NPC Natural Product Communications

2012
Vol. 7
No. 8
1015 - 1020

Antioxidant and Anti-cholinesterase Activity of *Globularia meridionalis* Extracts and Isolated Constituents

Rosa Tundis[a,*], Marco Bonesi[a], Federica Menichini[a], Monica R. Loizzo[a], Filomena Conforti[a], Giancarlo Statti[a], Filippo M. Pirisi[b] and Francesco Menichini[a]

[a]*Department of Pharmaceutical Sciences, Faculty of Pharmacy and Nutrition and Health Sciences, University of Calabria, I-87030 Rende (CS) Italy*

[b]*Department of Toxicology, University of Cagliari, I- 09124 Cagliari, Italy*

tundis@unical.it

Received: May 4th, 2011; Accepted: June 25th, 2012

The antioxidant and anti-cholinesterase properties of *Globularia meridionalis* (Podp.) O. Schwarz (Globulariaceae) were investigated. *G. meridionalis* aerial parts and roots were extracted with methanol, and the total extract successively partitioned with *n*-hexane. The methanol fraction of the roots showed the highest antioxidant activity with IC_{50} values of 8.0 and 2.1 μg/mL in the DPPH and β-carotene bleaching tests, respectively. From this fraction, three flavonoids, namely pectolinarigenin 7-*O*-β-glucoside, apigenin 7-*O*-β-glucoside and luteolin, and two phenylethanoids, namely acteoside and isoacteoside, were isolated. Potential anti-cholinesterase effects were assessed through the acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes inhibition assay. The *n*-hexane fraction of the roots exhibited the highest activity against both AChE and BChE with IC_{50} values of 65.5 and 70.4 μg/mL, respectively.

Keywords: *Globularia meridionalis*, Flavonoids, Phenylethanoids, Antioxidant activity, Anti-cholinesterase activity.

Alzheimer's disease (AD) is a devastating neurodegenerative disorder. The pathogenesis of AD has been linked to a deficiency in the brain neurotransmitter acetylcholine. Subsequently, acetylcholinesterase (AChE) inhibitors were introduced for the symptomatic treatment of AD. Nevertheless, in late AD stages, levels of AChE have declined by up to 85% and butyrylcholinesterase (BChE) represents the predominant cholinesterase in the brain. BChE, primarily associated with glial cells, but also with specific neuronal pathways, cleaves ACh in a manner similar to AChE to terminate its physiological action [1]. Both enzymes therefore represent therapeutic targets for ameliorating the cholinergic deficit considered to be responsible for the declines in cognitive, behavioural and global functioning characteristic of AD [1].

In recent years, considerable data have accrued indicating that the brain in AD is under increased oxidative stress and this may have a role in the pathogenesis of neuron degeneration and death in this disorder. Direct evidence supporting increased oxidative stress in AD are: increased brain Fe, Al and Hg, capable of stimulating free radical generation; increased lipid peroxidation and decreased polyunsaturated fatty acids; increased protein and DNA oxidation; diminished energy metabolism and decreased cytochrome c oxidase [2].

Numerous plant extracts have been used to treat cognitive disorders [3]. Therefore, ethno-pharmacological screening of plants may provide useful leads for the discovery of new drugs for AD therapy.

The genus *Globularia* consists of plants which are herbs, chamaephytes or shrubs, common in the Mediterranean regions, Europe and North East Africa, some members of which are known for their medicinal properties [4]. *G. alypum* is used as a diuretic, laxative, carminative, tonic and hypoglycaemic, *G. trichosantha* for the treatment of haemorrhoids [5,6], and *G. orientalis* exhibited significant radical scavenging activity [7].

The present investigation aimed 1) to evaluate the radical scavenging activity, antioxidant and anticholinesterase properties of *G. meridionalis* aerial parts and roots and 2) to correlate the bioactivity to the chemical composition.

Aerial parts and roots of *G. meridionalis* were extracted with methanol in order to obtain a total extract. This extract was tested to evaluate the total phenol content by Folin-Ciocalteau assay, the flavonoid total content using a method based on the formation of a flavonoid-aluminum complex, and the iridoids total content according to a spectrophotometric method based on the Trim and Hill reaction (Table 1).

Table 1: Total phenols, flavonoids and iridoids content of *G. meridionalis* aerial parts and roots.

	Total phenols (mg/g extract)	Total flavonoids (mg/g extract)	Total iridoids (mg/g extract)
G. meridionalis			
Aerial parts	410.3 ± 3.2	72.7 ± 2.6	90.3 ± 1.1
Roots	292.0 ± 1.5	125.0 ± 1.3	22.4 ± 0.7

Values represent means (n = 3) ± S.D.

