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Hybanthus parviflorus (Violaceae): Insecticidal activity of a South American plant

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ABSTRACT

Hybanthus parviflorus is a perennial shrub, widely distributed in the tropical and subtropical regions of America, and is known as 'violetilla' in Argentina. Previous phytochemical studies of this species led us to the isolation and determination of the primary structure of a novel macrocyclic polypeptide, the cyclotide hypa A. Here the insecticidal activities of extracts of *H. parviflorus* were determined against *Ceratitis capitata* Wied., the Mediterranean fruit fly or 'Medfly'. Mortality in the different life stages of Medfly, total mortality and modifications of the insect's physiology caused by 50% EtOH and CH_2Cl_2 extracts of *H. parviflorus* were evaluated. In addition, we determined the occurrence of ursolic acid, β-sitosterol and the polyphenols quercetin, quercetin-3-methyl ether, apigenin, luteolin, kaempferol, rutin, caffeic acid and chlorogenic acid. The promising insecticidal activity of 50% EtOH extracts of *H. parviflorus* and its purified fractions was related to the presence of cyclotides. The insecticidal activity of CH_2Cl_2 extracts could be related to the presence of polyphenols, ursolic acid and β-sitosterol.

1. Introduction

Both plants and herbivorous animals have co-evolved over hundreds of millions of years and as a result of this evolution, plants have acquired defense mechanisms against nematodes, insects, birds and mammals (Jongsma and Bolter, 1997). It is only when these herbivorous animals can adapt to these defense mechanisms that they may potentially become serious pests.

The Violaceae plant family occurs in temperate regions worldwide. It comprises 22 genera and 900 species. Violaceae are generally perennial herbaceous plants (Hoffmann et al., 1992). *Hybanthus parviflorus* (Mutis ex L.f.) Baill. (Violaceae) is a minor shrub which grows at 0–1000 m above sea level. It is a branchy erect plant no more than 50 cm high (Cabrera, 1965). This plant is found in Argentina, Bolivia, Brazil, Chile, Paraguay and Uruguay (Zuloaga and Morrone, 1999) and blossoms and fruits in autumn i.e. in April–May (Marzocca, 1997). *H. parviflorus* has adapted to grow in undisturbed soils. It is a weed of crops that are cultured annually and it is mainly found in fallows and in direct sown soy fields. It also grows in many types of stubble, on wastelands, on walls and amongst rubbles (Marzocca, 1997). The conservation of sites with natural vegetation, usually better adapted, helps to keep within the plant community herbicides-susceptible genotypes and also to limit the proliferation of the less susceptible ones. Some species that are considered to be weeds, such as *H. parviflorus*, when ground is fallows, can make a significant contribution to this fact, mainly during the initial stages of the implementation of an agricultural system (Papa, 2000).

H. parviflorus is a plant species that produces biotypes which are resistant to the herbicides commonly employed for the control of weeds in fallows and during the cultivation cycle, such as glyphosphate and the 2, 4–D. For this reason, *H. parviflorus* is cited among those plants belonging to the category of weeds displaying varying degrees of resistance to glyphosphate (Marzocca, 1993; Pengue, 2001; Faccini, 2000).

Primary chemical studies indicate the presence of an alkaloid called violine in its roots (Durañona and Dominguez, 1928), having similar properties to the isoquinoline alkaloid emetine, present *in Psychotria ipecacuana* (Rubiaceae) and which acts mostly as emetic and amebicide (Girault, 1987). For this reason, the roots of *H. parviflorus* can be used as a substitute for the ipecac root (Dominguez, 1903; Pizarro, 1966; Mateu Amengual, 1980; García Barriga, 1992).

The flavonoids quercetin, kaempferol, luteolin and apigenin have also been detected in the leaves (Harborne and Williams, 1975; Saxena, 1975; Mateu Amengual et al., 1981).

