MICROMORPHOLOGY AND BIOLOGICAL ACTIVITIES OF LEAVES AND STEM BARK OF *DIOSPYROS VILLOSA* (L.) DE WINTER

BY

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BY

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Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in the School of Life Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal

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PREFACE

The use and development of traditional herbal medicine could be traced down the history. Currently, the local folks in Africa embrace the use of medicinal herbs as a typical curative and management approach to common illnesses and diseases. There are many plants with different medicinal applications in South Africa and many of them are used in the treatment and management of diseases and infections. Despite the beneficial effect associated with the medicinal plant, they seem not to be incorporated to western drug form due to their undesirable side effects. To overcome this discrimination, in our laboratory, we further explored and evaluated antioxidant, antibacterial, quorum sensing inhibitory potential, anticancer as well as other significant potential of the synthesized nanoparticles of *Diospyros villosa* leaves and stem bark. This study further added to the existing records of plants having medicinal applications in South Africa. This effort was further directed to gain the holistic therapeutic insight rendered by *Diospyros villosa* in disease condition.

DECLARATION

I, Oluwatosin Temilade Adu (217081105), openly declare that the dissertation titled

'**Micromorphology and biological activities of leaves and stem bark of *Diospyros villosa (L.) de Winter'***

Is the result of experimental investigation and research and this dissertation has never been submitted either in parts or full for any degree in this university or any other institution. In situations where works of other scientists were used, they were truly acknowledged in the text. This whole experimental research study was thoroughly supervised by Prof. Yougasphree Naidoo.

Name: Oluwatosin Adu **Signature:** [redacted] **Date: 12/07/2021**

Supervisor: Prof Yougashree Naidoo **Signature:** [redacted] **Date: 22/07/2021**

Co-Supervisor: Prof. Lin Johnson **Signature:** [redacted] **Date: 20/07/2021**

DEDICATION

This research is fully dedicated to the God, the supreme and everlasting being.

ACKNOWLEGMENTS

First and foremost, I would like to appreciate Prof. Yougasphree Naidoo for her counsel, encouragement and support during the course of carrying out the studies. She was always available to look my way especially when I found nowhere to turn during my darkest moments in the course of carrying out this study. This study would not have been possible without her guidance.

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To all my seniors and colleagues in the laboratory, I am eternally grateful. The story of achieving this doctorate degree is not complete without you.

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And finally to my lovely husband (Temitope) and my babies (Taraoluwanimi and Oluwaseteminire), I would like to appreciate them for their support, endurance and understanding especially for coping with my time management at home.

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LIST OF ABBREVIATIONS

%	Percentage
HLCC-A549	Human Lung cancer cells
AHL	Acyl homoserines lactone
Ag^{o}	Reduced silver ion
Ag^{+}	Silver ion
AgO	Silver II oxide
$AgNO_3$	Silver trioxonitrate V
AgNPs	Silver nanoparticles
ANOVA	Analysis of variance
AOCC	African Orphan Crops Consortium
ATCC	American Type Collection Culture
ATP	Adenosine triphosphate
β-sitosterol	Beta-sitosterol
^{o}C	Celcius
cm	centimetre
C=O	Carbonyl functional group
Co	Company
CV	*Chromobacterium violaceum*
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
D. chloroxylon	*Diospyros chloroxylon*
D. chryophyllos	*Diospyros chryophyllos*
D. crassiflora	*Diospyros crassiflora*
D. dendo	*Diospyros dendo*

D. ebenaster	*Diospyros ebenaster*
D. ebenum	*Diospyros ebenum*
D. haplostyl	*Diospyros haplostyl*
D. hirsute	*Diospyros hirsuta*
D. kaki	*Diospyros kaki*
D. melanoxylon	*Diospyros melanoxylon*
D. mespiliformis	*Diospyros mespiliformis*
DNA	Deoxyribonucleic acid
D. perrieri	*Diospyros perrieri*
DPPH	1, 1- diphenyl-2-picrylhydrazyl
D. rubra	*Diospyros rubra*
D. villosa	*Diospyros villosa*
E. coli	*Escherichia coli*
EDX	Elemental Dispersion X-ray
FBS	Foetal Bovine Serum
FEG-SEM	Field Emission Gun- Scanning Electron Microscopy
$FeCl_3$	Ferric trichloride
FRAP	Ferric reducing antioxidant potential
FTIR	Fourier Transform Infrared
g	gram
GAE	Gallic Acid Equivalence
GI	Growth Inhibition
GN10	Gentamicin
HEK293	Human Embryonic Kidney cells
hr	hour

HPLC	High Performance Liquid Chromatography
IC_{50}	Inhibitory concentration
KBr	Potassium bromide
K. pneumonia	*Klebsiella pneumoniae*
keV	kiloelectron volts
KV	Kilovolts
LB	Luria-Bertani
LRT	Leaves nanoparticles at room temperature
m	metre
M	$moldm^{-3}$
MBC	Minimum bactericidal concentration
MCF-7	Breast cancer cells
MIC	Minimum inhibitory concentration
MDR	Multi Drug Resistance
mg	Milligram
ml	Millilitre
MH	Mueller Hilton
min	Minute
mM	millimolar
MRSA	methicillin-resistant *Staphylococcus aureus*
nm	nanometre
NaOH	Sodium hydroxide
NPs	nanoparticles
N-H	nitrogen bond
NRF	National Research Foundation

OD	Optical density
O-H	hydroxyl group
PDGFR	Platelet derived growth factor receptors
pH	hydrogen ion scale
QS	Quorum sensing
QSI	Quorum sensing inhibition
R	Resistant
ROS	Reactive Oxygen Species
S	Sensitive
S. aureus	*Staphylococcus aureus*
SEM	Scanning Electron Microscopy
S. epidermidis	*Staphylococcus epidermidis*
SPR	Surface Plasmon Resonance
SRT	Stem nanoparticles at room temperature
TEM	Transmission Electron Microscopy
TLC	Thin Layer Chromatography
TPC	Total Phenolic Content
TWAS	Third World Academy of Science
UK	United Kingdom
USA	United States of America
µg	Microgram
µL	Microliters
µm	Micrometre
UV	Ultraviolet
×g	gravity

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Chapter 4

ABSTRACT

Review Article: The genus *Diospyros* consists of many species which are distributed throughout the world. There is no elaborate information about the holistic importance of the plants. This review aims to delve for more information about the usefulness of the plants for humans. A detailed study of the genus *Diospyros* with a targeted focus on *Diospyros villosa* (L.) De Winter plant was carried out information on the plants' morphology was taken as observed during harvest. Other information with respect to geographical distribution, secretory structures, chemical composition, embedded bioactive constituents and the associated mechanism of action of the bioactive constituents were acquired. The genus *Diospyros* yields products of medicinal importance throughout the world. This study gives some detailed information about the genus *Diospyros* as well as the potential use of the species as functional medicinal plants with bioactive compounds.

Manuscript 1: This study evaluated the phytochemical constituents, antioxidant and antimicrobial potentials of *Diospyros villosa* (L.) De Winter leaves and stem bark. The extracts were obtained using different media (methanol, chloroform and hexane). DPPH and FRAP methods were used to investigate the antioxidant potentials of the crude extracts. The antimicrobial potency of *Diospyros villosa* extracts against five pathogenic bacteria was determined using MIC, MBC and agar well diffusion methods. Flavonoids, alkaloids and phenols were identified in *D. villosa* extracts. The mean concentrations of methanol extracts *Diospyros villosa* leaves and stem against DPPH providing 50% inhibition were 9.53 ± 0.25 μg ml^{-1} and 9.52 ± 0.30 μg ml^{-1} respectively. The methanolic leaves extracts further showed promising antimicrobial activity against *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* and *Staphylococcus aureus* with inhibition zones which are almost as high as the control. The antimicrobial methods also demonstrated that the leaves and stem bark extracts had wide antimicrobial abilities. The results revealed that *D.*

villosa leaves and stem bark contain reasonable amount of bioactive compounds. Hence, these compounds may serve as natural antioxidants and antibacterial agents for the treatment of bacterial infections and diseases.

Manuscript 2: Nanoparticles are synthesized through wet chemical techniques. Meanwhile, the chemicals used often are toxic and flammable. While considering safe substances, *Diospyros villosa* leaves and stem bark are both observed to be suitable for the biosynthesis of silver nanoparticles. In this research study, we described a less expensive and environmental-friendly technique for the biosynthesis of silver nanoparticles from silver nitrate ($AgNO_3$) solution and *Diospyros villosa* extracts. The obtained silver nanoparticles were characterized using the UV-vis absorption spectroscopy, FT-IR, EDX, SEM and TEM, DPPH scavenging ability, ferric reducing antioxidant potential, antimicrobial susceptibility and quorum sensing inhibition tests. The biosynthesized silver nanoparticles showed good antibacterial activity against *Escherichia coli, Staphylococcus aureus, Staphylococcus epidermis and Enterococcus faecalis.* The *D. villosa* nanoparticles demonstrated potential antioxidant and quorum sensing inhibition and may thus represent a source of antioxidant as well as quorum sensing inhibition candidate for infection and disease control.

Manuscript 3: The structural adaptation, histological compatibility and ecological functions of trichomes in *Diospyros villosa,* however remain largely unclear. This study aimed to interpret the structure and histochemical analysis of the leaves and stem bark of *Diospyros villosa* using light and electron microscopy and different histochemical tests as well as to elucidate the composition of the secretory products. Trichome density and length were also determined in three developmental stages of the leaves. The results of histochemical tests also revealed the presence of alkaloids and phenolic compounds which are medicinally important and used to treat multiple ailments.

Manuscript 4: *Diospyros villosa* is traditionally used for processing anti-bacterial properties. Its cytotoxic effects have not been studied before. Therefore, this study aimed to examine the nutritional properties as well the cytotoxic effects of *D. villosa*. The leaves and stem barks were subjected to three different extraction methods (methanol, chloroform and hexane) and their nanoparticles were synthesized at two different temperatures (room temperature and at 80 ºC). Thereafter, extracts were assessed using the associated AOCC protocols, for their nutritional content (moisture, fibre, proteins, lipid, ash and hydrolysable carbohydrates). *Diospyros villosa* extracts and their corresponding nanoparticles were then incubated overnight with cancerous and noncancerous cell lines to evaluate their cytotoxic potential.

STUDY OUTLINE

This dissertation is presented in a manuscript format. It consists of four research studies and seven chapters. The chapter one which is the background chapter is included so as to bring the reader up to speed in understanding the aim and objectives of this study. Chapter two is the review article which discusses the specific topics covered in the study and how the current study sought to fill the gaps in literature. Chapter three contains the first research study. This work has been submitted for publication and 'Under Review' in the Turkish Journal of Botany. Authors: Adu Oluwatosin Temilade, Naidoo Yougasphree, Lin Johnson, Sadashiva Channangihalli and Sivaram Venkatamegowda. Chapter four is the second research study. Chapter five is the third research study. Chapter six is the fourth research study. Chapter seven is the synthesis; this chapter specifically explains the four research chapters in line with the aims of the project.

CHAPTER ONE

1.0 Introduction

World Health Organization (WHO) described medicinal plant as any plant which any of its parts (like leaves, stem, root, flower etc.) can be used either as prophylactic or curative regimen for a particular ailment or disease. The parts of such plant are regarded to contain certain bioactive compounds which are responsible for the healing process. There are various naturally-existing plants which are widely used for the purpose of treating and managing ailments and diseases (Duraipandiyan et al., 2006). WHO reported that improper management of infectious diseases contributes to the world mortality rate and considered deadly as the count is becoming proportional with the increase in world population (WHO, 2014). Their resistance to antibiotic medications has reached a peak and is now becoming a public health major threat. There were 25,000 death cases in the United States of America owing to the fact that the prescribed medication to certain bacterial infection is becoming less potent and ineffective. These bacteria include *Escherichia coli* (*E. coli*), *Klebsiella pneumonia* (*K. pneumonia*) and *Streptococcus pneumonia* (*S. pneumonia*) (Garau, 2010). The inclusion criteria for the report involved infants, toddlers, adolescents and the adults. The researchers further reported that age and sex classifications can generate technical errors in the course of data collection for the report for diagnosis of these infections especially in children. In Africa, there are many problems faced with the estimation of the prevalence of infectious diseases both in children and adults (Owoaje et al., 2016, Troeger et al., 2018). These challenges include the difficulty in providing an appropriate and definitive approach for the diagnosis, the magnified occurrence of bacterial manifestation of the disease, the shortcoming of a standard case definition and the inefficient treatment and treatment given to patients having such infection compared to other diseases. The diagnosis of bacterial infection is particularly problematic. There is tendency that the current problems with

bacterial infections will continue for the next decade if not properly managed with appropriate diagnostic tests and accurately prescribed medications. Recently, the latest WHO report encourages researchers to investigate the use of wide spectrum antibiotics against both Gram-negative and Gram-positive bacteria. Based on a WHO report in 2017, there is need to classify bacterial infections as non-curative until novel antibiotics are discovered. Following different local susceptibility tests, there may be hope for an excellent therapy with the use of traditional and complementary medicine.

Disease progression in humans is characterized by concurrent decline of cellular functions and reduced ability of the body's immune system to counteract both external and internal stressors (Panossian et al., 2021). The disease progression has linked up with generations of free radicals as a result of oxidative stress (Seyedsadjadi and Grant, 2021). Interestingly, several infections and diseases have been linked up with the increase in free radical species (ROS) and subsequently, oxidative stress (Khoder-Agha and Kietzmann, 2021). Not only that but oxidative stress and inflammation have been reported to be closely related to the pathophysiological processes (Song et al., 2021). Many studies have further emphasized the antioxidant potential of naturally occurring bioactive compounds which are also referred to as '**phytochemical compounds**' (Kumar et al., 2017, Forni et al., 2019, Salehi et al., 2021). High concentrations of phytochemicals that protect against free radical damage are found in plants and, specifically, in the herbs, fruits and vegetables (Nazir et al., 2021). The phytochemical compounds as found in different plants are considered active and are transformed into drug forms as food supplements. These supplements assist the physiological systems in each individual by acting as natural antioxidants when ingested.

Cancer is a known non-curable disease and poses a big threat to physiological functioning of cells, tissues, organs and different systems in humans. Therapeutic measures like gene therapy, nanomedicine and immunotherapy have been widely used by medical practitioner

for the treatment and management of cancer. Chemotherapy, surgery and even radiotherapy have been used as curative measures for cancer (Kiong et al., 2021). In fact, genetic engineering as a form of advancement to molecular biology is recently used to treat cancer by targeting the metastasized cells while other healthy cells remain unaffected (Orafaie et al., 2021). Many precisely designed anti-cancer drugs have been shown to destroy normal cells alongside with the cancer cells as a result of being cytotoxic. The bioactive compounds which are naturally obtained from the plant products have been hypothesized to be effective alternative anti-cancer agents since they are prone to produce better biosafety factor compared to synthetic drugs (Aniqa et al., 2021). The bioactive compounds obtained from plant products are efficient, safe and less toxic to normal cells compared to synthesized drugs. This is as extrapolated from the fact that quite a little amount of natural compounds produces beneficial effects than a single purified product (Lichota and Gwozdzinski, 2018, Dehelean et al., 2021). In addition, the naturally obtained plant products attenuate the growth of cancer cells through different mechanisms like expression of antioxidant as well as anti-mutagenic activities (Kim et al., 2020) due to the presence of bioactive ingredients like alkaloids, polyphenols, flavonoids etc.

Scientists have concerns about the safety use of synthetic phytochemicals due to the adverse effects on human health like liver damage as a result of their toxicity and carcinogenicity. Traditional medicinal plants are natural sources of phytochemicals that possess antioxidant properties (Lopa et al., 2021). Most phytochemicals are often beneficial because they facilitate good health by exhibiting both prophylactic and curative properties on pathologic conditions like neurogenic disorder, cardiovascular diseases and cancer. (Subedi et al., 2021, Tshabalala et al., 2021). WHO reported that quite a large number of world's population use herbal medicine to maintain their health status on daily basis (Sen and Chakraborty, 2017). Natural products are known to play important roles in the treatment or prevention of

infectious diseases and many approved drugs that are currently in public circulation are obtained from the traditional plants. Several studies have reported that medicinal plants play major roles in the management of many diseases due to their potency with lesser side effects and cost implications (Xie et al., 2018, Durgawale et al., 2019, Albaser et al., 2020). The enhancement of human health by the plant-based novel drugs is primarily the significant contribution of traditional medicinal plant (Durgawale et al., 2019).

Of the many identified traditional medicine for promoting health is the phenolic compound. The biological activities of phenolic compounds are widely reported in different studies. The functionality of the phenols is due to the presence of the phenol ring, with the existing number of both free and conjugated hydroxyl group attached to the aromatic ring. Apart from the phenols, sub-classes of flavonoids (flavanones, flavonols, isoflavones, flavan-3-ols, and anthocyanidins), have attracted a lot of attention because of their positive effects on diseases (Chiocchio et al., 2021). Flavonoids are known antioxidants and are able to scavenge free radicals (Benot-Dominguez et al., 2021). Research studies have been limited to whether or not plant extracts could cause bacterial growth inhibition or death. Meanwhile, this is just a single aspect of a plant's curative potential. Of recent, there have been many calls for research studies which should be designed to investigate natural products as a non-toxic and a novel source for therapeutic and anti-pathogenic agent (Baloyi et al., 2019). Apart from the conventional antibacterial activities of medicinal plants, the quorum sensing inhibitory (anti-pathogenic) potential of these plants is a prominent function that is expected to be equally explored for the treatment of diseases. Medicinal plants are technically becoming important in developing countries for the treatment of diseases (Manandhar et al., 2019). There is need for better attention on quorum sensing inhibitory (QSI) potential of the bioactive ingredients found in the medicinal plants (Baloyi et al., 2019). There is a proper documentation of South African medicinal plants that are used to treat illness and diseases (Cock et al., 2018, Baloyi

et al., 2019, Vambe et al., 2018) and these plants are available in local markets within the country.

The active products from traditional plants are gaining broad interest among the scientists due to their inherent bioactive compounds and their nutritional and health related abilities (Nikmaram et al., 2018) which are quite beneficial to human beings. Medicinal plants are sources of raw materials for pharmaceutical drug formulations (WHO, 2014). In Africa, medicinal plants are accessible and very affordable to the rural populace compared to the highly expensive western medicine. Individuals living in rural communities depend mostly on medicinal plants as a source of nourishing diet (Mujuru et al., 2020). The biological active compounds like phytochemicals and nutrients are known to have beneficial effects on the human body's physiological functions (Williamson, 2017). Nearly 50% of mass population witin the age bracket (55-70) years the developing countries relies on herbal supplements which are incorporated into daily diets in order to meet their health demands (Tripathi et al., 2019). Presently, vegetables and fruits are the least expensive and readily available nutritional sources of proteins, vitamins and minerals in Nigeria (Adeyeye et al., 2018) and could add value to the health status in humans through their medicinal properties. Proximate analysis of medicinal plants and vegetables plays a valuable role in the assessment of plants' nutritional significance (Alam et al., 2020). Analysis of mineral elements is important to understand the nutritional values of medicinal plants. The elements present either at major or trace amounts are vital for the maintenance of good health status and their intake either in excess or in limited amounts can result into systemic malfunctioning and eventually a diseased state (Rocha et al., 2019).

Among different sources of living organisms like bacteria, fungi and plants, medicinal plants have emerged as one of the advantageous sources for the synthetic process of nanoparticles. Nano-technology is a newly explored and rapidly growing field of science and engineering.

This field has to do with the biosynthesis, design and structural manipulation of particle size within the range of 1-100 nm. There are many sectors where the use of nanoparticles are applicable such as food industries, telecommunication service, environmental management, hospitals, chemical industries and other health-care services providers etc. (Temizel-Sekeryan and Hicks, 2020). The obtained nanoparticles are particularly dependent on the type of metallic ion adopted in the course of biosynthesis and these include silver, gold and copper oxide. The adoption of plant extracts for the synthesis of nanoparticles is more advantageous compared to the use of biological materials as it does not involve the extensive maintenance of cell culture (Arya et al., 2018). The outstanding properties of metallic nanoparticles like good conductivity and stability as well as their effective antimicrobial properties cannot be under-emphasized. The researchers are getting attracted to silver nanoparticles for scientific purposes because of its observed biological activities which include agglomeration of the particles, surface chemistry, molecular shape and size, particle composition and morphology, surface chemistry and capping (Bélteky et al., 2019). Both physical and chemical properties of nanoparticles account for the bioavailability of therapeutic agents (Sepúlveda-Rivas et al., 2019, Campos et al., 2020). Therefore, the size and morphology are given consideration in the synthesis and development of silver nanoparticles in its industrial applications. Similarly, the synthesis of nanoparticles from the plant crude extracts is commonly and widely accepted among the scientists because of its advantages such as biosafety factor, less toxic and non-pathogenic, highly economical and ecologically friendly. The biomolecules found in the crude extracts of plant such as proteins, vitamins, enzymes, alkaloids and so on determine the silver ion reduction and stabilization in the biosynthesis of nanoparticles.

The green synthesis of various metallic nanoparticles was reported to exhibit various antimicrobial properties (Nisar et al., 2019). The precise mechanism through which nanoparticles exhibit the antimicrobial properties is yet to be scientifically identified.

However, some hypotheses indicate the possibilities the surface of the biosynthesized nanoparticles may be releasing toxic metallic ion (Nisar et al., 2019). There is another conceptualized idea that the released metallic ion from the surface of the nanoparticles may become highly reactive and thereby causing oxidative stress (Gao et al., 2021). At the moment, there are many studies revolving around the antimicrobial properties of biosynthesized nanoparticles especially the silver and gold metallic ions. The interaction of silver nanoparticles with the bacterial cell wall was reported to initiate the killing of bacterial cells (Rai et al., 2009). The Multidrug Resistance (MDR) of the pathogens is another challenge that needs to be overcome as the antibiotics already in circulation seem to have lost their efficacy. One major characteristic of the multidrug resistance bacteria is the formation of biofilm through cell-to-cell communication. Perhaps, there is need for an alternative and more effective therapy. An emerging method for production of a more potent antibiotic drug is the adoption of 'quorum sensing'. Quorum sensing inhibition (QSI) can help to inhibit the formation of biofilm. Therefore, focusing on a QS system is another avenue to explore as it appears important for effective antibacterial therapy. The synthesis of nanoparticles and quorum sensing inhibition may look more potent to initiate the production of a novel antibiotic drug but there is still the need to investigate their potential pharmacological applications

Many secondary metabolites found in medicinal plants are widely known for their medicinal activities. Alkaloids (Labanca et al., 2018), terpenoids (Bergman et al., 2019) and phenolic compounds give direct evidence of naturally-occurring plant bioactive products as a typical origin of the commonly circulated western medications. Some review articles give an overview on sample preparations (Tyc et al., 2017, Sakamoto et al., 2018) and characterization (Beran et al., 2019, Li et al., 2020). The selected plant species in the review articles were specifically known to contain secondary metabolites like alkaloids, phenols,

tannins etc. The expected secondary metabolites were obtained with the help of appropriate extraction and isolation paradigms.

Plants as well as the plant- extracted drugs are regarded as the oldest source of medications and have provided humanity with diverse and useful compounds towards the maintenance of good health status in human beings (Jain et al., 2020). Plants possess different types of bioactive compounds which are extracted, isolated and further incorporated into drug formulation and dosages (Baishya et al., 2020). Certain techniques like histochemistry and electron microscopy are now used to identify not just the compounds but also the morphological and molecular events that may be responsible for the mechanisms of action of the bioactive compounds as present in the traditional or medicinal plants (Khadhraoui et al., 2018). Histochemical analysis is quite useful for the study of plant secretory structures and the composition of their secretions. Each secretory structure has a tendency to produce more than one compound. Different types of histochemical tests can be performed to ascertain many metabolites in a plant (Badria and Aboelmaaty, 2019). These compounds are stored in specialized secretory plant structures (Huchelmann et al., 2017). Secretory structures are reported to be in the form of trichomes, glands, lactifiers and nectaries and are located in the reproductive and vegetative parts of the plant. Conformational changes occur in the secretory structures especially at different stages of plant development (Gigli-Bisceglia et al., 2020) and these changes may not just be observed with the naked eye only, but with the use of either light or electron microscope (Shao et al., 2020). The use of the electron microscope further helps to analyse the micro-morphological changes in different micro-features in plants (Ahmad et al., 2018). Both the scanning electron microscope (SEM) and transmission electron microscope (TEM) are known to provide high resolution imaging and thereby produce morphological structural and elemental information from the plants at high magnification.

Many studies emphasize the authenticity of the plant with much significance to the embedded morphological features (Evdokimova, 2017, Cetin et al., 2018, Amitrano et al., 2021). Identification of morphological features is very important for studying biological activities more importantly for the purpose of classification and determination of functional traits which mediate environmental interactions like gaseous exchange, water conductance and photosynthesis (Evdokimova, 2017). Several studies have shown that the micromorphology of leaf trichomes is taxonomically informative (Choi et al., 2020, Pereira et al., 2020, Song et al., 2020). However, trichome alone is reported not to be solely responsible for the secretion and storage of essential metabolites found in plants (Mishra et al., 2021). The species diversity of plants is reflected in the leaf trait and it is affected by environmental pressures or phylogenetic history (Bruy et al., 2018). Emanation of evolutionary convergence in plant species could be responsible for resemblance in leaf traits and technically dependent on environmental factors (Pickersgill, 2018). Morphological parameters of the different parts of plants are significant in plant taxonomy. Considering this, there are still some taxonomic disagreements among the plant scientists based on possession of special adaptive features in some group of plants (Bateman et al., 2021). The ultra-structures of medicinal plant-like secretory cells, trichomes and other specialized cells are important in plant taxonomy and phylogeny (Ohikhena et al., 2017).

Over the years, western medicine is only approved by the government management responsible for drug administration and control. However, the medical treatment for many ailments is very expensive and with side effects which are sometimes severe. Therefore, the use of alternative medicine helps to reduce the financial burden compared to the pressures from the western medicine competitors. In fact, many plants have been used as sources of alternative medicine and form part of human healthcare in the modern era (Shakya, 2016, Jamshidi-Kia et al., 2018). The plant family '*Ebenaceae*' has been reported for its medicinal

use with the exception of *Diospyros villosa* (*D. villosa*). The root of *D. villosa* plant has been found to be useful as a curative measure for oral and bowel disease. Meanwhile, this therapeutic potency of this plant is yet to be scientifically proven. Research on *D. villosa* is limited in comparison to others in the same genus. A comprehensive documentation of the genus *Diospyros* is yet to be done and likewise the possible application in phytomedicine has not been carried out. This, together with the dearth in knowledge focusing on *D. villosa* in South Africa, was the basis of the first rationale for this study.

Hence, this study sought to provide a wider experimentation into the phytochemical screening, functional biology and the morphological characteristics of *D. villosa*. This experimental study on *D. villosa* is novel and will contribute knowledge to the medicinal database of plants in South Africa. Observations made from this research study will further contribute to the existing knowledge and perhaps opens another pathway for medicinal plant research study.

1.1 Rationale of the study

Quite a number of antioxidants/antibacterial drugs are used for the treatment of infections and diseases. Most of them are not accessible to individuals with lower income. In fact, many individuals come down with side effects when administered the western drugs. The plant-based medicine may be another promising option to manage infectious diseases and its accompaning symptoms and complications, not solely for the readily availability but also for the lower cost implication and fewer or no side effects. As a result of the cost and side effects of many synthetic drugs, most of the developing countries have resulted in the use of natural products, particularly from medicinal plants as an alternative in the treatment of bacterial infection. Hence, there is need to verify and exploit the possible antibacterial and anticancer actions of this medicinal plant.

1.2 Scope and limitation of the study

This study was geared towards the folkloric and undocumented claims among the local Southern African women that *D. villosa* plant is used in the treatment of microbial infections with a view to validate the mechanism of actions and to identify the embedded bioactive compounds which may possibly be responsible for the antimicrobial/ antibacterial activities. The study further investigated the antimicrobial and quorum sensing inhibitory activities of the biosynthesized silver nanoparticles of *D. villosa* leaves and stem bark at different temperatures using *in vitro* models.

However, the study did not investigate the antimicrobial activities of *D. villosa* in an *in vivo* experimental paradigms due to the COVID-19 pandemic.

1.3 Aim and Objectives of this study

The aim of this research study was to investigate antioxidant, antimicrobial and anticancer effects of *D. villosa* leaves and stem bark using multi-mode studies and identify the possible bioactive ingredients in both its leaves and stem bark. Our hypothesis was that *D. villosa* plant will contain bioactive compounds secreted by special cells located either on the leaves or stem bark which may further have beneficial effects on some disease causative agents (bacteria).

The specific objectives are:

1. Phytochemical screening, antioxidant and antimicrobial activities of *D. villosa* leaves and stem extracts.
2. To evaluate the antioxidant, anti-microbial and quorum sensing inhibitory potential using green synthesis of silver nano particles from *D. villosa*.

3. To ascertain the micromorphology and histochemistry of the secretory structures of *D. villosa* leaves and stem bark.
4. To evaluate the cytotoxic effects of *D. villosa* leaves and stem bark extracts on cancer cells.

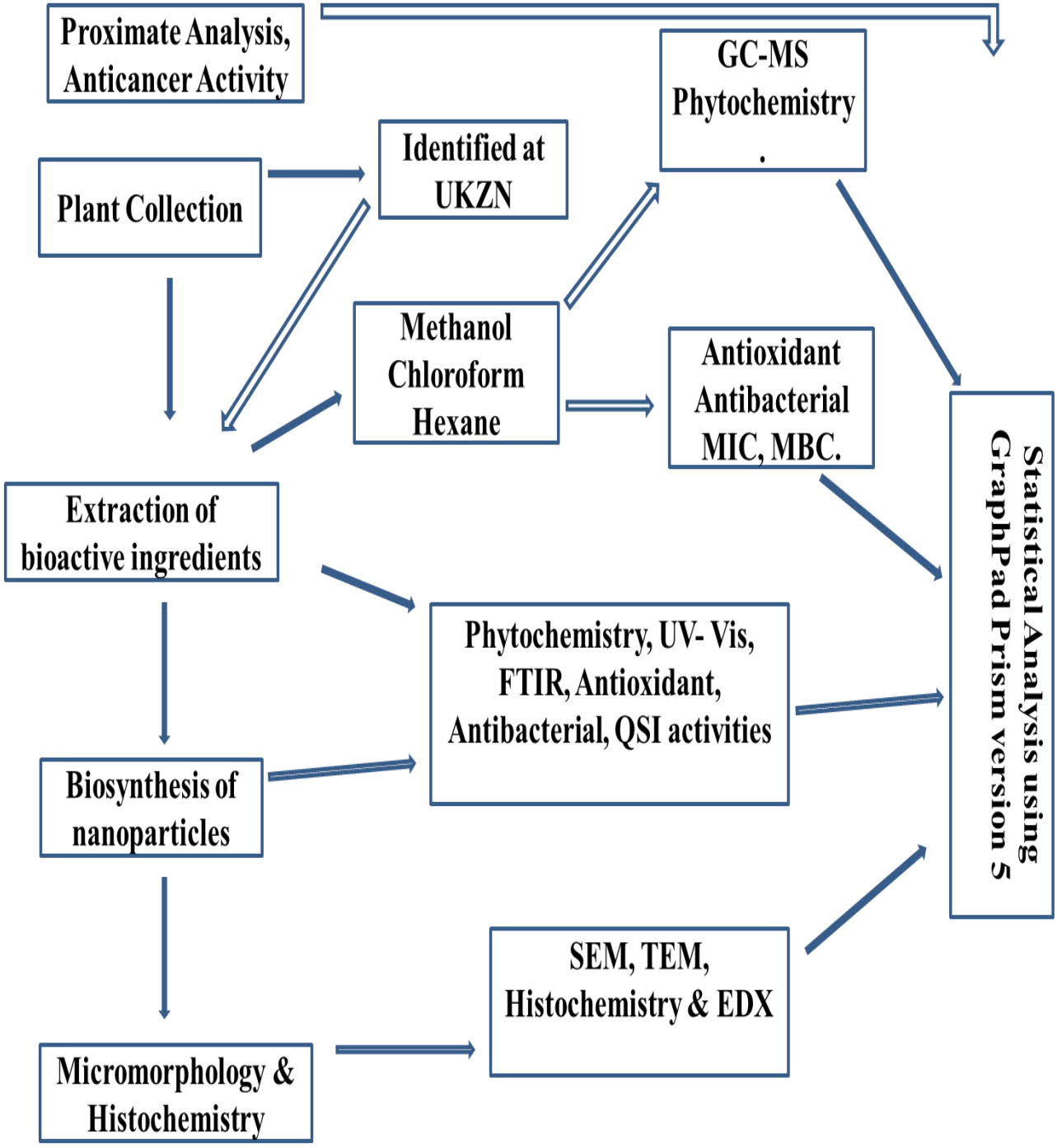

Figure 1: Flow diagram of the methodology employed in this study.

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CHAPTER TWO

Prologue

Diospyros villosa (L.) de Winter is a species that are widely distributed throughout the world but with limited record on its use for human purposes. This review elaborates more on the geographical distribution, other species list in the genus, morphology and anatomy, secretory ducts and trichomes, medicinal and chemical constituents, bioactive ingredients and their medicinal activities as well as the mechanism of action of the bioactive compounds.

‘**A review on the genus Diospyros with focus on *Diospyros villosa* (L.) De Winter’**

(This review is written in a manuscript form to be submitted to a journal)

A Review on the genus *Diospyros* with focus on *Diospyros villosa* (L.) De Winter.

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ABSTRACT

The genus *Diospyros* consists of many species which are distributed throughout the world. *Diospyros villosa* is a specie of the genus which is traditionally used in Mozambique and South Africa. There is no elaborate information about the holistic importance of the plants. A detailed study of the genus *Diospyros* with a targeted focus on *Diospyros villosa* plant was carried out. Information on the plants' morphology was taken as observed during harvest. Other information with respect to geographical distribution, secretory structures, chemical composition, embedded bioactive constituents and the associated mechanism of action of the bioactive constituents were acquired primarily by using the internet and scientific publications as well as many conference proceedings. Large spectra of phytochemicals with naphthoquinone origin were observed in the genus *Diospyros* and further identified with various medicinal and pharmacological activities. Different species of the genus were reported to be used for the treatment of ailments and diseases in humans. The genus *Diospyros* is of great medicinal importance throughout the world. This study provides a summarized insight into the genus and the potential use of the species as functional medicinal plants with bioactive compounds. *Diospyros villosa* is poorly studied in the genus and there is a need for further research to explore the useful holistic details of the species.

Keywords: *Diospyros villosa*; naphthoquinone; secretory; bioactive

2.0 Introduction

Quite a number of diseases are known to be threats to human health. The use of western medicine to cure these diseases seems unproductive in some cases. Even the productive conventional medications that are often used to manage human illness are costly and pose many side effects (Anand et al., 2017, Parvathaneni et al., 2019). Many individuals rather prefer to adopt the phytomedicine option than the western medication for the treatment and management of the illness and diseases (Amuka et al., 2013). The escalating interest in the use of medicinal plants is becoming competitive with the western medicine due to the positive outcomes, lesser side effects and cost-effectiveness of the traditional medicine. Plant extracts have been used over the years to cure ailments (Zhang et al., 2013). Exploiting traditional medicine systems has been identified as an acceptable and promising approach in this time of global rising costs of western medicine. There were debates concerning the herbal preparations in Madagascar, *Artemisia afra* which is very common throughout Africa as a potential cure for COVID-19 (Dandara et al., 2020). There is urgent need for curative measures for this disease which may involve opening to a wider array of medicinal plants with natural and herbal origin (Thomford et al., 2018).

Many plants were reported to produce secondary metabolites, also termed 'phytochemicals' (Mendoza and Silva, 2018, Kazmi et al., 2019, Lara et al., 2020). These are biologically active compounds which are required for the plants' metabolic reactions but also significantly involved in the pharmacological/ therapeutic activities of the plant (Alviano and Alviano, 2009). These secondary metabolites are either synthesized, stored or secreted in small quantities in specialised cells, termed secretory structures in the form of glands and trichomes (Kortbeek et al., 2019). The comprehensive description of the physiological-anatomy of the secretory vesicle where the metabolites are released usually helps to understand the medicinal properties of the metabolites (Bandu et al., 2019).

The family '*Ebenaceae*' (also known as the persimmon or ebony family) has four genera and about 490 species (Duangjai et al., 2009). This is further justifiable as Piedrahita and Synantherologist (2014) reported that there are almost 768 species in the family. *Ebenaceae* was first described in 1891 by Otto Kuntze (Index Nominum Genericorum) and it was one out of the 23 families in the order Ericales. *Diospyros villosa* was primarily assigned to the genus *Maba*. This genus was eventually included in the genus *Diospyros*. However, the name was conserved and regarded legitimate because the name '*Ebenaceae*' has become widely known and already used in botanical references such as Bentham and Hooker's Genera Plantarum, Engler (Bentham, 1883) and Prantl's Naturlichen Pflanzenfamilien and Hutchinson's Families of Flowering Plants (Munir, 1977).

Ebenaceae comprises mainly the trees and shrubs and are naturally distributed across Africa and other warmer regions of the world (Jahanbanifard et al., 2020). The significant characteristics of Ebenaceae encapsulate the astringency of the fruit at harvest and variable pollination (Mapes and Basurto, 2016, Hofmann, 2018, Sainge et al., 2019). Astringency is simply the acidic taste sensation produced by tannins in the leaves, bark or fruits of tree when chewed (Yahia, 2011). Species of the Ebenaceae are of diverse medicinal importance. *Diospyros abyssinica* is one of the species of the trees in the Ebenaceae family that grows in the southern part of Africa especially Mozambique and Zimbabwe. *Diospyros abyssinica* was reported to grow in the north-west region of Africa like Angola and Guinea (Mallavadhani et al., 1998). A type of concoction made from the *Diospyros abyssinica* plant is used as remedy for snakebites. Some known bioactive compounds isolated compounds from *Diospyros abyssinica* are the triterpenoids, betulin, betulinic acid and lupeol (Moghaddam et al., 2012). These bioactive compounds are known for different medicinal purposes (Kawase et al., 2003).

Diospyros mespiliformis is a known protected species of *Diospyros* in South Africa (Janick and Paull, 2008). The distribution of this plant spans from South Africa to Sudan and grows not only on fertile riverine areas but also on anthill soils (Janick and Paull, 2008). Subsistence farmers usually leave it standing in crop fields owing to its multiple uses (Palgrave et al., 1981). A research study on the potential uses of *Diospyros mespiliformis* focused mainly on the ethnomedical potential of the tree's leaf, bark and root extracts (Adzu et al., 2002). There are still several unresearched species of the Ebenaceae family which may have substantial scientific values/ importance. *Diospyros villosa* is a known species found in South Africa and in Mozambique. Till date, not many research studies have been reported about the importance of this plant species. In Mozambique, its root is reported to be used as a tooth-brush, for the treatment of oral infections (Cirera et al., 2010b), as purgatives (Watt and Breyerbrandwijk, 1932) and for the healing of fractures (Mallavadhani et al., 1998). This review, therefore, focuses on the phytochemistry, morphology and mechanisms of action of this genus with specific emphasis on *Diospyros villosa*.

2.1 An overview of the genus

The genus, *Diospyros* comprises more than 350 species (Mallavadhani et al., 1998) and is considered most important due to its economic importance when compared with others in the same family of Ebenaceae. *Diospyros* consists of both trees and shrubs and widely distributed across the world. Almost 41 species are found in India and mostly in evergreen forests of Deccan, Bengal and Assam, and a few are in northern part of India (Nematollahi et al., 2012, Ganapaty et al., 2006). The species of *Diospyros* generally known for its fruit-yielding is the '*Diospyros kaki*' (*D. kaki*). *D. kaki* originated in China and it has been cultivated in Japan for several years (Yonemori et al., 2008). The *D. kaki* fruits are large orange-red berries, very astringent until fully ripe. This is as a result of complete conversion of liquid tannin content

into crystals. The juice from the ripe fruit is sweet and palatable. Another notable fruit yielding species are *D. virginiana* in the United States of America (Visconti et al., 2017), *D. ebenaster* in Mexico (Baños et al., 2000), *D. lotus* in Central Asia and Middle East (Rauf et al., 2014), *D. mespiliformis* in Africa (Koné and Atindehou, 2008) and *D. melanoxylon* in India (Rath et al., 2009). The species are used for decoration purposes. The economic importance of *D. kaki* is obvious with its use in the manufacture of wooden boxes, furniture and mosaic in Japan. The trunk of *Diospyros* species can also be transformed into useful wooden pulps (Uphof, 1968). The fine pulps are made by shedding off the bark of felled trees. Fine ebonies can also be achieved from *D. dendo*, *D. mespiliformis*, *D. crassiflora*, *D. ebenum*, *D. melanoxylon*, *D. perrieri*, *D. haplostylis* and *D. celebica* (Mallavadhani et al., 1998). The duramen of some *Diospyros* species provides attractive colours. For instance, *D. chloroxylon* as found in India was green; *D. rubra* found in Mascarene Islands was red, *D. chryophyllos* found in Mascarene Islands was white and *D. hirsuta* was found variegated (Uphof, 1968).

Table I: Species List of the genus Diospyros (Mallavadhani et al., 1998)

Species	Country	Pharmacological uses
D. virginiana	America	i. Antihelmintic
		ii. Antifungal
		iii. Astringent
		iv. For internal haemorrhage
D. crassinervis	Bahamas	i. For bedwetting in children
D. paralea	Brazil	—

Species	Country	Pharmacological uses
D. decandra	Cambodia	i. It is used for insomnia
		ii. It is used for stimulating menstrual flow
		iii. It is used for producing vermicide and vermifuge
D. kaki	China	It is used for managing hiccups
D. lotus	Africa	i. It is used as sedative
		ii. It is used as antipyrexia
		iii. It is used to facilitates secretions
D. peregrina	Africa	i. It is used as antibacterial ii. It is used as anti-amoebic iii. It is used as an antiviral
D. loureiriana	East Africa	It is used as chewing stick
D. mespiliformis	Guinea	It is used as bactericidal agent
D. kaki	Japan	i. It is used against hiccups
		ii. It is used as an antihypertensive
		iii. It is used to treat Dyspnoea
D. gracilipes	Madagascar	It may cause skin lesion
D. ebenum	Malaysia	It is used in making astringent lotion
D. ismailii		It is used for curing skin diseases
D. kaki		i. It is used for treating snake bites

Species	Country	Pharmacological uses
		ii. It inactivates bacterial toxins iii. It increases life span iv. It decreases brain haemorrhage iv. It is used as free radical scavenger v. It inhibits lipid peroxidation vi. It gives hypotensive activity against urethane anaesthetized rats.
D. lotus		It is used to treat cough
D. rufa		It is used in the treatment of skin diseases
D. siamang		It is used in the treatment of skin diseases
D. toposoides		It is used in the treatment of skin diseases
D. wallichi		It is used in the treatment of skin diseases
D. usambarensis	Malawi	It is used to cure schistosomiasis
D. zombensis		It is used to cure schistosomiasis
D. leucomelas	Mauritius	It is used as anti-inflammatory agent
D. melanoxylon		It is used as an antibacterial
D. embryopteris	Phillippine	It reduced libido in 100% male rats
D. samoensis	Samoan Islands	i. It is used in making astringent lotion ii. It is used to treat tetanus and apoplectic attack
D. heudelotii	Sierra Leone	It is used as chewing stick

Species	Country	Pharmacological uses
D. hirsute	South Africa	i. It is used as purgative
		ii. It is used to treat gonorrhoea
D. lucida		It is used to relieve excessive menstrual pain
D. loureiriana		It is used as chewing stick
D.villosa		i. It is used as purgative
		ii. It is used for healing fractures
D. quaesita	Sri Lanka	It is used against asthma
D. morrisiana	Taiwan	It is used as an antibiotic
D. mespiliformis		It is used as bactericidal agent
D. verrucosa		It is used as antileprosy
D. mollis	Thailand	It is used as an anthelmintic agent
D. rhodocalyx		i. It is used as antidiarrhoeal
		ii. It is used as an antidiuretic agent
		iii. It is used to control bleeding
		iv. It is used against abdominal discomfort
		v. It is used for symptomatic relief of leucorrhoea
		vi. It is used to treat parasitic infection, abscess and renal disease
D. barteri	West Africa	It is used as chewing stick

Species	Country	Pharmacological uses
D. elliotii		It is used as chewing stick
D. tricolor		It is used as chewing stick
D. lycioides	Zambia	It is used as chewing stick

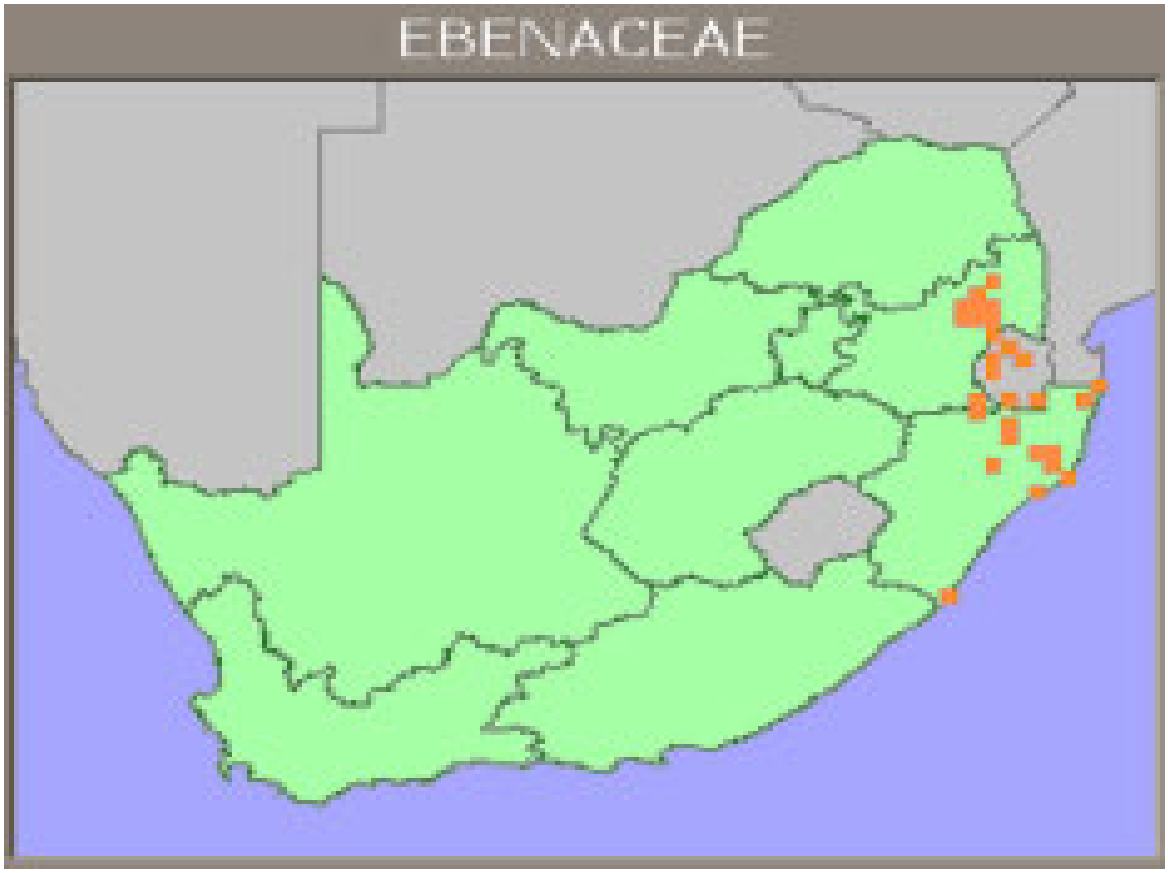

Figure 1: Distribution of genus *Diospyros* in South Africa (www.redlist.sanbi.org)

2.2 Classification of *Diospyros villosa* (Foden and Potter, 2005; www.ispotnature.org)

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Ericales

Family: Ebenaceae

Genus: *Diospyros*

Species: *Diospyros villosa*

Common names: Hairy Star-apple, Flora of Mozambique

2.3 Morphology and Anatomy.

Diospyros villosa is a perennial, bushy evergreen plant (Fig. 2) with a height range 1-4 m or greater or a rhizomatous suffrutex c. 0.5 m high. The leaves are chartaceous, drying dull brown above and much paler beneath. The dimension of the leaves' lamina is averagely 3 cm long, 1·5- 6·5 cm wide and 3·5 cm long. The shape of the leaves is always obovate but sometimes appears oblong. The leaves apex is usually broadly rounded and slightly emarginated and sometimes obtuse. The leaves' base is often in cordate or round shape. The lower surface of the leaves can either be sparsely or densely pubescent with long, slender, appressed or spreading hairs but rarely tomentose.

