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Modulation of Fatty Acids and Interleukin-6 in Glioma Cells by South American Tea Extracts and their Phenolic Compounds

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ABSTRACT

Dietary phenolic compounds are plant metabolites with beneficial effects on the central nervous system. Thus, our aim was to identify anti-inflammatory compounds from South American plants on glia, which regulates neuro-immune response. The compounds were extracted from *Lantana grisebachii* (LG), *Aspidosperma quebracho-blanco* (AQB), and *Ilex paraguariensis* (IP) teas and identified by HPLC-DAD-MS. Extracts (0–200 μg/ml) were tested on human T98-G and rat C6 glioma lines. Cellular viability (by the resazurin assay), fatty acid profile (by gas chromatography) and pro-inflammatory interleukin-6 release (IL-6 by ELISA) were determined. Data were analyzed by partial least-square regression to discriminate bioactive compounds. Twenty-one compounds were determined in LG, mainly iridoids, which were linked to ω-3 and ω-6 polyunsaturated fatty acids, but not to IL-6 release. Thirty-one compounds were found in AQB, mostly hydroxybenzoic derivatives, which were positively related to IL-6 release. Twenty-three compounds were identified in IP, including caffeoylquinic derivatives and mainly chlorogenic acid. They increased the ω-7 palmitoleic fatty acid, which was related to IL-6 decrease. These results enhances phytochemical knowledge of widely available plants, and suggest the lipid-related anti-inflammatory activity of IP phenolic compounds, which give nutritional relevance to the tea.

Introduction

Plants are dietary sources of phenolic compounds (or polyphenols), which constitute the largest group of non-energetic secondary metabolites with redox-modulating activities in mammalian cells (1,2). They are biosynthesized by the shikimic acid and polyacetate pathways, depending on the taxonomic species and on the environmental conditions. The first pathway produces aromatic amino acids, phenolic acids and their derivatives (e.g., hydroxybenzoic and hydroxycinnamic acids, lignans, etc.), while the second one produces quinones and xanthones, among others (1). Aqueous plant extracts contain these compounds, which confer anticancer activity (2).

Three South American plants were selected, *Lantana grisebachii* Stuck. (Verbenaceae), *Aspidosperma quebracho-blanco* Schltdl. (Apocynaceae), and *Ilex paraguariensis* A. St.-Hil. (Aquifoliaceae), because their teas modulate redox homeostasis of the immune and central nervous systems (3,4). They might thus contain molecules with neuro- immune potential to be assayed. Also, these species are accessible for human use given their broad geographical distribution (5), with *I. paraguariensis* being widely consumed as tea (6). Total phenols and flavonoids have been determined for these plants (3,4), but their detailed phenolic composition was unknown.

The principal cells involved in immune and inflammatory responses in the central nervous system (CNS) are glial cells (7), with gliomas representing the most frequent primary tumors of the CNS (8). They are thus possible targets of polyphenols, which, taken orally, could cross the blood-brain barrier and reach different brain regions (9).

It is also known that phenolic compounds can exert anti-inflammatory activity (10,11), and thus might mitigate the neuroinflammation involved in degenerative, metabolic and neoplastic diseases (11), promoted by pro-inflammatory mediators, such as interleukin-6 (IL-6) (7). On the other hand, cellular components determine the susceptibility to these responses, with fatty acids playing a recognized role as inflammatory intermediates (11).

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⊙ Supplemental data for this article can be accessed on the publisher's website.

However, precise mechanisms of polyphenol activity are not completely known. Our aim was to identify anti-inflammatory phenolic compounds from South American plants on glioma cells.

Materials and Methods

Plant Extraction

With the appropriate government consent (Cordoba MINCYT, Argentina), samples of *L. grisebachii* (LG, $n \geq$ 3) and *Aspidosperma quebracho-blanco* (AQB, $n \geq$ 3) were collected during the summer in the South American mountain zone of the phytogeographic Chaco region (GPS coordinates: -31.28, -64.44). Specimens were identified and stored in the RIOC Herbarium (UNRC, Argentina). The *I. paraguariensis* (IP, $n \geq$ 3) was from organic production in the Parana region. One gram of pulverized air-dried plant (leaves and stems) was extracted by adding 10 ml of $95°C$ water. These infusions (teas) were cooled at room temperature for 1 h, reaching a final temperature of $35–40°C$ (in darkness, with constant agitation). Finally, the extracts were recovered from infusions by filtration (using sterile 0.2-μm-pore polyethersulfone membranes for 4.5 bars) and then lyophilized, obtaining 0.259 g of LG extract, 0.194 g of AQB extract, and 0.497 g of IP extract, per gram of each plant.

Phytochemistry by HPLC-DAD-MS

The phenolic composition of the plant extracts was analyzed using a Hewlett-Packard 1100 series liquid chromatograph (Agilent Technologies, Waldbronn, Germany). A Spherisorb® S3 ODS-2 C18 reversed-phase, 3 μm, 150 × 4.6 mm column (Waters Corporation, Milford, MA, USA) was used with thermostat at $35°C$. The HPLC conditions were optimized for the analysis of these samples. The solvents employed were: (A) an aqueous solution (0.1%) of formic acid, (B) 100% HPLC-grade acetonitrile, establishing the following gradient: from 0% to 10% B for 3 min, from 10% to 14.5% B for 34 min, from 14.5% to 20% B for 3 min, from 20% to 35% B for 15 min and from 35% to 60% B for 5 min, at a flow rate of 0.5 ml/min. Spectra were recorded from 220 to 600 nm and detection was carried out at 250, 280, 330, and 370 nm as the preferred wavelengths. The mass spectrometer was connected to the HPLC system via the DAD cell outlet. Mass spectrometry was carried out using an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole linear ion trap mass analyzer controlled by Analyst 5.1 software. The mass spectrometry was performed in negative mode per the methodology used by García-Estévez [12] for the analysis of flavanols and phenolic acids: declustering potential -40 V, entrance potential -7 V, ion spray voltage 5000 V, GS1 40 psi, GS2 50 psi, curtain gas 20 psi, with collision gas set as "high." Two mass experiments were performed: full mass analysis (collision energy 10 V) and MS2 analysis (collision energy 25V). Moreover, to determine the most polar compounds in AQB, its extract was also analyzed using the chromatographic method used by García-Estévez et al. for the analysis of ellagitannins [13] and the corresponding mass spectrometry method for full mass analysis [14].

