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 (IC_{50} of 50 μg/ml). This activity is probably due to the phenolic fraction which shown an IC_{50} value of 22 μg/ml. A different result was obtained from the methanolic extract on the lipid peroxidation of liposomes (IC_{50} 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.[1] Several *Achillea* species are used for their pharmaceutical, cosmetic, and fragrance properties. The extracts exhibit pharmacologic activities such as antiinflammatory, analgesic, and antipyretic properties.[2] *Achillea ligustica* ALL., a member of the *Achillea nobilis* group, has been used as medicine mainly for the treatment of gastrointestinal disorders[3] and have been previously examined for flavonoids,[4] sesquiterpene lactones[5] and essential oils.[6] 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 (O_2^-).[7] 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.[8,9] 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.[10,11] 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, $FeCl_3$, ascorbic acid, butylated hydroxytoluene (BHT), propyl gallate, potato starch, sodium phosphate buffer, sodium chloride, α-amylase, sodium potassium tartrate and 3,5-dinitrosalicylic acid were obtained from Sigma-Aldrich S.p.a. (Milan, Italy). Methanol, ethanol, ethyl acetate, petroleum ether, diethyl ether, H_2SO_4, 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 Karaseva *et al*.[12] 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 g 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

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°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^{-4} 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 $FeCl_3$ (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 the 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 hyperglycemia. We investigated extracts from *A. ligustica* ALL. To determine the inhibitory effects on α-amylase (EC 3.2.1.1). The bioassay 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 from 1 mg/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:

$$\%\ \text{reaction} = \frac{[\text{maltose}]\ \text{test}}{[\text{maltose}]\ \text{control}} \times 100$$

$$\%\ \text{inhibition} = 100 - \%\ \text{reaction} \pm \text{S.D.}$$

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 $p \leq 0.05$. The 50% inhibitory concentration (IC_{50}) 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 of 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-ol, veridiflorol 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 (IC_{50} 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 (IC_{50} values of 27 μg/ml and 416 μg/ml, respectively). The assay for α-amylase inhibition showed that

Table 1. Content of *Achillea ligustica* Extracts and Yield % in Comparison to the Weight of the Plant

Extract	Weight (g)	Yield (%)
MeOH	39.960	10.454
n-Hexane	6.132	1.604
Phenolic	17.295	4.525

Table 2. Chemical Composition of *n*-Hexane Fraction from *Achillea ligustica*.

Compound[a)]	t_R[b)]	Composition (%)
Thujene	6.160	Tr.
Sabinene	6.966	Tr.
Linalool	8.915	2.925
α-Thujone	9.246	0.282
t-Pinocarveol	9.612	0.311
e-Borneol	9.989	0.962
Lynalyl propinate	10.292	2.541
Myrtenol	10.384	Tr.
c-Jasmone	12.870	Tr.
t-Caryophyllene	13.218	Tr.
β-Farnesene	13.401	Tr.
Alloaromadendrene	13.699	0.463
δ-Cadinene	14.264	0.263
Nerolidol	14.550	0.687
(−)-Spathulenol	14.927	0.427
Veridiflorol	15.099	5.078
Farnesol	15.247	0.503
γ-Gurjunene	15.699	2.149
Neophytadiene	17.082	0.751
Hexadecanoic acid methyl ester	17.802	0.836
Hexadecanoic acid	17.134	3.721
Octadecanal	18.164	Tr.
1-Octadecene	19.094	1.075
9,12-Octadecadienoic acid, methyl ester	19.220	0.818
Octadecanal	20.163	0.555
Tricosane	20.688	0.595
1-Octadecanethiol	21.483	0.407
Tetradecanal	21.831	0.392
Nonadecene	22.397	5.342
Octadecane	23.163	Tr.
Cyclohexdecane	23.426	Tr.
Eicosane	24.661	4.841
Eicosane	25.123	Tr.
Nonadecane	25.735	Tr.
Hexadecane, 1-(ethenyloxy)	26.135	Tr.
Cyclotetracosane	27.981	5.977
9-Tricosene	32.999	Tr.
Ergost-5-en-3β-ol	38.234	1.657
Stigmasta-5,22-dien-3-ol	39.435	3.670
Stigmast-6-en-3β-ol	42.052	7.524
β-Amyrin	43.750	4.470
Viminalol	46.024	3.125
Moretenol	51.820	17.228
Others not identified (18)		20.425

a) Compounds listed in order of elution from SE30 MS column. *b*) Retention time (as minutes). Tr.: Trace (<0.1%).

