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Research Article

Antioxidant, acetylcholinesterase inhibitory activity and cytotoxicity assessment of the crude extracts of *Boophane disticha*

Emmanuel A. Adewusi [a], Gerda Fouche [b], and Vanessa Steenkamp [a,*]

[a] *Department of Pharmacology, Faculty of Health Sciences, University of Pretoria, South Africa*
[b] *Natural Product Chemistry Group, Biosciences, Council for Scientific and Industrial Research, South Africa*

* **Corresponding author:** Department of Pharmacology, Faculty of Health Sciences, University of Pretoria, Private Bag X323, Arcadia 0007, South Africa; **Tel:** +27-12-3192547, **Fax:** +27-12-3192411, **Email:** vanessa.steenkamp@up.ac.za

Background: Traditional healers use *Boophane disticha* to treat several neurodegenerative diseases. Furthers studies need to be carried out to justify its use in traditional medicine.

Objective: The objective of this study was to evaluate the acetylcholinesterase (AChE) inhibitory activity as well as the antioxidant activity of the bulbs and roots of *B. disticha*. Cytotoxicity studies were carried out to evaluate its safety.

Methodology: Ethyl acetate and methanol extracts of the plant were screened for AChE inhibitory activity using the Ellman's colorimetric method. ABTS and DPPH radical scavenging assays were carried out to determine antioxidant activity. Cytotoxicity assessment was determined on human neuroblastoma (SH-SY5Y) cells using the MTT and neutral red uptake assays.

Results: All plant extracts tested exhibited AChE inhibitory activity, with the ethyl acetate extract of the bulb and methanol extract of the root showing the highest activity. Antioxidant activity was only observed with the methanol extract of the roots with IC_{50} values of 0.086 mg/ml and 0.13 mg/ml in both the ABTS and DPPH assays, respectively. The extract also exhibited low IC_{50} values in the cytotoxicity assays.

Discussion: These findings partially support the traditional use of *B. disticha* in the treatment of neurological disorders. Its use may however be limited by its toxicity.

Key words: Acetylcholinesterase, antioxidant, *Boophane disticha*, cytotoxicity, SH-SY5Y cells

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1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease and is characterized by memory impairment, cognitive dysfunction, behavioral disturbances and deficits in daily living (Konrath et al, 2012). Approaches to enhance cholinergic function in AD have included stimulation of cholinergic receptors or prolonging the availability of acetylcholine (ACh) released into the neuronal synaptic cleft by inhibiting ACh hydrolysis through the use of acetylcholinesterase (AChE) inhibitors (Howes and Houghton, 2003). Tacrine was the first widely used AChE inhibitor (Summers, 2006). Second generation AChE inhibitors with longer half-lives than tacrine, such as donepezil, galanthamine and rivastigmine, have since been developed and are currently in use (Shah et al, 2008). In addition, several lines of evidence indicate that reactive oxygen species are associated with the pathogenesis of AD, as some cellular characteristics of this disease are either causes or effects of oxidative stress (Zhu et al, 2004; Sultana et al, 2006; Konrath et al, 2012). Generally, the physiological role of antioxidant compounds is to attenuate the oxidation chain reactions by removing

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free-radical intermediates (Liu and Nair, 2010). Since a large amount of evidence demonstrates that oxidative stress is intimately involved in age-related neurodegenerative diseases, there have been a number of studies which have examined the positive effects of antioxidants in reducing or blocking neuronal death occurring in the pathophysiology of these disorders (Ramassamy, 2006). Consequently, the use of antioxidants has been explored in an attempt to slow AD progression and neuronal degeneration (Howes and Houghton, 2003).

Toxicity testing is an essential requirement for the development of modern pharmaceutical compounds. Medicinal plants are assumed to have low toxicity due to their long-term consumption by humans and animals (Luseba et al, 2007; Verschaeve and van Staden, 2008; Aremu et al, 2011). However, several studies have shown that many plants used as food or medicine, have potential toxic effects (Du Plooy et al, 2001; Barlow and Schlatter, 2010). Almost all known AChE inhibitors have several drawbacks, such as hepatotoxicity, short duration of biological action, low bioavailability, adverse cholinergic side effects in the periphery and narrow therapeutic windows (Lee et al, 2011). Some common synthetic antioxidants including butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been reported to be toxic (Aremu et al, 2011). Therefore, the search for new AChE inhibitors and antioxidants, particularly from natural products, with low toxicity and higher efficacy continues.

