An Antiviral Meliacarpin from Leaves of *Melia azedarach* L.

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Bioassay guided purification of the ethyl acetate extract of leaves of *Melia azedarach* led to the isolation of the limonoid 1-cinnamoyl-3,11-dihydroxymeliacarpin, which showed IC_{50} values of 6 μM and 20 μM for vesicular stomatitis (VSV) and herpes simplex (HSV-1) viruses, respectively. Its structure was established by spectroscopic methods.

Key words: Melia azedarach L., Limonoids, Antiviral Activity

Introduction

Melia azedarach L. (*Meliaceae*), also known as Chinaberry or Persian lilac tree, is a deciduous tree that is native to India and has long been recognized for its medicinal and insecticidal properties (Bohnenstengel *et al.*, 1999). Although the fruits are the poisonous part of the tree, they have been used traditionally for the treatment of a variety of diseases, especially dermatitis and rubella (del Mendez *et al.*, 2002; Kim *et al.*, 1999). In addition, a number of potent pharmaceutical limonoids and triterpenoids have been isolated from fruits and bark (Lee *et al.*, 1999).

Chinaberry was widely introduced in Argentina as an ornamental shade tree. We have previously reported that plant extracts from *Melia azedarach* L. inhibited the multiplication of Junin, Pichinde, Tacaribe, Sindbis, vesicular stomatitis (VSV), polio and herpes simplex (HSV) viruses in cell cultures (Wachsman *et al.*, 1982). The antiviral activity has been ascribed to a partially purified inhibitor designated as meliacine (Andrei *et al.*, 1988).

In the present paper, we report the isolation of 1-cinnamoyl-3,11-dihydroxymeliacarpin (**1**) from leaf extracts of *Melia azedarach* L. that inhibits VSV and HSV-1 multiplication *in vitro* when added after infection, with no cytotoxic effect.

Results and Discussion

Lyophilized leaves of *M. azedarach* L. were extracted with water and the aqueous solution was further extracted with EtOAc. The ethyl acetate extract was purified by CC over silica gel and preparative TLC to give a fraction exhibiting antiviral activity. Silica gel CC purification and reversed phase HPLC of this fraction afforded the antiviral 1-cinnamoyl-3,11-dihydroxymeliacarpin (**1**). Although this C-*seco* limonoid has previously been isolated from the fruits of *Melia azedarach* (Lee *et al.*, 1991), no spectroscopic nor physical data have been reported for its structure.

Compound **1** was obtained as a white amorphous powder. The molecular formula was determined by accurate mass measurements as $C_{36}H_{42}O_{13}$. The peaks at *m/z* 131 ($C_9H_7O^+$) and 147 ($C_9H_7O_2^+$) in the EIMS suggested the presence of a cinnamoyl moiety. In the aromatic and olefinic region, the ^{1}H NMR spectrum displayed a pair of doublets (J = 16.0 Hz) at 6.37 and 7.70 ppm and two multiplets at 7.37 and 7.47 ppm, typical for a *trans* cinnamoyl ester. The presence of the cinnamoyl group was also indicated by ^{13}C NMR and DEPT signals at δ 134.0 (C-1′, s), 128.1 (C-2′ and C-6′, d), 128.9 (C-3′ and C-5′, d), 130.6 (C-4′, d), 146.1 (C-7′, d), 117.1 (C-8′, d), and 165.7 ppm (C-9′, s), and confirmed by cross-peaks at δ 7.47/128.1 (H-2′, H-6′/C-2′, C-6′), 7.37/128.9 (H-3′, H-5′/C-3′, C-5′), 7.37/130.6 (H-4′/C-4′), 7.70/146.1 (H-7′/C-7′) and 6.37/117.1 (H-8′/C-8′) in the HETCOR spectrum. A system of two doublets (J = 2.9 Hz) at 5.01 and 6.41 ppm, a singlet at 5.69 ppm and four methyl singlets at 3.70, 2.16, 1.71 and 0.96 accounted for a meliacarpin skele-

