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Pentacyclic triterpenoids with antimicrobial activity from the leaves of *Vernonanthura patens* (Asteraceae)

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Abstract

Vernonanthura patens (Kunth) H. Rob, is native to Mexico and South America and found in dry or wet forests or pine forests. They are reaching in height from 0 to 1865 meters above sea level (masl). They are distributed throughout South America, in Central America: Mexico, Belize, Costa Rica, El Salvador, Guatemala, Honduras, Nicaragua, Panama, and northern South America: Venezuela, western South America: Bolivia, Colombia, Ecuador and Peru. Three pentacyclic triterpenoids from the leaves were isolated by chromatographic (Colum Chromatography (CC) and Thin Layer Chromatography (TLC)) techniques. The structures of these isolated compounds were identified by 1H NMR and 13C NMR spectroscopic and MS analysis as lupeol, epilupeol and acetyl lupeol. Lupeol and epilupeol showed antifungal activity against *Fusarium oxysporum* and *Penicillium notatum*, whereas acetyl lupeol was inactive.

Key words: Pentaciclyc triterpenoids, Lupeol, Epilupeol, Acetyl lupeol, Antimicrobial, *Vernonanthura patens*

Introduction

Vernonanthura patens (Kunth) H. Rob (Asteraceae) is a wild plant broadly distributed throughout America. It grows from 0 to 2200 meters above sea level in the Ecuadorian region. Its leaves are cooked and used against malaria, for postpartum treatment and for healing infected wounds of animals as part of a plant mixture (Blair, 2005; Kvist, 2006). It is also used against headaches, treatment of leishmaniasis (Gacheta, 2010), preparation of antivenos (Tene, 2007), and to combat athlete's foot (Valadeau, 2009). Its usefulness for treating certain types of cancer has also been referred to by indigenous healers. However, there are scarce biological and chemical studies on this species. The only results published so far refer to the antimalarial activity against *Plasmodium falciparum*, Itg2 strain (Blair, 2005), anti-*Leishmania* activity (Valadeau, 2009) of the leaves and lack of antiprotozoal activity against different strains of Leishmania (Fournet, 1994). On the chemical composition of the species, there are reports of sesquiterpene lactones and sesquiterpenes present in the aerial parts (Mabry, 1975: Jakupovic, 1986). There are some references on *Vernonanthura* species that shows the presence of diterpene compounds (Portillo, 2005; Valadeau, 2009), flavonoids (Borkosky, 2009; Mendonça, 2009), triterpenes (Tolstikova, 2006; Gallo, 2009), saponins (Borkosky, 2009) and sesquiterpene lactones. In addition, different biological activities have been described assuming that certain chemical groups could be responsible for the therapeutic properties attributed to species of this genus (Pollora, 2003, 2004; Portillo, 2005; Bardón, 2007). In this work, we performed the extraction and fractionation of leaves of the species in the vegetative state using column chromatography increasingly polar solvent and thin layer

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chromatography. Through these procedures, three triterpenoids isolated, which were identified using Nuclear Magnetic Resonance Spectroscopy of ^{1}H and ^{13}C. For these compounds, antimicrobial activity was determined against *Fusarium oxysporum* and *Penicillium notatum*.

Materials and Methods

Plant material

Mature leaves of *Vernonanthura patens* (laritaco) were collected at the vegetative state in the Marcabelí town, province of El Oro, Ecuador, with the following coordinates S 3° 47' 06,14" W 79° 54' 32.66", to 316,58 m. Leaves were collected in the early morning in February 2011. A voucher specimen is on deposit in the National Herbarium of Ecuador (QCNE) and a duplicate sample (CIBE37) is kept as a herbal witness in the laboratory of the CIBE-ESPOL Bioproducts.

Extraction and isolation

Coarsely ground leaves (67 g) were extracted by maceration, with two liters of methanol (HPLC grade) in a closed container and in the absence of light and with agitation in a New Brunswick Scientific shaker model C-10. The extraction time was 8 days and was conducted until total depletion of plant material. The crude extract was evaporated to dryness on a rotary evaporator Heidolph brand Laborota 4001 model with reduced pressure at 50°C.