Many plants are reputed to have 'anti-ageing' or 'memory-enhancing' effects and are used traditionally to treat several neurodegenerative diseases (Howes and Houghton, 2003). One such plant, *Boophane disticha* (L.f.) Herb. belongs to the family Amaryllidaceae. It is an attractive, deciduous bulbous plant with a thick covering of dry scales above the ground and is widely distributed in Africa, ranging from Sudan in the north to the Western Cape Province in the south (Wrinkle, 1984). Decoctions of bulb scales are given to sedate violent, psychotic patients while bulb infusions are reported to be used to treat mental illness (van Wyk and Gericke, 2000; Sobiecki, 2002). Bulb decoctions are also used in the treatment of headaches, abdominal pain, weakness, sharp chest pains and persistent bladder pains, as well as treatment of varicose ulcers, relief of urticaria, and cancer (Botha et al, 2005). This study was aimed at evaluating the AChE inhibitory and antioxidant activity of the bulbs and roots of *B. disticha* to partially justify its traditional use in treatment of neurodegenerative diseases. The safety of using this plant in traditional medicine was also investigated by assessing its toxicity using the MTT and neutral red assays.

2. Methods

2.1 Reagents and Chemicals

Acetylthiocholine iodide (ATCI), acetylcholinesterase (AChE) type VI-S from an 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), trolox, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT), and neutral red dye, were all purchased from Sigma Aldrich (South Africa). Ham's F-12 medium, fetal calf serum (FCS) and other cell culture reagents were obtained from Gibco Invitrogen Corporation. All organic solvents (analytical grade) were purchased from Merck (South Africa).

2.2 Plant collection and extract preparation

The bulbs and roots of *Boophane disticha* (L.f.) Herb. (Amaryllidaceae) were a gift from the South African National Biodiversity Institute (Tshwane). The root and bulb were separated, cut into small pieces and air-dried at room temperature. The dried material was ground to a fine powder using an Ika Analytical Mill (Staufen, Germany), and stored at ambient temperature in the dark 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 and evaporated to dryness using a rotary evaporator. All extracts were stored at -20 °C prior to analysis. The residues were re-dissolved 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 as modified by Eldeen et al (2005). 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 $MgCl_2.6H_2O$), 50 µl of Buffer B (50 mM, pH 8, containing 0.1% bovine serum albumin) and 25 µl of plant extract (0.007, 0.016, 0.031, 0.063 or 0.125 mg/ml). Absorbance was determined spectrophotometrically (Labsystems Multiscan EX type 355 plate reader) at 405 nm at 45 sec intervals, three times consecutively. Thereafter, AChE (0.2 U/ml) was added to the wells and the absorbance measured five times consecutively every 45 sec. Galanthamine served as the positive control. Any increase in absorbance due to the spontaneous hydrolysis of the substrate was corrected by subtracting the absorbance before addition of the enzyme from the absorbance after addition of the enzyme. The percentage inhibition for each test solution was then calculated using the equation:

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

Where A_{sample} is the absorbance of the sample extracts and $A_{control}$ is the absorbance of the blank [methanol 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 Antioxidant activity

DPPH radical scavenging activity

The effect of the extracts on DPPH radical was estimated using the method of Liyana-Pathirana and Shahidi (2005), with minor modifications. A solution of 0.135 mM DPPH in methanol was prepared and 185 µl of this solution was added to 15 µl of varying concentrations of the extract (0.007, 0.016, 0.031, 0.063 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:

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

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 (IC_{50}) was obtained by plotting inhibition percentage versus extract concentration.

ABTS radical scavenging activity

The method of Re et al (1999) was adopted for the ABTS assay. The stock solution which was allowed to stand in the dark for 16 hr 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, 0.016, 0.031, 0.063 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:

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

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.

