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Phytochemical analysis and anti-allergic study of *Agave intermixta* Trel. and *Cissus sicyoides* L.

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Abstract

Agave intermixta Trel. (Maguey) and *Cissus sicyoides* L. (Bejuco caro) are Caribbean plant species from the Dominican Republic used locally in traditional popular medicine that have shown an anti-inflammatory effect in experimental animal models. A phytochemical analysis on these species allowed us the isolation and identification of the steroidal sapogenins hecogenin and diosgenin from Maguey and the hydroxystilbene resveratrol from Bejuco caro. The effects of these plant extracts and their isolated constituents on compound-48/80-induced histamine release from peritoneal mast cells were investigated. Significant inhibition was produced by 0.5 mg mL^{-1} of a methanolic extract of Bejuco (41.1%) and by its constituent resveratrol (82.4%) at a dose of 250 μM. However, none of the steroidal sapogenins from *A. intermixta* showed a significant inhibitory effect on histamine release from mast cells. From these results, it can be deduced that the in-vitro anti-allergic activity towards the release of histamine from mast cells shown by the methanolic extract of C. *sicyoides* may be mediated by its constituent resveratrol and might contribute to the anti-inflammatory activity shown by this species.

Introduction

Agave intermixta Trel. (Agavaceae) is a species from tropical America, commonly known as Maguey or Maguey de bestia (Gupta 1995). This species is cultivated in the Dominican Republic because of its uses as a medicinal plant (Garcia 1972). The therapeutic uses of the aqueous extracts of its leaves include the treatment of inflammations, arthritis, tumours and infections (Gupta 1995). The species of the genus *Agave* constitute an important source of steroidal sapogenins, mainly hecogenin (Bedour et al 1979; Gupta 1995).

Cissus sicyoides L. (Vitaceae), so-called Bejuco caro or Bejuco de parra in the Dominican Republic, is a climber frequently found in the island where it is used in popular medicine as a diuretic, anti-inflammatory and anti-influenza agent (Carvajal et al 1983; De Mena 1994). From phytochemical studies it is well-known that the genus *Cissus* contains sterols, quinones and phenolic compounds in the leaves and anthocyanins in the fruits (Toledo 1983; Robineau 1997; Beltrame et al 2002). The traditional use as anti-inflammatory agents of other species of this genus (e.g. *C. trifoliata* in Mexico) has been validated in several acute and chronic models of inflammation in rats and mice (Pérez et al 1993). On the other hand, a mixture of decoctions of these tropical species is consumed in the Dominican Republic in the treatment of cancer in traditional popular medicine (Quílez 1998).

Previous investigations carried out by our group on these plants (Quilez 1998; García et al 1999; Sáenz et al 2000) demonstrated the effective in-vivo anti-inflammatory activity of both species and their mixture on hind-paw oedema in rats and ear swelling in mice caused by carrageenan and tetradecanoylphorbol acetate (TPA), respectively. In addition, a reduction in neutrophil infiltration into inflamed tissues was associated with their topical anti-inflammatory effect as detected by the measure of the activity of myeloperoxidase enzyme, a neutrophil specific marker (Garcia et al 2000).

This study was undertaken to evaluate whether the extracts or their constituents might inhibit the induced allergic inflammatory response of histamine release from

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isolated rat mast cells, as a possible mechanism of action implicated in their anti-inflammatory effects.

Material and Methods

Plant material

The leaves of *Agave intermixta* Trel. (Agavaceae) were collected in Arroyo Santiago (Monte Plata, Dominican Republic) and identified by Dr F. Jimenez at the National Botanic Garden of Santo Domingo (JBSD). The stems of *Cissus sicyoides* L. (Vitaceae) were collected and identified by Dr J. Garrido from paraje Mirador (Monte Plata, Dominican Republic). A voucher of both specimens was deposited in the National Botanic Garden of Santo Domingo (JBSD), Dominican Republic.

