sciendo

HRVATSKO FARMACEUTSKO DRUŠTVO
CROATIAN PHARMACEUTICAL SOCIETY

Acta Pharm. **71** (2021) 383–398
https://doi.org/10.2478/acph-2021-0029

Original research paper

Ilex paraguariensis extract as an alternative to pain medications

LUCIANA C. NOWACKI[1]
JOSÉ STECHMAN-NETO[2]
ELBERTH M. SCHIEFER[3]
ANDRESSA F. SANTOS[4]
ANDRÉA E. M. STINGHEN[5]
GUILHERME L. SASSAKI[6]
LAURO M. DE SOUZA[7]
KILLIAN E. CRISTOFF[2]
WESLEY M. DE SOUZA[4,*]

[1] *Pharmacology Department, Tuiuti University of Paraná, Curitiba, PR, Brazil*

[2] *Postgraduate Program in Communication Disorders, Tuiuti University of Paraná, Curitiba PR, Brazil*

[3] *Graduate Program in Electrical and Computer Engineering, Federal Technological University of Paraná Curitiba, PR, Brazil*

[4] *Clinical Analysis Department, Federal University of Paraná, Curitiba, PR, Brazil*

[5] *Basic Pathology Department, Federal University of Paraná, Curitiba, PR, Brazil*

[6] *Biochemistry Department, Federal University of Paraná, Curitiba, PR, Brazil*

[7] *Pequeno Principe College, Pelé Pequeno Principe Research Institute, Curitiba, PR, Brazil*

Accepted September 22, 2020
Published online November 10, 2020

Pain is a common and distressing symptom of many diseases and its clinical treatment generally involves analgesics and anti-inflammatory drugs. This study evaluated the toxicity of *Ilex paraguariensis* A. St.-Hil. (Aquifoliaceae) aqueous extract (leaves, petioles and branches) and its performance in a nociceptive response. Hepatotoxicity, psychostimulant test and evaluation of enzyme markers for liver damage were also tested. Chromatographic analysis by UPLC-MS demonstrated a series of isomeric monocaffeoylquinic acids, isomers of dicaffeoylquinic acid, flavonol glycosides, and saponins. Phase I and II of nociception were obtained for meloxicam, dexamethasone and aqueous *Ilex paraguariensis* extract. *Ilex paraguariensis* extract concentration was negatively correlated ($R = -0.887$) with alanine aminotransferase ($p < 0.05$) in acetaminophen-induced hepatotoxicity test, indicating hepatoprotective activity of this extract. *Ilex paraguariensis* extract also presented analgesic properties equivalent to drugs that already have proven efficacy. Notably, the administration of multiple doses of *Ilex paraguariensis* extract was considered safe from the therapeutic point of view.

Keywords: nociception, pain, hepatoprotection, toxicity, *Ilex paraguariensis,* aqueous extract, UPLC-MS

Pain is a debilitating and highly prevalent clinical condition, which directly interferes with the person's quality of life and the global economy (1). Pain can manifest from a variety of sources or conditions and is most frequently classified as acute or chronic in nature, with chronic pain representing a condition that has persisted beyond normal healing time. Chronic pain affects one-third to fifty percent of the world population and its management remains a major clinical challenge because the mechanisms that cause pain are poorly understood and there is a lack of effective treatments (1, 2).

* Correspondence; e-mail address: weslysouza@ufpr.br

The clinical treatment of pain is a primary concern of health professionals and is based on the use of analgesics. The relief caused by these drugs may occur through blocking painful stimuli before they reach the brain or by interfering with the way the brain interprets these stimuli. However, according to information from the Annual National Health Service (NHS), painkillers are one of the most dangerous drug groups legally used in the world and can have serious side-effects including death (3, 4).

The aminotransferases are intracellular enzymes and their elevated plasma levels indicate lesions in cells rich in enzymes. Aspartate aminotransferase (AST) is found in different organs and tissues, including the liver, heart, skeletal muscle, and erythrocytes. Alanine aminotransferase (ALT) is found predominantly in the liver, but with moderate amounts in the kidney and small amounts in the heart and skeletal muscle (5). In general, elevated aminotransferases are due to liver disease, although they can reflect damage in other organs as well.

Currently, orofacial pain models have been developed in animals, looking for a representation of inflammatory or neuropathic orofacial pain. The most densely innervated area of the body innervated by the trigeminal nerve is the orofacial region, where the most common acute pains are described. The assessment of both, behavioral and neurophysiological, aspects of orofacial pain is performed by the orofacial formalin test, being a widely accepted tonic pain model (6).

The use of medicinal plants for pain relief is becoming increasingly popular because of their relatively few side-effects and has been supported by professional and official programs worldwide. New natural drugs, capable of reducing pain and chronic inflammation safely and effectively, have been steadily sought by the scientific community. Aa an example dried leaves and twigs from *Ilex paraguariensis* A.St.-Hil., Aquifoliaceae (yerba mate), used to prepare a local tea known as "mate", a widely consumed beverage in several South American countries.

Studies indicate that yerba mate has several chemical compounds with therapeutic and pharmacological properties. Caffeine and theobromine are alkaloids that can affect the central nervous system (6). *Ilex paraguariensis* also has saponins, which gives it an astringent flavor with hemolytic properties while the presence of polyphenolic compounds is responsible for antioxidant capacity (6, 7). However, despite *I. paraguariensis* being widely studied from a chemical and pharmacological point of view, there are few and inconclusive studies dealing with the analgesic effect specifically.

The purpose of this study was, therefore, to access the *in vivo* effect of *I. paraguariensis* (leaves, petioles and branches) aqueous extract in nociceptive response and compare it with the effects presented by conventional drugs, evaluating both, the hepatoprotective and analgesic effects of yerba mate.

EXPERIMENTAL

Chemicals

Dexamethasone disodium phosphate and meloxicam were purchased from Aché Pharmaceutical Laboratories (Brazil), imipramine hydrochloride from Teuto (Brazil), acetaminophen from Janssen Cilag (Belgium), acetylsalicylic acid (ASA), sodium chloride

0.9 %, formic acid from Sigma-Aldrich (USA), formalin and acetic acid from Vetec (Brazil), saccharose from Biotec (Brazil), and β-carotene 10 % from Formédica (Brazil). HPLC-grade solvents, acetic acid and triethylamine were acquired from Tedia (Brazil) and acetonitrile from J. T. Baker (USA).

Standards of chlorogenic acid (purity ≥ 95 %), theobromine (≥ 98 %), caffeine (99 %) and rutin (≥ 94 %) were purchased from Sigma-Aldrich (USA).

Water was Milli-Q from Millipore (USA). Other solvents were purchased from Merck (USA).

Plant material, preparation of extract and chemical analysis

Leaves, petioles and branches of *Ilex paraguariensis* A. St. Hil., Aquifoliaceae (3 kg) were crushed according to the standard procedure were donated by the food industry Leão Junior® (Brazil) in February 2007. A voucher specimen was deposited in the Prof. Carlos Stellfeld herbarium (Pharmacognosy Laboratory of the Federal University of Paraná, Curitiba, PR, Brazil).

