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*Short Communication*

# Acetylcholinesterase inhibitory activity of plants used as memory-enhancers in traditional South African medicine

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**In traditional South African medicine there are a few plants used to improve memory and to treat Alzheimer's disease. Aqueous and ethanol extracts of five of these plants: *Malva parviflora* (leaves), *Boophane disticha* (leaves and bulbs), *Albizia adianthifolia* (stem bark), *Albizia suluensis* (root bark) *and Crinum moorei* (bulbs) were investigated. They were screened for acetylcholinesterase (AChE) inhibiting activity using thin-layer chromatography (TLC) and microtitre plate assays. Inhibition of AChE is an important approach in treating Alzheimer's disease. Promising results were obtained with bulbs of *B. disticha* and *C. moorei*. The aqueous and ethanol extracts of *B. disticha* (0.1mg ml$^{-1}$) yielded 38% and 27% inhibition in the microplate assay, respectively, while the ethanol extract of *C. moorei* had good dose-dependent inhibiting activity with 67% inhibition at the highest concentration. Aqueous and ethanol extracts of *C. moorei* and *B. disticha* showed AChE inhibiting activity in the TLC assay.**

Alzheimer's Disease (AD) is one of the most important diseases of cognitive dysfunction in the elderly (Shetty and Woodhouse 1999). AD has a gradual onset, and it progresses slowly. The major initial symptom is forgetfulness. In the Alzheimer-afflicted brain, the cells that use acetylcholine (ACh) are damaged or destroyed, resulting in lower levels of the neurotransmitter. Based on the cholinergic hypothesis that memory impairments in patients suffering from AD result from a defect in the cholinergic system, an important approach to treat this disease is to enhance the ACh level in the brain by inhibiting acetylcholinesterase (AChE).

Plants species traditionally used to increase memory and treat Alzheimer's disease, were selected based on information in a database, constructed at the University of KwaZulu-Natal. The database is mainly based on published literature (Watt and Breyer-Brandwijk 1962, Mabogo 1990, Hutchings *et al.* 1996, Van Wyk *et al.* 1997, Sobiecki 2002). The plants investigated are listed in Table 1. Plants were collected in KwaZulu-Natal and Lesotho (*A. adianthifolia*, *B. disticha* and *M. parviflora* during January 2002, *A. suluensis* and *C. moorei* in March–April 2003). Voucher specimens are housed in the University of Natal Herbarium. Two assays were use to detect and quantify AChE activity, Thin-Layer Chromatography (TLC) bioautographic assay and microtitre plate assays. Both assays utilise the fact that AChE cleaves Acetylthiocholine Codide (ATCI) to give thiocholine, which reacts with 5,5´-dithiobis-(2-nitrobenzoic acid) (DTNB) and produces a yellow product (5-thio-2-nitrobenzoate).

One gram of dried powdered plant material was extracted with 10ml water or ethanol for 1h in an ultra-sound bath. The extracts were filtered and the solvent was removed under vacuum. The residues from the aqueous and ethanol extracts were dissolved in water and methanol respectively to give a final concentration 10mg ml$^{-1}$. Thereafter 5µl and 10µl of each sample and 5µl of 0.1mM galanthamine hydrobromide (Sigma) in methanol were spotted on a Merck Silica gel 60 $F_{254}$ TLC plate (0.2mm) and developed in chloroform:methanol 8:2. After developing the TLC plate, enzyme inhibitory activity of the developed spots was detected using Ellman's method (Kiely *et al.* 1991, Rhee *et al.* 2001). The TLC plate was sprayed with 5mM acetylthiocholine iodide (ATCI) and 5mM 5,5´-dithiobis-(2-nitrobenzoic acid) (DTNB) in 50mM Tris-HCl, pH 8 until the silica was saturated with the solvent. It was allowed to dry horizontally for 3–5min and then sprayed with 3U ml$^{-1}$ acetylcholinesterase (AChE) from electric eel (type VI-s, lyophilised powder, 317U mg$^{-1}$ solid, 495U mg$^{-1}$ protein) (Sigma) dissolved in 50mM Tris-HCl, pH 8 at 37°C. After ~5min a yellow background appeared, with white spots for AChE inhibiting compounds. These were

