Chemical investigation of some species of Amaryllidaceae from the Greater Cape Region of South Africa as a source of bioactive compounds

A thesis submitted in fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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By

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Co-supervisor: Professor Ahmed Mohammed

Co-supervisor: Professor Christopher Nelson Cupido

October 2020

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DECLARATION

I, **Abobaker Saleh Mohamed Ibrakaw**, hereby declare that **"**Chemical investigation of some species of Amaryllidaceae from the Greater Cape Region of South Africa as a source of bioactive compounds" is my original work and to the best of my knowledge, it has not been, submitted before for any degree or assessment in any other University, and all the sources that, I used or quoted have been indicated and acknowledged by means of complete references.

02/11/2020

Signed **Date**

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ABSTRACT

The family Amaryllidaceae is widely distributed in the southern hemisphere. Members of the family are well known for their content of pharmacologically active alkaloids and represent an important epicentre of Amaryllidaceae-alkaloid diversity. Other metabolites from Amaryllidaceae, such as phenolics including flavonoids, lignans, chromones, and acetophenones, in addition to terpenoids and ceramides have been reported. *Boophone haemanthoides* (BHE), *Crossyne flava* (CRO), *Clivia miniata* (CME) and *Nerine humilis* (NHE) are members of Amaryllidaceae that have shown biological activity. Parkinson's disease (PD) is a neurodegenerative disease that progresses with increasing age and some of its major symptoms include tremors, postural and movement related difficulties. To 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 BHE and CRO along with the isolated compounds of BHE and CRO. The neuroprotective potential was investigated in MPP+-induced neurotoxicity in SH-SY5Y neuroblastoma cells. Cell viability was determined using the 3-(4,5-dimethyithiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and cell morphology was analysed using light microscopy. Furthermore, the effects of the extracts on apoptosis and ATP production were investigated using caspase 3/7 apoptosis kit and the Promega Mitochondrial ToxGlo ATP assay kit, respectively.

The chemical investigation of the nonpolar constituents of a methanol extract of the bulbs of BHE yielded thirteen known compounds. The compounds were identified as stigmast- 4-ene-3,6-dione (**1**); cholest-4-en-3-one (**2**); (22*E*)-stigmast-4,22-dien-3-one (**3**); stigmast-4-en-3-one (**4**); 6β-hydroxystigmast-4-en-3-one (**5**); 6β-hydroxycholest-4-en-3-one (**6**); cycloartenol (**7**); Acetovanillone (**8**); tyrosol (**9**) and 3-hydroxy-1-(4-hydroxyphenyl)-1-propanone (**10**); distachamine (**11**); 1α,3α-diacetylnerbowdine (**12**); Hippadine (**13**).

The isolated compounds of the BHE showed improved cell viability, increased ATP generation in the cells as well as inhibition of MPP+-induced apoptosis.

On the other hand, four alkaloids [pancratinine B (**14**), bufanidrine (**15**), buphanisine (**16**) and epibuphanisine (**17**)] were isolated and identified from *Crossyne flava*. The compounds attenuated ATP levels in the cells and inhibited apoptosis induced by MPP+.

The thesis also includes the biological evaluation of selected members of family Amaryllidaceae for their cytotoxicities and neuroprotection potential. *Amaryllis belladonna* total

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extracts showed cytotxicity below 30 µg/mL, while *Haemanthus pubescens* extracts had an IC_{50} value less than 30 µg/mL for the U87 and SH-SY5Y cells but not for the U251 cells.

In addition, *Clivia miniata* and *Nerine humilis* were investigated for their neuroprotective potential in MPP+-induced neurotoxicity in SH-SY5Y neuroblastoma cells. Both extracts improved cell viability as well as cell morphology by inhibiting the toxicity induced by MPP+. The extracts also improved ATP levels in cells and attenuated the apoptosis induced by MPP+.

In conclusion, the members of the Amarylldaceae investigated yielded compounds with high neuroprotection potential, and some of them showed potent cytotoxicity. Further studies will be required to isolate more compounds from Amaryllidaceae with special focus on neuroprotection activity.

Keywords

Cape floristic region

Amaryllidaceae

Boophone haemanthoides

Crossyne flava

Neuroprotective effect

Alkaloids

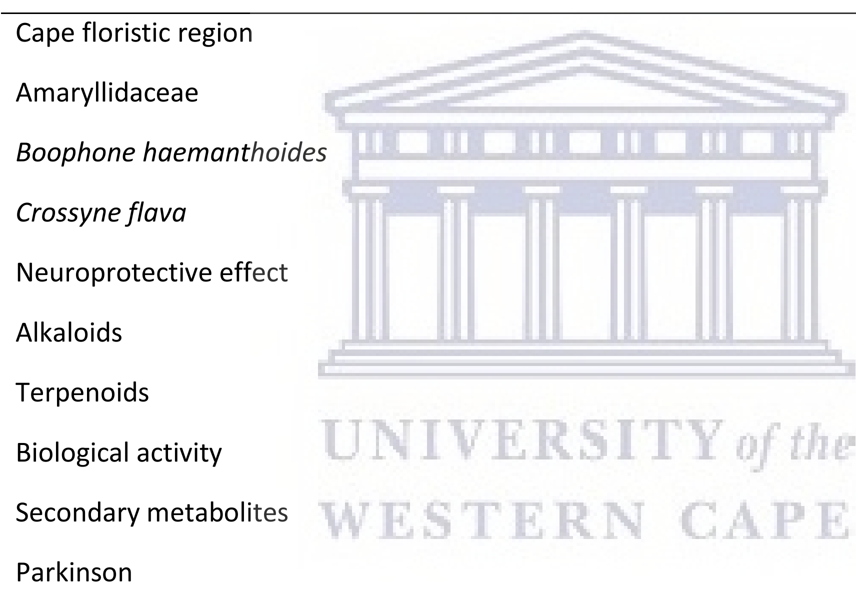
Terpenoids

Biological activity

Secondary metabolites

Parkinson

Cytotoxicity



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I would like to thank our **Libyan government** and **embassy of Libya** for the financial support that has afforded me the opportunity to explore not only my research skills toward a PhD, but also a new country and culture.

I would like to thank my beloved wife **Saada** whom I owe my deepest gratitude for her patience and support which has given me the strength to persevere. I will forever be indebted to you for understanding and inspiring to finalize my project. Also, to my lovely daughters **Najla**, **Rawasi**, **Hadil**, **Ragad** and my prince **Mohamed** for helping me by being my shining light and motivation.

To my late father **Saleh Mohamed Ibrakaw** may God's mercy be with him, my beloved mother **Fadoula Ali Ibrahim**, may she be spared a long life and to my siblings (brothers and sisters) may God preserve your life greater favour.

Thanks to the rest of **My Family** for their love and more particularly **My Brothers** and **Sisters**, for them unconditional and unwavering support.

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I thank you all for your prayers and motivation during my PhD research project. I really appreciate that.

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DEDICATION

This research work is dedicated to my beloved wife **Saada** whom I owe my deepest gratitude for her patience and support which has given me the strength to persevere.

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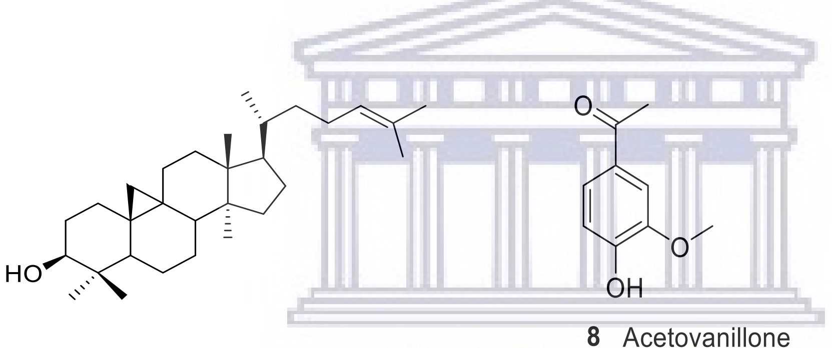
LIST OF ISOLATED COMPOUNDS

1	R_1=O, R_2=Et	Stigmast-4-ene-3,6-dione
2	R_1=H, R_2=H	Cholest-4-en-3-one
3	R_1=H, R_2=Et; Δ^{22}	(22E)-Stigmasta-4,22-dien-3-one
4	R_1=H, R_2=Et	Stigmast-4-en-3-one
5	R_1=OH, R_2=Et	6β-Hydroxystigmast-4-en-3-one
6	R_1=OH, R_2=H	6β-Hydroxycholest-4-en-3-one



8 Acetovanillone

7 Cycloartenol

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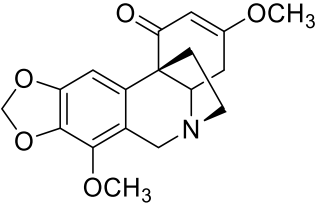
9 Tyrosol

10 3-Hydroxy-1-(4'-hydroxphenyl)-1-propanone



11 Distachamine

12 1α,3α-diacetylnerbowdine

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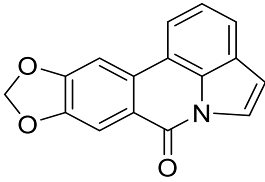

13 Hippadine

14 Pancratinine B

15 Bufandrine, R_1 = αOMe, R_2 = OMe
16 Buphanisine, R_1 = αOMe, R_2 = H
17 Epibuphanisine, R_1 = βOMe, R_2 = H

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LIST OF PUBLICATIONS

1) **Ibrakaw, A.S.,** Mohammed, A.A., Akinfenwa, A.O., 2020. A comprehensive review on nonalkaloidal chemical constituents from Amaryllidaceae (**Manuscript submitted to South African Journal of Botany/under revision).**

2) **Ibrakaw, A.S.,** Boatwright, J.S., Lesch, T., Cupido, C.N., Hussein, A.A., 2020. Triterpenes and other minor chemical constituents of *Boophone haemanthoides* F.M. Leight (Amaryllidaceae), South African Journal of Botany 2020, In press, https://doi.org/10.1016/j.sajb.2020.06.025.

3) **Ibrakaw, A.S.,** Omoruyi, S.I., Ekpo, O.E., Hussein, A.A., 2020. *Boophone haemanthoides* (Amaryllidaceae) and its bioactive compounds attenuate MPP+-induced toxicity in an in vitro Parkinson's disease model. Molecules 25, 5376; doi:10.3390/molecules25225376

4) Sylvester I. Omoruyi[a], Joshua Delport[b], Tusekile S. Kangwa[b], **Abobaker S. Ibrakaw[c]**, Christopher N. Cupido[d], Okobi E. Ekpo[b],*, Ahmed A. Hussein[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, In press, https://doi.org/10.1016/j.sajb.2020.06.028.

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5) Sylvester I. Omoruyi[a], Tusekile S. Kangwa[b], **Abobaker S. Ibrakaw[c]**, Christopher N. Cupido[d], Jeanine L Marnewick[e], Okobi E. Ekpo[b,*,] Ahmed A. Hussein[a]. Cytotoxic activities of selected plants of the family Amaryllidaceae on brain tumour cell lines. South African Journal of Botany 2020, In press. https://doi.org/10.1016/j.sajb.2020.09.015.

6) Sylvester I. Omoruyi[1], **Abobaker S. Ibrakaw[2]**, James S. Boatwrighta[2], Okobi E. Ekpo[3], Christopher N. Cupido[4], Ahmed A. Hussein[1]*. Biological activities of *Crossyne flava* and Amaryllidaceae alkaloids: Implications for Parkinson's disease (under publication)

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RESEARCH ARTICLE 1

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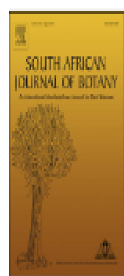

Triterpenes and other minor chemical constituents of *Boophone haemanthoides* F.M. Leight (Amaryllidaceae)

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ABSTRACT

The chemical investigation of the nonpolar constituents of a methanol extract of the bulbs of *Boophone haemanthoides* yielded ten known compounds as minor constituents. The compounds were identified as stigmast-4-ene-3,6-dione (**1**); cholest-4-en-3-one (**2**); (22*E*)-stigmast-4,22-dien-3-one (**3**); stigmast-4-en-3-one (**4**); 6*β*-hydroxystigmast-4-en-3-one (**5**); 6*β*-hydroxycholest-4-en-3-one (**6**); cycloartenol (**7**); acetovanillone (**8**); tyrosol (**10**) and 3-hydroxy-1-(4-hydroxyphenyl)-1-propanone (**11**). The isolation of compound **7** with other cholestane (**2, 6**) and stigmastane (**1, 3–5**) derivatives aligned with the biosynthetic pathway of plant steroids through **7** from squalene 2,3-epoxide. This is the first report on the isolation and identification of these compounds from *B. haemanthoides* and **1–6** and **11** for the family Amaryllidaceae.

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Ibrakaw A.S., Boatwright J.S., Hussein, A.A and Cupido, C.N. Title: ”Bioactive compounds from *Boophone haemanthoides”* SAC/RSC Young Chemists' Symposium, University of Cape Town, 2018.

Ibrakaw, A.S., Badmus, J.A., Boatwright, J.S., Cupido, C.N and Hussein, A.A. Title: “Bioactive compounds of Amaryllidaceae for treatment of Alzheimer's disease” Natural Products in Drug Discovery and Human Health, PSE meeting, 28-31 July 2019, Faculty of Pharmacy, University of Lisbon, Portugal.

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LIST OF ABBREVIATIONS

^{13}C-NMR	Carbon-13 nuclear magnetic resonance
1D-NMR	One-dimensional nuclear magnetic resonance
^{1}H-NMR	Proton nuclear magnetic resonance
2D-NMR	Two-dimensional nuclear magnetic resonance
AAE/g	Ascorbic acid per gram
AAPH	2, 2'-Azobis (2-methylpropionamidine) dihydrochloride, perchloric acid
ABTS	2, 2'-Azino-bis (3-ethylbenzo thiazoline-6-sulfonic acid) diammonium salt
AChE	Acetylcholinesterase
AD	Alzheimer disease
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BChE	Butyl cholinesterase
BHE	*Boophone haemanthoides*
BL6	Melanoma cells
BS	*Bacillus subtilis*
CA	*Candida Albicans*
CEM	Human acute lymphoblastic
$CDCl_3$	Deuterated chloroform
CME	*Clivia miniata*
CRO	*Crossyne flava*
D10	Chloroquine-sensitive
d	Doublet
DCM	Dichloromethane
DIW	De-ionized water

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DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC	*Escherichia coli*
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechingallate
ESI	Electrospray ionization
EtOAc	Ethyl acetate
EH	*Entamoeba hystolytica*
FAC 8	Chloroquine-resistant
Fig	Figure
FRAP	Ferric-ion reducing antioxidant power
FT-IR	Fourier transform infrared
g	Gram
H_2SO_4	Sulphuric acid
HaCaT	Human keratinocyte cell
HeLa	Cervical adenocarcinoma
HMBC	Heteronuclear multiple bond coherence
HPLC	High pressure liquid chromatography
HRMS	High resolution mass spectroscopy
HuAChE	Human Acetylcholinesterase
HuBuChE	Human Butyrylcholinesterase
IC_{50}	Half maximal inhibitory concentration
IZ	Inhibition zone
IR	Infrared
J	Coupling constant in Hz
KCl	Potassium chloride
KP	*Klebsiella pneumoniae*

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L	Litre
LD	*Leishmania donovani*
MAO-B	Mono amine oxidase B
MCF-7	Breast adenocarcinoma
MIC	Minimum inhibition concentration
MeOH	Methanol
mg	Milligram
mL	Millilitre
min	Minute
MPP+	1-Methyl-4-phenylpyridinium
MPTP	Methylphenyltetrahydropyridine
NHE	*Nerine humilis*
NMR	Nuclear magnetic resonance
ORAC	Oxygen radicals absorbance capacity
PCD	Programmed cell death
PD	Parkinson's disease
PF	*Plasmodium falciparum*
POP	Propyl oligopeptidase
q	Quartet
ROS	Reactive oxygen specie
Rt	Retention time
s	Singlet
SA	*Staphylococcus aureus*
SANA	N-succ-(Ala) 3-nitroanilide
SANBI	South African National Biodiversity Institute
SD	Standard deviation
SERT	Serotonin transporter
SK-MEL-1	Human Melanoma cell

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spp.	Species
TBARs	Thiobarbituric acid reagents
TBR	*Trypanosoma brucei* Rhodesiense
TC	*Trypanosoma cruzi*
TCA	Trichloroacetic acid
td	Triplet of doublets
TEAC	Trolox equivalent absorbance capacity
TE/g	Trolox equivalent per gram
TLC	Thin layer chromatography
TPTZ	(2,4,6-tri[2-pyridyl]-*s*-triazine, Iron (III) chloride hexahydrate
Trolox	6-Hydroxyl-2, 5,7,8-tetramethylchroman-2-carboxylic acid
USA	United States of America
UV	Ultra-violet
U87 and U251	Human glioblastoma cell lines

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CHAPTER ONE

1.1 Introduction

General overview of the use of medicinal plants as source of medicines

Plants are well-known to provide basic needs of man such as shelter, food, clothing and most importantly medicine against several illnesses (Beyene, 2016). Natural plant materials such as leaves, barks and roots have been recognized as sources of therapeutic agents for the myriads of diseases that plaque humanity from time immemorial (Fidler *et al.*, 2003). World Health Organization (WHO) estimation showed that about 80% of developing country populations rely on the plant as a source of primary health care delivery and about 25% of synthetic drugs derived from structural analogue of compounds from natural plant sources (Beyene, 2016; Rao *et al.*, 2004). A number of new drugs are products of plant natural phytochemicals which were isolated with unique structures that are out of human comprehension (Shelar and Shirote, 2011).

However, despite the huge potential and past successes attributed to natural products, pharmaceutical companies in the 1990s reduced the use of natural products for drug discovery (Cragg and Newman, 2013). This is not only as a result of perceived difficulties in access and supply, the complexity of natural product chemistry, slowness of getting desired product and concern in intellectual property right but also the introduction of combinatorial chemistry and computer based molecular modeling design (High Throughput Screening) (Luo *et al.*, 2014). The results obtained from these new methods of screening unfortunately did not provide expected outcome with increase declined approval rate of new drugs and this led to resurgence of natural product (Atanasov *et al.*, 2015).

Furthermore, the advent of robust, state of the art equipment have reinforced the understanding that the complex chemical entities in natural products hold future for most drug discovery. However, without prejudice to the achievement of synthetic drugs, their inability to effectively eradicate/or treat immunodeficiency syndromes such as arthritis,

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cancer, diabetes, ROS-induced diseases without adverse drug reaction has led to an increase search for natural derived drugs (Shelar and Shirote, 2011). Drugs discovered from natural sources such as penicillin, tetracycline, erythromycin, avermectin, quinine, artemisinin, lovastatin and analogs, cyclosporine, rapamycins, paclitaxel and irinotecan have revolutionized to large extent health care delivery (Harvey, 2008). However, about 100 drugs from natural products are at different stages of clinical trials for the therapeutic evaluations against cancer, cardiovascular, gastrointestinal, inflammation and metabolic diseases (Harvey, 2008). Ingenol 3-*O*-angelate a polyhydroxy diterpenoid ingenol derivative isolated from the sap of *Euphorbia peplus* is utiluzed for skin cancers, a semi-synthetic analogue of triptolide (14-succinyl triptolide sodium salt) isolated from *Tripterygium wilfordii*, an indigenous to the People's Republic of China is employed for autoimmune and inflammatory diseases and Combretastatin A-4 phosphate a stilbene derivative from the South African Bush Willow. *Combretum caffrum* acts as an anti-angiogenic agent is currently under different phases of clinical trials (Cragg and Newman, 2005; Fidler *et al.*, 2003; Kedei *et al.*, 2004; Kiviharju *et al.*, 2002). Nevertheless, the earth is replete with natural plants of different families and some are indigenous, while others are widespread. However, very few in the range of about 6% of existing medicinal plants species have been investigated for their biological and pharmacological portability (Cragg and Newman, 2013). This shows that there is still a large gap between natural endowment/diversity and exploration of the same for the human health challenges (Wiland-Szymańska, 2009). The present study seeks to evaluate biologically different species belong to the family of Amaryllidaceae, and the chemical constituents and their possible biological activities of *Boophone haemanthoides* and *Crossyne flava* that.

1.2 South African Flora

There are ca. 24.400 different plants species in South Africa (Cowling *et al.*, 1996). This makes up to nearly 10% of all the plant species on earth. South Africa is the only country

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that can claim to have one of the world's six floral kingdoms completely within its borders (Stoll-Kleemann and O'Riordan, 2002).

The diversity and abundance of South African plants is impressive, and this is one of the reasons that the South Africa flora is considered to be an excellent pool of natural products, which can be used for drug discovery. Numerous plants have been used since ancient time for their medicinal qualities by the indigenous people (Vargas, 1981).

1.3 Amaryllidaceae

Plants of the family Amaryllidaceae are widely distributed in warm temperate and tropical regions worldwide, with major centres of diversity in South America (28 genera) and South Africa (18 genera) (Du Plessis and Duncan, 1989; Snijman and Meerow, 2010). It was estimated that 263 species are native to southern Africa (the region with the highest diversity of amaryllides in the world), with 210 endemic species. An additional three genera and four species are naturalised, and an additional 13 genera and 43 species are cultivated in southern Africa (Snijman and Meerow, 2010).

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Members of this family have been used for thousands of years as herbal remedies (Atanasov *et al.*, 2015). The alkaloids from their extracts have been the subject of active chemical investigation for nearly 200 years. Over the past three decades many have been isolated, screened for different biological activities, and synthesized by a number of research groups (Nair *et al.*, 2013). Furthermore, a huge number of plants are traded for traditional medicines. Africans use the bulbs and leaves as poultices and decoctions for treating sores and digestive disorders, but in large dosages they are extremely poisonous (Snijman and Meerow, 2010). The Zulu people of South Africa also use rhizomes of *Clivia* species as protective charms (Nair *et al.*, 2013). In Peru, the Inca people frequently depicted flowers of Amaryllidaceae (*Ismene*, *Pyolirion* and *Stenomesson*) on ceremonial drinking vessels (Vargas, 1981).

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Amaryllidaceae alkaloids represent a large group of compounds (over 300 alkaloids have been isolated) and is an ever expanding group of biogenetically related isoquinoline alkaloids that are found exclusively in plants belonging to this family. In spite of their great variety of pharmacological and/or biological properties, only galanthamine is used therapeutically. This alkaloid is a long-acting, selective, reversible and competitive inhibitor of acetylcholinesterase, and is used for the treatment of AD (Snijman and Meerow, 2010).

1.4 Problem Statement

Multiple drug resistance or Multidrug resistance is a condition enabling a disease-causing organism to resist drugs or chemicals of a wide variety of structure and function targeted at eradicating the organism. Organisms that display multidrug resistance are typically bacterial and neoplastic (tumor) cells. Although antibiotics are widely used for the treatment of various infection/diseases, their uses are frequently limited by microorganisms displaying multidrug resistance (MDR). Additionally, the ageing diseases like Parknison represent another heavy burden confront the scientific community to find a new treatment (Geula *et al.*, 2008).

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The aim of this project is directed toward the screening of different species form Amaryllidacea for thei toxicity and neuro protection activity including BHE, CRO and NHE. Additionally, to isolate different chemical constituents (mainly, alkaloids and triterpenoids) from BHE and CRO as well as the biological profiling of the total extracts and the isolated compounds for their potential antioxidative stress, cytotoxicity and of aging-related diseases such as parkinson.

1.5 Hypothesis

South Africa occupies only 2% of the world's land surface, yet contains a disproportionately large share of global biodiversity, being home to nearly 10% of the planet's plant species. Its extraordinary plant diversity helps to rank South Africa as the country with the fifth highest number of plant species in the world.

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The South African flora an especially Amaryllidaceae family harbours natural compounds that may be used directly or indirectly as lead compounds for the development of innovative drugs in different field such as neurodegenerative diseases.

1.6 Aim(s) of the project

The main aim of the study is to discover new and potent natural products from selected members of Amaryllidaceae against different biological targets, i.e. micro-organism, cancer and neuroprotection.

1.7 Objectives

The main objectives of this study are:

1- Collection and documentation of different species belonging to Amaryllidaceae in the Greater Cape Region.

2- Extraction and screening of different species against the human cancer cell lines, neuroprotection and selection of the most active extracts.

3- Isolation and structural elucidation of the active compounds from active extract(s).

4- Biological evaluation of the isolated compounds.

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1.8 Expected Result

1. Chemical documentation of some South African plants belonging to Amaryllidaceae for the first time.
2. Isolation and chemical characterization of new bioactive compounds that are biologically active.
3. Explore the potential of South African flora as an important source for drug discovery.
4. Increase the documentation of the therapeutic value of South African flora.

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CHAPTER TWO A

LITERATURE REVIEW

2.1 GENERAL OVERVIEW

Amaryllidaceae is a large family of bulbous flowering plants consisting of almost 1000 species in 79 genera that are widely distributed throughout the tropical regions of the earth (Meerow and Snijman, 1998). The family is known to occur abundantly in South Africa, South America and the Mediterranean. They are traditionally used in agriculture, horticulture, ethnobotany, folklore medicine and pharmacology (Bastida *et al.*, 2006; Nair *et al.*, 2013). Amaryllidaceae have almost one-third of the members present in South Africa and are widely distributed through the Capensis floral kingdom of the Western Cape. South African members of this family belong exclusively to two tribes of Amaryllidaceae, Haemantheae and Cyrtantheae, out of the 14 known tribes (Meerow and Clayton, 2004). South African natives have been using extracts of the bulb of Amaryllidaceae for over two hundred years for medicinal purposes (Watt and Breyer-Brandwijk, 1962).

Abundant presence of diverse groups of alkaloids is a characteristic of Amaryllidaceae. The alkaloids from the family represent unique types of alkaloids that are not known to occur in other families of plants (Bastida *et al.*, 2011), especially isoquinoline not known to occur in any other plant family (Bastida *et al.*, 2006).

The alkaloids of Amaryllidaceae have been the subject of isolation and investigation for different biological activities in the last 200 years. The alkaloids inherent in the plants have been related to folklore medicinal uses over a thousand years ago (Bastida *et al.*, 2011). The fascinating structural features of the alkaloids have attracted significant interest for the evaluation of their medicinal importance (Tallini *et al.*, 2017). A study conducted by Bay-Smidt *et al.* (2011) to screen out candidate plant for the discovery of anti-Alzheimer's disease showed that *Apodolirion* does not contain alkaloids. The study concludes that the absence of the alkaloids in the genus is responsible for the non-activity against Acetylcholinesterase (AChE). This implies that the presence of alkaloids in the plant is responsible for anti-

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Alzheimer's disease ability in most of the genus. However, to date over 300 alkaloids have been isolated from the family with significantly biogenetically related different structures (Sandager *et al.*, 2005).

The peculiar arrangements of the alkaloid skeleton and wide array of medicinal benefits have elicited a lot of medicinal evaluations (De Andrade *et al.*, 2012). The interest in alkaloids from Amaryllidaceae started in 1877 and has increased because of the anti-proliferative and antiviral properties (Cook and London, 1952).

The diverse structure of Amaryllidaceae alkaloids, however, is categorized into nine skeletons with representative alkaloids (norbelladine, lycorine, homolycorine, crinine, haemanthamine, narciclasine, tazettine, montanine and galanthamine) (Ghosal *et al.*, 1985). The three main discernible skeletons of alkaloids that form the fundamental Amaryllidaceae alkaloids structural diversity is from alternative oxidative phenolic coupling of major intermediate metabolite (Barton and Cohen, 1956).

Biogenetical formation of Amaryllidaceae alkaloids is by intramolecular oxidative coupling of the main metabolite *O*-methylnorbelladine obtained from phenolic amino acids L-phenylalanine and L-tyrosine (De Andrade *et al.*, 2012). The ortho-para coupling of *O*-methylnorbelladine gives lycorine-type skeleton while para-ortho phenolic oxidative coupling lead to galanthamine-type skeleton and the crinine-, haemanthamine-, tazettine-, narciclasine- and montanine-type structures are products of para-para coupling (Barton and Cohen, 1956).

Lycorine type of alkaloids from Amaryllidaceae has been shown to have a myriad of biological activities. Inhibitor of ascorbic acid synthesis, cell proliferation and organogenesis in higher plants are part of the alkaloid's potential. Antiviral, anti-inflammatory, antifungal and anti-protozoan activities have also been adduced to lycorine type alkaloids (Bastida *et al.*, 2006). Anti-proliferative effect of lycorine against HL-60 leukemia cell line has been attributed to cell cycle arrest and apoptosis induction. Antitumor activity has also been revealed to have no apparent adverse effects in the treated animals (Liu *et al.*, 2009). Apart

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from pharmacological activity of Lycorine alkaloids, it also serves as precursor for modification into other compounds of biological importance. The modification of C-8 of lycorine produced stronger inhibition of acetylcholinesterase and also incompleteness of D-ring and presence of aromatic C-ring enhance activity of lycorine (Lee *et al.*, 2007).

Galanthamine-type is para-ortho coupling synthesized alkaloids. Galanthamine alkaloids of Amaryllidaceae have been shown to be selective inhibitors of the acetylcholinesterase and allosteric potentiating ligand in nicotinic acetylcholine receptors. It is known to facilitate cholinergic synapses in the management of Alzheimer disease because of its ability to increase acetylcholine (Maelicke *et al.*, 2001; Heinrich and Teoh, 2004). These characters of galanthamine led to its use as drug for the management of Alzheimer's and neurological disease and eventual approval in 2001 by the FDA (Food and Drug Administration) for the management of the disease. However, there has been increased interest in the biological evaluation of Amaryllidaceae as source of novel compounds for the therapeutic potential (Maelicke *et al.*, 2001).

Haemanthamine and crinamine of para-paraphenolic coupling alkaloids have been shown to be strong apoptotic enhancers in cancerous cells. The antimalarial of this compound has also been reported against chloroquine-sensitive strain of malarial pathogen as well as hypotensive and antiretroviral activity (McNulty *et al.*, 2007).

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2.2 *Amaryllis belladonna*

Amaryllis belladonna is a member of Amaryllidaceae, which is widely and mainly distributed in southern Africa. Twenty six Amaryllidaceae alkaloids were identified in *belladonna* bulbs, but only three were isolated from the bulb by Tallini *et al.* (2017). The three alkaloids isolated are 1-*O*-acetylcaranine (**9**), 3-*O*-acetylhamayne (**83**) and buphanamine (**64**). Anti-protozoa activity of the three alkaloids was tested against *Trypanosoma cruzi, T. brucei rhodesiense, Leishmania donovani* and *Plasmodium falciparum*. Alkaloid 3-*O*-acetylhamayne (**83**) showed significantly higher activities against all the tested protozoans but the activity was

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found to be non-selective because of exhibition of cytotoxicity against L6 cells (rat skeletal myoblasts) (Tallini *et al*., 2017).

The study of Wahyuni *et al*. (2013) screened 33 flower bulbs for allelochemical activity against weeds. They identified narciclasine (**103**) and lycorine (**11**) as the active component in *A. belladonna* and *Narcissus*, respectively, for the inhibition of germination and growth of *Senecio vulgaris* and *Lolium perenne*. The IC_{50} values of radicle growth inhibition were 0.1 and 0.93 µM for Narciclasine (**103**) and lycorine (**11**) respectively as compared with 0.11 µM of synthetic chlorpropham (Wahyuni *et al*., 2013).

(+) 8-*O*-demethylmaritidine (**71**) was isolated from the flower of *A. belladonna*. (+) 8-*O*-demethylmartidine (**71**) was obtained from chloroform extract of the bulb in the pre-flowering stage. However, (+) 8-*O*-demethylmaritidine (**71**) did not show remarkable cytotoxicity activity (Abou-Donia *et al*., 2005b).

A novel alkaloids containing the pyrrolo[de]phenanthridine ring system named (-)-amarbellisine (**15**) and five known alkaloids lycorine (**11**), (-)-pancracine (**93**), (+)-vittatine (**73**), (+)-11-Hydroxyvittatine (**74**) and (+)-hippeastrine) (**34**) were isolated from the *A. belladonna L* bulbs grown in Egypt. (-)-Amarbellisine (**15**), (-)-pancracine (**93**), (+)-vittatine (**73**) and (+)-11-hydroxyvittatine (**74**) showed antibacterial activity against Gram-positive *Staphylococcus aureus*. The zone of inhibition was found to be the highest in both amarbellisine (**15**) and (-)-pancracine (**93**) in Gram-positive *Staphylococcus aureus* while amarbellisine (**15**) and vittatine (**73**) only exhibited antibacterial against Gram-negative *Escherichia coli*. Interestingly all isolated compounds displayed anti-fungal activity against *Candida albicans* (Evidente *et al*., 2004).

2.3 *Ammocharis coranica*

Ammocharis coranica is a bulbous plant of five species widely distributed in the summer-rainfall regions of southern Africa. It is well known agent of hallucination and arrow poison in Zululand. Its bulb scales are burned for traditional headings or used as putty and adhesive and used traditionally for the treatment of mental disorders (Hulme, 1954).

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Koorbanally *et al.* (2000) extensively isolated six alkaloids from them bulb of *A.coranica* but those compounds have investigated for biological evaluation. The study identified 6 alkaloid compounds. Lycorine (**11**), acetylcaranine (**13**), crinamine (**80**), 1-O-acetyllycorine (**14**), hippadine (**17**) and 6a-hydroxypowelline (**48**) are other alkaloids isolated from the bulb. The bulb crude ethanolic extract and fractions of ethyl acetate and butanol showed significant anticholinesterase activity as reported by Elisha *et al.*, (2012). Bioassay-guided fractionation of the fraction led to the isolation of lycorine (**11**). Lycorine (**11**) was to be responsible for anticholinesterase activity observed in and fractions with IC_{50} value of 29.3 + 3.15 µg/mL (Elisha *et al.*, 2012).

2.4 *Boophone disticha*

This genus contains two species of *Boophone disticha* and *Boophone haemanthoides* (Meerow and Snijman, 1998). It is a well-known member of Amaryllidaceae for its unique therapeutic and toxicological potential. It is used both in formal and informal sectors of traditional medicine (Louw *et al.*, 2002). It is distributed along East Africa, ranging from Sudan in the north to the Western Cape in South Africa (Wrinkle, 1984). It is extensively used in South Africa for circumcision and initiation rituals.

Boophone disticha is one of the important medicinal plants of southern Africa origin. It is a poison bulb of Amaryllidaceae used for the treatment of wounds, headaches, abdominal pains, eye conditions and traditional hallucinogen (De Smet, 1996; Gadaga *et al.*, 2011). It is commonly used in traditional folkore medicine for ailments pertaining to the central nervous system (CNS), wounds and infections and inflammatory conditions (Nair and Van Staden, 2014). Anxiolytic studies of hydroethanolic extracts of *B. disticha* showed that the extracts produced good anxiolytic-like activity in the Suok test, Open field and Elevated plus Maze. The activity is related to presence of isoquinoline alkaloids qualitatively assessed (Pote *et al.*, 2018).

Buphanamine (**64**), buphanidrine (**44**), buphanisine (**42)** and distichamine (**65**) are four alkaloids isolated by Neergaard *et al.*, (2009) through the use of HPLC-UV separation. The

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four alkaloids were found to have compelling affinity for serotonin transporter (SERT) with IC_{50} values of 55, 62, 199 and 65 μM respectively. Buphanidrine (**44**) and distichamine (**65**) were found to have activity in functional assay with IC_{50} values of 513 and 646 μM (Neergaard *et al*., 2009). Likewise, Sandager *et al*., (2005) reported on the isolation of buphanidrine (**44**) and buphanamine (**64**) from *B. disticha* using bioassay guided fractionation on TLC and preparative TLC. The two alkaloids were found to possess seratonin affinity in rat brain. However, they showed low affinity to the $5HT_{1A}$ receptor (Sandager *et al*., 2005).

Bioassay guided fractionation of the bulb methanolic extract afforded AChE inhibitor 6-α-hydroxycrinamine (**81**). The IC_{50} for the AChE activity was 445 μM and the compound also showed cytotoxicity activity when evaluated using both MTT assay and neutral red with IC_{50} values of 54.5 and 61.7μM, respectively (Adekanmi Adewusi *et al*., 2012). Cytoxicity evaluation also showed that the compound present low toxicity with IC_{50} values of 173 and 223 μM respectively (Adekanmi Adewusi *et al*., 2012). However, while Adewusi and Colleagues (2012) reported the isolation of crinine (**40**) alkaloids, Cheesman and colleagues (2012) also reported the isolation of crinine (**40**) alkaloids known as buphanidrine (**44**) and distichamine (**65**). The isolated crinine (**40**) alkaloid was found to have broad range of activities against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae* and *Bacillus subtilis*. The activity against *Bacillus subtilis* was found to be 2 folds lesser than the other bacteria (Cheesman *et al*., 2012; Nair and Van Staden, 2014).

2.5 *Boophone haemanthoides*

The scientific evaluation of *Boophone haemanthoides* is limited. The only published work to date on the genus is the research paper of Nair *et al*. (2013). The study isolated eight alkaloids (lycorine (**11**), crinine (**40**), buphanisine (**42**), buphanidrine (**44**), ambelline (**55**), undulatine (**53**), distichamine (**65**) and distichaminol (**66**)). The distichaminol (**66**) alkaloid was reported for the first time. However, lycorine (**11**) and distichamine (**65**) were reported to have remarkable activities against human acute lymphoblastic (CEM), breast

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adenocarcinoma (MCF-7) and cervical adenocarcinoma (HeLa) cell with IC_{50} values of (1.8, 9.2, 8.9 μM) and (5, 2.3, 4) respectively (Nair *et al.*, 2013).

2.6 *Brunsvigia gregaria*

Brunsvigia is endemic to South Africa with about 20 species that grow in semi-arid regions. The alkaloids reported from species are crinamidine (**51**), crinine (**40**), flexinine (**50**), haemanthidine (**75**), lycoramine (**18**), lycorine (**11**), tazettine (**86**) and undulatine (**53**), none of these alkaloids tested for any possible biological activity (Quenckenberg *et al.*, 1995). The isolated alkaloids covered different types of alkaloids that are peculiar to Amaryllidaceae family. Crinine (**40**), lycorine (**11**), haemanthamine (**76**) and tazettine-types of alkaloids found in this genus and have been identified as potent compounds for anticancer, anti-inflammatory, antifungal, anti acetylcholinesterase (De Andrade *et al.*, 2012). It is interesting to note that majority of alkaloids isolated from *B. orientalis* belong to the 5, 10b-ethanophenanthridines with many having methoxy group at the C-7 position as it is found in *B. josephinae*. Major compounds isolated from *Brunsvigia orientalis* are crinamidine (**51**), undulatine (**53**), 6-hydroxycriname (**77**), crinamine (**80**), 1-*epi*-Bowdensine (**60**), 1-*epi*-Demethoxybowdensine (**63**) and 1-*epi*-Deacetylbowdensine (**62**). Petit *et al.* (1984) described undulatine (**53**) as a cytotoxic agent from *A. belladonna* bulbs. Antiplasmodial activity of crinamine (**80**) has also been reported from the bulb of *Brunsvigia radulosa* (Campbell *et al.*, 2000). Also, remarkable cytotoxic and moderate antimalarial activities had been reported for crinamine (**80**) (Likhitwitayawuid *et al.*, 1993).

In the other hand, *Brunsvigia radulosa* bulbs yielded novel alkaloids, 1-*O*-acetylnorpluviine (**23**) with other known alkaloids (1-*epi*-deacetylbowdensine (**62**), crinamine (**80**), crinine (**40**), hamayne (**82**), lycorine (**11**), anhydrolycorin-6-one (**8**) and sternbergine (**22**) as reported by Campbell *et al.*, (2000). The isolated compounds were tested against two strains of malarial pathogens (chloroquine sensitive (D) and chloroquine resistant (FAC8)) and cytotoxicity against BL6 mouse melanoma cells. The 1-*O*-acetylnorpluviine (**23**) showed outstanding cytotoxic and moderate antiplamodial activity. Anhydrolycorin-6-one (**8**) Crinamine

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(**80**),hamayne (**82**), and sternbergine (**22**) showed moderate activity in both tests. Campbell *et al*., (2000). The work of (Crouch *et al*., 2002) on the ethanolic extract of the bulbs of *B. radulosa* also produced lycorine (**11)**, crinamine (**80**), crinine (**40**), hamayne (**82**), 1-*O*-acetyllycorine (**14**) and anhydrolycorinium (**7**). Some of the alkaloids reported have been isolated in other genus of Amaryllidaceae family. Anhydrolycorinium (**7**) from *A. belladonna* had been reported to have potent antineoplastic activity against murine P-388 lymphocytic leukaemia by (Petit *et al*., 1984).The Isolation of anhydrolycorinium (**7**) provides scientific evidence for the traditional use of *A. belladonna* for the treatment of abdomina pain.

