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Original article

Bioactive dicaffeoylquinic acid derivatives from the root extract of *Calea urticifolia*☆

Ivan Felipe Mijangos-Ramos[a,1], Hiatzy E. Zapata-Estrella[a,1], Javier A. Ruiz-Vargas[a], Fabiola Escalante-Erosa[a], Noemi Gómez-Ojeda[a], Karlina García-Sosa[a], Valdir Cechinel-Filho[b], Nara L. Meira-Quintão[b], Luis M. Peña-Rodríguez[a,*]

[a] *Laboratorio de Química Orgánica, Unidad de Biotecnología, Centro de Investigación Científica de Yucatán, Mérida, Yucatán, Mexico*
[b] *Núcleo de Investigações Químico-Farmacêuticas, Universidade do Vale do Itajaí, Itajaí, SC, Brazil*

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ABSTRACT

Calea urticifolia (Mill.) DC., Asteraceae, is a native plant of the Yucatan Peninsula used in traditional medicine to treat inflammation and pain. The bioassay-guided purification of the ethanol root extract allowed the isolation of the main bioactive metabolites, which were identified as an inseparable mixture of thymol (**1**) and 3-methyl-4-isopropylphenol (**2**), together with 3,4-*O*-dicaffeoylquinic acid methyl ester (**3**), 3,4-*O*-dicaffeoyl-*epi*-quinic acid methyl ester (**4**), 3,5-*O*-dicaffeoyl-*epi*-quinic acid methyl ester (**5**) and 3,5-*O*-dicaffeoylquinic acid (**6**). The results showed that the analgesic activity detected in the root extract of *C. urticifolia* could be attributed mainly to the mixture of **1** and **2** and to the novel 3,4-*O*-dicaffeoyl-*epi*-quinic acid methyl ester (**4**). Alternatively, the similarity on the antiinflammatory and antioxidant activities of the dicaffeoylquinic acid derivatives **3**–**5** suggests that the former might be related to their ability as radical scavengers.

Introduction

One of the native plants commonly used in the practice of Yucatecan traditional medicine to alleviate pain and inflammation is *Calea urticifolia* (Mill.) DC., Asteraceae, commonly known as "amargo del monte" and used as a traditional remedy for the treatment of stomach ache, malaria and gastric ulcers (Méndez-González et al., 2010). Previous phytochemical studies of *C. urticifolia* have resulted in the isolation of sesquiterpene lactones, germacranolides, heliangolides, as well as isoeugenol and phloroglucinol derivatives (Del Castillo et al., 1981; Yamada et al., 2004). Additionally, sesquiterpene lactones from *C. urticifolia* have been reported to show various biological activities including antioxidant (Umemura et al., 2008), inhibition of NF-*k*B (Hehner et al., 1999), induction of apoptosis (Nakagawa et al., 2005), suppression of adipocyte differentiation (Matsuura et al., 2005), and inhibition of melanin biosynthesis (Ohguchi et al., 2009).

Recently, as part of our search for new analgesics and antiinflammatory agents from medicinal plants of the native flora of the Yucatan peninsula, we detected the presence of both antioxidant and analgesic activities in the root extract of *C. urticifolia* (Zapata-Estrella et al., 2014); here we wish to report on the isolation and identification of the bioactive metabolites responsible for the biological activities detected in the organic crude extract of the plant.

Materials and methods

General experimental procedures

FTIR spectra were recorded in $CHCl_3$ or MeOH (film) using an FT-Nicolet Magna Protégé 460 spectrophotometer. GC–MS analyses were carried out using an Agilent Technologies 6890N Gas Chromatograph coupled to a 5975B Mass Selective Detector (GC conditions: J&C Ultra 1 column 25 m long, ID 0.32 mm, film 0.5 mm), flow rate (He) of 1 ml/min and a temperature program of T_1 = 75 °C (4 min), T_2 = 200 °C (11 min), gradient = 5 °C/min.

