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Antimicrobial activities of diterpene labdane from seeds of *Byrsonima crassifolia*

[Actividad antimicrobial de diterpenos labdanos de la semilla de *Byrsonima crassifolia*]

Alethia MUÑIZ-RAMIREZ[1], Rosa Martha PEREZ-GUTIERREZ[2], Efren GARCIA-BAEZ[3] & Jose Maria MOTA-FLORES[2]

[1]*Departamento de Biotecnologia y Bioengenieria, Cinvestav-IPN, Av. IPN 2508, Col. San Pedro Zacatenco, Mexico D.F., CP 07360.*
[2]*Laboratorio de Investigacion de Productos Naturales, Escuela Superior de Ingeniería Química e Industrias Extractivas, IPN. Av. Instituto Politecnico Nacional s/n, Unidad Profesional Adolfo Lopez Mateos, Col. Zacatenco, CP 07758, Mexico D.F.*
[3]*Departamento de Biotecnologia, UPIBI-IPN, Col. San Pedro Zacatenco, Mexico D.F., CP 07360.*
Contactos | Contacts: Rosa Martha PEREZ-GUTIERREZ - E-mail address: rmpg@prodigy.net.mx

Abstract: Two compounds from the hexane extract of seeds of *Byrsonima crassifolia* were isolated and their structures elucidated using extensive spectroscopic analyses. These compounds are derived from the new labdane diterpene Labda-17-(1) and the known antimicrobial Labda-8 (17)-(2). The present study was aimed to study the effect antimicrobial of novel diterpene 1 against bacterial pathogens showed a moderate activity with MIC values 18.79-70.12 µg/ml and a MBC ranging between 250-1000 µg/ml against all assayed microorganisms.

Keywords: *Byrsonima crassifolia*, diterpene, labdane, antimicrobial

Resumen: Se aislaron dos compuestos del extracto de hexano de semillas de *Byrsonima crassifolia* y sus estructuras se dilucidaron por medio de extensos análisis espectroscópicos. Estos compuestos derivados del labdano corresponden al nuevo diterpeno Labda-17- (1) y el conocido antimicrobiano Labda-8(17)-(2). En el presente estudio se estudió el efecto antimicrobiano del nuevo diterpeno 1 sobre algunas bacterias patógenas mostrando sobre de estas una actividad moderada, con valores de MIC de 18.79-70.12 µg/ml y un rango de MBC que oscila entre 250-1000 µg/ml frente a todos los microorganismos ensayados.

Palabras Clave: *Byrsonima crassifolia*, diterpeno, labdano, antimicrobiano

INTRODUCTION

Byrsonima crassifolia is a tropical tree, commonly known as "nanche", distributed widely in México, Central and South America (Holmgren *et al.,* 1990). Nanche fruit is edible and bright yellow when after ripened, it has sweet taste and slightly bitter taste. In México, nanche is consumed as juice, liquor, jelly and candy. Since prehispanic times it has been used as medicine, reports on ethnobotanical uses include: bark, to promote bleeding in females, to facilitate childbirth and snakebite treatment; aerial parts, used as diuretic, febrifuge, to expel placenta and for diarrhea treatment; fruit, used for fever treatment and to induce a pleasant dizziness; bark and branches, assist in tightening loose teeth and fish poison; and finally, seeds for dysentery, infections, wound healing and anti-inflammatory (Bejar and Malone, 1993). Phytochemical studies indicate that nanche plant contains esters (Alves and Franco, 2003), epicatechins (Geiss *et al.,*1995), glycolipids (Rastrelli *et al.,*1997). A pharmacological study of leaf and bark extracts displayed spasmogenic effects (Bejar and Malone, 1993). In another study, a chloroformic extract from bark showed anti-inflammatory activity when evaluated by the Croton oil model (Silva *et al.,* 2007). Furthermore, the antioxidant activity of extracts from leaves, fruits and bark has been determinate (Maldini *et al.,* 2009). The ethyl-acetate extract of roots exhibited antibacterial activity (Martinez-Vazquez *et al.,* 1999). Also, the aqueous extract of leaves inhibited some dermatophytes (Caceres *et al.,* 1993). The ethanol extract of leaves showed trypanocidal activity against *Leishmania mexicana* promastigotes (Berger *et al.,* 1998). The hypoglycemic activity of hexane extract have previously been reported (Perez-Gutierrez *et al.,* 2010). Based on traditional uses of seeds from *B. crassifolia* and given the lack of scientific studies on its potential pharmacological properties, the aim of this study was to investigate the potential antibacterial activity of a new diterpene labdane from seed of *B. crassifolia*.

