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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-diacetylnerbowdine (**2**), hippadine (**3**), stigmast-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-tetrahydropyridine (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 (6-0HDA)-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 MSD5977E. Samples of ~ 1.0 mg were dissolved in 1.0 mL of CH_2Cl_2 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 m. 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 Departmen, 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:H_2O 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   ($C_{18}H_{19}NO_5$), m/z: 398.5, 285.3, 207.1, 137.1; $[\alpha]_{25}^D$ −45.4 (c 0.1 in CH_2Cl_2). ^{1}H NMR (400 MHz, CDCl$_3$): 2.17 (1H, ddd, J = 13.0, 10.4, 6.7 Hz, H_{11} exo), 2.30 (1H, ddd, J = 12.8, 8.5, 3.6 Hz, H_{11} endo), 2.38 (1H, ddd, J = 17.3, 11.5, 1.1 Hz, H_4), 2.49 (1H, dd, J = 17.3, 6.7 Hz, H_4), 2.86 (1H, ddd, J = 15.0, 13.4, 6.7 Hz, H_{12} endo), 3.38 (1H, ddd, J = 13.0, 10.4, 3.6 Hz H_{12} exo), 3.51 (1H, dd, J = 10.8, 6.8 Hz, H_{4a}), 3.74 (3H, s, 3-OCH$_3$), 3.73 (1H, d, J = 17.4 Hz, H_6), 3.95 (1H, s, 7-OCH$_3$), 4.12 (1H, d, J = 17.4 Hz, H_6), 5.37 (1H, d, J = 1.1 Hz, H_2), 5.84/5.85 (2H, d/each, J = 1.5 Hz; OCH$_2$O), 7.71 (1H, s, H_{10}). ^{13}C NMR (100 MHz, CDCl$_3$): 30.3 (t, C_4), 41.4 (t, C_{11}), 49.8 (s, C_{10b}), 52.5 (t, C_{12}), 55.6 (q, 3-OCH$_3$), 57.7 (t, C_6), 58.8 (q, 7-OCH$_3$), 66.1 (d, C_{4a}), 100.2 (d, C_{10}), 100.3 (t, OCH$_2$O), 102.0 (d, C_2), 116.6 (s, C_{6a}), 133.4 (s, C_{10a}), 135.3 (s, C_8), 139.8 (s, C_7), 147.5 (s, C_9), 173.2 (s, C_3), 198.7 (s, C_1).

$1\alpha,3\alpha$-diacetylnerbowdine (**2**): GC-MS: Rt 35.508 min; MS 403.4   ($C_{21}H_{25}NO_7$), m/z: 344.5, 284.3, 254.2, 204.2; $[\alpha]_{25}^D$ −16.1 (c 0.1 in CH_2Cl_2). ^{1}H NMR (400 MHz, CDCl$_3$); ^{13}C NMR (100 MHz, CDCl$_3$) see table 1.

Hippadine (**3**), GC-MS: Rt 34.807 min; Mass: 263.06 ($C_{16}H_{10}NO_3$), m/z: 205.2, 177.1, 131.2; ^{1}H NMR, $_H$ 8.03 (d, H_{12}, J = 3.5 Hz), 7.97 (s, H_7), 7.91 (d, H_1, J = 7.6 Hz), 7.74 (d, H_3, J = 7.6 Hz), 7.65 (s, H_{10}), 7.46 (t, H_2, J = 7.6 Hz), 6.88 (d, H_{11}, J = 3.5 Hz), 6.15 (s, OCH$_2$O) [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% CO_2 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	$1\alpha,3\alpha$-diacetylnerbowdine
3	Hippadine
4	Stigmast-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. haementhoids 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.
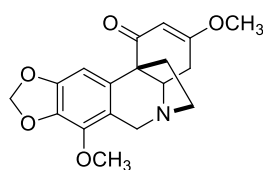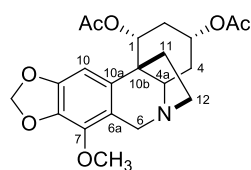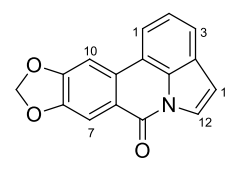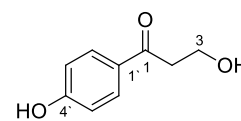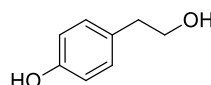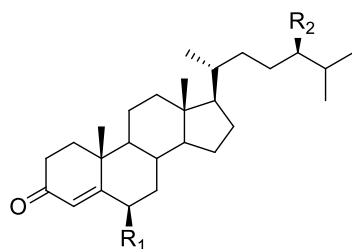

Distichamine (**1**)   1α,3α-diacetylnerbowdine (**2**)   Hippadine (**3**)

R_1 = =O; R_2 = Et, Stigmast-4-ene-3, 6-dione (**4**)   Tyrosol (**6**)   3-Hydroxy-1-(4`-hydroxyphenyl)-1-propanone (**7**)

R_1 = H; R_2 = H, Cholest-4-en-3-one (**5**)

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

Compound **3** (hippadine) was isolated as minor constituent and identified according to its ^{1}H NMR and GC-MS analysis data which compared with available data in literature[30].

