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Relevant activities of extracts and constituents of animals used in traditional Chinese medicine for central nervous system effects associated with Alzheimer's disease

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Abstract

The centipede *Scolopendra subspinipes mutilans* L. Koch ('Wugong'), the beetle *Mylabris phalerata* Pallas ('Ban mao') and the earthworm *Pheretima aspergillum* Chen ('DiLong') have a reputation in traditional Chinese medicine for reducing symptoms of central nervous system decline, including memory loss. A series of extracts of all three organisms was tested for acetylcholinesterase (AChE) inhibition and copper ion binding effects, the latter likely to reduce oxidative damage caused by excess copper. The beetle and centipede chloroform extracts showed the strongest AChE inhibitory effects (30.6% inhibition at 105 μg mL^{-1} and 32.3% inhibition at 167 μg mL^{-1}, respectively) and, in the case of the centipede, this was traced to the unsaturated fatty acids present using bioassay-guided fractionation. Cantharidin from the beetle was shown to have AChE activity (31% inhibition at 1 μM, 0.196 μg mL^{-1}), making it a major contributor to the activity of the beetle extract. The earthworm showed no AChE inhibitory activity. Since unsaturated fatty acids have not been previously reported to have AChE inhibitory activity, a series of related compounds was tested to determine structure–activity relationships. It was found that activity existed where there was a chain length of more than 16 C atoms with at least one unsaturated bond in the chain. The carboxylic acid group was also necessary for activity. The fatty acids present in the centipede also showed the ability to bind copper ions when tested using a novel thin layer chromatography method designed to detect copper-binding compounds. The activities reported give some support to the use of the beetle and centipede in traditional Chinese medicine for improving cognitive function.

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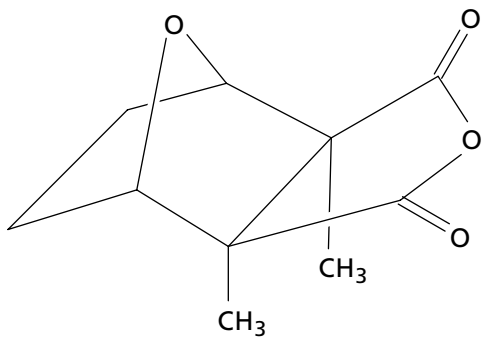
Introduction

The archetypal cholinesterase inhibitor is physostigmine from the West African ordeal poison Calabar bean *Physostigma venenosum*, which was discovered over 100 years ago. The application of naturally occurring acetylcholinesterase (AChE) inhibitors, especially galantamine, in the symptomatic relief of the early stages of Alzheimer's disease (AD) has generated renewed interest in AChE inhibitors from the plant kingdom, especially those plants used in traditional medicine for improvement of memory and other cognitive functions in old age (Howes & Houghton 2003; Howes et al 2003).

As part of our investigations into such products, our attention was drawn to some materials used in traditional Chinese medicine that were derived from animals rather than plants. These were recommended for mental alertness in old age by traditional Chinese medicine practitioners in the Peoples' Republic of China, canvassed by one of our group, Yuhao Ren. The animals in question were the beetle *Mylabris phalerata* Pallas ('Ban mao'), the centipede *Scolopendra subspinipes mutilans* L. Koch ('Wugong'), and the earthworm *Pheretima aspergillum* Chen ('DiLong'). There are many descriptions in traditional Chinese medicine of the dried body of *M. phalerata* having a reputation for improvement of heart and brain function. *M. phalerata* is known as the 'blister beetle' since the powdered insect produces blistering on contact with human skin. The blistering agent is due to the bicyclic terpenoid



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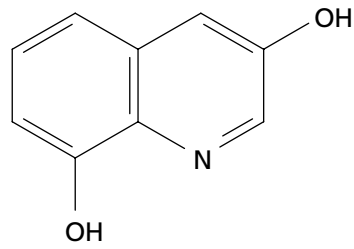

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Figure 1 Structure of cantharidin (**1**) and 3,8 dihydroxyquinoline (**2**).

cantharidin (Figure 1), which is found in all stages of the insect and is thought to play a role as a defensive substance. It has been shown to inhibit phosphatases 1 and 2A (McCluskey et al 2000), and thus affects cellular signal transduction, moderating such diverse functions as neurotransmission, T-cell activation and other processes (Knapp et al 1998).

