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RESEARCH ARTICLE

Inhibition of angiotensin converting enzyme activity by five *Senecio* species

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Abstract

In our continuous search of biological properties of *Senecio* species (Compositae), we investigated *S. ambiguus* subsp. *ambiguus* (Biv.) DC, *S. gibbosus* subsp. *gibbosus* DC, *S. leucanthemifolius* Poiret, *S. inaequidens* DC, and *S. vulgaris* L. for their angiotensin converting enzyme (ACE) inhibitory activity through an *in vitro* bioassay based on the enzymatic cleavage of the chromophore-fluorophore labelled substrate dansyltriglycine into dansylglycine, which is quantitatively measured by HPLC. Among analyzed extracts, ethyl acetate demonstrated the highest activity with IC_{50} values of 192.1 and 219.1 µg/mL for *S. ambiguus* subsp. *ambiguus* and *S. inaequidens*, respectively. Flavonoids were detected in these extracts on TLC sprayed with Natural Products reagent - polyethylene glycal reagent (NP/PEG).

Keywords: ACE inhibition*; Senecio ambiguus* subsp. *ambiguus; Senecio gibbosus* subsp. *gibbosus; Senecio inaequidens; Senecio leucanthemifolius; Senecio vulgaris*

Introduction

Hypertension is a common and often progressive disorder that poses a major risk for cardiovascular and renal disease (Chalmers, 1999; Odama & Bakris, 2000). Recent data have revealed that the global burden of hypertension is an important and increasing public health problem worldwide and that the level of awareness, treatment, and control of hypertension varies considerably among countries (Kearney et al., 2005). From a pathophysiological point of view, it is important to note that hypertensive disease involves changes in at least one of three hemodynamic variables (cardiac output, arterial stiffness, or peripheral resistance) that determine the measurable blood pressure (Perticone et al., 2001; Rizzoni et al., 2003). Each of these variables is a potential therapeutic target, and it is likely that changes in these variables also contribute to heterogeneity in the pharmacologic response of patients with hypertension.

Therefore, modern treatment strategies should not only target blood pressure reduction but also normalize vascular structure and function. Angiotensin converting enzyme (ACE) inhibitors are widely used in therapy, demonstrating their efficacy in reducing blood pressure, reversing abnormalities of vascular structure and function in patients with essential hypertension, and ultimately preventing “global cardiovascular risk” (Brown & Hall, 2005). ACE is a cell membrane peptidase, working as an ecto-enzyme, with its catalytic site exposed at the extracellular surface of the cell; it catalyzes conversion of angiotensin I into the active angiotensin II. This octapeptide is directly or indirectly involved in bradykinin metabolism. Inhibition of ACE is an effective screening method in the search of new antihypertensive agents (Wagner, 1991; Hansen et al., 1995; Somanadhan et al., 1996).

For a long time medicinal plants have been used for the treatment of many diseases, in most cases without a scientific background supporting their use. At present there is increasing emphasis on determining the scientific evidence and rationale for the use of preparation from medicinal plants.

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Senecio is the largest and complex genus in the tribe Senecionae (Compositae) and more than 1300 species have been reported (Loyola et al., 1985). This genus is rich in pyrrolizidine alkaloids (PAs) and sesquiterpenes with a furanoeremophilane skeleton while just few studies report about components like chalcones or flavonoids (Bohlmann et al., 1986; Urones et al., 1988; Pérez et al., 1991).

Senecio species have been used in folk medicine in the treatment of wounds and as antiemetic, anti-inflammatory and vasodilator preparations (Rose, 1972; Bautista Perez et al., 1991; Pérez et al., 1999). In a previous study, dihydroeuparin, a compound with strong antihypertensive activity was isolated from *S. graveolens* used as tea infusion for the treatment of mountain sickness (Loyola et al., 1985).

