Phytochemical and Pharmacological Analyses of *Embelia ruminata* (E.Mey. ex A.DC.) Mez

by

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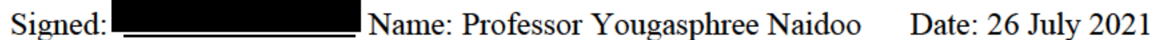
As the candidate's supervisors, we have approved this dissertation for submission.



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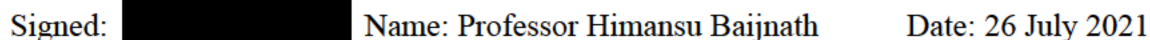

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PREFACE

The research contained 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 research did not receive any research funding.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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DECLARATION 1: PLAGIARISM

I, Neervana Rambaran, declare that:

(i) the research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work;

(ii) this thesis has not been submitted in full or in part for any degree or examination to any other university;

(iii) this thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons;

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a) their words have been re-written but the general information attributed to them has been referenced;

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(v) where I have used material for which publications followed, I have indicated in detail my role in the work;

(vi) this thesis is primarily a collection of material, prepared by myself, published as journal articles or presented as a poster and oral presentations at conferences. In some cases, additional material has been included;

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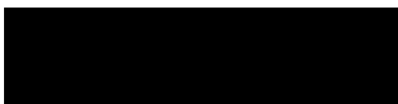

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DECLARATION 2: PUBLICATIONS

Publication 1

Neervana Rambaran, Yougasphree Naidoo, Farzana Mohamed, Hafizah Y. Chenia, Himansu Baijnath

Antimicrobial and anti-quorum sensing activities of the different solvent extracts of *Embelia ruminata*

Contributions: Neervana Rambaran collected, prepared extracts, analysed data and wrote up the manuscript. Farzana Mohamed and Dr Chenia assisted with experimental work for the antibacterial, anti-QS investigations and with proofreading. Prof Naidoo and Prof Baijnath supervised and edited the manuscript.
(Publication in preparation)

Publication 2

Neervana Rambaran, Yougasphree Naidoo, Depika Dwarka, Channangihalli Thimmegowda Sadashiva, Himansu Baijnath

Analyses of the phytochemical profile, *in vitro* antioxidant, radical scavenging and cytotoxicity activities of the vegetative and reproductive organs of *Embelia ruminata* (Primulaceae)

Contributions: Neervana Rambaran performed the experimental work, recorded data and wrote the manuscript. Dr Sadashiva assisted with the collecting of specimens and provided guidance with experimental techniques. Dr Dwarika assisted with the cytotoxicity experiments as well as proofreading. Prof Naidoo and Prof Baijnath supervised and edited the manuscript.
(Publication in preparation)

Publication 3

Phytosynthesis of silver nanoparticles architecture using *Embelia ruminata*: Synthesis, characterisation, antibacterial and anti-quorum sensing properties.

Neervana Rambaran, Yougasphree Naidoo, Farzana Mohamed, Hafizah Y. Chenia, Himansu Baijnath

Contributions:

Neervana Rambaran collected, prepared extracts, synthesised nanoparticles, analysed data and wrote up the manuscript. Farzana Mohamed and Dr Chenia assisted with the antibacterial, anti-QS investigations and with proofreading. Prof Naidoo and Prof Baijnath supervised and edited the manuscript.
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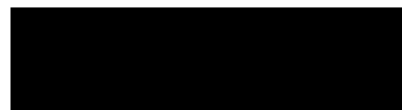

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ABSTRACT

The discovery of novel phytoconstituents to treat a plethora of ailments has become urgent as the demand for phyto-resourced products has intensified. To complement the search for new phytoceutical products, the current dissertation propelled an investigation into the phytochemical and biological potential of a South African plant, *Embelia ruminata* (E.Mey. ex A.DC.) Mez. The vegetative structures (leaf and stem bark) and the reproductive organs (fruit and seed) of *E. ruminata* were sequentially extracted using hexane, chloroform and methanol. The subsequent phytochemical analyses, which included phytochemical tests, Fourier transform infrared (FTIR) and gas chromatography-mass spectroscopy (GC-MS) of the crude extracts revealed the presence of various pharmacologically bioactive compounds. Furthermore, the data from the radical scavenging investigations demonstrated that the methanolic seed and stem bark extracts (IC_{50} of 3.54 and 37.47 µg/mL, respectively) displayed potent scavenging activities compared with the standard butylated hydroxytoluene (IC_{50} of 91.09 µg/mL). Evidently, the radical scavenging results corroborated with the cytotoxic effects of the crude extracts, which indicated that the methanolic seed and stem bark extracts had strong anticancer activities against the cancer cell lines, i.e., breast cancer (MCF-7) and human lung cancer (A549). Additionally, silver nanoparticles (AgNPs) using the aqueous extracts of the leaf, stem bark and fruit of *E. ruminata* were synthesised and characterised by adopting a series of standard tests. The antibacterial potential of both the AgNPs and the crude extracts were evaluated and were distinctively more effective against the Gram-positive than the Gram-negative bacterial strains, with the AgNPs of fruit extracts synthesised at room temperature (23±2 °C) and the methanolic stem bark crude extracts showing the most promising activity. Two biomonitor strains, *Chromobacterium subtsugae* CV017 (short chain) and *Chromobacterium violaceum* ATCC 12472 (long chain), were used to test the quorum sensing (QS) violacein inhibition capacity of the respective extracts. Overall, the AgNPs and crude extracts displayed more effective QS inhibition against the long chain than the short chain biomonitor strain. Interestingly, the chloroform leaf, hexane and methanol seed extracts showed QS violacein inhibitory activities against both biomonitor strains, indicating the potential of these extracts against multiple bacterial strains. These findings provide evidence that *E. ruminata* is a possible source of potential medicinal compounds.

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Om Shanti

'Life is not about reaching a destination, it's about enriching yourself through the journey.'

Brahma Kumaris

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LIST OF ABBREVIATIONS

A549	Adenocarcinomic human alveolar basal epithelial cells (Lung cancer)
AA	Ascorbic acid
AAA	Abdominal aortic aneurysm
AAE	Ascorbic acid equivalents
Abs	Absorbance
ABTS	2,2´-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
$AgNO_3$	Silver nitrate
AgNPs	Silver nanoparticles
AHL	N-acyl-homoserine lactones
AI	Autoinducer
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
a.u.	Absorbance units
BHT	Butylated hydroxytoluene
CFU	Colony-forming units
Chl	Chloroform
Chl-F	Chloroform fruit
Chl-L	Chloroform leaf
Chl-Se	Chloroform seed
Chl-St	Chloroform stem bark
CIP5	Ciprofloxacin
COVID-19	Coronavirus disease 19
csp	counts per second
CVD	Cardiovascular disease
DMEM	Dulbecco's modified Eagle's medium

DMSO	Dimethyl sulfoxide
DPPH	2, 2-Diphenyl 1-picryl hydrazyl radical
dw	Dry weight
EDX	Energy dispersive X-ray analysis
EPM	Elevated plus maze
eV	Electron volt
FBS	Foetal Bovine Serum
Fe^{3+}	Ferric ion
FRAP	Ferric reducing antioxidant power
FTIR	Fourier transform infrared
g	Grams
$GABA_A$	Gamma aminobutyric acid Type A
GAE	Gallic acid equivalent
GC-MS	Gas chromatography-mass spectroscopy
GI	Growth inhibition
GN10	Gentamicin
H	Hydrogen
h	hours
H_2SO_4	Sulphuric acid
Hex	Hexane
Hex-F	Hexane fruit
Hex-L	Hexane leaf
Hex-Se	Hexane seed
Hex-St	Hexane stem bark
HR-TEM	High-resolution transmission electron microscope
HIV	Human immunodeficiency virus
HO-1	Heme oxygenase-1
HSD	Honestly significant difference
HSV-1	Herpes simplex virus-1

HEK293	Human Embryonic Kidney cells
Hz	Hertz
IC_{50}	Half-maximal inhibitory concentration
IE	Ionisation energy
KeV	Kiloelectron-volt
MCF-7	Michigan Cancer Foundation-7 (breast cancer)
MDA	Malondialdehyde
Me	Methanol
Me-F	Methanol fruit
Me-L	Methanol leaf
Me-Se	Methanol seed
Me-St	Methanol stem bark
MH	Mueller-Hinton
Min	Minutes
mol	Mole
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
m/z	Mass-to-charge ratio
LB	Luria-Bertani
NF-kB	Nuclear factor kappa-B
NIST	National Institute Standard and Technology
NOR	Novel object recognition
NP	Nanoparticles
NrF-2	Nuclear factor erythroid 2-related factor
NTA	Nanoparticle Tracking Analysis
NTDs	Neglected tropical diseases
OD	Optical density
OM	Outer membrane
PBS	Phosphate buffered saline
PQ	Paraquat

QE	Quercetin equivalent
QS	Quorum sensing
QSI	Quorum sensing inhibition
Rt	Room temperature
RXR	Retinoid X receptor
SAED	Selected area electron diffraction
SD	Standard deviation
SPR	Surface plasmon resonance
STAT3	Signal transducer and activator of transcription 3
TAC	Total antioxidant capacity
tBHQ	tert-Butylhydroquinone
TFC	Total flavonoid content
TPC	Total phenolic content
UV	Ultraviolet
XIAP	X-linked inhibitor of apoptosis protein
ZOI	Zone of inhibition

CHAPTER 1
Introduction

1.1 Rationale for the research (nature and scope)

The practice of using plants for curative purposes is deeply engrained in the history of mankind and is an essential part of most civilizations across the world. The pillars for the use of medicinal plants rely on the experience-based knowledge of indigenous tribes and communities. This ancient knowledge is being supported by modern-day science by carrying out pharmacological research to understand and verify the efficacy of the chemical constituents and cellular principles of medicinal plants (Efferth, 2019). The unequivocal importance of medicinal plants has been advocated in the successful treatment as well as the prevention of various diseases (Krishnaprabu, 2020; Unuofin and Lebelo, 2020).

South Africa possesses a diverse population of flowering plant species, many of which have not yet been explored for their phytotherapeutic properties (Erhabor et al., 2019; Mahavy et al., 2020). This highlights a gap in the knowledge and the potential discovery for new phyto-resourced products. Furthermore, popular medicinal plants are under increasing pressure from climatic changes, development and over-utilisation (Kling, 2016). Hence, there is an urgent need to source other plants and their possible medicinal properties. This premise instituted the primary rationale for this study, which focused on unravelling the phytochemical and pharmacological properties of a local South African plant species, *Embelia ruminata* (E.Mey. ex A.DC.) Mez.

Embelia ruminata belongs to the family Primulaceae, which consists of 136 species distributed worldwide (The Plant List, 2013). Although there are a number of species in this genus, most studies have focused on a few key species. One such species is the Asian species, *Embelia ribes* Burm.f., which has great eminence as a medicinal plant and is commonly used in Ayurvedic practices (Othman et al., 2020; Xavier and Kani, 2021). In addition, there are also ethnobotanical studies documented using the African species *Embelia schimperi* Vatke (Togue et al., 2020). In contrast, there is a lack of previous studies that have been reported on *E. ruminata* and its possible phytomedicinal properties. Thus, an attempt was made to elucidate the therapeutic properties of *E. ruminata* with the intent of adding knowledge to this vast genus.

Despite established studies and advancements made, diseases such as cardiovascular diseases (CVD), inflammatory and metabolic disorders are prevalent and widespread in society (Umer et al., 2017; Einarson et al., 2018; Hamjane et al., 2020; Payab et al., 2021). From a global perspective, as the Coronavirus disease 2019 (COVID-19) pandemic has spread rampantly across continents, a high mortality rate has been associated with COVID-19 patients with underlying comorbidities such as CVD (Delgado-Roche and Mesta, 2020; de Lucena et al., 2020; Nishiga et al., 2020). In light of such developments, there is an urgent need to find novel compounds that can scavenge free radicals and serve as reducing agents to prevent the onset of underlying conditions. In this regard, the current study steered to establish the radical scavenging and antioxidant capacity of *E. ruminata*.

The escalating incidence of morbidity and mortality from cancer presents a serious public health challenge. According to the review by Ferlay et al. (2021), the global cancer statistics for the year 2020 indicated that the most commonly diagnosed cancers were female breast cancer (2.26 million cases), while the most common cause of cancer deaths was lung cancer. Despite various treatment options, the success rate is low and often associated with severe side effects (Gezici and Şekeroğlu, 2019). Thus, in the search for new natural treatment options, the exploration of medicinal plants as novel anticancer agents has gained momentum (Martínez-Aledo et al., 2020; Cilwyn et al., 2021). In view of this, the crude extracts of *E. ruminata* were evaluated for their efficacy as anticancer candidates. Furthermore, according to Twilley et al. (2020), South African plants that can be used to treat cancer have been poorly documented. Therefore, this study served to provide information on a South African plant species as a potential anticancer agent.

The rise of antibiotic resistance has resulted in an intense drive to search for novel antibacterial agents as alternatives to conventional antibiotics with researchers exploring the efficacy of various antibacterial compounds derived from plant sources (Ng et al., 2021). The present study intended to support this inquest by investigating the antibacterial potential of the extracts of *E. ruminata*. Additionally, the bactericidal and mutagenic mechanisms of conventional antibiotics exert evolutionary pressure on bacterial strains, in essence, provoking bacterial resistance (Ahmed et al., 2019). Research has orientated to find agents that instead target the pathogenesis and virulence process of the bacterium. One such approach is the attenuation of quorum sensing (QS) present in many bacteria (Piewngam et al., 2020). In microbial communities, the expression of virulence and pathogenicity is controlled by quorum sensing, a cell-to-cell

communication system ubiquitously used by microbes to monitor their population density (Deryabin et al., 2019). Predominantly, interest has geared towards finding natural products, known as QS inhibitors that can disrupt the QS process. In this study, the effectiveness of *E. ruminata* extracts to inhibit quorum sensing in *Chromobacterium subtsugae* and *Chromobacterium violaceum*, two biomonitor bacterial strains, were investigated.

To further elaborate the aim mentioned above, the efficiency of silver nanoparticles (AgNPs) as antibacterial and anti-QS agents was investigated. The propensity of AgNPs as effective alternatives to drug therapy has gained interest from researchers (Foroohimanjili et al., 2020; Gul et al., 2021; Shanmugapriya et al., 2021). To achieve the goal, an investigation focused on the green synthesis of silver nanoparticles (AgNPs) using aqueous *E. ruminata* leaf, stem bark and fruit extracts with optimisation of the synthesis process at room temperature (23± 2°C) and 80 °C was undertaken. The synthesised nanoparticles solutions were then tested for their antibacterial potential and ability to inhibit QS using *C. subtsugae* CV017 and *C. violaceum* ATCC 12472 biomonitors.

1.2 Aim of the study

The aim of the study was to investigate the phytochemical and pharmacological potential of the South African plant species, *Embelia ruminata*.

1.3 Objectives of the study

- To identify the phytochemical classes, the functional groups and major compounds present in the crude extracts of *E. ruminata* using preliminary phytochemical tests, Fourier transform infrared (FTIR) analysis and gas chromatography-mass spectroscopy (GC-MS), respectively.
- To quantitatively evaluate the total phenolic and total flavonoid content by means of the Folin-Ciocalteu and aluminum chloride assays, respectively.
- Assess the antibacterial potential of the different solvent extracts of *E. ruminata* leaf, stem bark, fruit and seeds using the Kirby-Bauer disk diffusion method.
- To qualitatively and quantitatively determine the anti-quorum sensing capacity of extracts employing the violacein inhibitory assay.
- Measure the radical scavenging ability of extracts employing 2, 2-diphenyl 1-picryl hydrazyl radical (DPPH) assays.

- To determine the antioxidant properties of extracts by applying the total antioxidant capacity (TAC) and ferric reducing antioxidant power (FRAP).
- To determine the cytotoxic effects of plant extracts on cancer cell lines, i.e., breast cancer (MCF-7) and human lung cancer (A549) as well as on normal cells (HEK293) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.
- To synthesise AgNPs and determine the optimal conditions for synthesis, i.e., the effect of temperature and time on the AgNPs fabrication process.
- Characterise the silver nanoparticles using UV-Visible spectroscopy, high-resolution transmission electron microscope (HR-TEM), energy dispersive X-ray (EDX) analysis, Fourier transformed infrared spectroscopy (FTIR) and zeta potential analyses.
- Establish the antibacterial and anti-QS activity of synthesised silver nanoparticles using the Kirby-Bauer disk diffusion method and QS violacein inhibitory assays, respectively.

1.4 Outline of dissertation structure

Chapter 1: Discusses the rationale of the study, the overview of the aims and objectives including the general outline of the dissertation methodology.

Chapter 2: Literature review provides an insight into the genus *Embelia* Burm.f. including an overview of traditional uses and recent scientific research developments.

Chapter 3. The antibacterial activity, including the ability of the crude extracts to inhibit quorum sensing in *Chromobacterium* spp, has been investigated.

Chapter 4: In this chapter, the compounds in the extracts of *E. ruminata* were determined. Subsequently, the radical scavenging and antioxidant activity were investigated and discussed. Thereafter, the cytotoxic potential of crude extracts was analysed.

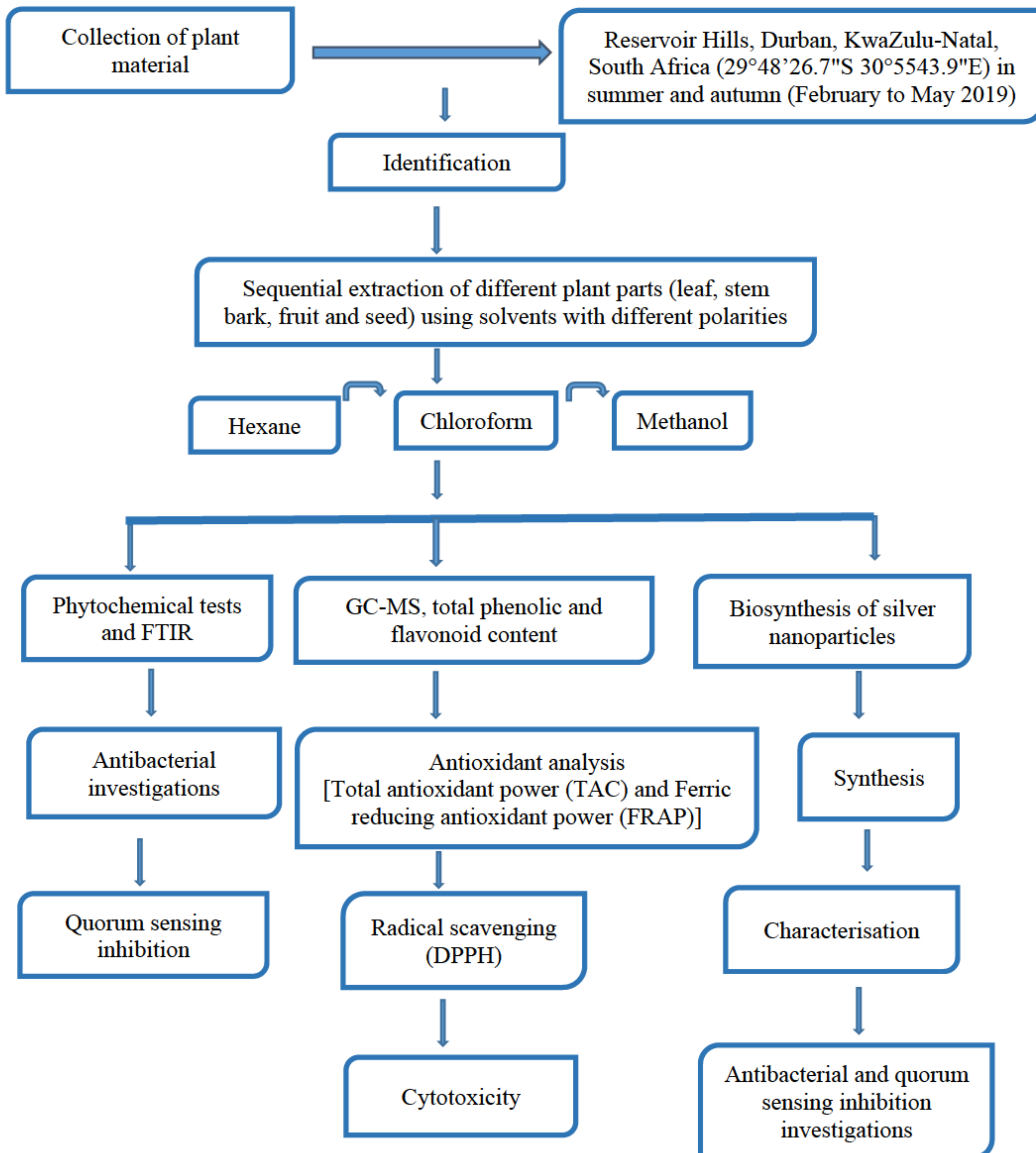
Chapter 5. The green synthesis of silver nanoparticles was undertaken. The antibacterial activity, as well as the ability of the synthesised AgNPs to inhibit quorum sensing, were determined.

Chapter 6. This chapter concludes the study with major findings, challenges that were faced and future research endeavours.

The chapters 3 to 5 has been presented as research papers.

1.5 Outline of the dissertation methodology

A basic overview of how the research was conducted has been outlined below:



1.6 References

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CHAPTER 2
Literature Review

2.1. Overview of the genus *Embelia* Burm.f.

2.1.1 Introduction

The genus *Embelia* Burm.f. belongs to the family Primulaceae. This family of dicotyledonous flowering plants (Xu and Chang, 2017) comprises 2500 species with 55 accepted genera (Royal Botanic Gardens Kew, 2021). The genus *Embelia* was originally placed in the family Myrsinaceae. Following a redefinition that was established on plastid molecular data, Myrsinaceae and two other closely related families: Maesaceae and Theophrastaceae were found to be nested within the family Primulaceae (Anderberg et al., 1998; Källersjö et al., 2000; Mast et al., 2001). Thus, these three families are now classified as belonging to the family Primulaceae in the order Ericales.

The benzoquinone derivatives are of particular importance in some genera of the family Primulaceae and are considered chemotaxonomic markers (Otegui et al., 1998; de Luna et al., 2017; Costa et al., 2020). Some examples of these benzoquinone derivatives include embelin, rapanone, vilangin, macrophyllin, maesaquinone, bhaogatin, maesanin, ardisianone A, B and ardiasiaquinone A, B and C (Otegui et al., 1998).

Nicolaas Laurens Burman, in his 1768 publication, Flora Indica, first described the genus *Embelia* using *Embelia ribes* Burm. f. as the type (Burman, 1768; Dubéarnès et al., 2015). Later, Mez (1902) revised the family Myrsinaceae, where he described 92 species of *Embelia*, with 8 subgenera (Mez, 1902; Dubéarnès et al., 2015). According to The Plant List (2013), there are approximately 156 species in the genus *Embelia* with 136 species that are taxonomically accepted and 20 unresolved species.

2.1.2 Distribution

Different species of the genus *Embelia* are found distributed in the tropical and subtropical areas of Africa, eastern Asia and Australia (Atlabachew et al., 2017) (Figure 2.1).

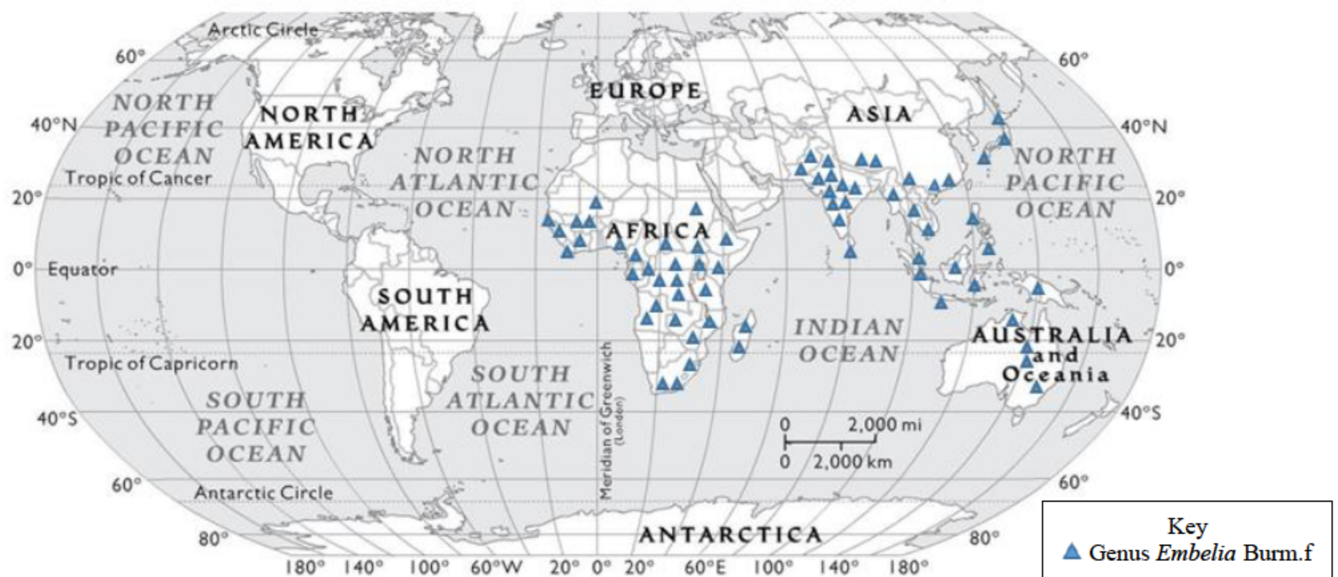

Figure 2.1 Geographical distribution of species in the genus *Embelia* Burm.f. (National Geographic Society, 2009 and Royal Botanic Gardens Kew, 2021)

2.1.3 Classification (GBIF, 2019)

Kingdom: Plantae

Phylum: Tracheophyta

Class: Magnoliopsida

Order: Ericales

Family: Primulaceae

Genus: *Embelia* Burm.f.

2.1.4 Morphological Description

The following description of the genus *Embelia* has been adapted from Hyde et al. (2021); Royal Botanic Gardens Kew (2021) and World Flora Online (2021).

Shrubs, becoming lianous at maturity or lianas. Leaves alternate; distichous or pseudoverticillate; margin crenate or entire. Inflorescence racemose, paniculate, umbellate or corymbose; bracteate at base. Pedicel subtended by one persistent floral bract. Flowers pedicellate or sessile, 4- or 5-merous; bisexual or functionally unisexual, plants dioecious, polygamous or monoecious. Calyx 4-5 parted or lobed with lobes united at base, free or shortly connate, imbricate, usually marked with resiniferous dots. Petals free or united at base, shortly connate, elliptic, obovate or oblong, the inner surface and

sometimes the outer margins densely papillose. Stamens adnate to base of petals, shorter or longer than corolla, filaments free, long, slender and well developed; anthers dehiscing longitudinally, connectives usually punctate abaxially with dark spots. Ovary superior. Ovary of female flower subglobose or ovoid, often pilose; long or short style; stigma disciform, entire or rarely lobed; placenta with few ovules arranged in 1 row. Ovary of male flowers much reduced, conical, tongue-like or stipiform. Fruit drupe, prominently punctate, 1-seeded; up to ± 14 mm in diameter, pericarp fleshy, endocarp crusty or rarely bony. Seeds solitary, subglobose with a small to large basal cavity, covered by membranous remnants of placenta; endosperm ruminate; embryo terete or transverse.

2.1.5 Phytocompounds of the genus *Embelia*

A diverse reservoir of biologically active chemicals are present in the plants (Ingle et al., 2017). These phytochemicals have a wide range of pharmacological activities that have been used to treat various maladies (Egbuna et al., 2018). The extraction of bioactive compounds from plants and their quantitative and qualitative estimation is essential for exploring new biomolecules that the pharmaceutical and agrochemical industry may use directly or as lead molecules to synthesise more potent analogues (Egbuna et al., 2018).

The principal compound established in the genus *Embelia* is embelin (Kaur et al., 2015; Rondevaldova et al., 2015; Vijayan and Raghu, 2021). Embelin is a benzoquinone derivative, which has been shown to have a diverse range of biological activities, including anti-inflammatory, antioxidant, antibacterial and anti-obesity (Atlabachew et al., 2017; Kundap et al., 2017; Bansal et al., 2020). Embelin (Figure 2.2) is a chemical species that comprises both quinone and hydroquinone functional groups plus a long hydrophobic tail (Caruso et al., 2020).

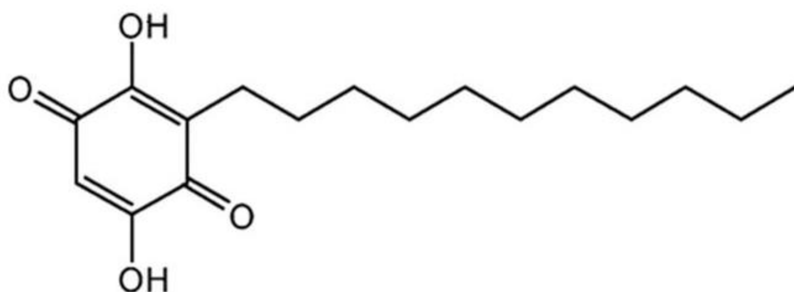

Figure 2.2 An illustration of 2,5-dihydroxy-1,4-benzoquinone compound, commonly referred to as embelin. Source: Mazlan et al. (2019).

From initial studies by Kaul et al. (1929), embelin was isolated from the fruit of *Embelia ribes*. Later work done by Chopra et al. (1996) showed that the seeds of *E. ribes* contain embelin (2.5–3.1%), quercitol (1.0%), fatty ingredients (5.2%), the alkaloid christembine, resinoid compounds, volatile oil, fixed oil, colouring matter and tannins.

Further phytochemical investigations conducted by Haq et al. (2005) resulted in the isolation of three compounds identified as embelinol, embeliaribyl ester and embeliol along with the known embelin. In another study, Lin et al. (2006) isolated a nitrogen-containing 3-alkyl-1,4-benzoquinone and a gomphilactone derivative together with sitosterol and daucosterol from the roots of *E. ribes*. Dang et al. (2014) isolated 12 compounds from the stem bark of *E. ribes*, which were categorised as resorcinol derivatives, benzoquinone, neolignan and phenylpropanoid compounds. All 12 compounds showed α-glucosidase inhibitory activity (Dang et al., 2014). Their results were further substantiated by investigations carried out by Chen et al. (2020).

In other studies, phytochemical investigations using *Embelia schimperi* Vatke have led to the isolation of flavonoids (Manguro and Williams, 1997), benzoquinones (Kiprono et al., 2004) and pentacyclic triterpenoids (Manguro et al., 2006). More recently, an alkylbenzoquinone, embeliquinone was characterised from the methanolic leaf extracts of *Embelia rowlandii* Gilg., which exhibited moderate anticancer activity against A549 cancer cell lines with the IC_{50} value of 21.8 μM (Bouzeko et al., 2019). Additionally, three alkenylresorcinols were isolated from the methanolic leaf extracts of *E. schimperi*, which exhibited moderate cytotoxic activity against human cervical cancer (Togue et al., 2020).

Guo et al. (2020) identified 56 compounds that included 16 phenolics, 16 flavonoids, 4 coumarins and 5 fatty acids in *E. ribes*. Unfortunately, the authors did not clearly indicate which plant organ was used for their analyses. In another study, Qin et al. (2020) isolated three flavonoid glycosides, embeliaflavoside A, B and C from the fruit of *E. ribes*. These compounds displayed radical scavenging activity with IC_{50} values of 2.52–9.78 µM and 7.56–26.47 µM for 2,20-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2, 2-diphenyl 1-picryl hydrazyl radical (DPPH) assays, respectively (Qin et al., 2020).

2.1.6 Traditional uses

Historical medical texts, archaeological remains, documents from ancient medicinal practices and their applications provide substantial information about the history of medicine (Magner and Kim, 2017; Gökalsın et al., 2019). Deeply embedded in these traditions is the utilisation of plants to treat various health-related issues as well as the maintenance of general well-being (Salmeron-Manzano et al., 2020).

Synchronously, some species of the genus *Embelia* are firmly entrenched in many traditional practices to treat a wide variety of ailments. This ethnomedicinal application has provided the impetus for many of the scientific studies that have unfolded subsequently. Table 2.1 (on page 29) reviews the traditional therapeutic uses employed by ethnic tribes using the different species of *Embelia*.

The Asian species, *E. ribes* is highly esteemed for its medicinal properties and has been used in the ancient healing practices of Ayurveda, Unani, Siddha and Tibetan therapeutic systems to treat inflammatory diseases such as rheumatism and fever (Kundap et al., 2017; Bansal et al., 2020). The ancient Ayurvedic practitioners described the fruit as an antihelminthic, tonic and recommended its use together with liquorice root to rejuvenate the body to hinder the effects of ageing (Akbar, 2020).

Another species, *Embelia tsjeriam-cottam* (Roem. & Schult.) A.DC., native to eastern countries, is used in different regions for a variety of ethnopharmacological remedies, which include: treatment of diarrhoea, dyspepsia, chest complaints, snake bites, piles, sore throat, bronchitis, spleen enlargement, arthritis as well as vermifuge, wound healing and as a carminative (Natarajan and Paulsen, 2000; Misra, 2004; Somkuwar et al., 2013;

Prashanth and Shiddamallayya, 2016; Thu et al., 2018; Bhat et al., 2019) (Table 2.1).

The tropical African species, *E. schimperi*, is commonly used to treat helminth infections (Teka et al., 2020; Alemneh and Imran, 2021). The different plant organs such as the leaves, stem bark, fruit, seeds or roots are powdered to make an oral decoction that is ingested. Other species reported to treat helminthiasis include: *E. rowlandii* (Focho et al., 2009; Tachama et al., 2015), *E. ruminata* (Mabona et al., 2013), *Embelia scandens* (Lour.) Mez (Ghorbani et al., 2011), *Embelia sessiliflora* Kurz (Tangjitman et al., 2013; Phumthum and Balslev, 2020) as well as *E. tsjeriam-cottam* (Misra, 2004; Thu et al., 2018).

Several scientific studies have also validated the use of *Embelia* as a treatment for helminth infections (Khan et al., 2011; Mohandas et al., 2013; Patil et al., 2019; Phumthum and Balslev, 2020). Furthermore, the Ayurvedic formulation, Vidangadi churna that contains *E. ribes* showed potent *in vitro* antihelminthic activity against adult earthworm *Pheritima posthuma* (Mohandas et al., 2013). Likewise, Khan et al. (2011) evaluated the herbal anti-round worm medicine: Kemol, 500 mg tablet, which contains 125 mg of *E. ribes* and was found to be economical, safe and an effective anti-round worm remedy.

In recent years, the focus of scientific analysis has diversified mainly due to the discovery that the compounds of *Embelia*, especially the embelin compound has several other pharmacological properties.

2.1.7 Scientific studies: Recent developments

There are published reviews in the past five years that have detailed the activity of mostly *E. ribes* and embelin (Kundap et al., 2017; Gaman et al., 2018; Ko et al., 2018; Badmanaban et al., 2019; Othman et al., 2020; Xavier and Kani, 2021). Henceforth, the account below has steered to focus on new developments and biological studies, although most of these studies have been performed using *E. ribes* and isolated embelin. This may emphasise the need to explore other species within the genus *Embelia* for their therapeutic properties. Below is an account of some recent developments using the genus *Embelia*.

2.1.7.1 Antioxidant

Oxidative stress plays a pivotal role in the onset of chronic conditions such as cardiovascular diseases, diabetes, neurodegenerative diseases and cancer (Sharifi-Rad et al., 2020). Medicinal plants such as those belonging to the genus *Embelia* have been explored for their antioxidant mechanisms to help alleviate such diseases (Guo et al., 2020; Qin et al., 2020).

Caruso et al. (2020) conducted in-depth studies using embelin as antioxidant agents. The authors found that embelin scavenges the superoxide radical by quenching its electron and thereby releasing molecular oxygen. Thus, molecular oxygen is released as embelin accepts an electron from the radical instead of releasing a hydrogen (H) atom to the radical (Caruso et al., 2017). This action of embelin, thereby, stabilises the radical, which becomes ineffective to scavenge and cause cellular damage.

The herbicide paraquat (PQ) is a fast-acting and effective agent used throughout the world to eliminate weeds (Rashidipour et al., 2020). However, PQ poisoning is a severe hazard to human health that can result in pulmonary toxicity, neurotoxicity and inflammation (Li et al., 2020). To explore strategies to circumvent the effects of PQ on human health, SreeHarsha (2020) investigated the protective effects of embelin against paraquat-incited lung damage in conjunction with its antioxidant and anti-inflammatory action. Their investigations showed that embelin significantly decreased oxidative stress markers, like malondialdehyde (MDA), leading to reduced lung tissue damage in the PQ-intoxicated rats.

On a similar note, Bansal et al., 2020 showed that embelin could ameliorate oxidative stress in high-fat diet-fed obese C57BL/6 mice leading to an alleviation of obesity by regulating the nuclear factor erythroid 2-related factor (NrF-2) and nuclear factor kappa-B (NF-kB) pathways.

2.1.7.2 Antiviral activities

Elias et al. (2021) investigated the *in vitro* antiviral activity of embelin using Vero cells infected with a recombinant strain of *Herpes simplex* virus-1 (HSV-1). Their experiments demonstrated that the treatment of HSV-1 virions with embelin resulted in a 98.7-100%

inhibition of the infection. Furthermore, it was postulated from their investigations that embelin was able to inhibit the HSV-1 virions attaching and penetrating the host cells. Thus, the authors concluded that embelin could decrease oxidative damage caused by HSV-1 infection and was an effective antiviral agent with the potential to reduce the infection of HSV-1 in cultured Vero cells (Elias et al., 2021). This study supported the concept that the inhibitory activities of embelin were attributed to its role as an antioxidant.

2.1.7.3 Psychotropic potential

Scopolamine is a non-selective post-synaptic muscarinic receptor blocker that can induce significant deficits in cognitive performance (Lu et al., 2018). The effectiveness of embelin as a nootropic and neuroprotective agent in scopolamine-induced amnesia in rats was demonstrated by Bhuvanendran et al. (2018). Their study showed that embelin significantly improved the recognition index and memory retention in the novel object recognition (NOR) and elevated plus maze (EPM) tests, respectively. These findings were further supported by a study by Nuthakki et al. (2019), where authors concluded that embelin could be a lead compound in treating Alzheimer's disease. Their research showed that embelin played a role in stopping the formation of amyloid-β oligomers, improved cholinergic-transmission and increased amyloid-β clearance (Nuthakki et al., 2019).

In the treatment of afflictions associated with central nervous system (CNS) disorders, other studies have shown that embelin acts by inhibiting pro-inflammatory cytokines by the modulation of the sodium channel, chloride conductance and gamma aminobutyric acid type A ($GABA_A$) receptors (Liu et al., 2018; Qian et al., 2018; Stalin et al., 2020). A review by Kundap et al. (2017) details the effectiveness of embelin and highlights that although embelin proved to be effective in laboratory experiments against various CNS disorders, it has not yet translated to human studies. Recently, Visentin et al. (2020) showed that embelin is the only known *in vitro* inhibitor to date that can prevent neuroserpin polymerisation, a severe and lethal neurodegenerative disease. Likewise, future studies are warranted, especially in human trials to fully realise the medicinal potential of these phytoceuticals.

Embelin possesses a flexible hydrophobic alkyl chain together with a hydrophilic benzoquinone unit that could advantageously increase cell permeability (Caruso et al.,

2017). These characteristics, together with their low molecular weight and lipid-soluble properties, may enable embelin to transverse the blood-brain barrier by diffusion across the transmembrane (Janicka et al., 2020), hence, eliciting an effect on the CNS (Caruso et al., 2017).

2.1.7.4 Anticancer and anti-inflammatory activities

Cisplatin is used as the first-line treatment for different types of solid tumours, such as lung, prostate, bladder, colon, ovarian, neck and oesophageal cancers (Giacomini et al., 2020). However, this platinum-containing drug has some serious side effects that include activating the apoptotic pathways, inflicting cellular damage via oxidative stress, inflammation, acute kidney injury and tubular injury resulting in electrolyte wasting (Manohar and Leung, 2018).

Subsequently, investigations conducted by Qin et al. (2019) showed that embelin had an influential free radical scavenging action, inhibited oxidative stress and elevated antioxidant defenses, which lead to decreased inflammation in the kidneys of rodents administered with cisplatin. According to the authors, embelin was able to regulate NrF-2 and heme oxygenase-1 (HO-1) signalling pathways and concluded that embelin might be an efficient nephroprotective agent (Qin et al., 2019).

One of the hallmarks of cancer is the evasion of apoptosis (Delbue et al., 2020). Ko et al. (2018) reviewed the anticancer properties of embelin and concluded that embelin induces apoptosis by targeting several signalling pathways, which differ considerably based on the origin of the cancer. This conclusion was further supported by analyses conducted by Xu et al. (2020), which revealed that embelin might exert dramatic effects on multiple cancers with diverse underlying mechanisms.

In addition, X-linked inhibitor of apoptosis protein (XIAP) is overexpressed in a variety of cancers and are instrumental in the resistance of cancer cells to treatment (Hussain et al., 2017; Seo et al., 2020). Embelin has been shown to exhibit chemopreventive activities by promoting apoptosis by binding to XIAP and blocking its interactions (Hussain et al., 2017; Prabhu et al., 2017). Additionally, the transcription factors, NF-κB and signal transducer and activator of transcription 3 (STAT3), play a key role in the resilience of

cancer by stimulating the inflammatory signalling network (Rius-Pérez et al., 2020). Liu et al. (2018) demonstrated that embelin was able to suppress NF-κB and STAT3 in abdominal aortic aneurysm (AAA) model mice.

Dwivedi et al. (2019) studied the anti-inflammatory effects of the ethanolic fruit extracts of *E. ribes* on formalin-induced paw oedema in rats. Their study found that the paw oedema was reduced by pretreatment with the plant extracts showing the significant anti-inflammatory potential of *E. ribes*. A review by Gaman et al. (2018) details the superiority of embelin over various phytoceutical compounds in helping to normalise pancreatic tissue since it favours apoptosis with respect to necrosis. Furthermore, Lu et al. (2016) provided a comprehensive account of embelin and its therapeutic role in chronic disease.

2.1.7.5 Antihelminthic

Helminthiasis, classified as neglected tropical diseases (NTDs), is mainly associated with poverty and predominant in the most impoverished populations of the developing countries (Patil et al., 2019). Helminthiasis is estimated to affect more than a quarter of the population of the world. Most of the drugs available to treat these diseases may result in severe side effects, poor patient compliance and the evolution of drug-resistance parasites are a severe threat (de Moraes and Geary, 2020). Thus, there is an urgent need for natural therapeutic remedies.

As mentioned previously, there are several species of *Embelia* that have been used, traditionally, to treat helminth infections. Hydroalcoholic extracts of *E. schimperi* and the isolated embelin showed both *in vitro* and *in vivo* antihelminthic activity against hookworm larvae of *Necator americans* and *Hymenolepis nana,* respectively (Debebe et al., 2015). These results are in line with previous investigations performed by Bogh et al. (1996), that reported the antihelminthic efficacy of extracts of *E. schimperi* against *Echinostoma caproni*, *Heligmosomoides polygyrus* and *Hymenolepis microstoma* in mice and also against *Hymenolepis diminuta* in rats.

More recently, Zebeaman and Gebeyehu (2018) investigated the wormicidal effects of fruit of *E. schimperi* and found effective activity. Tessema et al. (2018) found that other constituents like fatty acids and resorcinol derivatives might also contribute to the

observed antihelminthic activity, supporting the traditional use of the plant as a deworming agent.

2.1.7.6 Antimalarial

The malarial parasite, *Plasmodium*, produces an insoluble crystalline pigment, hemozoin, upon digesting host haemoglobin inside red blood cells (Lautenschlager et al., 2020). The accumulation of hemozoin in malaria-infected patients is associated with the severity and development of malarial complications (Pham et al., 2021). Thus, the inhibition of hemozoin formation is one of the main targets for antimalarial drugs (de Villiers and Egan, 2021). It has been proposed that embelin acts as an antimalarial agent with an inhibitory activity similar to that of 4-aminoquinoline chloroquine (an antimalarial drug), by impeding hemozoin formation (Bezu et al., 2015). Bezu et al. (2015) concluded that embelin and the semi-synthetic analogues showed significant *in vivo* antimalarial activity with 47.8-74.7% parasite depression at tested doses of 100-400 mg/kg in infected mice.

2.1.7.7 Embelin complexes

To investigate the proclivity as an antidiabetic agent, Alam et al. (2018) administered nanoformulated embelin-loaded niosomes to streptozotocin induced diabetic Wistar rats. This action resulted in a gradual decrease in blood glucose levels, without severe hypoglycaemia. Additionally, the maximum hypoglycaemic peak was noted at 8 h. By converse, the antidiabetic drug, repaglinide, showed maximum blood-glucose-lowering effect at 24 h. Thus, the investigation concluded that the embelin-loaded niosome formulation acted more effectively than repaglinide, which could be due to increased intestinal absorption of embelin after encapsulation in niosomes. Their study also confirmed the antioxidant efficacy of the formulation as significant increases in superoxide dismutase, catalase and glutathione levels were observed, along with decreased levels in the lipid peroxidation (Alam et al., 2018).

Nidhi et al. (2017) developed embelin-loaded enteric-coated microspheres to investigate their pharmacological potential in acetic acid-induced ulcerative colitis in rats. The optimised formulation of embelin-loaded microspheres displayed a steady release of embelin, leading to a reduction in the ulcer activity score, oxidative stress and inflammatory damage in the colon of the rats.

Arthanareeswari et al. (2020) formulated embelin-Fe complexes, which may have enhanced bioactivity. These complexes could play a role as chelating agents and function as alternative inorganic catalysts amplifying the pharmacological actions. These complexes may also prove effective in bypassing the outer membrane of Gram-negative bacteria, thereby enhancing uptake and antibacterial potency (Negash et al., 2019).

