UNIVERSITY OF KWAZULU-NATAL ™

INYUVESI YAKWAZULU-NATALI

# The secretory scales and medicinal properties of *Combretum erythrophyllum*


By:

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Submitted in fulfilment of the academic requirements for the degree of:

Doctor of Philosophy,


School of Life Sciences

College of Agriculture, Engineering and Science

University of KwaZulu-Natal, Durban

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Examiners Copy

02 December 2021

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## DECLARATION 1 - PLAGIARISM

I, ……Sahejna Bantho……. declare that

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Publications that form part and include research presented in this dissertation:

**Publication 1**

Bantho, S., Naidoo, Y., 2018. Secretory scales of *Combretum erythrophyllum* leaves. South African Journal of Botany 115(1), 316.

*Author contributions*: The candidate (first author) has conducted all experimental work. In lieu, the candidate has generated an abstract and presented the pertinent outcomes during an oral and poster presentation.

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**Publication 2**

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**Publication 3**

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*Author contributions*: The candidate (first author) has conducted all the experimental work. In lieu, the candidate has generated a research paper whereby the pertinent research outcomes were presented and published. The input received from the second and third authors have been fundamental in conceptualizing and developing the research protocols required for the project. In addition, the second and third authors have also assisted by proofreading this paper.

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*Author contributions*: The candidate (first author) has conducted all experimental work. In lieu, the candidate has generated an abstract and presented the pertinent outcomes during an oral and poster presentation.

**Publication 5:**

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*Author contributions*: The candidate (first author) has conducted all the experimental work. In lieu, the candidate has generated a research paper whereby the pertinent research outcomes were presented and published. The input received from the second and third authors have been fundamental in conceptualizing and developing the research protocols required for the project. In addition, the second and third authors have also assisted by proofreading this paper.

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# ABSTRACT

Medicinal plants are known to contain phytometabolites that could effectively improve an individual's state of health. Species of *Combretum* are highly valued in Africa due to the plethora of their traditional medicinal uses. *Combretum erythrophyllum.* Burch. Sond., commonly known as the river bushwillow, is known to contain medicinally important phytometabolites. Traditionally, the foliage is used to treat venereal diseases and abdominal pain, whilst the bark is used to alleviate sores, infertility, and labour pains. Although *C. erythrophyllum* has numerous traditional medicinal uses, there is limited scientific knowledge on the micromorphological structures and the associated exudate. Thus, this study aimed to characterize the micromorphological features of leaf and stembark secretory apparatus of *C. erythrophyllum*, using light and electron microscopy. Furthermore, a histo-phytochemical analysis was conducted to determine the presence and localization of phytometabolites within the trichomes and exudate. The antioxidant, antibacterial, apoptotic and cytotoxic potential of the leaf and stembark extracts were also evaluated. The micromorphological analysis identified the presence of peltate scales and non- glandular trichomes across surfaces. Peltate scales were comprised of a sunken basal cell, bicellular stalk, and a multicellular head. Head cell count appeared to increase upon leaf maturation. The granulocrine pathway was identified as a possible mode of secretion for *C. erythrophyllum* due to the extensive presence of vesicles, vacuoles, and electron dense material within the peltate scales. Preliminary histo-phytochemical analyses revealed the presence of carbohydrates, sterols, lipids, phenolic compounds, total proteins, alkaloids, and essential oils. Thin-layer chromatography allowed for the visualization of 36 compound classes while gas chromatography-mass spectrometry showed 266 compounds present. Fourier-transform infrared spectroscopy analysis confirmed the presence of phenols, alkenes, amines, alcohols, and esters among many. The antioxidant ability of the generated extracts were evaluated using the 2,2-diphenyl-1-picryl-hydrazyl-hydrate antioxidant assay and Ferric Reducing Antioxidant Power assay. A positive correlation between % inhibition and extract concentrations, was noted in both. In both instances, the methanol stembark extracts performed the best, (Leaf- 5,2866 and Stembark- 4,2866 µg/mL). Furthermore, the results obtained from the total flavonoid assay correlated with the trend observed through the total phenolic assay, whereby methanolic extracts yielded most promising results. Additionally, this study aimed to generate silver nanoparticles using crude extracts. A novel protocol for the synthesis of silver nanoparticles (AgNPs) using the leaf and stembark extracts of *C. erythrophyllum* was established. The generated AgNPs were characterized and evaluated for its potential antibacterial activity. Methanolic extracts inhibited the growth of *Pseudomonas aeruginosa*, *Bacillus subtilis*, Methicillin Resistant *Staphylococcus aureus*, and *Staphylococcus aureus*. Lastly, the generated crude extracts displayed promising results when evaluated for their cytotoxic and apoptotic abilities however upon nano-

encapsulation the cytotoxic and apoptotic capabilities increased significantly. In correlation with the above, the AgNPs appeared to intensify the overall inhibition activity. Based on the findings of the study, *Combretum erythrophyllum* has a reservoir of unexplored allopathic potential which could revolutionize the medicinal world.

Keywords: AgNPs, antibacterial, antioxidants, apoptosis, *Combretum erythrophyllum*, cytotoxicity, ethnobotany, micromorphology, trichomes, phytometabolites

# PREFACE

The experimental work described in this dissertation ['thesis' for the MSc, which was subsequently converted into a PhD] was carried out in the School of Life Sciences, University of KwaZulu Natal, Durban, from February 2017 to December 2021, under the supervision of Professor Yougasphree Naidoo.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

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# CHAPTER 1: INTRODUCTION

## 1.1 Ethnobotany

"*All medicinal preparations were derived from plants, whether in the simple form of raw plant material or in the refined form of crude extracts or mixture"*.

(Farnsworth and Soejarto, 1991)

Plants are said to have originated over 400 million years ago as the initial source of nutrition, clothing and shelter to all living organisms (Kenrick and Crane, 1997; Vishwanathan, 2018; Lee, 2021). The additional use of certain plant species in traditional and commercial medicine was also a practice of high interest (Dubey and Sao, 2018.).

Among several cultures and ethnic groups around the world, astute plant species are used, primarily, for their therapeutic properties (Hoareau and Da Silva, 1999; Thakuria et al., 2018; Dirir et al., 2021). Ethnomedicine involves the global comparative analysis of various traditional medicinal treatment methods, with emphasis being placed on compounds extracted from plants. There are three main disciplines associated with the study of ethnomedicine, namely, ethnopharmacology, ethnobiology and most importantly ethnobotany (Adeleye et al., 2021). The use and study of these plant species is described as Ethnobotany (Abebe et al., 2018; Kalayou et al., 2021). Ethnobotany is the study of plants and its associated medicinal use, primarily based on the knowledge gained from local cultures and people (Ludwinsky and Hanazaki, 2018). The use of plant species for their suggested healing properties is a prominent technique seen to be passed over generations (Balunas and Kinghorn, 2005; Vishwanathan, 2018; Kalayou et al., 2021). Practices such as Ayurveda and Chinese medicinal treatments are commonly utilized in the Asian continent while, traditional healers practicing similar approaches (eg. Sangoma's) are found in Africa (Rupani and Chavez, 2018; Weng, 2018; Nair et al., 2021). For the underprivileged, the use of cost-effective natural products surpasses the use of expensive, commercially produced pharmaceuticals (Bennett et al., 2018; Tan, 2018; Biswas et al., 2021). Generally, the use of plants to treat ailments as an alternative to conventional medicine was mostly linked to developing countries and communities that have limited access to conventional medicine (Atinga et al., 2018; Biswas et al., 2021), however, this trend has now changed.

In developed countries such as Germany, 90% of the population are turning towards registered plant-based medication in comparison to allopathic drugs (Willis, 2017). This is due to the vast benefits and minimal side effects associated with naturally derived medication (Verma and Singh, 2008; Tan, 2018; El- Saadony et al., 2021). In accordance, the WHO has mentioned that almost 75% of global beings depend on traditional healing methodologies rather than those commercially utilized, as their primary source of medicinal aid (Maluleka and Ngoepe, 2018; Alotaibi et al., 2021). Globally, the

current use of medicinal flora is not restricted or associated to those less fortunate but rather a trend to follow amongst all economic classes (Mutembei et al., 2018).

Medicinal plants are known to contain compounds that may effectively improve an individual's state of health (Bernardini et al., 2018; Bantho et al., 2020). Within plant species, there are two structurally and functionally defined metabolite groups (McGaw and Eloff, 2008; Ahman et al., 2018; Panchawat et al., 2021). Primary metabolites comprising of amino acids, nucleic acids, and sugars, are essential to cellular development, functioning, and reproduction (Pichersky and Raguso, 2018; Hayami et al., 2021). While exuded secondary metabolites are crucial to plant defence and include metabolites such as phenols, tannins, alkaloids, saponins, and other such organic metabolites (Ascensão and Pais, 1987; Santos et al., 2015). The medicinal use of these metabolites may prove to be remarkably advantageous to mankind (Kumari, 2018; Allemailem, 2021). In accordance, alkaloids are highly beneficial in treating a wide array of ailments (such as fevers and dysentery) yet may be toxic and lethal if administered in incorrect dosages (Patel et al., 2012; Roberts and Strack, 2018, Albuquerque et al., 2021). While the use of plants containing phenols and tannins may exhibit antimicrobial and anti-oxidant impressions upon the host (Wink, 1998; Mahata and Mandal, 2018). Certain plant species exhibits these beneficial effects upon the host when used in conjunction with traditional techniques such as boiling and grinding (Kidane et al., 2018). Limited knowledge on the impact of plant-based concoctions on human immunology may prove to be hazardous therefore, research pertaining to dosage regulations must be reflected upon (Farnsworth, 1994; Kahaliw et al., 2018; Bose et al.,2021). The impact of incorrect therapeutic dosages and the presence of harmful or toxic metabolites within certain plants is unknown, hence the study of these species is imperative (Van Wyk et al., 1997; Kahaliw et al., 2018; Bose et al.,2021).

Over the past two decades, a developing interest in the study of medicinal plants has led to the exuberant boost of its economic value in Africa (Joy, 1998; Van Wyk, 2008; Maluleka and Ngoepe, 2018, Schultz et al., 2021). South Africa comprises of almost 10% of the world's total plants and fauna species occupying an estimated land surface area of only 1.2 million $km^2$ (2% of the earth's total land surface) (Germishuizen and Meyer, 2003, Perera et al., 2021). The South African Government, along with various other institutions, has placed importance on the conservation of biodiverse regions (Maroyi, 2018, Shumba et al., 2021).

There are many key institutions such as the South African National Biodiversity Institute (SANBI), the World-wide fund for nature (WWF), the conservation of South Africa association (CSA) and the South African National Parks (SANParks) that are committed to the conservation of South Africa's biodiversity, as well as aiding in protecting and sustaining hotspot regions. The three globally recognized South African hotspot regions include the Cape Floral Kingdom, the Succulent Karoo and the Maputaland Pondoland Albany (Forest et al., 2018; Pieterse et al., 2018; Cunningham and

Beazley, 2018, Perera et al., 2021). These hotspots are known for their biodiverse ecosystems. Comprised of an estimated 20000 plant species, a large portion (approximately 50%) of these species are unique to these hotspots and are yet to be explored in a medicinal context (Ekoungoulou et al., 2018, Kumar et al., 2021).

Species of these areas are known to boost the plant economic market due to its medicinal properties and aesthetic worth (De Wit, 2006, Perera et al., 2021). South Africa prides itself on approximately 5700 different plant species, with more than 50% currently being utilized for its medicinal value (Mulholland, 2015, Aboyewa et al., 2021). An example of a native species of high economic and medicinal importance is the *Aspalathus linearis.* (Pringle et al., 2018, Khumalo et al., 2021). More commonly known as rooibos or red bush, this caffeine-free plant is popular for the ability to speed up metabolic rates and for its profound anti-oxidant and anti-inflammatory properties (Pretorius, 2007; Muller et al., 2018, Khumalo et al., 2021). Products comprised of this refined plant extract are currently on sale worldwide, in the form of tonics, teas, and creams (Chhikara et al., 2018; Smith et al., 2021). *Aloe vera (L.) Burm.* f is also characterized by its medicinal properties and is commonly utilized worldwide (Gao et al., 2018, Riaz et al., 2021). This species is known for its anti-oxidant, anti-inflammatory, and wound healing properties (Fox et al., 2017, Alven et al., 2021). Another such plant is *Moringa oleifera L. (moringa.),* also known as the drumstick tree, this plant is said to treat bacterial, fungal impurities, wounds, and headaches (Isingoma et al., 2018; Gupta et al., 2018, Arora and Arora, 2021). The utilization of this plant has been promoted over the years by the production of drugs using phytometabolites isolated from it (Choubey et al., 2018, Bhat et al., 2021).

There are an estimated 300 000 plant species found worldwide, with only a mere 28 187 plant species explored for their medicinal properties (Baber et al., 2018). Thus, leaving multitudes of potentially beneficial species yet to be evaluated for its pharmacological value.

### 1.2 The Family Combretaceae

Combretaceae is a large angiosperm and medicinally important family comprising approximately 20 genera and 600 species worldwide (Exell, 1970; Moura et al., 2018, Bantho et al., 2020). This family of rivine species is prominent in north-eastern parts of South Africa and are known to thrive in both subtropical as well as tropical regions (Lima et al., 2012).

Combretaceae is characterized by secretory hairs that can be either glandular or scales (Lima et al., 2012; Bantho and Naidoo, 2018, Bantho et al., 2020). The glandular trichomes are short and capitate whereas the scales are sub-sessile and peltate (Fahn, 1988, Siadati et al., 2020). These secretory hairs are rich in secondary metabolites that may aid in the treatment of various medicinal ailments (Beigi et al., 2018, Bantho et al., 2020). Combretastatin, a phytometabolite unique to this family, is an anti-cancer compound that was successfully isolated from *Combretum caffrum* (Eckl. & Zeyh.) Kuntze

about 21 years ago (Pettit and Singh, 1987; Lamaa et al., 2018, Maphutha et al., 2021). Ongoing clinical trials indicate the positive impact delivered from this phytometabolite (Atanasov et. al., 2018, Alam et al., 2021).

In addition, species such as *Combretum molle* R.Br ex G. Don and *Combretum roxburghii* Spreng. are known to have a wide array of medicinal uses (Bhatnagar et al., 2012; Naidoo et al., 2012, Aumeeruddy and Mahomoodally, 2020). These species have been evaluated in micromorphological, phytochemical and pharmacological studies. Pharmacological studies of these species indicate anti-inflammatory, hypotensive and antifungal properties at the least (Mcgaw et al., 2001; Anato and Ketema, 2018; Das et al., 2018), with substantial evidence suggesting the presence of antimicrobial compounds in at least 12 other species (Alexander et.al., 1992; Bhatnagar et al., 2012; Naidoo et al., 2012; Bantho at al., 2020).

*Combretum molle* is known for the salubrious antifungal and molluscicidal effects, and aids in combating bacterial infections (Fyhrquist et al., 2002; Hamza et al., 2021). Histochemical research shows that scales of *C. molle* contain secondary metabolites (terpenoids, phenolics, flavonoids and alkaloids) that are proposed to provide antimicrobial and antiherbivore protection (Bhatnagar et al., 2012; Naidoo et al., 2012; Ally and Mgonja, 2021). *Combretum roxburghii*, an Indian native species, is also shown to have noteworthy medicinal properties (Das et al., 2018). Interestingly, a variation of compounds was found in this species over different seasons. Samples collected and tested during February and October were high in antioxidant content compared to those of May and August, thus, suggesting that climatic conditions could play a vital role in influencing secretion levels (Bhatnagar et al., 2012). Histochemical research shows the secretory scales of *C. roxburghii* to be comprised of saponins, flavonoids and tannins (Bhatnagar et al., 2012). The extracted flavonoids are said to have antioxidant, anti-estrogenic and anti-proliferative properties (Cutler et al., 2008; Mgonja, 2021).

### 1.3 Ethnopharmacological applications of *Combretum* species in Africa

Traditional African medicine is said to be among the oldest and most diverse holistic medicinal systems in the world (Adeleye et al., 2021). This system encompasses the use of native medicinal plant species to alleviate and cure various ailments. Furthermore, this system incorporates religious, cultural and spiritual beliefs, with local knowledge for optimum results (WHO, 2008; Okaiyeto, and Oguntibeju et al., 2021). The herbal drug research unit, a branch of the South African Medical Research Council, incorporates scientific techniques to extract the full pharmacological value of the traditional African medical system. Their main aim is to develop drugs by incorporating medicinally important phytometabolites derived from native medicinal plants (SAMRC, 2021). Till date, a few drugs have been discovered and developed through the knowledge from traditional African

medicine, with many avenues left unexplored. Specifically in Africa, Combretaceae is a medicinally important family used extensively to treat various ailments (Maphutha et al., 2021).

Species from the *Combretum* genus, such as *Combretum* vendae A.E. van Wyk, *Combretum zeyheri* Sond., *C. molle, C. caffrum and C. quadrangulare* been utilized for their ethnopharmacological use, within this system. The ethnopharmacological use of *C. molle* includes: the treatment of bacterial infections (i.e.. gonorrhoea, syphilis) stomach ailments, hypertension, diarrhoea, possibly cancer and fever (Asita, et al., 2018). *Combretum vendae* is known to treat bacterial infections, leprosy, eye problems, and oxidative- stress related diseases (Mtunzi et al., 2017). Traditionally, *C. zeyheri* is commonly utilized in the treatment of bacterial infections, eye ailments, haemorrhoids, nosebleeds, gallstones and kidney pains (Moura, et al., 2018). Furthermore, *C. quadrangulare* is said to treat skin disease, while acting as an antipyretic, antidysenteric and anthelmintic agent. This species is also known to display antiparasitic, antitrypanosomal, antibacterial, antiviral and antihepatotoxicity activity (Nguyen et al., 2021.; Lima, et al., 2012).

### 1.4 Botanical description and ethnopharmacological properties of *Combretum erythrophyllum* (Burch.)

*Combretum erythrophyllum* (Burch.) Sond.; also known as the river bushwillow; is a riverine species known for its high traditional medicinal value (Mtunzi et al., 2017; Bantho et al., 2020). This is a deciduous, indigenous tree that thrives in areas with good groundwater level (Jordaan et al., 2011). It is adapted to grow in a wide array of climatic conditions and can be found in abundance across the eastern part of South Africa (Fig. 1.1) (Jordaan et al., 2011; SANBI, 2012).

Gelfland et al., (1985) describes the tree as being multi-stemmed, with dense foliage and a flaking bark which is light brown in colour (Fig. 1.2a). The branches grow in an outward, horizontal direction, with upright branches that characteristically originate for low lying branches (Fig 1.2). Foliage is simple and opposite with a entire margin, acute apex and base. Leaf colour appears to deepen as maturation occurs (Fig. 1.2b). Younger leaves are bright green while mature leaves are deep green in colour (Fig. 1.2b). In relation to the scientific name of *C. erythrophyllum (erythrophyllum* is defined as ‘red leaf’ in *Greek)*, leaves appear red during Autumn (Martini and Eloff, 1998)

Traditionally, the foliage (Fig. 1.2b) of *C. erythrophyllum* is used to treat and prevent venereal diseases and abdominal pain, whilst the bark (Fig. 1.2c) is used to treat sores, infertility and labour pains (Mawoza and Ndove, 2015; Mtunzi et al., 2017). In addition, roots are used to induce a purgative effect and the gum is used to treat sores (Mawoza and Ndove, 2015; Mtunzi et al., 2017; Bantho et al., 2020). Martini and Eloff (1998) highlighted the superior antibacterial efficiency of *C. erythrophyllum* in comparison to chloramphenicol and ampicillin. Martini et al. (2004) characterized the presence of seven beneficial flavonoids in *C. erythrophyllum*. as well.

Although some information regarding the medicinal potential of *C. erythrophyllum*, is available, there are many avenues yet to be explored.

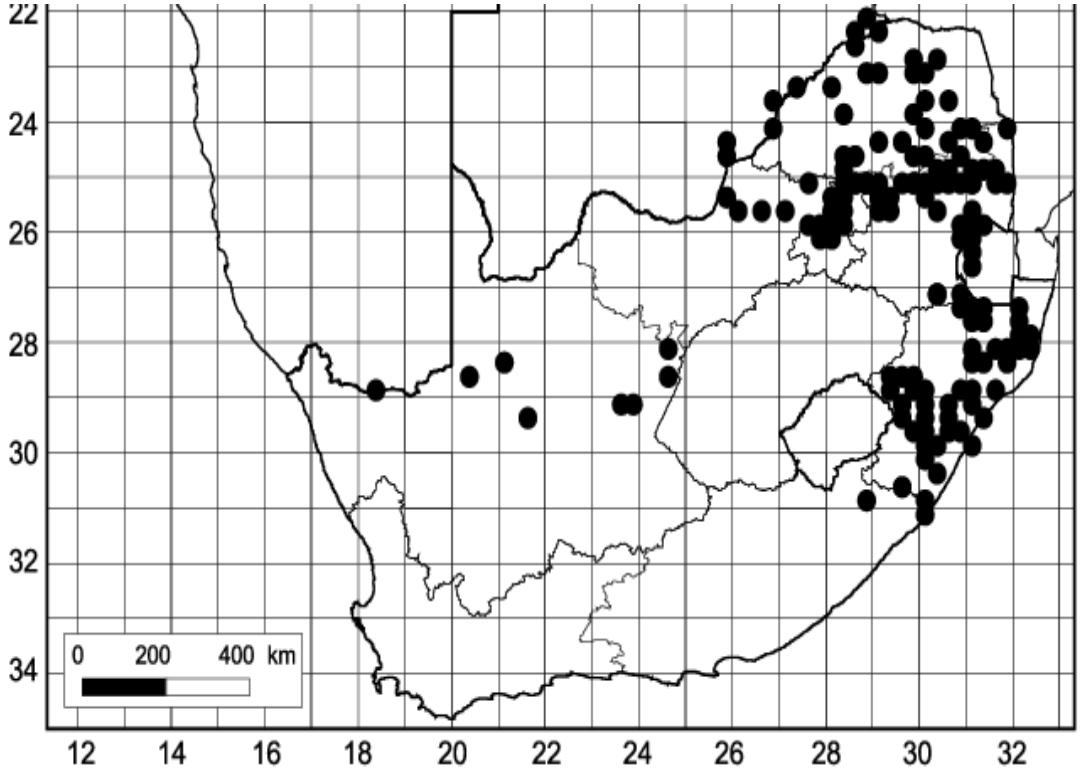



Figure 1.1: Geographical distribution of *Combretum erythrophyllum* along South Africa (Jordaan et al., 2011).

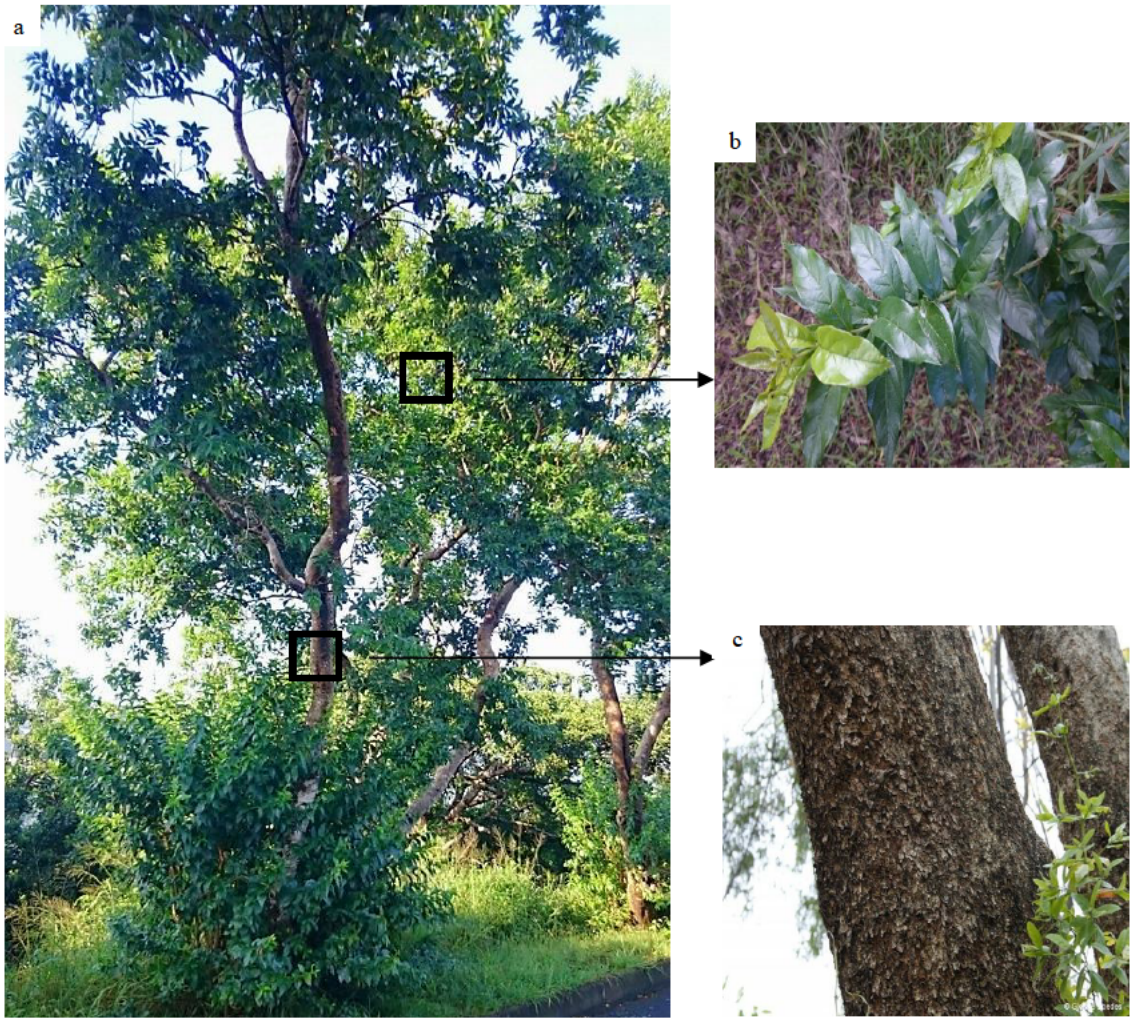



Figure 1.2: *Combretum erythrophyllum* tree growing at the University of Kwa Zulu-Natal, Westville Campus. a) whole tree, indicating the presence of low-lying branches, b) foliage of different developmental stages, indicating the light green colour in younger leaves and deep green colour in mature leaves and c) flaking bark.

## 1.5 Rationale for the research

Medicinal properties are attributed to secondary metabolites found in certain plant species (A'attiyyah et al., 2018, Bantho et al., 2020). Synthesized primary metabolites are crucial to cell functioning (Pichersky and Raguso, 2018; Hayami et al., 2021). These compounds play important roles in growth and development, whilst secondary metabolites are plant specific and are produced as part of the plant defence system against pests and pathogens (Rao and Ravishankar, 2002; Wink, 2018, Pang et al., 2021). These phytometabolites are actively secreted by specialized secretory structures, such as trichomes (Wittstock and Gershenzon, 2002; Feng et al., 2021).

Leaf surfaces of many plant species are known to have trichomes which are minute hair growths from the leaf epidermis (Munien et al., 2015; Bantho et al., 2020). These structures are known to have evolved over years deterring plants inability to 'fight' against predators (Champagne and Boutry, 2016; Wang et al., 2021). Trichome structures are indispensable for the production, accumulation and exudation of phytometabolites. They are the sites of synthesis as well as the reservoir of accumulated biological substances (Naidoo et al., 2012; Kalicharan et al., 2018; Bantho et al., 2020). Trichomes are classified according to their structure in relation to their given function. There are two categories of trichomes namely: non-glandular and glandular trichomes (Chang et al., 2018., Wang et al., 2021). Non-glandular trichomes act as a physical barrier and are known to aid in preventing water loss of the plant (Kariyat et., 2018., Shahzad et al., 2021). Whilst glandular trichomes are characterised by the presence of an exuding glandular head structure (Zager and Lange, 2018, Wu et al., 2021). Specifically, secretion from glandular trichomes play a large role in plant defence (Fahn, 1988; Kalicharan et al., 2018, Watts and Kariyat, 2021).

*Combretum* species are highly sought after in southern Africa due to its extensive use in traditional medicine (Mawoza and Ndove, 2015, Zwane and Bamford, 2021). Phytochemical analysis of *C. erythrophyllum* leaves indicated the presence of polyphenols, flavonoids, triterpenoids and saponins (Mawoza and Ndove, 2015). Pharmacological studies have shown this genus to have antibacterial, anti-inflammatory, antifungal, genitor-urinary, cytotoxic and mutagenic properties (Mtunzi et al., 2017, Forid et al., 2021). Valuable studies on the phytochemical constituents of the leaf of this species have been published, however, there are no studies conducted on the micromorphological and histochemical properties of *C. erythrophyllum*. Hence this study aims to evaluate the micromorphology and histochemistry of *C. erythrophyllum*. Trichomes are the source of these phytochemical constituents, hence a study investigating trichome structure in relation to their function and the importance of its exudate would be highly beneficial. Furthermore, the emanating study aims to quantify the total phenolics, total flavonoids, antioxidant, antibacterial, cytotoxic and apoptotic potential of the leaves and stembark of *C. erythrophyllum*.

The ‘State of World’s Plants’ (2017), mentioned, a measly <16% of plants have been evaluated for its medicinal properties. Further studies proving the true medicinal worth of traditionally utilized species will be highly beneficial, not only for further use in the modern drug development industry but also to communities. This study intends on elucidating the presence and location of beneficial and potentially harmful compounds within *C. erythrophyllum*, thus further building on the knowledge of its medicinal properties.

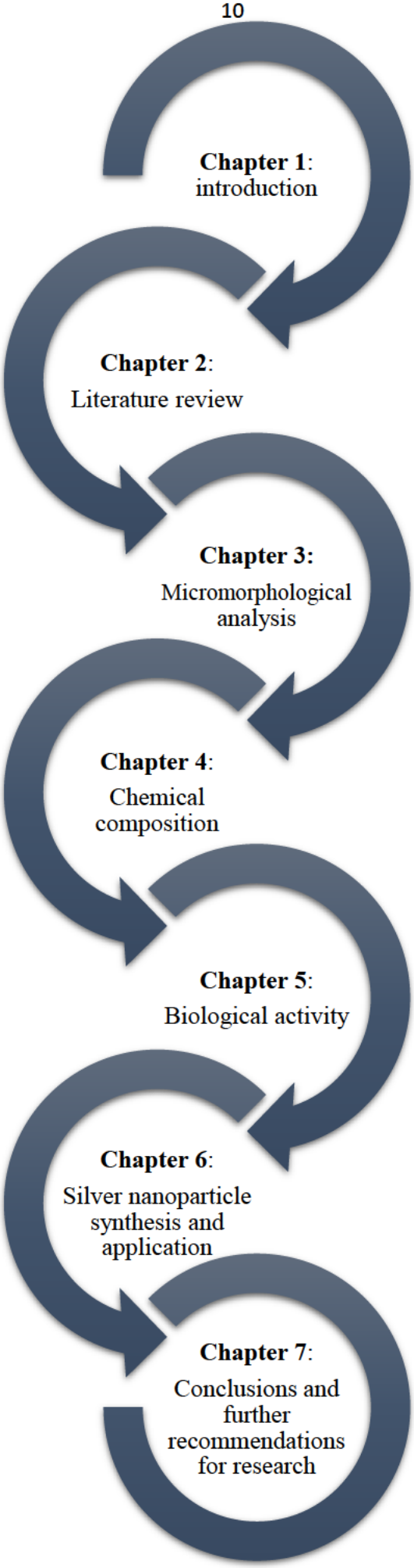
### 1.6 Aims

1. To investigate the micromorphology of stem and foliar developmental stages of *C. erythrophyllum*.
2. To examine mode of secretion and the secretory structures found on the stem and foliage of *C. erythrophyllum*.
3. To characterize secretory structures based on a histo-phytochemical analyses of *C. erythrophyllum* foliage and stem.
4. To determine the presence and chemical composition of metabolites from the foliage and stembark extracts of *C. erythrophyllum*.
5. To investigate the antioxidant potential of the foliage and stembark extracts of *C. erythrophyllum*.
6. To investigate the potential antibacterial and cytotoxic properties of foliage and stembark extract of *C. erythrophyllum.*
7. To generate a novel protocol for silver nanoparticle biosynthesis from the foliage and stembark extracts of *C. erythrophyllum.*

### 1.7 Objectives

1. To study the micromorphology of stem and foliar developmental stages, emergent, young and mature, of *C. erythrophyllum* using stereomicroscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM).
2. To investigate trichome density trends between stem and foliar developmental stages of *C. erythrophyllum.*
3. To conduct histo-phytochemical analyses of *C. erythrophyllum* stembark and foliage to determine the presence and localization of phytochemical groups.
4. To determine the presence and chemical composition of metabolites from the foliage and stembark extracts of *C. erythrophyllum* by performing a phytochemical and GC- MS analyses.
5. To investigate the invitro antioxidant potential of the foliage and stembark extracts *of C. erythrophyllum*.
6. To screen the crude foliage and stembark extracts of *C. erythrophyllum,* for their potential antibacterial and cytotoxic properties.
7. To biologically synthesize silver nanoparticles using the crude extracts.

## 1.8 Outline of thesis



## 1.9 References

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# CHAPTER 2: LITERATURE REVIEW

Secretory tissues are found in abundance upon foliar surfaces (Barthlott et al., 2017). The variation amongst morphology, exudate and function of secretory tissues are used to classify and distinguish between tissue types and classes (Wagner et al., 2004; Choi and Kim, 2013; Hayami et al., 2021). Fahn (1988) highlighted the structural differences between secretory tissues that occur in plants, ranging from single cells to complex multicellular structures. Svoboda and Svoboda (2000) suggest that secretory structures are classified based on their mode of secretion and topographic position. This method of classification identifies two distinct types. The first type involves the secretion of exudate within the plant vascular tissues and common examples include: resin ducts, mucilage ducts and laticifers (Fahn, 1988). While the second type is characterised by the release of exudate via aerial secretory structures to the outer surface, hence the exudate is transferred to extracellular spaces. Common examples include: salt glands, hydathodes and trichomes (Fahn, 1988; Svoboda and Svoboda, 2000).

Trichomes are minute protuberances from the leaf epidermis and are said to be the site of synthesis as well as the reservoir of accumulated biological substances (Naidoo et al., 2012a; Naidoo et al., 2012b; Beigi et al., 2018; Kalicharan et al., 2018; Hayami et al., 2021). Trichomes range from simple hairs to specialized tissues with varying functions and may be widely spread across both the vegetative and reproductive parts of the plant (Fahn, 1988; Chang et al., 2018). Furthermore, as highlighted by numerous authors, the important functions served by trichomes include protection against herbivores, regulation of leaf temperature and assistance in pollination (Kelsey et al., 1984; Beigi et al., 2018; Kaur et al., 2018; Bantho et al., 2020). In addition, trichome structures are known as primitive tools in plant taxonomic classification due to their distinct structure and its relative function (Chowdhury et al., 2018). Classification of plant species based on trichome presence was introduced many years ago (Behnke, 1984). Botanical literature highlights the variation in trichome types found, with more than 300 morphologically diverse structures noted (Wagner, 1991; Khokhar et al., 2012; Bose et al., 2021).

## 2.1. Trichome morphology, development and its relative function

Trichomes can be described as diverse epidermal outgrowths that differ in structure, function and form (Esau, 1977; Wagner, 1991; Chang et al., 2018). There are 2 notable categories of trichomes namely glandular and non-glandular, as highlighted by Fahn (1988). Trichomes are either unicellular- or multicellular in structure and are derived from basal and stalk cells, whilst certain plant species have unique trichomes with secreting head cells (Tian et al., 2017). Trichome cell walls are composed of cellulose lignin and, in some instances, a waxy cuticle (Liu et al., 2018; Takenaka et al., 2018). Certain trichomes are known to contain calcium carbonate within the cell walls which, when accumulated, could lead to crystal idioblast formation (Webb, 1999; Nicolás-Bermúdez et al.,

2018). The main functions of these secretory tissues include plant defence against herbivores and pathogens, protection against water loss and extreme temperatures as well as storage and secretion of essential oils (lipophilic substances) (Levin 1973; Martini et al., 2004; Dalin et al., 2008; Galdon-Armero et al., 2018). Specifically, secretion from glandular trichomes plays a large role in defence against pests and pathogens (Fahn 1988; Pichersky and Raguso, 2018). Besides a role in ecological defence, the secretion is known to provide medicinal and economic benefits (Kaur et al., 2018).

Trichome classification based upon shape and appearance was first introduced by Payne (1978). The author describes trichome morphology in common terms such as multicellular, shaggy or forked amongst many (Fig. 2.1). The morphological characters of trichomes are of key essence to the identification and classification of species (Payne, 1978; Demarco, 2017).

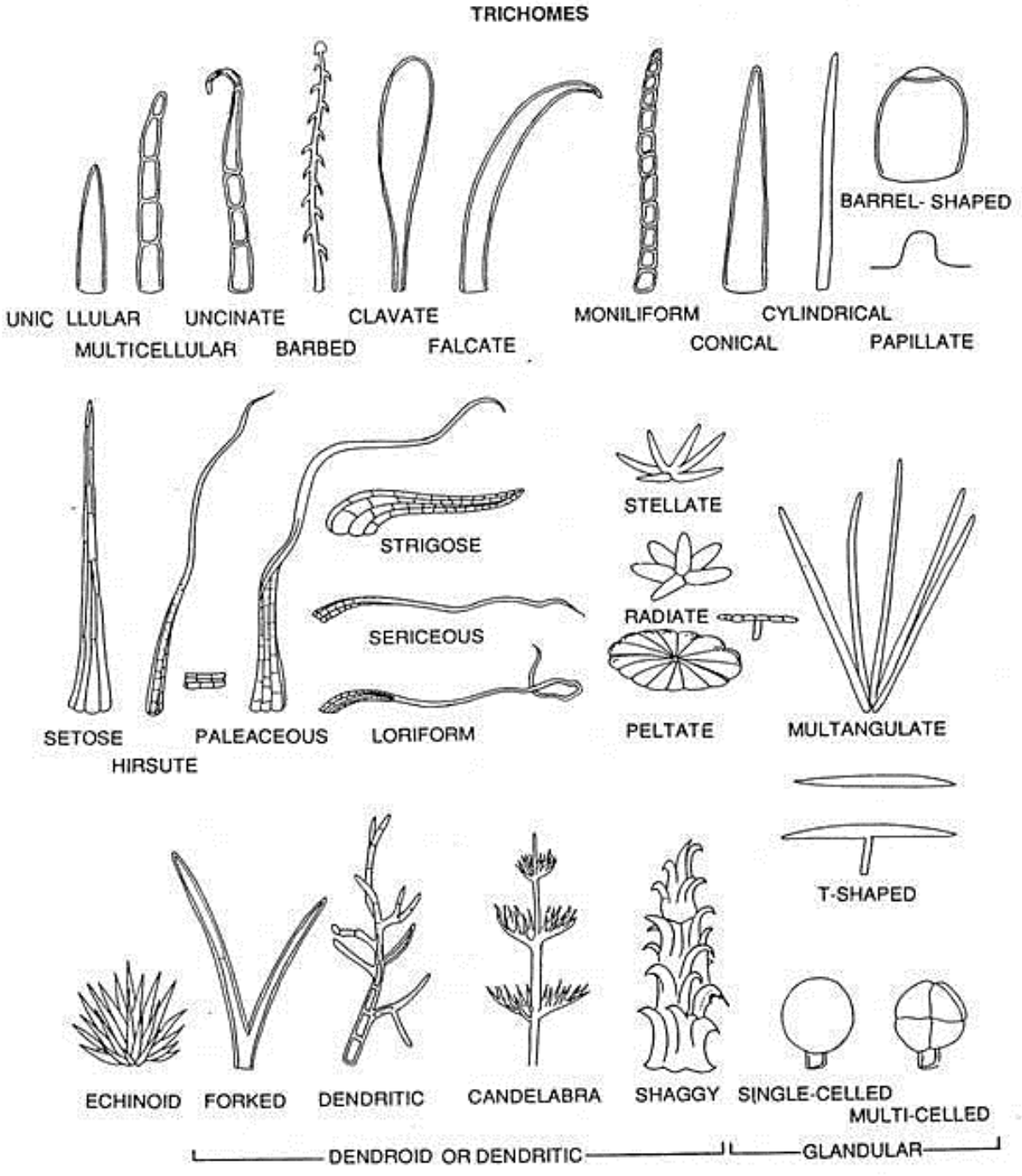



Figure 2.1: Trichome classification based on morphological characteristics. (adopted from Gupta, 2016).

### 2.1.1. Non- Glandular trichomes

Non-glandular trichomes can be found in abundance on foliar surfaces yet may differ in structure and function (Fahn, 1988; Hayami et al., 2021). They are categorised as unicellular or multicellular structures, that are either simple, branched or starred (Xiang et al., 2010). Furthermore, multicellular trichomes can be distinguished according to their cell count, i.e., uniseriate (single row of cells) and multiseriate (numerous rows of cells). Fahn (1988) classified trichome structures based on morphological characteristics, such as being either branched, hooked or pointed (Seyedi and Salmaki, 2015). Numerous authors have deduced that the main functions of non-glandular trichomes include preventing excessive moisture loss, protection from sunlight (which could lead to damage to foliage structures) and as a protective defence mechanism against pest and pathogens (Peter and Shanower, 1998; Werker 2000; Choi and Kim 2013; Bose et al., 2021).

### 2.1.2. Glandular trichomes

Glandular trichomes are structurally diverse secreting tissues that aid in plant defence and protection (Tian et al., 2017). These functionally specialized tissues produce secondary metabolites that are stored within aerial appendages on foliar surfaces (Duffey, 1986; Kaur et al., 2018). Glandular trichomes are further classified into peltate and capitate trichomes based on their morphological characters (Tian et al., 2017). Structurally, peltate trichomes are comprised of a large head with broad secretory cavities, a short stalk and a basal cell (Fig. 2.3c) (Ekeke and Agbagwa, 2017). Capitate trichomes, in turn, are distinguished by their long stalk, small head and narrow globular secretory cavities (Choi et al., 2011). Naidoo et al. (2012a) evaluated the structure and relative function of peltate scales found within *Combretum molle*. These 'scales' characteristic of the genus Combretum and are composed of a basal epidermal cell, a stalk (comprised of 2 cells) and a bulbous secretory head (comprised of 8- 24 cells) (Table 1, Fig. 2.3a). The basal epidermal cell is responsible for the provision and translocation of necessary substances to the stalk and multicellular head above (Naidoo et al., 2012a). Huang et al. (2008) highlighted the difference in exudate secreted via each trichome type. This research elucidated that exudate from peltate trichomes are lipophilic in nature whilst capitate trichomes exude polysaccharide substances (Huang et al. 2008). Substances released within this exudate may include mucilage, resin and secondary metabolites amongst many (Huang et al. 2008; Beigi et al., 2018). In addition, Duke (1994) highlights the medicinal value (antibacterial and antioxidant properties), secondary metabolites are said to have. Similarly, Bantho et al. (2020) published research evaluating the micromorphology, ultrastructure and histochemistry of the secretory scales found within *Combretum erythrophyllum*. The emanating micromorphological analysis resulted in the identification of peltate scales and non- glandular trichomes. The peltate scales were composed of a basal epidermal cell, a bicellular stalk and a multicellular head (comprised

of 8 to 19 cells) (Fig. 2.3b and c). Preliminary histochemical analyses indicated the predominant presence of alkaloids, phenolic compounds and essential oils within the exudate.

Naidoo et al. (2012b) emphasized he relationship between trichome development and the storage and release of exudate. It is noted that capitate trichomes release their exudate early in plant maturation hence they are 'short tetermwhilst peltate trichomes are termed as 'long term' structures due to their ability to store/accumulate exudate and only release it when the trichome is triggered/ raptured or mechanically damaged (Naidoo et al.,2012b) .

### 2.1.3. Functions

#### 2.1.3.1. Defence

Trichomes play an important role in defending foliage against biotic factors such as herbivores, pathogens and insects (Chahil et al., 2018). Research suggests that trichomes aid in reducing or preventing herbivore attacks by the presence of physical surface structures and by the release of chemical inhibiting substances (Umah et al., 2017; Lattanzio et al., 2006).

Ambrosio et al. (2008) explained the relationship between trichome density and effective herbivore resistance. Effective protection is often linked to high trichome coverage across the plant surface. A study has shown that within the Salicaceae family, *Salix cinerea* is known to have large quantities of non-glandular simple hair trichomes (Bjorkman and Ahrne, 2005). A common herbivory predator to this species is *Phratora vulgatissima*, the larva of this beetle is known to have devastating consequences upon *Salix* species. Research suggests that upon foliage interaction with the adult beetle, *S. cinerea* induced an immediate response resulting in the production of leaves with a higher trichome count (Dalin and Bjorkman, 2003; Bjorkman and Ahrne, 2005). Due to the increase in trichome density, beetle larvae movement was inhibited, making them more vulnerable to predators rather than the foliage. Additionally, larvae were unable to stick to leaf surfaces and appeared to fall off (Dalin and Bjorkman, 2003; Bjorkman and Ahrne, 2005). Plant trichomes are composed of high cellulose and lignin content (Chahil et al., 2018). Levin (1973) suggested that this may decrease the overall nutritional value associated to the plant thus being undesirable to predators.

Fahn (1988) suggested that non- glandular trichomes (uniseriate, hook-like) provide a structural defence mechanism against attacking herbivores trichomes. Szyndler et al. (2013) observed an extremely effective defence mechanism of *Phaseolus vulgaris* against *Cimex lectularius* (bed bug). Research shows that *P. vulgaris* has a dense coverage of uniseriate trichomes spread across its foliage surfaces (Szyndler et al., 2013). The presence of these trichomes allows for a successful defence mechanism against predators. This involves the physical entrapment of the insect via non-glandular uniseriate (hook-like) trichomes (Fig. 2.2a). Upon micromorphological examination, the entrapment of the insect legs was clearly indicated (Fig. 2.2b; Szyndler et al., 2013). The tip of the trichome is

bent and sharply pointed hence entrapment as well as the simultaneous piercing of the insect is caused (Szyndler et al., 2013).

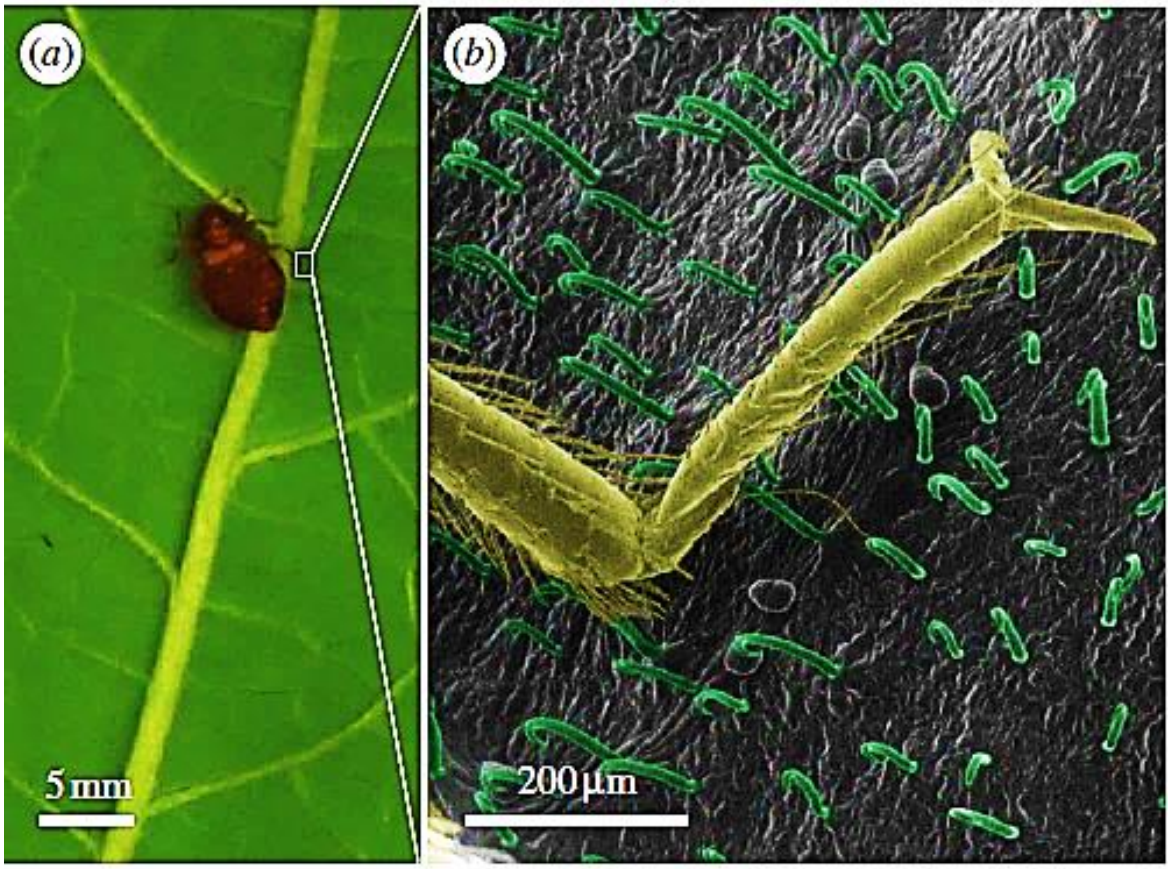



Figure 2.2. *Phaseolus vulgaris* leaves with hooked-like trichomes: a) stereo micrograph indicating the presence of a bed bug on the leaf surface and b) SEM micrograph indicating the presence of hook-like trichomes which are trapping the insect (Szyndler et al., 2013).

Glandular trichomes act as effective defence mechanisms due to the compounds they exude (Duffey, 1986; Tian et al., 2017). Many authors provide data which shows that glandular trichomes aid in defence against herbivores via the secretion of compounds (secondary metabolites) (McGaw et al., 2001; Ujowundu et al., 2017, Ogbole et al., 2018). These act as a natural repellent and in some cases may also encapsulate the attacker (Lattanzio et al.,2013). Secondary metabolites are plant specific and is produced as part of the plant defence system against pests and pathogens (Rao and Ravishankar, 2002; Zager and Lange, 2018). Phytometabolites such as phenols, alkaloids, tannins, terpenoids or flavonoids act as herbivore and pathogen repellents (Tian et al., 2017; Ogbole et al., 2018).

*Lycopersicon hirsutum* f. glabratum C.H.Muell. has several ketone groups as well as major volatile compounds (Lin et al., 1986). In a study carried out by Lin et al. (1986), the effectiveness of naturally occurring insecticides or repellents within wild tomato species, such as *L. hirsutum*, were examined against two common pests, *Spodoptera exigua* (Hübner) and *Keiferia lycopersicella* (Walsingham) larvae. In both instances, larvae were almost effectively destroyed due to the toxic terpenoid enriched exudate. Onkokesung et al. (2014) explored Arabidopsis species which contains trichomes rich in flavonoids. This research indicated that high flavonoid levels can effectively offer resistance towards, *Pieris brassicae L.* Another common form of defence is the secretion of mucilage via trichomes which allows for the entrapment of feeding insects (Lattanzio et al., 2013). In conjunction, Naidoo et al. (2014) reported *Solanum polyadenium*'s Greenm. ability to secrete a sticky mucilage exudate to encapsulate its common predator, *Empoasca fabea* Harris.(potato leafhopper).

Tannins are a group of commonly found polyphenols, which are proven to have a positive repellent effect on phytophagous insects (Kergunteuil et al., 2018). Effects include the hindrance of growth and development as well as decrease in the nutrient level of the insect. This bitter compound acts as a feeding deterrent against common predators. In the case of *Quercus robur* L., its most commonly known predator is *Operophtera brumata* L., the plant defends itself by the production of condensed tannins that target insect larvae growth whilst damaging the adult insect midguts (Van Asch and Visser, 2007).

#### 2.1.3.2. Protection against abiotic factors

Abiotic factors may introduce a plethora of negative impacts upon plant species (Wagner, 2004; Bjorkman et al., 2008). Periods of extreme temperatures, limited water supply and overexposure are some environmental stresses that could prove to be fatal to plants (Karabourniotis et al., 1995; Zandalinas et al., 2018). As a result of evolution, many species have adapted to develop structures that aid in decreasing the impact of the above-mentioned stresses (Fahn, 1988).

Non- glandular trichomes aid in reducing transpiration rates by reflecting light off foliar surfaces thus reducing overall water loss (Anandan et al., 2018). Larcher (2000) has reported the presence of

an indumentum of non-glandular trichomes across the foliar surface of *Oleae europeae* subsp. Cuspidate serving a similar purpose. This process also assists in reducing foliar temperature whilst overall environmental temperature increases. In addition, a dense coverage of trichomes results in the formation of a condensed system between the leaf surface and the aerial appendages, allowing for limited water evaporation and increased humidity levels (Larcher, 2000).

González-Villagra et al. (2018) highlighted the impact of glandular secreting trichomes have in the reduction of water loss. They suggested that exudate in the form of flavonoids, triterpenoids and waxes decelerate water loss. Crisp (1963) found a correlation between trichome density and effective prevention of water loss from a plant. This research indicated that trichomes appeared to trap water droplets, thus keeping foliar surfaces moist and hydrated. John and Hasenstein (2017) further mentioned that the occurrence of trichomes decrease water diffusion rates via the stomata. Bjorkman et al. (2008) explored trichome density and coverage across different environmental conditions. They found that shaded mesic environments accounted for decreased trichome production and coverage whereas those plants which are fully exposed to sunlight and are found in semi-arid environments, are rich in trichomes.

UV-B radiation is known to damage plant DNA thus negatively affecting plant growth and development (Skalta et al., 1994). Phenolic compounds absorb incoming UV -B rays and protect underlying cellular constituents (Wei et al., 2018; Oksanen, 2018). Barnes et al. (1996) investigated plant defensive strategies in *Arabidopsis thaliana* against UV-B radiation. They concluded that trichome production increased upon exposure to UV-B radiation, as trichomes were shown to absorb incoming rays of radiation.

In addition, domatia is characteristic to certain members of *Combretum* (Jongkind, 2018). Domatia describes the formation of hair-like chambers which develops on the lower mature leaf surface of numerous plant species (Izzo et al., 2018). Romero and Benson (2005) suggest that the presence of domatia in plants serves as a means of mechanical protection against predators by providing a breeding site for smaller insects such as mites. Recent studies have indicated that domatia has proved to be beneficial to not only the resident arthropod but also to the host plant itself. Resident mites appear to reduce overall herbivory attacks on the plants by deterring or eating herbivory pests and pathogens (Weber et al., 2016). Domatia provide protection against fungal pathogens and phytophagous arthropods that occur on the leaf surface yet could cause leaf necrosis and yellowing of the leaf (Levin, 1973; Pena and Bullock, 1994). Many species of the *Combretum* genus are known to have these structures which may prove fruitful or detrimental to the species (Jongkind, 2018).

#### 2.1.3.3. Pollination

Although trichomes have a crucial role in plant defence and protection, they do also serve an additional role of aiding in pollination via the attraction of known pollinators (Stpiczyńska et al., 2018). Fahn (1998), suggested that trichomes are densely spread across reproductive and vegetative parts of foliage, each trichome may be structurally and functionally different. Giuliani et al. (2018) investigated the role that trichomes and secondary metabolites play in aiding pollination within the Lamiaceae family. The emanating micromorphological analyse of *Salvia verticillate* indicated that the glandular hairs formed a dense indumentum on foliage surfaces (Giuliani et al., 2018). These hairs aided in the synthesis of terpenes thus allowing for the release of volatile organic compounds and essential oils. The presence of essential oils attracts common pollinators, such as *Apis mellifera*, allowing for successful pollination (Giuliani et al., 2018). A study conducted by Van der Meer et al. (1992) indicated that the presence of flavonoids within plant species aid in pollination by promoting the production of yellow pollen which has a large light spectrum visibility. The yellow colour found in numerous species is possibly present due to flavonoids as these pigments maybe responsible for colour compilations (Van der Meer et al., 1992).