The dried body of *S. subspinipes mutilans* is prescribed in traditional Chinese medicine to relieve cognitive dysfunction, as well as for other conditions (Noda et al 2001). Quinolines with a structure typical of a compound that might bind iron or copper, for example 3,8 dihydroxyquinoline (Figure 1), have been isolated from this insect (Noda et al 2001) but no work has been performed to determine if they have such a binding effect.

Various species of *Pheretima* are used in China for the prevention and treatment of high blood pressure and dementia in the elderly (Wang 2004). *P. aspergillum* Chen is the most common species and was used in the present study. Although macromolecular components have been isolated from *Pheretima* spp., no small molecules have been isolated and no tests for biological activity have been carried out.

Decline in cognitive function is commonly associated with old age and is particularly severe in the group of neurodegenerative conditions, the most common being AD. The aetiology of AD and similar conditions is still not known but there is increasing evidence that damage to the neurons caused by oxygen free radicals and inflammatory processes are causative factors. Depletion of cholinergic neurons and of acetylcholine (ACh) levels, and excess levels of some metal ions, notably copper, have also been noted in some AD sufferers post mortem (Lovell et al 1998).

The treatment of symptoms of AD with drugs is still a comparatively new development and, until recently, has relied very much on the use of cholinesterase inhibitors as a means by which the low levels of ACh, characteristic of AD, may be increased (Perry et al 1978; Giacobini 1990). It should be noted that the only other therapeutic strategy has focused on glutamate-mediated neurotoxicity in AD, with the introduction of memantine, an *N*-methyl-D-aspartate receptor antagonist.

Inhibition of AChE, the enzyme principally involved in the catabolism of ACh, results in increased levels of ACh in the central nervous system such that the deficiency associated with AD is reversed. In-vitro and in-situ colorimetric and thin layer chromatography (TLC) tests have been developed for AChE inhibition (Perry et al 2000; Rhee et al 2001, 2003; Marston et al 2002) and these have facilitated the bioassay-guided isolation of quite a large number of naturally occurring AChE inhibitors in recent years (Houghton & Howes 2005). Although the strongest activity is seen with alkaloidal compounds, a large number of plant-derived non-nitrogenous compounds has been discovered, and it is thought that these have a different inhibitory mechanism from the alkaloids. Few AChE inhibitors have been discovered in animals, but fasciculin, a peptide found in the venom of the green mamba snake, is a potent AChE inhibitor (Bui et al 2004).

In addition to AChE inhibition, other in-vitro tests relevant to factors associated with incidence, or reduced incidence, of AD have been utilized, and these include the formation of pro-inflammatory compounds (such as the eicosanoids), antioxidant properties, nicotinergic effects and the effect of agonist binding to β-oestrogen receptors (Perry et al 2001). The postulated epidemiological links between women on oestrogen replacement therapy and a decreased incidence of AD have since been discredited and so the oestrogen receptor binding effect is now discredited.

In recent years, damage due to an excess of reactive oxygen species (ROS) has been postulated to underlie the aetiology of a large number of diseases, including neurodegenerative conditions such as AD (Deschamps et al 2001). Naturally occurring antioxidants form part of the diet and have been extensively researched for their possible preventive effects. Several in-vitro tests have been developed for various aspects of antioxidant activity, including the prevention of the formation of ROS and scavenging of ROS before they are able to adversely affect membranes, enzymes or DNA. Formation of ROS is linked with inflammation and, to some extent, ROS formation occurs in the presence of large amounts of metal ions, especially Cu^{2+}.