For the production of the crude extract, 32 g of the ground yerba mate were placed in a Soxhlet extractor and refluxed with 130 mL of distilled water (6 h). Then the extract was concentrated in a rotary evaporator, frozen and freeze-dried with a yield of 31.0 % (*m*/*V*) of an aqueous crude extract of *Ilex paraguariensis* (CEIP). In all experiments, CEIP was diluted to the targeted concentrations in sterile 0.9 % NaCl just before the start of the tests. The doses were chosen according to the initial results of the acute toxicity test.

UPLC (ultra-performance liquid chromatography) was performed on Acquity-UPLC™ (Waters, USA) instrument, composed of a binary solvent manager, column oven and a photodiode array (PDA) and HR-MS detection systems. The analysis was carried out on the HSS T3 C18 column, 100 × 2.1 mm with a particle size of 1.7 μm, Waters (USA), and mobile phase composed of ultra-pure water and acetonitrile, both containing 0.1 % formic acid (*V*/*V*). The separation was developed with an acetonitrile gradient starting from 0 to 14 % in 5 min, then to 60 % in 13 min, returning to the initial condition in 14 min with 3 additional minutes for the system to re-equilibrate (adapting from refs. 8–10). The sample was prepared in MeOH/H_2O (1:1, *V*/*V*) and 5 μL was injected. Detection was provided by the high-resolution mass spectrometry (HR-MS) employing an LTQ-OrbiTrap XL, Thermo Scientific (USA), operating at atmospheric pressure ionization (API), with ion source at 350 °C and nitrogen for sample desolvation (sheath gas at 344.74 kPa and auxiliary gas at 68.95 kPa) and helium 6.0 (purity 99.9999 %) in the linear trap. For negative ionization, the energy of spray voltage was set at 3.5 kV with –10 V on the capillary and –125 V on the tube lens or 4 kV, 30 V and 100 V, resp., in positive ionization. Tandem-MS experiments were obtained by higher-energy collision dissociation-mass spectrometry (HCD-MS), using helium as collision gas and energy of 55 keV (normalized collision energy). For mass accuracy, external calibration was performed before sample analysis and resolution was set at and 15.000 full widths at half maximum (FWHM) in LC-MS mode.

Animals

Male Swiss albino mice (25–40 g) were used in the acute toxicity experiments, such as hepatotoxicity evaluation, orofacial pain induced by formalin test, open-field test and

acetic acid-induced writhing. For the measurement of hepatic markers, male Wistar rats (150–220 g) were selected. All animals were obtained from Immunobiological Research and Production Center in Piraquara (PR), Brazil, and kept for two weeks at 21 ± 2 °C, with light-dark cycles of 12 h each and free access to food and water. The experiments were performed with the approval of the animal ethical committee from Tuiuti University of Paraná, Curitiba, Brazil.

Acute toxicity method

CEIP was administered to mice both orally and intraperitoneally in doses of 250, 500, 1000, 2000, and 3000 mg kg^{-1} in 12 groups (5 male Swiss albino mice per group). 0.9 % NaCl was administered to the control groups. The general behavior of mice was observed continuously for 1 h after treatment and periodically for a period of 24 h with particular attention during the first 4 h. The mice were observed for 14 days for signs of toxicity, death, and death latency (11).

Acetaminophen-induced hepatotoxicity

The hepatoprotective activity of CEIP was determined as described by Manda and Bhatia (12). Mice were divided into seven groups (5 male Swiss albino mice per group) and treated for six consecutive days orally. The first group (control) received 40 % saccharose solution (*p.o.*), the second group distilled water (*p.o.*), the third group β-carotene (30 mg kg^{-1}, *p.o.*) and the 4^{th} to 7^{th} groups of animals were treated orally with CEIP (250–2000 mg kg^{-1}). On the 7^{th} day, all groups, except the control, received a single dose of acetaminophen (ACE, 400 mg kg^{-1}, *i.p.*). After 24 h of ACE administration, the animals were sedated with isoflurane, euthanized by cervical dislocation, and then the blood was collected in tubes containing 18 mg per 100 μL of EDTA. The samples were centrifuged (1500×*g* for 15 min) and the supernatants (plasma) were collected to be used for alanine aminotransferase (ALT) determination according to specifications from the UV-Labtest® Kit (Brazil) on a Thermo-Plate TP-Analyzer Plus Biochemical Analyzer® (Termoplate, China).

Evaluation of hepatic markers

In the evaluation of hepatic markers, animals were divided into five groups (5 male Wistar rats per group). The control group was treated with 0.9 % NaCl, whereas CEIP-treated groups received doses of 125, 250, 500 and 1000 mg kg^{-1} CEIP. The treatments lasted for 30 consecutive days. After this period, the animals were sedated with isoflurane, euthanized by cervical dislocation, and then the blood was collected in tubes. The tubes were centrifuged (1500×*g* for 15 min) and the serum kept refrigerated until it was analyzed using the Thermo-Plate TP-Analyzer Plus® equipment. The determination of biochemical parameters (AST and ALT) was performed following respective kit instructions (Labtest, Brazil).

Orofacial pain induced by formalin test

The orofacial pain test consisted of the treatment of seven groups of mice (5 male Swiss albino mice per group) with 2.5 % formalin (20 μL) in the upper lip of mice. The volume and the percentage of formalin were selected from our pilot studies which showed

a pain-related biphasic behavioral response (face-rubbing) of greater intensity at periods of 0–5 min (first phase) and 15–30 min (second phase). The nociceptive intensity was determined for each period by counting (in seconds) the time that the animal rubbed the injected area with the hind and/or front paws, indicative of pain (12). To evaluate the analgesic effect, seven groups of mice were treated with saline, 0.1 mL per 10 g (*i.p.*), dexamethasone, 0.5 mg kg^{-1} (*s.c.*), meloxicam, 5 mg kg^{-1} (*i.p.*) (10), and with concentrations of 250, 500, 1000 and 2000 mg kg^{-1} (*p.o.)* CEIP, 60 min prior to formalin.

Acetic acid-induced writhing test

The acetic acid-induced writhing test was performed as previously described by Koster *et al*. (13). Animals were divided into 15 groups (5 male Swiss albino mice per group). Muscular contractions were induced by intraperitoneal injection (*i.p.*) of a 0.6 % solution of acetic acid (10 mL kg^{-1}). The muscular contractions were then counted for 20 min after the injection and the percent inhibition of the number of writhes was calculated. The CEIP was administered in doses of 250, 500, 1000 and 2000 mg kg^{-1} *via* oral and intraperitoneal routes, while doses of 250, 500 and 1000 mg kg^{-1} were also administered *via* the subcutaneous route. Each control group received only 0.9 % NaCl (10 mL kg^{-1}) obeying the respective administration route and the reference drug (ASA, 400 mg kg^{-1}) was administered *via s.c.* route. The mice from all of the groups received the respective dose 60 min before the application of 0.6 % acetic acid.

Open-field test

To assess the possible effects of CEIP on motor activity, mice were tested in the open-field test, according to the method described by Holland and Weldon (14). Seven groups of mice (5 male Swiss albino mice per group) were individually placed in an acrylic box (50 × 40 × 20 cm) with the floor divided into 9 equal squares (10 cm^2). The number of squares crossed with the four paws was registered during a period of 2 min. Animals were treated with saline (10 mL kg^{-1}), imipramine (20 mg kg^{-1}), caffeine (30 mg kg^{-1}) and with CEIP (125, 250, 500, and 1000 mg kg^{-1}) orally. The mice were treated for 14 days and on the 15th day they were submitted to the open field-test (once a day, last dose 1 h before tests) (15).

