

RESEARCH ARTICLE

Antinociceptive compounds and LC-DAD-ESIMSⁿ profile from *Dictyoloma vandellianum* leaves

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Abstract

Limonoids, quinolone alkaloids and chromones have been reported as constituents of *Dictyoloma vandellianum* Adr. Juss. (Rutaceae). Although those compounds are known for their biological activities, only the anti-inflammatory activity of chromones isolated from the underground parts has been evaluated. There are no studies of the pharmacological properties of the aerial parts of *D. vandellianum*. The present study was carried out to determine the phytochemical profile and antinociceptive activity of the methanol extract, fractions and isolated compounds of leaves of *D. vandellianum*. The phytochemical profile was performed by HPLC-DAD-ESIMSⁿ and pure substances obtained were characterized by MS and NMR spectroscopy. The antinociceptive activity was assessed using the formalin assay in mice, and the motor function in the rotarod test. ME and all the fractions obtained from ME produced antinociceptive effects. Among them, the ethyl ether fraction was the most active. Data from HPLC-DAD-ESIMSⁿ showed that the ethyl ether fraction presented 42 compounds. The major compounds isolated from this fraction—gallic acid, methyl gallate and 1,2,6-tri-O-galloyl-β-D-glucopyranose—were tested and produced antinociceptive effects. Gallic acid, methyl gallate and 1,2,6-tri-O-galloyl-β-D-glucopyranose at antinociceptive doses did not affect the motor performance in mice in the rotarod test. This work is the first report of the occurrence of gallotanins in *D. vandellianum*. In addition, the pharmacological study showed that *D. vandellianum* leaves present antinociceptive activity, probably induced by gallic acid, methyl gallate and 1,2,6-tri-O-galloyl-β-D-glucopyranose.

Introduction

Pain is an unpleasant sensation that affects 20% of adults worldwide and it is associated with a wide range of diseases and tissue damage [1]. Currently, pharmacological management of pain

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is performed by drugs that are often not able to completely alleviate pain and induce frequent adverse effects [2]. In fact, all currently available drugs present poor analgesic efficacy in around half of treated chronic pain patients, stressing the importance of the development of new analgesics [3]. Historically, natural products have been a relevant source of chemical entities with analgesic properties, which can be clearly demonstrated by the key role of alkaloid morphine, and other opioids, in contemporary therapeutics [4]. Widespread use of anti-inflammatory analgesics, a class originating from the natural compound salicylic acid, also illustrates vast contribution of natural molecules to the pharmacotherapy of pain. Based on this, bioactive compounds of natural origin have been considered strategic options in the drug discovery process for analgesics [5].

Family Rutaceae includes about 160 genera and 1900 species, with a wide array of secondary chemical compounds presenting biological activities, such as coumarins [6–8], flavonoids [9–11] and alkaloids [12]. The genus *Dictyoloma* contains two species, *Dictyoloma peruvianum* Planch., which occurs in Peru and Bolivia, and *Dictyoloma vandellianum* A.D. Juss., popularly known as “tingui-preto” in Brazil [13]. Previous phytochemical studies of the fruits, stem, leaves and roots of *D. vandellianum* have led to the isolation of several limonoids, quinolone alkaloids and chromones [14–17]. These classes of compounds display a wide range of biological effects, such as anti-tumor, anti-malarial, anti-leishmania, anti-microbial, anti-inflammatory and anti-viral [17–22]. Recently, relevant anti-inflammatory properties of chromones isolated from *D. vandellianum* were demonstrated. Chromones exhibit in vitro and in vivo anti-inflammatory effects, probably due to glucocorticoid receptor activation and inhibition of the transcriptional activity of NF- κ B [23]. Considering that anti-inflammatory compounds frequently also exhibit analgesic properties, the present work was designed to investigate the hypothesis that *D. vandellianum* has constituents with antinociceptive action. Therefore, the phytochemical profile of *D. vandellianum* was obtained by HPLC-DAD-ESIMSⁿ and extract, fractions and isolated compounds were tested to antinociceptive effects in the formalin assay. This test, described by Dubuisson and Denis in 1977 [24], is one of the most valuable and widely used nociception assays in the preclinical identification of compounds with analgesic potential [25]. The formalin test is a model of pain with two distinctive phases that may indicate different types of pain. The early phase is a result of direct stimulation of nociceptors, while the late phase is caused by local inflammation with a release of inflammatory and hyperalgesic mediators [26,27]. These two phases of formalin test have obvious differential mechanisms, and therefore this test is useful not only for assessing analgesic effect of substances, but also for elucidating the mechanism of analgesia [27]. On the other hand, although the behavioral response in the formalin test reflects changes in nociception, other factors that influence behavior, such as changes in motor function, may affect this response [25]. Therefore, the motor performance of mice was also evaluated by using the rotarod test, in order to corroborate the antinociceptive properties demonstrated in the formalin assay.

Materials and methods

General

Nuclear magnetic resonance ¹H and ¹³C (single and two-dimensional) spectra were obtained on Varian spectrometer, model Gemini-500 (Varian, Inc., Palo Alto, CA, USA) (¹H: 500 MHz and ¹³C: 125 MHz); using CD₃OH solvent and TMS as internal standard.

All chromatographic separations were performed on octadecyl-functionalized 200–400 mesh (Sigma-Aldrich), using opened columns (40 × 4.5 cm, 45 × 5 cm, 15 × 8 cm). Thin-layer chromatography (TLC) was performed on glass plates covered with silica gel PF₂₅₄ Merck 60 and revealed with iodine vapor and/or UV light (254 and 365 nm).

All solvents (analytical/HPLC grade) were purchased from Quimex (São Paulo, Brazil) and Tedia (Rio de Janeiro, Brazil) and used without further purification. Indomethacin and dimethyl sulfoxide were obtained from Sigma Chemical Company (St. Louis, MO, USA). Indomethacin was dissolved in Tris HCl 0.1 M pH 8.0 plus saline. The chloroform fractions were dissolved in 5% DMSO plus saline, and the remaining fractions and compounds were dissolved directly in saline.

Plant material

The specimens were collected in March 2005 in Piatã (Inubia district, Bahia, Brazil), at an altitude of about 1304 m above mean sea level. Access registration in the National Management System of Genetic Patrimony and Associated Traditional Knowledge (SISGEN) was obtained under number A737BB9. The plant species were identified by Prof. Maria Lenise Silva Guedes, and deposited at the Herbarium Alexandre Leal Costa (ALCB), Institute of Biology, Federal University of Bahia with the registrations 69,163 (13° 14' 43" S, 41° 45' 28" W) and 88,951 (13° 04' 25" S, 41° 04' 51" W). The dried leaves and extracts were stored in a freezer at -8 °C until used.

Preparation of extracts, fractions and isolation of compounds

The leaves (1.2 kg) from *D. vandellianum* were macerated three times in methanol (6 L) for 7 days. The combined extracts were concentrated under reduced pressure, suspended in water (500 mL) and extracted successively with chloroform (5 × 150 mL), ethyl ether (5 × 150 mL) and ethyl acetate (5 × 150 mL). An aliquot (4 g) of the ether fraction (22 g) was subjected to a C₁₈ reversed-phase open-column chromatography using a gradient of water and methanol as eluent. Fractions of 100 mL were collected. Fractions 14, 23 and 28 resulted in the gallic acid (350 mg), methyl gallate (130 mg) and 1,2,6-tri-*O*-galloyl-β-D-glucopyranose (230 mg), respectively.