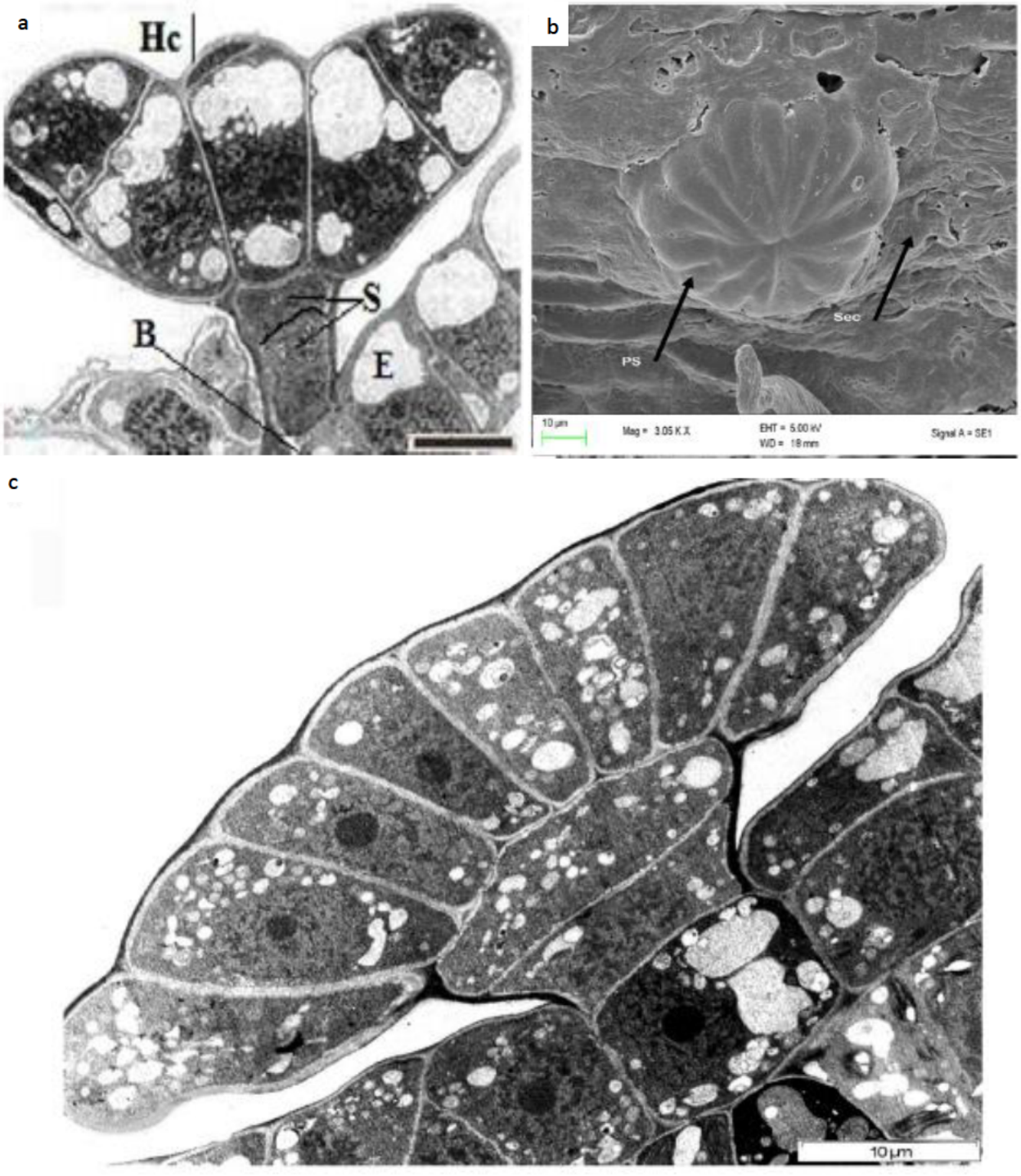



Figure 2.3. (a) TEM micrograph of a sunken scale of *C. molle* (Naidoo et al. 2012a), (b) SEM micrograph of the abaxial surface of the mature leaf from *C. erythrophyllum* indicating a mature peltate scale, (c) TEM micrograph of the peltate scale found upon *C. erythrophyllum*, longitudinal section of umbrella like scale (Bantho et al. 2020). Hc= Head cell, B= Basal cell, S= stalk, E= epidermal cell, PS = Peltate scale, Sec = Secretion and C = Crystal.

Table 2.1: Trichomes found in selected species from *Combretum*.

| Species: | Trichome(s) found: | Reference: |
|---|---|---|
| *C. collinum* | Glandular trichome with a multicellular head and uniseriate stalk<br>Glandular trichome with a unicellular head and uniseriate stalk<br>Cylindrical uniseriate trichome | Ekeke and Agbagwa, 2017 |
| *C. cuspitatum,* | Glandular conical trichome<br>Cylindrical uniseriate clavate trichome | Ekeke and Agbagwa, 2017 |
| *C. hispidum,* | Glandular conical trichome<br>Cylindrical uniseriate clavate trichome<br>Cylindrical uniseriate trichome<br>Glandular trichome with a multicellular head and uniseriate stalk | Ekeke and Agbagwa, 2017 |
| *C. paniculatum* | Glandular conical trichome<br>Cylindrical uniseriate clavate trichome | Ekeke and Agbagwa, 2017 |
| *C. platypterum* | Glandular conical trichome<br>Cylindrical uniseriate clavate trichome<br>Glandular trichome with a multicellular head and uniseriate stalk | Ekeke and Agbagwa, 2017 |
| *C. erythrophyllum* | Non-glandular trichomes<br>Peltate scales | Bantho et al., 2020 |
| *C. molle* | Non-glandular trichomes<br>Peltate scales | Naidoo et al., 2012a |
| *C. paniculatum* | Glandular conical trichome<br>Cylindrical uniseriate clavate trichome | Ekeke and Agbagwa, 2017 |
| *C. platypterum* | Glandular conical trichome<br>Glandular trichome with a multicellular head and uniseriate stalk<br>Cylindrical uniseriate clavate trichome | Ekeke and Agbagwa, 2017 |
| *C. racemosum* | Glandular conical trichome<br>Glandular trichome with a multicellular head and uniseriate stalk | Ekeke and Agbagwa, 2017 |
| *C. zenkeri* | Glandular conical trichome<br>Cylindrical uniseriate clavate trichome | Ekeke and Agbagwa, 2017 |

## 2.2. Taxonomy: *Combretum*

Kingdom: Plantae
Phylum: Tracheophyta
Class: Magnolipsida
Order: Mrytales
Family: Combretaceae
Genus: *Combretum*
Species: *Combretum erythrophyllum*

The order (Myrtales) consists of 14 families, 380 genera and an estimated 12000 species (Schönenberger and Conti, 2003). A noticeable trait of this order is the presence of two cotyledons and tricolpate pollen grains which further characterises Myrtales as a eudicot (Lucas et al., 2007). In addition, encapsulation of the xylem vessels with phloem tissue is a distinguished trait. Combretaceae stands out as one of the most utilized medicinal plant family within the order (Moura et al., 2018). As per Stace (1969), Combretaceae forms part of the first major linage of the order and consists of approximately 20 genera and 600 species (Moura et al., 2018). This family is mainly comprised of deciduous trees or shrubs that are prominent in north-eastern parts of Africa (McGaw et al., 2001). The medicinal use of plant species belonging to the family Combretaceae, is of high interest on the African continent (Mawoza and Ndove 2015). A combined molecule analysis (combined plastid and nuclear transcribed spacer data) was conducted by Maurin et al. (2010) to analyse the phylogenetic relationships of members of Combretaceae. The resulting analysis produced 184 trees. Figure 2.4 indicates the most parsimonious tree representing a monophyletic relationship (species which share a common ancestor) within *Combreteae* tribe (96/1.0). Three main subgenera were identified, subgenera *Combretum*, subgenera *Cacoucia* (Aubl .) Exell &Stace and subgenera *Apetalanthum* Exell & Stace (Stace, 1981). Within group 1, section Angustimaginata Engl. And Diels (100/ 1.0) was noted (Fig. 2.4a). *Combretum erythrophyllum* is represented within the *Combretum* subgenus which is part of the Angustimaginata section. The clade comprising of *C. erythrophyllum*, *Combretum caffrum* (Eckl. & Zeyh.) Kuntze and *Combretum nelsonii* Dümmer are sister clades to the entire parsimonious tree created from the acquired data (Fig. 2.4a).

*Combretum* is the largest genus within the family Combretaceae, comprising over 370 species (Mtunzi et al., 2017). This genus is being extensively studied for its use in pharmacology (Rogers and Verotta, 1996; Ujowundu et al., 2017; Ogbole et al., 2018). Noticeable characteristics of the genus include opposite leaf growth in a scattered arrangement, presence of domatia within mature leaves, the absence of stipules and inflorescences which carry bisexual flowers. These flowers may be characterised as petal formations in 4 or 5 arrangements (Stace, 1965). Specifically, Combretaceae

is characterized by secretory hairs that can be either glandular or scales (Lima et al., 2012; Bantho and Naidoo, 2018). These scales are known to be embedded within the gelatinous like exudate, which may comprise of phytochemicals. *Combretum erythrophyllum* is a species known to contain medicinally important phytochemicals (Martini et al., 2004; Mawoza and Ndove, 2015; Mtunzi et al., 2017).

*Combretum erythrophyllum*, also known as the river Bushwillow, is a deciduous, indigenous tree that thrives in areas with good groundwater level (Gelfand et al., 1985). This tree is characterised by having dense foliage ranging from 2 cm - 11 cm in length, younger leaves which are lighter green in colour, mature leaves which are firmer, slightly darker green in colour and a flaking brown bark. In addition, the flowers bloom in spring and are light yellow in colour. In autumn the leaves tend to turn red in colour.

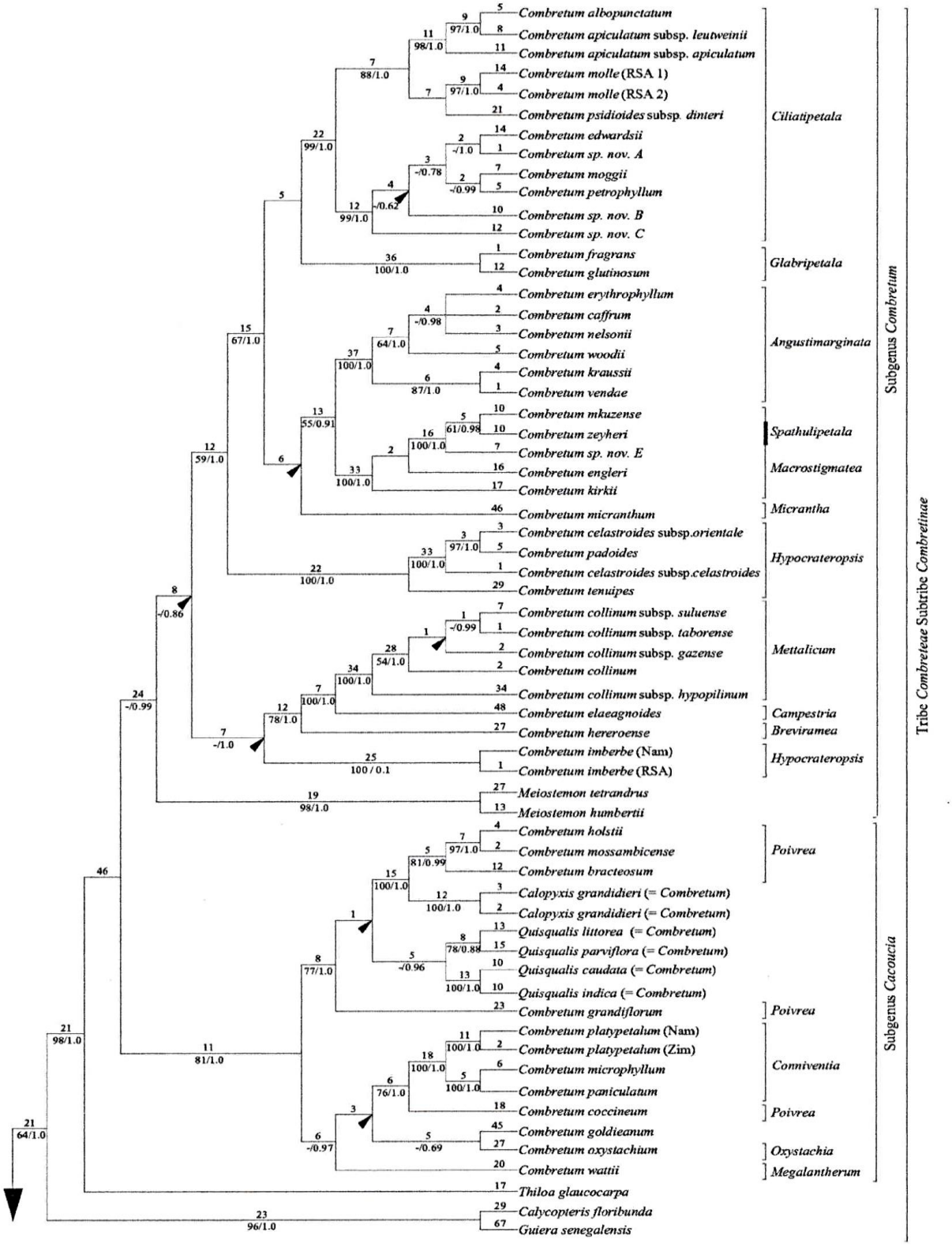



Figure 2.4a: Parsimonious tree created from the acquired combined plastid and nuclear transcribed spacer data, indicating the sections and subgenera classification of species of the *Combreteae* tribe (Maurin et al., 2010)

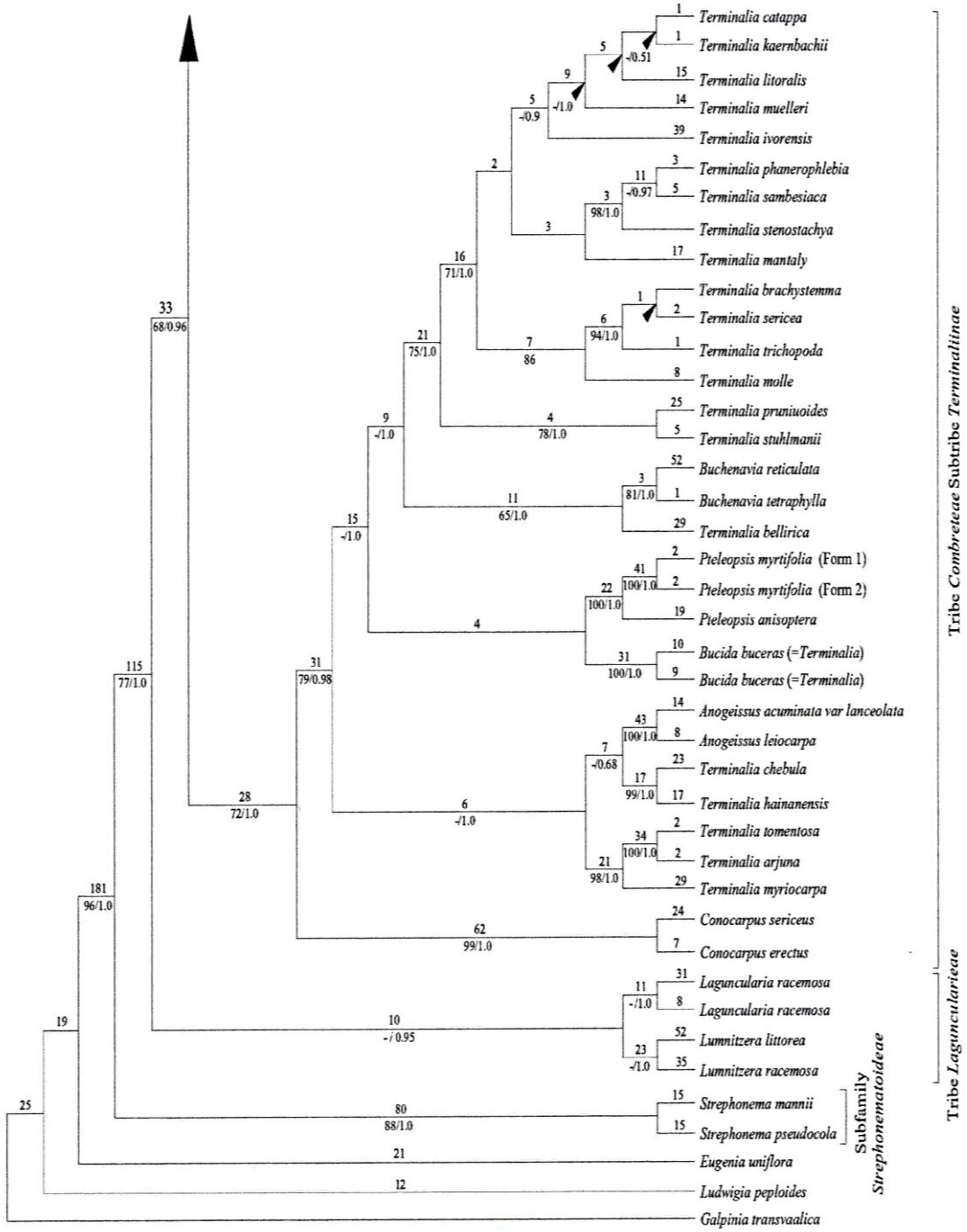



Figure 2.4b (continuation): Parsimonious tree created from the acquired combined plastid and nuclear transcribed spacer data indicating the sections and subgenera classification of species of the *Combreteae* tribe (Maurin et al., 2010)

## 2.3. Ethnopharmacological applications, biological activity and bioactive compounds found within *Combretum* species.

Medicinal properties of plants are attributed to the secondary metabolites found within. Secondary metabolites are classified into three main classes, namely alkaloids (nitrogen-containing compounds), terpenes and phenolic compounds (Gupta et al., 2018). The measure of the medicinal value of a plant is directly linked to its phytochemical constituents (Raks et al., 2018; Feng, et al., 2021). Fixed oils and fats, tannins, flavonoids, sterols and alkaloids were detected within *Combretum* species.

Alkaloids are structurally diverse compounds which consist of one or more heterocyclic nitrogen atom(s) and are key components in plants used for traditional medicine (Bribi, 2018; Raks et al., 2018). Research suggests that the therapeutic use of alkaloids can be dated back to the 19th century, where substances were isolated mainly for their narcotic and analgesic components (Shang et al., 2018). Pharmacological activities of alkaloids include their use as muscle relaxants, analgesics, antitussive agents, local anaesthetics as well as anti-cancer, anti-hypertensive and antiseptic agents (Bribi, 2018, Karabourniotis et al., 2019; Kong et al., 2021). Most commonly isolated alkaloids from plants include quinine, morphine, caffeine, strychnine and cinchonine (Roberts and Strack, 2018). *Combretum* species such as *C. molle, C. erythrophyllum, Combretum micrathum* G. Don., *Combretum lamprocarpum* Diels., *Combretum sokodense* Engl., *Combretum dolichopetalum* Engl. and Diels. and *Combretum racemosum* P. Beauv. tested positive for the presence of alkaloids in the crude extracts (Onocha et al., 2005; Ibrahim et al., 2017; Kizito et al., 2020; *Nwuke, 2020*). Novel alkaloids Combretine A, B, C and Betaine (1) were isolated from *C. micrathum* (Brossi, 1987).

Flavonoids are the largest phytometabolite group in the phenolic compound class (Karim et al., 2018). Thus far, over 9,000 flavonoids have been identified and isolated (Wang et al., 2018). Flavonoids are known to exhibit anti-inflammatory, antioxidant, anti-allergic, antiviral and low toxicity properties (Lesjak et al., 2018; Nile et al., 2018). Structurally, flavonoids can occur in three types, namely glycosides, aglycones and methylated forms (Wang et al., 2018; Yang et al., 2018). Thus far, flavonoids were isolated from *Combretum erythrophyllum, Combretum apiculatum* and *Combretum leprosum* (Rogers and Verotta, 1996; Martini et al., 2004; Sousa et al., 2021). These flavonoids are known to encompass high antimicrobial properties. In particular, two phenanthrenes and a dihydrostilbene, isolated from *C. apiculatum,* showed total inhibition of *Penicillium expansum* (Grayer and Harborne, 1994).

Combretastatin is a phenol which may be present within members of the *Combretum.* Pettit et al. (1982) first isolated combretastatin from a common bushwillow tree known as *Combretum caffrum* and analysed the benefits of this molecule in reducing cancerous effects. This molecule is shown to inhibit blood flow to cancerous tumours, eventually destroying it by damaging the endothelial cells,

both mature and new. Although the results proved promising, the simultaneous use of conventional methods were also advised (Pettit et al., 1982).

*Combretum molle* is said to have a wide array of medical properties, including antifungal and molluscicidal effects as well as in combating bacterial infections such as gonorrhoea and syphilis (Fyhrquist et al., 2002). Elucidating research has indicated that the secretory structures of *C. molle* is composed of multiple secondary metabolites such as terpenoids, phenolics, flavonoids and alkaloids. They are proposed to provide antimicrobial and antiherbivore protection (Bhatnagar et al., 2012; Naidoo et al., 2012a). *Combretum racemosum* is an indigenous shrub found in tropical and pan tropical regions on the African continent and is extensively used in traditional medicine (Kamou et al., 2017). Onocha et al. (2005) conducted a preliminary phytochemical analysis, as a result saponins, alkaloids and tannins were found. Furthermore, this species is commonly utilized in traditional medicine for its antimicrobial, antifungal activity as well as in treating coughs, tooth aches and haemorrhoids which suggests that this species could have additional unexplored medicinal value.

Benoit (1996) isolated metabolites from *Combretum micranthum* G. Don. These metabolites showed total antimalarial inhibition against antibiotic resistant strains of *Plasmodium falciparum*. *Combretum glutinosum* is known to treat dysentery, typhoid fever and antipyretics, amongst many ailments. These species are known to thrive in tropical regions of the African continent, hence its popularity amongst native traditional healers (Nwaeze and Abarikwu, 2006). Through the phytochemical analysis, tannins, triterpenoids, alkaloids, flavonoids and glucosides were identified. Nascimento et al. (2000) highlighted the use of integrating natural products derived from this species, with commercially produced medicine. Commercial medications for the treatment of snake bites, malaria and blood pressure are currently being developed using products of *C. glutinosum* as a key component.

Asres (2001) isolated phytometabolites from *C. paniculatum*. These phytometabolites showed promising results against HIV-1 and HIV-2 replication. *Combretum roxburghii*, an Indian native species, is shown to have noteworthy medicinal properties. Interestingly, seasonal change was seen to have a direct effect on the compounds exudated by this species. Samples obtained and tested during February and October were high in antioxidant content compared to those of May and August (Bhatnagar et al., 2012). Histochemical research shows that the secretory scales of *C. roxburghii* contain of saponins, flavonoids and tannins (Bhatnagar et al., 2012). The extracted flavonoids are said to have antioxidant, anti-estrogenic and anti-proliferative properties (Gretchen et al., 2008). Martini et al. (2004) identified the presence of these beneficial flavonoids in *C. erythrophyllum* as well.

*C. erythrophyllum* is known as the river Bushwillow and is well acquainted to the southern African community due to its high medicinal value. This species is used to treat venereal disease, abdominal

pain and sores, among others (Mawoza and Ndove, 2015). It is a deciduous, indigenous tree that thrives in areas with good groundwater level as it is a riverine species (Jordaan et al., 2011). Martini et al. (2004) has identified seven different flavonoids present in *C. erythrophyllum* which are believed to be responsible for their antibacterial effect. Phenols are known to have numerous health benefits, mainly their antioxidant and anti- microbial activities.

### 2.3.1 Pharmacological activity:

Microbial pathogens are continuously evolving, hence, advanced remedial methods need to be introduced (Lee et al., 2018). Scientists are now turning towards medicinal plant research to curb microbial pathogen growth. Continued research and analysis of current pathogens are crucial in order to develop efficient, naturally derived plant drug delivery methods (Mtunzi et al., 2017). Hence, the pharmacological use of medicinal plant species has become of prime interest (Kemper et al., 2008; Willis, 2017; Winkleman, 2018). The presence of phenolic compounds, flavonoids, glycosides, alkaloids and saponins has been associated with the antimicrobial properties derived from certain plants (Thorat, 2018, Nwuke, 2020). The medicinal use of plant species from the family Combretaceae is of high interest on the African continent (Mawoza and Ndove, 2015; Bantho, et al., 2020). Pharmacological studies have shown that species of *Combretum* have antibacterial, anti-inflammatory, antifungal, genitourinary, cytotoxic and mutagenic properties (Fyhrquist et al., 2002; Mawoza and Ndove, 2015; Mtunzi, et al., 2017) (Table 2.2 and 2.3).

#### 2.3.1.1. Antibacterial activity

Williamson et al. (2000) mentioned that *Combretum* species are characterized by high flavonoid and phenolic compound levels, denoting to their potential antibacterial properties. A recent study conducted by Mtunzi et al. (2017) indicated the prominent antibacterial effects of *C. erythrophyllum* leaf extract against multiple bacterial strains (*Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis* etc.). Species such as *Combretum apiculatum, Combretum albidum, Combretum leprosum, Combretum dolichopetalum and Combretum album* have also been noted for their high antioxidant and antimicrobial properties (Katerere et al., 2012; Chandar and Ramasamy, 2016; Burman et al., 2018; McGaw et al., 2001; Nwuke, 2020; Sousa et al., 2021). *Combretum sokodense* leaf, stembark and root extracts also showed inhibitory activities against *S. aureus* and *E. coli.* Results obtained from both leaf and stem extracts of *C. erythrophyllum* showed that this species had antibacterial activity against Methicillin-resistant *Streptococcus aureus* (MRSA). MRSA is one such bacterium known to be resistant towards current antibiotic treatments (Davies, 1994; Holmes and Howden, 2014).

### 2.3.1.2 Antiviral activity

Antiviral drug development is a tedious process due to the rapid incline of resistance noticed against viral variants of existing drugs and their low selective toxicity (Kilonzo et al., 2021). As a result, natural products, derived from medicinal plant species, are being evaluated for its antiviral activity as they are known to contain heterogeneous polysaccharides of high molecular weight (Aslam et al., 2021; Ray et al., 2021). These polysaccharides are known to effectively break the replication cycle of the viruses with minimal side effects. Naturally derived antiviral drugs have been used to effectively treat the following viruses: herpes simplex virus types 1 and 2, cytomegalovirus, influenza A virus, respiratory syncytial virus, human immunodeficiency virus type 1,2 (HIV-1,2), papilloma viruses and hepatitis B and C (Musarra-Pizzo et al., 2021; Vougogiannopoulou et al., 2021). *Combretum* species such as: *C. molle, C. quadrangulare, C. micranthum, C. glutinosum* and *C. paniculatum* have been evaluated for their antiviral potential. Particularly, *C. paniculatum* and *C. micranthum* have shown high antiviral activity against HIV-2 and Herpes simplex 1 and 2, respectively (Ferrea et al., 1993, Asres et al., 2001).

### 2.3.1.3. Antifungal activity

Research conducted by Masoko et al. (2007) showed that acetone-based leaf extractions of *C. erythrophyllum* yield higher antifungal rates against *Candida albicans, Cryptococcus neoformans, Sporothrix schenckii, Aspergillus fumigatus* and *Microsporum canis* in comparison to methanol, hexane and dichloromethane-based extractions. High antifungal activity is denoted to the presence of acidic triterpenoids and glycosides (Rogers and Verotta, 1996, Mawoza and Ndove, 2015). Masoko et al. (2007) evaluated the antifungal activity of *C. loefl*. This species was seen to have high antifungal activity which was attributed to the presence of tannins and saponins found in copiousness within *C. loefl*. Cholest-5-en-3-ol, 2-phyten-1-ol, Gallocatechin and Apigenin were isolated from *C. paniculatum*. These compounds were seen to have high antifungal activity against *Sporobolomyces salmonicolour* SBUG 549 and *Penicillium notatum* JP 36. Baba-Moussa et al. (1999) related the increased presence of tannins and saponins to intensified antifungal activity within the species from *Combretum*. Recently, species such as *C. erythrophyllum, C. padoides, C. zeyheri, C. psidioides* and *C. molle*, have been duly noted for their antifungal activity (Seepe et al., 2021; Fyhrquist et al., 2020; Rademan and Lall, 2020).

### 2.3.1.4. Antioxidant potential of plant extracts

Free radicals are naturally occurring by-products generated in the body as a result of the use of oxygen (Diniyah, et al., 2020). Environmental factors (Cigarette smoke, pesticides, car fumes etc.) may also lead to the accumulation of these radicals, posing extreme harm to the host (Eddaikra and Eddaikra, 2021; Mathaiyan et al., 2021). Accumulation of these radicals leads to oxidative stress

within the body, resulting in the distortion of cellular membranes and disintegration of the cellular components within (Dutta, 2021). This, in turn, leads to cell death (tissue damage) which could result in cancer, diabetes or atherosclerosis. To restore the balance between free radicals and antioxidant defence mechanisms within the body, naturally derived antioxidant drugs are favoured (Dinesh, 2021). These natural products (secondary metabolites such as phenolics, flavonoids, stilbenes, tannins and alkaloids) are known to display high levels of antioxidant activity. These products are said to have high levels of free radical scavenging molecules which work in restoring the homeostasis nature of the body, with minimal side effects. Research has shown that methanol-based extracts of species such as *C. woodii, C. collinum* and *C. hereroense* have displayed higher antioxidant activity in comparison to hexane- based extractions (Photolo et al., 2020). Phenolic compounds are more readily dispersed from plant material when extracted using methanol as the solvent of choice. This is due to the polarity of methanol, denoting to the increased antioxidant activity of methanol-based extracts. Of recent, species such as *C. leprosum, C. album, C. micranthum, C. albidum* and *C. indicum*, have been duly noted for their antioxidant activity (Sousa et al., 2021; Burman and Chandra, 2021; Mashi et al., 2021; Sharma et al., 2021; Forid et al., 2021).

### 2.3.1.5 Cytotoxity potential of plant extracts

Globally, the importance of incorporating plant-based medicine for the treatment of cancer has been highlighted as conventional treatments are proving less effective, with numerous side-effects (Pant, et al., 2021). Researchers are consistently identifying/ extracting natural products with known extensive cytotoxic activity as an alternative to treat/ cure this ailment. Many *Combretum* species are known for their profound cytotoxic activity. Species such as *C. fruticosum, C. erythrophyllum, C.quadrangulare, C. fragrans, C. leprosum, C. apiculatum, C. paniculatum* and *C. woodii* etc. have been extensively researched for their potential cytotoxic activity (Wende et al., 2021; Sousa et al., 2021; Nguyen et al., 2021; Gade et al., 2021; Maphutha et al., 2021).

Emanating research conducted by Silva-Filho et al. (2020), evaluated the cytotoxic activity of 3β,6β,16β-trihydroxylup-20(29)-ene, a triterpene isolated *from C. leprosum*. This isolate was seen to express high cytotoxic activity. Furthermore, the research intended to evaluate the impact nanoparticle encapsulation would have on the cytotoxic efficiency of the isolate. It was noted that nanoparticle encapsulation significantly increased the selectivity of the triterpene isolate in comparison to the tested cells.

Of recent, Morris et al. (2021) evaluated the integrated use of conventional cancer drugs with compounds isolated from *C. zeyheri* and C. *platypetalum* against Jurkat T Cells and HL-60 Cells. As a result, the above-mentioned combination inhibited the growth of these cells significantly.

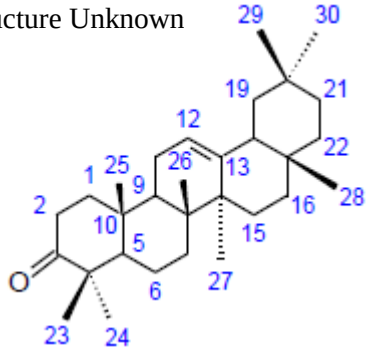
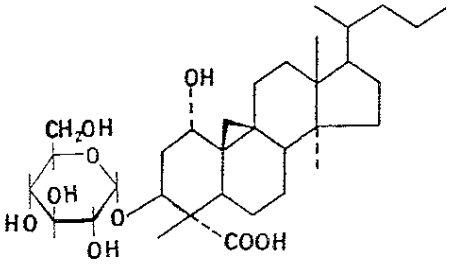
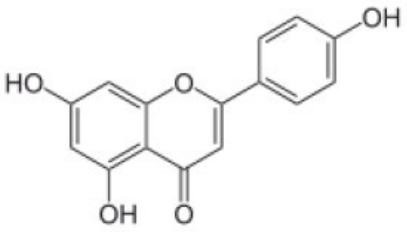
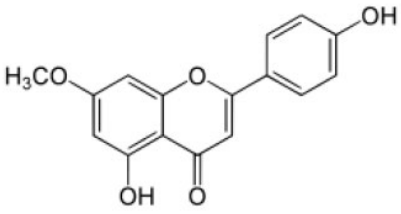
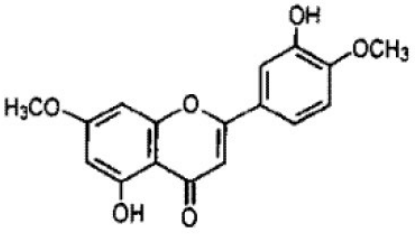
The integrative effect of the conventional drugs with compounds isolated from medicinal plants is a novel approach which yields promising results. This avenue certainly needs to be explored further.

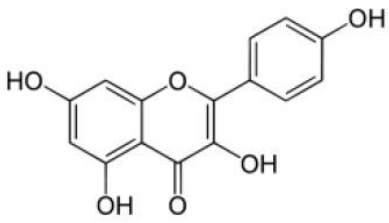
Table 2.2: Ethnopharmacological applications and biological activity of selected species from *Combretum* genus.

| Species Name: | Ethnopharmacological applications/ Biological activity | References: |
|---|---|---|
| *C. album.* | Malaria, fever | Burman et al., 2018 |
| | Antioxidant activity, cytotoxic activity | Lima, et al., 2012 |
| *C. apiculatum* | Conjunctivitis, leprosy, abdominal pain, snake bite | |
| | Inhibition of topoisomerase, anthelmintic, Antischistosomal, | Rogers and Verotta, 1996 |
| | Antibacterial, antifungal, anti-inflammatory activity, cytotoxic | Lima, et al., 2012 |
| | Activity | |
| *C. collinum* | Diuretic, purgative, gastro-intestinal problems | Songca et al., 2013 |
| | Antiparasitic, antibacterial activity, cytotoxic activity, anti-inflammatory | Lima, et al., 2012 |
| *C. edwardsii* | Anti-inflammatory, antioxidant, antiparasitic, antibacterial activity, cytotoxic activity | McGaw et al., 2001 Chukwujekwu and van Staden, 2016 Lima, et al., 2012 |
| *C. erythrophyllum* | Abdominal pain, wounds, venereal diseases | McGaw et al., 2001 |
| | Inhibition of topoisomerase, antibacterial activity, Geniturinary activity, spasmolytic activity, | Mawoza and Ndove, 2015 |
| | Inhibition of topoisomerase, anti-inflammatory activity, | Lima, et al., 2012 |
| | Anti-parasitic activity, cytotoxic activity, Mutagenic activity | |
| *C. fruticosum* | Bacterial infections | Braga et al., 2000 |
| | Inhibition of ACE, cytotoxic activity | Moura et al., 2018 Lima, et al., 2012 |
| *C. hartmannianum* | Antipyretic, diuretic, yellow fever, hepatic disorder, tuberculosis | Eldeen and Van Staden, 2007 |
| | Inhibition of acetylcholinesterase, antibacterial activity, Anti- | Maydell, 1990 Marid et al., 2006 |
| | parasitic activity, cytotoxic activity | Lima, et al., 2012 |

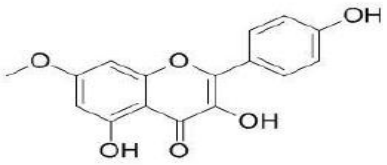
| | | |
|---|---|---|
| *C. hereroense* | Colds, Wounds, venereal diseases, dysentery, Infertility | Moura et al., 2018 |
| | Antibacterial activity, anti-parasitic activity, Hypoglycemic activity | Lima, et al., 2012 |
| *C. imberbe* | Diarrhoea, bilharziasis, Stomach aches | Venter and Venter, 1996 |
| | Antiparasitic, antibacterial activity, cytotoxic activity | Lima, et al., 2012 |
| *C. kraussii* . | Labour related ailments | Brookes et al., 1999 |
| | Antiparasitic, antibacterial activity, cytotoxic activity | Lima, et al., 2012 |
| | | McGaw et al., 2001 |
| | | Naidoo, et al., 2012 |
| | | Asita, et al., 2018 |
| *C. molle* | Gonorrhoea, syphilis, diarrhoea, Hypertension | Atindehou, et al., 2004 |
| | Antiparasitic activity, antibacterial, antifungal, Anti-inflammatory, antiviral, molluscicidal | Gansané, et al., 2010 |
| | | McGaw, et al., 2001 |
| | | Lima, et al., 2012 |
| *C. paniculatum.* | Ringworm, wounds, diarrhoea, infections | |
| | Antiparasitic, antibacterial activity, cytotoxic activity, CNS activity | Lima, et al., 2012 |
| *C. quadrangulare* | Skin disease | |
| | Antipyretic, antidysenteric, anthelmintic agent | Park et al., 2003 |
| | Antiparasitic activity, antitrypanosomal, Antibacterial activity, | Lima, et al., 2012 |
| | Antiviral activity, antihepatotoxicity | |
| *C. woodii* | Antiparasitic, antibacterial activity, cytotoxic activity, anti-inflammatory activity | Lima, et al., 2012 |
| *C. zeyheri* | Anaemia, abdominal ulcers, wounds, body pains, Infertility, | Moura, et al., 2018 |
| | Snakebite | Lima, et al., 2012 |
| | Antiparasitic activity, antibacterial | |

Table 2.3: Bioactive compounds isolated from selected species of the *Combretum* genus.

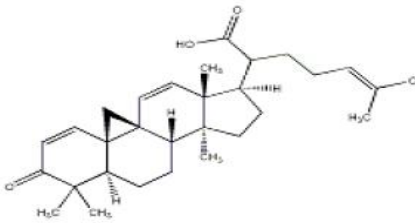
| Species: | Isolate: | Bioactivity: | Structure of compound: | Reference: |
|---|---|---|---|---|
| *C. collinum* | 1. olean-12-ene-3-one<br>2. Unknown | Antibacterial activity | 1. .<br>2. Structure Unknown  | Songca et al., 2013 |
| *C. edwardsii*<br>*C. molle* | 1. 1α-hydroxycycloartenoid mollic acid α-l-arabinoside | Hypoglycaemic and antidiabetic properties |  | (Rogers, 1989) Ojewole, and Adewole, 2008 |
| *C. erythrophyllum* | 1. apigenin,<br>2. genkwanin<br>3. 5-hydroxy-7,4'-dimethoxyflavone<br>4. kaempferol,<br>5. rhamnocitrin,<br>6. rhamnazin<br>7. quercetin-5,3'-dimethylether etc. | Cytotoxic activity molluscicidal, antifungal, anti-inflammatory activity |  **Apigenin**  **Genkwanin**  **5-hydroxy-7,4'-dimethoxyflavone** | Martini *et al*. (2004) Rogers (1998) (de Morais Lima *et al*., 2012) Mawoza and Ndove 2015 |



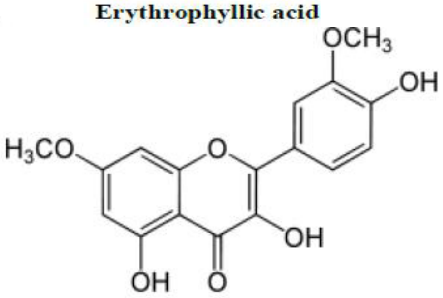

**Kaempferol**



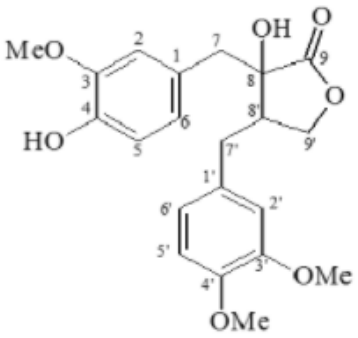
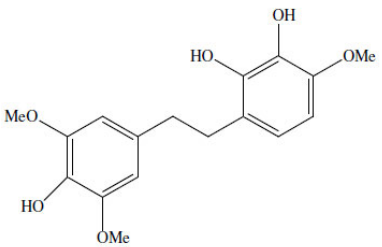

**Rhamnocitrin**

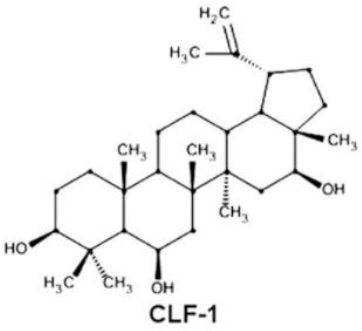
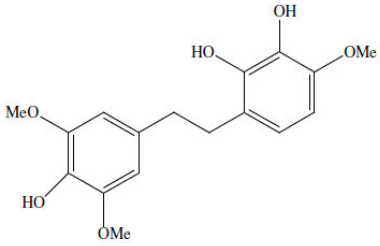
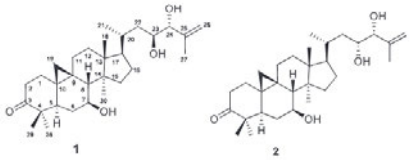



**Erythrophyllic acid**




**Rhamnazin**

| | | | | | |
|---|---|---|---|---|---|
| *C. fruticosum* | 1. | (−)-Trachelogenin | Cytotoxic activity |  | Moura, et al., 2018 |
| *C. kraussii* | 1. | stilbene 2’,3’,4-trihydroxyl-3,5,4’-trimethoxybibenzyl (combretastatin B5) | Antibacterial activity |  | Eloff, e al., 2005 |

| | | | | | |
|---|---|---|---|---|---|
| *C. leprosum.* | 1. | 3β, 6β, 16β-trihydroxylup-20(29)-ene | treatment of skin lesions wounds Antibacterial |  CLF-1 | do Nascimento-Neto et al., 2015 Cruz et al., 2019 de Araújo, et al., 2021. |
| *C. woodiie* | 1. | stilbene 2',3',4-trihydroxyl-3,5,4'-trimethoxybibenzyl (combretastatin B5) | Antibacterial activity |  | Eloff, e al., 2005 |
| *C. quadrangular* | 1. | Two new cycloartanes, combretanones G and H (1and2) | Cytotoxic activity |  | Nguyen, et al., 2021 |

**2.4. Nanotechnology: Biological synthesis and the associated application**

To evaluate plant species for its true medicinal worth, a species specific micromorphological and histo-phytochemical analyses is crucial in determining the presence, localization and source of compounds of interest. Subsequently, techniques enabling the isolation, manipulation and industrial use of these compounds should be evaluated.

Nanotechnology is a flourishing avenue yet to be thoroughly explored in medicinal plant research (Demirdogen et al., 2018). The biological synthesis of nanoparticles, utilizing plant extracts and other biological organisms as capping agents, is deemed as an economic, environmentally friendly and spontaneous method (Chinnasamy et al., 2017).

Plant extracts are known to contain biomolecules that aid in the reduction of metal ions that are present in nanoparticles (Duhan, 2017). This reduction occurs during the biological synthesis process. Metal ions are reduced to a base metal via water soluble plant metabolites. Hence nanoparticles impregnated with plant extract are formulated (Mittal et al., 2013). Specifically, the generation of silver nanoparticles using plant extracts involves the use of plant extract, silver nitrate solution and a heat source (Fig. 2.5, Khatoon et al., 2017). The biosynthesized nanoparticles may be screened for its potential biological activity, using numerous bioassays such as antimicrobial, antioxidant and anti-inflammatory assays, among many (Hu et al., 2019). As per Hedaginal and Taranath (2017), a crude extract using the leaves of *Thunbergia alata* Bojer ex Sims. and distilled water was generated. A substantial measure of the crude extract was added to an aqueous silver nitrate solution and incubated at 40 -60 $^{0}$C (Hedaginal and Taranath, 2017). After 24 hours, a characteristic colour change indicated the generation of silver nanoparticles (Hedaginal and Taranath, 2017). This formulation was then exposed to several bacterial strains and showed zones of inhibition towards *Staphylococcus aureus, Bacillus subtills* and *Escherichia coli* (Hedaginal and Taranath, 2017).

Alexander (2009) emphasized the ancient use of silver for its antibacterial properties. Minimal exposure to silver may be lethal to microbial pathogens whilst causing no impact or side effect to humans (Khatoon et al., 2017). Silver nanoparticle (AgNPs) synthesis combined with plant extracts, provides a nontoxic, practical and safe dispense system and are used in antimicrobial, anticancer, antifungal and antiprotozoal treatment (Huang et al., 2007).

The chemistry, morphology and distribution of synthesised AgNPs open a plethora of applicational uses in diverse fields (Demirdogen et al., 2018). Possible applications include use in UV blocking textiles, treatment of wastewater, water disinfection and further extrapolating into the biomedical field with utilization in cell imaging, possible cancer therapy, pathogen detection, gene and drug delivery (Tran and Le, 2013). Due to the antibacterial efficiency of AgNPs their use in environmental remediation and biomedical industry is justified.


Figure 2.5: Process followed to synthesize and characterise nanoparticles generated using plant extracts (Khatoon et al., 2017)

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# CHAPTER 3: MICROMORPHOLOGY, ULTRASTRUCTURE AND HISTOCHEMISTRY.


## Abstract

Combretaceae is a large rivine family prevalent on the Africa continent. Traditional healers commonly utilize species of this family to treat or cure illnesses and diseases. Medicinal plants are known to contain secondary metabolites which may effectively improve an individual's state of health. In addition, medicinal properties are attributed to secondary metabolites exuded by specialized micro-secretory structures, such as trichomes, laticifers or glands, Therefore, this study investigated the micromorphology ultrastructure and histochemistry of the secretory structures found in the leaves (emergent, young and mature) and stems of *Combretum erythrophyllum.* After an extensive micromorphological analysis, two distinct trichome types were identified, namely, peltate scales and non- glandular trichomes. Morphologically, the peltate scales are comprised of a sunken basal cell, bicellular stalk and a multicellular head. Head cell count appeared to increase with leaf maturation and ranged from 8 to 19 cells per a head. Trichome number was seen to be fairly constant across leaf developmental stages, hence suggesting the leaf expansion theory. The granulocrine pathway was identified as a possible mode of secretion for *C. erythrophyllum* due to the extensive presence of vesicles, vacuoles and electron dense material within the peltate scales. Preliminary histochemical analysis revealed the presence of lipids, phenolic compounds, total proteins, alkaloids and essential oils within *C. erythrophyllum*. The conducted study indicated the predominant presence of alkaloids, phenolic compounds and essential oils within *C. erythrophyllum*. These secondary metabolites may serve a crucial ecological role in preventing herbivory attack and protecting leaf/ stem surfaces against potentially harmful abiotic factors. While, certain secondary metabolites are known to exhibit anti-oxidant and anti-inflammatory properties. In accordance, these beneficial compounds may be further developed for use in medicinal applications.


Keywords: *Combretum erythrophyllum*, Micromorphology, Peltate, Scales, Trichomes, Ultrastructure

## 3.1 Introduction

Combretaceae is a large angiosperm family comprising of approximately 20 genera and 600 species worldwide (Moura et al., 2018). This family is prominently found in Asia, Australia and Africa as these species thrive in both tropical and subtropical regions (Lima et al., 2012). *Combretum* is the largest and most documented genus of this family (Mtunzi et al., 2017). Species of *Combretum* are highly valued in Africa due to the plethora of traditional medicinal uses they may offer (Mawoza and Ndove, 2015). The leaves, stems and roots are most commonly utilised to treat ailments such as gonorrhoea, tuberculosis, ulcers, eczema, stomach pains, colds and skin infections (Chinsembu et al., 2015; Cock and Van Vuuren 2015; Moura et al., 2018). Pharmacological studies have shown this genus to have antibacterial, anti-inflammatory, anti-fungal, genitor-urinary, cytotoxic and mutagenic properties (Mawoza and Ndove, 2015). In addition, species of *Combretum*, are currently being extensively studied to evaluate their potential use in commercial medicine (Ujowundu et al., 2017; Ogbole et al., 2018).

*Combretum erythrophyllum*, commonly known as the river bushwillow or Umdubu (Zulu), is a species reported to contain secondary metabolites that are of medicinal importance (Martini et al., 2004; Beigi et al., 2018). This is a deciduous, indigenous tree that thrives in areas with good groundwater levels (Jordaan et al., 2011). Traditionally, the root and leaves are used to treat venereal diseases and abdominal pain, whilst the bark is used to treat leprosy and sores (Mawoza and Ndove, 2015). The above pertains to the use of this tree as a medicinal plant.

Medicinal plants are known to contain metabolites that may effectively improve an individual's state of health (Kaur et al., 2018). Synthesized primary metabolites are crucial to cell functioning, whilst secondary metabolites are plant specific and are produced as part of the plant defence system against pests and pathogens (Rao and Ravishankar, 2002; Pichersky and Raguso, 2018). Medicinal properties are attributed to secondary metabolites found in certain species (Svoboda and Svoboda, 2000; Beigi et al., 2018). These are exuded by specialized secretory structures, such as trichomes, laticifers or glands (Wittstock and Gershenzon, 2002; Chang et al., 2018).

Trichomes are aerial protrusions from the plant epidermis (Chang et al., 2018). These are said to be the sites of synthesis as well as the reservoir of accumulated biological substances (Naidoo et al., 2012a). Trichomes may be widely spread across both the vegetative and reproductive parts of the plant while varying in morphology, function and chemical composition (Wagner, 2004; Fahn, 1988; Chang et al., 2018).

Combretaceae is characterized by two types of secretory structures, glandular trichomes and peltate scales (Lima et al., 2012). The glandular trichomes are short and capitate whereas the scales are sub-sessile and peltate (Fahn, 1988; Tian et al., 2017). In relation to the traditional medicinal uses of *Combretum* species, literature has reported the presence of secondary metabolites such as: fixed oils and fats, tannins, sterols and alkaloids, that may substantiate the plants medicinal worth (McGaw et al., 2001; Ujowundu et al., 2017, Ogbole et al., 2018). According to Mawoza and Ndove (2015), a phytochemical analysis of *C. erythrophyllum* revealed the presence of polyphenols, flavonoids, triterpenoids and saponins within leaf extract.

Martini and Eloff (1998) had found that *C. erythrophyllum* contained at least 14 antibacterial compounds. More recently, Martini et al., (2004) has reported the presence of seven different flavonoids to possibly substantiate the anti- bacterial properties of this species. The presence of various phytochemical constituents within the leaves of *Combretum erythrophyllum* have been reported (Mawoza and Ndove, 2015). The presence and accumulation of phytocompounds are possibly attributed to the presence trichomes, yet there has been no studies conducted on the micromorphological properties of *C. erythrophyllum*. Therefore, this study aims to investigate the micromorphology, ultrastructure and histochemistry of the leaves and stems of *C. erythrophyllum* using light and electron microscopy.

## 3.2 Materials and methods

### Collection of plant material

Plant material (leaves and stems) of *Combretum erythrophyllum* was harvested from the University of Kwa-Zulu Natal, Westville campus, Durban, South Africa (‘29º49’S;30º59’E). Species was confirmed by comparison to the previously submitted voucher specimen (Hennessy, 464) at the herbarium, University of KwaZulu-Natal, Westville Campus. Leaves of three developmental stages: emergent, young, mature (Fig. 3.14), and stems were used for the purpose of this study. The emergent stage may be defined as the first fully formed leaves, ± 1 -2 cm in length, light green in colour. Young leaves are usually smaller, ± 5 -7 cm in length, lighter in colour and softer in texture whilst mature leaves are larger, ± 9-11 cm in length, slightly deeper in colour and tend to stiffen over time.

#### 3.2.1 **Stereomicroscopy**

In order to examine leaf and stem topology (surface structures), both the abaxial and adaxial surface of fresh leaf material were viewed with the AZ- LED ring furnished stereomicroscope. Images were taken using the Nikon AZ100 stereomicroscope. This presented a general overview of the distribution and types of trichomes present.

#### 3.2.2 **Electron microscopy**

##### 3.2.2.1. **Scanning electron microscopy**

Fresh leaves and young stem were pre- washed in a glass beaker with distilled water and a few drops of Bio-Rad tween- 20 solution. A Glutinous- like layer was seen to conceal surface structures hence biological material was pre-washed to remove this layer. After being thoroughly rinsed using distilled water, sections were excised (~ 0.5mm$^2$) and fixed using 2.5% Glutaraldehyde in 0.1M $PO_4$ buffer, pH 7.2. for 24 hours. Three 5- min buffer washes and post fixation in 0.5% osmium tetroxide (this allows for sample preservation and structural stability) for 2 hours followed. Due to the light sensitive nature of osmium tetroxide, the post fixation stage was carried out in a dark cupboard. In order to remove water from cell structures, samples were exposed to a graded series of alcohol dehydration, 25%, 50%, 70% and then 100%. Samples were left to dry in the Quorum K180 critical point dryer.

Dried samples were secured onto aluminium stubs using carbon conductive tape and sputter coated with gold, in the QurumQ150 RES gold coater. These samples were then viewed with the LEO 1450 SEM (SmartSEM). Multiple images, per surface, per development stage were analysed.

3.2.2.2 **Transmission electron microscopy (TEM)**

Sections were excised and fixed using 2.5% Glutaraldehyde in 0.1M $PO_4$ buffer, pH 7.2. for 24 hours. Three 5- min buffer washes and post fixation in 0.5% osmium tetroxide (this allows for sample preservation and structural stability) for 2 hours followed. Due to the light sensitive nature of osmium tetroxide, the post fixation stage was carried out in a dark cupboard. In order to remove water from cell structures, samples were exposed to a graded acetone series of 25%, 50%, 70% and then 100%. Samples then underwent filtration in 50% propylene oxide and resin for 24 hours. Polymerisation of samples in 100% resin in oven at 85$^{o}$C for 8 hours, followed. Resin blocks were then newly cut-glass knives (prepared using the LKB 7801A) and Leica EM UC7 Ultra microtome. Survey sections of 10µm thick were cut, stained for a minute using toluidine blue and viewed using the Nikon eclipse, 80i light microscopy.

In addition, ultrathin sections were cut and stained for 10 minutes using acetyl acetate and rinsed with warm water. Thereafter, stained for 10 minutes using lead citrate, rinsed and viewed using the transmission electron microscope Jeol 1010 (iTEM).

3.2.3 **Light microscopy**

3.2.3.1 **Histochemistry**

Fresh leaf sections, supported with dental wax, were cut using the Oxford vibratome sectioning system (100µm thin) and stained with various histochemical stains, whilst hand cut stem sections were used. Compounds and cellular structure visibility were enhanced (Demarco, 2017)

The appropriate controls were conducted, of which the results are not shown.

a) *Toluidine Blue- Metachromatic, general stain* (Demarco, 2017)

Sections were immersed in the stain for 1 minute. Excess stain was then washed away using distilled water.

b) *Sudan IV- Lipids (Demarco, 2017)*

Sections were submerged in the stain for 30 minutes. Thereafter samples were briefly rinsed with 80% ethanol. Excess stain was then washed away using distilled water.

c) *Sudan Black- Lipids and cutin/suberins* (Demarco, 2017)

Sections were immersed in the saturated stain for 20 minutes. Excess stain was blotted off, simultaneously, 70% ethanol was added to the sections to rinse off. Excess stain was then washed away using distilled water.

d) *Phloroglucinol- Lignin aldehydes* (Demarco, 2017)

Sections were mounted in a drop of saturated aqueous solution of Phloroglucinol in 20% HCL, thereafter viewed.

e) *Nile Blue- Acidic lipids and fatty acids* (Demarco, 2017)

Sections were placed in 1% Nile blue at 37oCfor 1 minute, followed by adding 1% acetic acid at 37ºC for 1 minute. These sections were then rinsed off with distilled water.

f) *Ferric trichloride- Phenolic compound*s (Demarco, 2017)

Sections were placed in an aqueous solution of ferric trichloride and sodium carbonate for 15 minutes. Sections were rinsed off with distilled water and viewed.

g) *Bromophenol blue- Total proteins (Demarco, 2017)*

Sections were immersed in an aqueous bromophenol blue solution for 15 minutes. Sections were then rinsed using 0.5% acetic acid for 20 minutes. Sections with then treated with a 0.1M sodium phosphate buffer for 3 minutes before being viewed.

h) *Wagner's and Dittmars reagent- Alkaloids* (Demarco, 2017)

Sections were initially stained using with Wagner's reagent for 10 minutes followed by 10 minutes using Dittmars reagent.

i) *Naphthol and N,N-dimethyl-p-phenylene diamine (NADI) Reagent- Essential oils* (Furr and Mahlberg, 1981)

Sections were incubated for 60 minutes with the NADI reagent, in the dark. Sections were then thoroughly rinsed with a 0.1M sodium phosphate buffer, for 2 minutes.