Statistical analysis

Statistical analyses were performed using the JMP statistical packages (version 8.0, SAS Institute Inc., Cary, N.C., USA) and GraphPad (version Prism 4.1, USA). Results were expressed as mean ± standard error of the mean (SEM), and an ANOVA was performed followed by Dunnett's test. Statistical significance was considered to be $p < 0.05$. Pearson correlations were also performed using Python.

RESULTS AND DISCUSSION

Identification of compounds

The UPLC-MS analysis of the aqueous crude extract of *Ilex paraguariensis* (CEIP) revealed the presence of isomers of chlorogenic and dicaffeoylquinic acids, flavonol glycosides and several saponins (Fig. 1).

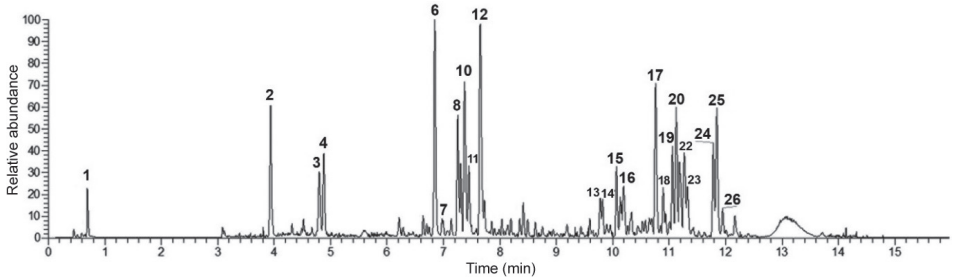


Fig. 1. UPLC-PDA fingerprint of aqueous crude extract of *Ilex paraguariensis* (CEIP). For peak codes see Table I.

Compound **1**, with a deprotonated ion at *m*/*z* 191.056, was consistent with quinic acid. The series of isomeric monocaffeoylquinic acids were observed in chromatograms, producing ions at *m*/*z* 353.088 $[M-H]^-$ with main fragments at *m*/*z* 135.045, 173.046, 179.035, and 191.056 appearing at different ratios, characteristic of each individual isomer, allowing their identification as *neo*-chlorogenic acid (compound **2**), chlorogenic acid (compound **3**), and *crypto*-chlorogenic acid (compound **4**). Caffeine (compound **5**), was found as a protonated ion at *m*/*z* 195.087, and confirmed with authentic standard. Similarly, isomers of dicaffeoylquinic acids were found in chromatogram with ions at *m*/*z* 515.119 $[M-H]^-$, producing fragments at *m*/*z* 135.045, 173.046, 179.035, 191.056, and 353.088, being identified as 3,4-dicaffeoylquinic acid (compound **8**), 3,5-dicaffeoylquinic acid (compound **10**) and 4,5-dicaffeoylquinic acid (compound **12**) (9).

Flavonol glycosides were also present in the preparation, appearing at *m*/*z* 609.146 $[M-H]^-$ and 611.160 $[M+H]^+$. Fragments were better observed from protonated ion, at *m*/*z* 465.103 and 303.050 consistent with rutin (compound **6**). Compound **7**, at *m*/*z* 465.103 with a fragment at *m*/*z* 303.050 was consistent with a quercetin-hexoside. Kaempferol-rutinoside (compound **9**) was also observed at *m*/*z* 593.151 $[M-H]^-$ and 595.166 $[M+H]^+$, with fragments from protonated ion at *m*/*z* 449.108 and 287.055 as previous observation (9).

As previously observed (8), many isomeric saponins were found in the present extract. In the negative ionization, they appeared adducted with Cl^- and $HCOO^-$, but only low fragments were produced. Regardless of the low abundance of the fragments, they were enough to compare with the previous studies (8, 10), allowing a partial identification. In contrast to the abundance of isomeric saponins, many peaks were observed with similar glycosylation pattern, which might be explained by the presence of isomeric aglycones. Two main triterpenes are found in saponins of *I. paraguariensis*, such as ursolic acid and oleanolic acid, with a minor proportion of hydroxylated triterpenes. To the aglycone, on the hydroxyl position C3, matesaponins are attached by an α-L-arabinopyranose, whereas on the carboxyl position C28, these saponins are attached by a β-D-glucopyranosyl unit (Fig. 2). These carbohydrate branches can be elongated by other monosaccharides, such as rhamnopyranose, glucopyranose and, at minor amounts, xylopyranose, appearing on the position C3, but only glucopyranosyl units were found elongating the oligosaccharide chain on the position C28 (8). In the present study, we found the oligosaccharide chain being elongated on the C3 position with glucopyranosyl (R^1) and/or rhamnopyranosyl (R^2) units. The oligosaccharide on the C28 position, can be formed with another unit of glucopyranosyl (R^3) (Fig. 2).

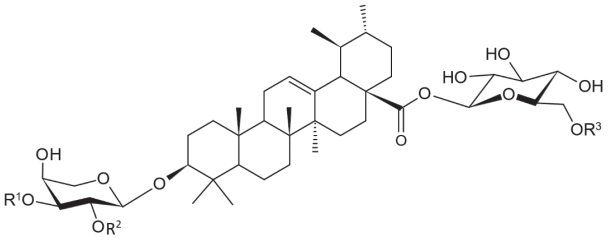


Fig. 2. The general structure of matesaponins, depicted here with an ursolic acid attached by an **α**-L-arabinopyranosyl unit on the hydroxyl at C3, and a **β**-D-glucopyranosyl, attached to the carboxyl C28.

Two isomers (compounds **13** and **14**) appeared at *m*/*z* 1119.559, and these compounds were differentiated based on their fragments, since different MS spectra profiles were observed. Compound **13** had fragments at *m*/*z* 957.506 and 811.442, consistent with losses of glucose and rhamnose units, and being consistent with matesaponin with a hydroxylated aglycone. However, compound **14**, with two consecutive losses of glucose units (*m*/*z* 957.506 and 795.453), exhibited a different oligosaccharide chain. A less abundant fragment fount at *m*/*z* 633.401 indicated the presence of non-hydroxylated aglycone, linked to the arabinopyranosyl unit.

The compound **16**, at *m*/*z* 1265.618, gave fragments at *m*/*z* 941.511 and 795.453. The first bond breakdown occurs in the ester linkage on the position C28, leading to the loss of two glucosyl units, which was accompanied by the loss of rhamnosyl unit, indicating that this compound has a general structure composed of Glc-(Rha)-Ara-Agly-$(Glc)_2$ as in previous findings (8, 10).

Compounds **17**, **19** and **20** appeared at *m*/*z* 1103.564. Compound **17** could be distinguished from **19** and **20** based on the fragmentation-MS profile, which was observed at *m*/*z* 779.459 and 633.401. Like **16**, the simultaneous loss of 2 glucosyl units [neutral loss (NL) of 324.105 atomic mass units (a.m.u.)] was consistent with the loss of a disaccharide from the position C28. However, compounds **19** and **20** had similar fragments, observed at *m*/*z* 941.511 and 795.453, consistent with sequential losses of a glucosyl unit from C28, followed by the loss of the rhamnosyl residue. Thus, the difference between compounds **19** and **20** must be in the aglycone moieties, *i.e.*, the presence of ursolic and oleanolic acid, that could not be differentiated in MS spectra.

