-diphenyl-1-picrylhydrazyl), gallic acid, (+)-catechin, vanillin, TPTZ (2,4,6-tris (2-pyridyl)-S-triazine), ferric chloride hexahydrate, and quercetin were obtained from Sigma-Aldrich (São Paulo, Brazil). Hydrochloric acid, sodium acetate, potassium ferricyanide, ascorbic acid, isobutanol, absolute ethanol, methanol and acetic (glacial) acid were purchased from Vetec (Rio de Janeiro, Brazil). Sodium hydroxide was purchased from Synth (Diadema, Brazil). Aqueous solutions were prepared using ultrapure water. For the formulation of popsicles, mineral water (Royal Fit, Ponta Grossa, Brazil), organic sucrose (Native, Sertãozinho, Brazil), emulsifying and stabilizing agents (Duas Rodas, Jaraguá do Sul, Brazil. Composition: mixture of fatty acid monoacylglycerols, potassium stearate, sorbitan monostearate and polyoxyethylene sorbitan monostearate), cocoa powder (Marfil, Curitiba, Brazil), concentrated bovine whey protein (80 g/100 g) and concentrated rice protein (73 g/100 g, Growth Supplements, Bombinhas, Brazil) were used.

Chemicals for HPLC analysis of phenolic compounds were purchased from different suppliers and their purity was ≥98%. Gallic acid, 5-caffeoylquinic acid, epicatechin, epicatechin gallate, gallocatechin, epigallocatechin, and epigallocatechin gallate were purchased from Sigma-Aldrich (Espoo, Finland). Procyanidin B2 was purchased from Extrasynthese (Lyon, France). Kaempferol, myricetin and quercetin were acquired from Carl Roth GmBH (Karlsruhe, Germany). Butylated hydroxyanisole (BHA) and formic acid were obtained from Sigma-Aldrich (Espoo, Finland). Phosphoric acid and hydrochloric acid were obtained from Merck (Darmstadt, Germany) and Fisher Scientific (Vantaa, Finland), respectively. Methanol (LC-MS) was obtained from J.T. Baker (Gliwice, Poland) and acetonitrile (LC-MS) from VWR Chemicals (Helsinki, Finland).

2.2. Plant material and obtaining the lyophilized mixed extract (LME)

Organic white tea (*Camellia sinensis* var. *sinensis*), fermented rooibos (*Aspalathus linearis*), and toasted matte (*Ilex paraguariensis*) were purchased from CLIPPERP (Germany), Matte Leão (Brazil), and Simon Lévelt (Netherlands), respectively. The material was ground, and the particle size standardized on 60 Tyler mesh. A mixture containing 82.9% white tea, 10.9% mate tea and 6.2% rooibos tea was extracted (80 °C for 10 min) with mineral water at a 1:50 (w/v) ratio, totaling 5 L of tea (Santos et al., 2018). Then, the aqueous extract was freeze-dried at 1,200 μHg at −50 °C for 120 h (Terroni LD, model LD1500, São Paulo, Brazil). The lyophilized herbal extract (LME) was stored under vacuum until analysis.

2.3. Phenolic composition and antioxidant profile of LME

The total phenolic content was determined by the Prussian blue method and the results are expressed in mg gallic acid equivalents per 100 g (mg GAE/100 g). The content of condensed tannins was determined by the method using H_2SO_4 and vanillin and expressed in mg of catechin equivalents per 100 g (mg CE/100 g). The ferric reducing antioxidant power, FRAP, and the free-radical scavenging activity in relation to DPPH radical were performed and expressed in mg ascorbic acid equivalents per 100 g LME (mg AAE/100 g). The total reducing capacity, TRC, was evaluated by the modified Folin-Ciocalteu method and expressed in mg of quercetin equivalents per 100 g (mg QE/100 g). Analyses were performed in quadruplicate. The description of the analytical methods employed was previously reported by Santos et al. (2018).

Table 1. Formulation of popsicles according to the protein source and addition of an herbal extract containing antioxidants

Formulations	Bovine milk whey protein (g/100 g)	Rice protein (g/100 g)	Lyophilized herbal extract (g/100 g)[1]
F1	5	0	0
F2	5	0	1
F3	0	5	0
F4	0	5	1
F5	2.5	2.5	0
F6	2.5	2.5	1

[1]Herbal extract composed of 82.9% white tea + 10.9% mate tea + 6.2% fermented rooibos tea.

An Acquity UPLC–Xevo G2 QTOF high resolution mass spectrometer (Waters, Milford, MA, USA) operated by Waters MassLynx 4.1 software was used for the characterization of phenolic compounds in LME. Compounds were separated on Waters Acquity BEH C_{18} (1.7 µm, 2.1 mm × 150 mm) column using a gradient of 0.1% formic acid in H_2O (A) and of 0.1% formic acid in acetonitrile (B). The gradient program was as follows: 2% of B in 0–2 min, 2–75% of B in 2–27 min, 75–99% of B in 27–32 min, held at 99% of B for 2 min, 99–2% in 2 min and held at 2% of B for 4 min. The flow rate was 0.55 mL/min, temperature of the column oven was 45 °C, and the injection volume was 1.0 µL. An electrospray ionization (ESI) was used with capillary voltage of −1 kV in negative and + 0.5 kV in positive mode. The sampling cone was set to 35 V and extraction cone to 4 V. The cone and desolvation nitrogen gas flows were 15 and 990 L/h, respectively. The desolvation temperature was 550 °C and the source temperature was 150 °C. Argon was used as the collision gas. MS analyses were conducted by data independent acquisition (MS^E) centroid data mode in a full scan *m/z* 50–1,500 with 0.2 sec scan time. In the MS^E function, the precursor ions from the low-collision energy MS-mode were fragmented using high collision energy ramped up from 25 to 45 V (Pihlava et al., 2018).

