CHAPTER 2: MATERIALS AND METHODS

2.1 Reagents and 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), trolox, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT), neutral red dye, dimethyl sulfoxide (DMSO), amyloid beta ($A\beta_{25-35}$), catechin, butylated hydroxytoluene (BHT), ascorbic acid, tannic acid, tris base, fetal bovine serum, sodium chloride, magnesium chloride hexahydrate and quercetin were all purchased from Sigma. Vanillin was procured from BDH chemicals Ltd., whereas Folin-Ciocalteus's phenol reagent and sodium carbonate were obtained from Merck Chemical supplies (Damstadt, Germany). Human neuroblastoma (SH-SY5Y) cells were purchased from American cell type collection culture (ATCC CRL-2266, Rockville, MD, USA). Ham's F-12 medium, fetal calf serum (FCS) and other cell culture reagents were obtained from Gibco Invitrogen Corporation. Methanol and all other organic solvents (analytical grade) were purchased from Merck. All chemicals for UPLC-MS work were of ultra-pure LC-MS grade and purchased from Fluka (Steinheim, Germany) while ultra-pure solvents were purchased from Honeywell (Burdick & Jackson, Muskegon, USA). Ultra-pure water was generated from a Millipore Elix 5 RO system and Millipore Advantage Milli-Q system (Millipore SAS, Molsheim, France).

2.2 Plant material

2.2.1 Plant collection

Plants were collected from different places all over South Africa. These include the Council for Scientific and Industrial Research (CSIR), Pretoria {plants with voucher numbers starting with 'P'}; the South African National Biodiversity Institute (SANBI), Pretoria {*}; Venda, Limpopo {plants with voucher numbers starting with 'LT'}; Makhado, Limpopo {plants with voucher numbers starting with 'NH'}; and University of Witwatersrand Botanical Gardens, Johannesburg {plant with voucher number starting with 'HANKEY'}. The identities of the plants were confirmed by a botanist. Voucher specimens of plants with voucher numbers starting with 'P' and * were deposited at SANBI, 'LT' at the Herbarium in Onderstepoort, 'NH' at Soutpanbergensis Herbarium and 'HANKEY' at the University of Witwatersrand Herbarium.

Selection was based on their documented ethno-medicinal use in improving memory, to treat insomnia, calm agitated people, and other neurological disorders (Table 2.1). In all, 44 extracts from 17 different plants were screened for activity.

Table 2.1 Plants investigated in the present study with documented ethno-medicinal use in treatment of neurological disorders.

Species	Family	Plant part	Voucher number	Traditional use
Adenia gummifera (Harv.) Harms.	Passifloraceae	Root	NH1912	Infusions made from the root are administered for depression and to treat madness and epilepsy (Bryant, 1966; Gelfand et al., 1985)
Boophane disticha (L.f.) Herb.	Amaryllidaceae	Root and bulb	*	Weak decoctions of bulb scales are given to sedate violent, psychotic patients; bulb infusions are used to treat mental illness (van Wyk and Gericke, 2000; Sobiecki, 2002)
Buddleja salviifolia (L.) Lam.	Buddlejaceae	Whole plant	P01281	*Buddleja* species are used together with *Heteromorpha trifoliate* and *Cussonia paniculata* by Sotho in South Africa to treat early nervous and mental illnesses (Watt and Breyer-Brandwijk, 1962)
Chamaecrista mimosoides L. Greene	Caesalpiniaceae	Root	P08814	Cold water root infusions of *C. mimosoides* are reported to be taken to remember forgotten dreams by Zulu (Hulme, 1954)
Crinum bulbispermum (Burm.f.) Milne-Redh. & Schweick.	Amaryllidaceae	Root and bulb	*	*Crinum* species are used as anticonvulsants (Oloyede and Farombi, 2010)
Ficus capensis Thunb.	Moraceae	Fruits	LT14	*Ficus* species are used to treat epilepsy, and to calm agitated people, and have been studied for behavioural effects in psychotic patients (Hutchings et al., 1996; Gamaliel et al., 2000)
Lannea schweinfurthii (Engl.) Engl.	Anacardiaceae	Root	LT19	Decoctions from the root are used to improve memory and as a sedative (Mabogo, 1990; van Wyk and Gericke, 2000)
Piper capense L.f.	Piperaceae	Root	LT16	Tuber or root reported to cause sleepiness (van Wyk and Gericke, 2000)

