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Phytochemical Study and Evaluation of Antioxidant, Neuroprotective and Acetylcholinesterase Inhibitor Activities of *Galeopsis ladanum* L. extracts

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ABSTRACT

The antioxidant activity, neuroprotective effect and acetylcholinesterase activity of the dichloromethane, ethyl acetate, methanol and water extracts of seeds, leaves and roots of *Galeopsis ladanum* L. were investigated. Ethyl acetate, methanol and water extracts of leaves showed the highest antioxidant activity (DPPH). Methanol and water extracts of seeds and the water extract of roots showed neuroprotective effect on hydrogen peroxide induced apoptosis in rat pheochromocytoma PC12 cells. Some dichloromethane, ethyl acetate and water extracts exhibited antiacetylcholinesterase activity by TLC.

KEYWORDS: *Galeopsis ladanum*; antioxidant activity; neuroprotective effect; PC12 cells; acetylcholinesterase inhibitors.

INTRODUCTION

In recent years considerable attention has been devoted to medicinal plants with antioxidant properties reducing free radical produced tissue injury. In living organisms the reactive oxygen species (ROS) and reactive nitrogen species (RNS) are known to cause damage to lipids, proteins, enzymes, and nucleic acids leading to cell or tissue injury implicated in the processes of aging as well as in wide range of degenerative diseases including inflammation, cancer, atherosclerosis, diabetes, liver injury, Alzheimer, Parkinson, and coronary heart pathologies, among others (1).

In the present study, we examined the protective effects of *G. ladanum* extracts on H_2O_2 induced cytotoxicity in cultured rat pheochromocytoma PC12 cells that retain dopaminergic characteristics and have been widely used for neuroprotective studies (2–4).

Alzheimer's disease (AD) is the most common cause of senile dementia in elderly humans. Actually acetylcholinesterase inhibitors (AChEI) are the most important compounds for the treatment of the AD symptoms, increasing the neurotransmitter acetylcholine levels at cerebral cortex synapses. Current efforts to identify new AChEI are mostly focused on alkaloids, but day after day are more studies of non-alkaloids compounds. There have been described flavonoids, xanthones, chalcones, cumarins and terpenoids with this activity (5–7).

Galeopsis ladanum L. was previously examined for chemical constituents (8–10). In these studies were identified 16 apigenin, luteolin, scutellarein, isoscutellarein and hipolaetin derived flavonoids, caffeic acid, pseudochlorogenic acid, stachydrine, harpagoside and 8-O-acethylharpagoside. Flavonoids as linariin and isolariin that showed AChEI activity (11–13), have similar chemical structure to flavonoids presents in *G. ladanum*.

MATERIALS AND METHODS

Preparation of the Extracts

Seeds, leaves and roots of *Galeopsis ladanum* L. (*Lamiaceae*) were collected in Congosto de Valdavia, Palencia (Spain). The plant was identified by Dr. R.Y. Cavero, Department of Plant Biology of the University of Navarra, Spain. Dry powdered plant material (seeds, leaves and roots) was extracted by sequential cold maceration using dichloromethane, ethyl acetate, methanol and water.

Preliminary Phytochemical Analysis

All extracts were subjected to preliminary phytochemical screening for the determination of major chemical groups by TLC (14) and HPLC. Determination of Total Phenols was performance employing Prussian Blue method (15), recommended by European Pharmacopoeia.

Free Radical Scavenging Activity

Antioxidant activity of plant extracts was determined through spectrophotometry using 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging radical assay (16). Extracts were prepared in their correspondent solvent to obtain concentrations of 10, 20, 50, 100, 200, 400, 800, 1200 and 1600 µg/ml; and standards at concentrations of 0.25, 0.5, 1, 2, 5, 10, 20, 40 and 100 µg/ml. 150 µl of a 0.04 mg/ml MeOH solution of DPPH were added to 150 µl of diluted solutions. The absorbance was determined at different times (0, 15, 30, 45, 60 and 75 min) at 517 nm. % inhibitions were plotted against respective concentration to obtain correspondent IC_{50}. These results were plotted against respective time to obtain IC_{50max} (IC_{50} of maximum inhibition) and t_{max} (time of maximum inhibition). The experiment was performed in quadruplicate. Vitamin C and BHA were used as positive controls.