Several HPLC methods were used for quantification of individual phenolics. LME samples were dissolved in 65% methanol (aq) and filtered into HPLC ampoules. Phenolic acids were determined by using an Agilent 1290 Infinity Series ultra-high-performance liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a diode array detector and a fluorescence detector. Phenolic acid separation was done with a Zorbax Eclipse Plus C_{18} (2.1 × 50 mm, 1.8 µm) column (Agilent Technologies Inc.) with a C_{18} guard column. The temperature of the column oven was set at 35 °C. A gradient elution was employed with a mobile phase consisting of 50 mM H_3PO_4 at pH 2.5 (A) and acetonitrile (B) as follows: 5% of B in 0–1.2 min, 5–15% of B in 1.2–4.25 min, 15–20% of B in 4.25–10 min, 20–50% of B in 10–15 min, 50% of B in 15–16.2 min, 50–5% of B in 16.2–17 min, post-time 2 min before the next injection. The flow rate of the mobile phase was 0.4 mL/min, and the injection volume was 2.0 µL. UV spectra of peaks were recorded between 190 and 400 nm.

Flavan-3-ols were determined by the same UHPLC-DAD-FLD device as phenolic acids using the same column. The binary mobile phase consisted of 0.5% formic acid (A) and acetonitrile (B): 2% of B in 0–2 min, 2–5% of B in 2–5 min, 5–15% of B in 5–12 min, 15–20% of B in 12–15 min, 20–35% of B in 15–20 min, 35–90% of B in 20–21 min, and back to the starting point in 2 min. The post-time was 2 min before the next injection. The flow rate was 0.5 mL/min and the injection volume was 2 µL. Elution was monitored by diode array detection (DAD; λ1 = 270 nm, λ2 = 280 nm) and fluorescence detection (FLD; λex = 275 nm, λem = 324 nm).

Flavonols were determined after acid-hydrolysis as aglycons. The LME sample (10–15 mg) was dissolved in 20 mL of 62.5% methanol (aq) containing 0.2% of BHA, after which 5 mL of 6 M HCl were added. The sample was refluxed under an inert atmosphere (argon) for 2 h. Hydrolyzed and cooled sample was brought to a volume of 50 mL and filtered into the HPLC ampoule. An Agilent 1100 HPLC-DAD device equipped with Nova-Pak C_{18} column (3.9 × 150 mm, 4 µm) was used. The binary mobile phase consisted of 50 mmol/L H_3PO_4 at pH 2.4 (A) and methanol (B): 5–58% of B in 0–50 min, 58–90% of B in 50–56 min, 90% of B in 56–68 min, 90–5% of B in 68–71 min. The post-time was 10 min before the next injection. The flow rate was 0.9 mL/min and the injection volume 10 µL. UV spectra of peaks were recorded between 190 and 400 nm and flavonols were quantified at 370 nm.

All phenolic compounds were quantified using calibration curves of authentic compounds when available. 5-Caffeoylquinic acid (chlorogenic acid) was used as the reference standard for all forms of caffeoylquinic acids. Gallolyated dimeric procyanidin was quantified as epicatechin gallate. Results were expressed as mg/100 g of LME.

2.4. Popsicle manufacturing

Six formulations of edible ice cream were prepared with 100 mL of water, 10 g/100 g of sucrose, 2 g/100 g of emulsifying and stabilizing agents, and 4 g/100 g of cocoa powder as the base formulation. The six popsicle formulations are shown in Table 1.

The technological process applied consisted of mixing all components in a blender (Mondial, Power NL26) with a capacity of 1.5 L at 500 W for 5 min. The incorporation of the emulsifying and stabilizing agents and cocoa powder was performed slowly to facilitate dissolution. Subsequently, the formulations were pasteurized at 65 °C/30 min. After cooling to 10 °C the mixture was beaten in an electric mixer (KitchenAid, Stand Mixer Bowl Kitchen Aid KEA30CEPNA, USA) with a capacity of 4.3 L at 300 W for 5 min. Then, the mixture was added in polyvinyl chloride molds and subjected to rapid freezing in an ultra-freezer at –74 °C. Finally, the popsicles were removed from the molds, individually wrapped and stored until analysis.

2.5. Proximal composition of formulations added of LME

To characterize the popsicles with added of lyophilized mixed extract, the contents of protein, moisture and ash were determined according to the AOCS methods n°. 920.87; 925.09; 923.03, respectively (AOAC, 2005). The lipid content was determined according

to the AOAC method n°. 989.05 (AOAC, 1990). The pH, total titratable acidity and total soluble solids were determined according to methods No. 981.12, 942.15 and 932.12, respectively (AOAC, 1997). The carbohydrate + fiber content of edible ice cream formulations was calculated by the difference between 100 and the sum of the protein, fat, moisture and ash content. The average caloric value of edible ice cream was calculated using the factors Atwater (proteins and carbohydrates = 4 kCal/g and lipids = 9 kCal/g), and the results expressed in Ccal/100 g.

2.6. Total phenolic compounds, total condensed tannins and in vitro antioxidant activity of popsicles

To determine the total phenolic content and antioxidant activity of the popsicles, an extraction was performed under constant agitation in a vortex tube shaker for 10 min, of 4 g of the popsicles in the presence of 4 mL of methanol. Subsequently, the mixture was centrifuged at 700 x g for 20 min, and the upper phase was collected and analyzed immediately. The total phenolic compounds, total condensed tannin content, as well as FRAP activity, antioxidant action against the DPPH radical and total reducing capacity, were evaluated as described in item 2.3, and the results were expressed in mg of the standard used for each analysis equivalent per 100 g of popsicle.