2.5 Cells and cell culture

The neuroblastoma cell line, SH-SY5Y (ATCC CRL-2266) was selected for this study as it has been widely used in experimental neurological studies, including analysis of neuronal differentiation, metabolism and function related to neurodegenerative and neuronadaptive processes, neurotoxicity and neuroprotection (Xie et al, 2010). It was purchased from American Type Culture Collection.

Cells were cultured in Ham's F-12 supplemented with 2% heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37 °C in a humidified incubator at 95% air and 5% CO_2.

For use in the assay, the cells were trypsin-treated for 10 min, decanted from culture flasks and centrifuged (200*g*, 10 min). The pellet was re-suspended in 1 ml fetal calf serum-supplemented Ham's F-12 medium and enumerated by staining with trypan blue. The cells were diluted to a concentration of 1×10^5 cells/well in Ham's F-12 medium and 100 µl of the cell suspension plated into each of the wells of a 96-well microtiter plate. 80 µl of Ham's F-12 medium was added whereafter plates were incubated for 1 hr at 37 °C in a humidified incubator at 95% air and 5% CO_2 to allow for cellular re-attachment.

2.6 Cytotoxicity studies

MTT assay

The MTT assay as described by Mossmann (1983) was used to measure cell viability. The principle of the assay is based on generation of formazan, a blue product, in the mitochondria of active cells which is measured by photometric techniques (Hansen et al, 1989). The cells were plated into 96-well culture plates, as described above, and treated with various concentrations (100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 µg/ml) of the plant extracts for 72 hr. Thereafter, 20 µl of MTT solution (5 mg/ml) was added to the wells and further incubated for 3 hr. 50 µl of solution containing 30% (w/v) *N,N*- dimethylformamide and 20% SDS in water was then added to breach the cells and dissolve the formazan crystals.

The plates were incubated overnight at 37 °C, after which absorbance was measured at 570-630 nm using a microtiter plate reader (Labsystems Multiscan EX type 355). Wells without cells were used as blanks and were subtracted as background from each sample. Cell viability was expressed as a percentage of the control values. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage versus extract concentration.

Neutral red assay

The neutral red uptake assay as described by Borenfreund and Puerner (1984) was also used to assess cell viability. This method is based on the determination of the accumulation of the neutral red dye in the lysosomes of viable, uninjured cells. After treatment and incubation of the cells (as described above for the MTT assay), 150 µl of neutral red dye (100 µg/ml) dissolved in serum free medium (pH 6.4), was added to the culture medium for 3 hr at 37 °C. Cells were washed with Phosphate Buffered Saline (PBS), and 150 µl of elution medium ($EtOH/AcOH/H_2O$, 50%/1%/49%) was added followed by gentle shaking for 60 min, so that complete dissolution could be achieved.

Absorbance was recorded at 540-630 nm using a microtiter plate reader (Labsystems Multiscan EX type 355). Cell viability was expressed as a percentage of the control values. A graph of percentage cell viability against extract concentration was plotted, and extract concentration providing 50% inhibition (IC_{50}) of cell death was calculated from the graph.

2.6 Statistical analysis

Tests were carried out where possible at least in triplicate and on three different occasions. The results are reported as mean ± standard deviation (S.D.). Standard curves were generated and calculation of the 50% inhibitory concentration (IC_{50}) values was done using GraphPad Prism Version 4.00 for Windows (GraphPad Software Inc.). Cytotoxicity results are