To date, no biological activities of the species *H. parviflorus* have been detected. Some years ago, phytochemical search of cyclotides led us to the isolation and determination of the structure of a novel

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macrocyclic polypeptide code named hypa A from this species (Broussalis et al., 2001). The insecticidal activity is a remarkable property of cyclotides from both ecological and environmental points of view, having technological and economical-commercial potential (Trabi and Craik, 2002).

The possible role of cyclotides as plant-defense mechanisms against microorganisms and insects has been mentioned (Craik et al., 1999). Besides, the antifeedant effect of ursolic acid on lepidopterous insects, e.g. *Spodoptera litura* (the tobacco pest) has been reported (Shukla et al., 1996). Beta-sitosterol has also been demonstrated to have an antifeedant effect (Chandramu et al., 2003).

Taking into account the presence of cyclotides, *H. parviflorus* was selected for the assessment of its insecticidal activity, studying mainly the insecticidal activity of three of its extracts and two purified fractions.

Ceratitis capitata, commonly known as the Mediterranean fruit fly or 'Medfly' is a worldwide pest and, in Argentina, it is one of the most important pests of fruit crops, especially citrus. It directly affects the fruit during the maturation process on the tree. *C. capitata* has a short life cycle, it is easy to manipulate and has a high fecundity, all features that make it suitable for biological assays (Bado et al., 2004). For this reason, the lethal and sublethal effects to the fruit fly of the 50% EtOH extracts, the tannin-free ethanolic extract and its butanolic and aqueous solutions and the acetonitrile (ACN) and CH_2Cl_2 extract of *H. parviflorus* were investigated.

2. Materials and methods

2.1. Preparation and purification of extracts

2.1.1. Extraction of the plant material

H. parviflorus [*syn. Ionidium glutinosum* Ventetat, *Viola parvifolia* Roemer and Shultes (Ballard and Jorgensen, 1997)], family Violaceae was collected, in part, in the intersection of the Nº 12 National Road and the Feliciano stream, La Paz Department, in the Province of Entre Ríos, Argentina in October 1998. The plant was identified by Dr. Juan de Dios Muñoz, keeping a voucher specimen – Muñoz 1514 (ERA) – in the Herbarium of the School of Agricultural Sciences, National University of Entre Ríos, Paraná city, Argentina.

The method employed for obtaining and purifying the extracts was described by Claeson et al. (1998) with the aim of obtaining peptide-enriched extracts. The vegetable material was dried under sunlight and in a forced air oven at a temperature below 40 °C, in order to preserve it and to avoid any enzymatic degradation of the compounds present. The dried and ground aerial parts of *H. parviflorus* (30.9 g) were extracted by maceration with CH_2Cl_2 (300 ml) for 1 h under continuous shaking. This procedure was repeated 7 times changing the solvent each time. The plant residue was then macerated in 50% v/v EtOH. The remains of the extraction were then macerated in 25% ACN – 0.1% TFA. Both, the ethanolic and ACN extracts were acidified with 2% AcOH and eluted through a polyamide column to remove the tannins The columns were eluted with 2% AcOH with a subsequent rinsing with 50% EtOH/2% AcOH to elute those peptides that were insoluble in 2% AcOH. Afterwards, the ethanolic tannin-free extract was lyophilized. A portion of it (42 mg) was dissolved in water and partitioned 3 times against n-BuOH. The butanolic phases were combined and the solvent removed, yielding 0.84 mg of the butanolic fraction. The remaining water of the butanolic extraction was named 'the aqueous fraction' (Broussalis et al., 2001).