G. meridionalis aerial parts were characterized by the highest content of phenols and iridoids with values of 410.3 mg/g and 90.3 mg/g of extract, respectively. Roots showed an interesting total for flavonoids content (125.0 mg/g of extract).

The total extract was partitioned with *n*-hexane and the obtained fraction was analysed by GC-MS. As shown in Table 2, the *n*-hexane fraction of the aerial parts was characterized by vitamin E, α-amyrin, β-sitosterol, γ-terpinene, methyl palmitate, methyl linoleate and methyl linolenate as major constituents. Some methyl esters of fatty acids, namely methyl linoleate, methyl linolenate, methyl stearate and methyl behenate, were the most abundant compounds identified in the *n*-hexane fraction of the roots, together with vitamin E, β-sitosterol and the triterpenes α- and β-amyrin.

Table 2: Major components in the *n*-hexane fraction of *G. meridionalis* aerial parts and roots.

Compound	Aerial parts[a]	Roots[a]	Identification[b]
Methyl benzoate	0.9	0.7	C
Benzoic acid	0.7	0.6	C
Tetradecanal	0.9	0.7	B
Methyl myristate	2.6	2.8	C
6,10,14-Trimethyl-2-pentadecanone	0.8	0.9	C
γ-Terpinene	4.4	0.9	A
Neophytadiene	2.7	0.5	B
Methyl palmitate	4.7	3.8	A
14-Methyl-hexadecanoic acid, methyl ester	2.6	3.8	C
1-Octadecene	1.4	1.7	C
Methyl linoleate	6.5	7.8	A
Methyl linolenate	5.8	4.9	A
Methyl stearate	4.1	5.4	B
Methyl arachidate	1.9	1.7	B
1-Nonadecene	0.6	1.5	C
Methyl behenate	3.1	4.3	B
9-Tricosene	1.6	1.8	C
Cyclotetracosane	1.8	1.9	C
Vitamin E	10.8	8.5	A
α-Amyrin	4.6	4.8	C
β-Amyrin	3.1	5.4	C
β-Sitosterol	6.9	7.4	A
Stigmasterol	4.2	3.6	C
Stigmast-6-en-3.β.-ol	3.3	3.7	C
(24R)-4-Stigmasten-3-one	1.6	1.7	C

[a] Abundance calculated as % peak area mean values, mean ± standard deviation (n = 3).
[b] The constituents identification is indicated by the following: A, mass spectrum and retention time agreed with standard; B, mass spectrum and retention time agreed with database or literature; C, mass spectrum agreed with mass spectral database.

The methanol soluble fraction was subjected to repeated chromatographic purification over a silica gel column to afford three flavonoids, namely pectolinarigenin 7-*O*-β-glucoside, apigenin 7-*O*-β-glucoside and luteolin, and two phenylethanoids namely verbascoside (acteoside) and isoacteoside. Several studies have previously showed the genus *Globularia* to be a rich source of phenolic compounds. In particular, in a flavonoid survey of the Tubiflorae, Harborne and Williams found 6-hydroxyluteolin to be the most abundant phenolic aglycon in all the *Globularia* species they examined [8]. Furthermore, other flavonoid derivatives, such as apigenin, luteolin and quercetin, have been reported from *Globularia* species [9]. A new flavone glycoside, aphyllanthoside, was isolated from the methanol extract of the aerial parts of *G. aphyllanthes* [10].

Globularia species are also characterized by the presence of phenylethanoids. Verbascoside was previously isolated from *G. trichosantha*, *G. davisiana*, *G. dumulosa*, *G. orientalis* and *G. alypum* [7,11-14], and isoacteoside from *G. trichosantha* and *G. davisiana* [11,14].

The antioxidant properties of *G. meridionalis* were evaluated using DPPH and β-carotene bleaching test. The scavenging effects in the DPPH test were examined at concentrations ranging from 50 μg/mL to 1000 μg/mL. *G. meridionalis* aerial parts and roots were able to reduce the DPPH radical to the yellow-colored 2,2-diphenyl-1-picrylhydrazyne in a concentration-dependent manner. The total extract and the methanol fraction of *G. meridionalis* roots showed the strongest radical scavenging activity with IC_{50} values of 9.0 μg/mL and 8.0 μg/mL, respectively (Table 3). Antioxidant activity, which reflected the ability of the samples to inhibit the bleaching of β-carotene, was measured and compared with that of a control, which contained no antioxidant component. It was demonstrated that addition of total extracts and methanol fractions of *G. meridionalis* inhibit oxidation of linoleic acid (Table 3, Figure 1).

