Comparative Radical Scavenging and Antidiabetic Activities of Methanolic Extract and Fractions from Achillea ligustica All.

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The yield of methanolic extract and total phenol and non polar content of flowered parts from Achillea ligustica All. are reported. GC-MS analysis of the non polar fraction showed that the triterpene moretenol was the major constituent (17.228%) followed by stigmast-6-en-3β-ol, veridiflorol and β-amyrin (7.524%, 5.078% 4.470%, respectively). The antioxidant activities of the methanolic extract and its fractions from A. ligustica were carried out using two different in vitro assays, 2,2-diphenyl-1-picrylhydrazyl (DPPH) test and lipid peroxidation of liposomes assay. Methanolic extract showed higher radical scavenging activity on DPPH (IC50 of 50 μg/ml). This activity is probably due to the phenolic fraction which shown an IC50 value of 22 μg/ml. A different result was obtained from the methanolic extract on the lipid peroxidation of liposomes (IC50 of 416 μg/ml). The α-amylase inhibition assay was applied to evaluate antidiabetic activity. The methanolic extract showed weak activity (28.18% at 1 mg/ml) while the n-hexane fraction showed 74.96% inhibition at 250 μg/ml.

Key words Achillea ligustica; phenolic content; antioxidant; α-amylase

The genus Achillea (Asteraceae) is represented by 26 species in Italy of which nine are endemic. Several Achillea species are used for their pharmaceutical, cosmetic, and fragrance properties. The extracts exhibit pharmacologic activities such as antiinflammatory, analgesic, and antipyretic properties. Achillea ligustica All., a member of the Achillea nobilis group, has been used as medicine mainly for the treatment of gastrointestinal disorders and have been previously examined for flavonoids, sesquiterpene lactones and essential oils. It has long been recognized that naturally occurring substances in higher plants have antioxidant activity, particularly flavonoids. Recently, there has been growing interest in oxygen-containing free radicals in biologic systems and their implied roles as causative agents in the etiology of a variety of chronic disorders.

Diabetes is a major risk factor for premature atherosclerosis, and oxidative stress plays an important role since diabetic monocytes produce increased superoxide anion (O2•−). Recent advances in understanding of the activity of α-amylase and α-glucosidase have led to the development of new pharmacologic agents. Amylase inhibition has gastrointestinal and metabolic effects that may aid not only in the treatment of diabetes but also of obesity. Accordingly, attention is being focused on the protective biochemical functions of naturally occurring antioxidants and inhibitors of α-amylase in the cells of organisms containing them. To the best of our knowledge, there are no reports on the in vitro antioxidant and α-amylase inhibitory activities of A. ligustica in the literature. In the present study, we used two different in vitro assays, 2,2-diphenyl-1-picrylhydrazyl (DPPH) test and lipid peroxidation of liposomes assay, to evaluate the antioxidant activity and an assay to evaluate α-amylase inhibition of different extracts from A. ligustica.

MATERIALS AND METHODS

Chemicals Thiobarbituric acid (TBA), phosphate buffered saline (PBS), bovine brain extract, FeCl3, ascorbic acid, butylated hydroxytoluene (BHT), propyl gallate, potato starch, sodium phosphate buffer, sodium chloride, α-amylase, sodium potassium tartrate and 3,5-dinitrosalicicylic acid were obtained from Sigma-Aldrich S.p.a. (Milan, Italy). Methanol, ethanol, ethyl acetate, petroleum ether, diethyl ether, H2SO4, chloroform, HCl, KOH, I-butanol, silica gel 70—230 mesh, and thin-layer chromatography (TLC) plates were obtained from VWR International s.r.l. (Milan, Italy).

Plant Materials The flowered parts of Achillea ligustica used in this study were collected (July 2003) in Calabria (Italy) and authenticated by Prof. Dimitar Uzunov, Botany Department, University of Calabria (Italy). A voucher specimen was deposited in the Botany Department Herbarium at the University of Calabria (CLU).

