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Effects of Bioavailable Phenolic Compounds from *Ilex paraguariensis* on the Brain of Mice with Lung Adenocarcinoma

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Abstract

Lung carcinoma is one of the most common cancers and has a high mortality. Recently, we showed that it produces neurological paraneoplastic syndrome, with *Ilex paraguariensis* extract (IP) exerting palliative effects due to its content of phenolic compounds. It is possible, therefore, that these diet agents can arrive at the brain and exert neuroprotection, after the oral intake of IP. Here, the aim was to investigate the protective role of bioavailable IP compounds on the telencephalon and diencephalon in lung adenocarcinoma-bearing BALB/cJ males. Mice aged 2-months were treated for 3 weeks with 0-100 IP mg/Kg/day. HPLC-UV revealed the presence of chlorogenic acid and quercetin in brain regions, liver and tumour, in an IP dose-dependent manner. Brain was also evaluated histologically, and IL-6 was measured by ELISA. Chlorogenic acid was the major compound found in brain, whereas quercetin was observed at the diencephalon to a lesser extent. Both compounds were involved in IP dose-dependent diencephalic interleukin-6 reduction. Histology suggested cellular protection with less apoptosis in chlorogenic-exposed areas. Taken together, chlorogenic acid and quercetin from dietary IP were bioavailable and bioactive in brain, thereby attenuating lung cancer-related neuroinflammation and damage. These findings support plant-based strategies to improve prognosis.

Keywords

Chlorogenic acid; High performance liquid chromatography; Interleukin 6; Paraneoplastic syndrome; Quercetin.

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Introduction

Lung cancer is worldwide one of the most frequent and lethal human neoplasias. This tumour affects the central nervous system (CNS) directly by metastasis, and indirectly by paraneoplastic syndromes (Jurado G3mez et al, 2001; Giglio et al, 2010; Koike et al, 2015). Moreover, it has been shown to induce highly deteriorating neuroinflammation and brain damage at the early onset of the condition in several patients (Islami and Sobue, 2015; Sateia et al, 2017). Although primary prevention of this illness is through tabaquism control, there are few preventive resources available to avoid or at least delay its progression and complications, which is known as secondary and tertiary prevention.

Plants are sources of a myriad of bioactive compounds, which can be incorporated into the human diet to exert chemoprevention of neurological complications (Miranda et al, 2017). Polyphenols, which are found in most plants, are phytochemicals that exert systemic immunomodulation and cytoprotection by multitarget mechanisms, such as regulation of the redox status and biological pathways, thereby improving human health (Miranda et al, 2017; Cittadini et al, 2018). Thus, they have potential as cancer chemopreventive and neuroprotective agents.

Ilex paraguariensis A. St.-Hil. (Aquifoliaceae) (IP) is a South American plant widely consumed as a tea, known as "yerba mate" or "mate" in the Spanish language, with dietary use and availability growing around the world (Bracesco et al, 2011). This plant and its derivatives, such as tea and its extract, are enriched in bioactive compounds which reach the CNS and induce desired effects (Miranda et al, 2017; Cittadini et al, 2018; Bracesco et al, 2011; Orosz, 2018; Albrecht et al, 2017). The IP extract mainly contains phenolic compounds, such as quinic acid, 3-caffeoylquinic acid, chlorogenic acid (the major compound), 4-caffeoylquinic acid, 1-

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caffeoylquinic acid, caffeoyl acid glucose, quercetin rutinoside, and isorhamnetin rutinoside, among others (Cittadini et al, 2018; Bracesco et al, 2011).

Although the capacity of phenolic compounds to cross the blood-brain barrier has been reported (Andrade and Assuncao, 2012; Cittadini et al, 2015), experimental designs often apply pure molecules by unusual ways of administration (El Mohsen et al, 2002; Ishisaka et al, 2011). Thus, it is necessary to have a better understanding of how these compounds affect the organism based on more common conditions, such as diet supply. Consequently, we carried out a preclinical study using lung adenocarcinoma (LAC-1)-bearing male BALB/cJ mice, which is an excellent model to investigate cancer evolution and neuroprotective effects of these compounds on the brain (Piegari et al, 2017).

