The Morphological Characterization, Chemical Composition and Biological Activity of *Barleria albostellata* (Acanthaceae)

BY

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ABSTRACT

Herbal preparations of plants continue to present mankind with novel remedies as many of these plants contain important secondary metabolites. Medicines manufactured by pharmaceutical companies are largely synthetic. The fear for ineffectiveness, adverse side-effects and toxicity, has brought about further scientific investigations on the potential usage of medicinal plants. Plants of the family Acanthaceae are rich in bioactive phytochemicals. Several plant species are being utilized for their ethnomedicinal properties based on their phytocompounds they acquire, with *Barleria* (Acanthaceae) being one of such genera. *Barleria albostellata* C.B. Clarke (Acanthaceae) is a shrub indigenous to South Africa. Herbal use of this plant has not been fully documented, however, several species of *Barleria* are used in traditional medicine. Little or no investigations have been undertaken to evaluate the micromorphology secretory mechanisms, through which such phytochemicals are synthesized and secreted; floral biology; phytochemical profile; antibacterial and antioxidant activity, cytotoxicity and synthesis of nanoparticles using the leaf and stem extracts of *B. albostellata*. Therefore, this study aimed at bridging these gaps by first characterizing the morphology, chemical composition and biological activity of leaves and stems of *B. albostellata*, using various microscopy techniques and biological assays. Floral biology of the plant was conducted using stereo- and scanning electron microscopic (SEM) techniques. ImageJ was used to measure the length and diameter of the different trichome types and pollen structures. Histo-phytochemical, thin-layer chromatography (TLC), fluorescence microscopy and gas-chromatography mass spectrometry (GC-MS) analysis were performed on crude extracts (hexane, chloroform and methanol) to determine the composition of the compounds that may be of medicinal importance. Biological (antibacterial and antioxidant) analyses were also conducted on the crude extracts. Cytotoxicity of the crude extracts were evaluated established using 3-[(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) assay in the human embryonic kidney (HEK293), cervical cancer (HeLa), and breast adenocarcinoma (MCF-7) cell lines. Silver nanoparticles (AgNPs) were synthesized using methanolic, aqueous- powdered and -fresh leaf and stem extracts. These AgNPs were characterized using UV–visible spectroscopy, high resolution transmission electron microscopy (HRTEM), energy-dispersive X-ray (EDX), Fourier transform infrared (FTIR) spectral analysis and nanoparticle tracking analysis (NTA). Antibacterial analysis of the synthesized AgNPs was assessed using the disk diffusion method. Cytotoxicity of the synthesized nanoparticles were investigated using the MTT assay on the three cell lines. Histochemically stained sections, stereo, SEM and TEM micrographs revealed a dense indumentum with the presence of five morphologically distinct glandular capitate trichome types, multangulate-dendritic branched (MDB) non-glandular trichomes and a glandular head attached to a branched non-glandular trichome. Both glandular and non-glandular trichomes in the leaves and stems of *B. albostellata* are important diagnostic features of this species. The secretory head of glandular typed trichomes varied from 20.72 ± 1.36 µm to 54.23 ± 2.08 µm. Additionally, the length of these stalks varied from 15.11 ± 3.89 µm to 101.92 ± 1.78 µm, and the width

from 11.62 ± 1.03 µm to 35.32 ± 3.87 µm. Multangulate-dendritic branched trichomes had a stalk length of 554.10 ± 92.27 µm and width of 28.55 ± 2.48 µm. Stained sections and crude extracts indicated the presence of important medicinal compounds such as alkaloids and phenolics. The histochemical tests indicated the presence of various phytocompounds that have medicinal importance and aid in defense against pathogens and herbivores. Pollen grains of *B. albostellata* are complex, intricate and display reticulate sculpturing, with a diameter of 77.53 ± 5.63 µm and aperture of 14.31 ± 0.59 µm. This will contribute significantly to our growing understanding on the floral and pollen biology of this species. Qualitative phytochemical screening, GC-MS and florescence microscopy of the leaf and stem extracts revealed various biologically active compounds and the presence of different colours in the leaf and stem powder. Different colour intensities obtained from TLC suggested concentrations of the separated compounds were varied. Additionally, the phyto-constituents found in the leaf and stem crude extracts of could inhibit the growth of various pathogenic strains. Results from this study revealed the medicinal potential of *B. albostellata* in the treatment of various bacterial diseases Ultraviolet spectra, NTA and EDX, revealed varying absorption peaks, size distribution, and elemental Ag in all extracts. Crude extracts and synthesized AgNPs displayed varying degrees of antioxidant, antibacterial and cytotoxic activities. Significance was established at $P < 0.05$ for all concentrations and treatments for antibacterial activity. The evaluated crude extracts may protect against FR and oxidative damage occurring in various pathological mechanisms. The observed *in vitro* cytotoxicity (IC_{50} values of crude extracts and AgNPs synthesized were > 63 µg/mL and >9 µg/mL, respectively) may be due to the presence of flavonoids, phenols, and antioxidant activity in the different parts of this species. Synthesized AgNPs showed possible bacteriostatic effects against Gram-positive and -negative human pathogenic bacteria. Their broad spectrum of bioactivity suggested that they may be as promising agents in fighting infections. This study ultimately proved that leaf and stem extracts of *B. albostellata* contained numerous biologically active compounds such as alkaloids and phenolics. These results are suggestive that the leaves and stems of *B. albostellata* are rich in bioactive compounds which, could be a possible source of antibacterial agents in treating several diseases .There is a great potential for *B. albostellata* as this plant displays valuable biological activities. Future studies on this plant are recommended, as this could advance the use of indigenous herbal medicine or product novel drug leads.

PREFACE

The research described in this dissertation was completed by the candidate while based in the Discipline of Biological Sciences, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville, South Africa. The study was financially supported by the National Research Foundation.

The contents of this study represents the original work by the author and have not been submitted in any form to another tertiary institution. Where use has been made of the work of others it is appropriately acknowledged in the text.

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DECLARATION 1- PLAGIARISM

I, Serisha Gangaram, declare that:

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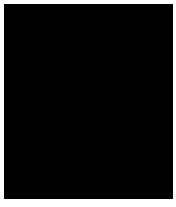

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DECLARATION 2- PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, in press and published and give details of the contributions of each author to the experimental work and writing of each publication.

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Publication 4: Evaluation of the antioxidant and cytotoxic activities of the leaf and stem crude extracts of *Barleria albostellata*. (Publication in preparation).

Publication 5: The green synthesis of silver anoparticles using *Barleria albostellata* extracts: Characterization, antibacterial and cytotoxcitity evaluation. (Publication in preparation).

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CONFERENCE CONTRIBUTIONS FROM THIS DISSERTATION

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5. The analysis of the phytochemical compounds and antibacterial activity in the leaves and stems of *Barleria albostellata*. Postgraduate Research and Innovation Symposium 2021 research day. (College of Agriculture Engineering and Sciences).

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LIST OF ABBREVIATIONS AND SYMBOLS

2C	Two capitate trichomes
A	Anther
AA	Ascorbic acid
Abs	Absorbance
ABTS	(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid))
AChE	Acetylcholinesterase enzyme
AD	Alzheimer's disease
Ag	Silver
$AgNO_3$	Silver nitrate
AgNPs	Silver nanoparticles
AIDS	Acquired Immune Deficiency Syndrome
$AlCl_3$	Aluminium chloride
ALT	Alanine aminotransferase
AP	Aperture
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
Au	Gold
Bc	Basal cell
BS	*Bacillus subtilis*
C	Carbon
CCL_4	Carbon tetrachloride
Ca	Calcium
CaOx	Calcium oxalate
CAT	Catalase
CIO	Carrageenan-induced oedema
Cl	Chlorine
cm	Centimetre
cm^2	Square centimeters
CO_2	Carbon dioxide
COX	Cyclooxygenase
CRS	Cold-restraint stress
Ct	Cuticles
CTC	Condensed tannin contents
Cu	Copper

Cys	Cystolith
DCys	Double cystolith
DLA	Dalton’s lymphoma Ascites
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2, 2′-diphenyl-1-picrylhydrazyl
DW	Dry weight
Ec	Epidermal cell
EC	*Escherchia coli*
EDX	Energy-dispersive X-ray
EMEM	Eagle’s Minimum Essential Medium
Ep	Epidermis
ER	Endoplasmic reticulum
F	Filament
FBS	Foetal bovine serum
FCA	Freund's complete adjuvant
Fe	Iron
$FeCl_3$	Iron (III) chloride
FEGSEM	Field emission gun scanning electron microscope
FL	Fresh leaf
FRAP	Ferric reducing antioxidant power
FR	Free radicals
FS	Fresh stem
FTC	Ferric thiocyanate method
FTIR	Fourier transform infrared (FTIR) spectroscopy
g	Gram
G	Granules
GA	Gallic acid
GAE	Gallic acid equivalents
GB	Golgi body
GC-MS	Gas chromatography-mass spectrometry (GC-MS) analysis
GCT	Glandular capitate trichome
GHBNG	Glandular head attached to a branched non-glandular trichome
GNG	Glandular head attached to a branched non-glandular trichome
GSTs	Glandular secreting trichomes
GT	Ground tissue

GV	Golgi vesicles
h	Hour
HEK293	Human embryonic kidney
HeLa	Cervical cancer
HRTEM	High resolution transmission electron microscopy
H_2SO_4	Sulfuric acid
IAW	Indian adult worm
IUCN	International Union for the Conservation of Nature
K	Potassium
L	Lumina
La	Lamina
LC	Leaf chloroform
LH	Leaf hexane
LM	Leaf methanol
Lm	Lamellar body
M	Mitochondria
MCF-7	Breast adenocarcinoma
MDB	Multangulate-dendritic branched
MDBT	Multangulate-dendritic branched trichomes
MFC	Minimum fungicidal concentration
Mg	Magnesium
mg	Milli gram
MHA	Mueller-Hilton agar
MIC	Minimum inhibitory concentration
ML	Methanol leaf
mL	Milli litre
mM	Milli molar
MMP-9	Matrix Metalloproteinase-9
MMU	Microscopy microanalysis unit
MNPs	Metal nanoparticles
MPO	Myeloperoxidase
MRSA	*Methicillin*-resistant *Staphylococcus aureus*
MS	Methanol stem
MTT	3-[(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide]
mV	Milli Volt
MW	Molecular weight
N	Nuclei

Na	Sodium
NaOH	Sodium hydroxide
NQO1	(NAD(P)H dehydrogenase [quinone] 1)
NTA	Nanoparticle tracking analysis
NC	Neck cell
nm	Nano meter
NP	Nanoparticle
NPs	Nanoparticles
NIST	National Institute of Standards and Technology library
Nrf2	Nuclear factor erythroid 2–related factor 2
O	Oxygen
P	Plastids
PA	*Pseudomonas aeruginosa*
PBS	Phosphate Buffered Saline
PCB	Polychlorinated biphenyls
Ph	Phloem
Pl	Pylorus ligation
PL	Powdered leaf
PM	Palisade mesophyll
POX	Peroxidase
PS	Powdered stem
QE	Quercetin equivalents
R	Resistant
R_f	Retention factor
ROS	Reactive oxygen species
S	Stripped
SA	*Staphylococcus aureus*
SANBI	South African National Biodiversity Institute
SC	Stem chloroform
SD	Standard deviation
Sec	Secretion
SEM	Scanning electron microscopy
Sh	Secretory head
SH	Stem hexane
Si	Silica
Sm	Stomata

SM	Stem methanol
SMA	Stigma
SOD	Superoxide dismutase
SPR	Surface plasmon resonance
SRB	Sulphorhodamine B
St	Stalk
ST	Style
STs	Simple trichomes
T	Trichome
TAF	Iridoid-enriched fraction
TBA	Thiobarbituric acid method
TEM	Transmission electron microscopy
TFC	Total Flavonoid Content
TIC	Total iridoid content
TLC	Thin-layer chromatography
TPC	Total Phenolic Content
TPTZ	2,4,6-tripyridyl-s-triazine
µg	Micro gram
µg/mL	Microgram per milli litre
µg/µL	Micro gram per Micro litre
µL	Micro Litre
UT	Unicellular non-glandular trichome
UV	Ultraviolet light
V	Vacuole
Vs	Vesicle
WD	Working distance
WHO	World Health Organisation
X	Xylem
˚	Degree
%	Percent
v/v	Volume to Volume ratio
α	Alpha
β	Beta
λ	Wavelength (lambda)

DEDICATION

This dissertation is dedicated to my late grandfather Mr. Khanailal Dookie and my parents Dino & Shamilla Gangaram, whose love, guidance and sacrifice is why I am here today.

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CHAPTER 1
INTRODUCTION

1.1 Traditional medicine

The World Health Organisation (WHO) defines traditional medicine as the knowledge, skill and practices which are built on the beliefs and experiences that are indigenous to various cultures. Information on traditional medicine has been generally passed on verbally through generations, and this information is used in diagnosing, preventing, treating or improving mental and physical illnesses (WHO, 2014). Traditional medicine is an ancient practice which is nearly as old as the existence of mankind. This declaration is backed by evidence obtained from studies of the older civilizations of human settlements where paleontologists discovered bunches of medicinal herbs among the fossilized remains of Neanderthal ancestors (Mayeng, 1996). Previously, man depended solely on higher plants for medicine, and this dependence is still apparent in the present era (Balandrin et al., 1993; Jackson, 2018; Walsh, 2018; Kerdel-Vegas, 2019)

Traditional preparations of plants (leaves, roots and bark) continue to offer mankind novel remedies. Plants are rich in a diversity of secondary metabolites such as alkaloids, flavonoids, terpenoids and tannins which have been found to contain antimicrobial properties (Cowan, 1999; Lewis and Ausubel, 2006; Rai et al., 2014; Umashankar, 2020). Plant extracts have treated various infectious diseases throughout the history of mankind (Rabe and Van Staden, 1997; Buwa and Van Staden, 2006; Singh et al., 2020) by means of herbal preparations. These include, concoctions, decoctions, infusions and teas (Van Wyk and Wink, 2004). Ancient texts of the Vedas and the Bible have described some of these traditional practices, using traditional herbs (Hoareau and Edgar, 1999; Shaila and Begum, 2021). A great deal of conventional medicine have also originated from plant extracts, with some of the effective drugs being plant-based, such as aspirin from the bark of the willow tree (Vickers et al., 2001).

Medicines manufactured by pharmaceutical companies are largely synthetic (Akerele, 1993; Schmid et al., 2001; Vlieghe et al., 2010). The fear for ineffectiveness, adverse side-effects and toxicity, has brought about further scientific investigations on the potential usage of medicinal plants (Redo et al., 1989; Silver and Bostian, 1993; Ahmad et al., 2006; Wood, 2017). Free radicals and reactive oxygen species present countless threats to cells (Hochstein and Atallah, 1988). Oxidative damage develops in the human body and certain tissues of organs, such as the brain. This is due to the increased utilization of oxygen (20% of overall inhaled body oxygen) and poorly developed antioxidant protective mechanisms (Ahaskar et al., 2007; Sisodia et al., 2008). Therefore, exploring medicinal plants for beneficial phytochemicals that could possibly serve as nutritional supplements, remove the reactive energy of free radicals and singlet oxygen, has increased (Sisodia et al., 2008).

Plants continue to play a role in human existence which makes ethnobotany an important research field (Sumner, 2000). The plant kingdom has many unique bioactive compounds of medicinal importance (Talhouk et al., 2007; Valdés-Jiménez et al., 2021). In recent years, scientists have attempted to determine where these medicinal and chemical properties arise from within the plant and which structures are involved in conferring the pharmacological action in medicinal plant species (Svoboda et al., 2000). In many instances, specialised structures are found to synthesize, store and/or secrete secondary metabolites for the ultimate purpose of disease prevention, anti-herbivory tactics and protection against water loss (Svoboda et al., 2000; Schmid et al., 2014). Those structures are known as trichomes and occur on the plant surface as hairs or external glands. Trichomes may be family or species-specific and can vary in their chemical components (Svoboda et al., 2000). Besides functioning as biochemical factories, plant secretory structures also aid as taxonomic characters, assisting in the identification of plant families (Svoboda et al., 2000).

Globally, the demand of herbal medicines is increasing rapidly due to their low cost and higher safety margins (Aiswarya and Ravikumar, 2014). The dependence on plants as an alternative source of medicine is dominant in developing countries, especially where traditional medicine plays a vital role in health care (Farnsworth, 1984; Hosseinzadeh et al., 2015). Herbal treatment is well established in many traditions and cultures. This is the way of life for almost 80% of the individuals that reside in rural areas, particularly those in Latin America, Asia, Africa (Banquar, 1993) and more specifically pertaining to this study in South Africa (Mukhtar et al., 2008). The use of traditional plants around the world represents an important reservoir of knowledge and a great potential of yet "undiscovered" use of natural resources (Zizka et al., 2015).

1.2 Traditional medicine in South Africa

Africa is regarded as the cradle of mankind with a rich cultural and biological diversity and with healing practices varying in regions (Gurib-Fakim, 2006). More than 80% of the population in Africa use traditional medicine (Kasilo and Trapsida, 2010). African traditional medicine has a longer history than Western medicine as there is a profound cultural trust among many Africans towards traditional medicine and their medicinal practitioners (Truter, 2007; Gyasi et al., 2016). A large proportion of the South African population practice traditional medicine for their psychological and physical health needs (Rabe and Van Staden, 1997).

In southern Africa, Van Wyk and Gericke (2000) estimated that there are about 3000 medicinal plants used frequently and from these plants approximately 38 indigenous species are commercialised, to a certain extent, and are available as processed materials in various dosage forms such as tablets, capsules, ointments or teas (Van Wyk, 2008). South Africa has one of the most diverse temperate floras with an estimated 24000 plant taxa making it one of the richest countries on earth in terms of floral wealth and biodiversity (Low and Rebelo, 1996; Van Staden, 2008). This diverse flora represents 10% of the

world's plant diversity (Fourie et al., 1992). For example, in KwaZulu-Natal, there are more than 1032 plant species from about 147 families that are used in traditional medicine (Light et al., 2005).

South Africa, like many developing countries, has traditional medicine as an alternative to the rural healthcare scheme due to lack of adequate healthcare services, conventionally-trained medical doctors and equipment in many rural areas (Van Wyk et al., 1997; Light et al., 2005). The majority of plants used traditionally in South Africa for medicinal purposes are collected from the wild and are yet to be completely examined for their bioactive compounds (Taylor and Van Staden, 2001). The movement towards commercialisation of medicinal plants has increased in South Africa, resulting in overharvesting and near-extinction of various valued indigenous plant species (Williams et al., 2000). Medicinal plant research in South Africa is challenged by many factors. These include climate change, declining wild flora due to habitat degradation (Jäger and Van Staden, 2000) and human urbanisation (Williams et al., 2013). This has resulted in the decline of natural populations of several medicinally significant species (Taylor et al., 2001).

The root of *Pelargonium sidoides* (medicinal plant indigenous to South Africa) has been extensively collected in large quantities to meet the demands for local and export trade. The increased number of plant gatherers and overharvesting has made its exploitation unsustainable (Lewu et al., 2006). Trade of the roots and tubers of wild ginger (*Siphonolochilus aethiopicus*) has occurred since the early 1800's and, from then, there has been an unprecedented increase in the commercial exploitation of medicinal plants (Cunningham, 1988). In KwaZulu-Natal, the African Wild ginger has been destroyed by destructive harvesting procedures by removing the roots and rhizomes (Zschocke et al., 2000). This species is an annual plant that has a slow germination rate, and harvesting the rhizomes in natural populations could lead to reduced genetic diversity or susceptible extinction (Zschocke et al., 2000).

Thus, the identification, examination and screening of traditionally consumed African medicinal plants are essential for conservation schemes. The ongoing threat to biodiversity and the verified record of natural products being used in drug discovery have provided compelling motives for extending the exploration of nature in order to identify unique active compounds as promising leads for effective drug development (Cragg et al., 1997; Amoo et al., 2009). Knowledge of the use of plants in traditional medicine is beneficial to healers and the pharmaceutical industry. Also, the validation of the pharmacological effects using new scientific approaches can benefit a large number of individuals (Cragg et al., 1997).

1.3 Drug discovery from medicinal plants

Various techniques have been used in acquiring compounds for the production of drugs. These include the isolation of compounds from plants and other natural sources, molecular modelling, synthetic and combinatorial chemistry (Balunas and Kinghorn, 2005). The significance of plants as one of the natural

sources of medicines can never be over-emphasized, as approximately 25% of prescribed drugs worldwide originate from plants (Rates, 2001). Fifty five categorized human diseases such as cancer, parasitic and microbial infections were researched by Newman et al. (2003). It was found that 87% of medications used for treatment were derived from natural products extracted from plants. Fabricant and Farnsworth (2001) showed that 122 bioactive compounds from approximately 94 plant species were consumed as clinical drugs. Drugs that arise from medicinal plants not only serve as new drugs but can also aid as templates for the development of synthetic drugs (Balunas and Kinghorn, 2005). Plant-derived medicines have been used for centuries and large quantities of drugs are directly originated from phyto-pharmacological substances (Boumendjel et al., 2013). Some well-known examples are as follows:

Atropine is an alkaloid found in *Atropa belladonna* leaves (Rajput, 2013) and roots (Bhandary et al., 1969). This compound has been used to induce pupil dilation during eye examinations (Duncan and Collison, 2003); to therapeutically treat Parkinson disease (Adler, 2008) and effectively treat certain cardiovascular conditions (Mirakhur and Dundee, 1980).

Morphine is a codeine-alkaloid acquired from roots and aerial parts of *Papaver somniferum* (Nessler and Mahlberg, 1978). Both compounds are the most important and effective analgesics used in medicine, worldwide (Allen et al., 2004).

Quassinoids is a triterpenoid from *Quassia amara*. This compound is effective against colic- associated stomach aches (Felter, 1922).

Quinine is an alkaloid that is present and obtained from the bark of *Cinchona pubescence*, an effective remedy used for malaria for more than 300 years (Martin and Gandara, 1945).

Taxol is a diterpenoid (Wani et al., 1971) from the bark of *Taxus brevifolia* (Schiff and Horwitz, 1980). This compound is highly promising as an antitumor agent in ovarian and breast cancer (Schiff et al., 1979; Rowinsky et al., 1990). Although there has been great progress in pharmaceutical industries in the search of important medicinal compounds from plants, a considerable amount of the plant biodiversity still remains unstudied (Cragg and Newman, 2007). There is a great potential for medicinal plant research, as future discoveries from plants could advance the use of indigenous herbal medicine or lead to new drug discoveries.

1.4 Nanotechnology: emerging benefits in the development of new drugs

Nanotechnology is an emerging field that focuses on the synthesis and application of small materials called nanoparticles (<100 nm) (Albrecht et al., 2006; Mittal et al., 2014; Sigamoney et al., 2016; Devi and Selvan, 2017; Khatoon et al., 2017). The physical properties of nanoparticles such as their size, shape, morphology and their large surface-area-to-volume ratio have optimised their activity in various fields i.e. chemistry, medicine and agriculture (Iravani, 2011; Safavi, 2012; Vanaja and Annadurai, 2012). There has been significant development in the study of metal-derived nanomaterials for their

therapeutic and biomedical applications (Liao et al., 2006). The development of multiple drug resistant microorganisms poses a worldwide threat to public health (Rai et al., 2009). The inappropriate use of antibiotics allow microorganisms to develop mutations making them resistant to conventional biocides (Kim et al., 2007; Rai et al., 2009; Sigamoney et al., 2016). Treatment of diseases caused by drug resistant pathogens can lead to increased morbidity and mortality (Huh and Kwon, 2011; Sousa et al., 2011; Bhatt et al., 2015). For these reasons, research in nanotechnology for an effective treatment against drug resistant bacteria is necessary (Morones et al., 2005).

Scientists have explored the use of plant extracts to synthesize metal nanoparticles (MNPs) which is more environmentally friendly and cost-effective (Salem et al., 2014; Salunke et al., 2014; Mollick et al., 2015), than conventional chemical or physical synthesis techniques (Bar et al., 2009; Thakkar et al., 2010; Vanaja and Annadurai, 2012; Sigamoney et al., 2016). Metals used to synthesize nanoparticles from plants include gold (Asmathunisha and Kathiresan, 2013), copper (Lee et al., 2011) and silver (Kim et al., 2007; Rai et al., 2009; Asmathunisha and Kathiresan, 2013; Sigamoney et al., 2016). Among the various MNPs, there has being significant interest in silver nanoparticles (AgNPs) as they are readily available, safe, nontoxic (Jha et al., 2009; Chandirika and Annadurai, 2018) and have numerous applications in therapeutics (such as antidiabetic, antimicrobial, anticancer, anti-inflammatory and antioxidant activities) (Lampe, 1999; Rahuman et al., 2000; Dipankar and Murugan, 2012; Vasanth et al., 2014).

Silver nanoparticles have been used as antiviral agents against herpes, hepatitis B and respiratory syncytial virus (Lu et al., 2008; Sun et al., 2008; Baram-Pinto et al., 2009). Medicinal plants have a variety of secondary metabolites which can reduce, cap and stabilise silver ions (Mallikarjuna et al., 2011; Chinnasamy et al., 2017; Khatoon et al., 2017; Maddila and Hemalatha, 2017). Nanostructured systems are assumed to enhance the action of plant extracts, thus improving their biological activity and possibly reducing adverse side effects (Bonifácio et al., 2014; Salam et al., 2014). Therefore, synthesized AgNPs from plant extracts have a broad spectrum of bioactivity which enables them as promising therapeutic agents to fight infections and lead to new drug discoveries.

1.5 Botanical description of *Barleria albostellata*

The genus *Barleria* belongs to the Acanthaceae family (Table 1.1) with about 300 species worldwide, comprising of herbs and shrubs (Makholela et al., 2003). The greatest representation of *Barleria* is in Africa where the diversity is in two centers; one in tropical east Africa (approximately 80 species) and the other in southern Africa (approximately 70 species) (Balkwill and Balkwill, 1998). *Barleria albostellata* C.B. Clarke, also known as 'grey *Barleria*', is the plant of interest in this study (Figure 1.1), which under suitable conditions grows up to 1.5 meters in height. This shrub thrives in semi-shade to full sun in woodland areas of South Africa (Froneman and Le Roux, 2007). Under subtropical and tropical conditions, *B. albostellata* is evergreen. However, in colder regions it can become deciduous

to semi-deciduous (Froneman and Le Roux, 2007). The distribution of *B. albostellata* extends from Limpopo, Gauteng and Mpumalanga to Durban (Figure 1.2). The genus name *Barleria* was derived from a French botanist and Dominican monk, Jacques Barrelier (Froneman and Le Roux, 2007).

The species name *albostellata* (Latin) means 'having white-like hairs which completely covers the leaves and stems' (Figure 1.1 C). This shrub flourishes from September to May and beautiful white flowers emerge sporadically (Figure 1.1 A). Flowers appear from a dense compound inflorescence and are surrounded by four leafy-bracts (Balkwill and Balkwill, 1997). The blossoming flowers are white in colour and have a tinge of purple on the bracts. In comparison to the flowers, the leaves are grey-green and have an abundant amount of velvety hairs. This plant develops fairly fast and attains maturity in about three years (Froneman and Le Roux, 2007; Figure 1.1 B).

Table 1.1 Taxonomic hierarchy of *Barleria albostellata*

Classification	**Name**
Kingdom	Plantae
Sub-kingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Sub-class	Asteridae
Order	Scrophulariales
Family	Acanthaceae
Genus	*Barleria*
Species	*Barleria albostellata*

Figure 1.1: *Barleria albostellata* growing alongside a pathway at the University of KwaZulu-Natal, Westville Campus. (A) White, tubular flowers emerge sporadically in spring and summer; (B) Mature plants grow up to 1.5 meters within 3 years; (C) Leaves and stems are covered completely with velvety greyish-white hairs.

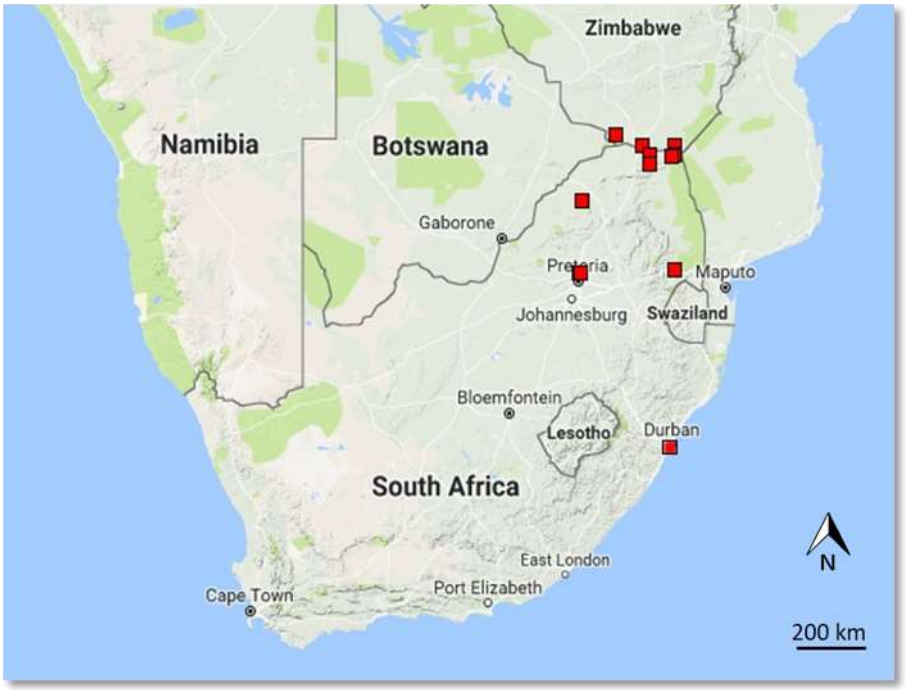

Figure 1.2: Distribution map of *B. albostellata* in southern Africa. Image adapted from the South African National Biodiversity Institute (SANBI): Botanical Research and Herbarium Management Software website. (http://newposa.sanbi.org/sanbi/Explore).

1.6 Ethnomedicinal uses of the genus *Barleria*

Barleria albostellata contains medicinal properties which was verified by Amoo et al. (2009). It was found that several extracts from this plant exhibited excellent anti-inflammatory properties and a broad-spectrum of antibacterial activity. This plant has a relatively high flavonoid content, with an added effect from tannin and iridoid compounds (Amoo, 2011). Although *B. albostellata* has no recorded practice in traditional medicine, many species of *Barleria* have been used in traditional medicine and were confirmed to contain various compounds possessing biological effects such as analgesic, anti-inflammatory, antileukemic, antihyperglycemic, antitumor, anti-amoebic, antibiotic and virucidal activities (Yosook et al., 1999; Wang et al., 2001; Jassim and Naji, 2003; Suba et al., 2004, 2005; Chomnawang et al., 2005). Traditionally, there is great medicinal potential for the genus *Barleria*. Thus, further investigation on *B. albostellata* is important as this will provide baseline information on the potential usage of extracts from this plant. This information is important in establishing the safe and efficient use of this plant in traditional medicinal practices.

1.7 Rationale of the study

Certain species within *Barleria* have been extensively examined for their pharmacognostic and phytochemical properties but few are micromorphologically evaluated for the secretory mechanisms by which such phytochemicals are synthesized and exuded. Secretory structures documented within *Barleria* include non-glandular and glandular peltate and capitate trichomes. Foliar trichomes derive from epidermal cells and may differ in size, appearance and distribution in various plant species (Werker, 2000). Glandular trichomes secrete bioactive compounds which can be responsible for the therapeutic properties of the plant. Categorizing these therapeutic compounds is imperative, as is understanding the mode of action of these plant structures in which they are confined and secreted (Naidoo et al., 2009).

Preliminary research has been conducted on the antibacterial properties of *B. albostellata* leaves and stems. Crude extracts positively inhibited both Gram-positive and -negative bacteria (Amoo et al., 2009). Antioxidant potential, cytotoxicity assays and the synthesis of nanoparticles from plant extracts of *B. albostellata* and analysing its antibacterial activity has not been attempted previously. In this regard, the study was novel. Little or no investigations have been undertaken to evaluate the micromorphology secretory mechanisms, through which such phytochemicals are synthesized and secreted. If structure is linked to function (Zimmerman and Brown, 1971; McNeil et al., 1984), then it is probable that microscopic investigations of the leaves and stems of *B. albostellata* could provide a better understanding of the potential therapeutics of this plant? Therefore, this study aimed to provide a greater insight into the structure, chemistry and biology of *B. albostellata*. Preliminary research conducted in this study will contribute to the South African medicinal plant database.

1.8 Aims and objectives

1.8.1 Chapter 3

Aim: To examine the micromorphology, distribution and chemical composition of trichomes present on the leaves and stems of *B. albostellata*.

Objective 3.1: To characterize the micromorphology distribution and ultrastructure of the foliar trichomes on leaves and stems using light, stereo, scanning and transmission electron microscopy.

Objective 3.2: To evaluate the changes in trichome density that occur in the stems and emergent, young and mature leaves using stereo and scanning electron microscopy.

Objective 3.3: To locate the site and composition of chemical compounds produced by trichomes using a range of histochemical tests with light and fluorescence microscopy.

1.8.2 Chapter 4

Aim: To examine the floral biology and pollen micromorphology of *B. albostellata* using stereo and scanning electron microscopy.

Objective 4.1: To provide a taxonomical description of the different floral structures present in *B. albostellata.*

Objective 4.2: To identify the type of pollen present in *B. albostellata* and compare it to other species within the genus.

1.8.3 Chapter 5

Aim: To investigate the phytochemical composition and antibacterial efficacies of the leaves and stems of *B. albostellata*.

Objective 5.1: To identify the presence and composition of phytochemical compounds produced using phytochemical tests and thin-layer chromatography (TLC).

Objective 5.2: To identify the presence of chemical compounds using powdered florescence microscopy.

Objective 5.3: To characterize key chemical compounds in crude extracts (hexane, chloroform and methanol) using gas chromatography-mass spectrometry (GC-MS) analysis.

Objective 5.4: To examine the antibacterial activity of leaf and stem crude extracts (hexane, chloroform and methanol) against various pathogenic strains of bacteria.

1.8.4 Chapter 6

Aim: To examine the antioxidant properties and cytotoxic effects of the crude extracts of *B. albostellata*.

Objective 6.1: To conduct a biological evaluation of the antioxidant properties present in the leaves and stems crude extracts using 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH), Ferric reducing antioxidant power assay (FRAP), total phenolic and flavonoid assays.

Objective 6.2: To perform an in vitro biological evaluation of the cytotoxicity activity of the crude extracts using agar well diffusion, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) against various tumour cell lines.

1.8.5 Chapter 7

Aim: To biosynthesize, characterize and evaluate the chemical nature and bioactivity of silver nanoparticles (AgNPs) using leaf and stem extracts (methanol, fresh and powdered) of *B. albostellata*.

Objective 7.1: To biosynthesize AgNPs using leaf and stem crude extracts.

Objective 7.2: Characterize AgNPs using UV–visible spectroscopy, scanning- high resolution transmission electron microscopy, energy-dispersive X-ray analysis, Fourier transform infrared spectral analysis.

Objective 7.3: Characterization of AgNPs and their nanocomplexes by measuring the zeta potential and sizing using NTA analysis.

Objective 7.4: To examine the antibacterial activity of synthesized AgNPs from leaf and stem extracts against various pathogenic strains of bacteria.

Objective 7.5: To perform an *in vitro* biological evaluation of the cytotoxicity activity of the crude extracts synthesized AgNPs using agar well diffusion, and 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay *in vitro*.

1.8.6 Chapter 8

Aim: To provide an overall conclusion and recommendations for future research.

1.9 Outline of dissertation

An overview on the background information of traditional medicine, the selected plant species *B. albostellata* (indigenous to South Africa), along with the aims and objectives of the study is described in Chapter 1. Chapter 2 provides an in depth understanding of the family, genus and species of *B. albostellata* along with the secretory structures present in the plant. Chapter 3 focuses on the foliar micromorphology, ultrastructure and histochemical analysis of the secretory structures present on the

leaves and stems of *B. albostellata*. These secretory structures are analyzed using various microscopy techniques. Chapter 4 focuses on evaluating and describing the floral biology of *B. albostellata* using stereo- and scanning electron microscopy. Chapter 5 evaluates the organoleptic and phytochemical properties, thin layer chromatography, fluorescence analysis, energy dispersive X-ray spectroscopy, gas chromatography-mass spectrometry and antibacterial activity of the leaf and stem extracts. Chapter 6 examines the total flavonoid, phenolic contents, antioxidant and cytotoxicity activities of the crude hexane, chloroform and methanol extracts at various concentrations. Chapter 7 involves the synthesis, characterization, antibacterial and cytotoxicity activities of leaf and stem extracts synthesized AgNPs. Chapter 8 provides a general conclusion and recommendations for future research.

1.10 Outline of dissertation methodologies

Comprehensive methodologies for each chapter are outlined accordingly in the various chapters. A basic overview of the research methodologies is as follows:

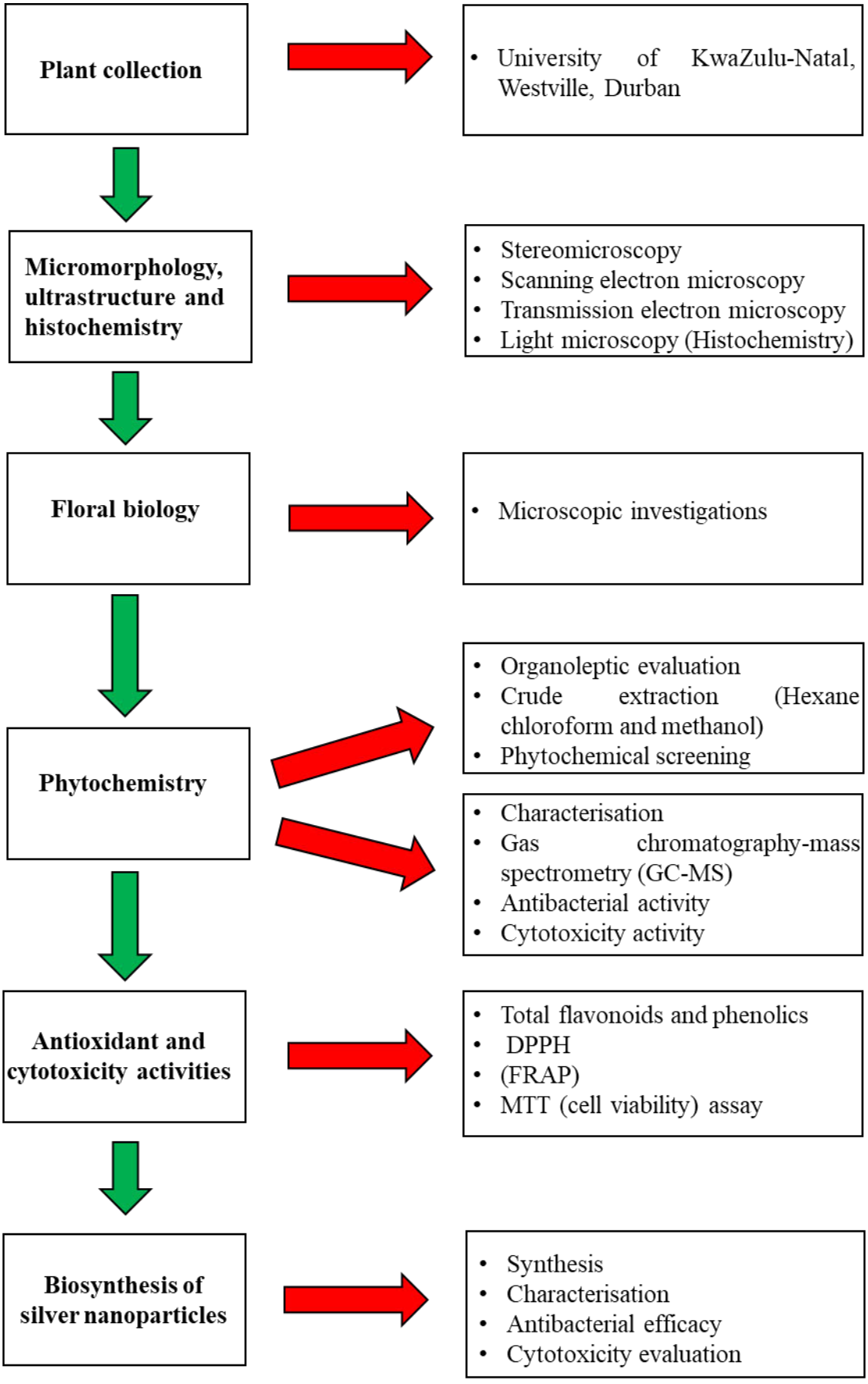

Figure 1.3: Diagrammatic representation of methodologies completed in the study.

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CHAPTER 2

REVIEW OF THE GENUS *Barleria* AND SPECIES *albostellata*: BIOLOGY, TAXONOMY, PHYTOCHEMISTRY, ETHNOPHARMACOLOGY, PHARMACOLOGY AND MORPHOLOGY

Abstract

Plant species belonging to the family Acanthaceae are globally known to possess various medicinal properties and have cultural and economic importance in both traditional medicine and horticulture. The plant kingdom contains various novel biologically active compounds, several of which could potentially have a greater medicinal value when compared to current medications. They are important to both animals and humans and are used as food or for ornamental purposes worldwide. *Barleria* is the third largest genus in the family Acanthaceae. A few of the highly important and reported species of *Barleria* include *B. prionitis*, *B. cristata*, *B. grandiflora*, and *B. lupulina*. The flowers, leaves, stems, roots, and seed extracts of plants belonging to this genus are rich in bioactive compounds and have exhibited significant medicinal potential for the treatment of various ailments and infections. Evidence derived from several studies has demonstrated the antioxidant, antibacterial, antifungal, anti-inflammatory, anticancer, antidiabetic, an-tiulcer, hepatoprotective, analgesic, antiamoebic, antihelminthic, antiarthritic, antihypertensive, and antiviral properties, in addition to inhibition of acetylcholinesterase activity and biosynthesis of nanoparticles, of the plant and seed extracts of species belonging to *Barleria*. Traditionally, the genus *Barleria* has significant medicinal potential; however, there is a scarcity of information on various species that are yet to be evaluated. This review provides a comprehensive report on existing literature, concerning the taxonomy, phytochemistry, biological activities and morphological studies of the genus *Barleria* and species *albostellata*.

Keywords: Acanthaceae; *Barleria*; Bioactive Compounds; Medicine; Trichomes

2.1 Introduction

Since prehistoric times, man has used herbal products, such as plants, in medicines to improve and treat diseases. Fossil records traced the usage of plants in medicine at least 60 000 years back (Fabricant and Farnsworth, 2001; Shi et al., 2010). Evidence of mankind's dependence on medicinal plants and their uses are found in ancient documented pharmacopeia's (Page et al., 1997; Cragg and Newman, 2001). The use of herbal medicines has presented a great challenge to early humans. It is highly likely that when pursuing for food, early humans consumed venomous plants, which resulted in diarrhoea, vomiting, or other poisonous reactions and possibly even death. However, in this manner, early humans acquired knowledge about natural medicines and edible materials (Gao et al., 2007).

The increasing use of medicinal plants in various cultures has prompted scientific studies into natural products. These studies are aimed at evaluating whether their traditional practices are supported with pharmacological effects or if their use is simply based on folklore (Sparg et al., 2002). Due to the growing interest in the use of traditional medicine, it is essential to meet some of the concerning challenges such as: the overall lack of research, evidence of safety, efficiency and good quality of natural products, patenting rights and, the need to maximize and integrate natural products as possible sources of remedies in primary health care. These challenges need to be quantified for the appropriate use of traditional medicine in modern therapeutics (Gamaniel and Jsselmuiden, 2004; Muhammad and Awaisu, 2008).

Plants have been extensively used for medicine, along with their availability (>250 000 higher plant species), there is a great likelihood of discovering beneficial bioactive compounds in the plant kingdom (Newman and Cragg, 2007). A single herb in traditional medicine may contain several phytochemical compounds, such as alkaloids, flavonoids, phenols, terpenoids etc. These chemicals can act alone or in combination with one another to deliver the desired pharmacological impact (Parasuraman et al., 2014). Through advances in the therapeutic principles, theoretical background, associated technologies, and knowledge of life sciences, a deeper understanding of the role of bioactive compounds in traditional medicine has become possible (Dong, 2013).

Acanthaceae is a large family of dicotyledonous flowering plants. Many plant species of Acanthaceae possess great therapeutic potential, whilst some are unexplored to-date (Khan et al., 2017). Plant species of this family play an important role to both man and animals as they are used for food, medicine or as ornamentals (Fongod et al., 2013; Koekemoer et al., 2014; Kar et al., 2017) and contain many essential secondary metabolites, some include, alkaloids, terpenoids, phenols, tannins, quinones and flavonoids (Khan et al., 2017). Several plant species are being utilized for their ethnomedicinal properties based on their phytocompounds they acquire, with *Barleria* (Acanthaceae) being one of such genera.

Several species within *Barleria* possess several pharmacological properties including, anti-inflammatory, antimicrobial, antiviral and anticancer activities (Yosook et al., 1999; Wang et al., 2001; Jassim and Naji, 2003; Suba et al., 2004[a], 2004[b], 2005; Chomnawang et al., 2005; Shukla and Gunjegaokar, 2018). There is very limited documented information on *Barleria albostellata* (C.B. Clark). There is no formal pharmacopoeia outlining the ethnobotanical use of the plant and distribution and the ethnobotanical records are either scarce or inconsistent. The current study aims at reviewing the taxonomy, phytochemistry, ethnopharmacology, pharmacology and morphology studies of the family Acanthaceae, genus *Barleria* and species *albostellata* using existing literature.

2.2 Taxonomy and historical review

The family name "Acanthus" originated from the Greek word, "acanth-" which meant "thorny" or "spiny" (Stearn, 1983) which, was consistent with the hairy, thorny or spiny appearance of most members of this family. Acanthaceae is a large, diverse angiosperm family of dicotyledonous plants that are distributed throughout tropical and subtropical regions (Mabberley, 1997; McDade et al., 2000; Xu and Chang, 2017). This family is distributed mainly in Africa and Asia (Balkwill and Balkwill, 1998; Darbyshire, 2010) and comprises of 250 genera and approximately 3520 species (McDade, 1984; Bhatt et al., 2010; Xu and Chang, 2017). Acanthaceae is known as the third largest family after Myrtaceae and Melastomataceae (Grant, 1955). At least 159 species of Acanthaceae were recorded by the International Union for the Conservation of Nature (IUCN) as endangered or critically endangered and 130 were vulnerable or near-threatened (IUCN, 2018). In South Africa, there are approximately 43 genera with 373 known species located in summer-rainfall and semi-arid areas (Balkwill and Welman, 2000; Koekemoer et al., 2014).

There are various estimated infrafamilial classifications for Acanthaceae within the Lamiales *sensu lato* (McDade et al., 2000). Lindau (1895) separated the family into subfamilies; Acanthoideae, Nelsonioideae, Thunbergioideae and Mendoncioideae. In order to delimit the four subfamilies from each other, one would consider the presence or absence of the retinacula, retinacula ovule numbers and shape. Genera grouped under Acanthoideae were characterised by retinaculate fruits. Genera that lacked retinaculate fruits were categorised as Nelsonioideae, Thunbergioideae and Mendoncioideae (Lindau, 1895). Based on the shared existence of a type of fruit found among angiosperms, Acanthaceae *sensu strict* has been accepted widely as a monophyletic group (McDade et al., 2000). The exact delimitation of the family has been controversial due to the appearance of three smaller lineages that do not share this type of fruit but appears to be evidently connected with Acanthaceae (McDade et al., 2000).

These small lineages are Mendoncioideae, Thunbergioideae and Nelsonioideae (McDade et al., 2008). Scotland and Vollesen (2000) used representative genera and generated a combined morphological matrix tree (Figure 2.1). Three distinct subfamilies, namely Nelsonioideae, Thunbergioideae and Acanthoideae were illustrated. Acanthoideae was divided into two tribes namely Acantheae and

Ruellieae. Pollen morphology was used to further classify taxa within the two groups. There were eleven types of pollen grains that were identified. Acanthaceae is known for the remarkable diversity of its pollen shape, size, exine structure, apertures, and ornamentation (Graham, 1988; Daniel, 1998; Bhatt et al., 2010; Choopan and Grote, 2015; House and Balkwill, 2017). Ruellieae and Barlerieae were classified under the group, Contortae. Within the Contortae group, mature "ribbed", "spiky", "sponge", and/or "honeycomb" pollen occurred. Ruellieae and Barlerieae were documented as having "honeycomb pollen" (Lindau, 1895). The latter tribe (Ruellieae) was grouped into five smaller subtribes. The genus *Barleria* was placed within the subtribe Barlerieae (Scotland and Vollesen, 2000). Cystoliths (also known as calcium oxalate crystals) are generally present in the tribe Ruellieae and the genus *Barleria*. These structures are identifiable on the lamina of dried leaves as white streaks (Clarke, 1885; Balkwill and Welman, 2000; Scotland and Vollesen, 2000; Shendage and Yadav, 2010; Sridharan and Gounder, 2016). In *Barleria*, cystoliths are found in the epidermal tissue, and are frequently arranged and paired in groups (Ahmad, 1975).

Cystoliths are defined as silicified bodies that contain cellulosic skeletons, which are characteristic of many Acanthaceae species (Metcalfe and Chalk, 1950; Lawrence, 2008; Gal et al., 2012). These crystals form an essential component in the taxonomic account of Acanthaceae, as this is the only family found within the Lamiales that comprise of cystoliths (Metcalfe and Chalk, 1950; Scotland and Vollesen, 2000). They differ in nature, size, shape, colour, and occurrence throughout the family and can occur singly, double or triple (Patil and Patil, 2011). Cystoliths, can serve as a deterrent against insects and large grazing herbivores, and the quantity of the crystals can determine defence resilience and efficiency (da Costa et al., 2009). The occurrence of cystoliths and stomata which are caryophyllaceous in Acanthaceae, is a diagnostic characteristic to the family (Inamdar, 1970; Metcalfe and Chalk, 1950; Scotland and Vollesen, 2000; Patil and Patil, 2011; Choopan and Grote, 2015).

Plants in this family demonstrate a thriving diversity of ecological and morphological characteristics. These include a large range of pollinator relationships and floral morphologies (McDade et al., 2000; Daniel et al., 2008; McDade et al., 2018). The Acanthaceae has cultural and economic importance in both traditional medicine and horticulture. Plant species within Acanthaceae are important to both animals and man since they are used as food, medicine or as ornamentals worldwide (Fongod et al., 2013; Koekemoer et al., 2014; Kar et al., 2017). The ethnobotanical uses of several South African plant families and/or species among the Zulu, Xhosa and Sesotho cultures, were investigated by Hutchings (1989). He found that various species within Acanthaceae were used in traditional medicine by these cultures.

This family has great therapeutic potential (Ismail et al., 2017), mainly due to the presence of alkaloids (Sharma and Kumar, 2016) and phenolics (Matos et al., 2018) in the leaves. The leaves, bark and roots of plant species in Acanthaceae are used for treating numerous ailments (Kosmulalage et al., 2007;

Tamboli and More, 2016[a]). Many plants of this family were reported to possess pharmacological activities such as antimicrobial, antidiabetic (Janakiraman et al., 2014), anticancer (Komalavalli et al., 2014), hepatoprotective (Kirtane et al., 2013), anti-inflammatory (Desu et al., 2011) and anti-pyretic (Ismail et al., 2017).

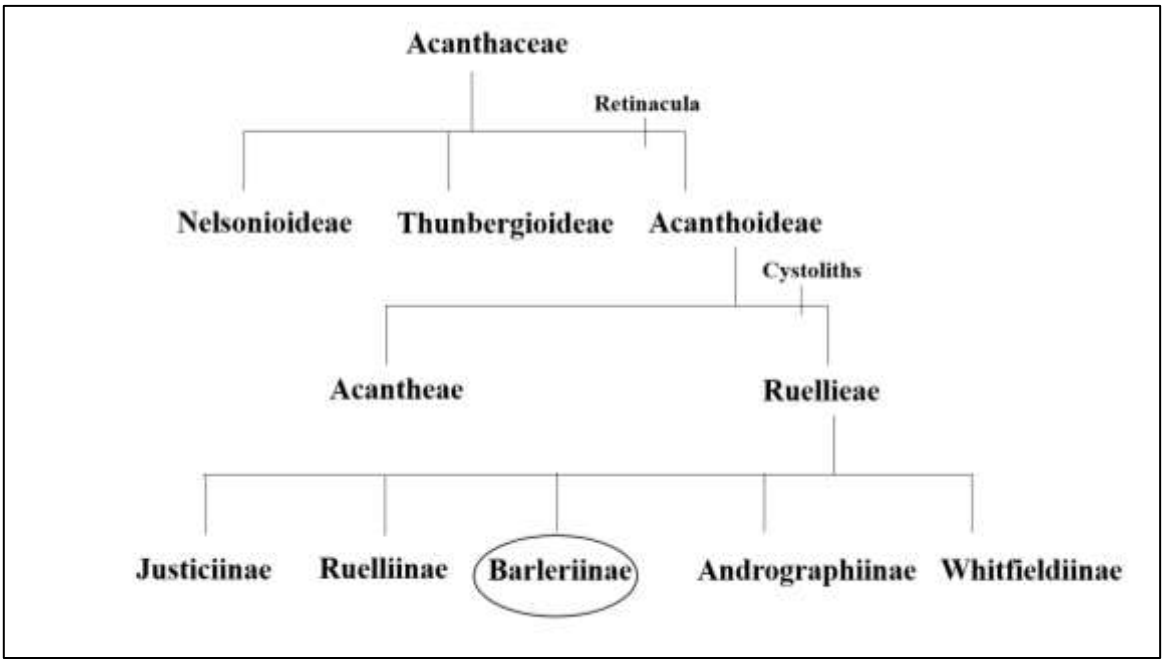

Figure 2.1: Taxonomy of Acanthaceae sensu adapted from Scotland and Vollesen, 2000 and Manktelow et al., 2001.

2.3 The Genus *Barleria*

2.3.1 Taxonomy and distribution of *Barleria*

The genus name *Barleria* was given by a French botanist and Dominican monk, Jacques Barrelier (1606-1673), who dedicated his spare time to botany (Pooley, 2005). *Barleria* is the third largest genus in the family Acanthaceae after *Justicia* and *Ruellia* (Grant, 1955; Balkwill and Balkwill, 1997; Kumar et al., 2018) and is the most species-rich genus in *Barlerieae* (Darbyshire et al., 2019). This genus has approximately 300 species of shrubs and herbs that are distributed in the subtropical and tropical regions of the world (Mabberley, 2008; Darbyshire, 2010; Darbyshire, 2015; Kumari et al., 2017; Al-Hakimi et al., 2018). Members of this genus originated from the Far East of Japan, through southern Asia, Arabia, India, Africa, Madagascar to as far west of Central America and Mexico (Balkwill and Balkwill, 1998; Kumari et al., 2017). *Barleria* is predominantly an 'Old World genus' (a term used in the West to refer to Africa, Asia and Europe), with its greatest species diversity in east tropical Africa followed by South Africa (Balkwill and Balkwill, 1998). The distribution of *Barleria* throughout Africa is illustrated in Figure 2.2. In southern Africa, there are 70 species of *Barleria* of which approximately 65% are endemic to the region (Singh et al., 2015[b]; Al-Hakimi et al., 2018).

2.3.2 Biological description

Barleria is easily distinguishable from other genera within Acanthaceae on the basis of three features: (i) a four-partite calyx comprising of two outer large segments and two smaller inner ones; (ii) globular, honeycombed pollen, and (iii) the prevalence of double cystoliths located in the epidermal cells (Balkwill and Balkwill, 1998; Darbyshire, 2010; Champluvier, 2011). The fruits of *Barleria* are hygrochastic (Hughes et al., 2007), meaning that the opening of the fruit is initiated by moisture or water (Bremekamp, 1926; Martínez-Berdeja et al., 2015). In *Barleria*, cystoliths are always double and lie in two adjacent cells. These structures are scattered over the leaf lamina and often lie parallel on the midrib (Obermeijer, 1933; Bhogaonkar and Lande, 2012; Tripp and Fekadu, 2014). Several species of *Barleria* are recognized for their medicinal or ornamental values (Kumar and Singh, 2013; Tamboli and More, 2016[c]; Kumar et al., 2018).

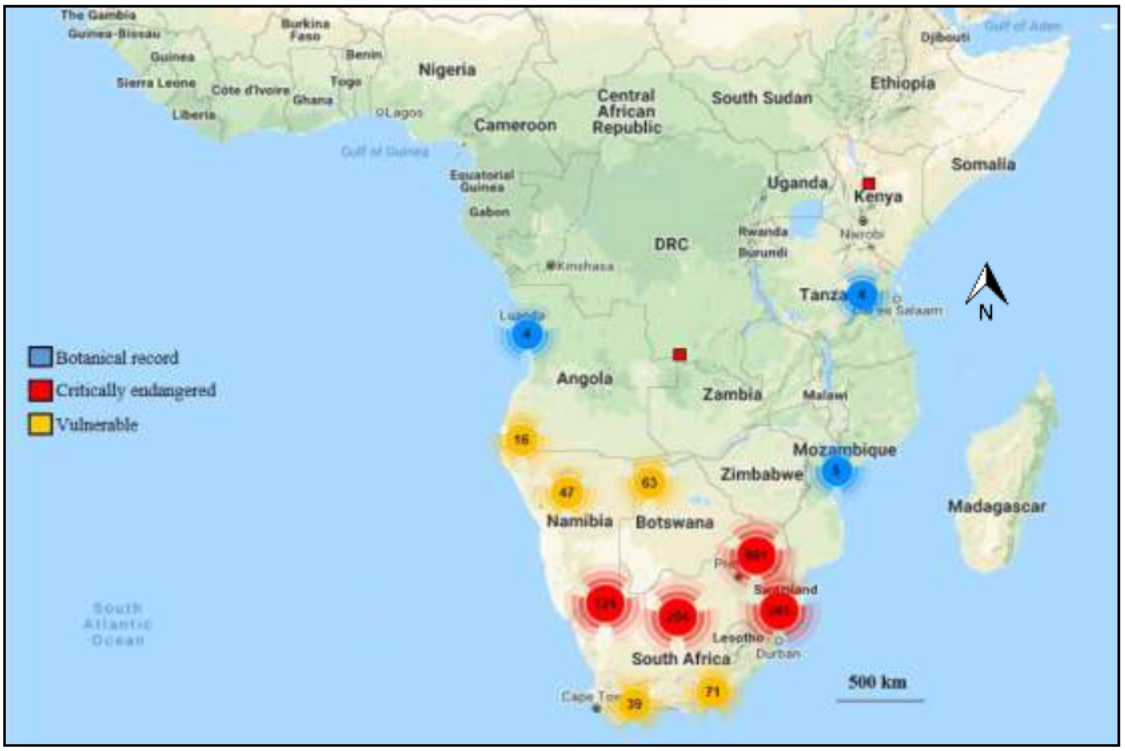

Figure 2.2: Distribution map of species of *Barleria* in Africa. Adapted from SANBI BRAHMS online (http://newposa.sanbi.org/sanbi/Explore).

2.3.3 Phytochemicals isolated from *Barleria*

Plants have the ability to synthesize various secondary metabolites, among which at least 12,000 already have been isolated (Cowan, 1999). It is important to determine the relationship between the phytochemical compounds of a medicinal plant and its pharmacological activity. A few of the highly important species of *Barleria* includes *B. greenii*, *B. prionitis*, *B. cristata*, *B. albostellata*, *B. grandiflora B. strigose*, *B. gibsoni* and *B. tomentosa* and *B. lupulina* (Kumar and Singh, 2013; Kumari et al., 2017). Several authors have reported that species belonging to this genus exert biological effects including antibacterial, antifungal, anti-inflammatory, anticancer, antidiabetic, antiulcer, hepatoprotective, analgesic, anti-amoebic, antihelminthic, antiarthritic, antihypertensive, antiviral activities and inhibition of acetylcholinesterase activity (Yosook et al., 1999; Wang et al., 2001; Jassim and Naji, 2003; Suba et al., 2004[a], 2004[b], 2005; Chomnawang et al., 2005; Shukla and Gunjegaokar, 2018).

Studies have reported that bioactive compounds such as quercetin, quinones, iridoid, iridoid glycosides, immunostimulant protein 'sankaranin' and antibiotics isolated from *Barleria* species are responsible for the above mentioned biological activities (Amoo et al., 2009; Ata et al., 2009; Gambhire et al., 2009[a]; Chetan et al., 2011; Chowdhury et al., 2013). Jäger et al. (1996) suggested that when bioactive compounds are detected in one plant species, it is possible that numerous species of the same genus contain active compounds of a comparable nature. It has been reported that *Barleria* consists of various secondary metabolites that have been primarily isolated from the flowers, leaves, stems, roots, and seeds of the plant (Table 2.1). The important phytochemical compounds isolated from *Barleria* are iridoids, phenolic acids/glycosides/lignans, flavonoids, terpenoids, and phytosterols (Table 2.1).

Table 2.1 Chemical compounds isolated compounds from the Genus *Barleria*

Plant species	Plant part	Chemical Group	Chemical Compounds/ Phytoconstituents	Reference
B. acanthoides	Whole	Phenolic glycosides	Barlerisides A	Karim et al., 2009
			Barlerisides B	
		Caffeoyl phenylethanoid glycoside	Verbascoside	
		Phenolic acid	*p*-hydroxycinnamic acid	
		Neolignan diglycoside	Barlericin	Karim et al., 2010
B. cristata	Whole	Aromatic compound	4-hydroxy-*trans*-cinnamate derivatives	Chowdhury et al., 2013
		Terpenoid	Oleanolic acid	
	Bark	Flavonoid	6-*O*-α-L-rhamnopyranoside-3,7,3'-*O*-trimethylated-8-hydroxyquercetin	Salib et al., 2013
			6-*O*-α-L-rhamnopyranoside quercetagetin	
			3-methoxyquercetin	
			Gossypetin 8-methyl ether	
			Quercetagetin	
			Tamarixetin	
			Gossypetin	
			Quercetin	
	Leaves	Phenolic acids	*p*-Coumaric acid	Hemalatha et al., 2012
			α-Tocopherol	
		Flavonoid	Luteolin	
			7-methoxy luteolin or 2-(4,5-dihydroxy phenyl)-5-hydroxy-7-methoxy-4*H*-chromen-4-one	
		Iridoid glycosides	Barlerin	
			Shanzhiside methyl ester	
		Phenylethanoid glycosides	Desrhamnosyl acteoside	El-Mawla et al., 2005
			Poliumoside	
			Acteoside (verbascoside)	
B. dinteri	Leaves	Iridoid glycosides	Barlerin	Gololo et al., 2017
B. lupulina	Aerial		8-*O*-acetylipolamiidic acid	Damtoft et al., 1982; Byrne et al., 1987; Tuntiwachwuttikul et al., 1998; Kanchanapoom et
			8-*O*-acetyl-6-*O*-(*p*-methoxy-*cis*-cinnamoyl)shanzhiside	
			8-*O*-acetyl-6-*O*-(*p*-methoxy-*trans*-cinnamoyl) shanzhiside	

			6-*O*-*p*-methoxy-*cis*-cinnamoyl-8-*O*-acetylshanzhiside methyl ester	al., 2001; Lans et al., 2001; Suksamrarn et al., 2003; Widyowati et al., 2010
			6-*O*-*p*-methoxy-*trans*-cinnamoyl-8-*O*-acetylshanzhiside methyl ester	
			6-*O*-*p*-cis-coumaroyl-8-*O*-acetylshanzhiside methyl ester	
			6-*O*-*p*-*trans*-coumaroyl-8-*O*-acetylshanzhiside methyl ester	
			Ipolamiide	
			Ipolamiidoside	
			Shanzhiside	
			Shanzhiside methyl ester	
			8-*O*-acetylshanzhiside	
			Barlerin	
			6-*O*-acetylshanzhiside methyl ester	
			Acetylbarlerin	
			Mussaenosidic acid	
			Phlorigidoside	
		Iridoid diglucoside	Lupulinoside	
		Phenylethanoid glycosides	Forsythoside	
			Poliumoside	
		Lignan glucosides	(+)-lyoniresinol 3-α-*O*-β-glucopyranoside	
		Fatty acyl glycoside lipid molecule	1-octen-3-yl-β-primeveroside	
		Organic compound/ O-glycosyl compounds	Benzyl β-primeveroside	
B. noctiflora	Leaves	Phenylethanoid glycoside	Barlerinoside	Yadav et al., 2016
B. prionitis	Aerial	Terpenoid	Balarenone	Kosmulalage et al., 2007
		Phenylethanoid glycoside	Barlerinoside	Ata et al., 2009
		Caffeoyl phenylethanoid glycoside	Verbascoside	Chen et al., 1998
		Iridoid glycosides	Barlerin	Ata et al., 2009; Shukla and Gunjegaokar, 2018
			Acetylbarlerin	

			Shanzhiside methyl ester	Chen et al., 1998
			6-*O*-*trans*-*p*-coumaroyl-8-*O*-acetylshanzhiside methyl ester	
			6-*O*-*cis*-coumaroyl-8-*O*-acetylshanzhiside methyl ester	
			7-methoxydiderroside	Ata et al., 2009
			Lupulinoside	
		Terpenoid	Pipataline	Kosmulalage et al., 2007
			Lupeol	
		Phytosterols	13,14-seco-stigmasta-5,14-diene-3-β–ol	
	Roots		β-sitosterol	Singh and Gupta, 2016
	Aerial	Flavonoid	Apigenin 7-*O*-β-D-glucoside	Kosmulalage et al., 2007; Mabry et al., 2012; Taneja and Tiwari, 1975; Damtoff et al., 1982
	Leaves		6-hydroxyflavone	Daniel, 2006
			Scutellarin	
	Aerial		Luteolin-7-*O*-β-D-glucoside	Gupta and Saxena, 1984
	Leaves	Phenolic acid	Melilotic acid	Daniel and Sabnis, 1987
			Syringic acid	Daniel, 2006
			Vanillic acid	
			p-hydroxybenzoic acid	
B. strigosa	Whole plant	Phenylethanoid glycoside	4-hydroxyphenylethyl 4-*O*-β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranoside	Kanchanapoom et al., 2004
		Caffeoyl phenylethanoid glycoside	Verbascoside	
		Iridoid glycoside	10-*O*-*trans*-coumaroyl-eranthemoside	
			Isoverbascoside	
			Decaffeoylverbascoside	
			Lyoniresinol 3 α -*O*- β-D-glucoside	
			7-*O*-acetyl-8-epi-loganic acid	
			(3R)-1-octen-3-ol-3-*O*-β-D-xylosyl-(1→6)-β-D-glucoside	
		Flavonoid	Apigenin 7-*O*- α -Lrhamnosyl-(1→6)-*O*- β-D-glucoside	
B. trispinosa	Aerial	Iridoid glycosides	6-α-L-rhamnopyranosyl-8-*O*-acetylshanzihiside methyl ester	Harraz et al., 2009
			Acetyl barlerin	

	Barlerin
	Shanzhiside methyl ester

2.3.3.1 Iridoids

Chemical compounds such as iridoids are monoterpenes that are biosynthesized from isoprene and are also identified to be precursors in the biosynthesis of alkaloids (Didna et al., 2007; Tundis et al., 2008; Amoo et al., 2011; Salim et al., 2013). Like glycosides, iridoids are generally found in various medicinal plants and are most often bound to glucose (Didna et al., 2007; Tundis et al., 2008). Iridoids that are isolated and purified exhibit a broad spectrum of bioactivities, including choleretic, antihepatotoxic, cardiovascular, hypoglycemic, anti-inflammatory, antiviral, antimutagenic, antitumor and analgesic activities (Didna et al., 2007; Tundis et al., 2008). Some important iridoid medicinal compounds found in *Barleria* include, *Barlerin*, Shanzhiside methyl ester, ipolamiide, acetylbarlerin, phlorigidoside, lupulinoside, 7-methoxydiderroside, isoverbascoside, decaffeoylverbascoside, 10-*O*-*trans*-coumaroyl-eranthemoside (Table 2.1).

2.3.3.2 Phenolic compounds (acids/ glycosides/lignans/neolignans)

Phenolic acids are natural compounds that are prevalent throughout the plant kingdom. They are involved in a variety of biological activities, namely antimicrobial, anti-inflammatory, antioxidant, antidiabetic, hepatoprotective and anticancer properties (Marcucci et al., 2001; Polya, 2003; Hosseinimehr et al., 2007; Saibabu et al., 2015; Amoo et al., 2011). Phenolic acids can be categorized into benzoic acids that consist of seven carbon atoms and cinnamic acids that comprise nine carbon atoms (C6-C3). Phenolic compounds derived from plants are different in their molecular structure and are typically characterized by their hydroxylated aromatic rings (Mandal et al., 2010). In several plants, phenolic compounds are polymerized into large molecules such as lignins and proanthocyanidins (condensed tannins). The antioxidant capacity of phenolic compounds has attracted the attention of researchers, as these compounds can reduce the risk of developing several diseases and protect the human body from free radicals (Halliwell, 1996). The important phenolic acids found in *Barleria* included *p*-hydroxycinnamic acid, *p*-Coumaric acid, α-Tocopherol, melilotic acid, syringic acid, vanillic acid and *p*-hydroxybenzoic acid (Table 2.1). The phenolic glycosides found in this genus are barlerisides A and B (Table 2.1).

Lignans and neolignans are a group of large, naturally occurring phenols that are derivatives from the shikimic acid biosynthetic pathway and have a wide distribution within the plant kingdom (Bernini et al., 2009). Both compounds exhibit dimeric structures that are formed by a β,β'-linkage between the two phenyl propane units and a altered degree of oxidation in the side chain (Bernini et al., 2009). One of the main ecological functions of lignans, is protecting the plants that synthesize them against herbivores and microorganisms (Teponno et al., 2016). One lignan glucosides, (+)-lyoniresinol 3α -*O*-β-glucopyranoside, was isolated from the aerial plant parts of *B. lupulina* (Table 2.1). In *B. acanthoides*, one type of neolignan diglycoside, barlericin, was isolated from the entire plant (Table 2.1).

2.3.3.3 Flavonoids

Flavonoids are present in the leaves, flowers and pollen of several plants and comprise a group of polyphenolic compounds (Larson, 1988). Flavones, flavanones, flavonols, isoflavones, and anthocyanins are the main classes of flavonoids that have been reported to possess a broad spectrum of biological and therapeutic activities (Jucá et al., 2020). Studies have also reported that flavonoids or flavonoid-rich extracts exhibit antioxidant, antimicrobial and anti-inflammatory activities (Burda and Oleszek, 2001; Havsteen, 2002; Tunalier et al., 2007; Pattanayak and Sunita, 2008; Wu et al., 2015). Flavonoids play a vital role in iinhibiting the activity of important enzymes in mitochondrial respiration and protection against heart diseases (Aust et al., 2001). This compound has the potential to prevent early stages of cancer due to its ability to scavenge free radicals (Cushnie and Lamb, 2005). A total of 14 flavonoids (Table 2.1) have been isolated from various plant parts of *Barleria*, including 6-*O*-α-L-rhamnopyranoside-3,7,3'-*O*-trimethylated-8-hydroxyquercetin, 3-methoxy quercetin, 6-*O*-α-L-rhamnopyranoside quercetagetin, quercetagetin, , gossypetin 8-methyl ether, tamarixetin, gossypetin, quercetin, luteolin, 7-methoxy luteolin or 2-(4,5-dihydroxy phenyl)-5-hydroxy-7-methoxy-4*H*-chromen-4-one, apigenin 7-*O*-β-D-glucoside 6-hydroxyflavone, apigenin 7-*O*-α-Lrhamnosyl- (1→6)-*O*- β-D-glucoside, scutellarin, luteolin-7-*O*-β-D-glucoside.

2.3.3.4 Terpenoids

Terpenoids are the most frequent and structurally diverse organic compounds that are derived from five-carbon isoprene units (Zwenger et al., 2008). Terpenoids are classified based on the number of isoprene units, such as; hemiterpenoids (C5); monoterpenoids (C10), sesquiterpenoids (C15), diterpenoids (C20), sesterterpenoids (C25), and triterpenoids (C30) (Zwenger et al., 2008; Prakash, 2018). Several terpenoids are known for their secondary metabolites that important roles in plant-plant and plant–environment interactions (Yu and Utsumi, 2009; Dudareva et al., 2013). In addition, four terpenoid compounds, oleanolic acid, balarenone, pipataline and lupeol, were isolated from the aerial parts and the entire plant of *Barleria* (Table 2.1). The terpenoids isolated from plant extracts are known to possess antiviral, antifungal, antibacterial, anti-inflammatory, antihyperglycemic, anticancer and insecticidal properties (Thoppil and Bishavee, 2011).

2.3.3.5 Phytosterols

Phytosterols are an important family of lipids that are typically found in plants and fungi and are essential to humans because of their nutritional and medicinal value. Phytosterols also function as precursors in the production of essential bioactive compounds such as steroidal glycoalkaloids, steroidal saponins, brassinosteroids and phytoecdysteroids (Moreau et al., 2018). They are grouped into 24-ethylsterols and 24-methylsterols (Zhang et al., 2020). Some examples of 4-desmethylsterols that are abundantly found in most plants are campesterol, sitosterol and stigmasterol (dos Santos et al., 2014).

Only two isolated phytosterols have been reported in *B. prionitis*; 13,14-seco-stigmasta-5,14-diene-3-β –ol and β-sitosterol (Table 2.1).

2.3.3.6 Phenylethanoid glycosides and aromatic compounds

Phenylethanoid glycosides are a group of water-soluble compounds prsent in the plant kingdom, and the majority of them have been isolated from medicinal plants (Georgiev et al., 2011; Kirmizibekmez et al., 2012). This group is categorized by a phenethyl alcohol (C6-C2) moiety that is attached to a β-allopyranose by a glycosidic bond. Phenylethanoid glycosides have been described to possess novel structures with diverse bioactivities (Jimenez et al., 1994, Fu et al., 2008). Six phenylethanoid glycosides, viz., desrhamnosyl acteoside, poliumoside, acteoside, forsythoside, barlerinoside and 4-hydroxyphenylethyl 4-*O*-β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranoside have been isolated from serveal species of *Barleria*. However, only one caffeoyl phenylethanoid glycoside, verbascoside, was isolated from *B. acanthoides*, *B. prionitis* and *B. strigosa* (Table 2.1). The primary role of plant aromatic compounds is protection from insects and pathogens (Zemek et al., 1987). The main role of plant aromatic compounds is for protection fagainst insects and pathogens (Zemek et al., 1987). The only aromatic compound 4-hydroxy-*trans*-cinnamate derivatives found in *Barleria* was isolated from *B. cristata* (Chowdhury et al., 2013).

2.3.4 Biological activities of extracts, fractions and isolated compounds from *Barleria*

2.3.4.1 Antioxidant properties

Antioxidants are defined as substances that inhibit or delay oxidative damage to a specific molecule (Yildirim et al., 2001). Oxidative stress is a key contributor to various chronic diseases (Ames et al., 1993). It implies a disruption in the imbalance between reactive oxygen species (ROS), free radicals (FR) and the endogenous antioxidant defence mechanisms (McCord, 2000). When antioxidant molecules encounter single FR, they neutralise them by donating one of their own electrons, which in turn ends the carbon-stealing reaction (Sa´nchez-Moreno et al., 1998; Hyldgaard et al., 2012). The antioxidant defence mechanisms in plants exist as enzymatic and non-enzymatic. The enzymatic defence mechanism includes, catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD). These antioxidants efficiently alleviate cell damage against ROS. The non-enzymatic antioxidant mechanisms consists of carotenoids, vitamin C, vitamin E and flavonoids (Jacob, 1995; Willcox et al., 2004; Stepien and Klobus, 2005). There is substantial evidence that FR cause oxidative damage to biomolecules (nucleic acids, lipids and proteins), which eventually results in ageing, atherosclerosis, diabetes mellitus, cancer, acquired immunodeficiency syndrome (AIDS), inflammation and various degenerative diseases in humans (Choi et al., 2002). Plants are a source of natural antioxidants, including phenols, flavonoids, ascorbic acid, and carotenoids. Ascorbic acid and β -carotene are one of the widely used antioxidants (McCall and Frei, 1999).

The reported antioxidant properties of various extracts and isolated compounds of *Barleria* are summarized in Table 2.2. Various methods have been used to evaluate the anti-oxidant activities of aqueous, acetone, benzene, butanol, chloroform, dichloromethane, ethanol, ethyl acetate, hexane, hydroalcoholic, methanol, and petroleum ether extracts and those of the isolated compounds barlerisides A and B, 6-*O*-*trans*-*p*-coumaroyl-8-*O*-acetylshanzhiside, methyl ester, shanzhiside methyl ester, acetylbarlerin, barlerin, lupulinoside and 7-methoxydiderroside. Antioxidant activity was observed and reported in all plant extracts by several researchers using various assays. The most frequently investi-gated species within the genus is *B. prionitis*. Amoo et al. (2011) examined the methanolic extracts of the different parts of *B. prionitis* using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay and reported that the extracts exhibited free radical scavenging activity, with the EC_{50} values varying from 6.65 to 12 µg/ml. In addition, they evaluated the ferric reducing antioxidant power and the β-carotene bleaching rate of the extracts and found that the extracts reduced the ferric ion complex to the ferrous form and decreased the carotenoid bleaching rate. The findings of their study suggest the occurrence of antioxidant compounds in the methanol extracts, which are capable of donating electrons and hydrogen atoms in their reactions (Amoo et al., 2011). Moreover, Jaiswal et al. (2010) evaluated the total phenolic content (TPC), the β-carotene bleach-ing potential, and the hydroxyl radical scavenging activity of the ethanolic extracts of *B. prionitis*. They found the highest TPC of 67.48 ± 0.72 mg/g in the leaf extracts and a β-carotene bleaching rate of 79.20 ± 1.26% compared to those of flower and stem extracts. The leaf extract exhibited strong free radical scavenging activities, with the IC_{50} values be-ing 336.15 ± 7.21 µg/ml for DPPH and 568.65 ± 6.11 µg/ml for the hydroxyl radical. Various species within the genus *Barleria* exhibit excellent antioxidant properties. Therefore, the antioxidants found in *Barleria* plant extracts exhibiting free radical scavenging activities may play an important role as therapeutic agents in numerous diseases that are related to oxidative stress (Ramchoun et al., 2009).

