

***In vitro* screening for acetylcholinesterase inhibition and
antioxidant activity of medicinal plants from southern
Africa**

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Running title: AChEI and antioxidant activity of herbals

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Abstract

Objective: To determine the acetylcholinesterase inhibitory (AChEI) and antioxidant activity of the ethyl acetate and methanol extracts of 12 traditional medicinal plants used in the treatment of neurological disorders.

Methods: AChEI activity was determined spectrophotometrically using the Ellman's colorimetric method. Antioxidant activity was carried out by determining the ability of the extracts to scavenge 2,2-diphenyl-1-picryl hydrazyl (DPPH) and 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radicals. The levels of total phenols, flavonoids and flavonols were determined quantitatively using spectrophotometric methods.

Results: AChEI was observed to be dose-dependent. *Lannea schweinfurthii* (Engl.) Engl. and *Scadoxus puniceus* (L.) Friis & I. Nordal. root extracts showed the lowest IC₅₀ value of 0.0003 mg/ml for the ethyl acetate extracts while *Zanthoxylum davyi* (I. Verd.) P.G. Watermann had the lowest IC₅₀ value of 0.01 mg/ml for the methanol extracts in the AChEI assay. The roots of *Piper capense* L.f., *Lannea schweinfurthii* (Engl.) Engl., *Ziziphus mucronata* Willd., *Zanthoxylum davyi* (I. Verd.) P.G. Watermann and *Crinum bulbispermum* (Burm.f.) Milne-Redh. & Schweick. showed noteworthy radical scavenging activity and good AChEI activity.

Conclusions: Five plants showed good antioxidant and AChEI activity. These findings support the traditional use of the plants for treating neurological disorders especially where a cholinesterase mechanism and ROS are involved.

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1. Introduction

Neurological disorders primarily affect the elderly population. Alzheimer's disease (AD), the most common neurodegenerative disorder is characterized clinically by progressive memory deficits and impaired cognitive function^[1,2]. AD is estimated to account for between 50 and 60% of dementia cases in persons over 65 years of age and according to the United Nations, the number of people suffering from age-related neurodegeneration, particularly from AD, will exponentially increase from 25.5 million in 2000 to an estimated 114 million in 2050^[3]. It is a major public health concern in developed countries due to the increasing number of sufferers, placing strains on caregivers as well as on financial resources^[2].

A deficiency in levels of the neurotransmitter acetylcholine (ACh) has been observed in the brains of AD patients, and inhibition of acetylcholinesterase (AChE), the key enzyme which hydrolyses ACh, is a major treatment option for AD^[4]. Traditionally used plants have been shown to be good options in the search for AChE inhibitors. Galantamine, originally isolated from plants of the Amaryllidaceae family, has become an important treatment of AD^[5]. The AChE inhibitory activity of this drug is the principal mode of action to provide symptomatic relief. Galantamine increases the availability of ACh in the cholinergic synapse by competitively inhibiting the enzyme responsible for its breakdown, AChE. The binding of galantamine to AChE slows down the catabolism of ACh and, as a consequence, ACh levels in the synaptic cleft are increased^[6-9]. It is licensed in Europe for AD treatment and was well tolerated and significantly improved cognitive function when administered to AD patients in multi-center randomized-controlled trials^[10]. To date, several plants have been identified as containing AChEI activity^[11].

Reactive oxygen species (ROS) generated from activated neutrophils and macrophages have been reported to play an important role in the pathogenesis of various diseases, including neurodegenerative disorders, cancer and atherosclerosis^[12,13]. Oxidative processes are among the pathological features associated with the central nervous system in AD. Oxidative stress causes cellular damage and subsequent cell death especially in organs such as the brain. The brain in particular is highly vulnerable to oxidative damage as it consumes about 20% of the body's total oxygen, has a high content of polyunsaturated fatty acids and lower levels of endogenous antioxidant activity relative to other tissues^[14-16]. The brain of patients suffering from AD is said to be under oxidative stress as a result of perturbed ionic calcium balances within their neurons and mitochondria^[17,18]. Herbal products are reported to possess the ability to act as antioxidants, thereby reducing oxidative damage^[19]. Among the natural phytochemicals identified from plants, flavonoids together with flavonols, and phenols represent important and interesting classes of biologically active compounds. Evidence suggests that these compounds are effective in the protection of various cell types from oxidative injury^[20].

