Cycloartanes from *Krameria pauciflora* and Their *In Vitro* PLA₂, COX-1, and COX-2 Enzyme Inhibitory Activities

**Authors**
M. Ángeles Ramírez-Cisneros¹, María Yolanda Ríos¹, Ramiro Ríos-Gómez², A. Berenice Aguilar-Guadarrama¹

**Affiliations**
¹ Centro de Investigaciones Químicas, Universidad Autónoma del Estado de Morelos, Cuernavaca, Morelos, México
² Unidad de Investigación en Sistemática Vegetal y Suelo, FES Zaragoza, UNAM, México D. F., México

**Abstract**

*Krameria pauciflora* is a species belonging to the Krameriaceae family. It has been used to treat inflammatory disorders in folkloric Mexican medicine; however, chemistry and pharmacological studies have not been carried out on this species. In this work, from the dichloromethane root extract of *K. pauciflora*, five cycloartane-type triterpenoids were isolated: cyclomargenyl-3-0-β-coumaroyl ester (1), cyclomargenyl-3-0-β-caffeoyl ester (2), cyclomargenyl-3-0-β-coumaroyl ester (3), cyclomargenol (4), polystichicol, and cyclomargenone (5). Additionally, the lignane 6′-methoxyratiophenol was isolated. To the best of our knowledge, compounds 1–3 are new natural products, whereas compounds 4 and 5 are isolated for the first time in the *Krameria* genus and the Krameriaceae family. The structures of all of these compounds were established by 1D and 2D NMR spectroscopy including 1H, 13C, DEPT, COSY, HSQC, and HMBC experiments, as well as by EI mass spectrometry. There is an incomplete previous report about the spectroscopic data of compounds 4 and 5. However, in this work, a complete and unambiguous assignment has been realized. Due to the traditional use of this plant and other species from this genus, such as *K. lappacea*, cycloartanes isolated herein were evaluated by their inhibition of phospholipase A₂, cyclooxygenase-1, and cyclooxygenase-2 enzymes. Cyclomargenyl-3-0-β-caffeoyl ester (1) showed inhibition of phospholipase A₂, cyclooxygenase-1, and cyclooxygenase-2 target enzymes for non-steroidal anti-inflammatory drugs. Both cyclooxygenases were inhibited by cyclomargenol (4); however, cyclomargenyl-3-0-β-feruloyl ester (2) showed inhibition only on cyclooxygenase-1.

**Abbreviations**

EI: electronic impact
HREIMS: high-resolution electronic impact mass spectrometry
PLA₂: phospholipase A₂
COX-1: cyclooxygenase-1
COX-2: cyclooxygenase-2
MPLC: medium pressure liquid chromatography
CC: column chromatography

**Supporting information** available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

**Introduction**

The Krameriaceae family includes a single genus, *Krameria*, which is comprised of about 17 species. In traditional Mexican folk medicine, the roots of some of these species are used as anti-inflammatory, anti-diarrheal, and antibacterial agents. These roots are also used as an acne treatment and as astringents, among other uses [1,2]. In previous studies realized by Achenbach and collaborators [3–5], mostly lignane-type compounds were isolated from the roots of *Krameria* species. However, until recently, there were no phytochemical studies concerning the nonpolar extract from *Krameria pauciflora* MOC et. Sessé ex DC. The aim of this research was to establish the chemical composition of root extracts via dichloromethane extraction and to assess the *in vitro* inhibitory activity on prostaglandin synthesis through PLA₂, COX-1, and COX-2 enzymes of pure secondary metabolites. Our research group has previously studied other plants containing diterpenes and sesquiterpenes with anti-inflammatory activity [6,7]. In this study, we characterized...
five cycloartane-type triterpenes, which are compounds that have previously been determined to have anti-inflammatory [8], cancer chemopreventive [9], and other activities. Therefore, the in vitro inhibitory activity on prostaglandin synthesis from compounds 1, 2, 4, and 5 was evaluated by investigating the ability of these compounds to inhibit enzymes involved in inflammation, such as PLA2, COX-1, and COX-2. Our findings suggest that 1, 2, and 4 have inhibitory activity against these enzymes similar to indomethacin.