MATERIALS AND METHODS
General experimental procedures
IR spectra were recorded on a Perkin-Elmer FTRI 1720X. Optical rotations were measured by a Perkin-Elmer 192 polarimeter equipped with a sodium lamp (589 nm). ^{1}H-NMR and ^{13}CNMR spectra were taken at a Bruker DRX-300 NMR spectrometer, with UXNMR software package, was used for NMR experiments; chemical shifts are reported in *δ* (ppm), downfield relative to TMS as an internal standard. The NMR experiments were carried out using the conventional pulse sequences as described in the literature. HREIMS were measured on a JEOL HX 110 mass spectrometer. Precoated TLC silica gel 60 F254 aluminum sheets and Sephadex LH-20 from Sigma-Aldrich (St. Louis, USA) were used. Column chromatography was carried on Silica gel 60 (230-400 mesh, Merck Co. New Jersey (USA), solvents used as eluents from Fermont (California, USA).

Plant material
B. crassifolia L. belong to the Malpighiaceae family, fruit were collected in Morelos state and were taxonomically authenticated in the Herbarium of Escuela Nacional de Ciencias Biologicas, Instituto Politécnico Nacional. A voucher specimen of the plant is stored for reference (No. 8976).

Extraction, isolation and characterization of the B. crassifolia diterpene derived
Seeds was air dried and the ground (4 kg) was extracted twice with hexane for 3 h. The seeds extracts were combined and evaporated *in vacuum* to generate a residue (225 g). The resulting extract was loaded onto a silica gel column chromatography and eluted with petroleum ether-acetone-hexane 2:1:0.5 and 7 fractions (F1-F-7) have been obtained. These fractions were then tested for antimicrobial activity. Active fractions were pooled together according to their similarities provides by thin layer chromatography analysis. The fraction F3 was the fraction that showed antimicrobial properties. F3 was subjected to silica gel column chromatography eluted with $CHCl_3$-EtOAc (11:2) to produce five fractions (F3-1 to F3-5). The active fraction F3-4 was subjected to chromatographed over silica gel column using CH_2Cl_2-$CHCl_3$ 3:1.5 to yield four subfractions (F34-1 to F34-4). The F34-1 fraction was further purified by preparative plate using petroleum ether-EtOAc 1:4 and visualized with UV at 254 nm. Fraction F341-2 was separated by Sephadex LH-20 using a gradient of $CHCl_3$–MeOH (from 10:1 to 5:1) to yield **1** (80 mg) and **2** (67 mg).

Compound **1**:
Yellowish oil; $[\alpha]_D^{24}$ -21.4º ($CHCl_3$, c 0.68); IR ν_{max} 2924, 2854, 1710, 1645, 1460, 1375, 1163, 1096, 895 cm^{-1}; *m/z* 588.9254 [M+H]$^+$, (calc. for $C_{38}H_{68}O_4$, 588.9217); ^{1}H NMR (CDCL$_3$, 300 MHz) δ: 1.55 (m, H-1a), 1.76