Compound **2** 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 $C_{21}H_{25}NO_7$. ^{1}H NMR Showed an aromatic signal appeared at δ_H 6.11 (*s*, H_{10}); two signals of methylenedioxy protons at 5.83 *d* (*J* = 1.3 Hz); 5.79 *d* (1.3) (OCH_2O); 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 4.13 *d* (*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). ^{13}C NMR showed 21 carbon signals classified using DEPT-135 and HSQC into a methylenedioxy carbon (δ_C 100.5), a quaternary carbon (δ_C 46.8, C_{10b}), six aromatic carbons including a methine (δ_C 117.2, 140.4, 133.2, 148.4, 97.1), a methoxy (δ_C 59.1), six methylene (δ_C 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_2O respectively), and two oxygenated methines (δ_C 68.3 and 68.0), and two acetates (21.2/170.5, 21.3/170.1).
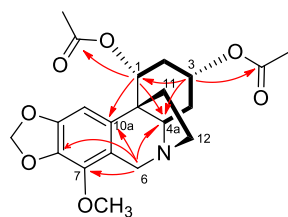

Figure 2. Important COSY (**—**) and HMBC (**H→C**) correlations of compound **2.**

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_1, C_3; H_1/ C_2, C_{10b}, C_3, C_{4a}, C_{10a}, CO; H_3/ C_2, C_4, C_{4a}, C_1, CO and H_{4a}/ 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_{10}/C_{10a}, C_{6a}, C_{10b}, C_7, C_9 and H_6/ C_{12}, C_{4a}, C_{6a}, C_{10a}, C_7. Careful literature review proposed the structure as given in figure 1.
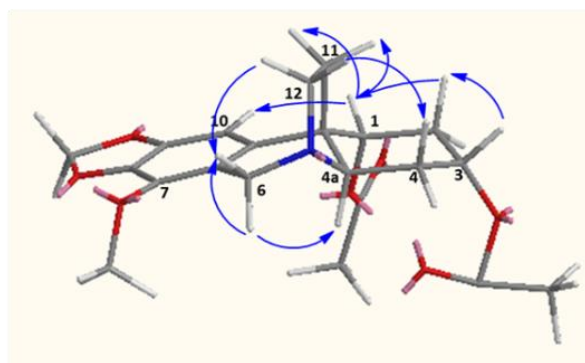
Figure 3. Important NOESY correlations of compound **2**

The beta orientation of C_{11} and C_{12} 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 C_1 and C_3 partially proved by the weak coupling of the equatorial protons at C_1 and C_3. On the other hand, NOESY spectra showed correlations between $H_{1\beta}$ / H_{10}, $H_{2\alpha}$, $H_{2\beta}$, H_{11exo}, and H_{11endo}; while $H_{3\beta}$ showed correlation with $H_{2\alpha}$, $H_{2\beta}$, $H_{4\alpha}$ and $H_{4\beta}$ and these indicate the equatorial protons (at C_1 and C_3) 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*-methylnorbelladine [33].

Table 2. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) data for compound **2** in CDCl$_3$

C	δ$_C$	δ$_H$ (*J$_{Hz}$*)	HMBC (H→C)
1	68.3 *d*	5.71 *br t* (2.6)	C_2, C_{10b}, C_{4a}, C_3, CO
2	28.6 *t*	1.95* (2α)	
		2.29 *dq* (2.6, 16.2) (2β)	C_4, C_{10b}, C_{4a}, C_1, C_3
3	68.0 *d*	5.15 *br quint* (2.6)	C_2, C_4, C_{4a}, C_1, C_3
4	30.5 *t*	2.08*, (4α)	
		1.46 *ddd* (3.3, 12.2, 15.5), (4β)	C_{10b}, C_{4a}
4a	59.9 *d*	3.48 *dd* (5.5, 12.2)	C_4, C_{11}, C_{12}, C_6, C_{10a}
6	57.5 *t*	4.13 *d* (17.3) (6α)	C_{11}, C_{12}, C_{10}, C_{6a}, C_8, C_{10a}, C_7, C_9
		3.76 *d* (17.3), (6β)	C_{12}, C_{4a}, C_{6a}, C_8, C_{10a}, C_7, C_9
6a	117.2 *s*		
7	140.4 *s*		
8	133.2 *s*		
9	148.4 *s*		
10	97.1 *d*	6.11 *s*	C_{10a}, C_{6a}, C_{10b}, C_7, C_9
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*	C_6

		2.76 *ddd* (13.6, 9.0, 6.3) *endo*	C_{4a}, C_{11}, C_6
1-**CO**CH₃	170.5 *s*		
1-CO**CH₃**	21.2 *q*	1.90** *s*	CO(C_1)
3-**CO**CH₃	170.1 *s*		
3-CO**CH₃**	21.3 *q*	1.97* *s*	CO(C_3)
OCH₃	59.1 *q*	3.95 *s*	C_7
OCH₂O	100.5 *t*	5.83 *d* (1.3); 5.79 *d* (1.3)	C_8, C_9

*, ** overlapped signals; *s* singlet; *d* doublet; *ddd* doublet of doublet of doublet; *br* broad; *quint* quintet; *t* triplet; *q* quartet.

