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RESEARCH ARTICLE

Metabolite profile and *in vitro* activities of *Phagnalon saxatile* (L.) Cass. relevant to treatment of Alzheimer's disease

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Abstract

The present study describes for the first time the *in vitro* properties (inhibition of NO production and anti-cholinesterase) of *Phagnalon saxatile* (L.) Cass. (Asteraceae). The methanolic extract showed antioxidant activity that was measured by DPPH assay and β-carotene bleaching test. The same extract inhibited NO production in the murine monocytic macrophage cell line RAW 264.7. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition was assessed by modifications of Ellman's method. Purification of the MeOH extract of *P. saxatile* allowed the isolation of phenolic compounds. Among them, the compounds that most effectively inhibited lipopolysaccharide-induced NO production were caffeic acid and methylchlorogenic acid, with IC_{50} values of 7 µg/mL and 12 µg/mL, respectively. Luteolin and 3,5-dicaffeoylquinic acid exhibited the most promising activity against AChE with an IC_{50} of 25.2 and 54.5 µg/mL, respectively, while caffeic acid and luteolin exhibited higher activity against BChE with an IC_{50} of 32.2 and 37.2 µg/mL, respectively.

Keywords: Phagnalon saxatile*; Asteraceae; phenolic compounds; inhibition of NO production; Alzheimer's disease*

Introduction

Oxidative damage, caused by the action of free radicals, may initiate and promote the progression of a number of chronic diseases, including cancer, cardiovascular diseases, inflammation, diabetes, and Alzheimer's disease[1]. Nitric oxide ($NO^{\bullet}$) is a diatomic free radical produced from L-arginine by constitutive and inducible nitric oxide synthase (cNOS and iNOS) in numerous mammalian cells and tissues. Nitric oxide (NO), superoxide (O^{2-}), and their reaction product peroxynitrite ($ONOO^-$) may be generated in excess during the host response against viral and antibacterial infections and contribute to some pathogenesis by promoting oxidative stress, tissue injury, and even cancer[2,3]. Recently, the effects of selected polyphenolics on NO production in lipopolysaccharide (LPS)-activated RAW 264.7 macrophage cells have been reported. These studies have shown that some polyphenolics or methanol extracts of plants inhibit NO production[4–7]. Most clinically important medicines belong to steroidal or non-steroidal anti-inflammatory chemical therapeutics for treatment of inflammation-related diseases. Though these have potent activity, long-term administration is required for the treatment of chronic disease. Furthermore, these drugs have various and severe adverse effects.

In recent years, oxidative stress has been described in the pathological changes that occur in Alzheimer's disease (AD)[8,9]. Acetylcholine (ACh) is found in the synapse of the cerebral cortex, and a deficiency in the cerebral cortex is one of the major features seen in sufferers of AD[10]. Acetylcholinesterase (AChE) inhibitors such as tacrine, donepezil, and the natural products rivastigmine and galantamine are currently the only effective treatment for AD. These approved drugs are limited in use due to their adverse side-effects, such as gastrointestinal disturbance, and bioavailability problems[11–13]. The continuing search for novel anticholinesterases from plants as therapeutic

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agents for dementia and other central nervous system disorders is motivated by the current need for new substances of natural origin targeted to the brain areas affected, with effectiveness in the described fields of application and low degrees of toxicity and side-effects for man.

The genus *Phagnalon* (Asteraceae) is represented by about 30 species distributed worldwide, six of which are typical of the Mediterranean region[14]. Different *Phagnalon* sp. are used in popular medicine, and a variety of extracts have been examined[15–21]. Among the Bedouins of the Negev desert, the bark of *P. rupestre* (L.) DC. is widely used to induce deliberate burns for the healing of various ailments[15], while in the Palestinian area the whole plant is used to treat asthma and headache, and as an analgesic for toothache. The aqueous and ethanolic extracts of this plant showed antimicrobial activity against both positive and negative bacteria and *C. albicans*[16]. Phytochemical investigations on this species are mainly devoted to the study of *P. rupestre*, and report the presence of terpenoids[22], flavonoids[23], hydroquinone glycosides, and caffeoylquinic acid derivatives[24]. Pharmacological studies have been led only on *P. rupestre*: prenylhydroquinone glucosides and caffeoylquinic esters from this plant have been studied for a variety of biological activities.