(dd, *J* = 13.1, 3.4 Hz, H-1b), 1.46 (dd, *J* = 13.1, 3.4 Hz, H-2a), 1.66 (m, H-2b), 1.22 (m, H-3a), 1.58 (m, H-3b), 1.34 (m, H-5), 1.70 (m, H-6), 1.33 (1H, m, 7a), 1.39 (m, H-7b), 1.84 (m, H-8), 1.96 (m, H-9), 1.39 (m, H-11a), 1.59 (m, H-11b), 2.16 (m, H-12a), 1.76 (m, H-12b), 5.3 (t, *J*= 6.4 Hz, H-14), 3.74 (2H,d, *J*= 7.2 Hz, H-15), 0.96 (3H, s, H-16), 4.08 (2H,d, *J*= 2.8 Hz, H-17), 1.17 (s, H-18), 1.18 (s, H-19), 0.75 (s, H-20), 2.02 (s, COOMe), 2.04, (s, COOMe), 1.18-1.25 (CH$_2$); ^{13}C NMR (CDCL$_3$, 100 MHz) δ: 33.21 (C-1), 18.23 (C-2), 34.65 (C-3), 41.64 (C-4), 52.24 (C-5), 25.71 (C-6), 39.89 (C-7), 37.82 (C-8), 54.32 (C-9), 36.81 (C-10), 24.16 (C-11), 43.21 (C-12), 129.89 (C-13), 131.18 (C-14), 68.72 (C-15), 16.52 (C-16), 62.54 (C-17), 33.71 (C-18), 22.35 (C-19), 14.23 (C-20), 172.13 (COOMe), 20.98 (COOMe), 173.07 (CH$_2$-COO-CH$_2$).

Screening for antibacterial activity

Compound **1** was dissolved in DMSO at a final concentration of 10 mg/ml and serial dilutions were prepared using the same solvent which were aseptically dispensed onto sterilized 6 mm filter paper discs and dried. The disk-diffusion method (Gaydos, 1982) was used to measure the antimicrobial activity of the compounds. The different organisms were seeded over previously sterilized Mueller-Hinton agar for bacteria. The zones of growth inhibition were observed around dried disks (Whatman 6 mm diameter). Each plate was inoculated with 0.1 ml of a bacterial culture directly from the 24 h broth culture diluted to match 0.5 MacFarland standard (108 Colony Forming Units (CFU)/ml). Inoculums on the plates were aseptically spread using a sterile glass rod to avoid contamination and allowed to stand for 2 - 3 minutes in a refrigerator at 4° C. The discs loaded with **1** were then placed onto the seeded plates. The bacterial cultures were incubated at 37° C for 24hr. After the incubation period, the zones of inhibition were measured and recorded in mm as described by Elgayyar *et al.,* (2000). Negative controls were done by using sterile 6mm discs soaked in 10 ml of DMSO and dried.

Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC)

Compound **1** from the antibacterial screening was tested for Minimum Inhibitory Concentration (MIC) and   Minimum Bactericidal Concentration (MBC). The MIC was determined on 96-well microdilution plates   according the published protocols (Micheal *et*

al., 2003). The MBC were determined by sub-culturing 0.1 ml of all the tubes showing no growth on Nutrient Agar (NA). After 24 h incubation at 37° C, the first plate showing no growth was recorded as the MBC. The MICs accuracy was checked against standard antibiotics. Sterility of the media was controlled by use of broth only in a negative control tube, and growth of the organism was checked by broth plus microorganism in question in a positive control tube. All the controls were subjected to the same conditions as the tests. Antimicrobial sensitivity and resistance were confirmed by use of standard discs containing ciprofloxacin (10 µg/ml).