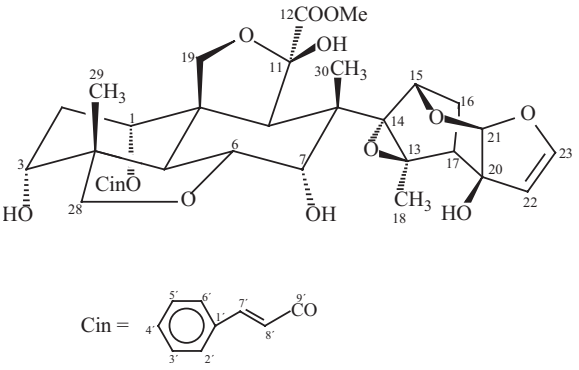

Fig. 1. Chemical structure of 1-cinnamoyl-3,11-dihydroxymeliacarpin isolated from the leaves of *Melia azedarach*.

ton. The presence of a hydroxyl group at C-11 was deduced from the signal of the hemiacetal carbon at 104.0 ppm (Kraus *et al.*, 1987), shifted upfield by 2.8 ppm with respect to the substitution with a methoxyl group at this position (Takeya *et al.*, 1996). ^{1}H and ^{13}C NMR spectra of **1** were similar to those of 1-cinnamoylmeliacarpin derivatives (Bohnenstengel *et al.*, 1999) except for the presence of a free hydroxyl group at C-3 instead of an acetyl, a cinnamoyl or a metacrylyl residue. This substitution pattern at ring A has previously been found in 1-cinnamoyl-3-hydroxy-11-methoxymeliacarpinin, isolated from the root bark of *Melia azedarach* (Takeya *et al.*, 1996). ^{1}H-^{1}H COSY, HETCOR and NOESY spectra allowed the assignment of all proton and carbon resonances. The substitution pattern at ring A, namely the presence of 1α-cinnamoyl and 3α-hydroxyl groups was deduced from the chemical shifts of H-1β and H-3β at δ 4.78 and 3.80 ppm, respectively, very similar to those (δ 4.90 and 3.85 ppm) in 1α-cinnamoyl-3α-hydroxy-11-methoxymeliacarpinin. The equatorial configuration for both protons was deduced form their coupling constant values. These assignments were supported also by the downfield shifts of H-28a (*ca.* 0.5 ppm), C-4 (0.9 ppm) and C-2 (2.5 ppm) in **1**, compared with those in 1α,3α-dicinnamoyl- and 1α-cinnamoyl-3α-metacrylyl-11-hydroxymeliacarpin derivatives (Bohnenstengel *et al.*, 1999) and the NOE of H-3β/Me-29. The upfield shift of the singlet at 3.34 ppm (H-9) corroborated the absence of a 1,3-diaxial relationship between H-9 and a 1α-hydroxyl group (Huang *et al.*, 1996), confirming the substitution pattern at ring A. The presence of a methyl group at C-4 instead of the typical carboxymethyl group in azadirachtin (Kraus *et al.*, 1987) was deduced from the quaternary signal at δ 19.3 ppm in the ^{13}C NMR spectrum and the cross-peak at δ 0.96/19.3 in the HETCOR spectrum of **1**.

To investigate if compound **1** is responsible for the antiviral effect detected along the purification procedure, we determined the IC_{50} values of **1** (concentration of **1** that inhibits 50% of viral infectivity) *in vitro*. A dose-dependent inhibition of virus replication was observed in drug-treated cultures infected with either of both viruses. Fig. 2 shows about 1.5 log units of reduction of VSV yield at 16 or 32 μM of **1**, and 1 to 2.5 orders-of-magnitude of yield inhibition in the case of HSV-1 when 32 or 64 μM of **1** were used, respectively. The IC_{50} values of **1** were 6 μM for VSV and 20 μM for HSV-1. The results of *in vitro* therapeutic indices, calculated by dividing the CC_{50} by the IC_{50}, were superior to 86 and 26 for VSV and HSV-1, respectively. In consequence, 1-cinnamoyl-3,11-dihydroxymeliacarpin proved to be an antiviral compound that reduced both VSV and HSV-1 infectivity in *in vitro* conditions. Since the antiviral activity detected in partially purified leaf extracts from *Melia azedarach* L. (meliacine) prevents the development of herpetic stromal keratitis in mice

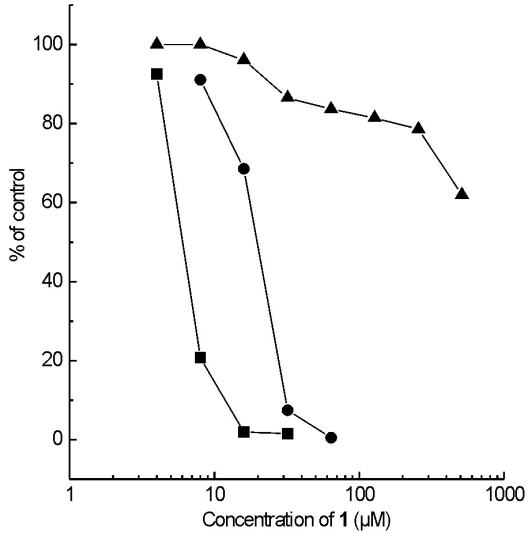

Fig. 2. Cytotoxic and antiviral effects of compound **1**. ▲ Vero cells; ● HSV-1 and ■ VSV.