Phagnalon saxatile (L.) Cass. is one of five suffruticose chamaephyte species growing wild in Italy, mostly in southern Italy[25]. A recent ethnobotanical study classifies it as a nutritional plant utilized for medicinal purposes[26]. A survey of the literature shows the presence in the aerial parts of the plant of an essential oil constituted mainly of sesquiterpenes, fatty acids, and waxes[27], 3,3-dimethylallyl-*p*-benzoquinone[28], and flavonoids such as apigenin, apigenin-7-glucoside, and luteolin[22], but phytochemical data are incomplete and pharmacological information on the plant and its metabolites is lacking. The objectives of the present study were (1) to investigate the phytochemical characteristics of *P. saxatile* flowering aerial parts, which exhibit significant antioxidant, inhibition of NO production, and anticholinesterase activities, and (2) to relate these activities to pure isolated compounds.

Materials and methods

General experimental procedures

Nuclear magnetic resonance (NMR) spectra were acquired on a Bruker DRX-600 (Billerica, MA, USA) (^{1}H at 599.19 MHz, ^{13}C at 150.86 MHz) spectrometer: δ (ppm), *J* in Hz, spectra referred to CHD_2OD as internal standard. Mass spectra were recorded with an API 2000 (Applied Biosystems, Foster City, CA, USA). Ultraviolet (UV) spectra were measured on a UV/VIS Jasco V530 spectrophotometer. Reverse-phase high performance liquid chromatography (HPLC) was performed with a TSP SpectraSeries P 100 equipped with a rheodyne injector and a refractive index detector, using a column C_{18} β-Bondapack (Waters, Milford, MA, USA; p.s. 10 μm, 300 × 7.8 mm, flow rate 2.5 mL/min). Thin layer chromatography (TLC) was performed on plates coated with silica gel 60 F_{254} (Merck, Whitehouse Station, NJ, USA), 0.25 mm. Elemental analysis was carried out with a PerkinElmer 240 apparatus. All solvents (analytical and deuterated grade) were purchased from Carlo Erba Reagenti, Milan, Italy.

Plant material

The flowering aerial parts of *Phagnalon saxatil*e (L.) Cass. were collected in June 2005, in Cefalù, capo Playa (Sicily, Southern Italy). A voucher specimen of the plant (SN 49) is kept in the Herbarium Neapolitanum (NAP), Faculty of Pharmacy, University of Naples "Federico II".

Extraction procedures and isolation of compounds

Plant material (452.5 g) was chopped and then sequentially extracted by cold maceration with 3 × 2.5 L of petroleum ether, $CHCl_3$, and CH_3OH. The methanolic solution was concentrated under reduced pressure obtaining a residue (15.8 g) that was chromatographed in 2 g lots on a Sephadex LH-20 (Pharmacia) column, eluting with CH_3OH. Fractions of 20 mL were collected and analyzed by TLC using BuOH/CH_3COOH/H_2O (60:15:25, v/v/v) as eluent and $Ce(SO_4)_2$ in H_2SO_4 as spray reagent. Fractions 12–14 (2.20 g), 15, 16 (1.14 g), 17 (0.178 g), 18 (0.263 g), 19–22 (1.09 g), and 23–27 (0.525 g) were gathered according to TLC analysis and further purified by reverse HPLC: fraction 12–14 (CH_3OH/H_2O, 40:60, as eluent) yielded compound (**1**) (6.6 mg; Rt = 9 min) and compound (**2**) (1.4 mg; Rt = 10.5 min); fraction 15, 16 (CH_3OH/H_2O, 40:60, as eluent) yielded compounds (**3**) (55.2 mg; Rt = 6.5 min), (**4**) (15.0 mg; Rt = 9 min), and (**5**) (13.4 mg; Rt = 13.5 min); fraction 17 (CH_3OH/H_2O, 55:45, as eluent) yielded compound (**6**) (10.6 mg; Rt = 10.5 min); fraction 18 (CH_3OH/H_2O, 60:40, as eluent) yielded compound (**7**) (0.8 mg; Rt = 6.5 min); fraction 19–22 (CH_3OH/H_2O, 60:40, as eluent) yielded compounds (**8**) (130.5 mg; Rt = 4.5 min) and (**9**) (22.4 mg; Rt = 12.5 min); fraction 23–27 (CH_3OH/H_2O 70:30 as eluent) yielded compounds (**10**) (4.7 mg; Rt = 6.5 min) and (**11**) (4.3 mg; Rt = 7.5 min). The structures of the compounds were determined by comparison of their NMR and mass spectrometry (MS) data with literature values[24].

