

Chemical Constituents of *Psychotria nemorosa* Gardner and Antinociceptive Activity

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Through dereplication strategies using gas chromatography-mass spectrometry (GC-MS) and ultra fast liquid chromatography-tandem mass spectrometry (UFLC-MS/MS), the ethanol extract from *Psychotria nemorosa* leaves (Rubiaceae) showed to be composed of: cinnamic acid, dihydroactinidiolide, 4-hydroxy- β -ionone, phytol, isophytol, 4,8,12,16-tetramethylheptadecan-4-olide, lupeol, a mixture of α/β -amyrin, the keto and acetylated derivatives, besides stigmast-4-en-3-one, campesterol, stigmasterol and γ -sitosterol by GC-MS. Likewise, by UFLC-MS/MS, the main compounds identified were: butin, resveratrol, rutin, kaempferol 7-O- β -D-glucopyranoside, deacetylasperuloside, epiloganin, hordenine, strictosidine, *N*-methyl-1,2,3,4-tetrahydro- β -carboline and *N*-formyl-tryptamine. The antinociceptive activity of the crude extract and its fractions was reported.

Keywords: *Psychotria nemorosa*, hyphenated chromatography-mass spectrometry, antinociceptive activity

Introduction

Herbal drugs have been used since ancient times as medicines, for the treatment of a range of diseases. Many modern drugs have been evolved from ethnopharmacological approaches.^{1,2} Discovery of novel bioactive compounds from plants is a challenging, laborious, expensive process and requires long time to isolate and characterize an active compound. If the isolated bioactive is a known compound, then the time and resources spent on it will be quite a wasted effort.³ To overcome these issues, new techniques for direct identification of bioactive natural products from extracts were developed. Recent high throughput assays technologies to study plant extracts for biological activity has intensified the need for appropriate dereplication strategies.⁴ Dereplication is the process that allows a rapid identification of secondary metabolites in crude extracts by distinguishing previously identified compounds from novel ones. The dereplication process involves separation of single metabolites by chromatographic methods,

identification by spectroscopic methods, bioassay for evaluation of the biological activity, and search in databases to verify the novelty of these compounds.⁵

Hyphenated chromatographic techniques possess characteristics that include efficient separation and rapid identification, among several other advantages in comparison with other analytical methods. These techniques bring significant improvements in the identification of novel natural compounds in complex matrices.^{6,7}

Psychotria comprises an important genus of Rubiaceae family and contains approximately 1700 species of flowering plants with tropical and subtropical distribution.⁸ The polyphyly of the genus has been demonstrated through recent worldwide molecular phylogenetic studies. Razafimandimbison *et al.*⁹ established that *Psychotria* includes all its allied genera, rendering the tribe Psychotrieae monogeneric. It is commonly used in folk herbal medicine for regulating menstrual disturbance, fever, against microbial infections, anti-inflammatory, analgesic, anticonvulsant, anti-rheumatic and even as cardiovascular, mental and eating disorders.¹⁰⁻¹³ *Psychotria* genus is a source of alkaloids, but other compounds like terpenes, flavonoids, tannins, coumarins and cyclotides (circular peptides) has also been isolated.¹⁴⁻¹⁷

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[†]In memoriam

Psychotria nemorosa Gardner (Rubiaceae), popularly known as “casca d’anta”, is a native terrestrial substrate shrub measuring 1-2 m in height with endemic growth in Brazil, found in Rain Forests in the states of Bahia, Paraíba (Northeast), Goiás (West-Central), Espírito Santo, Minas Gerais, Rio de Janeiro, São Paulo (Southeast), Paraná, Santa Catarina and Rio Grande do Sul (South).¹⁸ It is characterized by persistent bilobed stipules containing 8 to 10 arched secondary ribs on each side and visible cross linked tertiary ribs. Flowering and fruiting occurs in January and March, respectively.¹⁹

Literature review showed that the extracts of many *Psychotria* species have anti-inflammatory and analgesic activity, and preliminary tests pointed to alkaloids as the major responsible for the effect, besides other compounds.^{20,21} Pain is a protective mechanism, triggered by potentially injurious stimuli, that influences the quality of life and is directly related to a high amount of medical licenses all over the world.^{22,23} Usually, the therapeutic profile is based on analgesic and anti-inflammatory drugs that present several side effects as ulcers, vomiting, tolerance or dependence.²⁴ Due to these problems, there is continuous interest in the search of new substances that could act with lesser side effects.

As far as we know, no record on phytochemical studies nor biological approach of *P. nemorosa* is described in scientific literature. So, in this work, our objective was to investigate the chemical composition of the *P. nemorosa* leaves on a metabolomic approach and its antinociceptive property in mice.

Experimental

General experimental procedures

All solvents were high-grade purity purchased from Tedia (Brazil). Infrared (IR) spectra were recorded on a Nicolet Magna IR 760 spectrometer using KBr pellets. Column chromatography was performed over silica gel (25 g, 200-300 mesh, Silicycle, Canada) and thin layer chromatography (TLC) on precoated silica gel GF₂₅₄ aluminum plates (Merck, Germany). Spots were visualized by spraying with acidic cerium (IV) sulfate reagent followed by heating at 110 °C and usual specific reagents for different classes of natural product.

Plant material

Leaves of *P. nemorosa* Gardner were collected in November (2012) at Serra dos Órgãos (Rio de Janeiro State, Southeast of Brazil, -22.464042, -43.011746). The access

authorization number is 27035-4 emitted by Ministério do Meio Ambiente. A voucher specimen (Trovó 389 RB) was deposited at the herbarium of the Jardim Botânico do Rio de Janeiro and identified by Marcelo Trovó Lopes de Oliveira.

Extraction and isolation

Dried leaves (60 g) were exhaustively extracted with a 90% aqueous ethanol (EtOH) solution at room temperature for 7 days. The resulting extracts were combined and concentrated under low pressure at 40 °C in a rotaevaporator to yield a dark green liquid (crude ethanolic extract (EE), 7 g). EE (1.0 g) was dissolved in MeOH and subjected to silica gel column chromatography (CC) (21 × 2.5 cm) eluted with hexane, a gradient of hexane/ethyl acetate (EtOAc) (5, 10, 20, 50 and 100%) and methanol (MeOH) to afford thirty-five fractions, which were combined in twelve resulting fractions according to TLC analysis (Table S1, Supplementary Information).

Gas chromatography-mass spectrometry (GC-MS)

GC-MS analyzes were performed on a gas chromatograph coupled to a mass spectrometry (GCMS-QP 2010 Ultra, Shimadzu, Kyoto, Japan) equipment with a quadrupole mass analyzer, electron impact ion source (at 70 eV), auto sampler AOC-20s and auto injector AOC-20i. Analyzes were carried out using a DB-1 capillary column of 30 m × 0.25 mm × 0.25 µm, helium as carrier gas at 1.0 mL min⁻¹. The GC temperature was programmed as: 60 °C (2 min), 60-120 °C (6 °C min⁻¹), 120-290 °C (15 °C min⁻¹) then 290 °C for 17 min, according to Patitucci *et al.*²⁵ Injector, ion source and interface temperatures were 280, 230 and 290 °C, respectively. Data acquisition was performed by Lab Solutions Software and data analysis by NIST MS search database. The sample injection volume was 1.0 µL, all diluted with EtOAc. The triterpenes were identified by comparison of the retention time with authentic standards. *N*-Octadecane was used as internal standard in gas chromatography coupled to a flame ionization detector (GC-FID) with the same conditions described above.

Oxidation of triterpenes

β/α-Amyrin acetate (20a, 20b)

β/α-Amyrin (31a, 31b) (0.05 g), acetic anhydride (1.5 mL) and DMAP (0.001 g) were mixed, stirred and heated under reflux. The reaction was monitored by TLC (2 h), then diluted with H₂O (4 mL) and extracted with dichloromethane (CH₂Cl₂) (3 × 10 mL). The combined organic phases were dried over Na₂SO₄.²⁶ After evaporation,

a white solid of β/α -amyrin acetate (**20a**, **20b**) was obtained (49.0 mg, 98%). IR (KBr) $\nu_{\max}$ / cm^{-1} : 2949 (=C–H), 1734 (C=O), 1379, 1367 (C–H), 1246 (C–O); β -amyrin acetate (**20a**) MS m/z (%): 468 (M^+ , 3), 218 (100), 203 (52), 189 (24); α -amyrin acetate (**20b**) MS m/z (%): 468 (M^+ , 4), 218 (100), 203 (23), 189 (27). Lupeol acetate (48 mg, 96%). IR (KBr) $\nu_{\max}$ / cm^{-1} : 2943 (=C–H), 1732 (C=O), 1639 (C=C), 1454, 1367 (C–H), 1249 (C–O); MS m/z (%): 468 (M^+ , 20), 408(20), 218(36), 204(44), 189(100).

β/α -Amyrone (**18a**, **18b**)

Jones' reagent was added dropwise to the β/α -amyrin (**31a**, **31b**) (0.05 g) in acetone (5.0 mL) at room temperature until the red-orange color of the oxidant persisted. Drops of isopropanol were added until the appearance of a blue-green color, followed by water addition (3.0 mL), a Na_2CO_3 saturated solution and extraction with CH_2Cl_2 (3×10 mL).²⁷ The combined organic layers were dried over Na_2SO_4 and evaporated to give off-white crystals of amyrones (48.5 mg, 97%). IR (KBr) $\nu_{\max}$ / cm^{-1} : 2948 (=C–H), 2853 (C–H), 1706 (C=O), 1456, 1380 (–C–H); β -amyrone (**18a**) MS m/z (%): 424 (M^+ , 11), 218 (100), 203 (57), 189 (19); α -amyrone (**18b**) MS m/z (%): 424 (M^+ , 11), 218 (100), 203 (23), 189 (19).

