Bactericidal and Fungicidal Activities of *Calia secundiflora* (Ort.) Yakovlev

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Calia secundiflora (Ortega) Yakovlev (Fabaceae) is considered a medicinal plant in Mexico but has scarcely been used because of the toxicity of its quinolizidine alkaloids. Several quinolizidine alkaloids have shown bactericidal, nematicidal, and fungicidal activities. The purpose of this study was to identify the alkaloids in the seeds and evaluate the activity of the organic extract on several phytopathogenic fungi and bacteria. An *in vitro* bioassay was conducted with species of the following phytopathogenic fungi: *Alternaria solani, Fusarium oxysporum* and *Monilia fructicola;* and of the following bacteria *Pseudomonas* sp., *Xanthomonas campestris* and *Erwinia carotovora.* Cytisine, lupinine, anagyrine, sparteine, *N*-methylcytisine, 5,6-dehydrolupanine, and lupanine were identified by liquid chromatography-mass spectrometry in the extract of seeds; the most abundant compound of the extract was cytisine. It was observed that the crude extract of *Calia secundiflora* was moderately active on bacteria and more potent on phytopathogenic fungi. In contrast cytisine showed the opposite effects.

Key words: Calia secundiflora, Quinolizidine Alkaloids, Toxicity

Introduction

Quinolizidine alkaloids are characteristic features of plants of the Fabaceae family, and the genus *Calia* is especially abundant in them. *Calia secundiflora* (Ortega) Yakovlev [syn. *Sophora secundiflora* (Ortega) Lag. ex DC] (Kite and Pennington, 2003) is considered a toxic species due to the presence of these alkaloids. The phytochemical aspects of *C. secundiflora* in North America and Asia have been widely studied, and its medicinal and toxicological properties have been documented since the 1970s (Hatfield *et al*., 1977; Murakoshi *et al*., 1986).

C. secundiflora is a bushy plant, distributed throughout Africa, America and Asia. On the American continent, it is found from southeastern North America (Hatfield *et al*., 1977) to southern Mexico. In Mexico, it is usually found in the eastern Sierra Madre and in the meridional mountains of the coastal plains (Schultes and Hofmann, 1982) of the states of Sonora, Chihuahua, Coahuila, Nuevo León, San Luis Potosí, Tamaulipas, Zacatecas, Querétaro and Hidalgo.

García-Mateos *et al.* (2007) pointed out that the leaves and roots of plants of the state of Hidalgo, México accumulate a range of alkaloids similar to that found in the seeds, with cytisine and/or *N*-methylcytisine being most abundant. Thus, individual variability in alkaloid profiles would constitute a chemical defense mechanism which impedes or prevents the selection of resistant strains of a phytophagous insect pest (Kinghorn and Balandrin, 1983). Wink (1992) suggested that quinolizidine alkaloids are general antipredator feeding deterrents which serve as defense compounds (Kinghorn and Balandrin, 1983). *C. secundiflora* relies substantially on quinolizidine alkaloids for the chemical defense against herbivores and to a minor degree against microorganisms and competing plants (Wink, 1992, 1993a; Ohmiya *et al*., 1995). However, few references exist on their bactericidal and fungicidal effects. Very little work has been done to study these activities of the quinolizidine alkaloids.

Therefore, the objectives set for this study were to isolate and identify the quinolizidine alkaloids present in seeds of *Calia secundiflora* by LC-MS

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and to evaluate the activity of the crude alkaloid extract on the phytopathogenic fungi *Alternaria solani, Fusarium oxysporum* and *Monilia fructicola*, and on three bacteria, *Pseudomonas* sp., *Xanthomonas campestris* and *Erwinia carotovora.*

Materials and Methods

Gathering of material

Plant material was collected randomly in two municipalities of the state of Hidalgo: Cardonal-Santuario and Cardonal, which are located at 20° 36′ N and 99° 07′ W at an altitude of 2130 m.