The crude methanol extract was subjected to CC over activated silica gel (60 - 200 mesh) using *n*-hexane, *n*-hexane: EtOAc (90:10, 80:20) and EtOAc, taking 150 mL fractions each time. Four fractions were obtained: Fr 1 *n*-hexane (79 mg), Fr 2 *n*-Hex/EtOAc (90:10) (1370 mg), Fr 3 *n*-Hex/EtOAc (80:20) (0.60 mg) and Fr 4 EtOAc (0.21 mg). From fractions three and four, on further purification by preparative layer chromatography, compounds 1 (57 mg), 2 (20 mg) and 3 (90 mg) were obtained, respectively.

Mass spectra were performed on an Agilent 7890A gas chromatograph, with Agilent 5975 detector (Avondale, PA.USA).

NMR analyses **[**^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz)] were recorded at room temperature with a Bruker DRX500 spectrometer with an inverse multinuclear 5 mm probe head equipped with a shielded gradient coil. The spectra were recorded in $CDCl_3$, and the solvent signals (7.26 and 77.0 ppm, respectively) were used as reference.

Bioassays

The diffusion method with potato dextrose agar (PDA) was used to determine the antifungal activity of three isolated pure compounds from *V. patens* leaves at 100 and 200 µg/mL (Avello, 2009). Dilutions were made with 10% dimethylsulfoxide (DMSO). Strains of *Fusarium oxysporum* and *Penicillium notatum* were isolated from infected *Pinus radiata* and *Citrus sinense* fruits and maintained in the Collection of Fungi at the University of Concepcion-Chile.

Holes of 5 mm diameter were made in the agar with a sterile cork borer and filled with 20 µL of each concentration of pure compound. DMSO 10% was used as a negative control in each plate. A disc (5 mm diameter) of already grown fungus was placed in the center of Petri dishes and incubated at 22°C. Radial growth assessment was made during 2 weeks.

Experimental design was completely randomized and each assay was performed in triplicate. Descriptive statistics of the experimental data were made in order to represent and point out its most important features.

Results and Discussion

Compound 1

Compound 1 was isolated as a white amorphous solid. The mass spectrum suggested formula $C_{30}H_{50}O$ (M m/z 426). Its $^{1}HNMR$ spectrum (Cl_3CD, 300 MHz), exhibited seven signals as singlet to δ 0.76 (3H, s, Me-28), 0.78 (3H, s, Me-25), 0.83 (3H, s, Me-24), 0.94 (3H, s, Me-23), 0.96 (3H, s, Me-27), 1.03 (3H, s, Me-26) and at lower fields other to δ 1.67 (3H, s, Me-30) assignable to a methyl group on double bond; a multiplet at δ 1.94 (2H, m, H-21); a double doublet to δ 2.4 (1H, dd, J=11.01 and 5.7 Hz, H-19) to be assigned to a methynic proton; a double doublet to δ 3.20 (1H, dd, J=10.9 y 5.3 Hz, H-3) assignable to a geminal proton of a secondary alcohol, two doublets for proton to an exocyclic methylene to δ 4.55 (2H, d, J=2.0 Hz, H-29) and 4.67 (2H, d, J=2.0 Hz, H-29). $^{13}CNMR$ (Cl_3CD, 125 MHz), showed 30 carbon signals; vinyl carbon signal occurs at 109.5 ppm, the signal corresponding to methylene - methylidene at 151.1 ppm and the carbon bound to the hydroxyl group appears at 79.1 ppm. By comparing the chemical shifts with those reported in the literature concluded that the compound is a pentacyclic triterpene lupeol (Jamal, 2008).

