An investigation into the phytochemistry and biological activity of *Pappea capensis* used against cancer

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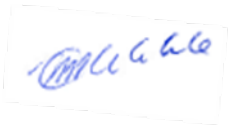
Doctor of Health Sciences: Biomedical Technology

Promoter: Prof S.S. Mashele, PhD

DECLARATION OF INDEPENDEDNT WORK

I, PAKISO MOSES MAKHOAHLE, identity number _____ and student number _____, do hereby declare that this research project submitted to Central University of Technology-Free State, for the degree DOCTOR OF HEALTH SCIENCES: BIOMEDICAL TECHNOLOGY, is my independent work, and complies with the ode of academic integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology-Free State; has never been submitted before to any institution by myself or any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification.



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List of Abbreviations

<- less than

>- greater than

µg- micro gram

µL- micro liter

3- HMG-CoA 3-hydroxymethylglutaryl coenzyme A

AB control- antibiotic control

Abs-Absorbance

AIDS-Aicquired immune-deficiency syndrome

$BaCl_2$- Bariun dichloride

C. albicans- Candida labicans

CO_{2-} Carbon dioxide

CoQ- coenzyme Q

ddH_20- double distilled water

DF cells- dermal fibroblast cells

DMEM- Dulbecco's modified eagle's medium

DMSO- Dimethyl sulfoxide

DNA: Deoxyribo nucleotide acid

DPPH- 2,2-diphenyl-1-picrylhydrazyl

E. faecalis- Enterococcus faecalis

Extract 6- ethanoic extraction

Extract 7- water/aqueous extract

Extract 8- methanolic extract

Fas-associated death domain (FADD)

FC-Folin-Ciocalteu

Fig- figure

g- gram

GC-MS- Gas Chromatography Mass Spectroscopy

H_2SO_4- Sulphuric acid

HCL-hydrochloric acid

HCT-15- Human carcinoma cells15

HP-5 GC Column- (5%-phenyl(- methylpolysiloxane

IF-γ- interferon gamma

iNOS- inducible nitric oxide synthase

INT - Iodonitrotetrazolium chloride ()

LPS – lipopolysaccharides

MCF7 -human breast cancer cell line 7

MIC- minimum inhibition concentration

mL- millilitre

ml-1- per millilitre

MLL gene- myeloid/lymphoid or mixed-lineage leukemia

MTT assay- (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay

Mueller-Hinton (MH) agar

Na_2CO_3- Sodium carbonate

NCI-National Cancer Institute

Nm- nano meter

NMR- nuclear magnetic resonance

NO- nitric oxide

NSAIDs- non-steroidal anti-inflammatory drugs

PBS (+Ca +Mg)

PMB- polymyxin B

PI- propidium iodide

PC3- prostate cancer cell line

ROS- reactive oxygen species

S. aureus- Staphylococcus aureus

SERMs-selective estrogen receptor modulators

Std Dev- standard deviation

TGF-β transforming growth factor

TLC-thin layer chromatography

UC- unidentified compounds

ug- microgram

USA-United states of America

UV- Ultra violet

VCR- vincristine'

VDS- vindesine'

VLB- vinblastine'

VRLB- vinorelbine'

WHO- world health organisation

Dedication

To

My family

For being Strong and gentle souls that taught me to trust God and my ancestors, believed in hard work and that so much could be done with little. Wings beneath my wings to wake me up even though sometimes it's tough

My Grandparents (Ntate-Moholo Matala Shadrack and Nkgono Madile Rebecca) Makhoahle

For being my first teachers

My Parents

For earning an honest living for us and for supporting and encouraging me to believe in myself. Mohlouoa would always say don't worry about money just go to school, I'll pay everything

Lastly, to my wife "Manguna" and my kids (Bohlale and Bonolo) for their sleeplessnights and support, I really appreciate your presence in my life. Thanks to the Almighty God for keeping us safe

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An investigation into the phytochemistry and biological activity of *Pappea capensis* used against cancer

Summary of the study

The aim of the study was to prepare crude plant extracts of *Pappea capensis*, broadly establish the bioactivity and describe the phytochemical properties of *Pappea capensis* water extract.

Therefore, *in vitro* antioxidant activity was carried out with ethanoic, water and methanolic *Pappea capensis* wood extracts by the DPPH-free radical scavenging method. The DPPH-free scavenging activity of the three extracts of *Pappea capensis* displayed antioxidant activity comparable to the used control. So, free radicals were evidently trapped. Henceforth, further investigation to establish the existence of phytochemicals and phenolic compounds as contributing a factor was necessary. In this study the different phytochemical compounds and phenols were identified from the wood of *Papea capensis* which suggested possession of pharmacological benefits which could be used in traditional medicines. The extracts were then subjected to further tests such as the antibacterial activity test. Cytotoxicity test and many more others should be done to ascertain the medical benefits and safety of the woody part of this plant.

The sensitivity testing of the plants plays a critical role in ascertaining the safety use of the plant extracts. In this study, antimicrobial screening showed that different extraction methods isolated the active compound which the solvent type depended on. The ethanoic and methanolic extracts were showed to have active compounds against Gram positive and Gram-negative organisms. Both extracts (ethanoic and methanolic) indicated that potential antimicrobial compounds were in the high polarity fraction. Water was found to be the only solvent mostly used by Sangomas and traditional healers to make medicinal mixtures for their patients. Then antimicrobial activity was extended by including other organisms. The *Staphylococcus aureus* and *Klebsiella pneumoniae* were found to be susceptible to ethanoic and methanolic extracts. However, in chapter 5 methanolic extract

showed broad antimicrobial activity against two organisms known as part of the normal flora (*Staphylococcus epidermidis* and *Enterococcus faecalis)* which are both Gram positives. The water extract showed antimicrobial activity against *Klebsiella pneumoniae*, although no antimicrobial activity was observed against all Gram-positive organism. The *Pappea capensis* plants extract were screened for cytotoxity against the three cell lines (vero cells, MCF7 and PC). Upon comparing the used control and the only extract showing slight toxicity, it's conclusive to say the high dose of methanolic extract is physiologically inappropriate. All extracts that are not active (cytotoxic) enough to suggest *Pappea capensis* can be taken into further anticancer testing. The extracts were further screened for genotoxicity. Two extracts were considered none genotoxic and the ethanoic extract could be considered genotoxic. The micronucleated cells of 30% were determined at the high concentration (200ug/ml) of ethanoic extract. A further test should be done to predict what mechanism is affected by compounds in ethanoic extract. The results indicate the potential use of this plant against cancer, despite the moderate genotoxicity at 200ug/ml.

The *in vitro* screening results for anti- and pro-inflammatory assays are shown in fig 8.1 (A and B) for the analysis of NO production and the cell viability analysis on LPS-activated macrophages treated with the different extracts concentrations. There was an increased NO production which correlated with each extract cytotoxicity in LPS- activated macrophages (fig 8.1 B). Resveratrol, a known inhibitor of iNOS expression served as a positive control to the regime. In fig 8.2 there was macrophage activation observed which was extracts concentrations depended. This response to treatment with different concentrations of *Pappea capensis* extracts corresponded with NO production in fig 8.2

and a similar trend was observed with the analysis of cell viability (fig 8.2 A and B) which predicts the corresponding cytotoxicity of all extracts. However, there was a high NO production and observable macrophage activation in the presence of water extract no. 7 (fig 8.2). A confirmatory test was done to ascertain if this effect is owed to plant constituents or due to endotoxin contamination. Then, NO production was analysed in the presence and absence of polymyxin B (PMB) as shown in fig 8.3. It can be concluded that PMB counteracts the pro-inflammatory effect suggesting that this activity is possibly a result of endotoxins in this part of the plant. The determination of endotoxin as a cofactor perpetrating pro-inflammatory activity tested with polymyxin B was used and indicated the presence of a toxin in water extract. The low anti-inflammatory effect seen in the three extracts at 200ug/ml are not physiologically active.

