

Inhibition of Acetylcholinesterase by Extracts and Constituents from *Angelica archangelica* and *Geranium sylvaticum*

Steinthor Sigurdsson* and Sigmundur Gudbjarnason

Science Institute, University of Iceland, Vatnsmyrarvegur 16, IS-101 Reykjavik, Iceland.
Fax: +354 525 48 86. E-mail: sts@raunvis.hi.is

* Author for correspondence and reprint requests

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The aim of this study was to explore the acetylcholinesterase (AChE) inhibition of several Icelandic medicinal herbs. Ethanolic extracts of *Angelica archangelica* seeds and the aerial parts of *Geranium sylvaticum* proved effective, with IC_{50} values of 2.20 mg/ml and 3.56 mg/ml, respectively. The activity of imperatorin and xanthotoxin from *A. archangelica* was measured. Xanthotoxin proved much more potent than imperatorin, with an IC_{50} value of 155 μ g/ml (0.72 mM) but that for imperatorin was above 274 μ g/ml (1.01 mM). However, furanocoumarins seem to have a minor part in the total activity of this extract. Synergistic interaction was observed between the extracts of *A. archangelica* and *G. sylvaticum*. Several medicinal herbs (*Achillea millefolium*, *Filipendula ulmaria*, *Thymus praecox* and *Matricaria maritima*) did not show AChE inhibitory activity.

Key words: Acetylcholinesterase, *Angelica archangelica*, *Geranium sylvaticum*

Introduction

Alzheimer's disease (AD) is an irreversible and chronic neurodegenerative disorder afflicting more than 20 million people worldwide (Kamal *et al.*, 2006). It is the most common cause of dementia in the elderly (Ellis, 2005). Among its characteristics is the presence of extra-neuronal amyloid deposits and progressive deficits in several neurotransmitter systems, of which the cholinergic is affected earliest, associated with a decline in levels of acetylcholine (Kamal *et al.*, 2006). Since the discovery of cholinergic deficits in patients suffering from AD, acetylcholinesterase (AChE) has been the main target for the treatment of it (Hodges, 2006). The AChE inhibitors tacrine, donepezil, galanthamine and rivastigmine are currently available as drugs for general clinical treatment of AD (Akhondzadeh and Abbasi, 2006).

Angelica archangelica has been long and widely used in folk medicine, and it is one of the most respected medicinal herbs in Nordic countries, where it was cultivated during the Middle Ages and exported to other parts of Europe. The most characteristic secondary metabolites of its fruits are essential oils and furanocoumarins (Newall *et al.*, 1996).

Imperatorin (8-isopentenylloxypsoralen) is the major furanocoumarin of *A. archangelica* seeds

and has been reported to have an inhibitory effect on AChE from mouse brain (Kim *et al.*, 2002). Xanthotoxin (8-methoxypsoralen) is one of the major furanocoumarins in *A. archangelica*. It differs from imperatorin only by having a methoxy-group replacing the isopentenylloxy group at position 8. It has been reported to inhibit AChE from *Drosophila melanogaster* with an IC_{50} value of 125 μ g/ml, and it has been suggested that this inhibition is responsible for its insecticidal activity (Miyazawa *et al.*, 2004).

Geranium sylvaticum (woodland geranium) is widespread in Europe, but is seldom referred to as a medicinal herb in the recent literature. It has not been studied previously with respect to its acetylcholinesterase activity.

Synergy, that is the observation that a combination of compounds or extracts is more effective than can be expected from the activity of its components, has been documented for various phyto-medicines (Williamson, 2001). Synergistic effect on acetylcholinesterase has been suggested by alkaloids of Amaryllidaceae plants (Orhan and Sener, 2003). The isobole method is the method of choice for demonstrating synergy (Williamson, 2001) and was used in this study.

The purpose of this study was to explore the acetylcholinesterase inhibition of extracts from several medicinal herbs growing in Iceland.