The 'green' nanotechnology using plants and their phytoconsistuents to synthesise nanocomposites has attracted interest in the biomedical field due to their multifunctional potential (Rana et al., 2020). The antioxidant, cytotoxic and antimicrobial potential of gold and silver nanoparticles synthesised from *E. ribes* were conducted by Dhayalan et al. (2017). Further studies by Othman and Sekar (2019) showed that silver nanosynthesised (AgNPs) embelin displayed potent antioxidant activity against free radicals and had significant effects against the human osteoblastic line MG-63 cancer cells. Similarly, Manikandan et al. (2019) concluded that AgNPs of *E. ribes* were potential candidates as anticancer and antibacterial agents.

Embelin derivatives were synthesised through a multicomponent reaction by Martin-Acosta et al. (2020). The synthesised derivatives showed no effect against Gram-negative bacteria. Contrastingly, several embelin synthesised compounds showed activity against Gram-positive strains. Parallel to antibacterial studies, alternative strategies such as quorum sensing are being explored due to the evolutionary pressure exerted by the bactericidal activity of antibacterial agents.

2.1.7.8 Quorum Sensing

Bacteria communicate via a network of chemical molecules known as autoinducers (AI) and is collectively referred to as quorum sensing (QS) (Vadakkan, 2020). In Gram-negative bacteria, N-acyl-homoserine lactones (AHL) are a typical class of AI (Hong et al., 2021). The attenuation of virulence factors can render the bacteria non-pathogenic by inhibiting their QS circuit (Luo et al., 2016). Phytomolecules present in plant extracts can act as quorum sensing inhibitors and may elicit a biofilm inhibitory effect (Erhabor et al., 2019; Tako et al., 2020; Zhong et al., 2020). Dwivedi and Singh (2016) showed that embelin had potential biofilm-inhibition capabilities against *Streptococcus mutans*, a common agent of oral infections such as dental caries.

2.1.7.9 Pharmacokinetic properties

Lipinski's rule of five predicts that for a compound to be considered for drug development, the compound should have the following properties: molecular mass less than 500 Dalton, high lipophilicity, less than 5 hydrogen bond donors, less than 10 hydrogen bond acceptors and molar refractivity should be between 40-130 m^{-3} mol^{-1} (Lipinski, 2004). According to Xu et al. (2020), the pharmacokinetic properties of embelin met all the requirements of Lipinski's rule of five and hence can be considered for drug development.

Li et al. (2019) investigated the bioavailability of embelin after intravenous and oral administration to rats and found that embelin rapidly reached the maximum plasma concentrations *in vivo* and the elimination occurred quickly as well. Furthermore, the authors found that the concentration of embelin in plasma was low and the absorption was incomplete. Similarly, the oral bioavailability was low, possibly due to the low solubility of embelin (Li et al., 2019).

2.1.7.10 Other

Košulič et al. (2018) evaluated the biopesticidal effectiveness of the leaf crude extracts of *E. ribes* together with two commercial pesticides azadirachtin (a natural, commercially available product) and amitraz (a synthetic acaricide). Biopesticides may be highly effective in killing pests but their side effects on non-target organisms must be minimal. The authors measured the lethal and sublethal effects of the predatory potential of the lynx spider *Oxyopes lineatipes* as a biological non-target agent. It was found that the spider's mortality increased with rising concentrations of both commercial products, but not with increasing concentrations of the extracts from *E. ribes*. Their study concluded that due to the absence of harmful effects on *O. lineatipes* in regards of mortality and predatory activity, the plant extracts from *E. ribes* could be a promising candidate as a new biopesticide (Košulič et al., 2018).

2.2 *Embelia ruminata* (E.Mey. ex A.DC.) Mez

2.2.1 Distribution

This South African plant species is distributed along the coastal and slightly inland forests of the Eastern Cape, extending northwards to the forests of Zululand in KwaZulu-Natal (Dyer, 1966) (Figure 2.3).

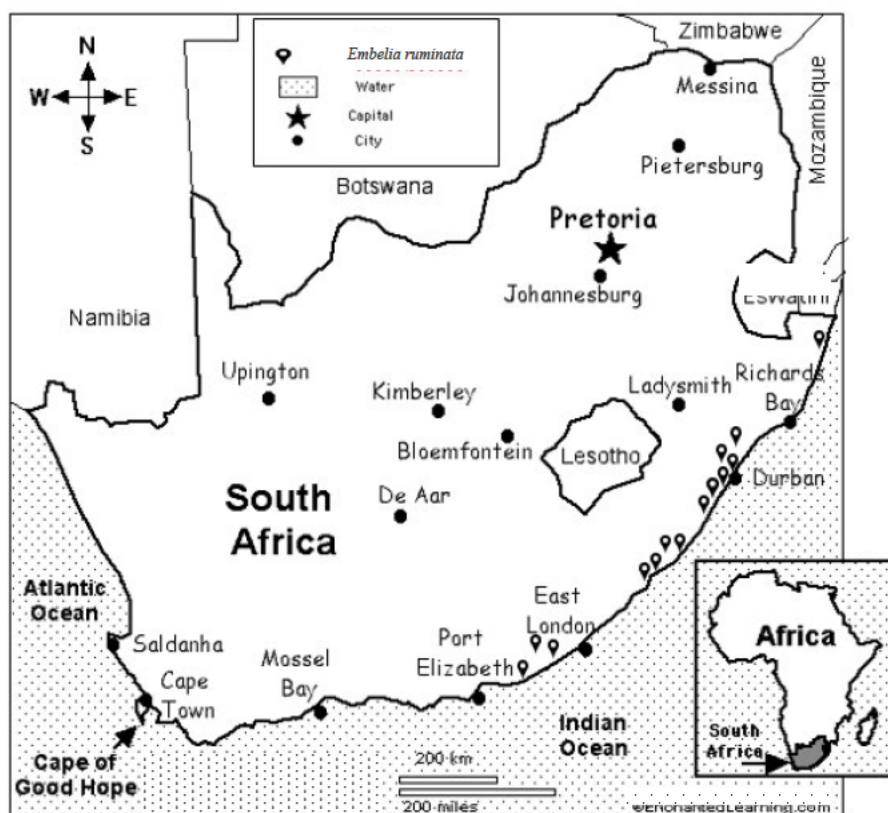

Figure 2.3 Geographical distribution of *Embelia ruminata* (African Plant Database, 2012 and Enchanted Learning, 2021).

2.2.2 Morphological Description

The following description of *E. ruminata* has been adapted from Harvey and Wright (1909) and Dyer (1966).

A scandent shrub or liana in bush or forest characterised by branchlets often reflexed minutely at first; somewhat glandular pubescent, becoming glabrous. Leaf arrangement alternate, distichous or pseudoverticillate; margin entire, with reflexed edges, often recurved when dry; obovate to obovate-oblong; base cuneate; gland-dotted but not always evident; fine lateral venation; shortly petiolate, sometimes appearing slightly winged, canaliculate, occasionally minutely pubescent with semi-glandular hairs, comparatively large leaf scars. Inflorescence short, few-flowered, axillary racemes up to 2.5 cm long from axils of leaf scars; bracts concave, oval, shorter than pedicels. Flowers white often unisexual. Calyx small, cup-shaped, 4-5-lobed or toothed. Petals white, 4-5, free, oblong, imbricate in bud, minutely, densely papillose within. Stamens 4-5, opposite, adnate to

petals below middle; filaments shorter than petals, flattish subulate; anthers medifixed with introrse dehiscence. Ovary on a small 5-crenate disc, unilocular with one basal ovule; style filiform with discoid or bifid stigma. Fruit (approximately 5 mm in diameter), drupaceous, globose or compressed-globose, 1-seeded, greenish at first becoming scarlet when ripe. Seeds subglobose, covered by membranous remnants of placenta; endosperm ruminate; embryo terete, transverse.

In Figure 2.4(a) and (b), the *E. ruminata* plant is depicted as a woody climber on a *Ficus* sp. tree, at the collection site in Reservoir Hills, Durban. Long trailing branches with short hardened lateral branches aid the climbing of the liana (Hyde et al., 2021). Figure 2.5(a) and (b) shows the adaxial and abaxial surface of a young leaf of *E. ruminata*.

Figure 2.6(a) and (b) illustrates the fruit in different stages of development. A fragile pericarp covers the seed [Figure 2.6(c)]. When the pericarp is removed, a yellow-orange coloured substance can be seen present in the cavities located on the outer surface of the seed coat (Ferreria and Laddha, 2013; Sudhakaran, 2016) [Figure 2.6 (d)]. The yellow powdery substance can be removed using a dissecting needle [Figure 2.6 (d) inset].

Figure 2.4(a) and (b) shows *Embelia ruminata* entwined onto a *Ficus* sp. tree located in Reservoir Hills, Durban, KwaZulu-Natal, South Africa (29°48'26.7"S 30°5543.9"E) with white arrows showing the stem bark.

Figure 2.5 Stereomicrographs of an emergent leaf of *Embelia ruminata*. Labels (a) = adaxial and (b) = abaxial surface

Figure 2.6 Fruit and seeds of *Embelia ruminata*. Labels (a) and (b) = twigs with fruit in different stages of development, (c) = fragile pericarp covering seed, (d) = seed after removal of pericarp, (d) inset = yellowish powder removed from the indentations of the seeds.

2.2.3 Traditional uses

The local Zulu tribes use the leaves, ripe fruit and roots as an antihelminthic against tapeworms (Bryant, 1966). The leaves are also chewed as a bitter tonic and eaten for refreshment (Gerstner, 1938). The local Zulu names for *E. ruminata* include ibhinini, ibinini, inbhinini and umoyushisa (Hutchings et al., 1996).

2.2.4 Pharmacological uses

Mabona et al. (2013) investigated the dermatological relevance of *E. ruminata* using a range of relevant pathogens. Their study showed that noteworthy results were obtained

against the following bacterial strains: gentamycin methicillin-resistant *Staphylococcus aureus* ATCC 33592, *Staphylococcus epidermidis* ATCC 2223 and *Pseudomonas aeruginosa* ATCC 27858. To our knowledge, besides this study, there have been no further investigations performed regarding the pharmacological importance of *E. ruminata*. Thus, the cornerstone of this project was to address this knowledge gap and investigate the potential therapeutic activity of *E. ruminata*.

Table 2.1 A list of the species within the genus *Embelia* and their ethnomedicinal application

Scientific name	Locality (Tribe)	Part used and vernacular name	Ethnomedicinal application	References
Embelia angustifolia (A.DC.) A.DC.	Reunion Island and Mauritius	Leaves, Liane savon	Diuretic, used in the treatment of kidney disorders, nephritis, inflammation of the bladder and gastritis.	(Lund et al., 1998)
Embelia concinna Baker	Antananarivo, Madagascar	Leaves, Tanterakala	Treatment for intestinal parasites and erectile dysfunction	(Randriamiharisoa et al., 2015)
Embelia coriacea Wall. ex A.DC.	Long Nah village of East Kalimantan, Indonesia	Roots and leaves, Serapat	Used as an energizing tonic for hard work (roots boiled, consumed in morning for 3 days), pain relief after birth (leaves are rolled, pressed, squeezed and consumed in the morning before breakfast for 3 days).	(Suharjito et al., 2014)
Embelia drupacea (Dennst.) M.R.Almeida & S.M.Almeida	Kolhapur District, Maharashta, India	Bark of roots and leaves, Ambati	Used to treat toothache, a decoction of leaves given orally for sore throat.	(Mahadkar and Jadhav, 2013; Naik et al., 2017)
Embelia impressa H.R. Fletcher	Hill tribes of Northern Thailand	Roots, Ma- ko-e	Roots boiled and liquid consumed by mothers having lactation problems.	(Anderson, 1986)
Embelia laeta (L.) Mez	Various regions of Taiwan and China	Whole plants, no vernacular name recorded	Widely used in Chinese medicine.	(Guo et al., 2020)
	Yunnan, China (Hani)	Roots, A na le go zi	Consumed orally for the treatment of diarrhoea.	(Ghorbani et al., 2011)
Embelia oblongifolia Hemsl.	Jinping, China (Red-headed Yao People)	Fruit and the whole plant, Ze hu pei	Used in medicinal baths to treat numbness of limbs, rheumatoid numbness and injuries from falls.	(Long and Li, 2004; Li et al., 2006)

Scientific name	Locality (Tribe)	Part used and vernacular name	Ethnomedicinal application	References
Embelia palauensis Mez	Morobe Province, Papua New Guinea (Anga tribe)	Young leaves, no vernacular name recorded	The crushed leaves are heated and squeezed. About 50 ml of juice is drunk daily for about three days to treat an enlarged spleen, probably caused by malaria.	(Holdsworth, 2008)
Embelia parviflora Wall. ex A.DC.	Yunnan, China (Hani)	Roots and leaves, Wu jia na ba	An oral decoction is used to treat bone fractures, wounds, stomach ache and bleeding.	(Ghorbani et al., 2011)
	Myanmar, India, China	Leaves and stem barks, Ju ho pen	Treatment for pain experienced in the waist and legs.	(Li et al., 2006)
Embelia pergamacea A.DC.	Gunung Gede Pangrango National Park, , Indonesia	No indication of part used, Kicemang gede	Used as a mouthwash.	(Arbiastutie et al., 2017)
Embelia philippinensis A.DC.	Federation of Malaysia, northernmost part of Borneo (Muruts of Sabah)	Young leaves, Papaling	Young leaves added to salads.	(Kulip, 2003)
	Kabayan, Cordillera Administrative Region (Ibaloi)	Leaves, stem barks and flowers, Besudak	Used in mummification process.	(Teodora, 2017)
	Philippines (Talaandig)	Roots, no vernacular name reported	Used as a sedative, anti-hypertensive remedy, prominently used for eliminating tumours or cysts.	(Odchimar et al., 2016)

Scientific name	Locality (Tribe)	Part used and vernacular name	Ethnomedicinal application	References
Embelia ribes Burm.f.	Northern India	Seeds, Jheum	Used to treat skin diseases and leprosy.	(Kala et al., 2006)
	Assam, north-east India between the foothills of eastern Himalayas and the Patkai and Naga Hills	Fruit, Biranga	Used to treat carbuncle.	(Saikia et al., 2006)
	Lohit and Dibang districts of Arunachal Pradesh, India.	Fruit and roots, Vaibidang	Used to treat worms infestation, liver disorders and as a tonic.	(Shankar and Rawat, 2008)
	Arunachal Pradesh, northeastern India (Tagin tribe)	Leaves and fruit, no vernacular name recorded	Used to treat stomach disorders, curing skin diseases and leprosy.	(Goswami et al., 2009)
	Eastern region of Shimoga district, Karnataka, India	Leaves and roots, Vayuvilanga (Vidanga)	Coughs and colds: A handful of powdered roots are mixed in lemon juice or buttermilk, 1–3 teaspoons of juice are taken orally with sugar, daily twice for 2 days. Paralysis: Leaves are powdered with roots of *Withania somnifera* (L.) Dun. and *Asparagus racemosus* Willd. to make a paste, taken orally, twice a day for 1 month.	(Rajakumar and Shivanna, 2009)
	India	Seeds, no vernacular name recorded	Equal parts of powdered seeds of *E. ribes,* fruit of *Piper longum L.* and borax powder has been utilised as an Ayurvedic contraceptive.	(Zaveri et al., 2010)

Scientific name	Locality (Tribe)	Part used and vernacular name	Ethnomedicinal application	References
	Gwalior forest division in Madhya Pradesh, India	Fruit, Baividang	One gram of fruit powder is consumed with water twice daily for about a month to expel intestinal worms (hookworms, threadworms).	(Anis et al., 2011)
	Vietnam	Stem barks, Ngu linh chi	Antibiotic, hypertension, diuretic and anti-inflammation.	(Dao et al., 2012)
	Amarkantak region, Madhya Pradesh, India	Fruit, Vaibidang, Bevrang	Fruit are used in the treatment of piles, sore throat and dyspepsia.	(Srivastava et al., 2012)
	Arunachal Pradesh, eastern Himalaya, India (Adi-Minyong tribe)	Tender leaves, Hinkong	Tender shoots and leaves are cooked and have been reported to act as a stomachic.	(Baruah et al., 2013)
	India	Fruit, seeds, and roots, Vidanga	Acts as ascaricidal, antihelminthic, purgative, diuretic, anti-inflammatory, antibacterial, febrifuge and blood-purifying agent. Found to be estrogenic and slightly progestogenic. Pulp is purgative. Fresh juice is cooling, diuretic and laxative. The roots are used as anti-diarrhoeal remedy. The seeds have spermicidal, oxytoxic and diuretic action. Aqueous fruit extracts showed antihelminthic effects against tapeworms.	(Byadgi, 2013)
	Sabah, Malaysia (Kadazan and Dusun communities)	Fruit, Vidanga	Antihyperlipidemic activity.	(Koriem, 2014)

Scientific name	Locality (Tribe)	Part used and vernacular name	Ethnomedicinal application	References
	Hassan district, Karnataka, India	Fruit and seeds, Vayu vidanga	Used to treat worm infestation, oedema, fever, anorexia, urinary calculi, polyuria, rigidity in lower limbs, fistula, pain, vomiting, abdominal disorders.	(Prashanth and Shiddamallayya, 2016)
	West Java, Indonesia	Kicemang beurit	Sprue treatment.	(Arbiastutie et al., 2017)
	India	Dried, mature fruit, Vidang	Used as anti-fertility, analgesic, antibacterial, anti-inflammatory, antidiabetic and cardiprotective agents.	(Mukherjee et al., 2017)
	Sri Lanka	Seeds, no vernacular name recorded	Used in antidiabetic Siddha preparations.	(Sathasivampillai et al., 2017)
	Kuala Kangsar, Malayasia	Fruit, Akar sulur kerang	Used to treat intestinal worm infections.	(Nor Azman et al., 2018)
	India	Fruit, no vernacular name recorded	Used in Ayurvedic formulations for treatment of hepatitis B.	(Zanwar and Wajpeti, 2019)
Embelia robusta Roxb.	Pind Dadan Khan, district Jhelum, Punjab, Pakistan	Fruit and root barks Baobreng	It is an expectorant and vermifuge. Dried bark used to treat toothache. The powder along with curd reported to be used to kill the tapeworms.	(Iqbal et al., 2011)
	Dindori forest division of Madhya Pradesh, India	Root bark, fruit, Bidang	Used as an antiseptic, carminative and antihelminthic. The dried root bark used to treat toothache.	(Mudaiya et al., 2015)
	India	Fruit, Vidanga	Used in Ayurvedic formulations to treat the blood disorder thalassemia.	(Mori et al., 2018)

Scientific name	Locality (Tribe)	Part used and vernacular name	Ethnomedicinal application	References
Embelia rowlandii Gilg	Wabane Subdivision, Lebialem Division, Cameroon (Mundani)	Fruit, leaves and roots, Nphenyate	Used to treat worms, gastritis and urinary tract infections.	(Focho et al., 2009; Tachama et al., 2015)
Embelia ruminata (E.Mey. ex A.DC.) Mez	South Africa, (Zulu tribes)	leaves, ripe fruit and roots, Ibhinini, Ibinini, Inbhinini and Umoyushisa	Antihelminthic against tapeworms, leaves are also chewed as a bitter tonic and eaten for refreshment.	(Gerstner, 1938; Bryant, 1966; Hutchings et al., 1996)
Embelia scandens (Lour.) Mez	China	Roots, Spiny rattan	Used in the treatment of rheumatism, bone pain, cramps, phthisis and cough. A root decoction is used for treating tuberculosis, diarrhoea, coughs and tracheitis.	(The Editorial Committee of Chinese Materia Medica, 1999; Dai et al., 2018)
	Naban River Watershed, Yunnan, China (Hani)	Stem barks, A chu a ha	An oral decoction is taken to treat ascariasis.	(Ghorbani et al., 2011)
Embelia schimperi Vatke	Tanzania and Kenya (Masai)	Dried fruit and roots, no vernacular name recorded	Taken orally by the Masai people as an antihelminthic treatment against human intestinal tapeworms.	(Bogh et al., 1996)
	Ankober District, Ethiopia	Fruit and seeds, Inkoko	Used to treat taeniasis, diarrhoea, ascariasis and constipation.	(Lulekal et al., 2014)
	Kenya	Fruit, no vernacular name recorded	Widely used across ethnic groups of Kenya as antihelminthics. Fruit chewed as both vermifuge and purgative. Dried fruit and roots are boiled or soaked in water and the	(Midiwo et al., 2002)

Scientific name	Locality (Tribe)	Part used and vernacular name	Ethnomedicinal application	References
			infusion taken as a treatment for intestinal worms.	
	Northwestern part of Ethiopia: Dibatie and Guangua (Amhara)	Fruit, Inkoko	Used as a taeniacide.	(Giday et al., 2007)
	Ethiopia (Amhara)	Fruit and seeds, Enqoqo, Enkiko	A handful of fruit are made into a cup of juice and are given orally on an empty stomach in the morning for seven days to treat taeniasis.	(Ragunathan and Weldegerima, 2007)
	Meinit Goldya and Meinit-Shasha districts, southwest Ethiopia (Meinit ethnic group)	Roots and fruit, Qamjach	Used to treat taeniasis and ascariasis. Additionally, used to heal gastrointestinal complaints.	(Giday et al., 2009)
	Wonago Woreda, Gedeo Zone, Ethiopia	Roots, no vernacular name recorded	Powdered fresh root mixed with water consumed for several days to treat leprosy.	(Mesfin et al., 2009)
	Southwest Ethiopia (Sheko ethnic group)	Roots and fruit, Qoqu	An oral decoction is given to patients with taeniasis or ascariasis on an empty stomach and a meal must not be taken until the proglottids were expelled. Due to potential adverse effects, decoction not recommended for children under the age of fifteen years.	(Giday et al., 2010)
	Southern Rwanda,	Leaves, no vernacular	A handful of crushed fresh leaves were	(Mukazayire et al.,

Scientific name	Locality (Tribe)	Part used and vernacular name	Ethnomedicinal application	References
	Umukaragata	name recorded	macerated with 1 L of water and one glass taken 3 times a day for one month to treat hepatitis.	2011)
	South Nandi District, Kenya.	Seeds, Kibong'ong'inik	Used to treat malaria.	(Pascaline et al., 2011)
	Nigeria	Leaves and stem barks, Kiru	Used as antispasmodic and anthelmintic agents.	(El-Ghani, 2016)
	Gurage zone, Southern Nations, Nationalities and Peoples Region, Ethiopia.	Seeds, Enqueqwe and Enqoqo	Treatment of taeniasis.	(Teka et al., 2020)
	South west and north west Cameroon	Leaves, no vernacular name recorded	Traditional healers use the leaves for the treatment of intestinal worms and diarrhoea.	(Togue et al., 2020)
	West Gojjam Zone, Amhara Region, Ethiopia	Fruit, Enkoko	Used to treat abdominal bloating, *Ascaris* and *Taenia saginata* infections.	(Alemneh and Imran, 2021)
	Mbulu District, Tanzania	Fruit and stem bark, Qaytla (Iraqw’)	Study reported the ethnomedical uses related to human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome AIDS-related conditions.	(Alphonce et al., 2021)
	Ambo town, western Ethiopia	Fruit, no vernacular name recorded	Treatment of tapeworm infections.	(Tolossa, 2021)
Embelia sessiliflora Kurz	Chiang Mai province, Northern	Fruit, Blea blor	Use as an antihelminthic agent.	(Tangjitman et al., 2013)

Scientific name	Locality (Tribe)	Part used and vernacular name	Ethnomedicinal application	References
	Thailand			
	Northern and western Thailand	No part used and vernacular name recorded	Effective treatment of helminth worm infections.	(Phumthum and Balslev, 2020)
Embelia subcoriacea (C.B.Clarke) Mez	Western Mizoram, India	Leaves, Tling	Decoction of leaves is used in medicinal baths to treat smallpox and injury after childbirth.	(Lalfakzuala et al., 2007; Rai and Lalramnghinglova, 2010)
	Sancharurn village in Nan Province, Thailand (Mien)	Leaves and stem barks, Toe poe tow sui	Use in treating chickenpox by using a decoction of leaves and stem bark in medicinal baths.	(Panyaphu et al., 2011)
	Yuan in northern Thailand	Leaves, Ma pong khuea	Treatment for muscular pain.	(Inta et al., 2013)
Embelia tsjeriam-cottam (Roem. & Schult.) A.DC.	Thane District, Maharashtra, India	Roots, Phatangri	An aqueous paste of roots is wrapped in a cloth and squeezed twice into the nostrils. This treatment was given for poisonous snake bites. It has been claimed that the person will start to vomit within 20–25 min, expelling the poison.	(Natarajan and Paulsen, 2000)
	Gandhamardan hill range, Orissa, India	Seeds, Nununia and Bidang	The dried seeds are chewed on an empty stomach in the morning for expelling tapeworms, claimed to cure dyspepsia and skin diseases.	(Misra, 2004)
	Dodamarg Region, India	Roots, Wawding	Treatment of piles, sore throat and dyspepsia.	(Somkuwar et al., 2013)

Scientific name	Locality (Tribe)	Part used and vernacular name	Ethnomedicinal application	References
	Hassan district, Karnataka, India	Fruit, Mara harive	Used as a carminative and antispasmodic agent.	(Prashanth and Shiddamallayya, 2016)
	Madhya Pradesh, India	Leaves, Bai birangi	To treat diarrhoea and chest complaints.	(Gupta et al., 2018)
	Chin state, Myanmar	Fruit, no vernacular name recorded	Used as a vermifuge, carminative, stimulant, stomachic, antimalarial and wound healing remedy. The fruit are utilised to cure chronic bronchitis and spleen enlargement.	(Thu et al., 2018)
	Karnataka, India	Leaves and roots, Vaayuvilanga	Treatment of arthritis.	(Bhat et al., 2019)
Embelia vestita Roxb.	Mizoram, India	Leaves, Tling	Decoction of leaves is used externally to treat chickenpox, itching, other skin diseases and urinary disorders.	(Hazarika et al., 2012)
Embelia whitfordii Merr.		Stem barks, Malaumau	Used to treat sudden cough; mouth sores and asthma.	(Ong and Kim, 2014)

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CHAPTER 3

Antimicrobial and anti-quorum sensing activities of the different solvent extracts of *Embelia ruminata*

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3.1 Abstract

On a global scale, the increasing incidence of multi-drug resistant pathogens has stimulated research towards discovering phytomedicinal compounds that can offer novel therapeutic remedies. In this study, as an initial step, the phytochemical profile of the South African plant species, *Embelia ruminata* (E.Mey. ex A.DC.) Mez, was investigated by performing various phytochemical tests and Fourier transform infrared (FTIR) analyses. Subsequently, the antibacterial potential of the different solvent extracts of the leaf, stem bark, fruit and seeds was assessed using Gram-negative and Gram-positive bacteria. Additionally, to explore potential anti-virulence strategies, the qualitative and quantitative quorum sensing (QS) inhibitory activities of *E. ruminata* extracts using *Chromobacterium subtsugae* CV017 (short chain) and *Chromobacterium violaceum* ATCC 12472 (long chain) biomonitor strains were investigated. The results of the qualitative phytochemical tests showed the presence of various phytochemical classes such as alkaloids, triterpenoids, phytosteroids, phenolic compounds, flavonoids and benzoquinones, while the FTIR analysis confirmed the presence of various functional groups. Extracts tested at 400 and 800 µg produced limited antibacterial activity against the Gram-negative bacteria, with the different solvent extracts of fruit demonstrating the most activity against some of the bacterial strains. The methanolic stem bark extracts showed promising results against selected Gram-positive strains, particularly methicillin-resistant *Staphylococcus aureus*. Notably, the chloroform fruit and methanolic seed extracts demonstrated greater antibacterial inhibitory activity than the antibiotic controls ciprofloxacin and gentamicin against methicillin-resistant *Staphylococcus aureus* ATCC 700698 and vancomycin-resistant *Enterococcus faecalis* ATCC 51299, respectively. In the anti-QS violacein inhibition assay, the chloroform leaf, hexane and methanolic seed extracts displayed inhibition of both biomonitor strains suggesting that these extracts of *E. ruminata* possess broad-spectrum QS activity. Furthermore, the methanolic seed extracts demonstrated the most potent violacein inhibitory activity against both biomonitor strains compared to other plant organs. Overall, the extracts showed more effective inhibition against the long than the short chain autoinducers. The results also indicated that the non-polar and polar solvents used as an extractant, solubilised different phytoconstituents from the plant matrices depending on their polarity. This study provides insight into the bioactivity of the extracts of *E. ruminata* as possible antibacterial agents and potential broad-spectrum QS inhibitors targeting resistant bacterial strains.

Keywords: *Embelia ruminata*, antibacterial activity, quorum sensing, *Chromobacterium subtsugae, Chromobacterium violaecum*, violacein

3.2 Introduction

The discovery of antibiotics has revolutionised modern medicine, transforming the methods used to treat many infectious diseases of human and animal origin (Aslam et al., 2018). Primarily, due to their indiscriminate use, antibiotic therapy has been globally jeopardised by the marked increase in antibacterial resistance among common bacterial pathogens (Nazir, 2020).

This has led to an intense drive to find novel agents that can alleviate the rise of antibiotic resistance. Research exploring the mechanistics of medicinal plants as an alternative to conventional medicine has dramatically increased in recent years (Erhabor et al., 2019; Mulat et al., 2019). Since plants do not possess a developed cellular and biochemical immune system, it is suggested that they would have evolved intrinsic methods to overcome bacterial infections (Bacha et al., 2016). Thus, the inherent phytochemicals in medicinal plants may represent a valuable reservoir of therapeutic products that display varying effectiveness against bacteria, sometimes even at low concentrations (Bouyahya et al., 2017). In addition to having antimicrobial properties, plants have been examined for their role in disrupting the bacterial infection process (Khan et al., 2018; Erhabor et al., 2019). Conventional antibiotics produce bactericidal and mutagenic action, an attribute that has culminated in the expansion of multi-resistant pathogens that are difficult to treat (Ahmed et al., 2019). The approach of interfering with bacterial pathogenesis provides an attractive alternative that advantageously does not necessarily favour the evolution of bacterial resistance (Saeki et al., 2020).

One such approach is the attenuation of quorum sensing (QS) present in many bacteria (Piewngam et al., 2020). In microbial communities, the expression of virulence and pathogenicity is controlled by QS, a cell-to-cell communication system ubiquitously used by microbes to monitor their population density (Deryabin et al., 2019). In the QS process, small signalling molecules, autoinducers (AI), initiate the expression of specific genes, which in turn activate the receptors that orchestrate the production of a myriad of virulence factors (Mahavy et al., 2020). Thus, AI concentration plays a fundamental role in determining virulence gene expression in several pathogenic bacteria (Banerji et al., 2020). In Gram-negative bacteria such as *Chromobacterium*, N-acyl-homoserine lactones (AHL) are a typical class of AI produced by LuxI/LuxR-type synthases (Papenfort and Bassler, 2016). In the QS system of *Chromobacterium*, when the concentration threshold

of the growth phase is reached, AHL signal molecules bind to receptors to form a complex that modulates the expression of target genes, which produces an insoluble, purple pigment called violacein (Venkatramanan et al., 2020). The production of violacein and other virulence factors such as metalloprotease, type II and type III secretion systems, swarming motility, exopolysaccharide, lipase, flagellar proteins, collagenase, chininase and cytolytin toxins are involved in the establishment of an infection in the host (Venkatramanan et al., 2020).

The attenuation of the above-mentioned bacterial factors can render the bacteria non-pathogenic by the inhibition of their QS circuit either by inhibiting signal molecules from being synthesised, inhibiting the activity of AHL-producing enzymes or by degrading signal receptors such as LuxR (Luo et al., 2016; Tako et al., 2020). Phytomolecules present in plant extracts can act as QS inhibitors (Erhabor et al., 2019; Tako et al., 2020; Zhong et al., 2020). They structurally mimic AHL signal molecules and alter QS-regulated behaviours in bacteria (Welsh and Blackwell, 2016; Saeki et al., 2020; Saipriya et al., 2020). Thus, plant extracts and their metabolites may provide an innovative disease control strategy (Bouyahya et al., 2017; Munir et al., 2020).

Many South Africans (up to 80%) rely on local medicines, mostly from plants, to manage their diseases and general healthcare needs (Erhabor et al., 2019). Low-income communities are predicted to be hit the hardest by drug-resistant infections (Ashley et al., 2020). The proportion of people succumbing to such infections is escalating, with the current pace of drug development being insufficient to mitigate the surge in antibiotic resistance (Petchiappan and Chatterji, 2017; Kuehl et al., 2020; Streicher, 2021). To address this knowledge gap, the plant species *Embelia ruminata* (E.Mey. ex A.DC.) Mez belonging to the family Primulaceae was investigated as a potential antibacterial agent and/or QS inhibitor. The medicinal properties of other species from the genus *Embelia* Burm.f. that have been reported include: antidiabetic (Durg et al., 2017), antiviral (Hossan et al., 2018), antibacterial (Mahendran and Hazirah, 2018; Bouzeko et al., 2019), anti-Alzheimer agent (Nuthakki et al., 2019), antioxidant (Caruso et al., 2020) and anticancer (Togue et al., 2020).

In the investigations undertaken in the current study, the phytochemical classes and functional groups were identified, followed by the analyses of the antibacterial properties of three different solvent (hexane, chloroform and methanol) extracts of *E. ruminata* leaf, stem bark, fruit and seeds using a range of Gram-negative and Gram-positive bacteria. Additionally, the potential of *E. ruminata* extracts to inhibit QS was studied qualitatively and quantitatively using *C. subtsugae* short chain AHL producer CV017 (Chernin et al., 1998) and long chain AHL producer *C. violaceum* ATCC 12472 (Morohoshi et al., 2008).

3.3 Material and Methods

3.3.1 Collection and identification of plant material

The leaves, stem bark and fruit of *E. ruminata* were collected from Durban, KwaZulu-Natal, South Africa (29°48'26.7 "S 30°5543.9"E), from February to May 2019. Professor H. Baijnath confirmed the species identity. A voucher specimen (Rambaran 1 with accession number 18256) was deposited in the Ward Herbarium, School of Life Sciences (Biology), University of KwaZulu-Natal, Durban, South Africa.

3.3.2 Preparation and extraction of plant material

The leaves, stem bark and fruit of *E. ruminata* were separated and sorted. A proportionate amount of the whole fruit was decorticated and the seeds retained. Thereafter, the leaves, stem bark, fruit and seeds were air-dried separately, pulverised to a fine powder and refrigerated in airtight bottles until extraction.

Extractants with increasing polarity, i.e., hexane, chloroform and methanol were used sequentially in the extraction process. The resultant solutions were filtered, concentrated to dryness under reduced pressure using a rotary evaporator and then stored in airtight vials at 4 °C for further analyses. To calculate the percentage yield, the formula below was used (equation 3.1):

$$\text{Extract yield (\%)} = \text{weight of dried extract (g)}/\text{ weight of fresh material} * 100 \quad \textbf{(3.1)}$$

3.3.3 Phytochemical screening

The outer surface of the decorticated seeds of *E. ruminata* has distinct cavities consisting of a yellow powdery substance (Figure 3.1 inset (a) on page 65). The powder from 10 seeds was removed and treated with 25% ammonia solution (Ferreria and Laddha, 2013).

This test was used to verify the presence of the benzoquinone compounds such as embelin, which is considered to be the principal compound of the genus *Embelia* (Vijayan and Raghu, 2021). Upon treatment with ammonia, the formation of deep pink coloured complexes positively confirms the presence of embelin in the extracts (Ferreria and Laddha, 2013). To further substantiate the presence of benzoquinone compounds, the different solvent extracts were dissolved with petroleum ether and treated with diluted ammonia. The formation of a bluish-violet precipitate indicates the presence of embelin (Kaur et al., 2015; Vijayan and Raghu, 2021).

Additionally, qualitative screening was performed using the hexane, chloroform and methanol extracts of *E. ruminata* employing standard protocols as described by Harborne (1973); Sofowora (1993) and Trease and Evans (2009).

3.3.4 Fourier transform infra-red (FTIR) analysis

The powdered sample was loaded in a FTIR spectroscope (Perkin-Elmer Spectrum 100 FTIR, USA) with a scan range from 400 to 4000 cm^{-1} and a resolution of 4 cm^{-1} at 25±2 °C (Akwu et al., 2019). The peak frequencies obtained were analysed according to the reference literature to determine the functional groups present (Coates, 2000; Stuart, 2004; Merck, 2020).

3.3.5 Antibacterial susceptibility testing

The Kirby-Bauer disc diffusion assay was used to assess the antibacterial activity of *E. ruminata* extracts. Four Gram-negative microorganisms, viz., susceptible *Escherichia coli* ATCC 25922, TEM-β-lactamase-producing *E. coli* ATCC 35218, extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* ATCC 700603 and *Pseudomonas aeruginosa* ATCC 27853, as well as seven Gram-positive microorganisms, viz, susceptible *Enterococcus faecalis* ATCC 29212, vancomycin-resistant *E. faecalis* ATCC 51299, susceptible *Staphylococcus aureus* ATCC 29213, methicillin-resistant *S. aureus* ATCC 33591, *S. aureus* ATCC 43300, *S. aureus* ATCC 700698 and *Staphylococcus epidermidis* ATCC 12228, were incubated on Mueller-Hinton (MH) agar plates at 37 °C for a duration of ± 16 h. Aliquots of the respective cultures equivalent to a 0.5 McFarland were swabbed onto MH agar plates (Chenia, 2013). Thereafter, 400 µg and 800 µg of the respective extracts (resuspended in 10% dimethyl sulfoxide, DMSO) were used to dose

blank discs (5mm diameter), placed onto the surface of the swabbed MH agar plates, followed by an incubation for 24 h at 37 °C. Antibiotic controls (Ciprofloxacin, CIP5 and Gentamicin, GN10) and solvent control [(10% dimethyl sulfoxide (DMSO)] were used. Following incubation, samples exhibiting zone of inhibition (ZOI) with diameters >15 mm were regarded as strong antibacterials, zone diameters between 11-15 mm as possessing intermediate activity and zone diameters <10 mm were considered weak antibacterial agents.

3.3.6 Qualitative quorum sensing inhibition

The quorum sensing inhibition potential of *E. ruminata* extracts was investigated using the agar overlay diffusion assay. The two biomonitor strains used were: *C. subtsugae* CV017 to detect short chain (C4-C6) AHL inhibition (Chernin et al., 1998) and *C. violaceum* ATCC 12472 to detect long chain (C10+) AHL inhibition (Morohoshi et al., 2008). An aliquot of 150 µL of the respective *Chromobacterium* spp strains grown overnight at 30 °C in Luria-Bertani (LB) broth was used to inoculate 5 mL of molten LB agar. The resultant agar-culture solution was then poured over the surface of pre-warmed LB agar plates and allowed to solidify (Chenia, 2013). Thereafter, 400 and 800 µg of the respective extracts were applied to blank discs with a diameter of 5 mm, placed onto the surface of the agar plates and incubated overnight at 30 °C. Discs dosed with 400 and 800 µg of vanillin were used as the positive control, while those dosed with 10% DMSO represented the negative control. Following incubation, the opaque zone (QS inhibition) diameters and clear (bactericidal) zone diameters were recorded. Extracts exhibiting opaque zones (QS inhibition) with diameters >15 mm were regarded as having strong inhibitory potential, zone diameters between 11-15 mm as possessing intermediate potential, and zone diameters <10 mm were considered weak QS inhibitors.

3.3.7 Quantitative quorum sensing inhibition

The quorum sensing inhibition potential of *E. ruminata* leaf, stem bark, fruit and seeds was quantified using *C. subtsugae* CV017 and *C. violaceum* ATCC 12472 as the indicator microorganisms (Chenia, 2013). One hundred microliters of the respective *Chromobacterium* strains were cultured in 3 mL of LB broth and incubated at 30 °C with increasing concentrations of each extract ranging from 0, 200, 400, 600, 800 and 1000 µg/mL. For this assay, growth (optical density $(OD)_{600}$ nm) and violacein production

(OD_{560} nm) were assessed following an overnight incubation. To precipitate insoluble violacein, 1 mL of the respective overnight, incubated, treated and untreated cultures of *Chromobacterium* was centrifuged (Labnet Prism Microcentrifuge) at 10 000 rpm for 10 min. The resultant supernatant was disposed of and the pellet homogenised in 1 mL of DMSO (Truchado et al., 2012; Chenia, 2013). To separate the cells, the solution was then centrifuged again at 10 000 rpm for 10 min. The precipitated violacein was quantified at OD_{560} nm using the Glomax Multi+ Detection System (microtitre plate reader; Promega). The percentage of violacein inhibition was calculated using the following formula (equation 3.2):

% Violacein inhibition= (control OD_{560} nm – test OD_{560} nm / control OD_{560} nm)*100 **(3.2.)**

(Packiavathy et al., 2011; Chenia, 2013).

3.3.8 Statistical analysis

Statistical analyses were conducted using SPSS versions 26 and 27. Data are indicated as a mean of triplicate replicates (*n*=3). The difference in the mean values of violacein inhibition between extracts and concentrations was determined using one-way analysis of variance (ANOVA) with $p \leq 0.05$ considered significant with Tukey honestly significant difference (HSD) *post hoc* test (Moradi et al., 2020).

3.4 Results and Discussion

Medicinal plants and their phytocomponents dictate their therapeutic effectiveness (Oladeji, 2016). The polarity and type of solvent used during the extraction process have an intense effect on the medicinal properties, including the yield of the resultant extracts due to the solubility of the phytocompounds in the various solvents (Dowlath et al., 2020). In the present study, the extracting solvents hexane, chloroform and methanol were used. Methanol resulted in the highest yield, possibly indicating that the phytoconstituents of *E. ruminata* are more polar in nature. This was followed by hexane, while chloroform produced the lowest yield (Table 3.1). Similarly, the methanolic extracts of *Mentha piperita* L. (19.5%) and *Glycyrrhiza glabra* L. (18.2%) produced the highest yield in studies conducted by Cosa et al. (2019).

Table 3.1: Percentage yield of crude extracts of the different plant organs of *Embelia ruminata*

Solvent	Percentage yield (%)			
	Leaf	Stem bark	Fruit	Seeds
Hexane	1.41	1.11	1.14	2.33
Chloroform	1.06	0.46	0.94	2.08
Methanol	5.38	6.66	6.03	2.42

The phytochemical tests, qualitatively confirmed the presence of different classes of phytochemicals (Table 3.2) known to impart medicinal characteristics of the plant. When treated with ammonia solution, the yellow powder from the decorticated seeds turned a deep pink to red colour, indicating a positive confirmation for the presence of embelin [Figure 3.1 inset (b)]. Furthermore, the treatment of the different solvent extracts with petroleum ether and diluted ammonia yielded positive results for the presence of embelin in the hexane and chloroform fruit and seed extracts. Similarly, the dosing of extracts with concentrated sulphuric acid (H_2SO_4) displayed a pronounced presence of quinone compounds in methanolic leaf, stem bark, fruit and seed extracts (Table 3.2). Qualitatively, these tests confirm the presence of benzoquinone compounds, possibly embelin in the extracts of *E. ruminata*. A study by Otegui et al. (1998) stated that the presence of benzoquinone derivatives can be used to chemotaxonomically characterise the family Myrsinaceae (redefined as family Primulaceae). This statement was supported by subsequent studies conducted by de Luna et al. (2013). Analytical studies are necessitated in the future to isolate pure compounds, thereby pinpointing the exact compounds responsible for the biological activity.

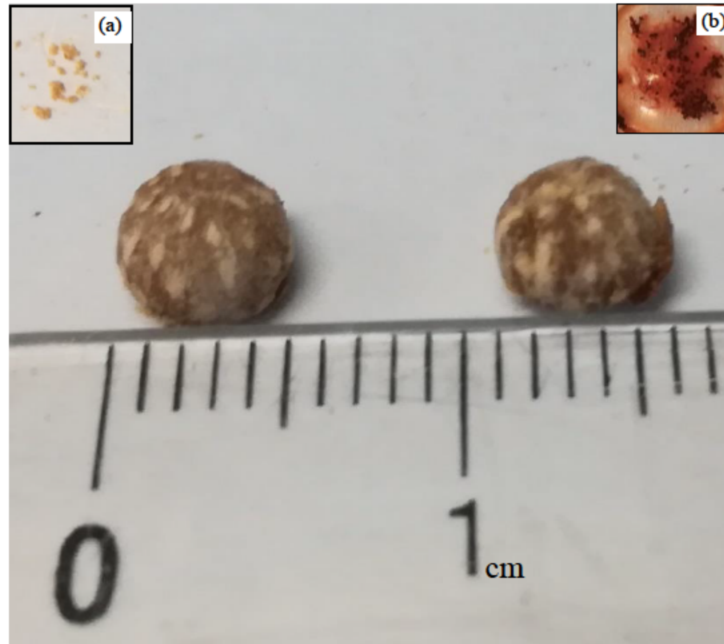


Figure 3.1. Seeds of *Embelia ruminata* after removal of pericarp. Labels inset (a) depicting yellow powder removed from the cavities of the seeds, inset (b) showing colour change after treatment with dilute ammonia solution.