In addition, the calyx of the leaves is always up to 0·7 cm long, cleft almost to the base. The lobes are either ovate or lanceolate, fulvous or tomentose hairs and the margins are often reduplicated. Its corolla is usually up to 1·2 cm long and deeply divided. The lobes of the corolla are oblong, reflexed and broadly acute at the apex. The filaments are usually 0.05 cm long and glabrous while anthers are 0.35 cm long, lanceolate and densely strigose-setose. The pistillodes are described as conoidal, fulvous-tomentose. There are ten locules having common style, short, stout, densely and puberulous. The five branches are long and glabrous distally. The female locules are usually 10 having common style, short, stout, densely

puberulous, the five branches are long and glabrous distally. The female flowers are similar to the male but smaller in size. There are ten staminodes of about 0.2 cm long with glabrous filaments. The ovary is 0.3 cm long, ovoid or conoidal in shape, five angled with densely strigose-tomentose hairs, especially on the angles. Its fruit is 3 cm long and 3.8 cm wide. It is further described as depressed-globose, tardily dehiscent densely hispid–tomentose hairs with long, deciduous bristles. Its fruit is depressed-globose, tardily dehiscent, with densely hispid-tomentose hairs having long, deciduous bristles and 3 cm long and 3.8 cm wide. Fruiting calyces are strongly accrescent, closely surrounding but not completely concealing the fruit except when young. Its lobes are up to 3cm long and 2 cm wide, ovate in shape and densely puberulous with prominent longitudinal nerves. The seeds are also 3-8 in number and up to 0.3 cm long, dull brown and the endosperm is considered smooth.

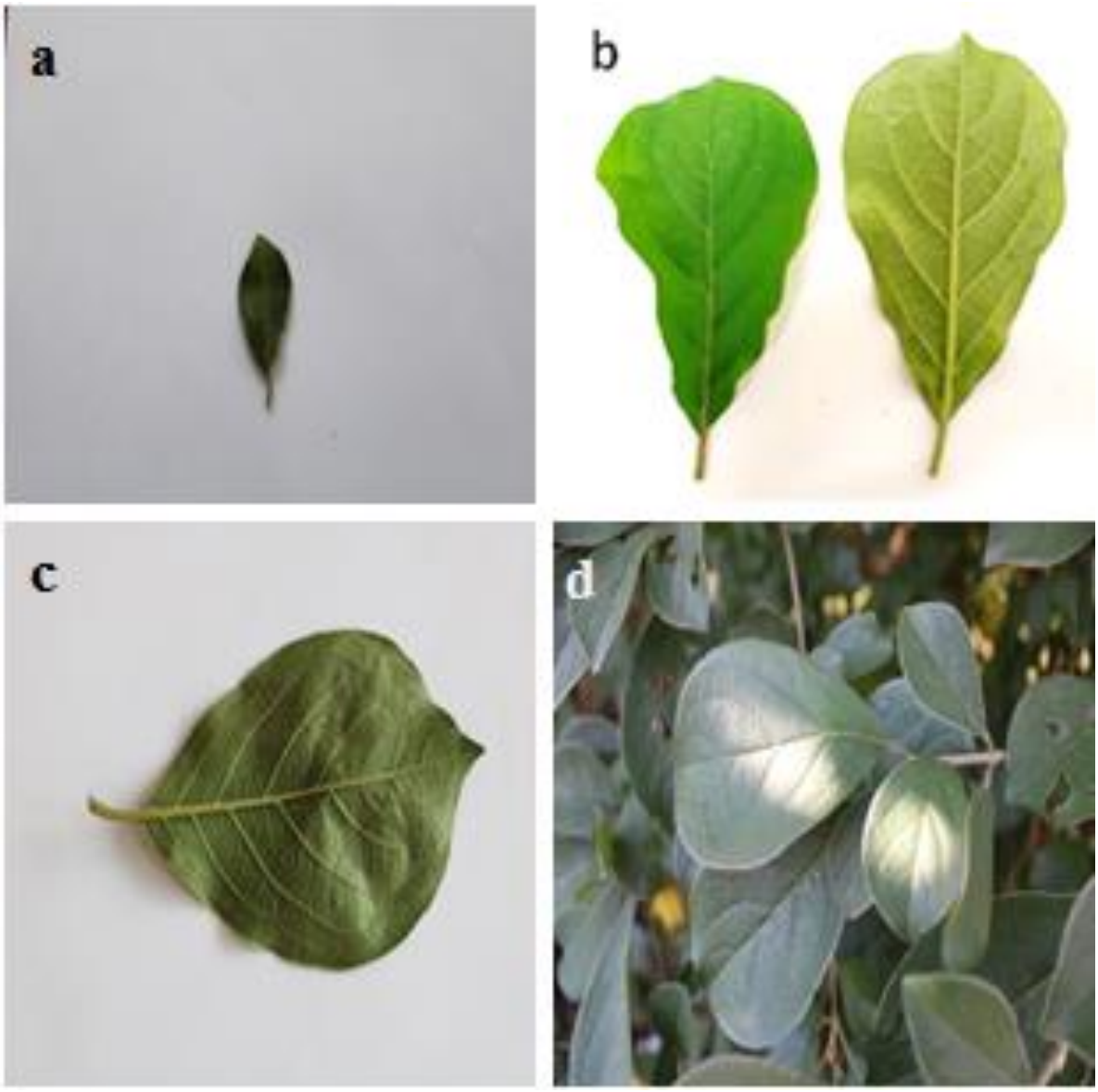

Figure 2: *Diospyros villosa*: a) Emergent leaves b) Young leaves c) Mature leaves d) Bushy evergreen tree image captured at the University of KwaZulu-Natal, Westville, Durban, South Africa.

2.4 Secretory Structures

Various substances synthesised in the plants are stored in the special cells and are released by the plant when necessary. The physiological importance of these secretory products is unquantifiable. One major role of the products is to allow the release of metabolites at a targeted area on the plant where activities for either plant's defense or nourishment would be

required. The inherent characteristic of the secretory structure is specific to the plant family (Figueiredo et al., 2014). Nevertheless, different research studies reported that each plant has the tendency to have more than a single type of secretory structure (Lange, 2015). Also, these secretory structures do not always develop synchronously and neither do they always release the same metabolites. There is possibility that they have different secretory processes. Sometimes, the released secretory constituents may be the same. The location of the secretory structure is a primary determinant to the function of the released secretory products (Maffei, 2010).

Table II: Secretory ducts in named *Diospyros* genus

External secretory ducts	
Trichomes	*Diospyros kaki* (Paudel and Heo, 2018)
Gums ducts	*Diospyros melanoxylon Roxb* (Singh and Bothara, 2014).
Lactiferous ducts	*Diospyros mespiliformis* (Upadhyay, 2015)
Internal secretory ducts	
Idioblasts	*Diospyros ehretioides* (Utsunomiya et al., 1998) *Diospyros ramulosa* (Kluge and Tessmer, 2018)
Cavities	*Diospyros inaki* (George et al., 1994) *Diospyros blancoi* (Jo et al., 2016)
Ducts/Canal	*Diospyros crassifolia* (Giachi et al., 2016)

2.5 Trichomes

Trichomes are fine, delicate structures that differentiate from the epidermal cells in the aerial parts of the plants. They are often referred to as the 'epidermal appendages' and the descriptive criteria for the trichomes are quite enormous. They can either be single-celled or

multicellular, curved or straight and non-secretory or glandular (Werker, 2000). However, the most typical basis for the classification of trichomes is the presence or absence of glands. Glandular trichomes encapsulate varieties of shapes and structures but with the frequent significant presence of metabolically active cells that secrete or store large metabolites. The glandular trichomes are further sub-divided into different types, viz peltate and capitate trichomes. The secretory products present in the cytoplasm of secretory cells were reported to be transport through the rough endoplasmic reticulum and further passage of these products to the external environment allows the fusion of dictyosomal vesicles with the cell membrane (Huang et al., 2008). There also were research studies compared the efficiency of different trichomes and suggested that the defensive strategy against arthropods pests is a function of glandular trichomes efficiency (Smith and Clement, 2012, Wheeler Jr and Krimmel, 2015). In addition, some studies further reported that the trichomes are involved in the secretion of wax which is majorly made up of flavonoid aglycones (Wollenweber and Schneider, 2000). Glandular trichomes are predominantly found in the angiosperms and are found in several plants prototypes (Barthlott et al., 2017). Capitate trichomes can be sub-divided into two based on the stalk's dimensions, the glandular head morphology and the secretion process (Chiniga Kemparaju, 2018). The capitate trichomes with short stalk have a single basal cell. The short stalk cell is better described to have hardened lateral walls with head having either ovoid or globoid shape. The secretory product in the short-stalked capitate trichomes accumulates within the apical cells and in a subcuticular space and exuded through cuticle micropores (Ranjini, 2015). Neither cuticle rupture nor pores do occur in the short-stalked capitate trichomes. Capitate trichomes possess a single basal cell, long celled stalk and a bulb-shaped head. The lower stalk cells are conical in appearance and are thick-walled, exhibiting a smooth or warty surface. The secretory head which is usually supported by a neck-cell of narrow diameter and cutinized side-wall develops a large and spherical

subcuticular space where the secretion accumulates temporarily. However, cuticle detachment occurs only at the globular region of the glandular cell. The cuticle adheres closely to the cell wall towards the base, especially at the point which corresponds to the narrowest region of the cell. Also, the long neck comprises the neck cell and a narrow lower region. At maturity, the short-stalked capitate trichomes are usually about 40 μm in height whereas long-stalked is about 150 μm. The underneath diameters of trichomes' heads are 25 and 35 μm respectively.

Classification of glandular trichomes is also dependent on the nature and type of secreted compounds (Barthlott et al., 2017). It can be hydrophilic, lipophilic, proteins, poly or monosaccharides. The embedded compounds in turn, are released once the cuticle is ruptured naturally or by the influence of surrounding insects (Tissier, 2012). On the contrary, capitate trichomes secrete non-volatile compounds directly onto the surface of the trichome (Schilmiller et al., 2008). The sticky droplets, in many cases are conspicuous at the tip of the trichomes and attractive to insects and other small arthropods (Fobes et al., 1985).

Digitiform trichome is another prototype of glandular trichome and it consists of three to four cells when viewed under electron microscopy (Tissier, 2012). However, its glandular apical cells have rounded tips and thin walls, unlike the basal cells that are always thick-walled and more vacuolated (Gazzoni, 2016). Meanwhile, non-glandular trichomes can as well be found existing on the aerial vegetative parts and within the plant tissues. They can be described as flattened, branched unicellular or multicellular hairs. They can also be characterised as branched and shaggy hairs because of the presence of the base and two or more contiguous rows of cells (Barboza et al., 2016). There is a tendency that different types of trichomes occur concurrently in a plant. The diversity of trichomes is solidly based on the metabolic characteristics of compounds produced.

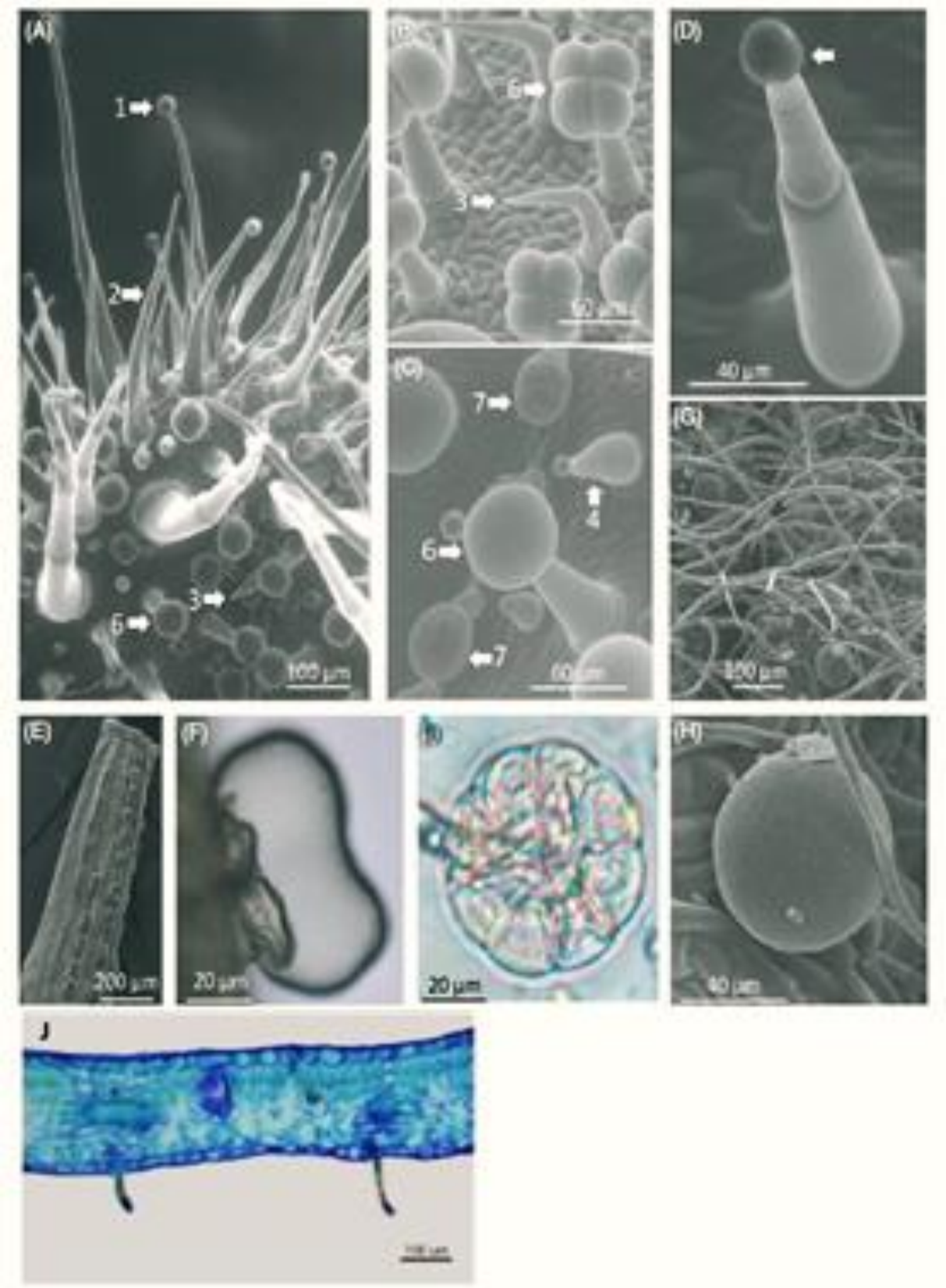

Figure 3: A sample of plant glandular trichome diversity.

A. Scanning electron microscopy (SEM) image of a young wild leaf. This picture illustrates the complexity of the leaf trichome landscape. Five different types of trichomes. Type 1, tall glandular trichomes with a single secretory cell; Type 2, tall non-glandular trichome; Type 3, short hooked non-glandular trichome; Type 6, glandular trichomes with four head cells; Type 7, short-glandular trichomes.
B. Detail of a Type 6 and Type 3 trichomes.
C. Detail of a Type 4, Type 6 and Type 3 trichomes. The four glandular cells of Type 6 trichomes are enveloped in a peri-cellular cuticle.
D. Capitate trichomes, similar to Type 4 trichomes. A droplet of exudate can be seen on the side of glandular head (white arrow).
E. SEM image showing files of glandular trichomes.
F. A close-up view of the same type of glandular trichome in light microscopy showing large subcuticular cavity where hydrophobic volatile compounds are stored, similar to the peltate trichomes.
G. SEM image of a leaf, showing peltate trichomes in a network of hairs.
H, I. Detailed view of a peltate trichome, showing the envelope delimiting the subcuticular storage space (H) and the plate of eight glandular cells (I).
J. Cross section of leaf showing unicellular trichome in *Diospyros kaki* (Paudel and Heo, 2018).

2.6 Chemical Composition

The use of medicinal plants is not only restricted to the narrow boundaries of foods and ornaments only but also attributed to therapeutic applications (Araújo et al., 2018). Plants naturally produce phytochemicals that can significantly contribute to the general healing process (Airaodion et al., 2019). There are variety of phytochemicals, including tannins, glycosides, terpenoids, naphthoquinones and phenols. However, this review is limited to phytochemicals that were reported to be found in *Diospyros* genus specifically *D. villosa*.

2.7 Active compounds

Out of the numerous identified species of *Diospyros*, more than 130 species have been screened for bioactive compounds. Certain bioactive compounds were reported to be identified in the *D. villosa* root, namely naphthoquinones, triterpenes, tannins and β-Sitosterol (Cirera et al., 2010a).

Table III: Bioactive compounds present in the root extracts of *Diospyros villosa*

Compounds	Medicinal/ Pharmacological uses
1,4-Naphthoquinones	Antineoplastic (Qiu et al., 2018); antifungal (de Oliveira et al., 2017); antidiabetic (Bansal and Anurag, 2019).
Triterpenes	Neuroprotective (Zhang et al., 2018); antibacterial (El-Sayed et al., 2016); antinociceptive and anti-inflammatory (Bednarczyk-Cwynar et al., 2016)
Tannins	Antimicrobial (Shukla and Bhathena, 2016); antioxidant (Sagbo et al., 2017); hepatoprotective (Kinoshita et al., 2007); cardioprotective (Smeriglio et al., 2017).
β-Sitosterol	Antidiabetic (Gupta et al., 2011); antihelmintic (Deepak et al., 2002); anti-inflammatory (Loizou et al., 2010); anti-Alzheimer (Ayaz et al., 2017); analgesics (Dighe et al., 2016); anticancer (Novotny et al., 2017).
Diospyrin	Antileishmanial(Hazra et al., 2013); antitumor (Pal et al., 1996).

2.7.1 Naphthoquinones

1, 4-naphthoquinone is a precursor to many natural compounds found in the *Diospyros* plant species (Lillie et al., 1976, Suwama et al., 2018). In the genus *Diospyros*, 1, 4-naphthoquinones is a very active compound with a wide array of biological potentials (Nematollahi et al., 2012). Nearly all the naphthoquinones of this genus consist mostly of monomers, dimers and few of them are trimmers and tetramers (Nematollahi et al., 2012). These 1, 4-naphthoquinones compounds are identified with peculiar characteristics for their pharmacological/ medicinal effects. 5-Hydroxy-2-methyl-1 and 4-naphthoquinone, also

referred to as ‘Plumbagin’ is a compound isolated from certain Diospyros species, having unique antibacterial activity against both Gram-negative and Gram-positive bacteria (Rasamison et al., 2016). Similar to Plumbagin is ‘Diospyrin’- a bisnaphthoquinonoid compound derived from *Diospyros* species with a broad spectrum of antibacterial effect (Sharma, 2017). In addition to these compounds is the ‘Isodiospyrin’. Isodiospyrin is unsymmetrical dimer of 7-methyljuglone which was also found in the species of *Diospyros* (Shwe et al., 2019). The Gram-positive antibacterial activity of Isodiospyrin was much more potent than diospyrin (Ajose et al., 2017). Diosquinone, Isodiospyrol, Maritinone, 7-methyljuglone and more of the naphthoquinone derivatives have been identified in *Diospyros* species and further reported to possess strong pharmacological effects.

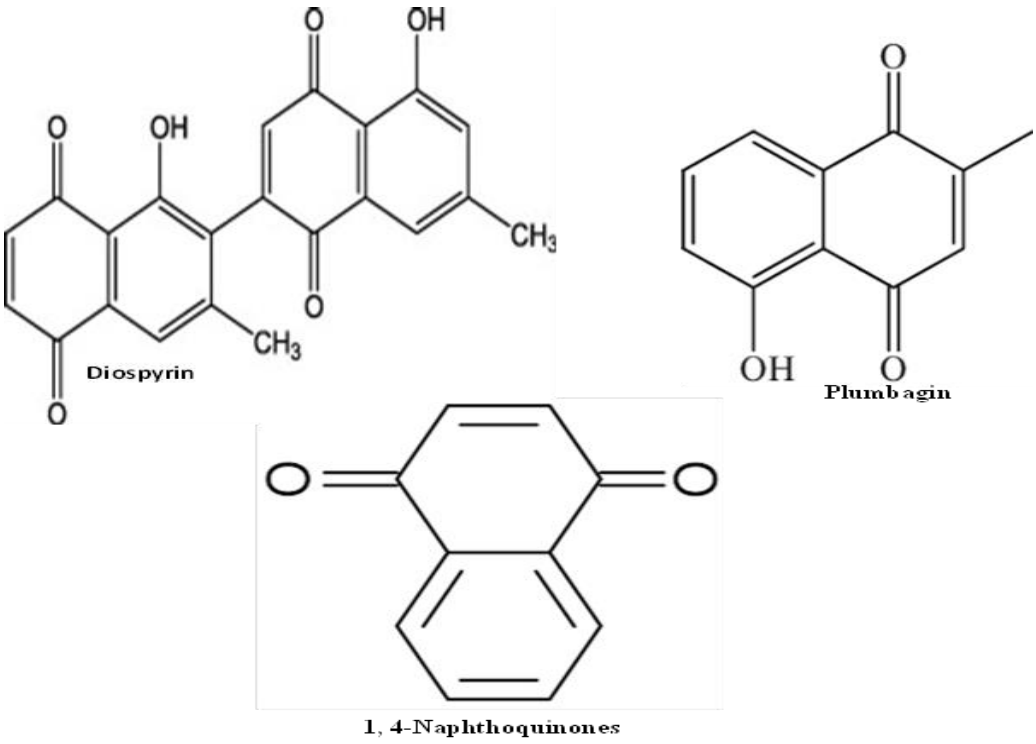

Figure 4. Structure of 1,4-Naphthoquinone and its derivatives (pubchem.ncbi.nlm.nih.gov)

2.7.2 Mechanism of actions of Naphthoquinones

The primary mechanism by which naphthoquinone exerts its action is via the release of apoptotic inducing factor (Pieretti et al., 2013). The apoptotic activity of the naphthoquinone appears to be mediated through a combined enhancement of radical oxygen species (ROS) and topoisomerase inhibition which ultimately leads to cell death and DNA damage (Ríos-Luci et al., 2012). A further multi-target profile for the naphthoquinone derivatives revealed its potent defence mechanism with their cytotoxicity like the inhibition of ororate synthesis, inhibition of thymidine incorporation to DNA replication by topoisomerase I and/or topoisomerase II cleavage (Babula et al., 2009).

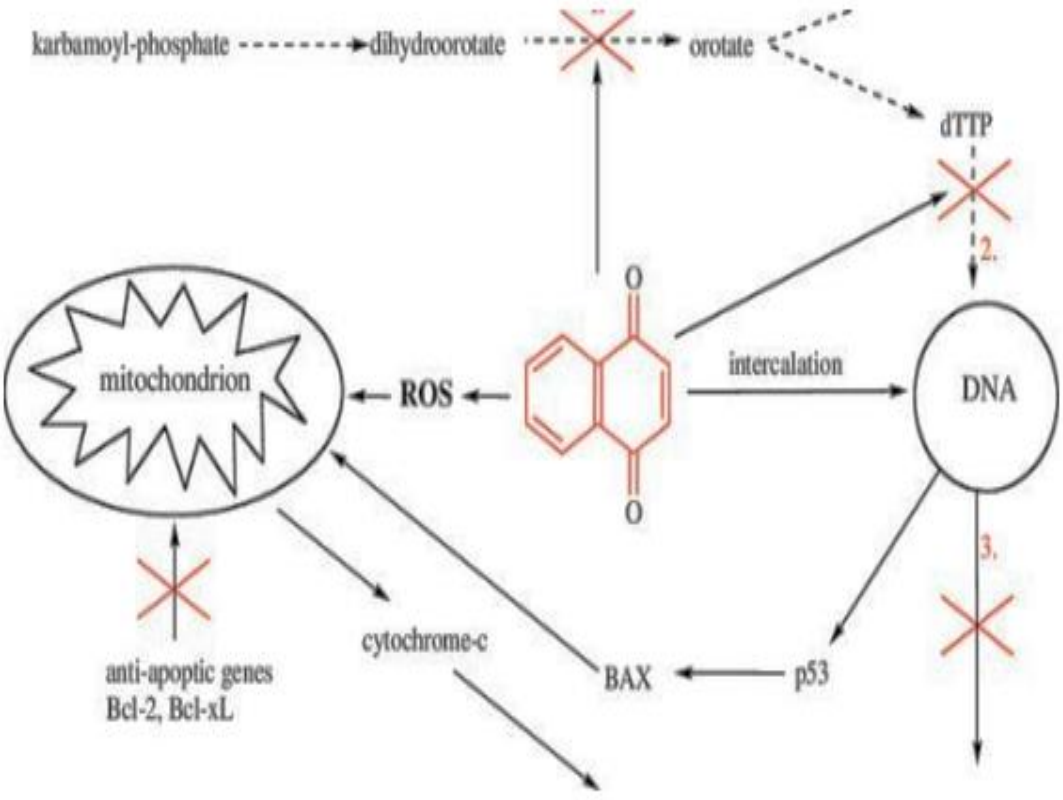

Figure 5: Mechanism of action of 1,4-Naphthoquinone derivatives (Babula et al., 2009)

2.7.3 Cytotoxic activities

Redox cycling, as well as the potent electrophilicity of naphthoquinones were reported to be responsible for their oxidative reactions against prokaryotic cells. Several studies further explained the redox reaction of the naphthoquinones through the one-electron reduction by flavoenzymes (Maldonado et al., 2016, Smithson et al., 2016). This reduction process perhaps shifts the toxicity level of the naphthaquinones through the production of semiquinones . Meanwhile, the biological activities are maintained through oxidation by transferring the electron to oxygen (Maldonado et al., 2016). A recent publication exists on the cytotoxic effect of naphthoquinones (Rivera-Ávalos et al., 2019).

2.7.4 Leishmanicidal activities

The findings of antileihmanial activities of 1,4-naphthoqinones suggested that this compound initiated apoptosis-like death in Leishmania. This mechanism indicated a new target for the treatment of Leishmanial disease (Mendonça et al., 2018). Further studies have also shown the leishmanicidal property of naphthoquinones. Eight substitutes of bis-2-hydroxy-1, 4-naphthoquinone derivatives indicated the inhibition of *L.amazonensis* and *L.braziliensis* promastigotes (De Araújo et al., 2014). In addition, another derivative of naphthoquinone as reported by Souza-Silva et al. (2014) showed that the parasitic effect of *Leishmania sp*. both morphological phases was entirely suppressed (Souza-Silva et al., 2014).

2.7.5 Trypanocidal activities

Krishnan et al. reported that certain derivatives of naphthoquinones exhibit the effects partly through the alkylation of the exposed thiol residues on topoisomerase II-DNA complexes which are quite independent of ATP (Krishnan and Bastow, 2001). It was further explained that the naphthoquinone derivatives inhibited the aforementioned enzyme by inducing its

detachment and religation from DNA in the presence of ATP. The standard mechanism of naphthoquinone derivatives inhibiting topoisomerase II indicated a novel pathway to target the enzyme with potential for anticancer drug design.

2.7.6 Antifungal activities

Bis-naphthoquinone is known to possess two naphthoquinones moieties and was reported to show potent activity against *Candida albicans* (ATCC 25555) with its minimum concentration at 0.09 μg/mL and its minimal fungicidal concentration at 0.17 μg/mL with both fluconazole and ketoconazole used as the standard (Elansary et al., 2016). The physiological mechanism of action is via the oxidative stress through the formation of reactive oxygen species. The naphthoquinones were reported to alter the integrity of the plasma membrane as well as the permeability (Allochio et al., 2016). The large alkyl chain of the naphthoquinone molecules was further identified to have fostered the direct interaction with the membrane.

2.7.7 Antioxidant activities

Soto-Maldonado et al. (2019) reported that the naphthoquinone derivatives were capable of inhibiting free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) by 35.8% to 85.2%. The 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ABTS scavenging ability of the naphthoquinones was further reported higher than the ascorbic acid (Mathiyazhagan et al., 2017). The free radical scavenging ability was attributed to the presence of phenolic –OH group. The naphthoquinones are irreversibly oxidized in redox processes and their potential to further generate reactive oxygen species (ROS) in reactions indicated by the colour change spectrophotometry showed the property that render these compounds reliable and useful for the development of biomolecules that may be later used in various therapeutic settings (Masek et al., 2019).

2.8 Medicinal constituents of plant extracts.

In spite of the strong medicinal values of *Diospyros* genus, there were just a few reports on the therapeutic use of *D. villosa* plant extracts. However, Cirera et al. (2010) reported that *D. villosa* root was used as a toothbrush to treat oral infection in Mozambique and sold in domestic market under the appearance of a chopped root (Cirera et al., 2010b). In the polar extract of *D. villosa* root extract, two naphthoquinones as well as five triterpenes and β-Sitosterol were identified by TLC (Cirera et al., 2010b). Not only that, the HPLC profile further identified four phenolic compounds along with the already identified naphthoquinones and triterpenes. In addition to these compounds, two naphthoquinones were found in the non-polar extract of the *D. villosa* root extract.

2.9 Conclusion

The genus *Diospyros* has many medicinally important species throughout the world. However, there is a lack in knowledge on certain species within the genus which in turn allows the chance for a future research. This review gives a positive impression about *D. villosa* as a useful medicinal plant, especially with the embedded phytochemicals. This species is used in-most parts of the world. Meanwhile, there is no scientific report about the medicinal efficacies of the plant. *D. villosa* presented a broad range of therapeutic applications for treating human ailments. Hence, there is need to explore and identify traditional therapeutic knowledge as well as its biological effects. Also, use of traditional medicine is becoming conventional and a better therapeutic alternative for disease management due to the lesser side effects, its availability and cost implication compared with the western medicine.

2.10 Acknowledgement

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2.11. Conflict of interest

The authors declare that there are no conflicts of interest.

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CHAPTER THREE

Prologue

Manuscript 1

Following a report of the use of *Diospyros villosa* (L.) de Winter as tooth brush for dental care in certain areas of Southern Africa, this scientific research was conducted in an effort to unveil the phytochemical constituents of *D. villosa* leaves and stem bark as well as to determine its potency against free radical species. We further investigated the antimicrobial activities of this plant as well the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

"Phytochemical screening, antioxidant and antimicrobial activities of *Diospyros villosa* (L.) De Winter leaves and stem bark extract"

The current manuscript has been sent for publication in **Turkish Journal of Botany.** We are now awaiting the outcome of the publication of the article by the editor. The chapter is written in the style of the journal as per the guide to authors document from the journal

Phytochemical screening, antioxidant and antimicrobial activities of *Diospyros villosa* (L.) De Winter leaves and stem bark extracts.

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ABSTRACT

This study aimed to evaluate the phytochemical composition, antioxidant capacity and antimicrobial effects of *Diospyros villosa* (L.) De Winter leaves and stem bark. The extracts were obtained using different media (methanol, chloroform and hexane). DPPH and FRAP methods were used to evaluate the antioxidant activity and Folin-Ciocalteu method was used to determine the total phenolic contents of the crude extracts. The antimicrobial effect of the extracts against five pathogenic bacteria was determined using MIC, MBC and agar well diffusion methods. Flavonoids, alkaloids and phenols were identified in *D. villosa* extracts. The mean concentrations of the methanolic leaves and stem bark extracts against DPPH providing 50% inhibition were 9.53 ± 0.25 μg ml^{-1} and 9.52 ± 0.30 μg mL^{-1} respectively. Also, total phenolic content within the test range of concentration was found to be 28.45 ± 0.50 mg of gallic acid equivalents per g of sample extract [mg g^{-1} (GAE)] (methanolic leaves extract) and 4.88 ± 0.36 mg g^{-1} (GAE) (methanolic stem bark extract). The methanolic leaves extracts further showed promising antimicrobial activity against *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* with inhibition zones of 18.0 ± 0.58, 23.5 ± 0.58, 20.0 ± 0.88 and 17.0 ± 2.0 mm respectively which are almost as high as the control. The results are suggestive that *D. villosa* leaves and stem bark are rich in bioactive compounds which could be a potential source of natural antioxidants and antibacterial agent for the treatment of pathogenic bacterial infections.

Keywords: DPPH; Free radicals; Minimum inhibitory concentration; Phenol content

3.0 Introduction

Accumulation of free radicals at high levels is attributed to many pathological conditions and chronic disease in humans (Sugamura and Keaney Jr, 2011). In this situation, humans race relies on the exogenous sources of anti-oxidants for additional support and therapeutic measures. Many studies have further shown that various plant species can circumvent the effects of oxidative damage incurred by the generation of free radicals (White et al., 2009, Ali et al., 2018, Das et al., 2019). High concentration of antioxidants in some plant species serve as the natural scavengers of free radicals (Pandey and Rizvi, 2009) whilst the identified anti-oxidants; flavonoids and phenolic compounds have a wide range of structural characteristics and diverse physiological effects (Balasundram et al., 2006). Bioactive compounds are also recognized for other multiple biological effects including anti-microbial/bactericidal properties (Jagani et al., 2009, Borrás-Linares et al., 2014).

Rashed et al. (2012) indicates that *Diospyros lotus* contains both gallic acid ($C_7H_6O_5$) and quercetin ($C_{15}H_{10}O_7$). *Diospyros montana* is also reported to contain 8-hydroxydiospyrin ($C_{22}H_{14}O_6$) (Rauf et al., 2017). These active compounds have diverse defensive responses against different microbial strains through the generation of hydrogen peroxide (Kumar et al., 2020) and through altering the permeability of the microbial membrane (Górniak et al., 2019, Singh and Singla, 2020). Following these reports, the idea of investigating a novel South African plant for its anti-microbial activity and generation of free radicals comes to mind. Perhaps, such plants may stand a better chance to alleviate the generation of free radicals and microbes in disease state.

Diospyros villosa (L.) De Winter is an African plant that occurs naturally in southern parts of the continent. *D. villosa* is a perennial, bushy evergreen plant, with a height range 1- 4 m. The leaves are chartaceous, drying dull brown above and much paler beneath. The dimension of the leaves' lamina is averagely 3 cm long and 1·5 cm - 6·5 cm wide. The shape of the leaves

is always obovate but sometimes appears oblong. The leaves apex is usually broadly rounded and slightly emarginated and sometimes, obtuse. The leaves' base is often in cordate or round shape. The roots of the plant are used locally as tooth-brush and to treat oral infection (Cirera et al., 2010b). This report gave an insight to the concept of this study, in such a way to provide a scientific evidence for the medicinal use of *D. villosa* plant in the pathogenesis of infection along the oral cavity. *Escherichia coli* was reported to be among the top pathogens that manifests in infections, which further results in cases of diarrhoeal illness (Brando et al., 2008). Other aerobic gram-negative bacteria like *Pseudomonas aeruginosa* and *Klebsiella pneumonia* were reported as opportunistic bacteria that further contribute to dental caries and sinusitis in orthodontic patients (Kitada et al., 2009, Flynn, 2019). Gram-positive bacteria like *Staphylococcus aureus* were reported to co-exist with other microbial strains in the inflamed cavities of the immunocompromised individuals and further induce bacterial reactivation in the infected cells (Vila et al., 2020). Vellappally et al. established the fact that streptococci were not the only Gram-positive bacteria responsible for the bloodstream infection but also, *S. aureus* and methicillin resistant *S. aureus* (MRSA) and were often isolated in the hollow cavities of the body. In fact, the scourge MRSA accounted for almost 20- 30% of all cases of oral infections (Vellappally et al., 2017).

At present, there are no studies using *D. villosa* plant extracts against these bacteria strains. Hence, this research study was then geared towards making a significant contribution to the present search being carried out to combat the microbial strains involved in microbial infections. And perhaps, contribute to the existing knowledge on antioxidant and antimicrobial properties of *D. villosa* extracts. To this end, the study is aimed at investigating the probable effect of *D. villosa* leaves and stem bark extracts on identified gram-negative and gram-positive bacteria strains in close association with human body infections.

3.1 Materials and Methods

3.2 Chemicals and reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH), gallic acid, acetic acid, Gentamicin and Streptomycin were purchased from Sigma-Aldrich (St. Louis, USA). Mueller Hilton agar media, Hexane, Methanol and Chloroform were purchased from Merck Chemical Co. (Durban, South Africa).

3.3 Plant collection

Fresh samples of mature leaves and stem bark of *D. villosa* were collected from KwaZulu-Natal, Durban, South Africa (29º 84′ 33.6″S, 31º 4′ 12″E). The plant was identified and a voucher specimen was deposited in the Ward Herbarium (01/18257) at the School of Life Sciences, University of KwaZulu-Natal. The collected plant parts (leaves and stem bark) were air-dried and pulverized into fine powder. The powdered samples were kept in a cool dry place for extraction purposes.

3.4 Plant extraction

Powdered samples of the plant weighing 8 g were heated to a temperature of 40 °C for 15min with 100 mL of 95% methanol in a round bottom flask attached to a Soxhlet apparatus. The crude extract was retained and the process was repeated thrice. Successive extractions using chloroform and hexane, respectively, were carried out after 30min intervals. The condensate was further evaporated to dryness under reduced pressure at 40 ºC in a rotary evaporator. The crude extract was stored at 4 ºC and used within 48 h for further tests. The extraction yield (%) = $\frac{Weight\ of\ the\ dry\ extract\ (g)}{Weight\ of\ sample\ used\ for\ the\ extracton\ (g)}$.

3.5 Qualitative phytochemical tests

The qualitative phytochemical constituent screening of the different extracts of the leaves and stem bark obtained from *D. villosa* was conducted using standard qualitative protocols (Harborne, 1973, Treare and Evans, 1985, Sofowora, 1993, Evans, 1997).

3.6 Gas chromatography-mass spectrometry (GC-MS) analysis

The extracts were filtered using Whatman filter paper No. 4 and thereafter through 0.22 µL membrane filter. The GC-MS analysis of the extracts were carried out using a GCMS-QP2010 Plus Shimadzu instrument, fitted with capillary chromatographic column of 30 cm × 0.25 mm ID × 0.25 µm film thickness of 5% phenylmethylsiloxane. The instrument was set to an initial temperature of 50 ºC, maintained for 1.5 min, then increased to 200 ºC at the rate of 4 ºC min^{-1}, then increased up to 300 ºC at the rate of 10 ºC min^{-1} held for 7 min. The injector and interface temperature were 240 and 220 ºC respectively. The helium flow rate was 1.2 mL min^{-1} and 2 µL of each of the crude extracts was solubilized and diluted ($\frac{1}{100}$) in their various extractant solvents (≥ 99%, GC grade, Sigma-Aldrich) and filtrated using a 0.22 µm filter, which was injected into the 'splitless' mode system. The mass spectral scan mode range was 40- 500 m/z, with a total running time of 30 min. The components were identified by comparing their mass spectra with data published in the data (Adams, 2007) and the Mass Spectral Search Program database of the National Institute of Standards and Technology, Washington DC, USA, (NIST 07).

3.7 Fourier Transform Infrared (FT-IR) analysis

FT-IR measurements were carried out to characterize the functional groups in the *D. villosa* leaves and stem bark extracts that are responsible for biochemical and molecular potential of the plants using spectrophotometer (Perkin Elmer 100 FT-IR, USA). The spectra were

scanned in the range of 4000- 400 cm^{-1} ranges at a resolution of 4 cm^{-1}. The samples were prepared by dispersing the extracts uniformly in a matrix of dry KBr, compressed to form transparent disc. KBr was used as a standard to analyse the samples.

3.8 Sensitivity test of leaves and stem bark extracts of *D. villosa* on test microorganisms

Antimicrobial activities of methanolic, chloroformic and hexanolic leaf and stem bark extracts of *D. villosa* were carried out using the agar well diffusion method. The discs were prepared using Whatman No. 1 and obtained by punching and putting in vial-bottles which were further sterilized in an oven at 150 °C for 15 min. The test microorganisms were resuscitated on the nutrient agar broth and further incubated at 37 °C overnight. The test microorganisms were also standardized at optical density of 0.1 at 625 nm using a UV-vis Spectrophotometer (Agilent Cary 60 Spectr., USA). Following this, 0.2 mL of the standardized test culture was added to 20 mL of molten Muller-Hilton agar and homogenized. This was then poured into sterile plates and allowed to solidify. Thereafter, the wells were aseptically bored into the inoculated Muller- Hilton agar plates using 6mm sterile cork-borer. The test solutions of extracts (100 µL) at graded concentration of 0.625, 1.25, 2.5, 5 and 10 mg mL^{-1} already dissolved in 10% DMSO were then introduced into each of the designated well on each plate, ensuring that no spillage occurred. A standardized amount (10 μgmL^{-1}) of Gentamycin and Streptomycin was introduced into the remaining cups on each plate to act as control for Gram-negative and Gram-positive bacteria strains respectively. The plates were left at room temperature for 1hr, allowed to diffuse into the medium, turned upside-down and thereafter incubated at 37°C for 16-18 hr in an incubator. Clear zones of inhibition were observed. Each extract's activity was tested in triplicate and the diameters of zones of inhibition were measured and recorded in millimetre.

3.9 Antioxidant activity

3.9.1 DPPH scavenging activity

The free radical scavenging activity of the extracts was determined by DPPH (1, 1-diphenyl-2-picrylhydrazyl) radicals as described (Barros et al., 2008). An aliquot of 3 ml of 0.004% DPPH solution in 95% ethanol and 0.1 mL of each plant extract and ascorbic acid at concentrations of 15, 30, 60, 120 and 240 μg mL^{-1} were mixed. The mixture was shaken well and allowed to reach a steady state at room temperature for 30 min. The decolourization of DPPH was determined by measuring the absorbance at 517 nm. The control was prepared using 0.1 mL of each constituents and double distilled water in the place of the plant extract or ascorbic acid. The percentage DPPH radical scavenging activity by the plant extracts was determined as thus: $\frac{Absorbance\ of\ Control - Absorbance\ of\ Test\ samples}{Absorbance\ of\ Control} \times 100.$

3.9.2 Ferric Reducing Antioxidant Potential (FRAP) Assay

The Ferric Reducing Potential assay was carried out according to the procedure described previously (Juntachote and Berghofer, 2005). Different concentrations (15, 30, 60, 120 and 240 μg mL^{-1}) of the extract (1 mL, in triplicates) were separately added to 2.5 mL of 0.2 M phosphate buffer pH 6 and 2.5 mL of potassium ferricyanide (1% w/v). The mixture was incubated for 20min at 50°C after which 2.5 ml of 10% trichloroacetic acid was added. An aliquot of 2.5 mL of each mixture was diluted twice with deionized water before adding 0.5 ml of 0.1% (w/v) $FeCl_3$. Absorbance was measured at 700 nm after 30min. Ascorbic acid was used as the positive control. The extract concentration providing 50% of absorbance (IC_{50}) was calculated from the graph of absorbance against the concentrations of the extract. Results were generated as thus: Scavenging Effect

$$(\%) = [\frac{Absorbance\ of\ Control\ at\ 700\ nm - Absorbance\ of\ sample\ at\ 700\ nm}{Absorbance\ of\ control\ at\ 700\ nm}] \times 100.$$

3.9.3 Total Phenolic Content (TPC)

TPC was determined by using the Folin-Ciocalteu colorimetric method with minor modifications (Shao et al., 2014). A volume of 0.1 mL of each sample extract was diluted to 3 mL with distilled water and 0.5 ml of freshly prepared Folin-Ciocalteu reagent was added to each sample extract. The mixture was incubated at room temperature for 3min, 2 mL of 20% sodium carbonate was added and the mixture was further incubated for 30min at room temperature. Total phenolic content was measured at 725 nm using spectrophotometer. Gallic acid was used as the positive control. Total phenol values were expressed as mg of gallic acid equivalents (GAE)/g of sample extracts.

3.10 Test microorganisms

The bacteria strains used were identified as American type collection culture strains obtained from the School of Pharmacy and Pharmacological Sciences, University of KwaZulu-Natal. Three Gram-negative bacteria namely: *E. coli* (ATCC 35218), *P. aeruginosa* (ATCC 27853), *K. pneumoniae* (ATCC 700603) and 2 gram-positive bacteria; *S. aureus* (ATCC 33591), MRSA (ATCC 43300) were used in this study.

3.10.1 Determination of minimum inhibitory concentrations of the *D. villosa* extracts on test microorganisms

The minimum inhibitory concentration (MIC) of the extracts was determined using the method as described by Akinpelu and Onakoya (2006). Two-fold dilution of the extract was prepared and 2 ml of different concentrations of the solution was added to 18 ml of pre-sterilized molten Mueller Hilton agar to give final concentrations regimes of 0.313 mg ml^{-1} to 0.01 mg ml^{-1}. The medium was then poured into sterile petri dishes and allowed to set. The

surface of the medium was allowed to dry before streaking with 18hrs old standardized bacterial cultures. The plates were then incubated at 37ºC for 48hrs, after which they were examined for the presence or absence of growth. The MIC was taken as the lowest concentration that prevents the bacterial growth.

3.10.2 Determination of minimum bactericidal concentration of the extracts on test microorganisms

The minimum bactericidal concentration of the extract was determined using (Ferrazzano et al., 2017). The inoculum was taken on the line of streaks without visible growth in the MIC assay and sub-cultured on freshly prepared nutrient agar and incubated at 37ºC for 48hr. The plates were later examined for the presence or absence of growth. The lowest concentration of the extracts that did not show any growth on a new set of plates was taken as the minimum bactericidal concentration of the extracts.

3.11 Statistical analysis

Results were expressed as means ± standard error. Statistical analysis was performed using Graph Pad Prism 5 (Graph Pad Software Inc., USA). All outcomes were compared with the control using both one-way and two-way analysis of variance (ANOVA) followed by Bonferroni Post Hoc analysis. Effects were considered statistically significant at p value $\leq$ 0.05.

3.12 Results

3.12.1 Phytochemical analysis

The yield of different extracts of *D. villosa* leaves and stem bark are given in Table I. It was observed that methanol extraction in the leaves produced a maximum yield of phytochemicals about 10.8%, whereas chloroform and hexane extraction in the leaves yielded 8.4% and 7.1% respectively. Similarly, the methanol extraction in the stem bark produced a yield of 9.2% meanwhile, chloroform and hexane extraction yielded 7.9% and 10.3% respectively. The results of the qualitative phytochemical screening of *D. villosa* leaves and stem bark extracts were shown in Table II. Phytochemical screening showed the presence of alkaloids, terpenoids and phenols in the methanol extracts of *D. villosa* leaves. In addition, the presence of flavonoids was further observed in the methanol extracts of *D. villosa* stem bark. Also, terpenoids, flavonoids and phenols were shown to be present in both the chloroformic and hexanolic stem extracts of *D. villosa*. All the extracts from the plant's leaves and stem cumulatively contain steroids, alkaloids, carbohydrates and saponins.

Table I: Yield of extracts of *D. villosa* leaves and stem bark

	Leaves extraction yield (%)	Stem bark extraction yield (%)
Methanol	10.8	9.2
Chloroform	8.4	7.9
Hexane	7.1	10.3

Table II: Phytochemical screening of the methanol, chloroform and hexane extracts of both leaves and stem bark of *D. villosa*.