Table 2.2: Antioxidant activity of extracts and compounds isolated from *Barleria*

Plant species	**Plant part**	**Extract/compound**	**Antioxidant activity/ models/assays**	**Reference**
B. acanthoides	Whole	Barleriside A and barleriside B	Superoxide scavenging activity and xanthine oxidase activity	Karim et al., 2009
B. albostellata	Leaves and stems	Methanol	DPPH, FRAP and β-Carotene-linoleic acid model system	Amoo et al., 2011
B. argillicola	Aerial and roots	Methanol	β-Carotene–linoleic acid model system, DPPH, TPC, TIC, TFC and CTC	Amoo and Van Staden, 2013
B. courtrallica	Leaves	Petroleum ether, benzene, ethyl acetate, methanol and ethanol	DPPH, hydroxyl radical scavenging activity, superoxide radical scavenging activity, ABTS and FRAP	Sujatha et al., 2018
B. cristata	Leaves	Ethanol and aqueous	DPPH, superoxide anion and nitric oxide radical scavenging activity and hydrogen peroxide scavenging activity	Amutha and Doss, 2012
	Leaves	Ethanol and aqueous	DPPH, ABTS and TPTZ	Narmadha and Devaki, 2012
	Leaves	Hexane, chloroform, acetone and methanol	DPPH, nitric-oxide reducing and FRAP	Pathy et al., 2015
	Leaves	Ethanol and petroleum ether	DPPH and FRAP	Vasanth et al., 2018
B. dinteri	Leaves	Hexane, dichloromethane, acetone and methanol	DPPH	Gololo et al., 2016
B. gibsoni	Leaves	Ethanol	TPC (gallic acid equivalence), TFC, DPPH and nitric oxide radical scavenging activity	Tamboli and More, 2016[b]
B. grandiflora	Leaves	Aqueous and hydro-alcoholic	FTC method and TBA method	Sawarkar et al., 2009

B. greenii	Leaves, stems and roots	Methanol	DPPH, FRAP and β-Carotene-linoleic acid model system	Amoo et al., 2011
B. lupulina	Leaves and stems	Methanol and aqueous	TPC (colorimetric) and DPPH	Kumari et al., 2017
B. montana	Leaves	Aqueous and ethanol	DPPH, FRAP and nitric oxide scavenging activity	Sriram and Sasikumar, 2013
	Leaves	Methanol	TPC (gallic acid and tannic acid), DPPH and hydrogen peroxide method	Banu et al., 2011
B. mysorensis	Leaves	Aqueous	DPPH, metal chelating activity, TPC	Mathew et al., 2012
B. noctiflora	Leaves and roots	Methanol	DPPH, TPC, TFC, FRAP, Fe^{2+} chelating activity, nitric oxide scavenging activity, ABTS, superoxide anion and hydrogen peroxide radical scavenging activity	Yadav et al., 2012
	Aerial and roots	Ethanol	DPPH	Manjula and Ganthi, 2018
	Aerial	Ethanol and aqueous	TPC, TFC, DPPH, ABTS, scavenging of hydrogen peroxide (H_2O_2), lipid peroxidation inhibitory activity, hydroxyl radical scavenging activity *p*-NDA method and superoxide radical scavenging activity by alkaline DMSO method	Arumugam et al., 2015
B. prionitis	Leaves, stems and roots	Methanol	DPPH, FRAP and β-Carotene-linoleic acid model system	Amoo et al., 2011
	Leaves, flower and stems	Ethanol	β-carotene bleaching, DPPH, hydroxyl radical scavenging activity and TPC	Jaiswal et al., 2010

	Aerial	Shanzhiside methyl ester, 6-O-trans-p-coumaroyl-8-Oacetylshanzhiside methyl ester, barlerin, acetylbarlerin and 7-methoxydiderroside, lupulinoside	DPPH	Ata et al., 2009
	Whole	Butanol, ethyl acetate, chloroform, hexane and methanol	DPPH	Kapoor et al., 2014
	Leaves and stems	Ethanol, methanol, acetone and aqueous	TPC, TFC, FRAP, DPPH and ABTS	Ranade et al., 2016
	Leaves and stems	Hexane, ethyl acetate, methanol and aqueous	TPC, DPPH and FRAP	Sharma et al., 2014
	Bark and leaves	Methanol and petroleum ether	DPPH	Kumar et al., 2013
	Leaves	Ethanol and aqueous	TPC, TFC, FTC, TBA, scavenging of hydrogen peroxide radicals and DPPH	Sawarkar et al., 2018
	Whole	Ethanol and aqueous	DPPH, ABTS, hydroxyl radical scavenging activity, FRAP and nitrous oxide reducing method	Chavan et al., 2011
	Flower	Hydro-alcoholic	DPPH	Shukla, 2019
B. strigosa	Roots	Ethyl acetate, methanol and hydro-alcoholic	TPC, TFC, ABTS, nitric oxide quenching, FRAP and DPPH	Deepak et al., 2021
	Leaves	Methanol	DPPH	Prapalert et al., 2017

*__DPPH__- (1,1-Diphenyl-2-picrylhydrazyl) free radical-scavenging activity; __FRAP__- Ferric Reducing Antioxidant Power, __TPC__- Total Phenolic Content (colorimetric Folin-Ciocalteu method); __TFC__-Total Flavonoid Content (aluminium-chloride colorimetric method); __TIC__- Total iridoid content (colorimetric method); __CTC__- condensed tannin contents (butanol–HCl method); __ABTS__- (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) free radical-scavenging activity; __FTC__- Ferric thiocyanate method; __TBA__- Thiobarbituric acid method; __TPTZ__-2,4,6-tripyridyl-s-triazine radical scavenging assay and __TBA__-Thiobarbituric acid method.

2.3.4.2 Ethnopharmacology

The reported ethnobotanical uses of some species within *Barleria* have been documented for a wide range of illnesses throughout various parts of Africa and India (Table 2.3). Snake bites are a serious issue in the Nandi community in Kenya. This tribe uses the leaves of *B. grandicalyx* (Lindau) as a paste to treat inflamed areas around the wound (Jeruto et al., 2008). In South Africa, the leaves of *B. dinteri* (Oberm.) are used in traditional medicine to treat some infectious diseases, intestinal tumours and relieve joint pain (Gololo et al., 2017). Specifically, the Vhavenda tribe in South Africa burn the roots and leaves of *B. randii* (S. Moore) and the smoke released is directed towards the genitalia of an infertile woman (Van Wyk and Gericke, 2000). The remainder of this medicinal plant is also prepared as either an infusion or a decoction and is taken orally (Van Wyk and Gericke, 2000) (Table 2.3).

Table 2.3: Reported ethnomedicinal uses of several species of *Barleria* in various countries

Species	Parts used	Ailment/uses	Country	Reference
B. dinteri	Leaves	Treatment of intestinal tumours and joint pain	South Africa	Gololo et al., 2017
B. gibsoni	Leaves, bark and roots	Ulcers, boils and hemorrhoids	India	Tamboli and More, 2016
B. grandicalyx	Leaves	Snake bites.	Kenya	Jeruto et al., 2008
B. lupulina	Leaves and roots	Dog bites, snakebites, bleeding wounds, cough, fever, eczema, scabies and rheumatism	India and Bangladesh	Kumari et al., 2017; Rahmatullah et al., 2018
B. montana	Leaves	Diabetes, cough and possess hepatoprotective activity	India	Sridharan and Gounder, 2016
B. prionitis	Bark, leaves and flowers	Toothache, fever, inflammation, and gastrointestinal disorders	India and Sri Lanka	Chopra et al., 1956; Kosmulalage et al., 2007; Aneja et al., 2010
B. randii	Roots and leaves	Infertility and anti-abortifacient	South Africa	Van Wyk and Gericke, 2000

2.3.4.3 Antibacterial activity

Infectious diseases are a serious concer in Africa (Van Vuuren and Muhlarhi, 2017). One of the primary causes of ill health and death are from bacterial infections (Elbashiti et al., 2011; Ncube et al., 2012; Islam et al., 2015). The extensive use of antibiotics to treat bacterial infections has encouraged researchers to screen medicinal plants for antibacterial activity (Srivastava et al., 2013, 2014). Plant species belonging to the genus *Barleria* (Acanthaceae) are known to exhibit exceptional antibacterial properties (Khobragade and Bhande, 2012). Numerous studies have validated the antibacterial activity of extracts and isolated compounds of *Barleria* (Table 2.4). The antibacterial activity of the various plant extracts has been evaluated against the Gram-positive bacteria *Bacillus cereus*, *B. pumilus*, *Bacillus* sp., *B. subtilis*, *Enterococcus faecalis*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus sporogenes*, *Micrococcus luteus*, *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus mutans*, and *S. pyogenes* and the Gram-negative bacteria *Comamonas acidovorans*, *Citrobacter* sp., *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *P. fluorescens*, *Proteus mirabilis*, *Providencia* sp., *Pseudomonas* sp, *Proteus vulgaris*, *Salmonella paratyphi*, *S. typhi*, *Shigella dysenteriae*, *Serratia marcescens*, *Vibrio cholera*, and *Xanthomonas oryzae*.

The most commonly investigated species for the antibacterial activity within the genus is *B. prionitis*. Amoo et al. (2009) examined the minimum inhibitory concentration (MIC) of the petroleum ether, di-chloromethane, and ethanol extracts of *B. prionitis*. These authors found that these extracts exhibited a broad spectrum of antibacterial activity. The MIC values ranged from 0.781 to 3.125 mg/ml for *B. subtilis*, *S. aureus*, *E. coli*, and *K. pneumoniae*. Their findings demonstrated the therapeutic potential of *B. prionitis* as an antibacterial agent. Furthermore, Aneja et al. (2010) evaluated the antibacterial activity of the acetone, ethanol, methanol, and aqueous extracts of *B. prionitis* bark. Their study results suggested that the methanolic bark extract was the most effective against all four oral bacteria with varying inhibition zones (*S. mutans* (15.65 ± 0.57 mm), *S. aureus* (16.32 ± 0.57 mm), *Pseudomonas* sp. (19.32 ± 0.57 mm), and *Bacillus* sp. (28.65 ± 0.57 mm).

Table 2.4: Antibacterial activities of extracts from species within *Barleria*

Plant species	**Plant part**	**Extract**	**Antibacterial activity**	**Reference**
B. acuminata	Leaves	Acetone, aqueous, dimethyl ether, chloroform and ethanol	*B. cereus, B. subtilis, E. faecalis, S. aureus, S. epidermidis, E. coli, K. pneumonia, P. mirabilis, S. typhi* and *S. dysentriae*	Bency et al., 2018
B. albostellata	Leaves and stems	Petroleum ether, dichloromethane and ethanol	*B. subtillis, S. aureus, E. coli* and *K. pneumoniae*	Amoo et al., 2009
B. argillicola	Aerial and roots	Petroleum ether, dichloromethane and methanol	*E. coli, P. aeruginosa* and *S. aureus*	Amoo and Van Staden, 2013
B. cristata	Bark	Ethanol	*S. aureus, B. subtillis* and *S. mutans*	Salib et al., 2013
	Leaves	Methanol	*K. pneumonia, S. aureus, E. coli* and *S. paratyphi*	Amutha and Doss, 2012
		Methanol and aqueous	*S. pyogenes* and *E. coli*	Sulthana et al., 2017
		Petroleum ether, chloroform and aqueous	*X. oryzae, B. subtilis, E. coli, P. aeruginosa* and *P. fluorescences*	Myint et al., 2020
B. dinteri	Leaves	n-hexane, dichloromethane, acetone and methanol	*E. coli, E. faecaelis, S. aureus* and *P. aeruginosa*	Gololo et al., 2016
B. grandiflora	Aerial	Ethanol and aqueous	*S. aureus* and *S. mutans*	Sawarkar et al., 2016
B. greenii	Stems and roots	Petroleum ether, dichloromethane and ethanol	*B. subtillis, S. aureus, E. coli* and *K. pneumoniae*	Amoo et al., 2009
B. lupulina	Whole	Chloroform, ethyl acetate and methanol	*Propionibacterium acnes*	Chomnawang et al., 2005
	Leaves	Methanol	*S. aureus, E. coli; P. aeruginosa, K. pneumoniae* and *S. typhi*	Kumari et al., 2017
	Leaves and stems	Acetone and methanol	*S. aureus, E. coli, P. aeruginosa, K. pneumoni* and *S. typhi*	Kumari and Dubey, 2016
	Leaves	Methanol	*S. aureus* and *B. pumilus*	Moin et al., 2012

	Leaves	Isolated essential oil	*B. pumilus* and *S. aureus*	Sarmad et al., 2012
B. montana	Leaves	Acetone, chloroform, dichloromethane, ethanol, methanol and aqueous	*E. coli*, *S. typhi*, *P. aeruginosa*, *K. pneumoniae*, *P. vulgaris*, *B. subtilis*, *S. pneumoniae*, *S. aureus* and *E.coli*	Natarajan et al., 2012
	Aerial	Methanol	*B. subtilis*, *B. cereus*, *B. pumilis*, *S. aureus*, *E. coli*, *P. aeuriginosa*, *P. vulgaris* and *S. marceseans*	Tulliballi and Seru, 2013
	Leaves	Aqueous, ethanol, methanol and chloroform	*E. aerogenes*, *E. coli*, *S. pneumoniae*; *B.subtilis* and *P. vulgaris*	Sridharan and Chinnagounder, 2012
B. prionitis	Leaves and stems	Petroleum ether, Di-chloromethane and ethanol	*B. subtilis*, *S. aureus*, *E. coli* and *K. pneumoniae*	Amoo et al., 2009
	Bark	Acetone, ethanol, methanol and aqueous	*S. mutants*, *S. aureus*, *Pseudomonas sp.* and *Bacillus sp.*	Aneja et al., 2010
	Leaves	Chloroform	*S. typhi*, *B. subtilis*, *V. cholera*, *M. luteus*, *Providencia sp.*, *L. sporogenus* and *Citrobacter sp.*	Gangopadhyay et al., 2012
		Aqueous, petroleum ether, chloroform and acetone	*L. rhamnosus*	Diwan and Gadhikar, 2012
		Petroleum ether , chloroform, ethanol and aqueous	*S. typhi*, *B. subtilis*, *S. aureus*, *V. cholera* and *E. coli*	Amit et al., 2014
	Leaves and stems	Ethyl acetate	*B. pumilus*, *B. subtilis*, *S. pyogenes*, *B. cereus*, *S. marcescens*, *C. acidovorans* and *P. aeruginosa*	Patel et al., 2015
	Leaves	Methanol	*S. mutants*, *S. aureus*, *L. acidophilus* and *Pseudomonas sp.*	Kumari et al., 2013

		Petroleum ether, chloroform and aqueous	*B. subtilis*, *E. coli*, *P. fluorescens* and *X. oryzae*	Myint et al., 2020
	Leaves	Petroleum ether, chloroform, ethanol and aqueous	*S. aureus*, *B. subtilis*, *P. vulgaris*, *K. pneumonia*, *E. coli* and *P. aeruginosa*	Chavan et al., 2010[b]
	Aerial	Ethanol	*B. cereus* and *P. aeruginosa*.	Kosmulalage et al., 2007
B. strigosa	Leaves	Methanol and butanol	*B. subtilis*, *S. aureus and M. luteus*	Manapradit et al., 2015

** Bacillus cereus; Bacillus pumilus; Bacillus subtilis; Bacillus* species.; *Comomonas acidovorans; Citrobacter* species; *Enterobacter aerogenes, Enterococcus faecalis; Escherichia coli; Klebsiella pneumoniae; Lactobacillus acidophilus; Lactobacillus rhamnosus; Lactobacillus sporogenus; Micrococcus luteus; Proteus mirabilis; Proteus vulgaris; Pseudomonas aeruginosa; Pseudomonas fluorescences; Pseudomonas sp.; Psuedomonas vulgaris; Salmonella paratyphi; Salmonella typhi; Serratia marceseans; Shigella dysentriae; Staphylococcus aureus; Staphylococcus epidermidis; Streptococcus mutants; Streptococcus pneumoniae*; *Streptococcus pyogenes*; *Streptococcus* species; *Vibrio cholera* and *Xanthomonas oryzae*.

2.3.4.4 Antifungal activity

Opportunistic fungal infections can become fatal to individuals with immuno-compromised conditions (Jankowska et al., 2001), in particular cancer (Awasthi et al., 2017) and HIV/AIDS patients (Denning, 2016). Management of these infections has become complex due to the limited number of cost-effective antifungal agents, toxicity of the accessible agents, relapse of infections, and resistance to these commonly used agents (Hamza et al., 2006; Doddanna et al., 2013). Consequently, it has become critical to explore naturally occurring antifungal agents. *Barleria* being one of such genera, has displayed excellent antifungal properties. Numerous studies have validated the antifungal activity of extracts and fractions of *Barleria* (Table 2.5). Aneja et al. (2010) evaluated the antifungal activity of acetone, ethanol, and methanolic plant extracts of *B. prionitis* and found that the extracts significantly reduced the growth of fungi, with the maximum zone of inhibition being observed for *Candida albicans* strain 1 (13.65 ± 0.57 mm, 12.94 ± 1 mm, and 15.31 ± 0.57 mm), *C. albicans* strain 2 (16 ± 0,52 mm, 11.31 ± 0.57 mm, and 16.96 ± 1 mm), and *Saccharomyces cerevisiae* (11.64 ± 0.57 mm, 11.31 ± 0.57 mm, and 13.95 ± 1 mm). Furthermore, Amoo et al. (2011) demonstrated the fungicidal activity of extracts derived from different parts of *B. prionitis* against *C. albicans*. They reported a minimum fungicidal concentration range of 4.7–6.3 mg/ml for the extracts of stems and roots.

Table 2.5: Antifungal activities of extracts from species within *Barleria*

Plant species	**Plant part**	**Extract**	**Antifungal activity**	**Reference**
B. albostellata	Leaves and stems	Petroleum ether, dichloromethane and ethanol	*C. albicans*	Amoo et al., 2011
B. cristata	Leaves	Saponin fraction	*C. albicans*, *A. flavous*, *Penicillium sp.*, *A. niger* and *Trichophyton sp*	Chellathai, 2015
		Saponin fraction	*A. flavous* and *A. niger*	Amutha and Doss, 2012
		Petroleum ether, chloroform, ethyl acetate, acetone, ethanol, methanol and aqueous	*A. flavous* and *C. albicans*	Myint et al., 2020
B. grandiflora	Leaves	Aqueous	*A. fumigatus*	Kumari et al., 2015
	Aerial	Ethanol and aqueous	*C. albicans*	Sawarkar et al., 2016
	Leaves	Ethanol and aqueous	*C. albicans*	
B. greenii	Leaves, stems and roots	Petroleum ether, dichloromethane and ethanol	*C. albicans*	Amoo et al., 2011
B. montana	Aerial	Petroleum ether, chloroform and methanol	*A. niger*, *R. stolonifera*, *S. cerevisiae* and *P. chrysogenum*	Tulliballi and Seru, 2013
B. prionitis	Bark	Acetone, ethanol and methanol	*S. cerevisiae* and *C. albicans*	Aneja et al., 2010
	Roots and stems	Petroleum ether, dichloromethane and ethanol	*C. albicans*	Amoo et al., 2011
	Leaves, stems and roots	Chloroform, acetonitrile and ethanol	*A. fumigatus*, *C. vaginitis*, *C. neoformans*, *C. albicans* and *B. dermatitidis*	Panchal and Singh, 2015
	Aerial	Ethanol and aqueous	*C. albicans*	Sawarkar et al., 2016
	Leaves	Ethanol and aqueous	*C. albicans*	
	Aerial	Methanol	*C. albicans*	Ghule and Yeole, 2012
		Petroleum ether, acetone, methanol and distilled water	*C. albicans* and *A. niger*	Singh et al., 2016

** Aspergillus flavous; Aspergillus fumigatus; Aspergillus niger; Blastomyces dermatitidis; Candida albicans; Candidal vaginitis; Cryptococcus neoformans; Pencillium chrysogenum; Penicillium* species; *Rhizopus stolonifera; Saccharomyces cerevisiae* and *Trichophyton* species.

2.3.4.5 Anti-inflammatory Activity

Several deteriorating diseases such as gouty arthritis, shoulder tendonitis, rheumatoid arthritis, polymyalgia rheumatica, asthma, cancer, heart disease, and inflammatory bowel disease are related to inflammatory processes (Polya, 2003; Iwalewa et al., 2007). Scientific research and pharmaceutical companies have been showing a growing interest in identifying novel anti-inflammatory compounds in medicinal plants. This can potentially lead to the production of new drugs in treating pain-related ailments with no side-effects (Fawole et al., 2010). This can potentially lead to the production of novel drugs in treating pain-related ailments with no side-effects (Fawole et al., 2010). Several studies have validated the anti-inflammatory activity of extracts and fractions of *Barleria* (Table 2.6). Amoo et al. (2009) analysed the anti-inflammatory activity of petroleum ether, dichloromethane, and ethanolic extracts using the cyclooxygenase COX-1 and COX-2 assays. They reported that petroleum ether extracts (leaf (72.5 ± 1.26%) and root (77.2 ± 1.41%) and dichloromethane extracts leaf (79.7 ± 1.55%)) of *B. prionitis* exhibited promising activity (>70%) in COX-1 assay. Moreover, in COX-2 assays, the root, petroleum ether (78.5 ± 1.90%), and dichloromethane extracts (70.4 ± 1.80%) of *B. prionitis* demonstrated the best activity (>70%). The non-polar extracts (petroleum ether and dichloromethane) exhibited greater activity than ethanolic extracts.

Cos et al. (2006) reported that compounds that are strong inhibitors of enzymes fail *in vitro* to settle against the entire organism, as their passage toward the cell membrane is restricted. In addition, Zschocke and Van Staden (2000) explained that the activity exhibited by nonpolar extracts is of significant interest because the lipophilic compounds extracted from these solvents exhibit better resorption through the cell membrane. Overall, their study results demonstrated that the anti-inflammatory activity of these extracts correlated to their inhibition of cyclooxygenase enzymes, following the inhibition of prostaglandin synthesis. Singh et al. (2003) examined the anti-inflammatory activity of methanol–water fractions (TAF) of *B. prionitis* on different acute and chronic animal test models. They observed that the ir-idoid-enriched fraction (TAF) demonstrated activity against histamine, carrageenan, and dextran-induced inflammation models. Marked inhibitory effect was exhibited by TAF in a dose-dependent manner on carrageenan-induced edema (normal rats), with the ED_{50} values being 89.70 and 143.51 mg/kg (11.93%–44.56%) in adrenalectomized rats. The oral administration of TAF inhibited histamine and dextran-induced edema, with the ED_{50} values being 333.52 mg/kg (12.16%–36.14%) and 467.19 mg/kg (12.35%–34.05%), respectively.

Table 2.6: Anti-inflammatory activities of extracts, fractions and isolated compounds from species within *Barleria*

Plant species	**Plant part**	**Extract**	**Anti-inflammatory activity/assays/model**	**Reference**
B. albostellata	Leaves and stems	Petroleum ether, dichloromethane and ethanol	COX-1 and COX-2	Amoo et al., 2011
B. cristata	Leaves	Aqueous	CIO in rat paws, prostaglandins inhibitory activity, and acetic acid induced capillary permeability in mice	Gambhire et al., 2009[a]
		Methanol	Inhibited oedema produced by histamine and serotonin in rats. Reduction in the increased peritoneal vascular permeability in mice	Gambhire et al., 2009[b]
B. greenii	Stems and roots	Petroleum ether, dichloromethane and ethanol	COX-1 and COX-2	Amoo et al., 2011
B. lupulina	Aerial	Aqueous	Activated the Nrf2 cell defense pathway in human dermal microvascular endothelial cells	Senger et al., 2016
	Aerial	Methanol	Acute and sub-acute inflammation models of albino rats	Suba et al., 2005
	Whole	Methanol and acetone	CIO in rat paws and ethyl phenylpropiolate-induced ear oedema in rats	Wanikiat et al., 2008
B. montana	Leaves	Ethanol	Formalin induced inflammation in male albino wistar rats	Sridharan et al., 2015
B. prionitis	Leaves, stems and roots	Petroleum ether, dichloromethane and ethanol	COX-1 and COX-2 assays	Amoo et al., 2011
	Whole	Methanol-water fractions (TAF)	CIO in adrenalectomised rats, activity in acute	Singh et al., 2003

			inflammation induced by carrageenan, histamine and dextran in rats	
	Roots	Aqueous fractions	CIO in rat paw model	Khadse and Kakde, 2011
	Aerial	Shanzhiside methyl ester, 8-O-acetyl shanzhiside methyl ester and iridoid glycosides rich monoterpenoidal fraction	Stimulated rat neutrophils by inhibiting MPO, elastase and MMP-9 enzymes	Ghule et al., 2020

* Cyclooxygenase (COX); Carrageenan-induced oedema (CIO); Nuclear factor erythroid 2–related factor 2 (Nrf2); Matrix Metalloproteinase-9 (MMP-9) and Myeloperoxidase (MPO).

2.3.4.6 Anticancer/ cytotoxcity activity

Worldwide, cancer has been considered as the most critical disease in humans due to its high morbidity and mortality rates (Jemal et al., 2011). Radiotherapy, surgery, and chemotherapy are the primary therapies used to treat cancer. Although these therapies have saved the lives of several patients with cancer, the severe side effects and the high relapse rates have rendered them only moderately effective to control and in certain cases cure cancers. Therefore, there is an urgent need to develop more diverse and effective therapies from several sources (Jemal et al., 2011). Compared with synthetic chemotherapeutic drugs, natural chemicals derived from plants are relatively less toxic and possess high target specificity (El-Halawany et al., 2018). Therefore, the potential usage of medicinal plants as anticancer drugs is important. In this regard, *Barleria* has demonstrated significant potential for anticancer activity, with several studies reporting the potent activity of extracts and isolated compounds against tumor cell lines (Table 2.7). In addition to *B. prionitis*, *B. cristata* and *B. grandiflora* have been frequently reported to exhibit potent anticancer activities (Table 2.7).

El-Halawany et al. (2018) examined the anticancer effects of phenolic compounds (verbascoside, isoverbascoside, dimethoxyverbascoside, apigenin-7-*O*-glucoside and p-hydroxybenzoic acid) isolated from *B. cristata*. They found that preliminary treatment of Hepa-1c1c7 cells with 3.125 μM of the tested isolated compounds inhibited the cytotoxic effect caused by menadione. Among the tested compounds, the best results were observed for isoverbascoside, which potently induced the activity of the enzyme in a dose-dependent manner. Isoverbascoside exhibited the strongest effect in protecting Hepa-1c1c7 cells against the toxicity of menadione (quinone substrate for NQO1), causing an 8.8-fold induction of NQO1 activity at 25 μM (compared with vehicle control activity level). In addition, Manglani et al. (2014), evaluated the anticancer activity of the leaf extracts *B. grandiflora* on various normal and cancerous cell lines such as human lung cancer cells (A-549), Dalton's lymphoma ascites (DLA tumour cells) and African green monkey kidney (Vero) normal cells. They found that alcoholic leaf extracts exhibited cytotoxic effects against the cancerous cell lines A-549 (IC_{50} values (alcoholic extract 143.4 μg/mL, aqueous extract 210.8 μg/mL) and DLA (IC_{50} values (alcoholic extract 137.2 μg/mL, aqueous extract 217.8 μg/mL). Both extracts were less potent to the Vero cell line, with the IC_{50} values being 148.7 μg/mL (alcoholic) and 52.6 μg/mL (aqueous). Their study showed that the alcoholic extracts were less toxic to the human cells and exhibited significant *in vitro* and *in vivo* antitumour activity against DLA cells.

Table 2.7: Anticancer activities of extracts and isolated compounds from species within *Barleria*

Plant species	Plant part	Extract/ compounds	Assays/Cell lines	Reference
B. cristata	Aerial	Verbascoside, isoverbascoside, dimethoxyverbascoside, p-hydroxybenzoic acid and apigenin-7-O-glucoside	NQO1 and murine hepatoma cell line hepa-1c1c7	El-Halawany et al., 2018
	Leaves and bark	Hexane, chloroform, acetone and methanol	Brine shrimp lethality assay and brine shrimp cysts	Pathy et al., 2015
B. gibsoni	Leaves	Petroleum ether, chloroform, acetone, ethyl acetate and ethanol	SRB \, MDA, MB 4355 (Human breast cancer) and hep G2 (Liver cancer cell line)	Tamboli and More, 2015
B. grandiflora	Leaves	Alcoholic and aqueous	A-549 (human lung cancer) cells, DLA tumour cells and Vero (African green monkey kidney) normal cells	Manglani et al., 2014
		Ethanol and aqueous	MTT, human gingival fibroblast cell lines and human dermal fibroblast cell lines	Sawarkar et al., 2016
B. lupulina	Leaves	Ethanol	MTT and cancerous THP-1 cell lines	Kumari and Kumar, 2020
		Ethanol and aqueous	MTT and hepG2 cells	Kumari and Dubey, 2016
B. prionitis	Leaves	Ethanol and aqueous	MTT, human gingival fibroblast cell lines and human dermal fibroblast cell lines	Sawarkar et al., 2016
		Ethanol	SRB, breast (MCF-7), colon (DLD-1), lung (A549), breast metastatic (MDMAMB-468), lung	Panchal et al., 2018

			metastatic (NCIH358) and colon metastatic (SW620)	
B. strigosa	Leaves	Methanol, hexane, dichloromethane, ethyl acetate and butanol	MTT colorimetric assay, human hepatocellular carcinoma (HepG2), human breast adenocarcinoma (MCF7), human oral epidermoid carcinoma (KB), human colon adenocarcinoma (HT29), murine lymphocytic leukemia (P388), human cervical carcinoma (HeLa) as well as two normal cell lines including African green monkey kidney (Vero) and mouse subcutaneous connective tissue (L929)	Manapradit et al., 2015

*MTT- (3-(4, 5-dimethylthiazolyl-2)-2,. 5-diphenyltetrazolium bromide); DLA- Dalton's lymphoma Ascites; NQO1- (NAD(P)H dehydrogenase [quinone] 1) and SRB- Sulphorhodamine B.

2.3.4.7 Antidiabetic activity

Based on folkloric claims, people with diabetes have been treated orally with various medicinal plants or their extracts since ancient times (Akhtar et al., 1980). Hypoglycemic synthetic agents can produce severe side-effects, including liver and kidney function distubuances and hematological coma (Larner, 1985). Therefore, the search for more safe and effective antidiabetic agents in plants has continued to be a critical area for research. *Barleria* species have also demonstrated antidiabetic activities as shown in Table 2.8. According to Singh et al. (2012), oral administration of ethanolic seed (200 mg/kg) extracts from *B. cristata* for 7 days decreased blood glucose levels in a model of alloxan-induced diabetes in rats. Furthermore, Vasanth et al. (2018) investigated the ethanol and petroleum ether leaf extracts of *B. cristata* for their antidiabetic activity and found that both extracts exhibited dose-dependent increases in the inhibitory activities of α-glucosidase (inhibition: ethanol extract 47% and petroleum ether extract 44%) and α-amylase (inhibition: ethanol extract 67% and petroleum ether extract 61%) at a concentration of 100 µl. Overall, the best results were obtained with ethanol extracts that demonstrated the maximum *in vitro* antidiabetic activity compared with petroleum ether extract. Reema and Pradeep (2010), reported about the antidiabetic properties of *B. prionitis* and observed a reduction in glycosylated hemoglobin ($P < 0.01$) and blood glucose ($P < 0.01$) levels in alloxan-induced diabetic rats treated with the ethanolic leaf extract. A further observation in their study was an increase in liver glycogen and serum insulin levels but a decrease in body weight. In experimental animals, the root ethanolic extract exhibited a moderate but non-significant antidiabetic activity. The above-described results have thus confirm the antidiabetic potential of the various species of *Barleria*.

Table 2.8: Antidiabetic activities of extracts and fractions from species within *Barleria*

Plant species	**Plant part**	**Extract**	**Antdiabetic activity/assays/models**	**Reference**
B. bispinosa	Aerial	Methanol	Male Wister rats and streptozotocin induced diabetic rats	Ezzat et al., 2014
B. cristata	Seeds	Ethanol	Wistar rats and alloxan-induced diabetic rats	Singh et al., 2012
	Leaves and roots	Ethanol and petroleum ether	Inhibition of α-amylase enzyme assay and inhibition of α-glucosidase enzyme assay	Vasanth et al., 2018
B. lupulina	Aerial	Methanol	Male Wister rats and streptozotocin-diabetic rats	Suba et al., 2004[a]
B. montana	Aerial	Methanol	Wistar albino rats and streptozotocin induced diabetic rats	Shyam et al., 2013
B. noctiflora	Aerial	Ethyl acetate and *n*-butanol	Wister rats and streptozotocin induced type-2 diabetes in rats	Arumugam and Natesan, 2016
	Aerial and roots	Ethanol	*In-vitro* antidiabetic activity was determined by inhibition of α-glucosidase and inhibition of α-amylase studies	Manjula and Ganthi, 2018
	Aerial	Ethyl acetate fraction	Wister rats and streptozotocin induced diabetic rats	Arumugam et al., 2016
B. prionitis	Leaves and roots	Ethanol	Adult albino rats and alloxan-induced diabetic rats	Dheer and Bhatnagar, 2010
	Leaves, stems and roots	Alcohol and aqueous	Albino rats and alloxan-induced hyperglycemic rats	Geetha and Wahi, 2001

2.3.4.8 Antiulcer activity

Gastric hyperacidity is a common problem that affects millions of individuals worldwide due to an imbalance between protective and aggressive factors (Alkofahi and Atta, 1999). Peptic ulcers are generally treated using proton pump inhibitors, H2 receptor antagonists, and antimuscarinics. However, the majority of these agents produce adverse effects such as impotence, arrhythmia, gynecomastia, hypersensitivity, and hematopoietic disorders (Peskar and Maricic, 1998). Therefore, it is crucial to explore plants containing natural antiulcer and antioxidant compounds that can be used as safer treatment alternatives with less side effects. *Barleria species* have also demonstrated antiulcer activities as shown in Table 2.9. Kumar and Singh (2013) investigated the antiulcer activity of the methanolic leaf extracts of *B. prionitis*. They reported a statistically significant reduction ($P = 0.05$) of ulcer index in the treated animals in comparison with control groups in both models. Substantial changes were observed only in the total acidity at a dose of 500 mg/kg, and changes were significant in the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) at both doses in the ethanol-induced gastric ulcer model.

Jaiswal et al. (2014) examined the gastroprotective effect of iridoid fractions obtained from the leaves of *B. prionitis* against various gastric ulcer models in rats. They observed that the fractions displayed a dose-dependent ulcer-protective effect in ulcer models induced by aspirin (24.65%–63.25% protection), pylorus ligation (PL) (18.67%–66.26% protection), cold-restraint stress (CRS) (20.77%–59.42% protection), and ethanol (16.93%–77.04% protection). The iridoid fractions derived from *B. prionitis* demonstrated antiulcerogenic properties by decreasing the acid-pepsin secretions in rat models of gastric ulcer (Jaiswal et al., 2014). The fractions reduced the ulcer index by significantly decreasing the lipid peroxidation product ($P < 0.01–0.001$) and superoxide dismutase activity ($P < 0.01–0.001$) and increasing the catalase activity in the CRS-induced model.

Table 2.9: Antiulcer activities of extracts and fractions from species within *Barleria*

Plant. species	**Plant part**	**Extract**	**Antiulcer activity/ gastric cytoprotective activity/models**	**Reference**
B. buxifolia	Whole	Methanol	Wistar rats, PL- and aspirin induced ulcers	Khadeerunnisa et al., 2020
B. gibsoni	Leaves	Ethanol	Wistar rats, PL-induced ulcer models	Tamboli and More, 2016[c]
B. lupulina	Aerial	Methanol	Albino (Wistar) rats, PL ulceration in rats, stress-induced ulceration, drug-induced gastric ulcer and duodenal ulcers in rats	Suba et al., 2004[b]
B. prionitis	Leaves	Methanol	Wistar rats, ethanol induced gastric mucosal lesions and indomethacin induced ulcer models	Kumar and Singh, 2013
		Iridoid fraction	Male Sprague–Dawley rats and female Swiss albino mice, PL- induced ulcers, aspirin- induced ulcers, CRS-induced ulcers and ethanol-induced ulcer	Jaiswal et al., 2014
		Methanol	Ethanol, indomethacin and induced ulcer models	Manjusha et al., 2013
		Chloroform	Rodent experimental models (indomethacin and pylorus ligation)	Choudhary et al., 2014

*CRS- cold-restraint stress and PL- Pylorus ligated.

2.3.4.9 Hepatoprotective Activity

Liver diseases (acute and chronic) are a global concern (Lee et al., 2007) and their treatment is difficult to achieve because none of the available drugs have been effective in stimulating liver function or aiding the liver to regenerate hepatic cells (Chattopadhyay, 2003; Jain et al., 2012). In addition, hepatotoxic chemicals cause damage to liver cells by accelerating lipid peroxidation and other oxidative injuries (Recknagel, 1983; Wendel et al., 1979; Dianzani et al., 1991; Subramaniam et al., 2015). Hence, due to increasing incidences of chemically induced hepatotoxicity, there is a demand for safe protective agents (Jain et al., 2012). Conseqeuntly, it is essential to explore alternative drugs from plant sources that are safe and efficient in treating liver diseases. Therefore, several medicinal plants, especially within the genus *Barleria*, have been screened for hepatoprotective activity by various researchers (Table 2.10). For instance, Balaji et al. (2013) investigated the hepatoprotective activity of the ethanolic leaf extracts of *B. cristata* against CCl_4 (0.7 ml/kg, i.p) induced hepatic damage in Wistar albino rats (at dose levels of 100–200 mg/kg). The ethanolic extract significantly ($P< 0.001$) decreased the serum levels of specific liver enzymes such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, total protein, total bilirubin, triglycerides, and cholesterol levels. They used a known hepatoprotective drug, silymarin (25 mg/kg), for comparison that displayed significant activity ($P< 0.001$). The ethanolic extract did not cause any mortality in the Wistar rats (up to a dose level of 200 mg/kg). Overall the results indicated that the ethanolic extract exhibited hepatoprotective properties, which may be due to the presence of flavonoids and alkaloids (Balaji et al., 2013).

Singh et al. (2005) also evaluated the iridoid-enriched fractions obtained from the ethanol–water leaf and stem extracts of *B. prionitis* for hepatoprotective activity in Charles Foster rats and Swiss albino mice. These fractions exhibited hepatoprotective activity in several chronic and acute animal test models. After a single dose of drug administration, the oral LD_{50} value was found to be >3000 mg/kg, with no signs of deformities or mortality (for a duration of 15 days). However, the intraperitoneal LD_{50} was found to be 2530 ± 87 mg/kg in mice. The extracts provided significant hepatoprotection against against hepatotoxicity induced by galactosamine, carbon tetrachloride, and paracetamol. Overall, their study reported significant and concentration-dependent hepatoprotective activity of the iridoid-enriched fractions, as most of the altered hepatic parameters in experimental rodents (liver damage) were reversed. Hence, extracts derived from *Barleria* have hepatoprotective properties that can serve as an effective treatment for acute hepatic diseases.

Table 2.10: Hepatoprotective activities of extracts and fractions from species within *Barleria*

Plant species	**Plant part**	**Extract**	**Hepatoprotective activity/assays/models**	**Reference**
B. cristata	Leaves	Ethanol	Wistar albino rats and CCl_4 induced hepatic damage in rats	Balaji et al., 2013
B. cuspidata	Leaves	Methanol	Wistar albino rats and CCl_4 induced hepatotoxicity in rats	Tabassum et al., 2020
B. gibsoni	Aerial	Aqueous and ethanol	Wistar albino rats, inducing agent paracetamol in Carboxy methyl cellulose and silymarin	Lakshman Kumar et al., 2015
B. montana	Leaves	Methanol	Wistar albino rats and ethanol-induced rat hepatic injury	Banu et al., 2012
	Aerial	Methanol	CCl_4 induced hepatotoxicity on rats	Tulliballi and Seru, 2013
B. prionitis	Leaves and stems	Fractions from ethanol-water	Charles Foster rats, Swiss albino mice, acute and chronic animal test models CCl_4 toxicity, cetaminophen (APAP) toxicity and D-GalN induced hepatotoxicity	Singh et al., 2005

*CCL_4- Carbon tetrachloride

2.3.4.9 Analgesic activity

Unbearable and long-term pain is one of the primary causes for poor quality of life, and therefore, several researchers are investigating the mechanisms and causes of pain and are exploring novel drugs in plants to reduce pain with less to no side effects. Although analgesic drugs are available and efficient in reducing pain, their repetitive application can cause several side effects such as tolerance and physical dependence (Gilron and Coderre, 2007; Manchikanti et al., 2013). Regarding the genus *Barleria*, the literature reports only one study conducted by Begum et al. (2017), who investigated the effects of chloroform, petroleum ether and ethanol extracts of the aerial parts of *B. prionitis* in Swiss albino mice at doses of 200 and 400 mg/kg. Overall, their study indicated that all the three extracts demonstrated significant analgesic ef-fects in mice, with inhibition proportions of 30.36%, 59.40%, and 33.70% when tested at 400 mg/kg.

2.3.4.10 Anti-amoebic activity

A common intestinal infection occurring in humans in developing countries is amoebiasis, which is caused by the protozoan parasite *Entamoeba histolytica*. Trophozoites from *E. histolytica* invade the intestinal mucosa, resulting in dysentery, and thereafter sporadically migrate to the liver, triggering abscesses (Martínez-Palomo, 1987; Samie et al., 2012). Although the drugs used in the treatment of amoebiasis are effective, they induce various side effects such as nausea, stomatitis, gastrointestinal discomfort, and vomiting (Kapoor et al., 1999; Hanna et al., 2000; Upcroft and Upcroft, 2001; Bansal et al., 2004; Toumi et al., 2009). Therefore, it is essential to identify new compounds in plants possessing antiamoebic activity that are safe for human usage. To date, only one study has been conducted on the anti-amoebic activity of a species of *Barleria*. Sawangiaroen et al. (2006) evaluated the antiamoebic activities of the chloroform leaf and stem extracts of *B. lupulina* at a concentration of 1000 μg/ml against the *E. histolytica* strain. They observed that the chloroform extracts derived from *B. lupulina* stem exhibited the best anti-amoebic activity (IC_{50} 78.5 μg/ml) against *E. histolytica*. Extracts were classified as "active", with an IC_{50} value of <100 μg/ml (Sawangiaroen et al., 2006). The IC_{50} of a standard drug, metronidazole, was 1.1 μg/ml.

2.3.4.11 Antihelminthic activity

Helminths are parasitic worms that are infectious to humans in developing countries (Hotez et al., 2008). These worms exist in the gastrointestinal tract and can burrow into the liver and other organs. Infected individuals excrete helminth eggs in their fecal matter, causing the contamination of soil in areas with poor sanitation (Idika et al., 2012). The drugs used to treat these infections have common side effects such as vomiting, nausea, abdominal pain, allergic reactions, expulsion of ascaris from mouth or nose, body ache, and fever (Tripathi, 2008). Consequently, the search for plants exhibiting antihelminthic activity with no side effects is critical. There is a scarcity of research on the genus *Barleria* exploring

the antihelmintic activity of its plant extracts; Table 2.11 displays the few studies investigating this activity reported in the literature. Chander et al. (2014) examined the antihelmintic activity of *B. buxifolia* aqueous and ethanolic leaf extracts against *Pheretima posthuman* worms. They found that the ethanolic extract at 100 mg/ml produced a significant effect (P <0.001) compared with the aqueous extract. The aqueous extract caused a dose-dependent paralysis that varied from loss of motility, loss of response to stimuli, and ultimately progressed to death. In the *P. posthuma* worms, the ethanolic extract took 37.75 ± 2.06 min for paralysis and 89.00 ± 1.82 min for death, whereas the duration for the aqueous extract was 64.00 ± 2.16 min for paralysis and 150.50 ± 2.64 min for death. Chavan et al. (2010[a]) also evaluated the antihelmintic activity (paralysis and time of death) of whole ethanolic and aqueous extracts of *B. prionitis* against *P. posthuma*. They reported that both aqueous and ethanolic extracts significantly demonstrated paralysis (P <0.01) in worms at lower doses (50, 75, and 100 mg/ml) and resulted in death at a high concentration of 100 mg/ml compared with albenadazole (standard).

Table 2.11: Anthelmintic activities of extracts from species within *Barleria*

Plant species	**Plant part**	**Extract**	**Anthelmintic activity/assays/models**	**Reference**
B. buxifolia	Leaves	Aqueous and ethanol	IAW *P. posthuma*	Chander et al., 2014
B. gibsoni	Leaves	Aqueous and ethanol	IAW *P.* posthuma	Tamboli and More, 2016[a]
B. prionitis	Whole	Aqueous and ethanol	IAW *P. posthuma*	Chavan et al., 2010[a]
		Petroleum ether, chloroform, ethanol and aqueous	IAW *P. posthuma*	Kaur et al., 2015

*IAW- Indian adult worm and *Pheretima posthuma*.

2.3.4.12 Antiarthritic activity

Rheumatoid arthritis is an autoimmune disease that is categorized by synovial membrane inflammation, pain, peripheral joint inflammation, damage of the articular tissue, and joint movement restriction (Paval et al., 2009; Banji et al., 2011; Patil et al., 2012). This disease can affect an individual's ability to conduct daily tasks and causes premature death (Murugananthan et al., 2013). Irrespective of the progress made in the management of this disease, the treatments fail to generate long-term benefits, thus resulting in adverse effects such as renal morbidity, gastrointestinal ulcers, hematological toxicity, and cardiovascular complications (Campbell, 1988; Nandi et al., 2008). This necessitates identifying alternative methods that cause less to no adverse effects. Therefore, it is essential to explore drugs from plant sources that exhibit antiarthritic activity. Table 2.12 summarizes the reported antiarthritic properties of various extracts and fractions of *Barleria*. A study conducted by Choudhary et al. (2014) investigated the antiarthritic potential of ethyl acetate fractions from the leaves of *B. prionitis* against

Freund's complete adjuvant-induced chronic arthritis and formaldehyde-induced acute nonimmunological arthritis in rats. They reported significant inhibition of edema in Sprague Dawley rats in acute and chronic models. The fraction used at a dose of 250 mg/kg exhibited potent and significant ($P \leq 0.05–0.01$) inhibition of paw edema. Ethyl acetate fraction was found to decrease the histo-pathological changes induced by Freund's complete adjuvant. Overall, their study results disclosed the potential use of *B. prionitis* fraction in protecting the synovial membrane through hematinic parameters, thus demonstrating promising antiarthritic activity.

Table 2.12: Antiarthritic activities of extracts and fractions from species within *Barleria*

Plant species	**Plant part**	**Extract**	**Antiarthritic activity/assays/models**	**Reference**
B. lupulina	Leaves	Methanol	Albino male mice, female Sprague Dawley rats, formalin-induced arthritis, adjuvant induced arthritis, collagen type II-induced arthritis and monosodium iodoacetate induced osteoarthritis	Mazumder et al., 2012
B. montana	Leaves	Ethanol	Male Albino Wistar rats and Complete Freund's *in vivo* method in induced rats	Basini and Sathrapalli, 2014
	Leaves	Ethyl acetate fraction	Sprague Dawley rats, formaldehyde induced arthritis and FCA-induced arthritis rat model	Choudhary et al., 2014
	Whole	Hexane, chloroform, *n*-butanol fractions and aqueous	Charles Foster rats, Swiss albino mice and adjuvant-induced polyarthritis in rats	Singh et al., 2003
B. prionitis	Whole	Methanol	Complete Freund's induced rat model	Sivakumar and Sivakumar, 2019

*FCA- Freund's complete adjuvant.

2.3.4.13 Antihypertensive activity

Hypertension, also defined as high blood pressure, is an ailment in which blood vessels persistently increase the blood pressure of an individual (WHO, 2013). This ailment contributes to the burden of premature mortality, heart diseases, disability, stroke, and kidney failure. Although several conventional antihypertensive drugs are used for hypertension treatment, they have adverse side effects such as extreme tiredness, dizziness, cramps, dehydration, and abnormal heart rate (Singh et al., 2015[a]). Therefore, researchers are focusing on herbal drugs as a source of treatment. Moreover, it is important

to examine plants and their d-rivatives for antihypertensive activity. In this context, the methanolic leaf extracts of *B. prionitis* at doses of 200 and 400 mg/bw were found to exhibit antihypertensive effects and displayed 103 ± 2.54 mm Hg, 100.5 ± 2.74 mm Hg, and 105.5 ± 2.35 mm Hg of diastolic blood pressure and 136.5 ± 2.51 mm Hg, 146 ± 2.21 mm Hg, and 143 ± 3.11 mm Hg of systolic blood pressure after a 6-week treatment period (Marya and Bothara, 2013).

2.3.4.14 Antiviral activity

Viral infections are the primary causes of diseases because of their complexity and diversity. This makes it difficult to counteract their diffusion and effects, which often result in pandemic events (Drexler, 2010). Moreover, the increased frequency of global travel, urbanization, and migration have rendered virus outbreaks a challenging issue for public health, specifically when antiviral therapies and vaccines are not available (Neiderud, 2015). In addition, the unsuccessful rate of numerous conventional drugs against viral infections and the onset of viral resistances have resulted in a growing interest in plants for promising antiviral agents (Irwin et al., 2016). Yosook et al. (1999) analyzed the leaf extracts of *B. lupulina* for intracellular activities against HSV-2 and five clinical HSV-2 isolates. Their study results demonstrated that the extracts exhibited activity against all the five clinical HSV-2 isolates. Chen et al. (1998) also reported about the isolation of iridoid glycosides (6-*O*-trans-*p*-coumaroyl-8-*O*-acetylshanzhiside methyl ester and *cis* isomer) from the methanolic extracts of *B. prionitis*, and these extracts were found to exhibit potent *in vitro* activity against the respiratory syncytial virus (EC_{50} 2.46 μg/mL, IC_{50} 42.2 μg/mL) (Chen, 1998).

2.3.4.15 Inhibition of acetylcholinesterase

Acetylcholine is a neurotransmitter at all parasympathetic, preganglionic autonomic and sympathetic postganglionic nerve endings, as well as at the neuromuscular junction and at some central nervous system synapses. Acetylcholinesterase (AchE) inhibitors comprise several compounds of diverse structures and have the ability to inhibit the ace-tylcholine neurotransmitter (Rosini et al., 2005; Khan, 2009). AchE inhibitors are the most common drugs used in the treatment of diseases such as Parkinson's, Alzheimer's, senile dementia, and ataxia (Ahmad et al., 2003). However, drugs such as rivastigmine, galantamine, and donepezil have limitations for medical use due to their adverse side effects (Ferreira et al., 2006). Therefore, it is necessary to explore the plant kingdom for drugs that may inhibit acetylcholinesterase. The various extracts and isolated compounds of *Barleria* with reported acetylcholinesterase inhibitory activity are summarized in Table 2.13.

Amoo et al. (2011) evaluated the acetylcholinesterase inhibitory activity of the methanolic extract of *B. prionitis* and found that it exhibited a dose-dependent inhibition action. At a higher concentration of extract (0.625 mg/ml), the leaf and stem of *B. prionitis* demonstrated greater inhibitory activity than its root extract. Kosmulalage et al. (2007) also reported about the isolation of various compounds from the

ethanolic extracts of *B. prionitis* and their potential in inhibiting acetylcholinesterase. Balarenone, along with lupeol, pipata-line, and 13,14-seco-stigmasta-5,14-diene-3-α-ol, isolated from ethanolic extracts demonstrated moderate inhibitory activity against AChE (Kosmulalage et al., 2007). Three distinct derivatives of pipataline, viz., 8-amino-7-hydroxypipataline, 7,8-epoxypipataline, and 7,8-dibromopipataline, were further synthesized to evaluate their inhibitory potential against acetylcholinesterase. Among the tested compounds, the best results were observed with 8-amino-7-hydroxypipataline, which exhibited significant acetylcholinesterase inhibitory activity with an IC_{50} value of 36.8 μM. Therefore, plant species within the genus *Barleria* demonstrate significant potential in inhibiting acetylcholinesterase activity.

Table 2.13: Acetylcholinesterase inhibition of extracts and isolated compounds from species within *Barleria*

Plant species	**Plant part**	**Extract/compound**	**Inhibition of acetylcholinesterase/**	**Reference**
B. albostellata	Leaves, stems and roots	Methanol	Microtitre plate assays based on the colorimetric method and AChE inhibition activities by galanthamine	Amoo et al., 2011
B. greenii	Leaves, stems and roots	Methanol	Microtitre plate assays based on the colorimetric method and AChE inhibition activities by galanthamine	Amoo et al., 2011
B. prionitis	Leaves, stems and roots	Methanol	Microtitre plate assays based on the colorimetric method and AChE inhibition activities by galanthamine	Amoo et al., 2011
	Aerial	Balarenone, pipataline and lupeol, 13,14-seco-stigmasta-5,14-diene-3-β-ol	Modified Ellman's assay and photometric method	Kosmulalage et al., 2007
		Shanzhiside methyl ester, 6-*O-trans-p*-coumaroyl-8-Oacetylshanzhiside methyl ester, barlerin, acetylbarlerin and 7-methoxydiderroside, lupulinoside	Ellman's assay	Ata at al., 2009

* AChE- acetylcholinesterase

2.3.4.16 Toxicology/safety of extracts of Barleria

Narmadha and Devaki (2013) evaluated the acute toxicity and effective dose determination of the ethanolic leaf extract of *B. cristata* L. in wistar albino rats. Based on their body weight (250, 500, 1000 and 2,000 mg/kg), the ethanolic leaf extract were administered orally as a single dose to rats. Results showed that the administration of the ethanolic leaf extract at all doses (up to 2000 mg kg) did not produce any sign of acute toxicity or instant death in rats while tested during the period of observation. Singh et al. (2005) evaluated the induced hepatotoxicity of the ethanol-water extract of the leaves and stems of *B. prionitis* in various experimental models, CCl_4, D-GalN and paracetamol. In the safety evaluation study the oral LD_{50} was found to be >3000 mg/kg, with no signs of mortality after a single dose of drug administration. Kumari et al. (2017) determined the toxicity of the methanol leaf and stem extracts by selecting different concentration of doses administered to albino rat (% mortality by using standard test). No mortality of albino rats (200, 400 and 600 mg/kg body weight) was recorded in both treatments of extracts. There is a scarcity of information on the toxicology and safety of extracts of *Barleria*, thus further studies are required.

2.3.5 Biosynthesis of nanoparticles from plant extracts of species within *Barleria*

Nanotechnology is an evolving field that focuses on the synthesis and application of small materials called nanoparticles (<100 nm) (Albrecht et al., 2006; Mittal et al., 2014; Sigamoney et al., 2016; Devi and Selvan, 2017; Khatoon et al., 2017). The physical properties of nanoparticles (NPs) such as their size, shape, morphology, and their large surface-area-to-volume ratio have optimised their activity in various fields such as chemistry, medicine, and agriculture (Iravani, 2011; Vanaja and Annadurai, 2012). Significant development has been made in the study of metal-derived nanomaterials for their therapeutic and biomedical applications (Liao et al., 2006). The development of multiple drug-resistant microorganisms poses a worldwide threat to public health (Rai et al., 2009). Incorrect use of antibiotics allow microorganisms to develop mutations, thereby making them resistant to conventional biocides (Kim et al., 2007; Rai et al., 2009; Sigamoney et al., 2016). Treatment of diseases caused by drug-resistant pathogens can lead to increased rates of morbidity and mortality (Huh and Kwon, 2011; Bhatt et al., 2015). Therefore, there is a need for widespread research in nanotechnology for identifying an effective treatment against drug-resistant bacteria (Morones et al., 2005).

Synthesis of NPs from plants has received substantial attention due to their efficient use as reducing and capping agents of metals and their range of pharmacological applications (Savithramma et al., 2011). Plants are widely accessible and less toxic, making this technique environmentally friendly and cost effective (Lee et al., 2011; Khalil et al., 2018). Medicinal plants are an abundant source of biologically active compounds. It is assumed that the bioreduction of NPs using plant extracts is merely due to the occurrence of phytochemicals such as flavones, organic acids, polyphenols, and quinones (Chinnasamy et al., 2017; Maddila and Hemalatha, 2017). The most frequently used metal nanoparticle for plant biosynthesis is silver (Salunke et al., 2014; Ahmed et al., 2016). Silver nanoparticles (AgNPs) are extremely toxic to multidrug resistant bacteria (Jain et al., 2009). The various extracts of *Barleria* with reported biological activity of synthesized NPs activity are summarized in Table 2.14.

Govindarajan and Benelli, (2016) examined the toxicity of AgNPs synthesized from *B. cristata* leaf extracts against the larvae of *Aedes albopictus* (LC_{50} value 12.46 µg/mL), *Culex tritaeniorhynchus* (LC_{50} value 13.49 µg/mL), and *Anopheles subpictus* (LC_{50} value 15.01 µg/mL) (vectors of mosquitoes). The biosynthesized AgNPs demonstrated acute toxicity at low dosages against the various larvae of mosquitoes (Govindarajan and Benelli, 2016). Overall, their study results emphasized that AgNPs synthesized from *B. cristata* are promising and ecofriendly agents that can be used against the vectors of mosquito. In addition, Gomathi et al. (2018) reported that AgNPs synthesized from the leaf extracts of *B. cristata* exhibited potent antimicrobial activity. The NPs demonstrated extremely promising antibacterial activity against *E. coli* and *S. aureus* (Gomathi et al., 2018). These studies have shown that the phytochemical compounds present in leaf extracts could serve as reducing and capping agents of silver nitrate ($AgNO_3$), a frequently used precursor in AgNP synthesis. Medicinal plants are considered

as a promising biological route for the synthesis of biocompatible metal nanoparticles. The development of green protocols for the synthesis of AgNPs has become a novel field of science and is known as green nanotechnology. There is a scarcity of scientific information on the synthesis of AgNPs from plants extracts of species within *Barleria*. Therefore, it is necessary to screen more plant extracts for the biosynthesis of AgNPs as these particles have promising use in the nanotechnology industry and can be used as an affordable, environmentally friendly alternative to conventional medicine.

Table 2.14: Biological activity of synthesized nanoparticles from extracts of species of *Barleria*

Plant species	**Plant part**	**Extract**	**Nanoparticles synthesized**	**Reported activity/phytochemicals**	**Reference**
B. cristata	Leaves	Aqueous	Ag	Mosquitocidal potential	Govindarajan and Benelli, 2016
			Ag	Antibacterial activity against *E. coli* and *S. aureus*	Gomathi et al., 2018
B. longiflora	Leaves	Aqueous	Ag	Antimicrobial activity, inhibition of *Enterococcus* sp., *Streptococcus* sp, *B. megaterium*, *P. putida*, *P. aeruginosa*, *S. aureus* and potential application in photocatalytic dye degradation processes	Cittrarasu et al., 2019
B. prionitis	Leaves	Aqueous	Ag	Diverse groups of phytochemicals, polyphenols, starch, reducing sugars, ascorbic acid and citric acid using GC-MS analysis	Ghosh et al., 2016

*Ag- Silver

2.4 *Barleria albostellata*

In South Africa, *B. albostellata* is known as 'The grey *Barleria'* or in Afrikaans the 'Bosviooltjie' (Froneman and Le Roux, 2007). According to Balkwill and Balkwill (1998), *B. albostellata* is a small shrub with an herbaceous calyx, anterior lobe divided and posterior lobe with sharp, curved apiculus (Figure 2.3 A). Bracts are highly modified, chartaceous, foliaceous, with reticulate venation being prominent; margins are entire, serrate or irregularly dentate (Figure 2.3 B-C). Bracteoles are similar to bracts but smaller (Figure 2.3 D). The inflorescence is a compound, terminal synflorescence, capitate or strobilate with units of solitary flowers (Figure 2.3 E). The lateral walls of seed capsules remain intact upon the splitting of the fruit (Figure 2.3 F). The leaves are discoloured, usually darker on the

adaxial than the abaxial surface. Leaves are densely covered with dendroid or stellate hairs, particularly the abaxial leaf surface (Figure 2.3 G) (Balkwill and Balkwill, 1998).

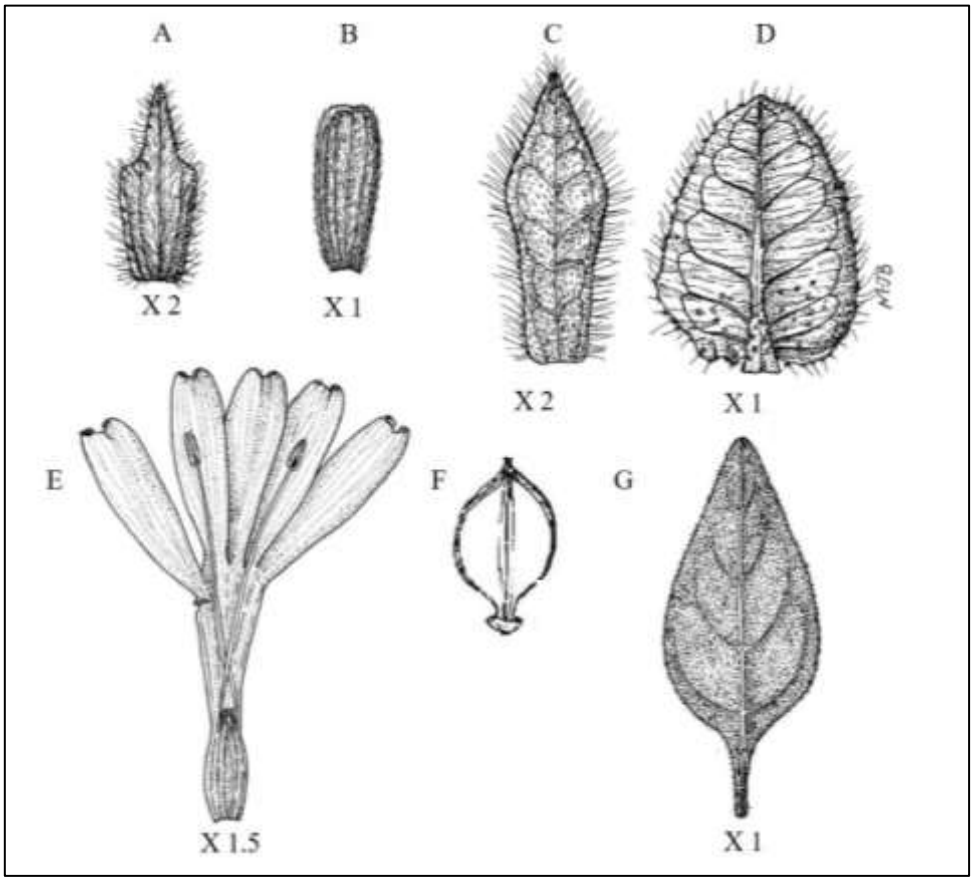

Figure 2.3: Illustrations of *B. albostellata* (Adapted from Balkwill and Balkwill, 1997). (A) Posticous calyx lobes outer surface; (B-C) Floral bracts abaxial surface; (D) Bracteoles abaxial surface; (E) Opened flower slit along the lower corolla lobe; (F) Unopened seed capsule; (G) Adaxial leaf surface.

2.4.1 The medicinal properties of *B. albostellata*

The medicinal value of plants relates to the presence of bioactive phytochemicals that exhibit positive physiological results on humans. This may then be used in the treatment of various diseases (Hoque et al., 2018). Bacteria and fungi have been involved in the pathology of several diseases. For example, Candidiasis is an opportunistic fungal infection caused by *Candida albicans* (Shai et al., 2008). This infection has been reported frequently among immune-compromised individuals with AIDS (Motsei et al., 2003; Shai et al., 2008; Berberi and Noujeim, 2015). Amoo et al. (2011) showed that phytochemical extracts of leaves and stems of *B. albostellata* exhibited fungistatic and fungicidal activities, especially against *C. albicans* (Amoo et al., 2011).

Bacteria such as *E. coli*, *K. pneumoniae* and *S. aureus* are known to be involved in respiratory or gastroenteritis infections (Moise and Schentag, 2000; Sun et al., 2006). Additionally, Amoo et al. (2009) also reported on the antibacterial activity of leaf extracts of *B. albostellata* against *B. subtilis*, *E. coli*, *S. aureus* and *K. pneumonia*. The infectious manifestation of new and re-emerging diseases with no effective therapies, along with the development of pathogen resistant strains to certain currently used drugs require the continuous search for efficacious and potent antimicrobial compounds (Cragg et al., 1997).

Alzheimer's disease (AD), a neurodegenerative disorder, is characterised by cognitive dysfunction, impaired memory, and at advanced stages, depression, language deficit and psychosis (Houghton et al., 2007; Amoo et al., 2011; Voorhees et al., 2017). Inhibition of the acetylcholinesterase enzyme (AChE), which is important in the maintenance of levels of acetylcholine and improved cholinergic function, is one of the standard approaches in the treatment of AD (Howes and Houghton, 2003; Vinutha et al., 2007; Gocer et al., 2016). The therapeutic use of some Ache inhibitors (tacrine), can lead to harsh side effects such as hepatotoxicity has necessitated further exploration for more potent drugs (López et al., 2002; Howes and Houghton, 2003; Ferreira et al., 2006). The AChE inhibitory activity of leaves and stem extracts of *B. albostellata* was significantly high, compared to other plant extracts. In *B. albostellata*, the highest inhibitory activity, as defined by the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values were recorded in stems in comparison to the leaves (Amoo et al., 2011).

It is essential however, to consider that, antibacterial, antifungal and antimicrobial activities vary depending on the plant parts used (Mahesh and Satish, 2008). Microscopically distinguishable plant structures can either secrete or excrete important active compounds (Cutter, 1978; Thanh et al., 2017). These secreted substances can form part of the plants natural defence system (Umah et al., 2017) and have the potential to be used for medicinal gain (Cutter, 1978; Gurib-Fakim, 2006). Medicinal compounds present in plant material can be located in glandular structures of epidermal hairs, also known as trichomes (Levin, 1973; Barthlott et al., 2017).

2.5 Secretory structures

Various important natural chemicals, used by man throughout the centuries, are produced by vascular plants secretory tissues (Fahn, 1988). Secretory tissues can be divided into two main categories: 1. Secretory tissues which are located on plant surfaces and are typically exuded from specialised secretory cells to the external surface of the plant (eg. Glandular trichomes) (Fahn, 1988; Khan, 2017). 2. Internal plant secretory tissues that secrete into specialised intercellular spaces (Fahn, 1987). These structures may be produced at various stages of a plant's life and can differ in type and quantity (Fahn, 1988). Such intraspecific variation can be due to the changes in the genetic make-up, habitat, growth condition and the plant organ being investigated (Figueiredo et al., 2008).

Secretory structures play a role in the metabolic pathway of plants, and in the plant's interrelation with the external environment. These structures can include mucilage secreting cells, salt secreting glands and trichomes (Fahn, 1979; Umah et al., 2017). There is great deviation in the structure and function of secretory tissues in different species of plants. The development and composition of secretion in these structures can also vary. The source of bioactive compounds within plants are often considered as secondary metabolites (Balandrin et al., 1993; Umah et al., 2017). These compounds have a variety of functions in plants and are known to be synthesized for various reasons. These reasons include, lack of nutrients; attraction of pollinators in seed dispersal and discouragement of herbivorous animals (Fahn, 1988; Cannell, 1998; Speed et al., 2015). This study focuses on the most diverse group of external secretory structures, plant trichomes.

2.6 Trichome development and morphology

Trichomes are multicellular or unicellular appendages derived from cells found on the aerial epidermis (Werker, 2000). The development of a trichome begins when single protodermal cells cease to divide and grow outward in the plane of the leaf surface (Szymanski et al., 1998, 1999; Tian et al., 2015). Committed cells then proceed through a series of distinguishable stages of trichome differentiation. The first stage is categorised by the radial expansion in the plane of the leaf; the second stage is the growth out of the plane of the leaf; third stage, the development of branches; fourth stage, branch expansion; fifth stage, diffuse expansion and the last stage is cell wall maturation (Szymanski et al., 1998, 1999; Marks et al., 2008).

Scientific interest in trichomes is based on their taxonomic and functional importance as well as on the economic efficacy of trichome secreted substances (Choi and Kim, 2013). Trichomes vary significantly in location, morphology, mode of secretion and ability to secrete (Serna and Martin, 2006). Though the morphology of trichomes differs significantly, they can be categorized into two types: glandular secreting trichomes (GSTs) and simple trichomes (STs) also referred to as "non-glandular" (presumably non-secreting) (Wagner et al., 2004; Huchelmann et al., 2017). Leaves of various plants are heavily

protected with glandular and non-glandular trichomes which are derived from the epidermal cells (Valkama et al., 2004; Huchelmann et al., 2017). Trichomes occur in various forms, being straight, tortuous, hooked, spiral, stellate, simple, peltate or capitate (Levin, 1973). In the leaves of Acanthaceae, two types of trichomes occur: (a) glandular and (b) non-glandular (Ahmad, 1978).

2.6.1 Glandular trichomes

Glandular trichomes comprise a variety of glands that synthesize, accumulate and secrete active compounds (Croteau, 1987; Gershenzon et al., 1989; Huchelmann et al., 2017). The structure of glandular trichomes usually consist of a unicellular or a multicellular stalk (Jani et al., 2013), with the glandular head being either unicellular or multicellular (Werker, 2000). Glandular trichomes comprise of a stalk, base and a terminal secretory head, which all may consist of one or many cells (Fahn, 1988; Werker, 2000). A neck cell can be found occasionally, between the secretory head and stalk cells, and is morphologically different from the two. Glandular trichomes can be found throughout dicotyledonous angiosperms (Metcalfe and Chalk, 1950; Levin, 1973; Bhatt et al., 2010). Glandular trichomes of 39 genera (109 species) of Acanthaceae are illustrated in Figure 2.4 (Ahmad, 1978).

Glandular capitate trichomes are variable in structure among different species and several trichomes are seen in *B. prionitis* (Bhogaonkar and Lande, 2012). Two general types of capitate trichomes were identified by Werker (1993, 2000). The first comprised of a uni-, bi- or multicellular head cell with a subcuticular space for the storage of exudates, a neck cell, unicellular stalk and a basal epidermal cell. The second is similar to the first type except that it has a unicellular, bulbous head cell with a subcuticular space and with one or two basal epidermal cells (Maleci-Bini and Giuliani, 2006; Jia et al., 2012). These trichomes are considered as short-term trichomes, as the duration of secretion is brief, and is only active during the early development of organs (Giuliani and Maleci-Bini, 2008).

2.6.2 Non-glandular trichomes

Non-glandular trichomes may be unicellular, multicellular or branched (Fahn, 1988; Gairola et al., 2008; Kryvych et al., 2011; Osman, 2012). Non-glandular trichomes of 39 genera (109 species) of Acanthaceae are illustrated in Figure 2.5 (Ahmad, 1978). Certain species of *Barleria* have characteristic non-glandular trichomes which are unicellular (Ahmad, 1978). Multicellular trichomes can be classified further as uniseriate (single row of cells) (Werker, 2000), as seen in *Asystasia chelonoides* and *B. lawii* of Acanthaceae (Ahmad, 1978); or multiseriate, (several rows of cells) as noted in *B. ventricosa* (Balkwill and Balkwill, 1997). Non-glandular trichomes in plant species found within Acanthaceae vary in density in the different developmental stages of leaves (Ahmad, 1978). Trichome diversity observed in *Barleria* includes the occurrence of sunken glands present on the abaxial surface of leaves, stellate, dendroid, anvil-shaped and biramous trichomes, as well as adpressed non-glandular trichomes with multiseriate bases (Balkwill and Balkwill, 1997).

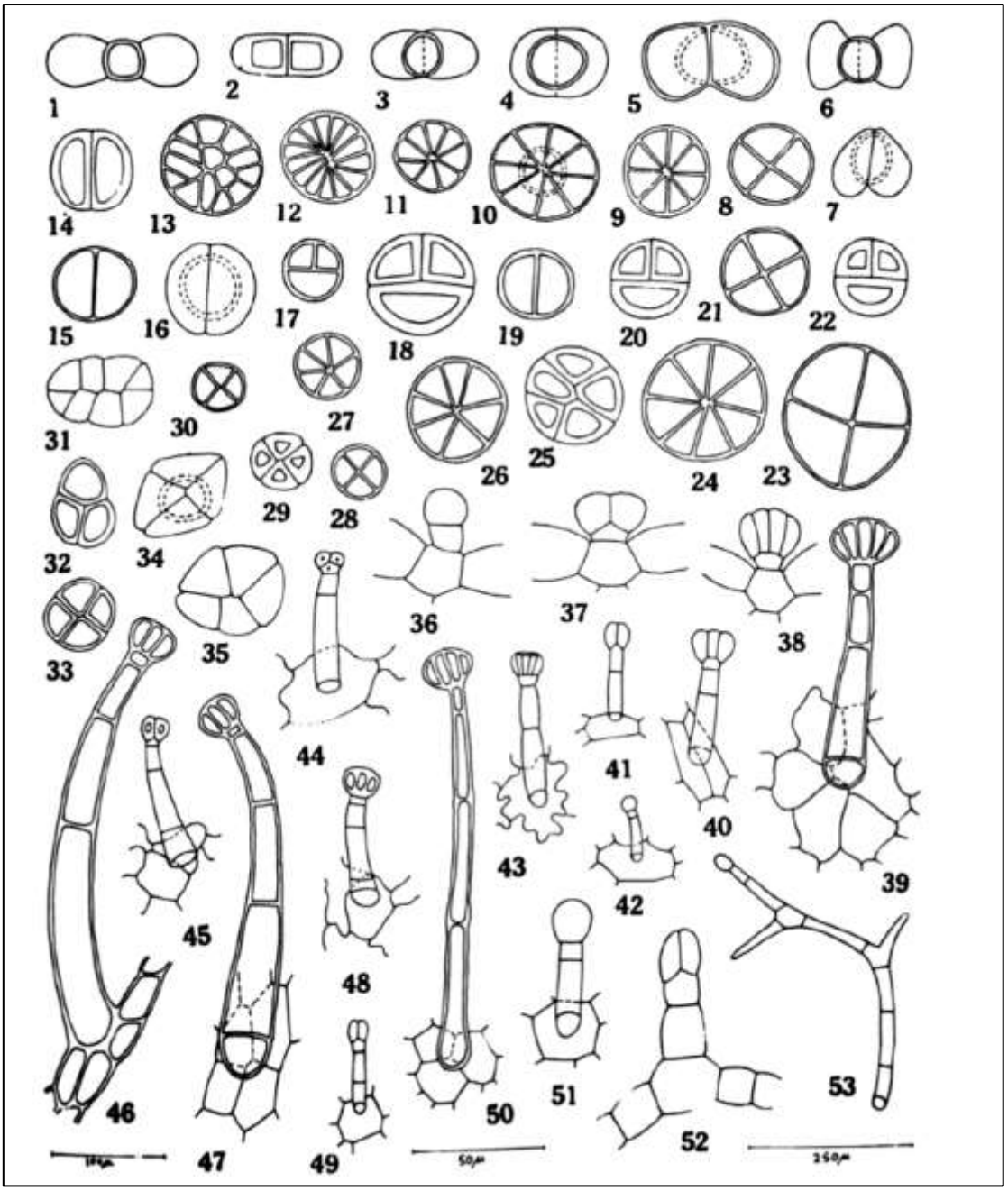

Figure 2.4: Glandular trichomes of Acanthaceae (adapted from Ahmad, 1978). (A) Sub-sessile (short-stalked) trichomes with two-celled panduriform glandular head (1-7, 36); (B) Sub-sessile (unicellular short-stalked) trichomes with two- or more celled, globular or disc-shaped head (8-35, 37, 38); (C) Long stalked trichomes with 1-5 celled uniseriate stalk terminated by one- or more-celled, globular or hemispherical, glandular head (39-53); (D) Branched trichome with a glandular head on one branch (53).

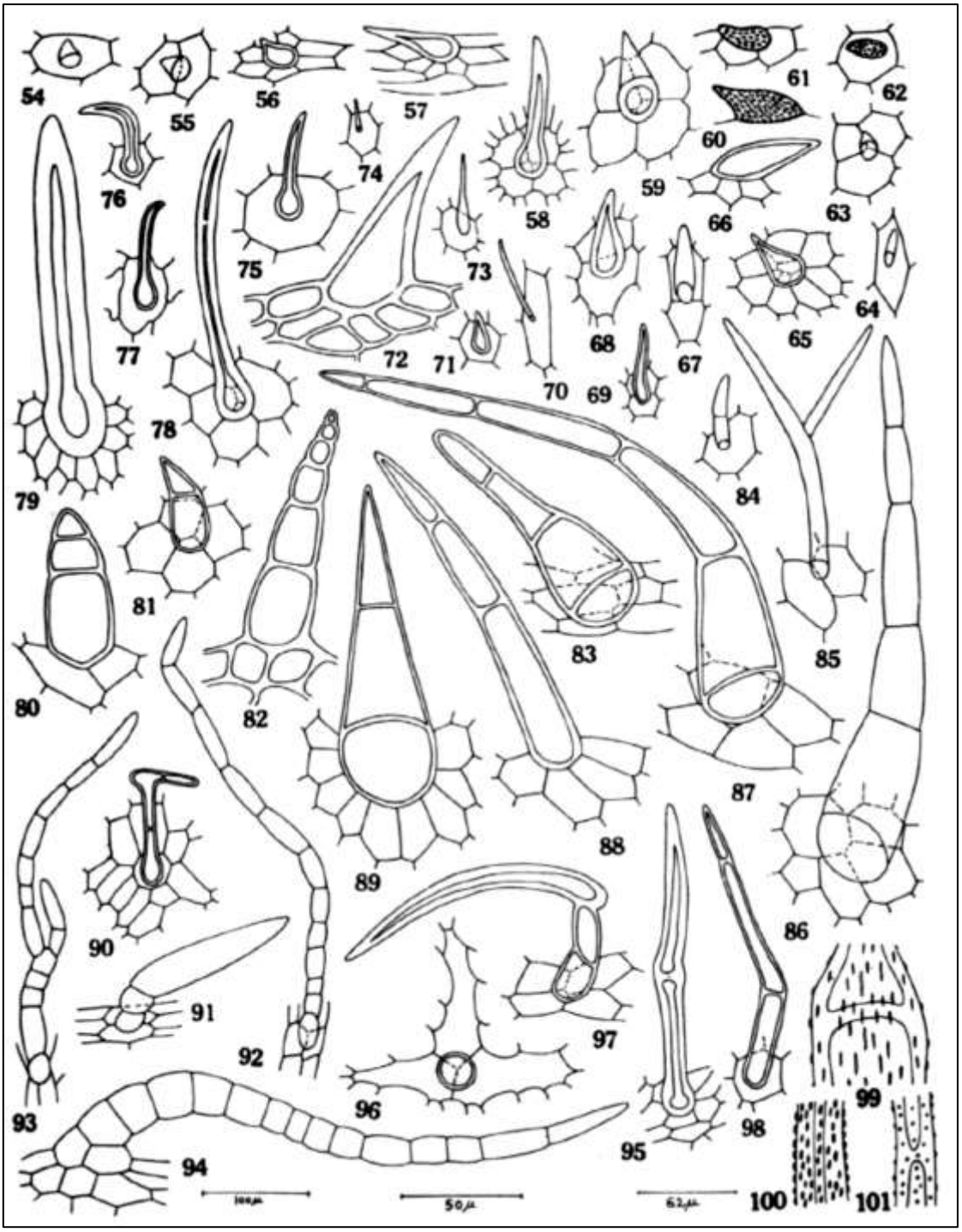

Figure 2.5: Non-glandular trichomes of Acanthaceae (adapted from Ahmad, 1978). (A) Unicellular trichomes (54-79); (B) Multicellular non-glandular trichomes are generally 2-6 celled (80-98); (C) Portions of non-glandular trichomes magnified illustrates wall ornamentation (99-101).

2.7 Trichome function

2.7.1 Plant defence

Plants can negatively affect insect survival and fertility, thus demonstrating a practical need for trichome research, specifically in economically important crop species (Levin, 1973). The success and proliferation of trichomes in various plant families lies in its ability to perform several functions. These functions can include plant defence (Wagner et al., 2004; Figueiredo et al., 2013), and the ability to modify trichome density and type through eco-geographically and biologically driven selection (Johnson, 1975; Wellso and Hoxie, 1982). Plants may be protected against herbivores and destructive insects both by morphological structures, such as trichomes and thorns, and by secondary compounds ("chemical defences") (Chapman, 1977; Southwood, 1986; Agren and Schemske, 1993; Margineanu et al., 2014). Trichome secretions can poison some pests, whilst others are rendered harmless by immobilisation in the secretion per se (Levin, 1973).

In *B. prionotis*, phytochemical constituents in leaves and stems such as tannins, alkaloids and flavonoids are secondary metabolites that serve as defence mechanisms against the predation of herbivores, insects and microorganisms (Chavan et al., 2010[b]). Some plants produce trichomes constitutively, while others react to injury by increasing trichome density in emergent leaves (Dalin et al., 2008). Trichome defences (both glandular and non-glandular) can detrimentally affect natural enemies by hindering movement (Keller, 1987; Krips et al., 1999). Glandular trichomes can differ in structure and secrete a variety of secondary substances (Levin, 1973). These trichomes are involved in the sequestration, production and accumulation of specific phytochemicals that often contain antioxidant and antimicrobial properties (Duke, 1994; Schilmiller et al., 2008). Glandular trichomes can be involved in both physical and chemical defence (Duffey, 1986; Soroka et al., 2011; Oksanen, 2018). Several compounds such as essential oils, resins, tannins and mucilage substances can be found in the glandular exudates of numerous plant genera and families (Uphof, 1962).