The aim of the present study was to determine the acetylcholinesterase inhibitory (AChEI) and antioxidant activity of the ethyl acetate and methanol extracts of 12 plants, traditionally used in the treatment of neurological disorders.

2. Material and methods

2.1 Chemicals

Acetylthiocholine iodide (ATCI), acetylcholinesterase (AChE) type VI-S, from electric eel, 5,5'-dithiobis [2-nitrobenzoic acid] (DTNB), galanthamine, 1,1-Diphenyl-2-picrylhydrazyl (DPPH),

2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and trolox were purchased from Sigma. Methanol and all other organic solvents (analytical grade) were purchased from Merck.

2.2 Plant collection and extract preparation

Specimens investigated in this study were identified and voucher specimens deposited at the South African National Biodiversity Institute (SANBI), Tshwane. The plant samples were cut into small pieces and air-dried at room temperature. Dried material was ground to a fine powder and stored at ambient temperature till use. Six grams of the powdered plant material was extracted with 60 ml of either methanol or ethyl acetate for 24 h while shaking. The extracts were filtered, concentrated using a rotary vacuum evaporator and then further dried *in vacuo* at ambient temperature for 24 h. All extracts were stored at -20 °C prior to analysis. The residues were redissolved in either MeOH or ethyl acetate to the desired test concentrations.

2.3 Micro-plate assay for inhibition of acetylcholinesterase

Inhibition of acetylcholinesterase activity was determined using Ellman's colorimetric method^[21] as modified by Eldeen et al.^[22]. Into a 96-well plate was placed: 25 µl of 15 mM ATCI in water, 125 µl of 3 mM DTNB in Buffer A (50 mM Tris-HCl, pH 8, containing 0.1 M NaCl and 0.02 M MgCl₂.6H₂O), 50 µl of Buffer B (50 mM, pH 8, containing 0.1 % bovine serum albumin) and 25 µl of plant extract (0.007 mg/ml, 0.016 mg/ml, 0.031 mg/ml, 0.063 mg/ml or 0.125 mg/ml). Absorbance was determined spectrophotometrically (Labsystems Multiscan EX type 355 plate reader) at 405 nm at 45 s intervals, three times consecutively. Thereafter, AChE (0.2 U/ml) was added to the wells and the absorbance measured five times consecutively every 45 s. Galantamine served as the positive control. Any increase in absorbance due to the spontaneous hydrolysis of the substrate was corrected by subtracting the absorbance before adding the

enzyme from the absorbance after adding the enzyme. The percentage inhibition was calculated using the equation:

$$\text{Inhibition (\%)} = 1 - (A_{\text{sample}}/A_{\text{control}}) \times 100$$

Where A_{sample} is the absorbance of the sample extracts and A_{control} is the absorbance of the blank [methanol/ethyl acetate in 50 mM Tris-HCl, (pH 8)]. Extract concentration providing 50% inhibition (IC_{50}) was obtained by plotting the percentage inhibition against extract concentration.

2.4 Determination of total phenolics

Total phenolic content was determined using the modified Folin-Ciocalteu method of Wolfe et al. [23]. The extract (1mg/ml) was mixed with 5 ml Folin-Ciocalteu reagent (diluted with water 1:10 v/v) and 4 ml (75g/l) sodium carbonate. The mixture was vortexed for 15 s and allowed to stand for 30 min at 40°C for color development. Absorbance was measured at 765 nm using a Hewlett Packard UV-VIS spectrophotometer. Total phenolic content is expressed as mg/g gallic acid equivalent and was determined using the equation based on the calibration curve: $y = 6.993x + 0.037$, where x is the absorbance and y is the gallic acid equivalent (mg/g).