Liquid chromatography-mass spectrometry instrumentation and conditions

A Shimadzu[®] (Kyoto, Japan) High Performance Liquid Chromatography System, coupled with an Amazon X or micrOTOF II (Bruker Daltonics, Billerica, MA, USA) with an electrospray ion (ESI) source, was used to perform the ESI-MSⁿ and HRESIMS analysis, respectively. The LC System consisted of a LC-20AD solvent pump unit (flow rate of 600 μL·min⁻¹); a DGU-20A₅ online degasser; a CBM-20A system controller and a SPD-M20A (190–800 nm) diode array detector. The LC separation was performed on a Kromasil C-18 5 μm 100Å, 250 × 4.6 mm (Kromasil, Bohus, Sweden) analytical column. Injections (20 μL) were performed using an autosampler (SIL-10AF). The mobile phase consisted of 0.1% formic acid in water (solvent A) and methanol (solvent B). Exploratory linear gradient (5 × 100% B) was performed to elution in 90 min. The analysis parameters are as follows: capillary 4.5 kV, ESI in negative mode, final plate offset 500 V, 40 psi nebulizer, dry gas (N₂) with flow rate of 8 mL/min and a temperature of 300 °C. CID fragmentation, in Amazon X, was achieved in auto MS/MS mode using enhanced resolution mode for MS and MS/MS mode. The spectra (*m/z* 50–1000) were recorded every 2 s.

Animals

Experiments were performed on male Swiss Webster mice (20–25 g) obtained from the Animal Facilities of the Gonçalo Moniz Institute, FIOCRUZ. Mice were housed in temperature-

controlled rooms (22–23 °C), under a 12:12 h light-dark cycle, with access to water and food ad libitum. Environmental enrichment was obtained with mouse igloos. Animal care and handling procedures were in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The present protocol was approved by the Institutional Animal Care and Use Committee, Ethics Committee for Animal Experimentation of FIOCRUZ (CEUA/FIOCRUZ. Permit Number: L-IGM-015/2013). Every effort was made to minimize the number of animals used and any discomfort. Accordingly, the animals were only used once and were sacrificed immediately after experimentation with isoflurane overdose. Behavioral tests were performed without knowing to which experimental group each mouse belonged.

Antinociceptive activity—Formalin test

The mice were placed in an open Plexiglas observation chamber for 30 min to acclimate to their surroundings. They were then removed and gently restrained while 20 μ L of 2.5% formalin (1:100 dilution of stock formalin solution, 37% formaldehyde in 0.9% saline) was injected subcutaneously into the dorsal surface of the hind paw using a 30 gauge needle. Following injection, the mice were returned to the observation chamber for a 30 min observation period. The nociceptive score was determined by counting the time the animal spent licking the injected paw during the early phase (0–10 min) and the late phase (10–30 min) [28]. The effects of ME (1.95–125 mg/kg) and its ether, ethyl acetate and chloroform fractions (100 mg/kg) were evaluated on the formalin test. Next, gallic acid, methyl gallate and 1,2,6-tri-*O*-galloyl- β -D-glucopyranose (0.19–200 mg/kg) obtained from the ethyl ether fraction were also evaluated. All treatments were administered once, by intraperitoneal route, 40 min before the injection of formalin. Indomethacin (10 mg/kg) and morphine (5 mg/kg) were used as the reference drugs. Vehicle group was treated with saline or 5% DMSO plus saline, as appropriate.

Motor function assay—Rotarod test

To evaluate possible non-specific muscle-relaxant or sedative effects of the treatments, immediately before the formalin assay, the mice were submitted to the rotarod test, in a modified form as previously described [29]. The rotarod apparatus (Insight, Ribeirão Preto, SP, Brazil) consisted of a bar with a diameter of 3 cm, subdivided into five compartments. The bar rotated at a constant speed of eight revolutions per min. Mice were trained 24 h before the experiment to remain on the bar for 120 s. Those not remaining on the bar for two consecutive periods of 120 s were not included in the study. Forty minutes after the intraperitoneal injection of diazepam (10 mg/kg, reference drug), ME (200 mg/kg), gallic acid (200 mg/kg), methyl gallate (200 mg/kg), 1,2,6-tri-*O*-galloyl- β -D-glucopyranose (200 mg/kg) or vehicle, the animals were placed on the rotating rod and the latency to fall was measured for up to 120 s. The results are expressed as the average time(s) the animals remained on the rotarod in each group.

Statistical analysis

Data are presented as means \pm standard error of the means (SEM) of measurements made on six animals in each group. Comparisons between three or more treatments were made using one-way ANOVA with Tukey's post hoc test. All data were analyzed using Prism 5 Computer Software (GraphPad, San Diego, CA, USA). Statistical differences were considered to be significant at $p < 0.05$. The ED₅₀ (dose of an agonist that produces 50% of the maximal possible effect of that agonist) values were expressed with a confidence interval (CI). Individual dose-response curves were fitted with the Hill logistic equation. ED₅₀ values were obtained as the

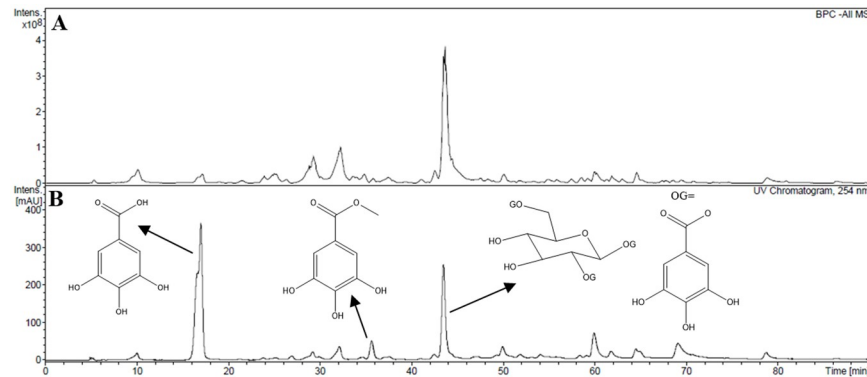


Fig 1. Base peak chromatograms (BPC) in negative ion mode (A) and DAD chromatogram at 254 nm (B) of ethyl ether fraction from leaves of *D. vandellianum* by HPLC-DAD-ESIMSⁿ. In (B), the peaks corresponding to gallic acid, methyl gallate and 1,2,6-tri-O-galloyl- β -D-glucopyranose was demonstrated.

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dose at which a half maximal reduction in nociceptive score occurred, calculated with 95% confidence limits.

Results

Phytochemical study

The HPLC-DAD-ESIMSⁿ analysis detected the presence of 42 compounds in the ether ethyl fraction (see Fig 1 and Table 1). The three known compounds that were isolated from this fraction had their structures confirmed by ¹H and ¹³C NMR and mass spectrometry. The isolated compounds were identified as gallic acid, methyl gallate and 1,2,6-tri-O-galloyl- β -D-glucopyranose.