2.7. Sensory analysis

Sensory analysis was performed according to the approved terms of the Ethics Committee of UEPG, CAAE: 65493717.9.0000.0105. A total of 444 untrained evaluators, 149 men and 295 women between ages of 18 and 50 years were used after signing the informed consent form. The edible ice cream, 15 g, was served in plastic packaging identified with three random digits. The nine-point structured hedonic scale ranging from 1 (extremely disliked) to 9 (extremely liked) was used to assess acceptability in terms of odor, taste, color and overall impression. The acceptance index for each sample was calculated using the average of the global impression attribute multiplied by 100 and divided by the maximum score, that is, 9. The results were expressed as a percentage of acceptance. The acceptance index for each of the 6 formulations was compared using the Marascuillo procedure, based on the *z* test with $p < 0.05$. Additionally, the evaluators were requested to answer how much more they would pay (R$ 0 to 2.00–US$ 0 to 0.37) for a "chocolate popsicle with the addition of extract rich in natural antioxidant compounds" compared to "commercial popsicle without the addition of natural antioxidants" , respectively. The evaluators were also asked (yes/no) whether they would buy the popsicle if it was marketed.

2.8. Statistical analysis

The analytical methods were performed in triplicate (unless otherwise stated) and the data were presented as mean ± standard deviation. To compare the responses, differences between the mean values were assessed by the one-way analysis of variance (ANOVA) followed by the Fisher test to compare the means. To compare two samples (presence or absence of extract) the paired *t*-Student test was applied. Probability values lower than 0.05 were used to reject the null hypothesis (Granato et al., 2014). The software TIBCO Statistica v. 13.3 (TIBCO Statistica Inc., USA) was used for all statistical analyzes.

3. Results and discussion

3.1. Phenolic composition and antioxidant activity of the lyophylized herbal extract (LME)

The average total phenolic content in the LME was 30,401 mg GAE/100 g and the total amount identified by chromatography was 11,736 mg/100 g. The condensed tannin content determined in the LME was 6,844 mg CE/100 g, which is much higher than the total flavan-3-ol content determined by chromatography. Flavonols, which include quercetin, kaempferol and their glucosides accounted for 65.4%, while flavan-3-ols accounted for 21.6% and phenolic acids for 13.0% of the total phenolic content in LME (Table 2).

Epigallocatechin, epigallocatechin gallate, gallic acid, and 5-caffeoylquinic acid were the most abundant phenolic compounds in LME. *C. sinensis* is the major source of tannins, such as flavan-3-ols, and contributed mainly to the total flavan-3-ol content of LME (Zeng et al., 2020). All flavan-3-ols identified in the present study have been previously reported in *C. sinensis* previously (Jiang et al., 2015; Wang et al., 2012). Fermented rooibos is a source of flavonols and other flavonoids (both aglycons and glucosides), such as isoquercitrin, rutin, pinobanksin, quercetin, isorhamnetin, luteolin, orientin, aspalathin, hyperoside, and vitexin (Santos et al., 2016; Walters et al., 2017). Tea leaves have also been recognized as a rich source of flavonols, especially quercetin, but also kaempferol and myricetin (Jeszka-Skowron, et al., 2018; Wang et al., 2012) Roasted mate is a source of caffeoylquinic acid derivatives (3-caffeoylquinic acid, 4-caffeoylquinic acid, and 5-caffeoylquinic acid, dicaffeoylquinic acids), other phenolic acids, and some flavonols, such as rutin, quercetin glucoside, kaempferol-rhamnoglucoside and kaempferol glucoside (Markowicz Bastos et al., 2007; Mateos et al., 2018). Santos et al. (2020) identified hesperidin, epigallocatechin gallate, isoquercitrin, rutin and hesperidin as the main compounds in LME. In addition, methylxanthines, caffeine and theobromine were detected by UHPLC-QTOF in positive mode, but these compounds were not quantified.

LME was evaluated for its *in vitro* antioxidant capacity by three different methods. FRAP and DPPH values were 70,375 ± 2,575 and 43,189 ± 630 mg AAE/100 g, respectively. Similarly, the total reducing capacity of LME, which encompasses both lipophilic and water-soluble antioxidants was 43,790 ± 2,238 mg QE/100 g. Ali et al. (2014) used pomegranate, asparagus, salep orchid and green asparagus powder (1 to 4 g/100 g) to manufacture ice creams and observed that the antioxidant activity measured by the DPPH and FRAP assays increased in a dose-dependent manner. The antioxidant activity observed in LME is directly related to the content of phenolic compounds. Recently, Santos et al. (2020) observed that LME also presented Folin-Ciocalteu reducing capacity (~1,800 mg GAE/L of tea) and Cu^{2+} chelating ability (~72% of pyrocatechol violet-Cu^{2+} complex formation). LME was also shown to decrease mechanical hemolysis of human erythrocytes and inhibited the induced oxidation of Wistar's rat brain homogenate. Morais et al. (2020) evaluated the antioxidant activity and bioaccessibility of phenolic compounds in edible flowers–*Rosa chinensis* Jacq., *Torenia fournieri* (F.) Lind., *Bellis annua* L., *Clitoria ternatea* L., *Cosmos sulphureus* Cav., *Dianthus chinensis* L., *Begonia tuberhybrida* Voss. and *Tagetes patula* L. and observed high and positive correlation values (r = 0.90) between total phenolic content and FRAP in the methanolic extract.