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.
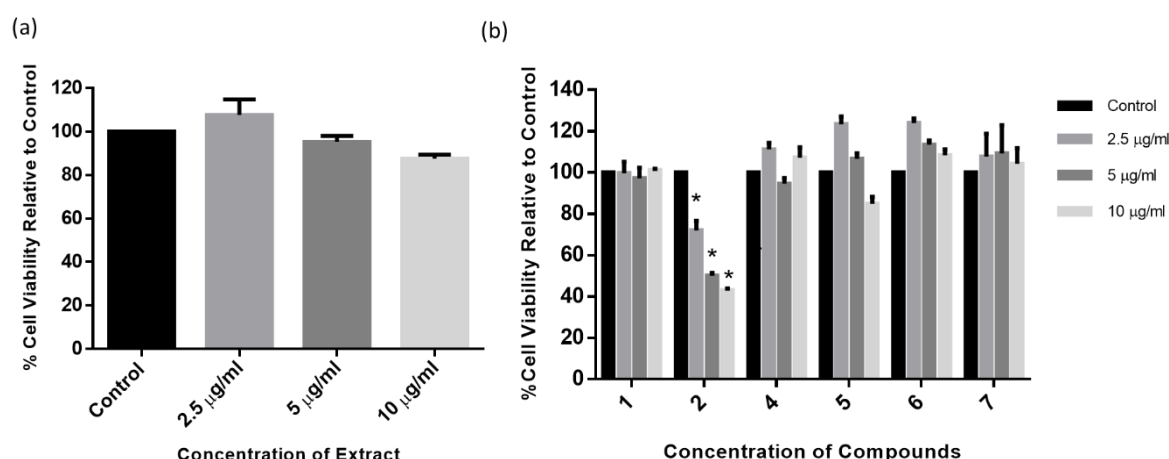

Figure 4. Dose-response of BHE and compounds. MTT assay cytotoxicity on SH-SY5Y cells treated with increasing concentrations (2.5, 5 and 10 μg/mL) of (a) BHE and (b) compounds for 24 hours and each bar represents mean cell viability expressed as percentage of control. * indicate significance at *p*< 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 reduced 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].
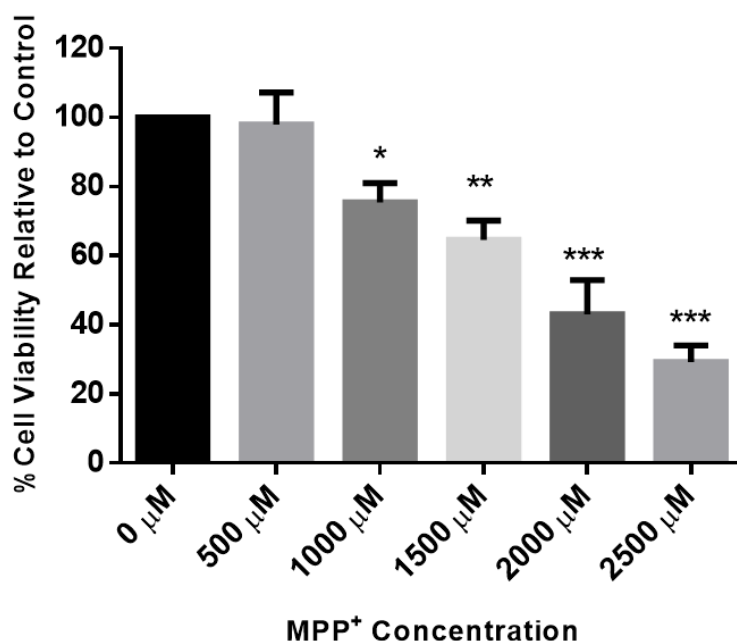
Figure 5. MPP$^+$ induced cytotoxicity in SH-SY5Y cells. Cells were exposed to increasing concentrations of MPP$^+$ (500µM – 2500 µM) and allowed to incubate for 24 hours. MTT assays were performed and results showed a concentration-dependent decrease in cell viability when compared to control. Each 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 MMP$^+$ 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.

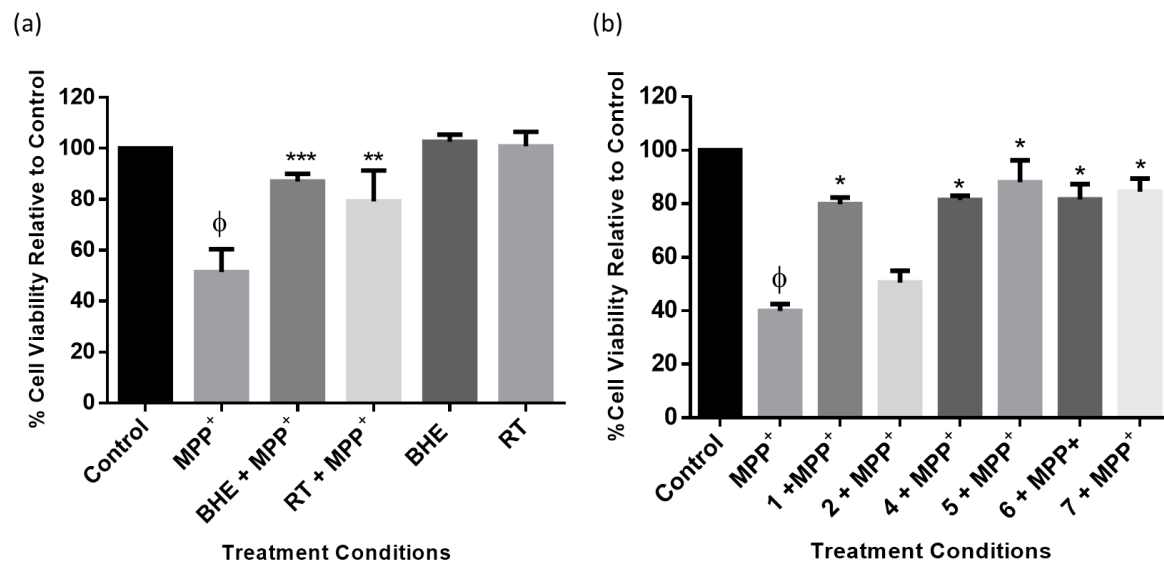(a)   (b)