2.5 Determination of total flavonoids

Total flavonoid content was determined using the method of Ordonez et al. [24]. A volume of 0.5 ml of 2% $AlCl_3$ ethanol solution was added to 0.5 ml of sample solution (1 mg/ml). After one hour at room temperature, the absorbance was measured at 420 nm using a Hewlett Packard UV-VIS spectrophotometer. A yellow color is indicative of the presence of flavonoids. Total flavonoid content was calculated as quercetin equivalent (mg/g), using the equation based on the calibration curve: $y = 0.025x$, where x is the absorbance and y is the quercetin equivalent (mg/g).

2.6 Determination of total flavonols

Total flavonol content was assessed using the method of Kumaran and Karunakaran [25]. To 2 ml of sample (1 mg/ml), 2 ml of 2% AlCl₃ ethanol and 3 ml (50 g/l) sodium acetate solution were added. The samples were incubated for 2.5 h at 20°C after which absorbance was determined at 440 nm. Total flavonoid content was calculated using the equation based on the calibration curve: $y = 0.0255x$, where x was the absorbance and y is the quercetin equivalent (mg/g).

2.7 Antioxidant activity

2.7.1 DPPH radical scavenging activity

The effect of the extracts on DPPH radical was estimated using the method of Liyana-Pathirana and Shahidi [26], with minor modifications. A solution of 0.135 mM DPPH in methanol was prepared and 185 μ l of this solution was mixed with 15 μ l of varying concentrations of the extract (0.007 mg/ml, 0.016 mg/ml, 0.031 mg/ml, 0.063 mg/ml or 0.125 mg/ml), in a 96-well plate. The reaction mixture was vortexed and left in the dark for 30 min (room temperature). The absorbance of the mixture was determined at 570 nm using a microplate reader. Trolox was used as the reference antioxidant compound. The ability to scavenge the DPPH radical was calculated using the equation:

$$\text{DPPH radical scavenging activity (\%)} = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$$

where A_{control} is the absorbance of DPPH radical + methanol and A_{sample} is the absorbance of DPPH radical + sample extract/standard. The extract concentration providing 50% inhibition (IC₅₀) was obtained by plotting inhibition percentage versus extract concentration.

2.7.2 ABTS radical scavenging activity

The method of Re et al. [27] was adopted for the ABTS assay. The stock solution which was allowed to stand in the dark for 16 h at room temperature contained equal volumes of 7 mM ABTS salt and 2.4 mM potassium persulfate. The resultant ABTS^{*+} solution was diluted with methanol until an absorbance of 0.706 ± 0.001 at 734 nm was obtained. Varying concentrations (0.007 mg/ml, 0.016 mg/ml, 0.031 mg/ml, 0.063 mg/ml or 0.125 mg/ml) of the extract were allowed to react with 2 ml of the ABTS^{*+} solution and the absorbance readings were recorded at 734 nm. The ABTS^{*+} scavenging capacity of the extract was compared with that of trolox and the percentage inhibition calculated as:

$$\text{ABTS radical scavenging activity (\%)} = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$$

where A_{control} is the absorbance of ABTS radical + methanol and A_{sample} is the absorbance of ABTS radical + sample extract/standard. The extract concentration providing 50% inhibition (IC_{50}) was obtained by plotting inhibition percentage versus extract concentration.

2.8 Statistical analysis

All determinations were carried out on three occasions in triplicate. The results are reported as mean \pm standard deviation (S.D.). Calculation of IC_{50} values was done using GraphPad Prism Version 4.00 for Windows (GraphPad Software Inc.).