Materials and Methods

Extract preparation

I. paraguariensis A. St.-Hil. (*Aquifoliaceae*) was obtained from commercial agroecological culture (Jactancia, Argentina), and prepared as a tea: 1 g of pulverised air-dried sample was extracted by adding 10 mL of 83°C water, which was left to cool to room temperature for 1 h under constant agitation. After this, the blend was centrifuged, and the extract was recovered from the supernatant by filtration (using 0.2-mm-pore polyethersulphone membranes for 4.5 bars) followed by 24-h lyophilisation at <-50°C vacuum. The infusion was shown by HPLC analysis to have 237 mg of polyphenols per gram of extract (85.9% quinic acid derivatives; 4.35% flavonols, 2.24% hydroxycinnamic acid derivatives and 7.51% unknown) (Cittadini et al, 2018).

Experimental conditions

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This study was carried out according to ethical and technical guidelines (approval by the Institutional Committee for the Care and Use of Laboratory Animals: UNC-FCM-SECYT-CICUAL-2014-09-02, with authors adhering to the ARRIVE guidelines). Animals (~23 g) were bred under standard laboratory conditions with *ad libitum* commercial diet (200±13 g/Kg/day; Cargill SACI, Argentina) and potable water (150±10 mL/Kg/day; Aguas Cordobesas SA, Argentina). Two-month-old male BALB/cJ mice (The Jackson Laboratory) received 1.4x10⁶ LAC-1 cells/animal by subcutaneous inoculation, which caused a lung adenocarcinoma to develop, and they were divided into three experimental groups (n≥9): Control received water, IP50 were treated with 50 mg/Kg/day of water-dissolved IP extract, and IP100 were treated with 100 mg/Kg/day of water-dissolved IP extract. A toxicity assay was previously tested to determine the safety of the dose (Cittadini et al, 2018). Animals were treated by oral intake for three weeks, with the assay being started on the same inoculation day. After treatment, the mice were sacrificed, and the organs were removed and weighed. The brain regions (telencephalon and diencephalon), liver and tumour were kept for experiments. In addition, a telencephalon fraction was separated and preserved for histological analysis. All tissues were mechanically homogenised with Milli-Q water and conserved to -80°C until undergoing biochemical analysis.

Analysis of phenolic compounds

Analytical grade phenolic standards: chlorogenic, gallic, caffeic, ferulic and coumaric acids, quercetin and β-glucuronidase/sulphatase enzymes were acquired from Sigma-Aldrich Química SA (Madrid, Spain). HPLC-grade solvents were used (formic acid, methanol, ethanol and acetic acid), which were supplied by Prolabo (Madrid, Spain). Lyophilised samples of telencephalon, diencephalon, liver and tumour were homogenised with phosphate buffered saline (PBS) by using a IKA T-18 Basic Ultra Turrax Homogenizer, and then centrifuged at 1000 g for 10 min with an

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Eppendorf 5415r centrifuge and analysed to determine their polyphenol profiles after β -glucuronidase/sulphatase treatment. Homogenates (200 μ L) were mixed with 0.58 N acetic acid (20 μ L) to prevent degradation, which were then analysed.

Samples were hydrolysed with 14 μ L of β -glucuronidase/sulphatase enzyme at 37°C for 30 min, with methanol (1 mL) being added to complete the reaction. After this, samples were centrifuged for 10 min, and solvents were evaporated using a SpeedVac concentrator at room temperature (30 min). Finally, samples were reconstituted with 0.20 mL Milli-Q water: ethanol (1:1, v/v), mixed on a vortex, passed through a 0.45 μ M PVDF filter (polyvinylidene-difluoride) and analysed by HPLC-UV. The standards and supernatants (20 μ L) obtained were analysed using an Agilent 1200 Series liquid chromatograph with a quaternary pump, a photodiode array detector (DAD) and an Ultrabase C18 column (5 μ m; 4.6 mm x 150 mm), which was set thermostatically at 25°C. Solvents used for the analysis were formic acid 0.1% (A); HPLC-grade acetonitrile with 0.1% of formic acid (B); ultra-pure water (C) and acetonitrile (D) at a flow rate of 1 mL/min. The elution program was started with 90% A to 10% B, and the gradient was the following: from 70% A to 30% B in 30 min, from 65% A to 35% B in 35 min, from 55% A to 45% B in 40 min, and then returning to the initial conditions in 50 min (Hidalgo et al, 2010). Standards were analysed at different UV wavelengths (260, 280, 306 and 320 nm), and their retention time (R_t) was recorded with DAD after HPLC separation. Quantification of the pure compounds ferulic, coumaric, and caffeic acids was performed at 320 nm, while this was carried out for gallic and chlorogenic acids at 280 nm. Quercetin was also observed at 320 nm. The HPLC peaks of the phenolic compounds in the tissues were identified by comparing their retention times and UV absorption spectra with those of pure standards injected under the same conditions (Supplementary material 1).