Similarly, the other 3 isomers appeared at *m*/*z* 957.507 (compounds **21**, **22** and **23**). These were also examined based on their fragmentation profile, indicating that the compound **21** exhibited the same loss pattern of NL 324.102 a.m.u., as in other matesaponins containing a di-glucosyl attached on C28. Like compounds **19** and **20**, compounds **22** and **23** had the same fragmentation profile, indicating a difference in the aglycone composition.

Two other isomers (compounds **24** and **25**) appeared at *m*/*z* 941.511, with the same fragments, at *m*/*z* 779.458 and 633.401. These matesaponins are composed of rhamnose and arabinose on position C3, and a unit of glucose on position C28. Differences are also attributed to the aglycone. The last saponin examined (compound **26**) appeared at *m*/*z* 999.517 with the main fragment at *m*/*z* 837.464. Different from others, compound **26** is composed of a glucose unit at C28 and a disaccharide Glc-Ara on C3, however, an additional acetyl group was found. This type of modification was previously described by Souza *et al*. (8)

Table I. Phytochemical composition of aqueous crude extract of Ilex paraguariensis (CEIP) obtained by UPLC-MS

Peak	t_R (min)	MS[1]	Diagnostic fragments	Identification	Ref.
1	0.69	191.056 $[M-H]^-$	–	Quinic acid	16
2	3.94	353.088 $[M-H]^-$	191.056, 179.035, 135.045	neo-chlorogenic acid	17
3	4.80	353.088 $[M-H]^-$	191.056	Chlorogenic acid	9
4	4.88	353.088 $[M-H]^-$	191.056, 179.035, 173.046, 135.045	crypto-chlorogenic acid	17
5	5.08	195.087 $[M+H]^+$	–	Caffeine[a]	9
6	6.84	611.160 $[M+H]^+$	465.103, 303.050	Rutin	9
7	6.99	465.103 $[M+H]^+$	303.050	Quercetin-hexoside	18
8	7.25	515.119 $[M-H]^-$	353.088, 191.056, 179.035, 173.046, 135.045	3,4-Dicaffeoylquinic acid	17
9	7.30	595.166 $[M+H]^+$	449.108, 287.055	Kaempferol-rutinoside	17
10	7.37	515.119 $[M-H]^-$	53.088, 191.056, 179.035	3,5-Dicaffeoylquinic acid	17
11	7.45	451.329 $[M-H]^-$ 487.306 $[M+Cl]^-$	–	Unknown	–
12	7.65	515.119 $[M-H]^-$	353.088, 191.056, 179.035, 173.046	4,5-Dicaffeoylquinic acid	17
13	9.78	1119.559 $[M+HCOO]^-$	957.506, 811.442	Glc-(Rha)-Ara-(OH)Agly-Glc	8
14	9.82	1119.559 $[M+HCOO]^-$	957.506. 795.453, 633.401	$(Glc)_2$-Ara-Agly-Glc	8
15	10.06	809.433	–	Unknown	–
16	10.19	1265.618 $[M+HCOO]^-$	941.511, 795.453	Glc-(Rha)-Ara-Agly-$(Glc)_2$	8
17	10.76	1103.564 $[M+HCOO]^-$	779.459, 633.401	Rha-Ara-Agly-$(Glc)_2$	8
18	10.89	777.407	–	Unknown	–
19	11.06	1103.564 $[M+HCOO]^-$	941.511, 795.453	Glc-(Rha)-Ara-Agly-Glc	8
20	11.12	1103.565 $[M+HCOO]^-$	941.511, 795.453	Glc-(Rha)-Ara-Agly-Glc	8
21	11.21	957.507 $[M+HCOO]^-$	633.401	Ara-Agly-$(Glc)_2$	8
22	11.27	957.507 $[M+HCOO]^-$	795.453	Glc-Ara-Agly-Glc	8
23	11.32	957.506 $[M+HCOO]^-$	795.453	Glc-Ara-Agly-Glc	8
24	11.78	941.511 $[M+HCOO]^-$	779.458, 633.401	Rha-Ara-Agly-Glc	8
25	11.84	941.511 $[M+HCOO]^-$	779.458, 633.401	Rha-Ara-Agly-Glc	8
26	11.94	999.517 $[M+HCOO]^-$	837.464	Glc-(Ac)Ara-Agly-Glc	8

Agly – aglycone (ursolic or oleanolic acids), Ara – arabinose, Glc – glucose, Rha – rhamnose

[a] Caffeine was detected in the positive mode.

and the acetyl group was found attached to the arabinosyl moiety. A description of the matesaponins found in the current work is summarized in Table I.

Evaluation of acute toxicity

Animals treated with 500, 1000, 2000, and 3000 mg kg^{-1} CEIP showed increased respiratory rate, myosis, piloerection and motor agitation without loss of leg seizures during the first 4 h. These effects were more pronounced in larger doses tested (2000 and 3000 mg kg^{-1}), administered intraperitoneally as well as orally. Our results suggest that CEIP generates signs of excitability in animals, making them more active.

We observed that the CEIP administered orally or intraperitoneally caused no death during the observation period suggesting that the extract might be considered safe (19, 20). However, adverse symptoms were observed with doses of 500 mg kg^{-1} and higher, partially corroborating with Andrade *et al*. (19), who used a single dose of the extract (2 g kg^{-1}) in rats. Pasquali *et al*. (21) demonstrated that there was no mortality or significant changes in overall behavior or other physiological activity in rats treated with a single dose of *Ilex paraguariensis*.

Acetaminophen-induced hepatotoxicity

For the evaluation of acetaminophen-induced hepatotoxicity, levels of alanine aminotransferase (ALT) were evaluated as shown in Table II.

In the control group, treated with 40 % saccharose, the enzyme activity was 45.80 ± 4.33 IU L^{-1}. The use of acetaminophen (ACE) greatly increased the ALT activity compared to the control (156.95 ± 7.57 IU L^{-1}). For the group treated previously with β-carotene, the enzymatic activity of ALT was reduced (68.79 %) compared to the values provided by ACE. The groups treated with CEIP 250, 500, 100 and 2000 mg kg^{-1} showed a reduced ALT activity for 77.7, 76.4, 83.3 and 84.9 %, resp.

*Table II. ALT activity in acetaminophen-induced hepatotoxicity in mice treated with CEIP (*p.o.*)*

Treatment	ALT (IU L^{-1})
Saccharose 40 % (control)	45.80 ± 4.33
ACE 400 mg kg^{-1}	156.95 ± 7.57*
β-carotene 30 mg kg^{-1} + ACE 400 mg kg^{-1}	48.97 ± 6.23
CEIP 250 mg kg^{-1} + ACE 400 mg kg^{-1}	34.98 ± 3.24
CEIP 500 mg kg^{-1} + ACE 400 mg kg^{-1}	37.00 ± 9.14
CEIP 1000 mg kg^{-1} + ACE 400 mg kg^{-1}	26.23 ± 5.52
CEIP 2000 mg kg^{-1} + ACE 400 mg kg^{-1}	23.74 ± 1.63

ACE – acetaminophen, CEIP - aqueous crude extract of *Ilex paraguariensis*

Mean ± standard error of the mean (n = 5).