Table 3. IC_{50} Values of Radical Scavenging on 2,2-Diphenyl-1-picrylhydrazyl and Inhibition of Lipid Peroxidation of *Achillea ligustica* Extracts ±S.D. (n=3)

Extract	IC_{50} (mg/ml)	
	DPPH	Lipid peroxidation
MeOH	0.050±0.832	0.416±1.021
Phenolic	0.022±0.561	0.027±0.325
n-Hexane	NA	NA
Propyl gallate	—	0.007±0.213
Ascorbic acid	0.002±0.161	—

NA: no activity.

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 μg/ml and 33.15% at 125 μg/ml

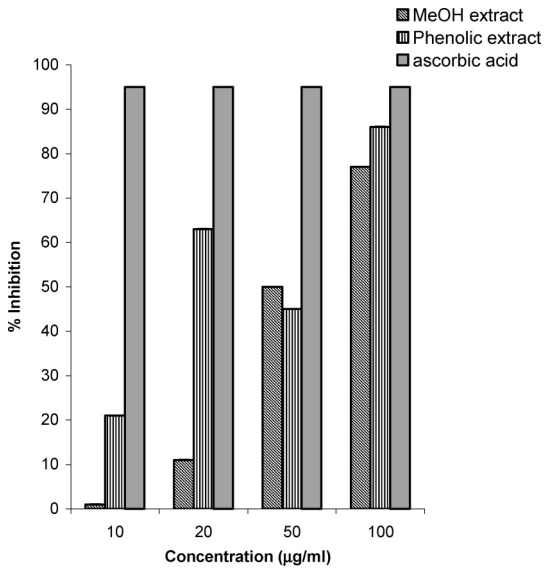

Fig. 1. Scavenging Effects of *Achillea ligustica* Extracts on the DPPH Free Radical

Data are means±standard deviation of three determinations; ascorbic acid was used as a positive control. *n*-Hexane extract showed no activity.

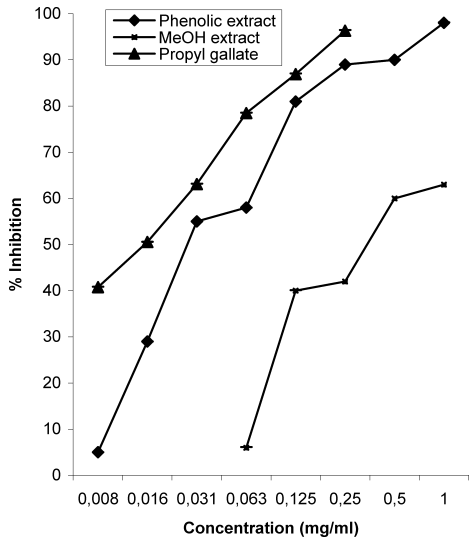

Fig. 2. % Inhibition of Lipid Peroxidation of *Achillea ligustica* Extracts

Data are means±S.D. of three determinations; propyl gallate was used as a positive control. *n*-Hexane extract showed no activity.

Table 4. Inhibitory Activity of Various Extracts of *Achillea ligustica* L. against α-Amylase±S.D. (n=3)

Extract	Concentration (mg/ml)	% Inhibition
MeOH	1	28.18±0.071
	0.5	NA
Phenolic	1	NA
	0.5	NA
n-Hexane	1	89.30±0.89
	0.5	85.03±0.95
	0.25	74.96±0.76
	0.125	33.15±0.85
Acarbose	0.050	50.00±0.90

All determinations were carried out in triplicate and averaged. The α-amylase inhibitory activity (%) was defined as the percent decrease in the maltose production rate over the control. Acarbose was used as positive control. NA: no activity.

(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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