Alkaloid extraction

A crude alkaloid extract was prepared following the method described by Harris and Wilson (1988). The mature *C. secundiflora* seeds were previously ground in a Thomas-Wiley mill (Thomas Scientific, Swedesboro, NJ, USA). The powder (1300 g) was defatted with hexane in a Soxhlet apparatus for 24 h. After evaporation, it was shaken for 24 h with 5% trichloroacetic acid and filtered. The resulting mixture was basified to pH 10 by adding 10 M sodium hydroxide. The final supernatant was extracted with dichloromethane (3×100 ml). The organic phase was recovered and dried by evaporating the solvent in a Büchi rotary evaporator at 40–45 °C under reduced pressure. The extract obtained weighed 6.8 g. Preliminary identification of the alkaloids in the extract was made using thin layer chromatography.

Alkaloids purification

The crude alkaloid extract (6.8 g) was separated by column chromatography (CC) with silica gel (G 60 Merck 70–230 mesh). A dichloromethane/methanol gradient (100:0 to 89:11 v/v) was used to collect 384 fractions; similar ones were identified using TLC and combined. The solvent was evaporated under vacuum. Cytisine was obtained in the fractions 101–144 (dichloromethane/methanol 96:4 v/v); identification was based on spectroscopic analysis.

Identification by liquid chromatography-mass spectrometry (LC-MS)

The analyses were done in reverse phase in a Waters 600 high resolution liquid chromatograph attached to a Finnigan Mat LCQ mass spectrometer. A 250 mm × 4.6 mm C18 Sun Discovery column was used for chromatography. The flow speed was 1 ml/min, and a 0.1% (pH 4.7) ammonium acetate/MeOH/ACN (75:20.5:45.5) mixture was used as the mobile phase with the UV detector at 230 nm. Mass spectrometry (electronic impact source at 70 eV; temperature at 180 °C; registered at 0.75 s/scan at the 38–600 *m/z* interval) was used to confirm and identify the alkaloids, comparing their spectra with those of standard samples and those of the spectra library (Wink, 1993b; Wink *et al.*, 1999).

Evaluation of the extract on phytopathogenic fungi

A potato-dextrose-agar (PDA) culture medium was used: 300 g potato, 10 g dextrose and 10 g agar per liter. The mixture was sterilized in an autoclave for 18 min at 120 °C and 120 psi. A suspension of 10^5 spores ml^{-1} was prepared for each of the following species of fungi: *Alternaria solani, Fusarium oxysporum,* and *Monilia fructicola.* The test of spores was determined in a Neubauer counting chamber. In a laminar flow chamber, 1 ml of the spore suspension of each species was added to the sterile medium in 10-cm Petri dishes.

Seven 6 mm diameter discs of Whatman No. 1 filter paper were then placed equidistantly in each Petri dish. Each disc was saturated with different concentrations of the extract and cytisine and controls (treatments). The Petri dishes were incubated at 27 °C. The treatments applied to each species for the first test were 0, 1.5, 3 and 6 mg ml^{-1} of the alkaloid extract and 6 mg ml^{-1} of the commercial products benomyl (methyl-1-butylcarbamoyl-2-benzimidazol carbamate) and chlorothalonil (tetrachloroisophthalonitrile). For the second test, five discs were saturated with of 5, 7.5 and 10 mg ml^{-1} of cytisine isolated from the plant, and the same control treatments (benomyl and chlorothalonil, 6 mg ml^{-1}) were applied.

The variable evaluated was the diameter of the inhibition halo, which was measured with a graduated ruler. Three measurements at different angles were taken and an average determined.

A complete random blocks design was used with a divided plot arrangement and three replications. Each Petri dish contained a complete set of treatments. The factor species was considered the large plot and treatment was considered the small plot.

Evaluation of the extract on phytopathogenic bacteria

The species used in this evaluation were *Pseudomonas* sp., *Xanthomonas campestris* and *Erwinia carotovora*. As in the previous test, the disc diffusion technique was used. For the preparation of the bacteria culture medium, 23 g l^{-1} of nutritive agar (Bioxon, Becton Dickinson, Franklin Lakes, NJ, USA) were used. It was poured into Petri dishes in equal amounts and sterilized at 120 °C for 18 min. Once solidified and cooled, the culture medium was impregnated with a solution of 10^6 bacteria ml^{-1}.

For the first test, the applied concentrations of each species were 0, 1.5, 3 and 6 mg ml^{-1} the organic extract and 6 mg ml^{-1} of the commercial product agrimicin (Pfizer Inc., New York, NY, USA), for the second test concentrations were 5, 7.5 and 10 mg ml^{-1} of cytisine, besides agrimicin at 6 mg ml^{-1}.