Cytotoxicity and Neuroprotective Effect

Cytotoxicity and neuroprotective effect were performed in rat pheochromocytoma PC12 cells. For the determination of the cytotoxicity, cells were seeded in 96 multiwell plates at a density of 20.000 cells/well and incubated during 48 hours. 100 µl of different extract solutions (1, 10, 50, 100, 250, 500 y 1000 µg/ml in DEMEM supplemented with horse serum 1 %) were added to the wells and incubated 24 hours. 3-(4,5-Dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT) is an indicator of the mitochondrial activity of living cells. MTT is reduced to a coloured compound (formazan) by mitochondria. The free-cell medium was removed and 100 µl of MTT-PBS horse serum 1 % solution (0.4 mg/ml final concentration) was added. After 1 h of 37 °C incubation, MTT solution was removed and formazan was dissolved in 100 µl of DMSO (17). MTT reduction in living cells was quantified at 570 nm wavelength using a Power Wave XS microplate reader. The experiment was performed in quadruplicate. For the determination of the neuroprotective activity, cells were treated for 30 min with 100 µl of PBS containing H_2O_2 (0.2 mM) 24 hours after the addition of the extracts employing the same conditions that in the cytotoxicity test and using the MTT assay for the determination of cell survival (18).

Acetylcholinesterase Inhibitor Activity

Antiacetylcholinesterase activity was studied by TLC with galanthamine as reference compound using Ellman's method (19). TLC plates were sprayed DTNB or 5,5'-dithiobis(2-nitrobenzoic acid)/ATCI or acetylthiocholine iodide 1:1 (1 mM DTNB and 1 mM ATCI in tris-HCl 50 mM buffer pH 8). It was allowed to dry for 3–5 min and then 3 U/ml of acetylcholinesterase (AchE) solution was sprayed. In order to verify whether the positive results shown using Ellman's method were due to enzyme inhibition or to chemical inhibition, we employed the false positive method developed by Rhee et al (20). Firstly TLC plates were sprayed with a 1 mM solution of DTNB followed by the thiocholine spray. Tiocholine was obtained incubating for 15 min at 37°C a mixture of 3 U/ml of AchE and 1 mM ATCI in tris-HCl 50 mM buffer (pH 8). Galanthamine was used as standard and the results were compared with the results of Ellman's method.

RESULTS AND DISCUSSION

The phytochemical analysis by TLC and HPLC showed the presence of flavonoids, phenolic acids, alkaloids, iridoids and saponins in different extracts (Table 1). Fatty acids are the major compounds in seeds. Alkaloids were only present in methanolic extracts of all plant organs, while saponins only in aqueous extracts. Flavonoids were detected principally in ethyl acetate and methanolic extracts of leaves and roots, and phenolic acids in ethyl acetate, methanolic and aqueous extracts of all plant organs.

Flavonoids were and identified by HPLC-UV as isoscutellarein derivates (5,7,8,4'-tetrahydroxyflavone), λ_{MeOH} = .277, 305, 326 sh, and hypolaetin derivatives (5,7,8,3',4'-pentahydroxyflavone), (λ_{MeOH} = 277, 300 sh, 340).

Ethyl acetate (16.5 µg/ml), methanol (21.3 µg/ml) and water (37.1 µg/ml) extracts of leaves showed the highest antioxidant activity against DPPH radical. High antioxidant activity of these extracts is due to the presence of flavonoids and phenolic acids (Table 1).

Table 1 Radical scavenging activity against DPPH of *Galeopsis ladanum* L. extracts.