Excess metal ions can be removed by chelators or copper-binding compounds, as long as the chelator is able to penetrate the blood–brain barrier. Tests for the ability to bind metal ions have tended to rely on colorimetric spectrophotometry, but in this study we describe an approach involving detection on TLC plates.

This study describes the testing of extracts of beetle, centipede and earthworm species for AChE inhibition and copper ion binding effects, and subsequent structure–activity studies on AChE inhibition by long-chain fatty acids.

Materials and Methods

The beetle, centipede and earthworm species were purchased in the dried state from Heping Clinic, Zhengzhou city, Henan, China. They were confirmed to be the correct species by staff at Nanjing University of Traditional Chinese Medicine, Nanjing, China. Voucher specimens are stored in the museum of the pharmacognosy group, King's College London, and the voucher numbers assigned were *M. phalerata* B2004a, *S. subspinipes mutilans* Wg2001.7.6S and *P. aspergillum* Dl2001.6.8A.

Extraction

Extraction of approximately 25 g (accurately weighed) of the ground dried body of each animal species was performed using a Soxhlet apparatus with 330 mL hexane, 330 mL chloroform and 330 mL 96% ethanol sequentially for 5 h each time to achieve extraction of constituents with a wide range of polarity. Each extract was filtered and evaporated by reduced pressure using a rotary evaporator to give percentage yield as shown in Table 1. The residue from each extraction was redissolved with acetone to give a series of concentrations ranging from 50 $\mu g\,mL^{-1}$ to 500 $\mu g\,mL^{-1}$. An aqueous extract was obtained by refluxing the residue remaining from the sequential solvent extractions with distilled water for 2 h, filtering, freeze-drying and dissolving the residue in water.

Enzyme assay

The phosphate buffer was made up by mixing appropriate volumes of Solution A ($Na_2HPO_4.7H_2O$, 28.4 $g\,L^{-1}$) and Solution B (NaH_2PO_4, 27.4 $g\,L^{-1}$), both stored at 4°C.

For buffer solution pH 8.0, distilled water (500 mL) and Solution A (470 mL) were mixed and then Solution B was added until pH 8.0 was reached. The mixture was stored at 4°C and fresh solutions were prepared each week. Buffer solution pH 7.0 was made up and stored in the same way.

Physostigmine hemisulfate solution (0.018 mM; 15 mg dissolved in 2.5 mL distilled water) was made fresh every day and stored in an ice bath before use.

The 0.06-mM acetylthiocholine iodide solution was also made fresh every day.

For the 0.05-mM DTNB solution, 40 mg sodium bicarbonate was dissolved in 5 mL phosphate buffer pH 7.0 and then 100 mg 5,5-dithio-bis-(2-nitrobenzoic acid) was added.

AChE solution (0.86 $U\,mL^{-1}$) consisted of 12 mg bovine erythrocyte AChE (EC3.1.1.7) (0.36 units mg^{-1}) dissolved in 5 mL phosphate buffer pH 8.0. The fresh solution was stored on ice before use and fresh solutions were prepared for every independent experiment.

The stock solutions of extracts or fractions were prepared in acetone and diluted with 100 mM phosphate buffer pH 8.0, so that the final amount of acetone in the

Table 1 Yield of extracts from the animal species used and inhibition of bovine erythrocyte acetylcholinesterase

Animals	Solvent extract	Percentage yield w/w	Maximum concentration tested ($\mu g\,mL^{-1}$)	Inhibition (%)
Centipede	Hexane	5.3	103	30.6 ± 5.8
Scolopendra	Chloroform	5.3	167	32.3 ± 4.3
subspinipes mutilans	Ethanol	4.1	256	44.7 ± 5.6*
	Water	2.5	274	0 ± 2.6
Beetle	Hexane	5.2	96	29.8 ± 6.8
Mylabris phalerata	Chloroform	4.7	105	30.6 ± 5.4
	Ethanol	3.9	212	49.6 ± 4.9*
	Water	2.8	282	0 ± 3.4
Earthworm	Hexane	4.7	101	29.6 ± 4.3
Pheretima aspergillum	Chloroform	5.1	106	28.7 ± 5.9
	Ethanol	4.7	211	27.4 ± 5.1
	Water	2.9	172	0 ± 2.4
Positive control	Physostigmine	–	0.07	50 ± 1.8