2.7 *Clivia caulescens*

Clivia genus is endemic to South Africa region and represented by three species (*C. miniata, C.nobilis* and *C. caulescences*) found mostly in inland forest. Genus of *Clivia* are used by traditional healer in South Africa as cure for physiological ailment or of spiritual origin and also implicated in human poisoning (Crouch *et al*., 2003). The array of large number of isoquinoline alkaloids have been linked to its biological activity. Isolation of compounds from the bulbs of *C. caulescens* R.A, Dyer, revealed isoquinoline alkaloids such as hippeastrine (**34**), haemanthamine (**76**), 11-hydroxyvittatine (**74**), lycorine (**11**) sterbergine (**22**), haemanthamine (**76**) and haemanthidine (**75**) (Crouch *et al*., 2003). Lycorine (**11**) is the most abundant compound in the *C. caulescens* bulb. Lycorine (**11**) is known to cause scorbutic symptoms in experimental animals and acts as a respiratory stimulant (Crouch *et al*., 2003). Moderate antitumor activity, antiviral properties and weak protozoicide had been reported previously for lycorine (**11**) (Crouch *et al*., 2003). Tazettine (**86**) isolated from *C. gardenii* has been shown to have a LD_{50} of 71 which is lower than that of lycorine (**11**) with 41 mg in dog. However, Crouch *et al*. (2003) found that roots and leaves of the crude extract of *Clivia miniata* Regel had profound antiviral activity. The phytochemical isolation of the extract from *C. miniata* produces lycorine (**11**), clivimine (**35**), clivonine (**30**) and cliviamartine (**36**). Further study revealed that lycorine (**11**) was responsible for the observed antiviral effect because other isolated compounds showed no antiviral property (xiao-Yu Bai, Li-Da Du and

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Guan-Hua Du., 2018). Lycorine (**11**) showed poliomyelitis virus inhibition at 1 µg/mL while at 25 µg/ml cytotoxicity ensured. Further, (Shawky, 2016) reported isolation of alkaloids from the *C. nobilis* cultivated in Egypt with their biological activity. The study reported isolation of four alkaloids, lycorine (**11**) with pyrrolo{de} phenanthridine nucleus (lycorine-type) which is the common alkaloid of the Amaryllidaceae family, clivatine (**31**) and nobilisine (**33**), both with [2]benzopyrano (3,4-g) indole nucleus (lycorenine-type) and (+) 8-*O*-demethylmaritidine (**71**) with 5,10b-ethanophenanthridine nucleus (crinine-type). Nobilisine (**33**) was found to produce exceptional activity against the Gram-negative *Pseudomonas aeruginosa* comparable to standard used. Evidente *et al*. (2004) also isolated compounds from *C. nobilis* cultivated in Egypt. They isolated two alkaloids with pyrrolo-[de]phenanthridine and [2]benzopyranol[3,4-g]indole ring systems. The previously known alkaloids such as lycorine (**11**), nobilisine (**33**), nobilisitine (**32**A) and (**32**B) were also reported. The extract and other isolated alkaloids except lycorine (**11**) and clivimine (**35**) had antibacterial activity against Gram-positive *S. aureus*. Nobilisitine (**32**B) had antifungal activity against *C. albicans* while (**32**B) showed remarkable activity against *S. aureus* and *C. albicans* with minimum inhibition concentration (MIC) at 78 and 312 µg/mL respectively.

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2.8 *Crinum*

Crinum is an important genus of Amaryllidaceae with diverse geological distribution. They are found in tropics, subtropics and warm temperate regions of the world. The genus comprises about 130 species distributed throughout the World. African region housed most of the species with Southern Africa having 22 endemic species (Benson, 1970). Significant percentage of these species are threatened with extinction due to either that they are endemic and naturally rare or experiencing significant habitat loss due or over exploited as ornamental plants and herbal medicine (Maroyi, 2016). It has traditional medicinal values such as antitumor, immunostimulating, analgesic, antiviral, antimalarial, antibacterial and antifungal (Refaat *et al*., 2012). Phytochemical screening resulted in the identification of classes of compounds such as alkaloids, phenolic and non-alkaloids constituents (Benson,

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1970). The alkaloids are related to the group of alkaloids that are consistent with Amaryllidaceae family.

2.8.1 *Crinum bulbispermum*

Crinum bulbispermum naturally grows along rivers and streams or in damp depressions (Maroyi, 2016). The bulb is traditionally used to treat aching joints, rheumatism, varicose veins, backache, kidney or bladder infections and as poultices for septic sores and abscesses in South Africa (Roberts, 1990).

The lycorine (**11**) obtained from *C. bulbispermum* was found to have anticancer property with IC_{50} values ranging from 0.3 to1.6 μg/mL against battery of cancer cell lines. The anti-proliferative activity of the compound was reported to be through induction of apoptosis (Hajiz *et al*., 1991). Elgorashi *et al*. (2003) isolated 15 alkaloids from both *C. bulbispermum* and *C. moorei*. The anti-inflammatory activities of the alkaloids were tested *in vitro* and showed inhibitory activity against COX-1 and COX-2. 8α-ethoxyprecriwelline (**90**) had moderate inhibitory activity against both COX-1 and -2. Others showed moderate inhibitory only against COX-1 excerpt cherylline (**105**) that did not show any activity against both COX-1 and -2. The other alkaloids isolated are *N*-desmethyl-8α-ethoxypretazettine (**87**), *N*-desmethyl-8β-ethoxypretazettine (**88**), bulbispermine (**78**), 1-*O*-acetyllycorine (**14**), *epi*-vittatine (**79**), crinamine (**80**), 3-*O*-acetylhamayne (**83**), 6-hydroxycrinamine (**77**), *epi*-buphanisine (**43**), powelline (**41**), crinine (**40**), crinamidine (**51**) and 1-epi-deacetylbowdensine (**62**) (Elgorashi *et al*., 2003). crinine (**40**), crinamine (**80**) and lycorine (**11**) were isolated from the bulb and showed AChE inhibitory activity with IC_{50} values of 461 μM, 300 μM and 213 μM respectively (Elgorashi and Van Staden, 2009). Van Dyk *et al.* (2009) showed that lycorine (**11**) isolated from the bulb has IC_{50} value of 0.03 μg/mL against chloroquine-resistant *Plasmodium falciparum* (FCR-3).

Extracts of different parts of the plant have been shown to have strong antioxidant potential. Bulb extract was reported to have IC_{50} value of 68.5μg/mL against ABTS radicals. High phenolic contents, radical scavenging prowess, metal chelating, hydrogen donor and singlet

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oxygen quenchers properties of the plant have been reported (Adekanmi Adewusi *et al.*, 2012; Bhandare *et al.*, 2010). Like other Amaryllidaceae members alkaloids have been identified as major compounds in the bulbs and mostly responsible for its pharmacological action (Adekanmi Adewusi *et al.*, 2012; Seoposengwe *et al.*, 2013). However, both alkaloids of Amaryllidaceae type and non-alkaloids have been isolated from the plant. Six diverse isoquinoline alkaloids such as lycorine (**11**), galanthamine (**100**), Cherylline (**105**), tazettine (**86**), augustamine (**101**) and trispaeridine (**102**) were identified (Maroyi, 2016).

2.8.2 *Crinum lugardiae*

Crinum lugardiae is a native to South Africa, Namibia and Botswana. The study of the bulbs and roots of the plant led to the isolation of lycorine (**11**), crinine (**40**) and hamayne (**82**) (Elgorashi and Van Staden, 2001). The compounds isolated from this species have been reported for different biological activities from other members of the Amaryllidaceae (Ding *et al.*, 2017).

2.8.3 *Crinum macowanii*

Crinum macowanii is locally referred to as umduze by the Ndebele people; South Africa. It is locally used against sexually-transmitted diseases, backache and also to increase lactation in animal and human mothers. It is used traditionally as a remedy for boils, diarrhoea, fever, inflammation, respiratory system problems, skin rashes, tuberculosis, wounds and urinary tract problems (Maroyi, 2016). It's antiviral against RNA viruses and antifungal against *Candida albicans* have been reported by Duri *et al.*, (1994). Hydro ethanolic extract of *C. macowanii* at 40 mg/kg was reported to have reversed significantly the scopolamine induced amnesia in Balb/c mice, activity which is comparable to the positive control Donepezil (Chen *et al.*, 2015).

The extraction of ethanolic bulb extract of *C. macowanii* afforded eleven alkaloids such as macowine (**39**), 4a-Dehydroxycrinamabine (**58**), lycorine (**11**), cherylline (**105**), crinine (**40**), krepowine (**67**), powelline (**41**), buphanidrine (**44**), crinamidine (**51**), undulatine (**53**) and 1-

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epi-deacetylbowdensine (**62**) (Nair *et al*., 2000). Lycorine (**11**) was the only compound that showed mild activity against *plamodium falciparum* with IC_{50} value of 0.34 µg/mL (Nair *et al*., 2000). None also showed activity against *Leishmania donovani* and *Trypanosma cruzi* (Nair *et al*., 2000). Hippadine (**17**), an alkaloid isolated from *C. macowanii* has been found to play a laudable role in male spontaneous hypertensive Wistar rats by lowering blood pressure and heart rate. The activity of hippadine (**17**) was related to inhibition of alpha1 and beta1 adrenoceptor (Maroyi, 2016; Mugabo *et al*., 2014). Lycorine (**11**) and hamayne (**82**) were isolated from the bulb and showed dose dependent Aβ42 lowering activity in HeLa cell line (Kwon *et al*., 2011). Screening for mutagenicity activity of 51 South African medicinal plant, Elgorashi *et al*. (2003) reported that *C. macowanni* extract only showed mutagenicity in strain TA98 with and without metabolic activation. *C. macowanii* has been shown however, to be more related to *C. bulbispermum* than *C. moorei* according to the types of alkaloids constituents (Elgorashi *et al*., 2003).

2.8.4 *Crinum moorei*

Crinum moorei is one of the Amaryllidaceae that are gradually going to extinction because of the overwhelming uses of the bulb. It is difficult to propagate using conventional method coupled with its exploitation for medicinal use and horticultural aggravate the problem of the extinction (Koopowitz, 1986). Elgorashi *et al*. (2003) isolated two new alkaloids named 3-[4-(8-aminoethyl) phenoxyl] bulbis-permine (**84**) and mooreine (**16**) among the thirteen known Amaryllidaceae alkaloids from *C. moorei*. Ten alkaloids *viz* lycorine (**11**), crinine (**40**), cherylline (**105**), crinamidine (**51**), crinine (**40**), *epi*-buphanisine (**43**), powelline (**41**), undulatine (**53**), 1-*epi*-deacetylbowdensine (**62**) and 3-*O*-acetylhamayne (**83**) were identified from the *in vitro* propagated bulblet of *C. moorei* (Fennell *et al*., 2003). Anti-proliferative activity of bulbispermine (**78**) a crinine type Amaryllidaceae alkaloids isolated from *C. moorei* against apoptosis resistant cell line showed IC_{50} values of 9 µM for T98G and 38 µM for U373 and 11 and 8 µM against Hs683 and HeLa cells respectively (Luchetti *et al*., 2012).

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2.8.5 *Crinum stuhlmannii*

Crinum stuhlmannii is one of the Genus *Crinum* found in Africa to treat health challenges like coughs, cold, renal and hepatic problems, backache and sexually transmitted diseases (Fennell and van Staden 2001). Lycorine (**11**), 9-*O*-demethylpluvine (**20**), kirkine (**24**), ambelline (**45**), crinamine (**80**), crinine (**40**), hamayne (**82**) and amabiline (**55**) are the alkaloids isolated by Machocho *et al*., (1998).

2.9 *Crossyne*

Crossyne is new genus that consists of two species *viz C. flava* and *C. guttata*. These species were previously believed to be *Boophone* but differ in their capsules and seeds. The genus is native to Western Cape Province of South Africa and in the red list of South African plants South African National Biodiversity Institute (SANBI). A few scientific evaluations on this genus were only done on *Crossyne flava* by Viladomat *et al*., (1995) under the name *Boophone flava* while *Crossyne guttata* has not been scientifically evaluated.

2.9.1 *Crossyne flava*

Fourteen alkaloids have been isolated from the bulb, in which lycorine (**11**), augustine (**52**) and crinamine (**80**) are the main cytotoxic and antimalarial. However, at the period of reporting their work on this plant, Viladomat *et al*., (1995) show that montabuphine (**96**). Buflavine (**97**) and 8-*O*-demethylbluflavine (**97**) are the first alkaloids with eight heterocyclic rings. Crinine (**40**), buphanisine (**42**), undulatine (**53**), hamayne (**82**), 3-*O*-acetylhamayne (**83**), *epi*-vittatine (**79**) and *epi*-buphanisine (**43**) were also isolated from the plant (Viladomat *et al*., 1995).

2.10 *Cyrtanthus*

Cyrtanthus species are well distributed in the Western and Eastern provinces of South Africa while it is sparsely distributed in KwaZulu-Natal province. The species are used to treat scrofula, chronic cough, headache, cystitis and leprosy (Elgorashi and Van Staden, 2004). Genus *Cyrtanthus* is most diverse in terms of colour, morphology and orientations which

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made it an attractive for ornamental. It has potential as a potted plant for commercial importance (Du Plessis and Duncan 1989). The genus has about 50 bulbous species of which seven are used in South African traditional medicines. It is a known largest genus of southern Africa's Amaryllidaceae with almost 90% of its species resides in South Africa. The genus is known by their rapid flowering response to natural bush fire. *C. breviflorus, C. contractus, C. mackenii, C. sanguineus, C stenanthus* and *C. tuckii* bulbs are used as infusion by the Zulu traditional healers as protection against storms and evil spirits (Elgorashi and Van Staden 2009). *C. obliquus* root infusions are used for stomach ache and treatment of leprosy. *C. breviflorus* bulbs are for roundworms and tapeworm treatment while *C. sanguineus* is used to facilitate labour in women (Jäger *et al.*, 1996).

2.10.1 *Cyrtanthus breviflorus*

Cyrtanthus breviflorus is a widespread species from coastal region to upland and used by Zulu to treat intestinal worms (Viladomat *et al.*, 1997). The work of Tanahashi et al., (1990) showed that Amaryllidaceae alkaloids galanthamine (**100**) used as anti-Alzheimer is produced by some of the species of this genus. The alkaloids isolated by the same group from the bulb are lycorine (**11**), haemanthamine (**76**), crinamine (**80**) and tazettine (**86**) (Crouch *et al.*, 2005).

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2.10.2 *Cyrtanthus contractus*

Cyrtanthus contractus is commonly used by foklore medicinal practional of South African to treat different ailments including mental illness and age dependent dementia (Ncube *et al.*, 2015). The alkaloids contents of the plants have been shown to vary according to the period of collection. Of significance is the collection between May -September shown to have significant total alkaloids and related significantly to the bioactivity of the plant (Ncube *et al.*, 2015). The plant afforded the isolation of narciprimine (**104**) and was found to have activity against AChE with IC_{50} value of 78.9 µM (Nair *et al.*, 2011).

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2.10.3 *Cyrtanthus elatus*

Zephyranthine (**5**), 1,2-*O*-diacetylzephyranthine (**6**), galanthamine (**100**), haemanthamine (**76**) and haemanthidine (**75**) were isolated from the bulb of *C. elantus*. (Herrera *et al*., 2001). Haemanthamine (**76**) showed activity against *Entamoeba hystolytica* and *Plasmodium falciparum* with IC_{50} values of 0.75 and 0.67 μg/mL respectively while haemanthidine (**75**) showed 0.70, 1.1, and 1.58 μg/mL against *P. falciparum*, *Trypanosoma brucei rhodesiense* and *T. cruzi* respectively (Herrera *et al*., 2001).

2.10.4 *Cyrtanthus faicatus*

Cyrtanthus faicatus is a globes bulb of about 50-100 mm found majorly in the Natal midlands (Reid and Dyer., 1984). It is found useful by the Zulu folklore medicine for the treatment of scrofula, chronic coughs, headahce, cystitis, leprosy, roundworm and tapeworm (Hutchings *et al*., 1996). Alkaloids such as papyramine (**70**), epipapyramine (**72**), maritidine (**68**), *O*-methylmaritidine (**69**) and tazettine (**86**) were isolated from *C. falcatus* (Elgorashi and Van Staden, 2003).

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2.10.5 *Cyrtanthus mackenii*

Lycorine (**11**), tazettine (**86**) and 11-hydroxyvittatine (**74**) were isolated from the bulb of *C. mackenii* (Snijman and Meerow, 2010). The pharmacological screening of crude extract of Amaryllidaceae family showed that *C. mackenii, C. suaveolens* and *C. falcatus* have anti-inflammatory activity and COX-1 and COX-2 inhibition. *C. falcatus* and *C. suaveolens* have been shown to have mutagenic effects in the *Sailmonella* (Elgorashi and Van Staden, 2004).

2.10.6 *Cyrtanthus obliquus*

Four alkaloids obliquine (**85**) 11-hydroxygalanthine (21), 3-epi-macronine (**89**), narcissidine (**19**) and tazettine (**86**) have been isolated from the bulbs (Brine *et al*., 2002). Substantial in vitro antioxidants were reported for the isolated compounds (Mahlangeni *et al*., 2015). The entire isolated compound did not however show cytotoxicity against cell lines of mammalian origin at 100 μg/mL (Brine *et al*., 2002).

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2.11 *Gethyllis*

Gethyllis is an endanger genus that comprises of 32 species distributed widely in the Western and Northern Cape. The genus chemistry and biological activities is limited (Du Plessis and Duncan, 1989). *Gethyllis ciliaris* is used traditionally for flatulence and indigestion by the people in the Cape. The fruit pods of the species are good for stomach disorders and the flower is used for toothache (Watt and Breyer-Brandwijk, 1962).

2.12 *Haemanthus*

Haemanthus genus comprises of 22 species widespread in southern Africa (Snijman, 2000).

2.12.1 *Haemanthus albiflos*

Haemanthus albiflos is used as a poultice over fractures and its infusion to manage chronic coughs by South African locals (Dold and Cocks, 2002). The extract of this bulb showed antiviral and cytotoxicity activities. The Zulu used the bulb or *H. deformis* as a decoction with *Scadoxus puniceus* and *C. macowanii* to treat amenorrhea and pregnancy related problem (Crouch *et al*., 2005). The alkaloids contents of *H. albiflos*, *H. pauculifolius* and *H. deformis* were investigated by Crouch *et al*., (2005). The work resulted in the isolation of homolycorine (**25**), albomaculine (**26**) and *O*-methyl-lycorenium salt (**38**). *H. albiflos* and *H. pauculifolius* afforded homolycorine (**25**), Paucamine (**37**), quaternary salt of homolycorine (**25**), montanine (**94**) and manthidine (**95**). *H. deformis* was found to yield coccinine (**96**), montanine (**94**) and salt of manthidine (**95**) (Crouch *et al*., 2005). Hydroalcoholic extract of *H. albiflos* was shown to delay in viral RNA synthesis at concentration below 0.5 µg/mL while the higher concentration blocked the RNA synthesis of poliovirus propagated in HeLa cells (Husson *et al*., 1993). The alkaloids, montanine (**94**) and coccinine (**96**) are the major bioactive principles in *H. coccineus*, *H. montanus* and *H. sanguineus*. The affinity for the serotonin transport protein (SERT) with IC_{50} values for the extracts of *H. coccineus* (2.0 ± 1.1 µg/mL), *H. montanus* (6.8 ± 1.0 µg/mL) and *H. sanguineus* (28.7 ± 1.1 µg/mL) depicting the highest activity for *H. coccineus*. These activities are more potent compared to the activities of the major active constituents, coccinine (**96**) and montanine (**94**). The results

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from the study did conclude that the observed activity for the extract did not correlate with relative proportion of the two major alkaloids but due to more potent unidentified minor constituents (Stafford *et al.*, 2013).

2.13 *Nerine*

Nerine genus has the second largest species of Amaryllidaceae family. The bulbs are used by Sotho and Zulu tribes to treat coughs and colds, renal and hepatic problems and for infertility (Cahlíková *et al.*, 2019). *Nerine* represents one of the seven sub-tribes known as Amaryllidinae, which is mostly found in temperate region of South Africa (Snijman and Linder, 1996).

2.13.1 *Nerine bowdenii*

Nerine bowdenii is native to the Eastern Cape Province and KwaZulu-Natal Drakensberg. It is an endemic species (Van Rijn *et al.*, 2010). Bioassay guided work of Van Rijn *et al.* (2010) showed that *N. bowdenii* toluene extract has strong activity as AChE inhibitor. 11-*O*-acetylambelline (**46**), filifoline (**47**), undulatine (**53**), buphanisine (**42**) and ambelline (**45**) were identified from the bulbs. All the isolated alkaloids were able to inhibit AChE but not as active as galanthamine (**100**) (Van Rijn *et al.*, 2010). The alkaloids isolated from this bulb belong to the belladine (**1**). (belladine (**1**), 6-*O*-demethylbelladine (**4**) and 4'-*O*-Demethylbelladine (**2**)), crinine (**40**), (undulatine (**53**), 11-O-acetylambelline (**46**), buphanidrine (**44**), buphanisine (**42**), Ambelline (**45**), deacetylbowdesine (**61**), buphanamine (**64**), 6a-hydroxyundulatine (**54**), 1-*O*-acetylbulbisine (**57**), crinamidine (**51**), powelline (**41**), (haemanthamine (**76**) and hamayne (**82**) lycorine (**11**) acetylcaranine (**13**), caranine (**10**) and 1-*O*-acetyllycorine (**14**) and tazettine (**86**) (Vaněčková *et al.*, 2016). Undulatine (**53)** and powelline (**41**) showed highest AChE inhibitory activity. Contrary to the previous findings, the work showed activity for 1-*O*-acetyllycorine (**14**) against BChE activity. The authors conclude that the activity observed for the compound greatly relate to the source of the

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compounds. Buphanisine (**42**) showed remarkable cytotoxicity activity against P53-mutated Caco-2 and HT-29 colorectal adenocarcinoma cells (Vaněčková *et al.*, 2016).

2.13.2 *Nerine filifolia*

Nerine filifolia was reported to have IC_{50} values activity of 18.5±0.8 and 58.6±1.3 μg/mL against AChE and BChE respectively (Cahlíková *et al.*, 2012). In 2005, Nair *et al* reported isolation of novel compounds N-demethylbelladine (**3**), 6α-methoxybuphanidrine (**49**) and filifoline (**47**). Filifoline (**47**) did not show cytotoxic to myoblast (L6) cells and no exhibition of in vitro anti-protozoal activity (Nair *et al*, 2005).

2.13.3 *Nerine filamentosa*

Nerine filamentosa extract has been reported to show anti acetylcholinesterase and anti butylcholinesterase activity with IC_{50} values of 21.6 and 13.0 μg/mL compared with *Nerine filifolia* extract that 18.5 and 58.6 μg/mL respectively (Cahlíková *et al.*, 2012). The pattern of *N. filamentosa* alkaloids has been shown to be dominated by crinine-type Amaryllidaceae alkaloids like undulatine (**53**), buphanamine (**64**), ambelline (**45**) and parkamine (**12**). *N. bowdenii* alkaloids content has been reported to be similar to the alkaloids constituents of *N. filamentosa*. This implies that modest activity of *N. bowdenii* can be related to the possible activity of *N. filamentosa* (Cahlíková *et al.*, 2011).

2.13.4 *Nerine huttoniae*

The compounds isolated from *N. huttoniae* are 6-*O*-methylkrigeine (**28**), krigeine (**27**) and oxokrigenamine (**29**). Oxokrigenamine (**29**) showed anticholinesterase activity with IC_{50} value of 34±11 μg/mL whereas 6-*O*-methylkrigeinen (**28**) failed to show activity with concentration as high as 50 μg/mL (Molander *et al.*, 2011).

2.13.5 *Nerine sarniensis*

Nerine sarniensis is restricted to the Western Cape of South Africa and it also known as the Guernsey Lilly. Extract of this bulb showed potent adulticidal and larvicidal activity against

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Aedes aegypti (Masi *et al.*, 2016). Sarniensine (**91**), lycorine (**11**), 3-*epi*-macronine (**89**) and tazettine (**86**) were isolated from the bulbs. Sarniensine (**91**) alkaloids showed remarkable adulticidal activity with IC_{50} value of 1.38+0.056 µg/mL (Masi *et al*, 2016). Crinsarnine (**56**) and Sarniensinol (**92**) bowdensine (**59**), Sarniensine (**91**), hippadine (**17**) and 1-*O*-acetyl-lycorine (**14**).were isolated from *N. sarniensis,* only crinsarnine (**56**) showed adulticidal activity with an LD_{50} of 2.29 +0.049 µg/mosquito (Masi *et al.*, 2016).

2.14 *Scadoxus puniceus*

Methanolic extract of *S. puniceus* showed anti-apoptotic, restored intracellular contents of glutathione in the cell line which implies that the extract has neuroprotective capacity (Seoposengwe *et al.*, 2013). Ethyl acetate fraction of *S. puniceus* showed anti AChE activities with IC_{50} value of 0.3 µg/mL (Adekanmi Adewusi *et al.*, 2012).

S. puniceus water extract exhibited over 70 % inhibitory activity of COX-1 enzyme (Ndhlala *et al.*, 2011). Alkalods isolated from the plant are haemanthamine (**76**) and haemanthidine (**75**) with remarkable AChE inhibitory activity of IC_{50} values of 23.1 and 23.7µM respectively (Naidoo *et al.*, 2018).

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Table 1: List of Alkaloids isolated from SA Amaryllidaceae and their biological activities

<table>
<tr><th>No</th><th>Name of plant</th><th>Isolated alkaloids</th><th>Biological Activities</th><th>Reference</th></tr>
<tr><td>1</td><td>Amaryllis belladonna</td><td>9,83,69</td><td>antiprotozoal and cytotoxic activities (IC_{50} of µg/mL)
<table>
<tr><th></th><th>T. b. R STIB 900</th><th>T. C T. C4 LacZ</th><th>L. D. MHOM-ET67/L82</th><th>P. F. NF54</th><th>Cytotoxic L6</th></tr>
<tr><td>9</td><td>1.97</td><td>35.9</td><td>>100</td><td>3.21</td><td>14.2</td></tr>
<tr><td>83</td><td>1.51</td><td>8.25</td><td>17.91</td><td>1.14</td><td>17.2</td></tr>
<tr><td>69</td><td>28.2</td><td>62.9</td><td>> 100</td><td>25.9</td><td>> 100</td></tr>
</table></td><td>Tallini et al., 2017</td></tr>
<tr><td>2</td><td></td><td>11,103</td><td>allelochemical activity against weeds
11: showed IC_{50} measured for radicle growth inhibition at 0.93µM.
103: has IC_{50} of 0.10µM</td><td>Wahyuni et al., 2013</td></tr>
<tr><td>3</td><td></td><td>71</td><td>71: showed anticancer activity of (MCF7 of 100µM), (NCI-H460 of 82µM) and (SF-268 of 69µM).</td><td>Abou-Donia., 2005b</td></tr>
<tr><td>4</td><td></td><td>15,34,73,74,93</td><td>Antibacterial activity (IC_{50} of µg/mL)
<table>
<tr><th></th><th>AS (MIC)</th><th>EC (MIC)</th><th>PA (MIC)</th><th>CA (MIC)</th></tr>
<tr><td>15</td><td>22</td><td>22</td><td>--------</td><td>24</td></tr>
<tr><td>34</td><td>--------</td><td>--------</td><td>--------</td><td>25</td></tr>
<tr><td>73</td><td>19</td><td>22</td><td>--------</td><td>17</td></tr>
<tr><td>74</td><td>17</td><td>--------</td><td>--------</td><td>20</td></tr>
<tr><td>93</td><td>22</td><td>--------</td><td>16</td><td>24</td></tr>
</table></td><td>Evidente et al., 2004</td></tr>
<tr><td>5</td><td>Ammocharis coranica</td><td>11,13,14,17,48,80</td><td></td><td>Koorbanally et al., 2000</td></tr>
<tr><td>6</td><td>Boophone disticha</td><td>42,44,64,65</td><td>Antibacterial activity (IC_{50} of µg/mL)
<table>
<tr><th></th><th>BS (MIC)</th><th>SA (MIC)</th><th>EC (MIC)</th><th>KP (MIC)</th></tr>
<tr><td>44</td><td>0.13</td><td>0.063</td><td>0.063</td><td>0.063</td></tr>
<tr><td>65</td><td>0.13</td><td>0.063</td><td>0.063</td><td>0.063</td></tr>
</table></td><td>Neergaard et al., 2009</td></tr>
<tr><td>7</td><td></td><td>81</td><td>81: anti-AChE activity of IC_{50} of 445±30 µM, and cytotoxicity against MTT value of IC_{50} of 54.5±2.6µM</td><td>Adewusi et al., 2013</td></tr>
<tr><td>8</td><td></td><td>40</td><td>40: showed Anticancer activity against HL-60/Dox IC_{50} value of 14.04µM</td><td>Nair and Van Staden., 2014</td></tr>
<tr><td>9</td><td>Boophone haemanthoides</td><td>11,40,42,44,53,55,65,66</td><td>Cytotoxic activity
<table>
<tr><th></th><th>human acute lymphoblastic (CEM)</th><th>breast adenocarcinoma (MCF-7)</th><th>cervical adenocarcinoma (HeLa)</th></tr>
<tr><td>53</td><td>> 50</td><td>> 50</td><td>> 50</td></tr>
<tr><td>55</td><td>> 50</td><td>> 50</td><td>> 50</td></tr>
</table></td><td>Nair et al., 2013</td></tr>
</table>

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<table>
<tr><td></td><td></td><td></td><td><table><tr><td>66</td><td>> 50</td><td>> 50</td><td>> 50</td></tr></table></td><td></td></tr>
<tr><td>10</td><td>Brunsvigia gregaria</td><td>11,18,40,50,51,53,75,86</td><td></td><td>Quenckenber et al., 1995</td></tr>
<tr><td>11</td><td>Brunsvigia orientalis</td><td>76</td><td></td><td>De Andrade et al., 2012</td></tr>
<tr><td>12</td><td></td><td>60,63,77</td><td></td><td>Pettit et al., 1984</td></tr>
<tr><td>13</td><td>Brunsvigia radulosa</td><td>8,22,23,62,80,82</td><td>Antiplasmodial and cytotoxic activity (IC_{50} of µg/mL)<table>
<tr><td></td><td>chloroquine-sensitive (D10)</td><td>chloroquine-resistant (FAC8)</td><td>Cytotoxic with melanoma cells (BL6)</td></tr>
<tr><td>8</td><td>6.1</td><td>6.41</td><td>3.3</td></tr>
<tr><td>22</td><td>3.9</td><td>4.8</td><td>3.5</td></tr>
<tr><td>23</td><td>28.3</td><td>34.2</td><td>1.6</td></tr>
<tr><td>62</td><td>> 50</td><td>> 50</td><td>> 100</td></tr>
<tr><td>80</td><td>2.8</td><td>3.4</td><td>1.8</td></tr>
<tr><td>82</td><td>15.6</td><td>18.2</td><td>9.34</td></tr>
</table></td><td>Campbell et al., 2000</td></tr>
<tr><td>14</td><td></td><td>7,14</td><td></td><td>Crouch et al., 2002</td></tr>
<tr><td>15</td><td>Clivia caulescens</td><td>22,34,74,75,76,86</td><td></td><td>Crouch et al., 2003</td></tr>
<tr><td>16</td><td>Clivia miniata</td><td>11,30,35,36</td><td>Antiviral activity

11: showed poliomyelitis virus inhibition at 1µg/mL while at 25 µg/mL.</td><td>Leven et al., 1982</td></tr>
<tr><td>17</td><td>Clivia nobilis</td><td>31, 33,71</td><td>Antibacterial activity (IC_{50} of µg/mL)<table>
<tr><td></td><td>AS (MIC)</td><td>AS (MBC)</td><td>PA (MIC)</td><td>PA (MBC)</td></tr>
<tr><td>31</td><td>125</td><td>250</td><td>250</td><td>500</td></tr>
<tr><td>33</td><td>31.25</td><td>62.5</td><td>62.50</td><td>125</td></tr>
<tr><td>71</td><td>31.25</td><td>62.5</td><td>125</td><td>250</td></tr>
</table></td><td>Shawky, E., 2016</td></tr>
<tr><td>18</td><td></td><td>32</td><td>Antibacterial activity

showed (MIC IC_{50} of 78µg/mL against SA and 312µg/mL. against CA</td><td>Evidente et al., 2004</td></tr>
<tr><td>19</td><td>Crinum bulbispermum</td><td>11</td><td>Anticancer activity

11: showed anticancer IC_{50} of 0.3 ± 1.6 µg/mL</td><td>Abd El Hafiz et al., 1991</td></tr>
<tr><td>20</td><td></td><td>14,40,41,43,51,62,77,78,79,80,83,87,88,90,104</td><td>Anti-inflammatory activity using COX-1 and COX-2<table>
<tr><td></td><td>COX-1</td><td>COX-2</td></tr>
<tr><td>43</td><td>4(%)</td><td>0(%)</td></tr>
<tr><td>78</td><td>23(%)</td><td>0(%)</td></tr>
<tr><td>79</td><td>17(%)</td><td>0(%)</td></tr>
</table></td><td>Elgorashi et al., 2003</td></tr>
</table>

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<table>
<tr><td></td><td></td><td></td><td><table><tr><td>87</td><td>32(%)</td><td>0(%)</td></tr><tr><td>88</td><td>24(%)</td><td>0(%)</td></tr></table></td><td></td></tr>
<tr><td>21</td><td></td><td>86,100,101,102,105</td><td></td><td>Maroyi et al., 2016</td></tr>
<tr><td>22</td><td>Crinum lugardiae</td><td>11,40,82</td><td></td><td>Elgorashi and van Staden., 2001</td></tr>
<tr><td>23</td><td>Crinum macowanii</td><td>11,39,40,41,44,51,53,58,62,67,104</td><td>Acetylcholinesterase activity

105: showed IC_{50} 407 ±32 μM</td><td>Elgorashi et al., 2003</td></tr>
<tr><td>24</td><td></td><td>17,82</td><td>Antiprotozoal activity

17: showed Antiprotozoal against T.B. rhodesiense STIB-900 (IC_{50} 8.4μg/mL).</td><td>Mugabo et al., 2014</td></tr>
<tr><td>25</td><td>Crinum moorei</td><td>16,84</td><td></td><td>Elgorashi et al., 2003a</td></tr>
<tr><td>26</td><td></td><td>14,40,41,43,51,62,77,78,79,80,87,88,92,105</td><td>Acetylcholinesterase activity

14: has IC_{50} 0.96 μM</td><td>Elgorashi et al., 2001</td></tr>
<tr><td>27</td><td></td><td>11,53,83</td><td></td><td>Fennell et al., 2003</td></tr>
<tr><td>28</td><td>Crinum stuhlmannii</td><td>11,20,24,40,45,55,80,82</td><td>11, 80, 45 showed activity against Entamoeba hystolytica (strain HK9(IC_{50} value of 0.23, 0.53 and 0.28 μg/mL respectively</td><td>Machocho et al., 1998</td></tr>
<tr><td>29</td><td>Crossyne flava</td><td>11,40,42,43, 52,53,79,80,82,83,97,98,99</td><td></td><td>Viladomat et al., 1995</td></tr>
<tr><td>30</td><td>Cyrtanthus breviflorus</td><td>11,76,80,86</td><td></td><td>Crouch et al., 2005</td></tr>
<tr><td>31</td><td></td><td>100</td><td></td><td>Tanahashi and Co., 1990</td></tr>
<tr><td>32</td><td>Cyrtanthus contractus</td><td>104</td><td></td><td>Nair et al., 2011</td></tr>
<tr><td>33</td><td>Cyrtanthus elatus</td><td>5,6,75,76,100</td><td>Antibacterial activity IC50 of μg/mL<table><tr><td></td><td>EH (HK9)</td><td>PF (HK9)</td></tr><tr><td>75</td><td>0.70</td><td>1.1</td></tr><tr><td>76</td><td>75</td><td>0.67</td></tr></table></td><td></td></tr>
<tr><td>34</td><td>Cyrtanthus falcatus</td><td>68,69,70,72,86</td><td></td><td>Elgorashi and van staden., 2003</td></tr>
<tr><td>35</td><td>Cyrtanthus mackenii</td><td>11,86,74</td><td></td><td>Snijman and meerow., 2010</td></tr>
<tr><td>36</td><td>Cyrtanthus obliquus</td><td>19,21,85,86,89</td><td></td><td>Brine et al., 2002</td></tr>
<tr><td>37</td><td>Haemanthus albiflos</td><td>25,26,37,38,94,95,96</td><td>94: showed cytotoxicity activity against Calcein-AM IC_{50} of 0.78 ± 0.16μM.</td><td>Crouch et al., 2005</td></tr>
<tr><td>38</td><td>Nerine bowdenii</td><td>1,2,4,10,11,13,14,40,41,44,51,54,57,61,64,76,82,86,100</td><td>HuAChE, HuBuChE, and POP inhibitory activities IC_{50} (μM)<table><tr><td></td><td>HuAChE</td><td>HuBuChE</td><td>POP</td></tr></table></td><td>Vaneckøva et.al 2016</td></tr>
</table>

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1	699.2 ± 19.4	315.3 ± 10.5	>100.
2	606.8 ± 74.2	30.7± 4.0	0.37 ± 0.03
4	223.3±23.6	115.7±10.1	0.66 ± 0.09
10	>1000	187.6 ± 51.3	1.99 ± 0.33
13	443.7 ± 62.4	141.2 ± 12.6	0.65 ± 0.04
14	> 1000	176.2 ± 14.2	0.45 ± 0.05
41	29.1 ± 1.6	394.0 ± 4.8	0.77 ± 0.02
54	> 1000	624.8±95.0	Nm
64	236.5 ± 32.3	626.2 ± 67.9	3.11 ± 0.36
86	>1000	>1000	>100
100	1.7 ± 0.1	42.3 ± 1.3	>100

39		**42,45,46,47,53,**	Acetylcholinesterase activity **45**: IC_{50} 1270 μM **46:** IC_{50} 1160 μM	Van Rijn *et al.*, 2010
40	*Nerine filifolia*	**3,47,49**	cytotoxicity activity 47: (L6) cells IC_{50} 0.002μg/mL)	Nair *et al.*, 2005
41	*Nerine filamentosa*	**12,45,53,64**		Cahlikova *et al.*, 2011
42	*Nerine huttoniae*	**27,28,29**	**29**: has anti- AChE activity, IC_{50} 34±11 μg/mL	Molander *et al.*, 2011
43	*Nerine sarniensis*	**11,86,89,99,92**	**92**: showed adulticidal activity LD_{50} 1.38+0.056 μg/mosquito	Masi *et al.*, 2016
44		**14,17,56,59,100**	The following compounds showed differen mortality % at 5 μg/mosquito **14**: 23 ± 6 ; **17**: 33 ± 6; **56**: 97 ± 6; **59**: 33 ± 6; **100** 33 ± 6	Masi *et al.*, 2016
45	*Scadoxus puniceus*	**75,76**	Acetylcholinesterase activity **75**: IC_{50} 23.1 μM **76**: IC_{50} 23.7 μM	Naidoo *et al.*, 2018

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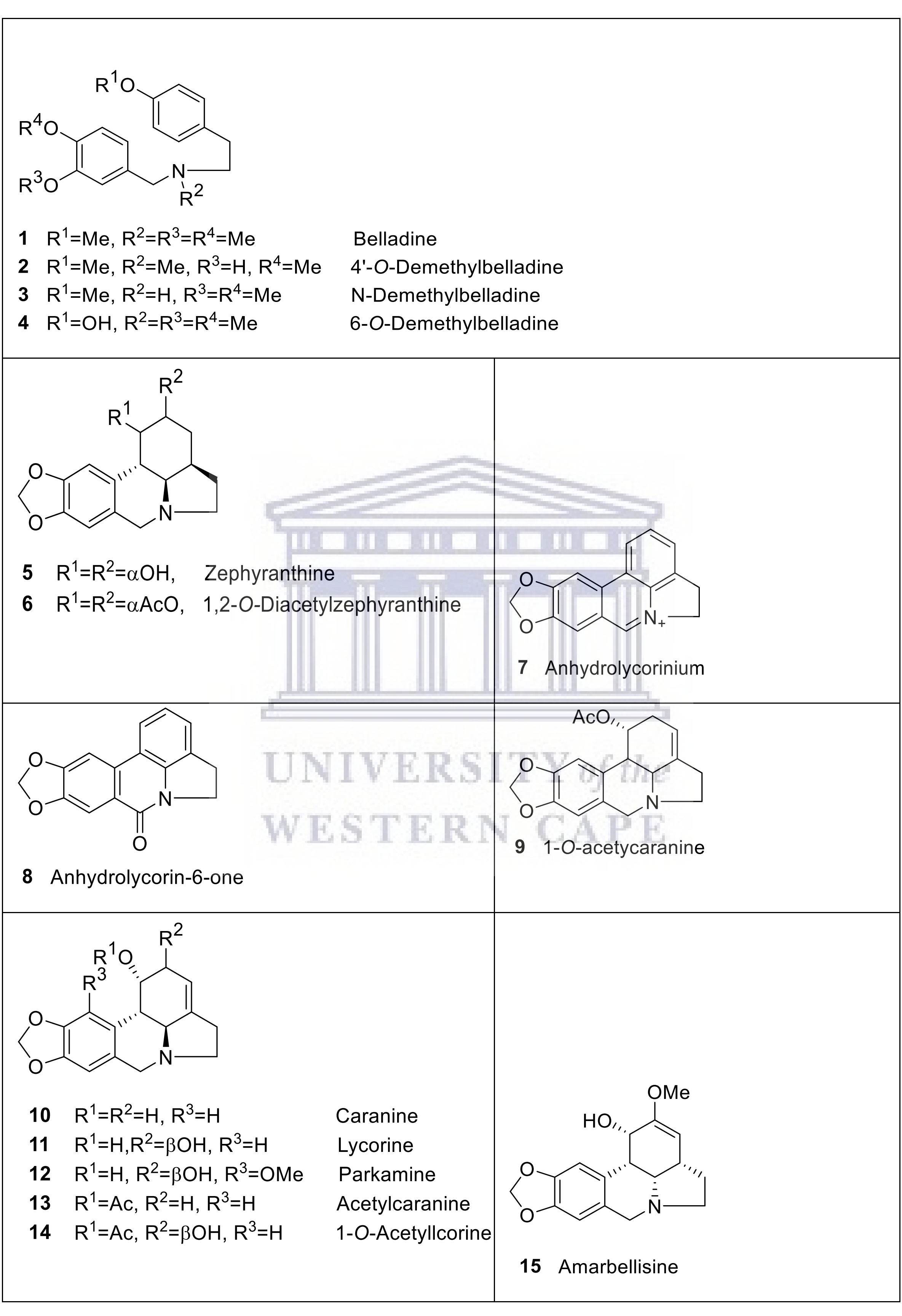
Table 2: The Chemical structures of the alkaloids isolated from South African Amaryllidaceae



16 Mooreine

17 R=H Hippadine
18 R=OMe Lycoramine

19 Narcissidine

20	R^1=R^2=R^3=H, R^4=Me, R^5=H	9-O-Demethylpiuviine
21	R^1=H, R^2=OMe, R^3=αOH, R^4=R^5=Me	11-Hydroxygalanthine
22	R^1=Ac, R^2=OH, R^3=R^4=H, R^5=Me	Sternbergine
23	R^1=Ac, R^2=R^3=R^4=H, R^5=Me	1-O-Acetylnorpluviine
24	R^1=R^2=R^3=R^4=H, R^5=Me	Kirkine