1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay

This experimental procedure was adapted from Wang *et al.*[29], modified by our workgroup as reported previously[30]. The absorbance was measured using a PerkinElmer Lambda 40 UV/VIS spectrophotometer at 517 nm against a blank, which was without DPPH. All tests were run in triplicate. Ascorbic acid was used as a positive control. A decrease of DPPH solution absorbance indicates an increase of DPPH radical scavenging activity. This activity is given as % DPPH radical-scavenging, calculated by the equation:

$$\%\text{DPPH radical} - \text{scavenging} = [1 - (\text{sample absorbance with DPPH} - \text{sample absorbance without DPPH/control absorbance})] \times 100$$

β-Carotene bleaching test

Antioxidant activity was determined using a modified β-carotene bleaching test[31]. Briefly, 1 mL of β-carotene solution (0.2 mg/mL in chloroform) was added to 0.02 mL

of linoleic acid and 0.2 mL of 100% Tween 20. After evaporation of chloroform and dilution with water, 5 mL of the emulsion was transferred into different test tubes containing 0.2 mL of samples in 70% ethanol at different concentrations. Standard (propyl gallate) at the same concentration as samples was used for comparison. The tubes were then gently shaken and placed at 45°C in a water bath for 60 min. The absorbance of the samples, standard, and control was measured at 470 nm using a PerkinElmer Lambda 40 UV/VIS spectrophotometer against a blank, consisting of an emulsion without β-carotene. The measurement was carried out at initial time ($t = 0$) and successively at 30 and 60 min. All samples were assayed in triplicate and results averaged. The antioxidant activity (AA) was measured in terms of successful bleaching of β-carotene by using the following equation:

$$AA = \left(1 - \frac{A_0 - A_t}{A^{\circ}{}_0 - A^{\circ}{}_t}\right) \times 100$$

where A_0 and $A^{\circ}{}_0$ are the absorbance values measured at the initial incubation time for samples/standard and control, respectively, while A_t and $A^{\circ}{}_t$ are the absorbance values measure in the samples/standard and control, respectively, at $t = 30$ min and $t = 60$ min.

Cell culture

The murine monocytic macrophage cell line RAW 264.7 (European Collection of Cell Cultures, London, UK) was grown in a plastic culture flask in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimitotic solution (penicillin/streptomycin) under 5% CO_2 at 37°C. After 4–5 days cells were removed from the culture flask by scraping and were centrifuged for 10 min at 1500 rpm. The medium was then removed and the cells were resuspended with fresh DMEM. Cell counts and viability were assessed using a standard trypan blue cell counting technique. The cell concentration was adjusted to 1×10^6 cells/mL in the same medium, and 100 µL of the above concentration was cultured in a 96-well plate for 1 day to become nearly confluent. Concentrations ranging from 10 to 100 µg/mL of the samples were prepared from stock solutions by serial dilution in DMEM to give a volume of 100 µL in each well of a microtiter plate (96-well). Then cells were cultured with vehicle, *P. saxatile* MeOH extract, and isolated compounds in the presence of 1 µg/mL LPS for 24 h.

Assay for cytotoxic activity

Cytotoxicity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay reported by Tubaro *et al.*[32], with some modification. The assay for each concentration of samples was performed in triplicate and the culture plates were kept at 37°C with 5% (v/v) CO_2 for 1 day. After 24 h of incubation, 100 µL of medium was removed from each well. Subsequently, 100 µl of 0.5% (w/v) MTT (Sigma, Italy), dissolved in phosphate buffered saline, was added to each well and allowed to incubate for a further 4 h. After that, 100 µL of dimethylsulfoxide (DMSO) was added to each well to dissolve the formazan crystals. Absorbance values at 550 nm were measured with a microplate reader (GDV DV 990 B/V; Roma, Italy). Cytotoxicity was expressed as CD_{50}, which is the concentration to reduce the absorbance of treated cells by 50% compared with the control (untreated cells).