Table 3.2 Phytochemical analyses of different solvent extracts of *Embelia ruminata* leaf, stem bark, fruit and seed extracts

Phytochemical compound	Test performed	Leaf			Stem bark			Fruit			Seed		
Benzoquinone	Diluted ammonia										+++		
		Hex	Chl	Me	Hex	Chl	Me	Hex	Chl	Me	Hex	Chl	Me
Benzoquinone (embelin)	Pet ether and diluted ammonia	-	-	-	-	-	-	+	++	-	++	+++	-
Alkaloids	Mayer’s	-	-	-	-	-	+++	-	-	+	+	-	+
	Dragendoff’s	-	-	-	-	-	+++	++	-	++	+	-	+
	Wagner's	+++	-	-	++	-	-	+++	-	-	+	-	+
Triterpenoids	Salkowsi	-	-	-	++	++	-	++	++	++	+	+	+
Phytosteroids	Salkowsi	-	-	+	-	+	+	+	+	+	-	-	+
	Libermann -Burchard	-	-	+	+	+	-	+	+	+	-	-	-
Flavonoids	10% lead acetate	-	-	+	-	-	+	-	-	+	+++	++	+
	Alkaline reagent	-	+	+	+	+	+	+	-	-	+	-	+
Phenolic compounds	5% aqueous Ferric chloride	-	+++	+++	+++	+++	+++	+++	+++	+++	+	++	+
	Gelatin Test	-	+	-	-	-	+	-	-	+	-	-	++
Coumarins	10% sodium hydroxide	-	-	-	-	-	-	-	-	-	-	-	-
Quinones	Conc. H_2SO_4 test	-	-	+++	-	-	+++	-	-	+++	-	-	+++
Anthraquinone	Borntrage’s reaction	-	-	-	-	-	-	-	-	+	-	+	-
Resins	Acetic anhydride, conc. H_2SO_4 test	+	++	+++	+	++	+++	+	++	+++	+	++	+++

- = negative result, + = mildly present, ++ = distinctly present, +++ = very strongly present, Hex= Hexane, Chl = Chloroform, Me = Methanol, Pet= Petroleum, Conc.=concentrated

In the FTIR analysis, the following functional groups were identified: alcohols, aliphatic primary amides, aliphatic primary amines, aliphatic ketones, alkanes, alkenes, alkyne, amines, anhydrides, aromatic compounds, carboxylic acid, conjugated alkenes, cyclic alkene, esters, nitro compounds and quinone compounds [Table 3.3, the corresponding Figures included in the Appendix Section: A3.1 and A3.2 on pages 185 and 186]. It was noted that the vegetative plant parts displayed similar profiles that were dependent on extracting solvent rather than the plant organ. For example, the hexane leaf and stem bark crude extracts showed similar absorbance peaks at 3550-3200 cm^{-1}, possibly indicating a N-H bond stretching due to the presence of an amide (Khatua et al., 2018). Similarly, two bands in the region of 3000-2840 cm^{-1} could be attributed to aliphatic C-H stretching (Majewska et al., 2017), confirming the presence of alkanes in the sample. Bands in the region of 2000-1650 cm^{-1} could indicate a C-H bending for aromatic compounds. Similarly, in the methanolic vegetative extracts, the most predominant peaks were observed between 3550 and 3200 cm^{-1}, which showed the presence of O-H bonds typical of alcohols (Khatua et al., 2018). Interestingly, the peaks noted in the region of 1650-1600 cm^{-1} could be the C=O bond of a quinone compound (Kacmaz et al., 2019). This data concurred with the phytochemical test results for quinone compounds, which showed a prominent presence of quinone compounds in methanolic extracts.

Table 3.3 FTIR peak values and functional groups of the different solvent extracts of *Embelia ruminata* leaf, stem bark, fruit and seeds

Serial number	Wave number (cm^{-1})	Frequency range (cm^{-1})	Functional class	Assignment
Hexane Leaf				
	3409.03	3550-3200	Aliphatic primary amide	N-H
	2920.68	3000-2840	Alkane	C-H stretching
	2850.78	3000-2840	Alkane	C-H stretching
	1736.8 1710.01	2000-1650	Aromatic compounds	C-H stretching
	1452.21	1450	Alkane	
Chloroform Leaf				
	3380	3400-3300	Aliphatic primary amide	N-H
	2920.22	3000-2840	Alkane	C-H stretching
	2851.38	3000-2840	Alkane	C-H stretching
	1705.59	1725-1705	Aliphatic ketone	C=O stretching
	1596.84	1650-1580	Amine	N-H bending
	1159.62	1250-1020	Amine	C-N stretching
Methanol Leaf				
	3344.37	3550-3200	Alcohol	O=H stretching
	2928.98	3200-2700	Alcohol	O=H stretching
	2076.33	2000-1650	Aromatic compound	C-H bending
	1623.69	1650-1600	Quinone compound	C=O bond
	1406	1420-1330	Alcohol	O-H bending
	1049.30	1050-1040	Anhydride	CO-O-CO stretching
Hexane Stem bark				
	3394	3500-2400	Aliphatic primary amide	N-H

	2916.37	3000-2840	Alkane	C-H stretching
	2848.97	3000-2840	Alkane	C-H stretching
	1735.96 1712.41	2000-1650	Aromatic compound	C-H bending
	1623.69	1650-1600	Conjugation alkene	C=C
Chloroform Stem bark				
	3380	3400-3300	Amine	N-H stretching
	2916.84	3000-2840	Alkane	C-H stretching
	2849.38	3000-2840	Alkane	C-H stretching
	1713.02	1725-1705	Aliphatic ketone	C=O stretching
	1622.36	1650-1580	Amine	N-H bending
	1462.36	1465	Alkane	C-H bending
	1157.98	1250-1020	Amine	C-N stretching
Methanol Stem bark				
	3237.69	3550-3200	Alcohol	O=H stretching
	2923.58	3200-2700	Alcohol	O=H stretching
	1606.77	1650-1600	Quinone compound	C=O bond
	1519	1550-1500	Nitro compound	N-O compound
Hexane Fruit	3307.23	3400-3300	Aliphatic primary amine	N–H stretching
	3009.27	3000-2840	Alkane	C–H stretching
	2921.69	3000-2840	Alkane	C–H stretching
	2853.37	3000-2840	Alkane	C–H stretching
	1744.63	1750-1735	Ester	C=O stretching

	1614.25	1650-1600	Quinone	C=O bond
	1462.75	1465	Alkane	C–H bending
	1160.67	1210-1163	Ester	C–O stretching
Chloroform Fruit				
	3308	3200-2700	Aliphatic primary amine	N–H stretching
	3008	3000-2840	Alkane	C–H stretching
	2921.84	3000-2840	Alkane	C–H stretching
	2852.55	3000-2840	Alkane	C–H stretching
	1744.30	1750-1735	Ester	C=O stretching
	1616.33	1650-1600	Quinone	C=O bond
	1463.86	1465	Alkane	C–H bending
	1160.16	1210-1163	Ester	C–O stretching
Methanol Fruit				
	3290.89	3550-3200	Alcohols	O=H stretching
	2930.20	3000-2840	Alkane	C–H stretching
	1717.74	1725-1705	Aliphatic ketone	C=O stretching
	1599.42	1650-1566	Cyclic alkene	C=C bending
	1403.53	1420-1330	Alcohol	O–H bending
Hexane Seed				
	3465.81 3303.78 3310.07	3500-2400	Alkene	=C–H stretching
	2922.28	3000-2840	Alkane	C–H stretching
	2853.11	3000-2840	Alkane	C–H stretching
	1827.33	2000-1650	Aromatic compound	C–H bending

	1743.71	1725-1705	Aliphatic ketones	C=O stretching
	1644.96 1613.33	1650-1600	Quinone	C=O bond
	1459.17	1470-1430	Alkane	C–H bend
	1160.03	1210-1163	Ester	C–O stretching
Chloroform Seed				
	3305.39	3300-2500	Carboxylic acid	O–H stretching
	3009.24	3000-2840	Alkane	C–H stretching
	2920.14	3000-2840	Alkane	C–H stretching
	2851.26	3000-2840	Alkane	C–H stretching
	2172.96	2260-2100	Alkyne	C≡C stretching
	1744.71	1780-1710	Carboxylic acid	C=O stretching
	1612.26	1650-1600	Quinone	C=O bond
	1461.89	1460	Alkene	C–H bend
	1168.79	1210-1163	Ester	C–O stretching
Methanol Seed				
	3298.09	3550-3200	Alcohol	O–H stretching
	2923.31	3000-2840	Alkane	C–H stretching
	2852.99	3000-2840	Alkane	C–H stretching
	1609.84	1650-1600	Quinone	C=O bond
	1439.93	1420-1330	Alcohol	O–H bending
	1104.24	1124-1087	Alcohol	C–O stretching
	1043.78	1050-1040	Anhydride	CO–O–CO
	990.38	995-985	Alkene	C=C bending

Aligned to this, it was noted that the FTIR spectrum for the chloroform fruit extracts displayed a high degree of similarity in reference to their peak pattern to published reports for the isolated, pure embelin compound (Badamaranahalli et al., 2015; Kaur et al., 2015; Shaikh et al., 2016; Pundarikakshudu et al., 2016). Kaur et al. (2015) reported the following peaks for pure embelin: 3309 cm^{-1} because of the stretching vibration of the O-H group, 2920 cm^{-1} due to C-H stretching, 2848 cm^{-1} owing to the stretching vibration of the methyl C-H group, 1615 cm^{-1} due to C=O vibration and 1194 cm^{-1} on account of the C-O bond. These reported peaks are analogous to the peak pattern identified in the chloroform fruit extracts (Figure 3.2 and 3.3). Furthermore, some of the functional groups distinctive for embelin were also identified in hexane fruit and seed as well as chloroform seed extracts. It is possible that these similarities may indicate the presence of the embelin compound in these extracts.

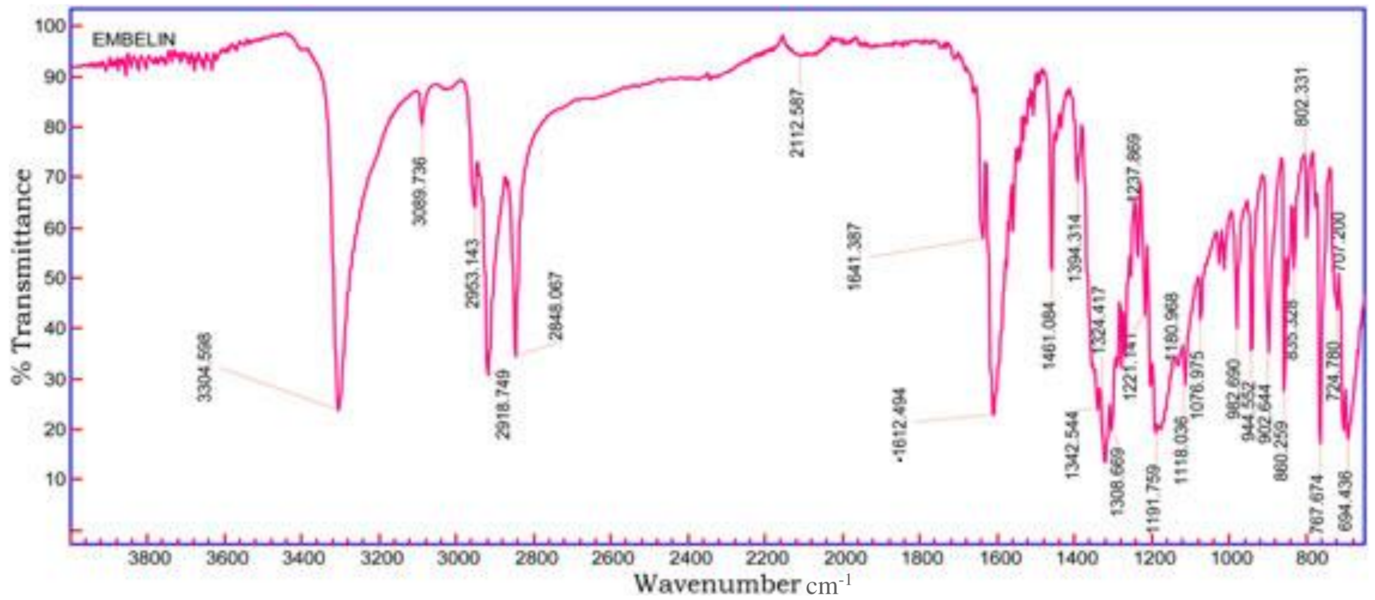


Figure 3.2 FTIR of pure embelin. Source: Shaikh et al. (2016).

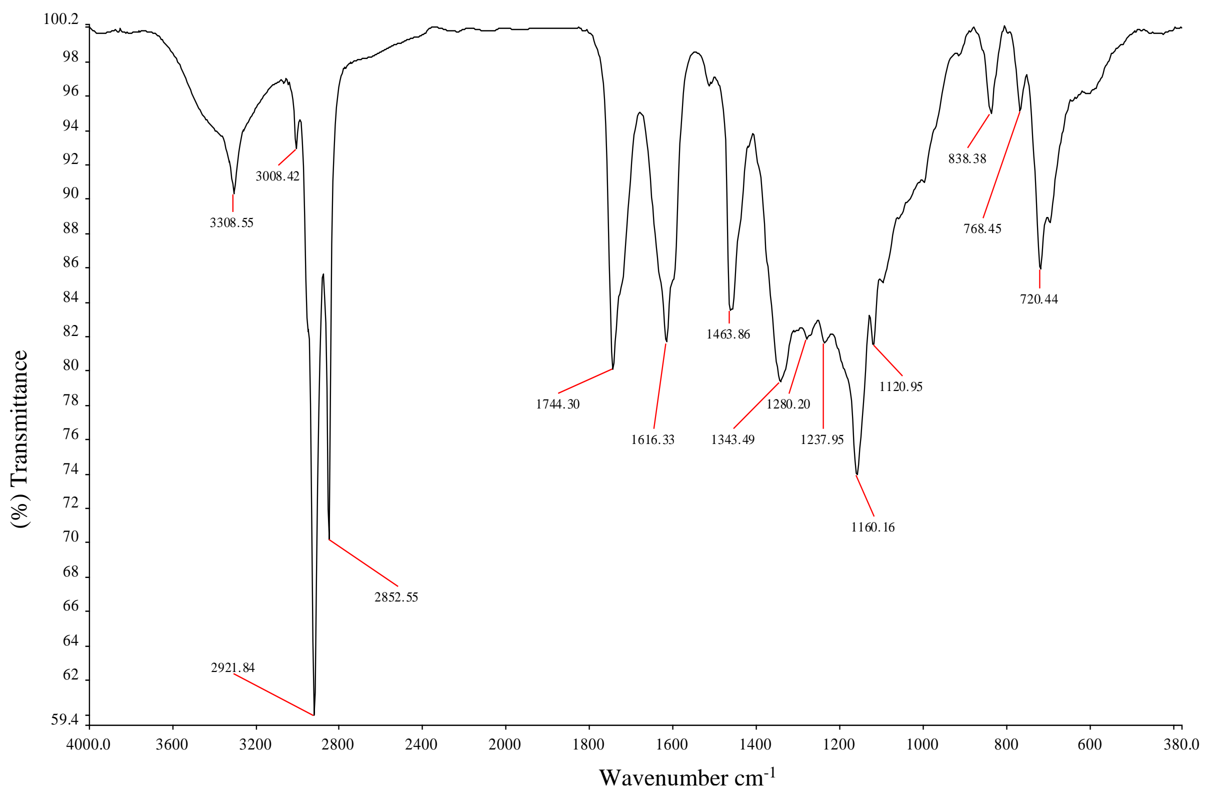


Figure 3.3 FTIR of the crude chloroform fruit extracts of *Embelia ruminata*.

Globally, emphasis has been placed on identifying new strategies to circumvent the escalation of antibiotic resistance. As the efficacy of the current antibiotic therapy decreases and the discovery of new antibiotic drugs stagnates, exploring alternative therapies could offer a much-needed solution (Streicher, 2021). Renewed interest has been invigorated towards utilising medicinal plants and their derivatives to incapacitate the bacterial virulence process without impacting viability (Casciaro et al., 2019; Mickymaray, 2019; Streicher, 2021). In the current study, the South African species, *E. ruminata* was evaluated for its potential as an anti-virulence agent.

The antibacterial activity of *E. ruminata* extracts revealed that apart from the chloroform leaf extracts, which demonstrated a zone of inhibition of 8 mm at 800 µg against *K. pneumoniae* ATCC 700603, the fruit extracts were the only extracts to exhibit antibacterial activity against the Gram-negative bacteria (Table 3.4). The hexane and chloroform fruit extracts demonstrated weak antibacterial activity at concentrations of 400 and 800 µg against the TEM β-lactamase-resistant *E. coli* ATCC 35218, while the methanolic fruit extracts displayed weak inhibition at 400 and 800 µg against extended-spectrum β-lactam producing *K. pneumoniae* ATCC 700603. Thus, it can be deduced that the different solvent extracts of the fruit were most effective against Gram-negative bacteria compared to the antibacterial activity of the other plant organs.

It is widely known that Gram-negative bacteria possess a formidable outer membrane (OM) that is composed of an asymmetric bilayer of phospholipids and lipopolysaccharides (Delcour, 2009; Mishra et al., 2020). The OM of Gram-negative bacteria is a major factor that enables bacterial resistance to a wide range of antibiotics, including β-lactams, quinolones, colistins and other antibiotics (Breijyeh et al., 2020). To circumvent such challenges, alternative strategies such as conjugating antibacterial agents to siderophore molecules that chelate iron (Fe^{3+}) ions have been investigated (Dauner and Skerra, 2020). Bacteria require Fe^{3+} for proliferation and may take up the conjugates of the siderophores and the antibacterial agents into its periplasm or cytoplasm (Kong et al., 2019). The benzoquinone compound, embelin possesses a long, non-polar, hydrophobic alkyl saturated chain (Zhang et al., 2016), which imparts an important lipid character to the embelin compound, potentially useful for membrane insertion (Caruso et al., 2020). This characteristic might afford the embelin compound to be an ideal siderophore conjugating agent that could bypass the protective OM bilayer of the Gram-negative

bacteria (Arthanareeswari et al., 2020). Future studies exploring these aspects together with the isolation of lead compounds from *E. ruminata* fruit extracts may result in a greater potency against Gram-negative bacteria.

Table 3.4 Antibacterial profile showing the zones of inhibition (mm) obtained using the different solvent extracts of *Embelia ruminata* extracts against selected Gram-negative bacteria

Extract	*E. coli* ATCC 25922		*E. coli* ATCC 35218		*K. pneumoniae* ATCC 700603		*P. aeruginosa* ATCC 27853	
	400 µg	800 µg	400 µg	800 µg	400 µg	800 µg	400 µg	800 µg
Hexane Leaf	0	0	0	0	0	0	0	0
Chloroform Leaf	0	0	0	0	0	**8**	0	0
Methanol Leaf	0	0	0	0	0	0	0	0
Hexane Stembark	0	0	0	0	0	0	0	0
Chloroform Stembark	0	0	0	0	0	0	0	0
Methanol Stembark	0	0	0	0	0	0	0	0
Hexane Fruit	0	0	**7**	**9**	0	0	0	0
Chloroform Fruit	0	0	**9**	**10**	0	0	0	0
Methanol Fruit	0	0	0	0	**8**	**10**	0	0
Hexane Seed	0	0	0	0	0	0	0	0
Chloroform Seed	0	0	0	0	0	0	0	0
Methanol Seed	0	0	0	0	0	0	0	0
Control								
Ciprofloxacin (CIP5)	30		37		26		32	
Gentamicin (GN10)	19		20		17		19	

Antibacterial activity classified as follows: ≤10 mm = weak, 11-15 mm = intermediate and >15 mm = strong

In the antibacterial susceptibility assays using Gram-positive bacteria, the different solvent extracts of *E. ruminata* leaf, stem bark, fruit and seeds demonstrated weak (ZOI ≤10 mm) to intermediate (ZOI 11-15 mm) antibacterial activity (Table 3.5). The methanolic stem bark extracts produced the most promising results showing intermediate activity against the nosocomial pathogen, *E. faecalis* ATCC 29212, susceptible *S. aureus* ATCC 29213, methicillin-resistant *S. aureus* ATCC 33591 and ATCC 700698. *Staphylococcus aureus* strains are associated with a plethora of diseases, such as mild skin and soft tissue infections, infective endocarditis, osteomyelitis, bacteraemia and fatal pneumonia, with the multidrug-resistant strains presenting a severe challenge in hospital-acquired infections (Guo et al., 2020; Kollef et al., 2021). Consistent with the results obtained from the current investigations, ethnobotanical studies have documented that tribal groups of India, especially in the northern and eastern areas of the country, use the fruit and seeds of *Embelia ribes* Burm.f. to treat skin diseases, including carbuncles, which are generally caused by *S. aureus* infections (Kala et al., 2006; Saikia et al., 2006; Goswami et al., 2009). Rondevaldova et al. (2015) showed the anti-staphylococcal capacity of *Embelia schimperi* Vatke and embelin synergistically enhanced the susceptibility of *S. aureus* to β-lactam antibiotics, oxacillin and tetracycline. Similarly, Mazlan et al. (2019) reported an increase in the antibacterial activity with the combinatory effect of embelin with antibiotics, nalidixic acid, ciprofloxacin, vancomycin and tetracycline against *S. aureus*. Likewise, the synergistic interactions of *E. ruminata* methanolic stem bark extracts combined with antibiotics may serve as newly formulated anti-staphylococcal products and could be a worthy target for future studies.

Interestingly, for *S. aureus* ATCC 700698, the following extracts displayed a higher antibacterial activity than the antibiotic control ciprofloxacin: the methanolic stem bark, chloroform and methanolic fruit at 400 µg, chloroform leaf and methanolic seed at 800 µg respectively. Correspondingly, at a concentration of 800 µg, the methanolic stem bark and seed extracts displayed a greater inhibition than the antibiotic control gentamicin, while the chloroform fruit extracts showed an equivalent inhibition to gentamicin for *S. aureus* ATCC 700698 (Table 3.5). The weak activity of both the antibiotic controls may indicate that these commercial antibiotic drugs are no longer capable of exerting an effect on *S. aureus* ATCC 700698 strain.

Enterococcus faecalis is also prevalent in the hospital environment as nosocomial infections (Abdulgader et al., 2020; Danelli et al., 2020). The genomic plasticity of enterococci, together with their natural resistance to several antimicrobials, including an aptitude to acquire and transfer genetic resistance determinants, has contributed to their adaptation to a hospital environment (Sparo et al., 2018). The assayed plant extracts showed that the chloroform fruit and methanolic seed extracts displayed a higher antibacterial activity at 400 and 800 µg than the antibiotic control gentamicin for *E. faecalis* ATCC 51299 (Table 3.5). Possibly the results indicate the ineffectiveness of gentamicin against *E. faecalis* while potentially highlighting that the chloroform fruit and methanolic seed extracts may possess lead compounds that could be utilised in the augmentation of novel antibacterial drugs against *E. faecalis*.

Staphylococcus epidermidis was inhibited by the methanolic leaf, stem bark and seed extracts, as well as the hexane stem bark extracts (Table 3.5). This bacterial strain was previously considered part of the normal microbiota of human and animal skin. It has evolved into an opportunistic pathogen causing healthcare-acquired infections in patients with indwelling medical devices (Widerström, 2016; Ahmadunissah et al., 2021). Ethnopharmacological usage and previous studies have shown the effectiveness of *E. ribes* in treating skin diseases and wounds (Sharma et al., 2002; Swamy et al., 2007; Mabona et al., 2013). Hence, the methanolic extracts of the leaf, stem bark and seeds including the hexane stem bark of *E. ruminata* may possess lead compounds that could hold the promise of being a therapeutic agent restraining the spread of *S. epidermidis*.

A comparison of the antibacterial activity of the different solvent extracts of each plant organ showed that methanolic leaf, stem bark and seed extracts, including the chloroform fruit extracts, had the most pronounced effect against the Gram-positive strains compared to the other solvent extracts of the respective plant organs. From this it can be deduced that methanol as an extracting solvent effectively solubilised phytocompounds with antibacterial activity. This can be supported by other studies that acquired similar results (Padalia et al., 2017; Seleshe and Kang, 2019; Ekşi et al., 2020). Overall, the chloroform fruit extracts performed the best in the antibacterial assays as these extracts showed notable antibacterial activity against Gram-negative and Gram-positive bacteria. A possible reason may be the presence of benzoquinone compounds in the chloroform fruit extracts. Thus, this may be in line with the phytochemical tests and FTIR assays that

showed positive results and the presence of functional groups for benzoquinone compounds such as embelin.

Table 3.5 Antibacterial profile showing the zones of inhibition (mm) obtained using the different solvent extracts of *Embelia ruminata* extracts against selected Gram-positive bacteria

Extracts	*E. faecalis* ATCC 29212		*E. faecalis* ATCC 51299		*S. aureus* ATCC 29213		*S. aureus* ATCC 33591		*S. aureus* ATCC 43300		*S. aureus* ATCC 700698		*S. epidermidis* ATCC 12228	
	400 µg	800 µg	400 µg	800 µg	400 µg	800 µg	400 µg	800 µg	400 µg	800 µg	400 µg	800 µg	400 µg	800 µg
Hexane Leaf	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chloroform Leaf	0	0	0	0	0	0	**10**	**13**	0	0	0	**8**	0	**8**
Methanol Leaf	**8**	**9**	0	0	0	0	0	**8**	0	**8**	0	0	**8**	**12**
Hexane Stembark	**8**	**9**	0	0	0	0	0	**8**	0	**8**	0	0	0	**8**
Chloroform Stembark	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Methanol Stembark	**9**	**9**	0	0	**9**	**10**	**11**	**12**	0	0	**9**	**12**	**10**	**13**
Hexane Fruit	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chloroform Fruit	**11**	**13**	**11**	**13**	0	0	0	**9**	0	0	**9**	**11**	0	0
Methanol Fruit	0	0	0	0	0	0	0	**9**	0	0	**8**	**9**	0	0
Hexane Seed	0	0	0	0	0	0	0	**9**	0	0	0	0	0	0
Chloroform Seed	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Methanol Seed	0	0	**7**	**8**	0	**9**	0	**12**	0	0	0	**12**	0	**9**
Controls														
Ciprofloxacin (CIP5)	33		38		23		22		23		6		28	
Gentamicin (GN10)	18		0		19		16		9		11		20	

Antibacterial activity classified as follows: ≤10 mm = weak, 11-15 mm = intermediate and >15 mm = strong

The ability of the hexane, chloroform and methanol extracts of *E. ruminata* leaf, stem bark, fruit and seeds to inhibit quorum sensing was tested using *Chromobacterium* agar overlay assay. *Chromobacterium subtsugae* CV017 and *Chromobacterium violaceum* ATCC 12472 produce a purple pigment violacein, a marker attribute during QS and alterations in violacein production are associated with QS activity. Thus, in the qualitative assay, the inhibition of violacein was indicated by the formation of colourless, opaque halos surrounding the dosed discs, while bactericidal activity was demonstrated by clear killing zones. For the CV017 biomonitor strain, two extracts, the chloroform leaf and methanol seed extracts showed violacein inhibition at 400 and 800 µg without any bactericidal activity [Table 3.6 and the corresponding Figures have been incorporated in the Appendix section: A3.3(a_1) to (a_{12}) on pages 187 and 188]. In contrast, hexane stem bark, methanol fruit and hexane seed extracts demonstrated violacein inhibition at 800 µg.

With the ATCC 12472 biomonitor strain, the chloroform leaf and fruit extracts were able to inhibit violacein production at 400 and 800 µg without any bactericidal activity [Table 3.6 and the corresponding Figures have been included in the Appendix section A3.3(b_1) to (b_{12}) on page 187 and 188]. In comparison, this effect was observed in the methanolic stem bark extracts at 800 µg. Six of the extracts (i.e., methanol leaf, hexane and chloroform stem bark, methanol fruit, hexane and methanol seed), were bactericidal at 800 µg rather than demonstrating violacein inhibitory potential. The chloroform leaf extracts appeared to be most effective in establishing an inhibition of short and long chain AHL autoinducers. There was an observed increase in the diameter of the colourless halos of QSI zones in a concentration-dependent manner for both short and long chain biomonitors.

In another study, Cosa et al. (2019) reported that the methanolic extracts of *Melissa officinalis* L. showed violacein inhibitory activity against *C. violaceum* ATCC 12472 producing a QSI zone of 9 mm. Similarly, from the data of the current study, the methanolic stem bark extracts exhibited a QSI halo of 9 mm.

Table 3.6 Qualitative inhibitory effects of the hexane, chloroform and methanol extracts of *Embelia ruminata* leaf, stem bark, fruit and seeds on quorum sensing (QS) violacein production using *Chromobacterium* biomonitor strains

	C. subtsugae CV017						*C. violaceum* ATCC 12472					
Extract	400 µg			800 µg			400 µg			800 µg		
	Total zone diameter (mm)	Clear zone diameter (mm)	QSI halo (mm)	Total zone diameter (mm)	Clear zone diameter (mm)	QSI halo (mm)	Total zone diameter (mm)	Clear zone diameter (mm)	QSI halo (mm)	Total zone diameter (mm)	Clear zone diameter (mm)	QSI halo (mm)
Hexane Leaf	0	0	0	0	0	0	0	0	0	0	0	0
Chloroform Leaf[#]	7	0	7	9	0	9	7	0	7	8	0	8
Methanol Leaf	0	0	0	8	8	0	0	0	0	8	8	0
Hexane Stembark[#]	0	0	0	8	0	8	6	6	0	11	11	0
Chloroform Stembark	0	0	0	9	9	0	0	0	0	7	7	0
Methanol Stembark[#]	0	0	0	11	11	0	0	0	0	9	0	9
Hexane Fruit	0	0	0	0	0	0	0	0	0	0	0	0
Chloroform Fruit[#]	9	9	0	10	10	0	7	0	7	8	0	8
Methanol Fruit[#]	0	0	0	10	0	10	10	10	0	12	12	0
Hexane Seed[#]	0	0	0	8	0	8	0	0	0	8	8	0
Chloroform Seed	0	0	0	0	0	0	0	0	0	0	0	0
Methanol Seed[#]	7	0	7	9	0	9	0	0	0	8	8	0
Controls												
Vanillin	11	0	11	11	0	11	9	0	9	9	0	9

= bactericidal effects

= quorum sensing inhibition (QSI)

QSI activity considered weak ≤10 mm, intermediate 11-15 mm and strong >15 mm

[#]indicates the extracts selected for the quantitative analyses

Based on data from the agar overlay assay, seven selected extracts (chloroform leaf, hexane stem bark, methanol stem bark, chloroform fruit, methanol fruit, hexane seed and methanol seed) were subjected to the quantitative violacein inhibition assay (Figure 3.4). QSI was considered noteworthy when the percentage violacein inhibition ≥50% and % growth inhibition (GI) was <40%.

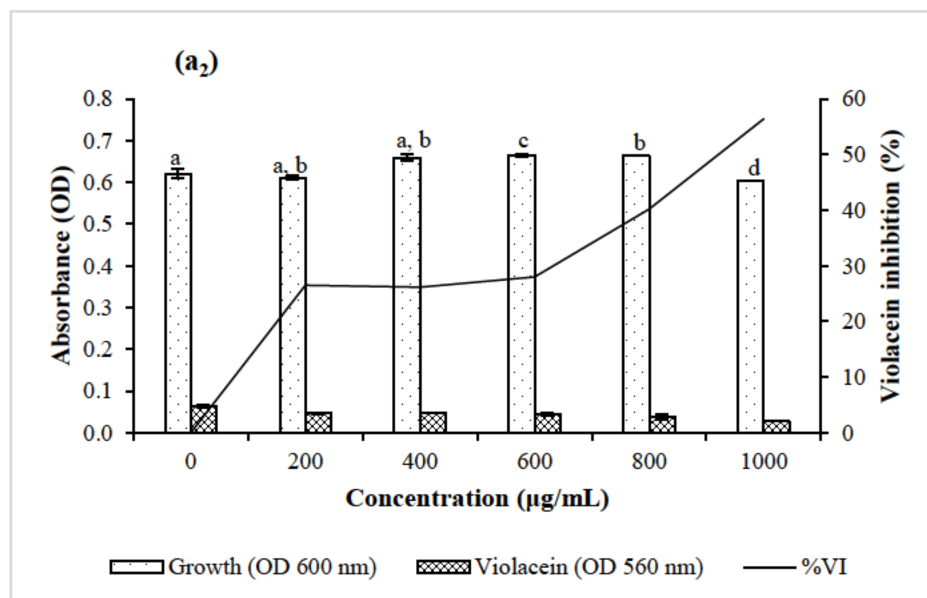
In the assay with the short chain AHL-producing CV017 biomonitor strain, a decrease in violacein production was observed with increasing concentrations for three extracts of *E. ruminata* viz. chloroform leaf, methanolic and hexane seed. The chloroform leaf and hexane seed extracts demonstrated violacein inhibition of 56.44 and 49.52% with GI of 2.77 and 26.76%, respectively, at 1000 µg/mL [Figure 3.4(a_2) and (a_{10})]. An IC_{50} of 4.89 µg/mL was obtained for the methanolic seed extract, which exhibited the most potent activity with a violacein inhibition of 55.68% (GI of 39.02%) at a concentration of 1000 µg/mL [Figure 3.4(a_{12})].

Using the long chain AHL-producing *C. violaceum* ATCC 12472 biomonitor strain, the methanolic seed extracts demonstrated violacein inhibition of 78.50% at 800 µg/mL with GI of 26.30% but were bactericidal at 1000 µg/mL [Figure 3.4(b_{12})]. Similarly, the hexane seed extracts displayed inhibition of 53.31-81.52% between 200-600 µg/mL with a GI of 5.04-32.00% [Figure 3.4(b_{10})]. Growth inhibition accompanied violacein inhibition at higher concentrations of the methanol and hexane seed extracts, suggesting that these extracts possess QSI properties at the lower concentrations and antibacterial activity at the higher concentrations. The chloroform and methanol fruit extracts displayed violacein inhibition of 51.07-83.53% at 800-1000 and 400-1000 µg/mL, respectively, with GI of 9.40-25.11% with no bactericidal effects [Figure 3.4(b_8) and (b_9)]. This biomonitor produces long chain AHL signals; thus, apart from the hexane stem bark, all six extracts showed disruption of the long chain AHL-based QS system. As with the *C. subtsugae* CV017 biomonitor results, the methanolic seed extracts with an IC_{50} of 2.51 µg/mL exhibited the most effective QSI activity compared to the other extracts. Shukla and Bhathena (2016) observed that seeds of *E. ribes* weakly influenced QS of *C. violaecum* ATCC 12472. Their *E. ribes* extracts had a very low range of concentration at which it could prevent QS, as the difference between its minimum inhibitory concentration (4 mg/mL) and minimum QS inhibitory concentration (~1 mg/mL) was only 4-fold. This is in disagreement with the findings of the present study and may be due to the difference

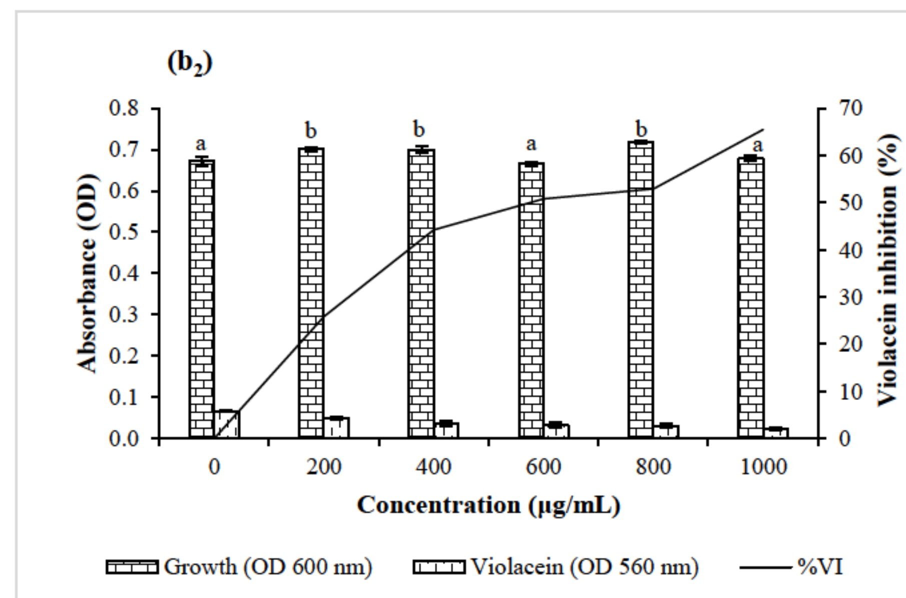
in methodologies employed and *Embelia* species used.

Statistically, the mean values among extracts were greater than expected by chance and demonstrated a significant difference at $p \leq 0.05$. Overall, the extracts were more effective against the long than the short chain autoinducers. Possibly the phytocompounds of *E. ruminata* could compete with the long chain signals more effectively. Phytocompounds such as flavonoids, furanones and lactones can bind to the autoinducer signal receptor sites and block signal transduction (Paczkowski et al., 2017; Roychoudhury, 2020; Zhou et al., 2020).

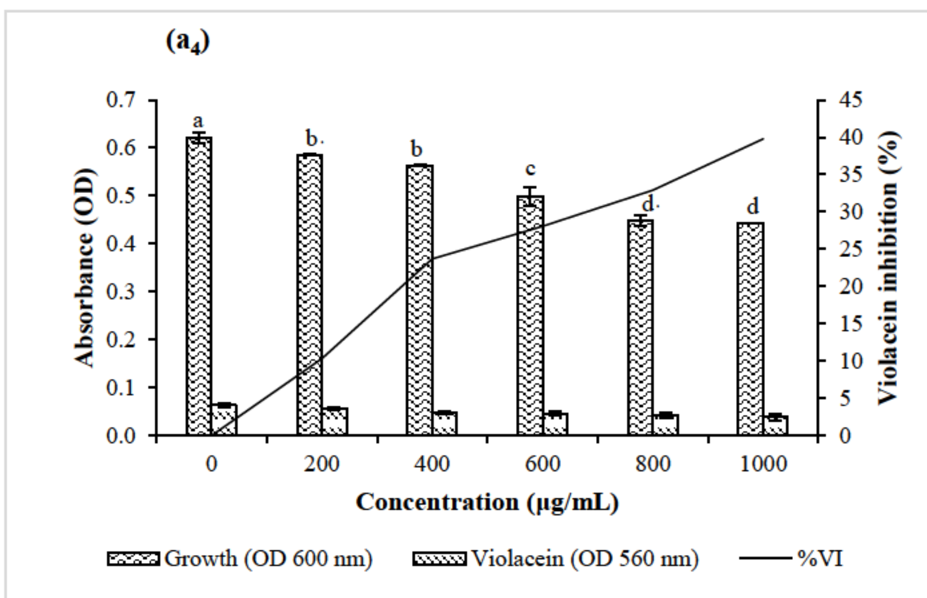
The chloroform leaf, hexane and methanolic seed extracts displayed QS antagonistic activity against both biomonitor strains, suggesting broad-spectrum activity against short and long chain AHL-based bacterial strains. Future assessments of these extracts, verifying principal compounds that mediate broad-spectrum QSI, needs to be undertaken.



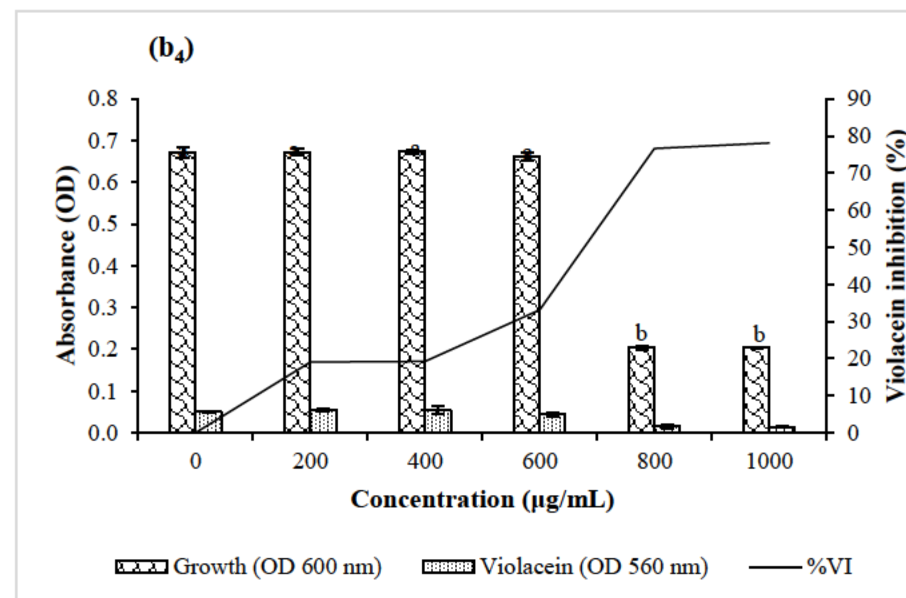
(a2)
Absorbance (OD)
Violacein inhibition (%)
Concentration (µg/mL)
0
200
400
600
800
1000
a
a, b
a, b
c
b
d
Growth (OD 600 nm)
Violacein (OD 560 nm)
%VI



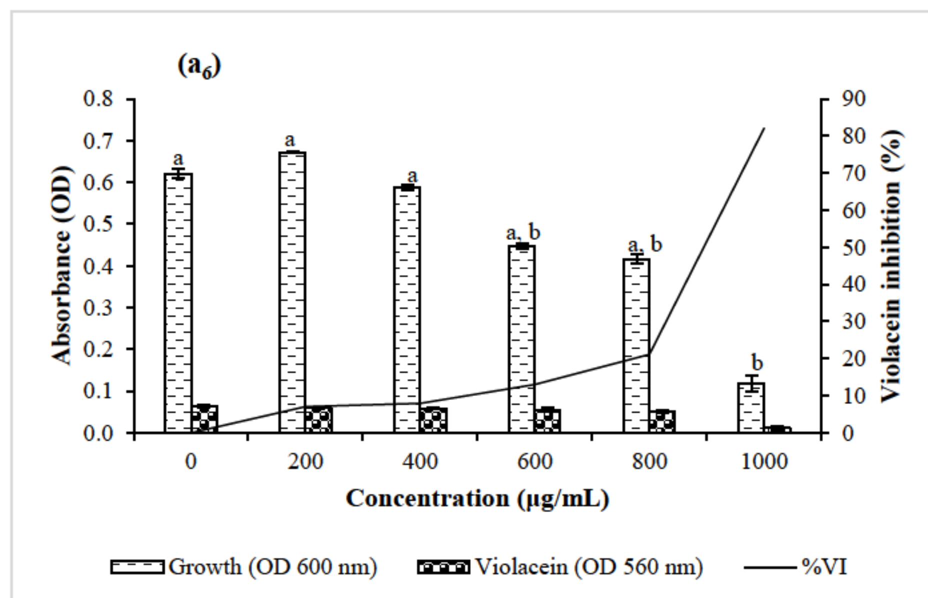
(b2)
Absorbance (OD)
Violacein inhibition (%)
Concentration (µg/mL)
0
200
400
600
800
1000
a
b
b
a
b
a
Growth (OD 600 nm)
Violacein (OD 560 nm)
%VI

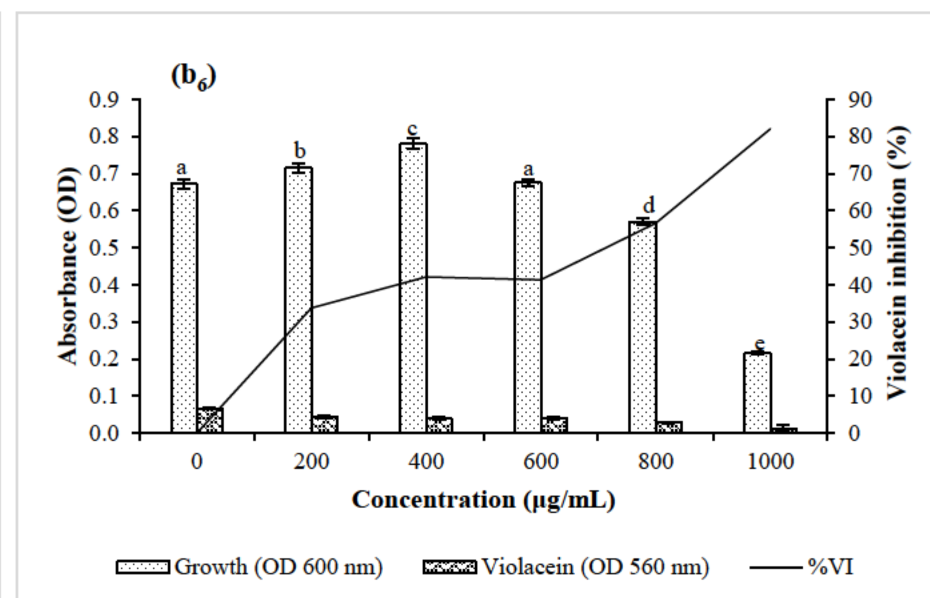


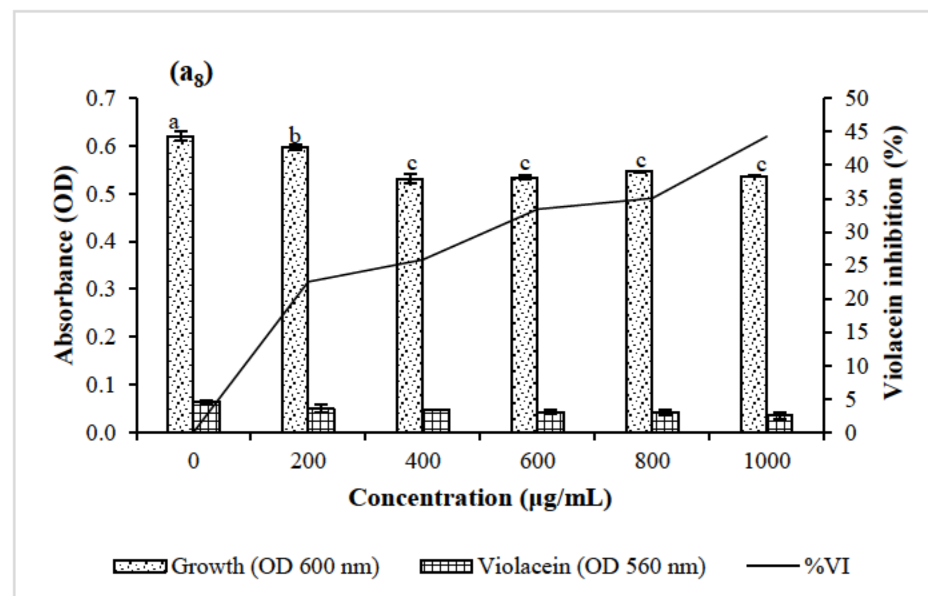
(a4)
Absorbance (OD)
Violacein inhibition (%)
Concentration (µg/mL)
0
200
400
600
800
1000
a
b
b
c
d
d
Growth (OD 600 nm)
Violacein (OD 560 nm)
%VI