Preparation of plant extracts

Aqueous extracts of Maguey and Bejuco were prepared according to the Spanish Pharmacopeia method for decoctions (Real Farmacopea Española 1997). The mixture of both species was made following the folk medicine method, namely, six fingers of *A. intermixta* and six inches of stems of *C. sicyodes*. The aqueous extracts were filtered and evaporated and the residues were obtained with yields of 1.56% for *A. intermixta* Trel. (Maguey), 8.82% for *C. sicyoides* L. (Bejuco) and 4.90% for the mixture of both species.

Bejuco methanolic extract was obtained from the dry Bejuco stem pieces. They were kept on maceration in petroleum ether–ethyl ether–methanol (1:1:1) and the solvent was renewed every 24 h. The extraction fluids obtained were added up and concentrated under reduced pressure until dry residue. This residue was re-dissolved in methanol 80% and kept at 4°C for 24 h for removal of waxes and chlorophylls. The methanolic solution was concentrated until dry residue, yielding 0.4%.

Isolation of pure compounds

Hecogenin and diosgenin
The isolation of the genins was carried out from the acidic juice of *A. intermixta* as proposed by Srinivasulu & Rao (1983). Maguey leaves were liquidized by a mechanical juice squeezer (Type 140.6.03). The chromatographic separation of the dry residue was performed using a Sephadex LH 50 (Merck) column and methanol as eluent. Hecogenin and diosgenin were detected in the 3–4 and 4–5 fractions, respectively. Both compounds were purified by preparative thin-layer chromatography (TLC) (Merck silica gel plates; 0.2–0.5 mm) using n-hexane–ethyl ether (7:3).

Resveratrol
The methanolic extract of Bejuco was fractionated using a Silicagel 60 (0.063–0.200 mm; Merck) chromatographic column and n-hexane, dichloromethane and dichloromethane–methanol (several proportions). The hydroxystilbene resveratrol was recovered from the (3:7) dichloromethane–methanol fraction.

For the identification of the compounds, instrumental techniques included fusion point (Thermovar HT 1B11), mass spectra (Kratos MS 80 RFA) and NMR (Bruker AMX-500) analysis.

Reagents and solutions for bioassay

Dimethyl sulfoxide (DMSO), compound 48/80, *o*-phthaldehyde (OPT), bovine serum albumin (BSA), sodium cromoglicate, toluidine blue, heparin sodium and phosphate buffer were supplied by Sigma Chemical Company and NaOH and HCl by Panreac.

Animals

Male Wistar rats, 8–10 weeks old, 250–300 g, were housed at 24 ± 2°C and 60 ± 20% relative humidity, on a 12-h light–dark cycle. Rats were given free access to a diet of standard chow and water. All the experiments were performed according to the guidelines for the ethical treatment of animals of the European Union.

Mast cell isolation

Peritoneal mast cells were isolated as previously reported (Wang & Teng 1990). Mixed rat peritoneal cells were collected by peritoneal lavage with heparinized Tyrode solution and were purified by centrifugation through a 30% BSA density gradient.

Purified mast cells were washed and re-suspended in Tyrode's solution with glucose (composition in mM: NaCl 137, KCl 2.7, $NaHCO_3$ 12, NaH_2PO_4 0.3, $MgCl_2$ 1.0, $CaCl_2$ 1.0, glucose 5.6 and BSA 0.1%). For the determination of the concentration of mast cells, 50 μL of toluidine blue 0.05% in saline was added to an equal volume of the cell suspension and was adjusted to 1×10^6 cells/mL for the experiments.

Effect of extracts and compounds on histamine release from mast cells

The mast cell samples (0.5 mL) in triplicate were prewarmed at 37°C for 10 min with 2 μL of vehicle (DMSO) or test substances (0.5 mg mL^{-1} of the extracts or 250–500 μM of hecogenin, diosgenin and resveratrol). Sodium cromoglicate at 500 μM was used in these experiments as reference compound. The histamine release reaction was triggered by the addition of 2 μL of compound 48/80 at a final concentration of 10 μg/mL for an incubation time of 20 min at 37°C. The reaction was stopped by the addition of ice-cold Tyrode's solution and the mixture was centrifuged for 10 min at 1000 *g*.