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 ф (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.
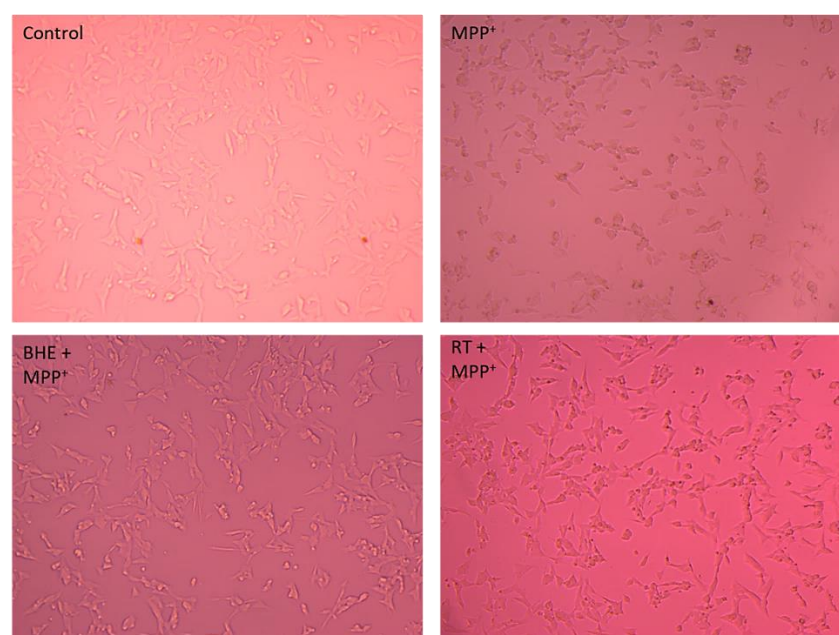

Figure 7. BHE and compounds inhibit SH-SY5Y morphological changes induced by MPP+. Cellular morphology of SH-SY5Y cells pre-treated with BHE and compounds (2.5 µg/mL) prior to exposure to 2000 µM MPP+ for 24 hours and images were captured using the light microscope at 100X magnification.

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.
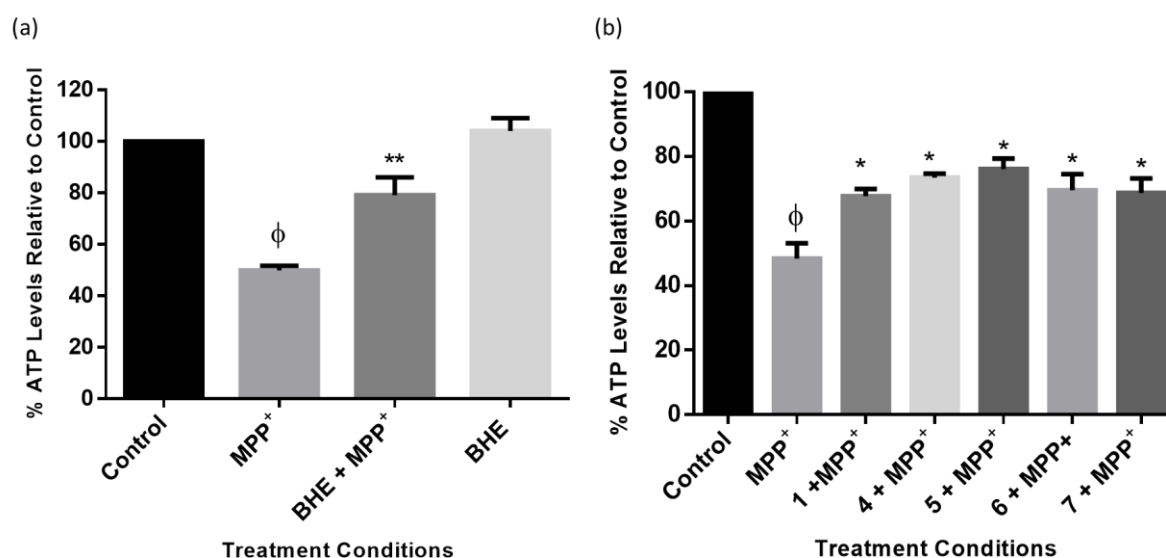