Salvia tiliifolia Vahl.	Lamiaceae	Whole plant	P03649	*Salvia* species have been reported to be used for memory-enhancing purposes in European folk medicine (Perry et al., 2003)
Scadoxus puniceus (L.) Friis & I. Nordal.	Amaryllidaceae	Root and bulb	*	Known to cause CNS excitation or depression (Veale et al., 1992)
Schotia brachypetala Sond.	Fabaceae	Root and bark	P06300 (root); P08514 (bark)	The bark and roots of *S. brachypetala* are reported to be used for nervous conditions (van Wyk and Gericke, 2000)
Tabernaemontana elegans Stapf.	Apocynaceae	Root	NH1920	*Tabernaemontana* species have been used as traditional rejuvenation remedies, to improve memory and as a central nervous system stimulant (Taesotikul et al., 1998; Ingkaninan et al., 2003)
Terminalia sericea Burch. ex DC.	Combretaceae	Root	NH1878	Roots are used for general weakness and epilepsy (Hutchings et al., 1996; Gelfand et al., 1985)
Tulbaghia violacea Harv.	Alliaceae	Root and bulb	*	Rhizome infusion is administered for fits (Hutchings et al., 1996)
Xysmalobium undulatum (L.)W.T.Aiton.	Apocynaceae	Root	HANKEY 1653	Roots are administered to treat hysteria (Hutchings et al., 1996)
Zanthoxylum davyi (I. Verd.) P.G. Watermann	Rutaceae	Root	LT4	Used as infusions and decoctions to treat epilepsy and febrile conditions (Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996)
Ziziphus mucronata Willd.	Rhamnaceae	Root	NH1909	*Ziziphus* species are used to nourish the heart and calm the spirit. It is often used to aid sleep or calm the mind (Gomes et al., 2009)

*obtained from SANBI, Pretoria

2.2.2 Extract preparation

Plant material was cut into small pieces and air-dried at room temperature. 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, 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 re-dissolved in either MeOH or ethyl acetate to the desired test concentrations.

Plant specimens obtained from the CSIR were already prepared as an extract in dichloromethane/methanol (1:1) or water. These specimens were diluted to the desired test concentrations using DCM/MeOH (1:1) or water, respectively.

2.3 Determination of acetylcholinesterase inhibition

2.3.1 Micro-plate assay

Inhibition of acetylcholinesterase activity was determined using Ellman's colorimetric method as modified by Eldeen et al. (2005). Three buffers were prepared for the assay; Buffer A (50 mM Tris-HCl, pH 8), Buffer B (50 mM, pH 8, containing 0.1 % bovine serum albumin) and Buffer C (50 mM Tris-HCl, pH 8, containing 0.1 M NaCl and 0.02 M $MgCl_2.6H_2O$). Into a 96-well plate was placed: 25 µl of 15 mM ATCI in water, 125 µl of 3 mM DTNB in Buffer C, 50 µl (72.5 µl for isolated compounds/fractions) of Buffer B and 25 µl (2.5 µl for isolated compounds/fractions) of plant extract. Absorbance was measured spectrophotometrically (Labsystems Multiscan EX type 355 plate reader) at 405 nm every 45 s, three times

consecutively. Thereafter, AChE (0.2 U/ml) was added to the wells and the absorbance measured five times consecutively every 45 s. 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 adding the enzyme from the absorbance after adding the enzyme. The percentage inhibition was calculated using the equation:

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

Where A_{sample} is the absorbance of the sample extract and $A_{control}$ is the absorbance of the blank [extraction solvent in Buffer A (50 mM Tris-HCl, pH 8)]. Extract concentration providing 50% inhibition (IC_{50}) was obtained from the graph of the percentage inhibition against extract concentration.

2.3.2 TLC assay

Plant extracts (10 µl), fractions or isolated compounds (3 µl) were applied to TLC plates (silica; 10 cm × 10 cm). After developing, the plates were sprayed with 5 mM ATCI and 5 mM DTNB in 50 mM Tris-HCl buffer, pH 8 until the silica was saturated with the solvent. The plates were then sprayed with 3 U/ml AChE which was dissolved in 50 mM Tris-HCl buffer (pH 8). Galanthamine was used as a positive control, as it is a known AChE inhibitory compound (Heinrich and Teoh, 2004). A yellow background with white spots was considered indicative of AChE inhibiting fractions/compounds.