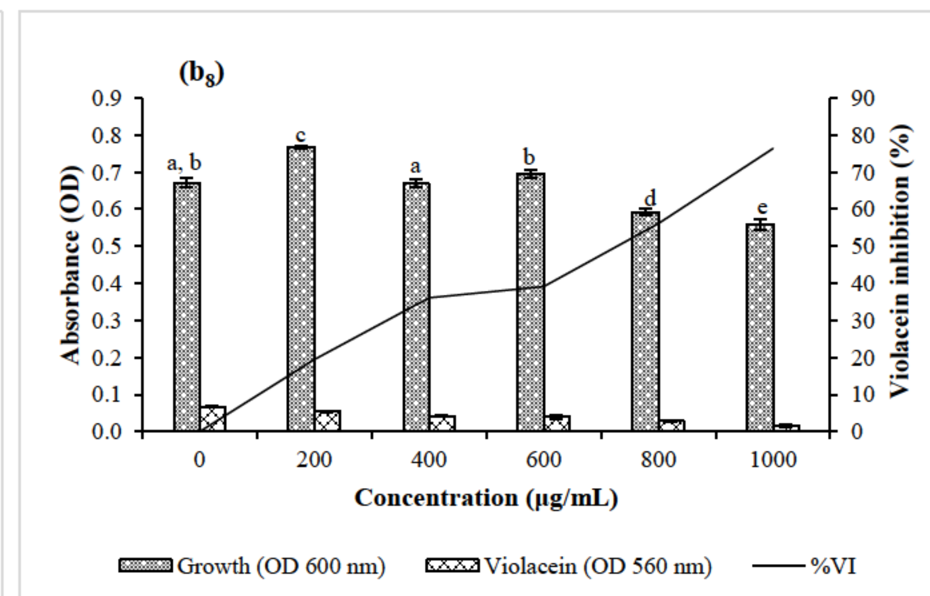


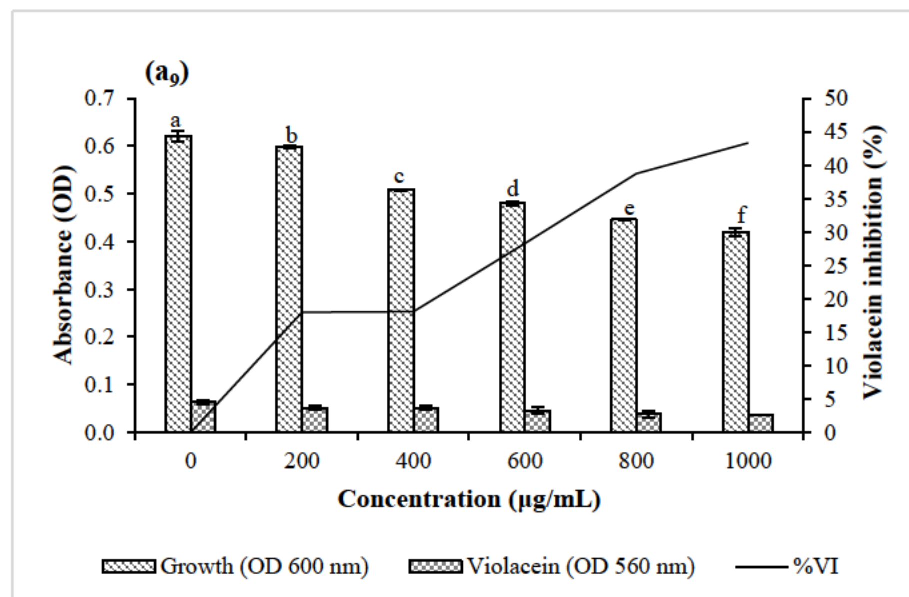
(b4)
Absorbance (OD)
Violacein inhibition (%)
Concentration (µg/mL)
0
200
400
600
800
1000
b
b
Growth (OD 600 nm)
Violacein (OD 560 nm)
%VI

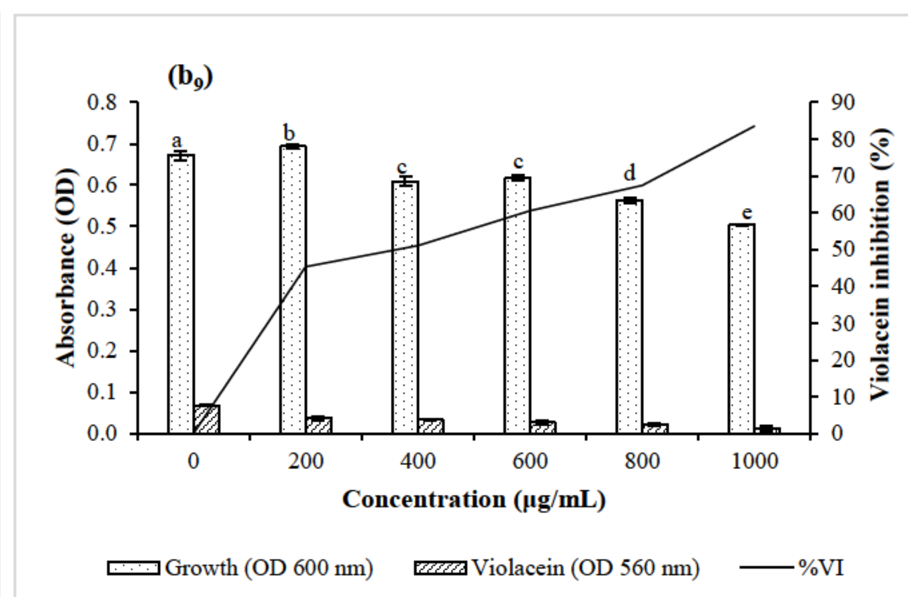

(a6)
Absorbance (OD)
Violacein inhibition (%)
Concentration (µg/mL)
Growth (OD 600 nm)
Violacein (OD 560 nm)
%VI

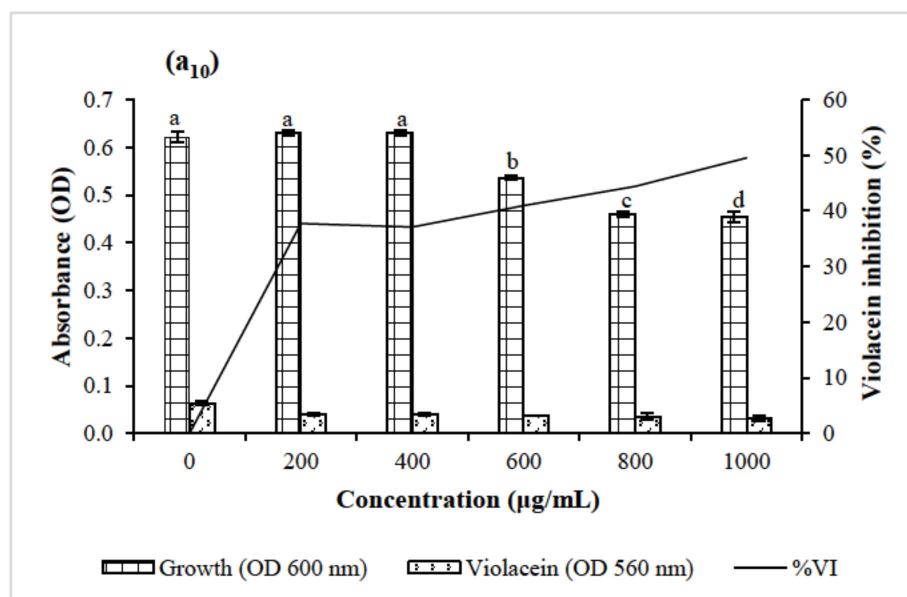

(b6)
Absorbance (OD)
Violacein inhibition (%)
Concentration (µg/mL)
Growth (OD 600 nm)
Violacein (OD 560 nm)
%VI

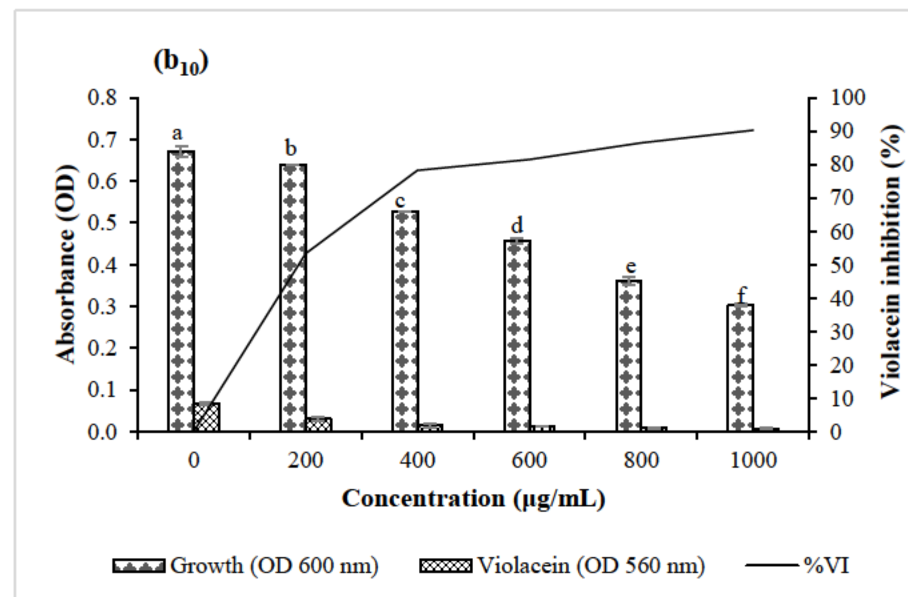

(a8)
Absorbance (OD)
Violacein inhibition (%)
Concentration (µg/mL)
Growth (OD 600 nm)
Violacein (OD 560 nm)
%VI


(b8)
Absorbance (OD)
Violacein inhibition (%)
Concentration (µg/mL)
Growth (OD 600 nm)
Violacein (OD 560 nm)
%VI


(a9)
Absorbance (OD)
Violacein inhibition (%)
Concentration (µg/mL)
Growth (OD 600 nm)
Violacein (OD 560 nm)
%VI


(b9)
Absorbance (OD)
Violacein inhibition (%)
Concentration (µg/mL)
Growth (OD 600 nm)
Violacein (OD 560 nm)
%VI


(a10)
Absorbance (OD)
Violacein inhibition (%)
Concentration (µg/mL)
Growth (OD 600 nm)
Violacein (OD 560 nm)
%VI


(b10)
Absorbance (OD)
Violacein inhibition (%)
Concentration (µg/mL)
Growth (OD 600 nm)
Violacein (OD 560 nm)
%VI

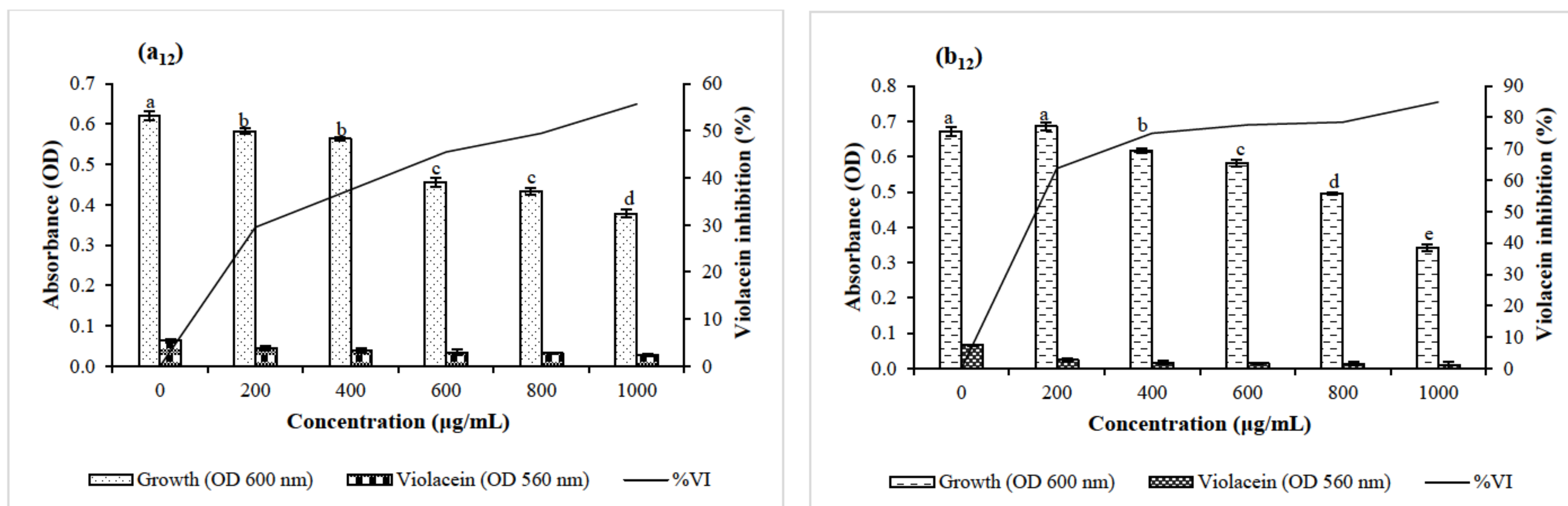


Figure 3.4 Quantitative analysis of the concentration-dependent inhibitory effects of the hexane, chloroform and methanol extracts using *Embelia ruminata* leaf, stem bark, fruit and seeds, on growth and violacein production by *Chromobacterium* spp. Labels (a_2) and (b_2) = chloroform leaf, (a_4) and (b_4) = hexane stem bark, (a_6) and (b_6) = methanol stem bark, (a_8) and (b_8) = chloroform fruit, (a_9) and (b_9) = methanol fruit, (a_{10}) and (b_{10}) = hexane seed, (a_{12}) and (b_{12}) = methanol seed using biomonitors CV017 and ATCC 12472, respectively. Results are the average of three triplicate independent experiments and standard deviations are shown. Significance at $p \leq 0.05$ is indicated by different alphabetic letters.

In terms of the extracting solvents, methanol as an extractant produced effective inhibition against Gram-positive bacteria and in the QSI investigations. Published literature has attributed the greater susceptibility of microbial strains to methanolic extracts due to the polarity of the solvent, which enabled the extraction of a greater degree of phytoceuticals (Ramalingam and Rajaram, 2018; Borges et al., 2020; Babaeekhou and Ghane, 2021). The activity of the methanolic stem bark extracts stands out in the assays with the Gram-positive bacterial strains. Accordingly, the methanolic seed extracts displayed the most dominant inhibitory activity in the anti-QS tests. Thus, it can be ascertained that methanol was effective as an extractant for bioactive metabolites with anti-virulence properties from *E. ruminata*. Likewise, alkaloid compounds such as 2-(pent-4-ol)-N-methyl, which were characterised from the methanolic extracts *E. schimperi*, were shown to have a wide spectrum of antibacterial activity (Duke, 2017). Guyasa et al. (2018) isolated two flavans from the methanolic stem bark of *E. schimperi*, of which one compound, epicatechin displayed comparably greater antibacterial activity against *S. aureus* than the control, antibiotic gentamicin. Additionally, their findings support the results acquired in the present study, which showed the susceptibility of *S. aureus* ATCC 29213 to the methanolic stem bark (Table 3.5).

Other compounds such as alkylresorcinols, benzoquinones derivatives, benzofurans and polyphenol such as gallic acid have been isolated from this plant genus (Ganesan et al., 2009; Dang et al., 2014; Chen et al., 2018). Previous studies have reported that these compounds have antibacterial properties (Bouarab-Chibane et al., 2019; Carcamo-Noriega et al., 2019; Siheri et al., 2019; Xu et al., 2019) and may be present in the methanolic extracts of the stem bark and seed, enabling their anti-virulence action.

Parallel to this, the two other extracts of *E. ruminata*, chloroform leaf and fruit extracts also produced notable antagonistic effects against selected Gram-negative and Gram-positive bacteria (Tables 3.4 and 3.5). In the QSI tests, the chloroform leaf extracts exhibited inhibitory effects against both short and long chain autoinducers in qualitative and quantitative analyses, while the chloroform fruit extracts inhibited the long chain autoinducer. In the case of hexane as an extractant, there was appreciable activity of the hexane seed extracts in the QSI assays.

Overall, it can be deduced that different compounds of *E. ruminata* were solubilised in the different solvents depending on their chemical properties. Following the principle of similarity

and intermiscibility, the more similar the solvent and the polarity of compound, the faster the dissolution of compounds from the plant matrix (Thouri et al., 2017). Thus, it is possible that the anti-QS activity of the extracts of *E. ruminata* was due to various phytochemical components that were extracted in the respective solvents. To optimise the extraction process, the exploration utilising other solvents as extractants should be performed.

3.5 Conclusion

This study provided a foundation for future studies with *E. ruminata* extracts demonstrating potential as anti-infective agents. The chloroform fruit and methanolic seed extracts, demonstrated greater inhibitory activity than the antibiotic controls ciprofloxacin and gentamicin against methicillin-resistant *Staphylococcus aureus* ATCC 700698 and vancomycin-resistant *Enterococcus faecalis* ATCC 51299, respectively. These extracts could have lead compounds that can be exploited as antibacterial agents. The results of the anti-QS investigations indicated that more effective activity was displayed against long chain AHLs as evidenced by *C. violaecum* ATCC 12472 data than short chain AHLs. Furthermore, the study indicated that the chloroform leaf, hexane and methanolic seed extracts have broad-spectrum activity. Thus, these extracts could serve as inhibitors of pathogenicity and virulence of AHL autoinducers in multiple bacterial QS systems. Future studies identifying principal compounds of *E. ruminata* that arrest the disease-causing mechanisms of bacteria as well as their mode of action and anti-pathogenicity in animal models need to be explored.

Conflict of Interest

The authors have no conflict of interest to declare.

3.6 Acknowledgements

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CHAPTER 4

Analyses of the phytochemical profile, *in vitro* antioxidant, radical scavenging and cytotoxicity activities of the vegetative and reproductive organs of *Embelia ruminata* (Primulaceae)

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4.1 Abstract

Medicinal plants and their bio-constituents play a pivotal role in the synthesis and formulation of new and novel therapeutic drugs. There is an urgent need to source, evaluate and document these phytomedicinal agents. Consequently, this study aimed to investigate some of the phytochemical and biological properties using different solvent extracts of the leaf, stem bark, fruit and seeds of *Embelia ruminata* (E.Mey. ex A.DC.) Mez. To commence the analyses, gas chromatography-mass spectroscopy (GC-MS) analysis was employed to investigate the phytochemical profile of *E. ruminata*. Total phenolic and flavonoid content were evaluated using the Folin-Ciocalteu and aluminum chloride method, respectively. The antioxidant effects were investigated using the total antioxidant capacity (TAC) and ferric reducing antioxidant power (FRAP) methods. The radical scavenging activity was established using 2, 2-diphenyl 1-picryl hydrazyl radical (DPPH) assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the cytotoxic effects of plant extracts on cancer cell lines, i.e., breast cancer (MCF-7) and human lung cancer (A549) as well as on normal cells (HEK293). In the results of the GC-MS analyses, twenty-eight bioactive compounds were identified. The total phenolic and flavonoid content ranged from 0.31 to 33.81 µg/mL with chloroform stem bark extracts showing the highest content of flavonoids. In the FRAP assay, the methanolic seed extracts exhibited the most effective reducing capacity of 67.63 mg AAE/g dry weight, while the chloroform seed extracts showed the highest TAC capacity of 38.50 mg AAE/g dry weight. Potent radical scavenging activity was exhibited by the methanolic seed extracts (IC_{50} of 3.54 µg/mL) in comparison to the standard butylated hydroxytoluene, which produced an IC_{50} of 91.09 µg/mL. As evidenced from the cytotoxicity data, the methanolic stem bark and seed extracts displayed notable anticancer activity with IC_{50} values of 0.74 and 3.96 µg/mL on MCF-7 and 0.26 and 5.58 µg/mL on A549 cell lines, respectively. These findings provide evidence that *E. ruminata* is a possible source of some important medicinal compounds.

Key words: *Embelia ruminata*, phytochemical, antioxidant activity, phenolic compounds, flavonoid, GC-MS, cytotoxicity

4.2 Introduction

The therapeutic use of plants for medicinal purposes has been an integral part of ancient traditional systems since antiquity to date. Medicinal plants have assumed a central stage in public health care as a natural alternative to contemporary drugs, with an estimated 65-80% of the world population living in developing countries relying on plant-based traditional medicine (Dwivedy et al., 2019).

To complement the search for new phyto-resourced compounds, a South African plant species, *Embelia ruminata* (E.Mey. ex A.DC.) Mez, was investigated in this study. This plant species belongs to the genus *Embelia* Burm.f. (family Primulaceae), which comprises approximately 136 species (GBIF, 2019) occurring in China, India, Southeast Asia, Australia, Polynesian islands, Africa and Madagascar (Dubéarnès et al., 2015). In South Africa, *E. ruminata* occurs as a scandent shrub or climbing liana, distributed along the coastal and slightly inland forests of the Eastern Cape, extending northwards to the forests of KwaZulu-Natal (Dyer, 1966).

Locally, Zulu tribes refer to *E. ruminata* as ibinini and traditionally use the leaves, fruit and roots as an antidote against tapeworm infections (Bryant, 1966; Hutchings et al., 1996). The Asian species, *Embelia ribes* Burm.f., is widely used in Ayurvedic formulations and has great eminence for its medicinal properties (Kundap et al., 2017; Kamble et al., 2020; Othman et al., 2020). Some major chemical constituents of this genus include embelin, christembine, homoembelin, vilangine and quercitol (Lu et al., 2016).

In essence, with the perspective of the vital importance of the phytoconstituents of plants, this study focused on unravelling the bioactive properties of this Primulaceae species, *E. ruminata*. From a conservation standpoint and simultaneously with the aim of exploring plant organs where the highest concentration of active substances can be found, the vegetative plant organs (leaf and stem bark) and reproductive organs (fruit and seeds) of *E. ruminata* were utilised in this study.

To date, little is known about the phytochemical and pharmacological activities of this species. Thus, this study was designed to investigate the phytochemical profile using GC-MS analyses. Additionally, the total phenolic and flavonoid content, including the antioxidant and radical scavenging capacity of the different solvent extracts of *E. ruminata* leaf, stem bark, fruit and

seeds were established. Furthermore, the cytotoxic potential was investigated to determine the proficiency of *E. ruminata* extracts as possible anticancer agents.

4.3 Materials and Methods

4.3.1 Collection, preparation and extraction of plant material

Embelia ruminata was collected from Reservoir Hills, Durban, KwaZulu-Natal, South Africa (29°48'26.7 "S 30°5543.9"E), from February to May, 2019. Professor H. Baijnath confirmed the species identity and the voucher specimen (Rambaran 1 with accession number 18256) was deposited in the Ward Herbarium, School of Life Sciences (Biology), University of KwaZulu-Natal, Westville Campus, South Africa.

The leaves, stem bark and fruit of *E. ruminata* were separated and air-dried for a duration of approximately two weeks. To obtain the seed, a portion of the dried fruit was decorticated, the seed was retained and allowed to dry. Thereafter, the plant material was pulverised to fine powder and refrigerated in airtight bottles until extraction.

The extraction process was carried out by employing the consecutive extraction method using 100 g of either leaf, stem bark, fruit or 15 g of seeds. To maximise the solubilisation of phytocompounds, each extraction was performed for 3 cycles for a duration of three hours using 200 mL (leaf, stem bark and fruit) and 60 mL (seeds) of solvents with increasing polarity i.e., hexane, chloroform and methanol. After each extraction, the resultant solution was filtered using Whatman No. 4 filter paper. The filtrates were concentrated to dryness using a vacuum rotary evaporator (Heidolph Instruments) and then stored in airtight vials at 4 °C for further analyses.

4.3.2 Gas chromatography-mass spectrophotometer (GC-MS) analysis

The different solvent extracts were diluted (1/100) in their respective extractant solvents (≥99%, GC grade, Sigma-Aldrich) and filtered using a 0.22 μm membrane filter. The GC-MS-QP2010 Plus Shimadzu instrument equipped with a capillary chromatographic column of 30 m × 0.25 mm ID × 0.25 μm film thickness of 5% phenylmethyl siloxane was used to conduct the analysis. The operating conditions were as follows: using 70 eV ionisation voltage, the GC temperature programme was set to a temperature of 50 °C, maintained for 1.5 min; thereafter

the temperature was increased to 200 °C at a rate of 4 °C $min^{-1}$and then increased to 300 °C at a rate of 10 °C min^{-1} and held for 7 min. The injector and interface temperatures were 240 and 220 °C, respectively. The helium flow rate of 1.2 mL min^{-1} was employed. Two microlitres of each crude extract was injected into the "splitless" mode system with a mass spectral scan mode range of 40-500 m/z.

A comparison of the spectrum of the unknown components and of known components stored in the National Institute Standard and Technology (NIST) library was undertaken. Using the NIST and other public repositories or databases, such as the Human Metabolome Database (Wishart et al., 2018), FoodB (2019) and PubChem (Kim et al., 2019), the name, molecular weight and structure of the compounds were ascertained. The relative quantity of the phytocompounds present in each of the extracts was expressed as a percentage based on peak height % produced in the chromatogram.

4.3.3 Quantification of total phenolic content (TPC)

The total phenolic content of *E. ruminata* crude extracts was evaluated using the Folin-Ciocalteu colorimetric method described by Singleton and Rossi (1965). Briefly, a calibration curve was prepared by adding 1 mL of Folin-Ciocalteu reagent (diluted ten-fold) to 200 µL aliquots of different concentrations of methanolic gallic acid solutions (10, 20, 40, 80, 160 µg/mL), vortexed and followed by the addition of 800 µL of 0.7 M sodium carbonate. After incubation at room temperature (23±2 °C) for 30 min, the absorbance was measured at 765 nm using the UV-Vis spectrophotometer (Shimadzu, Japan) against a reagent blank. The reagent blank constituted all reagents except the extract or standard and 95% methanol was added as an equivalent. A similar process was carried out to assess the total phenolic content of the crude extracts. All determinations were done in triplicate. Total phenolic content was recorded as milligrams of gallic acid equivalent (GAE) per gram of dry weight of extract using the formula (equation 4.1):

$$C_{tp} = C^{*}\,{}^{V}\!/_{m} \qquad \textbf{(4.1)}$$

where C_{tp} = total phenolic content (mg/g) in GAE (gallic acid equivalent), C = concentration of gallic acid obtained from calibration curve in mg/mL, v = volume of extract (mL) and m = mass of extract in grams (g) of dry weight (dw).

4.3.4 Determination of total flavonoid content (TFC)

Total flavonoid content of the different solvent extracts of *E. ruminata* leaf, stem bark, fruit and seeds was measured spectrophotometrically using the aluminum chloride assay (Ebrahimzadeh et al., 2008). In brief, the standard quercetin (10, 20, 40, 80, 160 µg/mL) prepared in 95% methanol was used to create the calibration curve. Aliquots of 0.5 mL of the standard were placed in a glass vial, followed by the addition of 1.5 mL methanol, 0.1 mL potassium acetate (1 M), 0.1 mL aluminum chloride (10%) and 2.8 mL of distilled water. Incubation was carried out at room temperature (23±2 °C) for 40 min. The absorbance of the solution was evaluated at 415 nm using a UV-Vis spectrophotometer (Shimadzu, Japan) against a reagent blank. The reagent blank included all reagents with 95% methanol substituting the extract or standard. The same process was repeated for the plant crude extracts. Results were expressed as the mean value of triplicate replicates in mg quercetin equivalent (QE) per gram dry weight using the formula (equation 4.2):

$$C_{tf} = C^{*}\,{}^{V}\!/_{m} \qquad \textbf{(4.2)}$$

where C_{tf} = total flavonoid content (mg/g) in QE, C = concentration of quercetin obtained from calibration curve in mg/mL, v = volume of extract (mL) and m = mass of extract in grams (g) of dry weight (dw).

4.3.5 Total antioxidant capacity (TAC) by the phosphomolybdenum method

The total antioxidant activity of the hexane, chloroform and methanolic extracts of *E. ruminata* leaf, stem bark, fruit and seeds was evaluated by performing the phosphomolybdenum assay (Prieto et al., 1999). A concentration gradient of 20-320 µg/mL, dissolved in 95% methanol, was prepared for the respective plant extracts. An aliquot of 0.3 mL of the respective plant concentration was mixed with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and incubated in a 95 °C water bath for 90 min. Thereafter, the samples were allowed to cool to room temperature (23±2 °C). The development of green colour was measured spectrophotometrically (UV-Vis Spectrophotometer, Shimadzu, Japan) at 695 nm against a blank. In the blank, all reagents were added except the plant extract or standard, which was replaced by 95% methanol. Ascorbic acid was used as a standard. The experiment was conducted in triplicates and results expressed as a mean value in milligrams of ascorbic acid equivalents per gram of the dry weight (dw) of extract (mg AAE/g dw).

4.3.6 Ferric reducing antioxidant power (FRAP)

The reducing power of the different solvent extracts of *E. ruminata* was evaluated according to the method by Benzie and Strain (1996), with some modifications. Briefly, the hexane, chloroform and methanol extracts of *E. ruminata* leaf, stem bark, fruit and seeds were dissolved in 95% methanol to prepare concentrations of 20, 40, 80, 160 and 320 µg/mL. Thereafter, 2 mL of 0.2 M phosphate buffer (pH 6.6) and 2 mL of 1 % aqueous potassium ferricyanide were added to the respective concentrations. After a 30 min incubation at 50 °C, 2 mL of trichloroacetic acid (10%) was added, centrifuged (3000 rpm, 10 min) and 2 mL of the resultant supernatant collected. To the supernatant, a volume of 2 mL distilled water and 0.4 mL of 0.1% (w/v) ferric chloride were added and allowed to stand for 10 min. The absorbance was measured at 700 nm against the reagent blank, which comprised all reagents except the extract or standard and 95% methanol was added as an equivalent. Ascorbic acid was employed as a standard. The results were recorded as milligrams ascorbic acid equivalent per gram dry weight (mg AAE/g dw).

4.3.7 Radical scavenging activity by the DPPH method

The free radical scavenging capacity of the hexane, chloroform and methanol extracts of *E. ruminata* leaf, stem bark, fruit and seeds, was investigated using the DPPH assay described by Blois (1958), with some modifications. In brief, 1 mL of 0.1 mM methanolic stock solution of DPPH was added to 1 mL of plant extract with concentrations of 20, 40, 80, 160 and 320 µg/mL. All mixtures were vigorously shaken for 5 min and allowed to stand in the dark for 30 min at room temperature (23±2 °C). Thereafter, the absorbance was measured at 517 nm using a UV-Vis Spectrophotometer (Shimadzu, Japan). Methanol was used as a blank and butylated hydroxytoluene (BHT) was used as a standard. The experiments were carried out in triplicates and the percentage scavenging activity of the crude extracts on DPPH was calculated using the formula below (equation 4.3):

$$\% \text{ Scavenging activity } = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}} * 100 \tag{4.3}$$

Dose-response curves were plotted between the percentage of scavenging activity (y-axis) and the logarithmic transformation of the concentrations (x-axis), using Microsoft Office Excel, 2016. The half-maximal inhibitory concentration (IC_{50}) value was calculated from the antilogarithmic values of the linear regression analysis. The IC_{50} is the concentration of extract that inhibits the formation of DPPH radicals by 50%.

4.3.8 Cytotoxicity Assay

Cytotoxicity was assessed using two cancer cell lines, i.e., breast cancer (MCF-7) and human lung cancer (A549) and a non-cancerous cell line (HEK293). Cells were grown in a humidified incubator maintained at 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS) including a low dose of antibiotics (10 000 U/mL of penicillin and streptomycin sulphate, respectively) (Thumbrain et al., 2020). Cells were grown to 80% confluency, trypsinised and subcultured. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine anticancer activities of the crude extracts following a previously described procedure used by Dwarka et al. (2017), with some modifications. Briefly, 96 well flat-bottom plates were seeded with 50 µL of cells (1.2 $\times 10^3$ cells/mL) together with 50 µL of DMEM. Thereafter, the plates were incubated in a humidified incubator at 5% CO_2 maintained for 24 h at 37 °C. Cells were then dosed with 50 µL of different concentrations of crude extracts (7.8 to 1000 µg/mL) prepared in 5% dimethyl sulfoxide (DMSO). After a 24 h incubation, 20 µL of MTT reagent [5 mg/mL in phosphate buffered saline (PBS)] was added to individual wells and then incubated at 37 °C for 4 h. To solubilise formazan, 100 µL of DMSO was added to each well and the optical density read at 570 nm using a micro plate spectrophotometer (Multiscan Go, Thermo Scientific) for both the treated and untreated cells. Camptothecin was used as a positive control. The % viability was derived using the following formula (equation 4.4.):

$$\%\ \text{Cell viability} = \frac{(\text{Absorbance of treated})}{\text{Absorbance of untreated}} * 100 \quad \textbf{(4.4)}$$

4.3.9 Statistical Analysis

Experimental data was expressed as mean ± standard deviation (SD) derived from triplicate replicates. For DPPH analysis, IC_{50} was acquired with the Microsoft Office Excel, 2016. The IC_{50} for the cytotoxicity assays was obtained using Graph Pad Prism 5 Software (San Diego CA, USA). Furthermore, data was subjected to statistical analysis using SPSS 26 for windows, IBM Corporation, New York, USA. The Tukeys honestly significant difference (HSD) test was used at $p<0.05$ to establish significance. In addition, the Pearson correlation coefficient was used to ascertain the relationship of the total phenolic and flavonoid content with radical scavenging as well as antioxidant activity with $p<0.01$.

4.4 Results and Discussion

Medicinal plants and their phytotherapeutic components play an integral role in the advancement of modern medicine. Over recent years, there has been a renewal of interest in traditionally used medicinal plants in southern Africa, thus propelling the exploration of these plants for new therapeutically active compounds (Abdalla and McGaw, 2018). This premise formed the foundation for the current study, i.e., to initialise investigations into the potential curative properties of the plant species, *E. ruminata*.

Collectively, there were 28 bioactive compounds identified from the GC-MS analysis using previously published literature. These compounds have been categorised according to their class and presented in Table 4.1. The most abundant compound was squalene, which was characterised in hexane seed extracts with an abundance of 51.76%. This was followed by 5-heptylresorcinol found in chloroform fruit extracts (37.84%), which is known to have α-glucoside inhibitory activity. Another resorcinol compound 1,3-Benzenediol, 5-pentadecyl, often referred to as adipostatin has been shown to act as a cyclooxygenase inhibitor (Kim et al., 2019), was found in the hexane stem bark and fruit as well as in the chloroform leaf, stem bark and fruit extracts.

Table 4.1 GC-MS analysis of the pharmacologically active phytocompounds identified in the different solvent extracts of *Embelia ruminata*

No.	Compound	Plant organ	Height %	Activity	Reference
	Fatty acyls				
1	1-Tridecanol $C_{13}H_{28}O$	Hex-St	2.36	Effective in controlling cariogenic bacteria.	Jabeen and Ranganathan (2019)
2	13-Docosenamide $C_{22}H_{43}NO$	Me-L	25.65	Endogenous bio-regulators to treat tumour growth, circulatory disease, inflammation, nociception, nervousness and depression.	Saranya et al. (2019)
3	cis-Vaccenic acid $C_{18}H_{34}O_2$	Hex-L	8.28	Antibacterial activity.	Semwal et al. (2018)
4	n-Hexadecanoic acid $C_{16}H_{32}O_2$	Hex-L	4.91	Antioxidant, hypocholesterolemic, nematicidal, pesticide, hemolytic and alpha-reductase inhibitor.	Pushpabharathi et al. (2018)
5	Hexadecanamide $C_{16}H_{33}NO$	Me-St Me-Fr	4.45 4.36	May be involved in cell signalling and act as a membrane stabilizer.	Wishart et al. (2018)
6	Oleamide $C_{18}H_{35}NO$	Me-St Me-Fr	21.26 19.28	Anti-inflammatory, antitumour, antihypertensive, antihyperglycemic, immunomodulatory as well as diuretic.	Tanvir et al. (2018)
7	Pentadecanal- $C_{15}H_{30}O$	Hex-St	2.94	Antibiofilm activity against *Staphylococcus epidermidis* ATCC 35984.	Ricciardelli et al. (2018)
8	Tridecanoic acid $C_{13}H_{26}O_2$	Hex-St	3.08	Antimicrobial and antibiofilm activity.	Jin et al. (2021)

9	Tetradecanamide $C_{14}H_{29}NO$	Me-L	5.62	May be involved in ocular surface signaling and the maintenance of the complex tear film.	Nichols et al. (2007)
	Fatty alcohols				
10	1-Heneicosanol $C_{21}H_{44}O$	Hex-St	6.78	Antimicrobial activity.	Balachandar et al. (2018)
11	1-Heptacosanol $C_{27}H_{56}O$	Chl-St	2.57	Nematicidal, anticancer, antioxidant and antimicrobial.	Venkata Raman et al. (2012)
	Hydrocarbons: Alkanes				
12	Eicosane $C_{20}H_{40}$	Chl-L Chl-St Hex-Fr	3.23 2.16 1.59	Anti-inflammatory, antinociceptive, antipyretic and antioxidant effects.	Aati et al. (2019)
13	Tetratetracontane $C_{44}H_{90}$	Hex-L Chl-L Hex-St Hex-Fr Me-Se	18.91 6.31 9.63 9.90 5.11	Antibacterial activity.	Amudha et al. (2018)
14	2,6,11-trimethyldodecane $C_{15}H_{32}$	Me-Se	5.53	Antibacterial activity.	Yue et al. (2017)
	Hydrocarbons: Benzene derivatives				
15	2,2'-Methylenebis (4-methyl-6-tert butylphenol) $C_{23}H_{32}O_2$	Me-Se	20.11	Antioxidant and shown to act as an autophaging molecule sensitising tumour cells to anticancer drugs.	Al Zoubi et al. (2017); Jang et al. (2017)

	Hydrocarbons: Iodinated				
16	Hexadecane, 1-iodo- $C_{16}H_{33}I$	Hex-Fr	0.73	Anticancer activity.	Swantara et al. (2019)
	Hydroquinones				
17	tert-Butylhydroquinone $C_{10}H_{14}O_2$	Me-St	0.99	Antioxidant, prevents oxidative stress, apoptosis and anti-obesity.	Zeng et al. (2017)
18	1,4-Benzenediol, 2-methyl- (Toluhydroquinone) $C_{21}H_{36}O_2$	Chl-St	27.18	Angiogenesis inhibitor, anti-inflammatory and antibacterial,	Hwang et al. (2015); Kim et al. (2015)
	Lipid and lipid-like molecules: Steroids and steroid derivatives				
19	Ergosta-7,22-dien-3-ol, (3.beta.,22E)- $C_{28}H_{46}O$	Hex-L	2.32	Anticancer, cholesterol-lowering, antidiabetic, antioxidant, nephroprotective, antiviral, antibacterial and immunomodulation.	Patel (2019)
20	Chondrillasterol $C_{29}H_{48}O$	Hex-Se	3.86	Antimicrobial activity.	Mozirandi et al. (2019)
21	Stigmasterol $C_{29}H_{48}O$	Hex-L Chl-St Hex-St Hex-Fr	2.38 3.32 10.13 5.41	Preparation of progesterone and other important steroids.	Kaur et al. (2020)
	Organooxygen compounds				
22	Methyl 2-O-benzylpentofuranoside $C_{13}H_{18}O_5$	Me-L	1.96	Antiviral potential against many enveloped DNA viruses.	Oladunmoye et al. (2019)

23	1,3-Benzenediol, 5 pentadecyl $C_{21}H_{36}O_2$	Chl-L Hex-St Chl-St Hex-Fr Chl-Fr	12.65 1.66 17.05 15.57 25.73	Adipostatin, anti-filarial, cyclooxygenase inhibitor, molluscicide, pesticide, antiproliferative agents and anticancer.	Teerasripreecha et al. (2012); FoodB (2019); Kim et al. (2019)
24	5-Heptylresorcinol $C_{13}H_{20}O_2$	Chl-L Hex- St Hex-Fr Chl-Fr Me-Fr	26.39 4.04 22.89 37.84 14.88	α-Glucoside inhibitory activity.	Hu et al. (2020)
	Prenol lipids: Triterpenoids				
25	Squalene $C_{30}H_{50}$	Hex-L Hex-St Hex-Fr Chl-Fr Hex-Se Chl-Se	2.85 2.62 16.85 4.26 51.76 7.71	Cardioprotective, antioxidant, antibacterial, antifungal, anticancer and detoxifying agent.	Lozano-Grande et al. (2018)
	Prenol lipids: Diterpenoids				
26	Phytol $C_{20}H_{40}O$	Hex-L Chl-L Me-L	20.35 17.32 1.75	Cholinesterase inhibitory activity, a precursor for the manufacture of synthetic forms of vitamin E and vitamin K1 and modulation of transcription in cells.	Elufioye et al. (2017), Ho et al. (2018)
27	trans-Geranylgeraniol $C_{20}H_{34}O$	Hex-L Hex-St Hex-Fr	4.27 6.2 2.17	Regulates testosterone production.	Ho et al. (2018)
	Prenol lipids: Tocopherol				
28	Vitamin E $C_{29}H_{50}O_2$	Hex-St Chl-St	13.38 2.66	Anti-dermatitic, anti-leukemic, antitumor, anti-ageing, analgesic, antidiabetic, anti-inflammatory and antioxidant.	Valentino et al. (2018)

Unknown compound	Hex-Fr	9.59
	Chl-Fr	19.37
	Hex-Se	22.93
	Chl-Se	92.29

Hex-L = Hexane leaf, Chl-L = Chloroform leaf, Me-L = Methanol leaf
Hex-St = Hexane stem bark, Chl-St = Chloroform stem bark, Me-St = Methanol stem bark
Hex-Fr = Hexane fruit, Chl-Fr = Chloroform fruit, Me-Fr = Methanol fruit
Hex-Se = Hexane seed, Chl-St = Chloroform seed, Me-Se= Methanol seed

Embelin, a naturally occurring quinone compound found to be the principal constituent in the genus *Embelia* (Kaur et al., 2015; Vijayan and Raghu, 2021), was not identified in any of the extracts from the GC-MS results. However, fatty acyls, hydroquinone and resorcinol derivatives were established in the different solvent extracts. Similarly, in a study using *Embelia schimperi* Vatke, the authors could not detect embelin in their samples but were able to characterise fatty acids and resorcinol derivatives (Tessema et al., 2018).

Interestingly, the GC-MS results showed that the unknown compound that was characterised in the hexane fruit and seed as well the chloroform fruit and seed extracts, had a mass spectrum of 294 *m/z* (corresponding Tables included in the Appendix section: Table A4.7, A4.8, A4.10 and A4.11 on page 192 and 193). The molecular weight for pure embelin is 294.4 g/mol with a chemical formula $C_{17}H_{26}O_4$ (Kim et al., 2019). Additionally, studies have shown that embelin isolated from the crude extracts manifest as distinct golden to orange-red coloured crystals (Kaur et al., 2015; Pundarikakshudu et al., 2016; Nuthakki et al., 2019). This distinctive feature can be seen in especially the chloroform seed extracts (Figure 4.1). This exemplifies the possibility of the presence of the embelin compound in the respective extracts. Future studies, executing the isolation of pure compounds will provide more tangible results regarding the phytoconstituents of *E. ruminata*.

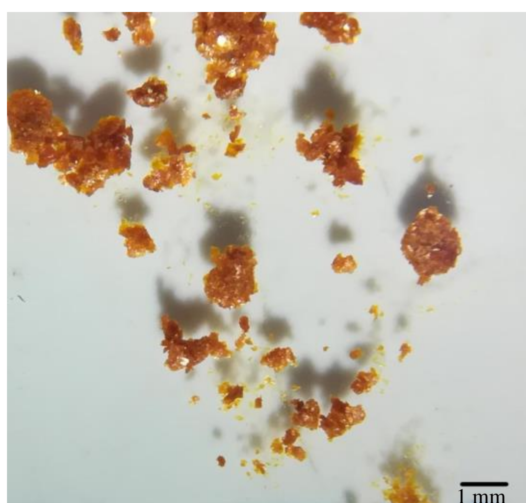

Figure 4.1 Image showing the golden to orange-red crude extract of the chloroform seed of *Embelia ruminata*.

In the quantification assays, the total phenolic content varied from 0.31 to 12.83 mg GAE/g dw (Table 4.2). The chloroform and methanolic seed extracts had the highest phenolic content of 12.83 and 11.04 mg GAE/g dw, respectively, while the methanol fruit extracts had the lowest phenolic content of 0.31 mg GAE/g dw. In the computation of total flavonoid content, a range from 0.86 to 33.81 mg QE/g dw was derived. The chloroform stem bark extracts displayed the highest flavonoid content of 33.81 mg QE/g dw, followed by the chloroform leaf extracts of 17.20 mg QE/g dw (Table 4.2). Kamble et al. (2020) reported total phenolic and flavonoid content of 18.18 mg GAE/g and 8.35 mg RE/g dw for the methanolic and ethanolic fruit extracts of *E. ribes*, respectively. Their results contrasts with the findings of the current study in which the methanolic fruit extracts displayed lower phenolic and flavonoid content. The dissimilar results may be due to the use of different species of *Embelia*, extraction protocols and methodologies. Additionally, Kamble et al. (2020) showed from their study that genotypes of *E. ribes* collected from different geographical locations displayed a distinct contrast in their total phenolic and flavonoid content. Likewise, the disparate findings between the respective studies may be due to the different geographical localities.