The GC-MS revealed the presence of 41 compounds which included 4-ethylbenzaldehyde, 2, 4-di-ter-butyl phenol, acetic acid and butanoic acid have the role in antioxidant, antimicrobial, antitumor, antifungal effects. The identified alkanes and aldehydes are possible poisonous compounds identified in fig 8.2A. Then it can be concluded the pro-inflammatory activity was indeed caused by toxin or poisonous compounds from the plant itself. Furthermore, seven (7) unknown bioactive chemical compounds were revealed regardless of their amounts, their existence could be having a positive impact on therapeutic agents and a source of the biological activities claimed for between traditional healers. Furthermore, many diverse chemical compounds identified and some quantified make it a holistic plant for use in traditional medicine and for aesthetic value between the local communities in Limpopo. More scientific tests are

needed to carry out bioassay guided fractionation of the three extracts to determine the purified compounds and identify the ones that are biologically active.

Medicinal plants, being the only sources that healers and Sangomas rely on for treatment of their patients have received tremendous attention in drug therapy, discovery and development. In conclusion, this study has shown that the *Pappea capensis* may serve as a new possible source of remedies due to the presence of these phytochemicals, bioactive compounds and antimicrobial activities.

Chapter 1

INTRODUCTION AND LITERATURE REVIEW

CANCER AND *PAPPEA CAPENSIS TREE*

1.1 Introduction

Cancer remains one of the leading morbidities and mortalities globally. Amongst the non-communicable diseases, cancer is found to be the second leading cause of deaths after cardiovascular disease (WHO, 2005; Mathers and Loncar, 2006; Lopez *et al.,* 2006, Hoyert *et al.,* 2006). Cancer is responsible for 25% of deaths globally more than AIDS, tuberculosis, and malaria together (Sener and Grey, 2005). It has been reported that cancer incidence and mortality are higher in North America, Australia, New Zealand and Western Europe compared to other parts of the world (Parkin *et al.,* 2001). In the United States of America, 25% of deaths is attributed to cancer (Jemal *et al.,* 2007). The number of cancer deaths is projected to increase from 7.1 million in 2002 to 11.5 million by 2030 globally (Mathers and Loncar, 2006). Sub-Saharan countries apprear to be among the 46 countries mostly affected by cancer (Bray *et al.,* 2018).

The basic treatment for cancer for decades has been relying on Chemotherapy. Since cancer cells lose many of the regulatory functions present in the normal cells, they divide continuously while normal cells don't. That makes cancer cells susceptible to chemotherapeutic drugs. Almost five decades of systemic drugs discovery and development have resulted in large collection of useful chemotherapeutic agents. However, chemotherapeutic agents are not devoid of their intrinsic problems such as different kinds of toxicities. Various kinds of toxicities which include 5-fuorouracil, a common therapeutic agent known to cause myelotoxicity, cardiotoxicity and has widely documented in few cases as vasospatic agent (Macdonald, 1999; Rastogi *et al.*, 1993*)*. Another globally used chemotherapeutic agent, doxorubicin causes cardiac, renal toxicity and myelotoxicity (Aviles *et al.,* 1993; Leo *et al.,* 1997; Gibaud *et al.,* 1994). Another well-known chemodrug, bleomycin is reported to cause pulmonary toxicity and cutaneous toxicity (Parvinen *et al.,* 1983*;* Karam *et al.,* 1995; Cohem *et al.,* 1973). Similarly, cyclophosphamide, a drug known to treat many lignant conditions has been reported to cause bladder toxicity in the form of

hemorrhagic, immunosuppression, alopecia and at high doses it causes cardiotoxicity (Fraiser *et al.,* 1991). The toxicity of chemotherapeutic drugs mostly creates significant problem in the treatment of cancer using allopathy or established medicine.

1.2 Literature review

The use of medicial plants for the teatemnt of cancer is driven by spiritual beliefs in african communities and westernides use plants to extract beneficial metabolites. Different therapies have been propounded for the treatment of cancer, many of which use plant derive products. Accessibility and affordability of the medicinal herbs have made them as a fundamental part of many people's life all over the world. The selection of medicinal plants is a conscious process, which has led to an enormous number of medicinal plants being used by plentiful cultures in the world (Heinrich *et al*., 2004). Surveys conducted in Australia and United State indicates that almost 48.5% and 34% of individuals had used at least one form of unconventional therapy (canceraustralia.gov.au/resources; healthcaredelivery.cancer.gov/nhis/). According to WHO, about 65-80% of the world's population in developing countries, due to poverty and lack of access to modern medicine, depends essentially on plants for their primary health care (Calixto, 2005). Indeed, evaluation of effective plants to cure certain diseases has been recommended by WHO due to the lack of safe modern drugs.

In recent years, efficacy of herbal medicines in diseases like inflammatory bowel disease (Achike and Kwan, 2003; Calixto, 2005; Rahimi *et al.,* 2009; Rezaie *et al.,* 2007), obesity

(Hasani-Ranjbar *et al.,* 2009a; Heber, 2003), diabetes (Edzard, 2005; Rahimi *et al.,* 2005), pancreatitis (Ara Tachjan *et al.,* 2010; Mohseni-Salehi-Monfared *et al.,* 2009), cancers (Angelo and Edzard, 2009; Boon and Wong, 2004; Calixto, 2000; Paduch *et al.,* 2007) and inflammatory and oxidant-related diseases (Hasani-Ranjbar *et al.,* 2009b; Rahimi *et al.,* 2010) has been systematically reviewed. Even in the early 2000's, 11% of 252 drugs considered as basic and essential by WHO was exclusively of flowering plant origin (Rates, 2001). It is estimated that close to 25% of the active compounds in currently prescribed synthetic drugs were first identified in plant resources (Halberstein, 2005) and 20,000 plants have been used for medicinal proposes of which, 4000 have been used commonly and 10% of those are commercial. Out of the 250,000–500,000 plant species on earth, only 1-10% have been studied chemically and pharmacologically for their potential medicinal value (Verpoorte, 2000). Plants posess metabolites which warrant further studying in other plants as they are useful for drug development.

The biosynthesis of secondary metabolites is derived from the fundamental processes of photosynthesis, glycolysis and the Krebs cycle to afford biosynthetic intermediates which, ultimately results in the formation of secondary metabolites normally called natural products (Dewick, 2002). It has been hypothesised that secondary metabolism utilizes amino acids, the acetate and shikimate pathways to produce "shunt metabolites" known as intermediates that have adopted an alternate biosynthetic route, leading to the biosynthesis of secondary metabolites (Sarker *et al*., 2006). Changes affecting the normal biosynthetic pathways that produce secondary metabolites are associated with the natural causes (e.g. viruses or environmental changes) or unnatural causes (e.g.

chemical or radiation) in an effort to adapt or provide longevity for the plant (Sarker *et al*., 2006).

1.3 Classification of plants' secondary metabolites

The palette of secondary metabolites can be divided into a number of distinct groups on the basis of their chemical structure and synthetic pathways. These groups can, in turn, be broadly differentiated in terms of the nature of their ecological roles and therefore their ultimate effects and comparative toxicity in the consuming animal (Kennedy and Whightman, 2011).The three main groups of secondary metabolites in plants that are of interest in this study are alkaloids, phenolic compounds, and terpenoids (Harbone, 1984; Firn and Jones, 2003; Wink, 2003).

The recorded use of alkaloids for medicinal purposes dates back some 5000 years ago (Goldman, 2001) and this class of molecules has contributed towards the majority of poisons, neurotoxins, and traditional psychedelics (e.g. atropine, scopolamine, and hyoscyamine, from the plant *Atropa belladonna*) and social drugs e.g. nicotine, caffeine, methamphetamine (ephedrine), cocaine, and opiates consumed by humans as indicated on Fig 1.1 (Zenk and Juenger, 2007).

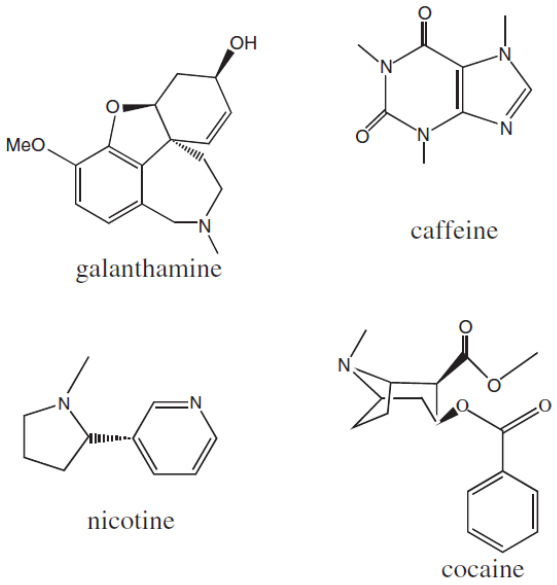

Figure 1.1. Alkaloids (Kennedy and Whightman, 2011).