False-positive reactions were eliminated by the method of Rhee et al. (2003). A TLC plate identical to the one described above was prepared. The developed TLC plate was sprayed with 5 mM DTNB in 50 mM Tris-HCl (pH 8). Plates were then sprayed with 5 mM ATCI and 3 U/ml

AChE in Buffer A. A yellow background with white spots was indicative of false positive reactions.

2.4 Antioxidant activity

2.4.1 DPPH radical scavenging activity

The effect of the extracts on DPPH radical scavenging 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 mixed with 15 µl of varying concentrations of the extract 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 (Labsystems Multiscan EX type 355 plate 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 calculated from the graph of inhibition percentage versus extract concentration.

2.4.2 ABTS radical scavenging activity

The method of Re et al. (1999) was followed for determination of radical scavenging ability. 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 of the extract were allowed to react with 2 ml of the $ABTS^{*+}$ solution and the absorbance readings were recorded at 734 nm using a Perkin-Elmer UV/Vis Lambda 2 spectrophotometer. 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. All tests were carried out on three separate occasions. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage versus extract concentration.

2.5 Phytochemical screening

The plants which showed good antioxidant activity (>50%) in both assays, were further evaluated for determination of level of phenolic compounds.

2.5.1 Determination of total phenolics

Total phenolic content in the extracts was determined using the modified Folin-Ciocalteu method of Wolfe et al. (2003). The extract (1 mg/ml) was mixed with 5 ml Folin-Ciocalteu reagent (diluted with water 1:10 v/v) and 4 ml (75 g/l) sodium carbonate. The mixture was vortexed for 15 s and allowed to stand for 30 min at 40^{o}C for colour development. Absorbance was measured at 765 nm using the Perkin-Elmer UV/Vis Lambda 2 spectrophotometer. Total phenolic content was expressed as mg/g tannic acid equivalent using the equation based on the calibration curve: y = 0.1216x, where x is the absorbance and y is the tannic acid equivalent (mg/g).

2.5.2 Determination of total flavonoids

Total flavonoid content was determined using the method of Ordonez et al. (2006). A volume of 0.5 ml of 2% $AlCl_3$ ethanol solution was added to 0.5 ml of sample (1 mg/ml). After 1 h incubation period at room temperature, the absorbance was measured at 420 nm using the Perkin-Elmer UV/Vis Lambda 2 spectrophotometer. A yellow colour 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 Compound isolation

Boophane disticha bulb contained very promising AChE inhibitory activity, was available in sufficient amounts and was therefore selected for isolation of active compounds.

2.6.1 Column chromatography

Different sized columns, ranging from 1.5-8 cm in diameter, were used depending on the amount of sample available and the purification stage. Separation of crude extracts was generally carried out on a column using silica gel 60 (0.063-0.2 mm).

2.6.2 Thin-layer chromatography

Thin-layer chromatography (TLC) was carried out on pre-coated glass plates (Merck, SIL G-25 UV_{254}, 20 cm x 20 cm). 10 µl (1 mg/ml) of the extract was loaded onto the plates and eluted with chloroform: methanol (9:1 or 8:2) for the methanol extracts and hexane: ethyl acetate (9:1 or 8:2) for the ethyl acetate extracts. The plates were first viewed under ultraviolet (UV) light (short-

wave 254 nm and long-wave 366 nm) and then sprayed with a vanillin-H_2SO_4 (1 g vanillin in 100 ml H_2SO_4) solution, and heated at 100ºC for few minutes, as a general qualitative test to detect the compounds present. The TLC plates were also sprayed with Dragendorff's reagent to detect the presence of alkaloids.

2.6.3 Preparative thin-layer chromatography

Compounds which were visible under UV light were further purified using preparative TLC. The glass plates were lined with the sample, 1.5 cm from the bottom of the plate. The samples were loaded onto the plates using a Pasteur pipette, from one end of the plate to the other. Plates were developed using the solvent systems described above (2.6.2). Compounds of interest were visualized under UV light. The bands were scraped off, dissolved in methanol or ethyl acetate and then filtered to remove the silica gel from the filtrate.

