ANTIFUNGAL ACTIVITY OF EXTRACTS AND TERPENE CONSTITUENTS OF AERIAL PARTS OF *Juniperus Lucayana.*

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(Received July 2010; Accepted December 2010)

ABSTRACT

Ethanolic and hexane extracts from the aerial parts of *Juniperus lucayana* were assayed against the phytopathogenic fungus *Botrytis cinerea* by the poisoned food technique. The hexane extract showed to have a higher antifungal activity than ethanolic extract. Fractionation of hexane extract by silica gel open column chromatography and HPLC afforded seven known compounds sandaracopimaric acid (**1**), 4-*epi*-dehydroabietic acid (**2**), oplopanone (**3**), oplodiol (**4**), nephtediol (**5**), 7α-hydroxycallitrisic acid (**6**), and 7-oxocallitrisic acid (**7**). Compound **1** showed a significative fungal growth inhibition during the assay. The effect of compounds **2**-**7** on spore germination fungus was also studied.

Keywords: *Juniperus lucayana*, terpenoids, antifungal activity, *Botrytis cinerea*, growth inhibition, spore germination.

RESUMEN

Los extractos etanólicos y n-hexánico obtenidos a partir de las partes aéreas de *Juniperus lucayana* fueron evaluados sobre el hongo fitopatógeno *Botrytis cinerea* por el método de envenenamiento del medio. El extracto n-hexánico mostró una mayor actividad antifúngica que el etanólico. El fraccionamiento del extracto n-hexánico mediante cromatografía de columna abierta sobre gel de silice y CLAE permitió el aislamiento de siete compuestos conocidos, el ácido sandaracopimárico (**1**), el ácido 4-*epi*-dehidroabiético(**2**), la oplopanona (**3**), el oplodiol (**4**), el neftediol (**5**), el ácido 7α-hidroxicallitrisico (**6**), y el ácido 7-oxocallitrisico (**7**). El compuesto **1** presentó una inhibición significativa del crecimiento del hongo durante el experimento. Adicionalmente se estudió el efecto de los compuestos **2**-**7** sobre la germinación de esporas del hongo.

Palabras clave: *Juniperus lucayana*, terpenoides, actividad antifúngica, *Botrytis cinerea*, inhibición del crecimiento, germinación de esporas.

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INTRODUCTION

In recent years, public pressure to reduce the use of synthetic fungicides in agriculture has increased (Shafique *et al.*, 2007). Concerns have been raised about both the environmental impact and the potential health risk related to the use of these compounds. In contrast, natural product-based fungicides have the ability to decompose rapidly, reducing risk to the environment with the added advantage in that they have both unique modes of action and low mammalian toxicity (Duke *et al.*, 2003; Ortiz *et al.*, 2008). During our search for antifungal substances from Cuban plants, we found that the ethanolic extracts from the wood and aerial parts of *Juniperus lucayana* Britton (Cupressaceae) exhibited antifungal activity against *Botrytis cinerea*, with IC_{50} values ranging from 125 to 250 µg/mL (Ortiz *et al.*, 2004). This fungus is a serious pathogen that attacks economically important crops such as lettuce, carrots, tobacco, strawberry and grapes (Aleu *et al.*, 2001; Daoubi *et al.*, 2005). *Juniperus* species have been extensively investigated as a source of natural products with potential antimicrobial, acaricidal and insecticidal activities (Karaman *et al.*, 2003; Schmidt, 2004; Barrero *et al.*, 2005). To our knowledge, no fungicidal studies had been carried out on this species. In previous works we reported the bioassay-guided fractionation of ethanolic extract from the wood of *J. lucayana* which afforded three new sesquiterpenes along with six known sesquiterpenes and two flavonoids (Ortiz *et al.*, 2007). In this work, we reported the antifungal activity of the n-hexane and ethanolic extracts from the aerial parts of *J. lucayana*, as well as fractionation of the n-hexane extract by open column chromatography and HPLC to afford the isolation of seven compounds, first time reports for this species.