Material and Methods

General experimental procedures

All purification processes carried out with MPLC were performed on Büchi® equipment with a pump controller C-610 and a C-601 pump unit. NMR spectra were recorded using 200 MHz Varian® and 400 MHz Varian-Unity® spectrometers. Samples were dissolved in a mix of CDCl3, acetone-d6, and benzene-d6 (1:1:1), and also CDCl3 as indicated in each case. IR spectra were obtained in film by a dichloromethane solution in a NaCl cell, with an IR Bruker Vector 22® device, with a fixed spectral window from 400–4000 cm⁻¹. Mass spectra were recorded on a high-resolution JEOL JMS – 700® spectrometer through the EI technique. GC-MS were recorded using a 6890 Agilent® device coupled with a 5973 Agilent® spectrometer. Cayman Chemical® kits, catalogue numbers 10004883 and 560131, were used for PLA2 and both COX enzymes to test for inhibitory activity, respectively, following the manufacturer’s instructions. Absolute ethanol was used in these evaluations. Absorbances of these samples were measured in a Bio-Rad 680® microplate reader.

Plant material

Krameria pauciflora MOC et. Sesé ex DC. roots were collected in Durango, México, in November 2005 by M.C. Ramiro Ríos Gómez from Facultad de Estudios Superiores Zaragoza (FEZA), UNAM. A specimen (voucher # 9545) was deposited in the herbarium of this same faculty.

Extraction and isolation

Dry and powdered K. pauciflora roots (3.6 kg) were extracted with dichloromethane (3 × 4 L × 48 h). The solvent was recovered using a rotary evaporator to obtain 28.2 g of extract. The purification process was performed with CC and MPLC, using silica gel 230–400 mesh as the stationary phase unless otherwise specified. Then 23.5 g of extract was adsorbed on 20 g of silica gel 60 F254 and subjected to CC in a 1.7 i.d. open column packed with 5” silica gel humidified with the mobile phase. Eluates of 130 mL were collected using a dichloromethane-acetone stepwise solvent system (5%, 10%, 15%, 20%, 25%, 50%, 100% acetone and, finally, with 100% MeOH) affording 90 fractions. The chromatographic process was monitored by TLC. TLC sheets (Silica gel 60 F254; Merck®) were observed with UV light and subsequently developed using ceric ammonium sulfate. On the basis of TLC similarities, fractions were pooled into four groups (A–D). Group A (0.5 g) was not purified further since it was constituted mainly of fatty acids identified in GC-MS as palmitic, stearic and oleic acids, oleic acid methyl ester, palmitic acid, isopropyl ester, and stigmasterol. Group B (2.5 g) was resolved into pure compounds by means of column chromatography in a 0.8 i.d. column with 6” silica gel eluted with the n-hexane-acetone system (0–50%, acetone) collecting 250 fractions of 210 mL. Fractions of this chromatographic process were classified into five subgroups (BI–BV).

Further CC purification of the BII group in a 0.4” i.d. column packed with 6” of silica gel collecting 2 mL fractions eluted with ethyl ether (100%) and then with n-hexane (100%) yielded pure 5 (10 mg); this process finished in fraction 25. The BII group was purified on a TLC sheet eluted with a dichloromethane-acetone system (98:02, rf 0.5), yielding 3 (5 mg). Purification on the BIV group by CC (0.4” i.d., 6” silica) with the n-hexane-acetone system (0%–5%, acetone) collecting 55 eluates of 2 mL and then applied on a TLC sheet and eluted with the n-hexane-acetone system (90:10, rf 0.3) achieved the isolation of 5 mg of the lignane E-3,5-dimethoxy-4-(6-prop-1-enyl)benzofuran-2-yl-phenol.