**Table 1:** Percentage inhibition of acetylcholinesterase using the microtitre plate assay by extracts of plants traditionally used to treat memory-related ailments

| Family | Species | Voucher specimen | Plant part analysed | Extraction solvent | Inhibition (%) | | |
|---|---|---|---|---|---|---|---|
| | | | | | 0.1mg ml$^{-1}$ | 0.05mg ml$^{-1}$ | 0.025mg ml$^{-1}$ |
| Amaryllidaceae | *Boophane disticha* (L. f.) Herb. | *Stafford 53* NU | Leaves | Water | 2 | 0 | 0 |
| | | | | Ethanol | 15 | 16 | 17 |
| | | | Bulb scales | Water | 37 | 0 | 0 |
| | | | | Ethanol | 30 | 29 | 21 |
| | *Crinum moorei* Hook. f. | *Stafford 54* NU | Bulb | Water | 13 | 0 | 15 |
| | | | | Ethanol | 67 | 58 | 27 |
| Fabaceae | *Albizia adianthifolia* (Schumach.) W.F. Wight | *Stafford 55* NU | Stem bark | Water | 14 | 11 | 5 |
| | | | | Ethanol | 8 | 0 | 0 |
| | *Albizia suluensis* Gerstn. | *Stafford 56* NU | Root bark | Water | 13 | 16 | 17 |
| | | | | Ethanol | 15 | 0 | 0 |
| Malvaceae | *Malva parviflora* L. | *Stafford 57* NU | Leaves | Water | 16 | 2 | 0 |
| | | | | Ethanol | 13 | 14 | 21 |

observed and recorded within 15min because they disappeared after 20–30min.

To test for false-positive reactions a TLC plate identical to the one in the TLC assay was prepared (Rhee *et al.* 2003). After developing the TLC plate, it was sprayed with 5mM DTNB in 50mM Tris-HCl, pH 8 and allowed to dry horizontally for 3–5min. Then it was sprayed with 5mM ATCI and 3U ml$^{-1}$ AChE in 50mM Tris-HCl, pH 8 at 37°C. After ~5min a yellow background appeared; occurrence of white spots indicated false-positive reactions.

The detection limit was measured by spotting 2.5µl of various concentrations (5mM–5µM) of galanthamine on a TLC plate which was then processed as above for the AChE assay. The minimum concentration which could be recognised by the naked eye was considered as the detection limit.

AChE activity was also measured using a 96-well microplate assay (Ingkaninan *et al.* 2000, Rhee *et al.* 2001) based on Ellman's method. The yellow product, 5-thio-2-nitrobenzoate, can be detected at 405nm. In the 96-well plates, 25µl of 15mM ATCI in Millipore water, 125µl of 3mM DTNB in 50mM Tris-HCl, pH 8, 0.1M NaCl, 0.02M $MgCl_2$ x 6 $H_2O$, 50ml of 50mM Tris-HCl, pH 8, 0.1% bovine serum albumin, 25ml of plant extract at concentrations of 0.1, 0.05 and 0.025mg ml$^{-1}$ were added and the microplate was read five times at 405nm every 13s in a Labsystems Multiscan EX type 355 plate reader. Then 25µl of 0.22Uml$^{-1}$ AChE were added to the wells and the microplate was read again eight times at 405nm every 13s. The rate of reaction was calculated by Multiskan EX software version 1.0 and Microsoft Excel. Any increase in absorbance due to the spontaneous hydrolysis of substrate was corrected by subtracting the rate of the reaction before adding the enzyme. Percentage of inhibition was calculated by comparing the rates for the samples to the blank (10% methanol in buffer A). To measure the detection limit, 10µM, 1µM, 0.1µM, 0.01µM and 0.001µM of galanthamine in methanol were used as samples and the assay was performed as above.

The detection limits for galanthamine in the TLC and the microplate assay were found to be 4.6ng for one spot in the TLC assay and 0.3µM, equivalent to 2.8ng, in the microplate assay, the microplate assay thus being more sensitive than the TLC assay. The results from the TLC assay are shown in Figure 1. Table 1 shows the results from the microplate assay. Both aqueous and ethanol bulb extracts of *B. disticha* and *C. moorei* showed inhibiting activity in these assays, with the ethanol extracts having better activity. However, the aqueous extract of *B. disticha* (0.1mg ml$^{-1}$) showed 37% inhibition in the microplate assay while the ethanol extract showed 30% inhibition. The highest inhibition (67%) was obtained with the ethanol extract of *C. moorei,* which had good dose-dependent activity. The aqueous extract from the same plant showed weak inhibition in the microplate assay. Both aqueous and ethanol extracts of *C. moorei* and *B. disticha* showed AChE inhibiting activity in the TLC assay. The TLC plate for *B. disticha* had three white inhibiting zones for the ethanol bulb extract, whereas the plate for *C. moorei* yielded four inhibiting zones for the ethanol extract (Figure 1A). In addition to the four inhibiting zones of *C. moorei* two false positive zones were observed (Figure 1B). The ethanol extract of *A. adianthifolia* showed only a weak inhibiting zone in the TLC assay (Figure 1), this weak activity was confirmed in the microplate assay (8% inhibition at 0.1mg ml$^{-1}$) for the ethanol extract and 14% inhibition for the water extract. The ethanol extracts of *M. parviflora* and *A. suluensis* showed inhibiting activity both in the TLC and microplate assay (Table 1 and Figure 1A). However, this activity proved due to false positive reactions (Figure 1B).