**Fluorescence microscopy**

j.) *Acridine orange- trichome viability* (Demarco, 2017)

Fresh cut sections were placed into the acridine orange solution and left for 10 minutes. Sections were thoroughly rinsed, mounted on a clean slide and viewed under UV light.

Stained material was mounted on clean glass slides and viewed using the Nikon compound and fluorescence microscope, Nikon eclipse, 80i and Nikon DS-Fi1 microscopy (NIS-Elements D).

## 3.3 Results and Discussion

**Micromorphology highlights**

Trichomes are said to be the site of synthesis as well as the reservoir of accumulated biological substances (Naidoo et al., 2012a; Kalicharan et al., 2018). Through the conducted micromorphological analysis of *Combretum erythrophyllum*, two types of trichomes were observed, namely: non- glandular unicellular trichomes and peltate scales. These secretory scales followed a general morphology including a broad sunken basal cell, 2 stalk cells and multicellular (8-19) cell head structure. Peltate scales are characteristic to the members of Combretaceae (Stace, 2007; Ekeke and Agbagwa, 2017). Literature has suggested the presence of this type of trichome in species such as: *Combretum acutum* Laws., *Combretum bauchiens* Hutch. and Dalz., *Combretum fuscum* Planch. ex Benth., *Combretum ghasalense* Engl. and Diels, *Combretum glutinosum* Perr. Ex DC., *Combretum micranthum* G. Don and *Combretum molle*, which are all native to Africa (Ekeke and Agbagwa, 2017). Although they follow the same general morphology, these scales may differ in cellular and chemical composition (Fahn, 1988).

**Stereomicroscopy:**

A trichome distribution pattern was seen across leaf samples of different developmental stages (Fig. 3.1 and 3.2). Trichomes were observed in higher concentrations along the well-developed, mid and lateral vein regions on the abaxial and adaxial surface. The configuration of trichomes along the mid and lateral veins could suggest a possible pathway for raw materials to travel to the head of glandular trichomes (Naidoo et al., 2012a; Naidoo et al., 2012b). Well-developed regions are dense in epidermal cells and have thick cell walls. In addition, the stem was seen to be densely covered in non- glandular trichomes (Fahn, 1988). Emergent leaves (Fig. 1a and b) were seen to be densely covered with non- glandular trichomes, found mainly along the mid and lateral vein regions. In younger leaves (Fig. 3.1), the abaxial surface (Fig. 3.1a) appeared to be richer in non- glandular trichomes in comparison to adaxial surface (Fig. 3.2b). Minimal non- glandular trichomes were observed on both the abaxial and adaxial surfaces of mature leaves (Fig. 3.2c) and d), while the visualization of peltate scale structures and stomata were noted. Scales appeared to be colourless, while those in the post secretory phase appeared to be brown in colour.

Domatia was present on the abaxial surface of mature leaves, within these hair-tuffs, arthropods and their eggs were visualized (Fig. 3.2d). Domatia, is the term given to the small hollow chambers that forms on the abaxial surface of mature leaves of numerous plant species (Walter, 1996; Jongkind, 2018). Research has shown that the main function of domatia is to serve as a means of protection against predators by providing a breeding site for smaller insects such as mites (Romero and Benson, 2005). Broad mites were commonly seen roaming mature leaf surfaces and the presence of their eggs

prove the above statement. Numerous authors have authenticated the benefits associated to the presence of these hair tuffs on foliage. Domatia may also display a mutualistic relationship between the resident arthropod and the host plant itself by providing protection against fungal pathogens and phytophagous arthropods that occur on the leaf surface. However, this relationship may result in leaf necrosis and yellowing of the leaf (Levin, 1973; Pena and Bullock 1994; Lucas et al., 2018; Prior and Palmer, 2018; Keller et al., 2018).

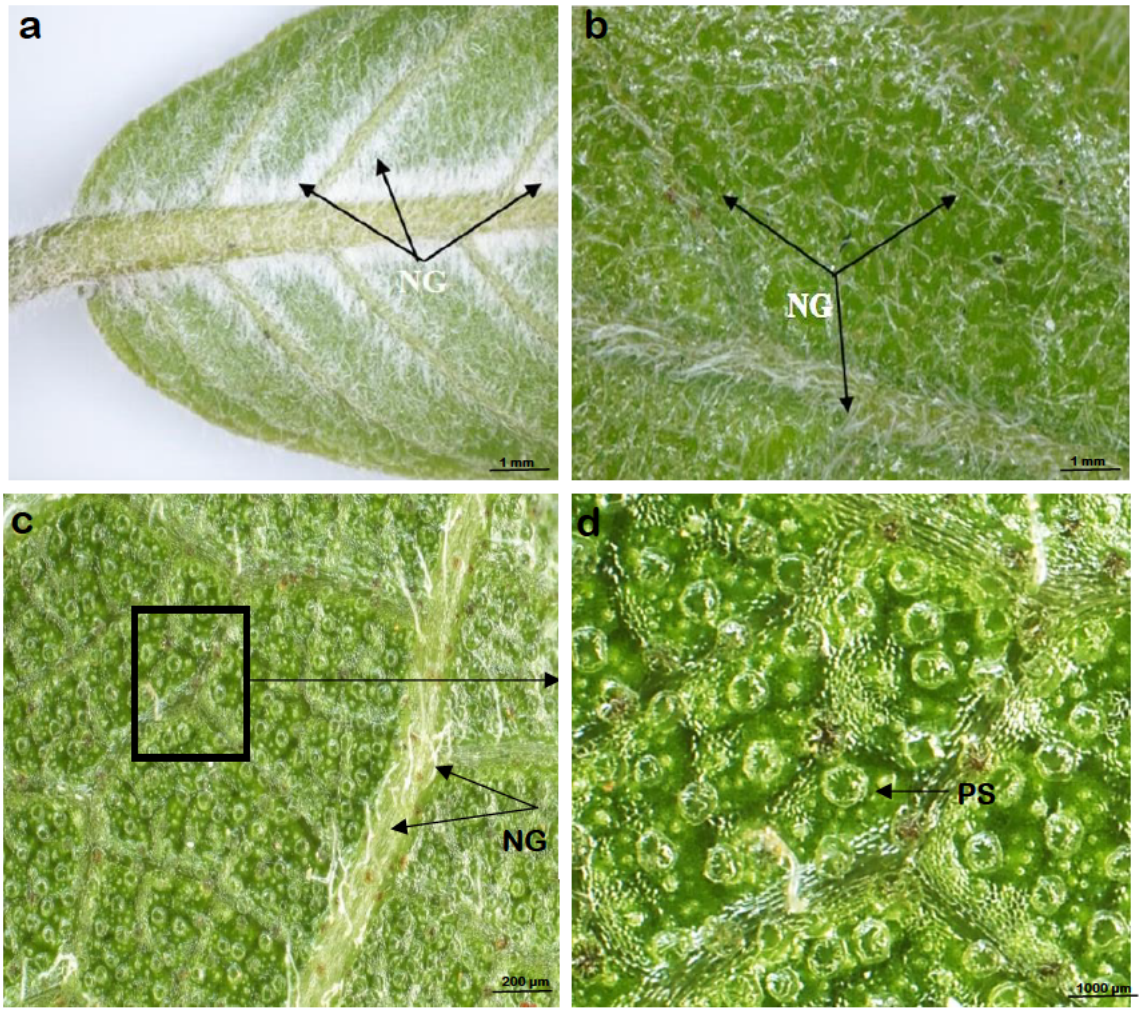


Figure 3.1: Stereographs depicting the leaf topology of the a) abaxial surface, b) adaxial surface of the emergent leaf with dense non-glandular trichome coverage along the mid and lateral veins, c) and d) abaxial surface of the young leaf, indicating peltate scales with a secretion around the structures, of *C. erythrophyllum* NG= Non- glandular and PS= Peltate scales.

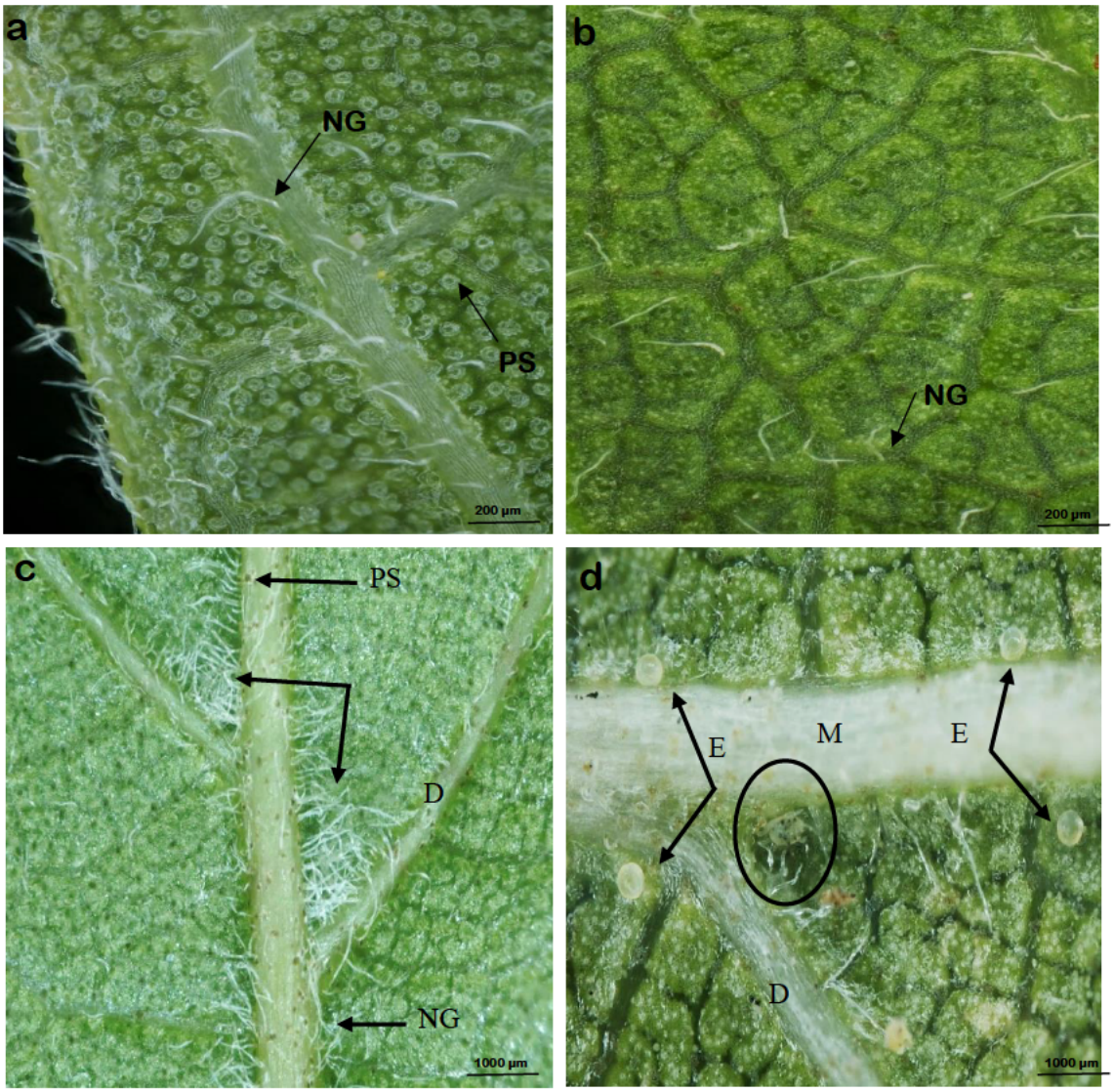


Figure 3.2: Stereomicrograph showing the leaf topology of the a) abaxial surface of the young leaf, b) adaxial surface, c) and d) abaxial surface of the mature leaf, indicating the tiny hair tuffs (domatia), a mite (indicated by the circle) and eggs laid on the leaf surface, of *C. erythrophyllum*, D= Domatia, NG= Non-glandular, PS= Peltate scales, E=eggs and M-Mite

**Histochemistry**

Synthesized primary metabolites are essential to cell functioning (Rao and Ravishankar, 2002; Pichersky and Raguso, 2018). These compounds play important roles in growth and development, whilst secondary metabolites are plant specific and are produced as part of the plant defence system against pests and pathogens (Rao and Ravishankar, 2002; Zager and Lange, 2018). Histochemical analyses were conducted using fresh-hand and vibratome cut sections of mature leaf and stem material. Through this evaluation, the composition and localisation of the exudate were assessed (Fig. 3.3- 3.8). The analysis indicated the presence of secondary metabolites such as: alkaloids, phenols and essential oils (Table 3.1). Appropriate controls were conducted (results not shown).

A reddish/brown colour change within the head cells and exudate of the peltate scale indicated the presence of alkaloids when sections were stained using Wagner's and Dittmar reagent (Fig. 3.5a-d). Research suggests that the therapeutic use of alkaloids can be dated back to the 19th century (Shang et al., 2018). Alkaloids are a diverse group of nitrogen-containing phytometabolites that have been used in traditional medicine for their narcotic and analgesic components for centuries (Bribi, 2018; Raks et al., 2018). Thus, the detection of alkaloids within the scales of *C. erythrophyllum* emphasized the importance of their reported traditional use in treating sores and wounds, amongst many other applications in medicine (Eloff et al., 2008; Mtunzi et al., 2017). The presence of alkaloids has also been associated to other *Combretum* species (Naidoo et al., 2012a; Burman et al., 2018; Chittasupho and Athikomkulchai, 2018; Welch et al., 2018; Burger et al., 2018).

Phenolic compounds were identified by the presence of dark black green deposits within the head of peltate scales and palisade cells when stained with ferric trichloride (Fig. 3.4a-b). Literature reported the presence of phenolic compounds within *Combretum decandrum* and *C. molle* among many (Uzor et al., 2014; Naidoo et al., 2012a; Welch et al., 2018). Thus, suggesting its profound presence in species of the genus. Phenolic compounds are a structurally diverse group of secondary plant metabolites, comprising of numerous groups such as tannins, flavonoids, combretastatin and quinones, amongst serval others (Lattanzio, 2013; Thorat, 2018). The presence of phenols in both leaf and stem segments correlates with findings from Harborne and Williams (2000), suggesting that increased polyphenol levels were a direct defence mechanism against UV damage. Medicinally, phenols are known to express anti-mutagenic, anti-inflammatory, apoptosis-inducing, anti-carcinogenic, and anti-oxidant activity (Garcia- Macias et al., 2007; Santangelo et al., 2018).
Essential oils are hydrophobic, volatile compounds, known of their role in plant defence and high medicinal value (Morone-Fortunato et al., 2010; Chinou, 2018). NADI regent was used to identify the presence of essential oils (brown) and resin acids (red). Essential oils were positively identified within peltate scales and resin acids were seen in non- glandular trichomes (Fig. 3.3a-d). Essential

oils are mainly comprised of esters, hydrocarbons, oxides, ketones, and aldehydes (Lull et al., 2018). Esters, oxides, and aldehydes are known to exhibit antimicrobial, antiseptic, and spasmolytic properties, while hydrocarbons and ketones are known for their cell- regenerating, sedative, neurotoxic, antitumor, and antibacterial capabilities (Tiwari and Rana, 2015; Winkleman, 2018; Abdel-Hameed et al., 2018). In addition, certain essential oils emit strong scents which act as a deterrent against pests and pathogens (Blowman et al., 2018).

Neutral lipids within palisade cells appeared red in colour when stained with Nile blue. While, acidic lipids present in the head cells of peltate scales, stained blue (Fig. 3.7c). Sudan III and IV and Sudan black indicated the presence of lipids (red) and suberin (bluish), respectively, within peltate scales, cuticle and epidermal cells (Fig. 3.6 a-b and c-d). Toluidine blue stained for polyphenols (blue), within peltate scales, while hydroxylated polysaccharides (unstained- green) and carboxylated polysaccharides (pink) within epidermal cells (Fig. 3.7d).

With the use ruthenium red, the head cells of peltate scales stained deep red in colour while the exudate stained light pink, indicating the presence of mucilage and pectin (Fig. 3.4c-d). In addition, the exudate positively stained for the presence of phenolic compounds and essential oils (Fig. 3.3-3.5). The exudate was seen in conspicuous amounts across emergent, young and stem surfaces. Secreting glandular trichomes act as effective defence mechanisms due to the secondary metabolites they exude (Wagner, 1991; Santos et al., 2015; Kalicharan et al., 2018). Secondary metabolites are plant specific and is produced as part of the plant's defence system against pests and pathogens (Rao and Ravishankar, 2002; Zager and Lange, 2018). These metabolites act as a natural repellent and in some cases, cause the encapsulation of the attacker (Lattanzio, 2013). In addition, the mucilage-rich exudate allows for the reflection of light from the leaf surface eliminating water loss and the formation of a gel-like covering (lubricating action) across the leaf surface aiding in growth and expansion (Werker, 2000; Anandan et al., 2018).

Furthermore, the presence of lignified cell walls lining the neck of the stalk cells were visualized when stained with phloroglucinol (Fig. 3.7a and b). Research suggests the presence of lignified cells wall within a few *Combretum* species (Tilney, 2002). Lignified cell walls of the peltate scales may prevent the apoplastic backflow of exudate (Duan et al., 2018). This mechanism is highly beneficial as it may prevent plant degradation caused by the back flow of exudate within the plant (Fahn, 1988). Ecologically, the presence of a lignified cell wall promotes trichome protection against herbivores as lignin cannot be digested by animals (Chahil et al., 2018). Furthermore, lignin acts as a reinforcement to ensure the upright stability of the cellular structure (Liu et al., 2018; Takenaka et al., 2018).

Through the formulated survey sections, it was observed that the leaves of *C. erythrophyllum* have periclinal epidermal cell walls, and palisade spongy parenchyma with tubular extensions (Fig. 3.8d). Similar findings were noted within *Combretum bracteosum, Combretum coriiflium. Combretum caffrum, Combreum molle* and *Combretum andenogonium* (Ekeke and Agbagwa, 2017).

**Fluorescence microscopy:**

Sections were stained with aqueous acridine orange and then viewed using fluorescence. Within peltate scales, the neck of the bicellular stalks reflected a yellow greenish colouration, indicating the presence of lignified cell walls (Fig. 3.8 a-b). Unstained sections were allowed to auto-fluoresce in its natural state, emersed in water. The peltate scales auto- fluoresced blue across the lining of the bicellular stalk and head, which indicated the presence of phenols (Fig. 3.8c). Higher concentration can be noted at the polar ends of the head (Fig. 3.8c), which possibly indicates the use of phenolic compounds in protection against herbivores (Kergunteuil et al., 2018). While deep red deposits indicated the presence of chlorophyll within the palisade and spongy mesophyll cells (Fig. 3.8c). In addition, the walls of the stalk cells fluoresced white demonstrating the presence of lignin. This finding correlates with the results obatined through the phloroglucinol and acridine orange test.

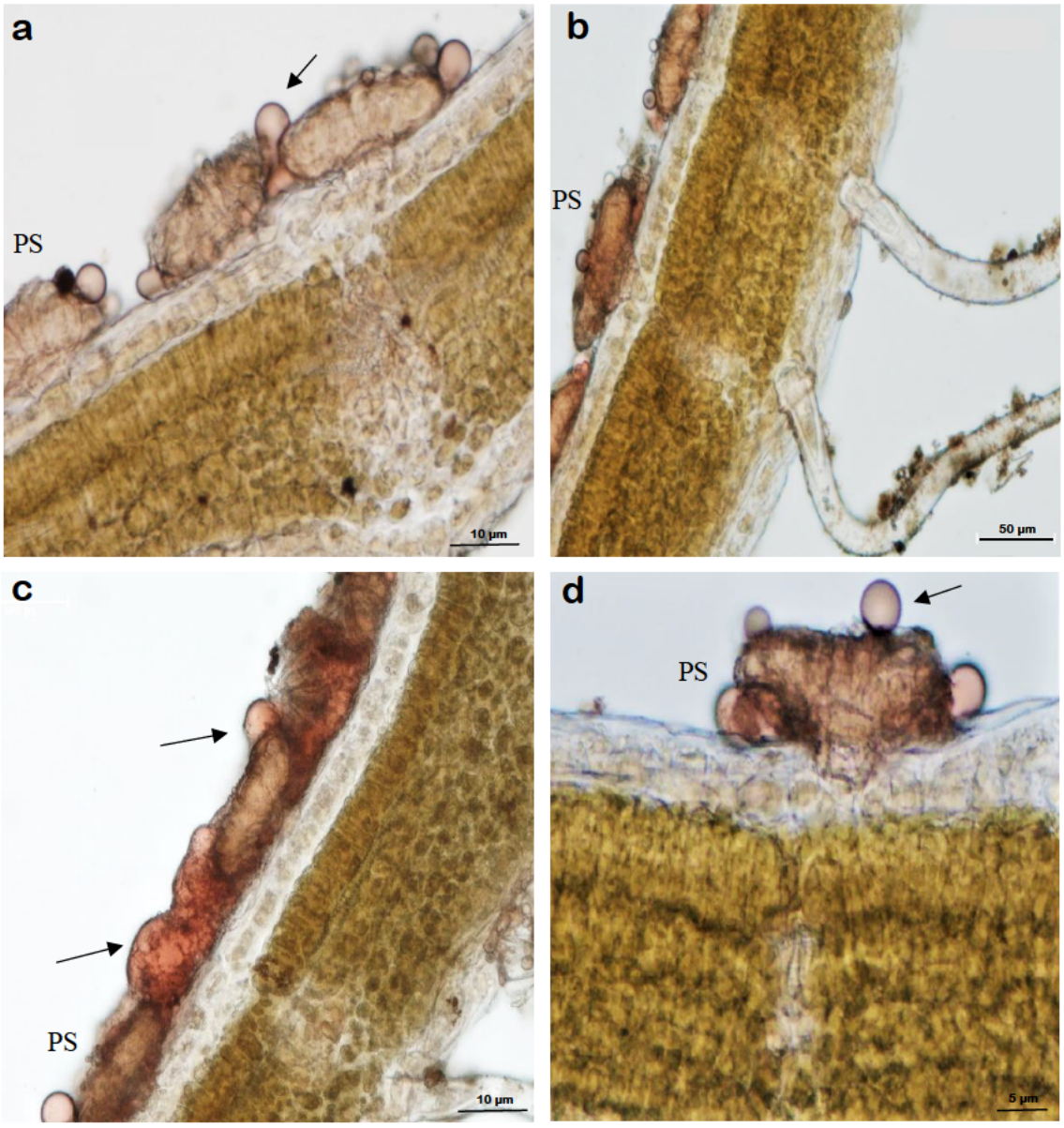


Figure 3.3: a)-d) Light micrographs of a young leaf of *C. erythrophyllum*, stained using: NADI reagent indicating the presence of essential oils (brown) and resin acids (red) within the head cells and the exudate of the peltate scales (exudate- indicated by arrow), PS=Peltate scales.

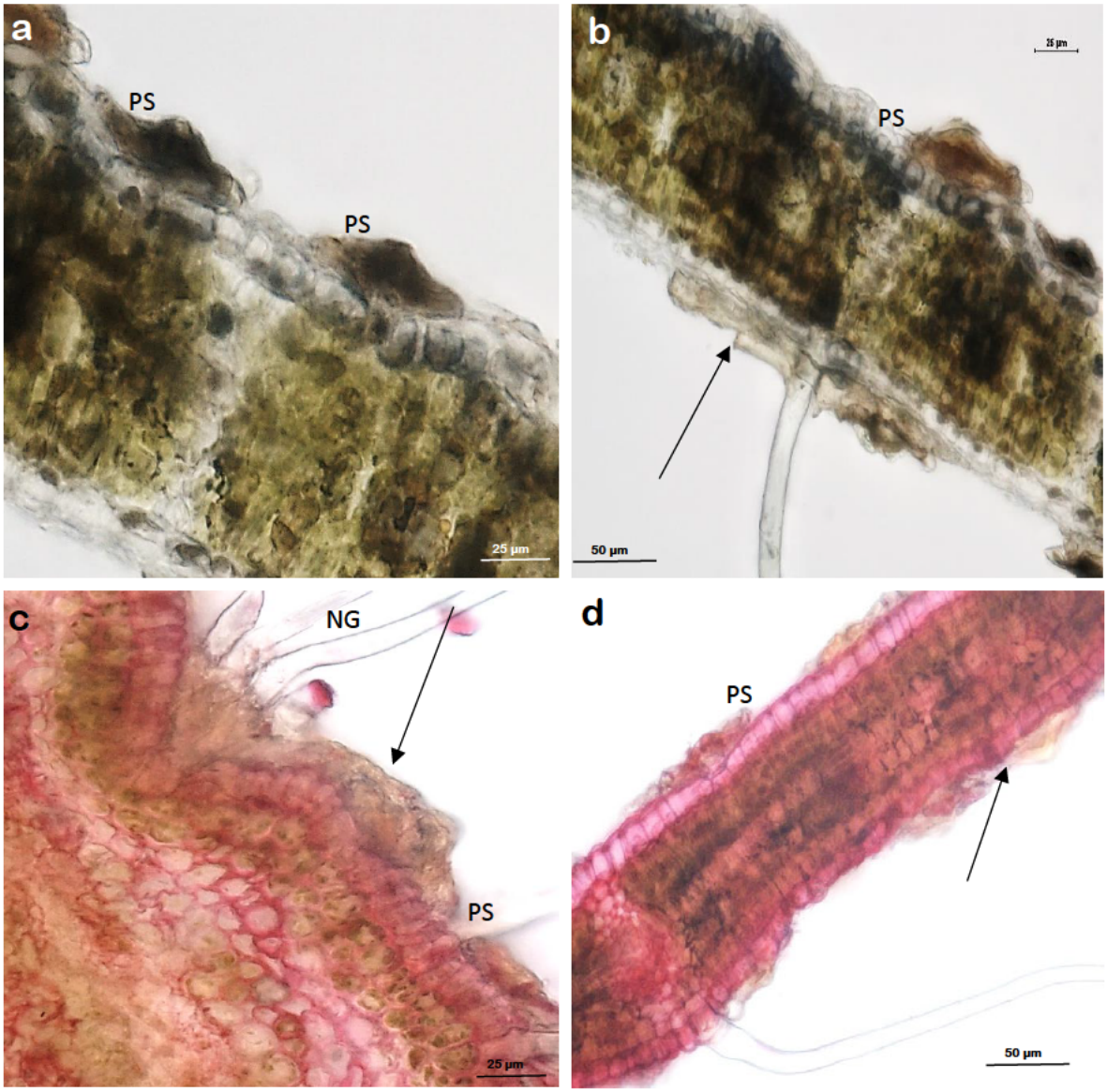


Figure 3.4: Light micrographs of *C. erythrophyllum* showing, hand cut mature leaf sections stained with: a) -b) Ferric trichloride: indicating the presence of phenolic compounds within the head and stalk of the peltate scales, in addition, the glutinous secretion (arrows) upon the leaf surface appeared to stain light brown in colour and c)-d) Ruthenium red indicating the presence mucilage and gums within the peltate scale and exudate (arrows), PS= Peltate scales, non- glandular = NG.

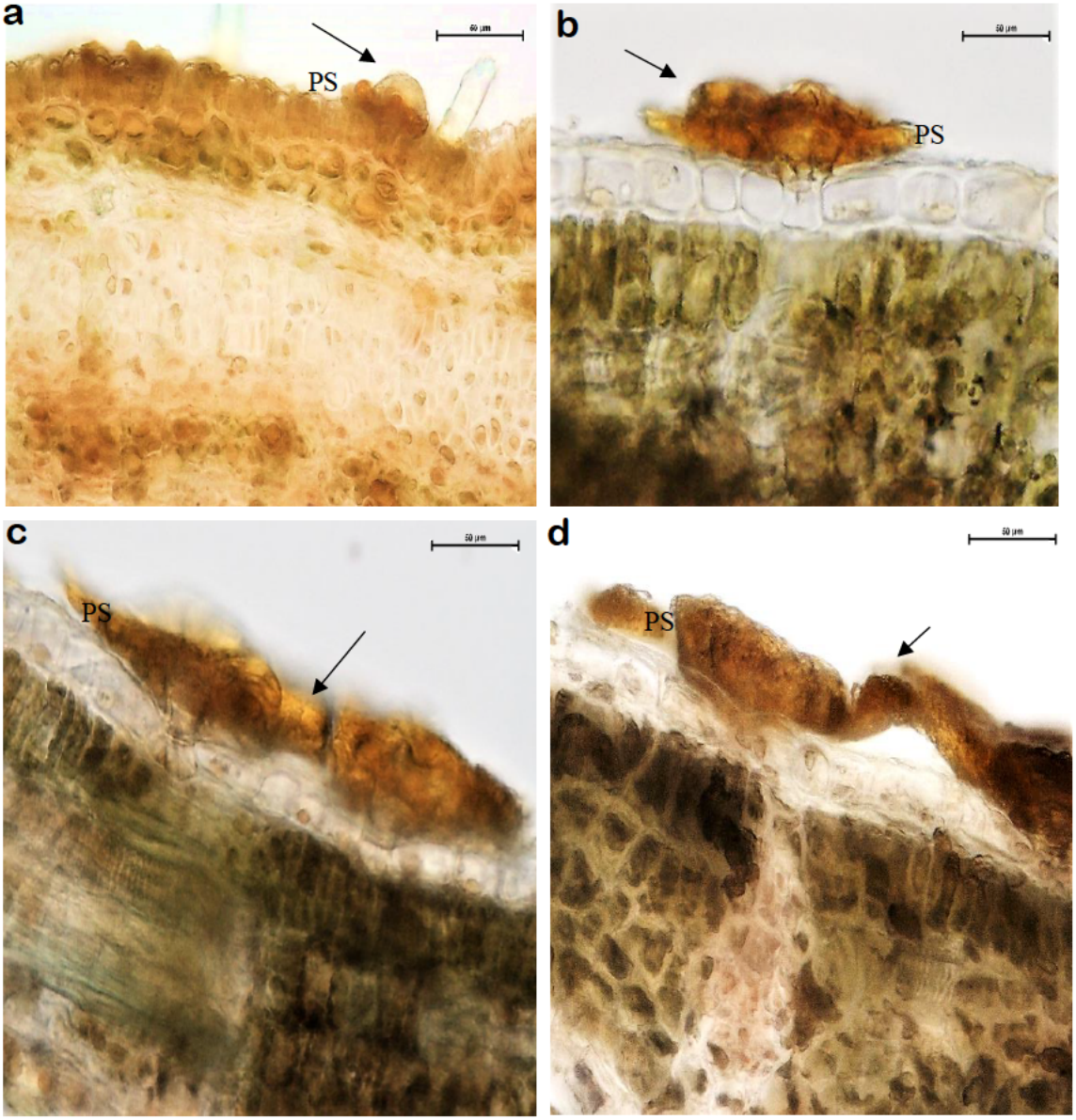


Figure 3.5: Light micrographs of hand cut cross sections of *C. erythrophyllum*: a) stem and b-d) leaf indicating the presence of alkaloids within peltate scales when stained using Wagners and Dittmars reagent, the head of the peltate scale as well as the exudate (arrows) stained brown in colour, PS= Peltate scales.

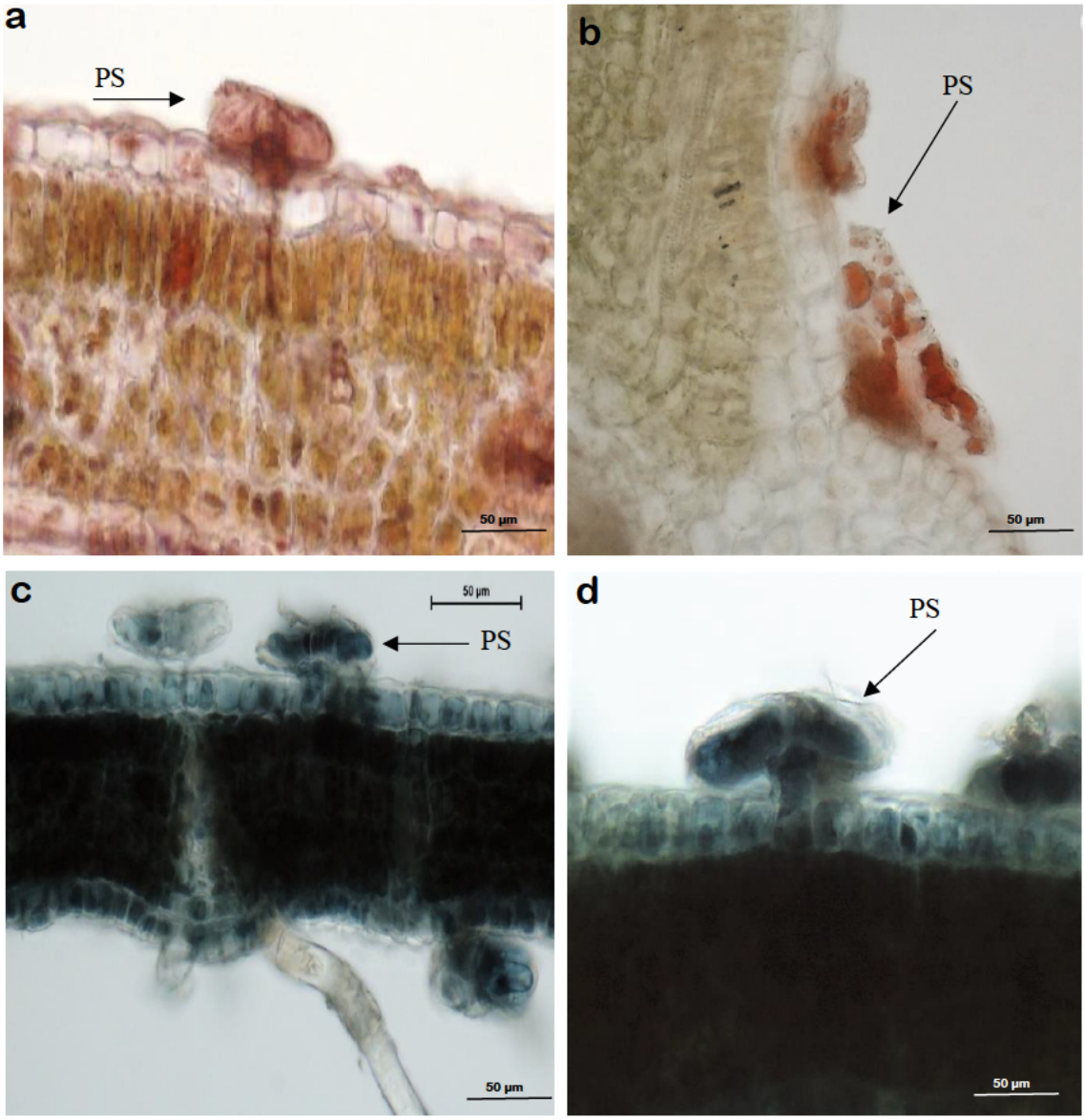


Figure 3.6: Light micrographs of hand cut cross sections of *C. erythrophyllum* showing peltate scales, stained with: a)- b) Sudan III and IV: Cutinized walls and lipids stained red and c)-d) Sudan black indicating the presence of suberin (bluish) and cutin (green/brown), PS= Peltate scale.

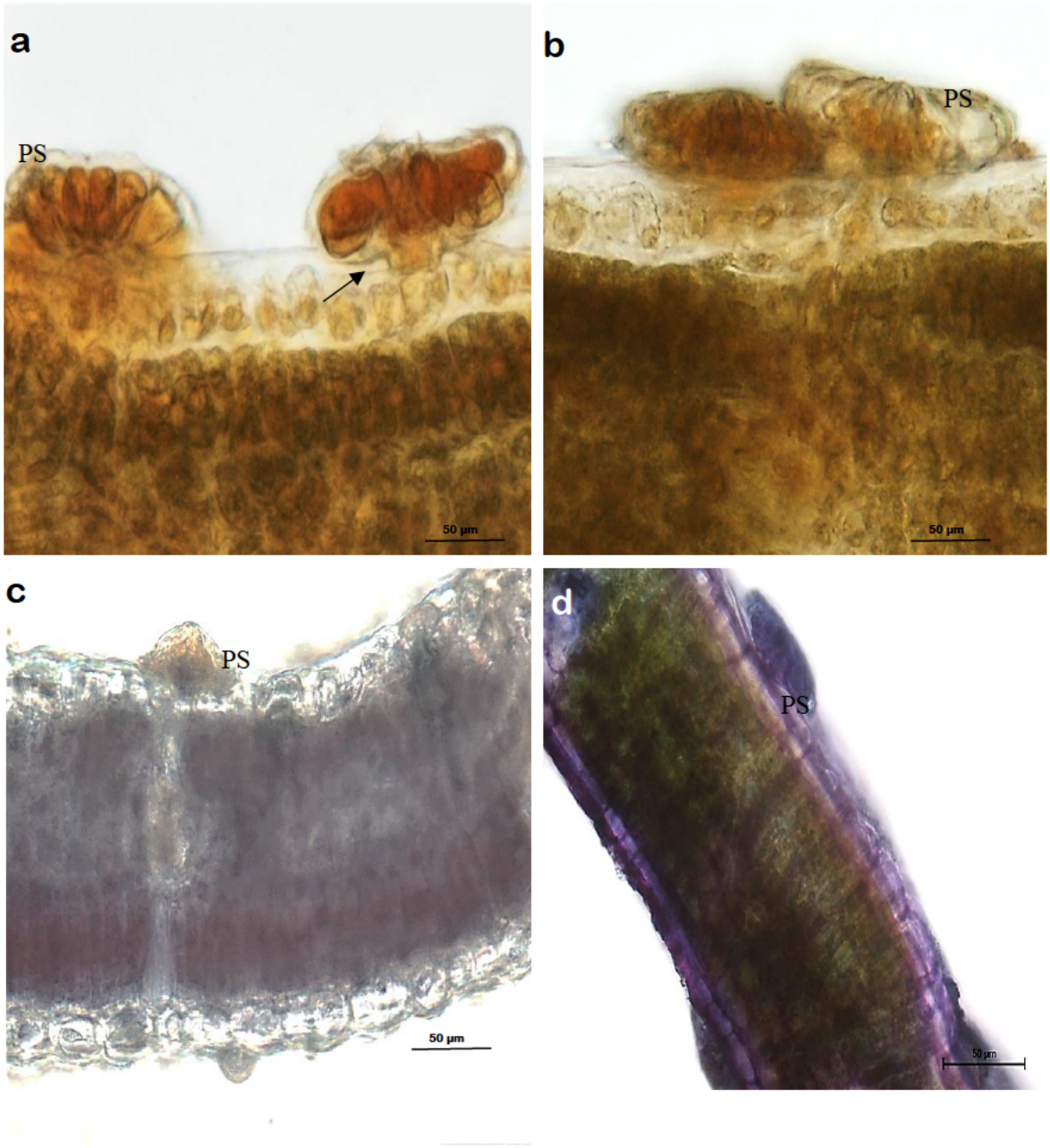


Figure 3.7: Light micrographs of hand cut cross sections of *C. erythrophyllum* showing peltate scales, stained with: a)- b) Phloroglucinol indicating lignified cell walls along the neck of the stalk cells (arrow) , c) Nile blue indicating the presence of acidic lipids within the head cell and d) Toluidine blue: showing polyphenols (blue), hydroxylated polysaccharides (unstained- green) and carboxylated polysaccharides (pink) within the peltate scale head, PS=Peltate scale.

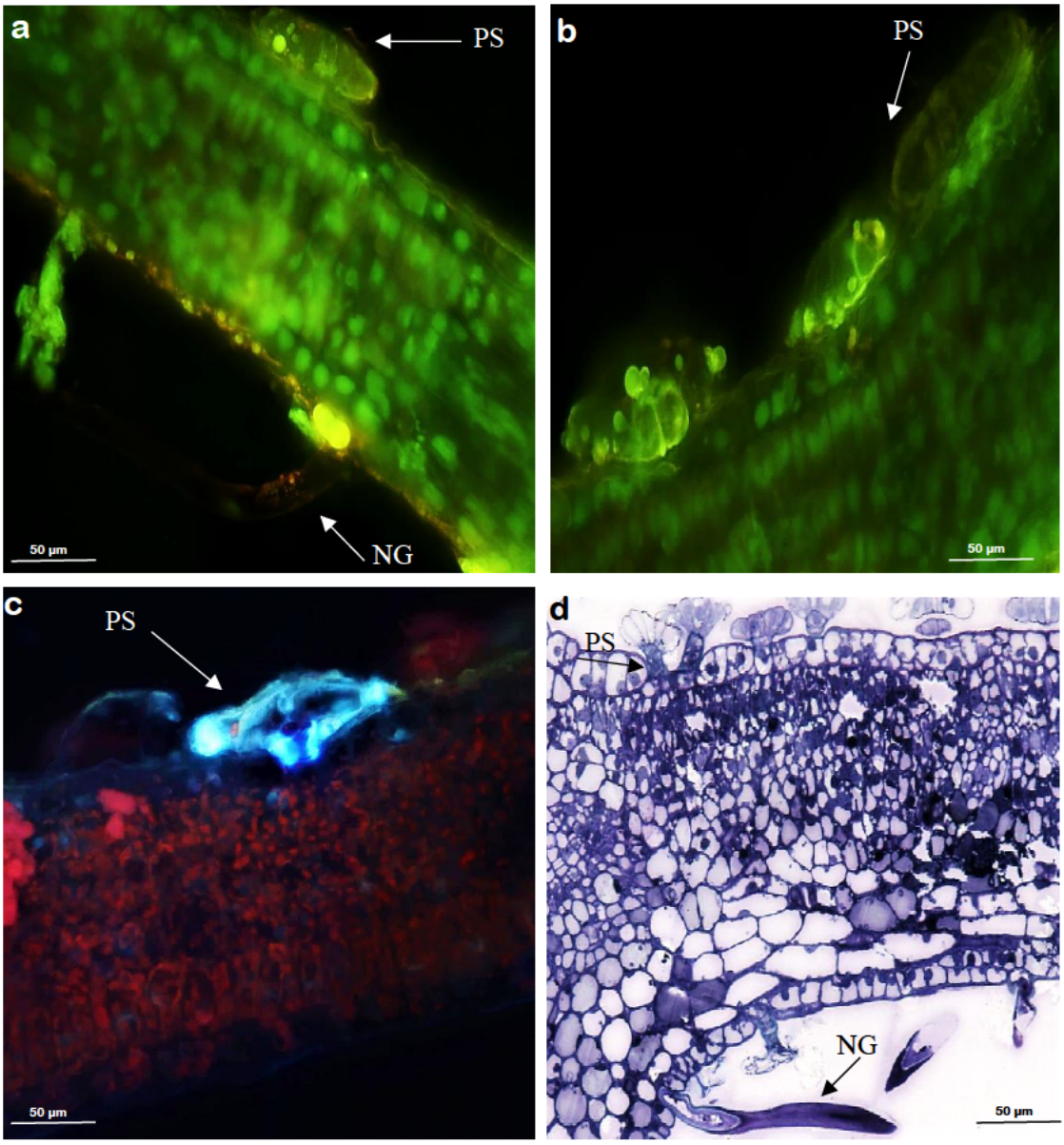


Figure 3.8: Light micrographs of hand cut cross sections of *C. erythrophyllum* showing peltate scales, stained with a)-b) Acridine orange showing lignified cell walls (yellow greenish), c) autofluorescence showing lignified cell walls, phenolics and chloroplasts (white, blue and red respectively) and d) survey section of the young leaf, PS= Peltate scale, NG= Non- glandular.

Table 3.1: Results of the histochemical analysis, indicating the presence of compounds within the peltate scales in leaf and stem material of *C. erythrophyllum*.

| COMPOUND CLASS | TEST | LEAVES | STEMS |
|---|---|---|---|
| **Metachromatic staining** | Toluidine blue | ++ | + |
| **Proteins** | Bromophenol blue | + | + |
| **Lipids** | Sudan black b | ++ | - |
| | Sudan iv | ++ | ++ |
| | Nile blue | + | + |
| **Lignin aldehydes** | Phloroglucinol | + | + |
| **Phenolic compounds** | Ferric trichloride | ++ | + |
| **Alkaloids** | Wagner's and Dittmar reagent | ++ | ++ |
| **Essential oils** | NADI reagent | ++ | + |
| **Cell viability** | Acridine orange | ++ | ++ |

*Intensity of reaction: + present, ++ intense presence

-Appropriate controls were conducted (results not shown).

**Scanning electron microscopy (SEM):**

SEM analysis of *C. erythrophyllum* leaf material, showed the presence of unicellular non-glandular trichomes and peltate scales. Structurally, the non-glandular trichomes appeared to have striations (Fig. 3.9c) and the peltate scales had a multicellular head. The head cell number was seen to increase as leaf maturation occurred. Within the emergent, young and mature leaves, head cell count was 8-11 (Fig. 3.9), 11-14 (Fig. 3.10) and 14- 19 (Fig. 3.11), respectively. Striations were also noted within *C. imberbe, C. collinum* and *C. padoides* (Tilney, 2002).

Surfaces of emergent leaves (Fig. 3.9a and b) were densely covered in unicellular non- glandular trichomes which partially concealed the peltate scales. The emergent and young leaf sections, peltate scales were seen to be embedded within a glutinous-like secretion (Fig. 3.9 and 3.10). The magnitude of this secretion appeared to diminish upon leaf maturation. This trend is unique to *Combretum erythrophyllum* and has yet to be reported within literature (Fig. 3.9 and 3.10). Exudate was noted mainly around the scale structures (Fig. 3.10a). The release of exudate appeared to be from the sides of the peltate scale. This is indicative of a weak region or break within the scale structure (Kalicharan et al., 2018). Research by Naidoo et al., (2012a) based on *C. molle*, noted a unique mode of secretion, within the *Combretum* genus. Initially, the lateral ostiole is utilized as a pathway to exude secretion (Naidoo et al 2012a). The subcuticular space then acts as a reservoir to the exudate following exudation through the central main pore (Mashimbye et al., 2018).

In comparison to the emergent leaves, the younger leaves appeared to have a dense covering of non-glandular trichomes, concealing the multitudes of peltate scales embedded below. These peltate scales appeared to be in a pre- secretory phase of development (Fig. 3.9c-d). Stomata appeared to be closed on the adaxial surface and opened on the abaxial surface of young leaves. Mature leaf surfaces appeared to have minimal visible levels of secretion, peltate scales and non- glandular trichomes (Fig 3.11 a-b). In addition, numerous peltate scales appeared to be shrivelled and condensed, ie. in the post- secretory phase. Although an observable change in trichome density was noted, the change in trichome density was not significant. Trichome density remained fairly constant in relation to leaf maturation and an increase in leaf area. This is linked to the leaf expansion theory (Duke, 1994; Singh, 2017).

Duke (1994), explored the link between leaf area and trichome density. As leaves expand, trichomes become further separated due to ongoing cellular divisions, increase in epidermal and stomata cell formations, hence the increased appearance of stomata to trichomes in *C. erythrophyllum*. Notably, in fully matured leaves, peltate scales were seen to be fewer with more in the post- secretory stage of development. The density of trichomes may remain constant throughout the developmental stages

and instead the leaf surface area expands (Singh, 2018). Suggesting that trichome formation and density is established early in development. A similar trend was noted in research conducted on *C. molle* (Naidoo et al., 2012a). Emergent and younger leaves are highly vulnerable to pathogenic attacks hence the early establishment of trichomes provide a key defence mechanism in protection against pests and pathogens (Duke, 1994).

Through the SEM analysis of the stem material, peltate scales and an indumentum of non-glandular trichomes were seen (Fig. 3.11 c-d). The peltate scales appeared to be relatively smaller in size as compared to those seen in the leaves. In addition, a minimal glutinous-like secretion layer was noted upon the stem surface. This is possibly due to the less vulnerable nature of the stem in comparison to the leaf hence minimal exudate-based chemical protection is required (Rodriguez-Dominguez et al., 2018). Chemical protection is provided by the exudate which is released through peltate scales (Naidoo et al., 2012b). It may be said that scale size is relatively proportional to the amount of exudate produced and released. Thus, a minimised amount of the glutinous -like secretion was noted upon the stem surface.

Furthermore, the presence of crystal idioblasts were seen within stem and mature leaf segments (Fig. 3.11b). Thus far, crystals have been found within *Combretum wattii* Exell. and *Combretum decandrum* Roxb. (Tilney, 2002). Currently, literature suggests the presence of crystals within 215 plant families with evidence linking crystal formation and location as a taxonomic classification tool (Yang et al., 2018). Calcium oxalate crystal formation is a genetic plant mechanism formulated to remove excess calcium or oxalate found within a plant (Nicolás-Bermúdez et al., 2018). The mechanism gives rise to crystal idioblasts which differ in shape, size and location. The main functions of these crystals include, promoting calcium homeostasis, structural support, removal of excess calcium and oxalate (Schmitt et al., 2018). In addition, certain crystals are known to be toxic in nature hence aiding in the protection against herbivores (Dubey et al., 2018). When these crystals become excessively large in size, the organelles around them begin to degenerate, which could prove to be detrimental to the plant (Mhinana et al., 2010; Navarro-León et al., 2018). The presence of crystals within *C. erythrophyllum* supports the concept of crystal presence as a deterrent mechanism against herbivores.

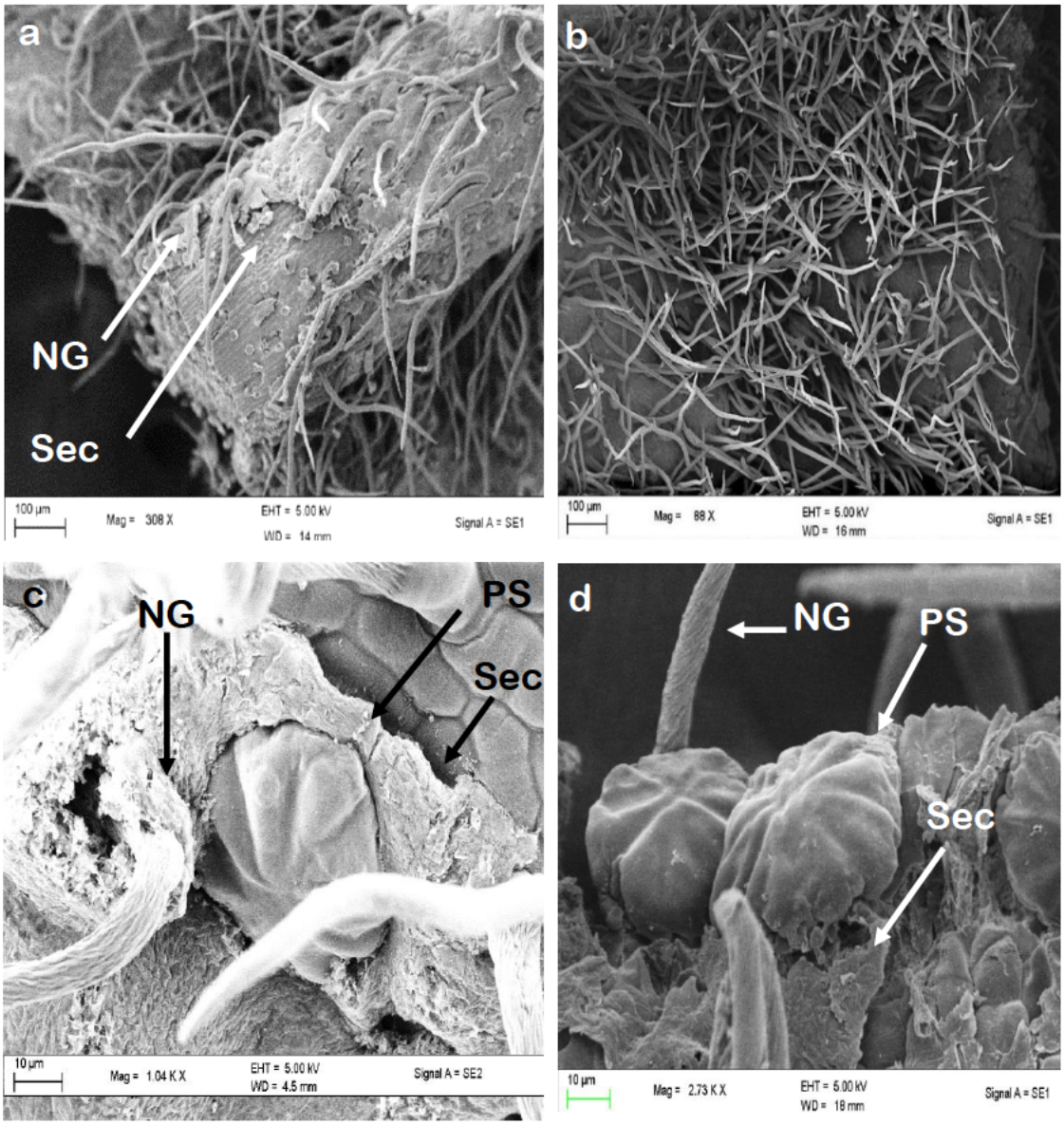


Figure 3.9: SEM micrographs of the emergent leaf of *C. erythrophyllum* a) abaxial leaf surface showing the presence of numerous non- glandular trichomes and a glutinous– like covering across the leaf surface b) adaxial surface indicating dense non- glandular trichome coverage, c) and d) abaxial surface indicating a peltate scale in the post secretory phase of development, PS= Peltate scale, NG= Non-glandular and Sec= Secretion.