3.2. Proximate composition and physicochemical properties of popsicles

The physicochemical properties and proximate composition of the

Table 2. Phenolic compounds in the lyophilized herbal extract characterized by UHPLC-QTOF MS in negative mode and quantified by HPLC/UHPLC methods

Phenolic compounds	UHPLC-QTOF MS: $[M-H]^-$ (calculated mass)	MS^2	Amount (mg/100 g)
Gallic acid ($C_7H_6O_5$)	169.0123 (169.0140)	125.053	815 ± 2
3-Caffeoylquinic acid ($C_{16}H_{18}O_9$)	353.0857 (353.0873)	191.051, 179.031	170 ± 3
5-Caffeoylquinic acid ($C_{16}H_{18}O_9$)	353.0852 (353.0873)	191.053, 179.035	207 ± 5
4-Caffeoylquinic acid ($C_{16}H_{18}O_9$)	353.0865 (353.0873)	179.031, 191.052	61 ± 4
Dicaffeoylquinic acid 1 ($C_{25}H_{24}O_{12}$)	515.1176 (515.1190)	353.086, 191.052, 179.031	<LOQ
Dicaffeoylquinic acid 2 ($C_{25}H_{24}O_{12}$)	515.1199 (515.1190)	353.086, 191.053, 179.031	138 ± 2
Dicaffeoylquinic acid 3 ($C_{25}H_{24}O_{12}$)	515.1198 (515.1190)	353.086, 179.031, 191.053	136 ± 2
Total phenolic acids			*1,527 ± 19*
Catechin ($C_{15}H_{14}O_6$)	289.0675 (289.0712)		19 ± 1
Epicatechin ($C_{15}H_{14}O_6$)	289.0704 (289.0712)		233 ± 12
Gallocatechin ($C_{15}H_{14}O_7$)	305.0638 (305.0661)		19 ± 1
Epigallocatechin ($C_{15}H_{14}O_7$)	305.0648 (305.0661)		1,060 ± 80
Epigallocatechin gallate ($C_{22}H_{18}O_{11}$)	457.0769 (457.0771)	305.064, 169.010	877 ± 18
Procyanidin B2 ($C_{30}H_{26}O_{12}$)	577.1340 (577.1350)	425.086, 289.070	18 ± 1
Gallolyated dimeric procyanidin ($C_{37}H_{30}O_{16}$)	729.1453 (729.1456)	577.098, 289.067, 169.006	310 ± 29
Total flavan-3-ols			*2,536 ± 141*
Myricetin-hexoside ($C_{21}H_{20}O_{13}$)	479.0827 (479.0826)	317.026	323 ± 38 (myricetin, total amount)
Myricetin-rhamnoside-hexoside ($C_{27}H_{30}O_{17}$)	625.1410 (625.1405)	479.081, 317.026	
Quercetin-hexoside ($C_{21}H_{20}O_{12}$)	463.0879 (463.0877)	301.032	6,340 ± 310 (quercetin, total amount)
Quercetin-pentoside ($C_{20}H_{18}O_{11}$)	433.0750 (433.0771)	301.031	
Quercetin-rhamnoside-hexoside 1 ($C_{27}H_{30}O_{16}$)	609.1467 (609.1456)	301.032	
Quercetin-rhamnoside-hexoside 2 ($C_{27}H_{30}O_{16}$)	609.1471 (609.1456)	301.032	
Quercetin-rhamnoside-dihexoside 1 ($C_{33}H_{40}O_{21}$)	771.1996 (771.1984)	301.033	
Quercetin-rhamnoside-dihexoside 2 ($C_{33}H_{40}O_{21}$)	771.2012 (771.1984)	301.033	
Quercetin-coumroyl-rhamnoside-hexoside	755.1804 (755.1823)	301.032	
Kaempferol-hexoside	447.0905 (447.0927	285.039	
Kaempferol-rhamnoside-hexoside ($C_{27}H_{30}O_{15}$)	593.1507 (593.1506)	285.083	1,010 ± 80 (kaempferol, total amount)
Kaempferol-rhamnoside-dihexoside ($C_{33}H_{40}O_{20}$)	755.2042 (755.2035)	285.038	
Kaempferol-dirhamnoside-hexoside ($C_{33}H_{40}O_{19}$)	739.2075 (739.2086)	285.037	
Apigenin-C,C-dihexoside 1 ($C_{27}H_{30}O_{15}$)	593.1493 (593.1506)	353.063	<LOQ (apigenin, total amount)
Apigenin-C,C-dihexoside 2 ($C_{27}H_{30}O_{15}$)	593.1512 (593.1506)	413.085	
Apigenin-C,C-pentoside-hexoside ($C_{26}H_{28}O_{14}$)	563.1412 (563.1401)	353.063	
Total flavonols			*7,673 ± 428*
Total phenolic content quantified			*11,736*

Table 3. Proximal composition and physicochemical properties of popsicles manufactured with different protein sources, with or without the addition of the lyophilized herbal extract (LME) containing antioxidants

Evaluated parameters	Bovine whey protein + LME	Rice protein + LME	Bovine whey protein + rice protein + LME
Proximal composition			
Lipid (g/100 g)	1.02 ± 0.04[a]	1.02 ± 0.03[a]	1.00 ± 0.05[a]
Ash (g/100 g)	0.71 ± 0.07[a]	0.62 ± 0.03[a]	0.47 ± 0.02[b]
Protein (N × 6.38; g/100 g)	7.41 ± 0.47[a]	7.54 ± 0.02[a]	6.11 ± 0.13[b]
Moisture (g/100 g)	82.51 ± 0.14[a]	76.95 ± 0.32[b]	82.90 ± 1.94[a]
Carbohydrates + fibers (g/100 g)	8.35 ± 0.57[b]	13.87 ± 0.28[a]	9.52 ± 2.02[ab]
Mean energy value (kCal/100 g)	72	95	71
Physicochemical properties			
pH	6.29 ± 0.03[a]	6.71 ± 0.01[a]	6.61 ± 0.01[a]
Titratable acidity (g/100 g)	0.63 ± 0.03[a]	0.18 ± 0.01[c]	0.28 ± 0.01[b]
Total soluble solids (°Brix)	24	14	18