For the phytochemical characterization of each plant, 1 mg/ml of dry extract of LG and IP was used and 1.5 mg/ml of dry extract of AQB, since previous testing indicated the existence of phenolic compounds in low concentrations in the latter. The compounds were identified by their retention times, UV spectra and molecular and fragment ions supplied by the mass spectrometry (MS) analysis. The differences in relative abundance of the fragment ions detected for each compound were used to differentiate between isomers by comparison with the fragmentation patterns reported in literature [15,16]. Each identified compound was quantified based on its molecular structure (see section results: Tables 1–3). The standards used to identify and quantify the identified compounds (as bracketed) were gallic acid (compounds 1, 4, 14 of LG; 1–3, 7, 9, 10, 13–18, 26, 28, 30 of AQB and 2, 3, 12 of IP), protocatechuic acid (compounds 6 and 12 of AQB), chlorogenic acid (compound 3 of LG; 5, 11 of AQB and 1, 4–6, 9, 10, 11, 13, 14, 16, 17, 19–23 of IP), caffeic acid (compounds 9, 10, 12, 15–17 of LG and 7–8 of IP), vanillic acid (compound 4 of AQB), syringic acid (compound 8 of AQB), p-coumaric acid (compound 6 of LG), ellagic acid (compound 31 of AQB), geniposidic acid (compounds 2, 5, 7, 8, 11, 13, 20, 21 of LG), epicatechin (compound 27 of AQB), catechin (compound 24 of AQB), quercetin 3-O-glucoside (compound 19 of AQB); quercetin 3-O-rutinoside (compound 25 of AQB and 15 of IP), isorhamnetin 3-O-glucoside (compounds 18–19 of LG and 22–23 of AQB), isorhamnetin 3-O-rutinoside (compound 29 of AQB and 18 of IP), and kaempferol 3-O-glucoside (compounds 20–21 of AQB).

Cell Culture and Treatment

Human T98-G cells (ATCC® n° CRL-1690™) and rat C6 cells (ATCC® n° CCL-107™) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with fetal bovine serum (10%), penicillin G sodium (100 IU/ml) and gentamycin sulphate (40 mg/ml), at $37°C$ and 5% CO_2. After these cells were seeded in

Table 1. Chromatographic characteristics (t_R), mass spectral data, UV spectrum (λ_{max}), tentative identification and concentration of phenolic compounds (mg/g of dried extract) in *Lantana grisebachii* extract.

Compound	t_R (min)	[M-H]$^-$ (m/z)	Fragment ions (m/z)	UV λ_{max} (nm)	identification	Group	mg/g of dried extract	%
1	5.56	282	150, 133	254, 274(sh)	unknown	—	2.94	1.75
2	5.86	391	167, 123, 149	236	Dihydrotheveside	Iridoid	26.75	15.91
3	6.24	353	191	326, 298(sh)	3-Caffeoylquinic acid	QA derivatives	1.64	0.98
4	8.04	375	169, 151, 213	238	Unknown	—	3.80	2.26
5	9.01	373	167, 211, 123, 149, 193	232, 314	Geniposidic acid	Iridoid	1.77	1.05
6	10.11	487	179, 135, 161	332, 300(sh)	Coumaroyl–hesperidoside	HA derivatives	1.19	0.71
7	11.09	389	345, 121, 165, 209, 139	236	Theveside	Iridoid	41.69	24.79
8	17.36	403	371, 127, 191, 121, 139	234	Theviridoside	Iridoid	15.47	9.20
9	19.20	639	621, 161, 179, 459, 529	330, 296(sh)	β OH-Acteoside[*]	HA derivatives	2.93	1.74
10	19.49	639	621, 161, 179, 459, 529	330, 296(sh)	β OH-Acteoside[*]	HA derivatives	3.04	1.81
11	23.18	537	163, 373, 313, 331, 119, 149	232, 296(sh), 312	Coumaroyl-geniposidic acid	Iridoid	3.71	2.20
12	23.51	623	461, 161, 315, 179	246, 294(sh), 332	Verbascoside isomer	HA derivatives	20.56	12.22
13	24.58	567	193, 373, 343, 149, 361	326, 296(sh)	Feruloyl-geniposidic acid	Iridoid	4.59	2.73
14	25.08	637	315, 301	364, 300 (sh)	Isorhamnetin *O*-rhamnosyl-glucuronide	Flavonol	4.84	2.88
15	25.83	623	461, 161, 315	328, 292(sh)	Verbascoside isomer	HA derivatives	2.15	1.28
16	26.19	623	461, 161, 315, 179	328, 292(sh)	Verbascoside isomer	HA derivatives	1.96	1.17
17	26.52	623	461, 161	328, 292(sh)	Verbascoside isomer	HA derivatives	2.89	1.72
18	26.89	491	315	360, 299 (sh)	Isorhamnetin 3-*O*-glucuronide	Flavonol	6.67	3.96
19	27.46	477	315	362, 299 (sh)	Isorhamnetin 3-*O*-glucoside	Flavonol	8.51	5.06
20	28.42	557	373, 149, 133	230, 326	Geniposidic acid derivative	Iridoid	5.55	3.30
21	30.12	559	373, 149, 185	230, 296(sh), 330	Geniposidic acid derivative	Iridoid	5.53	3.29

[*]Hydroxylated verbascoside.
[QA]: Quinic acid derivatives.
[HA]: Hydroxycinnamic acid derivatives.

sterile clear flat bottom polystyrene 96-well plates (50,000 cells/cm^2) and incubated for 24 h to achieve maximum attachment, they were treated for 72 h with LG, AQB or IP (dose range: 0 -control-, 50, 100, 200 μg/ml), with each phenolic compound being quantified and used as statistical predictor. Eight separate experiments were performed in triplicate assaying each one of the three plant extracts.

Cellular Viability

Cellular viability was measured and confirmed by the resazurin-based assay. Briefly, viable cells were stained with resazurin (0.05 mg/ml in culture medium for 6 h) to record absorbance at 600 nm (GloMax-Multi microplate reader, Promega Corp., USA) (17). Cellular viability was a necessary condition to release IL-6 and consequently included in statistical models.