1.4 Phenolic compounds

Phenolic compounds are based on phenol (an oxygen linked to a fully saturated C6 ring), the simplest member of this class of plant substances. Medically important phenolic compounds include flavonoids and tannins (Harborne and Williams, 2000). Phenols are characterised as compounds containing a hydroxyl group (—OH) directly binding to an aromatic ring. Phenolic compounds, in particular flavonoids, are generally involved in the protection of plants from attack by microbes and insects (Cushnie and Lamb, 2005; Friedman, 2007). Special classes of plant phenolics are the tannins, characteristically astringent, bitter plant polyphenols that are toxic to herbivores due to their capacity to either bind and precipitate or shrink proteins and other macromolecules (Cushnie and Lamb, 2005). Some phenols are used as chemopreventive agents for example Resveratrol (3, 5, 4-trihydroxystilbene), an oligomeric polyphenol found as dimer, trimer and tetramer. This molecule is been reported to prevent cancer and cardiovascular diseases in vasoprotection and neuroprotection (Ates *et al*., 2007; Delmas *et al*., 2006;

Vitrac *et al*., 2004). Acetylsalicylic acid (aspirin) a time honoured analgesic and antipyretic drug was derived from salicylic acid which occurs in the willow tree. Some other important salicylic acid derivatives are methyl salicylate, a common ingredient of liniments (Dewick, 2002). Eugenols extracted from cloves is used as an anesthetic and antiseptic in pharmaceutical and dental preparations (Daniel *et al*., 2009).

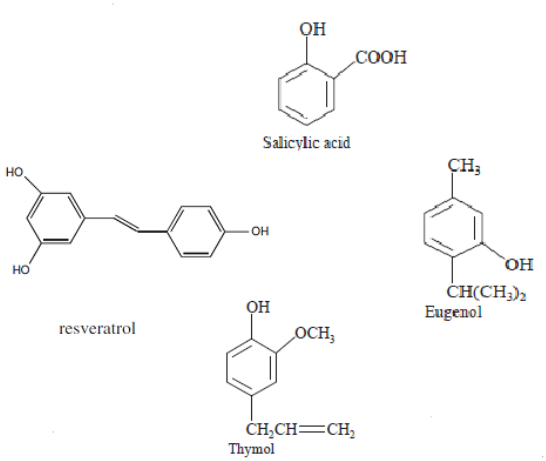

Figure 1.2. Phenols (Aggarwal and Shishodia, 2006; Dewick, 2002)

1.5 Terpenoids

Terpenoids are dimers or combinations of isoprene, a common organic compound that is highly volatile due to its low boiling point (Zwenger and Basu, 2008). This class of secondary metabolites is diverse and consists of more than 30,000 lipid-soluble compound groups. Their structural characteristics include 1 or more 5-carbon isoprene units, which are ubiquitously synthesized by all organisms via two potential pathways which are the mevalonate and deoxy-d-xylulose pathways (Rohmer, 1999). The common

chemical structure of terpens consist of ($C_{10}H_{16}$), and they can occur as diterpenes, triterpenes, and tetraterpenes (C_{20}, C_{30}, and C_{40}), as well as hemiterpenes (C_5) and sesquiterpenes (C_{15}) (Arif *et al*., 2011).

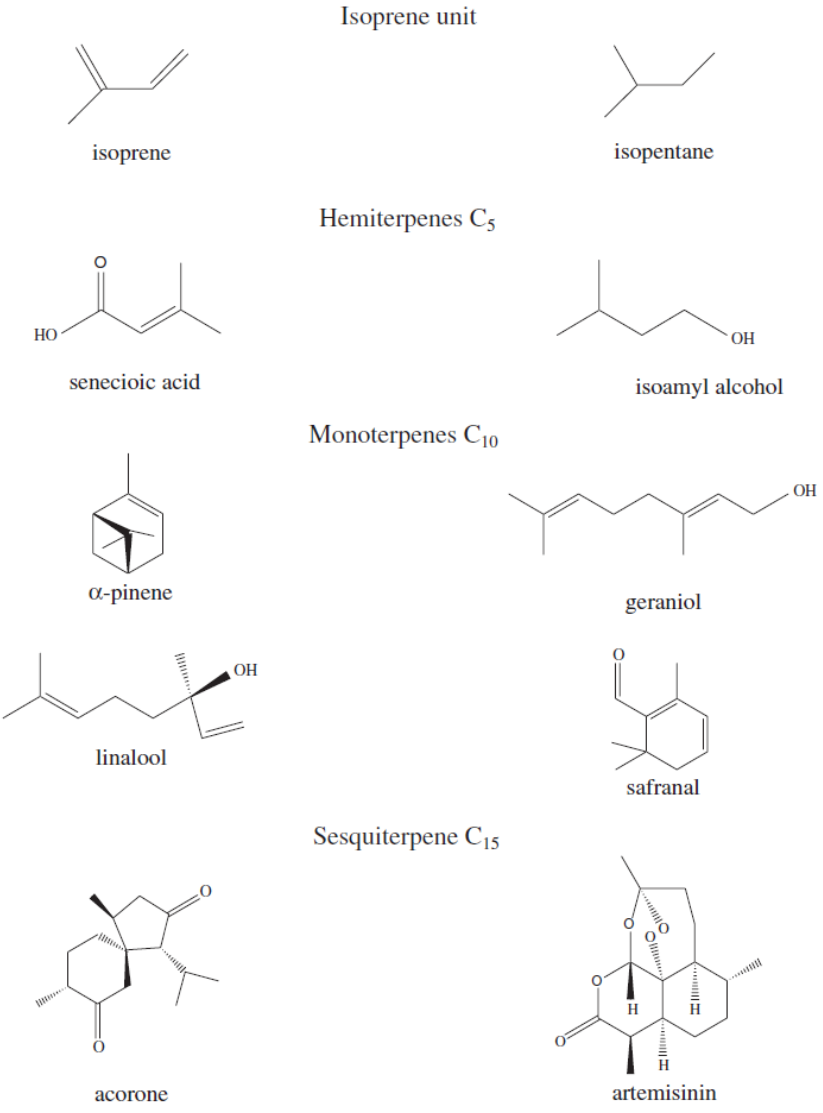

Figure 1.3. Basic building unit and various classes of terpenes (Hao *et al.*, 2013,).

Geraniol, an acyclic dietary monoterpene, is the only monoterpene that has been studied *in vitro* against liver cancer cells. Geraniol was found to inhibit the growth of HepG2 human hepatic carcinoma cells by decreasing 3-hydroxymethylglutaryl coenzyme A

(HMG-CoA) reductase, the major rate-limiting enzyme in cholesterol biosynthesis in mammals (Polo and Bravo, 2006). Combination of ardisiacrispin (A+B), a triterpenoid saponin mixture in the fixed proportion 2:1 of ardisiacrispin A and ardisiacrispin B, was derived from *Ardisia crenata*. This mixture exerted cytotoxic activity against Bel-7402 liver cancer cells through pro-apoptotic, anti-proliferative, and microtubule disruptive activities (Li *et al*., 2008).

1.6 Medicinal plants in South Africa

South Africa, which comprises of less than 1% of the world's land surface, contains 8% of its plant species (Coetzee *et al*., 1999). South Africa is considered to be a "hotspot" for biodiversity and more than 22,000 plant species occur within its boundaries (Coetzee *et al*., 1999). This rich plant biodiversity, with over 20,000 different species, is a great source of interest to the scientific community for the discovery of novel based products (Fouche *et al*., 2006). About 70% of these species are endemic to South Africa. It is estimated that there are more than 100,000 practicing traditional healers in South Africa, with a liable industry worth about R500 million per annum (Mander and Le Breton, 2005). It is estimated that 27 million indigenous medicine consumers live in South Africa with a large supporting industry (Mander, 1998). Annually, an average of 700,000 tons of plant materials are consumed, of which most of them are collected in the wild for local use and international trade (Cunningham, 1988; Lange, 1997). Plants harvested from a diverse range of vegetation types, including Valley Thicket, Afromontane Forest, Coastal Forest and Moist Upland Grassland.