when supplied before or after infection, with no toxic effects (Alché *et al.*, 2000; Pifarré *et al.*, 2002), 1-cinnamoyl-3,11-dihydroxymeliacarpin deserves to be assayed in *in vivo* experimental models.

Experimental

General procedures

^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Bruker ACE-200 and AM 500 spectrometers. Carbon substitution degrees in the ^{13}C NMR spectrum was established by DEPT multiple sequence. Mass spectra were collected on a TRIO-2 VG mass spectrometer. The IR spectrum was obtained on a Nicolet Magna-550 FTIR spectrometer. Optical rotation was determined on a Perkin-Elmer 343 polarimeter. Preparative HPLC was carried out on an SP liquid chromatograph equipped with a Spectra Series P100 solvent delivery system, a Rheodyne manual injector, and an UV detector (λ 245 nm) using a C_{18} Bondclone 10μ column (30 cm × 21.2 mm *i.d.*) and MeOH:H_2O (60:40 v/v). TLC was performed on precoated Si gel F254 ($CHCl_3$-MeOH (90:10 v/v)).

Plant material

Fresh green leaves of *M. azedarach* L. (Meliaceae) were collected during late spring and summer 2001. The specimens harvested from trees grown in Buenos Aires city were identified at the Department of Botany of the Faculty of Exact and Natural Sciences, University of Buenos Aires, where a voucher specimen was kept and registered as BAFC 1432 (Argentina).

Extraction and isolation

The leaves (5.4 kg) were collected, washed immediately with distilled water and stored at −20 °C until processed. The thawed leaves were cut into small pieces and extracted in a Waring blender with 10 mM potassium phosphate buffer (pH 7.2) (13.5 l) containing 0.35 M KCl. The sap obtained was pressed through a cheesecloth and centrifuged at 10000 × *g* for 1 h. Pellets were discarded and the supernatants lyophilized. All operations were carried out at 4 °C. The lyophilized extract (241 g) was suspended in distilled water (1.2 l), shaken overnight and centrifuged at 10000 × *g* for 1 h. The pellet was discarded and the aqueous solution was extracted with EtOAc. The ethyl acetate extract was concentrated to give a residue (3.35 g), which was subjected to column chromatography on silica gel, using $CHCl_3$ and a $CHCl_3$-MeOH gradient of increasing polarity. The fraction (644 mg), eluted with $CHCl_3$-MeOH (95:5 v/v), showed antiviral activity. This fraction was further purified by preparative silica gel TLC, developed with $CHCl_3$-MeOH (90:10 v/v), to afford a fraction (288 mg) that was subjected to dry flash column chromatography on reversed-phase C_{18} silica gel and eluted with H_2O, mixtures of H_2O-MeOH with increasing amounts of MeOH and finally MeOH. Fractions eluting with MeOH-H_2O (80:20) and MeOH-H_2O (65:35 v/v) showed the highest antiviral activity and were further pooled (244.7 mg) and subjected to repeated reversed-phase HPLC using MeOH-H_2O (60:40 v/v) to yield compound **1** (41.4 mg).

1-cinnamoyl-3,11-dihydroxymeliacarpin (**1**)