Inhibition of nitric oxide (NO) production in LPS-stimulated RAW 264.7 cells

The presence of nitrite, a stable oxidized product of NO, was determined in cell culture media using Griess reagent (1% sulfanamide and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% H_3PO_4)[33]; 100 µL of cell culture supernatant was removed and combined with 100 µL of Griess reagent in a 96-well plate, followed by spectrophotometric measurement at 550 nm using a microplate reader (GDV DV 990 B/V). The nitrite concentration in the supernatant was determined by comparison with a sodium nitrite standard curve.

Microtiter cholinesterase inhibition assay

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition was assessed by modification of Ellman's method[34], which is based on the reaction of released acetyl- or butyrylthiocholine to give a colored product with a chromogenic reagent. AChE or BChE 20 µL (0.20 U/mL in buffer pH 8) and *P. saxatile* isolated compounds (25 µL) were added to 50 µL of buffer, pH 8, and pre-incubated in an ice bath at 4°C for 30 min. Duplicate wells were also treated this way with 20 µL of physostigmine (0.1 mM) to allow interference of the test substances in the assay to be assessed, and to control for any hydrolysis of acetylcholine not due to enzyme activity. The reaction was started by adding dithionitrobenzene (DTNB) solution (125 µL of 0.05 mM in buffer, pH 7) and acetylthiocholine (ATCI) or butyrylthiocholine BTCI (25 µl, 0.018 mM in buffer, pH 7), and tubes were incubated in a water bath for 20 min at 37°C. The reaction was stopped by placing the assay solution tubes in an ice bath and adding physostigmine (20 µL, 0.018 mM in buffer, pH 7). Blanks were used of reagents without compounds, and a positive control was set up which was the same as the blank except that physostigmine (20 µL, 0.018 mM in buffer, pH 7) was added. The production of yellow anion was immediately recorded on a spectrophotometer at 405 nm. The inhibition rate (%) was calculated by the equation:

$$\text{Inhibition}(\%) = \frac{[(\text{Blank} - \text{Blank positive control}) - (\text{Experiment} - \text{Experiment control})]}{(\text{Blank} - \text{Blank positive control})}$$

Results and discussion

Compound characteristics in P. saxatile MeOH extract

The major constituents in the MeOH extract were isolated using several repeated procedures of RP-18

silica gel HPLC. The structures of the known compounds (Figure 1), 1-*O*-β-glucopyranosyl-2(3′,3′-dimethylallyl) hydroquinone (**1**), 1-*O*-β-glucopyranosyl-2(3′-hydroxymethyl-3′-methylallyl) hydroquinone (**2**), chlorogenic acid (**3**), caffeic acid (**4**), methylchlorogenic acid (**5**), apigenin 7-*O*-β-glucopyranoside (**6**), 3,5-di-*O*-caffeoylquinic acid methyl ester (**7**), 3,5-di-*O*-caffeoylquinic acid (**8**), luteolin-4′-*O*-glucopyranoside (**9**), luteolin (**10**), and apigenin (**11**), were determined by comparison of their NMR and MS data with literature values[22]. All these compounds have been previously investigated for their antioxidant activity. In fact, Chiang *et al.*[35] have accredited to chlorogenic acid, caffeic acid, and 3,5-di-*O*-caffeoylquinic acid a very high antioxidant activity. The antioxidant activity of flavonoids was confirmed by Bae *et al.*[36]. The compounds present in a sufficient amount (**3–6**, **8–11**) were investigated for their anti-inflammatory and anticholinesterase activities in order to relate biological activities of the extract to pure isolated compounds.