3. Results

Twelve plant species: roots of *Adenia gummifera* (Harv.) Harms (Passifloraceae), *Piper capense* L.f. (Piperaceae); *Zanthoxylum davyi* (I. Verd.) P.G. Watermann (Rutaceae), *Xysmalobium*

undulatum (L.)W.T.Aiton. (Apocynaceae), *Lannea schweinfurthii* (Engl.) Engl. (Anacardiaceae), *Terminalia sericea* Burch. ex DC. (Combretaceae), *Ziziphus mucronata* Willd. (Rhamnaceae), *Tabernaemontana elegans* Stapf. (Apocynaceae), *Crinum bulbispermum* (Burm.f.) Milne-Redh. & Schweick. (Amaryllidaceae), *Scadoxus puniceus* (L.) Friis & I. Nordal. (Amaryllidaceae), *Tulbaghia violacea* Harv. (Alliaceae) and fruits of *Ficus capensis* Thunb. (Moraceae) were investigated for AChEI as these plants have been reported to treat various neurological conditions^[28-39]. Ten of the plant species showed some level of inhibitory activity against AChE as indicated by their IC₅₀ values (Table 1). At the highest concentration (0.125 mg/ml), 40% showed good (>50% inhibition), 50% moderate (30-50% inhibition) and 10% low (<30% inhibition) AChE inhibition^[40]. *Lannea schweinfurthii* and *Scadoxus puniceus* root extracts showed the lowest IC₅₀ values for the ethyl acetate extracts while *Zanthoxylum davyi* had the lowest IC₅₀ value for the methanol extracts (Table 1). Generally, inhibition of AChE was dose dependent and the ethyl acetate extracts were more active than the methanol extracts.

The ethyl acetate extracts of all the plants with the exception of *T. sericea* showed either no activity or very low radical scavenging activity in both the DPPH and ABTS assays as indicated by their IC₅₀ values (Table 1). As the methanol extract showed higher activity, it would appear as if very polar solvents are able to extract compounds containing antioxidant activity. Methanol extracts of the roots of five plants and ethyl acetate of one plant showed radical scavenging activity < 50%.

The extracts which showed good DPPH and ABTS radical scavenging ability (> 60%) were further evaluated for their phenolic composition (Table 2). The levels of these phenolic compounds are an indication of the potential antioxidant activity of the plant extracts. The methanol extracts of *T. sericea* roots contained the highest flavonoid and flavonol content.

4. Discussion

Zanthoxylum davyi roots showed good AChEI with IC₅₀ values of 0.01 mg/ml and 0.012 mg/ml for the methanol and ethyl acetate extracts respectively (Table 1). Seven benzo[c]phenanthridine alkaloids have been isolated from the stem-bark of *Z. davyi*^[41], and these or similar alkaloids may be responsible for its observed inhibition of acetylcholinesterase. In addition, anticonvulsant activity has been reported for both the methanol and aqueous leaf extracts of *Z. capense*^[42]. As convulsion is a neurologic disorder, similar compounds present in the roots of *Z. davyi* may be responsible for its activity and this supports the traditional use of the plant in the treatment of neurologic diseases. *Z. capense* leaves have also been shown to contain triterpene steroids and saponins and these compounds are known to exhibit neuroprotective activity^[43]. The ethyl acetate extracts of *C. bulbispermum* bulbs showed an IC₅₀ value of 0.039 mg/ml for AChEI (Table 1), which may be ascribed to several alkaloids which have been isolated from the plant^[44]. In addition alkaloidal extracts from *Crinum jagus* and *C. glaucum* have been demonstrated to possess AChEI activity which has been ascribed to hamayne (IC₅₀ - 250µM) and lycorine (IC₅₀ - 450µM)^[45]. Furthermore, the alkaloids; haemanthamine and lycorine, isolated from *C. ornatum*, have been shown to contain anticonvulsant activity^[46]. It is possible that the presence of these or similar alkaloids may be responsible for the activity observed. The ethyl acetate extract of *Piper capense* was observed to show inhibition of AChEI with IC₅₀ value of 0.041 mg/ml (Table 1). Amide alkaloids with activity in the CNS have been identified from the roots of *P. guineense*^[28]. *P. methysticum* has been reported to possess local anaesthetic, sedating, anticonvulsive, muscle-relaxant and sleep-stimulating effects which is due to the presence of kavopyrones^[28]. *P. capense* contains the amide alkaloids; piperine and 4,5 – dihydropiperine, which have previously been shown to have CNS activity^[47]. Also, piperine has been reported to improve memory impairment

