Received: 24 May 2022 | Revised: 11 August 2022 | Accepted: 23 August 2022

DOI: 10.1002/jsf2.78

RESEARCH ARTICLE

JSFA Reports

Patagonian ñire (*Nothofagus antarctica*) combined with green tea - Novel beverage enriched in bioactive phytochemicals as health promoters

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Funding information
Fondo para la Investigación Científica y Tecnológica, Grant/Award Number: PICT-2018-2668; Instituto Nacional de Tecnología Agropecuaria, Grant/Award Numbers: PE-2019-I114-A007, PE-2019-I140; Fundación Carolina; Consejo Nacional de Investigaciones Científicas y Técnicas

Abstract

Background: Polyphenol-enriched tea is an interesting product for food industries under the current consumer demands. This study aimed at evaluating the *Nothofagus antarctica* (*NA*) species as a valuable source of bioactive compounds as well as obtaining an optimized organic green tea (OGT)-*NA* blend. *NA* leaves of different developmental stages and several mixture proportions were analyzed. HPLC–DAD–ESI/MSn was used to identify and quantify the polyphenol compounds. Antioxidant capacity and the type of interaction occurring between constituents were assessed.

Results: *NA* infusions exhibited high polyphenol diversity and some differences in the individual concentration were found between *NA* leaves. *NA* polyphenol profile showed great complementarity to the one present in green tea. Besides, *NA* impacted positively the antioxidant strength in the mixtures. Finally, the 67% OGT-33% *NA* blend exhibited an optimized performance in relative and total polyphenol contents and antioxidant properties, and thus, it could be recommended as a novel beverage.

Conclusions: *N. antarctica* is a valuable source of polyphenol compounds and its combination with green tea, in a blend, could represent an interesting food product. In addition, this new non-wood product constitutes a novel productive strategy for adding value to the silvopastoral systems with positive socio-economic impact.

KEYWORDS
green tea, health benefits, natural resources, non-wood product, novel infusion, polyphenols

INTRODUCTION

Temperate forests of South America constitute are valuable resources of biodiversity with potential as natural reservoirs of bioactive phytochemicals for human wellbeing and health. *Nothofagus antarctica*, known as ñire, is a relevant resource with one of the greatest distribution areas of all native trees in the Patagonian region, covering 751,640 ha from 36° S to 55° S in Argentina. This species has a similar distributional range to the west of the Andes mountain range in Chile. ñire is a pioneer species that shows the greatest ecological range of the South American *Nothofagus* genus.[1] It grows from valley bottoms, steep slopes with shallow soils, floodplain environments, and post-fire scrub towards the most xeric limit of the Patagonian forests.[2] In addition, this species develops different morphological variants probably related to the environmental conditions where the population is established[1] that could be the result of locally adapted ecotypes,[3] an expression of plasticity or a combination of both factors.[4] Furthermore, *N. antarctica* forests are central for developing of livestock

production in the region[5] and approximately 70% of these forests have been used under silvopastoral systems.[6] In the last years, "Forest Management with Integrated Livestock (Manejo de Bosque con Ganadería Integrada, MBGI)" has been implemented based on the provision of ecosystem services by *N. antarctica* forests, and on an adaptive management scheme to define the interventions.[5] The strategy of producing added value of wood, non-wood, and animal products are key factors for silvopastoral system development.

In the last decade, the phytomedicinal properties of *N. antarctica* gained interest among scientists and farmers, increasing the ecological and economical potential of this tree species in the region. Barbosa et al.[7] reported the traditional use of ñire as febrifuge by the Patagonian native inhabitants as well as its cytotoxic activity. In addition, the antioxidant capacity of infusions elaborated from *N. antarctica* leaves and the essential oil profile of its hydrodestilated has been reported for the first time by Gastaldi et al.[8] In these studies, a sesquiterpene alfa-agarofurane was identified as the main component of the species-essential oil, yielding from 61% up to 84% of the total volatile compounds. Differences in essential oil content and the identity of minor volatile compounds have been reported among populations with contrasting growing conditions.[9–10] Regarding its biological activity, a preliminary report suggested antiproliferative effects of *N. antarctica* aqueous extract over the HT-29 and Caco-2 cells derived from colon cancer.[11] Currently, *N. antarctica* leaves have been recently registered in the Argentinean Food Codex and used as a part of culinary recipes, including infusions, and spirit beverages.[12–13] Recent studies supported that diets rich in natural antioxidants play an important role in preventing human diseases.[14–15] In this regard, alternatives to increase antioxidant content in the daily diet include the use of polyphenols-rich plant substrates as complement on primary food matrices.[16–19] Nevertheless, it is important to take into account that the resulting antioxidant activity is influenced by several factors, such as substrate ratio, proportion of the phenolic compounds added, and the interaction among them.[20–21] For this reason, the optimal substrate proportion in the mixture should be determined in order to maximize food or beverage beneficial health properties.

The aims of the present work were to determine the polyphenol profile of *N. antarctica* infusions and to obtain an optimized green tea-*N. antarctica* blend that could represent an interesting food product. For this, the total phenolic content (TPC) and antioxidant capacity of the individual infusions and sequential mixture ratios between *N. antarctica* and green tea were assessed. In addition, comparisons with others popularly known beverages, such as infusions elaborated with organic green tea (OGT) and yerba-mate (*Ilex paraguariensis*), were done.