Due to high demand of medicinal plants the Forest Biome was the vegetation type found to be most threatened by over-harvesting (Dold and Cocks, 2002). In an intensive investigation in South Africa, Fouche *et al* reported that among 7,500 randomly selected plant extracts representing 700 taxa, 32 extracts demonstrated potent anticancer activity, representing 24 different plant taxa, which is a high rate of 3.4% based on the number of taxa screened (Fouche *et al*., 2006). *The* current demand for numerous plant species resulting in intensive harvesting of indigenous plant stocks (this is in association with the lack of main resource management and plant production) has resulted in the rareness of various indigenous medicinal plants (Fuku *et al*., 2013, Mashele *et al*., 2010)

1.7 Plant-derived anticancer agents

Plants have been used for decades in the treatment of cancer. It was estimated that about 67% of pharmaceutical products approved between 1974 and 1994, for human cancer therapy were derived from natural sources (Richard *et al.,* 2005) of which plants have been a main source of several clinically convenient anticancer agents. Approximately five decades passed until several natural and synthetic antineoplastic were discovered. It was discovered that there are remarkable numbers of plant sourced agents in clinical trials for the treatment of cancer (Cragg and Newman, 2005). Some are being investigated as direct cytotoxins, whereas others are being studied from the aspect of their potential role as inhibitors of particular cell cycle enzymes, proteins, or pathways. The search for anticancer agents from plant sources started in earnest in the 1950s with the discovery

and development of the vinca alkaloids, ‘vinblastine’ (VLB) and ‘vincristine’ (VCR), and the isolation of the cytotoxic ‘podophyllotoxins’ (Cragg and Newman, 2005).

1.9 Alkaloids and anticancer properties

Alkaloids are a group of naturally occurring chemical compounds that contain mostly basic nitrogen atoms. This group also includes some related compounds with neutral and even weak acidic properties. In addition, some synthetic compounds of similar structure are attributed to alkaloids. In addition to carbon, hydrogen and nitrogen, alkaloids may also contain oxygen, sulfur and more rarely other elements such as chlorine, bromine, and phosphorus. Plant alkaloids make up a group of chemotherapy medications used to treat cancer. Alkaloids block cell division by preventing microtubule function which is vital for cell division. Alkaloids are divided into three major subgroups based on the type of plant origin;

“Vinca alkaloids” bind to specific sites on tubulin, inhibiting the assembly of tubulin into microtubules (M phase of the cell cycle). The first agents to advance into clinical use were (VLB) and (VCR), isolated from the Madagascar periwinkle, *Catharanthus roseus* G. Don. (Apocynaceae) which was used by various cultures for the treatment of diabetes (Gueritte and Fahy, 2005). Recently, ‘vinorelbine’ (VRLB) and ‘vindesine’ (VDS) have been developed as the semi-synthetic analogs of these agents (Lee and Xiao, 2005).

“Podophyllotoxin” is a plant-derived compound, which help with digestion as well as used to produce two other cytostatic drugs, ‘etoposide’ and ‘teniposide’(Lee and Xiao, 2005). These compounds prevent the cell from entering the G1 phase (the start of DNA

replication) and the replication of DNA (the S phase) (Lee and Xiao, 2005). Etoposide and teniposide; semi-synthetic derivatives of the natural product, 'epipodophyllotoxin' (an isomer of podophyllotoxin), could be considered as links to a plant originally used for the treatment of cancer (Lee and Xiao, 2005).

1.10 Coumarins and anticancer properties

Plant coumarins are structurally distinct, non-anticoagulant compounds that have significant medicinal activity (Yarnell and Abascal, 2009). Coumarins, also known as benzopyrones, are present in remarkable amounts in plants, although their presence has also been detected in microorganisms and animal sources (Borges *et al.,* 2005). The pharmacological and biochemical properties and therapeutic applications of simple coumarins depend upon the pattern of substitution on the original structure (Kostova *et al.,* 2006). Coumarins are aromatic compounds with a specific ring structure (Kostova *et al.,* 2006). The structural diversity found in this family of compounds led to the division into different categories, from simple coumarins to many other kinds of policyclic coumarins, such as furocoumarins and pyranocoumarins. Coumarins have attracted intense interest in recent years because of their diverse pharmacological properties. The coumarins have been recognized to possess anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities (Kostova *et al.,* 2006). 'Coumarin' is a natural substance that has shown anti-tumor activity *in vivo*, with the effect believed to be due to its metabolites (e.g. 7-hydroxycoumarin). A recent study has shown that 7-hydroxycoumarin inhibits the release of Cyclin D1, which is overexpressed in many types of cancer. 'Esculetin' (6,7-dihydroxycoumarin) inhibits

growth and cell cycle progression by inducing arrest of the G1 phase in HL-60 leukemia cells, resulting from the inhibition of retinoblastoma protein phosphorylation (Aoife and O'Kennedy, 2004; Cho *et al.,*2014).

1.11 Flavonoids and anticancer properties

Flavonoids (or bioflavonoids) (derived from the Latin word flavus meaning yellow), are a class of plant secondary metabolites or yellow pigments having a structure similar to that of flavones. Flavonoids are polyphenolic compounds that are ubiquitously in plants. Flavonoids can be classified into: flavonoids, isoflavonoids, neoflavonoids, which are all ketone-containing compounds. Over 5000 naturally occurring flavonoids have been characterized from various plants. They have been shown to possess a variety of biological activities at nontoxic concentrations in organisms (IUPAC Compendium of Chemical Terminology, 1997). The role of dietary flavonoids in cancer prevention is widely discussed. Compelling data from laboratory studies, epidemiological investigations, and human clinical trials indicate that flavonoids have important effects on cancer chemoprevention and chemotherapy. Many mechanisms of action have been identified, including carcinogen inactivation, antiproliferation, cell cycle arrest, induction of apoptosis and differentiation, inhibition of angiogenesis, antioxidation and reversal of multidrug resistance or a combination of these mechanisms (Ren *et al.*, 2003).

Flavonoids were found to be strong topoisomerase inhibitors and induce DNA mutations in the (myeloid/lymphoid or mixed-lineage leukemia) MLL gene, which are common findings in neonatal acute leukemia (Strick *et al*., 2000). The DNA changes were increased by treatment with flavonoids in cultured blood stem cells (Van Doorn-

Khosrovani *et al.*, 2007). A high flavonoid-content diet in mothers is suspected to increase risk particularly of acute myeloid leukemia in neonates (Ross, 1998; Ross, 2000; Spector *et al*., 2005). Polyphenols (flavonoids and delphinidin) were found to be strong topoisomerase inhibitors, similar to some chemotherapeutic anticancer drugs including etoposide and doxorubicin. This property may be responsible for both an anticarcinogenic-proapoptotic effect and a carcinogenic DNA damaging potential of the substances (Bandele *et al*., 2008; Esselen *et al*., 2009). 'Flavopiridol' (synthetic polyhydroxylated flavone) (cycline-dependent kinases (CDKs) inhibitor) synthesized by the Indian subsidiary of Hoechst (Aventis) following the isolation and synthesis of the plant-derived natural product, rohitukine, were in Phase III clinical trials both as a single agent and in combination with other agents, particularly with paclitaxel and cis-platinum (Cragg and Newman, 2005). Quercetin (3,3',4',5,7-pentahydroxyflavone) passed Phase I clinical trial with antiproliferative activity *in vitro* and is known to inhibit signal transduction targets including tyrosine kinases, protein kinase C, and phosphatidyl inositol-3 kinase (Ferry *et al*., 1996). 'Genistein' is a well-known isoflavone and is a tyrosine kinase inhibitor. Studies have indicated that genistein elicits inhibitory effects on cell growth of various carcinoma cell lines and may be a potential candidate for cancer therapy (Aoife and O'Kennedy, 2004; Liu *et al.,* 2016).