Antinociceptive activity

The antinociceptive properties of test compounds were investigated in the formalin test in mice. Initially, the effect of systemic injection of methanol extract of *D. vandellianum* leaves (ME) on the formalin-induced nociception was evaluated (Fig 2). Administration of formalin in control mice induced a biphasic flinching response, with early phase ranging from 0 to 10 min and late phase from 10 to 30 min after the injection. Intraperitoneal injection of ME (7.8–125 mg/kg), 40 min prior to the formalin injection, produced a significant antinociceptive effect in both early and late phases of the test (Fig 2A and 2B, respectively). ME at 1.95 mg/kg did not induce antinociception in the formalin test. In the late phase of the test, the ME-induced antinociceptive effect showed a dose-dependent profile, with ED₅₀ value of 8.03 mg/kg (CI 4.62 to 13.94).

Antinociceptive effects of the three fractions (chloroform, ethyl ether and ethyl acetate) obtained from ME were evaluated next (Fig 3). A decrease in nociceptive score was induced by the systemic injection of ethyl ether (FEE; 100 mg/kg) and ethyl acetate (FEA; 100 mg/kg) fractions in both early and late phases of formalin test. Inhibitory effect of the ethyl ether fraction was of 76% and 95% on early and late phases of formalin test, respectively. Ethyl acetate fraction induced an inhibitory effect of 63% and 75% on the early and late phases, respectively. On the other hand, the pretreatment with chloroform fraction (FCHCl₃, 100 mg/kg) produced antinociceptive effect only in late phase of the test, displaying 66% of nociception inhibition.

Considering that FEE induced a relevant antinociceptive effect in the formalin assay, its phytochemical profile was next evaluated to identify constituents within this fraction with

Table 1. Characterization of the compounds tentatively identified by HPLC-ESI-MSⁿ in *Dictyoloma vandellianum*.

Peak No.	t _R (min.)	m/z [M - H] ⁻	Molecular formula	Calcd.	error (ppm)	MS ² /MS ³	Tentative assignment	Reference
1	5.3	331.0671	C ₁₃ H ₁₆ O ₁₀	331.0659	-3.4	MS ² [331]: 313 (7.63); 271 (51.21); 253 (3.47); 241 (3.82); 211 (28.15); 193 (38.24); 169 (100); 125 (13.75)	Galloylhexose I	[30,31]
2	10.1	355.0284	C ₁₄ H ₁₂ O ₁₁	355.0295	3.3	MS ² [355]: 337 (100); 293 (1.32); 249 (1.43)	Chebolic acid	[31]
3	16.7	169.0125	C ₇ H ₆ O ₅	169.0131	3.8	MS ² [169]: 125 (100); 81 (0.25)/MS ³ [169 → 125]: 97 (73.07); 81 (100); 69 (17.81)	Galic acid ^a	[31]
4	17.9	343.0658	C ₁₄ H ₁₆ O ₁₀	343.0659	0.5	MS ² [343]: 191 (100); 169 (7.01)/MS ³ [343 → 191]: 173 (84); 127 (100); 93 (62.43); 85 (78)	5-O-galloylquinic acid	[32]
5	18.8	331.0653	C ₁₃ H ₁₆ O ₁₀	331.0659	2.0	MS ² [331]: 271 (100); 211 (1.35); 169 (0.70)/MS ³ [331 → 271]: 211 (100); 169 (8.45); 125 (2.08)	Galloylhexose II	[30,31]
6	20.1	331.0671	C ₁₃ H ₁₆ O ₁₀	331.0671	-3.4	MS ² [331]: 313 (1.90); 271 (100); 241 (1.53); 211 (1.79); 169 (1.4)/MS ³ [331 → 271]: 211 (100); 169 (9.14); 125 (1.72)	Galloylhexose III	[30,31]
7	20.6	343.0671	C ₁₄ H ₁₆ O ₁₀	343.0659	-3.4	MS ² [343]: 191 (20.96); 173 (18.96); 169 (100); 125 (6.67)/MS ³ [343 → 169]: 125 (100)	4-O-galloylquinic acid	[32]
8	22.7	483.0753	C ₂₀ H ₂₀ O ₁₄	483.0769	3.4	MS ² [483]: 331 (45.67); 313 (100); 271 (17.19); 241 (6.17); 211 (7.33); 193 (14.17)/MS ³ [483 → 313]: 253 (8.22); 169 (100); 151 (8.44); 125 (7.21)	Digalloyl-hexoside I	[30,31]
10	25.5	483.0751	C ₂₀ H ₂₀ O ₁₄	483.0769	3.8	MS ² [483]: 331 (33.47); 313 (100); 271 (13.45); 211 (7.48); 193 (10.11)/MS ³ [483 → 313]: 295 (4.46); 169 (100); 151 (7.10); 125 (16.19)	Digalloyl-hexoside II	[30,31]
11	26.3	495.0760	C ₂₁ H ₂₀ O ₁₄	495.0769	1.9	MS ² [495]: 477 (3.88); 343 (100); 325 (47.37); 245 (3.84); 193 (7.70)/MS ³ [495 → 343]: 191 (27.41); 169 (100); 125 (8.13)	Digalloylquinic acid I	[32]
12	29.3	483.0749	C ₂₀ H ₂₀ O ₁₄	483.0769	4.2	MS ² [483]: 423 (92.69); 405 (16.88); 331 (17.16); 313 (69.26); 295 (18.25); 271 (100); 241 (24.9); 211 (63.65); 193 (50.25)/MS ³ [483 → 271]: 253 (3.38); 211 (100); 193 (12.41); 169 (9.2); 125 (2.80)	Digalloyl-hexoside III	[30,31]
13	32.1	483.0745	C ₂₀ H ₂₀ O ₁₄	483.0769	5.0	MS ² [483]: 423 (92.28); 405 (22.22); 331 (14); 313 (68); 295 (24.61); 271 (100); 241 (23.71); 211 (79.58); 193 (61.39)/MS ³ [483 → 271]: 253 (4.47); 211 (100); 193 (15.81); 169 (13.34); 125 (2.11)	Digalloyl-hexoside IV	[30,31]
14	34.8	495.0737	C ₂₁ H ₂₀ O ₁₄	495.0769	6.5	MS ² [495]: 343 (100); 325 (5.52); 191 (5.57)/MS ³ [495 → 343]: 191 (100); 167 (90.45); 125 (5.55)	Digalloylquinic acid II	[32]
15	35.1	635.0880	C ₂₇ H ₂₄ O ₁₈	635.0878	-0.2	MS ² [635]: 483 (53.16); 465 (100); 313 (19.11); 271 (3.83)/MS ³ [635 → 465]: 313 (100); 295 (11.42); 235 (8.74); 169 (9.84)	Tri-galloyl-hexoside I	[30]
16	35.8	183.0308	C ₈ H ₈ O ₅	183.0297	-4.9	MS ² [183]: 168 (100); 124 (68)	Methylgallate ^a	[30]
17	36.1	483.0760	C ₂₀ H ₂₀ O ₁₄	483.0769	1.9	MS ² [483]: 465 (18.8); 439 (14.74); 423 (43.94); 331 (12.62); 313 (28.82); 271 (100); 241 (4.5); 211 (51.02); 193 (53.30)/MS ³ [483 → 271]: 253 (1.89); 211 (100); 169 (11.26); 125 (2.56)	Digalloyl-hexoside V	[30,31]
18	37.3	495.0746	C ₂₁ H ₂₀ O ₁₄	495.0769	4.7	MS ² [495]: 343 (100); 325 (23.17); 289 (3.73); 245 (2.03); 193 (13.47)/MS ³ [495 → 343]: 191 (13.22); 169 (100); 125 (7.58)	Digalloylquinic acid III	[32]
19	37.3	635.0848	C ₂₇ H ₂₄ O ₁₈	635.0878	4.9	MS ² [635]: 483 (50.15); 465 (100); 423 (2.73); 313 (20.03); 297 (2.41); 271 (3.71); 251 (1.24)/MS ³ [635 → 465]: 313 (100); 295 (16.37); 169 (29.37)	Tri-galloyl-hexoside II	[30]
20	38.6	321.0235	C ₁₄ H ₁₀ O ₉	321.0241	1.9	MS ² [321]: 169 (100); 125 (9.36)/MS ³ [321 → 169]: 125 (100)	Digallic acid	[30]
21	39.9	321.0235	C ₁₄ H ₁₀ O ₉	321.0241	1.9	MS ² [321]: 169 (100); 125 (9.36)/MS ³ [321 → 169]: 125 (100)	Digallic acid II	[30]
22	42.5	647.0855	C ₂₈ H ₂₄ O ₁₈	647.0878	3.7	MS ² [647]: 495 (100); 477 (10.15); 343 (16.29); 325 (8.14); 307 (1.06)/MS ³ [647 → 495]: 477 (3.11); 343 (100); 325 (43.34); 307 (3.08); 289 (3.19); 245 (2.83); 193 (5.84)	3,4,5-tri-O-galloylquinic acid	[32]
23	43.6	635.0890	C ₂₇ H ₂₄ O ₁₈	635.0878	-1.7	MS ² [635]: 483 (82.99); 465 (100); 423 (8.61); 313 (41.91); 295 (9.50); 271 (11.02)/MS ³ [635 → 465]: 313 (100); 295 (12.96); 169 (11.64)	1,2,6-tri-O-galloyl-β-D-glucopyranose ^a	[30]
24	49.2	197.0451	C ₉ H ₁₀ O ₅	197.0444	-3.3	MS ² [197]: 169 (100); 125 (9.88)/MS ³ [197 → 169]: 125 (100)	Ethyl gallate	[33]
25	50.0	953.0927	C ₄₁ H ₃₀ O ₂₇	953.0890	-3.8	MS ² [953]: 935 (21); 801 (7.45); 633 (17.85); 615 (10.65); 589 (7.38); 481 (10.9); 463 (100)	Chebularic acid	[31]
26	51.2	787.1050	C ₃₄ H ₂₈ O ₂₂	787.0988	-7.8	MS ² [787]: 635 (100); 617 (62.74); 483 (9.37); 465 (26.41); 313 (4.3)/MS ³ [787 → 635]: 483 (41.72); 465 (100); 313 (57.95)	Tetra-O-galloylhexoside	[30]