Different letters in the same line represent a statistical difference between popsicles ($p < 0.05$).

popsicles are shown in Table 3. There was no significant difference in the lipid content and pH between the different formulations ($p > 0.05$). Regarding the ash and protein contents, there was a significant difference between the popsicles added with only one protein source (either animal or vegetable) in relation to the mixture of the two proteins ($p < 0.05$). The ash values ranged from 0.71 ± 0.07 (bovine whey protein) to 0.47 ± 0.02 g/100 g (50% bovine whey protein and 50% rice protein). Protein contents ranged from 7.54 ± 0.02 (rice protein) to 6.11 ± 0.13 g/100 g (50% animal protein and 50% vegetable protein).

Significant difference was observed ($p < 0.05$) in the moisture content between the popsicle added with rice protein and the other two formulations ($p < 0.05$), ranging from 82.90 ± 1.94 (50% bovine whey protein and 50% rice protein) to 76.95 ± 0.32 (rice protein). Regarding the carbohydrate content, a significant difference ($p < 0.05$) existed between the formulation added with bovine whey protein (8.35 ± 0.57 g/100 g) and rice protein (13.87 ± 0.28 g/100 g). For the total titratable acidity, there was a significant difference ($p < 0.05$) between all formulations, and values ranged from 0.63 ± 0.03 (bovine whey protein) to 0.18 g/100 g (rice protein).

3.3. Total phenolic content and condensed tannins in popsicles

Figure 1a shows the total phenolic content of the popsicles with and without the addition of the LME with animal and/or vegetable protein. Obviously, the higher total phenolic contents ($p < 0.001$) were observed in popsicles added with 1 g of LME/100 g of popsicle. It is also observed that the animal and/or vegetable proteins did not negatively affect the solubility and incorporation of phenolic compounds in the popsicle model. Aqueous herbal extracts are recognized sources of phenolic compounds. The incorporation of this type of extract in foods has become an alternative to enhance the antioxidant activity of foods and beverages (Granato et al., 2018).

Figure 1b shows the content of total condensed tannins and a similar behavior to that of the total phenolic content was observed. Popsicles with the addition of LME had values between 50 to 55 mg CE/100 g, while popsicles without the addition of LME did not present tannins. As LME is composed of flavan-3-ols originating from *the C. sinensis* extract, it is plausible that popsicles added with LME would be the source of condensed tannins (Santos et al., 2018).

Our results are in line with those reported by Fidelis et al. (2020) who added a lyophilized tannin-rich extract from camu-camu (*Myrciaria dubia*) seeds in yogurt. This extract had a total phenolic content of 43,598 mg GAE/100 g and a condensed tannins content of 5,766 mg CE/100 g. When added to yogurt (from 0.25 to 1 g/100 g), DPPH and FRAP values increased considerably ($p < 0.05$) in a dose-dependent manner compared to the control (without the tannin-rich extract). Martins et al. (2018) developed popsicles added with concentrated watermelon juice as source of natural flavor and bioactive compounds and found a total phenolic content of 12.5 mg GAE/100 g, lycopene content of about 3 mg/100 g, and antioxidant activity measured by the DPPH assay.

As a final comment on the total phenolic content of foods added with natural extracts, any alleged health benefits to humans should be assessed using clinical trials as it is still debatable whether a higher ingestion of phenolic compounds provides any additional benefits for the overall health (Granato et al. 2020a; Granato et al., 2020b).

3.4. In vitro antioxidant activity of edible ice cream

Figure 2 shows the results of the antioxidant activity of the popsicle formulations. Regarding DPPH and FRAP, there was a significant difference ($p < 0.001$) between the different formulations with or without the addition of LME. Regarding the total reducing capacity, the popsicles without the addition of LME did not present any quantifiable antioxidant activity using the applied methodology.

Figure 2a shows that the popsicles added with 1 g of LME/100 g had a FRAP mean value of 260 ± 8 mg AAE/100 g, while popsicles without the addition of LME had 28 ± 1 mg AAE/100 g, thus representing an 8.75-fold increase. This disparity between popsicles with and without the addition of LME was also observed for the DPPH assay (Figure 2b). Popsicle added with 1 g of LME/100 g had a DPPH mean value of 157 ± 8 g of AAE/100 g while a DPPH value of 12 ± 1 mg AAE/100 g was obtained for the popsicles without the addition of LME. The total reducing capacity (Figure 2c) ranged from 93 ± 7 mg QE/100 g quantified in the edible ice cream added with animal protein to 80 ± 6 mg QE/100 g in the ice cream