1.12 Saponins and anticancer properties

Saponins are a group of naturally occurring plant glycosides, characterized by their strong foam-forming properties in aqueous solution. The presence of saponins has been

reported in more than 100 families of plants out of which at least 150 kinds of natural saponins have been found to possess significant anticancer properties. There are more than 11 distinguished classes of saponins including dammaranes, tirucallanes, lupanes, hopanes, oleananes, taraxasteranes, ursanes, cycloartanes, lanostanes, cucurbitanes and steroids (Man *et al.,* 2010). Saponins are amphipathic glycosides grouped by the soap-like foaming they produce when shaken in aqueous solutions, and by their composition of one or more hydrophilic foamside moieties combined with a lipophilic triterpene derivative. The aglycone (glycoside-free portion) of the saponins are termed ‘sapogenins’. The subset of saponins that are steroidal have been termed ‘saraponins’ (Hostettmann and Marston, 1995).

The pharmaceutical applications of saponins are varied as to their origins and chemical structures. Just to mention a few: a number of saponins or saponin-rich mixtures have found use as anti-inflammatory, antidiuretic, antipyretic and analgesic agents, central nervous system depressants and as treatment for ulcers (Cheeke, 1989). Due to the great variability of their structures, saponins always display anti-tumorigenic effects through varieties of antitumor pathways (Man *et al.,* 2010). The proposed mechanisms of anticarcinogenic properties of saponins include direct cytotoxicity, immunemodulatory effects, bile acid binding properties and normalization of carcinogen-induced cell proliferation (Rao and Sung, 1995). Extracts from *Maytenus diversifolia* were found to inhibit growth of leukemic lymphocytes *in vivo* (Cheeke, 1989). Rao and Sung, (1995) showed that soybean saponins at the concentration of 150-600 ppm had a dose-dependent growth inhibitory effect on human carcinoma cells (HCT-15). Viability was also significantly reduced. Shibata, (2001) reported that tetracyclic triterpenoid saponins

isolated from ginseng, the root and rhizome of *Panax ginseng* C.A. Meyer (Araliaceae), showed the anticarcinogenic activity in two-stage anti-cancer-promotion experiments *in vitro* and *in vivo* (Shibata, 2001; Yu *et al,* 2019).

1.13 Terpenes and anticancer properties

Triterpenoids demonstrated a range of unique and potentially usable biological effects. The history of the use of plants with high saponin/triterpenoid content can be found in the first written herbariums (Dzubak *et al.,* 2006). Terpenes are naturally occurring substances produced by a wide variety of plants and animals. Terpenes are biosynthetically derived from isoprene units with the molecular formula C5H8. The basic formula of all terpenes is $(C_5H_8)n$, where 'n' is the number of linked isoprene units (Gao and Singh, 1998). The most common forms of terpenes are the monoterpenes (C10) and sesquiterpenes (C15), but hemiterpenes (C5), diterpenes (C20), triterpenes (C30), and tetraterpenes (C40) also exist. A terpene containing oxygen is called a terpenoid (Bakkali *et al.,* 2008). It has been reported that terpenes in high concentrations can be toxic and considered as key weapons against herbivores and pathogens. A broad range of the biological properties of terpenoids include; anticancer, cancer chemopreventive effects, antimicrobial, antifungal, antiviral, antihyperglycemic, anti-inflammatory and antiparasitic activities (Dzubak *et al.,* 2006; Paduch *et al.,* 2007). Bardon *et al.,* (2002) claimed that various terpenes can act as inhibitors in a dose-dependent manner, to prevent the development of mammary, liver, skin, lung, colon, fore-stomach, prostate and pancreatic carcinomas. Epidemiological studies suggest that dietary monoterpenes may be helpful

in the prevention and therapy of cancers, of which among them, D-limonene and perillyl alcohol have been shown to possess chemopreventive and therapeutic properties against many human cancers (Paduch *et al.,* 2007). Chemotherapeutic activities towards human pancreatic cancers have also been shown for other terpenes, such as farnesol and geraniol (Burke *et al.,* 2002). Concerning more than 20000 triterpenoids, known to occur in nature; oleanolic acid (OA), ursolic acid (UA), synthetic oleananetriterpenoids (SO) and rexinoids are highly effective for the prevention and treatment of cancer in many animal models, but as yet are not definitive agents in clinical practice. To improve potency, many modifications of the basic triterpenoid structure have been made (Liby *et al.,* 2007).

1.14 Mechanisms of action of terpenes'cytotoxicity

Many attempts have been made to prove the influence of terpenoids on inducing apoptosis as a desired strategy of controlling cancers (Liby *et al.,* 2007; Paduch *et al.,* 2007; Yogeeswari and Sriram, 2005; Zhang *et al.,* 2004). Apoptosis is a process that develops in several phases: 1) an initiation phase, in which the biochemical pathways participating in the process depend on the apoptosis-inducing agent; 2) a decision phase, during which the cell "decides" to commit suicide; and 3) a common degradation phase, which is characterized by the activation of catabolic hydrolases (caspases and nucleases). Although the activation of caspases and nucleases is necessary for the acquisition of the full apoptotic morphology, it appears that the inhibition of such enzymes does not inhibit cell death induced by a number of different triggers: Bax, Bak, c-Myc, PML), Fas-associated death domain (FADD), glucocorticoid receptor occupancy, tumor

necrosis factor, growth factor withdrawal, CXCR4 cross-linking, and chemotherapeutic agents (Costantini *et al.,* 2000).

The activation of apoptosis by terpenes occurs via intrinsic cell death pathway, also known as the mitochondrial apoptotic pathway. Yang and Ping Dou, (2010) explained the activation of apoptosis triggered by inhibition of the fas-associated death domain and NF-αB pathways. It has been well documented that suppression of the ubiquitinproteasome and NF-αB pathways are essential for induction of tumor cell apoptosis (Paduch *et al.,* 2007). A proposed mechanism expressed that monoterpenes may activate transforming growth factor (TGF)-β signaling which is produced in a latent form. This activation increases mRNA synthesis encoding (TGF)-β receptors and is closely associated with elevated synthesis of pro-apoptotic proteins (Bax, Bak, and Bad) without influencing Bcl-2 expression. Moreover, through TGF-β, the cell cycle is down-regulated, influencing the production of cyclin and cyclin-dependent kinases or their reciprocal interactions. In consequence, it leads to G1-phase arrest and cell apoptosis (Ahn *et al.,* 2003). Additional studies revealed that some monoterpenes can influence tumor cells by inhibiting the synthesis of coenzyme Q (CoQ), an important element of mitochondrial respiratory metabolism. The reduction of CoQ in cell membranes may, therefore, limit cellular signal transduction and metabolism and induce apoptosis of tumor cells (Ahn *et al.,* 2003; Gould, 1995; Paduch *et al.,* 2007).

In another study, Ulrich, (2007) showed treatment with ursolic acid (pentacyclic triterpenoid) leads to a significant time- and dose-dependent cell growth inhibition of colorectal cancer cells, coincident with the up regulation of the cell cycle1 regulators cyclin E, p21WAF1/Cip1 and p27Kip1. In addition, ursolic acid significantly induces apoptosis,

which is mediated by an increase of BAX/Bcl-2-protein-ratio as well as an upregulation of TRAIL protein which meets in an induction of caspase-3 activity.

1.15 Chemoprevention of terpenes

Cancer chemoprevention, as first defined in 1976 by Sporn, is the use of natural, synthetic, or biologic chemical agents to reverse, suppress, or prevent carcinogenic progression (Sporn, 1976). According to the National Cancer Institute (NCI), five classes of mainly chemically synthesized chemopreventive agents are characterized of high priority: selective estrogen receptor modulators (SERMs), non-steroidal anti-inflammatory drugs (NSAIDs), calcium compounds, glucocorticoids and retinoids. In parallel, the NCI identified about 40 edible plants possessing potential chemopreventive compounds, globally known as phytochemicals (National Center for Health Statistics, 2005). For instance, among dietary monoterpenes, D-limonene and perillyl alcohol have been shown to possess chemopreventive and therapeutic properties against many human cancers. There are multiple mechanisms of monoterpene chemopreventive actions. They may act during the initiation phase of carcinogenesis, preventing interaction of carcinogens with DNA, or during the promotion phase, inhibiting cancer cell development and migration. The chemopreventive and therapeutic activities of monoterpenes in later stages of carcinogenesis include induction of cancer cell apoptosis, re-differentiation of tumor cells, and influence on molecular mechanisms regulating their functions. The most important mechanism that monoterpenes influence is post-translational isoprenylation of proteins regulating the growth of cells (Riva *et al.,* 2019).