and neurodegeneration in the hippocampus of animal models with AD^[48]. The ethanol extracts of *Xysmalobium undulatum* were found to exhibit good antidepressant-like effects in three animal models^[49]. The leaves of this plant have also been reported to have good selective serotonin reuptake inhibitory activity^[50]. The neuroprotective effect of the plant has been ascribed to several glycosides^[29], which may be responsible for its observed activity as its ethyl acetate extracts showed inhibition of the enzyme with IC₅₀ value of 0.0005 mg/ml (Table 1). Glycosides are among the class of compounds which show neuroprotective activity. Four pregnane glycosides; cynatroside A, cynatroside B, cynatroside C and cynascyroside D, have been isolated from *Cynanchum atratum*^[51-53]. These glycosides showed AChE inhibition with IC₅₀ values varying between 3.6 µM for cynatroside B and 152.9 µM for cynascyroside D^[51-53].

Polar solvents have been reported to extract compounds including alkaloids which show cholinesterase inhibitory activity^[22]. This explains the use of methanol and ethyl acetate as solvents for extraction in this study. As the ethyl acetate extracts showed better activity for most of the plants, it may appear as if the solvent is able to extract more of the compounds which inhibit AChE.

Several Anacardiaceae species including *Lannea velutina*, *Sclerocarya birrea* and *Harpephyllum caffrum* have been shown to be a source of natural antioxidants. This activity has been ascribed to the high levels of proanthocyanidins and gallotannins present in the plants^[54]. As *Lannea schweinfurthii*, belongs to the same family, similar compounds could be present and therefore responsible for its good antioxidant activity, as its methanol extracts showed an IC₅₀ value of 0.0036 mg/ml for inhibition of ABTS radicals (Table 1). *Piper capense* showed good antioxidant activity (IC₅₀ value of 0.0402 mg/ml and 0.0443 mg/ml for inhibition of ABTS and DPPH radicals) which has also been reported for other *Piper* species; *P. arboreum* and *P.*

tuberculatum^[55]. This activity has been ascribed to the flavonols; quercetin and quercitrin^[56]. The leaves and roots of *T. sericea* are reported to be used traditionally in treating several infections and diseases. Sericoside, the triterpenoidal saponin found in *T. sericea* has been reported to have anti-inflammatory and antioxidant activity^[57]. Sericoside acts by reducing neutrophil infiltration and decreasing superoxide generation due to its radical scavenging activity^[57] and it may be responsible for the antioxidant activity of the plant as observed in the study. *Crinum ornatum* bulbs have been shown to contain good inhibition of DPPH radicals and hydrogen peroxide as well as being able to inhibit peroxidation of tissue lipids in the malonaldehyde test^[30]. Similar to the AChEI activity, lycorine and haemanthamine have been reported to be responsible for the antioxidant activity^[46].

The total phenolic content of the methanol extracts of *P. capense* and *C. bulbispermum* roots were relatively high for both solvents tested. Phenolic compounds contribute to the antioxidant activity of plant extracts and they are well known as radical scavengers, metal chelators, reducing agents, hydrogen donors and singlet oxygen quenchers^[58].

Flavonoids have been reported to be partly responsible for antioxidant activity, as they act on enzymes and pathways involved in anti-inflammatory processes^[59]. Furthermore, the hydrogen-donating substituents (hydroxyl groups) attached to the aromatic ring structures of flavonoids enable them to undergo a redox reaction, which in turn, helps them scavenge free radicals^[60].

Flavonols are phytochemical compounds found in high concentrations in a variety of plant-based food and beverages^[56]. Consumption of flavonols has been associated with a variety of beneficial effects including an increase in erythrocyte superoxide dismutase activity, decrease in

lymphocyte DNA damage, decrease in urinary 8-hydroxy-2'-deoxyguanosine, and an increase in plasma antioxidant capacity^[56].