MATERIALS AND METHODS

Plant material

Leaves of 15 trees of *N. antarctica* were collected randomly in a population located at 41°8′ S and 71°29′ W during 2020. In each tree, leaves from different stratum (from 1 to 3 m in height) were harvested. In addition, two foliar development stages were included in the analysis: (1) fully expanded leaves with an intense green color (NA_1); and (2) leaves near senescence with orange-red color but still conserved turgor (NA_2). The harvested leaves were then freeze-dried by Christ Alpha 1–2 LD equipment (Christ, Osterode and Harz, Germany), grounded to a fine powder, and stored at −20°C until preparation of the aqueous extract. The commercial OGT "Naturesana Artemis Bio" (Herbes del Moli S.L., Spain) and the popular known Argentinean yerba-mate "Playadito" (Cooperativa Agrícola de la Colonia Liebig Ltda., Argentina) were included in the analysis.

Preparation of infusions

The infusions were elaborated following the methodology described by Domínguez-Perles et al.[22] One hundred milliliters of simmering water (90 ± 2°C) were added to 2 g of total dried material, left for 5 min with occasional stirring, and then filtered. Individual infusions of *N. antarctica* (*NA*) considering the two foliar development stages (NA_1 and NA_2), OGT, and yerba-mate (Ym) were prepared. In addition, OGT-*NA* blends with different mixture proportions were included in the analysis 67% OGT − 33% *NA* (2:1), 50% OGT − 50% *NA* (1:1), and 33% OGT − 67% *NA* (1:2). Three replicates of each infusion were included in the analyses.

Identification of phenolic compounds

The identification of phenolic compounds in the individual infusions of *N. antarctica*, OGT, and Ym was carried out by HPLC–DAD–ESI/MSn analyses. An Agilent HPLC 1100 series model equipped with a photodiode array detector and a mass detector in series (Agilent Technologies, Waldbronn, Germany) was used. The equipment consisted of a binary pump (model G1312A), an autosampler (model G1313A), a degasser (model G1322A), and a photodiode array detector (model G1315B). The HPLC system was controlled by Chem-Station software (Agilent, version 08.03). The mass detector was an ion trap spectrometer (model G2445A) equipped with an electrospray ionization interface. The ionization conditions were set as described by Migues et al.[23] The mass spectrometry data were acquired in the negative ionization mode, except for anthocyanin derivatives. The MSn was carried out in the automatic mode on the more abundant fragment ion in MS ($n - 1$).

Quantification of phenolic compounds

Twenty microliters of the filtered samples were injected in a reversed-phase HPLC-DAD Agilent 1100 system. Compounds were separated in a Luna C18 column (25 cm × 0.46 cm, 5 μm particle size; Phenomenex, Macclesfield; UK). The mobile phase was a mixture of water/formic acid (99:1 vol/vol) (A) and acetonitrile (B). The flow rate was 0.8 ml/min in a linear gradient. Individual compounds were tentatively identified following their characteristic UV–Visible spectra and peak

elution order (retention time—Rt), and compared with the peaks previously identified in the HPLC-DAD–ESI/MSn method. The following analytical standards were included in the analysis: catechin, chlorogenic acid, gallic acid, quercetin-rutinoside, and cyanidin 3-hexoside. A calibration curve for each standard was built considering a concentration range between 2–0.031 mM for each one. These calibration curves and the chromatograms obtained by HPLC-DAD were used for the polyphenol quantification. Flavanols were quantified as catechins at 280 nm, ellagic, caffeic, gallic acid derivatives as gallic acid at 280 nm, caffeoyl- quinic acid derivatives as chlorogenic acid at 320 nm, and flavonoids as quercetin-rutinoside at 360 nm. The sum of caffeoyl and quinic acid derivatives were mentioned as total of chlorogenic acid; whereas the sum of esters and glycosides of flavonoids were mentioned as total flavonoids. Three replicates of each infusion were quantified and the results were expressed as mg per 100 ml infusion and mg per g dried weight (DW).

Total phenolic content

TPC was determined using a rapid colorimetric method described by Medina-Ramón et al.[24] and based on the oxidation of the hydroxyl groups of phenols in basic media by the Folin–Ciocalteu reagent. This method is adapted to microscale in 96-well microplate (Nunc, Roskilde, Denmark). Briefly, 15 µl of each diluted infusion (1:5 vol/vol in water), a blank or a dilution of gallic acid (used as standard) were placed in an individual microplate-well, then 12 µl of Folin–Ciocalteu reagent and 30 µl of 20% sodium carbonate solution were subsequently added and the mixture was allowed to react in darkness for 1 min at room temperature. The absorbance was registered at 765 nm by an Infinitive® M200 microplate reader (Tecan, Grödig, Austria). All measurements were carried out by triplicate. The results are expressed as mg of gallic acid equivalents (GAE) per 100 ml of infusion, using a calibration curve over the range of 1–100 mg of gallic acid in 100 ml. Although this method is not specific to polyphenols because other reducing compounds could also be included in the quantification, it is highly useful for comparison among beverages.

In vitro analysis of antioxidant capacity

To assess the antioxidant capacity of the infusions, including individual tisanes and OGT-*NA* blends, the 2, 2-diphenyl-picyl-hydrazyl assay (DPPH•) and ferric reducing antioxidant power (FRAP) were carried out following the methodologies described by Migues et al.[23] and LLorach et al.,[25] respectively. Briefly, the antioxidant activity was evaluated by measuring the variation in absorbance at 515 nm after 35 min for DPPH• assay and at 593 nm after 40 min for FRAP. The reactions were scaled to a 96-well microplate. All samples were centrifuged at 10,000 × rpm for 10 min, at 4°C, and the reactions were started by adding 2 µl of the diluted sample (1:5 vol/vol in water) to the well containing 250 µl of DPPH• dissolved in methanol (absorbance ≈ 1) or FRAP solution. Absorbance measurements and the reaction kinetic was registered by an Infinitive® M200 microplate reader (Tecan, Grödig, Austria). Assays were performed in triplicate and the results are expressed in millimolar of equivalent Trolox® per 100 ml of infusion (mM TE/100 ml), using a calibration curve over the range of 0.25–5 mM Trolox.