3,11-Dioxo- β/α -amyrene (**21a**, **21b**)

tert-Butyl chromate (3.0 mL) was added to a solution of β/α -amyrone (**18a**, **18b**) (20 mg) in acetone (2.0 mL), acetic anhydride (3.0 mL) and acetic acid (6.0 mL), refluxed for 6 h and at the end diluted with H_2O (2.0 mL) and extracted with CH_2Cl_2 (3×10 mL). The organic phase was washed with aqueous oxalic acid solution 5% (2×10 mL), Na_2CO_3 saturated solution (10.0 mL) and H_2O (10.0 mL), then dried over Na_2SO_4 and evaporated.²⁸ The product was a yellowish oil (17.6 mg, 88%). IR (KBr) $\nu_{\max}$ / cm^{-1} : 2976 (=C–H), 2872 (–C–H), 1709, 1663 (C=O), 1457 (C=C); 3,11-dioxo- β -amyrene (**21a**) MS m/z (%): 438 (M^+ , 31), 273 (75), 232 (93), 217 (28), 135 (100); 3,11-dioxo- α -amyrene (**21b**) MS m/z (%): 438 (M^+ , 19), 273 (100), 232 (54), 217 (9), 135 (76).

Ultra fast liquid chromatography-mass spectrometry (UFLC-MS)

UFLC-MS analyzes were performed on a Shimadzu UFLC (UFLC Shimadzu System Proeminene, Kyoto, Japan), consisting of a binary pump solvent management system, an online degasser, an auto-sampler, a column oven and a photo diode array detector (DAD). Separation was performed on a Phenomenex Luna C18 (2) 100 A (250 $\times$ 4.6 mm i.d., 5 μm) column equipped with a Phenomenex Luna C18 guard column (4.3 $\times$ 5 mm; Phenomenex, California, USA). The mobile phase consisted of water (A) and acetonitrile (B) both

containing 0.1% formic acid (v/v), with a gradient elution: 0-50 min: 5-100% B (linear gradient); 50-55 min: 100% B (isocratic gradient); 55-60 min: 100-5% B (column washing); 60-65 min: 5% B (isocratic, column equilibration). The flow rate was 1.0 mL min^{-1} . The column temperature was set at 35 °C and the injection volume was 20 μL . The UV spectra were recorded from 200 to 800 nm. The UFLC system was coupled to a microTOF-II mass spectrometer equipped with an electrospray interface (ESI) (Bruker Daltonics Inc., Billerica, MA, USA) operating in both positive and negative ion mode using a capillary voltage of 3.5 kV, end plate 500 V to obtain the accurate mass data. The parameters for ESI coupled to quadrupole-time-of-flight mass spectra (ESI-QTOF-MS) were: drying gas temperature, 320 °C; drying gas flow, 10 L min^{-1} and nebulizing gas pressure, 60 psi. Detection was carried out within a mass range of 50-1300 Da. Nitrogen was used as drying, nebulizing and collision gas.

Similar UFLC equipment and chromatographic conditions were used, as described above, coupled to a mass spectrometer ESI-IT (Bruker Daltonics, Billerica, MA, USA), fitted with an electrospray ionization source operating in positive and negative mode and ion-trap analyzer. Tandem mass spectrometry (MS^2) analyzes were developed in this equipment. The mass spectrometer parameters used were: capillary voltage, 3.5 kV; desolvation temperature, 330 °C; gas flow, 10 L min^{-1} and pressure at 60 psi. Nitrogen was used as both drying and nebulizing gas. Amplitude fragmentation energy for MS/MS experiments was 0.70 V. Data analysis was carried out with Bruker Daltonics software version 4.1.

Animals

All experiments were performed with male Swiss Webster mice (20-25 g) obtained from our own animal facilities. Animals were kept in a room with controlled temperature 22 ± 2 °C for 12 h light/dark cycle with free access to food and water. Twelve hours before each experiment, the animals received only water, in order to avoid food interference with substances absorption. Animal care and research protocols were in accordance with the principles and guidelines adopted by the Brazilian College of Animal Experimentation (COBEA), approved by the Biomedical Science Institute/UFRJ, Ethical Committee for Animal Research, and received the number DFBCICB015-04/16.

Psychotria nemorosa fractions administration

P. nemorosa (leaves) fractions were dissolved in dimethyl sulfoxide (DMSO) in order to prepare a stock solution at a

concentration of 100 mg mL⁻¹. In all experiments, the final concentration of DMSO did not exceed 0.5% at which it has no effect *per se*. The crude extract (EE), hexane, hexane/EtOAc 5%, hexane/EtOAc 10%, EtOAc and MeOH fractions were administered by oral gavage at 10, 30 or 100 mg kg⁻¹ in a final volume 0.1 mL. The control group was composed by vehicle (ultrapure water), which was the same used to solubilize the EE and fractions that were administered on the day of the experiment.

Formalin-induced licking response

This procedure was similar to the method described by Gomes *et al.*²⁹ Mice received an injection of 20 µL of formalin (2.5% v/v) into the dorsal surface of the left hind paw. The time that the animal spent licking the injected paw was immediately recorded. The nociceptive and inflammatory response consists of the following two phases: the first phase lasts until 5 min after the formalin injection (first phase, neurogenic pain response), and the second phase occurs 15–30 min after the formalin injection (second phase, inflammatory pain response). The animals were pre-treated with oral doses of EE, hexane, hexane/EtOAc 5% and hexane/EtOAc 10% fractions, EtOAc and MeOH 60 min before the administration of formalin.

Statistical analysis

Each experimental group consisted of 6 mice. The results are presented as mean ± standard deviation (S.D.). The area under the curve (AUC) was calculated using Prism Software 5.0 (GraphPad Software, La Jolla, CA, USA). Statistical significance between groups was determined using the application of analysis of variance (ANOVA) followed by Bonferroni's test. *p*-Values less than 0.05 were considered to be significant.

Results and Discussion

The crude ethanolic extract (EE) from leaves of *P. nemorosa* was fractionated in a silica gel open column chromatography affording twelve fractions, all analyzed by TLC. Each fraction was evaluated by GC-MS or liquid chromatography coupled to mass spectrometry (LC-MS) on a metabolomic perspective based on their polarities. Mass spectrometry (MS) allows a direct screening of the compounds, providing structural information without the need of laborious isolation since each family of metabolites may have a characteristic mass fragmentation pattern and retention times in GC-qMS and typical UV absorption and MS/MS profile in LC-DAD-MS/MS systems.³⁰

In addition, fractions and EE were evaluated for antinociceptive properties on formalin-induced licking response in mice due the occurrence of two distinct phases of nociceptive behavior: one phase immediately after the injection, lasting for about 5 min, and the other phase starting approximately 20 min after the injection.³¹

The fractions resulting from the CC silica gel can be seen in Table S1 (Supplementary Information). Only those with sufficient amount to access biological activity were investigated in the phytochemical approach. To identify the less polar compounds, GC-MS was used and their profiles are shown in Figures 1A–1D.

GC-MS chemical compounds present in fractions 1, 5, 8 and 9 can be seen in Tables S2 and S3 (Supplementary Information). The MS of the compounds showed more than 90% of similarity with NIST database. These results attested to the major occurrence of cinnamic acid, sterols (campesterol, stigmasterol, γ -sitosterol and stigmast-4-en-3-one) and pentacyclic triterpenes such α/β -amyrone, α/β -amyrin acetate, 3,11-dioxo- α/β -amylene, lupeol and α/β -amyrin, besides some fatty acids, diterpene alcohols and hydrocarbons. Co-injection with authentic materials additionally confirmed the identification. It is important to report that fractions 15–22 were not investigated by GC-MS due to their increased polarity and also due to the small amounts for biological assays. Figure 2 shows the chemical structures of the compounds identified in this work.