The tissue phenolic content was measured at 750 nm, which depended on the reducing capacity, by incubating 25 μ L of supernatants with the 2 N Folin reactant (25 μ L), water (150 μ L) and a

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saturated sodium bicarbonate solution (50 µL) for 30 min in darkness at 37°C. The results were calculated as µg/g of tissue using a standard curve of gallic acid, and expressed with respect to control (Albrecht et al, 2017; Cittadini et al, 2015).

Redox bioactivity of these compounds was assessed in each organ by the Spearman’s correlation between phenolic concentrations and the lipoperoxide level determined as previously done (Cittadini et al, 2015).

Determination of interleukin 6 (IL-6)

The IL-6 levels were determined by ELISA using an OptEIA™ kit (BD Biosciences, USA) with absorbance at 450 nm. IL-6 was measured in the encephalic regions (telencephalon and diencephalon). IL-6 concentrations were calculated from a standard curve (15.6-1000 pg/mL) and expressed per g of tissue (A-Jin et al, 2015).

Histological analysis

Telencephalon treated with IP and control group samples was fixed in buffered paraformaldehyde for 2 h at room temperature and processed for histological analysis. Then, telencephalon histological sections were stained by Hoechst to locate apoptotic cells. After rehydrating the cuts with decreasing concentrations of ethanol: water (50:50), Hoechst staining was prepared in PBS (1 mL PBS + 0.5 mL of Hoechst), and incubation was carried out for 10 min at 37°C in darkness. Following three washes with PBS, staining was performed with eosin (1 mL PBS + 50 µL of eosin), and sections were incubated again for 5 min at 37°C in darkness. Finally, washing with PBS was repeated. Three slides were observed for each treatment, and the percentage of apoptotic cells was recorded at 400x magnification using a Leica videomicroscope with epifluorescence (E: 350 nm/M: 460 nm). Images were analysed by Image J 1.48 software®

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(USA). Also, fixed brains were stained with the classical hematoxylin/eosin technique and with the Kluver Barrera's staining (cresyl violet/luxol fast blue) to evaluate tissue and cytological changes.

Statistical analysis

Data were expressed as mean $\pm$ standard error (SE) from at least three separate experiments performed twice. ANOVA models were used to evaluate differences between treatments, after which, the Fisher test was performed for mean comparison ($p < 0.05$). The Spearman correlation was considered relevant for coefficients $> 50\%$. Analyses were performed using the InfoStat 2012 software (InfoStat Group, Argentina).

Results

Phenolic compounds and activity

The phenolic compounds identified by HPLC-UV were chlorogenic acid and quercetin, which were found at 7.17 and 29.28 min, respectively (Figures 1, Table 1). Other phenolic compounds, such as gallic, coumaric, caffeic and ferulic acids, were not included as only trace values were detected. Low concentrations of quercetin were also verified by HPLC-MS (Supplementary material 1).

The IP50 treatment resulted in a significant increase of chlorogenic acid in telencephalon, diencephalon, and tumour, whereas quercetin was not detected. After the IP100 treatment, this acid increased even more in telencephalon, diencephalon, tumour, and liver ($p < 0.05$), and the levels of quercetin rose in the diencephalon, and liver. No detectable compounds were found in the untreated control group (Table 1).

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LAC-1-bearing mice showed increasing IP dose-dependent concentrations of both compounds at the studied sites, where the chlorogenic concentration was higher than the quercetin concentration. Also, chlorogenic acid exhibited a differential tissue distribution with an increased concentration principally in the telencephalon, but also in the diencephalon, liver, and tumour, while quercetin was mainly distributed in the diencephalon (Table 1).

Increasing concentrations of chlorogenic acid, but not quercetin, in the brain were positively correlated to the tissue phenolic content found in the same organ, which was enhanced 1.23-1.47 times in telencephalon, and 1.83-2.02 times in diencephalon after the IP50 and IP100 treatments, respectively.

No significant Spearman's correlations between bioavailable phenolic concentrations and the lipoperoxide level were found ($p>0.1$).