Group C (8 g) was subjected to CC (dichloromethane-acetone system, 0–10% acetone) in a 1.7” i.d. column with 6” of silica gel. 90 eluates of 50 mL were collected and fractions were merged into four groups (CI–CIV). Compound 4 was obtained from CII MPLC purification (n-hexane-acetone system 0%–5%, acetone) using a 0.4” i.d. cartridge and a 5 mL/min flow, collecting a fraction of 40 mL with n-hexane; 10 mL of the next eluates were collected for a total of 190. Fractions 64–77 contained 4 (n-hexane-acetone 98:02, 35 mg). Additional TLC purification of fractions 78–106 of the previous process yielded 2 (15 mg, rf 0.35). CC of the CIII group (dichloromethane-acetone system, 0–10%, acetone) using a 1.7” i.d. column packed with 6” of silica gel collected a total of 80 fractions of 50 mL. Then, with the n-hexane-EtOAc system (5–40%, EtOAc) in a 0.8” i.d. column with 6” of silica gel, 127 fractions of 10 mL were collected. Finally, after purification in a Pasteur pipette packed with 2” silica gel (40 mL of the n-hexane fraction), elution was carried out with n-hexane-acetone (97:03) allowing us to furnish 1 (30 mg); a total of 85 fractions of 0.5 mL were collected.

Chemicals and enzymes

Cycloartanes purified from K. pauciflora were tested for their PLA2, COX-1, and COX-2 inhibitory capacity. Indomethacin was purchased from Sigma® (99%). Human sPLA2-type V, sPLA2 assay buffer (10x), sPLA2 DNTB, sPLA2 diheptanoyl thio-PC (substrate), prostaglandin screening EIA (enzyme immunoassay), anti-solvent, prostaglandin screening AChE (acetylcholinesterase), tracer, prostaglandin screening EIA standard, EIA buffer (10x), wash buffer (400x), Tween 20, mouse anti-rabbit IgG coated plate, ELL-man’s reagent, reaction buffer (10x), COX-1 (oxine), COX-2 (human recombinant), hem, arachidonic acid (substrate), potassiumhydroxide, hydrochloric acid, and stannous chloride were purchased from Cayman Chemical®.

PLA2 inhibitory activity

Cycloartanes were evaluated for their ability to inhibit PLA2 activity using the sPLA2 (type V) inhibitor screening assay kit following the manufacturer’s instructions. Briefly, PLA2 (0.0–5.0% solution) was diluted 1:50 with assay buffer, this solution was then used to performed the assay. The enzyme (10 µL) was added to all wells and mixed with 10 µL of cycloartanes dissolved in ethanol to give final concentrations of 10, 35, and 100 µM. The reactions final concentration of enzyme was 0.0–4.5 × 10⁻³%. The reaction buffer (10 µL) was mixed with 10 µL of ethanol as a control of no activity. Reactions were initiated adding 200 µL of substrate solution (diheptanoyl thio-PC) to all wells. The microplate was shaken for 30 seconds, covered, and incubated for 15 minutes at 25°C. After that, the color was developed by the addition of 10 µL of DTNB, the plate was shaken, and after a minute, the absorbance was determined at 415 nm.
COX inhibitory activity
Cycloartanes were tested for their ability to inhibit COX-1 and
COX-2 prostaglandin synthesis using a COX inhibitor screening
assay kit according to the reported method [6] and the manufac-
turer’s instructions.

Statistical analysis
All assays were performed in triplicate. A significant difference
was statistically determined by a variance analysis test (one-way
ANOVA). Tukey’s and/or Dunnett’s test(s) were performed. P
values ≤ 0.05 were considered to be statistically significant.