The antioxidant strength was determined by calculating the percentage of inhibition of the DPPH• radical (%*I*) and the amount of antioxidant needed to reduce the concentration of DPPH• to 50% (IC_{50}), as described Acosta-Otálvaro et al.[26] In addition, a combination index (CI) based on the DPPH• radical scavenging activity for binary mixture (OGT-*NA*) was calculated according to Enko and Gliszczynska-Swigło[20] in order to describe the type of interaction between substrates. An additive interaction occurs when the index is equal to 1; whereas an antagonist or synergist effects occur when the interaction index is higher or lower than 1, respectively.[20] In this study, a more precise range for CI values and interaction type was used following the suggestions of Chou[27]: 0.3–0.7 (synergetic), 0.7–0.85 (moderate synergetic), 0.85–0.90 (slight synergetic), 0.90–1.10 (nearly additive), 1.10–1.20 (slight antagonistic), 1.20–1.45 (moderate antagonistic), and 1.45–3.3 (antagonistic).

Data analysis

Statistical analyses for the concentration of polyphenol compounds, TPC, and antioxidant activity were carried out on R environment (R Core Team, 2015). Firstly, normality and homogeneity of variance for each variable were tested by running Shapiro–Wilk and Levene tests, respectively. Since both assumptions were corroborated, an analysis of variances (ANOVA) followed by the multiple comparison LSD test (p-value <0.05) was carried out. In addition, correlation tests were performed in order to determine the relationships between compound-type and total polyphenol concentration as well as between the polyphenol content and OGT proportion in the mixture and the antioxidant capacity (by both DPPH• assay and FRAP). When significant correlation was detected ($p < 0.01$), correlation coefficients were indicated.

RESULTS

A total of 23 compounds were detected at 320 nm by HPLC–DAD in infusions elaborated with both fully-expanded and near senescence leaves of *N. antarctica* (NA_1 and NA_2). A characteristic chromatogram for these samples is shown in Figure 1. The identity of the detected peaks was determined by HPLC–DAD–ESI/MSn considering the characteristic UV–Vis spectra, order of elution (Rt), parent ions (MS−), along with the MS/MS daughter spectra. Fifteen different phenolic compounds were identified in these infusions, including seven derivatives of phenolic acids and eight flavonoid derivatives (Table 1). It was detected a high diversity in the phenolic acid constituents, mainly represented by gallic, ellagic, quinic, caffeic, and coumaric acids. In the case of flavonoids, the variability was mainly restricted to myricetin and quercetin compounds. In addition, an extra compound corresponding to an anthocyanin was found in those infusions elaborated

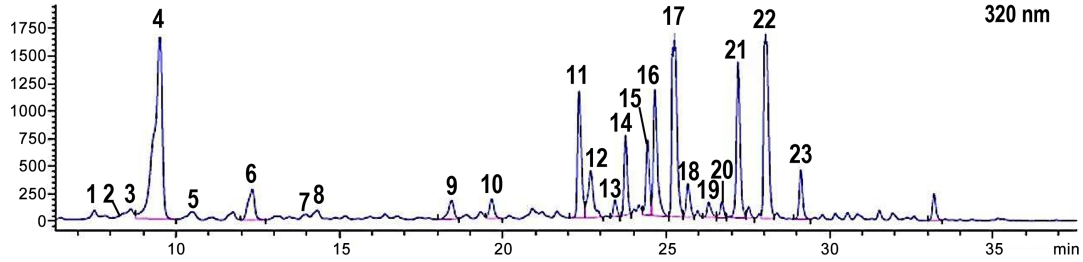

FIGURE 1 Chromatographic profile of *N. antarctica* infusions by HPLC–DAD at 320 nm. Numbers correspond to each identified polyphenol compound and the identity of each one are detailed in Table 1.

TABLE 1 Polyphenol profile and concentration (mg/100 ml of infusion) of the aqueous extract from *N. antarctica* leaves

ID	Rt	Tentative compound names	MS−	MSn	λ nm	NA_1		NA_2	
1, 2, 3, 5	6.3, 7.3, 8.2, 10.2	Di-HHDP-glucose	783	481, 301, 275	280	9.54 ± 2.60	a	6.04 ± 0.20	A
4	9.3	3-CQA	353	191, 179	320	7.35 ± 2.41	a	0.48 ± 0.01	B
6	11.9	3-p-Coumaroyl-QA	337	163	280	2.79 ± 0.47	b	6.42 ± 1.21	A
7	13.3	Caffeoyl-hexoside	341	179, 135	280	0.92 ± 0.32	a	n.d.	A
8	14.3	5-CQA	353	191	320	0.59 ± 0.34	a	0.59 ± 0.09	A
9	18.3	Digalloyl-HHDP-glucose	785	633, 483, 301	280	3.37 ± 0.49	b	6.29 ± 0.12	A
10	19.7	Tetragalloyl-glucose	787	635, 313, 169	280	3.66 ± 1.16	a	3.51 ± 1.30	A
11, 12	22.3, 22.5	Myricetin-hexoside	479	317, 271, 151	360	5.47 ± 0.40	a	4.55 ± 0.09	A
13, 15	23.5, 24.6	Myricetin-pentoside	449	317, 271, 151	360	7.11 ± 0.88	b	11.17 ± 0.98	A
14	23.8	Quercetin-Galloyl-hexoside	615	463, 301, 151	360	2.61 ± 0.20	a	3.35 ± 0.35	A
16	24.9	Myricetin-rhamnoside	463	317, 271, 179	360	Traces		Traces	
17, 18	25.5, 25.8	Quercetin-hexoside	927/463	463, 301, 151	360	19.24 ± 0.45	a	15.52 ± 2.24	A
19, 20, 21	26.4, 26.8, 27.3	Quercetin-pentoside	433	301, 151	360	9.44 ± 0.30	a	8.70 ± 0.14	A
22	28.2	Quercetin-rhamnoside	447	301, 179	360	4.43 ± 0.83	a	3.63 ± 0.26	A
23	29.2	Quercetin-Galloyl-pentoside	585	417, 301	360	2.49 ± 0.23	a	3.34 ± 0.73	A
		Total				79.00 ± 3.99	a	73.60 ± 5.92	A