Although *Psychotria* genus is characterized mainly as a source of alkaloids, the occurrence of terpenoids is well known. In *P. yunnanensis*, norisoprenoids and the monoterpenoid (6*S*)-menthiafolic acid were isolated, in addition to the ancorane sesquiterpene psycacoraone A.^{32,33} Volatile compounds were also detected in *P. leiocarpa*.³⁴

β -Sitosterol was found in *P. mariniana* and *P. hainanensis*, together with stigmasterol in *P. vellosiana*.^{17,35,36} These steroids, in the glycosylated form, were also reported.^{36–38}

Lupeol was found in *P. vellosiana* and *P. mariniana*.^{17,35} Phytochemical studies of *P. adenophylla* allowed the characterization of other triterpenes such as bauerenol, bauerenol acetate, friedelin, betulin, ursolic acid, traces of α -amyrin and betulinic acid.³⁹ Ursolic acid was isolated from *P. serpens*.⁴⁰ Besides *P. adenophylla*, α -amyrin was also found in *P. stachyoides*.³⁷ It should be emphasized that the occurrence of α/β -amyrone, α/β -amyrin acetate and 3,11-dioxo- α/β -amylene is being reported for the first time in *Psychotria* genus. α/β -Amyrone (m/z 424, [M]⁺) and α/β -amyrin acetate (m/z 468, [M]⁺) showed ions at m/z 218 due to a typical retro Diels–Alder reaction ([M]⁺ – C₁₄H₂₂O and [M]⁺ – C₁₆H₂₆O₂, respectively). Characteristic ions as m/z 203 ([M]⁺ – C₂H₅) and m/z 189

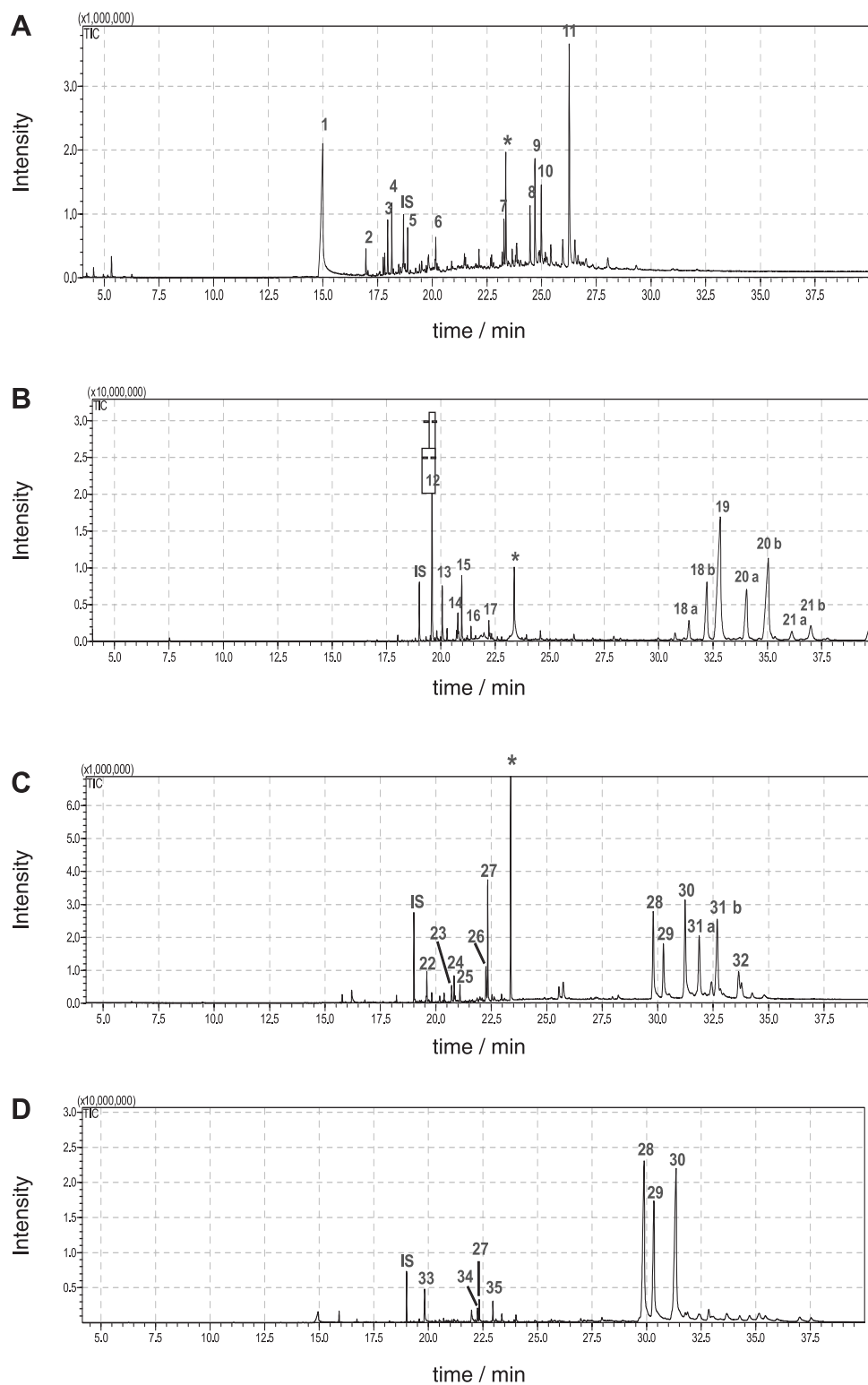


Figure 1. Total ion count mass spectrometry analysis of *P. nemorosa* leaves: (A) hexane; (B) hexane/EtOAc 5% and (C and D) hexane/EtOAc 10% fractions obtained by GC-MS. IS: Internal standard. *Plasticizer: bis-(2-ethylhexyl) phthalate.

($[M]^+ - CH_3$), derived from the ions m/z 218, were also observed. A McLafferty rearrangement in α/β -amyrin acetates ($[M]^+ - C_2H_4O_2$) at m/z 408 was also seen. The same takes place with 3,11-dioxo- α/β -amyrene

(m/z 438, $[M]^+$), generating the characteristic fragments at m/z 273 ($[M]^+ - C_{11}H_{17}O$), 232 ($[M]^+ - C_{14}H_{22}O$), 217 ($[M]^+ - C_{14}H_{22}O - CH_3$), 189 ($[M]^+ - C_{14}H_{22}O - CH_3 - CO$) and 135 ($[M]^+ - C_{11}H_{17}O - C_{10}H_{18}$).⁴¹

The mixture of α/β -amyrin (**31a**, **31b**), previously purified from *Protium* sp. resin,⁴² and lupeol (**19**), derived from *Vellozia* sp.,⁴³ were subjected to classic esterification and oxidation reactions to give derivatives in good yields (Schemes 1 and 2). These reference compounds were used in co-injection assays or for comparison of retention time as an additional tool to confirm GC-MS results (Supplementary Information).

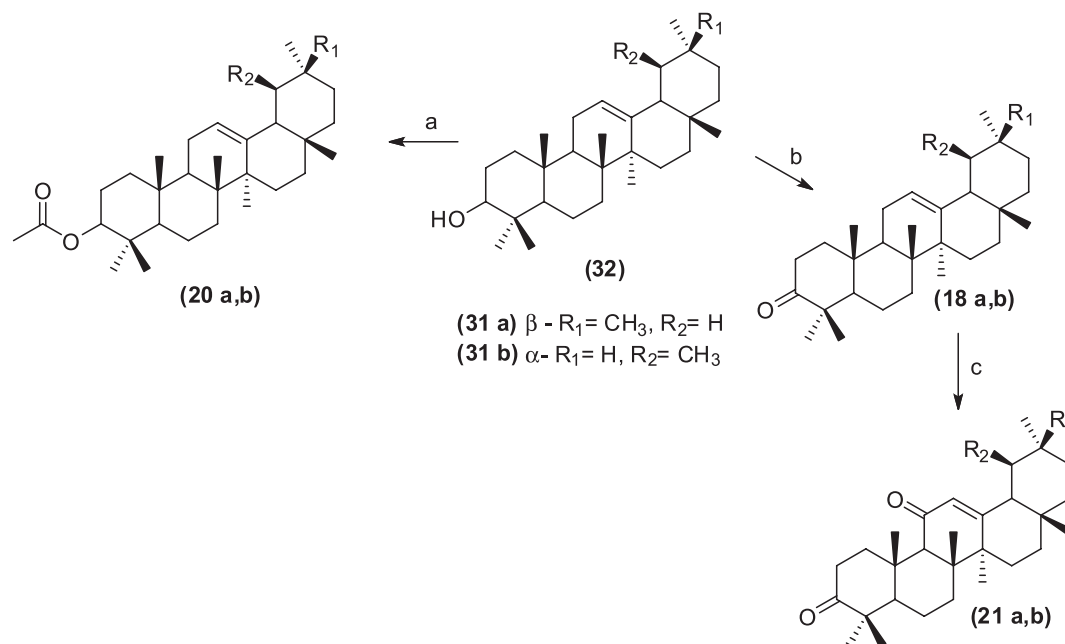
As α -amyrin acetate (**20b**) and lupeol acetate exhibit the same retention times in this condition (34.8 min), the mass fragmentation profile was determined to confirm the presence of α -amyrin acetate in fraction 5 (Supplementary Information).

Besides the triterpenes confirmation, GC-MS allowed us to detect a further series of hydrocarbons, long chain fatty acids and their esters.