	Name of test	Leaves			Stem bark		
		Methanol	Chloroform	Hexane	Methanol	Chloroform	Hexane
Alkaloids	Mayer's test	+++	-	-	+++	-	-
	Wagner's test	-	+	+	-	+	+
Protein	Dragendorff	-	+	+	-	+	+
Carbohydrates	Benedict's	+	-	-	+	-	-
Steroids	Lieberman-Burchard test	++	+	+	+	+	+
Coumarins	NaOH test	++	-	-	++	-	-
Saponin	Foam test	++	+	+	+++	+	+
Flavonoids	Lead acetate	+++	-	-	+++	-	++
Terpenoids	Salowski's	+++	-	-	++	+	++
Phenols	$FeCl_3$ test	+++	-	-	++	+	-

+ denotes present; - denotes absence

3.12.2 Gas chromatography-mass spectrometry (GC-MS) analysis

The GC–MS analysis of the crude extracts from the leaves and stem of *D. villosa* revealed the presence of a number of bio-active compounds such as phytol, n-hexadecanoic acid, Palmioleic acid, Eicosanoic acid and derivatives, ascorbic acid and derivatives etc. (Table III-VIII). The retention time (RT), compound name, molecular formular and peak height (%) are presented in Table III-VIII.

Table III: **Compounds identified in the chloroform extracts of *D. villosa* leaves by GC-MS spectral analysis**

S/N	RT	Compound Name	Molecular Formular	Height (%)
1.	3.801	1-Butene, 3-chloro-2-methyl-	C_5H_9cl	1.08
2.	4.254	1, 1-Dimethyl-3-chloropropanol	$C_5H_{11}clO$	0.87
3.	4.296	Pentanoic acid, 2-hydroxy-4-methyl-(S)-	$C_6H_{12}O_3$	7.45
4.	5.252	1-Butene,2,3,3-trimethyl	C_7H_{14}	0.93
5.	10.454	11-Methyldodecanol	$C_{13}H_{28}O$	1.14
6.	10.557	11-Methyldodecanol	$C_{13}H_{28}O$	1.21
7.	10.656	11-Methyldodecanol	$C_{13}H_{28}O$	1.02
8.	11.830	Vanillin	$C_8H_8O_3$	0.28
9.	12.397	10-Methylnonadecane	$C_{20}H_{42}$	0.67
10.	12.545	Eicosane	$C_{20}H_{42}$	0.53
11.	12.680	Heptadecanoic acid, heptadecyl ester	$C_{34}H_{68}O_2$	0.25
12.	12.869	Phenol, 2, 4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	5.54
13.	12.943	1-Dodecanol, 2-hexyl-	$C_{18}H_{38}O$	0.87
14.	13.015	Eicosane	$C_{20}H_{42}$	1.12
15.	13.125	1-Dodecanol, 2-hexyl-	$C_{18}H_{38}O$	0.98
16.	13.222	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4	$C_{11}H_{16}O_2$	2.52
17.	13.585	Fumaric acid, ethyl 2-methyl allyl ester	$C_{10}H_{14}O_4$	1.19
18.	13.697	Decane, 2,3,7-trimethyl-	$C_{13}H_{28}$	0.33
19.	14.193	Trans-3(10)-Caren-2-ol	$C_{10}H_{16}O$	1.04
20.	14.340	Decane, 2,3,7-trimethyl-	$C_{13}H_{28}$	0.33
21.	14.429	2,4a,8,8-Tetramethyldecahydrocyclopropa[d]$_n$	$C_{15}H_{26}$	1.23
22.	14.512	2-Butanone, 4-(2,6,6-trimethyl-1-cyclohexen-1	$C_{13}H_{22}O$	1.02
23.	14.677	Oxalic acid, 6-ethyloct-3-yl heptyl ester	$C_{19}H_{36}O_4$	0.39
24.	14.749	1-{2-[3-(2-Acetyloxiran-2-yl)-1,1-dimethyl propyl]cycloprop-2-enyl} ethanone	$C_{14}H_{20}O_3$	1.27
25.	14.981	11-Methyldodecanol	$C_{13}H_{28}O$	0.68

26.	15.304	11-Methyldodecanol	$C_{13}H_{28}O$	0.67
27.	15.440	Tetradecanoic acid	$C_{14}H_{28}O_2$	3.13
28.	15.550	11-Methyldodecanol	$C_{13}H_{28}O$	0.80
29.	15.685	1-Dodecanol, 2-Octyl	$C_{20}H_{42}O$	0.52
30.	15.879	6-Methyl-cyclodec-5-enol	$C_{11}H_{20}O$	3.88
31.	16.320	Phytol, acetate	$C_{22}H_{42}O_2$	4.14
32.	16.410	2-Pentadecanone,6,10,14-trimethyl-	$C_{18}H_{36}O$	3.77
33.	16.679	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	0.91
34.	16.761	Pentadecanoic acid	$C_{15}H_{30}O_2$	0.48
35.	16.970	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	1.41
36.	17.538	5,9,13-Pentadecatrien-2-one,6,10,14-trimethy	$C_{18}H_{30}O$	0.64
37.	17.741	Hexadecanoic acid,methyl ester	$C_{17}H_{34}O_2$	1.74
38.	18.168	cis-13-Eicosenoic acid	$C_{20}H_{38}O_2$	0.63
39.	18.753	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	21.20
40.	21.024	Eicosanoic acid	$C_{20}H_{40}O_2$	0.40
41.	21.795	Methyl 10-trans,12-cis-octadecadienoate	$C_{19}H_{34}O_2$	0.45
42.	21.991	Methyl 8,11,14-heptadecatrienoate	$C_{18}H_{30}O_2$	1.22
43.	22.537	Phytol	$C_{20}H_{40}O$	5.21
44.	22.935	cis,cis,cis-7,10,13-Hexadecatrienal	$C_{16}H_{26}O$	14.36
45.	24.190	Methyl 5,13-docosadienoate	$C_{23}H_{42}O_2$	0.41

Table IV: **Compounds identified in the chloroform extracts of *D. villosa* stem by GC-MS spectral analysis**

S/N	RT	Compound Name	Molecular Formular	Height (%)
1.	10.830	Octadecanoic acid	$C_{18}H_{36}O_2$	1.48
2.	10.925	Octadecanoic acid	$C_{18}H_{36}O_2$	3.05
3.	10.991	Undecanoic acid	$C_{11}H_{22}O_2$	3.04
4.	11.050	n-Decanoic acid	$C_{10}H_{20}O_2$	2.71
5.	13.890	Disulfide,di-tert-dodecyl	$C_{24}H_{50}S_2$	1.63
6.	14.524	Phytol,acetate	$C_{22}H_{42}O_2$	0.85

7.	16.326	Phytol,acetate	$C_{22}H_{42}O_2$	48.61
8.	16.682	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	10.00
9.	16.976	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	15.54
10.	18.586	1-(+)-Ascorbic acid 2,6-dihexadecanoate	$C_{38}H_{68}O_8$	2.79
11.	19.546	Eicosane	$C_{20}H_{42}$	3.17
12.	20.233	6,8a-Epidoxy-4a-methyl-2-oxo-3,4,4a,5,6,7,8	$C_{10}H_{14}O_4$	0.87
13.	22.405	E,E,Z-1,3,12-Nonadecatriene-5,14-diol	$C_{19}H_{34}O_2$	1.92
14.	22.435	2-(2-vinyloxy-ethoxy)-cyclohexanol	$C_{10}H_{18}O_3$	1.60
15.	22.620	Phytol	$C_{20}H_{40}O$	2.76

Table V: **Compounds identified in the methanol extracts of *D. villosa* leaves by GC-MS spectral analysis**

S/N	RT	Compound Name	Molecular Formular	Height (%)
1.	3.971	Toluene	C_7H_8	2.05
2.	4.349	Tetrachloroethylene	C_2cl_4	2.33
3.	13.240	Nonanedioic acid, dimethyl ester	$C_{11}H_{20}O_4$	1.24
4.	13.931	Ethyl-1-thio-beta-d-glucopyranoside	$C_8H_{16}O_5S$	0.66
5.	14.956	Methyl-21-methyldocosanoate	$C_{24}H_{48}O_2$	1.12
6.	16.131	Carda-4,20(22)-dienolide,3-(6-deoxy-3-O-methyl)	$C_{30}H_{44}O_9$	0.68
7.	16.185	Carda-4,20(22)-dienolide,3-(6-deoxy-3-O-methyl)	$C_{30}H_{44}O_9$	0.67
8.	16.325	Phytol acetate	$C_{22}H_{42}O_2$	9.80
9.	16.430	Cyclohexadecanone	$C_{16}H_{30}O$	1.10
10.	16.683	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	4.98
11.	16.785	Pseudosmilagenin bis(3,5-dinitrobenzoate)	$C_{41}H_{48}N_4O_{13}$	0.52
12.	16.972	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	7.28
13.	17.295	Acetamide,N-methyl-N-[4-(3-hydroxypyrrolidinyl)-2-butynyl	$C_{11}H_{18}N_2O_2$	0.20
14.	17.449	1-oxaspiro(2,5)octan-4-one,2,2,6-trimethyl-	$C_{10}H_{16}O_2$	0.65

		trans		
15.	17.669	Methylhexadec-9-enoate	$C_{17}H_{32}O_2$	2.60
16.	17.756	Pentadecanoic acid,14-methyl-,methyl ester	$C_{17}H_{34}O_2$	29.89
17.	17.925	Methyl 2,3,4-tri-O-acetyl-6,7-di-O-methyl-beta-D-glucoheptopyranoside	$C_{16}H_{26}O_{10}$	0.26
18.	18.635	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	7.65
19.	18.825	1,1,1,4,7,7,7-Heptamethyl-4-vinyltrisilethylene	$C_{13}H_{32}Si_3$	0.56
20.	21.820	9,12-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	6.48
21.	22.023	11,14,17-Eicosatrienoic acid, methyl ester	$C_{21}H_{36}O_2$	15.19
22.	22.964	Heptadecanoic acid, 16-methyl-, methyl ester	$C_{19}H_{38}O_2$	1.86
23.	23.450	Acetamide, N-(4-piperidinylmehyl)-	$C_8H_{16}N_2O$	0.32
24.	23.620	3-[3-[1-Aziridinyl]propoxy]-2,5-dimethylpyraz	$C_{11}H_{17}N_3O$	1.01
25.	23.641	3-Tridecen-1-yne,(Z)-	$C_{13}H_{22}$	1.01

Table VI: **Compounds identified in the methanol extracts of *D. villosa* stem by GC-MS spectral analysis**

S/N	RT	Compound Name	Molecular Formular	Height (%)
1.	3.965	Toluene	C_7H_8	2.71
2.	4.343	Tetrachloroethylene	C_2cl_4	3.17
3.	13.425	2,2,6,7-Tetramethyl-10-oxatricyclo[4,3,1,0(1,6)-decan-5-ol	$C_{13}H_{22}O_2$	0.34
4.	14.339	tau-Muurolol	$C_{15}H_{26}O$	2.41
5.	14.462	A-Cardinol	$C_{15}H_{26}O$	3.28
6.	15.858	2-Methyltetracosane	$C_{25}H_{52}$	1.53
7.	16.325	Phytol, acetate	$C_{22}H_{42}O_2$	0.99
8.	17.766	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	26.89
9.	17.905	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-methyl ester	$C_{18}H_{28}O_3$	3.35

10.	18.030	Borane, diethyl(1-ethyl-2-methyl-1-butenyl)-(Z)	$C_{11}H_{23}B$	0.73
11.	18.608	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	25.93
12.	19.040	2,7-dithiatricyclo[4,3,1,0(3,8)] decane,10-bromo	$C_8H_{11}BrS_2$	0.40
13.	19.146	Ethyl 15-methyl-hexadecanoate	$C_{19}H_{38}O_2$	1.51
14.	21.825	Methyl 10-trans, 12-cis-octadecadienoate	$C_{19}H_{34}O_2$	10.79
15.	22.037	6-Octadecenoic acid, methyl ester, (Z)-	$C_{19}H_{36}O_2$	8.20
16.	22.223	6-Octadecenoic acid, methyl ester, (Z)-	$C_{19}H_{36}O_2$	1.47
17.	22.957	Heptadecanoic acid, 16-methyl-, methyl ester	$C_{19}H_{38}O_2$	2.01
18.	23.469	1-[3-Aminopropyl]-2[1H]-pyridone	$C_8H_{12}N_2O$	0.91
19.	23.640	Bicyclo[3,3,2] decan-9-one	$C_{10}H_{16}O$	2.59
20.	23.710	1H-Inden-1-one,octahydro-7a-methyl-,trans	$C_{10}H_{16}O$	0.81

Table VII: **Compounds identified in the hexane extracts of *D. villosa* leaves by GC-MS spectral analysis**

S/N	RT	Compound Name	Molecular Formular	Height (%)
1.	3.836	3-Hexanone	$C_6H_{12}O$	0.86
2.	3.885	2-Hexanone	$C_6H_{12}O$	1.43
3.	3.985	Cyclopentanol,1-methyl-	$C_6H_{12}O$	1.03
4.	4.639	Cyclopentanone,3-methyl-	$C_6H_{12}O$	1.42
5.	5.425	2-Pentanethiol,2-methyl-	$C_5H_{14}S$	6.21
6.	5.678	Valeric acid,2-ethoxyethyl ester	$C_9H_{18}O_3$	10.80
7.	5.924	Pentanoic acid,2-propenyl ester	$C_8H_{14}O_2$	4.33
8.	6.061	2-Furanmethanol, tetrahydro-5-methyl-	$C_6H_{12}O_2$	3.55
9.	6.308	Oxalic acid, cyclohexyl ethyl ester	$C_{10}H_{16}O_2$	22.48
10.	6.611	2-Pentene,4,4-dimethyl-,(E)-	C_7H_{14}	2.55
11.	6.650	2-Pentene,4,4-dimethyl-,(E)-	C_7H_{14}	2.36
12.	6.740	Phosphorus dibromide, cyclohexyl-	$C_6H_{11}Br_2P$	0.86
13.	10.458	2-Isopropyl-5-methyl-1-heptanol	$C_{11}H_{24}O$	0.41

14.	10.659	2-Isopropyl-5-methyl-1-heptanol	$C_{11}H_{24}O$	0.26
15.	11.765	Octadecanoic acid	$C_{18}H_{36}O_2$	0.29
16.	11.845	Tetradecanoic acid	$C_{14}H_{28}O_2$	0.41
17.	11.948	Octadecanoic acid	$C_{18}H_{36}O_2$	1.00
18.	12.159	5,9-Undecadien-2-one,6,10-dimethyl-,(E)	$C_{13}H_{22}O_2$	0.45
19.	12.874	Phenol,2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	1.39
20.	13.022	Hexadecanoic acid, butyl ester	$C_{20}H_{40}O_2$	1.87
21.	13.155	Dodecane,4-methyl-	$C_{13}H_{28}$	0.40
22.	13.222	2(4H)-Benzofuranone,5,6,7,7a-tetrahydro-4,4	$C_{11}H_{16}O_2$	0.99
23.	15.473	Tetradecanoic acid	$C_{14}H_{28}O_2$	1.85
24.	16.336	Phytol,acetate	$C_{22}H_{42}O$	1.57
25.	16.432	2-Pentadecanone,6,10,14-trimethyl-	$C_{18}H_{36}O$	4.43
26.	16.690	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	0.27
27.	16.798	Pentadecanoic acid	$C_{15}H_{30}O_2$	0.23
28.	16.987	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	0.42
29.	17.554	5,9,13-Pentadecatrien-2-one,6,10,14-trimethyl	$C_{18}H_{30}O$	0.41
30.	17.675	Oxirane,hexadecyl-	$C_{18}H_{36}O$	0.14
31.	17.764	Pentadecanoic acid,14-methyl-,methyl ester	$C_{17}H_{34}O_2$	1.99
32.	18.204	Palmitoleic acid	$C_{16}H_{30}O_2$	0.45
33.	18.871	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	11.77
34.	19.235	2-Hexadecene,3,7,11,15-tetramethyl-,[R-[R	$C_{20}H_{40}$	0.17
35.	21.093	Eicosanoic acid	$C_{20}H_{40}O_2$	0.26
36.	21.846	9,12-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	0.70
37.	22.052	Methyl 8,11,14-heptadecatrienoate	$C_{18}H_{30}O_2$	1.40
38.	22.579	Phytol	$C_{20}H_{40}O$	2.52
39.	23.187	Butyl 9,12-Octadecadienoate	$C_{22}H_{40}O_2$	0.41
40.	24.158	cis,cis,cis-7,10,13-Hexadecatrienal	$C_{16}H_{26}O$	5.65

Table VIII: Compounds identified in the hexane extracts of *D. villosa* stem by GC-MS spectral analysis

S/N	RT	Compound Name	Molecular Formular	Height (%)
1.	3.881	2-Hexanone	$C_6H_{12}O$	1.08
2.	3.979	Cyclopentanol,1-methyl-	$C_6H_{12}O$	0.69
3.	4.636	Cyclopentanone,3-methyl-	$C_6H_{10}O$	1.07
4.	5.418	2-Pentanethiol,2-methyl-	$C_6H_{14}S$	5.96
5.	5.669	Valeric acid, 2-ethoxy ethyl ester	$C_9H_{18}O_3$	10.71
6.	5.916	Pentanoic acid, 2-propenyl ester	$C_8H_{14}O_2$	3.88
7.	6.054	2-Furanmethanol, tetrahydro-5-methyl-	$C_6H_{12}O_2$	3.59
8.	6.299	Oxalic acid, cyclohexyl propyl ester	$C_{11}H_{18}O_4$	21.93
9.	6.603	2-Pentene,4,4-dimethyl-,(Z)-	C_7H_{14}	2.26
10.	6.644	2-Pentene,4,4-dimethyl-,(Z)-	C_7H_{14}	2.04
11.	6.733	Cyclohexane,nitro	$C_6H_{11}NO_2$	0.77
12.	12.679	Heptadecane,2,6,10,15-tetramethyl-	$C_{21}H_{44}$	0.54
13.	12.868	Phenol,2,4-bis(1,1-dimethyl ethyl)-	$C_{14}H_{22}O$	1.30
14.	13.365	1,6,10-Dodecatrien-3-ol,3,7,11-trimethyl,(E)	$C_{15}H_{26}O$	0.68
15.	13.737	Carophyllene oxide	$C_{15}H_{24}O$	1.96
16.	14.014	12-Oxabicyclo[9,1,0]dodeca-3-7-diene,1,5,5,8-tetramethyl-[1R,3E,7E,11R]	$C_{15}H_{24}O$	0.69
17.	14.122	6-Isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydronaphthalene-2-ol	$C_{15}H_{24}O$	0.50
18.	14.156	Cubenol	$C_{15}H_{26}O$	0.39
19.	14.313	α-cadinol	$C_{15}H_{26}O$	2.44
20.	14.429	α-cadinol	$C_{15}H_{26}O$	3.86
21.	14.684	Octadecane,1-chloro	$C_{18}H_{37}cl$	0.62
22.	14.871	6-Isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydronaphthalene-2-ol	$C_{15}H_{24}O$	0.38
23.	15.450	Tetradecanoic acid	$C_{14}H_{28}O_2$	1.32
24.	16.327	Phytol, acetate	$C_{22}H_{42}O_2$	1.73
25.	16.419	2-Pentadecanone,6,1014-trimethyl-	$C_{18}H_{36}O$	2.36

26.	16.545	Cyclopentadecanone,2-hydroxy	$C_{15}H_{28}O_2$	0.31
27.	16.683	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	0.36
28.	16.780	Pentadecanoic acid	$C_{15}H_{30}O_2$	0.53
29.	16.979	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	0.55
30.	17.753	Pentadecanoic acid, 14-methyl-,methyl ester	$C_{17}H_{34}O_2$	2.41
31.	18.921	n-Hexadecanoic acid,ethyl ester	$C_{16}H_{32}O_2$	13.98
32.	19.155	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	0.32
33.	19.315	2-Bromotetradecane	$C_{14}H_{29}Br$	0.20
34.	21.079	Heptadecanoic acid	$C_{17}H_{34}O_2$	0.44
35.	21.828	9,12-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	0.65
36.	22.056	8-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	0.72
37.	22.600	Phytol	$C_{20}H_{40}O$	0.91
38.	22.971	Methylstearate	$C_{19}H_{38}O_2$	0.17
39.	23.840	1,E-11,Z-13-Octadecatriene	$C_{18}H_{32}$	2.69
40.	24.125	6-Octadecenoic acid,(Z)-	$C_{18}H_{34}O_2$	3.04

3.12.3 Antimicrobial effects

The zone of inhibition of *D. villosa* leaves and stem bark extracts at 10 mg mL^{-1} were found to be significantly higher compared to the control ($p < 0.05$), except for the activity of methanol stem bark extract against *E. coli* where the zone of inhibition at 10 mg mL^{-1} was found to be lower compared to the control (Table IX). At a concentration of 5 mg mL^{-1}, the zone of inhibition of methanol leaves extract as observed against *S. aureus*, methicillin-resistant *S. aureus* and *K. pneumoniae* were found to be higher compared to the control (Streptomycin and Gentamicin) (Table IX). The zone of inhibition of methanol stem bark extracts at 5 mg mL^{-1} as observed against *S. aureus* was found to be significantly higher compared to the control ($p < 0.05$).

There was an effect of chloroform extracts of *D. villosa* leaves on *S. aureus*. The zone of inhibition of the chloroform leaves extracts against *K. pneumoniae* was found to be higher compared to the control (Gentamicin) at a concentration of 10 mg mL^{-1} (Table IX). At 5 mg mL^{-1}, the zone of inhibition of chloroform stem bark extracts against *K. pneumoniae* was further observed to be higher compared to the control; although, not statistically significant. At all concentrations, the zone of inhibition of the hexane leaves extracts against *K. pneumoniae* was found to be lower compared to the control (Gentamicin). Meanwhile, the zone of inhibition against *K. pneumoniae* at a concentration of 10 mg mL^{-1} was higher compared to the control (Table IX). The zone of inhibition at all other doses were found to be lower compared to the control.

Following the serial dilution of 0.625 mg mL^{-1} to the least concentration of the extracts, all the bacterial strains were observed to be resistant to the activities of the methanolic leaf extracts of *D. villosa* at a concentration of 0.01 mg mL^{-1} (Table X). Also, the same trend was observed with the methanol stem bark extracts of the plant at 0.01 mg mL^{-1} (Table X). It was also observed that the bacterial strains were sparred as there was no reactivity at the concentration of 0.01 mg mL^{-1}.

Table IX: Zone of inhibition (mm) of the graded doses of *D. villosa* leaves and stem bark extracts against bacteria strains.

	Meth Lv. Extr.						Meth. Stem Extr.					
	Conc.(mg ml^{-1})						Conc(mgml^{-1})					
Bacteria strain	**10**	**5**	**2.5**	**1.25**	**0.625**	**Control**	**10**	**5**	**2.5**	**1.25**	**0.625**	**Control**
E. coli	18.3 ± 0.67	19 ± 0	19.5 ± 0.5	19.67 ± 1.28	0.90 ± 0.10	21 ± 3.08	16.3 ± 0.88	15 ± 0.88	13.3 ± 1.05	12.3 ± 1.05	0.8 ± 0	23.3 ± 0.77

P. aeruginosa	18.0 ± 0.58	13.0 ± 0.58	12.0 ± 1.53	13.33 ± 0.88	0.90 ± 0	11.67 ± 0.77	14.3 ± 0.67	11.0 ± 0.58	0.9 ± 0	10.67 ± 0.33	0.9 ± 0.1	10.33 ± 0.29
S. aureus	20.3 ± 0.88	15.3 ± 0.33	10 ± 1.15	12.33 ± 0.67	0.83 ± 0.10	15 ± 1.15	14.0 ± 0.58	11.3 ± 0.88	12.50 ± 0.50	10.33 ± 0.33	0.90 ± 0	5.45 ± 4.55
MRSA	17 ± 2	15.5 ± 1.50	12.67 ± 0.67	11.67 ± 0.88	0.2 ± 0	17.67 ± 1.45	16 ± 0	15.33 ± 0.88	13.33 ± 0.33	11.33 ± 0.67	0.63 ± 0.09	19 ± 1.15
K. pneumonia	23.50 ± 0.50	21.5 ± 0.50	16.67 ± 3.33	13.67 ± 1.20	10.67 ± 5.46	19.3 ± 2.91	20.33 ± 1.33	17.33 ± 1.86	17.0 ± 2.08	11.67 ± 0.88	0.73 ± 0.12	21.67 ± 1.20

			Chl. Lv. Extr. Conc.(mg ml^{-1})						Chl.Stem Ext. Conc.(mg ml^{-1})			
Bacteria strain	**10**	**5**	**2.5**	**1.25**	**0.625**	**Control**	**10**	**5**	**2.5**	**1.25**	**0.625**	**Control**
K. pneumonia	24.50 ± 0.5	22 ± 1.0	21.50 ± 1.50	20 ±1.0	15.0 ± 2.0	20 ± 0	20.33 ± 1.33	20 ± 0	18.0 ± 0.58	16.33 ± 1.33	14.33 ± 2.33	19 ± 0.88

			Hex. Lv. Extr. Conc.(mg ml^{-1})						Hex. Stem bark Extr. Conc.(mg ml^{-1})			
Bacteria strain	**10**	**5**	**2.5**	**1.25**	**0.625**	**Control**	**10**	**5**	**2.5**	**1.25**	**0.625**	**Control**
K. pneumonia	17.50 ± 0.50	15 ± 2.0	14 ± 0	13 ± 1.0	0.85 ± 0.05	19.5 ± 0.50	24.0 ± 1.0	21.0 ± 2.0	20.5 ± 3.50	15.50 ± 3.50	13.50 ± 3.50	21.50 ± 1.50

Table X: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *D. villosa* leaves and stem bark extracts against different bacterial strains.

	Met. Leav. Extr. Conc. (mg ml^{-1})		Met. Stem Extr. Conc. (mg ml^{-1})		Chl. Leav. Extr. Conc. (mg ml^{-1})		Chl. Stem Extr. Conc. (mg ml^{-1})	
Bacteria strain	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
K. pneumonia	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
P. aeruginosa	0.01	0.01	0.01	0.01				
S. aureus	0.01	0.01	0.01	0.01				
MRSA	0.01	0.01	0.01	0.01				
E. coli	0.01	0.01	0.01	0.01				

3.12.4 DPPH radical scavenging activity

The radical scavenging activity and IC_{50} values of leaves and stem bark extracts were summarized in Fig.1-4 respectively. The simultaneous occurrence of higher percentage radical scavenging activity and lower IC_{50} values indicated a higher antioxidant activity. Among the different leaves extracts, the methanol leaves extract showed higher scavenging activity (85.29%) compared to other extracts, whereas ascorbic acid at the same concentration showed 92.82% which is slightly close to each other (Figure 1a). Similarly, the methanol leaves extract showed to be an excellent DPPH radical scavenging activity with IC_{50} value of 9.53 μg mL^{-1} compared to that of ascorbic acid (10.3 μg mL^{-1}) (Figure 1b). On the other hand, both chloroform and hexane leaves extracts showed weak antioxidant behaviour with higher IC_{50} values 10.7 μg mL^{-1} and 11.8 μg mL^{-1} respectively when compared to that of ascorbic acid. The methanol and hexane stem bark extracts also showed high scavenging activity at 85.76% and 87.22% respectively, but not as high as the ascorbic acid (92.82%) (Figure 2a). The IC_{50} of both methanol (9.53 μg mL^{-1}) and hexane (9.53 μg mL^{-1}) stem extracts were found to be lower compared to that of ascorbic acid (10.0 μg mL^{-1}) (Figure 2b).

Meanwhile, the chloroform stem bark extract indicated a higher IC_{50} compared to the ascorbic acid.

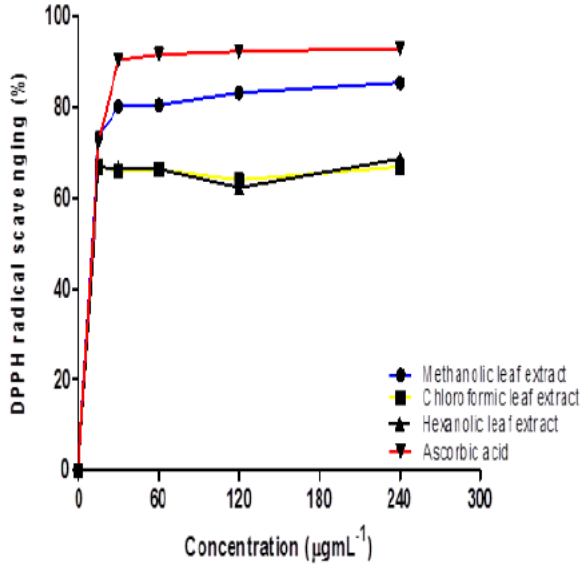

Figure 1a: Comparison of DPPH radical scavenging (%) of *D. villosa* leaf extracts.

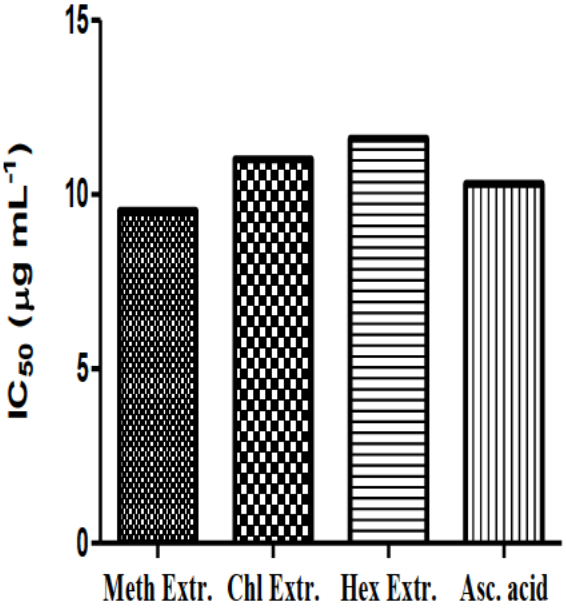

Figure 1b: IC_{50} values of *D. villosa* leaf extracts in DPPH scavenging assay.

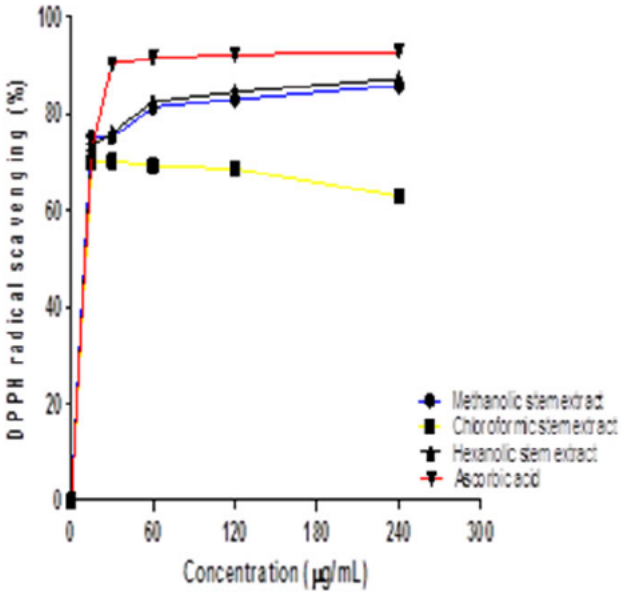

Figure 2a: Comparison of DPPH radical scavenging (%) of *D. villosa* stem bark extract

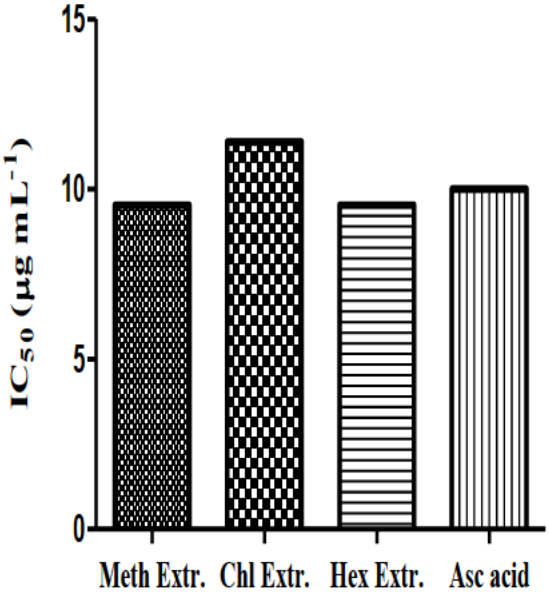

Figure 2b: IC_{50} values of *D. villosa* stem extracts in DPPH radical scavenging assay

3.12.5 Ferric Reducing Antioxidant Potential

The ferric reducing potential and IC_{50} values of leaves and stem bark extracts of *D. villosa* were summarized in Fig.3-4 respectively. Among the different leaves extracts, the hexanic leaves extracts showed high scavenging activity (85.2%), whereas ascorbic acid at the same concentration showed 79.3% (Figure 3a). The chloroform and methanol leaves extracts further showed higher reducing power compared to ascorbic acid. Similarly, the methanol leaves extracts showed ferric reducing power with IC_{50} value of 112 μg mL^{-1} compared to that of ascorbic acid (143 μg mL^{-1}) (Figure 3b). On the other hand, both chloroform and hexane leaves extracts showed excellent antioxidant behaviour with IC_{50} values 11.0 μg mL^{-1} and 13.3 μg mL^{-1} respectively when compared to that of ascorbic acid (Figure 3b). The methanol and hexane stem bark extracts also showed high scavenging activity at 85.8% and 87.22% respectively, but not as high as the ascorbic acid (92.8%) (Figure 4a). The IC_{50} of both methanol and chloroform stem extracts were found to be lower compared to that of ascorbic acid (141.0 μg mL^{-1}) (Figure 4b). Similarly, the hexane stem bark extract further indicated a higher IC_{50} compared to the ascorbic acid.

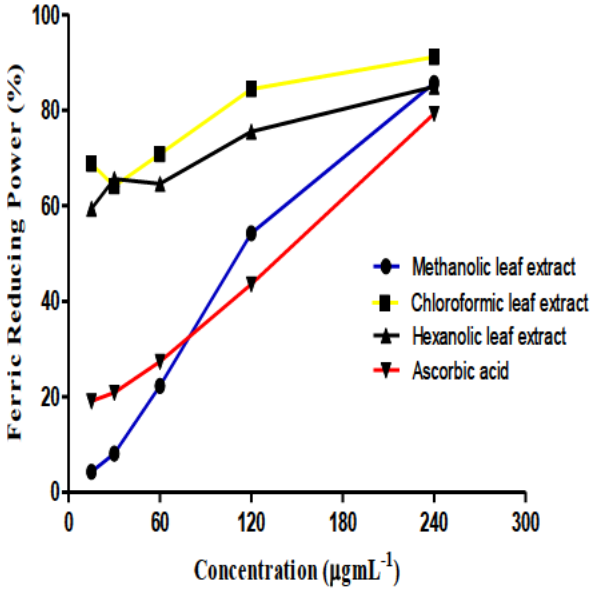

Figure 3a: Comparison of ferric reducing power of *D. villosa* leaves extract.

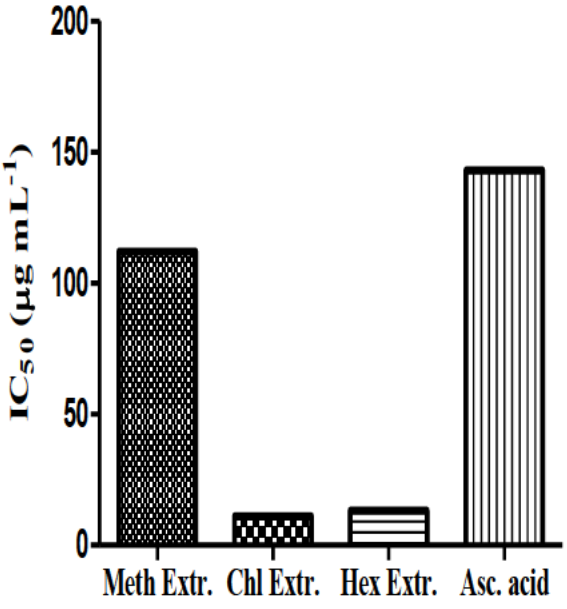

Figure 3b: IC_{50} values for *D. villosa* extracts in ferric reducing antioxidant potential

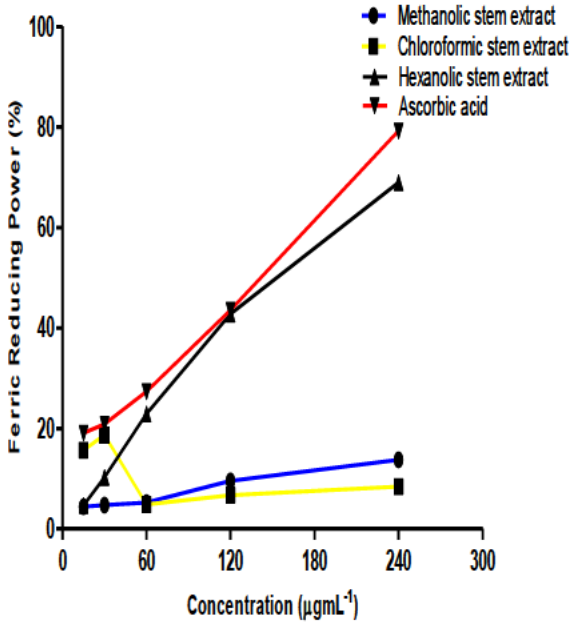

Figure 4a: Comparison of ferric reducing power in (%) of *D. villosa* stem bark extract.

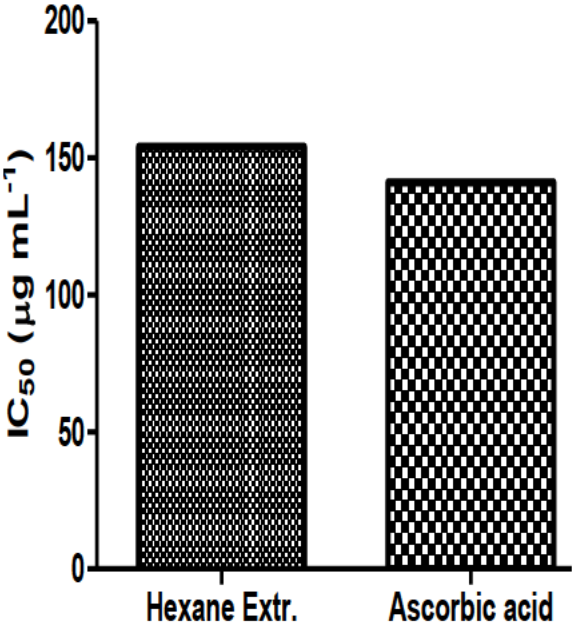

Figure 4b: IC_{50} values for *D.villosa* stem ferric reducing antioxidant potential.

3.12.6 Total Phenolic Content

The TPC in the leaves and stem bark of *D. villosa* was estimated and analysed. One-way analysis of variance showed that there was a significant difference in the total phenol contents in *D. villosa* leaves and stem bark $F_{(2, 6)} = 225.8$, $p \leq 0.001$. The highest TPC in *D. villosa* leaves was found in methanol extracts (28.45 ± 0.50) mg gallic acid equivalent per gram of dry weight. Meanwhile, the highest TPC in the stem bark was found in the hexane extracts (14.40 ± 0.58) mg gallic acid equivalent per gram of dry weight (Figure 5).

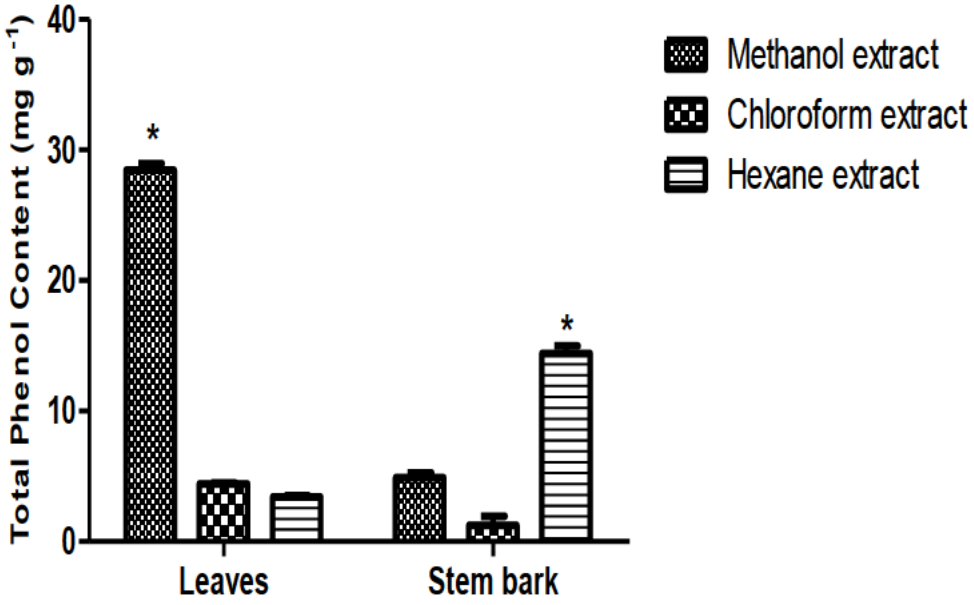

Figure 5: Total Phenol content of the solvent extracts of both *D. villosa* leaves and stem bark. $F_{(2, 6)} = 225.8$, $p \leq 0.0001$. *(methanol vs. chloroform), $p < 0.05$. *(hexane vs. methanol), $p < 0.05$. Values are expressed as means ± SEM; n = 3/group.

3.12.7. Fourier Transform Infrared (FT-IR) spectral analysis

The FT-IR spectra of *D. villosa* leaves and stem bark extracts using methanol, chloroform and hexane as the media for extraction are presented in Figure 6. The spectra showed the

vibrational frequencies of the various functional groups present in the crude extracts. Absorption peaks with a broad range 2500-3300 cm^{-1} were characteristic of hydroxyl (O-H) group particularly from carboxylic acid (Figure 6a & b). The peaks around 1650-1750 cm^{-1} were assigned to carbonyl (C=O) group. The stretching peaks at 1599 and 1605 cm^{-1} (at Figure 6a & b) occurred due to the presence of (C≡N) nitrile. The C-H (hydrocarbon) stretches appeared at 2927 cm^{-1} (Figure 6c & d). The peak at 2849 cm^{-1} was meant to further characterize (C-H) stretching vibration. The strong peaks absorbed at 1735 and 1617 cm^{-1} (Figure 6c & d) were the (C=O) stretches in aldehydes and ketones. The peaks at 3369 and 3309 cm^{-1} (Figure 6c & d) occurred due to the presence of (O-H) functional group. Also, the strong peaks at 2916 and 2848 cm^{-1} as well as at 2917 and 2849 cm^{-1} (in the Figure 6e & f) were due to the presence of (C-H) functional group.

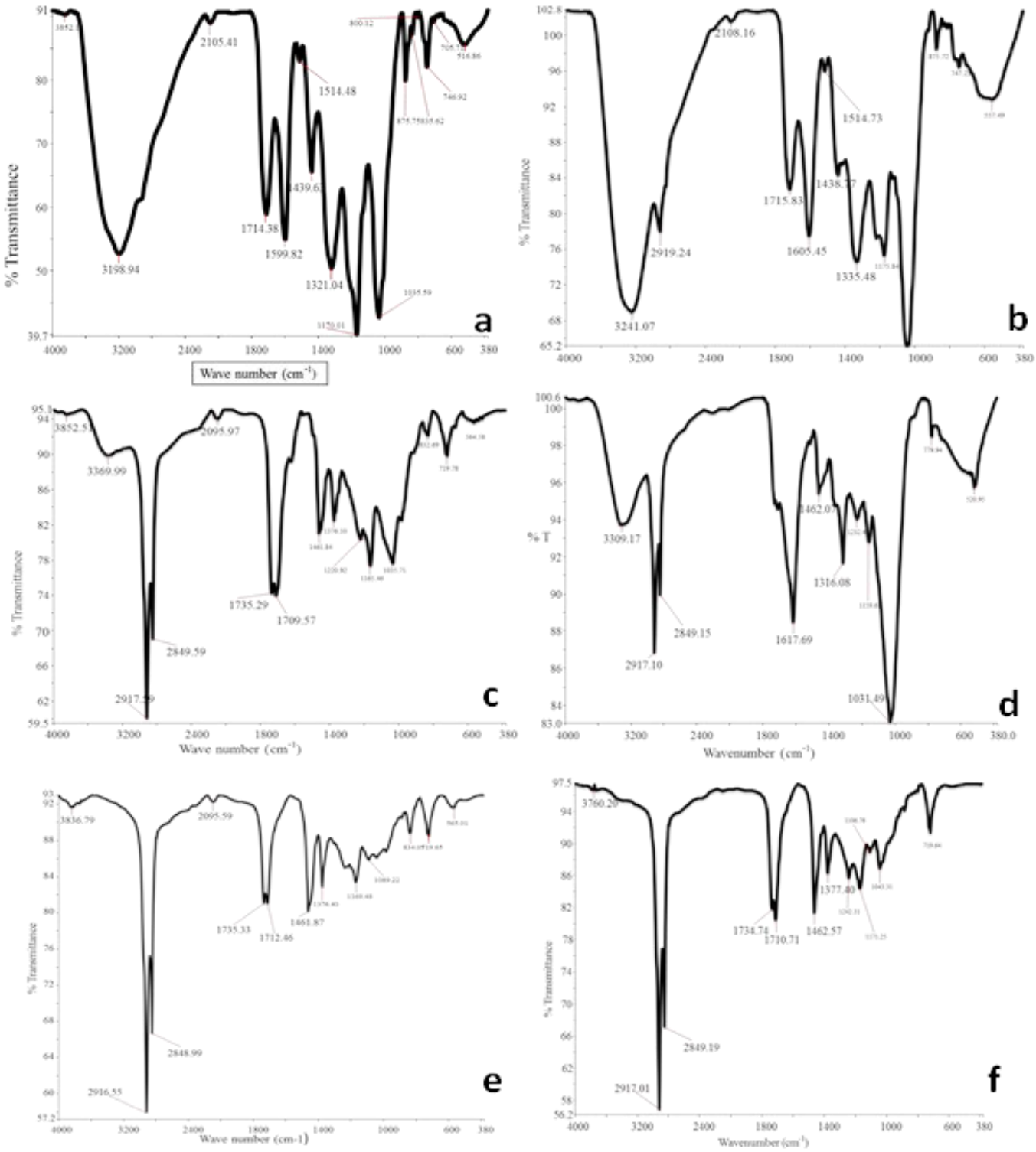

Figure 6: FT-IR spectra of crude plant extracts of *D. villosa* **a**) crude leaf methanol extract; **b**) crude stem bark methanol extract; **c**) crude leaf chloroform extract; **d**) crude stem bark chloroform extract; **e**) crude leaf hexane extract; **f**) crude stem bark hexane extract at room temperature (24 ºC).

3.13 Discussion

Several studies have reported that herbal antioxidants act against free radicals (Wangia et al., 2016, Dintcheva et al., 2017, Tatipamula et al., 2017). The presence of secondary metabolites like terpenoids, alkaloids, flavonoids and phenolic compounds have been implicated as antioxidant factors in different plant materials (Muchirah et al., 2018). It is interesting to note that the methanol extract of *D. villosa* (both leaves and stem bark) showed the presence of flavonoids and alkaloids. Following the qualitative phytochemical analysis, methanol leaves extract presented with colour intensity to have indicated for the presence of terpenoids, flavonoids and even phenolic compounds. It is with no doubt that the confirmed compounds (terpenoids, flavonoids and phenols) would provide the justifiable underlying factors for the antioxidant activity of *D. villosa*. This agrees with Echeverría et al. (2017) that antioxidant activity of natural flavonoids of *Chilean flora* (Flora of Chile) is as a result of the embedded hydroxyl group for oxygenation- substitution pattern. The presence of hydroxyl group in the *D. villosa* leaves and stem bark supported hydroxylation as the plant's mechanism to exhibit its antioxidant function and thereby confer stability to free radicals. Also, this study revealed the presence of alkaloids in the methanol leaves and stem bark extracts only. Many alkaloids are to be potent antioxidants and are used for the treatment and/or management of the risk of skin cancer (Ahsan et al., 2007). The detection of alkaloids in the plant extracts is in line with Dangoggo et al. (2012) who revealed that the aqueous extract of *Diospyros mespiliformis* (ebony diospiros) leaves were quite rich in alkaloids and may further be responsible for the effective inhibitory effect on DPPH, thereby helping in detoxification of the generated radical oxygen species.