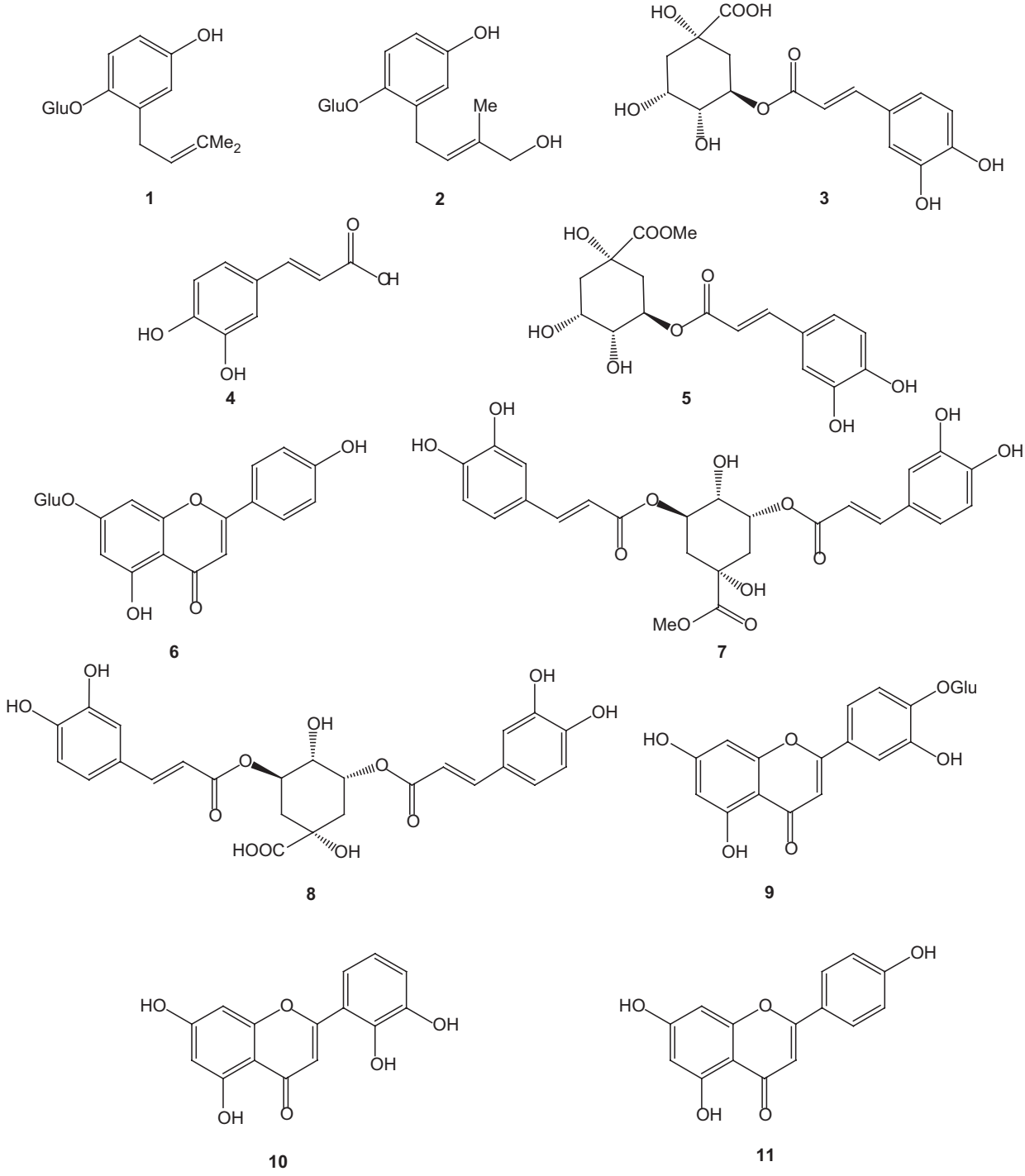

Figure 1. Structures of isolated compounds from *P. saxatile*.

Free radical scavenging and antioxidant activity

The DPPH radical-scavenging activity of all samples was measured. The effect of antioxidants on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was thought to be due to their hydrogen-donating ability. The scavenging effects of the methanolic extract and fractions on DPPH were examined at different concentrations (10, 25, 50, 100, 250, 500, and 1000 μg/mL). As shown in Table 1, the methanolic extract showed significant activity (IC_{50} 25 μg/mL). Antioxidant activity, which was demonstrated by the ability of the samples to inhibit the bleaching of β-carotene, was measured and compared to that of the control, which contained no antioxidant component. The methanolic extract of *P. saxatile* inhibited oxidation of linoleic acid. As shown in Table 1, the methanolic extract showed antioxidative activity after 30 min of incubation as well as after 60 min of incubation (IC_{50} 14 μg/mL and 41 μg/mL, respectively), indicating that its activity was not correlated with time of heating.