The restricted movement of insects on leaf surfaces can be additionally inhibited by the occurrence of a sticky exudate associated with various glandular trichomes (Elsey, 1974; Belcher and Thurston, 1982; Obrycki and Tauber, 1984; Sarria et al., 2009). Glandular trichomes exudate can affect natural enemies by reducing their dwelling time on plants (Obrycki and Tauber, 1984; Romeis et al., 1999; Lovinger et al., 2000; Serna and Martin, 2006), entrapping small-bodied individuals (Obrycki and Tauber, 1984; Gruenhagen and Perring, 1999), and can act as a toxin (Kennedy, 2003). In addition to poisoning and trapping insects, non-volatile exudates from trichome such as simple or complex phenolics and alkaloids

may serve as gustatory repellents. This is proposed by the feeding response of insects to intact tissue or specific chemicals (Levin, 1973).

Non-glandular trichomes are recognized exclusively for their physical protection in plants against biotic and abiotic stresses (Werker, 2000; Wagner et al., 2004), as well as discouraging feeding and ovipositing insects (Levin, 1973; Baur et al., 1991; Szyndler et al., 2013). These trichomes are presumed to arise early in leaf development and deteriorate with maturity (Wagner et al., 2004). This may suggest that non-glandular trichomes may play a role in the protection of emergent leaves until there is a buildup of defence chemicals upon maturation (Johnson, 1975). Expression of genes involved in anthocyanin, glucosinolate and flavonoid pathways can nonetheless be detected in non-glandular trichomes, indicating the roles of these trichomes in defence and in the biosynthesis of secondary compounds (Wang et al., 2002; Jakoby et al., 2008). The density of non-glandular trichomes in young *Alnus incana* (L.) trees after the attack of *Agelastica alni* (L.) beetles was observed by Baur et al. (1991). The number of trichomes increased in the leaves after the attack. The non-glandular trichomes were positively correlated with the amount of defoliation caused to the plant.

2.7.2 Pollination and attraction

In addition to plant defence, trichomes play a role in plant-insect interactions. Several trichome-derived compounds are used as attractants for species-specific pollination (Caissard et al., 2004). Trichomes are also involved in specialised mechanisms of insect capture for pollination (Oelschlägel et al., 2009). *Aristolochia* flowers trap their pollinators using various mechanisms. These include a waxy surface, narrowing of the utricle and directional trichomes. These mechanisms guide insects into the entrance of the flowers but hinder their escape until post-pollination, when flowers modify their inner surface (Oelschlägel et al., 2009). A study conducted by Vogel (1975) reported on trichomes that mimicked pollen and attracted pollinators by deceit.

2.7.3 Protection against water loss, ultraviolet-B and light damage

In cases of elevated temperatures or drought, trichomes can reflect light. This lowers the temperature over the leaf surface, thus reducing water loss through transpiration (Abdulrahaman and Oladele, 2011). Dense trichomes, thick leaf cuticles and surface waxes in plants (Van der Merwe et al., 1994) are believed to have evolved in response to water stress (Stock et al., 1992). This progression is known to be beneficial in improving ultraviolet-B (UV) stress. Leaf trichomes are known to accumulate flavonoids and UV-B-absorbing phenolic compounds (Karaboumiotis et al., 1993[a]; Karaboumiotis et al., 1993[b]; Skaltsa et al., 1994; Yan et al., 2012).

Trichomes prominent on the leaf apex and veins are commonly seen in angiosperms (Oppenheimer, 1959). This adaptation may limit incoming UV light and hence protect vascular tissue (Bhatt et al., 2010). Several South African plants have high foliar alkaloid levels (Watt and Breyer-Braisdwijk, 1962), which contain UV-B screening properties. This development can protect the plant against UV-B radiation (Yan et al., 2012) and limit photosynthetic damage to the leaf (Tevini et al., 1991). Research studies indicated that UV-B radiation can produce an increase in trichome density in *Arabidopsis thaliana* and *Nicotiana tabacum*. This is important in the protection of the underlying plant tissues against the damaging effects of UV-B radiation (Barnes et al., 1996; Yan et al., 2012).

2.8 Internal plant secretory tissue

Apart from external secretory structures or trichomes, Fahn (1988) reported on the occurrence of numerous secretory tissues located within plant organs that secrete endogenously. Some of these tissues are mucilage ducts, mucilage cells, resin, latex, oil idioblasts and crystal idioblasts. These internal secretory cells are common in several angiosperm families (Metcalfe, 1983). This study focuses on the internal secretory tissue, crystal idioblasts.

2.8.1 Crystal idioblasts

Crystals can be found in any organ or tissue in plants and is usually formed in the vacuoles of specialised cells called crystal idioblasts (Foster, 1956; Gebura and Winiarczyk, 2016). Crystal idioblasts are specialised cells that contain calcium oxalate (CaOx) crystals (Franceschi and Horner, 1980; Webb, 1999). Calcium oxalate is a biomineral in plants and occur as crystals of various shapes (Webb, 1999; Franceschi, 2001; Gebura and Winiarczyk, 2016). These crystals are formed in a defined shape and spatial location in plants. This property can be a useful feature in plant systematics and taxonomy (Horner and Wagner, 1995; Lersten and Horner, 2000; Monje and Baran, 2002). Crystals are abundant in some angiosperms and gymnosperms (Franceschi and Nakata, 2005). There are several hypotheses made by researchers regarding the function of crystal idioblasts. These may include plant protection, calcium regulation, detoxification (e.g. oxalic acid or heavy metals), ion balance, tissue support and light gathering and reflection (Schürhoff, 1908; Franceschi and Horner, 1980, Franceschi, 2001). Crystals may play a role in the structural support of leaves and stems as suggested by Schneider (1901). He reported that the gelatinous or mucilaginous residue that surrounds these crystals may act as a bumper between the cell wall and the crystal (Schneider, 1901).

A recent study by Tooulakou et al. (2016) presented findings on the potential use of CaOx crystals. Drought, high temperature and light intensity represents important environmental stress factors that inhibits the plant survival and productivity. Distressed photosynthesis occurring in several plants can result in the use of large CaOx crystals as dynamic internal carbon pools, thus preventing water loss (Tooulakou et al., 2016). An

example of crystals playing a role as an active deterrent mechanism was observed in the plant *Tragia ramose* (Thurston, 1976). This plant is covered with stinging hairs, which are made up of elongated stinging cells that comprise of needle-shaped crystals. When a human or an animal brushes against these hairs, the tip of the cell breaks, allowing the crystals to puncture the dermis of the animal (Thurston, 1976). In plants, several forms of CaOx crystals have been identified, and their only occurrence in higher plants is what makes them a fascinating taxonomic tool (Al-Rais et al., 1971). There is a great potential for the discovery of therapeutic compounds from traditionally used plants in South Africa. Using microscopy techniques, this investigation intends to create a complete understanding of the functioning of secretory structures in *B. albostellata*. The findings from this study are expected to serve as a preliminary phase in confirming *B. albostellata* as a medicinal plant, and to contribute to the growing ethnopharmacological field in South Africa.

2.9 Conclusion and future perspectives

This review describes a comprehensive account of the taxonomy, phytochemical constituent's ethnopharmacology, biological activities and mophology of plants belonging to the genus *Barleria*. This review will aid taxonomists in identifying species within the genus *Barleria* using various characteristics (pollen crystals, trichomes etc.) presented. Several bioactive compounds isolated from *Barleria* species, such as iridoids, phenolics, flavonoids, terpenoids, phytosterols, phenylethanoid glycosides, and aromatic compounds, possess various biological properties of medicinal importance. Moreover, these bioactive compounds have demonstrated several biological activities, including antioxidant, antibacterial, antifungal, anti-inflammatory, anticancer, antidiabetic, antiulcer, hepatoprotective, analgesic, antiamoebic, antihelmintic, antiarthritic, antihypertensive, antiviral, and acetylcholinesterase activity inhibition properties and the ability to synthesize AgNPs. Further investigations are recommended to explore more about the species within *Barleria* to identify new therapeutic compounds or drug leads, as most of them have not yet been subjected to chemical and biological assessment. Therefore, further research on the bioactive compounds and pharmacological activities of plants within this genus will provide a basic understanding of the importance of these species as medicinal plants and a potential source of novel and useful drugs.

2.10 References

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CHAPTER 3

FOLIAR MICROMORPHOLOGY, ULTRASTRUCTURE AND HISTOCHEMICAL ANALYSIS OF *Barleria albostellata*

Abstract

Barleria albostellata C.B. Clarke (Acanthaceae), a species indigenous to South Africa, is a valuable medicinal plant with a broad spectrum of antibacterial and anti-inflammatory activities. This study aimed to characterise the micromorphology, distribution, and chemical composition of the trichomes present on the leaves and stems of *B. albostellata* using light and electron microscopy and histochemistry. Morphological observations using stereo and scanning electron microscopy (SEM) revealed a dense indumentum bearing numerous non-glandular trichomes on the leaves and stems of *B. albostellata*. The histochemical and SEM analyses revealed the presence of five morphologically distinct glandular capitate trichome types, multangulate-dendritic branched non-glandular trichomes and a glandular head attached to a branched non-glandular trichome. The secretory head of glandular typed trichomes varied from 20.72 ± 1.36 µm to 54.23 ± 2.08 µm. Additionally, the length of these stalks varied from 15.11 ± 3.89 µm to 101.92 ± 1.78 µm, and the width from 11.62 ± 1.03 µm to 35.32 ± 3.87 µm. Multangulate-dendritic branched trichomes had a stalk length of 554.10 ± 92.27 µm and width of 28.55 ± 2.48 µm. The secretory head of type V capitate trichome had an average diameter of 16.88 ± 0.63 µm with the length and width of the stalk measuring 22.06 ± 1.14 µm and 9.57 ± 0.82 µm, respectively. Transmission electron micrographs showed that numerous plastids, mitochondria, and endoplasmic reticulum cisternae were actively involved in the secretory process. The stained leaf and stem sections indicated the presence of alkaloids and phenolics as the major medicinal compounds in the glandular and non-glandular trichomes. To our knowledge, this study represents the first detailed report describing key micromorphological features of the foliar structures of *B. albostellata* as well as the preliminary chemical composition of the secretions produced by these structures.

Keywords: Capitate trichomes; Chemical compounds; Multangulate-dendritic branched non-glandular trichome

3.1 Introduction

Barleria albostellata is an evergreen shrub that thrives in semi-shade to full-sun environments in the woodland areas of South Africa. It is commonly known as 'grey barleria' and belongs to the family Acanthaceae (Froneman and Le Roux, 2007). This shrub is widely distributed from Limpopo, Gauteng, and Mpumalanga to KwaZulu-Natal (Balkwill and Balkwill, 2002; Froneman and Le Roux, 2007). *Barleria albostellata* is known for its medicinal properties (Amoo et al., 2009). Phytochemical compounds such as iridoids, phenolics, flavonoids, gallotannins, proanthocyanidins from its leaves and stems exhibited a broad-spectrum of antibacterial activities and excellent anti-inflammatory properties. Although, there are no documented reports on the use of *B. albostellata* in traditional medicine, numerous reports have been published on the analgesic, anti-inflammatory, antitumor, anti-hyperglycemic, antileukemic, anti-amoebic, antibiotic, and virucidal activities of *Barleria* plant extracts (Yosook et al., 1999; Wang et al., 2001; Jassim and Naji, 2003; Suba et al., 2004, 2005; Chomnawang et al., 2005; Amoo et al., 2009; Shukla and Gunjegaokar, 2018). Thus, the genus *Barleria* has a great medicinal potential.

Medicinally important active compounds are found in microscopically distinct plant structures known as trichomes (Levin, 1973; Cutter, 1978; Pickard, 2008; Barthlott et al., 2017; Thanh et al., 2017). Trichomes are unicellular or multicellular appendages derived from the cells located on the aerial epidermis (Werker, 2000). They vary considerably in location, morphology, mode of secretion, and their ability to secrete (Serna and Martin, 2006). Although the morphology of trichomes varies greatly, they can be categorised into two types: glandular trichomes (secreting) and non-glandular (presumably non-secreting) (Wagner et al., 2004; Huchelmann et al., 2017). The complete description of trichome morphology will be a useful diagnostic tool for the identification of the species of the Acanthaceae family. Even though anatomical descriptions are accessible for some species of *Barleria* (Bhogaonkar and Lande, 2012; Sridharan and Gounder, 2016), the anatomy of the leaves and stems of *B. albostellata* have not yet been described.

The micromorphology of secretory structures, which synthesize and secrete phytochemicals, has been rarely studied and is, therefore, poorly understood. Thus, it is important to investigate the micromorphology of the secretory structures of *B. albostellata* in order to provide baseline information on the potential usage of the extracts from this plant. The objective of this study was to characterise the micromorphology of trichomes present on the leaves and stems surfaces of *B. albostellata* as well as to determine the possible site of synthesis and chemical composition of its secretions.

3.2 Materials and methods

3.2.1 Plant materials

Leaves and stems of *B. albostellata* were collected from the University of KwaZulu-Natal, School of Life Sciences, Westville Campus (29° 49′ 51.6″ S, 30° 55′ 30″ E), Durban, South Africa. A voucher specimen (7973000) was deposited in the Ward Herbarium of the University of KwaZulu-Natal, Life Sciences, Westville Campus. Three stages of leaf development: emergent, young and mature and stems were compared using microscopy techniques. The development stages of leaves were based on their length/expansion, texture, and colour. Leaves were classified based on their length as emergent (6–8 mm), young (9–30 mm) and mature (50 mm).

3.2.2 Stereomicroscopy

Fresh leaves and stems were examined using the Nikon AZ100 stereomicroscope (Nikon Corporation, Yokohama, Japan) equipped with a Nikon Fiber Illuminator and photographed using the NIS-Elements Software (NIS-elements D 3.00). The adaxial and abaxial surfaces of fresh leaves at the three developmental stages were imaged with an emphasis on their mid-vein and stem. Trichomes were physically removed from the stems with the use of cellophane tape and imaged.

3.2.3 Scanning electron microscopy (SEM)

The micromorphology of the chemically–fixed samples of both leaf surfaces for each developmental stage and stems were examined in detail. The initial step of preparation involved the primary fixation of fresh leaf sections (± 5 mm^2) in 2.5% glutaraldehyde for 18–24 h. Thereafter, the samples were rinsed thrice (for 5 min each) with 0.1 M sodium phosphate buffer (pH 7.2) and were subjected to post-fixation in 0.5% osmium tetroxide for 3 h at 24°C. The samples were again washed thrice (for 5 min each) using the sodium phosphate buffer and were dehydrated by exposing them to increasing concentrations of ethanol (30%, 50%, 75%, 100%) for two sessions, each of 5 min, followed by exposure to 100% ethanol for two sessions, each of 10 min. The dehydrated samples were critically point-dried using the Quorum K850 Critical Point Dryer (Quorum Technologies Ltd., Laughton, East Sussex, UK) with a vertical chamber. The samples were then mounted onto small aluminium stubs using double-sided adhesive carbon tape and sputter coated with a layer of gold using the Quorum 150 RES (Quorum Technologies Ltd.), a combined system for carbon and sputter coating. The samples were viewed and photographed using the LEO 1450 SEM at a working distance (WD) of 12–15 mm. Images were captured using the SmartSEM image software (Zeiss, Jena, Germany). (Protocol adapted from the microscopy and microanalysis unit).

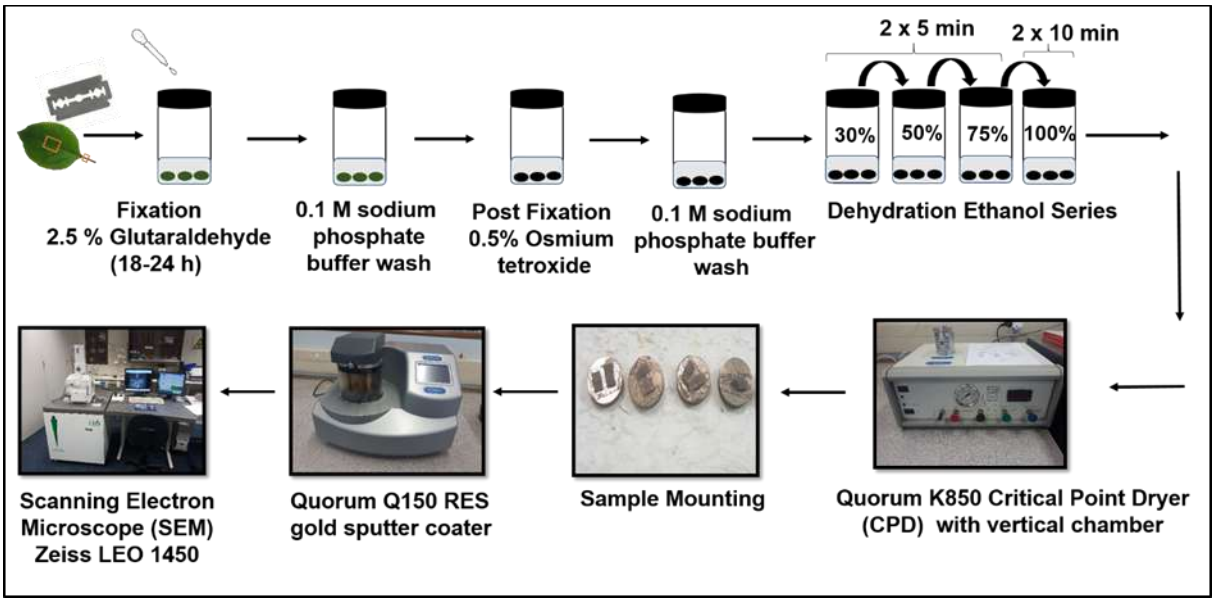

Figure 3.1: Scanning electron microscopy protocol for leaf and stem sections of *B. albostellata*.

3.2.4 Morphometric analysis of trichomes

Selected images of trichomes obtained from SEM were analysed using the ImageJ software program (Fiji, http://fiji.sc/Fiji; Schindelin et al., 2012). The diameter of the trichome head, length, and width of the stalk for each type was measured.

3.2.5 Transmission electron microscopy (TEM)

The ultrastructure of leaf tissue was viewed, analysed and imaged using TEM. Leaf sections from the three developmental stages (±2 mm^2) were excised and primarily fixed in 2.5% glutaraldehyde for 24 h. The sections were rinsed thrice in a 0.1 M phosphate buffer (pH 7.2) and thereafter post-fixed in 0.5% osmium tetroxide for 3 h. Samples were rinsed thrice for 5 min; using the phosphate buffer. The samples were then dehydrated using a graded acetone series (for 5 min each in 30%, 50%, 75%) and two sessions of 10 min each in 100% acetone. After dehydration, the samples were transferred to the clearing agent, propylene oxide, for 15 min and then gradually infiltrated using a graded series of Spurr's low-viscosity epoxy resin in propylene oxide solution (25%, 50%, 75% and 100%) (Spurr, 1969). The samples were embedded in equal parts of Spurr's resin and acetone for 4 h and then in 100% resin for 24 h at 70°C (Spurr, 1969). Subsequently, the samples were transferred to silicon molds and polymerised for 8 h at 70°C.

Glass knives were prepared on the LKB Knifemaker 7801A (Elekta, Stockholm, Sweden) and were used to section the resin blocks. Ultrathin resin-embedded sections were obtained using the Reichert Jung ultra-microtome (Leica, Wetzlar, Germany). The sections were surveyed in order to determine the regions of interest. They were stained with 1% Toluidine Blue, placed on slides, and viewed using the Nikon Eclipse 80i light microscope (Nikon Corporation), equipped with a Nikon DS-Fi1 camera and the NIS-Elements imaging software package. The ultrathin sections were then excised at 90–110 nm using the Reichert Jung ultra-microtome and placed on copper grids. The sections were stained with 2.5% uranyl acetate for 10 min at 23°C, rinsed with distilled water and then stained with lead citrate for 10 min. The copper grids were rinsed with distilled water, viewed, and photographed using the JEOL 1010 TEM (JEOL, Tokyo, Japan) equipped with the iTEM software. (The protocol was adapted from the microscopy and microanalysis unit).

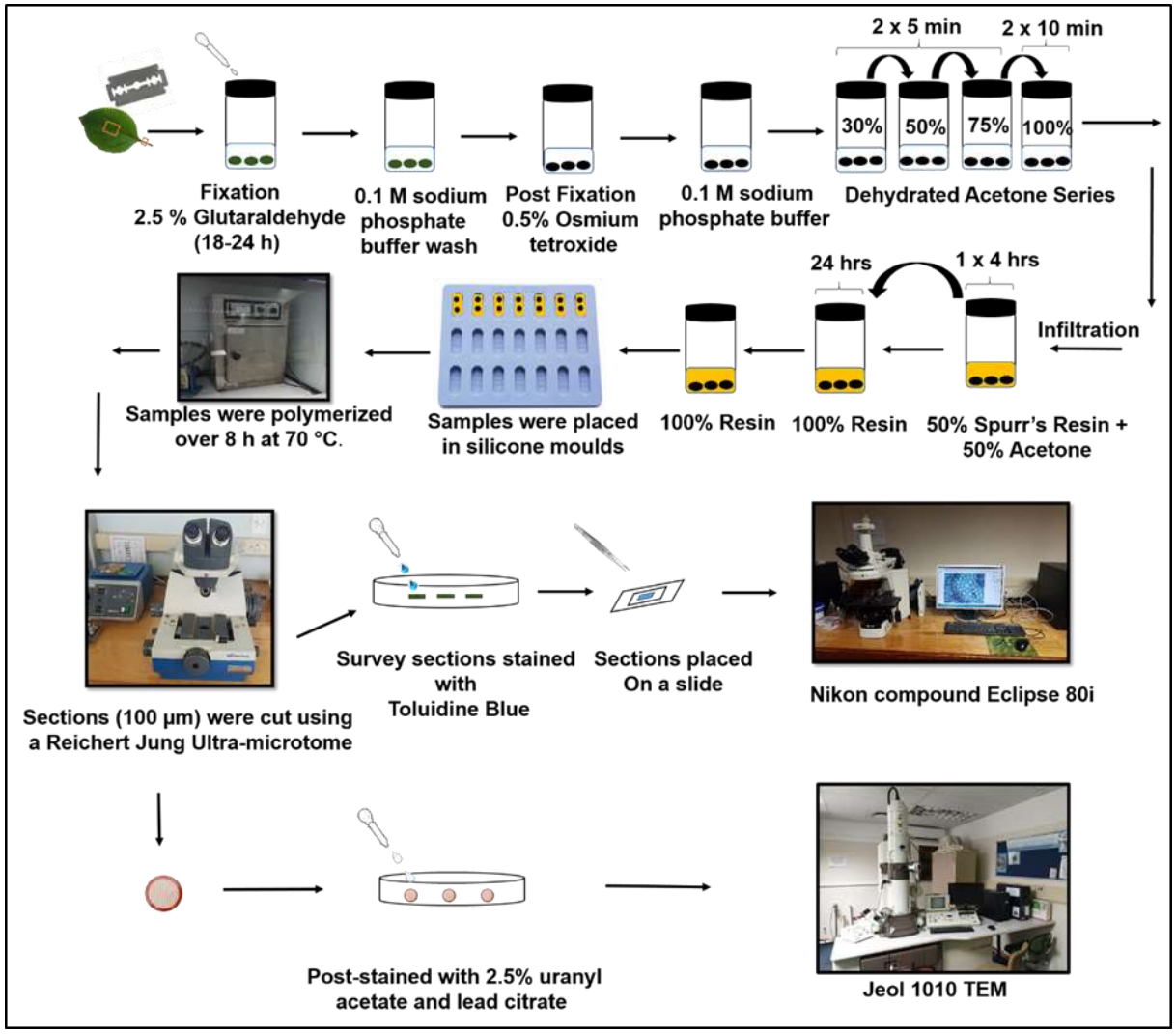

Figure 3.2: Transmission electron microscopy protocol for leaf and stem sections of *B. albostellata*.

3.2.6 Histochemistry

A section of fresh leaf material was positioned between two segments of dental wax and safeguarded in an Oxford® Vibratome Sectioning System (The Vibratome Company, St. Louis, MO, USA). Leaf and stem sections of 90–110 μm were obtained and histochemically stained as described below. The unstained sections served as controls (results not shown). The stained sections were viewed and photographed with the Nikon Eclipse 80i compound light microscope equipped with the Nikon DS-Fi1 compound microscope (Nikon).

3.2.6.1 Alkaloids

Ditmars and Wagners: Sections were stained separately for 10 min each with both Ditmars and Wagners staining reagents, rinsed with distilled water, mounted and viewed. A deep brown-orange colour indicated a positive reaction (Furr and Mahlberg, 1981).

3.2.6.2 Cellulose

Fast green: Sections were dipped in the stain for 1 min and rinsed thoroughly with distilled water. A bright green colour indicated a positive reaction (cell walls) (Tos et al., 1980).

3.2.6.3 Lipid, lignin cutin and suberin

Sudan Black B: Sections were stained for 30 min, rinsed with 70% ethanol and washed in distilled water. Sections were mounted onto a slide with glycerol. A blue-black staining of tissues indicated a positive reaction (Pearse, 1985; Demarco, 2017).

Nile blue: Sections were immersed in 1% Nile Blue at 60°C for 5 min and then in 1% acetic acid for an additional 1 min. Sections were rinsed with distilled water, mounted and viewed. Acidic lipids stained blue (Cain, 1947; Demarco, 2017).

Safranin: Sections were dipped in the stain for 30 sec and rinsed with distilled water. A bright red staining indicated a positive reaction (cell walls) (Bond et al., 2008).

3.2.6.4 Monochromatic staining

Toluidine Blue: Sections were immersed in the stain for 1 min and thereafter rinsed with distilled water, mounted and viewed. A bright pink-purple colour indicated positively for caboxylated polysaccharides and polyphenols stained blue to green. Phosphate groups present on macromolecules stained purple to blue (O'Brien et al., 1964; Sridharan and Shankar, 2012).

3.2.6.5 Mucilages and polysaccharides

Ruthenium red: Sections were placed in 0.1% of the solution for 5 min, washed twice in distilled water, mounted in glycerol and viewed. A pink to red colour indicated a positive reaction (Gregory and Baas, 1989; Demarco, 2017).

3.2.6.6 Nuclei acids

Methylene blue: Sections were immersed in the stain for 1 min and washed thoroughly with distilled water. Acidic cell parts (nucleus) stain blue (Grace and Stribley, 1991).

3.2.6.7 Phenolic compounds

Ferric trichloride: Sections were placed in 10% Ferric trichloride stain and a drop of aqueous sodium carbonate was added to the section for 15 min at room temperature. Deep black deposits produced, indicated a positive reaction (Johansen, 1940).

3.2.6.8 Total proteins

Coomassie Blue: Sections were immersed in 0.25% Coomassie blue for 15 min and differentiated in 7% acetic acid. Sections were then rinsed briefly in distilled water and mounted in glycerol. A blue staining of tissues indicated a positive reaction (Fisher, 1968).

3.2.7 Fluorescence microscopy

Fresh sections were obtained using free hand sectioning methods and the Oxford® vibratome and viewed using the Nikon DS-Fi1 fluorescence compound microscope (Nikon, Japan) equipped with the NIS Elements D software. These sections were viewed using ultra-violet light with an excitation wavelength of 330 nm and DM wavelength of 400 nm. Nikon DS-Fi1 compound microscope (Nikon, Japan) equipped with NIS Elements D software.

3.2.7.1 Cellulose

Calcofluor White: Sections were placed into 0.01% calcofluor white solution for 10 min. Stained sections were rinsed and then mounted in distilled water. Cellulose in cell walls fluoresces light blue under UV light (Hughes and McCully, 1975; Demarco, 2017).

3.2.7.2 Cell viability

Acridine orange: Sections were stained in 0.01% acridine orange for 20 min and mounted with distilled water. This fluorescence dye binds to cell DNA, indicating cell viability (yellow-green colour) (Ruzin, 1999; Winter et al., 2007; Demarco, 2017).

3.2.7.3 Phenolic compounds

Autofluorescence: Sections were mounted in distilled water and viewed. Plant tissues have many autofluorescence components. Phenolic compounds emit a blue fluorescence (Ascensão and Pais, 1987; Talamond et al., 2015).

3.3 Results and discussion

3.3.1 Analysis of secretory structures via stereomicroscopy

The leaf and stem surfaces of *B. albostellata* were characterised by a dense indumentum of non-glandular and glandular trichomes on both adaxial and abaxial surfaces (Figure 3.3 A–F). However, both trichome types differed in density and location on the leaves and stems. The leaves of *B. albostellata* appeared highly pubescent at all stages of development, especially at the emergent stage (Figure 3.3 A and B). This was due to the higher density of non-glandular trichomes than glandular trichomes on the leaf surface (Figure 3.3 and 3.4). It is assumed that non-glandular trichomes play a vital role in the protection of a leaf, especially during the earliest stages of development (Bhatt et al., 2010). Emergent leaves have relatively high nutritional value and may require further protection from trichomes, as these leaves are greatly susceptible to insect and pathogen attack (Duke, 1994; Chaurasiya et al., 2007). Non-glandular trichomes on the emergent leaves were highly dense, making it difficult to view the glandular trichomes and leaf surface. These trichomes, could be possibly protecting the glandular trichomes from insect attack (Gangaram et al., 2020).

Non-glandular trichomes were highly concentrated on the leaf midrib and veins at all stages of leaf development (Figure 3.3) (Gangaram et al., 2020). A greater density of trichomes found on the leaf veins and apex is a typical trend observed in angiosperms (Oppenheimer, 1959). Non-glandular trichomes aggregated along the midrib and veins protect vascular tissue from UV damage (Bhatt et al., 2010; Tozin et al., 2017). The abaxial leaf surface (Figure 3.3 B, D and F) appeared to have a higher density of trichomes than the adaxial surface (Figure 3.3 A, C and E). This was probably because the abaxial leaf surface might have responded to insect damage by increasing the trichome density (Gangaram et al., 2020).

In all developmental stages, non-glandular trichomes appeared intermingled and transparent at higher magnifications (Figure 3.3 C). The presence of dense silvery hairs found on leaves and stems increases the reflectance of solar radiation and efficiently reduces leaf temperatures (Downs and Black, 1999; Werker, 2000; Dai et al., 2010). Non-glandular trichomes were classified as multangulate-dendritic branched (MDB) (Figure 3.3 C). The density of both glandular and non-glandular trichomes appeared to have decreased with the progression of leaf development (Figure 3.3 C-F). Leaf cells expand during growth, thus causing the number of trichomes to decrease relative to the increase in surface area (Ascensão and Pais, 1987; Werker et al., 1993; Duke, 1994; Gairola et al., 2008; Bhatt et al., 2010). At the mature stages of leaf expansion, when the epidermis is fully developed, the functional role of trichomes becomes insignificant; thus, they often shed and senesce (Valkama et al., 2004).

A spider was observed to be trapped between non-glandular trichomes, restricting its movement on the lamina (Figure 3.4 A). Non-glandular trichomes can act as a mechanical barrier, preventing the movement of insects (Baur et al., 1991). The MDB branched trichomes covered the entire stem (Figure 3.4 B-D) making it difficult to view the surface. Therefore, trichomes were removed from a section of the stem region illustrating the lamina surface of the leaf (Figure 3.4 E). However, with trichome removal, the lamina appeared bare and no glandular trichomes were observed.

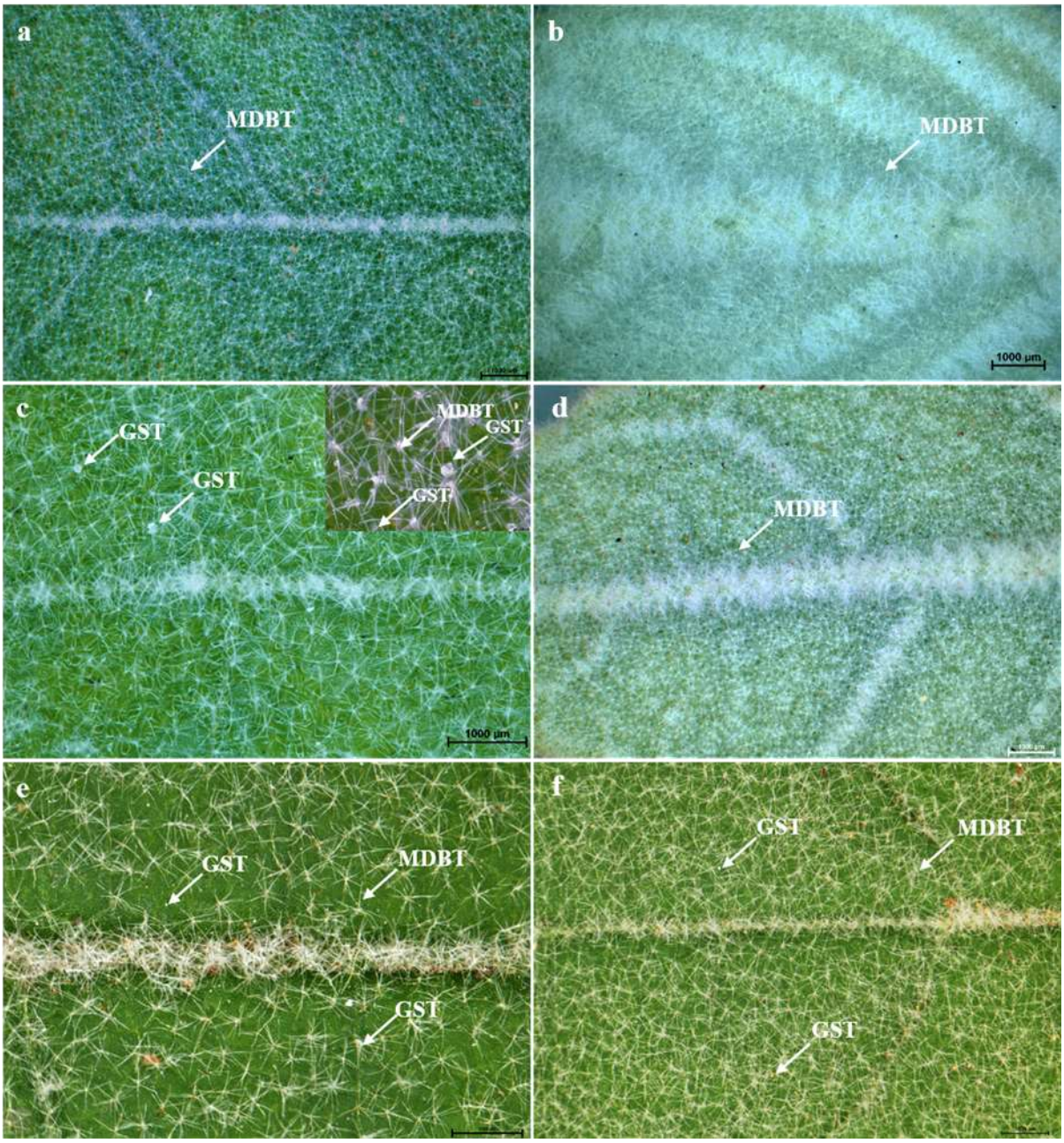

Figure 3.3: Stereomicrographs of multangulate-dendritic branched non-glandular and glandular secreting trichomes on the leaf surface of *B. albostellata*. (A) Adaxial surface of emergent leaf; (B) Abaxial surface of emergent leaf; (C) Adaxial surface of young leaf; (D) Abaxial surface of young leaf; (E) Adaxial surface of mature leaf; (F) Abaxial surface of mature leaf.

Abbreviations: MDBT= Multangulate-dendritic branched; GST= Glandular secreting trichome.

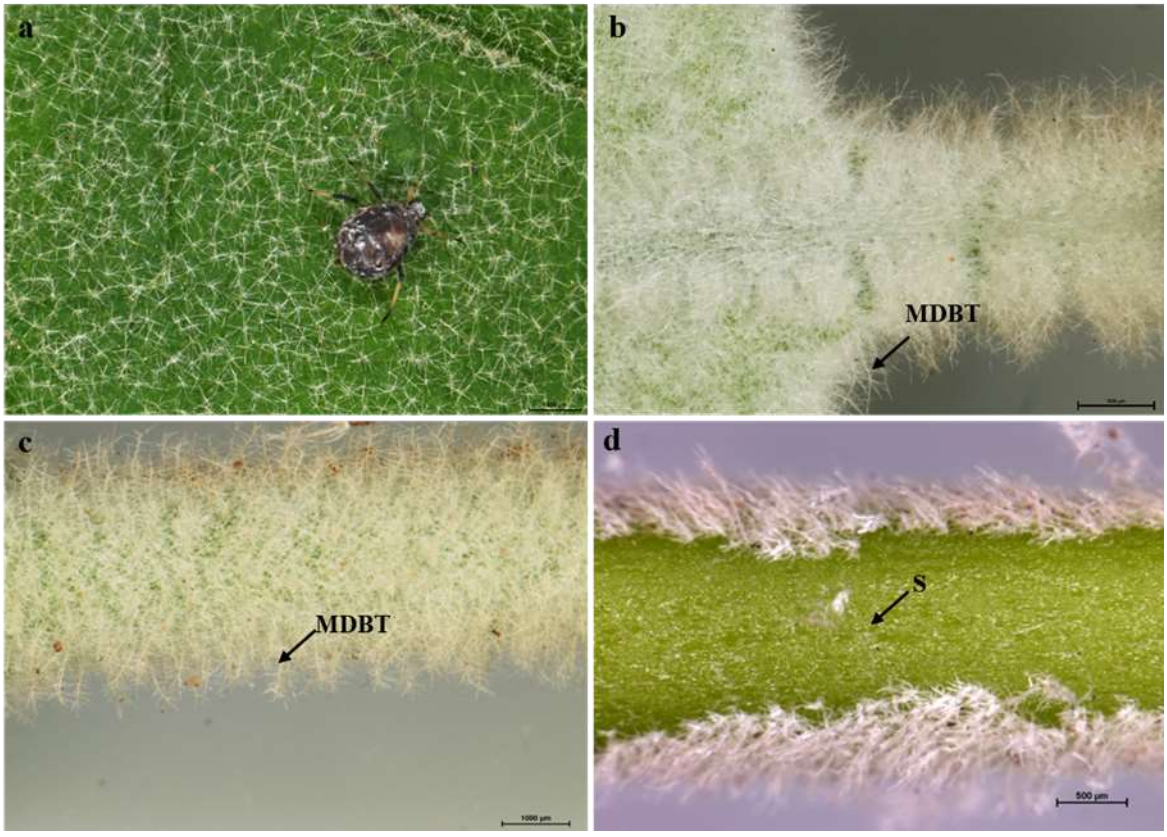

Figure 3.4: Stereomicrographs of multangulate-dendritic branched non-glandular and glandular secreting trichomes on the stem surface of *B. albostellata*. (A) A spider trapped between trichomes; (B) Emergent surface; (C) Young surface; (D) Mature surface; (E) Stripped stem section.

Abbreviations: MDBT= Multangulate-dendritic branched; GST= Glandular secreting trichome; S= Stripped.

3.3.2 Secretory structures observed via scanning electron microscopy

Two methods were used to prepare samples for scanning electron microscopy, i.e., chemical fixation and freeze drying. However, when samples were submerged in liquid nitrogen, plant structures collapsed; hence, chemical fixation was the preferred method (Gangaram et al., 2020). The scanning electron micrographs of glandular trichomes showed great morphological diversity (Figures 3.5, 3.6 and 3.7). Five types of capitate trichomes were observed and designated I, II, III, IV, and V. These trichomes differed in shape and size and occurred throughout all stages of leaf and stem development. Trichomes were densely distributed on both adaxial and abaxial leaf surfaces. Type I capitate trichomes were composed of a 4-celled broad secretory head and a short stalk (unseen) embedded in the epidermal layer (Figure 3.5 A). The

secretory head of type I trichome had an average diameter of approximately 21.44 ± 4.49 µm, whilst the length and width of the stalk could not be measured (unseen). The accumulation of secretion was observed at the apex of the head of the short-stalked capitate trichomes (Figure 3.5 B and C). Glandular trichomes might release secretory substances at fragile areas on the secretory head via cuticle rupture (Jia et al., 2012; Naidoo et al., 2013), micropores (Ascensão et al., 1999; Naidoo et al., 2012) or by diffusion through the cuticle (Caissard et al., 2012). Mechanical damage, low humidity, or high temperature might cause cuticle rupture (Ascensão et al., 1995; Ventrella and Marinho, 2008; Baran et al., 2010). Trichome secretion can also protect plant photosynthetic tissues, as the secretion can reflect extreme solar radiation and aid in scattering the absorbed heat (Tozin et al., 2017).

Type II capitate trichomes consisted of a 2–5-celled secretory head and a 1–2-celled short stalk (Figure 3.5 B–D). The average diameter of the secretory head of type II trichome was 20.72 ± 1.36 µm, with the length and width of the stalk measuring 15.11 ± 3.89 µm and 11.62 ± 1.03 µm, respectively. Type III capitate trichomes had a long-stalk (53.61 ± 3.20 µm in length and width of 12.79 ± 0.65 µm), and a bulbous secretory head which had an average diameter of 21.03 ± 2.98 µm (Figure 3.6 A). Furthermore, type I, II, and III capitate trichomes were consistent with the trichomes found in both leaves and stems of *B. prionitis* L. (Bhogaonkar and Lande, 2012), whilst type I capitate trichomes were found only in *B. montana* Nees (Sridharan and Gounder, 2016). Type IV capitate trichomes comprised of a broad secretory head with several cells (an average diameter of 54.23 ± 2.08 µm), a neck cell, and a 3-celled long stalk (Figure 3.6 B) measuring 101.92 ± 1.78 µm in length and 35.32 ± 3.87 µm in width. This trichome shared similarities with the capitate trichome found in *Hygrophila serpyllum* T. Anderson (Acanthaceae) (Ahmad, 1978). The thickened neck cells present in certain capitate trichomes are assumed to prevent the apoplastic backflow of secreted products, the possible phytotoxic composition that can pose a risk to the entire plant if released (Serrato-Valenti et al., 1997; Werker, 2000; Baran et al., 2010).

Glandular trichomes were found scattered all over the leaf surface and concealed under the MDB non-glandular trichomes (Figure 3.6 C) (Gangaram et al., 2020). Non-glandular trichomes were found to 'arch over' the glandular trichomes. Multangulate-dendritic branched trichomes had a stalk length of 554.10 ± 92.27 µm and width of 28.55 ± 2.48 µm. Owing to the longer length of the MDB non-glandular trichomes and their close proximity to the glandular trichomes, it is probable that these structures might provide physical protection to the glandular trichomes. Stomata were observed on the surface topography of both leaves and stems (Figure 3.6 C). The densely arranged non-glandular trichomes on the leaf surface were thought to protect the stomata from extensive heat during the dry and hot seasons. Two capitate trichomes developing on top of each other below the MDB trichomes were observed (Figure 3.6 D). Two type V, glandular capitate trichomes that were attached to each other appeared branched (Figure 3.7 A). The

secretory head of type V capitate trichome had an average diameter of 16.88 ± 0.63 µm with the length and width of the stalk measuring 22.06 ± 1.14 µm and 9.57 ± 0.82 µm, respectively. Various morphological structures of certain capitate trichomes might be related to the production of numerous chemical substances with specific functional roles (Werker et al., 1985).

The MDB non-glandular trichomes were observed at all stages of leaf and stem development (Figure 3.7 B). However, multicellular non-glandular trichomes were found in the leaves and stems of *B. montana* (Banu et al., 2012), *B. prionitis* (Bhogaonkar and Lande, 2012), and *B. gibsonii* Dalzell (Tamboli and More, 2016). The glandular head (an average diameter of 20.27 ± 1.05 µm) attached to a branched non-glandular trichome found in this study (Figure 3.7 C–E) shared similarities with that found in *Dyschoriste vagans* (Wight) Kuntze (Acanthaceae) (Ahmad, 1978). The average length and width of GHBNG trichomes were 49.98 ± 5.22 µm and 11.73 ± 0.53 µm. It has been hypothesised that glandular trichomes develop phylogenetically from non-glandular trichomes in several plant species (Uphof, 1962; Fahn and Shimony, 1977; Fahn, 1979). In certain species of the Labiatae family, both branched non-glandular trichomes and trichomes with one of the branches carrying a glandular head occur (Azizian and Cutler, 1982; Werker et al., 1985). No reported studies have investigated the secretory structures of *B. albostellata* and very few have investigated the secretory structures in the genus *Barleria* (Ahmad, 1978; Bhogaonkar and Lande, 2012).

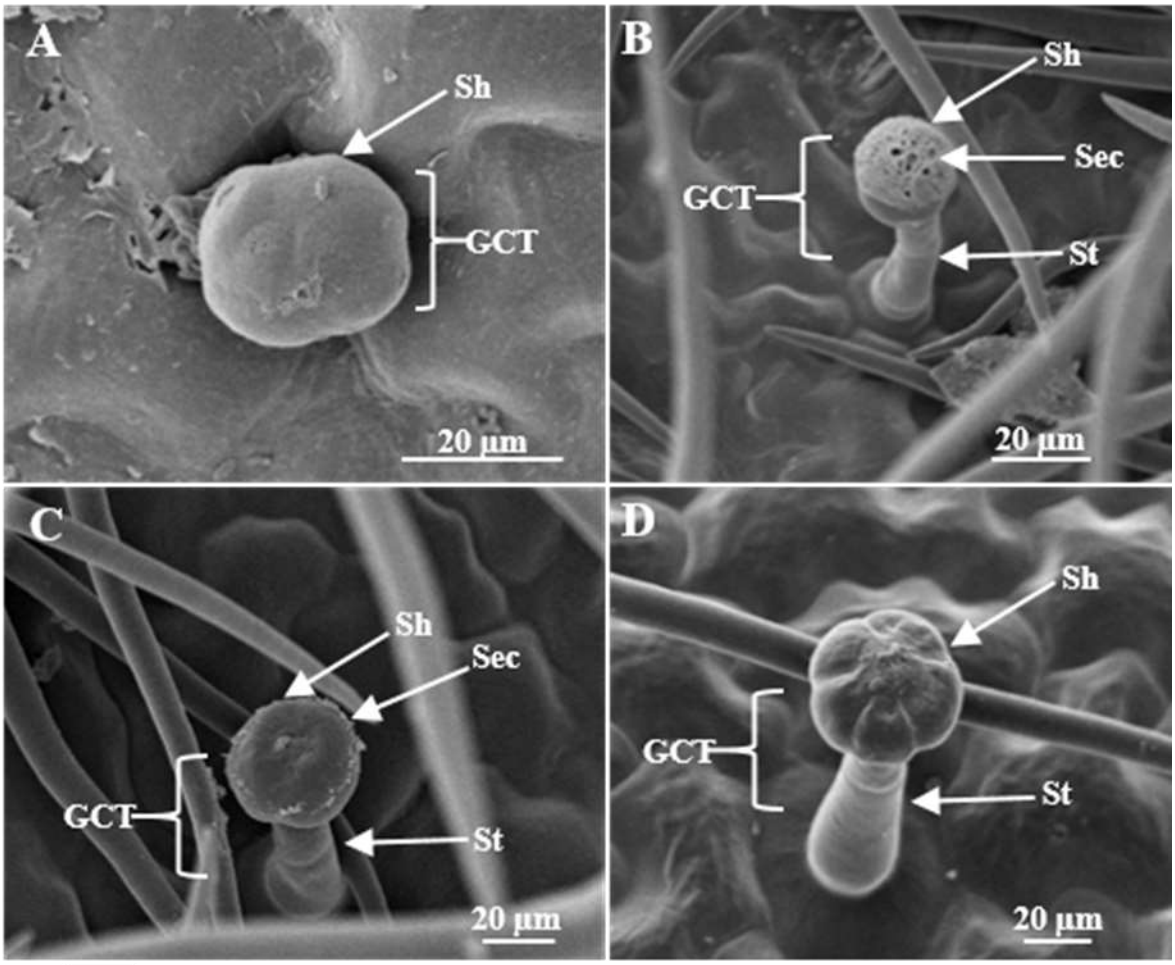

Figure 3.5: Scanning electron micrographs of glandular capitate trichomes found at all stages (emergent, young and mature) in both the leaves and stems of *B. albostellata*. (A) Glandular capitate trichome with a short stalk cell (unseen) and a broad secretory head; (B-C) Glandular capitate trichome with a two-celled stalk and a secretion covering the secretory head; (D) Glandular capitate trichome with a 2-celled stalk and a five-celled secretory head.

Abbreviations: GCT= Glandular capitate trichome; Sh= Secretory head; St= Stalk; Sec= Secretion.

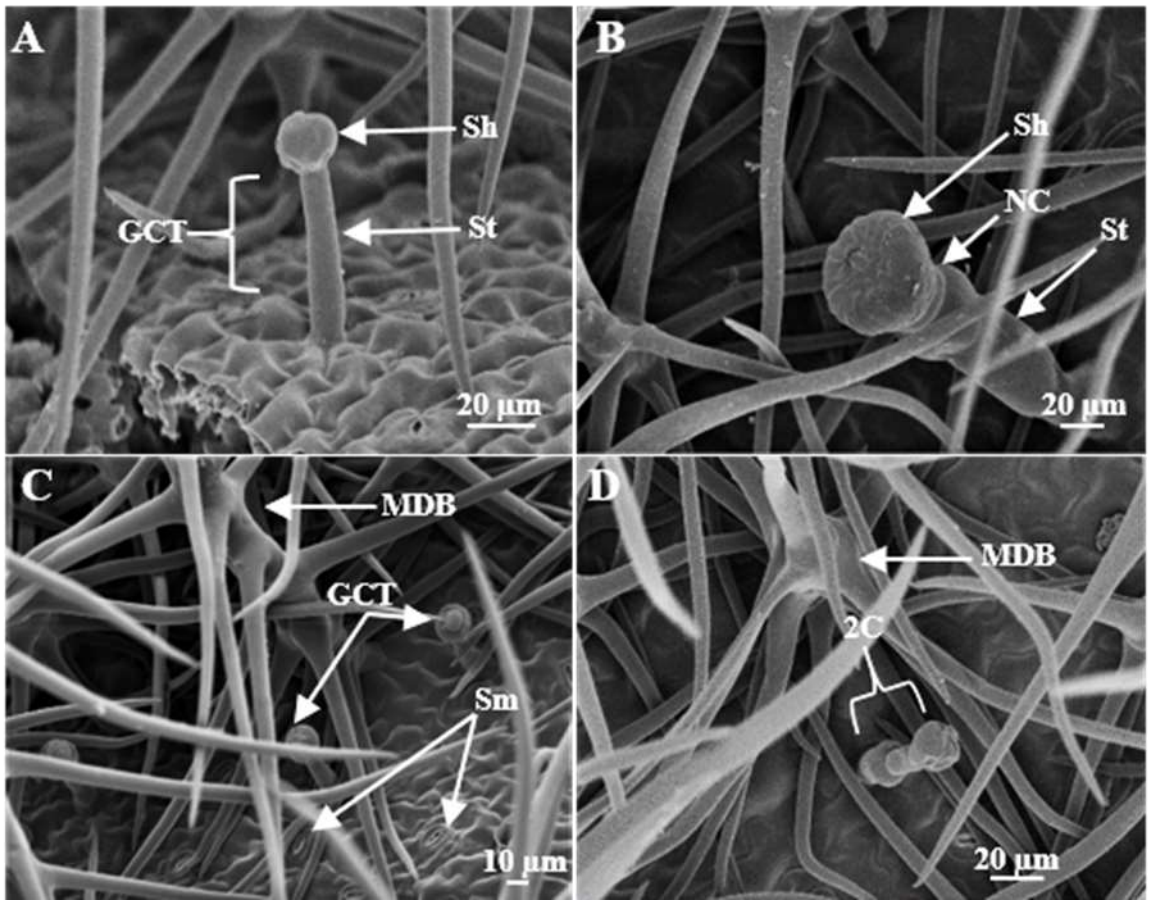

Figure 3.6: Scanning electron micrographs of glandular and non-glandular trichomes found at all stages (emergent, young and mature) of the leaves and stems of *B. albostellata*. (A) Glandular capitate trichome with a single-celled long stalk and a one- or more-celled globular shaped secretory head; (B) Glandular capitate trichome with a 3-celled long stalk and a neck cell attached below the globular several-celled secretory head; (C) Multangulate-dendritic branched non-glandular trichome, glandular capitate trichomes hidden below and stomata are visible on the surface; (D) Multangulate-dendritic branched trichome and two capitate trichomes are found developing one on top of the other.

Abbreviations: GCT= Glandular capitate trichome; St= Stalk; Sh= Secretory head; NC= Neck cell; MDB= Multangulate-dendritic branched; Sm= Stomata; 2C= Two capitate trichomes.

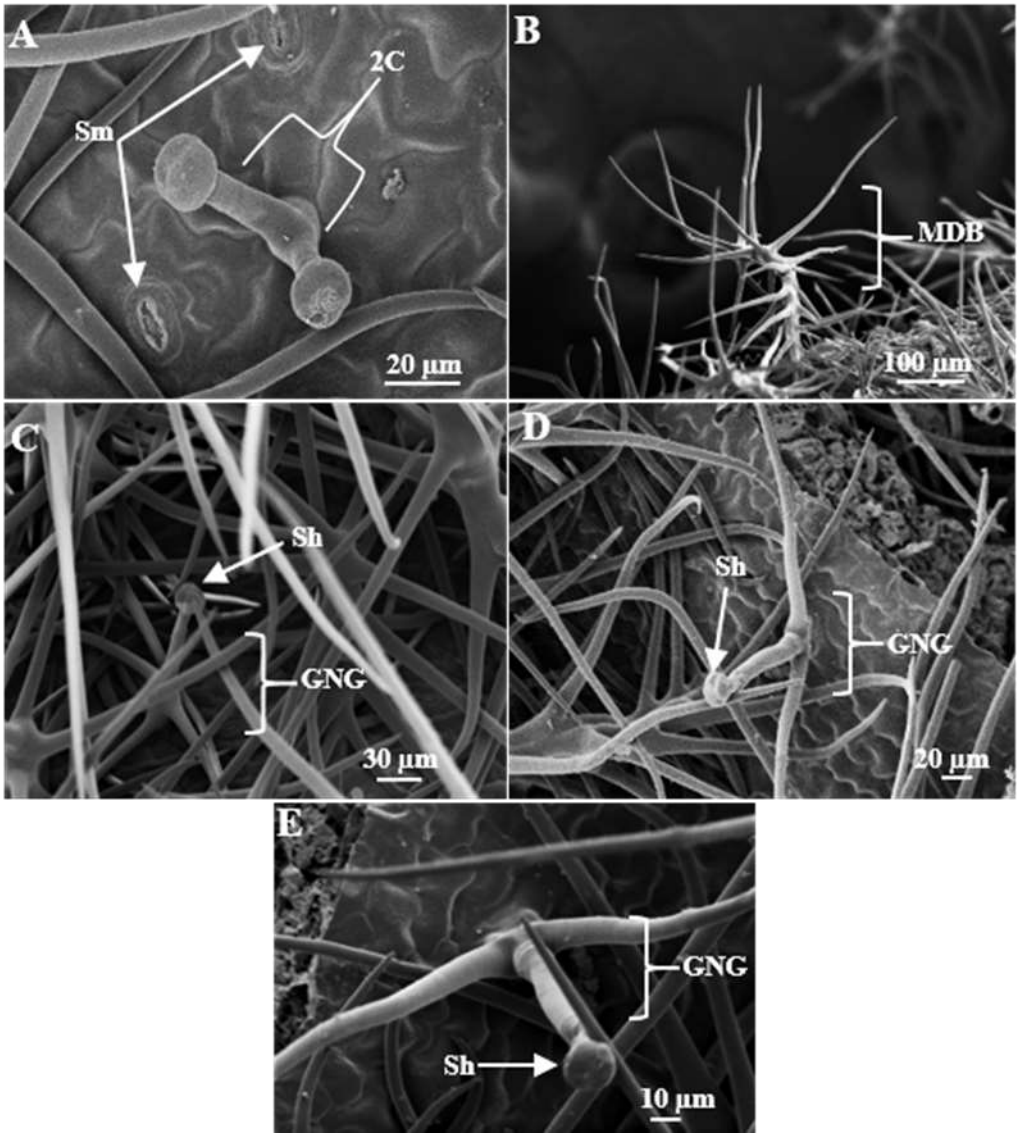

Figure 3.7: Scanning electron micrographs of different types of trichomes found at all stages (emergent, young and mature) of the leaves and stems of *B. albostellata*. (A) Two branched glandular capitate trichomes and stomata are visible; (B) Multangulate- dendritic branched non-glandular trichome (MDB); (C-E) Glandular head attached to a branch of a non-glandular trichome.

Abbreviations: 2C= Two capitate trichomes; Sm= Stomata; MDB= Multangulate-dendritic branched; Sh= Secretory head; GNG= Glandular head attached to a branched non-glandular trichome.

3.3.3 Survey sections of resin embedded leaf and stem material

The lamina of *B. albostellata* was chartaceous and puberulous. The adaxial surface of the midrib comprised of a short wide bulge, whilst the abaxial surface was a wide and long semicircular part (Figure 3.8 A). The lamina was clearly dorsiventral, with the adaxial epidermal cells appearing thick and wide and the abaxial, narrow and cylindrical. Ground tissue, palisade mesophyll cells, phloem and xylem are displayed in the transverse section of the leaf midrib (Figure 3.8 A). Palisade mesophyll cells appeared as a single adaxial row, which was thick and wide (Figure 3.8 A). Whereas the spongy mesophyll cells appeared cylindrical and were about 4 layers (Figure 3.8 A). A thick layer of thin-walled epidermal cells (squarish in size) was observed on the midrib (Figure 3.8 A). The vascular bundle appeared thick and bowl-shaped (U-shaped). It comprised of long compressed rows of thick-walled xylem elements and small layers of phloem elements (Figure 3.8 A and B). Along the abaxial side of the xylem, the phloem occurs as a continuous arc (Figure 3.8 A and B). Similar anatomical descriptions of the lamina and midrib were observed in *B. montana* (Sridharan and Gounder, 2016), *B. noctiflora* L.f. (Arumugam and Natesan, 2015), *B. gibsonii* (Tamboli and More, 2016) and *B. prionitis* (Bhogaonkar and Lande, 2012).

The raphide calcium oxalate (CaOx) crystals were scattered in the ground tissue of the resin–embedded stem and leaf sections (Figure 3.8 B and D). These crystals were also observed in the ground tissue of the leaf sections of *B. prionitis* (Bhogaonkar and Lande, 2012). Several hypotheses were proposed by researchers concerning the function of these crystals. These functions include, calcium regulation, plant protection, ion balance, tissue support and detoxification of heavy metals (Franceschi and Horner, 1980; Franceschi, 2001). Tooulakou et al. (2016) presented findings on the possible use of CaOx crystals, that is, when photosynthesis is distressed in plants, large crystals are used as dynamic internal carbon pools, preventing water loss. Numerous forms of crystals have been identified in plants, and their occurrence only in higher plants makes them a fascinating taxonomic tool (Al-Rais et al., 1971).

Elongated cystoliths were observed in specialised, wide epidermal cells called lithocysts (Figure 3.8 C and D). These structures are mostly observed in pairs in leaf sections and are found in *B. montana* (Sridharan and Gounder, 2016). Cystoliths are silicified bodies that comprise of cellulosic skeletons and are a diagnostic characteristic of Acanthaceae (Metcalfe and Chalk, 1950; Lawrence, 2008; Gal et al., 2012; Choopan and Grote, 2015). Type I (Figure 3.9 A and B), type II (Figure 3.9 C) and type III (Figure 3.9 D) capitate trichomes were observed on leaf and stem resin embedded sections.

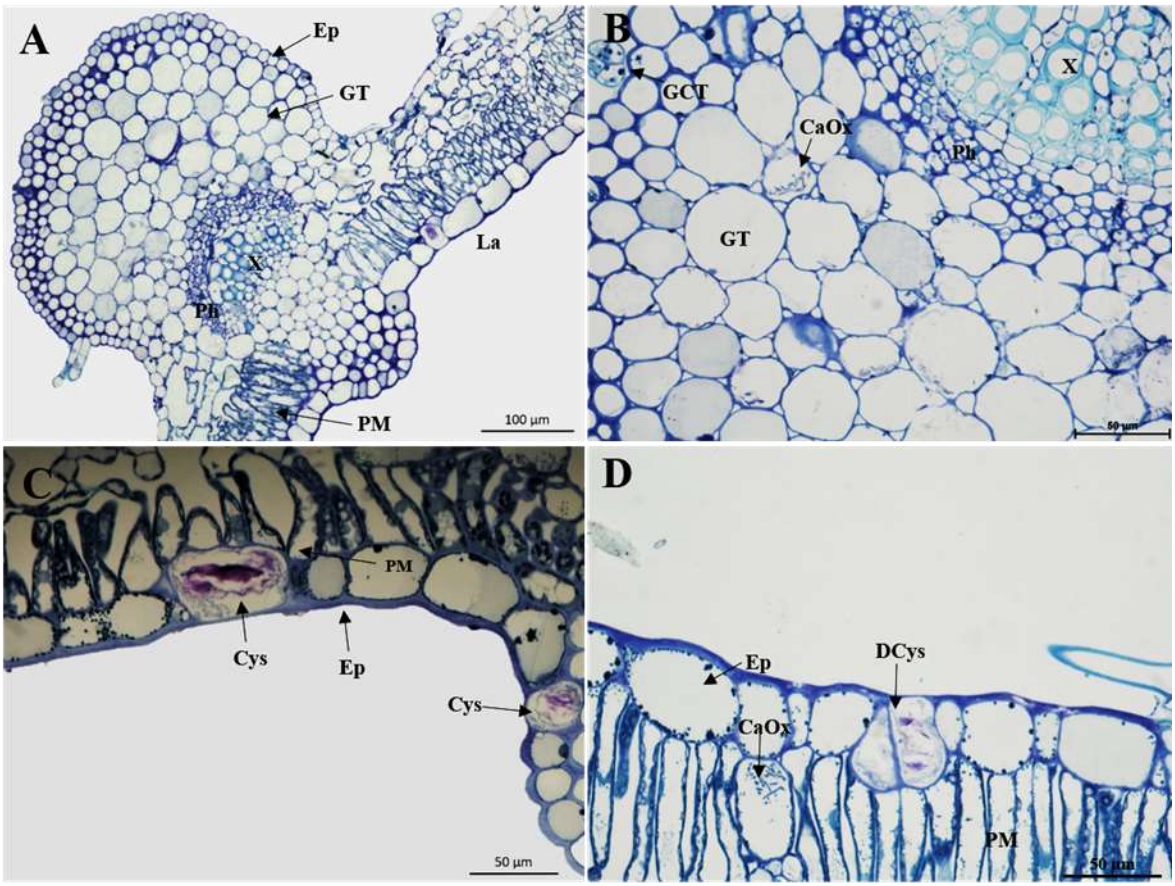

Figure 3.8: Light micrographs illustrating survey sections of resin embedded plant material of *B. albostellata*. (A) Midrib of an emergent leaf; (B) Section of stem material; (C) Upper epidermal layer of a young leaf; (D) Upper epidermal layer of a mature leaf.

Abbreviations: Ep= Epidermis; GT= Ground tissue; La= Lamina; PM= Palisade mesophyll; Ph= Phloem; X= Xylem; GCT= Glandular capitate trichome; CaOx= Calcium oxalate crystals; Cys= Cystolith; DCys= Double cystolith.

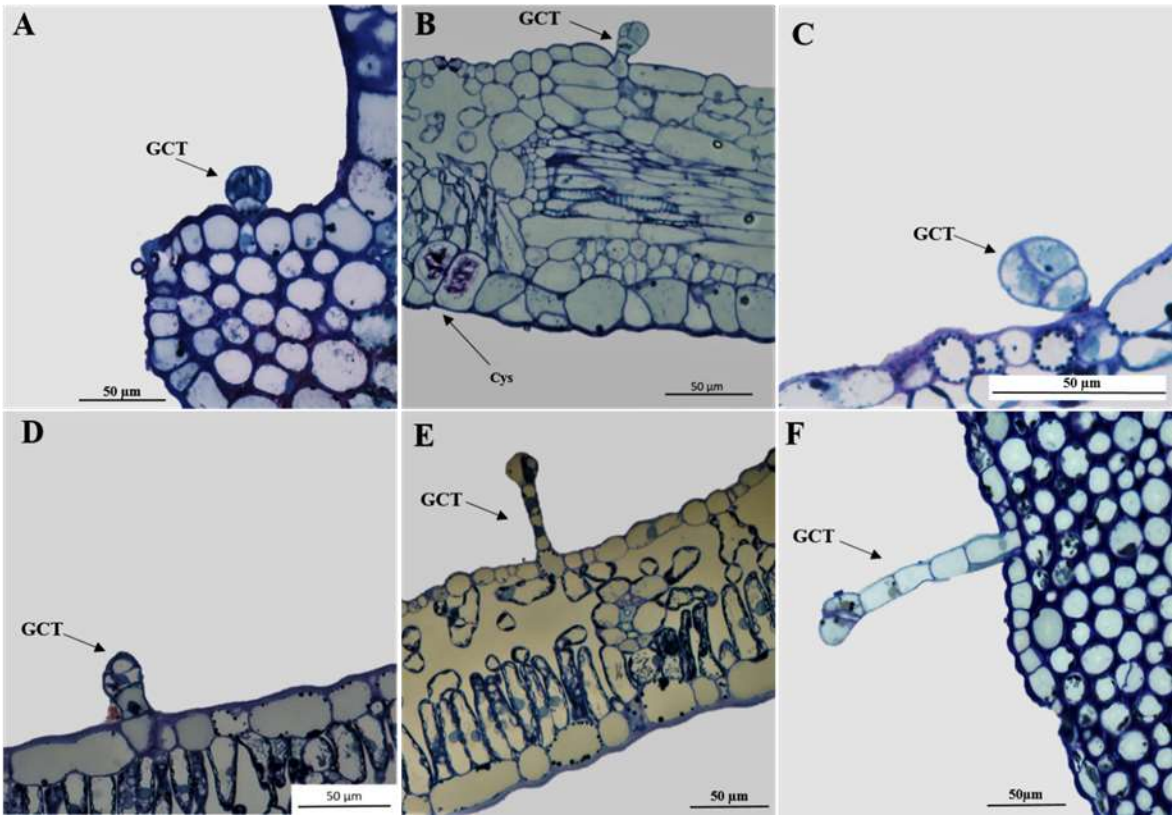

Figure 3.9: Survey sections of resin embedded glandular trichomes found in *B. albostellata*. (A-C) Capitate trichomes with a sunken or short stalk; (D) Capitate trichome with a single celled stalk and a 2-celled head; (E) Capitate trichome with a long 3-celled stalk and a 2-celled head; (F) Capitate trichome with a long 4-celled stalk and a 2-celled head.

Abbreviations: GCT= Glandular capitate trichome; Cys= Cystolith.

3.3.4 Secretory structures observed via transmission electron microscopy

Ultrastructural studies indicated that the type I glandular trichome head cells possessed large nuclei, vacuoles of varying sizes, electron-dense vesicles and plastids (Figure 3.10 A and B). The plastids present in trichomes aid in the synthesis, accumulation, and secretory processes of various compounds (Turner et al., 2000; Pyke and Howells, 2002). Clear cutinisation of the lateral cell walls of trichomes are illustrated in Figure 3.10 A and B. The cutinisation of secretory trichomes might prevent the leakage of secreted material back through the apoplast (Fahn, 1988; Ascensão and Pais, 1998). Several plasmodesmata were visible in the connecting cell walls of the trichome (Figure 3.10 B).

Glandular capitate trichomes (type I) showed dense cytoplasm with Golgi bodies (Figure 3.10 C and D), endoplasmic reticulum cisternae and many mitochondria containing a dense matrix (Figure 3.10 E and F).

The presence of plastids, mitochondria, and endoplasmic reticulum cisternae in secretory head cells indicated that these cells exhibit the typical active metabolism of secretory systems (Naidoo et al., 2012). The head cells of type I *B. albostellata* appeared to be extremely vacuolated with certain vacuoles containing lamellar material (concentric membranous concentric) (Figure 3.10 G). The basal and stalk cells of the MDB trichomes appeared vacuolated (Figure 3.11 A). The vesicles, mitochondria, vacuoles, cuticles, and endoplasmic reticulum were visible in non-glandular trichomes (Figure 3.11 A–C). Sample preparation using the standard protocol for transmission electron microscopy was a challenge as resin blocks were brittle due to poor infiltration, which made it difficult to section and view the plant material, resulting in distorted images. Thus, the protocol for chemical fixation of the plant material had to be slightly adapted and new blocks had to be prepared.

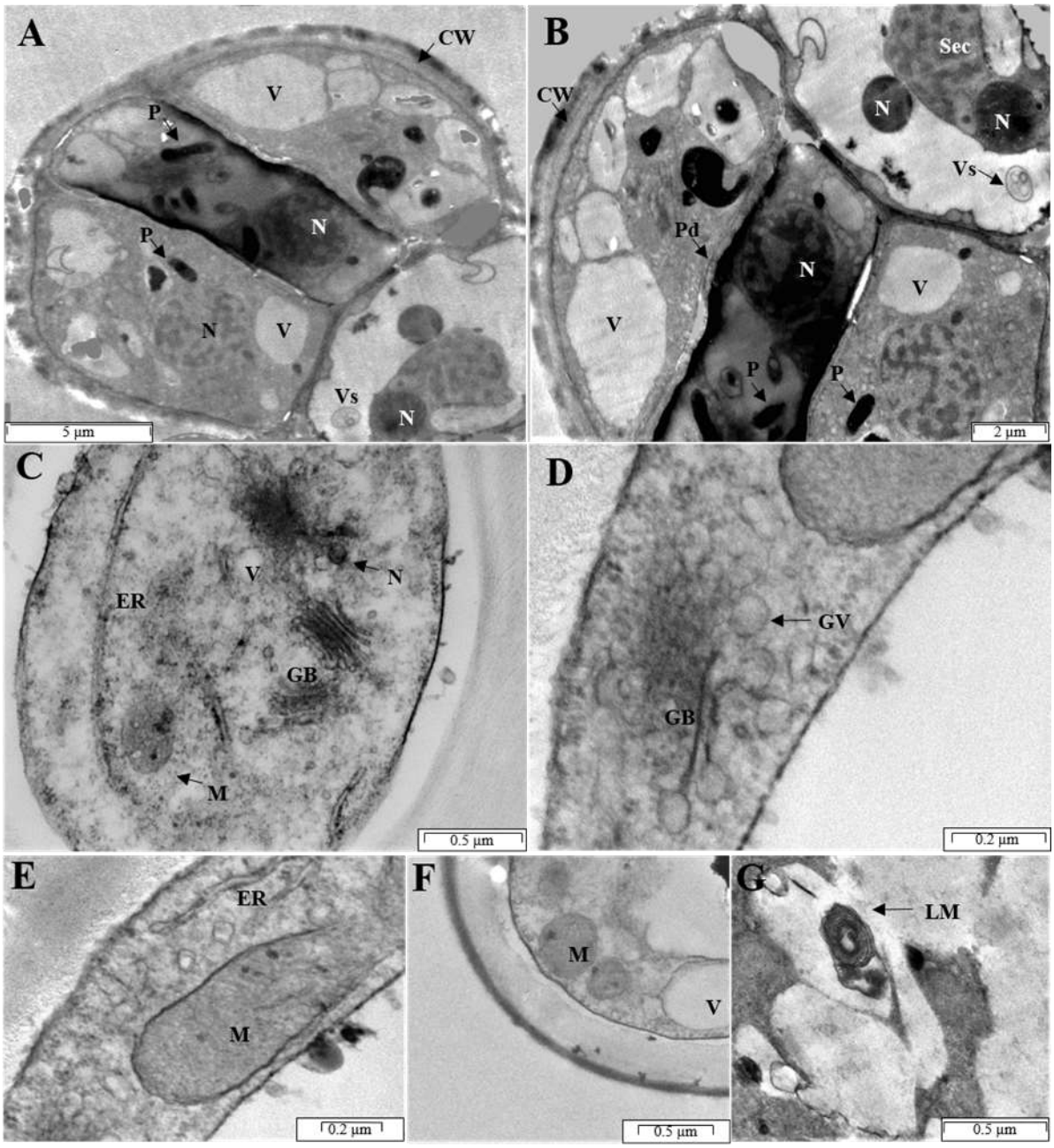

Figure 3.10: Transmission electron micrographs of secretory heads of glandular capitate trichomes. (A-B) Vacuoles (V), electron dense plastids (P), numerous nuclei (N), a vesicle (Vs), plasmodesmata (Pd) and secretory material (Sec) are visible in the heads of capitate trichomes; (C-F) a dense cytoplasm with endoplasmic reticulum (ER), mitochondria (M), vacuole (V), nuclei, golgi body (GB) and Golgi vesicles (GV) were observed at various locations within the trichome head; (G) Lamellar body (LM) observed within the head cells.

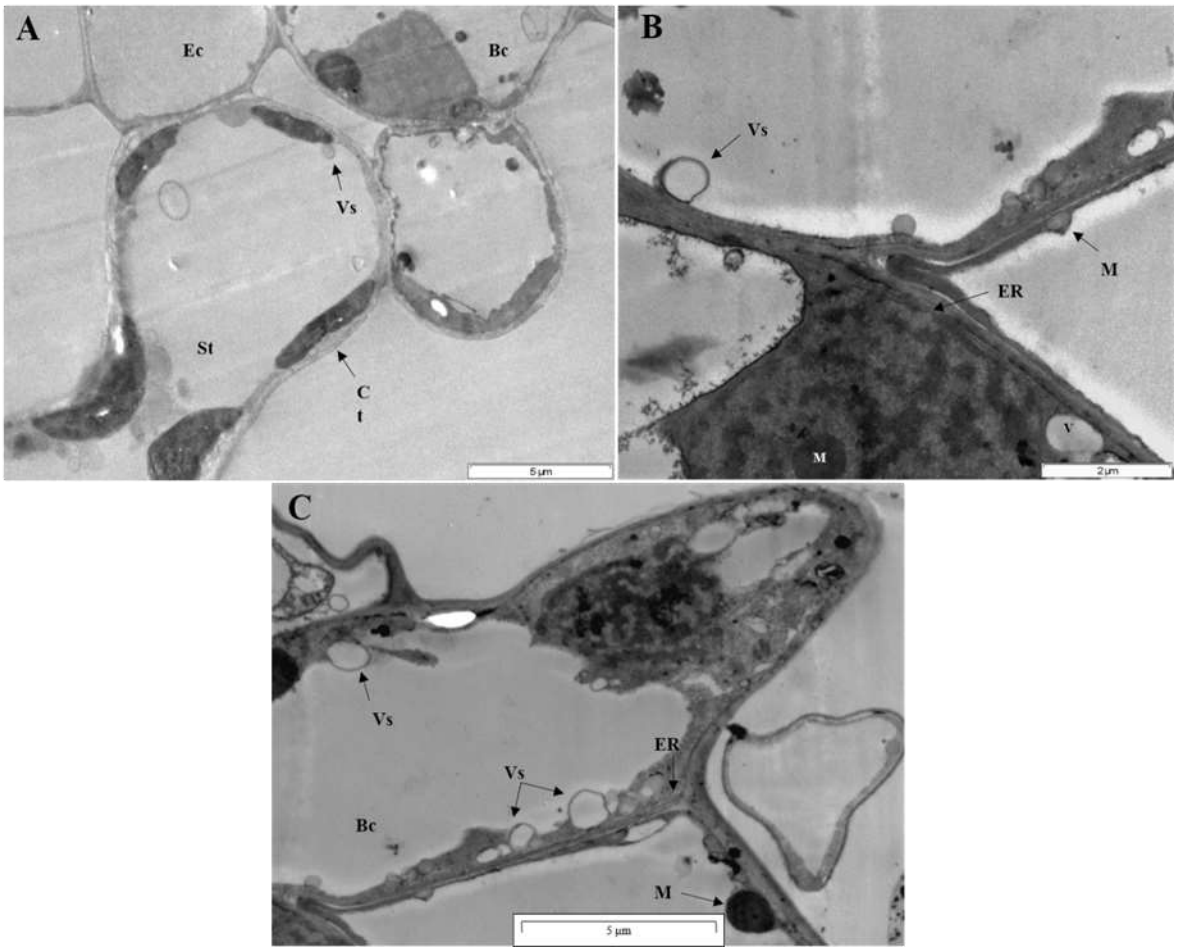

Figure 3.11: Transmission electron micrographs of non-glandular trichomes. (A) Epidermal cell (Ec), basal cell (Bc), stalk cells (St), vesicles (Vs) and cuticles (Ct) found within the trichome were visible; (B-C) Vesicles (Vs), mitochondria (M), vacuole (V) and endoplasmic reticulum (ER) were observed in the trichome.

3.3.5 Chemical composition of compounds located within trichomes

Histochemical tests are valuable tools used to detect and localise important metabolites present in plant secretions. Glandular trichomes stained positively with Wagner's and Ditmar's reagents (for alkaloids), fast green (for cellulose), Sudan Black, (for lipids) Nile Blue (for lignin, cutin and suberin) Safranin (for mucilage), Ruthenium Red (for mucilage), Methylene blue (nuclei acids), Ferric trichloride (for phenolics) and Coomassie Blue (for proteins) (Figures 3.12, 3.13 and 3.14). Table 3.1 illustrates the reactions of the different types of glandular trichomes to the various histochemical tests. There were no observed differences in the results from the histochemical tests between the trichome types (Table 3.1).

Alkaloids detected in trichomes might be linked to the chemical defense of the plant against various herbivores as these substances are toxic to insects (Levin, 1973; Wagner, 1991; Fordyce and Agrawal, 2001). Lignin is found in the cell walls and is important for structural support in plants (Boerjan et al., 2003). The cutin present in trichomes can prevent the backflow of exudates into the leaf (Fahn, 1988; Naidoo et al., 2014), and it occurs above the basal cell that is believed to aid structural support (Payne, 1978). Suberised body cells in trichomes have been related to the control of the movement of aqueous metabolites to the apoplast. This permits the stalk cells to control the directional passage of metabolites to the glandular head cells (Dell and McComb, 1979; Werker, 2000; Lange and Turner, 2013).

Polyphenols are considered to be plant protectors against pathogens and herbivores (Hättenschwiler and Vitousek, 2000). Mucilaginous secretions have a shiny appearance because of which they play a role in light reflection, water loss by transpiration, and reduction of leaf temperature (Dell and McComb, 1979; Fahn, 1979; Werker and Fahn, 1981; Gaff, 1997; Ascensão et al., 1999; Wagner et al., 2004; Machado et al., 2012). Phenols are produced when non-toxic glycosides become hydrolysed; this process releases phenolics, which are harmful to microbial pathogens, from trichomes (Omwirhiren et al., 2017).

