Acetyl-cholinesterase Inhibition by Extracts and Isolated Flavones from *Linaria reflexa* Desf. (Scrophulariaceae)

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Extracts, linariin, isolinariin A and B obtained from *Linaria reflexa* Desf. (Scrophulariaceae) were tested for acetylcholinesterase inhibition activity using Ellman's method. A dose-response relationship was observed for all extracts and isolated compounds. Flavones exhibited IC_{50} values ranging from 0.27 μM to 0.30 μM. The structure-activity relationship was briefly discussed.

Keywords: *Linaria reflexa* Desf., Scrophulariaceae, flavones, Acetyl-cholinesterase (AChE), Ellman's method.

Alzheimer's disease (AD) is characterized by profound memory loss sufficient to interfere with social and occupational functioning. It is the most common form of dementia, affecting more than 20 million people worldwide. AD is characterized by an insidious loss of memory, associated functional decline and behavioural disturbances.

Neuroimaging of the patient with AD or other dementias may reveal atrophy of the brain, such as enlarged ventricles and sulci and narrowed gyri, although these features are not always present [1]. Neuronal loss is the main neuropathologic feature underlying the symptoms of AD. Microscopically, AD is characterized by the presence of senile plaques and neurofibrillary tangles (NFTs). Plaques are extracellular deposits of filamentous 3-amyloid, a protease cleavage product of amyloid precursor protein [2,3].

The first neurotransmitter defect discovered in AD involved acetylcholine (ACh). Because cholinergic function is required for short-term memory function, it was determined that cholinergic deficit in AD was also responsible for much of the short-term memory deficit [4]. The resultant decrease in ACh-dependent neurotransmission is thought to lead to the functional deficits of AD, much as dopaminergic deficits underline Parkinson's disease and its clinical manifestations [5,6]. Clinical trials in patients with AD have focused on drugs that augment levels of ACh in the brain to compensate for losses of cholinergic function in the brain. These drugs have included ACh precursors, muscarinic agonists, nicotinic agonists, and cholinesterase inhibitors. The best-developed and most successful approaches to date have used cholinesterase inhibition [7]. The majority of FDA-approved drugs for Alzheimer's disease (AD) are tacrine, donepezil, rivastigmine, and galanthamine. Peripheral cholinergic adverse effects for currently used acetylcholinesterase (AChE) inhibitors are common as well as other side effects such as hepatotoxicity, gastrointestinal disturbance and bioavailability problems [8, 9].

Numerous herbal extracts, containing several active constituents and often more than one plant species, have been used to treat CNS-related disorders [10,11]. From many of these species, AChE inhibitors have been isolated in recent years. Linarin, isolated from *Mentha arvensis,* exhibited twice the potency of galanthamine against AChE [12]. It was therefore considered of interest to investigate *Linaria reflexa* Desf. (Scrophulariaceae), a likely source of linarin-like flavonoids. This is one of 20 species of *Linaria* reported in Italy, while 200 species are distributed worldwide [13]. *Linaria* species have been used in folk medicine as laxative, spasmolitic, cholagogic and anti-inflammatory drugs [14] and have been the subject of recent biological studies [15].

Since a large amount of evidence demonstrated that oxidative stress was intimately involved in age-related neurodegenerative diseases, there have been a great number of studies which have examined the positive benefits of antioxidants such as flavonoids to reduce or to block neuronal death occurring in the pathophysiology of these disorders [16].

Table 1: Acetyl-cholinesterase inhibition effects of *L. reflexa* extracts and isolated flavones on TLC.

Extracts	AChE inhibition
MeOH	++
n-Hexane	++
AcOEt	+++
Flavones	
Linariin	++
Isolinariin A	++
Isolinariin B	++

+++ strong inhibitory effect; ++ clear inhibitory effect; Positive control: Physostigmine +++.

To evaluate the potential of this plant, a preliminary screening on TLC was performed using the methods first described by Rhee *et al.* [17,18] (Table 1). All extracts and isolated flavones exerted activity on the enzyme. In order to evaluate the IC_{50} value, an acetyl-cholinesterase inhibition assay based on Ellman's method was performed (Table 2). A dose-response relationship was observed for all extracts and isolated compounds (Figure 1a,b).