Gas Chromatography of Fatty Acids

Total lipids were extracted and partitioned from cells placed in PBS by the Folch method (18). The phase containing phospholipids was treated with sodium methoxide at 4°C overnight to obtain fatty acid methyl esters, which were extracted and dried with nitrogen (19). The separation, quantification and identification of esters were performed using a capillary column (BPX 20 m longitude, 0.25 mm ID, 0.25 μm film, SUPELCO$^©$, USA) in a Clarus 500$^©$ (Perkin-Elmer$^®$) gas-liquid chromatograph with a flame ionization detector (19). Fatty acids (FA) were grouped into their ω families: saturated FA: myristic (14:0), palmitic (16:0), stearic (18:0), lignoceric (24:0); ω3 FA: α-linoleic (18:3), eicosatrienoic (20:3), eicosapentaenoic (20:5), docosapentaenoic (22:5), docosahexaenoic (22:6). ω6 FA: linoleic (18:2), γ-linoleic (18:3), eicosadienoic (20:2), arachidonic (20:4). ω7 FA: palmitoleic (16:1). ω9 FA: oleic (18:1), eicosanoic (20:1), erucic (22:1), nervonic (24:1). Their percentage amounts were used as continuous variables in statistical models.

IL-6 Determination

IL-6 was measured in media by immuno-ELISA (450 and 570 nm) by a commercial kit (BD Biosciences) (7). This interleukin was a response variable in statistical models.

Statistical Analysis

Extract doses and cellular responses were analyzed by linear regression in both cell lines. Partial least squares regression (PLS) was used to discriminate compound effects and response relations, by modelling compound doses as predictors, cellular viability, fatty acids and IL-6 release as responses, and cell lines C6 and T98 as categorical data (Infostat v.2012 software). This method reports standardized correlations to be graphed by linear charts (20,21).

Table 2. Chromatographic characteristics (t_R), mass spectral data, UV spectrum (λ_{max}), tentative identification and concentration of phenolic compounds (mg/g of dried extract) in *Aspidosperma quebracho blanco* extract.

Compound	t_R (min)	[M-H]$^-$ (m/z)	Fragment ions (m/z)	UV λ_{max} (nm)	Identification	Group	mg/g of dried extract	%
1*	7.31	295	161	260	unknown	—	0.71	0.80
2*	7.43	237	191, 135, 121	250	unknown	—	4.80	5.40
3	7.54	315	153, 109	240, 306	Gentisoyl-hexoside	HBA derivatives	2.52	2.83
4*	8.22	151	109, 133	268	Vanillin	HBA derivatives	1.21	1.35
5	8.62	353			Chlorogenic acid	QA derivatives	0.37	0.42
6	8.91	315	153, 109	260, 294	Protocatechuic acid-hexoside	HBA derivatives	0.66	0.74
7*	9.08	169	125	270	Gallic acid	HBA derivatives	6.70	7.52
8*	9.79	197	153, 135	274	Syringic acid	HBA derivatives	0.36	0.40
9*	11.05	389	345, 121, 165, 209	234, 326	Phthalic acid derivative	HBA derivatives	0.50	0.56
10*	12.99	331	169, 151	271	Galloyl-glucose	HBA derivatives	0.14	0.16
11	13.72	353	191	326, 298(sh)	1-Caffeoylquinic acid	QA derivatives	10.89	12.24
12*	14.62	153	109	259, 294	Protocatechuic acid	HBA derivatives	2.12	2.38
13*	15.4	315	153, 109	263, 317	Gentisoyl-hexoside	HBA derivatives	6.66	7.49
14	15.56	403	357, 125, 151, 191	246	unknown	—	4.34	4.88
15*	16.74	375	213, 169, 107		Trihydroxy-phthalic acid hexoside	HBA derivatives	7.34	8.24
16	17.36	371	249, 121, 353, 231	232, 274	unknown	—	3.28	3.69
17	17.80	403	371, 121, 191, 223	236	unknown	—	5.24	5.89
18*	18.54	477	153, 109		Gentisoyl-dihexoside	HBA derivatives	6.69	7.51
19	19.61	771	301	364, 300(sh)	Quercetin glucosyl-rhamnosyl-glucoside	Flavonol	3.73	4.20
20	21.38	775	285	365, 300(sh)	Kaempferol glucosyl-rhamnosyl-galactoside	Flavonol	1.05	1.18
21	21.53	775	285	364, 298(sh)	Kaempferol glucosyl-rhamnosyl-glucoside	Flavonol	1.85	2.08
22	21.70	785	315	361, 302(sh)	Isorhamnetin glucosyl-rhamnosyl-galactoside	Flavonol	2.16	2.43
23	21.87	785	315	361, 302(sh)	Isorhamnetin glucosyl-rhamnosyl-glucoside	Flavonol	1.63	1.83
24	23.63	289	245, 137	278	Catechin	Flavanol	0.76	0.85
25	24.42	609	301	368	Quercetin rutinoside	Flavonol	2.20	2.47
26	25.80	507	463, 153, 353	306	unknown	—	1.86	2.09
27	27.40	289	245, 137, 151	270	Epicatechin	Flavanols	1.34	1.51
28	27.50	417	181, 387, 167, 151, 403	262, 306	unknown	—	4.68	5.26
29	29	623	315	362, 298(sh)	Isorhamnetin 3-*O*-rutinoside	Flavonol	0.93	1.05
30	35.12	389	181, 275, 345,285	274, 308, 376	unknown	—	1.37	1.53
31	38.73	301	257, 153, 109	268, 344	Ellagic acid	HBA derivatives	0.93	1.05

*These compounds were determined from the chromatogram obtained in the analysis of the sample using the chromatographic method described in García-Estévez et al. (REF) (see section HPLC).
HBA: Hydroxybenzoic acid derivatives.
QA: Quinic acid derivatives.

Results

Phytochemistry

The phenolic composition results were quantified in mg/g of plant and then converted into mg/g of dry extract (Tables 1–3).

LG: The phenolic profile of LG (Table 1) showed high concentrations of iridoid glycosides, which reached 50% of the total phenolic compounds identified (**compounds 2, 5, 7–8, 11, 13, 20–21**). **Compound 7** was the most abundant in this sample, reaching 10.4 mg/g. It was identified as the iridoid theveside, based on its UV spectra and on the results of the mass analysis, which showed a pseudomolecular ion at m/z 389 with a fragmentation pattern with a significant peak at m/z 345. **Compound 2** was detected at a level of 6.93 mg/g of plant and was identified as dihydrotheveside (pseudomolecular ion at m/z 391). The third most abundant compound (4.01 mg/g of plant) was theviridoside (**compound 8**), which showed a [M-H]$^-$ ion at m/z 403. These iridoid glycosides exhibited UV spectra with a maximum (λ_{max}) at ~234 to 236 nm.