2.1.2. TLC and HPLC analysis of flavonoids

The polyphenolic compounds (flavonoids and caffeylquinic acids) present in the CH_2Cl_2 and 50% EtOH extracts of *H. parviflorus* were studied by thin-layer chromatography (TLC) and HPLC. The TLC systems used were 1) Stationary phase (SP): silicagel; Mobile phase (MP): AcOEt, formic acid, AcOH, water (100:11:11:27); 2) SP: cellulose; MP: AcOH 15% V/V, and 3) SP: cellulose; MP: AcOH 40% V/V. All chromatograms were observed under UV light (254 and 366 nm) and revealed with natural product reagent (Wagner et al., 1984). They were then examined again under UV light. The CH_2Cl_2 and 50% EtOH extracts and the fraction eluted with 25% ACN–0.1% TFA of the tannin-free butanolic fraction of the 50% EtOH extract were analyzed by HPLC–DAD employing a C18 column. The butanolic fraction of 50% EtOH extract eluted with 25% ACN–0.1% TFA that had been kept for the analysis of flavonoids, was subjected to semi-preparative RP–HPLC employing 25% ACN–0.1% TFA as mobile phase. To analyze this fraction, the solvents: A: H_2O–AcOH (98:2) and B: MeOH–AcOH (98:2), were employed as the mobile phase. The injection volume was 100 μl and the flow rate 1.3 ml/min.

The 50% EtOH extract was subjected to HPLC using a Phenomenex™ C18 analytical column and employing apigenin, luteolin, kaempferol, quercetin, quercetin-3-methyl ether and rutin as reference flavonoids. The mobile phase was a mixture of solvents A: H_2O–AcOH (98:2) and B: MeOH–AcOH (98:2) eluted in a linear gradient from 85% to 60% of A during 10 min, 60% to 25% of A during 40 min, 25% to 15% of A during 5 min. The injection volume was 20 μl and the flow 1.3 mL/min.

2.1.3. Presence of β-sitosterol, oleanoic and ursolic acids

The presence of β-sitosterol in the CH_2Cl_2 extract of de *H. parviflorus* was determined by TLC and GC employing a β-sitosterol standard.

The TLC systems employed were a) SP: silicagel; MP: CH_2Cl_2, acetone (95:5), b) SP: silicagel; MP: ciclohexane, AcOEt (1:1), c) SP: silicagel; MP: toluene, acetone, AcOH (100:3:0.07). All chromatograms were revealed with sulfuric acid 50% EtOH V/V. Oleanoic and ursolic acids were analyzed by TLC in two chromatography systems (b and c). The GC analysis of the CH_2Cl_2 extract of *H. parviflorus* employing standards of oleanoic and ursolic acid and the co-chromatography of the extract using the corresponding standards, allowed confirmation of the presence of such acids in the extract.

2.2. Insecticidal experimental design

The insecticidal activity of the CH_2Cl_2, 50% EtOH, the tannin-free ethanolic extracts and the butanolic and aqueous solutions as well as the ACN extract were investigated. The concentrations employed were: CH_2Cl_2 extract: 1000 and 100 ppm; 50% EtOH extract: 1000 and 100 ppm; tannin-free 50% EtOH extract and its butanolic and aqueous solutions: each 200 ppm and ACN extract: 200 ppm. The mortality at each stage of the life cycle of the fly as well as the overall mortality were assessed. The delay in the development of the insect produced by the CH_2Cl_2 and 50% EtOH extracts was also evaluated. The effect of 50% EtOH and tannin-free 50% EtOH extracts, butanolic and aqueous solutions and ACN extract were also evaluated on the pre-pupation mortality of the fruit fly. Each solution was assayed at 200 ppm.

2.3. Insecticidal activity assay

C. capitata Weid. (Diptera, Tephritidae) larvae from an established laboratory colony reared in Chair of Agricultural Zoology, Faculty of Agronomy, University of Buenos Aires, Argentina, with a Teran artificial diet (Teran, 1977) and environmental standard conditions (25 ± 2 °C, 60 ± 5% RH, in darkness), were assayed. Cohorts of one-day-old 10 larvae were reared in plastic vessels containing the artificial diet previously mixed with ethanolic solutions of *H. parviflorus* extracts and fractions, depending upon the treatments to obtain the corresponding final concentration.