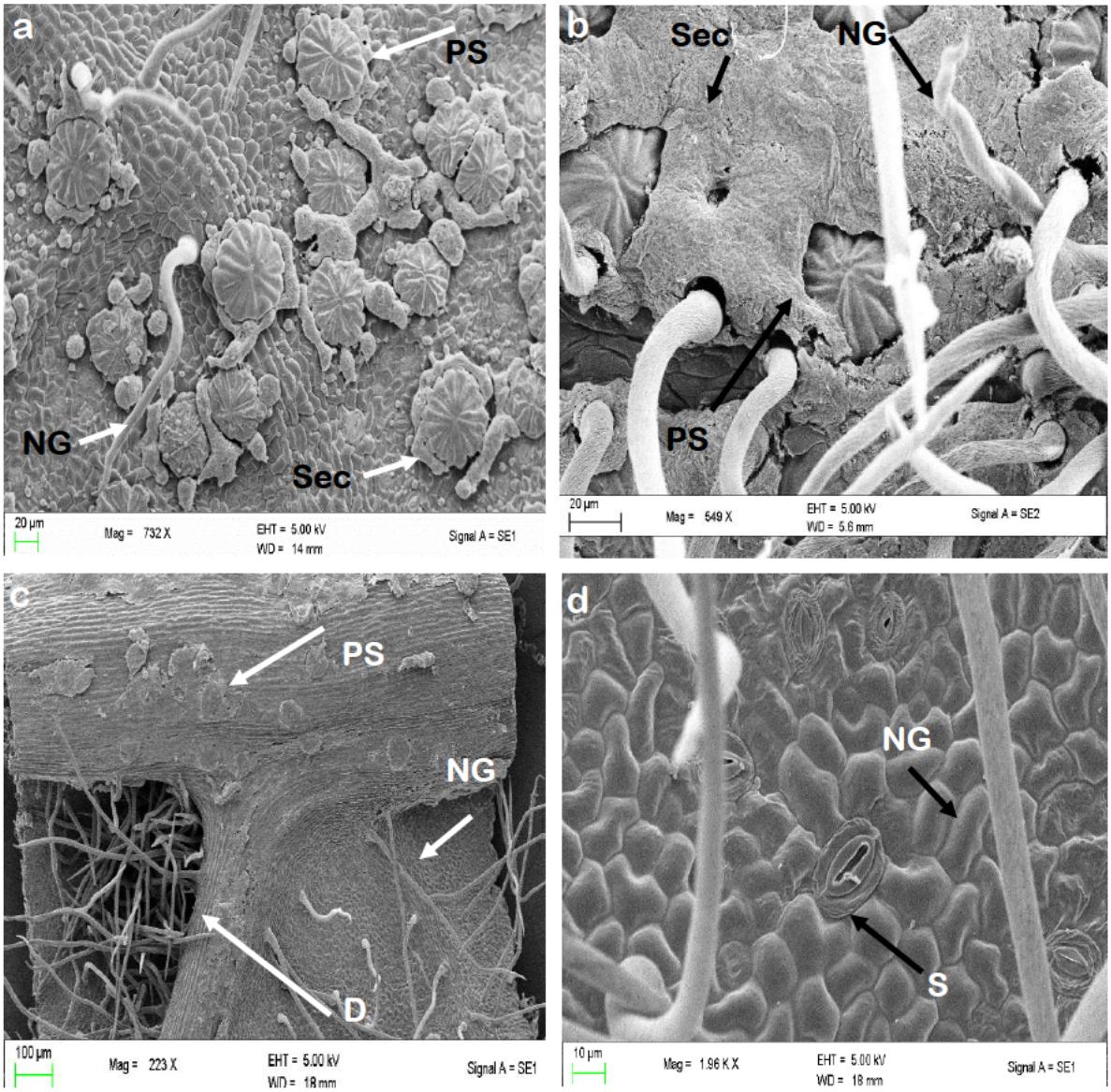


Figure 3.10: SEM micrographs of *C. erythrophyllum* indicating the a) young leaf abaxial surface, b) young leaf adaxial surface showing the presence of many peltate scales concealed below a glutinous like secretion, c) mature leaf abaxial surface showing hair like tuffs and d) mature leaf adaxial surface showing the presence of minimal secretion and multiple stomata, PS= Peltate scale, NG= Non-glandular, S= Stomata, D= Domatia and Sec= Secretion.

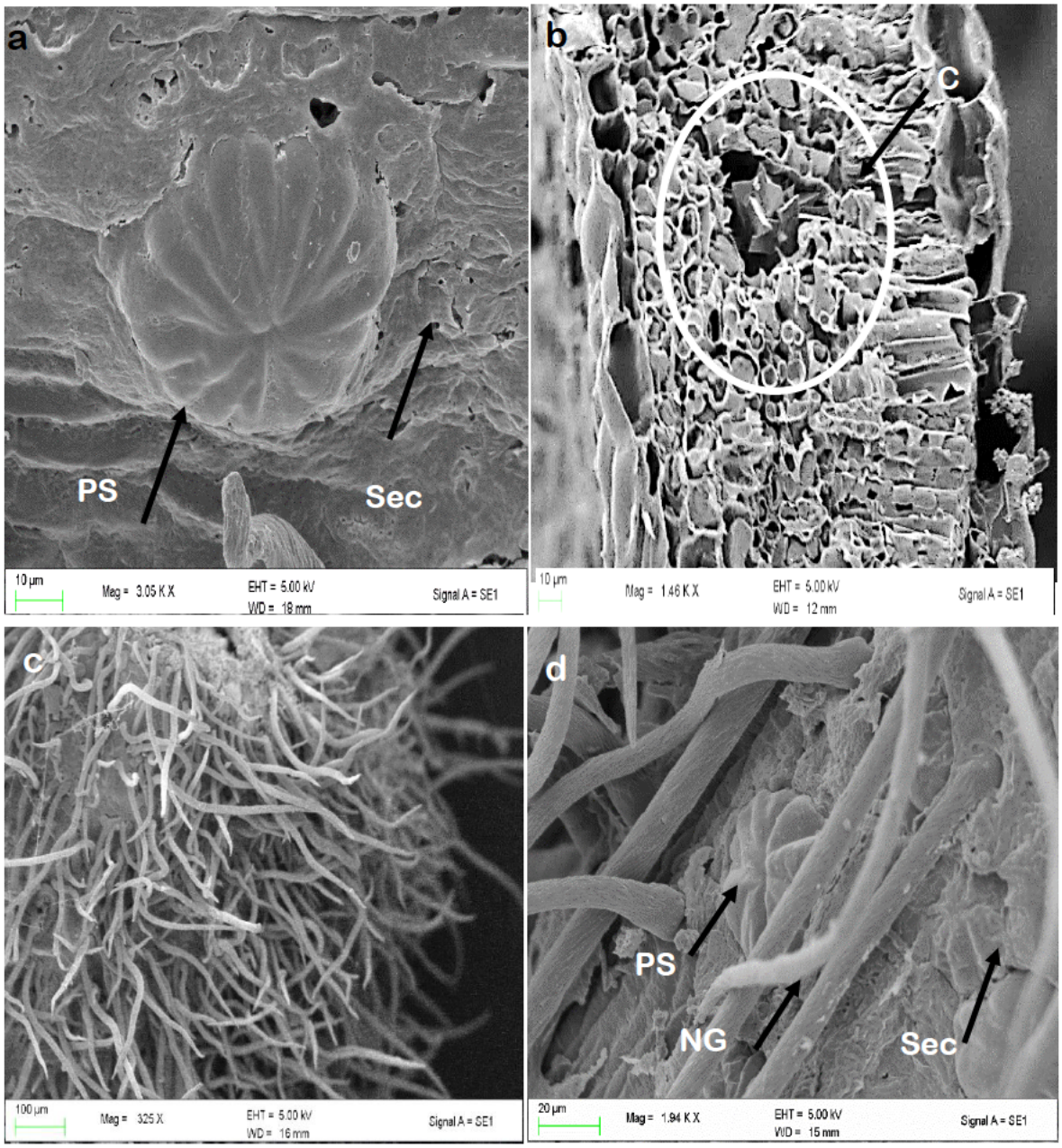


Figure 3.11: SEM of the mature leaf a) abaxial b) transverse section showing crystal idioblast- c) and d) stem surface, indicating peltate scales that appear smaller in size PS= Peltate scale, NG= Non-glandular, Sec= Secretion and C= Crystal.

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**Transmission electron microscopy (TEM):**

Peltate scales were analysed using TEM. The trichome appeared to be comprised of a broad basal cell, a bicellular stalk and an umbrella-like head, encompassing multiple head cells (Fig. 3.12a). Furthermore, a conspicuous amount of electron dense lipophilic material was seen within the head cells (Fig. 3.13b). This material hindered the visualisation of certain cellular components, but vesicles, vacuoles, mitochondria, dictyosomes, endoplasmic reticulum cisternae and nuclei were observed (Fig. 3.12-13). The electron dense lipophilic material was visualized within the stalk cells, vesicles and vacuoles within the head cells. In addition, the stalk cells noticeably contained more plastids and vacuoles in comparison to the head cells (Fig. 3.12b). Numerous plasmodesmatal connections were seen between the basal, stalk and head cells (Fig. 3.12b and 3.12c). This indicated the possible symplastic transport of exudate and smaller molecules (sugars etc.) from the stalk cells towards the head cells (Tharanya et al., 2018). This mode of transport involves the free movement of solutes, between cells, by the aid of numerous plasmodesmata (Lu et al., 2018).

The basal epidermal cell forms the collecting cell of the peltate scale. This cell appears to be a large, rigid and sunken structure composed of vacuoles and vesicles (Fig. 3.12c). A large vacuole and medium sized nucleus were characteristic of the basal cell. The endoplasmic reticulum is smooth, while plastids are few and distributed around the nucleus (Fig. 3.13c). An apparent multi-membranous structure appears to disintegrate which allows for the formation of exudate and subsequently, the formation of vesicles in the cytoplasm (Fig. 3.12c). The produced exudate can be stored in the newly formed vesicles and vacuoles, thus proposing a mechanism for the production and storage of exudate before exocytosis.

The bicellular stalk appeared to be composed of multiple vacuoles and a medium sized nucleus (Fig. 3.13a). A dense cytoplasm with numerous plastids and ribosomes were also observed. Plastids were seen to contain lipophilic material and were indicated by the formation of dark black deposits within. Notably, the cytoplasm was seen to have less electron dense material in comparison to the head and basal cells. High presence of vacuoles were observed but these were not impregnated with electron dense material as previously observed in the basal cell (Fig. 3.13c). Mitochondria, endoplasmic reticulum cisternae and dictyosomes were seen surrounding the centralised nucleus. A thickening of the cell wall lining the stalk cells was observed (Fig. 13.12-13). This is a mechanism to prevent apoplastic backflow of the exudate through the walls which is termed as the transfusion zone (Singh et al., 2018).

The head cells were comprised of a complex network of mitochondria, plastids, dictyosomes and endoplasmic reticulum cisternae (Fig. 3.13b-c). This indicated the presence of a highly active

metabolic system (Stpiczyńska et al., 2018a). These organelles are large, predominant and plentiful within the cytoplasm, near vacuoles. Plastids, vacuoles and vesicles were seen in abundance along the periphery of the cell (Fig. 3.13a). In addition, these organelles also appeared to be surrounding the centralised nucleus. Numerous vacuoles stained darker in colour indicating the presence of lipophilic material. This may be indicative of the continuous multimembrane degradation process. This process leads to the formation of exudate which is stored in the vesicles and vacuoles. The subcuticular space was observed along the periphery of the head cell (Fig 3.13b). The separation of the trichome cuticle from its cell wall lead to the simultaneous formation of the subcuticular space (De Vargas et al., 2018). The cell walls of the head cells appeared to be thicker and composed of loose microfibrils in comparison to the bicellular stalk and basal cell (Fig 3.13b -c). The loose arrangement of the microfibrils allows for the movement of exudate through the wall (Machado et al., 2012; De Vargas et al., 2018).

Although a small subcuticular space was noted along the periphery of head cells, it is assumed that majority of the exudate is stored in the surrounding numerous vesicles and larger vacuoles. Thus, substantiating the presence of numerous electron dense vesicles and vacuoles within the head cells (Fig.3.13a). The above findings suggest that of *C. erythrophyllum* follows a possible granulocrine pathway as its the proposed mode of secretion (Stpiczyńska et al., 2018b). In accordance, the characteristic cellular components of a granulocrine pathway includes the presence of dictyosomes, endoplasmic reticulum and vesicles (Rehman and Asif Hanif, 2016). This pathway involves the accumulation and storage of exudate in surrounding vesicles followed by the vesicle mediated fusion with the plasma membrane allowing for the exocytosis of the exudate (Ballego-Campos et al., 2018).

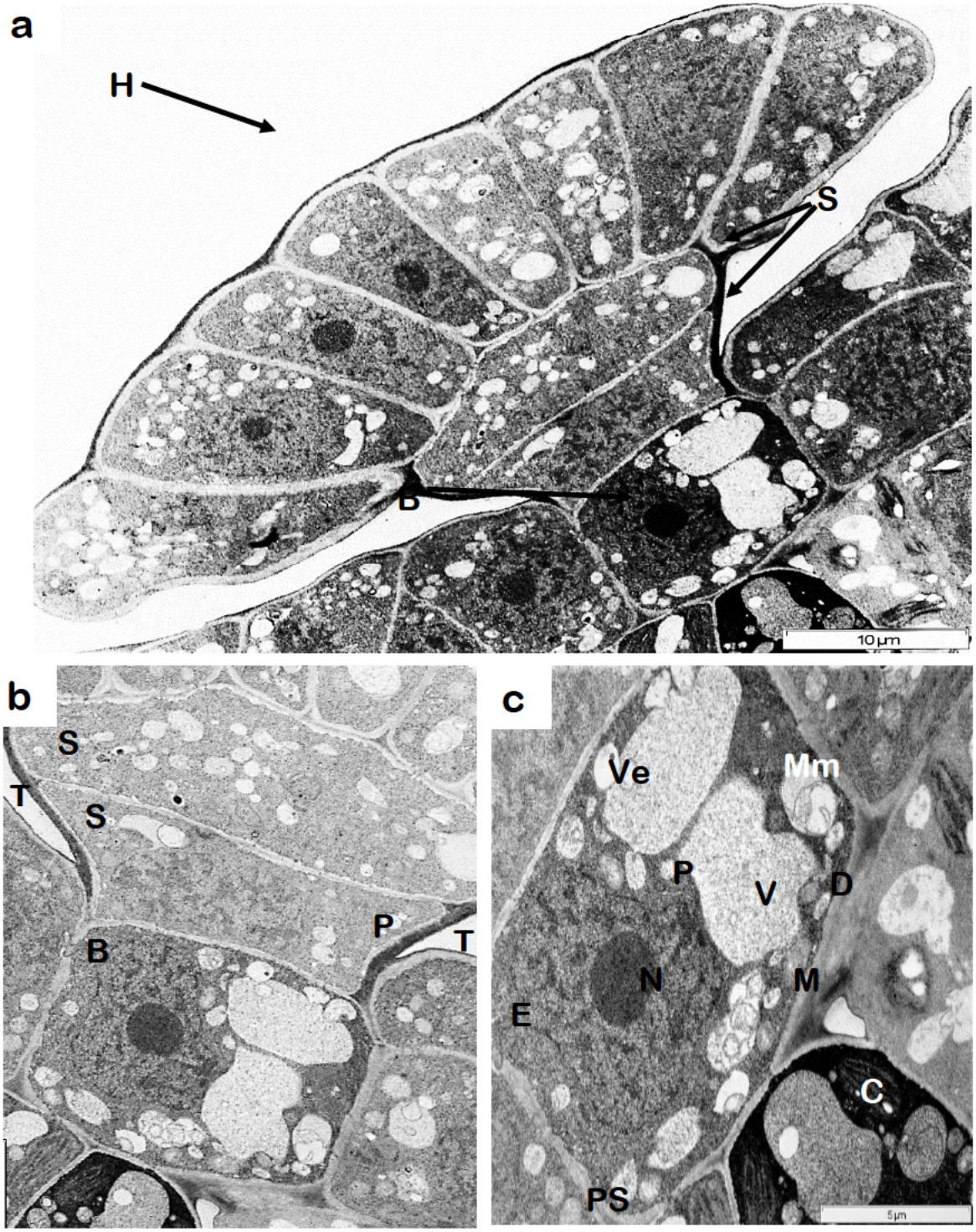


Figure 3.12: TEM micrographs of the peltate scale found within *C. erythrophyllum,* a) cross section of umbrella like scale b) Stalk cell indicating presence of transfusion zone and c) basal cell indicating plasmodesmatal connections from adjacent cells, H= head, S= Stalk cell, B=Basal cell, T= transfusion zone, V= Vacuoles, Ve= vesicles, PS= Plasmodesmata, P= Plastids, PS= Plasmodesmata, M= Mitochondria, D= Dictyosomes, Mm=Multimembranous organelle and C= Chloroplast.

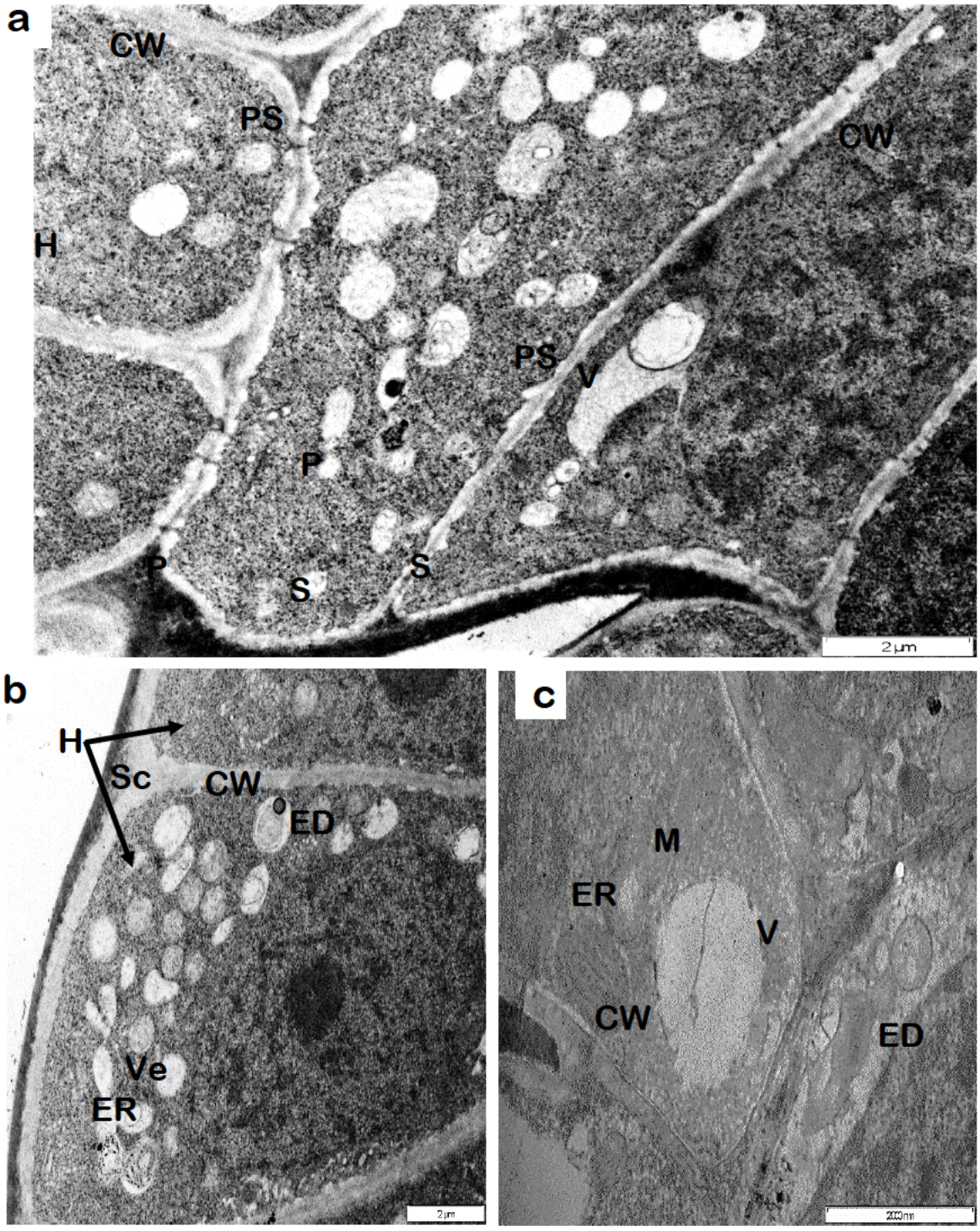


Figure 3.13: TEM micrographs of the peltate scale found within *C. erythrophyllum* a) Stalk cells, indicating plasmodematal connections from the stalk to head cells, b) Head cell, plasmodematal connections from the adjacent head cells, cell wall comprising of loose fibres and the presence of numerous vesicles, and c) Head cell indicating the presence of a mitochondrion and electron dense material H= Head, S= Stalk cell, B= Basal cell, PS= Plasmodesmata, CW= Cell wall, Electron dense material, P= Plastid, V= Vacuoles and Ve= Vesicle.

**3.4 Conclusion**

This study investigated the micromorphology, ultrastructure and histochemistry of the leaves and stems of *Combretum erythrophyllum*. After an extensive micromorphological analysis, two distinct trichome types were identified, peltate scales and non- glandular trichomes. A preliminary ultrastructural analysis of the peltate scales identified a granulocrine pathway as the mode of secretion. In addition, the presence of alkaloids, phenols and oils indicate the potential medicinal use of *C. erythrophyllum* as a antimicrobial and anti- oxidant agent.

The overall findings suggest that *C. erythrophyllum* is indeed a plant worthy to be considered for its medicinal properties. Further studies, incorporating biological assays and possible trichome isolation should be explored. In addition, biotechnological processes should be evaluated in order to increase plant yield in a contracted time period.

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## 3.6 Appendix

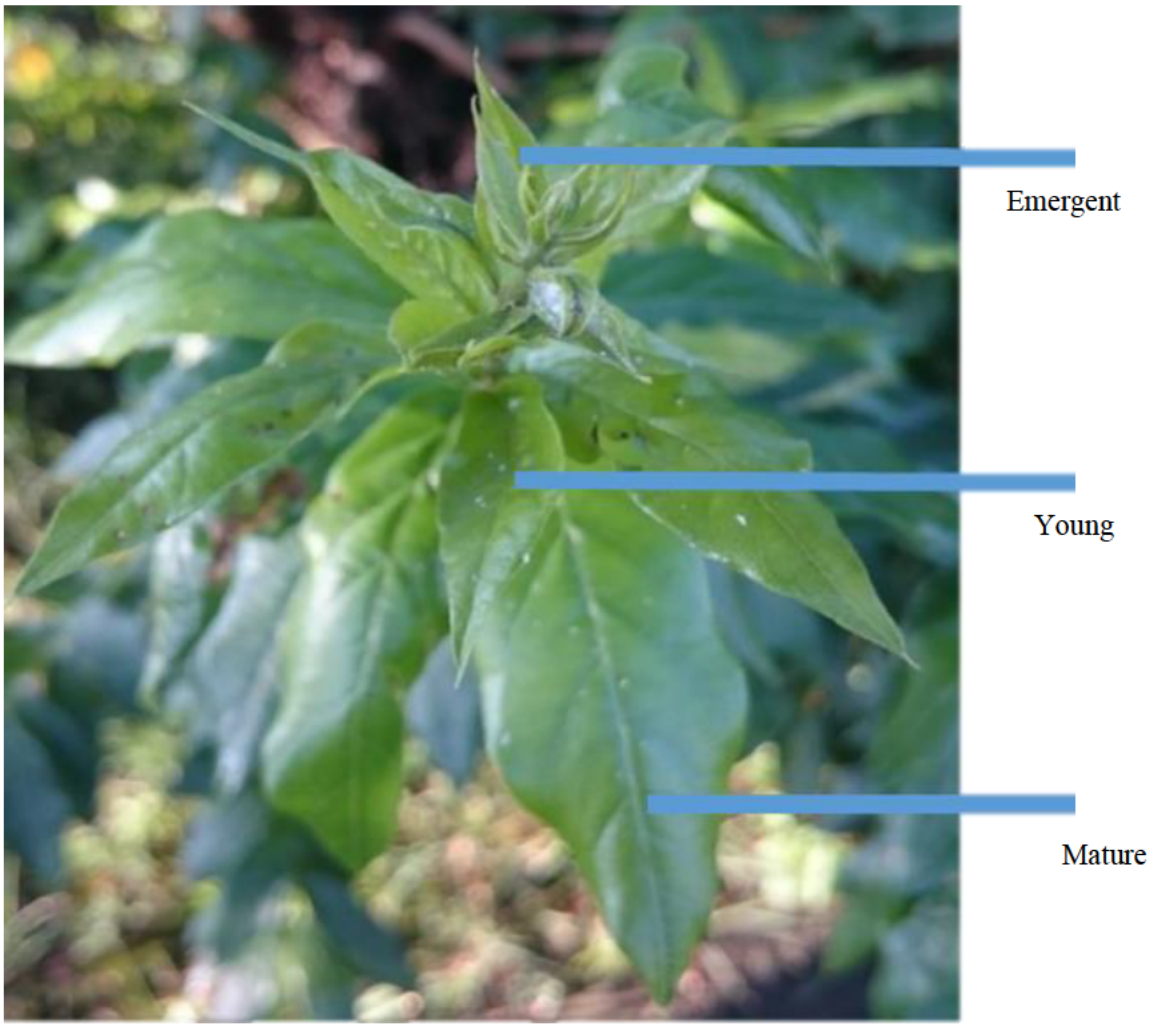


Figure 3.14: Leaves of *C. erythrophyllum* showing visible differences among the emergent, young and mature leaves.

# CHAPTER 4: CHEMICAL COMPOSITION OF COMBRETUM ERYHTROPHYLLUM: PHYTOCHEMICAL ANALYSIS, EDX, GC-MS, TLC AND FLUORESCENCE MICROSCOPY OF THE LEAF AND STEMBARK.


## Abstract

Combretaceae is a large Angiosperm family that is highly sought after because of its pronounced medicinal value. *Combretum* is recognized as the largest genus, prevalent in southern Africa and extensively used in traditional medicine. This study aimed to provide a comparative analysis of the phytochemical constituents and possible pharmacogenetic properties of the leaf and stembark extracts of *Combretum erythrophyllum.* Crude leaf and stembark extracts were generated using hexane, chloroform and methanol as the solvents of choice. Preliminary phytochemical tests indicated the presence of multiple phytocompounds, including carbohydrates, alkaloids, sterols, phenols, fixed oils and fats. Flavonoids were found within the leaf extracts only, while saponins, mucilage and gums were specifically identified within the stembark extracts. The chemical composition of *C. erythrophyllum* leaf and stembark extracts using gas chromatography-mass spectroscopy is being reported here for the first time; the analysis led to an identification of 266 compounds. Major phytocompounds such as, sitosterol and lupeol, which may have possible anti-cancer and anti-inflammatory properties, were identified. Furthermore, a pharmacogenetic evaluation was conducted. As a result of the evaluation, both the leaf and stembark material were seen to fluoresce a wide array of colours (brown, red, green and blue colourations), indicating possible presence of beneficial phytometabolites and their use in the medicinal world. Given the wide array of proposed medicinal benefits associated with the presence of phytocompounds identified within *C. erythrophyllum*, this species should be considered further for its medicinal importance. The isolation and extraction of these beneficial compounds opens further avenues into their use in the pharmaceutical industry.


Keywords: *Combretum erythrophyllum*, Bioactivity, Phytochemistry, Phytometabolites, Traditional medicine

## 4.1. Introduction

Among several cultures and ethnic groups around the world, medicinal plant species are known to act as an ancient therapeutic tool (Kayser, 2018; Gökbulut, 2021). Medicinal flora is known to contain metabolites that may effectively improve an individual's state of health (Kaur et al., 2018; Bantho et al., 2020). Due to extensive research into the benefits of medicinal plants, first world countries are now integrating naturally-derived medicines into mainstream health care systems.

A growing interest in the study of medicinal plants has led to the exuberant boost of its global economic value (Van Wyk, 2008). The global medicinal plant industry was valued at an estimated 60 billion USD in 2003, whereas in 2012, a single avenue of the industry, traditional Chinese medicine, was valued at $83 billion hence, proving its exponential growth among communities (Braun, and Pitt, 2018, Scheffers et al., 2012, Pimm and Joppa, 2015). The World Health Organization mentions that 80% of the global population rely on the utilization of medicinal plants for the treatment of numerous ailments (Willis, 2017, Kumar and Navaratnam, 2013, Rupani and Chavez, 2018). Thus, it has become of utmost importance to promote extensive studies integrating the safe use and conservation of these medicinal plants (Scheffers et al., 2012, Castillo-Pérez et al., 2012, Kemper et al., 2008). The pharmacological use of medicinal plant species has numerous benefits with minimal side effects (Kumar and Navaratnam, 2013, Winkleman, 2018, Verma and Singh, 2008). Known benefits associated with this naturally-derived form of medication includes cost efficiency, accessibility and a role in physiological effectiveness (Wink and Schimmer, 2018).

Medicinal properties are attributed to the secondary metabolites found in plants (Rao and Ravishankar, 2002). Synthesized primary metabolites aid in cell functioning, plant growth and development (Wink and Schimmer, 2018). Comparatively, secondary metabolites are plant-specific and are produced as part of the plant defense system against pests and pathogens (Rao and Ravishankar, 2002, Wink, 2018). Secondary metabolites are classified into three main classes, namely, alkaloids (nitrogen-containing compounds), terpenes and phenolic compounds (Gupta et al., 2018). They are exuded by specialized secretory tissues such as resin ducts, mucilage ducts, laticifers, salt glands, hydathodes and trichomes (Fahn, 1988, Svoboda and Svoboda, 2000, Wittstock and Gershenzon, 2002).

Microbial pathogens are continuously evolving, hence, advanced remedial methods need to be introduced (Lee et al., 2018). Scientists are now turning towards medicinal plant research to curb microbial pathogen growth. Continued research and analysis of current pathogens is crucial in order to develop efficient, naturally-derived, plant drug delivery methods (Mtunzi et al., 2017).

The medicinal use of plant species from the family Combretaceae is of high interest on the African continent (Mawoza and Ndove, 2015). Literature suggests the hardy nature and predominant presence of medicinally important phytometabolites within these plants (Eloff et al., 2008, Lima et al., 2012). Traditionally, *Combretum erythrophyllum* is known to proclaim high medicinal value (Mtunzi et al., 2017). This tree is commonly known as the river bushwillow, and is a deciduous, indigenous tree that thrives in areas with good groundwater levels. Traditional uses include treatment of venereal diseases, abdominal pain and sores, among many (Mawoza and Ndove, 2015).

Pharmacological studies have shown that species of *Combretum* have antibacterial, anti-inflammatory, antifungal, genitourinary, cytotoxic and mutagenic properties (Mtunzi et al., 2017, Fyhrquist et al., 2002). Martini et al. (2004) associated the possible antibacterial effects of *C. erythrophyllum* to the presence of several flavonoids and phenolic compounds found within it. In addition, epidemiological studies highlight the occurrence of antioxidant compounds within the species (Mtunzi et al., 2017). A recent study conducted by Mtunzi et al. (2017) indicated the prominent antibacterial effects of *C. erythrophyllum* leaf extract against multiple bacterial strains.

Phytochemical analysis can be conducted to detect the presence of phytometabolites of therapeutic importance. A detailed analysis of a plant's biologically active metabolite profile can prove to be beneficial in new drug discoveries (Thorat, 2018). The measure of the medicinal value of a plant is directly linked to its phytochemical constituents, such as alkaloids, phenols and essential oils (Raks et al., 2018). Further studies demonstrating the true medicinal worth of traditionally utilized species will be highly beneficial, not only for further use in the modern drug development industry, but also to communities. There have been studies published on the phytochemical constituents and possible biological activity of the leaves of *Combretum erythrophyllum*, however, there have been limited or no studies published on the phytochemical constituents and biological activity of the stembark. Hence this study aims to provide a comparative analysis of the phytochemical constituents and possible pharmacogenetic properties of both the leaf and stembark extracts of *C. erythrophyllum*, by conducting a phytochemical analysis, energy- dispersive X-ray (EDX), gas chromatography-mass spectrometry (GC-MS), thin layer chromatography (TLC) and powder microscopy analysis. Information regarding both beneficial and potentially harmful compounds present within *C. erythrophyllum* was elucidated through the emanating study.

## 4.2. Materials and methods

### 4.2.1. Phytochemistry

#### 4.2.1.1. Plant collection

Fresh leaf (with attached petioles) and stembark material of *Combretum erythrophyllum* was obtained from the University of Kwa-Zulu Natal (Westville), Durban, South Africa (29°49′S; 30°59′E). A voucher specimen was previously submitted to the Herbarium (13476/2), University of KwaZulu-Natal, Westville Campus. The collected fresh material was left to dry at ambient temperature, ~25ºC for six weeks. The dried leaves were then crushed using a commercial Philipps HR7762, China, blender to obtain a fine powder-like material. Because of the hardy nature of stembark, samples were subsequently freeze-dried using liquid nitrogen and crushed. The material was stored away until further use.

#### 4.2.1.2. Extraction

Approximately 25 g of crushed leaf and stembark material were added to 125 ml of chloroform (organic solvent) in separate round-bottom flasks. These were attached to the reflux apparatus (set up as per Fig. 4.7), placed within an Electrothermal EMV0250/CE MK5 heating mantle and distillation commenced via the electrical application of heat. After approximately three hours of heating at 35ºC, the extract was filtered using a funnel and filter paper. This process was repeated three times to allow maximum extraction of compounds. Thereafter, this process was repeated using hexane, followed by methanol as the organic solvent of choice. The crude extract was then utilized for phytochemical analysis and thin layer chromatography.

#### 4.2.1.3. Phytochemical analysis

Various chemicals were added to portions of crude extracts to detect the presence of bioactive phytocompounds (adapted from Jaradat et al., 2015).

a.) *Fixed oils and fats*

Spot test: Crude extract (0.1 ml) was spotted on a sheet of filter paper and left to dry. The presence of an oil ring indicated a positive detection of fixed oils.

b.) *Saponins*

Foam test: Crude extract (0.5 ml) was added to 10 ml of distilled water and shaken rapidly, by hand or 15 min. The presence of saponin was indicated by a 1-2 cm layer of foam on the surface of the solution.

Froth test: Crude extract (2 ml) was diluted using 20 ml of distilled water and shaken rapidly, by hand for 15 min. A froth layer of 1 cm was an indicator of the presence of saponin.

c.) *Phenols*

Ferric chloride test: Two drops of $FeCl_3$ were mixed with 1 ml of crude extract. The presence of phenol was indicated by a green/black colour change.

d.) *Carbohydrates*

- Molisch: A 0.2 ml solution of alcoholic α-naphthol was added to 1 ml of crude extract and swirled. Concentrated $H_2SO_4$ (0.5 ml) was gently added to the solution and the visualization of a purple/red ring between solvent layers indicated the presence of carbohydrates.
- Benedict's: Crude extract (2 ml) was combined with 1 ml of Benedict's reagent. The solution was then boiled in a water bath at 60°C for 2 min. Reducing sugars were indicated by the formation of an orange-red precipitation.
- Fehling's: Crude extract (1 ml) and Fehling's A and B (1 ml) solutions were added to a test tube. The resulting solution was boiled in a water bath. Reducing sugars were indicated by the formation of a red precipitation.

e.) *Glycosides*

Salkowski's test: Two drops of Salkowski reagent were added to 2 ml of crude extract. Glycosides were observed by the formation of a red ring.

f.) *Sterols*

Chloroform (3 ml) and sulfuric acid (two drops) were added to 2 ml of crude extract. The visualization of a red and green ring between the layers indicated the presence of sterol and cholesterol, respectively.

g.) *Alkaloids*

- Mayer's: Mayer's regent (1 ml) was added to 2 ml of crude extract. A yellow precipitate was an indication of the presence of alkaloids.
- Wagner's: Wagner's reagent (1 ml) was added to 2 ml of crude extract. A brown/ red precipitate was indicative of the presence of alkaloids.
- Dragendorff's: Dragendorff's reagent (1 ml) and 2 ml of crude extract were combined. A red precipitate indicated the presence of alkaloids.

*h.) Flavonoids:*

Lead acetate: A 5% lead acetate solution (1 ml) and 5 ml of crude extract were combined. Generation of a white precipitate indicated the presence of flavonoids.

*i.) Mucilage and gums:*

Crude extract (1 ml) was infused with two drops of 0.5% Ruthenium Red. Mucilage and/or polysaccharides were positively identified by a red colour change.

#### 4.2.1.4. Thin layer chromatography (TLC)

The prepared extracts were evaluated using thin layer chromatography. This generalized analysis was conducted to identify phytometabolic compound classes found in the extracts. Using a pencil, a base line was drawn onto the edge of a silica gel 60 F254 plate. The plate was spotted with a droplet of each crude extract and placed into a beaker containing a mobile solution of 9 parts toluidine: 1-part ethyl acetate: 0.5-part formic acid: 0.5-part glacial acetic acid. The beaker was covered with foil and the solution was left to move up the plate. By active capillary action of the solution moving up the silica plate, a number of compound classes were visualized. A non-polar compound is known to travel faster in comparison to a polar compound(Meyers, 2000). The plate was viewed under ultraviolet light at wavelengths of 254 and 360 nm. The plate was then exposed to a solution comprising: 0.5 ml anisaldehyde solution, 10 ml glacial acetic acid, 85 ml methanol, and 5 ml sulfuric acid. After exposure to this solution, the plate was left in an oven at 105°C for 5-10 min. The apparatus was set up as shown in Figure 4.8.

Retention values (Rf) were calculated using the following formula:

$$\mathrm{Rf} = \frac{\text{Distance travelled by solvent}}{\text{Distance of the solvent front}}$$

#### 4.2.1.5. Gas chromatography-mass spectrometry (GC-MS)

The generated extracts were analyzed using GC-MS (QP-2010 SE Shimadzu, Japan), with an Rx_5Sil MS (Restek, Rxi-GC columns) capillary column. Helium was used as the carrier gas and all relevant flow rates were noted. Total flow rate was 4.9 ml $min^{-1}$ while the column and purge flow rates were recorded as 0.96 ml $min^{-1}$ and 3.0 ml $min^{-1}$, respectively. Temperatures of the injection port, detector, and oven were maintained at 220°C, 320°C, and 150°C, respectively. After a holding period of 2 min, the oven temperature was increased and held at 295°C. The applied injection volume was 1 µl and scan speed was a recorded to be 3333m. The sensitivity and accuracy of the equipment was tested using relative standards and calibration methods. The extracts were run in splitless mode for a duration of 37 min. As a result, molecular structures and mass were key features analyzed in compound identification. The National Institute of standards and Technology (NIST) database was used for compound identification (Sermakkani and Thangapandian, 2012; Adeyinka and Moodley, 2018).

#### 4.2.1.6. Energy- dispersive X-ray (EDX)

An energy dispersion X-ray analysis was performed to determine the elemental composition of leaf and stembark material. 0.2 mgs of powdered leaf and stembark material were placed on the surface

of individual sterile aluminum stubs. These stubs were then sputter coated with gold using the Quarum Q150 RES gold coater. The samples were subsequently viewed and analyzed using the Field Emission Gun Scanning Electron Microscope Zeiss FEGSEM Ultra Plus (Carl Zeiss, Germany), AzTec software (Oxford Instruments, UK).

**4.2.2. Fluorescence microscopy**

Powdered leaf and stembark material were utilized for this analysis. A portion of dried leaf/stembark material was sprinkled onto a clean glass slide and submerged within 1-2 drops of the prepared reagent of choice. The prepared slide was then gently swirled and left to incubate in a cool, dry, dark environment for 2- 3mins (until all the reagent was absorbed). The slides were viewed using the Nikon compound and fluorescence microscope, Nikon eclipse, 80i and Nikon DS-Fi1 microscopy (NIS-Elements D) using both brightfield light and ultraviolet 2a (330/380nm). The following reagents were utilized for the purpose of this study: water, sulfuric acid, acetic acid, aqueous sodium hydroxide, hydrochloric acid, ethanol, ethyl acetate, hexane, chloroform, methanol, petroleum ether, diethyl ether and acetone.

## 4.3. Results and Discussion

In the *Combretum* genus, exuded bioactive secondary metabolites are associated with the presence of trichomes (Mtunzi et al., 2017, Fahn, 1988). Trichomes are minute protuberances from the epidermis of plants and are said to be the sites of synthesis, as well as the reservoir of accumulated biological substances (Naidoo et al., 2012). The variations in morphology, quality, quantity or composition of exudate and function of secretory tissues are used to classify and distinguish between trichome types (Wagner et al., 2004, Choi and Kim, 2013). The exudate is of prime interest due to the presence of bioactive phytocompounds with possible medicinal properties; hence research into evaluating the presence of phytocompounds in trichome exudates is essential.

**Phytochemical analysis**

Preliminary phytochemical tests indicated the presence of multiple phytocompounds (Table 1). Polarity of compounds differ; hence, a variety of organic solvents were utilized to extract a maximum number of compounds present (Meyers, 2000). The following compounds were detected within the crude leaf extracts namely: carbohydrates, alkaloids, flavonoids, sterols, phenols, as well as fixed oils and fats (Table 1). Phytochemical analysis of the stembark extracts indicated the presence of the following compounds: carbohydrates, alkaloids, sterols, phenols, saponins, mucilage and gum, as well as fixed oils and fats (Table 1). Comparative analysis between the dried leaf and stembark samples showed a wider compound variety in the stembark. The leaf (hexane) extract and stembark (methanol) extract showed the highest number of phytometabolites present within this species.

Eloff et al. (2008) highlighted the presence of alkaloids, tannins, phenols and saponins in numerous *Combretum* species. In lieu, the presence of the above mentioned phytometabolites were noted in C. *erythrophyllum* (Table 1). Alkaloids were indicated by the formation of a reddish/brown ring produced upon addition of Dragendorff's reagent. The colour change indicates the formation of ion pairs, which occurs between metal atoms (of the reagent) and the nitrogen molecules (of alkaloids) present (Parbuntari et al., 2018). The detection of alkaloids in both leaf and stembark extracts of *C. erythrophyllum* (Table 1) denotes to its prominent use in traditional medicine (Eloff et al., 2008). Alkaloids are a diverse group of nitrogen-containing phytometabolites that have been key components in traditional medicine for centuries (Raks et al., 2018, Bribi, 2018). Research suggests that the therapeutic use of alkaloids can be dated back to the 19$^{th}$ century, where substances were isolated mainly for their narcotic and analgesic components (Shang et al., 2018). Continued research has broadened the utilization of alkaloids in the pharmaceutical industry (Roberts, 2013). Pharmacological activities of alkaloids include their uses as muscle relaxants, analgesics, antitussive agents, local anesthetics, as well as anti-cancer, anti-hypertensive and antiseptic agents (Bribi, 2018). Thus, the detection of alkaloids in extracts of *C. erythrophyllum* (Table 1) emphasizes the importance of their reported traditional use in treating sores and wounds, among many other applications in

medicine (Eloff et al., 2008). *Combretum* species such as *Combretum sokodense* and *Combretum racemosum* also tested positive for the presence of alkaloids in generated crude extracts (Onocha et al., 2005, Ibrahim et al., 2017). Although beneficial, incorrect alkaloid dosages could be lethal, hence further research into evaluating the minimal lethal administrative concentration of plants dense in alkaloids is required (Eloff et al., 2008). A range of alkaloids have been isolated and used in commercial medicine these include: quinine, morphine, caffeine, strychnine and cinchonine (Roberts and Strack, 2018). Although medically important, in plants these compounds aid in chemical defense and successful pollination (Matsuura and Fett-Neto, 2017).

Using the spot test, the presence of fixed oils and fats (essential oils) were identified by the production of an oil ring on filter paper. Table 1 indicates the presence of essential oils in the tested crude extracts. Thus, highlighting the possible medicinal value of this species, in relation to other members in this genus, which also contains essential oils (Mtunzi et al., 2017). Essential oils are hydrophobic, volatile compounds that are found in an estimated 10% of the world's known flora (Morone-Fortunato et al., 2010). The biological activity of essential oils is based on their chemical constituents (Chauke et al., 2018). They consist mainly of esters, hydrocarbons, oxides, ketones and aldehydes. Esters, oxides and aldehydes are known to exhibit antimicrobial, antiseptic and spasmolytic properties, while hydrocarbons and ketones are known for their cell-regenerating, sedative, neurotoxic, antitumor and antibacterial capabilities (Winkleman, 2018, Tiwari and Rana, 2015). The positive result for oils in leaf and stembark extracts of *C. erythrophyllum* (Table 1) emphasizes the link between their reported traditional use in treating bacterial diseases and the identified metabolite (Mtunzi et al., 2017). In addition, these oils are commonly known for their enchanting scents. In plants, the scent is used to ward off preying herbivores and to protect against pathogens (Chauke et al., 2018).

The stem extracts tested positive for saponins, mucilage and gums, which were not detected in the leaf extracts (Table 1). In addition, the presence of a foam layer above the diluted solution indicated the presence of saponins in the stembark extract. Saponins are amphipathic compounds that are classified by their structural differences (Thorat, 2018). These compounds are characteristically identified according to the formation of foam upon agitation, within an aqueous solution (Faizal and Geelen, 2013). Desai et al. (2009) elaborated on the plethora of medicinal properties associated to saponins. These include antibacterial, antifungal and anti-inflammatory properties, in addition to lowering of cholesterol levels and improving host immune systems. With reference to the above, pharmacological studies have found *C. erythrophyllum* to have antibacterial, anti-inflammatory, antifungal, genitourinary, cytotoxic and mutagenic properties (Mawoza and Ndove, 2015). *Combretum* species such as *Combretum sokodense* and *Combretum racemosum* also tested positive for the presence of saponins in the stem extract (Onocha et al., 2005; Ibrahim et al., 2017). In

addition, saponins are known to protect leaf surfaces by acting as deterrents to pathogens and preventing the growth of mould as surface agents(Faizal and Geelen, 2013).

Glycosides are naturally occurring compounds found in flora and were present in both the leaf and stembark extracts (Table 1). These are non-volatile, amorphous compounds which are highly prized in the medicinal industry, because of their numerous benefits (Thorat, 2018). In addition, glycosides protect plants from diseases and pests (Khan et al., 2018). Glycosides isolated from members of the *Combretum* genus are known to exhibit antifungal, anti-inflammatory and molluscicidal effects (Thorat, 2018). Promising results obtained from previous research of C*ombretum* species such as *Combretum racemosum*, show that glycosides are beneficial phytometabolites (Onocha et al., 2005; Chaudhari and Mengi, 2006; Dawe et al., 2013).

Phenolic compounds are structurally diverse secondary plant metabolites comprising of numerous groups such as tannins, flavonoids, combretastatin and quinones, amongst several others (Thorat, 2018; Lattanzio, 2018). The presence of tannins and phenols was indicated in the generated crude extract by a black/green colour change upon the addition of $FeCl_3$. Phenolic compounds were positively identified across all tested extracts (Table 1). These compounds are known to express the following major effects: anti-mutagenic, anti-inflammatory, apoptosis-inducing, anti-carcinogenic and antioxidant activity (Garcia-Macias et al., 2007). In addition, Masoko et al. (2007) associates the presence of tannins and saponins in *Combretum loefl*, to its profound anti-fungal activity.

Combretastatin is a phenol which may be present in members of the *Combretum* genus. Pettit et al. (1982) first isolated combretastatin from a common bushwillow tree known as *Combretum caffrum* and analyzed the benefits of this molecule in reducing cancer effects. Preliminary phytochemical analysis conducted on *Combretum albidum*, *Combretum racemosum* and *Combretum sokodense* extracts also indicated the presence of phenolic compounds (Onocha et al., 2005; Ibrahim et al., 2017; Chandar and Ramasamy, 2016).

Additionally, flavonoids were identified by the generation of a white precipitate upon the addition of lead acetate to the crude leaf extracts. As shown in Table 1, leaf extracts showed the presence of flavonoids, that were absent from the stembark extracts. Flavonoids are the largest phytometabolite group in the phenolic compound class (Karim et al., 2018). Thus far, over 9,000 flavonoids have been identified and isolated (Wang et al., 2018). Common flavonoids include kaempferol, quercetin and naringin (Wang et al., 2018). Flavonoids are known for their anti-cancer, antiviral, anti-allergic, anti-inflammatory, cholesterol-reducing and most importantly, antibacterial properties (Baron, 2018; Bujang, 2018). Current research suggests the use of flavonoids in reducing the symptoms of menopause as well as lowering the risk of osteoporosis (Monteiro et al., 2018; Kaleem and Ahmad, 2018). In a study by Berkoff (1998), it was observed that flavonoids reduced the release of inflammatory mediators within the cell membrane; thus, exhibiting anti-inflammatory effects. With

*C erythrophyllum*, Martini et al. (2004) attributes the plant's antibacterial effects to the presence of seven different flavonoids. Another study highlighted the presence of flavonoids within plant cell tissues, which serve as chemical barriers against microorganisms, possibly due to the permeability of flavonoids (Williamson et al. 2000). This supports the traditional use of *C. erythrophyllum* leaves to treat inflammation and disease (Mawoza and Ndove, 2015; Schmelzer and Gurib-Fakim, 2013).

In plants, these compounds are known to serve a role in flavor profile development, fruit colouration and defense (Harborne and Williams, 2000). Studies show that exposure to high levels of ultraviolet (UV) radiation results in the overall increase in the presence of flavonoids and phenols (Garcia-Macias et al., 2007). As seen in Table 1 the intense presence of phenols in both leaf and stembark extracts correlates with findings from Harborne and Williams (2000), suggesting that increased polyphenol levels were a direct defense mechanism against UV damage.

Table 4.1: Phytochemical analysis of *C. erythrophyllum* leaves and stembark.

| **Test** | **Leaves** | | | **Stembark** | | |
|---|---|---|---|---|---|---|
| | **Hexane** | **Chloro form** | **Methanol** | **Hexane** | **Chloro form** | **Methanol** |
| **Carbohydrates:** | | | | | | |
| • Molisch | ++ | ++ | ++ | ++ | ++ | ++ |
| • Benedicts | - | ++ | ++ | - | - | ++ |
| • Fehlings | ++ | ++ | - | + | ++ | ++ |
| **Alkaloids:** | | | | | | |
| • Mayers | ++ | - | ++ | ++ | + | + |
| • Wagners | ++ | ++ | - | ++ | + | - |
| • Dragensdorffs | ++ | - | ++ | ++ | + | + |
| **Flavonoids:** | | | | | | |
| • Lead acetate | ++ | + | + | - | - | - |
| **Saponins:** | | | | | | |
| • Froth | - | - | - | - | + | - |
| • Foam | - | - | - | + | - | + |
| **Glycosides**: | | | | | | |
| • Sulfuric acid | + | ++ | + | - | - | ++ |
| **Sterols:** | | | | | | |
| • Sterols | ++ | - | + | ++ | + | + |
| • Chloroform | - | + | - | + | - | ++ |
| **Phenols:** | | | | | | |
| • Ferric trichloride | + | + | ++ | + | + | ++ |
| **Mucilage and Gums:** | | | | | | |
| • Ruthenium red | - | - | - | - | - | ++ |
| **Fixed Oils and fats:** | | | | | | |
| • Filter paper | ++ | - | + | - | - | ++ |

Intensity of reaction: (-) negative reaction, (+) slight colour change) and (++) Intense colour change

**Gas Chromatography-Mass Spectrometry (GC-MS):**

Gas chromatography is a compound identification technique which allows for the qualitative and quantitative evaluation of samples (Kuppuswamy et al., 2013). In comparison, mass spectrometry allows for an extremely accurate molecular analysis, hence, the combination of both techniques allows for the best form of identification and separation of molecules (Chandar and Ramasamy, 2016). Compounds identified through this study are known to have numerous pharmacological properties that are prevalent in plant medicinal research.

The chemical constituents of *C. erythrophyllum* leaf and stembark extracts (hexane, chloroform and methanol) were evaluated using GC-MS. This was the first reported GC-MS analysis of *C. erythrophyllum*. GC-MS screening of the leaf and stembark extracts resulted in the cumulative identification of 45, 25 and 196 phytocompounds in the hexane, chloroform and methanolic extracts, respectively (Tables 4.2 – 3, Fig 4.1-3). Highest number of phytocompounds were detected in the methanolic extracts hence making it of prime interest. From the leaf methanolic extract, 107 phytocompounds were identified, whereas 95 phytocompounds were noted in the stembark extract. The analysis revealed many novel compounds. Within the leaf extract, the compound which exhibited the highest peak area (14.44%) was 9-octadecen-1-ol, (Z), whereas, in stembark extract, Lup-20(29)-en-3-ol acetate (3. beta) had the highest peak area (15.51%). Overall, an abundance of phytocompounds was detected across all tested extracts. Notably, literature has highlighted the medicinal importance of tetratetracontane, eicosane, octadecanoic acid, terephthalic acid, lupeol and phytol (Wal et al., 2011; Kumar et al., 2010).

Tetratetracontane had the highest peak in the hexane leaf extract (retention time: 26.745, peak area: 6.74%), this compound is highly prized for its antibacterial and antioxidant properties (Hajar and Gumgumjee, 2014; Rhetso et al., 2020). Eicosane from the hexane stembark extract had the highest peak (retention time: 23.236, peak area: 5.10%). This is an alkane compound, known for its prominent use in cosmetic, lubricant and petroleum industry. In addition, this compound maybe utilized in medicine as an antioxidant, antibacterial and anti-fungal agent (Ahsan et al., 2017).

Octadecanoic acid had the highest peak area in the chloroform leaf extract (retention time: 19.678, peak area: 5.39%), this compound is known for its use as a cosmetic, lubricant and fragrant. In addition, octadecanoic acid is known for its possible medicinal use as an antioxidant and antimicrobial agent, while also known to lower cholesterol levels (Sharma et al., 2021). Terephthalic acid had the highest peak area in the chloroform stembark extract (retention time: 24.578, peak area: 32.93%). Plants containing this compound are known for its prominent use in traditional medicine as an antioxidant agent with hypocholesterolemic activity (Osuntokun and Cristina, 2019).

Lupeol is known for its anti-inflammatory properties and is an effective therapeutic tool in aiding cancer treatment (Hassan et al., 2018). Other known pharmacological activities include prevention

or treatment of arthritis and diabetes (Wal et al., 2011). Phytol is known to exhibit antioxidant, anticancer, hypocholesterolemic, anticoronary, antimicrobial, antiarthritic, anti-inflammatory, antidiabetic and immunostimulatory properties (Kumar et al., 2010; Rhetso et al., 2020).

Such findings indicate possible pharmacological activities associated with this species and the phytocompounds found in.