Note: ID: identification number; Rt: retention time in minutes; MS−: parent ions in the ESI− mode; MSn: daughter spectra; λ nm: maximum absorbance in nanometers. HHDP: hexahydroxydiphenic acid; CQA: caffeoyl quinic acid. NA_1: infusions elaborated with *N. antarctica* green leaves, NA_2: infusions elaborated with *N. antarctica* colorful leaves. Lowercase letters correspond to a posteriori test of ANOVA. In the same row, different letters mean that exist signifcant differences.

with *N. antarctica* leaves close to senescence that ranged from bright orange to dark red colors.

In summary, seven compounds exhibited a maximum absorbance close to 280 nm corresponding to ellagic, caffeic, and gallic acid derivatives (peaks **1**, **2**, **3**, **5**, **7**, **9**, and **10**), other three close to 320 nm corresponding to quinic acid derivatives (peaks **4**, **6**, and **8**) and 13 compounds close to 360 nm involving quercetin and myricetin derivatives (peaks from **11** to **23**). The identified phenolic compounds were grouped based on their constituent nature as follows:

Ellagic acid derivatives

Ellagic acid derivatives are esters of hexahydroxydiphenic acid (HHDP) and a polyol, usually glucose, or gallic acid.[28] In the *NA* infusions, four compounds (peaks **1**, **2**, **3**, **5**) exhibited a mass spectra of [M]− ions at *m/z* 783 in the ESI− mode, and generated fragment ions at *m/z* 481 corresponding to an HHDP-glucose, 301 corresponding to the HHDP residue, and 275 by decarboxylation of the HHDP moiety. Therefore, these compounds were identified as *bis-HHDP-hexoside*[29] (Table 1). In addition, an extra compound (peak **9**) was found and identified as an ellagitanin derivative since it exhibited [M]− ions at *m/z* 785 and characteristic fragment ion spectra at *m/z* 633 by loosing of galloyl (*m/z* 152) moiety, 483 corresponding to digalloyl-glucose residue, and 301 corresponding to the HHDP residue. This compound was named as *digalloyl-HHDP-glucose*.

Gallic acid derivatives

In *NA* infusions, three galloylated-type compounds were identified including one ester of gallic acid with glucose polyols and two esters

of gallic acid with flavonoids (peaks **10**, **14**, **23**) (Table 1). The **peak 10** was named tetragalloyl-glucose because exhibited a mass spectra of [M]− ions at *m/z* 787 in the ESI− mode. Its fragment ion spectra at *m/z* 635 corresponding to the trigalloyl-glucose residue,[28–30] 313 corresponding to the loosing of H_2O molecule to the monogalloyl-glucose residue (*m/z* 331)[31] and 169 corresponding to the deprotonated ion of gallic acid moieties. **Peaks 14** and **23** corresponded to esters of gallic acid with glycosides of flavonoids, which showed a mass spectra of [M]− ions at *m/z* 615 and 585, respectively. In the first case, the compound was identified as *quercetin-galloyl-hexoside* based on its MS/MS daughter ions at *m/z* 463 and 301 corresponding to a loss of a galloyl moiety and hexose moiety, respectively, as well as the typical daughter ions at *m/z* 301 and 151 that indicated the existence of a quercetin moiety.[32–33] In the second case, it was detected fragmentation ions spectra at *m/z* 417 and 301, corresponding to the loss of gallic acid and the presence of quercetin-pentoside residue, and thus it was named as *quercetin-galloyl-pentoside*.[34]

Caffeoyl and quinic acid derivatives

Four caffeoyl and quinic acid derivatives were found in the novel beverage base on *N. antarctica* leaves, including two esters of caffeic and quinic acids (peak **4**, **8**), also known as chlorogenic acids, one ester of 3-p-coumaroyl and quinic acid (peak **6**), and one ester of caffeic acid and hexose polyol (peak **7**) (Table 1). The identified caffeoyl quinic acid (*CQA*) compounds showed [M]− ions at *m/z* 353 in the ESI− mode and MS/MS daughter ions at *m/z* 191 (base peak) and 179, corresponding to deprotonated *CQA* and caffeic acid fragments, respectively. Based on the relative intensity of the secondary ion at *m/z* 179, the peak **4** was identified as *3-CQA* (*m/z* 179 > 5%); whereas the peak **8** was named as *5-CQA* since the intensity of the secondary ion was less than 5%.[35] Respected to the *3-p-coumaroyl quinic acid* (*3-p-CoQA*) ester (peak **6**), it exhibited [M]− ions at *m/z* 337 in the ESI− mode and MS/MS daughter ions at *m/z* 163 related to 3-p-coumaric acid residue. Finally, an extra caffeoyl derivative was identified and named as *caffeoyl-hexose* (peak **7**) since it exhibited a mass spectra of [M]− ions at 341 and a characteristic fragment ion at *m/z* 179 and 135 related to caffeic acid residue and the loss of CO_2 from the phenolic acid moiety.