Materials and Methods

Plant material and extracts

Achillea millefolium (yarrow, flowers and leaves separately), *Filipendula ulmaria* (meadowsweet), *Thymus praecox* (creeping thyme), *Matricaria maritima* (scentless chamomille), *Angelica archangelica* (seeds) and *Geranium sylvaticum* (woodland geranium) were collected in Iceland. The plant material was dried and stored at room temperature. Subsequently, it was extracted with 45% ethanol in water (v/v) for a minimum of 10 d.

Furanocoumarins

Imperatorin was precipitated from a 45% ethanolic extract of *A. archangelica*, growing in Iceland, by adding water, and recrystallized twice from diethyl ether until its purity was more than 95% (monitored by HPLC). Xanthotoxin was purchased from Aldrich. An *A. archangelica* seed extract with reduced furanocoumarin content was made by evaporating 100 ml of the extract on a rotary evaporator until 35 ml remained. The resulting concentrate was centrifuged for 5 min at $14,000 \times g$. The supernatant was collected and used in AChE inhibition assays.

Enzyme assay

The AChE assay was performed by the modified method of Ellman *et al.* (1961). Mouse brains, either fresh or stored at -20°C , were homogenized in 5 volumes of buffer [10 mM tris(hydroxymethyl)-aminoethane-HCl, pH 7.2, 1 M NaCl, 50 mM MgCl_2 and 1% triton X-100] and then centrifuged for 30 min at $14,000 \times g$. All extraction steps were carried out on ice or at 4°C . The enzyme extract was either subsequently used or stored at -70°C . The assays were carried out in 96-wells microtiter plates. 0.25 ml of sample was diluted with 1.21 ml buffer (50 mM sodium phosphate buffer, pH 8). 70 μl of the resulting mixture were added to 10 μl of enzyme solution and thoroughly mixed for 1 min at 37°C , before adding 10 μl of reagent mixture (3.5 mM acetylthiocholine and 7 mM dithiobisnitrobenzoic acid). The final concentrations of the reagents were thus 0.39 mM of acetylthiocholine and 0.78 mM of dithiobisnitrobenzoic acid. Upon adding the reagents, the solution was mixed and incubated at 37°C , and the absorbance at 412 nm was measured every 30 s for 5 min. Typically, the rate was linear between 1 and 3 min from the start of the reaction, and those

rates were used for subsequent calculations. Galanthamine was used as positive control, and had an IC_{50} value of 10.8 μM in this assay.

In the case of imperatorin and xanthotoxin, up to 2% triton X-100 was added to the buffer to enhance the poor solubility of the furanocoumarins, particularly of imperatorin. The effect of the added triton X-100 on the rate of the enzymatic reaction was negligible, but control samples were examined containing the same amount of the detergent.

HPLC

In order to establish the concentration of dissolved furanocoumarin in the assay, immediately upon completion of the enzymatic assays, the test solutions were filtered using 0.45 μm syringe filters (Schleicher & Schuell) and measured by HPLC, upon appropriate dilution. A HP-1100 instrument with a quaternary pump was used for the mobile phase. The column was from Pecosphere, contains a C18 stationary phase with a particle size of 3 μm (33×4.6 mm I. D.). Gradient elution was from 0–8 min from 40–60% MeOH in water, detection at 305 and 309 nm at room temperature, with a flow rate of 1.0 ml/min. The signal at 305 nm was monitored and compared to standard curves with known amounts of xanthotoxin. The standard curve was applied to other furanocoumarins by correcting for different molecular weights and the respective extinction coefficients at their maxima at about 300 nm (Lee and Soine, 1969).

Results

45% Ethanolic extracts

The 45% ethanolic extracts of *A. archangelica* and *G. sylvaticum* inhibited AChE dose-dependently, with IC_{50} values of 2.20 mg/ml and 3.56 mg/

Table I. IC_{50} values of the inhibition of AChE by extracts from *G. sylvaticum* and *A. archangelica* and by 5 mixtures of the extracts.