☆ Even though no *in vivo* assay results are detailed in this report; all procedures referred to in the discussion followed the "Principles of Laboratory Animal Care" from NIH publication no. 85–23 and were approved by the Animal Ethics Committee of UNIVALI (Protocol numbers 599/2007 UNIVALI). The number of animals and the intensity of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the treatments.

* Corresponding author.
E-mail: lmanuel@cicy.mx (L.M. Peña-Rodríguez).

[1] Both authors contributed equally to this work.

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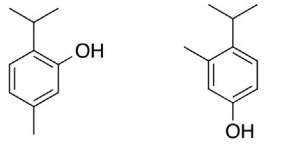
Mass spectra were obtained using a JEOL-JMS-SX102 mass spectrometer. NMR spectra (^{1}H, ^{13}C) and two-dimensional experiments (COSY, HSQC and HMBC) were acquired on a JEOL GSX 270 MHz (Jeol Europe, France) and Avance III 500 system (Bruker, Germany). HRESIMS were measured using an LC (D)ESI/LTQ Orbitrap (Thermo Scientific). Analytical TLC analyses were carried out using aluminum-backed silica gel (60F254) plates (E.M. Merck, 0.2 mm thickness); the plates were first examined under UV light (254 and 366 nm) and the various components in the chromatograms were visualized by dipping the plates in a solution of phosphomolybdic acid (20 g) and ceric sulfate (2.5 g) in 500 ml of sulfuric acid (5%), followed by drying and gentle heating. For the detection of components with radical scavenging activity, the plates were sprayed with a 0.2% ethanolic solution of 2,2-diphenyl-1-picrylhydazyl (DPPH). Open column chromatography purifications were performed using silica gel (Aldrich, 70–230 mesh); TLC-grade silica gel 60 GF_{254} (E.M. Merck) was used for vacuum liquid chromatography (VLC).

Plant material

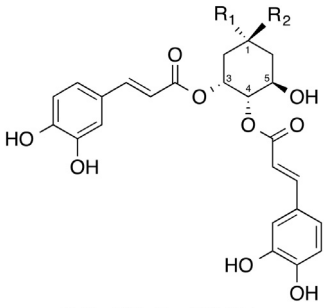
Plant material, *Calea urticifolia* (Mill.) DC., Asteraceae, was collected in November 2009 at the 9.5 km mark of the Yaxcabá-Libre Unión highway in Yucatan, México, and identified by field specialist Paulino Simá-Polanco; a voucher specimen was deposited in the Herbarium of Unidad de Recursos Naturales, Centro de Investigación Científica de Yucatán under collection number PSIMA-3010.

Extraction and isolation

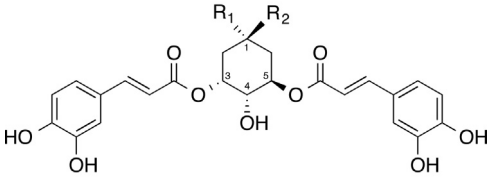
Roots of *C. urticifolia* (738 g) were dried for three days at room temperature, followed by three days in an oven at 50 °C. The dry and ground plant material was macerated (three times) with EtOH (14 l) at room temperature for 72 h. Evaporation of the solvent under reduced pressure yielded 46 g (6.23%) of the crude extract (CUR). The CUR extract was suspended in 370 ml of H_2O–MeOH (3:2) and the resulting suspension was partitioned successively between hexane (2.5 l) and EtOAc (2.5 l). The active hexane fraction (23.79 g) was successively fractionated and purified using VLC [gradient elution with hexane–CH_2Cl_2 (1 l)] and open column chromatography [hexane–Me_2CO 9:1 (800 ml)], resulting in the isolation of a fraction (140.7 mg) containing an inseparable mixture of components identified by GC-MS as 2-isopropyl-5-methylphenol (thymol, **1**) and 3-methyl-4-isopropylphenol (**2**) (Figs. S-1 and S-2 in Supplementary Material). The active EtOAc fraction (8.59 g, 18.67%) was fractionated by VLC [gradient elution with CH_2Cl_2–hexane–MeOH (3 l) and CH_2Cl_2–MeOH–H_2O (1.5 l)] to produce eight major fractions (2A-H). Fraction 2G (6.52 g) was purified using VLC ($CHCl_3$–MeOH–EtOAc–H_2O 2:2:4:1) and open column chromatography ($CHCl_3$–MeOH–H_2O 70:30:10 + 50 μl of formic acid for each 10 ml) to produce 98.2 mg of 3,4-*O*-dicaffeoylquinic acid methyl ester (**3**), 50.2 mg of 3,4-*O*-dicaffeoyl-*epi*-quinic acid methyl ester (**4**), 108 mg of 3,5-*O*-dicaffeoyl-*epi*-quinic acid methyl ester (**5**), and 30.9 mg of 3,5-*O*-dicaffeoylquinic acid (**6**). Structures of metabolites **3**, **5**, and **6** were confirmed by comparing their spectroscopic data with those reported in the literature (Zhang et al., 2000; Wald et al., 1989; Pauli et al., 1998) (see Supplementary Material for detailed spectroscopic data of metabolites **3**, **5**, and **6**).