**Table 4.2.** GC-MS phytochemical analysis of *C. erythrophyllum* leaf extract.

| No | Phytochemical compound | CAS NO. | Solvent | RT | Peak (%) | Pharmacological activity and References |
|---|---|---|---|---|---|---|
| 1 | Phenol, 2,4-bis(1,1-dimethylethyl)- | 96-76-4 | Chloroform<br>Hexane | 12.715<br>12.719 | 2.17<br>2.77 | Antibacterial activity (Jaradat et al., 2015) |
| 2 | *n*-Pentadecanol | 629-76-5 | Chloroform | 16.901 | 1.23 | Antioxidant and antidiabetic (Ali et al., 2021) |
| 3 | Phytol, acetate | 0-00-0 | Methanol | 17.052 | 3.09 | Unknown |
| 4 | n-Heptadecanol-1 | 1454-85-9 | Methanol | 17.522 | 6.13 | Anti-oxidant (Duke, 1994; Van Wyk, 2008) |
| 5 | Pentadecanoic acid | 1002-84-2 | Chloroform<br>Hexane<br>Methanol | 17.770<br>17.773<br>18.327 | 2.82<br>2.93<br>3.80 | Possible cancer prevention (Wang et al., 2018; Duke, 1994)] |
| 6 | Phytol | 150-86-7 | Hexane | 19.124 | 1.05 | Antimicrobial, anti-inflammatory and possible cancer prevention (Quijano-aviles et al., 2021) |
| 7 | 9-Octadecen-1-ol, (Z)- | 143-28-2 | Methanol | 19.287 | 14.44 | Emollien and delivery of medication (Duke, 1994) |
| 8 | cis,cis,cis-7,10,13-Hexadecatrienal | 56797-43-4 | Hexane | 19.447 | 2.68 | Antioxidant, antifungal and antibacterial (Alade et al., 2021) |
| 9 | *n*-Nonadecanol-1 | 1454-84-8 | Methanol | 19.509 | 6.33 | Antioxidant, anti-inflammatory and possible cancer prevention (Elamin et al., 2021; Kumar et al.,2021) |
| 10 | Eicosanoic acid | 506-30-9 | Hexane | 19.670 | 1.51 | Anti-inflammatory, anti-diabetic, antibacterial and anti-oxidant (Raks et al., 2018) |
| 11 | Octadecanoic acid | 57-11-4 | Hexane<br>Chloroform | 19.670<br>19.678 | 4.82<br>5.39 | Lowers cholesterol, antimicrobial, and anticancer activity [Wang et al., 2018, 38] |
| 12 | Phytol | 150-86-7 | Methanol | 19.741 | 4.10 | Antimicrobial, anti-inflammatory and anticancer [Quijano-aviles et al., 2021] |
| 13 | Thiophene, 2-butyl-5-hexyl- | 4806-12-6 | Methanol | 24.534 | 2.74 | Unknown |
| 14 | 13-Docosenamide, (Z)- | 112-84-5 | Hexane<br>Chloroform | 24.606<br>24.634 | 1.31<br>2.36 | anti-oxidant and antimicrobial (Joy et al., 1998) |
| 15 | Tetratetracontane | 7098-22-8 | Hexane | 26.745 | 6.74 | Cytoprotective and antioxidant (Kuppuswami et al., 2013) |
| 16 | 1-Heptacosanol | 2004-39-9 | Methanol | 27.334 | 2.98 | Membrane stabilizer [Duke, 1994, Van Wyk, 2008) |
| 17 | beta-Sitosterol | 83-46-5 | Hexane<br>Methanol | 28.384<br>29.247 | 2.21<br>9.06 | Chronic wound treatment, anti-inflammatory and anti-proliferation [Duke, 1994] |
| 18 | 9,19-Cyclolanost-25-en-3-ol, 24-methyl-, (3.b,24S) | 511-61-5 | Methanol | 30.022 | 2.78 | Unknown |

**Table 4.3**. GC-MS phytochemical analysis of *C. erythrophyllum* stembark extract.

| No | Phytochemical compound | CAS NO. | Solvent | RT | Peak (%) | Pharmacological activity and References |
|---|---|---|---|---|---|---|
| 1 | Phenol, 2,4-bis(1,1-dimethylethyl) | 96-76-4 | Hexane | 12.716 | 1.95 | Antibacterial activity (Shang et al., 2018) |
| 2 | *n*-Pentadecanol | 629-76-5 | Chloroform | 16.906 | 1.38 | Antioxidant and antidiabetic (Verma and Singh, 2008) |
| 3 | n-Heptadecanol-1 | 1454-85-9 | Methanol | 17.523 | 3.57 | Antioxidant (Singh et al., 2014; Van Wyk, 2008) |
| 4 | Pentadecanoic acid | 1002-84-2 | Hexane | 17.738 | 3.56 | Possible cancer prevention (Raks et al., 2018; Singh et al., 2014) |
| 5 | 9-Octadecen-1-ol, (Z)- | 143-28-2 | Methanol | 19.285 | 9.31 | Anti-neoplastic, Antioxidant, Natural moisturiser (Singh et al., 2014) |
| 6 | *n*-Nonadecanol-1 | 1454-84-8 | Methanol | 19.509 | 4.28 | Anti-acne, anti-inflammatory and possible cancer prevention [Wang et al., 2018, Williamson et al., 2000] |
| 7 | Octadecanoic acid | 57-11-4 | Hexane<br>Chloroform | 19.675<br>19.644 | 2.15<br>0.68 | Lowers cholesterol, antimicrobial, and anticancer activity [Raks et al., 2018; Sermakkani and Thangapandian, 2012] |
| 8 | Decanedioic acid, dibutyl ester | 109-43-3 | Hexane | 19.667 | 1.23 | Antimicrobial, Antifouling activity (Van Wyk, 2008) |
| 9 | 2-methyloctacosane | 0-00-0 | Hexane | 19.936 | 1.39 | Unknown |
| 10 | Eicosane | 7098-22-8 | Hexane | 23.236 | 5.10 | Antioxidant (Umarani and nethaji, 2021) |
| 11 | Phthalic acid, di(4-methylhept-3-yl) ester | 117-81-7 | Chloroform | 24.525 | 31.17 | Antioxidant, Antimicrobial Allelopathy (Wal et al., 2011) |
| 12 | Terephthalic acid, dodecyl 2-ethylhexyl ester | 6422-86-2 | Chloroform | 24.578 | 32.93 | Antioxidant, Hypocholesterolemic activity, Antimicrobial (Wagner et al., 2004) |
| 13 | 13-Docosenamide, (Z)- | 112-84-5 | Hexane<br>Chloroform<br>Methanol | 24.593<br>24.687<br>25.169 | 2.70<br>0.996<br>2.62 | Anti-oxidant and antimicrobial (Thorat, 2008) |
| 14 | Tetratetracontane | 7098-22-8 | Hexane | 26.080 | 3.38 | Cytoprotective and antioxidant (Schmelzer and Gurib-Fikim, 2013) |
| 15 | 1-Heptacosanol | 2004-39-9 | Methanol | 26.783 | 1.02 | Treats Diabetes, Potential anticancer activity, Antioxidant, Antimicrobial(Van Wyk, 2008) |
| 16 | Silane | 0-00-0 | Hexane | 28.901 | 1.39 | Unknown |
| 17 | beta-Sitosterol | 83-46-5 | Methanol | 29.247 | 4.20 | Chronic wound treatment, anti-inflammatory and anti-proliferation (Singh et al., 2014) |
| 18 | 4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11, | 0-00-0 | Methanol | 29.748 | 2.59 | Unknown |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 12,12a,14,14a,14b-octadecahydro-2H-picen-3-one | | | | | |
| 19 | Androst-5-en-17-ol, 4,4-dimethyl | 0-00-0 | Methanol | 30.088 | 2.08 | • Unknown |
| 20 | Lupeol | 545-47-1 | Methanol | 30.221 | 11.32 | Treats Arthritis, Treats Diabetes, Potential anticancer activity (Raks et al., 2018) |
| 21 | Lup-20(29)-en-3-ol, acetate, (3.beta.) | 1617-68-1 | Methanol | 32.432 | 15.51 | TreatsDiabetes,,,Potential anticancer activity (Raks et al., 2018) |

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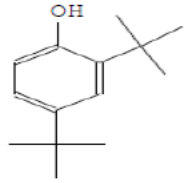

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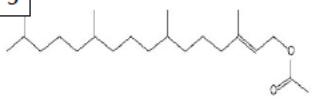

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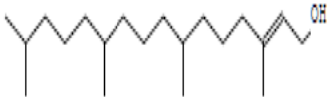

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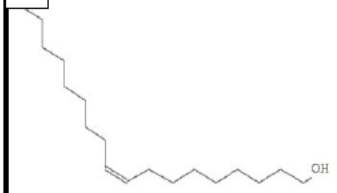

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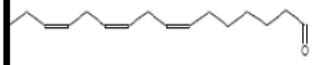

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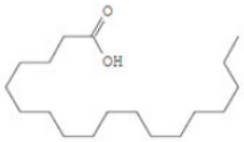

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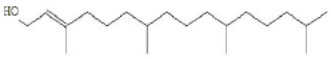

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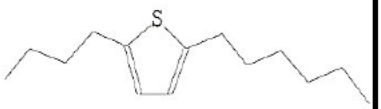

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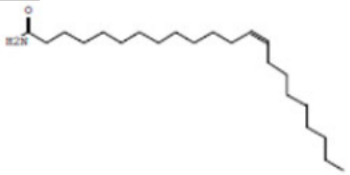

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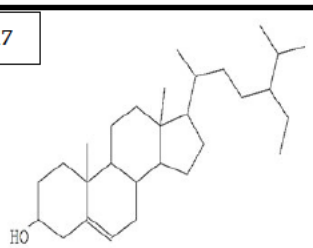

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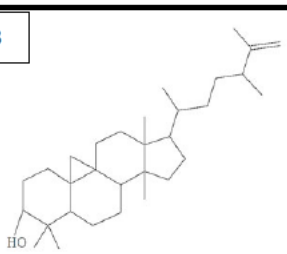

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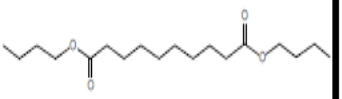

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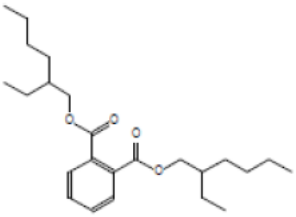

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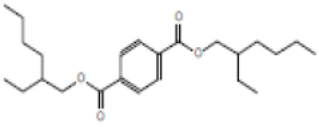

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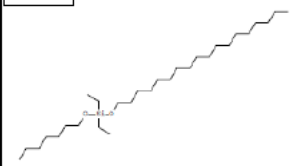

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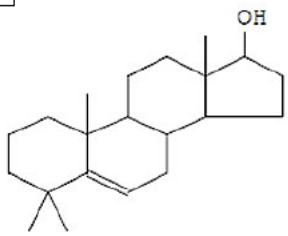

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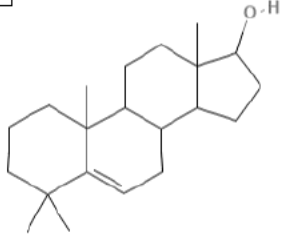

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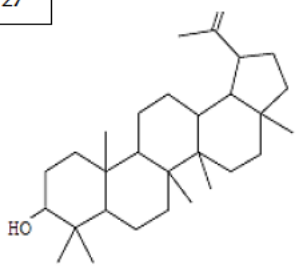

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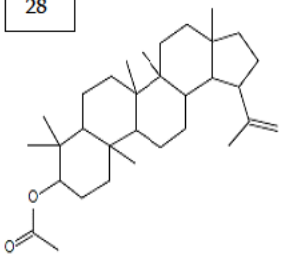

Figure 4.1. Chemical structures of compounds found in the leaf and stembark extracts of *C. erythrophyllum* (1, Phenol, 2,4-bis(1,1-dimethylethyl); 2, n-Pentadecanol; 3 Phytol,acetate;4,n-Heptadecanol-1;5, Pentadecanoic acid; 6, Phytol; 7, 9-Octadecen-1-ol, (Z)-; 8, cis,cis,cis-7,10,13-Hexadecatrienal; 9, n-Nonadecanol-1; 10, Eicosanoic acid; 11, Octadecanoic acid; 12, Phytol; 13, Thiophene, 2-butyl-5-hexyl-;14, 13-Docosenamide, (Z)-;15, Tetratetracontane;16, 1-Heptacosanol; 17, beta-Sitosterol; 18, 9,19-Cyclolanost-25-en-3-ol, 24-methyl-, (3.b,24S); 19, Decanedioic acid, dibutyl ester; 20, 2-methyloctacosane; 21, Eicosane;22, Phthalic acid, di(4-methylhept-3-yl) ester; 23, Terephthalic acid, dodecyl 2-ethylhexyl ester; 24, Silane; 25, 4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydro-2H-picen-3-one;26, Androst-5-en-17-ol, 4,4-dimethyl-;27 Lupeol and Lup-20(29)-en-3-ol, acetate, (3.beta.)

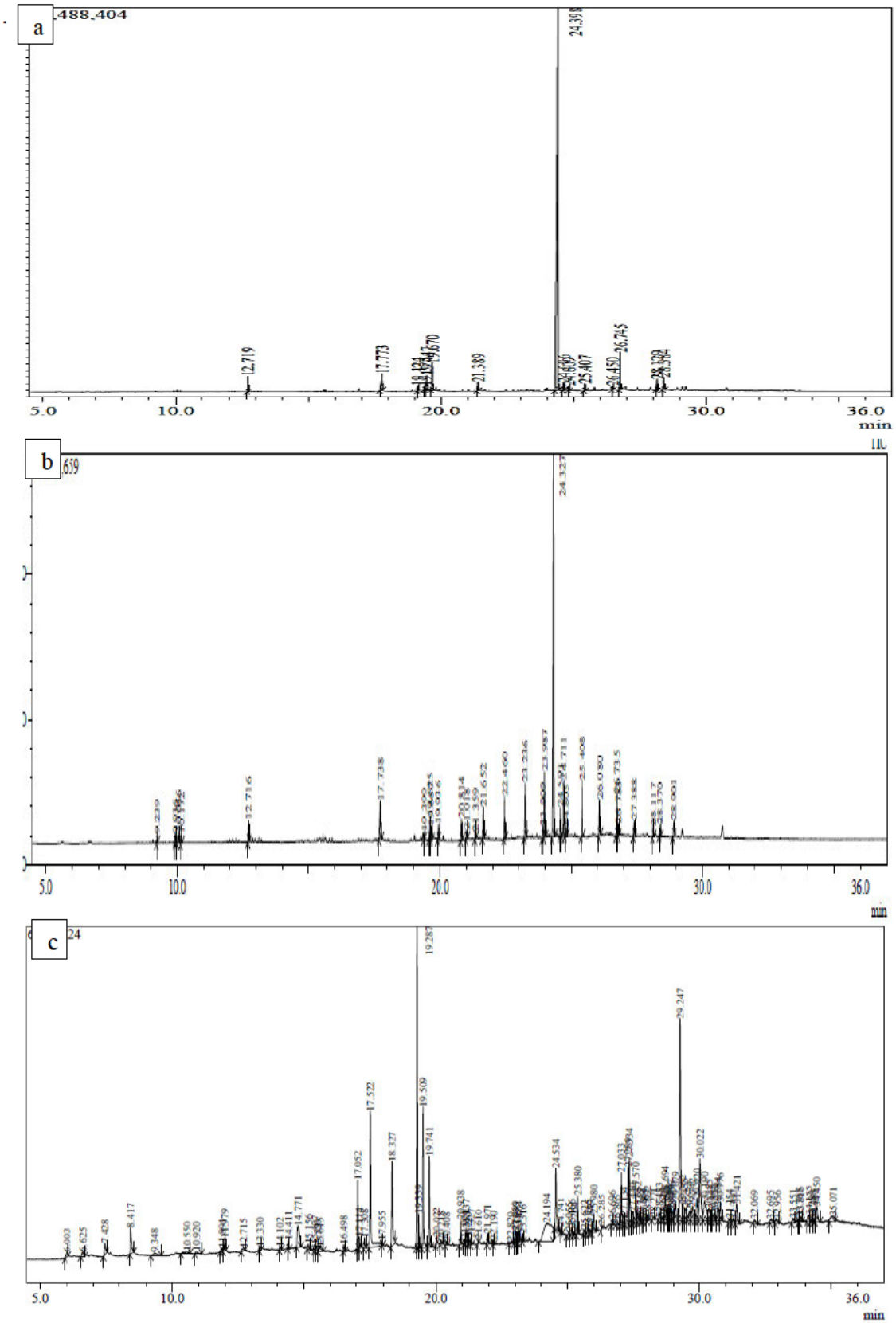


Figure 4.2: Total ion chromatograph of the a) hexanolic, b) chloroform and c) methanolic leaf extracts of *Combretum erythrophyllum*.

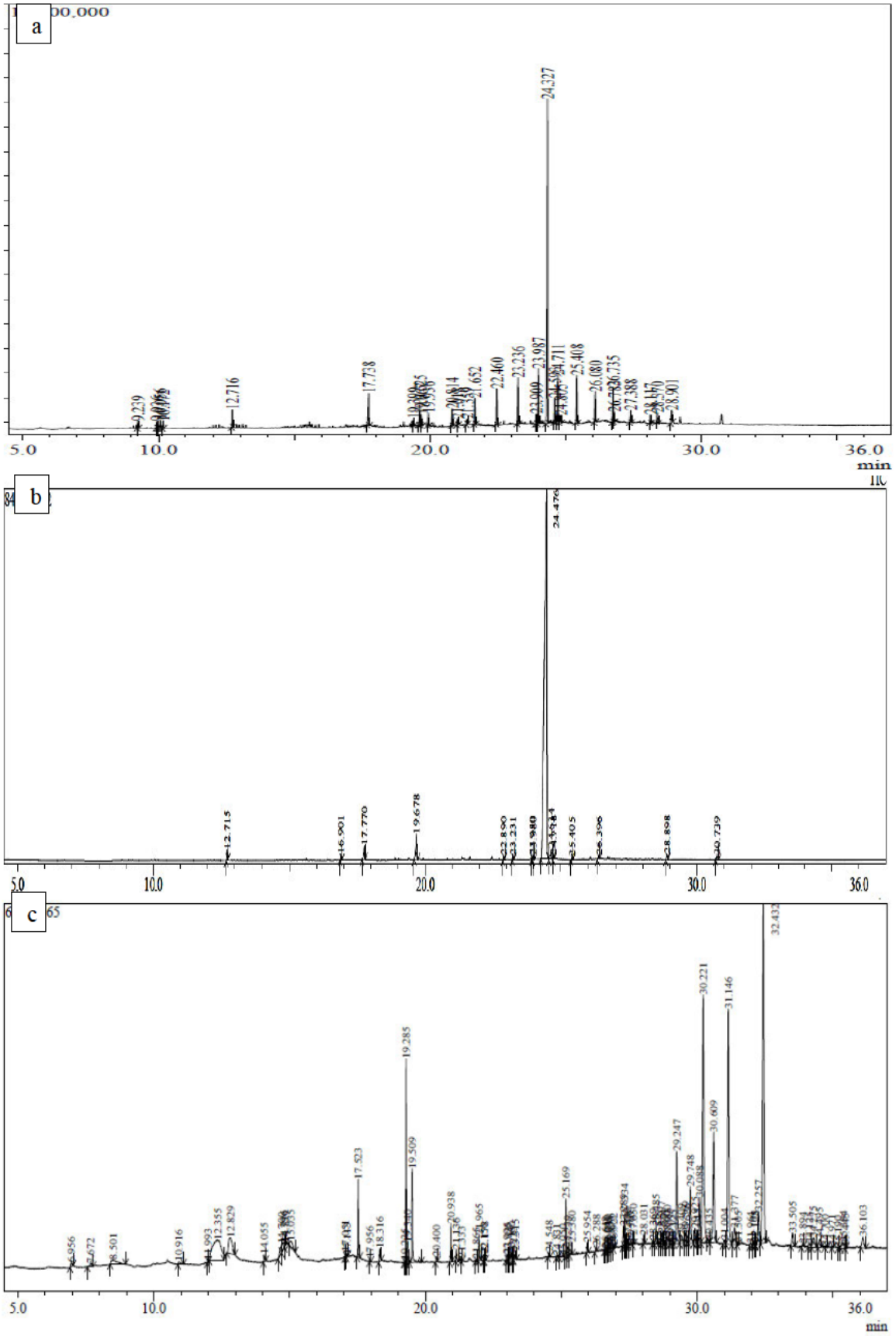


Figure 4.3: Total ion chromatograph of the a) hexanolic, b) chloroform and c) methanolic stembark extracts of *Combretum erythrophyllum*.

**Thin Layer Chromatography (TLC)**

TLC analysis resulted in the visualization of approximately 36 different compound classes (Fig. 4.4), when viewed under UV light (254 nm and 360 nm) and after exposure to anisaldehyde solution. Different classes of compounds were noted by the appearance of bands that resulted from the upward movement of the mobile solution on the plate. Compounds found closer to the base line are more polar than those compounds which have travelled further up (Meyers, 2000). A compound from the leaf hexane extract had the highest Rf value of 0.89 while a compound class from the stembark chloroform extract had the lowest Rf value of 0.06 thus indicating their possible polarity and distance travelled in the plate.

As compounds move up the plate, many colourless bands were formed; thus, the TLC plates were viewed under UV light of different wave lengths to aid visualization. Under normal light (Fig. 4.4), hexane, chloroform, and methanol leaf extracts were shown to have eight, one and three bands, respectively, whereas hexane, chloroform and methanol stembark extracts revealed eleven, two and five bands, respectively. When viewed under UV light (Fig. 4.4 b and c), six additional bands were indicated in the chloroform and methanol leaf extracts, as well as in the methanol stembark extract.

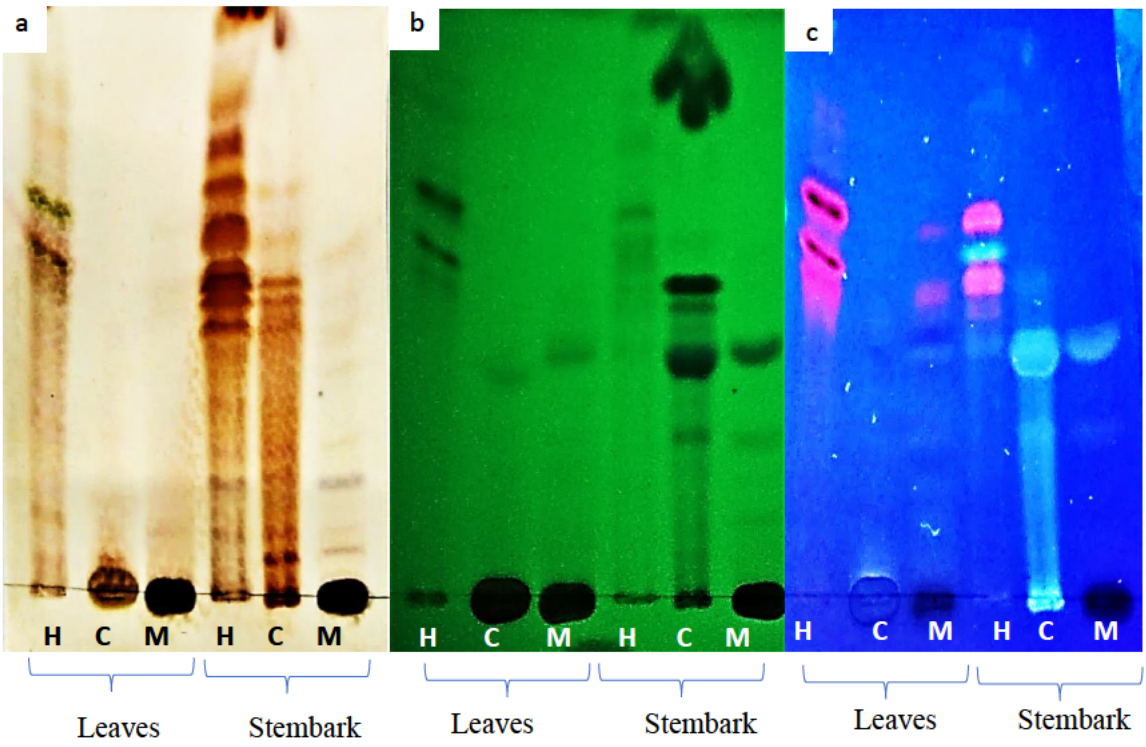


Figure 4.4: Thin layer chromatographs showing the separation of the hexane, chloroform and methanolic extract of the leaves and stembark of *C. erythrophyllum* using solvent system 9 toluidine: 1 ethyl acetate: 0.5 formic acid: 0.5 glacial acetic acid a.) after spraying with anisaldehyde solution, b.) under 254nm c.) under 360nm.

**Energy- dispersive X-ray (EDX)**

The elemental composition of powdered (dried) leaf and stembark material were characterized using the EDX analysis. Carbon (C), Oxygen (O), Chlorine (Cl), Potassium (K) and Calcium (CA) were found in both the extracts (Table 4.8).

Fundamentally, the presence of these elements are justified as they are essential for plant growth and development (Wink and Schimmer, 2018). The presence of C, O and Cl indicates an active metabolizing system (Braun and Pitt, 2018). The dense presence of calcium in the stembark (26.08%) may be related to the presence of crystals found (Paiva, 2021). In addition, the dense presence of chlorine may indicate the presence of a gelatinous covering across leaf and stembark surfaces (Balaz et al., 2021). Overall, the leaf extract was shown to encompass a wider variety of elements as opposed to the stembark (Fig. 4.5)

Furthermore, the powdered leaf material was characterized by the presence of Magnesium (Mg), Aluminum (Ag) and Silicon (Si). Notably, the presence of Mg, Al and K (essential nutrients) is well established in medicinal plants (Kumar et al., 2021). The distinct presence of these compounds in the leaf material, suggest that the leaf may be more medicinally inclined in comparison to the stembark. The presence of silicon in the leaf material is highly beneficial to the plant. Ecologically, this element may decrease transpiration rates (which in turn, prevents water loss) and protects the plant against a range of biotic and abiotic stresses (Kumar et al., 2021).

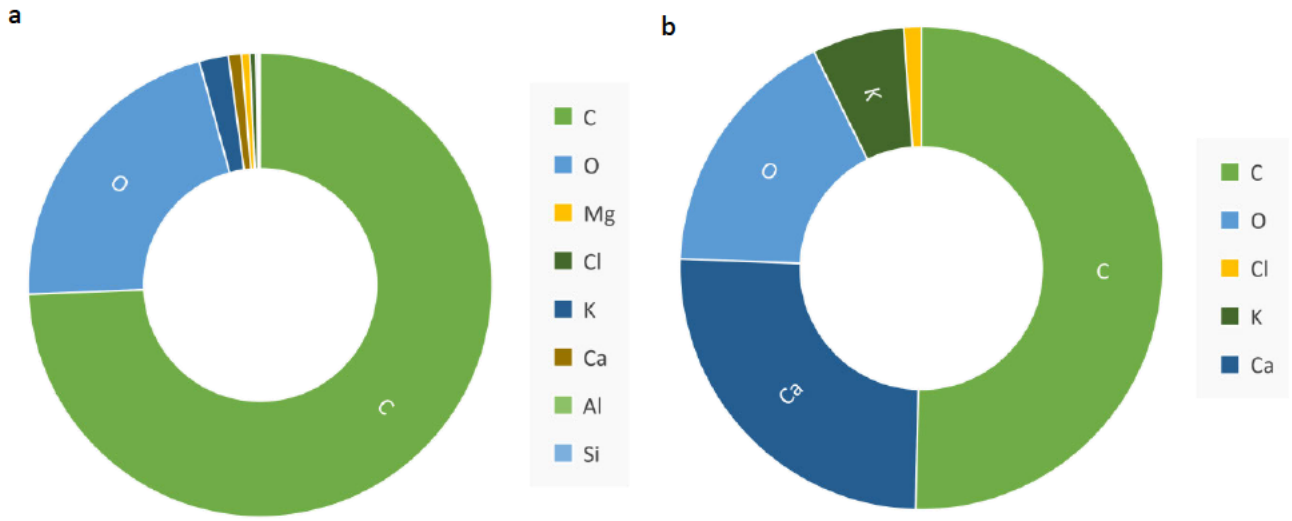


Figure 4.5: a) Elemental composition, Leaf, b) Elemental composition, Stembark.

Table 4.4: EDX elemental composition of leaf and stembark.

| ELEMENT: | % COMPOSITION: | |
|---|---|---|
| | LEAF | STEMBARK |
| **C** | 74,67 | 52,075 |
| **O** | 21,51 | 17,62 |
| **CL** | 0,43 | 1,22 |
| **K** | 2,05 | 6,37 |
| **CA** | 0,92 | 26,08 |
| **AL** | 0,14 | - |
| **MG** | 0,56 | - |
| **SI** | 0,17 | - |

**Fluorescence microscopy**

Powdered leaf and stembark material were utilized for this analysis. The fluorescence classification of crude powdered samples were evaluated under bright and UV2a (330/380nm) light. The emanating assay revealed various and distinct colour changes for the different samples (Table 4.5). On the addition of different reagents, noticeable colour changes were observed at various wavelengths. The colour change occurs when molecules emit a light as they return from an excited to non-excited state (Najar et al., 2021). Excited molecules scatter the absorbed light while fluorescing (Najar et al., 2021). Research indicates that various phytocompounds fluoresce at different wavelengths, many can do so under normal bright light whilst others, such as alkaloids, require the aid of UV intervention (Khan et al., 2016). The noticeable colour change of the crude powered samples were distinct and highly beneficial in identifying the possible phytochemical compounds of the sample (Singh et al., 2014). Furthermore, the emanating analysis clearly depicts the presence of two trichome types (peltate scale and non-glandular), various cellular components and the distinguishable colour changes noted when viewed using the UV2a (330/380nm) adapter (Fig 4.6 and Table 4.5). Overall, this analysis is a cost effective means of classification and identification of proposed medicinal plants and its phytocompounds. In order to establish the true ethnobotanical value of a plant, its botanical characterization needs to be fully explored (Najar et al., 2021). Hence, the information retrieved via this analysis is prudent in establishing the true ethnobotanical value of *C. erythrophyllum*.

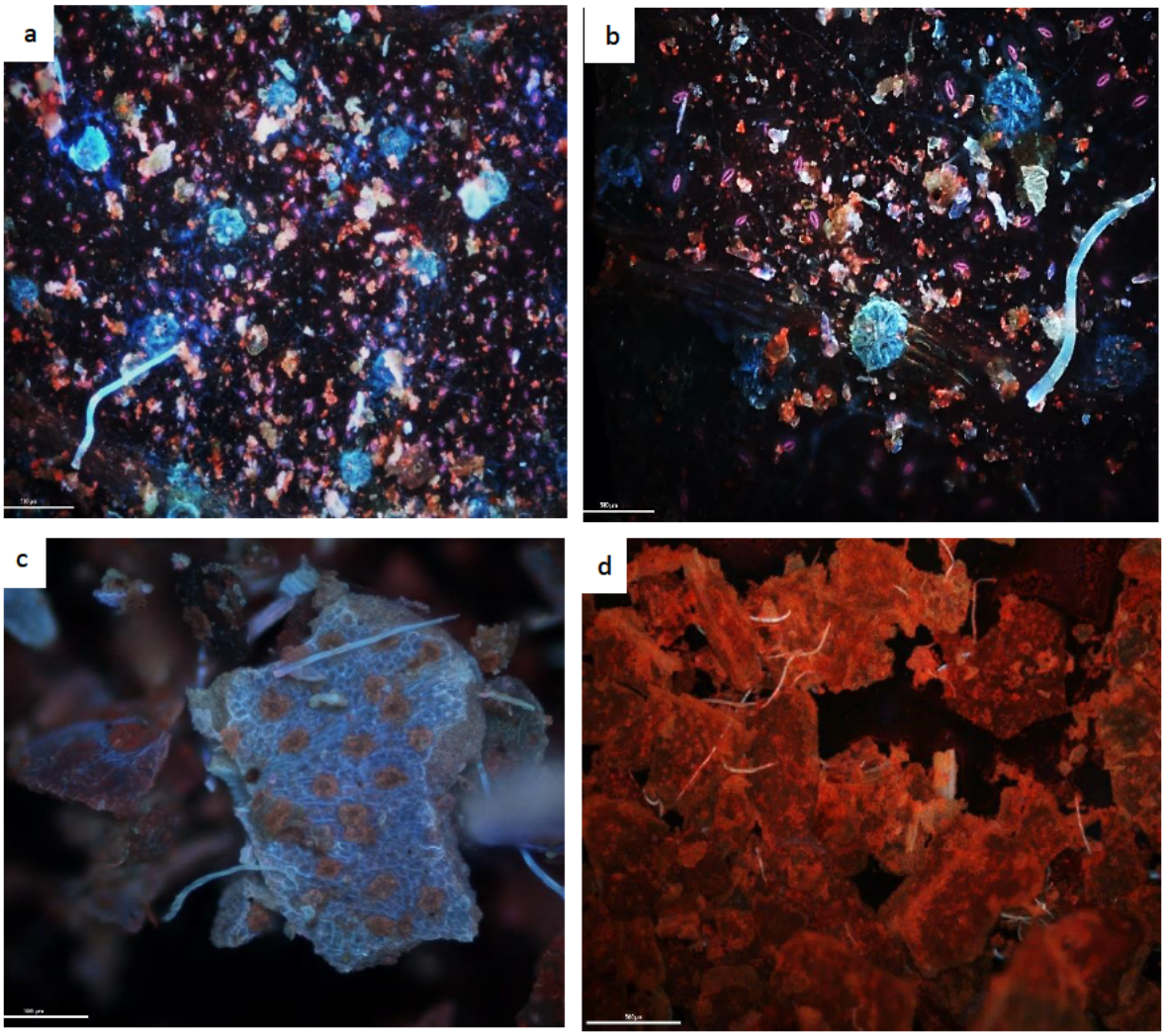


Figure 4.6: Fluorescence micrographs of powder leaf and stembark material viewed under UV2a (330-380nm a) powdered stembark material emersed in water, b) powdered leaf material emersed in water, c) powdered leaf material emersed in acetic acid and d) powdered leaf material emersed in ethanol.

Table 4.5: Powder microscopical analysis of the leaves and stembark of *Combretum erythrophyllum.*

| | COLOUR CHANGE | | | |
|---|---|---|---|---|
| | **Leaves** | | **Stembark** | |
| | **Bright light** | **UV (ex330/380)** | **Bright light** | **UV (ex330/380)** |
| **POWDER ONLY** | Dark green | Blue, red | Brown | Green, blue |
| **POWDER + WATER** | Greenish brown | Orange, blue | Brown | Green, blue, red |
| **POWDER + 50% $H_2SO_4$** | Brown | Orange, blue | Brown, red | Green, blue |
| **POWDER + ACETIC ACID** | Brown | Red, blue | Brown | Green, blue |
| **POWDER ONLY + AQUEOUS NAOH** | Brown | blue | Brown | Green, blue, red |
| **POWDER + HCL** | Greenish brown | Orange, blue | Brown, red | Green, blue, red |
| **POWDER + ETHANOL** | Greenish brown | Orange, blue | Brown, red | Green, blue, red |
| **POWDER + ETHYL ACETATE** | Greenish brown | Orange, blue | Brown, red | Green, blue, red |
| **POWDER + HEXANE** | Brown | Green, blue | Brown | Green, blue, red |
| **POWDER + CHLOROFORM** | Greenish brown | Green, blue, red | Green, brown, red | Green, blue, red |
| **POWDER + METHANOL** | Greenish brown | Orange, blue | Brown, red | Green, blue, red |
| **POWDER + PETROLEUM ETHER** | Greenish brown | Orange, blue | Brown, red | Green, blue, red |
| **POWDER + DIETHYL ETHER** | Greenish brown | Orange, blue | Brown, red | Green, blue, red |
| **POWDER + ACETONE** | Greenish brown | Orange, blue | Brown, red | Green, blue, red |

## 4.4 Conclusion

This study aimed to investigate the presence and potential biological activity of phytometabolites from the leaf and stembark extracts of *C. erythrophyllum*. Through the analyses, it can be concluded that *C. erythrophyllum* is indeed a plant worthy to be considered for its medicinal properties. The presence of phenols, sterols, flavonoids, saponins and alkaloids possibly indicate antiviral, antibacterial and antioxidant properties in the plant. However, further studies into identifying individual compounds, isolation, phytometabolite quantification and the feasibility of utilizing these extracts as beneficial medicinal extracts should be explored. In addition, anticancer, antioxidant and antimicrobial assays should be conducted to evaluate the biological activity of this species. It is possible that this plant could be the future panacea for the ills of people worldwide.

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4.6 Appendix


Figure 4.7: Crude extract generation protocol.

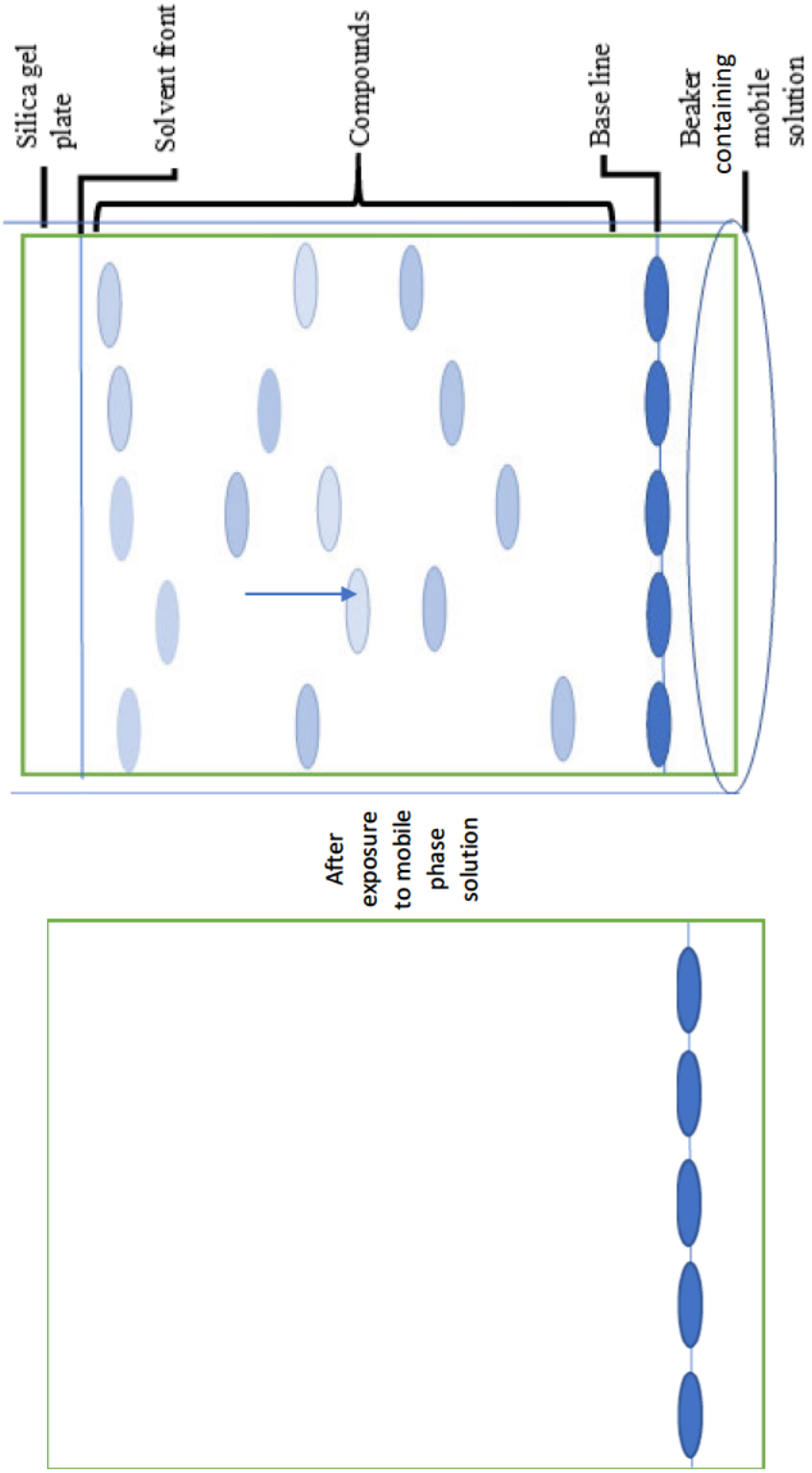


Figure 4.8: Thin layer chromatography setup.

# CHAPTER 5: BIOLOGICAL ACTIVITY OF *COMBRETUM ERYTHROPHYLLUM*: TOTAL PHENOLICS, ANTIOXIDANT ASSAYS, APOTOSIS ASSAY, CYTOTOXIC AND ANTIBACTERIAL SCREENING OF THE LEAF AND STEMBARK EXTRACT.

**Abstract**

Species of *Combretum* are highly valued in Africa due to the plethora of traditional medicinal uses they may offer. Species such as *Combretum erythrophyllum, Combretum molle, Combretum album, Combretum apiculatum, Combretum caffrum* and *Combretum quadrangulare* are commonly utilized and are known to contain medicinally important phytometabolites. Traditionally, *C. erythrophyllum* is used to treat bacterial infections, venereal diseases, abdominal pain, sores, infertility and labour pains, while displaying, anti-viral, anti-parasitic, cytotoxic and mutagenic activity. There are numerous published works available on the bioactivity of phytometabolites of the leaf extracts of *C. erythrophyllum*, however there has been limited or no studies published on the bioactivity of the stembark. Hence this study aimed to provide a comparative analysis of the biological activity of the leaf and stembark extracts of *C. erythrophyllum*. The following characters were evaluated through the emanating study: total flavonoid and phenolic content, antioxidant, antibacterial, cytotoxic and apoptosis activity of the leaf and stembark extract. Methanolic extracts appeared to have the highest possible antioxidant potential among all tested extracts and displayed the lowest IC50 values (leaf- 5,2866 and stembark- 4,2866 µg/mL) when evaluated using the DPPH assay, the methanolic extracts appeared to quantify the largest amount of compositional phenolic content (1341,05± 4,4mg / GAE/g). A positive correlation between % inhibition and extract concentrations, was noted for all assays. The extent/level of antioxidant activity was seen to be directly proportional to flavonoid and phenolic content. Extracts with the highest total phenolic content appeared to display strongest antibacterial and cytotoxic activity. This study integrated the use of fluorescence microscopy with acridine orange staining in order to accurately determine the viability of cells. A direct correlation was observed between the results obtained from the cytotoxicity and apoptosis assay. Focus should now be placed on isolating phytocompounds of importance from the best performing extracts. The transformation of an isolate into a drug of pharmacological importance has yet to be appraised on a large scale. Therefore, further evaluation of this species and particularly the transformation of the isolates needs to be explored as this species has shown immense medicinal potential.

Key words: Phytometabolites, Bioactivity, Bio-nanotechnology, Antibacterial activity, *Combretum erythrophyllum*

## 5.1. Introduction

Worldwide, the rate at which infectious diseases are spreading are of utmost concern (Soyemi, 2021). More importantly, a large percentage of these emerging diseases, mainly caused by pathogenic bacteria (eg. Methicillin resistant *Staphylococcus aureus(MRSA))* and viruses (eg. Human immune deficiency virus), are showing signs of drug resistance, rendering most conventional treatments ineffective (Sharaf et al., 2021). Furthermore, signs of drug resistance have also been noted in chemotherapy, a conventional method used in treating numerous cancer types (More et al., 2021). Due to the inefficiencies of current conventional treatments, researchers have had to divert attention towards alternate, natural methods of treatment which pose minimal side effects i.e., treatments derived from natural products (Hussain et al., 2021).

Pathogenic microbes are rapidly evolving, which makes the accurate and effect treatment of the diseases they cause highly difficult (Bottery et al., 2021). In addition, these evolved strains appear to display signs of drug resistance, hence rendering the use of most common antibiotics invalid. Generally, conventional antibiotics are derived from a specific bacterium designed to compete with and dissolve the pathogen in question (mechanism deployed: parasitism) (Vakhidova et al., 2021). A parasitic symbiotic relationship is created between the two bacteria. However, evolution has now favoured the survival of pathogenic bacteria by making them antibiotic resistant in nature. This phenomenon is facilitated by a series of rapid genetic mutations and the easy transfer of resistant genes between the microbes. Walsh (2000), identified three levels of antibiotic resistance. Firstly, the inactivation of the antibiotic by the disintegration of its chemical composition; secondly, controlled mutagenesis of the macromolecular target molecule and lastly the disruption of the antibiotic distribution pathway to the infection site. Alternate treatments which could nullify the effect of antibiotic resistance, needs to be explored. Recently, research evaluating the use of conventional drugs and plant extract to treat MRSA has been performed. In lieu, Sharaf et al., (2021) have evaluated the incorporative use of penicillin and isolates from *Zygophyllum album* L.fil. against MRSA. This combination proved highly successful, deeming promising inhibitory activity against MRSA. Incorporating conventional and alternate, natural treatment methods could be the way to a richer pharmaceutical future, opening avenues to discovering novel drug delivery systems.

Research suggests that cancer is one of the leading causes of fatality in the current age. This is mainly due to the sporadic growth and migration of these aggressive invasive cancer cells. Due to the nature of these cells, they are known to pose a great obstacle in conventional cancer treatment methods (such as chemotherapy) (More et al., 2021). Conventional methods of treating this ailment have proven to be highly detrimental to the host, with extreme side effects as chemotherapeutic agents are extremely toxic in nature (Paladini et la., 2016). These methods are not cost or time effective, bearing minimal results, in most cases. The main problem associated with conventional therapy is drug

resistance or multi drug resistance (MDR) (Zheng et al., 2021). Medical professionals have reported that some cancer cells have shown insensitivity to chemotherapeutic agents. Avenues to overcome this insensitivity are continuously being explored with much success discovered in the medicinal plants world. Bioactive compounds, such as quercetin, curcumin and tetrandrine, extracted from medicinal plants, have shown to restore chemotherapy sensitivity, hence incorporating plant-based therapy with conventional cancer treatment holds much promise (Sundaram et al., 2021; Tagde et al., 2021).

Medicinal plants are highly valued for their phytochemical constituents that can be used in drug development (Kayser, 2018). Certain plant species are known to thrive against microbial disruptions due to the presence of bioactive compounds with antimicrobial potential. These bioactive compounds are traditionally known to treat and cure infections and diseases within humans (Burman et al., 2018). The presence of phenolic compounds, flavonoids, glycosides, alkaloids and saponins has been associated with the antibacterial properties derived from certain plants (Thorat, 2018). Martini and Eloff (1998) stated that C. *erythrophyllum* is known to contain numerous antibacterial compounds, each with high biological activity.

Further studies demonstrating the true medicinal worth of traditionally utilized species will be highly beneficial, not only for further use in the modern drug development industry, but also to communities. Reports generated by the WHO, suggests that there have been no novel drug discoveries since 1961, but rather modifications of existing drugs (Singh et al., 2021). There is no balance between the number of evolving microbes and relevant drug discovery (Phuyal et al., 2020). Thus, further research evaluating the use of medicinal plant species to treat these ailments is prudent. There have been studies conducted on the phytochemical constituents and possible biological activity of the leaves of *Combretum erythrophyllum*, however, there have been limited or no studies conducted on the phytochemical constituents and biological activity of the stembark. Hence this study aims to provide a comparative analysis of the biological activity of the leaf and stembark extracts of *C. erythrophyllum*. The following will be elucidated from the emanating study: total phenolic content, total flavonoid content, antioxidant properties, apoptosis, cytotoxic and antibacterial screening of the leaf and stembark extract.

## 5.2. Materials and method

### 5.2.1. Generation of crude extract

#### 5.2.1.1. Plant collection

Plant material of *C. erythrophyllum* was obtained from the University of Kwa-Zulu Natal (Westville), Durban, South Africa (29°49′S; 30°59′E). A voucher specimen was previously submitted to the Herbarium (13476/2), University of KwaZulu-Natal, Westville Campus. The collected fresh material was left to air dry at ambient temperatures of 23-25°C for six weeks. The dried leaf and stembark material were then crushed into a fine powder using a blender (Philipps HR7762, China) and stored in cool and dark conditions until further use.

#### 5.2.1.2. Extraction

Approximately 10 g of crushed leaf material was added to 100 ml of analytical reagent grade hexane (organic solvent) in a round bottom flask, attached to the reflux apparatus and placed in a heating mantle. The mantle was set at a low heat and distillation commenced. After approximately three hours of heating, the extract was filtered using a funnel and filter paper (Whatman No. 1). This process was repeated three times to allow maximum extraction of compounds. This process was then repeated using chloroform, followed by methanol, as the organic solvents of choice. Thereafter, the crushed stembark material was processed following the above method. Generated extracts were utilized for nanoparticle synthesis, characterisation and various assays evaluating the biological activity of the synthesised material.

### 5.2.2. Antioxidant assays

#### 5.2.2.1. Extract concentrations

One mg/mL stock solution, of each extract, was generated for the purposes of these assays. Subsequently, aliquots of each extract were diluted to obtain final concentrations of 15, 30, 60, 120 and 240 µg/mL.

These concentrations were utilized for a range of antioxidant, total phenolics and total flavonoids assays.

#### 5.2.2.2. DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) scavenging activity

A sterile 96 well microplate was inoculated with 50 µL a 0.3 mM solution of DPPH (dissolved in methanol) and 100 µL of each extract. The solutions were thoroughly blended and the microplates wrapped in foil and placed in a dark environment at ambient temperatures for 30 minutes. Ascorbic acid was utilized as the standard of choice (positive control). After the incubation period, the absorbance was read at 517 nm using the Synergy HTX multimode microplate reader, BioTek

Instruments Inc., (Winooski, USA), equipped with the Gen5 software for data collection and analysis.

The DPPH scavenging activity was calculated using the following equation:

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

#### 5.2.2.3. Ferric ($Fe^{+3}$) reducing antioxidant power

A sterile 96 well microplate was inoculated with 25 µL of a 0.2 M sodium phosphate buffer, 50 µL of 1% potassium ferricyanide (dissolved in distilled water) and 25 µL of each extract. The solutions were thoroughly blended and microplates incubated at ambient temperatures for 30 minutes. Gallic acid was utilized as the positive control. After the incubation period, 25 µL of 10% trichloroacetic acid (TCA), 25 µL of distilled water and 5 µL of 0.1% ferric chloride was introduced into each well. The solutions were thoroughly blended, incubated for a further 10 minutes and the absorbances read at 700 nm using the Synergy HTX multimode microplate reader, BioTek Instruments Inc., (Winooski, USA), equipped with the Gen5 software for data collection and analysis. The Ferric ($Fe^{+3}$) reducing antioxidant power was calculated using the following equation:

$$\text{\% Inhibition} = \left({}^{\text{Abs of sample}}/_{\text{Abs of Gallic acid}}\right) \times 100$$

#### 5.2.2.4. Total phenolic content

In order to quantify the total phenolic content of the tested extract, the Folin–Ciocalteu reagent assay was carried out. A sterile 96 well microplate was inoculated with 150 µL of 10% diluted Folin-Ciocalteau reagent, 120 µL of 0.7 M $Na_2CO_3$ and 30 µL of the extracts. The solutions were thoroughly blended and microplates incubated with shaking on a rotary shaker, at ambient temperatures for 30 minutes. Gallic acid was utilized as the positive control. The solutions were thoroughly blended, and then the absorbances were read at 765 nm using the Synergy HTX multimode microplate reader, BioTek Instruments Inc., (Winooski, USA), equipped with the Gen5 software for data collection and analysis. The total phenolic content was calculated using the following equation:

$$C_{tp} = Concentration * {}^{\text{Volume}}/_{\text{mass}}$$

**5.2.2.5. Total flavonoid content**

A sterile 96 well microplate was inoculated with 40 µL of plant extract, 200 µL of water, 15 µL of 5% sodium nitrate, 15 µL of 10% aluminum chloride and 15 µL of sodium hydroxide. Quercetin was utilized as the positive control. The solutions were thoroughly blended, and the absorbances read at 510 nm using the Synergy HTX multimode microplate reader, BioTek Instruments Inc., (Winooski, USA), equipped with the Gen5 software for data collection and analysis.

The total flavonoid content was calculated using the following equation:

$$C_{tf} = \text{Concentration} * {}^{\text{Volume}}/_{\text{mass}}$$

**5.2.3. Antibacterial screening**

The antibacterial properties of the generated extracts (Concentrations: 10, 12.5, 25, 50 and 100 mg/mL) were screened against 5 different bacterial strains: *Escherichia coli* (ATCC 35218*)*, *Pseudomonas aeruginosa (*ATCC 25783*), Bacillus subtilis (ATCC:6633)*, *Staphylococcus aureus (*ATCC: 25923*)* and Methicillin-Resistance *Staphylococcus aureus* (ATCC: 43300). In addition, positive controls were used. Streptomycin was used against gram positive bacteria and gentamycin was used against gram negative bacteria.

The medium was prepared using Biolab Mueller Hilton agar, South Africa (38 g) and distilled water (1L). Following this, 20 ml of media were poured into sterile Petri dishes and allowed to set. A prepared nutrient broth (yeast extract, 5 g; tryptone powder, 10 g; NaCl, 10 g) was used to grow bacterial cultures (incubation period of 24 h). A 0.5 McFarland turbidity standard was used as a guide to manipulate the turbidity of the bacterial cultures. This standard provided an optimum density at which a comparable range of bacterial cell growth was found. This method provided a standardized platform on which the growth or inhibition of different microorganisms were compared. The optical density of each bacterial strain was measured at 620 nm using the Agilent Technologies Cary 60 UV-Vis. The concentrations of the bacterial broths were adjusted to obtain a resulting optical density of 0.1. The bacterial cultures were then evenly spread over the agar with a sterile loop and spreader. All relevant materials and media were autoclaved. 1cm sterile filter paper discs were inoculated 90 µL of extract and left to dry. These discs were then systemically placed on the agar and the plates were incubated for a period of 24 h. Gram positive bacterial strains were incubated at 30°C while gram negative strains were incubated at 37°C.

These plates were then analyzed for apparent zones of inhibition.

### 5.2.4. Cytotoxicity assay

#### 5.2.4.1. Preparation of extracts: Concentrations

A five mg/mL stock solution was generated for the purpose of this assay. Portions of these extracts were, subsequently, diluted, using dimethylsulphoxide (DMSO), to formulate the concentrations mentioned below.

Concentrations evaluated: 15, 30, 60, 120 and 240 µg/mL.

#### 5.2.4.2. MTT assay

The cytotoxic effect of the generated extracts was evaluated against three cell lines, human embryonic kidney cells (HEK293), human cervical carcinoma cells (HeLa) and human breast adenocarcinoma cells (MCF-7) (All cell lines sourced from ATCC, Manasas, VA, USA). Cells were incubated in sterile 96 well plates, (cell densities: 250000-300000 cells per well) overnight at 37˚C. Thereafter the medium was replaced with fresh medium, followed by addition of 100µL of each extract at their varying concentrations. The plate was then incubated for 48 hours at 37°C. This was done in triplicate.

The growth medium was removed and replaced with 0.1 ml of medium containing 10% MTT solution (5 mg/mL in PBS). Cells were incubated at 37˚C for a further 4 hours. The MTT containing medium was then removed and 0.1 ml DMSO was added to the wells and the absorbances measured at 570 nm using a Mindray MR-96A microplate reader (Vacutec, Hamburg, Germany).

### 5.2.5. Apoptosis assay:

In order to evaluate the apoptotic potential of the generated extracts, an apoptosis analysis using acridine orange/ ethidium bromide (AO/EB) staining was performed. Cells were incubated in sterile 96 well plates, (cell densities: 250000-300000 cells per well) overnight at 37°C. The growth medium was then aspirated and substituted with 250 µL of complete medium. Thereafter, 150 µL of extract (most toxic cytotoxic concentration was tested 240 µg) was added to each well and the plate was then incubated for 24 hours at 37°C. Subsequently, all remaining growth medium was removed and the cells were briefly rinsed (twice) using 200 µL of PBS. Cells were then stained using 10 µl of the dye (100 µg/ml acridine orange, 100 µg/ml ethidium bromide in PBS). The plate was shaken for 5 minutes on a Stuart Scientific platform rocker at 30 rev/min. The cells were then washed with PBS and all cells viewed using an inverted fluorescence microscope (Olympus CKX41, Tokyo, Japan) at excitation and emission wavelengths of 490 nm and 516 nm respectively. In order to quantify the apoptotic properties of the extracts against the tested cell lines, the apoptotic index was calculated using the equation listed below.

$$Apoptotic\ Index = \frac{\text{Number of apoptotic } cells}{\text{Number of total cells counted}}$$

### 5.2.6. Statistical analysis

The emanating study was performed in triplicate. All generated data was subjected to a statistical analysis using the IBM SPSS, statistical analysis software version 27, IBM CORP1997, USA.

A *post hoc*, tukey's- honest significant difference test was performed. All data was normalised and a significant difference was noted among all data sets, $P<0.05$.

## 5.3. Results and discussion:

### 5.3.1. Antioxidant assays

Singh et al. (2021) have stressed the importance of the controlled presence of antioxidants within the human body. It was further suggested that antioxidants help maintain an homeostatic environment within the body by regulating the quantity of both free radicals and antioxidants present within. A deficit or excess of either may lead to health conditions, such as oxidative stress related ailments. As the body ages, its natural ability to restore this balance diminishes hence alternative measures are required to prevent the onset of further health issues. Conventional treatments, such as vitamins (with added antioxidant potential) may aid in restoring the balance but could result in additional harm, hence modern alternatives incorporating herbal extracts are being explored (Van der Goot et al., 2016). Interest in incorporating medicinal plants with antioxidant capabilities and conventional treatment methods has increased. Literature suggests that medicinal plants are a rich source of phytochemicals and in turn a possible source of naturally occurring antioxidants (Bantho et al., 2020). These naturally occurring antioxidants are known to inhibit free radicals while disrupting oxidative chain reactions within cells. Due to the naturally occurring antioxidants found in plants, research suggest that those prone (elderly and vulnerable) to oxidative stress related ailments, incorporate a richer plant-based diet (Kitts et al., 2000). Phytochemicals such as, triterpenes, phenols, flavonoids and resins are known to exhibit radical scavenging activity (Annadurai et al., 2021; Akwu et al., 2019). There are numerous assays that may be performed in order to quantify the antioxidant potential of plant extracts, namely: DPPH, FRAP, ABTS etc. (Neri et al., 2021).