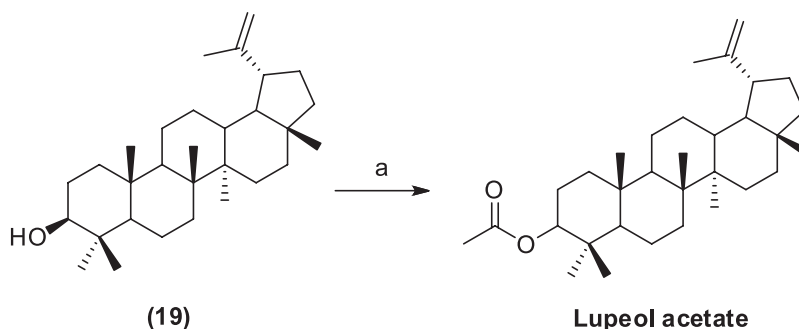
Nowadays, ultra fast liquid chromatography/tandem mass spectrometry (UFLC-MS/MS) is one of the most

convenient techniques for online characterization, due to its superior sensitivity, high selectivity, short run time and resolution power which allow direct screening of natural products.⁴⁴ UFLC coupled to quadrupole-time-of-flight (Q-TOF) is widely applied in herb research and has brought a big convenience for qualitative analysis, allowing quick and effective data acquisition. In terms of the accurate mass measurement, it gives characteristic ions for the identification of molecular formula and the gas phase decomposition reactions activated by collision induction dissociation (CID) may furnish key information for a safe structural elucidation.^{45,46}

The most polar fractions eluted with EtOAc and MeOH from the silica gel column chromatography of EE were analyzed by UFLC coupled with a Q-TOF and a quadrupole ion trap MS apparatus, to obtain high mass accuracy measurements of both parent and fragment ions, as also to deliver sufficient information to detect and reliably identify



Scheme 1. (a) Ac_2O , DMAP, reflux, 2 h (98%); (b) Jones reagent, acetone (97%); (c) di-*tert*-butyl chromate, Ac_2O , AcOH, acetone, reflux, 6 h (88 %).²⁶⁻²⁸



Scheme 2. (a) Ac_2O , DMAP, reflux, 2 h (96%).²⁶

compounds in this complex mixture. Tables 1 to 4 show retention times, formulae, UV features, compound names, mass spectrum features in both positive and negative ions modes, and fragment ions results produced by MS².

In the EtOAc fraction, it was possible to identify some compounds previously described in other *Psychotria* species, such as: loliolide, in *P. cadigensis*⁴⁷ and *P. yunnanensis*;³³ butin, also in *P. yunnanensis*;³³ epiloganin, as part of the alkaloid brachycerin in *P. brachyceras*⁴⁸ and deacetylasperuloside, in *P. leiocarpa*.¹⁵ The fraction eluted with MeOH yielded rutin and kaempferol-7-*O*-glucopyranoside, as in *P. haianensis*,³⁶ and epiloganin. Phenolic compounds closely related with syringol/vanillic alchool/hydroxytyrosol were described in *P. yunnanensis*.³³

Characteristic UV spectra existed in different types of compounds, and can be an important tool to precisely identify isomers with distinguishable chromophores. Data from mass spectra, such as $[M - H]^-$, $[M + H]^+$, $[M + Na]^+$, $[M + K]^+$ ions with their characteristic fragment ions reinforce identification. In our analyzes, butin (m/z 273.1108, $[M + H]^+$) was detected in positive mode showing that loss of H₂O (m/z 255.09 $[M + H - H_2O]^+$) is characteristic of two OH groups in *ortho*. Ions at m/z 245.05 $[M + H - CO]^+$ and m/z 229.08 $[M + H - CO_2]^+$ were observed, consistent with the literature.⁴⁹ MS and UV data are according to literature^{50,51} (Table 1). Loliolide (m/z 197.1163, $[M + H]^+$) was identified by comparison of its mass spectra with previously reported values. Strong UV-Vis absorption at 218 nm suggested an α - β unsaturated ester/lactone.⁵² Ions at m/z 179.06 and m/z 111.92 confirmed the loss of H₂O $[M + H - H_2O]^+$, and also the loss of the ring adjacent to the lactone $[M + H - H_2O - C_5H_8]^+$ (Table 1).

The ion at m/z 385.1258 $[M + Na]^+$ was related to a gibberellin nucleus, with a fragmentation similar to that reported by Takahashi *et al.*⁵³ The ions at m/z 316.2820 $[M + H]^+$ and m/z 318.2974 $[M + H]^+$ led to the identification of dehydrophytosphingosine and phytosphingosine, respectively, according to the literature.^{54,55} The ceramide 2-amino-1,3,4-docosanetriol (m/z 412.3161, $[M + K]^+$) and 2-palmitoylglycerol (m/z 353.2645, $[M + Na]^+$), a glycerolipid, were observed. The mass spectra data are in agreement with previous reports^{56,57} (Table 1).

The ESI-MS spectra (negative ion mode) of fraction EtOAc (Table 2) revealed 7-epiloganin, resveratrol, deacetylasperuloside, ferulic and azelaic acids. Based on the fragmentation characteristics of iridoid-*O*-glycosides, the assignment of 7-epiloganin (m/z 389.1169, $[M - H]^-$) was consistent with fragments at m/z 357.20 and m/z 226.95 in the MS/MS spectrum, due to the loss of methoxy group and glucose moiety from the

precursor ion, respectively. Elimination of methyl ester from m/z 226.95 $[M - H - C_2H_4O_2]^-$ with subsequent dehydration showed the major product ion at m/z 151.11 $[M - H - C_2H_4O_2 - H_2O]^-$.^{58,59} The UV spectrum and fragmentation patterns of $[M - H]^-$ of resveratrol (m/z 227.0646) is consistent with previous reports.^{60,61} The fragment ion at m/z 185.05 $[M - H - CHCOH]^-$ corresponds to a loss of 42 Da with H rearrangement from the precursor ion (m/z 227.0646). The ions at m/z 159.08 $[M - H - C_3O_2]^-$ and m/z 183.18 $[M - H - CO_2]^-$ were formed by a cyclization reaction of the precursor ion with loss of C₃O₂ and OH rearrangement with loss of CO₂, respectively.

Based on literature published data, the presence of deacetylasperuloside (m/z 371.0950 $[M - H]^-$) was suggested by the following ions: m/z 353.04 $[M - H - H_2O]^-$, m/z 209.06 $[M - H - Glc]^-$, m/z 165.06 $[M - H - Glc - CO_2]^-$ and m/z 147.05 $[M - H - Glc - CO_2 - H_2O]^-$.^{62,63}

Ferulic acid was identified through the deprotonated molecule $[M - H]^-$ (m/z 193.0458) that gave the major fragment ion at m/z 148.92 $[M - H - CO_2]^-$. Other fragments were at m/z 178.08 $[M - H - CH_3]^-$ and m/z 134.12 $[M - H - CH_3 - CO_2]^-$.^{64,65} The UV values correspond to this phenolic compound.⁶⁶

The deprotonated molecule at m/z 187.0930 $[M - H]^-$ for azelaic acid produced the fragment ions at m/z : 169.03 $[M - H - H_2O]^-$, 143.10 $[M - H - CO_2]^-$ and 124.98 $[M - H - CO_2 - H_2O]^-$ according to mass bank record and UV spectrum.^{67,68}

In the polar fraction eluted with MeOH it was detected hordenine, eusterol, 8-amino-7-oxononanoic acid, syringol/vanillic alchool/hydroxytyrosol, stryctosidine, *N*-methyl-1,2,3,4-tetrahydro- β -carboline and a pyrogallol related compound, in positive mode. 7-Epiloganin, rutin, kaempferol 7-*O*- β -D-glucopyranoside and *N*-formyl-tryptamine were identified in the negative mode (Tables 3 and 4). The alkaloids stryctosidine, *N*-methyl-1,2,3,4-tetrahydro- β -carboline and the flavonoids rutin and kaempferol 7-*O*- β -D-glucopyranoside have been reported in other *Psychotria* species.^{36,69,70}

Hordenine yielded $[M + H]^+$ at m/z 166.1215 and a major fragment at m/z 121.01 $[M + H - HN(CH_3)_2]^+$, which then loses H₂O yielding an ion at m/z 103.07 $[M + H - HN(CH_3)_2 - H_2O]^+$. Stryctosidine, already reported in genus *Psychotria*, was detected as $[M + H]^+$ at m/z 531.2283, that loses OH $[M + H - OH]^+$ yielding a fragment at m/z 514.19 and at m/z 369.17, due to loss of the sugar moiety $[M + H - Glc]^+$.⁷¹ The alkaloid *N*-methyl-1,2,3,4-tetrahydro- β -carboline $[M + H]^+$ at m/z 187.1226 yielded ions at m/z 158.09 and m/z 144.16 due to loss of CH₃N $[M + H - CH_3N]^+$ and C₂H₅N $[M + H - C_2H_5N]^+$ fragments, respectively.⁷²

The flavonoid rutin was detected as the deprotonated molecule $[M - H]^-$ at m/z 609.1453. The fragment ions, resulting from the cleavage of the glycosidic bond with the loss of 308 Da corresponds to a rhamnose (146 Da) plus a glucose (162 Da) moiety followed by loss of CH_2O fragment, at m/z 301.06 $[M - H - Rham - Glc]^-$ and 271.10 $[M - H - Rham - Glc - CH_2O]^-$, respectively. All spectral data, mass and UV, are in according to Tiberti *et al.*⁷³ and Lopes-Lutz *et al.*⁷⁴ The molecular formula $C_{21}H_{20}O_{11}$ ($[M - H]^-$ at m/z 447.1827) was associated to kaempferol 7-*O*- β -D-glucopyranoside, that gave an ion at m/z 285.08 $[M - H - Glc]^-$ due to known *O*-glucosides fragmentation pattern.⁷⁵ Consistent with the UV spectra and fragmentation behavior, a homolytic cleavage is observed, generating an aglycone ion at m/z 284.13.⁷⁶

The alkaloid *N*-formyl tryptamine was detected in negative mode ($[M - H]^-$ at m/z 187.0934) and exhibited two fragments: one major at m/z 159.05 due the loss of

CO moiety $[M - H - CO]^-$, and other at m/z 130.11 after a supposing loss of $CHNH_2$ $[M - H - CHNH_2]^-$.

The occurrence of hordenine, resveratrol and ferulic acid are being reported for the first time in *Psychotria* genus.