The GC–MS analysis showed the presence of quite a number of biologically active compounds e.g. phytol, n-hexadecanoic acid, palmitoleic acid, oxalic acid, 3, 7, 11, 15-Tetramethyl-2-hexadecen-1-ol, acetate, alpha-cadinol, tau-Muurolol, eisosane, vitamin E.

The major compounds found in the methanol leaf extracts were 11, 14, 17-Eicosatrienoic acid, methyl ester (15.19%), Pentadecanoic acid, methyl ester (29.89%) while in the methanol stem extracts, n-Hexadecanoic acid (25.93%), hexadecanoic methyl ester (25.93%) and methyl 10-trans, 12-cis octadecadienoate (10.79%) were present. In the chloroform leaf extract, the major compounds were n-Hexadecanoic acid (20.12%) and cis, cis, cis-7, 10, 13-Hexadecatrienal (14.36%) while phytol acetate (48.61%) and 3, 7, 11, 15-tetramethyl-2-hexadecen-1-ol (15.54%) were found in the chloroform stem extract of *D. villosa*. Similarly, oxalic acid, cyclohexyl ethylester (22.48%) and n-hexadecanoic acid (11.77%) were found in the hexane leaf extract while in the hexane stem extract, oxalic acid, cyclohexylpropyl ester (21.93%) and n-hexadecanoic acid, ethyl ester (13.98%) were found in high proportion. The high concentration of valeric acid in the hexane extracts of the leaves and stem bark may be associated with the antioxidant activity of the extracts. This in accordance with Vishwarkarma et al.(2016) where valeric acid, isolated from *Valeriana wallichii* was scientifically proven to have anti-inflammatory properties by reducing lipid peroxidation as well as restoring glutathione level in intracerebrovascular streptozotocin induced neurodegeneration and further suggests that it could be used in the management of inflammatory diseases.

Phytol is a diterpene compound and it may act as an antimicrobial, anti-inflammatory, anti-cancer and diuretic. Phytol acetate as found in the *D. villosa* extracts was revealed to be in high concentration and could be used as a novel class of pharmaceuticals for the treatment of rheumatoid arthritis and possibly chronic inflammatory diseases. This is further corroborated by Ogunlesi et al. (2009) where phytol increased oxidative burst *in vivo* and thereby corrected the effect of the genetic polymorphism in translational model of arthritis. Phytol may further be considered a novel class of pharmaceuticals for the treatment of chronic inflammatory diseases. In fact, phytol is an acyclic diterpene alcohol that can be used as a precursor for the

manufacture of synthetic forms of vitamin E (Ogunlesi et al., 2009). Among other identified phytochemicals, n-Hexadecanoic acid, Hexadecanoic acid and Palmitic acid have the antioxidant, hypocholesterolemic, nematicide, pesticide and lubricant properties (Bai et al. 2006). n-Hexadecanoic acid and ethyl ester act as antifungal, antitumour and antibacterial. Hexadecanoic acid as found in *D. villosa* possess antioxidant, haemolytic properties as well as being effective pesticide.

Similarly, Rathee et al. (2006) reported that methanolic extract of *Mentha longifolia* (L) ssp. plant showed an impressive antioxidant activity via the ability to scavenge various biologically relevant reactive oxygen species and inhibit lipid peroxidation. In this study, methanol extracts of *D. villosa* (both leaves and stem bark) showed a very good antioxidant activity, in comparison with the reference drug (ascorbic acid). This activity could further be associated to the presence of phenolics, flavonoids and alkaloids content. The observed lower IC_{50} values of these extracts supported the significance of *D. villosa* leaves and stem bark as promising natural sources of antioxidants and hence they can be used for the prevention of free radical-mediated diseases. Furthermore, the result of DPPH radical scavenging ability showed that methanol leaves and stem bark extracts can prevent radical-induced oxidative damage. This is even reflected with the phenol content in the methanol leaf and stem bark extracts. Accordingly, Saeed et al. (2012) established the correlation between health benefit of polyphenolic- rich plants and their antioxidant properties and the possible mechanism behind the phenolic activity could be the redox properties of their hydroxyl group.

The functional groups in any bio-organic compound expressed influence on the biological activities of such compound. The influence was as a result of the contribution of the embedded functional groups to the inherent properties of the compounds like solubility, stereochemistry, partition coefficient, acid-base properties etc. All these properties were supposed to influence metabolic extraction, absorption, distribution and toxicity of bioactive

molecules (Olivares-Vicente et al., 2018). Hence, functional group analysis plays a vital role in understanding the physicochemical properties of the extracts. Identification of the functional groups therefore, helped to evaluate the structure-function relationship of the bio-organic compound. In this study, FT-IR spectral analysis of the leaves and stem bark extracts of *D. villosa* showed the presence of phytochemicals carrying hydrogen bonded –OH functional group. It is well established that hydroxyl functionality is an integral part of most of the phenolic phytochemicals such as flavonoids and tannins (Diaz et al., 2012).

Although, the mechanism of antibacterial activity could not be ascertained in this study, it was however noted that higher zones of inhibition were produced by the graded doses of methanol leaves extracts compared to that of stem bark. It will not be a mere coincidence that the chloroform and hexane extracts showed antibacterial activity against *K. pneumonia* only. The presence of alkaloids in the methanol extracts may be identified as an additional key factor for the antibacterial activity of *D. villosa* plant. This is supported by Bai et al. (2006) with evidence to confirm the antibacterial activity of alkaloids as well as the mechanism of action through intercalation with bacteria DNA. There has been a lot of scope and importance for development of new antimicrobials in treatment of microbial infection (Fair and Tor, 2014). The latest trend showed that the plant-based antimicrobial agents have an enormous therapeutic potential since they showed no major side effects on human beings (Anand et al., 2019). The fact that the plant extracts produced zones of inhibition against different bacteria strains indicated its antimicrobial activity and further confirmed its use as anti-infection agent. Also, the production of zones of inhibition against both gram-negative and gram-positive bacteria showed its tendency for a wide spectrum of activity. The methanol extract of *D. villosa* leaves further indicated a higher zone of inhibition against *S. aureus*, *P.aeruginosa* and *K.pneumoniae* compared to the conventional antibiotics at high concentration. Although, the mechanism of actions of *D. villosa* is yet to be ascertained, there is no doubt that the

chemical contents of the plants like phenols, flavonoids and alkaloids are much likely to be responsible for the antimicrobial activities. This is similar to Linuma et. al (1994), where the presence of flavonoids in the extract was revealed to be a typical phytochemical responsible for microbial inhibition. In addition, the methanol stem bark extract of *D. villosa* further showed reaction against these strains but not as high as the control drug. The observed reduction in the degree of inhibitory activity may be attributed to lesser concentration of phenolic content in the stem bark extract of *D. villosa*. This is also in agreement with Vaquero et al. (2007) where phenolic compounds in wines were found to have possessed high antibacterial effects. Other research study by Majhenič et al. (2007) found that methanol extracts of *Paullinia cupana* (guarana) seed had higher antibacterial activity as a result of high phenolic content (Majhenič et al., 2007). Hence, it can be expected that this plant possessed unique phytochemicals which are responsible for inhibition of bacterial metabolism.

3.14 Conclusion

The present study revealed that the leaves and stem bark extracts of *D. villosa* displayed the presence of alkaloids, phenols, flavonoids and even terpenoids. The leaves of the plant further revealed strong antioxidant activity owing to the high concentration of total phenolic content as well as the absorption peaks with a wide range for hydroxyl group. In addition, the plant showed strong antimicrobial activities against Gram-negative and Gram-positive bacteria strains with the minimum inhibitory concentration at 0.01 mg/ml. Therefore, this study suggested that the leaves and stem bark extracts of *D. villosa* are a potential source of natural antioxidant and antimicrobial agents. It is hoped that the present study would lead to the designing of some compounds that could be used to investigate new and more potent

antioxidant and antimicrobial agents of the plant origin. Further research is needed to isolate active molecules from the crude extract and also to evaluate in details in vivo biological activities of such isolated compounds.

3.15 Authors' contributions

This serves to narrate the contributions of each author in this manuscript titled 'Phytochemical screening, antioxidant and antimicrobial activities of *Diospyros villosa* (L.) De Winter leaves and stem bark extracts. **A. O** was involved with the plant identification, plant extraction, phytochemical tests, FTIR analysis, antioxidant tests, antimicrobial tests, statistical analysis as well as the write-up of the manuscript. **N. Y** was involved in the conception of the idea and all the experimental paradigms as well as the write up. Also, **L. J** was involved with in the antioxidant and antimicrobial tests as well as the write-up of the manuscript. **S. C** was much involved with the plant identification, plant extraction and the write-up of the manuscript. **S. V** gave intellectual inputs on the write-up of this manuscript.

3.16 Acknowledgements

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3.17 Conflict of Interest

The authors have declared no conflict of interest.

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CHAPTER FOUR

Prologue

Manuscript 2

Diospyros villosa leaves and stem bark are found to be a suitable source for the biosynthesis of silver nanoparticles. In the present study, the synthesized silver nanoparticles were characterized by using the UV-vis absorption spectroscopy, FT-IR, EDX, SEM and TEM, DPPH scavenging ability, FRAP, antimicrobial susceptibility and quorum sensing inhibition tests. The formation of silver nanoparticles from *Diospyros villosa* leaves is confirmed by surface plasmon resonance, determined by UV-vis spectra at 400- 435 nm. The FT-IR spectra revealed the presence of O-H stretching vibration as well as aromatic hydrocarbon vibration in the nanoparticles of both leaves and stem bark. The SEM analysis showed the spherical structure of the silver nanoparticles with some agglomeration in the leaves as compared with the stem bark extracts. The synthesized silver nanoparticles further showed good antibacterial activity against human pathogens such as *Escherichia coli, Staphylococcus aureus, Staphylococcus epidermis and Enterococcus faecalis. D. villosa* nanoparticles demonstrated potential antioxidant and anti-quorum sensing activity. Hence, the plant may thus represent a source of antioxidant as well as anti-quorum sensing therapeutic candidate for the control of existing and emerging infectious disease.

"Green synthesis of silver nanoparticles using *Diospyros villosa* extracts and evaluation of antioxidant, antimicrobial and anti-quorum sensing potential"

Green synthesis of silver nanoparticles from *Diospyros villosa* extracts and evaluation of antioxidant, antimicrobial and anti-quorum sensing potential.

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ABSTRACT

Metallic nanoparticles are traditionally synthesized by wet chemical techniques, in which the chemicals used are often toxic and flammable. In this present study, environmentally friendly technique for the biosynthesis of silver nanoparticles from *Diospyros villosa (D. villosa)* leaves and stem bark extracts using 1 mM silver nitrate ($AgNO_3$) is described. The synthesized silver nanoparticles were characterized using UV-Vis absorption spectroscopy, Fourier Transform Infrared (FT-IR) Analysis, Elemental Dispersion X-ray (EDX) analysis, Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging ability, antimicrobial susceptibility and the quorum sensing inhibitory activity was assessed quantitatively using the *Chromobacterium violaceum* biosensor systems. The stem bark nanoparticles (NPs) of *D. villosa* synthesized at 80 ºC (S80) showed good scavenging activity with a lower IC_{50} value at 8.75 µg/ml compared to ascorbic acid (9.58 µg/ml). The total phenol content of the S80 NPs was measured and found to be 10.22 ± 0.14 mg/g gallic acid equivalence (GAE). The synthesized silver nanoparticles demonstrated good antibacterial activity against human pathogens, i.e., *Escherichia coli, Enterococcus faecalis* and more *importantly Staphylococcus aureus* and *Staphylococcus epidermidis.* Methanol extracts of *D. villosa* leaves and stem bark indicated an outstanding anti-quorum sensing activity in almost all the tested concentrations and importantly at 1000 *µg/ml*. Similarly, the bacterial growth inhibition (% GI) and violacein inhibition (% VI) against *C. violaceum* CV 017 by S80 at concentration of 80 µg/ml *were* 10.08% *and 58.83% respectively.* L80 at concentration of 160 µg/ml showed % GI (39.68%) and (% VI) 79.83% respectively against *C. violaceum* ATCC 12472. SRT at a concentration of 160 µg/ml also indicated % GI (13.83%) and % VI (65.97%) against *C. violaceum* CV 017. Although, *D. villosa* extracts showed excellent QS inhibition activities, L80 and SRT at concentration of 160 µg/ml may also be considered as QS inhibitors following their

performance against *C. violaceum* ATCC 12472 and *C. violaceum* CV 017 respectively. *D. villosa* may thus represent a source of antioxidant as well as anti-quorum sensing therapeutic candidate for the control of Gram-negative bacterial infections.

Keywords: DPPH, Electron Microscopy, Nanoparticles, Quorum sensing

4.0 Introduction

The ability of active biomolecules of plant for the limitation of infectious diseases has been reported (Sharifi-Rad et al., 2018, Ayaz et al., 2019, Gyebi et al., 2020) and the series of resilience observed with bacteria have resulted into continuous search for a new plant-based compounds with diverse mode of antibacterial activities for therapeutic purposes. The commonly used antibiotics exhibit bactericidal effects either by blocking bacterial enzymes activities, thereby rendering the bacteria susceptible to the antibiotic effect or by attacking the bacteria directly (Liu et al., 2019). The reduced potency of some antibiotics is becoming more obvious with recurrent ailments and disease. The pathogens have been shown to exhibit antibiotic resistance. Quorum sensing (QS) is an intercellular communication pathway in bacteria which involves synchronised genetic expression in response to cellular concentration (Krishnamurthy et al., 2016). It has been identified as an emerging focus towards the detection of anti-virulent compounds against bacteria (Welsh and Blackwell, 2016, Fleitas Martínez et al., 2019).

Green synthesis of nanoparticles (NPs) in recent years has been extensively utilized in the field of nanotechnology (Rafique et al., 2017, Khani et al., 2018, Ahmad et al., 2019). Among other materials, synthesis of silver nanoparticles (AgNPs) has gained awareness due to its active resistance against microbes (Anandaradje et al., 2020, Hamed et al., 2020). The synthesized AgNPs have more antibacterial advantages compared with their antibiotic counterpart (Kasithevar et al., 2017, Rasheed et al., 2017). Out of the various mechanisms of action identified with AgNPs, it was reported to halt the replicative functions of bacterial cells by interrupting the membrane permeability, resulting into cell apoptosis by allowing the weakening of cell development (Zhang et al., 2020). The identified mechanisms involve attachment with protein functional groups leading to protein denaturation and apoptosis, stoppage of DNA duplication, cell membrane disruption as well as leakage of its cellular

content with respect to protein denaturation and cell death (Salleh et al., 2020). There are reports on quorum sensing and biofilm inhibition of nanoparticles synthesized from plants (Ali et al., 2017, Satish et al., 2017, LewisOscar et al., 2021) and there is a need for further investigation of traditional plants already used in the environment for medicinal purposes but with no scientific documentation.

The synthesis of nanoparticles with the help of organic products have gained scientific attention due to its safety to living organisms and eco-friendly (Agarwal et al., 2017). Plants are rather considered for the synthesis of silver nanoparticles due to the appreciable amount of phytochemicals with strong antioxidant properties than reactive metallic ions (Nephawe, 2015, Sridhar, 2019). It is no longer news in the scientific environment that naturally occurring plants are used locally to treat diseases (Mustafa et al., 2017, Salehi et al., 2018, Anand et al., 2019). *Diospyros villosa* (*D. villosa*) is a plant which naturally occurs in South Africa. *D. villosa* is a perennial, bushy evergreen plant, with a height range 1- 4 m. The roots of the plant are used as a purgative remedy for gastrointestinal complications (Cirera et al., 2010a), as a tooth-brush and to treat oral infection (Cirera et al., 2010b). Dougnon et al. (2020) further reported that the medicinal efficacy of various plants found in the genus *Diospyros* for the treatment of illnesses like fever, whooping cough and diarrhoea.

This research study was geared towards assessing the potential use of *D. villosa* leaves and stem bark extracts as a bio-reduction agent for NP formation. The objectives were to characterize AgNPs synthesized from *D. villosa* leaves and stem bark extract, determine their antioxidant potential and explore their quorum sensing inhibitory activity using *Chromobacterium violaceum* biosensor system.

4.1 Materials and methods

4.2 Chemicals and reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH), gallic acid, acetic acid, Gentamicin and Ciprofloxacin were purchased from Sigma-Aldrich (St. Louis, USA). Mueller Hilton agar media, hexane, methanol and chloroform were obtained from Merck Chemical Co. (Durban, South Africa).

4.3 Plant collection

Fresh samples of mature leaves and stem bark of *D. villosa* were collected from KwaZulu-Natal, Durban, South Africa (29º 84′ 33.6″S, 31º 4′ 12″E). The plant was identified and a voucher specimen was deposited in the Ward Herbarium (01/18257) of the School of Life Sciences, University of KwaZulu-Natal. The collected plant parts (leaves and stem bark) were air-dried and pulverized into fine powder. The powdered samples were kept in a cool dry place for extraction purposes.

4.4 Plant extraction

Powdered samples of each plant part weighing 8 g were heated to a temperature of 40 °C for 15 min with 100 ml of Millipore ^{TM} water for 15 min. The solution was then filtered through Whatman No. 1 paper. The filtrate was further evaporated to dryness under reduced pressure at 40 °C in a rotary evaporator. The crude aqueous extracts were stored at 4 ºC and used within 48 h.

4.5 Synthesis of silver nanoparticles (AgNPs)

The method as described by Bodede et al. (2017) was used in the synthesis of AgNPs. A prepared 5 ml aliquot of the plant extracts was thoroughly mixed with 50 ml silver nitrate ($AgNO_3$) solution having concentration of 1 mM. The resulting solution was vigorously shaken together for 10 min so as to allow perfect formation of the nanoparticles. The solution was later allowed to be stationary and incubated at room temperature (25 ºC) and at 80 ºC using a water bath for 1 h. The appropriate formation of NP was indicated by dark brownish colouration of the solution. The colour intensity of the solution was closely monitored and container bearing the solution was removed once the colour was observed to be completely bright. This was done to prevent formation of NP clusters which could occur almost as soon as the deepest brownish colour was observed (Moodley et al., 2018). The solution was later stored in a dark environment to disallow the reduction of silver ion (Ag^+) to Ag^o in the presence of sunlight (Kannan et al., 2013, Sharma et al., 2013). Similarly, 5 ml of distilled water was added to a 50 ml aliquot of aqueous $AgNO_3$ in the preparation of control and the same procedure as described above was carried out. All analyses were carried out in triplicate.

4.6 Quantification of AgNPs

The AgNP solutions obtained from the *D. villosa* leaves and stem bark at different temperatures (room temperature and at 80 °C) were differently poured into already weighed microcentrifuge tubes and allowed to centrifuge for 2 h at 1650 ×*g* and at 4 °C using an Eppendorff microcentrifuge (5804/5804 R, USA). The resulting supernatant from the solutions were decanted and the insoluble residues were modified in 20 ml of distilled water and further allowed to centrifuge for three more times in order to remove the inert substances.

The samples were subsequently oven-dried for a period of 24 h at 40 °C. The microcentrifuge tubes were weighed again to calculate the yield of the synthesized AgNPs.

4.7 UV-Vis spectra analysis

The fractional portion of the yield was intermittently harvested to ascertain that the bioreduction of Ag^{+} in the solution was fully completed. The obtained samples were further mixed with 2 ml of distilled water. The mixture was later taken to a spectrophotometer (Shimadzu UV-2600, Japan) for UV visible (Vis) spectra analysis, with the range of wave-lengths between 200 and 800 nm, having a resolution of 1 nm. The corresponding peaks were observed and recorded.

4.8 Fourier Transform Infrared (FT-IR) analysis

FT-IR analysis was meant for the detection of embedded biomolecules which are likely to be accountable for the reduction and stability of AgNPs as well as the surrounding state of the ligands used as capping agent on top of the nanoparticles (Moteriya and Chanda, 2017). FT-IR was carried out after bio-reduction by scraping-off the residue that was attached to the capping ligand. The dehydrated nanoparticle was further analysed so to identify the functional groups of the biosynthesized silver nanoparticles. The infrared spectra for the samples were achieved using spectrophotometer (Perkin Elmer 100 FT-IR, USA) which is further equipped with ATR testing accessory.

4.9 Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray (EDX) Analysis

Nearly 40 µl of the obtained sample was placed on the glass which was already connected to a brass stub having carbon tape with adhesive strip on both sides. The affixed sample was

later allowed to dry with the aid of a mercury lamp for 60 min. A flat and thin in-built metal in an automated Quorum (Q15OR ES) module sputter coater (vaccum of 0.1 Torr for 2.5 min) was further placed twice over the sample for 10 min period. The morphological examination on the sample was made possible with the aid of a Zeiss Ultra-Plus field emission gun scanning electron microscope (FEG-SEM) with acceleration voltage value at 5 kV. The identified features were digitally captured by the NIS-D image software. The Zeiss Ultra-Plus having an energy dispersive X-ray (EDX) spectrometer and attached to Astronomical Thermal Emission camera (*Aztec* 1.2) was also used to determine the elemental composition of the sample at an acceleration voltage of 20kV.

4.10 Transmission Electron Microscopy (TEM)

The determination of the particulate nature (size and shape) of the biosynthesized AgNPs was done using the transmission electron microscopy (TEM). A single drop of the AgNPs was deposited on a formvar-coated copper grid and allowed to dry for 10 min. The captured images were viewed under Joel TEM 1010 (Japan) at 200 kV.

4.11 DPPH scavenging activity

The free radical scavenging ability of the biosynthesized nanoparticles was tested and measured against DPPH (2,2-diphenyl-1-picrylhydrazyl) (Braca et al., 2001). An admixture of 3 ml aliquot of 0.004 % DPPH solution in 95 % ethanol, 0.1 ml of AgNPs and different concentration of vitamin C were prepared. The prepared mixture was sonicated thoroughly and eventually made static for a period of 30 min. The strength of DPPH decolorization was confirmed at an absorbance of 517 nm. The control was also prepared following the already analysed protocol and specifically using 0.1 ml of each constituent as well as the distilled water in replacement to AgNPs or ascorbic acid. The percentage inhibition of DPPH by the

samples was analysed by comparing both the absorbance values of the control and that of experimental samples. The higher the absorbance, the lower the scavenging ability (Salleh et al., 2020).

4.12 Ferric Reducing Antioxidant Potential (FRAP) Assay

The Ferric Reducing Potential assay was carried out according to the procedure described previously (Juntachote and Berghofer, 2005). One ml of multiple concentrations (15, 30, 60, 120 and 240 μg ml^{-1}) of NPs was prepared in triplicates. Each extract was also mixed with phosphate buffer (0.2M, 25ml, pH = 6) and potassium ferricyanide (1% w/v, 2.5 ml). The prepared mixture was incubated at a temperature of 50 °C for 20 min and further mixed with trichloroacetic acid (10%, 2.5 ml). A fraction of 2.5 ml from the prepared solution was further diluted twice with distilled water. Then, ferric trichloride (0.1% w/v, 0.5ml) was eventually introduced into the each mixture. Each prepared solution was finally taken to microplate reader after 30 min for the measurement of absorbance at 700 nm. The positive control used in this experiment was gallic acid. The concentration of extract producing 50% absorbance (IC_{50}) was extrapolated from the graph where absorbance was plotted against the concentrations of the extract. Results were generated as thus: Scavenging effect (%) = $[\frac{Absorbance\ of\ Control\ at\ 700\ nm - Absorbance\ of\ sample\ at\ 700\ nm}{Absorbance\ of\ control\ at\ 700\ nm}] \times 100$.

4.13 Total Phenolic Content (TPC)

Total phenol content was performed by using the Folin-Ciocalteu colorimetric principle with minor readjustment (Atanassova et al., 2011, Shao et al., 2014). Each NP (0.1 ml) at multiple concentrations (15, 30, 60, 120 and 240 μg ml^{-1}) was mixed with distilled water (3 ml). A freshly prepared Folin-Ciocalteu reagent (0.5 ml) was further added to each sample extract. The mixture was rendered static for 3 min at room temperature. This was followed by the addition of sodium carbonate (20%, 2 ml) and further incubated at room temperature for 30

min. Total phenolic content was measured at 725 nm using spectrophotometer. Gallic acid was also used as the positive control. Total phenol concentration was expressed as mg of gallic acid equivalents (GAE)/g of sample extracts.

4.14 Antimicrobial susceptibility test

The Kirby Bauer disc diffusion assay was used to carry out the antimicrobial susceptibility tests. Four Gram-negative microorganisms, *viz*., *Escherichia coli* ATCC 35218, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella pneumoniae* ATCC 700603, as well as seven Gram-positive microorganisms: *Enterococcus faecalis* ATCC 51299, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* ATCC 33591, *Staphylococcus aureus* ATCC 43300, *Staphylococcus aureus* ATCC 700698, and *Staphylococcus epidermidis* ATCC 12228, were grown at 37 °C overnight on Mueller-Hinton (MH) agar plates. Inocula equivalent to a 0.5 McFarland were used to swab the surface of the MH plates (Chenia, 2013). Thereafter, blank discs were impregnated with 200 µg and 400 µg of the respective nanoparticles and placed on 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. Following incubation, samples exhibiting zone diameters >15 mm were regarded as being strong antimicrobials, zone diameters between 11-15 mm were regarded as possessing intermediate activity and zone diameters <10 mm were considered weak antimicrobial agents.

4.14.1 Qualitative quorum sensing inhibition

Qualitative evaluation of QS inhibition by the extracts and nanoparticles was carried out based on the capacity to prevent the formation of violacein (purple in colour) by *C. violaceum* CV017 and *C. violaceum* ATCC 12472 (Chenia, 2013). Lack of purple pigment formation is an indication of violacein inhibition. About five millilitres of liquefied Luria-Bertani (LB)

agar was inoculated with 150 µl of the respective *C. violaceum* strains. Strains were grown at a temperature of 30 °C overnight in LB broth. The agar-culture was then poured on pre-warmed LB agar plates and allowed to solidify. Thereafter, blank discs were impregnated with 100 µg and 200 µg of the respective nanoparticles and positioned on the agar plates and further incubated overnight at 30 °C. Discs impregnated with 100 µg and 200 µg of vanillin were used as the positive control. Following incubation, opaque zone diameters indicative of QS inhibition and clear zone diameters indicative of bactericidal activity were recorded.

4.14.2 Quantitative quorum sensing inhibition

The quorum sensing inhibition of the prepared samples was quantified using *C. violaceum* ATCC 12472 and CV017 as the indicator microorganisms (Chenia, 2013). One hundred microliters of *C. violaceum* strains were cultured in 3 ml of LB broth and incubated at 30 °C with increasing concentrations of each AgNP sample, i.e., 0; 20; 40; 80; 160 and 320 µg/ml. For this assay, growth (OD_{600} nm) and violacein production (OD_{560} nm) was determined following overnight incubation. One ml of the *C. violaceum* cultured overnight was then centrifuged with centrifugal force at 13 000 $\times g$ for 10 min so to precipitate insoluble violacein. The obtained supernatant was decanted and the pellets were further suspended in dimethyl sulfoxide (DMSO, 1 ml) (Truchado et al., 2012, Chenia, 2013). The newly prepared solution was centrifuged once more at 13 000 $\times g$ for 10 min. The second centrifugation was targeted towards separating the cells and the precipitated violacein was measured at OD_{560} nm using the Glomax Multi+ Detection System (microtitre plate reader) (Promega) (Chenia, 2013). The percentage violacein inhibition = $\frac{Control\ OD_{560}\ nm - Test\ OD_{560}\ nm}{Control\ OD_{560}\ nm} \times 100$ (Abraham et al., 2011, Chenia, 2013).

4.15 Statistical analysis

Data is presented as mean ± SEM. Statistical analysis was done using GraphPad Prism version 5 (Graph Pad Software Inc., USA). All outcomes were compared with control using analysis of variance (ANOVA) followed by Bonferroni post hoc analysis. Effects were considered statistically significant at p value less than or equal 0.05.

4.16 Results

4.16.1 Synthesis and quantification of AgNPs

The percentage yield of biosynthesized AgNPs from *D. villosa* leaves and stem bark are presented in Figure 1. The two-way ANOVA showed that there was a significant interaction between the temperature and variation in the parts of the plant ($F_{(1, 8)} = 33.94$, $p < 0.001$). The percentage yield of AgNPs using the leaves extract at RT (LRT) was found to be significantly higher compared to that of 80°C (L80) ($p < 0.001$). The percentage yield of AgNPs using stem bark at RT (SRT) was observed not to be statistically different compared to that of 80°C (S80).

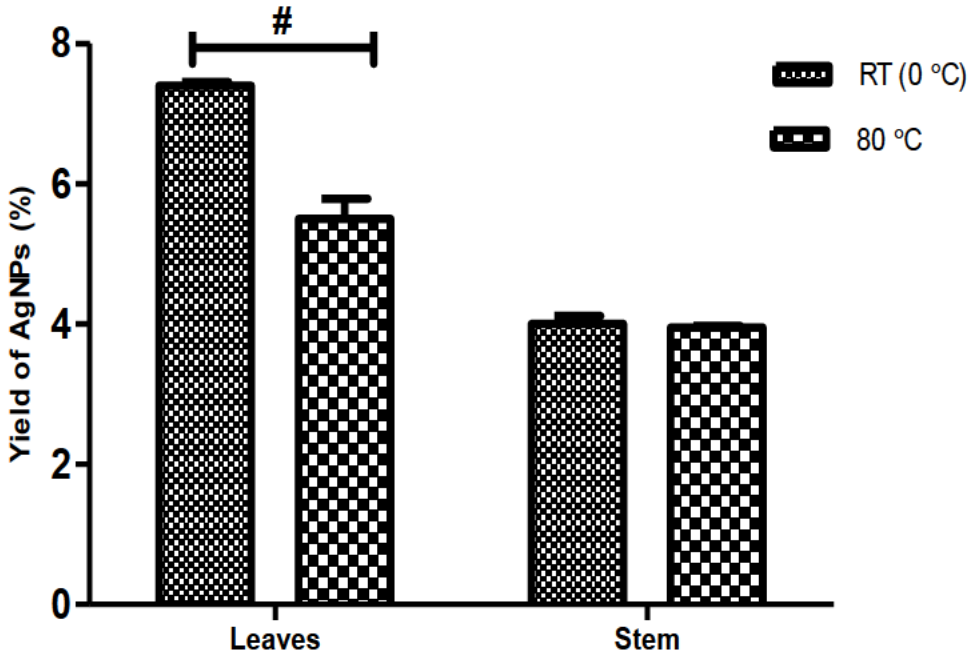


Figure 1: Percentage yield of AgNPs obtained from leaves and stem bark of *D. villosa* at room temperature (RT) and at 80 °C. $F_{(1, 8)} = 33.94$. #(Leaves RT vs. Leaves 80 °C), $p < 0.001$.

4.16.2 UV- visible spectra analysis

For the biosynthesized AgNPs using leaves extract at RT and at 80 °C, the absorption peaks were found to be at 424 nm and 417 nm respectively. Meanwhile, the absorption peaks were noted to be at 367 and 369 nm for the biosynthesized AgNPs using stem bark extracts at RT and at 80 °C respectively. It was also observed that AgNPs biosynthesized from the leaves extract produced higher absorption peaks at RT compared to 80 °C. Similarly, the AgNPs synthesized from the leaves had higher intensities compared to the stem bark extract.

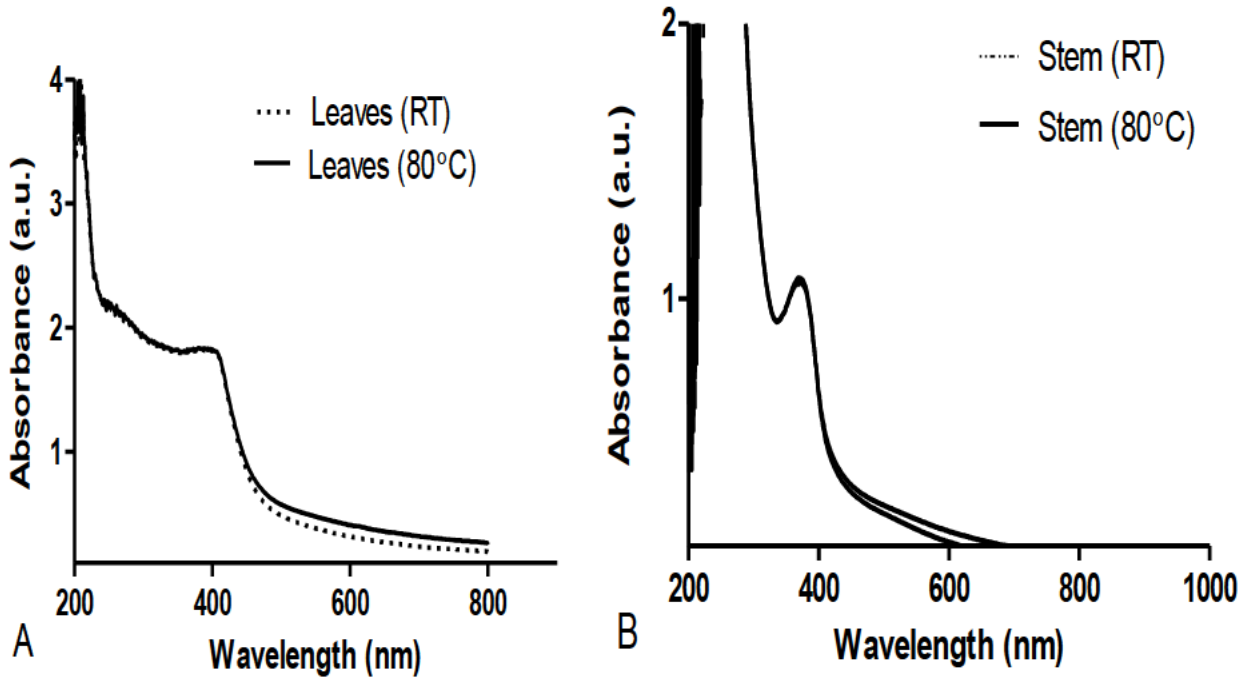


Figure 2: UV-visible spectra of *D. villosa* leaves (A) and stem (B) nanoparticles at RT and at 80 °C

4.16.3 Fourier Transform Infrared (FT-IR) Analysis

The FT-IR spectra of *D. villosa* leaves and stem bark and their corresponding nanoparticles are presented in Figure 3a-f. The spectra showed the vibrational frequencies of different functional groups found in both the crude extracts and in the nanoparticles. The spectrum of the aqueous extract of the leaves and leaves nanoparticles synthesized at 80 °C as well as stem nanoparticles synthesized at both room temperature and 80 °C showed characteristic absorption bands for C-H stretching at 2929.37, 2937.87, 2937.87 and 2926.54 cm^{-1} (Figure 3a, 3c, 3e and 3f). Similarly, a strong O-H stretching band at 3369.91 and 3287.75 cm^{-1} was observed in the crude stem extracts and stem nanoparticles at 80 °C (Figure 3d and 3f). There was also a strong absorbance band for carbonyls (C=O) stretching band at 1606, 1609.17 and 1602.09 cm^{-1} in both the crude leaves and stem extract as well as stem nanoparticles synthesized at 80 °C (Figure 3a, 3d and 3f). It was also observed that a weak absorbance band for alkynes appeared within the range of 2365 and 2371.26 cm^{-1} in the nanoparticles synthesized at RT, crude leaf extract and stem nanoparticles at 80 °C.

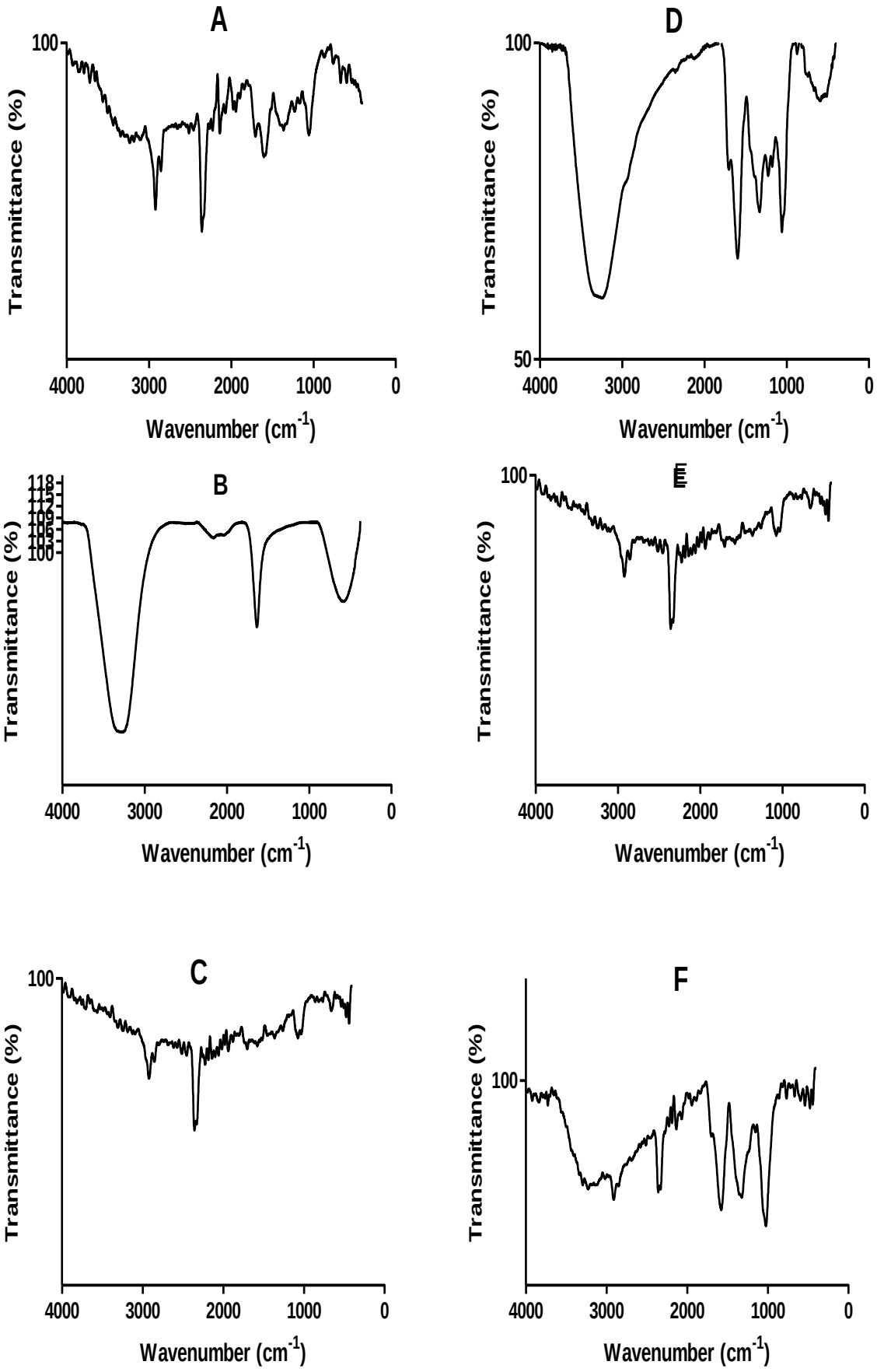

A
100
Transmittance (%)
4000
3000
2000
1000
0
Wavenumber (cm-1)
D
100
50
Transmittance (%)
4000
3000
2000
1000
0
Wavenumber (cm-1)
B
118
100
Transmittance (%)
4000
3000
2000
1000
0
Wavenumber (cm-1)
E
100
Transmittance (%)
4000
3000
2000
1000
0
Wavenumber (cm-1)
C
100
Transmittance (%)
4000
3000
2000
1000
0
Wavenumber (cm-1)
F
100
Transmittance (%)
4000
3000
2000
1000
0
Wavenumber (cm-1)

Figure 3: FT-IR spectra of crude extracts and the biosynthesized AgNPs of *D. villosa* plant. (a) crude aqueous leaf extract; (b) leaves nanoparticles at RT (LRT); (c) leaves nanoparticles at 80 °C (L80); (d) crude aqueous stem-bark extract; (e) stem-bark nanoparticles at RT (SRT); and (f) stem-bark nanoparticles at 80 °C (S80).

4.16.4 Scanning Electron Microscopy (SEM) Analysis

The SEM images of the *D. villosa* leaves and stem bark nanoparticles are presented in Figure 4. The images showed that the nanoparticles of the *D. villosa* leaves synthesized at room temperature and at 80 °C were agglomerated and widely distributed. Meanwhile, the nanoparticles of the stem extract (at 80 °C) were of similar sizes, spherical and well distributed (Figure 4d). The spherical morphology of the nanoparticles was more distinct in the stem bark extract compared to that of the leaves.

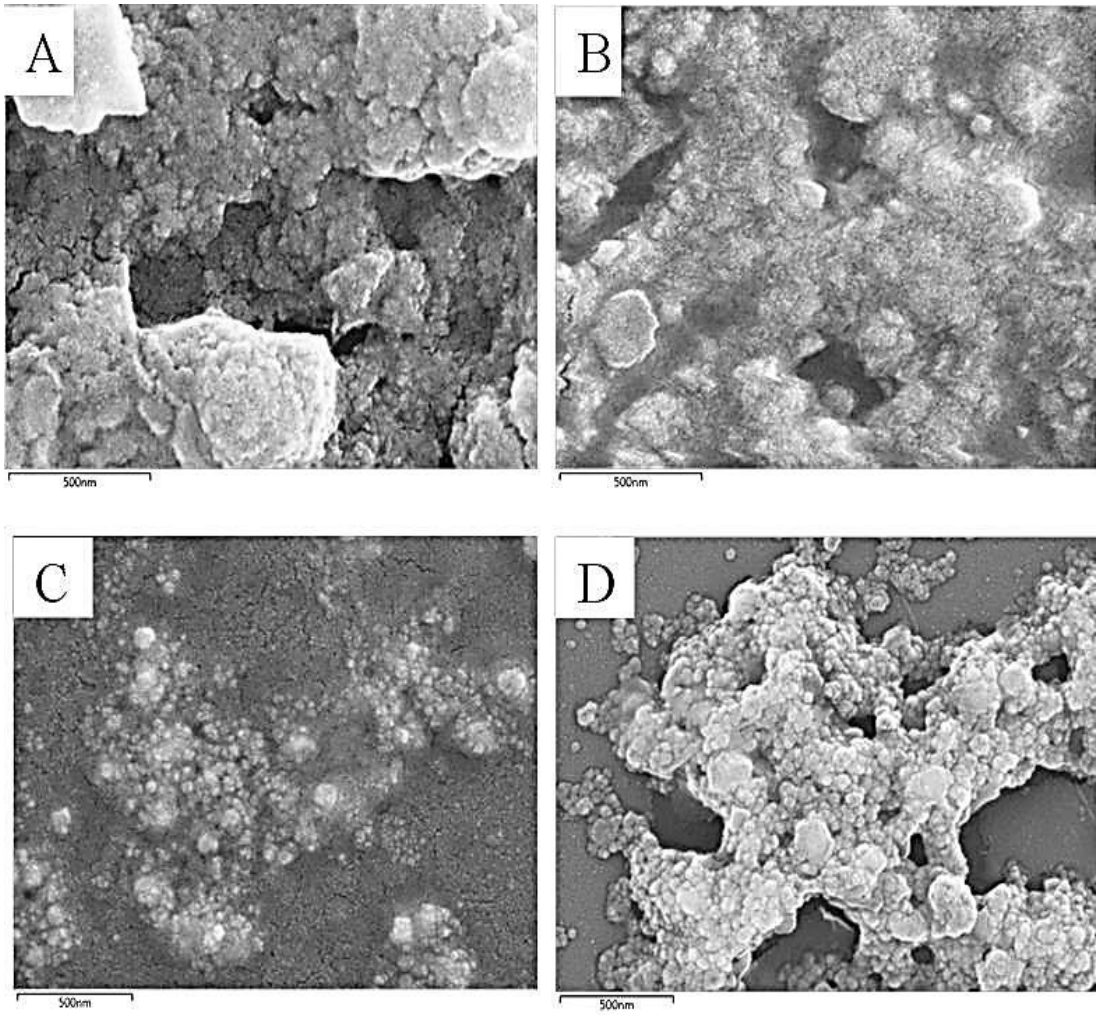


Figure 4: SEM images of *D. villosa* leaves (A-B) and stem bark (C-D) nanoparticles synthesized at RT and at 80 °C, respectively.

4.16.5 Energy Dispersive X-ray (EDX) Analysis

EDX analysis spectra showed the existence of sodium and zinc salts within the synthesized nanoparticles from *D. villosa* leaves and stem bark. The concentrations of both zinc and sodium salts were found higher in the stem bark nanoparticles synthesized at room temperature (SRT) compared to that of leaves extract nanoparticles at same temperature (LRT). Other trace elements like oxygen, magnesium, potassium and carbon were further observed to exist in the *D. villosa* leaves and stem bark nanoparticles. The high concentration of silicon was as a result of glass slide on which the nanoparticles were placed. Also, the Ag^{+} was quite visible at 3 keV.

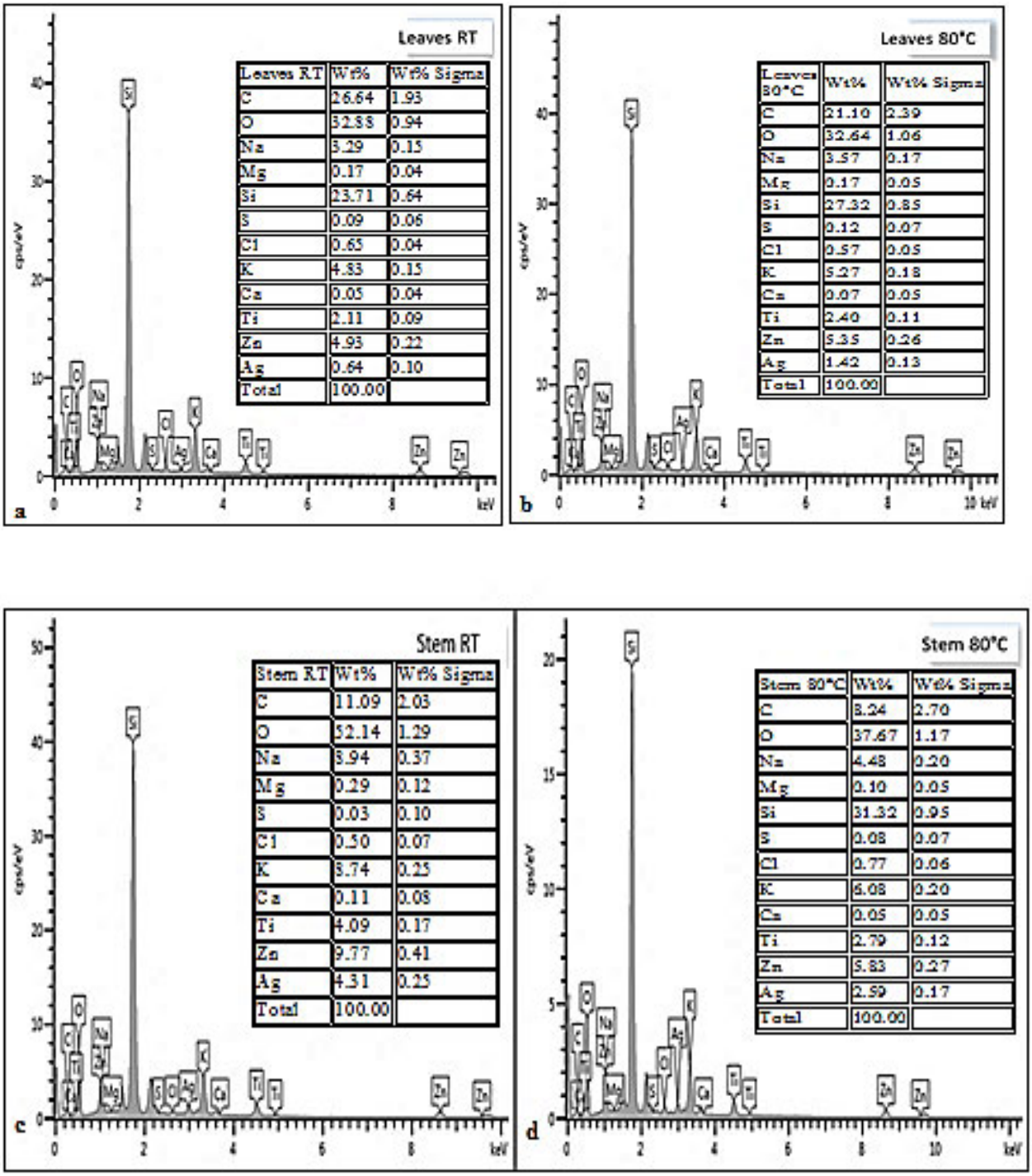


Figure 5: EDX spectra of *D. villosa* leaves nanoparticles synthesized at RT (**a**), at 80 ºC (**b**) *D. villosa* stem bark nanoparticles at (RT) (**c**), and at 80 ºC (**d**) respectively.