Table 3: Radical scavenging and antioxidant activity of *G. meridionalis* [IC_{50} (μg/mL)].

Extract and fractions	DPPH	β-Carotene bleaching test	
		30 min	60 min
Aerial parts			
Total	21.0 ± 0.8**	2.4 ± 0.1^	3.0 ± 0.1^
Methanol	22.0 ± 0.8**	3.6 ± 0.1^	4.0 ± 0.2^
n-Hexane	893.0 ± 3.8**	5.7 ± 0.2**	8.9 ± 0.3**
Roots			
Total	9.0 ± 0.3**	2.3 ± 0.1^	4.3 ± 0.2^
Methanol	8.0 ± 0.3**	2.1 ± 0.1^	3.7 ± 0.1^
n-Hexane	847.0 ± 3.8**	98.4 ± 1.6**	> 100
Positive control			
Ascorbic acid	2.0 ± 0.1	-	-
Propyl gallate		1.0 ± 0.1	1.0 ± 0.1

IC_{50} ± S.D. (n = 3). DPPH: One-way ANOVA test: ***p < 0.0001; Multicomparison Dunnett's test: ** p< 0. 01. β-Carotene bleaching test: 30 and 60 min: One-way ANOVA test: ***p < 0.0001; Multicomparison Dunnett's test: ** p< 0. 01, ^ p> 0. 05 .

The methanol fraction from the roots showed greater antioxidant potency than other fractions with an IC_{50} value of 2.1 μg/mL after 30 min of incubation. Both roots and aerial parts total extracts showed an interesting activity with IC_{50} values of 2.3 μg/mL and 2.4 μg/mL, respectively, after 30 min of incubation. Previously, the antioxidant effects of *G. alypum* extract and its constituents have been reported [9]. The results showed that the activity towards the DPPH free radical was mainly due to the flavonoid and phenylethanoid constituents. Several studies reported the relationships between phenolic content and antioxidant activity. On the basis of these considerations, the strong activity of the roots could be due to the higher level of flavonoids.

Perusal of the literature revealed the pharmacological role of phenylethanoids. Lee *et al.* [15] have shown that phenylethanoid glycosides from leaves of *Callicarpa dichotoma* significantly attenuate neurotoxicity induced by the muscarinic antagonists glutamate and scopolamine resulting in a considerable increase in cognitive ability in rats with a pronounced memory deficit.

Among isolated compounds from *G. meridionalis*, acteoside and isoacteoside were recently tested for their antioxidant activity in the DPPH and ALP assays [16]. Both phenylethanoids presented good activity of the same order of magnitude as the positive control quercetin. Acteoside was evaluated also using the total oxidant scavenging capacity (TOSC) assay against peroxynitrite [17]. Results showed that the phenylethanoid glycoside possessed a strong antioxidant activity with a 9.9-fold TOSC value compared with the positive control Trolox. This compound also seems able to inhibit the growth and proliferation of various cancer cells. An *in vitro* study examined the effects of acteoside on telomerase activity, telomere length and cell cycle of human gastric carcinoma cells MKN45 [18].

The beneficial properties of these metabolites seem to be strongly related to their antioxidant and free radical scavenging properties. In recent years, considerable data have accrued indicating that the brain, in neurodegenerative disorders, is under increased oxidative stress and this may have a role in the pathogenesis of neuron degeneration and death in this disorder. Reversible inhibitors of cholinesterase are currently used in clinical trials examining the treatment of AD. Cholinesterase inhibitors may interact with the central cholinergic system to improve memory and cognitive deficits of the patients by diminishing the breakdown of acetylcholine at the synaptic site in the brain.

Table 4: Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activity [IC_{50} (μg/mL)[a]] of *G. meridionalis*.

Extract and fractions	AChE	BChE
Aerial parts		
Total	194.2 ± 0.1**	156.4 ± 0.1**
MeOH	374.9 ± 0.1**	734.9 ± 0.2**
n-Hexane	89.1 ± 0.2**	145.4 ± 0.3**
Roots		
Total	172.7 ± 0.1**	82.9 ± 0.2**
MeOH	429.8 ± 0.1**	374.0 ± 0.1**
n-Hexane	65.5 ± 1.6**	70.4 ± 0.1**
Isolated compounds		
Luteolin	25.2 ± 0.4**	37.2 ± 0.5**
Pectolinarigenin-7-*O*-β-glucoside	203.2 ± 2.3**	271.1 ± 2.3**
Apigenin-7-*O*-β-glucoside	172.6 ± 1.4**	221.4 ± 2.5**
Acteoside	>500	>500
Isoacteoside	>500	>500

IC_{50} ± S.D. (n = 3). One-way ANOVA test: ***p < 0.0001; Multicomparison Dunnett's test: ** p< 0. 01. Physostigmine was used as positive control (IC_{50} of 0.07 μg/mL and 0.17 μg/mL against AChE and BChE, respectively).