Flavonoids

In the novel *N. antarctica* beverage, 11 glycosides of flavonoids were identified (peak **11**, **12**, **13**, **15**, **16**, **17**, **18**, **19**, **20**, **21**, **22**) without including the two esters with gallic acid reported as gallic acid derivatives. From the total, half compounds corresponded to myricetin-glycosides and the remaining to quercetin-glycosides. With respect to the first group, peak **11** and **12** were identified as *myricetin-hexose*, peak **13** and **15** as isomers of *myricetin-pentose*, and peak **16** as *myricetin-rhamnoside* since they showed [M]− ions at *m/z* 479, 449 and 469 in the ESI− mode, respectively. In all cases, they yielded fragmentation ion spectra at *m/z* 317, 271, and 151 or 179 corresponding to deprotonated myricetin aglycone residue, the loss of CH_2O unit, and the typical retro Dies-Alder (RDA) reactions of flavon-3-ols, respectively.[33–36] In the second group, peak **15**, **16**, **17**, **18**, **19**, **20**, and **21** exhibited [M]− ions at *m/z* 927/463, 443 and 447 in the ESI− mode, and they were identified as *quercetin-hexoside*, *quercetin-pentoside*, and *quercetin-rhamnoside*, respectively. All of them showed MS/MS daughter ions at *m/z* 301 and 151 or 179 related to the quercetin moiety.

Anthocyanin derivatives

In the infusions elaborated with the *N. antarctica* leaves from the final growing season (bright orange to dark red with conserving turgor), it was found and extra phenolic-type compound, specifically an anthocyanin. This compound was identified as *Cyanidin-3-hexoside* (*Cy3-glc*) since it exhibited a maximum absorbance at 520 nm, a typical [M +- H] + ions at *m/z* 449 in the ESI+ mode, and MS/MS daughter spectra at *m/z* 287 of the cyaniding aglycone. Furthermore, these infusions had a characteristic red color that allows distinguishing them from those elaborated with green leaves, and this particular color is attributed to the presence of the Cy3-glc.

The qualitative analysis showed an almost complete complementary between OGT and *NA* aqueous extracts since the majority of compounds are specific to each beverage (see Supplementary material S1). Compared with yerba mate infusions, it was observed greater similarity based on the presence of derivatives of caffeoyl-quinic acid and quercetins (see Supplementary material S1). In the quantitative analysis, significant differences were found in some of the identified compounds between *NA* infusions (Table 1). In particular, chlorogenic acid (*3CQA*) was significantly higher in the infusion elaborated with *NA* green leaves than those using colorful leaves (close to senescence); whereas *coumaroyl quinic acid* (*3pCoQA*) and *myricetin-pentoside* (*Myr-pent*) compounds exhibited an opposite performance. These differences were not found when total polyphenol content was considered (Table 1). Ym and OGT infusions showed superior polyphenol contents than *NA* infusions (Table 2). However, the phenolic profile of *NA* infusions exhibited higher constituent diversity and flavonoid-type compounds than other infusions. A cup of *NA* infusion (100 ml) elaborated with green leaves is providing 10.72 (±2.33) mg of quinic acid derivatives, 17.49 (±2.34) mg of gallic, caffeic, and ellagic acid derivatives, and 50.79 (±0.92) mg of flavonoids. These yields were similar to those obtained by *NA* infusions elaborated with colorful leaves (Table 2). Flavonols were the main constituents in *NA* infusions (64.29 ± 1.16% of total); whereas catechins derivatives stood out as the most abundant in OGT (62.27 ± 1.05% of total). Furthermore, *NA* infusions exhibited up to 13.57 (±2.95) % of chlorogenic acids, which were not present in OGT (Supplementary material S1). OGT-*NA* blends reached a similar polyphenol content to those obtained in OGT, independently of the proportion mixture (Table 2). Nevertheless, significant differences in the concentration of each polyphenol-type were found among the mixtures. As it increased the OGT

TABLE 2 Comparison of polyphenol concentrations among *N. antarctica* infusions and popularly known beverages (organic green tea and yerba-mate) as well as among OGT-*Na* blends

	Na$_1$	*Na*$_2$	OGT	Ym	OGT-*Na*$_1$ (2:1)	OGT-*Na*$_2$ (2:1)	OGT-*Na*$_1$ (1:1)	OGT-*Na*$_2$ (1:1)	OGT-*Na*$_1$ (1:2)	OGT-*Na*$_2$ (1:2)	*Cor*
Chlorogenic	10.72 ± 2.33 B	7.49 ± 1.11 B	n.d. C	199.72 ± 5.25 A	17.53 ± 0.95 c	14.84 ± 0.11 c	25.05 ± 0.67 bc	19.75 ± 0.57 ab	27.80 ± 1.64 a	25.95 ± 0.15 ab	0.72 *
Flavonoids	50.79 ± 0.92 A	50.26 ± 3.83 A	21.88 ± 1.99 B	17.69 ± 1.09 C	48.81 ± 1.91 b	43.96 ± 1.78 b	61.61 ± 2.16 a	53.48 ± 2.04 ab	64.53 ± 2.41 a	62.73 ± 2.37 a	n.s.
Catechins	n.d. B	n.d B	78.81 ± 1.33 A	n.d B	40.93 ± 0.79 a	42.29 ± 0.73 a	32.06 ± 0.5 b	25.76 ± 1.84 c	19.34 ± 1.36 d	14.70 ± 0.25 d	0.86 *
Others	17.49 ± 2.34 B	15.84 ± 0.98 B	25.88 ± 0.20 A	0.87 ± 0.03 C	17.50 ± 0.57 a	18.65 ± 0.81 a	14.70 ± 0.71 b	15.50 ± 0.21 ab	13.72 ± 1.27 b	12.35 ± 1.13 b	n.s.
Total	79.00 ± 3.99 C	73.60 ± 5.92 C	126.57 ± 0.46 B	218.28 ± 6.36 A	124.77 ± 2.62 B	119.74 ± 3.43 B	133.43 ± 2.86 B	114.49 ± 0.98 B	125.39 ± 3.97 B	115.73 ± 1.34 B	