25 R=H Homolycorine
26 R=OMe Albomaculine

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27 R=H Krigeine **28** R=Me 6-*O*-Methylkrigeine **29** R=O Oxokrigenamine	**30** R=H Clivonine **31** R=CH_3 Clivatine **32** R=OH Nobilisitine
33 Nobilisine	**34** Hippeastrine
35 Clivimine	**36** Cliviamartine
37 R=OH Paucamine **38** R-OMe O-Methyl-lycorenium	**39** Macowine

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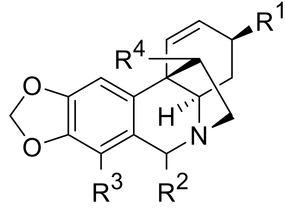
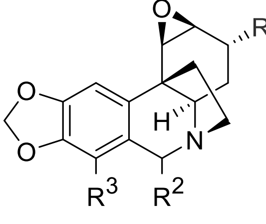


40 R^1=αOH, R^2=R^3=H, R^4=H — Crinine
41 R^1=αOH, R^2=H, R^3=OMe, R^4=H — Powelline
42 R^1=αOMe, R^2=R^3=H, R^4=H — Buphanisine
43 R^1=βOMe, R^2=R^3=H, R^4=H — *epi*-Buphanisine
44 R^1=αOMe, R^2=H, R^3=OMe, R4=H — Buphanidrine
45 R^1=αOMe, R^2=H, R^3=OMe, R^4=OH — Ambelline
46 R^1=αOMe, R^2=H, R^3=OMe, R^4=OAc — 11-*O*-Acetylambelline
47 R^1=αOMe, R^2=H, R^3=OMe, R^4=ONic — Filifoline
48 R^1=αOH, R^2=αOH, R^3=OMe, R^4=H — 6α-Hydroxypowelline
49 R^1=αOMe, R^2=αOMe, R^3=OMe, R^4=H — 6α-Methoxybuphanidrine



50 R^1=OH, R^2=R^3=H — Flexinine
51 R^1=OH, R^2=H, R^3=OMe — Crinamidine
52 R^1=OMe, R^2=R^3=H — Augustine
53 R^1=OMe, R^2=H, R^3=OMe — Undulatine
54 R^1=OMe, R^2=αOH, R^3=OMe — 6a-Hydroxyundulatine

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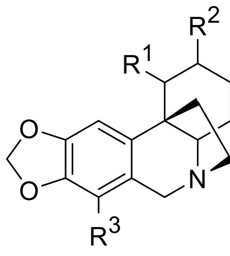

55 R^1=R^2=αOH, R^3=H — Amabiline
56 R^1=αOAc, R^2=βOMe, R^3=OMe — Crinsarnine
57 R^1=βOAc, R^2=βOH, R^3=OMe — 1-*O*- Acetylbulisine
58 R^1=R^2=βOH, R^3=H — 4a-Dehydroxycrinamabine
59 R^1=αOAc, R^2=βOAc, R^3=OMe — Bowdensine
60 R^1=R^2=βOAc, R^3=OMe — 1-*epi*-Bowdensine
61 R^1=αOH, R^2=βOH, R^3=OMe — Deacetybowdensine
62 R^1=R2=βOH, R^3=OMe — 1-*epi*-Deacetylbowdensine
63 R^1=R^2=βOAc, R^3=H — 1-*epi*-Demethoxybowdensine

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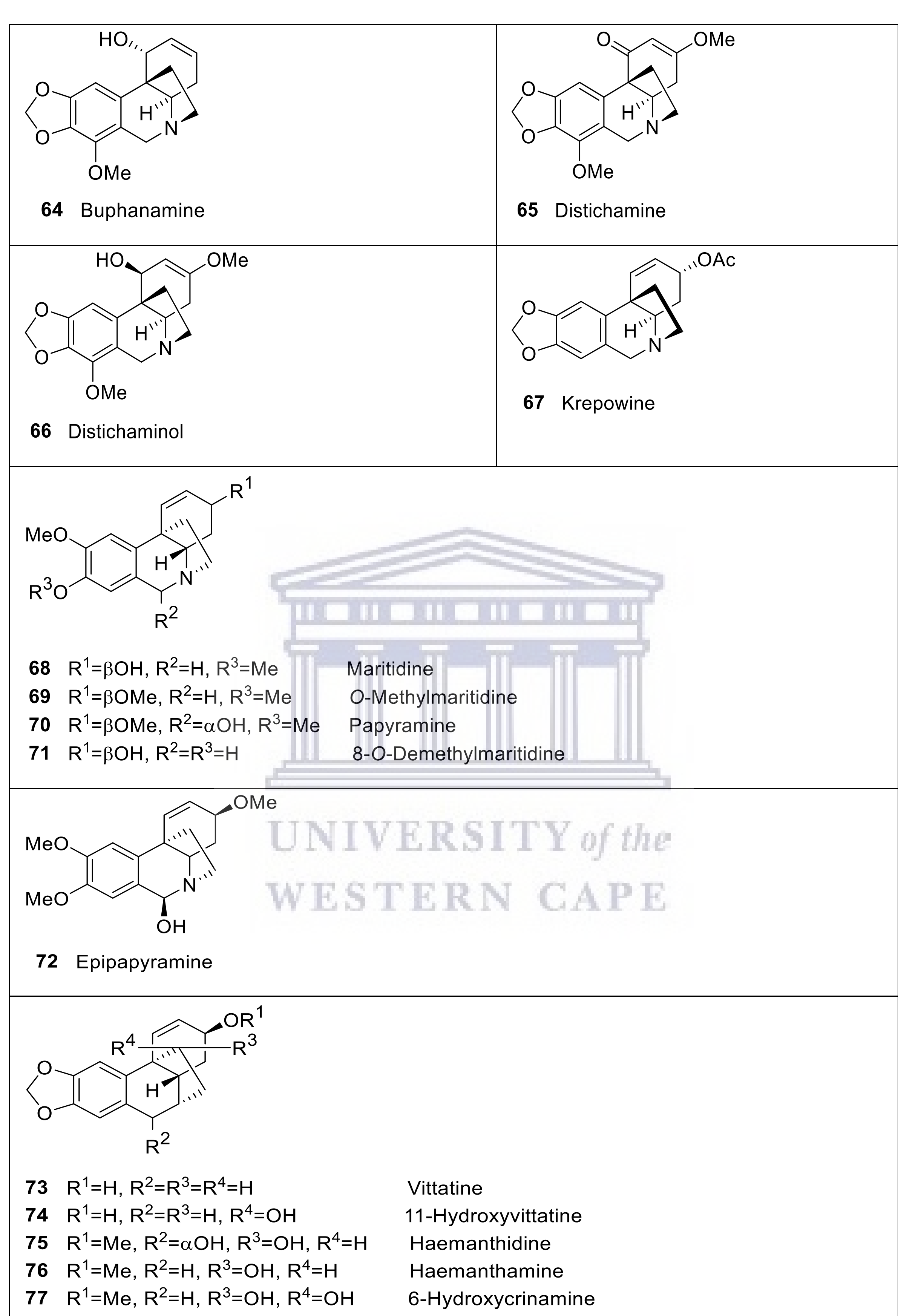

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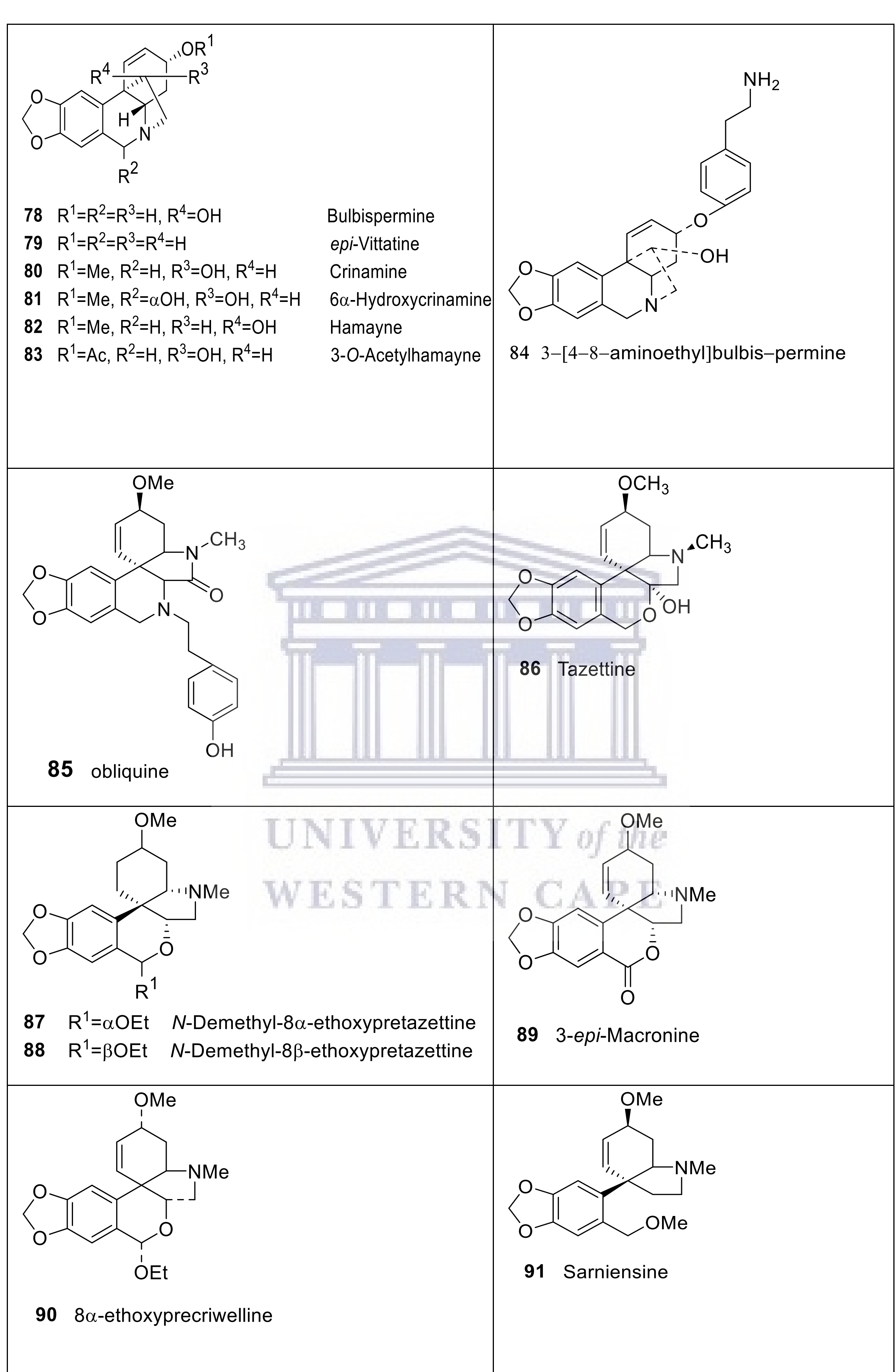

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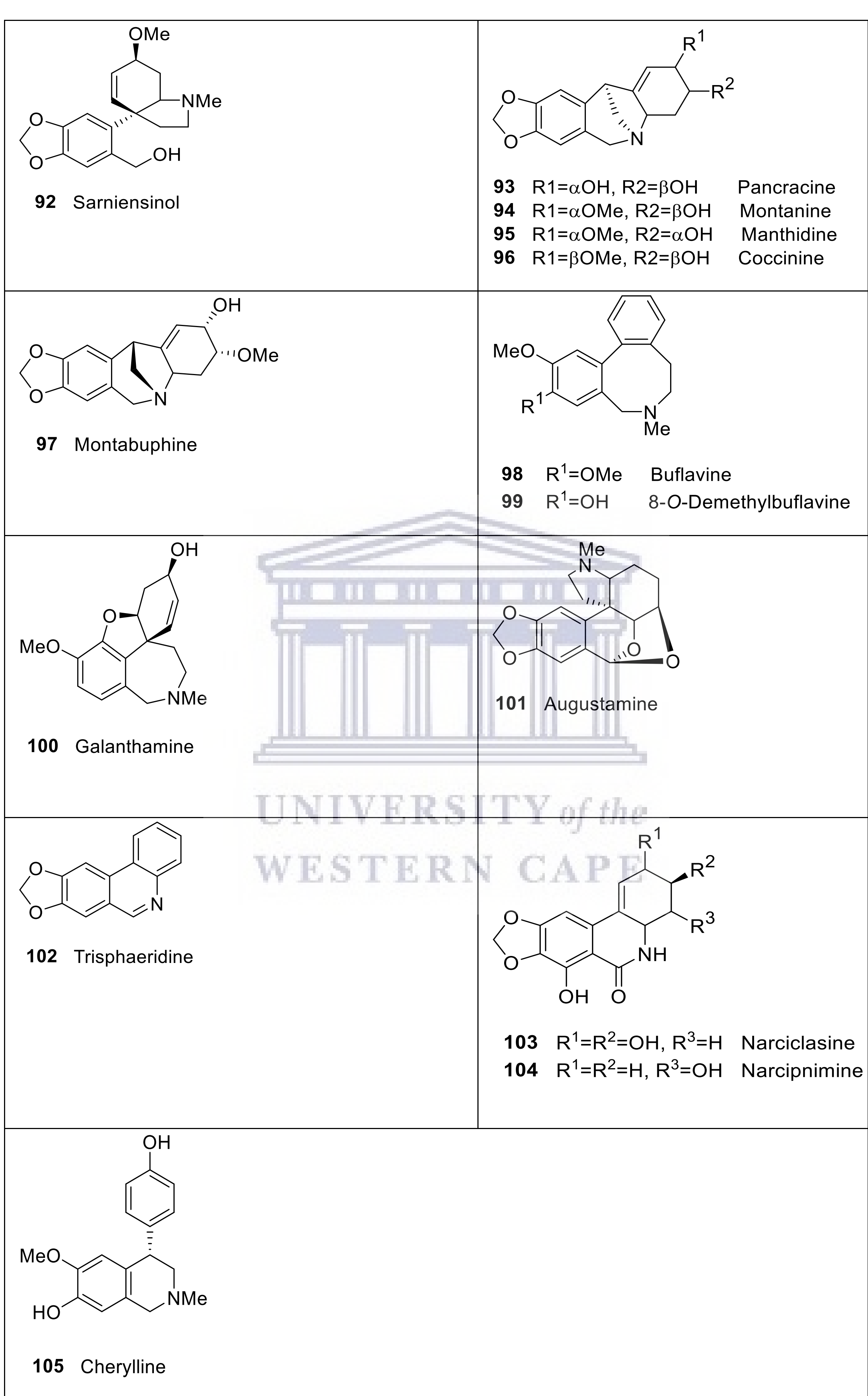

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CHAPTER TWO B

A comprehensive review on non-alkaloidal chemical constituents from Amaryllidaceae

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Abstract

Amaryllidaceae is a subfamily belonging to the Amaryllidaceae and is widely distributed in the southern hemisphere. The subfamily is well known for its content of pharmacologically active alkaloids and represents an important epicentre of Amaryllidaceae -alkaloid diversity. Other metabolites from Amaryllidaceae such as phenolics including flavonoids, lignans, chromones, and acetophenones, in addition to terpenoids and ceramides have been reported and received less attention compared to the alkaloids. Currently, 223 non-alkaloidal compounds have been isolated and identified from ~7 % of the subfamily members. Many of the isolated compounds showed interesting biological activities. Isolation of certain compounds such as flavans and phytosterols from Amaryllidaceae have significant taxonomical activity among the Amaryllidaceae subfamilies. This paper represents an extensive review of the non-alkaloidal chemical constituents of Amaryllidaceae and their biological activities including a brief discussion of their chemotaxonomical importance.

Keywords

Amaryllidaceae , non-alkaloidal content, terpenoids, flavonoids, phenolics.

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Abbreviations

AN	*Aspergillus niger*
A549	Human lung cancer
B16F10	Murine melanoma
BuChE	Butyrylcholinesterase enzyme
CA	*Candida albicans*
CN	*Cryptococcus neoformans*
CP	*Candida parapsilosis*
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC	*Escherichia coli*
FRAP	Ferric-ion reducing antioxidant power
HCT116	Human colon carcinoma
HeLa	Human cervical carcinoma
HepG2	Hepatoma
HUVECs	Human umbilical vein endothelial cells.
IC_{50}	The half maximal inhibitory concentration
LH-60	Human promyelocytic leukemia cells
Molt 4	Child T-cell leukemia
NF-κB	Nuclear factor kappa B
PA	*Pseudomonas aeruginosa*
PC-3M	Metastatic human prostate cancer cells
RAW264.7	Mouse macrophages
M. tuberculosis H37Rv	*Mycobacterial tuberculosis* H37Rv
StA	*Staphylococcus aureus*
SF	*Shigella flexneri*

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SH-SY5Y	Human neuroblastoma cell line
SK-BR-3	Human breast cancer
SPB	*Salmonella paratyphi* B
ST	*Salmonella typhi*
TR	*Trichophyton rubrum*

Introduction

Amaryllidaceae belong to a family of flowering plants included in the monocot order Asparagales. The family was originally created in 1805, and according to the current phylogenetic work by Fay and Chase (Chase *et al*., 2016) who developed the broader (*sensu lato*) concept of the family, and the plant list web-site (The plant list, June 2020), the family contains about 2362 accepted species, divided into about 80 genera, 17 tribes and three subfamilies, the Agapanthoideae (only one genus agapanthus), Allioideae (with around 20 genera) and Amaryllidaceae (~ 59 genera) (Meerow and Snijman 1998). The Amaryllidaceae (formerly recognized as a separate family, Amaryllidaceae J.St.-Hil.) is a widely distributed subfamily of about 850 species. It has a centre of diversity in the southern hemisphere especially South Africa, South America, and in the Mediterranean (Nair *et al*., 2017; Berkov *et al*., 2020).

Plants of the Amaryllidaceae are used in traditional medicine to treat mental problems, primarily in Southern Africa. The traditional use of plants of Amaryllidaceae has been related to their unique alkaloidal contents (Watt and Breyer 1962; Viladomat *et al*., 1997; He *et al*., 2015; Nair *et al*., 2017).

The potent activity of the Amaryllidaceae was recognized by traditional healers who used different species as a poison, and the treatment of grave diseases such as cancer and mental health issues.

Amaryllidaceae can be described as the epicentre of most alkaloids due to its characteristic nature that every member of the family contains bioactive alkaloidal

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compounds (Kintsurashvili and Vachnadze, 2007; Bay-Smidt *et al.*, 2011; He *et al.*, 2015, Nair *et al.*, 2013, 2017). The chemical profile of this subfamily contains over 636 alkaloids which have been subdivided into 42 major skeletons biogenetically derived from belladine (Jin and Yao 2019; Berkov *et al.*, 2020). The plant extracts and chemical compounds possess different biological activities such as anti-proliferative, anti-inflammatory, anti-hypertensive, neurodegeneration prevention, cancer and antibacterial (Murali and Kuttan, 2016, Nair *et al.*, 2017). The wide distribution of alkaloids among these family members indicated its chemotaxonomical importance. However, only relatively few reports have been published on other chemical constituents such as terpenoids and flavonoids (Refaat *et al.*, 2012; Maroyi, 2016; Nair *et al.*, 2017). In continuation of the previous reviews on the genera *Crinum* and *Zephyranthes* which reflected the presence of non-alkaloidal chemical constituents (Tram *et al.*, 2002; Katoch *et al.*, 2015) this review is designed to compile chemical and biological information on non-alkaloidal chemical constituents from plants belonging to the Amaryllidaceae subfamily and to discuss the chemotaxonomical importance in relation to other subfamilies among the Amaryllidaceae.

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Review methodology

Relevant works of literature on non-alkaloid chemical constituents of Amaryllidaceae subfamily were searched through Sci-Finder, Medline, Google Scholar, and Science Direct databases. Nitrogen-containing compounds, fatty acids and volatile oils were excluded. Compounds identified using LC-MS and similar techniques were mentioned briefly.

Results

Table 3 summarises the distribution of non-alkaloidal different classes of the isolated compounds and lists compounds isolated, plant species, parts, and origin. Figures 1-11 illustrate the chemical structures and names of the isolated compounds.

Flavonoids

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Flavonoids are an important class of natural products that have a polyphenolic nature and are widely distributed in higher plants. Flavonoids play a variety of biological roles such as the colour and aroma of flowers, protection from different biotic and abiotic stresses and act as unique UV filters, function as signal molecules, allopathic compounds, phytoalexins, detoxifying agents and antimicrobial defensive compounds (Chacón *et al.*, 2013; Wink, 2013).

Chemically, Flavonoids are derived from phenylpropanoid systems and have the basic carbon skeleton of C_6-C_3-C_6. Different sub-classes of these compounds include flavans, flavanols, chalcones, flavanones, flavones, flavonols, and isoflavones.

Flavans and Flavan-3-ol:

Flavans are the most common flavonoid-type distributed among Amaryllidaceae members (Figure 1). The lipophilic character is reflected by the oxidation pattern of the isolated compounds which is limited to positions C7, C3\` and C4\` and occasionally C2\`. Only two glucosylated compounds (**13** and **14**) were identified from *Zephyranthes candida* (Zhan *et al.*, 2016). The C2 position is mostly in the *S* configuration. The rare C-8 methylflavanes (**18-22**) were isolated from a host of different species (see figure 1).

A limited number of 3-flavanols (**23-30**) have been isolated. The presence of C3\` C4\` methylenedioxy ring (compounds **28-30**) increases the lipophilicity of the isolated compounds.

The structure of **26**, **33**, and **34** were confirmed by single-crystal X-ray diffraction analysis. Tazettones A-D (**31-34**) with an unusual rearranged flavan skeleton, were identified in *N. tazetta* var. *chinensis*. Biogenetically compounds **31-34** have been suggested to be derived from the rearrangement of 8-methylflavan derivatives (Fu *et al.*, 2013, 2016).

Flavans (1) **Flavanols (2)**

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1 7-Hydroxyflavan, R_1=R_2=R_3=H

2 7,4`-Dihydroxyflavan, R_1=R_2=H, R_3=OH

3 7,4`-Dimethoxyflavan, R_1=Me, R_2=H, R_3=OMe

4 2(*S*)-4`-Hydroxy-7-methoxyflavan, R_1=Me, R_2=H, R_3 =OH

5 2(*S*)-3',4'-Dihydroxy-7-methoxyflavan, R_1=Me, R_2=R_3=OH

6 7,4`-Dihydroxy-3'-methoxyflavan, R_1=H, R_2 =OMe, R_3=OH,

7 (2*S*)-3`,7-Dihydroxy-4'-methoxyflavan, R_1=H, R_2=OH, R_3=Me

8 (2*S*)-7-Hydroxy-3`,4`-dimethoxyflavan, R_1=H, R_2=R_3=OMe

9 3`-Hydroxyl- 4`,7-dimethoxyflavan, R_1=Me, R_2 =OH, R_3 =OMe

10 7,4`-Dihydroxy-3`-vinyloxyflavan, R_1=H, R_2=OCH=CH_2, R_3=OH

11 (2*S*)-4`Hydorxy-7,3`-dimethoxyflavan, R_1=Me, R_2=OMe, R_3=OH

12 (2*S*)-7,3`,4`-Trimethoxyflavan, R_1=Me, R_2=R_3=OMe

13 (2*S*)-7,3`-Hydroxy-4`-methoxyflavan 3`-*O*-glucoside,

R_1=H, R_2 =OH, R_3 =OGlu

14 (2*S*)-7,3`-Hydroxy-4`-methoxyflavan 7-*O*- glucoside,

R_1= Glu, R_2 =H, R_3 =OH

15 7-Hydroxy-3`,4`-methylenedioxyflavan, R_1=R_2=H;

16 7-Glucosyloxy-3`,4`-methylenedioxyflavan, R_1=Glu, R_2=H

17 7-Methoxy-2`-hydroxy-4`,5`-methylenedioxyflavan, R_1=Me; R_2=OH

23 (2*R*, 3*R*)-3,4`,7-trihydroxyflavan, R_1=R_2=H, R_3=OH

24 (2*R*, 3*R*)-3,7-dihydroxyflavan, R_1=R_2= R_3=H

25 (2*R*, 3*R*)-3,4`-dihydroxy-7-methoxy-flavan, R_1=Me, R_2=H, R_3 =OH

26 (2*R*,3*R*)-7-methoxy-flavan-3-ol, R_1=Me, R_2=R_3=H

27 4'-Hydroxy-7-methoxy-3'-methylflavanol, R_1=R_2=Me, R_3=OH

28 (2*S*,3*S*)-3-Hydroxy-7-methoxy-3`,4`-methylenedioxy flavan, R=Me

29 (2*R*,3*R*)-3-Hydroxy-7-methoxy-3`,4`-methylenedioxy flavan, R=Me

30 (2*R*,3*R*)-3,7-Dihydroxy-3`,4`-methylenedioxyflavan, R=H

31 Tazettone A, R=Me, R_1= =O, R_2=R_3=H

32 Tazettone B, R=Me, R_1= =O, R_2=OH, R_3=Me

33 Tazettone C, R=H, R_1=βOH, R_2=R_3=H

34 Tazettone D, R=Me, R_1=βOH, R_2=R_3=H

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18 (2*R*)-7,4`-Dihydroxy-8-methylflavan, R_1=R_4=R_2=R_3=H

19 3`,7-Dihydroxy-4`-methoxy-8-methylflavan, R_1=R_2=H, R_4=Me, R_3=OH

20 4`-Hydroxy-5,7-dimethoxy-8-methylflavan, R_1=Me, R_2=OMe, R_3=R_4=H

21 (-)-4`-Hydroxy-7-methoxy-8-methylflavan, R_1=Me, R_2=R_3=R_4=H

22 Tazettone F, R_1=R_2=R_4=H R_3=OH

Figure 1: Chemical structures of Flavans and Flavanols

Flavanones, Dihydroflavonols, Chalcones and Flavones

Seven flavanones and one dihydroflavonol have been reported within the period covered in this review (Table 3 contains the references for the listed compounds, Figure 2) and include the C6 and C8 methylated derivatives, farrerol (**38**) and cyrtomintin (**40**). The C8-methyl derivatives of naringenin (**41**) and the C6 derivative of 7-methoxy aromadendrin (**49**) have also been identified. Several chalcones and dihydrochalcones have been isolated and it has been suggested that compound **46** (2',4,4'-trihydroxy-3'– methylchalcone) is the possible precursor to many of the different C8 methylated derivatives **18-22** (Abdel-Hafiz *et al.*, 1990). The C8 (syzalterin, **51**) and C8, C6 (sideroxylin/ sucalyptin, **53/54**) methylated derivatives were isolated.

Flavanones (3)

Chalcones (4)

35 Isolarrien, R_1=OMe, R_2=OH R_3=R_4=R_5=R_6=H

36 Liquiritigenin, R_1=R_3=R_4= R_6=H, R_2=R_5=OH

42 2`,4`-Di-*O*-methyldavidigenin, R_1=R_2=OMe, R_3=H

43 2,4,4`-Trihydroxydihydrochalcone, R_1=R_3=OH, R_2=H

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37 7-Hydroxyflavanone, R_2=OH, R_1=R_3=R_4=R_5=R_6=H

38 Farrerol, R_1=R_3=Me, R_2=R_4=R_5=OH, R_6=H

39 Liquiritigenin 7-methylether, R_1=R_3=R_4=R_6=H, R_2=OMe, R_5=OH

40 Cyrtominetin, R_1=R_3=Me, R_2=R_4=R_5=R_6=OH

41 8-Methylnaringenin, R_1=Me, R_3=H, R_2=R_4=R_5=OH, R_6=H

44 Davidigenin, R_1=R_2=OH, R_3=H

Dihydroflavonols (5)

49 6-*C*-,7-*O*-Dimethylaromadendrin

45 Isoliquiritigenin, R=R_1=R_2=R_3=H

46 2',4,4'-Trihydroxy-3'-methylchalcone, R=Me, R_1=R_2=R_3=H

47 4,4`-Dihydroxy-2-methoxychalcone, R=R_2=R_3=H, R_1=OMe

48 3,2',4'-Trihydroxy-4-methoxychalcone, R=R_1=H, R_2=OH, R_3=Me

Flavones (6)

50 4',7-Dihydroxyflavone, R=R_1=R_2=R_3=R_4=R_5=H

51 Syzalterin, R=Me, R_1=H, R_2=OH, R_3= R_4= R_5=H

52 4`-Hydroxy-7-methoxyflavone, R=R_2=R_3=R_4=R_5=H, R_1=Me

53 Sideroxylin, R=R_1=R_2=Me, R_3=OH, R_4=R_5=H

54 Eucalyptin, R=R_1=R_2=R_5=Me, R_3=OH, R_4=H

55 5,6,3`-Trihydroxy-7,8,4`-trimethoxyflavone, R=OMe, R_1=R_5=Me, R_2=R_3=R_4=OH

56 Luteolin bioside, R= R_2= R_5=H, R_3= R_4= OH R_1=Man-Glu

Figure 2: Flavanones, Dihydroflavonols, Chalcones and Flavones

Flavonols

The glycosidic forms of kaempferol, quercetin and isorhamnetin constituted the majority of these isolated compounds in which it was found that the flowers are the main source of the flavonols (Table 3, figure 3). None of the isolated compounds contain a methyl group at C6 and/or C8 which indicates that the flowers accumulate different flavonoids from the bulbs. Rutin (**73**) is the most common flavonol glycoside isolated. The glycosylation, in general,

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occurs at C-3. However, C-7 (**60**), C-3` (**69**) and C4` (**63**) have been isolated (Schönsiegel *et al.* 1969; Abou-Donia *et al.*, 2008).

Anthocyanins

The color chemistry of the Amaryllidaceae flowers have been subjected to an interesting analysis which illustrated that they are linked with certain compounds belonging to the carotenoids, flavones and/or anthocyanins (He *et al.*, 2011; Li *et al.*, 2015). The colors of different *Hippeastrum* cultivar flowers have been attributed to the presence of cyanidin 3-*O*-rutinoside (**80**) and pelargonidin 3-*O*-rutinoside (**81**). According to the color space analysis "CIELab" **80** contributes to the red color while **81** contributes to the orange color (Byamukama *et al.*, 2006).

Studies using mainly LC-MS identified different anthocyanins in different Amaryllidaceae flowers. Glycosides of pelargonidin and/or cyanidin have been identified in the genera *Lycoris* (Arisumi, 1971; He *et al.*, 2011), *Nerine* (Arisumi and Shioya, 1970), and *Hippeastrum* (Hrazdina, 1988). Different color hues such as red, purple or blue depend not only on the chemical constituents (the pigments) but also on the pH of the media and additionally due to co-pigmentation among others (Hada *et al.*, 2003; Steyn *et al.*, 2002).

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Homoisoflavanones

The detection of isoflavonoids using LC-MS in different species of Amaryllidaceae showed the presence of daidizin in addition to 14 common isoflavonoids in different parts of the studied plants (Mikšátková *et al.*, 2014). The rare homoisoflavanones derivatives (**82-85**) were isolated from *Cyrtanthus obliquus* bulbs (Table 3, figure 3) (Mahlangenia *et al.*, 2015). Flavans **13**, **14**, and **26** were isolated from *Zephyranthes candida* collected in China and displayed significant inhibitory effects on the LPS-induced NO production in RAW264.7 mouse macrophages with IC_{50} values of 17.34, 16.14, and 21.52 μM, respectively (Zhan *et al.*, 2016). Compound **15** was isolated from *Zephyranthes candida* collected in Nigeria and demonstrated anti-poliovirus activity with an IC_{50} of 0.2384 μg/mL and with a selectivity index

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>151 (Oluyemisi *et al.*, 2015). However, the true structure of the compound needs careful revision according to the reported spectral data.

Compound **4** was isolated from *Crinum bulbispermum* and showed a cytotoxic effect on Molt 4 cells (ED_{50} 42 μg/mL) and inhibited the incorporation of ^{3}H-thymidine in Molt 4 cells at IC_{50} <10 μg/mL. (Abdel-Hafiz *et al.*, 1991).

The flavans **2**, **4**, **5**, **7** and **12** were isolated from *Narcissus tazetta* var. *chinensis* and demonstrated moderate to weak cytotoxicity. Compound **4** demonstrated weak cytotoxic activity against A549 (IC_{50} 36.47 μg/mL), HCT116 (28.48 μg/mL), SK-BR-3 (16.82 μg/mL), and HepG2 (28.71 μg/mL) cell lines, while **7** was active against SK-BR-3 (26.81 μg/mL) and HepG2 (26.50 μg/mL). On the other hand, compounds **2** and **12** showed activity against HCT1 (28.96, 29.18 μg/mL) and HepG2 (34.36, 38.51 μg/mL) respectively (Fu *et al.*, 2013). Compound **5**, in comparison to **7**, **38**, **39**, and **40**, had a more potent antioxidant activity than the positive control, vitamin E and protects SH-SY5Ycells against H_2O_2-induced impairment (Fu *et al.*, 2016).

Compounds **19** (from *Lycoris albiflora*), **1**, **7** and **15** (from *Habranthus brachyandrus*) showed moderate cytotoxic activity against the LH-60 cell line with IC_{50} values of 6.67, 27.9, 42.6 and 19.0 μg/mL, respectively (Jitsuno *et al.*, 2009; 2011).

Compounds **38** and **53** (from *Scadoxus pseudocaulus*) demonstrated moderate antibacterial and antifungal activity (MIC = 8–128 μg/mL) and **38** showed antioxidant activity using the DPPH assay (IC_{50} 58.2 μM) (Pagning *et al.* 2016).

Compounds **61**, **70**, and **71** were isolated from *Crinum bulbispermum* and showed moderate antibacterial activity against CA (Abou-Donia *et al.*, 2005a). In addition, compounds **3** and **11** (from *Crinum distichum*) displayed moderate antibacterial activity (Koagne *et al.*, 2018).

Cyrtanthus obliquus bulbs have been used by South African traditional healers for many diseases such as pregnancy-related ailments, cystitis, age-related dementia and leprosy. The antioxidant evaluation of the isolated compounds (**82-85**) was performed using DPPH and FRAP chemical assays and showed strong activity for compounds **82** and **84** (Mahlangenia *et al.*, 2015).

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Flavonols (7)

57 Kaempferol, R=R_1=R_4=R_3=R_5=H, R_2=OH

58 Kaempferol 3-*O*-glucoside, R=R_1=R_3=R_4=R_5=H, R_2=O-Glu

59 Kaempferol-3-*O*-arabinoside, R=R_1=R_3=R_4=R_5=H, R_2=O-Ara

60 Kaempferol 3-,7-*O*-diglucoside, R=Glu, R_1=R_3=R_4=R_5=H, R_2=O-Glu

61 Kaempferol-3-*O*-xylosyl(1→3)-glucoside, R=R_1=R_3=R_4=R_5=H, R_2=O-Glu-Xyl

62 Kaempferol-3-*O*-xyloside, R=R_1=R_3=R_4=R_5=H, R_2=O-Xyl

63 Kaempferol-3-,4`-*O*-dixyloside, R=R_1=R_3=R_4=H, R_2=O-Xyl, R_5=Xyl

64 Kaempferol-3-*O*-rutinoside, R= R_1=R_3=R_4= R_5=H, R_2=O-rutinoside

65 Kaempferol-3-*O*-sophorodside R=R_1=R_3=R_4=R_5=H, R_2=O-Glu-(2→1)Glu

66 Leucoside, R=R_1=R_3=R_4=R_5=H, R_2=O-Glu-(2→1)Xyl

67 Leucovernide R=Glu, R_1=R_3=R_4=R_5=H, R_2=O-Glu-(2→1)Xyl

68 Quercetin-3-*O*-glucoside, R_3=OH, R_2=O-Glu, R=R_1=R_4=R_5=H

69 Quercetin-3`-*O*-glucoside, R_3=O-Glu, R_2=OH, R=R_1=R_4=R_5=H

70 Quercetin-3-*O*-galactoside, R_2=O-Gla, R_3=OH, R=R_1=R_4=R_5=H

71 Qurecetin-3-*O*-arabinoside, R_3=OH, R=R_1=R_4=R_5=H, R_2=O-Ara

72 Quercetin-3-(6-acetylglucose) (1→3)-glucoside, R_3=OH, R_2=6-AcetylGlu(1→3)Glu, R=R_1=R_4=R_5=H

73 Rutin, R_3=OH, R_2=O-rutinoside, R=R_1=R_4=R_5=H

74 Rhamnetin-3-*O*-rutinoside, R=Me, R_2=O-rutinoside, R_3=OH, R_1=R_4=R_5=H

75 Isorhamnetin-3-*O*-glucoside, R_3=OMe, R_2=O-Glu, R=R_1=R_4=R_5=H

76 Isorhamnetin-3-*O*-arabinoside, 7-glucoside, R_3=OMe, R_2=O-Ara, R=Glu, R_1=R_4=R_5=H

77 Isorhamnetin 3-*O*-glucoside, 7-glucoside, R=Glu, R_3=OMe, R_2=O-Glu, R_1=R_4=R_5=H

78 Isorhamnetin-3-*O*-arabinoside, R_3=OMe, R_2=O-Ara, R=R_1=R_4=R_5=H

79 Isorhamnetin-3-*O*-rutinoside (Narcissin), R_3=OMe, R_2=O- rutinoside, R=R_1=R_4=R_5=H

Anthocyanins (8)

80 Cyanidin 3-*O*-rutinoside, R=H

81 Pelargonidin 3-*O*-rutinoside, R=OH

Homoisoflavanones (9)

82 5,7-Dihydroxy-6-methoxy-3-(4'-methoxybenzyl) chroman-4-one, R=Me

83 Dihydroxy-6-methoxy-3-(4'-hydroxybenzyl) chroman-4-one, R=H

84 5,7-Dihydroxy-6-methoxy-3-(4'-methoxybenzylidene) chroman-4-one, R_1=OMe, R_2=Me

85 5,7-Dihydroxy-3-(4'-hydroxybenzylidene) chroman-4-one, R_1=R_2=H

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Figure 3: Flavanols, Anthocyanins and Homoisoflavanones

Lignans

The furofurane lignans (+)-epipinoresinol (**86**) and (+)-pinoresinol (**87**) were isolated from *Galanthus nivalis* subsp. *cilicicus* collected in Turkey (Kaya *et al.* 2004) (Table 6, Figure 4). Three neolignans (**89−91**) were identified from *Lycoris albiflora* (Jitsuno *et al.*, 2011) while the 4`-glucoside derivative of pinoresinol (**88**) was from *Scadoxus pseudocaulus* (Pagning *et al.*, 2020). It is interesting to note that **90** showed moderate activity against LH-60 (Jitsuno *et al.*, 2011).

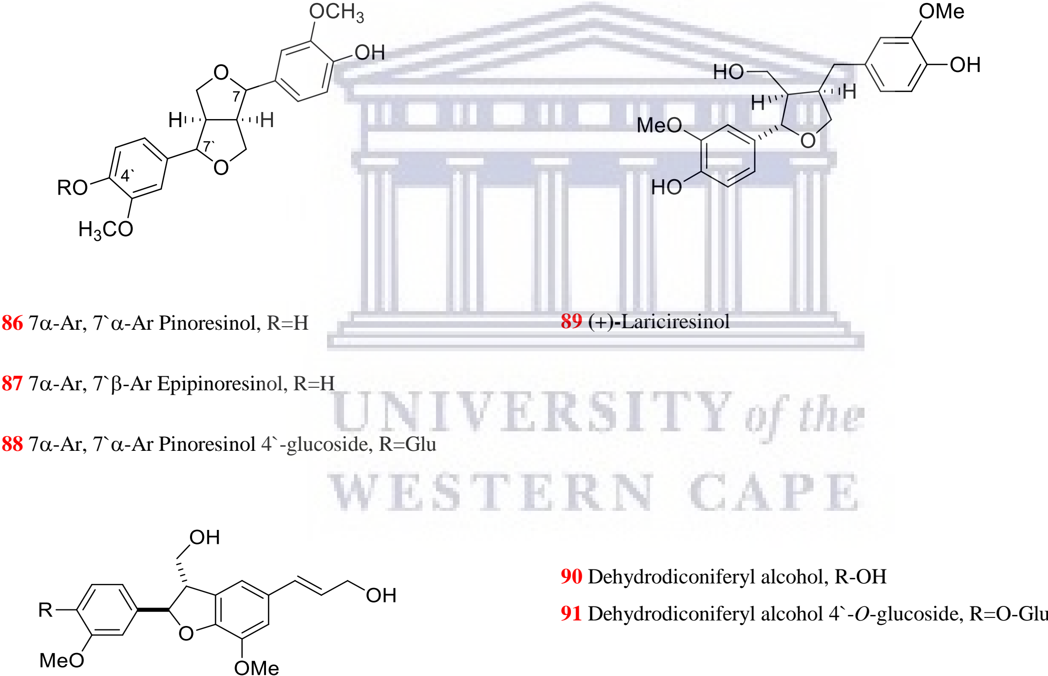

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Figure 4: Chemical structure of Lignans

Chromones/coumarins

Chromones derivatives **93-103** were isolated from different species of Amaryllidaceae (Table 6, Figure 5). Glucosylated derivatives at C7 viz., **100** and **101**), at C5 (**99**), and the rare C6-*C*-glucose derivative (**102**, biflorin) were isolated from *Pancratium biflorum* (Ghosal et al., 1983). Compound **101** displayed weak anti-migratory and good anti-proliferative activities against the highly metastatic human PC-3M cells at 50 μM (Ghosal *et al.*, 1982;

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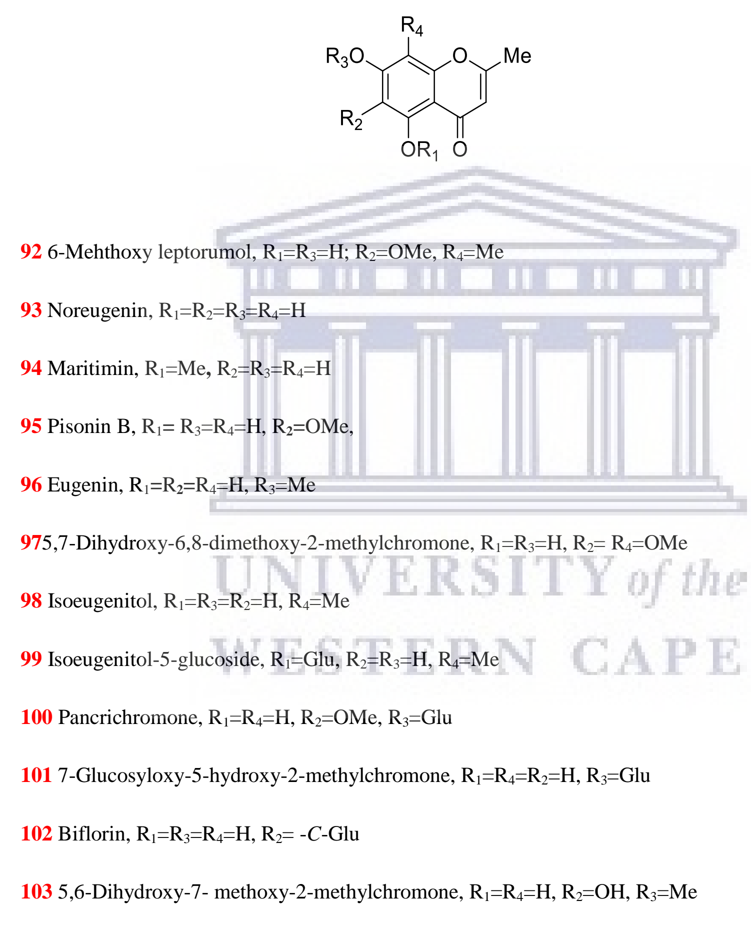
1983). Biflorin (**102**) showed a wide spectrum of biological activities especially against cancer (for more information; Wisintainer *et al*., 2014).