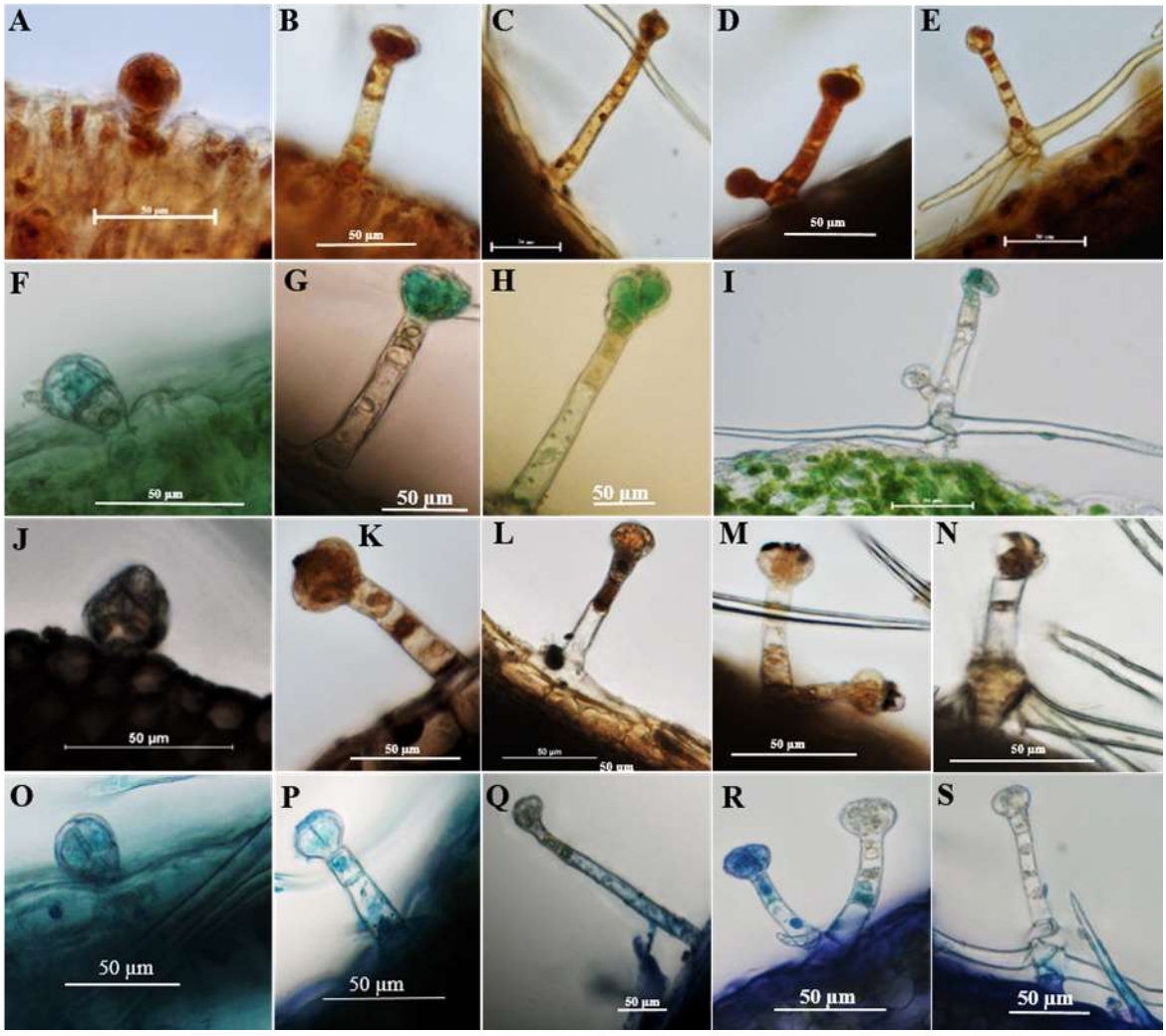

Figure 3.12: Light micrographs of transverse sections of histochemically stained trichomes found on both leaves and stems of *B. albostellata.* (A-E) Wagner's/Dittmar's reagent stained alkaloids brown in head and stalk cells of trichomes; (F-I) Fast green stained cellulose bright green in head cells of trichomes; (J-N) Sudan Back stained lipids, cutin and suberin black in head and stalk cells; (O-S) Nile blue stained acidic lipids blue in head and stalk cells. (A, F, J and O) Glandular capitate trichome with a short stalk embedded; (B, G, K and P) Glandular capitate trichome with a short stalk; (C, H, L and Q) Glandular capitate trichome with a thin long stalk; (D, M and R) Two glandular capitate trichomes attached to each other; (E, I, N and S) Glandular capitate trichome attached to a non-glandular dendritic trichome.

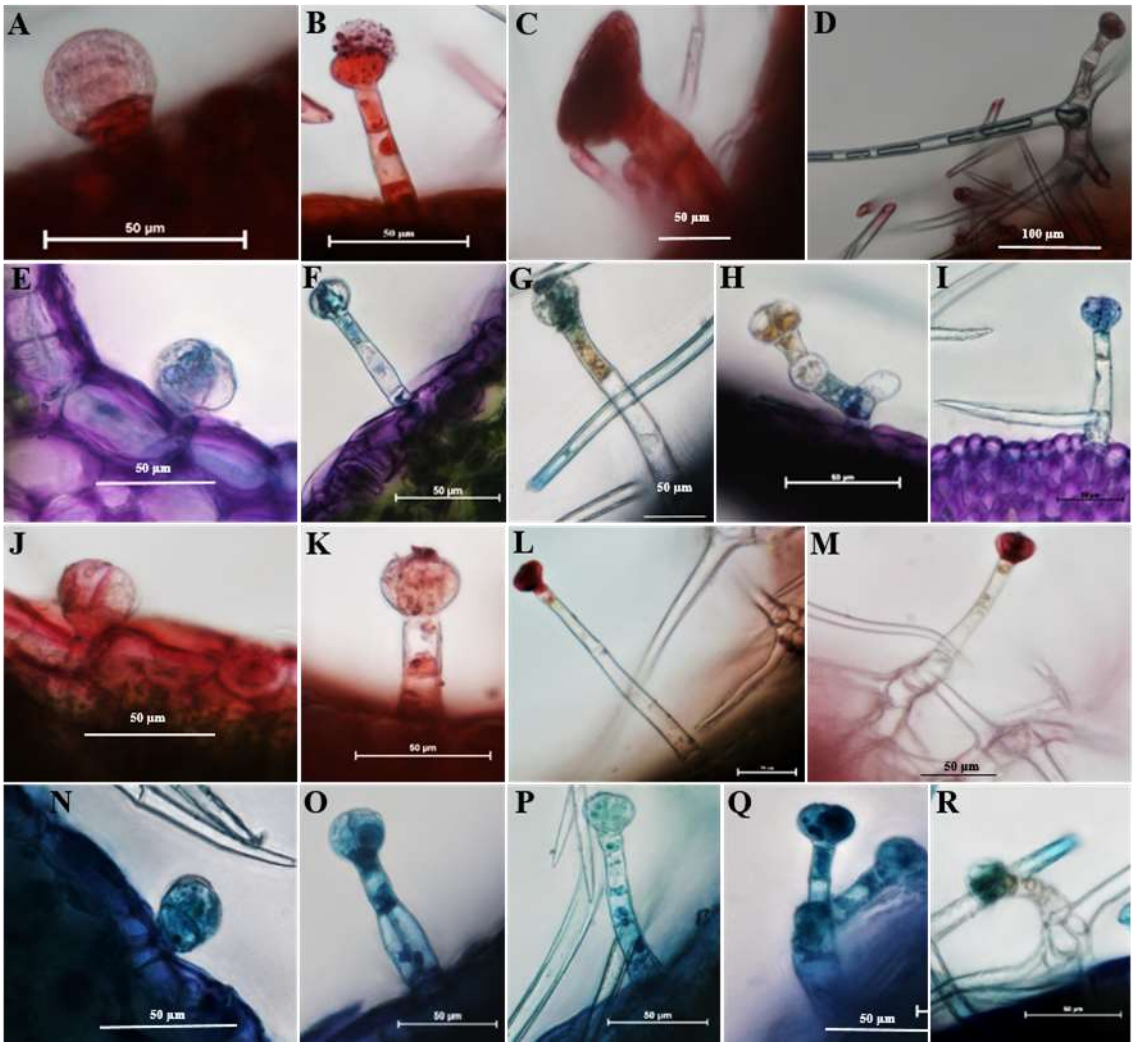

Figure 3.13: Transverse sections of histochemically stained trichomes found on both leaves and stems of *B. albostellata*. (A-D) Safranin stained lignin, cutin and suberin red in head and stalk cells; (E-I) Toluidine Blue stained caboxylated polysaccharides pink-purple and polyphenols blue in head and stalk cells; (J-M) Ruthenium red stained positively for mucilage in head and stalk cells; (N-R) Methylene blue stained positively for nuclei acids in head and stalk cells. (A, E, J and N) Glandular capitate trichome with an embedded stalk; (B, F, K and O) Glandular capitate trichome with a short stalk; (C) Capitate trichome with a thick stalk, neck cell and large head; (G, L and P) Capitate trichome with a thin long stalk and globular head; (D, I, M and R) Glandular capitate trichome attached to a non-glandular dendritic trichome; (H and Q) Two glandular capitate trichomes attached to each other.

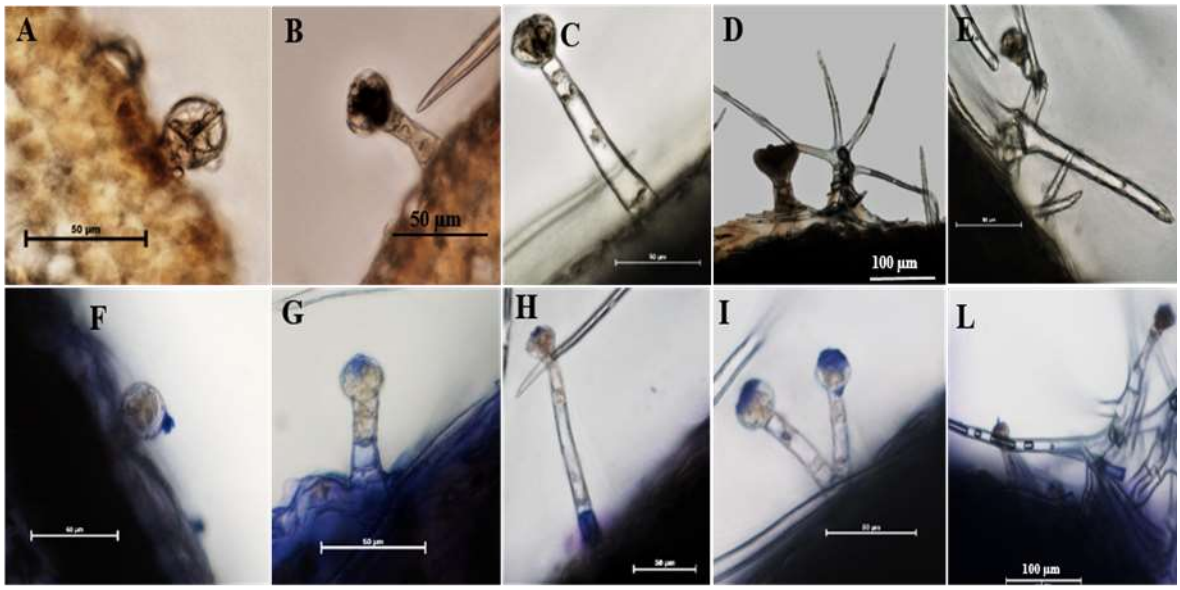

Figure 3.14: Transverse sections of histochemically stained trichomes found on both leaves and stems of *B. albostellata*. (A-E) Ferric trichloride stained phenolic deposits black in capitate head cells; (F-L) Coomassie blue stained proteins blue in head and stalk cells. (A, F) Glandular capitate trichome with an embedded stalk; (B and G) Glandular capitate trichome with a short stalk; (C and H) Glandular capitate trichome with a thin long stalk; (D) Glandular capitate trichome with a thick stalk and large head and a multangulate-dendritic branched non-glandular; (I) Two glandular capitate trichomes attached to each other; (E and L) Glandular capitate trichome attached to a non-glandular dendritic trichome.

Table 3.1: Observations of histochemical tests on the glandular trichomes of leaf and stem sections of *B. albostellata*.

Compound group	Stain/s	Glandular trichomes						Reaction observed
		Type I	Type II	Type III	Type IV	Type V	Glandular head attached to a branched non-glandular trichome (GHBNG)	
Alkaloids	Wagner's and Dittmar	+	+	+	+	+	+	Head and stalk cells of glandular trichomes and the GHBNG trichomes stained orange-brown and a deeper colour was observed in type V trichomes.
Lipids, cutin and suberin	Sudan black B	+	+	+	+	+	+	Head and stalk cells of glandular and GHBNG trichomes stained dark black deposits.
Acidic lipids	Nile blue	+	+	+	-	+	+	Glandular trichomes stained blue except for type IV, whereas only the basal cells of GHBNG trichomes stained blue.
Lignin, cutin and suberin,	Safranin	+	+	+	+	0	+	Head and stalk cells of glandular trichomes stained pink-red and a deeper colour was observed in type IV trichomes; whilst only head cells of GHBNG stained pink-red. Type V trichome was challenging to locate on sections.
Mucilage and polysaccharides	Ruthenium red	+	+	+	+	0	+	Head and stalk cells of type I, II and IV glandular trichomes stained a light pink-red colour; whilst only head cells of type III and GHBNG trichomes stained a darker pink-red. Type V trichome was challenging to locate on sections.
Phenolic compounds	Ferric trichloride	+	+	+	+	-	+	Head cells of type I, II, III and the GHBNG, stained dark black; whilst type V did not stain, and type IV head and stalk cells stained dark black.
Total proteins	Coomassie blue	+	+	+	0	+	+	Head and stalk cells of type I, II, III and V trichomes stained a light blue, whilst head cells of GHBNG stained blue. Type IV trichome was challenging to locate on sections.

+/- indicates presence/absence of compound group; 0 indicates absence of trichome on a plant section.

Several classes of compounds were identified histochemically, inside the cells of non-glandular trichomes. All cells of the MDB non-glandular trichome contained alkaloids (Figure 3.15 A). Safranin stained both the stalk and branches of the non-glandular trichomes, indicating the presence of lignin, cutin and suberin (Figure 3.15 B). The non-glandular trichome sections stained positively with Toluidine Blue, an indication of polyphenols (Figure 3.15 C). Polyphenols are considered plant protectors against pathogens and herbivores (Hättenschwiler and Vitousek, 2000). Total proteins were detected in the branches of the non-glandular trichomes (Figure 3.15 D). Phenolic compounds were detected in the base, stalk cells, and branches of the MDB non-glandular trichomes (Figure 3.15 E).

The reactions of the MDB non-glandular trichomes to the different histochemical tests are summarized in Table 3.2. According to Werker (2000), non-glandular trichomes were considered not to play a role in the production and storage of biologically active chemical compounds as these plant structures are known for protecting plants against biotic and abiotic stresses. However, our results contradict the findings of Werker (2000), as various chemical compounds were observed to accumulate in non-glandular trichomes, indicating that these trichomes showed secretory activity and might play an important role in chemical defense against insects and pathogens. Non-glandular trichomes consist of living cells that can synthesize and store biologically active compounds (Santos-Tozin et al., 2016). The histochemically stained leaf sections of the Lamiaceae and Verbenaceae species comprised of non-glandular trichomes that stained positively for various chemical compounds (Santos-Tozin et al., 2016).

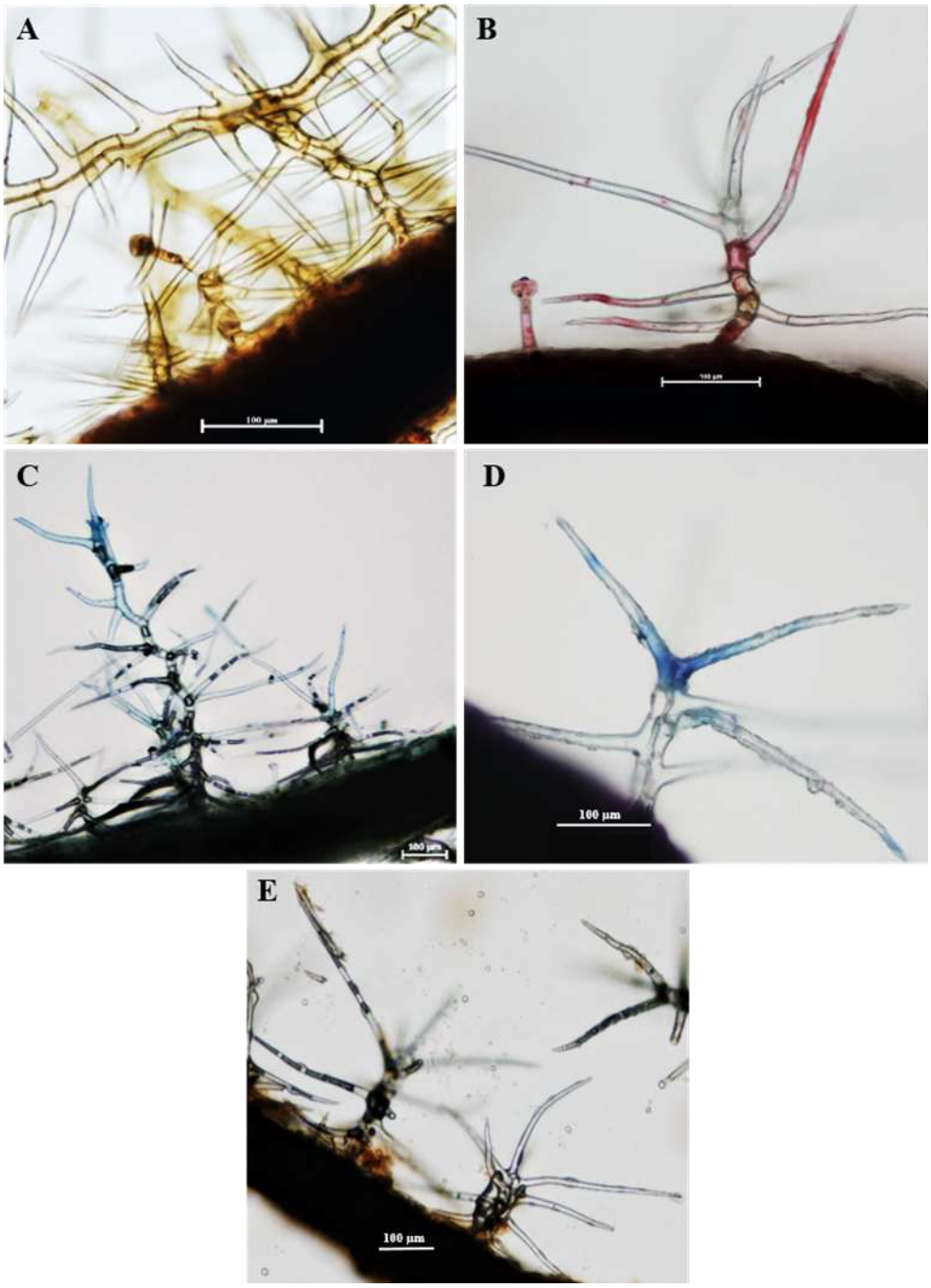

Figure 3.15: Transverse sections of histochemically stained multangulate-dendritic branched non-glandular trichomes found on both leaves and stems of *B. albostellata*. (A) Section stained with Wagners's and Dittmar's; (B) Section stained with safranin; (C) Section stained with Toluidine Blue; (D) Section stained with Coomassie blue; (E) Section stained with Ferric Trichloride.

Table 3.2: Observations of histochemical tests on the non-glandular trichomes of leaf and stem sections of *B. albostellata*

Compound group	Stain/s	Multangulate-dendritic branched	Reaction observed
Alkaloids	Wagner's and Dittmar	+	Branches and basal cells of the non-glandular trichomes stained orange-brown
Lignin, cutin and suberin,	Safranin	+	Branches and basal cells of the non-glandular trichomes stained pink-red
Carboxylated polysaccharides and polyphenols	Toluidine Blue	+	Branches and basal cells of non-glandular trichomes stained dark blue
Total proteins	Coomassie blue	+	Branches of non-glandular trichomes stained blue
Phenolic compounds	Ferric trichloride	+	Branches and basal cells of non-glandular trichomes stained black

+/- indicates presence/absence of compound group

The leaves, stems, and trichomes stained with calcofluor white indicated a positive reaction for carbohydrates (Figure 3.16 A–D (A- type I; B- type II and C-D type III)). The sections stained with acridine orange illustrated trichome viability of the glandular and non-glandular trichomes by exhibiting yellow-green fluorescence (Figure 3.16 E–I (E- type I; F- type II and G-H- type III) and non-glandular trichomes (I- MDB)). Acridine orange is used to stain lysosomes, which comprise of macromolecule-disintegrating enzymes; and is, therefore, used to determine cell viability (Pierzynska-Mach et al., 2014). Although non-glandular trichomes are regarded as non-secretory, the leaf sections stained positive with acridine orange indicated the viability of the basal stalk cells and branches of these trichomes (Figure 3.16 I). The autofluorescence of fresh leaf sections emitted a red and blue fluorescence under UV light (Figure 3.16 J–L (J- type I; K- type II and L- MDB non-glandular trichomes)). The red fluorescence emitted by the stained sections indicated the presence of plastids within the cells (Ascensão and Pais, 1987); whereas the blue fluorescence indicated the presence of phenolic compounds in the cells (Ascensão and Pais, 1987).

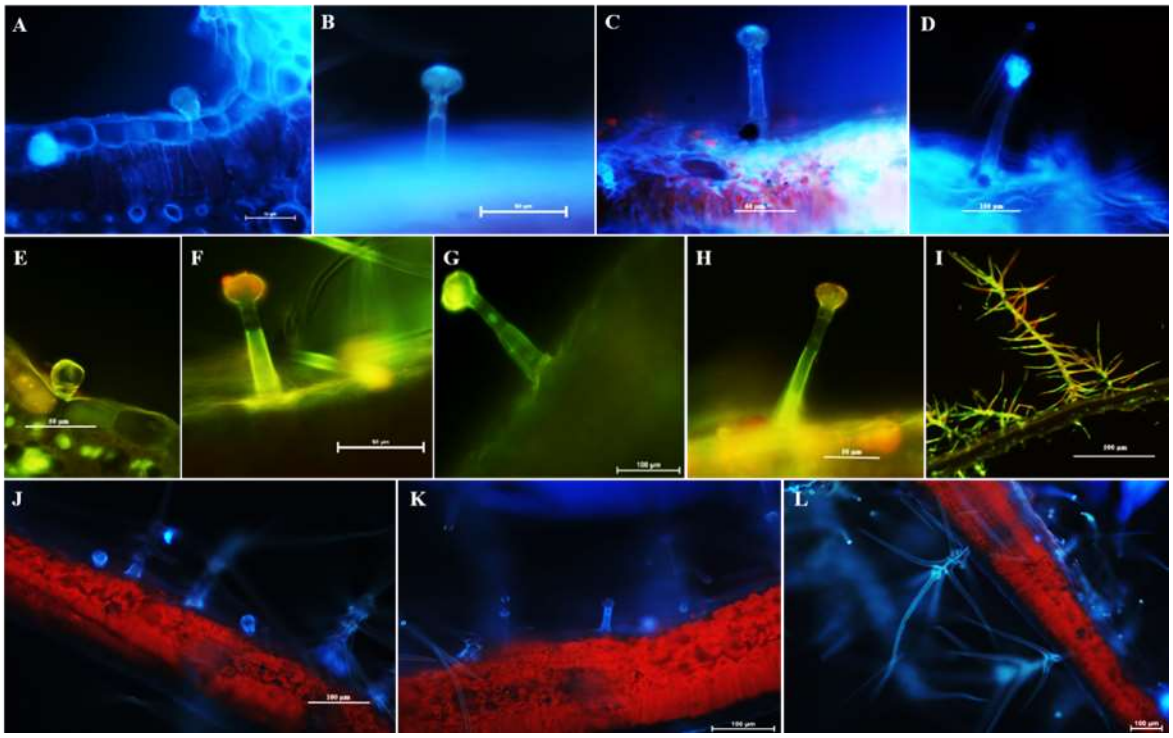

Figure 3.16: Fluorescence micrographs of trichomes found on *B. albostellata*. (A-C) Leaf sections stained with calcofluor white; (D) Stem section stained with calcofluor white; (E) Stem section stained with acridine orange showing trichome viability; (F-I) Leaf section stained with acridine orange showing trichome viability; (J-L) Autoflorescence of a leaf section.

This study characterised the micromorphology, distribution, and chemical composition of the trichomes present on the leaves and stems of *B. albostellata* using light and electron microscopy and histochemistry. Microscopic techniques revealed a dense indumentum of non-glandular and glandular trichomes on both adaxial and abaxial surfaces and is assumed to play a vital role in the protection of a leaf and stem surfaces from insect attack (Gangaram et al., 2020). Using exisiting literature, the various types of glandular and non-glandular trichomes found were compared to other species of *Barleria* (Banu et al., 2012; Bhogaonkar and Lande, 2012; Tamboli and More, 2016), however, these types were found to be important diagnostic features of this species. Stained sections indicated the presence of important medicinal compounds such as alkaloids and phenolics. The presence of various phytocompounds found in the various trichomes may have medicinal importance and aid in defense against pathogens and herbivores (Fordyce and Agrawal, 2001; Omwirhiren et al., 2017).

3.4 Conclusions and future perspectives

The combination of various microscopic techniques facilitated the identification of five different types of glandular trichomes, which are involved in the secretion of various compounds. Non-glandular and glandular trichomes were found on both leaf and stem surfaces and across all developmental stages of the *B. albostellata* leaves. Interestingly, two branched capitate trichomes attached to each other and a glandular capitate trichome head attached to the branch of a non-glandular trichome were observed. *Barleria prionitis* (Bhogaonkar and Lande, 2012; Datta and Biswas, 1968), *B. lupulina* Lindl., *B. lawii* T. Anderson, *B. cristata* L. and *B. strigosa* Willd. (Ahmad, 1975), shared both type I and II trichomes only with *B. albostellata*. According to Sridharan and Gounder (2016), the only type of trichome found in *B. montana* was peltate, however, these trichomes were absent in *B. albostellata*. Arumugam and Natesan (2015) reported on *B. noctiflora* to share type I and III trichomes with *B. albostellata*. Unicellular, long non-glandular trichomes were the only reported trichome found in *B. gibsonii* (Tamboli and More, 2016). Non-glandular trichomes found in *B. prionitis* (Bhogaonkar and Lande, 2012; Datta and Biswas, 1968), *B. lupulina*, *B. lawii*, *B. cristata*, and *B. strigosa* (Ahmad, 1975), were bicellular, pointed, unicellular, long, short and multicellular. According to reported literature, these species of *Barleria* lacked type III, IV, V, MDB non-glandular and GHBNG trichomes. Our results showed that both glandular and non-glandular trichomes in the leaves and stems of *B. albostellata* are important diagnostic features of this species. The histochemical tests indicated the presence of various phytocompounds that have medicinal importance and aid in defense against pathogens and herbivores. The information provided in this study improves our understanding of the role of these compounds in plant survival and defense. Further studies may focus on the length of trichomes across all developmental stages as this is a useful tool in the monitoring the pattern of trichome growth. The micromorphology of seedlings, flowers, and roots can be further evaluated as these plant parts also contain trichomes. To our knowledge, this study is the first report combining the morphology, ultrastructure, and histochemistry of the trichomes of *B. albostellata*; thus, there is a wide scope for future research in this field.

3.5 References

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CHAPTER 4

FLORAL BIOLOGY OF *Barleria albostellata* (Acanthaceae)

Abstract

Barleria albostellata C.B. Clarke (Acanthaceae) is an indigenous shrub to South Africa and is relatively understudied. This shrub is a valuable medicinal plant with a wide spectrum of antibacterial and anti-inflammatory activities. Detailed studies on the floral and pollen morphology on *B. albostellata* are rare. This study was conducted to observe the morphology of the flower and pollen grains using stereomicroscopy and scanning electron microscopy (SEM). Morphological observations using stereomicroscopy and scanning electron microscopy showed numerous non-glandular trichomes on the bracteoles and bracts of *B. albostellata.* Three types of trichomes were identified on these structures, I- unicellular and II- multangulate-dendritic branched non-glandular trichomes, and III- capitate glandular trichomes. A taxonomical description of the floral structures using the stereo and SEM micrographs are provided. SEM micrographs revealed the pollen grains as globose tricolporate with a rough honeycomb exine, and tiny granules within the lumina. The diameter of the pollen grains was 77.53 ± 5.63 µm, while the aperture of these grains was 14.31 ± 0.59 µm. This study provides insight into the floral biology of *B. albostellata*, and results presented in this study will add to the body of knowledge and encourage further research on this species.

Keywords: Microscopy; Morphology; Pollen grains; Trichomes

4.1 Introduction

Flower receptiveness plays an important role in pollination variability, reproductive success and plant productivity (Zhang et al., 2018). These active traits include timing of the opening of the anther, position of the anther, pollen appearance, stigma position, receptiveness and morphology (Harder et al., 2001). Pollination involves the transferal of pollen from the anther to the stigma of the flower (McMullen and Close, 1993), and the rate of success is highly dependent on pollen viability (the capability of pollen to efficiently seed set) (Dafni and Firmage, 2000). Furthermore, the reproductive success of a plant can depend on its ability in attracting flower visitors. These visitors that aid in pollination may exert selection on specific floral traits that are attractive to them (Kay and Sargent, 2009; Sletvold et al., 2016). Several species of *Barleria* are recognised for their floral diversity.

Barleria albostellata, an evergreen shrub, thrives in semi-shade to full-sun woodland areas of South Africa, and under suitable conditions, grows up to 1.5 meters in height (Figure 4.1). However, in colder regions, they can become deciduous to semi-deciduous (Froneman and Le Roux, 2007). *Barleria albostellata*, generally known as 'grey barleria', belongs to the family Acanthaceae (Froneman and Le Roux, 2007); and is widely distributed from Limpopo, Gauteng, and Mpumalanga to KwaZulu-Natal (Froneman and Le Roux, 2007). This shrub flourishes from September to May, with beautiful white flowers appearing sporadically (Figure 4.1). Flowers appear from a dense compound inflorescence and are surrounded by four leafy-bracts (Balkwill and Balkwill, 1997). The blooming flowers are white in colour and have a tinge of purple on the bracts. In contrast to the flowers, the leaves are grey-green and have an abundance of velvety hairs. This plant develops fairly fast and attains maturity in about three years (Figure 4.1) (Froneman and Le Roux, 2007).

Acanthaceae is regarded as a eurypalynous family (Raj, 1961), with significant diversity of its pollen shape, size, exine structure, apertures and ornamentation (Graham, 1988; Daniel, 1998; Bhatt et al., 2010; Choopan and Grote, 2015; House and Balkwill, 2017). Members of *Barleria* are easily recognised for its globose, tricolporate pollen with roughly reticulate (also referred to as the honeycomb-patterned) and interapertural exine (Balkwill and Balkwill, 1997; McDade et al., 2008; Darbyshire et al., 2010, 2015). Characterizing the morphology of pollen grains is useful in plant systematics and this can further add to the body of knowledge within the genus and family. Little is known on the floral and pollen morphology of *B. albostellata*, however substantial amount of work has been done in the family Acanthaceae (Scott, 1872) and in other species of *Barleria*.

Previous studies have found that members of this genus are pollinated by moths (Pooley, 1998; Scott-Shaw et al., 2007), or attract various species of butterflies (Froneman, 2010). Additionally, it was noted that the flowers of *B. albostellata* were also pollinated by insects and butterflies (Froneman and Le Roux, 2007). On a regular basis, carpenter bees were also observed to visit the flowers of *B. albostellata*. Plants within this genus produce copious amounts of nectar which attract bumble bees (Froneman, 2010). Several morphological features of the *B. albostelata* flower and pollen grains are largely unexplored. Therefore, the present study aims to describe the floral morphology and pollen of *B. albostellata* using stereo and scanning electron microscopy.

Figure 4.1: *Barleria albostellata* found along a pathway at the University of KwaZulu-Natal, Westville Campus. (A-B) White, tubular flowers emerge sporadically in spring and summer.

4.2 Materials and methods

4.2.1 Plant materials

Flowers and seeds of *B. albostellata* were collected from the University of KwaZulu-Natal, School of Life Sciences, Westville Campus (29° 49′ 51.6″ S, 30° 55′ 30″ E), Durban, South Africa. A voucher specimen (7973000) was deposited in the Ward Herbarium of the University of KwaZulu-Natal, Life Sciences, Westville Campus. Five replicates of flowers were analysed using microscopy techniques.

4.2.2 Floral morphology

4.2.2.1 Stereomicroscopy

Fresh flowers were eamined using the Nikon AZ100 stereomicroscope (Nikon Corporation, Yokohama, Japan) equipped with a Nikon Fiber Illuminator and photographed using the NIS-Elements Software (NIS-elements D 3.00).

4.2.2.2 Scanning electron microscopy (SEM)

Flower

The micromorphology of chemically–fixed flowers of *B. albostellata* were examined in detail. The initial step of preparation involved dissecting the different parts of the flower: petal, stigma, style, anther, and filament and thereafter primary fixating the sections (± 10 mm^2) in 2.5% glutaraldehyde for 18–24 h. After primary fixation, samples were rinsed for 5 min each (thrice) with 0.1 M sodium phosphate buffer (pH 7.2) and then post-fixed in 0.5% osmium tetroxide for 3 h at room temperature. Samples were washed thrice (for 5 min each) using a sodium phosphate buffer and dehydrated by subjecting them to increasing concentrations of ethanol (30%, 50%, 75%, 100%) for two sessions, each of 5 min, followed by exposure to 100% ethanol for two sessions, each of 10 min. Dehydrated samples were critically point-dried using the Quorum K850 Critical Point Dryer (Quorum Technologies Ltd., Laughton, East Sussex, UK) with a vertical chamber. Samples were mounted onto aluminium stubs using double-sided adhesive carbon tape and sputter coated with a layer of gold using the Quorum 150 RES (Quorum Technologies Ltd.), a combined system for carbon and sputter coating. The samples were then viewed and photographed using the LEO 1450 SEM at a working distance (WD) of 12–15 mm. Images were captured using the SmartSEM image software (Zeiss, Jena, Germany). (Protocol adapted from the microscopy and microanalysis unit, University of KwaZulu-Natal, Westville).

Pollen

With respect to the stigma of the flower, pollen grains were dusted from the stigma onto aluminium stubs using double-sided adhesive carbon tape and sputter coated with a layer of gold using the Quorum 150 RES (Quorum Technologies Ltd.), a combined system for carbon and sputter coating. Images of pollen grains were captured using the SmartSEM image software (Zeiss, Jena, Germany). Diameter of pollen grains were determined using ImageJ software Java 1.53e.

4.3 Results and discussion

4.3.1 Analysis of floral structures via stereomicroscopy

Bracteoles of *B. albostellata* vary from narrowly ovate to ovate, with glabrous or hairy surfaces and margins that are spiny, with scanty teeth (Figure 4.2 A-D). Long white hairs are prominent on the surface and margins of the floral bracts, (Figure 4.2 A-B) upper, and lower bracteoles (Figure 4.2 C-D) and a posterior lobe with sharp, curved apiculus (Figure 4.3 A). Flowers of *B. albostellata* (2-4 flowers) are bisexual with a nectariferous disc, zygomorphic and in cymes (a wide, flat-topped, distinct flower cluster in which the central flowers are opened first) (Palmer and Pitman, 1972; Schmidt et al., 2002). Flowering is an important phenological event, which impacts the reproductive success of a species (Fenner, 1998). The flowers are enclosed by four leafy, hairy, purple-tinged bracts. Purple-tinged bracts are assumed to contain some sort of nectar (Figure 4.2 A-D). The corolla (petals, 1+4) is irregular, thin, tubular and gamopetalous (Figure 4.3 B). The scent of the flowers of *B. albostellata* is produced nocturnally, with the strongest smell produced by mature, unopened buds, than with the open flowers. Floral visitors observed to interact with flowers of *B. albostellata* were butterflies and bees. Similar observations were reported by Balkwill et al. (1990) in flowers of *B. greenii*. Generally, flowers of *Barleria* are pollinated by butterflies and was described by Burkhardt (1964), and Faegri and van der Pijl (1979), as large, white and tubular, comprising deep nectaries/ nectariferous discs which function as nectar guides. Bumblebees were observed to frequently visit *B. greenii* and remove nectar from outside of the flower, by creating a narrow slit at the base of the corolla tube (Balkwill et al., 1990).

The fertile stamens (anther + filament), inserted on the corolla, are usually present in pairs (Figure 4.3 C) and are not didynamous. Filaments are long and may appear as twisted and can cross over each other and are usually hairy at the base (Figure 4.3 C). Anthers are basifixed and longitudinally dehisce, whilst the style is terete. The stigma is filiform and is found beyond the level of the dehisced anthers, while the style arches upward and is terete (Figure 4.3 C). Similar observations were reported by Balkwill et al. (1990) in flowers of *B. greenii*. Makholela et al. (2004) also observed these similarities in *B. saxatilis*. They suggested that the position of the stigma above the anthers promotes autonomous self-pollination. Slit along the lower corolla lobe revealed the growing stamen with hairy trichomes attached to the lower region of the filament (Figure 4.3 D), a characteristic of species within *Barleria* (Obermeijer, 1933).

Figure 4.2: Stereomicrographs of the floral bracts of *B. albostellata*. (A-B) Floral bracts abaxial surface; (C) Upper bracteole abaxial surface; (D) Lower bracteole abaxial surface.

Abbreviations: UT= Unicellular non-glandular trichome.

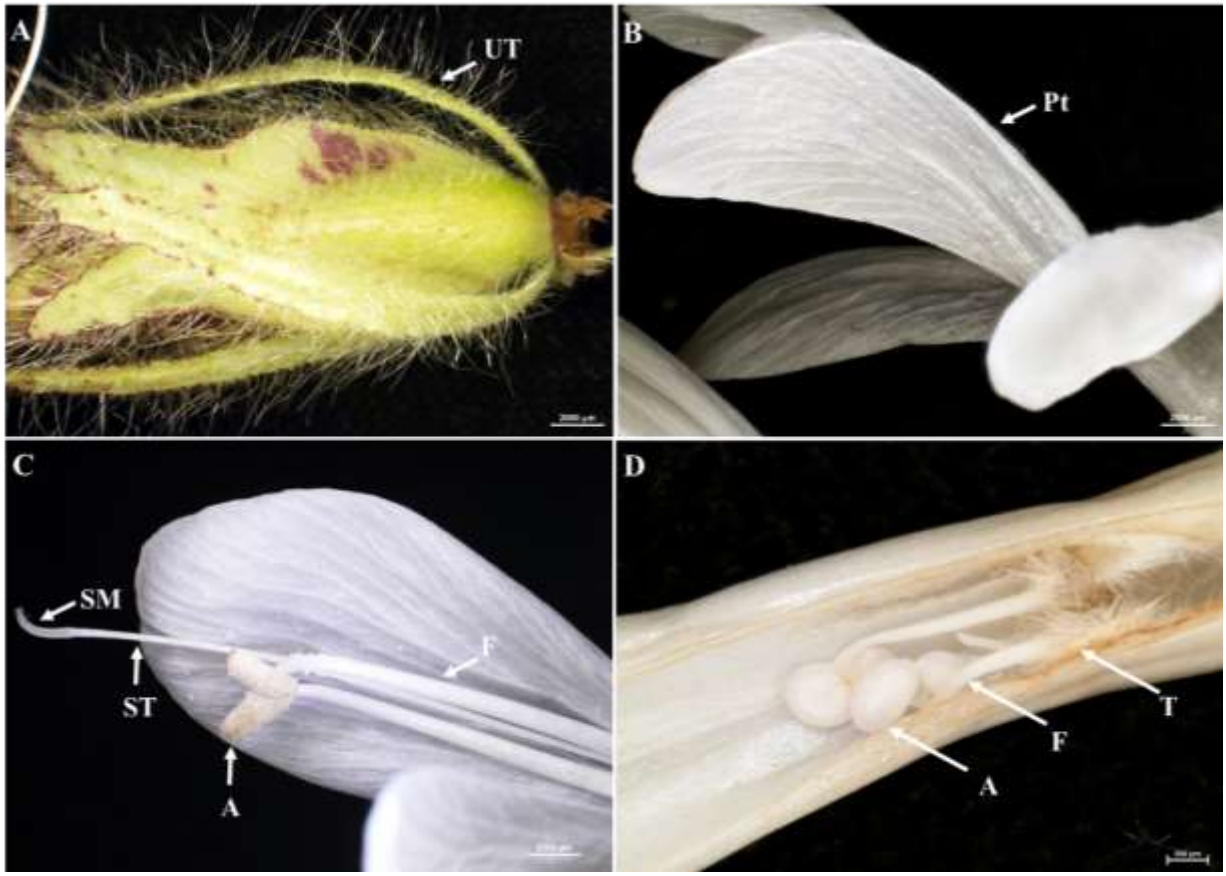

Figure 4.3: Stereomicrographs of the bracts and petals of *B. albostellata*. (A) Posticous calyx lobes of bracts, outer surface; (B) Petals of the flower; (C) Stamen, stigma and style; (D) Slit along the lower corolla lobe.

Abbreviations: UT= Unicellular non-glandular trichome; T= Trichome; SM=Stigma; ST= Style; A= Anther; F= Filament; Pt= Petal.

4.3.2 Floral structures observed via scanning electron microscopy

Floral bracts were heavily pubescent with non-glandular and glandular trichomes (Figure 4.4 A and B). Unicellular non-glandular trichomes were highly dense, long, pointed and located on the serrated edges of the floral bracts or occurring along the mid-region. Similar observations were reported by Darbyshire (2008) in *B. aristata* floral bracts. Perhaps the edges of the floral bracts might have responded to insect damage, therefore increasing the trichome density. Glandular capitate trichomes were few and scattered all over the floral bracts, while the multangulate-dendritic branched (MDB) non-glandular trichomes were predominant (Figure 4.4 A and B). In certain cases, the MDB non-

glandular trichomes were found to ‘arch over’ the glandular trichomes. Due to its close proximity, the MDB non-glandular trichomes may provide some sort of physical protection to these glandular tichomes. The adaxial and abaxial surfaces of a petal contained several grooves and appeared as coarse and pitted (Figure 4.4 C and D), with epidermal cells in an irregular shape. High magnification of a section of the surface of the stigma displayed parallel striations (Figure 4.5 B). The cap of the anther is curved and round (protection of pollen), with a slit in the middle (Figure 4.5 C).

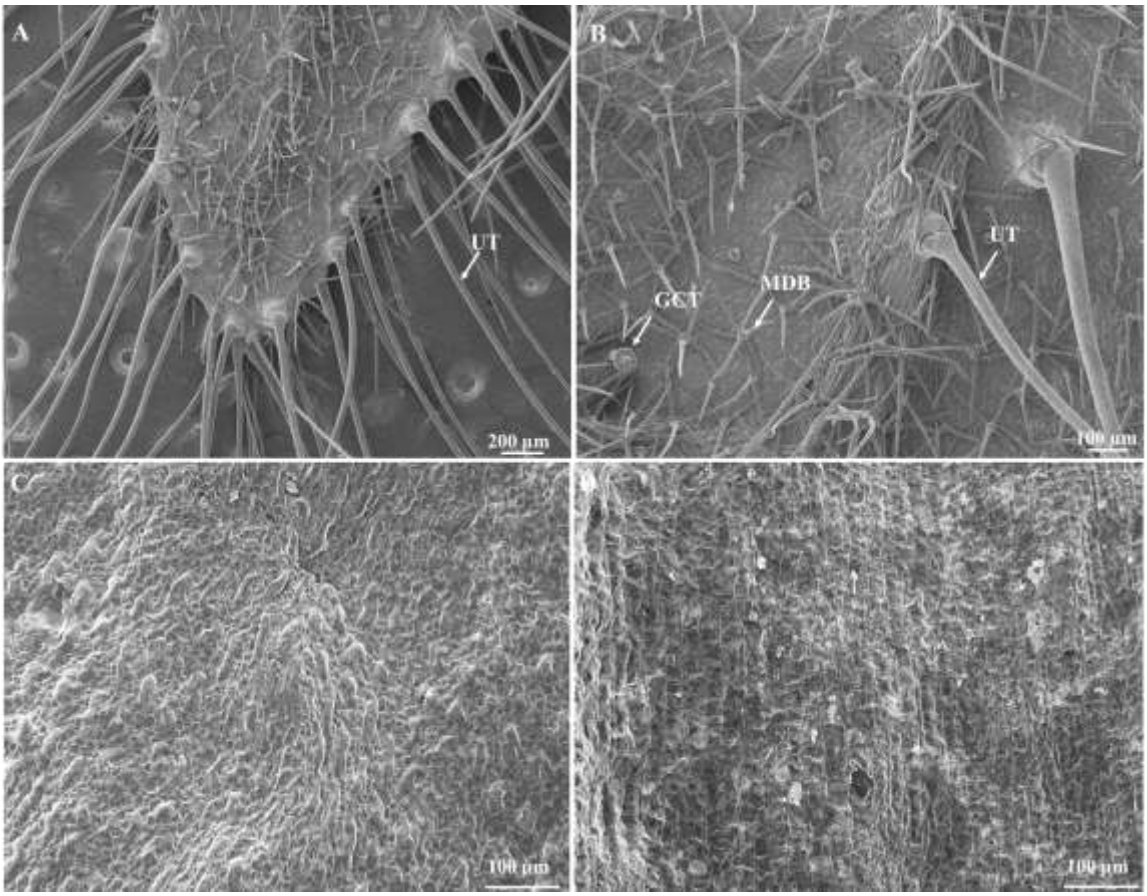

Figure 4.4: Scanning electron micrographs of the floral morphology of *B. albostellata*. (A) Floral bract; (B) Glandular and non-glandular trichomes, on the floral bracts; (C) Adaxial surface of a petal; (D) Abaxial surface of a petal.

Abbreviations: UT= Unicellular non-glandular trichome; MDB= Multangulate-dendritic branched non-glandular trichome; GCT= Glandular capitate trichome.

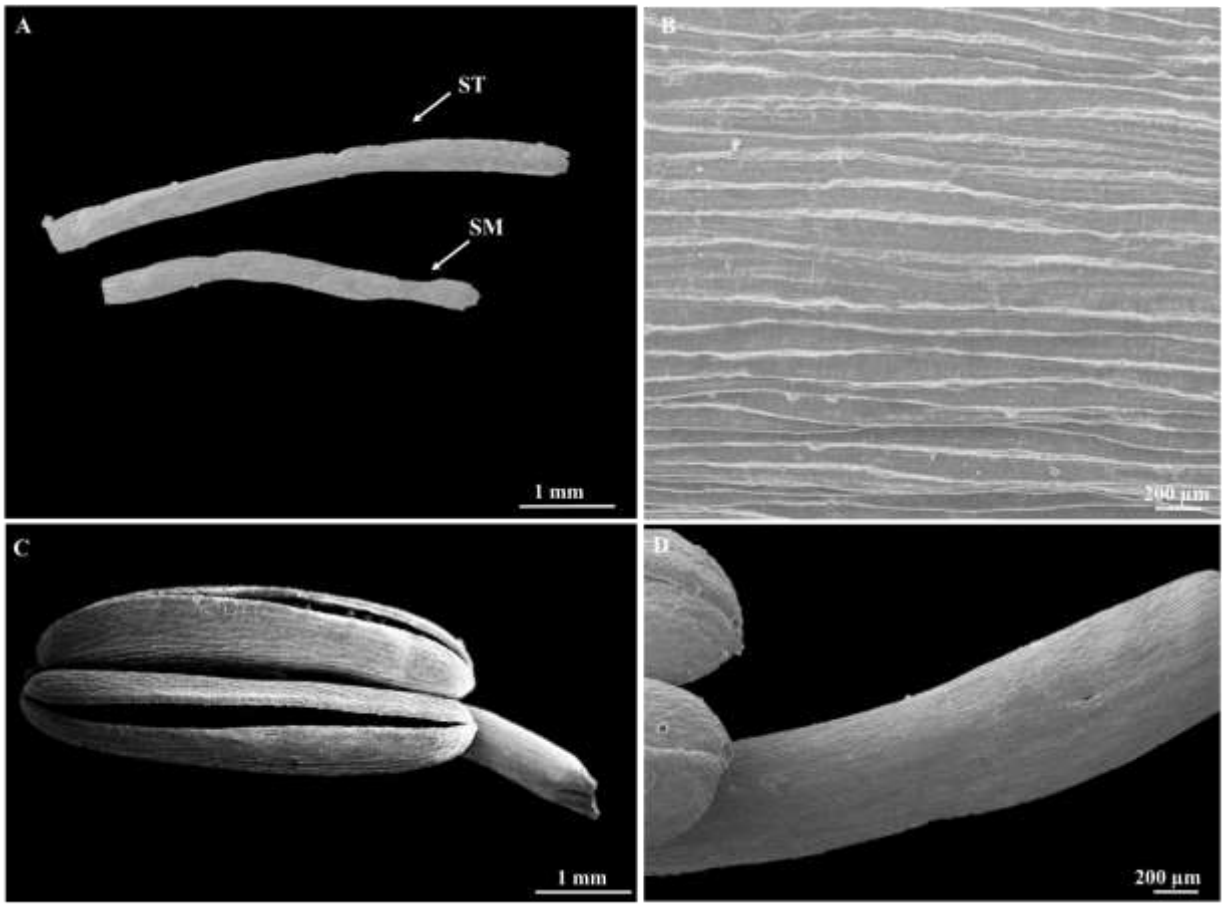

Figure 4.5: Scanning electron micrographs of the floral morphology of *B. albostellata*. (A) Low magnification image of a dissected section of the style and stigma; (B) High magnification image of a section of the stigma; (C) Anther; (D) Filament.

Abbreviations: SM= Stigma; ST= Style.

4.3.3 Pollen morphology

Pollen micromorphological features have contributed beneficial phylogenetic information in the accurate identification of species within Acanthaceae (Al-Hakimi et al., 2018; Raza et al., 2020). Scanning electron micrographs of pollen grains had an open reticulate tectum and appeared as globose tricolporate in equatorial view, honeycombed-shaped, with intense, coarse reticulation of the interapertural exine (Figure 4.6 A), these characteristics are specific to pollen found in species of *Barleria* (Obermeijer, 1933; Balkwill and Balkwill, 1997; McDade et al., 2008; Darbyshire et al., 2010; Gosavi et al., 2013; Darbyshire et al., 2015; Ravikumar et al., 2016). Pollen grains in *B.*

albostellata had a diameter of 77.53 ± 5.63 μm (Figure 4.6). This measurement varied from 60.5 ± 0.3 μm in *B. parviflora* to 81.5 ± 1 μm in *B. orbicularis*. Similar pollen grain measurements noted in *B. albostellata* was documented for *B. ventricosa* (74.9 ± 0.7 μm) and *B. proxima* (79.1 ± 1 μm) (Al-Hakimi et al., 2018). Before pollination at maturity, pollen grains are located inside the cap of the anther for protection (Figure 4.6 B-D). Tiny granules are observed inside lumina of the pollen grain (Figure 4.6 A). This was also noted in *B. parviflora*, *B. ventricosa* (Al-Hakimi et al., 2018), *B. prionitis* and *B. hochstetteri* (Scotland and Vollesen, 2000). The aperture of pollen grains in *B. albostellata* appeared circular in shape (Figure 4.6 E-F), which was also noted in *B. bispinosa* (Al-Hakimi et al., 2018). The aperture width of pollen grains in *B. albostellata* was 14.31 ± 0.59 μm. The width varied from 9 μm in *B. acanthoides* and *B. aculeata*, 13 μm in *B. tetracantha*, 16 μm in *B. ventricosa* and *B. bispinosa*, to 23 μm in *B. prionitis* (Al-Hakimi et al., 2018). Pollen grains in various families are recognized by distinct morphological features represented in their exine (Tripathi et al., 2017). Similar pollen grains characteristics to that of *B. albostellata* were found in *B. grootbergensis* (Darbyshire et al., 2012), *B. durairajii* (Ravikumar et al., 2016). Pollen grains in *Barleria* are characteristic to the family Acanthaceae, however their reticulate ornamentation displays close resemblances with those found in their associated genera such as Lepidagathis, Crabbea, and Ruellia (Shendage and Yadav, 2009).

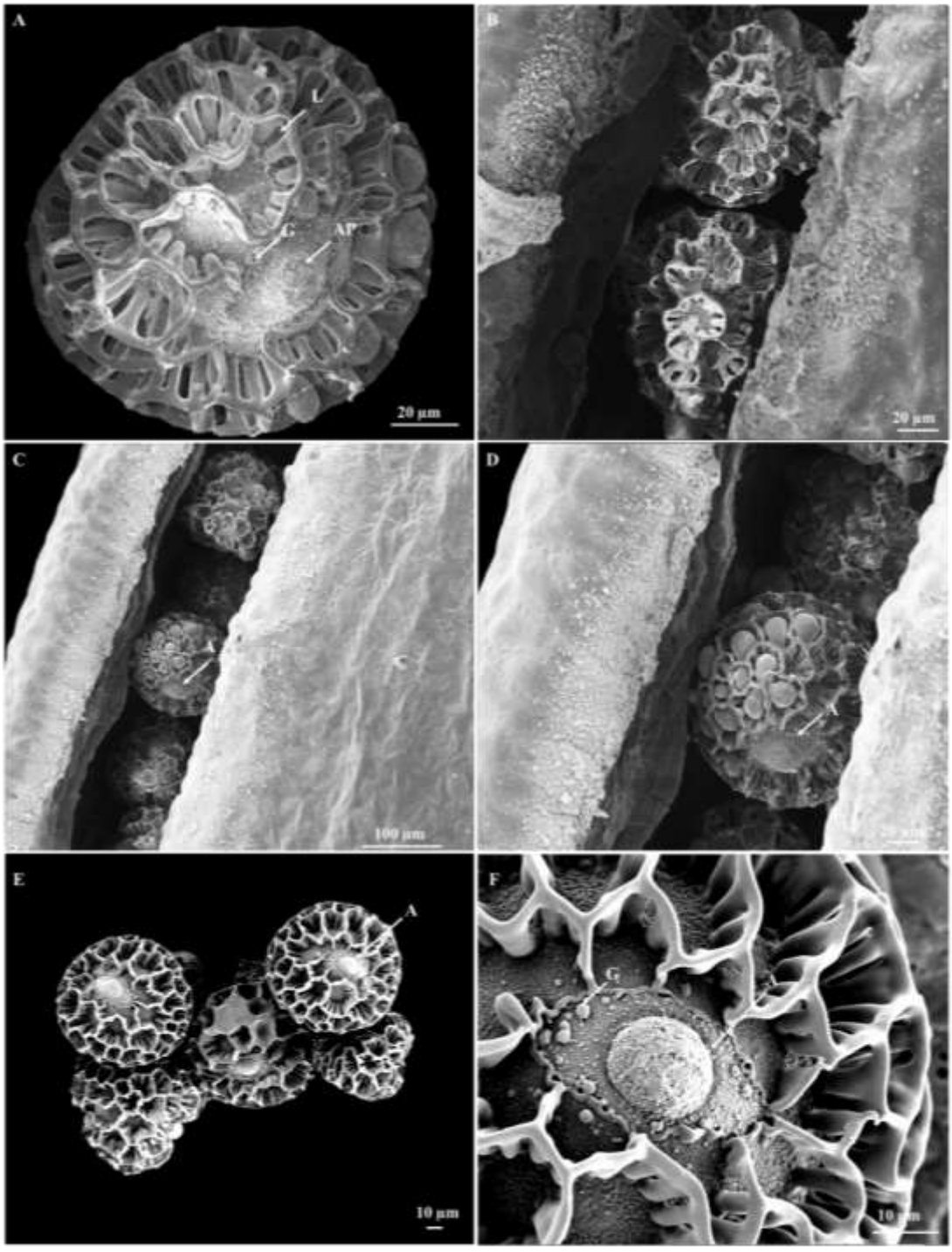

Figure 4.6: Scanning electron micrographs of the pollen micromorphology of *B. albostellata*. (A) Single pollen grain, equatorial view; (B-E) Pollen grains found within the anther; (F) Aperture of pollen grain.

Abbreviations: AP= Aperture; G= Granules; L= Lumina.

The morphology of the flower and pollen grains using various microscopic techniques showed numerous non-glandular trichomes on the bracteoles and bracts of *B. albostellata*. Three types of trichomes were identified on these structures and were found in other species of *Barleria* (Obermeijer, 1933; Balkwill et al., 1990; Makholela et al., 2004; Darbyshire, 2008). MDB non-glandular trichomes may provide some sort of physical protection to the glandular capitate tichomes. Pollen micromorphological features found are characteristic to species of *Barleria* (Obermeijer, 1933; Balkwill and Balkwill, 1997; McDade et al., 2008; Darbyshire et al., 2010; Gosavi et al., 2013; Darbyshire et al., 2015; Ravikumar et al., 2016).

4.4 Conclusion and future perspectives

The combination of stereo- and scanning electron microscopy facilitated the identification of the floral and pollen morphology of *B. albostellata*. Knowledge on the floral biology and pollen morphology of *B. albostellata* using microscopy techniques is lacking. Floral structures identified were compared to previously reported information on the species. Pollen grains of *B. albostellata* are complex, intricate and display reticulate sculpturing. Thus, the results presented in this study contributes significantly to our growing understanding on the floral and pollen biology of *B. albostellata*. In this regard, the study is novel. Furthermore, results from this study will also assist taxonomists in identifying *B. albostellata* using their SEM micrographs of their distinct pollen structures. Additional ultrastructural studies on the floral structures should be conducted to further examine the internal features of the cells and organelles. Further studies may also focus on evaluating the micromorphology of the seeds and roots of *B. albostellata*.

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CHAPTER 5

THE ANALYSIS OF PHYTOCHEMICAL COMPOUNDS AND ANTIBACTERIAL ACTIVITY IN *Barleria albostellata*

Abstract

Many infectious microbes have displayed resistance to many accessible medicines. Plants offer mankind an alternative source of medicines as they are widely accessible and highly effective against various infectious microorganisms. *Barleria albostellata* C.B. Clarke (Acanthaceae) is a shrub that is indigenous to South Africa and is relatively understudied. However, plants within this genus are well-known for their medicinal and ethnopharmacological properties. This study aimed at characterising the phytochemical compounds and antibacterial efficacies of *B. albostellata*. Phytochemical analysis, thin-layer chromatography (TLC), fluorescence microscopy and gas-chromatography mass spectrometry (GC-MS) analysis were performed to determine the composition of compounds that may be of medicinal importance. Energy-dispersive X-ray (EDX) analysis was additionally used to detect important trace elements present in the plant material. Crude leaf and stem extracts (hexane, chloroform and methanol) were subjected to an antibacterial analysis against several pathogenic microorganisms. The qualitative phytochemical screening of leaf and stem extracts revealed the presence various compounds. The different colour intensities obtained from TLC suggested that the concentrations of separated compounds were varied, with Rf values ranging from 0.03 cm to 1.02 cm. Fluorescence microscopy qualitatively assessed the leaf and stem powdered material, which displayed various colours under bright- and UV-light. GC-MS chromatograms represents 10-108 peaks of various compounds detected in the leaf and stem crude extracts. Major pharmacologically active compounds found in the extracts were alpha-amyrin, flavone, phenol, 2,4-bis(1,1-dimethylethyl)-, phytol, phytol acetate, squalene and stigmasterol. Crude extracts positively inhibited Gram-positive and Gram-negative bacteria. Significance was established at $P < 0.05$ for all concentrations and treatments. These results indicate that the leaves and stems of *B. albostellata* are rich in bioactive compounds, which could be a potential source of antibacterial agents treating various diseases linked to the pathogenic bacteria studied. Future discoveries from this plant could advance the use of indigenous traditional medicine and provide novel drug leads.

Keywords: Antibacterial activity; Energy-dispersive X-ray analysis; Gas-chromatography mass spectrometry; Phytochemical analysis; Thin-layer chromatography

5.1 Introduction

The dependence on plants as a source of medicine is prevalent in developing countries, especially where traditional medicine plays an important role in health care (Farnsworth, 1984; Hosseinzadeh et al., 2015; Bhat et al., 2019). As defined by the World Health Organisation (WHO), traditional medicine is the knowledge, skill and practices based on the beliefs and experiences in various cultures (WHO, 2014). The demand for herbal medicines worldwide is rapidly increasing due to their availability, low cost and higher safety margins (Gurib-Fakim, 2006; Aiswarya and Ravikumar, 2014; Eddouks et al., 2017). The adverse side-effects of conventional medicine are related to certain pharmacological compounds; therefore, creating different therapies with greater effectiveness and bioavailability, with fewer side-effects, is essential (Croteau et al., 2000; Salehi et al., 2018). Natural compounds isolated from plants have been assumed to remain an essential part of the exploration for new medicines against human diseases (Semenya et al., 2018; Alagawany et al., 2019).

Africa is considered the cradle of humankind comprising a rich cultural and biological diversity and with healing practices varying in regions (Gurib-Fakim, 2006; Eddouks et al., 2017; Sadgrove, 2020). Infectious diseases are a serious problem in Africa (Van Vuuren and Muhlarhi, 2017; Nkengasong et al., 2020), with one of the leading causes of morbidity and mortality arising from bacterial infections (Gram-positive and -negative bacteria) (Elbashiti et al., 2011; Ncube et al., 2012; Islam et al., 2015; Prasad et al., 2021). However, with the use of herbal medicine, certain bacterial infections have been reported to be under control, while others are resistant (Wise, 2008). According to WHO, by 2050, there will be approximately 10 million deaths arising from common diseases such as respiratory- and urinary tract infections and drug-resistant pathogens, surpassing the number of deaths resulting from cancer (Walsh, 2014; WHO, 2020). Therefore, it is crucial to find alternative solutions, such as herbal extracts, to overcome future threats in the medical field (Yang et al., 2018).

Throughout the history of mankind, plant extracts have been used to treat various ailments through herbal preparations (Rabe and Van Staden, 1997; Buwa and Van Staden, 2006; Ekor, 2014; Yuan et al., 2016). These preparations include concoctions, decoctions, infusions and teas (Van Wyk and Wink, 2004; Nafiu et al., 2017). Traditional preparations of various plant parts offer us novel remedies. Plants are rich in several of naturally-occurring phytochemicals such as alkaloids, flavonoids, tannins and terpenoids which have been found to contain antimicrobial properties. These secondary metabolites are important components of a plants' natural defence mechanisms and are products of primary metabolism (Cowan, 1999; Lewis and Ausubel, 2006; Al-tameme et al., 2015;

Al-Marzoqi et al., 2016; Hadi et al., 2016; Umah et al., 2017; Davies et al., 2020; Hatcher et al., 2020).

In South Africa, about 3000 medicinal plants were reported to be used regularly, and from these plants, 38 indigenous species were commercialised (Van Wyk and Gericke, 2000; Van Wyk, 2011). These commercialised plant species are available as processed material in various forms, such as capsules, ointments, tablets or teas (Van Wyk, 2008; Amoo et al., 2014). The verified record of natural products being used in drug discovery (Fabricant and Farnsworth, 2001; Shen, 2015) have provided compelling evidence for increasing the exploration of nature to identify unique active compounds as promising leads for effective drug development (Cragg et al., 1997; Rybicki et al., 2012; Newman and Cragg, 2016). There has been significant progress in pharmaceutical industries in search of important plant based medicinal compounds, however, a significant amount of the plant biodiversity remains unexplored (Cragg and Newman, 2007; Wangchuk, 2018). There is a growing need in linking the phytochemical compounds of a medicinal plant with its pharmacological activity. Screening plants for biologically active compounds has resulted in the development of new therapeutic drugs to treat several diseases (Vidhya and Udayakumar, 2015).

Barleria albostellata is an indigenous South African species of the Acanthaceae family. This shrub extends from Limpopo, Gauteng and Mpumalanga to KwaZulu-Natal (Froneman and Le Roux, 2007). Although *B. albostellata* has no recorded practice in traditional medicine, many species within the genus have been widely used in traditional medicine and were confirmed to contain various compounds possessing biological effects such as analgesic, anti-inflammatory, antileukemic, antihyperglycemic, antitumor, anti-amoebic, antibiotic and virucidal activities (Yosook et al., 1999; Wang et al., 2001; Jassim and Naji, 2003; Suba et al., 2004, 2005; Chomnawang et al., 2005).

Thus, further investigation on *B. albostellata* is important as this study will provide baseline information on the potential usage of extracts from this plant. This information is vital for establishing the safe and efficient use of this plant in traditional medicinal practices. There is a scarcity of scientific data on the phytochemical compounds of the leaf and stem extracts of *B. albostellata* and its potential antibacterial activity against human pathogens. This study was therefore carried out to bridge these gaps.

5.2 Materials and methods

5.2.1 Plant materials

Leaves and stems of *B. albostellata* were collected from the University of KwaZulu-Natal, Westville campus (29° 49′ 51.6″ S, 30° 55′ 30″ E), Durban, South Africa. A voucher specimen (7973000) was deposited in the Ward Herbarium of the University of KwaZulu-Natal, Life Sciences, Westville campus.

5.2.2 Organoleptic evaluation

The evaluation of crude leaf and stem material was completed with the aid of sensory organs following standard methods (Sen and Datta, 1982). This protocol uses colour, odour, taste and texture. Organoleptic assessment is accomplished using organs of sense and describing specific features of the material. This assessment is regarded as a first step towards establishing the identity and degree of purity of the sample (Jarald and Jarald, 2007).

5.2.3 Preparation of crude extract

For the preparation of the crude extract, leaves and stems were oven-dried for 2 weeks at 35°. The dried materials were crushed to a fine powder with the aid of a mechanical blender (Russel Hobbs, model: RHB315). The powdered material underwent sequential extraction using various solvents (hexane, chloroform and methanol) in a Soxhlet apparatus. Approximately 10 g of powdered leaves were placed into a round bottom flask containing 100 ml of hexane, the appropriate solvent, and boiled for 3 h at 40°C. The extracted solution was filtered (Whatman® No. 1 filter paper) and retained. This procedure was conducted in replicates. Consecutive extractions of chloroform followed by methanol were achieved. Each solvent extraction followed the same process as mentioned above. Successive extractions were performed on the leaf and stem material.

5.2.3.1 Evaporation and concentration

The concentration of each extract was left to evaporate in a dark fume-hood, at room temperature. The dried extracts were stored in airtight, labelled glass jars, to prevent the material from reacting with the atmospheric humidity. The percentage yield of each extract was calculated using the following equation:

$$\text{Extract Yield (\%)} = \frac{\text{Weight of dried extract (g)}}{\text{Weight of plant material (g)}} \times 100$$

5.2.4 Phytochemical tests

The intensity of the colour reactions was illustrated by symbols, (-) for no observed changes, (+-) for low intensity, (++) for medium intensity and (+++) for high intensity. Preliminary phytochemical screening was carried out on the powdered material and chemically tested for the presence of various constituents using standard methods (Harborne, 1973; Trease and Evans, 1978; Sofowora, 1993; Tiwari et al., 2011; Baskaran and Karthikeyan, 2019) as described below.

5.2.4.1 Test for alkaloids

Two drops of Mayers chemical reagent (potassium iodide was added to mercury (II) chloride solution) was added were transferred to 1 ml of extract in a test tube. The formation of a yellow precipitate indicated a positive reaction.

Two drops of Wagners chemical reagent (iodine was added to potassium iodide) were transferred to 1 ml of extract in a test tube. A positive reaction was indicated by the formation an orange-brown precipitate.

Two drops of Draggendorff's reagent (potassium iodide solution was added to bismuth subnitrate and glacial acetic acid) were mixed with 1 ml of extract in a test tube. The formation of a reddish precipitate indicated a positive reaction.

5.2.4.2 Test for amino acids and proteins

One drop of Ninhydrin solution (ninhydrin was added to ethanol) was transferred to 1 ml of extract in a test tube. An observed colour change to purple indicated a positive reaction.

5.2.4.3 Tests for carbohydrates

One drop of Molisch reagent (α-naphthol solution) was transferred to 1 ml of extract in a test tube. After the solution was thoroughly mixed, 0.5 ml of concentrated sulphuric acid was decanted slowly along the sides of the test tube to settle above the solution. The development of a violet or deep purple ring indicated a positive reaction.

Approximately 1 ml of each extract was mixed with 1 ml of Fehling's solution A (aqueous solution of copper sulfate) and B (potassium sodium tartrate solution in sodium hydroxide) and left undisturbed in a water bath to boil. The formation of a red precipitate indicated a positive reaction.

Approximately 1 ml of Benedict's reagent (solution of sodium citrate, sodium carbonate, and copper sulfate pentahydrate) was added to a test tube containing 1 ml of extract, the solution was mixed and boiled for 2 min in a water bath. The formation of a precipitate varying in colour from yellow to red indicated a positive reaction.

5.2.4.4 Test for fixed oils and fats

One drop of the extract was dispensed onto a filter paper (Whatman No. 1). The appearance of an oil stain on the filter paper indicated a positive reaction.

5.2.4.5 Test for flavonoids

One ml of 5% lead acetate solution was added to 5 ml extract. The formation of a white precipitate indicated a positive reaction.

5.2.4.6 Tests for mucilage

A total amount of two drops of 0.5% ruthenium red solution were added to 1 ml of extract. A colour change of pink to red indicated a positive reaction.

5.2.4.7 Tests for phenolics

Two drops of 10% ferric trichloride were transferred to 1 ml of extract in a test tube. The appearance of a green or black colour indicated a positive reaction.

5.2.4.8 Test for saponins

Froth test: Three ml of the extract was mixed with 10 ml of distilled water. The solution was shaken for 15 min. The formation of a 1 cm layer of froth indicated a positive reaction.

Foam test: Two ml of water was added to 0.5 ml of extract and shaken. The formation of foam that persisted for 10 min indicated a positive test for saponins.

5.2.4.9 Test for terpenoids

Two ml of chloroform was added to 5 ml plant extract, followed by decanting 3 ml of concentrated sulphuric acid along the side of the test tube to form a layer. A reddish-brown colour indicated a positive reaction.

5.2.4.10 Test for sterols

Three ml of chloroform was mixed with 2 ml of extract, followed by the addition of 2-3 drops of sulphuric acid down the side of the test tube. The appearance of a red ring between the solvent layers and a fluorescent green ring below indicated a positive reaction.

5.2.5 Thin layer chromatography (TLC)

Thin layer chromatography (TLC) is an affinity-based technique used to separate compounds in a mixture. Leaf and stem extracts were examined using TLC. Glass capillaries were used to spot small amounts of each extract (hexane, chloroform and methanol) separately onto a pre-coated silica gel 60 F_{254} TLC plate (Merck). The plate was positioned upright in a beaker which contained a mobile solution containing 8 ml toluene and 2 ml ethyl acetate (8:2 v/v). The solvent was allowed to run up the plate to a distance of 8 cm. Images of the plate were taken under ultra-violet (UV) light (254 and 366 nm), to compare the chemical profiles of the various extracts. The plate was then sprayed with an anisaldehyde-sulphuric acid reagent, heated at 90°C for 5 min and photographed with a digital camera. Anisaldehyde-sulphuric acid is a universal reagent for natural products, which allows for colour differentiation. Plates sprayed with anisaldehyde-sulphuric acid are expected to react with the various compounds affecting the colour intensity than when observed with UV light alone (without anisaldehyde) (Alebiosu et al., 2015). The retention factor (R_f) values of the active compounds were calculated by the given formula:

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by the solvent front}}$$

5.2.6 Fluorescence analysis

Fluorescent analysis of the dried powdered plant material plays an important role in the determining the quality and purity of the tested drug. A small quantity of the dry plant powder (leaves and stems) was placed separately onto clean microscope slides. Two drops of each prepared reagent were dispensed, mixed gently by slanting the slide, and allowed to stand for 3 min for the thorough absorption of the solution by the plant powder. The slides were then viewed using a Nikon Eclipse microscope, using bright field light and UV-2A (excitation 320/380) illumination. The colours attained by the application of various reagents were recorded. Fluorescence analysis of the leaf and stem powder was carried out using the standard method (Kumar et al., 2012; Chanda, 2014; Akwu et al., 2019).

5.2.7 Energy-dispersive X-ray spectroscopy (EDX)

Energy-dispersive X-ray spectroscopy is an analytical technique used for the elemental analysis (composition) of a sample. The EDX analysis was conducted at the Microanalysis Microscopy Unit (MMU) at the University of KwaZulu-Natal, Westville. Fresh leaves and stems were harvested and placed in a mortar. Liquid nitrogen slush (-210°C) was added to the plant material and crushed with the aid of a pestle and mortar. A fine powder resulted after 3 min and was oven dried for 48 h. A small amount of the powdered material was placed onto carbon conductive tape on aluminium stubs and sputter-coated with gold with the Quorum 150 RES gold coater. Elements were identified using the Aztec analysis software on the Ultra Plus FEG-SEM (Carl Zeiss, Germany) at 5kV.

5.2.8 Gas chromatography-mass spectrometry (GC-MS)

This analysis is used to examine liquid, gaseous or solid samples and produce several different peaks in the gas chromatogram. Each peak generates a specific mass spectrum which is used for compound identification. Leaf and stem methanolic extracts were analysed using the GC-MS (QP-2010 Ultra Shimadzu, Japan) instrument, with an Rx_5Sil Ms capillary column (0.25 μm internal diameter and 0.25 μm film thickness) from Restek. The carrier gas, helium, had a flow rate of 0.96 ml/min and a total flow of 4.9 ml/min, and a linear velocity of 36.7 cm/sec at a purge flow of 3.0 ml/min. The injection temperature was set at 250°C. The oven temperature was set at 50°C and held for 1 min, increased to 310°C and held for a further 10 min. Chemical compounds (analytes) were identified by relating their retention times with those of the polychlorinated biphenyl (PCB) standards found in the

National Institute of Standards and Technology (NIST) library. This analysis was conducted at the Department of Chemistry at the University of KwaZulu-Natal, Westville campus.

5.2.9 Antibacterial bioassay

5.2.9.1 Preparation of crude extracts

Crude (hexane, chloroform and methanol) leaf and stem extracts were transferred to Eppendorf centrifuge tubes, dissolved in 10% dimethyl sulfoxide (DMSO) at various concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 mg/mL, and homogenised using a vortex. The prepared sample was stored at - 4°C until further use.

5.2.9.2 Test microorganisms

The prepared crude extracts were subjected to antibacterial assays. The antibacterial activity of leaf and stem samples were tested against 5 strains, the Gram-positive bacteria: *Bacillus subtillus* (ATCC 6633), *methicillin*-resistant *Staphylococcus aureus* (ATCC 43300), *Staphylococcus aureus* (ATCC 25923); and Gram-negative bacteria: *Pseudomonas aeruginosa* (ATCC 25783), and *Escherichia coli* (ATCC 35218). These bacterial strains were supplied by Professor Johnson Lin, School of Life Sciences (Microbiology Department), University of KwaZulu-Natal, and maintained in 75% glycerol at −80 °C.

5.2.9.3 Preparation of culture media and bacterial cultures

Mueller-Hinton agar (MHA) (Biolab, South Africa) was prepared by suspending 38 g of the agar into 1 L of distilled water. The medium was mixed on a stirrer (15 min), heated in a microwave (10 min) and autoclaved (Model: HL-320) at 121°C for 1 h. The agar was poured into sterile Petri plates (90 mm) and was allowed to set at room temperature (23°C).

Both Gram-positive and –negative bacteria from stock cultures were sub-cultured onto fresh agar plates, and incubated overnight (24 h) at 37°C. Glass test tubes containing distilled water (15-20 ml) were autoclaved at 121°C for 1 h. Colonies of bacteria, from each Petri plate were removed with a sterile loop and inoculated by transferring 5 $\mu g.\mu L^{-1}$ into the glass test tubes containing 15 ml of sterile distilled water (0.5 McFarland scale). The absorbance of each bacterial culture was measured, adjusted, and diluted to attain a viable cell count using the Cary 60 UV-Vis spectrophotometer.

Each bacterial strain was separately smeared uniformly over the surface of the MHA plates with a sterile cotton swab. The disc diffusion method was used to evaluate the antibacterial activity of each crude extract. Approximately, 20 µL of the prepared crude extracts were dispensed onto sterile filter paper discs (Whatman no. 1 filter paper) (6 mm in diameter) using sterile micropipette tips. The sterile discs were loaded with each extract of varying concentrations and allowed to dry overnight. Discs were then placed onto Petri plates containing bacteria. Petri plates were sealed and incubated for 24 h at 37 °C. Zones of inhibition evident around the filter paper were taken as positive results. The diameters of inhibition were measured and photographed within 18-24 h after incubation, to determine if the extract exhibited any antibacterial activity. Filter paper discs loaded with streptomycin and gentamycin were used as positive controls, and 10% DMSO as the negative control (Okla et al., 2021). The analyses were conducted in triplicates, and data were presented as mean ± standard deviation.

5.2.10 Statistical analysis

All experiments conducted for the antibacterial assay were carried out in triplicate. Values were expressed as mean ± standard deviation (significant at $p < 0.05$ level). Antibacterial data were statistical analysed using the one-way analysis of variance (ANOVA). *In vitro* cytotoxicity data were subjected to Tukey's-honest significant difference multiple range post hoc tests.

5.3 Results and discussion

5.3.1 Organoleptic characteristics and crude extract yield of *B. albostellata*

Organoleptic evaluation is a conventional, qualitative method whereby an individual uses their sight, smell, taste, and touch to document the characteristic features of crude drugs. These assessments may serve as a baseline for preliminary phytochemical and pharmacological screening of a plant (Selvam, 2015). The organoleptic features of *B. albostellata* were evaluated by using sensory organs (Table 5.1). The following features were noted on both adaxial and abaxial surfaces, the leaves were grey-green in colour but lighter on the lower surface. The stems appeared as 'yellow-buff' on the upmost internodes and white/cream below. The odour of the leaves was slightly aromatic, whilst the stems were inodourous. The taste was acrid for both leaves and stems. Both surfaces of the leaves were velvety, whereas the stems were woody and glabrescent. According to Shaheen et al. (2014), organoleptic studies are important taxonomic parameters, assisting in the verification of several medicinal plant species.

Table 5.1: Organoleptic features of different parts of *B. albostellata*

Organoleptic features	**Leaf**	**Stem**
Colour	Grey-green on both surfaces but lighter on the lower side	yellow-buff on uppermost internodes, white or cream below
Odour	Slightly aromatic	Inodourous
Taste	Acrid	Acrid
Texture	Velvety	Woody, glabrescent

The highest percentage yield of the crude extracts from *B. albostellata* was obtained from the methanolic extract of the leaves (16.78%), followed by 9.38% from the methanolic stem extract (Table 5.2). The lowest percentage yield was observed in the hexane stem extract (1.94%). Overall, this suggests that the percentage yield of phyto-compounds in *B. albostellata* were greater in the leaf crude extract than from the stem. Furthermore, this infers that there may be more polar compounds in the leaf extracts and a smaller amount of non-polar compounds in the stem. Therefore, the yield obtained indicates the polarity of the different solvents is related to the plants' pharmacological importance (Abubakar et al., 2017; Chintalapani et al., 2018). Each crude extract (hexane, chloroform and methanol) displayed distinct colours (Table 5.2). Hexane extracts were oily upon evaporation of the solvent, whereas chloroform and methanol dried to a hard-sticky solid.

Table 5.2: Percentage yield of the leaf and stem crude extracts of *B. albostellata*

Crude extract	**Leaves**	**Stem**	**Leaves**	**Stem**	**Leaves**	**Stems**
	Dried extract yield (g)		**Percentage yield (%)**		**Colour**	
Hexane	0.139	0.194	1.39	1.94	Dark yellow	Light yellow
Chloroform	0.265	0.219	2.65	2.19	Dark green	Light green
Methanol	1.678	0.938	16.78	9.38	Dark brown	Light yellow

5.3.2 Phytochemical screening for major classes of compounds in extracts of *B. albostellata* using qualitative colour tests, TLC and fluorescence analysis

Phytochemicals of the various extracts were established by qualitative colour tests and TLC (Table 5.3, Figures 5.1 and 5.2). Major compounds identified in leaf and stem extracts of *B. albostellata* were alkaloids, amino acids, carbohydrates, flavonoids, mucilage and gums, phenols, saponins, terpenoids and sterols (Table 5.3). Fixed oils and fats were present in leaves and absent in the stems extracts. The intensity of compounds in the leaf extracts was greater in comparison to the stem. These compounds can act as defence mechanisms against various microorganisms, herbivores and insects (Murugan et al., 2013; Wink, 2018; Yactayo-Chang et al., 2020).

Various phytochemicals have been known to contain diverse activities that may help protect against chronic diseases (Liu, 2003; Singh and Sharma, 2020). Amoo et al. (2011) reported the presence of phenols, iridoids, gallotannins, flavonoids and condensed tannin in the leaves and stems of *B. albostellata*. These authors also found the total iridoid content to be the highest in the leaves of *B. albostellata*. Important pharmacological alkaloids can be found in iridoids, as this compound is known to be a precursor in the biosynthesis of alkaloids (Didna et al., 2007; Tundis et al., 2008). Similar compounds were also found in extracts of other *Barleria* species including, *B. acuminata* (Bency et al., 2018), *B. dintteri* (Semenya et al., 2018), *B. cristata* (El-Mawla et al., 2005; Hemalatha et al., 2012; Chowdhury et al., 2014), *B. longiflora* (Baskaran and Karthikeyan, 2019) and *B. prionitis* (Shukla and Gunjegaonkar, 2018).

Metabolites such as alkaloids and terpenoids (Table 5.3) were reported to contain antimicrobial, anti-cancer and anti-malarial properties (Mahato and Sen, 1997; Dahanukar et al., 2000; Russo et al., 2013; Usman et al., 2013; Cushnie et al., 2014; Kaur and Ahmed, 2021). Additionally, alkaloids have organic and natural constituents with sedative and analgesic roles (Paul et al., 2017). Amino acids, carbohydrates and fixed oils and fats (Table 5.3) were reported to contain antioxidant properties (Olsson et al., 2004; Agoramoorthy et al., 2007; Dalle-Donne et al., 2009). Flavonoids and phenols (Table 5.3) possessed anti-inflammatory, anti-apoptosis, anti-carcinogen and anti-aging properties (Han et al., 2007; Kunle and Egharevba, 2009; Gopalakrishnan and Udayakumar, 2017; Nguyen et al., 2020).