In our continuing screening on the antihypertensive activity of *Senecio* species (Tundis et al., 2005), we report for the first time the ACE inhibitory activity of *S. ambiguus* subsp. *ambiguus* (Biv.) DC*, S. gibbosus* subsp. *gibbosus* DC*, S. leucanthemifolius* Poiret*, S. inaequidens* DC*,* and *S. vulgaris* L. extracts.

Materials and methods

Plant material

The aerial part of *Senecio ambiguus* subsp. *ambiguus* (Biv.) DC*, Senecio gibbosus* subsp. *gibbosus* DC*, Senecio leucanthemifolius* Poiret, *Senecio inaequidens* DC, and *Senecio vulgaris* L. were collected in southern Italy during flowering season 2003 in their natural habitat. Plant materials were taxonomically identified by N.G. Passalacqua and L. Peruzzi of the Natural History Museum of Calabria and Botanical Garden of University of Calabria, Italy. Voucher specimens were deposited in the Botany Department Herbarium at the University of Calabria (CLU), Italy. All species were dried in a dark place at room temperature and coarsely powdered before extraction.

Extraction procedure

Dried and powdered aerial parts of *S. ambiguus* subsp. *ambiguus* (Biv.) DC*, S. gibbosus* subsp. *gibbosus* DC*, S. leucanthemifolius* Poiret*, S. inaequidens* DC*,* and *S. vulgaris* L. (200 g) were extracted exhaustively with methanol (3 × 5 L) at room temperature to give the crude extract. Crude extract was suspended in H_2O and partioned with *n*-hexane, dichloromethane (CH_2Cl_2) and ethyl acetate (EtOAc). The residue was acidified with 2.5% H_2SO_4 and stirred overnight with Zn powder to reduce pyrrolizidine alkaloids (PAs) *N*-oxides. The aqueous acid solution was basified, and extracted with chloroform ($CHCl_3$). The combined organic solutions were dried over anhydrous sodium sulphate and evaporated to dryness. Yield of extracts are reported in Table 1.

Composition of extracts

In order to identify the inhibitory compounds present within the *Senecio* extracts silica gel Thin Layer Chromatography (TLC) was carried out and specific spray reagents used for various compound types (Wagner & Bladt, 1996).

The *n*-hexane extracts were developed with *n*-hexane:acetone (6:4) and then treated with vanillin-sulphuric acid reagent (VS) highlighting the presence of terpenoids. The EtOAc extracts were developed with EtOAc:HCOOH:AcOH:H_2O (100:10:10:20) and then sprayed with Natural Products reagent-polyethylene glycol reagent (NP/PEG) revealing the presence of flavonoids when examined under UV light 365 nm in all *Senecio* species except in *S. leucanthemifolius*. Alkaloids were detected in chloroform extracts eluting with CH_2Cl_2-MeOH-NH_3 85:14:1, immediately followed by spraying with Dragendorff's reagent (DRG).

ACE-inhibition test

The *in vitro* ACE inhibitory activity was measured using the method described by Elbl and Wagner (1991), which was later modified by Hansen et al. (1995). Briefly, the chromophore-fluorophore labeled substrate dansyltriglycine was cleaved by angiotensin I-converting enzyme preparation from rabbit lung (EC 3.4.15.1) into dansylglycine, which is quantitatively measured by HPLC.

The test extract (1 mg) was dissolved in 1 mL HEPES assay buffer, to obtain the final concentration from 330 to of 100 µg/mL of inhibitors solution. The ACE solution (25 µL) was pre-incubated in 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 the reaction was stopped by adding a solution of 0.1 N Na_2EDTA (50 µL). The dansylglycine

Table 1. Content (g) of methanol extracts and fractions of *Senecio* species.

