Boophone haemanthoides (Amaryllidaceae) and its bioactive compounds attenuate MPP*-induced toxicity in an in vitro Parkinson’s disease model.

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Abstract: Parkinson’s disease (PD) is a neurodegenerative disease that progresses with increasing age and some of its major symptoms include tremor, postural and movement related difficulties. Till date, the treatment of PD remains a challenge because available drugs only treat the symptoms of the disease or possess serious side effects. In light of this, new treatment options are needed, hence this study investigates the neuroprotective effects of an organic Boophone haemanthoides extract (BHE) and its bioactive compounds using an in vitro model of PD involving the toxin 1-methyl-4-phenylpyridinium (MPP*) and SH-SY5Y neuroblastoma cells. A total of seven compounds were isolated from BHE viz: distichamine (1), 1α,3α-diacetylmobowdine (2), hippadine (3), stigmaster-4-ene-3, 6-dione (4), cholest-4-en-3-one (5), tyrosol (6), and 3-hydroxy-1-(4`-hydroxyphenyl)-1-propanone (7). Six compounds (1, 2, 4, 5, 6, 7) were investigated and five showed neuroprotection alongside the BHE. This study gives insight into the bioactivity of the non-alkaloidal constituents of Amaryllidaceae since the isolated compounds and the BHE showed improved cell viability, increased ATP generation in the cells as well as inhibition of MPP*-induced apoptosis. Together, these findings support the claim that the Amaryllidaceae plant family could be a potential reserve of bioactive compounds for the discovery of neuroprotective agents.

Keywords: Amaryllidaceae; Boophone haemanthoides; Alkaloids; Terpenoids, Parkinson’s disease; Neuroprotection; Apoptosis.

1. Introduction

Parkinson’s disease (PD) is a neurodegenerative disease that worsens with increasing age and affects about 10 million people worldwide. The initial manifestations of the disease occur at approximately 60 years of age with females being less susceptible to the disease than males [1,2]. Although the incidence of PD has been strongly linked to age, a cross sectional study showed that approximately 30% of PD patients are younger than 65 years of age at the time of diagnosis [3]. The major symptoms of PD include tremor, postural and gait related challenges, bradykinesia (slowness of movements), hypokinesia (reduction in movement amplitude) as well as akinesia which is the absence of normal unconscious movements [2]. These symptoms result from the profound and selective loss of dopaminergic neurons in the substantia nigra pars compacta of the midbrain and the formation of Lewy bodies in the cytoplasm of neuronal cells [4].

Although the etiology of PD is not fully understood, studies have shown that the loss of dopaminergic neurons can be associated with a number of factors, key among which are oxidative...
stress and mitochondrial dysfunction [5,6]. Biochemically, the earliest signs of PD involve the impairment of the mitochondrial electron chain impairment, alteration of mitochondrial dynamics as well as an imbalance in calcium and iron homeostasis [7]. Following these changes, there is increased reactive oxygen species (ROS) generation in the mitochondria of neuronal cells, leading to a defect in the functioning of mitochondrial complex I which is believed to be a major contributor to dopaminergic neuronal cell degeneration in PD [8,9]. In addition, the synthesis of adenosine triphosphate (ATP) is negatively affected and, the reduction of cellular ATP in turn, drives dopaminergic neurons into programmed cell death (PCD) [10].

To understand the progression of PD in laboratory studies, the non-toxic chemical 1,2,3,6-methyl-phenyl-tetrahydropyrididine (MPTP) is often used to model the disease in vivo [11,12]. Upon crossing the blood-brain barrier (BBB), MPTP is converted by the enzyme mono amine oxidase B (MAO-B) in astrocytes, into the toxic metabolite 1-methyl-4-phenylpyridinium (MPP⁺), which leads to mitochondrial dysfunction [13]. MPP⁺ is known to cause the mitochondrial permeability transition pore to open which in turn, leads to a change in the mitochondrial membrane potential, increased ROS accumulation in the cells which alters ATP levels and eventually induce apoptosis [7].

Although there is no specific cure for PD, levodopa which is a dopamine-replacement therapy, is currently in use for the treatment of PD symptoms. However, the prolonged use of levodopa has been shown to be associated with some side effects, including the enhancement of oxidative stress and the acceleration of degeneration of residual dopaminergic neurons in PD patients using this medication [10], thus necessitating the search for alternative treatment options. A number of in vitro and in vivo studies have shown that herbal medicines, phytochemicals as well as other plant-derived bioactive compounds and dietary supplements could ameliorate the effects of PD [14-16].