Mucilage and Gums (Table 5.3) are used in the treatment of gastric ulcers, for wound healing, as cytoprotective agents, and also contain antipyretic and antiseptic properties (Jani et al., 2009; Wadhwa et al., 2013; Bhutada et al., 2017; Dhingra et al., 2021). Saponins present in plant extracts

(Table 5.3) are believed to contain anticancer, antioxidant, antiviral and anti-inflammatory properties (Shi et al., 2004; Qadir et al., 2015). Additionally, saponins display several hepatoprotective and antimicrobial activities (Prakash and Vedanayaki, 2019). Sterols were found to reduce cholesterol levels and contain anti-inflammatory and antioxidant properties (Table 5.3) (Prieto et al., 2006; Marangoni and Poli, 2010; Baskar et al., 2012; Trautwein et al., 2018; Martianto et al., 2021).

Table 5.3: Preliminary phytochemical screening for major classes of compounds in hexane, chloroform and methanolic leaf and stem extracts of *B. albostellata*

Compound group	Phytochemical test	Leaves			Stems		
		Hexane	**Chloroform**	**Methanol**	**Hexane**	**Chloroform**	**Methanol**
Alkaloids	Dragendorffs	+++	+++	+++	++	+++	+++
	Mayers	+++	-	++	-	-	+-
	Wagners	+++	+++	+-	+-	+++	+-
Amino acids	Ninhydrin	+-	-	+-	-	-	++
Carbohydrates	Benedicts	-	-	+++	-	++	-
	Fehlings	-	+++	+-	+++	+++	++
	Molisch	-	+-	+-	-	+-	-
Fixed oils and fats	Filter paper	++	+-	+-	-	-	-
Flavonoids	Lead acetate	+++	+++	+-	+++	+++	+-
Mucilage and Gums	Ruthenium	+++	+++	+++	++	+++	+++
Phenols	Ferric trichloride	+-	++	+++	+-	+-	+-
Saponins	Froth	+-	++	+-	+-	+-	++
	Foam	+-	+-	+++	+++	+++	++
Terpenoids	Chloroform	++	++	++	+-	+-	++
Sterols	Salkowski's	-	++	+-	-	-	+-

*Intensity of reaction: (-) No observed changes, (+-) low intensity, (++) medium intensity, (+++) high intensity.

Thin-layer chromatography illustrates the separation of compounds from the plant extract. As the solvent progressed up the plate, the bands appeared in different colours (Figures 5.1 and 5.2). After spraying the TLC plate with anisaldehyde (universal indicator) and incubation, more compounds were identified from the plant (Figure 5.1 A) than when observed under UV light alone (without anisaldehyde) (Figure 5.1 B). For the leaves, 6 bands migrated from the hexane extract, 7 from the chloroform extract and 5 from the methanol extract. In the stems, 8 bands migrated from the hexane extract, 1 band from the chloroform extract and none from the methanol extract. The leaf extracts contained more bands than the stem extract, which may indicate that there are many compounds present (Figure 5.1). The colours of the spot regions varied throughout the extracts, from green, orange-brown, tinges of purple to dark black (Figure 5.1 A).

Fluorescence bands viewed under UV-light at 254 nm appeared yellow and blue for chloroform leaf extracts only (Figure 5.1 B). Fluorescence bands observed under green UV-light at 366 nm (without anisaldehyde) appeared black (Figure 5.2 A), and at blue UV-light at 366 nm (without anisaldehyde) bands were red and blue for chloroform leaf extracts only (Figure 5.2 B). Blue, yellow and green fluorescent bands (366 nm) were observed at the spot regions for the other extracts (Figure 5.2 B). Spots of red, blue and green viewed on the TLC plate at UV (366 nm) may correspond to several classes of secondary metabolites (Yahyaoui et al., 2017). Wagner et al. (1996) reported that characteristic blue and green fluorescence viewed under UV light (366 nm) might indicate the presence of flavonoids. Thin-layer chromatography screens bioactive compounds in plant extracts (Frum, 2006; Muthukrishnan and Sivakkumar, 2018).

The difference in the colour intensities on the TLC plate implied that the concentrations of compounds separated were different. Retention factor (R_f) values measure the movement of the solvent up the plate. The R_f values of the separated compounds are reflected in Table 5.4. The chloroform extract in the leaves and hexane extract from the stems had the most bands (Table 5.4). Bands closer to the migration region had a lower R_f value, whilst values furthest away had a higher value (Table 5.4). Depending on the solvent system, phytochemicals produce R_f values ranging from 0.03 cm to 1.02 cm (Table 5.4). According to Dheer et al. (2019), the methanolic leaf extracts of *B. prionitis* displayed R_f values ranging from 0.57-0.77 cm, whilst the hexane and methanol extracts of *B. dinteri* displayed values ranging from 0.22-0.95 cm and 0.26-0.66 cm, respectively (Semenya et al., 2018). Ghule et al. (2020) found isolated fractions from *B. prionitis* leaf methanol extracts to display R_f values at 0.32 cm and 0.51 cm, an indication of iridoids. The difference in the R_f values of the phytochemicals indicated the polarity of the compounds (Francis and Sudha, 2017; Semenya et al., 2018). Non-polar compounds move up the plate rapidly while polar substances travel slower up the plate or not at all. Leaf extracts contained more non-polar compounds than polar substances and the opposite was true for the stem extracts except for the hexane stem extract (Figure 5.1).

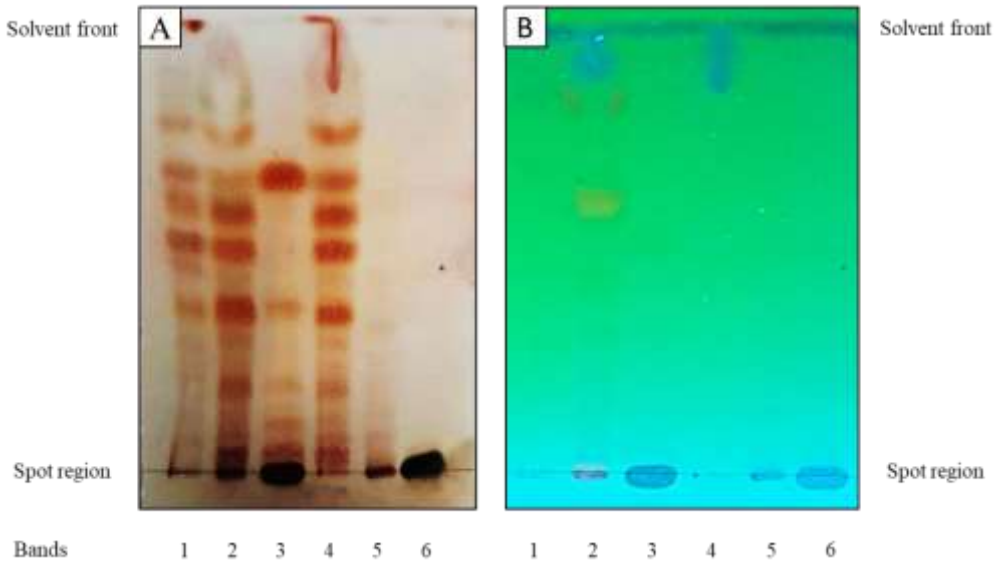


Figure 5.1: TLC profile of crude extracts of *B. albostellata* viewed under visible light (with anisaldehyde reagent) (A) and UV-light at 254 nm (without anisaldehyde) (B). Bands: 1- Hexane extract of leaves, 2- Chloroform extract of leaves, 3- Methanol extract of leaves, 4- Hexane extract of stems, 5- Chloroform extract of stems, 6- Methanol extract of stems.

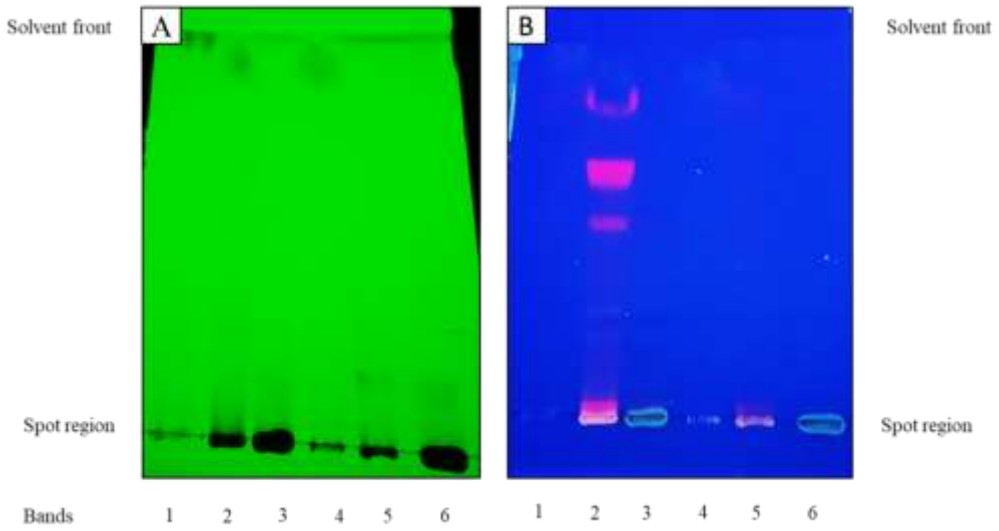


Figure 5.2: TLC profile of crude extracts of *B. albostellata* viewed under green UV-light at 366 nm (without anisaldehyde) (A) and blue UV-light at 366 nm (without anisaldehyde) (B). Bands: 1- Hexane extract of leaves, 2- Chloroform extract of leaves, 3- Methanol extract of leaves, 4- Hexane extract of stems, 5- Chloroform extract of stems, 6- Methanol extract of stems.

Table 5.4: TLC profile of *B. albostellata* crude extracts with retention factor (R_f) values

	Extract R_f values (cm)					
	Leaves			**Stem**		
Bands	Hexane	Chloroform	Methanol	Hexane	Chloroform	Methanol
1	0.38	0.04	0.04	0.03	0.35	-
2	0.52	0.20	0.12	0.18	-	-
3	0.61	0.36	0.20	0.35	-	-
4	0.70	0.51	0.36	0.51	-	-
5	0.84	0.60	0.65	0.58	-	-
6	1.02	0.69	-	0.66	-	-
7	-	0.83	-	0.77	-	-
8	-	-	-	0.90	-	-

A technique used in the qualitative assessment of natural products or crude drugs is fluorescence analysis, which is an important tool for pharmacognostic evaluation (Zhao et al., 2011; Andrews et al., 2020). The powdered and fluorescence characteristics of the leaf and stem powder of *B. albostellata* are presented in Table 5.5 and Figures 5.3-5.6. The powdered leaf and stem material treated with several reagents displayed various colours when observed under bright light, and this is compared to the colours observed under UV-light (Table 5.5). It should be noted that the colours indicated for the powdered leaf and stem material viewed under bright light, was described according to the overall appearance. The purity and quality of crude drugs are occasionally authenticated using standard fluorescence characteristics, as certain natural products display no fluorescence in daylight but do so under UV-light (Pandavadra and Chanda, 2014). Natural products such as berberine alkaloids exhibit fluorescence under UV-light and none in daylight (Kumar et al., 2013; Zheng et al., 2020). As most crude drug materials do not fluorescence, these materials are converted either into fluorescent decomposition- or by-products with the aid of several reagents (Zhao et al., 2011; Chanda, 2014; Andrews et al., 2020). Furthermore, fluorescence analysis can be used to preserve the quality and effectiveness of crude drug materials by easily detecting adulterants and substituents (Carvalho et al., 2011; Zhao et al., 2011; Folashade et al., 2012; Andrews et al., 2020).

The most prominent UV colour that stands out in both leaves and stems powder was blue (Table 5.5, Figures 5.4, 5.6). This colour was observed in multiple plant samples where different reagents were used. According to Chase and Pratt (1949), several drugs display duplication of colours, as there are sometimes more than four drugs found in a particular colour group. According to Sridharan and

Gounder (2016), powdered leaves of *B. montana* were separately exposed to 24 h of light with the addition of various reagents. These included, powder + water, + ethanol, + ethyl acetate, + hexane, + chloroform, and + acetone. Colours observed from the various regents after 24 h were orange, green, pale and light green, respectively. When these samples were exposed to UV-light, colours observed were greenish-orange, light and dark green, pale yellow, yellowish-orange and pale red. Similar fluorescence results listed in Table 5.5, were detected for certain reagents in the powdered leaf material for *B. noctiflora* (Arumugam and Natesan, 2015) and *B. gibsoni* (Tamboli and More, 2016).

Table 5.5: Fluorescence analysis of the leaf and stem powder of *B. albostellata*

Plant sample	**Leaves**		**Stem**	
	Bright light	**UV**	**Bright light**	**UV**
1. Powder	Dark green	Blue	Brown and red	Blue
2. Powder + Water	Dark green and black	Blue and brown	Brown	Blue
3. Powder + H_2SO_4	Reddish-brown	Blue	Reddish-brown and black	Blue and green
4. Powder + Acetic acid	Dark green and brown	Pink and purple	Brown and yellow	Blue and purple
5. Powder + Aqueous NaOH	Dark brown and orange	Blue	Brown and yellow	Blue
6. Powder + HCl	Dark green	Light yellow-green	Brown	Yellow-green
7. Powder + Ethanol	Light green	Light yellow-green	Brown	Blue and white
8. Powder + Ethyl acetate	Light green and brown	Blue	Brown and grey	Blue and white
9. Powder + Hexane	Dark green and brown	Blue	Brown, grey and black	White and blue
10. Powder + Chloroform	Dark green	Blue	Brown and yellow	Blue
11. Powder + Methanol	Dark green	Blue and green	Brown and grey	Blue and white
12. Powder + Petroleum ether	Dark green and brown	Blue and purple	Brown and yellow	Blue and green
13. Powder + Diethyl ether	Green and brown	Blue	Brown and grey	Blue
14. Powder + acetone	Green and brown	Blue	Brown and grey	Blue

***** H_2SO_4= Sulfuric acid; NaOH= Sodium hydroxide.

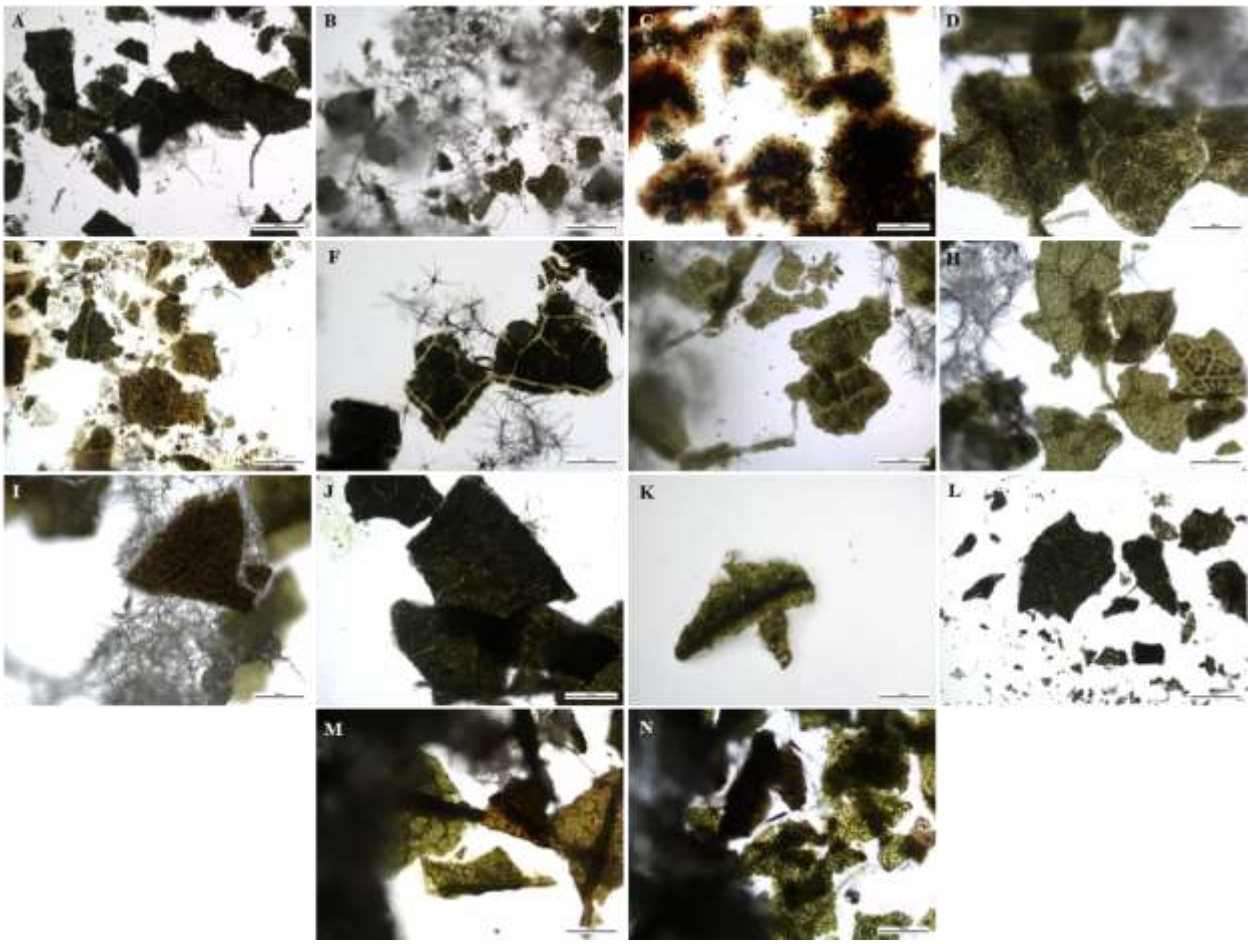


Figure 5.3: Powdered analysis (bright light) of leaf powder from *B. albostellata*. (A) Powder; (B) Powder + water; (C) Powder + H_2SO_4; (D) Powder + acetic acid; (E) Powder + aqueous NaOH; (F) Powder + HCl; (G) Powder + Ethanol; (H) Powder + ethyl acetate; (I) Powder + hexane; (J) Powder + chloroform; (K) Powder + methanol; (L) Powder + petroleum ether; (M) Powder + diethyl ether; (N) Powder + acetone.

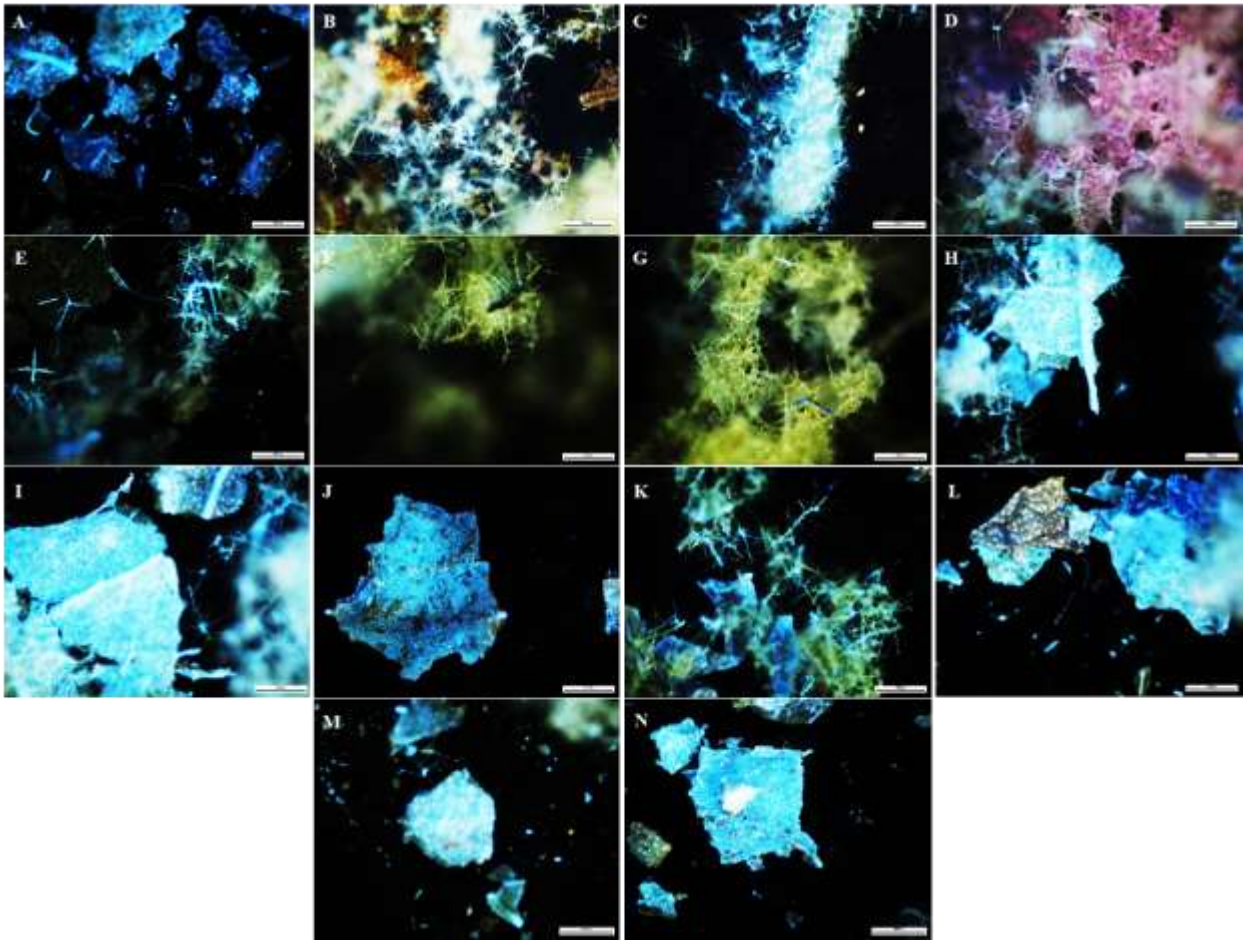


Figure 5.4: Fluorescence analysis (UV-light) of leaf powder from *B. albostellata*. (A) Powder; (B) Powder + water; (C) Powder + H_2SO_4; (D) Powder + acetic acid; (E) Powder + aqueous NaOH; (F) Powder + HCl; (G) Powder + Ethanol; (H) Powder + ethyl acetate; (I) Powder + hexane; (J) Powder + chloroform; (K) Powder + methanol; (L) Powder + petroleum ether; (M) Powder + diethyl ether; (N) Powder + acetone.

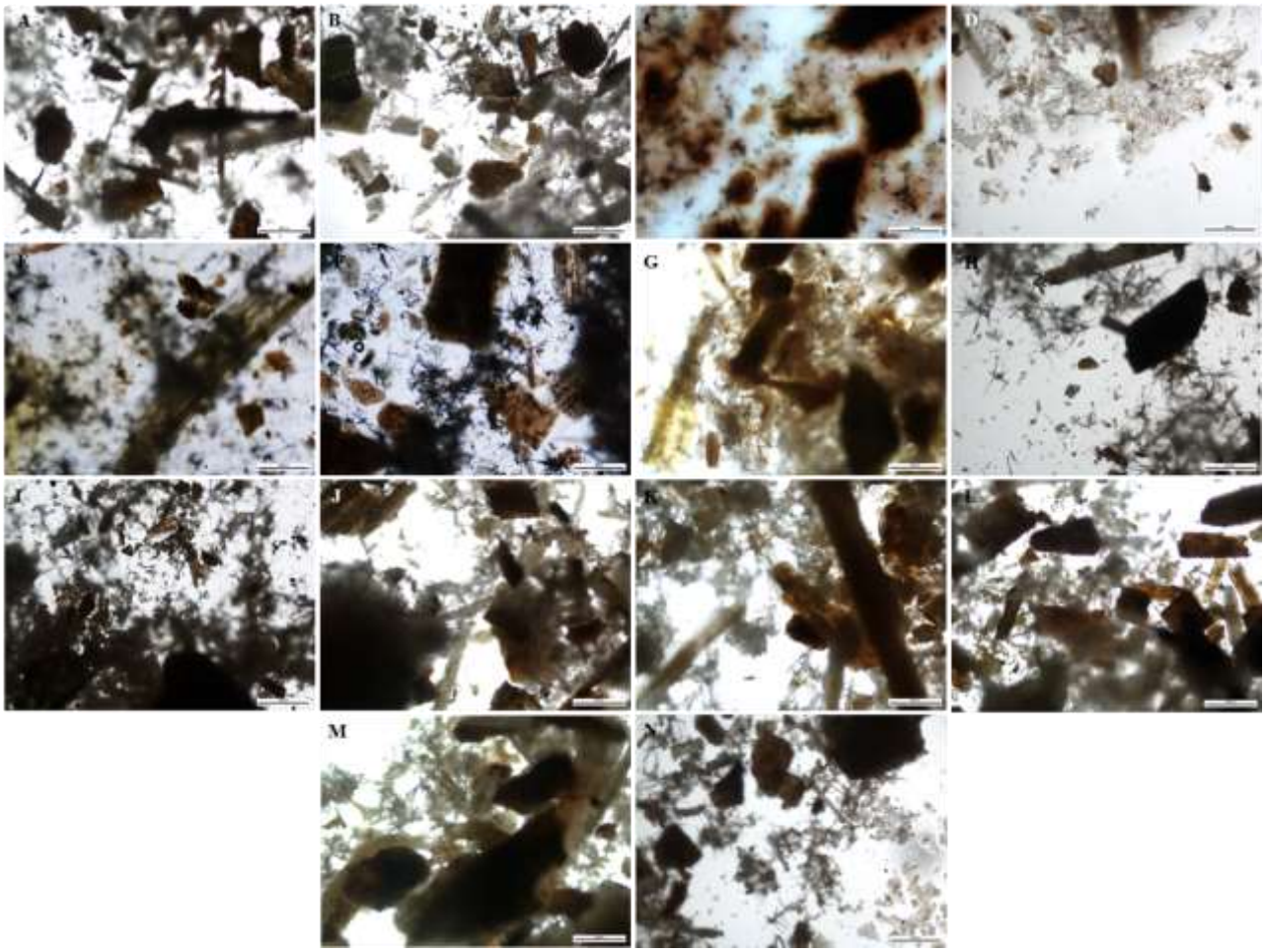


Figure 5.5: Powdered analysis (bright light) of stem powder from *B. albostellata*. (A) Powder; (B) Powder + water; (C) Powder + H_2SO_4; (D) Powder + acetic acid; (E) Powder + aqueous NaOH; (F) Powder + HCl; (G) Powder + Ethanol; (H) Powder + ethyl acetate; (I) Powder + hexane; (J) Powder + chloroform; (K) Powder + methanol; (L) Powder + petroleum ether; (M) Powder + diethyl ether; (N) Powder + acetone.

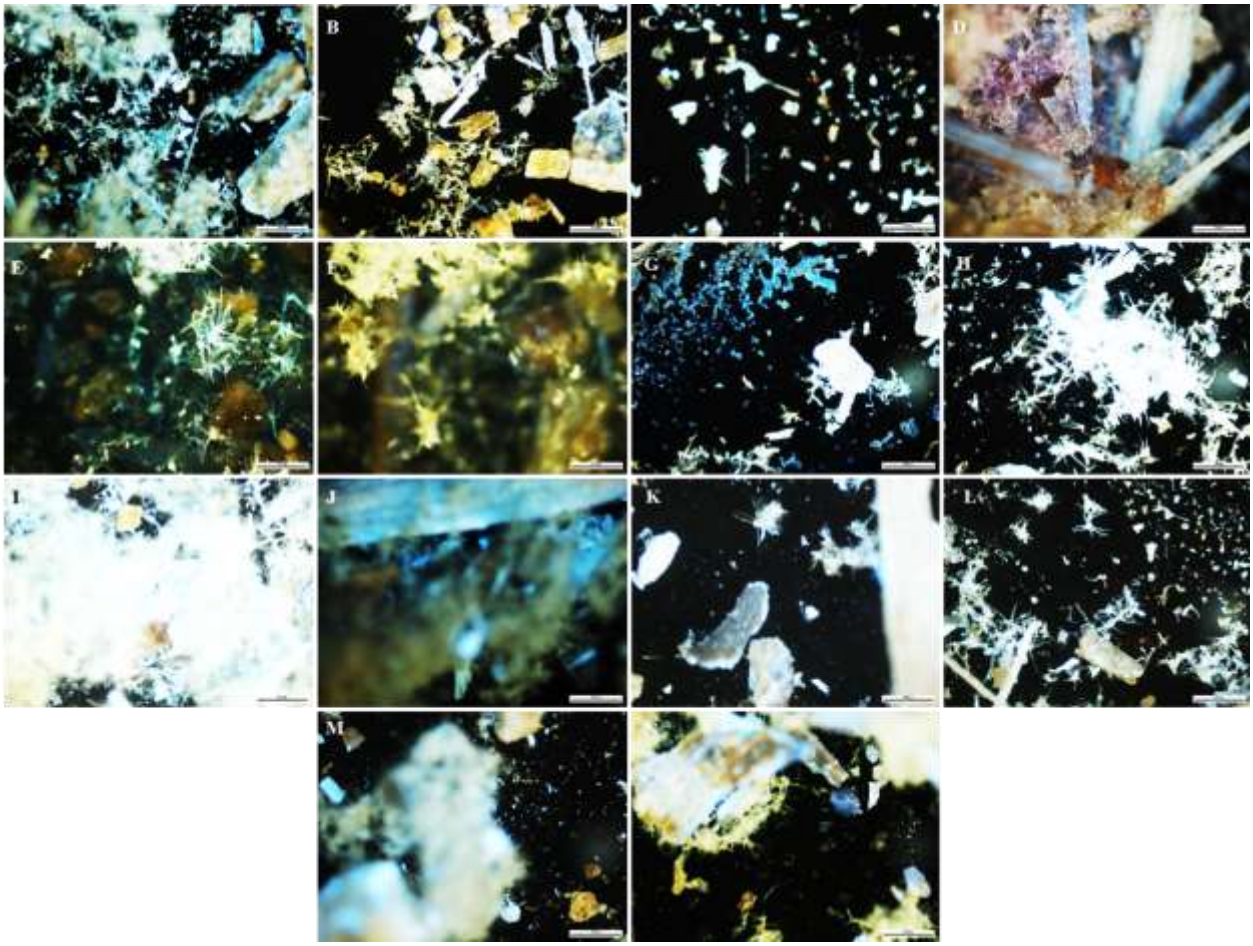


Figure 5.6: Fluorescence analysis (UV-light) of stem powder from *B. albostellata*. (A) Powder; (B) Powder + water; (C) Powder + H_2SO_4; (D) Powder + acetic acid; (E) Powder + aqueous NaOH; (F) Powder + HCl; (G) Powder + Ethanol; (H) Powder + ethyl acetate; (I) Powder + hexane; (J) Powder + chloroform; (K) Powder + methanol; (L) Powder + petroleum ether; (M) Powder + diethyl ether; (N) Powder + acetone.

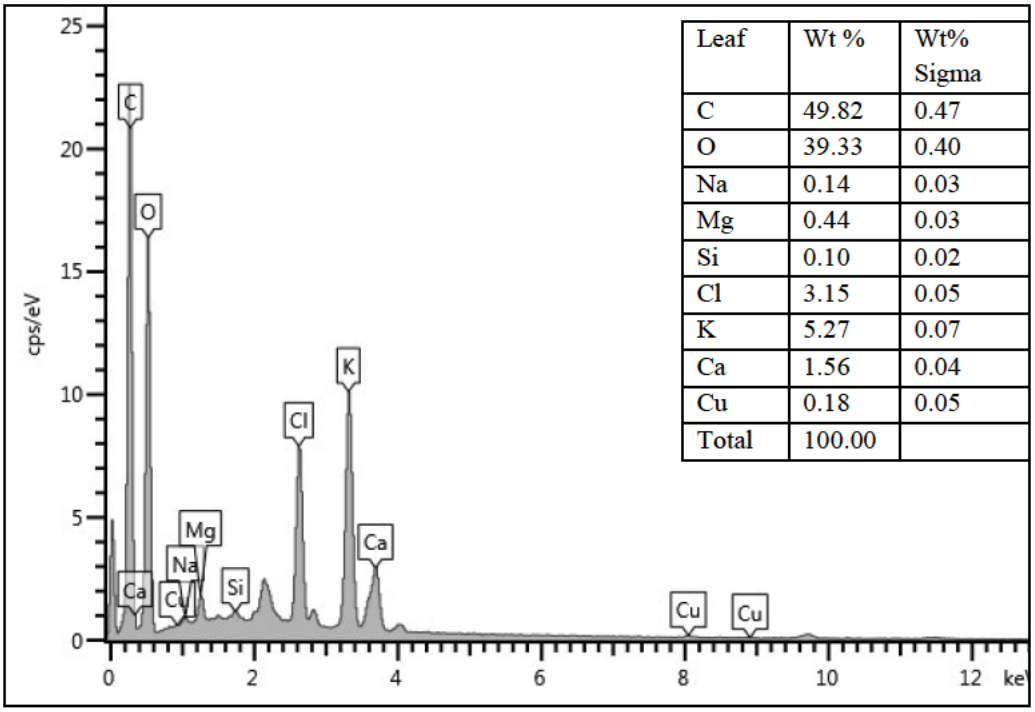
5.3.2 Energy-dispersive X-ray (EDX) spectroscopy

The EDX spectroscopy of dried leaf material detected the presence of carbon (C), oxygen (O), sodium (Na), magnesium (Mg), silica (Si), chlorine (Cl), potassium (K), calcium (Ca) and copper (Cu) (Figure 5.7). The same compounds were present in the stem material except for Na and Si (Figure 5.8). Carbon was the highest trace element found in both leaves and stems with a percentage weight of 49.82% and 54.37%, respectively. The various percentage weights observed in the leaf and stem material may be due to the composition of minerals in the soil and its surrounding climatic conditions (Underwood, 2012). Knowledge of the elemental composition present in plants via EDX analysis is important in determining the safety/toxicity of the plant (Obiajunwa et al., 2002; Scimeca et al., 2018).

Metals such as Cu, are an essential micronutrient for plants and are required for the proper structure and function of many proteins, regulation of transcription factors and redox reactions (Marschner, 2012;

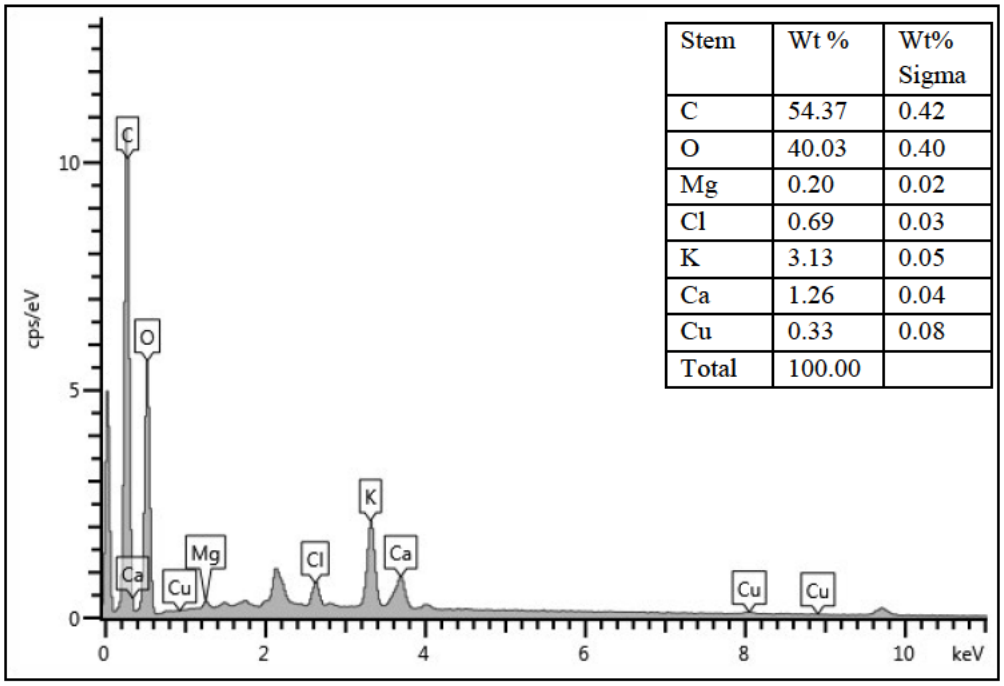
Andresen et al., 2018). Metals such as Mg, K and Cu act as antioxidants (Gupta and Sharma, 2006). Additionally, micronutrients have an essential role in the plant responses against abiotic (dangerous weather conditions) and biotic stress (pathogens) (Aznar et al., 2015; Andresen et al., 2018; Shafi and Zahoor, 2020). An important pathway in transporting of metals into humans is from the soil to the plant and from the plant to the human (Starlin et al., 2012[a]; Yan et al., 2020). This microanalysis is also used to detect of heavy metal pollutants (Scimeca et al., 2014; Scimeca et al., 2018; Quevedo et al., 2020).

Sodium is vital for the regulation of osmotic pressure of the body and helps to maintain acid-base and water stability of the body (Figures 5.7 and 5.8) (Lokhande et al., 2014). Magnesium and potassium are responsible for carbohydrate metabolism, enzymatic activity and ionic balance (Griffin, 1981). Chloride works with Na and K to transmit an electrical charge in bodily fluids and helps regulate pH (Starlin et al., 2012b). Calcium is used to enhance the quality of teeth, bones and cardiac functions (Obiajunwa et al., 2002; Lokhande et al., 2014, Bachheti et al., 2012). Copper plays an important role in iron (Fe) metabolism (Figure 5.7 and 5.8), with a deficit of this element resulting in spontaneous rupture of vessels and fragile bone cortices (Obiajunwa et al., 2002). The roles minerals play in living organisms include the preservation of certain physicochemical processes that are related to chromoproteins, metalloproteins, nucleoproteins and lipoproteins (Ekinci et al., 2004). To date, there is little or no information on the EDX analyses of plant material in species of *Barleria*.



Leaf	Wt %	Wt% Sigma
C	49.82	0.47
O	39.33	0.40
Na	0.14	0.03
Mg	0.44	0.03
Si	0.10	0.02
Cl	3.15	0.05
K	5.27	0.07
Ca	1.56	0.04
Cu	0.18	0.05
Total	100.00	

Figure 5.7: EDX spectroscopy of fresh leaf material from *B. albostellata*.



Stem	Wt %	Wt% Sigma
C	54.37	0.42
O	40.03	0.40
Mg	0.20	0.02
Cl	0.69	0.03
K	3.13	0.05
Ca	1.26	0.04
Cu	0.33	0.08
Total	100.00	

Figure 5.8: EDX spectroscopy of fresh stem material from *B. albostellata*.

5.3.3 Phytochemical screening for major classes of compounds in *B. albostellata* extracts using GC-MS

The GC-MS chromatogram represents 10 peaks (Figure 5.9), 10 peaks (Figure 5.10), and 108 peaks (Figure 5.11) of various compounds detected in the leaf hexane, chloroform and methanolic extract, respectively. Additionally, 10 peaks (Figure 5.12), 10 peaks (Figure 5.13) and 104 peaks (Figure 5.14) were identified in the stem hexane, chloroform and methanolic extract. This analysis is used for the qualitative and quantitative examination of active compounds in plants (Agarwal et al., 2017; Yogashree et al., 2021). Several overlapping peaks were observed in the middle stages of most chromatograms, Figure 5.9 (19-30 min), Figure 5.11 (14-33 min), Figure 5.12 (19-20 min), Figure 5.13 (19-25 min) and Figure 5.14 (19-33 min). Each peak in the chromatogram (Figure 5.9–5.14) represents a signal produced when a compound is washed out with a solvent from the gas-chromatography column into the detector (Hameed et al., 2016; Falaki, 2019).

Numerous small peaks were observed throughout the chromatogram. Major chemical compounds with high peaks and area percentages greater than one were selected and identified in leaf and stem extracts (hexane, chloroform and methanol) (Tables 5.6–5.11). In certain circumstances, compounds with an area percentage less than one, were only mentioned if they played an important role in the genus. The methanolic extracts for both leaf and stem revealed the highest number of compounds. A total of 17 compounds with an area percentage greater than one was found in the leaf and stem methanolic extracts (Tables 5.8 and 5.11).

For the leaf hexane chromatogram, the highest peak identified was bis(2-ethylhexyl) phthalate, which had the highest percentage area of 85.76 (Table 5.6). Furthermore, this compound had the greatest percentage area for the following extracts: leaf chloroform, 61.29 (Table 5.7); stem chloroform, 93.41 (Table 5.10) and stem methanol, 27.82 (Table 5.11), respectively. Kumari and Dubey et al. (2016) investigated the GC-MS profile of the leaf and stem extracts of *B. lupulina*. These authors found bis(2-ethylhexyl) phthalate in the acetone-soluble stem extract, with an area percentage of 95.80. Habib and Karim (2009) confirmed the antibacterial activity of the isolated compound, bis(2-ethylhexyl) phthalate, which inhibited both Gram-positive (*Bacillus subtilis*, *Sarcina lutea* and *Staphylococcus aureus*) and Gram-negative (*Escherchia coli*, *Shigella dysenteriae*, *Shigella shiga* and *Shigella sonnei*) bacteria (Table 5.12). The lowest peak identified in the leaf hexane (Table 5.6), stem hexane (Table 5.9), and chloroform (Table 5.10) chromatogram was pentadecanoic acid, with an area percentage of 1.02, 1.00 and 1.00, respectively. It should be noted that pentadecanoic acid has not been reported in any species of *Barleria*; however, this compound is a fatty acid, and is found in the milk fat of cows, regulates hormones, improves the immune system and boosts metabolism (Hansen et al., 1954; Smedman et al., 1999).

Octadecanoic acid, 2,3-dihydroxypropyl ester and tetratetracontane displayed the lowest peaks in the leaf chloroform chromatogram, with an area percentage of 1.00 (Table 5.7). These compounds have not been reported in any species of *Barleria*, though, octadecanoic acid, 2,3-dihydroxypropyl ester displays anticancer and antimicrobial activities (Arora and Kumar, 2018), while, tetratetracontane promotes plant growth, antioxidant and cytoprotective activities (Siddiquee et al., 2012; Mallick and Dighe, 2014). In the leaf methanolic chromatogram, diisooctyl phthalate, exhibited the highest peak, with an area percentage of 16.69 (Table 5.8). Baskaran et al. (2016), analysed the ethanolic leaf extracts of *B. longiflora* using GC-MS. These authors found diisooctyl phthalate at an area percentage of 4.39 in the leaf extracts and described the compound as exhibiting both antimicrobial and anti-fouling properties. The 13-docosenamide, (Z)- displayed the lowest peak, with an average percentage of 2.46, and has not been reported in any species of *Barleria*, but was reported to exhibit antimicrobial properties in *Ludwigia perennis* (Sharmila et al., 2017).

The highest peak for the stem hexane chromatogram was terephthalic acid Dodecyl 2-ethylhexyl ester had an average percentage of 86.67 (Table 5.9). This compound was reported to exhibit antioxidant and hypocholestero-lemic activities (López-Lázaro, 2009; Osuntokun et al., 2017) as well as, bacteriostatic and bactericidal properties (Osuntokun and Omotuyi, 2018). Additionally, the lowest peak on the stem methanol chromatogram was tributyl acetyl citrate (Table 5.9). Al-Rubaye et al. (2017) examined the methanolic leaf extracts of *Sinapis arvensis* for its medicinal properties. These authors found tributyl acetyl citrate to display antioxidant and anti- inflammatory activities.

The identified compounds illustrated in Table 5.12, possessed various biological properties of medicinal importance. Several compounds found in the extracts of *B. albostellata* were also noted in other species of *Barleria*. Phyto-compounds such as phenol, 2,4-bis(1,1-dimethylethyl), found in *B. albostellata* (Table 5.12), were identified in *B. prionitis* (Ghosh et al., 2016), *B. montana* (Sriram and Sasikumar, 2012) and *B. lupulina* (Kumari and Dubey, 2016). The 9,12,15-octadecatrienoic acid, (Z,Z,Z) (Table 5.12) was only prominent in *B. buxifolia* (Tamil et al., 2017). Kumari and Dubey (2016) reported on octadecanoic acid (Table 5.12), in the extracts of *B. lupulina*, while, Sriram and Sasikumar (2012) found this compound in *B. montana*. Squalene found in *B. albostellata* (Table 5.12) was also identified in *B. montana* (Natarajan et al., 2012), *B. longiflora* (Baskaran et al., 2016), *B. courtallica* (Sujatha et al., 2017), *B. lupulina* (Kumari, and Dubey, 2016) and *B. grandiflora* (Kumari et al., 2015).

Eicosane, a solid n-alkane (Table 5.12), was found in extracts of *B. courtallica* (Sujatha et al., 2017), *B. prionitis* (Pandey et al., 2018) and *B. dinteri* (Semenya et al., 2018). In the extracts of *B. courtallica* (Sujatha et al., 2017) and *B. lupulina* (Kumari, and Dubey, 2012), phytol and acetate (Table 5.10), was identified. Furthermore, phytol (Table 5.12) was reported in *B. montana* (Natarajan et al., 2012), *B. longiflora* (Baskaran et al., 2016), *B. courtallica* (Sujatha et al., 2017), *B. lupulina* (Kumari and Dubey, 2016), *B. strigosa* (Manapradit et al., 2015), *B. buxifolia* (Tamil et al., 2017) and *B. prionitis* (Pandey et al., 2018). Vitamin E (Table 5.12), a fat-soluble vitamin was only noted in *B. courtallica* (Sujatha et al., 2017). Flavones, a class of flavonoids, found in the extracts of *B. albostellata* (Table 5.12), were also reported in *B. prionitis* (Banerjee et al., 2020) and *B. acanthoides* (Karim et al., 2009).

Campesterol found in *B. longiflora* (Rao et al., 1999), stigmasterol, in *B. courtallica* (Sujatha et al., 2017), *B. montana* (Natarajan et al., 2012), *B. longiflora* (Baskaran et al., 2016), *B. cristata*, *B. prionitis* (El-Emary et al., 1990, Choudhary et al., 2014.), *B. lupulina* (Wanikiat et al., 2008) and beta-sitosterol identified in *B. prionitis* (Dheer et al., 2019), *B. courtallica* (Sujatha et al., 2017), *B. montana* (Natarajan et al., 2012) and *B. longiflora* (Baskaran et al., 2016); are three characteristic phytosterols found in *B. albostellata* (Table 5.12). Stigmasta-3,5-dien-7-one has only been reported in *B. albostellata* (Table 5.12), while, 13,14-seco-stigmasta-5,14-diene-3α-o was noted in *B. prionitis* (Kosmulalage et al., 2007). Additionally, alpha-amyrin was noted in *B. cristata* and *B. prionitis* (El-Emary et al., 19 90, Pandey et al., 2018). Sujatha et al. (2017), and Kumari and Dubey (2016), reported the presence of 9,12-octdecadienoic acid (Z, Z) (Table 5.12) in the extracts of *B. courtallica* and *B. lupulina*, respectively.

To date, 1-heptacosanol; l-(+)-ascorbic acid 2,6-dihexadecanoate; tridecanoic acid; decanedioic acid, dibutyl ester; 1,2,3,5-cyclohexanetetrol; 1,2-15,16-diepoxyhexadecane; 1,4-benzenedicarboxylic acid, bis(2-ethylhexyl) ester; simiarenol; dichloroacetic acid, tridec-2-ynyl ester; 4,4,6a,6b,8a,11,11,14b-octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydro-2H-picen-3-one; alpha. amyrenone; acetic acid, 3-hydroxy-6-isopropenyl-4,8a-dimethyl-1,2,3,4,5,6,7,8 and cholest-4-en-3-one, found in *B. albostellata*, were not reported in any species of *Barleria*. Although, GC-MS analysis

identified the phytochemical constituents present in the hexane, chloroform and methanolic extracts, it should be noted that the most compounds were found in the leaf (Table 5.8) and stem methanolic (Table 5.11) extracts.

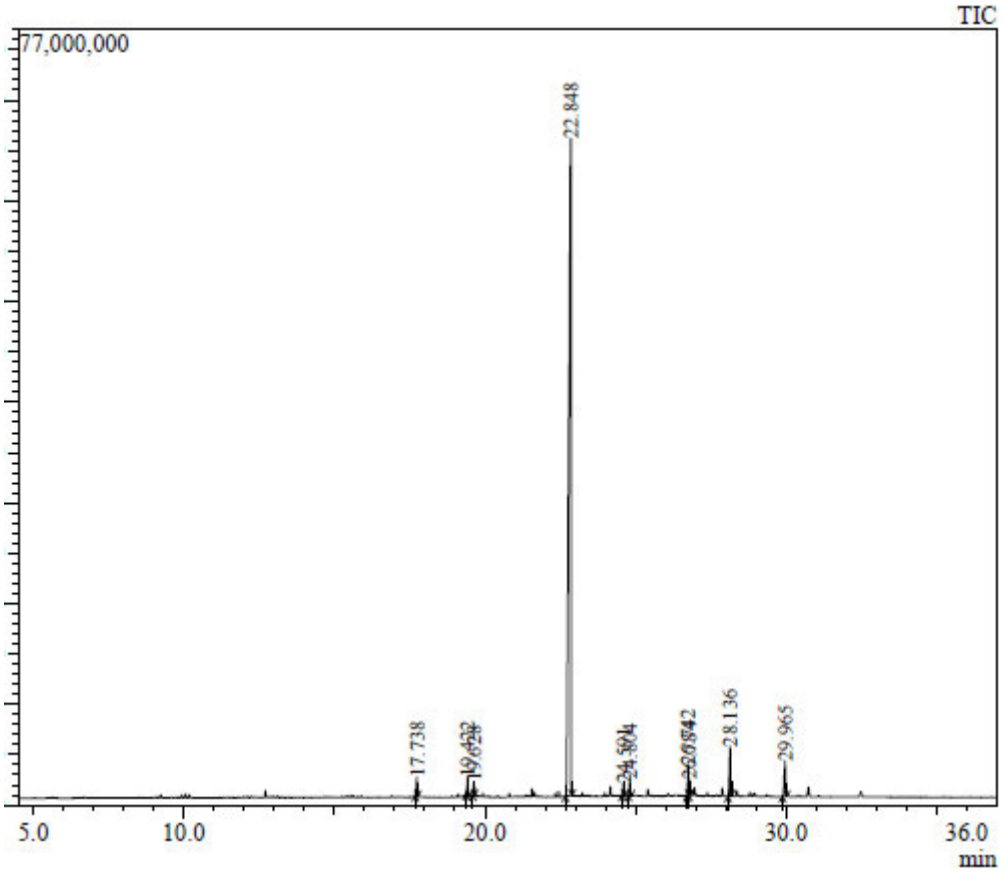


Figure 5.9: GC-MS chromatogram of leaf hexane extract of *B. albostellata*.

Table 5.6: Phytochemical compounds identified in leaf hexane extracts of *B. albostellata* by GC-MS analysis

Peak	Retention time	Phytochemical compound	Molecular formula	Molecular weight	CAS NO	Area %
1	17.738	Pentadecanoic acid	$C_{15}H_{30}O_2$	242	1002-84-2	1.02
2	19.422	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	$C_{18}H_{30}O_2$	278	463-40-1	1.25
3	19.628	Octadecanoic acid	$C_{18}H_{36}O_2$	284	57-11-4	1.09
4	22.848	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390	117-81-7	85.76
5	24.591	13-Docosenamide, (Z)	$C_{22}H_{43}NO$	337	112-84-5	1.12
6	24.804	Squalene	$C_{30}H_{50}$	410	111-02-4	1.06
7	26.742	Eicosane	$C_{20}H_{42}$	282	112-95-8	1.39
8	26.784	1-Heptacosanol	$C_{27}H_{56}O$	396	2004-39-9	1.21
9	28.136	Tetratetracontane	$C_{44}H_{90}$	618	7098-22-8	3.25
10	29.965	l-(+)-Ascorbic acid 2,6-dihexadecanoate	$C_{38}H_{68}O_8$	652	28474-90-0	2.85

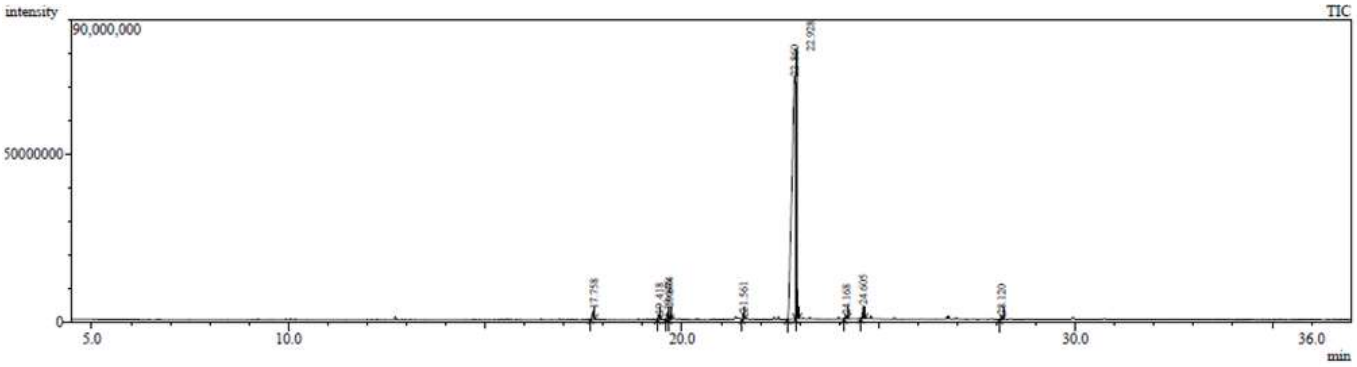


Figure 5.10: GC-MS chromatogram of leaf chloroform extract of *B. albostellata*

Table 5.7: Phytochemical compounds identified in leaf chloroform extracts of *B. albostellata* by GC-MS analysis

Peak	Retention time	Phytochemical compound	Molecular formula	Molecular weight	CAS NO	Area %
1	17.738	Pentadecanoic acid	$C_{15}H_{30}O_2$	242	1002-84-2	1.02
2	19.418	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	$C_{18}H_{30}O_2$	278	463-40-1	1.04
3.	19.646	Tridecanoic acid	$C_{13}H_{26}O_2$	214	638-53-9	1.01
4	19.674	Decanedioic acid, dibutyl ester	$C_{18}H_{34}O$	314	109-43-3	1.02
5	22.860	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390	117-81-7	61.29
6	22.928	Terephthalic acid, dodecyl 2-ethylhexyl ester	$C_{28}H_{46}O_4$	446	0-00-0	30.59
7	24.168	Octadecanoic acid, 2,3-dihydroxypropyl ester	$C_{21}H_{42}O$	443	123-94-4	1.00
8	24.605	13-Docosenamide, (Z)-	$C_{22}H_{43}NO$	338	112-84-5	1.03
9	28.120	Tetratetracontane	$C_{44}H_{90}$	619	7098-22-8	1.00

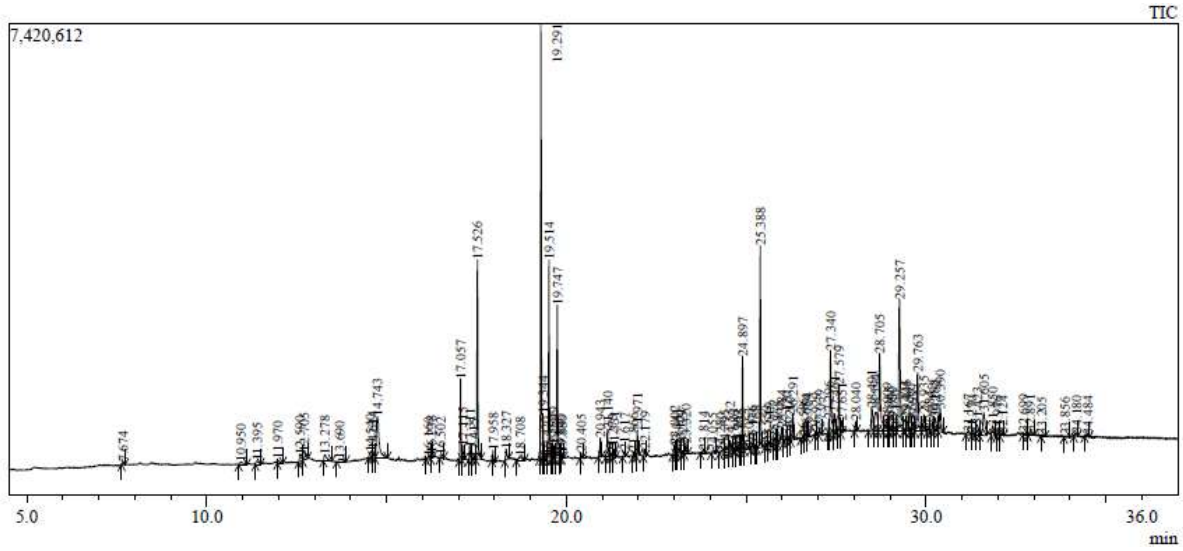


Figure 5.11: GC-MS chromatogram of leaf methanol extract of *B. albostellata*

Table 5.8: Phytochemical compounds identified in leaf methanol extracts of *B. albostellata* by GC-MS analysis

Peak	Retention time	Phytochemical compound	Molecular formula	Molecular weight (g/mol)	CAS NO	Area %
1	14.743	1,2,3,5-Cyclohexanetetrol	$C_6H_{12}O_4$	619	53585-08-3	3.63
2	17.057	Phytol, acetate	$C_{22}H_{42}O$	339	0-00-0	7.29
3	19.514	*n*-Nonadecanol-1	$C_{19}H_{40}O$	285	1454-84-8	5.35
4	19.747	Phytol	$C_{20}H_{40}O$	297	150-86-7	4.66
5	21.140	1,2-15,16-Diepoxyhexadecane	$C_{16}H_{30}O_2$	254	0-00-0	3.10
6	22.778	Diisooctyl phthalate	$C_{24}H_{38}O_4$	390	131-20-4	16.69
7	24.591	13-Docosenamide, (Z)-	$C_{22}H_{43}NO$	337	112-84-5	2.46
8	24.897	1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	$C_{24}H_{38}O_4$	391	6422-86-2	6.46
9	25.388	Squalene	$C_{30}H_50$	410	111-02-4	5.39
10	27.340	1-Heptacosanol	$C_{27}H_{56}O$	397	2004-39-9	4.27
11	27.579	Vitamin E	$C_{29}H_{50}O_2$	431	59-02-9	3.67
12	27.723	Flavone, 4',5-dihydroxy-6,7-dimethoxy-	$C_{17}H_{14}O_6$	314	6601-62-3	11.69
13	28.491	Campesterol	$C_{28}H_{48}O$	401	474-62-4	5.16
14	28.705	Stigmasterol	$C_{29}H_{48}O$	413	83-48-7	4.01
15	29.257	Beta-Sitosterol	$C_{29}H_{50}O$	415	83-46-5	6.70
16	29.763	Alpha-Amyrin	$C_{30}H_{50}O$	427	638-95-9	3.22
17	30.390	Simiarenol	$C_{30}H_{50}O$	427	1615-94-7	4.25

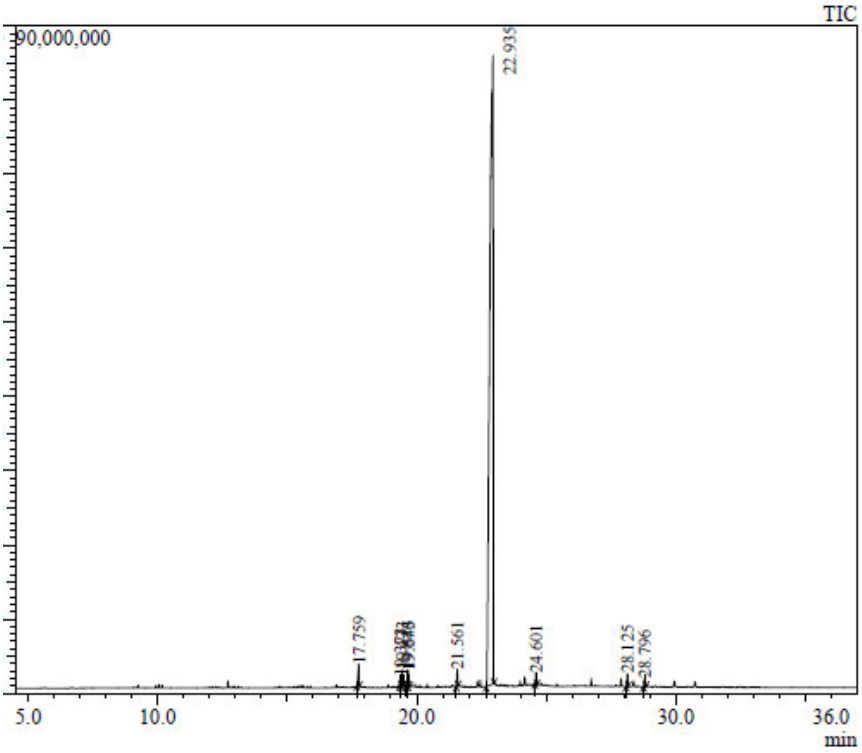


Figure 5.12: GC-MS chromatogram of stem hexane extract of *B. albostellata*

Table 5.9: Phytochemical compounds identified in stem hexane extracts of *B. albostellata* by GC-MS analysis

Peak	Retention time	Phytochemical compound	Molecular formula	Molecular weight	CAS NO	Area %
1	17.759	Pentadecanoic acid	$C_{15}H_{30}O_2$	242	CAS:1002-84-2	1.00
2	19.373	9,12-Octdecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	280	CAS:60-33-3	2.33
3	19.433	Dichloroacetic acid, tridec-2-ynyl ester	$C_{15}H_{24}C_{l2}O_2$	306	CAS:0-00-0	1.52
4	19.646	Octadecanoic acid	$C_{18}H_{36}O_2$	284	CAS:57-11-4	1.52
5	19.673	Decanedioic acid, dibutyl ester	$C_{18}H_{34}O_4$	314	CAS:109-43-3	1.39
6	22.935	Terephthalic acid, dodecyl 2-ethylhexyl ester	$C_{28}H_{46}O_4$	446	CAS:0-00-0	86.67
7	24.601	13-Docosenamide, (Z)-	$C_{22}H_{43}NO$	337	CAS:112-84-5	1.32
8	28.125	Tetratetracontane	$C_{44}H_{90}$	618	CAS:7098-22-8	1.45
9	28.796	4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydro-2H-picen-3-one	$C_{30}H_{48}O$	424	CAS:0-00-0	2.35

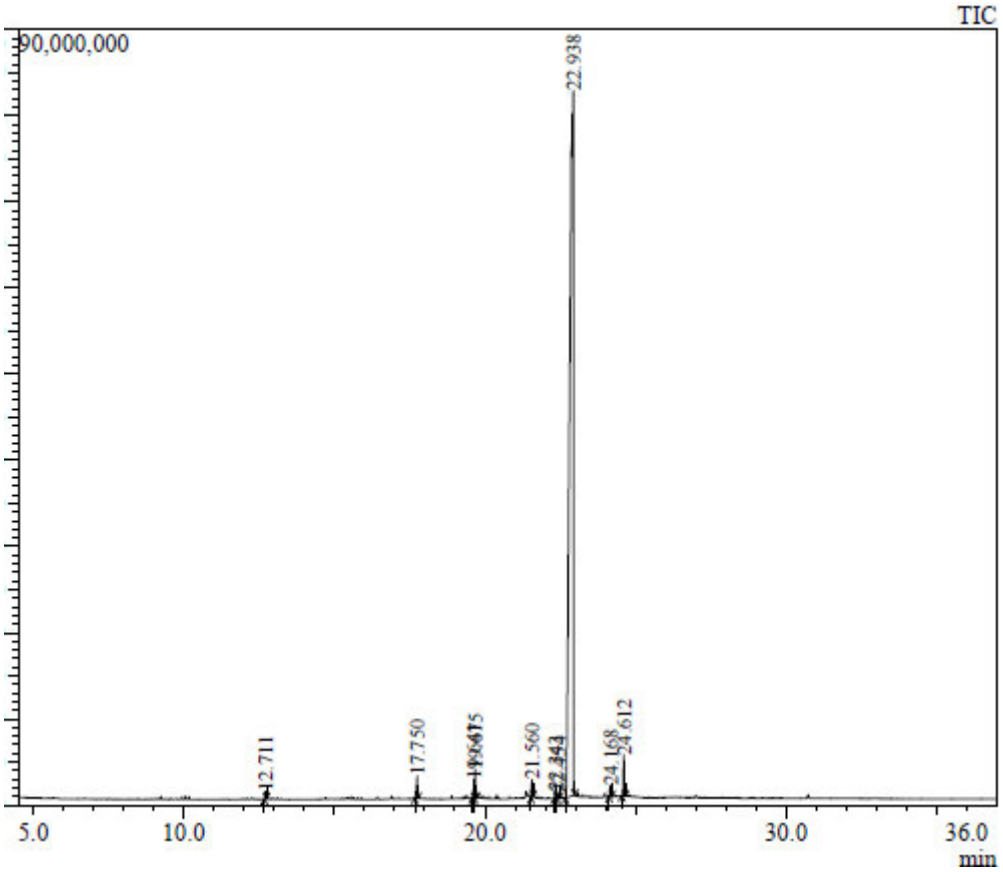


Figure 5.13: GC-MS chromatogram of stem chloroform extract of *B. albostellata*

Table 5.10: Phytochemical compounds identified in stem chloroform extracts of *B. albostellata* by GC-MS analysis

Peak	Retention time	Phytochemical compound	Molecular formula	Molecular weight	CAS NO	Area %
1	12.711	Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	206	CAS:96-76-4	1.20
2	17.750	Pentadecanoic acid	$C_{15}H_{30}O_2$	242	CAS:1002-84-2	1.00
3	19.641	Octadecanoic acid	$C_{18}H_{36}O_2$	284	CAS:57-11-4	1.06
4	19.675	Decanedioic acid, dibutyl ester	$C_{18}H_{34}O_4$	314	CAS:109-43-3	1.07
5	22.343	Diisooctyl phthalate	$C_{24}H_{38}O_4$	390	CAS:131-20-4	1.10
6	22.938	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390	CAS:117-81-7	91.73
7	24.168	Octadecanoic acid, 2,3-dihydroxypropyl ester	$C_{21}H_{42}O_4$	358	CAS:123-94-4	1.03
8	24.612	13-Docosenamide, (Z)-	$C_{22}H_{43}NO$	337	CAS:112-84-5	1.21

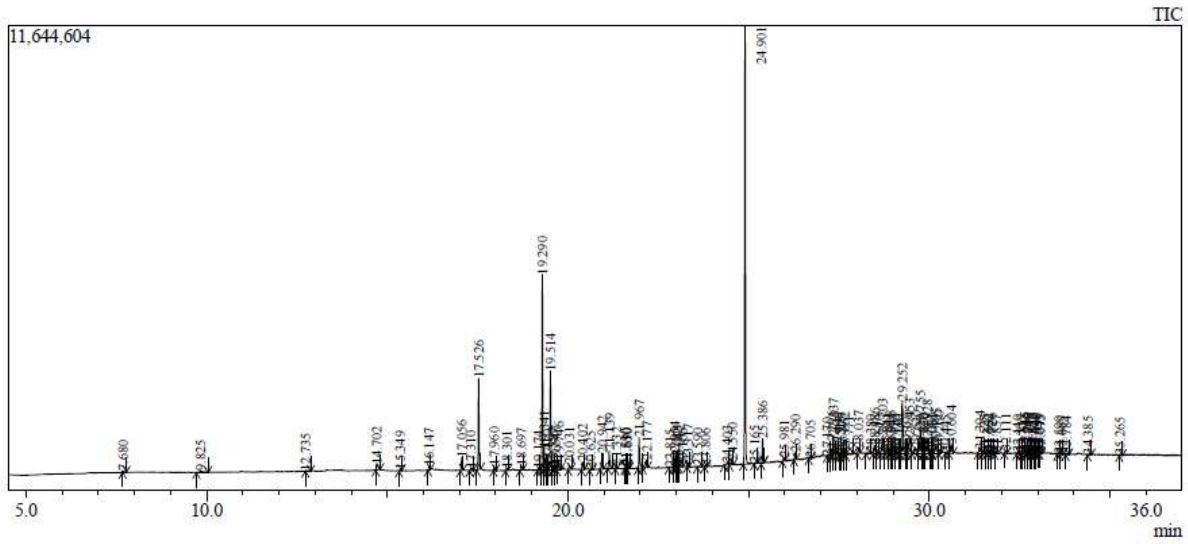


Figure 5.14: GC-MS chromatogram of stem methanol extract of *B. albostellata*

Table 5.11: Phytochemical compounds identified in stem methanol extracts of *B. albostellata* by GC-MS analysis

Peak	Retention time	Phytochemical compound	Molecular formula	Molecular weight	CAS NO	Area %
1	19.514	n-Nonadecanol-1	$C_{19}H_{40}O$	285	1454-84-8	5.84
2	20.942	Tributyl acetylcitrate	$C_{20}H_{34}O_8$	402	77-90-7	1.05
3	21.139	1,2-15,16-Diepoxyhexadecane	$C_{16}H_{30}O_2$	254	0-00-0	1.23
4	21.967	9-Octadecenamide	$C_{18}H_{35}NO$	281	301-02-0	2.19
5	22.340	Diisooctyl phthalate	$C_{24}H_{38}O_4$	390	131-20-4	1.20
6	22.848	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390	117-81-7	27.82
7	24.588	13-Docosenamide, (Z)-	$C_{22}H_{43}NO$	337	112-84-5	1.14
8	24.901	1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	$C_{24}H_{38}O_4$	391	6422-86-2	25.68
9	25.386	Squalene	$C_{30}H_{50}$	410	111-02-4	1.39
10	27.337	1-Heptacosanol	$C_{27}H_{56}O$	397	2004-39-9	1.72
11	28.703	Stigmasterol	$C_{29}H_{48}O$	413	83-48-7	1.89
12	29.252	Beta-Sitosterol	$C_{29}H_{50}O$	415	83-46-5	5.68
13	29.453	Alpha. Amyrenone	$C_{30}H_{48}O$	425	0-00-0	2.18
14	29.755	Alpha-Amyrin	$C_{30}H_{50}O$	427	638-95-9	3.16
15	29.928	Acetic acid, 3-hydroxy-6-isopropenyl-4,8a-dimethyl-1,2,3,4,5,6,7,8	$C_{17}H_{26}O_3$	278	0-00-0	2.01
16	30.195	Stigmasta-3,5-dien-7-one	$C_{29}H_{46}O$	411	2034-72-2	1.51
17	30.604	Cholest-4-en-3-one	$C_{27}H_{44}O$	385	601-57-0	1.13

Table 5.12: Pharmacological activities of compounds found in *B. albostellata*

No	Phytochemical compound	Pharmacological action	References
1	Pentadecanoic acid	Flavouring agent, lubricants, adhesive agents, ability to regulate hormones, improve the immune system, boost metabolism and inhibits production of uric acid	Hansen et al., 1954; Smedman et al., 1999; Arora et al., 2017; Bekinbo et al., 2020; Janaki et al., 2021
2	9,12,15-Octadecatrienoic acid, (Z,Z,Z)	Antioxidant, anti-inflammatory, antimicrobial, diuretic, anticancer, antitumor, chemopreventive properties used in vaccine formulations and reduced complications in Covid-19 patients	Prabhadevi et al., 2012; Sen and Batra, 2012; Rampilla and Khasim, 2020; Weill et al., 2020
3	Octadecanoic acid	Antimicrobial activity	Kumaradevan et al., 2015; Ganesh and Mohankumar, 2017; Pavani and Naika, 2021.
4	Bis(2-ethylhexyl) phthalate	Antibacterial activity	Habib and Karim, 2009; Mohamad et al., 2020
5	13-Docosenamide, (Z)-	Antimicrobial activity	Sharmila et al., 2017; Olusola et al., 2020
6	Squalene	Cosmetics, skin ointments, antioxidant, antitumor, anticancer, chemo-preventive and sun-screen properties	Ezhilan and Neelamegam, 2012; Rao and Anisha, 2018; Nainggolan and Sinaga, 2021
7	Eicosane	Antitumour, antifungal activity and bronchodilators	Sivasubramania and Brindha, 2013; Ahsan et al., 2017; Subramanian et al., 2020
8	1-Heptacosanol	Nematicidal, anticancer, antioxidant and antimicrobial properties	Raman et al., 2012; Chowdhary and Kaushik, 2018; Pei-Xia et al., 2020
9	Tetratetracontane	Plant growth production, antioxidant, cytoprotective, and anti-inflammatory activities	Siddiquee et al., 2012; Mallick and Dighe, 2014; Agarwal et al., 2017; Rajisha and Fernandes, 2020

10	l-(+)-Ascorbic acid 2,6-dihexadecanoate	Antioxidant food addictive, antimetastatic, anti-invasive, cancer, cardio protective and anti-infertility	Al-Marzoqi et al., 2015; Kadam and Lele, 2017; Khan et al., 2020
11	Tridecanoic acid	Antifungal, antibacterial and larvicidal	McGraw et al., 2002; Sivakumar et al., 2011; Kushwaha et al., 2019
12	Decanedioic acid, dibutyl ester	Antimicrobial, antispasmodic and anti-inflammatory effects	Sahi, 2016
13	Terephthalic acid, dodecyl 2-ethylhexyl ester	Antioxidant, hypocholestero-lemicactivity, bacteriostatic and bactericidal properties	López-Lázaro, 2009; Osuntokun et al., 2017; Osuntokun and Omotuyi, 2018; Anbukumaran et al., 2021
14	Octadecanoic acid, 2,3-dihydroxypropyl ester	Anticancer, antimicrobial, acidifier, acidulant, arachidonic acid inhibitor and inhibits production of uric acid	Arora and Kumar, 2018; Janaki et al., 2021
15	1,2,3,5-Cyclohexanetetrol	Antioxidant, antimicrobial and anti-inflammatory properties	Sarumathy et al., 2011
16	Phytol, acetate	Anti-inflammatory, antileishmanial, anti-trypanosomal, antimicrobial, anticancer and diuretic	Kalaisezhiyen and Sasikumar, 2012; Al-Marzoqi et al., 2016; Nisha et al., 2018; Vinoth et al., 2021
.17	n-Nonadecanol-1	Antimicrobial and cytotoxic properties	Hsouna et al., 2011; Kuppuswamy et al., 2013
18	Phytol	Anticancer, antimicrobial, anti-inflammatory, antioxidant activity, diuretic, cosmetics and used in the fragrance industry	Grover and Patni, 2013; Janaki et al., 2021
19	1,2-15,16-Diepoxyhexadecane	Antitumor and anti-inflammatory properties	Shareef et al., 2016

20	Diisooctyl phthalate	Antimicrobial and antifouling activity	Tyagi and Agarwal, 2017
21	1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	Anticancer properties	Save et al., 2015; Vijay et al., 2020
22	Vitamin E	Skin repair, enhancing the immune system and contains anticancer, antitumor and antioxidant properties	El Sohaimy et al., 2015; Rao and Anisha, 2018; Abraham et al., 2019
23	Flavone	Antibacterial, antimutagenic, antiviral and antioxidant activity	Buening et al., 1978; Buening et al., 1981; Sivaraj et al., 2020
24	Campesterol	Anti-inflammatory and anticancer activity	Santhanamari et al., 2016; Beschi et al., 2021
25	Stigmasterol	Anti-inflammatory, anti-asthma, anti-cancerous, anti-inflammatory, antiarthritic, hypoglycemic, antioxidant and thyroid inhibiting properties. Analgesic, antiosteoarthritic and antimutagenic activity	Jegajeevanram et al., 2014; Panda et al., 2009; Janaki et al., 2021
26	Beta-Sitosterol	Reduces cholesterol levels; androgen blocker, anti-amyloid beta and anticancer properties	Bharathy et al., 2012; Janaki et al., 2021
27	Alpha-Amyrin	Alpha amylase and glucosidase inhibitor, antioxidant, antibacterial and anti-inflammatory properties	Okoye et al., 2014; Rao and Anisha, 2018
28	Simiarenol	Antinociceptive activity	Kuroshima et al., 2005
29	9,12-Octdecadienoic acid (Z,Z)-	Antiinflammatory, antibacterial, antiarthritic, hepatoprotective, anti-histaminic, anticoronary and anti-cancer properties	Arora and Kumar, 2018; Chinnadurai et al., 2019; Thirumalai et al., 2021
30	Dichloroacetic acid, tridec-2-ynyl ester	Cosmetic treatments, anticancer, antimicrobial, antioxidant activity	Roy et al., 2019; Francis et al., 2021
31	4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydro-2H-picen-3-one	Anti-bacteria, antioxidant, antitumor and cancer preventives	Duan et al., 2011; Durairaj et al., 2014

32	Phenol, 2,4-bis(1,1-dimethylethyl)-	Antibacterial and anti-inflammatory activities	Amaral et al., 2014
33	Tributyl acetylcitrate	Anticancer and antimicrobial activities	Hussein et al., 2016; Hugar et al., 2017
34	9-Octadecenamide	Antimicrobial activity	Khan et al., 2019
35	Alpha. Amyrenone	Antibacterial and antimalarial activities	Manjari et al., 2014; Vashisht et al., 2017
36	Acetic acid, 3-hydroxy-6-isopropenyl-4,8a-dimethyl-1,2,3,4,5,6,7,8	Antimicrobial activity	Shanmugavel et al., 2015; Hase et al., 2017
37	Stigmasta-3,5-dien-7-one	Anti-diabetic and anticancer properties. Free radical scavenging activity	Delazar et al., 2010; Balogun et al., 2013; Chouni et al., 2021
38	Cholest-4-en-3-one	Anti-obesity and an intestinal metabolite of cholesterol	Suzuki, 1993; Kanjikar et al., 2017

5.3.4 Antibacterial activity of leaf and stem extracts of *B. albostellata*

The current interest in herbal plants as therapeutic agents has increased in several parts of the world. This is due to the ever-increasing occurrence of drug-resistant bacteria and the influx of new pathogenic bacterial strains. Active phytochemicals found in hexane, chloroform and methanolic extracts of *B. albostellata* were subjected to antibacterial assays. Various concentrations (3.125, 6.25, 12.25, 25, 50, and 100 mg/mL) were tested against the Gram-positive, *B. subtillus*, methicillin-resistant *S. aureus*, *S. aureus* and Gram-negative, *E. coli* and *P. aeruginosa*. The zone of inhibition of the growth of bacteria was used to evaluate the antibacterial potential of the various extracts. Results presented in Table 5.13 of certain leaf and stem extracts showed significant inhibition compared to streptomycin and gentamicin (positive controls) (Table 5.13). Clear zones of inhibition were observed in the leaf and stem crude extracts against the various strains. Significance was established at $P < 0.05$ for all concentrations and treatments.

As the concentration increased, the zone of inhibition against various bacterial strains also increased. The highest inhibitory activity was observed at 100 mg/mL for both leaf and stem extracts for *B. subtillus* and *S. aureus*. The leaf hexane extracts were resistant to MRSA, *E. coli* and *P. aeruginosa*, whilst the stem hexane extracts displayed no inhibition against *E. coli* and *P. aeruginosa* only (Table 5.13). Concentrations at both 3.125 and 6.25 mg/mL for all extracts were resistant to both Gram-positive and –negative bacteria. Amoo et al. (2009) verified the antibacterial activity of *B. albostellata* against *B. subtillis, S. aureus* and *E. coli*. However, low activity was observed against Gram-negative bacteria (Amoo et al., 2009). Matu and Van Staden (2003) suggested that a thick murein layer present in the structure of Gram-negative bacteria may prevent the entry of inhibitors. The differences in the bacterial inhibition varied for each crude extract. The leaf methanolic extracts at 100 mg/mL displayed the highest inhibition against all tested bacterial strains. However, the stem methanolic extracts had the highest inhibition against *S. aureus* and *P. aeruginosa* only.