Histamine in the supernatant was determined fluorimetrically (Hakanson & Ronnberg 1974) after condensation with *o*-phthaldehyde (1% methanol). The emitted fluorescence was measured in a fluorescence microplate reader (Spectra Fluor Tecan) ($\lambda_{excitation}$ 360 nm, $\lambda_{emission}$ 450 nm). To estimate the spontaneous release of histamine, exactly the same procedure was followed but without adding samples or compound 48/80. The release of histamine

was expressed as a percentage of total of the maximum level (cells stimulated with compound 48/80).

Statistical analysis

Differences between treatments were statistically examined using the Kruskal–Wallis test in conjunction with an appropriate post-hoc test. In all cases $P < 0.05$ denoted significance.

Results

Identification of compounds from Maguey juice

Hecogenin

m.p. 258–260°C. The development of TLC silica gel with oleum reagent gave a dark-yellow spot at R_f 0.89, 0.5 and 0.08 with the mobile phases dichloromethane–methanol (7:3 v/v), n-hexane–ethyl acetate (5:5 v/v) and n-hexane–ethyl ether (7:3 v/v), respectively.

E.M. m/z (%) (430 (M^+, 32), 371 (8), 361 (5), 358 (21), 316 (40), 287 (6), 273 (38), 139 (100), 129 (55), 69 (46).

Diosgenin

m.p. 202–204°C. The development of TLC silica gel with oleum reagent gave a greenish-grey spot at R_f 0.92, 0.75 and 0.17 with the mobile phases dichloromethane–methanol (7:3 v/v), n-hexane–ethyl - acetate (7:3 v/v) and n-hexane–ethyl - ether (7:3 v/v), respectively.

E.M. m/z (%) 414(M^+, 8), 346 (5), 342 (5), 300 (21), 282 (40), 271 (18), 143 (7), 139 (100), 115 (13).

In both cases, the presence of a base peak in the mass spectra at m/z 139 $[C_9H_{15}O]^+$ indicated the spiro ring system break. Hecogenin showed the molecular ion at m/z 430 $[C_{27}H_{42}O_4]$ and diosgenin at 414 $[C_{27}H_{42}O_3]$.

Identification of compound from the methanolic extract of Bejuco

Resveratrol

m.p. 255–260°C. The development of TLC silica gel with oleum reagent gave a brown spot at R_f 0.25 and 0.42 with the mobile phases n-hexane–ethyl ether (7:3 v/v) and n-hexane–ethyl acetate (7:3 v/v), respectively, and a blue colour at λ 366 nm under UV light.

^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.31 (2H, d, J = 8.5 Hz, H-3′,5′), 6.89 (1H, d, J = 16 Hz, H-β), 6.71 (1H, d, J = 16 Hz, H-α), 6.70 (2H, d, J = 8.5 Hz, H-2′,6′), 6.39 (2H, d, J = 2.5 Hz, H-2,6), 6.11 (1H, d, J = 2.5 Hz, H-4).

^{13}C NMR($CDCl_3$, 500 MHz): δ 158.77 (C-3,5), 157.42 (C-4′), 140.72 (C-1), 129.89 (C-1′), 129.06 (C-β) ,128.44 (C-2′,6′), 125.70 (C-α), 116.13 (C-3′,5′), 105.56 (C-2,6), 102.34 (C-4).

E.M. m/z (%) 228 (M^+, 100), 211 (10), 199 (6), 181 (9), 157(5), 153 (4), 114 (3).

Antihistamine activity

The effects of the plant extracts and their constituents on compound 48/80-induced histamine release from rat peritoneal mast cells are show in Table 1. The spontaneous release of histamine from mast cells was approximately 20% and the maximum histamine level of the mast cells was that of the cells treated only with 10 μg mL^{-1} of compound 48/80 (100% of release).