Crinum latifolium is a rare species growing in Vietnam. The local people used the plant for treatment of cancer. The coumarin derivative 4-[(senecioyloxy)methyl]-6,7-dimethoxycoumarin (**104**) was isolated as a bioactive constituent which showed strong antiangiogenic activity and inhibited 76.6 % of the tube-like formation of HUVECs at 3.0 µg/mL with no toxicity against B16F10 and HCT116 cell lines (Nam *et al*. 2004).



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H_3CO

H_3CO

O O

O

O

104 4-[(Senecioyloxy)methyl]-6,7-dimethoxycoumarin

Figure 5: Chromones/coumarins

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Phenylethanoids and phenylpropanoids

Phenylethanoids and phenylpropanoids are important building blocks in the biosynthesis of many natural products. Figure 6 illustrates the compounds reported from Amaryllidaceae. Non-glycosylated free forms such as tyrosol (**105**) and/or glucosyl/methyl/ethyl ethers have also been isolated. Mono (**108**, **109**, **112**, **113**, **118**, **121**) and diglucoside (**110**, **111**, **114**, **119**, **120**) derivatives were among the isolated compounds.

{[7*S*]-7-(4-Hydroxyphenyl)-7-hydroxypropyl}-2`-methylbenzene-3`,6`-diol (**123**) isolated from *Lycoris aurea* showed neuroprotection against H_2O_2/$CoCl_2$-induced neuronal cell death in dopaminergic neuroblastoma SH-SY5Y cells (Jin el al., 2014).

The extract of flowers of *Narcissus tazetta* var*. chinensis*, showed anti-melanogenic activity. Pure compounds **1068**, **111**, **114**, **118** and **120** were isolated as active constituents and exhibited potent activity against theophylline-stimulated melanogenesis in B16 melanoma 4A5 cells at non-toxic concentrations (Morikawa, *et al.* 2016).

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Phenylethanoids

105 Tyrosol, R=H

106 Tyrosol acetate, R=Ac

108. Tazettoside A, R=H, R_1=O-Glu, R_2=R_3=H.

109 Tazettoside B, R=O-Glu, R_1=OMe, R_2=R_3=H.

110 Tazettoside C, R=O-Glu(1→6)Glu, R_1=OMe, R_2=R_3=H.

111 Tazettoside D, R=O-Glu(1→6)Glu, R_1=R_2=H, R_3=OMe.

112 2-Phenylethyl glucoside, R=O-Glu, R_1=R_2=R_3=H.

113 Salidroside, R=O-Glu, R_1=R_2=H, R_3=OH.

114 2(3,4-Dimethoxyphenyl)ethyl diglucoside, R=O-Glu(1→6)Glu, R_1=H, R_2=R_3=OMe.

107 Tazettone E

115 1-(3`,4`-dimethoxyphenyl)-1,2-ethandiol

Phenylpropanoids

116 3-Hydroxy-1-(4`-hydroxyphenyl)-1-propanone

122 1-Ethoxy-3-(4`-glucosyloxy-3`-methoxy)-phenyl-2*E*-propenyl

117 Tazettone G

123 ((7*S*)-7-(4-Hydroxyphenyl)-7-hydroxypropyl) -2`-methylbenzene-3`,6`-diol

118 3-Phenylpropyl glucoside, R=O-Glu, R_1=H

119 3-Phenylpropyl diglucoside, R=O-Glu(1→6)Glu, R_1=H

120 Cinnamyl diglucoside, R=O-Glu(1→6)Glu, R_1=H, Δ^1

121 Tazettoside E, R=O-Glu, R_1=OMe

Figure 6: Phenylethanoids and phenylpropanoids

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Phenolic acids

The majority of isolated phenolic acids incorporate the phenylpropanoid skeleton (Figure 7). The bulbs of *C. asiaticum* L. var. *sinicum* Baker have been used to treat abscesses, aching joints and sores in China. The benzoic acid derivatives (**125**, **126)** with other cinnamic acid derivatives (**137**, **139**, **140)** were isolated from the bulbs of *C. asiaticum* L. var. *sinicum* Baker (Sun *et al*. 2008; 2009). Caffeic (**137**) and dihydrocaffeic (**131**) acids were isolated from the flowers of *Hippeastrum vittatum* (Youssef 2005).

Chlorogenic (**128**), and neochlorogenic (**129**) acids were isolated from the flowers of *Narcissus tazetta* var. *chinensis* (Morikawa, *et al*., 2016), and piscidic acid (**127**) was isolated from the bulbs of *Narcissus poeticus* (Okumura *et al*., 1955).

Neochlorogenic acid methyl ester (**130**) was isolated from *Pancratium maritimum* and showed moderate inhibitory activity (43%) against *M. tuberculosis* H37Rv at 12.5 µg/mL, and potent cytotoxicity against HeLa cells with IC_{50} = 1.0 µg/mL (Youssef 2003).

The chlorogenic acid family belongs to that of the caffeoyl acid esters of quinic acid being widely distributed in nature and showed interesting biological activities. (for more information: Clifford *et al*., 2017; Naveed *et al*., 2018)

Detection of phenolic acids by LC-MS and HPTLC was reported; **126**, **131, 137,** protocatechuic, vanillic, syringic and ferulic acids were detected in the extracts of *Pancratium maritimum* L*., Sternbergia colchiciflora* W. K., *Galanthus nivalis* L*., Galanthus elwesii* Hook., and *Leucojum aestivum* L*.,* (Nikolova and Gevrenova 2005). Chlorogenic acid (**128**) was found in the flowers of *Narcissus* cultivars and bulbs of *Crinum woodrowii* Baker (Li *et al*., 2015; Jagtap, 2015). In addition, **135**, ferulic and coumaroyl quinic acid derivatives were detected in *Pancratium maritimum* L*.* (Rokbeni *et al*., 2016). Furthermore, **137**, protocatechuic, vanillic, syringic, gallic, m-hydroxybenzoic, sinapic, genistic, and salicylic acids were detected in *Cryptostephanus vansonii* and *Scadoxus puniceus* (Moyo *et al*., 2017; Naidoo *et al*., 2018). Vanillic and sinapic acids were identified as the major phenolic acids from of the leaves, flowers and shoots of an Iranian *Galanthus transcaucasicus* Fomin (Karimi *et al*., 2018). The acids ferulic and genistic were detected in the aerial parts of *Galanthus nivalis* L., *Narcissus pseudonarcissus* L., *N. poeticus* L. and *Leucojum vernum* L. (Benedec *et al*., 2018).

O R1 R

HO O OMe HO

124 Benzoic acid, R=H, R_1=OH

125 *p*-Hydroxybenzoic acid, R=R_1=OH

126 Ethyl 4-hydroxybenzoate, R=OH; R_1=OEt

131 Dihydrocaffeic acid methyl ester

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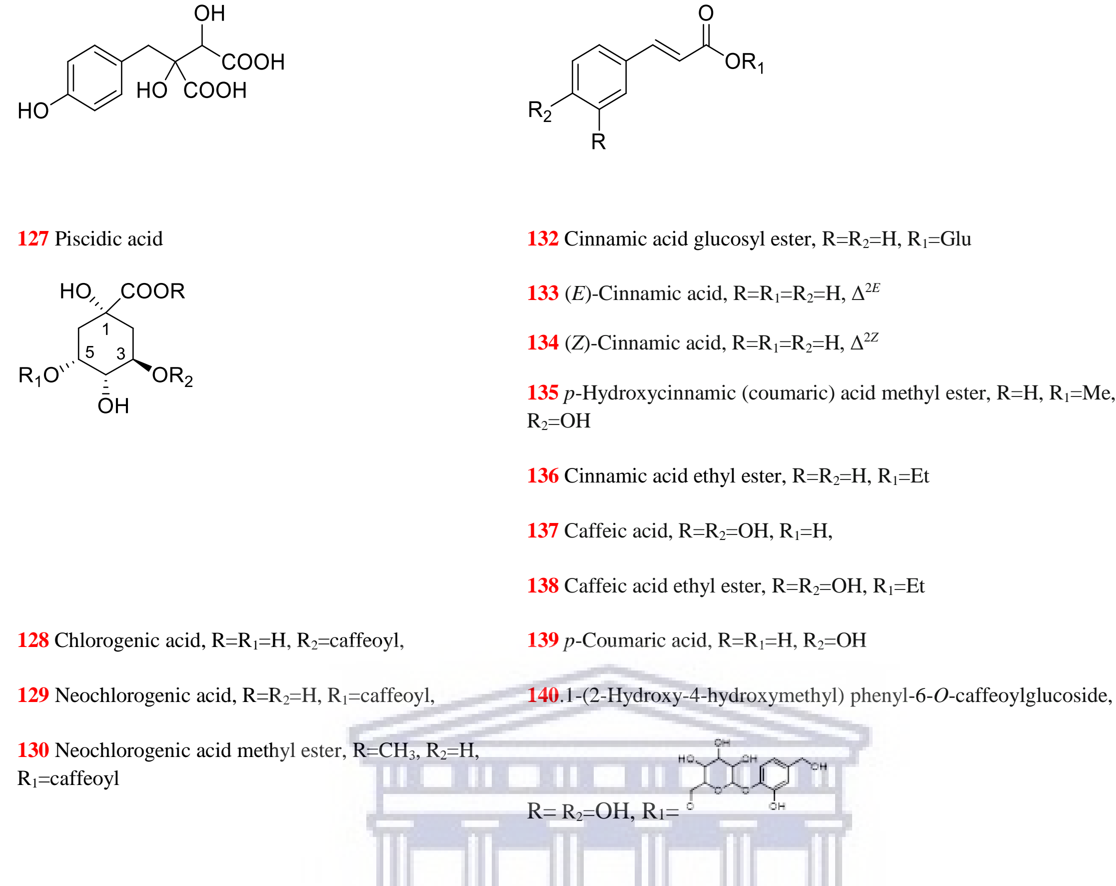

Figure 7: Chemical structure of Phenolic acids

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Acetophenones

4-Hydroxyacetophenone has been isolated as either its methoxylated, and/or glycosylated derivative from Amaryllidaceae (Table 6, Figure 8). Piceol (**147**) represents the simplest structure of the acetophenones and was isolated from *B. disticha*, *Crinum buphanoides* and *C. graminicola* (Tagwireyi *et al.*, 2017; Masi *et al.*, 2018). Nine glycosylated acetophenones (**148-156**) were isolated from *Haemanthus multiflorus* and **148** showed cytotoxic activity against the HL-60 cell line (IC_{50} 20.1 μM) (Yokosuka *et al.*, 2017). From *Pancratium biflorum* **146**, **155**, and **156** were isolated and it was found that **155** and **156** showed growth stimulating activity and enhanced the viability of Ehrlich ascites tumour cells. However, these same two compounds had no activity on the production of prostaglandin synthetase and 5-lipoxygenase (Ghosal *et al.*, 1983, 1989).

R_3O, R_4, Me, R_2, R_1, O

141 2,4-Dihydroxy-6-methoxy-3-methylacetophenone, R_1=OH, R_2=Me, R_3=H, R_4=OMe

142 2,6-Dimethoxy-4-hydroxyacetophenone, R_1=R_4=OMe, R_2=R_3=H

143 Acetovanillone, R_1=R_3=R_4=H, R_2=OMe

144 4-Methoxy-3-hydroxyacetophenone, R_1=R_4=H, R_3=Me, R_2=OH

145 2-Hydroxy-4,6-dimethoxyacetophenone, R_1=OH, R_2=H, R_3=Me, R_4=OMe

146 2,4,6-Trimethoxyacetophenone, R_1=R_4=OMe, R_3=Me, R_2=H

147 Piceol, R_1=R_2=R_3=R_4=H

148 2,4-Dihydroxy-6-methoxy-3-methylacetophenone 4-*O*-rhamnosyl-(1→6)-glucoside, R_1=OH, R_2=Me, R_4=OMe, R_3=Rha-(1→6)-Glu

149 2,4-Dihydroxy-6-methoxy-3-methylacetophenone 4-*O*-apiosyl-(1→6)- glucoside, R_1=OH, R_2=Me, R_4=OMe, R_3=Api(1→6)Glu

150 2,4-Dihydroxy-6-methoxy-3-methylacetophenone 4-*O*-glucosyl-(1→6)-glucoside, R_1=OH, R_2=Me, R_4=OMe, R_3=Glu(1→6)Glu

151 2,4-Dihydroxy-6-methoxyacetophenone 4-*O*-glucosyl-(1→6)-glucoside, R_1=OH, R_2=H, R_4=OMe, R_3=Glu(1→6)Glu

152 2,4-Dihydroxy-6-methoxyacetophenone 4-*O*-arabinosyl-(1→6)-glucoside, R_1=OH, R_2=H, R_4=OMe, R_3=Ara(1→6)Glu

153 2,4-Dihydroxy-6-methoxyacetophenone 4-*O*-rhamnosyl-(1→6)-glucoside, R_1=OH, R_2=H, R_4=OMe, R_3=Rha(1→6)Glu

154 2,4-Dihydroxy-6-methoxyacetophenone 4-*O*-apiosyl-(1→6)-glucoside, R_1=OH, R_2=H, R_4=OMe, R_3=Api(1→6)Glu

155 2,6-Dimethoxyacetophenone 4-*O*-glucoside, R_1=OMe, R_2=H, R_4=OMe, R_3=Glu

156 4,6-Dimethoxyacetophenone 2-*O*-glucoside, R_1=O-Glu, R_2=H, R_3=Me, R_4=OMe

Figure 8: Chemical structure of Acetophenones

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Terpenoids

Terpenoids have been poorly studied among the Amaryllidaceae, especially mono-, sesqui- and diterpenoids. A few compounds belonging to different classes of the triterpenes were reported in addition to the sesquiterpene (**184**). To the best of our knowledge, 28 terpenoids have been reported so far. Triterpenes having the lupane, ursane and oleanane skeletons have been identified (see Table 6). In addition, terpenes with different skeletons of the phytosterols having different substitution patterns at C-4, C-14 and C-24 have also been reported (see Table 6, figure 9).

The phytosterols (C-24 alkyl-substituted sterols) e.g. ergostane and stigmastane are the most abundant sterols in the plant kingdom. Cholestane is commonly accumulating in animals. However, some related compounds have been reported from certain species of higher plants. The first examples of cholestanes (**158**, **162**) isolated from Amaryllidaceae have recently been from *Boophone haemanthoides*, (Ibrakaw *et al.*, 2020).

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Compounds **165** and **169** were isolated from *Boophone disticha* bulbs and both compounds showed low activity against human neuroblastoma (SH-SY5Y) cells with IC_{50} values of 173.0 and 223.0 µM respectively (Adewusi *et al*., 2013).

The β-sitosterol 3-glucoside (**172**) was isolated from *Crinum purpurascens* and showed weak antibacterial activity against ST and SPB (MIC 200, 250 µg/mL) (Nkanwen *et al*., 2009).

The sesquiterpene, parthenicin (**184**) was isolated from *Crinum ensifolium* and showed strong cytotoxic activity against a selection of cancer cell lines and strongly inhibited NF-κB activity with an IC_{50} value of 1.82 µM (Khoi *et al*. 2011).

157 Stigmast-4-ene-3,6-dione, R_1= =O, R_2=Et

158 Cholest-4-en-3-one, R_1=R_2=H

159 (22*E*)-Stigmasta-4,22-dien-3-one, R_1=H, R_2=Et; Δ^{22}

160 Stigmast-4-en-3-one, R_1=H, R_2=Et

161 6β-Hydroxystigmast-4-en-3-one, R_1=OH, R_2=Et

162 6β-Hydroxycholest-4-en-3-one, R_1=OH, R_2=H

163 Cycloartenol

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164 24-Methylenecycloartan-3β-ol, R_1=R_2=Me, R=βOH

165 Cycloeucalenol, R_1=H, R_2=Me, R=βOH

166 Cycloeucalenone, R_1=H, R_2=Me, R= =O

167 24-methylenepollinastanone, R_1=R_2=H, R= =O

168 Cyclolaudenol, R=Me

169 31-Norcyclolaudenol, R=H

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170 Dihydrositosterol, R=H

171 β-sitosterol, R=H, Δ^5

172 β-sitosterol-3-*O*-glucoside, R=Glu, Δ^5

173 Stigmasterol, R=H, $\Delta^{5,22}$

174 Stigmasta-4-en-3β-ol, R=H, Δ^4

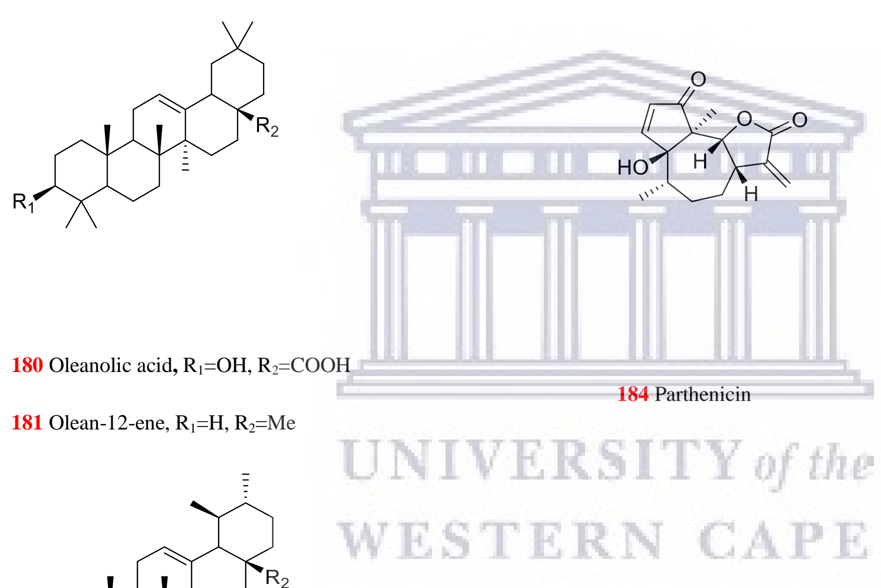
175 Lupeol, R_1=βOH, R_2=H, R_3=Me

176 Lupenone, R_1= =O, R_2=H, R_3=Me

177 Glochidone, R_1= =O, R_2=H, R_3=Me, Δ^1

178 3β,27-Dihydroxylup-20(29)-ene, R_1=βOH, R_2=OH, R_3=Me

179 Betulinaldehyde, R_1=βOH, R_2=H, R_3=CHO



180 Oleanolic acid**,** R_1=OH, R_2=COOH

181 Olean-12-ene, R_1=H, R_2=Me

184 Parthenicin

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182 Ursan-12-ene, R_1=H, R_2=Me

183 Ursolic acid, R_1=βOH, R_2=COOH

*The conventional numbering system is followed in this article

Figure 9: Chemical structure of Terpenoids

Glycolipids and ceramides

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Sphingolipids are derived from long-chain 1,3-dihydroxy-2-amino bases and display good potential therapeutic effects. Glycolipids **196** and **197** were isolated from *Hippeastrum vittatum* (Wang *et al*., 2006), in addition to the five ceramide glucosides (**191**-**195**) (Wang *et al*., 2005) (Table 6, Figure 10).

Ceramides **185**-**190** were isolated from *Zephyranthes candida*. It was found that **185** and **187** displayed moderate antibacterial activity against StA, EC, AN, CA and TR (Wu *et al*., 2009a, 2009b).

185 Candidamide A, R=H, X_1=15, X_2=1, X_3=7

186 Zephyranamide D, R=OH, X_1=17, X_2=11, X_3=3

191 Soyacerebroside I, X_1=6, X_2=7, Δ^{8E}

192 Soyacerebroside II, X_1=6, X_2=7, Δ^{8Z}

193 Dracontioside A, X_1=8, X_2=7, Δ^{8Z}

194 JCer 1, X_1=8, X_2=7, Δ^{8E}

195 2-[(2*R*-2-Hydroxyoctadecanoyl)amido]-4,8-octadecadiene-1,3-diol 1-*O*- glucoside, X_1=10, X_2=7, Δ^{8Z}

187 Candidamide B, R=H, X_1=9, X_2=1, X_3=15

188 Zephyranamide C, X_1=10, X_2=14, X_3=2

196 1-Linenoyl-3-*O*-galactosyl-*sn*-glycerol, X=3,

197 1-Oleoyl-3-*O*-galactosyl-*sn*-glycerol, X=7

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189 Zephyranamide A, X_1=15, X_2=6, X_3=3, Δ^{13}

190 Zephyranamide B, X_1=20, X_2=6, X_3=1

Figure 10: Chemical structure of Glycolipids/Ceramides

Miscellaneous compounds

Vanillin (**202**) was isolated from *Crinum bulbispermum* (Watt and Breyer 1962) and its 4-glucoside derivative, amabiloside (**203**), was isolated from *Crinum amabile* (Likhitwitayawuid *et al*., 1993). Aliphatic hydroxyketones (**219**-**223**) were isolated from *Crinum augustum* growing in Egypt. (Abdel-Hafiz, 1990, 1991) (Table 6, Figure 11).

6-Hydroxy-2*H*-pyran-3-carbaldehyde (**209**) was isolated from *Crinum yemense* and showed strong inhibition of the tyrosinase enzyme at a concentration of 42.2 μM (Abdel-Halim *et al*., 2008). In addition, benzyl glucoside **(204),** isolated from *Narcissus tazetta* var*. chinensis* showed potent anti-melanogenesis activity in the theophylline-stimulated B16 melanoma 4A5 cells with an IC_{50} of 59.0 μM, (Morikawa *et al*. 2016).

4-(Hydroxymethyl)-5-hydroxy-2*H*-pyran-2-one (**211**) was isolated from *Scadoxus pseudocaulus* and showed antibacterial activity against EC, PA, CA, CN, CP and SF (MICs range 4–64 μg/mL) and antioxidant activity using the DPPH assay (IC_{50} of 59.5 μM), and inhibited BuChE with an IC_{50} of 23.5 μM (Pagning *et al*., 2016, 2020).

198 2-(3,4-Dihydroxyphenyl)-5-formyl-1,3-benzodioxole

199 4,5-Methylenedioxy-4'-hydroxy-2-aldehyde-(1,1'-biphenyl)

200 5-Hydroxylmethyl-2-furancarboxaldehyde, R=H

202 Vanillin, R=Me, R_1=H

204 Benzyl glucoside, R=Glu

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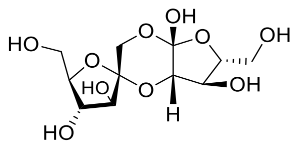
201 5-Formylfurfuryl acetate, R=Ac

203 Amabiloside, R=H, R_1=Glu

205 Benzyl diglucoside, R=Glu(1→6)Glu



206 Di-D-fructofuranose 1,2':2,3' dianhydride

207 2-(4-hydroxybenzyl) malic acid

208 3,5-Dimethoxyphenyl 1-*O*-arabinosyl-(1→6)-glucoside

209 6-Hydroxy-2*H*-pyran-3-carbaldehyde

210 Calleryanin

211 4-(Hydroxymethyl)-5-hydroxy-2*H*-pyran-2-one

212 Ambretollide

213 Methyl (*S*)-3-(glucosyloxy) butyrate.

214 1,1`-Bis(1,1`-carboxy-ethyl) ether

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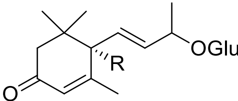

215 9-Hydroxymegastigma-4,7-dien-3-one 9-glucoside, R=H

216 Roseoside II, R=OH

217 Pancricin

218 Ethylglucoside

219 5-Hydroxyhexacosan-9-one, X_1=3, X_2=3, X_3=16

220 5-Hydroxytriacontane-9-one, X_1=3, X_2=3, X_3=20

221 5-Hydroxyoctacosan-9-one, X_1=3, X_2=3, X_3=28

222 5-Hydroxydotriacontan-9-one, X_1=3, X_2=3, X_3=22

223 23-Hydroxyhentriacontan-29-one, X_1=21, X_2=5, X_3=1

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Figure 11: Miscellaneous compounds

Table 3: Non-alkaloidal chemical constituents isolated from Amaryllidaceae

	Species	Isolated compounds*	Parts **/ Origin***	Reference
1.	*Amaryllis belladonna* L.	**Fla [64, 73] / PA [135]**	L-F /Eg	Abou-Donia et al., 2005b
2.	*Ammocharis coranica* (Ker Gawl.) Herb.	**Ter [164-167]**	B/SA	Koorbanally et al., 2000
3.	*Boophone disticha (*L.f.) Herb.	**AcP [147]**	B/SA	Tagwireyi et al., 2017
		Ter [165, 169]	B/SA	Adewusi et al., 2013
4.	*Boophone haemanthoides* F.M.Leight.	**PP [105, 116] / Ter [157-163]**	B/SA	Ibrakaw et al., 2020
5.	*Crinum amabile* Donn ex Ker Gawl.	**Ot [203]**	B/Tha	Likhitwitayawuid et al., 1993
6.	*Crinum americanum* L.	**Fla [1, 4, 27, 77]**	B/Eg	Ali et al., 1988b
7.	*Crinum asiaticum* L.	**Fla [1, 4, 36, 45, 54]**	B/Eg	Mohamed 2000
8.	*Crinum asiaticum* L. *var. japonicum* Baker	**Fla [4]**	B/Ko	Min et al., 2001
		Ter [163, 168, 169, 173]	B/Jp	Takagi and Yamaki, 1977
9.	*Crinum asiaticum* L. *var. sinicum* (Roxb. ex Herb.) Baker	**Fla [1, 2, 7, 25, 37, 50] / PA [125, 126, 137, 139, 140]**	B/Ch	Sun et al., 2009, 2008
		Ot [198]	B/Ch	Qian et al., 2009
10.	*Crinum augustum* Rox ex Ker Gawl. a synonym of *Crinum asiaticum* var. *asiaticum*	**Fla [4, 21, 45, 46] / Ter [164, 165, 172** mixture of **171/173, 183] / Ot [219-223]**	B/Eg	Ali et al., 1988a; Abdel-Hafiz et al., 1990; 1991; Ramadan, 1998; Refaat et al., 2009;
11.	*Crinum biflorum* Rottb	**Fla [7, 8, 9, 15, 26, 28, 29, 38, 57] / Ter [171, 172, 180, 175]**	WP/Ca	Kianfé et al., 2019
12.	*Crinum bulbispermum* (Burm.f.) Milne-Redh. and Schweick.	**Ter [173, 170]**	B/Eg	Ali and Abdel-Hafiz 1985
		Fla [5, 25, 35, 36, 42, 45] / Ot [199]	B/Eg	Ramadan et al., 2000
		Fla [4]	B/Eg	Abdel-Hafiz et al., 1991
		Fla [47, 52] / PP [106, 115]	B/Eg	Khalifa 2001
		Fla [58, 61, 670, 72, 75]	F/Eg	Abou-Donia et al., 2005a
		Fla [62]	L/Eg	Ali et al., 1981

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		Ot [202]		Watt and Breyer 1962.
13.	*Crinum buphanoides* Welw. ex Baker	**AcP [143, 147]**	B/SA	Masi et al., 2018
14.	*Crinum distichum* Herb.	**Fla [3, 4, 11]**	WP/Ca	Koagne et al., 2018
15.	*Crinum ensifolium* Roxb. ex Ker Gawl.	**Ter [184]**	B/Ch	Khoi et al., 2011
16.	*Crinum graminicola* I.Verd.	**AcP [147]**	B/SA	Masi et al., 2018
17.	*Crinum jagus* (J.Thomps.) Dandy	**Fla [57]**	L/Ca	Taiwe et al., 2016
18.	*Crinum latifolium* L.	**PA [125, 126, 132 - 134] / Ter [171, 172]**	- /Ch	Qian 2010
		Fla [2, 10, 27, 57, 58] / PA [135, 136, 138]	L/Vt	Nam and Vung 2006; Tri et al., 2005; Thanh et al., 2010
		Fla [2, 6, 10, 44, 55] / CC [104] / Ter [163]	L/Vt	Nam et al., 2004
19.	*Crinum macowanii* Baker	**AcP [143, 144]**	B/SA	Kouama et al., 2014
20.	*Crinum moorei* Hook F.	**Fla [1, 4] / Ter [171] / CC [92, 93, 96,]**	B/Eg	Kamel 1996
21.	*Crinum purpurascens* Herb.	**Ter [172]**	L/Ca	Nkanwen et al., 2009
22.	*Crinum x powelli*	**Fla [4] / Ot [210]**	B/Sz	Kissling et al., 2005
23.	*Crinum yemense* Deflers	**PA [124] / Ot [209, 214]**	B/Ym	Abdel-Halim et al., 2008
24.	*Cyrtanthus breviflorus* Harv.	**Ter [175-179]**	B/SA	Crouch et al., 2005
25.	*Cyrtanthus obliquus* (L.f.) Aiton	**Fla [82, 83, 84, 85]**	B/SA	Mahlangenia et al., 2015
26.	*Galanthus caucasicus* (Baker) Grossh.	**Fla [56, 70, 73]**	WP/Uz	Tsakadze et al., 1988
27.	*Galanthus nivalis* L.	**Fla [65]**	F/--	Hörhammer et al., 1967
28.	*Galanthus nivalis subsp. cilicicus* (Baker) Gottl.-Tann.	**Li [86, 87]**	WP/Tk	Kaya et al., 2004
29.	*Gethyllis ciliaris* (Thunb.) Thunb.	**CC [98, 99]**	B/SA	Elgorashi et al., 2007
30.	*Habranthus brachyandrus* (Baker) Sealy	**Fla [1, 2, 7, 15 (2*S*), 23-25, 30, 43] / Li [90] / AcP [145] / Ot [213]**	B/Jp	Jitsuno et al., 2009
31.	*Haemanthus multiflorus* Martyn	**AcP [148-156] / Ot [208]**	B/Jp	Yokosuka et al., 2017
32.	*Hippeastrum ananuca* Phil.	**Fla [26, 28]**	B/Chl	Pacheco et al., 1981

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33.	*Hippeastrum* cultivars	**Fla [80, 81]**	F/Ug	Byamukama et al., 2006
34.	*Hippeastrum vittatum* (L'Hér.) Herb.	**PA [131, 137]**	F/Eg	Youssef 2005.
		Ce [191-197]	WP/Ch	Wang et al., 2005, 2006
35.	*Hippeastrum x hortorum*	**Fla [7, 15, 18, 48]**	B/Gr	Wink and Lehmann 1996
36.	*Hymenocallis littoralis* (Jacq.) Salisb.	**Fla [2, 7, 19, 38] / CC [93, 95 - 97]**	WP/Vt	Anh et al., 2014
		Fla [69, 73]	B/Eg	Abou-Donia et al., 2008
37.	*Leucojum vernum* L.	**Fla [65 - 67]**	F/--	Chari and Wagner 1976, Hörhammer et al., 1967
38.	*Lycoris albiflora* Koidz.	**Fla [18, 19] / Li [89, 90, 91] / AcP [145, 146]**	B/Jp	Jitsuno et al., 2011
39.	*Lycoris aurea* (L'Hér.) Herb.	**PP [123]**	B/Ch	Jin et al., 2014
40.	*Lycoris radiata* (L'Hér.) Herb.	**PP [122] / Ot [206]**	B/Ch	Li et al., 1997, 2013
		Ot [207]	B/Ch	Koizumi et al., 1976
41.	*Narcissus poeticus* L.	**PA [127]**	B/--	Okumura et al., 1955
		Fla [58, 60]	B/--	Schönsiegel et al., 1969
42.	*Narcissus pseudonarcissus* L.	**Fla [1, 2, 18]**	B/--	Coxon et al., 1980
		Fla [58, 59, 70, 71, 75-78]	B/--	Schönsiegel and Egger 1969
43.	*Narcissus tazetta* L.	**Fla [78]**	B/--	Kubota and Hase1956
44.	*Narcissus tazetta* var. *chinensis* (M. Roem.) Masam. and Yanagih	**Fla [2, 4, 5, 7, 12, 18, 22, 31, 32, 33, 34, 38, 39, 40, 41] / PE [107] / PP [117]**	B/Ch	Fu et al., 2013, 2016
		Fla [73, 74, 79], / PE [1068-114] / PP [118-121] / PA [128, 129] / Ot [204, 205, 215, 216]	F/Jp	Morikawa et al., 2016
45.	*Pancratium biflorum* Roxb	**CC [93, 101, 102, 103] / AcP [146, 155, 156]**	WP, B/In	Ghosal et al., 1982, 1983, 1989
46.	*Pancratium littorale* Jacq.	**Fla [18]**	S/Pa	Ioset et al., 2001
47.	*Pancratium maritimum* L	**Fla [36, 38, 45, 51] CC [94] / PA [130] / AcP [141, 142]**	B, F/Eg	Youssef 1998, 2003
		CC [101, 100] / AcP [141] / Ot [201, 217, 218]	B/Eg	Ibrahim et al., 2014
		Fla [4, 20, 62, 63] / CC [92, 93, 96],	L, B/Eg	Ali et al., 1981, 1990
		PA [137]	WP/Tu	Rokbeni et al., 2016

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48.	*Scadoxus pseudocaulus* (I.Bj¢rnstad and Friis) Friis and Nordal	**Fla [38, 49, 53] / Li [88] / Ot [200, 211]**	WP/Ca	Pagning et al., 2016, 2020
49.	*Stenomesson variegatum* (Ruiz and Pav.) J.F.Macbr.	**Ter [174, 181, 182]**	B/Pe	Ruggeri et al., 1991
50.	*Zephyranthes candida* (Lindl.) Herb.	**Fla [64, 73]**	F/Jp	Nakayama et al., 1978
		Fla [7, 13, 14, 26]	WP/Ch	Zhan et al., 2016
		Fla [15]	WP/Ng	Oluyemisi et al., 2015
		Fla [2(2*S*), 4(2*S*), 7(2*S*), 15, 73] / Ter [171, 172] / Ce [185-190] / Ot [212]	B/Ch	Wu et al., 2009a, 2009b
51.	*Zephyranthes flava* (Herb.) G.Nicholson	**Fla [6, 15, 16, 17]**	B/In	Ghosal et al., 1985

*Compounds: **Flav**: flavonoids; **Li**: Lignans; **CC**: Chromones and Coumarins; **PP**: Phenylpropanoinds and Phenylethanoids; **PA**: Phenolic acids; **AcP**: Acetophenones; **Ter**: terpenoids; **Ce**: Ceramides; **Ot**: Others.
Part extracted: **L: leaves; **F**: flowers; **B**: Bulbs; **S**: Stem; **WP**: Whole Plant.
***Origin (country): **Ca**: Cameroon; **Ch**: China; **Chl**: Chile; **Eg**: Egypt; **Gr**: Germany; **In**: India; **Jp**: Japan; **Ko**: Korea; **Ng**: Nigeria; **Pa**: Panama; **Pe**: Peru; **SA**: South Africa; **Sz**: Switzerland; **Tha**: Thailand; **Tk**: Turkey; **Tu**: Tunisia; **Ug**: Uganda; **Uz**: Uzbekistan; **Vt**: Vietnam; **Ym**: Yemen.

Conclusion

The enzymatic systems in living organisms (including higher plants) have the ability to construct different metabolites, especially secondary metabolites, with unlimited diversity and complexity from carbon dioxide, water and light. The exact function of the numerous secondary metabolites is still not comprehensively understood. However, some functions of these secondary metabolites have been proved through experimental evidence such as defence, communication, nitrogen storage, UV protection and as antioxidative agents (Wink, 2013; Chacón *et al.*, 2013).

Secondary metabolites have been recognised to be considered as an important factor in plant classification, especially flavonoids and alkaloids which proved to be suitable for analysis using simple techniques to be easily identified. (Mabry *et al* 1970; Marin 1996).

Plants belonging to the Amaryllidaceae subfamily have a unique alkaloidal chemical composition making them different from two Amaryllidaceae subfamilies viz., “Agapanthoideae and Allioideae”. Alkaloids from Amaryllidaceae have a potent pharmacological profile and one member of these compounds, *viz* galanthamine was

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approved by the FDA for treatment of mild memory impairments (Berkov *et al*., 2020). Up to date, the chemical profile of this subfamily contains over 636 alkaloids and have been subdivided into 42 major skeletons biogenetically derived from belladine (Jin and Yao 2019; Berkov *et al*., 2020).

The above reasons make the Amaryllidaceae very attractive for further exploring potential bioactive alkaloidal compounds. This situation has resulted in other metabolites being overlooked. Currently, about 223 non-alkaloidal compounds (as listed above) have been identified and belonging to different classes of compounds. Most importantly, different chemical trends among the isolated compounds have been identified, which make this subfamily more unique not only for its alkaloidal content, but also for the other metabolites like flavonoids, terpenoids, and ceramides.

The biosynthesis of phenylmethanoids (C6-C1), phenylethanoids (C6-C2), phenylpropanoids (C6-C3), acetophenones (C6-C2), chromones (C6-C4) and flavonoids (C6-C3-C6) could be derived from the shikmic acid pathway and directly related to the alkaloid biosynthesis through belladine (C6-C1-N-C2-C6) which is the commonly accepted precursor of the Amaryllidaceae alkaloids.

Although relatively fewer terpenoids have been isolated, the presence of different skeletons of phytosterols including cholestane is very significant. The phytosteroidal saponins were reported from the subfamily Allioideae as active constituents and taxonomical biomarkers. The presence of saponin aglycons in the Amaryllidaceae subfamily may represent a limited but important chemical-bridge between the two subfamilies.

Among the flavonoids, many lipophilic flavans/flavanols were isolated from both aerial parts and bulbs. The lipophilic character increases the bioavailability of the flavans in the human diet and thereby boosts the therapeutic effects. On the other hand, mono-, di- and triglycosides of flavonol and acetophenones were isolated mainly from flowers.

The reported 223 non-alkaloidal compounds were isolated from less than 7% (54 plants) of the subfamily species which indicates that a reasonable concentration of these metabolites is present in comparison to alkaloids. The presence of such a wide range of important

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biologically active non-alkaloidal compounds should encourage a more comprehensive investigation in the near future to fully understand the full chemical profile of this subfamily and thereby discover their biological potential for human health benefits.

Declaration of Competing Interest

The authors declare no conflict of interest.

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CHAPTER THREE

Triterpenes and other minor chemical constituents of *Boophone haemanthoides* F.M. Leight (Amaryllidaceae*)*

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Abstract

The chemical investigation of the nonpolar constituents of a methanol extract of the bulbs of *Boophone haemanthoides* yielded ten known compounds as minor constituents. The compounds were identified as stigmast-4-ene-3,6-dione (**1**); cholest-4-en-3-one (**2**); (22*E*)-stigmast-4,22-dien-3-one (**3**); stigmast-4-en-3-one; 6b-hydroxystigmast-4-en-3-one (**5**); 6b-hydroxycholest-4-en-3-one (**6**); cycloartenol (**7**); acetovanil-lone (**8**); tyrosol (**10**) and 3-hydroxy-1-(4-hydroxyphenyl)-1-propanone (**11**). The isolation of compound 7 with other cholestane (**2**, **6**) and stigmastane (**1**, **3**, **5**) derivatives aligned with the biosynthetic pathway of plant steroids through 7 from squalene 2,3-epoxide. This is the first report on the isolation and identification of these compounds from *B. haemanthoides* and **1—6** and 11 for the family Amaryllidaceae

***Keywords**:* Amaryllidaceae*, Boophone haemanthoides,* biosynthesis, cholestanes
non-alkaloidal content, phytosterols

Abbreviations:

CEM, Human acute lymphoblastic;

^{13}C NMR, Carbon-13 nuclear magnetic resonance;

^{1}H NMR, Proton nuclear magnetic resonance;

C18, Reversed phase with Octadecyl silane as adsorbent layer;

d, Doublet;

DCM/CH_2Cl_2, Dichloromethane;

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dd, Doublet of doublet;

DEPT-135, Distortionless Enhancement by Polarization Transfer-135;

GC—MS, Gas Chromatography coupled with Mass spectrometer detector;

HeLa, Cervical adenocarcinoma;

HPLC, High performance liquid chromatography;

J, Coupling constant;

m/z, Mass/charge ratio;

MCF-7, Breast adenocarcinoma;

Me, Methyl;

MeOH, Methanol;

Prep-TLC, Preparative Thin Layer Chromatography;

R_t, Retention time;

s, Singlet;

t, Triplet;

TLC, Thin Layer Chromatography;

TMS, Tetramethyl silane;

UV, Ultraviolet

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3.1 Introduction

The small African genus *Boophone* Herb. is a member of the bulbous plant family Amaryllidaceae. The family is here used in a narrow sense that is equivalent to the subfamily Amaryllidaceae within the broadly circumscribed family that includes the subfamilies Agapan- thoideae and Allioideae (Chase *et al*., 2009). It is particularly diverse in southern Africa where 20 genera and 266 species are found (Koekemoer *et al*., 2014). *Boophone* commonly known as 'gifbol' comprises two species, *B. disticha* (L.f) Herb. and *B. haemanthoides* F.M. Leight (Meerow and Snijman, 1998). They occur from southern Africa to tropical East Africa with *B. disticha* more widely distributed than *B. haemanthoides*, which is limited to parts of southern Namibia and the South African winter rainfall areas of Namaqualand, Nieuwoudtville, Calvinia and West Coast to Saldanha (Wrinkle, 1984).
Plants of the genus have unique therapeutic and toxicological profiles with *B. haemanthoides* having limited traditional uses compared to the more common *B. disticha*, for example, the papery bulb scales are used to treat asthma and as a compress for aching knees (De Beer and Van Wyk, 2011). The chemical composition of

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the Amaryllidaceae is very diverse. It has a rich alkaloidal content with different skeletons and some of these isolated alkaloids show promising biological activities (Nair *et al.*, 2013a; Ding *et al.*, 2017). Other chemical constituents such as terpenoids and flavonoids are rarely reported for the Amaryllidaceae. Previous chemical studies on *B. haemanthoides* reported eight alka- loids namely, crinine, buphanisine, buphanidrine, ambelline, undula- tine, distichamine, distichaminol and lycorine. The last two compounds showed cytotoxic activity against CEM (human acute lymphoblastic), MCF-7 (breast adenocarcinoma) and HeLa (cervical adenocarcinoma) cells (Nair *et al.*, 2013b). Our study reports the iso- lation and identification of the non-alkaloidal constituents from *B. haemanthoides*.