**Boophone haemanthoides** is a deciduous, winter-growing bulb plant that survives in almost all weather conditions including during the moist winter season as well as in hot and dry summer temperatures. It belongs to the Amaryllidaceae plant family and is endemic to the winter and rainfall regions of South Africa and Namibia [17]. The Amaryllidaceae plant family which comprises of over 800 species and 80 genera is well distributed in the tropical regions of the world and found in abundance in the Southern Africa region of Africa [18,19]. Plants in this family are well known for their alkaloids and so far, more than 630 alkaloids have been isolated from these plants, many of which are known to possess a number of biological activities including antibacterial, anti-cancer and neuroprotective activities [20-22].

*B. haemanthoides* was reported to be used traditionally by the Khoi-San tribe in the Northern Cape Province of South Africa for the treatment of asthma and for relieving knee pain [23]. Furthermore, a number of bioactive Amaryllidaceae alkaloids have been isolated from *Boophone disticha*, another member of the genus *Boophone*, including distichamine, buphanidrine, buphanisine, crinine and distichaminol [24-26]. In one study, *B. disticha* was reported to show neuroprotective activities in 6-hydroxydopamine (6-OHDA)-induced SH-SY5Y toxicity by inhibiting ATP degeneration [27] while distichamine, buphanidrine and buphanisine have also been reported to show a strong affinity for the neurotransmitter, serotonin [21]. Importantly, the approval of galanthamine, an Amaryllidaceae alkaloid for the treatment of Alzheimer’s disease by the US food and drug administration (FDA) has made alkaloids from this plant family potential sources of novel neuroprotective agents [28]. Thus, the present study investigates the neuroprotective activities of *B. haemanthoides* and its isolated compounds on MPP⁺-induced neuronal toxicity in SH-SY5Y neuroblastoma cells.

2. Materials and Methods

Organic solvents such as acetonitril (ACN, HPLC grade), methanol, dichloromethane, ethyl acetate, and hexane, were supplied by Merck (Cape Town, South Africa). Thin layer chromatography (TLC) was performed on normal-phase (Merck) Silica gel 60 PF254 pre-coated aluminum plates. Column chromatography was conducted on silica gel 60 H (0.040–0.063 mm particle size, Merck, Cape Town, South Africa) and Sephadex LH-20 (Sigma-Aldrich, Cape Town, South Africa).
NMR spectra were recorded on an Avance 400 MHz NMR spectrometer (Bruker, Rheinstetten, Germany) in deuterated chloroform, using the solvent signals as the internal reference. GC-MS analysis was performed using an Agilent Technologies 7820A coupled with MSDB977E. Samples of ~1.0 mg were dissolved in 1.0 mL of CHCl₃ and 1.0 μL was injected directly into the GC-MS operating in the electron ionization (EI) mode at 70 eV and utilizing HP5 MS column (30m 0.25mm i.d., film thickness 0.25 μm). The temperature gradient performed was adjusted as 40-80 °C (8 min), 80-220 °C (10 °C/min), hold at 220 °C for 5 min, 220-300 °C (20 °C /min) and 10 min hold at 300 °C. The injector and detector temperatures were both at 250 °C, with source and MS Quad at 230 °C and 150 °C, respectively, and the flow-rate of carrier gas (He) was 1.5 mL/min. A split ratio of 1:3 was applied.

2.2. Identification and collection of plant material

Bulbs of *B. haemanthoides* were collected from cultivated plants and their identities were authenticated by Prof Christopher Cupido, Botany Department, University of Fort Hare. A voucher specimen (UFH 2020-3-01) of the plant was deposited in the Giffen Herbarium of the University of Fort Hare.

2.3. Isolation of compounds

Fresh bulbs (~3.2 kg) were blended and extracted with methanol for 2 days. The total extracts (BHE) were combined and evaporated under reduced pressure at 40 °C to yield ~150 g. The extract (~120 g) was loaded on a silica gel column (18 X 35 cm) and eluted with a gradient mixture of hexane and ethyl acetate of increasing polarity, similar fractions were pooled together according to their TLC profile to give 20 main fractions. The chromatographic manipulation of fractions 4, 7, 8, 10 and 12 yielded 10 known compounds in small quantities except for compounds 4-7 included in this study. More experimental details are contained in our recently published paper [29]. Other fractions containing alkaloids were subjected to chromatographic purification and resulted in the isolation of compounds 1-3 as follows: fraction 13 (1.1 g) was chromatographed on sephadex using isocratic 10 % aqueous ethanol, and prep-TLC using DCM:MeOH (95:5) to yield compound 1 (40 mg). Fraction 14 chromatographed under the same conditions to yield compound 2 (27 mg). Fraction 6 was subjected to HPLC purification using ACN:H₂O gradient (from 50 to 100 ACN in 30 minutes) to yield compound 3 (~1.0 mg)