As shown in Table 4, the *n*-hexane fraction of the roots exhibited the highest activity, with IC_{50} values of 65.5 and 70.4 μg/mL for AChE and BChE, respectively. The *n*-hexane fraction of the aerial parts had corresponding IC_{50} values of 89.1 and 145.4 μg/mL, respectively.

A different activity was evidenced for the methanol fraction from the aerial parts and roots. In particular, the methanol fraction of the aerial parts showed a major selectivity against AChE with an IC_{50} value of 374.9 μg/mL, while the roots methanol fraction was more active against BChE.

These fractions were characterized by the presence of phenylethanoids and flavonoids. Among them, luteolin exhibited the highest activity with IC_{50} values of 25.2 and 37.2 μg/mL against AChE and BChE, respectively [19]. A lower activity was obtained with apigenin 7-*O*-β-glucoside that exhibited IC_{50} values of 172.6 and 221.4 μg/mL against AChE and BChE, respectively. A similar trend was observed with pectolinarigenin 7-*O*-β-glucoside (IC_{50} values of 203.2 and 271.1 μg/mL against AChE and BChE, respectively).

The phenylethanoids acteoside and isoacteoside were inactive against both enzymes at the maximum concentration tested (500 μg/mL). In our previous work we tested linariin, and isolinariin A and B obtained from *Linaria reflexa* for AChE inhibition activity. Flavones exhibited IC_{50} values ranging from 0.27 μM to 0.30 μM [20]. Others flavonoids have been reported to inhibit AChE, including the flavanone naringenin, which has been shown to mitigate amnesia *in vivo* and some chalcones with IC_{50} values ranging from 28.2 to 134.5 μM [21,22]. 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 glucuronised, so it is quite possible that a glycosidal form reaches the central nervous system (CNS) [23]. In particular, Youdim *et al.* [24] 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. In spite of the lack of anti-cholinesterase activity of the phenylethanoids acteoside and isoacteoside, it is noteworthy that previous studies have shown that acteoside has neuroprotective activities. In fact, acteoside was demonstrated to inhibit neuronal death induced by the 1-methyl-4-phenylpyridinium ion (MPP^+) and glutamate, and to protect SH-SY5Y cells against β-amyloid-induced cell injury by attenuating ROS production and modulating the apoptotic signal pathway through the Bcl-2 family, cytochrome c and caspase-3 [25,26].

