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In vitro antioomycete activity of *Artemisia ludoviciana* extracts against *Phytophthora* spp.

[Actividad antioomiceto *in vitro* de extractos de *Artemisia ludoviciana* contra *Phytophthora* spp]

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Abstract

Artemisia ludoviciana Nutt. ("Estafiate", common name) is widely used in traditional Mexican medicine to relieve pain and stomach problems, and was studied to determine its potential antioomycete activity against *Phytophthora* spp. The wild oomycete isolates tested were *P. cactorum*, *P. capsici*, *P. cinnamomi*, *P. infestans* and *P. mirabilis*, all of them were sensible to crude extracts of the aerial parts of the plant. From this extract was obtained a fraction by TLC method (R_f =0.72) that contained essential oils capable of inhibiting oomycete growth with a minimum inhibitory concentration (MIC) in a range of 0.2 to 0.4 mg·ml^{-1}. The major compounds in the microbicidal fraction were borneol (16.28 %), camphor (7.41 %) and *cis*-verbenol (1.69 %). It was observed that only a mixture of them (63:28:6.5 µg·ml^{-1}) inhibited the growth of five *Phytophthora* species with a similar effect to the raw extract and the active fraction.

Keywords: Antioomicete; *Phytophthora*; Borneol; Camphor; *cis*-Verbenol.

Resumen

Artemisia ludoviciana Nutt ("Estafiate" nombre común), ampliamente usada en la medicina tradicional mexicana para aliviar el dolor y problemas estomacales, fue estudiada para investigar la actividad antioomiceto contra *Phytophthora* spp. Los aislados silvestres fueron *P. cactorum*, *P. capsici*, *P. cinnamomi*, *P. infestans* and *P. mirabilis*. Todos fueron sensibles al extracto crudo de la parte aérea de la planta. De estos extractos se obtuvo una fracción por TLC (R_f = 0.72) que contuvo aceites esenciales capaces de inhibir el crecimiento de los oomicetos con una concentración mínima inhibitoria (MIC) en el intervalo de 0.2 a 0.4 mg·ml^{-1}. Los compuestos mayoritarios en la fracción microbicida fueron borneol (16.28 %), camfor (7.41 %) y *cis*-verbenol (1.69 %). Se observó que únicamente una mezcla de ellos inhibió el crecimiento de las cinco especies de *Phytophthora* con un efecto similar al del extracto crudo y al de la fracción activa.

Palabras Clave: antioomiceto; *Phytophthora*; Borneol; Camfor; *cis*-Verbenol.

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INTRODUCTION

The *Phytophthora* genus is related to heterokontas and brown gold algae. It shows different ways of interaction with host plants and its control is different to that applied to real fungi and they are different phylogenetically taking into account their classification in the Chromist (Chromalveolata) kingdom (Van der Peer and De Wachter, 1997). It includes more than 50 phytopathogen species for more than 150 economically important crops and is responsible for blight, late mildew and rooting diseases. Depending on the species it can infect the leaves (*P. infestans* and *P. cactorum*), roots, stem (*P. cinnamomi*) and even the fruit. Interest in controlling this pathogen was renewed when some aggressive stocks were detected in west Mexican, in the avocado producing region, and also because of the detection of Mexican aggressive strain A2 (mate type) from *P. infestans* that affects significantly the potato crops in Europe and other parts of the world (Hohl and Iselin, 1984; Goodwin, 1997; Goodwin and Drenth, 1997). All of this has motivated the search for alternative methods to conventional chemical control, in order to obtain efficient and eco-friendly antioomycete substances (Damian Badillo *et al*., 2005). An alternative is the use of medical plants as it is the case of *Ocimum adscendes*. Their essential oil had a protective effect against fungi in stored *Capsicum annum* seed, which was more efficient than conventional fungicides (Asthana *et al*., 1989). Also, it has been reported that the raw extract from *Eucalyptus citriodora* and the essential oils from other plant species inhibited the mycelia growth in oomycetes such as *P. infestans* (Schwan-Estrada, 1998; Mine Soylu *et al*., 2006).

Potentially, *Artemisia* plants may be a source of toxic compounds against oomycete from the *Phytophthora* genus. Some species from this plant have been widely studied from the phytochemical point of view, mainly due to its use in traditional medicine for stomach illnesses. Compounds such as, camphor, germacrene D, *trans*-pinocarveol, *β*-selinene, *β*-cariofillene, artemisia cetone, *z*-epoxyocimene, crisantenyl acetate, *z*-epoxyocimene and *β*-thujone have been identified in and purified from *Artemisia annua, A. absinthium, A. santonicum* and *A. spicigera*. All of them show antifungal activity, while others like arteanuine B and artemisinin, have toxic effects against intestinal protozoon, *Entamoeba histolytica* and *Giardia lamblia*. Other have unknown biological functions such as the sesquiterpene lactones from *A. ludoviciana* (Jakupovic *et al*., 1991; Juteau *et al.*, 2002; Ramos-Guerra, 2004; Kordali *et al*., 2005a).