% <i>G. sylvaticum</i>	IC_{50} [mg/ml]		
	Total	<i>G. sylvaticum</i>	<i>A. archangelica</i>
0.00	2.20	0.00	2.20
37.79	2.03	0.77	1.26
47.68	2.02	0.96	1.06
64.57	2.10	1.36	0.74
78.47	2.04	1.60	0.44
84.54	2.22	1.88	0.34
100.00	3.56	3.56	0.00

ml, respectively (Table I). The 45% ethanolic extracts of the aerial parts of *A. millefolium*, *F. ulmaria*, *T. praecox* and *M. maritima* showed little inhibition (< 24%) at concentrations of 3.1 mg/ml and higher.

Supernatant of concentrated *A. archangelica* extract

The supernatant of the 45% ethanolic extract of *A. archangelica* seeds, which had been concentrated in a rotary evaporator and centrifuged, was assayed. 100 ml had been concentrated to 35 ml and the precipitating material removed. The sample was tested in quintuplicate at the concentration 2.88 mg/ml, and inhibited the enzyme by (51.0 ± 4.4)%.

Furanocoumarin content in enzyme assays

The furanocoumarin content in an enzyme assay with maximal content of *A. archangelica* seed extract was measured, after pooling the reaction mixtures from six identical wells (90 µl each). The total furanocoumarin content was 55.2 µg/ml, consisting of 23.3 µg/ml of imperatorin, 11.6 µg/ml xanthotoxin, 8.1 µg/ml isoimperatorin, 6.3 µg/ml isopimpinellin and 6.0 µg/ml bergapten. The furanocoumarin content of the assay with the supernatant of the evaporated extract was assayed and found to contain 3.8 µg/ml of xanthotoxin, whereas other furanocoumarins were not detectable.

Furanocoumarins

Imperatorin showed no significant inhibition activity on AChE at the maximal concentration measured, 274 µg/ml, i.e., $IC_{50} > 274 \mu\text{g/ml}$. The IC_{50} value of xanthotoxin was found to be 155 µg/ml.

Synergy of combined extracts

The extracts from *A. archangelica* and *G. sylvaticum* were mixed together in five different ratios and assayed. The dose-response curve of the mixture containing 84.54% of *G. sylvaticum*, and those of both extracts, are shown in Fig. 1. The IC_{50} values are shown in Table I. Fig. 2 shows the isobole curve of 50% inhibition of the extracts from *A. archangelica* and *G. sylvaticum*. The IC_{50} value of each component is shown on the axes, and the additional five points show the concentration of each

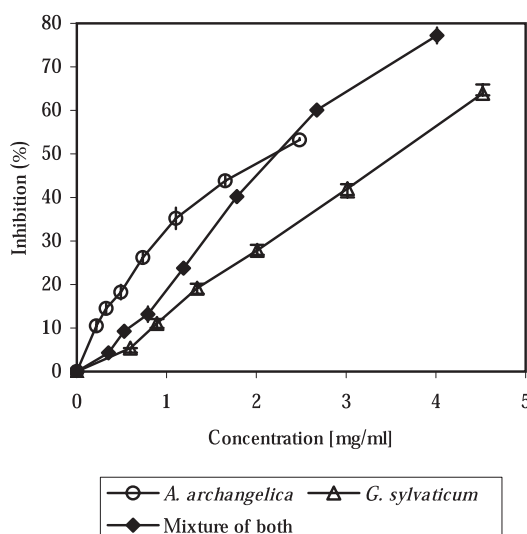


Fig. 1. Dose-dependent inhibition of AChE by the extracts from *A. archangelica* and *G. sylvaticum* and a mixture containing 84.54% *G. sylvaticum* extract and 15.46% *A. archangelica* extract. Each point represents the average of two separate measurements, each done in triplicate, and the standard error of the mean. The synergistic activity was prominent above 2 mg/ml.