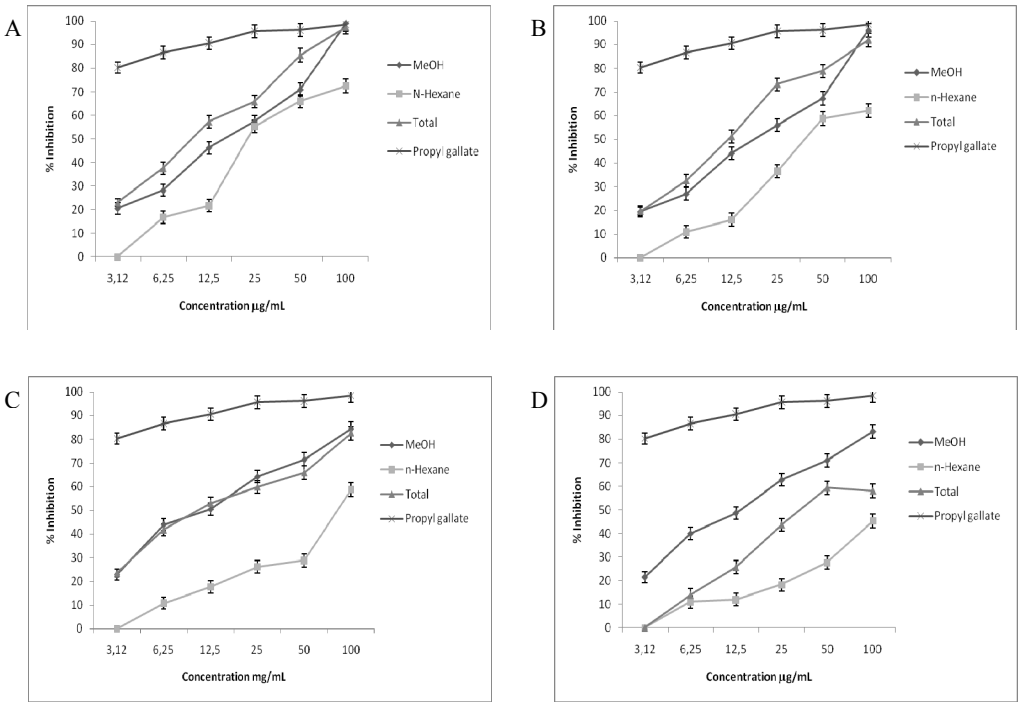

Figure 1: Lipid peroxidation inhibition using β-carotene-linoleic acid system of *G. meridionalis* aerial parts after A) 30 min of incubation; B) 60 min of incubation, and *G. meridionalis* roots after C) 30 min of incubation and D) 60 min of incubation. Data are mean ± S.D. (n = 3).

In summary, the present work showed for the first time the antioxidant activity and the inhibition of AChE and BChE, two enzymes frequently targeted for the treatment of AD, by *G. meridionalis* aerial parts and roots. We also observed that *G. meridionalis* could be a good source of phenolic compounds, which are one of the most bioactive groups of secondary constituents in plants. Therefore, we propose here the potential benefits of *G. meridionalis* on the basis of the phytochemical characteristics and the observed bioactive properties. Further studies of the extracts and/or the identified compounds on the pharmacokinetics and mode of action are warranted.

Experimental

Chemicals and reagents: Methanol, dichloromethane, and *n*-hexane were obtained from VWR (Milan, Italy). Sodium phosphate buffer, acetylthiocholine iodide (ATCI), 5,5'-dithiobis(2-nitrobenzoic-acid) (DTNB), butyrylthiocholine iodide (BTCI), Folin-Ciocalteau reagent, $AlCl_3$, physostigmine, acetylcholinesterase from *Electrophorus electricus* (EC 3.1.1.7, Type VI-S), butyrylcholinesterase from equine serum (EC 3.1.1.8), butylated hydroxytoluene (BHT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Tween 20, β-carotene, quercetin, chlorogenic acid, and aucubin were obtained from Sigma-Aldrich S.p.A. (Milan, Italy).

Plant material: Aerial parts and roots of *Globularia meridionalis* (Podp.) O. Schwarz (Globulariaceae) were collected in Calabria (Italy) in May 2004, and authenticated by Dr Lorenzo Peruzzi of the Biology Department at the Pisa University (Italy). A voucher specimen has been retained at the Herbarium of the University of Calabria (CLU) under the reference no 12492.

Extraction and isolation: Aerial parts (440 g) and roots (200 g) of *G. meridionalis* were powdered and extracted with methanol for 48 h and 72 h, respectively, through maceration (3×5 L). Combined methanol solutions were concentrated under reduced pressure and dried to obtain the total extract (yield 12.4% and 5% for aerial parts and roots, respectively). In order to operate a separation of lipophilic compounds, the total extract was solubilized with methanol and extracted with *n*-hexane. The *n*-hexane solutions were combined and dried to obtain the *n*-hexane fraction (yield 0.6% and 0.3% for aerial parts and roots, respectively). The remaining methanol fraction of the roots was subjected to CC over silica gel 60 (0.040-0.063 mm) using a CH_2Cl_2/MeOH gradient system to yield fractions A-G. Column fractions were assayed according to their TLC profile on silica gel and detected by UV light at 254 and 365 nm, natural products reagent, polyethylene glycol reagent, and H_2SO_4 50% v/v. Fractions E, F and G were further purified by silica gel CC (silica gel 0.040-0.063 mm) using a CH_2Cl_2/MeOH gradient system and preparative TLC (silica gel, CH_2Cl_2/MeOH 8:2, 7:3, 5:5) to afford 3 flavonoids, namely pectolinarigenin 7-*O*-β-glucoside (8 mg), apigenin 7-*O*-β-glucoside (11 mg), and luteolin (9 mg), and two phenylethanoids, namely acteoside (5 mg) and isoacteoside (6 mg). The methanol fraction of the aerial parts was subjected to the same separation by CC affording acteoside (12 mg), isoacteoside (8 mg), and apigenin 7-*O*-β-glucoside (7 mg). The structures of the isolated compounds were elucidated through spectroscopic methods, as previously described [9,13, 27,28].

Determination of total phenol content: The total phenol content of the total extract of aerial parts and roots was determined by the Folin-Ciocalteau method, as previously described [29]. The extract was mixed with Folin-Ciocalteau reagent and distilled water and the flasks were vigorously shaken. Subsequently, 15% sodium carbonate solution was added and the mixtures were mixed thoroughly again. The mixtures were allowed to stand for 2 h, protected from light. The absorbance of the blue color produced was measured with a spectrophotometer (UV-Vis Jenway 6003) at 765 nm. Chlorogenic acid was used as a standard and the total phenolics content was expressed as chlorogenic acid equivalents in mg/g of extract.