Compound 2

Compound 2 was isolated as a white amorphous solid. The mass spectral analysis has allowed establishing the molecular formula

$C_{32}H_{52}O_2$ from the molecular ion M m/z 468. The ^{1}HNMR spectrum (Cl_3CD, 300 MHz), shows the presence of eight tertiary methyl groups singlets at values of δ 0.77(3H, s, H-27), 0.79 (3H, s, H-26), 0.85 (3H, s, H-24), 0.87 (3H, s, H-23), 0.94 (3H, s, H-28), 1.05 (3H, s, H-25) and 1.65 (3H, s, H-30) and a methyl acetate to 2.07 (3h, s, H-2´) ppm. Singlets at the δ 4.62 (1H, s, H-29a) and 4.72 (1H, s, H-29b) indicate the presence of a exomethylene group. Moreover double doublet at δ 4.25 (1H, dd, J_{axax}=9.8, J_{axeq}=4.2, H-3) was caused by the proton bonded to carbon was connected to the functional group acetate. ^{13}CNMR (Cl_3CD, 125 MHz), shows a carbonyl group at δ 171.2 ppm and its connection to C-3 to δ 81.0 ppm. Are also located two olefinic carbons C-20 (151.2 ppm) and C-29 (109.5 ppm), connected to the single double bond exocyclic methylene group present in the structure. Spectrum signals have coincided with those reported for lupeol acetate (Bracho, 2009).

Compound 3

Compound 3 was isolated as a white amorphous solid. The mass spectrum suggested formula $C_{30}H_{50}O$ (M m/z 426). Its ^{1}HNMR spectrum (Cl_3CD, 300 MHz), exhibited too seven signals as singlet to δ 0.79 (3H, s, Me-28), 0.80 (3H, s, Me-25), 0.83 (3H, s, Me-24), 0.94 (3H, s, Me-23), 0.96 (3H, s, Me-27), 1.03 (3H, s, Me-26) and at lower fields other to δ 1.68 (3H, s, Me-30) assignable to a methyl group on double bond; a multiplet at δ 1.93 (2H, m, H-21); a double doublet to δ 2.4 (1H, dd, J=11.01 and 5.7 Hz, H-19) to be assigned to a methynic proton; a double doublet to δ 3.40 (1H, dd, J=10.9 y 5.3 Hz, H-3) assignable to a geminal proton of a secondary alcohol, this indicates that the hydroxyl group is in the alpha position, and that when the hydroxyl is in beta, this chemical shift occurs at 3.20 ppm. Two doublets for proton to an exocyclic methylene to δ 4.57 (2H, d, J=2.1 Hz, H-29) and 4.69 (2H, d, J=2.1 Hz, H-29). 13CNMR (Cl3CD, 125 MHz), showed 30 carbon signals; two signals are characteristic of a double bond in compounds having skeletons derived from lupane (109.6, 151.2ppm) which exhibit an isopropenyl group with specific chemical shifts those of 19.5, 151.2 and 109.6 ppm for the carbons C-30, C-20 and C-29, respectively. The spectrum showed a characteristic signal of carbon oxygen ported 76.4 ppm, assignable to hydroxyl group at position 3-α. By comparing the chemical shifts with those reported in the literature concluded that the compound is a pentacyclic triterpene epilupeol (Souza, 2001).

In Tables 1 and 2, the spectroscopic data for lupeol and epilupeol are presented for the identified compounds compared with those reported in the literature.

The most relevant antifungal activity was observed in the pure compounds 1 and 3 at both concentrations tested. The pure compounds showed selective inhibition properties and a certain concentration dependence on its antifungal activity. Compound 1 showed a rate of inhibition of 50 and 90% (100 and 200 µg/mL respectively) against *Penicillium notatum*, while compound 3 was capable to inhibit 80 and 100% of *Fusarium oxysporum* growth for each assayed concentrations. Compound 2 showed no antifungal activity against the microorganisms tested. Statistical differences ($P \leq 0.05$) with negative controls indicated that DMSO did not influence the results of biological evaluation (Figure 1).