Preparation of the Extracts The air-dried and ground sample (382 g) was extracted with MeOH through maceration (48 h×3 times). The resulting extract was dried under reduced pressure to give 39.96 g. Total phenols and lipophilic fraction were determined from methanolic extract. Total phenols were estimated using the method described by Karas et al. Five grams of the methanolic extract was suspended in water (260 ml), acidified with conc. HCl (20 ml) and extracted with I-butanol. The extract was treated with aqueous KOH (32 g, 400 ml). The alkaline solution was acidified with HCl (20 ml) until the pH became 2 and extracted with I-butanol. The I-butanol extract was washed with water and concentrated until dry. A total of 2.164 g of phenolic compounds was obtained (17.295 g in the total methanolic extract; 4.525% in comparison to the weight of the plant).

In order to isolate the lipophilic fraction, 30 g of the methanolic extract was suspended in a methanol/water (9 : 1) mixture and extracted with n-hexane to give 4.604 g (6.132% in the total methanolic extract; 1.604% in comparison to the weight of the plant).

GC-MS Analysis The lipophilic fraction analysis was performed using a Hewlett-Packard (HP) gas-chromatograph, model 5890, equipped with a mass spectrometer, model 5972 series II, and controlled by HP software equipped with a capillary column 30 m×0.25 mm, static-phase SE30, using programmed temperature from 60 to 280
1°C (rate 16°/min); the detector and the injector were set to a temperature of 280°C and 250°C, respectively (split vent flow 1 ml min⁻¹). Compound identification was verified based on the relative retention time and mass spectra with those of Wiley 138 library data of the GC-MS system (HP). The results were also confirmed by the comparison of the compound elution order with their relative retention indices in non-polar phases reported in the literature.13)

**DPPH Assay** This experimental procedure was adapted from Wang et al.14) In an ethanol solution of DPPH radical (final concentration 1.0×10⁻⁵ M), test extracts were added, and their concentrations were 10, 20, 50, and 100 μg/ml. The reaction mixtures were shaken vigorously and then kept in the dark for 30 min. The absorbance of the resulting solutions was measured in 1 cm cuvettes using a Perkin Elmer Lambda 40 UV/VIS spectrophotometer at 517 nm against a blank, in which DPPH was absent. All tests were run in triplicate and averaged. Ascorbic acid was used as a positive control.

**Lipid Peroxidation of Liposomes Assay** The in vitro antioxidant activity tests were carried out using the TBA test.15) The TBA reaction is based on the fact that peroxidation of most membrane systems leads to the formation of small amounts of free malonaldehyde (MDA). MDA reacts with TBA to yield a coloured product, which in an acid environment absorbs light at 532 nm and is readily extractable into organic solvents. The intensity of colour is a measure of the MDA concentration. Absorbance at 532 nm was determined on a Perkin Elmer Lambda 40 UV/VIS spectrophotometer. The incorporation of any antioxidant compound in the lipid peroxidation assay reaction mixture will lead to a reduction in the extent of peroxidation. The methanolic extract and phenolic and n-hexane fractions of *A. ligustica* were tested for their antioxidant activity against liposomes that were prepared from bovine brain extract in PBS (5 mg/ml). Peroxidation was started by adding FeCl₃ (1 mM) and ascorbic acid (1 mM), followed by incubation at 37°C for 20 min. Ascorbic acid is a well-known antioxidant but it also has prooxidant properties in the presence of certain transition metal ions, such as Fe or Cu. BHT in ethanol was added to prevent lipid peroxidation during the TBA test itself. Propyl gallate (0.1 mM) was used as a positive control.16)