3.2 Material and methods

3.2.1 Chemical and instrumentation conditions

The organic solvents methanol (HPLC grade), ethanol, ethyl acetate, and hexane used for extraction and column chromatography were general purpose reagents and were obtained from Merck Chemicals (Pty) Ltd (Cape Town, South Africa). ^{1}H, ^{13}C, and DEPT-135 NMR spectra were recorded on a Bruker spectrometer operating at 400 (for ^{1}H) and 100 MHz (for ^{13}C). The chemical shift values were reported in ppm relative to TMS as the internal standard. Final purifications were performed using Agilent Technologies 1200 series, equipped with UV detector, manual injector, quaternary pump (G1311A), vacuum degasser (G1322A), column compartment (G1316A) and reversed phase C18 column SUPELCO (25 cm x 10 mm, 5 µm). The flow rate was set at 1.5 mL/min, and detection wavelength at λ254 nm. GC-MS analysis was performed utilizing an Agilent Technologies 7820A coupled with MSD5977E. Samples of ~ 1.0 mg were dissolved in CH_2Cl_2 and 1 mL was injected directly into the GC-MS operating in the electron ionization (EI) mode at 70 Ev and utilizing HP5 MS column (30 m 0.25 mm i.d., film thickness 0.25 m). The temperature gradient performed was as follows: 8 min at 40 °C, 80 220 °C at 10 °C/ min and 5 min hold at 220 °C, 220 300 °C at 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 and the injection volume was 1 mL.

3.2.2 Plant material

Bulbs of *B. haemanthoides* were collected in Nieuwoudtville in the Northern Cape Province, South Africa, in December 2016. The plants were identified by the Curator of the Hantam National Botanical Garden, Mr. Eugene Marinus and one of the co-authors

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(CNC). A voucher specimen (UFH 2020 3 01) was deposited in the Giffen Herbarium (UFH) at the University of Fort Hare.

3.2.3 Extraction and fractionation of the total extract

Fresh bulbs (3.2 kg) were blended with methanol and extracted for 2 days, after filtration, the remaining plant materials were re-extracted with fresh methanol. The total extracts 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. Fraction 4 (100 mg) was purified on HPLC using MeOH:H_2O (60% MeOH to 100 MeOH in 40 min) to yield compounds **1** (R_t 45.91 min.), **2** (46.88), **3** (51.04), **4** (54.03) and **7** (52.82) (~ 1.0 mg/each compound), purification of fraction 7 (30.0 mg) under the same conditions gave compounds **5** (R_t 24.775 min.) and **6** (26.147) (1.0 mg/each). Fractions 8 (45 mg), 10 (100 mg) and 12 (96 mg) were purified on Prep-TLC using silica gel plate (F254, Merck) and DCM:MeOH (97:3) as solvent system to yield compounds 8 (1.0 mg); 10 (5 mg); and 11 (7.0 mg) respectively. Purity of the isolated compounds were confirmed using ^{1}H NMR as well as GC–MS analysis.

3.2.4. Physical and spectroscopic data of the isolated compounds

Compound **1**. MS; 426.5 ($C_{29}H_{46}O_2$), 398.5, 285.3, 207.1, 137.1; ^{1}H NMR: 6.15 (*d*, *J* = 0.7 Hz, H-4), 2.65 (*dd*, *J* = 3.9, 15.9 Hz, H-7a), 1.15 (*s*, Me-19), 0.91 (*d*, *J* = 6.5 Hz; Me-21), 0.83 (*t*, *J* = 7.4 Hz, Me-29), 0.82 (*d*, *J* = 7.2 Hz, Me-27), 0.80 (*d*, *J* = 7.1 Hz, Me-26), and 0.70 (*s*, Me-18).

Compound **2**. MS; *m/z*: 384.5 ($C_{27}H_{44}O$), 342.5, 261.4, 229.3, 124.3; ^{1}H NMR: 5.70 (*s*, H-4), 1.16 (*s*, Me-19), 0.89 (*d*, *J* = 6.7 Hz; Me-21), 0.845 (*d*, *J* = 6.7 Hz, Me-27), 0.842 (*d*, *J* = 6.6 Hz, Me-26), and 0.67 (s, Me-18).

Compound **3**. MS; *m/z*: 410.5 ($C_{29}H_{46}O$), 341.1, 281.0, 253.2, 207.1, 147.2. ^{1}H NMR: 5.71 (*d*, 0.7 Hz, H-4), 5.13 (*dd*, *J* = 15.1; 8.5 Hz, H-22), 4.99 (*dd*, *J* = 15.1; 8.5 Hz, H-23), 1.16 (*s*, Me-19), 1.00 (*d*, *J* = 6.9 Hz; Me-21), 0.83 (*d*, *J* = 7.6 Hz, Me-26); 0.78 (*t*, *J* = 7.2 Hz, Me-29), 0.77 (*d*, *J* = 6.3 Hz, Me-27), and 0.71 (*s*, Me-18).

Compound **4.** MS: 412.5 ($C_{29}H_{46}O$), 370, 281.2, 299.3, 207.1, 124.1. ^{1}H NMR: 5.69 (*s*, H-4), 1.15 (*s*, Me-19), 0.89 (*d*, *J* = 6.5 Hz; Me-21), 0.82 (*t*, *J* = 7.4 Hz, Me-29), 0.81 (*d*, *J* = 7.2 Hz, Me-26), 0.79 (*d*, *J* = 6.7 Hz, Me-26), and 0.70 (*s*, Me-18).

Compound **5**. MS *m/z*: 428.6 ($C_{29}H_{46}O_2$), 398.5, 285.3, 207.1, 137.1, 98.2. ^{1}H NMR: 5.79 (*s*, H-4), 4.32 (*br s*, H-6a), 1.35 (*s*, Me-19), 0.90 (*d*, *J* = 6.4 Hz; Me-21), 0.82 (*t*, *J* = 7.6 Hz, Me-29), 0.81 (*d*, *J* = 7.1 Hz, Me-26), 0.79 (*d*, 6.4 Hz, Me-26), and 0.72 (*s*, Me-18).

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Compound **6**. MS: *m/z*: 400.5 ($C_{27}H_{44}O_2$). 341.2, 287.3, 245.2, 207.1, 137.1. ^{1}H NMR: 5.79 (*s*, H-4), 4.32 (*br s*, H-6a), 1.35 (*s*, Me-19), 0.89 (*d*, *J* = 6.5 Hz; Me-21), 0.845 (*d, J* = 6.6 Hz, Me-27), 0.842 (*d*, *J* = 6.6 Hz, Me-26), and 0.71 (*s*, Me-18)

Compound **7**. ^{1}H NMR: 5.08 (*br t*, *J* = 7.4 Hz, H-24), 3.26 (*m*, H-3), 1.66 (*s*, Me-26), 1.58 (*s,* Me-27), 0.95 (6H; *s*, Me-30, 18), 0.87 (*s,* Me-32), 0.86 (*d*, *J* = 6.5 Hz, Me-21), 0.79 (*s,* Me-31), 0.53 (*d, J* = 4.4 Hz, H-19 *endo*), and 0.31 (*d, J* = 4.4 Hz, H-19 *exo*).

Compound **8**. ^{1}H NMR: 7.51 (*m*, H-2, 6), 6.92 (*d*, *J* = 8.2 Hz, 3.92 (*s,* OMe), 2.53 (*s,* Me).

Compound **10**. MS: *m/z*: 138.2, 107.1, 91.1, 77.1. ^{1}H NMR; 6.92 (2H, *d, J* = 8.4 Hz, H-2, 6), 6.60 (2H, *d, J* = 8.4 Hz, H-3, 5), 3.57 (t, *J* = 7.2 Hz, CH_2 8), 2.61 (*t*, *J* = 7.2 Hz CH_2 7). ^{13}C NMR 155.3 (C-4), 129.5 (C-1, 2, 6), 114.7 (C-3, 5), 63.2 (C-8), and 38.0 (C-8). Compound **11**. MS: *m/z*: 166.1, 121.1, 93.0. ^{1}H NMR: 7.89 (2H, *d, J* = 8.8 Hz, H-2`, 6`), 6.84 (2H, *d, J* = 8.8 Hz, H-3`, 5`), 3.93 (*t*, *J* = 6.1 Hz CH_2 3), 3.16 (*t*, *J* = 6.1 Hz, CH_2 2). ^{13}C NMR 198.4 (C-1), 162.5 (C-4`), 130.5 (C-2`, 6`), 128.9 (C-1`), 114.9 (C-3`, 5`); 57.5 (C-3) and 40.3 (C-2).

3.3 Results and discussion

The chromatographic purifications of the nonpolar fractions of *B. haemanthoides* total extracts using different techniques including semi-prep HPLC, resulted in the isolation and identification 10 known compounds (figure 9). These were identified as stigmast-4-ene- 3,6-dione (**1**); cholest-4-en-3-one (**2**); (22*E*)-stigmasta-4,22-dien-3-one (**3**); stigmast-4-en-3-one (**4**); 6b-hydroxystigmast-4-en-3-one (**5**); 6b-hydroxycholest-4-en-3-one (**6**); cycloartenol (**7**); acetovanillone (8); tyrosol (10) and 3-hydroxy-1-(4-hydroxyphenyl) 1-propanone (**11**).

Compound **1** (stigmast-4-en-3,6-dione was purified from the main fraction 4 using semiprep-HPLC. The GC-MS analysis showed a single peak at 43.113 min with *m/z*: 426.5 which indicated a molecular formula $C_{29}H_{46}O_2$. The ^{1}H NMR spectrum showed a typical 3,6-diketone steroid, the low field olefinic signal at 6.15 ppm indicated the presence a double bond between two carbonyl groups (Kontiza *et al*., 2006).

Further, the aliphatic region showed signals of six methyls, two of them appeared as singlets at 1.14 and 0.70 (Me-19 and 18 respectively), and four appeared at 0.91 *d* (6.5 Hz; Me-21); 0.83 *t* (7.5 Hz; Me-29); 0.82 *d* (7.4 Hz; Me-26); 0.79 d (7.4 Hz; Me-27). Stig-mast-4-en-3,6-dione (compound **1**) is widely distributed in nature and was identified in many plant extracts e.g. *Colubrina asiatica* and *Fallopia convolvulus* (Li *et al*., 2019; Wei *et al*., 2004; Cui *et al*., 2009; Della Greca *et al*., 1990).

The GC-MS analysis of compound **4** (stigmast-4-en-3-one), showed a single peak with *m/z*: 412 ($C_{29}H_{46}O$) with 14 amu less than that of compound 1. On the other hand, the ^{1}H

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NMR spectrum of compound **4** showed similar signals with that of compound **1**, except the for high chemical shift of H-4 to δ_H 5.69 ppm which indicated the absence of a C-6 carbonyl group. (Kontiza *et al.*, 2006; Prachayasittikul *et al.*, 2009; Georges *et al.*, 2006). The presence of compound **4** (also known as β-sitostenone) was previously reported for *Spilanthes acmella*, *Anacardium occidentale*, and *Morinda citrifolia* (Prachayasittikul et al., 2009; Alexander-Lindo et al., 2007; Saludes et al., 2002). The GC-MS analysis of compound 5 (6b-hydroxystigmast-4-en-3-one) showed a single peak at Rt 43.165 min with m/z: 428.6 ($C_{29}H_{46}O_2$). The 1H NMR spectrum of this compound showed similarity with compound 4, except for the appearance of a signal at 4.32 belonging to H-6, which indicates the presence of a hydroxy group at C6. The chemical shift of H-4 (dH 5.79) slightly differs from compounds 1 and 4. (Kontiza *et al.*, 2006; Georges *et al.*, 2006). Compound 5 was previously isolated from *Microcos tomentosa* (Kaennakam *et al.*, 2013) and the red alga *Jania adhaerens* (Alarif *et al.*, 2012).

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Stigmast-4-ene-3, 6-dione (**1**): R_1 = =O; R_2 = Et
Cholest-4-en-3-one (**2**): R_1 = H; R_2 = H
(22*E*)-Stigmasta-4,22-dien-3-one (**3**): R_1 = H; R_2 = Et; Δ^{22}
Stigmast-4-en-3-one (**4**): R_1 = H; R_2 = Et
6β-Hydroxystigmast-4-en-3-one (**5**): R_1 = OH; R_2 = Et
6β-Hydroxycholest-4-en-3-one (**6**): R_1 = OH; R_2 = H

Cycloartenol (**7**)

Acetovanillone (R = Me; **8**)
Vanillin (R = H; **9**)

Tyrosol (**10**)

3-Hydroxy-1-(4`-hydroxyphenyl)-1-propanone (**11**)

Figure 12: Chemical structure of compounds 1—11.

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The GC-MS analysis of compound **3** (22*E*)-stigmast-4,22-dien-3-one) showed a single peak at R_t 39.807 min with *m/z*: 410 ($C_{29}H_{46}O$). It showed ^{1}H NMR signals closely related to compound **4**, except for the presence of a double bond between C22-C23 which was con- firmed by the appearance of signals at 5.13 and 4.99 (*dd*, *J* = 15.1; 8.5 Hz/H-22, H-23) (Kontiza *et al.*, 2006; Georges *et al.*, 2006). Similar to Compound **1**, this compound is also widely distributed in nature and was identified in many plant extracts e.g. *Croton palanostigma* and *Vigna luteola* (Mochiutti *et al.*, 2019; Lam *et al.*, 2019).

The GC-MS analysis of compound **2** (cholest-4-en-3-one) showed a single peak at R_t 38.319 min with *m/z*: 384 ($C_{27}H_{44}O$). The ^{1}H NMR profile showed signals that are closely related to that of compound **4**, except for the presence of a side aliphatic chain. In compound **2** the C-24 ethyl group is absence and the MS spectra showed a difference of 29 amu, which supports this absence. The methyl pattern demonstrated by compound **2** is a typical C27-cholestane skeleton (Kontiza *et al.*, 2006; Rebelo *et al.*, 2004; Mushfiq and Rehman, 2010; Georges *et al.*, 2006). As in the case of compound **5**, this compound was also recently isolated from *Microcos tomentosa* (Kaennakam *et al.*, 2013).

The GC-MS analysis of compound **6** (6β-hydroxycholest-4-en-3-one) showed a single peak in GC-MS analysis at 40.021 min with *m/z*: 400.5 ($C_{27}H_{44}O_2$). The ^{1}H NMR spectrum is closely related to com- pound 5 except for the absence of the ethyl group signals at C-24, this was supported by the MS spectra as mentioned earlier. The methyl ^{1}H NMR pattern showed a typical 27-cholestane skeleton that is similar to compounds **2**. However, the presence of the 6β-OH causes the Me-19 to shift to δ_H 1.36 (from 1.16 of compound **4**) (Kontiza *et al.*, 2006; Georges *et al.*, 2006). This compound is common in nature and was previously identified from *Bombax ceiba*, *Microcos tomentosa*, and *Tinospora sinensis* (Wang *et al.*, 2017; Kaennakam *et al.*, 2013; Lam *et al.*, 2018).

The stereochemistry at C-24 (compounds **1**, **3**, **5**) could not be ascertained from the given data, however, it is expected that they have 24a-configuration (24*R*) based on biosynthetic ground since higher plants predominantly produce this configuration (Sica *et al.*, 1984; Adewusi *et al.*, 2013).
Compound **7** was identified as cycloartenol based on ^{1}H NMR spectra which showed a signal at 5.08 ppm (*br t*, *J* = 7.1 Hz, H-24); a signal a proton adjacent to hydroxyl group at 3.26 (*m*, H-3); two high field shifted two protons at 0.53, and 0.31 both are *d* (*J* = 4.1 Hz; 19- *endo* and *exo* respectively) in addition to signals of seven methyl's six of them are singlet at 1.66 and 1.58 (Me-25 and 26), 0.94 *s* (6H, Me-18, 30); 0.87, 78 (Me-32, 31), and a doublet 0.86 (6.3 Hz; Me-21). This compound is widely distributed in nature and was identified from *Crinum asiaticum* (Takagi and Yamaki, 1977).

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Acetovanillone (**8**) was isolated as white aromatic crystals and has similar structure of vanillin (**9**) (Lee *et al.*, 2015). The GC-MS analysis showed a single peak at R_t 20.861 min with *m/z* 166.2 ($C_9H_{10}O_3$). The ^{1}H NMR is matching with the data reported for the same compound in literature (Win, Z.Z., 2011), and it was isolated from *B. disticha* (Tag-wireyi and Majinda, 2017), *Crinum buphanoides* and *C. graminicola* (Masi *et al.*, 2018). Tyrosol (10) was isolated as amorphous white powder.

The GC-MS analysis of **10** showed a single peak at R_t 20.104 min with *m/z* 138.2 (M^+) corresponding to $C_8H_{10}O_2$, which showed fragment at *m/z* 107.1 (base peak) and *m/z* 91.1.The ^{1}H NMR spectra showed 1,4-disubstituted benzene as indicated by the signals at dH 6.92 (2H, *d, J* = .4 Hz, H-2,—6) and 6.60 (2H, *d, J* = 8.4 Hz, H-3, 5); two isolated methylene groups at 3.57 (CH_2 8) and 2.61 (CH_2 7) (*t*/each; *J* = 7.2 Hz). The ^{13}C NMR (with DEPT-135) showed signals of 8 carbons that could be classified as 6 aromatic carbons at 155.3 (C-4), 129.5 (3C; C-1, -2, -6), 114.7 (2C; C-3, -5), and two methylene at 63.2 (C-8) and 38.0 (C-8), with one of them (δ_H 3.57/dC 63.2) being attached to a hydroxyl group. The above profile is identical with that of tyrosol, a common phenolic alcohol. Tyrosol was previously isolated from olives and *Vitis vinifera* (Covas *et al.*, 2003). Tyrosol demonstrated antioxidant (Puerta *et al.*, 2001; Bertelli *et al.*, 2002), anti-inflammatory (Giovannini *et al.*, 2002), antidiabetic (Dejadisai *et al.*, 2017), and neuroprotective (Bu *et al.*, 2007) activities.

3-Hydroxy-1-(4`-hydroxyphenyl) 1-propanone (**11**) was isolated as amorphous white powder. The GC MS showed a single peak at R_t 23.875 min with *m/z* 166.1 (M^+) corresponding to $C_9H_{10}O_3$, it and fragments at *m/z* 121 (base peak) and *m/z* 93. The ^{1}H NMR spectra deduced 1,4-disubstituted benzene as indicated from the signals at dH 7.89 and 6.84 (2H/each; *d*/each; *J* = 8.8 Hz); two isolated methylene groups at 3.93 (CH_2-3) and 3.16 (CH_2-2) (*t*/each; *J* = 6.1 Hz). The ^{13}C NMR (with DEPT-135) showed signals of 9 carbons that could be classified as 6 aromatic carbons at 162.5 (C-4`), 130.5 (2C; C-2`, 6`), 128.9 (C-1`), 114.9 (2C; C-3`, 5`); a carbonyl group at 198.4 (C-1) and two methylene at 57.5 (C-3) and 40.3 (C-2), one of the methylene group is hydroxylated while the other is next to the carbonyl group. The above data indicate that compound **11** has a chemical structure of 3-hydroxy-1-(4`-hydrox- yphenyl) 1-propanone (Fig. 12), which is supported by recently published data for *Ailanthis altissima* (Ni *et al.*, 2019).

Except for Compounds **7** and **8**, all other isolated compounds are here reported for the first time for the Amaryllidaceae. The phytosterols (C-24 alkyl-substituted sterols) e.g. ergostane, and stigmastane are the most abundant sterols in the plant kingdom, while the cholestane is commonly accumulating in animals. However, some compounds related to cholestane have been reported for certain species of higher plants. Sterols play important roles in the physiology and biochemistry of most living organisms.

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The presence of acylated cholestanes (mainly glycosides) from the genus *Allium*, which is a member of the Alliaceae, a family that is closely related to the Amaryllidaceae, are well-documented (Sobolewska *et al.*, 2016). On the other hand, the isolation of cholestane related structures (compounds 2 and 6) for the South African Amaryllidaceae is reported for the first time in this study.

3.4 Conclusion

This study provides an overview of the chemistry of *B. haemanthoides* and a newly isolated class of compounds not previously described for the Amaryllidaceae. However, the triterpenoids are poorly described for this family, it is considered as an important class of secondary metabolites and is contributing to the chemical profile of the species. New phytochemical studies for other Amaryllidaceae members will be important to discover other metabolites in addition to the alkaloids.

Declaration of Competing Interest

The authors declare no conflict of interest in this work.

Author Contributions

AH conceived the research idea. AI carried out the isolation of the compounds, AH and AI elucidated the structures. TL ran all the GC MS analysis, CNC identified the plant material. All authors read, edited and contributed to the manuscript. JSB, CNC, and AH are AI supervisors.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.sajb.2020.06.025.

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CHAPTER FOUR

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.

4.1 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

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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-methylphenyltetrahydropyridine (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

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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.

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4.2 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). Alkaloids compounds isolated were tested with dragendorffreagent. 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

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°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.

4.2.1. 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.

4.2.2. 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 ºC 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** (40mg). 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).

4.2.3 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, $H_{4\beta}$), 2.49 (1H, *dd*, *J* = 17.3, 6.7 Hz, $H_{4\alpha}$), 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

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(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_2O), 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_2O), 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).

1α,3α-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; $[\alpha]^{D}_{25}$ −16.1 (c 0.1 in CH_2Cl_2). ^1H NMR (400 MHz, $CDCl_3$); ^{13}C NMR (100 MHz, $CDCl_3$) see table 1.

Hippadine (**3**), GC-MS: R_t 34.807 min; Mass: 263.06 ($C_{16}H_{10}NO_3$), *m/z*: 205.2, 177.1, 131.2; ^1H NMR, H 8.03 (*d*, *J* = 3.5 Hz, H_{12}), 7.97 (*s*, H_7), 7.91 (*d*, *J* = 7.6 Hz, H_1), 7.74 (*d*, *J* = 7.6 Hz, H_3), 7.65 (*s*, H_{10}), 7.46 (*t*, *J* = 7.6 Hz, H_2), 6.88 (*d*, *J* = 3.5 Hz, H_{11}), 6.15 (*s*, OCH_2O) [30].

4.2.4 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).

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4.2.5 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 4). 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

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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 4: Table showing list of compounds isolated from BHE

Compound	Name
1	Distachamine
2	1α,3α-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

4.2.6 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 [64]. The percentage cell viability was calculated using the formula below:

$$\% \text{ Cell Viability} = \frac{\text{Absorbance of treated well}}{\text{Absorbance of Untreated well}} \times 100$$

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The concentration required to kill 50% of the cells (IC50) was determined via a survival curve using GraphPad Prism6 software (GraphPad software, San Diego, CA, USA).

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4.2.7 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 EVOS M7000 (ThermoFisher Scientific,Waltham, MA USA) with 10X objective lens. Images were captured using the Zeiss software version 2.3.

4.2.8 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 [65]. 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 [65].

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4.2.9 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 [66].

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4.3 Results

4.3.1 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 10) 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**) Stigmast-4-ene-3, 6-dione (**4,** R_1 = =O; R_2 = Et,), Cholest-4-en-3-one (**5,** R_1 = H; R_2 = H) Tyrosol (**6**) 3-Hydroxy-1-(4`-hydroxyphenyl)-1-propanone (**7**).

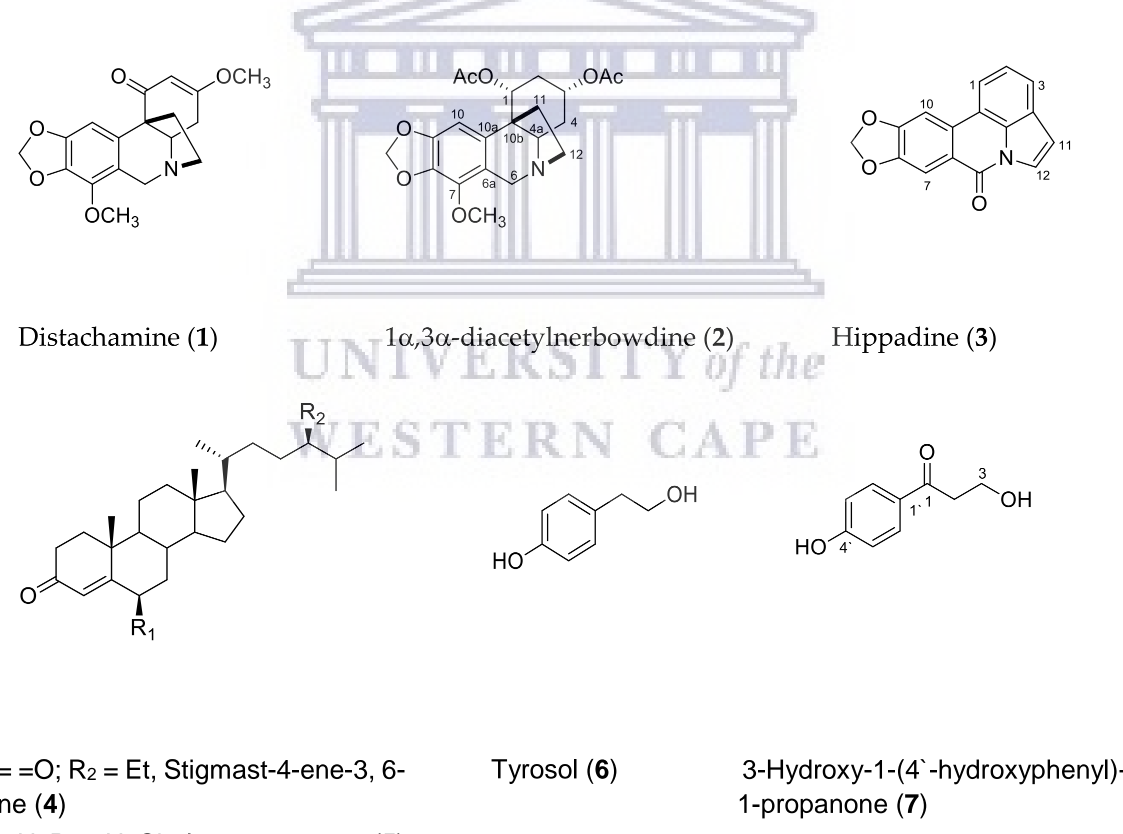

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Figure 13: Chemical structures of compounds 1- 7 isolated from *B. haementhoids.*

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. The GC-MS analysis

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showed a single peak at R_t 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 5). ^{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).

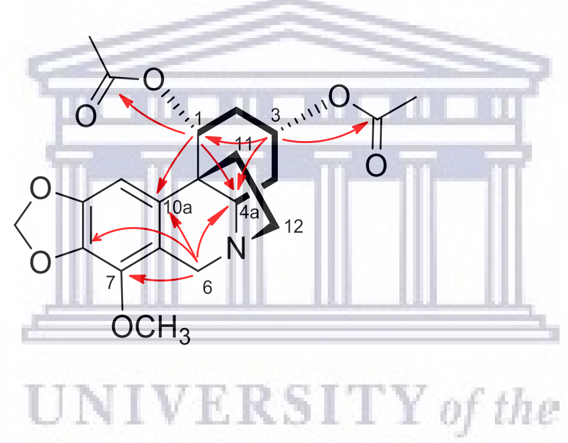

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Figure 14: Important COSY (—) and HMBC (H→C) **correlations of compound** 2.

Comparison of the given NMR data with literature indicated a crinane alkaloid with two acetoxy and a methoxy group as shown in figure 13. 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 14) 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 $C_{4a\alpha}$ which indicated the positions of the two acetate groups at C_1 and C_3. The positions were further confirmed by HMBC spectra (Figure 14) 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 15.

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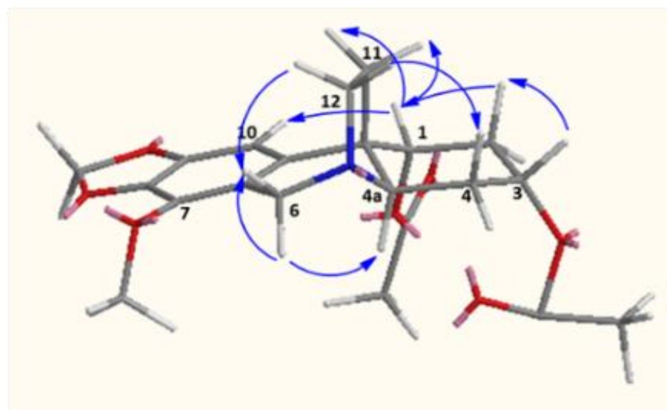

Figure 15: Important NOESY correlations of compound **2**

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 C1 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 15). 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 5: ^{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

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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**CH_3	170.5 *s*		
1-CO**CH_3**	21.2 *q*	1.90** *s*	CO(C_1)
3-**CO**CH_3	170.1 *s*		
3-CO**CH_3**	21.3 *q*	1.97* *s*	CO(C_3)
OCH_3	59.1 *q*	3.95 *s*	C_7
OCH_2O	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.

4.3.2. 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 16a 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 16b). 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.

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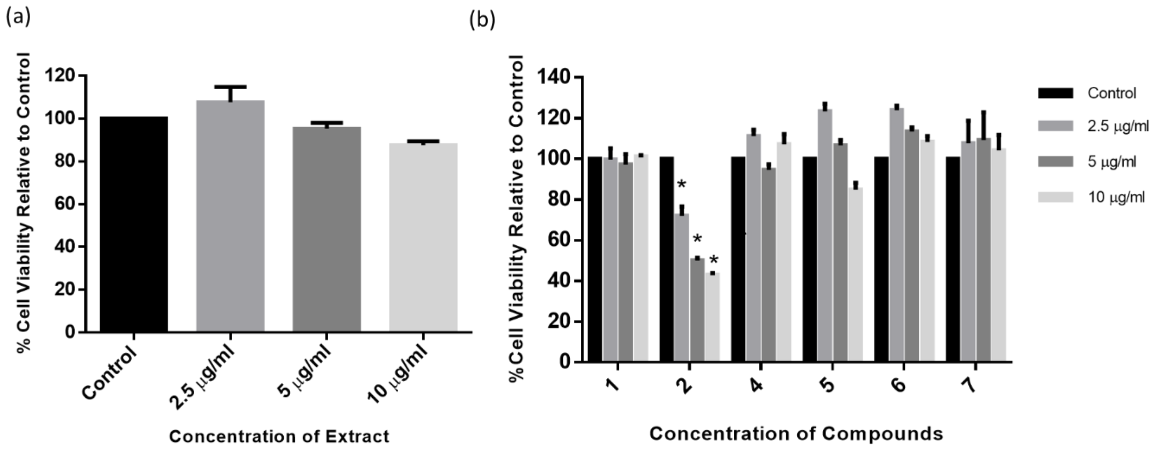

* indicate significance at $p < 0.05$.

Figure 16: 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

4.3.3. 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 17 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].

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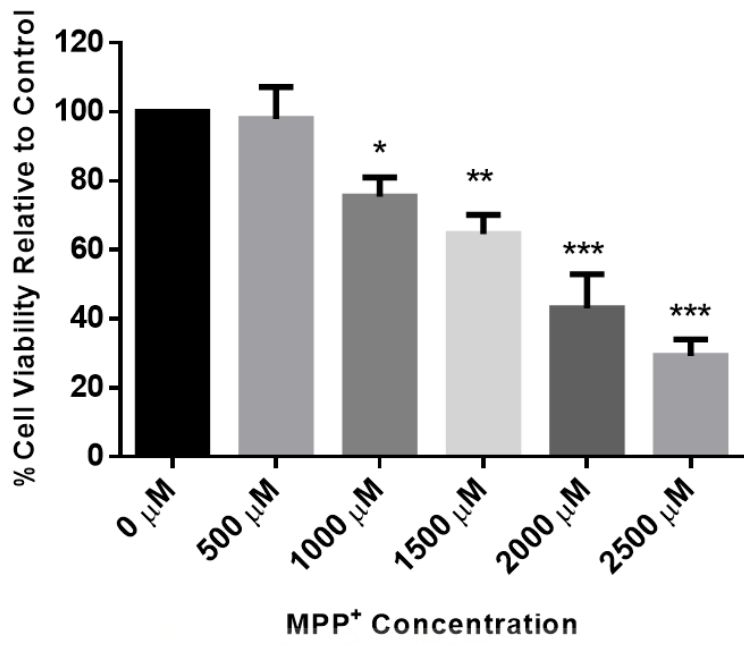

Figure 17: MPP+ induced cytotoxicity in SH-SY5Y cells. Cells were exposed to increasing concentrations of MPP+ (500μM – 2500 μM) and allowed to incubate for 24 hours. MTT assays were performed and results showed a concentration-dependent decrease in cell viability when compared to control. Each bars 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)**

4.3.4. 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 18a 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 18b). Together, these results suggest that BHE and the isolated compounds could attenuate MPP+-induced toxicity in SH-SY5Y cells.

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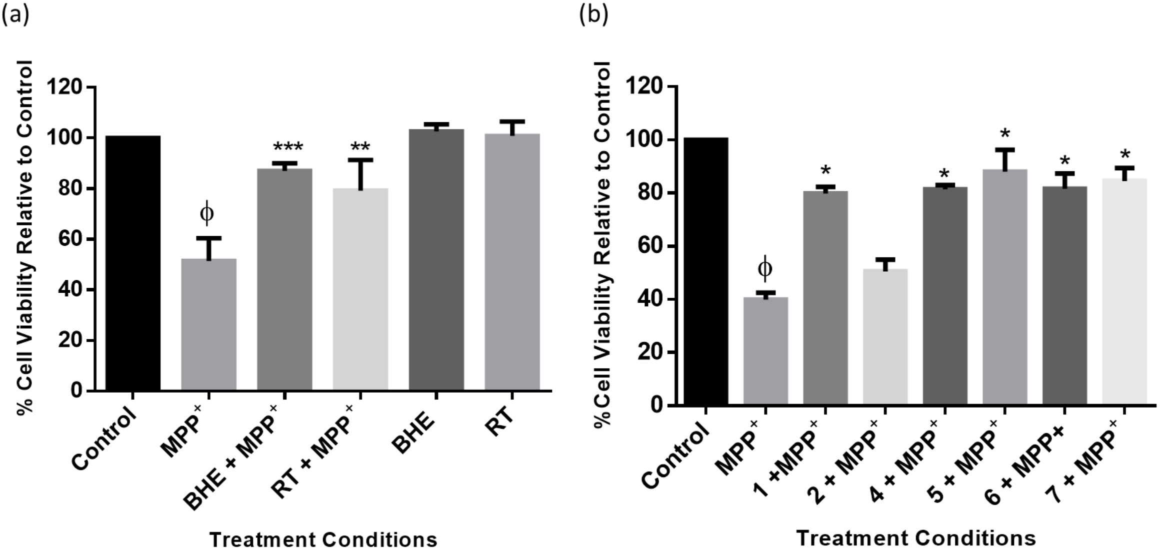

Figure 18: BHE and compounds show protection in SH-SY5Y cells. Cells were pre-treated with extracts (a) and compounds (b) before exposure to MPP+ for 24 hours. Each bar represents mean percentage cell viability relative to control and significance of difference indicated with * (*p*<0.05), ** (*p*<0.01) and * (*p*<0.001) when extract/compounds are compared to MPP+ and ϕ (MPP+ vs control)**

4.3.5. 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 19 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.

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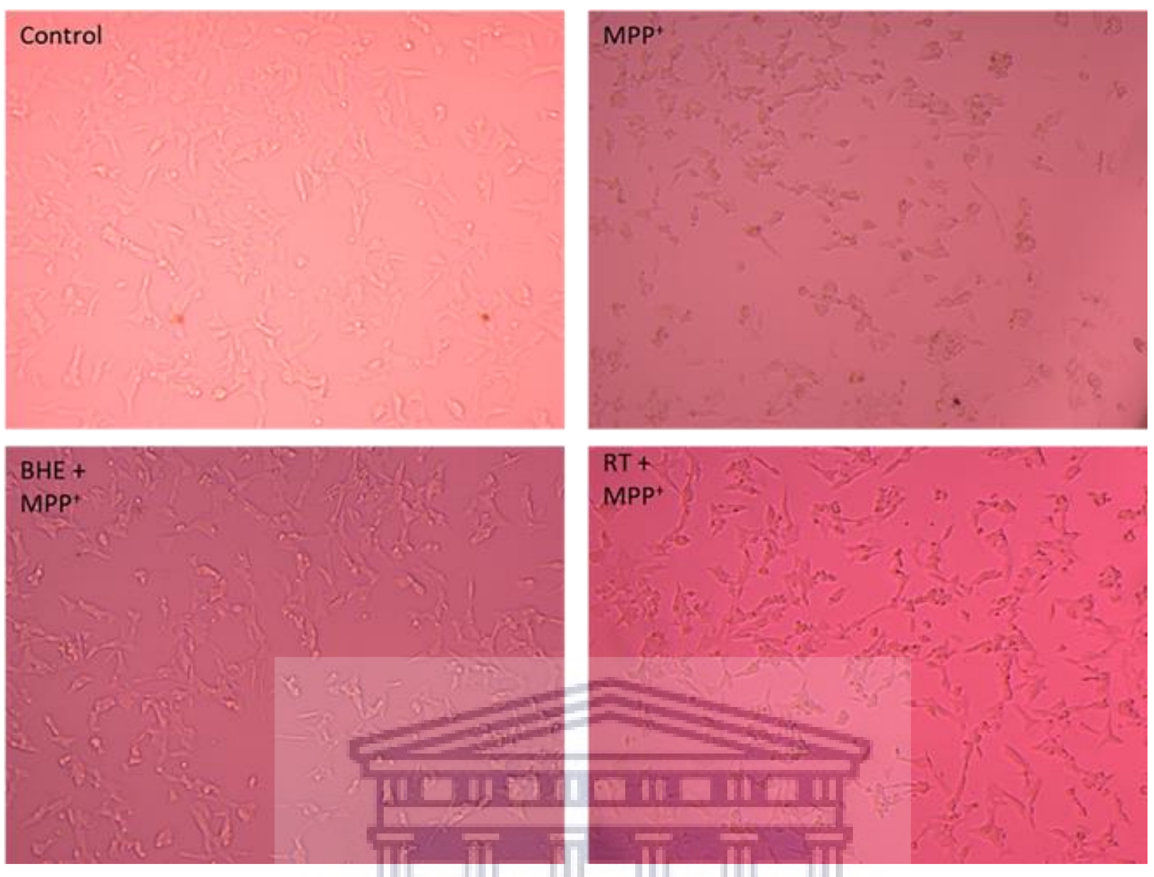

Figure 19: BHE and compounds inhibit SH-SY5Y morphological changes induced by MPP+. Cellular morphology of SH-SY5Y cells pre-treated with BHE and compounds (2.5 µg/mL) prior to exposure to 2000 µM MPP+ for 24 hours and images were captured using the light microscope at 100X magnification

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4.3.6. 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 20a). Additionally, a similar trend was observed for the cells pre-treated with compounds (Figure 20b). Together, these results indicate that BHE and the compounds could rescue SH-SY5Y cells from MPP+-induced ATP depletion.

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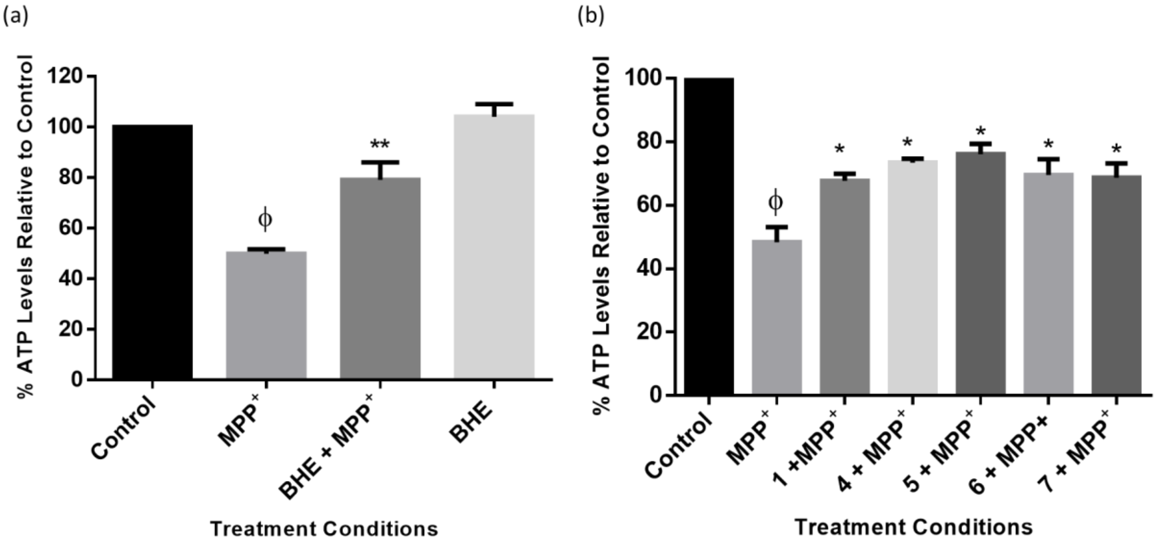

Figure 20: BHE and compounds inhibit MPP+-induced ATP degeneration. Cells were pre-treated with 2.5 µg/mL of extracts (a) and compounds (b) before exposure to 2000 µM of MPP+ for 24 hours and ATP levels assessed. Each bar represents mean percentage level relative to control and significance of difference indicated with * (*p*<0.05) and ** (*p*<0.01) when extract/compounds are compared to MPP+ and ϕ (MPP+ vs control).