A. ludoviciana is a widely spread species distributed throughout Mexican territory and is commonly known as "estafiate", with medical properties and a traditional use similar to other species from the same genus, as described above. It has been reported that it also has antifungal activity against plant and vertebrates pathogens (Damián-Badillo *et al.*, 2008a). Therefore, in the context of using Mexican medical plants for phytopathogen control, specifically oomycetes, the purpose of this work was to evaluate the *in vitro* antioomycete activity from *A. ludoviciana* extracts against *Phytophthora* spp.

MATERIALS Y METHODS

Plant material

A. ludoviciana Nutt., (Asteraceae), specimens were collected from the estafiate crop in the Instituto Nacional de Investigaciones Forestales Agricolas y Pecuarias at the Uruapan Campus at Michoacan State and were identified in the Facultad de Biologia Herbarium, Universidad Michoacana de San Nicolas de Hidalgo. The material plant was collected at the early flowering stage (March-July of 2006-2007) and was dried.

One specimen was prepared for identification in the UMSNH herbarium (Voucher number 03309). Material was dried at room temperature and roots, aerial parts (stems and leaves) and flowers were separated and pulverized, then kept protected from direct light until the extraction process.

Plant extracts

The plant extracts were obtained according to the Damian Badillo *et al*., method (2008b). Briefly, a mixture of CH_3Cl_3:MeOH (1:2 v/v) was added to each 100 g of dry powder from the different plant parts and left five days in maceration at 4 °C and then filtered. The solvent was removed and the extract was dissolved in ethanol. To the aerial plant extract obtained with ethyl acetate and a soxhlet equipment was used for 2 h at 74 ºC, it was filtered, the solvent removed and 1 g was dissolved in 1 mL of ethanol and maintained at 4 ºC until the moment of the bioassays.

Thin layer chromatography (TLC).

A. ludoviciana chloroform-methanol extract fractioning was carried out by thin layer

chromatography (silica gel 60, Sigma®; 20 x 20 cm plaques) with a solvent system of CH_3Cl_3:MeOH (2:1 v/v) in a chromatographic chamber. Plaques were dried to room temperature and revealed with ultraviolet light at 254 nm, marking the bands corresponding to the fractions. The R_f for each of the fractions was calculated. Six different fractions were obtained and eluted with 10 ml of a mixture of chloroform-methanol (2:1 v/v). Solvent from each fraction was removed using a rotary evaporator at 45 °C and the residue was dissolved in 1 ml of absolute ethanol for bioassays and methanol for gas chromatography/mass spectrometry (GC/MS).

Essential oils: Plant extract and active fraction analysis were performed using the Damian Badillo *et al*. method (2008b), a Hewlett Packard 6890 chromatographer, with a HP 5973 mass detector, with an Equity 5 (30m x 20 mm) capilar column. The temperature programming condition of the oven was from 50 to 200 °C at 13 °C·min^{-1}, 200 to 300 °C and 300 °C·5 min^{-1} and programmed at 250 °C by 2 °C·min^{-1}. Initial injector temperature was 40 °C, increasing from 2 °C to 250 °C. The mass spectrum was taken at 70 eV with a mass range from 20 to 450 amu. Compound identification was done comparing the mass spectra and the retention time with the spectral data basis NIST, with a reliability percentage of 94 %.

Oomycete culture

P. cactorum, P. capsici, P. cinnamomi, P. infestans and *P. mirabilis* wild strains were isolated from sick tissues of host plants (strawberry, chili, avocado and potato, respectively). Phytopathologist Silvia Fernandez Pavia PhD identified them according to the *Phytophthora* taxonomic keys of CABI Biosciences Database (2003), Erwin and Ribeiro (1996) and Cooke *et al*. (2000). They were grown in dextrose potato media and once the mycelium grew, they were maintained in potato dextrose agar (PDA) (Difco, USA) and grown at 19 or 22 °C for 7 to 15 days depending on the species.

Reagents

All substances were reactive grade and the pure essential oils camphor, borneol and *cis*-verbenol, were acquired from Sigma Co.