Discs of Whatman No.1 filter paper, 6 mm in diameter and impregnated with each test species, were placed equidistantly in the Petri dishes, which were incubated at 27 °C. The evaluated variable was the diameter of the inhibition halo, following the same procedure as described for the previous test.

The experimental design was done with random blocks in divided plots with three replications. Each Petri dish contained a complete set of treatments. The factor species was the large plot and treatment was the small plot.

Statistical analysis

The data obtained was analyzed with the ANOVA and a Tukey test of comparison of means using Statistical Analysis System (SAS) software, version 8.0, 2002. A correlation test was done on the variables evaluated.

Results and Discussion

Using the method described by Harris and Wilson (1988), 7.72 g of organic extract (0.6% of the original sample) were obtained from 1.3 kg of ground *C. secundiflora* seeds.

An alkaloid extract was fractioned by means of preparative column chromatography, and cytisine was the most abundant alkaloid (1.67 g, 0.13% of original sample); other minor alkaloids were not evaluated. Zavala-Chávez *et al.* (2006) found that the leaves and roots of plants from Hidalgo accumulated a similar range of alkaloids in the seeds, where cytisine and/or *N*-methylcytisine were the most abundant.

According to Robinson (1979), a plant is considered a source of alkaloids if it contains more than 0.05% alkaloids by dry weight. Hegnauer (1963) lowered the minimum to 0.01%. Alkaloid contents in *Lupinus* species range between 0.6 and 6% dry weight with comparatively high values in flowers and fruits which are important for reproduction and survival (Körper *et al.*, 1998). The *C. secundiflora* alkaloid concentrations obtained in the present work were below the minimums listed by these authors, possibly because quinolizidine alkaloids are synthesized in diurnal rhythm in chloroplasts and before transported mainly to roots and seeds (Tyski *et al.*, 1988). Zamora-Natera *et al.* (2005) pointed out that the relation to the total content was variable in *L. exaltatus* depending on the analyzed organ, stage of growth and period of cultivation, but there exist no findings on *C. secundiflora*.

Waller and Nowacki (1978) considered that mature legume fruits have the highest concentrations of alkaloids since legumes tend to accumulate alkaloids in the fruit. Wink (1984) pointed out that the concentrations of alkaloids of the lupin plant are generally in the range where the alkaloids express effective antimicrobial activity or more higher, and that it is difficult to obtain data which unequivocally show that the intact plant should be protected against microbial attack *in vivo*. This is fulfilled for *Lupinus* and other Fabaceae (*Sophora, Cytisus*) which accumulate quinolizidine alkaloids in nearly all tissues and scarcely acquire a microbial infection.

Table I shows the profile of the alkaloids identified in *C. secundiflora* with their relative retention times resulting from the analysis made by LC-

Table I. Chromatographic and spectrometric characteristics of the alkaloids identified in *C. secundiflora*.

Alkaloid	Percentage	*m/z* [M^+]	Retention time [min]
Anagyrine	2.99	244	22.2
N-Methylcytisine	7.98	204	15.6
Cytisine	49.13	190	16.3
Sparteine	13.97	234	12.7
Lupanine	2.00	248	18.9
5,6-Dehydrolupanine	5.98	246	18.4
Lupinine	17.95	169	6.6

MS. The mass spectra of the alkaloids show fragmentation patterns characteristic of quinolizidine alkaloids (Ohmiya *et al*., 1995; Kinghorn *et al.*, 1982; García-Mateos *et al*., 2007). Some of the alkaloids previously described in the literature for *C. secundiflora* were identified, such as cytisine, lupanine, lupinine, anagyrine, *N*-methylcytisine, 5,6-dehydrolupanine and sparteine. Hatfield *et al*. (1977) described a similar profile for *Sophora secundiflora.*

Cytisine did not produce significant differences among any of the species evaluated, suggesting that the activity of this compound is less than of the organic extract. The organic extract greatly inhibited the mycelium development at concentrations of 6 mg ml^{-1}, surpassing benomyl at the same dosage. Its fungicidal activity, however, was not greater than that of chlorothalonil, which, at the same concentration (6 mg ml^{-1}), was more effective concerning the inhibitory activity. Cytisine showed fungicidal activity at 7 mg ml^{-1}, showing less control than benomyl, without surpassing the fungicidal power of chlorothalonil, which was more effective in controlling the mycelium growth (6 mg ml^{-1}) (Table II).