2.4. Physical and spectroscopic data of the isolated compounds

**Distichamine (1) GC-MS: Rt 28.048 min; MS 329.2 (C₂₃H₂₂NO₅), m/z: 398.5, 285.3, 207.1, 137.1; [α]D²⁵ −45.4 (c 0.1 in CHCl₃).¹H NMR (400 MHz, CDCl₃): 2.17 (1H, ddd, J = 13.0, 10.4, 6.7 Hz, H₂ exo), 2.30 (1H, ddd, J = 12.8, 8.5, 3.6 Hz, H₃ endo), 2.38 (1H, ddd, J = 17.3, 11.5, 1.1 Hz, H₄ endo), 2.49 (1H, dd, J = 17.3, 6.7 Hz, H₄ endo), 2.86 (1H, ddd, J = 15.0, 13.4, 6.7 Hz, H₂ endo), 3.38 (1H, ddd, J = 13.0, 10.4, 3.6 Hz H₂ exo), 3.51 (1H, dd, J = 10.8, 6.8 Hz, H₆ endo), 3.74 (1H, d, J = 17.4 Hz, H₇ endo), 3.92 (1H, d, J = 17.4 Hz, H₇ endo), 4.12 (1H, d, J = 17.4 Hz, H₇ endo), 5.37 (1H, d, J = 1.1 Hz, H₆), 5.84/5.85 (2H, d/each, J = 1.5 Hz, OCH₂O), 6.91 (1H, s, H₁₀).¹³C NMR (100 MHz, CDCl₃): 30.3 (t, C₃), 41.4 (t, C₁₀), 49.8 (s, C₁₁), 52.5 (t, C₁₂), 55.6 (q, 3-OCH₃), 57.7 (t, C₆), 58.8 (q, 7-OCH₃), 66.1 (d, C₁₀a), 100.2 (d, C₁₀), 100.3 (t, OCH₂O), 102.0 (d, C₆), 116.6 (s, C₉), 133.4 (s, C₁₀a), 135.3 (s, C₈), 139.8 (s, C₇), 147.5 (s, C₇), 173.2 (s, C₇), 198.7 (s, C₁₇).

¹α,3α-diacetylnelbowdine (2): GC-MS: Rt 35.508 min; MS 403.4 (C₂₅H₂₅NO₅), m/z: 344.5, 284.3, 254.2, 204.2; [α]D²⁵ +16.1 (c 0.1 in CHCl₃).¹H NMR (400 MHz, CDCl₃); ¹³C NMR (100 MHz, CDCl₃) see table 1.

**Hippadine (3), GC-MS: Rt 34.807 min; Mass: 263.06 (C₁₅H₁₆NO₃), m/z: 205.2, 177.1, 131.2; ¹H NMR, δ 8.03 (d, H₂, J = 3.5 Hz), 7.97 (s, H₃), 7.91 (d, H₁, J = 7.6 Hz), 7.74 (d, H₃, J = 7.6 Hz), 7.65 (s, H₁₀a), 7.46 (t, H₂, J = 7.6 Hz), 6.88 (d, H₁n, J = 3.5 Hz), 6.15 (s, OCH₂O) [30].

2.5. Cell culture and maintenance
The human neuroblastoma SH-SY5Y cells were generously donated by the Blackburn Laboratory, University of Cape Town, South Africa. Cells were grown in Dulbecco’s Modified Eagle’s medium (DMEM, Gibco, Life Technologies Corporation, Paisley, UK), supplemented with 10% fetal bovine serum, (FBS, Gibco, Life Technologies Corporation, Paisley, UK), 100 U/mL penicillin and 100 µg/mL streptomycin (Lonza Group Ltd., Verviers, Belgium). Cultures were incubated at 37 °C in humidified air with 5% CO₂ and the medium was changed every three days. Cells were sub-cultured when 70 to 80 percent confluency was attained, using a solution of 0.25 % trypsin EDTA (Lonza Group Ltd., Verviers, Belgium).

2.6. Treatments

Stock solutions of 40 mg/mL of BHE as well as the isolated compounds were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis MO, USA) from which final concentrations were made in cell growth medium. To determine the optimum concentration of BHE and compounds to be used for the neuroprotection studies, SH-SY5Y cells were plated at a density of 10,000 cells/well and treated with concentrations (2.5, 5 and 10 µg/mL) of BHE as well as the compounds (1, 2, 4-7) (Table 1). The vehicle-treated cells (cells treated with the same concentration of DMSO similar to that of the highest concentration of extract) were used as control. Additionally, a stock solution of 50 mM MPP⁺ (Sigma-Aldrich, St Louis MO, USA) was prepared in un-supplemented DMEM and further dilutions were made in supplemented growth medium to obtain a final concentration range of 500 to 2500 µM, which were added to the cells, while the cells that were not exposed to MPP⁺ served as control. All treatments lasted for 24 hours and the 2.5 µg/mL concentration was selected for neuroprotection studies. Thus, cells were plated as above and pre-treated with 2.5 µg/mL of BHE and the compounds for 2 hours prior to the addition of 2000 µM MPP⁺ and the treatments were incubated for 24 hours. The untreated cells served as control and 25 µM of rutin (RT), a known neuroprotective agent, was used as positive control.