Table 1
Mortality at each stage of the life cycle of *C. capitata* and overall mortality.

Extract (ppm)	Without puparium formation (%)	Adult emergence (%)	Without adult emergence (%)	Overall mortality (%)
Control	0a	100	0	0a
CH_2Cl_2 (1000)	53b	10	37	90b
CH_2Cl_2 (100)	32b	18	50	100b
50% EtOH (1000)	30b	15	55	100b
50% EtOH (100)	53b	10	37	95b

a and b: significant differences with respect to control ($p < 0.05$).

Untreated larvae were used as controls. Four replicates of each treatment were assayed. Each replicate was put inside a larger plastic vessel containing sterilized sand for pupation. Mortality until adult emergence (%) was recorded, EC_{50} values – the concentration needed to avoid development in 50% of larvae – was calculated assaying three concentrations.

2.4. Statistical analysis

The percentages of puparia, expressed in relation to the number of exposed larvae, were used to calculate PT_{50} – the time needed for 50% of larvae to pupate – and EC_{50} values were calculated by Probit analysis using a computer program (Finney, 1971). Statistical differences in mortality ($p \leq 0.05$) were calculated with ANOVA and Tukey's multiple range test (Steel and Torrie, 1993).

3. Results

3.1. Phytochemical analysis

The TLC analysis of the polyphenolic compounds (flavonoids and caffeylquinic acids) present in the CH_2Cl_2 and 50% EtOH extracts of *H. parviflorus* allowed determination of the presence of rutin, the principal compound, and chlorogenic acid in the 50% EtOH extract by means of the three chromatographic systems employed (1–3).

The analysis of the UV spectra of the compounds present in extracts and fractions subjected to HPLC–DAD allowed the presence of other flavonoids and caffeylquinic acids to be determined and the principal flavonoid rutin to be quantified.

The following flavonoids were identified by comparison of their retention times (rt) and UV spectra with those of the corresponding commercial standards: quercetin (rt 26.59 min), luteolin (rt 28.04 min), quercetin-3-methyl ether (rt 28.47 min), kaempferol (rt 30.48 min), apigenin (rt 31.32 min) and the glycoside rutin (rt 18.62 min). Quercetin-3-methyl ether and rutin were identified for the first time in *H. parviflorus.* Since the amount of rutin was considerably higher than the other flavonoids, it was quantified. The rutin content, determined by HPLC, was 1.34 g/100 g EtOH 50% extract (1.34%) or 0.24% referred to the dried and powdered plant material.

Upon analyzing the UV spectra of the butanolic fraction of the 50% EtOH extract eluted with 25% ACN–0.1% TFA, the presence of flavonoids could be demonstrated. Under these experimental conditions, a triglicoside of the flavonoid rutin, named QT, was isolated.

3.2. Insecticidal activity

3.2.1. Mortality at each stage of the life cycle of C. capitata and overall mortality

The effects of the CH_2Cl_2 and 50% EtOH extracts were evaluated on the puparium and on the overall mortality(Fig. 1). The effect of extracts on insect mortality was also recorded as emergence or non-emergence of the adult stage (Table 1).

Table 2
Delay in the development of the insect.

Extract (ppm)	Pupation time PT_{50} (days)	Confidence interval
Control	5.6a	5.3–5.7
CH_2Cl_2 (100)	5.6a	5–6.1
50% EtOH (1000)	11.3b	9.8–19.6
50% EtOH (100)	12b	10.2–25

PT: pupation time. Values followed by the same letter do not differ significantly in Tukey's test ($P = 0.05$).

3.2.2. Delay in the development of the insect

The effects of the CH_2Cl_2 and 50% EtOH extracts on the delay in the development of the fruit fly, recorded as pupation time, were evaluated (Table 2).