4.16.6 Transmission Electron Microscopy (TEM) Analysis

The TEM images revealed different shapes and sizes of the particles. TEM images further showed that the shape of the nanoparticles was predominantly spherical. The diameter of the nanoparticles was observed to be in the range 5 nm and 28 nm. Particles size distribution obtained from *D. villosa* stem bark extract at room temperature was found within 19.74 nm and 27.85 nm. Also, few lower sizes (9.45 nm) were found in the stem bark nanoparticles synthesized at 80 °C.

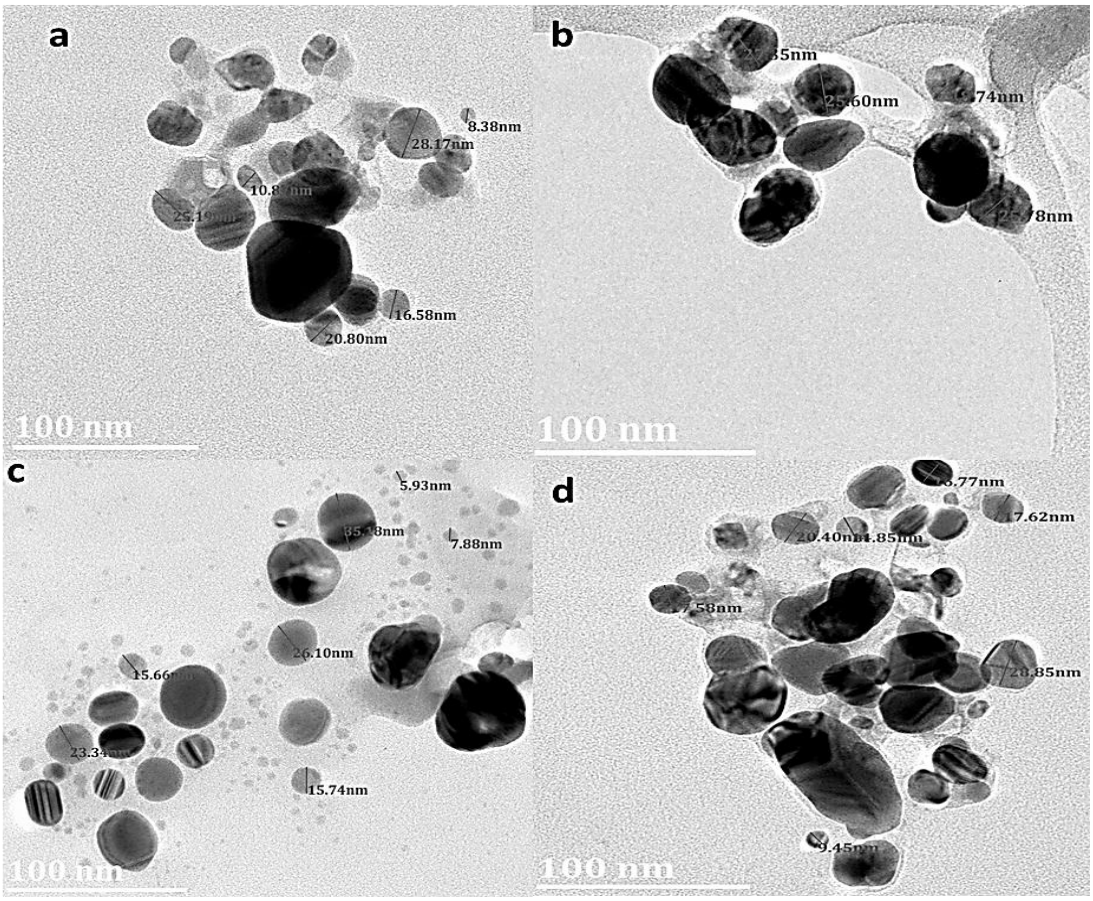
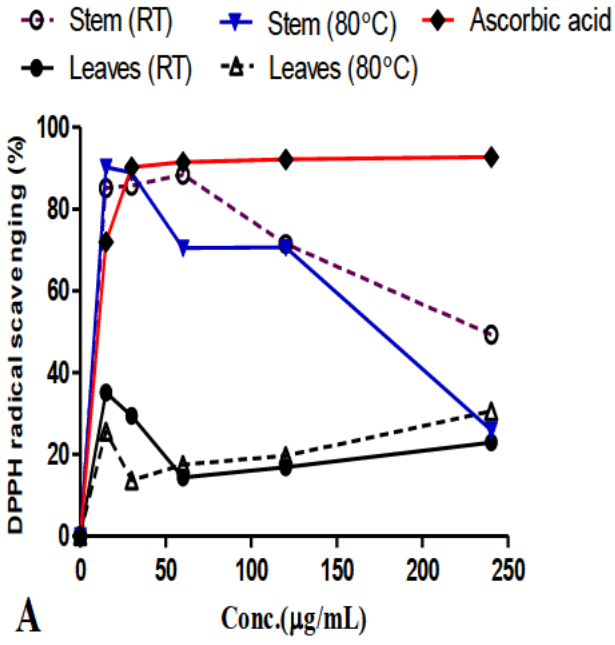


Figure 6: TEM images of *D. villosa* leaves nanoparticles synthesized at RT (**a**) and at 80 ºC (**c**), stem nanoparticles synthesized at RT (**b**) and at 80 ºC (**d**) respectively.

4.16.7 DPPH Scavenging activity

The radical scavenging activity and IC_{50} values of *D. villosa* stem bark nanoparticles are summarized in Fig. 7a and b, respectively. Normally, the higher the % radical scavenging activity and lower IC_{50} values indicate a higher antioxidant activity (Wijewardhana et al., 2019). Amongst all the synthesized nanoparticles used in this study, stem bark nanoparticles synthesized at 80 °C showed an excellent scavenging activity of 90.8%, while ascorbic acid at the same concentration indicated 72.0%. Similarly, the stem bark nanoparticles of *D. villosa* at 80 °C showed an improved radical scavenging activity with IC_{50} found to be at 8.75 µg/ml. This value is significantly lower compared to the ascorbic acid with IC_{50} value of 9.58 µg/ml. Meanwhile, the *D. villosa* stem bark nanoparticles synthesized at RT showed weak antioxidant behaviour with an IC_{50} value of 235 µg/ml compared to ascorbic acid (control). Although, the antioxidant activity of the stem bark nanoparticles synthesized at RT was higher compared to ascorbic acid, it is not statistically significant. The antioxidant activity of the leaves nanoparticles was observed not to be significant compared to the control.



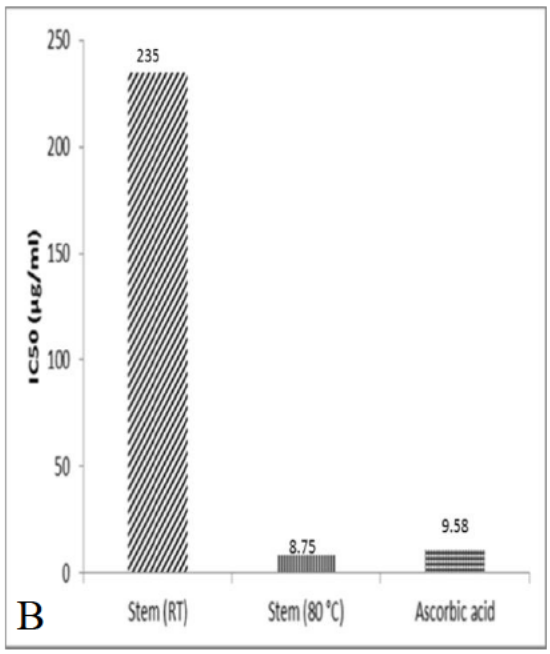


Figure 7: Comparison of percentage DPPH radical scavenging activities (A) and the IC_{50} values (B) of *D. villosa* leaves and stem bark nanoparticles at room temperature (RT) and 80 °C.

4.16.8 Ferric Reducing Antioxidant Power

The ferric reducing power of nanoparticles synthesized from *D. villosa* leaves at RT and at 80 °C as well as their IC_{50} values is presented in Fig. 8a and 8b respectively. Generally, the IC_{50} value is inversely proportional to antioxidant activity. The antioxidant results showed that the nanoparticles synthesized from *D. villosa* leaves at high temperature (80 °C) showed an effective reducing capacity with a lower IC_{50} value of 164.0 µg/ml, which was slightly lower compared to that of ascorbic acid which showed an IC_{50} value of 170.0 µg/ml. However, gallic acid displayed an excellent activity with an IC_{50} value of 91.8 µg/ml. The antioxidant activity of the stem bark nanoparticles was further observed to be lower compared to the control.

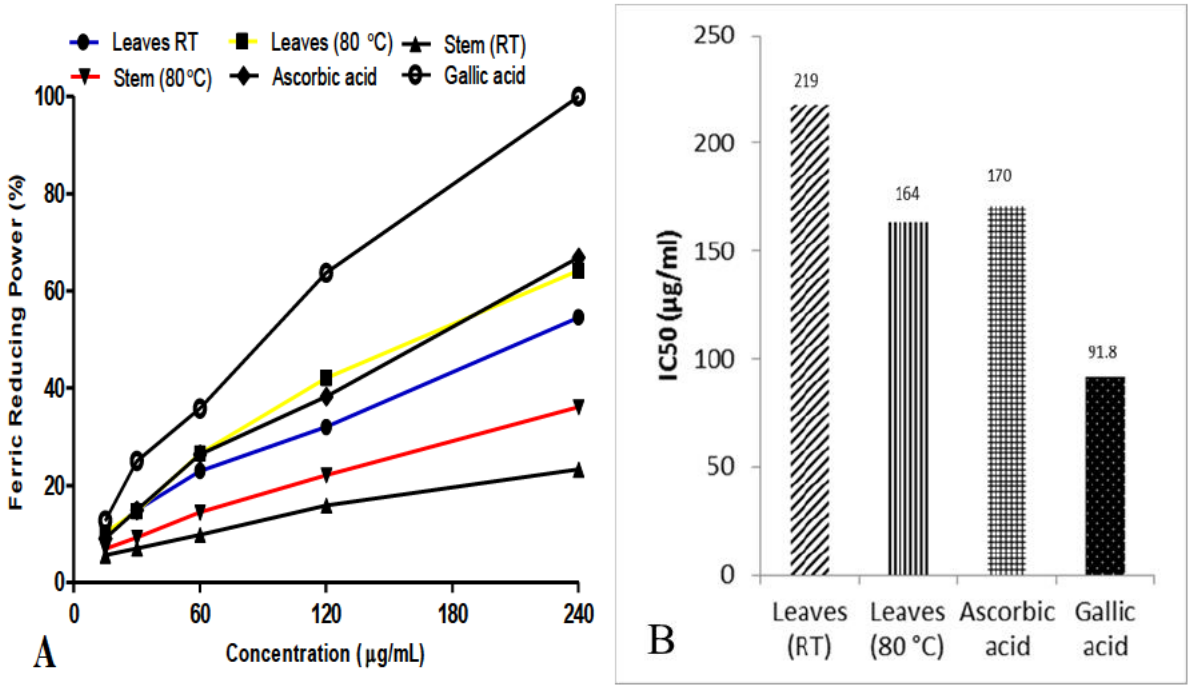


Figure 8: Comparison of percentage ferric reducing power (**A**) and the IC_{50} values (**B**) of *D. villosa* leaves nanoparticles at room temperature (RT) and 80 °C.

4.16.9 Total Phenol Content

The total phenol content (TPC) in the *D. villosa* leaves and stem bark nanoparticles was estimated and analysed. Two-way analysis of variance also revealed the temperature has a significant effect on TPC in the synthesized nanoparticle from *D. villosa* leaves and stem bark $F_{(1, 8)} = 219$, $p \leq 0.0001$. The TPC in the leaves and stem bark nanoparticles synthesized at 80 °C was found to be (20.81 ± 0.098) and (10.22 ± 0.14) mg equivalent per gram of dry weight, respectively. Meanwhile, the TPC in the leaves and stem bark nanoparticles (synthesized at RT) was found to be (17.44 ± 0.36) and (6.324 ± 0.29) mg gallic acid equivalent per gram of dry weight, respectively.

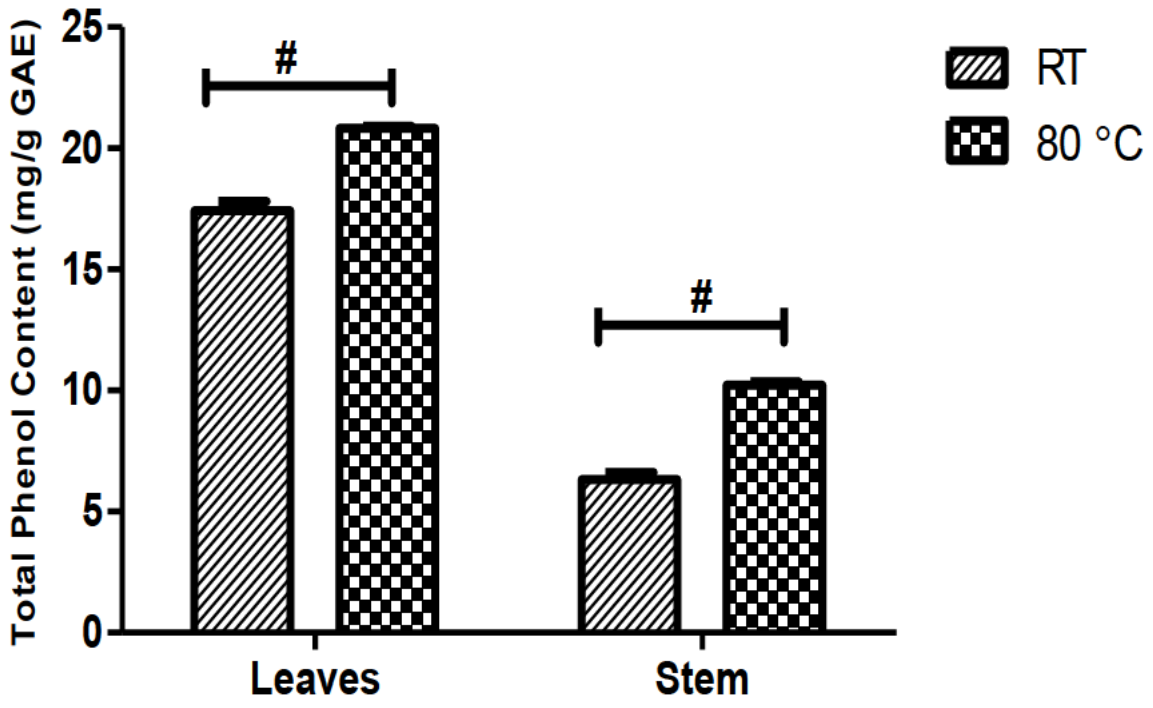


Figure 9: Comparison of total phenol content (mg/g) of *D. villosa* leaves and stem nanoparticles at room temperature and at 80 °C. $F_{(1, 8)} = 219$, $p < 0.0001$.$^{\#}$(RT vs. 80 ºC), $p < 0.001$.

4.16.10 Antibacterial activity

In the antimicrobial susceptibility tests, the stem bark nanoparticles at 100 µg and 200 µg showed weak activity against both strains of *E. coli* and *K. pneumoniae* compared to the antibiotic controls (Table I). At 200 µg, the stem bark nanoparticles indicated a weak activity against *P. aeruginosa* compared to the control. The leaves nanoparticles also demonstrated weak activity against the tested Gram-negative bacteria strains at the concentration of 100 µg and 200 µg. Intermediate activity was however, observed with the leaves nanoparticles synthesized at room temperature against *E. coli* ATCC 25922 at concentration of 100 µg and 200 µg.

Table I: Zone of inhibition (mm) of *D. villosa* leaves and stem nanoparticles against Gram-negative bacteria strains

	E. coli **ATCC 25922**		***E. coli*** **ATCC 35218**		***K. pneumoniae*** **ATCC 700603**		***P. aeruginosa*** **ATCC 27853**	
AgNPs Extracts	**100 µg**	**200 µg**	**100 µg**	**200 µg**	**100 µg**	**200 µg**	**100 µg**	**200 µg**
L RT	11	15	7	8	0	0	0	0
L 80	8	9	7	9	0	0	0	0
SB RT	0	8	7	9	8	8	8	10
SB 80	0	9	7	8	8	8	8	10
Control								
CIP5	30 (S)		37 (S)		26 (S)		32 (S)	
GN10	19 (S)		20 (S)		17 (S)		19 (S)	

Weak ≤ 10 mm; Intermediate (11- 15) mm; Strong > 15 mm.
CIP5: Ciprofloxacin; GN10: Gentamicin
Response to tested compounds is indicated by S (sensitive) and R (resistant).

The stem bark nanoparticles showed weak activity against all the tested Gram-positive bacteria except at a concentration of 200 µg where *S. epidermidis* exhibited intermediate activity (Table 2). The leaves nanoparticles at 100 µg and 200 µg showed weak activity against *E. faecalis* ATCC 29212, *E. faecalis* ATCC 51299, *S. aureus* ATCC 29213 and methicillin-resistant *S. aureus* ATCC 43300 compared to the antibiotic controls (Table 2). However, the leaves nanoparticles demonstrated intermediate activity at 100 µg and 200 µg against methicillin-resistant *S. aureus* ATCC 33591. Similarly, an intermediate activity was observed with the leaves nanoparticles at 100 µg against methicillin-resistant *S. aureus* ATCC 700698 and *S. epidermis* ATCC 12228. Stronger activity was observed, however, with leaves nanoparticles at a concentration of 200 µg against methicillin-resistant *S. aureus* ATCC 700698 and *S. epidermis* ATCC 12228.

Table II: Zone of inhibition (mm) of *D. villosa* leaves and stem NPs against Gram-positive bacterial strains.

AgNP	***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**	
Quantity (µg)	**100**	**200**	**100**	**200**	**100**	**200**	**100**	**200**	**100**	**200**	**100**	**200**	**100**	**200**
L RT	9	10	8	9	9	9	12	14	9	9	11	16	12	16
L 80	9	10	8	9	8	7	11	14	8	8	12	18	12	14
SB RT	7	8	0	8	0	8	0	8	7	8	0	8	9	12
SB 80	8	9	0	9	0	10	8	10	0	7	0	10	8	11
CIP5	33 (**S**)		38 (**S**)		23 (S)		22 (S)		23 (S)		6 (R)		28 (S)	
GN10	18 (R)		0 (R)		19 (R)		16 (S)		9 (R)		11 (R)		20 (S)	

Weak ≤ 10 mm; Intermediate (11- 15) mm; Strong > 15 mm.
CIP5: Ciprofloxacin; GN10: Gentamicin
Response to control antibiotics is indicated by S (sensitive) and R (resistant).

In the current study, *D. villosa* leaves and stem bark nanoparticles synthesized at RT and 80 ºC were evaluated for their QS inhibition against the *C. violaceum* ATCC 12472 and CV 017 strains. Using the agar overlay method, all NPs synthesized at both RT and 80 ºC (100 µg and

200 µg) displayed QS inhibition which was in accordance with the loss of purple pigmentation (Figure 10). NPs inhibited the violacein production with QS inhibition halos ranging from 3-5 mm (Table III). The QS inhibition appeared more predominant for CV 017 compared to ATCC 12472 (Figure 10), with both QS inhibition and halos as well as translucent zones indicating some bacterial activity. The highest QS inhibition was obtained with 200 µg of all the tested NPs against CV 017 (Table 3) as well as ATCC 12472.

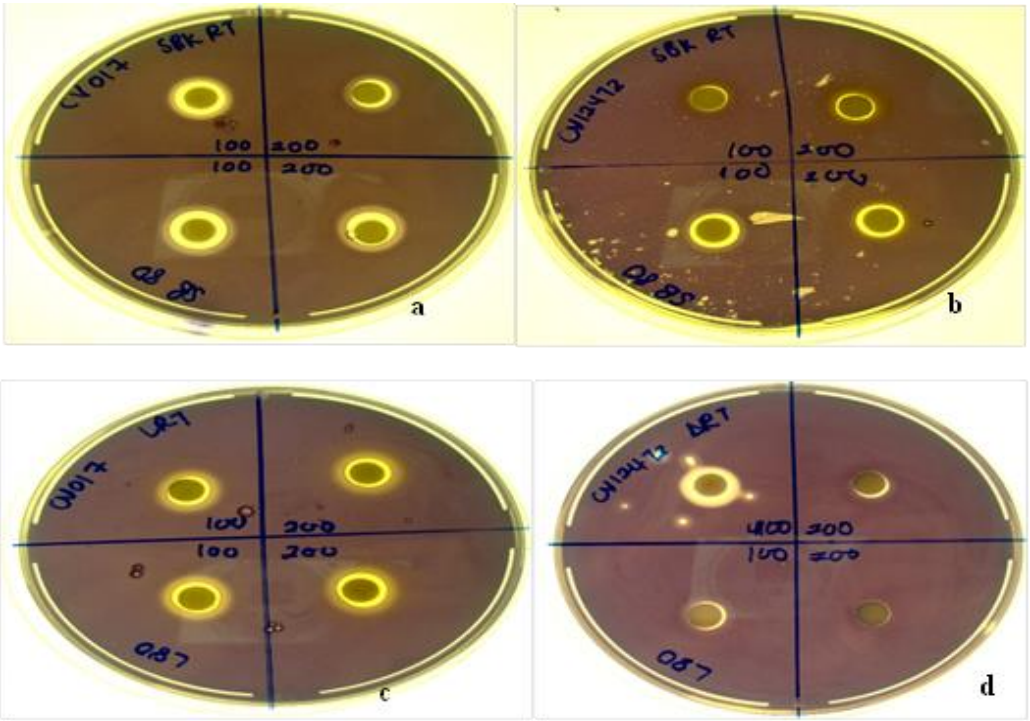


Figure 10: Qualitative agar overlay bioassay of *D. villosa* stem bark NPs, synthesised at RT and at 80 °C, using *C.violaceum* (a) CV017 and (b) ATCC 12472 as well as *D. villosa* leaves nanoparticles at RT and at 80 °C using *C. violaceum* (c) CV017 and (d) ATCC 12472, displaying quorum sensing inhibition (non-pigmented zones) and antimicrobial activities (translucent zones).

Table III: Zone of inhibition (mm) of *D. villosa* leaves and stem bark AgNPs against *C. violaceum* strains.

AgNPs	***C. violaceum* ATCC 12472**						***C. violaceum* CV017**					
	100 µg			**200 µg**			**100 µg**			**200 µg**		
	TZD	**CZD**	**QSI**	**TZD**	**CZD**	**QSI**	**TZD**	**CZD**	**QSI**	**TZD**	**CZD**	**QSI**
L RT	13	8	5	14	9	5	13	8	5	14	9	5
L 80	12	8	4	13	8	5	13	8	5	14	9	5
SB RT	11	7	4	14	9	5	13	10	3	14	9	5
SB 80	12	8	4	15	10	5	15	11	4	14	9	5
Vanillin (400 µg)	**9**			**9**			**11**			**11**		

TZD = Total zone diameter in mm

CZD = Clear zone diameter in mm

QSI = Quorum sensing inhibition zone in mm

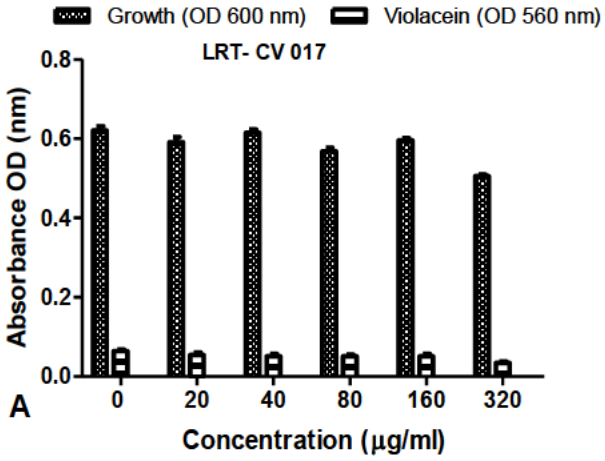
The QS inhibition of the leaves and stem nanoparticles synthesized at both RT and 80 ºC were quantified using *C. violaceum* CV 017 as the indicator microorganisms (Figure 11). The % violacein inhibition (%VI) of *C. violaceum* CV 017 by *D. villosa* leaves nanoparticles synthesized at RT (LRT) were less than 50% at all concentrations tested (Figure 11E). However, at a concentration of 80 µg/ml, the %GI and %VI of leaves nanoparticles synthesized at 80 ºC (L80) were found to be 10.08 µg/ml and 58.83 µg/ml, respectively (Figure 11B and F). At 160 µg/ml, L80 demonstrated %GI and %VI of 85.82% and 89.39% respectively (Figure 11F), while at 320 µg/ml, both %GI and %VI were observed to be 86.28% and 82.27% respectively.

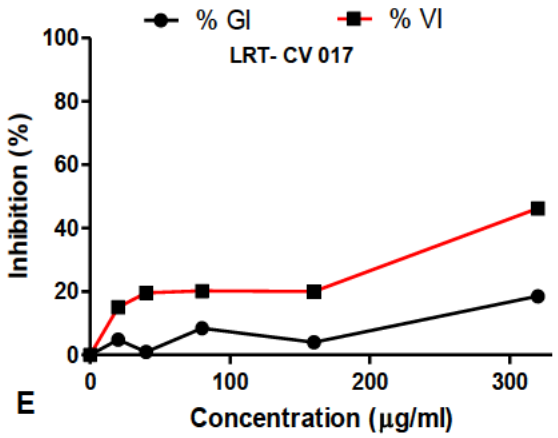
The %GI and %VI of *D. villosa* stem nanoparticles synthesized at RT (SRT) when tested at 20 - 80 µg/ml were < 50% (Figure 11G). However, at 160 µg/ml, the %GI and %VI values of SRT against CV017 were found to be 13.83% and 65.97%, respectively. (Figure 11G). Furthermore, the %GI and %VI of SRT at concentration of 320 µg/ml against CV017 were found to be 84.74% and 71.63% (Figure 11G). SRT showed a good QS inhibition at 160 µg/ml while its activity at 320 µg/ml may be attributed to its antimicrobial potency.

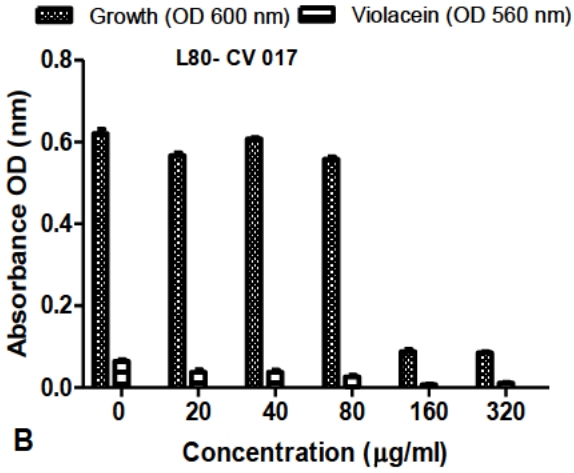
Diospyros villosa stem nanoparticles synthesized at 80 ºC (S80), in contrast, showed negligible %GI as well as %VI < 30% at all the concentrations used (Figure 11H).

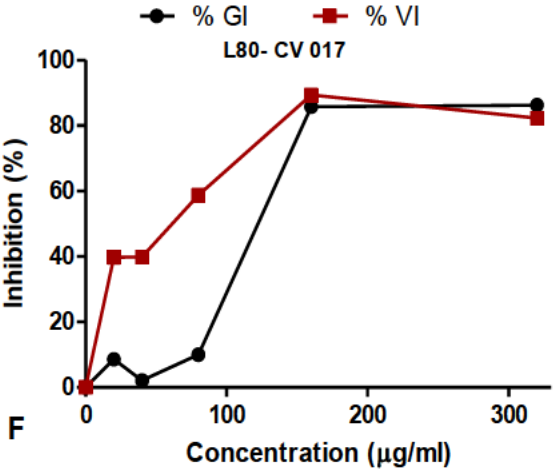
The QS inhibition of the leaves and stem nanoparticles synthesized at both RT and 80 ºC were quantified using *C. violaceum* ATCC 12472 as the indicator microorganisms (Figure 12). The %GI and %VI of *D. villosa* LRT against ATCC 12472 were found to be <50% at 20 – 160 µg/ml (Figure 12E). However, at 320 µg/ml, the %GI and %VI of LRT against *C. violaceum* ATCC 12472 was 29.07% and 56.53% (Figure 12E). The %GI and %VI of L80 nanoparticles against *C. violaceum* ATCC 12472 at 20 – 80 µg/ml was less than 50% (Figure 12F). However, the %GI and %VI of L80 at concentration of 160 µg/ml against ATCC 12472 was found to be 39.68% and 79.83% (Figure 12F).

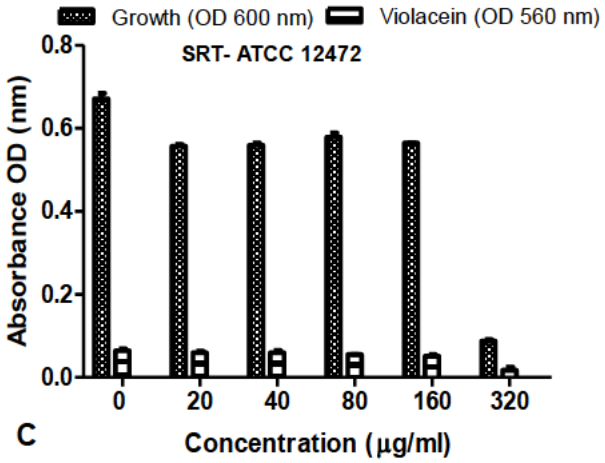
The %GI and %VI of *D. villosa* stem nanoparticles synthesized at RT (SRT), against ATCC 12472 at the concentration of 320 µg/ml was observed to be 87% and 73.26% respectively (Figure 12G). The SRT at concentration of 320 µg/ml may rather be considered a good antimicrobial agent than QS inhibitor. The *Diospyros villosa* stem nanoparticles synthesized at 80 ºC (S80) demonstrated %VI of <50% at all tested concentrations against ATCC 12472 (Figure 12H).

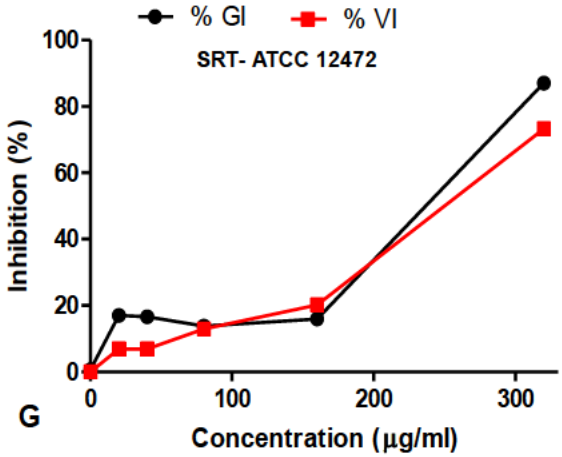

Growth (OD 600 nm)
Violacein (OD 560 nm)
LRT- CV 017
Absorbance OD (nm)
0.8
0.6
0.4
0.2
0.0
0
20
40
80
160
320
Concentration (µg/ml)
A


% GI
% VI
LRT- CV 017
Inhibition (%)
100
80
60
40
20
0
0
100
200
300
Concentration (µg/ml)
E


Growth (OD 600 nm)
Violacein (OD 560 nm)
L80- CV 017
Absorbance OD (nm)
0.8
0.6
0.4
0.2
0.0
0
20
40
80
160
320
Concentration (µg/ml)
B


% GI
% VI
L80- CV 017
Inhibition (%)
100
80
60
40
20
0
0
100
200
300
Concentration (µg/ml)
F


Growth (OD 600 nm)
Violacein (OD 560 nm)
SRT- ATCC 12472
Absorbance OD (nm)
0.8
0.6
0.4
0.2
0.0
0
20
40
80
160
320
Concentration (µg/ml)
C


% GI
% VI
SRT- ATCC 12472
Inhibition (%)
100
80
60
40
20
0
0
100
200
300
Concentration (µg/ml)
G

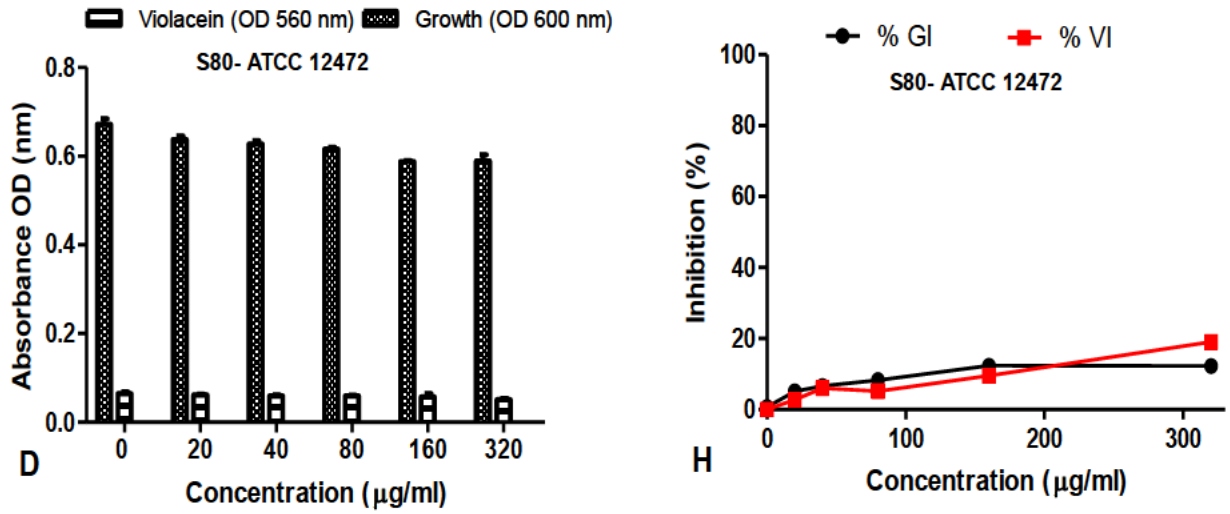
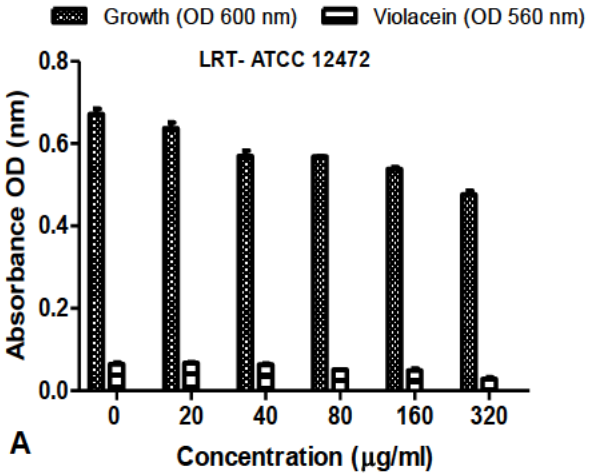
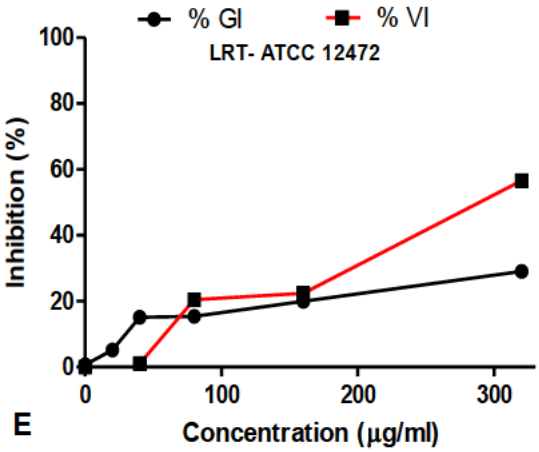
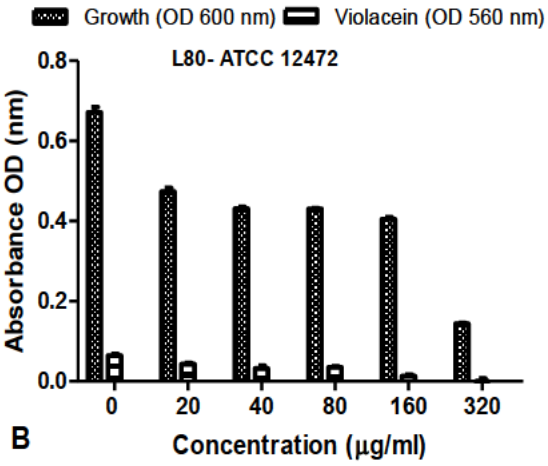
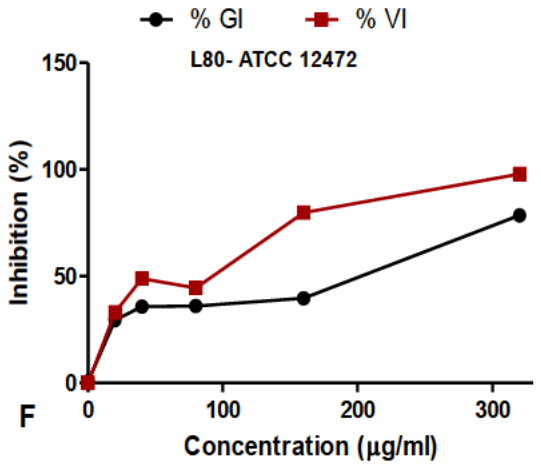


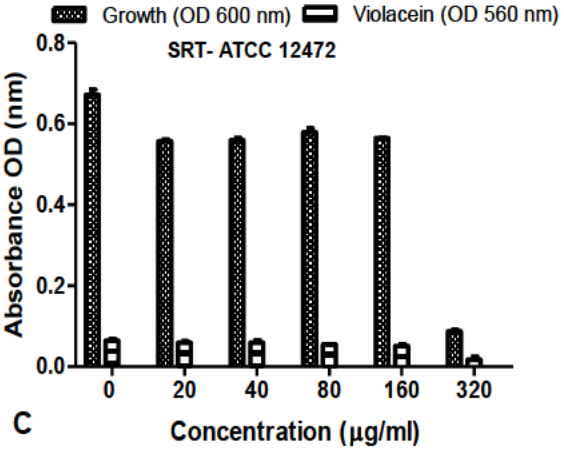
Fig. 11: Quantitative analysis of the inhibitory effect of different concentrations of *D. villosa* leaves and stem bark nanoparticles synthesized at both room temperature (RT) and at 80 ºC (80) on production of violacein by CV017. Cultures were grown in the presence of 0-320 µg/ml of respective leaves and stem bark nanoparticles at RT and 80 ºC. Data were the average of triplicate independent experiments and SD.

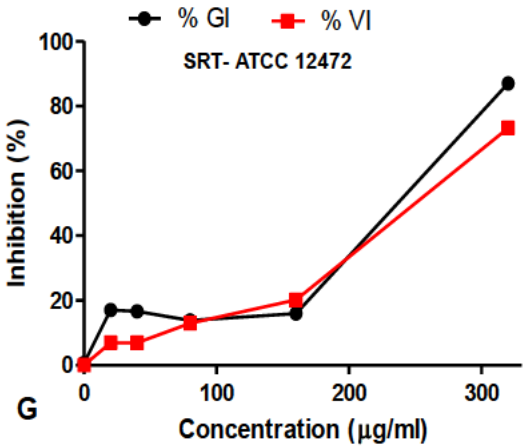

Growth (OD 600 nm)
Violacein (OD 560 nm)
LRT- ATCC 12472
Absorbance OD (nm)
0.8
0.6
0.4
0.2
0.0
0
20
40
80
160
320
Concentration (µg/ml)
A


% GI
% VI
LRT- ATCC 12472
Inhibition (%)
100
80
60
40
20
0
0
100
200
300
Concentration (µg/ml)
E


Growth (OD 600 nm)
Violacein (OD 560 nm)
L80- ATCC 12472
Absorbance OD (nm)
0.8
0.6
0.4
0.2
0.0
0
20
40
80
160
320
Concentration (µg/ml)
B


% GI
% VI
L80- ATCC 12472
Inhibition (%)
150
100
50
0
0
100
200
300
Concentration (µg/ml)
F


Growth (OD 600 nm)
Violacein (OD 560 nm)
SRT- ATCC 12472
Absorbance OD (nm)
0.8
0.6
0.4
0.2
0.0
0
20
40
80
160
320
Concentration (µg/ml)
C


% GI
% VI
SRT- ATCC 12472
Inhibition (%)
100
80
60
40
20
0
0
100
200
300
Concentration (µg/ml)
G

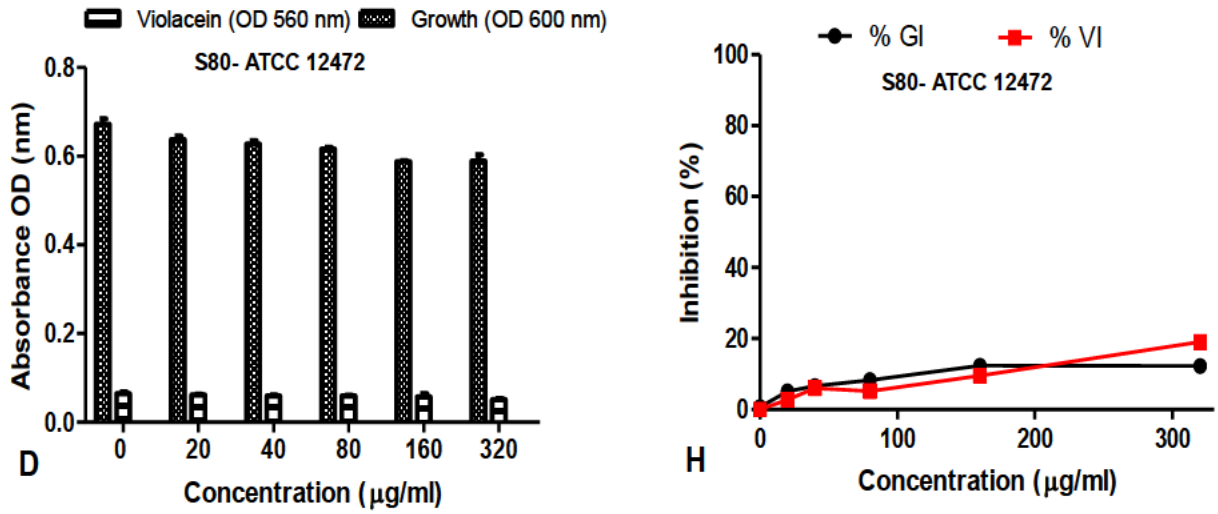


Figure 12: Quantitative analysis of the inhibitory effect of different concentrations of *D. villosa* leaves and stem bark nanoparticles synthesized at both room temperature (RT) and at 80 ºC (80) on production of *C. violaceum* ATCC 12472. Cultures were grown in the presence of 0-320 µg/ml of respective leaves and stem bark nanoparticles at RT and 80 ºC. Data were presented as the mean value and SD.

4.17 Discussion

The synthesized nanoparticles as used in this study were synthesized from *D. villosa* plant leaves and stem bark extracts with the reaction temperature being varied, i.e., room temperature vs. 80 ºC. Distilled water was used as the reactional medium while the plant metabolites were used as the reducing and capping agents. The *Diospyros villosa* leaves and stem bark NPs were characterised and investigated for their anti-oxidant, quorum sensing inhibitory and antibacterial activities.

The chemical compositions of the extracts were known to influence the properties of nanoparticles and the reduction process during NPs synthesis. This may be due to different types and amount of reducing and stabilizing agents as contained in the plant extracts

(Bandeira et al., 2020). There is variation in the bioactive ingredients of the plant extracts and factors like extraction solvents and concentration, time, temperature, solubility are accountable for such occurrence (Cvetanović et al., 2019, Ullah et al., 2020). The effect of extraction temperature condition on the percentage yield in the synthesis of *D. villosa* leaves NPs was clearly significant ($p < 0.001$). Dhanani et al. (2017) suggested that different extraction temperature yielded different extracts composition. It is expected that extraction temperature must be chosen accurately for the maximum extraction of the targeted compounds which are further responsible for reduction and capping of the NPs. The appearance of the brown colour was an indication of the silver ionic reduction and formation of AgNPs (Zayed et al., 2019). This observation was further proven by UV-vis spectral analysis.

The UV-vis spectra of biosynthesized AgNPs from *D. villosa* leaves and stem bark at different temperatures are shown in Figure 2. It was quite obvious in Figure 2a that *D. villosa* leaves extract revealed a peak matching the surface plasmon resonance (SPR) band of AgNPs (Baruah et al., 2020). In support of this, SEM showed spherical shape of the NPs; but a better morphological characterization of NPs was achieved using the TEM. Transmission electron microscopy provided the evidence that the nanoparticles were predominantly spherical in shape; less agglomerated and distributed particularly in the temperature enhanced biosynthesized AgNPs (Figure 6c and d). The difference in the degree of agglomeration in the temperature-enhanced biosynthesis as compared with that of room temperature was suggested to occur due to the variety of bio-reducing compounds. Vinod et al. (2017) suggested that the NPs have propensity to aggregate together forming micro-size particles that are more stable. The high reactivity of aqueous leaves extract (at room temperature) could be ascribed the presence of water soluble heterocyclic compounds and other natural reducing agents. Therefore, the use of water facilitated the extraction of water-soluble

compounds (Reddy et al., 2010) which are further considered to facilitate the synthesis of NPs such as the obtained AgNPs from the leaves extract at both RT and 80 ºC .

The nature of the biomolecules present in the *D. villosa* leaves and stem bark was proven by FT-IR analysis (Figure 3). The widened peaks in the range 3200- 3400 cm^{-1} were ascribed to O-H stretching band of poly-phenolic compounds. The sharp peaks at 1600- 1700 cm^{-1} emanated from C=O stretching vibrations (Singh et al., 2014). From these results, it can be deduced that the observable bands at 3369 cm^{-1} could be ascribed to hydroxyl stretching vibrations. This is in agreement with Ayepola et al. (2018) where the same specified range was assigned to O-H stretching vibration with further explanation that the O-H are bonded to sites through strong bond interaction. Hence, the characteristics of O-H stretching as shown with the absorption peak at 3369.91 cm^{-1} further suggested the presence of aromatic acids (Sara et al., 2018). In addition, the spectral analysis depicted that O-H group in the AgNPs biosynthesized extract of *D. villosa* was involved in the reduction of silver ion. The biological molecules of the extract especially the hydrogen contents could act as the reducing agent for the AgNPs. Similarly, the observable bands at 1620 cm^{-1} were assigned to C=O aromatic vibrations. This support the FTIR reported by Tarekegne et al. (2020) where the peak at the same range with our observable value was attributed to symmetric C=O stretch. There is no major difference in the embedded functional groups of the biomolecules while considering the biosynthesis of NPs temperature differences.