The alkaloid extract showed important activity in inhibiting the development of the three species. However, *Xanthomonas campestris* was the most sensitive strain, and *E. carotovora* was the most resistant to the organic extract. Cytisine was similar for the three species, inhibiting *Xanthomonas campestris* to a higher degree than the organic extract (Table II).

Table II. Comparison of means of the diameter of inhibition of the mycelial growth in each species.

Species	Diameter of inhibition [mm]	
	Alkaloid extract	Cytisine
Fusarium oxysporum	7.55 a	5.83 a
Monilia fructicola	7.00 a	5.50 a
Alternaria solani	1.33 b	5.22 a
LSD	0.94	0.69
Xanthomonas campestris	8.13 a	9.20 a
Pseudomonas sp.	7.26 b	7.66 b
Erwinia carotovora	6.26 c	5.80 c
LSD	0.81	1.09

Values having similar letters within columns are not significantly different at $P \leq 0.05$.
LSD, least significant difference.

Tyski *et al*. (1988) pointed out that there are no significant differences in the activity of pure alkaloid compounds: sparteine, lupanine, angustifoline and 13-OH-lupanine, compared to the ethanol extract obtained from *Lupinus angustifolius* against pathogenic bacteria.

Statistically, the concentrations of the alkaloid extract of 3 and 6 mg ml^{-1} were the best for microbial control after the commercial product agrimicin, which had the largest diameter of inhibition. The most effective concentration of cytisine was 10 mg ml^{-1}, which exhibited an inhibitory activity statistically different to that of agrimicin.

These results support the assumption that quinolizidine alkaloids have a broad spectrum of biotoxic properties. Several alkaloids affect more than one of the basic molecular targets tested (DNA intercalation, inhibition of DNA and RNA enzymes and inhibition of protein biosynthesis, membrane stability) in microbial and animals cells (Wink *et al*., 1999; Körper *et al*., 1998). Ohmiya *et al*. (1995) and Kinghorn and Balandrin (1983) described the biological activity of several alkaloids. Among those detected in Fabaceae plants, especially in the subfamily Papilionaceae, were sparteine, lupanine, anagyrine, cytisine and 5,6-dehydrolupanine as inhibitors of cell growth as well as of the action of the enzymes β-glucoronidase and acetylcolinesterase. It is likely that these activities are responsible for the phytotoxic effects that are exhibited by the alkaloids. There is still very little known about their mechanism of action in plants (Wink *et al*., 1999). Körper *et al*. (1998) described that the two major alkaloids usually are lupanine and sparteine which occur in many members of the family Fabaceae (*Lupinus*) in concentrations high enough to effectively interfere with the molecular targets and prevent a fungal or bacterial infection in plants (Wink, 1984).

The results underscore the importance of alkaloids as an alternative source of natural products for the control of phytopathogenic fungi. It can be assumed that quinolizidine alkaloids are synergistic and may be therefore also important in the antimicrobial defense (Tyski *et al*., 1988). However, there is sufficient evidence that quinolizidine alkaloids of many Fabaceae inhibit the growth of bacteria and phytopathogenic fungi and the germination of others plants, but there are only few studies on cytisine (Wink, 1984; Wippich and Wink, 1985; Tyski *et al*., 1988). These properties of alkaloids or

extracts can be used to protect several crops by spraying them with exogenous alkaloids.

It can be assumed that the crude alkaloid extract of *Calia secundiflora* is moderately active as a bactericide and more potent as a fungicide. In contrast, the alkaloid cytisine showed more bactericidal activity than the organic extract. These results support the assumption that quinolizidine alkaloids have a broad spectrum of biotoxic properties. The analysis made by LC-MS identified seven alkaloids: cytisine, lupanine, lupinine, anagyrine, *N*-methylcytisine, 5,6-dehydrolupanine and sparteine. The results underscore the importance of *Calia secundiflora* as an effective antimicrobial agent and an alternative source of natural products for the control of phytopathogenic bacteria and fungi.

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