2.6.4 Isolation of compounds from the methanol extract

The methanol extract (1.4 g) was subjected to silica gel column chromatography (66.7 g; particle size 0.063 - 0.2 mm). The separation and purification was carried out using a stepwise gradient mixture of chloroform, $CHCl_3$: MeOH starting from 100:0 until 70:30 as eluent to give 50 fractions. Fractions were collected every 5 min at a rate of 1 ml/min. The fractions were pooled together based on the similarity in their R_f values on a thin-layer chromatography plate to give four sub-fractions. Each sub-fraction was tested for their inhibition of AChE on the TLC plate as described in section 2.3.2 above. Sub-fractions 2 (3-16) and 3 (17-21), were the only active sub-fractions.

Sub-fraction 2 was further chromatographed on a silica gel column using a stepwise gradient mixture of $CHCl_3$: MeOH starting from 95:5 until 75:25 as eluent to give another set of 30 fractions and tested for activity. Of this sub-fraction, only fractions 1, 4 and 8 were active.

Sub-fraction 3 was also further chromatographed on a silica gel column using a stepwise gradient mixture of $CHCl_3$: MeOH starting from 95:5 until 75:25 as eluent, to give 23 fractions which were also tested for activity. Fractions 5, 14, 15 and 16 were active and had similar R_f values as fractions 1, 4 and 8 from sub-fraction 2, and so these seven fractions were combined and further purified. Preparative TLC yielded **compound 1** or **EAM 3,16 1-4** (20 mg). Compound 1 was analysed using the UPLC-QTOF (mass spectroscopy determination) and Nuclear Magnetic Resonance spectroscopy (1D and 2D experiments) for its structure determination.

Fractions 21 and 22 from sub-fraction 3 were combined as they had similar R_f values and showed activity for inhibition of AChE on the TLC plate. However, the yield (0.5 mg) was very low and this fraction could not be purified further. It was named **EAM 17-21 21,22** and reserved for the quantitative assay.

A schematic diagram of the fractionation and isolation of the methanol extract is provided in Figure 2.1.