1 **2**



3 R_1=OH; R_2=CO_2CH_3
4 R_1=CO_2CH_3; R_2=OH



5 R_1=CO_2CH_3; R_2=OH
6 R_1=OH; R_2=CO_2H

Spectroscopic data

3,4-*O*-Dicaffeoyl-*epi*-quinic acid methyl ester (**4**): yellow powder; R_f 0.52 ($CHCl_3$/MeOH/H_2O 70:30:10 + 50 μl of formic acid for each 10 ml of solvent); IR (film) λ_{max}: 3390, 2955, 1695, 1598 cm^{-1}; ^{1}H NMR (500 MHz, CD_3OD) δ 2.18 (2H, m, H-2), 2.33 (2H, dd, *J* = 3.85, 14.45 Hz, H-6), 4.32 (1H, ddd, *J* = 4.3, 8.45, 8.40 Hz, H-5), 5.03 (1H, dd, *J* = 3.20, 8.20 Hz, H-4), 5.62 (1H, dd, *J* = 3.70, 5.10 Hz, H-3), 6.26 (1H, d, *J* = 15.90 Hz, H-8″), 6.26 (1H, d, *J* = 15.85 Hz, H-8″), 6.73 (1H, d, *J* = 8.15 HZ, H-5′), 6.76 (1H, d, *J* = 8.20 Hz, H-5″), 6.88 (1H, dd, *J* = 1.60, 8.20 Hz, H-6″), 6.91 (1H, dd, *J* = 1.65, 8.20 Hz, H-6′), 7.02 (1H, d, *J* = 1.85 Hz, H-2″), 7.03 (1H, d, *J* = 1.80 Hz, H-2′), 7.54 (1H, d, *J* = 15.85 HZ, H-7″), 7.55 (1H, d, *J* = 15.85 Hz, H-7′); ^{13}C NMR (125 MHz, CD_3OD): δ 176.12 (C-7), 168.52 (C-9′), 168.44 (C-9″), 149.68 (C-4′), 149.64 (C-4″), 147.39 (C-3′, 3″), 146.81 (CH, C-7′, 7″), 127.70 (C-1′), 127.63 (C-1″), 123.25 (CH, C-6′), 123.14 (CH, C-6″), 116.53 (CH, C-5′), 116.46 (CH, C-5″), 114.94 (CH, C-2′, 2″), 114.80 (CH, C-8′, 8″), 75.56 (C-1), 75.12 (CH, C-4), 69.82 (CH, C-3), 66.03 (CH, C-5), 53.00 (CH_3, C-8), 41.00 (CH_2, C-6), 36.40 (CH_2. C-2); HRESIMS (positive ion mode) *m/z* 569.1055 [M+K]$^+$ (Calcd. for $C_{26}H_{26}O_{12}K$: 569.1061).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical reduction assay

The assay was carried out as previously described (Zapata-Estrella et al., 2014).