Table 1 Histamine release from rat peritoneal mast cells and inhibition of histamine release by tested extracts and isolated compounds

Group	Histamine release (%)	Inhibition of release (%)
Unstimulated cells	20.8 ± 0.1**	—
Cells with compound 48/80	100	—
Sodium cromoglicate 500 μM	61.7 ± 1.4*	38.2 ± 1.4
Hecogenin 500 μM	97.1 ± 3.5	2.8 ± 3.5
Diosgenin 500 μM	100.6 ± 5.5	−0.6 ± 5.5
Resveratrol 250 μM	17.5 ± 0.1***	82.4 ± 0.1
Maguey aqueous extract 0.5 mg mL^{-1}	91.8 ± 3.2	8.1 ± 3.2
Bejuco aqueous extract 0.5 mg mL^{-1}	82.6 ± 1.3	17.3 ± 1.3
Maguey + Bejuco aqueous extract 0.5 mg mL^{-1}	78.1 ± 1.5	21.8 ± 1.5
Bejuco methanolic extract 0.5 mg mL^{-1}	58.9 ± 0.5*	41.1 ± 0.5

Data are represented as the mean ± s.e., n = 4. Statistical significance was assessed using a non-parametric multiple comparison Kruskal–Wallis test with Dunn's post-hoc test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs 10 μg mL^{-1} of compound 48/80-treated control group.

Concerning the pure compounds, resveratrol was the only one that inhibited histamine release from mast cells, producing an inhibition of 82.4% ($P < 0.001$) at a dose of 250 μM (Table 1). The steroidal compounds hecogenin and diosgenin did not affect the release. On the other hand, although none of the aqueous extracts produced a significant effect on the release of histamine, the mixture of both aqueous extracts presented a rather higher inhibitory effect (21.8%) than each of the extracts on its own. However, the methanolic extract of Bejuco (0.5 mg mL^{-1}) was the most potent among the tested extracts, inhibiting histamine release by 41.1%.

Discussion

Although the steroidal compounds hecogenin and diosgenin have been isolated from several species of *Agave* (Alemán & Roquel 1973; Bedour et al 1979; Cuellar & Diaz 1979a, b; Compañía Española de Esteroides 1980; Varshney et al 1982; Sati & Pant 1983; Srinivasulu et al 1985; Gbolade 1988), for the first time, we have identified them from *A. intermixta* Trel. In spite of the previously documented anti-inflammatory activity of the aqueous extract of this plant (Garcia et al 2000), in our investigations, this extract and the isolated compounds hecogenin and diosgenin did not show any significant inhibitory effect on histamine release from mast cells, as has also

been reported for some fitosterols (Navarro et al 2001). However, a slight inhibitory effect could be observed when Maguey aqueous extract was assayed mixed with Bejuco aqueous extract – the form in which the plants are consumed in Caribbean popular medicine.

On the other hand, resveratrol (3,4′,5-hydroxyestilbene) is a representative of the hydroxystilbene compound class that occurs in many plants and is especially abundant in grapevine (Vitaceae). It has received special attention in many investigations since it is known to have a cancer chemopreventive activity (Jang et al 1997; Radford 1997) and a variety of biological activities, including an anti-inflammatory effect due to the inhibition of cyclooxygenase (Jang et al 1997; Shin et al 1998) and leukotriene generation (LTB4) (Huang et al 2001).

In previous works, resveratrol has demonstrated significant anti-allergic activity upon the release of β-hexosaminidase (IC50 (50% inhibitory concentration): 15 μM) (Cheong et al 1999). The authors suggested that such an effect might be related to the presence of two benzene rings with an appropriate distance in its stilbene structure, as also described for flavonoids, natural products whose anti-allergic activity has been firmly established (Fewtrell & Comperts 1977; Foreman 1984).

In this work, the most significant inhibition was produced by the methanolic extract of Bejuco (41.1%) and by its constituent resveratrol (82.4%). These results indicate that the anti-allergic activity of the methanolic extract from *Cissus sicyoides* may be caused, at least in part, by its constituent resveratrol, although the effects of other phenolic components identified in this plant (Toledo 1983; Robineau 1997, Beltrame et al 2002) warrant further investigations.

Conclusion

From these results, it can be deduced that the in-vitro anti-allergic activity towards the release of histamine from mast cells shown by the methanolic extract of C*issus sicyoides* may be mediated by its constituent resveratrol and might contribute to the anti-inflammatory activity shown by this species.

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