4.3.7 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 21a 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 21b). Altogether, these results indicate that the inhibition of apoptosis by BHE and the compounds is a neuroprotection mechanism.

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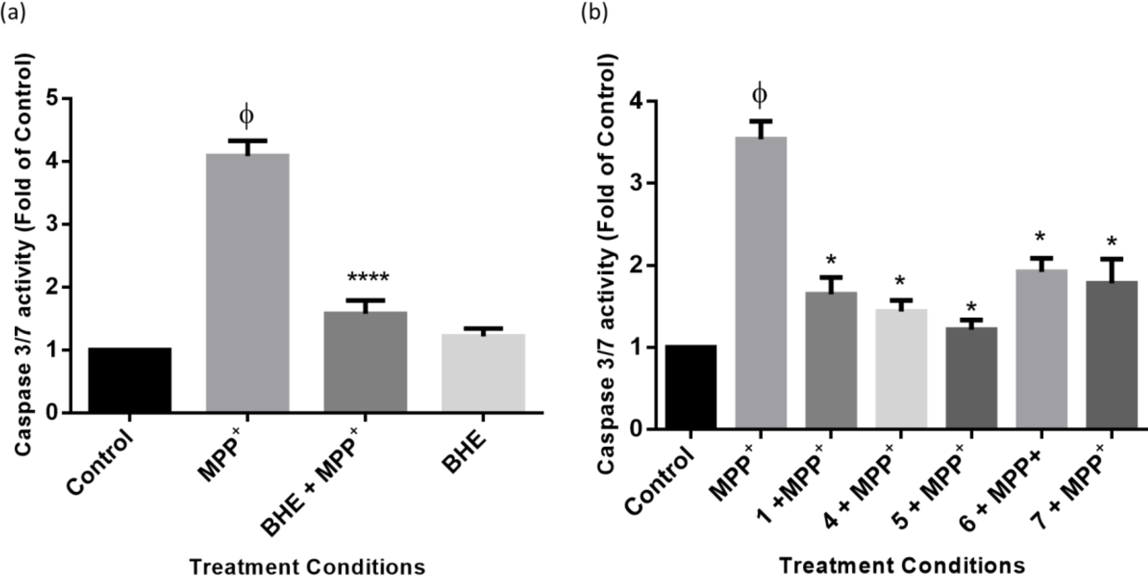

Figure 21: BHE and compounds reduce MPP+-induced caspase 3/7 activity. Cells were pre-treated with 2.5 µg/mL of extracts (a) and compounds (b) before exposure to 2000 µM of MPP+ for 24 hours and activity of caspase 3/7 was determined. Each bar represents mean percentage level relative to control and 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)**

4.4 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 4 and Figure 13). Furthermore,

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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 16b and 18b), 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 an 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.

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4.5 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

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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Conflicts of Interest

“The authors declare no conflict of interest.”

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CHAPTER FIVE

In vitro neuroprotective potential of *Clivia miniata* and *Nerine humilis* (Amaryllidaceae*)* in MPP+-induced neuronal toxicity in SH-SY5Y neuroblastoma cells

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Abstract

Parkinson's disease (PD) is one of the most common neurodegenerative diseases (NDD) and mainly affects the ageing population. A significant feature of PD pathogenesis is the progressive loss of dopaminergic neurons in the substantia nigra pars compacta part of the midbrain in affected persons. This neuronal loss occurs partly due to the increased generation of reactive oxygen species (ROS) in the mitochondrial and the depletion of adenosine triphosphate (ATP) in affected neurons. In this study, the methanolic extracts of *Clivia miniate* (CME) and *Nerine humilis* (NHE) belonging to the plant family Amaryllidaceae , were investigated for their neuroprotective potential in MPP+-induced neurotoxicity in SH-SY5Y neuroblastoma cells. Cell viability was determined using the 3-(4,5-dimethyithiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays and cell morphology was analysed using light microscopy. Furthermore, the effects of the extracts on apoptosis and ATP production were investigated using caspase 3/7 apoptosis kit and the Promega Mitochondrial ToxGlo ATP assay kit respectively. Additionally, the antioxidant contents of extracts were evaluated using routine laboratory procedures. The results show that pre-treatment of cells with the extracts at 2 and 4 mg/mL concentrations improved cell viability as well as cell morphology by inhibiting the toxicity induced by MPP+. The extracts also improved ATP levels in cells and attenuated the apoptosis induced by MPP+. Furthermore, antioxidant assays showed that both extracts had low antioxidant activity. Findings from this study indicate that CME and NHE may be promising as neuroprotective agents for PD and warrant further investigation to determine the bioactive components of the plants that may be responsible for the observed effects.

Keywords: 1-methyl-4-phenylpyridinium (MPP+), Amaryllidaceae*,* *Clivia miniate*, *Nerine humilis*, Neuroprotection, Parkinson's disease

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Abbreviations:

MTT, 3-(4,5-dimethyithiazol-2-yl)-2,5-diphenyltetrazolium bromide;

MPP+, 1-methyl-4-phenylpyridinium;

FRAP, ferric-ion reducing antioxidant power;

TEAC, trolox equivalent absorbance capacity;

ROS, reactive oxygen species;

TE, Trolox equivalents;

AAE, Ascorbic acid equivalents;

GAE, Gallic acid equivalents;

EGCG, Epigallocatechin gallate

5.1 Introduction

Parkinson's disease (PD) is a common age-related neurodegenerative disease which progresses with age and affects over 10 million people worldwide (Srivastav *et al.*, 2017). PD is associated with a number of motor impairments including 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 (Lopes *et al.*, 2010). In addition, such non-motor symptoms as pain, sleep disturbance, depression and cognition-related challenges have been described in PD patients (Gao, *et al.*, 2019). The classic pathological hallmark of PD is the loss of dopaminergic neurons in the substantia nigra pars compacta partly due to adenosine triphosphate (ATP) depletion following increased generation of reactive oxygen species (ROS) in the mitochondrial (Blesa *et al.*, 2015; Sarrafchi *et al.*, 2016). In addition, the accumulation of Lewy bodies (abnormal protein aggregates containing a-synuclein) could be toxic to the dopamine-producing neurons and result in microglial activation or microgliosis (Xicoy *et al.*, 2017). Although the aetiology of PD is not fully understood, there is evidence that besides ageing, environmental and genetic factors have been implicated in the disease (De Lau and Breteler, 2006; Johnson and Bobrovskaya, 2015; Pang *et al.*, 2019). Research into the treatment of PD involves genetic-based, cell culture, in vivo and ex vivo experimental models as well as clinical trials. However, for most laboratory studies to investigate PD treatment, the toxin 1-methyl-4-phenylpyridinium (MPP+) is routinely used in SH-SY5Y neuroblastoma cells as an in vitro model (Shishido *et al.*, 2019; Limboonreung *et al.*, 2020; Zhang *et al.*, 2020). MPP+ is a toxic metabolite of 1-methyl-4phenyl-1,2,3,6-tetrahydropyridine (MPTP), which is a by-product in the synthesis of 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP), a synthetic analogue of heroin, which induces severe parkinsonism in humans when injected intravenously (Ziering and Lee, 1947; Langston *et*

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al., 1984). Considering the complex nature of PD, there is no specific cure at the moment and efforts are geared towards treating the associated symptoms. The most common treatment is the use of the dopamine replacement therapy, levodopa (Dauer and Przedborski, 2003). At the start of treatment, motor symptoms of PD may initially respond positively to levodopa but after a while, patients are usually plagued with complications and disabling side effects known to affect the quality of life (Dauer and Przedborski, 2003; LeWitt, 2008; Shulman *et al*., 2008). It is therefore important to search for novel therapies for PD with potentially minimal side effects which medicinal plants could represent, considering the rich chemical composition and other bioactive compounds (Jain and Jain, 2018; Freyssin *et al*., 2018).

The bulbous plant family Amaryllidaceae worldwide consist of about 800 species assigned to 80 genera that are mainly distributed in the tropics and temperate regions. Home to 266 species in 20 genera, southern Africa is particularly rich in members of this family. In this region, the highest concentration of species is in the Western Cape and along the West Coast of South Africa into southern Namibia (Koekemoer *et al*., 2013). Plants in the family Amaryllidaceae*,* are known to contain a number of unique alkaloids with relevant cholinesterase inhibitory activity, and extracts and/or active constituents from these plants have been reported to show interesting neuroprotective effects (Hung *et al*., 2011). Two members of the family *Nerine humilis* (Jacq.) Herb. and *Clivia miniata* (Lindl.) Regel are the focus of this investigation. Nerine Herb. with approximately 23 species is widespread, ranging from the Cape Peninsula to the northwest and northeast of South Africa, Botswana, Lesotho, Namibia and Swaziland (Zonneveld and Duncan, 2006; Snijman and McMaster, 2019). *N. humilis* occurs from Clanwilliam (in southern Namaqualand) to Worcester, Bredasdorp, and Montagu to Baviaanskloof in the southern Cape where it grows in rocky and mountainous areas with loamy and sandy to gravelly soils in full shade or partial sun. Plants of some Nerine species were documented for their traditional uses for the treatment of coughs and colds, renal, and hepatic conditions, to relieve back pain and as a remedy for infertility (Nair *et al*., 2005). Chemical investigation on the genus was focused on the alkaloidal content, where 53 alkaloids were reported from 9 species belonging to this genus with 50% belonging to crinane skeleton (Cahlíková *et al*., 2019). *Clivia* Lindl. on the other hand is a small genus of some six species that grows in the forests of Kwazulu-Natal, Eastern Cape, Mpumalanga and Swaziland (Snijman and McMaster, 2019). *C. miniata* is traditionally used as an emetic remedy for fevers, as uterine tonics, treatment for snakebites, barrenness and urinary complaints (Broster, 1982; Bryant, 1909; Varga and Veale, 1997). Overdose of *Clivia* species is reported to cause toxicity, and are capable of causing dermatitis (Hutchings, 1996). Amaryllidaceae alkaloids have been isolated from the genus including *C. miniata* (Viladomat *et al*., 1997; Crouch *et al*., 2003; Nair *et al*., 2005). The

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present study to investigate the neuroprotection activity of *N. humilis* and *C. miniata* in an MPP+ PD model was therefore motivated by the above previous studies.

5.2 Materials and method

5.2.1. Identification and collection of plant material

Bulbs of *Nerine humilis* and *Clivia miniata* were collected from cultivated plants and their identities were authenticated by the coauthor, Christopher N. Cupido, a Plant Systematist and former Curator of the Compton Herbarium at the South African National Biodiversity Institute, using standard plant identifications methods. Voucher specimens of each species (*N. humilis*-UFH 2020-4-01, *C. miniate*-UFH 2020-4-02) were deposited in the Giffen Herbarium of the University of Fort Hare.

5.2.2. Preparation of plant material

Approximately 200 g of each fresh plant material (bulbs), were homogenized separately with methanol and extracted for two days at room temperature. After filtration, the extracts were evaporated under reduced pressure at 45 °C and the solvent-free extracts of *C. miniata* (CME) and *N. humilis* (NHE) were kept at 4 °C till further use.

5.2.3. Cell culture and maintenance

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The human neuroblastoma SH-SY5Y cells were generously donated by the Blackburn Laboratory, University of Cape Town. 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 mg/mL streptomycin (Lonza Group Ltd., Verviers, Belgium). Cultures were incubated at 37 °C in humidified air with 5% CO_2 with a medium change every three days. Cells were sub-cultured when they attained 70 to 80 percent confluency using a solution of 0.25 % trypsin EDTA (Lonza Group Ltd., Verviers, Belgium).

5.2.4. Treatments

Stock solutions of 40 mg/mL of plant extracts were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis MO, USA) from which dilutions of 2, 4, 6, 8 and 10 mg/mL concentrations were made in cell growth medium. To determine the concentration of CME and NHE that will confer neuroprotection in SH-SY5Y cells, the cells were plated at a density of 10, 000 cells/well and treated with concentrations (2-10 mg/mL) of CME and NHE while

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the vehicle-treated cells were used as control (cells treated with the same concentration of DMSO similar to that of the highest concentration of extract). Similarly, 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 mM which were added to cells, while cells not exposed to MPP+ served as control. All treatments lasted for 24 h and the concentrations 2 and 4 mg/mL were selected for neuroprotection studies for both extracts as they either increased viability or showed minimal cytotoxicity to the cells (Calabrese, E.J., 2018). For neuroprotection experiments (Calabrese, E.J., 2018), cells were plated in 96-well plates at a density of 10 000 cells per well and were left overnight to attach. Thereafter, cells were pre-treated for 2 h with 2 and 4 mg/mL of CME and NHE followed by the addition of 2000 mM MPP+ and treatments were incubated for 24 h. The untreated cells served as control.

5.2.5. 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. This assay seeks to ascertain mitochondrial activity of living cells on the basis of their ability to reduce the yellow MTT tetrazolium salt into a purple formazan crystal mainly by mitochondrial dehydrogenases. After treatment, 10 or 20 mL (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 h. At the end of the incubation, the medium containing the MTT dye was removed and the resulting purple formazan formed was solubilized with 100 mL of DMSO. After that, absorbance was measured using a microplate reader (BMG Labtech Omega_ POLARStar) at a wavelength of 570 nm and percentage cell viability was calculated relative to the control (Van Meerloo et al., 2011).

5.2.6. Adenosine triphosphate assay

The Mitochondrial ToxGlo ATP assay kit (Promega, USA) was used to investigate ATP levels in the cells (Olanow *et al.*, 2004). 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.

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5.2.7. 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 CME and NHE before the addition of 2000 mMMPP+. Treatments lasted for 24 h 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.

5.2.8. Cell morphology

To visualize changes in cell morphology of the SH-SY5Y cells following the respective treatments, cells were seeded in a 96 well plates at a density of 10 000 cells per well and were pre-treated with 2 and 4 mg/mL of NHE and CME for 2 h before the introduction of 2000 mM MPP+. After the 24 h 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.

5.2.9. Antioxidant assays

5.2.9.1. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out in accordance to the method described previously (Benzie and Strain, 1996). In a 96-well plate, 10 mL of the stock solution (1 mg/mL w/v) of the total extracts were mixed with 300 mL FRAP reagent. The FRAP reagent was prepared by mixing (10:1:1, v/v/v) of acetate buffer (300 mM, pH 3.6), tripyridyl triazine (TPTZ) (10 mM in 40mM HCl) and $FeCl_3.6H_2O$ (20 mM), Incubation commenced at room temperature for 30 min, and the plate was read at a wavelength of 593 nm in a Multiskan spectrum plate reader (Thermo Fisher Scientific). L-Ascorbic (Sigma Aldrich, South Africa) was used as a standard with concentrations varying between 0 and 1000 mM. Further dilutions were done to the samples that were highly concentrated and such dilution factors were recorded and used for calculations of the affected samples. Epigallocatechnin gallate was used as antioxidant positive control. The results were expressed as mM ascorbic acid equivalents per milligram dry weight (mM AAE/g) of the test samples.

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5.2.9.2. Total phenolic content

The evaluation of total amount of phenolic compounds in CME and NHE was done with slight modifications to method by Salar *et al.*, (2012). Following prescribed protocols, plates containing the extracts were read at 593 nm and the results were expressed as gallic acid equivalent (GAE).

5.2.9.3. Trolox equivalent absorbance capacity (TEAC) assay

This assay was done by adopting a combination of two previously described protocols (Re *et al.*, 1999; Arts *et al.*, 2004).

5.2.10. Statistical analysis

Data generated from this study was analysed using the Graph-Pad Prism 6 statistical package and expressed as means ± standard error of means (SEM) of three independent experiments performed in quadruplicate wells. One-way analysis of variance (ANOVA) was used to determine levels of significance and data set with p value less than 0.05 was considered significant when MPP+-treated cells were compared to either the extract-treated or control cells.

5.3. Results

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5.3.1. Confirmation of MPP+-induced toxicity in SH-SY5Y cells

The MPP+ toxin is widely used to induce neurotoxicity in in vitro PD models. To evaluate the concentration of MPP+ that reduced cell viability significantly to about 50%, cells were seeded in 96-well plates and exposed to increasing concentrations of MPP+ (500 - 2500 mM) and MTT assays were performed. Fig. 22 shows that MPP+-treatment induced a dose-dependent reduction of cell viability in SH-SY5Y cells when compared to the control. The 2000 mM MPP+ concentration that reduced cell viability to approximately 45% was selected as the concentration of choice for further investigation.

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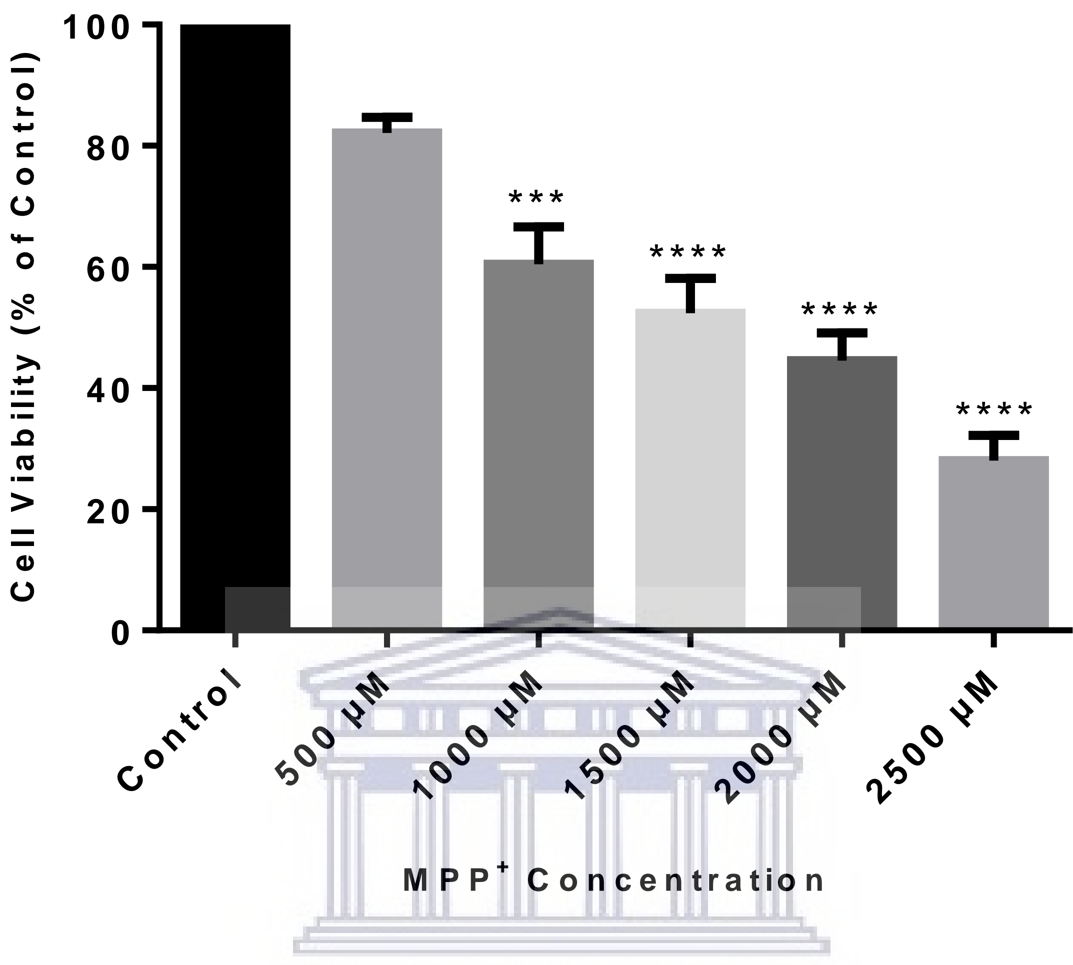

Figure 22: MPP+ induced cytotoxicity in SH-SY5Y cells. Cells were exposed to increasing concentrations of MPP+ (500mM- 2500 mM) and allowed to incubate for 24 h. MTT assays were performed and results showed a concentration-dependent decrease in cell viability when compared to control. Bars represent means ± SEM of three independent experiments and significance of difference indicated with * (p<0.001), and **** (p<0.0001)**

5.3.2. SH-SY5Y viability following exposure to CME and NHE.

To determine whether CME and NHE will cause toxicity, SH-SY5Y cells were seeded in 96-well plates and thereafter treated with 2, 4, 6, 8 and 10 mg/mL of the extracts. Results showed that the CHE did not induce any significant cytotoxicity in SH-SY5Y cells at all concentrations. The slight increase in cell viability observed following treatment with the 2 and 4 mg/mL concentrations was not significant (Fig. 23A). On the other hand, SH-SY5Y cells treated with NHE showed no significant reduction in cell viability at the 2, 4 and 6 mg/mL concentrations but at 8 and 10 mg/mL concentrations, cell viability was significantly inhibited (Fig. 23B). However, to maintain consistency, the 2 and 4 mg/mL concentrations of both CME and NHE were selected for the neuroprotection studies.

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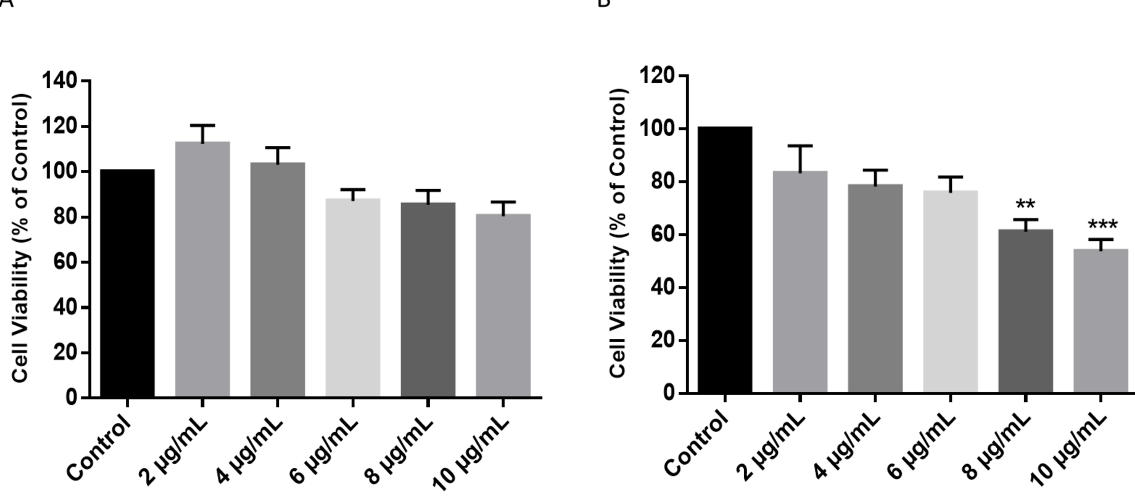

Figure 23: Cytotoxicity of CME and NHE on SH-SY5Y cells. SH-SY5Y cells were exposed to increasing concentrations (2 mg/ml - 10 mg/ml) of CME (A) and NHE (B) for 24 h incubation and MTT assays were performed and treated cells were expressed as percentage of control. Bars represent means ± SEM of three independent experiments and significance of difference indicated with ** ($p<0.01$) and * ($p<0.001$)**

5.3.3. CME and NHE protect SH-SY5Y cells from MPP+-induced cytotoxicity

To determine the effects of NHE and CME on MPP+-induced cytotoxicity (2000 mM), SH-SY5Y cells were pre-treated with 2 and 4 mg/mL concentrations of both plant extracts for 2 h before the addition of MPP+ for 24 h. Fig. 24 shows that at all concentrations tested, the plant extracts significantly improved the viability of SH-SY5Y cells exposed to MPP+ when compared to the control. Cell viability for the MPP+-treated cells reduced to 45% while that of CME-pre-treated cells was 71.8% and 77.5% for 2 and 4 mg/mL concentrations respectively (Fig. 24A). Similarly, the NHE-pre-treated cells also showed 74.8% and 89.8% viability for the 2 and 4 mg/mL concentrations respectively compared to the 45% obtained in the MPP+-treated cells (Fig. 24B).

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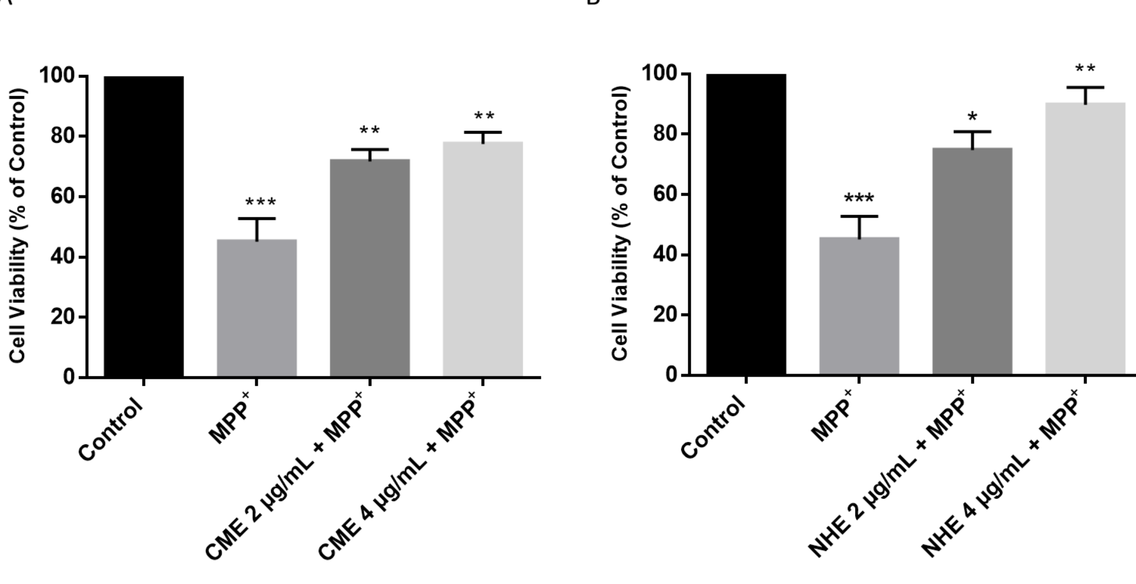

Figure 24: CME and NHE induced protection in SH-SY5Y cells. To investigate the protective activity of CME and NHE plant extracts, cells were pre-treated with the plant extracts before being exposed to MPP+ followed by a 24 h incubation. MTT assay results show that whereas treatment with MPP+ significantly decreased cell viability, pre-treatment with CME (A) and NHE (B) significantly improved cell viability. Bars represent means ± SEM of three independent experiments and significance of difference indicated with * ($p<0.05$), **($p<0.01$) and * ($p<0.001$).**

Taken together, these results show that both plant extracts could mitigate the MPP+-induced toxicity in the SH-SY5Y cells.

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5.3.4. CME and NHE inhibit ATP degeneration in SH-SY5Y MPP+- induced toxicity.

A mechanism of MPP+-induced neuronal cell death is the depletion of ATP in the cells caused by the inhibition of mitochondrial complex I (Meredith and Rademacher, 2011). To investigate whether CME and NHE could attenuate ATP depletion in MPP+-treated SH-SY5Y cells, the SH-SY5Y cells were pre-treated with 2 and 4 mg/mL concentrations of the plant extracts and later exposed to a 2000 mM concentration of MPP+. Results obtained showed that MPP+ treatment significantly reduced cellular ATP levels while pre-treatment with the extracts appeared to prevent such reduction when compared to control. In specifics, cellular ATP levels stood at 39.7% in the MPP+- treated cells while pre-treatment with CME at both 2 and 4 mg/mL increased ATP levels to 66.8 and 72.8% respectively (Fig. 25A). Similar results were obtained for the NHE-treated cells which had ATP levels of 74.8 and 80.2% at both 2 and 4 mg/mL concentrations respectively compared to the MPP+ treated cells (Fig. 25B). Taken together, these results show that the inhibition of ATP depletion by CME and NHE following MPP+ toxicity is a possible neuroprotection mechanism.

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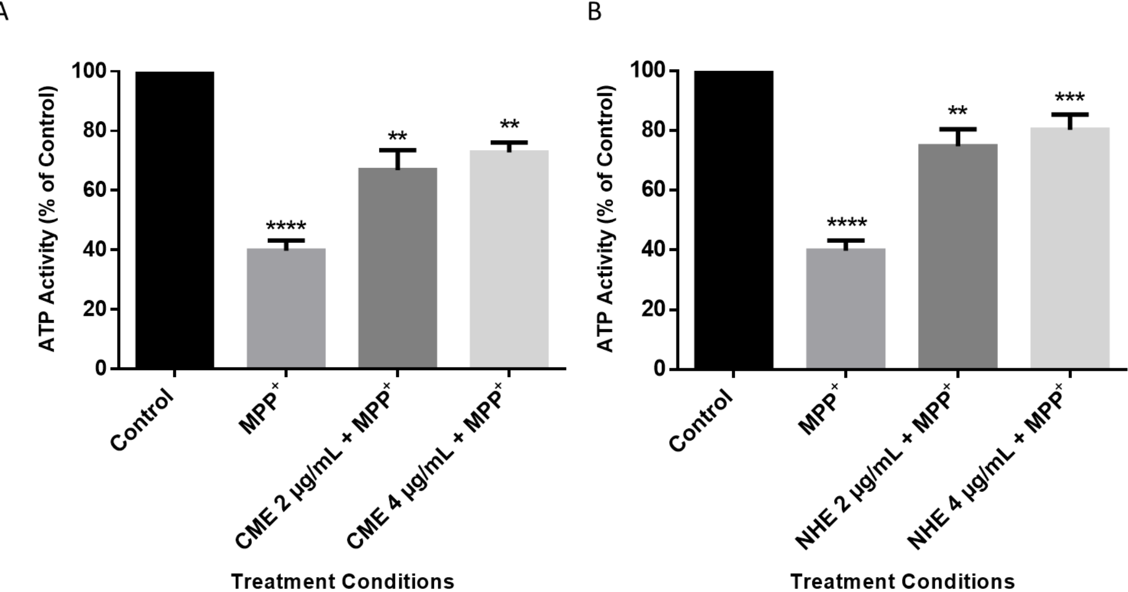

Figure 25: Effect of the extracts on MPP+-induced depletion of ATP. SH-SY5Y cells pretreated with 2 and 4 mg/mL of CME (A) and NHE (B) and exposed to 2000 mM MPP+ for 24 h and Mitochondrial ToxGlo ATP assay was used to determine ATP levels. Treated cells were expressed as percentage of the control and level of significance was determined by comparing extract treated cells to the MPP+ group. Bars represent means ± SEM of three independent experiments and significance of difference indicated with ** (p<0.01), * (p<0.001) and **** (p<0.0001)**

5.3.5. CME and NHE protect cells from MPP+- induced apoptosis

Programmed cell death (PCD) in the form of apoptosis has been shown to be activated following MPP+-induced toxicity in SH-SY5Y cells (Jovanovic-Tucovic *et al.*, 2019; Yuan *et al.*, 2019). To investigate apoptosis in cells pre-treated with CME and NHE before exposure to MPP+, caspase 3/7 activity was measured. Caspases are critical mediators of apoptosis and caspase 3 and caspase 7 are known as effector or executioner caspases as they cleave a number of cellular structural and repair proteins (Slee *et al.*, 2001; Yuan *et al.*, 2016). Results show that at all concentrations tested, pre-treatment with CME and NHE mitigated MPP+-induced apoptosis in SH-SY5Y cells (Fig. 26).

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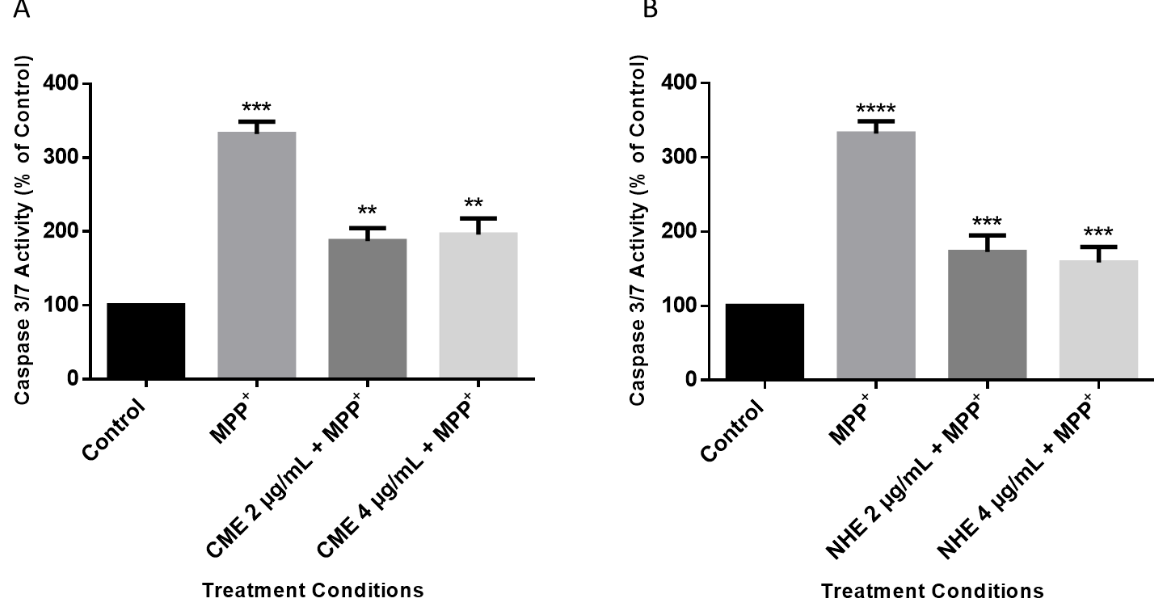

Figure 26: Effect of the extracts on MPP+-induced apoptosis. Caspase 3/7 activity was measured in cells pretreated with CME (A) and NHE (B) prior to exposure to MPP+. Results were expressed as percentage of control and level of significance was determined by comparing extract treated cells to the MPP+ group. Bars represent means ± SEM of three independent experiments and significance of difference indicated with ** (p<0.01), * (p<0.001) and **** (p<0.0001).**

In particular, while caspase 3/7 activity in the control cells was set at 100%, MPP+-treated cells had 332% caspase 3/7 activity and the CME pretreated cells had reduced caspase 3/7 activity of 187 and 195.9% respectively for the 2 and 4 mg/mL concentrations. Similarly, the cells treated with NHE also had reduced caspase 3/7 activity with values at 172.9 and 158.5% respectively for the 2 and 4 mg/mL concentrations. Together, these results indicate that both the CME and NHE protect SH-SY5Y neuroblastoma cells from MPP+-induced apoptosis.

5.3.6. CME and NHE inhibit morphological changes induced by MPP+

In order to visualize morphological changes in SH-SY5Y cells following the respective treatments, cells were processed as stated above for neuroprotection experiments and morphology was visualised using the light microscope. Images obtained showed that treatment with MPP+ only, induced significant changes in cell morphology reminiscent of a cell undergoing programmed death, compared to the control. Some of the observed changes include cell shrinkage as well as loss of neuronal projections. However, following pre-treatment with 2 and 4 mg/mL concentrations of CME and NHE, an improvement of cell morphology was evident (Fig. 27). These findings suggest that CME and NHE protects SH-SY5Y cells from deleterious morphological changes induced by MPP+.

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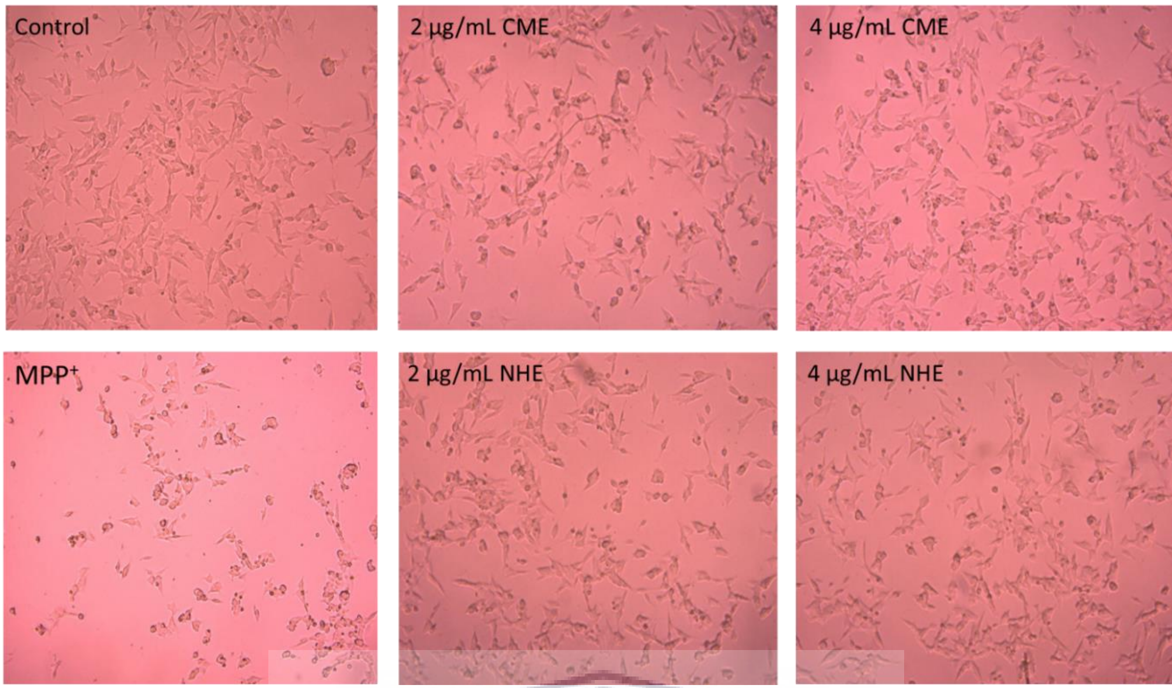

Figure 27: Effect of the extracts on MPP+-induced changes in cell morphology. Cellular morphology of SH-SY5Y cells pre-treated with CME and NHE (2 and 4mg/mL) prior to exposure to 2000 mM MPP+ for 24 h and images were captured using the light microscope at 100X magnification.

5.3.7. Antioxidant activity

The antioxidant activity of the CME and NHE was determined by measuring their ferric-ion reducing antioxidant power (FRAP), and trolox equivalent absorbance capacity (TEAC). The TEAC and FRAP are based on a single electron transfer (SET) in which the antioxidant transfers an electron to the corresponding cationic radical to neutralize it mechanism (Perez-Fons *et al.*, 2010). The bulbs extracts showed low phenolic content (0.027; ~0.060 mg/g for CME and NHE respectively) when compared with *Aspalathus linearis* (60.7 mg/g) (Bhebhe *et al.*, 2015). The results (Table 6) showed weak antioxidant activity for both plant extracts, and this was evident by the low phenolic contents in each of the plant extracts.

Table 6: Antioxidant status of the extracts

Sample	Polyphenols (mg GAE/g)	FRAP (mmol AAE/g)	TEAC (mmol TE/g)
Clivia miniata	27.54 ± 1.20	16.87 ± 1.06	234.3 ± 4.67
Nerine humilis	59.98 ± 2.99	53.69 ± 2.24	338.70 ± 0,03
EGCG*		7525.0 ± 4.9	4146.4 ± 19.8

* Epigallocatechnin gallate (antioxidant positive control)

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5.4. Discussion

The first ever publication on PD (initially called the shaking palsy and later named after the author) marked its 200th anniversary in 2017 (Obeso *et al.*, 2017; McDonald *et al.*, 2018). Many years later, pharmacological treatment for this neurodegenerative disease is still far-fetched and only palliative treatment for its many symptoms is known. The only approved treatment options in current use for PD include surgical approaches such as deep brain stimulation to relieve motor symptoms as well as the FDA-approved drug levodopa known to cause many undesired outcomes (Lee *et al.*, 2018). Medicinal plants and natural products have shown promising activities for the treatment of PD and in the present study we show for the first time that CME and NHE protect SH-SY5Y cells from MPP+-induced neuronal toxicity. Our findings show that exposure of cells to MPP+ for 24 h induced a reduction in viability (Fig. 22) while treatment with the CME and NHE had no significant effects on cell viability (Fig. 23). Furthermore, pre-treatment of cells with CME and NHE at 2 and 4 mg/mL concentrations significantly protected SH-SY5Y cells from the deleterious effects of MPP+ while NHE at 4 mg/mL appears to offer the best neuroprotection (Fig. 24). This provides some evidence that both extracts used in this study, and possibly plants in the family Amaryllidaceae, have the potential of preventing or slowing down the effects of dopaminergic neuron degeneration in the substantia nigra pars compacta of the midbrain which is a main cause of PD. A previous study revealed that plants of the family Amaryllidaceae are receiving attention for their possible neuroprotective properties (Nair and van Staden, 2013). Our laboratory recently reported on the neuroprotective potential of *Crossyne guttata*, a member of the family Amaryllidaceae in an in vitro PD model (Omoruyi *et al.*, 2019).