Several notable bioactive compounds found in the leaf and stem extracts of *B. albostellata* using GC-MS analysis were reported to display antibacterial efficiency. The presence of phytol and flavone found in the leaf methanolic extracts of *B. albostellata* could be responsible for the antibacterial effects against the several tested strains. Phytol was reported to severely damage the deoxyribonucleic acid (DNA) of bacteria by inducing oxidative stress (Lee et al., 2016). The presence of flavonoids, block important enzymes that play a significant role in the reproduction, growth, cell rupture, or functional modification in bacteria (Awolola et al., 2014). Stigmasterol, another compound found in the leaf methanolic extract was reported to act as a lactamase inhibitor, which prevented antibacterial resistance by restoring the vulnerability of the antibiotic resistant bacteria to antibiotics (Yenna et al., 2017). Phthalates found in all extracts except the stem hexane, were reported to contain a permeability barrier, which is essential to several cellular functions, including sustaining the energy status of the cell, solute transport,

membrane-coupled energy transducing system and metabolic regulation (Sikkema et al., 1995; Bajpai et al., 2015). The mode of action used by most bioactive compounds in treating microbial infections is by interacting with the microbial enzyme system, interfering with nucleic acids, cell wall and cell membrane (Neu, 1992; Mukhopadhyay and Peterson, 2006; Tenover, 2006).

Additionally, the antibacterial efficiency in the various extracts may be due to greater solubility of phyto-compounds in polar than non-polar solvents (Kumari and Dubey, 2016). It was recommended that the inability of plant extracts of other solvent systems to display antibacterial activity against the various bacterial strains could be due to these strains exhibiting some sort of resistance mechanism, e.g., alteration of target sites, enzymes inactivation, reduced drug accumulation, or the amount of bioactive compounds present is very low (Schwarz and Noble, 1999). Extracts of *B. acuminata* (Bency et al., 2018), *B. cristata* (Kumar et al., 2018), *B. greenii* (Amoo et al., 2009), *B. prionitis* (Chavan et al., 2010) and *B. montana* (Natarajan et al., 2012) exhibited antibacterial activity against *B. subtillis* and *S. aureus*. However, *B. cristata* displayed low inhibition against *E.coli* (Kumar et al., 2018) and *B. montana* (Natarajan et al., 2012) moderate activity against *E.coli* and *P. aeruginosa*. According to Kumari and Dubey (2016), ethanolic leaf extracts of *B. lupulina* inhibited the growth of *E. coli*, *S. aureus* and *P. aeruginosa*, whereas methanolic extracts displayed zones of inhibition against *S. aureus*, and no inhibition against *E. coli* and *P. aeruginosa* (Moin et al., 2012). Various medicinal plant extracts were reported to display greater activity against Gram-positive bacteria as opposed to Gram-negative bacteria (Amoo et al., 2009; Tekwu et al., 2012; Rubio-Moraga et al., 2013). Therefore, extracts of this plant were effective against both Gram-positive and –negative bacteria.

Table 5.13: Antibacterial activity of crude extracts from leaves and stem of *B. albostellata* against human pathogenic strains (zone of inhibition mm)

Strain	Concentration (mg/mL)	Treatments/extracts						Positive control (mg/mL)	
		Leaf hexane	Leaf chloroform	Leaf methanol	Stem hexane	Stem chloroform	Stem methanol	L	S
BS	**3.125**	R	R	R	R	R	R		
	6.25	R	R	R	R	R	R		
	12.5	R	R	R	R	R	R		
	25	R	8.33±1.53	8.00±1.00	R	7.33±0.58	8.00±0.00	9.00±1.00	11.00±1.00
	50	7.67±2.08	7.67±0.58	9.33±0.58	7.67±2.08	8.00±1.00	8.67±0.58		
	100	9.00±3.46	7.00±0.00	10.00±2.00	8.67±1.52	10.00±3.61	9.67±0.58		
MRSA	**3.125**	R	R	R	R	R	R		
	6.25	R	R	R	R	R	R		
	12.5	R	R	R	R	R	R		
	25	R	8.67±0.58	R	R	R	R	9.33±0.58	9.00±1.00
	50	R	9.00±0.00	8.67±2.08	R	7.33±0.58	8.00±1.00		
	100	R	10.00±0.00	11.00±2.65	10.67±2.31	8.00±1.00	9.00±2.00		
SA	**3.125**	R	R	R	R	R	R		
	6.25	R	R	R	R	R	R		
	12.5	R	R	7.33±0.58	R	R	7.00±0.00		
	25	R	7.67±0.58	8.00±0.00	8.00±1.00	7.33±0.58	8.00±1.00	9.67±0.58	10.00±1.00
	50	7.33±0.58	8.67±0.58	8.67±1.53	9.00±0.00	8.33±0.58	10.00±1.73		
	100	9.33±0.58	9.33±0.58	10.33±1.53	10.33±1.53	9.00±1.73	11.00±2.65		
EC	**3.125**	R	R	R	R	R	R		
	6.25	R	R	R	R	R	R		
	12.5	R	R	R	R	R	R		
	25	R	9.67±0.58	R	R	9.33±0.58	R	8.67±0.58	9.33±0.58
	50	R	10.67±1.15	9.00±3.46	R	10.00±1.00	9.67±2.08		
	100	R	12.33±2.08	12.67±0.58	R	11.33±1.15	11.33±1.15		
PA	**3.125**	R	R	R	R	R	R		
	6.25	R	R	R	R	R	R		
	12.5	R	R	R	R	R	R		
	25	R	8.67±1.53	7.33±0.58	R	7.00±0.00	9.33±1.15	9.33±0.58	8.67±1.15
	50	R	9.00±0.00	8.67±1.52	R	8.67±0.58	10.67±2.87		
	100	R	10.00±3.00	14.33±1.53	R	9.67±1.15	12.33±0.58		

BS= *B. subtillus,* MRSA= methicillin-resistant *S. aureus,* SA= *S. aureus*, EC= *E. coli*, PA= *P. aeruginosa*, R= resistant, Positive controls (Streptomycin 10 mg/mL, Gentamicin 10 mg/mL,), Negative control= DMSO, (n = 3).

Qualitative phytochemical screening, GC-MS and TLC revealed various biologically active compounds which have been known to contain diverse activities that may help protect against chronic diseases (Liu, 2003; Singh and Sharma, 2020). Several compounds found in the extracts of *B. albostellata* were also noted in other species of *Barleria* (Baskaran et al., 2016; Ghosh et al., 2016; Kumari and Dubey, 2016; Natarajan et al., 2012; Sujatha et al., 2017; Sriram and Sasikumar, 2012). Additionally, the phyto-constituents found in the leaf and stem crude extracts of could inhibit the growth of various pathogenic strains. Various medicinal plant extracts were reported to display greater activity against Gram-positive bacteria as opposed to Gram-negative bacteria (Amoo et al., 2009; Tekwu et al., 2012; Rubio-Moraga et al., 2013). The antibacterial efficiency in the various extracts may be due to greater solubility of phyto-compounds in polar than non-polar solvents (Kumari and Dubey, 2016).

5.4 Conclusions and future perspectives

It is evident from the present study that the qualitative colour tests, TLC, fluorescence and GC-MS analysis that the leaves and stems of *B. albostellata* possess biologically active compounds. Additionally, the phyto-constituents found in the hexane, chloroform and methanol leaf and stem extracts of *B. albostellata* could inhibit the growth of various pathogenic strains. Results from this study revealed the medicinal potential of *B. albostellata* in the treatment of various bacterial diseases. Other solvents such as ethanol and acetone can be used in extracting phytochemical compounds from the leaves and stems. These extractions can be subjected to antibacterial assays in order to evaluate its potency against various pathogenic strains. Further studies should be conducted on the isolation, identification and characterisation of the bioactive compounds in *B. albostellata* that may be responsible for its bioactivity. The is important to further understand the mechanisms involved in the antibacterial activity. Additionally, other parts of the plant such as the flowers and roots should assessed for their safety and bioactivity and to identify any new therapeutic compounds or drug leads.

5.5 References

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CHAPTER 6

EVALUATION OF THE ANTIOXIDANT AND CYTOTOXIC ACTIVITIES OF THE LEAF AND STEM CRUDE EXTRACTS OF *Barleria albostellata*

Abstract

Barleria albostellata C.B. Clarke (Acanthaceae), is a plant indigenous to South Africa and is relatively understudied. Species within this genus are recognized for their ethnopharmacological and phyto-medicinal values. This study evaluated the total flavonoid, phenolic contents, antioxidant and cytotoxic activities of the crude hexane, chloroform and methanol extracts at five different concentrations (15, 30, 60, 120 and 240 µg/mL). *In vitro* antioxidant activity of the crude extracts of *B. albostellata* was done using the 2, 2′-diphenyl-1-picrylhydrazyl (DPPH) scavenging and ferric reducing antioxidant power (FRAP) assays, while the phenolic content was measured using the Folin-Ciocalteu's assay. The cytotoxicity of the crude extracts was established using the 3-[(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) assay in the human embryonic kidney (HEK293), cervical cancer (HeLa), and breast adenocarcinoma (MCF-7) cell lines. The highest total flavonoid content was found in the methanolic leaves (42.39 ± 1.14 mg GAE/g DW), compared to other solvents. Additionally, total phenolic content was greatest in the methanol leaf extract (6.05 ± 0.09 GAE/g DW), followed by the methanol stem extracts (2.93 ± 0.73 GAE/g DW). The methanolic leaf and stem extracts needed for 50% inhibition (DPPH) was 16.95 µg/mL and 14.27 µg/mL, respectively, whereas for FRAP, the reducing power of all extracts were considerably lower than the ascorbic acid standard. The IC_{50} values of crude extracts tested in the three cell lines were > 63 µg/mL. Results from this study are indicative that the leaves and stems of *B. albostella* are rich in several bioactive compounds which may be a possible source of natural antioxidants and may have the potential to treat certain diseases. Using bioassay-guided fractionation, the isolation of the bioactive compounds from the leaves and stems of *B. albostellata* and the assessment of its safety, will be essential for further investigation into this species in the search of potential novel therapeutic drug leads. To the best of our knowledge, this is the first report on the cytotoxic activities of the leaf and stem crude extracts of *Barleria albostellata*.

Keywords: Antioxidant; Bioactive compounds; Cancer; Cytotoxicity; Radical scavenging activity

6.1 Introduction

Reactive oxygen species (ROS) are free radicals (FRs) that are produced continuously by an organism's regular use of oxygen (Tiwari, 2004), either by exogenous stimuli (Juan et al., 2021) or endogenously (Ziech et al., 2010). There has been considerable evidence indicating that an imbalance between the formations of these FRs can trigger a pathological condition called oxidative stress (Folorunsho et al., 2018; Jamshidi-Kia et al., 2020). Reactive oxygen species can destroy DNA, lipids and proteins (Ziech et al., 2010; Yan and Zaher, 2019), and are linked to more than 100 diseases including inflammation (Mangge et al., 2014), neurodegenerative disorders (Gandhi and Abramov et al., 2012; Madireddy and Madireddy, 2020), and carcinogenesis (Li et al., 2015; Majumder et al., 2020). Molecules known as antioxidants are employed by the human body to counteract these FRs (superoxide, hydroxyl, peroxyl and nitric oxide radicals), thereby repairing radical damage by initiating cell regeneration (Jaouad and Torsten, 2010; Bhattacharya, 2015; Majumder et al., 2020). The human body has an intrinsic antioxidant mechanism, and various biological functions such as anti-aging, antimutagenic, and anti-carcinogenic responses originate from this property (Göçer and Gülçin, 2011; Gülcin, 2012).

Scientists have taken an interest in appreciating the use of antioxidants in the maintenance of human health and in the prevention and treatment of diseases (Halliwell and Gutteridge, 1981; Majumder et al., 2020). Society is moving away from using synthetic antioxidants due to their adverse reactions, including carcinogenicity. Their long-term toxicological effects on humans has increased over the years, thus creating a demand for natural antioxidants, specifically from plants, for use in the food, cosmetic and pharmaceutical sectors (García-Alonso et al., 2004; Ferreira et al., 2006; Sylvie et al., 2014; Kapadiya, 2016). Naturally occurring antioxidants found either in raw plant extracts or in their chemical constituents are effective in preventing the destructive processes caused by oxidative stress (Zengin et al., 2011; Hassan et al., 2017). Studies on plants have indicated the presence of various antioxidants such as flavonoids, phenolics, proanthocyanidins and tannins (Saeed et al., 2012; Adebiyi et al., 2017). These secondary metabolites alleviate oxidative stress by scavenging FRs (Madikizela and McGaw, 2019). Even though the toxicity profile of most medicinal plants has not been comprehensively evaluated, it is commonly accepted that medicines produced from plants are safer and cheaper than their synthetic counterparts (Vongtau et al., 2005; Oluyemi et al., 2007). A novel approach in finding effective anticancer drugs may be seen in the development of agents with antioxidant action that can overcome the resistance and suppress the adverse effects caused by oxidative stress in cancer cells (Verpoorte, 2000; Cragg and Newman, 2005; Tauchen et al., 2019). Therefore, plant derived drug candidates with potent antioxidant activities may be ideal anticancer agents.

As stated by the World Health Organisation (WHO), the key cause for morbidity and mortality is cancer, with approximately 8 million cancer-related deaths and 14 million new cases in 2012 (Forman and Ferlay, 2014) and 9.6 million deaths in 2018 (WHO, 2018). However, this number is expected to further

increase to 75 million prevalent cases, 27 million incident cases and 17 million cancer-associated deaths by 2030 (Adeloye et al., 2016). In South Africa, over one hundred thousand cancer cases are reported each year. The most predominant cancers found amid South African men are lung, prostate, esophageal and colorectal cancer, and Kaposi sarcoma, while in women the most predominant cancers are cervical, breast, colorectal cancer, melanoma, and Kaposi sarcoma (Madhuri and Pandey, 2009; CANSA, 2017).

Regardless of the therapeutic developments made in understanding the procedures involved in carcinogenesis, cancer has turned out to be one of the most critical medical problems (Singh et al., 2016). Despite efforts in increasing awareness, early prognosis and novel medicinal interventions, the incidence of drug resistance, high costs of treatments and increased reports of secondary toxicity of anticancer synthetic drugs have delayed the progress made (Niraula et al., 2014; Singh et al., 2016). Furthermore, current chemotherapeutic drugs bring about adverse drug reactions such as, musculoskeletal pain, nausea, vomiting, headache, anorexia, gastritis, oral ulceration, diarrhoea, constipation, alopecia and neuropathy (Singh and Singh, 2018), resulting in additional counteractive treatments which further increases the overall cost of therapy. As a result, many patients in developed and developing countries depend purely on phytochemicals and plant extracts to combat cancer (Shukla and Mehta, 2015).

Cancer is amongst the most challenging human diseases, creating an increase in scientific and commercial interest for the discovery of novel anticancer agents from plant sources (Mbaveng et al., 2017). Since ancient times, humans have acquired knowledge on the use of medicinal plants (El-Seedi et al., 2013; Ouelbani et al., 2016). Traditional medicinal plants are frequently used in the treatment of cancer for many people in Africa, due to their lower income or spatial distance from the urban treatment centres (Kabbaj et al., 2012). Approximately 60% of medical drugs used in the treatment of cancer have been isolated from natural products (Reddy et al., 2003; Gordaliza, 2007). This includes chemotherapeutic drugs, such as etoposide phosphate, homoharringtonine, phenolics, podophyllum lignans, *Taxus* diterpenes, terpenoids, vinblastine and vincristine (Cragg and Newman, 2005; Tan et al., 2006; Kaur et al., 2011; Atanasov et al., 2015; Newman and Cragg, 2016).

Investigations on the ethnopharmacological use of medicinal plants in cancer treatment has been reviewed using scientific databases (Tariq et al., 2017), but the identification of unknown plants for their medical use in cancer is still an important topic. The main approaches used in the selection of plant species in cancer drugs discovery include random screening, information on the chemotaxonomy of the plant, and ethnopharmacological knowledge (Alonso-Castro et al., 2011; Solowey et al., 2014). Several studies have investigated the effect of plant extracts as anticancer agents, due to their low toxicity and side effects (Jain and Jain, 2011; Mulla and Swamy, 2012; Newman and Cragg, 2016). Therefore, the search for medicinal plants with natural antioxidant and anticancer properties as safer treatment alternatives, with least side effects is crucial. One such genus, *Barleria* (Acanthaceae) has displayed

great potential for antioxidant and anticancer activity (Manglani et al., 2014; Choudhury et al., 2015; Kumari and Dubey, 2016; Panchal et al., 2018).

Barleria albostellata (Acanthaceae) C.B. Clarke, also known as 'grey *Barleria*', is an evergreen shrub of South Africa (Froneman and Le Roux, 2007) and is broadly distributed from Limpopo, Gauteng, and Mpumalanga to KwaZulu-Natal (Balkwill and Balkwill, 2002; Froneman and Le Roux, 2007). This plant is recognized for its medicinal properties (Amoo et al., 2009), as phytochemical compounds extracted from its leaves and stems, such as flavonoids, iridoids, phenolics, gallotannins, proanthocyanidins displayed a wide range of antibacterial activities and anti-inflammatory properties. In traditional medicine, there are no documented reports on the use of *B. albostellata*, however, several reports have been published on the anti-inflammatory, analgesic, antitumor, antileukemic, anti-hyperglycemic, anti-amoebic, antibiotic, and virucidal activities of species within the genus *Barleria* (Yosook et al., 1999; Wang et al., 2001; Jassim and Naji, 2003; Suba et al., 2004, 2005; Chomnawang et al., 2005; Amoo et al., 2009; Shukla and Gunjegaokar, 2018). Thus, the genus *Barleria* has great medicinal potential. The main goals of this study were to evaluate the antioxidant activity of crude leaf and stem extracts of *Barleria albostellata* using various assays; and to test the cytotoxicity of these extracts on selected mammalian cancer cell lines viz. HEK293, HeLa and MCF-7. To the best of our knowledge, there are no published reports on the antioxidant and anticancer activity of the crude extracts of *Barleria albostellata*.

6.2 Materials and methods

6.2.1 Plant materials

Leaves and stems of *B. albostellata* were collected from the University of KwaZulu-Natal, Westville campus (29° 49′ 51.6″ S, 30° 55′ 30″ E), Durban, South Africa. A voucher specimen (7973000) was deposited in the Ward Herbarium of the University of KwaZulu-Natal, Life Sciences, Westville campus.

6.2.2 Preparation of crude extract

Before preparing the crude extract, leaves and stems material were oven-dried for 2 weeks at 35°C. The dried plant materials were crushed to powder with the aid of a mechanical blender (Russel Hobbs, model: RHB315). The ground material underwent sequential extraction using various solvents (hexane, chloroform and methanol) in a Soxhlet apparatus. An amount of 10 g of crushed leaves were placed into a round bottom flask containing 100 ml of hexane, the appropriate solvent, and boiled for 3 h at 40°C. The extracted solution was filtered (Whatman® No. 1 filter paper) and retained. This procedure was conducted in replicates. Consecutive extractions of chloroform followed by methanol were

completed. Each solvent extraction followed the same procedure as mentioned above. Consecutive extractions were performed on the leaf and stem material.

6.2.2.1 Evaporation and concentration

Each extract was left at room temperature to evaporate in a dark fume-hood. Dried extracts were stored in sealed, labelled glass jars, in order to avert the material from reacting with the atmospheric humidity. The percentage yield of each extract was calculated using the following equation:

$$\text{Extract Yield (\%)} = \frac{\text{Weight of dried extract (g)}}{\text{Weight of plant material (g)}} \times 100$$

6.2.2 Total flavonoid, total phenolic content and *in vitro* antioxidant assay

6.2.2.1 Estimation of total flavonoid content

The total flavonoid content was determined using the assay described Arruda et al. (2018), with modification. Approximately 25 µL of each extract of varying concentrations (15, 30, 60, 120 and 240 µg/mL) was dispensed into a 96-well microtiter plate (F-Bottom, Greiner Bio-One). To each extract, 100 µL of ultrapure water and 7.5 µL of 5% (w/v) sodium nitrite ($NaNO_2$) was added. After 5 min, 7.5 µL of 10% (w/v) aluminum chloride ($AlCl_3$) was added to the reaction mixture of each extract and allowed to stand for 6 min. Finally, 50 µL of 1M sodium hydroxide (NaOH) and 60 µL of ultrapure water were added to each extract and thoroughly mixed. The absorbance was measured at 510 nm against a blank using the Synergy HTX Multi-mode reader, Bio. Tek Instruments Inc., Winooski, USA. The calculation of the total flavonoid content was done using a quercetin standard curve and results were displayed as mg quercetin equivalents (QE) per gram of dry weight (DW) using the formula below:

$$C_{tf} = C * \frac{V}{m}$$

C_{tf} = Total flavonoid content (mg/g) in quercetin equivalent

C= Concentration of quercetin acquired from the calibration curve in mg/mL

V= Volume of extract in mL

m= Mass of extract in gram

6.2.2.2 Estimation of total phenolic content

The total phenolic content was determined using the Folin–Ciocalteu assay, as described by Liu and Yao (2007). Using a 96-well microtiter plate (F-Bottom, Greiner Bio-One), a total amount of 150 µL of 10% diluted Folin-Ciocalteau reagent and 120 µL of 0.7 M sodium carbonate (Na_2CO_3) was added to each extract (30 µL) of varying concentrations (15, 30, 60, 120 and 240 µg/mL). The microtiter plate was placed on a mechanical shaker (VEVOR Orbital Rotator Shaker) and incubated for 30 min at room temperature. The absorbance for each well was measured at 765 nm using the Synergy HTX Multi-mode reader, Bio. Tek Instruments Inc., Winooski, USA. Results were displayed as mg of gallic acid equivalents (GAE) per gram of dry weight (DW) using the formula below:

$$C_{tp} = C * \frac{V}{m}$$

C_{tp} = Total phenolic content (mg/g) in GAE (gallic acid) equivalent

C= Concentration of gallic acid acquired from the calibration curve in mg/mL

V= Volume of extract in mL

m= Mass of extract in gram

6.2.2.1 DPPH scavenging activity

The radical scavenging activity of 2,2'-diphenyl-1-picrylhydrazyl (DPPH) was described by Braca et al. (2002). A total amount of 50 µL of 0.1 mM DPPH was prepared in methanol and added to 100 µL of each extract of varying concentrations into a 96-well microtiter plate (F-Bottom, Greiner Bio-One) and mixed well. The plate was allowed to incubate in the dark for 30 mins at room temperature (24°C). The standard used was ascorbic acid and the absorbance was measured at 517 nm using the Synergy HTX Multi-mode reader, BioTek Instruments Inc., (Winooski, USA). The IC_{50} value (the concentration of the antioxidant agent that gives rise to 50% inhibition of the oxidant) was calculated in order to evaluate the *in vitro* antioxidant activity. This value can be obtained from the inhibition curve, by plotting the percentage inhibition values against the concentration logarithmic scale. The following equation was used to calculate the extracts scavenging abilities:

Abs= Absorbance

Absorbance of DPPH and methanol: Abs control

Absorbance of DPPH radical + sample (standard or compound): Abs sample

$$\text{DPPH Scavenging activity (\%)} = \left[\frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}}\right] \times 100$$

6.2.2.2 Ferric (Fe^{3+}) reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power of the extracts was accomplished according to Benzie and Strain (1996), with modifications. Approximately 50 µL of each extract of varying concentrations (15, 30, 60, 120 and 240 µg/mL) was dispensed into a 96-well microtiter plate (F-Bottom, Greiner Bio-One). To each extract, 50 µL of 0.2 M sodium phosphate buffer (pH 6.6) and 100 µL of 1% potassium ferricyanide was added and mixed thoroughly. The solution was incubated for 30 min, at 50°C. To stop the reaction, 10 µL of 0.1% iron (III) chloride ($FeCl_3$), 50 µL of distilled water and 50 µL of 10% trichloroacetic acid was added to each solution and thoroughly mixed. The subsequent solution was left for 10 min to stand and thereafter the absorbance was measured at 700 nm using the Synergy HTX Multi-mode reader, BioTek Instruments Inc., Winooski, USA. Results were displayed as a percentage of the absorbance of the crude extract to that of gallic acid, using the below formula:

$$\% \text{ inhibition} = \left(\frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{gallic acid}}}\right) \text{x } 100$$

Abs= Absorbance

6.2.3 *In vitro* cytotoxicity/ MTT assays

6.2.3.1 Preparation of crude sample

Crude hexane, chloroform and methanol leaf and stem extracts were dissolved in 10% dimethyl sulfoxide (DMSO) at various concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 mg/mL. Prepared samples were transferred to 3 ml Eppendorf Tubes® and stored at 4°C until further use.

6.2.3.2 Cell cultures

The cytotoxicity of the crude extracts were evaluated in the human embryonic kidney (HEK293), cervical cancer (HeLa), and breast adenocarcinoma (MCF-7) cells. Cryopreserved cells were acquired from the American Type Culture Collection (ATCC), Manassas, VA, USA. All experimental work performed on cell cultures were carried out in sterile class II biohazard hood. Cell lines were cryopreserved and stored in a -80°C Nuaire biofreezer. Before analysis, cells were removed and thawed, by rapidly placing them in a 37˚C water bath. Cell suspensions were transferred aseptically into centrifuge tubes and centrifuged (Eppendorf benchtop centrifuge) at 1000 rpm for 5 mins. Thereafter the supernatant of each suspension was discarded. The remaining pellet (cells) was then re-suspended in 1 ml of complete sterile medium (Eagle's Minimum Essential Medium (EMEM) with the addition of 1% antibiotics (100 units/mL penicillin, 100 µg/mL streptomycin) and 10% foetal bovine serum (FBS). Each cell suspension was transferred and grown in a 25 cm^2 tissue culture flask, containing 4 ml

of complete sterile medium. Subsequently, cells were incubated (Thermo-Electron Corporation, Waltham, Massachusetts, USA) at 37°C (comprising 5% CO_2) and observed daily using an inverted microscope (Nikon TMS-F 6V, Tokyo, Japan). The medium was changed routinely, until the cells reached confluency (Daniels and Singh, 2019).

6.2.3.3 MTT (cell viability) assay protocol

The cells metabolic activity and its ability to reduce MTT 3-[(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide] to formazan via the succinate- tetrazolium reductase system was measured by the MTT assay (Mosman, 1983). Cells were trypsinized (Trypsin-EDTA, Sigma) and seeded into 96-well microtiter plates, and thereafter incubated overnight at 37˚C, permitting the attachment of cells. The growth medium was then substituted with fresh medium (EMEM +10% FBS + 1% antibiotics) (Daniels and Singh, 2019), and cells were treated with varying concentrations of the *B. albostellata* extracts (15, 30, 60, 120 and 140 µg/ mL) and incubated for 48 h at 37˚C. The growth medium in each well was then aspirated, followed by the addition of 100 µl medium comprising 10 µl of the MTT solution (5 mg/ml in Phosphate Buffered Saline (PBS) solution) and thereafter incubated for 4 h at 37°C. The medium containing MTT was then removed and substituted with 100 µl of DMSO to ensure that the formazan crystals had solubilized. The amount of these crystals present is suggestive of cellular viability (Vinken and Blaauboer, 2017). Upon the addition of DMSO, the solution changed purple in colour. Absorbance of each extract was recorded at 570 nm using the Mindray M-R-96A microplate reader (Vacutec, Hamburg, Germany), with DMSO as a blank. Positive controls (containing cells only), were recorded as 100% survival (Daniels and Singh, 2019). This assay was performed in triplicates and graphs generated using Microsoft Excel 2019 ™ were used to calculate the concentration at which 50% cell death was achieved (IC_{50}).

6.2.4 Statistical Analysis

Experimental analyses were performed in triplicate. Values were displayed as mean ± standard deviation and subjected to statistical analysis using R statistical computing software, 2020, version 3.6.3. Data were statistical analysed using the One-Way Analysis of Variance (ANOVA) followed by Tukey's-honest significant difference multiple range post hoc tests. Data were expressed as mean significant at $p<0.05$ level.

6.3 Results and discussion

6.3.1 Percentage yield of crude extracts of *B. albostellata*

The percentage yield of the crude extracts was highest from the leaf methanolic extract (16.78%), followed by 9.38% from the stem methanolic extract (Table 6.1). The lowest yield was observed in the hexane stem extract (1.94%). This suggests that the percentage yield of the phytochemical compounds

in *B. albostellata* was greater in the crude leaf than the stem extract. In addition, this suggests that there may be more polar compounds in the leaf extracts and a lesser amount of non-polar compounds in the stem. Thus, the yield obtained indicated that the polarity of the various solvents correlated to the plants' pharmacological importance (Abubakar et al., 2017; Chintalapani et al., 2018). Each extract (hexane, chloroform and methanol) displayed various colours (Table 6.1). Upon evaporation of the solvent, it should be noted that the hexane extracts were oily, while chloroform and methanol extracts dried to a hard-sticky solid.

Table 6.1: Percentage yield of the leaf and stem crude extracts of *B. albostellata*

Crude extract	**Leaves**	**Stem**	**Leaves**	**Stem**	**Leaves**	**Stems**
	Dried extract yield (g)		**Percentage yield (%)**		**Colour**	
Hexane	0.139	0.194	1.39	1.94	Dark yellow	Light yellow
Chloroform	0.265	0.219	2.65	2.19	Dark green	Light green
Methanol	1.678	0.938	16.78	9.38	Dark brown	Light yellow

6.3.2 Evaluating the total flavonoid and total phenolic content of crude extracts

One antioxidant assay is unable to display all aspects of the activities of natural products. This is because the antioxidant properties of plants can be linked to a number of various mechanisms (Grauzdytė et al., 2018). Flavonoids are secondary metabolites with low molecular weight and are distributed throughout the plant kingdom. This phytochemical compound is produced by several plants in high quantities. The concentrations of flavonoids evaluated at 240 ug/mL in the crude extracts, measured as quercetin equivalents, are presented in Table 6.2. The highest total flavonoid content was observed in the methanolic leaves (42.39 ± 1.14 mg QE/g DW), compared to other solvents (Table 6.2). This may indicate that more flavonoids could be extractable with these solvents. The flavonoid content of all extracts of *B. albostellata* were satisfactory, this may partly be responsible for the different pharmacological activities observed in the extracts. The lowest flavonoid content was found in the hexane leaves (11.22 ± 0.22 mg QE/g DW). According to Amoo et al. (2011), the greatest and lowest flavonoid content was documented in the leaves of *B. greenii* and *B. albostellata* stems, respectively. With exception of the hexane leaves, the total flavonoid content observed in the leaves and stems of *B. albostellata* were comparable (Table 6.2). It was also noted that as the polarity increased from hexane (non-polar) to methanol (polar), so did the total flavonoid content (mg QE/g DW) (Table 6.2). Aside from their quantity, the nature or quality of the flavonoid present in the leaves and stems extracts may differ in its therapeutic potential (Amoo et al., 2011). According to Ren et al. (2010), the leaves of *B. prionitis* contained a naturally occurring flavonoid, 6-hydroxyflavone, which is a promising drug candidate in treating anxiety-like disorders. Flavonoids exhibit a noteworthy range of biochemical and pharmacological properties, with the most notable being their antioxidant, antimicrobial and anti-

inflammatory activities (Middleton et al., 2000). The antioxidant activity observed from flavonoids are due to numerous different mechanisms, such as scavenging of FRs, inhibition of enzymes that cause free radical (FR) generation and chelation of metal ions (Sawarkar et al., 2018). Reliant on their structure, flavonoids may scavenge nearly all known ROS (Maryam et al., 2009).

Table 6.2: Total flavonoid content of the crude extracts of the leaves and stem of *B. albostellata*

Crude extracts	**Total flavonoid (mg QE/g DW)**	
	Leaves	**Stem**
Hexane	11.22 ± 0.22	31.79 ± 0.59
Chloroform	34.38 ± 0.28	36.20 ± 0.685
Methanol	42.39 ± 1.14	37.10 ± 0.95

Data displayed as mean ± SD of triplicate

The most widely spread secondary metabolite in the plant kingdom is phenolics. This compound has the potential to be a natural antioxidant and have the ability to act as an efficient radical scavenger (Kapoor et al., 2014). Phenolic compounds react with active oxygen FRs for instance, superoxide anion, hydroxyl, and lipid peroxyl radicals (Afanasiev et al., 1989). These composites have a wide spectrum of biological and chemical activities, including FR scavenging properties (Makhafola et al., 2016). The total phenolic content assessed at 240 ug/mL in each of the evaluated plant extracts are presented in Table 6.3. The methanol leaf extract had the highest amount of total phenolics (6.05 ± 0.09 GAE/g DW), followed by the methanol stem extracts (2.93 ± 0.73 GAE/g DW). This may indicate that more phenolic compounds could be extractable with these solvents. Similar results were reported by Amoo et al. (2011), the total phenolics content observed in the methanolic leaves of *B. albostellata* were greater (5.27 ± 0.324 mg GAE/g DW) then the methanolic stems extracts (3.76 ± 0.084 mg GAE/g DW). According to Table 6.3, leaf extracts contained more phenol content than the stems. Amoo et al. (2011) reported the greatest phenolic content in the leaves of *B. prionitis*, *B. greenii* and *B. albostellata*, compared to other plant parts. Jaiswal et al. (2010) reported similar findings earlier, as phenols detected in the leaves were higher than the stems of *B. prionitis*. Whereas, Kumari et al. (2017), reported *B. lupulina*, stem extracts to contain more phenolic content than the leaves. An important factor for the antioxidant activity of phenolic compounds is its redox property, which allows them to act as hydrogen donors, singlet oxygen quenchers and reducing agents (Samak et al., 2009). Manian et al. (2008) suggested the FR scavenging activity of crude extracts may be linked to the nature of phenolic compounds present, therefore assisting in the hydrogen donating ability/electron transfer. The therapeutic use of this compound could assist in the control of FR disorders such as, inflammation, heart disease, stroke, cancer and diabetes mellitus (Ghasemzadeh and Ghasemzadeh, 2011). Total phenolics

are regarded as more potent antioxidants than carotenoids *in vitro*, Vitamin C and E (Chintalapani et al., 2018).

Table 6.3: Total phenolic content of the crude extracts of the leaves and stem of *B. albostellata*

Crude extracts	**Total phenols (mg GAE/g DW)**	
	Leaves	**Stem**
Hexane	1.15 ± 0.56	1.06 ± 0.03
Chloroform	2.51 ± 0.27	1.25 ± 0.28
Methanol	6.05 ± 0.09	2.93 ± 0.73

Data displayed as mean ± SD of triplicate

6.3.3 Antioxidant screening of crude extracts using DPPH and FRAP assays

The DPPH FR scavenging activity was evaluated by the decrease in absorbance at 516 nm, which is induced by antioxidants (Manjula and Ganthi, 2018). This assay is not specific to any precise class of antioxidants, and therefore provides the general antioxidant capacity of the extract (Tepe et al., 2004). Figure 6.1 presents the percentage FR scavenging activity of crude extracts of the leaves and stems of *B. albostellata*. The radical scavenging activities present in extracts of the leaves and stems were compared with ascorbic acid as a standard. The radical scavenging activity of the crude extracts was studied by its ability to reduce DPPH (stable radical) and any molecule that may donate a hydrogen or electron to DPPH (Patel et al., 2012). The electron donating ability of *B. albostellata* is most commonly determined using DPPH FR scavenging tests due to its reliability. For all crude extracts there was a dose-dependent change in radical scavenging activities. Overall, in all extracts, with increasing concentration there was an increase in the DPPH radical scavenging activity (Figure 6.1). Statistical analysis showed all extracts had significantly different activity across all concentrations ($P < 0.05$) when compared to the ascorbic acid, 15-240 µg/mL.

The methanolic leaf and stem extracts had more effective radical scavenging activity than the hexane and chloroform plant extracts, with inhibition at 90.37% and 90.43%, respectively. Dose–response radical scavenging activities were also observed in the methanolic extracts of different parts of *B. prionitis*, *B. greenii* and *B. albostellata* (Amoo et al., 2011). These solutions had discoloured from purple to a faded solution. A purple-coloured solution visible in the DPPH assay accepts electrons, which then converts to a discoloured solution. The point of colour change is linked to the effectiveness and concentration of antioxidants present (Herrera-Calderon et al., 2018). The amount of discoloration, indicates the FR scavenging action (Kumar et al., 2014). The scavenging activity of the methanolic extracts compared with the standard ascorbic acid suggests that the leaves and stems of *B. albostellata*

are also an effective scavenger of FRs. Higher radical scavenging activity values were recorded at lower IC_{50} values (Table 6.4). Vasanth et al. (2018) found maximum DPPH radical-scavenging activity at 100 µg/ml in the ethanol and petroleum ether leaf extracts of *B. cristata* with percentage inhibition values of 76.01 and 70.57, respectively.

The concentration of the methanolic leaf and stem extracts needed for 50% inhibition (IC_{50}) was 16.95 µg/mL and 14.27 µg/mL, respectively. These results were compared with the IC_{50} value of ascorbic acid for the leaves (4.03 µg/mL) and stems (1.50 µg/mL) (Table 6.3). The radical scavenging activity of DPPH is influenced by the polarity of a medium, chemical structure of the scavenger, pH of the reaction, concentration of sample and reaction time (Sujatha et al., 2018). Free radicals reactions are linked in the pathology of several diseases such as cancer, Alzheimer's and inflammation (Houghton et al., 2007). Kumari et al. (2017) observed the DPPH radical-scavenging activity of the methanolic leaf and stem extracts of *B. lupulina*. These authors found the IC_{50} values of the methanol leaves and stems as 48.86 µg/mL and 60.82 µg/mL, respectively. Overall, the results obtained in this study indicated that the chloroform and methanol extracts displayed good radical scavenging activity, which was a low amount when compared to the standard ascorbic acid.

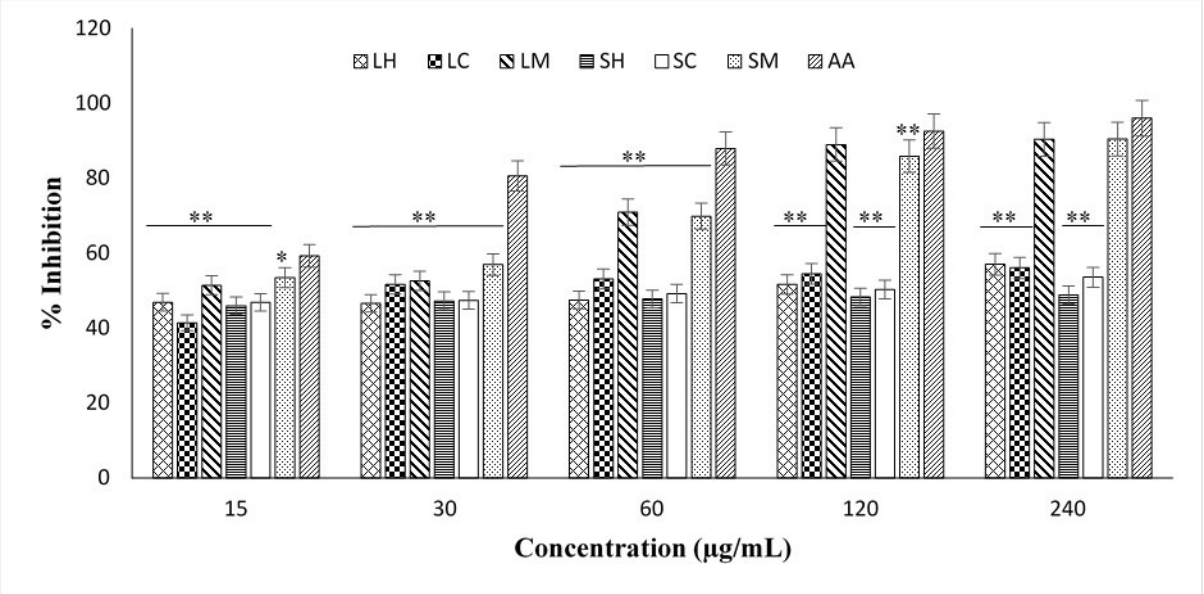

Figure 6.1: *In vitro* antioxidant activity (% inhibition DPPH) of crude extracts from the leaves and stems of *B. albostellata*. (*P <0.05 and **P <0.001 above each bar were considered statistically significant when comparing each extract to the ascorbic acid at different concentrations, 15-240 µg/mL). Data are presented as means ± SD, n = 3 and displayed as a percentage of the control sample. LH- Leaf hexane; LC- Leaf chloroform; LM- Leaf methanol; SH- Stem hexane; SC- Stem chloroform; SM- Stem methanol; AA- Ascorbic acid.

Table 6.4: IC_{50} values of the DPPH radical scavenging activity of the leaves and stem extracts of *B. albostellata*

	DPPH (µg/mL)	
Extract	**Leaves**	**Stems**
Hexane	61.53	697.75
Chloroform	44.99	73.38
Methanol	16.95	14.27
Ascorbic acid	4.03	1.50

Data displayed as mean, n=3, of triplicate determinations.

The FRAP assay is established on an electron transfer reaction (Huang et al., 2005). The presence of antioxidants (reductants) in the tested extracts leads to the reduction of ferricyanide/ferric ion compound to its ferrous form, by distinctive formation of 'Perl's Prussian blue' and is measured spectrophotometrically (Chung et al., 2002). According to Haung et al. (2005), the degree of colour variation is directly proportional to the concentrations of antioxidants present in the extracts. Figure 6.2 illustrates the reducing power of extracts from *B. albostellata*. All crude extracts assessed demonstrated an overall dose-dependent response. As the extract concentration increased the reducing power activity decreased. The extracts reducing power were significantly lower than the ascorbic acid standard (Figure 6.2). Similar results were observed for the different parts of *B. prionitis*, *B. greenii* and *B. albostellata* (Amoo et al., 2011). Statistical analysis indicated that all extracts had significantly different activity across all concentrations ($P < 0.05$) compared to ascorbic acid, 15-240 µg/mL.

The leaf and stem hexane extracts displayed the lowest reducing power (Figure 6.2). Similar results were observed in the leaf and stem hexane extracts of *B. prionitis* (Sharma et al., 2014). Low-to moderate reducing power activity was also observed in the ethanol and petroleum ether leaf extracts of *B. cristata* (Vasanth et al., 2018). The results from Figure 6.2 suggests the presence of antioxidant compounds in the various extracts with electron-donating ability, in which this assay is recognised to measure semi-quantitatively (Amarowicz et al., 2004; Rumbaoa et al., 2009). The occurrence of these compounds may be in smaller amounts or in an impure form, which can be responsible for the low activity exhibited by the extracts (Amoo et al., 2011).

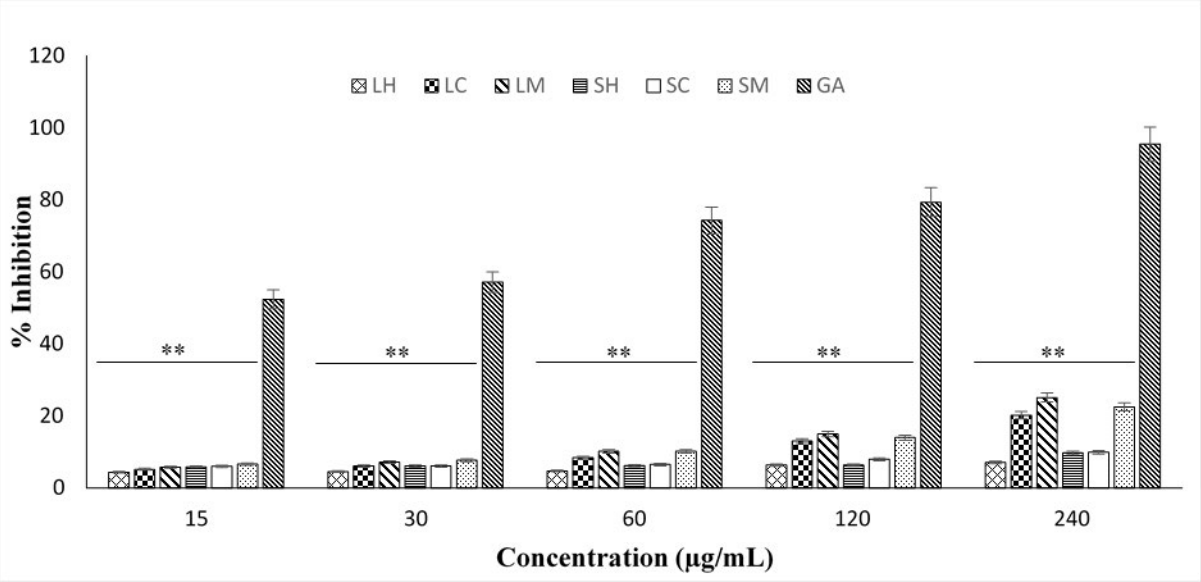

Figure 6.2: *In vitro* antioxidant activity (% inhibition FRAP) of crude extracts from the leaves and stems of *B. albostellata*. (*P <0.05 and **P <0.001 above each bar were considered statistically significant when comparing each extract to the Gallic acid, at different concentrations, 15-240 µg/mL). Data are presented as means ± SD, n = 3 and displayed as a percentage of the control sample. LH- Leaf hexane; LC- Leaf chloroform; LM- Leaf methanol; SH- Stem hexane; SC- Stem chloroform; SM- Stem methanol; GA- Gallic acid.

Table 6.5: IC_{50} values of the FRAP reducing power activity of the leaves and stem extracts of *B. albostellata*.

	FRAP (µg/mL)	
Extract	**Leaves**	**Stems**
Hexane	1.20×10^{20}	9.12×10^{17}
Chloroform	>90000	1.05×10^{15}
Methanol	>15000	>58000
Gallic acid	14.96	14.90

Data displayed as mean, n=3, of triplicate determinations

6.3.4 *In vitro* cytotoxicity effect of crude extracts of *B. albostellata*

New therapeutic approaches against cancerous cell lines can mediate the initiation of apoptosis (Motadi et al., 2020). The MTT cellular viability assay depends on the viable cells' mitochondrial metabolic capacity (Grauzdytė et al., 2018). As displayed in Figure A-C, the percentage cell survival for all crude extracts were dose-dependent. Results indicated the anti-proliferative effect decreases with increase in the concentration of the crude extract. All crude extracts at various concentrations showed low-to moderate cytotoxicity, and was lowest when treated with the stem methanolic extract (Figure 6.3 A). Crude extracts demonstrated moderate cytotoxicity at high

concentrations (240 µg/mL). The highest cellular viability for the HeLa cells was observed at 15 µg/mL of the leaf methanol extract (Figure 6.3 B), while the lowest viability was observed at 240 µg/mL with the leaf chloroform extract (Figure 6.3 B). For MCF-7 cells, the percentage cell viability was greatest at 15 µg/mL for the leaf chloroform extract, and lowest at 240 µg/mL for the stem methanol extract (Figure 6.3 C). At varying concentrations, cellular viability was > 35% for all crude extracts (Figure 6.3 A-C). As the concentration of each crude extract increased, so did its toxicity become more significant in each cell line. This slow decrease in cellular viability in all extracts may be due to the occurrence of some compounds that can inhibit cell proliferation (Gordanian et al., 2014).

Sawarkar et al. (2016) evaluated the cytotoxicity of ethanolic extracts of *B. prionitis* and *B. grandiflora*. These authors observed 50% cytotoxicity for human dermal fibroblast and human gingival fibroblast cell lines at >1000 µg/mL. Cytotoxic agents found in extracts may destroy the cell membrane, leading to cell lysis, cause necrosis, or induce apoptosis by triggering many biochemical mechanisms (Alonso-Carrillo et al., 2017). Additionally, the differences in cytotoxicity among the various extracts of *B. albostellata* may be attributed to the level of antioxidants present, or related to the inhibitory effects through other signalling pathways (Sammar et al., 2019). Furthermore, it is well recognized that temperature, solvent type, methods, and time of extraction can disturb the extraction of phytochemical compounds (Tuntiwachwuttikul et al., 1998).

Statistical analysis indicated crude extracts across all concentrations had significantly different activities ($P < 0.05$).The IC_{50} values of the crude extracts in the three mammalian cell lines are represented in Table 6.6. In the HEK293 and HeLa cells, low cytotoxic activity was observed for all extracts. The hexane leaf extract had the highest IC_{50} value of 294.44 µg/mL for HEK293, an indication of poor cytotoxicity. Additionally, low cytotoxicity for HeLa was observed for the stem hexane extract (IC_{50} value of 376.70 µg/mL). Low to moderate cytotoxicity was observed in the stem hexane (HEK293) and leaf methanolic extracts (HeLa) with IC_{50} values of 95.28 µg/mL and 98.86 µg/mL, respectively. Flavonoids found in these extracts may be responsible for its cytotoxicity. There is ongoing evidence that several flavonoids exert anticancer activity, though, the mechanisms responsible for its effect have not been fully explained (Kopustinskiene et al., 2020).

In the MCF-7 cells, low cytotoxicity was observed in the leaf methanol extracts (IC_{50} value of 239.88 µg/mL), whereas moderate cytotoxic levels were observed for the hexane stem extracts (IC_{50} value of 63.10 µg/mL). According to Manapradit et al. (2015), the highest cytotoxicity of the leaf butanolic extracts of *B. strigosa* was found in the HeLa and MCF-7 cells. Kumari and Dubey (2016) treated Hep G2 cells with both aqueous and ethanolic leaf extracts of *B. lupulina* and demonstrated the growth inhibition, cell shrinkage, vacuolation and cell lysis due to the extracts. Therefore, it is possible that any cytotoxic effects induced in the cancer cells could be due to active phytochemical compounds found in the various extracts of the plant.

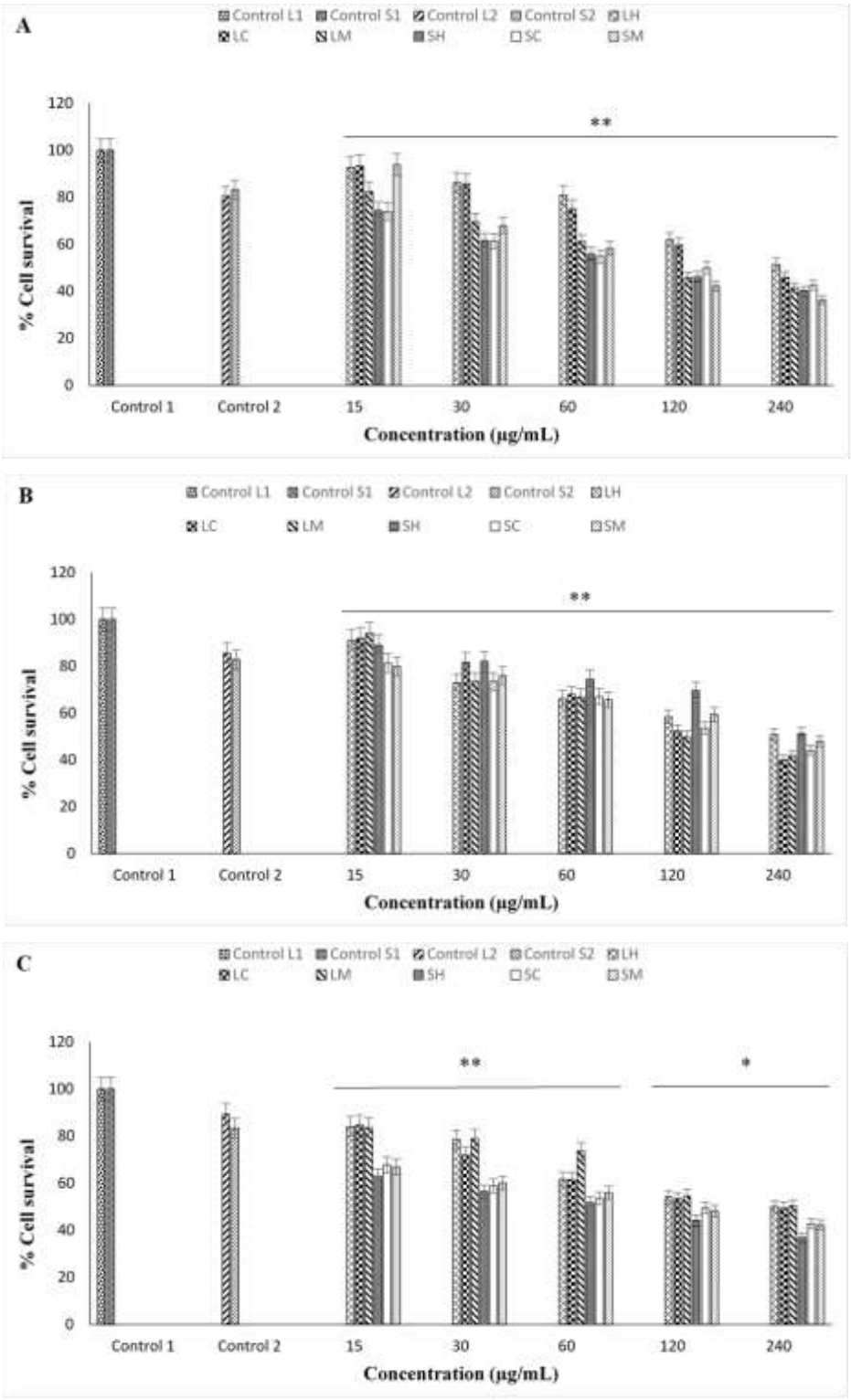

Figure 6.3: *In vitro* cytotoxicity activity (% cell survival) of crude leaves and stems extracts of *B. albostellata*. (A) Human embryonic kidney (HEK293); (B) Cervical cancer cells (HeLa), (C) Breast adenocarcinoma (MCF-7). (*P <0.05 and **P <0.001 were considered statistically significant within the different concentrations, 15-240 µg/mL). Data are presented as means ± SD, n = 3 and displayed as a percentage of the control sample. Control Leaves 1- cells only; Control Stems 1- cells only; Control Leaves 2- DMSO only; Control Stems 2- DMSO only; LH- Leaf hexane; LC- Leaf chloroform; LM- Leaf methanol; SH- Stem hexane; SC- Stem chloroform; SM- Stem methanol.

Table 6.6: IC_{50} values of the cytotoxicity activity of hexane, chloroform and methanol leaves and stem extracts of *B. albostellata*.

Cell lines	Extracts	Cytotoxicity (µg/mL)	
		Leaves	Stems
HEK293	Hexane	294.44	95.28
	Chloroform	210.86	110.15
	Methanol	116.15	96.61
HeLa	Hexane	220.80	376.70
	Chloroform	143.55	165.58
	Methanol	98.86	232.27
MCF-7	Hexane	194.98	63.10
	Chloroform	181.97	102.33
	Methanol	239.88	102.33

Data displayed as mean, n=3, of triplicate determinations

The antioxidant activity observed from flavonoids in *Barleria* are due to numerous different mechanisms, such as scavenging of FRs, inhibition of enzymes that cause free radical (FR) generation and chelation of metal ions (Sawarkar et al., 2018). The total flavonoid content observed in the leaves and stems of *B. albostellata* may differ in its therapeutic potential. Other species of *Barleria* contained a naturally occurring flavonoid, 6-hydroxyflavone, which is a promising drug candidate in treating anxiety-like disorders. Phenols found in the methanolic extracts of *B. albostellata* has the potential to be a natural antioxidant and have the ability to act as an efficient radical scavenger (Kapoor et al., 2014). Various authors have reported the greatest phenolic content in the leaves of species of *Barleria*, compared to other plant parts (Jaiswal et al., 2010; Amoo et al. 2011). The evaluated crude extracts may protect against FR and oxidative damage occurring in various pathological mechanisms. The *in vitro* cytotoxicity observed in the extracts of *B. albostellata*, may be due to the presence of flavonoids, phenols, and antioxidant activity in the different parts of this species. Additionally, the differences in cytotoxicity among the various extracts of B. *albostellata* may be attributed to the level of antioxidants present, or related to the inhibitory effects through other signalling pathways (Sammar et al., 2019).

6.4 Conclusion and future perspectives

Overall, the results of this study highlight the therapeutic potential of *Barleria albostellata*. The present study evaluated the antioxidant and cytotoxicity measures of *B. albostellata*, which can be useful in establishing its therapeutic value. All extracts, to some degree, presented with good antioxidant properties. This could suggest that the evaluated crude extracts of *B. albostellata* may protect against FR and oxidative damage occurring in various pathological mechanisms. Antioxidants in the human body are essential in controlling the damaging consequences of FRs. The link between radical-scavenging agents in crude extracts and their cytotoxicity in cancer cells could be beneficial to data screening projects that explore for natural products with cytotoxicity potential. The observed *in*

vitro cytotoxicity may be due to the presence of flavonoids, phenols, and antioxidant activity in the different parts of this species. Through bioassay-guided fractionation, the isolation of specific bioactive compounds from the leaves and stems and the evaluation of its safety, will be necessary in the further exploration of this species for potentially new therapeutic drug leads. This could perhaps aid in underpinning the precise compounds responsible for the various pharmacological activities. To the best of our knowledge, this is the first report on the cytotoxic activities of the leaf and stem crude extracts of *Barleria albostellata*. Findings from this study would significantly contribute to the advancement of natural compounds for potential use in the healthcare sectors.

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CHAPTER 7

THE GREEN SYNTHESIS OF SILVER NANOPARTICLES USING *Barleria albostellata* EXTRACTS: CHARACTERIZATION, ANTIBACTERIAL AND CYTOTOXICITY EVALUATION

Abstract

Silver nanoparticles (AgNPs) have progressively gained popularity due to their unique physicochemical and biological properties. Synthesized AgNPs, using leaves and stems of *B. albostellata* C.B. Clarke, were characterised using UV-visible spectroscopy, scanning electron microscopy (SEM), high resolution transmission electron microscopy (HRTEM), energy-dispersive X-ray (EDX) analysis, Fourier transform infrared (FTIR) spectral analysis and nanoparticle tracking analysis (NTA). Preliminary antibacterial analysis of the synthesized AgNPs was assessed using the disk diffusion method. The cytotoxicity of the synthesized AgNPs was established using the 3-[(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) assay in the human embryonic kidney (HEK293), cervical cancer (HeLa), and breast adenocarcinoma (MCF-7) cell lines. The results suggest that *B. albostellata* extracts can aid as environmentally friendly biofactories for AgNPs synthesis. UV–vis spectroscopy of the leaf and stem extracts displayed absorption peaks between 400–450 nm, confirming AgNP synthesis. Elemental Ag was highest in the methanol leaf extracts (16.87 ± 0.89%) and lowest in the powdered stem extracts (7.13 ± 1.44%). Synthesized AgNPs were predominantly spherical in shape. HRTEM revealed the AgNPs synthesized from the methanolic stem extracts (34.32 ± 16.99 nm) were larger in size, while those from the powdered stem extracts were smaller (16.57 ± 5.55 nm). AgNPs synthesized from both the leaf and stem extracts exhibited zeta potential values between -8.8 and -32.1 mV, with a hydrodynamics diameters ranging from 34.3 to 111.3 nm. FTIR spectroscopy confirmed the presence of various functional groups on the AgNPs and the presence of the capping and stabilising agents from the respective extracts. AgNPs synthesized from leaf and stem extracts displayed significant antibacterial activity and were sensitive to both Gram-positive and –negative bacteria. Synthesized AgNPs exhibited selective *in vitro* cytotoxicity against HEK293, HeLa and MCF-7 cell lines. The IC_{50} values of the AgNPs synthesized from the various extracts were all above 9 µg/mL. Overall, AgNPs synthesized from *B. albostellata* extracts comprised novel antibacterial and anticancer agents, and warrant further investigation. Bio-synthesized AgNPs have great potential in the area of nanotechnology and may be used as an affordable, eco-friendly alternatives for the delivery of conventional therapeutics.

Keywords: Antibacterial activity; Biofactories; Biosynthesis; Cytotoxicity; Elemental Ag^{+}

7.1 Introduction

Nanotechnology is an emerging field focusing on the synthesis and application of small particles called nanoparticles (NPs) (<100 nm) (Albrecht et al., 2006; Mittal et al., 2014; Devi and Selvan, 2017; Khatoon et al*.,* 2017; Pirtarighat et al., 2019; Jadoun et al., 2020). The term "Nano" is a Latin word, which means dwarf (Bhatia, 2016). The advancement of molecular technology has allowed for atoms and molecules of some material, for example; plants, to be reduced to NPs of 1-100 nm in size (Ahmed et al., 2016). This field has various applications in biology, chemistry, energy science, medical science and pharmaceutics (Iravani, 2011; Vanaja and Annadurai, 2012; Singh et al., 2019). The use of naturally occurring reagents such as biodegradable polymers, sugars, microorganisms and plant extracts as reductants and capping agents can be considered desirable for nanotechnology (Kharissova et al., 2013; Ahmed et al., 2014; Ahmed and Ikram, 2015; Pal et al., 2019).

Nanomedicine links the gap between the physics of nanostructures and the biology that leads to their medicinal importance (Ghosh et al., 2016). Green syntheses are environmentally friendly processes used in the field of chemistry and are becoming progressively popular as they can assist in alleviating global environmental concerns (Thuesombat et al., 2014; Pal et al., 2019). The use of natural resources (organic systems) and model solvent systems is necessary to achieve this goal (Singh et al., 2018). Even though the microbial path of preparing NPs is regarded as eco-friendly, the use of plant material in their production may be more favourable since the microbial route possesses complex steps for surface modification and microbial screening is time consuming (Ahmed et al., 2017; Pal et al., 2019).

Biosynthetic methods using plant extracts act as reducing and capping agents (Gardea-Torresdey et al., 2002; Shankar et al., 2003; Chandran et al., 2006; Pal et al., 2019) and have progressed as a simple, feasible and environmentally friendly alternative to more complicated chemical synthetic processes of nanomaterials (Ahmad et al., 2003; Husseiny et al., 2007; Smitha et al., 2009; Zhu et al., 2018). Even the precise mechanism of synthesising NPs using plant extracts is unclear, it has been shown that biomolecules such as alkaloids, phenols and flavonoids found in these extracts play an important role in reducing the metal ions and capping the biosynthesized NPs (Krishnaraj et al., 2010; El-Seedi et al., 2019). There has been significant interest in metal NPs such as gold (Au) (Asmathunisha and Kathiresan, 2013; Kuppusamy et al., 2016; MR and Iyer, 2020), copper (Cu) (Lee et al., 2011; Kuppusamy et al., 2016) and silver (Ag) (Kim et al., 2007; Rai et al., 2009; Asmathunisha and Kathiresan, 2013; Kuppusamy et al., 2016; Azócar et al., 2019) in treating various ailments, because of their size, shape and biological properties (Lee and El-Sayed, 2006).

Cancer is a leading cause of death in Africa, with 70% of deaths occurring in low- to middle income countries (WHO, 2021). Despite this, the burden of infectious diseases has taken precedence over cancer in the public health sector of various countries in Africa (Jamison et al., 2006). Chemotherapy drugs used in treating cancer are challenging, as they are non-specific and result in multidrug resistance. This

in turn leads to chemotherapy associated toxicities, causing patients to succumb to the side effects of the drug and not the disease (Zhang et al., 2011; Nikolaou et al., 2018). Nano materials are anticipated to revolutionise the treatment of cancer via advances in initial detection, prognosis and treatment. NPs are predicted to offer site-specific delivery, improved effectiveness of the bound therapeutics agents, and the capability to withstand multidrug resistance (Robert et al., 1985; Singh and Lillard, 2009; Mousa and Bharali, 2011; Anselmo and Mitragotri, 2014; Wang et al., 2020). NPs are important antibacterial agents, as disease-causing microorganisms are rapidly mutating and are increasingly exhibiting resistance to conventional biocides (Kim et al., 2007; Rai et al., 2009), therefore creating an urgency in modifying or developing alternative treatments and antimicrobial compounds (Mandal et al., 2014; Pirtarighat et al., 2019).

Among metal NPs, more attention has been directed towards silver NPs (AgNPs) as they are widely available (Jha et al., 2009; Chandirika and Annadurai, 2018) and have various applications in therapeutics (such as antimicrobial, antidiabetic, anticancer and antioxidant activities) (Lampe, 1999; Rahuman, 2000; Dipankar and Murugan, 2012; Vasanth et al., 2014; Hembram et al., 2018). Globally, the most commercialised nano-material is Ag, as five hundred tons of AgNPs are produced per year (Larue et al., 2014). The application of AgNPs in artificial implants, diagnostics, tissue engineering, imaging, sensing, gene and drug delivery are associated with its distinct optical, electrical and thermal properties (Sharma et al., 2013; Begum et al., 2017). Several phytochemicals found in medicinal plants were reported to reduce, cap and stabilise Ag^{+} ions (Chinnasamy et al., 2017; Khatoon et al., 2017; Maddila and Hemalatha, 2017). Plant-mediated biosynthetic methods are widely used for the rapid production of AgNPs with polar solvents (methanol and water) being reported to be highly effective in their synthesis (Salam et al., 2012). The morphological limitations of synthesising NPs (e.g., shape and size) can be controlled by changing the reaction conditions and concentrations of chemicals (e.g., pH and temperature) (Singh et al., 2018).

Barleria albostellata (Acanthaceae) is an indigenous shrub that is distributed in the tropical and subtropical regions of South Africa (Froneman and Le Roux, 2007). This shrub is known for its medicinal properties, as the leaf and stem extracts exhibit antibacterial and anti-inflammatory properties (Amoo et al., 2009). There is a scarcity of scientific information on the use of *B. albostellata* in traditional medicine. However, several species within the genus have been used in traditional medicine due to their analgesic, anti-inflammatory, antileukemic, antitumor, anti-amoebic, antibiotic and virucidal activities (Yosook et al., 1999; Wang et al., 2001; Jassim and Naji, 2003; Suba et al., 2004, 2005; Chomnawang et al., 2005; Amoo et al., 2009; Shukla and Gunjegaokar, 2018). The metabolite profile of plants can vary across plant organs (Akula and Ravishankar, 2011). Considering the vast capability of plants as sources for green synthesis, this study aimed at synthesising, characterizing and evaluating the chemical nature and bioactivity of AgNPs using leaf and stem methanolic, aqueous fresh and powdered extracts of *B. albostellata*. To our knowledge, there are no previous reports on the

biosynthesis of NPs from extracts of *B*. *albostellata*. Thus, investigation on the synthesis of AgNPs using *B*. *albostellata* is essential to elucidate its potential use in the nanotechnology industry.

7.2 Materials and methods

7.2.1 Plant materials

Leaves and stems of *B*. *albostellata* were collected from the University of KwaZulu-Natal, Westville campus (29° 49′ 51.6″ S, 30° 55′ 30″ E), Durban, South Africa. A voucher specimen (7973000) was deposited in the Ward Herbarium, of the University of KwaZulu-Natal, Westville campus.

7.2.2 Preparation of the methanolic crude extract

For preparation of the crude extract, leaves and stems were oven-dried at 35°C for 2 weeks. Dried plant material was removed and ground to a fine powder, using a mechanical blender (Russel Hobbs, model: RHB315), then extracted using methanol in a Soxhlet apparatus. Approximately 10 g of powdered leaves were placed into a round bottom flask containing 100 ml of methanol, and boiled for 3 h at 40°C. The extracted solution was filtered (Whatman® No. 1 filter paper) and retained. This procedure was repeated thrice. Consecutive extractions were carried out on both leaf and stem material. Extracts were transferred into glass jars and stored at 4°C until further use.

7.2.3 Fresh aqueous extract

The fresh material was extracted according to Govindarajan and Benelli, (2016), with modifications. Approximately 35 g of washed and cut, leaf and stem material were added to 600 ml beakers. Sterile distilled water (100 ml) was added to each beaker, and placed in an oven at 60°C for 30 min. Thereafter, the extracts were filtered into conical flasks, transferred into glass jars and stored at 4°C until further use.

7.2.4 Powdered aqueous extract

Approximately 10 g of powdered leaf and stem material was added to a 250 ml Erlenmeyer flask, followed by the addition of 100 ml sterile distilled water. Solutions were mixed and heated in an oven at 60°C for 3 h. Thereafter, the samples were filtered and transferred to glass jars and stored at 4°C until further use.

7.2.5 Synthesis of AgNPs

Silver nitrate ($AgNO_3$, 1 mM) (Merck) aqueous solution was prepared with deionised water and used for the synthesis of AgNPs. The reduction of Ag^+ was achieved by adding 10 ml of the crude extract (leaves and stems) to 90 ml of $AgNO_3$ solution (Premasudha et al., 2015; Cittrarasu et al., 2019). The solution was incubated at 80°C for 3 h, until a colour change was observed. The formation of AgNPs

was indicated by a brownish colour (Asmathunisha and Kathiresan, 2013; Gunasekaran et al., 2017). Once the colour of the solution intensified, the conical flasks were removed to avoid agglomeration of the AgNPs which usually occurs when the solution achieves a very dark colour (Moodley et al., 2018). All analyses were carried out in triplicates.

7.2.6 Quantification of AgNPs

After synthesis, the reaction mixture (methanolic, fresh- and powdered aqueous) of leaf and stem extracts were dispensed into centrifuged cups. Solutions were topped with 20 ml of distilled water and centrifuged (BECKMAN COULTER, Avanti® J-E Centrifuge, USA) at 10,000 rpm at 4°C for 20 min, in order to obtain a concentrated pellet. The supernatant from each solution was discarded and the pellet was topped with deionised water. Centrifugation was repeated 3 times in order to remove unreacted material that is either in a form of a compound or biomass residue i.e., any free enzyme/protein molecules that are not bound to the AgNPs. The pellet was dispersed in deionised water and vortexed for 5 min (Vortex Mixer Model VM-1000). The resulting suspension of each extract was then oven-dried at 50°C for approximately 7 days. The yield of the synthesized AgNPs of each extract was determined using the following equation and thereafter characterised:

$$\text{Extract Yield (\%)} = \frac{\text{Weight of dried extract (g)}}{\text{Weight of plant material (g)}} \times 100$$

7.2.7 Characterisation of AgNPs

7.2.7.1 UV-visible spectroscopy

AgNPs synthesis was determined by examining each colloidal solution (1 ml) after synthesis using the SHIMADZU UV-1800 Spectrophotometer (Germany) at a range of 200–800 nm at a medium speed, using 1 mM $AgNO_3$ solution as a blank. The absorption spectra of each sample was correlated to that found in literature, confirming their successful synthesis. Prior to analysis, all synthesized samples were sonicated (SONICLEAN, sonication bath) and vortexed for 5 min, in order to ensure uniformity of the solution.

7.2.7.2 Scanning electron microscopy (SEM)

Approximately 2 ml of each synthesized NPs solution was pipetted separately into 3 ml Eppendorf Tubes® and sonicated (SONICLEAN, sonication bath) for 20 min. Thereafter, approximately 20 µL of each solution was pipetted onto aluminium stubs and left to dry under a mercury lamp for 60 min. The stubs were sputter coated with gold using the Quorum 150 RES. Samples were viewed and analyzed on an Ultra Plus field emission gun scanning electron microscope (FEGSEM) (Carl Zeiss, Germany). Images were captured using the SmartSEM imaging software.

7.2.7.3 Energy-dispersive X-ray spectroscopy (EDX)

Energy dispersive X-ray spectroscopy analytically identifies the elemental composition present in any material.The synthesized NPs solutions of the leaves and stems were sonicated (SONICLEAN, sonication bath) for 20 min. Thereafter, approximately 20 µL of each solution was dispensed onto a glass coverslip attached to an aluminium stub containing double-sided adhesive carbon tape. Solutions were dried under a mercury lamp for about 60 min. Elemental composition of NPs produced from leaf and stem methanolic extracts were identified using the Aztec analysis software on the Ultra Plus FEGSEM (Carl Zeiss, Germany) at 5kV.

7.2.7.4 High resolution transmission electron microscopy (HRTEM)

The shape, size and distribution of the synthesized AgNPs produced from leaves and stems were analysed with the HRTEM. Each solution was sonicated (SONICLEAN, sonication bath) to ensure that the AgNPs were evenly distributed. Formvar carbon coated grids (400-mesh) (Ted Pella Inc. Redding, USA), were dipped in each solution and placed under a lamp for 1 h at room temperature, to allow the solvent to evaporate. Samples were viewed under the JEOL JEM HRTEM 2100 (Tokyo, Japan) at 200 kV. Size of the AgNPs were determined using ImageJ software Java 1.53e.

7.2.7.5 Nanoparticle Tracking Analysis (NTA)

In order to accurately represent the size distribution, zeta potential and nano-complexes of all synthesized AgNPs, NTA (Nanosight NS-500, Malvern Instruments, UK) analysis was performed at 25°C. Approximately 2 ml of each NP (methanol, aqueous- fresh and –powdered AgNPs) solution was removed, and dispensed into Eppendorf tubes (3 ml). About 1 ml of a 1:500 dilution (in 18 Mohm water/ Ultrapure Millipore water) of each extract was prepared in a suspension and analysed, in order to determine the hydrodynamic diameter. All samples were analysed in triplicate. Images were viewed, captured and analyzed using the NTA 3.2 analytical software.

7.2.7.6 Fourier transform infrared spectroscopy (FTIR)

In order confirm the presence of capping functional groups of AgNPs, infrared spectroscopy of the leaves and stem dried extracts and their synthesized AgNPs were achieved using the Agilent Cary 630 spectrometer using Agilent MicroLab PC 5.1.22. Resolution Pro 5.0.0.395 was used to process the data for peaks. Data was collected using ATR Diamond^{-1} Bounce with 30 background scans and 30 sampling scans with a resolution of 4 cm^{-1}. Detection of peaks displayed by the various function groups were scanned at a range between 3800-800 cm^{-1}. The analysis was conducted at the Chemistry Department at the University of KwaZulu-Natal, Pietermaritzburg campus.

7.2.8 Antibacterial bioassays

7.2.8.1 Test microorganisms

The antibacterial activity of both leaf and stem AgNPs samples were tested against 5 strains. These strains were Gram-positive: *Bacillus subtillus* (ATCC 6633), *methicillin*-resistant *Staphylococcus aureus* (ATCC 43300), *Staphylococcus aureus* (ATCC 25923); and Gram-negative bacteria: *Pseudomouas aeruginosa* (ATCC 25783), *Escherichia coli* (ATCC 35218). These bacterial strains were supplied by Professor Johnson Lin, School of Life Sciences (Microbiology Department), University of KwaZulu-Natal, and maintained in 75% glycerol at −80 °C.

7.2.8.2. Preparation of sample

Synthesized NPs from the methanolic, aqueous- fresh and –powdered leaf and stem extracts were dissolved in 10% dimethyl sulfoxide (DMSO) at various concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 mg/mL. Prepared samples were transferred to 3 ml Eppendorf Tubes® and stored at 4°C until further use.

7.2.8.3 Preparation of culture media and bacterial cultures

Mueller-Hinton agar (MHA, 38 g) (Biolab, South Africa) was suspended in 1 L of distilled water, stirred for 15 min, heated in a microwave (10 min) and autoclaved (Model HL-340) for 1 h at 121°C. The medium was dispensed into sterile Petri plates (90 mm) at room temperature (23°C). Each bacterial strain (Gram-positive and –negative) from stock cultures were sub-cultured onto fresh agar plates and incubated overnight (24 h) at 37°C. Glass test tubes containing distilled water (15-20 ml) were autoclaved at 121°C for 1 h. Bacterial colonies from each plate were inoculated by transferring 5 $\mu g.\mu L^{-1}$ of the strain into the glass test tubes containing 15 ml of sterile distilled water (0.5 McFarland scale). The absorbance of each bacterial culture was measured, adjusted, and diluted to attain a viable cell count using a Cary 60 UV-Vis spectrophotometer.

Freshly cultured bacterial strains were smeared over the MHA plates with an L-shaped metal spreader (hockey stick). The disk diffusion method was used to assess the antibacterial activity of the synthesized AgNPs. Approximately, 20 µL of AgNPs prepared with the crude methanolic and aqueous– fresh and powdered extracts were pipetted onto sterile filter paper discs (Whatman® no. 1 filter paper) (6 mm in diameter). Sterile discs loaded with AgNPs of varying concentrations were allowed to dry overnight and thereafter placed onto Petri plates containing bacteria. Petri plates were sealed and incubated for 24 h at 37°C, overnight. Zones of inhibition visible around the filter paper were taken as positive results, and diameters were measured and photographed within 18-24 h after incubation, in order to determine if the synthesized AgNPs exhibited any antibacterial activity. Clear zones of inhibition were measured and tabulated. Filter paper discs loaded with streptomycin and gentamycin were used as the positive

controls and 10% DMSO as the negative control. The analyses were conducted in triplicate and data was presented as mean ± standard deviation.

7.2.9 *In vitro* cytotoxicity/ MTT assays

7.2.9.1 Cell cultures

The cytotoxicity of AgNPs were assessed in the human embryonic kidney (HEK293), cervical cancer cells (HeLa) and breast adenocarcinoma (MCF-7) cells. Cryopreserved cells were obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA. Experimental work on cell cultures were carried out in a sterile class II biohazard hood. Cryopreserved cell lines stored in a -80°C Nuaire biofreezer were removed and promptly placed in a 37˚C water bath to thaw. Cell suspensions were aseptically transferred into centrifuge tubes and centrifuged (Eppendorf benchtop centrifuge) at 1000 rpm for 5 min, and the supernatant discarded. The pellet (cells) was then re-suspended in 1 ml of complete sterile medium (Eagle's Minimum Essential Medium (EMEM) supplemented with 1% antibiotics (100 units/mL penicillin, 100 µg/mL streptomycin) and 10 % Foetal Bovine Serum (FBS)). Cell suspensions were transferred and grown in a 25 cm^2 tissue culture flask comprising 4 ml of complete sterile medium. Thereafter, cells were incubated (Thermo-Electron Corporation, Waltham, Massachusetts, USA) at 37°C (comprising 5% CO_2) and examined daily using an inverted microscope (Nikon TMS-F 6V, Tokyo, Japan). The sterile complete medium was renewed regularly, till the cells were grown to confluency (Daniels and Singh, 2019).

7.2.9.2 MTT (cell viability) assay protocol

The metabolic activity of cells and their ability to reduce MTT 3-[(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide] to formazan via the succinate- tetrazolium reductase system was measured by this assay (Mosman, 1983). Cells were trypsinized and seeded into 96-well microtiter plates and incubated at 37˚C overnight to allow cells to attach. Thereafter, the growth medium was replaced with fresh medium (EMEM +10% FBS + 1% Antibiotics) (Daniels and Singh, 2019), and cells were treated with varying concentrations of AgNPs synthesized from *B. albostellata* (15, 30, 60, 120 and 140 µg/ mL) and incubated at 37˚C for 48 h. The growth medium in each well was then aspirated, followed by the addition of 100 µl of medium containing 10 µl of the MTT solution (5 mg/ml in Phosphate Buffered Saline (PBS) solution) and incubated for 4 h at 37°C. The medium containing MTT was then removed from each well and replaced with 100 µl of DMSO to ensure total solubility of the formazan crystals. The occurrence of these crystals is indicative of cellular viability (Vinken and Blaauboer, 2017). Upon DMSO addition, the solution turned purple in colour. Absorbance was recorded at 570 nm using the Mindray M-R-96A microplate reader (Vacutec, Hamburg, Germany), with DMSO as a blank. Positive controls (containing cells only), were recorded as 100 % survival (Daniels and

Singh, 2019). This assay was performed in triplicate and graphs generated using Microsoft Excel 2019 ™ were used to calculate the concentration at which 50% cell death was achieved (IC_{50}).

7.2.10 Statistical Analysis

Experimental analyses were carried out in triplicate. Values were expressed as mean ± standard deviation and subjected to statistical analysis using R statistical computing software, 2020, version 3.6.3. Data were statistical analysed using the One-Way Analysis of Variance (ANOVA) followed by Tukey's-honest significant difference multiple range post hoc tests. Data were expressed as mean significant at $p< 0.05$ level.

7.3 Results and discussion

7.3.1 Synthesis of AgNPs and UV characterisation

The reduction of $AgNO_3$ in leaf and stem extracts after 3 h of incubation at 80°C were visually evident from the colour change (brownish-yellow) of the reaction mixture (Figure 7.1). These observed colour changes are indicative of the production of AgNPs (Vanaja and Annadurai, 2012; Kannan et al., 2013). The observed colour change was due to the reduction of Ag^+ to Ag^0 by numerous biomolecules present in the plant extract (Govindarajan and Benelli, 2016). Results showed that the brown colour intensity increased with the duration of the incubation period. However, after 3 h, there were no visible colour change in the various solutions. This may be due the reduction of $AgNO_3$ and the excitation of the surface plasmon resonance (SPR) effect (Mulvaney, 1996). This effect is a unique optical phenomenon of metal NPs that can be easily detected under UV-vis light and arises due to the surface plasmon oscillation of free electrons (Jensen et al., 1999). AgNPs synthesized from plant extracts often display intense colour changes following incubation (Heemasager et al., 2014). An intense brown colour was also observed in aqueous leaf extracts of *B. prionitis* (Ghosh et al., 2016).