Figure 8. BHE and compounds inhibit MPP+-induced ATP degeneration. Cells were pre-treated with 2.5 μg/mL of extracts (a) and compounds (b) before exposure to 2000 μM of MPP+ for 24 hours and ATP levels assessed. Each bar represents mean percentage level relative to control and significance of difference indicated with * ($p<0.05$) and ** ($p<0.01$) when extract/compounds are compared to MPP+ and ɸ (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.
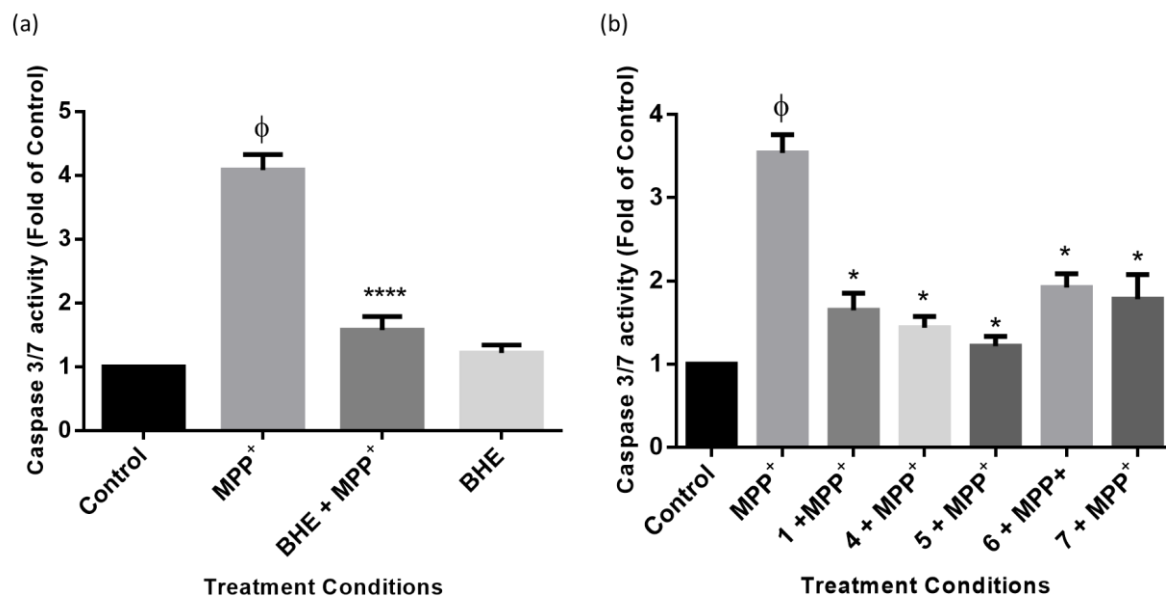
Figure 9. BHE and compounds reduce MPP^+-induced caspase 3/7 activity. Cells were pre-treated with 2.5 µg/mL of extracts (a) and compounds (b) before exposure to 2000 µM of MPP^+ for 24 hours and activity of caspase 3/7 was determined. Each bar represents mean percentage level relative to control and significance of difference indicated with * ($p<0.05$), ** ($p<0.01$) and **** ($p<0.0001$) when extract/compounds are compared to MPP+ and φ (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 Amrayllidaceae members for treatment of mental and neuro-related diseases [45].

Furthermore, all the isolated compounds showed neuroprotective activity except for 1α, 3α-diacetylnerbowdine (**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.

Conflicts of Interest: "The authors declare no conflict of interest."

References

1. Dauer, W.; Przedborski, S. Parkinson's disease: mechanisms and models. *Neuron* **2003**, *39*, 889-909.

2. Lopes, F.M.; Schröder, R.; da Frota Júnior, M.L.C.; Zanotto-Filho, A.; Müller, C.B.; Pires, A.S.; Meurer, R.T.; Colpo, G.D.; Gelain, D.P.; Kapczinski, F. Comparison between proliferative and neuron-like SH-SY5Y cells as an in vitro model for Parkinson disease studies. *Brain Research* **2010**, *1337*, 85-94.

3. Wickremaratchi, M.M.; Perera, D.; O'Loghlen, C.; Sastry, D.; Morgan, E.; Jones, A.; Edwards, P.; Robertson, N.; Butler, C.; Morris, H.R. Prevalence and age of onset of Parkinson's disease in Cardiff: a community based cross sectional study and meta-analysis. *Journal of Neurology, Neurosurgery & Psychiatry* **2009**, *80*, 805-807.

4. Simunovic, F.; Yi, M.; Wang, Y.; Macey, L.; Brown, L.T.; Krichevsky, A.M.; Andersen, S.L.; Stephens, R.M.; Benes, F.M.; Sonntag, K.C. Gene expression profiling of substantia nigra dopamine neurons: further insights into Parkinson's disease pathology. *Brain* **2009**, *132*, 1795-1809.

5. Fernandez-Moriano, C.; Gonzalez-Burgos, E.; Gómez-Serranillos, M.P. Mitochondria-targeted protective compounds in Parkinson's and Alzheimer's diseases. *Oxidative medicine and cellular longevity* **2015**, *2015*.

6. Onyango, I.G. Mitochondrial dysfunction and oxidative stress in Parkinson's disease. *Neurochemical Research* **2008**, *33*, 589-597.

7. Requejo-Aguilar, R.; Bolaños, J.P. Mitochondrial control of cell bioenergetics in Parkinson's disease. *Free Radical Biology and Medicine* **2016**, *100*, 123-137.

8. Jenner, P.; Olanow, C.W. The pathogenesis of cell death in Parkinson's disease. *Neurology* **2006**, *66*, S24-S36.

9. Yan, M.H.; Wang, X.; Zhu, X. Mitochondrial defects and oxidative stress in Alzheimer disease and Parkinson disease. *Free Radical Biology and Medicine* **2013**, *62*, 90-101.