Table 1. Table showing list of compounds isolated from BHE

| Compound | Name                                      |
|----------|-------------------------------------------|
| 1        | Distachamine                              |
| 2        | 1α,3α-diacetylnerbowdine                  |
| 3        | Hippadine                                 |
| 4        | Stig mast-4-ene-3, 6-dione                |
| 5        | Cholest-4-en-3-one                        |
| 6        | Tyrosol                                   |
| 7        | 3-Hydroxy-1-(4-hydroxyphenyl)-1-propanone |

2.7. Cell viability assays

The MTT (Sigma-Aldrich, St Louis MO, USA) cell viability assay was used to determine the viability of cells following treatment with both plant extracts and MPP⁺. Cells were seeded in 96-well plates and treated as stated above after which the MTT assay was performed. After treatment, 10 or 20 µL (depending on well volume) of 5mg/mL MTT solution in PBS (Lonza Group Ltd., Verviers, Belgium) was added to each well and left to incubate in the dark at 37 °C for 4 hours. After incubation, the medium containing the MTT dye was discarded and the MTT formazan was solubilized with 100 µL of DMSO for absorbance reading using a microplate reader (BMG Labtech Omega® POLARStar) at a wavelength of 570 nm. Cell viability was calculated and expressed as percentage of control.

2.8. Cell morphology

To visualize changes in the morphology of the SH-SY5Y cells following the respective treatments, cells were seeded in 96 well plates at a density of 10 000 cells per well and were pre-
treated with 2.5 µg/mL of BHE and 25 µM of rutin for 2 hours prior to the addition of 2000 µM MPP⁺. After the 24 hours treatment, changes in morphology for the various treatment conditions were observed using the Zeiss inverted light microscope with 10X objective lens. Images were captured using the Zeiss software version 2.3.

2.9. Adenosine triphosphate assay

The Mitochondrial ToxGlo ATP assay kit (Promega, USA) was used to investigate ATP levels in the cells. Briefly, cells were plated at a density of 10 000 cells per well in a white 96-well plate and after attachment, cells were treated as per neuroprotection assay above. After treatment, cells were processed according to the manufacturer’s protocol and luminescence intensity was read using the microplate reader (BMG Labtech Omega® POLARStar) and readings were expressed as percentages of control.

2.10. Caspase 3/7 apoptosis assay

To investigate apoptosis in the cells, the Caspase 3/7 assay kit (Promega, USA) was used to estimate levels of caspase 3/7 activity in the cells according to manufacturer’s instructions. Briefly, cells were plated in a white 96-well plate at a density of 10 000 cells per well and allowed to attach overnight, after which cells were pre-treated with BHE and compounds before the addition of 2000 µM MPP⁺. Treatments lasted for 24 hours and at the end of the experiments, equal volumes of Caspase 3/7 assay mix were added to each well and luminescence intensity was read with a microplate reader (BMG Labtech Omega® POLARStar). Luminisence intensities of treated cells were expressed as percentages of control.

3. Results

3.1. Isolation and identification of the chemical constituents

*B. haemenroids* collected in South Africa were previously investigated and eight alkaloids including distichamine (1) were described [26]. Recently, triterpenes and other minor constituents including compounds 4-7 were described in our previous publication [29]. In this study we report on the isolation and identification of three additional known alkaloids *viz:* distichamine (1), 1α,3α-diacetylnerbowdine (2) and hippadine (3) (Figure 1) as well as the neuroprotection potential of compounds 1, 2, 4-7 against MPP⁺-induced toxicity in an *in vitro* PD model.