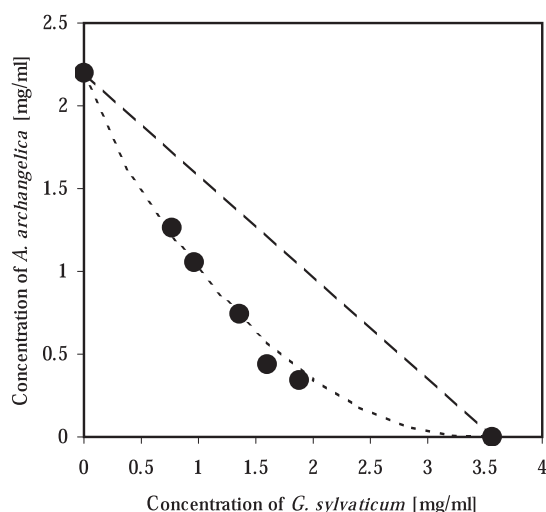


Fig. 2. Isobole curve for the concentrations of extracts from *G. sylvaticum* and *A. archangelica* at 50% inhibition of AChE. The points show the concentration of each component at IC_{50} for the individual extracts and for the five mixtures.

component at IC_{50} for different mixtures. If the components do not interact, the quantity of each component at IC_{50} of the mixtures should appear on the straight line between the IC_{50} values of the

components (Williamson, 2001). Fig. 2 shows, that in the mixtures the IC_{50} values appear under the straight line between the IC_{50} values of the components. This shows synergy, and that the effect is greater than expected from their individual dose-response curves. In all mixtures, the synergy is much more prominent in higher doses (above ca. 2 mg/ml) than in lower doses, as can be seen for the mixture containing 84.54% of *G. sylvaticum* and 15.46% of *A. archangelica* in Fig. 1.

Discussion

Acetylcholinesterase inhibition activity was demonstrated for extracts of *A. archangelica* and *G. sylvaticum*. Other plant materials tested, of *A. millefolium*, *F. ulmaria*, *T. praecox* and *M. maritima*, had no significant inhibitory activity.

AChE inhibition has previously been ascribed to furanocoumarins (Kim *et al.*, 2002; Miyazawa *et al.*, 2004). Therefore, it was safe to assume, that the inhibition of the *A. archangelica* seed extract was due to their presence. The two most abundant furanocoumarins in the reaction mixture when the extract was assayed were imperatorin and xanthotoxin.

Imperatorin is the most abundant furanocoumarin in the extract, comprising 40–50% of its total furanocoumarin content (results not shown). As it is very poorly soluble in water, 2% triton X-100 was added to the reaction buffer, after establishing that this had negligible effect on the enzymatic reaction, to enhance the solubility. Further, as it could not be assumed, that all of the added imperatorin was in solution, nor that the initially dissolved imperatorin would remain dissolved, the reaction mixture containing the highest quantity of imperatorin was filtered after the assay and measured by HPLC. The results showed, that though up to 274 $\mu\text{g/ml}$ (1.01 mM) was dissolved, imperatorin was without any significant effect on the enzymatic reaction in our test system. This is in contrast with a previous study (Kim *et al.*, 2002). However, in that study, the test system was different, with the sample being incubated with the enzyme for 30 minutes before the reaction buffer was added.

Xanthotoxin is a prominent furanocoumarin of the extract, and the second most abundant in the reaction mixture in the assay of the seed extract. It was treated in the same manner as imperatorin, although its solubility was less affected by the detergent. The IC_{50} value was established as 155 $\mu\text{g/}$

ml (0.72 mM). As could be anticipated, this is somewhat different from the value that has been established for AChE from *Drosophila melanogaster* (Miyazawa *et al.*, 2004), namely 0.58 mM (125 $\mu\text{g/ml}$).