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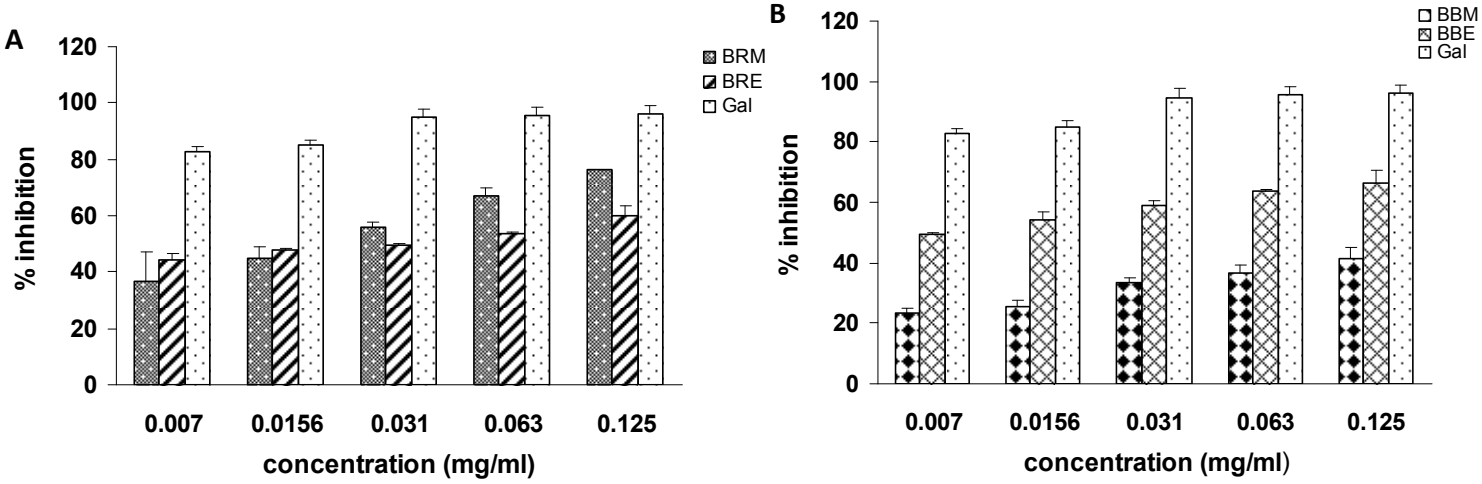
expressed as the percentage cell survival compared to the untreated control using a dose response curve.

3. Results and Discussion

The results of the AChE inhibitory activities of the extracts of *B. disticha*, as well as the positive control, galanthamine, are provided in **Figure 1**. All the plant extracts tested contained some level of inhibition against AChE. The ethyl acetate extract of the bulb and methanol extract of the root showed the highest activity with AChE inhibitory values of 66.4% and 76.3%, respectively, at the highest concentration tested (0.125 mg/ml). The IC_{50} values, of the plant extracts, indicating AChE inhibitory activity are presented in **Table 1**. With the exception of the methanol extracts of the bulbs, all other extracts tested had low IC_{50} values. Although the IC_{50} of these plant extracts were higher than that of galanthamine (0.00053 mg/ml), they possess good AChE inhibitory activity considering they are still mixtures containing various compounds.

Figure 1 Percentage inhibition of acetylcholinesterase by the ethyl acetate and methanol extracts of (A) roots and (B) bulbs of *Boophanedisticha*. **BRM**, *B. disticha* root methanol extract; **BRE**, *B. disticha* root ethyl acetate extract; **BBM**, *B. disticha* bulb methanol extract; **BBE**, *B. disticha* bulbs ethyl acetate extract; **Gal**, Galanthamine.



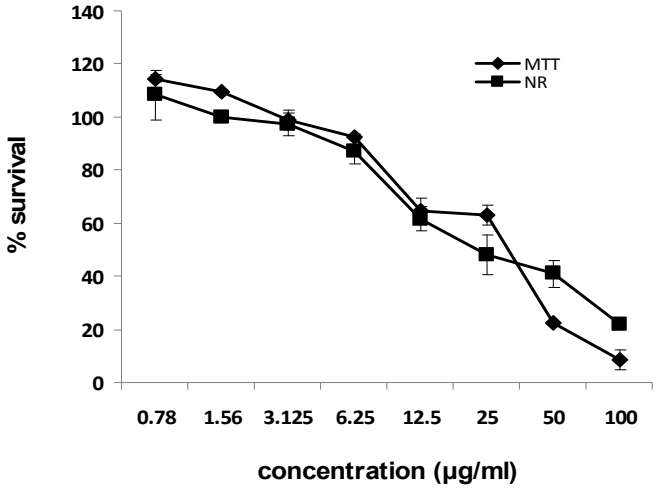


Figure 2 Effect of the methanol extract of *B. disticha* roots on the viability of SH-SY5Y cell lines as measured by MTT and neutral red uptake assays after 72 hr of incubation

Table 1 AChE inhibitory activity of extracts of *B. disticha* as represented by their IC_{50} values.