Distachamine (1) 1α,3α-diacetylnerbowdine (2) Hippadine (3)
Compound 3 described here as a natural product for the first time. It showed a typical positive reaction of alkaloids with dragendorff reagent on TLC. The GC-MS analysis showed a single peak at Rt 36.405 minute, with m/z 403.4, corresponding to molecular formula C_{21}H_{25}NO_{7}. ¹H NMR Shows an aromatic signal appeared at δH 6.11 (s, H_{10}); two signals of methylenedioxy protons at 5.83 d (J = 1.3 Hz); 5.79 d (1.3) (OCH_{2}O); two proton signals of two methines appeared at 5.71 br t (J = 2.6 Hz) and 5.15 br quint (J = 2.6 Hz) of H_{1} and H_{3} respectively; two signals of a methylene group at 5.71 br t (J = 17.3 Hz) and 3.76 d (J = 17.3 Hz) and belong to C_{6}; a proton signal at 3.48 dd (J = 5.5, 12.2 Hz); two acetoxy groups at 1.97 and 1.90; a methoxy at 3.95, in addition to signals of four methylene groups (C_{11}, C_{12}, C_{2} and C_{4}) (Table 2). ¹³C NMR showed 21 carbon signals classified using DEPT-135 and HSQC into a methylenedioxy carbon (δC 100.5), a quaternary carbon (δC 46.8, C_{10b}), six aromatic carbons including a methine (δC 117.2, 140.4, 133.2, 148.4, 97.1), a methoxy (δC 59.1), six methylenes (δC 28.6, 30.5, 57.5, 38.2, 51.0, 100.5 of C_{2}, C_{4}, C_{6}, C_{11}, C_{12} and OCH_{2}O respectively), and two oxygenated methines (δC 68.3 and 68.0), and two acetates (21.2/170.5, 21.3/170.1).

Figure 1. Chemical structures of compounds 1-7 isolated from B. haementhoids.

Comparison of the given NMR data with literature indicated a crinane alkaloid with two acetoxy and a methoxy group as shown in figure 1. Other 2D NMR experiments (COSY, HMBC, and NOESY) confirmed the structure and the positions of the two acetates and the methoxy groups. The COSY spectra (Figure 2) showed correlations (coupling) of the methylene protons at C_{2} with the methine protons at C_{1} and C_{3}, while the H_{1} only coupled with H_{2} protons, also, the methylene protons at C_{4} has correlations with the methines protons at C_{3} and C_{4a}, which indicated the positions of the two acetate groups at C_{1} and C_{3}. The positions were further confirmed by HMBC spectra (Figure 2) which showed correlations between H_{2} and carbons C_{4}, C_{10b}, C_{7}, C_{5}, H_{1}/C_{2}, C_{10b}, C_{3}, C_{4a}, C_{10a}, CO; H_{2}/C_{2}, C_{4}, C_{4a}, C_{1}, CO and H_{2a}/C_{4}, C_{11}, C_{12}, C_{6}, C_{10a}. On the other hand, the methoxy group was allocated at C_{7} from the HMBC correlations of the methoxy protons with C_{7}; H_{2a}/C_{10a}, C_{4a}, C_{10b}, C_{7}, C_{3} and H_{6}/C_{12}, C_{4a}, C_{6a}, C_{10a}, C_{7}. Careful literature review proposed the structure as given in figure 1.
The beta orientation of C₁₁ and C₁₂ were proposed tentatively due the fact that, the compound has the same optical rotation with nerbowdine, which was isolated from the same species and the precursor of 2. The alpha orientation of the acetate groups at position C₁ and C₃ partially proved by the weak coupling of the equatorial protons at C₁ and C₃. On the other hand, NOESY spectra showed correlations between H₁β/ H₁0, H₂α, H₃β, H₁exo, and H₁endo; while H₃β showed correlation with H₂α, H₃β, H₆α and H₉β and these indicate the equatorial protons (at C₁ and C₃) are in β positions (Figure 3).

This is the first report on the isolation of the diacetate from a natural source. However, the same compound (2) was described in literature as the diacetate synthetic derivative of nerbowdine (also called haemanthine, hemanthine, and buphantine) [31].

Distichamine has been previously reported from B. haementhoides [26], B. distichia [21] and Amaryllis belladonna [32]. The isolation of mixed alkaloidal skeletons such as crinine (compounds 1, 2) and lycorine (3) types is a common feature of the alkaloid biosynthesis from all members of the amarylloideae subfamily, which reflects the ability of the dynamic enzymatic systems of these subfamily members to biosynthesize different skeletons from the 4’-O-methylnorbeladine [33].