The concentration of xanthotoxin in the seed extract reaction mixture at IC_{50} is less than 11.6 $\mu\text{g/ml}$. As the IC_{50} value of isolated xanthotoxin is 155 $\mu\text{g/ml}$, it is clear that other compounds in the extract explain most of the activity. The total concentration of furanocoumarins at IC_{50} is less than 55 $\mu\text{g/ml}$ – more than 40% of which is imperatorin. Thus, if furanocoumarins were the reason for the overall activity of the extract, the remaining furanocoumarins would have to be much more active than xanthotoxin.

Another experiment designed to answer the question if furanocoumarins were the main active components was to test the extract from which most of the furanocoumarins had been removed. The furanocoumarins in question have very limited solubility in water, and precipitate when the ethanol content is reduced considerably. When 65% of its volume was evaporated, most or all of the ethanol had been removed, and only a small proportion of xanthotoxin remained in the mixture. However, in a sample containing only 3.8 $\mu\text{g/ml}$ of furanocoumarins, all of which was xanthotoxin, in the assay about 50% inhibition was attained. Thus, it is clear that most of this activity can be ascribed to other compounds, unknown at present. Essential oils can be excluded as active components in this case, as they evaporate during concentration.

This is the first study that assesses the effect of *G. sylvaticum* on AChE. The active component or components are unknown at present. However, it provides some clues to their nature, that an extract with 96% ethanol was less active than the extract with 45% ethanol (results not shown).

Synergistic activity between the extracts of *A. archangelica* and *G. sylvaticum* was demonstrated by the isobole method. All tested mixtures had IC_{50} values similar to that of the *A. archangelica* extract, that is much lower than for the *G. sylvaticum* extract, even when the latter accounted for more than 84% of the resulting mixture. This effect was most pronounced in mixtures in which about 80% of the sample is derived from *G. sylvaticum*. The synergistic effect is mainly visible in doses at about 2 mg/ml or higher. At lower concentrations the dose-response curve lies between those of its constituents.

- Akhondzadeh S. and Abbasi S. H. (2006), Herbal medicine in the treatment of Alzheimer's disease. *Am. J. Alzheimers Dis. Other Demen.* **21**, 113–118.
- Ellis J. M. (2005), Cholinesterase inhibitors in the treatment of dementia. *J. Am. Osteopath. Assoc.* **105**, 145–158.
- Ellman G. L., Courtney K. D., Andres V., and Featherstone R. M. (1961), A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7**, 88–95.
- Hodges J. R. (2006), Alzheimer's centennial legacy: origins, landmarks and the current status of knowledge concerning cognitive aspects. *Brain* **129**, 2811–2822.
- Kamal M. A., Al-Jafari A. A., Yu Q. S., and Greig N. H. (2006), Kinetic analysis of the inhibition of human butyrylcholinesterase with cymserine. *Biochim. Biophys. Acta* **1760**, 200–206.
- Kim D. K., Lim J. P., Yang J. H., Eom D. O., Eun J. S., and Leem K. H. (2002), Acetylcholinesterase inhibitors from the roots of *Angelica dahurica*. *Arch. Pharm. Res.* **25**, 856–859.
- Lee K. H. and Soine T. O. (1969), X. Spectral studies on some linear furanocoumarins. *J. Pharm. Sci.* **58**, 681–683.
- Miyazawa M., Tsukamoto T., Anzai J., and Ishikawa Y. (2004), Insecticidal effect of phthalides and furanocoumarins from *Angelica acutiloba* against *Drosophila melanogaster*. *J. Agric. Food Chem.* **52**, 4401–4405.
- Newall C. A., Andersen L. A., and Phillipson J. D. (1996), *Herbal Medicines. A Guide for Health Care Professionals*. The Pharmaceutical Press, London.
- Orhan I. and Sener B. (2003), Bioactivity-directed fractionation of alkaloids from some Amaryllidaceae plants and their anticholinesterase activity. *Chem. Nat. Comp.* **39**, 383–386.
- Williamson E. M. (2001), Synergy and other interactions in phytomedicines. *Phytomedicine* **8**, 401–409.