Compound 5 was identified as geniposidic acid, which was found at a lower amount of 0.45 mg/g of plant. Based on the mass spectrometric features of this compound (Table 1), **compounds 11 and 13**, which showed deprotonated molecular ions at m/z 537 and m/z 567, respectively, were identified. **Compound 11** showed a fragmentation pattern with major peaks at m/z 163 ([coumaric acid-H]$^-$) and 373 ([geniposidic acid-H]$^-$), identifying it as coumaroyl-geniposidic acid (0.96 mg/g of plant). Similarly, **compound 13** showed major fragment ions at m/z 193 ([ferulic acid-H]$^-$) and m/z 373 ([geniposidic acid-H]$^-$), thus identifying this compound as feruloyl-geniposidic acid (1.19 mg/g of plant). **Compounds 20** (t_R 28.42 min, pseudomolecular ion at m/z 557) and **compound 21** (t_R 30.12 min, pseudomolecular ion at m/z 559), with fragment ions at m/z 373 and m/z 149, were also identified as geniposidic derivatives, although their identity was not fully determined. These compounds were detected at levels of 1.44 mg/g and 1.43 mg/g of plant, respectively.

Compounds 6, 9, 10, 12, 15–17, showed UV spectra characteristics of hydroxycinnamic acids. Among these,

Table 3. Chromatographic characteristics (t_R), mass spectral data, UV spectrum (λ_{max}), tentative identification and concentration of phenolic compounds (mg/g of dried extract) in *Ilex paraguariensis* extract.

Compound	t_R (min)	[M-H]⁻ (m/z)	Fragment ions (m/z)	UV λ_{max} (nm)	Identification	Group	mg/g of dried extract	%
1	4.47	191	111, 129	266	Quinic acid	QA derivatives	3.28	1.38
2	4.88	371	191, 173, 135	252, 278	Unknown	—	0.59	0.25
3	5.018	473	189, 351, 221, 311	294	Unknown	—	0.61	0.26
4	7.41	353	191(100), 179(49), 135(27)	326, 298(sh)	3-Caffeoylquinic acid	QA derivatives	2.01	0.85
5	8.04	353	191(100), 179(50), 135(30)	326, 296(sh)	5-Caffeoylquinic[1]	QA derivatives	57.80	24.37
6	8.50	391	217, 179, 135	272	3-Disuccinoyl quinic acid	QA derivatives	7.44	3.13
7	8.83	341	161, 179, 203, 135	328, 300(sh)	Caffeoyl acid glucose	HA derivatives	2.39	1.01
8	10.67	341	179, 161, 221, 135	328, 300(sh)	Caffeoyl acid glucose	HA derivatives	2.06	0.87
9	12.33	353	173(100), 179(90), 191(75), 135(70)	326, 298(sh)	4-Caffeoyl quinic acid	QA derivatives	23.49	9.91
10	12.89	367	193(100), 134(15)	324, 294(sh)	3-Feruloylquinic acid	QA derivatives	4.40	1.85
11	13.31	353	191(100), 179(4)	326, 300(sh)	1-Caffeoylquinic acid	QA derivatives	35.52	14.98
12	16.96	427	161, 179	274	Unknown	—	12.99	5.48
13	18.43	367	173(100), 191(29)	322, 292(sh)	4-Feruloylquinic acid	QA derivatives	2.37	0.99
14	20.12	367	191(100), 173(12)	324, 296(sh)	5-Feruloylquinic acid	QA derivatives	2.99	1.26
15	24.49	609	301	364, 300(sh)	Quercetin rutinoside	Flavonol	12.36	5.21
16	26.12	515	353(100), 173(60), 335(20)	326, 298(sh)	3,4-Dicaffeoylquinic acid	QA derivatives	8.62	3.63
17	27.80	515	353(100), 191(80), 179(60)	328, 298(sh)	1,3-Dicaffeoylquinic acid	QA derivatives	37.43	15.78
18	29.18	623	315	362, 300(sh)	Isorhamnetin rutinoside	Flavonol	0.69	0.29
19	30.66	515	353(100), 173(90), 179(70), 191(50), 203(20)	328, 300(sh)	1,4-Dicaffeoylquinic acid	QA derivatives	14.03	5.92
20	34.53	529	367(100), 193(80), 134(20)	324, 294(sh)	3,5-Caffeoyl-feruloylquinic[2]	QA derivatives	1.86	0.79
21	35.15	529	353(100), 191(80), 179(60)	324, 294(sh)	3,5-Caffeoyl-feruloylquinic[3]	QA derivatives	1.32	0.56
22	35.55	515	353(100), 173(70), 179(50)	326, 298(sh)	4–5 Dicaffeoylquinic acid	QA derivatives	1.25	0.53
23	37.19	529	367(100), 173(80)	328, 300(sh)	3,4-Caffeoyl-feruloylquinic[4]	QA derivatives	1.67	0.70

QA: Quinic acid derivatives; HA: hydroxycinnamic acid derivatives.
[1]Chlorogenic acid; 2 3,5-Caffeoyl-feruloylquinic acid(3F,5C).
3 3,5-Caffeoyl-feruloylquinic acid(3C,5F).
4 3,4-Caffeoyl-feruloylquinic acid(3C,4F).

compounds 12 and 15–17 were identified as verbascoside isomers. **Compound 12** was the most abundant, reaching 5.33 mg/g of plant. These four compounds presented the same UV spectra and the same pseudomolecular ion ([M-H]⁻) at m/z 623, with similar fragmentation patterns (fragments ions at m/z 461, 161, 315, and/or 179). **Compounds 9 and 10** were identified as the hydroxylated verbascoside called β OH-acteoside (0.76 mg/g of plant and 0.79 mg/g of plant, respectively). Its molecular ion was found at m/z 639 with a major fragment ion at m/z 621, followed by another at m/z 161.