Results are expressed as the mean ± s.d. of three different experiments. *Significantly greater inhibition compared with the hexane, chloroform and water extracts of the same animal species ($P < 0.05$, Dunn's test). The extracts of the centipede and beetle were significantly more active than those of the earthworm ($P < 0.05$ Kruskal–Wallis test).

assay was less than 1%. This amount of acetone was shown to have no effect on the activity of AChE (results not shown).

The modified Ellman method was used to assess AChE inhibition using published procedures (Perry et al 2000). The production of the yellow anion was recorded for 10 min on a Shimadzu UV/VIS 2101 double beam spectrophotometer at 412 nm at 20°C. A positive control was set up by adding physostigmine (20 μL of 0.018 mM buffer pH 7.0) at the start of the experiment in order to control the non-enzyme hydrolysis of ATC. A blank comprising reagents without extract was used. A blank positive control was set up, which was the same as the blank except physostigmine (20 μL of 0.018 mM buffer pH 7.0) was added. The inhibition rate (%) was calculated by the equation:

$$\begin{aligned}\text{inhibition}\% = &((\text{blank} - \text{blank positive control})\\ &- (\text{experiment} - \text{experiment control}))/\\ &(\text{blank} - \text{blank positive control})\end{aligned}$$

Results were calculated as the mean ± s.d. of three different experiments (duplicate tube for each experiment).

This inhibitory assay was carried out on the extracts and their fractions on a series of fatty acids and with linoleic acid in the absence and presence of 0.5% Triton X.

All tests were carried out in triplicate and values are expressed as means ± s.d. For pure compounds, a series of concentrations was tested and the percentage inhibition plotted against concentration. The program Graphpad Prism 2.0 was used to calculate the IC50 values.

Copper-binding experiment

Crotonic acid, 8-hydroxyquinoline, boron trifluoride, ethylenediaminetetraacetic acid (EDTA), polyoxyethylene (10) iso-octyl phenyl ether (Triton X-100) were purchased from Fisher Scientific, Loughborough, UK. Myristoleic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, gamma-linolenic acid, linoleic acid, arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid and methyl linolenate were purchased from Sigma, Poole, Dorset, UK.

Development of TLC assay

Many metal ions can form chelates with suitable donor molecules. The metal–chelate complex usually extends the absorption wavelength compared with the metal ions on their own, and this was exploited in designing a detection spray containing Cu^{2+} ions for compounds likely to chelate with copper. The known copper chelators 8-hydroxyquinoline and EDTA (1.5 mg mL^{-1}) were used as positive controls.

Each sample (2.5 μL) was spotted on the silica gel TLC plate. Extracts and compounds were analysed by TLC using an appropriate mobile phase to give discreet zones, then sprayed with a solution of 40 mg $CuSO_4 \cdot 5H_2O$ dissolved in 2.5 mL distilled water and 2.5 mL methanol and examined in daylight after spraying.

Fractionation of extraction of the centipede *S. subspinipes mutilans*

Dried and powdered *S. subspinipes mutilans* (280 g) was extracted with 95% MeOH, filtered and evaporated under reduced pressure to give an extract (65 g). Treatment with acetone gave two portions, A and B, for acetone soluble and acetone insoluble, respectively (A 34.0 g and B 23.1 g, after removal of solvent under reduced pressure). Since A showed chelating zones on the TLC system, it was fractionated by silica gel vacuum liquid chromatography (gradient elution using 50-mL aliquots of toluene 100%, dichloromethane, ethyl acetate:methanol 2:1) to yield fractions F1–F3, eluents combined after TLC examination of fractions. Fractions were tested on the TLC system silica gel/chloroform:methanol 19:1, but only the toluene fraction (9.5 g) showed chelating zones. F1 was subjected to normal phase column chromatography on silica gel using dichloromethane and fractions examined by TLC (silica gel/dichloromethane:methanol 9:1 spraying with acidic anisaldehyde). Four sub-fractions were obtained, F1A (1.6 g), F1B (554 mg), F1C (557 mg) and F1D (548 mg), but only F1A showed activity. F1A appeared to be oily and could not be further fractionated by silica chromatography. It was suspected to contain fatty acids and so was analysed by gas chromatography/mass spectrometry (GC-MS).