RESULTS AND DISCUSSION

Two compounds were isolated from hexane extract of seeds of *B. crassifolia* after purification through silica gel column chromatography. Compound **1** showed a molecular ion at *m/z* 588.9254 in its HREIMS and the presence of 38 carbons on the ^{13}C NMR spectrum suggested a molecular formula C$_{38}$H$_{68}$O$_4$, DEPT experiments classified the protonated carbon signals to six methyls, twenty three methylenes, four methines and five quaternary carbons. The   IR spectrum of **1** showed absorption bands at ν$_{max}$ 1645 cm^{-1} to double bond and 1710 and 1163 cm^{-1} corresponding to an ester group, it was supported by the presence of signal of quaternary carbons at δc 172.13 and δc 173.07 in its ^{13}C NMR spectrum. Its ^{1}H NMR spectrum showed signals for four tertiary methyl groups (δ$_H$ 0.96, s; 1.17, s; 1.18, s; 0.75, s), one trisubstituted double bonds (δ$_H$ 5.3, t, *J* = 6.4 Hz) and two acetoxyl groups (δ$_H$ 2.02, s and 2.04, s). In the ^{13}C NMR spectrum, two acetoxy carbonyl carbons (δ$_C$ 172.13, and 173.07), and the one 129.89, trisubstituted double bonds characterized by the presence of the two olefinic carbons (δ$_C$ and 131.18). Inspection of the 2D NMR data (^{1}H–^{1}H COSY, ^{1}H–^{1}H TOCSY, HSQC and HMBC) allowed the assignments of all the signals observed in the 1D NMR spectra and revealed the labdane skeleton of the diterpene **1** (Bohlmann and Czerson, 1979; Timmermann *et al.,* 1986). Connectivities in the HMBC spectrum from H-17 to C-7, C-8 and C-9 (δ$_C$ 54.32) corroborated the location of the side chain to be at C-9, whose methine proton H-9 (δ$_H$ 1.96) formed an isolated proton spin system with H-11 (δ$_H$ 1.39 and δ$_H$ 1.59) and H-12 (δ$_H$ 1.76 and 2.16) as deduced from the COSY and TOCSY spectra (Itokawa *et al.,*1988). A trisubstituted double bond was located at C-13 (δ$_C$ 129.89) and C-14 (δ$_C$ 131.18) on the basis of ^{2}J$_{C–H}$ and ^{3}J$_{C–H}$ HMBC correlations from

Me-16 (δ_H 0.96) to C-12 (δ_C 43.21), C-13 and C-14, and from the olefinic proton H-14 (δ_H 5.3) to C-12 (Figure 1). The double bond at C-13 was assigned as the E configuration because of the ROESY correlation observed between H-15 and Me-16, and by comparison of NMR data with those of related compounds (Urones *et al.,* 1995; Su *et al.,* 1996). Large number of methylene groups (22.7-34.4), suggesting the presence of fatty acid esterified with the labdane derivative at C-15. It is supported by the HMBC correlation between methylene protons H$_2$-15 (δ_H 4.08, 2H, d, J = 7.2 Hz) and methylene chain and the quaternary carbon δc 173.07. Analysis of ^{1}H-^{1}H COSY showed clear correlation between H5/H6, H6/H7, H11/H12 (Figure 2). One methyl group (δc16.52), and large number of methylene groups (δc

22.6-34.5) (DEPT) suggesting the presence of monoenic fatty acid esterified with a labdane derivative at C-15. The NMR data of the long-chain moiety were in good accordance with those for Δ9-18:1 *cis* fatty acid esters (Mannina *et al.,* 1999). Therefore, compound **1** was identified as labda-17-acetoxi-13E-en-15-palmitate. All data of ^{1}H NMR and ^{13}CNMR spectra, ^{1}H-^{1}H, COSY and HMBC connectivities of **2** allowed the identification of labda-8(17),13E-dien-19-carboxy-15-yl palmitate which was previously isolated from *Cretan propolis* (Peopova *et al.,* 2009) exhibited a weak antimicrobial activity against *Staphylococcus aureus, Staphylococcus epidermidis, Klebsiella pneumonia, Escherichia coli,Candida tropicalis* and *Candida glabrata.*