10. Olanow, C.W.; Agid, Y.; Mizuno, Y.; Albanese, A.; Bonucelli, U.; Damier, P.; De Yebenes, J.; Gershanik, O.; Guttman, M.; Grandas, F. Levodopa in the treatment of Parkinson's disease: current controversies. *Movement Disorders* **2004**, *19*, 997-1005.

11. Zhao, Y.; Luo, D.; Ning, Z.; Rong, J.; Lao, L. Electro-Acupuncture ameliorated MPTP-induced parkinsonism in mice via TrkB neurotrophic signaling. *Frontiers in neuroscience* **2019**, *13*, 496.

12. Ren, Z.-l.; Wang, C.-d.; Wang, T.; Ding, H.; Zhou, M.; Yang, N.; Liu, Y.-y.; Chan, P. *Ganoderma lucidum* extract ameliorates MPTP-induced parkinsonism and protects dopaminergic neurons from oxidative stress via regulating mitochondrial function, autophagy, and apoptosis. *Acta Pharmacologica Sinica* **2019**, *40*, 441-450.

13. Kim, H.-Y.; Jeon, H.; Kim, H.; Koo, S.; Kim, S. *Sophora flavescens* Aiton decreases MPP(+)-induced mitochondrial dysfunction in SH-SY5Y cells. *Frontiers in Aging Neuroscience* **2018**, *10*, 119-119.

14. Yi, F.; He, X.; Wang, D. Lycopene protects against MPP+-induced cytotoxicity by maintaining mitochondrial function in SH-SY5Y cells. *Neurochemical research* **2013**, *38*, 1747-1757.

15. Kim, D.; Jeon, H.; Ryu, S.; Koo, S.; Ha, K.-T.; Kim, S. Proteomic analysis of the effect of Korean red ginseng in the striatum of a Parkinson's disease mouse model. *PLoS One* **2016**, *11*, e0164906.

16. Chang, H.-C.; Liu, K.-F.; Teng, C.-J.; Lai, S.-C.; Yang, S.-E.; Ching, H.; Wu, C.-R. Sophora tomentosa extract prevents MPTP-induced parkinsonism in C57BL/6 mice via the inhibition of GSK-3β phosphorylation and oxidative stress. *Nutrients* **2019**, *11*, 252.

17. Wrinkle, G. An introduction to the genus *Boophane*. *Herbertia* **1984**, *40*, 77-82.

18. Meerow, A.; Snijman, D. Amaryllidaceae. In *Flowering plants· monocotyledons*, Springer: 1998; pp. 83-110.

19. Meerow, A.W.; Snijman, D.A. Phylogeny of Amaryllidaceae tribe Amaryllideae based on nrDNA ITS sequences and morphology. *American Journal of Botany* **2001**, *88*, 2321-2330.

20. Nair, J.J.; Rárová, L.; Strnad, M.; Bastida, J.; Cheesman, L.; van Staden, J. Crinane alkaloids of the Amaryllidaceae with cytotoxic effects in human cervical adenocarcinoma (HeLa) cells. *Natural Product Communications* **2014**, *9*, 1934578X1400900406.

21. Neergaard, J.S.; Andersen, J.; Pedersen, M.E.; Stafford, G.I.; Staden, J.V.; Jäger, A.K. Alkaloids from Boophone disticha with affinity to the serotonin transporter. *South African Journal of Botany* **2009**, *75*, 371-374.

22. Cheesman, L.; Nair, J.J.; van Staden, J. Antibacterial activity of crinane alkaloids from Boophone disticha (Amaryllidaceae). *Journal of Ethnopharmacology* **2012**, *140*, 405-408.

23. De Beer, J.J.J.; Van Wyk, B.E. An ethnobotanical survey of the Agter–Hantam, Northern Cape Province, South Africa. *South African Journal of Botany* **2011**, *77*, 741-754.

24. Hauth, H.; Stauffacher, D. The Alkaloids from *Buphane disticha* (L. f.) Herb . 2. Communication on amaryllidaceae alkaloids. *Helvetica Chimica Acta* **1961**, *44*, 491-502.

25. Nair, J.; Manning, J.; Van Staden, J. Distichamine, a chemotaxonomic marker for the genus Boophone Herb.(Amaryllidaceae). *South African Journal of Botany* **2012**, *83*, 89-91.

26. Nair, J.J.; Rárová, L.; Strnad, M.; Bastida, J.; van Staden, J. Alkaloids from *Boophone haemanthoides* (Amaryllidaceae). *Natural Product Communications* **2013**, *8*, 1934578X1300801211.

27. Lepule, K.H.; Cordier, W.; Steenkamp, P.; Nell, M.; Steenkamp, V. The ability of three African herbal remedies to offer protection against an in vitro model of Parkinson's disease. *South African Journal of Botany* **2019**, *126*, 121-131.

28. Heinrich, M.; Teoh, H.L. Galanthamine from snowdrop—the development of a modern drug against Alzheimer's disease from local Caucasian knowledge. *Journal of Ethnopharmacology* **2004**, *92*, 147-162.

29. Ibrakaw, A.; Boatwright, J.; Lesch, T.; Cupido, C.; Hussein, A. Triterpenes and other minor chemical constituents of *Boophone haemanthoides* FM Leight (Amaryllidaceae). *South African Journal of Botany* **2020**.in press.