Plant part analyzed	Extraction solvent	AChE inhibition IC_{50} (mg/ml)
Bulb	Methanol	*
	Ethyl acetate	0.0073 ± 0.002
Root	Methanol	0.0199 ± 0.009
	Ethyl acetate	0.0230 ± 0.007
Galanthamine		$5.3 \times 10^{-4} \pm 1.0 \times 10^{-5}$

* *represents extracts with maximum inhibition below 50% at the highest concentration tested*

Leaf extracts of *B. disticha* have been shown to contain neuroprotective activity through possession of affinity for the serotonin transporter in rat brain (Nielsen et al, 2004). A number of biologically active alkaloids, including buphanidrine, buphanamine, crinine, lycorine, distichamine and lycorine have been isolated from *B. disticha*. Several of these compounds have been reported to show moderate to high affinity for the serotonin transporter (Elgorashi et al, 2006). Some of these alkaloids, as well as similar alkaloids, isolated from other plants in the Amaryllidaceae family have been reported to contain AChE inhibitory activity. Crinine, epibuphanisine, crinamidine and epivittatine have been isolated from *Crinum moorei* (Elgorashi et al, 2001a); hamayne from *C. macowanii* (Elgorashi et al, 2001b) and 3-O-acetylhamayne and crinamine from *C.*

bulbispermum. Inhibition of AChE (IC_{50}) was shown to be 461 ± 14 µM, 547 ± 5 µM, 300 ± 27 µM, 239 ± 9 µM, 553 ± 3µM, 594 ± 8 µM, and 697 ± 12 µM for each of these alkaloids, respectively (Elgorashi et al, 2004). The presence of these or related alkaloids may be responsible for the good AChE inhibitory activity observed with the methanol and ethyl acetate extracts of the bulbs and root of *B. disticha*.

The ethyl acetate extract of the bulb and root, and methanol extract of the bulb did not show any antioxidant activity (results not shown). Antioxidant activity was however observed, with the methanol extract of the root in both the ABTS and DPPH assays with IC_{50} values of 0.086 mg/ml and 0.13 mg/ml respectively. The extract scavenged the ABTS and DPPH radicals in a dose-dependent manner, though antioxidant activity observed was lower than that of trolox (positive control) which had IC_{50} values of 0.013 mg/ml and 9.6×10^{-6} mg/ml for both the ABTS and DPPH assays, respectively. Lycorine and haemanthamine have been reported to contain antioxidant activity (Oloyede et al, 2010), and the presence of these or similar Amaryllidaceae alkaloids may be responsible for the observed antioxidant activity in the methanol extract of the root of *B. disticha*. This is the first report on the antioxidant activity of crude extracts of *B. disticha*.

The methanol extract of the root of *B. disticha* was further tested for its toxicity as it showed good AChE inhibitory and antioxidant activity. The effect of the extract on the viability of SH-SY5Y cell lines using the MTT and neutral red assay is presented in **Figure 2**. Results obtained from both cytotoxicity assays were comparable and the extract showed IC_{50} values of 23.3 µg/ml and 27.8 µg/ml for the MTT and neutral red assays respectively. The low IC_{50} values obtained showed that the extract is toxic and this is not surprising as the toxic effect of *B. disticha* is documented in literature and this is mainly due to its alkaloidal content (Botha et al, 2005). The Amarrylidaceae alkaloids lycorine, crinamine and augustine have been reported to demonstrate significant cytotoxic activity in 12 different cell lines (Likhitwitayawuid et al, 1993). Also, crinafolidine and crinafoline have been observed to produce significant reduction in the viability and *in vivo* growth of S-180 ascites tumor cells (Tram et al, 2002).

4. Conclusion

The present study showed that the ethyl acetate and methanol extracts of the bulbs and roots have good AChE inhibition. In addition, the methanol extract of the roots contains antioxidant activity which partially supports the traditional use of the plant for treating neurological disorders. However, its use may be limited by its toxicity.

Conflict of Interest declaration

The authors declare no conflict of interest

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