This preliminary phytochemical screening by GC-MS and LC-MS proved to be an useful tool to identify secondary metabolites from *P. nemorosa* leaves, both polar and nonpolar bioactive compounds successfully mapped (Figure 2).

Antinociceptive activity

As shown in Figure 3, the EE of *P. nemorosa* leaves exhibited a significant antinociceptive activity on formalin-induced licking response test in mice, but the EE from branches did not (data not shown). The injection of formalin (2.5%) leads to a biphasic licking response of the injected

Table 1. Characterization of the chemical composition of EtOAc fraction of *P. nemorosa* leaves by UFLC-DAD-ESI-MS² in positive mode

Peak	R_t / min	m/z experimental	m/z calculated	Error [ppm]	Protonated molecule $[M]^+$	Molecular formula	MS/MS fragments	λ_{max} / nm	Proposed compound
36	14.8	273.1108	273.1112	-1.5	$[M + H]^+$	$C_{15}H_{12}O_5$	255, 245, 229	274; 318	butin
37	16.8	197.1163	197.1172	4.6	$[M + H]^+$	$C_{11}H_{16}O_3$	179, 164, 112	217	loliolide
38	19.9	385.1259	385.1258	0.4	$[M + Na]^+$	$C_{19}H_{22}O_7$	353, 327, 309	210	gibberellin related compound
39	25.4	316.2820	316.2842	-8.2	$[M + H]^+$	$C_{18}H_{37}NO_3$	298, 280, 250	223	dehydrophytosphingosine
40	26.5	318.2974	318.3003	9.1	$[M + H]^+$	$C_{18}H_{39}NO_3$	300, 282, 265	223	phytosphingosine
41	28.9	412.3161	412.3188	6.4	$[M + K]^+$	$C_{22}H_{47}NO_3$	394, 376, 346	223	2-amino-1,3,4-docosanetriol
42	31.8	353.2645	353.2662	-5.0	$[M + Na]^+$	$C_{19}H_{38}O_4$	335, 262, 248	224	2-palmitoylglycerol
43	34.4	629.3601	629.3601	0	$[M + Na]^+$	$C_{41}H_{50}O_4$	611, 447, 429	223	n.i.

n.i.: not identified.

Table 2. Characterization of the chemical composition of EtOAc fraction of *P. nemorosa* leaves by UFLC-DAD-ESI-MS² in negative mode

Peak	R_t / min	m/z experimental	m/z calculated	Error [ppm]	Deprotonated molecule $[M]^-$	Molecular formula	MS/MS fragments	λ_{max} / nm	Proposed compound
44	6.5	389.1169	389.1175	1.6	$[M - H]^-$	$C_{17}H_{26}O_{10}$	357, 227, 169, 151	235	7-epiloganin
45	7.6	227.0646	227.0647	0.1	$[M - H]^-$	$C_{14}H_{12}O_3$	185, 183, 159	279; 305	resveratrol
46	9.5	379.0677	379.0671	-1.8	$[M - H]^-$	$C_{17}H_{16}O_{10}$	333, 249, 161	208	n.i.
47	12.1	431.1885	431.1896	2.5	$[M + Cl]^-$	$C_{21}H_{32}O_7$	385, 223, 161	213	n.i.
48	13.6	371.0950	371.0984	9.1	$[M - H]^-$	$C_{16}H_{20}O_{10}$	353, 209, 165, 147	233	deacetylasperuloside
49	14.2	475.1759	475.1762	0.8	$[M - H]^-$	$C_{28}H_{28}O_7$	429, 289, 235, 163	218	n.i.
50	15.3	363.0713	363.0722	2.2	$[M - H]^-$	$C_{17}H_{16}O_9$	317, 267, 241	220	n.i.
51	15.9	193.0458	193.0506	-25.0	$[M - H]^-$	$C_{10}H_{10}O_4$	178, 149, 134	285; 322	ferulic acid
52	17.7	187.0930	187.0976	-24.7	$[M - H]^-$	$C_9H_{16}O_4$	169, 143, 125	205	azelaic acid
53	18.9	401.1200	401.1220	-5.0	$[M + Cl]^-$	$C_{15}H_{26}O_{10}$	369, 325, 193	220	n.i.
54	32.4	721.3543	721.3526	2.4	$[M - H]^-$	$C_{41}H_{54}O_{11}$	675, 397	224	n.i.
55	36.5	559.3058	559.3065	-1.2	$[M - H]^-$	$C_{35}H_{44}O_6$	513, 379, 277	230	n.i.

n.i.: not identified.

Table 3. Characterization of the chemical composition of MeOH fraction of *P. nemorosa* leaves by UFLC-DAD-ESI-MS² in positive mode

Peak	R _t / min	<i>m/z</i> experimental	<i>m/z</i> calculated	Error [ppm]	Protonated molecule [M] ⁺	Molecular formula	MS/MS fragments	λ _{max} / nm	Proposed compound
56	3.2	166.1215	166.1226	-7.2	[M + H] ⁺	C ₁₀ H ₁₅ NO	121, 103, 91	278	hordenine
57	4.4	328.1719	328.1731	-3.6	[M + Na] ⁺	C ₁₄ H ₂₇ NO ₆	166, 121	—	n.i.
58	6.0	164.1054	164.1046	4.7	[M + H] ⁺	C ₁₀ H ₁₃ NO	147, 119, 91	260	eusterol
59	6.4	310.1254	310.1261	2.3	[M + Na] ⁺	C ₁₃ H ₂₁ NO ₆	292, 264, 166	206	n.i.
60	6.8	210.1111	210.1101	-4.9	[M + Na] ⁺	C ₉ H ₁₇ NO ₃	192, 166	215	8-amino-7-oxononanoic acid
61	8.0	244.0309	244.0330	8.5	[M + K] ⁺	C ₆ H ₁₁ N ₃ O ₅	205, 202	202	sugar containing azide
62	8.5	360.1757	360.1765	2.3	[M + H] ⁺	C ₁₅ H ₂₅ N ₃ O ₇	315, 297, 191	202	n.i.
63	11.2	177.0533	177.0546	7.2	[M + Na] ⁺	C ₈ H ₁₀ O ₃	145, 159	229; 282	syringol/vanillic alcohol/ hydroxytyrosol
64	11.5	207.1367	207.1356	-5.4	[M + Na] ⁺	C ₁₁ H ₂₀ O ₂	190, 152, 139	209	n.i.
65	11.8	374.1917	374.1922	-1.1	[M + H] ⁺	C ₁₆ H ₂₇ N ₃ O ₇	329, 297, 209, 173	214	n.i.
66	14.1	531.2283	531.2313	5.7	[M + H] ⁺	C ₂₇ H ₃₄ N ₂ O ₉	514, 369	217	stryctosidine
67	16.6	261.1085	261.1081	1.7	[M + H] ⁺	C ₁₀ H ₁₆ N ₂ O ₆	243, 201, 177	219	n.i.
68	17.5	461.1913	461.1918	-1.1	[M + H] ⁺	C ₂₃ H ₂₈ N ₂ O ₈	371, 299, 158	219	n.i.
69	18.6	187.1226	187.1233	2.2	[M + H] ⁺	C ₁₂ H ₁₄ N ₂	158, 144	219	<i>N</i> -methyl-1,2,3,4-tetrahydro- β-carboline
70	20.4	149.0207	149.0209	1.8	[M + Na] ⁺	C ₆ H ₆ O ₃	132, 131	275	pyrogallol related compound

n.i.: not identified.

Table 4. Characterization of the chemical composition of MeOH fraction of *P. nemorosa* leaves by UFLC-DAD-ESI-MS² in negative mode

Peak	R _t / min	<i>m/z</i> experimental	<i>m/z</i> calculated	Error [ppm]	Deprotonated molecule [M] ⁻	Molecular formula	MS-MS fragments	λ _{max} / nm	Proposed compound
71	3.1	361.0893	361.0907	3.9	[M + Cl] ⁻	C ₁₂ H ₂₂ O ₁₀	325, 265, 163	—	n.i.
72	5.4	385.1311	385.1293	-4.8	[M - H] ⁻	C ₂₁ H ₂₂ O ₇	339, 307	—	n.i.
73	6.6	435.1216	435.1216	-0.1	[M + Cl] ⁻	C ₂₂ H ₂₄ O ₇	389	205	n.i.
44	7.0	389.1175	389.1168	-3.5	[M - H] ⁻	C ₁₇ H ₂₆ O ₁₀	357, 227, 169, 151	235	7-epiloganin
74	9.0	609.1453	609.1469	-2.1	[M - H] ⁻	C ₂₇ H ₃₀ O ₁₆	301, 271, 179	258; 357	rutin
75	9.5	447.1827	447.1832	-0.9	[M - H] ⁻	C ₂₁ H ₂₀ O ₁₁	325, 285, 284	228; 284; 349	kaempferol 7- <i>O</i> -β-D- glucopyranoside
76	12.1	501.1568	501.1573	-1.0	[M - H] ⁻	C ₁₇ H ₃₀ N ₂ O ₁₅	483, 441, 307, 217	205	n.i.
77	12.5	449.1985	449.1988	0.8	[M - H] ⁻	C ₁₅ H ₃₄ N ₂ O ₁₃	403, 319, 283	212	n.i.
78	13.4	695.2114	695.2134	-2.9	[M - H] ⁻	C ₃₉ H ₃₆ O ₁₂	635, 532, 429, 387, 325, 235	216	n.i.
79	15.5	387.1059	387.1045	3.5	[M - H] ⁻	C ₁₅ H ₂₀ N ₂ O ₁₀	369, 343, 284, 193	217	n.i.
80	18.1	187.0934	187.0949	7.9	[M - H] ⁻	C ₁₁ H ₁₂ N ₂ O	159, 130	230; 290	<i>N</i> -formyl-tryptamine
81	20.7	401.1213	401.1202	1.9	[M - H] ⁻	C ₁₆ H ₂₂ N ₂ O ₁₀	369, 325, 282	220	n.i.