Flavonols were also identified in LG. **Compounds 18 and 19** showed pseudomolecular ions at m/z 491 and 477, respectively, with a major fragment ion at m/z 315 ([isorhamnetin-H]⁻), i.e., losses of 176 and 162, respectively. Therefore, taking into account that the normal position of glycosylation of flavonols is the hydroxyl group in the C-3, they were identified as isorhamnetin 3-O-glucuronide (1.73 mg/g of plant) and isorhamnetin 3-O-glucoside (2.20 mg/g of plant), respectively. **Compound 14**, which showed a pseudomolecular ion at m/z 637 and a major fragment ion at m/z 315 ([isorhamnetin-H]⁻), was identified as isorhamnetin rhamnosylglucuronide (1.25 mg/g of plant). All these compounds showed the characteristic UV spectrum of flavonols (Table 1). Only **compound 3** was identified as a quinic derivative, since its molecular ion was detected at m/z 353 with fragment ion at m/z 191. It was denominated 3-caffeoylquinic acid (0.42 mg/g of plant).

AQB: In AQB, 31 phenolic compounds were identified (Table 2), with hydroxybenzoic derivatives predominating (**compounds 3, 4, 6–10, 12, 13, 15, 18, 31**).

Compounds 3 and 13, defined as gentisoyl hexoside (0.49 1.29 mg/g of plant, respectively), showed a pseudomolecular ion at m/z 315, with fragment ions at m/z 153 ([gentisic acid-H]⁻) and m/z 109. **Compound 18**, showing a pseudomolecular ion at m/z 477 and the same fragmentation pattern (m/z 153 ([gentisic acid-H]⁻) and m/z 109) was identified as gentisoyl dihexoside (1.30 mg/g of plant). **Compound 9** (molecular ion at m/z 389) and **compound 15** (molecular ion at m/z 375) presented characteristics of phthalic derivatives, the latter being identified as trihydroxy-phthalic acid hexoside (respective concentrations: 0.10 and 1.43 mg/g of plant).

Within hydroxybenzoic acid derivatives, protocatechuic acid was identified (**compound 12**) showing a pseudomolecular ion at m/z 153 ([M-H]⁻), and **compound 6** was identified as protocatechuic acid hexoside ([M-H]⁻) at m/z 315 with a main fragment ion at m/z 153 ([protocatechuic acid-H]⁻). Their concentrations were 0.41 and 0.13 mg/g of plant, respectively.

Compound 7 was identified as gallic acid ([M-H]⁻ at m/z 169). Its concentration was 1.30 mg/g of plant. Similarly, **compound 10**, identified as galloyl glucose (M-H]⁻ at m/z 331 with main fragment ions at m/z 169

([gallic acid-H]⁻) and at m/z 151 ([gallic acid-H$_2$O]⁻), was also found but in lower amounts (0.03 mg/g of plant).

Other compounds identified as among hydroxybenzoic acids were **compounds 4, 8, and 31**. At t_R 8.21 min, **compound 4** showed a [M-H]⁻ at m/z 151 and was identified as vanillin (0.23 mg/g of plant). Syringic acid (**compound 8**, 0.07 mg/g of plant) was identified by its pseudomolecular ion at m/z 197 ([M-H]⁻) and its fragmentation pattern (fragment ions at m/z 153 and 135). Finally, ellagic acid (**compound 31**, ([M-H]⁻ at m/z 301, with fragment ions at m/z 257, 153, and 109) reached 0.18 mg/g of plant.

Two compounds of AQB extract were identified as quinic acid derivatives. **Compound 5**, at a concentration of 0.07 mg/g of plant, was defined as chlorogenic acid (5-caffeoylquinic acid) based on its retention time and on its pseudomolecular ion at m/z 353 ([M-H]⁻). **Compound 11** was identified as 1-caffeoyl-quinic acid because its molecular ion was m/z 353 with fragment ion at m/z 191.

Several compounds identified in AQB (**compounds 19–23, 25, and 29**) exhibited characteristics of flavonols (spectra UV λ_{max} ~360 nm). **Compounds 19** and **25** showed a pseudomolecular ion at m/z 771 and 609, respectively, and in both cases, the main fragment ion was detected at m/z 301 ([quercetin-H]⁻). Based on their fragmentation patterns, they were defined as quercetin 3-O-glucosyl-rhamnosyl-glucoside (0.73 mg/g of plant) and quercetin 3-O-rutinoside (0.43 mg/g of plant), respectively. In turn, considering their relative chromatographic retention times, UV-vis spectra and mass spectrometry behavior, **compounds 20 and 21** were identified as kaempferol 3-O-glucosyl-rhamnosyl-galactoside (from the first chromatographic elution) and kaempferol 3-O-glucosyl-rhamnosyl-glucoside, which showed a pseudomolecular ion at m/z 775 with a main MS^2 fragment ion at m/z 285. Their concentrations were 0.20 and 0.36 mg/g of plant. **Compounds 22** (0.42 mg/g of plant) **and 23** (0.32 mg/g of plant) showed a molecular ion at m/z 785 with a main fragment ion at m/z 315 ([isorhamnetin-H]⁻). They were isorhamnetin 3-O-glucosyl-rhamnosyl-galactoside and isorhamnetin 3-O-glucosyl-rhamnosyl-glucoside (flavonols), respectively, according to their fragmentation pattern and chromatographic retention time. **Compound 29**, which presented a pseudomolecular ion at m/z 623 with MS^2 fragments at m/z 315 ([isorhamnetin-H]⁻), was identified as isorhamnetin 3-O-rutinoside (0.18 mg/g of plant).

Monomeric flavanols were also found in the AQB. **Compound 24**, showing a pseudomolecular ion at m/z 289 and fragment ions at m/z 245 and 137, was identified as catechin (0.15 mg/g plant), and **compound 27** as epicatechin (0.26 mg/g of plant), which showed the same molecular ion and fragment ions at m/z 245, 137, and 151.