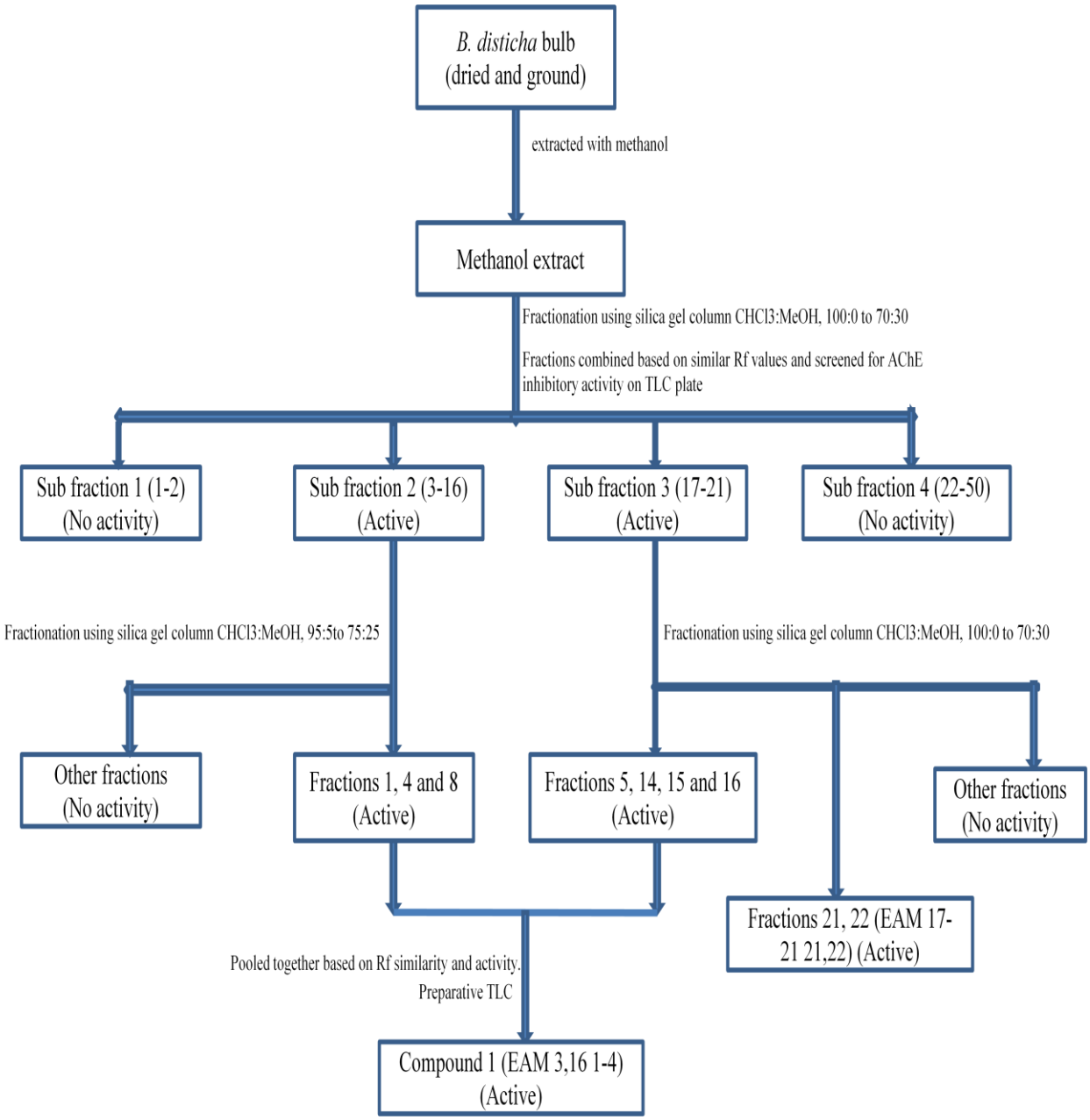

Figure 2.1 Schematic diagram of the fractionation and isolation of compounds from the methanol extracts of *Boophane disticha*.

2.6.5 Isolation of compounds from the ethyl acetate extract

The ethyl acetate extract (1.4 g) was subjected to silica gel column chromatography (65 g; particle size 0.063 - 0.2 mm). The separation and purification was done using a stepwise gradient mixture of hexane: ethyl acetate starting from 100:0 until 0:100 as eluent to give 70 fractions. Fractions were collected every 5 min at a rate of 1 ml/min. The fractions were pooled together based on the similarity in their R_f values on a thin-layer chromatography plate to give four sub-fractions. All four sub-fractions were tested for activity and only sub-fraction 2 (20-41) was active.

Sub-fraction 2 was subjected to further silica gel column chromatographic purification and subsequently eluted using a stepwise gradient mixture of hexane: ethyl acetate, starting from 90:10 until 0:100, to give another set of 120 fractions. These fractions were pooled together based on the similarity in their R_f values on a thin-layer chromatography plate and again tested for activity. Fraction 104 (EAE 104) showed a major spot on TLC but it was not active in the AChE inhibition assay, and the NMR data showed it to be impure so structural elucidation could not be carried out. Furthermore, the yield obtained was very low (0.2 mg), making further purification impossible.

Fractions 82-100 were active and were combined and loaded onto a column for further purification and isolation of the compounds. Compounds were eluted using a stepwise gradient mixture of hexane: ethyl acetate, starting from 80:20 until 0:100. Fraction 1 obtained from this separation was found to be a pure and active compound (**compound 2** or **EAE 1** or **F1**). However, this compound was very unstable, degraded, and lost its activity before NMR analysis was done. Fraction 9 (**compound 3** or **EAE 9**) was found to be pure, but inactive. This fraction

was further analysed using the UPLC-QTOF (mass spectroscopy determination) and Nuclear Magnetic Resonance spectroscopy (1D and 2D experiments) for its structure elucidation. Fraction 11 (**EAE 11**) was active for inhibition of AChE, but could not be further purified because of its very low yield (0.4 mg). However, it was reserved for the quantitative assay.

A schematic diagram of the fractionation and isolation of the ethyl acetate extract is provided in Figure 2.2.

The active compound and fractions were evaluated quantitatively, for their AChE inhibitory activity using the method described in section 2.3.1.