Determination of total flavonoid content: The total flavonoids content was determined using a method based on the formation of a flavonoid-aluminum complex [29]. One mL of the extracts was added to a 10 mL volumetric flask. Distilled water was added to make a volume of 5 mL. At zero time, 0.3 mL of 5% (w/v) sodium nitrite was added to the flask. After 5 min, 0.6 mL of 10% (w/v) $AlCl_3$ was added and then at 6 min 2 mL of 1 M NaOH was also added to the mixture, followed by the addition of 2.1 mL distilled water. The absorbance at 510 nm was read immediately. Quercetin was chosen as a standard and the levels of total flavonoid content were determined in triplicate and expressed as quercetin equivalents in mg/g of extract.

Determination of total iridoid content: The content of iridoids was determined according to a colorimetric method based on the Trim and Hill reaction [30]. In this assay, 400 μL of extract was mixed with 4.0 mL of Trim-Hill reagent (acetic acid/0.2% CuSO4/HCl concentration 10:1:0.5). After the sample had been heated at 100°C for 5 min, the absorption was read at 609 nm a blue color indicating the presence of iridoids. Aucubin was chosen as a standard and the levels of total iridoids content were determined in triplicate and expressed in mg/g of extract.

Gas chromatography/mass spectrometry (GC/MS) and gas chromatography (GC) analysis: *n*-Hexane fractions were analysed using a Hewlett-Packard 6890 gas chromatograph equipped with an HP-5 capillary column (30 m length, 0.25 mm i.d., 0.25 μm film thickness) and interfaced with a Hewlett Packard 5973 Mass Selective. Ionization of the sample components was performed in electron impact mode (EI, 70 eV), with helium as the carrier gas. The analytical conditions were: oven temperature 5 min isothermal at 50°C, then 50-250°C at a rate of 13°C/min, then held isothermal for 10 min. Injector and detector were maintained at 250°C and 280°C, respectively. Tentative identification of the compounds was achieved through retention indices (*I*), with those of the literature or with those of authentic compounds available in our laboratory [31]. The retention indices were determined in relation to a homologous series of *n*-alkanes (C_8-C_{24}) under the same operating conditions. Further identification was made by comparison of their MS with those stored in Wiley 138, Wiley 275 or NIST98 Libraries or with MS from literature [31]. GC analysis were performed on a Shimadzu GC17A gas chromatograph equipped with a flame ionization detector (FID) and controlled by Borwin Software. The samples were analysed on a fused silica 30 m SE-54 capillary column with an internal diameter of 0.25 mm and a film thickness of 0.25 μm. Nitrogen was used as the gas vector. Injector and detector were maintained at 250°C and 280°C, respectively. The oven temperature programming was 50°C during injection, then increased from 50 to 280°C at the rate of 13°C/min. Component relative concentrations were calculated based on GC peak areas without using correction factors. The quantification of the components was performed on the basis of their GC peak areas and the percentages of the characterized components were as given in Table 2.

DPPH assay: In an ethanol solution of DPPH radical (final concentration was 1.0 x 10^{-4} M), test samples were added at different concentrations (50-1000 μg/mL) [29]. 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 blank, which was without DPPH. All tests were run in triplicate and averaged. Ascorbic acid was used as a positive control.

β-Carotene bleaching test: Antioxidant activity was determined using the β-carotene bleaching test, with some modifications [29]. One 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 100 mL of water, 5 mL of the emulsion was transferred into different test tubes containing 0.2 mL of sample in 70% ethanol at different concentrations. Propyl gallate was used as positive control. The tubes were 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 Perkin Elmer 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 the mean value calculated.

Cholinesterase inhibition assay: Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibiting activities were measured by slightly modifying a spectrophotometric method based on the reaction of released thiocholine to give a colored product with a chromogenic reagent [19]. *Electrophorus electricus* (EC3.1.1.7, Type VI-S) AChE and equine serum (EC3.1.1.8) BChE were used, while acetylthiocholine iodide and butyrylthiocholine iodide, respectively, were used as substrates of the reaction. The 5,5'-dithiobis(2-nitrobenzoic-acid) (DTNB) was used for the measurement of the cholinesterase activity. In this procedure, AChE or BChE (0.20 U/mL in buffer pH 8) and samples at final concentrations ranging from 20 to 500 μg/mL (20 μL) were added to 2 mL of buffer, pH 8, and pre-incubated in an ice bath at 4°C for 30 min. The reaction was started by adding DNTB solution (20 μL of 0.05 mM in buffer pH 7) and either acetylthiocholine iodide (ATCI) or butyrylthiocholine iodide (BTCI) (20 μL 0.018 mM in buffer pH 7), and the tubes were placed in a water bath for 20 min at 37°C. The reaction was halted by placing the assay solution tubes in an ice bath and adding physostigmine (20 μL 0.018 mM in buffer pH 7). The hydrolysis of acetylthiocholine and butyrylthiocholine was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released during enzymatic hydrolysis, and immediately recorded on a spectrophotometer (Jenway 6300) at 405 nm. All the reactions were performed in triplicate.

