BIOACTIVE COMPONENTS OF *Hedera helix*

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The composition of a methylene chloride extract from the leaves of *Hedera helix* growing on S. Miguel island (Azores) was investigated. Fourteen compounds were identified. The extract was rich in methyl esters of several carboxylic acids, stigmasterol and α– and β–amyrin and promising antithrombin activity. The active components were identified as β–amyrin, stigmasterol and hexadecanoic acid. β–amyrin also presented activity on a brine shrimp (*Artemia salina*) bioassay.

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INTRODUCTION

Hedera helix L. ssp. *canariensis* (Willd) Cout. is a climbing plant with wooden stems. The leaves, which are mostly wider than long, are dark evergreen. The flowers are yellow-green and the fruits black (SJÖGREN 1984).

Hedera helix is known to contain saponins (SCHLOSSER 1973; RAO et al. 1974; BALANSARD et al. 1980; KRAJEWSKA 1981; ELIAS et al. 1991; MAJESTERSAVORNIN et al. 1991; CRESPIN et al. 1995; TRUTE et al. 1997; BEDIR et al. 2000). α-Hederin, β-hederin and δ-hederin (SCHLOSSER 1973; MAJESTER et al. 1991; TRUTE et al. 1997a; STEGLICH et al. 2000), hederasaponins A, B, C, D, E, F, G, H, I (SCHLOSSER 1973; ELIAS et al. 1991; MAJESTER et al. 1991), hederagenin (PIO FONT QUER 1979; TRUTE et al. 1997a), 3-O-β-D-glucopyranosylhederagenin (CRESPIN et al. 1995), helixosides A and B, 3-O-β-D-glucopyranosyl-(1>2)-β-D-glucopyranosyl oleanolic acid 28-O-β-D-glucopyranosyl-(1>6)-β-glucopyranosyl esters (BEDIR et al. 2000), oleanolic and echinocystic acids and their glycosides (KRAJEWSKA 1981; FAVEL et al. 1994; GRISHKOVETS et al. 1999) are examples of triterpenoid saponins known to the present in this plant. Other terpene compounds like monoterpepenes and sesquiterpenes also have been identified in this plant. α-Pinene, β-pinene, limonene, sabinene, β-caryophyllene and germacrene D were identified on the essential oil of stems and leaves (TUCKER et al. 1994) and abscisic acid in the acid fraction of leaf extracts of *Hedera helix* (HILLMAN et al. 1974). Phenolic compounds have also been identified in *H. helix* (TRUTE et al. 1997a). Acids like chlorogenic acid, neochlorogenic acid, 4,5- and 3,5-O-dicaffeoylquinic acid, rosmarinic acid, caffeic acid and protocatechuic acid (TRUTE et al. 1996; 1997b) and flavonoids (KRAJEWSKA 1981) like rutin, kaempferol, isoquercitrin, astraglin, quercetin (TRUTE et al. 1997b) and 3-rhamnoglycosyl kaempferol (REYNAUD et al. 1982) were isolated from this plant. Alkaloids like emetine have also been isolated from alcoholic extracts of *H. helix* (MAHRAN et al. 1975). Long chain unsaturated alcohols, such as falcarinol, were isolated from the stems and petioles of *H. helix* (STEGLICH et al. 2000). Falcarinol exhibits antibacterial and cytotoxic (antitumor) activities as well as antifungal and analgesic activities.

This plant has been employed as medicinal plant for curing several human and animal diseases (CHICHIRICCO et al. 1980). Indeed, *H. helix* has shown several biological activities which can be used for agricultural or medicinal purposes. Crude plant extracts have shown molluscicidal activity against *Biomphalaria alexandrina* (EL-EMAM et al. 1990; SHOEB et al. 1994), as well as, antifeedant activity against the corn wireworm , *Melanotus communis* (VILLANI et al. 1985) or *Mythimna unipuncta*, *Mamestra brassicae* and *Spodoptera frugiperda* (HUBRECHT 1988). The plant also showed cytotoxic effects (MERZABANI et al. 1979).

Saponins of the plant are reported to have antifungal, anthelmintic, molluscicidal, antileishmanial, antipasmodic, antimicrobial, antitrypanosomial, antimutagenic and cytotoxic activities (CIOAKA et al. 1978; MARGINEANU et al. 1978; HOSTETTMANN 1980; TRUTE et al. 1997a; ELIAS et al. 1990; 1991; TEDLAOUTI et al. 1991; QUETIN et al. 1992). α-Hederin and β-hederin were shown to inhibit the growth of a wide range of fungi (SCHLOSSER 1973; BALANSARD et al. 1980). α-Hederin and hederasaponin C showed antifungal activity against *Candida abicans*, *Microsporum canis*, *Epidermophyton floccosum* and *Tricophyton rubrum* (TIMON et al. 1980; FAVEL et al. 1994). α-Hederin also showed anthelminthic activity against *Fasciola hepatica* and *Dicrocoelium* spp. (JULIEN & al. 1985). The effects of this monodesmosidic triterpenoid saponin on cultured mouse B16 melanoma cells and noncancer mouse 3T3 fibroblasts were investigated by DANLOY (et al. 1994). α-Hederin was cytotoxic and inhibited the proliferation of both cell types. The compound also induced vacuolisation of the cytoplasm and membrane alterations resulting in cell death.