Inhibition of NO production

The anti-inflammatory activity of *P. saxatile* MeOH extract and isolated compounds was studied *in vitro*, analyzing their inhibitory effects on chemical mediators released from macrophages. Once activated by inflammatory stimulation, macrophages produce a large number of cytotoxic molecules. Treatment of RAW 264.7 macrophages with LPS (1 μg/mL) for 24 h induces NO production, as assessed by measuring the accumulation of nitrite, a stable metabolite of NO, in the medium, based on the Griess reaction. NO, a macrophage-derived mediator, is considered to play a key role in the inflammatory response, based on its occurrence at inflammatory sites and its ability to induce many of the hallmarks of the inflammatory response. The beneficial effect of *P. saxatile* extract on inhibition of the production of inflammatory mediators in macrophages can be mediated through oxidative degradation of the products of phagocytes, such as O^{2-} and HOCl. As shown in Table 2, incubation of RAW 264.7 cells with the methanol extract and isolated compounds of *P. saxatile* induced a significant inhibitory effect on LPS-induced nitrite production. The MeOH extract of *P. saxatile* showed significant inhibition of LPS-induced NO

Table 1. Free radical scavenging and lipid peroxidation inhibition of methanolic extract from *P. saxatile*.

		IC_{50} (μg/mL)[a]		
			β-Carotene bleaching test	
	% Inhibition	DPPH	30 min	60 min
DPPH assay (μg/mL)		25 ± 0.18		
1000	92.5			
500	93.9			
250	93.6			
100	92.0			
50	86.9			
25	50.8			
10	21.8			
β-Carotene bleaching test[b]			14 ± 0.08	41 ± 0.21
100	78.1/76.0			
50	59.6/59.7			
25	50.8/43.7			
10	36.7/38.8			
5	13.1/22.0			
1	11.0/18.5			
Propyl gallate[c]			1 ± 0.01	1 ± 0.01
Ascorbic acid[c]		2 ± 0.01		

[a]Values are given ± SEM ($n = 3$).
[b]Inhibition values are 30 min/60 min.
[c]Propyl gallate and ascorbic acid were used as positive controls.

Table 2. Inhibition of NO production in LPS-induced RAW 264.7 macrophages by methanolic extract and compounds isolated from *P. saxatile*.

Compound	Concentration (μg/mL)	% Inhibition	IC_{50}
MeOH extract	100	65 ± 1.4**	82.09 ± 1.6**
	50	30 ± 0.8**	
	25	27 ± 0.7**	
	10	25 ± 0.5**	
Chlorogenic acid (**3**)	100	95 ± 1.9**	17.59 ± 0.75**
	50	50 ± 1.2**	
	25	33 ± 0.7**	
	10	22 ± 0.5**	
Caffeic acid (**4**)	100	97 ± 1.9**	7.21 ± 0.63**
	50	65 ± 1.5**	
	25	29 ± 0.6**	
	10	40 ± 0.7**	
Methylchlorogenic acid (**5**)	100	99 ± 2.2**	12.86 ± 0.71**
	50	57 ± 1.3**	
	25	48 ± 0.9**	
	10	29 ± 0.7**	
Apigenin 7-*O*-β-glucopyranoside (**6**)	100	99 ± 2.1**	25.35 ± 0.87**
	50	43 ± 0.9**	
	25	26 ± 0.7**	
	10	15 ± 0.4**	
3,5-Dicaffeoylquinic acid (**8**)	100	10 ± 0.5**	—
Luteolin-4′-*O*-glucopyranoside (**9**)	100	90 ± 1.9**	30.55 ± 0.91**
	50	38 ± 0.8**	
	25	26 ± 0.6**	
	10	10 ± 0.3**	
Luteolin (**10**)	100	91 ± 1.8**	20.76 ± 0.82**
	50	38 ± 0.8**	
	25	36 ± 0.7**	
	10	15 ± 0.4**	
Apigenin (**11**)	100	90 ± 2.0**	16.75 ± 0.72**
	50	37 ± 1.0**	
	25	22 ± 0.6**	
	10	18 ± 0.5**	
Indomethacin	200	95 ± 2.1**	52.8 ± 1.2**
	100	66 ± 1.3**	
	50	46 ± 1.1**	
	25	5 ± 0.3**	
	12.5	2.5 ± 0.2**	