Cyclomargenol-3-β-caffeoyl ester (1): amorphous white solid;
UV (CH2Cl2) λmax (log ε) 318 (1.73), 293 (1.57), 308 (1.56), 230
(1.23), 246 (0.94) nm; [α]D + 30.5 (c 0.4; CH2Cl2); IR νmax (film)
3070 (CH2 cyclopropane ring), 2926, 2854 (CH2 and CH3), 1709
(=C-C-O–-system), 1682 (C=O unsaturated), 1605 (double
bonds), 887 (terminal CH2), 850 and 810 (aromatic ring trisub-
tuted) cm⁻¹; 1H and 13C NMR data were recorded on 400 and
100 MHz, respectively, in a 1:1:1 CDCl3-acetone mixture (Table 1).
1D and 2D spectra are available as Supporting Information.

Results and Discussion

Dichloromethane extract from K. pauciflora roots was fraction-
ated by CC, MPLC using silica gel as the stationary phase or TLC
to obtain five cycloartane-type triterpenes and a lignan (Fig. 1).
Cyclomargenol-3-β-caffeoyl ester (1), cyclomargenol-
3-β-feruloyl ester (2), and cyclomargenol-3-β-coumaroyl

Supporting information
Original spectra for compounds 1–5 are available as Supporting
Information.

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* Spectroscopic data acquired in a mix of deuterated solvents in order to obtain a
more defined spectra.
ester (3) are novel natural products. Therefore, we elucidated their total molecular structures, whereas cyclomargenol (4, polystichol) and cyclomargenone (5) are compounds that have been previously reported in the literature [10–14]; however, their structures were not previously completely assigned by $^1$H NMR and $^{13}$C NMR spectroscopic data. In our research, we were able to assign their structures unequivocally, based on the detailed analysis of COSY, HSQC, and HMBC experiments.

Compound 1 was obtained as a white, amorphous solid, and its molecular formula, $C_{41}H_{60}O_4$, was deduced based on HREIMS m/z 616.4487 [M]+, which indicates twelve degrees of unsaturation. $^{13}$C NMR spectrum showed forty-one carbon signals, whereas the DEPT experiment found only 7CH$_3$, 13CH$_2$, and 11CH signals. Thus, we established that ten of the carbons should be quaternary. The IR spectrum showed an absorption band at 3070 cm$^{-1}$, which belongs to the methylene group of a cyclopropane ring. The presence of this methylene group is further corroborated by two signals at $\delta_{H}$ 0.18 (1H, $d$ = 4.0 Hz) and $\delta_{H}$ 0.45 (1H, $d$ = 4.0 Hz) in the $^1$H NMR spectrum, which are characteristic signals for $\beta/\beta$,19-cycloartane triterpenoids. Accordingly, seven methyl signals (a triple, a double and five single signals) were observed in the range of $\delta_{H}$ 0.80 and $\delta_{H}$ 1.5. A triple signal corresponding to methyl group H-32 ($\delta_{H}$ 0.83, $\delta_{C}$ 12.8) was found up-field, and its multiplicity indicated an interaction with methylene H-31 ($\delta_{C}$ 26.7). In the HMBC experiment (Fig. 2), H-32 showed long-range correlation peaks with C-31 and C-24, thus confirming the presence of the ethyl group on C-24. Of the six remaining methyls, five were attached to a quaternary carbon, one of which (H-26, $\delta_{H}$ 1.55) was a vinyl methyl group according to its chemical shift and which had a long-range correlation peak with C-25 ($\delta_{C}$ 148.3) and C-27 ($\delta_{C}$ 112.4). The hydrogens ($\delta_{H}$ 4.74 and $\delta_{H}$ 4.80) of C-27 correlated with C-24 and C-26, corroborating the C25–C27 unsaturation.