Part used	Extract	Yield (%)	Phytochemical screening	Total phenol mg/100 mg	DPPH IC_{50} (µg/ml)
Seeds	DCM	44.7	Fatty acids	ND	ND
	EtOAc	0.6	Flavonoids, phenolic acids	1.9 ± 0.3	ND
	MeOH	2.9	Phenolic acids, alkaloids	2.0 ± 0.2	100.0±0.4
	H_2O	0.8	Phenolic acids, saponins	ND	164.5±4.6
Leaves	DCM	6.3	Essential oils	ND	345.2±11.5
	EtOAc	1.2	Flavonoids, phenolic acids, iridoids	3.8 ± 0.3	16.5±0.1
	MeOH	20.5	Flavonoids, phenolic acids, alkaloids, iridoids	4.1 ± 0.2	21.3±0.1
	H_2O	3.8	Flavonoids, phenolic acids, saponins, iridoids	1.8 ±0.1	37.1±2.1
Roots	DCM	1.0	Fatty acids	ND	304.6±0.8
	EtOAc	3.4	Flavonoids, phenolic acids, iridoids	4.0 ± 0.3	91.1±0.1
	MeOH	8.8	Flavonoids, phenolic acids, alkaloids, iridoids	3.2 ± 0.2	81.5±0.5
	H_2O	3.6	Phenolic acids, saponins, iridoids	ND	153.8±5.0
Standards	Vitamin C	—		—	2.2±0.1
	BHA	—		—	3.0±0.1

ND: no detected; BHA: butylated hydroxyanisole.

Table 2. Cytotoxicity of *G. ladanum* extracts (% PC12 cell survival).

C (µg/ml)		1	10	50	100	250	500	1000
DCM	SGL	92.0±5.6	92.3±2.8		94.4±1.3	76.1±3.3	81.0±6.6	86.7±9.9
	LGL	91.4±7.5	95.6±9.7	86.1±6.2	79.0±0.8	73.0±2.3	85.5±1.9	82.7±1.7
	RGL	98.5±5.3	111.5±8.8	93.8±9.2	117.7±2.4	103.1±5.9	111.4±1.1	76.7±10.3
EtOAC	SGL	106.3±12.8	111.6±1.6	115.2±0.5	101.1±7.9	81.0±2.2	74.9±2.7	67.3±8.4
	LGL	110.2±13.9	109.4±7.9	113.4±8.7	105.4±2.1	93.5±1.5	47.2±7.8	22.1±0.8
	RGL	100.6±10.7	89.8±0.7	97.4±5.0	104.1±2.8	94.9±8.7	96.2±5.5	98.7±4.3
MeOH	SGL	96.4±0.8	96.9±0.2	84.3±2.8	88.7±1.0	93.1±1.2	97.5±1.0	77.1±3.2
	LGL	98.6±2.7	101.1±4.1	91.6±15.7	104.8±7.5	99.3±14.8	91.4±13.8	112.5±15.0
	RGL	99.5±0.3	100.0±13.9	103.8±2.4	100.4±2.7	107.9±3.5	107.9±2.5	84.2±0.6
H_2O	SGL	98.5±1.1	90.4±6.3	86.3±4.6	96.7±0.6	81.6±8.4	96.5±2.9	88.4±5.7
	LGL	103.1±1.1	102.9±8.5	104.3±17.4	98.9±3.5	108.2±5.0	99.5±7.7	103.2±9.0
	RGL	100.6±10.7	89.8±0.7	97.4±5.0	104.1±2.8	94.9±8.7	96.2±5.5	98.7±4.3

SGL: seeds; LGL: leaves; RGL: roots of *Galeopsis ladanum* L.

Table 3. % PC12 cell survival against hydrogen peroxide induced apoptosis.