IP: Most of the phenolic compounds (**compounds 1, 4–6, 9–11, 13, 14, 16–23**) in this plant showed the UV spectrum characteristic of quinic acid derivatives ($\lambda_{máx}$ ~326 nm) (Table 3). **Compounds 4, 5, 9, and 11** showed a pseudomolecular ion at m/z 353 and were identified as caffeoylquinic acid isomers. Their identities were assigned depending on the different relative intensities of fragmentation (22). **Compound 4** showed fragment ions at m/z 191(100), 79(49), 135(27) and was seen to be 3-caffeoylquinic acid (1 mg/g of plant), and **compound 5**, which showed fragment ions at m/z 191(100), 179(5), and 135(3), was 5-caffeoylquinic acid (chlorogenic acid). This phenolic compound was the most abundant (28.82 mg/g of plant). **Compound 11** also showed fragments of 191(100) and 179(4) and, given the absence of other fragments, was identified as 1-caffeoylquinic acid (17.71 mg/g of plant). Finally **compound 9** showed greater intensity in fragment 173(100), 179(90), 135(70), and lowest in 191(75), and therefore was identified as 4-caffeoylquinic acid (11.71 mg/g of plant).

Compounds 16, 17, 19, and 22 presented a pseudomolecular ion at m/z 515 and a main fragment ion at m/z 353(100), which can correspond to the characteristics of dicaffeoylquinic acids. Given their different intensities of fragmentation, they were identified by comparing them with those reported in literature (15,16). **Compound 16**, which showed fragment ions at m/z 353(100), 173(60), 335(20), was defined as 4,3-dicaffeoylquinic acid (4.3 mg/g of plant), and **compound 17**, with fragment ions at m/z 353(100), 191(80), 179(60), was 1,3-dicaffeoylquinic acid (18.66 mg/g of plant). **Compound 19** also showed fragmentation of m/z 353(100), 173(90), 179(70), 191(50), 203(20), and it was identified as 1,4-dicaffeoylquinic acid (6.99 mg/g of plant). Finally, **compound 22** was defined as 4,5-dicaffeoylquinic acid (0.62 mg/g of plant) based on its fragmentation pattern (fragment ions at m/z 353(100), 173(70), and 179(50)).

Compounds 10, 13 and 14 showed a pseudomolecular ion at m/z 367, which is characteristic of feruloylquinic acids. Based on their fragmentation patterns (15,16), they were identified, respectively, as 3-feruloylquinic acid (fragments ions at m/z 193(100) and 134(15)), 4-feruloylquinic acid (fragment ions at m/z 173(100) and 191(29)) and 5-feruloylquinic acid (MS^2 ions at m/z 191(100) and 173(12)). The observed amounts of each isomer were 2.19, 1.18, and 1.49 mg/g of plant, respectively.

Compounds 20, 21, and 23 showed a molecular ion at m/z 529, which can be related to caffeoyl-feruloylquinic acid structures. The different isomers were

identified by comparing their MS^2 fragmentation pattern with those previously reported (15,16). **Compound 20**, identified as 3,5-caffeoyl-feruloylquinic acid (3F, 5C), showed fragment ions at m/z 367(100), 193(80) ([ferulic acid-H]$^-$) and 134(20) and its concentration was 0.93 mg/g of plant. **Compound 21** was identified as 3,5-caffeoyl-feruloylquinic acid (3C, 5F), since it showed fragment ions at m/z 353(100) ([(caffeoylquinic acid-H]$^-$), 191(80) ([quinic acid-H]$^-$) and 179(60) ([caffeic acid-H]$^-$). Its concentration was 0.66 mg/g of plant. Finally, **compound 23**, with values of 0.83 mg/g of plant, was defined as 3,4-caffeoyl-feruloylquinic acid (3C, 4F) because of its fragmentation pattern (m/z 367(100) and 173(80)).

Compounds 7 and 8, identified as hydroxycinnamic derivatives, were defined as caffeoyl acid glucose (1.19 and 1.03 mg/g of plant, respectively). These presented [M-H]$^-$ at m/z 341 and MS^2 fragmentation at m/z 179, 161, and 135.

Flavonols were also found in the extract of *IP*. **Compound 15** showed a molecular ion at m/z 609 with a main fragment ion at m/z 301 ([quercetin-H]$^-$), so it was defined as quercetin rutinoside (6.16 mg/g of plant). Similarly, **compound 18** was defined as isorhamnetin rutinoside (0.34 mg/g of plant) based on its molecular ion at m/z 623 and fragment ion at m/z 315 ([isorhamnetin-H]$^-$).

Bioactivity

Cellular viability of C6 and T98-G lines did not show a significant response to the extracts (AQB, IP, and LG) in the linear regression analysis; thus, they did not show toxicity (Figure 1).

The AQB extract induced IL-6 release by both cell lines in a dose-dependent manner, whereas the LG extract reduced IL-6 release by C6 cells in a non-significant way, without activity on T98-G cells. The IP extract significantly reduced IL-6 release by C6 cells, without activity on T98-G cells. Thus, AQB was pro-inflammatory, LG was not active, and IP was anti-inflammatory (Figure 1).

The lipid profile of C6 cells treated with LG included ω-9, saturated, ω-6, and ω-3 fatty acids in decreasing amounts. Similar results were found in AQB-treated cells, although they showed higher amounts of the first ones (ω-9, and saturated fatty acids). The ω-7 fatty acids were the major lipids in IP-treated cells, followed by ω-9, saturated, ω-6, and ω-3 acids (Figure 2).

On the other hand, T98-G cells treated with LG mainly showed ω-9 acids followed by ω-6, ω-7, saturated, and ω-3 ones. IP and AQB enhanced the ω-9 content, followed by ω-7, saturated, ω-6, and ω-3 ones (Figure 2).