The results of this screening indicate that some of the plants investigated do have acetylcholinesterase inhibiting activity, especially the bulbs of *B. disticha* and *C. moorei* which should be further investigated. The compounds responsible for the activity are presently unknown, but both species belong to the Amaryllidaceae, known for alkaloids with AChE-inhibiting activity (Rhee *et al.* 2001, López *et al.* 2002). The results indicate that water is not the best extraction solvent for obtaining the active compounds, something which has to be considered in relation to the use of plant material in traditional medicine.

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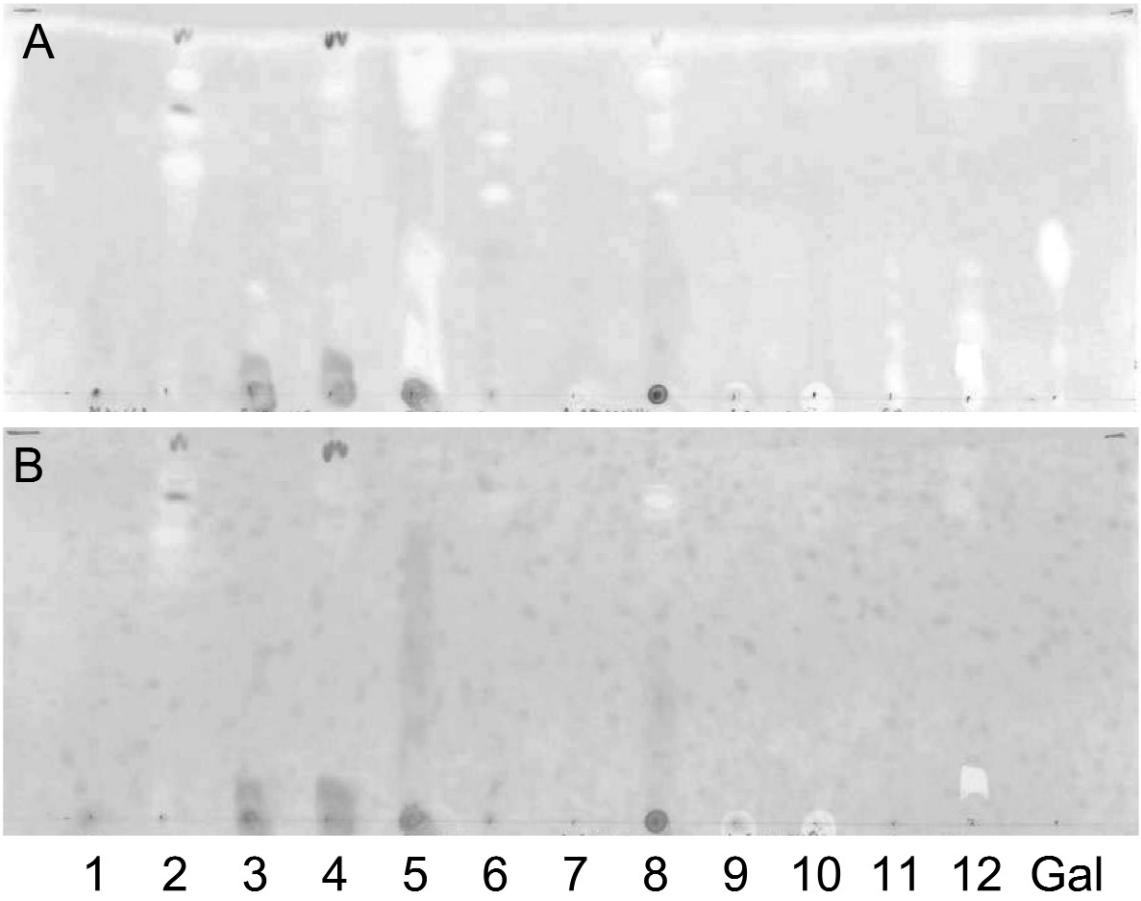


**Figure 1:** (A) Acetylcholinesterase inhibitory activity using the TLC assay, (B) false-positive control plate. (1) aqueous and (2) ethanol leaf extracts of *Malva parviflora*, (3) aqueous and (4) ethanol leaf extracts of *Boophane disticha* (5) aqueous and (6) ethanol bulb extracts of *B. disticha*, (7) aqueous and (8) ethanol bark extracts of *Albizia adianthifolia*, (9) aqueous and (10) ethanol root bark extracts of *Albizia suluensis*, (11) aqueous and (12) ethanol bulb extracts of *Crinum moorei*. Reference: galanthamine. White spots occurring only on plate (A) indicated acetylcholinesterase inhibition

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