MATERIAL AND METHODS

General experimental procedures

The melting points (m.p.) were determined on a Reichert-Thermovar apparatus. The optical rotations were measured in $CHCl_3$ solution on a Perkin–Elmer 341 polarimeter. IR spectra were recorded on a Mattson Genesis spectrophotometer, series FT-IR. ^{1}H and ^{13}C NMR measurements were obtained on Varian Inova 400 and 600 MHz NMR spectrometers, using $SiMe_4$ as the internal reference. HPLC was performed with a Hitachi/Merck L-6270 apparatus equipped with an UV/Vis detector (L 4250) and a differential refractometer detector (RI-71). TLC was performed on Merck Kiesegel 60 F254 layers, 0.2 mm thick. Sephadex LH-20 and Si-gel (Merck) were used for column chromatography. Semi-preparative HPLC purification was conducted using a Si-gel column Lichrospher Si-60 column (10 µm, 1 cm wide, 25 cm long).

Plant material

J. lucayana was collected at the Institute of Fundamental Research on Tropical Agriculture "Alexander von Humboldt" in Santiago de las Vegas, Cuba, in March, 2005 and identified by Dr. Pedro Sánchez. A specimen of this plant is deposited in the herbarium of the "Instituto de Ecología y Sistemática de las plantas en Cuba" with the number HAC-42498.

Extraction and isolation

The air-dried powdered aerial parts (460 g) of *J. lucayana* were extracted using successively n-hexane and ethanol in a Soxhlet apparatus, for 4 h. The solvent was evaporated under reduced pressure to yield 31.7 g of an ethanol crude extract (**JFetd**). The n-hexane extract was cooled at –10 ^{0}C for 24 h, yielding an insoluble fraction. The defatted hexane extract (**JFhex**, 15.0 g) was fractionated by Sephadex LH-20 column chromatography using a mixture of n-hexane, $CHCl_3$, and MeOH (2:1:1) yield-

ing six fractions (F_{1-6}). TLC revealed that fractions F_{-3} and F_{-4} contained the same products. These fractions were purificated by means of column chromatography on Si-gel with $CHCl_3$, and MeOH and $CHCl_3$ mixtures containing increasing percentages of MeOH to give the compounds **1** (91.2 mg) and **2** (19.0 mg). Fraction F_{-1} was subjected to Sephadex LH-20 column yielding three fractions (F_{7-9}), Fraction F_{-8} was purificated by column chromatography on Si-gel eluting with MeOH and $CHCl_3$ mixtures containing increasing percentages of MeOH to give 15 fractions (F_{10-24}). Finally, fractions F_{-11}, F_{-19}, and F_{-20} were thus purificated by means of semi-preparative HPLC column [mixture of acetone and n-hexane (20:80), 2.8 mL. min^{-1}] to afford compounds **3** (8.4 mg), **4** (4.3 mg), **5** (11.2 mg), **6** (1.9 mg), and **7** (4.4 mg).

Microorganism

B. cinerea culture used in this work, Bc 2100, was obtained from the "Colección Española de Cultivos Tipos (CECT)", Universidad de Valencia, Facultad de Biología, Spain, where a culture of this strain is on deposit.

Antifungal assays

Poison food technique

Extracts and fractions (F_{1-6}) were dissolved in ethanol to give final of 500 mg/L for fractions and concentrations ranging from 125 to 500 mg/L for extracts. Compound **1** was evaluated at 150 mg/L. Antifungal assays were then carried out in accordance with the poison food technique (Soundharrajan *et al*, 2003). The solutions were added to a glucose–malt–peptone–agar [61 g/L of glucose (20 g)-malt (20 g)-peptone (1 g)-agar (20 g), pH 6.5–7.0]. The final ethanol concentration was identical in both control and treated cultures. The medium was poured into sterile plastic Petri dishes measuring 9 cm in diameter and 1.0-cm diameter mycelial discs of fungus cut from an actively growing culture were placed at the center of the agar plates. Inhibition of radial growth was measured during 6 days. Growth inhibition was calculated as the percentage of inhibition of radial growth relative to the negative control. Two independent assays were conducted, each in triplicate. The results are shown as mean values of colony diameters; (±SD).

Spore germination

Compounds **2-7** were dissolved in ethanol-water (1:1) at 100 and 200 mg/L. *B. cinerea* conidia used for this experiment were collected from cultures of fungi growing in the PDA media. The conidia were collected from the plates with 1 mL sterile distilled water, passed through a glass wool filter to remove hyphae, diluted, counted, and immediately used in the bioassay. Spore germination assays were carried out on multi-well microscope slides containing 10 wells. Each well was filled with 3 μL of the compound solutions at 100 and 200 mg/L, sterilized by filtration, together with conidia (2 μL, to a final concentration of 1×10^5 conidias/mL) of the fungus to be tested. Assays with sterile distilled water and water-ethanol, were used as controls. The plates were incubated at 25 °C and after 8 h, numbers of germinated and non-germinated conidias were counted in a light microscope and the spore germination percentage was calculated. Three wells per compounds concentration were prepared, and three areas per well were recorded (each containing 50 conidia). The experiment was performed by duplicate.