(Continued)

Table 1. (Continued)

Peak No.	t_R (min.)	m/z [M - H] ⁻	Molecular formula	Calcd.	error (ppm)	MS ² /MS ³	Tentative assignment	Reference
27	52.8	635.0906	C ₂₇ H ₂₄ O ₁₈	635.0878	-4.3	MS ² [635]: 577 (9.3); 483 (66.3); 465 (100); 423 (10.36); 313 (41.42); 271 (6.98)/MS ³ [635 → 465]: 447 (4.15); 313 (100); 295 (8.7); 169 (7.23)	Tri-galloyl-hexoside IV	[30]
28	56.7	447.0931	C ₂₁ H ₂₀ O ₁₁	447.0921	-2.0	MS ² [447]: 429 (18.9); 357 (72.2); 327 (100)/MS ³ [447 → 327]: 299 (100); 284 (14.53); 191 (2.23)	Isoorientin	[34]
29	57.4	635.0883	C ₂₇ H ₂₄ O ₁₈	635.0878	-0.6	MS ² [635]: 599 (33); 483 (74.18); 465 (100); 313 (35.37); 301 (5.28); 271 (10)/MS ³ [635 → 465]: 447 (5.74); 313 (100); 295 (12.61); 169 (3.68)	Tri-galloyl-hexoside V	[30]
30	58.5	431.1002	C ₂₁ H ₂₀ O ₁₀	431.0972	-6.8	MS ² [431]: 341 (7.93); 311 (100); 283 (4.91)/MS ³ [431 → 311]: 283 (100); 191 (1.52)	Vitexin	[34]
31	60.8	615.1010	C ₂₈ H ₂₄ O ₁₆	615.0980	-4.8	MS ² [615]: 463 (100); 301 (43.71); 271 (2.5)/MS ³ [615 → 463]: 301 (100); 271 (1.9)	Galloylquercetin hexoside	[34]
32	61.9	583.1111	C ₂₈ H ₂₄ O ₁₄	583.1082	-4.9	MS ² [583]: 431 (100); 413 (23.24); 311 (10.88); 293 (5.64)/MS ³ [583 → 431]: 341 (8.91); 311 (100); 283 (7.04)	(Iso)vitexin galloyl	[34]
33	61.9	431.0984	C ₂₁ H ₂₀ O ₁₀	431.0972	-2.6	MS ² [431]: 413 (7.23); 341 (37.47); 311 (100); 283 (2.25)/MS ³ [431 → 311]: 283 (100)	Isovitexin	[34]
34	64.6	583.1113	C ₂₈ H ₂₄ O ₁₄	583.1082	-5.3	MS ² [583]: 431 (100); 413 (11.98); 311 (4.73)/MS ³ [583 → 431]: 341 (27.95); 311 (100); 283 (3.4)	(Iso)vitexin galloyl	[34]
35	65.1	463.0893	C ₂₁ H ₂₀ O ₁₂	463.0871	-4.7	MS ² [463]: 301 (100); 271 (2.53); 179 (3.27)/MS ³ [463 → 301]: 271 (81.54); 255 (54.71); 179 (100); 151 (83.54)	Quercetin-O-hexoside	[35]
36	66.0	599.1075	C ₂₈ H ₂₄ O ₁₅	599.1031	-7.3	MS ² [599]: 447 (17.67); 357 (9.74); 327 (14.52); 313 (27.64); 285 (100); 271 (2.19)/MS ³ [599 → 285]: 217 (86.4); 199 (64.36); 175 (100); 151 (41.27)	Astragalín-O-gallate I	[33]
37	67.3	599.1040	C ₂₈ H ₂₄ O ₁₅	599.1031	-1.4	MS ² [599]: 447 (79.39); 357 (4.12); 327 (6.12); 313 (100); 285 (78.65); 271 (3.04)/MS ³ [599 → 313]: 241 (16.56); 169 (100); 125 (21.48)	Astragalín-O-gallate II	[33]
38	69.2	301.0026	C ₁₄ H ₆ O ₈	301.0037	3.9	MS ² [301]: 257 (100); 229 (88.42); 185 (63.06); 157 (5.74)/MS ³ [301 → 257]: 229 (83.14); 213 (13.31); 201 (12.95); 185 (100); 157 (3.98)	Ellagic acid	[36]
39	70.8	447.0931	C ₂₁ H ₂₀ O ₁₁	447.0921	-2.0	MS ² [447]: 285 (100); 255 (46); 227 (8.7); 179 (1.43)/MS ³ [447 → 285]: 267 (10.26); 255 (100); 227 (15.75)	Kaempferol-O-hexoside	[35]
40	78.8	301.0351	C ₁₅ H ₁₀ O ₇	301.0342	-2.7	MS ² [301]: 273 (18.47); 179 (100); 151 (84.4)/MS ³ [301 → 179]: 169 (20.13); 151 (100)	Quercetin	[37]
41	80.9	593.1295	C ₃₀ H ₂₆ O ₁₃	593.1289	-0.9	MS ² [593]: 447 (11.79); 285 (100)/MS ³ [593 → 285]: 257 (100); 185 (31.12); 151 (73.39)	Tribuloside	[33]
42	86.4	285.0404	C ₁₅ H ₁₀ O ₆	285.0393	-3.6	MS ² [285]: 257 (67.73); 243 (77.95); 229 (100); 185 (78.03); 169 (72.37); 151 (52.27)	Kaempferol	[30]