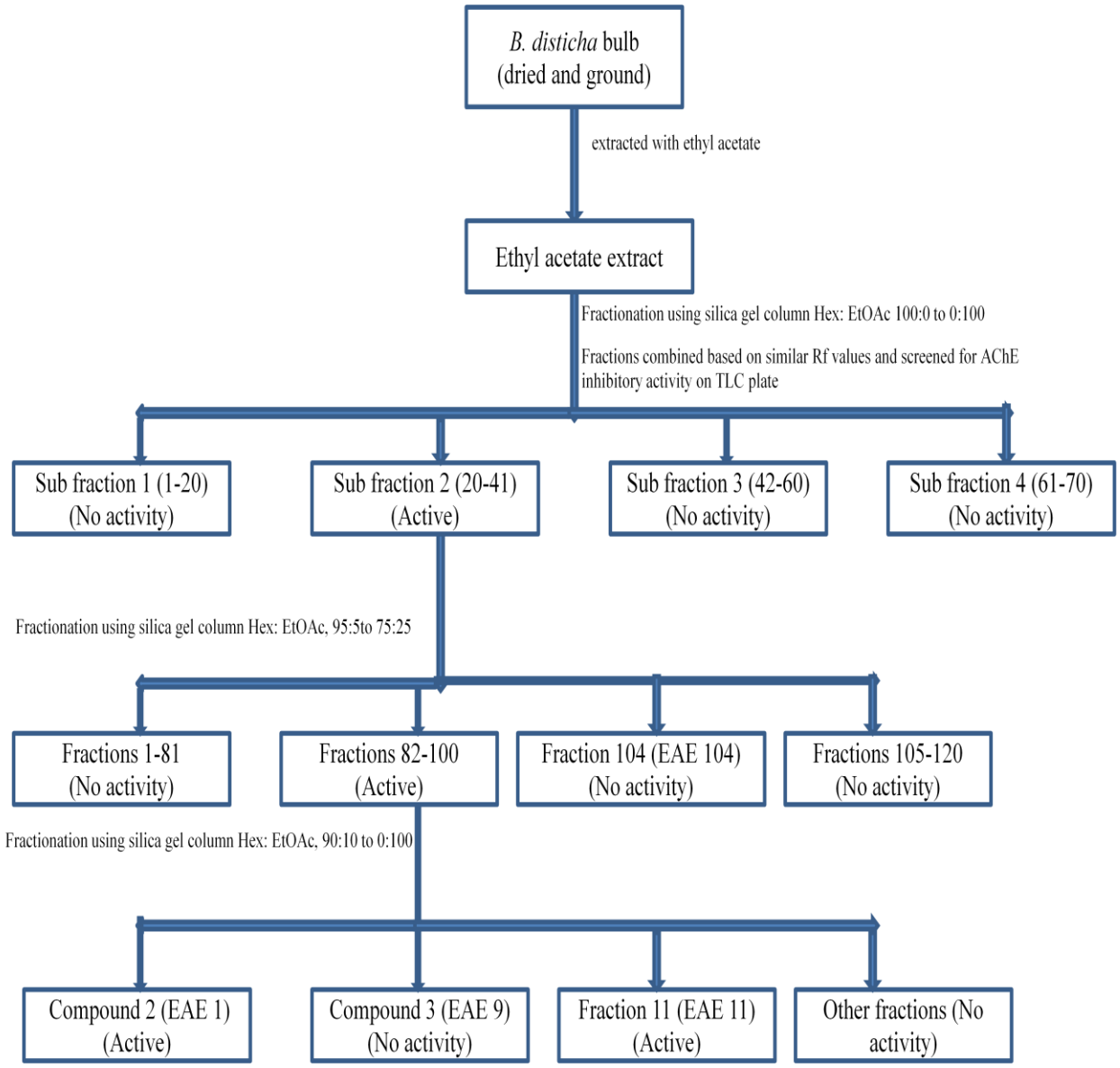

Figure 2.2 Schematic diagram of the fractionation and isolation of compounds from the ethyl acetate extracts of *Boophane disticha*.