Note. Values are mean ± SD ($n = 3$); multicomparison Dunnett's test: ** $p < 0.01$. Indomethacin was used as positive control

production in RAW 264.7 cells in a dose-dependent manner, with an IC_{50} value of 82 μg/mL. The pure compounds that most effectively inhibited LPS-induced NO production were caffeic acid (**4**) and methylchlorogenic acid (**5**), with IC_{50} values of 7 μg/mL and 12 μg/mL, respectively (Table 2). Although the anti-inflammatory activity of caffeic acid has already been studied[37], methylchlorogenic acid was investigated for the first time. In contrast with Wang and Mazza[38], who showed that chlorogenic acid has no inhibitory effect, but on the contrary may induce the production of NO, we found that both chlorogenic acid and its methyl ester showed anti-inflammatory activity. As regards flavonoids, several articles[39,40] have discussed their anti-inflammatory activity, particularly of apigenin and luteolin. No data are available for the glucopyranosides of these compounds, which in our study showed significant activity on NO production. In fact luteolin-4′-glucopyranoside (**9**) showed an IC_{50} value of 30 μg/mL, while apigenin-7-glucopyranoside (**6**) showed an IC_{50} value of 25 μg/mL. Phenolic compounds are one of the most important groups of secondary metabolites in edible plants. Epidemiological studies have indicated that naturally occurring phenolic compounds may play an important role in the maintenance of human health and prevention of several diseases[41]. This is also believed to be correlated to the anti-oxidative properties of phenolics. In particular, flavonoids have a broad spectrum of biological properties, e.g. antinflammatory. It has been previously reported that the presence of free hydroxyls on a flavonoid nucleus enhances the inhibition of inflammation, whereas substitution by methoxy groups or sugars diminishes the activity[42]. Based on structure and activity relationship analysis, we observed that substitution of the C4′ hydroxyl group with glucose, as in the case of luteolin-4′-*O*-glucopyranoside (**9**), and substitution of the C7 in the case of apigenin 7-*O*-β-glucopyranoside (**6**), resulted in an approximately two-fold increase in IC_{50} values for inhibition of NO production relative to that of luteolin (**10**) or apigenin (**11**), containing free hydroxyl groups in the C4′ and C7 position, respectively.

Table 3. Cholinesterase inhibitory activity of methanolic extract and compounds isolated from *P. saxatile*.

	Concentration (μg/mL)	% InhibitionAChE	IC_{50}AChE	% InhibitionBChE	IC_{50}BChE	SI(IC_{50} BChE/AChE)
MeOH extract	1000	—	>1000	100	523.75 ± 2.6**	—
	750			55.1		
	500			49.2		
	250			21.9		
	100			0		
Chlorogenic acid (**3**)	—		—		—	1.48
Caffeic acid (**4**)	100	100	32.4 ± 1.7**	100	32.2 ± 1.5**	0.99
	75	88.7		88.6		
	50	52.4		52.3		
	25	46.9		47.0		
	10	23.8		24.1		
Methylchlorogenic acid (**5**)	—		—		—	—
Apigenin 7-*O*-β-glucopyranoside (**6**)	200	100	102.8 ± 2.5**	100	85.7 ± 1.4**	0.83
	100	50.3		57.6		
	50	25.9		41.3		
	25	10.6		36.4		
	10	0		9.6		
3,5-Dicaffeoylquinic acid (**8**)	100	100	54.5 ± 1.6**	100	73.2 ± 1.4**	1.3
	75	67.4		51.7		
	50	46.9		42.4		
	25	31.2		20.8		
	10	23.5		0		
Luteolin-4′-*O*-glucopyranoside (**9**)	100	100	66.1 ± 2.3**	50.3	99.5 ± 2.3**	1.50
	75	56.4		34.2		
	50	44.3		10.9		
	25	23.4		0		
	10	5.5		0		
Apigenin (**11**)	100	100	27.5 ± 1.3**	100	42.7 ± 1.6**	1.55
	75	76.4		79.2		
	50	52.9		52.8		
	25	46.6		40.8		
	10	10.8		12.6		
Physostigmine			0.07 ± 0.02		0.17 ± 0.04	2.42

Note. Values are mean ± SD ($n = 3$); multicomparison Dunnett's test: ** $p < 0.01$.