IL-6 concentration

IL-6 decreased after IP50 treatment in telencephalon (30%), whereas it increased after IP100 (23%) with respect to control ($p<0.05$). In diencephalon, both IP50 and IP100 resulted in a decreased IL-6 concentration with respect to control (9% and 29%, respectively, $p<0.05$), with a dose-dependent response being revealed (Figure 2). Moreover, the IL-6 levels in diencephalon were negatively correlated to the chlorogenic and quercetin concentrations, whereas this outcome was not found in telencephalon. Diencephalon also exhibited a higher IL-6 concentration than telencephalon under the three treatments.

Histology

Several microscopic fields of brain grey matter were evaluated at 400x magnification. A growing number of cells were found in mice treated with IP50 and IP100 with respect to control ($p<0.05$).

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Furthermore, mice under both treatments, but mainly IP100, presented better neuronal cytological characteristics in their brains (Figure 3, Supplementary material 2). These findings were associated with fewer apoptotic cells (C=29%, IP50=27%, and IP100=18%), characterised by condensed nuclei in Hoechst-stained brains, and lower pyknosis (Supplementary material 2). Neurons showed improvement of cresyl violet staining and cytology by increasing IP doses (Figure 3: rectangle), whereas cellular retraction (Figure 3: oval) and gliosis foci (preliminary data not shown) were found without treatment.

Discussion

The extract of *Ilex paraguariensis* (IP) supplied two phenolic compounds: chlorogenic acid and quercetin, which were identified in an oral IP dose-dependent manner at different organic sites of LAC-1-bearing mice after intake. In agreement with the present study, pharmacological research has established that both compounds are metabolised in a highly inducible way, which explains their low permanence in the liver. They are also able to cross the blood-brain barrier to reach the brain (Andrade and Assuncao 2012; Manach et al, 2005). Related to this, several molecules that are bioactive *in vitro* are less effective *in vivo*, because they cannot cross this boundary (De Oliveira et al, 2017). Another important factor is that brain regions can exhibit differential selectivity in order to obtain phenolic compounds from the bloodstream (El Mohsen et al, 2002). Chlorogenic acid was constantly found by HPLC-UV in treated mice, in accordance with its high concentration in this extract, whereas the rest of phenolic acids (gallic, ferulic, caffeic, and coumaric acids) were inconstantly found in trace levels. On the other hand, the flavonoid quercetin found in lower concentrations was additionally confirmed by mass spectroscopy (Supplementary material 1), which is an original result in murine brain after oral supply of a phytoextract.

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IP is a good candidate as an oral phenolic source, because its polyphenols are available after digestion, thus enhancing intestinal absorption (Albrecht et al, 2017). The levels of chlorogenic acid and quercetin found in the brain can be related to kinetic properties (e.g. accomplishment of the Lipinski's rules), tissue affinity, and the biochemical stability of the cerebrospinal fluid and the encephalic microenvironment, which is highly regulated by the blood-brain barrier and glia (De Oliveira et al, 2017; Kumar et al, 2018). Chlorogenic acid was the major compound found after IP intake, in agreement with previous studies that reported its high concentration and predominance in *Ilex paraguariensis* (Cittadini et al, 2018; Albrecht et al, 2017). Furthermore, this phenolic acid was also observed at the tumours for both IP doses, which may be advantageous given its cancer modulating potential (Yamagata et al, 2018). Therefore, the correlation observed in telencephalon and diencephalon between chlorogenic and phenolic concentrations was espectral. Although unpublished data of our group suggest variable redox effects in different organs of LAC-1-bearing mice, the current work did not show redox involvement of the identified bioavailable compounds as lipoperoxide-modulating agents. The antioxidant capacity of IP has demonstrated to be neuroprotective under different conditions (Colpo et al, 2017; De Lima et al, 2017). In the case of lung cancer, additional deleterious responses are triggered in the brain and its cells, such as inflammation (Cittadini et al, 2018; unpublished data), which was controlled here.