L. reflexa extracts are able to inhibit the AChE in a dose-dependent manner. The crude extract showed an IC_{50} of 241.0 μg/mL while the *n*-hexane extract had a value of 220.7 μg/mL. This extract was characterized by the presence of fatty acids (Table 3). It is of interest that some such compounds have recently
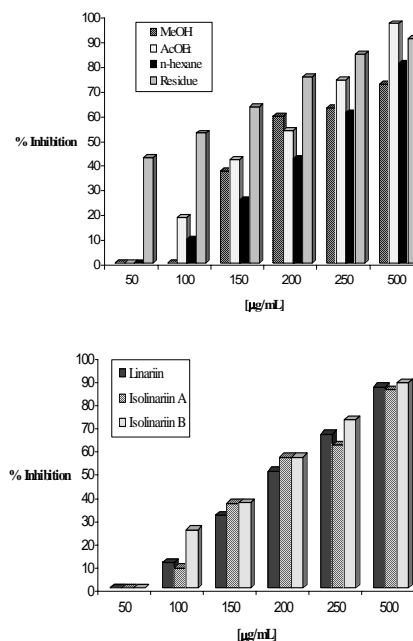

Figure 1: Dose-dependent inhibition of acetyl-cholinesterase by *L. reflexa* extracts and isolated flavones.

been shown to have relatively weak AChE inhibitory properties [19]. The most active extract was the EtOAc with an IC_{50} of 185.6 μg/mL. From this extract we have isolated three flavones, linariin and isolinariins A and B. IC_{50} values of 0.27 μM, 0.28 μM and 0.30 μM were found for isolinariin A and B and linariin, respectively.
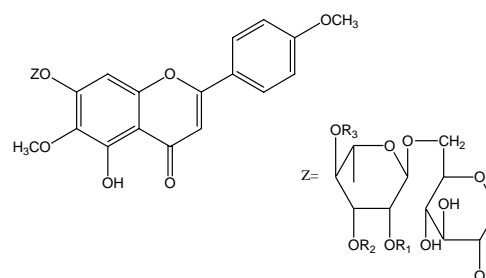
Flavones from *L. reflexa*	R	R₁	R₂
Linariin	H	H	Ac
Isolinariin A	Ac	H	H
Isolinariin B	H	Ac	H

Figure 2: Flavones from *Linaria reflexa*.

The IC_{50} values against AChE are comparable to the commercial drug physostigmine. Structurally, linariin has the same rhamnoglycoside moiety as linarin, but has an additional methoxyl group in position 6 in the aglycone. The presence of rhamnoglycoside in linarin has been shown to play a part in its interaction with the enzyme [12]. Apart from linarin, only a few flavonoids and derivatives have been reported to inhibit AChE including the flavanone naringenin,

which has been shown to mitigate amnesia *in vivo* [20] and some chalcones with IC_{50} values ranging from 28.2 to 134.5 μM) [21].

Table 2: Acetyl-cholinesterase inhibition activity (IC_{50}) of *L. reflexa* extracts and isolated flavones.

Extracts	IC_{50} (μg/mL)
MeOH	241.0± 1.6**
n-Hexane	220.7 ± 1.8**
AcOEt	185.6 ± 1.2**
Flavones	**IC_{50} (μM)**
Linariin	0.30 ± 0.05**
Isolinariin A	0.27 ± 0.02**
Isolinariin B	0.28 ± 0.03**

Physostigmine was used as positive control (IC_{50} 0.25 μM)).Data are given as the mean of at least three independent experiments ± S.D. Differences within and between groups were evaluated by one-way analysis of variance (ANOVA) test completed by a with a multicomparison Dunnett's test. **$p< 0.01$ compared with the positive control experiment.

Table 3: GC/MS analysis of *n*-Hexane extract from *L. reflexa*.

Identification	t_R^a
Myristic acid	17.15
6,10,14-trimethyl-2-Pentadecanone	17.83
Methyl palmitate	18.45
Palmitic acid	18.67
Methyl stearate	19.92
Stearic acid	20.22
Methyl linoleate	20.38
Methyl arachidate	20.98
Eicosane	21.25
9-Octadecyne	21.42
Tricosane	21.47
Tetracosane	21.73
Behenic acid	22.87
Pentacosane	22.37
Heptacosane	23.88
Nonacosane	25.96
α-Tocopherol	32.13
β-Sitosterol	34.51
γ-Tocopherol	34.59

[a] t_R: Retention times (min) on the non polar SE-30 column.

Flavonoids are reported to possess numerous biological activities, including antioxidant and anti-inflammatory properties [22]. These activities may contribute to the treatment of AD and other neurodegenerative diseases. In fact, with aging the brain may be vulnerable to oxidative stress, since it exhibits reduced free radical scavenging ability and utilizes high amounts of oxygen [23,24]. Recent studies have examined the permeability of flavonoids and their known circulating metabolites across the blood brain barrier (BBB). Flavonoids are hydrolysed in the gut and then glucuronised, so it is quite possible that a glycosidal form reaches the central nervous system (CNS) [25]. In particular, Youdim *et al*. 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 [26]. In conclusion this work provides evidence that linariin, isolinariin A and B obtained from *L. reflexa* can contribute to the treatment of neurodegenerative diseases such as Alzheimer by acting as pro-drugs.