PLS regression was used to correlate responses of both cell lines exposed to extract compounds, and to
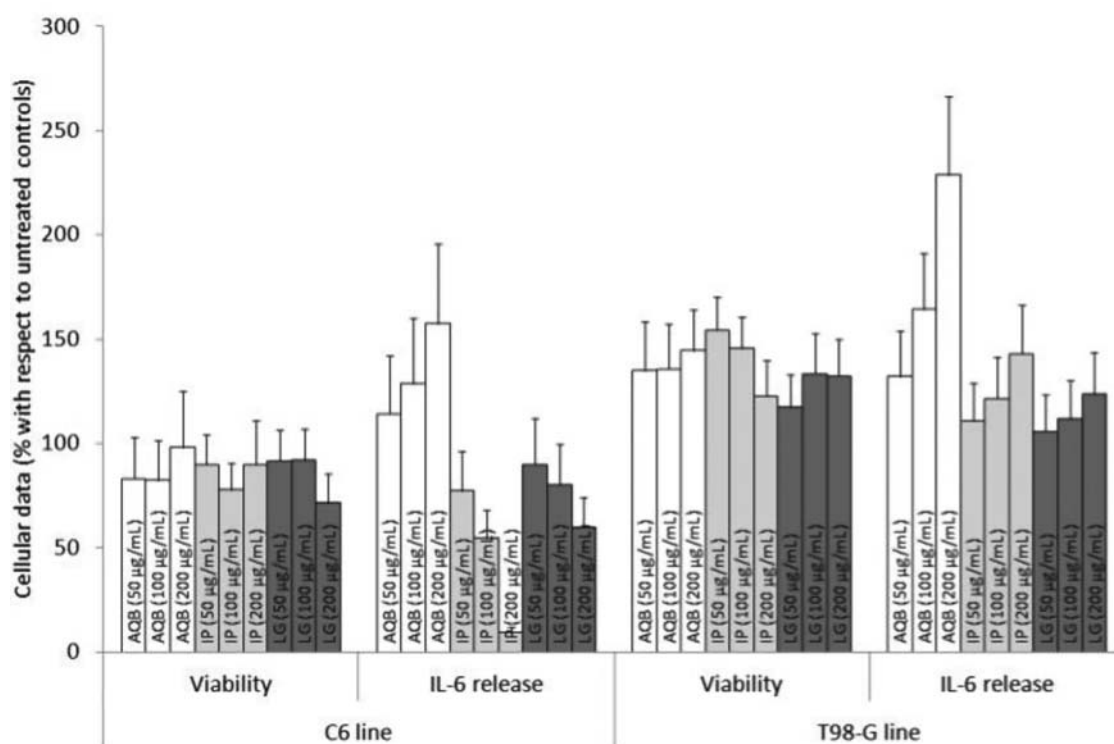
Figure 1. Cellular viability and IL-6 release of the C6 and T98-G cell lines treated with LG, AQB, and IP (expressed as mean percentage and standard error with respect to untreated controls: C = 100%).
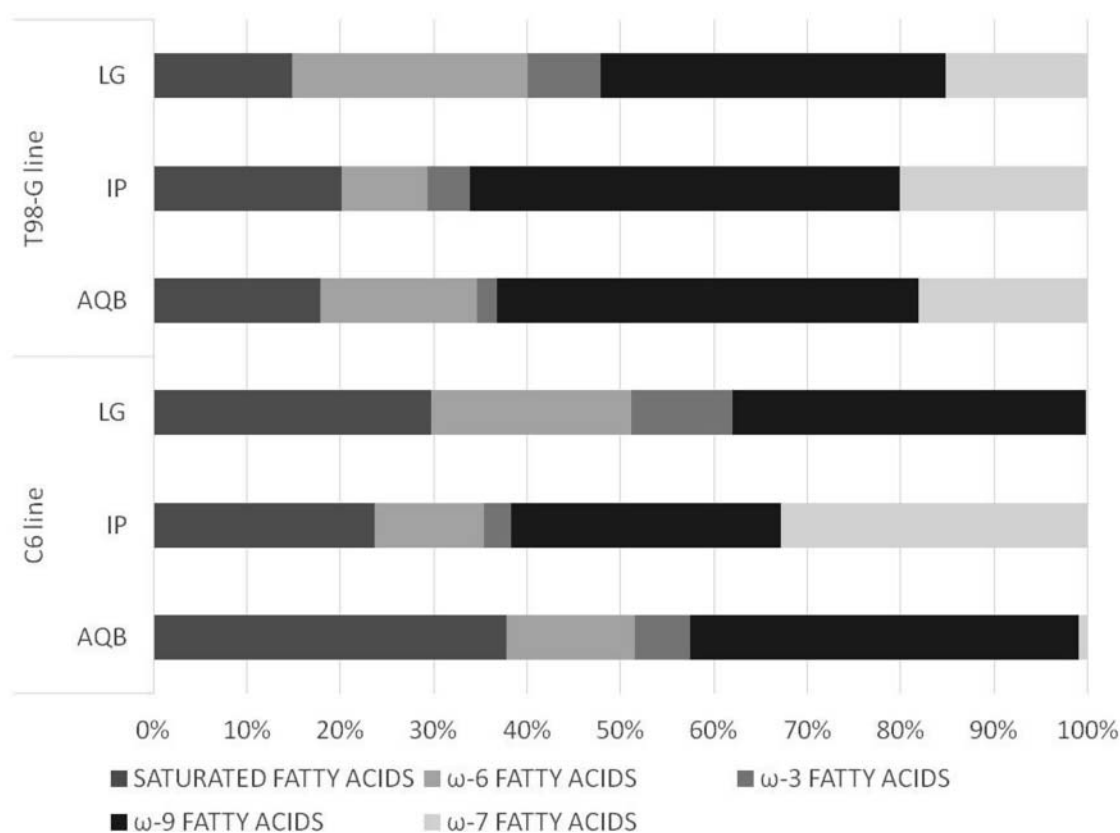
Figure 2. Fatty acid families of the C6 and T98-G cell lines treated with LG, AQB, and IP. Descriptive bars showed relative content of each one (%).

discriminate effects of each compound. Levels of IL-6 release were inversely related to levels of ω-7 fatty family, which was represented by the palmitoleic acid (Figure 3).

Compounds of LG were related to polyunsaturated ω-6 and ω-3 fatty acids, without further association with IL-6 release. Compounds of AQB, with T98-G cells being responsive, were directly related to IL-6 release and less strongly to ω-9 and saturated fatty acids (Figure 3).

Compounds of IP were mainly related to ω-7 levels, followed by ω-9 and saturated acids. Also, these compounds were inversely related to IL-6 release. The chlorogenic acid was identified as the main active compound on C6 cells, among the major phenolic acids of IP (1-caffeoylquinic, 5-caffeoylquinic, 1,3-dicaffeoylquinic) (Figure 3).

Discussion

This study analyzed numerous phenolic compounds in tea extracts of three South American plants: LG, AQB, and IP, which were assayed in cultures of two cell lines (murine C6 and human T98-G). These cells were chosen given glia plays a key role in IL-6-mediated neuroinflammation, which is involved in glioma response (23).