GC-MS analysis of F1A

The methyl esters of the fatty acids present were obtained by dissolving 205.4 mg F1A in 5 mL chloroform and 1 mL of this solution was treated with 1 mL 14% boron trifluoride in MeOH and the mixture was held in a 100°C water bath for 30 min. Samples (40 mg) of palmitic acid, stearic acid and oleic acid were treated in the same way to serve as reference solutions.

The mixture was analysed by GC-MS using a Fisons MD800 mass (quadrupole) GC8000 series instrument equipped with a flame ionization detector. MS scanning: m/z 40–650. Column capillary (30 m × 0.25 mm i.d.) coated with OV1 (SGE Ltd, Milton Keynes, UK). Carrier gas: helium (20 psi) at a flow rate of 0.5 mL min^{-1}. Column temperature: initially 40°C for 4 min, then increasing to 280°C (8°C min^{-1}) and holding at 280°C for 20 min. Injector and detector temperatures: 270°C and 250°C, respectively. Injection: 0.5 μL.

Since the F1 fraction rich in fatty acids showed some inhibition of AChE, a series of fatty acids was tested for this activity.

Statistical analysis

Statistical analysis of the % inhibition of AChE shown by the different solvent extracts for each animal species was carried out using the Kruskal–Wallis test (SPSS 9.0 for Windows; SPSS Inc. Chicago, IL, USA). Individual differences between the various

extracts were then examined using Dunn's test (SPSS 9.0 for Windows). Statistical analysis of the % inhibition of AChE shown by the different fatty acids was carried out using the Kruskal–Wallis test. Individual differences between the various acids were then examined using Dunn's test. Comparison of the effects of fatty acid alone, fatty acid plus Triton X and positive control was statistically analysed using the Kruskal–Wallis test. Individual differences were then compared using Dunn's test. All determinations were carried out in triplicate and in each experiment $P < 0.05$ was considered as significant.

Results and Discussion

The yield of extracts of *S. subspinipes mutilans*, *P. aspergillum* and *M. phalerata* and percentage inhibition of bovine erythrocyte AChE are shown in Table 1.

Moderate AChE inhibition was seen for the *M. phalerata* and *S. subspinipes* chloroform and ethanol extracts. The ethanol extracts appeared to have greater activity ($P < 0.05$), although this was at a higher concentration than the chloroform extracts, which may in fact be more active. More detailed studies would have to be carried out to determine if this is the case. A shortage of material and the hazards involved precluded bioassay-guided fractionation of the beetle *M. phalerata*, but its major terpenoidal constituent, cantharidin, was tested and found to exhibit weak activity ($31.2 \pm 3.4\%$ at $1\,\mu$M, i.e. $0.2\,\mu g\,mL^{-1}$). Although the concentration of cantharidin in the beetle sample was not measured in this study, it is generally accepted to contain less than 1% cantharidin (Pharmacopeia of the Peoples' Republic of China 1992). Assuming the chloroform extracted all the cantharidin present, the $105\,\mu g\,mL^{-1}$ dose of the chloroform extract would contain no more than $1.05\,\mu g\,mL^{-1}$ cantharidin and had a similar inhibition of 30.6%, so it is likely that cantharidin is the major contributor to the AChE inhibition.

Chelating activity

EDTA gave a dark blue zone after spraying, while 8-hydroxyquinoline gave an orange/brown zone. In the case of the dichloromethane extract of *S. subspinipes mutilans*, the system silica gel G(Merck)/methanol was used and the extract showed a band with a high Rf value giving an intense blue colour, thus indicating chelating effects; the extract of *M. phalerata* showed no such zones.