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Decreased levels of ATP have been implicated in the pathogenesis of PD (Meredith and Rademacher, 2011). The toxicity of MPP+ has been reported to have adverse effects on mitochondria by disrupting mitochondrial respiration which eventually results in the decline of the mitochondrial DNA content as well as ATP production (Kim and Park, 2018; Kim *et al.*, 2018). Findings from the present study (Fig. 25) show that both CME and NHE increased the ATP levels which suggests their potential as a treatment agent for PD. Our findings corroborate those from a previous study which indicated that *Boophone disticha*, a member of the family Amaryllidaceae, protected SH-SY5Y cells from 6-hydroxydopamine-induced toxicity by inhibiting ATP degeneration (Lepule *et al.*, 2019). Apoptosis is reported to play an integral role in the loss of neurons in PD as demonstrated by the presence of DNA fragmentation, elevated levels of caspases and apoptotic chromatin changes in dopaminergic neurons of PD patients in post-mortem studies (Tompkins *et al.*, 1997; Hartmann *et al.*, 2000; Mogi *et al.*, 2000). The intrinsic mitochondrial apoptotic pathway appears to be predominant in PD and this is characterised by the release of cytochrome C

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which stimulates the activation of caspases which eventually leads to cell death (Mattson, 2006; Fernandez-Moriano *et al.*, 2015). It is established that MPP+-induced apoptosis in neuroblastoma cells leads to the activation of the executioner caspases 3/7 and these are considered neuronal markers in PD (Jellinger, 2000). From our findings (Fig. 26 and 27), the MPP+ on its own increased the activity of the caspase 3/7 as well as induced changes in cell morphology reminiscent of cells undergoing apoptosis. Furthermore, pre-treatment with CME and NHE showed a reduction in the elevated levels of caspase 3/7 activity caused by MPP+ treatment as well as improved cell morphology, thus validating the anti-apoptotic properties of CME and NHE in a neuronal cell model. This anti-apoptotic activity is of importance as the ability to halt the progression of apoptosis is critical for the acceptability of potential neuroprotective agents (Perier *et al.*, 2012).

The wide spectrum of biological activities of different members of the Amaryllidaceae family is generally believed to be attributed to their alkaloidal contents. As mentioned earlier, more than 53 alkaloidal compounds were reported from the genus *Nerine*, some of which showed interesting biological activities like cholinesterase inhibition (Cahlíkova *et al.*, 2019). In support of this, the alkaloidal contents of plants have been reported to exhibit neuroprotection in various cellular and animal models of neurodegenerative disease (Wang *et al.*, 2010; Bi *et al.*, 2015; Xi *et al.*, 2019). Additionally, more than 15 alkaloidal compounds have been identified from *C. miniate* (Viladomat *et al.*, 1997; Crouch *et al.*, 2003). These compounds may have contributed directly or indirectly to the bioactivity of the plant extracts as observed in this study.

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In conclusion, this study investigated the neuroprotective activity of CME and NHE in an in vitro PD model using SH-SY5Y neuroblastoma cells. These extracts demonstrated neuroprotection against MPP+-induced toxicity in these cells as seen in their ability to maintain ATP production and attenuate apoptosis in the SH-SY5Y cells. In addition, morphological analysis corroborates the neuroprotective effects induced by the extracts. All the above findings tend to suggest that CME and NHE could slow down the progression of neuronal damage. Although the data from this study appears promising, further studies are suggested to elaborate the mechanisms of action as well as elucidate the bioactive compounds responsible for these activities.

Declaration of Competing Interest

The authors declare no conflict of interest.

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CHAPTER SIX

Cytotoxic activities of selected plants of the family Amaryllidaceae on brain tumour cell lines

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Abstract

Malignant primary brain tumours are reported to be the leading cause of death from solid tumours in children and the third leading cause of death from cancer in adolescents and adults. Current treatment options include surgery, radiation and chemotherapy. Despite these treatment options, patient survival still remains poor. The Amaryllidaceae family contain alkaloids which have shown several biological activities including the treatment of Alzheimer's disease. This study investigates the cytotoxic activity of the methanolic extracts of fourteen plants belonging to the Amaryllidaceae family in brain tumour cell lines. The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay was used to determine the effects of plant extracts on cell viability while routine antioxidant assays were conducted to determine the antioxidant activities of the extracts. Results showed that of the fourteen extracts, five (*Cyrtanthus breviflorus, Amaryllis belladonna, Crinum variable, Haemanthus pubescens, Nerine filifolia*) showed cytotoxicity in all the cell lines tested with IC_{50} values under 100 µg/mL. Six extracts (*Crinum moorei, Clivia miniata, Haemanthus amarylloides, Crossyne guttata, Nerine humilis,* and *Ammocharis longifolia*) showed varying levels of cytotoxicity in the cell lines tested and were unable to induce 50% reduction in cell viability across the lines tested at the highest concentration of 100 µg/mL. Furthermore, three plant extracts (*Brunsvigia bosmaniae, Boophone disticha, Strumaria truncata*) had minimal or no cytotoxic effects on all cell lines tested when compared to control. The extracts also showed varying degree of antioxidants but were not as potent as the positive control. Findings from this study suggest that species of the Amaryllidaceae family may be useful in the treatment of central nervous system cancers and should be further explored in animal models of central nervous system (CNS) and other cancer types.

Keywords: Amaryllidaceae, Cell viability, Cytotoxicity, Glioblastoma, Neuroblastoma

***Abbreviations*:**

CNS, central nervous system;

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FDA, United States Food and Drug Administration;

AChE, acetylcholinesterase;

MTT, 3-(4,5-dimethyithiazol-2-yl)-2,5-diphenyltetrazolium bromide;

NADPH, Nicotinamide adenine dinucleotide phosphate;

FRAP, ferric-ion reducing antioxidant power;

TEAC, trolox equivalent absorbance capacity;

AAE, Ascorbic acid equivalents;

GAE, Gallic acid equivalents;

EGCG, Epigallocatechin gallate.

6.1 Introduction

The central nervous system (CNS) which is one of the divisions of the nervous system consists of the brain and the spinal cord and has a complex framework that is susceptible to approximately 130 primary neoplasms (Ogun *et al.*, 2016). Malignant primary brain tumours are reported to be the leading cause of death of all solid tumours in children and are the third leading cause of cancer-related deaths in adolescents and adults aged 15 to 34 years (Buckner *et al.*, 2007; Fernandez and Loddenkemper, 2017). These tumours range from the very uncommon, non-invasive and surgically curable tumours such as pilocytic astrocytomas to the highly invasive, virtually incurable and most common adult intraparenchymal tumours such as the glioblastoma multiforme (Nabors *et al.*, 2013). Another notable primary CNS tumour is the neuroblastoma (NB) which is a rare intracranial tumour affecting children mainly in the early years of life and is one of the leading causes of cancer-associated death in children (Mishra *et al.*, 2018). The NB tumours can also be extracranial and this type is the most common extracranial solid malignant tumours in infants and children arising from primitive sympathetic ganglion cells and may present as an adrenal mass or more commonly with localised or metastatic disease (Fisher and Tweddle, 2012). NB accounts for 15% of all cancer related deaths in children and the survival rate is less than 50% (Pugh *et al.*, 2013). Malignant brain tumours such as the Glioblastoma multiforme (GBM) originate from the glial cells of the brain and are known to have a poor prognosis with a 5-year survival rate of about 5‑6% (Carlsson *et al.*, 2014). As much as it can occur in all ages, the peak age of glioblastoma incidence is 55‑60 years with an incidence ratio of 1.6 times higher in men compared to women (Ostrom *et al.*, 2013; Thakkar *et al.*, 2014). Due to the invasive nature of glioblastoma, the surgical approach of treatment is nearly impossible and mostly unsuccessful. Thus, less-invasive treatment is often preferred, leading to the discovery and development of new treatment approaches which target the tumour without affecting non-cancerous cells (Aquino *et al.*, 2014). Treatment of CNS tumours (GBM and

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NB) involve a combination of surgery, radiation and chemotherapy depending on the extent of malignancy (Merchant *et al.*, 2010). Although some currently available chemotherapeutic agents are designed to target the DNA of cancerous cells to significantly lower the burden of these cancers, most have been associated with many side-effects including chronic neurocognitive and endocrine deficits in children (Mulhern *et al.*, 2004; Laughton *et al.*, 2008). The presence of the blood-brain barrier (BBB) which is a selectively permeable barrier also makes treatment difficult as some cytotoxic agents do not cross the barrier (Bhowmik *et al.*, 2015; Weidle *et al.*, 2015). Thus, there is the need to explore more therapeutic options capable of suppressing the growth of cancerous cells with minimal effects on normal cells. Research has shown that herbal medicine and natural products have contributed significantly to the development of new drugs for the treatment and management of various maladies (Thomford *et al.*, 2018; Otvos *et al.*, 2019). In support of this, about 80% of all drugs approved by the United States Food and Drug Administration (FDA) in the last thirty years for cancer therapy are either natural products or derived from natural products (Bishayee and Sethi, 2016). Plants from the family Amaryllidaceae have long been known for their medicinal properties (Fennell and van Staden, 2001). This angiosperm family consists mainly of bulbous perennial or biennial plants, which are largely found in the tropical and the warm parts of the temperate regions of the world. Worldwide, the Amaryllidaceae consist of about 800 species classified in 60 genera. In Africa, they are abundantly found in southern Africa with 266 known species in 20 genera concentrated in the Western Cape and along the West Coast through Namaqualand into southern Namibia (Koekemoer *et al.*, 2013). It is therefore not surprising that the indigenous people of the region such as the Khoi, San, Sotho, Tswana, Xhosa, and Zulu explore plants of the Amaryllidaceae for medicinal use. Documented uses include the treatment of ailments such as stomach pain, skin diseases, headaches, dizziness, wounds, snake bites, infertility among others (Hutchings, 1996). Furthermore, plants of the Amaryllidaceae family have also been reported to be used traditionally for the treatment of cancer in regions where the plant is endemic including South Africa. Notable among such plants is the *Narcissus species, Boophone disticha* and *Amaryllis belladonna* which is included in this study (Pettit *et al.*, 1984; Botha *et al.*, 2005; Kornienko and Evidente, 2008). Chemically, the Amaryllidaceae has gained prominence for their alkaloidal content called Amaryllidaceae alkaloids. The Amaryllidaceae alkaloids constitute an important group of naturally occurring bases possessing a diversity of functionality and structure (Martin, 1987). These alkaloids have been reported to possess diverse biological properties including anti-viral, anti-hypertensive, neuroprotective, and anti-cancer, activities (Gabrielsen *et al.*, 1992; Schmeda-Hirschmann *et al.*, 2000; Shawky, 2017; Pellegrino *et al.*, 2018; Cole *et al.*, 2019). Importantly, the approval of galanthamine by the United States FDA for the treatment of Alzheimer's disease due to its acetyl cholinesterase (AChE) inhibitory activity, suggest that alkaloids from the plant family can cross the blood-

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brain barrier which poses a threat to the treatment of brain tumours (Maelicke *et al.*, 2001). Considering this, in the present study, we aim to characterise the cytotoxic activities of fourteen species of the Amaryllidaceae plant family in the glioblastoma cell lines U251 and U87 as well as the SH-SY5Y neuroblastoma cell line.

6.2 Materials and methods

6.2.1. Identification and collection of plant material

Plants used in this study were cultivated and sourced from the Karoo Desert National Botanical Garden in Worcester Western Cape Province, except for *Clivia miniata*, which was obtained from a private garden. The identities of all species were authenticated by one of the co-authors (CNC) and voucher specimens were deposited in the Giffen Herbarium (UFH) of the University of Fort Hare. The list of study species along with their accession numbers are given in Table 7.

Table 7: Study species and herbarium accession numbers.

	Plant name	**Accession number**
1	*Crinum moorei* Hook.f.	UFH 2020–4–07
2	*Brunsvigia bosmaniae* F.M.Leight.	UFH 2020–4–09
3	*Cyrtanthus breviflorus* Harv.	UFH 2020–4–10
4	*Clivia miniata* (Lindl.) Bosse	UFH 2020–4–02
5	*Amaryllis belladonna* L.	UFH 2020–4–11
6	*Crinum variable* Hochst. ex A.Rich.	UFH 2020–4–08
7	*Strumaria truncata* Jacq.	UFH 2020–4–12
8	*Crossyne guttata* (L.) D.Mull.-Doblies and U.Mull.-Doblies	UFH 2020–4–13
9	*Haemanthus amarylloides* Jacq.	UFH 2020–4–06
10	*Boophone disticha* (L.f.) Herb.	UFH 2020–4–14
11	*Nerine humilis* (Jacq.) Herb.	UFH 2020–4–01
12	*Nerine filifolia* Baker	UFH 2020–4–03
13	*Ammocharis longifolia* (L.) Herb.	UFH 2020–4–04
14	*Haemanthus pubescens* L.f.	UFH 2020–4–05

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6.2.2. Preparation of plant material

Approximately 200 g of each of the fourteen fresh plant material (*C. breviflorus, A. belladonna, C. variable, H. pubescens, N. filifolia, C. moorei, C. miniata, H. amarylloides, C. guttata, N. humilis, A. longifolia, B. bosmaniae, B. disticha and S. truncata*) were homogenised separately with methanol and extracted for two days at room temperature and after filtration, the extracts were evaporated under reduced pressure at 45 °C. The solvent-free methanolic extracts were kept at 4 °C till further use.

6.2.3. Cell culture and maintenance and treatments

The U251 and U87 human glioblastoma cells (World Health Organisation Stage IV) and the human neuroblastoma SH-SY5Y 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 with a medium change every three days. Cells were sub-cultured when they attained 70‑80% confluency using a solution of 0.25% trypsin EDTA (Lonza Group Ltd., Verviers, Belgium) prior to experiments. For cell treatment, a stock solution of 40 mg/mL of each plant extract was made in Dimethyl sulfoxide (DMSO, Sigma Aldrich, St Louis, USA) and diluted in medium to concentration of 1 mg/mL from which final concentrations of 25, 50, 75 and 100 μg/mL were made by further dilution in cell growth medium. As a positive control, Doxorubicin (Dox, Sigma Aldrich, St Louis, USA) was made in sterile water and exposed to cells with similar to cells in similar concentrations as extracts.

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6.2.4. Cytotoxicity evaluation of plant extracts

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used in this study to determine the cytotoxicity of plant extracts. This assay measures the ability of viable cells which contain Nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidoreductase enzymes, to reduce the MTT reagent to formazan (Van Meerloo *et al.*, 2011). Yellow MTT salt solution is reduced to purple formazan in the mitochondria of living cells (Van Meerloo *et al.*, 2011). The cells were seeded in a 96-well plate at an optimum established density (SH-SY5Y 10,000 cells/well; U87 and U2515000 cells/well) in 100 μL of culture medium, and then left to attach overnight. After attachment, the cells were treated for 24 h by replacing the culture medium with 25, 50, 75 and 100 μg/mL concentrations of the different plant extracts or Dox and the vehicle treated cells (cells treated with the same amount of DMSO in the highest concentration of the extract) or untreated cells (cells treated with medium only in the case of Dox) served as control. After

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treatment, 10 μL of the MTT solution (Sigma-Aldrich, USA) was added to each well and incubated for 4 hours before being read at 570 nm with the BMG Labtech multi-well plate reader. Thereafter absorbance readings obtained were expressed as percentages of the control and the maximal inhibitory concentration required to kill 50% of the cells (IC50) was calculated from sigmoidal plots on GraphPad Prism 6.

6.2.5. Cell morphology

To investigate morphological changes, cells were plated in 96 well plates and allowed to attach overnight before exposure to the IC50 of the extracts for 24 h. Thereafter, cellular morphology was investigated using the Zeiss inverted light microscope with 10X objective lens. Images were captured using the Zeiss software version 2.3.

6.2.6. Antioxidant assays

6.2.6.1. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was done as previously described with a slight modification (Benzie and Strain, 1999). Briefly, 10 μL of the stock solution (1 mg/mL w/v) of the total extracts were mixed with 300 μL FRAP reagent (10:1:1, v/v/v of acetate buffer at 300 mM, pH 3.6, tripyridyl triazine (TPTZ) at 10 mM in 40 mM HCl, and $FeCl_3.6H_2O$ at 20 mM) in a 96-well plate and incubated for 30 min at room temperature. Thereafter, absorbance was measured at 593 nm and the results were expressed as micromole ascorbic acid equivalents per gram of dry weight (μM AAE/g DW) of the samples.

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6.2.6.2. Total phenolic content

The evaluation of the total amount of phenolics was done with slight modifications to method by Salar *et al*., (2012). Following prescribed protocols, plates containing the extracts were read at 593 nm and the results expressed as gallic acid equivalents (GAE).

6.2.6.3. Trolox equivalent absorbance capacity (TEAC) assay

This assay was done by adopting a combination of two previously described protocols (Re *et al*., 1999; Arts *et al*., 2004). Statistical analysis Data generated from this study was analysed using the GraphPad Prism 6 statistical package and expressed as means ± standard error of means (SEM) of three independent experiments.

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6.3. Results

6.3.1. Cytotoxicity of extracts

The MTT cytotoxicity assay was used to establish the effect of the plant extracts on the viability of U251, U87 glioblastoma cells and the SH-SY5Y neuroblastoma cells. Cells were seeded in 96 well plates and treated with concentrations of plant extracts ranging from 25, 50, 75 and 100 μg/mL for 24 h. Results from the MTT assays performed showed that extracts of the Amaryllidaceae family differentially induced a reduction in cell viability in the cells at the concentrations tested. Notably, of the fourteen (14) extracts tested, five (*C. breviflorus, A. belladonna, C. variable, H. pubescens and N. filifolia*) and the positive control (Dox) showed cytotoxicity in all the cell lines tested with IC50 values under 100 μg/mL (Fig. 28).

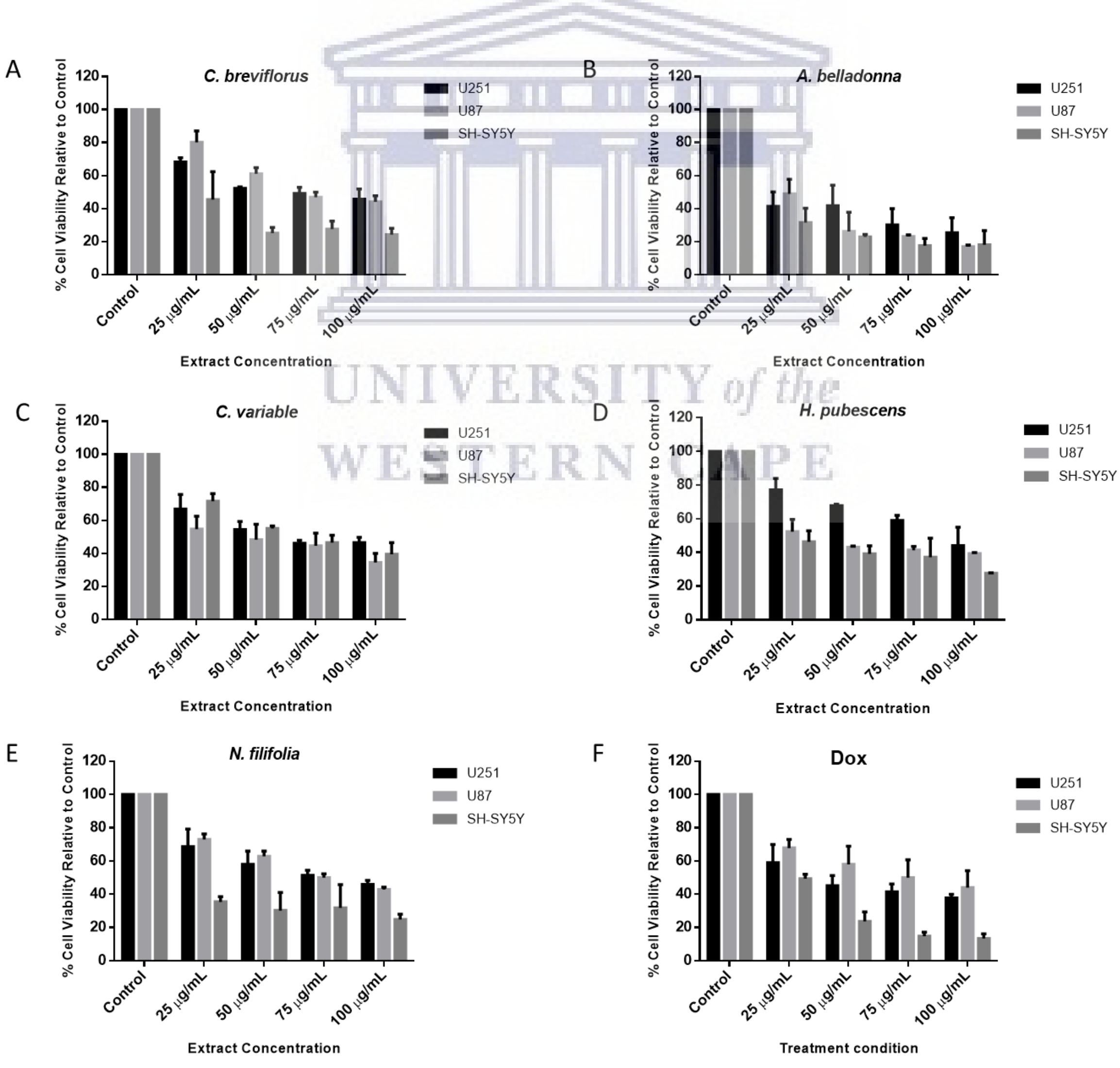

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Figure 28: Methanolic extracts of *C. breviflorus* (A) *A. belladonna* (B) *C. variable* (C), *H. pubescens* (D), *N. filifolia* (E) and Dox (F) induced concentration dependent cytotoxicity in U251, U87 and SH-SY5Y cells.

Additionally, six extracts (*C. moorei, C. miniata, H. amarylloides, C. guttata, N.e humilis,* and *A. longifolia*) showed varying levels of cytotoxicity in the cell lines tested and were unable to induce 50% reduction in cell viability across the lines tested at the highest concentration of 100 μg/mL (Fig. 29).

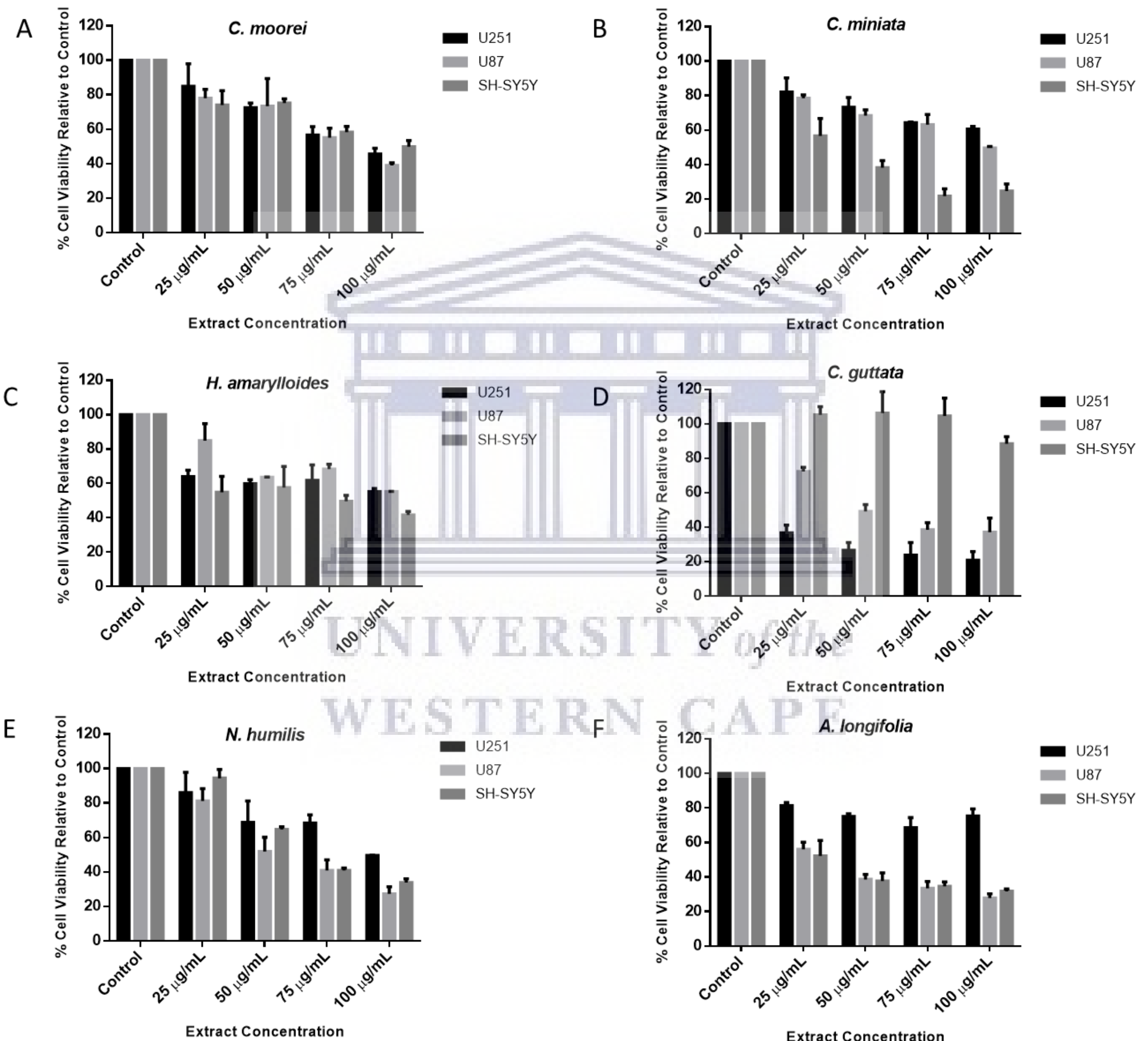

Figure 29: Effect of methanolic extract of *C. moorei* (A), *C. miniata* (B), *H. amarylloides* (C), *C. guttata* (D), *N. humilis* (E), and *A. longifolia* (F) on U251, U87 and SH-SY5Y cell lines.

Furthermore, three plant extracts (*B. bosmaniae, B. disticha* and *S. truncata*) had no marked effects in the cell viability of all cell lines tested (Fig. 30).

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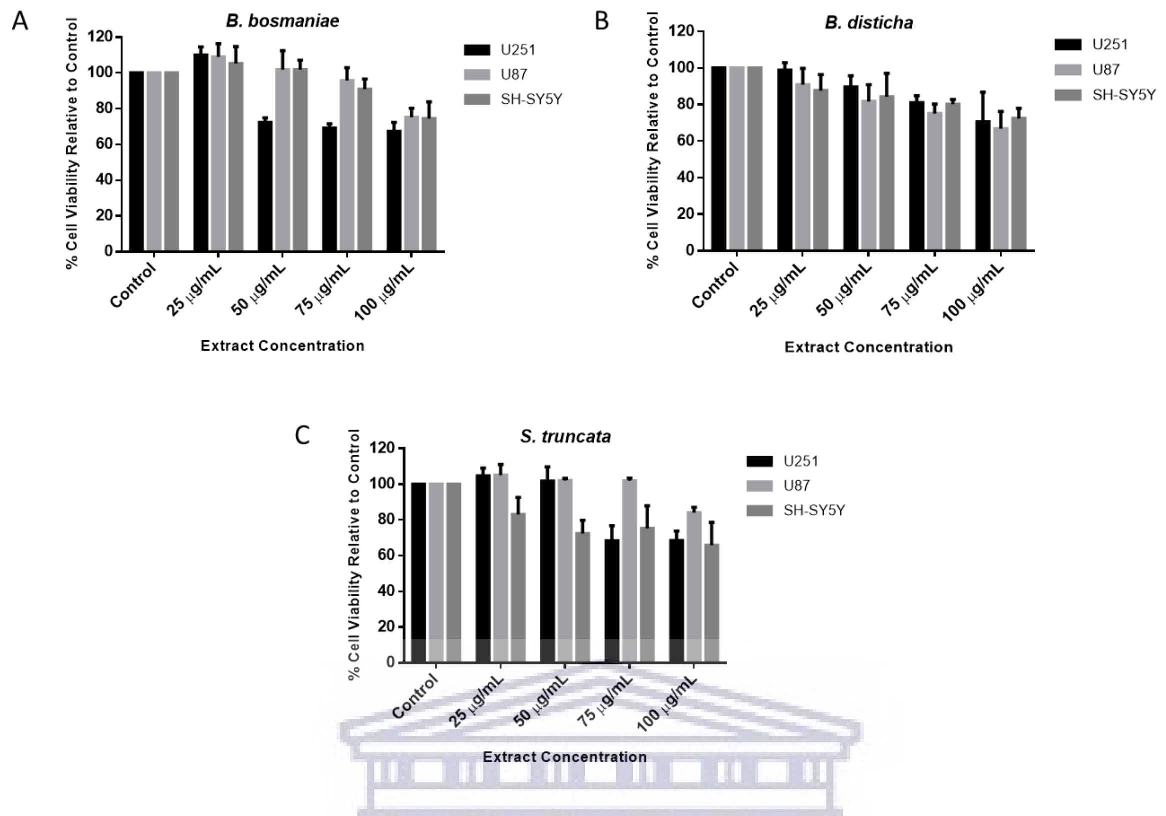

Figure 30: Effect of methanolic extract of *B. bosmaniae* (A), *B. disticha* (B), and *S. truncata* (C) on the cell viability of U251, U87 and SH-SY5Y cell lines as obtained from MTT assays

Taken together, the plant extracts of the Amaryllidaceae family showed differential effects on the cell viability of the U251 and U87 glioblastoma cells as well as the SH-SY5Y neuroblastoma cells. Importantly, some of the extracts, had lower IC_{50} values when compared to the Dox as shown in Table 8.

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Table 8: Summary of IC_{50} values

S/N	Plant extract	IC_{50} (µg/mL)		
		U251	U87	SH-SY5Y
1	*C. moorei*	90.51	82.39	>100
2	*B. bosmaniae*	>100	>100	>100
3	*C. breviflorus*	70.56	74.59	16.73
4	*C. miniata*	>100	>100	31.27
5	*A. belladonna*	15.64	23.09	6.65
6	*C. variable*	69.23	39.47	63.88
7	*S. truncata*	>100	>100	>100
8	*C. guttata*	9.369	53.79	>100
9	*H. amarylloides*	>100	>100	61.62
10	*B. disticha*	>100	>100	>100
11	*N. humilis*	>100	56.41	67.90
12	*N. filifolia*	79.99	76.83	25.87
13	*A. longifolia*	>100	32.01	26.73
14	*H. pubescens*	92.03	29.25	20.16
15	Dox (Positive control)	42.18	74.21	24.10

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6.3.2. Morphological evaluation

To further confirm the cytotoxicity induced by extracts, morphological changes in cells following exposure to IC50 values of extracts were observed. The results show a reduction in cell proliferation and varying changes in cell morphology ranging from roundedness in some of the cells to loss of neuronal projections in the U87 and SH-SY5Y cells (Fig. 31). These changes are reminiscent of cells undergoing apoptosis.

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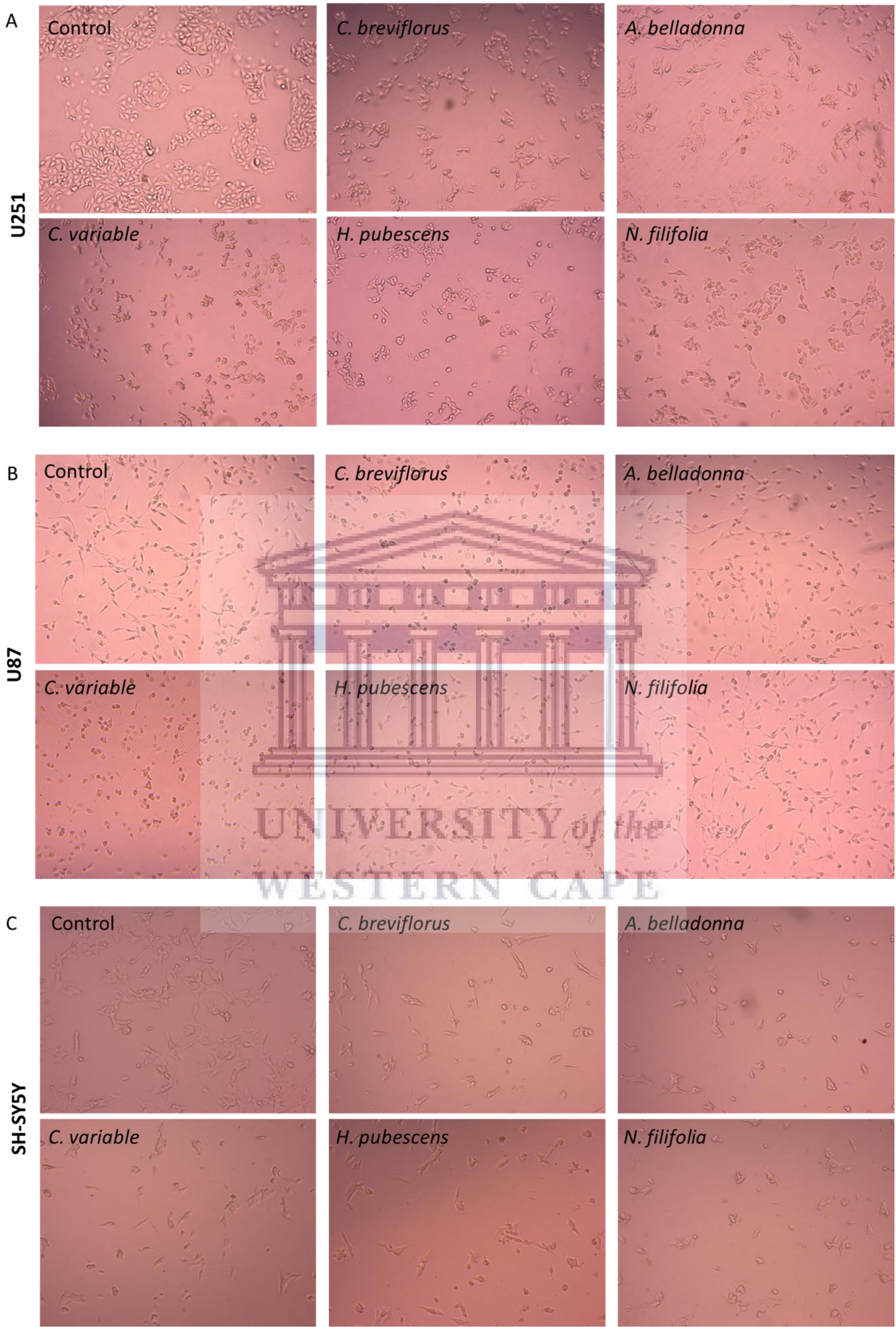

Figure 31: Morphological changes in U251 (A), U87 (B) and SH-SY5Y (C) cells after 24 h exposure to IC_{50} of CBE, ABE, CVE, HPE and NFE. Images were captured using the light microscope at 100X magnification

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6.3.3. Antioxidant activities of extracts

The antioxidant activity of the plant extracts was determined by measuring their ferric-ion reducing antioxidant power (FRAP), and trolox equivalent absorbance capacity (TEAC). Additionally, polyphenolic content was measured and expressed as gallic acid equivalent. The results showed weak antioxidant activity for plant extracts, and this was evident by the low phenolic contents in each of the plant extracts (Table 9).

Table 9: Table showing antioxidant activities of plant extracts

S/N	Sample	Polyphenols (mg GAE/g)	FRAP (μmol AAE/g)	TEAC (μmol TE/g)
1	*C. moorei*	75.71 ± 2.35	51.77 ± 2.14	416.9 ± 6.28
2	*B. bosmaniae*	4.85 ± 0.73	1.36 ± 0.07	78.12 ± 4.04
3	*C. breviflorus*	19.88 ± 1.36	4.57 ± 0.26	216.4 ± 3.70
4	*C. miniata*	27.54 ± 1.20	16.87 ± 1.06	234.3 ± 4.67
5	*A. belladonna*	55.65 ± 3.98	38.42 ± 2.55	274.2 ± 9.47
6	*C. variable*	23.15 ± 2.74	9.08 ± 0,43	203.2 ± 7.71
7	*S. truncata*	22.52 ± 1.92	11.56 ± 0.73	203.3 ± 2.80
8	*Crossyne guttata*	38.57 ±3.07	16.47 ± 0.781	322.9 ± 6.83
9	*H. amarylloides*	9.15 ± 0.57	1.32 ± 0.19	99.08 ± 4.96
10	*B. disticha*	11.32 ± 1.59	1.89 ± 0.15	122.0 ± 1.43
11	*N. humilis*	59.98 ± 2.99	53.69 ± 2.24	338.70 ± 0.03
12	*N. filifolia*	20.50 ± 0,81	1.95 ± 0,21	145.2 ± 7.63
13	*A. longifolia*	53.67 ± 2,09	34.58 ± 1,91	358.6 ± 24,71
14	*H. pubescens*	108.3 ± 6,69	98.34 ± 7,94	440.0 ± 0,93
	EGCG*		7525.0 ± 4.9	4146.4 ± 19.8

*Epigallocatechine gallate, positive control

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6.4. Discussion

Plants have been explored for thousands of years and traditionally used by various cultures. It is estimated that to-date, about 70‑95% of developing countries still use herbal medicines (Fridlender *et al*., 2015). The Amaryllidaceae family consists of species that have been known for their medicinal and toxic properties (Ingrassia *et al*., 2008). Their biological activities include anti-inflammatory, anti-microbial, anti-malarial, anti-tumour as well as AChE-inhibiting activities (Breiterova *et al*., 2020). The anticancer properties of many medicinal plants is extensively being explored. With the Amaryllidaceae family's wide range of biological activities, it is not surprising that these plants have been exploited in the history of traditional medicine (Bastida *et al*., 2006). In the present study, fourteen species of the Amaryllidaceae family were screened for their cytotoxic effects on glioblastoma and neuroblastoma cell lines. Findings from this study showed that extracts of the Amaryllidaceae family differentially induced cytotoxicity in the cell lines tested following 24 h' exposure time. It is possible that the observed effects will differ if the exposure time is increased to 48 or 72 h. However, it is worthy to note that the IC_{50} obtained for the *A. belladonna* extract was below 30 μg/mL for all cell lines tested. This finding is of immense interest as the American National Cancer Institute (NCI) guidelines set the 50% inhibition (IC_{50}) limit to less than 30 μg/mL after 72 h for a crude extract to be considered promising for further characterisation (Wang *et al*., 2011; Abdel-Hameed *et al*., 2012). Similar results were also obtained for the *H. pubescens* extract which had an IC_{50} value less than 30 μg/mL for the U87 and SH-SY5Y cells but not for the U251 cells. Thus, both *A. belladonna* and *H. pubescens* will require further mechanistic studies to establish their potential use as anti-cancer agents. Importantly, some of the extracts also showed specificity for particular cancers and this was more pronounced in the cells treated with *C. guttata*, where the U87 and U251 glioblastoma cells were more sensitive and the SH-SY5Y cells showed minimal sensitivity. The Amaryllidaceae have been widely shown to be good sources of alkaloids with unique chemical structures and various biological activities (Kornienko and Evidente, 2008; Cimmino *et al*., 2017). Due to the unique nature of their alkaloid contents, these plants may contribute greatly to the discovery of phytochemical-based drugs. In the present study, we showed that the chemical composition of the extracts with respect to their phenolic components, FRAP as well as TEAC which are quite low when compared to the positive control. These findings exclude the role of phenolics and/or flavonoids for the observed biological activity. Thus, it is therefore plausible to suggest that the alkaloid contents may be responsible for most of the biological activities demonstrated by Amaryllidaceae (Dalecka *et al*., 2013; He *et al*., 2015; Jin, 2016). The alkaloids from this family including lycorine, amarbellisine, haemanthamine, narciclasine and pancratistatin, which have been demonstrated to possess anti-cancer activities through the inhibition of cell proliferation and

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triggering of apoptosis in various cancer cell lines (McLachlan *et al*., 2005; Liu *et al*., 2009; Cedron *et al*., 2015; Habartova *et al*., 2016; Nair *et al*., 2016). Thus, the cytotoxic activities observed in this study could largely be attributed to the chemical contents of the Amaryllidaceae.

In conclusion, our findings on the cytotoxic activities of the fourteen species of Amaryllidaceae investigated tend to support the claim that this plant family could provide new insights into the treatment and management of brain tumours if explored further. In future, we hope to purify the extracts that showed promising activities with the aim of isolating the pure compounds that could be responsible for the observed effects of the extracts and test such extracts/compounds on normal, non-cancerous cells to determine selectivity.

Declaration of Competing Interest

The authors declare no conflict of interest.

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CHAPTER SEVEN

Biological activities of *Crossyne flava* and Amaryllidaceae alkaloids: Implications for Parkinson's disease

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Abstract

Parkinson's disease (PD) is one of the most common neurodegenerative diseases that affects approximately 6.3 million people worldwide and it's characterized by dopaminergic neuronal loss in the substantia nigra pars compacta, part of the mid-brain. Till date treatment for PD remains challenging and available treatment options may be associated with serious side effects, hence the need for new treatment options. The Amaryllidaceae plant family as well as alkaloids from the plant species have been reported to have neuroprotective potentials. This study, therefore, investigates the biological activities of *Crossyne flava* and isolated alkaloids in 1-methyl-4-phenylpyridinium (MPP^+) *in vitro* SH-SY5Y PD model. Four ompounds, pancratinine B (**1**), bufanidrine (**2**), buphanisine (**3**) and epibuphanisine (**4**) were isolated from the plant and their impact on cell viability and neuroprotection alongside the extract was assessed using MTT assays. The effect of compounds and extract on apoptosis and adenosine triphosphate activities were also determined. Results show that both extract and compounds protected SH-SY5Y cells from neurotoxicity induced by MPP^+. Indeed, pre-treatment with extract and compounds inhibited ATP depletion and apoptosis induced by MPP^+ in the cells. Findings from this study show that Amaryllidaceae plant family and their alkaloids may be very useful in the discovery of novel treatments for the treatment of neurodegenerative diseases and this also validates their earlier reported use in traditional settings.

Keywords: Amaryllidaceae; *Crossyne flava*; Alkaloids; Parkinson's disease, 1-methyl-4-phenylpyridinium (MPP^+); Neuroprotection

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7.1 Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease that affects approximately 6.3 million people worldwide and its characterized by dopaminergic neuronal loss in the substantia nigra pars compacta, part of the mid-brain (Ferreira and Romero-Ramos, 2018). Parkinsonism is a motor standpoint for the clinical diagnosis of PD as it encompasses four PD symptoms which are rest tremor, rigidity, bradykinesia and postural instability (Massano and Bhatia, 2012). Other than the dopaminergic loss, PD is pathologically marked by the presence of intraneuronal proteinaceous cytoplasmic inclusions and these inclusions are composed of alpha-synuclein named as Lewy neurites and Lewy bodies (Kaidery and Thomas, 2018, Ma *et al.*, 2019). Inadequate dopamine (a very vital brain monoamine known to function primarily as an inhibitory neurotransmitter) levels makes it hard to regulate the striatal neurons excitability leading to degeneration hence making the ability to control movements very difficult for patients (Maiti *et al.*, 2017).