As part of a program whose main objective has been the discovery of potential medicinal uses of the forest biomass of the Azores, the methylene chloride extract of *Hedera helix* has shown to possess antithrombin activity (MEDEIROS et al. 2000). On the other hand, the alcoholic extract showed almost no activity. So, the purpose of this study was to investigate the composition of the methylene chloride extract of this plant as, the chemical nature of the compounds responsible for the antithrombin activity showed previously, remains unknown until now. Considering the potential medicinal uses of plants of the Azores, it was considered appropriate to test the isolated antithrombin active compounds also on a general bioassay to detect a broad spectrum of pharmacologic activivities as the brine shrimp bioassay (MEYER et al.1982).

MATERIALS AND METHODS

Extraction of Plant Material

Hedera helix was collected in September-October 2000, and selected at random from plants growing on S. Miguel Island. Two sets of voucher herbarium specimens were made. One set was sent to the Museum Carlos Machado Herbarium (Azores), and the other was kept at the laboratory of Institute of New Technologies of Azores (INOVA-5).

The air-dried plant material (5 kg) was ground into coarse pieces and was extracted with methylene chloride (24 h) in a soxhlet apparatus. The solvent was removed *in vacuo* to give 100 g of crude material.

Chromatography

Ten grams of the crude methylene chloride extract were separated by open column chromatography. The column size was 7x100 cm with 300 g of silica gel (E. Merck 7734). One hundred millilitres of hexane was collected for each fraction, and the separation was performed by gradually increasing the solvent polarity with chloroform. A total of 540 fractions were collected, and the combined fractions were monitored by thin-layer chromatography (TLC) using UV light (254 nm) as detecting agent.

Instrumentation

Infrared (IR) spectra were recorded on a Perkin-Elmer Model 1600 infrared spectrometer. Solid samples were prepared as potassium bromide (KBr) pellets. Ultraviolet (UV) spectra were

obtained on a Perkin Elmer Lambda 6, UV-VIS spectrometer. Nuclear magnetic resonance (^{1}HNMR and ^{13}CNMR) was performed on a Varian 300 MHz. Gas chromatographic (GC) analyses were obtained from a VARIAN model 3400 using a flame ionization detector (FID). A 15 m RTx 5 column (5% diphenyl, 95% dimethylsiloxane) was used. The temperature of the column was programmed to change from 90°C to 130°C at a rate of 1°C/min and then to 280°C at a rate of 4°C/min. The temperature of the injector and detector was 250°C. The GC-MS analyses were performed on a gas chromatograph-mass spectrometer, Hewlett Packard model 6890 Series GC System with a HP 5973 MS detector (EI mode, 70eV). A column type, HP-5 (5% phenylmethylsiloxane) with a length of 30 m and an inside diameter of 2.5 μm and a film thickness of 0.25 μl was used. The temperature of the column was programmed to change from 90°C to 130°C at a rate of 1°C/min and then to 280°C C at a rate of 4°C/min. The temperature of the injector and detector were 250°C and 170°C, respectively. Kovats indices (21, 22) were calculated by using a standard mixture of C_5-C_{30} n-alkanes (Aldrich) and using the same chromatographic conditions described above. High resolution mass spectra were obtained on a VG7070EHF mass spectrometer (Perkin Elmer). Melting points were observed on a Mel-Temp melting point apparartus and are uncorrected.

Antithrombin Activity

The chromogenic bioassay (MEDEIROS et al. 2000) was used to determine the antithrombin activity of the fractions and pure compounds. 1 pt (part per thousand) samples were prepared using Tris-NaCl buffer (Sigma Chemical Company). After mixing equal volumes of the sample solution and of a thrombin solution (reconstituting 500 units of bovine plasma Lyophilized powder from Sigma Chemical Company), the chromogenic reagent (D-PHE-L-PIPECOYL-ARG p-nitroanilide from Sigma Chemical Company) was added. The absorbance at 405 nm (A_{405}) was measured continuously over a 5 min period (t') in a Molecular Devices kinetic microplate reader. Pure methanol was used as negative control. Heparin (Aldrich) was used as positive control at 1 pt. Three replicates were performed for each test and control samples, and the average percent activity (A%) was calculated using the formula:

