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Chemical Composition of and Inhibition of Angiotensin-Converting Enzyme by *Senecio samnitum huet*

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Abstract

Extracts of *Senecio samnitum* Huet and derived methyl ester of chlorogenic acid have been shown to inhibit angiotensin-converting enzyme (ACE) by using an *in vitro* bioassay based on the enzymatic cleavage of the chromophore-fluorophore labeled substrate dansyl-triglycine into dansylglycine, which is quantitatively measured by high-performance liquid chromatography (HPLC). GC/MS and NMR identified compounds present within the studied *S. samnitum* extracts. The most effective fraction was obtained in ethyl acetate, which gave $52.56 \pm 0.23\%$ (SD) inhibition at $300\,\mu g/ml$. The major constituent of this fraction, the methyl ester of chlorogenic acid, showed significant ACE inhibition of $56.78 \pm 0.25\%$ at a concentration of $82.5\,\mu g/ml$.

Keywords: ACE, angiotensin-converting enzyme, Compositae, *Senecio samnitum*.

Introduction

The large genus *Senecio* (Compositae) is represented by about 1300 species (Loyola et al., 1985). Pyrrolizidine alkaloids and sesquiterpenes with a furanoeremophilane skeleton are the major components in the *Senecio* genus (Bohlmann et al., 1986; Urones et al., 1988; Pérez et al., 1991). *Senecio* species have been used in folk medicine for the treatment of wounds and as antiemetic, anti-inflammatory, and vasodilator preparations (Rose, 1972; Bautista et al., 1991; Pérez et al., 1999). The traditional use of *Senecio graveolens* (Compositae) for the treatment of mountain sickness and the isolation of dihydroeuparin, a compound with a strong antihypertensive activity, stimulated us to investigate the angi-tensin-converting enzyme (ACE) inhibition properties of *Senecio samnitum*, Huet a species native to southern Italy (Loyola et al., 1985). The primary causative determinants of hypertension remain elusive, but it is generally thought that it develops as a result of disturbances of the body's blood pressure regulating systems. By exploring the means by which normotension is maintained by the renin angiotensin system (RAS), an understanding of the treatment of hypertension using ACE inhibitors can be achieved. In its classic definition, the RAS maintains blood pressure through angiotensin II, a potent vasoconstrictor (Lee et al., 1993). ACE inhibitors act by inhibiting the conversion of angiotensin I to angiotensin II, and several compounds with this activity (captopril, enalapril, ramipril, etc.) are used clinically, being considered effective and safe for the treatment of hypertension; their antihypertensive effect being enhanced by a low-salt diet. (Abrams et al., 1984). They are also well tolerated and have a good safety profile.

To our knowledge, no previous studies have been undertaken on the phytochemistry and biological properties of *S. samnitum* Huet. This paper deals with the isolation of compounds from *S. samnitum* and investigation of the ACE inhibitory properties of *n*-hexane, dichloromethane, ethyl acetate, and *n*-butanol extracts and methyl ester of chlorogenic acid of *S. samnitum* aerial parts.

Materials and Methods

Plant material

The aerial parts of *Senecio samnitum* Huet were collected in the flowering season in Calabria (Italy) in July 2002. The voucher specimen was authenticated by Dr. L. Bernardo (Department of Botany, University of Calabria, Italy) and deposited at the Herbarium of University of Calabria (CLU), Italy, under the reference number 4512.

General experimental procedures

NMR spectra were recorded on Bruker Avance 300 and 400 MHz spectrometers. Electron Ionization Mass Spectrometry (EIMS) (70 eV) analysis was recorded on a Hewlett-Packard 6890N gas chromatograph equipped with a methyl silicone SE-30 capillary column (30 m×0.25 mm id × 0.25 μm film thickness) and interfaced with a Hewlett Packard 5973N Mass Selective Detector. Si gel (Merck, 200–400 mesh) was used for column chromatography. Thin-layer chromatography analysis was carried out on silica gel GF254 plates (Merck, Italy).

Extraction and isolation

Dried and powdered aerial parts (345 g) of *S. samnitum* were extracted with methanol (15 l) three-times at room temperature, and the solutions were combined and concentrated under reduced pressure to obtain 39.18 g residue (11.35% yield). The methanol extract was dissolved in water and fractionated by liquid-liquid partition with *n*-hexane (2.47 g, 0.72% yield), dichloromethane (1.23 g, 0.36% yield), and ethyl acetate (2.39 g, 0.69% yield), each extract being taken to dryness under reduced pressure.

The *n*-hexane, dichloromethane, and ethyl acetate extracts were investigated by capillary gas chromatography-mass spectrometry (GC-MS). Carrier gas was helium. The "solvent delay," the time gap of a given analysis in which the mass spectrometer is turned off, was 3 min. One microliter of sample dissolved in appropriate solvent was injected into the gas chromatograph. The injector and detector temperatures for the gas chromatograph were 250°C and 280°C, respectively. The analytical conditions worked with the following program: oven temperature was programmed from 60°C to 280°C at a rate of 16°C/min; the final temperature of 280°C was held for 10 min.