Table 4.2 Total phenolic content, total flavonoid content, total antioxidant capacity (TAC), ferric reducing antioxidant power (FRAP) and radical scavenging capacity (DPPH) of *Embelia ruminata* leaf, stem bark, fruit and seeds

Plant organ	Solvent	Phenolic (mg GAE/g dw)	Flavonoid (mg QE/g dw)	TAC (mg AAE/g dw)	FRAP (mg AAE/g dw)	DPPH IC_{50} value (µg/mL)
Leaf	Hexane	0.35 ± 0.08^{a}	$8.02 \pm 0.08^{b, c}$	8.62 ± 0.28^{a}	2.88 ± 0.01^{a}	$629.22 \pm 0.7^{*}$
	Chloroform	1.78 ± 0.04^{b}	17.20 ± 0.08^{d}	21.88 ± 0.61^{b}	8.26 ± 0^{b}	$>1000^{*}$
	Methanol	0.38 ± 0.27^{a}	1.49 ± 0.22^{a}	1.79 ± 0.14^{c}	2.08 ± 0.01^{c}	$193.95 \pm 0.32^{*}$
Stem Bark	Hexane	0.38 ± 0.09^{a}	$8.14 \pm 0^{b, c}$	5.58 ± 0.14^{d}	2.58 ± 0^{d}	>1000
	Chloroform	4.75 ± 0.13^{c}	33.81 ± 0^{e}	24.49 ± 0.75^{e}	22.84 ± 0.02^{e}	$194.58 \pm 0.22^{*}$
	Methanol	1.74 ± 0.19^{b}	0.86 ± 0.08^{a}	3.56 ± 0.14^{f}	10.34 ± 0.06^{f}	$37.47 \pm 0.07^{*}$
Fruit	Hexane	1.04 ± 0.04^{f}	$5.18 \pm 0.16^{a,b}$	12.40 ± 0^{g}	5.06 ± 0.01^{g}	95.61±1.69
	Chloroform	2.57 ± 0.18^{g}	$7.66 \pm 0.53^{b, c}$	15.14 ± 0.51^{h}	12.57 ± 0.03^{h}	126.96±0.27
	Methanol	0.31 ± 0.09^{a}	1.03 ± 0.14^{a}	1.85 ± 0^{c}	1.19 ± 0.02^{i}	$108.07 \pm 0.32^{*}$
Seed	Hexane	1.37 ± 0.01^{d}	$12.83 \pm 3.99^{c, d}$	21.89 ± 0.14^{b}	7.45 ± 0.01^{j}	$908.43 \pm 0.34^{*}$
	Chloroform	12.83 ± 0.01^{h}	$9.50 \pm 0.14^{b, c}$	38.50 ± 0.73^{i}	52.56 ± 0.01^{k}	$43.20 \pm 0.17^{*}$
	Methanol	11.04 ± 0.08^{i}	7.90 ± 0.3^{b}	22.90 ± 0.14^{j}	67.63 ± 0.01^{l}	$3.54 \pm 0.09^{*}$
Standard	BHT					$91.09 \pm 0.45^{*}$

Each value is expressed as a mean ± standard deviation with n=3 replicates. Mean values with different superscript letters ($^{a-l}$) in the columns are significantly different from one another at $p < 0.05$. * indicates significant difference at $p < 0.05$ between DPPH IC_{50} values of crude extracts and BHT IC_{50} values. Abbreviation AAE = ascorbic acid equivalent, BHT = Butylated hydroxytoluene, DPPH = 2, 2-diphenyl 1-picryl hydrazyl radical, dw = dry weight, GAE = gallic acid equivalent and QE = quercetin equivalent.

In the TAC assays, the results indicated that extracts were able to reduce phosphomolybdenum MO (VI) to MO (V) in a variable range of 1.79 to 38.50 mg AAE/g dw (Table 4.2). The highest activity of 38.50 mg AAE/g dw was established in chloroform seed extracts. It was noted that the increase in total antioxidant capacity was dose-dependent. A significant Pearson correlation was found between the total phenolic and flavonoid content with TAC (Table 4.3). Laraib et al. (2020) reported higher TAC activity for the methanolic fruit than the leaf extracts for *Myrsine africana* L., which belongs to the family Primulaceae. Likewise, these findings were reciprocated by Al-Abd et al. (2017) for another Primulaceae species, *Ardisia elliptica* Thunb. Surprisingly, there were few studies that investigated the TAC of the decorticated seeds. The data of the current study showed that the seed extracts had the highest TAC activity compared to the other plant organs.

The FRAP analysis showed that all extracts of *E. ruminata* demonstrated the ability to reduce Fe^{3+} to Fe^{2+} in varying degrees (Table 4.2). The methanolic seed extracts displayed the highest ferric reducing power of 67.63 mg AAE/g dw. Extracts displayed a concentration-dependent activity, i.e., the ferric reducing power increased as the concentration of the extract increased. Pearson correlation analyses showed a significant correlation between total phenolic and FRAP (Table 4.3). Kamble and Gaikwad (2019) reported that the methanolic stem bark of *E. ribes* had higher FRAP activity than the methanolic leaf extracts (72.22±0.31 and 66.66±0.27 mg Fe^{+2}/g dw, respectively). Although their study reported a much higher activity than the current study, a similar trend was observed where the methanolic stem bark of *E. ruminata* showed higher activity than the methanolic leaf extracts. The difference in the reported antioxidant activities between the respective studies could be due to different species of *Embelia* and geographical localities.

Table 4.3 Correlation of total phenolic and total flavonoid content with DPPH, total antioxidant capacity (TAC) and ferric reducing capacity (FRAP)

Pearson correlation	DPPH	TAC	FRAP
Phenolic	-0.308	0.792**	0.971**
Flavonoid	0.157	0.563**	0.171

** Correlation represents significance at the 0.01 level (2-tailed).

The different solvent extracts of *E. ruminata* exhibited varying degrees of radical scavenging activity. Overall, superior scavenging activity was observed for the methanolic extracts [Figure 4.2 (a-d)]. The methanolic seed extracts showed the most convincing radical scavenging capacity with an IC_{50} of 3.54 µg/mL compared to BHT, which had an IC_{50} of 91.09 µg/mL (Table 4.2). The GC-MS analysis identified the compound 2,2'-methylenebis(4-methyl-6-tert-butylphenol) in the methanolic seed extracts, which is used extensively in the manufacturing industry as a synthetic antioxidant (Takagi, 1994; Al Zoubi et al., 2017; Jang et al., 2017). It is possible that the natural form of this compound amplified the capacity of the methanolic seed extracts to produce such potent radical scavenging results. Additionally, the methanolic seed extracts displayed the highest ferric reducing capacity of 67.63 mg AAE/g dw and a total antioxidant capacity of 22.90 mg AAE/g dw.

Likewise, a hydroquinone compound, tert-butylhydroquinone (tBHQ) was identified in the methanolic stem bark extracts. Tert-butylhydroquinone is commonly used as a synthetic food antioxidant to prevent oils and fats from oxidative deterioration and rancidity (Carocho et al., 2018). This is noteworthy in respect to the present study, which produced results showing that the methanolic stem bark extracts had an influential radical scavenging activity with an IC_{50} of 37.47 µg/mL in comparison to BHT (IC_{50} 91.09 µg/mL). The bio-constituents of the methanolic stem bark extracts may have acted synergistically to produce potent radical scavenging effects.

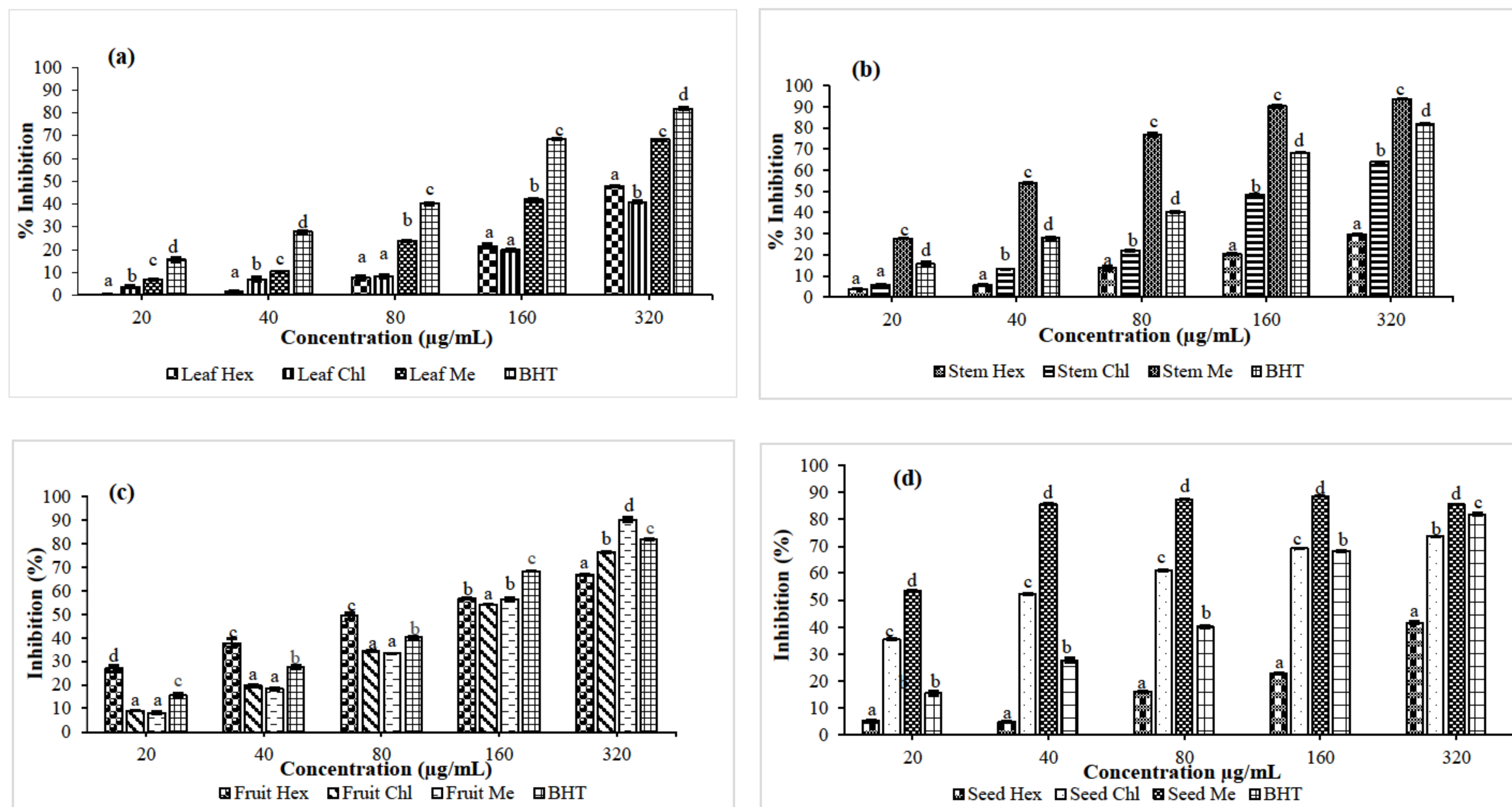

Figure 4.2 (a-d) Graph showing the DPPH radical scavenging activity of *Embelia ruminata*. Labels: (a) = Leaf, (b) = Stem bark, (c) = Fruit and (d) = Seed extracts. Superscript letters ($^{a\text{-}d}$) denote concentrations that are significantly different from each other (Tukey's range test at $p<0.05$). Hex=Hexane, Chl= Chloroform, Me= Methanol and BHT= Butylated hydroxytoluene.

Against this backdrop, it is generally assumed that the phenolic and flavonoid content of a plant gives it the robustness to have effective radical scavenging capacity (Batool et al., 2019). However, the interaction of other compounds apart from phenolics and flavonoids may influence the radical scavenging capacity (Dzoyem and Eloff, 2015). This rationale may explain the lack of a significant correlation that was found between DPPH, total phenolic and flavonoid content (Table 4.3). Other studies have also reported on the dysfunctional relationship between DPPH and the polyphenolic content (Dzoyem and Eloff, 2015; Odchimar et al., 2016; Haron and Jusoh, 2019; Lahlou et al., 2019; Papathanasiou et al., 2020). Therefore, from this study, it can be deduced that the presence of other radical scavenging compounds, which often comprise complex mixtures of different kinds of active compounds and the presence of compounds other than phenolics, should not be neglected in the determination of radical scavenging properties. On the same rationale, an insignificant relationship between the total flavonoid content and FRAP was found. There is a possibility that other compounds present in the extracts were able to reduce Fe^{3+} to Fe^{2+}.

Cancer is a serious metabolic syndrome and is one of the major causes of mortality and morbidity worldwide (Iqbal et al., 2017). Plants and their reservoir of phytoceutical compounds may serve as ideal candidates for anticancer drug development due to their multifaceted mechanisms that could target various elements that play a pivotal role in carcinogenesis (Iqbal et al., 2017; Twilley et al., 2020). In the cytotoxicity investigations of the present study, two cancer cell lines (MCF-7 and A549) and one non-cancer cell line (HEK293) were used. Overall, all extracts showed convincing effects on the cancer cell lines (Figure 4.3 to 4.5).

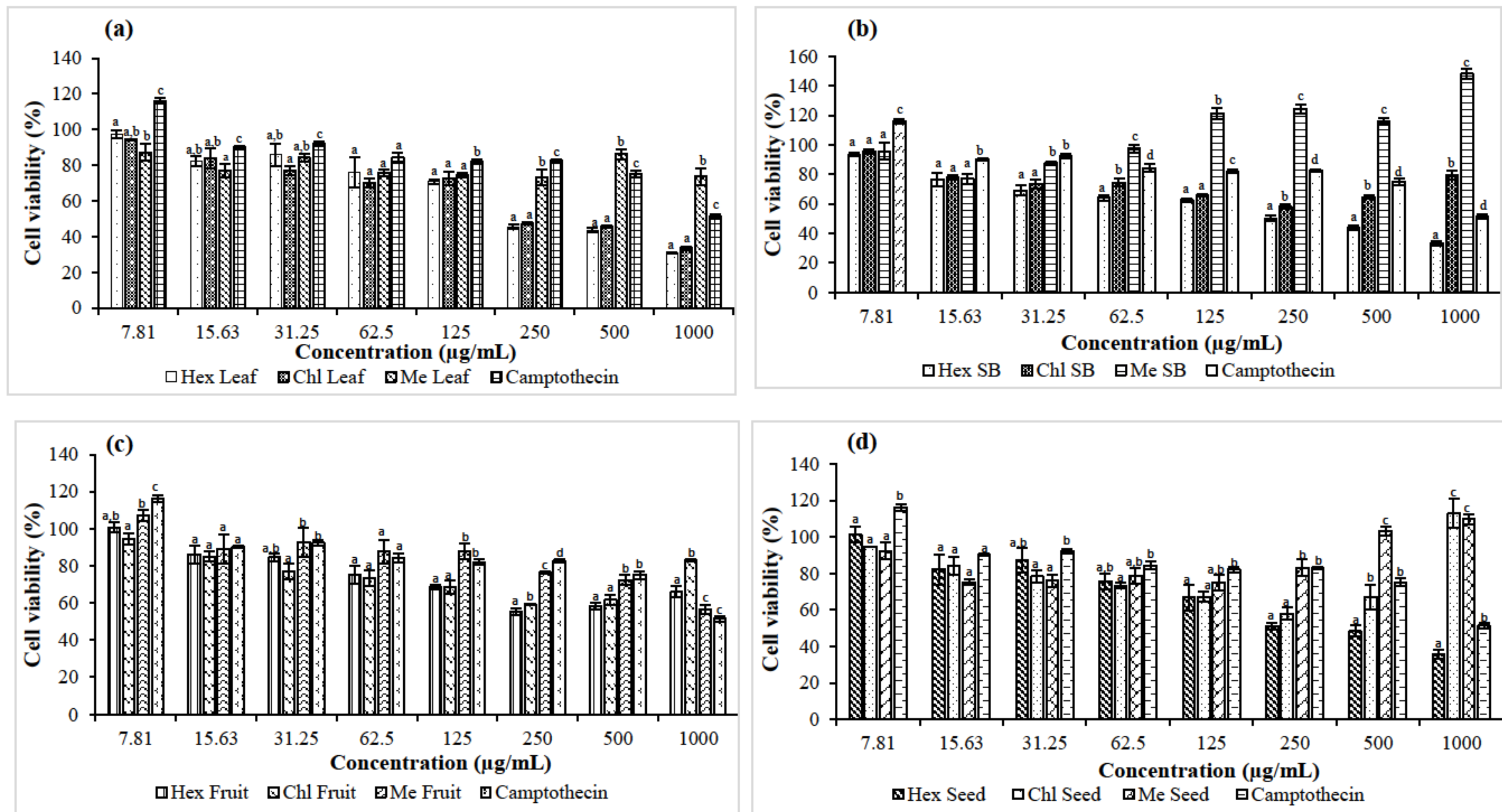

Figure 4.3 Cytotoxicity of different solvent extracts of *Embelia ruminata* on the cancer cell lines MCF-7. Labels (a) = Leaf, (b) = Stem bark, (c) = Fruit and (d) = Seed. Bars denote standard deviation. Different alphabetic superscript letters ($^{a-d}$) indicate significance at $p < 0.05$ with n = 3 replicates. Hex = Hexane, Chl = Chloroform, Me = Methanol and SB = Stem bark

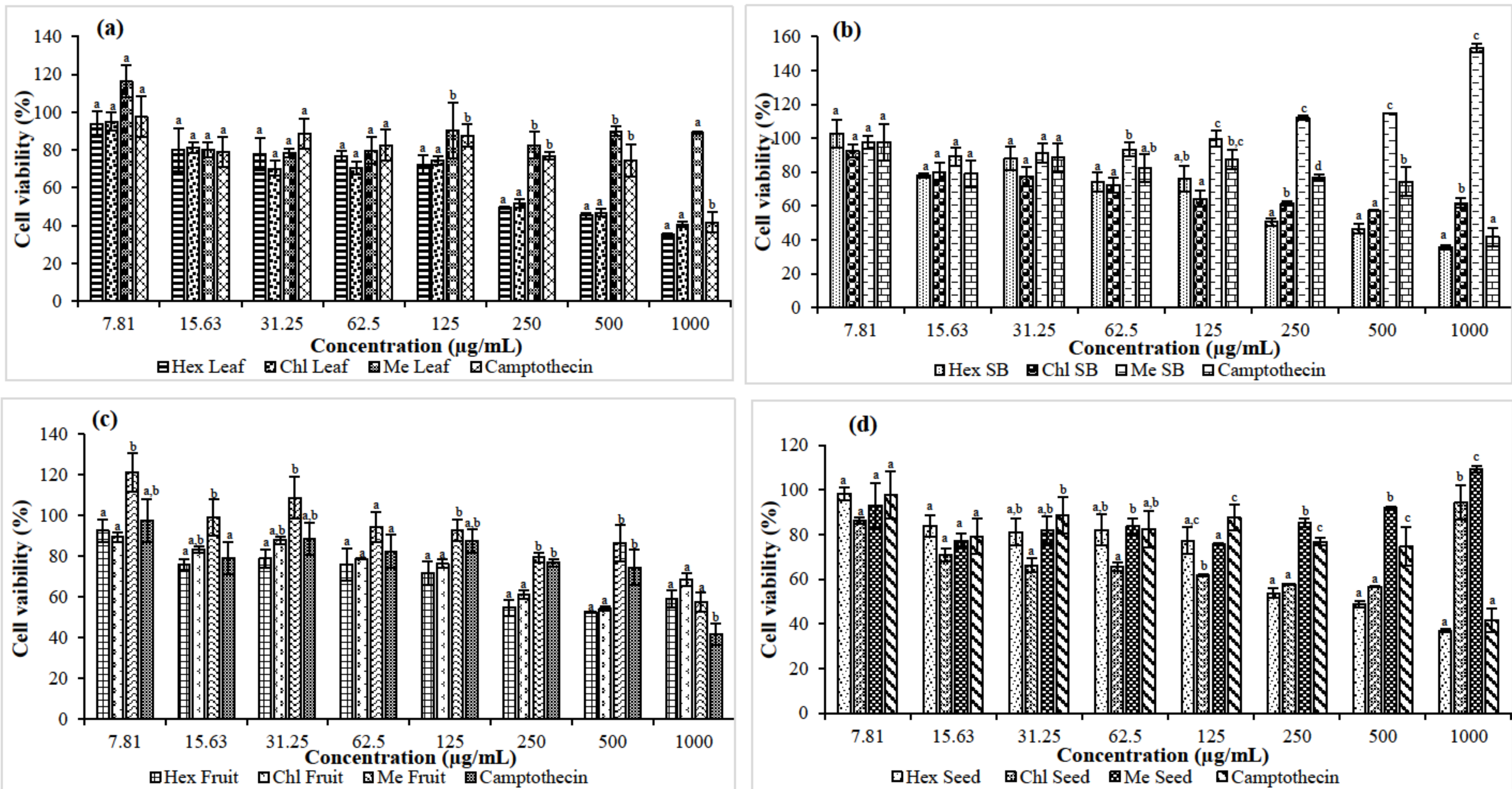

Figure 4.4 Cytotoxicity of different solvent extracts of *Embelia ruminata* on the cancer cell lines A549. Labels (a) = Leaf, (b) = Stem bark, (c) = Fruit and (d) = Seed. Bars denote standard deviation. Different alphabetic superscript letters ($^{a\text{-}c}$) indicate significance at $p < 0.05$ with $n = 3$ replicates. Hex = Hexane, Chl = Chloroform, Me = Methanol and SB = Stem bark

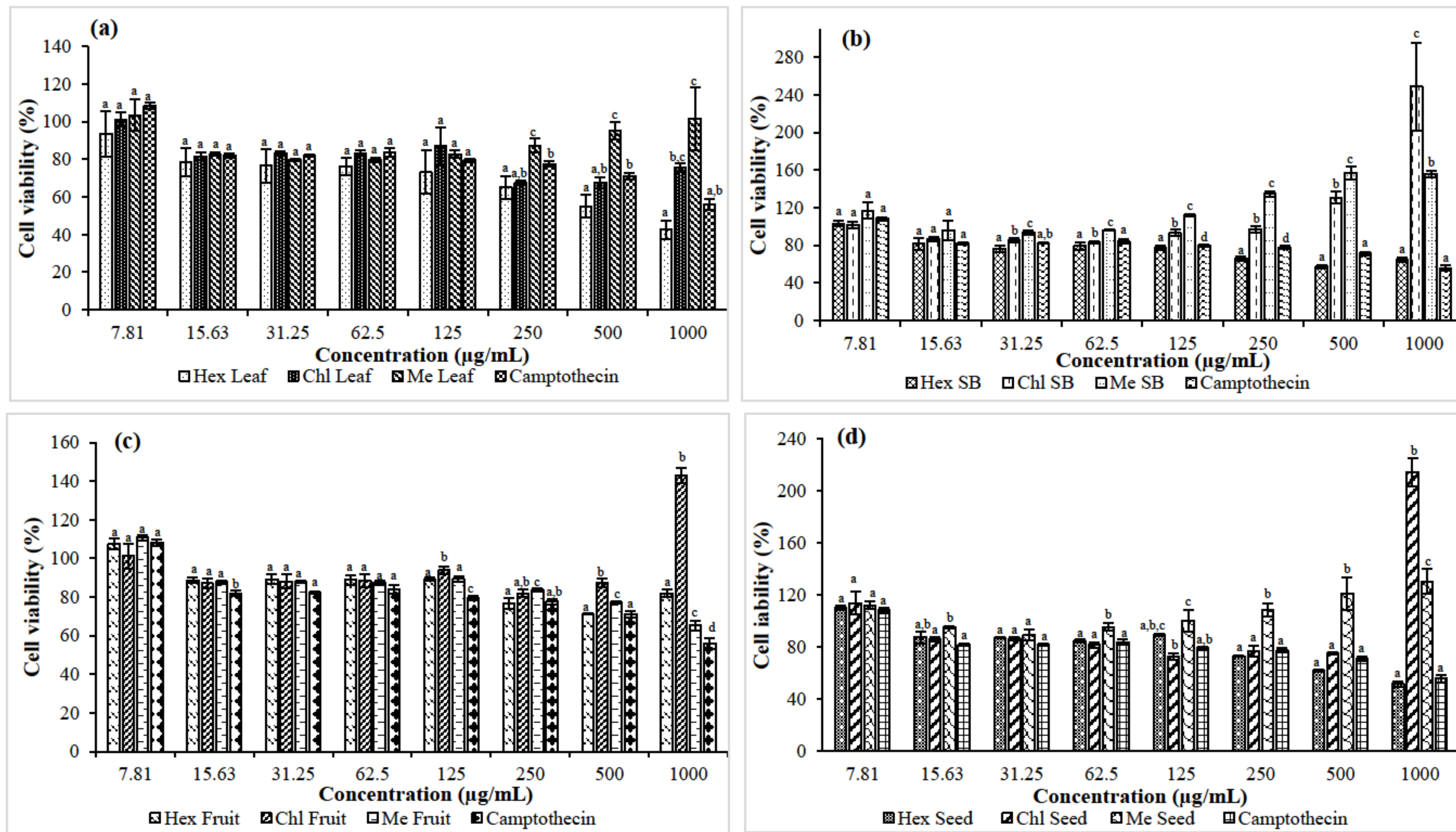

Figure 4.5 Cytotoxicity of different solvent extracts of *Embelia ruminata* on the cancer cell lines HEK293. Labels (a) = Leaf, (b) = Stem bark, (c) = Fruit and (d) = Seed. Bars denote standard deviation. Different alphabetic superscript letters ($^{a\text{-}d}$) indicate significance at $p < 0.05$ with n = 3 replicates. Hex = Hexane, Chl = Chloroform, Me = Methanol and SB = Stem bark

The most notable results were that of the methanolic stem bark and seed extracts in comparison to the positive control camptothecin. Methanolic stem bark and seed extracts displayed potent IC_{50} values of 0.74 and 3.96 µg/mL on MCF-7 and 0.25 and 5.58 µg/mL on A549 cell lines, respectively (Table 4.4). This data concurred with the radical scavenging results, which showed that the methanolic stem bark and seed extracts had strong scavenging activity compared to the control BHT. In addition, the compound, tert-butylhydroquinone identified from the GC-MS assay in the methanolic stem bark, has been reported to have apoptotic effects (Zeng et al., 2017). Likewise, the compound 2,2'-methylenebis(4-methyl-6-tert-butylphenol) identified in the methanolic seed extracts has been shown to have antitumor effects by activating autophagy and apoptosis (Jang et al., 2017). Thus, it is likely that these compounds may have contributed to the anticancer activities of the methanolic stem bark and seed extracts.

The cytotoxicity activity of the methanolic stem bark and seed extracts was followed by the methanolic leaf extracts with IC_{50} of 8.47 µg/mL for both cancer cell lines and chloroform seed extracts with IC_{50} of 18.42 and 12.2 µg/mL on cell lines MCF-7 and A549, respectively. The methanolic fruit extracts showed the least cytotoxic activity with IC_{50} of 123.9 and 127.3 µg/mL, respectively. Despite displaying minimal activity, the methanolic fruit extracts on A549 cell lines, showed higher cytotoxic activity than the control camptothecin. In other studies, Kaur et al. (2015) reported an IC_{50} of 80 µg/mL for embelin isolated from the fruit of *E. ribes*, while Antoney et al. (2016) recorded an IC_{50} of 49.98 µg/mL for the methanolic leaf extracts of *E. ribes* on MCF-7 cancer cells. Comparatively, these reported IC_{50} values are much higher than the IC_{50} values obtained in the current study, which may be due to different species of *Embelia*, climatic conditions and experimental protocols.

The selectivity index (SI) was calculated by evaluating the cytotoxic effects of *E. ruminata* extracts on normal cell lines. The SI value is defined as the ratio of the IC_{50} of normal cells to the IC_{50} of cancer cell lines. High selectivity for cancer cells is considered if the SI ratio exceeds 2 (Wang et al., 2019). The selectivity index of *E. ruminata* crude extracts were in the low range i.e., below 2, suggesting that the extracts were non-selective (Table 4.4.). Furthermore, this may indicate that although the extracts may have a pronounced effect on the cancer cells, the extracts negatively impacted the normal cells (Table 4.4).

Table 4.4 The IC_{50} and selectivity index (SI) values of *Embelia ruminata* extracts on cell lines

Extracts	Solvent	MCF-7 IC_{50} (µg/mL)	Selectivity index	A549 IC_{50} (µg/mL)	Selectivity index	HEK293 IC_{50} (µg/mL)
Leaf	Hexane	$106.9^{a, b}$	1.24	88.5^{a}	0.98	86.38^{a}
	Chloroform	$85.56^{a, b}$	0.48	60.6^{a}	0.67	$40.90^{a, b, c}$
	Methanol	$8.47^{d, e}$	1.25	8.47^{c}	1.26	$10.63^{c, d, e}$
Stem Bark	Hexane	54.91^{a}	0.75	$95.42^{a,b}$	0.43	$41.39^{a, b}$
	Chloroform	$25.64^{c, d}$	0.01	$26.31^{a, b}$	0.01	$0.14^{g, h}$
	Methanol	0.74^{g}	0.31	0.25^{d}	0.92	0.23^{h}
Fruit	Hexane	$48.47^{c, d}$	0.95	$39.47^{a, b}$	1.17	$46.18^{b, c, d}$
	Chloroform	31.29^{d}	0.02	$67.62^{a, b}$	0.01	$0.64^{d, e, f}$
	Methanol	$123.9^{e, f}$	0.52	127.3	0.50	$64.03^{b, c, d}$
Seed	Hexane	$93.1^{b, c}$	1.03	$119.5^{a, b}$	0.80	$95.67^{a, b, c}$
	Chloroform	$18.42^{d, e}$	0.02	$12.2^{a, b}$	0.03	$0.36^{e, f}$
	Methanol	3.96^{f}	0.23	5.58^{c}	0.16	$0.91^{f, g}$
Control	Camptothecin	$95.26^{e, f}$	0.68	$288.8^{b, c}$	0.22	$64.95^{a, b, c}$

Different alphabetic superscript letters ($^{a-h}$) indicate significance at $p < 0.05$, down each column, results expressed as n=3 replicates.

Likewise, it was observed from the SI values that camptothecin displayed cytotoxic effects on the normal cells. This is a concern as camptothecin is a commercially available anticancer drug. The results from this study indicate that camptothecin may cause collateral damage to non-cancerous cells opposing its therapeutic use. This may further substantiate the need to source new curative anticancer remedies.

Previous studies have attested to the relevance of embelin from *E. ribes* as anticancer agents by the downregulation of X chromosome-linked inhibitor-of-apoptosis protein (XIAP) and nuclear factor-κB (NF-κB), which are involved in tumour cell survival, proliferation, invasion and angiogenesis (Dai et al., 2009; Jiang et al., 2013; Wang et al., 2014; Nigam et al., 2015; Ko et al., 2018; Qian et al., 2018). Collectively, due to the excellent anticancer activity displayed by the extracts of *E. ruminata*, further investigations may need to be undertaken to extrapolate the therapeutic properties of the plant species.

Additionally, the current study demonstrated that the seed extracts incarnated a more pronounced biological activity than the fruit extracts. Interestingly, it has been postulated that many fleshy fruits contain secondary metabolites, at levels equivalent to or surpassing those in vegetative plant parts (Whitehead et al., 2014). Many of these secondary metabolites serve as a deterrent or are toxic to potential fruit pests and herbivory. However, it has been claimed that with the ripening of the fruit, an alteration in the chemistry of the fruit occurs with a reduction in the overall concentration of metabolites (Tsahar et al., 2002; Whitehead and Bowers, 2013; Maynard et al., 2020). This potentially aims to minimise the adverse effects of the secondary metabolites on a likely seed dispersal agent. Based on this proposition, it is conceivable that secondary metabolites of the ripe fruit of *E. ruminata* receded with ripening, thus, producing fewer convincing results than that of the seed extracts (Table 4.2 on page 117).

4.5 Conclusion

The present study revealed that methanolic seed and stem bark extracts of *E. ruminata* produced convincing free radical scavenging effects on stable DPPH. Moreover, the results demonstrated that radical scavenging capacity might not be dependent on the presence of phenolic and flavonoid compounds but also on other plant metabolites. Concurrently, the data evidenced from the cytotoxicity studies showed that the methanolic seed and stem bark extracts had potent anticancer activity but these extracts were non-selective. The FRAP and TAC studies showed that the reducing power and total antioxidant capacity were dose-dependent. The GC-MS studies identified compounds that could be utilised for various pharmaceutical purposes. Further studies to isolate and characterise individual compounds are required to assign the resultant pharmacological activities to specific compounds. This research has created a scope for future investigations. Overall, the findings from the current study provide evidence that *E. ruminata* leaf, stem bark, fruit and seeds are possible sources of some medicinally important compounds.

Declaration of Competing Interest

The authors declare no conflict of interest.

4.6 Acknowledgements

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CHAPTER 5

Phytosynthesis of silver nanoparticles architecture using *Embelia ruminata*: Synthesis, characterisation, antibacterial and anti-quorum sensing properties

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5.1 Abstract

Innovations in green nanotechnology using plants to biosynthesise nanostructures are revolutionising the medicinal field. This study focused on the green synthesis of silver nanoparticles (AgNPs) using aqueous *Embelia ruminata* leaf, stem bark and fruit extracts with optimisation of the process at room temperature (Rt) and 80 °C. The bioreduction process was confirmed visually by colour changes that occurred in the solutions. The resultant solutions were then subjected to characterisation using standard methods, which confirmed that the synthesised AgNPs were spherical to near-spherical in shape, with a size range of 21.06-32.15 nm, polydispersed and crystalline. FTIR confirmed the presence of functional groups that acted as reducing and stabilising agents. The AgNPs solutions were tested for their antibacterial potential and ability to inhibit quorum sensing (QS) using *Chromobacterium subtsugae* CV017 and *Chromobacterium violaceum* ATCC 12472 biomonitors. The AgNPs displayed varying degrees of antibacterial activity against selected bacterial strains ranging from a weak to an intermediate response with the fruit Rt extracts eliciting the greater antibacterial activity than the other plant organs. Using the qualitative agar well diffusion assay, stem bark 80 °C and Rt AgNPs were most effective, displaying violacein inhibition with no bactericidal effects at 200 µg for CV017 and ATCC 12472 biomonitors, respectively. In the quantitative analyses, the fruit 80 °C AgNPs demonstrated the most significant violacein inhibition using the CV017 biomonitor, while the leaf and fruit Rt AgNP extracts demonstrated the most noteworthy violacein inhibition using the ATCC 12472 biomonitor. The investigations carried out in this study are the first report for this plant species, thus establishing the groundwork for future research.

Keywords: *Embelia ruminata*, silver nanoparticles, green synthesis, antimicrobial, quorum sensing

5.2 Introduction

The green synthesis of metal nanoparticles (NPs) is gaining increasing popularity due to their biocompatibility and eco-friendly properties (Jemilugba et al., 2019). At the forefront of this current trend is the utilisation of silver for nanosynthesis owing to its extraordinary properties that include chemical stability, conductivity, catalytic and biological activities such as antibacterial, antifungal, antiviral and anti-inflammatory (Rao et al., 2016). Furthermore, the biological synthesis of silver nanoparticles (AgNPs) using plant extracts has become ubiquitous (Leema et al., 2019; Castillo-Henríquez et al., 2020; Vanlalveni et al., 2021). They are considered a rich source of phytoconstituents such as flavonoids, alkaloids, terpenoids, phenolic compounds, saponins, amino acids, proteins, and vitamins. These biomolecules can act as reducing and stabilising agents for the synthesised silver nanoparticles (Ghotekar et al., 2020).

The resultant size, shape, structure and dispersity of the nanostructures play a crucial role in influencing the physicochemical and biological attributes of the NPs (Jarzębski et al., 2017; Khan et al., 2019; Belloni et al., 2020). Congruently, it has been documented that reaction parameters such as temperature and time could play a key role in controlling growth, shape and size of nanoparticles (Vijayaraghavana and Ashokkumar, 2017).

The antibacterial activity of AgNPs is well known and has been exploited against a broad range of human pathogens (Elbahnasawy et al., 2021). Research has shown that AgNPs are inclined to accumulate at the site of the bacterial membrane forming aggregates that lead to perforations in the membrane (Varier et al., 2019). Furthermore, the small size of AgNPs with a larger surface area enables a more pronounced effect, possibly not only interacting with the surface of the membrane but also penetrating into the bacteria causing cellular death (Vanlalveni et al., 2021).

As an adjunct to finding new and novel strategies to combat antibiotic resistance, the focus has shifted to anti-virulence strategies such as quorum sensing inhibition (QSI). Quorum sensing (QS) is cell-to-cell communication in microorganisms. It is mediated by small, diffusible signal molecules (autoinducers) generated by the bacteria to detect cell densities (Moradi et al., 2020). In Gram-negative bacteria, QS signals are expedited *via* the N-acylhomoserine lactones (AHL) (Mion et al., 2021). Of particular interest are

QS inhibitors that target and disrupt the QS process of bacteria without inhibiting bacterial growth, hence, reducing the development of resistance as minimal selective pressure is exerted on the pathogen (Al-Shabib et al., 2016; Borges and Simoes, 2019). Many natural products, including plant extracts, possess bacterial QS system modulating properties, thereby reducing the virulence and pathogenicity of bacterial strains (Khan et al., 2019; Mostafa et al., 2020; Tchatchedre et al., 2020).

Embelia ruminata (E.Mey. ex A.DC.) Mez, (Primulaceae) is a scandent shrub or liana found in bushes or forests. In South Africa, the plant extends along the coastal and slightly inland forests of the Eastern Cape northwards to the forests of Zululand in KwaZulu-Natal (Dyer, 1966). A benzoquinone derivative (3-undecyl 2,5-dihydroxy, 1,4- benzoquinone), also referred to as embelin has been reported to be the principal compound found in the genus *Embelia* Burm.f. (Vijayan and Raghu, 2021). Several investigations have reported on the pharmacological properties of embelin such as antimicrobial (Chitra et al., 2003; Sivasankar et al., 2017), anticancer (Ko et al., 2018), anti-Alzheimer agent (Nuthakki et al., 2019), anti-inflammatory (Bansal et al., 2020), antioxidant (Caruso et al., 2020), antidiabetic (Chen et al., 2020), neuroprotective (Rao et al., 2020) and antiviral (Elias et al., 2021).

In this study, the suitability of *E. ruminata* aqueous leaf, stem bark and fruit extracts as reducing and stabilizing agents for AgNP synthesis was explored by employing the green synthesis methodology. The synthesised AgNPs were characterised using UV-Visible spectroscopy, high-resolution transmission electron microscopy (HR-TEM), energy dispersive X-ray analysis (EDX), Fourier transformed infrared spectroscopy (FTIR) and zeta potential analyses. In addition, the study aimed to optimise the temperature and duration of the AgNP fabrication process. Studies advocating the bioactivity potential of the other species of *Embelia* have led to the analysis of *E. ruminata* AgNPs extracts as antibacterial agents (Lulekal et al., 2014; Barbade and Datar, 2015; Dhayalan et al., 2017; Hossan et al., 2018; Togue et al., 2020).

To analyse the anti-QS properties, the AgNPs phytosynthesised from *E. ruminata* were tested for their ability to inhibit the QS process of the Gram-negative bacterium using two biomonitor strains, *Chromobacterium subtsugae* CV017 and *Chromobacterium*

violaceum ATCC 12472. Both biomonitor strains are known for its ability to produce an easily detectable, quantifiable, water-insoluble purple pigment called violacein (Sachu et al., 2020). The production of violacein is controlled by AHL signal molecules and plays an important role in understanding quorum sensing with the loss of the purple pigmentation, indicating QSI (Aliyu et al., 2020). The specificity of AHLs is determined by the length of the acyl chain (Saipriya et al., 2020). The *C. subtsugae* CV017 biomonitor produces short chain (C4-C6) AHL (Chernin et al., 1998) while *C. violaceum* ATCC 12472 produces long chain (C10 +) AHL signals (Morohoshi et al., 2008). The broad-spectrum QS inhibitors that disrupt the QS pathways of both long and short chain AHL signals are highly desirable, potentially limiting the degree of pathogenicity and hindering the production of virulence factors over an extended range. Investigations into the prospect of AgNPs extracts of *E. ruminata* serving as broad-spectrum QS inhibitors were thus undertaken.

The current study focused on the characterisation and elucidation of the therapeutic functionality of the biosynthesised AgNPs from aqueous extracts of *E. ruminata* leaf, stem bark and fruit.

5.3 Material and Methods

5.3.1 Collection and preparation of plant material

The leaves, stem bark and fruit of *E. ruminata* were collected from Reservoir Hills, Durban, KwaZulu-Natal, South Africa (29°48'26.7 "S 30°5543.9"E), from February to May, 2019. The species identity was confirmed using taxonomic keys and a voucher specimen (Rambaran 1 with accession number 18256) was deposited in the Ward Herbarium, School of Life Sciences, University of KwaZulu-Natal, Durban, South Africa. Plant samples were separated, sorted, air-dried, pulverised to a fine powder and stored in airtight bottles. For extraction, 20 g of the powdered plant material was added to 200 mL of deionised water in an Erlenmeyer flask and incubated in a water bath at 60 °C for 30 min. The resultant solution was cooled to room temperature (23±2 °C), filtered using Whatmann No.1 filter paper and stored at 4 °C until further use.

5.3.2 Synthesis of silver nanoparticles (AgNPs)

A 5 mL aliquot of either of the leaf, stem bark or fruit extracts of *E. ruminata* were added to 45 mL of 1 mM of silver nitrate ($AgNO_3$) solution for the bioreduction process (Singh et al., 2018). The reaction mixture was monitored visually for a change in colour at regular

intervals. To investigate the effect of temperature on the synthesis of AgNPs, samples were incubated for 1 h at the respective temperatures, i.e., at room temperature (Rt) (23 ± 2 °C) and at 80 °C in a waterbath. Thereafter, all samples were kept in the dark at room temperature for 24 h. The biosynthesised AgNP reaction mixtures were centrifuged at 10,000 rpm for 20 min at 4 °C using the Beckman Coulter Anvati J-E centrifuge. Supernatants were discarded and pellets washed thrice with distilled water and centrifuged. Purified pellets were transferred to glass vials, dried at 50 °C and stored at room temperature in the dark until further use (Verma et al., 2017).

5.3.3 Characterisation of biosynthesised *E. ruminata* AgNPs

5.3.3.1 UV-Visible absorbance spectroscopy

The progress of the reaction between the metal ions and respective extracts was monitored by measuring the optical absorption at different time intervals (0 min, 60 min and 24 h). The Shimadzu UV-2600 spectrophotometer having a resolution of 1 nm with a wavelength range of 200-800 nm was used (Guilger-Casagrande et al., 2019).

5.3.3.2 High-resolution transmission electron microscopy (HR-TEM) analysis

The structural characterisation, as well as the selected area electron diffraction (SAED) patterns of AgNPs, were acquired using the JEOL 2100 HRTEM (Japan) with an accelerating voltage of 200 kV (Singh et al., 2018). A drop of sonicated AgNPs was placed onto carbon-coated copper grids lined with formvar and allowed to dry by evaporation for 20 min at Rt (23 ± 2 °C). The size of the nanoparticles was analysed using iTEM (Soft imaging system, Germany Version 5.0). The elemental composition of the samples were identified using energy dispersive X-ray (EDX) analysis (Inca software coupled with an Oxford X-Max 80 mm detector, England).

5.3.3.3 Zeta potential determination

Nanoparticle Tracking Analysis (NTA) was used to evaluate the zeta (ζ) potential of the *E. ruminata* leaf, stem bark and fruit AgNPs. A 1:1000 dilution of each sample in double distilled water was analysed using the Nanosight NS500 (Malvern Instruments, Worchestershire, UK) equipped with a sCMOS camera with a laser wavelength of 430 nm. The NTA 3.2 analytical software was used to derive results.

5.3.3.4 Fourier transformed infrared analysis (FTIR)

FTIR analysis was conducted using crude *E. ruminata* extracts and their respective AgNPs to identify the possible functional groups responsible for reducing, capping and stabilising the phytosynthesised AgNPs. The Perkin-Elmer Spectrum 100 FTIR (USA) spectroscope with a scan range from 400 to 4000 cm^{-1} and a resolution of 4 cm^{-1} was used (Ahmad and Kalra, 2020).

5.3.4 Antibacterial susceptibility tests

Antibacterial susceptibility assays were carried out using the Kirby Bauer disc diffusion assay. Four Gram-negative microorganisms, viz., susceptible *Escherichia coli* ATCC 25922, TEM-β-lactamase-producing *E. coli* ATCC 35218, extended spectrum β-lactamase-producing *Klebsiella pneumoniae* ATCC 700603 and *Pseudomonas aeruginosa* ATCC 27853, as well as seven Gram-positive microorganisms, i.e., susceptible *Enterococcus faecalis* ATCC 29212, vancomycin-resistant *E. faecalis* ATCC 51299, susceptible *Staphylococcus aureus* ATCC 29213, methicillin-resistant *S. aureus* ATCC 33591, *S. aureus* ATCC 43300, *S. aureus* ATCC 700698, and *Staphylococcus epidermidis* ATCC 12228, were grown at 37 °C overnight on Mueller-Hinton (MH) agar plates. Inocula equivalent to 0.5 McFarland were used to swab surfaces of MH agar plates (Chenia, 2013). Thereafter, blank discs (5 mm diameter) were impregnated with 100 and 200 µg of the respective AgNPs and placed onto the surface of the swabbed MH agar plates. Agar plates were then incubated for 24 h at 37°C. Ciprofloxacin (CIP5) and Gentamicin (GN10) were used as the antibiotic controls and blank discs impregnated with 10% dimethyl sulfoxide (DMSO) were used as the solvent control. Following incubation, samples exhibiting zone diameters >15 mm were regarded as strong antibacterials, zone diameters between 11-15 mm were regarded as possessing intermediate activity and zone diameters <10 mm were considered weak antibacterial agents.