2.7 Structural elucidation of compounds

2.7.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopy was performed using a 600 MHz Varian NMR. All spectra were recorded at room temperature in either deuterated chloroform or deuterated methanol. The chemical shifts were reported in ppm relative to tetramethylsilane (TMS). Structural characterizations were carried out using a combination of 1D (^{1}H, ^{13}C) and various 2D (Distortionless Enhancement by Polarisation Transfer - DEPT, Correlation Spectroscopy - COSY, Heteronuclear Single Quantum Coherence - HSQC and Heteronuclear Multiple Bond Correlation - HMBC) experiments.

2.7.2 Mass Spectrometry

The various samples were analysed using a WATERS 2695 HPLC separation module. Two Atlantis T3 columns (10 x 250 mm, 5 µ particle size) connected in series were used for the separation. UV-VIS detection was done on a WATERS PDA scanning from 200 – 600 nm. Mass spectrometry detection was performed using a WATERS SQD scanning from 100 – 1200 *m/z* with polarity (+/-) switching.

The SYNAPT G1 mass spectrometer was used in V-optics and operated in electrospray mode. Leucine enkephalin (50 pg/mL) was used as reference calibrant to obtain typical mass accuracies between 1 and 3 mDalton. The mass spectrometer was operated in positive and negative mode with a capillary voltage of 2.0 kV, the sampling cone at 30 V and the extraction cone at 4 V. The scan time was 0.1 s covering the 100 to 1000 Dalton mass range. The source temperature was 120ºC and the desolvation temperature was set at 450ºC. Nitrogen gas was used as the

nebulisation gas at a flow rate of 800 L/h. The software used to control the hyphenated system and do all data manipulation was MassLynx 4.1 (SCN 704).

2.8 Cytotoxicity studies

Compounds and fractions isolated from the bulbs of *B. disticha*, together with four very promising plants {*Ziziphus mucronata* (roots), *Lannea schweinfurthii* (roots), *Terminalia sericea* (roots) and *Crinum bulbispermum* (roots)}, selected based on their good antioxidant activity, were further investigated for their cytotoxicity and determination of their ability to prevent cell death induced by $A\beta_{25-35}$. *Piper capense* had good antioxidant activity but was not selected for further studies as the extracts were very toxic to the SH-SY5Y cells (results not shown). Methanol extracts of these plants were prepared as described in section 2.2.2. Residues were re-dissolved in DMSO to the desired test concentrations.

2.8.1 Cells and cell culture

SH-SY5Y (ATCC CRL-2266) cell lines were used for the cytotoxicity studies. Ethical clearance was obtained to carry out studies on the commercially purchased cell line (Appendix D). 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^{o}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 FCS-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 and plates were then incubated for 1 h at 37°C in a humidified incubator at 95% air and 5% CO_2 to allow for cellular reattachment.

2.8.2 Cell viability

2.8.2.1 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 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 in section 2.8.1 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 h. Thereafter, 20 µl of MTT solution (5 mg/ml) was added to the wells and further incubated for 3 h. 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 plate reader). 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.

2.8.2.2 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 h at 37°C. Cells were washed with Phosphate Buffered Saline (PBS), and 150 µl of elution medium (EtOH/AcCOOH, 50%/1%) 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 plate reader). 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.

2.8.3 Treatment with $A\beta_{25-35}$

$A\beta_{25-35}$ was reconstituted in sterile water at a concentration of 1 mM. Aliquots were incubated at 37°C for 72 h to form aggregated amyloid. During the experiment, $A\beta_{25-35}$ was directly added to cultured medium to achieve a final concentration of 20 µM. Three concentrations of each of the plant extracts, compound or fractions that were not toxic or presented low toxicity (as determined from the tests above), were selected to assess their protective effects. The cells were plated as described above, pre-treated with the plant extracts for 2 h before $A\beta_{25-35}$ treatment and then further incubated for 72 h. The viability of the cells was determined by the MTT and neutral red assays. Cell viability was expressed as a percentage of the control values.

2.9 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 expressed as the

percentage cell survival compared to the untreated control using a dose response curve. The curve was created and IC_{50} calculated using GraphPad Prism. Data obtained from mass spectroscopy were analysed using MassLynx 4.1 (SCN 704) software and the fragmentation patterns of the compounds isolated were identified with the Agilent ChemStation software which has a National Institute of Standards and Technology (NIST) library of mass fragmentations.