I, II, III, IV and V Numbers used to discriminate putative individual isomers.

^a Identified by ¹H and ¹³C NMR

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antinociceptive properties. A phase-reverse chromatography of the fraction was performed, allowing isolation of gallic acid (GA), methyl gallate (MG) and 1,2,6-tri-O-galloyl-β-D-glucopyranose (TGG). GA at 12.5 ($p < 0.01$), 50 ($p < 0.001$) and 200 ($p < 0.001$) mg/kg, administered by intraperitoneal route 40 min before formalin injection, reduced the nociceptive behavior of mice in late, but not early phase of the test (Fig 4). GA at 3.12 and 0.78 mg/kg did not induce effect on formalin test. Pretreatment with indomethacin (10 mg/kg, ip), a standard nonsteroidal anti-inflammatory drug, produced a similar inhibition profile of late phase ($p < 0.001$). As expected, the pretreatment with morphine (5 mg/kg, ip), a gold standard opioid, inhibited both the early ($p < 0.01$) and late ($p < 0.001$) phase of the formalin test.

Intraperitoneal administration of MG (3.1–200 mg/kg), 40 min before formalin injection, inhibited the late phase of formalin test ($p < 0.001$; $p < 0.01$; $p < 0.05$). MG at 0.78 and 0.19

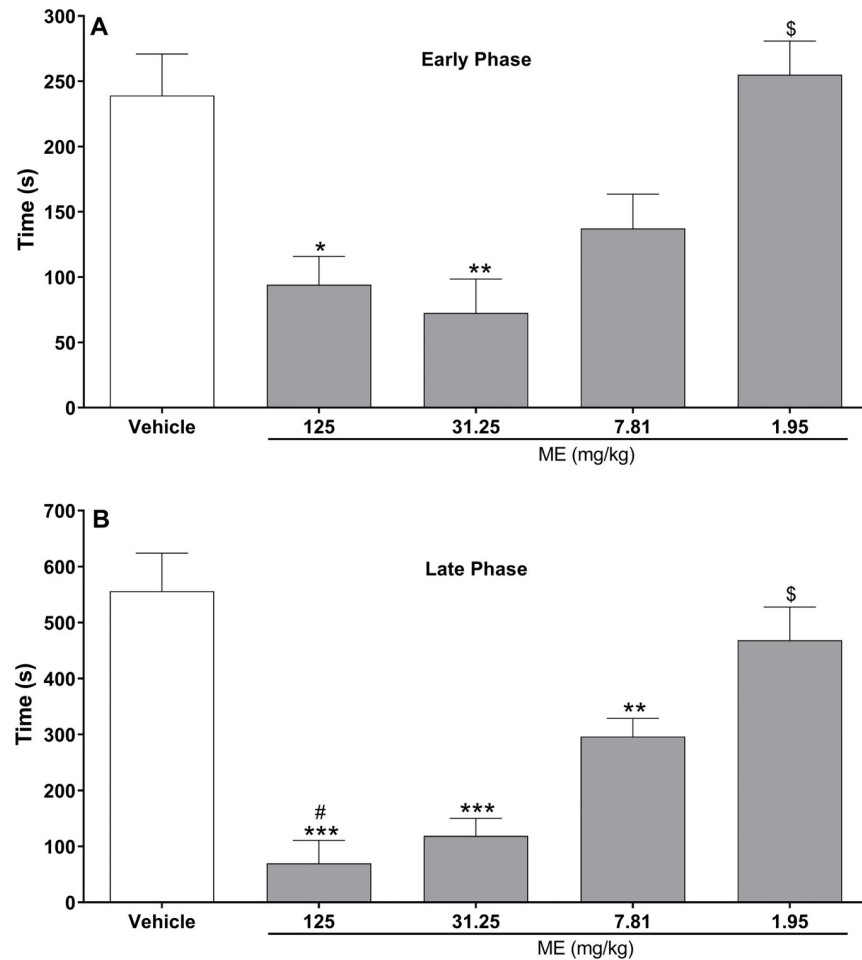


Fig 2. Effects of the methanol extract of *D. vandellianum* leaves (ME) on formalin test in mice. Mice were treated with vehicle (saline, control group) or ME (125–1.95 mg/kg) by intraperitoneal route 40 min before the intraplantar injection of formalin (injected at time zero). Mice were observed from 0 to 10 min (early phase; A) and from 10 to 30 min (late phase; B), and a nociceptive score was determined for each period by counting the time in seconds that the animal spent licking the injected limb during the observation time. Data are expressed as mean times \pm S.E.M.; $n = 6$ mice per group. Statistical significance relative to the control group: * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$). Statistical significance relative to the 7.81 mg/kg group: # ($p < 0.05$). Statistical significance relative to the 125 and 31.25 mg/kg groups: § ($p < 0.05$). ANOVA followed by Tukey's test.

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mg/kg showed no antinociceptive effect. MG, at all doses tested, did not induce a statistically significant reduction in the early phase of the formalin test. Systemic pretreatment with TGG at doses between 0.78 and 200 mg/kg inhibited the late, but not the early phase of the formalin test ($p < 0.001$; $p < 0.01$). TGG at 0.19 mg/kg had no effect in this assay. In the late phase of the formalin test, the TGG-induced antinociception was a dose-dependent effect, as indicated by the statistically significant difference between active doses ($p < 0.01$), displaying an ED_{50} value of 0.80 mg/kg (CI 0.22 to 2.57).