Results and discussion

The initial fractionation of the bioactive root extract of *C. urticifolia* produced two semi-purified fractions that showed a significant antioxidant activity when tested in the DPPH radical scavenging assay. The bioassay-guided purification of the hexane fraction (EC_{50} 2.069 ± 0.59 mg/ml) resulted in the isolation of a fraction showing a single bioactive spot on TLC; a GC–MS analysis of the purified fraction showed the presence of two components

at t_R 12.03 and 12.23 min (*ca.* 45% and 55%, respectively; Fig. S-1), both showing a parent ion peak at *m/z* 150 (Fig. S-2), but each having a different fragmentation pattern that, when compared to those in the database of the equipment, suggested their corresponding to 2-isopropyl-5-methylphenol (thymol, **1**) and 3-methyl-4-isopropylphenol (**2**). The identification of the two metabolites was confirmed by comparing their ^{1}H and ^{13}C NMR data with those reported in the literature (Aeschbach et al., 1994) and by comparing the retention time of **1** with that of an authentic sample. The ubiquitous thymol (**1**) has been reported to have antioxidant (Esmaeili and Khodadadi, 2011) analgesic (Mastelić et al., 2008) and antimicrobial (Uma et al., 2011) activities. Similarly, 3-methyl-4-isopropyl phenol (**2**), which has been previously isolated from *Plectranthus amboinicus*, is also reported as a bioactive metabolite with antimicrobial, antioxidant and analgesic activity (Uma et al., 2011).

A similar bioassay-guided purification of the EtOAc fraction (EC_{50} 0.467 ± 0.010 mg/ml) resulted in the isolation of three bioactive isomers of dicaffeoylquinic acid methyl ester (**3**–**5**) and a derivative of dicaffeoylquinic acid (**6**). The ESIMS analyses of **3**, **4** and **5** showed the same parent ion peak at *m/z* 530, corresponding to a molecular formula of $C_{26}H_{26}O_{12}$ for the three metabolites. Metabolite **3** was identified as 3,4-*O*-dicaffeoylquinic acid methyl ester (Zhang et al., 2000), a metabolite commonly isolated from Asteraceae plant species (Park, 2010; Akihisha et al., 2013) and recognized as having antioxidant and anti-inflammatory activities (Hung et al., 2006).

The ^{1}H and ^{13}C NMR spectra of metabolite **4** proved to be very similar to those of **3**, with key differences in the chemical shift values of C-1 (74·19 in **3**, 75.56 ppm in **4**) and the ester carbonyl carbon (173.66 ppm in **3**, 176.12 ppm in **4**), which coincided with those reported for the same carbons in 3,5-*O*-dicaffeoylquinic acid and its C-1 epimer (Wang, 2009). Furthermore, the small downfield chemical shift observed for C-3 in the ^{13}C NMR of **4** (67.00 ppm in **3**, 69.82 ppm in **4**) is in agreement with that resulting from a γ-steric effect that occurs when a heteroatom and the carbon in the gamma position are in a gauche conformation (Fig. 1) (Luo et al., 2013). This evidence allowed the identification of **4** as 3,4-*O*-dicaffeoyl-*epi*-quinic acid methyl ester, a dicaffeoyl-*epi*-quinic acid derivative that, to the best of our knowledge, has not been described before.

Metabolite **5** showed similar ^{1}H and ^{13}C NMR data to that of **4**, with the main differences observed in the HMBC experiment, specifically the ^3J correlations between H-3 and H-5 (δ 5.31 and 5.23) and the ester carbonyl carbons at 167.12 and 166.32 ppm, respectively. These correlations identified C-3 and C-5 as the esterified positions and allowed the identification of **5** as 3,5-*O*-dicaffeoyl-*epi*-quinic acid methyl ester (Zhang et al., 2000), a bioactive metabolite with antioxidant and anti-inflammatory activities previously isolated from Asteraceae plant species (Hung et al., 2006; Wang, 2009).