Numerous researchers have highlighted the antioxidant ability of multiple *Combretum* species (Hamza et al., 2021; Sousa et al., 2021; Coulidiati et al., 2009). These studies revealed that the quantity of phenolic compounds is directly proportional to the antioxidant activity associated with the plant. These results correlate with the findings obtained from this study. Species such as a *Combretum sericeum* and *Combretum acutum* exhibited high levels of antioxidant activity while simultaneously exhibiting high phenol and flavonoid content (Masoko and Eloff, 2007).

#### 5.3.1.1. Assay of 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH)

DPPH is a stable free radical that can attach to an unstable electron or hydrogen radical to stabilize the molecule (Singh et al., 2021). A colour change is observed when DPPH radicals react with complementary reducing agents. In this process electrons are absorbed and the colour change occurs, simultaneously. Fundamentally, this assay induces a violet to orange yellow colour change within the tested extract, distinctly indicating the free radical scavenging activity occurring within the extract. The free radical scavenging activity indicates the presence of possible antioxidant ability. In order to evaluate the antioxidant ability of the generated extracts, a DPPH (2,2-diphenyl-1-picryl-

hydrazyl-hydrate) assay was performed. This assay was performed on the crude hexane, chloroform and methanolic leaf and stembark extracts of *C. erythrophyllum* (at varying concentrations).

The DPPH analysis indicated that all extracts displayed varying free radical scavenging activity, implying possible antioxidant potential (Table 5.1, Fig. 5.1c). Furthermore, a positive correlation between % inhibition and extract concentrations, was noted. The chloroform leaf and stembark extracts displayed the highest IC50 values (120,7063 and 265,4076 µg/mL, respectively) indicating the lowest overall antioxidant potential from all extracts (Table 5.1). This was further substantiated by the level of colour change observed after the addition of DPPH (Fig. 5.1a and b). Extracts still displayed a light purple/ violet colour, indicating low free radical scavenging activity. The methanolic extracts displayed the lowest IC50 values (leaf- 5,2866 and stembark- 4,2866 µg/mL) indicating the highest possible antioxidant potential from all tested extracts (Table 5.1). This was further confirmed by the colour change observed after the addition of DPPH (Fig. 5.1a and b). Extracts displayed a distinctive orange yellow colour, indicating high free radical scavenging potential. Overall, IC50 values of the tested crude extracts were significantly lower than the positive control (ascorbic acid).

Recently, many C*ombretum* species have been evaluated for their potential antioxidant capabilities. The antioxidant potential of the roots of *Combretum album* Pers. was recently evaluated, with substantial antioxidant properties quantified from the methanolic extracts (highest antioxidant activity- IC50 12.98 µg/mL) (Burman and Chandra, 2021). *Combretum leprosum* Mart. was evaluated for a range of properties, with both the leaf and stembark fractions displaying significant antioxidant potential. However, the stembark fraction appeared to project immense antioxidant activity (Sousa et al., 2021). This in turn, correlates with the results from the emanating study, substantiating the increased antioxidant potential noted within the stembark extracts, which appears to be characteristic among some *Combretum* species.

Table 5.1: DPPH free radical scavenging activities (IC50 values) of the leaf and stembark extracts (hexane, chloroform and methanol) of *C. erythrophyllum* (tukeys' honest significant difference multiple range post Hoc test $P < 0.05$ IBM SPSS version 27).

| Extracts | IC50 (µg/ml) | |
|---|---|---|
| | Leaves | Stembark |
| Hexane | 31,2543 | 13,55683 |
| Chloroform | 120,7063 | 265,4076 |
| Methanol | 5,2866 | 4,2866 |

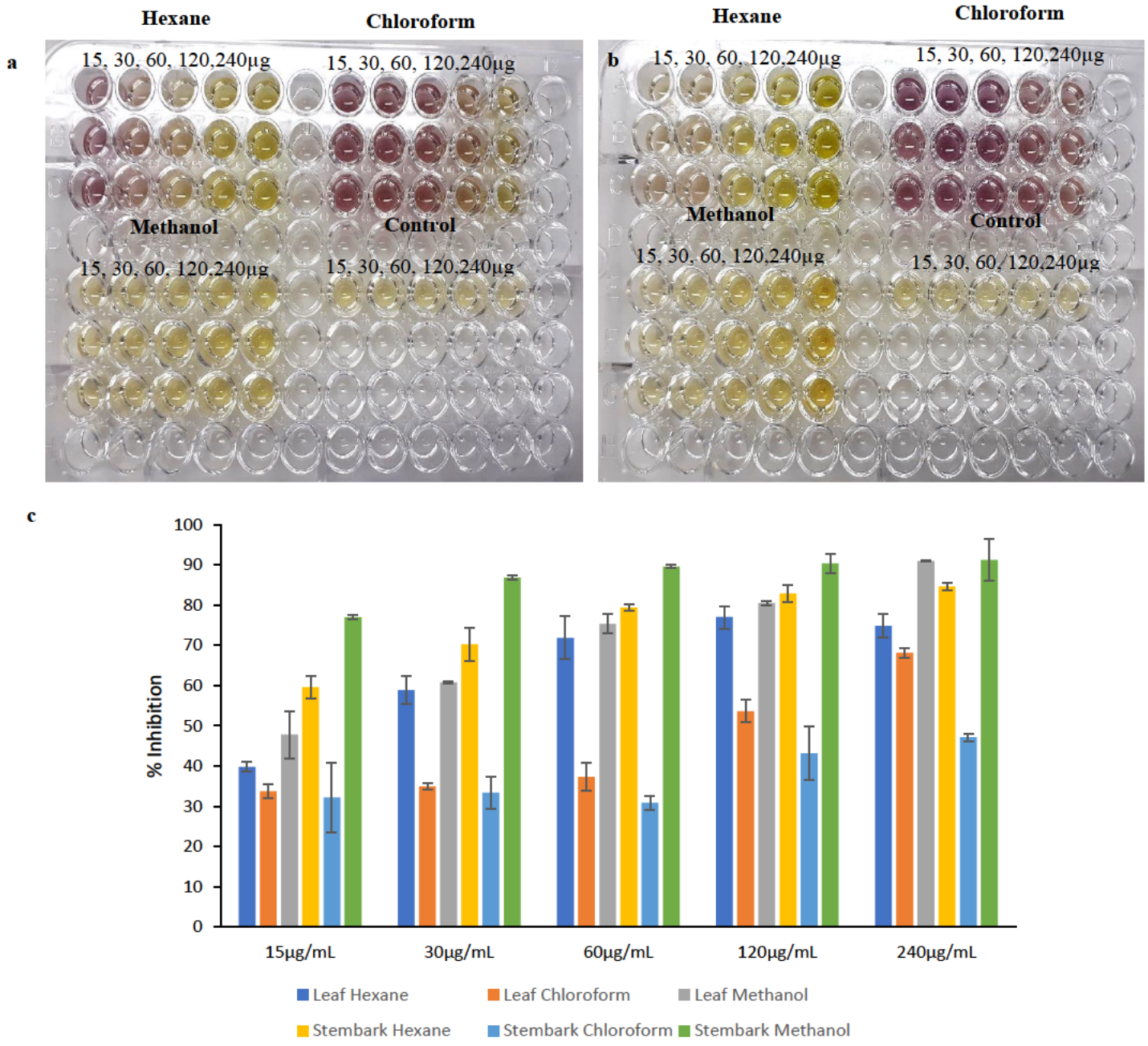


Figure 5.1: 96 well containing a) leaf extract (with all relevant chemicals utilized for the assay) and b) stembark extract (with all relevant chemicals utilized for the assay), after the incubation period, c) DPPH free radical scavenging activities of the leaf and stembark extracts (hexane, chloroform and methanol) of *C. erythrophyllum* (tukeys' honest significant difference multiple range post Hoc test $P < 0.05$ IBM SPSS version 27).

### 5.3.1.2. FRAP (Ferric Reducing Antioxidant Power) assay

In order to further evaluate the antioxidant ability of the generated extracts, a Ferric ($Fe^{+3}$) reducing antioxidant power assay was performed. This assay was performed on the crude hexane, chloroform and methanolic leaf and stembark extracts of *C. erythrophyllum* (at varying concentrations). Essentially, this assay quantifies the antioxidant potential of the tested extract, by the reduction of ferric iron ($Fe^{3+}$) to ferrous iron ($Fe^{2+}$). This reduction is facilitated by antioxidants that are possibly present within the extract. A colour change is anticipated during the reduction process (gain of electrons), from yellow, to green (antioxidant potential) and blue (high antioxidant potential), based on the antioxidant activity of the tested extract (Daniel and Temikotan, 2021).

The FRAP analysis indicated that all extracts displayed varying free radical scavenging activity, implying possible antioxidant potential (Table 5.2, Fig. 5.2). As noted, in the DPPH assay, a positive correlation between % inhibition and extract concentrations, was also noted. The chloroform leaf and stembark extracts displayed the highest IC50 values (over 500 µg/mL, respectively) indicating the lowest overall antioxidant potential from all extracts (Table 5.2). The methanolic extracts displayed the lowest IC50 values (<1µg/mL) indicating the highest possible antioxidant potential among all tested extracts (Table 5.2). This finding was further substantiated by the visual colour change observed upon the addition of the various chemicals associated to the assay (Fig 5.2a and b). The FRAP results obtained varied from 6% to 97% inhibition depending on the extract concentration and the extraction solvent (Fig. 5.2c). The results obtained from the emanating assay are in direct correlation with those obtained from the DPPH assay, indicating that the antioxidant activity is concentration dependant. In both instances, the methanol stembark extracts performed the best, with lowest IC50 recorded. Overall, IC50 values of the tested crude extracts were significantly lower than the positive control.

Interest in the antioxidant capabilities of species within the *Combretum* genus has been of high interest mainly due to their extensive use in traditional medicine (Coulidiati et al., 2009). Ghissing et al., (2021), conducted extensive research on the antioxidant ability of *Combretum indicum* (L.) DeFilipps. Results indicated that this species was indeed a rich source of antioxidant agents capable of neutralizing free radicals. Furthermore, *Combretum* species, such as *Combretum micranthum* G. Don, *Combretum glutinosum* Perr. Ex DC., *Combretum acutum* Laws. and *Combretum sericeum* G. Don, were also evaluated for their potential antioxidant capabilities using the FRAP assay, yielding favorable results (Ibrahim et al., 2017; Ladekan-Yayi et al., 2021; Coulidiati et al., 2011). These results are similar to those obtained from the current study. Furthermore, numerous *Combretum* species have indicated their possible ability to neutralize free radicals. Hence, further evaluation into the genus *Combretum* would be of pharmacological importance.

Table 5.2: FRAP free radical reducing activities (IC50 values) of the leaf and stembark extracts (hexane, chloroform and methanol) of *C. erythrophyllum* (tukeys' honest significant difference multiple range post Hoc test $P < 0.05$ IBM SPSS version 27).

| Extracts | IC50 (µg/ml) | |
|---|---|---|
| | Leaves | Stembark |
| Hexane | >1000 | 250,5053 |
| Chloroform | 593,4376 | >1000 |
| Methanol | <1 | <1 |

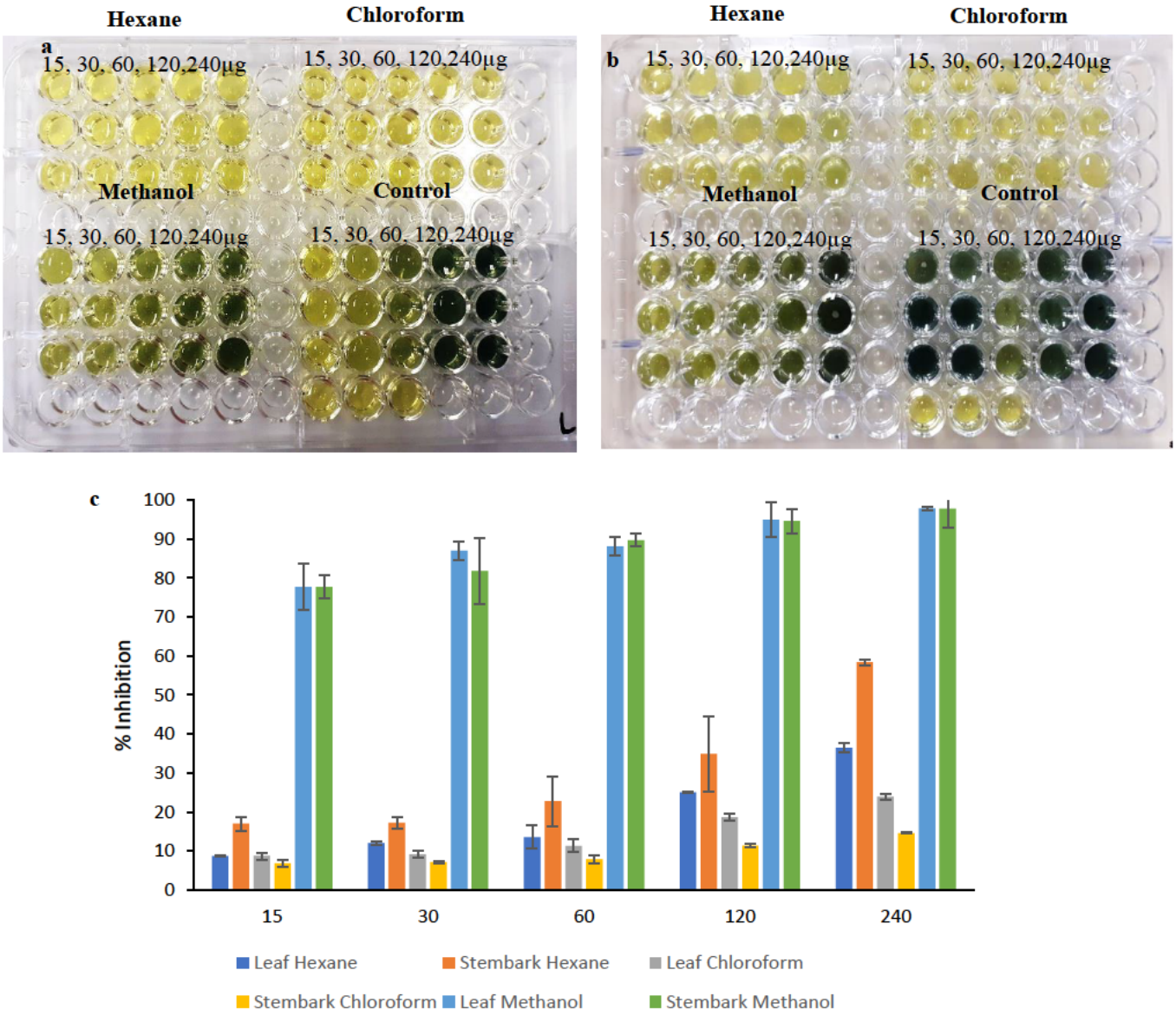


Figure 5.2: 96 well containing a) leaf extract (with all relevant chemicals utilized for the assay) and b) stembark extract (with all relevant chemicals utilized for the assay), after the incubation period, FRAP free radical reducing activities (IC50 values) of the leaf and stembark extracts (hexane, chloroform and methanol) of *C. erythrophyllum* (tukeys' honest significant difference multiple range post Hoc test $P < 0.05$ IBM SPSS version 27).

### 5.3.2 Total phenolic content

Phenolic compounds may be considered as the most copious phytometabolite found within plants (Bantho et al., 2020). These compounds are known to express anti-mutagenic, anti-inflammatory, apoptosis-inducing, anti-carcinogenic and antioxidant properties, mainly due to their phytoalexin potential (compounds produced within the plant, as a mechanism to alleviate biotic and abiotic stresses) (Garcia-Macias et al., 2007). In order to quantify the total phenolic content of the tested crude extract, the Folin–Ciocalteu reagent (FCR) assay was carried out. This assay was performed on the crude hexane, chloroform and methanolic leaf and stembark extracts of *C. erythrophyllum* (at varying concentrations). Upon addition of FCR, crude extracts enriched with high concentrations of phenolic compounds displayed a dark blue colour change (Phuyal et al., 2020).

From this study, it was deduced, that all extracts contained phenolic compounds (chloroform<hexane<methanol) (Table 5.3). The chloroform stembark appeared to have the lowest phenolic content (119,79 ± 1,90mg / GAE/g), whereas the methanolic extracts appeared to quantify the largest amount of compositional phenolic content (1341,05± 4,4mg / GAE/g). This finding correlates to the fact the polar solvents such as methanol (low boiling point and high penetration rate) allow for the maximum extraction of low molecular weight polyphenols (Do et al., 2014).

There are three main phytometabolite classes namely, terpenoids, phenols and alkaloids (Gupta et al., 2018). Phenols are the most researched and medicinally valued of the three (Phuyal et al., 2020). Current research suggests that numerous health benefits are associated to the consumption of plants rich in phenols. Williamson et al., (2000) mentioned that *Combretum* species are characterized by high flavonoid and phenolic compound levels. To date, a few *Combretum* species that have been quantified for its total phenolic content, viz., *C. leprosum, Combretum micranthum, Combretum lanceolatum, Combretum indicum* and *Combretum molle* (da Silva et al., 2021; Mashi et al., 2021; Sousa et al., 2021; Forid et al., 2021). These species were found to exhibit high levels of phenolic compounds which correlates to the results obtained from this research. Nascimento et al., (2000) highlighted the use of integrating natural products derived from these species, with commercially produced medicine.

Table 5.3: Quantification of the total phenolic content of the leaf and stembark extracts (hexane, chloroform and methanol) of *C. erythrophyllum.*

| Extracts | Total phenolic content (mg / GAE/g) | |
|---|---|---|
| | Leaves | Stembark |
| Hexane | 163,125 ± 2.22 | 253,125 ± 1,73 |
| Chloroform | 178,541 ± 1,63 | 119,79 ± 1,90 |
| Methanol | 713,125 ± 0,5 | 1341,05± 4,4 |

### 5.3.3. Total flavonoid content

Flavonoids are the largest phytometabolite group within the phenolic compound class (Karim et al., 2018). Flavonoids are known for their anti-cancer, antiviral, anti-allergic, anti-inflammatory, cholesterol-reducing, antioxidant and antibacterial properties (Phuyal et al., 2020; Fernandez et al.., 2021). In order to quantify the total flavonoid content of the tested extract, the aluminum chloride assay was carried out. Essentially, the use of aluminum chloride is fundamental in determining the presence of flavonoids within plant extracts as the aluminum ions tend to form a complex with the carbonyl and hydroxyl groups present within flavonoids thus inducing a yellow colouration (Wu et al., 2021). This assay was performed on the crude hexane, chloroform and methanolic leaf and stembark extracts of *C. erythrophyllum* (at varying concentrations).

From the emanating study, it was deduced, that all extracts contained flavonoids (Hexane < Chloroform <Methanol) (Table 5.4). The hexane stembark appeared to have the lowest flavonoid content (1,725 ± 0,46mg / QE/g) whereas the methanolic leaf extracts appeared to quantify the largest amount of compositional flavonoid content (39,42 ± 1,86mg/ QE/g). As previously mentioned, extraction of phenolic compounds and flavonoids are expected to be highest in methanolic extracts, primarily due to the polar nature of the solvent.

Structurally, flavonoids can occur in three types, namely glycosides, aglycones and methylated forms (Wang et al., 2018; Yang et al., 2018). Thus far, flavonoids were isolated from *C. erythrophyllum*, *Combretum apiculatum* and *Combretum leprosum* (Rogers and Verotta, 1996; Martini et al., 2004; Sousa et al., 2021). Furthermore, research shows that the secretory scales of *Combretum roxburghii* are enriched with saponins, flavonoids and tannins (Bhatnagar et al., 2012). The extracted flavonoids are said to have antioxidant, anti-estrogenic and anti-proliferative properties (Gretchen et al., 2008). Martini et al. (2004) has identified seven different flavonoids present in *C. erythrophyllum* which are believed to be responsible for their antibacterial effect, numerous health benefits, antioxidant and anti- microbial properties.

Table 5.4: Quantification of the total flavonoid content of the leaf and stembark extracts (hexane, chloroform and methanol) of *C. erythrophyllum.*

| Extracts | Total flavonoid content (mg / QE/g) | |
|---|---|---|
| | Leaves | Stembark |
| Hexane | 16,9875 ± 0,265 | 1,725 ± 0,46 |
| Chloroform | 22,495 ± 0,212 | 5,9375 ± 1,964 |
| Methanol | 39,42 ± 1,86 | 37,05± 17,8 |

### 5.3.4. Antibacterial screening

Preliminary antibacterial screening was conducted using the crude leaf and stembark using chloroform, hexane, and methanol extracts of *C. erythrophyllum*. The antibacterial properties of the generated extracts (concentrations: 10, 12.5, 25, 50 and 100 mg/mL) were screened against 5 different bacterial strains: *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus* and methicillin-resistance *Staphylococcus aureus*. Mechanisms that allow for the induction of inhibitory antibacterial activity include penetration of the cell wall, membrane disintegration and a hinderance of enzyme attachment during protein synthesis. Research suggests that upon the onset of any of the above events, antibacterial activity will be observed (Hanif et al., 2020).

All the leaf extracts displayed clear zones of inhibition against the five tested bacterial strains, whereas the stembark extracts failed to depict a zone of inhibition against *E. coli* (Gram negative). In addition, all zones of inhibition were compared against the positive controls which were used. Streptomycin was used against gram positive bacteria and gentamycin was used against gram negative bacteria. Tables 5.5-7 highlights the antibacterial properties exhibited by the leaf extracts, which showed intense activity against *S. aureus* (Gram positive), *E. coli*, and MRSA (Gram positive). Stembark extracts displayed moderate inhibitory activity against *S. aureus*, *P. aeruginosa* (ATCC 252152), and methicillin-resistant *S. aureus* (Gram positive). The extracts demonstrated inhibitory activity against Gram positive bacteria in comparison to Gram negative bacteria. *E. coli* showed no zone of inhibition against stembark extracts (Table 5.5-7). Research suggests that this phenomenon could be displayed due to the structure and permeability of the cell walls of Gram negative bacteria, which influences the inhibitory activity (Mtunzi et al., 2017). These factors contribute to the high resistance observed from Gram negative bacteria (Akwu et al., 2019). Among all extracts, methanolic extracts appeared to have the highest rate of inhibition against the tested bacteria (besides *E. coli*). Eloff (1998), stressed the efficiency of utilizing methanol as the solvent of choice for extractions. This research showed that within the *Combretum* species, the use of methanol allows for the maximum extraction of phytochemical compounds, such as terpenoids, tannins, flavones, phenols and saponins, which are known to have possible antibacterial activity (Eloff, 1998; Hanif et al., 2020). Thus, denoting to the possible high levels of inhibitory activity seen through the emanating study.

Williamson et al. (2000) mentioned that *Combretum* species are enriched with high flavonoid and phenolic compound levels, explaining their potential antibacterial properties. A recent study conducted by Mtunzi et al. (2017) indicated the prominent antibacterial effects of *C. erythrophyllum* leaf extract against multiple bacterial strains (*Escherichia coli, Pseudomonas aeruginosa, Bacillus*

*subtilis* etc.), which correlates with the findings from this emanating study. Furthermore, species such as, *Combretum albidum, Combretum leprosum, Combretum indicum, Combretum dolichopetalum and Combretum album* have also been noted for their high antioxidant and antibacterial properties (McGaw et al., 2001; Chandar and Ramasamy, 2016; Burman et al., 2018; Hanif et al., 2020; Nwuke, 2020; Sousa et al., 2021). In correlation with results obtained in this study, *Combretum sokodense* leaf, stembark and root extracts also showed inhibitory activities against *S. aureus* and *E. coli* (Ibrahim et al., 2017).

Toleman (2006), emphasized the importance of alternative antibiotic development against emerging drug-resistant bacteria. Currently, multi-drug resistant pathogenic bacteria are on the rise, posing extreme harm to the public (Holmes and Howden, 2014; Ibrahim et al., 2017). Methicillin-resistant *Streptococcus aureus* (MRSA), is one such bacterium known to be resistant towards current antibiotic treatments (Davies, 1994; Holmes and Howden, 2014). As noted in Tables 5.5-7, stembark extracts showed positive inhibitory activity towards MRSA, thus highlighting their potential use in drug development against this bacterium. Overall, results obtained from both leaf and stembark extracts of *C. erythrophyllum* showed that this species has antibacterial activity.

Table 5.5: Antibacterial activity: Zones of inhibition (mm) of *Combretum erythrophyllum* leaf and stembark hexanolic extract.

| | **Leaf extract** | | | | | | **Stembark extract** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Concentrations (mg/mL)** | | | | | | **Concentrations (mg/mL)** | | | | | |
| | *Control* | *10* | *12.50* | *25* | *50* | *100* | *Control* | *10* | *12.50* | *25* | *50* | *100* |
| *Escherichia coli* | 10 | 10 | 10.5 | 15.5 | 19.5 | 22 | 10 | 7 | 7 | 8 | 11 | 13 |
| *Pseudomonas aeruginosa* | 11 | 10 | 10 | 13 | 15 | 17 | 11 | - | - | - | - | - |
| *Staphylococcus aureus* | 9 | - | - | - | - | - | 9 | 7 | 7 | 9 | 13 | 16 |
| Methicillin Resistant *Staphylococcus aureus* | 15 | 7 | 7 | 8 | 10 | 11 | 15 | 7 | 7 | 9 | 12 | 18 |
| *Bacillus subtillus* | 8 | - | - | - | - | - | 8 | 7 | 7 | 8 | 12 | 17 |

Table 5.6: Antibacterial activity of *Combretum erythrophyllum* leaf and stembark chloroform extract.

| **Bacterial strain:** | **Leaf extract** | | | | | **Stembark extract** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Concentrations (mg/mL)** | | | | | **Concentrations (mg/mL)** | | | | |
| | *10* | *12.50* | *25* | *50* | *100* | *10* | *12.50* | *25* | *50* | *100* |
| *Escherichia coli* | 10 | 12 | 13 | 19.5 | 23 | 7 | 7 | 11 | 12 | 16 |
| *Pseudomonas aeruginosa* | 7 | 10 | 13 | 16 | 18 | 7 | 9 | 9 | 12 | 15 |
| *Staphylococcus aureus* | - | - | - | - | - | 7 | 7 | 9 | 11 | 15 |
| Methicillin Resistant *Staphylococcus aureus* | 7 | 7 | 9 | 10 | 13 | 7 | 9 | 9 | 15 | 18 |
| *Bacillus subtillus* | 7 | 10 | 11 | 13 | 20 | 7 | 7 | 9 | 11 | 15 |

Table 5.7: Antibacterial activity of *Combretum erythrophyllum* leaf and stembark methanol extract.

| **Bacterial strain:** | **Leaf extract** | | | | | **Stembark extract** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Concentrations (mg/mL)** | | | | | **Concentrations (mg/mL)** | | | | |
| | *10* | *12.50* | *25* | *50* | *100* | *10* | *12.50* | *25* | *50* | *100* |
| *Escherichia coli* | 10 | 12 | 20 | 26 | 25 | 10 | 12 | 17 | 20 | 23 |
| *Pseudomonas aeruginosa* | 7 | 10 | 16 | 17.5 | 20 | - | - | - | - | - |
| *Staphylococcus aureus* | 7 | 9 | 9 | 12 | 16 | 7 | 8 | 8 | 12 | 16 |
| Methicillin Resistant *Staphylococcus aureus* | 7 | 7 | 12 | 24 | 28 | 7 | 7 | | 16 | 20 |
| *Bacillus subtillus* | 9 | 9 | 15 | 18 | 20 | 7 | 7 | 10 | 15 | 20 |

### 5.3.5. Cytotoxicity:

The cytotoxic potential of the generated crude leaf and stembark extracts of *C. erythrophyllum* (hexane, chloroform and methanol) was evaluated against three cell lines HEK293, HeLa and MFC-7, using the MTT assay. The results obtained showed that the hexane, chloroform and methanolic extracts exerted a cytotoxic effect on the various cells. Noticeably, a high potent activity was seen from the methanolic stembark extracts when tested against HEK293 cells.

The US National Cancer Institute indicated that a crude plant extract with an IC50 value of less than 20 µg/ml is considered to display cytotoxicity activity (Alabsi et la.,2016). The emanating study revealed that the methanolic stembark extract appeared to have a IC50 value <20µg/mL, making it a favourable option to be further developed and considered as a conventional anti-cancer drug (Table 5.8). Due to the low IC50 value obtained from this study for this extract, further isolation of its bioactive compounds is key to discover its true pharmacology capabilities. As indicated in table 5.8, methanolic extracts were the best performing, with the overall lowest IC50 values when tested against HeLa and HEK293 cells (Leaf- 54,53 µg/mL and stembark 18,30µg/mL). The lowest cell viability (30%) was noted within the HeLa cell lines when treated with the methanolic stembark extract (at 240 µg/mL strongest cytotoxic effect). Furthermore, the highest cell viability (93%) was noted within the MFC-7 cell line when treated with the chloroform leaf extract (at 15µg/mL weakest cytotoxic effect). Overall, the crude methanolic extracts performed the best, yielding the lowest IC50 values.

Many *Combretum* species have been extensively researched for their potential cytotoxic activity, eg. *C. fruticosum, C. erythrophyllum, C.quadrangulare, C. fragrans, C. leprosum, C. apiculatum, C. paniculatum* and *C. woodii* etc. (Wende et al., 2021; Sousa et al., 2021; Nguyen et al., 2021; Gade et al., 2021; Maphutha et al., 2021). Wende et al. (2021) evaluated the integrated use of conventional cancer drugs with compounds isolated from *C. zeyheri* and C. *platypetalum* against Jurkat T Cells and HL-60 Cells. As a result, the above integration facilitated the inhibition of the growth of the cells significantly. The integrative effect of the conventional drugs with compounds isolated from medicinal plants is a novel approach which yields promising results. This novel approach needs to be expanded further by possibly incorporating the methanolic stembark extracts (IC50<20µg/mL) of *C. erythrophyllum* to unlock its true value in the pharmacological world.

Table 5.8: IC50 values of the cytotoxic analysis of the leaf and stembark extract of *C. erythrophyllum*

| Cell lines | Extracts | Cytotoxicity (ug/ml) | |
|---|---|---|---|
| | | Leaves | Stembark |
| HEK293 | Hexane | 90,89 | 78,40 |
| | Chloroform | 70,25 | 53,37 |
| | Methanol | 59,01 | 18,30 |
| HeLa | Hexane | 97,15 | 73,04 |
| | Chloroform | 72,34 | 50,92 |
| | Methanol | 54,53 | 39,23 |
| MCF7 | Hexane | 202,88 | 227,38 |
| | Chloroform | 1083,11 | 371,86 |
| | Methanol | 198,87 | 204,77 |

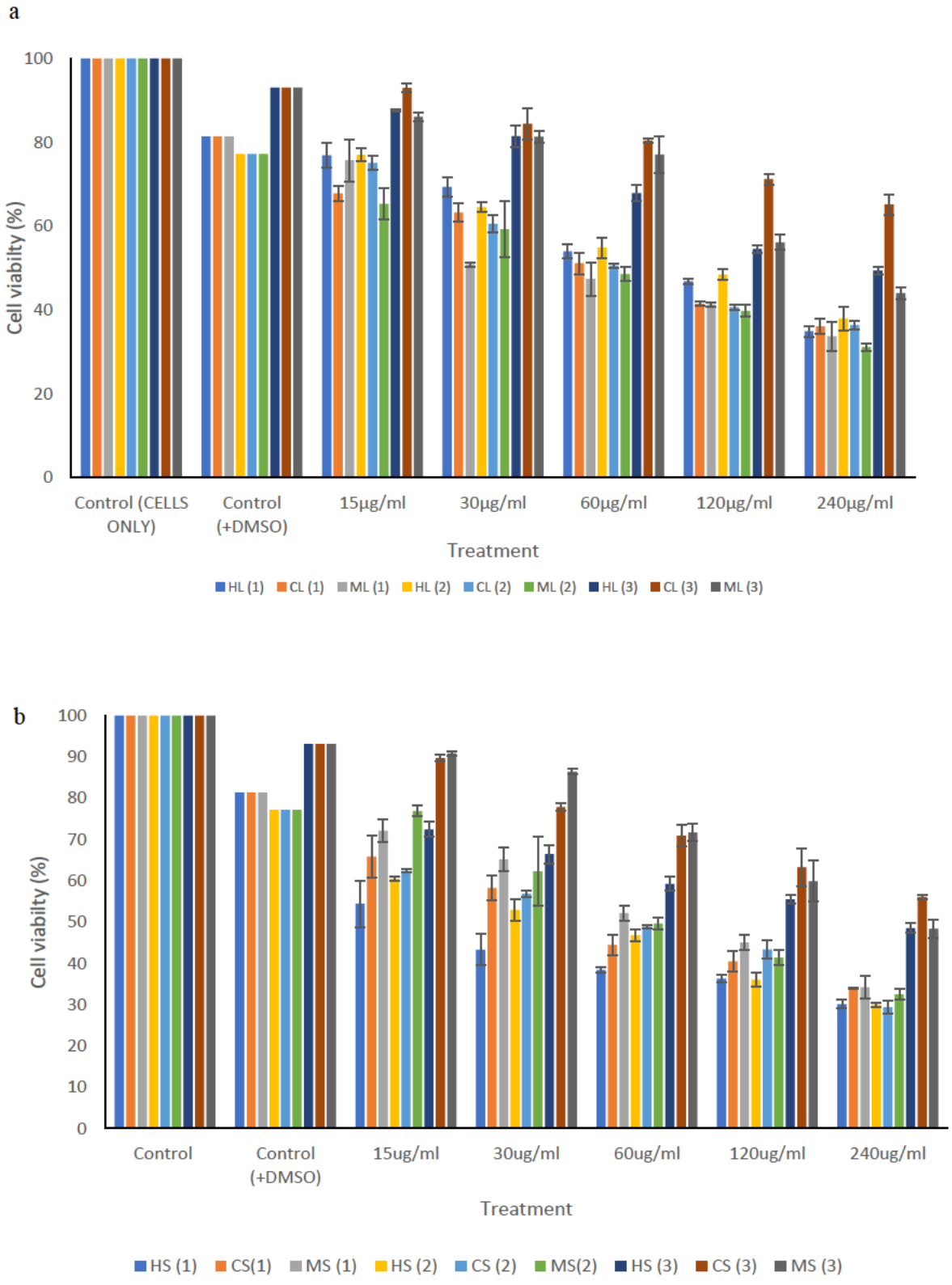


Figure 5.3: Cytotoxic activity of the a) leaf, b) stembark extracts of *C. erythrophyllum* (tukeys' honest significant difference multiple range post Hoc test $P < 0.05$ IBM SPSS version 27).

### 5.3.6. Apoptosis

Apoptosis is a genetically controlled process which may result in the disintegration of the nuclei structure and DNA, ultimately resulting in overall cell death (Al-Nasser et al., 2021). This process is primarily initiated by the presence of allelochemicals, which in turn results in the onset of oxidative stress within the cell, initiating apoptosis (Mobaraki et al., 2021). During apoptosis the following cellular variations are noticed: disintegration of the nucleus and chromatin network, distortion and shrinkage of the cell membrane (Ali et al., 2021). The importance of being able to influence cell death (apoptosis) is highly beneficial in pharmaceutical therapies (Basu, 2021). In ailments such as cancer (uncontrolled cell growth), mediators are required to eliminate the cancer cells while exhibiting slight impact on normal cells, as research indicates high cellular resistance seen against conventional treatments i.e. chemotherapy (Thapa et al., 2021). This may be naturally induced through apoptosis, which may be lacking (cancer) or over expressed (neurodegenerative diseases) in certain ailments. Phytometabolites, alkaloids and phenols, extracted from medicinal plants, may exhibit apoptotic activity, which can be integrated with conventional drug therapy for better results (Safarzadeh et al., 2014).

This study integrated the use of fluorescence microscopy with acridine orange staining in order to accurately determine the viability of cells. This method was favored as it is time and cost effective (Anitha et al., 2021). When stained with AO/EB, viable cells appear green, with a fully formed nucleus. Apoptotic cells, initially appear to have yellow green dots, signaling a disintegrating chromatin network. Upon reaching the completion of apoptosis, cells exhibit an orange nucleus, which shows some form of disintegration. Distinctively, necrotic cells at this stage also appear orange however display a structurally intact nucleus (Fig. 5.4-6).

A direct correlation can be seen between the results obtained from the cytotoxicity and apoptosis assay. At high IC50 concentrations, cell viability is high while the apoptotic index is low. Whereas, at low IC50 concentrations, cell viability is low and the apoptotic index is high, cell appears to stain orange in colour indicating their low cell viability (Fig. 5.4-6). This phenomenon is further confirmed by the results obtained from the apoptotic index (Fig. 5.7). Methanolic extracts displayed the greatest apoptosis, whereas the hexanoic extracts appeared to have least apoptotic activity (Fig. 5.7). This a novel technique deployed on *C. erythrophyllum*, however similar results were noted within species such as*: Combretum apiculatum, Combretum zeyteri, Combretum platypetalum, Combretum leprosum* and *Combretum fragans* (Silva et al., 2020; Gade et al., 2020, Maphutha et al., 2021; Wende et al., 2021).

Overall, the generated crude extracts from *C. erythrophyllum* showed promising cytotoxic activity against cancer cells, with the possibility of inducing controlled apoptosis within these cells. This ability will prove highly effective in possibly treating cancer.

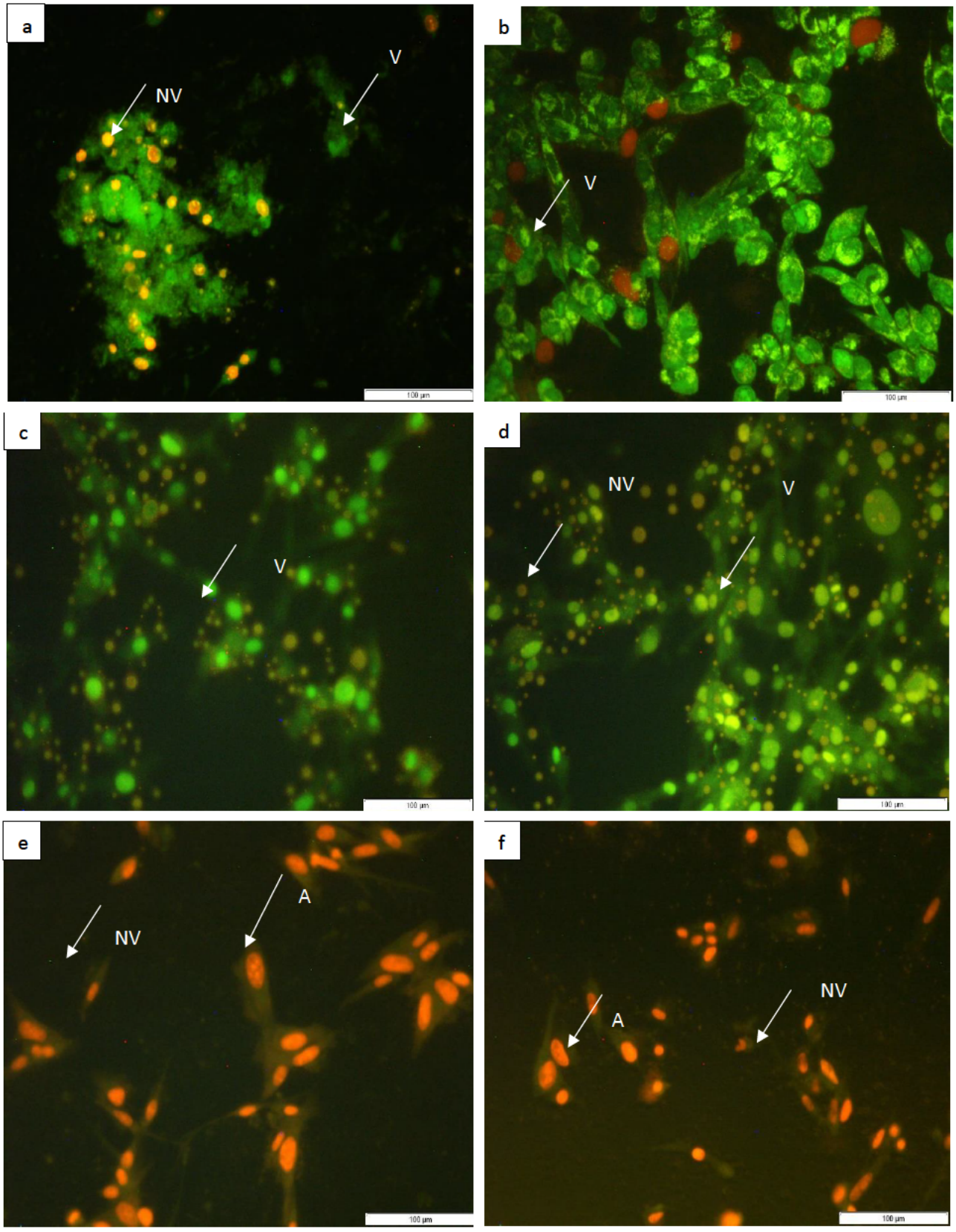


Figure 5.4: Light micrographs indicating the cell viability of HEK293 cells stained using acridine orange hexane a) leaf, b) stembark extract, chloroform c) leaf, d) stembark and methanolic e) leaf and f) stembark extracts of *C. erythrophyllum (A- Apoptotic cell, V-Viable cell, NV- non-viable cell)*.

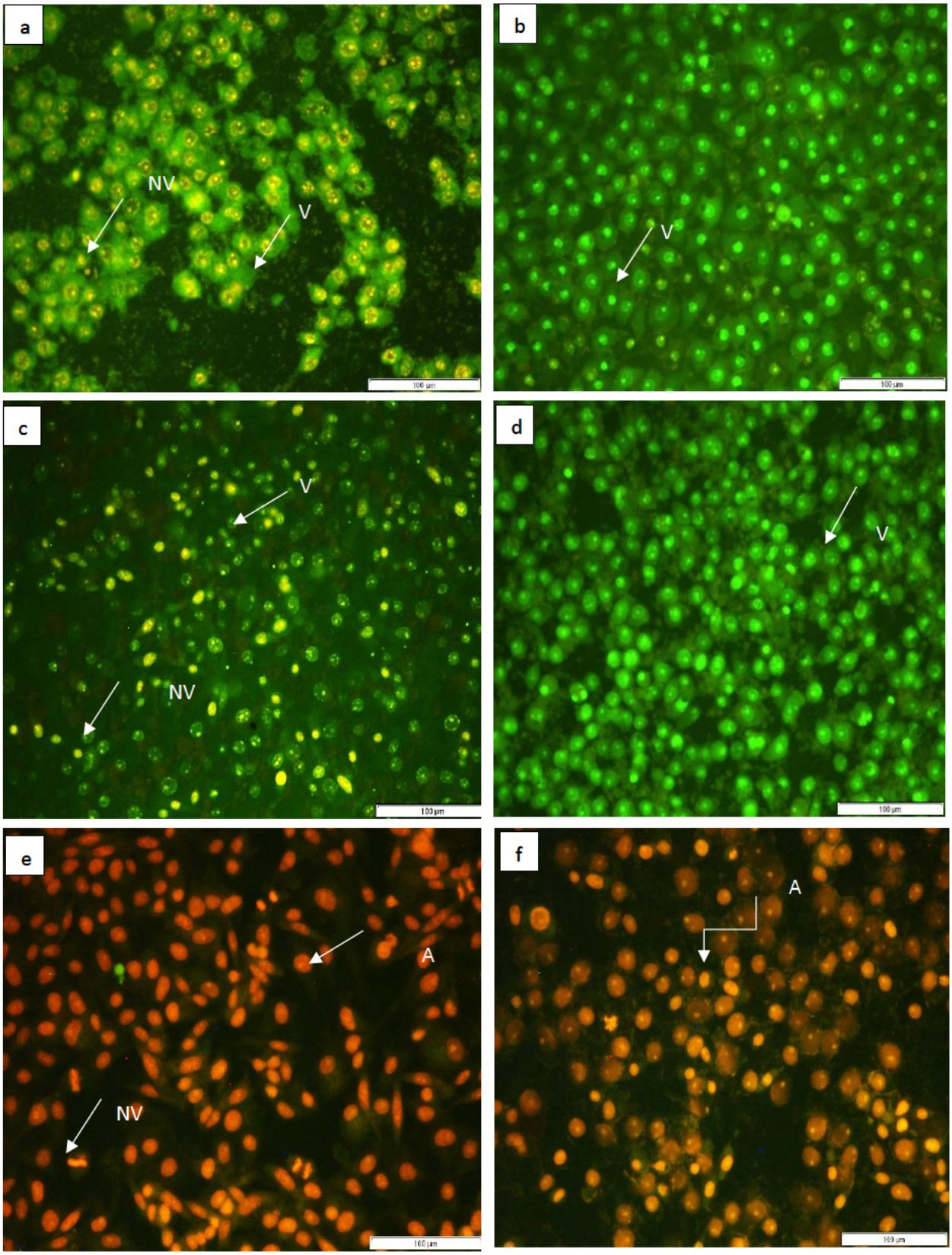


Figure 5.5: Light micrographs indicating the cell viability of HeLa cells stained using acridine orange hexane a) leaf, b) stembark extract, chloroform c) leaf, d) stembark and methanolic e) leaf and f) stembark extracts of *C. erythrophyllum (A- Apoptotic cell, V-Viable cell, NV- non-viable cell)*.

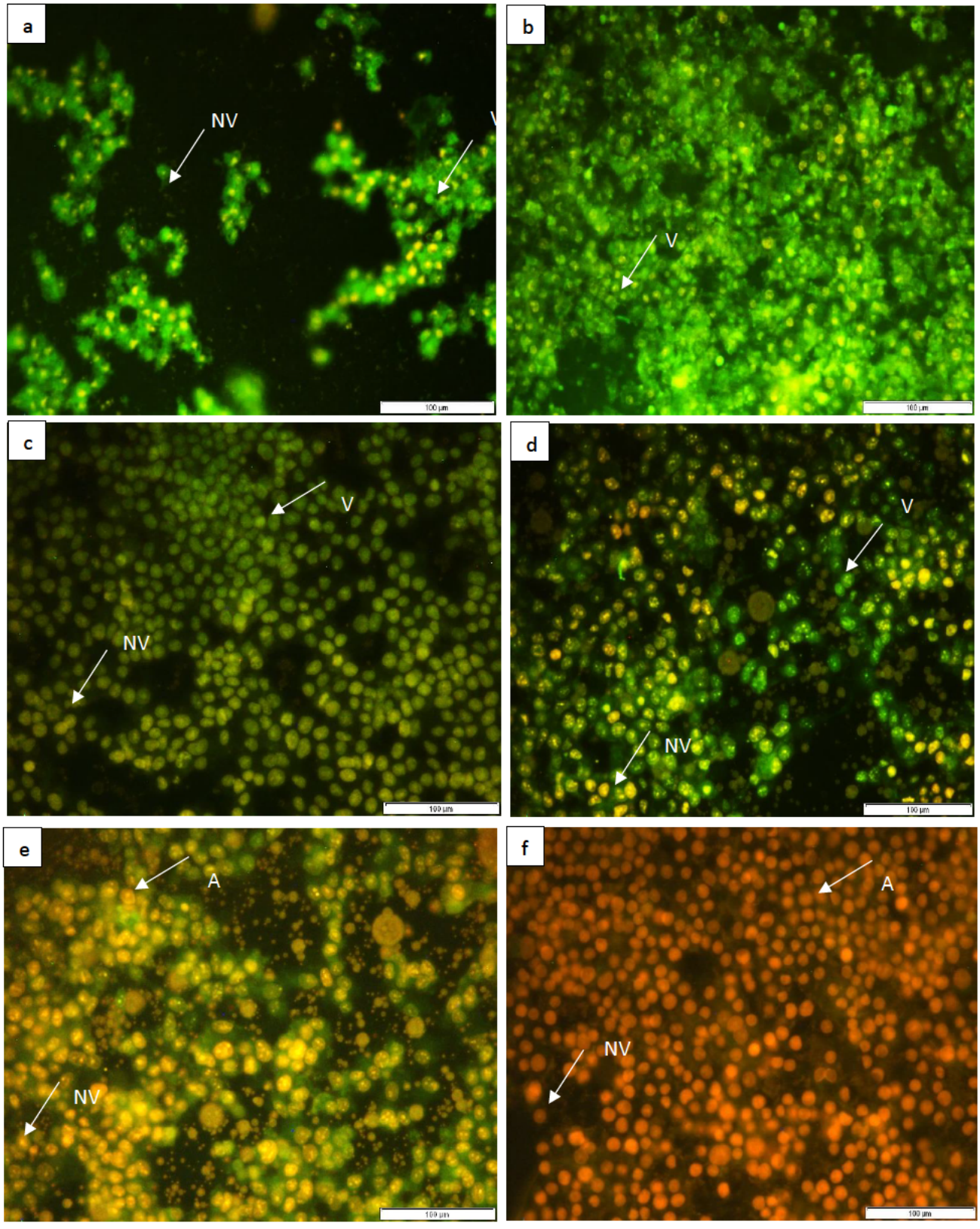


Figure 5.6: Light micrographs indicating the cell viability of MCF-7 stained using acridine orange hexane a) leaf, b) stembark extract, chloroform c) leaf, d) stembark and methanolic e) leaf and f) stembark extracts of *C. erythrophyllum (A- Apoptotic cell, V-Viable cell, NV- non-viable cell)*.

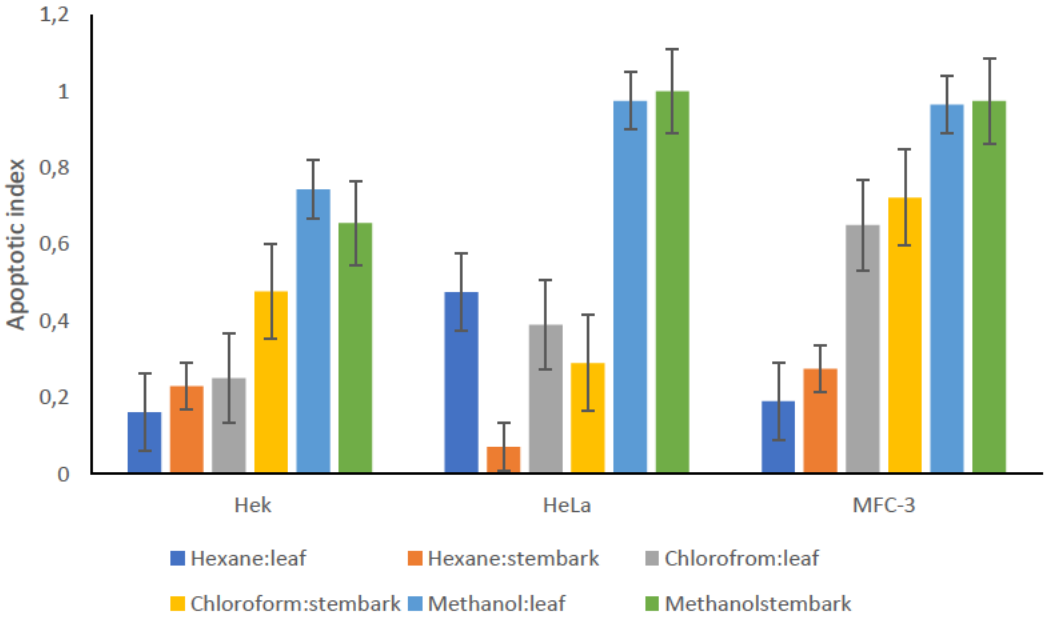


Figure 5.7: Apoptotic index of each cell line against various crude extracts (240 µg/mL). The mean results are displayed, ± SD, n = 3, (tukeys' honest significant difference multiple range post Hoc test $P < 0.05$ IBM SPSS version 27).

## 5.4. Conclusion

This study aimed to determine a comparative analysis of the biological activity of the leaf and stembark extracts of *C. erythrophyllum*. The following characters were elucidated from the emanating study: total flavonoid and phenolic content, antioxidant properties, apoptosis, cytotoxic and antibacterial activity of the leaf and stembark extract. The presence of phenols, sterols, flavonoids, saponins and alkaloids possibly indicate cytotoxic, antibacterial and antioxidant properties within *C. erythrophyllum*. The biological activity of this species was further substantiated by the results obtained from the emanating study. It may be concluded that the antioxidant properties, total phenolic and total flavonoid content are directly proportional to the apoptotic, cytotoxic and antibacterial activity expressed by the tested extracts. In lieu, the integration of conventional treatments with naturally derived compounds could prove to be pharmacologically beneficial and is indeed an avenue that needs to be further explored .

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# CHAPTER 6: SYNTHESIS, CHARACTERISTATION AND BIOLOGICAL ACTIVITY OF SILVER NANOPARTICLES GENERATED USING THE LEAF AND STEMBARK EXTRACT OF *COMBRETUM ERYTHROPHYLLUM*.


## Abstract

Medicinal plants are known to contain numerous phytometabolites with pharmacological value. Literature suggests that the medicinal use of phytometabolites in its natural state could have limited success due to poor absorption rates. Currently, the focus lies on synthesizing phytometabolites extracted from medicinal plants and silver ions to generate nano-scale carriers with specialized properties. Thus, the nano-synthesis of phytometabolites with silver ($Ag^{+}$) ions is proposed. The use of silver is promoted due to its known antibacterial and antioxidant effectiveness, among many. Nanotechnology allows for the green generation of nano-scaled particles that are able to penetrate target areas due to its size and unique structure. A novel protocol for the synthesis of silver nanoparticles (AgNPs) using the leaf and stembark extracts of *Combretum erythrophyllum* was established. The generated AgNPs were characterised using transmission electron microscopy (TEM), scanning electron microscopy (SEM), energy-dispersive X-ray (EDX), nanoparticle tracking analysis (NTA) and UV-visible spectrophotometry (UV-vis). Characterisation was based upon particle size, shape and elemental silver composition. Within the stembark extract, synthesised nanoparticles were large, spherical in shape and dense in elemental silver composition. While, synthesised nanoparticles of the leaf extract were small to medium in size, varied in shape and contained minimal quantities of silver. Furthermore, it was established that the synthesized nanoparticles exhibited high antibacterial, cytotoxic and apoptotic properties due to the conducted assays. The Fourier-transform infrared spectroscopy (FTIR) analysis revealed the presence of numerous compound classes associated with proposed pharmacological activity. Overall, the methanolic extracts appeared to have been most dense in phytometabolites and are worth studying further for its pharmaceutical value. Presently, antibiotic resistant bacteria are continuously evolving thus, posing as a threat to conventional drug delivery system. Nanotechnology provides a platform that enables the formulation of a low toxicity and hypersensitive drug delivery system. Further studies evaluating the biological activity of AgNPs synthesised using extracts of *C. erythrophyllum* could enhance its proposed pharmaceutical value.


Keywords: AgNPs, $Ag^{+}$, antibacterial activity, bio-nanotechnology, *Combretum erythrophyllum*

## 6.1. Introduction

The progressive field of bio-nanotechnology has revolutionized the modern medicinal world (Prasad et al., 2018). Bio-nanotechnology is a science which manipulates, and controls matter at an atomic and molecular level, in an eco-friendly method (Demirdogen et al., 2018). This technology makes use of nanoparticles which are minute units (0.1-100 nm) that differ in shape and structure (Gibb et al., 2018). These particles have a large surface area which enables the attachment of surface constituents and prompts drug delivery (Hu et al., 2019). Furthermore, interest has been generated towards nanoparticle synthesis of metals, such as silver or gold (Gibb et al., 2018). Research suggests that metals may be manipulated into nanosized particles with specialised properties that enables them to penetrate tissues and target specific host regions (Demirdogen et al., 2018).

Synthesized metal nanoparticles exhibit magnetic, catalytic and electrical properties, which differ from other materials (Sheoran and Kaur, 2018). Metal nanoparticle research is currently receiving attention due to their ability to enhance pharmaceutical applications by their unique size, shape and penetrating ability (Gibb et al., 2018). In accordance, antibiotic resistant pathogens are posing eminent risks within the medical industry, suggesting a need for alternative solutions. It has been elucidated from research that silver nanoparticles (AgNPs) is the one of the materials of choice in the biomedical field (Demirdogen et al., 2018). This is due to their proposed antibacterial properties and continuous ability to bind to inorganic molecules (Demirdogen et al., 2018; Li and Qu, 2018).