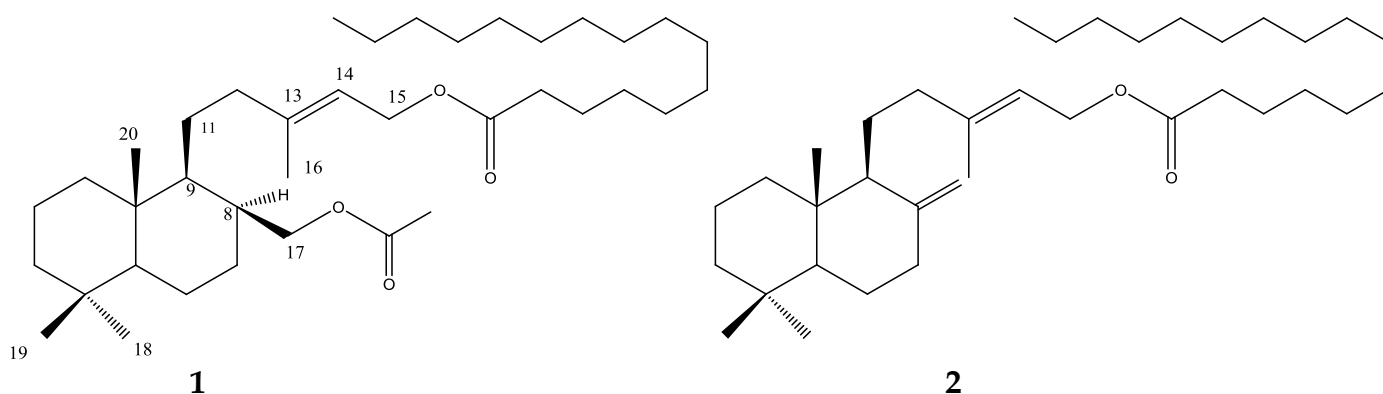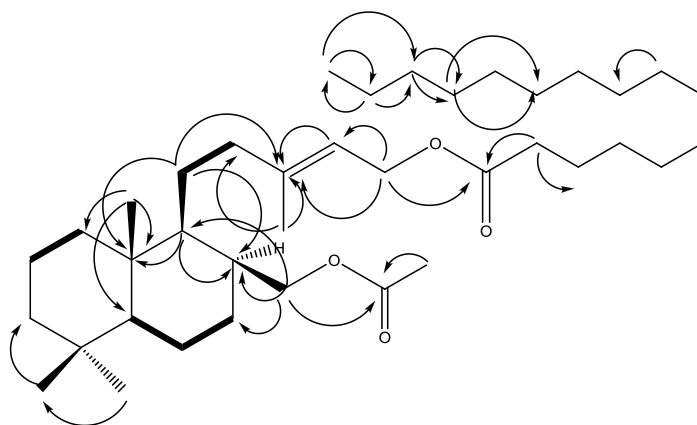**Figure 1**
Compounds 1 and 2 isolated from *B. crassifolia*

Figure 2
Selected ^{1}H-^{1}H COSY (bold) and ^{1}H-^{13}C HMBC (arrow) correlations of 1

Screening of the *in vitro* antimicrobial activity of diterpenoid **1** was studied against Gram positive microorganisms *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, *Staphylococcus aureus*, and Gram negative microorganisms as *Escherichia coli*, *Salmonella paratyphi*, *Pseudomonus aeruginosa*, *Salmonella typhi*, *Shigella boydii*, *Salmonella dysenteriae*, *Vibrio mimicus* and *Vibrio parahemolyticus* of significant importance using the filter paper disc agar diffusion technique. All the tested bacteria were standard strains from ATCC (American type culture collection). The results showed variations in the antimicrobial properties of diterpene labdane **1** (Table 1). Compound **1** had strong activity (inhibition zone ≥ 20 mm), moderate activity (inhibition zone < 20-12 mm) and no inhibition (zone < 12 mm). The most effective **1** was that of, *S. paratyphi* (23.87 ± 0.65 mm, zdi), followed by *P. aeruginosa* (21.45 ± 0.53 mm, zdi), *S. typhi* (20.72 ± 0.56 mm, zdi), *B. subtilis* (20.89 ± 2.90 mm, zdi). In contrast, showed a moderate activity against *B. megaterium* (19.20 ± 3.42 mm, zdi), *B.cereus* (18.76 ± 0.68), *Staphylococcus aureus* (17.95 ± 1.78), *Escherichia coli* (13.35 ± 1.43), and *S. dysenteriae (*16.52 ± 1.88*).* However, no signicant antimicrobial effect against *Vibrio mimicus* (12.08 ± 4.12), *Sarcinia lutea (*12.17 ± 1.23*), V. parahemolyticus* (11.87 ± 4.20) and *Shigella boydii* (0.0 mm) was observed. Gram-positive bacterial cells were, in general, more resistant to the presence of antimicrobial than Gram-negative.