The HRESIMS analysis of the last metabolite showed a protonated ion peak at *m/z* 517, indicating a molecular formula of $C_{25}H_{24}O_{12}$. The ^{1}H and ^{13}C NMR data proved to be very similar to those of **5**, except for the absence of the methoxyl group methyl signal at δ 3.60 which allowed its identification as the well-known 3,5-*O*-dicaffeoylquinic acid (**6**) (Tolonen et al., 2002; Pauli et al., 1998), a metabolite reported to have a strong antioxidant activity in both the DPPH radical scavenging assay (Akihisha et al., 2013; Wu et al., 2007; Hung et al., 2006) and the peroxynitrite scavenging assay (Nugrojo et al., 2009), as well as being neuroprotective (Nakajima et al., 2007), hepatoprotective (Wu et al., 2012), analgesic (Dos-Santos et al., 2005) and anti-inflammatory (Dos-Santos et al., 2010; Park et al., 2009; Hung et al., 2006).

The caffeoylquinic acid derivatives represent an important group of natural products, commonly found in plants of the Asteraceae family as mono- or dicaffeoylquinic acid derivatives, with the carboxylic acid at C-1 often methylated (Fraisse et al., 2011). Although quinic acid is most commonly found as the parent skeleton, *epi*-quinic acid derivatives can also occur (Nugrojo et al., 2009). The strong antioxidant activity observed for the dicaffeoylquinic acid methyl ester derivatives (**3**–**5**), when tested in the DPPH radical scavenging assay, suggested their being responsible for the antioxidant activity detected in both the crude root extract from *C. urticifolia* and the semipurified EtOAc fraction (Table 1). These results are in agreement with literature reports describing caffeoylquinic acid derivatives as the major antioxidant metabolites commonly isolated from Asteraceae plant species (Fraisse et al., 2011; Kim and Lee, 2005). It has also been reported that the catechol moiety, combined with the unsaturated ester moiety in the structure of the caffeoylquinic acid derivatives, is essential for the

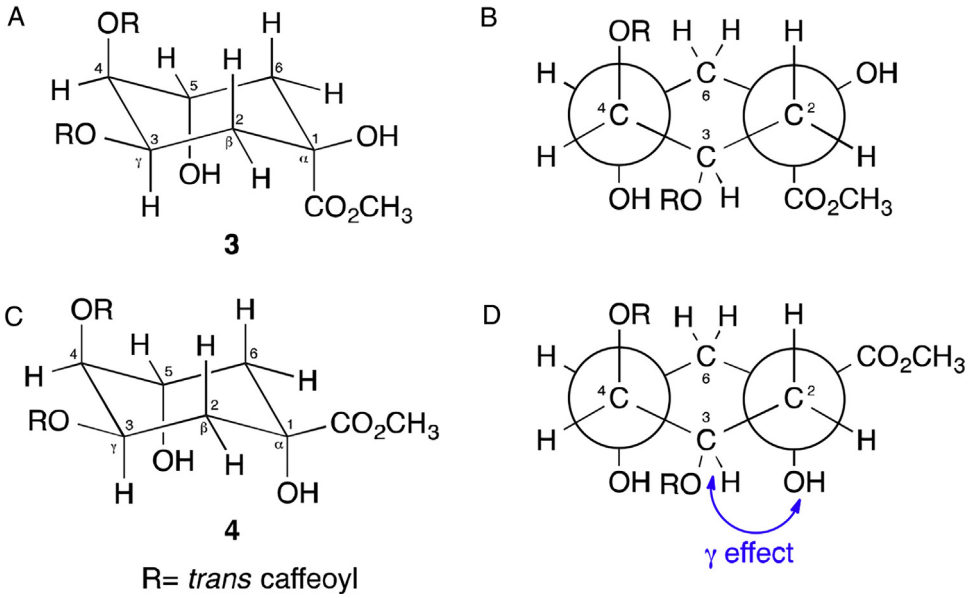


Fig. 1. Chair conformations (A, C) and Newman projections (B, D) of 3,4-*O*-dicaffeoylquinic acid methyl ester (**3**) and 3,4-*O*-dicaffeoyl-*epi*-quinic acid methyl ester (**4**), respectively, to show the γ steric effect between the hydroxyl group in C-1 and carbon C-3 in the structure of **4**.