5.3.5 Qualitative quorum sensing inhibition

The quorum sensing inhibition potential of *E. ruminata* AgNPs was investigated using the agar-overlay diffusion assay. Short chain AHL producer, *C. subtsugae* CV017 and long chain AHL producer, *C. violaceum* ATCC 12472, were used as the indicator organisms (Chenia, 2013). Five millilitres molten Luria-Bertani (LB) agar was inoculated with 150 µL of the respective *Chromobacterium* strains grown overnight at 30 °C in LB

broth. The agar-culture was then poured over the surface of pre-warmed LB agar plates and allowed to solidify. Thereafter, blank discs (5 mm diameter) were impregnated with 100 and 200 µg of the respective AgNPs, placed onto the surface of the agar plates and incubated overnight at 30 °C. Discs impregnated with 100 and 200 µg of vanillin were used as the positive control. Blank discs dosed with 10% DMSO were used as negative controls. Following incubation, opaque (QS inhibition) and clear (bactericidal) zone diameters were recorded. AgNPs samples exhibiting opaque zone (QS inhibition) diameters >15 mm were regarded as having strong inhibitory potential, zone diameters between 11-15 mm were regarded as possessing intermediate potential and zone diameters <10 mm were considered as weak QS inhibitors.

5.3.6 Quantitative quorum sensing inhibition

The quorum sensing inhibition potential of the biosynthesised AgNPs was quantified using *C. subtsugae* CV017 and *C. violaceum* ATCC 12472 as the indicator microorganisms (Chenia, 2013). One hundred microliters of the respective *Chromobacterium* strains were cultured in 3 mL LB broth and incubated at 30 °C with increasing concentrations of each AgNPs extract ranging from 0; 20; 40; 80; 160 and 320 µg/mL. For this assay, growth (optical density $(OD)_{600}$ nm) and violacein production (OD_{560} nm) were determined following overnight incubation. One mL of the respective overnight treated and untreated cultures of *Chromobacterium* were centrifuged (Labnet Prism Microcentrifuge) at 10 000 rpm for 10 min to precipitate insoluble violacein. The culture supernatant was discarded and pellet resuspended in 1 mL of DMSO (Truchado et al., 2012; Chenia, 2013). The solution was then centrifuged again at 10 000 rpm for 10 min to separate the cells and precipitated violacein was quantified at OD_{560} nm using the Glomax Multi+ Detection System (microtitre plate reader) (Promega). The percentage of violacein inhibition was calculated using the following formula (equation 5.1):

% Violacein inhibition = (control OD_{560} nm – test OD_{560} nm / control OD_{560} nm)*100 **(5.1)** (Packiavathy et al., 2011; Chenia, 2013).

5.3.7 Statistical analysis

Statistical analyses were conducted using SPSS versions 26 and 27. Data are presented as mean values of a triplicate series of replicates (*n*=3). The significance of the effect of temperature and time on the synthesis process as well as the difference in the mean values

of violacein inhibition between extracts and between concentrations was determined by one-way analysis of variance (ANOVA) followed by Tukey honestly significant difference (HSD) post hoc test using a 95% confidence interval with $p \leq 0.05$ being considered significant (Moradi et al., 2020).

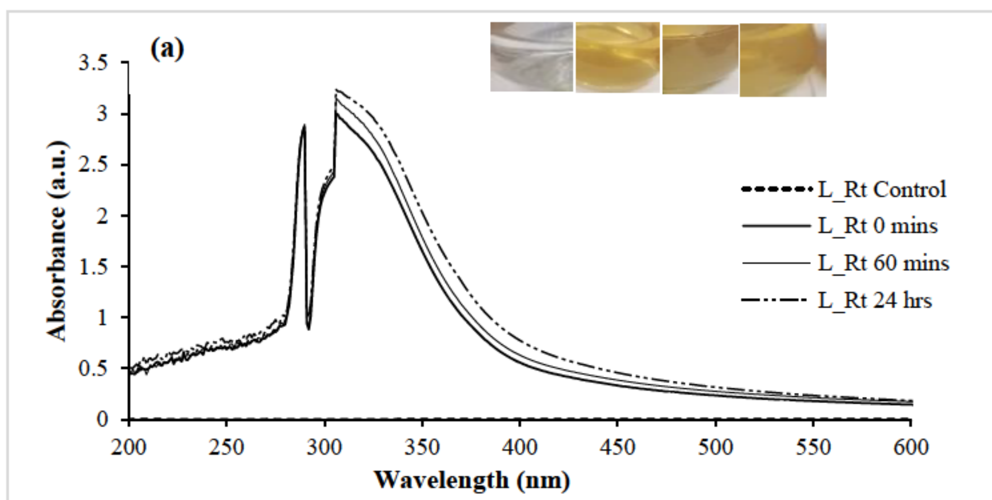
5.4 Results and Discussion

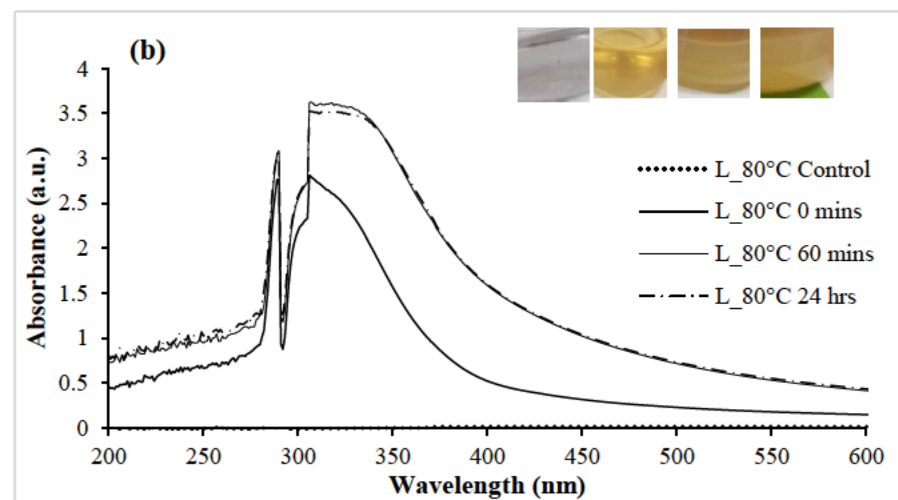
It has been documented that the reduction of silver ions to AgNPs results in a characteristic colour change in the aqueous medium due to the excitation of the surface plasmon resonance (SPR) on the synthesised particles (Pirtarighat et al., 2018). In this study, the addition of silver nitrate ($AgNO_3$) to the plant extracts produced a wide range of colour changes in the respective extracts [Figure 5.1(a) to (f) insets]. The leaf Rt extracts changed from a bright golden colour to a cloudy yellow solution. Correspondingly, the leaf 80 °C extracts displayed a similar colour change with more intensity after the 1 h incubation period. The colour changed from a bright golden colour to a cloudy brownish hue for the stem bark extracts, with the change being more prominent in the stem bark 80 °C extracts. For the fruit extracts, the colour changed from a faint pink to a dull ash colour. Overall, the extracts produced different tones of colours, signifying the reduction of Ag^+ to Ag.

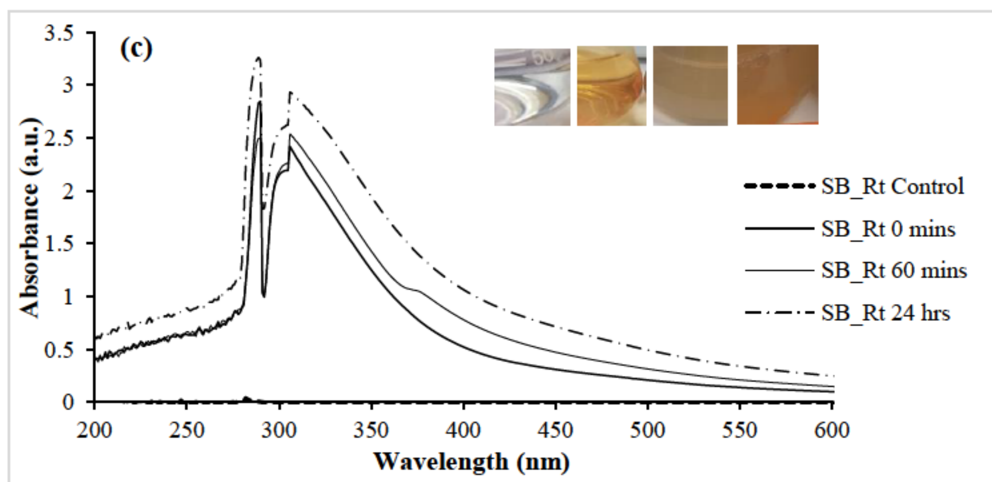
Furthermore, due to the SPR vibrations, the conversion of silver to nanostructures was monitored by UV spectroscopy, which displayed absorbance peaks between 290 and 320 nm for the AgNPs formulated using the aqueous extracted leaf, stem bark and fruit of *E. ruminata*. This further confirmed the biofabrication of AgNPs in the reaction mixtures [Figure 5.1(a) to (f)]. It is also recognised that the SPR band shift is dependent on the particle size, chemical surroundings and dielectric constant (Khan et al., 2019). The control silver nitrate solution, which contained $AgNO_3$ and distilled water showed an absence of an absorbance peak and no colour changes, indicating that there was no reduction of silver nitrate in the control. This supported the possibility that the phytocompounds present in *E. ruminata* were involved in the bioreduction of silver ions to AgNPs.

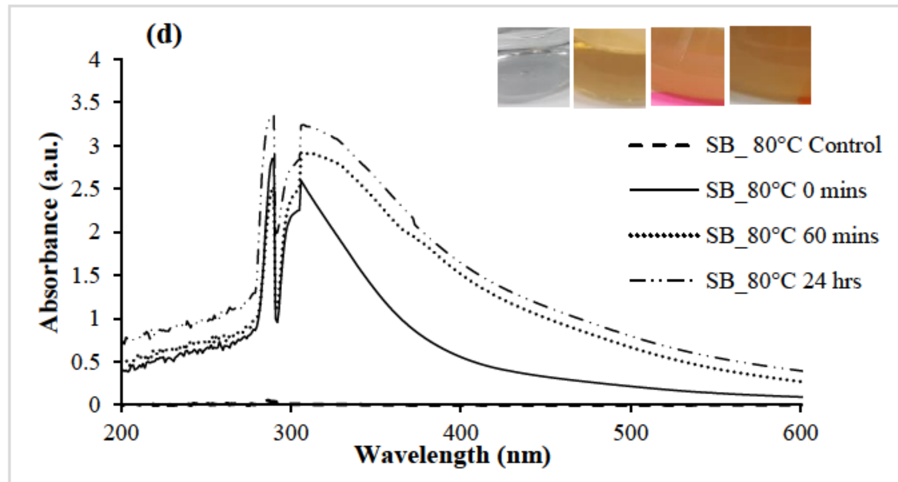
Notably, the intensity of the SPR peaks mostly increased in the different extracts from the initial time to 60 min to 24 h signifying the presence of AgNPs [Figure 5.1(a)-(f)] (Rao

and Tang, 2017; Velgosová and Mražíková, 2017). Moreover, the intensity of the colour changes in the reaction mixture was directly proportional to the incubation time (Osibe et al., 2018). This may imply that the synthesis of AgNPs increased with time as observed by the increase in absorbance of the SPR band (λ_{max}= 290 to 320 nm) (Elemike et al., 2017). Furthermore, Varghese et al. (2020) suggested from their study that an SPR peak at 290 nm may indicate proteins capped around the AgNPs that conversely act as stabilising agents for AgNPs.


(a)
Absorbance (a.u.)
Wavelength (nm)
L_Rt Control
L_Rt 0 mins
L_Rt 60 mins
L_Rt 24 hrs


(b)
Absorbance (a.u.)
Wavelength (nm)
L_80°C Control
L_80°C 0 mins
L_80°C 60 mins
L_80°C 24 hrs


(c)
Absorbance (a.u.)
Wavelength (nm)
SB_Rt Control
SB_Rt 0 mins
SB_Rt 60 mins
SB_Rt 24 hrs


(d)
Absorbance (a.u.)
Wavelength (nm)
SB_ 80°C Control
SB_80°C 0 mins
SB_80°C 60 mins
SB_80°C 24 hrs

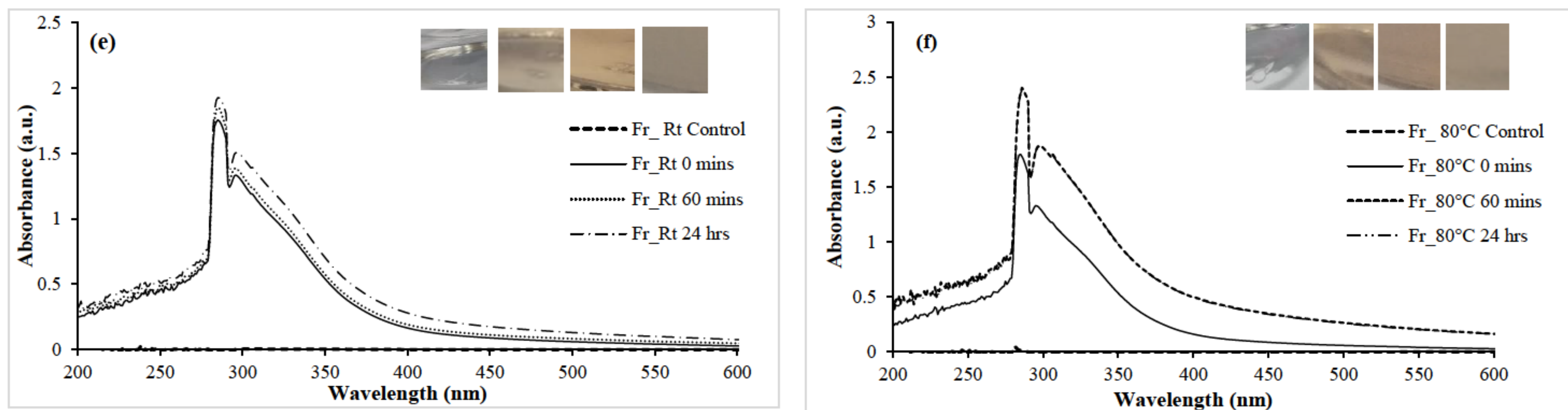


Figure 5.1(a)-(f). UV-Vis absorption of aqueous *Embelia ruminata* AgNPs biosynthesised at room temperature and 80 °C at different time intervals 0 min, 60 min and 24 h. Inset (from left to right) shows the different colour changes observed in the biosynthesised AgNPs at different time intervals (Control, 0 min, 60 min and 24 h). Labels: (a) L_Rt = Leaf Room temperature, (b) L_80 °C = Leaf 80 °C, (c) SB_Rt = Stem bark Room temperature, (d) SB_80 °C = Stem bark 80°C, (e) F_Rt = Fruit Room temperature, (f) F_80 °C = Fruit 80 °C.

Regarding the parameter of temperature, previous studies have indicated that at higher temperatures, the kinetic energy of the molecules increases and the elemental Ag^+ ions are consumed faster, leading to the formation of smaller nanoparticles (Ibrahim, 2015; Verma et al., 2017). In the present study, no significant difference was observed in the nanoparticle size for samples incubated at room temperature and 80 °C. This may indicate that *E. ruminata* extracts were able to achieve the resultant size of the synthesised nanocomposites at room temperature without the effect of increased temperatures.

The HR-TEM results confirmed the presence of AgNPs with variable shapes with no significant difference, most of them being spherical to almost spherical [Figure 5.2(a) to (f)], with a mean size range of 21.06-32.15 μm [Figure 5.3(a) to (f)] and polydispersed. The EDX analysis showed a peak at 3 keV, which verified the presence of elemental silver ions (Hemlata et al., 2020) [Figure 5.4 (a) to (c)]. The SAED results revealed that the silver ions reduced to AgNPs by *E. ruminata* were polycrystalline [Figure 5.4 (a) to (c) inset]. Manikandan et al. (2019) reported an average size of 30.2±2 nm and the polycrystalline nature of AgNPs synthesised using the fruit of *E. ribes,* while Singh et al. (2018) reported a size range of 15.22 to 29.48 nm for AgNPs synthesised using *Plumbago auriculata* Lam. leaf and calyx extracts at Rt and 60 °C, respectively.

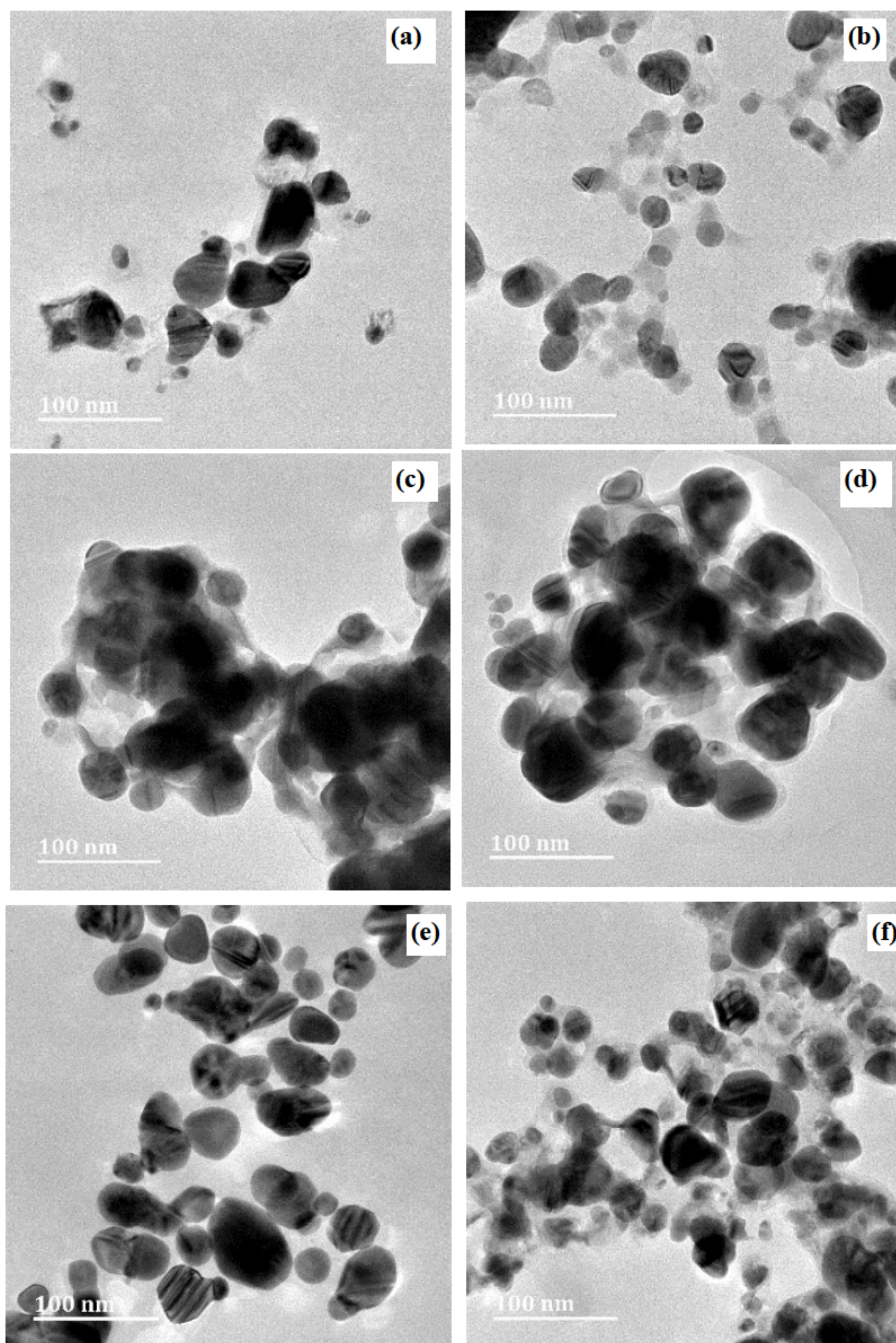
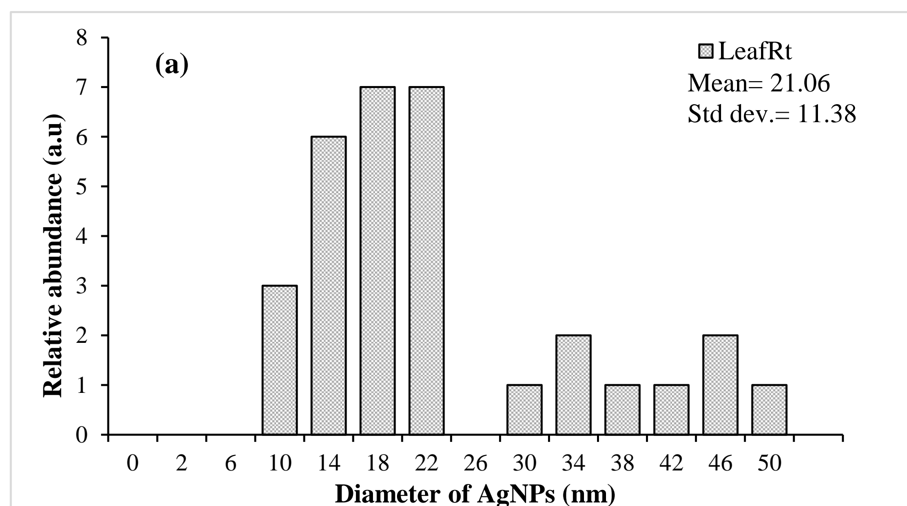
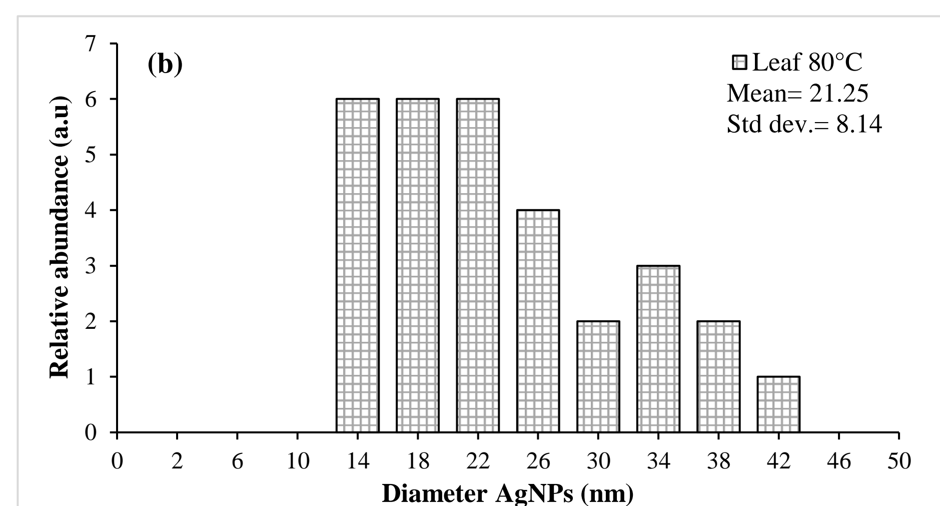


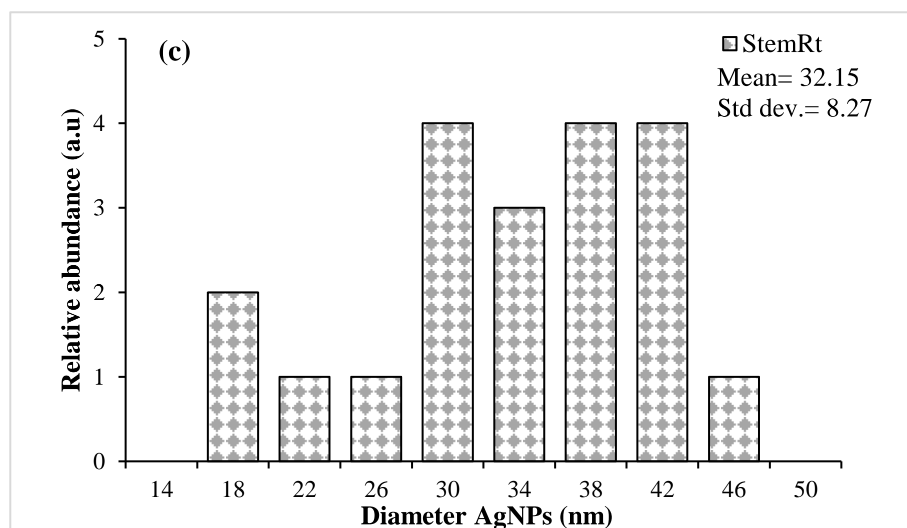
Figure 5.2 (a)-(f) HR-TEM micrographs of AgNPs synthesised from *E. ruminata* extracts. Labels: (a) Leaf extract at room temperature, (b) Leaf extract at 80 °C, (c) Stem bark extract at room temperature, (d) Stem bark extract at 80 °C, (e) Fruit extract at room temperature and (f) Fruit extract at 80 °C.



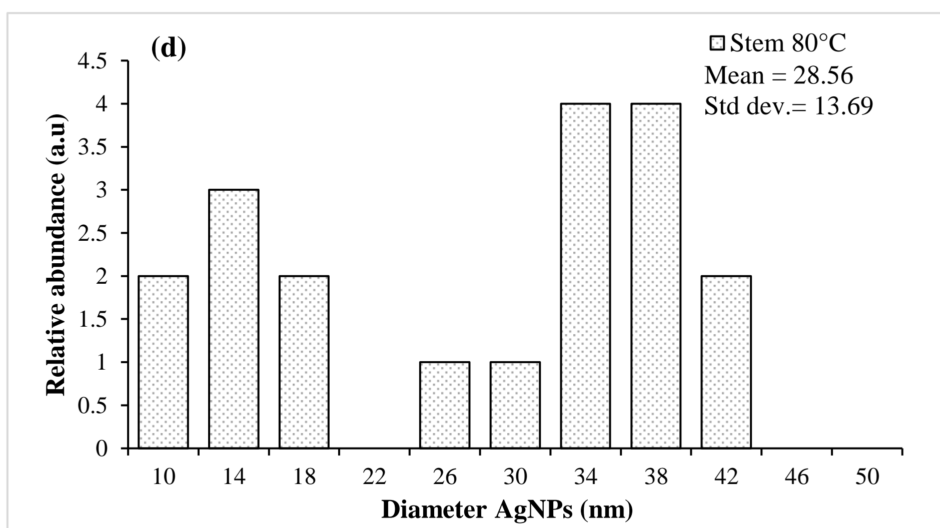
(a)
LeafRt
Mean= 21.06
Std dev.= 11.38
Relative abundance (a.u)
0
1
2
3
4
5
6
7
8
0 2 6 10 14 18 22 26 30 34 38 42 46 50
Diameter of AgNPs (nm)



(b)
Leaf 80°C
Mean= 21.25
Std dev.= 8.14
Relative abundance (a.u)
0
1
2
3
4
5
6
7
0 2 6 10 14 18 22 26 30 34 38 42 46 50
Diameter AgNPs (nm)



(c)
StemRt
Mean= 32.15
Std dev.= 8.27
Relative abundance (a.u)
0
1
2
3
4
5
14 18 22 26 30 34 38 42 46 50
Diameter AgNPs (nm)



(d)
Stem 80°C
Mean = 28.56
Std dev.= 13.69
Relative abundance (a.u)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
10 14 18 22 26 30 34 38 42 46 50
Diameter AgNPs (nm)

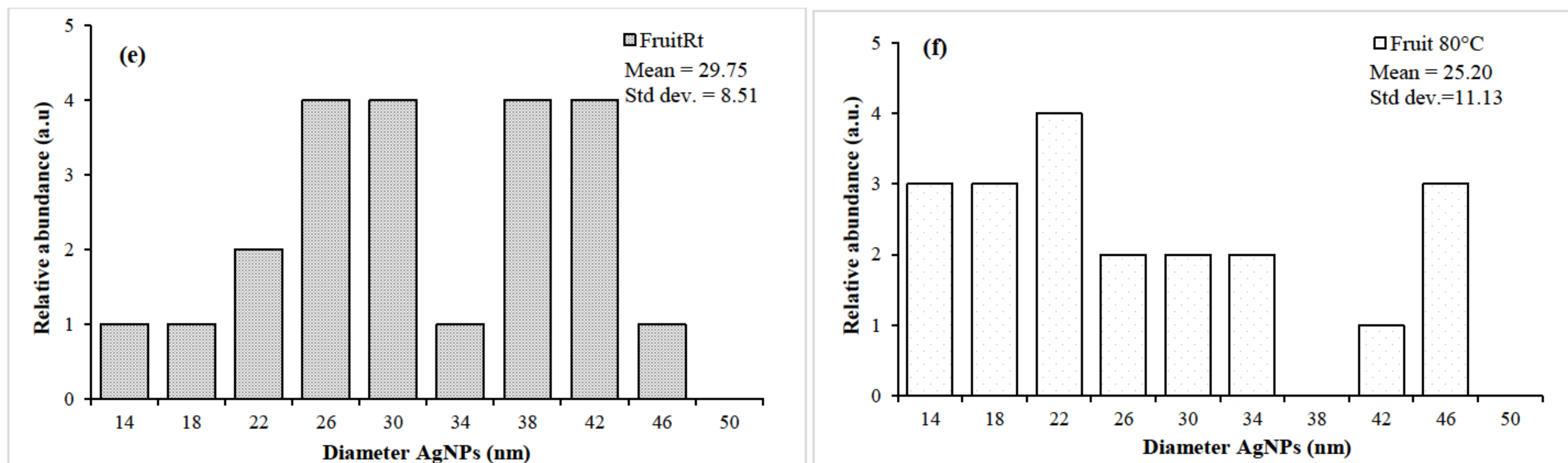


Figure 5.3(a)-(f) Nanoparticle size distribution histogram of AgNPs biosynthesised from *E. ruminata* leaf, stem bark and fruit extracts at room temperature (Rt) and 80 °C.

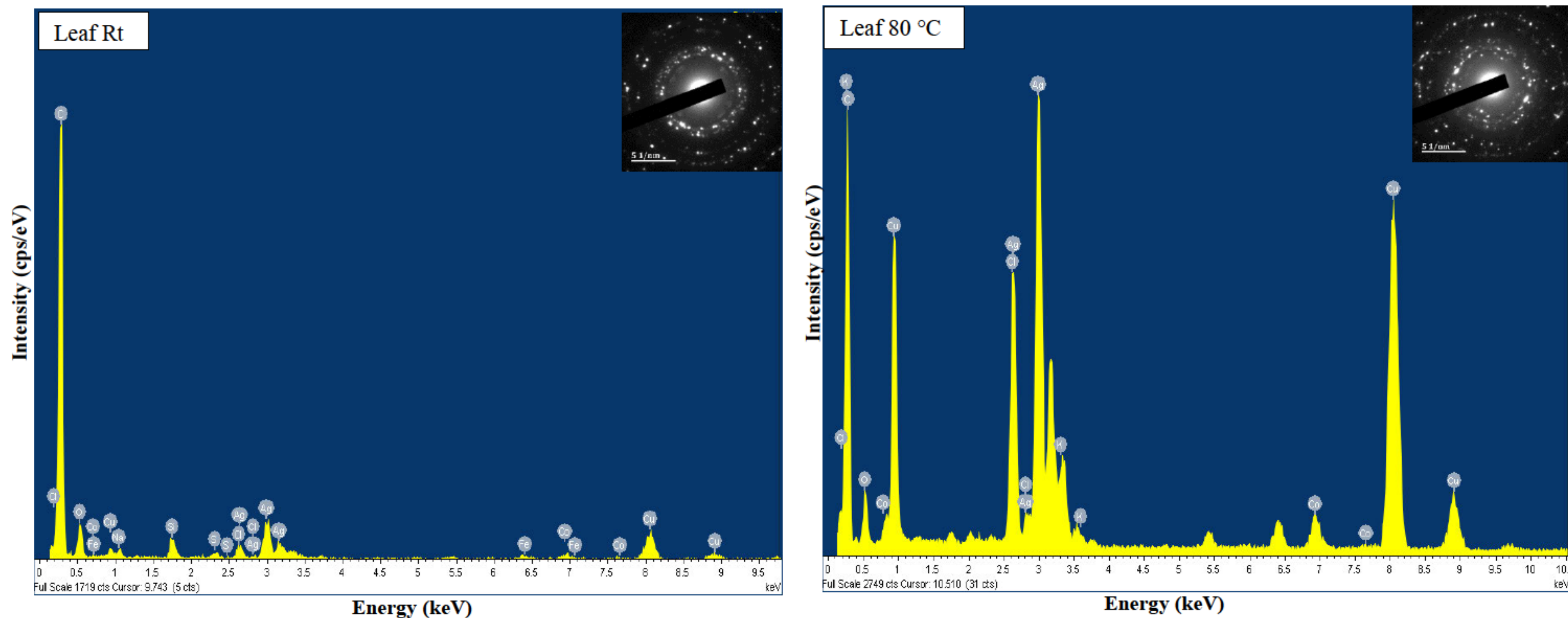


Figure 5.4(a) Energy dispersive X-ray (EDX) analysis after the formation of silver nanoparticles (AgNPs) synthesised at room temperature (Rt) and 80 °C using *E. ruminata* leaf extracts. Inset showing the selected area electron diffraction (SAED) patterns of AgNPs.

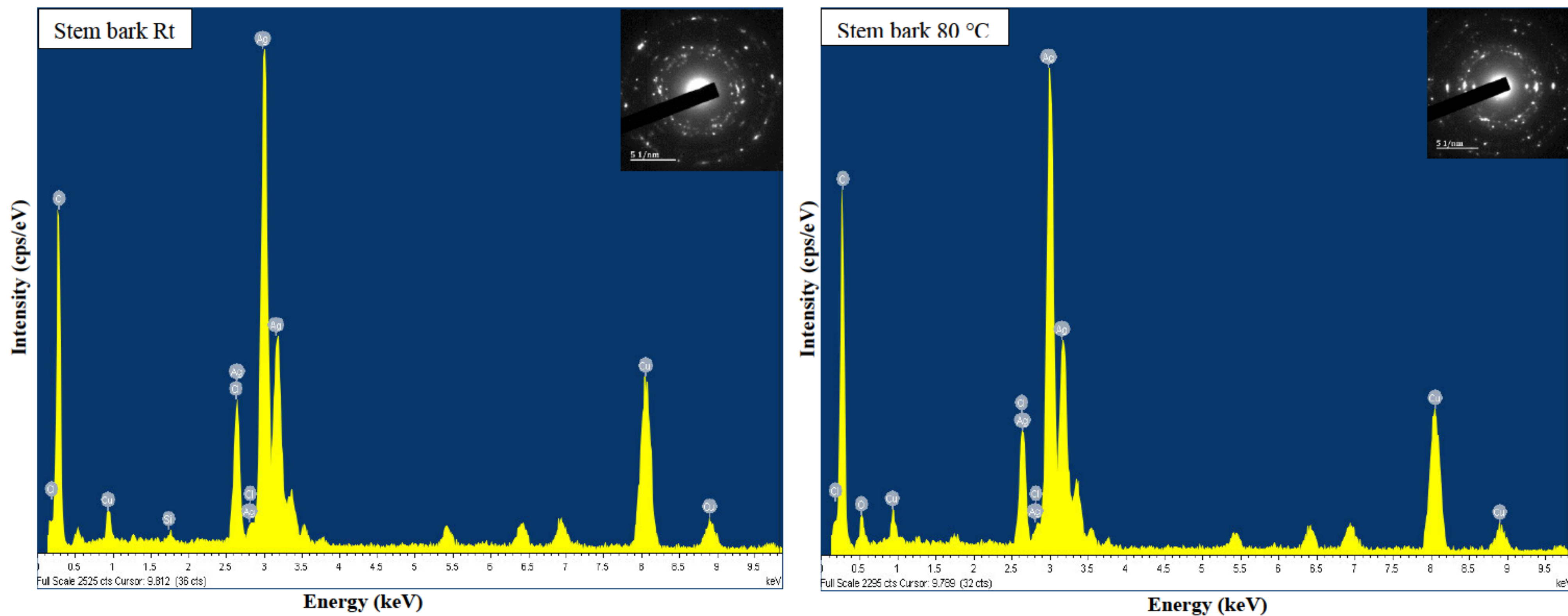


Figure 5.4(b) Energy dispersive X-ray (EDX) analysis after the formation of silver nanoparticles (AgNPs) synthesised at room temperature (Rt) and 80 °C using *E. ruminata* stem bark extracts. Inset showing the selected area electron diffraction (SAED) patterns of AgNPs.

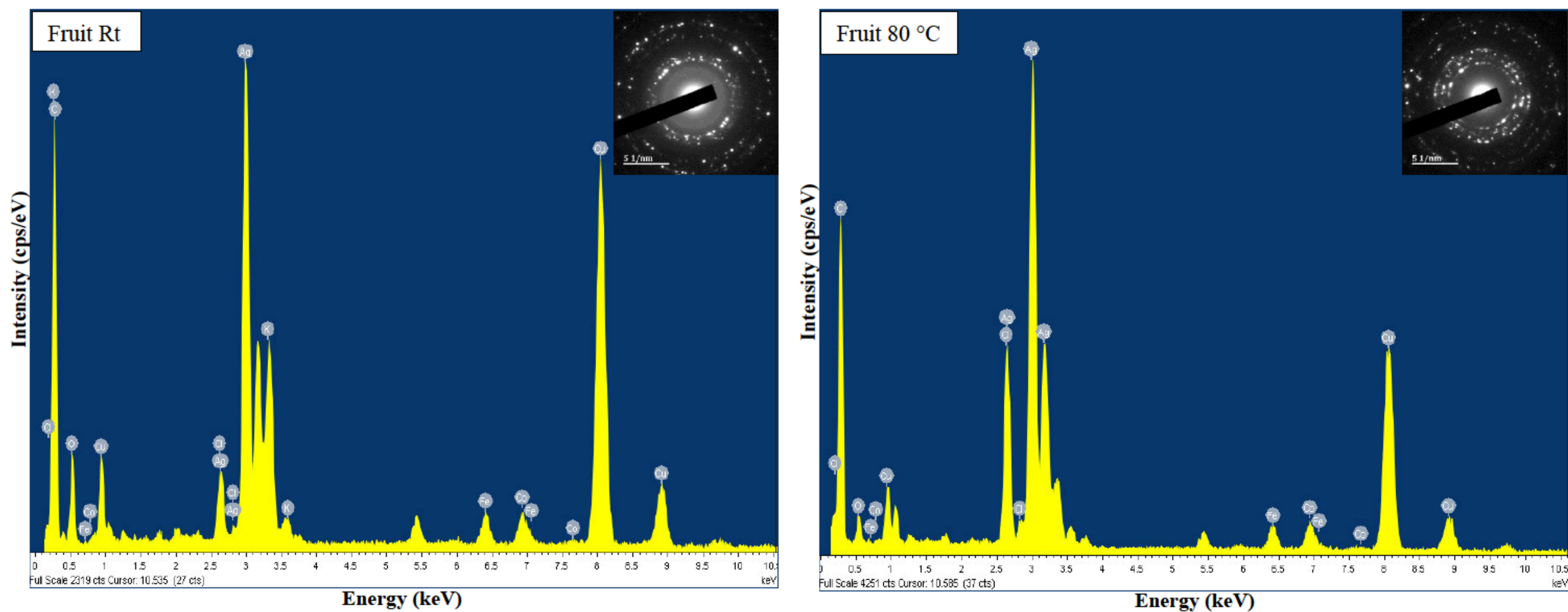


Figure 5.4(c) Energy dispersive X-ray (EDX) analysis after the formation of silver nanoparticles (AgNPs) synthesised at room temperature (Rt) and 80 °C using *E. ruminata* fruit extracts. Inset showing the selected area electron diffraction (SAED) patterns of AgNPs.

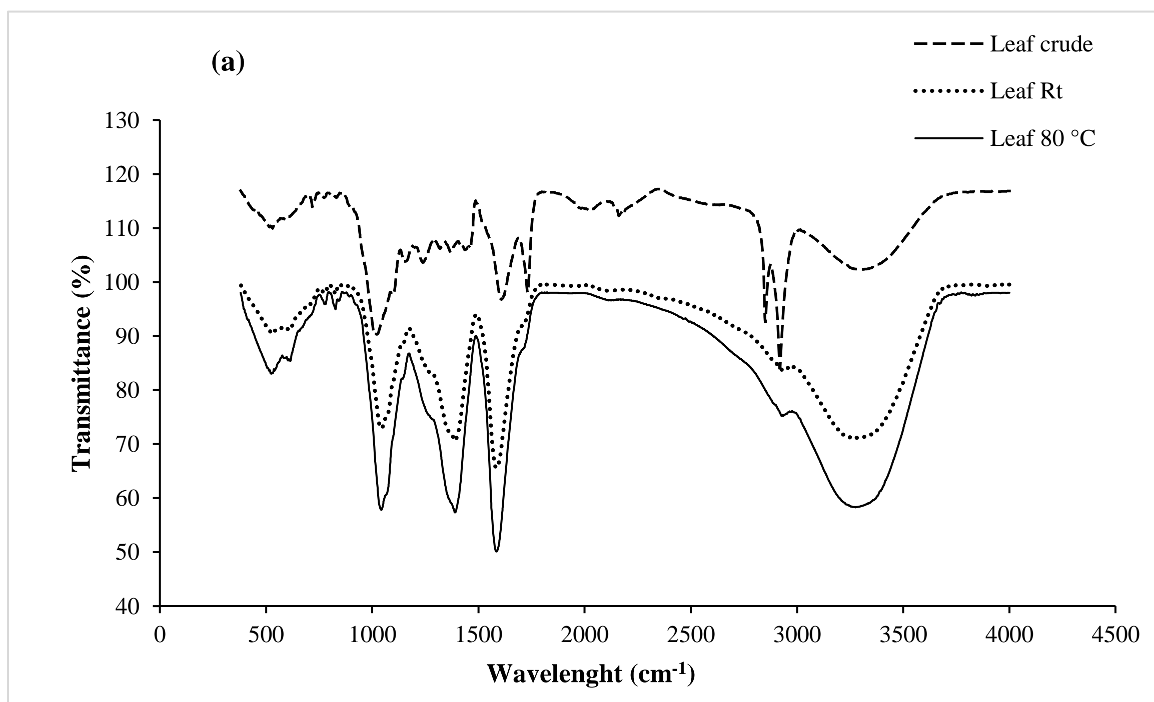
The ζ-potential showed a much larger size for AgNPs than the HR-TEM analysis (Table 5.1). This is probably because of agglomeration of smaller particles. The ζ-potential is indicative of the degree of repulsion between the charged particles in a nanodispersion (Samimi et al., 2019). Nanoparticles with a zeta potential higher than +30 mV or lower than -30 mV are regarded to be stable in the dispersion medium (Raval et al., 2019). A ζ-potential in this range implies that there is sufficient repulsive force between the charged particles to attain better colloidal stability (Joseph and Singhvi, 2019). Conversely, a low zeta potential value suggests particle aggregation and flocculation due to the van der Waals attractive forces that act upon the nanoparticles (Zhu et al., 2017). In the present study, all samples yielded a ζ-potential that ranged from -12.8-7.3 mV, indicating that AgNPs may incline towards a lower stability and may have a greater tendency to aggregate. However, Joseph and Singhvi (2019) stated that along with zeta potential, other variables such as material properties, presence of surfactants and the solution chemistry also influence the physical stability of the obtained nanosuspension. This infers that zeta potential is not an absolute measurement of nanoparticle stability (Samimi et al., 2019). Othman and Sekar (2019) reported a ζ-potential of -34.9 mV for AgNPs synthesised using embelin isolated from the fruit of *E. ribes*. Their results differ from the findings of the current study and may be due to the use of the isolated embelin compound for the AgNPs formulation in their study compared to crude extracts that were utilised in the current study.

Table 5.1 Comparison of the particle size of *Embelia ruminata* AgNPs at room temperature (Rt) and 80 °C using HR-TEM, nanoparticle tracking analysis and zeta potential

Samples	HR-TEM	NTA	
	Nanoparticle size (nm) ± SD	Nanoparticle size (nm) ± SD	ζ-Potential (mV) ± SD
Leaf Rt	21.06 ± 11.38	46.9 ± 1.4	-0.2 ± 0.2
80 °C	21.25 ± 8.14	102.0 ± 4.7	1.8 ± 0.7
Stem Rt	32.15 ± 8.27	58.3 ± 16.1	-12.8 ± 1.4
80 °C	28.56 ± 13.69	65.9 ± 9.4	7.3 ± 0.2
Fruit Rt	29.75 ± 8.51	160 ± 19.5	-8.4 ± 1.0
80 °C	25.20 ± 11.13	198 ± 12.9	-0.2 ± 1.7

HR-TEM= High-resolution transmission electron microscopy analysis, NTA = Nanoparticle Tracking Analysis, ζ = Zeta and SD = Standard deviation

The FTIR absorption spectra of the water-soluble extracts before and after the reduction of Ag^{+} ions have been presented in Figure 5.5 (a)-(c). Pronounced absorption peaks in the range of 3600-3000 cm^{-1} were observed for all biosynthesised AgNPs. These bands are characteristic of stretching vibrations of the O-H functional group in carboxylic acid and N-H stretching vibrations of amines in amino acids, peptides and proteins (Stuart, 2004; Cheng et al., 2014; Hamouda et al., 2019). Previous studies have indicated that hydroxyl groups serve as reducing agents and carboxyl groups support the size and shape of nanoparticles (Osibe et al., 2018; Singh et al., 2018). New spectral peaks were observed at 1586.42, 1585.8, 1582.5 and 1582.73 cm^{-1} in leaf Rt and 80 °C AgNP spectra, as well as in fruit Rt and 80 °C AgNP spectra. This could be attributed to the amide II, N-H bending vibration coupled with C-N stretching at 1044.58, 1042.89, 1046.76 and 1045.23 cm^{-1}, respectively (Talari et al., 2016; Merck, 2020). These absorption bands indicate that proteins are interacting with the AgNPs (Singh et al., 2018). It has been documented that the amide linkage of a protein can form a covering around AgNPs, preventing agglomeration, thus, stabilising the medium (Hamouda et al., 2019). Peak shifts at the wavelength of 1610.71 and 1606 cm^{-1} in the stem bark Rt and 80°C AgNPs were also noted. This could be attributed to the quinone compound, which has been shown to be responsible for the reduction of $AgNO_3$ and particle size (Nouri et al., 2020).