The IP-related IL-6 reduction is consistent with the antiinflammatory activity of its phenolic compounds (Cittadini et al, 2018). It is known that diencephalic functions are crucial for organic integration and depend on many factors, such as nutrition, immunity, and neurotransmission (Schreiner et al, 2017), with phenolic compounds being able to regulate these leading to beneficial local and systemic responses (Miranda et al, 2107). Previous data have reported effects of IP derivatives on LAC-1-bearing mice against neuroinflammation, demyelination, and

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cachexia (Cittadini and Repossi, 2016). Here, telencephalon revealed lower levels of IL-6 with a biphasic response (a decrease in the case of IP50, and an increase for IP100), which reflects the complex neurophysiological role and regulation of this cytokine. In addition, IL-6 participates in the response of mature neurons and glial cells in both normal and pathological conditions involving neurotrophin-like and neuroimmune modulating mechanisms (Erta et al, 2012). The different responses in diencephalon and telencephalon to IP are due to the CNS spatial heterogeneity (structure, function, and cellular composition). This confers a great complexity and multiple mechanisms with a predilection of xenobiotics in certain biochemical systems (Maurer and Philbert, 2015). In fact, diencephalon also incorporated quercetin, an antiinflammatory molecule (Ishisaka et al, 2011), which enhanced IL-6 reduction after IP100. In addition, glia and neurons respond differently to foreign molecules at different doses (Kapoor et al, 2006).

LAC-1 induces neurodegeneration in mice (Kapoor et al, 2006), whereas neuroprotective effects with an improvement in the cell count and morphology were found in the present study in brains treated with IP. In this sense, lung cancer can damage nervous tissue and induce cell death during its evolution (Giglio and Gilbert, 2010), whereas polyphenols have a protective potential (Silva and Pogacnik, 2017), with chlorogenic acid antiapoptotic activity also having been reported (Anggreani and Lee, 2017). Moreover, this IP-promoted neuroprotection agrees with other results already discussed, and with results found in another neuropathological model (De Lima et al, 2018).

In summary, our results showed that orally administered *Ilex paraguariensis* extract led to a dose-dependent brain accumulation of chlorogenic acid and quercetin in lung adenocarcinoma-bearing mice, where they exerted different neuroprotective effects on the telencephalon and diencephalon, to alleviate cancer-related neurological complications.

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Conflict of interest statement

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Table 1. Phenolic compounds in LAC-1-bearing male BALB/cJ mice ($\mu\text{g}/\text{mg}$ of dry tissue: mean $\pm$ SE) after three-week treatment

	Chlorogenic acid (280 nm HPLC-UV)	Quercetin (320 nm HPLC-UV)
Telencephalon		
Control	Nd	Nd
IP50	0.22 $\pm$ 0.11*	Nd
IP100	1.01 $\pm$ 0.15*	Nd
Diencephalon		
Control	Nd	Nd
IP50	0.07 $\pm$ 0.00*	Nd
IP100	0.24 $\pm$ 0.00*	0.02 $\pm$ 0.00*
Liver		
Control	Nd	Nd
IP50	Nd	Nd
IP100	0.09 $\pm$ 0.05	0.01 $\pm$ 0.01
Tumour		
Control	Nd	Nd
IP50	0.05 $\pm$ 0.02	Nd
IP100	0.20 $\pm$ 0.03*	Nd

References: Nd: not detected; * significant different at $p < 0.05$.

Oral treatments with *Ilex paraguariensis* (IP) extract: IP50: 50 mg/Kg/day, or IP100: 100 mg/kg/day.

Chlorogenic acid ($R_t \approx 7.17$ min, 280 nm HPLC-UV) in telencephalon (A), diencephalon (B), tumour (C) and liver (D) of lung adenocarcinoma-bearing male BALB/cJ mice treated orally for 3 weeks with 100 mg/Kg/day of *Ilex paraguariensis* extract (IP100 a) or 50 mg/Kg/day (IP50), and Quercetin ($R_t \approx 29.28$ min, 320 nm HPLC-UV) in diencephalon (A) and liver (B) (IP100 b).

129x200mm (96 x 96 DPI)

Tissue interleukin 6 (IL-6) concentration (mean $\pm$ SE) in telencephalon and diencephalon of lung adenocarcinoma-bearing male BALB/cJ mice treated orally for 3 weeks with 100 mg/Kg/day of *Ilex paraguariensis* extract (IP100), 50 mg/Kg/day (IP50), or 0 mg/Kg/day (C: untreated control). * Significant differences at $p < 0.05$.

Grey matter cells of lung adenocarcinoma-bearing male BALB/cJ mice treated orally for 3 weeks with 100 mg/Kg/day of *Ilex paraguariensis* extract (IP100), 50 mg/Kg/day (IP50), or 0 mg/Kg/day (C: untreated control). Representative microphotographs with histological stainings at 400x magnification. * Significant differences at $p < 0.05$ (mean $\pm$ SE, quantified using Fiji ImageJ v 1.51 software).

254x190mm (96 x 96 DPI)