The GC-MS experiments showed that F1A, obtained from the *S. subspinipes mutilans* extract, was a mixture of palmitic acid, oleic acid and stearic acid in an approximate ratio of 10:3:2. The individual fatty acids were shown to give a reaction characteristic of chelation when tested in the TLC system. Since they only contain one functional group likely to bond to copper, they are not true chelators but copper-binding molecules, which are nevertheless likely to have a protective effect similar to true chelators.

There are increasing reports that fatty acids are linked to AD. Since the fatty acids were found to contribute to the copper-binding activity of extracts of *S. subspinipes mutilans* and *M. phalerata*, we were interested in investigating the effect of these fatty acids on AChE.

AChE inhibitory activity of fatty acids

Fraction F1A had a high concentration of fatty acids and also showed some inhibition of AChE. When the dichloromethane extract of the centipede *S. subspinipes mutilans* was examined by TLC, it was shown to contain the same zone corresponding to the fatty acids, and so it was considered of interest to test the pure fatty acids for AChE inhibitory activity. The results are shown in Table 2. It can be seen that unsaturated fatty acids have a relatively moderate inhibitory activity on AChE, whereas saturated fatty acids and the esters of unsaturated fatty acids do not have this type of activity (Table 2). To our knowledge, this is the first time that this has been reported. Even among the unsaturated fatty acids, there was no inhibitory activity when the chain length was less than 16 C atoms, as evidenced by the lack of inhibition seen with crotonic and myristoleic acids. The strongest inhibitor among the fatty acids was docosahexaenoic acid, which was about 200-fold less potent than physostigmine.

The double bond, present in all active compounds, may have some interaction with the indole ring of AChE, since the double bond of galanthamine stacks with the indole ring of the enzyme (Greenblatt et al 1999). Since only fatty acids with more than 16 C atoms have an inhibitory effect on AChE, it may be that the enzyme has two ligand binding sites for these compounds, one being a peripheral site and the other the active site, at the bottom of the gorge, where interaction with the indole ring of tryptophan 84 occurs (Sussman et al 1991). It is noteworthy that methyl linolenate did not possess any activity, whereas linoleic acid showed inhibitory activity (Table 2), suggesting that the acid moiety is another important functional group for AChE inhibition and may form a hydrogen bond with the glutamic acid group of the catalysis centre, similar to the way in which the SH of PEG-SH interacts with AChE (Koellner et al 2002). If the fatty acids interact with the two sites of AChE, the chain for fatty acids may need to be long enough to dock AChE in the two different sites, one at the tryptophan and the 334 glutamic acid in the active site and the other at the lipophilic peripheral site. It is known that the strength of inhibition of other enzymes by fatty acids is related to their chain length and degree of unsaturation (Chen et al 2002).

Hydrophobic binding of long-chain fatty acids may be disrupted if the surface active agent Triton X-100 is also present in the system, as demonstrated by a reduction in the activity of territrem B (Chen et al 1999). Figure 2

Table 2 Inhibitory activity (IC50 μM ± s.d., n = 3) of fatty acids on bovine erythrocyte acetylcholinesterase