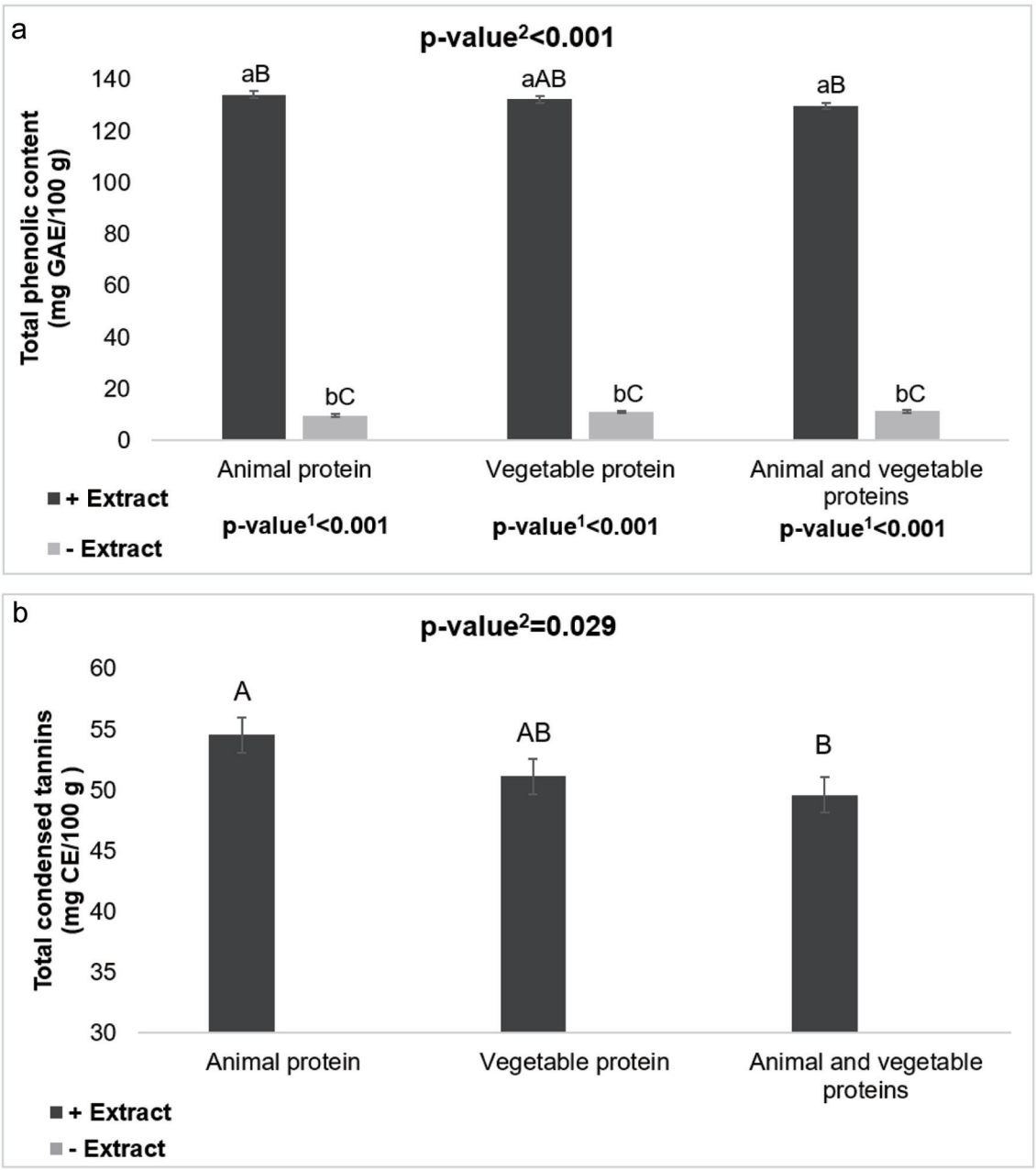
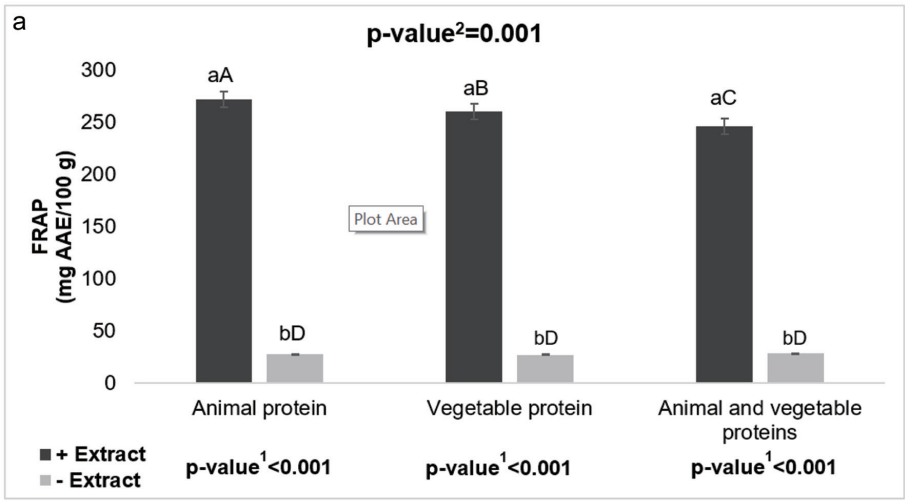