In the rotarod test, the intraperitoneal administration of ME, gallic acid, methyl gallate and 1,2,6-tri-O-galloyl- β -D-glucopyranose (200 mg/kg), did not reduce the run time of the mice, indicating that these treatments did not induce motor performance alterations (Fig 5). As expected, the central nervous system depressant diazepam (10 mg/kg) reduced the time of mice on the rotarod after 40 min of intraperitoneal treatment with this standard drug.

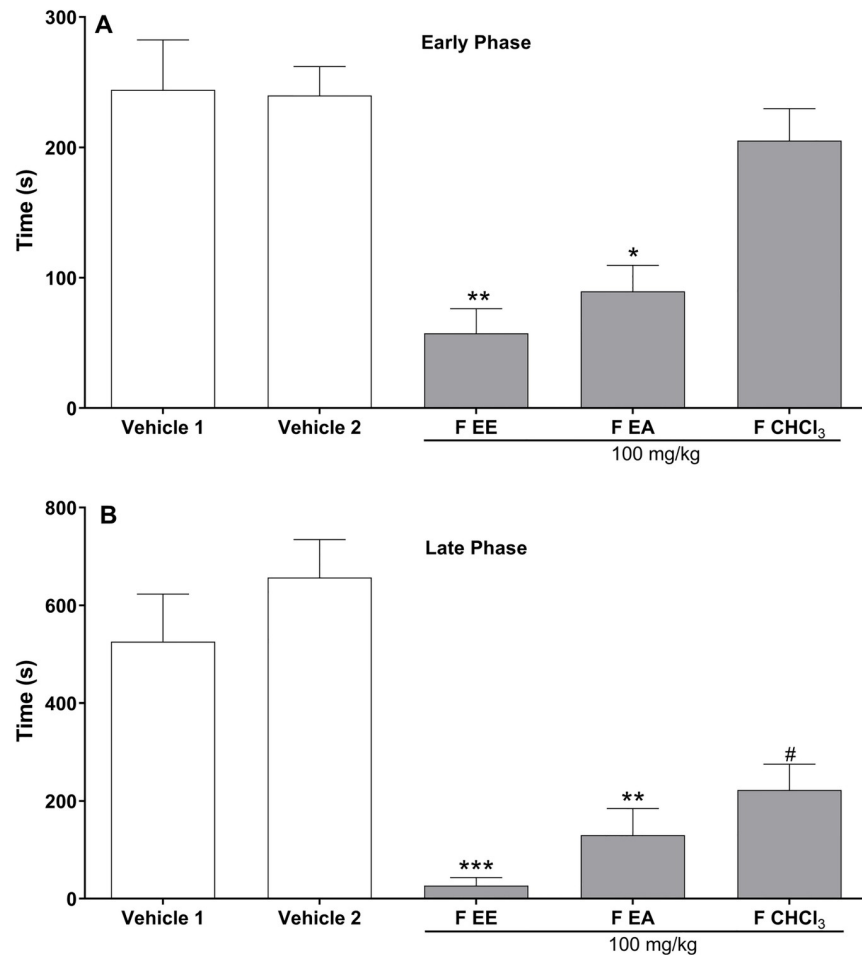


Fig 3. Effects of different fractions from methanol extract of *D. vandellianum* leaves (ME) on formalin test in mice. Mice were treated with vehicle (control groups) or fractions (100 mg/kg) by intraperitoneal route 40 min before the intraplantar injection of formalin (injected at time zero). FEE: ether fraction from ME (solubilized in saline); FEA: ethyl acetate fraction from ME (solubilized in 5% DMSO); FCHCl₃: chloroform fraction from ME (solubilized in 5% DMSO). Vehicle 1: saline (control group of FEE). Vehicle 2: 5% DMSO plus saline (control group of FEA and FCHCl₃). Mice were observed from 0 to 10 min (early phase; A) and from 10 to 30 min (late phase; B), and a nociceptive score was determined for each period by counting the time in seconds that the animal spent licking the injected limb during the observation time. Data are expressed as mean times \pm S.E.M.; $n = 6$ mice per group.

* Significantly different from vehicle 1 group ($p < 0.05$); **significantly different from vehicle 1 group ($p < 0.01$); *** significantly different from vehicle 1 group ($p < 0.001$); # significantly different from vehicle 2 group ($p < 0.01$).

ANOVA followed by Tukey's test.

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Discussion

Phytochemical profile

The 42 compounds were tentatively assigned in ethyl ether fraction from *D. vandellianum* by the interpretation of their fragmentation patterns obtained from mass spectra (HRESIMS, MS² and MS³ experiments). Data provided by isolated compounds and literature information was also employed for the comprehensive evaluation of samples. The retention times and mass spectrum data along with peak assignments for compounds identified using negative ionization are described in Table 1.

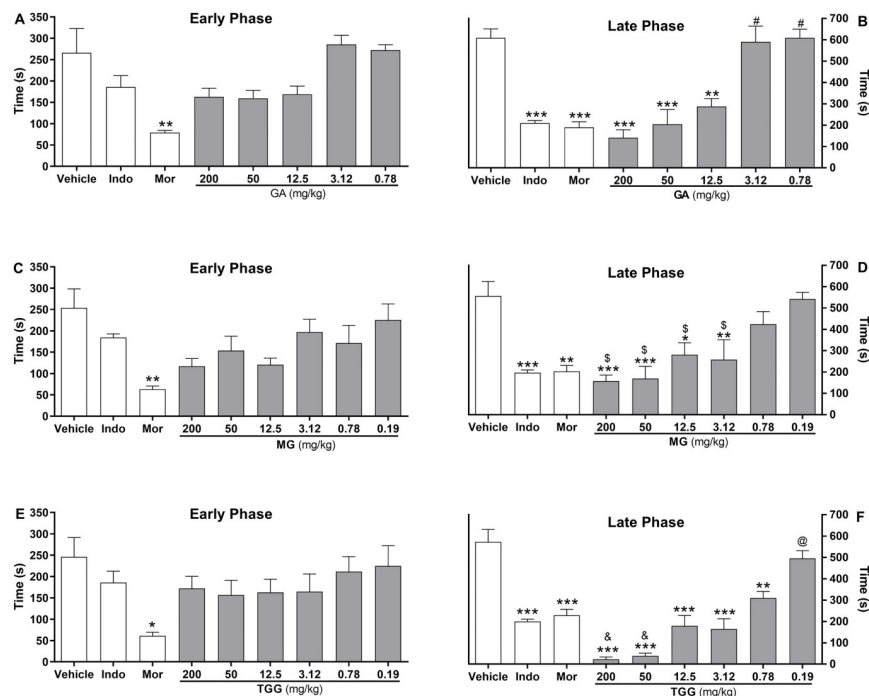


Fig 4. Effects of gallic acid, methyl gallate and 1,2,6-tri-O-galloyl- β -D-glucopyranose on formalin test in mice. Mice were treated with vehicle (saline, control group), indomethacin (Indo; 10 mg/kg, reference drug), morphine (Mor; 5 mg/kg, reference drug), gallic acid (GA; 0.78–200 mg/kg, panels A and B), methyl gallate (MG; 0.19–200 mg/kg, panels C and D) and 1,2,6-tri-O-galloyl- β -D-glucopyranose (TGG; 0.19–200 mg/kg, panels E and F) by intraperitoneal route 40 min before the intraplantar injection of formalin (injected at time zero). Mice were observed from 0 to 10 min (early phase, panels A, C and E) and from 10 to 30 min (late phase, panels B, D and F), and a nociception score was determined for each period by counting the time in seconds that the animal spent licking the injected limb during the observation time. Data are expressed as mean times \pm S.E.M.; $n = 6$ mice per group. Statistical significance relative to the control group: * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$). Statistical significance relative to the 200, 50 and 12.5 mg/kg groups: # ($p < 0.01$). Statistical significance relative to the 0.78 mg/kg group: \$ ($p < 0.001$). Statistical significance relative to the 0.19 mg/kg groups: & ($p < 0.05$). ANOVA followed by Tukey's test.