Statistical analysis: Data were expressed as means ± SD. Statistical analysis was performed by using Student's *t* test. Differences were considered significant at $P \leq 0.05$. The inhibitory concentration 50% (IC_{50}) was calculated from the Prism dose-response curve (GraphPad, Prism version 4.0 for Windows, GraphPad Software, San Diego, CA, USA) obtained by plotting the percentage of inhibition versus the concentrations.

Acknowledgments - Authors wish to acknowledge Dr L. Peruzzi, University of Pisa (Italy) for supplying the plant material.

References

[1] Loizzo MR, Tundis R, Menichini F, Menichini F. (**2008**) Natural products and their derivatives as cholinesterase inhibitors in the treatment of neurodegenerative disorders: an update. *Current Medicinal Chemistry*, ***15***, 1209-1228.
[2] Markesbery WR. (**1997**). Oxidative stress hypothesis in Alzheimer's disease. *Free Radical Biology & Medicine*, ***23***, 134-47.
[3] Houghton PJ, Ren Y, Howes MJ. (**2006**) Acetylcholinesterase inhibitors from plants and fungi. *Natural Product Reports*, ***23***, 181-199.
[4] Elbetieha A, Oran SA, Alkofahi A, Darmani H, Raies AM. (**2000**) *Phyllanthus amarus* extract administration increases the life span of rats with hepatocellular carcinoma. *Journal of Ethnopharmacology*, ***72***, 215-219.
[5] Sezik E, Tabata M, Yesilada E, Honda G, Goto K, Ikeshiro Y. Traditional medicine in Turkey. I. (**2001**) Folk medicine in northeast Anatolia. *Journal of Ethnopharmacology*, ***35***, 191-196.
[6] Jouad H, Maghrani M, Eddouks M. (**2002**). Hypoglycaemic effect of *Rubus fructicosis* L. and *Globularia alypum* L. in normal and streptozotocin-induced diabetic rats. *Journal of Ethnopharmacology*, ***81***, 351-356.
[7] Calis I, Kirmizibekmez H, Tasdemir D, Sticher O, Ireland CM. (**2002**) Sugar esters from *Globularia orientalis*. *Zeitschrift für Naturforschung*, ***57c***, 591-596.
[8] Harborne JB, Williams AC. (**1971**) 6-Hydroxyluteolin and scutellarein as phyletic markers in higher plants. *Phytochemistry*, ***10***, 367-378.
[9] Es-Safi NE, Khlifi S, Kerhoas L, Kollmann A, El Abbouyi A, Ducrot PH. (**2005**) Antioxidant constituents of the aerial parts of *Globularia alypum* growing in Morocco. *Journal of Natural Product*, ***68***, 1293-1296.
[10] Kırmızıbekmez H, Bassarello C, Piacente S, Akaydın G, Calis I. (**2009**) Flavonoid, phenylethanoid and iridoid glycosides from *Globularia aphyllanthes*. *Zeitschrift für Naturforschung*, ***64b***, 252-256.
[11] Çalis I, Kirmizibekmez H, Sticher O. (**2001**) Iridoid glycosides from *Globularia trichosantha*. *Journal of Natural Products*, ***64***, 60-64.
[12] Çalis I, Kirmizibekmez H, Tasdemir D, Ireland MC. (**2002**) Iridoid glycosides from *Globularia davisiana*. *Chemical & Pharmaceutical Bulletin*, ***50***, 678-680.
[13] Kirmizibekmez H, Akbay P, Sticher O, Calis I. (**2003**) Iridoids from *Globularia dumulosa*. *Zeitschrift für Naturforschung*, ***58c***, 181-186.
[14] Kırmızıbekmez H, Bassarello C, Piacente S, Calis I. (**2008**) Phenylethyl glycosides from *Globularia alypum* growing in Turkey. *Helvetica Chimica Acta*, ***91***, 1525-1532.
[15] Lee KY, Jeong HS, Lee HS, Kim YC. (**2006**) Acteoside of *Callicarpa dicotoma* attenuates scopolamine-induced memory impairments. *Biological & Pharmaceutical Bulletin*, ***29***, 71-74.
[16] Martin F, Hay AE, Quinteros Condoretty VR, Cressend D, Reist M, Gupta MP, Carrupt PA, Hostettmann K. (**2009**) Antioxidant phenylethanoid glycosides and a neolignan from *Jacaranda caucana*. *Journal of Natural Products*, ***72***, 852-856.
[17] Tai BH, Jung BY, Cuong NM, Linh PT, Tung NH, Nhiem NX, Huong TT, Anh NT, Kim JA, Kim SK, Kim YH. (**2009**) Total peroxynitrite scavenging capacity of phenylethanoid and flavonoid glycosides from the flowers of *Buddleja officinalis*. *Biological & Pharmaceutical Bulletin*, ***32***, 1952-1956.
[18] Zhang F, Jia Z, deng Z, Wei Y, Zheng R, Yu L. (**2002**) *In vitro* modulation of telomerase activity, tolomere length and cell cycle in MKN45 cells by verbascoside. *Planta Medica*, ***68***, 115-118.
[19] Conforti F, Rigano D, Formisano C, Bruno M, Loizzo MR, Menichini F, Senatore F. (**2010**) Metabolite profile and *in vitro* activities of *Phagnalon saxatile* (L.) Cass. relevant to treatment of Alzheimer's disease. *Journal of Enzyme Inhibition and Medicinal Chemistry*, ***25***, 97-104.