Antioxidants are known to defend living cells against harmful effect incurred through oxidative stress (He et al., 2017). The DPPH assay is based on the strength of deep violet colouration at a strong band of 517 nm which further directly points to the concentration. DPPH was reduced by accepting an electron from an antioxidant substance which resulted in the colour change to light yellow from violet with a consequent reduction in absorption. The tested biosynthesized *D. villosa* leaves and stem bark NPs showed free radical scavenging

properties, but to varying degrees. The biosynthesized NPs (at 80 °C) indicated better DPPH antioxidant activity compared to leaves and stem bark nanoparticles (at RT). Further results achieved with DPPH experiments were expressed as median inhibitory concentration (IC_{50}) for a better comparison of the antioxidant potential. The antioxidant potency is inversely proportional to the value of IC_{50}. It was found from the IC_{50} that the synthesized NPs under elevated temperature provided is in direct proportion to antioxidant activity compared to the extracts. Bharathi et al. (2018) similarly found that AgNPs synthesized from *D. montana* leaves extracts demonstrated higher DPPH scavenging activities compared to the crude extracts. This result also supported the DPPH radical scavenging assays that had been reported for AgNPs synthesized under enhanced temperature. In addition, phenols are recognised to be an essential bioactive compound because of their capacity as an antioxidant (Untea et al., 2018). Therefore, the presence of hydroxyl ion in the capping agent as shown in the FT-IR spectral analysis could be another reason for the effective antioxidant capacity of the nanoparticles. Putting these results together, *Diospyros villosa* can be suggested to be applicable in the production of potential AgNPs antioxidant for safe biomedical and healthcare practices.

Only the leaves NPs (LRT and L80) indicated a notable antibacterial activity. The activity thought to be noteworthy is revealed by inhibitory potential against either Gram negative and/or Gram-positive bacteria (Caleja et al., 2018). Only two pathogens *S. aureus* ATCC 700698 and *S. epidermidis* ATCC 12228 were absolutely vulnerable to the NPs. The Gram-positive bacteria were more vulnerable to the NPs synthesized from *D. villosa* leaves and stem bark. The leaves NPs displayed activities against *S. aureus* and *S. epidermidis* suggesting that leaves NPs may have application in topical ointments as a curative measure to *S. epidermidis* and *S. aureus* skin infections. The antimicrobial activity of some plants within the same genus with *D. villosa* has been reported against various pathogens (Bharathi et al.,

2018, Hamedi and Shojaosadati, 2019). Maridass and Ganapathy (2018) reported that NPs synthesized from *D. malabrica* resulted in diminished bacterial growth against *E. coli* and *S. aureus* respectively. This agrees with the obtained results in this study as the LRT revealed a very strong activity against *E. coli* ATCC 35218, *S. aureus* ATCC 33591, ATCC 700698 and *S. epidermidis* ATCC 12228. The L80 NPs also demonstrated similar activity against by revealing a strong reactivity against *S. aureus*. The slight variation observed of the NPs activity as reflected in the result of leaves nanoparticles is collaborated by the reports of Gontijo et al. (2020) with some selected NPs having non-significant activities while others yielded better activities. The varying degrees in reactional bioactivity may be a function of concerned metabolites.

All the synthesized AgNPs supported QS inhibition against short-chain acyl homoserine lactone (AHL) producer *C. violaceum* CV 017 at the tested concentrations. LRT and L80 further supported QS inhibition against long-chain AHL *C. violaceum* ATCC 12472 while, only LRT exhibited QS inhibition against *C. violaceum* ATCC 12472 (Table 3). The halos of growth inhibition indicated zones of 7-10 mm while QS inhibition was detected with zones having the diameter of 3-5 mm.

Traditional medicinal plants have been proven to interact with bacterial quorum sensing and attenuated pathogenicity of bacteria (Khan et al., 2018). However, growth inhibition ≥ 40% indicated bactericidal activity rather than QS inhibitory potential. The activity of the synthesized NPs is an additional and a clear significance of its potential for therapeutic significance. The SRT at the concentration of 160 µg/ml also displayed a good level of potency against short-chain AHL producing *C. violaceum* CV 017. The LRT (at a concentration of 320 µg/ml similarly displayed QS inhibition against *C. violaceum* ATCC 12472. *Diospyros villosa* NPs synthesized at RT were effective as QS inhibitors (at 160 – 320 µg/ml) although not as potent as the plant extracts (data not shown). The leaves NPs

synthesized at 80 ºC (L80) could be applied as a short-chain AHL QS inhibitor. Meanwhile, the leaves NPs were more effective as antimicrobial agents against Gram-positive bacteria, while the stem bark NPs could potentially be useful against Gram-negative bacteria.

4.18 Conclusion

A suitable and more reliable antioxidant potential was obtained with temperature enhanced AgNPs. The C=O as well as the N-H groups present in the *D. villosa* NPs were identified to be responsible for its stabilization and reduction. Furthermore, temperature enhancement in the NPs biosynthesis facilitates a more distinct morphology of *D. villosa* stem bark NPs and with better potency as a reliable antioxidant. Also, this experimental research study was done to determine the quorum sensing inhibitory potential of *D. villosa*. The promising QS and antimicrobial bioactivities of the *D. villosa* stem NPs suggests the potential of these NPs as antimicrobials and/or as alternative to current therapeutic agents.

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CHAPTER FIVE

Prologue

Manuscript 3

Diospyros villosa is characterized by different histological features which may be held responsible for the synthesis and production of different bioactive compounds. The synthesis and secretion of these compounds might be owing to secretory pores and sometimes, hairy projection of the surface of the leaves. To address the question of whether the secreted active compounds are products of the hairy projections, the micromorphology as well as the histology of *Diospyros villosa* (L.) de Winter was investigated.

"Micromorphology and histology of the secretory apparatus of *Diospyros villosa* (L.) de Winter leaves and stem bark"

Micromorphology and histology of the secretory apparatus of *Diospyros villosa* (L.) de Winter leaves and stem bark.

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ABSTRACT

Diospyros villosa (*D. villosa*) is a perennial species prominently acknowledged for its local medicinal applications. The native utilization of this species in traditional medicine may be ascribed to the presence of secretory structures and their exudate (comprised of phytochemicals). However, the morphological nature and optical features of the secretory structures in *D. villosa* remain largely unclear. This study was directed to ascertain the occurrence and adaptive features of structures found within the leaves and stem bark of *D. villosa* using light and electron microscopy techniques. The current study note the existence of trichomes and other secretory structures were noted. SEM indicated the presence of non-glandular hirsute trichomes with bulky stalk on both leaves and stem surfaces. Transverse stem sections revealed the existence of crystal idioblasts. Also, the presence of the main phytochemical groups and their localization within the foliage and stem bark was elucidated through various histochemical tests. The trichomal length and density were also assessed in leaves at different stages of development. The results indicated that the trichomal density at different stages of development of the *D. villosa* leaves and stem bark was not significantly different from one another, $F_{(3, 39)} = 1.183$, $p= 0.3297$. The average length of the non-glandular trichomes in the emergent, young and mature leaves as well as in the stem was recorded to be 230 ± 30.6 µm, 246 ± 40.32 µm, 193 ± 27.55 µm and 164 ± 18.62 µm, respectively. The perimeter and circumference of the observed trichomes in the developmental stages of *D. villosa* leaf and the stem-bark were not statistically different, $F_{(3, 39)} = 1.092$, $p = 0.3615$. The results of histochemical tests showed the existence of phenols alkaloids which are medicinally important and beneficial for treatment of diseases.
Keywords: Histochemistry, Microscopy, Secretory, Trichomes

5.0 Introduction

The genus *Diospyros* is comprised of more than 350 species (Mallavadhani et al., 1998) and is considered most important due to its high economic value (El-Hawary et al., 2020). *Diospyros* is made up of shrubs and trees which are distributed across the world. Almost 42 species can be found in India, within the Central Deccan Plateau dry deciduous forest, tropical dry deciduous forest at Assam and Bengal Safari forest (Ganapaty et al., 2006, Nematollahi et al., 2012). Furthermore, various species within the genus *Diospyros* are located around Africa and are highly important in both the traditional medicine and food industry (Lachenaud et al., 2017). *Diospyros villosa* (*D. villosa*) is a well-known species used in the maintenance of oral hygiene (Cirera et al., 2010b).

Diospyros villosa is a plant that occurs naturally throughout the African continent (Cirera et al., 2010a). *Diospyros villosa* is a perennial, bushy, evergreen plant which could be as tall as 1-4 m. The leaves are chartaceous, dry-dull brown in colour upon on the abaxial surface and much paler on the adaxial surface. The foliage are at average 3 cm long, 2.5 cm wide and are obovate/oblong in shape. The leaves apex is usually broadly rounded, slightly emarginated and sometimes obtuse whereas the base is often in cordate or round shaped (Cirera et al., 2010b). The foliage may contain secretory structures such as trichomes and bioactive phytochemicals which are possibly responsible for the medicinal potential of this species.

Trichomes are uni- or multi-celled structures which originate from epidermal cells of the aerial organs. These epidermal structures vary significantly in morphological characters, location, ability to secrete and type of secretion (Werker, 2000b). Trichomes have many functional roles within a plant. These include: protection to the plant from external stress or mechanical damage (Li et al., 2018), decrease the heat load of a plant, maximize freezing tolerance, participate in seed dispersal, retain water balance in plant leaves, deflect intense radiation of the sun and offer protection against herbivores (Xiao et al., 2017). Additionally,

the glandular trichomes offer chemical protection against different plant eating microorganisms and higher animals (Glas et al., 2012). In addition, the exudate may be of prime importance and use in the medicinal industry

There is a lack of scientific data on the trichome morphology for many '*Diospyros*' species. Similarly, the morphology and structure of trichomes and the exudate found in *D. villosa* have been scarcely studied, like *D. villosa*. The primary focus of this study was to investigate the histomorphology and histochemistry of the leaves and stem bark of *D. villosa*. The nature of secretory products in the leaves and stem bark were also investigated using histochemical assays in order to assess the existing constituents of the plant. Results from this study will contribute to the existing knowledge for *D. villosa*.

5.1 Materials and Methods

5.2 Plant collection

Freshly harvested foliage and stem bark material of *D. villosa* were collected from KwaZulu-Natal, Durban, South Africa (29º 84′ 33.6″S, 31º 4′ 12″E). The plant was identified and deposited in the herbarium with number (01/18257) at the School of Life Sciences, University of KwaZulu-Natal, Durban. These samples were utilized for histological staining and morphological assessment. The developmental stages of the leaf were categorized as emergent, young and mature. A total number of ten replicates were made for each stage of the leaves and stem bark.

5.3 Stereomicroscopy

The structures at the top surface (abaxial) and underneath (adaxial) of the leaf and stem bark of the plant material were observed with an AZ-LED ring furnished stereomicroscope. Images were captured and processed with a Nikon AZ100 stereomicroscope furnished with a camera and the Nikon NISD Elements Software (Version 3.00).

5.4 Electron microscopy

Electron microscopy was used to examine the trichomal morphology in the leaf and stem of *D. villosa*. The samples were studied systemically with the aid of Nikon AZ100 stereomicroscope, Japan, attached to Nikon Fibre Illuminator and images were taken using a Nikon DXM1200C digital camera. The images were taken using the NIS Element Software.

5.5 Scanning Electron Microscopy

The dirt observed to have blocked the surface of the samples was washed-off so as to reduce the ubiquity. Then, the leaves samples belonging to each developmental stage and the stem sections were washed with distilled water and subsequently with few drops of Bio-Rad tween-20 solution. The leaves and stem sections were once washed with distilled water. The sections were sectioned and fixed using glutaraldehyde (2.5%) in phosphate buffer (0.1 M, pH 7.2) for 24 h. The samples were preserved and made stable by allowing buffer washes thrice for 5 min and fixed in osmium tetroxide (0.5%) for 2 h in the absence of sunlight. Samples were later dehydrated in serial solutions (i.e. 25%, 50%, 70% and 100%). The Quorum K180 critical point dryer was used to dry the samples and later placed on aluminium stubs with the aid of carbon conductive tape and sputter-coated with gold in a QurumQ150 RES gold coater. The samples were viewed with LEO 1450 SEM (SmartSEM) and images were captured and analysed.

5.6 Transmission Electron Microscopy

Segments of leaf and stem tissues were excised and fixed in glutaraldehyde (2.5%) in phosphate buffer (0.1 M, pH 7.2) for 24 h. These sections were later washed with buffer thrice and fixed in osmium tetroxide (0.5%) in the absence of sunlight for 2 h. Following this, the sections were dehydrated in graded doses of acetone i.e. 25%, 50%, 70% and 100%. The sections were infiltrated with 50% propylene oxide (50%) and Spurr's resin for 24 h. This was further followed by allowing polymerisation of sections in 100% resin at a temperature

of 85 °C for 8 h. The resin blocks were sectioned using the LKB 7801A on a Leica EM UC7 microtome (Leica Microsystems, Germany). The thinner sections were collected and stained in uranyl acetate (2.5%) for 10 min. The sections were further rinsed with slightly warm water and stained with 2.5% lead citrate and finally rinsed before viewing under a Jeol 1010 transmission electron microscope.

5.7 Light microscopy

The sections from leaf and stem bark were obtained as described in the procedure for transmission electron microscopy. The Leica ultramicrotome EM UC7 (Leica Microsystems, Germany) was used for the sectioning and the sections were then stained with 1% toluidine blue for 1min. The stained sections were viewed under a Nikon eclipse, 80i light microscope.

5.8 Histochemistry

The excision of sections having 100 µm thickness was made possible with the help of dental wax while using an Oxford vibratome. The obtained sections were hydrated and subsequently stained accordingly. The sections were stained with toluidine blue to detect carboxylated polysaccharides (Mahadevan, 1964). The sections were also stained with mercuric bromophenol blue to indicate total proteins (Levy and Mazia, 1953). Sudan back as well as Sudan IV was further used for the confirmation of total lipids and fatty acids (Demarco, 2017). The confirmatory test for the presence of phenolic compound was done using ferric trichloride (Shalini and Sampathkumar, 2012). The ruthenium red was used for the detection of acidic polysaccharides (Demarco, 2017). Meanwhile, the confirmatory test for the presence of alkaloids was done using Wagner’s and Dittmar’s reagents (Ascensão and Pais, 1987).

5.9 Fluorescence Microscopy

Fresh hand cut sections of the leaf and stem bark were utilized for the purpose of this assay. The sections were mounted on the glass slide and viewed. The images were captured at various wavelengths (300nm, 330nm and 380nm) using the Zeiss LSM 710 microscope, Germany. The stem bark sections were stained with 2% acridine orange for a period of 2 min. The sections were later rinsed with distilled water. The prepared sections were placed on the Zeiss LSM 710 microscope and images were captured at 488 nm. Furthermore, the obtainable leaf and stem sections were stained with Calcofluor White for 2 min and rinsed using distilled water. The sections were further placed in water and viewed using an epifluorescence microscope (Nikon Eclipse ATI) at a wavelength of 365 nm. The histomorphology of the embedded structures in the plant was done with the aid of Calcofluor White (Flores-Félix et al., 2015). This stain may rather stain callose than being attached to the cellulose.

5.10 Energy Dispersive X-ray Microanalysis (EDX)

The elemental constituents of the leaves at developmental stages as well as the stem bark of *D. villosa* were determined. The elemental composition and quantification of both leaves and stem were detected by Oxford EDX detector (Oxford Instruments, UK) in a set-up of Zeiss Ultra Plus FEG-SEM (Germany) at 20 kV (Marguí et al., 2009). The leaves' area analyses were done to determine the chemical constituents of the secretory products from the plant.

5.11 Trichome Density, Length and Statistical Analysis

A good choice of images acquired from SEM was analysed using Image J software. The statistical package GraphPad Prism (GraphPad Software Inc., USA) was used for the data analysis. The trichomes on the leaves and stem surfaces were counted. Likewise, the trichomal density, average length, perimeter and circumference were analysed using Image J software. The quantified observations were further analysed using one-way analysis of

variance (one-way ANOVA) and Bonferroni test was used as the post-hoc analysis. The normality of data was assessed by Kolmogrov-Smirnov test and the acquired data were compared with one another using one way ANOVA when the expected requirements are duly met. P values less than 0.05 was standard as being significant.

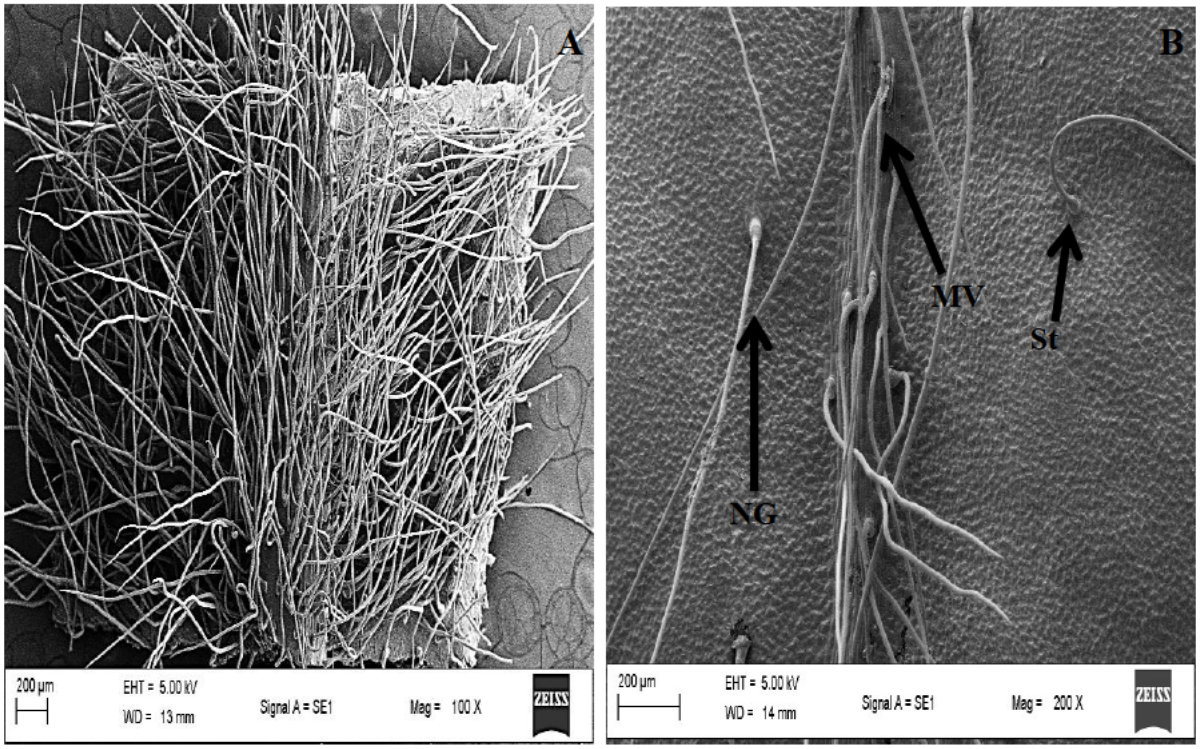
5.12 Results

5.12.1 Stereomicroscopy

Stereomicrographs revealed that the abaxial surface of the leaf was predominantly occupied by trichomes compared with the adaxial surface (Figure 1A and 1B). Stereomicrographs of *D. villosa* revealed the sole type of trichomes (non- glandular) which was found on the stem bark (Figure 2). The transverse section of *D. villosa* stem further revealed the presence of crystal idioblasts (Figure 2D).

Figure 1: Stereomicrograph indicating the leaf topology of *Diospyros villosa*. (A) Abaxial surface of the leaf with dense non-glandular trichomes along the mid and lateral veins (B)

Adaxial surface of the leaf showing fewer non-glandular trichomes. *NG = Non glandular trichomes*.



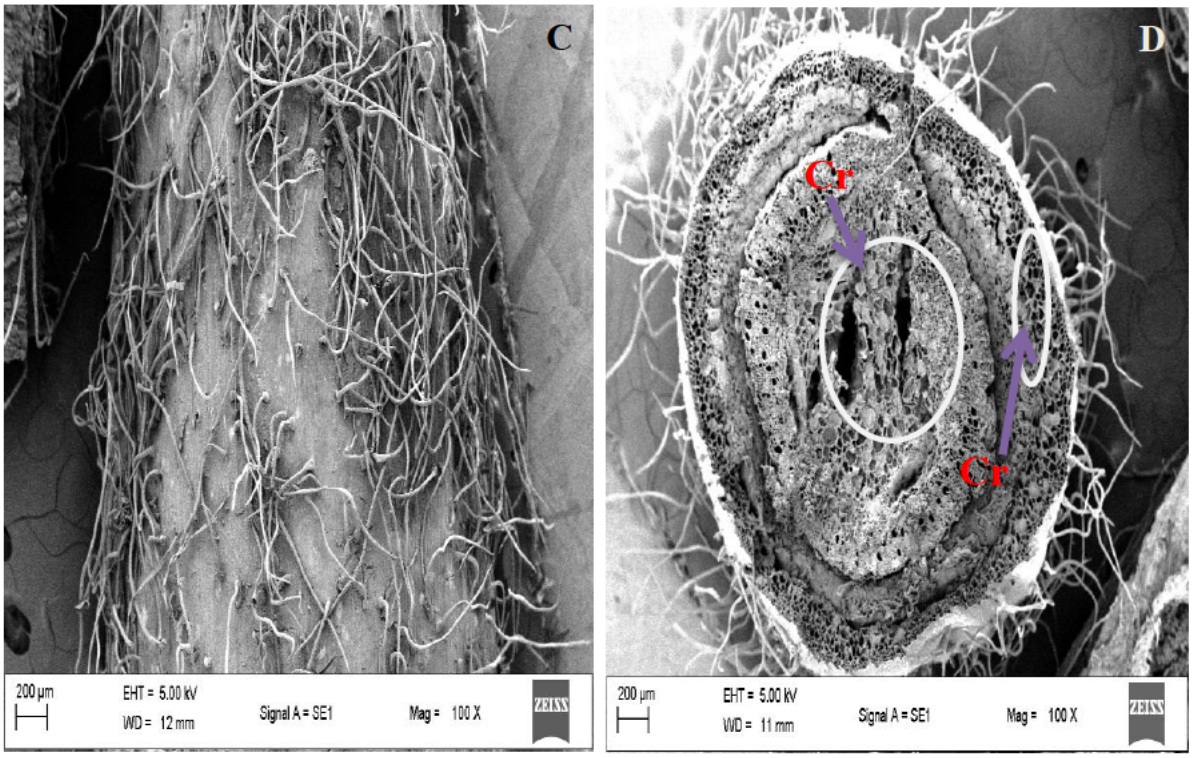
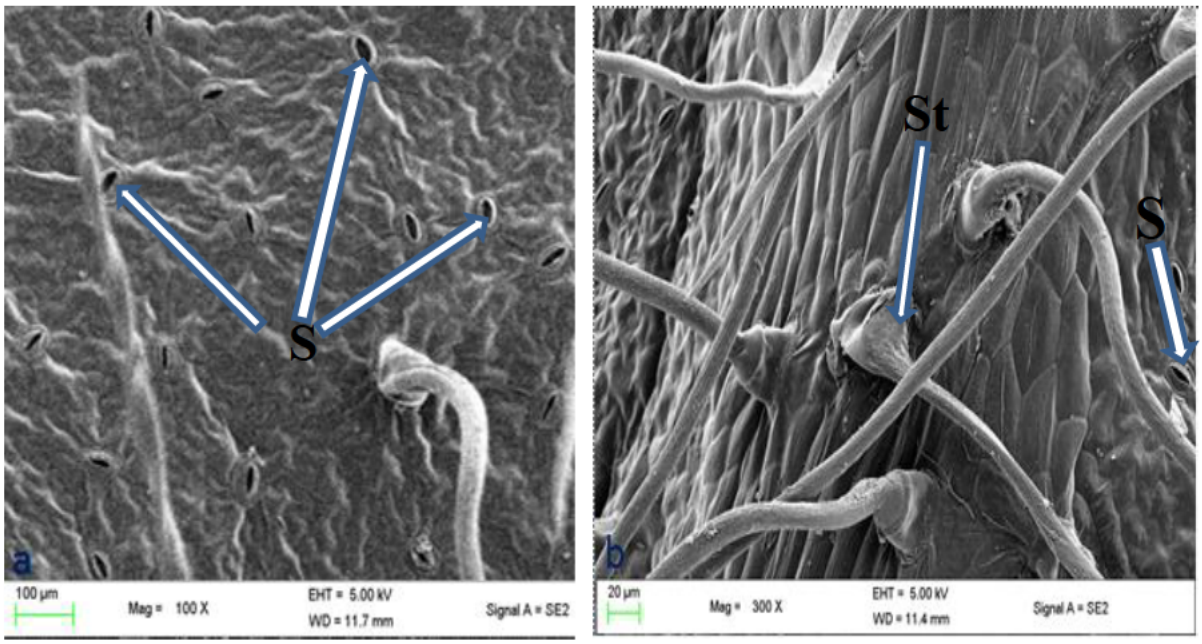


Figure 2: SEM micrograph of the leaf and stem bark of *Diospyros villosa* showing: A) Abaxial leaf surface showing the presence of numerous non-glandular trichomes across the leaf surface B) Adaxial leaf surface showing scanty non-glandular trichome coverage, C) Stem surface indicating the distribution of non-glandular trichomes and D) transverse sectional area of the stem bark. *NG = Non glandular, MV = Medial vein, St = Stalk/ base of the trichome, Cr = Crystal*

5.12.2 Scanning Electron Microscopy

Scanning electron micrographs of the leaves showed secretory pores on the epidermis (Figure 3a). Stomata/secretory pores appeared in abundance on the mature leaves' adaxial surface compared to both the emergent and young leaves. Micrographs further revealed a single non-glandular trichome type (Figures 3c & d). The non-glandular trichome can be regarded as hirsute (Payne, 1978) which comprises a long but stiff stalk.



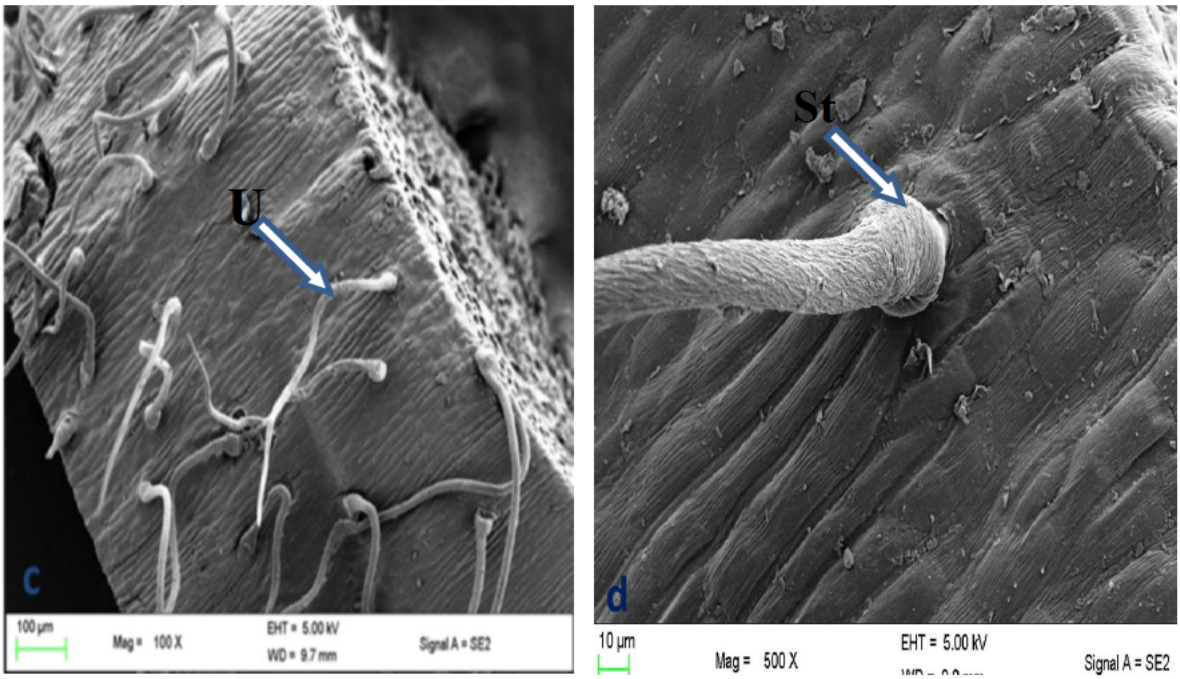


Figure 3: SEM of *Diospyros villosa* leaves showing: a) stomata (**S**) on mature leaf adaxial surface; b) non-glandular single-celled stalk on the adaxial surface of mature leaf (**St**); SEM of *D. villosa* stem showing: c) non-glandular hirsute (**U**) trichome on surface of the stem-bark, d) non glandular trichome with bulky stalk (**St**).

5.12.3 Transmission Electron Microscopy (TEM)

The *Diospyros villosa* leaves and stem bark sections were assessed using TEM. There is further observation of different molecular components such as endoplasmic reticulum, vesicles, vacuoles (large), mitochondria, starch granule, ribosome, chloroplast and nuclei which were observed (Figure 4). The leaf showed to consist of large vacuoles and a nucleus (Figure 4A). Also, plasmodesmatal connection was observed in the leaf (Figure 4B). Cytoplasm containing numerous plastids (Figure 4A) as well as ribosomes was also seen. Plastids were also observed to have lipophilic material, which was indicated by the presence of dark black deposits within. Furthermore, cytoplasm was seen to contain dense materials. The presence of a complex network of endoplasmic reticulum, mitochondria and plastids (Figure 4C) were further observed in the leaves. These organelles were mostly large and abundant in the cytoplasm. The vacuoles, chloroplast and vesicles were found sufficiently

along the cell wall periphery (Figure 4C). Similarly, the stem was observed to be filled with large vacuoles and well observed chloroplasts (Figure 5a & b).

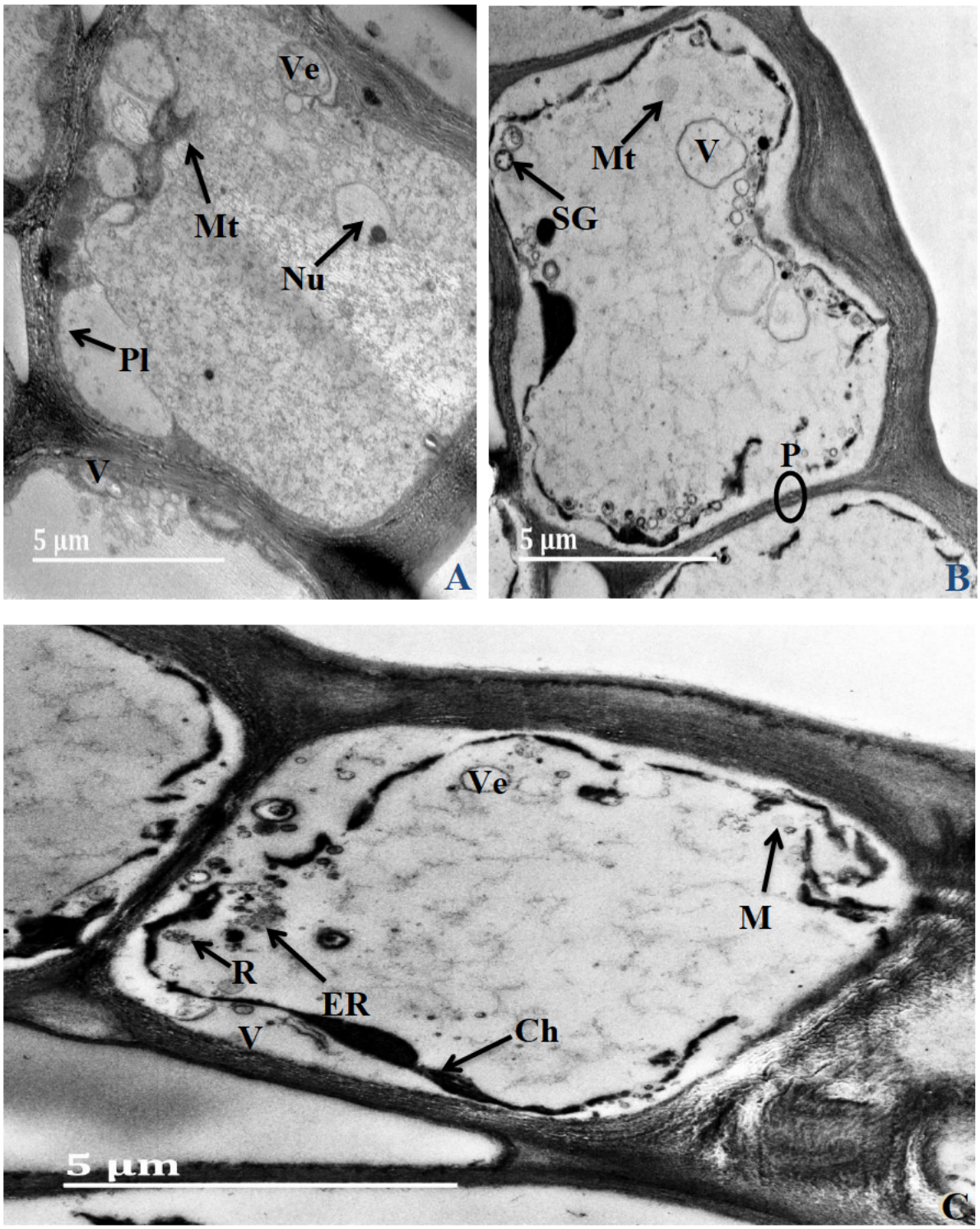


Figure 4: TEM micrograph of the *D. villosa* leaves at different developmental stages A) Mature, B) Young and C) Emergent. Ch = Chloroplast, Pl = Plastids, P = Plasmodesmata, Mt

= Mitochondria, V = Vacuole, Nu = Nucleus, SG = Starch granules, ER = Endoplasmic Reticulum and R = Ribosome.

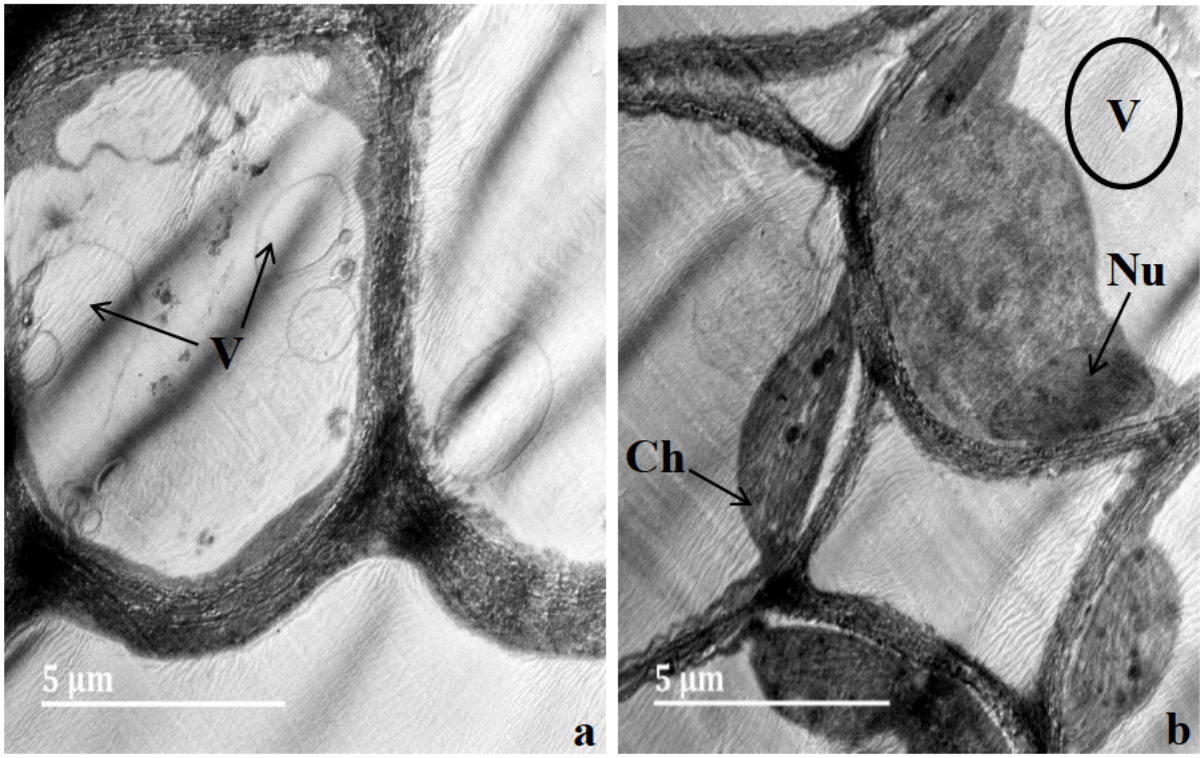


Figure 5: TEM micrograph of the *D. villosa* stem **(a)** and **(b)**. Ch = Chloroplast, V = Vacuole, Nu = Nucleus.

5.12.4 Trichome Density, Length, Perimeter and Circumference

Trichome density appeared to be similar across all developmental stages of the *D. villosa* leaf. Although the trichome density observed on the stem-bark appeared to be higher compared to the leaf surfaces (Figure 6), the ANOVA indicated that there were no statistical difference in the trichomal density among the developmental stages of *D. villosa* leaf and stem-bark, $F_{(3,\ 39)} = 1.183$, $p = 0.3297$. Similarly, the average length of the non-glandular trichomes were approximately 230 ± 30.6 µm, 246 ± 40.32 µm, 193 ± 27.55 µm and 164 ± 18.62 µm (Figure 7). One way ANOVA further showed that there was no significant

difference in the average trichomal length, $F_{(3, 39)} = 1.478$, $p = 0.2369$. The perimeter and circumference of the trichomes in the developmental stages of *D. villosa* leaf and the stem-bark were not statistically different, $F_{(3, 39)} = 1.092$, $p = 0.3615$ and $F_{(3, 39)} = 0.2717$, $p = 0.8454$ (Figure 8 and 9) respectively.

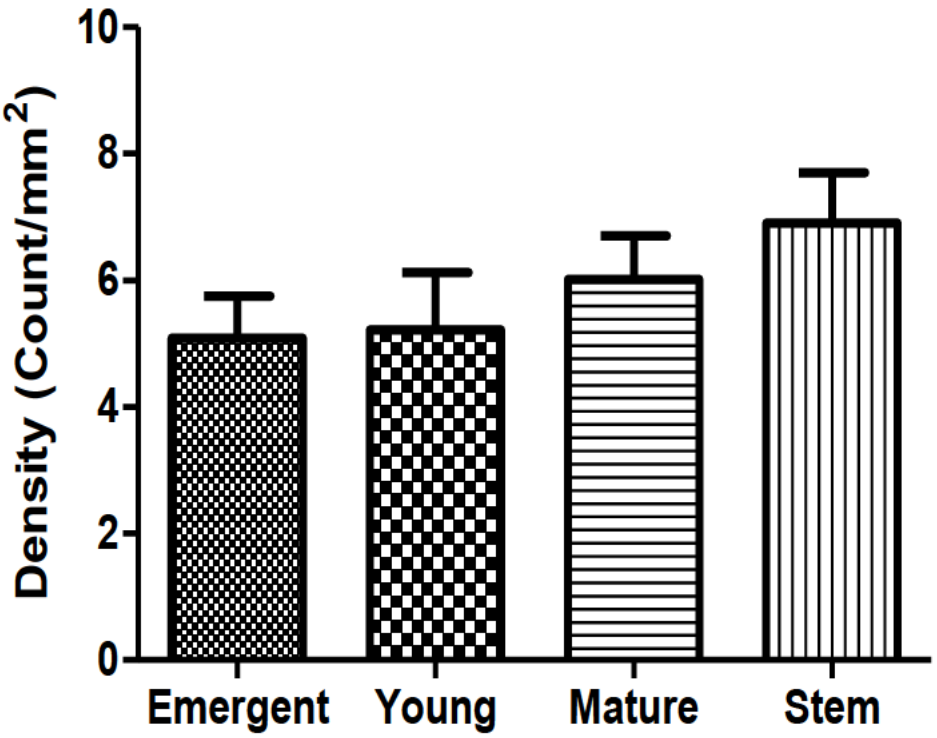


Figure 6: Density of non-glandular trichomes at different developmental stage of *D. villosa* leaves and stem bark

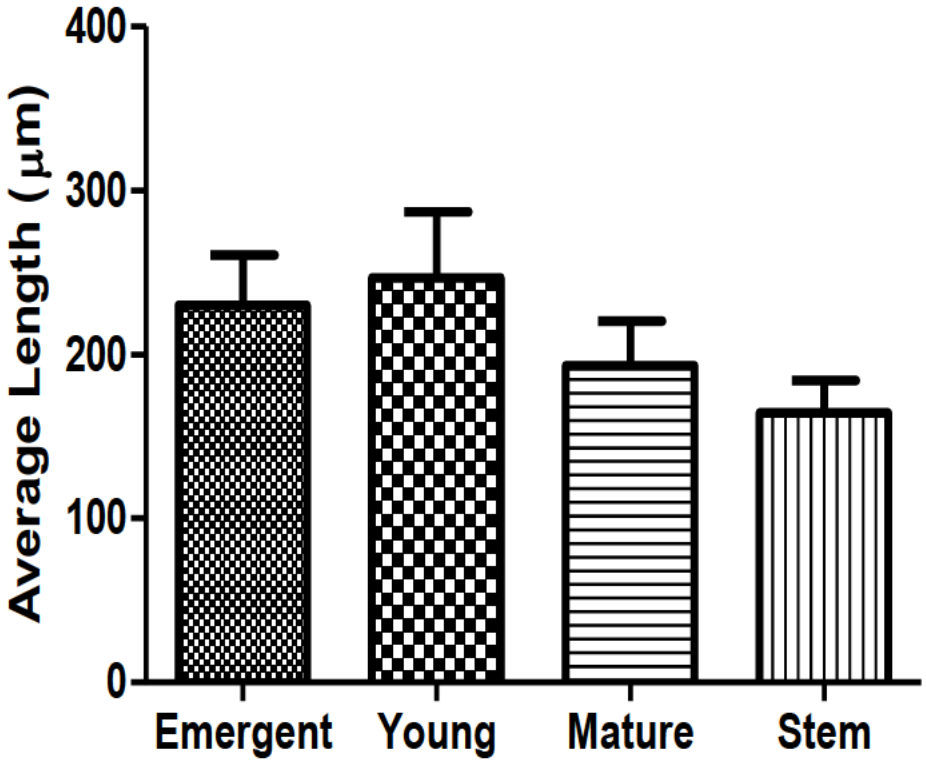


Figure 7: Average length of non-glandular trichomes at different developmental stage of *D. villosa* leaves and stem bark

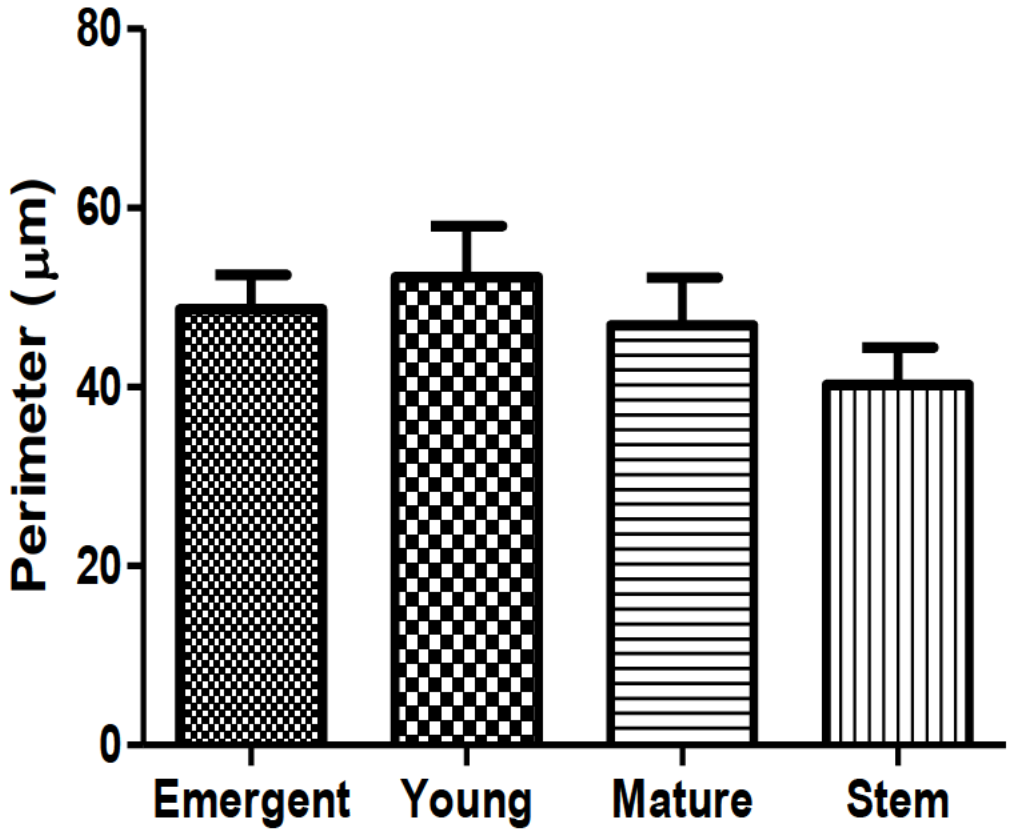


Figure 8: Perimeter covered by the non-glandular trichomes at the different developmental stage of *D. villosa* leaves and stem bark.