The highest percentage yield of the synthesized AgNPs from *B. albostellata* was obtained from the powdered stems solution (0.50%), followed by 0.32% from the methanolic leaf AgNPs (Table 7.1). The lowest percentage yield was obtained from NPs from the fresh stem solution (0.10%). Overall, this suggests that the percentage yield of phyto-compounds in *B. albostellata* were greater in the leaf synthesized extract than from the stem. NP yield can be effected by several factors, such as the type of material used, amount of phytocompounds and metals present in the extracts used for the synthesis (Chandran et al., 2006; Jiang et al., 2011). AgNPs obtained from the various extracts were characterised by UV-vis spectroscopy (Figure 7.2) and major peaks were found along the absorption band of the SPR (Mulvaney, 1996). Characteristic absorption peaks were observed at 416 nm (methanol leaves), 402 nm (methanol stems), 400 nm (fresh leaves), 398 nm (fresh stems), 450 nm (powdered leaves) and 438 nm (powdered stems), respectively. Most of these peaks confirmed the formation of AgNPs. Previous literature have stated that AgNPs have a λmax in the range of 400–450 nm (Bogireddy et al., 2016; De

Aragaˆo et al., 2019). Plasmon bands were extended in figure 7.2 with an absorption tail at longer wavelengths which may be associated with the distribution and size of NPs (Ahmad et al., 2003). Pirtarighat et al. (2019) suggested broad plasmon bands found in a specific spectrophotometric range may be due to several metabolites present in the synthesized AgNPs from the plant extract. AgNPs produced from the leaf and stem solutions had a maximum absorbance of 4.00 (Figure 7.2). AgNPs synthesized from *B. longiflora* and *B. cristata* leaf extracts displayed a maximum absorption at 443 nm and 449 nm, respectively (Govindarajan and Benelli, 2016), while synthesis using aqueous leaf extracts of *B. prionitis,* produced a maximum absorption at 420 nm (Ghosh et al., 2016).

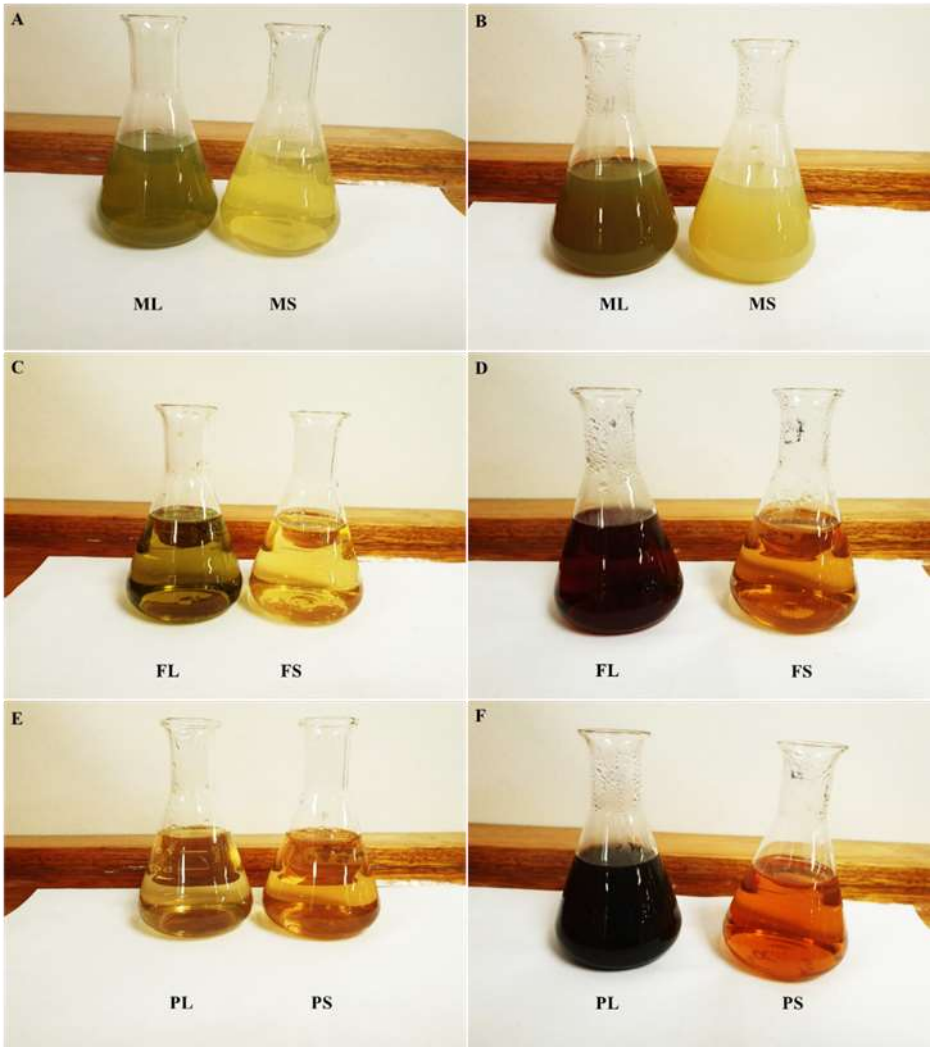

Figure 7.1: Silver nanoparticles synthesized from leaf and stem extracts of *B. albostellata*. (A) Leaf and stem methanolic extracts before AgNPs synthesis; (B) Leaf and stem methanolic extracts after incubation with $AgNO_3$; (C) Fresh leaf and stem extracts before AgNPs synthesis; (D) Fresh leaf and stem extracts after incubation with $AgNO_3$; (E) Powdered leaf and stem extracts before AgNPs synthesis; (F) Powdered leaf and stem extracts after incubation with $AgNO_3$.

Table 7.1: Percentage yield of the leaf and stem synthesized extracts of *B. albostellata*

Crude extract	Leaves	Stem	Leaves	Stem
	Dried AgNPs yield (g)		Percentage yield (%)	
Methanol	0.032	0.030	0.32	0.30
Fresh	0.030	0.010	0.12	0.04
Powdered	0.026	0.050	0.26	0.50

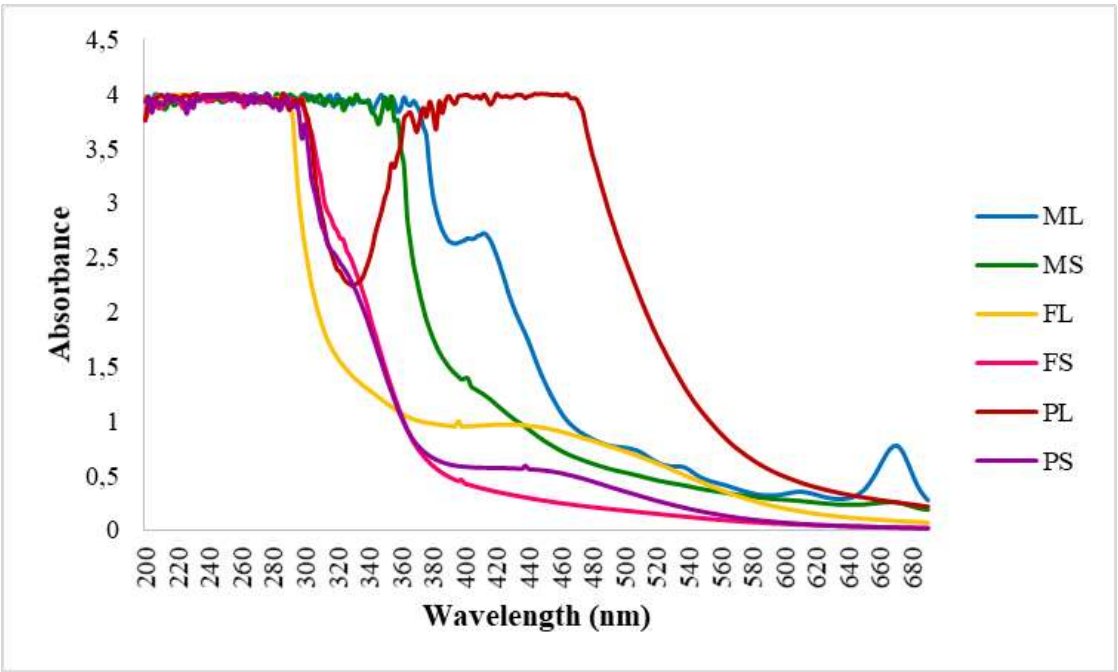

Figure 7.2: UV-visible spectroscopy of AgNPs synthesized from methanol, fresh and powdered leaves and stem extracts of *B. albostellata*, after 3 h incubation with $AgNO_3$. ML= Methanol leaf; MS= Methanol stem; FL= Fresh leaf; FS= Fresh stem; PL= Powdered leaf; PS= Powdered stem.

7.3.2 Scanning electron microscopy and EDX analysis

Synthesized AgNPs from the various extracts were distributed throughout the sample and showed some agglomeration (Figures 7.3A-7.8A). MubarakAli et al. (2011) reported that this could be due to a dehydration-induced aggregation of AgNPs. Micrographs confirmed that synthesized AgNPs were nano-sized (<100 nm) and appeared roughly spherical in shape (Figures 7.3A-7.8A). Synthesized AgNPs from *B. prionitis* (Ghosh et al., 2016) and *B. cristata* (Gomathi et al., 2018) were also reported to be predominantly spherical in shape. All prepared samples for SEM analysis displayed considerable NP agglomeration, thus SEM micrographs were not ideal for assessing NP size. Gomathi et al. (2018) suggested that AgNPs synthesized from the leaf extracts of *B. cristata* were agglomerated because the biological constituents may have gathered the small particles, forming larger knobs.

EDX confirmed the presence of AgNPs and displayed strong peaks at 3 keV (Figures 7.3 B - 7.8 B). These peaks confirmed the biosynthesis of AgNPs and the organic compounds which were present on the NPs (Bello et al., 2017). AgNPs synthesized from the leaf extracts of *B. prionitis* also displayed strong peaks at 3 keV (Ghosh et al., 2016). Several studies confirmed the presence of Ag^+ by observing the energy peak at 3 keV (Ahluwalia et al., 2014; Muthukrishnan et al., 2015). Overall, AgNPs from the methanolic leaf solution (Figure 7.3 B) displayed the highest percentage of elemental Ag^+ production (16.87 ± 0.89) and the lowest was found in the powdered stems (7.13 ± 1.44%) (Table 7.2). AgNPs from the methanolic stems had the second highest percentage of 14.78 ± 3.53. Fresh leaves and stems displayed Ag^+ production at 9.47 ± 1.38% and 8.12 ± 0.71%, while the powdered leaf and stem material exhibited percentages at 8.85 ± 1.09 and 7.13 ± 1.44, respectively (Table 7.2). Kumar et al. (2010) proposed that different plant parts vary in terms of the biochemical constituents they contain which in turn affects the synthesis, production, size and shape of AgNPs. The significantly higher production of AgNPs obtained using leaf extracts (relative to the stems) may be due to their high concentration of secondary metabolites, as opposed to the stems, which may promote metal ion reduction (Patel, 2013).

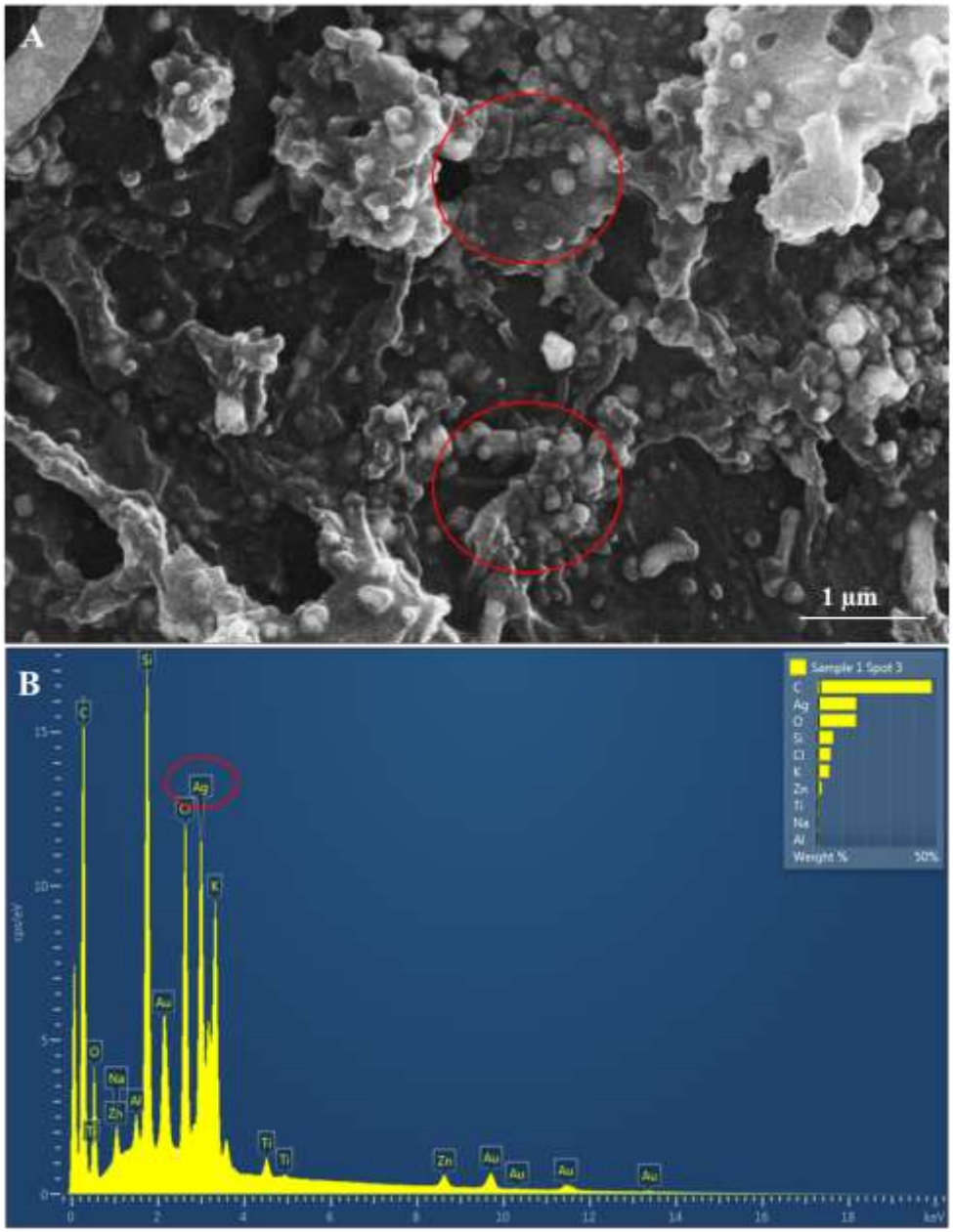

Figure 7.3: Micrographs of AgNPs synthesized from the methanol leaf extracts of *B. albostellata*. (A) Aggregated AgNPs; (B) EDX spectroscopy. Circles indicate AgNPs agglomeration.

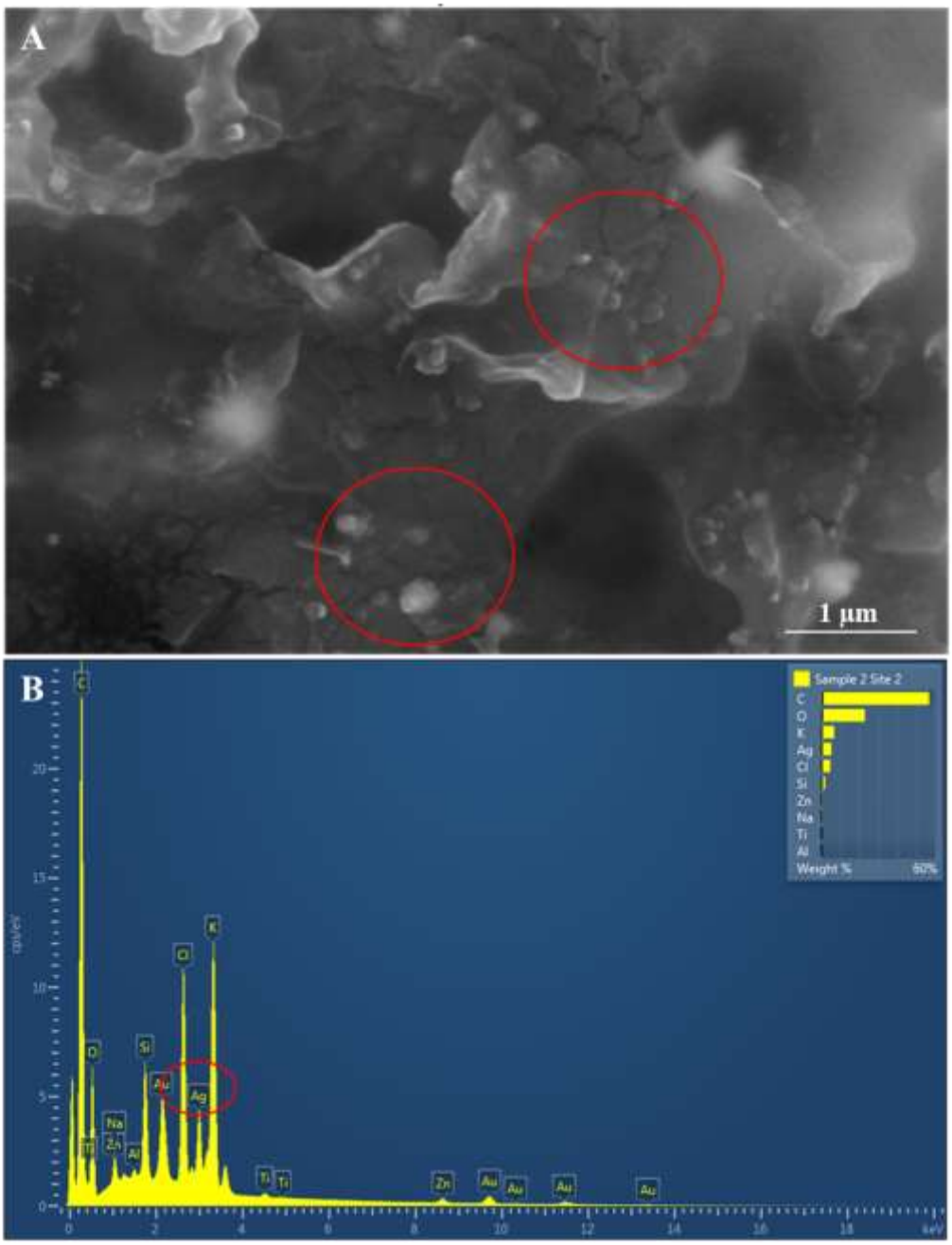

Figure 7.4: Micrographs of AgNPs synthesized from the methanol stem extracts of *B. albostellata*. (A) Aggregated AgNPs; (B) EDX spectroscopy. Circles indicate AgNPs agglomeration.

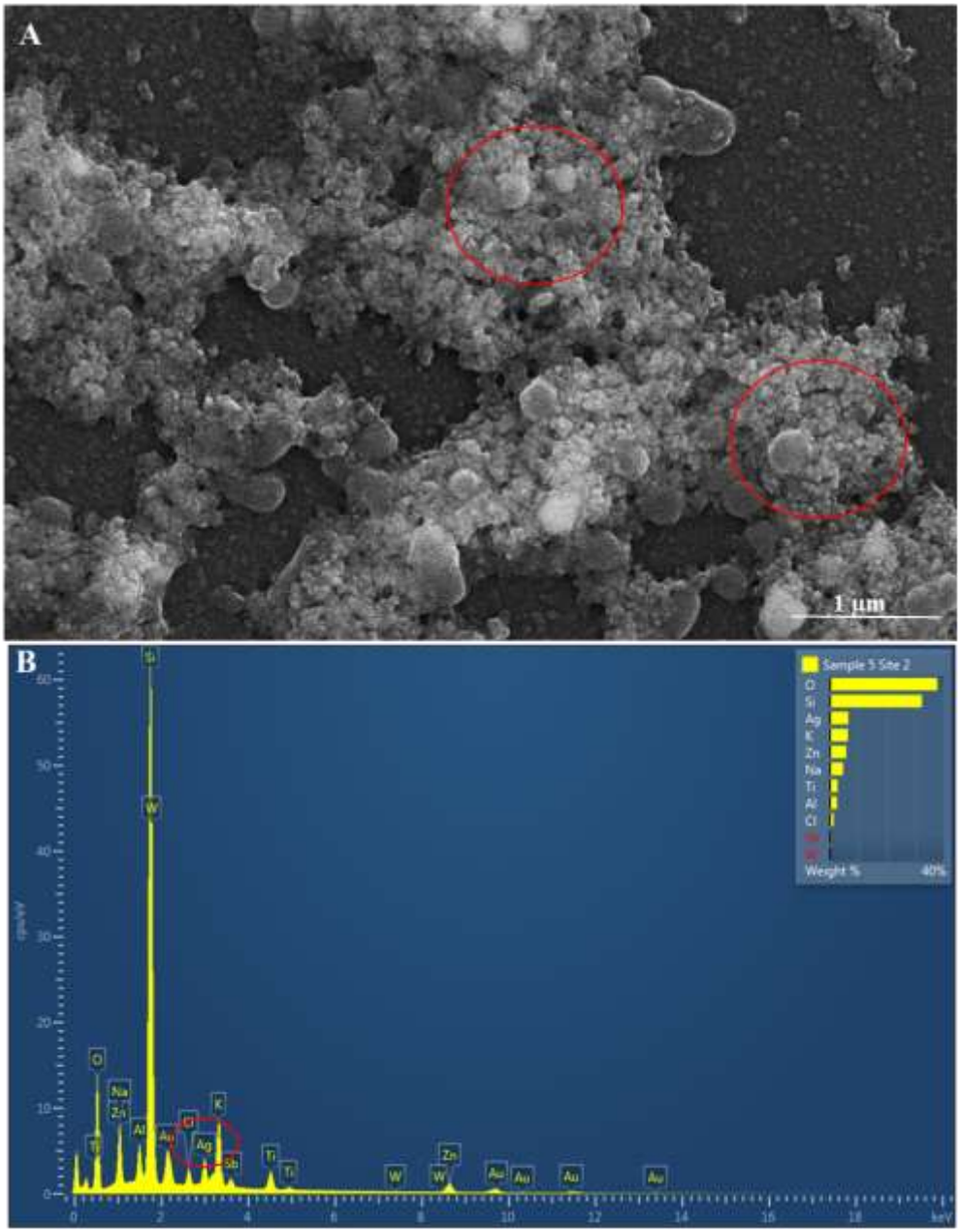

Figure 7.5: Micrographs of AgNPs synthesized from the fresh leaf extracts of *B. albostellata*. (A) Aggregated AgNPs; (B) EDX spectroscopy. Circles indicate AgNPs agglomeration.

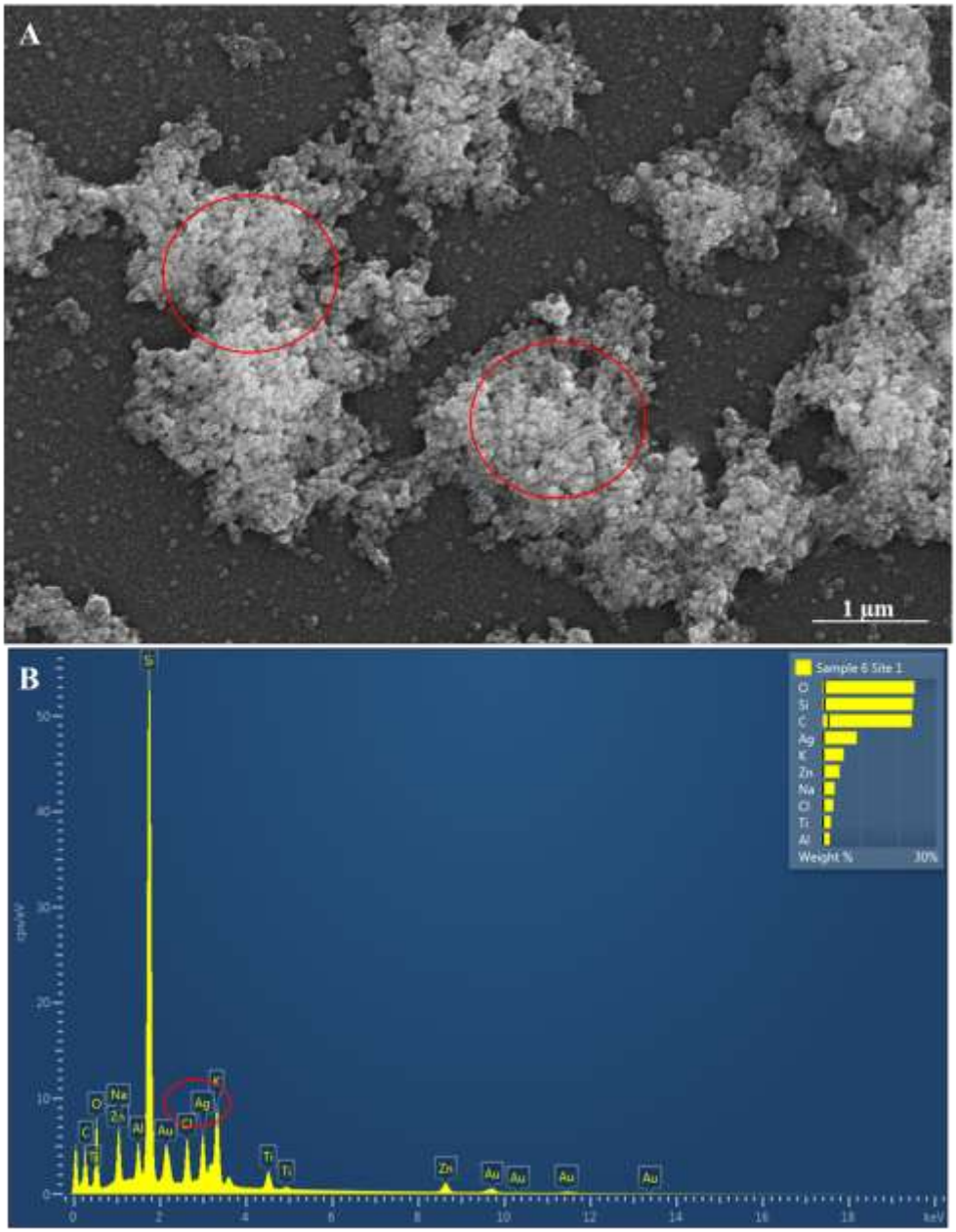

Figure 7.6: Micrographs of AgNPs synthesized from the fresh stem extracts of *B. albostellata*. (A) Aggregated AgNPs; (B) EDX spectroscopy. Circles indicate AgNPs agglomeration.

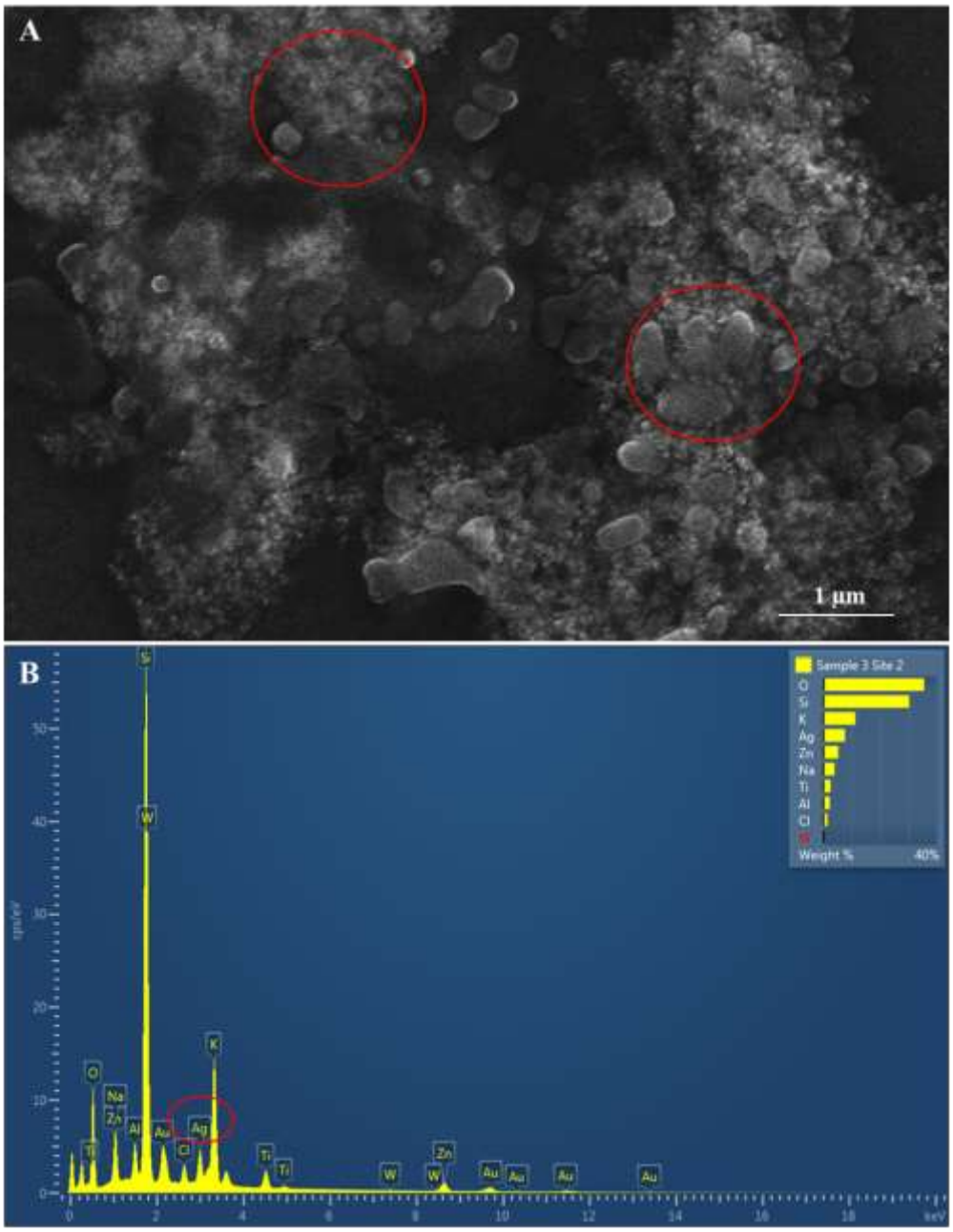

Figure 7.7: Micrographs of AgNPs synthesized from the powder leaf extracts of *B. albostellata*. (A) Aggregated AgNPs; (B) EDX spectroscopy. Circles indicate AgNPs agglomeration.

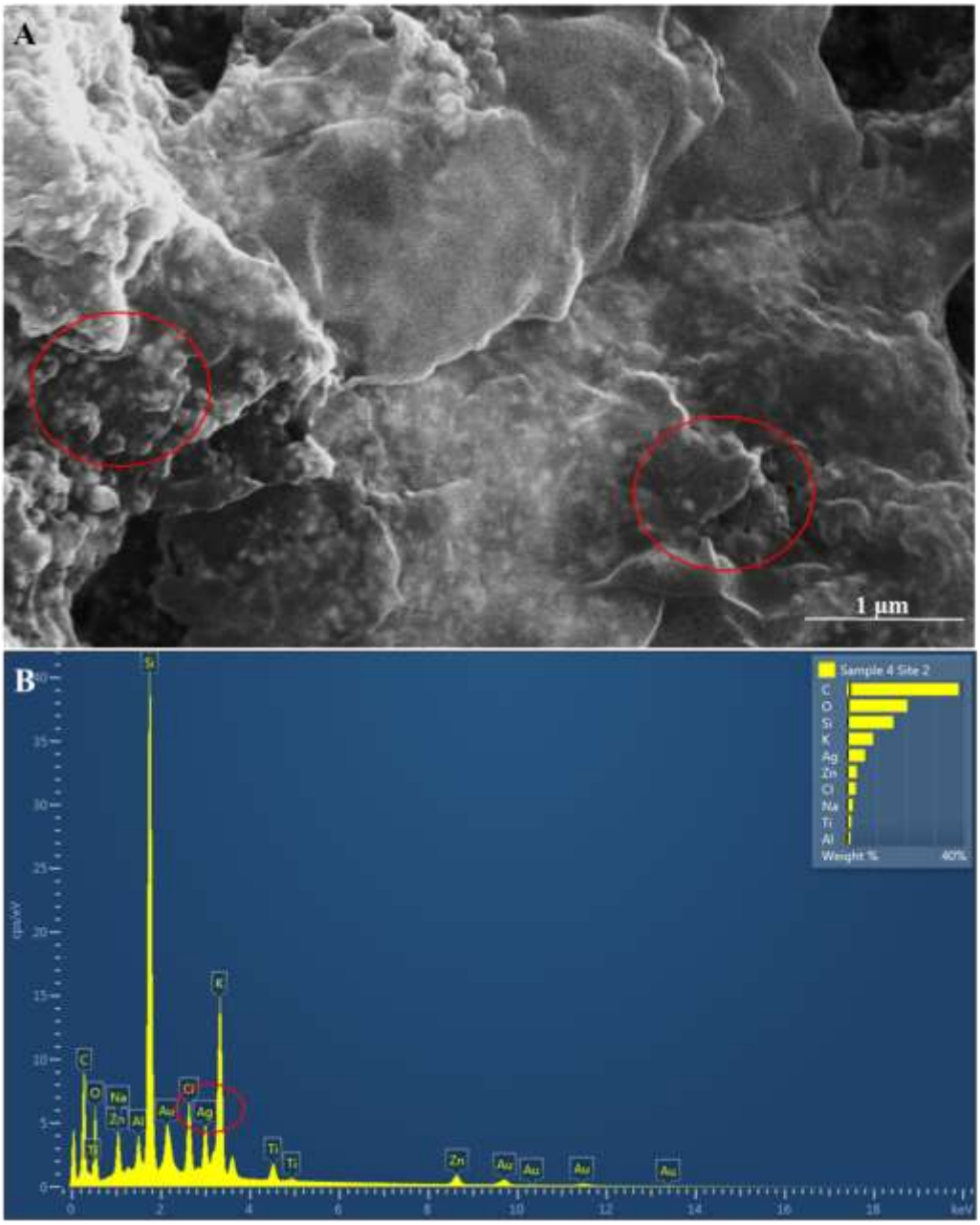

Figure 7.8: Micrographs of AgNPs synthesized from the powder stem extracts of *B. albostellata*. (A) Aggregated AgNPs; (B) EDX spectroscopy. Circles indicate AgNPs agglomeration.

Table 7.2: Average % weight of AgNPs synthesized from various leaves and stems extracts of *B. albostellata*

Type of extract	Average % weight
Methanol leaf	16.87 ± 0.89
Methanol stem	14.78 ± 3.53
Fresh leaves	9.47 ± 1.38
Fresh stems	8.12 ± 0.71
Powdered leaves	8.85 ± 1.09
Powdered stems	7.13 ± 1.44

Data displayed as mean ± SD of triplicate

7.3.3 High-Resolution Transmission electron microscopy of synthesized AgNPs

Transmission electron microscopy was used to examine the structure of the synthesized AgNPs. NPs were dispersed fairly and occasionally observed in agglomeration or to coalesce into nano-clusters. The slight aggregation may be as a result of the high surface energy that usually occurs when the preparing the NPs in a water medium (Shao et al., 2018). NPs synthesized from both leaf and stem extracts (Figures 7.9-7.11) were found to be small and roughly spherical in shape. Certain NPs were either spherical or triangular in shape. This variation in shape may be as a result of different groups of phytochemicals which aid in the reduction and stabilization of the AgNPs (Nayak et al., 2015). The kinetic energy of molecules increases at higher temperatures. This reaction speeds up the Ag ion consumption, thus reducing the likelihood for particle size development (Verma and Mehata, 2016). This could explain the small particle size found in the HRTEM micrographs.

According to Verma and Mehata (2016), the size of AgNPs can be altered by varying pH levels, concentration and temperature. A slight layer of film was observed around the AgNPs in the stem extracts (Figure 5.6 B). Mallikarjuna et al. (2011) observed similar films around the synthesized AgNPs and recognised them as the functional groups capping the AgNPs. This was further confirmed by Cittrarasu et al., (2019), who found similar films in the aqueous leaf extracts of *B. longiflora* and suggested the thin layer was the organic material capping of the AgNPs. Additionally, the capping may effectively prevent the agglomeration of AgNPs. Furthermore, Mittal et al. (2013) suggested that the capping of functional groups may provide added stability to the AgNPs in the solution.

According to the histogram for the mean particle size of synthesized AgNPs from the methanolic leaves and stems (Figures 7.12 and 7.13), aqueous fresh leaves and stems (Figures 7.14 and 7.15), and aqueous powdered leaves and stems (Figures 7.16 and 7.17) of *B. albostellata*, AgNPs were not uniform in size and varied across the different extracts; however, all particles were below 100 nm. Mean data of the

synthesized NPs from the leaf and stem extracts were 31.69 nm (Figure 7.12), 34.32 nm (Figure 7.13), 21.77 nm (Figure 7.14), 20.48 nm (Figure 7.15), 18.39 nm (Figure 7.16) and 16.57 nm (Figure 7.17), respectively. AgNPs of a similar size (15–30 nm) were observed in *B. cristata* (Gomathi et al., 2018). Additionally, NPs synthesized from the aqueous leaf extracts of *B. prionitis* were spherical and varied from 10 to 20 nm (Ghosh et al., 2016).

Data in Figure 7.12 were bimodal, as two peaks were observed, this meant that the size of majority of the AgNPs from the methanolic leaf extracts were between 20-40 nm. Additionally, data in Figures 7.13 and 7.17 were left skewed, with a large portion of the data on the right, and smaller observations trailing off to the left. In Figures 7.14 and 7.15, the data were right skewed, with majority set off to the left, while a few observations were trailing off to the right. Figure 7.16 displayed data that appeared almost bell-shaped, a large peak in the middle and tails that extend on either side at nearly the same frequency.

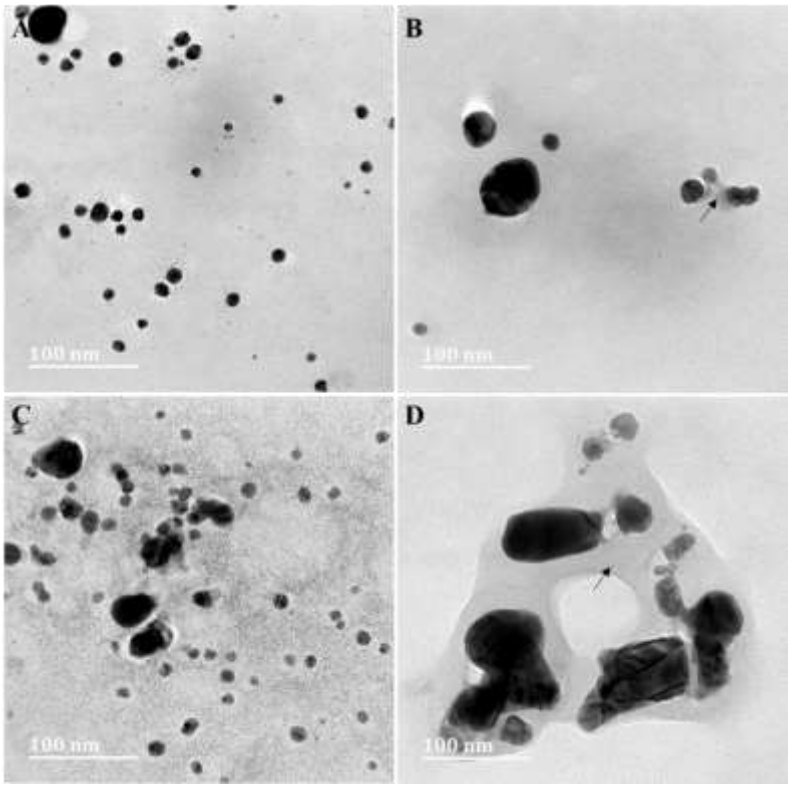

Figure 7.9: HRTEM micrographs of the AgNPs synthesized from, (A-B) methanolic leaf extracts; (C-D) methanolic stem extracts of *B. albostellata*. Arrowhead indicates film around AgNPs.

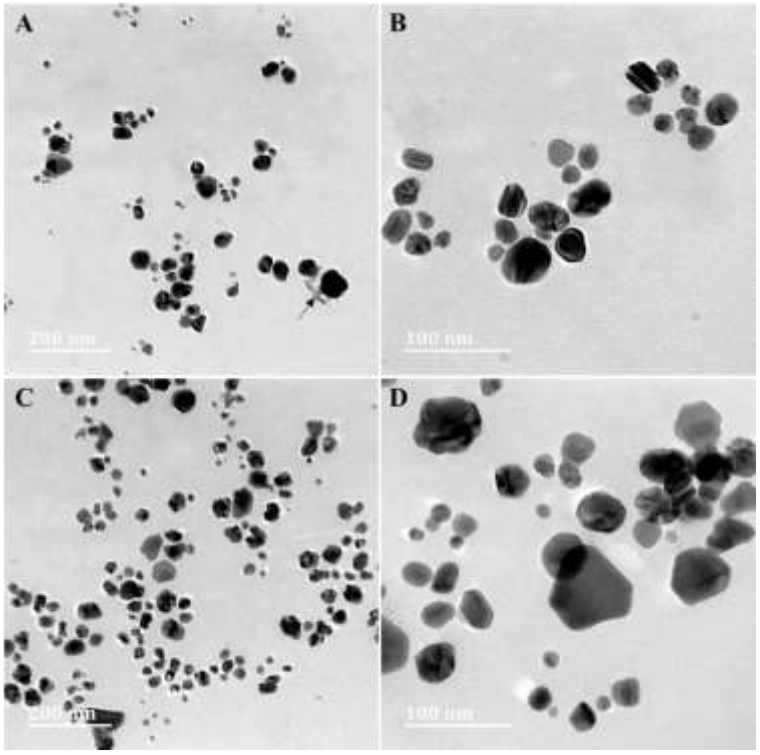

Figure 7.10: HRTEM micrographs of the AgNPs synthesized from, (A-B) fresh leaf extracts; (C-D) fresh stem extracts of *B. albostellata*. Arrowhead indicates film around AgNPs.

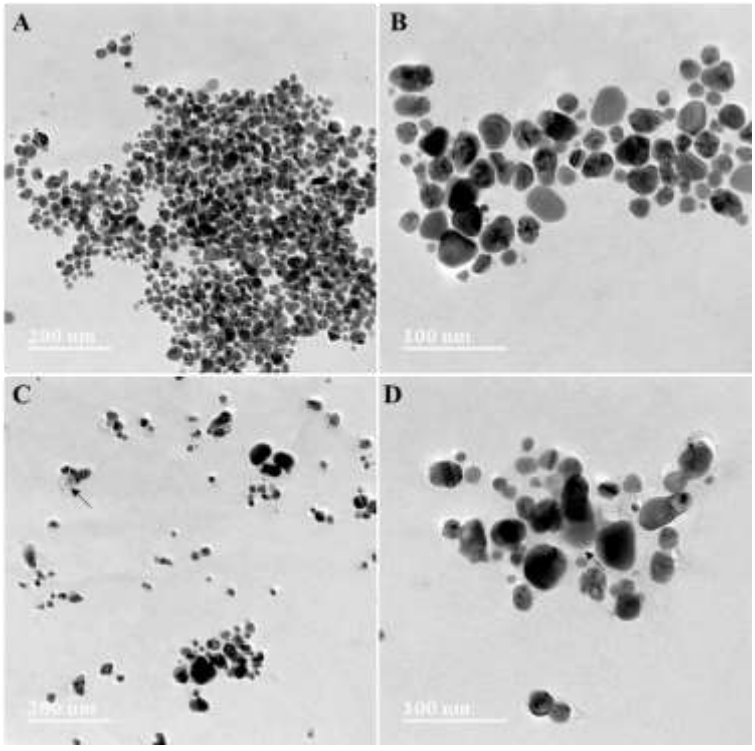

Figure 7.11: HRTEM micrographs of the AgNPs synthesized from, (A-B) powdered leaf extracts; (C-D) powdered stem extracts of *B. albostellata*. Arrowhead indicates film around AgNPs.

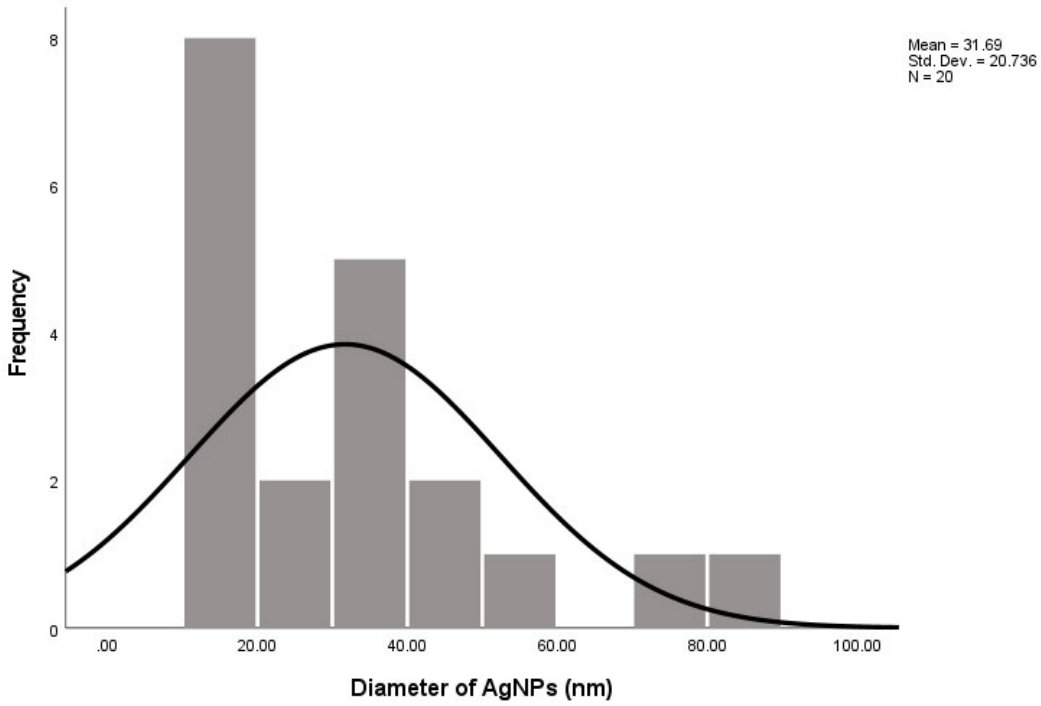

Figure 7.12: The mean particle size of synthesized AgNPs from the methanolic leaves of *B*. *albostellata*.

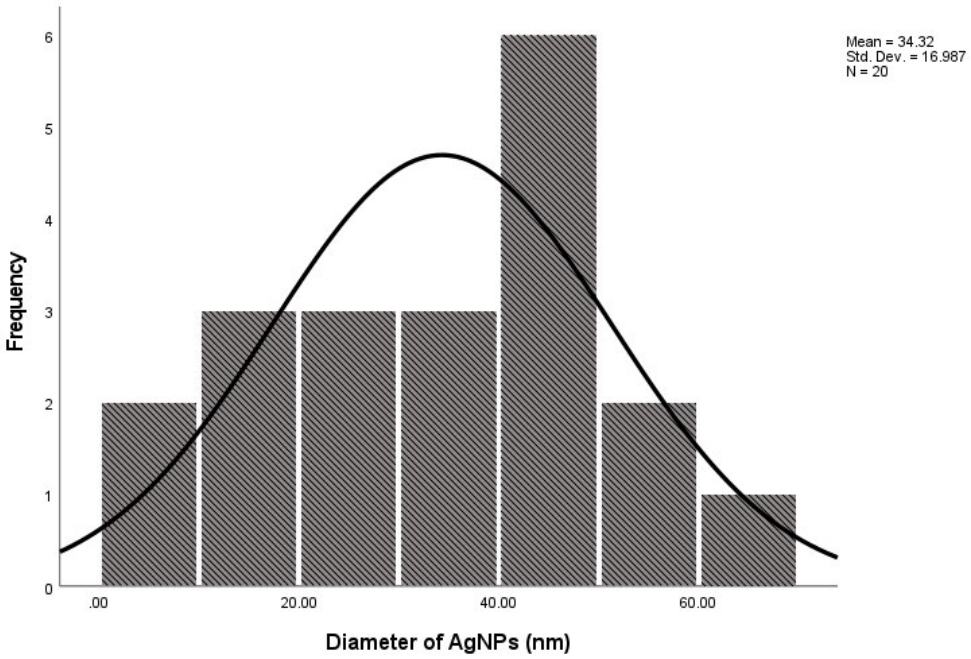

Figure 7.13: The mean particle size of synthesized AgNPs from the methanolic stems of *B*. *albostellata*.

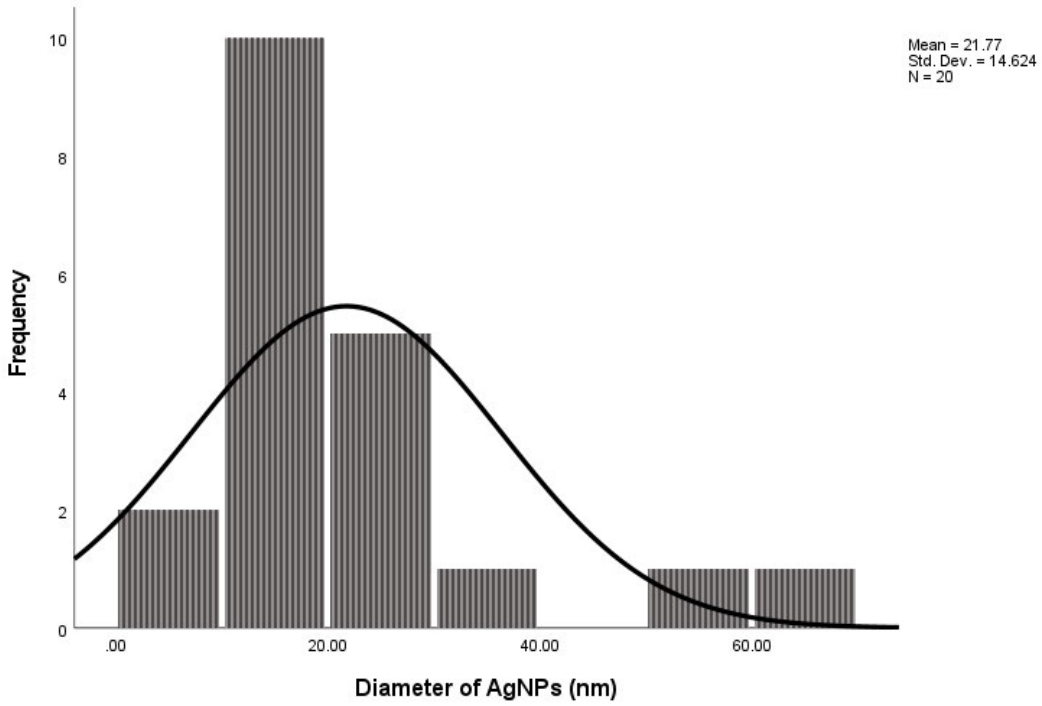

Figure 7.14: The mean particle size of synthesized AgNPs from aqueous fresh leaves of *B*. *albostellata*.

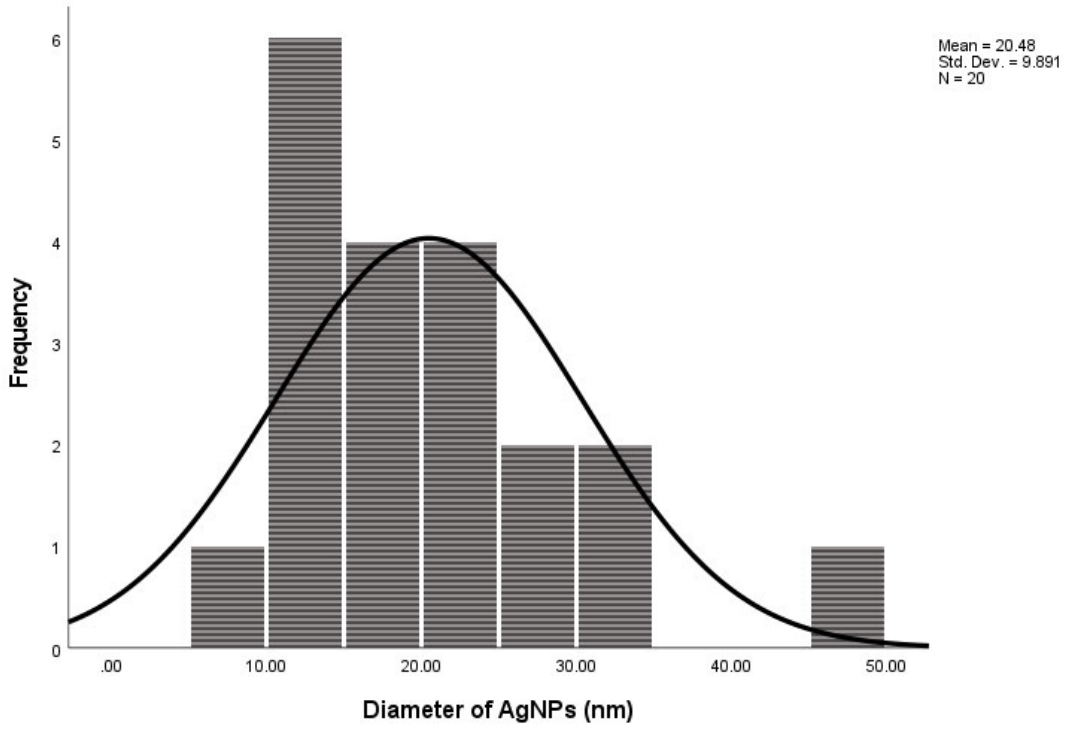

Figure 7.15: The mean particle size of synthesized AgNPs from aqueous fresh stem extracts of *B*. *albostellata*.

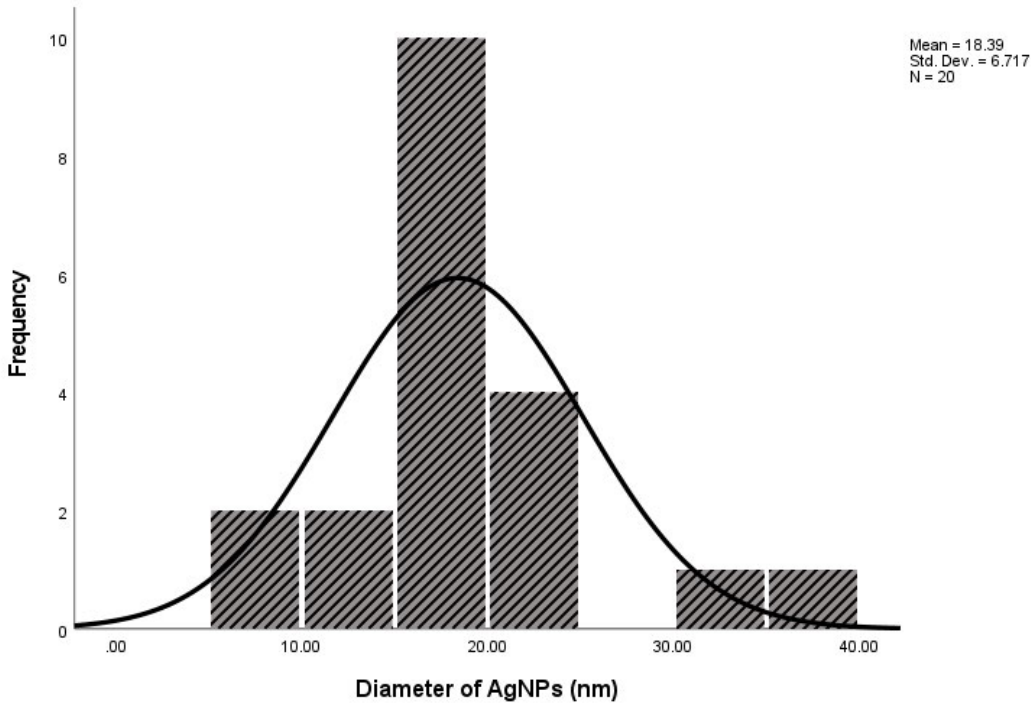

Figure 7.16: The mean particle size of synthesized AgNPs from powdered leaf extracts of *B. albostellata*.

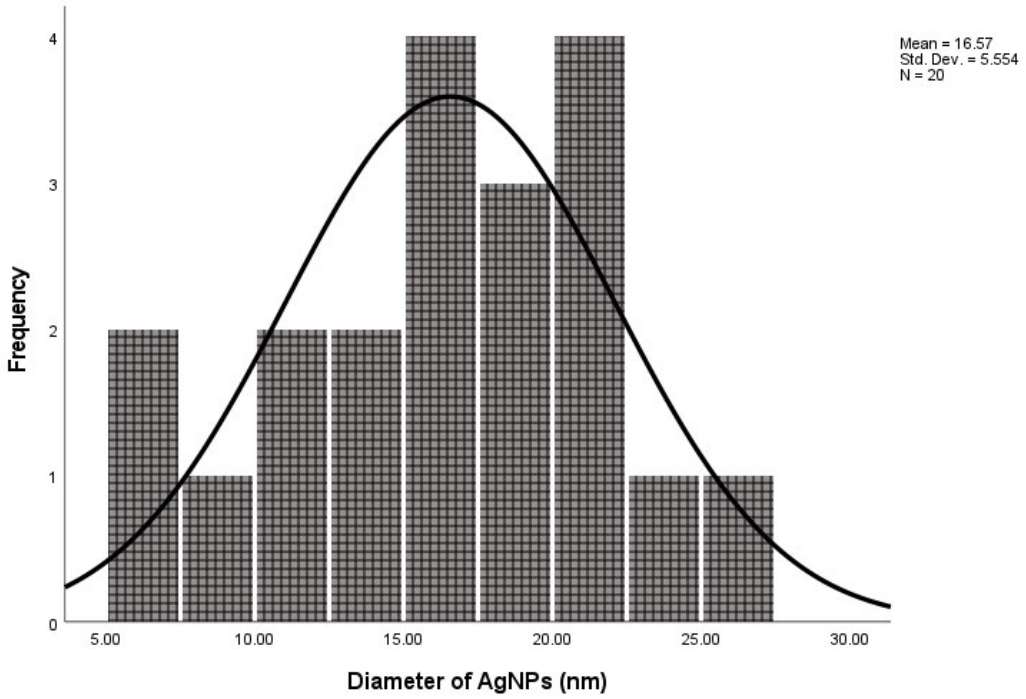

Figure 7.17: The mean particle size of synthesized AgNPs from powdered stem extracts of *B. albostellata*.

7.3.4 Nanoparticle Tracking Analysis (NTA)

NTA was used to precisely determine the size and distribution of the AgNPs, their colloidal stability and zeta potential, as shown in Table 7.3. These results indicated the AgNPs synthesized from the methanolic leaf and stem extracts had a mean diameter of 111.3 ± 4.4 nm and 110.7 ± 4.2 nm, which contradicts the HRTEM results. As stated previously, the various sizes of the synthesized AgNPs solutions were visualized using HRTEM and measured using IMAGEJ analysis, however, the sizes and zeta potential of the AgNPs, as well as their complexes were evaluated using NTA. The differences in results may be due to the fact that during HRTEM analysis, AgNPs solutions of the various extracts were pipetted onto copper grids, dried with aid of a UV lamp and thereafter visualized. However, in NTA, solutions are prepared in an aqueous suspension and analysed, resulting in a hydrodynamic diameter which is closer to what one might expect in an *in vivo* system. It has been reported from previous studies that sizes obtained from NTA are expectedly slightly larger than that seen under TEM (Akinyelu and Singh, 2018; Oladimeji et al., 2021). The zeta potential of a solution is regarded as the electrostatic value of the nanoparticle, and this then correlates to the surface charge of nanoparticles (Honary and Zahir, 2013).

Zeta potential has been commonly used by the pharmaceutical industry to evaluate their formulations for stability. A desirable zeta potential value less than −30 mV or greater 30 mV is usually considered to have adequate repulsive force, in order to achieve improved physical colloidal stability (Joseph and Singhvi, 2019). A zeta potential within this range is regarded as stable, as a result of the high levels of mobility within the solution, and greater levels of electrostatic repulsion, which minimises NP aggregation (Griffiths et al., 2011). Alternatively, a very small zeta potential value may result in the aggregation or flocculation of the NPs, as a result of van der Waals attractive forces acting upon them. This can cause physical instability of the NPs (Hunter, 2013; Freitas and Müller, 1998; Shah et al., 2014). Two factors that affect the zeta potential of a sample are pH and conductivity of the solution the NPs are suspended in (Chorom and Rengasamy, 1995). AgNPs synthesized from the leaf and stem methanolic extracts exhibited a high zeta potential of -33.2 ± 0.1 and -28.2 ± 0.0, respectively (Table 7.3). This indicates good long-term colloidal stability and potential suitability for *in vivo* application. The lowest zeta potential was observed in the AgNPs synthesized from the fresh leaf extracts (-8.8 mV).

Table 7.3: Size distribution and zeta potential analysis of AgNPs synthesized from various extracts

Sample (AgNPs)	Nanoparticle size (nm) (mean + standard error)	Zeta potential (mV) (mean + standard error)
Methanol leaf	111.3 ± 4.4	-33.2 ± 0.1
Methanol stem	110.7 ± 4.2	-28.2 ± 0.0
Fresh leaf	53.9 ± 8.4	-8.8 ± 0.2
Fresh stem	37.7 ± 0.9	-17.2 ± 0.0
Powdered leaf	57.9 ± 0.6	-17.0 ± 0.0
Powdered stem	34.3 ± 0.2	-16.8 ± 0.1

7.3.5 Fourier-transform infrared spectroscopy of synthesized AgNPs

The FTIR spectroscopy of synthesized AgNPs using the leaves and stems are presented in Figures 7.18-7.23, respectively. Prominent peaks were observed for the methanolic leaf extracts at 3273.31, 2927.50, 2346.67, 2119.29, 1622.25, 1393.67, 1333.55, 1287.63, 1034.01, 897.23, 813.23 cm^{-1} (Figure 7.18) and stems at 3330.48, 3276.33, 2347.91, 2119.13, 1619.30, 1474.26, 1392.44, 1332.30, 1041.01, 932.56, 895.77 cm^{-1} (Figure 7.19). Additionally, for the aqueous fresh leaf extracts, peaks were observed at 3397.89, 3370.78, 2331.58, 2122.09, 1911.28, 1599.40, 1389.56, 1323.17, 1070.81, 1039.08, 820.01, 722.42 cm^{-1} (Figure 7.20) and stems at 3269.77, 2929.85, 2348.90, 2110.30, 2094.08, 1614.30, 1391.17, 1330.95, 1038.69, 896.38, 821.34 cm^{-1} (Figure 7.21). Lastly, peaks for the aqueous powdered leaf extracts were detected at 3281.52, 3223.08, 2927.53, 2328.14, 2115.65, 1888.35, 1592.68, 1394.61, 1336.86, 1036.22, 808.04, 767.36 cm^{-1} (Figure 7.22) and stems at 3262.30, 2929.28, 2344.39, 2116.90, 1607.25, 1391.95, 1333.34, 1036.10, 766.74 cm^{-1} (Figure 7.23).

Absorbance peaks observed between 3550-3200 and 3200-2700 cm^{-1} are distinctive to a possible O-H stretching group of polyphenols/alcohol respectively (Dubey et al., 2010; Basnet et al., 2016). Shanmugam et al. (2014) proposed that these bonds may be due to the presence of enzymes, polysaccharides or proteins in the plant extract. A peak at 3330.48 cm^{-1} correspond to phenolic hydroxyl groups and secondary amines, a similar peak was observed in *B. longiflora* leaf aqueous extract (Cittrarasu et al., 2019). The presence of a moderate sharp peak at 2927 cm^{-1} was associated to C–H (methoxy compounds) stretching vibration (Marimuthu et al., 2011). The 2117 and 2120 cm^{-1} wavelengths display the C≡C stretch of alkynes. Bands appearing in the range of 1700–1600 cm^{-1} were linked to C–O stretching vibrations (Chand et al., 2020). Bands at 1619.30 and 1622.25 cm^{-1} were

characteristic to C=O stretching. According to Heneczkowski et al. (2001), a band at 1619 cm^{-1} was assigned to flavonoid and fisetin.

Peaks at 1650-1580 cm^{-1} may be linked to N-H bending (amine). Similar bending vibrations related to amine was observed in the leaf extracts of *B. prionitis* (Ghosh et al., 2016), while peaks at 1420-1330 cm^{-1} were related to O-H bending. Bands ranging from 1390-1380 cm^{-1} are characteristic to C-H bending of aldehyde. Aromatic amine, C-N stretching is related to peaks at 1342-1266 cm^{-1}. Aromatic esters are associated to C-O stretching at peaks of 1310-1250 cm^{-1}. Additionally, vibrational stretching of C-O was observed in the leaf extracts of *B. prionitis* (Ghosh et al., 2016). Peaks at 1250-1020 cm^{-1} relates to the C–N stretching vibration of amine (Devaraj et al., 2013). Peaks at 850-550 cm^{-1} are linked to C-Cl stretching, a halo compound. Peaks in the range of 880 ± 20 cm^{-1} and 810 ± 20 cm^{-1} corresponds to C-H bending. A peak at 755 ± 20 cm^{-1} represents C-H bending (1,2-disubstituted). According to Sutherland et al. (1994), terpenes show significant absorbance in regions below 700 cm^{-1}. Ghosh et al. (2016) reported the phytochemical diversity of the leaf extracts of *B. prionitis* comprising of phenols, ascorbic acid, citric acid, reducing sugars, alongside with several other compounds that may play an important role in both reducing and stabilizing the AgNPs. Majority of the absorbance bands from the synthesized extracts of *B. albostellata* showed characteristic functional groups of phenols, alcohols, terpenes, alkynes, aldehydes, primary and secondary amines. Therefore, these phyto-constituents present in the leaves and stems of *B. albostellata* have played an active role in the bioreduction of synthesized AgNPs.

Figure 7.18: FTIR spectra of AgNPs synthesized from leaf methanolic extracts of *B. albostellata*.

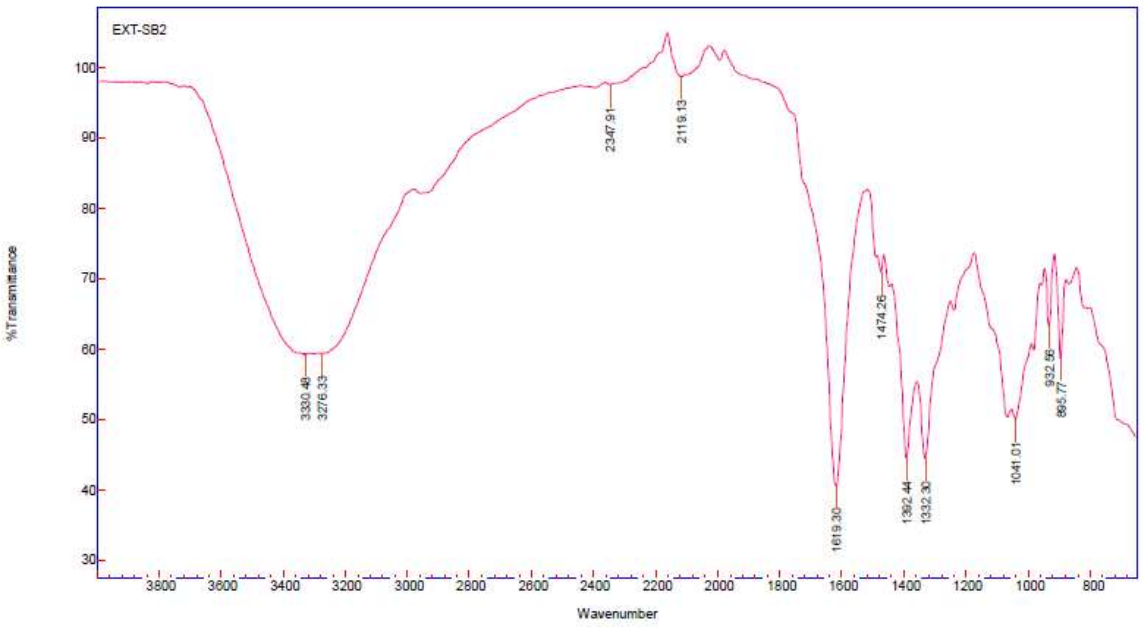

Figure 7.19: FTIR spectra of AgNPs synthesized from stem methanolic extracts of *B. albostellata*.

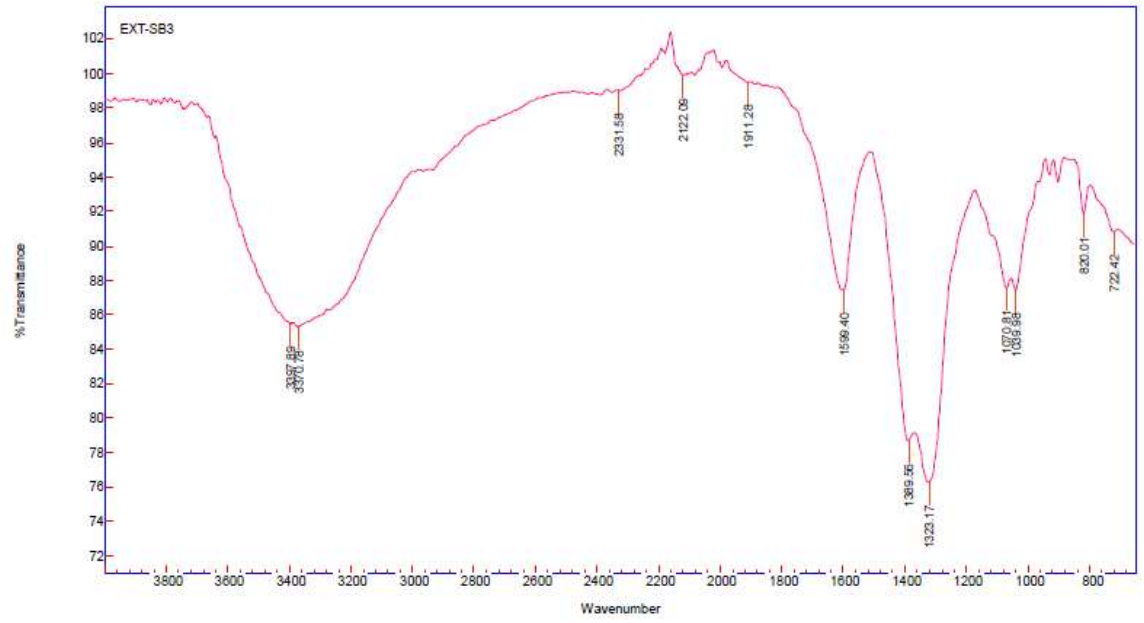

Figure 7.20: FTIR spectra of AgNPs synthesized from fresh aqueous leaf extracts of *B. albostellata.*

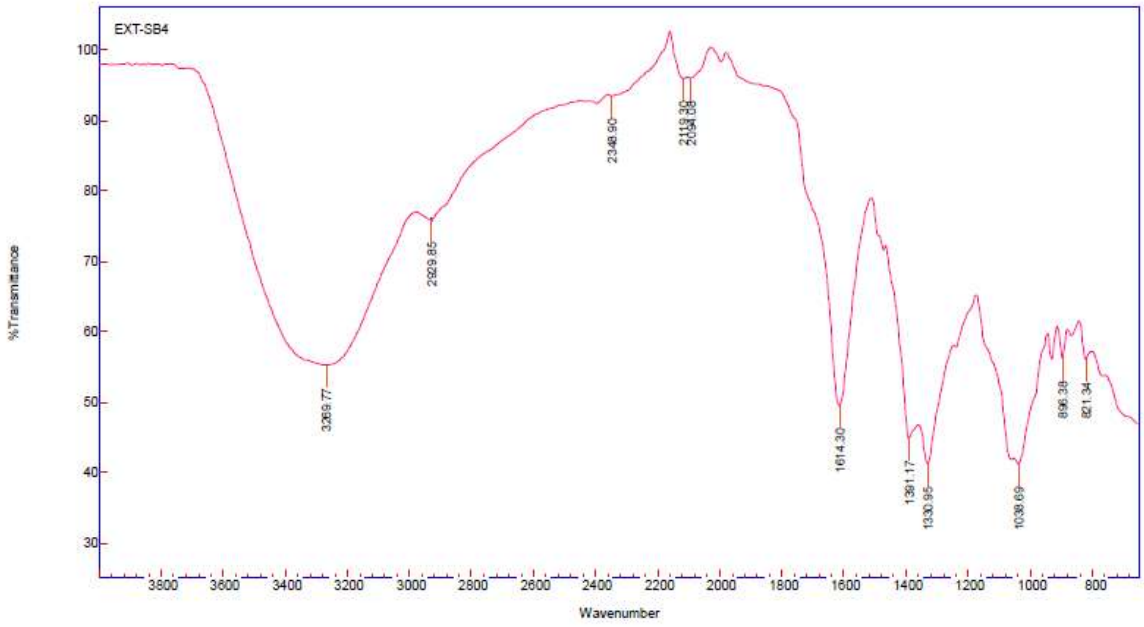

Figure 7.21: FTIR spectra of AgNPs synthesized from fresh aqueous stem extracts of *B. albostellata.*

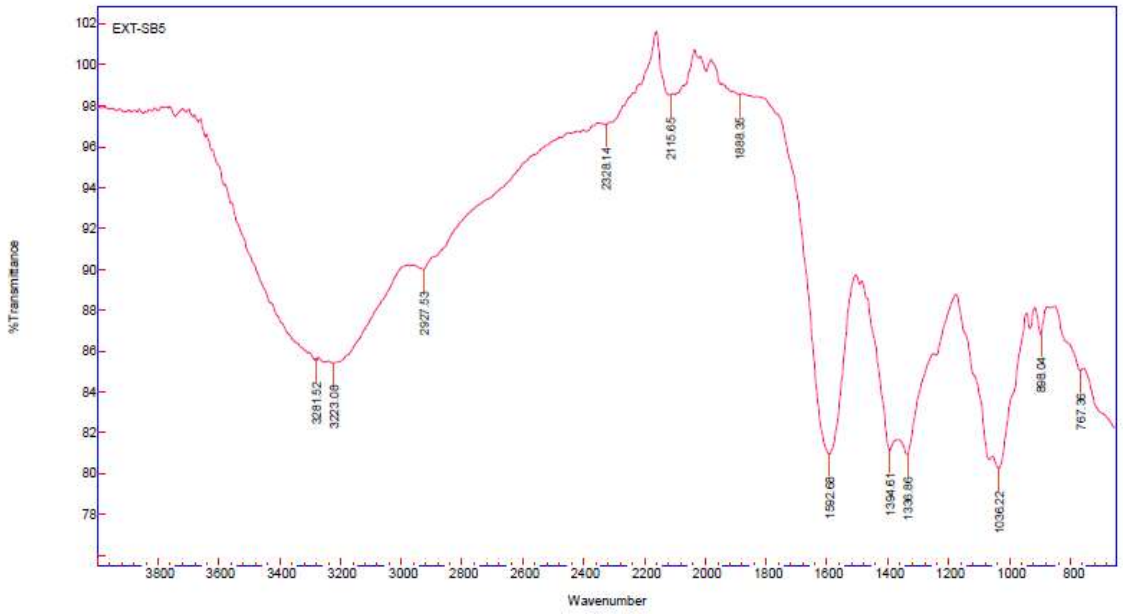

Figure 7.22: FTIR spectra of AgNPs synthesized from powdered aqueous leaf extracts of *B. albostellata.*

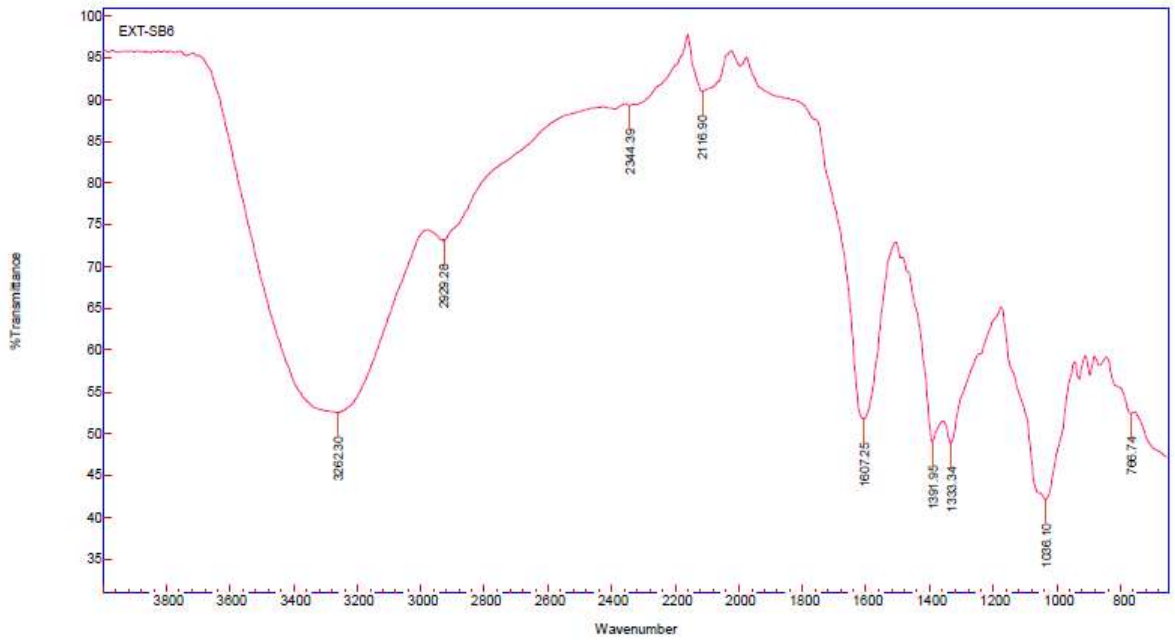

Figure 7.23: FTIR spectra of AgNPs synthesized from powdered aqueous stem extracts of *B. albostellata.*

7.3.6 Antibacterial activity of synthesized AgNPs from various leaf and stem extracts

The use of AgNPs in antibacterial assays is a fascinating strategy to overcome the problem of multidrug resistance by bacteria (Shahverdi et al., 2007). AgNPs synthesized from the leaf and stem extracts were subjected to antibacterial analysis. Various concentrations (100, 50, 25, 12.25, 6.25 and 3.125 mg/mL) were tested against the Gram-positive and –negative strains. Clear zones of inhibition were observed for the AgNPs synthesized from the leaf and stem methanolic, fresh and powdered extracts against; Gram-positive *B. subtillus, methicillin*-resistant *S. aureus*, *S. aureus* and Gram-negative, *E. coli* and *P. aeruginosa*.