Chemical name	Trivial name	Shorthand designation	IC50
3-Methylacrylic	Crotonic acid	4:0	> 1000
cis-9-Tetradecenoic	Myristoleic acid	14:1 (ω-5)	> 1000
Hexadecanoic	Palmitic acid	16:0	> 1000
cis-9-Hexadecenoic	Palmitoleic acid	16:1 (ω-7)	112.9 ± 17.5^{a}
Octadecanoic	Stearic acid	18:0	> 1000
cis-9-Octadecenoic	Oleic acid	18:1 (ω-9)	85.7 ± 4.3^{d}
9,12-Octadecadienoic	Linoleic acid	18:2 (ω-6)	59.9 ± 4.2^{bde}
9,12,15-Octadecatrienoic	Linolenic acid	18:3 (ω-3)	163 ± 12.7^{d}
6,9,12-Octadecatrienoic	Gamma-linolenic acid	18:3 (ω-6)	234 ± 84.6^{d}
5,8,11,14-Eicosatetraenoic	Arachidonic acid	20:4 (ω-6)	373 ± 28.2
5,8,11,14,17-Eicosapentaenoic	Eicosapentaenoic acid	20:5 (ω-3)	143 ± 13.58
4,7,10,13,16,19-Docosahexaenoic	Docosahexaenoic acid	20:6 (ω-3)	52.7 ± 4.97^{c}
Methyl *cis*, *cis*-9,12-octadecadienoic	Methyl linoleate	18:2 (ω-6)	> 1000
Ethanedioic	Oxalic acid	2:0	> 1000
Dodecanoic	Lauric acid	12:0	> 1000
Positive control	Physostigmine		0.25

[a]Significantly greater % inhibition ($P < 0.05$, Dunn's test) compared with palmitic acid; [b]significantly greater % inhibition ($P < 0.05$, Dunn's test) compared with oleic acid, linolenic acid and gamma-linolenic acid; [c]significantly greater % inhibition ($P < 0.05$, Dunn's test) compared with arachidonic acid and eicosapentaenoic acid; [d]significantly greater % inhibition ($P < 0.05$, Dunn's test) compared with stearic acid; [e]significantly greater % inhibition ($P < 0.05$, Dunn's test) compared with methyl linoleate. The individual IC50 values for crotonic acid, myristoleic acid, palmitic acid, stearic acid, palmitoleic acid, oleic acid, linoleic acid, linolenic acid, gamma-linolenic acid, arachidonic acid, methyl linoleate, oxalic acid, oxalic acid, lauric acid and eicosapentaenoic acid on acetylcholinesterase were significantly different from each other ($P < 0.05$, Kruskal–Wallis test).