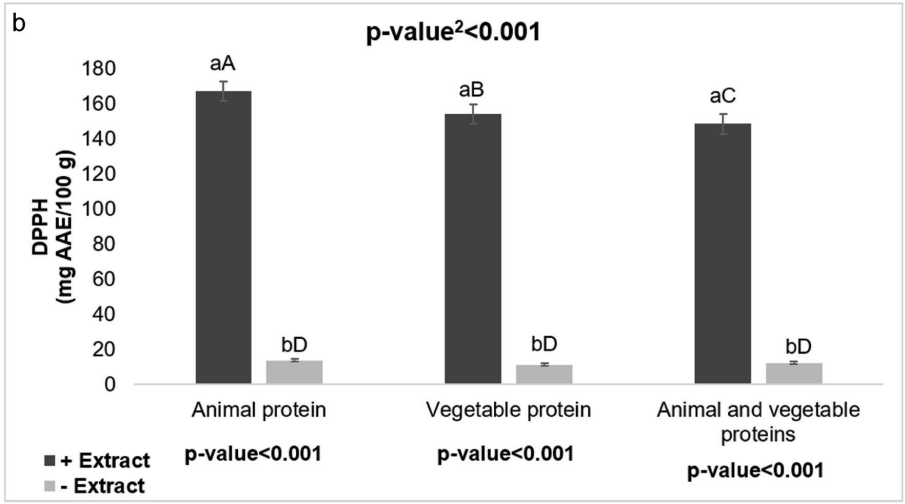
Figure 1. Total phenolic content (a) and condensed tannins (b) of popsicles manufactured with different sources of proteins, with or without the addition of the lyophilized herbal extract (LME). [1]Probability value obtained by the paired Student-t test; [2]Probability value obtained by one-way ANOVA; Different lower case letters indicate a significant difference between popsicle manufactured using the same protein source, with and without LME; Different capital letters indicate a significant difference among all popsicle formulations; +extract = popsicle with addition of 1 g/100 g of lyophilized mixed extract; −extract = popsicle without adding 1 g/100 g of lyophilized herbal extract.

added with protein animal and vegetable. Similarly, Gremski et al. (2019) added a lyophilized herbal extract (1 g/100 g) composed of 70% green mate (*Ilex paraguariensis*) and 30% lemon balm (*Melissa officinalis*) in ice cream. The herbal extract was mainly composed of flavonoids (quercetin-3-rutinoside, hesperidin and isoquercetin – 11 g/100 of lyophilized extract) which provided a total phenolic content, DPPH, FRAP, and total reducing capacity of 160 mg GAE/100 g, 81 mg AAE/100 g, 442 mg AAE/100 g, and 231 mg QE/100 g, respectively.

A similar trend observed herein was obtained by Escher et al. (2019) when a lyophilized extract from *Calendula officinalis* flower extract was added to a yogurt model. Yogurts added with the flower extract up to 1.5 g/100 g presented a respective total phenolic content and antioxidant activity (DPPH and total reducing capacity) up to 27 mg GAE/100 g, 13 mg AAE/100 g and 54 mg QE/100 g.

These results show that the addition of LME increased the antioxidant activity of popsicles formulated with rice and/or bovine





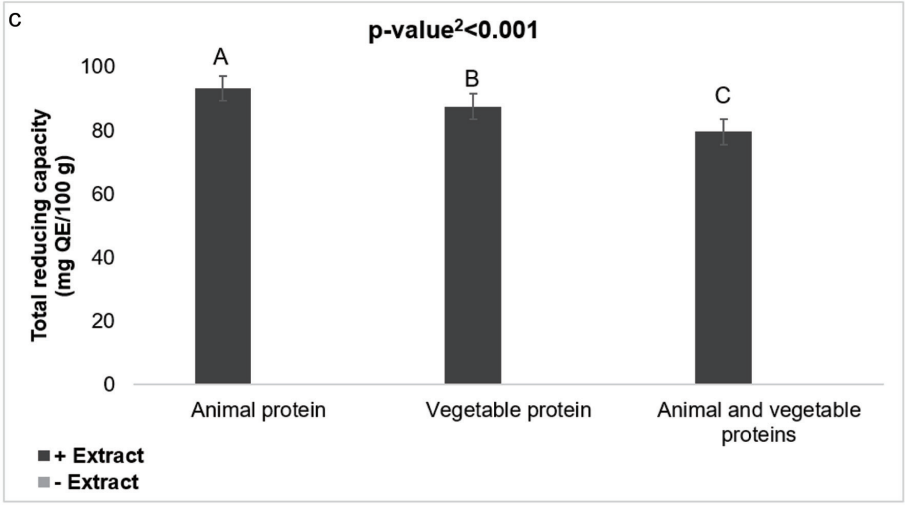


Figure 2. *In vitro* **antioxidant activity (FRAP, A; DPPH, B; TRC, C) of popsicles manufactured with different protein sources, with or without the addition of the lyophilized herbal extract (LME).** [1]Probability value obtained by the paired Student-t test; [2]Probability value obtained by one-way ANOVA; Different lower case letters indicate a significant difference between popsicle manufactured using the same protein source, with and without LME; Different capital letters indicate a significant difference among all popsicle formulations; +extract = popsicle with addition of 1 g/100 g of lyophilized mixed extract; −extract = popsicle without adding 1 g/100 g of lyophilized herbal extract.

Table 4. : Sensory data of popsicles manufactured with different protein sources, with or without the addition of an herbal extract containing antioxidants.

Formulations	Odor	Taste	Consistency	Color
Bovine whey protein – herbal extract	7.1 ± 1.5^{ab}	8.0 ± 1.3^{a}	7.9 ± 1.3^{a}	8.2 ± 1.0^{a}
Bovine whey protein + herbal extract	7.4 ± 1.6^{a}	7.7 ± 1.3^{ab}	7.6 ± 1.4^{ab}	8.2 ± 1.2^{a}
Rice protein – herbal extract	6.8 ± 1.8^{bc}	6.6 ± 1.9^{c}	6.5 ± 1.8^{c}	6.9 ± 1.7^{b}
Rice protein + herbal extract	6.4 ± 1.9^{c}	5.8 ± 2.0^{d}	6.3 ± 2.2^{c}	7.3 ± 1.7^{b}
Bovine whey protein + rice protein – herbal extract	7.5 ± 1.6^{a}	7.4 ± 1.5^{b}	7.3 ± 1.6^{b}	8.2 ± 1.3^{a}
Bovine whey protein + rice protein + herbal extract	7.1 ± 1.6^{ab}	6.8 ± 1.9^{c}	6.7 ± 1.9^{c}	8.3 ± 1.2^{a}
p-Value[1]	0.001	<0.001	<0.001	<0.001

[1]Probability values obtained by one-way ANOVA; Different letters in the same column represent statistical difference between popsicle formulations.

whey protein. Our results clearly show that the incorporation of natural extracts rich in antioxidant compounds in popsicle, which is a product widely consumed by all age groups, is feasible. This is in line with the global trend of consumers looking for foods that have more functional natural compounds and less synthetic additives (Granato et al., 2018; Kooijmans and Flores-Palacios, 2014; Katt and Meixner, 2020).