The roots of *P. capense*, *Z. capense*, *L. schweinfurthii*, *Z. mucronata* and *C. bulbispermum* showed good antioxidant and cholinesterase inhibitory activity. These findings support the traditional use of the plants for treating neurological disorders especially those where a cholinesterase mechanism and reactive oxygen species are involved. These novel leads require further investigation.

Conflict of interest statement

We declare that we have no conflict of interest.

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Legends to Tables

Table 1. AChEI, ABTS and DPPH radical scavenging activity of methanol and ethyl acetate extracts.

Table 2. Total phenol, flavonoid and flavonol contents of the methanolic plant extracts with antioxidant activity (> 60%).

Table 1

Species	Extraction solvent (plant part)	AChE inhibition IC ₅₀ (mg/ml)	ABTS radical inhibition IC ₅₀ (mg/ml)	DPPH radical inhibition IC ₅₀ (mg/ml)
<i>Adenia gummifera</i>	Ethyl acetate (root)	0.0189 ± 0.005	*	*
<i>Piper capense</i>	Methanol (root)	*	0.0402 ± 0.003	0.0443 ± 0.010
	Ethyl acetate (root)	0.0407 ± 0.012	*	*
<i>Zanthoxylum davyi</i>	Methanol (root)	0.0100 ± 0.004	0.0752 ± 0.021	*
	Ethyl acetate (root)	0.0116 ± 0.002	*	*
<i>Xysmalobium undulatum</i>	Ethyl acetate (root)	0.0005 ± 0.00	*	*
<i>Lannea schweinfurthii</i>	Methanol (root)	*	0.0036 ± 0.001	0.0151 ± 0.004
	Ethyl acetate (root)	0.0003 ± 0.00	*	*
<i>Terminalia sericea</i>	Methanol (root)	*	0.0031 ± 0.001	0.0147 ± 0.006
	Ethyl acetate (root)	*	0.0746 ± 0.017	*
<i>Ziziphus mucronata</i>	Methanol (root)	*	0.0187 ± 0.020	0.0291 ± 0.051
	Ethyl acetate (root)	0.0112 ± 0.003	*	*
<i>Ficus capensis</i>	Ethyl acetate (fruit)	0.0319 ± 0.005	*	*
<i>Scadoxus puniceus</i>	Ethyl acetate (bulb)	0.0003 ± 0.00	*	*
<i>Crinum bulbispermum</i>	Ethyl acetate (root)	0.0393 ± 0.014	*	*
	Methanol (bulb)	0.0148 ± 0.039	0.0685 ± 0.041	*
	Ethyl acetate (bulb)	0.0021 ± 0.007	*	*
Controls				
Galanthamine		5.3 × 10 ⁻⁵	N/A	N/A
Trolox		N/A	0.0131	9.6 × 10 ⁻⁶

* represents extracts with maximum inhibition below 50% at the highest tested concentration of 0.125 mg/ml

Table 2

Plant and part	Total Phenol^a	Total Flavonoid^b	Total Flavonol^b
<i>Z. davyi</i> roots	97.26 ± 0.40	8.66 ± 0.40	22.84 ± 0.10
<i>L. schweinfurthii</i> roots	101.27 ± 0.10	13.58 ± 0.30	17.29 ± 0.60
<i>T. sericea</i> roots	36.73 ± 0.21	73.05 ± 0.40	28.78 ± 0.50
<i>Z. mucronata</i> roots	73.86 ± 0.25	17.76 ± 0.20	15.53 ± 0.30
<i>C. bulbispermum</i> roots	202.38 ± 0.50	9.18 ± 0.50	20.79 ± 0.10
<i>P. capense</i> roots	237.60 ± 0.12	18.14 ± 0.20	12.90 ± 0.10

^aExpressed as mg tannic acid/g of extract

^bExpressed as mg quercetin/g of extract