Table 2. ¹H (400 MHz) and ¹³C NMR (100 MHz) data for compound 2 in CDCls

| C  | δc  | δH (JHz)    | HMBC (H→C) |
|----|-----|-------------|------------|
| 1  | 68.3  | 5.71 br t (2.6) | C₂, C₁₀b, C₄α, C₃, CO |
| 2  | 28.6 t | 1.95* (2α)       |            |
|    |       | 2.29 dq (2.6, 16.2) (2β) | C₄, C₁₀b, C₄α, C₁, C₃ |
| 3  | 68.0  | 5.15 br quint (2.6) | C₂, C₃, C₄α, C₁, C₃ |
| 4  | 30.5 t | 2.08*, (4α)       |            |
|    |       | 1.46 ddd (3.3, 12.2, 15.5), (4β) | C₁₀b, C₄α |
| 4a | 59.9  | 3.48 dd (5.5, 12.2) | C₁, C₁₁, C₁₂, C₇, C₁₀a |
| 6  | 57.5  | 4.13 d (17.3) (6α) | C₁₁, C₁₂, C₁₀a, C₈, C₁₀b, C₇, C₉ |
|    |       | 3.76 d (17.3), (6β) | C₁₂, C₄α, C₈, C₁₀b, C₇, C₉ |
| 6a | 117.2 | s            |            |
| 7  | 140.4 | s            |            |
| 8  | 133.2 | s            |            |
| 9  | 148.4 | s            |            |
| 10 | 97.1  | 6.11 s       | C₁₀a, C₈, C₁₀b, C₇, C₉ |
| 10a| 137.2 | s            |            |
| 10b| 46.8  | s            |            |
| 11 | 38.2 t | 1.99* exo   |            |
|    |       | 1.88** endo  |            |
| 12 | 51.0 t | 3.30 ddd (13.6, 10, 3.2) exo | C₆ |
3.2. Dose response of BHE and isolated compounds

In order to ascertain the optimum concentrations of BHE and isolated compounds to be used for neuroprotection studies, the MTT cytotoxicity assay was performed in SH-SY5Y cells treated with 2.5, 5 and 10 µg/mL of either extracts or individual compounds. Figure 4a shows that BHE had no impact on SH-SY5Y cell viability and the 2.5 µg/mL concentration increased cell viability the most (107.65%). Furthermore, all compounds showed either increased or had no significant effect in cell viability at all concentrations tested except for compound (2) which showed a significant reduction in cell viability at all treatment concentrations (Figure 4b). Taken together, these results indicate that BHE and isolated compounds show no cytotoxicity in SH-SY5Y cells at the tested concentrations and the 2.5 µg/mL concentration was chosen for further neuroprotection studies.

![Figure 4](image)

**Figure 4.** Dose-response of BHE and compounds. MTT assay cytotoxicity on SH-SY5Y cells treated with increasing concentrations (2.5, 5 and 10 µg/mL) of (a) BHE and (b) compounds for 24 hours and each bar represents mean cell viability expressed as percentage of control. * indicate significance at $p<0.05$.

3.3. Dose response of MPP⁺ in SH-SY5Y

To confirm the concentration of MPP⁺ to be used for evaluating neuronal toxicity in the SH-SY5Y cells, the MTT cytotoxicity assay was again performed following exposure of the cells to a range of 500 to 2500 µM of MPP⁺ for 24 hours. Figure 5 shows a concentration-dependent decrease in the viability of the cells and the 2000 µM concentration which was found to reduce cell viability to about 43% when compared to control was chosen for further neuroprotection studies. This is also similar to our previously published report on the effects of MPP⁺ in these cells [34].
Figure 5. MPP$^+$ induced cytotoxicity in SH-SY5Y cells. Cells were exposed to increasing concentrations of MPP$^+$ (500µM – 2500 µM) and allowed to incubate for 24 hours. MTT assays were performed and results showed a concentration-dependent decrease in cell viability when compared to control. Each bars represent mean percentage cell viability and significance of difference is indicated with* (p<0.05), ** (p<0.01) *** (p<0.001), and **** (p<0.0001).

3.4. BHE and isolated compounds protect SH-SY5Y cells from MPP$^+$-induced toxicity

To investigate the neuroprotective activities of BHE and isolated compounds, SH-SY5Y cells were plated and pre-treated with 2.5 µg/mL of either BHE or RT, the standard neuroprotective agent for 2 hours before exposure to 2000 µM of MPP$^+$ followed by MTT assays after 24 hours. Figure 6a shows that BHE at 2.5 µg/mL significantly improved cell viability following MPP$^+$ toxicity. Indeed, compared to control, cell viability reduced to about 51% in the MPP$^+$ treated group and following pre-treatment with BHE and RT, cell viability increased to 87% and 79% respectively. Similarly, cell viability was also improved in cells pre-treated with the compounds and as expected, the compound 2 showed no neuroprotective activity with cell viability at approximately 50% which discouraged further investigation (Figure 6b). Together, these results suggest that BHE and the isolated compounds could attenuate MPP$^+$-induced toxicity in SH-SY5Y cells.
Figure 6. BHE and compounds show protection in SH-SY5Y cells. Cells were pre-treated with extracts (a) and compounds (b) before exposure to MPP\(^+\) for 24 hours. Each bar represents mean percentage cell viability relative to control and significance of difference indicated with * (p<0.05), ** (p<0.01) and *** (p<0.001) when extract/compounds are compared to MPP+ and \(\phi\) (MPP+ vs control).