Identification of the compounds from *n*-hexane, dichloromethane, and ethyl acetate extracts was based on the comparison of the mass spectral data on computer matching against Wiley 138, Wiley 275, and NIST 98 and a home-made mass spectral library built from pure substances.

The dichloromethane extract (387.8 mg) was subjected to Si column chromatography (Merck) 20–45 μm (CH$_2$Cl$_2$:MeOH 85:15). Column fractions were investigated and combined according their TLC profiles (silica gel; CH$_2$Cl$_2$:MeOH 9:1) and the detection was done by Dragendorff's reagent and H$_2$SO$_4$ 50% v/v. Senecionine (6 mg) was crystallized from MeOH from the major fraction. The ethyl acetate extract (1.62 g) was subjected to Si-gel CC (Merck, 0.040–0.063 mm) eluting with an increasing polar gradient of CH$_2$Cl$_2$/MeOH. Column fractions were assayed and combined according to TLC separation (silica gel; CH$_2$Cl$_2$:MeOH 9:1) (NEU reagent and UV 256, 365 nm). From the major fraction, the methyl ester of chlorogenic acid (150 mg) was isolated for the first time in *Senecio* species. Due to small fraction sizes, further separation of the ethyl acetate extract was not successful, but a screening by TLC with the natural products polyethylene glycol reagent (NP/PEG) and UV showed the presence of flavonoids. These substances are presumed to be responsible for ACE-inhibition activity.

The structures of the compounds were determined on the basis of their spectral data (UV, IR, MS, ^{1}H NMR, and ^{13}C NMR) and were identical with those previously described (Liu & Roder, 1991).

ACE inhibition test

Inhibition of ACE is currently considered to be a useful therapeutic approach in the treatment of high blood pressure. Detection of inhibition of ACE by using an *in vitro* ACE-inhibition assay is an effective screening method in the search for new antihypertensive agents (Hansen et al., 1995). This method is based on the ACE-catalyzed cleavage of the chromophore-fluorophore labeled substrate dansyltriglycine into dansylglycine, which is quantitatively measured by HPLC. Solutions of inhibitors were made by dissolving 1 mg of test extract and 250 μg of methyl ester of chlorogenic acid in 1 ml HEPES assay buffer to obtain the final concentration of 330 μg/ml of extracts and 82.5 μg/ml of pure compound. Inhibitors extracted using ethanol were dissolved in HEPES assay buffer containing 10% ethanol. For these studies, a commercially available angiotensin I–converting enzyme preparation from rabbit lung (EC 3.4.15.1) has been used. The ACE solution (25 μl) was preincubated in microtiter plates with a test or control solution (25 μl) for 5 min at 37°C. The enzyme reaction was started by adding a combined solution (25 μl) of the substrate dansyltriglycine (7.86 mM), and the internal standard, dansyl-L-glutamine (0.353 mM). At the end of the incubation time (chosen by the plotting of a calibration curve), the reaction was stopped by adding a solution of 0.1 N Na$_2$EDTA (50 μl). The cleavage product (dansylglycine) and unreacted substrate (dansyltriglycine) were separated and quantified by reversed-phase HPLC with UV detection at 250 nm.

Instrumentation

HPLC Perkin Elmer Series 410 LC Pump. Injector, Perkin Elmer 20 μl loop. Detector, Perkin Elmer UV/VIS LC290 spectophotometric. Solvent system, ALTECH SN 1250-99, part no. 288215 BIN II 43, HYPERSIL ODS 5u lot no. 5002.150 mm × 4.6 mm SN:1250-99. Mobile phase, isocratic system-10 mM NaH_2PO_4 buffer (pH 7) (1.56 g NaH_2PO_4 in 900 ml of water; adjust the pH to 7.0 and make up to 1 liter. Acetonitrile (88.12), flow rate 2 ml/min, run time 30 min. Linear calibration curve for dansylglycine was plotting from 0.2 to 25 μg/ml. All materials were purchased from Sigma (London, UK).

The decreased concentration of dansylglycine in the test reaction compared with the control reaction was expressed as percentage inhibition and calculated from the equation:

$$\text{Inhibition (\%)} = 100 - \frac{(\text{dansylglycine}) \, T}{(\text{dansylglycine}) \, C} \times 100$$

where T = test reaction and C = control reaction. All experiments were carried out in triplicate. The therapeutic drug captopril was used as positive control.