The highest inhibitory activity was observed at 100 mg/mL in all AgNPs, for both Gram-positive and –negative bacteria. From all of the AgNPs tested against *B. subtillus*, the NPs from the fresh stem displayed the highest inhibitory activity (18.33 ± 3.21 mm), whilst the lowest was observed in the powdered leaf (12.33 ± 1.53 mm) (Table 7.4). No inhibitory activity was observed in the the leaf and stem AgNPs at 6.25 and 3.125 mg/mL against *B. subtillus* (Table 7.4). The powdered stem AgNPs displayed the highest activity (18.67 ± 3.21 mm) against *Methicillin*-resistant *S. aureus*, while the fresh leaf AgNPs demonstrated the lowest (12.00 ± 2.65 mm) (Table 7.4). Only the powdered leaf AgNPs at 6.25 mg/mL displayed no activity against *Methicillin*-resistant *S. aureus*. No observed activity was observed at 3.125 mg/mL against *Methicillin*-resistant *S. aureus* (Table 7.4). The methanolic leaf AgNPs showed the highest inhibitory activity against *S. aureus* (16.67 ± 2.52 mm), whereas the powdered leaf

AgNPs displayed the lowest (13.67 ± 2.52 mm). In *S. aureus*, no zones of inhibition were observed at 6.25 and 3.125 mg/mL for both powdered leaf and stem methanolic AgNPs (Table 7.4). Furthermore, at 3.125 mg/mL, the powdered stem AgNPs displayed no activity against *S. aureus* (Table 7.4). The powdered stem AgNPs demonstrated the highest activity (19.33 ± 1.15 mm) against *E. coli*, whereas the powdered leaf AgNPs showed the lowest activity (15.00 ± 1.00 mm). Zones of inhibition were observed at all concentrations for all AgNPs against *E. coli* (Table 7.4). According to the HRTEM analysis, AgNPs synthesized from the various extracts ranged from 34.32-16.57 nm. Numerous studies reported that AgNPs of smaller dimensions (<30 nm) have a greater ability to penetrate into bacteria (Mohammadi et al., 2011; Fellahi et al., 2013; Besinis et al., 2014; Tamayo et al., 2014; Wu et al., 2014). This suggests that smaller particles may engage with the contact surface of bacteria more frequently than large ones, therefore improving the antibacterial activity (Guzman et al., 2012; Sun et al., 2014). Additionally, the morphology and physicochemical characteristics of NPs have been recognized to exert an effect on their antimicrobial activities (Mohammadi et al., 2010; Seil and Webster, 2012). Kvitek et al. (2008) found smaller size AgNPs to display greater surface area then the larger particles, resulting in improved antibacterial activity. This statement was further validated by Collins et al. (2010), who suggested that smaller sized particles exhibited strong antibacterial activity, as they have a greater ability to easily enter the bacteria.

Pirtarighat et al. (2019) proposed that the bactericidal activity of AgNPs is perhaps due to the attachment of these particles to the cell wall. Ag^+ ions released from NPs promote antibacterial activity. Additionally, these positively charged ions react with the phosphorus and sulphur found in biomolecules such as DNA and RNA in the bacterial cells, thereby causing their disruption (Hajipour et al., 2012; Umashankari et al., 2012). According to various literature, the antibacterial potential of AgNPs are observed to denature the outer membrane of bacteria (Lok et al., 2006), cause gaps/pits in the membrane resulting in their destruction (Iavicoli et al., 2013; Yun et al., 2013), and bring about an interaction between the AgNPs and sulfhydryl/ disulphide groups of enzymes, hindering their metabolic processes and leading to cell death (Egger et al., 2009). The potency of synthesized AgNPs from the leaves of *B. cristata* (Gomathi et al., 2018) and *B. gibsoni* (Shao et al., 2018) showed antibacterial activity against *E. coli* and *S. aureus*. AgNPs are known to affect various biological processes within microorganisms, as they modify the structure and function of the cell membrane, rendering them permeable (Safavi, 2012). AgNPs were reported to accumulate on the membrane of *E. coli* cells, creating a gap in the integrity of the bilayer and increasing its permeability, resulting in bacterial cell death (Rai et al., 2014; Gomathi et al., 2018). For *P. aeruginosa*, AgNPs from the leaf methanolic extract displayed the highest inhibitory activity (21.67 ± 2.87 mm), and the lowest was observed in the powdered AgNPs (15.00 ± 4.00 mm). Additionally, no observed activity was observed at 3.125 mg/mL in the powdered leaf and stem methanolic AgNPs against *P. aeruginosa* (Table 7.4). Cittrarasu et al. (2019) reported on the antibacterial activity of *B. longiflora* aqueous leaf AgNPs. According to these authors, strong

antibacterial activity at 100 mg/mL was observed against *S. aureus* (14.5 ± 0.08 mm) and *P. aeruginosa* (18 ± 0.14 mm). Therefore, the biosynthesized AgNPs from the leaves and stem extracts of *B. albostellata*, exhibited antibacterial activity against both Gram-positive and Gram-negative bacteria.

Table 7.4: Antibacterial activity of synthesized AgNPs from *B. albostellata* against human pathogenic strains (zone of inhibition mm)

Strain	Concentration (mg/mL)	Treatments/synthesized AgNPs						Positive control (mg/mL)	
		Leaf methanol	Fresh leaves	Powder leaves	Stem methanol	Fresh stem	Powder stem	L	S
BS	**3.125**	R	R	R	R	R	R		
	6.25	R	R	R	R	R	R		
	12.5	6.67±0.58	7.33±0.58	6.67±0.58	6.67±0.58	6.67±1.15	7.00±0.00	9.00±1.00	11.00±1.00
	25	7.33±0.58	8.00±1.00	8.00±1.00	7.67±1.15	8.00±1.73	9.00±0.00		
	50	9.67±2.08	9.67±3.79	10.00±1.73	9.00±3.46	9.33±2.31	11.33±2.31		
	100	13.33±3.06	14.33±2.52	12.33±1.53	15.00±4.58	18.33±3.21	17.33±3.21		
MRSA	**3.125**	R	R	R	R	R	R		
	6.25	7.00±1.73	6.67±0.58	R	6.67±0.58	6.67±0.58	6.67±0.58	9.33±0.58	9.00±1.00
	12.5	9.33±0.58	7.00±0.00	6.00±1.00	7.33±1.15	7.67±1.53	9.00±1.00		
	25	10.67±0.58	8.00±1.00	7.33±0.58	9.33±1.15	9.67±0.58	11.67±1.53		
	50	12.67±2.52	9.00±1.00	10.00±3.00	12.67±2.52	12.00±2.00	14.00±4.00		
	100	14.67±0.58	12.00±2.65	11.00±2.00	15.00±1.00	14.00±3.00	18.67±3.21		
SA	**3.125**	7.33±1.53	7.33±0.58	R	R	6.67±0.58	R	9.67±0.58	10.00±1.00
	6.25	9.00±2.65	8.00±6.93	R	R	7.33±1.15	7.33±1.53		
	12.5	10.33±0.58	9.33±3.05	7.33±2.08	7.67±1.52	8.67±1.53	8.67±0.58		
	25	11.67±2.08	10.33±0.58	9.67±1.15	9.33±1.15	10.33±0.58	9.33±2.52		
	50	15.00±1.00	12.00±1.00	11.67±3.79	12.33±3.06	13.33±2.31	13.67±2.52		
	100	16.67±2.52	14.00±2.65	13.67±2.52	15.00±2.65	16.00±2.65	16.33±4.72		
EC	**3.125**	7.00±1.00	7.33±0.58	7.00±0.00	7.00±0.00	8.33±0.58	7.67±0.58	8.67±0.58	9.33±0.58
	6.25	8.67±0.58	9.33±0.58	8.67±0.58	9.33±0.58	9.67±0.58	9.33±0.58		
	12.5	10.33±0.58	11.33±1.53	10.00±0.00	10.33±2.52	11.33±0.58	10.67±1.53		
	25	12.00±2.65	13.67±1.53	11.67±0.58	12.33±2.31	13.00±2.65	12.33±1.15		
	50	14.00±2.65	14.00±1.00	13.67±4.16	14.00±1.73	15.33±0.58	14.00±1.00		
	100	18.67±3.51	15.67±1.53	15.00±1.00	16.67±2.31	17.00±3.00	19.33±1.15		
PA	**3.125**	7.33±1.53	7.00±0.00	R	R	7.00±0.00	7.00±0.00	9.33±0.58	8.67±1.15
	6.25	8.33±1.53	7.67±1.15	7.00±0.00	7.33±0.58	8.33±0.58	7.67±0.58		
	12.5	13.00±2.65	10.33±3.51	8.67±1.53	8.00±1.73	11.67±2.89	9.67±0.58		
	25	14.33±3.05	12.33±2.08	10.67±3.79	11.67±2.89	13.33±2.89	11.00±3.00		
	50	15.33±1.53	13.67±3.21	13.67±3.52	13.67±3.21	14.67±0.58	15.33±0.58		
	100	21.67±2.87	17.00±1.00	15.00±4.00	19.67±1.53	17.33±3.79	18.67±1.15		

BS= *B. subtillus,* MRSA= methicillin-resistant *S. aureus,* SA= *S. aureus*, EC= *E. coli*, PA= *P. aeruginosa*, R= resistant, Positive controls (Streptomycin 10 mg/mL, Gentamicin 10 mg/mL,), Negative control= DMSO, (n = 3).

7.3.7 *In vitro* cytotoxic effect on cancerous cell lines using biologically synthesized AgNPs

Preparation of NPs that eradicate cancerous cells while leaving normal cells unharmed, is crucial towards improving harsh toxicities linked with drug administration. *In vitro* cytotoxicity analysis is a valuable tool for screening synthesized compounds with potential anti-cancer activity. The MTT assay was carried out to evaluate the level of cell death. MTT is reduced in the mitochondria, and the absorbance measured is suggestive of the mitochondrial activity of the cell population, and hence the amount of viable cells (van Meerloo et al., 2011). As shown in Figure 7.24 A-C, the percentage cell survival for all extracts were dose-dependent. All AgNPs at various concentrations showed high cytotoxicity against all cell lines (Figure 7.24 A-C). NPs can efficiently enter the tumor micro-environment and prevent cancer cells from metastasizing (Parasuraman, 2011; Sutradhar and Amin, 2014; Fernandes et al., 2018). Controls 1, represented 100% of viable cells, while control 2 contained DMSO only. For HEK293, the highest cellular viability was observed at 15 µg/mL when treated with the powdered leaf and stem AgNPs. Furthermore, at 240 µg/mL, the lowest percentage cell viability was observed when treated with the fresh stem AgNPs (Figure 7.24 A). The synthesized NPs demonstrated medium-high cytotoxicity even at lower concentrations (15 µg/mL). The highest percentage cellular viability for HeLa was observed at 15 µg/mL when treated with the powdered stem AgNPs, whilst the lowest viability was observed at 240 µg/mL when exposed to the methanol stem AgNPs (Figure 7.24 B). For MCF-7, percentage viability was greatest at 15 µg/mL when treated with powdered stem AgNPs, and lowest at 240 µg/mL when treated with the methanol stem and fresh leaf AgNPs (Figure 7.24 C). Cell viabilities were above 30% for most NPs at varying concentrations (Figure 7.24 A-C). As the NP concentration increased, so did their toxicity on the cell lines become more significant. Statistical analysis showed that all extracts had significantly different activities across all concentrations ($P < 0.05$).

The IC_{50} values of the synthesized AgNPs in the three mammalian cell lines is represented in Table 7.5. Significant cytotoxic activity was observed in the HEK293 cell line upon exposure to the methanol leaf AgNPs, with an IC_{50} value of 9.02 µg/mL. However lower cytotoxicity was found in the stem AgNPs (61.66 µg/mL). Fresh leaf and stem AgNPs displayed moderate cytotoxicity in the HEK293 cell line (IC_{50} 47.86 and 33.04 µg/mL), while lower cytotoxic levels were observed for the powdered leaf and stem AgNPs (IC_{50} 100 and 61.24 µg/mL) (Table 7.5). Methanol leaf and stem AgNPs displayed the highest cytotoxicity in the HeLa cell line, with IC_{50} values of 5.87 and 12.58 µg/mL, respectively. Fresh leaf and stem AgNPs displayed moderate cytotoxicity in the HeLa cell line (IC_{50} 29.64 and 32.14 µg/mL), whereas lower cytotoxic levels were observed in the powdered leaf and stem AgNPs (IC_{50} 69.18 and 54.70 µg/mL) (Table 7.5).

Significant cytotoxic levels (IC_{50} 16.11 and 27.23 µg/mL) was observed on the MCF-7 cell line upon exposure to the methanolic leaf and stem AgNPs. Moderate cytotoxic levels were observed in the fresh

leaves and stems AgNPs (47.86 and 41.30 µg/mL), and lower levels in the powdered leaf and stem AgNPs (74.13 and 100 µg/mL) (Table 7.5). Chen and Schluesener (2008) proposed that AgNPs interact with the thiol groups of the inner membrane of the mitochondria, inhibiting the antioxidant defence mechanism, leading to the formation of reactive oxygen species (ROS). The accumulation of ROS results in an inflammatory response which initiates the destruction of the mitochondria, triggering the release of apoptogenic factors and inducing cell death.

NPs are small in size, allowing them to easily enter and interact with cancer cells, and ultimately disturbing cellular functions (Park et al., 2010). Additionally, Sanpui et al. (2011) proposed that AgNPs have the potential to interfere with genes associated with the cell cycle progression, thus inducing DNA damage and apoptosis in cancerous cells. Furthermore, Jeyaraj et al. (2013) suggested that there are different mechanisms for cytotoxicity of AgNPs, which includes the induction of ROS, apoptosis and Ag ion release. Hussain et al. (2005) observed an increase in the generation of ROS, as the NP concentration increased. Therefore, it can be expected that any cytotoxic effects induced in the cancer cells may be due to the active bound compounds capping the AgNPs.

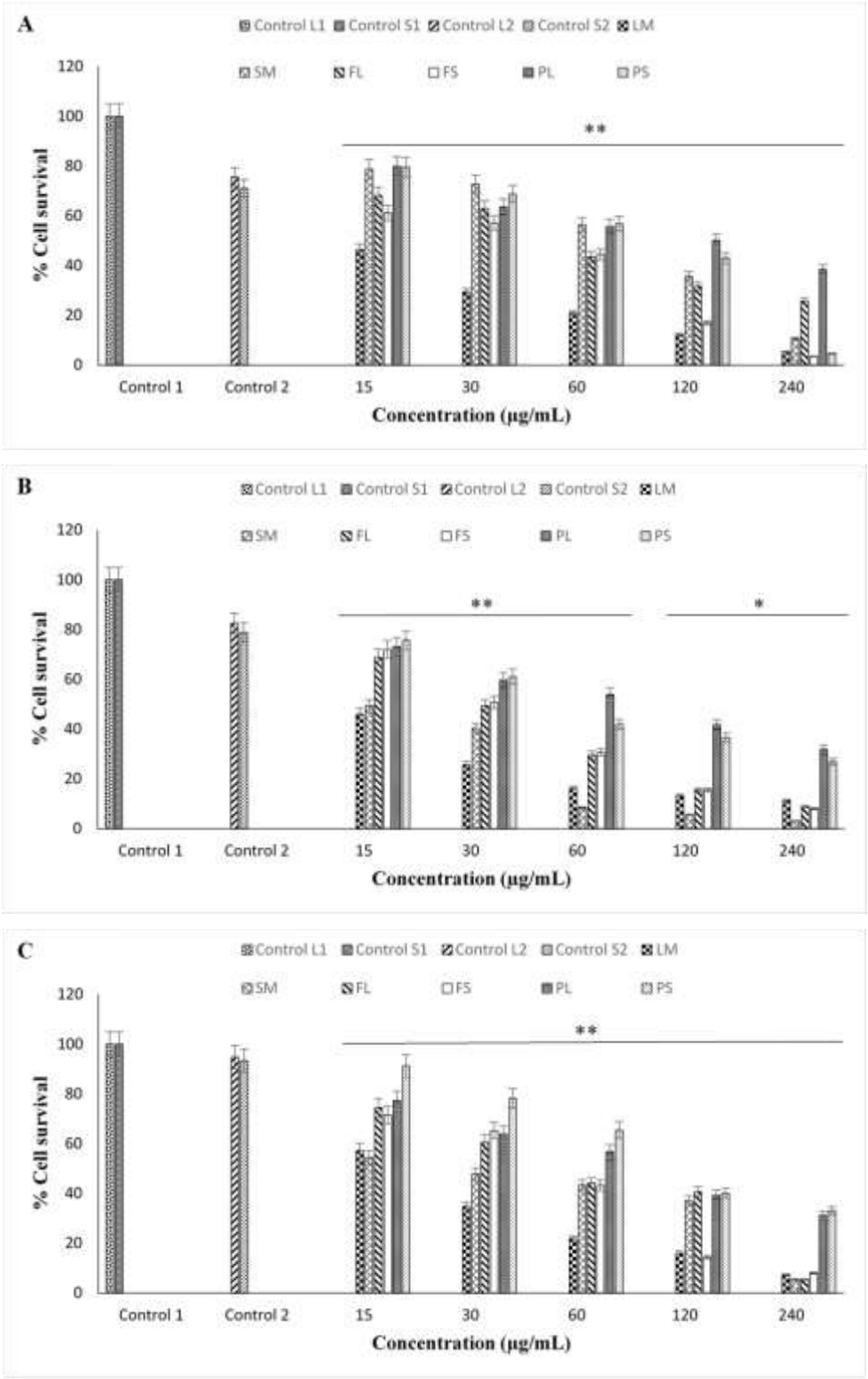

Figure 7.24: *In vitro* cytotoxicity activity (% cell survival) of AgNPs from the leaves and stems extracts of *B. albostellata*. (A) HEK293 human embryonic kidney; (B) HeLa cervical cancer cells, (C) MCF-7 breast adenocarcinoma. (*P <0.05 and **P <0.001 were considered statistically significant within the different concentrations, 15-240 µg/mL). Data are presented as means ± SD, n = 3 and displayed as a percentage of the control sample. Control Leaves 1- cells only; Control Stems 1- cells only; Control Leaves 2- DMSO only; Control Stems 2- DMSO; LM- Leaf methanol; SM- Stem methanol; FL- Fresh leaves; FS- Fresh stems; PL- Powdered leaves; PS- Powdered stems.

Table 7.5: IC_{50} values of the cytotoxicity activity of AgNPs from the methanol, fresh and powdered leaves and stem extracts of *B. albostellata*

Cell lines	Extracts	Cytotoxicity (µg/mL)	
		Leaves	Stems
HEK293	Methanol	9.02	61.66
	Fresh	47.86	33.04
	Powder	100.00	61.24
HeLa	Methanol	5.87	12.58
	Fresh	29.64	32.14
	Powder	69.18	54.70
MCF-7	Methanol	16.11	27.23
	Fresh	47.86	41.30
	Powder	74.13	100.00

Data are presented as mean. n=3.

Biosynthetic methods using plant extracts act as reducing and capping agents (Gardea-Torresdey et al., 2002; Shankar et al., 2003; Chandran et al., 2006; Pal et al., 2019). Using various microscopic techniques, AgNPS of similar shape and sizes found in *B. albostellata* were noted in other species of Barleria (Ghosh et al., 2016; Gomathi et al., 2018). The biosynthesized AgNPs from the leaves and stem extracts exhibited antibacterial activity against both Gram-positive and Gram-negative bacteria, this was also noted in several species of *Barleria* (Gomathi et al., 2018; Shao et al., 2018; Cittrarasu et al., 2019). The precise mechanism of synthesising NPs using plant extracts is unclear, it has been shown that biomolecules such as alkaloids, phenols and flavonoids found in these extracts play an important role in reducing the metal ions and capping the biosynthesized NPs (Krishnaraj et al., 2010; El-Seedi et al., 2019). Synthesized AgNPs exhibited selective *in vitro* cytotoxicity against HEK293, HeLa and MCF-7 cell lines. The size of the NPs, allow them to easily enter and interact with cancer cells, and ultimately disturbing cellular functions (Park et al., 2010). AgNPs have the potential to interfere with genes associated with the cell cycle progression, thus inducing DNA damage and apoptosis in cancerous cells. Any cytotoxic effects induced in the cancer cells may be due to the active bound compounds capping the AgNPs.

7.4 Conclusions and future perspectives

A fast, effective and environmentally-friendly synthesis of AgNPs was established using the *B. albostellata* leaf and stem extracts, which may be considered as an important alternative to chemical synthesis especially for the potential use in medical and pharmaceutical applications. This was highlighted by their favourable morphology, small sizes and zeta potential which augurs well for their biological applications. FTIR analysis confirmed that the bioactive compounds present in the *B.*

albostellata extracts were involved in the reduction and capping of the AgNPs. Diverse groups of phytochemicals play a critical role in both bioreduction and stabilization of the AgNPs.

Synthesized AgNPs showed possible bacteriostatic effects against Gram-positive and -negative human pathogenic bacteria. Their broad spectrum of bioactivity suggested that they may be as promising agents in fighting infections. All AgNPs exhibited cytotoxicity *in vitro*, with some selectivity to cancer cell lines, which warrants their future research as potential chemotherapeutic agents. As the NP surface charge affects their activity, it is thus suggested that moderately stable AgNPs be used for further evaluation. Further studies should be conducted on testing different ratios of silver nitrate to plant extract, in order to find the best ratio that yields the maximum amount of AgNPs. Additionally, further characterization such as nuclear magnetic resonance spectroscopy could be conducted to further characterise the metabolites present in plant extracts. To the best of our knowledge, this is the first report on the synthesis, characterisation, antibacterial and cytotoxic activities of the AgNPs synthesized from the extracts of *B. albostellata*. Findings from this study could contribute significantly to the advancement in the development of novel phytochemical-based green compounds for potential use in the healthcare sector.

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CHAPTER 8
CONCLUSIONS, CHALLENGES AND RECOMMENDATIONS FOR FUTURE RESEARCH

8.1 Conclusions

This study was undertaken to characterise the morphology, chemical composition and biological activity of the leaf and stem extracts of *B. albostellata*. Secretory structures documented within the leaves and stems of *B. albostellata* include glandular and non-glandular trichomes. The combination of different microscopic techniques allowed for the identification of five different glandular capitate trichomes, a multangulate-dendritic branched non-glandular trichome and a glandular head on a non-glandular trichome. These trichomes are involved in the secretion of various valuable compounds. Glandular and non-glandular trichomes were found on both leaf and stem surfaces and across all developmental stages of the leaves. Histochemical tests indicated the presence of alkaloids and phenolics in the glandular and non-glandular trichomes. These trichome types are reported for the first time in *B. albostellata*. Morphological observations of the flower and pollen grains using stereo- and scanning electron microscopy showed numerous non-glandular trichomes on the floral bracteoles and bracts of *B. albostellata.* Three types of trichomes were identified on these floral structures, I- unicellular and II- multangulate-dendritic branched non-glandular trichomes, and III- capitate glandular trichomes. SEM micrographs revealed pollen grains as tricolporate with a coarse reticulation of the exine. Little is known on the floral and pollen morphology of *B. albostellata*, with results from this floral biology study adding to the body of knowledge of this species. Preliminary phytochemical analysis indicated the presence of several phytocompounds (10 compounds). This study ultimately proved that leaf and stem extracts of *B. albostellata* contained numerous biologically active compounds such as alkaloids and phenolics. TLC suggested that the concentrations of the separated compounds differed. Leaf and stem powdered material displayed various colours under bright- and UV-light. Major pharmacologically compounds found in extracts were alpha-amyrin, flavone, phenol, 2,4-bis(1,1-dimethylethyl)-, phytol, phytol acetate, squalene and stigmasterol. Compounds identified, possess various biological properties of medicinal importance. Crude extracts positively inhibited Gram-positive, *B. subtillus*, methicillin-resistant *S. aureus*, *S. aureus* and Gram-negative, *P. aeruginosa* and *E. coli*. These results are suggestive that the leaves and stems of *B. albostellata* are rich in bioactive compounds which, could be a possible source of antibacterial agents in treating several diseases linked to the pathogenic bacteria studied. The detailed phytochemical profile provided in this study could be further exploited for plant-based drug development.

Crude extracts displayed varying degrees of antioxidant activities and were dose dependent. The observed *in vitro* cytotoxicity of crude extracts may be due to the presence of flavonoids, phenols, and antioxidant activity in the different parts of *B. albostellata*. This cytotoxicity of the leaf and stem crude extracts of *B. albostellata* were reported for the first time in this study. Preliminary results from this study revealed the potential of *B. albostellata* in effectively synthesising silver nanoparticles (AgNPs) from the leaf and stem extracts, with elemental Ag^{+} being the highest in the methanol leaf extract (16.87 ± 0.89%) and lowest in the powdered stems extracts (7.13 ± 1.44%). Mean particle size of the AgNPs were varied with the use of the different extracts. AgNPs from the leaf and stem extracts exhibited zeta potential values between -8.8 and -32.1 mV, with a hydrodynamics diameter ranging from 34.3 to 111.3 nm. FTIR analysis indicated that bioactive compounds present in these extracts are involved in the reduction and capping of AgNPs. It was observed that synthesized NPs possessed potent antibacterial activities against Gram-positive and negative bacteria. The AgNPs also exhibited selective *in vitro* cytotoxicity in cancer cell lines. The IC_{50} cytotoxicity values of AgNPs synthesized from the various extracts in HEK293, HeLa, and MCF-7 cells were > 9 µg/mL. Overall, synthesized AgNPs obtained from *B. albostellata* were novel antibacterial and anticancer agents, and warrants further investigation. The various pharmacological activities conducted in this study provide a basic understanding of the importance of the investigated medicinal plant as a potential source of novel and useful drug leads. Preliminary research achieved in this study will contribute to the growing ethnopharmacological field in South Africa. To our knowledge, this study will be the first report combining the morphology, chemical composition and biological activity of *B. albostellata*. Thus there is a wide scope for future research in this field.

8.2 Challenges

There have been various challenges that had impacted this project during the year 2019-2021. Sample preparation using the standard protocol for transmission electron microscopy was a challenge as resin blocks were brittle due to poor infiltration, making it difficult to section and view the plant material. Thus, the protocol for chemical fixation of the plant material had to be slightly changed and new blocks had to be prepared. Thereafter, several instruments required to complete experimental work in this project were not operational at the microscopy microanalysis from 2019 to date, specifically the scanning electron microscope and transmission electron microscope. In addition, a major set-back was due to a fire that had occurred in the Life Science building on the 5th floor at the University of KwaZulu-Natal. This fire had taken place in December, 2018 which destroyed the entire west wing of the 5th floor. This resulted in no lab space, equipment, chemicals, resources, and plant samples were destroyed or contaminated. This made it very difficult to complete work and conduct experiments throughout 2019 and 2020 (before closure of the University). To date, the 5th floor is not operational and is under construction. This was a major setback.

Furthermore, the beginning of the academic year for 2020 began with several challenges. Violent student protests commenced from the 27th of January until the 20th of February 2020. This disruption was a major setback as experimental work could not continue and the University had suspended the academic program. Whilst the academic program was suspended, postgraduate students were not allowed to continue their experimental work due to safety issues. For the academic year 2020, nearly 20 working days were lost due to the student protest action. This hindered experimental work as various assays could not be completed. Thereafter, the COVID-19 pandemic devastated South Africa, this resulted in the total suspension of academic activity and the closure of the University of KwaZulu-Natal on the 16th of March 2020 until July 2020. Upon returning to campus during COVID-19 and due to several protocols been in place for social distancing etc., there was limited access to research space and equipment. Strict access to various instruments/ lab space was only allowed to residing students of specific departments (Microbiology, Biochemistry and Chemistry).

8.3 Future perspectives

The genus Acanthaceae is of great medicinal importance throughout the world. However, there is a lack of knowledge of some species within the family which provides an opportunity for further studies. Ultrastructural studies on the floral structures should further examine the internal features of cells and organelles present on the floral structures. It will be important to investigate other plant parts such as the flowers and roots of *B. albostellata* for similar pharmacological activities to ensure proper use of the plant. Phytochemical screening of leaf and stems extracts revealed the presence of various compound classes of medicinal importance emphasising *B. albostellata* as a suitable candidate for further isolation and purification of medicinally active compounds which can lead to a sustainable drug development. Isolation and identification of phytocompounds in other parts of the investigated plant should also be undertaken and subjected to antibacterial, antioxidant and cytotoxicity assays.

Other solvents used in phytochemical extractions such as ethanol and acetone can also be subjected to antibacterial, antioxidant and cytotoxicity assays, in order to evaluate its potency. Further research should be conducted on the mechanisms involved in cell death induced by the extracts from *B. albostellata* on the various cancer cell lines. Plant extracts of *B. albostellata* successfully synthesized AgNPs. These extracts can further be utilized for the sysnthesis of other metal NPs such as gold or copper and subjected to the various pharmacological assays. More detailed antibacterial studies are required in order to determine minimum inhibitory concentration (MIC) values against the microorganisms. Overall, further investigations are necessary for full exploration of *B. albostellata* for novel therapeutic compounds or drug leads.

APPENDIX A1

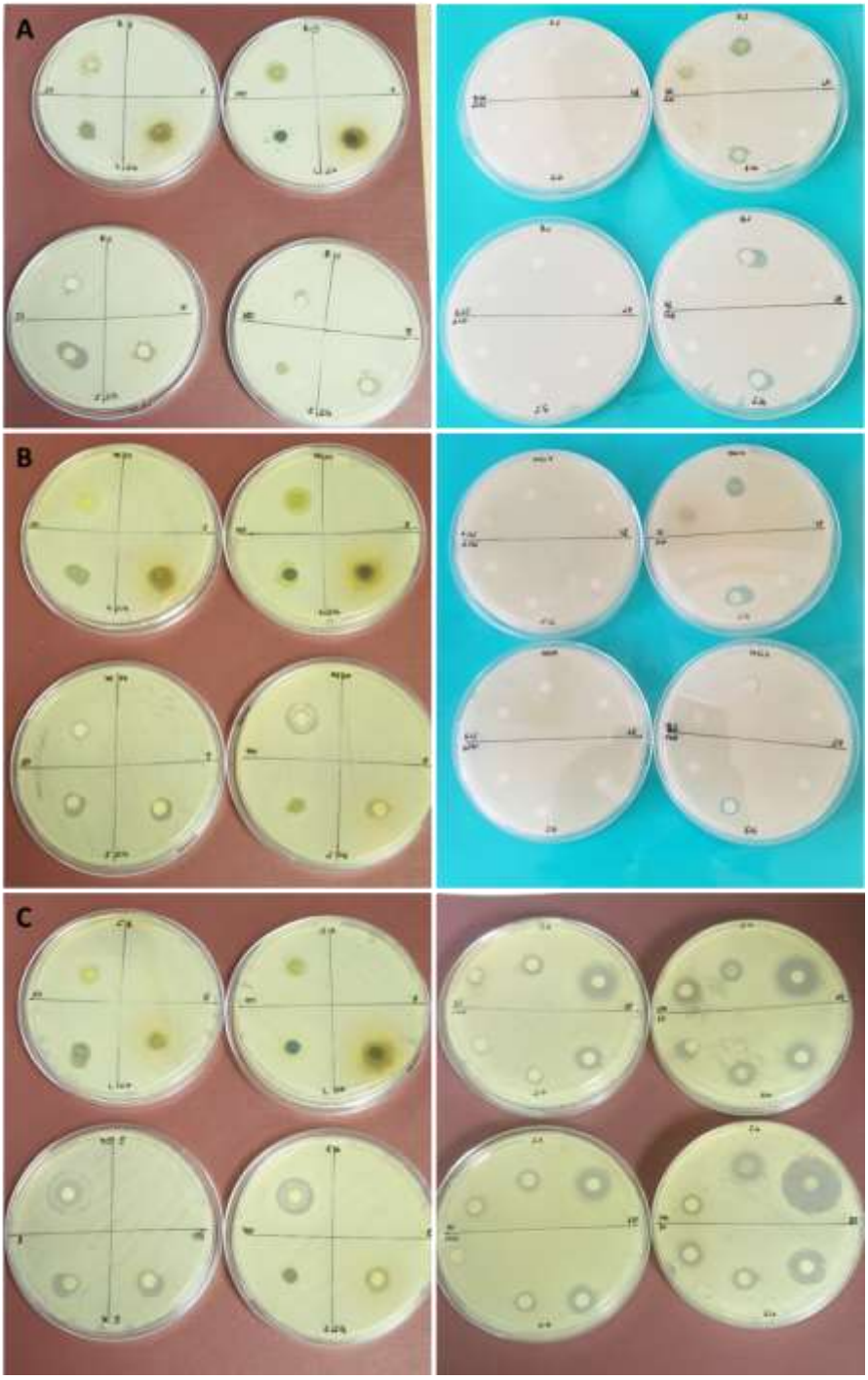


Figure 1: Plates displaying antibacterial activity of crude extracts (hexane, chloroform and methanol) from the leaves and stem of *B. albostellata*. (A) *B. subtillus*; (B) Methicillin-resistant *S. aureus*; (C) *S. aureus*.

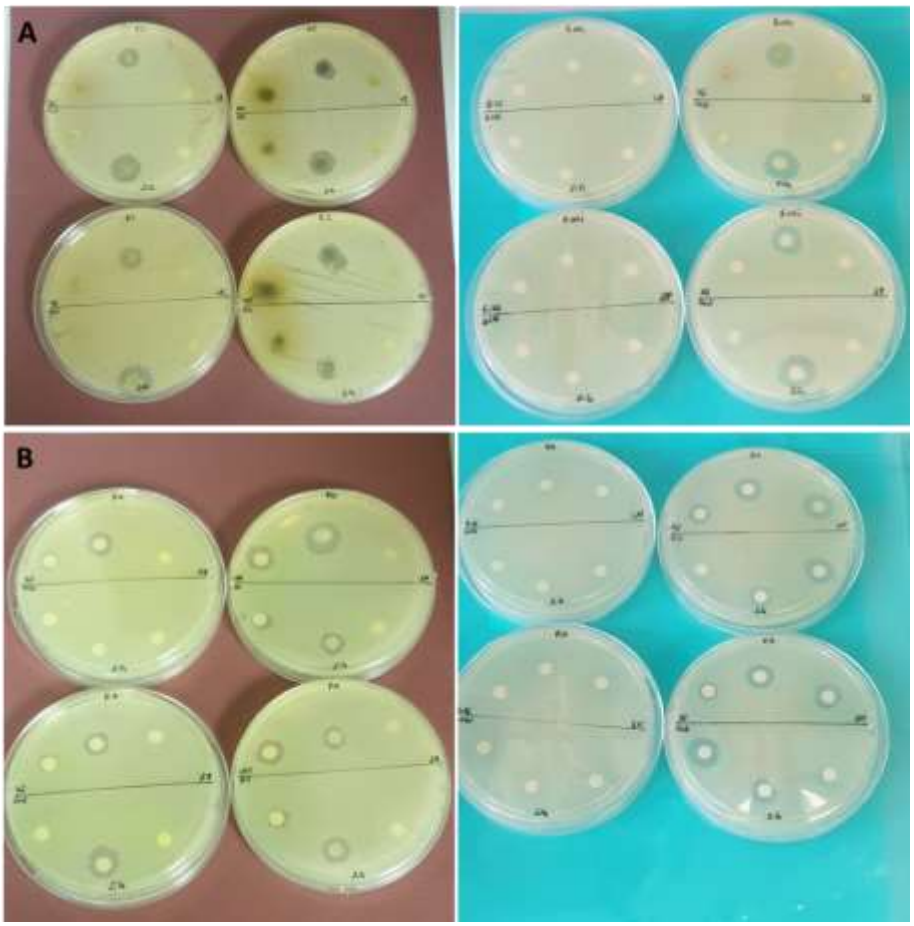


Figure 2: Plates displaying antibacterial activity of crude extracts (hexane, chloroform and methanol) from the leaves and stem of *B. albostellata*. (A) *E. coli*; (B) *P. aeruginosa*.

APPENDIX A2

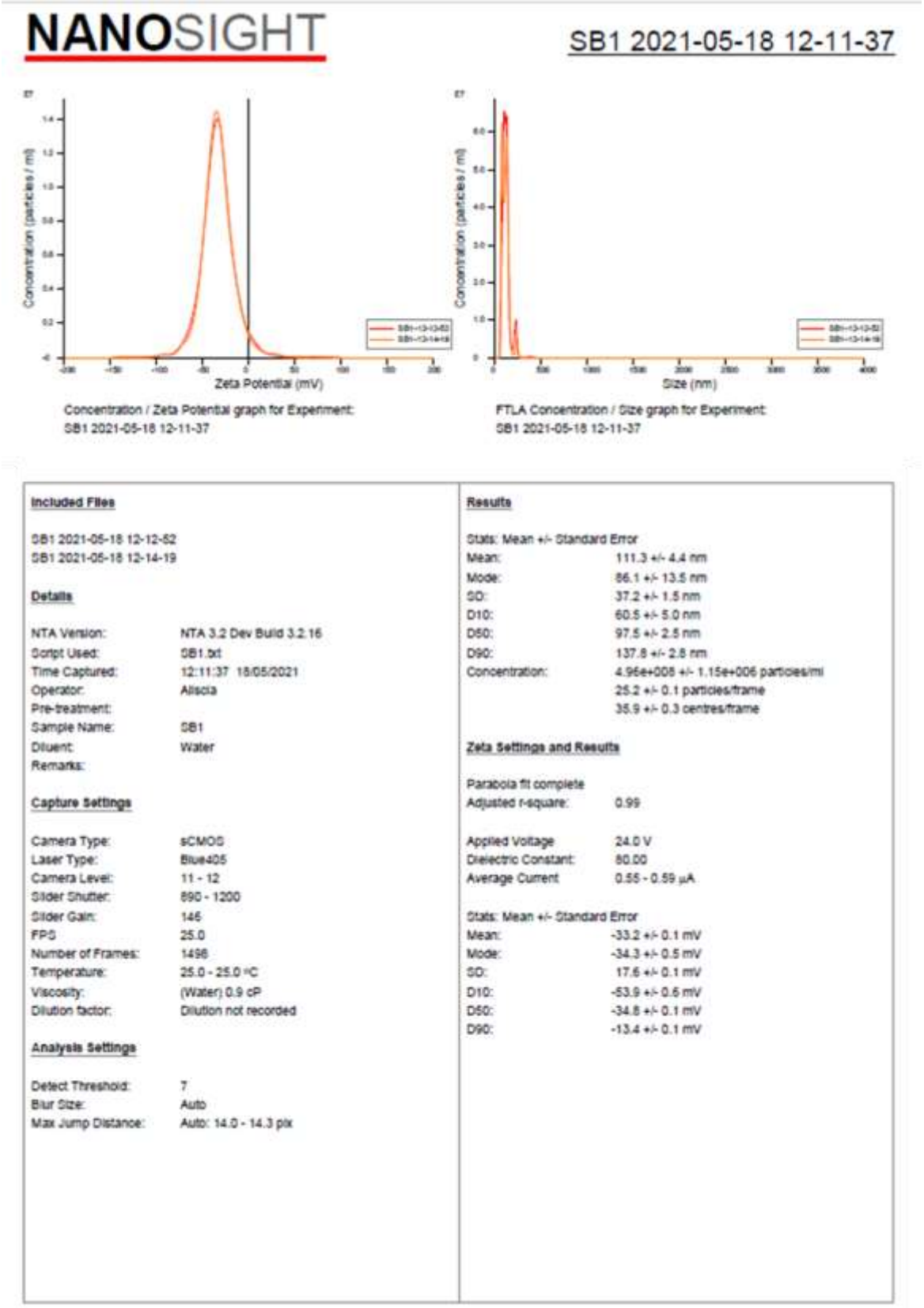


Figure 3: The size distribution and Zeta potential of AgNps synthesized from leaf methanolic extracts using NTA

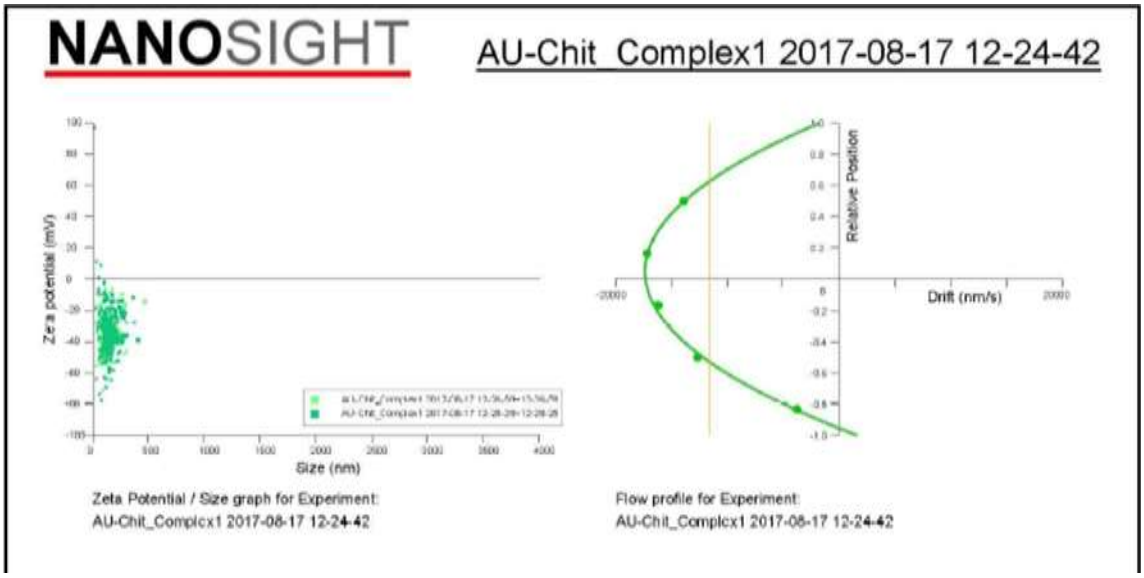
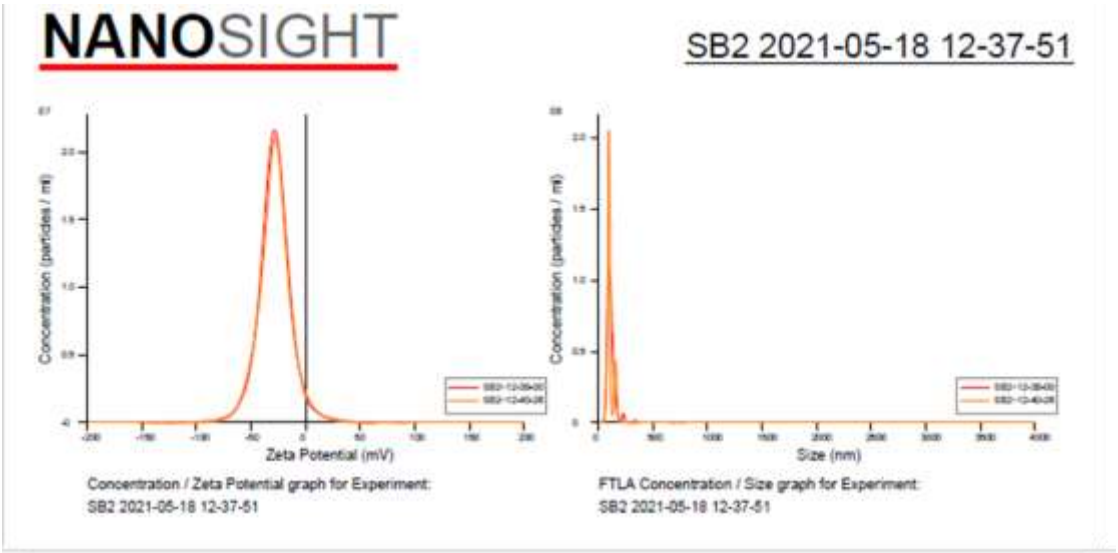


Figure 4: The size distribution and Zeta potential of AgNps synthesized from leaf methanolic extracts using NTA



Included Files

SB2 2021-05-18 12-39-00
SB2 2021-05-18 12-40-26

Details

NTA Version:	NTA 3.2 Dev Build 3.2.16
Script Used:	SB2.txt
Time Captured:	12:37:51 18/05/2021
Operator:	Alisia
Pre-treatment:	
Sample Name:	SB2
Diluent:	Water
Remarks:	

Capture Settings

Camera Type:	sCMOS
Laser Type:	Blue405
Camera Level:	12
Slider Shutter:	1200
Slider Gain:	146
FPS	25.0
Number of Frames:	1498
Temperature:	25.0 - 25.0 °C
Viscosity:	(Water) 0.9 cP
Dilution factor:	Dilution not recorded

Analysis Settings

Detect Threshold:	7
Blur Size:	Auto
Max Jump Distance:	Auto: 13.8 - 14.7 pix

Results

Stats: Mean +/- Standard Error

Mean:	110.7 +/- 4.2 nm
Mode:	97.2 +/- 0.4 nm
SD:	35.6 +/- 8.0 nm
D10:	70.7 +/- 0.2 nm
D50:	93.2 +/- 4.5 nm
D90:	143.0 +/- 3.5 nm
Concentration:	6.46e+008 +/- 1.52e+007 particles/ml
	32.8 +/- 0.8 particles/frame
	43.8 +/- 1.2 centres/frame

Zeta Settings and Results

Parabola fit complete

Adjusted r-square:	0.99
Applied Voltage	24.0 V
Dielectric Constant:	80.00
Average Current	0.71 - 0.85 µA

Stats: Mean +/- Standard Error

Mean:	-28.2 +/- 0.0 mV
Mode:	-28.1 +/- 0.3 mV
SD:	15.3 +/- 0.1 mV
D10:	-47.1 +/- 0.5 mV
D50:	-29.2 +/- 0.1 mV
D90:	-11.6 +/- 0.3 mV

Figure 5: The size distribution and Zeta potential of AgNps synthesized from stem methanolic extracts using NTA.

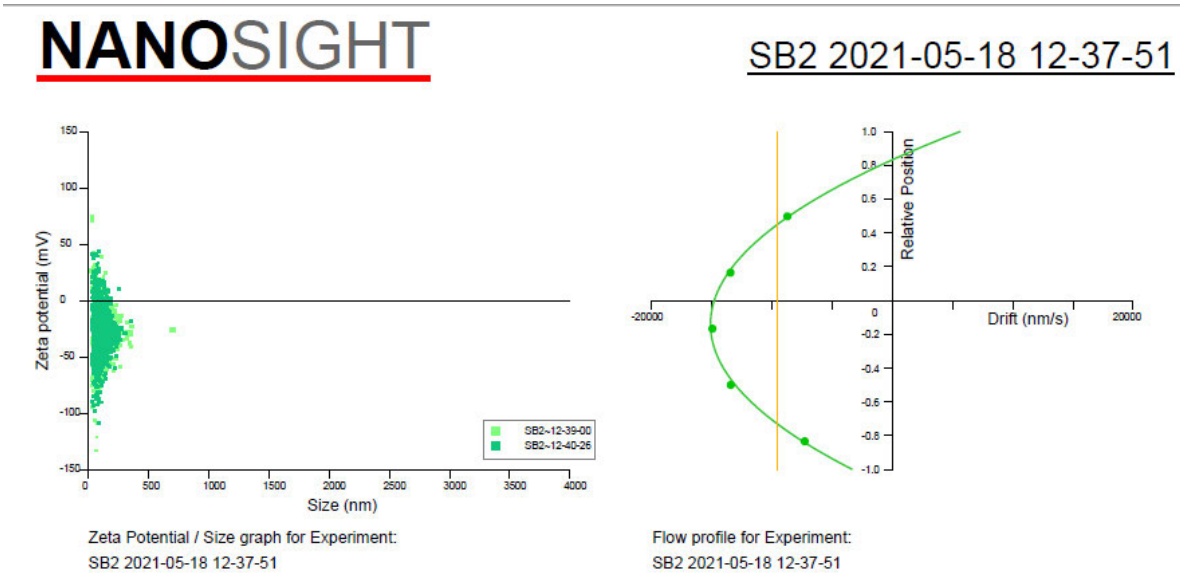
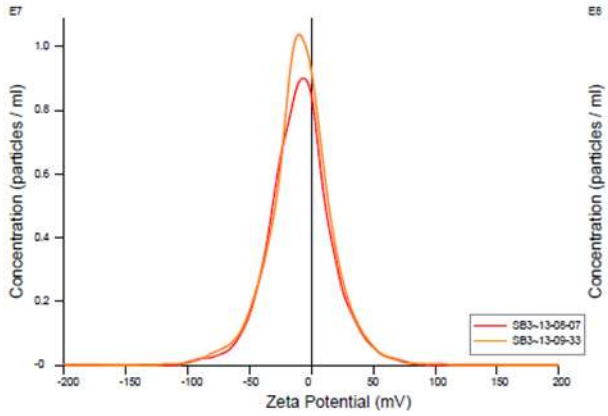


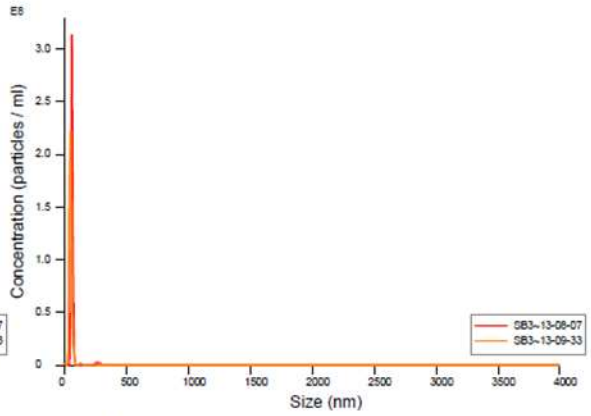
Figure 6: The size distribution and Zeta potential of AgNps synthesized from stem methanolic extracts using NTA.

NANOSIGHT

SB3 2021-05-18 13-06-56



Concentration / Zeta Potential graph for Experiment:
SB3 2021-05-18 13-06-56



FTLA Concentration / Size graph for Experiment:
SB3 2021-05-18 13-06-56

Included Files

SB3 2021-05-18 13-08-07
SB3 2021-05-18 13-09-33

Details

NTA Version:	NTA 3.2 Dev Build 3.2.16
Script Used:	SB.txt
Time Captured:	13:06:56 18/05/2021
Operator:	Alisola
Pre-treatment:	
Sample Name:	SB3
Diluent:	Water
Remarks:	

Capture Settings

Camera Type:	sCMOS
Laser Type:	Blue405
Camera Level:	11 - 12
Slider Shutter:	890 - 1200
Slider Gain:	146
FPS	25.0
Number of Frames:	1498
Temperature:	25.0 °C
Viscosity:	(Water) 0.9 cP
Dilution factor:	Dilution not recorded

Analysis Settings

Detect Threshold:	7
Blur Size:	Auto
Max Jump Distance:	Auto: 14.0 - 21.7 pix

Results

Stats: Mean +/- Standard Error

Mean:	53.9 +/- 8.4 nm
Mode:	49.0 +/- 6.5 nm
SD:	25.8 +/- 9.1 nm
D10:	30.8 +/- 8.5 nm
D50:	39.8 +/- 6.3 nm
D90:	52.6 +/- 4.8 nm
Concentration:	5.13e+008 +/- 2.26e+007 particles/ml
	26.0 +/- 1.1 particles/frame
	33.4 +/- 0.8 centres/frame

Zeta Settings and Results

Parabola fit complete

Adjusted r-square:	0.99
Applied Voltage	24.0 V
Dielectric Constant:	80.00
Average Current	0.43 - 0.48 µA

Stats: Mean +/- Standard Error

Mean:	-8.8 +/- 0.2 mV
Mode:	-8.3 +/- 1.8 mV
SD:	25.7 +/- 0.2 mV
D10:	-40.5 +/- 0.1 mV
D50:	-9.6 +/- 0.3 mV
D90:	20.8 +/- 0.0 mV

Figure 7: The size distribution and Zeta potential of AgNps synthesized fresh leaf aqueous extracts using NTA.

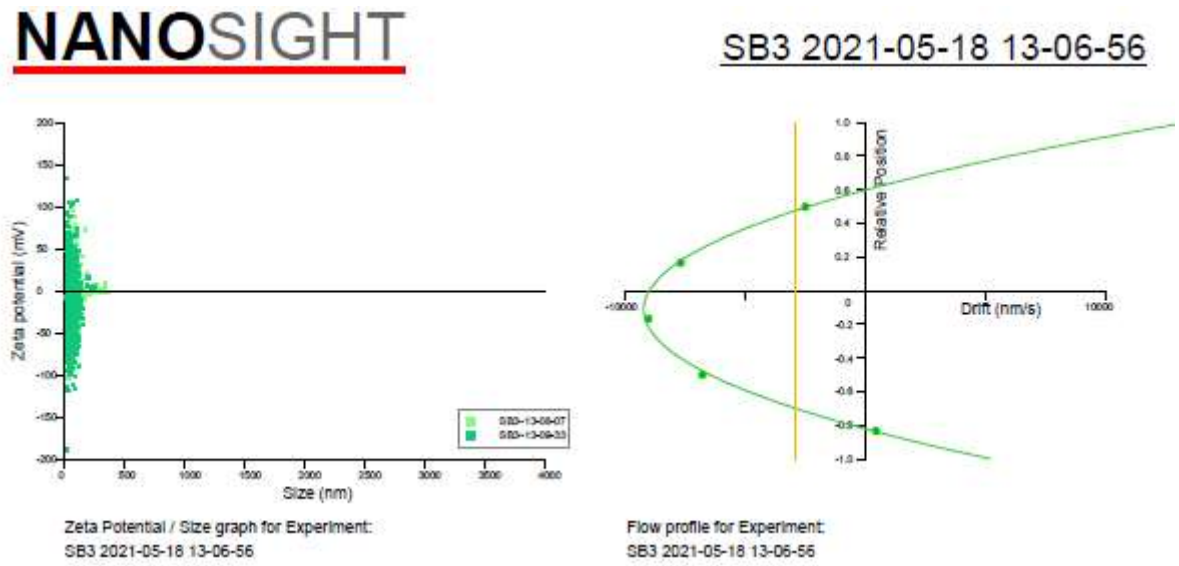
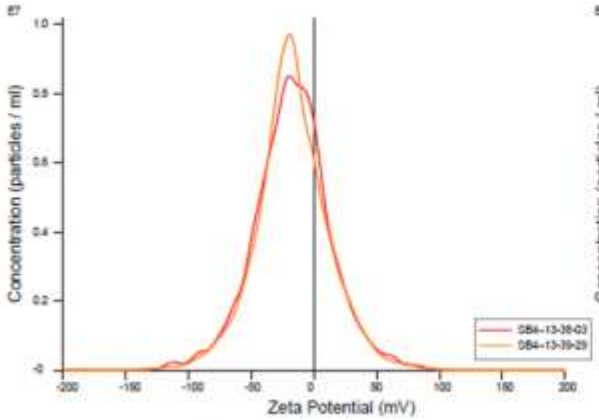


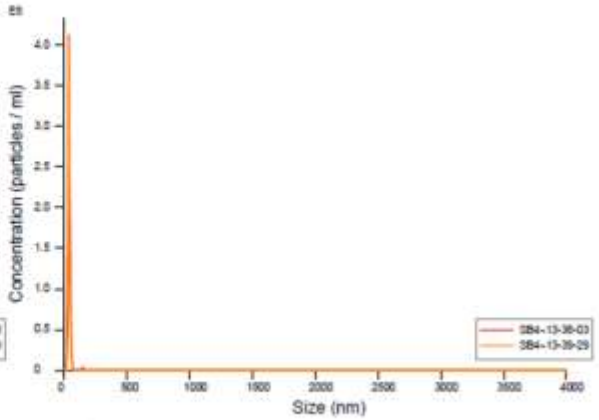
Figure 8: The size distribution and Zeta potential of AgNps synthesized fresh leaf aqueous extracts using NTA.

NANOSIGHT

SB4 2021-05-18 13-36-38



Concentration / Zeta Potential graph for Experiment:
SB4 2021-05-18 13-36-38



FTLA Concentration / Size graph for Experiment:
SB4 2021-05-18 13-36-38

Included Files

SB4 2021-05-18 13-36-03
SB4 2021-05-18 13-39-29

Details

NTA Version:	NTA 3.2 Dev Build 3.2.16
Script Used:	SB4.txt
Time Captured:	13:36:38 18/05/2021
Operator:	Aliscia
Pre-treatment:	
Sample Name:	SB4
Diluent:	Water
Remarks:	

Capture Settings

Camera Type:	sCMOS
Laser Type:	Blue405
Camera Level:	12
Slider Shutter:	1200
Slider Gain:	146
FPS	25.0
Number of Frames:	1498
Temperature:	25.0 - 25.0 °C
Viscosity:	(Water) 0.9 cP
Dilution factor:	Dilution not recorded

Analysis Settings

Detect Threshold:	7
Blur Size:	Auto
Max Jump Distance:	Auto: 23.7 - 24.3 pix

Results

Stats: Mean +/- Standard Error

Mean:	37.7 +/- 0.9 nm
Mode:	36.0 +/- 0.7 nm
SD:	10.1 +/- 3.1 nm
D10:	18.1 +/- 2.2 nm
D50:	26.5 +/- 0.7 nm
D90:	37.5 +/- 1.3 nm
Concentration:	5.70e+008 +/- 6.35e+006 particles/ml
	28.9 +/- 0.3 particles/frame
	38.1 +/- 1.1 centres/frame

Zeta Settings and Results

Parabola fit complete

Adjusted r-square:	0.99
Applied Voltage	24.0 V
Dielectric Constant:	80.00
Average Current	0.48 - 0.52 µA

Stats: Mean +/- Standard Error

Mean:	-17.2 +/- 0.0 mV
Mode:	-19.4 +/- 0.1 mV
SD:	30.2 +/- 0.7 mV
D10:	-54.3 +/- 1.1 mV
D50:	-16.4 +/- 0.6 mV
D90:	18.7 +/- 0.2 mV

Figure 9: The size distribution and Zeta potential of AgNps synthesized fresh stem aqueous extracts using NTA.

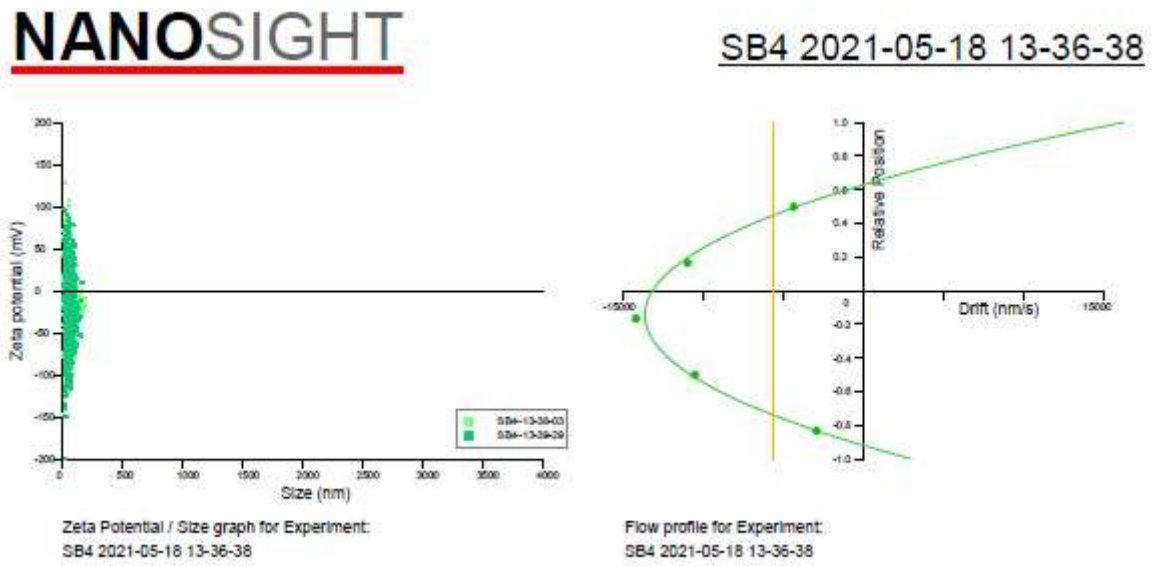
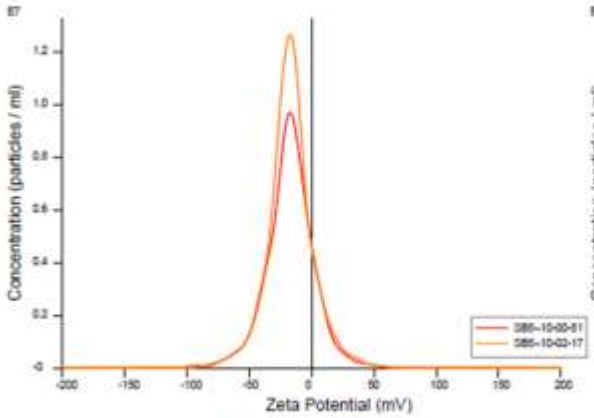


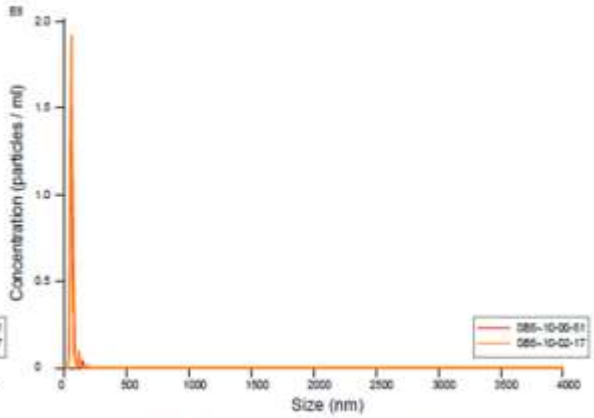
Figure 10: The size distribution and Zeta potential of AgNps synthesized fresh stem aqueous extracts using NTA.

NANOSIGHT

SB5 2021-05-19 09-59-38



Concentration / Zeta Potential graph for Experiment:
SB5 2021-05-19 09-59-38



FTLA Concentration / Size graph for Experiment:
SB5 2021-05-19 09-59-38

Included Files

SB5 2021-05-19 10-00-51
SB5 2021-05-19 10-02-17

Details

NTA Version:	NTA 3.2 Dev Build 3.2.16
Script Used:	SB5.txt
Time Captured:	09:59:38 19/05/2021
Operator:	Alisola
Pre-treatment:	
Sample Name:	SB5
Diluent:	Water
Remarks:	

Capture Settings

Camera Type:	sCMOS
Laser Type:	Blue405
Camera Level:	9
Slider Shutter:	607
Slider Gain:	15
FPS	25.0
Number of Frames:	1498
Temperature:	25.0 - 25.0 °C
Viscosity:	(Water) 0.9 cP
Dilution factor:	Dilution not recorded

Analysis Settings

Detect Threshold:	7
Blur Size:	Auto
Max Jump Distance:	Auto: 19.4 - 21.2 pix

Results

Stats: Mean +/- Standard Error

Mean:	57.9 +/- 0.6 nm
Mode:	54.4 +/- 1.8 nm
SD:	16.8 +/- 0.4 nm
D10:	32.9 +/- 0.3 nm
D50:	45.2 +/- 1.3 nm
D90:	62.2 +/- 3.1 nm
Concentration:	4.05e+008 +/- 3.45e+007 particles/ml
	20.5 +/- 1.8 particles/frame
	29.0 +/- 0.6 centres/frame

Zeta Settings and Results

Parabola fit complete

Adjusted r-square:	1.00
Applied Voltage	24.0 V
Dielectric Constant:	80.00
Average Current	0.42 - 0.43 µA

Stats: Mean +/- Standard Error

Mean:	-17.0 +/- 0.0 mV
Mode:	-17.3 +/- 0.1 mV
SD:	19.4 +/- 0.5 mV
D10:	-40.4 +/- 0.9 mV
D50:	-18.1 +/- 0.2 mV
D90:	4.6 +/- 0.3 mV

Figure 11: The size distribution and Zeta potential of AgNps synthesized powdered leaf aqueous extracts using NTA.

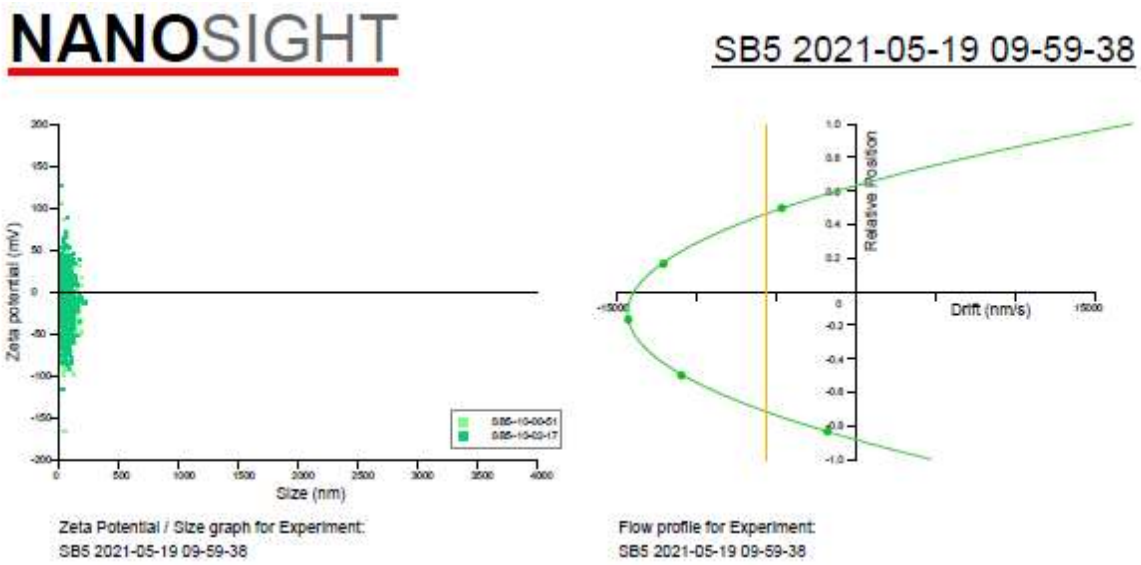
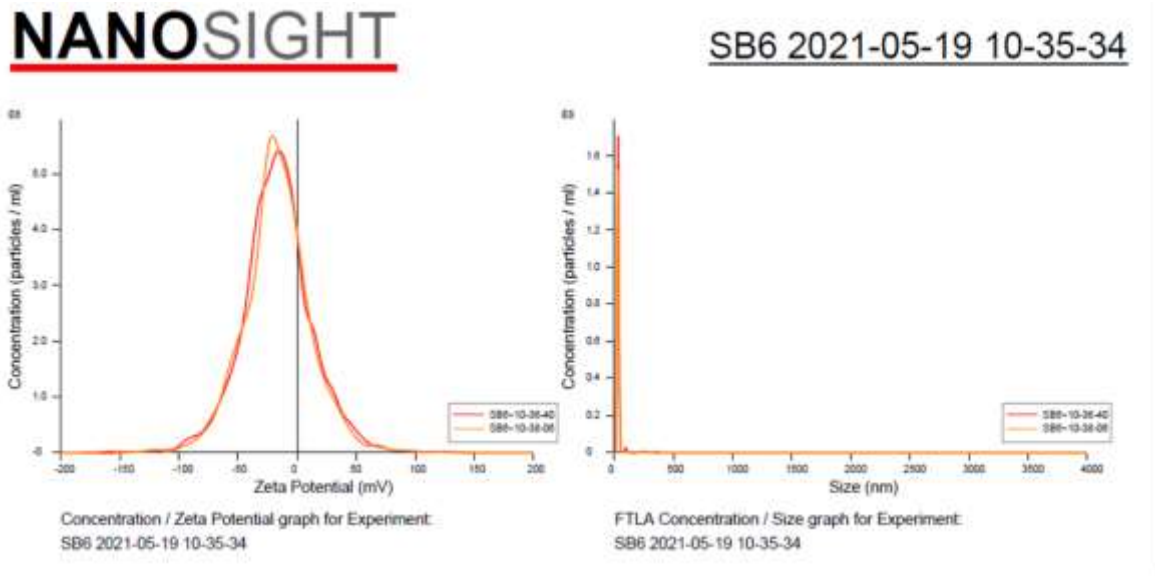


Figure 12: The size distribution and Zeta potential of AgNps synthesized powdered leaf aqueous extracts using NTA.



Included Files

SB6 2021-05-19 10-36-40
SB6 2021-05-19 10-38-06

Details

NTA Version:	NTA 3.2 Dev Build 3.2.16
Script Used:	SB6.txt
Time Captured:	10:35:34 19/05/2021
Operator:	Alisola
Pre-treatment:	
Sample Name:	SB6
Diluent:	Water
Remarks:	

Capture Settings

Camera Type:	sCMOS
Laser Type:	Blue405
Camera Level:	12
Slider Shutter:	1200
Slider Gain:	146
FPS:	25.0
Number of Frames:	1498
Temperature:	25.0 °C
Viscosity:	(Water) 0.9 cP
Dilution factor:	Dilution not recorded

Analysis Settings

Detect Threshold:	7
Blur Size:	Auto
Max Jump Distance:	Auto: 24.8 - 25.1 pix

Results

Stats: Mean +/- Standard Error

Mean:	34.3 +/- 0.2 nm
Mode:	32.0 +/- 0.2 nm
SD:	22.3 +/- 1.7 nm
D10:	12.5 +/- 0.1 nm
D50:	22.2 +/- 0.0 nm
D90:	31.6 +/- 1.5 nm
Concentration:	3.30e+008 +/- 4.11e+006 particles/ml
	16.8 +/- 0.2 particles/frame
	20.7 +/- 0.4 centres/frame

Zeta Settings and Results

Parabola fit complete
Adjusted r-square: 1.00

Applied Voltage	24.0 V
Dielectric Constant:	80.00
Average Current	0.42 - 0.44 µA

Stats: Mean +/- Standard Error

Mean:	-16.6 +/- 0.1 mV
Mode:	-18.2 +/- 2.6 mV
SD:	28.8 +/- 0.1 mV
D10:	-52.9 +/- 0.3 mV
D50:	-18.1 +/- 0.1 mV
D90:	17.5 +/- 0.9 mV

Figure 13: The size distribution and Zeta potential of AgNps synthesized powdered stem aqueous extracts using NTA.

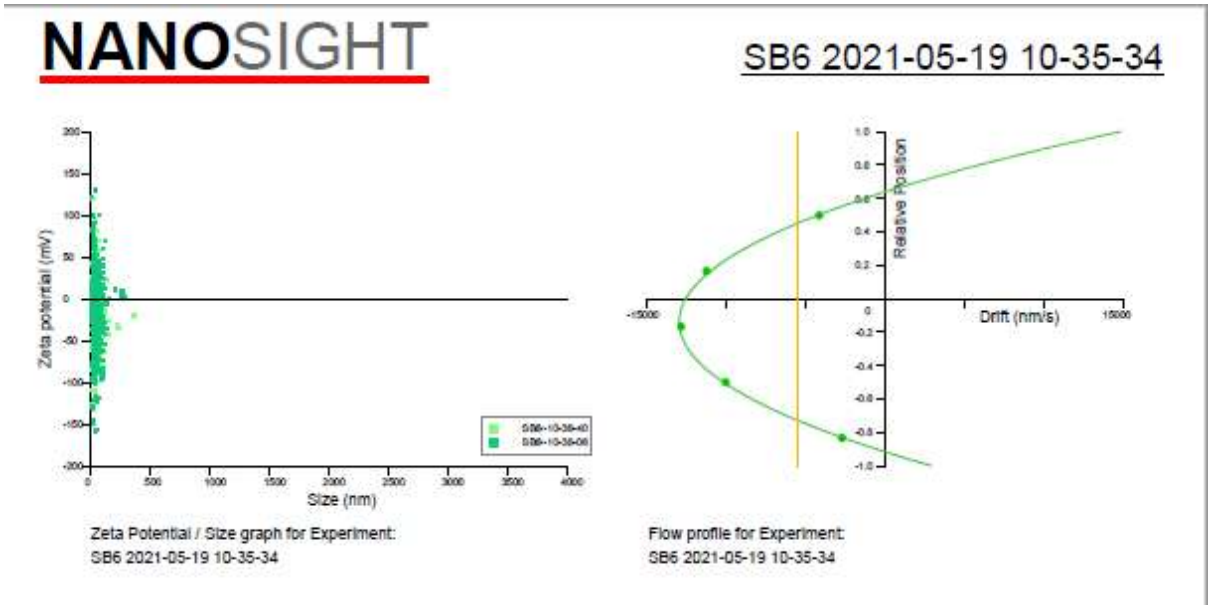


Figure 14: The size distribution and Zeta potential of AgNps synthesized powdered stem aqueous extracts using NTA.

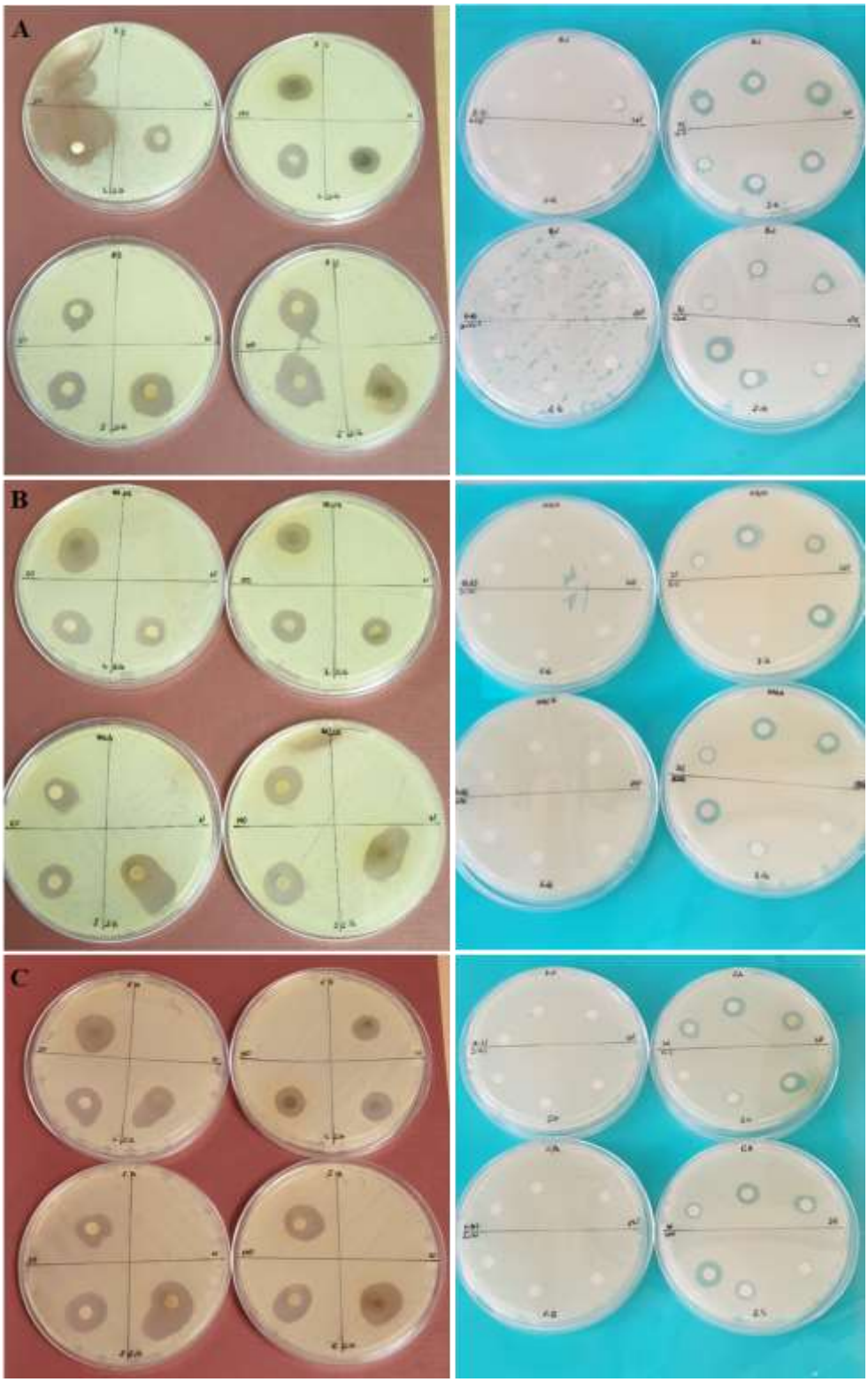


Figure 15: Plates displaying antibacterial activity of synthesized extracts (methanol, fresh and powdered) from the leaves and stem of *B. albostellata*. (A) *B. subtillus*; (B) Methicillin-resistant *S. aureus*; (C) *S. aureus*.

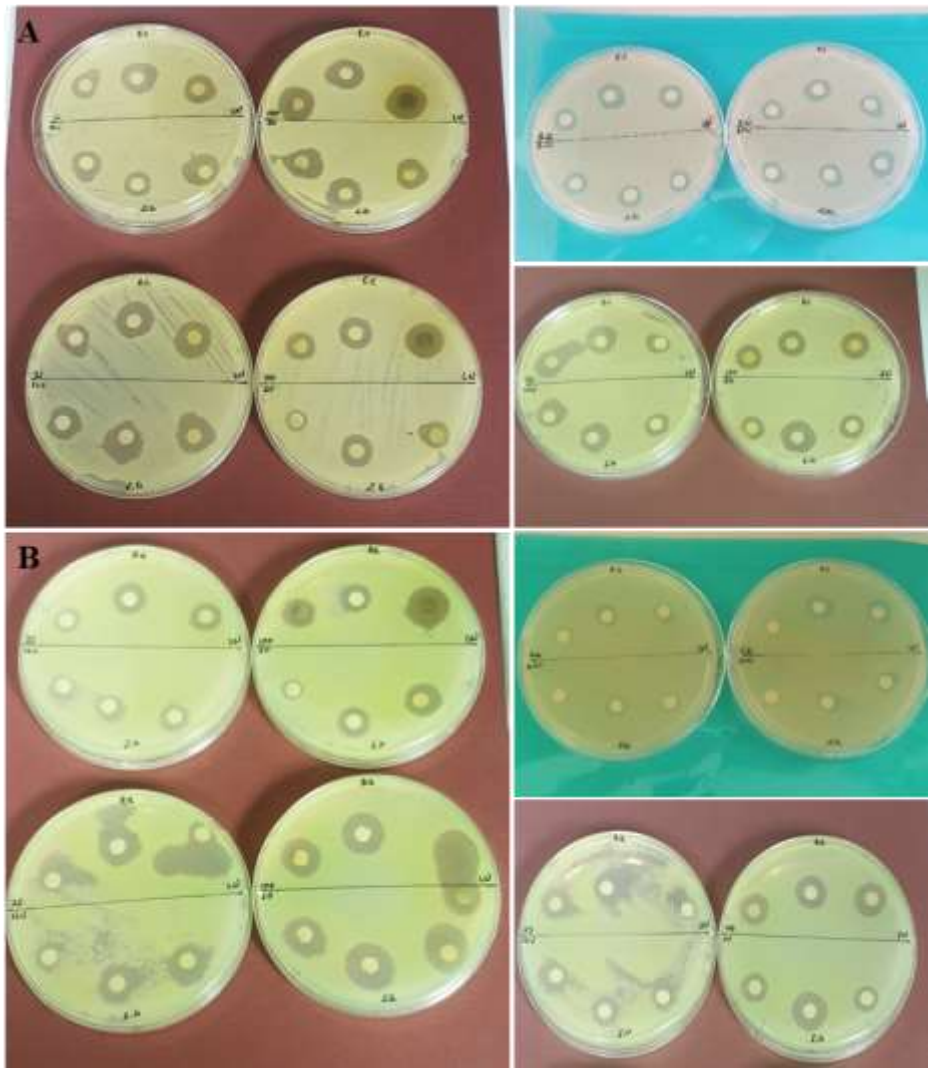


Figure 16: Plates displaying antibacterial activity of synthesized extracts (methanol, fresh and powdered) from the leaves and stem of *B. albostellata*. (A) *E. coli*; (B) *P. aeruginosa*.

APPENDIX B

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Foliar micromorphology, ultrastructure, and histochemical analysis of *Barleria albostellata* C.B. Clarke

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ABSTRACT

Barleria albostellata (Acanthaceae), is a valuable medicinal plant with a broad spectrum of antibacterial and anti-inflammatory activities. This study aimed to characterize the micromorphology, distribution, and chemical composition of the trichomes present on the leaves and stems of *B. albostellata* using light and electron microscopy and histochemistry. Morphological observations using stereo and scanning electron microscopy (SEM) revealed a dense indumentum bearing numerous non-glandular trichomes on the leaves and stems of *B. albostellata*. The histochemical and SEM analyses revealed the presence of five morphologically distinct glandular capitate trichome types, multangulate-dendritic branched (MDB) non-glandular trichomes and a glandular head attached to a branched non-glandular trichome. Transmission electron micrographs showed that numerous plastids, mitochondria, and endoplasmic reticulum cisternae were actively involved in the secretory process. The stained leaf and stem sections indicated the presence of alkaloids and phenolics as the major medicinal compounds in glandular and non-glandular trichomes. To our knowledge, this study represents the first detailed report describing the key micromorphological features of the foliar structures of *B. albostellata* as well as the preliminary chemical composition of the secretions produced by these structures.