$$A\% = 1 - \frac{(V_{max\ sample})}{(V_{max\ blank})} \times 100$$

$$V_{max} = \frac{(A^{405})_{t=t''} - (A^{405})_{t=0}}{t'}$$

Brine Shrimp Bioassay

The procedure for the brine shrimp bioassay was described before (MEYER et al. 1982; HARTL et al. 2000). Brine shrimp (*Artemia salina*) hatched in artificial seawater are pipetted into each well containing the sample (2 ppm). After 48 hours of incubation the number of dead nauplii was counted. Pure methanol was used as negative control. Diacetoxyscirpenol (DAS) was used as positive control at 2 ppm. Three replicates were performed for each test and control samples, and the average percent activity was calculated using the formula:

$$\%\ \text{Activity} = 100 - \%\ alife$$

$$\%\ \text{alife} = \left[test / negative\ control\right] \times 100$$

RESULTS AND DISCUSSION

Eleven compounds were identified and three isolated from six fractions. A mixture of several compounds was eluted with hexane (fractions 4-124). The components were identified by comparing their retention times and mass spectra (MS) with those of authentic samples (Aldrich) and the use of a computerized MS data bank (Wiley and NIST). They were the hydrocarbons pentacosane, heptacosane and hentriacontane. 1-eicosanol was eluted with hexane (fractions 298-302). It was identified by comparing their physical and spectral data (MS, IR, UV, ^{1}H NMR, ^{13}C NMR) with an authentic sample (Aldrich). Fractions 303-308 also eluted with hexane

afforded the steroid stigmasterol (0.12% w/w)after recystallization with 5% ethyl acetate in hexane and then methanol. It was identified by comparing their physical and spectral data (mixed mp, MS, IR, UV, ^{1}H NMR, ^{13}C NMR) with authentic samples (Aldrich). A mixture of α–amyrin (0.95% w/w) and β–amyrin (0.23% w/w) was eluted with hexane (fractions 310-317). These pentacyclic triterpenes were identified by comparing their retention times and MS with those of authentic samples (Aldrich) and the use of a computerized MS data bank (Wiley and NIST). Six methyl esters were identified from the 20% chloroform-hexane eluate (fractions 455-496) as methyl esters of hexadecanoic, eicosanoic, docosanoic, tetracosanoic, hexacosanoic and octacosanoic acids. The identification of these esters was also made by comparing their retention times and MS with those of authentic samples (Aldrich) and the use of a computerized MS data bank (Wiley and NIST). Hexacosanoic acid was eluted with 40% chloroform-hexane (fractions 521-540) after recrystallization from ethanol. This compound was identified by comparing authentic samples (Aldrich) physical and spectral data (mixed mp, MS, IR, UV, ^{1}H NMR, ^{13}C NMR) with the data of it.

All the fractions obtained were tested *in vitro* by the antithrombin bioassay test (MEDEIROS et al. 2000). The results of this chromogenic bioassay presented in Table 1 demonstrate the antithrombin activity of the fractions 310-317 (73%) from which the two amyrins were identified. The antithrombin activity of authentic samples of α–amyrin and β–amyrin was also tested under identical conditions. The results suggest that the activity of the fractions 310-317 can be attributed to the existence of β–amyrin which appear to possess similar activity (table 1). The same fraction presented activity when tested on the brine shrimp bioassay and β–amyrin was again the compound which presented activity (Table 1). Fractions 303-308 exhibited also antithrombin activity (56%) from which stigmasterol was isolated. The antithrombin activity exhibited by an authentic sample of stigmasterol tested under identical conditions appeared to be similar (69%). Fractions 521-540 also presented some antithrombin activity (51%). Hexadecanoic acid, isolated from this fraction showed similar activity when tested under identical conditions (table 1). Fractions 303-308 and 521-540 showed no activity when tested on the brine shrimp bioassay as well as stigmasterol and hexadecanoic acid.

Table 1.
Biological activity of the fractions from the methylene chloride extract of *Hedera helix*

Sample	Antithrombin activity (%)	Brine shrimp activity (%)
fractions 4-124	--	*20*
fractions 298-302	*23*	*0*
fractions 303-308	*56*	*0*
fractions 310-317	*73*	*40*
fractions 455-496	*32*	*0*
fractions 521-540	*51*	*0*
α-amyrin	*39*	*0*
β-amyrin	*78*	*90*
stigmasterol	*69*	*0*
hexadecanoic acid	*47*	*0*
heparin	*74*	--
Diacetoxyscirpenol	--	*100*

In summary, three of the fourteen compounds identified from *Hedera helix* L. ssp. *canariensis* (Willd) Cout. had biological activity when tested on the antithrombin and one of them (β–amyrin) also showed activity on the brine shrimp bioassay. Interestingly it has already been reported that acylated β–amyrin aglycones of *H. helix* showed antiviral activity (RAO et al. 1974). This is the first report of these biological activities of *H. helix* and the identification of β–amyrin, stigmasterol and hexadecanoic acid as the active components.

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