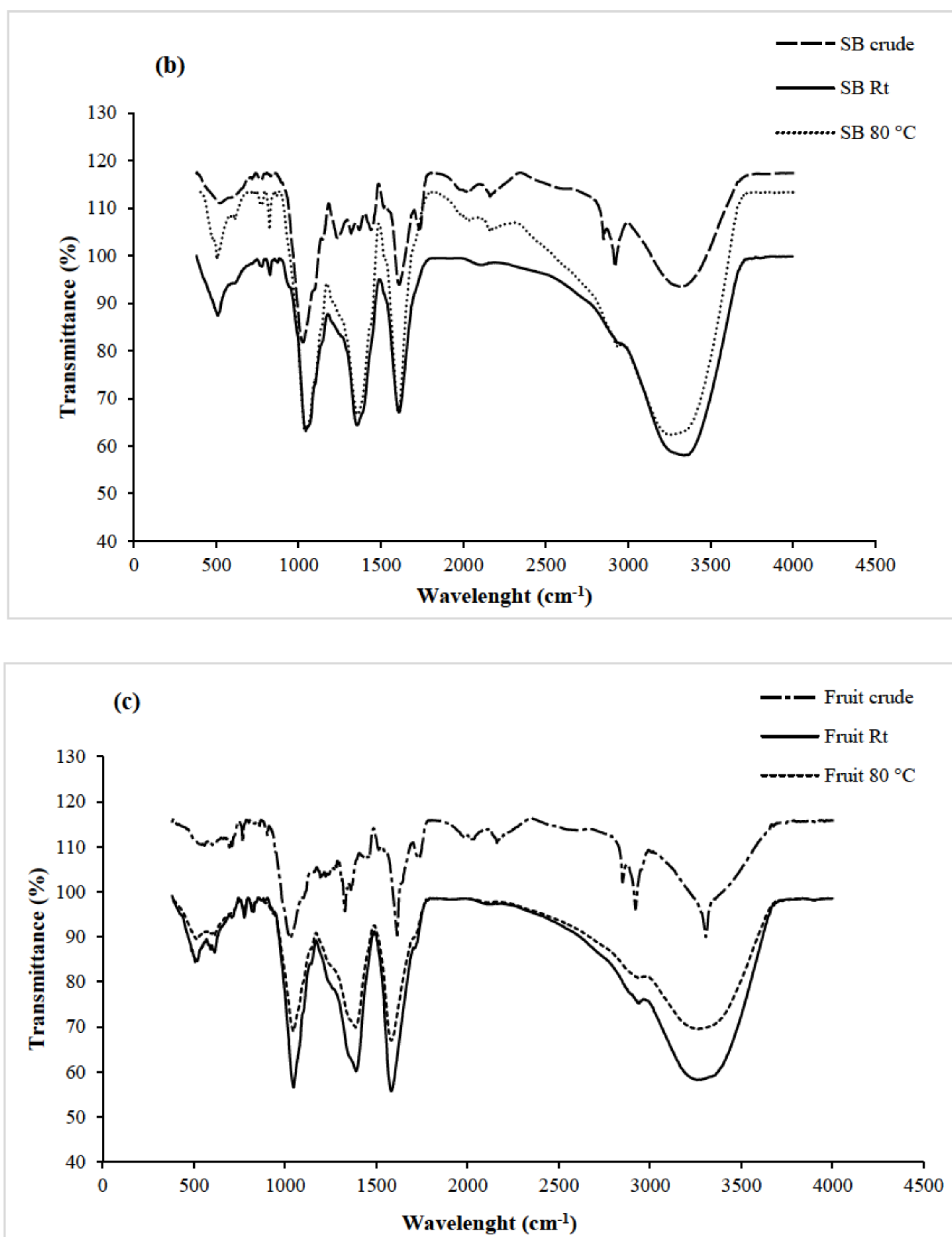


Figure 5.5(a)-(c) FTIR spectra of crude extracts and respective biosynthesised AgNPs of *Embelia ruminata*. Labels (a) aqueous crude leaf extract, AgNPs biosynthesised with leaf extract at room temperature and 80 °C; (b) aqueous crude stem bark (SB) extract, AgNPs biosynthesised with stem bark extract at room temperature and 80 °C, (c) aqueous crude fruit extract, AgNPs biosynthesised with fruit extract at room temperature and 80 °C.

The results for the antibacterial tests for the six AgNPs generated from *E. ruminata* extracts have been shown in Table 5.2. The fruit AgNPs appeared to be most promising with the fruit Rt AgNPs showing intermediate antibacterial activity against sensitive *E. coli* ATCC 25922 at 200 µg, multi-drug resistant *P. aeruginosa* ATCC 27853 at 200 µg and *S. epidermidis* ATCC 12228 at 100 and 200 µg (Table 5.2). Similarly, fruit 80 °C AgNPs demonstrated intermediate activity against methicillin-resistant *S. aureus* ATCC 33591 and ATCC 700698 at 200 µg as well as *S. epidermidis* ATCC 12228 at 100 and 200 µg.

Interestingly, apart from stem bark RT AgNPs, all other AgNPs samples showed antibacterial activity against *S. epidermidis* at 200 µg (Table 5.2). Conventionally regarded as a harmless skin commensal, studies have shown that *S. epidermidis* has emerged as a nosocomial pathogen infecting immunocompromised patients carrying medical devices (Lee et al., 2018). In a study by Dong et al. (2019), both biofilm-positive and -negative *S. epidermidis* induced potent pro-inflammation. Thus, the AgNPs extracts of *E. ruminata* that displayed antibacterial activity could be a potential candidate for preventing the pathogenesis of *S. epidermidis* infections. This can be further supported by a study by Swamy et al. (2007), where authors demonstrated that the crude extracts and embelin isolated from the ethanolic leaf extracts of *Embelia ribes* Burm.f., showed significant wound healing activity compared to the standard skin ointment, framycetin. Additionally, the results from the current study supports the ethnopharmacological use of the leaves as a paste applied topically to cure skin infections (Sharma et al., 2002).

An overall evaluation of the antibacterial results showed that AgNPs were more effective against Gram-positive than the Gram-negative bacteria. A plausible reason could be due to the outer membrane of Gram-negative bacteria, which poses a significant barrier to many compounds, including antibiotics. In comparison, the cell wall of Gram-positive bacteria is made up of several layers of peptidoglycan, enabling different compounds to mostly penetrate (Stefanović, 2018). This possibly indicates that the AgNPs derived from phytomolecules of *E. ruminata* could bypass the Gram-positive bacterial cell wall, resulting in a more pronounced antibacterial effect.

Table 5.2 Antibacterial profile of *Embelia ruminata* AgNPs against selected Gram-negative and Gram-positive bacteria showing zones of inhibition (mm)

	Gram-negative bacteria								Gram-positive bacteria													
Sample	*E. coli* ATCC 25922		*E. coli* ATCC 35218		*K. pneumoniae* ATCC 700603		*P. aeruginosa* ATCC 27853		*E. faecalis* ATCC 29212		*E. faecalis* ATCC 51299		*S. aureus* ATCC 29213		*S. aureus* ATCC 33591		*S. aureus* ATCC 43300		*S. aureus* ATCC 700698		*S. epidermidis* ATCC 12228	
	Concentration (μg)																					
NPs	100	200	100	200	100	200	100	200	100	200	100	200	100	200	100	200	100	200	100	200	100	200
Leaf Rt	0	**9**	0	**8**	0	**7**	0	**8**	**7**	**9**	**7**	**10**	**7**	**9**	**7**	**8**	**7**	**9**	**7**	**7**	**10**	**12**
Leaf 80 °C	0	0	0	**8**	**7**	**7**	0	**8**	**7**	**9**	**7**	**10**	**7**	**7**	**8**	**8**	**7**	**7**	**8**	**10**	0	**9**
Stembark Rt	0	0	0	0	0	0	0	0	0	0	0	**8**	0	0	0	0	**8**	**8**	0	0	0	0
Stembark 80 °C	0	**8**	0	0	0	0	0	0	0	**8**	0	**8**	0	0	0	0	0	0	0	0	**7**	**9**
Fruit Rt	**9**	**12**	**8**	**10**	0	**8**	**9**	**11**	**7**	**8**	**7**	**8**	**8**	**9**	**8**	**9**	**8**	**9**	**7**	**9**	**11**	**13**
Fruit 80 °C	0	0	0	**10**	0	**8**	**8**	**10**	**8**	**9**	**7**	**9**	**8**	**9**	**10**	**12**	**8**	**8**	**9**	**12**	**13**	**13**
Controls																						
Ciprofloxacin (CIP5)	30		37		26		32		33		38		23		22		23		6		28	
Gentamicin (GN10)	19		20		17		19		18		0		19		16		9		11		20	

Antibacterial activity classified as follows: ≤10 mm = weak, 11-15 mm = intermediate and >15 mm = strong

A qualitative assessment of QSI by *E. ruminata* AgNPs was undertaken with 100 and 200 µg of the respective AgNPs extracts. The transparent inner zones indicated inhibition of growth (bactericidal activity), while the opaque outer zones denoted QSI [Table 5.3 and the corresponding Figures have been included in the Appendix section A5.1(a) to (f), (a_1) to (f_1) on page 198]. Using the *C. subtsugae* CV017 biomonitor, 50% of the AgNPs, i.e., stem bark Rt, fruit Rt and 80 °C demonstrated QSI at 100 to 200 µg with bactericidal activity. At 200 µg, the leaf Rt AgNPs displayed QSI with killing, while the stem bark 80 °C AgNPs demonstrated only QSI activity. Using the *C. violaceum* ATCC 12472 biomonitor, all AgNPs displayed QSI activity at 200 µg (Table 5.3). The stem bark Rt AgNPs showed only violacein inhibition, while with the other AgNPs violacein inhibition was accompanied by growth inhibition. The objective of QSI is to disrupt the process and is not aimed at killing the bacteria. Thus, the most effective AgNPs were the stem bark 80°C and Rt AgNPs, which displayed QSI using the biomonitors CV017 and ATCC 12472 at 200 µg, respectively, with no bactericidal effect. Additionally, the AgNPs extracts appeared to be more effective against the *C. violaceum* ATCC 12472 than the C. *subtsugae* CV017 biomonitor. This possibly indicates that the phyto-AgNPs of *E. ruminata* were able to inhibit the mode of action of the long chain AHL molecules in a more influential manner than the short chain AHL molecules.

Ilk et al. (2020) reported QS zones of violacein inhibition of 16.22 to 21.48 mm for propolis bioformulated AgNPs using the ATCC 12472. These reported zones of violacein inhibition are higher than the diameters found in the present study. The variability in results may be due to the use of different species and methodologies.

Table 5.3 Qualitative inhibitory effects of the aqueous AgNPs extracts of *Embelia ruminata* leaf, stem bark and fruit on quorum sensing (QS) violacein production using *Chromobacterium subtsugae* and *Chromobacterium violaceum* biomonitors

	***C. subtsugae* CV017**						***C. violaceum* ATCC 12472**					
	100 µg			**200 µg**			**100 µg**			**200 µg**		
Extracts	**Total zone diameter (mm)**	**Clear zone diameter (mm)**	**QSI halo (mm)**	**Total zone diameter (mm)**	**Clear zone diameter (mm)**	**QSI halo (mm)**	**Total zone diameter (mm)**	**Clear zone diameter (mm)**	**QSI halo (mm)**	**Total zone diameter (mm)**	**Clear zone diameter (mm)**	**QSI halo (mm)**
Leaf RT	11	11	0	12	10	**2**	16	10	**6**	18	10	**8**
Leaf 80 °C	13	13	0	9	9	0	17	10	**7**	18	12	**6**
Stembark RT	14	9	**5**	16	9	**7**	13	8	**5**	12	0	**12**
Stembark 80 °C	0	0	0	**8**	0	**8**	13	8	**5**	13	9	**4**
Fruit RT	13	10	**3**	12	10	**2**	13	10	**3**	13	9	**4**
Fruit 80 °C	13	11	**2**	13	11	**2**	12	9	**3**	13	11	**2**
Controls												
Vanillin	**11**	0	**11**	**11**	0	**11**	**9**	0	**9**	**9**	0	**9**

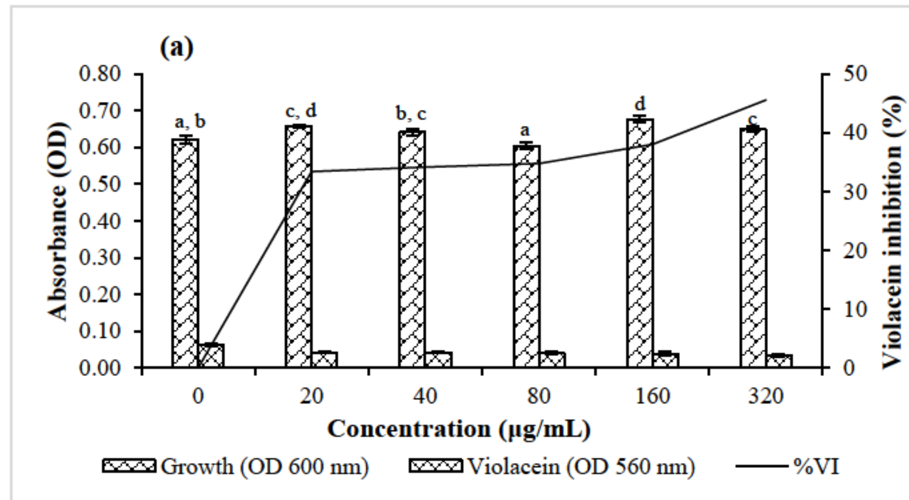
(light grey) = bactericidal activity
(grey) = QSI +bactericidal activity
(dark) = QSI activity
QSI considered weak ≤10 mm, intermediate 11-15 mm and strong >15 mm
RT= room temperature (23±2 °C)

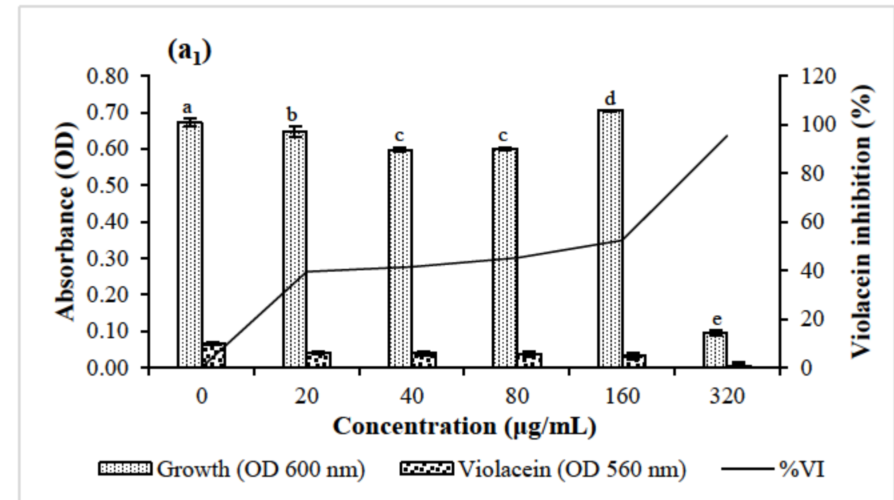
The quantitative violacein inhibition assay was based on the agar-overlay diffusion assay data and all six AgNPs extracts were tested. Quorum sensing inhibition was considered noteworthy when the % violacein inhibition was ≥50% and the % growth inhibition (GI) was <40% [Figure 5.6(a) to (f), (a_1) to (f_1)]. Using the CV017 biomonitor, fruit Rt AgNPs were bactericidal at 320 µg/mL with GI of 85.59% [Figure 5.6(e)], while the fruit 80 °C AgNPs demonstrated violacein inhibition of 50.47% at 160 µg/mL but were bactericidal at 320 µg/mL with the GI of 70.27% [Figure 5.6(f)]. This biomonitor produces short chain AHL signals, so inhibition indicates interference with short chain AHL-based QS.

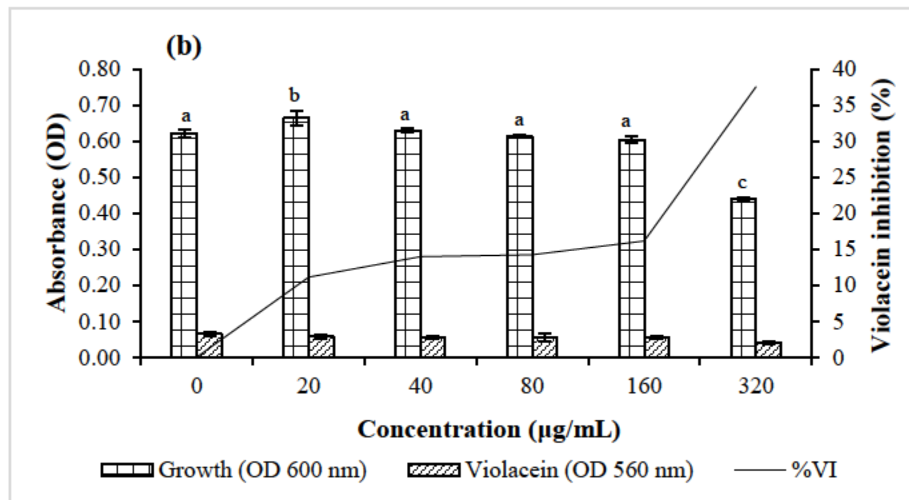
Using the ATCC 12472 biomonitor, leaf and fruit Rt AgNPs demonstrated QS inhibition ≥50% at 160 µg/mL with GI of -4.98 and 16.20% [Figure 5.6(a_1) and (e_1)], respectively. However, bactericidal activity was observed at 320 µg/mL with GI of 86.10 and 87.41%, respectively. This biomonitor produces long chain AHL signals, so inhibition indicates interference with long chain AHL-based QS.

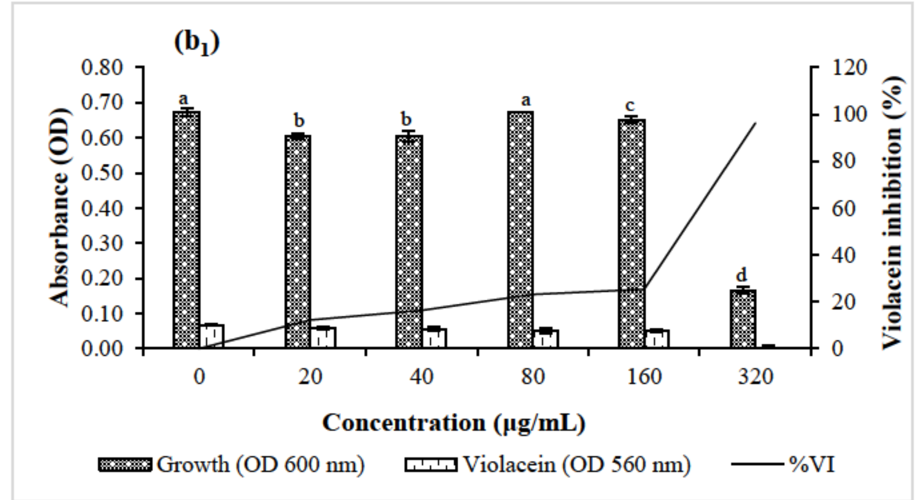
Shah et al. (2019) reported that AgNPs biosynthesised using *Piper betle* Linn. was able to reduce violacein production in *C. violaceum* ATCC 12472 with 75.25, 88.69 and 97.48% inhibition at concentrations of 10, 15 and 20 µg/mL, with no bactericidal effects. Their study showed more effective violacein inhibition at lower concentrations than the present study. The difference in findings between the studies may be due to different plant species and experimental protocols that were employed.

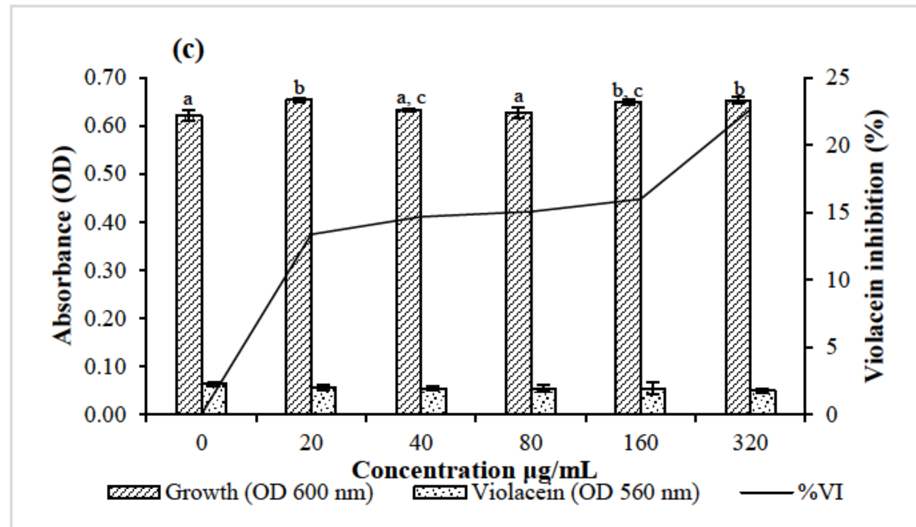
Similar to the qualitative QS tests, the AgNPs of *E. ruminata* had a more pronounced effect against long than short chain autoinducers. Studies have shown that NPs can form conjugates with phytocompounds that competitively bind to receptor sites, which can suppress the QS circuit (Rajkumari et al., 2017; Lu et al., 2019). Likewise, it is possible that the phytocompounds of *E. ruminata* AgNPs were able to bind to receptor sites, displacing AHL in the long chain biomonitor in a more effective manner than short chain biomonitor strain.

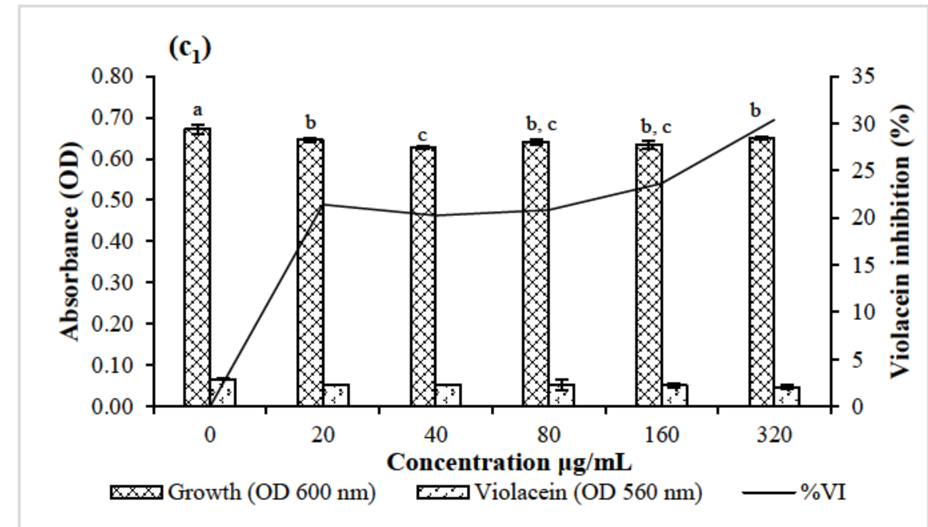

(a)
Absorbance (OD)
Violacein inhibition (%)
Concentration (µg/mL)
Growth (OD 600 nm)
Violacein (OD 560 nm)
%VI

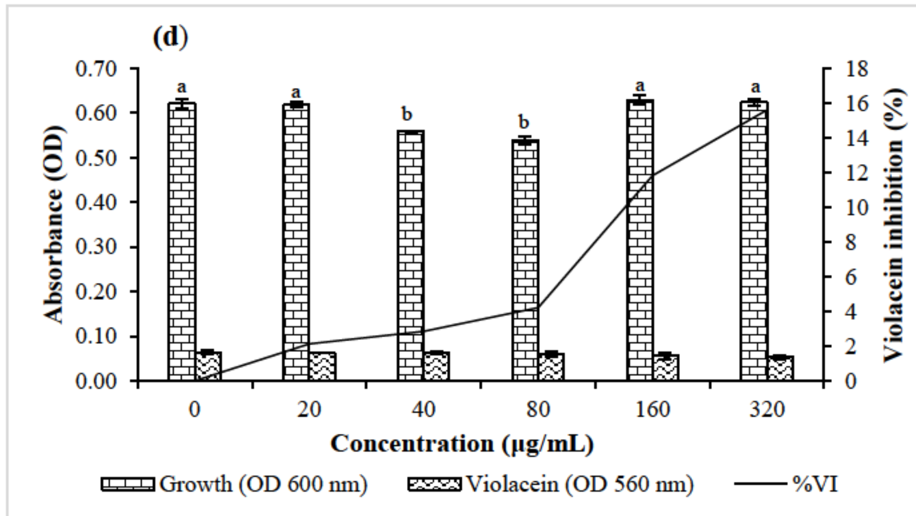

(a1)
Absorbance (OD)
Violacein inhibition (%)
Concentration (µg/mL)
Growth (OD 600 nm)
Violacein (OD 560 nm)
%VI

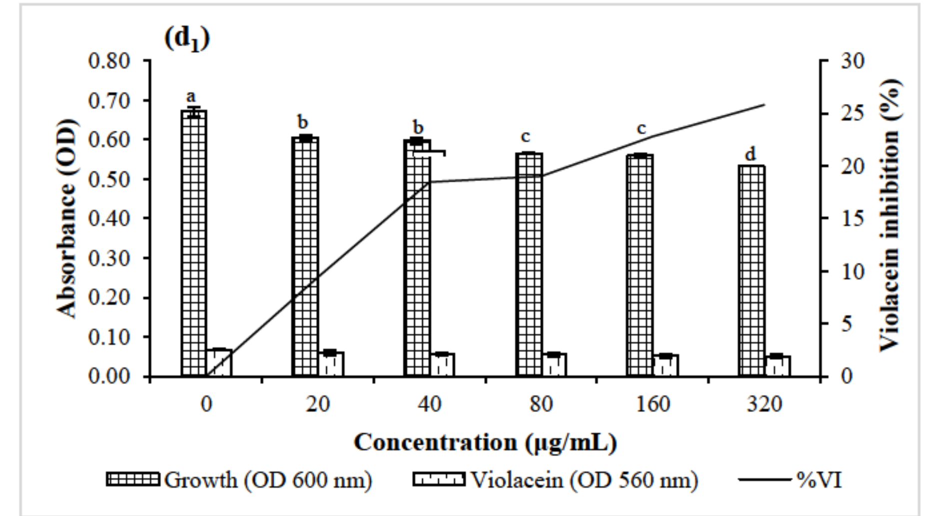

(b)
Absorbance (OD)
Violacein inhibition (%)
Concentration (µg/mL)
Growth (OD 600 nm)
Violacein (OD 560 nm)
%VI


(b1)
Absorbance (OD)
Violacein inhibition (%)
Concentration (µg/mL)
Growth (OD 600 nm)
Violacein (OD 560 nm)
%VI


(c)
Absorbance (OD)
Violacein inhibition (%)
Concentration µg/mL
Growth (OD 600 nm)
Violacein (OD 560 nm)
%VI


(c1)
Absorbance (OD)
Violacein inhibition (%)
Concentration µg/mL
Growth (OD 600 nm)
Violacein (OD 560 nm)
%VI


(d)
Absorbance (OD)
Violacein inhibition (%)
Concentration (µg/mL)
Growth (OD 600 nm)
Violacein (OD 560 nm)
%VI


(d1)
Absorbance (OD)
Violacein inhibition (%)
Concentration (µg/mL)
Growth (OD 600 nm)
Violacein (OD 560 nm)
%VI

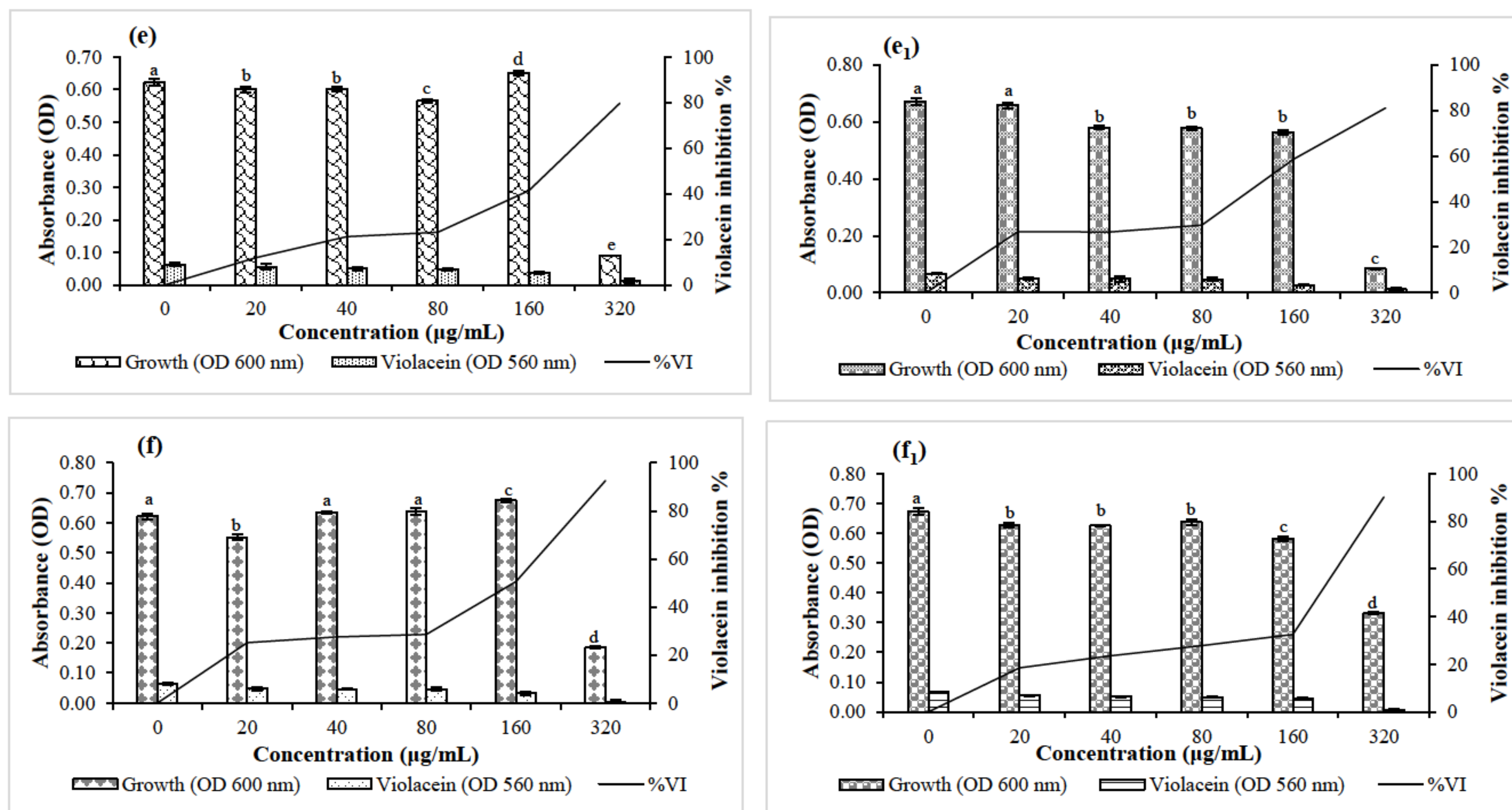


Figure 5.6 Quantitative analysis of the concentration-dependent inhibitory effects of AgNPs, using aqueous *Embelia ruminata* extracts, on growth and violacein production by *Chromobacterium* spp. CV017 and ATCC 12472. Labels (a) and (a_1) = Leaf Rt, (b) and (b_1) = Leaf 80 °C, (c) and (c_1) = Stem bark Rt, (d) and (d_1) = Stem bark 80 °C, (e) and (e_1) = Fruit Rt, (f) and (f_1) = Fruit 80 °C using biomonitors *C. subtsugae* CV017 and *C. violaceum* ATCC 12472, respectively. The average of three triplicate independent experiments and standard deviations are shown. Significance at $p \leq 0.05$ indicated by different alphabetic letters.

Overall, the AgNPs of fruit extracts showed notable results in the antibacterial assays and in both the qualitative and quantitative violacein inhibition assays. Statistical analysis showed that the mean values among extracts were greater than expected by chance and demonstrated a significant difference at $p \leq 0.05$. Future studies elucidating the mode of action of these extracts could effectuate novel anti-pathogenic agents that circumvent virulence of antibiotic resistant strains.

In a study by Dwivedi and Singh (2016), the authors found that embelin isolated from *E. ribes* could inhibit biofilm formation by *Streptococcus mutans,* possibly by disrupting the QS pathway. In another study, Leema et al. (2019) extrapolated from their studies that the antibacterial properties were enhanced in the embelin-synthesised AgNPs compared to pure embelin. Following this trend, further studies involving the isolation of principal compounds from *E. ruminata* to synthesise NPs may result in the derivatisation of valuable QSI and biofilm-inhibitory compounds.

5.5 Conclusion

This was the first study using *E. ruminata* to biosynthesise AgNPs, including the characterisation and biological assays. The biosynthesised AgNPs demonstrated their efficacy by producing inhibition against both short and long chain AHL autoinducer. The data of the present study suggest that AgNPs biosynthesised from *E. ruminata* extracts can act as inhibitors by antagonising QS behavior. Future research exploring fractionation of pure compounds could yield phytochemicals of pharmaceutical relevance. Thus, this study has created the foundation for prospective studies and revealed the therapeutic potential of AgNPs derived from *E. ruminata*.

Declaration of Competing Interest

The authors declare no conflict of interest.

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CHAPTER 6

Conclusions, challenges faced and recommendations for future research

6.1 Introduction

The current research project aimed to inaugurate and establish a foundational understanding of the phytochemical and biological activities of the plant species, *Embelia ruminata*. To initialise the investigations, the phytochemical profile that included the preliminary phytochemical tests, FTIR and GC-MS analyses were performed. These tests revealed the presence of various functional groups and phytocompounds that have been reported for the first time for this plant species. Some of the functional groups identified in the FTIR analyses were similar to those reported previously for embelin. In the GC-MS investigations, compounds such as fatty acids and resorcinol derivatives that have been reported for other *Embelia* species (Tessema et al., 2018) were identified.

An analysis of the radical scavenging and antioxidant capacity was undertaken, which produced interesting data, especially for the methanolic stem bark and seed extracts. In essence, the radical scavenging activity of the methanolic stem bark and seed extracts were 2.4 and 25 fold more potent than the standard butylated hydroxytoluene. As evidenced from the data, the potential of this plant species may lie in its antioxidant capacity. Several other studies that have used different species of *Embelia* have concluded that the basis of the therapeutic potential was due to its antioxidant capacity (Bansal et al., 2020; Caruso et al., 2020; SreeHarsha, 2020; Elias et al., 2021). Thus, a general conclusion may be that the ability of species of the genus *Embelia* to reduce oxidative stress may be the key driver for its potential as a curative agent. Concurrently, the strong cytotoxic activity on the cancer cell lines that was displayed by the methanolic stem bark and seed extracts may be affiliated with its inherent antioxidant action. While these extracts showed a lack of cytotoxic specificity to the normal cell lines, it is possible that the isolated, pure compounds of the respective extracts may elicit a more accurate response to cancer cell lines. Hence, further investigations are necessitated.

The investigations of the bacterial susceptibility activities showed *E. ruminata* had limited antibacterial activity. Parallel to this, the anti-quorum sensing (QS) tests demonstrated that the chloroform leaf, hexane and methanolic seed extracts may have potential as broad spectrum QS inhibitors due to their ability to inhibit both short and long chain autoinducer strains of *Chromobacterium* spp. As antimicrobial resistance becomes more prevalent on a global scale,

research is aiming to move away from bactericidal action, orientating towards interfering with the bacterial pathogenesis and virulence process. This route, advantageously, does not activate evolutionary pressure that gives rise to resistance in strains. Hence, the therapeutic potential of *E. ruminata* may be as QS inhibitors. Furthermore, there have been a small number of studies that have investigated QS inhibitory activity in the genus *Embelia*. Only two other studies were found, by Dwivedi and Singh (2016) and Shukla and Bhathena (2016) with both these studies using *E. ribes*. Hence, the results from this study using *E. ruminata* will provide a plausible reference for future studies.

In the nanosynthesis component, the aqueous extracts of *E. ruminata* were able to reduce silver to silver nanoparticles (AgNPs). Similar to the crude extracts, the AgNPs extracts displayed very mild antibacterial activity. A possible reason may be that the architecture of compounds such as benzoquinone compounds like embelin may afford superior antioxidant properties but may hinder antibacterial performance.

6.2 Challenges, drawbacks and recommendations

As mentioned above, the results from the antibacterial studies indicated that mild inhibitory activity was displayed by the extracts of *E. ruminata*. This may be due to the inadequate solubility of compounds in the relevant extracting solvents. Furthermore, the method of extraction may not have been suitable to enable solubilisation of antibacterial compounds. The GC-MS analysis indicated that most of the compounds quantified in the extracts of *E. ruminata* were insoluble in water. Eloff (2019) stated that because many of the antibacterial compounds in plant extracts are relatively non-polar, these compounds do not diffuse well in the aqueous agar matrix used in agar diffusion studies. Considering that most of the compounds in this study are not miscible in water, the poor antibacterial results may be due to the weak diffusion of compounds in the aqueous based agar medium. To bypass such drawbacks, future studies should focus on using alternative methods such as the resazurin microtiter assay (Reddy et al., 2021).

The sequential extraction method used in this project was lengthy and time-consuming. Volatile compounds and semi-volatile compounds would have been lost in the process. Furthermore, huge quantities of solvent and plant material were required (Falaki, 2019). Future studies should evaluate the feasibility of different extraction methodologies, such as microextraction that target analytes from the plant matrices (Diuzheva et al., 2020).

The seasonal availability of fruit was another issue encountered. The aim was to collect fruit that were in the same stage of development, i.e., mature fruit. Thus, employing an extraction technique that requires a small quantity of plant material may circumvent this drawback.

6.3 Further recommendations

The other plant organs such as the flowers and roots of *E. ruminata* need to be explored for their medicinal properties. This, together with other extractions protocols and solvents may produce products that can be potentially used in drug development. Innovative avenues such as tissue culture techniques for propagation should be investigated to ensure the sustainability of important phytocompounds of *E. ruminata*.

The antihelminthic properties should be investigated to authenticate the traditional use of *E. ruminata* by local Zulu inhabitants to treat helminth infections.

Moreover, prospects for future investigations may encompass the conjugation of pure compounds such as embelin to sideophore molecules that chelate Fe^{3+}. This conjugated complex could bypass the protective bilayer of the outer membrane of Gram-negative bacteria. Additionally, the synergistic combination of commercial antibiotics with pure compounds of *E. ruminata* may prove valuable as QS inhibitors rather than bactericidal agents.

Futhermore, the antifungal and antiviral activities of *E. ruminata* need to be explored. Elias et al., 2021 showed that embelin was effective in reducing the infection of *Herpes simplex* virus-1 (HSV-1) in cultured Vero cells. These investigations should be probed further using *E. ruminata* to optimise the exact mechanisms of action.

The diagnosis of individual compounds that regulate oxidative stress with translation to *in vivo* animal studies must be undertaken. The identified compounds can then be investigated for their potential in alleviating other oxidative stress-induced diseases such as cardiovascular disease, neurodegenerative and metabolic disorders. Additionally, it is well known that there is a strong association between obesity and cancer (Brown, 2021; Fang et al., 2021; Silveira et al., 2021). Embelin demonstrated effectiveness in mitigating obesity in high-fat diet-fed obese C57BL/6 mice (Bansal et al., 2020). In the current study, the methanolic stem bark and seed extracts of

*E. ruminat*a showed potent cytotoxicity on cancer cell lines. However, the extracts were, simultaneously, toxic to normal cells. Prospective studies should be undertaken to optimise the effect of isolated compounds from these extracts with the aim of reducing toxicity on normal cells. Coupled with this, the impact of pure compounds of *E. ruminata* on the amelioration of obesity biomarkers, is a worthy target.

6.4 Conclusion

The current study has produced relevant data regarding *E. ruminata* as a medicinal plant. The respective investigations that were undertaken in the present study will serve as a building block into the therapeutic properties of this plant species.

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APPENDIX

In this chapter, the additional information has been arranged according to each chapter in the following order: protocols, tables followed by figures, where relevant, for each of the chapter 3, 4 and 5.

Chapter 3:

Phytochemical screening:

Below are some of the protocols and recipes used in the phytochemical tests using the references: Harborne (1973), Trease and Evans (1989), Evans (1997) and Sofowora (1993).

Test for alkaloids

A small portion of the extract was stirred with a few drops of 1% hydrochloric acid, warmed in the water bath for 3 minutes at 30°C and filtered. The filtrate was carefully tested with various alkaloidal reagents.

•Mayer's test: To a few milliliters of plant extract, two drops of Mayer's reagent was added. To prepare Mayer's reagent, 1.36 g of mercuric chloride was added to 60 mL distilled water, 5 g of potassium iodide was added to 10 mL distilled water. Both solutions were combined. The appearance of a creamy white precipitate indicated the presence of alkaloids.

•Dragendoff's test: One mL of Dragendoff's reagent was added to 1 mL plant extract. To make up Dragendoff's reagent, 8 g bismuth (111) nitrate pentahydrate was dissolved in 20 mL liquid ammonia, 27.2 g potassium iodide was dissolved in 50 mL distilled water. Both solutions were mixed together. A brick red precipitate implied a positive result.

•Wagner's test: This was made up by adding 1.27 g of iodine, 2 g potassium iodide to 100 mL distilled water. One mL of this reagent were added to 1 mL of plant extract. A reddish brown precipitate denoted a positive result for alkaloids.

Test for Triterpenoids

Salkowsi test: Two mL extracts were treated with 2 mL chloroform and then filtered. Three mL of concentrated sulphuric acid (H_2SO_4) were added carefully to form a layer. A reddish-brown colouration of the interface showed conclusive results for the presence of triterpenes.

Test for Phytosteroids

Salkowsi test: Two mL of plant extracts were treated with 2 mL chloroform and then filtered. A few drops of H_2SO_4 was added along the side of test tube. It was then shaken and allowed to stand. The formation of a red colour in lower chloroform layer indicated a positive result.

Liebermann – Burchard test: One mL of extracts was treated with 1 mL of chloroform and then filtered. The filtrate was, in turn, treated with 1mL acetic anhydride, boiled and cooled. Thereafter, 1mL of concentrated H_2SO_4 was added along the side of the test tube. A characteristic brown ring at the junction indicates presence of phytosterols.

Test for Flavonoid

•10% lead acetate test: Filtered extracts were treated with 10% lead acetate. A resultant yellow precipitate was an affirmative result.

•Alkaline reagent test: Two mL of extracts were treated with 2mL 10% NaOH followed by treatment with dilute H_2SO_4. The formation of an intense yellow colour, which becomes colourless on addition of dilute acid indicates the presence of flavonoids.

Test for Phenolic compounds

•Aqueous 5% $FeCl_3$: One mL of distilled water was added to 1 ml of extract and filtered. Then, 3 drops of reagent were added. Greenish to black colour solution denoted a confirmatory result.

•Gelatin test: One mL of 1% w/v solution of gelatin containing 10% sodium chloride was added to 1 mL of extract. A white precipitate indicated the presence of phenolic compounds.

Test for Coumarins

One mL of extract was added to 1mL of 10% sodium hydroxide. The formation of a yellow colour indicates the presence of coumarins.

Test for Quinones

The formation of a red colour indicates presence of quinones when 1mL of concentrated H_2SO_4 was added to 1mL of extract.

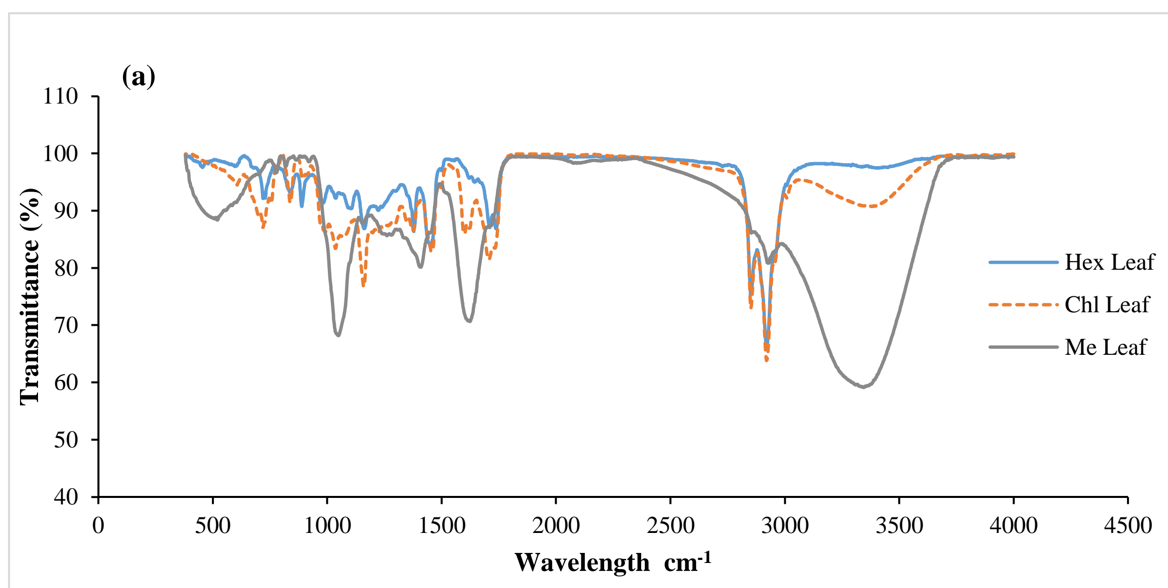
Test for Anthraquinones

To 3mL of extract, 3 mL of chloroform was added. This mixture was heated in a water bath for 5 min and filtered while hot. When cooled, 5% potassium hydroxide solution was added to the filtrate. The appearance of a violet to pink colour in the aqueous layer indicated a positive result.

Test for resin

One mL of the various solvent extracts was treated with 1mL of acetic anhydride followed by the addition of concentrated H_2SO_4. A positive result was indicated by the formation of a yellow to orange colouration.