Research has indicated the ancient use of silver as an antimicrobial agent in the treatment of wounds, infections and diseases (Bailey et al., 2018). Although beneficial, toxicity levels were of concern. However, with recent advancements in nanotechnology, the benefits of silver has been amplified when synthesised with nanoparticles, resulting in a quicker and more efficient form of antibiotic drug delivery. The formulation of AgNPs provides for a safe, inorganic compound with the ability to disintegrate around 650 pathogenic microorganisms (Hu et al., 2019).

The chemical and physical composition of AgNPs highlight its antimicrobial, antibacterial and cytotoxic properties (Gibb et al., 2018). Within the medical field these nanoparticles are also known to aid in diagnostic and therapeutic applications. There are three methods that have been used for the synthesis of AgNPs namely biological, physical and chemical (Demirdogen et al., 2018). Physical and chemical methods are costly and are environmentally harmful due to high toxicity levels (Iravani et al., 2014). The high use of toxic chemicals in these methods (mentioned above), limits their overall use in pharmaceutical therapy. Alternatively, the biological method allows for an environmentally friendly and cost-effective synthesis (Chandirika and Annadurai, 2018). This method of synthesis incorporates the use of plant, fungal or microorganismal extracts for AgNP synthesis. Specifically, a larger yield of AgNPs are generated with plant extracts rather than microorganismal extracts. This phenomenon is due to the abundance of bioactive secondary metabolites found within plants which

encourages the conversation of $Ag^+$s to AgNPs. The efficacy of AgNPs as an antimicrobial agent is due to their ability to provide larger contact area with the microorganism while attaching to the cell membrane (Chandirika and Annadurai, 2018). In bacterial cells, the AgNPs are able to penetrate the cell membrane while simultaneously releasing $Ag^+$. The interaction between the $Ag^+$ and sulphur containing proteins found within the cell membrane, allows for the inhibition of cell divisions leading to cell degradation. Demirdogen et al. (2018) indicated that the biologically synthesised AgNPs exhibited higher antimicrobial activity against tested pathogenic bacteria compared to the latter methods of synthesis i.e., physical and chemical.

The use of medicinal plant extracts for AgNP synthesis is highly favourable due to their general non-toxic stable nature, easy accessibility and presence of phytometabolites (which aid in $Ag^+$ reduction) (Chandirika and Annadurai, 2018). Phytometabolites aid in the reduction of metal ions present in nanoparticles (Duhan, 2017). Metal ions are reduced to a base metal via water soluble plant metabolites during the biological synthesis process. Hence nanoparticles impregnated with plant extract is formulated (Vázquez-Núñez et al., 2018). The generated AgNPs can be screened through numerous assays to evaluate its biological activity such as anti-inflammatory, anti-cancer, antioxidant, antibacterial and anti-protozoal assays. AgNPs were generated using a crude leaf extract of *Thunbergia alata*. An antibacterial assay indicated the presence of zones of inhibition towards *Staphylococcus aureus*, *Bacillus suptilis* and *Escherichia coli* (Hedaginal and Taranath, 2017). Hence proving the possible use of AgNPs synthesised from plant extract`s, in the pharmaceutical industry (Chung et al., 2016).

Research suggests that cancer is one of the leading causes of death in the current age (More et al., 2021). This is mainly attributed to the aggressive invasive nature of cancer-causing tumours. Furthermore, the growth and migration of cancer cells cannot be controlled, hence the treatment of this ailment is difficult. Conventional methods (ie. chemotherapy) of treating this ailment have proven to be highly detrimental to the host, with extreme side effects (Zheng et al., 2021). These methods are also highly costly and time-consuming bearing minimal results in most cases. The main problem associated with treating cancer conventionally, is chemotherapy resistance (multi drug resistance) (Sundaram et al., 2021; Tagde et al., 2021). Cancer cells can become insensitive to the chemotherapy being used to treat it. Interestingly, certain bioactive compounds, such as quercetin, curcumin and tetrandrine, which may be extracted from medicinal plants, are shown to restore chemotherapy sensitivity, which is an added benefit in incorporating plant-based therapy with conventional cancer treatment methods (Zheng et al., 2021). Attention has now been drawn towards the use of naturally derived products (plant based) for the treatment of this ailment. Over the past decade, countries such as the United Arab Emirates, Saudi Arabia, Egypt, Germany and Switzerland have been effectively utilising plant-based alternatives for the treatment of cancer. Therapies incorporating naturally derived products were seen to have minimal side effects with optimistic

results (Akwu et al. 2021). However, only 5% of the world's plants have been effectively evaluated for its potential use as a commercial drug alternative in cancer treatment (WHO). The detection and evaluation of the medicinal properties of bioactive compounds found within medicinal plants is yet to be fully explored. Many of these bioactive compounds are known to have anticancer properties which act as chemopreventative agents against many types of cancers. Plants such as: *Picrorhiza Kurroa* Royle ex Benth., *Gynandropsis pentaphylla* (L.) DC., *Nigella sativa* L., *Boswellia serrata* Roxb. and *Asparagus racemosus* (Wiilld.) have been used to successfully treat various types of cancer such as, hepatic, hepatoma, Lewis lung carcinoma, human epiderma carcinoma and epidermoid carcinoma, respectively.

The use of nanotechnology incorporating plant extracts is an avenue to be explored. *Combretum erythrophyllum* is recognized in southern Africa, because of its extensive use in traditional medicine. Mawoza and Ndove (2015) have elaborated on the traditional use of this species as a purgative and to treat venereal diseases and abdominal pain. Pharmacological studies have shown this species to have antibacterial, anti-inflammatory and antimicrobial properties (Mtunzi et al., 2017). Silver is known for its antimicrobial properties thus the synthesis of AgNPs using plants extracts may enhance overall antimicrobial action. To our knowledge, the synthesis of AgNPs using the stembark extracts of *C. erythrophyllum* has not been attempted. Therefore, this study aimed to generate a novel protocol for the synthesis of AgNPs using the leaf and stembark extracts of *C. erythrophyllum*. Furthermore, the antibacterial, cytotoxic and apoptotic properties of the generated AgNPs were evaluated.

## 6.2. Materials and method

### 6.2.1. Generation of crude extract

#### 6.2.1.1. Plant collection

Plant material of *C. erythrophyllum* was obtained from the University of Kwa-Zulu Natal (Westville), Durban, South Africa (29°49′S; 30°59′E). A voucher specimen was previously submitted to the Herbarium (13476/2), University of KwaZulu-Natal, Westville Campus. The collected fresh material was left to air dry at ambient temperatures of 23-25˚C for six weeks. The dried leaves were then crushed into a fine powder using a blender (Philipps HR7762, China) and stored in a cool and dark conditions until further use.

#### 6.2.1.2. Extraction

Approximately 10 g of crushed leaf material was added to 100 ml of HPLC grade hexane (organic solvent) in a round bottom flask, attached to the reflux apparatus (set up as per Fig. 6.13) and placed in a heating mantle. The mantle was set at a low heat and distillation commenced. After approximately three hours of heating, the extract was filtered using a funnel and filter paper (Whatmans' No. 1). This process was repeated three times to allow maximum extraction of compounds. This process was then repeated using chloroform, followed by methanol, as the organic solvents of choice. Thereafter, the crushed stembark material was processed following the above method. Generated extracts were utilized for nanoparticle synthesis, characterisation and various assays evaluating the biological activity of the synthesised material.

### 6.2.2. Nanoparticle synthesis

Deionized water was used to prepare a 1 mM $AgNO_3$ solution. A 1:19 ratio of the crude leaf extracts to the 1mM $AgNO_3$ solution was combined and heated in a hot water (60 ˚C) bath for 30 minutes. This was then repeated using the generated stembark extracts. Aliquots (1.5 ml) of each solution were centrifuged for 5 minutes 5 times at 20 ˚C and 16.0 rcf using the Eppendorf centrifuge 5415R. The pellet was suspended in water for further use for characterization.

#### 6.2.2.1. Characterization: UV-visible Spectrophotometry

Deionized water (0.5 ml) was used as a control to zero the spectrophotometer. Thereafter, 0.5 ml of each solution was aliquoted into glass cuvettes and loaded into a spectrophotometer (Shimadzu UV-1800, Japan). The generated AgNP solution was characterized within the 300-600 nm wavelength range.

#### 6.2.2.2. Characterization: Energy-dispersive X-ray (EDX)

An energy dispersion X-ray analysis (EDX) was performed to determine the chemical composition of synthesised nanoparticles. A 0.1 ml of each solution was placed on the surface of individual sterile aluminum stubs. These were left to dry for 2 hours followed by sputter coating in gold using the Quarum Q150 RES gold coater. The samples were then viewed and analyzed using the Field Emission Gun Scanning Electron Microscope Zeiss FEGSEM Ultra Plus, AzTec software.

#### 6.2.2.3. Characterization: Transmission electron microscopy (TEM)

Aliquots of the sonicated solutions were added onto separate copper grids and left to dry for 2 hours. The analysis was carried forth to authenticate the presence, shape and size of the biosynthesized AgNPs. Samples were viewed using the High-Resolution Transmission Electron Microscope JEOL 2100.

#### 6.2.2.4. Characterization: Nanoparticle stability

A Nanoparticle Tracking Analysis (NTA) was performed in order to obtain information on the size and zeta potential of the generated nanoparticles. This was done using the Nanosight NS500 (Malvern Instruments, Malvern, Worchestershire, UK), equipped with sample chamber (approximately 0.25 ml), a sCMOS camera and a laser wavelength of 430 nm. Prior to use, all extracts were vortexed (30 seconds), sonicated (20 minutes) and diluted in 18 MOhm water (1:100, 1 mL). Images were obtained and analysed using the NTA 3.2 analytical software.

#### 6.2.2.5. Characterization: Fourier-transform infrared spectroscopy:

The nanoparticle solutions were analyzed using the Agilent Cary 630 spectrometer with Agilent MicroLab PC 5.1.22 for data collection. The data was generated by a ATR Diamond-1 Bounce with 30 background scans and 30 sampling scans in the range 4000 – 650 $cm^{-1}$ with a resolution of 4 $cm^{1}$. The ResolutionPro 5.0.0.395 software was used to process the data obtained to generate peaks and smooths. The analysis was carried forth to determine the type of compound classes present within the solution. The data obtained was then analyzed using the IR Pal V 2.0 table driven infrared Application, by Dr .Wolf van Heeswijk, 2010.

### 6.2.3. Antibacterial screening

The antibacterial properties of the generated AgNP extracts (concentrations: 10, 12.5, 25, 50 and 100 mg/mL) were screened against five different bacterial strains: *Escherichia coli* (ATCC 35218*), Pseudomonas aeruginosa (*ATCC 25783*), Bacillus subtilis (ATCC:6633)*, *Staphylococcus aureus (*ATCC: 25923*)* and Methicillin-Resistance *Staphylococcus aureus* (ATCC: 43300).

A silver nitrate solution was used as the negative control. In addition, positive controls were used. Streptomycin was used against gram positive bacteria and gentamycin was used against gram negative bacteria.

The medium was prepared using Biolab Mueller Hilton agar, South Africa (38 g) and distilled water (1L). Following this, 20 ml of media were poured into sterile Petri dishes and allowed to set. A prepared nutrient broth (yeast extract, 5 g; tryptone powder, 10 g; NaCl, 10 g) was used to grow bacterial cultures (incubation period of 24 h). A 0.5 McFarland turbidity standard was used as a guide to manipulate the turbidity of the bacterial cultures. This standard provided an optimum density at which a comparable range of bacterial cell growth was found. This method provided a standardized platform on which the growth or inhibition of different microorganisms were compared. The optical density of each bacterial strain was measured at 620 nm using the Agilent Technologies Cary 60 UV-Vis. The concentrations of the bacterial broths were adjusted to obtain a resulting optical density of 0.1. The bacterial cultures were then evenly spread over the agar with a sterile loop and spreader. All relevant materials and media were autoclaved. Sterile filter paper discs (1 cm) were inoculated with 90 µL of extract and left to dry. These discs were then placed on the agar and the plates were incubated for a period of 24 h. Gram positive bacterial strains were incubated at 30$^{o}$C while gram negative strains were incubated at 37$^{o}$C. These plates were then analyzed for apparent zones of inhibition.

### 6.2.4. Cytotoxity assay

#### 6.2.4.1. Preparation of extracts: Concentrations

A 5 mg/mL stock solution was generated for the purpose of this assay. Portions of this extract was, subsequently, diluted, using distilled water, to formulate the concentrations mentioned below. Concentrations evaluated: 15, 30, 60, 120 and 240 µg/mL.

#### 6.2.4.2. MTT assay

The cytotoxic effect of the generated extracts was evaluated against three cell lines, human embryonic kidney cells (HEK293), human cervical carcinoma cells (HeLa) and human breast adenocarcinoma cells (MCF-7) (All cell lines were sourced from ATCC, Manasas, VA, USA). Cells were incubated in sterile 96 well plates, (cell densities: 250000-300000 cells per well) overnight at 37˚C. Thereafter, the growth medium was replaced with fresh medium, and 100 µL of each extract at their varying concentrations were placed into the respective wells. The cells were then incubated for 48 hours at 37$^{o}$C. This was done in triplicate. The growth medium was then removed and replaced with 0.1 ml of medium containing 0.01 ml of MTT solution (5 mg/ml in PBS), and cells incubated at 37˚C for a further 4 hours. The MTT and medium was then removed and 0.1 ml dimethylsulphoxide (DMSO) was added to the wells.

The absorbances were measured at 570 nm using a Mindray MR-96A microplate reader (Vacutec, Hamburg, Germany).

### 6.2.5. Apoptosis assay:

In order to evaluate the apoptotic potential of the generated extracts, an apoptosis analysis using acridine orange/ ethidium bromide (AO/EB) staining was performed. Cells were incubated in sterile 96 well plates, (cell densities: 250000-300000 cells per well) overnight at 37˚C. The growth medium was then aspirated and substituted with 250 µL of complete medium. The concentration at which the highest toxicity occurred were utilised. Approximately, 150 µL of extract (most toxic cytotoxic concentration was tested 240 µg) was added to each well and the plate was then incubated for 24 hours at 37˚C. Subsequently, all remaining growth medium was removed and the cells were briefly rinsed (twice) using 200 µL of PBS. Cells were then stained using 10 µl of the dye (100 µg/ml acridine orange, 100 µg/ml ethidium bromide in PBS). The plate was shaken for a duration 5 minutes on a Stuart Scientific platform rocker at 30 rev/min. The cells were then washed using PBS visualized using an inverted fluorescence microscope (Olympus CKX41, Tokyo, Japan) at excitation and emission wavelengths of 490 nm and 516 nm respectively. In order to quantify the apoptotic properties of the extracts against the tested cell lines, the apoptotic index was calculated using the equation listed below.

$$Apoptotic\ Index = \frac{\text{Number of apoptotic } cells}{\text{Number of total cells counted}}$$

### 6.2.6. Statistical analysis

The emanating study was performed in triplicate. All generated data was subjected to a statistical analysis using the IBM SPSS, statistical software version 27, IBM CORP1997, USA.

A *post hoc*, tukey's- honest significant difference test was performed. All data was normalised and a significant difference was noted among all data sets, $P<0.05$.

## 6.3. Results and discussion

The presence of medicinally important phytometabolites (bioactive compounds) within medicinal plants have been established through many phytopharmological studies (Chung et al., 2016). However, the overall medicinal effectiveness of these phytometabolites may be limited due to their absorption constraints (Hu et al., 2019). Most phytometabolites are highly soluble in water but do not have the ability to penetrate cellular lipid membranes due to their large molecular size therefore reducing their overall efficiency (Chinnasamy et al., 2017). Thus, the use of nanotechnology in medicine, is crucial in combating such issues. Nanotechnology allows for the phytometabolites to attached to nanosized particles therefore reducing their overall delivery size enabling better absorption rates and improving its overall efficiency (Demirdogen et al., 2018). Bio-nanotechnology allows for effective drug delivery via the establishment of a controlled environment by directing therapeutics to the target area and controlling the treatment period (Hu et al., 2019). Within this study, *C. erythrophyllum* (stembark and leaf extracts) had the ability to reduce $Ag^{+}$ therefore generating AgNPs. This is a novel protocol to this species and has not been reported before.

Hexane, chloroform and methanol were used as the solvents of choice for the extractions. Due to the polarity of methanol, methanolic extracts appeared to have the highest extraction rate of both polar and non-polar secondary metabolites within. Figure 6.1a depicts a mixture of the silver nitrate solution and the generated methanol stembark and leaf extracts of *C. erythrophyllum*, respectively. At this stage, no colour change was visualized. Figure 6.1b indicates the distinct colour change of the above-mentioned solutions after heating. The leaf solution changed from pale green to a dark brown colour, while the stembark solution changed from an opaque coloured solution to a distinctive deep red brown colour (Fig. 6.1b). A colour change is indicative of the reduction of Ag+ and the collation of excited electrons. A series of oxidation reduction-based reactions allows for the biosynthesis of AgNPs, hence a colour change signifies the presence of AgNPs. This was noted for all extracts, however a more intense colour change was noted for the methanolic extracts. This correlates with previous studies conducted on species such as *Combretum quadrangulare* and *Combretum indicum*, in which Ag+ were reduced by the extracts (capping/ reducing agents) and AgNPs were formulated (Bahuguna et al., 2016; Chittasupho and Athikomkulchai, 2018).

## Characterization: UV-visible Spectrophotometry (UV-vis)

Metal nanoparticles are easily identified due to their unique optical properties and surface plasmon resonance (Demirdogen et al., 2018). Surface plasmon resonance is a region parallel to the surface of the nanoparticles where excited electrons collide (Gibb et al., 2018). Electron movement is characterized by the relative size and shape of particles hence nanoparticles deflect specific spectrums within the UV region, indicating their presence (Hu et al., 2019). Nanoparticles are characterized by unique intense absorbance within the UV region, thus a visualization using UV-vis is key in characterizing AgNPs (Sheoran and Kaur, 2018).

The surface plasmon resonance (peak) were displayed at 420 nm and 418 nm for the leaf and stembark extracts, respectively (Fig. 6.1c and d). Research suggests that the presence of AgNPs is confirmed by an absorbance maximum between 418 nm – 460 nm (Nabikhan et al., 2010; Awwad et al., 2013; Vanaja and Annadurai, 2013). In both samples, an inversely proportional relationship was seen between the wavelength (nm) and relative absorption. In addition, an increase in wavelength was noted against a decreasing absorption rate, after the peak in absorption (Fig. 6.1c and d). This trend may indicate the presence of large AgNPs and the possible reduction in reaction rates between phytometabolites and silver nitrate.

Plant components may differ in their phytometabolite composition, hence affecting the characterisation of formulated nanoparticles (Chung et al., 2016). Nanoparticles produced from leaf extracts may differ from those produced from stembark extracts. It can be noted that areas within a plant that is exposed to higher levels of abiotic stress such as leaves, are known to contain higher levels of phytometabolites (Khatoon et al., 2017).

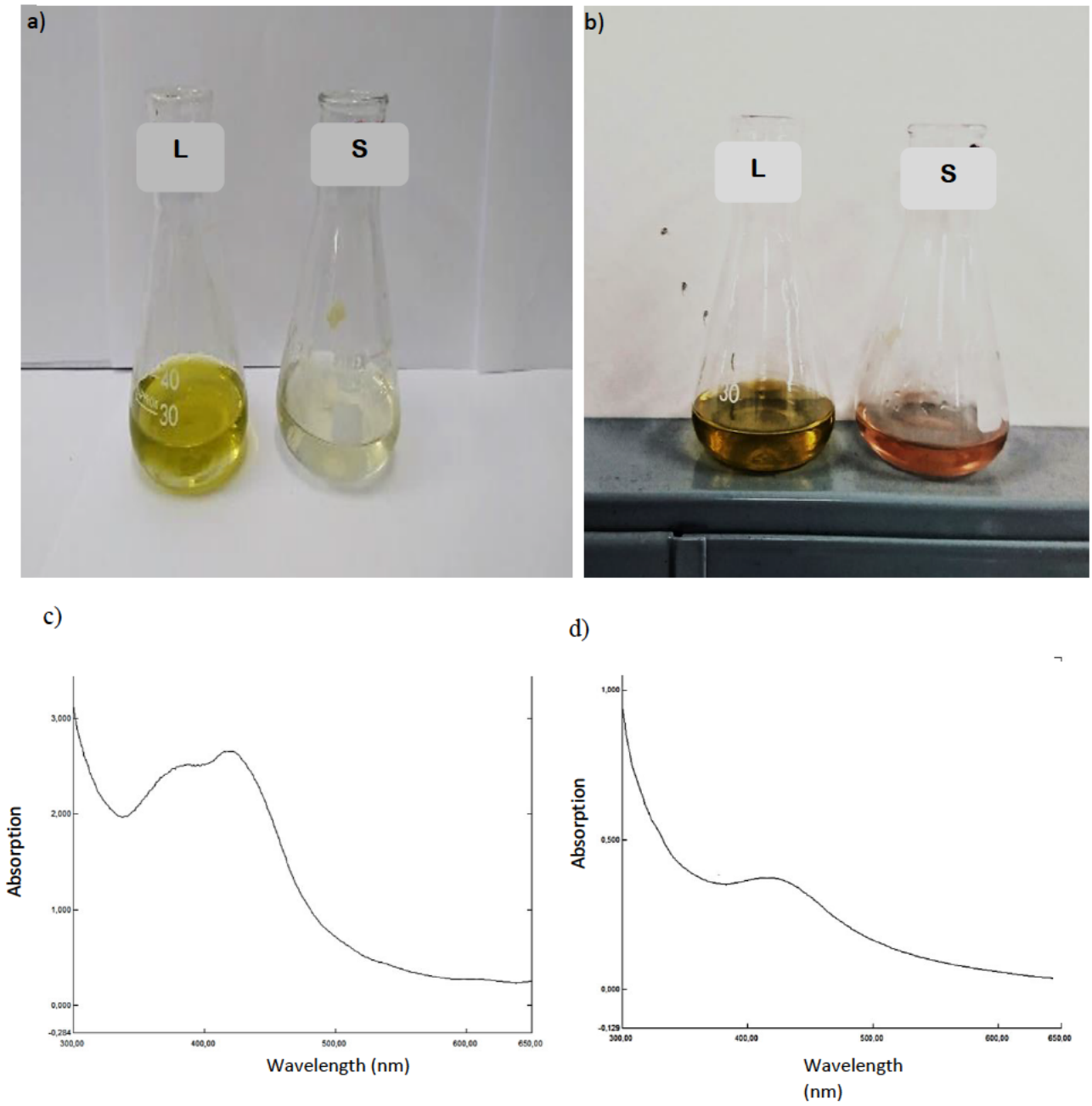


Figure 6.1: Methanol a) Leaf and stembark extract, with the $AgNO_3$ solution, before heating and b) leaf and stembark extract, with the $AgNO_3$ solution, after heating, c) UV- vis spectroscopy recorded after 30 minutes of heating, leaf $AgNO_3$ solution and d) stembark $AgNO_3$ solution L=Leaf and S=Stem bark.

## Characterization: Energy-dispersive X-ray (EDX)

EDX is a microanalysis which allows for the detection of elements within a sample using reflecting electrons emitted by the SEM (Paulkumar et al., 2017). Current research links the utilization of this technique to the study of drug delivery via the detection of nanoparticles (Hu et al., 2019). Within this technique atoms become ionized and gradually return to their ground state, releasing unique energy x-rays characteristic to a specific element. An EDX graph indicates a specific element's energy level in correspondence to its peak (Sharma, 2018).

The positive reduction of the AgNPs by the *C. erythrophyllum* extracts was validated by the EDX analysis. The distinct presence of silver was noted within all extracts, at 3 keV (Fig. 6.2-4). Within the leaf extract, a minimal amount of silver was noted, while the stembark extract was composed of higher percentages of silver (Fig 6.2-4). This result indicates possible higher antimicrobial activity within the stem bark. The presence of C, O and Cl indicates an active metabolizing stembark. Calcium was detected in the leaf extracts and may be related to the presence of calcium crystals (Bantho et al., 2020). Na and Zn were detected in most extracts, these are regarded as micronutrients and are also known for their ecological role of defense against pests and pathogens while aiding in providing a homeostatic metabolizing system in the plant (Honghong, 2018; Cabot et al., 2019). In addition, Cl may indicate the presence of a gelatinous covering across leaf and stembark surfaces (Yadav et al., 2016). Notably, the leaf extract contained Fe and K, which are essential nutrients and their presence is well established in medicinal plants (Sharma, 2018). The distinct presence of these compounds in the leaf, suggest that the leaf may be more medicinally inclined in comparison to the stem bark.

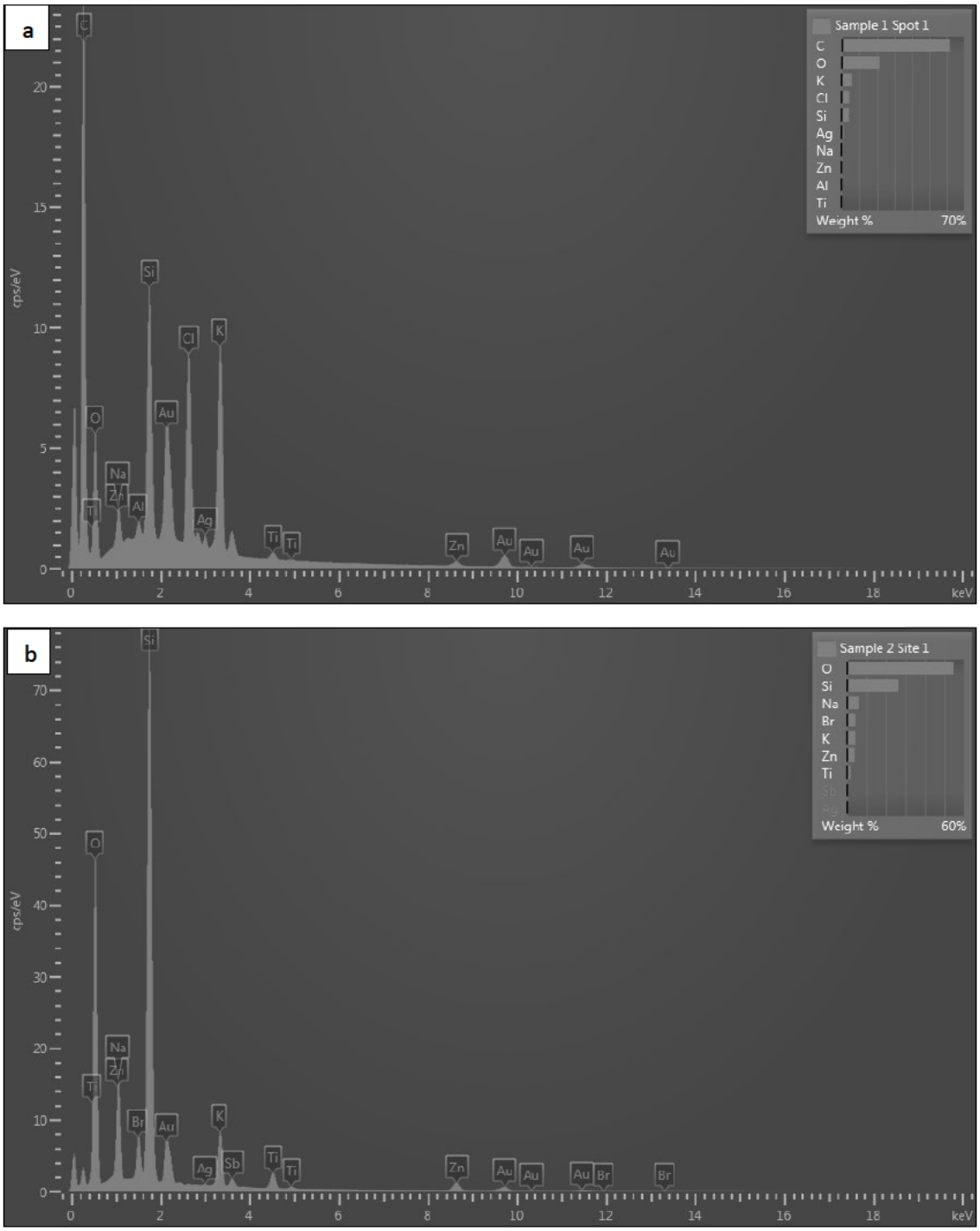


Figure 6.2: EDX analysis of the AgNPs hexane a) leaf and b) stembark solution.

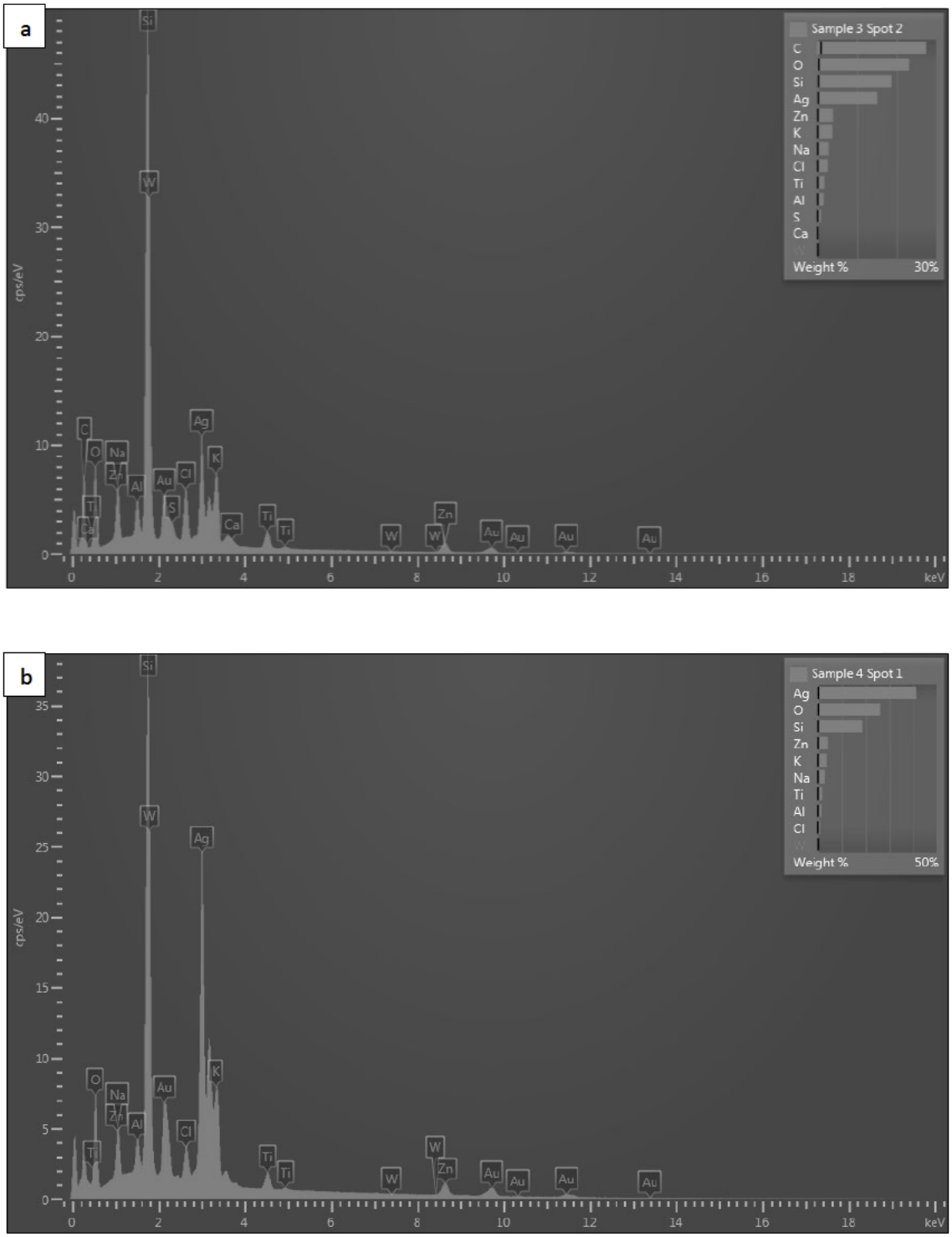


Figure 6.3: EDX analysis of the AgNPs chloroform a) leaf and b) stembark solution.

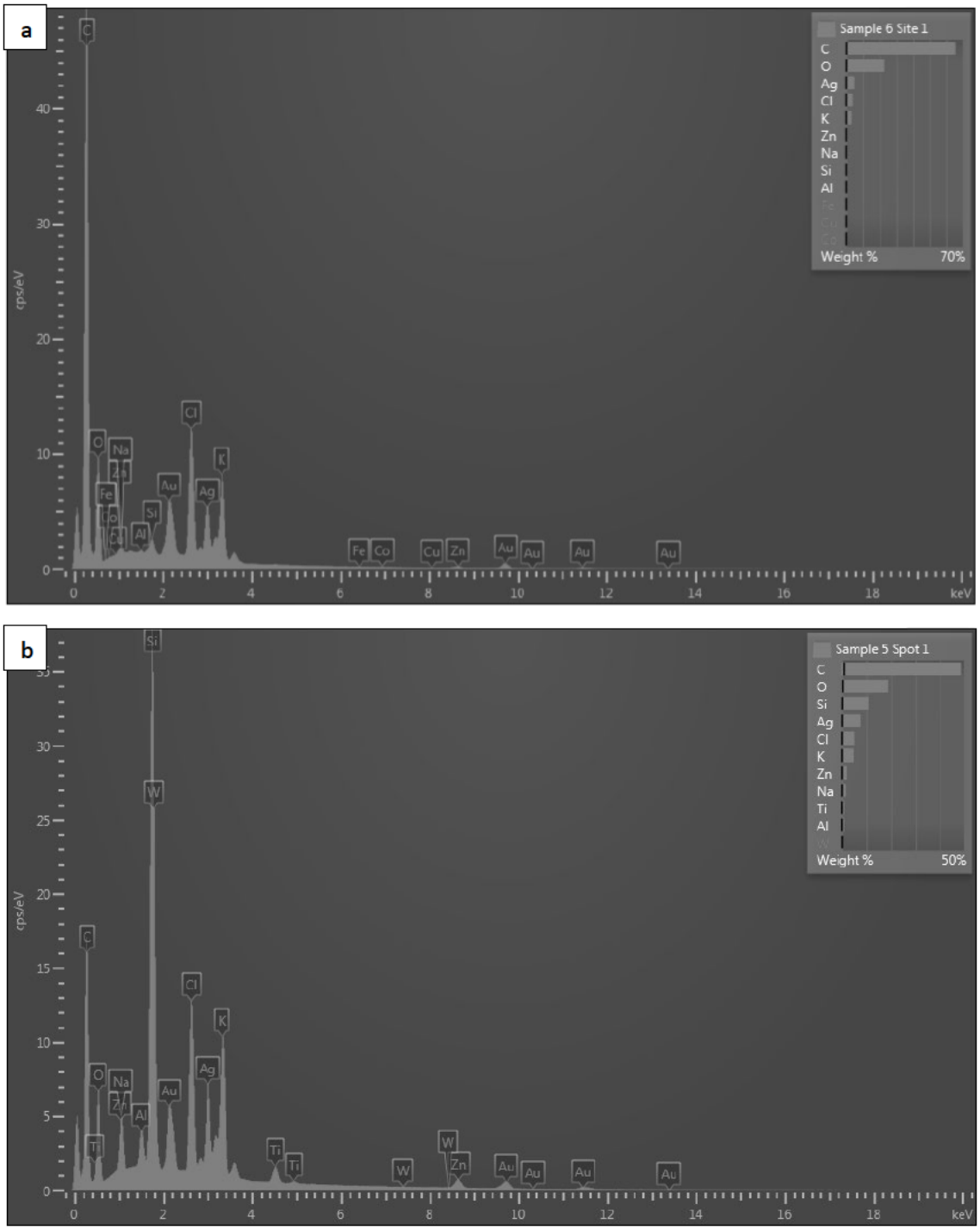


Figure 6.4: EDX analysis of the AgNPs methanol a) leaf and b) stembark solution.

## Characterization: Transmission electron microscopy (TEM)

Synthesized nanoparticles can be structurally and morphologically characterized using TEM. As electrons hit and interact with the sample, energy rays are emitted thus generating a 2-d image of high resolution. The image analysis software then uses this information to generate data of the individual particles. As a result, data based on the distribution, location, shape and other structural outliers can be noted (Srirangam and Rao, 2017).

The shape and structural features of the newly synthesis nanoparticles were visualized, using TEM (Fig. 6.5). Overall, stembark nanoparticles appeared to be significantly larger in size (23- 45nm) and oval (Fig. 6.5). The leaf nanoparticles appeared smaller in size with spherical and triangular shaped particles (20-25nm) (Fig. 6.5e). The formation of triangular shaped particles is due to the binding that occurs between stabilizing polymers and the crystal side of a particle (Granbohm et al., 2018). As a result, accelerated growth on the binding side is caused and a longer side of the particle is formed. Pal et al. (2007) indicated that triangular shaped nanoparticles are the most effective antimicrobial agents due to their shape and stability. Particles formed by the leaf extract ranged from 15 nm to 35 nm whereas particles of stembark extract ranged from 25 nm to 55 nm. Presence of spherical shaped nanoparticles were noted for extracts of *C. molle* (Sibiya and Moloto, 2018).

It was noted that the stembark is comprised of larger nanoparticles, with a higher $Ag^+$ content, as opposed to the leaf extracts. Granbohm et al. (2018) linked the size of nanoparticles to the concentration of $Ag^+$ ions present in the nanoparticles. It is suggested that larger nanoparticles contain a higher concentration of $Ag^+$, and hence have higher antimicrobial activity. Raza et al. (2016) indicated that the antimicrobial effect is predominant from nanoparticles that have a larger surface area enhancing microbe's interaction. The size of nanoparticles can also be related to the presence of phytometabolites found in extracts (Kumar et al., 2010). Kumar et al., (2010) implied that larger AgNPs are directly related to the increased presence of polyphenols. In relation to this, Mawoza (2015) indicated that the presence of numerous phytometabolites such as phenols, flavonoids, triterpenoids and saponins may enhance AgNP synthesis and particle size. In addition, a thin film was seen surrounding the stembark nanoparticles. Mallikarjuna et al. (2011) related the presence of a thin film around nanoparticles to the possible capping of the AgNPs by the chemical substitutes.

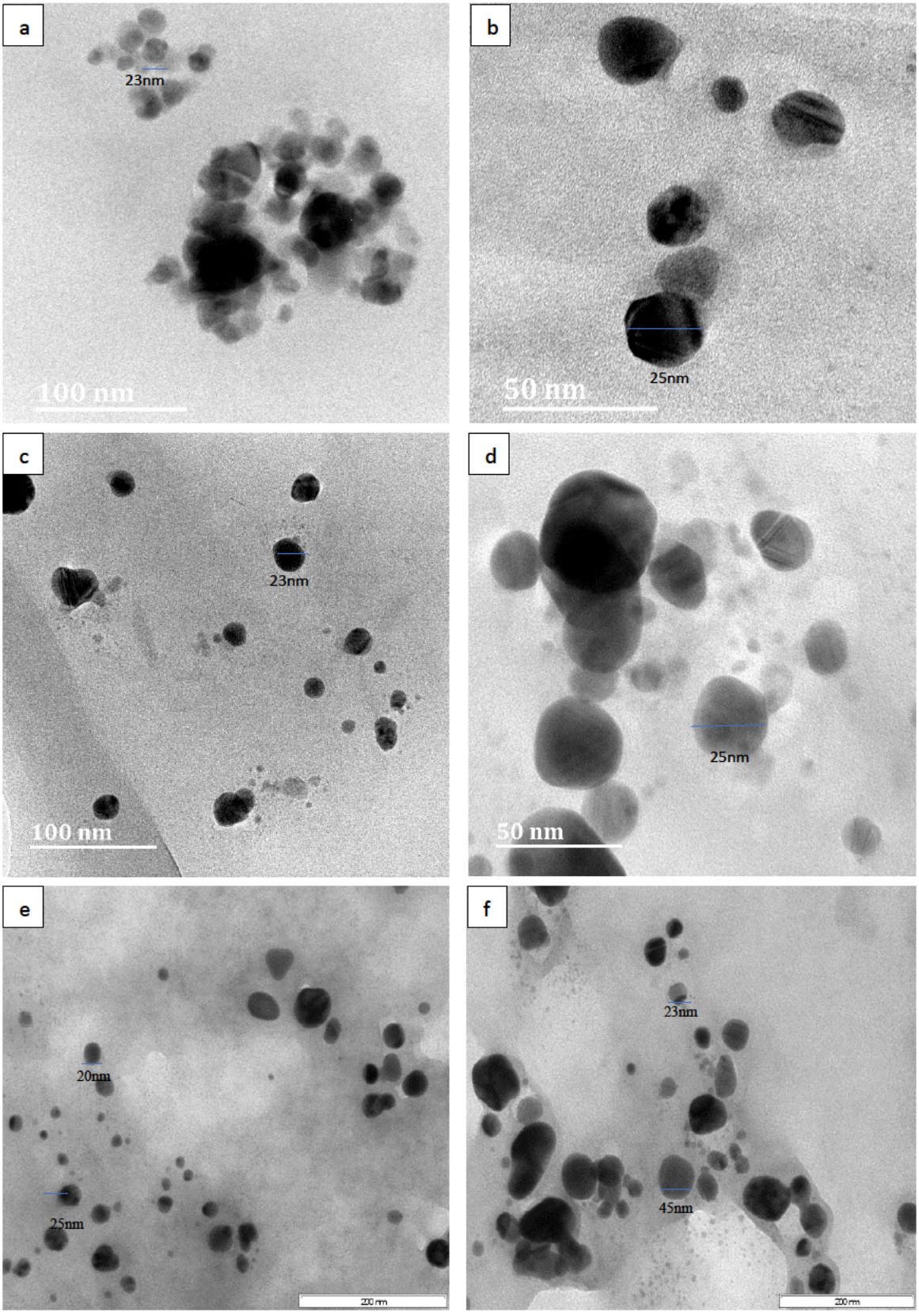


Figure 6.5: TEM micrographs indicating silver nanoparticles of different size and shape present in the hexane a) leaf, b) stembark extract, chloroform c) leaf, d) stembark and methanolic e) leaf and f) stembark extracts of *C. erythrophyllum*.

## Nanoparticle Tracking Analysis (NTA)

The synthesized nanoparticles were characterized using a nanoparticle tracking analysis (NTA). Information on the particle size, distribution and their relative zeta potential was elucidated from the emanating analysis.

A nanoparticle's delivery system effectiveness is greatly influenced by the size of the nanoparticle (Yugay et al., 2021). The size determines the distribution, circulation time, carrying capacity, penetration ability and cellular uptake. The zeta potential refers to the surface charge of a nanoparticle (Alabdallah and Hasan, 2021). This data provides crucial information regarding the colloidal stability of the nanoparticle which in turn influences the overall pharmacokinetic properties of the synthesized nanoparticle (Honary and Zahir, 2013). As noted above, TEM analysis revealed the approximate size of the AgNPs generated from the leaf and stembark extracts of *C erythrophyllum.* However, the NTA data provided more accurate information regarding the size of AgNPs particles in an aqueous medium as seen in an *in vivo* environment.

The emanating TEM analysis showed the particle size to range from (leaf 20-30 nm) and (stembark 25-5 0nm). The NTA data showed the particle size to range from (leaf 101-148 nm) and (stembark 107-137 nm). The noticeable differences in measurements between both methods of analysis could be due to the manner in which samples were prepared for the analysis. For the TEM analysis, liquid samples were dried upon a copper grid whereas for the NTA analysis, samples were viewed in liquid medium. Research suggests that results obtained via the NTA method are more accurate as the particles are subjected to a hydrodynamic environment (Akwu et al. 2021). The accurate determination of the size of a nanoparticle is crucial in determining the effectiveness of a drug delivery system (Yugay et al., 2021).

The zeta potential of a nanoparticle provides important information regarding the surface charge and colloidal stability of the nanoparticle (Alabdallah and Hasan, 2021). Griffith's et al., (2013) suggested that the ideal zeta potential of a nanoparticle should be higher than 25 mV or lower than -30 mV. Nanoparticles found with this spectrum are most stable given the high levels of electrostatic repulsion found in the solution resulting in less aggregation of the nanoparticles (Yugay et al., 2021). Results obtained showed that AgNPs generated using hexane (leaf), chloroform (leaf and stem bark) and methanol (leaf), had a negative zeta potential that fell within the desired range (less than -30 mV). Overall, these AgNPs have a high colloidal stability and do not need the addition of a polymer for further stabilization. Furthermore, it can also be noted that the extracts of the hexane stembark and methanol leaf had a zeta potential more than -30 mV, suggesting that the AgNPs generated in these

extracts are unstable, with high collision possibilities. In order to stabilize these nanoparticles, the addition of a polymer is required. The overall zeta potentials obtained indicated that all extracts exhibited a negative charge. Research suggests that nanoparticles exhibiting a negative zeta potential can penetrate cells effectively (Yugay et al., 2021). This is primarily because cell membrane absorption is not characterized by a negative or positive zeta potential however the nanoparticles should fall in the desired range unless accompanied by a polymer. Interestingly, negatively charged nanoparticles are favored due to their increased circulation rate and proposed lower cytotoxicity levels as opposed to positively charged nanoparticles (Alabdallah and Hasan, 2021).

Table 6.1: Average size and zeta potential, of the AgNPs generated using *C. erythrophyllum.*

| **Extract** | | **Size (nm) and ± SE** | **Zeta potential (mV) and SE** |
|---|---|---|---|
| **Hexane** | **Leaf** | 101.2 +/- 0.1 | -19.8 +/- 0.3 |
| | **Stem bark** | 114.0 +/- 4.7 | -31.9 +/- 0.0 |
| **Chloroform** | **Leaf** | 146.8 +/- 1.1 | -17.7 +/- 0.0 |
| | **Stem bark** | 135.2 +/- 6.6 | -25.3 +/- 0.0 |
| **Methanol** | **Leaf** | 133.6 +/- 1.6 | -38.9 +/- 0.0 |
| | **Stem bark** | 107.1 +/- 4.0 | -28.8 +/- 0.1 |

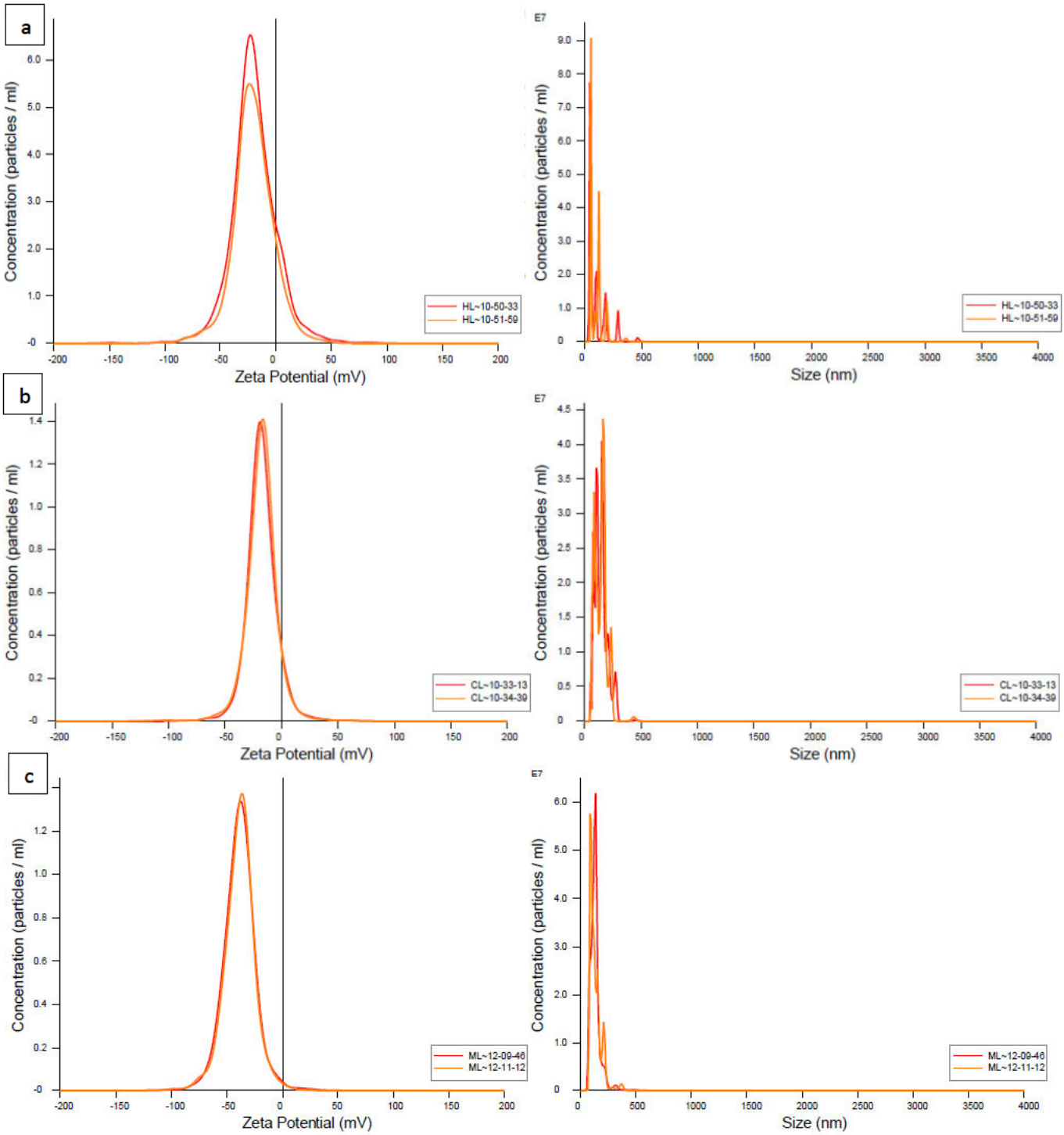


Figure 6.6: Size and zeta potential distribution of nanoparticles generated from a) leaf (hexane), b) leaf (chloroform) and c) leaf (methanol)

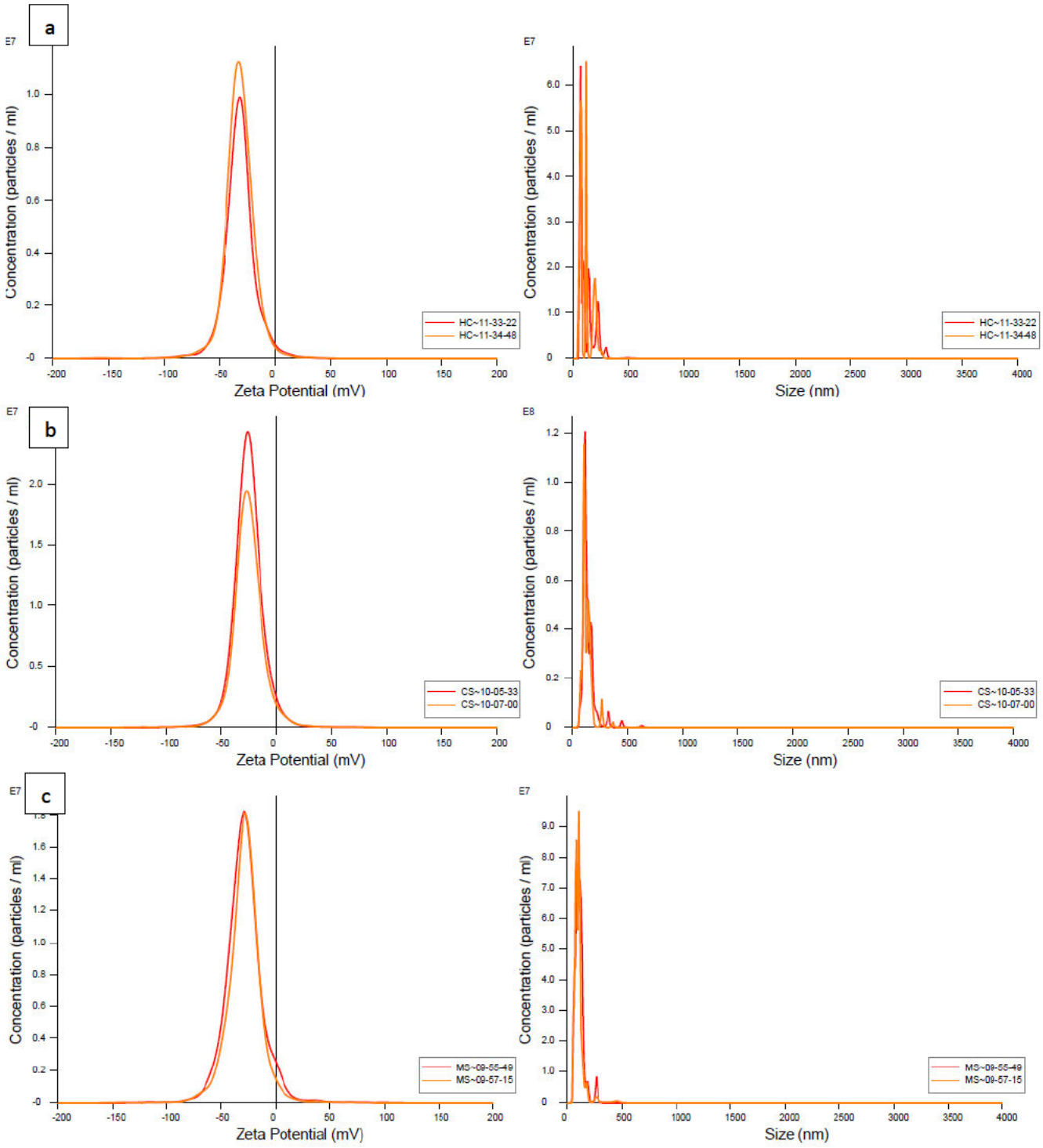


Figure 6.7: Size and zeta potential distribution of nanoparticles generated from a) stembark (hexane), b) stembark (chloroform) and c) stembark (methanol)

## Antibacterial screening

Preliminary antibacterial screening was conducted using the AgNPs synthesized from the leaf and stembark extracts. For the nano- leaf extracts, clear zones of inhibition were seen against most of the 5 tested bacterial strains, whereas the nano- stembark extracts failed to produce a zone of inhibition against *Pseudomonas aeruginosa*. Tables 6.2-4 highlights the antibacterial properties exhibited by the nano extracts. Overall, methanolic nano extracts appear to have performed the best. Largest zones of inhibition were displayed by the methicillin-resistant *S. aureus* (Gram negative), when treated using the methanolic leaf AgNPs.

The nano-leaf extracts showed intense activity against *E. coli*, *Bacillus subtillus*, Methicillin-Resistant *S. aureus* and *P. aeruginosa*. Nano- stembark extracts displayed intense inhibitory activity against *B. subtillus* and *E. coli*. The nano extracts demonstrated inhibitory activity against Gram positive bacteria in comparison to Gram negative bacteria; in particular, nano- stembark extracts showed no zone of inhibition against *E. coli* (Table 6.2).

Analysis indicated a higher overall antibacterial activity in leaf extract. Raza et al. (2016) revealed that a smaller sized nanoparticle has the highest bacterial efficiency against certain bacteria. This is due to the ability of the AgNPs to penetrate the cell membrane of bacterial cells (Kim et al., 2018). This mechanism causes an interaction between proteins present in the bacterium and released $Ag^{+}$ which results in cell degradation. AgNPs antibacterial action relies on their ability to release $Ag^{+}$ that disrupt cellular processes (Raza et al., 2016). Small nanoparticles have a smaller surface area in comparison to their volume. Hence, they can release a larger quantity of silver cations which allow them to destroy bacteria more easily (Yadav et al., 2016). The antibacterial property of the solution is determined by the AgNPs ability to release Ag+ (Demirdogen et al., 2018). If there is a slow release of $Ag^{+}$ then there is limited antibacterial effect (Patel, 2013).