[20] Loizzo MR, Tundis R, Menichini F, Bonesi M, Statti GA, Deguin B, Tillequin F, Menichini F, Houghton PJ. (**2007**) Acetyl-cholinesterase inhibition by extracts and isolated flavones from *Linaria reflexa* Desf. (Scrophulariaceae). *Natural Product Communications*, ***2***, 759-763.
[21] Heo HJ, Kim MJ, Lee JM, Choi SJ, Cho HY, Hong B, Kim HK, Kim E, Shin DH. (**2004**) Naringenin from *Citrus junos* has an inhibitory effect on acetylcholinesterase and a mitigating effect on amnesia. *Dementia and Geriatric Cognitive Disorders*, ***17***, 151-157.
[22] Hasan A, Khan KM, Sher M, Maharvi GM, Nawaz SA, Choudhary MI, Atta-ur-Rahman, Supuran CT. (**2005**) Synthesis and inhibitory potential towards acetylcholinesterase, butyrylcholinesterase and lipoxygenase of some variably substituted chalcones. *Journal of Enzyme Inhibition and Medicinal Chemistry*, ***20***, 41-47.
[23] Rice-Evans C. (**2004**) Flavonoids and isoflavones: absorption, metabolism, and bioactivity. *Free Radical Biology & Medicine*, ***36***, 827-828.
[24] Youdim KA, Shukitt-Hale B, Joseph JA. (**2004**) Flavonoids and the brain: Interactions at the blood brain barrier and their physiological effects on the CNS. *Free Radical Biology & Medicine*, ***37***, 1683-1693.
[25] Li Y, Lu J, Li Q, Zhao Y, Pu X. (**2008**) Pedicularioside A from *Buddleia lindleyana* inhibits cell death induced by 1-methyl-4-phenylpyridinium ions (MPP^+) in primary cultures of rat mesencephalic neurons. *European Journal of Pharmacology*, ***579***, 134-140.
[26] Wang H, Xu Y, Yan J, Zhao X, Sun X, Zhang Y, Guo J, Zhu C. (**2009**) Acteoside protects human neuroblastoma SH-SY5Y cells against beta-amyloid-induced cell injury. *Brain Research*, ***1283***, 139-147.
[27] Mabry TJ, Markham KR, Thomas MB. (**1970**) *The systematic identification of flavonoids*. Springer, New York.
[28] Markham KR. (**1982**) *Techniques of flavonoid identification*. Academic Press, London.
[29] Menichini F, Tundis R, Bonesi M, Loizzo MR, Conforti F, Statti G, de Cindio B, Houghton PJ, Menichini F. (**2009**) The influence of fruit ripening on the phytochemical content and biological activity of *Capsicum chinense* Jacq. cv Habanero. *Food Chemistry*, ***114***, 553-560.
[30] Galvez M, Martin-Cordero C, Houghton PJ, Ayuso MJ. (**2005**) Antioxidant activity of methanol extracts obtained from *Plantago* species. *Journal of Agricultural and Food Chemistry*, ***53***, 1927-1933.
[31] Adams RP. (**2001**) *Identification of essential oil components by Gas Chromatography/Quadrupole Mass Spectrometry*. Allured, Carol Stream, IL .