30. Robbins, D.W.; Boebel, T.A.; Hartwig, J.F. Iridium-catalyzed, silyl-directed borylation of nitrogen-containing heterocycles. *Journal of the American Chemical Society* **2010**, *132*, 4068-4069.

31. Hauth, H.; Stauffacher, D. 89. Die Struktur von Acetylnerbowdin: 4. Mitteilung über Amaryllidaceen-Alkaloide. *Helvetica Chimica Acta* **1963**, *46*, 810-812.

32.   Cho, N.; Du, Y.; Valenciano, A.L.; Fernández-Murga, M.L.; Goetz, M.; Clement, J.; Cassera, M.B.; Kingston, D.G. Antiplasmodial alkaloids from bulbs of Amaryllis belladonna Steud. *Bioorganic & Medicinal Chemistry Letters* **2018**, *28*, 40-42.

33.   Berkov, S.; Osorio, E.; Viladomat, F.; Bastida, J. Chemodiversity, chemotaxonomy and chemoecology of Amaryllidaceae alkaloids. In *The Alkaloids: Chemistry and Biology*, Elsevier: 2020; Vol. 83, pp. 113-185.

34.   Enogieru, A.B.; Omoruyi, S.I.; Ekpo, O.E. Aqueous leaf extract of *Sutherlandia frutescens* attenuates ROS-induced apoptosis and loss of mitochondrial membrane potential in MPP+-treated SH-SY5Y cells. *Tropical Journal of Pharmaceutical Research* **2020**, *19*, 549-555.

35.   Höglinger, G.U.; Carrard, G.; Michel, P.P.; Medja, F.; Lombès, A.; Ruberg, M.; Friguet, B.; Hirsch, E.C. Dysfunction of mitochondrial complex I and the proteasome: interactions between two biochemical deficits in a cellular model of Parkinson's disease. *Journal of Neurochemistry* **2003**, *86*, 1297-1307.

36.   Stennicke, H.R.; Renatus, M.; Meldal, M.; Salvesen, G.S. Internally quenched fluorescent peptide substrates disclose the subsite preferences of human caspases 1, 3, 6, 7 and 8. *Biochemical Journal* **2000**, *350*, 563-568.

37.   Denault, J.-B.; Salvesen, G.S. Caspases: keys in the ignition of cell death. *Chemical Reviews* **2002**, *102*, 4489-4500.

38.   Thornberry, N.A.; Rano, T.A.; Peterson, E.P.; Rasper, D.M.; Timkey, T.; Garcia-Calvo, M.; Houtzager, V.M.; Nordstrom, P.A.; Roy, S.; Vaillancourt, J.P. A combinatorial approach defines specificities of members of the caspase family and granzyme B Functional relationships established for key mediators of apoptosis. *Journal of Biological Chemistry* **1997**, *272*, 17907-17911.

39.   Bressenot, A.; Marchal, S.; Bezdetnaya, L.; Garrier, J.; Guillemin, F.; Plénat, F. Assessment of apoptosis by immunohistochemistry to active caspase-3, active caspase-7, or cleaved PARP in monolayer cells and spheroid and subcutaneous xenografts of human carcinoma. *Journal of Histochemistry & Cytochemistry* **2009**, *57*, 289-300.

40.   Obeso, J.; Stamelou, M.; Goetz, C.; Poewe, W.; Lang, A.; Weintraub, D.; Burn, D.; Halliday, G.M.; Bezard, E.; Przedborski, S. Past, present, and future of Parkinson's disease: A special essay on the 200th Anniversary of the Shaking Palsy. *Movement Disorders* **2017**, *32*, 1264-1310.

41.   McDonald, C.; Gordon, G.; Hand, A.; Walker, R.W.; Fisher, J.M. 200 Years of Parkinson's disease: what have we learnt from James Parkinson? *Age and Ageing* **2018**, *47*, 209-214.

42.   Lee, D.J.; Dallapiazza, R.F.; De Vloo, P.; Lozano, A.M. Current surgical treatments for Parkinson's disease and potential therapeutic targets. *Neural Regeneration Research* **2018**, *13*, 1342.

43.   Voon, V.; Napier, T.C.; Frank, M.J.; Sgambato-Faure, V.; Grace, A.A.; Rodriguez-Oroz, M.; Obeso, J.; Bezard, E.; Fernagut, P.-O. Impulse control disorders and levodopa-induced dyskinesias in Parkinson's disease: an update. *The Lancet Neurology* **2017**, *16*, 238-250.

44.   Espay, A.J.; Morgante, F.; Merola, A.; Fasano, A.; Marsili, L.; Fox, S.H.; Bezard, E.; Picconi, B.; Calabresi, P.; Lang, A.E. Levodopa-induced dyskinesia in Parkinson disease: Current and evolving concepts. *Annals of Neurology* **2018**, *84*, 797-811.

45.   Nair, J.J.; van Staden, J. Cytotoxicity studies of lycorine alkaloids of the Amaryllidaceae. *Natural Product Communications* **2014**, *9*, 1934578X1400900834.

46.   Nair, J.J.; van Staden, J. Pharmacological and toxicological insights to the South African Amaryllidaceae. *Food and Chemical Toxicology* **2013**, *62*, 262-275.

47.   Jin, A.; Li, X.; Zhu, Y.-Y.; Yu, H.-Y.; Pi, H.-F.; Zhang, P.; Ruan, H.-L. Four new compounds from the bulbs of *Lycoris aurea* with neuroprotective effects against $CoCl_2$ and H_2O_2-induced SH-SY5Y cell injuries. *Archives of Pharmacal Research* **2014**, *37*, 315-323.