Note: Chlorogenic: sum of caffeoyl and quinic acid derivatives; Flavonoids: sum of esters and glycosides of flavonoids; Catechins: total of catechin derivatives; Others: integration of total of ellagic, gallic and caffeic derivatives. *NA*$_1$: infusions elaborated with *N. antarctica* green leaves, *NA*$_2$: infusions elaborated with *N. antarctica* colorful leaves; OGT: organic green tea; Ym: infusion elaborated with yerba-mate (Ilex paraguariensis). Different letters correspond to significantly differences in the LSD test (p-value <0.01); capital letters correspond to differences among individual infusions and lowercase letters correspond to differences among the OGT-Na mixtures. Cor: correlation coefficient. n.s.: not significant (p > 0.05).
*Highly significant (p-value <0.01).

proportion in the mixture, the amount of catechins derivatives was significantly higher; meanwhile, the amount of chlorogenic acid and flavonoids in the mixture was significantly higher when *NA* proportion increased. The total polyphenol concentration positively correlated to the catechin- and chlorogenic-derivative contents. In addition, it was observed that the chlorogenic acid and flavonoids concentrations from *NA* part (mg/g *NA* DW) were highly superior to those yielded in the individual *NA* infusions (Figure 2). Moreover, these concentrations highly exceeded the theoretical amount of each compound when it is considered the *NA* proportion in the mixture.

Individual *NA* infusions achieved an average TPC (by Folin–Ciocalteu methodology) of 111.31 ± 1.68 mg of GAE/100 ml, considering both foliar developmental stages. These values were not significantly different from the obtained in OGT 109.56 (±0.44) mg GAE/100 ml. Non-significant differences in TPC were found between the OGT-*NA* blends and the individual infusions (i.e., OGT and *NA* separately): 112.68 (±0.56) mg/100 ml in the 2:1 OGT-*NA*, 110.74 (±1.04) mg/100 ml in the 1:1 OGT-*NA*, and 108.49 ± 0.47 mg/100 ml 1:2 OGT-*NA*.

With regard to the antioxidant capacity, significant differences were found among individual infusions (Figure 3a) where OGT exhibited the highest DPPH• radical scavenging capacity and FRAP value. Besides, the *NA* infusions elaborated with colorful leaves reached similar performance (by DPPH•) than those observed in OGT (Figure 3b). In the same way, 2:1 OGT-*NA*$_1$ achieved a radical scavenging capacity close to those of OGT and significantly greater than Ym. Correlation analyses showed that only catechin derivatives significantly correlated to the assessed antioxidant capacity (*cor* = 0.96 for DPPH and *cor* = 0.92 for FRAP) among all polyphenol compounds. In addition, OGT proportion in the mixtures also positively correlated to DPPH (*cor* = 0.70) and FRAP (*cor* = 0.94) values. In addition, significant differences were found for DPPH• inhibition percentage (%*I*) and IC$_{50}$ among infusions (Table 3). *NA* infusions elaborated with green leaves reached higher radical inhibition than OGT as well as the lowest amount of IC$_{50}$. However, infusions with *NA* colorful leaves obtained a %*I* similar to OGT and Ym and an IC$_{50}$ lower than OGT. The antioxidant strength in the mixtures increased as long as increased the *NA* proportion. In particular, %*I* in the 2:1 OGT-*NA* was similar to *NA*$_1$ and *NA*$_2$ infusions and IC$_{50}$ value was lower than OGT. The CI values showed that a synergistic interaction between OGT and *NA* is occurring in almost all mixtures (Table 3).

DISCUSSION

Teas are the most consumed beverages in the world after water, and thus a polyphenol-enriched tea (e.g., with *N. antarctica* leaves) could be an interesting product for food industries under the current consumer demands. The present work characterized a valuable source of bioactive compounds (i.e., polyphenols) from *N. antarctica* leaves that exhibit a high constituent diversity. Moreover, its polyphenol profile has a great complementarity with the compound naturally present in OGT. These findings support the strategy of combining them in an optimized blend in order to integrate the medicinal properties of each