Significant difference compared to control: *$p < 0.01$.

Alanine aminotransferase (ALT) activity values are in accordance with Manda and Bhatia (12), who observed an ALT value of 123.77 ± 29.61 IU L^{-1} in ACE treated group, indicating liver damage. The CEIP (250, 500, 1000, and 2000 mg kg^{-1}) reduced the ALT activity with values lower than those of the control group (β-carotene), demonstrating even hepatoprotective effect (22). ALT is an excellent liver damage marker, however, there is no reliable correlation between ALT levels and the degree of the injury (23).

A Pearson correlation between *Ilex paraguariensis* and ALT resulted in $R = -0.887$ and $p = 0.045$, which indicates a strong negative and significant correlation between CEIP concentration and ALT, indicating an increased hepatoprotection with higher concentrations of CEIP.

Evaluation of hepatic markers

CEIP administered orally (125, 250, 500, and 1000 mg kg^{-1}) did not change liver damage biomarkers, such as aspartate aminotransferase (AST) compared with the saline control group (227.2 ± 14.64 IU L^{-1}) and ALT (91.2 ± 4.45 IU L^{-1}), significantly, as shown in Table III.

Orofacial pain induced by formalin test

In the first phase of nociception (0–5 min), the control group had a rubbing time of 71.50 ± 14.41 s. Pre-treatment with CEIP orally (250, 500, 1000, and 2000 mg kg^{-1}) 60 min before formalin labial administration had significantly reduced rubbing time of the lip by 55.0 % (32.16 ± 7.00 s), 56.6 % (31.00 ± 8.72 s), 51.0 % (35.00 ± 7.94 s) and 55.9 % (31.50 ± 4.47 s), resp., compared to the control ($p < 0.05$). A significant reduction in nociception was also observed after treatment with meloxicam (56.0 %) as shown in Fig. 3a.

The second test phase (15–30 min) showed a rubbing time of 147.66 ± 7.61 s for the control group. Pre-treatment with CEIP orally with the same doses as above, 60 min prior to formalin, reduced the lip rubbing time by 50.3 % (73.33 ± 28.38 s), 15.7 % (124.50 ± 38.04 s), 65.3 % (51.20 ± 14.68 s) and 61.3 % (57.20 ± 11.76 s), resp., but significant reductions were observed at higher doses ($p < 0.05$). However, pre-treatments with dexamethasone and meloxicam significantly reduced rubbing time ($p < 0.05$) by 85.0 % (22.20 ± 4.70 s) and 62.7 % (55.10 ± 10.64 s), resp., as shown in Fig. 3b.

*Table III. Chronic treatment with CEIP (*p.o.*) through AST and ALT in rats*

Treatment	AST (IU L^{-1})	ALT (IU L^{-1})
Saline (control)	227.2 ± 14.6	91.2 ± 4.5
CEIP 125 mg kg^{-1}	189.4 ± 13.6	91.7 ± 9.0
CEIP 250 mg kg^{-1}	194.8 ± 9.0	104.2 ± 12.6
CEIP 500 mg kg^{-1}	198.4 ± 17.1	108.4 ± 11.2
CEIP 1000 mg kg^{-1}	225.5 ± 14.1	106.4 ± 3.6

CEIP – aqueous crude extract of *Ilex paraguariensis*

Mean ± standard error of the mean ($n = 5$).

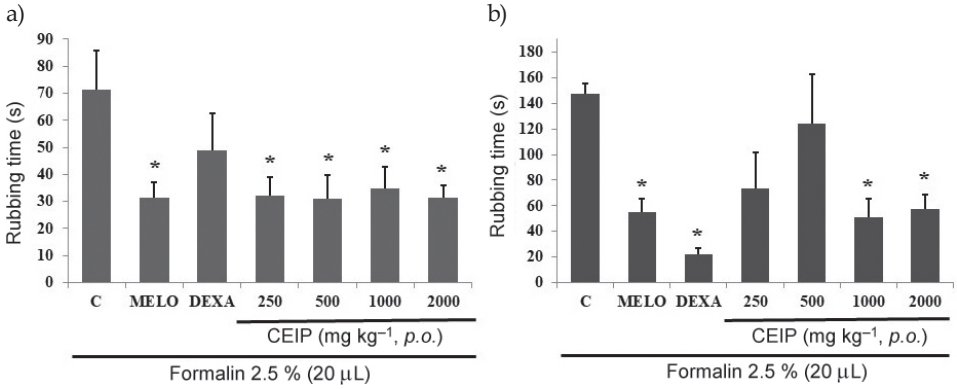


Fig. 3. Evaluation of the analgesic effect of CEIP in orofacial pain in: a) phase I and b) phase II. C – control: 2.5 % formalin + saline *i.p.*, MELO (5 mg kg^{-1}, *i.p.*), DEXA (0.5 mg kg^{-1}, *s.c.*), or orally with different doses of CEIP (250–2000 mg kg^{-1}). Mean ± standard error of the mean (n = 5). Statistically significant difference *vs.* control: *$p < 0.05$.

CEIP – aqueous crude extract of Ilex paraguariensis, DEXA – dexamethasone, MELO – meloxicam

It was demonstrated that the administration of meloxicam or dexamethasone had induced antinociceptive activity with different potentials in phases I and II of the formalin--induced orofacial test which is in agreement with other algesiometric studies (24). Meloxicam inhibits cyclooxygenase, an enzyme essential for prostaglandin production (25), which is consistent with the results found in this work where selective inhibition of COX_2 (26) was observed, suggesting prostaglandins participate already in the first stage of the pain induced by formalin.

Dexamethasone, an effective anti-inflammatory drug, inhibits the induction of COX_2 and cytokines, which are important in the inflammatory response, however, it has some side-effects (27, 28). The inhibitory effect of dexamethasone on COX_2 could indicate that prostaglandins induced by COX_2 are involved in nociception in the second phase of pain, corroborating previous studies (29). Our results agree with other models that demonstrate the dexamethasone anti-nociceptive effect at higher doses (26, 30).

The main findings reported in this work indicate that treatment with *Ilex paraguariensis* extract was able to relieve the orofacial pain induced by formalin in mice, being in agreement with studies suggesting that natural products have antinociceptive effects in experimental models (6, 26). The CEIP proved to have an antinociceptive effect that may be related to the presence of the flavonoids and phenolic acids (31, 32).

The first phase of pain is characterized by direct neural pain, the formalin acting as an aggressive nociceptive agent, which can only be withdrawn with opioid analgesics as shown in numerous studies (30). The second phase is characterized by an inflammatory process, where substances are normally released by cells, such as prostaglandins, cytokines, and bradykinins, among others (33). The CEIP prior administration demonstrated an analgesic effect in the first phase of formalin-induced orofacial pain, possibly due to the release of opioid substances or activation of opioid receptors directly. This is because the first test phase is characterized by the direct effect of formalin on nociceptors and may be inhibited by centrally acting analgesics (34). However, this effect did not remain in the second stage.