RESULTS AND DISCUSSION

Ethanolic and hexane extracts from the aerial parts of *Juniperus lucayana* were assayed against the phytopathogenic fungus *B. cinerea*, using the poisoned food technique (Soundharrajan *et al.*, 2003). As observed in **Figure 1**, the hexane extract (**JFhex**) proved to be more active than

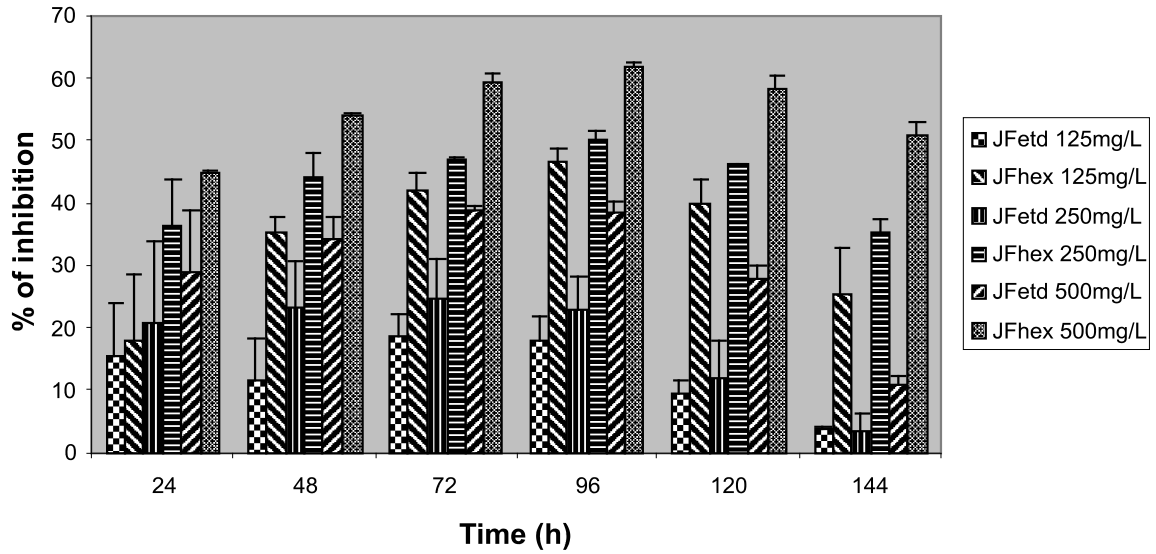

Figure 1. Antifungal effect of ethanolic and hexane extracts of aerial parts of *Juniperus lucayana*

ethanolic extract (**JFetd**), at different concentrations evaluated. The hexane extract exhibited a significant inhibition in fungal growth at concentrations of 250 and 500 mg/L (50.1 and 61.5%, respectively), while the ethanolic extract was less effective. In both extracts, in concentrations lower than 250 mg/L, a delay in fungal growth was observed.

Although the doses recorded in our experiments are high (125-500 mg/L) these results are highly relevant, since a fungicidal effect is not previous observed in products derived from aerial parts of *J. lucayana*.