3.5. Sensory analysis

The data for the sensory attributes of the popsicles – liking of odor, flavor, consistency and color–are shown in Table 4. There was a significant difference ($p < 0.05$) in all parameters evaluated. Formulations F1 and F2, which were added with bovine whey protein, were better perceived by the assessors for all their assessed sensory parameters. These results were similar for popsicles with or without the LME. Popsicles manufactured with rice protein (formulations F3 and F4, respectively) garnered the lowest hedonic scores, and LME negatively impacted on the liking of flavor.

Figure 3 shows the effects of adding 1 g of LME/100 g on the acceptance rate of popsicles. It was observed that the addition of LME in formulation did not decrease the overall acceptance of formulations ($p > 0.05$). Sacchi et al. (2019) developed ice creams added with extra virgin olive oil and found that although the product had 25 mg GAE/kg of total phenolics, it was well accepted by the taste panel for receiving high scores for "cut grass", "aromatic persistence", and "global aromatic intensity". Ice creams added with spray-dried microalgae up to 0.3 g/100 g (total phenolic content between 150 and 240 mg GAE/kg) did not differ from the control (no addition of microalgae) in terms of overall sensory acceptability and degree of liking of color, structure, taste, odor, melting, texture, and strange taste (Durmaz et al., 2020).

The overall acceptance indices for the popsicle formulations are shown in Figure 3. No significant ($p = 0.354$) difference in acceptance index was observed between F1 (91%) and F2 (88%). Popsicles manufactured with rice protein (F3 and F4) presented the lowest acceptance indices −73 and 69%, respectively. This result shows that the added protein is the determining factor in the acceptance of the product. Kurt and Atalar (2018) used quince seed flour up to 0.75 g/100 g (4.6 g/100 g of proteins) to develop ice creams and found that the protein content was not associated with the sensory acceptance of the formulations. Additionally, the degree of liking of flavor, texture and appearance was not different ($p > 0.05$) from the control (no addition of quince seed flour). Dos Santos Cruxen et al. (2017) developed ice creams with increased concentrations of butiá (*Butia odorata*) pulp (30 to 50%; total phenolic content between 49 and 67 mg GAE/100 g and DPPH values between 17 and 24% of inhibition). Sensory analysis showed that there was no difference in overall acceptability between treat-

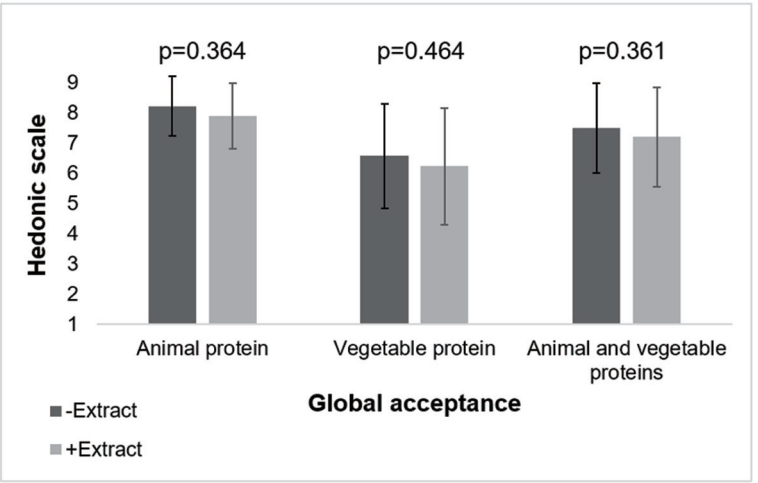


Figure 3. Overall sensory acceptance of popsicle formulations manufactured with different protein sources with or without the addition of the lyophilized herbal extract (LME). Note: The probability values are based on the paired Student-*t* test.

ments, but the degree of liking of color and flavor was higher for ice creams added with 50% of butiá pulp.

When the evaluators were asked about the purchase intention, 77% responded that they would buy popsicles with added proteins and natural extract. The assessors also gave their opinion on the amount paid more for a 75 g unit of "chocolate popsicle added with a natural extract rich in antioxidants" (R$ 0 to 2.00 – US$ 0 to 0.37) compared to a "commercial popsicle without the addition of a natural extract rich in antioxidants" (R$ 2.00 – US$ 0.37), respectively. The result was that 90% of the assessors would pay at least R$ 0.50 (US$ 0.09) more for the product rich in natural antioxidants. This result highlights the demand of current consumers, who are concerned not only with sensory issues, but also with the presence of natural compounds with possible bioactivities rand possible health benefits of the product.

4. Conclusions

The results presented in this work indicates that the addition 1 g/100 g of the optimized lyophilized extract containing white tea, roasted mate and fermented rooibos in popsicles provides a significant increase in antioxidant compounds, regardless of the added protein. The product formulated with animal protein and added with the optimized extract showed 88% acceptance of by the consumers, indicating its market potential. Rice protein was shown to be an adequate source of protein for the development of popsicles. Overall, popsicles that are sources of proteins and phenolic compounds may become a potential delivery system of high-quality nutritional proteins and bioactive compounds.

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