Growth inhibition experiment

Potato dextrose agar dishes were inoculated with a small piece of mycelia in the center of the petri dish and incubated from 7 to 15 days at 19 °C or 22 °C depending on the oomycete tested. When mycelia grew, 0.5 cm^2 were placed over filter paper wetted with 10 µl (0.1 mg·ml^{-1}) from the extracts, fractions or diluted compounds in ethanol and were cultivated under the conditions mentioned above. Methyl *N*-(methoxyacetyl)-*N*-(2,6-xylyl)-D-alaninate (Ridomil Gold EC™) was used as a positive control, at a concentration of 1 mg·ml^{-1}. The concentration of the major compounds was: borneol (63 µg·ml^{-1}), camphor (28 µg·ml^{-1}) and *cis*-verbenol (6.5 µg·ml^{-1}). Every 12 h for the next fifteen days the inhibition diameter in the cultures was measured, subtracting that of the paper (10 mm).

Statistics

The results obtained are presented as the mean ± SD of the inhibition zone ($I\ \% = [(C\text{-}T)\cdot C^{-1}]100$: $I\ \%$ = relative inhibition, C = control colonial diameter, T = colonial diameter from the treated oomycete). 100 µl of absolute ethanol or water were used as references for comparison. The maximum growth measured was 2 cm, which was considered 100 % growth. The halo was measured and the corresponding proportion was calculated for each of the treatments. 100 % inhibition corresponds to no growth at all. The minimum inhibitory concentration from the extract and vegetable oil required for complete control of pathogen growth (MIC) was expressed in mg·ml^{-1} and classified as biocide effect over *Phytophthora* spp. The minimal oomiceticide concentration (MOC) is equal to MIC.

All the experiments were done three times with three replicates for each treatment. The Statistic 7.0 program was used to calculate the significance of all the data by the Tukey test ($p < 0.001$).

RESULTS

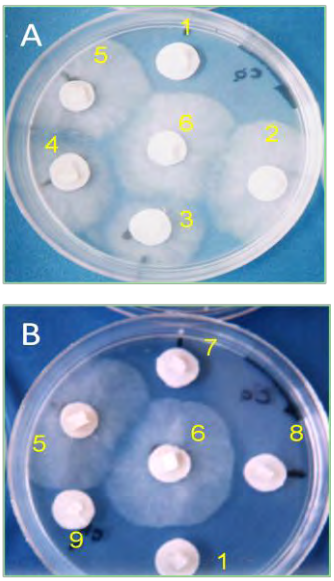
Screening of different extracts obtained from *A. ludoviciana* was carried out to find an antioomycete effect in this plant. It was observed that the chloroform-methanol extract from the green parts inhibited the growth 100 % in four of the oomycetes and in the case of *P. infestans* a 60 % inhibition was observed. Only *P. capsici* and *P. cinnamomi* were sensitive to the extracts obtained with ethyl acetate. It was also observed that the root extract does not contain oomycetes growth-affecting metabolites (Table 1). The leaf extract obtained with chloroform-methanol was fractionated by thin layer chromatography and six chromatographic signals were

observed, which were located by their R_f and were tested against five *Phytophthora* spp. Fraction III inhibited the growth of all the oomycetes with a minor MIC in a range of 0.2 to 0.4 mg·ml^{-1}, relating to the other growth inhibiting fractions, so, it was in this fraction that we sought plant metabolites causing *Phytophthora* sp growth inhibition (Table 2). The MIC value of the positive control Ridomil Gold EC™ was in a range of 0.1 to 0.25 mg·ml^{-1}.

To know the essential oil composition from the fraction, it was analyzed by gas chromatography coupled to mass spectrometer, giving as a result the identification of compounds, mainly terpenoids. It was observed that in contrast to fraction one, the rest of the fractions contain: borneol (16.2 %), camphor (7.4 %) and/or *cis*-verbenol (1,69 %) as major compounds (Table 3).