C (µg/ml)		1	10	50	100	250	500	1000
DCM	SGL	32.8±5.8	33.7±1.2	30.8±1.4	31.7±2.4	32.4±9.0	31.6±3.2	33.5±4.0
	LGL	31.8±6.3	25.6±2.1	26.4±1.1	25.8±2.2	29.6±1.5	23.2±6.1	25.9±3.9
	RGL	36.3±1.8	37.1±4.0	35.6±1.5	40.5±3.8	33.8±1.9	33.7±4.1	36.4±4.9
EtOAC	SGL	32.1±3.2	36.8±5.2	32.3±2.8	32.1±4.6	36.2±5.8	33.5±3.3	31.8±4.1
	LGL	31.9±1.5	28.6±6.3	29.3±4.9	28.8±6.7	29.6±3.1	23.1±2.0	21.6±5.9
	RGL	35.5±2.3	39.4±2.5	35.7±2.9	32.4±2.1	30.0±5.6	28.1±5.0	35.1±4.4
MeOH	SGL	30.2±1.4	33.1±5.8	95.0±7.1	109.1±3.1	112.2±4.3	117.7±3.7	116.5±4.1
	LGL	33.9±1.4	34.9±2.1	40.7±5.6	39.8±3.4	39.9±4.1	32.4±1.8	37.8±3.2
	RGL	35.2±3.3	37.1±6.8	33.8±4.0	36.7±4.2	38.0±3.3	41.4±3.6	39.9±3.3
H_2O	SGL	25.8±1.3	47.3±6.4	60.2±1.4	86.1±2.4	115.4±3.5	116.3±1.0	114.6±3.4
	LGL	24.8±2.8	25.6±1.4	30.3±5.2	38.2±6.6	40.1±3.6	46.0±6.6	48.1±4.1
	RGL	29.5±1.1	29.6±1.2	86.5±4.4	98.9±4.6	105.8±5.7	114.3±2.4	115.1±6.3

SGL: seeds; LGL: leaves; RGL: roots of *Galeopsis ladanum* L.

Only EtOAc extract of leaves showed cytotoxicity at high concentrations (500 and 1000 µg/ml) (Table 2). Extracts with a high content of phenolic acids, methanolic extract of seeds and aqueous extracts of seeds and roots, exhibited neuroprotective effect on hydrogen peroxide induced apoptosis in PC12 cells. DCM and EtOAc extracts showed no neuroprotective activity (Table 3).

DCM and EtOAc extracts of leaves and EtOAc extract of roots showed antiacetylcholinesterase activity by TLC. Three active compounds were detected: one in DCM extract of leaves (Fr=0.61), two in EtOAc extract of leaves and roots (Fr=0.30 and 0.59) (Figure 1).

CONCLUTION

Extracts of *Galeopsis ladanum* L. showed antioxidant activity against DPPH, neuroprotective effect in PC12 cells and antiacetylcholinesterase activity by TLC.

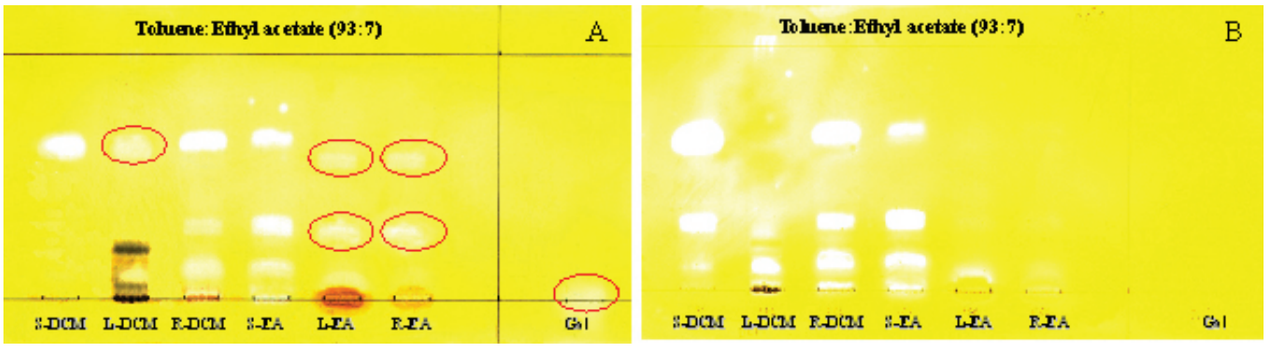

Figure 1: Antiacetylcholinesterase activity by TLC of *G. ladanum* extracts using Ellman's (A) and false positives methods (B). S:seeds; L:leaves; R:roots; DCM:dichloromethane; EA:ethyl acetate.

These results suggest that *Galeopsis ladanum* L. may be a potential candidate for the screening of new multipotent compounds for the treatment of Alzheimer's Disease.

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