As mentioned before, the hexane extract was more effective on *B. cinerea* than ethanolic extract. Therefore, we decided as a first step to isolate the active principles from hexane extract. The hexane extract was chromatographed over a Sephadex LH-20 column (n-hexane/$CHCl_3$/MeOH, 2:1:1). All fractions obtained, $F_{1\text{-}6}$, were also tested against *B. cinerea* using the same assay. Fractions $\mathbf{F_{-1}}$, $\mathbf{F_{-3}}$, and $\mathbf{F_{-4}}$ exhibited a 59.2, 71.5 and 70% inhibition in the growth of this fungus, respectively, at concentrations of 100 mg/L (see **Figure 2**). On the basis of their antifungal activity and their thin-layer chromatographic profiles, fractions $\mathbf{F_{-1}}$, $\mathbf{F_{-3}}$ and $\mathbf{F_{-4}}$ were purified by a combination of Si-gel column and semi-preparative HPLC analysis affording seven known compounds. The structures of the compounds were identified by physical and spectroscopic data measurement (mp, $[\alpha]_D^{20}$, IR, ^{1}H NMR, ^{13}C NMR and 2D NMR) and by comparing the data obtained with published values, such as sandaracopimaric acid ((-)-pimara-8(14), 15-dien-19-oic acid) (**1**) (Edwards *et al.*, 1960; Dang *et al.*, 2005), 4-*epi*-dehydroabietic acid (**2**) (San Feliciano *et al.*, 1992), oplopanone (2α-methyl-5α-isopropil-7α-oxomethylbiciclo[4.3.0]nonan-2β-ol) (**3**) (Su *et al.*, 1995; Kuo *et al.*, 2002), oplodiol (7-eudesmene-1β,4β-diol) (**4**) (Minato and Ishikawa, 1967; Tchuendem *et al.*, 1999), nephtediol ((1S, 4R, 7S)-germacra-5E, 10(15)-diene-1,4-diol) (**5**) (Kitagawa *et al.*, 1987), 7α-hydroxycallitrisic acid (7α-hydroxyabieta-8,11,13-trien-19-oic acid) (**6**) (De Pascual *et al.*, 1983) and 7-oxocallitrisic acid (7-oxoabieta-8,11,13-trien-19-oic acid) (**7**) (Prinz *et al.*, 2002) (see **Figure 3**).

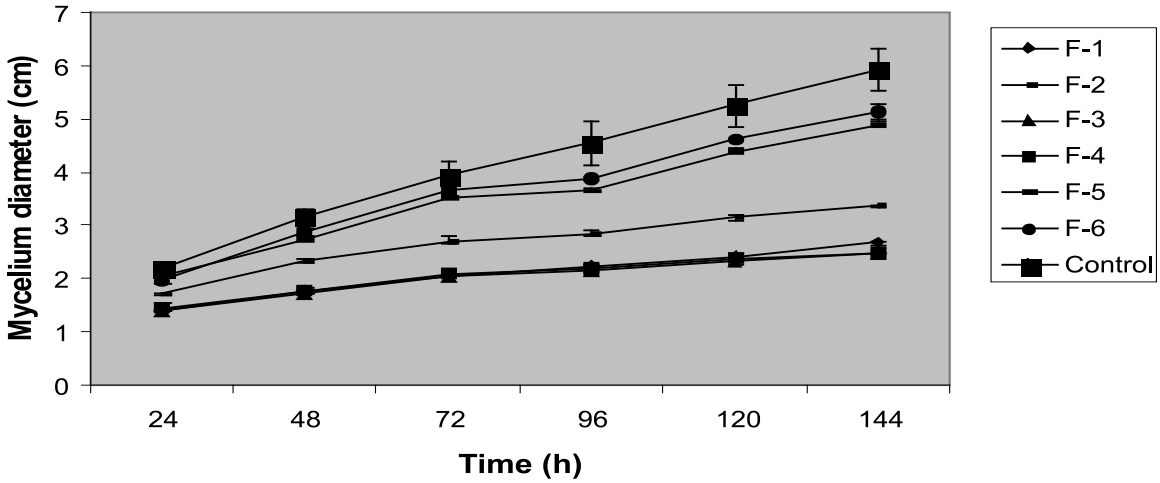

Figure 2. Antifungal activity of fractions F_{1-6} obtained from hexane extract on *B. cinerea*. Values represent means of two independent experiments with three replicates in each case. Bars represent SD of the mean.