Figures



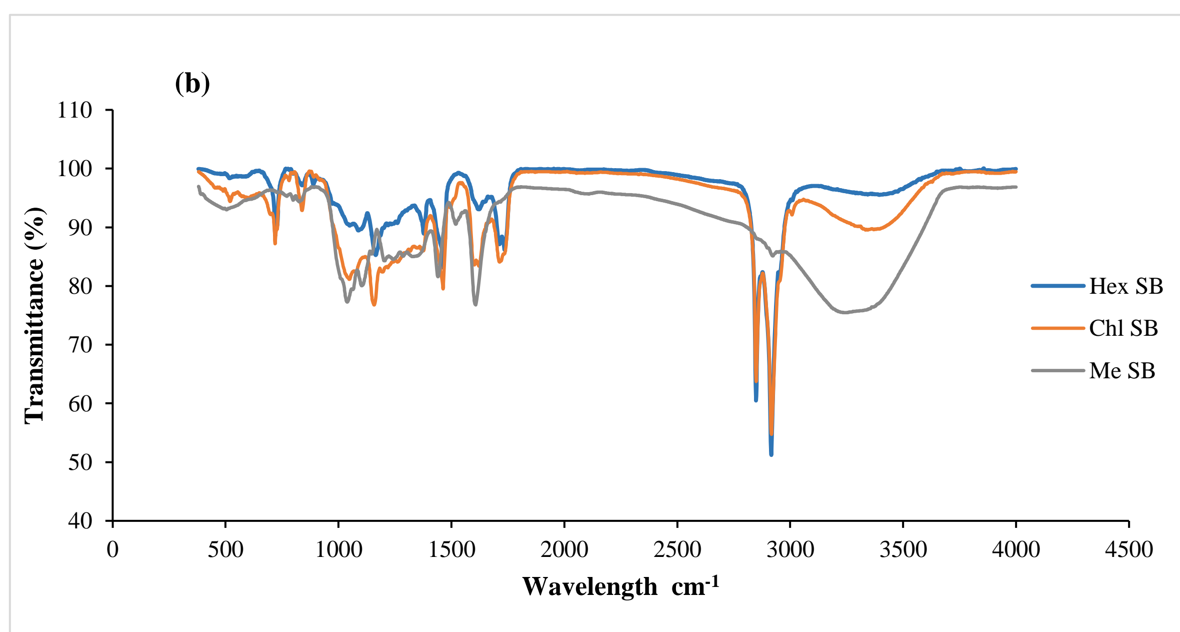
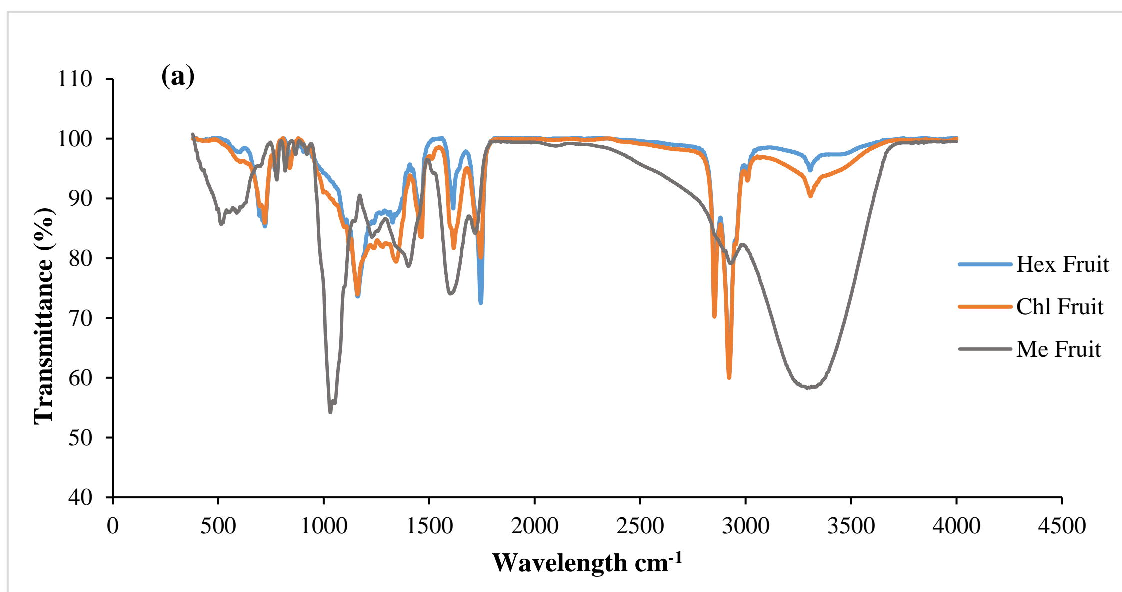


Figure A3.1 FTIR spectra of *Embelia ruminata* leaf (a) and stem bark (b). Hex = Hexane, Chl = Chloroform, Me = Methanol and SB = stem bark.



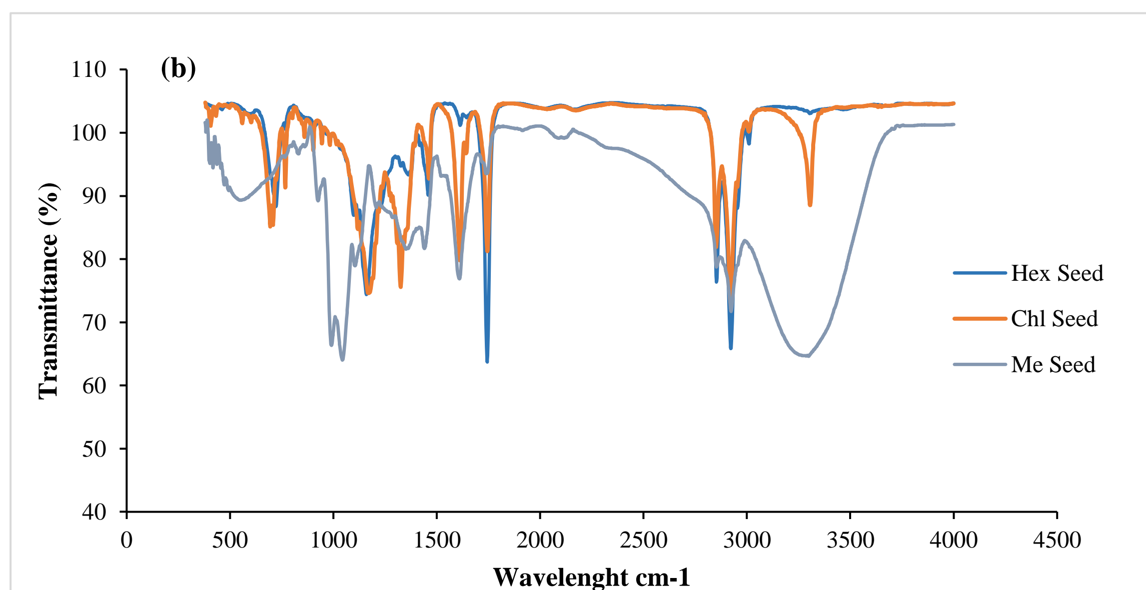
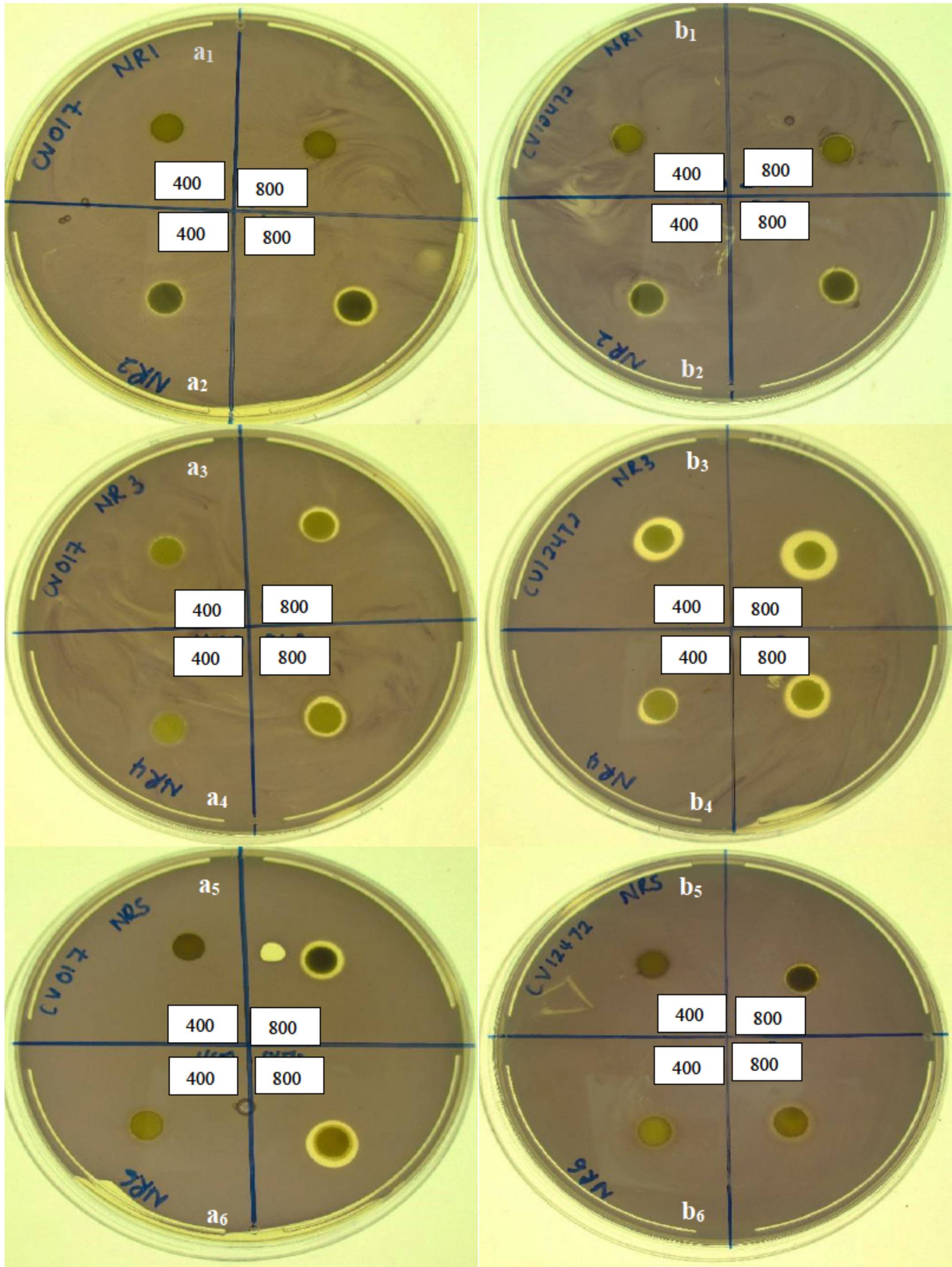


Figure A3.2 FTIR spectra of *Embelia ruminata* fruit (a) and seed (b). Hex = Hexane, Chl = Chloroform and Me = Methanol.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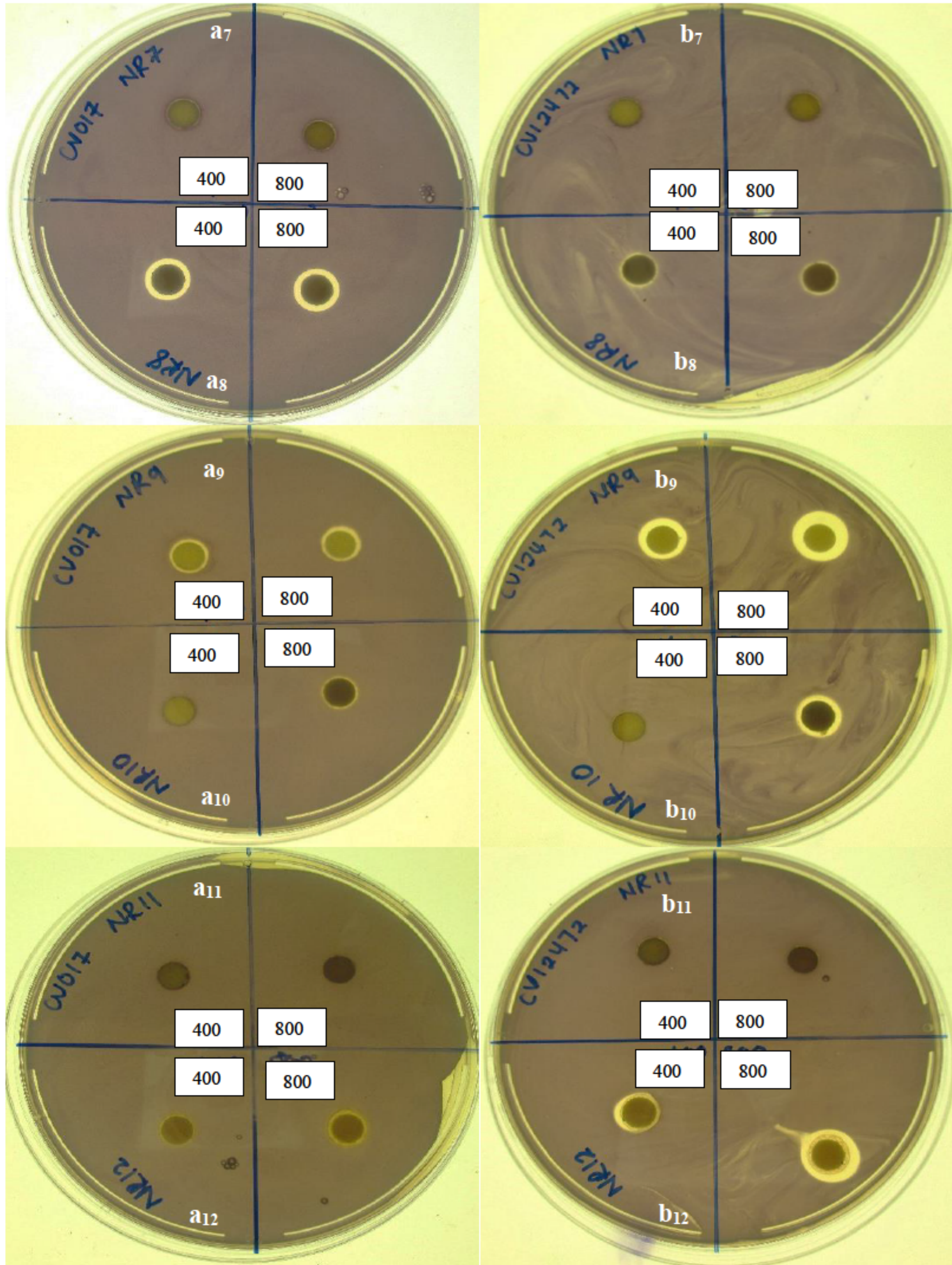

Figure A3.3 Qualitative inhibitory effects of the hexane, chloroform and methanol extracts of *Embelia ruminata* leaf, stem bark, fruit and seeds on violacein production using *Chromobacterium* spp. Label: a_1 and b_1 = hexane leaf, (a_2) and (b_2) = chloroform leaf#, (a_3) and (b_3) = methanol leaf, (a_4) and (b_4) = hexane stem bark#, (a_5) and (b_5) = chloroform stem bark, (a_6) and (b_6) =methanol stem bark#, (a_7) and (b_7) = hexane fruit, (a_8) and (b_8) = chloroform fruit#, (a_9) and (b_9) = methanol fruit#, (a_{10}) and (b_{10}) = hexane seed#, (a_{11}) and (b_{11}) = chloroform seed, (a_{12}) and (b_{12}) = methanol seed# using biomonitors CV017 and ATCC 12472, respectively. # indicates the extracts that were used in the subsequent quantitative inhibition assays.

Chapter 4:Tables

Table A4.1 GC-MS spectral analysis of phytocompounds identified in hexane leaf extracts of *Embelia ruminata*

S/N	R.time	Compound	Molecular formula	Molecular weight (g/mol)	Height %
1	58.72	*n*-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	4.91
2	63.20	Phytol	$C_{20}H_{40}O$	296.5	20.35
3	65.03	cis-Vaccenic acid	$C_{18}H_{34}O_2$	282.5	8.28
4	82.20	Heptadecane, 2,6,10,15-tetramethyl-	$C_{21}H_{44}$	296.6	1.91
5	85.12	Squalene	$C_{30}H_{50}$	410.7	2.85
6	93.12	Tetratetracontane	$C_{44}H_{90}$	619.2	18.91
7	95.65	Stigmasterol	$C_{29}H_{48}O$	412.7	2.38
8	97.01	Ergosta-7,22-dien-3-ol, (3.beta.,22E)-	$C_{28}H_{46}O$	398.7	2.32
9	97.90	2-methyloctacosane	$C_{29}H_{60}$	408.8	4.87
10	108.19	trans-Geranylgeraniol	$C_{20}H_{34}O$	290.5	2.59
11	113.84	cis-13-eicosenoic acid	$C_{20}H_{38}0_2$	310.5	4.27

Table A4.2 GC-MS spectral analysis of phytocompounds identified in chloroform leaf extracts of *Embelia ruminata*

Peak	R.time	Compound	Molecular formula	Molecular weight (g/mol)	Height %
1	63.13	Phytol	$C_{20}H_{40}O$	296.5	17.52
2	87.75	Eicosane	$C_{20}H_{42}$	282.5	3.23
3	89.90	5-Heptylresorcinol	$C_{13}H_{20}O_2$	208.3	26.93
4	90.00	1,3-Benzenediol, 5-pentadecyl-	$C_{21}H_{36}O_2$	320.5	12.65
5	92.98	Tetratetracontane	$C_{44}H_{90}$	619.2	6.31

Table A4.3 GC-MS spectral analysis of phytocompounds identified in methanol leaf extracts of *Embelia ruminata*

Peak	R.time	Compound	Molecular formula	Molecular weight (g/mol)	Height %
1	18.84	Phytol	$C_{20}H_{40}O$	296.5	1.75
2	19.48	Tetradecanamide	$C_{14}H_{29}NO$	227.39	5.62
3	20.13	Methyl 2-O-benzylpentofuranoside	$C_{13}H_{18}O_5$	254.28	1.96
4	21.07	13-Docosenamide	$C_{22}H_{43}NO$	337.6	25.65

Table A4.4 GC-MS spectral analysis of phytocompounds identified in hexane stem bark extracts of *Embelia ruminata*

Peak	R.time	Compound	Molecular formula	Molecular weight (g/mol)	Height %
1	58.25	Tridecanoic acid	$C_{13}H_{26}O_2$	214.34	3.08
2	64.53	1-Tridecanol	$C_{13}H_{28}O$	200.36	2.36
3	82.17	Heptadecane, 2,6,10,15-tetramethyl	$C_{21}H_{44}$	296.6	2.12
4	84.69	1,3-Benzenediol, 5-pentadecyl	$C_{21}H_{36}O_2$	320.5	1.66
5	85.09	Squalene	$C_{30}H_{50}$	410.7	2.62
6	87.75	Tetratetracontane	$C_{44}H_{90}$	619.2	9.63
7	89.72	5-Heptylresorcinol	$C_{13}H_{20}O_2$	208.3	4.04
8	91.42	Pentadecanal-	$C_{15}H_{30}O$	226.4	2.94
9	93.16	Vitamin E	$C_{29}H_{50}O_2$	430.7	13.38
10	95.63	Stigmasterol	$C_{29}H_{48}O$	412.7	10.13
11	96.5 1	Heneicosanal	$C_{21}H_{42}O$	310.6	8.49
12	98.05	1-Heneicosanol	$C_{21}H_{44}O$	312.6	6.78
13	98.39	2-Pentacosanone	$C_{25}H_{50}O$	366.7	5.09
14	108.18	trans-Geranylgeraniol	$C_{20}H_{34}O$	290.5	6.2

Table A4.5 GC-MS spectral analysis of phytocompounds identified in chloroform stem bark extracts of *Embelia ruminata*

Peak	R.time	Compound	Molecular formula	Molecular weight (g/mol)	Height %
1	52.63	Cyclopentane, 1,2-dimethyl-3-(1-methylethenyl)-	$C_{10}H_{18}$	138	1.59
2	84.73	1,3-Benzenediol, 5-pentadecyl-	$C_{21}H_{36}O_2$	320.5	17.05
3	87.71	Eicosane	$C_{20}H_{42}$	282.5	2.16
4	89.79	1,4-Benzenediol, 2-methyl- (Toluhydroquinone)	$C_7H_8O_2$	124.14	27.18
5	90.37	1,54-Dibromotetrapentacontane	$C_{54}H_{108}Br_2$	917.2	1.25
6	93.07	Vitamin E	$C_{29}H_{50}O_2$	430.7	2.66
7	95.56	Stigmasterol	$C_{29}H_{48}O$	412.7	3.32
8	98.01	1-Heptacosanol	$C_{27}H_{56}O$	396.7	2.57
9	98.37	2-Pentacosanone	$C_{25}H_{50}O$	366.7	2.57
10	108.13	3,8,11-Trioxatetracyclo[4.4.1.0(2, 4).0(7,9)] undecane, (1.alpha.,2.alpha.,4.alpha.,6 .alpha.,7.beta.,9.beta.)-	$C_8H_{10}O_3$	154.16	0.97

Table A4.6 GC-MS spectral analysis of phytocompounds identified in methanol stem bark extracts of *Embelia ruminata*

Peak	RT	Compound	Molecular formula	Molecular weight (g/mol)	Height %
1	11.544	Octane, 1,1,2,2-tetrafluoro-	$C_8H_{14}F_4$	186.19	0.34
2	15.167	1,4-Benzenediol, 2,3,5-trimethyl-	$C_9H_{12}O_2$	152.19	3.68
3	15.624	2-Allyl-1,4-dimethoxy-3-methyl-benzene	$C_{12}H_{16}O_2$	192.25	0.65
4	16.925	t-Butylhydroquinone	$C_{10}H_{14}O_2$	166.22	0.99
5	17.017	1,3-Benzenediol, 4,5-dimethyl-	$C_8H_{10}O_2$	138.16	2.88

6	17.386	Hexadecanamide	$C_{16}H_{33}NO$	255.44	4.45
7	19.908	α-Resorcylic aldehyde	$C_7H_6O_3$	138.12	0.71
8	21.063	Oleamide	$C_{18}H_{35}NO$	281.5	21.26
9	22.133	2-methylhexacosane	$C_{27}H_{56}$	380.7	0.37
10	23.155	1-Ethylpentyl acetate	$C_9H_{18}O_2$	158.24	1.31
11	25.802	1,3-Benzenediol, 5-pentadecyl-	$C_{21}H_{36}O_2$	320.5	0.74

Table A4.7 GC-MS spectral analysis of phytocompounds identified in hexane fruit extracts of *Embelia ruminata*

S/N	RT	Compound	Molecular formula	Molecular weight	Height %
1	65.91	Unknown			9.59
2	76.22	Hexadecane, 1-iodo-	$C_{16}H_{33}I$	352.34	0.73
3	84.96	1,3-Benzenediol, 5-pentadecyl-	$C_{21}H_{36}O_2$	320.5	15.57
4	85.13	Squalene	$C_{30}H_{50}$	410.7	16.85
5	87.78	Tetratetracontane	$C_{44}H_{90}$	619.2	9.90
6	89.82	5-Heptylresorcinol	$C_{13}H_{20}O_2$	208.3	22.89
7	92.93	Eicosane	$C_{20}H_{40}$	282.5	1.59
8	96.96	Stigmasterol	$C_{29}H_{48}O$	412.7	5.41
90	108.15	Trans-geranylgeraniol	$C_{20}H_{34}O$	290.5	2.17

Table A4.8 GC-MS spectral analysis of phytocompounds identified in chloroform fruit extracts of *Embelia ruminata*

S/N	RT	Compound	Molecular formula	Molecular weight	Height %
1	65.85	Unknown			19.37
2	85.05	Squalene	$C_{30}H_{50}$	410.7	4.26
3	89.813	5-Heptylresorcinol	$C_{13}H_{20}O_2$	208.3	37.84
4	89.95	1,3-Benzenediol, 5-pentadecyl-	$C_{21}H_{36}O_2$	320.5	25.73
5	92.58	2,2,6,6-Tetramethyl-4-piperidyl benzoate	$C_{16}H_{23}NO_2$	261.36	2.24

Table A4.9 GC-MS spectral analysis of phytocompounds identified in methanol fruit extracts of *Embelia ruminata*

S/N	RT	Compound	Molecular formula	Molecular weight	Height %
1	19.487	Hexadecanamide	$C_{16}H_{33}NO$	255.44	4.36
2	21.072	Oleamide	$C_{18}H_{35}NO$	281.5	19.28
3	21.26	Octadecanamide	$C_{18}H_{37}NO$	283.5	5.74
4	25.76	5-Heptylresorcinol	$C_{13}H_{20}O_2$	208.3	14.88

Table A4.10 GC-MS spectral analysis of phytocompounds identified in hexane seed extracts of *Embelia ruminata*

S/N	RT	Compound	Molecular formula	Molecular weight	Height %
1	62.76	Undecane, 3,8-dimethyl-	$C_{13}H_{28}$	184.36	2.97
2	65.88	Unknown			22.93
3	85.17	Squalene	$C_{30}H_{50}$	410.7	51.76
4	87.63	1,54-Dibromotetrapentacontane	$C_{54}H_{108}Br_2$	917.2	3.80
5	96.84	Chondrillasterol	$C_{29}H_{48}O$	412.7	3.86

Table A4.11 GC-MS spectral analysis of phytocompounds identified in chloroform seed extracts of *Embelia ruminata*

S/N	RT	Compound	Molecular formula	Molecular weight	Height %
1	66.24	Unknown			92.29
2	84.99	Squalene	$C_{30}H_{50}$	410.7	7.71

Table A4.12 GC-MS spectral analysis of phytocompounds identified in methanol seed extracts of *Embelia ruminata*

S/N	RT	Compound	Molecular formula	Molecular weight	Height %
1	21.753	2,2'-Methylenebis(4-methyl-6-tert-butylphenol)	$C_{23}H_{32}O_2$	340.5	20.11
2	22.445	2,6,11-trimethyldodecane	$C_{15}H_{32}$	212.41	5.53
3	26.066	Tetratetracontane	$C_{44}H_{90}$	619.2	5.11

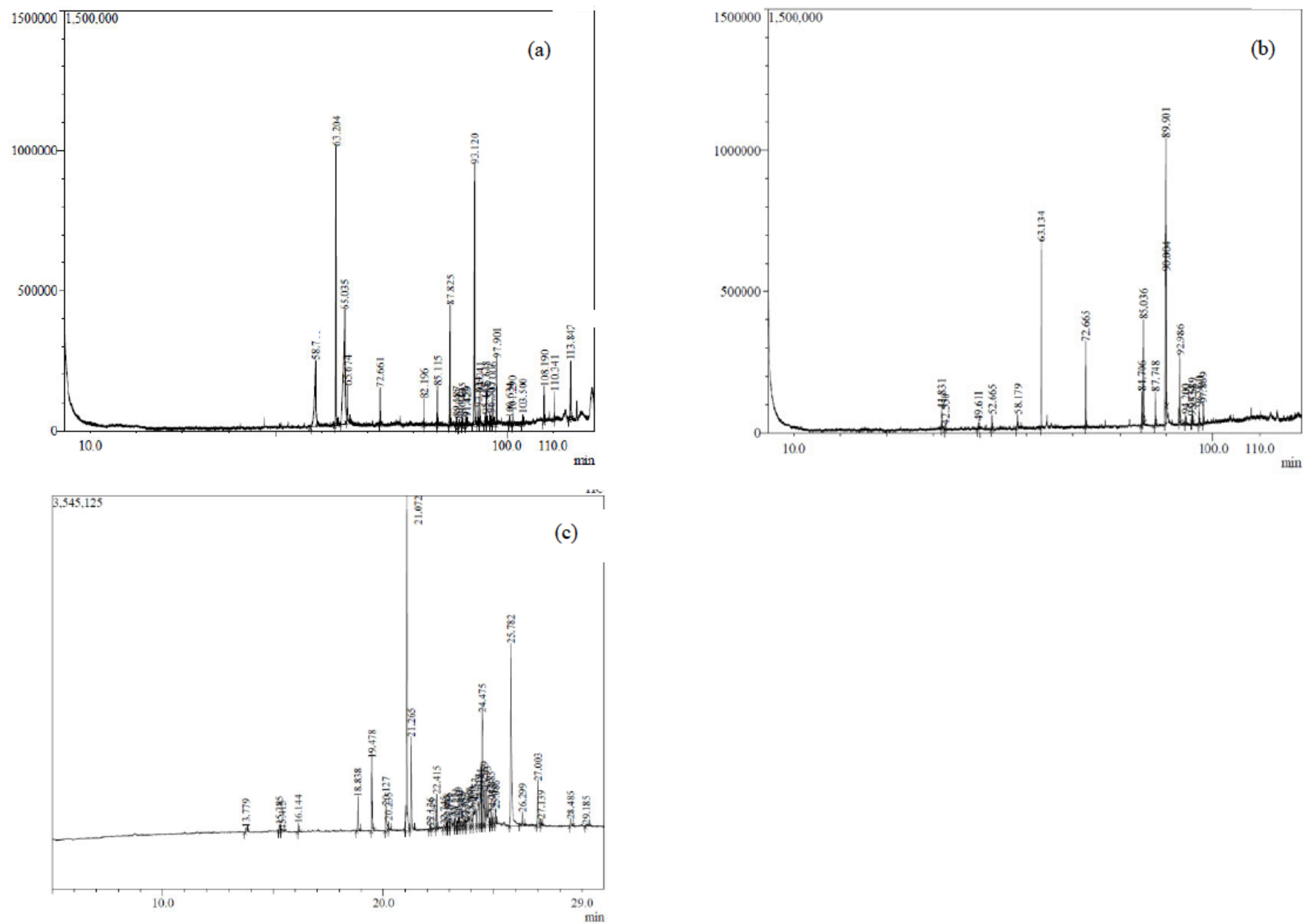

Figure A4.1 GC-MS chromatograph of *Embelia ruminata* leaf extracts. Labels (a) = Hexane, (b) = Chloroform and (c) = Methanol

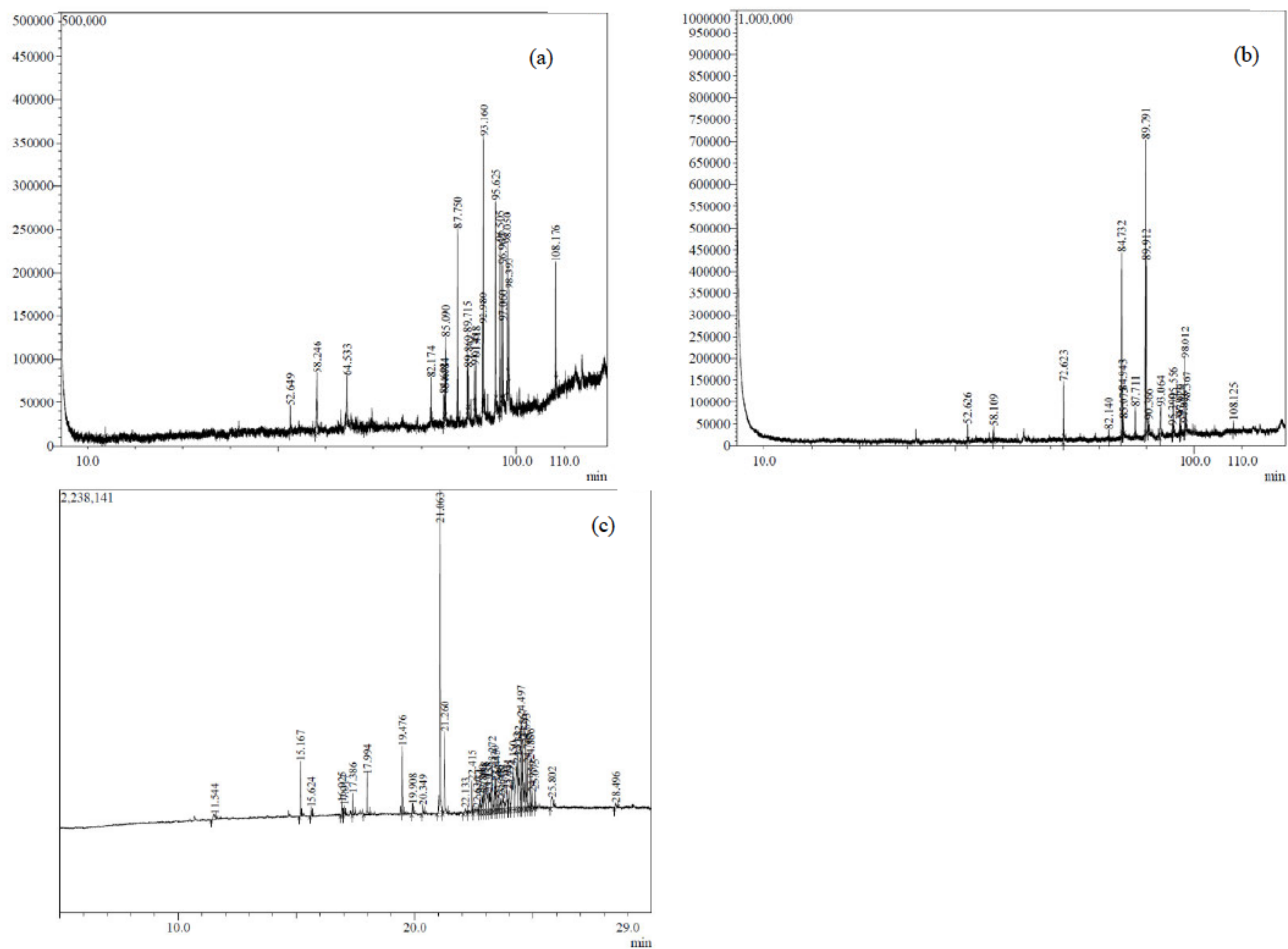

Figure A4.2 GC-MS chromatograph of *Embelia ruminata* stem bark extracts. Labels (a) = Hexane, (b) = Chloroform and (c) = Methanol

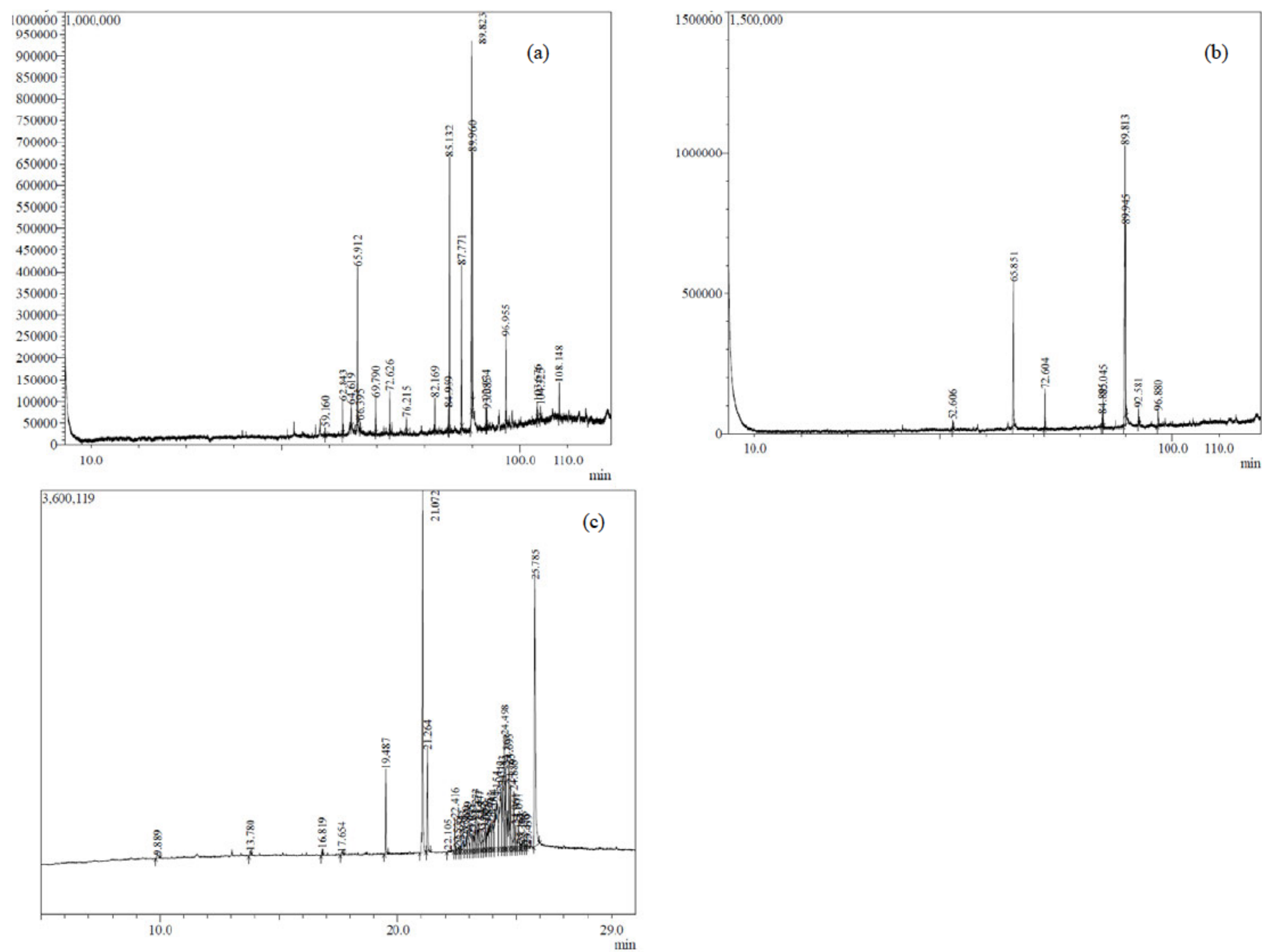

Figure A4.3 GC-MS chromatograph of *Embelia ruminata* fruit extracts. Labels (a) = Hexane, (b) = Chloroform and (c) = Methanol

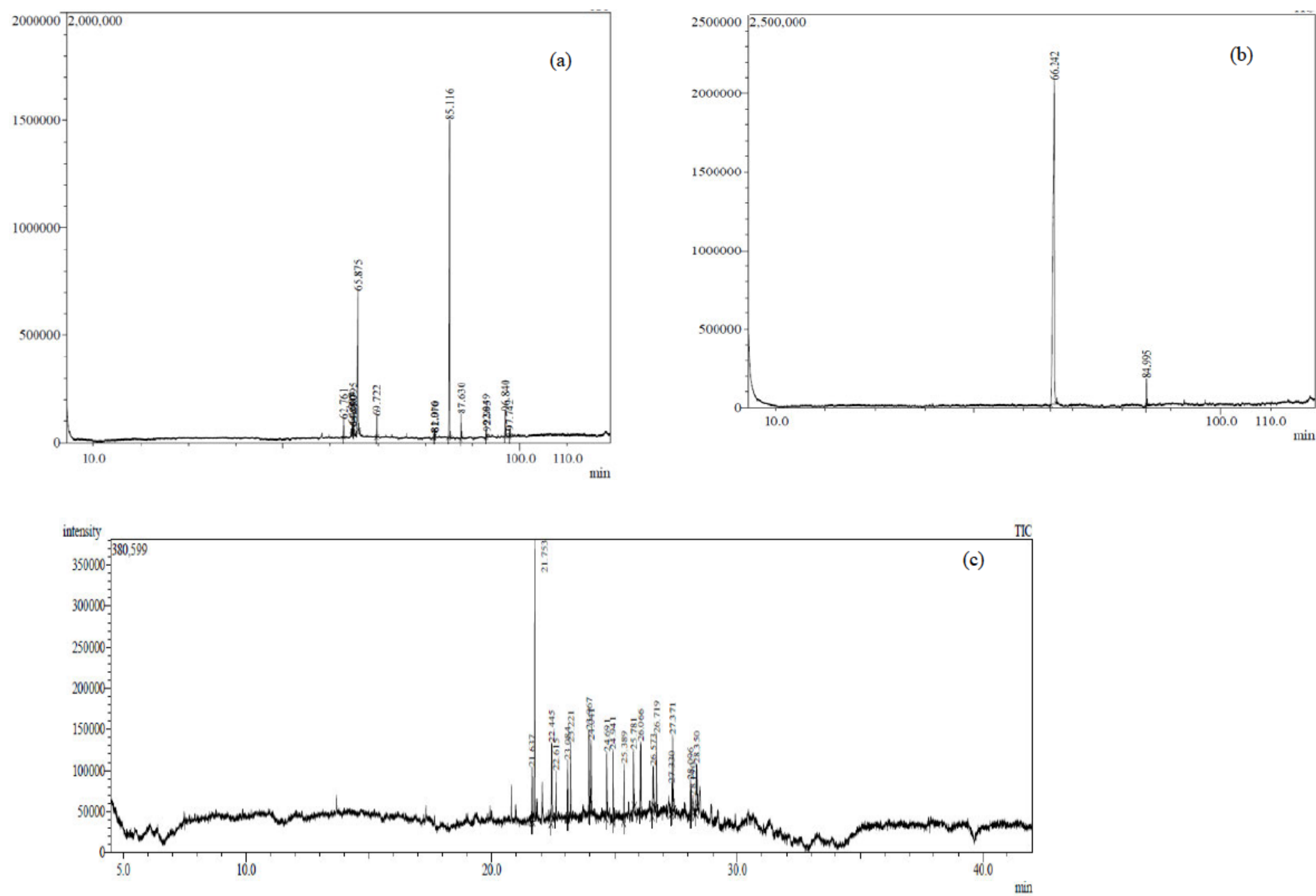

Figure A4.4 GC-MS chromatograph of *Embelia ruminata* seed extracts. Labels (a) = Hexane, (b) = Chloroform and (c) = Methanol

Chapter 5: Figures

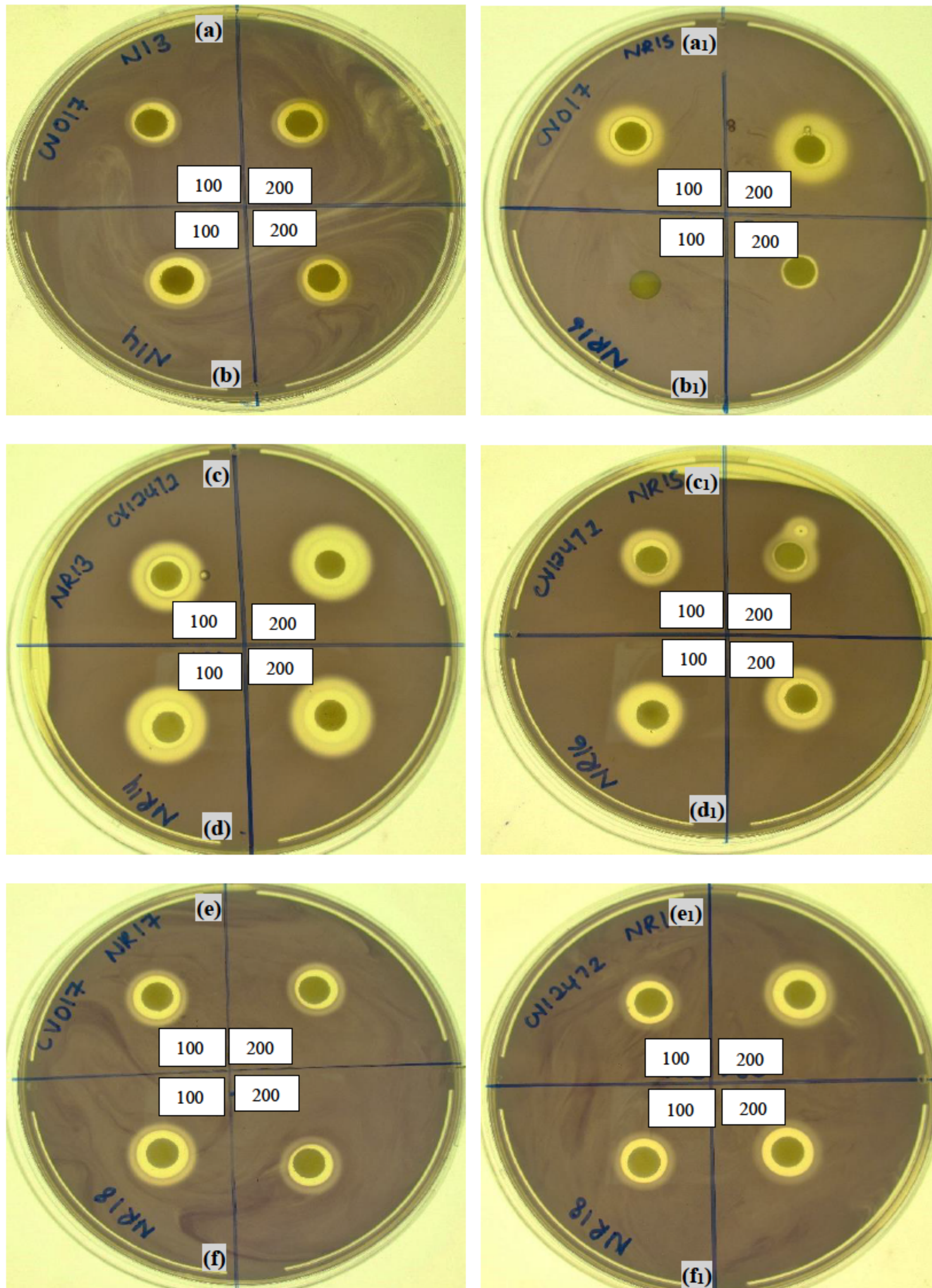

Figure A5.1 Inhibitory effect of AgNPs biosynthesised using aqueous extracts of *Embelia ruminata* on violacein production by *Chromobacterium subtsugae* CV017 and *Chromobacterium violaceum* ATCC 12472. Labels (a) and (a_1) = Leaf Rt, (b) and (b_1) = Leaf 80 °C, (c) and (c_1)=Stem bark Rt, (d) and (d_1)= Stem bark 80 °C, (e) and (e_1) = Fruits Rt, (f) and (f_1) = Fruits 80 °C using biomonitors CV017 and ATCC 12472, respectively.

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