Tannin test (eliminating false positives)

Extracts inhibiting ACE by 50% or more were subjected to the gelatin salt block test to eliminate false positives brought about by the presence of tannins. The tannin test was performed by extracting 5 g of dry plant material with 50 ml of water, ethanol (96%), or acetone. After evaporation of the solvents, the extracts were redissolved in 13 ml of hot water (90–100°C) and allowed to cool to room temperature. Two drops of 10% NaCl were added to "salt" out any nontannin compounds, which could cause a false positive reaction. After vaccum filtration, 3 ml of filtrate was added to each of four test tubes. The following solutions were then added to the test tubes: 4–5 drops of 1% gelatin solution; 4–5 drops of 1% gelatin + 10% NaCl solution; and 3–4 drops of 10% ferric chloride. For a negative control, water and no extract was used.

The test was considered negative if there was no precipitation in tubes 1 and 2 or if 3 showed no color formation and positive if there was precipitation in tubes 1 and 2 and color formation in 3 (either blue-black for hydrolyzable or brownish-green for condensed tannins). *S. samnitum* extracts produced a negative gelatin salt block test. All materials were purchased from Sigma.

Results and discussion

Previous studies showed that many plant-derived compounds have resulted in ACE inhibition: hydrolyzable tannins, phenylpropanes, proanthocyanidins, flavonoids, xanthones, fatty acids, and terpenoids (Nyman et al., 1998). Since a survey of the literature revealed that no studies on the potential angiotensin-converting enzyme inhibition activity of extracts and compounds isolated from *S. samnitum* had been undertaken, the aim of this study was to investigate the ACE-inhibition activity, according to the method described by Elbl and Wagner (1991), which was later modified by Hansen et al. (1995).

In order to identify the compounds present within the *S. samnitum* extracts, GC/MS analysis was performed. *S. samnitum* extracts were diluted to a final volume of 1 ml with methanol (approximately 1 mg/ml). One microliter of each solution was injected into the gas chromatograph and analyzed with a quadrupole mass spectrometric detector. The compounds detected are not new, but they are reported for the first time in *S. samnitum*. A total of 19 compounds were detected in the *n*-hexane, dichloromethane, and ethyl acetate extracts of the studied *S. samnitum*. The results from these analyses are summarized in Table 1, where indication of the sample from which each component has been identified is reported.

The current work showed the ACE inhibitory property of different extracts of *S. samnitum*. This may imply that *in vivo*, these extracts may have a hypotensive effect. The methanol extract of the aerial parts of *S. samnitum* showed a good ACE inhibition of $72.56 \pm 0.12\%$ at 330 μg/ml. The most effective fraction

Table 1. Compounds present within *Senecio samnitum* Huet extracts and identified by GC-MS.

Extracts	Identified compounds
n-Hexane extract	• Docosanoic acid • Octadecanoic acid • Hexadecanoic acid • 9,12-Octadecadienoic acid • α-Amyrin • β-Amyrin • Vitamin E • Stigmasta-5, 22-dien-3-ol (3β, 22*E*) • (22*R*,24*S*)-22,24-Dimethylcholesterol
Dichloro methane extract	• Benzenacetic acid, 4-hydroxy • 1-2-Benzenedicarboxylic acid, dibutyl ester • 10-Demethylsqualene • Senecionine • Seneciphylline • Integerrimine
Ethyl acetate extract	• Propanedioic acid, dimethyl ester • Butanedioic acid, dimethyl ester • Butanedioic acid, hydroxy-, dimethyl ester • 9,12,15-Octadecatrienoic acid, methyl ester

Table 2. ACE inhibitory of *Senecio samnitum* Huet extracts (tested concentrations 330 µg/ml) and methyl ester of chlorogenic acid (tested concentration 82.5 µg/ml).

Extracts/compound	Inhibition % ± SD
Total MeOH	72.56 ± 0.12
n-Hexane	N.A.
Dichloromethane	N.A.
Ethyl acetate	52.56 ± 0.23
Methyl ester of chlorogenic acid	56.78 ± 0.25

The values represents means of two different experiments under standard assay conditions described in the text ± SD. Captopril (38 nM) used as positive control. N.A., not active.

of the total methanol extract was that obtained with ethyl acetate, which gave 52.56 ± 0.23% inhibition at 330 µg/ml.

It was possible to isolate and identify the main component of this extract, which is the methyl ester of chlorogenic acid (9.8% of ethyl acetate extract), not isolated in other *Senecio* species. This compound showed significant ACE inhibition of 56.78 ± 0.25% at a concentration of 82.5 µg/ml. This is greater than that reported previously for the chlorogenic acid, which was 4% at 330 µg/ml (Lacaille-Dubois et al., 2001). All results are shown in Table 2.

We have not tested senecionine, which is a member of pyrrolizidine alkaloids known to be hepatotoxic, but it is easily removed from extracts of *S. samnitum* by dichloromethane (Bah et al., 1994; De Vivar et al., 1996).

In conclusion, the methanol and ethyl acetate extracts of *Senecio samnitum* have been shown to cause inhibition of angiotensin-converting enzyme; further studies will be useful in order to demonstrate possible therapeutic relevance of *S. samnitum* extracts.

Acknowledgments

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