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Gallotannins and ellagitannins were detected and tentatively assigned. They were distinguished by their characteristic fragment ion spectra yielding sequential losses of galloyl (m/z 152), gallate (m/z 170), and pexahydroxydiphenic acid (HHDP) or ellagic acid residues (m/z 301). Gallotannins composed of one to four units of galloyl esters of glucose were identified. The galloylhexose derivatives (**1**, **5** and **6**) were assinalated based on the $[M - H]^-$ ion at m/z 331 and MS/MS produced as typical product ions at m/z 271, 193, 169 and 125 [28, 29]. Five peaks (**8**, **9**, **12**, **13** and **17**) with the precursor ion at m/z 483 were assigned to digalloyl-hexoside relying on the MS and MS/MS spectra that showed product ions at m/z 331 $[M - H - 162]^-$ and 169 $[M - H - 162 - 152]^-$ corresponding to the neutral losses of hexose and galloyl moieties, respectively [28, 29]. Similarly, trigalloyl-hexoside (**15**, **19**, **23**, **27** and **29**) and tetragalloyl (**26**) were tentatively assignment [28, 29]. Furthermore, galloylquinic acid derivatives were identified from observation of the precursor's ions at m/z 343, m/z 495 and m/z 647, which were attributed to galloylquinic acids (**4** and **7**), digalloylquinic acids (**11**, **14** and **18**) and trigalloylquinic acids (**22**) respectively. The MS² and MS³ spectra indicated as products ions at m/z 191, 173, 169, 125 and 85, consistent with fragmentation behavior of galloylquinic acid derivatives. In addition, the compounds gallic acid (**3**), methyl gallate (**16**) and ethyl gallate (**24**) were identified based on $[M - H]^-$ ions at m/z 169, 183 and 197, respectively. MS² data of these ions

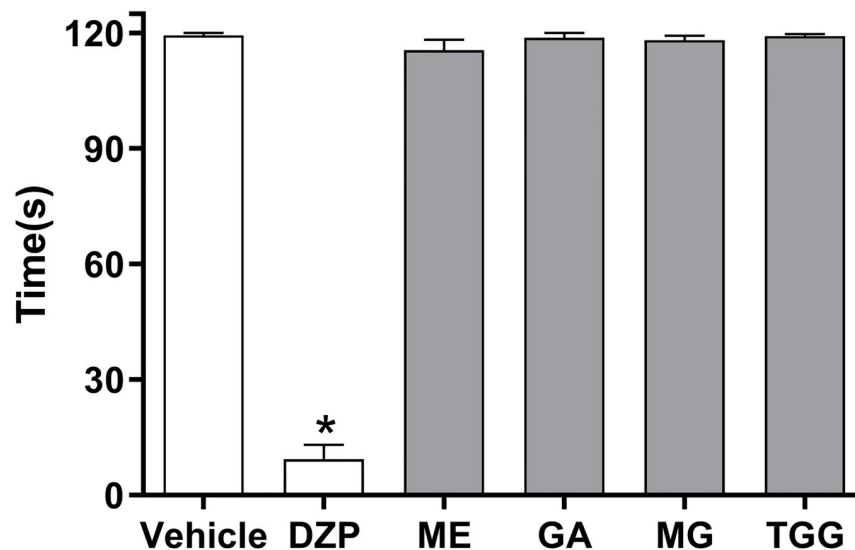


Fig 5. Effects of ME, gallic acid, methyl gallate and 1,2,6-tri-O-galloyl- β -D-glucopyranose from *D. vandellianum* on motor function in mice. Bar graph representing the run time on the rotarod, 40 min after intraplantar injection of ME (200 mg/kg), gallic acid (GA; 200 mg/kg), methyl gallate (MG; 200 mg/kg), 1,2,6-tri-O-galloyl- β -D-glucopyranose (TGG; 200 mg/kg), diazepam (DZP; 10 mg/kg, reference drug) or vehicle (saline; control group). Data are reported as means \pm SEM; $n = 6$ mice per group. * Significantly different from the vehicle group ($p < 0.001$). One-way ANOVA followed by the Tukey's test.

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were compared with literature data [28,29,31]. Ellagic acid (38) was identified based on the $[M - H]^-$ ion at m/z 301 that was confirmed by MS² spectrum with product ions at m/z 257, 229, 185 [34]. Moreover, others ellagitannins as chebulic acid and chebulagic acid were also identified [29].

Regarding to identification of flavonoids, they were classified in two groups: O-glycosides and C-glycosides, which were identified comparing their MS/MS spectra with those available in literature. It was observed two parent ions at m/z 301 and 285, both result as product an ion at m/z 151, identified as quercetin and kaempferol, respectively [28,35]. Besides parent ions at m/z 463 and 447 exhibited product ions at m/z 301 $[M-H-162]^-$ and 285 $[M-H-162]^-$, a characteristic loss of the hexose moiety, were identified as quercetin-O-hexoside (35) and kaempferol-O-hexoside (39), respectively [33]. Peaks 31 (m/z 615) and 36 (m/z 599) exhibited the same loss of 314Da characteristic of hexose and galloyl moieties, it originated fragment ions at m/z 301 and 285, which corresponds to quercetin and kaempferol. Thus, they were identified as galloylquercetin hexoside and astragalin-O-gallate, respectively [32,33]. Concerning to C-glycosides, fragments $[M-H-60]^-$, $[M-H-90]^-$ and $[M-H-120]^-$ work as characteristic diagnostic ions to glycine moiety. Peak 28 was identified as isoorientin, it presented $[M-H]^-$ ion at m/z 447 and MS/MS spectra at m/z 429, 357, 327 and 285 [32]. Peak 33, $[M-H]^-$ ion at m/z 433, produced fragment ions at m/z 413, 341 and 311 corresponding to C-glycoside fragmentation pattern, which was suggested as isovitexin [32]. Peak 30 was identified as your isomer, vitexin [32].

Of the 42 compounds observed by HPLC-DAD-ESIMSⁿ, three had their structures confirmed by NMR (gallic acid, methyl gallate, 1,2,6-tri-O-galloyl- β -D-glucopyranose).