Concerning their chemical composition, twenty-one compounds were measured in LG, which were mainly

iridoids such as dihydrotheveside and theveside. This deepens our knowledge of the genus, given that the scientific literature is often about compounds of *L. camara*, e.g., lantadenes (24). Moreover, the extract was a source of geniposidic derivatives, which have been described in different taxonomic families (25). Detection of verbascoside isomer, another major LG compound, was in accordance with phytochemical profile of *Verbenaceae* (26). Although some iridoids reduce inflammatory responses (27), LG compounds did not modify IL-6 in this work. Nonetheless, they were active and positively related to cellular content of polyunsaturated fatty acids, which was previously found [unpublished data].

Thirty-one compounds were found in AQB, which mainly contained hydroxybenzoic acid derivatives. It also presented different flavonoids, such as isorhamnetin glycosides. These results are original because previous research generally focused on its alkaloids (28). Although the gallic acid is widely distributed in *Plantae*, the major compounds trihydroxy-phthalic acid hexoside and gentisoyl-dihexoside are less frequent and found in certain families (29,30). Thus, phytochemical profile of AQB was extended by this work. Other minor compounds were in accordance with phytochemical profile of *Apocynaceae* (30). Although some minor compounds such as isorhamnetin exhibit anti-inflammatory potential (31),
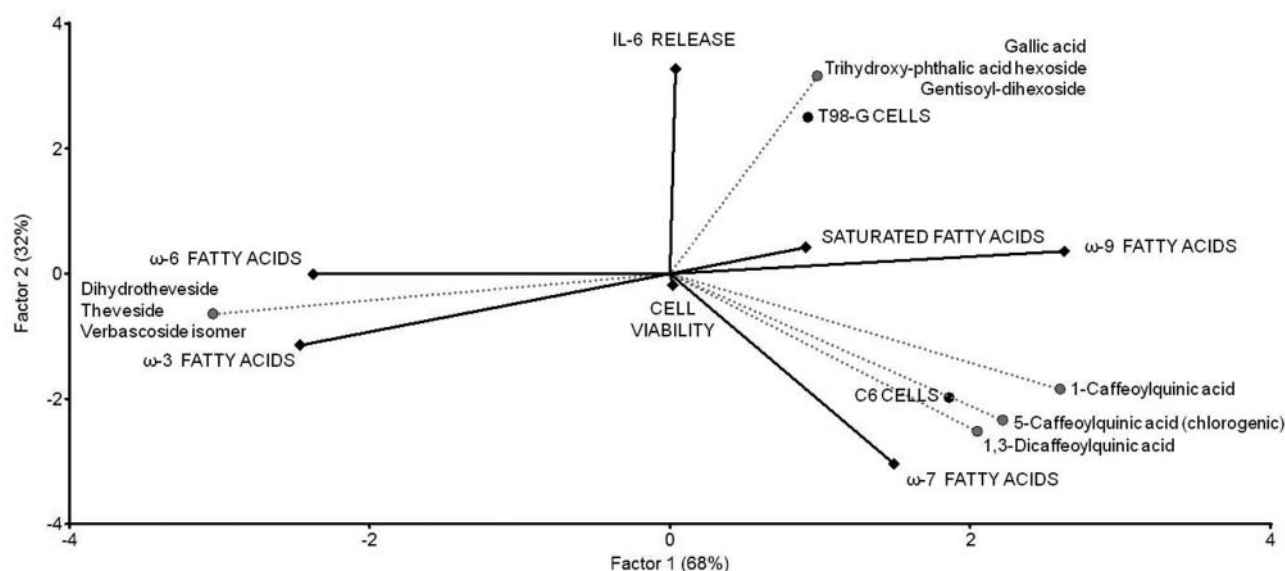
Figure 3. Response correlation according to extract compound doses (major compounds: LG: dihydrotheveside, theveside, verbascoside isomer; AQB: gallic acid, trihydroxy-phthalic acid hexoside, gentisoyl dihexoside; IP: chlorogenic acid, 1-caffeoylquinic acid, 1,3-dicaffeoylquinic acid) in the C6 and T98-G cell lines. Line length indicated relative variable intensity, and grades of line separation indicated variable correlation (i.e., $<90°$ = positive, $90°$ = null, $>90°$ = negative).

role of major AQB compounds in neuroinflammation is unknown. Actually, the extract promoted IL-6 release in this work.

In the case of IP, the infusion provided more compounds in amount per ml than the others ($>$LG$>$AQB). IP presented twenty-three identifiable compounds, including several caffeoylquinic acid derivatives. Although molecules of this plant have previously been identified (6,32), the current work found a remarkable content of chlorogenic acid. The reduction of IL-6 release found after exposure to these phenolic acids (1-, 5-, and 1,3- forms) was according to their anti-inflammatory activity (32,33).

The effects of IP compounds were mostly associated with the increase of ω-7 fatty family, represented by the palmitoleic acid. It therefore confirmed the anti-inflammatory effect of this fatty acid on glial cells, including reduction of IL-6 and other inflammatory mediators found in other cells (34,35). In addition to this activity, IL-6 is also involved in several biological processes in the brain, as regulator of synaptic plasticity, neural networks, cognitive responses and development of neuropathies (36). This indicated a regulatory role of phenolic acids on glial lipid metabolism (i.e. chlorogenic acid might up-regulate stearoyl-9-CoA desaturase, which was supported by ω-9 levels). This gives a new insight into a poorly understood fatty acid and its role as a target of neuro-immunomodulating agents. Furthermore, neuroprotective signalling promotes palmitoleic acid (37), with content of this ω-7 lipid being enhanced in murine brain by the IP infusion (38).

Polyphenols provided by each infusion can rise $\sim$10% to 25% of total daily polyphenols after being included into the diet, in accordance with results shown in Tables 1–3 and revised data (39), which supports their relevance. Moreover, IP concentration used in cultures provided relevant levels of chlorogenic acid, which has been detected in brain after oral consumption of the extract [unpublished data], under representative conditions of human intake (6).

Conclusion

This study provides a broad phytochemical characterization of three South American plants, which contain neuroactive molecules with importance for human nutrition and health. IP is commonly used as a tea called "mate," which provides high amounts of dietary phenolic compounds with anti-inflammatory activity on glioma cells, whereas LG and AQB did not. Also, results indicate that these compounds modify the lipid composition of glia and its immune function, with a specific role of the palmitoleic acid being found by the current work.

Acknowledgments

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