3.5. BHE improves cell morphology in SH-SY5Y after MPP\(^+\) insult

Furthermore, morphology of the cells was observed after treatments as per neuroprotection experiment and Figure 7 shows that compared to the control cells, MPP\(^+\) treatment indeed, induced loss of neuronal cells evidenced by the changes in cell morphology which include loss of neuron projections and roundness of cells. However, pre-treatment of cells with 2.5 µg/mL of BHE and 25 µM RT improved cell morphology to almost that of control cells.

Figure 7. BHE and compounds inhibit SH-SY5Y morphological changes induced by MPP\(^+\). Cellular morphology of SH-SY5Y cells pre-treated with BHE and compounds (2.5 µg/mL) prior to exposure to 2000 µM MPP\(^+\) for 24 hours and images were captured using the light microscope at 100X magnification.
3.6. BHE and isolated compounds mitigate MPP⁺-induced ATP depletion in SH-SY5Y cells

As a mechanism of toxicity, MPP⁺ induces ATP degeneration in neuronal cells by the inhibition of mitochondrial complex I [35]. Thus, to further elucidate the mechanism of neuroprotection induced by BHE and isolated compounds, levels of ATP were measured in the cells after treatment as per the neuroprotection experiment above. The results show that MPP⁺ depleted ATP levels in the cells to approximately 50% and following pre-treatment with BHE, ATP levels increased in the cells to approximately 79% (Figure 8a). Additionally, a similar trend was observed for the cells pre-treated with compounds (Figure 8b). Together, these results indicate that BHE and the compounds could rescue SH-SY5Y cells from MPP⁺-induced ATP depletion.

![Figure 8](image.png)

**Figure 8.** BHE and compounds inhibit MPP⁺-induced ATP degeneration. Cells were pre-treated with 2.5 µg/mL of extracts (a) and compounds (b) before exposure to 2000 µM of MPP⁺ for 24 hours and ATP levels assessed. Each bar represents mean percentage level relative to control and significance of difference indicated with * (p<0.05) and ** (p<0.01) when extract/compounds are compared to MPP⁺ and φ (MPP⁺ vs control).

3.7. BHE and isolated compounds inhibit MPP⁺-induced apoptosis in SH-SY5Y cells

To further ascertain the mechanism involved in the neuroprotection of BHE and compounds in SH-SY5Y cells, the levels of cellular apoptosis were assessed using caspase 3/7 as a marker. Caspases belong to the family of cysteine proteases which drive apoptosis in cells and carry out their function by the cleavage of substrates [36,37]. Caspases could be initiator caspases (caspases 8 and 9) or executioner caspases (caspase 3 and 7), the latter being frequently used as markers of apoptosis [38,39]. To investigate apoptosis, cells were treated as for the neuroprotection studies above and caspase 3/7 activities were measured. Figure 9a shows that BHE mitigates MPP⁺-increased levels of caspase 3/7 activity in the SH-SY5Y cells. In specifics, MPP⁺ increased levels of caspase 3/7 to about 4 times the value of the control and pre-treatment with BHE was found to reduce this activity to about 1.5 times the control. Furthermore, all the compounds also protected SH-Y5Y cells from MPP⁺-induced apoptosis as expected (Figure 9b). Altogether, these results indicate that the inhibition of apoptosis by BHE and the compounds is a neuroprotection mechanism.
Figure 9. BHE and compounds reduce MPP+-induced caspase 3/7 activity. Cells were pre-treated with 2.5 µg/mL of extracts (a) and compounds (b) before exposure to 2000 µM of MPP+ for 24 hours and activity of caspase 3/7 was determined. Each bar represents mean percentage level relative to control and significance of difference indicated with * (p<0.05), ** (p<0.01) and **** (p<0.0001) when extract/compounds are compared to MPP+ and ф (MPP+ vs control).

4. Discussion

The burden of PD remains an increasing global trend and since the first scientific report of PD related conditions over 200 years ago, the treatment of this condition has remained a challenge [40,41]. The current treatment for PD is the FDA-approved drug levodopa which appears to only address PD-associated symptoms [42]. However, the prolonged use of levodopa is known to be associated with some side effects [43,44]. Considering this, research into the discovery of safe and effective alternative treatment options with potent neuroprotective effects is plausible. Thus, plant-derived natural products that are capable of slowing down or protecting against neuronal cell death in PD, have gained attention in recent years. Plants of the Amaryllidaceae family have been reported to improve memory in traditional use for the treatment of neurological disorders [45] and these effects were confirmed through a series of pharmacological studies which indicated the neuroprotective potential of this family [46-49]. The unique family of alkaloids called Amaryllidaceae alkaloids are linked to the neuroprotective effects of this plant family [50-52]. Galanthamine which is the most notable of these alkaloids, is a known acetylcholinesterase inhibitor that has been approved for use in the treatment of Alzheimer’s disease in clinical settings [53]. In the present study, we investigated the neuroprotective potentials of BHE and its bioactive compounds in a MPP+ model of PD.