Acetic acid-induced writhing test

The administration of acetic acid by *i.p.* injection to the negative control group (saline) pre-treated orally, intraperitoneally or subcutaneously by CEIP, induced a number of writhings of 47.0 ± 6.0, 37.9 ± 3.3, and 53.7 ± 3.1, resp., counted during the observation period (20 min). The group of animals that received acetylsalicylic acid as a standard antinociceptive drug presented 97.5 % (*s.c.*) writhing inhibition compared with the respective control group (saline). On the other hand, CEIP (250, 500, 1000, and 2000 mg kg^{-1}, *i.p.*) significantly reduced the number of writhing movements induced by the 0.6 % acetic acid solution in a dose-dependent manner by 59.4, 72.9, 94.4 and 99.7 %, compared with the control ($p < 0.01$). Oral administration of CEIP (250, 500, 1000, and 2000 mg kg^{-1}) showed a non-dose dependent antinociceptive activity on the acetic acid-induced writhing test. The lowest dose (250 mg kg^{-1}) used elicited 15.7 % inhibition of writhing whereas the maximum antinociceptive effect of CEIP was observed at 500 mg kg^{-1}, which resulted in 42.6 % inhibition, and this effect was not altered by higher doses of CEIP. Subcutaneous treatment with 500 and 1000 mg kg^{-1} caused a significant and apparent dose-dependent inhibition of the acetic acid-induced writhes ($p < 0.05$). In addition, the inhibition (46.6 %) produced by CEIP at 1000 mg kg^{-1} (*s.c.*) was significantly less than that observed for acetylsalicylic acid (400 mg kg^{-1}, *s.c.*) and by *i.p.* administration of the same dose of CEIP (Table IV).

Table IV. Writhings induced by acetic acid in mice treated with CEIP or ASA

Treatment	Number of writhings (in 20 min)	Inhibition (%)
Saline *p.o.*	47.0 ± 6.0	–
CEIP 250 mg kg^{-1} *p.o.*	39.6 ± 3.8	15.7 ± 13.4
CEIP 500 mg kg^{-1} *p.o.*	27.0 ± 3.2*	42.6 ± 10.0
CEIP 1000 mg kg^{-1} *p.o.*	29.4 ± 3.3	37.4 ± 10.6
CEIP 2000 mg kg^{-1} *p.o.*	27.0 ± 3.7*	42.6 ± 10.7
Saline *i.p.*	37.9 ± 3.3	–
CEIP 250 mg kg^{-1} *i.p.*	15.4 ± 2.5**	59.4 ± 7.5
CEIP 500 mg kg^{-1} *i.p.*	10.2 ± 2.6**	72.9 ± 7.4
CEIP 1000 mg kg^{-1} *i.p.*	2.1 ± 1.6**	94.4 ± 4.2
CEIP 2000 mg kg^{-1} *i.p.*	0.1 ± 0.1**	99.7 ± 0.3
Saline *s.c.*	53.7 ± 3.1	–
ASA *s.c.*	1.3 ± 0.4**	97.5 ± 0.8
CEIP 250 mg kg^{-1} *s.c.*	36.3 ± 3.4	32.4 ± 7.4
CEIP 500 mg kg^{-1} *s.c.*	30.3 ± 10.0*	43.5 ± 19.0
CEIP 1000 mg kg^{-1} *s.c.*	28.7 ± 2.1*	46.6 ± 5.0

ASA – acetylsalicylic acid, CEIP – aqueous crude extract of *Ilex paraguariensis*

Mean ± standard error of the mean ($n = 5$).

Significant difference compared to the respective control: *$p < 0.05$, **$p < 0.01$.

The results indicate that CEIP has a significant antinociceptive effect in the chemical nociception model suggesting that the extract action is peripherally mediated, which can be obtained in all three routes of administration (*p.o.*, *i.v.* and *s.c.*) corroborating with Carvalho *et al*. (35) who used only the oral route.

Open field test

The groups treated orally with caffeine and CEIP (250, 500, and 1000 mg kg^{-1}) showed an increase in the spontaneous movement of the mice of 69.9, 63.8, 48.7 and 83.1 %, resp., when compared to the control group treated with saline ($p < 0.05$) as shown in Fig. 4.

In the psychostimulant test, CEIP demonstrated an increase in spontaneous movement probably due to the presence of alkaloids (36, 37). Caffeine acts in the central nervous system, produces alertness and mental activity, facilitates the motor capacity (38), which probably gives the mate the stimulating properties on the motor activity of the animals. It is believed that many of the caffeine effects occur through competitive antagonism at adenosine receptors. Carvalho *et al*. (35) suggest that the effects of *I. paraguariensis* are partially due to the noradrenergic pathway modulation by methylxanthines (*e.g.*, caffeine).

Furthermore, some of the pharmacological properties attributed to *Ilex paraguariensis* have been related to the high content of phenolic acids such as chlorogenic acids, 4,5-dicaffeoylquinic acid, and many others (39). Studies suggest that the administration of *Ilex paraguariensis* contributes to the body's defense against free radicals (40) and inhibits the oxidation of LDL cholesterol (39).

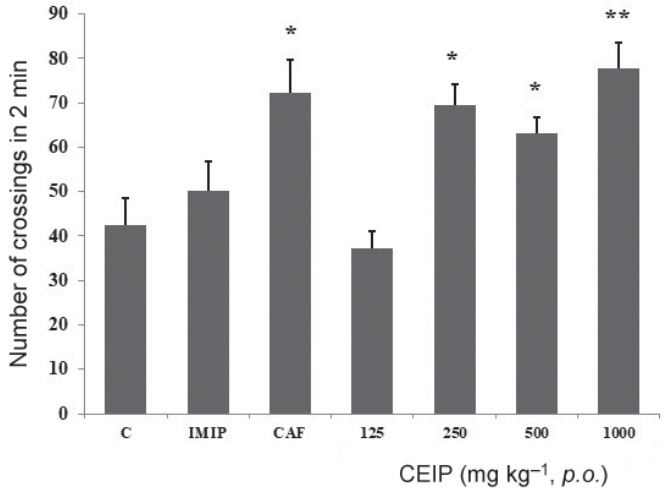


Fig. 4. Effect of aqueous crude extract of *Ilex paraguariensis* (CEIP) on spontaneous locomotion of mice. Mean ± standard error of the mean ($n = 5$). Statistically significant difference *vs*. control: *$p < 0.05$ and **$p < 0.01$.

C – control, saline (10 mL kg^{-1}), CEIP – aqueous crude extract of *Ilex paraguariensis*, IM – imipramine (20 mg kg^{-1}), CAF – caffeine (30 mg kg^{-1})

CONCLUSIONS

The administration of the aqueous crude extract of *Ilex paraguariensis* (CEIP) in repeated doses was considered therapeutically safe and results suggest both hepatoprotective and analgesic activities. UPLC-MS analysis revealed the presence of cyclic polyols, carbohydrates, polyphenolic compounds, methylxanthine alkaloids, flavonoids, and saponins. The analgesic mechanisms that CEIP produces, both in the first and the second stage, have certain limitations because the obtained efficiency in the first stage, at all the doses, was lost in the second phase, remaining only at higher doses tested. However, we hypothesize that the CEIP's dual role, in this case, could be due to: (*i*) an immediate stimulation of the opioid system, or (*ii*) acting similarly to carbamazepine which stabilizes the overexcited nerve membrane, inhibiting repetitive neuronal firing and reducing the propagation of synaptic excitatory impulses. The permanence in the second phase probably occurs through inhibition of inflammatory substances released at this late stage of pain since the *Ilex paraguariensis* demonstrated an antiedematogenic effect in the previous works.