In the HMBC experiment, the methyl signal at $\delta_{H}$ 0.98 ($\delta_{C}$ 18.9) was assigned as H-18 due to its correlation with carbons 12, 13, 14, and 17. Methyl group H-21 ($\delta_{H}$ 0.90, $d$, $6.4$ Hz, $\delta_{C}$ 19.0) showed a COSY cross-peak to H-20 and a heteronuclear long-range peak in HMBC with C-17, C-20, and C-22. Moreover, H-17 displayed a correlation to the methylene at $\delta_{C}$ 29.0 (C-16), and H-16 showed a correlation to C-17 and C-15. This structural fragment was corroborated by a COSY experiment where it was possible to observe the spin system correlation involving H-15–16–17. In accordance with additional evidence found in the HMBC experiment, it was possible to assign methyls H-29, H-28, and H-30 (Table 1) as well as a hydroxyl group on C-7 [23]. Otherwise, it is important to note that the coupling constants shown in H-3 ($J$ = 4.4 Hz ax-equatorial and 12.0 Hz ax-axial) are typical for pseudo-axial (β) orientation of oxygen and pseudo-axial (α) orientation of hydrogen attached to C-3. By means of the correlation observable at H-3 and H-5 with H-28 in the NOESY experiment, it was evident that all of them have an α orientation (Fig. 3).

Additionally, two vinyl protons at $\delta_{H}$ 6.38 (1H, $d$, $J$ = 16.0 Hz) and $\delta_{H}$ 7.78 (1H, $d$, $J$ = 16.0 Hz) were observed in $^1$H NMR. These chemical shifts and coupling constants suggest a trans-α,β-unsat-
urated carbonyl ($\delta_c$ 167.3 and 1683 cm$^{-1}$ IR absorption). Finally, an aromatic ring was deduced by the occurrence of three protons at $\delta_H$ 7.22 (1H, $d_J = 1.6$ Hz), $\delta_H$ 6.82 (1H, $d_J = 8.0$, 1.6 Hz), and $\delta_H$ 6.90 (1H, $d_J = 8.0$ Hz). These characteristics are indicative of eight degrees of unsaturation, up to now, and therefore a pentacyclic triterpene, including the cyclopropane ring (vide supra), led to a cycloartane structure. In $^{13}$C NMR, two aromatic ring carbon resonances were displaced at $\delta_c$ 147.9 and 146.0, suggesting the presence of hydroxyl groups on these carbons. Together with the coupling constants analysis, it corresponded to the caffeoyl group on C-3. Supporting this fact, in HMBC, a long-range crosspeak to methyne H-3 $\delta_H$ 4.85 ($\delta_c$ 80.7) with C-4, C-28, C-29 and C-1' ($\delta_c$ 167.3) carbonyl in the C6-C3 unit was observed. This allowed us to establish the structure of 1 as cycloargenyl-3-O-$\beta$-caffeoyl ester.

The natural product 2 was obtained as an amorphous powder. Its molecular formula was established on the basis of HREIMS as C$_{42}$H$_{62}$O$_4$, $m/z$: 630.4669 [M$^+$]. In the $^1$H NMR spectrum of 2, it is possible to observe similar signals, which suggests that they had the same aglycone skeleton of compound 1. The main difference between compound 2 and 1 is an additional methoxy group signal at $\delta_H$ 3.95, indicating that it must correspond to a methyl ether derivative of 1. According to the HMBC experiment, 2 is a furelic derivative on position 3 of aglycone, establishing that 2 corresponds to cycloargenyl-3-O-$\beta$-feruloyl ester. 2D NMR experiments are in accordance with the triterpene moiety of compound 1 (Tables 1S and 2S, Supporting Information). Compound 3 was obtained as a colorless solid with a molecular formula of C$_{41}$H$_{60}$O$_3$ on the basis of HREIMS, $m/z$: 600.4525 [M$^+$]. It was possible to infer the symmetry in this molecule by its $^{13}$C NMR spectrum, which presents only 39 carbon signals. The $^1$H NMR contains the same characteristic signals for cycloargenyl triterpenes (1 and 2). A unique AB system with integration for one proton in the aromatic proton resonance region, with an ortho coupling constant, establishes that 3 corresponds to cycloargenyl-3-O-$\beta$-coumaroylester. Complete assignment of compounds 1-3 NMR signals was established based on 1D and 2D NMR (Tables 1S and 2S and Fig. 15-19S, Supporting Information). Cycloargenyl-3-O-$\beta$-coumaroyl ester has been reported in the literature [24]; however, the $\beta$ orientation for the coumaroyl group attached to C-3 has been proposed, which is inconsistent with the informed coupling constant values ($J = 9.0$, 8.0 Hz). These coupling constant values correspond to a 45° dihedral angle between H-3 and both H-2s, indicating an $\alpha$ orientation for the coumaroyl group. Compound 3 isolated in this work showed coupling values of $J = 11.2$ and 4.4 Hz (ax-ax and ax-eq, respectively), making it clear that it is the $\beta$ orientation for the coumaroyl group [25]. Additionally, NOE correlations with H-5 and CH$_3$-28 confirm the $\alpha$ orientation of H-3.