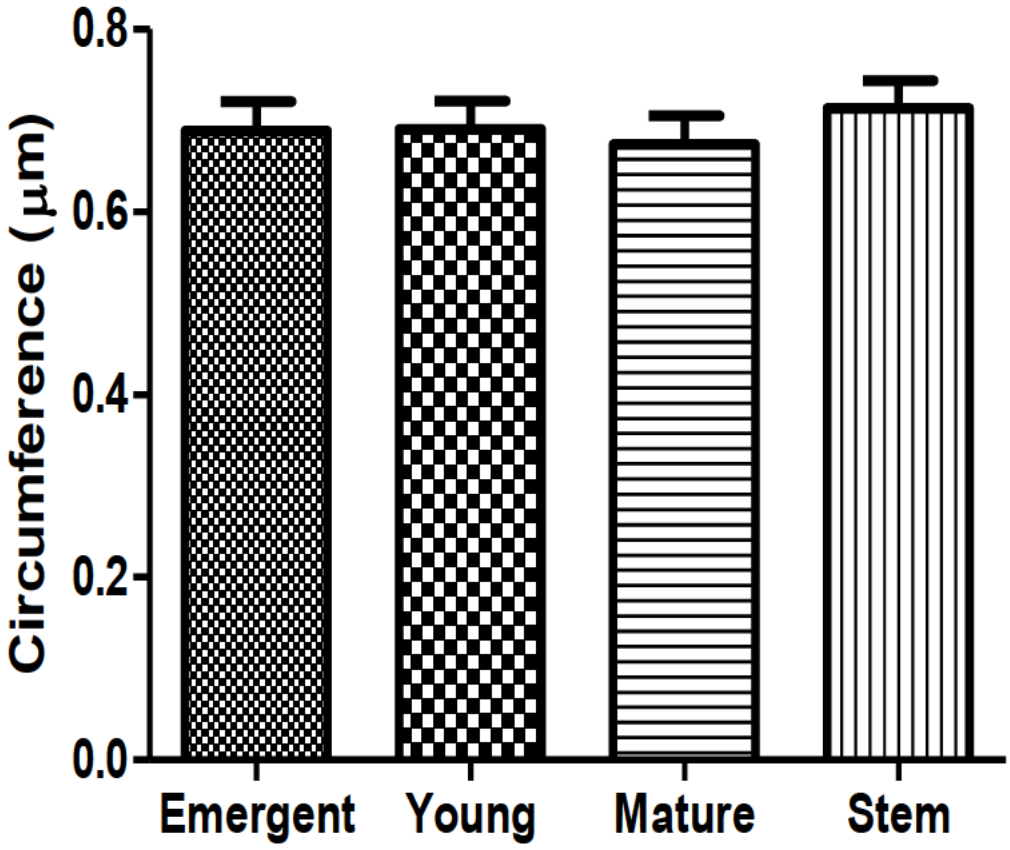
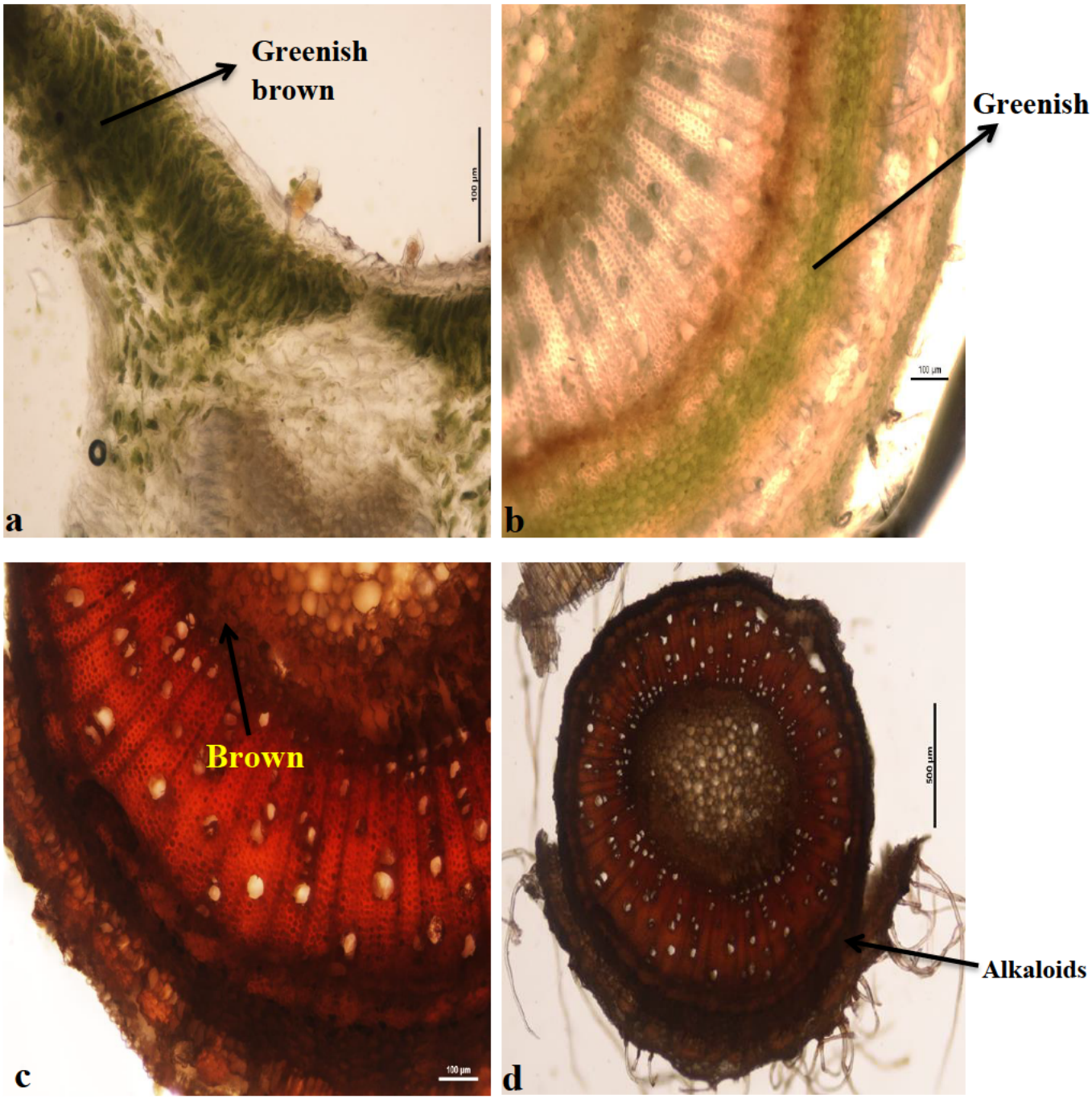


Figure 9: Circumference of the non-glandular trichomes at the different developmental stage of *D. villosa* leaves and stem bark

5.12.5 Histochemistry

The histochemical analysis showed that lipids, phenols and alkaloids were present in the leaves and stem bark (Figures 10 and 11). The greenish-brown colour further implied that the phenolic compounds (Figure 10a & b), the brown colouration as indicated in Figure 10c and d confirmed the presence of alkaloids and the black deposit indicated the presence of lipids (Figure 10e & f). These results agreed with the histochemical tests whereby various reactions indicated different colourations indicating the presence of different compounds as shown in Table 1.


Greenish brown
100 µm
a
Greenish
100 µm
b
Brown
100 µm
c
500 µm
Alkaloids
d

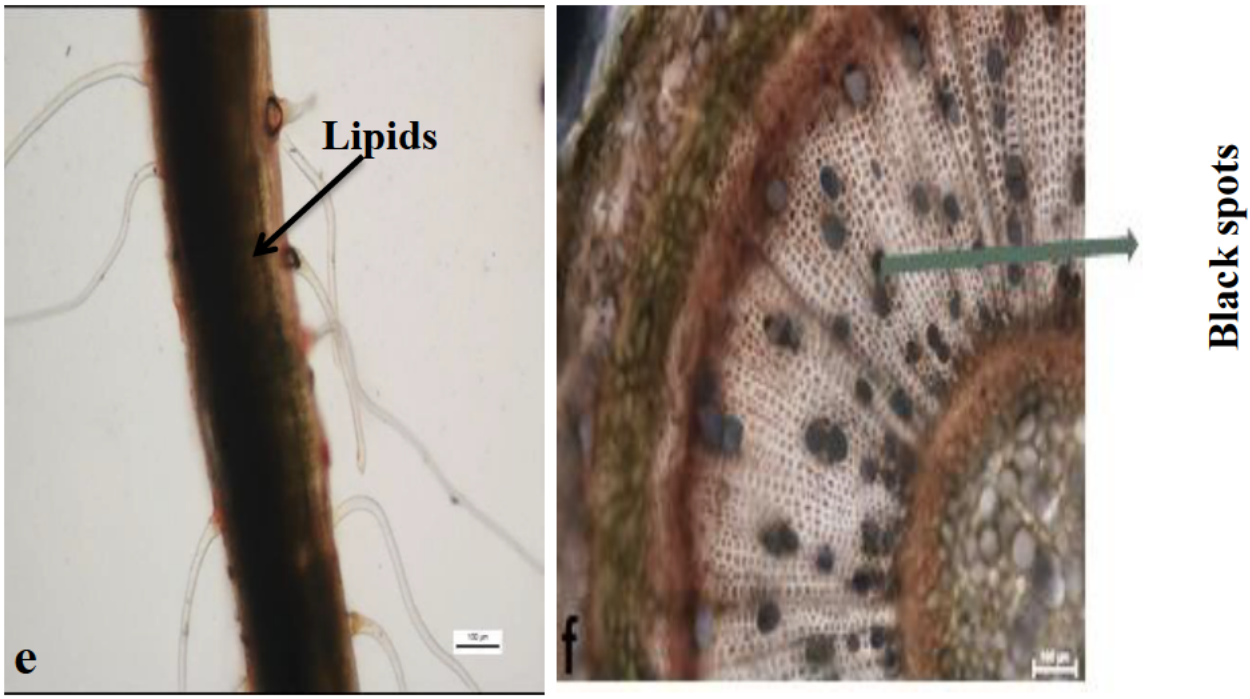


Figure 10: Light micrographs showing the histochemical staining characterization of both leaf and stem sections of *D. villosa*. a) Phenolic compounds stained brown on the leaf surface with Toluidine; b) Phenolic compounds stained greenish brown in the stem with Toluidine; c) Alkaloids stained brown colour on the *D. villosa* leaf with Dittmar reagent; d) Alkaloids stained brown on *D. villosa* stem with Dittmar reagent; e) Lipids stained with black stains on the leaf surface with Sudan black; f) Lipids stained with black on the cross section of the stem with Sudan black.

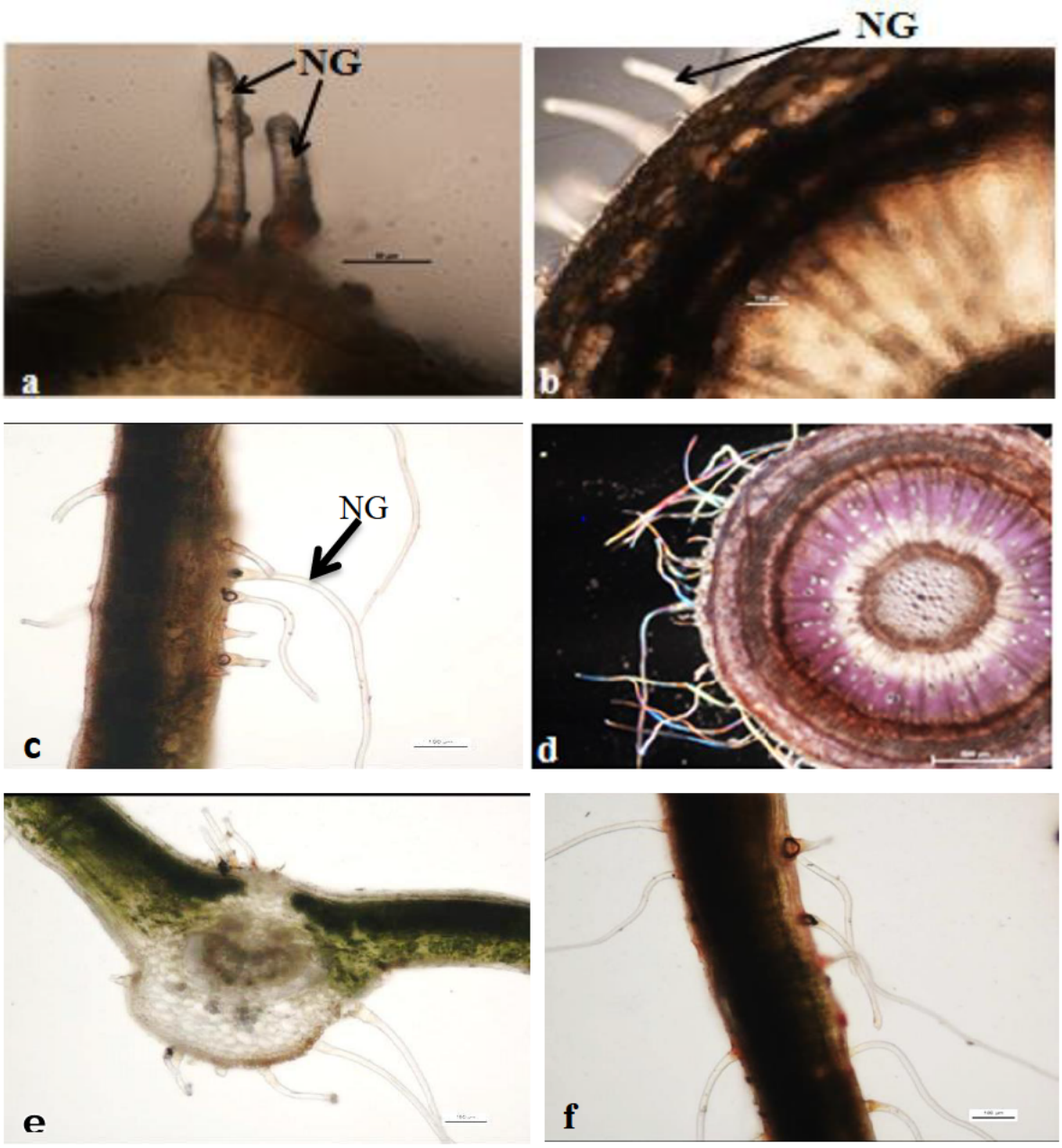


Figure 11: Light micrographs showing histochemical characterization of trichomes of *D. villosa*. a) alkaloids compounds in non-glandular trichomes stained brown with Dittmar reagent; b) phenolic compounds stained red-brown in the stem and non-glandular (NG) trichomes with ferric trichloride; c) lipids stained black in the leaf and non-glandular (NG) trichomes with Nile blue; d) total protein stained purple in the stem and non-glandular trichomal section with mercuric bromophenol blue; e) lipids stained yellowish black in non-glandular trichomes with Sudan III and IV; f) acidic polysaccharides stained purplish red in non-glandular trichomes and leaf with Ruthenium red scale bar = 100 µm.

Table I: Observations of histochemical tests on fresh leaf and stem bark sections of *D. villosa*

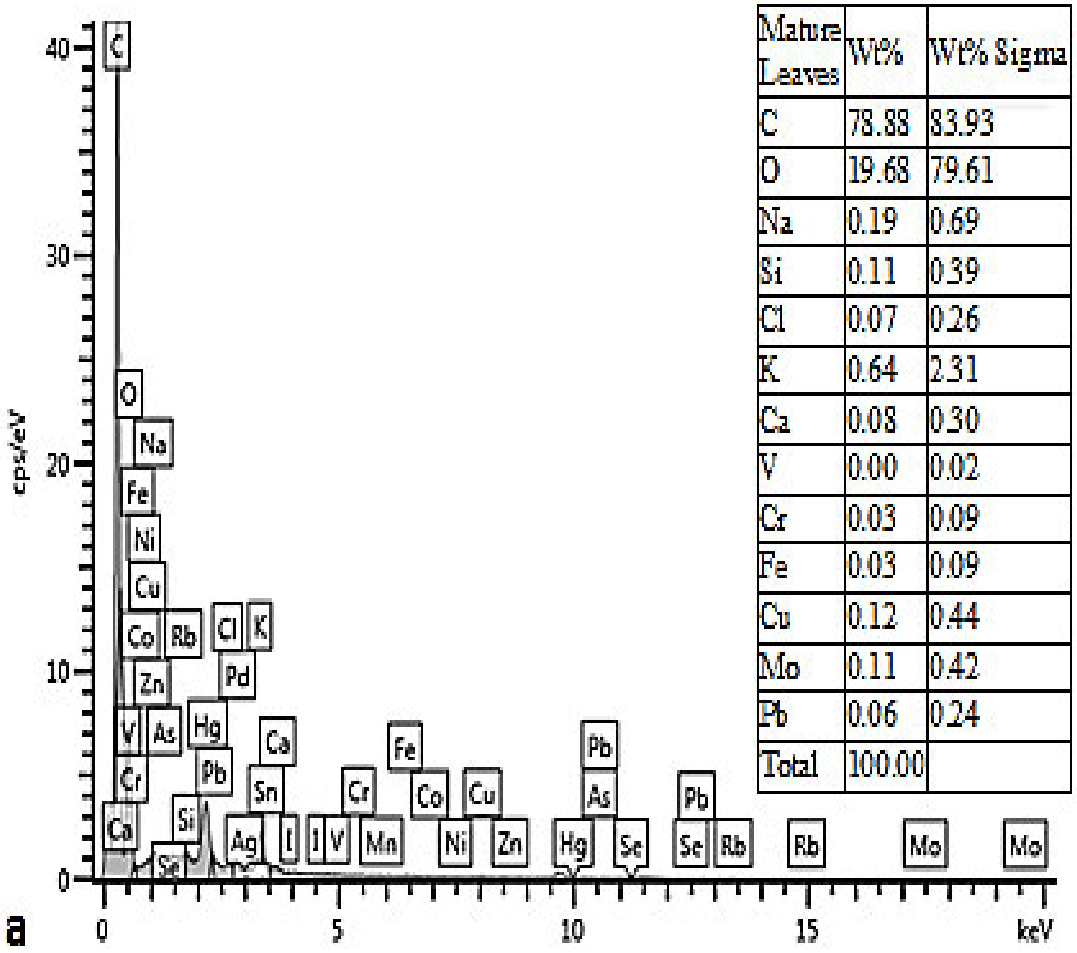
Compounds	Stains	Leaves/ Stem	Trichomes	Reactions observed
Alkaloids	Dittmar's	+	+	Brownish colouration in the stem as well as the trichomes
Lipids	Sudan III and IV	+	+	Cells in the leaf and stem sections stained black, the trichomes stained black as well
	Nile Blue	+	+	Black colouration in the leaf sections and non-glandular trichomes
Phenols	Ferric trichloride	+	+	Brown deposits on the cells of the leaf sections, the non-glandular cells further stained brown
Acidic Polysaccharides	Ruthenium red	+	+	Leaf and non-glandular trichomes stained purplish red
Total protein	Mercuric bromophenol blue	+	+	Stem cells and non-glandular trichomes stained purple
Polyphenols (lignin, tannins)	Toluidine blue	+	+	Leaf cells and non-glandular trichomes stained brown

(+) indicates presence of compounds

5.12.6 EDX

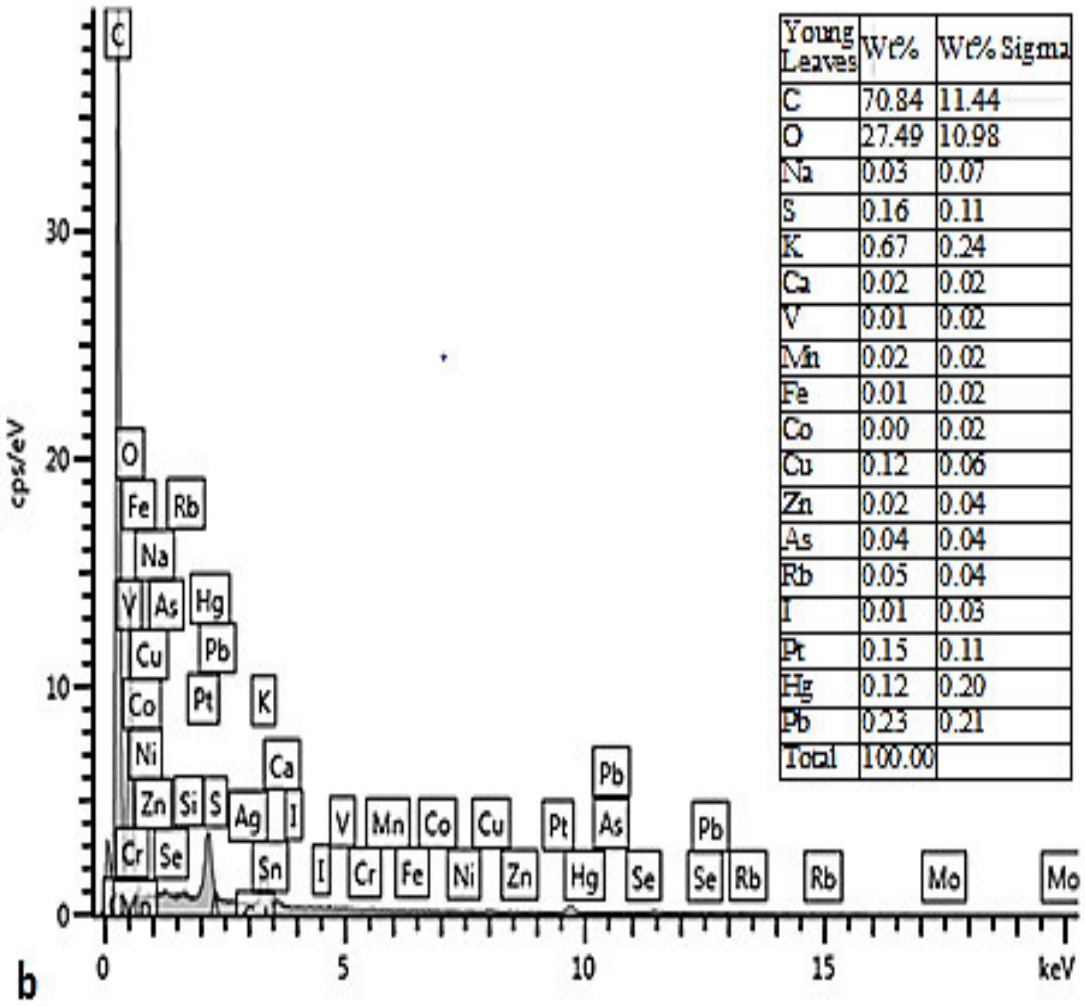
Energy Dispersive X-ray microanalysis showed that sodium and calcium are present in the leaf sections and the presence of sodium is observed to be higher than the calcium (Figure 12a & b). The mature leaves indicated the highest amount of sodium and calcium (Figure 12a) while the emergent leaves indicated the lowest concentration of salts (Figure 12c). The sodium salts were predominantly noted within the mature leaves (Figure 12a). Similarly, the

EDX spectral showed that calcium and sodium salts are present within the stem bark, meanwhile the existence of calcium was higher compared to that of sodium (Figure 12d).



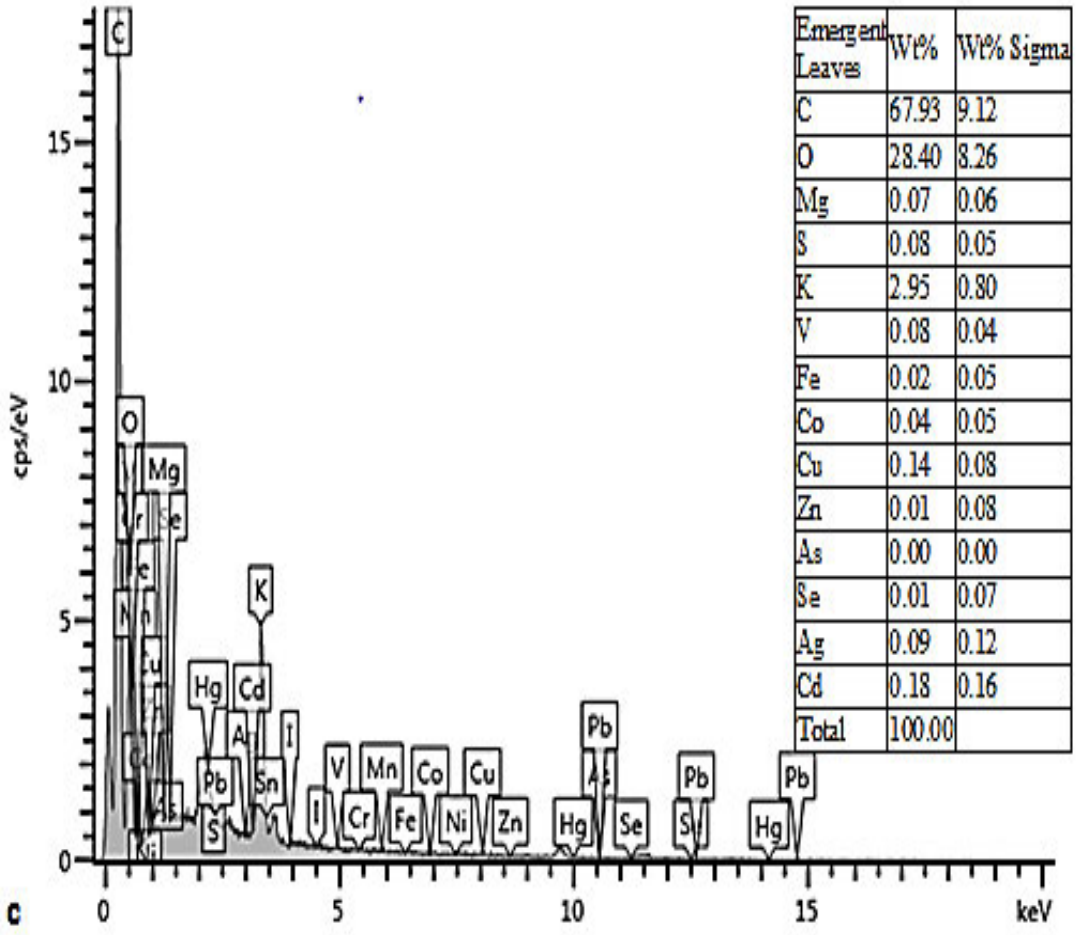
Mature Leaves	Wt%	Wt% Sigma
C	78.88	83.93
O	19.68	79.61
Na	0.19	0.69
Si	0.11	0.39
Cl	0.07	0.26
K	0.64	2.31
Ca	0.08	0.30
V	0.00	0.02
Cr	0.03	0.09
Fe	0.03	0.09
Cu	0.12	0.44
Mo	0.11	0.42
Pb	0.06	0.24
Total	100.00	

Figure 12a: EDX spectra showing the elemental composition of the secretions of *D. villosa* leaf at the mature developmental stage



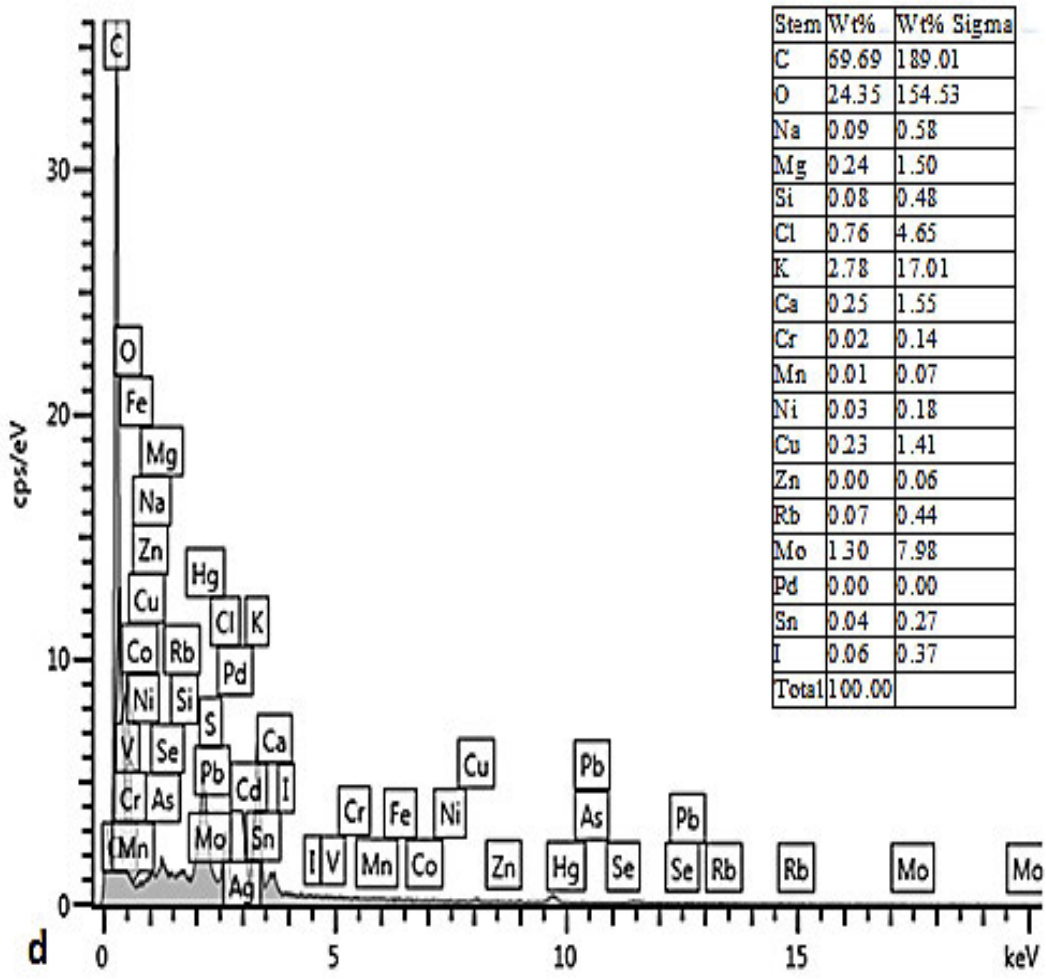
Young Leaves	Wt%	Wt% Sigma
C	70.84	11.44
O	27.49	10.98
Na	0.03	0.07
S	0.16	0.11
K	0.67	0.24
Ca	0.02	0.02
V	0.01	0.02
Mn	0.02	0.02
Fe	0.01	0.02
Co	0.00	0.02
Cu	0.12	0.06
Zn	0.02	0.04
As	0.04	0.04
Rb	0.05	0.04
I	0.01	0.03
Pt	0.15	0.11
Hg	0.12	0.20
Pb	0.23	0.21
Total	100.00	

Figure 12b: EDX spectra showing the elemental composition of the secretions of *D. villosa* leaf at its young developmental stage



Emergent Leaves	Wt%	Wt% Sigma
C	67.93	9.12
O	28.40	8.26
Mg	0.07	0.06
S	0.08	0.05
K	2.95	0.80
V	0.08	0.04
Fe	0.02	0.05
Co	0.04	0.05
Cu	0.14	0.08
Zn	0.01	0.08
As	0.00	0.00
Se	0.01	0.07
Ag	0.09	0.12
Cd	0.18	0.16
Total	100.00	

Figure 12c: EDX spectra showing the elemental composition of the secretions of *D. villosa* leaf at its emergent developmental stage



Stem	Wt%	Wt% Sigma
C	69.69	189.01
O	24.35	154.53
Na	0.09	0.58
Mg	0.24	1.50
Si	0.08	0.48
Cl	0.76	4.65
K	2.78	17.01
Ca	0.25	1.55
Cr	0.02	0.14
Mn	0.01	0.07
Ni	0.03	0.18
Cu	0.23	1.41
Zn	0.00	0.06
Rb	0.07	0.44
Mo	1.30	7.98
Pd	0.00	0.00
Sn	0.04	0.27
I	0.06	0.37
Total	100.00	

Figure 12d: EDX spectra showing the elemental composition of the secretions of *D. villosa* stem bark.

5.13 Discussion

The microscopical investigation of the leaves and stem bark of *D. villosa* revealed that the trichomes were unicellular, non-glandular and longitudinally elongated on the surface. Non glandular trichome has been previously reported for *D. sericea* and *D. hispida* by Beenken (2017) whereas the trichome types in *Diospyros* species were described as having unique, simple or bifurcated trichomes with walls often covered with longitudinally elongated warts and/ secretory cells. The microscopic analysis of trichomes in this study revealed the

presence of metabolites which further served as evidence for the presence of storage cells. Trichomes are perfect storage structures for secondary metabolites and often rupture and discharge the compounds at the time of damage (Wagner, 1991, Gonzáles et al., 2008). This typical attribute was noted for the *Diospyros* species i.e, the surface of trichome tips possess globular secreting cells as well as elongated spindle-shaped warts (Beenken, 2017). The trichomes in *D. villosa* showed a close resemblance to species within the genus like *D. mespiliformis*, *D. lotus,* etc., and were thus considered to significantly regulate leaf transpiration intensity by enhancing water retention of the leaf tissues at high leaf water deficit.

The discharge exudate released through the stomata and/or secretory pores provide defense to the leaf. It may be said that frequency of the stomatal pores is comparatively related to the amount of exudate produced and released. Furthermore, the presence of star-shaped crystal idioblasts was observed in the stem sections (Figure 2D). It may be suggested that the presence of crystals in the stem sections of *D. villosa* may serve as a taxonomical informative character (Yang et al., 2018) and, perhaps a mechanism by the plant to remove excess electrolyte storage within the plant (Jou et al., 2007). The mechanism allows identifying the crystal idioblasts which vary in shape and size. The primary function of the crystal includes promoting electrolyte homeostasis, cell support, removal of excess ions and electrolytes (Gao et al., 2021, Ghasemi-Omran et al., 2021). Similarly, crystal idioblasts are acknowledged to be toxic but rather facilitate the plant's protection against herbivores (Lambers and Oliveira, 2019). Provided that these crystals become extremely outsized, the surrounding cytoplasmic structures may start degenerating which is quite dangerous for the plant (Mhinana et al. 2010; Navarro-Leon et al. 2018). The existence of crystal within the *D. villosa* stem may support the mechanism promoting electrolyte homeostasis in the plant.

The size of non-glandular trichomes has its significant contribution to its relative function. These trichomes serve as a mechanical impediment to pests and a defence mechanism for the plant as well as act as a form of physical protection to the underlying secretory cells (Kasim, 2011). In this study, the non-glandular trichome type accumulated phytocompounds. Hence, the trichomes also played roles in the mechanical and other measures of defence against radiation and pathogens, respectively. Similar findings were reported by Beenken (2017) for *Diospyros* species. Therefore, it can be explained that trichome length varied among the families of *Diospyros Sp.* and further contributed to the significance of the family. In addition, the findings of this study supported that secretions from trichomes are associated with storage cells. This was further substantiated by the histochemical analysis which indicated the presence of chemical compounds in the trichomes. Trichomes are characterised not only by their morphology but also by their functioning (secretion, storage and mode of release). One may conclude that the trichomes of *Diospyros* differ in both morphology and physiology. EDX analyses indicated the presence of magnesium chloride in the stem bark of *D. villosa* (Fig. 9d).

Examination of the morphology, distribution and the phytochemistry of the secretion associated with leaves and stem bark of a plant could further assist in elucidating possible functions of the trichomes of this plant (Werker, 2000a, Dai et al., 2010). The physical attributes like density, size and trichomal arrangement on the leaf surface possibly promote the protection against pests and other plant damaging organisms such as the alkaloids and phenols observed to be present in the leaves and stem bark of *D. villosa*. Non-glandular trichomes could also reduce transpiration rates and curb surface leaf exposure to intense temperatures (Werker, 2000a, Dai et al., 2010). The typical non-glandular trichomes observed on *D. villosa* leaves and stem-bark would assume these roles as these trichomes were so dense on leaves that it is quite challenging to assess the leaf surface directly. Since *Diospyros*

villosa survive in dry territories, the observed trichomes are likely to serve a functional role in the conservation of water. This is quite similar to Hameed and Hussain (2011) where trichomes in plants with higher tolerance for survival in dry region were reported to enhance water conservation.

Although, the non-glandular trichomes were regarded as non-secretory, the microscopy of the stained leaf section indicated that the viability of both basal and stalk cells of the trichomes (Figure 3b and d). Histochemical analysis further buttressed that the leaves, stem bark and trichomes of *D. villosa* accumulated the phytocompounds. Therefore, these structures played significant role in the chemical defence against insect, herbivores and pathogens. The prominent chemical compounds responsible for these functional roles are phenols and alkaloids. These phytochemical compounds, in accordance with Soni et al. (2015), were of medicinal importance and repute. Alkaloids are nitrogenous chemical compounds which were reported to treat different ailments and diseases like inflammation (Aslam and Janbaz, 2019), oxidative stress and inflammation (Gutiérrez et al., 2014), asthma and fever (Agu and Okolie, 2017). Alkaloids appeared as active metabolites and natural repellents against insect herbivores and natural enemies (Ashihara et al., 2017, Kortbeek et al., 2019). In addition, phenolics were classified as abundant secondary metabolites (Vuolo et al., 2019). The phenolic compounds functioned adequately against pests and pathogens. Upon the release of phenols, they are further oxidized to quinones by polyphenol oxidase, thus facilitating the entrapment of insect on the leaf surface. The presence of phenolic compounds within the trichomes (Figure 11a & b) explained the insect entrapment ability of *D. villosa.* Also, phenolics promoted the plant's defense against pathogens and ultraviolet radiations (Lee et al., 2019). These compounds were abundant in all plant segments and contained potent antioxidant activities in comparison with other curative and pharmaceutical uses and are used in aesthetic and lumbering industry (Kumar and Goel, 2019).

5.14 Conclusion

The emanating study provided novel information regarding the micromorphology and functions of microstructures of *D. villosa* leaves and stem bark. Non-glandular trichomes were observed on both leaves and stem bark of *D. villosa*. The histochemical analysis further indicated the deposition of alkaloids and phenolic compounds in the leaves and stem bark of *D. villosa*. These compounds are of ecological and medicinal importance as they have a chemical defense mechanism against pathogens and are of use in the medicinal industry in treating a range of ailments. However, secretory products should be further evaluated and a comprehensive phytochemical screening should be conducted so as to establish all other phytochemicals in the plants.

5.15 Acknowledgement

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5.16 Conflict of Interest

The authors have declared no conflict of interest.

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CHAPTER SIX

Prologue

Manuscript 4

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"Cytotoxic effects of *Diospyros villosa* leaves and stem bark extracts on cancer cells"

Cytotoxic effects of *Diospyros villosa* leaves and stem bark extracts on cancer cells.

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ABSTRACT

Diospyros villosa is traditionally use for an anti-bacterial property. Its cytotoxic effects have not been studied before. Therefore, this study aimed to examine the nutritional properties as well the cytotoxic effects of *D. villosa*. The leaves and stem barks were subjected to three different extraction methods (methanol, chloroform and hexane) and their nanoparticles were synthesized at two different temperatures (room temperature and at 80 ºC). Thereafter, extracts were assessed using the associated AOCC protocols, for their nutritional content (moisture, fibre, proteins, lipid, ash and hydrolysable carbohydrates). *Diospyros villosa* extracts and their corresponding nanoparticles were then incubated overnight with cancerous and noncancerous cell lines to evaluate their cytotoxic potential. The nutritional analysis revealed that both young and mature leaves were rich sources of protein having values of 14.95% and 11.37% respectively. The moisture content was observed to be higher in all the leaf types with values (8.54 ± 0.75%, 9.67 ± 0.98% and 7.40 ± 0.80%) compared to the stem (2.13 ± 0.07%) respectively. The MTT cytotoxicity assay showed that the cell viability of MCF-7 cell lines were significantly lower when exposed to hexane and chloroform leaves extracts of *D. villosa* (IC_{50} of 26.64 and 26.07 µg ml^{-1}) respectively, compared to camptothecin (36.54 µgml^{-1}). Similarly, the MCF-7 cell viability was observed to be significantly lower when exposed to hexane and chloroform stem extracts of *D. villosa* (IC_{50} of 24.57 and 3.92 µg ml^{-1}), compared to camptothecin (36.54 µg ml^{-1}). The cell viability of A549 cell lines were also found lower when exposed to the hexane and chloroform extract (IC_{50} of 7.76 and 4.59 µg ml^{-1}) compared to camptothecin (IC_{50} of 19.26 µg ml^{-1}). Furthermore, the viability of A549 cell lines was found lower when exposed to hexane and chloroform stem extract of *D. villosa* (IC_{50} of 10.67 and 5.35 µg ml^{-1}) compared to camptothecin (19.26 µg ml^{-1}).The biosynthesized nanoparticles further displayed a good anticancer activity with IC_{50} value of 4.08 µg ml^{-1} when compared to the control (36.54 µg ml^{-1}). However, the HEK293 cell viability was observed to be significantly higher on exposure to hexane stem extract of *D. villosa* (IC_{50} of 158.5 µg ml^{-1}) compared to camptothecin (IC_{50} of 14.77 µg ml^{-1}). Therefore, *Diospyros villosa* leaves, stem bark and nanoparticles synthesized showed high potential for being considered as a candidate for anti-cancer regimen.

Keywords: Cell viability, Cytotoxicity, Camptothecin, *Diospyros villosa*, Nanoparticles

6.0 Introduction

Cancer is a complex disease of uncontrolled growth of tumour cells due to signalling failure of oncogenic expressions resulting in many different types of cancers based on the origin of tumours in the particular organ (Anusewicz et al., 2020). An estimate of 19.3 million new cancer cases and almost 10 million cancer deaths were reported in 2020 (Sung et al., 2021). In fact, almost 11.7% of all the new cancer cases was reported to be female breast cancer and identified to be the most common diagnosed cancer in women (Bray et al., 2018). Up till date, efforts are made to develop efficient approaches not only to diagnose cancer but also to treat the disease. A variety of therapeutic approaches including chemotherapy (Dickens and Ahmed, 2018), molecular targeted therapy (Piawah and Venook, 2019), gene therapy (Carrillo et al., 2018), radiotherapy (Brundha et al., 2019), immunotherapy (Dobosz and Dzieciątkowski, 2019), phototherapy (Chang et al., 2019), and embolotherapy (Kishore et al., 2020) have been extensively applied to treat cancers in clinic. All these therapeutic measures present with severe effects on the patients. However, there is still need to secure a more reliable, cheaper and readily available therapeutic measure, with limited side effects.

There are many medicinal plants with therapeutic properties that have been used traditionally in many countries and are also being researched by various groups in the form of extracts against different types of cancer for possible treatments (Kamble and Gacche, 2019, Goodarzi et al., 2020, Aumeeruddy and Mahomoodally, 2021). Additionally, dietary supplementation of phytonutrients is an emerging trend that provides multifaceted defensive mode against various maladies such as cancer by limiting tumour development by binding to cancer cell membrane or their receptors, thereby initiating cytotoxicity and apoptosis thus inhibiting tumour growth (Chen and Liu, 2018). These phytonutrients possess certain key advantages over alternative chemotherapy agents such as their affinity and level of tissue penetration, strong target specificity and low toxicity (Soldati et al., 2018). Medicinal plant therapeutic

agents also contributes indirectly by activating the endogenous defence systems to modulate cellular signalling processes (Marabini et al., 2020) and thereby enhancing the overall health status. There is abundance of medicinal shrubs, vegetables and trees in South Africa that are yet to be prodded meticulously for their health promoting properties. Similarly, nanoparticles synthesized from green plants have been reported to possess unique biological properties and hence it becomes useful in therapeutics and drug delivery (Chenthamara et al., 2019). The biosynthesized nanoparticles eradicate cancer cells by flow and penetration to different regions of tumours through blood vessels into the target cells (Kang et al., 2018).

Diospyros villosa (*D. villosa*) is an African plant which naturally occurs in southern parts of the continent. *D. villosa* root was reported to be used by group of herbalists found in the botanically diverse Western Cape of South Africa to treat gastrointestinal complaints, worm and flatulence (Philander, 2011). Also, the root of *D. villosa* plant was used in rural community of northern Maputaland to treat pain and dysmenorrhea (De Wet and Ngubane, 2014). *Diospyros ferrea* (Wild.) leaves nanoparticles were reported for its anti-cancer activities against MCF-7 cancer cell lines (Rajesh et al., 2017). Hence, this research study is then geared towards making a significant contribution to the present search being carried out to ascertain the nutritive contents of *D. villosa* leaves and stem bark and to investigate the anti-cancer properties of *D. villosa* leaves and stem bark as well as its nanoparticles on breast cancer cell lines (MCF-7), human embryonic kidney immortalized cell lines (HEK 293), and adenocarcinomic human alveolar basal epithelial cancer cells (A549).

6.1 Materials and methods

6.2 Plant collection

Fresh samples of mature leaves and stem bark of *D. villosa* were collected from KwaZulu-Natal, Durban, South Africa (29º 84′ 33.6″S, 31º 4′ 12″E). The plant was identified and a

voucher specimen was deposited in the Ward Herbarium (01/18257) at the School of Life Sciences, University of KwaZulu-Natal, Westville campus. The collected plant parts (leaves and stem bark) were washed, air-dried and pulverized into fine powder. The powdered samples were kept in a cool dry place for extraction purposes.

6.3 Plant extraction

Powdered samples of the plant weighing 8 g were heated to a temperature of 40 °C for 15 min with 100 ml of 95 % methanol in a round bottom flask attached to a Soxhlet apparatus. The crude extract was retained and the process was repeated thrice. Successive extractions using chloroform and hexane, respectively, were carried out after 30 min intervals. The condensate was further evaporated to dryness under reduced pressure at 40 ºC in a rotary evaporator. The crude extract was stored at 4 ºC and used within 48 hr for further tests.

The extraction yield (%) = $\frac{Weight\ of\ the\ dry\ extract\ (g)}{Weight\ of\ the\ sample\ used\ for\ the\ extraction\ (g)} \times 100$.

6.4 Proximate analysis

The proximate analysis of leaves and stem samples including the moisture, crude fibre, proteins, lipids, ash content and hydrolysable carbohydrates were assessed. Moisture content was determined by drying the leaves and stem samples at 80 ºC in an oven until constant weight was obtained (AOAC, 2002). Crude fibre was determined by the loss in weight on ignition of dried residue following the digestion of fat-free samples with 1.25% each of sulphuric acid and sodium hydroxide solutions (AOAC, 2002). The total protein content (N × 6.25) was estimated by the macro-Kjeldahl nitrogen assay method using a digestion apparatus combined with the photo-colorimetric method described by Baethgen and Alley (1989). The total lipids content was determined according to AOAC (1990), by n-hexane extraction using an automatic Soxhlet analyzer (Soxtherm 2000 Automatic, C. Gerhardt, UK).

6.5 Synthesis of silver nanoparticles (AgNPs)

Silver nitrate (1 M $AgNO_3$; Sigma Aldrich, South Africa) was prepared by dissolving 0.17 g in 100 ml of distilled water. Following this, 1 mM of $AgNO_3$ was prepared by diluting 10 ml in distilled water (90 ml). The reduction of Ag^+ was achieved by adding 5 ml of each *D. villosa* aqueous extract (leaves or stem bark) to 20 ml of 1 mM $AgNO_3$. The mixtures were incubated for 24 hr in the light at room temperature (RT; 24 °C). The procedure was repeated with the incubation at 80 °C by heating the extracts in a water bath for 60 min. The colour change from light yellow to dark brown was indicative of the presence of AgNPs (Kannan et al., 2013, Sharma et al., 2013). Syntheses were performed in triplicate.

6.6 Quantification of AgNPs

Each AgNP solution was subjected to centrifugation using an Eppendorff microcentrifuge (5804/5804 R, USA). The treatment solutions (leaves and stem bark at room temperature and at 80 °C) were separately transferred into pre-weighed microcentrifuge tubes and purified for 2 hr at 1650 ×g and at 4 °C. The supernatant from each solution was decanted and the insoluble residue was reconstituted in 20 ml sterile distilled water and centrifuged repeatedly three more times for effective removal of unreacted materials. Samples were then oven-dried at 40 °C for 24 hr after which the tubes were re-weighed to obtain the yield of the synthesised AgNPs.

6.7 Anticancer Activity

Human embryonic kidney (HEK293), breast cancer (MCF-7) and human lung cancer (A549) cells were donated by the Department of Biotechnology, Durban University of Technology. Cells were grown at 37 °C in a humidified incubator under 5% CO_2 in Dulbecco's modified

Eagle's medium (DMEM) containing 10% Foetal Bovine Serum (FBS) and antibiotics (Penicillin; 10000 U mL^{-1} and Streptomycin sulphate; 10000 U mL^{-1} [Penicillin/Streptomycin]). Antibiotics change the phenotype and morphology of cells; therefore, the use of the antibiotics should be in very low concentrations, thus for this study, 1% Penicillin/Streptomycin was used. The cells were grown until 80% confluence was reached with media replaced as necessary. After confluence was reached, cells were trypsinized and sub-cultured. The 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the cytotoxicity of the extracts. The MTT assay was conducted according to Dwarka et al. (2017) with minor modifications. Briefly, cells (50 µL of 1×10^2 cells mL^{-1}) as well as 50 µL of DMEM were seeded into a 96 well flat bottom plate and incubated (37 °C for 24 hr) in a humidified incubator under 5% CO_2. Cells were then treated with 50 µL of extracts at varying concentrations (7.8-1000 µg mL^{-1}) prepared in 5% DMSO and incubated for 24 hr. Camptothecin was used as a positive control. MTT reagent (20 µL, 5 mg mL^{-1}) was added to the cells and incubated for 37 °C for 4 h. One hundred microlitres of DMSO was then added to each well in order to solubilise the formazan salt formed, and absorbance was read at 570 nm on a micro plate spectrophotometer (Multiscan Go, Thermo Scientific) for both treated and untreated cells. The percentage viability was calculated using the following formula:

$$\% \text{ Cell Viability} = \frac{Absorbance\ of\ treated\ cells}{Absorbance\ of\ untreated\ cells} \times 100.$$

6.8 Statistical Analysis

Data are displayed as means ± SEM. Statistical analysis was performed using Graph Pad Prism 5 (Graph Pad Software Inc., USA). The results were compared using one-way ANOVA followed by Bonferroni post hoc tests. Also, two-way ANOVA followed by Bonferroni post hoc tests was used where necessary. Effects were considered statistically

significant at p value < 0.05. The half maximal inhibitory concentration (IC_{50}), the lower the IC_{50} on the cancerous cell lines the more potent the extract as an anticancer agent.