paw. The first phase lasts until 5 min after injection due to a direct stimulation of nociceptors, and the second phase occurs between 15 and 30 min after formalin injection due to a combination of an inflammatory reaction in the peripheral tissue and changes in central processing.³¹

The anti-inflammatory non-steroidal drug acetylsalicylic acid (ASA) reduced the time by 13.0% and the opioid analgesic morphine by 44.8%. Ethanol extract (EE) inhibited in 64.4, 62.5 and 67.2% the licking time at the doses of 10, 30, and 100 mg kg⁻¹, respectively (Figure 3),

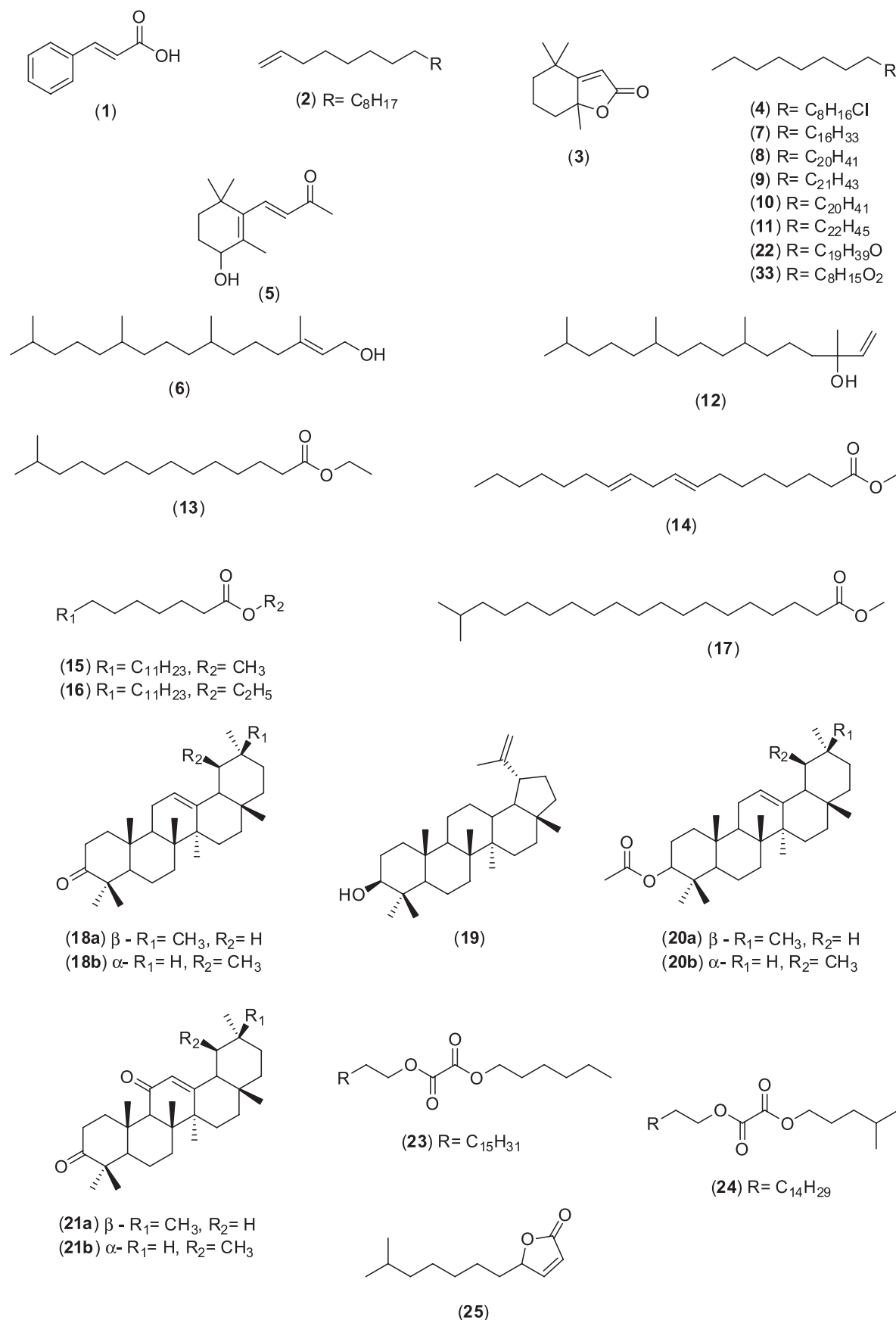


Figure 2. Structures of the identified compounds from *P. nemorosa* leaves.

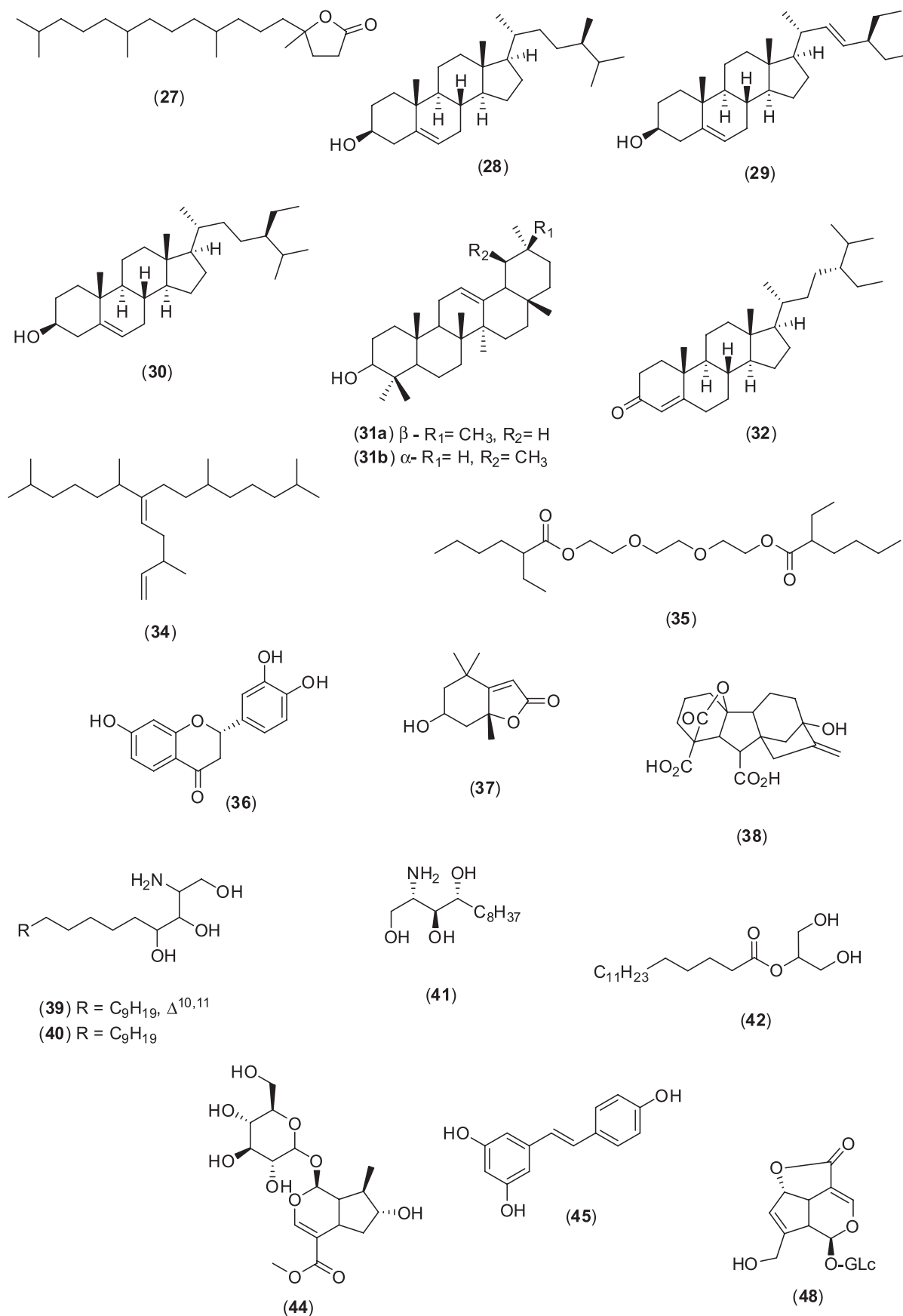


Figure 2. Structures of the identified compounds from *P. nemorosa* leaves. (cont.)