Experimental

Plant material: Fresh aerial parts of *L. reflexa* were collected in Calabria (Italy) during February 2003 in their natural habitat and were taxonomically identified by Prof. G. Cesca of the Natural History Museum of Calabria and the Botanical Garden of Calabria University, Italy. Voucher specimen (P1001) was deposited in the Botany Department Herbarium at the University of Calabria (CLU), Italy.

Extraction protocol and separation: The extraction of active principles from *L. reflexa* was conducted according to our previous report [15]. Briefly, *L. reflexa* aerial parts (1 kg) were extracted with methanol. After evaporation of the solvent under vacuum, the crude extract (80.82 g) was dissolved in methanol; upon cooling, a solid precipitate (X) (16 g) and a methanol fraction were obtained. This precipitate was dissolved in H_2O, acidified with HCl (1 N) and extracted with EtOAc, to give a precipitate (Y) and an EtOAc soluble fraction. The EtOAc soluble fraction was subjected to column chromatography over silica gel 20–45 μm (CH_2Cl_2/MeOH 85:15), to afford linariin (1.97 g), and a fraction of 70 mg. This fraction was purified by HPLC [JASCO RP-18 250 x 20 mm i.d.; the elution solvents used were A (aqueous 0.01 M phosphoric acid) and B (100% MeOH) with the following gradient: 5% B as initial condition; 50% B for 10 min; 70% B for 5 min; 80% B for 5 min and finally 100% in B for 5 min] to afford isolinariin A (30 mg) and isolinariin B (35 mg). The precipitate (Y) was crystallized from MeOH to afford pectolinariin (2.50 g). The compounds were identified on the basis of their spectral data (UV, IR, MS, ^{1}H NMR, and ^{13}C NMR), identical with those previously described [15]. The methanol fraction was subsequently partitioned with *n*-hexane (2.88 g) (Figure 1).

Thin layer chromatography (TLC) with bioassay detection for AChE inhibition: The bioassay detection for AChE inhibition was evaluated by TLC [18,19]. A 2.5 mm silica gel plate F254 no. 5554 was used as a stationary phase. The separation was carried out using CHCl$_3$/MeOH 2.5%/AcOH (aq) (65:35:5) as eluent. The non-selective cholinesterase inhibitor, physostigmine (0.01 µg/band), was used as a positive control. A 5 µL sample of isolated compound dissolved in methanol (1 mg/mL) was applied to the plate. After the plate had been developed, it was dried at room temperature and then sprayed with 30 mM acetylthiocholine iodide (ATCI) followed by 20 mM dithionitrobenzene (DTNB). The plate was dried at room temperature for 45 min, then sprayed with 10.17 U/mL AChE. After 20 min, the plate was observed under visible light. A positive spot indicating an AChE inhibitor was colourless on the yellow background. The result was compared to that from the TLC analysis of the same sample after spraying with anisaldehyde reagent [27].

Bioassay for anti-cholinesterase activity: Inhibition of AChE was assessed by a modified colorimetric Ellman's method [28]. AChE 40 µL (0.36 U/mL in buffer pH 8) and isolated compounds (20 µL) were added to 2 ml of buffer pH 8 and pre-incubated in an ice bath at 4°C for 30 min. Duplicate tubes 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 ACh not due to AChE activity. The reaction was started by adding DNTB solution (20 µL of 0.05 mM in buffer pH 7) and ATCI (20 µL

0.018 mM in buffer pH 7) and the tubes were put 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 absorbance at 412 nm was measured spectrophotometrically (Perkin Elmer Lambda 40 UV/VIS spectrophotometer) and the percentage inhibition was calculated. Physostigmine was used as positive control. The inhibition rate (%) was calculated by the equation:

Inhibition % = [(Blank – Blank positive control) - (Experiment – Experiment control)]/ (Blank – Blank positive control)

Statistical analysis: All experiments were carried out in triplicate. Data were expressed as means ± S.D. Differences were evaluated by the one-way analysis of variance (ANOVA) test completed by a multicomparison Dunnett's test. Differences were considered significant at **$p < 0.01$. The inhibitory concentration 50% (IC$_{50}$) was calculated by a nonlinear regression curve with the use of Prism Graphpad Prism version 4.0 for Windows, GraphPad Software, San Diego, CA, USA (www.graphpad.com). The dose–response curve was obtained by plotting the percentage of inhibition versus the concentrations.

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