Study in animal models demonstrated possible effects of CEIP at the peripheral, systemic and central levels. Studies investigating analgesic activity, histopathological and inflammatory markers production are required to understand the involved mechanisms.

Acknowledgements. – The authors thank Tuiuti University of Paraná and CAPES for the student scholarship awarded (A. F. Santos and E. M. Schiefer).

REFERENCES

1. M. Duenas, B. Ojeda, A. Salazar, J. A. Mico and I. Failde, A review of chronic pain impact on patients, their social environment and the health care system, *J. Pain Res.* **9** (2016) 457–467; https://doi.org/10.2147/jpr.s105892
2. B. B. Rahavard, K. D. Candido and N. N. Knezevic, Different pain responses to chronic and acute pain in various ethnic/racial groups, *Pain Manag.* **7** (2017) 427–453; https://doi.org/10.2217/pmt-2017-0056
3. R. Cregg, G. Russo, A. Gubbay, R. Branford and H. Sato, Pharmacogenetics of analgesic drugs, *Br. J. Pain* **7** (2013) 189–208; https://doi.org/10.1177/2049463713507439
4. A. D. Kaye, A. J. Garcia, O. M. Hall, M. G. Jeha, K. D. Cramer, A. L. Granier, A. Kallurkar, E. M. Cornett and R. D. Urman, Update on the pharmacogenomics of pain management, *Pharmacogen. Pers. Med.* **12** (2019) 125–143; https://doi.org/10.2147/pgpm.s179152
5. S. M. Gwaltney-Brant, *Chapter 7 – Nutraceuticals in Hepatic Diseases*, in *Nutraceuticals: Efficacy, Safety and Toxicity* (Ed. R. C. Gupta), Elsevier, Inc., Amsterdam 2016, pp. 87–99.
6. G. M. Nardi, E. F. Carvalho, S. K. Oliveira, V. K. Nardi, T. C. Gelinski, M. C. Bortoluzzi and M. Maraschin, *Ilex paraguariensis* promotes orofacial pain relief after formalin injection: involvement of noradrenergic pathway, *Pharmacog. Res.* **8** (2016) 31–55; https://doi.org/10.4103/0974-8490.178643
7. F. Pérez-Cano and M. Castell, Flavonoids, Inflammation and immune system, *Nutrients* **8** (2016) 659–663; https://doi.org/10.3390/nu8100659
8. L. M. D Souza, N. Dartora, C. T. Scoparo, T. R. Cipriani, P. A. J. Gorin, M. Iacomini and G. L. Sassaki, Comprehensive analysis of mate (*Ilex paraguariensis*) compounds: Development of chemical strategies for matesaponin analysis by mass spectrometry, *J. Chromatogr. A* **1218** (2011) 7307–7315; https://doi.org/10.1016/j.chroma.2011.08.047
9. N. Dartora, L. M. de Souza, A. P. Santana-Filho, M. Iacomini, A. T. Valduga, P. A. J Gorin and G. L. Sassaki, UPLC-PDA-MS evaluation of bioactive compounds from leaves of *Ilex paraguariensis* with

different growth conditions, treatments and ageing, *Food Chem*. **129** (2011) 1453–1461; https://doi.org/10.1016/j.foodchem.2011.05.112

10. P. G. Santiago, F. M. Gasparotto, K. S. Gebara, F. B. Bacha, F. A. dos Reis Lívero, M. A. Strapazon, E. L. Cardoso Junior, C. A. L. Kassuya, L. M. de Souza and A. Gasparotto Junior, Mechanisms underlying antiatherosclerotic properties of an enriched fraction obtained from *Ilex paraguariensis* A. St. – Hil., *Phytomedicine* **34** (2017) 162–170; https://doi.org/10.1016/j.phymed.2017.08.012
11. H. A. Twaij, A. Kery and N. K. Al-Khazraji, Some pharmacological, toxicological and phytochemical investigations on *Centaurea phyllocephala*, *J. Ethnopharmacol*. **9** (1983) 299–314; https://doi.org/10.1016/0378-8741(83)90037-5
12. K. Manda and A. L. Bhatia, Role of β-carotene against acetaminophen-induced hepatotoxicity in mice, *Nutr. Res*. **23** (2003) 1097–1103; https://doi.org/10.1016/S0271-5317(03)00103-9
13. R. Koster, M. Anderson and E. J. De-Beer, Acetic acid for analgesic screening, *Fed. Proc*. **18** (1959) 412–418.
14. H. C. Holland and E. A. Weldon, A note on a new technique of recording ambulation in the open field test and its validation, *Acta Psychol*. **28** (1968) 293–300; https://doi.org/10.1016/0001-6918(68)90020-6
15. M. M. Campos, E. S. Fernandes, J. Ferreira, L. B. Bortolaza, A. R. Santos and J. B. Calixto, Pharmacological and neurochemical evidence for antidepressant-like effects of herbal product Catuama, *Pharmacol. Biochem. Behav.* **78** (2004) 757–764; https://doi.org/10.1016/j.pbb.2004.05.024
16. L. Bravo, L. Goya and E. Lecumberri, LC/MS characterization of phenolic constituents of mate (*Ilex paraguariensis*, St. Hil.) and its antioxidant activity compared to commonly beverages, *Food Res. Int.* **40** (2007) 393–405; https://doi.org/10.1016/j.foodchem.2011.05.112
17. M. Carini, R. M. Facino, G. Aldini, M. Calloni and L. Colombo, Characterization of phenolic antioxidants from mate (*Ilex paraguariensis*) by liquid chromatography mass spectrometry and liquid chromatography tandem mass spectrometry, *Rapid Commun. Mass Spectrom*. **12** (1998) 1813–1919; https://doi.org/10.1002/(SICI)1097-0231(19981130)12:22<1813::AID-RCM379>3.0.CO;2-%23
18. L. M. Souza, T. R. Cipriani, C. F. Sant'Ana, M. Iacomini, P. A. J. Gorin and G. L. Sassaki, Heart-cutting two-dimensional (size exclusion x reversed phase) liquid chromatography–mass spectrometry analysis of flavonol glycosides from leaves of *Maytenus ilicifolia*, *J. Chromatogr. A* **1216** (2009) 99–105; https://doi.org/10.1016/j.chroma.2008.11.032
19. F. Andrade, C. A. C. Albuquerque, M. Maraschin and E. Silva, Safety assessment of yerba mate (*Ilex paraguariensis*) dried extract: Results of acute and 90 days subchronic toxicity studies in rats and rabbits, *Food Chem. Toxicol*. **50** (2012) 328–334; https://doi.org/10.1016/j.fct.2011.08.02
20. W. R. Sousa, B. H. L. B. Lourenço, M. P. Reis, G. Donadel, M. A. A. Marques, E. L. Cardozo Junior, E. Jacomassi, S. T. Belettini, F. A. R. Lívero and A. Gasparotto Junior, Evaluation of reproductive toxicology of aqueous extract of yerba mate (*Ilex paraguariensis* A. St.-Hil.), a traditional South American beverage, *J. Med. Food*. **22** (2019) 97–101; https://doi.org/10.1089/jmf.2018.0060
21. T. R. Pasquali, S. M. D. Macedo, S. S. Roman, V. Dal Prá, R. L. Cansian, A. J. Mossi, V. J. Oliveira and M. A. Mazzuti, Acute toxicity and anti-inflammatory effects of supercritical extracts of *Ilex paraguariensis*, *Afr. J. Pharm. Pharmacol*. **5** (2011) 1162–1169; https://doi.org/10.5897/AJPP11.234
22. S. H. Jang, M. A. Hossain, J. S. Lee, Md. A. Reza, S. P. Lee, J. Kang and S-C. Park, Hepatoprotective effects of *Ilex paraguariensis* st. Hilaire (yerba mate) extract in rats, *Indian J. Trad. Know.* **17** (2018) 707–715.
23. M. R. McGill, The past and present of serum aminotransferases and the future of liver injury biomarkers, *EXCLI J.* **15** (2016) 817–828; https://doi.org/10.17179/excli2016-800
24. D. Burukoglu, C. Baycu, F. Taplamacioglu, E. Sahin and E. Bektur, Effects of nonsteroidal anti-inflammatory meloxicam on stomach, kidney, and liver of rats, *Toxicol. Ind. Health* **32** (2014) 980–986; https://doi.org/10.1177/0748233714538484
25. V. Noriega, H. F. Miranda, J. C. Prieto, R. Sotomayor-Zárate and F. Sierralta, Involvement of NO in antinociception of NSAIDS in murine formalin hind paw assay, *Drug Res.* **70** (2020) 145–150; https://doi.org/10.1055/a-1095-5418