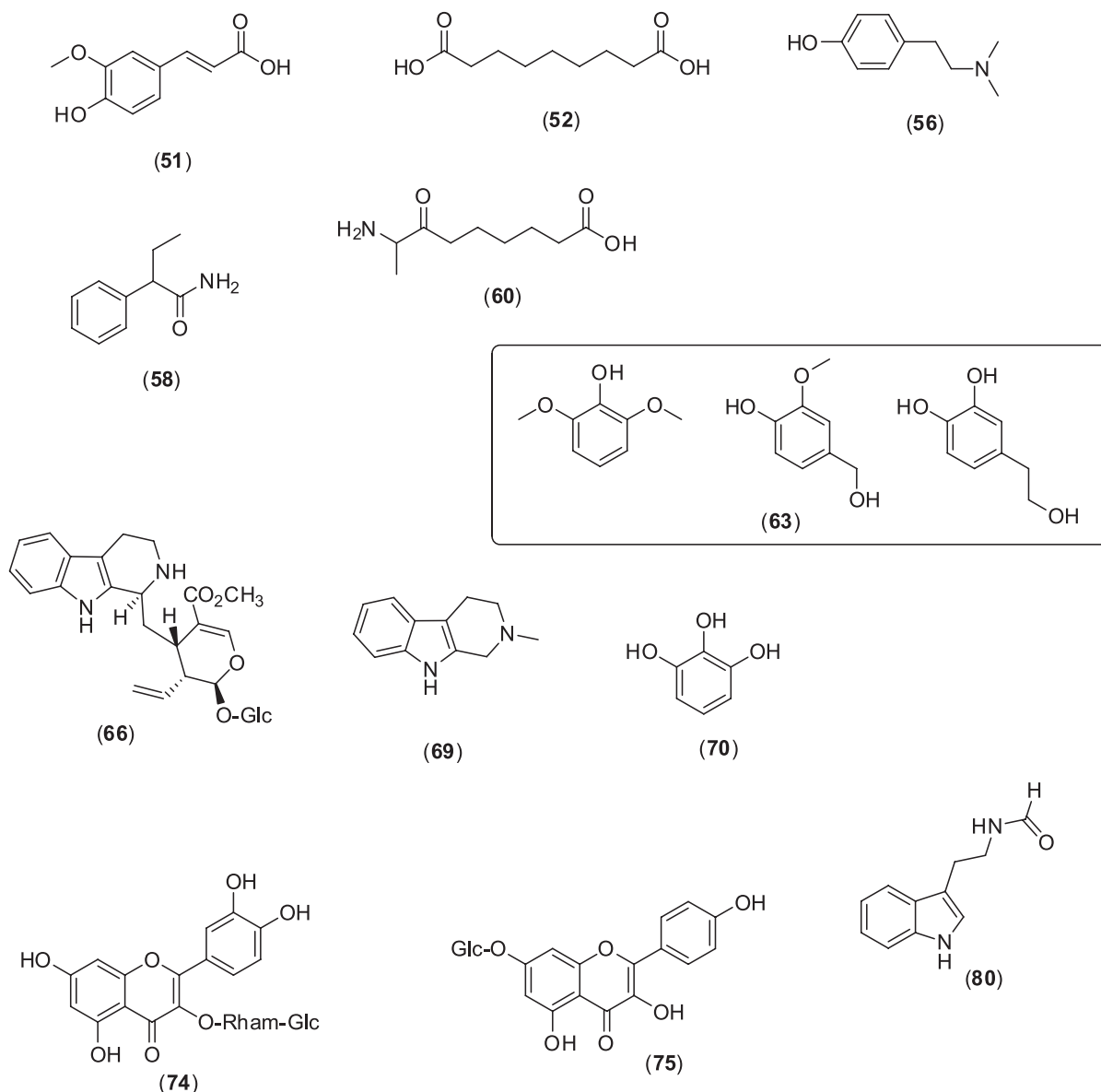


Figure 2. Structures of the identified compounds from *P. nemorosa* leaves. (cont.)

when compared with the vehicle group (42.4 ± 7.7) in the first phase. Analysis of the 2nd phase of the response to formalin showed a significant inhibition at doses of 30 and 100 mg kg⁻¹, with the following inhibition values: 44.2 and 62.4%, respectively. The positive control groups ASA and morphine showed 46.7 and 33.3% of reduction in the licking response, respectively.

In the search for compounds exhibiting antinociceptive activity, the fractions obtained from silica gel column chromatography were tested on formalin-induced licking response in mice, and all of them were able to inhibit the phases of response to formalin (Figures 4 and 5). Only hexane/EtOAc 10% (fraction 9 that contains the sterols) and hexane/EtOAc 20% were not tested due to the small amount.

Figure 4A shows that the hexane fraction (fraction 1) was able to significantly inhibit the first phase at the dose of 100 mg kg⁻¹ (41.3% of inhibition) and in the second phase at the dose of 30 mg kg⁻¹ (41.7% of inhibition). The hexane/EtOAc 5% fraction (fraction 5) was able to significantly inhibit only the first phase at the dose of 30 and 100 mg kg⁻¹ (45.7 and 40.1% of inhibition, respectively) (Figure 4B). The hexane/EtOAc 10% fraction (Fraction 8) was able to significantly inhibit the first phase at the doses of 30 and 100 mg kg⁻¹ (47.2 and 55.6% of inhibition, respectively) and in the second phase at the doses of 10 and 30 mg kg⁻¹ (50.0 and 55.3% of inhibition, respectively) (Figure 4C).

As previously mentioned, fraction 1 contains cinnamic acid, dihydroactinidiolide, 4-hydroxy- β -ionone and phytol, compounds that previously showed antinociceptive

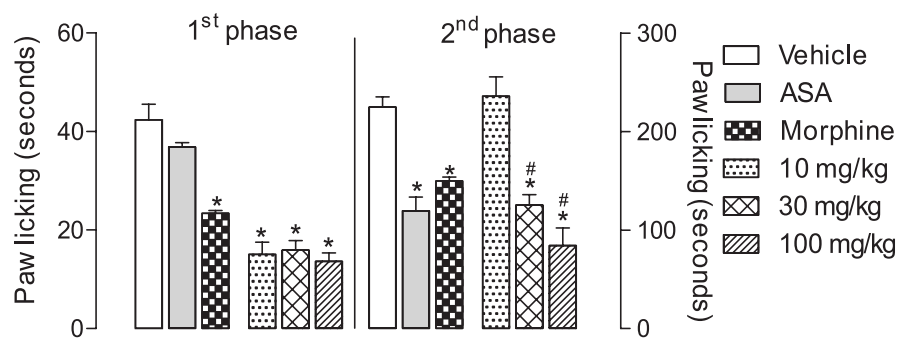


Figure 3. Effects of EE extract from *P. nemorosa* on formalin-induced licking response in mice. Animals were pre-treated with different doses (10, 30 or 100 mg kg⁻¹, p.o.) of EE, ASA (200 mg kg⁻¹), morphine (2.5 mg kg⁻¹) or vehicle. The results are presented as mean $\pm$ S.D. (n = 6) of the time that the animal spent licking the formalin-injected paw. Statistical significance was calculated by ANOVA followed by Bonferroni's test. * p < 0.05 when compared to vehicle-treated mice; # p < 0.05 when compared to morphine-treated mice.

activity.⁷⁷⁻⁸⁰ A mixture of oxidized and esterified triterpenes were observed in fraction 5, as also α/β -amyrin triterpenes in fraction 8, well known for their analgesic and anti-inflammatory roles.⁸¹⁻⁸⁴

Figure 5A shows that EtOAc fraction was able to significantly inhibit the first phase at the doses of 10, 30 and 100 mg kg⁻¹ (61.7, 46.7 and 63.7% of inhibition, respectively) and in the second phase at the dose of 100 mg kg⁻¹ (36.7% of inhibition). On the other hand, MeOH fraction was able to significantly inhibit the first phase and the second phase at the doses of 10, 30 and 100 mg kg⁻¹ (71.2, 59.7 and 52.4%; 41.7, 63.5 and 59.5% of inhibition, respectively) (Figure 5B). This effect may be attributed, at least partially, to the phenolic compounds butin,⁸⁵ resveratrol,⁸⁶ ferulic acid⁸⁷ and the glycosilated iridoid loganin,⁸⁸ present in EtOAc fraction. Next, in the MeOH fraction, the great inhibition may be due to the presence of alkaloids,^{21,89,90} flavonoids^{91,92} and phenolic compounds.^{79,93} We also tested whether the extracts could cause some effect on motor activity. The results obtained after treatment of the mice with doses of 100 mg kg⁻¹ did not show any effect on motor performance of mice (data not shown).