Cycloargenol (4) and cycloargenone (5) are compounds already reported in the literature [10-14, 26]; however, the spectroscopic data are not described completely for these two metabolites. For this reason, the present work assignment of NMR data on the basis of 1D and 2D experiments (Table 1S and 2S, Supporting Information) was completed. Cycloartane triterpenes have been isolated in K. parvifolia [5], K. grayi [4], and K. ixina [27]. On the other hand, E-3,5-dimethoxy-4-[6-(prop-1-enyl)benzofuran-2]-yl-phenol was isolated in this work and its spectroscopic data were compared with those reported in the literature. This lignane was previously found in the K. ramosissima extract [28].

Triterpene compounds, including cycloartane-type triterpenes, reportedly have anti-inflammatory and/or quimiopreventive activities [8,9,29-33]. Cycloartane triterpenoids from K. pauciflora exhibited in vitro inhibitory effects of the NSAIDs target enzymes PLA$_2$, COX-1, and COX-2 (Figs. 4-6). Compound 1 was assayed and compared with the control by PLA$_2$ inhibition, and reduction of about 50% of prostaglandin production was achieved when it was tested at 10 µM ($p \leq 0.05$), although 1 exhibited an effect that was not dependent on the concentration (Fig. 4). PLA$_2$ was also inhibited by compound 2 when it was tested at 10 µM (Fig. 4). Compounds 4 and 5 were not active in the present assay.

As we can see in Fig. 5, compounds 1, 2, and 4 exhibited similar COX-1 enzyme inhibitory activity. We can observe that COX-1...
was weakly inhibited by compounds 1, 2, and 4 at the concentrations tested. These three compounds exhibited an inhibition independent of the concentration and similar to indomethacin (10 µM, p ≤ 0.05) even when they were tested at 100 µM, which is a notable fact because COX-1 activity is important for gastrointestinal protection. Furthermore, compound 4 inhibited less than 50% of the prostaglandin synthesis through COX-1 even when it was assayed at 100 µM (Fig. 5). COX-2 was inhibited by compounds 1 and 4, although they did not show a concentration-dependent effect. When they were compared with indomethacin at the same concentration (10 µM), metabolites from *K. pauciflora* exhibited a more potent effect than indomethacin, which showed an inhibition of 40.8%, and compounds 1 and 4 showed an inhibition of 51.9 and 71.3%, respectively (Fig. 6). It should be mentioned that when compound 4 was tested at 100 µM, production of prostaglandins was reduced more than 80%, which is outside the standard curve. Compound 5 showed no inhibition against COX-1 and COX-2.

In summary, compound 1 inhibited the three enzymes, compound 2 inhibited COX-1 and PLA₂ only when it was tested at 10 µM, and compound 4 exhibited a significant reduction of activity of both COX enzymes at the concentrations tested. It is worth noting that when compound 4 was tested at 10 µM against COX-1 and COX-2, the inhibitory effect was greater for COX-2 than for COX-1.

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**Conflict of Interest**

All authors declare no conflict of interest.

**References**