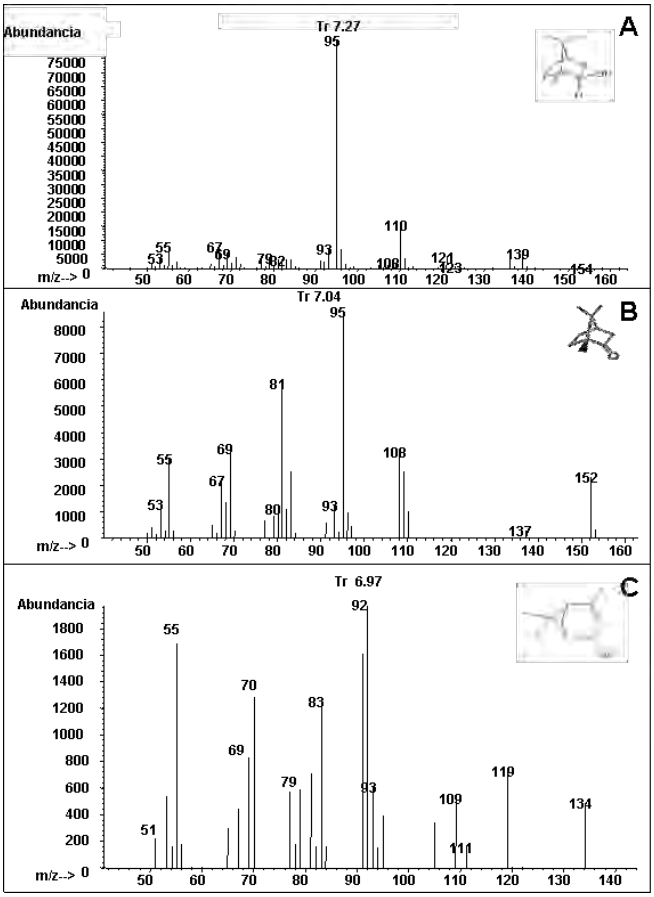
To know which of the major compounds detected on the *A. ludoviciana* leaf were responsible for the inhibitory effect, the oomycete were exposed to the purified compounds and a mixture of them. The results showed that purified compounds in isolation did not affect the growth of the oomycete tested. However, with the mixture of them, the inhibitory effect on *Phytophthora* sp growth was 100 % (Figure 1A), was similar to that observed with the raw extract and fraction III (Figure 2B).

Figure 1. Effect of essential oils from green parts from *A. ludoviciana* on *P. capsici* mycelial growth.



A. Before *A. ludoviciana* effect: **1**. Borneol, camphor and *cis*-verbenol mixture; **2**. Borneol; **3**. Camphor; **4**. *cis*-Verbenol. B. After *A. ludoviciana* effect: **7**. Chloroform-methanolic extract of green parts (leaves and stems); **8**. TLC-fraction III; **9.** Borneol and Camphor. The controls were 5. Ethanol and 6. Water.

Figure 2. Mass spectra of major metabolites identified from *A. ludoviciana* green parts.



A, Borneol. B, Camphor. C, *cis*-Verbenol.

Table 1. Effect of *A. ludoviciana* extracts on *Phytophthora* spp mycelial growth.

Tissue	Extraction solvent	Mycelial growth inhibition (%)				
		Pcac	Pcap	Pcin	Pinf	Pmir
Flower	Ethyl acetate	-	40	60	-	-
Leaves/stems	Ethyl acetate	-	7	-	-	-
Flower	Chloroform methanol	-	90	93	-	-
Leaves/stems	Chloroform methanol	100	100	100	60	100

Susceptibility to the plant extracts were done with only one concentration (0.1 mg·ml^{-1}) by the classic paper-disk agar diffusion assay. Pcac, *P. cactorum*. Pcap, *P. capsici*. Pcin, *P. cinnamomi*. Pinf, *P. infestans*. Pmir, *P. mirabilis*.

Table 2. Minimal inhibitory concentration (MIC) of the chloroform-methanol extract fractions from the green parts of *A. ludoviciana* on *Phytophthora* spp mycelial growth.

Fraction	R_f	MIC (mg·ml^{-1})				
		Pcac	Pcap	Pcin	Pinf	Pmir
I	0.79		3.9			
II	0.74		3.2		5.3	
III	0.72	0.4	0.2	0.2	0.2	0.28
IV	0.70		0.5		0.7	
V	0.58		0.5		1.1	
VI	0.57		1.6		2.3	

Susceptibility to the TLC fractions from the plant extracts were done under similar conditions to Table 1. The concentration range was 0.1 - 10 mg·ml^{-1} in the classic paper-disk agar diffusion assay. Pcac, *P. cactorum*, Pcap, *P. capsici*, Pcin, *P. cinnamomi*, Pinf, *P. infestans*, Pmir, *P. mirabilis*.