Table 1
DPPH radical scavenging activity (expressed as EC_{50}) of the organic crude extract (CUR), semipurified hexane and EtOAc fractions, and purified metabolites **1–6** from the root extract of *Calea urticifolia*.

Sample	EC_{50}[a]
CUR	0.54 ± 0.010 mg/ml
Hexane	2.07 ± 0.590 mg/ml
EtOAc	0.47 ± 0.010 mg/ml
Thymol + 3-methyl-4-isopropyl phenol (**1** + **2**)	0.14 ± 0.012 mg/ml
3,4-*O*-Dicaffeoylquinic acid methyl ester (**3**)	12 ± 0.10 μM
3,4-*O*-Dicaffeoyl-*epi*-quinic acid methyl ester (**4**)	89 ± 0.05 μM
3,5-*O*-Dicaffeoyl-*epi*-quinic acid methyl ester (**5**)	15 ± 0.10 μM
3,5-*O*-Dicaffeoyl-*epi*-quinic acid (**6**)	NE
Ascorbic acid	26 ± 0.01 M

NE, not evaluated.
[a] Each value represents the mean of three determinations ± S.E.M.

radical-scavenging activity of these metabolites (Parejo et al., 2004; Jie et al., 2008).

The terpenoid mixture (**1** and **2**) and the novel 3,4-*O*-dicaffeoyl-*epi*-quinic acid methyl ester (**4**) were the only metabolites that showed analgesic activity when tested in the persistent pain induced by carrageenan model (data not shown). These results coincide with previous reports about **1** exhibiting antinociceptive and local anesthetic effects (Haeseler et al., 2002) as well as anti-inflammatory activity when evaluated in the carrageenan-induced hindpaw edema model (Riella et al., 2012). Similarly, caffeoylquinic acid derivatives such as 4,5-*O*-dicaffeoylquinic acid and 3,4,5-*O*-tricaffeoylquinic acid have been reported to show analgesic activity when tested in the acetic acid writhing induction test (Dos-Santos et al., 2005). The analgesic activity of metabolite **4**, and the fact that 3,5-*O*-dicaffeoyl-*epi*-quinic acid has been reported to show analgesic activity when tested in the acetic acid writhing induction test (Dos-Santos et al., 2010), suggests that for the dicaffeoyl-*epi*-quinic acid methyl ester derivative **5**, esterification of the carboxylic acid results in a significant reduction of activity. Finally, the similar levels of anti-inflammatory activity detected for the dicaffeoylquinic acid methyl esters derivatives **3**–**5** when tested in the carrageenan-induced paw edema (data not shown), showed a clear correlation with their antioxidant activity (Table 1). This correlation suggests that the anti-inflammatory activity of the purified metabolites might be related to their ability as radical scavengers.

Conclusions

The results of this study suggest that the terpenoid mixture of metabolites **1** and **2**, and the dicaffeoylquinic acid derivatives **3**–**6**, are the metabolites responsible for the biological activities detected in the crude extract of the root of *C. urticifolia* and for the traditional use of the plant. The dicaffeoylquinic acid derivatives represent an important class of secondary metabolites with potential to develop novel ant-inflammation and analgesic agents.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contributions

LMPR, VCF, and NLMQ conceived and designed the experiments; IFMR, HEZE and NGO performed the experiments; LMPR, IFMR, HEZE, and JARV analyzed the data; FEE and KGS contributed reagents/materials/analysis tools; LMPR, IFMR, FEE and JARV wrote the paper.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2018.01.010.

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