Compound 1,2,6-tri-O-galloyl- β -D-glucopyranose was obtained as a yellowish brown amorphous powder. The ¹H NMR spectrum reveals three singlets, each one integrating for two protons at δ_H 7.10, 7.15 and 7.18, assignable to the aromatic protons of the three galloyl moieties the molecule. In the carbohydrate region, the spectrum clearly shows three downfield

hydrogens resonances: a doublet at δ_{H} 5.85 integrating one proton with large coupling constant, indicating a β -configuration of the anomeric proton; a triplet at δ_{H} 5.31, and two signals at δ_{H} 4.61 (d, $J = 12$ Hz) and 4.47 (dd, $J = 12, 4.4$ Hz) which were assigned to H-2 and H-6 glucose hydrogen [38]. The signals of these hydrogens are downfield compared to those of β -D-glucopyranose, indicating the location of galloyl units at these positions [39]. The structure was confirmed by the analysis of the ^{13}C -NMR spectrum. Three carbonyl ester signals at δ_{C} 166.9, 168.2 and 168.3, confirm the presence of the three galloyl moieties. Signals at δ_{C} 94.5 (C-1), 68.3 (C-2) and 62.9 (C-6) indicate that the hydroxyl groups at these positions are galloylated [35]. Its molecular formula was defined as $\text{C}_{27}\text{H}_{24}\text{O}_{18}$ by HRESIMS at m/z 635.0890 $[\text{M} - \text{H}]^-$ (calculated for $\text{C}_{27}\text{H}_{23}\text{O}_{18}$, 635.0879, $\Delta = -1.7$ ppm). The structure of this gallotanin was confirmed by comparison of the above described spectra data with the literature and was identified as 1,2,6-tri-*O*-galloyl- β -D-glucopyranose [40].

The isolated compounds gallic acid and methyl gallate also had their structures confirmed by comparing the ^1H and ^{13}C NMR and mass spectrometry data with the literature data [40–42]. In such a way, the known compounds gallic acid, methyl gallate, 1,2,6-tri-*O*-galloyl- β -D-glucopyranose were identified.

Antinociceptive activity

The present study demonstrates that systemic administration of methanol extract of *D. vandellianum* leaves produces a consistent antinociceptive effect. Fractionation of *D. vandellianum* leaves showed that this effect could be attributed, at least in part, to their bioactive constituents gallic acid, methyl gallate and 1,2,6-tri-*O*-galloyl- β -D-glucopyranose.

The formalin test is a useful screening tool for assessment of the analgesic properties of plant extracts and compounds. In the present study, data from this assay indicated that ME induces a dose-dependent antinociceptive effect. Afterwards, this methanol extract was fractionated and the obtained fractions (ether, ethyl acetate and chloroform) were also evaluated in formalin test. All tested fractions presented antinociceptive effects *in vivo*, but the ethyl ether fraction was the most active. In line with this result, ethyl ether fraction was selected for the phytochemical study, evidencing three major compounds: gallic acid, methyl gallate and 1,2,6-tri-*O*-galloyl- β -D-glucopyranose. In order to evaluate the eventual contribution of these compounds to antinociceptive action of ME, their biological properties were next evaluated in the formalin test.

The results obtained from the present work demonstrate that GA induces antinociceptive effects in the late phase of the formalin test. From a pharmacological point of view, it has been proposed that drugs that block the nociception transmission, such as opioid analgesics, suppress both phases of formalin-induced pain response, whereas nonsteroidal anti-inflammatory drugs, such as indomethacin, seem to suppress only the late phase [43]. Based on these concepts, it is possible to propose that GA-induced antinociception is associated with anti-inflammatory properties. In line with this idea, the antinociceptive effect of GA has been demonstrated in the carrageenan-induced inflammatory pain model [44]. In addition, this compound has also been reported to have relevant anti-inflammatory and immunomodulatory activities [45–47].

Like GA, methyl gallate presented an antinociceptive effect on the inflammatory phase of formalin test, displaying analgesic activity with a similar profile of NSAIDs. Supporting this hypothesis, it was previously demonstrated that MG has anti-inflammatory and cyclooxygenase-2 inhibitory activities [48,49]. Even though the antinociceptive properties of gallic acid ethyl ester have been previously described [50], the present data demonstrate the antinociceptive effect of MG.

Few studies have reported the pharmacological properties of TGG, and most of them describe its antibacterial and antiviral activities [40,51–53]. A previous study has demonstrated antinociceptive activity of methanolic extract of *Miconia minutiflora* (Bonpl.) DC., which contains gallotannin class in its composition [54]. The present study displays the direct pharmacological evidence for the antinociceptive activity of gallotannin. TGG exhibited antinociceptive effect on the inflammatory phase of formalin test, and was more efficacious than indomethacin, the analgesic anti-inflammatory drug used as a gold standard in this test. Corroborating this relevant effect of TGG on the inflammatory phase of formalin, Erdélyi and coworkers demonstrated by *in vitro* experiments, that this class of compound is able to reduce the inflammatory cytokines expression [55]. Importantly, gallic acid, methyl gallate and 1,2,6-tri-O-galloyl- β -D-glucopyranose at antinociceptive doses did not affect the motor performance in mice in the rotarod test. These results corroborate the antinociceptive properties pointed by formalin test.

Despite consistent antinociceptive effects induced by systemic treatment with GA, MG and TGG, the contribution of others bioactive molecules to ME-induced antinociception cannot be ruled out. The ether ethyl fraction of ME presented 39 minor compounds, and it is well accepted that minor constituents in plant extracts may contribute to pharmacological properties of these extracts through synergistic actions or independent pharmacological effects [56]. In addition, among the minor compounds identified in ME, some of them, such as kaempferol and quercetin, have well-established antinociceptive activity [57,58]. Antinociceptive properties of quercetin have been demonstrated in both experimental and clinical conditions. Quercetin produces dose-related antinociception in several models of chemical pain, such as acetic acid test, formalin test, nociception induced by glutamate and capsaicin [57], and oxaliplatin-induced neuropathic pain [9], showing also analgesic properties in clinical conditions [59]. Similarly, kaempferol treatment is found to attenuate neuropathic pain [60] and chemical pain [61] in pre-clinical studies. Thus, it is possible that these compounds contribute to the antinociceptive effect of the ether ethyl fraction of ME, however, this hypothesis has not been investigated here.

Conclusions

The present study, using a classic model of analgesic drug screening combined with reliable methods of structural analysis, demonstrated that the methanolic extract of *D. vandellianum* leaves shows antinociceptive properties on experimental inflammatory pain. The isolation indicated that gallic acid, methyl gallate and 1,2,6-tri-O-galloyl- β -D-glucopyranose, major constituents of the ether fraction from ME, are the antinociceptive components of *D. vandellianum*. The antinociceptive properties demonstrated in formalin test were corroborated by the results of rotarod test, which did not demonstrate motor deficits and nonspecific depression in the nervous system. To the best of our knowledge this is the first report of the antinociceptive activity of 1,2,6-tri-O-galloyl- β -D-glucopyranose. The occurrence of these compounds in *D. vandellianum* is also described for the first time.

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Validation: Marcelo Sobral da Silva, Cristiane Flora Villarreal, Eudes da Silva Velozo, Josean Fechine Tavares.

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