3.2.3. Pre-pupation mortality

The CH_2Cl_2 and the 50% EtOH extracts induced mortality at 100 and 1000 ppm but only the 50% EtOH extract induced delays in development time (Table 3).

4. Discussion and conclusions

All the extracts and fractions induced high percentage mortality. However, the mortality induced by the butanolic fraction obtained by the purification of the tannin-free 50% EtOH extract was higher (by 90%) than the mortality induced by the aqueous fraction (65%) and the ACN extract (58%). This result is in agreement with the greater proportion of cyclotides present in this fraction which displays a high solubility in BuOH. The aqueous fraction and ACN extract contain cyclotides but in smaller quantities, as determined by HPLC–DAD.

Cyclotides have different biological activities but their function in plants is still not elucidated. One might suspect that these molecules are involved in defense mechanisms (Jennings et al., 2005) since they are found in plant species which contain them (Craik et al., 1999).

All their biological activities assessed in mammal cellular systems seem to be related to interactions with membranes, a common feature of the defense molecules of plants (Felizmenio-Quimio et al., 2001). The insecticidal activity is a remarkable property of cyclotides from both ecological and environmental points of view, having technological and commercial prospects. Whichever the mechanism of action may be, these findings clearly demonstrate that the outstanding stability of the cystine knot structure (Craik et al., 1999) makes it an excellent framework on which a wide range of biological activities can be added, generating potential applications as insecticides in agriculture.

Cyclotides are found in plants in small quantities. For this reason we have investigated extracts and purified fractions enriched in these compounds. Furthermore, the ursolic acid and the β-sitosterol that have been isolated from many plant species, presented different

Table 3
Pre-pupation mortality.

Extract or fraction	Mortality (%)
Control	0a
50% EtOH	80c
Tannin-free 50% EtOH	100c
BuOH fraction	90c
Aqueous fraction	65b
ACN	58b

Values followed by the same letter do not differ significantly ($P = 0.05$).

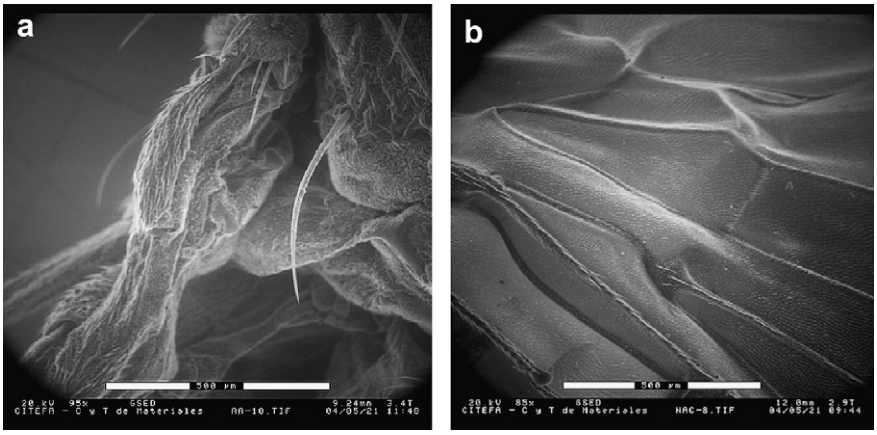

Fig. 1. Insecticide activity of the 50% EtOH extract of *H. parviflorus* on the fruit fly (*Ceratitis capitata*). a) Wing which failed to unfurl due to the 50% EtOH extract at 1000 ppm. b) Normal wing unfurled. Scale: 500 μm.

degrees of antifeedant activity on lepidopterous insects. These compounds, present in the CH_2Cl_2 extract of *H. parviflorus* could be in part or totally responsible for the insecticidal activity of this extract. By generating these products, a substantial part of the traditional insecticidal market would be redirected towards the biotechnological industry, as it has occurred with the first generation of transgenic plants. The global expenditure in the chemical control of insect pests is estimated to be more than 3 billion dollars per year.

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