**α-Amylase Inhibition Assay** It is widely accepted that the most challenging goal in the management of patients with diabetes is to achieve blood glucose levels as close to normal as possible.17) α-Amylases, endoglucanases that catalyze hydrolysis of the internal α-1,4-glucosidic linkage in starch and other related polysaccharides, have also been targets for the suppression of postprandial hyperglycaemia. We investigated extracts from *A. ligustica* ALL. To determine the inhibitory effects on α-amylase (EC 3.2.1.1). The biosay method was adopted and modified from Sigma-Aldrich.18) A starch solution (0.5% w/v) was obtained by stirring 0.125 g of potato starch (Sigma) in 25 ml of 20 mM sodium phosphate buffer 20 mM with sodium chloride 6.7 mM, pH 6.9 at 65°C for 15 min. The enzyme solution was prepared by mixing 0.0253 g of α-amylases in 100 ml of cold distilled water. *A. ligustica* ALL. extracts were dissolved in buffer to give final concentrations of 1 ng/ml to 125 μg/ml. The colorimetric reagent was prepared by mixing a sodium potassium tartrate solution (12.0 g of sodium potassium tartrate, tetrahydrate in 8.0 ml of NaOH 2 M) and 3,5-dinitrosalicylic acid solution 96 mM. Both control and plant extracts were added with starch solution and left to react with α-amylase solution under alkaline conditions at 25°C. The reaction was measured over 3 min. The generation of maltose was quantified by the reduction of 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid. This reaction (corresponding to the color change from orange-yellow to red) is detectable at 540 nm. In the presence of α-amylases inhibitors, maltose should be reduced and the absorbance value decreased. Preliminary experiments established that the optimal condition are: starch, 0.25 w/v%; α-amylases 1 unit/ml; and inhibitor concentration, /ml. The α-amylases inhibition was expressed as a percentage of inhibition and calculated from the equations:

% reaction = [(maltose) test - (maltose) control] × 100

% inhibition = 100 − % reaction± S.D.

Acarbose was used as a positive control.

**Statistical Analysis** Data were expressed as means±S.D. Statistical analysis was performed by using Student’s t-test. Differences were considered significant at p<0.05. The 50% inhibitory concentration (IC₅₀) was calculated from the Prism dose–response curve (statistical program) obtained by plotting the percentage of inhibition versus the concentrations.

**RESULTS AND DISCUSSION**

The methanolic extract yield and total phenol and non polar content of the flowered parts of *A. ligustica* are shown in Table 1. The high yield of phenolic content (4.525%) is of particular interest. GC-MS analysis of the non polar fraction resulted in the identification of 43 constituents (79.575% of the total fraction). The triterpene moretenol was the major constituent (17%), followed by stigmast-6-en-3β,veridi-florol and β-amyrin (7%, 5%, 4%, respectively) (Table 2). The antioxidant activities of the different extracts of *A. ligustica* were carried out using two different in vitro assays (DPPH and lipid peroxidation of liposomes assays). The effects of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen donating ability. The scavenging effects of plant extracts on DPPH were examined at four different concentrations (10, 20, 50, 100 μg/ml). As shown in Table 3 and Fig. 1, both the phenolic fraction and methanolic extract showed high activity (IC₅₀ values of 22 μg/ml and 50 μg/ml respectively). Different results were obtained on the lipid peroxidation of liposomes. As shown in Fig. 2 the phenolic extract showed greater antioxidative potency than the methanolic extract (IC₅₀ values of 27 μg/ml and 416 μg/ml, respectively). The assay for α-amylase inhibition showed that

<table>
<thead>
<tr>
<th>Extract</th>
<th>Weight (g)</th>
<th>Yield (%)</th>
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<tbody>
<tr>
<td>MeOH</td>
<td>39.960</td>
<td>10.454</td>
</tr>
<tr>
<td>n-Hexane</td>
<td>6.132</td>
<td>1.604</td>
</tr>
<tr>
<td>Phenolic</td>
<td>17.295</td>
<td>4.525</td>
</tr>
</tbody>
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Table 1. Content of *Achillea ligustica* Extracts and Yield % in Comparison to the Weight of the Plant
the methanolic extract possess 28.18% of inhibition at a concentration of 1 mg/ml. However \( n \)-hexane extracts showed 74.96% of inhibition at 250 \( \mu \)g/ml and 33.15% at 125 \( \mu \)g/ml.
(Table 4). This activity is due probably to the triterpene moretenol which was the major constituent of the extract. The present study elucidated for the first time the biologic properties of the methanolic extract and fractions from *A. ligustica*. Particularly important are the results on the antioxidant activity of the *A. ligustica* phenolic fraction and inhibition of α-amylase shown by *n*-hexane fraction.

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REFERENCES AND NOTES

18) www.sigmaaldrich.com