Table 1
Antimicrobial activity of the compound 1 obtained from seeds of *Byrsonima crassifolia*

Test microorganisms	Inhibition zone diameter (mm)	MIC (µg/ml)	MBC (µg/ml)
Gram positive bacteria			
Bacillus cereus ATCC 11778	18.76 ± 0.68	38.34 ± 1.87	500
B. megaterium ATCC 12872	19.20 ± 3.42	43.26 ± 2.76	500
B. subtilis ATCC 6633	20.89 ± 2.90	41.28 ± 3.29	500
Staphylococcus aureus ATCC 6538	17.95 ± 1.78	36.79 ± 1.87	500
Sarcinia lutea ATCC 9341	12.17 ± 1.23	58.84 ± 4.76	1000
Gram negative bacteria			
Escherichia coli ATCC 25922	13.35 ± 1.43	69.20 ± 2.76	1000
Pseudomonus aeruginosa ATCC 9027	21.45 ± 0.53	32.41 ± 4.11	500
Salmonella paratyphi ATCC 6539	23.87 ± 0.65	20.27 ± 1.04	250
S. typhi ATCC 43971	20.72 ± 0.56	18.79 ± 2.54	250
Shigella boydii ATCC 9207	0.0	-	-
S. dysenteriae ATCC 13313	16.52 ± 1.88	45.67 ± 3.87	500
Vibrio mimicus ATCC 33654	12.08 ± 4.12	66.78 ± 5.14	1000
V. parahemolyticus ATCC 43996	11.87 ± 4.20	70.12 ± 4.98	1000

Zone diameter inhibition (ZDI) of compound 1 (20 µg) per disk

However, more precise data on antimicrobial properties were obtained through determinations of bacteriostatic and bactericidal concentrations. The minimun inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) the results are shown in Table 1. The most bacteriostatic properties was against all Gram-negative strains assayed. Martinez-Vazques *et al.,* (1999) studied ethyl acetate, methanol extracts of roots and stems of *B. crassifolia* against *Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhi, Shigella fiexneri, Staphylococcus aureus, Staphylococcus epididermis, Streptococcus pneumoniae* and *Micrococcus luteus.* The ethyl acetate of roots was the most active. In another research had been previously studied methanol extract from *B. crassifolia* against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa,* and *Candida albicans* (Navarro *et al.,* 1996). The results obtained by us indicate the existence of antimicrobial compounds in this plant, and show a good correlation between the reported studies of *B. crassifolia* uses in traditional medicine against infectious diseases and the experimental data of such extracts toward the most

common pathogens. Also results indicate the existence of antimicrobial compounds in the hexane extract of *B. crassifolia,* the phytochemical isolation and identification of two active compounds indicates that the antimicrobial activity of the seed of *B. crassifolia* is due mainly to the presence of compounds labdane diterpene.

In conclusion the present study shows that antimicrobial activity is present in the seeds of *B. crassifolia.* Bioassay with the purified diterpene **1** indicated that exhibited strong activity against Gram negative bacterias. The results suggest that further investigations against other enteric Gram-positive and Gram-negative bacteria, which are frequently resistant to drugs commonly used in therapy, may be carried out.

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