Plants	Dried Plant	MeOH	CH_2Cl_2	*n*-Hexane	EtOAc
S. ambiguus subsp. *ambiguus*	536.7	67.1	5.0	6.7	5.6
S. gibbosus subsp. *gibbosus*	461	63.8	1.4	1.0	6.1
S. leucanthemifolius	974.5	147.9	3.9	10.5	9.1
S. inaequidens	300	13.4	0.4	1.6	0.6
S. vulgaris	733.2	80.4	1.4	10.5	3.0

and 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):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-Aldrich, Milan, Italy.

Tannin test

The 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 tannins test was performed by extracting 5 g of dried plant materials with 50 mL of water, ethanol (96%) or acetone. After evaporation of the solvents, the extracts were re-dissolved in 13 mL hot water (90-100°C) and allowed to cool to room temperature. Two drops of 10% NaCl are added to "salt" out any non-tannin compounds which could cause a false positive reaction. After vacuum 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 was used without extract. 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 hydrolysable or brownish-green for condensed tannins) (Nyman et al., 1998).

Results and discussion

Pyrrolizidine alkaloids (PAs) are the most characteristic secondary metabolites of *Senecio* species (Hartmann & Ober, 2000). PAs are ester alkaloids consisting of a necine base moiety, esterified with a necic acid. They may occur as monoesters, open-chain diesters, or macrocyclic diesters. In all *Senecio* species, senecionine *N*-oxide was identified as the primary product of biosynthesis. It is synthesized in the roots and translocated into the shoots, where it is transformed into the species-specific PA profiles (Pelser et al., 2005). Senecionine was proved to be incorporated into simple retronecine esters as seneciphylline; epoxides of retronecine esters as jacobine including jaconine, its product of chlorolysis, jacozine; and epoxides of otonecine esters as otosenine and florosenine.

Although pyrrolizidine alkaloids (PAs) are known for their hepatotoxicity, mutagenicity carcinogenicity, and teratogenicity (Fu et al., 2004), several biological activities including ACE inhibitory activity of some *Senecio* species have been reported (El-Shazly et al., 2002; Toma et al., 2004; Loizzo et al., 2004, 2005, 2006, 2007; Tundis et al., 2005; Conforti et al., 2006). For the above mentioned reason, PAs were removed from methanol extracts using chloroform following the procedure previously described. These extracts were not tested for ACE inhibition.

ACE inhibition was revealed through an *in vitro* bioassay based on the measure of the enzymatic cleavage of the chromophore-fluorophore-labeled substrate dansyltriglycine into dansylglycine and diglycine. The decreased concentration of dansylglycine in the test reaction compared with the control reaction was expressed as percentage inhibition and calculated from the equation:

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

where T = test reaction and C = control reaction.

The IC_{50} values of *S. ambiguus* subsp. *ambiguus, S. gibbosus* subsp. *gibbosus, S. leucanthemifolius, S. inaequidens, S. vulgaris* extracts are summarized in Table 2. Among the tested extracts, it is interesting to note that dicloromethane extracts did not exhibit any type of activity according to *S. samnitum* biological profile (Tundis et al., 2005). Among analysed *n*-hexane extracts, *S. ambiguus* subsp. *ambiguus* demonstrated considerable activity with an IC_{50} of 306.9 μg/mL. This ACE inhibitory property may be due to the presence of compounds such as linoleic acid, palmitic acid, γ-tocopherol, α- and β-amyrin, and campesterol, identified by GC/MS analysis in this species in our previous work (Tundis et al., 2005a). Differentially, all EtOAc extracts exhibited significant activity and dose-response curve was shown in Figure 1 in this work. In particular, *S. inaequidens* exhibited the highest ACE inhibitory with an IC_{50} value of 192.1 μg/mL. *S. ambiguus* subsp. *ambiguus* showed a strong activity with an IC_{50} of 219.1 μg/mL.

Table 2. ACE inhibitory activity (IC_{50}) of *Senecio* species (μg/mL).