Table 3. GC/MS analysis of the microbicide TLC fractions from the chloroform-methanol extract from *A. ludoviciana* green parts

TLC fraction	Components	Retention time (min)	Relative abundance (%)
I	Limonene	5.62	0.12
II	Camphor	7.04	0.30
	Borneol	7.27	0.91
III	Eucaliptol	5.6	0.53
	Terpineol	6.45	0.34
	cis-verbenol	6.97	1.69
	Camphor	7.04	7.41
	Borneol	7.27	16.28
	Mirtenal	7.59	0.34
	Espatulenol	7.62	0.42
	Cariofilene	11.6	0.55
	derivate	11.7	0.84
	Espatulenol derivate		
IV	Eucaliptol	5.67	0.26
	Terpineol	6.47	0.33
	cis-verbenol	6.97	1.29
	Camphor	7.04	4.27
	Borneol	7.27	12.51
	Espatulenol	11.60	0.44
	Cariofilene	11.68	0.61
	derivate	12.13	0.21
	Espatulenol derivate		
V	*cis*-verbenol	6.97	0.32
	Camphor	7.05	1.82
	Borneol	7.27	3.78
VI	Camphor	7.05	0.79
	Borneol	7.27	1.59

DISCUSSION

Secondary metabolites produced by plants in their different developing steps, in their natural competition for new ecological niches, or in their defence mechanisms against microorganisms and predators, are natural sources of research for alternative controls against microorganisms causing health problems to animals and plants, and causing biodeterioration of different materials. In this work volatile compounds from *A. ludoviciana* that inhibited *Phytophthora* sp. growth were researched. The results showed that chloroform-methanol leaf and stem extracts (green parts) from *A. ludoviciana* inhibited oomycete growth. The metabolite content in the different extracts differed from an organ to organ, since leaves and stem extracts showed the highest activity while those from the roots had no apparent effect.

While the other extracts inhibited only two species of *Phytophthora*, the results showed that species variability in the same genus was significant. It suggests that the more susceptible oomycete to this plant species extracts were *P. capsici* and *P. cinnamomi* even when they belong to different groups (II and IV, respectively according to Cooke et al., 2000) inside the phylogenetic tree of *Phytophthora* genus, while the rest were not. On the other hand, *P. cactorum* as well as *P. infestans* and *P. mirabilis* belong to group I, so this difference may be due to particular characteristics of the mentioned groups (Cooke et al., 2000).

When the chromatographic fractions were tested against the oomycete, only fraction III was toxic for the five *Phytophthora* species. Otherwise, *P. capsici* and *P. infestans* were sensitive to at least five fractions. This is an interesting observation, as it would be expected that *P. infestans* and *P. mirabilis* would have had the same behavior because they belong to the same group I, while *P. capsici* is found in the second group (Cooke *et al.*, 2000). A probable explanation is the metabolite concentration and each species sensibility to them.

This is the first report where it is showed that borneol, camphor and *cis*-verbenol, the main components of the essential oil of the aerial parts from *A. ludoviciana*, have antioomycete properties. Comparing the MIC values against the positive control, suggest that this essential oil mixture may be used as a versatile and potent oomiceticide agent. Essential oils have been reported in *A. dracunculus*, *A. absinthium*, *A. santonicum* and *A. spicigera*, which presented a high antifungal activity against 34

phytopathogen species, among them the *P. capsici* oomycete, and this activity has been attributed to monoterpenes among which only borneol and camphor were identified in our fraction (Meepagala *et al*., 2002*;* Kordali *et al.,* 2005b). Moreover, it also has been reported that the aerial part of *A. dracunculus* L. var. *dracunculus*, contains compounds that showed microbicidal activity against *Botrytis cinerea*, *Colletotrichum gloeosporioides*, *C. acutatum* and *C. fragaria*e phytopathogens, but neither correspond to the ones identified in this work (Meepegala, *et al.*,2002, 2003). Another report, related to microbicidal activity in the *Artemisia* genus, refers to the aerial extracts from *A. verlotorum* against the pathogenic oomycete, *Saprolegnia fera.* The compounds responsible for this activity were not mentioned (Macchioni *et al.*, 1999).

The mixture of borneol, camphor and *cis*-verbenol is essential to obtain the antioomycetic effect, because it is not found with the compounds alone; Shafi (2004), reported that borneol does not have antioomycetic activity against *P. capsici*. This last fact suggests that when bioassays are being done it is necessary to test pure compounds and the mixture of the rest of the metabolites that are present in one extract or the active fraction, because an effect may be the result of the synergism of several components.

This work besides presenting the antioomycetic effect of the chloroform-methanol extract of the green parts of *A. ludoviciana* and the importance of using mixtures of compounds generates new research aims to identify more efficient and effective compounds, as well as understand their mechanism of action.

CONCLUSIONS

The chloroform-methanol extract of the green parts of *A. ludoviciana*, contains the secondary metabolites, borneol, camphor and *cis*-verbenol. These essential oils showed antioomycetic properties as a mixture, so it can be affirmed that this plant is toxic to *Phytophthora* spp.

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