Table 6.2: Antibacterial activity: Zones of inhibition (mm) of the biosynthesized AgNPs from *Combretum erythrophyllum* leaf and stembark hexanolic extract.

| **Bacterial strain:** | **Leaf extract** | | | | | **Stembark extract** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Concentrations (mg/mL)** | | | | | **Concentrations (mg/mL)** | | | | |
| | *10* | *12.50* | *25* | *50* | *100* | *10* | *12.50* | *25* | *50* | *100* |
| *Escherichia coli* | 10 | 12.5 | 18.5 | 21.5 | 25 | 7 | 7 | 9 | 12 | 15 |
| *Pseudomonas aeruginosa* | 10 | 10 | 15 | 18 | 20 | - | - | - | - | - |
| *Staphylococcus aureus* | - | - | - | - | - | 7 | 7 | 9 | 12 | 15 |
| Methicillin Resistant *Staphylococcus aureus* | 7 | 7 | 10 | 12 | 15 | 7 | 7 | 9 | 15 | 20 |
| *Bacillus subtillus* | - | - | - | - | - | 7 | 7 | 9 | 12 | 18 |

Table 6.3: Antibacterial activity of the biosynthesized AgNPs from *Combretum erythrophyllum* leaf and stembark chloroform extract.

| **Bacterial strain:** | **Leaf extract** | | | | | **Stembark extract** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Concentrations (mg/mL)** | | | | | **Concentrations (mg/mL)** | | | | |
| | *10* | *12.50* | *25* | *50* | *100* | *10* | *12.50* | *25* | *50* | *100* |
| *Escherichia coli* | 10 | 10.5 | 15 | 21.5 | 25 | 7 | 7 | 9 | 12 | 15 |
| *Pseudomonas aeruginosa* | 10 | 10 | 15 | 18 | 20 | 7 | 9 | 11 | 12 | 15 |
| *Staphylococcus aureus* | - | - | - | - | - | 7 | 7 | 9 | 12 | 15 |
| Methicillin Resistant *Staphylococcus aureus* | 7 | 7 | 10 | 12 | 15 | 7 | 7 | 9 | 15 | 20 |
| *Bacillus subtillus* | 7 | 10 | 11 | 15 | 25 | 7 | 7 | 9 | 12 | 15 |

Table 6.4: Antibacterial activity of the biosynthesized AgNPs from *Combretum erythrophyllum* leaf and stembark methanol extract.

| **Bacterial strain:** | **Leaf extract** | | | | | **Stembark extract** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Concentrations (mg/mL)** | | | | | **Concentrations (mg/mL)** | | | | |
| | *10* | *12.50* | *25* | *50* | *100* | *10* | *12.50* | *25* | *50* | *100* |
| *Escherichia coli* | 12 | 12.5 | *20* | 25 | 28 | *12* | *12* | *17* | *23* | 25 |
| *Pseudomonas aeruginosa* | 10 | 10 | 15 | 18 | 20 | - | - | - | - | - |
| *Staphylococcus aureus* | 7 | 7 | 9 | 12 | 15 | 7 | 7 | 9 | 10 | 15 |
| Methicillin Resistant *Staphylococcus aureus* | 7 | 7 | 11 | 23 | 30 | 7 | 7 | 9 | 15 | 22 |
| *Bacillus subtillus* | *9* | 10 | 16 | 18 | 20 | 7 | 7 | *7* | 12 | 25 |

## Fourier-transform infrared spectroscopy (FTIR)

FTIR is a powerful tool which enables the identification of various functional groups within compounds (Poojitha et al.,2021). It works by interpreting the infrared absorption spectrum which is unique to each functional group (Burman, 2018).

The nano leaf and stembark extracts of *C. erythrophyllum* were characterized using FTIR. As a result major functional groups were indicated by the formation of a peak (IR band). FTIR analysis confirmed the presence of phenols, alkenes, amines, alcohols and esters among many others in the hexane, chloroform and methanolic extracts (Table 6.5 and 6). Interestingly, aromatics were only detected in the hexane nano- leaf and stembark and chloroform nano-stembark extracts. In the nano-leaf solution, major IR bands were seen at 3272.29, 1638.09 and at 574.62 cm $^{-1}$. For the generated nano-stembark solution, major IR bands were seen at 3271.83, 1638.91 and at 571.65 cm $^{-1}$(Fig. 6.4). The major IR bands seen are highly similar for both extracts. The band found around 3272 cm-1 is represented of a C-H stretch that is characteristic of the alkynes functional group (Fig. 6.5). In addition, phenols are also representative in this wavelength. The band found around 1638 cm$^{-1}$ is representative of both a C=C and C=O stretch which are characteristic of the alkene and amide functional groups respectively. Lastly, the band found around 571-574 cm$^{-1}$ region is representative of a C-Br stretch that is characteristic of alkyl halides. In the study, FTIR analysis confirmed the presence of functional groups within the generated extracts that could pertain to possible medicinal value (Sibiya and Moloto, 2018; Kulshreshtha et al., 2018, Poojitha et al.,2021).

Similarly, the chemical composition of *Combretum album* extracts were analyzed using FTIR. As a result, R-$CH_2$-OH groups, amines with NH and C-N stretches were noted (Burman, 2018). This research links the presence of compounds found through the FTIR analysis to its potential antibacterial efficacy.

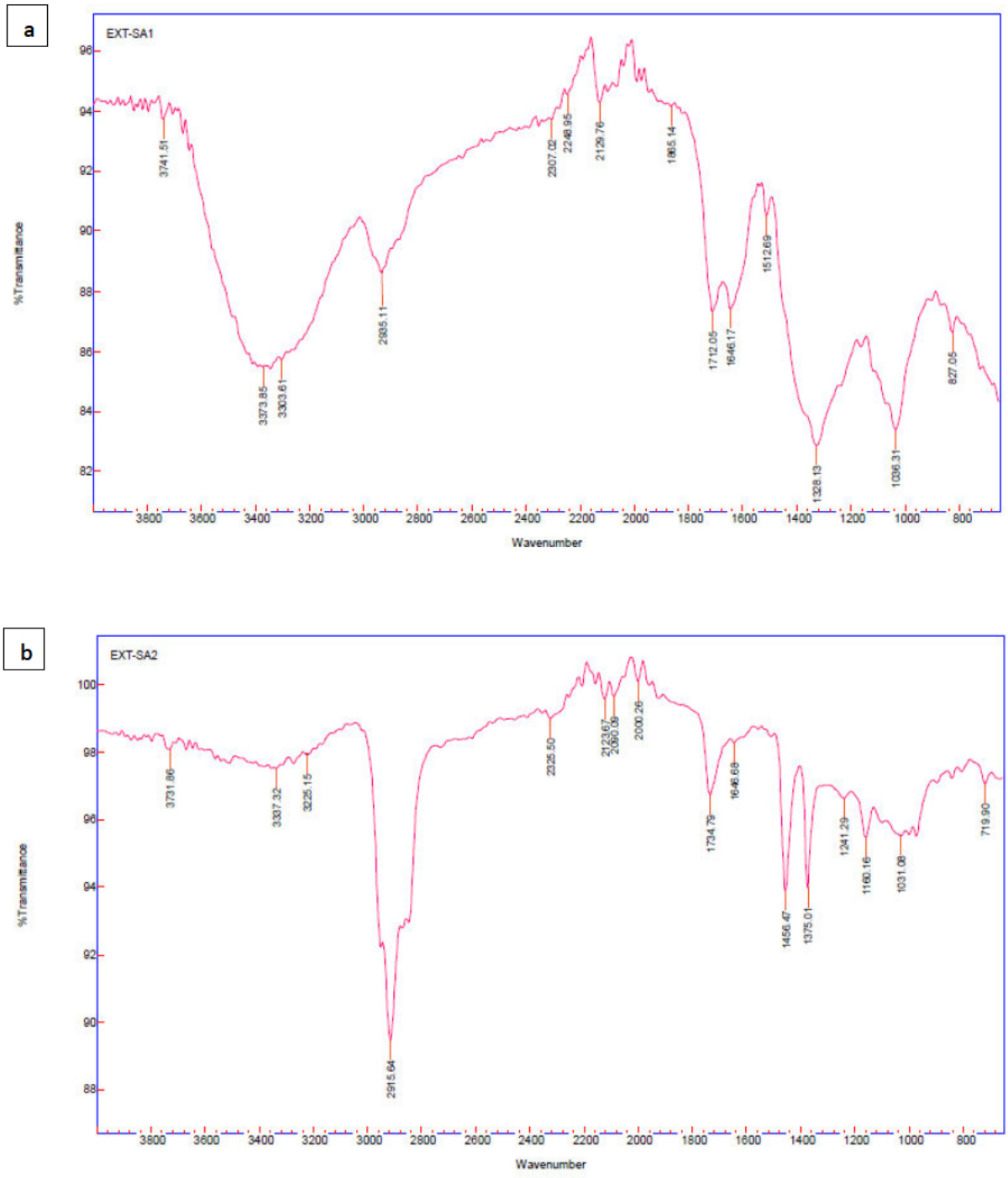


Figure 6.8: FTIR results obtained using the biosynthesized AgNPs from *Combretum erythrophyllum* a) leaf and b) stembark **hexane** extract.

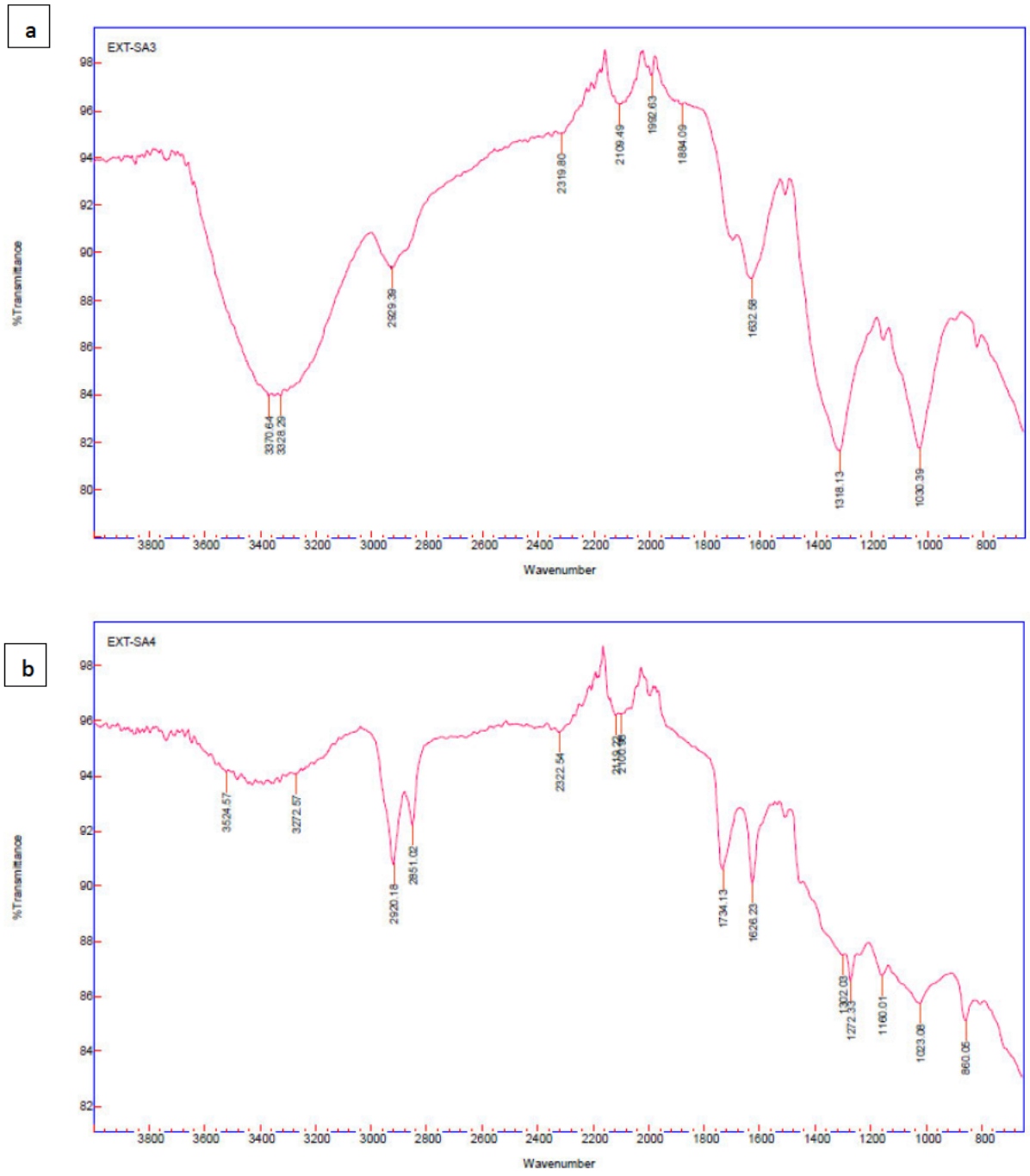


Figure 6.9: FTIR results obtained using the biosynthesized AgNPs from *Combretum erythrophyllum* a) leaf and b) stembark **chloroform** extract.

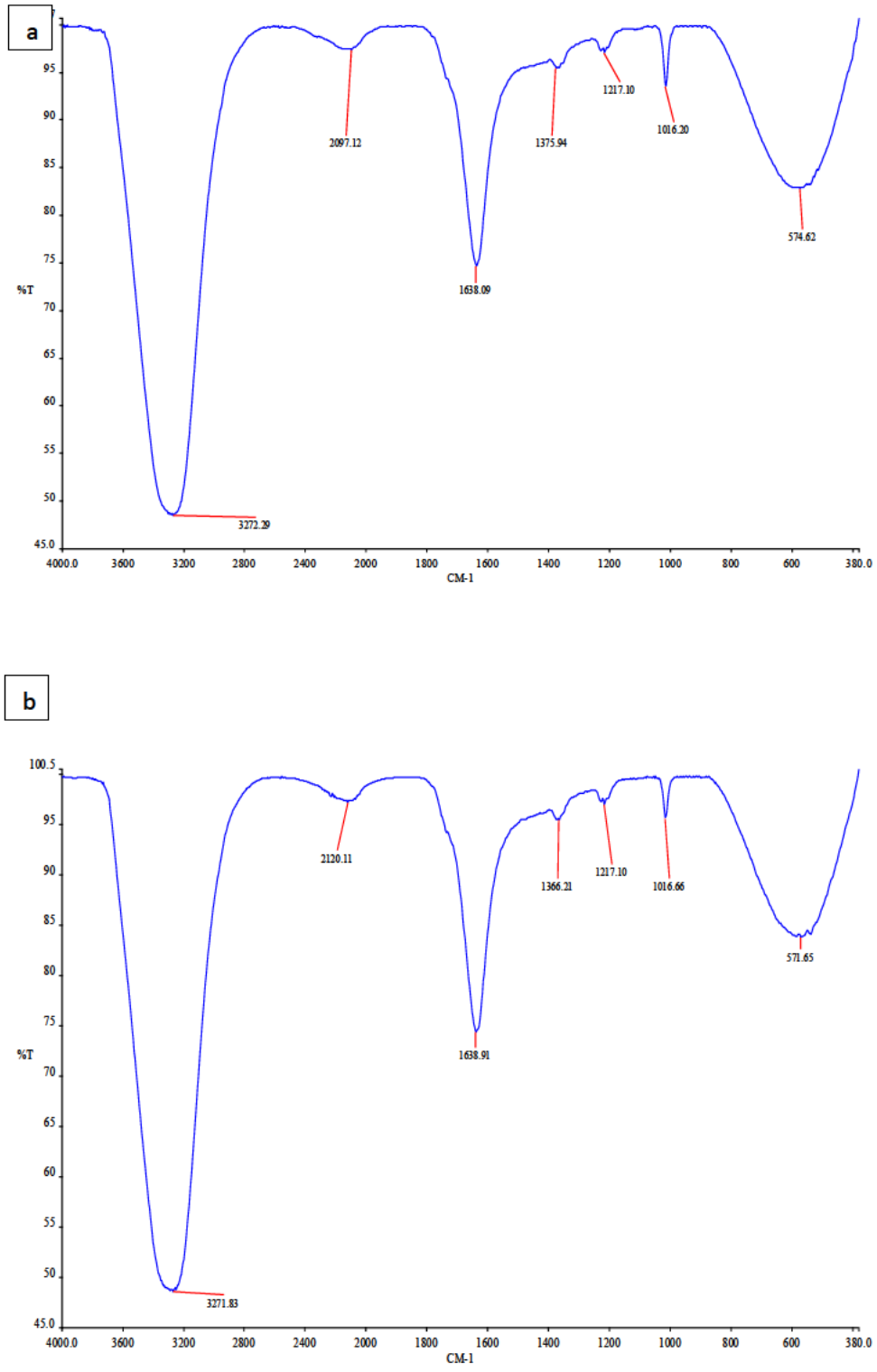


Figure 6.10: FTIR results obtained using the biosynthesized AgNPs from *Combretum erythrophyllum* a) leaf and b) stembark **methanolic** extract.

Table 6.5: FTIR results obtained the biosynthesized AgNPs from *Combretum erythrophyllum* leaf extract (Data analysed using IRPal 2.0 program).

| Wave number ($cm^{-2)}$ | Assignment | Possible compound class present | Extract |
|---|---|---|---|
| 3373.85 | RCO-OH | Carboxylic acids | Hexane, chloroform |
| | ArO-H -H | Phenols | |
| 3328.29 | RCONHR | Amides | Chloroform |
| | RCO-OH | Carboxylic acids | |
| | ArO-H -H | Phenols | |
| | RC#C-H stretch of C-H | Alkynes | |
| 3303.61 | RCO-OH | Carboxylic acids | Hexane |
| | ArO-H -H | Phenols | |
| | RC#C-H stretch of C-H | Alkynes | |
| 3272.29 | RC#C-H stretch of C-H | Alkynes | Methanol |
| | C =O stretch of COOH | Carboxylic acids | |
| | ArO-H -H | Phenols | |
| 2935.11 | RCO-OH | Carboxylic acids | Hexane, chloroform |
| | RCH2CH3 | Alkanes | |
| 2248.95 | RC#CR stretch of C#C | Alkynes | Hexane |
| 2129.76 | RC#CH stretch of C#C | Alkynes | Hexane, chloroform |
| | R-N-C | Miscellaneous | |
| | R-N=C=S | Miscellaneous | |
| 2097.12 | N=C stretch of R-N=C=S | Miscellaneous | Methanol |
| 1712.05 | R2CO | Ketones | Hexane |
| | RCO-OH | Carboxylic acids | |
| 1646.17 | R2C=CH2 | Alkenes | Hexane |

| | | | |
|---|---|---|---|
| | RCONHR Stretch of | Amides | |
| | C=O | | |
| | C=N | Miscellaneous | |
| 1638.09 | C=C Stretch | Alkenes | Methanol |
| | RCONHR Stretch of | Amides | |
| | C=O | Amines | |
| | $RNH_2$ $NH_2$ in plane | Miscellaneous | |
| | C=N | | |
| 1512.69 | N=O Nitroso | Miscellaneous | Hexane |
| | N-O nitro compound | Miscellaneous | |
| 1373.94 | $CH_2$ and $CH_3$ | Alkanes | Methanol |
| | S=O sulfate ester | Miscellaneous | |
| 1328.13 | R-F | Alkyl halides | Hexane |
| | S=O Sulfone | Miscellaneous | |
| | | | |
| 1318.13, | R-F | Alkyl halides | Chloroform |
| 1030.39 | S=O Sulfone | Miscellaneous | |
| | RC =OH stretch of C-O | Carboxylic acids | |
| | RCOOR stretch of C-O | Esters | |
| 1217.10 | R- F stretch of C-F | Alkyl halides | Methanol |
| | $RNH_2$ stretch of C-N | Amines | |
| | C =O stretch of COOH | Carboxylic acids | |
| | RCOOR stretch of C-O | Esters | |
| | P-H phosphine bending | Miscellaneous | |
| | of P-H | | |
| 1036.31 | R-F | Alkyl halides | Hexane |
| | RCO-OH stretch of C-O | Carboxylic acids | |

| | | | |
|---|---|---|---|
| | RCOOR’ stretch of C-O | Esters | |
| | S=O sulfoxide | Miscellaneous | |
| 1016.20 | R-R stretch of C-F | Alkyl halides | Methanol |
| | RCO-OH Stretch of C-O | Carboxylic acids | |
| | RCOOR stretch of C-O | Esters | |
| | Si-OR (broad) | Miscellaneous | |
| 827.05 | R2C=CHR | Alkenes | Hexane |
| | R-Cl | Alkyl halides | |
| | 1, 3, 5-trisub | Aromatics | |
| | S-OR esters | Miscellaneous | |
| 574.62 | R- Br stretch of C-Br | Alkyl halides | Methanol |

Table 6.6: FTIR results obtained for the biosynthesized AgNPs from *Combretum erythrophyllum* stembark extract (Data analysed using IRPal 2.0 program).

| Peak ($cm^{-2}$) | Assignment | Possible compound class present | Extract |
|---|---|---|---|
| 3524.57 | RCH2OH | Alcohols | Chloroform |
| 3271.83 | RC#CH Stretch of C-H | Alkynes | Methanol |
| | C=C stretch of CO-OH | Carboxylic acids | |
| | ArO-H-H | Phenols | |
| 3337.32 | C=C stretch of CO-OH | Carboxylic acids | Hexane, chloroform |
| | ArO-H-H | Phenols | |
| 2915.64 | RCO-OH | Carboxylic acids | Hexane, chloroform |
| | RCH2CH3 | Alkanes | |
| 2123.67 | RC#CH stretch of C#C | Alkynes | Hexane, chloroform |
| | R-N=C=S | Miscellaneous | |
| 2120.11 | RC#CH stretch of C#C | Alkynes | Methanol |
| | R-N=C=S | Miscellaneous | |
| 1734.79 | RCOOR’ | Esters | Hexane, chloroform |
| | RCOOR’ 6-ring | Esters | |

| | | | |
|---|---|---|---|
| 1638.91 | C=C stretch | Alkenes | Methanol, hexane |
| | CONHR stretch of | Amides | |
| | C=O | | |
| | C=N | Miscellaneous | |
| 1456.47 | RCH2CH3 | Alkanes | Hexane |
| | C-C ring | Aromatics | |
| 1366.21 | C-H rock | Alkanes | Methanol,hexane |
| | S=O | Miscellaneous | |
| 1302.03 | R-F stretch of C-F | Alkyl halides | Chloroform |
| | RCO-OH stretch of | Carboxylic acid | |
| | C-O | | |
| | RCOOR’ | Esters | |
| | N-O nitro compound | Miscellaneous | |
| 1241.21, 1160.16 | R-F stretch of C-F | Alkyl halides | Hexane, chloroform |
| | Ar-O-H | Ethers | |
| | RCO-OH stretch of | Carboxylic acid | |
| | C-O | | |
| | RCOOR’ | Esters | |
| | N-O amine oxide | Miscellaneous | |
| 1217.10 | R-F stretch of C-F | Alkyl halides | Methanol |
| | $RNH_2$ stretch of C-N | Amines | |
| | RCO-OH stretch of | | |
| | C-O | Carboxylic acid | |
| | RCOOR’ | | |
| | | Esters | |
| | | Miscellaneous | |

| | | | |
|---|---|---|---|
| 1031.08 | R-F stretch of C-F | Alkyl halides | Hexane, chloroform |
| | RCO-OH stretch of C-O | Carboxylic acid | |
| | RCOOR’ | Esters | |
| | P-OR esters | Miscellaneous | |
| 860.06 | 1,3,5- trisub | Aromatics | Chloroform |
| 719.90 | R-F stretch of C-F | Alkyl halides | Hexane |
| | RCO-OH stretch of C-O | Carboxylic acid | |
| | RCOOR’ | Esters | |
| | P-OR esters | Miscellaneous | |

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**Cytotoxity**

The cytotoxic potential of the generated nanoparticles was evaluated against 3 cell lines HEK293, HeLa and MFC-7, using the MTT assay. The results obtained from this study, showed that the AgNPs generated from the hexane, chloroform and methanolic extracts exerted a cytotoxic effect on the various cancer cells. Noticeably, a high potent activity was seen within the AgNPs (methanolic leaf extracts) when tested against MFC-7 cells.

The US National Cancer Institute has suggested that crude plant extracts with an IC50 value of less than 20 ug/ml is considered to have cytotoxicity activity (Alabsi et al., 2016). In lieu of this, the AgNPs (methanolic stembark extracts) appear to have a IC50 value <20µg/mL, making it an ideal candidate to be further developed and considered as a conventional anti-cancer drug. Due to the low IC50 value, this extract can be further refined to isolate bioactive compounds to enhance the possible pharmacology applications.

As indicated in table 6.7, AgNPs (methanolic extracts) were the best performing, with the overall lowest IC50 values when tested against HeLa cells (Leaf- 29,92351 µg/mL and stembark 16,1µg/mL). The lowest cell viability (6,7%) was noted within the HeLa cell lines when treated with the AgNPs (methanolic stembark extract) (strongest cytotoxic effect). Furthermore, the highest cell viability (90%) was noted within the HeLa cell line when treated with the AgNPs (chloroform leaf extract). Overall, the crude methanolic extracts performed the best, yielding the lowest IC50 values.

Table 6.7: IC50 values of the cytotoxic analysis of biosynthesized AgNPs from *Combretum erythrophyllum* leaf and stembark extract *C. erythrophyllum*

| Cell lines | Extracts | Cytotoxicity (ug/ml) | |
|---|---|---|---|
| | | Leaves | Stem bark |
| HEK293 | Hexane | 498,0948 | 103,22 |
| | Chloroform | 67,99929 | 142,68 |
| | Methanol | 52,08879 | 90,87 |
| HeLa | Hexane | 234,2229 | 157,43 |
| | Chloroform | 67,41607 | 79,50 |
| | Methanol | 29,92351 | 16,14 |
| MCF7 | Hexane | 372,5829 | 519,71 |
| | Chloroform | 97,9035 | 44,64 |
| | Methanol | 41,89544 | 17,45 |

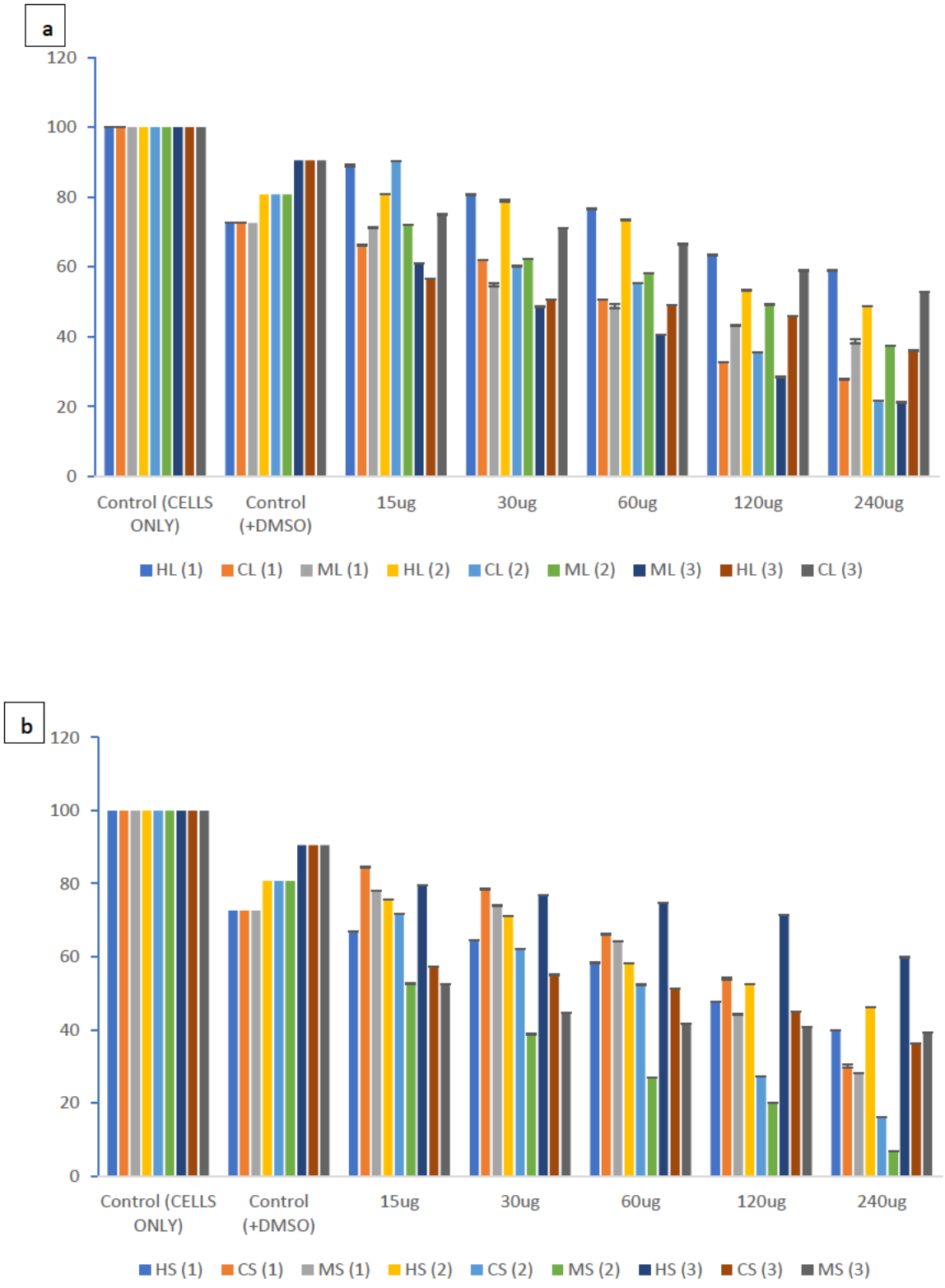


Figure 6.11: Cytotoxic activity of the a) leaf, b) stembark extracts of *C. erythrophyllum* (tukeys' honest significant difference multiple range post Hoc test $P < 0.05$)

**Apoptosis**

Apoptosis is a genetically controlled process which may result in the disintegration of nuclei structure and DNA, ultimately resulting in overall cell death (Al-Nasser et al., 2021). Within plants, apoptosis is primarily induced by the presence of chemicals. These chemicals are known to cause oxidative stress within the cell, inducing the initiation of apoptosis process (Mobaraki et al., 2021). During apoptosis the following cellular variations are noticed: disintegration of the nucleus and chromatin network, distortion and shrinkage of the cell membrane (Ali et al., 2021).

Induced apoptosis using plant generated AgNPs are of prime interest for cancer treatment methods (Jabir et al., 2021). Research suggests that nanoparticles generated from plant extracts could induce apoptosis at specific target areas within the human body (Ali et al., 2021). A plant extract with a proposed apoptotic ability, can be delivered to a tumor cell by means of a nanoparticle as a transport vesicle. Upon penetrating the cell, apoptosis is induced resulting in programmed tumor cell death (Jabir et al., 2021).

The emanating study used fluorescent microscopy coupled with acridine orange staining to evaluate the viability of tested cells. This method was favored as it is accurate, time and cost effective (Anitha et al., 2021). When stained with AO/EB, viable cells appear green, with a fully formed nucleus. Cells, in which apoptosis has initiated, appear to have yellow green dots, signaling a disintegrating chromatin network. Cells that are reaching the completion of apoptosis exhibit an orange nucleus that shows some form of disintegration. Importantly, necrotic cells at this stage also appear orange however display a structurally intact nucleus (Fig. 6.12).

In lieu of this, a direct correlation can be seen between the results obtained from the MTT and apoptosis assay. At high IC50 concentrations, cell viability is high and the apoptotic index is low. Whereas, at low IC50 concentrations, cell viability is low and the apoptotic index is high, as a result the cell appears to stain orange in colour indicating their low viability (Fig. 6.12-13). This phenomenon was further confirmed by the results obtained from the apoptotic index (Fig. 15). Overall, the AgNPs synthesized from *C. erythrophyllum* showed promising cytotoxic activity against cancer cells, with the possibility of inducing controlled apoptosis within these cells.

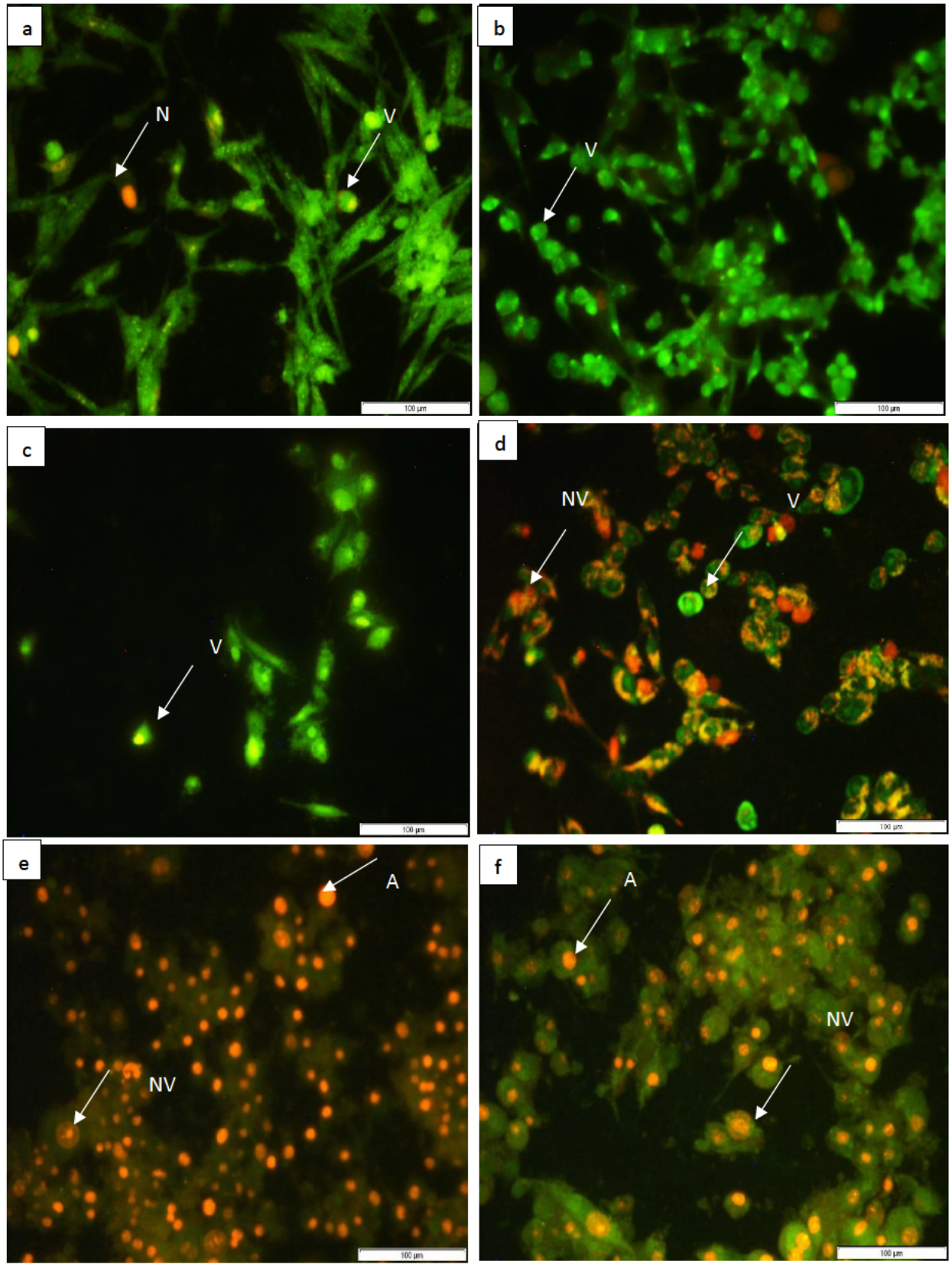


Figure 6.12: Light micrographs indicating the cell viability of HEK293 cells stained using acridine orange: ethidium bromide. AgNPs synthesized from hexane a) leaf, b) stembark extract, chloroform c) leaf, d) stembark and methanolic e) leaf and f) stembark extracts of *C. erythrophyllum (A- Apoptotic cell, V-Viable cell, NV- non-viable cell)*.

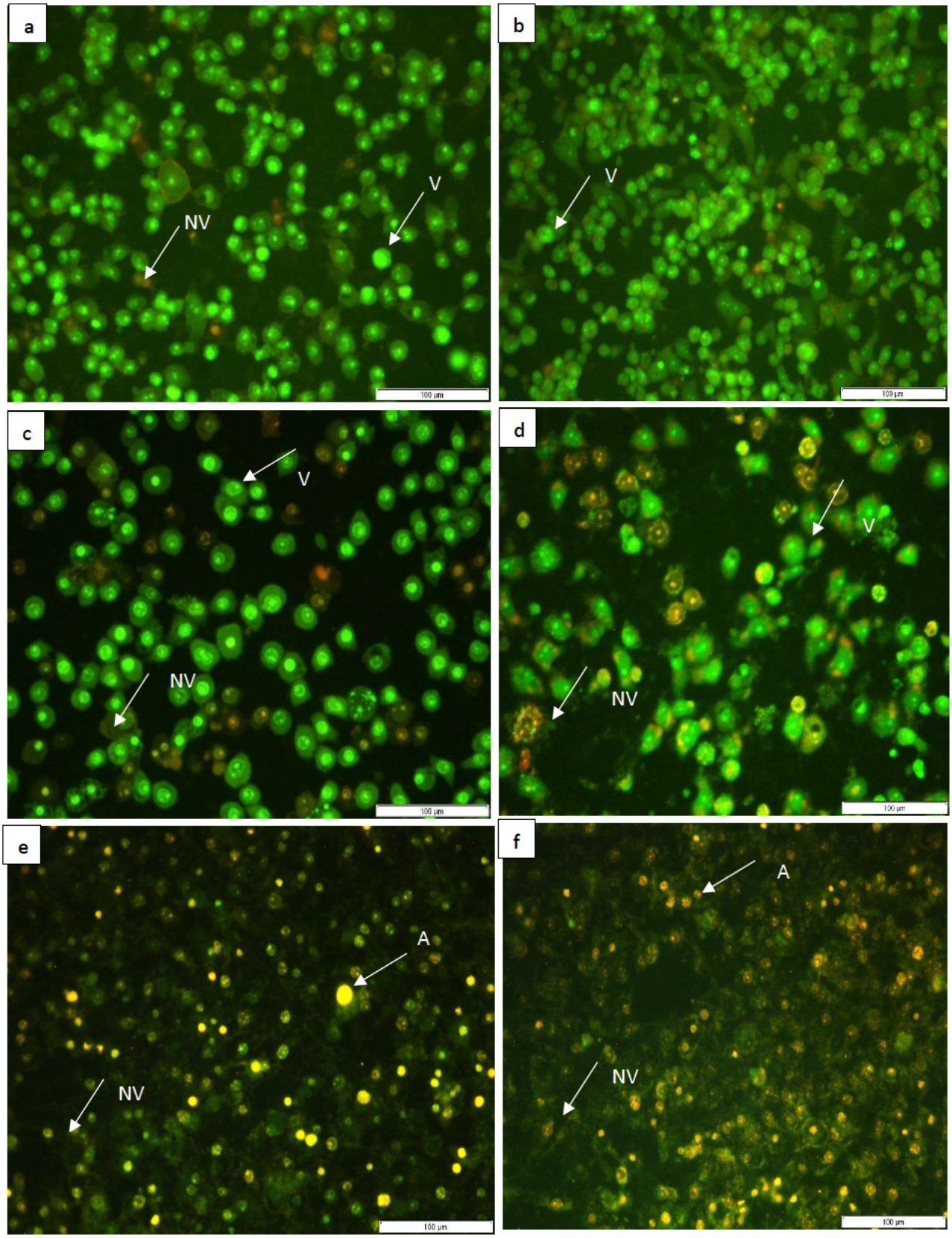


Figure 6.13: Light micrographs indicating the cell viability of HeLa cells stained using acridine orange: ethidium bromide. AgNPs synthesized from hexane a) leaf, b) stembark extract, chloroform c) leaf, d) stembark and methanolic e) leaf and f) stembark extracts of *C. erythrophyllum (A- Apoptotic cell, V-Viable cell, NV- non-viable cell)*.

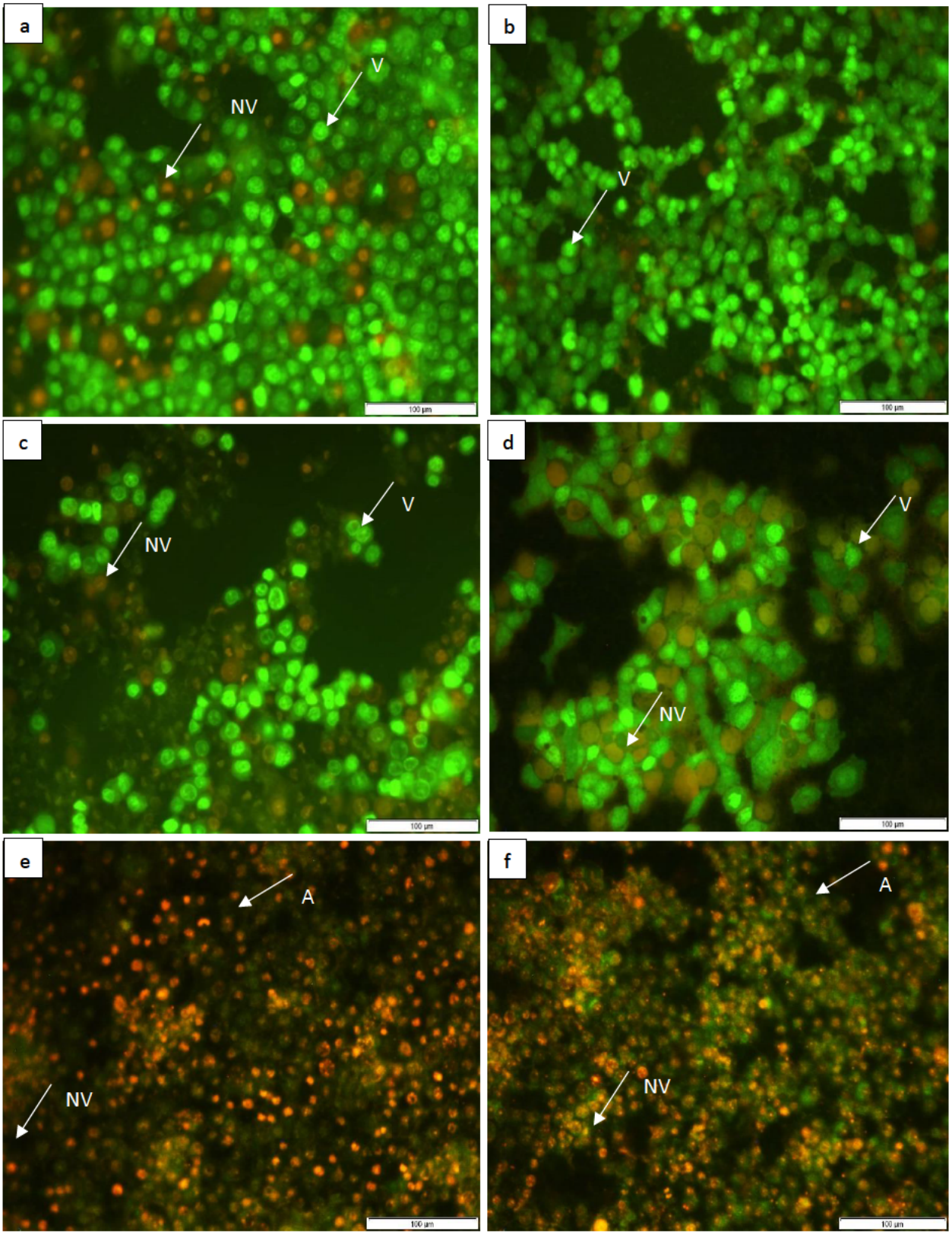


Figure 6.14: Light micrographs indicating the cell viability of MCF-7 stained using acridine orange: ethidium bromide. AgNPs synthesized from hexane a) leaf, b) stembark extract, chloroform c) leaf, d) stembark and methanolic e) leaf and f) stembark extracts of *C. erythrophyllum (A- Apoptotic cell, V-Viable cell, NV- non-viable cell)*.

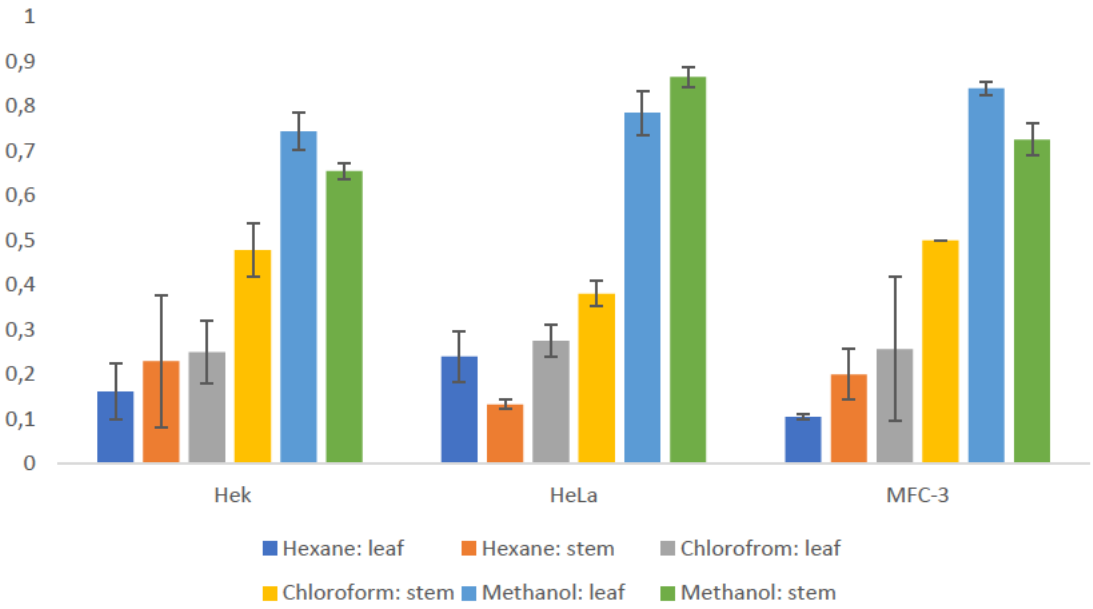


Figure 6.15: Apoptotic index of each cell line against biosynthesized AgNPs from *Combretum erythrophyllum* leaf and stembark extract (240µg/mL). The results are represented as a mean ± SD, n = 3 (tukeys' honest significant difference multiple range post Hoc test $P < 0.05$).

## 6.4. Highlights

Nanoparticle efficiency and antimicrobial effect is based on their size, shape and structure. Nanoparticle size and elemental $Ag^+$ content are directly proportional. In accordance with current EDX findings (Fig. 6.3), the stembark contained a higher elemental silver composition (32.82%) in comparison to the leaf (3.56%), justifying the presence of larger nanoparticles within the stembark (Fig. 6.4b). However, a large nanoparticle size does not imply greater antibacterial activity. Raza et al (2016) revealed that smaller sized nanoparticles have a greater inhibition activity against bacteria. In accordance, the conducted TEM analysis of *C. erythrophyllum* revealed that the leaf extract produced significantly smaller sized nanoparticles compared to the stem bark. This finding correlates with the conducted antibacterial assay in which the nano- leaf extracts demonstrated better inhibitory activity in comparison to the AgNPs from the stembark extract. The use of plant extracts in conjunction with conventional methods has proven to be highly beneficial and effective in enhancing the benefits of chemotherapy as it restores chemo sensitivity to multi drug resistant cancer cells by improving the overall penetration ability of drugs, within the effective cells. the methanolic stembark extracts appear to have an IC50 value <20µg/mL, making it an ideal candidate to be further developed and considered as a conventional anti-cancer drug. Due to the low IC50 value, this extract can be further refined to isolate bioactive compounds to enhance the possible pharmacology applications. Overall, the AgNPs synthesized from *C. erythrophyllum* showed promising cytotoxic activity against cancer cells, with the possibility of inducing controlled apoptosis within these cells.

## 6.5. Conclusion

Currently, the biomedical industry is striving towards creating a more efficient method of drug delivery. The proposed drug delivery system is aimed at establishing target specific delivery, using controlled dosages. The need arises due to the evolving strains of antibiotic resistant bacteria present. Nanotechnology provides a platform that enables the formulation of a low toxicity and hypersensitive drug delivery system. Currently, the focus is on synthesizing phytometabolites, extracted from medicinal plants, and $Ag^{+}$ to generate nano- scale carriers with specialized properties. These carries have the potential to enhance existing drug delivery systems by providing increased antioxidant, antimicrobial and antibacterial agents. A novel protocol for the synthesis of AgNPs using the leaf and stembark extracts of *C. erythrophyllum* was designed. Furthermore, it was established that the synthesized nanoparticles exhibited high antibacterial properties, with proposed apoptosis ability. Further studies evaluating the biological activity of extracts of *C. erythrophyllum* synthesized nanoparticles could possibly enhance its pharmaceutical value.

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# CHAPTER 7: CONCLUSIONS AND FURTHER RECOMMENDATIONS FOR RESEARCH.

## 7.1 Introduction

Local knowledge systems are imperative to advancements in the medicinal industry (Silva et al., 2018). The integrated use of local knowledge along with scientific assays allows researchers to discover novel phytocompounds with proposed allopathic value (Selvin and Durai, 2018).

*Combretum erythrophyllum* is known to have numerous traditional uses yet there is limited scientific knowledge on its micromorphological structures and exudate (Mtunzi et al., 2017). Micromorphological analysis of *C. erythrophyllum*, identified the presence of non- glandular unicellular trichomes and peltate scales, across surfaces (Chapter 3). Peltate scales are comprised of a multicellular head, bicellular stalk and a basal epidermal cell. Head cell count appeared to increase with leaf maturation. Across foliar developmental stages, trichome density appeared to remain constant. In addition, the presence of vesicles, vacuoles and electron dense material within the peltate scales suggests the granulocrine pathway as a possible mode of secretion.

Preliminary phytochemical tests indicated the presence of multiple phytocompounds, including carbohydrates, alkaloids, sterols, phenols, fixed oils, and fats (Chapter 4). These findings substantiate the results obtained from the histochemical analysis. The first reported gas chromatography-mass spectrometry (GC-MS) screening of *C. erythrophyllum* leaf and stembark extracts was conducted, identifying 266 phytocompounds. Major phytocompounds were identified that included, amongst many, sitosterol and lupeol, which may have possible anti-cancer and anti-inflammatory properties (Hidayathulla, 2018; Deshmukh and Sharma, 2018).

The antioxidant ability of the generated extracts was evaluated using the DPPH and FRAP assay (Chapter 5). These assays were performed on the crude hexane, chloroform and methanolic leaf and stembark extracts of *C. erythrophyllum* (at varying concentrations). A positive correlation between % inhibition and extract concentrations, was noted in both. The DPPH assay revealed that chloroform leaf and stembark extracts displayed the highest IC50 values (120,7063 and 265,4076µg/mL, respectively) indicating the lowest overall antioxidant potential among all extracts (Table 1). The methanolic extracts displayed the lowest IC50 values (leaf- 5,2866 and stembark- 4,2866 µg/mL) indicating the highest possible antioxidant potential among all tested extracts. The FRAP analysis further substantiated that all extracts displayed varying free radical scavenging activity, chloroform leaf and stembark extracts displayed lowest overall antioxidant potential and methanolic extracts displayed the highest possible antioxidant potential. In both instances, the methanol stembark extracts performed the best, with lowest IC50 recorded. Total phenolic content of the tested crude extract deduced, that all extracts contained phenolic compounds (chloroform<hexane<methanol).

The methanolic extracts appeared to quantify the largest amount of compositional phenolic content (1341,05± 4,4mg /GAE/g). This finding correlates to the fact the polar solvents such as methanol (low boiling point and high penetration rate) allow for the maximum extraction of low molecular weight polyphenols (Do et al., 2014). The results obtained from the total flavonoid assay correlated with the trend observed through the total phenolic assay, whereby methanolic extracts extracted the largest yield of both phenolics and flavonoids. Research has indicated that plants with increased levels of phenolic compounds, such as flavonoids, are a rich source of antioxidant agents and display immense antibacterial and cytotoxic activity (Phuyal et al., 2020).

A novel AgNPs biosynthesis protocol was introduced (Chapter 6). Synthesised AgNPs are quintessentially 1- 100nm in size, induce a distinctive colour change in solutions and produces a peak silver presence, at 3kv, when analysed using EDX (Paulkumar et al., 2017). In correlation with the above, the AgNPs synthesised using extracts of *C. erythrophyllum* are on par with literature. The efficacy of the generated AgNPs is based on the size, shape and structure. In accordance, elemental silver concentration is known to increase particle size. Within stembark extracts nanoparticles were large in comparison to the leaf extracts. This correlates to the results obtained from the EDX analysis whereby stembark extracts contained ~10x more silver than the leaf extract tested. The stability of generated nanoparticles were of optimum level which was confirmed by the UV- vis spectroscopic analysis. Overall the AgNPs had effective growth control over seven of the nine tested bacteria. The antibacterial activity of the generated methanolic extracts and synthesised AgNPs was evaluated. Although both extracts inhibited bacterial growth, AgNPs appeared to amplify inhibitory activity against *Bacillus subtills* and *Staphylococcus aureus*. Lasty, the generated crude extracts displayed promising results when evaluated for their cytotoxic and apoptotic abilities however upon silver nano encapsulation the cytotoxic and apoptotic capabilities increased significantly.

Due to the above obtained results, it is indeed a possibility that AgNPs generated using extracts of *C. erythrophyllum* could be effectively used in the pharmaceutical industry.

## 7.2 Aims and objectives

This study aimed to investigate the micromorphology, histo-phytochemistry, antioxidant, total phenolic and total flavonoid content of stembark and foliar developmental stages of *Combretum erythrophyllum*. Furthermore, a novel protocol was proposed for the biosynthesis of silver nanoparticles using the extracts *C. erythrophyllum*. The potential antibacterial, cytotoxic and apoptotic properties of foliage and stembark extract of *C. erythrophyllum* was also investigated. Collaboration of information obtained through this study will generate novel uses and future opportunities for medicinal research.

## 7.3 Challenges

Micromorphological analysis was difficult due to the presence of glutinous like secretion layer across foliage and stem surfaces. This layer hindered the visualization of aerial appendages. Numerous peltate scale were embedded within, hence structural evaluations were problematic and time consuming. Biological material was highly sensitive to change in environments. Samples had to be well hydrated in order to prevent collapsing and shrivelling of structures. Ultrathin sectioning was challenging due to the presence of crystals idioblasts in mature leaves. These caused the material to tear easily during sectioning.

## 7.4 Future possibilities

Trichome development could be tracked in order to determine which developmental stage would yield the highest concentration of exudate. In lieu, seasonal variation may alter phytometabolite quantities and this should be evaluated. Isolation and biological evaluation of medicinally important phytometabolites from the exudate can be explored. Hence allowing for possible drug precursors to be established. The biosynthesis of isolated phytometabolites and silver nitrate, to generate AgNPs can aid in drug development. These particles have the ability to deliver the proposed antibacterial properties of silver coupled with the benefits of the synthesised phytometabolites to target regions. This mechanism should be evaluated using the extracts of *C. erythrophyllum*. In addition, biotechnological processes should be evaluated in order to yield a large plant turnout in a contracted time period.

## 7.5 Conclusion

Currently, the biomedical industry is striving towards creating medicines with minimal side effects and efficient drug delivery. This study investigated the micromorphology, ultrastructure histo-phytochemistry and silver nanoparticle analyses of the leaves and stembark of *Combretum erythrophyllum*. The protocols and results obtained from the conducted nanoparticle synthesis micromorphological, antioxidant (stembark), total phenolic (stembark), total flavonoids (stembark), Cytotoxity (stembark), apoptosis (stembark), GC-MS, and FTIR analyses were novel to this species. Overall results indicate that *C. erythrophyllum* should be considered for its allopathic value.

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