Plants	*n*-Hexane	CH_2Cl_2	EtOAc
S. ambiguus subsp. *ambiguus*	306.9 ± 3.7	–	219. 4 ± 1.7
S. gibbosus subsp. *gibbosus*	–	> 330	309.4 ± 3.1
S. leucanthemifolius	–	> 330	–
S. inaequidens	> 330	–	192.1 ± 1.8
S. vulgaris	–	–	327. 8 ± 3.4

The values represents means of two different experiments under standard assay conditions described in the text ± s.d (n = 3). – : Not active. Captopril (38 nM) was used as a control positive.

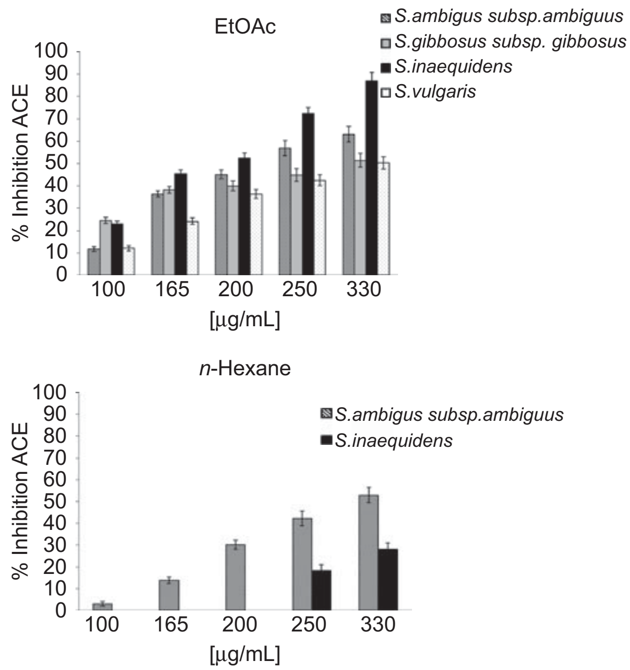

Figure 1. Dose-dependent inhibition of ACE by *Senecio* species EtOAc and *n*-hexane extracts. Each data point represents the mean ± SD ($n=3$).

The same bioactive profile was observed for *S. gibbosus* subsp. *gibbosus* and *S. vulgaris,* exhibiting ACE inhibition activity in only EtOAc extracts, with IC_{50} values of 309.4 and 327.8 µg/mL, respectively. Ferry (1977) isolated and identified quercetin, isoquercetrin, isorhamnetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside and some polyphenols such as phenolic acid, vanillic acid, and caffeic acid in *S. inaequidens* and *S. vulgaris*. We believe that flavonoids are responsible for the observed ACE inhibitory activity; in fact, Wagner et al. (1991) demonstrated how flavonoids can inhibit ACE through the generation of chelate complexes within the active center of ACE. Free hydroxyl groups of phenolic compounds are also suggested to be important structural moieties to chelate the zinc ions, thus inactivate the ACE activity (Chen & Lin, 1992). The non-flavonoid polyphenol compounds failed to inhibit ACE activity. This lack of effect on ACE activity does not allow the establishment of any structure-activity relationship, like that observed for membrane interaction and antioxidant capacity (Verstraeten et al., 2003; Erlejman et al., 2004).

On the contrary, *S. leucanthemifolius* EtOAc extract was inactive against ACE, possibly due to the lack of flavonoids in this species compared to the others analyzed in this work. A tannin test was done on EtOAc extracts in order to eliminate false positive. Moreover, in our previous investigation we reported the *S. inaequidens* EtOAc extract possesses greater antioxidant activity in comparison with *S. vulgaris* and this different biological profile was linked to the different flavonoid content (Conforti et al., 2006a).

The present work showed for the first time the anti-hypertensive properties of different *Senecio* species and scientifically supports the traditional use of *Senecio*. Among analyzed extracts, EtOAc exhibited the most promising activity, probably due to the presence of flavonoids. Further research relating to isolation of the active constituents is in progress in our laboratory.

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