White amorphous powder mp 208–209 °C (after crystallization in MeOH-H_2O). $[\alpha]_D^{20}$ – 41.4° ($CHCl_3$, *c* 0.29), IR ν_{max} cm^{-1} (KBr): 3400, 1738, 1717, 1630, 1600, 1370, 1055; ^{1}H-NMR (500 MHz): δ 0.96 (3H, s, H-29), 1.71 (3H, s, H-30), 2.09 (1H, dt, *J* = 16.6, 2.5 Hz, H-2ß), 2.16 (3H, s, H-18), 2.24 (1H, dt, *J* = 16.6, 2.5 Hz, H-2α), 2.36 (1H, d, *J* = 5.2 Hz, H-17), 3.12 (1H, d, *J* = 12.7 Hz, H-5), 3.34 (1H, s, H-9), 3.62 (1H, d, *J* = 7.9 Hz, H-28b), 3.70 (1H, s, **Me**O), 3.80 (1H, m, H-3), 3.79 (1H, d, *J* = 9.4 Hz, H-19a), 4.15 (1H, dd, *J* = 12.8, 2.6 Hz, H-6), 4.17 (1H, bd, *J* = 9.4 Hz, H-19b), 4.18 (1H, d, *J* = 7.9 Hz, H-28a), 4.63 (1H, d, *J* = 3.5 Hz, H-15), 4.70 (1H, bs, H-7), 4.78 (1H, bt, *J* = 2.8 Hz, H-1), 5.01 (1H, d, *J* = 2.9 Hz, H-22), 5.69 (1H, s, H-21), 6.37 (1H, d, *J* = 16.0 Hz, H-8′), 6.41 (1H, d, *J* = 2.9 Hz, H-23), 7.37 (3H, m, H-3′, H-4′ and H-5′), 7.47 (2H, m, H-2′ and H-6′), 7.70 (1H, d, *J* = 16.0 Hz, H-7′); ^{13}C NMR (125 MHz): δ 171.7 (Me**O**C = O), 165.7 (C-9′), 146.5 (C-23), 146.1 (C-7′), 134.0 (C-1′), 130.6 (C-4′), 128.9 (C-3′and C-5′), 128.1 (C-2′ and C-6′), 117.1 (C-8′), 108.9 (C-21), 107.4 (C-22), 104.0 (C-11), 83.6 (C-20), 76.2 (C-15), 76.9 (C-28), 75.2 (C-7), 72.6 (C-6), 72.0 (C-1), 70.0 (C-14), 69.9 (C-3 and C-19), 68.9 (C-13), 53.2 (**Me**O), 50.2 (C-10), 49.0 (C-17), 44.9 (C-8), 44.7 (C-9), 43.7 (C-4), 34.3 (C-5), 30.6 (C-2), 25.0 (C-16), 21.1 (C-30), 19.3 (C-29), 18.2 (C-18); HRMS: *m/z*: 682.2652 ([M]$^+$,

calcd for $C_{36}H_{42}O_{13}$: 682.2625). EIMS: *m/z* (rel. int.): 682 [M^+] (1), 546 (0.4), 501 (0.5), 478 (0.4), 456 (0.5), 147 (19.1), 131 (38.5).

Cells and viruses

Vero (African green monkey kidney) cells (ATCC CCL 81) were grown in Eagle's minimal essential medium supplemented with 5% inactivated calf serum (MEM 5%) and 50 μg/ml gentamycin and maintained in monolayer formation in MEM supplemented with 1.5% inactivated calf serum (MEM 1.5%). HSV-1 strain F and the Indiana strain of VSV were propagated at low multiplicity on Vero cell monolayers.

Bioguided procedure

The antiviral activity was assayed in the different steps along the protocol of purification. Vero cells grown in 96-well flat bottom plates received two-fold serial dilutions of a 2 mg/ml solution of each preparation in MEM 1.5% and were incubated at 37 °C in 4% CO_2 for 2 h (pre-treatment) Then, supernatants were eliminated and the cells were infected with 10^3 plaque forming units (PFU) of VSV/well. After 1 h of adsorption at 37 °C in 4% CO_2, Vero cells were maintained with MEM 1.5% until the appearance of cytopathic effect. All determinations were performed in triplicate.

Determination of the antiviral activity of **1**

Vero cells grown in 96-well culture plates were infected either with VSV or HSV-1, at a m.o.i. (multiplicity of infection) of 0.1, and treated or not with different concentrations of **1** in MEM 1.5%. All determinations were performed in triplicate. After 24–48 h of incubation at 37 °C in 4% CO_2, supernatants were collected and titrated by a plaque assay.

Cytotoxicity assay

Cell viability was determined using the cleavage of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma) by the mitochondrial enzyme succinate dehydrogenase to give a blue product (formazan) (Denizot and Lang, 1986). The absorbance of each well was measured on an Eurogenetics MPR-A 4i microplate reader (Eurogenetics, Belgium), using a test wavelength of 570 nm and a reference wavelength of 630 nm. Results were expressed as a percentage of absorbance of treated cell cultures with respect to untreated ones. The CC_{50} was defined as the concentration that caused a 50% reduction in absorbance.

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