48.   Castillo, W.O.; Aristizabal-Pachon, A.F.; Sakamoto-Hojo, E.; Gasca, C.A.; Cabezas-Fajardo, F.A.; Takahashi, C. *Caliphruria subedentata* (Amaryllidaceae) decreases genotoxicity and cell death induced by β-amyloid peptide in SH-SY5Y cell line. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **2018**, *836*, 54-61.

49.   Adewusi, E.A.; Fouche, G.; Steenkamp, V. Cytotoxicity and acetylcholinesterase inhibitory activity of an isolated crinine alkaloid from *Boophane disticha* (Amaryllidaceae). *Journal of Ethnopharmacology* **2012**, *143*, 572-578.

50.   Cimmino, A.; Masi, M.; Evidente, M.; Superchi, S.; Evidente, A. Amaryllidaceae alkaloids: Absolute configuration and biological activity. *Chirality* **2017**, *29*, 486-499.

51.   Ding, Y.; Qu, D.; Zhang, K.-M.; Cang, X.-X.; Kou, Z.-N.; Xiao, W.; Zhu, J.-B. Phytochemical and biological investigations of Amaryllidaceae alkaloids: a review. *Journal of Asian Natural Products Research* **2017**, *19*, 53-100.

52.   Hulcová, D.; Breiterová, K.; Siatka, T.; Klímová, K.; Davani, L.; Šafratová, M.; Hošťálková, A.; De Simone, A.; Andrisano, V.; Cahlíková, L. Amaryllidaceae alkaloids as potential glycogen synthase kinase-3β inhibitors. *Molecules* **2018**, *23*, 719.

53.   Li, D.-D.; Zhang, Y.-H.; Zhang, W.; Zhao, P. Meta-analysis of randomized controlled trials on the efficacy and safety of donepezil, galantamine, rivastigmine, and memantine for the treatment of Alzheimer's disease. *Frontiers in Neuroscience* **2019**, *13*, 472.

54.   Omoruyi, S.; Enogieru, A.; Hussein, A.; Ekpo, O. Preliminary investigation of the neuroprotective potentials of *Crossyne guttata* in MPP+-induced toxicity in SH-SY5Y. *Journal of African Association of Physiological Sciences* **2019**, *7*, 138-144.

55.   Omoruyi, S.I.; Delport, J.; Kangwa, T.S.; Ibrakaw, A.S.; Cupido, C.N.; Ekpo, O.E.; Hussein, A.A. In vitro neuroprotective potential of *Clivia miniata* and *Nerine humilis* (Amaryllidaceae) in MPP+-induced neuronal toxicity in SH-SY5Y neuroblastoma cells. *South African Journal of Botany* **2020**.

56.   Ago, Y.; Koda, K.; Takuma, K.; Matsuda, T. Pharmacological aspects of the acetylcholinesterase inhibitor galantamine. *Journal of Pharmacological Sciences* **2011**, *116*, 6-17.

57.   Son, I.H.; Park, Y.H.; Lee, S.I.; Yang, H.D.; Moon, H.-I. Neuroprotective activity of triterpenoid saponins from *Platycodi radix* against glutamate-induced toxicity in primary cultured rat cortical cells. *Molecules* **2007**, *12*, 1147-1152.

58.   Qiu, J.; Wang, X.; Song, C. Neuroprotective and antioxidant lanostanoid triterpenes from the fruiting bodies of Ganoderma atrum. *Fitoterapia* **2016**, *109*, 75-79.

59.   Schapira, A.; Cooper, J.; Dexter, D.; Clark, J.; Jenner, P.; Marsden, C. Mitochondrial complex I deficiency in Parkinson's disease. *Journal of Neurochemistry* **1990**, *54*, 823-827.

60.   Keeney, P.M.; Xie, J.; Capaldi, R.A.; Bennett, J.P. Parkinson's disease brain mitochondrial complex I has oxidatively damaged subunits and is functionally impaired and misassembled. *Journal of Neuroscience* **2006**, *26*, 5256-5264.

61.   Ho, P.W.-L.; Liu, H.-F.; Ho, J.W.-M.; Zhang, W.-Y.; Chu, A.C.-Y.; Kwok, K.H.-H.; Ge, X.; Chan, K.-H.; Ramsden, D.B.; Ho, S.-L. Mitochondrial uncoupling protein-2 (UCP2) mediates leptin protection against MPP+ toxicity in neuronal cells. *Neurotoxicity Research* **2010**, *17*, 332-343.

62.   Nakano, M.; Imamura, H.; Sasaoka, N.; Yamamoto, M.; Uemura, N.; Shudo, T.; Fuchigami, T.; Takahashi, R.; Kakizuka, A. ATP maintenance via two types of ATP regulators mitigates pathological phenotypes in mouse models of Parkinson's disease. *EBioMedicine* **2017**, *22*, 225-241.

63.   Chalmers-Redman, R.M.; Fraser, A.D.M.; Carlile, G.W.; Pong, A.; Tatton, W.G. Glucose protection from MPP+-induced apoptosis depends on mitochondrial membrane potential and ATP synthase. *Biochemical and Biophysical Research Communications* **1999**, *257*, 440-447.