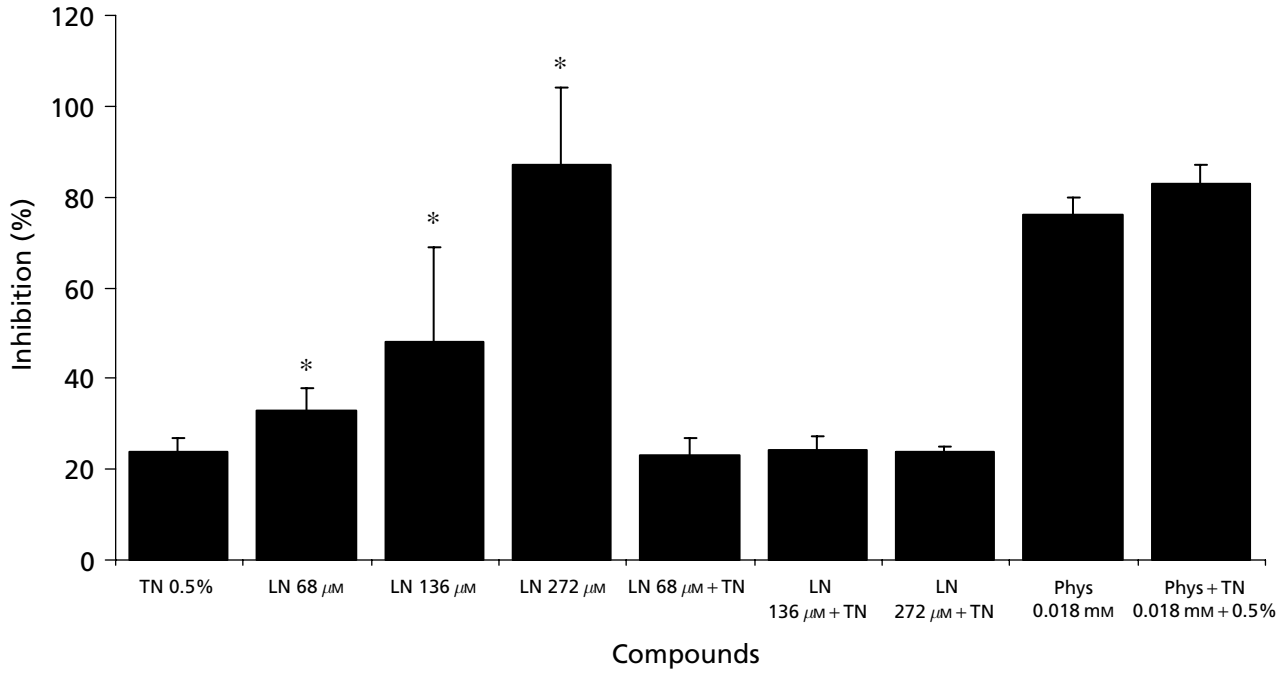

Figure 2 Acetylcholinesterase inhibition owing to linoleic acid in the absence and presence of Triton X-100. Results are calculated as the mean ± s.d. of three independent experiments (duplicate tube for each experiment). TN, Triton X-100; LN, linoleic acid; Phys, physostigmine. The % inhibition values of TN 0.5%, LN (19, 38 and 76 $\mu g\,mL^{-1}$), TN + LN (19, 38 and 76 $\mu g\,mL^{-1}$), Phys and Phys + TN were significantly different ($P < 0.05$, Kruskal–Wallis test). *Significantly different compared with the corresponding TN + LN ($P < 0.05$, Dunn's test).

shows that a significant dose-dependent inhibition of AChE for linoleic acid was observed. In contrast, when 0.5% Triton X-100 was present in the assay, linoleic acid did not show this dose-dependence and the inhibitory activity was significantly reduced. The inhibitory activity of physostigmine on AChE was almost unchanged in presence of Triton X-100. This indicates that physostigmine has different inhibitory activity compared with linoleic acid and that the fatty acid does not bind primarily at the active site at the base of the gorge in the enzyme. It is possible that the detergent Triton X-100 disturbed the hydrophobic interaction between linoleic acid and AChE that causes the inhibition.

There is growing evidence that essential fatty acid deficiency is an important risk factor for AD. Polyunsaturated fatty acids are primary components in the brain and are related to membrane integrity and fluidity. As humans cannot synthesize them, they must be obtained through the diet as essential fatty acids, which are derived from linolenic, linoleic and oleic acids. Reduced brain levels of long-chain polyunsaturated fatty acids such as arachidonic acid and docosahexaenoic acid are observed in elderly patients with AD (Farooqui & Horrocks 2001). The ratio between saturated fatty acids and unsaturated fatty acids, particularly, polyunsaturated fatty acids, influences various aspects of neurotransmission. High docosahexanoic acid consumption is associated with reduced AD risk in mouse models (Calon et al 2004), and docosahexanoic acid was shown to provide protection from impairment of learning ability in AD model rats (Hashimoto et al 2002). In addition, studies indicated that a high dietary intake of n-3 fatty acids and weekly consumption of fish can reduce the risk of incident AD in humans (Morris et al 2003), and an association between fish consumption and a low prevalence of AD has been noted (Kalmijn et al 1997). Some studies have also shown that docosahexaenoic acid gives protection against AD, related with the increase of transthyretin, an amyloid beta protein scavenger (Puskas et al 2003).

Two of the organisms tested, the beetle and the centipede, showed AChE inhibitory activity and, in the case of *S. subspinipes mutilans*, a copper-binding effect also, indicating antioxidant activity. Both of these activities would be useful in the prevention or symptomatic relief of AD and could lend some support to their traditional use for treating age-related cognitive dysfunction in old age. However, the need for caution in making such extrapolations should be stressed, since the activities are quite low and a considerable amount of either animal would need to be taken if in-vivo doses were calculated from the in-vitro results discussed here. In addition, the beetle has unpleasant blistering effects that prevent its use by anyone other than a skilled practitioner. In spite of these negative aspects, these findings are of interest from an ethnopharmacological point of view.

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