Cytotoxicity

The cytotoxic effect of all samples (*P. saxatile* MeOH extract and isolated compounds) in the presence of LPS (10 μg/mL) was evaluated. The *P. saxatile* MeOH extract and isolated compounds did not show any cytotoxicity at concentrations of 10–100 μg/mL.

Anticholinesterase activity

This article reports for the first time the cholinesterase inhibitory properties of *P. saxatile* and derived compounds (Table 3). Results evidenced that the MeOH extract was able to inhibit BChE (IC_{50} 523.75 μg/mL) but was unable to affect AChE activity (Table 3). As regards the isolated compounds, aglycons were more potent than glycosides in inhibition of the enzyme; in fact IC_{50} against AChE was 25.2 μg/mL for luteolin and 66.1 μg/mL for luteolin-4′-*O*-glucopyranoside, and 27.5 and 102.8 μg/mL for apigenin and apigenin-4-glycoside, respectively. Luteolin exhibited the same pattern also against BChE (IC_{50} of 37.2 and 99.5 μg/mL for aglycon and glycoside, respectively). Previously, our research group reported the ability of linariin and isolinariin A and B, three flavones isolated from *Linaria reflexa*, to inhibit AChE in a dose-dependent manner[43]. Moreover, recent studies have examined the permeability of flavonoids and their known circulating metabolites across the blood–brain barrier (BBB). Flavonoids are hydrolyzed in the gut and then glucuronized, so it is quite possible that a glycosidal form reaches the central nervous system (CNS)[44]. In particular, Youdim *et al.*[45] reported that flavonoids from particular families are able to permeate the BBB, whereas the entry of others is limited by the actions of efflux transporters expressed at the endothelium surface. However, it should be noted that penetration of the BBB does not necessarily equate to entry into neurons, where flavonoids are believed to elicit their neuroprotective effects. The inhibitory activity of caffeic acid against AChE and BChE is also reported (IC_{50} of 32.4 and 32.2 μg/mL, respectively). Previously, Borrelli *et al.*[46] showed the ability of caffeic acid phenethyl ester (CAPE) to inhibit AChE. Luteolin and 3,5-dicaffeoylquinic acid exhibited the most promising activity against AChE with IC_{50} of 25.2 and 54.5 μg/mL, respectively.

Conclusions

Oxidative damage may initiate and promote the progression of a number of chronic diseases, including inflammation and Alzheimer's disease. The present work showed for the first time the *in vitro* activity of *P. saxatile* methanolic extract in the treatment of inflammation and Alzheimer's disease and its metabolite profile. The beneficial effects may be attributable to the ability of *P. saxatile* extract to act in different ways. In this study we have demonstrated that the MeOH extract of *P. saxatile* exhibited significant antioxidant activity and an inhibitory effect on NO production (an inflammatory mediator) in macrophages. Furthermore, anticholinesterase assays on the methanolic extract and isolated phenolic compounds showed that luteolin was the most potent inhibitor of both AChE and BChE. We also observed that the extract of *P. saxatile* could be a good source of phenolic compounds, which are one of the most bioactive groups of secondary metabolites in plants. The metabolism and bioavailability of these phenolic compounds *in vivo* are suggested to correlate well to their observed antioxidant properties *in vitro*.

Therefore, we propose here the potential benefits of *P. saxatile* extract on the basis of the phytochemical characteristics and the observed bioactive properties. Naturally occurring phytocompounds possess antioxidative, anti-inflammatory, and anticholinesterase properties, which appear to contribute to their chemopreventive or chemoprotective activity. Further studies of the plant extracts and/or the identified compounds from *P. saxatile* on the pharmacokinetics or mode of action on mechanisms of chemopreventive properties are warranted.

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