Our findings show that a total of seven compounds including three known alkaloids, two triterpenes and tyrosol as well as 3-hydroxy-1-(4’-hydroxyphenyl)-1-propanone were isolated from BHE (Table 1 and Figure 1). Furthermore, the neuroprotective activity of the compounds 1, 2, 4-7 were determined alongside the total extract, BHE. Findings show that BHE and the compounds protected SH-SY5Y cells from MPP+-induced toxicity at the 2.5 µg/mL concentration. This suggests that in the presence of a neuronal insult, the extract under study as well as the isolated compounds could prevent loss of dopaminergic neurons in the substantia nigra pars compacta of the midbrain which is a classical hallmark of PD. This finding is consistent with what we have previously reported in our laboratory for Crossyne guttata, Nerine humilis and Clivia miniata belonging to the same Amaryllidaceae family [54,55] and support the traditional uses of Amaryllidaceae members for treatment of mental and neuro-related diseases [45].
Furthermore, all the isolated compounds showed neuroprotective activity except for 1α, 3α-diacetylnervobaridine (2) which showed toxicity to the SH-SY5Y cells even at the 2.5 µg/mL concentration. Interestingly, while most of the activities of the plants of the Amaryllidaceae family have been attributed to their alkaloids [20-22], the triterpenes isolated from the BHE also showed potent neuroprotective activity comparable to the amaryllidaceae alkaloids [56]. In specifics, the triterpene, Cholest-4-en-3-one showed the highest neuroprotective activity which is consistent with the cell viability data (Figure 4b and 6b), albeit not significant compared to other compounds. In support of our findings, previous studies have shown that pre-treatment with triterpenes protected rat primary cultures and SH-SY5Y cells against the toxicity induced by exposure to glutamate and 6-hydroxydopamine respectively [57,58].

As part of the pathogenesis of PD, neuronal cells show a reduction in ATP production as a result of changes in the mitochondrial electron transport chain [59,60]. As a mechanism of action, MPP+ is known to increase levels of ROS in the mitochondria of neuronal cells thus leading to reduced ATP levels in the cells [14]. It is also well established that an increase in the levels of intracellular ATP is an indication of improved mitochondrial function [61,62]. In the current study, there is evidence that BHE and the tested compounds improved ATP production in the cells, which suggests an improvement in mitochondrial function critical for cell survival. A previous study has reported that Boophone disticha, the other member of the Boophone genus, protected SH-SY5Y cells from 6-hydroxydopamine-induced dopaminergic neuronal death by restoring ATP levels in the cells [27]. As observed with the cell viability neuroprotection results, the triterpene 5 also had the best outcome as it increased ATP generation the most when compared to other compounds.

Furthermore, the MPP+-induced reduction of ATP levels, caused programmed cell death which in this case is apoptosis [63]. However, BHE and the compounds showed inhibition of the apoptotic pathway in the cells, as evidenced by the reduction of caspases 3/7 activity which is an indication of improved cell survival. Thus, the inhibition of apoptosis was found to improve cellular function.

5. Conclusions

In this study, the neuroprotective activity of BHE and isolated compounds was investigated in an in vitro PD model using MPP+. Seven compounds were isolated from B. haementhoids and six of the compounds were further investigated for their neuroprotective potentials. Our results show that whereas MPP+ induced cellular toxicity through the inhibition of cell viability, reduction in ATP levels and the induction of apoptosis, pre-treatment with BHE and the compounds attenuated these effects of MPP+. Furthermore, five of the six compounds investigated, displayed varying levels of neuroprotection. Due to the wide spectrum of activities demonstrated by the Amaryllidaceae alkaloids, other metabolites like triterpenes were overlooked. Surprisingly and interestingly, triterpenes and other non-alkaloidal metabolites showed strong neuroprotection activity with large safety margins when compared to alkaloids. Altogether, this study demonstrates that the Amaryllidaceae plant family may be useful in the exploration of potential neuroprotective agents and more mechanistic and in vivo studies will be required in future studies to further elucidate their activities.
**Author Contributions:** For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used “Conceptualization, AAH; methodology, ASA and SIO, validation, AAH, SIO, and OEE, formal analysis, SIO and AAH; investigation, ASI, and SIO; resources, AAH and OEE; data curation, AAH, SIO, and OEE; writing—original draft preparation, ASI and SIO; writing—review and editing, AAH, SIO, and OEE; supervision, AAH; project administration, AAH; funding acquisition, AAH. All authors have read and agreed to the published version of the manuscript.

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