1.16 The genus Pappea.

Derivation of name and historical aspects

The generic name *Pappea* is named after a German physician and plant collector Carl Pappe, while the specific name *capensis* refers to southern Africa. *Pappea capensis* belongs to the same family as the popular fruit, the litchi. The family is represented in South Africa most notably by the false currants (*Allophylus* spp.), the well-known and widely cultivated sand olive (*Dodonaea viscosa*) and the bushveld red-balloon (*Erythrophysa transvaalensis*). This species was previously known as two separate varieties (*Pappea capensis* var. *capensis* and *P. capensis* var. *radlkoferii*). However, it is now widely accepted that it was just regional variation which resulted in confusion between an arid form from drier areas and a lusher form from regions of higher rainfall (pza.sanbi.org/pappea-capensis).

The plant belongs to the family sapindaceae (litchi or soap-berry family), the most commonly used names are jacket plum, Indaba tree, bushveld cherry (English); doppruim (Afrrikaans); umQhokwane, umVuna, iNdaba (Zulu); iliTye, umGqalutye (Xhosa); mongatane, Mopsinyugane (Northern Sotho); liLetsa (Swati); Xikwakwaxu, Gulaswimbi (Tsonga). It consists of the red fruits which are a tasty treat for humans and a firm favourite with birds and animals (pza.sanbi.org/pappea-capensis). It also provides a fine oil that can be extracted from the seeds. The jacket plum is related to the litchi and is a natural addition for the bird or wildlife garden (pza.sanbi.org/pappea-capensis).

The jacket plum is a long-leafed, hardy, evergreen, and small to medium tree with a height of 2-8 m. It is easily cultivatedunder ideal conditions (hot and raininy climate) it can grow at a moderate rate but can be slow-growing under dry and/or cold conditions (pza.sanbi.org/pappea-capensis).

The leaves are simple and oblong, hard-textured and wavy. The leaf margin may vary from sharply toothed (especially in young growth) to almost smooth in mature growth. The greenish flowers are borne on catkins in the axils of the leaves, followed by round green velvety fruits which split open to reveal bright red flesh with a dark brown to black seed imbedded within (pza.sanbi.org/pappea-capensis).

Pappea capensis is widespread in southern Africa from the Northern Cape through the drier Karoo, Eastern Cape, KwaZulu-Natal, to the Northern provinces, as well as Mozambique, Zimbabwe and northwards into eastern and southern tropical Africa. It naturally occurs in bushveld, riverine thicket, wooded grassland and rocky outcrops in grasslands as well as scrub veld and is often found on termite mounds. Due to its wide distribution range it is well suited to cultivation in a wide variety of climatic conditions (pza.sanbi.org/pappea-capensis) .

The fruit is eaten by various frugivorous birds and animals which in turn distribute the seeds in their droppings. The leaves are browsed by game such as elephant, giraffe, kudu, nyala, bushbuck, and grey duiker as well as domestic stock animals. The jacket

plum has also been recorded as the larval food plant to the caterpillars of the following butterflies of southern Africa:

Common hairtail butterfly (*Anthene definita definita*). Brown playboy butterfly (*Virachola antalus*), pearlspotted charaxes (*Charaxes jahlusa*) and gold-banded forester (*Euphaedra neophron*). The sweetly scented flowers attract a wide variety of insects which in turn attract many birds. The seed is parasitized by a small, bright red bug (*Leptocoris hexophtalma*) which sucks the oil from the seed on the ground below the tree.

The delicious and very juicy fruit with a tart flavour is used to make and preserve jelly, vinegar and an alcoholic drink. Fragrant non-drying golden yellow oil is extracted from the roasted seeds. There are reports of it being used for oiling rifles. It is also used as a purgative and for lubrication, as a cure for ringworm, to restore hair as well as for making soap. Leaves, bark and the oil extracted from the seed are used medicinally against baldness, ringworm, nosebleeds, chest complaints, eye infections and venereal disease. Bark is also used in protective charms that are sprinkled on the ground. Some research has reported that the leaves are very effective in killing snails. Infusions of the bark are also used by Kenyan Masai warriors to gain courage as well as an aphrodisiac and a blood strengthening tonic. The root is used orally or as an enema and as a purgative for cattle. *Pappea capensis* known as the ancient *Lobengula's* Indaba tree in Zimbabwe was grown in the state house in Bulawayo in Zimbabwe, is an ancient specimen of *Pappea capensis* (pza.sanbi.org/pappea-capensis).

The wood is hard, light brown with a reddish tint, tough and heavy with a twisted grain. There is apparently little difference between the heartwood and the sapwood. The stems seldom attain significant girth and therefore do not yield much useable wood. It is, however, used to make sticks, poles, cattle yokes, kitchen utensils and furniture. This tree is still used as an important source of traditional medicine today for treatment of many dieases . The new leaves are an attractive pinky-bronze when they emerge in spring, this contrasts well with the dark green of the old leaves making an attractive display.

The trees flower from September to May (southern hemisphere) and the rather special fruit is produced from December to July. The dense crown is popular with nesting birds as it provides a concealed and sheltered nesting sites (pza.sanbi.org/pappea-capensis). Nursery grown plants adapt well to cultivation and respond well to organic and synthetic horticultural fertilizers. Saplings are slow-growing especially when young but growth increases as the tree matures. Growth is also considerably quicker in warmer climates or warmer positions of the garden.

Fig1.4: A and B shows a *Pappea capensis* tree where sample was collected

1.17 RATIONALE FOR THIS STUDY

Common scientific reports above indicate plant-derived agents which have medical benefits to different conditions. The antigenicity and toxicity of the plant extracts in the treatment of cancer remains unknown.

The slow diminishing of indigenous culture, in favour of western European-derived culture, is an accelerating process among indigenous people around the world (Prance, 1994). Consequently, the traditional knowledge that forms the basis of use of medicinal plants is under threat of being lost and warrants rigorous scientific investigation.

The current study was encouraged by case reports describing astonishing improvement of patients who have been critically ill due to progressive prostate cancer, the doctors around Mokopane area had no explanation for the improvement. Mrs RR Matabane (the traditional healer) confirmed that some doctors asked patients what they have used, these patients reported that they had been treated with an extract from a bark of a medicinal plant. This plant material was offered to us for an initial analysis for anti-cancer activity. After characterisation by a botanist, the plant was found to be *Pappea capensis* (fig. 1.4 (A) and (B)). Currently, the investigation of these claims, the efficacy of the use of *Pappea capensis*, as well as further investigation of its therapeutic potential has received less scientific investigation.

Objectives of the study: To prepare a crude plant extract of *Pappea capensis*, broadly establish the bioactivity and describe the phytochemical properties of *Pappea capensis* water extract.

Specific aims:

To conduct an *in vitro* anti-cancer screening of *Pappea capensis* extracts (ethanol, methanol and water extracts)

To evaluate the genotoxicity and cytotoxicity effect of *Pappea capensis* extracts

To evaluate the radical scavenger activities of *Pappea capensis* extracts

To evaluate the anti-microbial activity of *Pappea capensis* extracts

To examine the chemical composition of *Pappea capensis* extracts

To examine the pro and anti-inflammatory effect of *Pappea capensis* extracts

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Chapter 2

IN VITRO ANTIOXIDANT SCREENING OF *PAPPEA CAPENSIS* EXTRACTS

2.1 Introduction

Cancer remains one of the prominent and life-threatening diseases globally, with more than 100 different types occurring as a result of molecular changes within the cell (Shaikh *et al.,* 2014). It is ranking as the second killer globally, following cardiovascular and infectious diseases (Kelloff, 2008, WCR, 2014). The disease is widely prevalent in both males and females. Almost a third of the world population are at risk of developing cancer at some point time in their lives (WCR, 2014). Approximately 13% of the world population dies due to cancer (WCR, 2014), hence this triggered the scientific investigation to find medicinal plants that possess biological activities against cancer cells for more than three decades (John, 1984; Rafik, 2016, Poudyali and Singh, 2019). Studies have proved that different plants species have antioxidant activities owing this to the phenolic contents they possess (Hassas-Roudsari *et al.,* 2009; Lin *et al*., 2009; Gerber *et al.,* 2002).