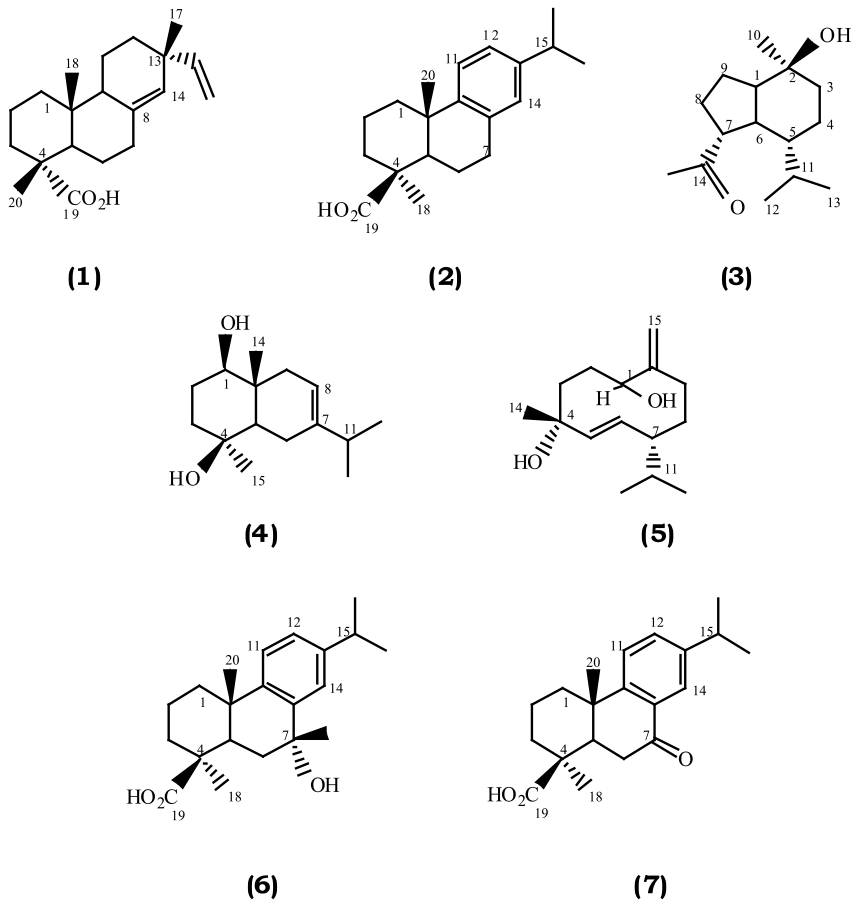

Figure 3. Compounds isolated from hexane extract of *J. lucayana.*

Compound **5** is isolated for the first time from a *Juniperus* species, meanwhile compounds **1**, **2**, **3**, **4**, **6**, and **7** have been previously obtained from the genus *Juniperus* (Kuo *et al.*, 1994; San Feliciano *et al.*, 1995; Fang *et al.*, 1996). The isolation of all this compounds from *J. lucayana* has not before been reported to the best of our knowledge.

The antifungal properties of the compounds **1**-**7** was evaluated for first time against *B. cinerea*, as described in the part experimental. The sandaracopimaric acid (**1**), evaluated by poison food technique, reduced the radial fungus growth (70.8% growth inhibition) in the first day of assay. However, it showed a weak activity after five days of experiment (52.7% growth inhibition) (see **Figure 4**). Compounds **2-7** did not inhibited the spore germination of *B. cinerea* at concentrations tested (**Table 1**).

Table 1. Spore germination activity of compounds **2-7.**

Treatment	Spore germination (%)	
	100 mg/L	200 mg/L
water	98.5	98.5
water-ethanol	96.5	96.5
1	ND	ND
2	97.2	98.0
3	95.0	97.0
4	97.8	94.1
5	100	100
6	98.2	100
7	98.0	98.2

ND: Not determined

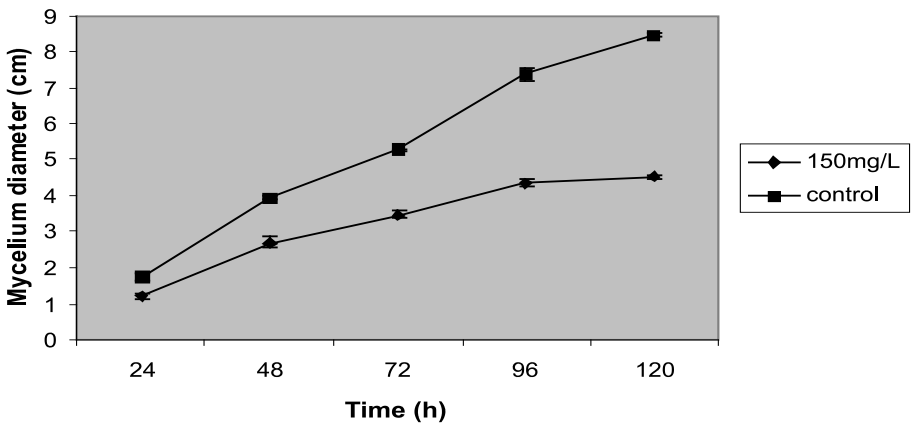

Figure 4. Antifungal activity of compound **1** on *B. cinerea.*

CONCLUSIONS

The ethanolic and hexane extracts from *J. lucayana* showed an important antifungal effect against *B. cinerea*. The presence of sandaracopimaric acid (**1**) contributes to this effect. Further studies on the isolated of additional compounds responsible of the activity of the extract are required.

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