26. A. Barden, M. Phillips, E. Mas, L. M. Hill, I. Mowat, P. Loh, T. Corcoran and T. A. Mori, Effects of antiemetic doses of dexamethasone on plasma mediators of inflammation resolution and pain after surgery in women, *Prostag. Other Lip. Med.* **149** (2020) Article ID 106427; https://doi.org/10.1016/j.prostaglandins.2020.106427
27. T. M. Z. Alhouzani, M. Saleem, S. Khadka, S. M. M. Al-Subari, D. B. Shrestha, M. Rizvi and I. A. Butt, Toxicity profiles of commonly used anti-inflammatory drugs in geriatrics, *Europasian J. Med. Sci.* **2** (2020) 2 (issue 3, 10 pages); https://doi.org/10.46405/ejms.v2i2.107
28. R. H. Patil, M. N. Kumar, K.M. Kiran, R. Nagesh, K. Kavya, R. L. Babu, G. T. Ramesh and S. C. Sharma, Dexamethasone inhibits inflammatory response via down regulation of AP-1 transcription factor in human lung epithelial cells, *Gene* **645** (2018) 85–94; https://doi.org/10.1016/j.gene.2017.12.024
29. R. S. Barreto, J. S. Quintans, R. K. L. Amarante, T. S. Nascimento, R. S. Amarante, A. S. Barreto, E. W. M. Pereira, M. C. Duarte, H. D. M. Coutinho and I. R. A. Menezes, Evidence for the involvement of TNF-α and IL-1β in the antinociceptive and anti-inflammatory activity of *Stachys lavandulifolia* Vahl. (Lamiaceae) essential oil and (–)-α-bisabolol, its main compound, in mice, *J. Ethnopharmacol.* **191** (2016) 9–18; https://doi.org/10.1016/j.jep.2016.06.022
30. S. Hunskaar and K. Hole, The formalin test in mice – dissociation between inflammatory and non inflammatory pain, *Pain* **30** (1987) 103–114; https://doi.org/10.1016/0304-3959(87)90088-1
31. S. M. A. Bahamonde, M. L. Flores, O. L. Cordoba, C. A. Taira and S. Gorzalczany, Antinociceptive and anti-inflammatory activities of an aqueous extract of *Chiliotrichum diffusum, Rev. Bras. Farmacog.* **23** (2013) 699–705; https://doi.org/10.1590/S0102-695X2013005000051
32. D. W. Lim, J. G. Kim, T. Han, S. K. Jung, E. Y. Lim, D. Han and Y. T. Kim, Analgesic Effect of *Ilex paraguariensis* extract on postoperative and neuropathic pain in rats, *Biol. Pharm. Bull*. **38** (2015) 1573–1579; https://doi.org/10.1248/bpb.b15-00360
33. G. E. Lombardo, S. Cirmi, L. Musumeci, S. Pergolizzi, A. Maugeri, C. Russo, C. Mannucci, G. Calapai and M. Navarra, Mechanisms underlying the anti-inflammatory activity of bergamot essential oil and its antinociceptive effects, *Plants* **9** (2020) 704–720; https://doi.org/10.3390/plants9060704
34. E. Tamaddonfard, S. Tamaddonfard and S. Cheraghiyan, Effects of intracerebroventricular injection of vitamin B12 on formalin-induced muscle pain in rats: role of cyclooxygenase pathway and opioid receptors, *Vet. Res. Forum* **9** (2018) 329–335; https://doi.org/10.30466/vrf.2018.33104
35. E. F. Carvalho, S. K. Oliveira, V. K. Nardi, T. C. Gelinski, M. C. Bortoluzzi, M. Maraschin and G. M. Nardi, *Ilex paraguariensis* promotes orofacial pain relief after formalin injection: involvement of noradrenergic pathway, *Pharmacogn. Res*. **8** (2016) 31–37; https://doi.org/10.4103/0974-8490.178643
36. W. A. Kukula-Koch and J. Widelski, *Chapter 9 – Alkaloids*, in *Pharmacognosy*, Elsevier Inc., Amsterdam 2017, pp. 163–198.
37. E. H. Al-Khateeb, G. A. Al-Assi, A. K. Shakya, N. Al-Rawi and N. Shalan, Antioxidant potential of *Pistacia Vera* L. fruit hull, *Anchusa Strigosa* flowers and *Ilex Paraguariensis* A. St.-Hil. leaves extract, *Orient. J. Chem.* **35** (2019) 982–986; https://doi.org/10.13005/ojc/350309
38. R. P. Barcelos, F. D. Lima, N. R. de Carvalho, G. Bresciani and L. F. Royes, Caffeine effects on systemic metabolism, oxidative-inflammatory pathways, and on exercise performance, *Nutr. Res.* **80** (2020) 1–17; https://doi.org/10.1016/j.nutres.2020.05.005
39. C. H. Blum-Silva, V. C. Chaves, E. P. Schenkel, G. C. Coelho and F. H. Reginatto, The influence of leaf age on methylxanthines, total phenolic content, and free radical scavenging capacity of *Ilex paraguariensis* aqueous extracts, *Rev. Bras. Farmacog.* **25** (2015) 1–6; https://doi.org/10.1016/j.bjp.2015.01.002
40. C. F. Silva, P. Nardin, M. Grings, J. N. Uecker, J. H. Cerqueira, J. A. Rincón, R. T. Abib, A. Schneider, C. C. Barros, R. Andreazza and S. Pieniz, Evaluation of the redox state of Wistar Rats submitted to high-fat diet supplemented with infusion of *Ilex paraguariensis, Braz. Arch. Biol. Technol.* **61** (2018) e18161189 (14 pages); https://doi.org/10.1590/1678-4324-2018161189