The determination of the bioactivity of plant extracts or natural products as well as synthetic compounds, being it antioxidant, antimicrobial, cytotoxic to name a few is a crucial element in the discovery of new and improved therapeutic entities. In this study, the anti-oxidant activity, antibacterial activity and total phenolic content of 3 plant extracts were determined.

Antioxidants are involved in the defence mechanism of organisms against pathogens (John, 1984). Many health benefits are associated with antioxidants and provide protection against the development of diseases caused by oxidative stress (Gerschi and

Negulescu, 2011). In addition to antioxidants, polyphenols have been emerging as one of the major natural products that are important to human health for the treatment of diseases (Sharma *et al.,* 2012; Tiwari *et al.,* 2011; Altemimi *et al.,* 2017). Evidence has shown that polyphenols are actually good antioxidants and are effective in preventing cardiovascular and inflammatory diseases as well as anti-cancer agents (Zhang *et al.,* 2006). A knowledge assessing students in the Limpopo province also indicated extensive use of the medicinal plants by the different ethnic groups in South Africa (Mongalo and Makhafola, 2018). Natural antioxidant compounds are believed to have pharmacological potent with low or less side effects (Benmehdi *et al.,* 2017). For these reasons, over the decades there has been an increase in the interest of potential antioxidant activity as well as the total phenolic content of plant and natural products. So, in this study the antioxidant potential of *Pappea capensis* extracts was determined based on their scavenging of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free racicals.

2.2 Materials and methods

2.2.1. Plant material

The plant material was collected once around September in 2015 at road R101. The plant material (*Pappea capensis*) was authenticated by Dr Zietzmann Bloemfontein museum and scientists at the National Botanical Gardens in Pretoria, South Africa *capensis* (PC Zietsman & A Makhoahle 5448 the specimen is housed at the herbarium of the National Museum, Bloemfontein (NMB)). The collected bark and wood materials were dried at

room temperature and pulverised by a mechanical mill and weighed. It was then stored at room temperature until analysis.

Three 360g wood samples were weighed out for extraction with 1080ml acetone, water or methanol, respectively. Volumes were adapted according to the consistency of the sample. Then the rest of the solvent was added, and solutions allowed to seep out for 24 hours.

Filtering was performed after 24 hours, then the solids were removed from each solution by filtration with a Millipore funnel with medium filter paper (Bright sign nr102) connected to a Millipore vacuum pump. Where needed, samples were centrifuged in 50ml conical tubes. Removing Solvents: Most extracts contained both aqueous and organic solvents, and both Freeze-drying steps were employed with a Virtis Freeze drier to remove aqueous solvent as well as a Rotary evaporate (55°C) to remove organic solvents. After repeated steps of both freeze-drying and vacuum evaporation the samples were moved to pre-weighed containers and the yield determined.

2.2.2 *In vitro* assessment of anti-oxidant activity using the DPPH assay:

In a 96 well plate 5 µL sample, 120 µL Tris-HCL buffer (50 mM, pH 7.4) and 120 µL freshly prepared DPPH (0.1 mM in ethanol) were added and allowed to incubate for 20 minutes in the dark at room temperature. The absorbance was measured at 513 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA) and the percentage radical scavenging activity was calculated as follows:

% DPPH scavenged = (blank-sample)/blank x 100

For the blank / control, buffer was substituted as the 5 µL sample. Plant extracts were tested at final concentrations of 250 and 500 µg/mL. Quercetin was used as a positive control at final concentrations of 6.25 - 25 µM.

2.3 *In vitro* assessment of antioxidant activity using the DPPH assay:

The DPPH radical scavenging assay was used as a preliminary screening of the extracts for their antioxidant activity. The proton radical scavenging activity is known as a significant mechanism of antioxidant. The results of this assay are indicated in Table 2.1, clearly showing the % DPPH scavenging activity of each extract.

Table 2.1: Antioxidant activity of extracts expressed as % DPPH scavenged

Extract	Concentration (µg/mL)	% DPPH scavenged	Std Dev
6	250	81.21	4.90
	500	69.63	2.62
7	250	77.14	5.09
	500	78.40	3.77
8	250	84.03	0.68
	500	79.18	3.74
Quercetin (positive control)	6.25 µM	48.65	9.71
	12.5 µM	84.35	1.08
	25 µM	86.43	0.65

Results are from a single experiment performed as quadruplicates

2.4 Discussion:

The DPPH scavenging assay was performed to determine the anti-oxidant activity of *Pappea capensis extracts*. The results presented in Table 2.1 evidently showed that all extracts possess anti-oxidant activity when compared to the positive control *(Quercetin*). The addition of the *Pappea capensis* extracts to the DPPH solution induced a rapid decrease in absorbance at 513 nm, indicating free radical scavenging activity of the extracts (table 2.1). The radical scavenging activity of the extract was concentration-dependent and similar to that of known antioxidants. The results of the three extracts showed a similar pattern to what has been reported to other studies done on different plants (Hassas-Roudsari *et al.,* 2009; Lin *et al*., 2009; Gerber *et al.,* 2002). All *Pappea capensis* extracts showed concentration dependent free radical scavenging activity at concentrations above 250 ug ml^{-1}, while *Quercetin* showed almost similar scavenging activity from 12.5uM.

2.5 Conclusion

In vitro antioxidant activity was carried out with ethanoic, water and methanolic *Pappea capensis* extracts by the DPPH free radical scavenging method. From the results of the DPPH it was found that the three extracts of *Pappea capensis* displayed antioxidant similar to the control. Henceforth, this warrant further investigation to establish the existence of phytochemicals and phenolic compounds as it is evidently clear that free radicals were trapped by the three extracts of *Pappea capensis.*

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Chapter 3

IN VITRO PHYTOCHEMICAL ANALYSIS AND TOTAL PHENOLICS SCREENING FROM *PAPPEA CAPENSIS* EXTRACTS USING FOLIN-CIOCALTEU (FC) ASSAY

3.1 Introduction

Phytochemicals (are derived from the Greek word “Phyto” meaning plant) are naturally occurring biological compounds that originate in plants (Saxena *et al.,* 2013). These compounds offer additional health benefit to humans than those attributed to micronutrients and macronutrients (Hasler *et al.,* 1999). Almost 150 phytochemicals have been studied fully and are found to accumulate in the different parts of the plants (King *et al.,* 1999*;* Costa *et al.,* 1999; *American Cancer Society.,* 2000). The level of this compound’s concentration differed from within the same plant and among the different plant species (King *et al.,* 1999).

Secondary metabolites possess multiple functionality and biological activities as a result of more than one functional group (Dey and Harborne, 1989). The classification of the metabolites is owed to their chemical structure, solubility, composition, and their pathways involved namely the original synthetic and biosynthetic pathways (Harbone *et al.,* 1986). Secondary metabolites have been categorised in six families namely flavonoids, alkaloids, steroids, phenolics, tannins and triterpenes.

Flavonoids

Flavanoids occurs naturally as compounds containing two benzene rings linked together with a pyrone ring, in the case of flavones, or a dihydropyrone ring in instances of flanones (Giuseppe *et al.,* 2007). They are regarded as the most common class of phenolics which

could also be found as monomers, dimers and oligomers. Flavonoids are soluble in water and contain 15 carbon atoms with low molecular weighed compound. They include the phenylbenzopyrones which are commonly found in all vascular plants. Various groups such as aurones, flavanones, chalcones, quercetin, rutinosides, isoflavanoids, flavones, flavanols, anthocyanins, cathechins, kempferols and leucoanthocyanidis represented by fig 3.1 showing typical flavonoids with the basic unit of ketone (Li *et al.,* 2018).

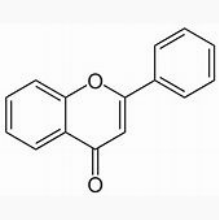

Fig. 3.1: Flavonoids with the basic unit of ketone (Li *et al.,* 2018).