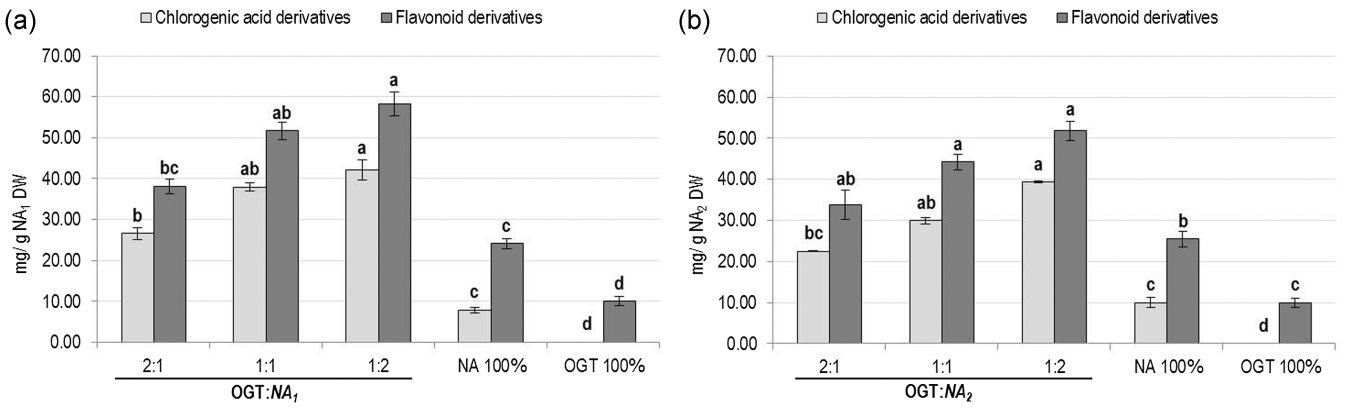

FIGURE 2 Chlorogenic and flavonoid concentrations (mg/g DW) among infusions. (a) Infusions elaborated with *N. antarctica* green leaves; (b) infusions elaborated with *N. antarctica* leaves close to senescence. Only the chlorogenic derivatives and flavonoids concentrations related to *N. antarctica* proportion were plotted out in the OGT-*NA* blends. Letters correspond to LSD test; different letters means significant differences (p-value <0.01).

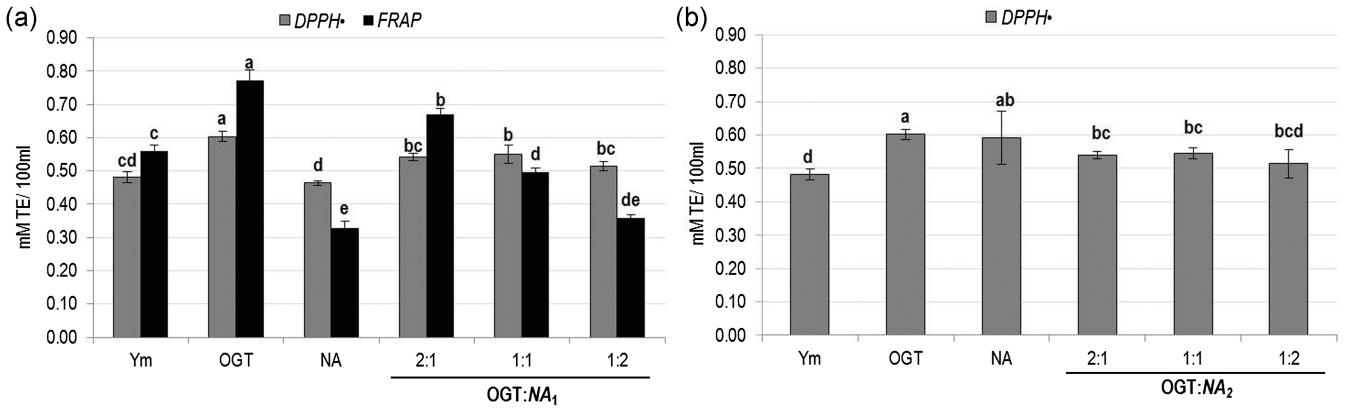

FIGURE 3 Antioxidant capacity in mM TE/100 ml (DPPH• and FRAP assays). (a) Infusions elaborated with *N. antarctica* green leaves; (b) Infusions elaborated with *N. antarctica* leaves close to senescence. Letters correspond to LSD test; different letters means significant differences (p-value <0.01).

kind of polyphenols, and therefore enhance the health benefits to humans. The addition of *N. antarctica* leaves in herbal blends contributes with a valuable source of *Myricetin* and *Quercetin* glycosides, but also caffeic, quinic and gallic acid derivatives and ellagitanins. Furthermore, it is important to consider the phenological stage of *N. antarctica* raw material (i.e., leaves) due to the existence of differences in the relative concentration of some compounds and antioxidant capacity. In particular, chlorogenic acid concentration was 15-fold higher in early stages; whereas *Cyanidin-3-hexoside* was only present in near senescent leaves and it conferred a characteristic red color to the infusions. Besides, *NA* infusion elaborated with green leaves evidenced the highest antioxidant strength (measured by IC_{50}) while the one elaborated with near senescent leaves showed similar DPPH• scavenging capacity than OGT. This was directly related to the molecular structure of phenolic compounds present in the food matrix that implies diverse antioxidant performances.[37] On the other hand, the provenances of *N. antarctica* materials are also important to obtain a high-quality product. In this regard, the TPC (mg GAE/100 ml) of the studied *NA* infusions was significantly higher than those reported by Gastaldi et al.,[11] who analyzed raw materials from populations located 800 km south from the present collection site. This highlights the importance of evaluating the chemotype existences among *N. antarctica* populations linked to the effect of environmental growing conditions (e.g., different latitude, climate, water stress conditions) on the concentration and composition of polyphenols.[38]