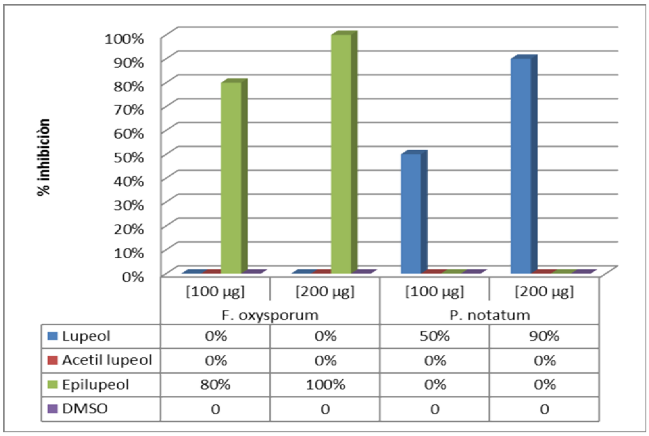

Figure 1. Percent Inhibition of isolated compounds *V. patents* against *F. oxysporum* and *P. notatum* at different concentrations.

Table 1. ^{1}HNMR shifts values of compounds 1 and 3 and reference compounds used as (δ_H).

H	1 $CDCl_3$	2 $CDCl_3$	Compound 1 $CDCl_3$	Compound 3 $CDCl_3$
3	3.20 m	3,40 m	-	-
19	2,28 m	2,38 m	-	-
21 a	-	-	-	-
21 b	-	-	-	-
23	-	-	0,94 s	0,96 s
24	-	-	0,83 s	0,78 s
25	-	-	0,78 s	0,85 s
26	1,03 s	1,03 s	1,03 s	1,02 s
27	-	-	0,96 s	0,93 s
28	0,79 s	0,79 s	0,76 s	0,82 s
29	4,57 s	4,57 s	4,65 m	4,67 m
	4.69 s	4,69 s	4,67 m	4,55 m
30	1,68 s	1,68 s	1,67 s	1,67 s

Legend:

1. Souza, 2001. 3β-hydroxy-lupeol
2. Souza , 2001 3α-hydroxy-lupeol

(-) unreported data

Tabla 2. ^{13}CNMR shifts values of compounds 1 and 3 and reference compounds used as (δ_C).

C	1 $CDCl_3$	2 $CDCl_3$	Compound 1 $CDCl_3$	Compound 2 $CDCl_3$
1	38,7	32,5	38,86	31,9
2	27,4	25,3	25,18	25,2
3	78,9	76,4	79,01	76,0
4	38,6	37,6	38,73	37,5
5	55,3	48,2	55,35	48,2
6	18,3	18,4	18,33	18,4
7	34,2	34,4	34,31	34,4
8	40,8	41,1	40,86	41,0
9	50,4	50,5	50,47	50,4
10	37,1	37,3	37,19	37,2
11	20,9	21,2	20,95	21,0
12	25,11	25,3	27,43	25,1
13	38,0	38,0	38,08	38,0
14	42,8	43,0	42,84	43,1
15	27,4	27,6	27,46	27,5
16	35,5	35,8	35,60	35,6
17	43,0	43,2	43,00	43,1
18	48,2	48,2	48,33	48,1
19	47,9	48,2	47,98	48,2
20	150,9	151,2	150.9	150,9
21	29,8	29,9	29,87	29,8
22	40,0	40,2	40,01	40,1
23	28,0	28,2	27,99	28,1
24	15,4	22,4	16,11	22,5
25	16,1	16,4	15,99	16,3
26	15,9	16,2	15,36	16,1
27	14,5	14,7	14,56	14,6
28	18,0	18,2	18,0	18,1
29	109,3	109,6	109,31	109,4
30	19,3	19,5	19,3	19,4

Legend:

1. Souza, 2001. 3β-hydroxy-lupeol
2. Souza , 2001 3α-hydroxy-lupeol

(-) unreported data

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