6.9 Results

The yield of different extracts of *D. villosa* leaves and stem bark are given in Table I. It was observed that methanol leaves extract produced a maximum yield of 10.8 %, whereas chloroform and hexane leaves extract yielded 8.4 % and 7.1 % respectively. Similarly, the methanol extraction in the stem bark produced a yield of 9.2 % meanwhile, chloroform and hexane extraction yielded 7.9 % and 10.3 % respectively. The yield obtained from leaves nanoparticles at room temperature and at 80 ºC was observed to be 7.4% and 5.5% respectively. Also, percentage yield from stem nanoparticles at room temperature and at 80 ºC was found to be 4.0% and 3.95% respectively.

Table I: Yield of extracts of *D. villosa* leaves, stem bark and nanoparticles

	Methanol (%)	Chloroform (%)	Hexane (%)	Nanoparticle at RT (g/g of dry plant material)	Nanoparticle at 80 ºC (g/g of dry plant material)
Leaves	10.8	8.4	7.1	0.07	0.055
Stem bark	7.2	7.9	10.3	0.04	0.039

The proximate analysis of different extracts of *D. villosa* leaves and stem bark are given in Table II. It was observed that the protein content of young and mature leaves of *D. villosa* were found significantly ($p < 0.05$) higher compared to the stem $F_{(3, 11)} = 51.45$, $p = 0.0009$. Similarly, the moisture content of the leaves (emergent, young and adult) were found significantly ($p < 0.05$) higher compared to the stem. The moisture contents in the leaves was

found significantly higher compared to the stem bark. Although, there were differences in the lipids, crude fibre and carbohydrate in the leaves and stem bark of *D. villosa*, not significant.

Table II: Nutritional content (%) of investigated *D. villosa* leaves and stem bark

	Emergent Leaves (%)	Young Leaves (%)	Mature Leaves (%)	Stem (%)
Protein	8.50 ± 1.61	11.37 ± 0.68*	14.95 ± 0.83*	5.22 ± 0.72
Lipids	11.97 ± 1.36	11.39 ± 1.77	14.37 ± 0.16	13.34 ± 0.28
Crude fibre	29.73 ± 2.71	36.4 ± 3.49	29.60 ± 2.77	40.17 ± 3.63
Ash	8.33 ± 0.44*	6.33 ± 0.60	6.67 ± 0.67	5.33 ± 0.60
Moisture	8.54 ± 0.75*	9.67 ± 0.98*	7.40 ± 0.80*	2.13 ± 0.07
Carbohydrate	32.93 ± 0.62	24.84 ± 0.91	27.01 ± 0.44	33.81 ± 0.35

Values are mean ± SD of carefully conducted triplicate experiments. *$p < 0.05$.

The MTT assay was used to determine cytotoxicity of the *D. villosa* leaves and stem using different extraction media as well as the biosynthesized AgNPs at different temperature (RT and 80 ºC) on cancerous and non-cancerous cell lines. For the extract to be a good anticancer, it should display toxicity on MCF-7, or A549 cancer cell and mild reactivity to HEK293 with a further supporting evidence of IC_{50}. The lower the IC_{50} values indicated, the higher the cytotoxic activity in cancerous cells.

Among the different leaf extracts, hexane showed noteworthy cytotoxic effect on MCF-7 cell line (IC_{50} 26.64 µg ml^{-1}) and chloroform extract showed significant cytotoxic effect (IC_{50} 26.07 µg ml^{-1}) (Table III). However, the methanolic leaves showed the best cytotoxic effect (IC_{50} 7.09 µg ml^{-1}) in MCF-7 cells. The hexane, chloroform and methanol leaves, all three extracts demonstrated greater anti-cancer activity than the standard camptothecin (IC_{50} 36.54 µg ml^{-1}).

Table III: IC_{50} values of methanol, chloroform and hexane extract of *D. villosa* leaf against MCF-7 cell.

	Methanol	Chloroform	Hexane	Camptothecin
IC_{50}	0.16	26.07	26.64	36.54

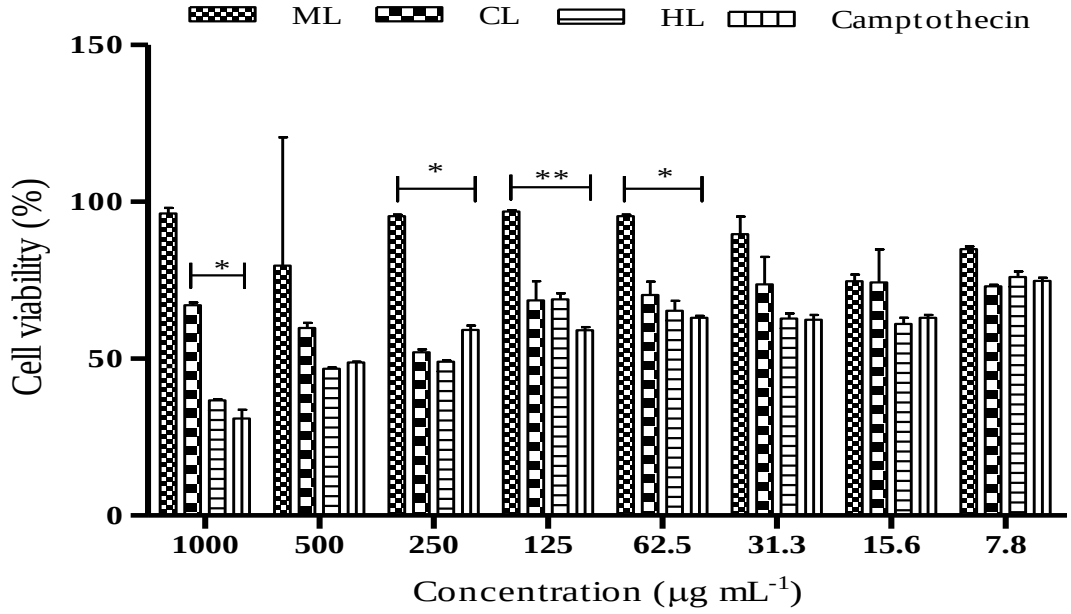

Figure 1: Cell viability of MCF-7 cancer cell line treated with different concentration of *D. villosa* leaves

The cytotoxicity of MCF-7 cells by both hexane (IC_{50} 24.57 µg ml^{-1}) and chloroform stem extracts (IC_{50} 3.919 µg ml^{-1}) (Table IV) were much greater compared with camptothecin (IC_{50} value of 36.54 µg ml^{-1}). However, the greatest anticancer activity produced in MCF-7 cells was demonstrated by the methanolic stem extract (IC_{50} 0.17 µg ml^{-1}).

Table IV: IC_{50} values of methanol, chloroform and hexane extract of *D. villosa* stem against MCF-7 cell.

	Methanol	Chloroform	Hexane	Camptothecin
IC_{50}	0.16	3.92	24.57	36.54

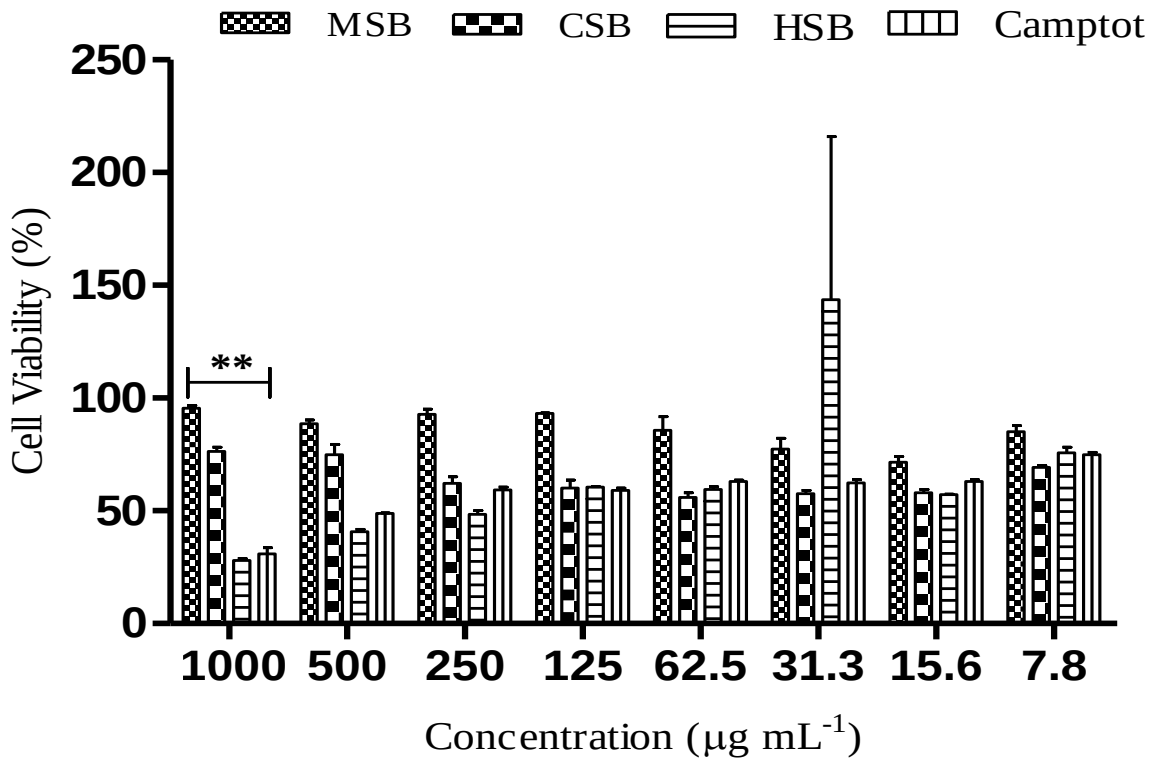

Figure 2: Cell viability of MCF-7 cancer cell lines treated with different concentration of *D. villosa* stem extract.

The *Diospyros villosa* stem nanoparticles biosynthesized at RT showed significant toxic effect on MCF-7 (4.08 µg ml^{-1}) compared to Camptothecin (36.54 µg ml^{-1}) (Table V and Figure 3). In addition, the leaves nanoparticle synthesized at RT showed significant toxic effect on MCF-7 cell lines (IC_{50} 2.03 µg ml^{-1}) The IC_{50} of the leaves and stem nanoparticles synthesized at 80 ºC was found to be 2.53 and 5.11 µg ml^{-1} respectively.

Table V: IC_{50} values of *D. villosa* leaves and stem bark nanoparticles at both RT and at 80 ºC against MCF-7 cell.

	Leaves (RT)	Stem (RT)	Leaves (80 ºC)	Stem (80 ºC)	Camptothecin
IC_{50}	2.03	4.08	2.53	5.11	36.54

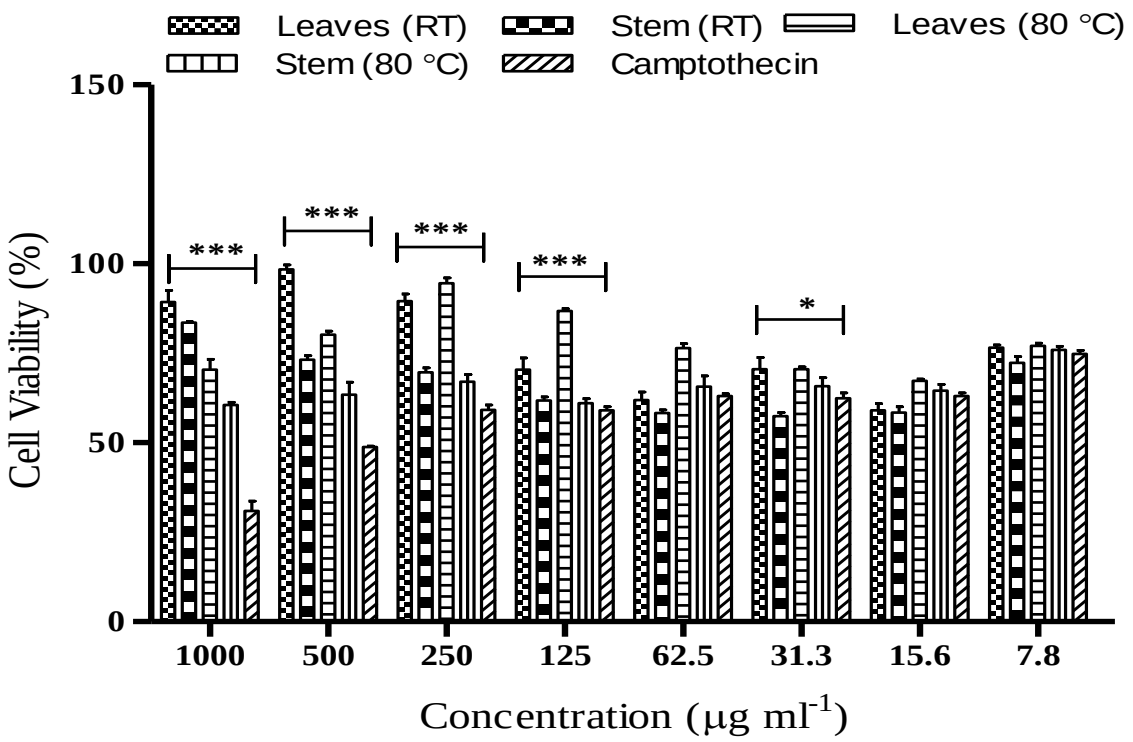

Figure 3: Cell viability of MCF-7 cancer cell lines treated with different concentration of nanoparticles synthesized from *D. villosa* leaves and stem extract at room temperature and at 80 ºC.

The viability of HEK 293 cell line was observed to be higher on exposure to methanolic leaves extract of *D. villosa* (IC_{50} 41.85 µg ml^{-1}), chloroform leaves (IC_{50} of 198.5 µg ml^{-1}) and hexane leave (IC_{50} of 158.5 µg ml^{-1}) compared to camptothecin (IC_{50} of 14.77 µg ml^{-1}) (Table VI).

Table VI: IC_{50} values of methanol, chloroform and hexane extracts of *D. villosa* leaves against HEK293 cell.

	Methanol	Chloroform	Hexane leaf	Camptothecin
IC_{50}	41.85	198.5	158.5	14.77

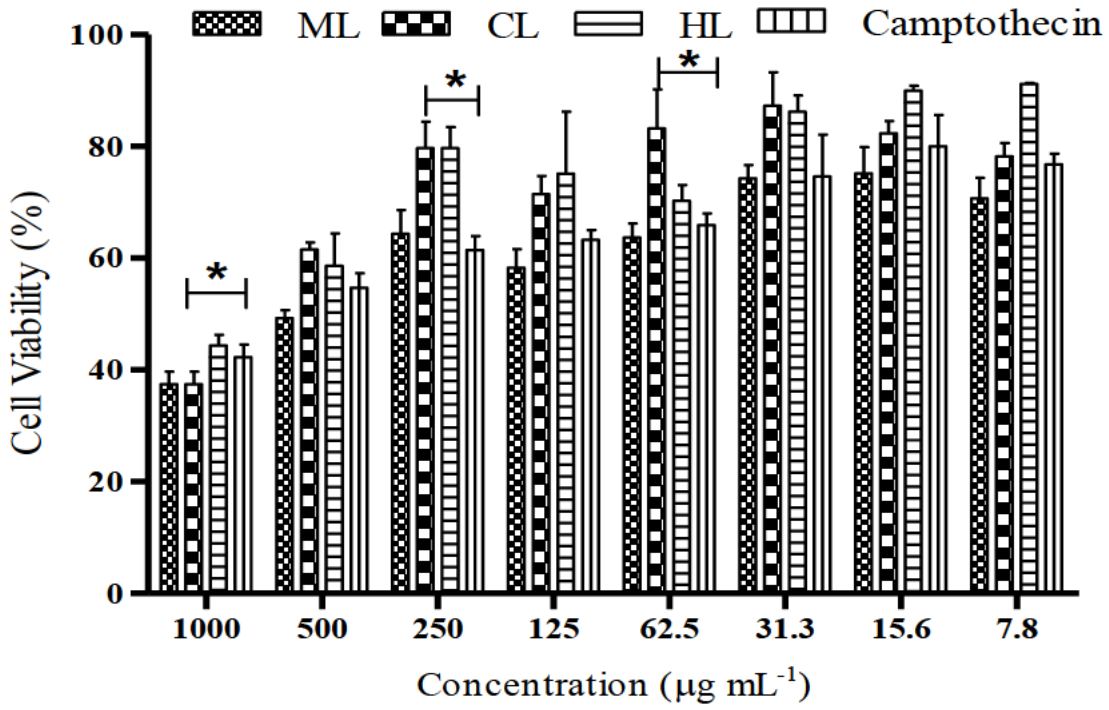

Figure 4: Cell viability of HEK293 cancer cell lines treated with different concentration of *D. villosa* leaves extract.

The viability of HEK293 cells was further observed to be greater when exposed to hexane stem extract of *D. villosa* (IC_{50} of 45.13 µg ml^{-1}) (Figure 5). However, the viability of the HEK293 cells was largely affected when treated with the chloroform (IC_{50} of 3.93 µg ml^{-1}) and methanolic (IC_{50} of 0.10 µg ml^{-1}) extracts of *D. villosa* (Figure 5). Camptothecin produced an IC_{50} of 14.77 µg ml^{-1}.

Table VII: IC_{50} values of methanol, chloroform and hexane extract sof *D. villosa* stem against HEK293 cell.

	Methanol	Chloroform	Hexane	Camptothecin
IC_{50}	45.1	3.93	0.10	14.77

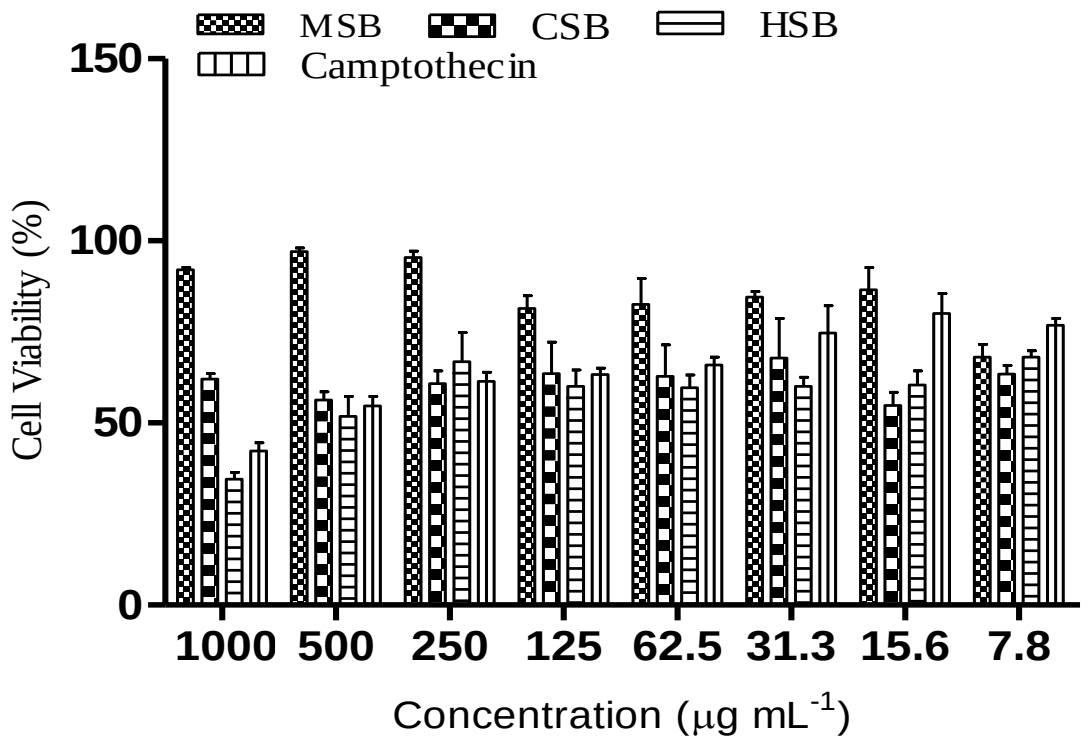

Figure 5: Cell viability of HEK293 cancer cell lines treated with different concentration of *D. villosa* stem extract.

Diospyros villosa leaves nanoparticles biosynthesized at RT showed significant toxic effect on HEK293 cell line (IC_{50} of 4.77 and 7.09 μg ml^{-1}) compared to camptothecin (14.77 μg ml^{-1}) (Table VIII). The viability of HEK293 cells were higher when exposed to leaves and stem nanoparticles (IC_{50} of 333.8 and 51.36 μg ml^{-1}) of *D. villosa* (synthesized at 80 ºC).

Table VIII: IC_{50} values of *D. villosa* leaves and stem bark nanoparticles at both RT and at 80 ºC against HEK293 cell.

	Leaves (RT)	Stem (RT)	Leaves (80 ºC)	Stem (80 ºC)	Camptothecin
IC_{50}	4.77	7.09	333.80	51.36	14.77

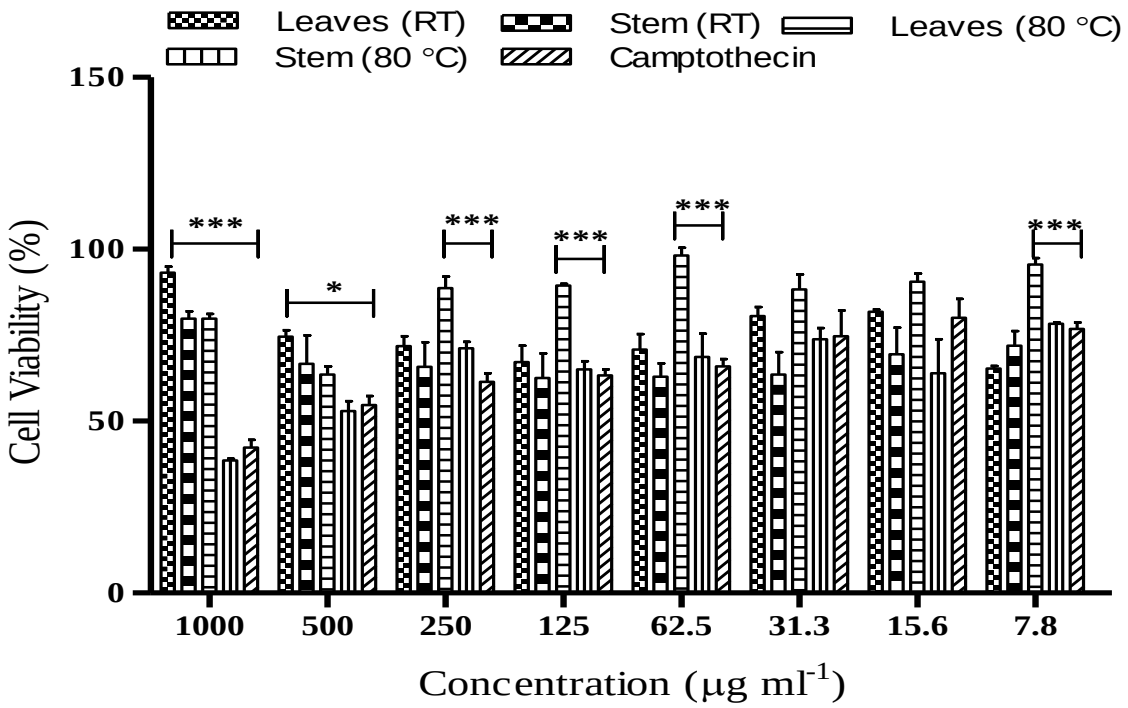

Figure 6: Cell viability of HEK 293 cancer cell lines treated with different concentration of nanoparticles synthesized from *D. villosa* leaves and stem extract at room temperature and at 80 ºC.

The cell viability of A549 cells on exposure to chloroform (IC_{50} of 4.592 µg ml^{-1}) and hexane leaves extracts (IC_{50} of 7.76 µg ml^{-1}) showed a greater anti-cancer effect compared to camptothecin (IC_{50} of 19.26 µg mL^{-1}) (Table 9).

Table IX: IC_{50} values of methanol, chloroform and hexane extracts of *D. villosa* leaf against A549 cell.

	Methanol	Chloroform	Hexane	Camptothecin
IC_{50}	4.59	7.09	7.76	19.26

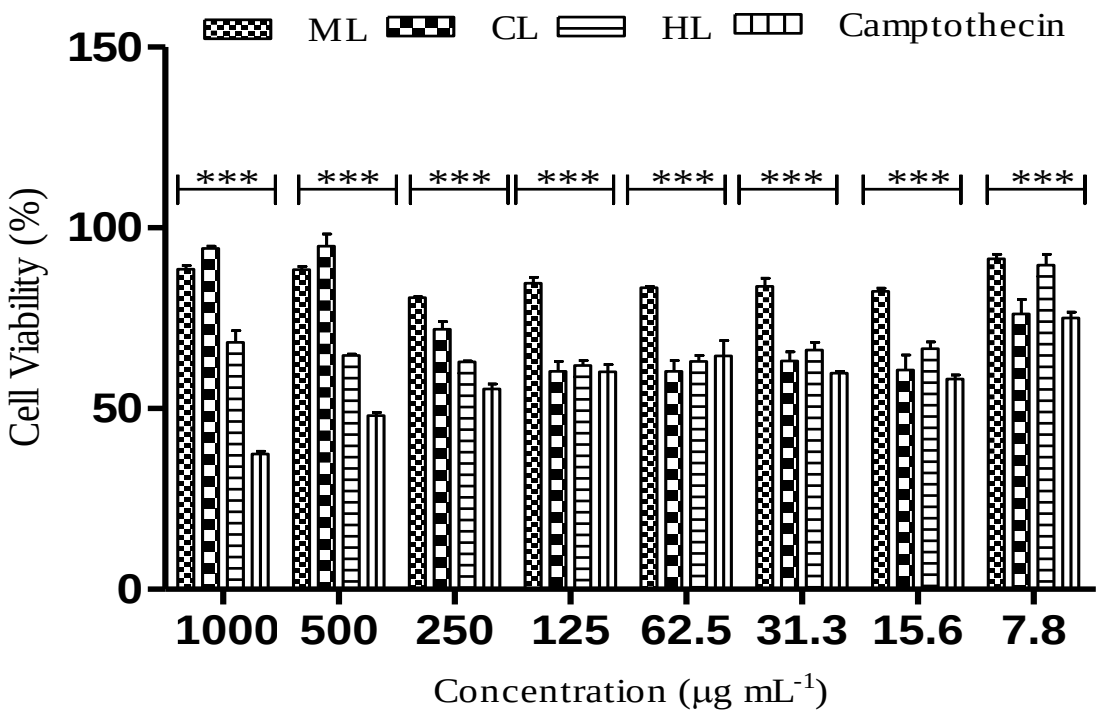

Figure 7: Cell viability of A549 cancer cell lines treated with different concentrations of *D. villosa* leaves extract.

In addition, the cell viability of A549 cells was observed to be lower on exposure to hexane stem extract (with IC_{50} value of 5.35 µg ml^{-1}, chloroform stem extract (IC_{50} of 10.67 µg ml^{-1}) and methanolic extract (13.48 µg ml^{-1}) of *D. villosa* compared to control (IC_{50} of 19.26 µg ml^{-1}) (Table X).

Table X: IC_{50} values of methanol, chloroform and hexane extract of *D. villosa* stem against A549 cell.

	Methanol	Chloroform	Hexane	Camptothecin
IC_{50}	13.48	10.67	5.35	19.26

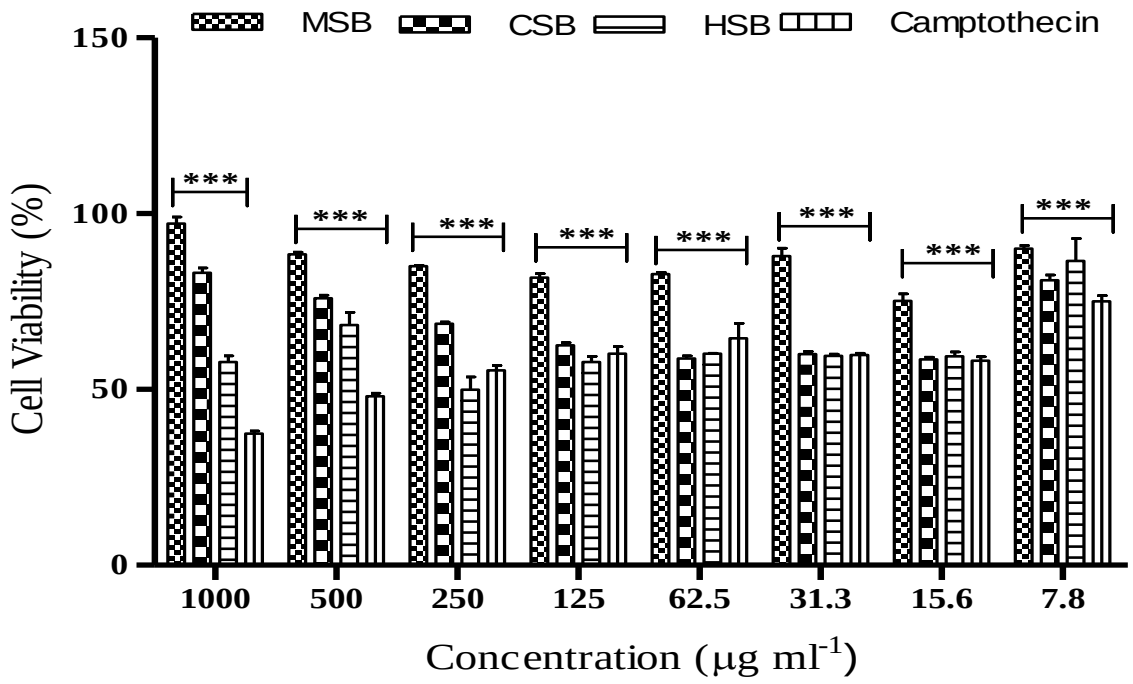

Figure 8: Cell viability of A549 cancer cell lines treated with different concentration of *D. villosa* stem extract.

The viability of A549 cells was observed to be lower on exposure to *D. villosa* stem nanoparticles 80 ºC and RT (IC_{50} values of 5.03 and 4.93 µg ml^{-1} respectively) compared to control (19.26 µg ml^{-1}) (Table XI).

Table XI: IC_{50} values of *D. villosa* leaves and stem bark nanoparticles at both RT and at 80 ºC against A549 cell.

	Leaves (RT)	Stem (RT)	Leaves (80 ºC)	Stem (80 ºC)	Camptothecin
IC_{50}	7.13	4.93	33.80	5.03	19.26

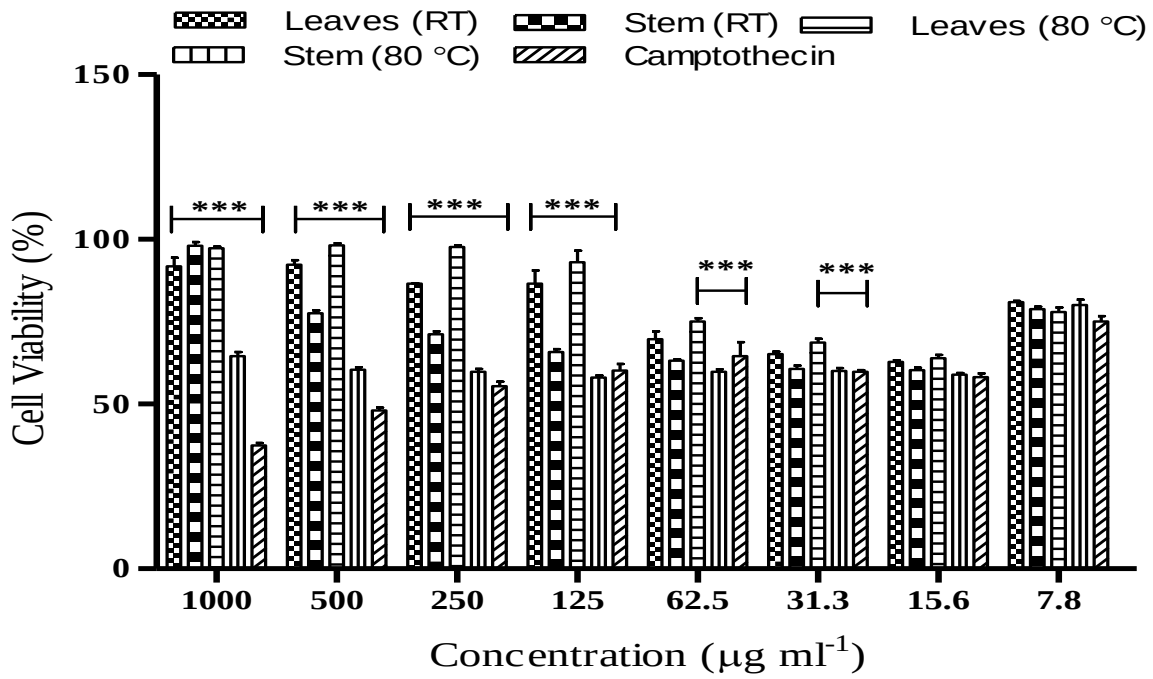

Figure 9: Cell viability of A549 cancer cell lines treated with different concentrations of nanoparticles synthesized from *D. villosa* leaves and stem extract at room temperature and at 80 ºC.

6.10 Discussion

It was observed that the methanol extract produced maximum extraction yield. This is in line with Abdullah et al. (2020) who reported previously that methanol extract of different plants usually yields significantly higher amount compared to chloroform and hexane extract of same plants and it was further explained that it may be due to occurrence of functional particles which are mostly polar organic phytochemical and are always available in most medicinal plants. For most studies, crude fibre, protein and good energy are considered as the main determinants of food types, and very few studies are available on the elemental composition of the *Diospyros* edible species. Crude fibre and protein in *Diospyros* leaves are well within the range as reported by earlier workers for other wild edibles (Tuncturk et al., 2015, Unuofin et al., 2017). The relatively high fibre content is an indication that the intake of *D. villosa* leaves could enhance peristalsis along gastrointestinal tract, digestion and even

prevent constipation (Nyamwamu et al., 2020). High fibre intake could lead to a reduced incidence of cohorts of metabolic syndrome disorder (Kim et al., 2020b). Dietary proteins are pivotal in the manufacturing and safeguarding of certain organic materials necessary for smooth functioning of human body (Unuofin et al., 2017). The relatively high protein content of *D. villosa* could make it a useful supplement to diets with small amount of proteins. Considering these nutritional values of *D. villosa*, the leaves seem to be fit for human consumption. However, there is still need for further identification and assessment of the protein make-up in the leaves and perhaps, the other vital and essential nutritive components of the plant.

Diospyros villosa leaves have long been recognized as a traditional medicinal plant. However, the putative anti-cancer effects of *D. villosa* leaves and their mechanisms of action have not been scientifically evaluated previously. As illustrated in Fig. 1 and 7, viability assays revealed that MCF-7 and A549 cells were more vulnerable to the plant extracts of *D. villosa* leaves than HEK293 cells. Similarly, a same trend was observed with *D. villosa* stem bark (Fig. 2 and 8). MCF-7 and A549 cells were more vulnerable to stem extracts of *D. villosa*. Thumbrain et al. pinpointed that if an extract should be a good anticancer agent, it should display toxicity on the A549 and MCF-7 cell lines while being somewhat less toxic to HEK293 cells. In this study, both leaves and stem bark exhibited strong cytotoxicity against MCF-7 and A549 cells but with disproportionate trend towards HEK293 cells. This shows that *D. villosa* extracts may not be toxic to normal cells, which makes it an ideal anticancer agent.

The exact mechanism of action through which the plant extracts exhibited its toxicity on cells was not established in this study. Earlier studies further demonstrated that the majority of proteins in typical African diet come from high quality plant protein (Sterling and Bowen, 2019). Elevated levels of protein and essential amino acids can inhibit the cancer progression

and growth. This is in concordance with Gao et al. who reported that plant proteins activated IGF-1 insulin signalling in order to regulate cancer growth and autophagy but to a very little extent. In addition, the presence of considerable amounts of protein in the plant may be considered an avenue for building a complex compound with them embedded functional phytochemicals, some of which are thought to stop carcinogenesis through their antioxidant properties by interfering with oxidative stress signalling pathway and suppressing DNA damage. This further agrees with Heon-Su et al. (2020) where it was pointed out that a member of *Diospyros* genus (*Diospyros kaki*) exhibited cell death via activation of platelet-derived growth factor receptors (PDGFRs) which serve as the active binding site for membrane auto-phosphorylation. Our experimental findings support the notion that the incorporation of *D. villosa* protein into supplements may play a role in cancer inhibition and retrogression.

The results further showed that *D. villosa* leaves, stem and nanoparticles synthesized at room temperature were able to inhibit cell growth *in vitro* with high efficiency. Even more, the extracts showed more potency in MCF-7 cancer lines, displaying high cytotoxicity. This is in line with Park et al. where *Diospyros kaki* (Thumb.) suppressed the proliferation of human cancer cell lines by decreasing cyclin D_1 expression. Although, the mechanism of *D. villosa* cancer inhibition may not have been achieved through cyclin D_1 expression, the excellent display of IC_{50} may be considered. In this present study, the *D. villosa* leaves, stem and biosynthesized nanoparticles with the highest anticancer activity presented IC_{50} showing potent inhibitory effect on the growth of MCF-7 and A549 cell lines. The results showed that the *D. villosa* plant presented the lower range of IC_{50} compared to a referenced anticancer medication, showing the higher potency. On the other hand, the methanolic extract of *D. villosa* plant showed the lowest potency in inhibiting cell growth in MCF-7 and A549 cells. Taking in account that the beneficial properties of Diospyros plants are related to a variety of

bioactive components that enhance antioxidant capacity and consequently anticancer activity (Sara et al., 2018, El-Hawary et al., 2020). *Diospyros villosa* may be strong candidates for future cancer studies, having high antioxidant and anticancer activity. The activities of both hexane leaves and stem extracts were quite promising as effective anticancer agents. The hexane stem extract did not just only inhibit the growth of MCF-7 cells but also possess a lower IC_{50} compared to standard. The lowest value of IC_{50} as produced by methanolic leaves extracts would have been considered the best, but higher percentage viability of MCF-7 cells further explained that the methanolic leaves extract may rather be considered a strong antioxidant/antibacterial than anticancer agent. The hexane stem extract displayed both lower IC_{50} value compared to the standard and a low percentage viability of A549 cells. In fact, the less toxicity of hexane leaves extract to HEK293 was observed as the IC_{50} values was quite higher. Both hexane stem extract and the synthesized stem nanoparticles at room temperature showed marked anticancer activities.

6.11 Conclusion

In this work, we have explored the nutritive contents and anticancer effect of the *D. villosa* leaves and stem bark as well as the nanoparticles against three cancer cell lines. The study revealed that *D. villosa* had high fibre and protein contents. *Diospyros villosa* may therefore be considered a plant with great potential as food supplement. It is further possible to conclude that *D. villosa* extracts potentially inhibited the viability of human breast carcinoma and lung carcinoma cells. Hence, its incorporation into nutritive supplements may provide a preventive measure to both breast and lung cancer.

6.12 Acknowledgement

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6.13 Conflict of Interest

The authors have declared no conflict of interest.

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CHAPTER SEVEN

Conclusion and Recommendation for Further Research

7.0 Introduction

The deficiency of information with focus on *D. villosa* in South Africa moulded the basis of this research study to create better awareness on the medicinal properties and secretory complex of *Diospyros villosa* by providing scientific evidence in coordinated approach as well as its promising roles in traditional medicine. A better perception of this plant is described by the secretory structures and products. The scientific exploration of the leaves and stem bark for the biosynthesis of nanoparticles and analysis of quorum sensing inhibition renders the study distinctive.

The family Ebenaceae has 4 genera and around 490 species (Duangjai et al., 2009). The assessment done on the species in this study gave quite important report on information on species distribution and emphasized the therapeutic significance of the *Diospyros* genus. *Diospyros villosa* also has high traditional and medicinal importance (Philander, 2011). Plants are loaded with enormous amount of secondary metabolites that provide therapeutic benefits against a wide range of human ailments (Baloyi et al., 2019). Green plants' application as an alternative curative measure for diseases rather than western medicine is now conventional (Phumthum et al., 2018). The therapeutic potentials of *D. villosa* were ascribed to the presence of bioactive ingredients and this was as reported by Cirera et al. (2010a).

At the inception of this study, the phytochemical analysis of *D. villosa* revealed the occurrence of bioactive compounds in the leaves and stem bark and are accountable for the therapeutic potentials of *D. villosa* plants. The results obtained from FTIR results further confirmed the occurrence of phytocompounds in the methanol, chloroform and hexane extracts of both leaves and stem bark of *Diospyros villosa*. Presence of several compounds in

the plant ascertained the report that *D. villosa* is locally used to treat ailments. This current study also provided information about the antioxidant and antimicrobial tendency of the phytocompounds against both Gram-negative and Gram-positive bacteria. The study further confirmed the important role for antibacterial purposes.

The following section of this experimental research was proposed to explore siver nanoparticles biosynthesis and assess the anti-quorum sensing activity. The methodology used for the silver nanoparticles biosynthesis from both leaves and stem extracts of *D. villosa* was described. The biosynthesized silver nanoparticles obtained through the bio-reduction method displayed unique attributes of nanoparticles (Mousavi et al., 2018). FT-IR results showed better tendency for the use of phytoconstituents as potent stabilizing and capping agents for nanoparticles. The synthesized silver nanoparticles from stem extract at 80 ºC demonstrated good quorum sensing inhibitory potential with corresponding violacein and bacterial growth inhibition. This enhanced outcome could be ascribed to the existence of many bioactive compounds as mentioned in this study. Temperature is implicated to potentiate the action of nanoparticles synthesized from plant extracts (Ruddaraju et al., 2019). This study confirmed the better quorum sensing inhibitory activity of the stem nanoparticles synthesized at 80 ºC compared to the nanoparticles synthesized at room temperature. The results from this present study further ascertained the procedure to be uncomplicated, quick, readily accessible, safe and less toxic. This particular study would definitely be useful in the use of AgNPs synthesized from stem extracts of *D .villosa* in the development of drug forms and dosaging for new sets of therapeutic medications.

Morphological examination as carried out in this experiment revealed the presence of trichomes in *D. villosa* leaves and stem bark. The observed trichomes were further confirmd to be non-glandular. The non-glandular trichome can also be categorized as hirsute (Payne, 1978), comprising long but stiff stalk and it is usually expected that this type of trichome

serves as a spontaneous obstruction to the plant's unfriendly agent and perhaps, a defence mechanism for the plant (Karabourniotis et al., 2020). This study reports the first scientific evidence about the length of the trichomes in the *D. villosa* plant. Histochemical test showed the occurrence of phenol as confirmed by ferric trichloride. The release of these compounds could be due to the secretory pores as found on the leaf surfaces.

The subsequent segment of the study was intended to determine the nutritional importance of the plant using proximate analysis as well as the anticancer properties of both the plant extracts and the biosynthesized nanoparticles. In the investigation, the anticancer activity was assessed through the determination of percentage viability of cancer cells (A549, HEK293 and MCF-7) by MTT assay (Dwarka et al., 2017). Proximate analysis showed that *D. villosa* leaves comprise considerable quantity of crude fibre and protein. In fact, the biosynthesized nanoparticles from *D. villosa* stem at high temperature inhibited the viability of human breast carcinoma and lung carcinoma cells. Just a moderate inhibition was observed with human embryonic kidney cells. This particular study would certainly be useful in the incorporation of bioactive compounds of *D. villosa* into food supplements and their use in drug production as a novel way to treat and manage newly evolved pathogens.

7.1 Aim and Objectives

This dissertation was designed holistically set to explore the medicinal properties and biological activities of *D. villosa* using an array of biological, chemical and microscopic methods. The intention to create a wider perception and further elaborate on the microstructure and physiological ability of the embedded structures, plant studies evolving round micromorphology are undertaken using different microscopic experimental paradigms like SEM, TEM and EDX. The biomolecular constituents of the leaves and stem bark of *D.*

villosa were analysed using biochemical and histological assays as well as FT-IR analysis so as to validate the medicinal uses of the plant. The DPPH, FRAP and total phenol content was utilised to determine the antioxidant potentials of the plant while antimicrobial screening and determination were evaluated using both MIC and MBC.

This study further delved to synthesize silver nanoparticles at different temperatures (RT and 80 ºC) under ideal environmental conditions using leaf and stem extracts of *D. villosa* with the intention to assess the anti-quorum sensing properties. In addition, biosynthesized nanoparticles were characterized with an array of techniques (UV-Vis Spectrophotometry, FT-IR, SEM, TEM, and EDX).

The micromorphological investigation aimed at identifying the special features responsible for the secretion of phytochemicals. The located secretory pores at the leaves surface were identified as well as non-glandular trichomes on the leaves and stem surfaces. Proximate analysis aimed at evaluating the nutritive content of the plant and further links with the anticancer activity and the synthesized AgNPs. This was achieved through the assessment of viability of the A549, HEK293 and MCF-7 cancer cell on exposure to both the extracts and AgNPs.

7.2 Challenges

The utmost uninteresting encounter while carrying out this experiment occurred during the determination of anticancer potential of both the leaf and stem extracts as well as the biosynthesized nanoparticles. Apart from being informed about the methodology, the available cancer cells were limited. The use of animal models is quite important while developing a basic knowledge with drug formulation and dosaging as anticancer regimen for

curative purposes. Although, the animal model for anticancer study of this plant was not done, the available cancer cell lines serve as an alternative, thus the challenge was eliminated. Besides the good knowledge of this study, the COVID-19 pandemic contributed a lot of disruptions to this study. Strict compliance to the pandemic guidelines was ensured and further served as a measure to overcome the challenge.

7.3 Future possibilities

The genus *Diospyros* is highly esteemed in traditional and medicinal practice throughout the world. This genus consists of almost 490 species. Meanwhile, only a few of the species has been examined for their medicinal impacts. This deficiency in the information about some of the species provides ample opportunity for expanded study.

Micromorphology of the trichomes and stomata contribute to the existing body of knowledge. However, the functionality and responses of the trichomes and stomatal pores are not well documented. Therefore, it would then be logical and noteworthy to investigate the plant's physiological and reactional mechanisms to environmental stress as well as plants' productivity and other characteristics of *D. villosa* plant.

Biochemical screening of *D. villosa* plant extracts showed the existence of many compounds with medicinal importance emphasizing *D. villosa* as an outstanding nomination for compound isolation and purification of embedded bioactive compounds towards drug development and dosaging. It is pertinent that more analyses like HPLC-PDA, HPLC-MS, GC-MS are necessary to further characterize the extracts and, if necessary, isolation and structure characterization of compounds by HRMS and NMR. The extracts of *D. villosa* perfectly synthesized silver nanoparticles. Adoption of this plant may also be examined for the biosynthesis of other nanometals like gold. Other studies can further investigate the use of biosynthesized nanoparticles on plant. There may be the need to provide molecular genetic

analysis which may also provide opportunity to resolve the relative contributions of various factors to observed morphological features of the plants.

7.4 Summary and Conclusion

Diospyros villosa is a traditional plant locally used as curative for several ailments. It could be inferred from this study that *D. villosa* leaves and stem bark contain some active compounds. It further provides a comprehensive information about the phytochemical outline and may further be used for the development of nutraceutical.

The leaf and stem bark extracts proved to be efficient in biosynthesis of AgNPs and further exhibited its potential as antibacterial agents against Gram negative and Gram positive bacteria. The biosynthesized stem extract nanoparticles at high temperature further showed a good anti-quorum sensing activity. This study has further augumented previous reports on the biosynthesis of metal nanopartcles using plant leaf and stem bark extracts.

Microscopical analysis of the leaves and stem bark also showed the occurrence of stomata and trichomes in the plant parts with captivating qualities. The anticancer properties of the plant leaves and stem bark was further confirmed in this study. Overall, *D. villosa* has a variety of phytochemical compounds which are essential and necessary to manage or cure diseases and therby preventing the deterioration of human health especially in pathological conditions.

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