Evolutionary advances in the past two decades have supported findings that progression in understanding PD is not only compromised by genetic factors but also the association of environmental toxins, formation of free radicals and oxidative stress (Dias *et al.*, 2013). These environmental factors involve tobacco use and chemical/pesticide exposure (Kieburtz and Wunderle, 2013, Chen and Ritz, 2018). A notable chemical is the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) which is a prodrug to the neurotoxin MPP+. The MPP+ is a metabolite of MPTP and it leads to permanent symptoms of PD by inducing death of dopaminergic neurons (Chun *et al.*, 2001). MPP+ exerts its neurotoxic activities though generation of free radicals which leads to the inhibition of the mitochondrial complex I electron transport chain and in turn depletion of adenosine triphosphate (ATP) (Li *et al.*, 2017). Apparently, the depletion of ATP leads to neuronal cell death as well as symptoms of PD (Hartmann et al., 2001).

Currently, there has been no successful disease-modifying treatments to halt progression of PD, hence current and upcoming research has its focus on discovering more therapeutic approaches to be used in the alleviation of motor symptoms of PD. These approaches are administered based on term temporal nature, severity and side effects of the disease (Zahoor *et al.,* 2018). The most common form of treatment is the use of levodopa but this in itself overtime has been shown to also lead to PD-like symptoms. This challenge has given room to more investigations of novel therapies which include both synthetic and plant-derived natural products.

For hundreds of years, the world at large has turned to medicinal plants for the safety, efficiency, acceptability and the lesser side effects as compared to chemical drugs and this has seen medicinal plants being utilized thoroughly by healthcare systems (Rabiei et al.,

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2019). Recent discoveries have increased the reliance on medicinal plants in both developing and developed countries as there has been a remarkable surge in the perception by the public of accepting herbal remedies not only in drug stores but supermarkets too thus they are being well integrated in communities as a primary source of healthcare (Ekor, 2014). With PD, herbal medicines have been used by specialists as an adjuvant treatment to lessen the dosage of dopaminergic drugs and the effects that come with prolonged usage of the drugs thus leading to the improvement of side effects caused by PD (Li et al., 2006, Freitas *et al*., 2017). A notable plant family that has exhibited neuroprotective activities is the Amaryllidaceae plant family which also has a lot of diverse biological activities (Fennell and van Staden, 2001).

Crossyne flava is a deciduous bulb plant which grows up to 50 cm in height form a bulb with size ranging from 9 cm to 13 cm and are majorly distributed in the West Coast of South Africa. This plant belong to the Amaryllidaceae family which are largely domiciled in the tropical and warm temperate regions of the world and they consist over 800 species with about 60 genera. (Koekemoer *et al*., 2013). The Amaryllidaceae are prominently known for their alkaloids which have been reported to have several biological activities including antiviral, antibacterial, antifungal, antimalarial, analgesic, anti-cancer and neuroprotective activities (Van Goietsenoven et al., 2010, He et al., 2015, Naidoo et al., 2020, Sibanyoni *et al*., 2020). Furthermore, the Amaryllidaceae alkaloid galanthamine has received approval for the United States Food and Drugs Administration (FDA) for the treatment of Alzheimer's disease (Heinrich and Teoh, 2004). Considering this, the Amaryllidaceae alkaloids are being exploited for novel bioactive compounds which may confer neuroprotective activities (Naidoo *et al*., 2020, Sibanyoni *et al*., 2020). This present study therefore investigates the neuroprotective activities of *C. flava* and its bioactive compounds on MPP+-induced neurotoxicity in SH-SY5Y cells.

7.2 Materials and methods

7.2.1 Chemical and reagents

Organic solvents such as methanol (HPLC grade), ethanol, 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).

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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 utilizing 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

7.2.2 Identification and collection of plant material

Bulbs of *C. flava* were collected in November 2018 in the Karoo National Gardens, Worcester, South Africa. The identity of the species was authenticated by one of the co-authors (CNC) and voucher specimens (No UFH 2020–4–XX) was deposited in the Giffen Herbarium (UFH) of the University of Fort Hare, South Africa.

7.2.3 Preparation of plant extract and isolation of compounds

Fresh bulbs (150 g) of *C. flava* were blended and extracted with methanol for 2 days. The total extracts were combined and evaporated under reduced pressure at 40 °C to give a yield of 21 g. For isolation of compounds, 20 g of total extract was loaded on a silica gel column (5 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 25 main fractions. Fraction XI (63.7mg) was chromatographed on sephadex using isocratic 5 % aqueous ethanol, then Prep-TLC to yield compound 1 (5.2 mg). Fraction XV (108.2 mg) was chromatographed under the same conditions to yield compound 166.6 (7.2 mg). Fraction XIV (100.3mg) was chromatographed on silica gel column (using isocratic elution of 5% DCM/MeOH), to yield compounds labelled as (170-1, 7.3mg), and (170-2, 5.3 mg) as indicated.

7.2.4 Cell culture and maintenance

The human neuroblastoma SH-SY5Y cells were generously donated by the Blackburn Laboratory, University of Cape Town. Cells were grown in Dulbecco's Modified Eagle's medium (DMEM, Gibco, Life Technologies Corporation, Paisley, UK), supplemented with

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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 with a medium change every three days. Cells were sub-cultured when they attained 70 to 80 percent confluency using a solution of 0.25 % trypsin EDTA (Lonza Group Ltd., Verviers, Belgium).

7.2.4.1 Treatments

Stock solutions of 40 mg/mL of *C. flava* extract as well as 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 CRO and compounds to be used for 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 *C. flava* extract and compounds (**1**, **2**, **3** and **4**). 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. All treatments lasted for 24 hours and the 2.5 µg/mL concentration was selected for neuroprotection studies. For neuroprotection experiments, cells were plated as above and pre-treated with 2.5 µg/mL of *C. flava* extract and compounds for 2 hours prior to the addition of 2000 µM MPP^+ and treatments were incubated for 24 hours. The concentration of MPP^+ used for this study was informed by a previous study from our laboratory (Omoruyi *et al*., 2020). The untreated cells served as control and 25 µM of rutin (RT) was used as positive control (a known neuroprotective agent).

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7.2.4.2 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 5 mg/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 (Van Meerloo et al., 2011).

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7.2.4.3 Cell morphology

To visualize changes in cell morphology of the SH-SY5Y cells following the respective treatments, cells were seeded in a 96 well plates at a density of 10 000 cells per well and were pre-treated with 2.5 µg/mL of *C. flava* extract 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.

7.2.4.4 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 (Olanow *et al.*, 2004).

7.2.4.5. 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 CRO 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 intensity of treated cells were expressed as percentages of control.

7.3 Statistical analysis

Data generated from this study were expressed as means ± standard error of means of at least three independent experiments analysed using GraphPad Prism Version 6. Significance between groups was determined using one way analysis of variance (ANOVA). A value of $p < 0.05$ was considered as significant.

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7.4 Results

7.4.1 Identification of compounds

Four known alkaloids (**1**-**4**), were isolated and identified based on NMR and GC-MS analysis (Table 10, Fig. 32). Compounds **2-4** were identified as bufanidrine (**2**), buphanisine (**3**) and epibuphanisine (**4**) (Viladomat et al., 1995a, Viladomat et al., 1995b). These compounds belong to crinine-type alkaloids, and according to the recent review by Berkov et al., (2020), there are 85 compounds isolated so far from Amaryllidaceae family and have crinine skeleton. The first compound (pancratinine B) belong to montanine-type alkaloids and isolated once from *Pancratium canariense* (Cedron et al., 2009).

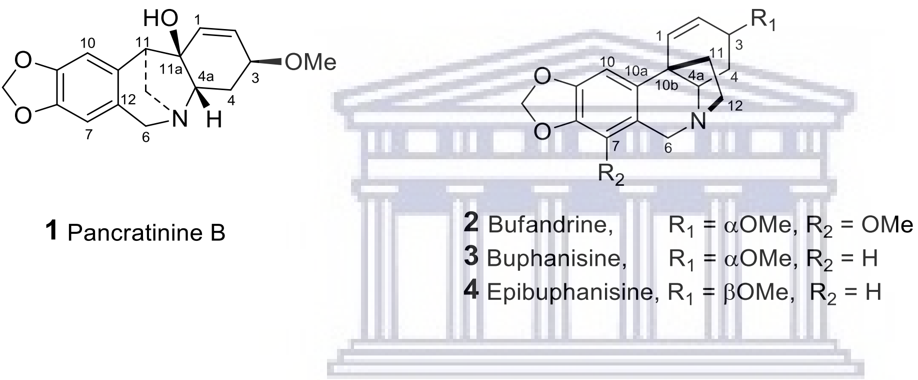

Figure 32: **Chemical structures of isolated compounds** 1-4 **from** ***C. flava***

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Table 10: ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of compounds **1-4**.

	Pancratinine B (**1**)		Buphandrine (**2**)		Buphanisine (**3**)		Epibuphanisine (**4**)	
No	**δ_C**	**δ_H, *J* (Hz)**	**δ_C**	**δ_H, *J* (Hz)**	**δ_C**	**δ_H, *J* (Hz)**	**δ_C**	**δ_H, *J* (Hz)**
1	133.4 *d*	6.03 *br d*, 10.4	132.9 *d*	6.58 *d*, 10.2	132.9 *d*,	6.58 *d*, 10.1	132.1 *d*	6.56 *d*, 9.5
2	131.7 *d*	5.70 *br d*, 10.4	125.3 *d*	5.95 *dd*, 10.2, 5.2	125.4 *d*,	5.95 *dd*, 10.1, 5.4	125.6 *d*	5.96 *dd*, 9.5, 4.9
3	72.3 *d*	3.94 *br dd*, 5.0, 10.8	72.6 *d*	3.82*	77.2 *d*	3.80 *ddd*, 5.4, 4.3, 2.0	72.3 *d*	3.81 *br s*
4	30.9 *t*	1.50 *ddd*, 4.6, 11.0, 12.8	28.7 *t*	2.08 *m*	28.7 *t*,	2.07 *ddd*, 13.3, 4.3, 2.0	28.2 *t*	2.20*
		2.35 *br d*12.9		1.57 *dt*, 13.7, 4.3		1.58 *td*, 13.3, 4.3		1.62 *td*, 13.4, 4.2
4a	67.5 *d*	2.95 *m*	62.6 *d*	3.31 *dd*, 13.7, 4.3	63.1 *d*	3.32*	63.3 *d*	3.41 *dd*, 13.4, 4.2
6	61.8 *t*	3.83 *d*, 16.8	58.6 *t*	4.24 *d*, 17.6	62.3 *t*	4.38 *d*, 16.3	61.8 *t*	4.54 *d*, 16.2
		4.26 *d*, 16.8		3.80 *d*, 17.6		3.76 *d*, 16.3		3.83 *d*, 16.2
6a	125.4 *s*		117.3 *s*		126.2 *s*		125.6 *s*	
7	106.8 *d*	6.51 *s*	140.9 *s*		106.9 *s*	6.45 *s*	106.9 *d*	6.46 *s*
8	145.9 *s*		133.4 *s*		145.7 *s*		145.9 *s*	
9	147.4 *s*		150.0 *s*		146.0 *s*		146.4 *s*	
10	109.9 *d*	6.63 *s*	96.6 *d*	6.81 *s*	102.9 *d*	6.81 *s*	103.0 *d*	6.81 *s*
10a	129.4 *s*		139.3 *s*		138.4 *s*		137.7 *s*	
10b			44.3 *s*		44.3 *s*		44.5 *s*	
11 (*exo*)	49.2 *t*	2.64 *d*, 2.2	44.0 *t*	2.15, *ddd*, 14.0, 9.0, 4.6	44.2 *t*	2.15 *ddd*, 12.4, 9.2, 4.3	43.6 *t*	1.89 *ddd*, 10.6, 10.6, 5.2
(*endo*)				1.90 *ddd*, 11.8, 10.6, 6.0		1.90 *ddd*, 12.4, 11.0, 6.0		2.20*

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11b	82.5 *s*							
12 (*exo*)	54.7 *t*	2.82 *d*, 11.8	53.6 *t*	3.37**	53.4 *t*	3.40*	53.3 *t*	3.46**
(*endo*)		2.97 *dd*, 2.2, 11.8		2.87, *ddd*, 14.0, 9.0, 6.0		2.93 *ddd*, 12.0, 9.0, 6.0		2.94 *ddd*, 14.0, 9.0, 6.0
OCH_2O	100.9 *t*	5.92 *s*	100.5 *t*	5.84 *d*, 5.2	100.7 *t*	5.86, 5.85, *d*, 1.3	100.9 t	5.88, 5.87 *s*/each
OMe-3	56.3 *q*	3.42 *s*	56.4 *q*	3.35 *s*	56.5 *q*	3.34 *s*	56.6 *q*	3.34 *s*
OMe-7			59.1 *q*	3.96 *s*				

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7.4.2 Dose response of *C. flava* and compounds

In order to determine the optimal dose of *C. flava* and compounds that will be used for neuroprotection studies, a cell viability assay was performed in the SH-SY5Y cells treated with 2.5, 5 and 10 µg/mL of either *C. flava* extract or compounds. Results show that the total extract induced a dose dependent reduction in cell viability, which was only significant at the 5 and 10 µg/mL concentration (**Figure 33A**). For the compound treated cells, compound **1** showed a moderate increase in cell viability at concentrations tested albeit not significant, while compound **2** showed a slightly concentration-dependent reduction in cell viability which was also not significant (**Figure 33B and C**). Additionally, compounds **3** and **4** also showed a concentration-dependent reduction in cell viability and was significant at 10 µg/mL for **3** and at both 5 and 10 µg/mL for **4** when compared to control (cells treated with a similar concentration of DMSO in the highest concentration of the compounds) (**Figure 33D and E)**. Together *C. flava* extract and compounds did not induce any marked change in cell viability of SH-SY5Y cells at the 2.5 µg/mL concentration and this was similar for the compounds. Hence, this concentration was selected as the optimum concentration to be used for further neuroprotection experiments.

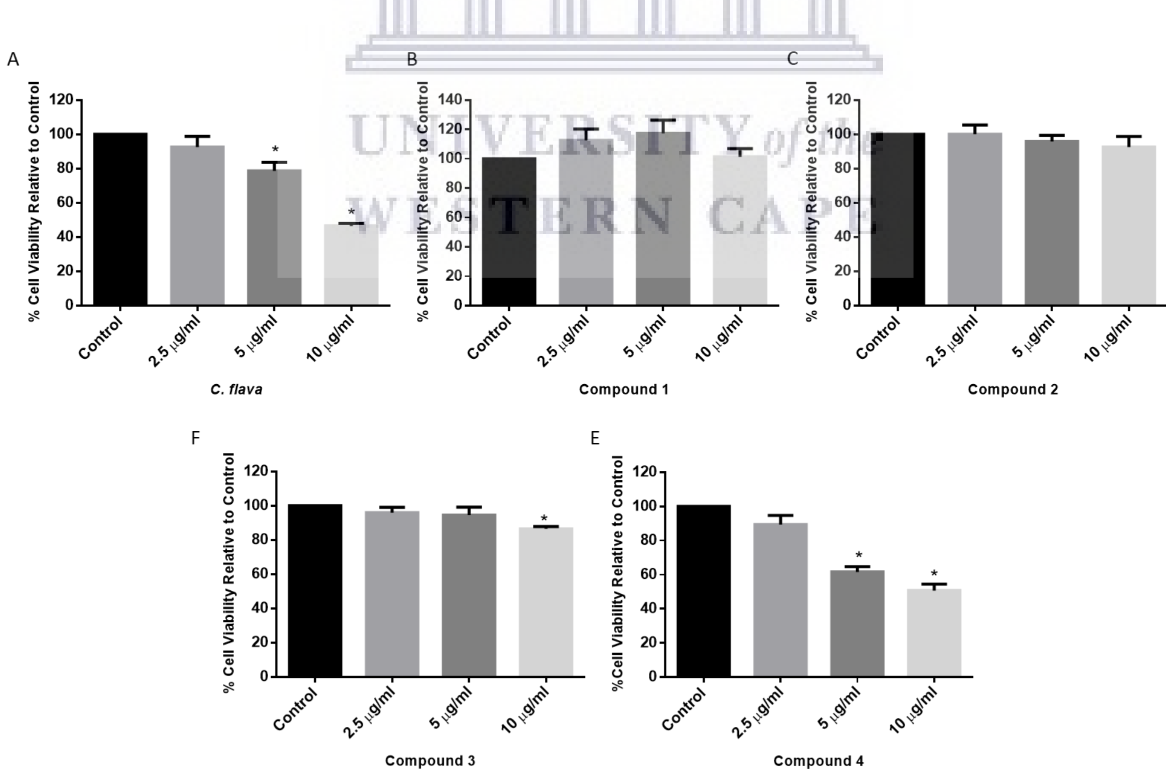

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Figure 33: Dose-response of *C. flava* and compounds. MTT assay cytotoxicity on SH-SY5Y cells treated with increasing concentrations (2.5, 5 and 10 µg/mL) of *C. flava* (A) Compounds 1, 2, 3, and 4 (B – E) for 24 hours and each bar represents mean cell viability expressed as percentage of control. * indicate significance at p< 0.05

7.4.3 *C. flava* and compounds mitigate MPP⁺-induced toxicity

In order to determine whether *C. flava* or compounds protect SH-SY5Y cells from the MPP⁺-induced toxicity, 10 000 cells were plated per well and treated with 2.5 µg/mL of either extract or compounds for 2 hours before the addition of 2000 µM MPP⁺ and thereafter MTT assay was performed after 24 hours. The results show that treatment of SH-SY5Y cells with MPP⁺ lead to a significant reduction of SH-SY5Y cell viability when compared to control. Indeed MPP⁺ reduced cell viability to about 40 to 50% while in the cells that were exposed to *C. flava* and compounds prior to the addition of MPP⁺ cell viability was seeing to be improving towards normal (**Figure 34A and B**). Similarly, rutin which was used as a positive control also showed neuroprotection which was comparable to the cells treated with *C. flava* and compounds. However, cells treated with extract alone or rutin alone showed no changes in cell viability. Together, *C. flava* and compounds confer neuroprotection in SH-SY5Y cells exposed to MPP⁺ toxicity.

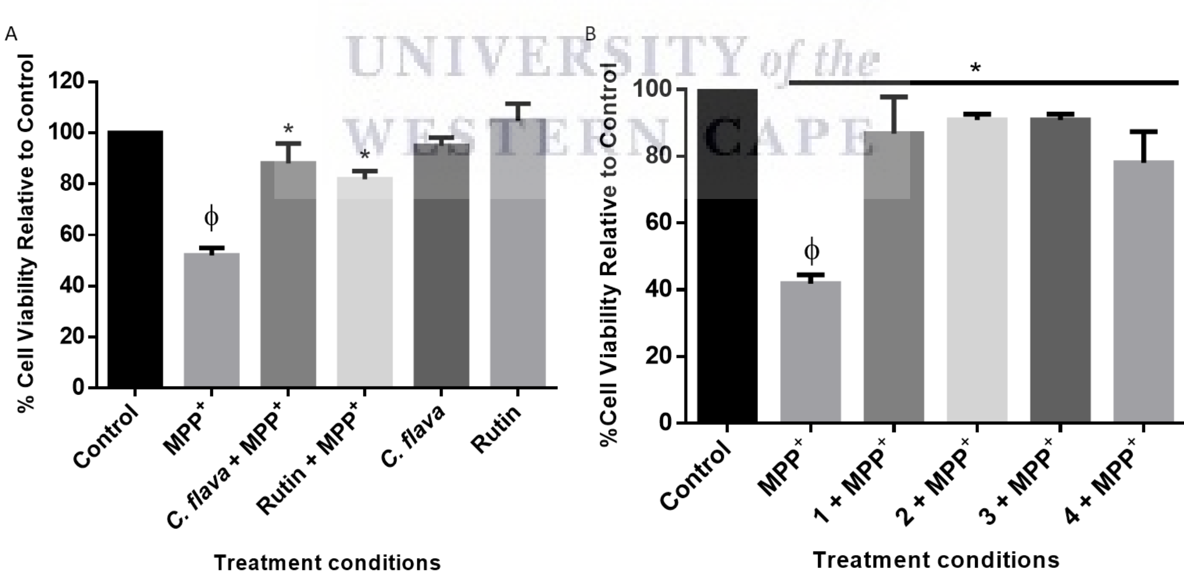

Figure 34: ***C. flava* and compounds show protection in SH-SY5Y cells. Cells were pre-treated with *C. flava* (2.5 µg/mL, 25 µM rutin (A) and compounds (B) before exposure to MPP⁺ for 24 hours. Each bar represents mean percentage cell viability relative to control and significance of**

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difference indicated with * ($p<0.05$) when extract/compounds are compared to MPP^+ treated cells and ϕ (MPP^+ vs control).

7.4.4 *C. flava* prevents MPP^+-induced alterations in cell morphology

Following neuroprotection experiment, we next investigated changes in cell morphology in the cells pre-treated with extract or compounds before addition of MPP^+ as well as cells treated with MPP^+ alone. The results show that MPP^+ as expected induced morphological changes in cells ranging from loss of neuronal projections, cell shrinkage to roundness of cells. However, cells pre-treated with *C. flava* and rutin showed a morphology with unremarkable changes compared to control (**Figure 35**). These results indicate restoration of cell morphology is involved in the neuroprotection *of C. flava*.

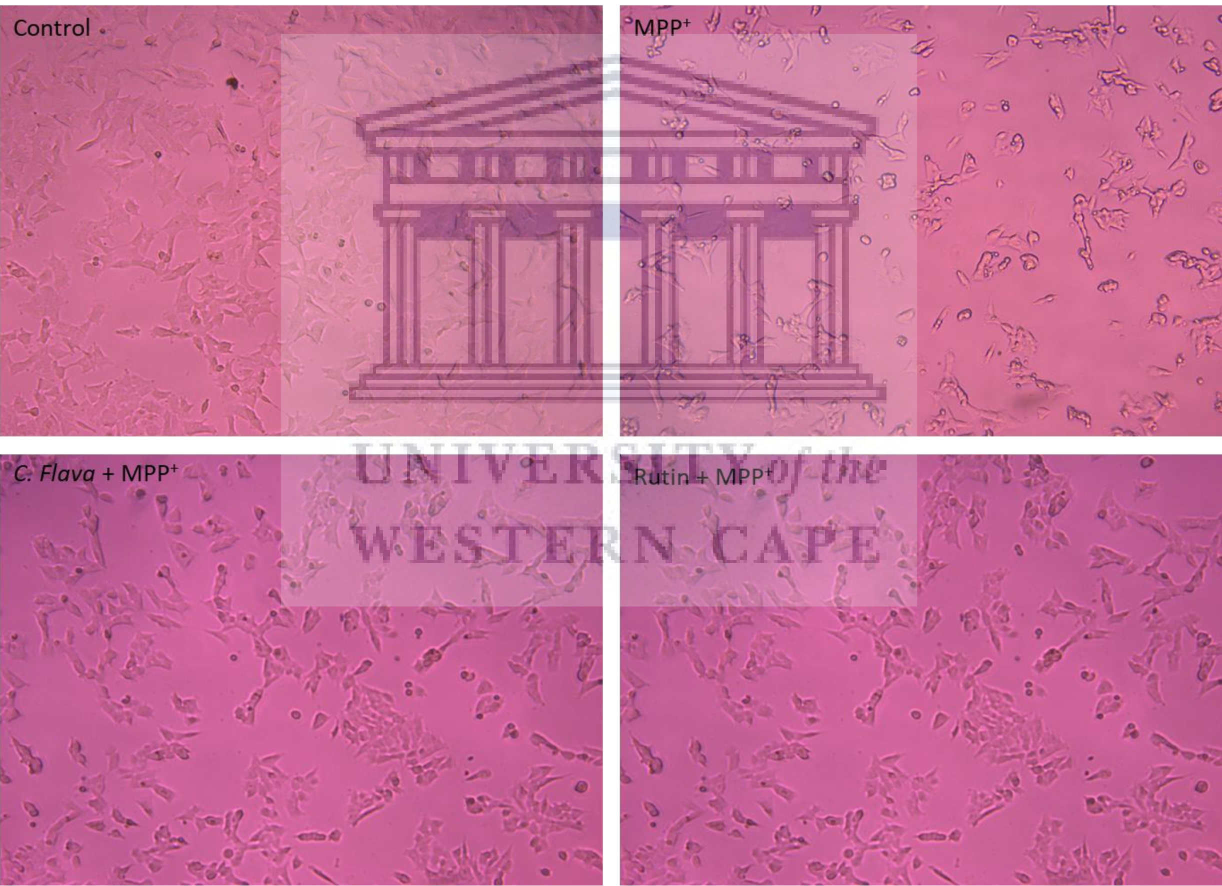

Figure 35: *C. flava* inhibit SH-SY5Y morphological changes induced by MPP^+. SH-SY5Y cells were pre-treated with *C. flava* (2.5 µg/mL) and rutin (2.5 µM) before exposure to 2000 µM MPP^+ for 24 hours. Cells were visualized and images acquired using the light microscope at 100X magnification.

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7.4.5 *C. flava* and compounds attenuate MPP^+-induced loss of ATP

As a pathology of PD, there is depletion of ATP following generation of ROS (Yan *et al.*, 2013, Requejo-Aguilar and Bolaños, 2016). Considering this we next investigated the changes in ATP levels following treatments to ascertain if *C. flava* extract and compounds will be able to inhibit depletion of ATP in the SH-SY5Y cells. Consistent with the mechanism of action of MPP^+, the results from this study shows that whereas MPP^+ significantly reduced ATP levels in the cells compared to control, cells pre-treated with extract and compounds had an opposing effect in ATP (**Figure 36**). Indeed, extract and compounds inhibited the reduction of ATP levels in the cells and this was significant for all treatment conditions.

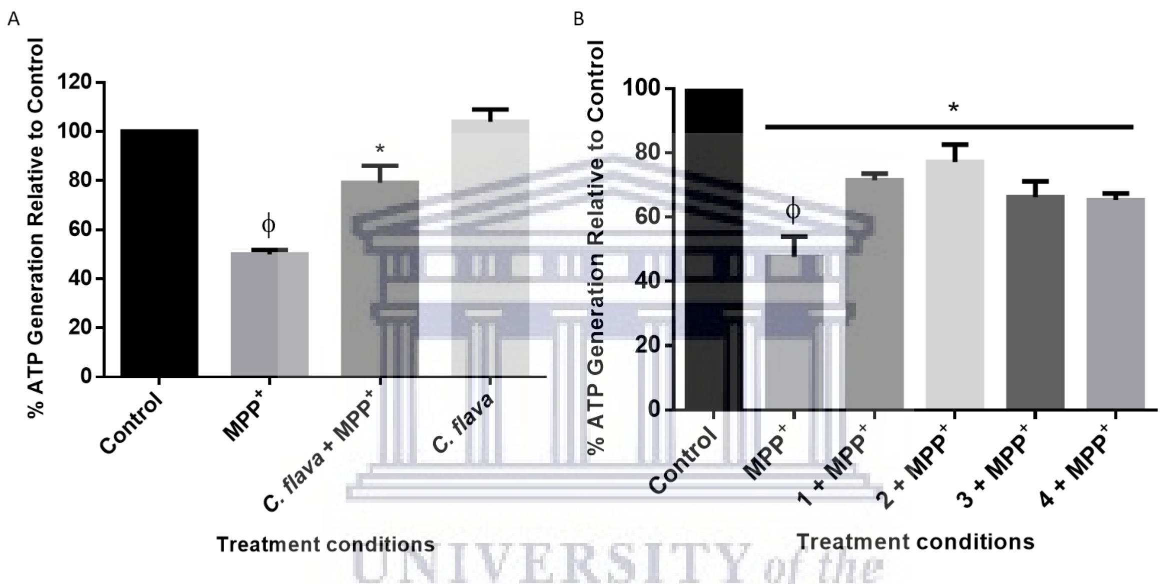

Figure 36: ***C. flava* and compounds attenuate MPP^+-induced ATP degeneration. Cells were pre-treated with 2.5 µg/mL of *C. flava* (A) and compounds (B) before exposure to 2000 µM of MPP^+ for 24 hours and ATP levels assessed. Each bar represents mean percentage level relative to control and significance of difference indicated with * when extract/compounds are compared to MPP^+ and ϕ (MPP^+ vs control).**

7.4.6 *C. flava* and compounds inhibit MPP^+-induced apoptosis

Consequent upon reduction of ATP in the pathology of PD, cell death eventually take place. This to further investigate the mechanism of neuroprotection of *C. flava* and compounds, cells were treated in accordance with neuroprotection experiments and the activities of caspase-3/7 was determined as an indicator for apoptosis. Caspases are belong to a family of cysteine proteases and their activities are connected to cell death as their levels are usually high during apoptosis (Nakajima and Kuranaga, 2017). Caspases could be initiators of apoptosis like the caspase 8 and 9 which are activated during the extrinsic and intrinsic apoptotic pathway

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respectively or executioner caspases (3 and 7) which lies down stream of the apoptotic pathway (Li and Yuan, 2008). The executioner caspases are most times used to determine apoptosis when the aim is not distinguish between the intrinsic or extrinsic apoptotic pathway (Thornberry *et al.*, 1997, Bressenot *et al.*, 2009). The results of this study shows that whereas MPP^+ induced apoptosis in the cells evident by an elevated caspase-3/7 activities, pre-treatment with *C. flava* and compounds reversed the activities of Caspase-3/7. Together, these results show that inhibition of Apoptosis is involved in the mechanism of neuroprotection from *C. flava* and its bioactive compounds.

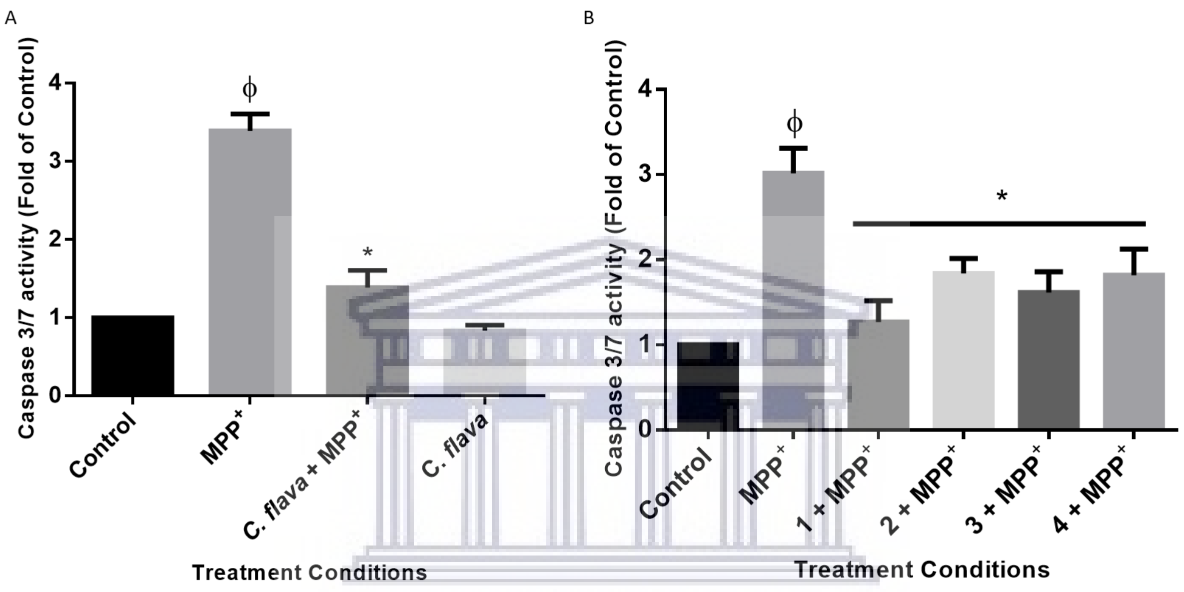

Figure 37: ***C. flava*** **and compounds inhibit MPP^+-induced caspase-3/7 activity. Cells were pre-treated with 2.5 µg/mL of extract (A) and compounds (B) before exposure to 2000 µM of MPP+ for 24 hours and activity of caspase-3/7 was determined. Each bar represents level of caspase-3/7 expressed as fold of control and significance of difference indicated with * when extract/compounds are compared to MPP^+ and ϕ (MPP+ vs control).**

7.5 Discussion

PD continues to pose a challenge to quality of life and despite the many years after this condition was first reported, treatment options are still lacking (Obeso *et al.*, 2017, McDonald *et al.*, 2018). The most widely used treatment for PD is levodopa which addresses only some of the symptoms and if used for too long, it may lead to some serious side effects *(Voon et al.*, 2017, Espay *et al.*, 2018, Lee *et al.*, 2018). The present study investigates the neuroprotective effects of *C. flava* and its bioactive alkaloids on an *in vitro* PD model. We provide first evidence of the neuroprotective activities of *C. flava* as well as it bioactive compounds; pancratinine B (**1**) bufanidrine (**2**), buphanisine (**3**) and epibuphanisine (**4**) in PD. Findings show that both *C.*

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flava and compound inhibited neurotoxicity induced by MPP^+. These findings are consistent with the traditional uses of amaryllidaceae plant family as a previous study reported that plants from the family were being used traditionally for the treatment of neurological disorders (Nair and van Staden, 2014). Further biological and pharmacological studies have also validated this traditional claim (Adewusi *et al.*, 2012, Nair and van Staden, 2013, Jin *et al.*, 2014, Omoruyi *et al.*, 2020).

Furthermore, the plant family also has unique family of alkaloids called and these alkaloids have been shown to offer neuroprotective effects (Cimmino *et al.*, 2017, Ding *et al.*, 2017, Hulcová *et al.*, 2018). In this study, the four alkaloids isolated and identified showed neuroprotective activities in the PD model. Interestingly bufanidrine (**2**) and buphanisine (**3**) have previously been shown to have a strong affinity for the serotonin reuptake transport protein (SERT) and this activity was suspected to be linked to the presence of 1,3-dioxole moiety in the Amaryllidaceae alkaloids which was alluded to be responsible for their neuroprotection in Alzheimer's disease (Sandager *et al.*, 2005, Elgorashi *et al.*, 2006, Neergaard *et al.*, 2009). Whether this moiety is also responsible for the anti-parkinsonism effect is not known but considering the similarity in pathways of the progression of both PD and Alzheimer's disease which can be linked to oxidative stress and mitochondrial dysfuntion (Crews and Masliah, 2010, Yan *et al.*, 2013, Huang *et al.*, 2016), it is plausible that it may play a role but this has to be validated. Similarly, epibuphanisine (**4**) has also been shown to bind to SERT and $GABA_A$-benzodiazepine receptor as well as inhibit acetylcholinesterase as a mechanism of action within the central nervous system and this can also be linked to their anti-anxiety and anti-Alzheimer's effects (Elgorashi *et al.*, 2004, Elgorashi *et al.*, 2006).

As a mechanism of the pathology of PD, there is mitochondrial oxidative stress which leads to the impairment of the mitochondrial complex I and subsequently depletion in ATP levels in the brain cells (Moon and Paek, 2015). In MPP^+ toxicity, decrease in ATP further increases the level of ROS production which in turn mitigates other mitochondrial complexes like III and IV as well as suppresses mitochondrial function including mitochondrial gene expression and protein expression as well as oxidative phosphorylation proteins (Burté *et al.*, 2011, Piao *et al.*, 2012, Jeong *et al.*, 2015). Also affected by ATP loss is dopamine and calcium signalling in the cells and all of these culminate to PD (Zhang *et al.*, 2010). Thus, the level of ATP generation in the cells is a critical test for the integrity of the mitochondria. Findings from the current study revealed that *C. flava* and compound attenuated ATP depletion in cells induced by MPP^+. This gives an indication that mitochondrial function and integrity was improved. Consistent with our findings, *Boophone disticha* belonging to the Amaryllidaceae family has been shown to repeal the effect of 6-hydroxydopamine (6-OHDA)-induced ATP loss in SH-SY5Y cells (Lepule *et al.*, 2019).

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Additionally, consequent upon ATP depletion in cells following neurotoxicity by MPP^+, cells proceed to cell death which could either be in the form of apoptosis or necrosis (Zhang et al., 2010, Ito *et al*., 2017). Apoptosis is a form of programmed cell death and involve a cascade of events which either go via the mitochondrial or intrinsic pathway or the extrinsic pathway (Li and Yuan, 2008). However, critical to both pathways downstream are the executioner caspases such as caspase-3/7 which are effector caspases and promote cleavage of cellular content and eventually cell death (Julien and Wells, 2017). Thus arresting apoptosis is critical for the normal functioning of cells in the PD progression and inhibition of apoptosis in neurodegenerative diseases has been suggested to be a therapeutic option (Waldmeier and Tatton, 2004). Findings from the current study showed that *C. flava* and compounds were able to rescue cells from apoptosis induced by MPP^+. Importantly, whether necrosis pathway was also activated, we cannot infer from this study but it will be worth investigating in future studies.

7.6 Conclusion

The study investigated the neuroprotective effects of *C. flava* and its bioactive compounds isolated from the plant in an *in vitro* model of PD. Four alkaloids were isolated and we present the first evidence of the activities of these compounds in PD alongside *C. flava* extract. Indeed, as a mechanism of action, both extract and compounds attenuated ATP levels in the cells and inhibited apoptosis induced by MPP^+. This further lends credence to the alleged usage of Amaryllidaceae family traditionally to treat nervous system disorder as well as show the potential of exploiting Amaryllidaceae alkaloids for novel drug candidates.

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CHAPTER EIGHT

GENERAL CONCLUSIONS AND RECOMMENDATIONS

8.1 General Conclusions

This study reflects the chemistry of *B. haemanthoides, Crossyne flava* and isolated compounds not previously described for the Amaryllidaceae. Although, the triterpenoids were poorly described; they are considered as an important class of secondary metabolites which contribute to the chemical profile of the species. The chromatographic purifications of the nonpolar fractions of *B. haemanthoides* total extracts using different techniques including semi-prep HPLC resulted in the isolation and identification 13 known compounds. These were identified as stigmast-4-ene-3,6-dione (**1**); cholest-4-en-3-one (**2**); (22*E*)-stigmasta-4,22-dien-3-one (**3**); stigmast-4-en-3-one (**4**); 6β-hydroxystigmast-4-en-3-one (**5**); 6β-hydroxycholest-4-en-3-one (**6**); cycloartenol (**7**); acetovanillone (**8**); tyrosol (**9**) and 3-hydroxy-1-(4-hydroxyphenyl) 1-propanone (**10**); Distachamine (**11**); 1α,3α-diacetylnerbowdine (**12**); Hippadine (**13**) from *B. haemanthoides*. Similarly, chromatographic manipulation of *C. flava* resulted in four other compounds; pancratinine B (**14**), bufanidrine (**15**), buphanisine (**16**) and epibuphanisine (**17**).

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

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to alkaloids. In summary, this study demonstrates the therapeutic potential of Amaryllidaceae plant family with focus on the exploration of its neuroprotection. Further mechanistic and in vivo studies will be required in future studies to elucidate their activities.

On the other hand, four alkaloids were isolated from *C. flava* and we present the first evidence of the activities of these compounds in PD alongside *C. flava* extract. Indeed, as a mechanism of action, both extract and compounds attenuated ATP levels in the cells and inhibited apoptosis induced by MPP+. This also supports the acclaimed traditional usage of Amaryllidaceae family to treat nervous system disorder and its potential as novel drug candidates in drug discovery.

The thesis also includes the biological evaluation of selected members of family Amrayllidaceae for their cytotoxicities and neuroprotection potential. This research study showed that extracts of the Amaryllidaceae differentially induced cytotoxicity in the cell lines tested following 24 hours exposure time. Noteworthy is that the IC_{50} obtained for the *Amaryllis belladonna* extract was below 30 µg/mL for all cell lines tested. Similar results were also obtained for the *Haemanthus pubescens* extract which had an IC_{50} value less than 30 µg/mL for the U87 and SH-SY5Y cells but not for the U251 cells. Thus, both *A. belladonna* and *H. pubescens* will require further mechanistic studies to establish their potential use as anti-cancer agents. Importantly, some of the extracts also showed specificity for particular cancers and this was more pronounced in the cells treated with *Crossyne guttata*, where the U87 and U251 glioblastoma cells were more sensitive and the SH-SY5Y cells showed minimal sensitivity.

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In addition, *Clivia miniata* and *Nerine humilis* were investigated for their neuroprotective potential in MPP+-induced neurotoxicity in SH-SY5Y neuroblastoma cells. The results showed that pre-treatment of cells with the extracts at 2 and 4 µg/mL concentrations improved cell viability as well as cell morphology by inhibiting the toxicity induced by MPP+. The extracts also improved ATP levels in cells and attenuated the apoptosis induced by MPP+. Furthermore, antioxidant assays showed that both extracts had low antioxidant activity. Findings from this study indicate that *Clivia miniate* and *Nerine humilis* may be promising as neuroprotective agents for PD and warrant further investigation to determine the bioactive components of the plants that may be responsible for the observed effects.

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8.2 Recommendations

This work details the chemical and biological investigations of selected members of the family Amaryllidaceae. The plants of the family Amaryllidaceae are a large group comprising over 60 genera and more than 1000 species. Amaryllidaceae plants have been widely used by local traditional healers and have been reported to have numerous pharmacological uses.

It is recommended that further studies be carried out on the isolation of compounds from this important family of plants to harvest the full potential especially non-alkaloidal secondary metabolites. The study may also be away of validating their use for many traditional purposes especially toxicity, neurodegenerative and antibacterial uses.

Plants belonging to Amaryllidaceae genera were investigated phytochemically, many of the compounds mentioned above have been isolated from the genus *B. haemanthoides* and *C. flava*. New phytochemical studies for other Amaryllidaceae members will be important to discover other metabolites in addition to the alkaloids.

Since, the extracts coming from two plants (*Clivia miniata* and *Nerine humilis*) showed potent neuroprotective abilities, it is recommended that, further chemical studies to be carried out to identify the bioactive constituents.

Also, it is recooemnded to study the active extracts and / or the bioactive compounds in-vivo animal models and check to determine the toxicity level in-vivo.

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