OG-*N. antarctica* mixtures analyzed in this study (67%–33%, 50%–50%, 33%–67%) resulted to be interesting binary combinations because they achieved similar total polyphenol concentrations (mg/100 ml) to OGT, but with higher compound diversity. Furthermore, all these mixtures showed enhancement in extraction yields of some *N. antarctica*-polyphenols (e.g., cholorogenic acid derivatives and flavonoids), which highly exceeded the expected amount (mg/g DW) in a proportion-dependent manner. A similar trend was observed in chlorogenic and sinapic acid derivatives measured in mixtures elaborated with OGT and broccoli by-products.[22] This suggests a relevant role of green tea in the relative extraction of some phenolic compounds. In agreement with this, the type of interaction between mixture constituents should be taken into account when determining the

TABLE 3 Antioxidant propierties by DPPH• assay

	Mixture proportion	DPPH•	%I	IC_{50}	CI	Type of interaction
OGT	100%	0.61 ± 0.02 a	38.97 ± 0.14 c	9.40 ± 0.68 a		
Ym	100%	0.49 ± 0.02 cd	45.53 ± 2.26 ab	5.42 ± 0.44 bcd		
Na_1	100%	0.45 ± 0.01 d	48.10 ± 1.39 a	4.98 ± 0.15 d		
Na_2	100%	0.59 ± 0.08 ab	41.21 ± 4.18 abc	6.79 ± 1.72 b		
OGT-Na_1	2:1	0.54 ± 0.02 bc	44.16 ± 0.68 ab	5.96 ± 0.16 bc	0.83 ± 0.02	Synergism
OGT-Na_1	1:1	0.55 ± 0.02 abc	40.38 ± 0.79 bc	6.79 ± 0.49 b	0.95 ± 0.08	Almost additive
OGT-Na_1	1:2	0.51 ± 0.02 cd	47.63 ± 1.66 ab	5.25 ± 0.30 cd	0.73 ± 0.05	Synergism
OGT-Na_2	2:1	0.53 ± 0.01 bcd	44.16 ± 0.68 ab	5.96 ± 0.07 bc	0.76 ± 0.04	Synergism
OGT-Na_2	1:1	0.54 ± 0.03 abc	40.38 ± 0.79 bc	6.65 ± 0.42 b	0.81 ± 0.12	Synergism
OGT-Na_2	1:2	0.51 ± 0.04 cd	47.63 ± 1.66 ab	5.75 ± 1.08 bc	0.73 ± 0.16	Synergism

Note: DPPH•: mM TE/100 ml infusion; *%I*: percentage of inhibition of the DPPH• radical. IC50: half maximal inhibitory concentration (mM TE); CI: combination index; NA_1: infusions elaborated with *N. antarctica* green leaves, NA_2: infusions elaborated with *N. antarctica* colorful leaves; OGT: organic green tea; Wt: white tea; Ym: infusion elaborated with yerba-mate (*Ilex paraguariensis*). Letters correspond to LSD test (p-value <0.05). In the same column, different letters mean that exist significant differences.

optimized combination in the OGT-*NA* blends. The antioxidant capacity of binary mixtures could not be predicted from their individual performance of each constituent[18–39] since it depends on the interaction among the phenolic compounds[19–21] and the substrate ratio in the mixture.[20] In OGT-*NA* blends analyzed here, the 2:1 ratio exhibits a good performance related to a radical scavenging capacity similar to OGT and greater than yerba-mate. It is important to note that catechins derivatives were the only polyphenol compounds that significantly correlated to the measured antioxidant capacity. Besides, antioxidant determinations carried out in this study were based on the same kind of redox reaction, specifically the electron transference.[40] For these reasons, despite DPPH and FRAP assays are the most widely used methods to evaluate the antioxidant interaction among phenolic compounds,[20,21] other methodologies should be considered in order to detect diverse antioxidant mechanisms in future studies. Lastly, 2:1 OGT-*NA* ratio stood out by its good antioxidant strength (high *%I* and low IC_{50}) and the synergistic interaction that is having place between constituents. Considering all, the 67% OGT – 33% *N. antarctica* mixture could be recommended as an optimized blend exhibiting a good balanced among relative and total polyphenol contents and antioxidant properties. Therefore, this blend can be rendered as a new beverage enriched in bioactive phytochemicals for further investigations on functionality including bioavailability and bioactivity as health promoter. The use of *N. antarctica* as a food product implies a novel productive strategy of adding value to the Patagonian silvopastoral systems and with positive socio-economic impacts.

The presented study reports a valuable source of polyphenol compounds from a native species (*N. antarctica*) of Patagonian forests, suggesting a new non-wood product with high economic value. It is also supported the strategy of combining this species and green tea in an optimized blend that integrates the health benefits of each one. In addition, the positive effect of *N. antarctica* on the beverage antioxidant strength was suggested. Effects of phenological stage and provenance of raw material on the polyphenol concentration and antioxidant capacity were also discussed.

ACKNOWLEDGMENTS

The authors thank Veronica Arana, Carolina Soliani, and Fabian Jaque for their assistance in material collection. In addition, the authors would like to acknowledge Encarnación Gómez Plaza for reviewing the manuscript and providing very helpful comments and suggestions.

FUNDING INFORMATION

This work was financially supported by Agencia Nacional de Promoción Científica y Tecnológica [grant numbers PICT-2018-2668] and Instituto Nacional de Tecnología Agropecuaria [project numbers PE-2019-I140 and PE-2019-I114], Argentina. The first author had a postdoctoral fellowship from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and received a research stay fellow from Fundación Carolina.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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How to cite this article: Mattera MG, Langenheim ME, Reiner G, Peri PL, Moreno DA. Patagonian ñire (*Nothofagus antarctica*) combined with green tea - Novel beverage enriched in bioactive phytochemicals as health promoters. JSFA Reports. 2022;2(9):442–51. https://doi.org/10.1002/jsf2.78