The model used to start the studies was the formalin-induced licking response, as it can discriminate pain in its central and/or peripheral components; this test studies moderate and tonic pain.⁹⁴ It has two distinct phases, which reflect two different types of pain. The first phase (neurogenic pain) is characterized by direct chemical stimulation of nociceptors, afferent fibers predominantly type C and, in part, type A δ , while the second phase (inflammatory pain) is characterized by the appearance of a local inflammatory process, where various proinflammatory chemical mediators are released and that can be inhibited by nonsteroidal anti-inflammatory drugs.^{95,96}

The results showed that the EE and MeOH fraction (10,

30 and 100 mg kg⁻¹) significantly inhibit both phases of the formalin-induced licking response. The fractions hexane (100 mg kg⁻¹), hexane/EtOAc 5%, 10% and EtOAc (10, 30 and 100 mg kg⁻¹) significantly inhibit the first phase. The results of the first phase suggest an antinociceptive activity that can result in both direct action on opioid receptors (predominantly type μ), present in primary afferent fibers (type C) or even an inhibition in the release of mediators such as serotonin, substance P, kinins, histamine and calcitonin gene-related peptide (CGRP). An inhibitory effect shown by EE (30 and 100 mg kg⁻¹), hexane (30 mg kg⁻¹) and hexane/EtOAc 10% (10 and 30 mg kg⁻¹), EtOAc (100 mg kg⁻¹) and MeOH (10, 30 and 100 mg kg⁻¹) in the second phase of formalin may suggest an inhibition in the formation and/or release of arachidonic acid metabolites such as prostaglandins and leukotrienes and other inflammatory mediators such as bradykinin, histamine and serotonin, as well as cytokine, eicosanoids, kinins, glutamate and nitric oxide (NO).^{94,95}

Conclusion

In summary, it was disclosed a concise and useful way to analyze both nonpolar and polar fractions from *P. nemorosa* leaves through dereplication strategy. Compounds as cinnamic acid, dihydroactinidiolide, 4-hydroxy- β -ionone, phytol, isophytol, and 4,8,12,16-tetramethylheptadecan-4-olide and mainly the triterpenoids α/β -amyrin, α/β -amyrone, lupeol, α/β -amyrin acetate, 3,11-dioxo- α/β -amylene and the steroids stigmast-4-en-3-one, campesterol, stigmasterol and γ -sitosterol were identified for the first time. The triterpenes were further confirmed by standard compounds comparison arising from classical esterification and oxidation reactions, as an additional tool. The polar compounds identified were resveratrol, butin (a stilbene compound), the flavonoids rutin and

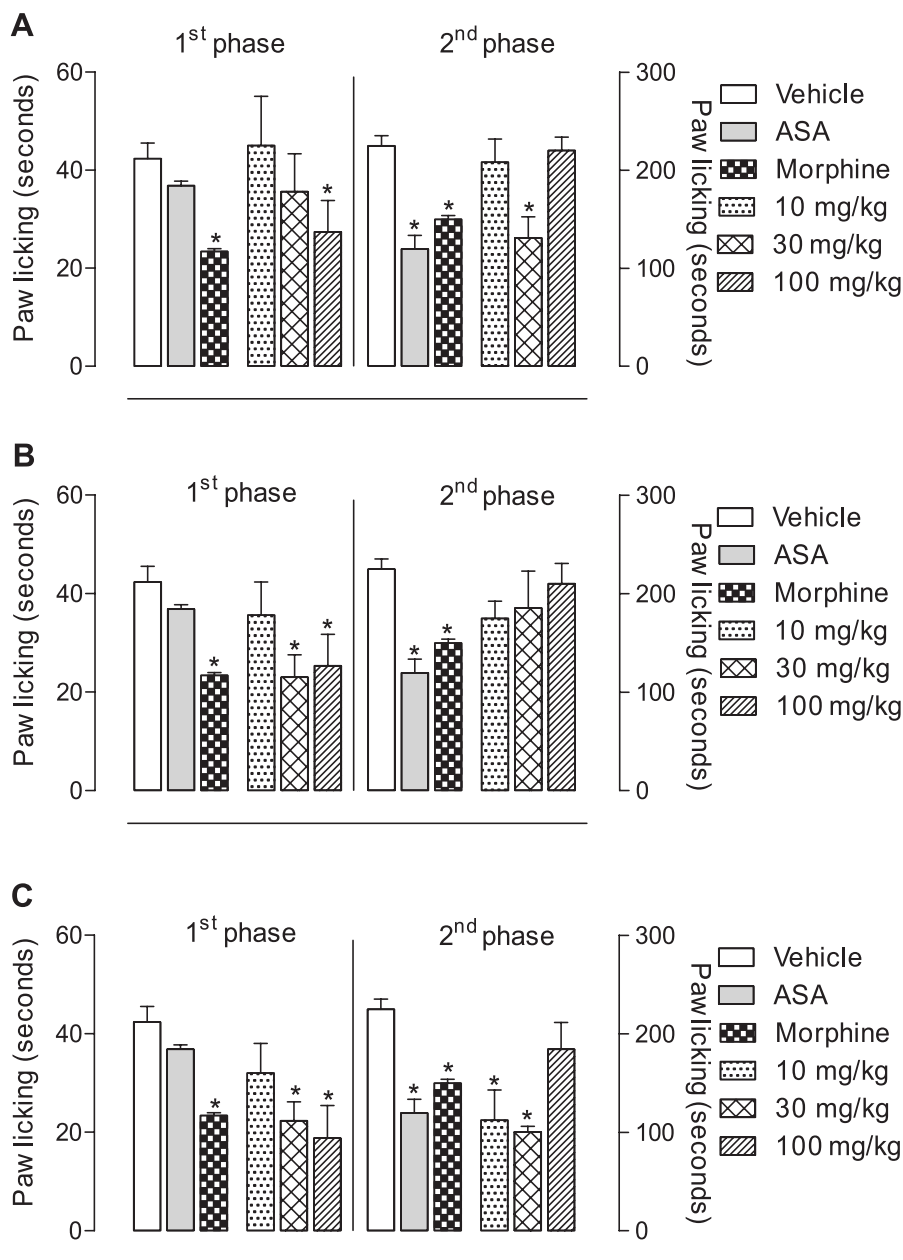


Figure 4. Effects of *P. nemorosa* (A) hexane fraction (fraction 1); (B) hexane/EtOAc 5% fraction (fraction 5) and (C) hexane/EtOAc 10% fraction (fraction 8), on formalin-induced licking response in mice. Animals were pre-treated with different doses (10, 30 or 100 mg kg⁻¹, p.o.) of fractions, ASA (200 mg kg⁻¹), morphine (2.5 mg kg⁻¹) or vehicle. The results are presented as mean $\pm$ S.D. (n = 6) of the time that the animal spent licking the formalin-injected paw. Statistical significance was calculated by ANOVA followed by Bonferroni's test. **p* < 0.05 when compared to vehicle-treated mice.

kaempferol 7-*O*- β -D-glucopyranoside; the iridoid glycosides deacetylasperuloside, epiloganin, and alkaloids as hordenine, stryctosidine, *N*-methyl-1,2,3,4-tetrahydro- β -carboline and *N*-formyl-tryptamine.

Furthermore, to the best of our knowledge, this is the first work describing the phytochemical profile and antinociceptive properties of *P. nemorosa* leaves. The extract and fractions were able to produce an oral antinociceptive effect in acute pain model in mice (formalin-induced licking response).

Supplementary Information

Supplementary data are available free of charge at <http://jbcs.sbq.org.br> as a PDF file.

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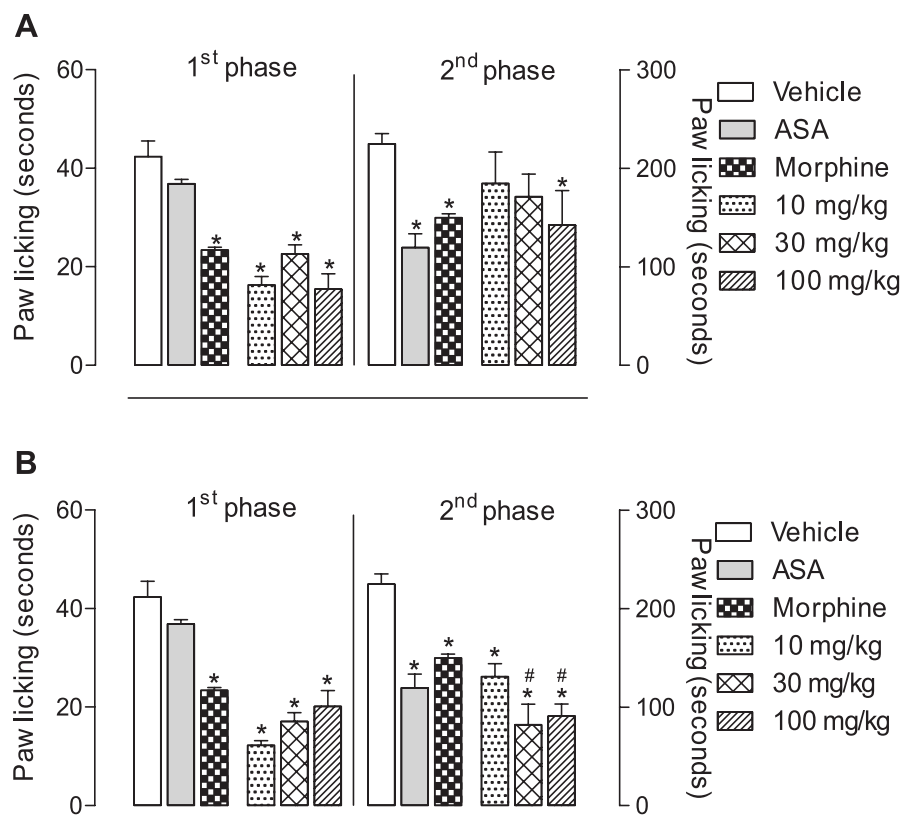


Figure 5. Effects of *P. nemorosa* (A) EtOAc and (B) MeOH fractions on formalin-induced licking response in mice. Animals were pre-treated with different doses (10, 30 or 100 mg kg⁻¹, p.o.) of fractions, ASA (200 mg kg⁻¹), morphine (2.5 mg kg⁻¹) or vehicle. The results are presented as mean $\pm$ S.D. (n = 6) of the time that the animal spent licking the formalin-injected paw. Statistical significance was calculated by ANOVA followed by Bonferroni's test. **p* < 0.05 when compared to vehicle-treated mice; #*p* < 0.05 when compared to morphine-treated mice.

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