All of which are produced by plants in different amounts and concentrations (van Wyk and Wink, 2004). Presences of phenolics have been identified in plants that have shown to possess antioxidants from the different plants; whereby upon screening there was reduction in DPPH scavenging activity in the presence of the plant extract (Hassas-Roudsari *et al.,* 2009; Lin *et al*., 2009; Gerber *et al.,* 2002). Different extraction methods such as ethanol and aqueous have been reported to show the presence of tannis and active phytochemicals (Fuku *et al.,* 2013). So, in chapter 2, the three extracts of the *Pappea capensis* proved to possess antioxidant activities, and that necessitated further screening of the presence of total phenolic content in this plant.

3.2 Materials and methods

3.2.1. Plant material

The plant material (*Pappea capensis*) was authenticated by Dr Zietzmann at the Bloemfontein museum, and scientists at the National Botanical Gardens in Pretoria, South Africa. The collected bark and wood materials were dried at room temperature and pulverised by a mechanical mill and weighed. It was then stored at room temperature until analysis.

Three 360g wood samples were weighed out for extraction with 1080ml acetone, water or methanol respectively. Volumes were adapted according to the consistency of the sample. Then the rest of the solvent was added, and solutions allowed to seep out for 24 hours. Filtering was performed after 24 hours, then the solids were removed from each solution by filtration with a Millipore funnel and medium filter paper (Bright sign nr102) connected to a Millipore vacuum pump. Where needed, samples were centrifuged in 50ml conical tubes.

Removing Solvents: Most extracts contained both aqueous and organic solvents and we employed both Freeze-drying steps with a Virtis Freeze drier to remove aqueous solvent as well as a Rotary evaporate (55°C) to remove organic solvents. After repeated steps of both freeze-drying and vacuum evaporation the samples were moved to pre-weighed containers and the yield determined.

3.2.2 Phytochemical screening

Chemical tests were carried out on the acetone, aqueous and methanol extracts of *Pappea capensis*. The samples were extracted and indicated above and then subjected to the chemical tests described below (Edoaga *et al*., 2005).

3.2.2.1 Alkaloid determination using Harborne (1973) method

Five grams (5 g) of the sample was weighed into a 250 ml beaker, and 200 ml of 10% acetic acid in ethanol was added, covered and allowed to stand for 4 hours. This was followed by filtering the solution passively through a Whatman® filterpaper. The extract was concentrated in a waterbath to one-quarter of the original volume. Concentrated ammonium hydroxide was added dropwise to the extracts until the precipitation was completed. The whole solution was allowed to settle and then the precipitate was collected and washed with dilute ammonium hydroxide and then filtered further. The residue was the alkaloid, which was air dried and weighed.

Test for tannins

About 0.5 g of the dried powdered samples was boiled in 20 ml of water in a test tube and then filtered through a Whatman® filter. A few drops of 0.1% ferric chloride was added and brownish green or a blue-black colouration was observed in acetone and water extracts only.

Test for saponin

About 2 g of the powdered sample was boiled in 20 ml of distilled water in a water bath (set at 100^{o}C) and filtered. Then 10ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion for all extracts.

Test for steroids

Two ml of acetic anhydride was added to 0.5 g ethanolic extract of each sample with 2 ml H_2S0_4. The colour changed from violet to blue or green in some samples indicated the presence of steroids in all extracts.

Test for terpenoids (Salkowski test)

Five ml of each extract was mixed in 2 ml of chloroform, and concentrated H2S04 (3 ml) was carefully added to form a layer. A reddish-brown colouration of the interface was formed to show positive results for the presence of terpenoids in all extracts.

3.2.2 Total phenolics determination using the Folin-Ciocalteu (FC) assay:

A standard curve was prepared using gallic acid at a concentration range of 6.25 µg/mL – 100 µg/mL. It is important to note that all extracts and the standard control (gallic acid) were prepared at 10 x the desired concentrations in consideration of the dilution factor of 1:10. Sample extracts (stock = 100 mg/mL) were prepared at a concentration of 10 mg/mL

and centrifuged at 12 000 xg for 10 minutes. Thereafter, 20 µL of supernatant was added to a well of a 96 well plate, in triplicate. A 1:1 and 1:10 dilution of the supernatant of each extract was also done. One hundred µL of FC reagent was added to each well and the plate was incubated for 5 minutes at room temperature. An aliquot of 80 µL of 7.5 % Na_2CO_3 was added slowly to each well and the plate was left to incubate at room temperature in the dark for 2 hours. The absorbance was then read at 750 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, USA).

3.3.1 Results of phytochemical analysis

Table 3.1: Phytochemicals from wood powder of *Pappea capensis*

Extract	Tannins	Alkolods	Saponins	Steroids	Phenols	Terpenoids	Flavonoids
Acetone	+	+	+	+	+	+	+
Aqueous	++	+	++	++	+	-	-
Methanolic	-	+	+ (Trace)	+	+++	++	+

Key: + slightly present; ++= moderately present; +++= highly present

The phytochemicals detected in the wood powders of *Pappea capensis* were tannis, alkaloids, saponnis, steroids, phenols, terpenoids and flavonoids as depicted above in table 3.1. Phytochemical screening results showed *Pappea capensis* is mostly rich in phenols extracted from all conditions. The phenols contents were further estimated using the gallic acid standard curve generated using values of gallic acid (fig.3.2)

3.3.2 Results of total phenolics determination:

A standard curve was done using gallic acid for the determination of the concentration of phenolics in each plant extract (Figure 3.1). Table 3.2 indicates the total phenolic concentration of each extract using the equation of the straight-line graph in Figure 3.1. (Refer to annexure 1)

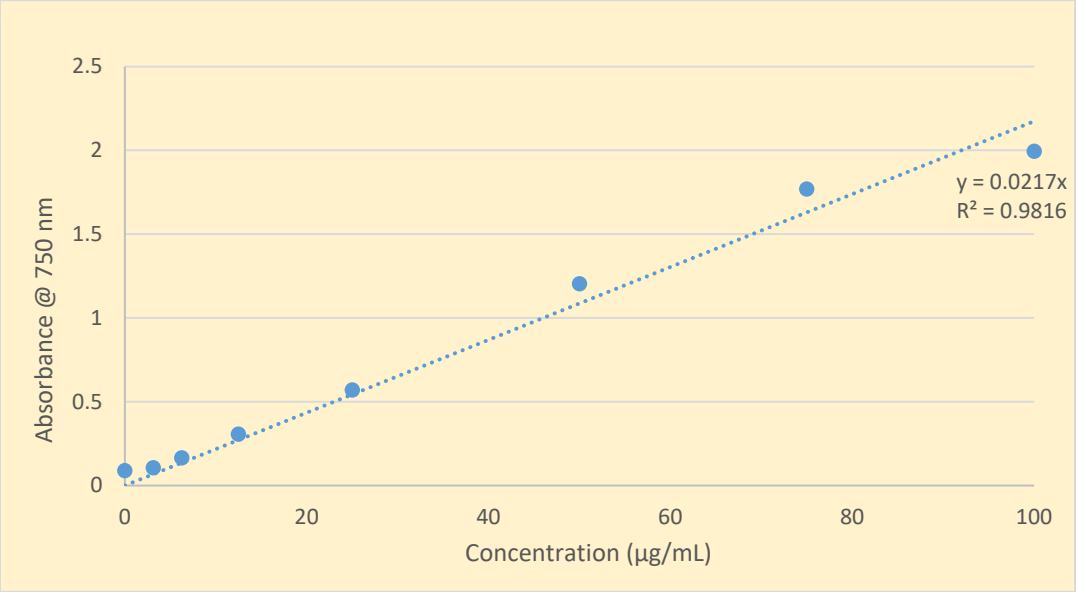

Figure 3.2: Standard curve for total phenolics determination using the FC assay and gallic acid as the standard control.

Table 3.2: Total phenolics determination using the FC method

Extract	Ave. Abs @ 750 nm	Resulting phenolic concentration (µg/mL)	Std dev of concentration
6	1.01	46.51	2.60
7	0.31	14.19	0.24
8	0.48	219.82	18.25

Results are from a single experiment performed as quadruplicates

3.4 Discussion:

Plants produce natural phytochemicals for protection and used them for defence against insect and the different microorganisms. Primary compounds produced by plants are proteins, common sugars and chlorophyll, while the secondary compounds include flavonoids, reducing sugars, terpenoids, tannins, terpenoids and phenols (Krishnaiah *et al.,* 2007). The phytochemicals detected from the wood powder of *Pappea capensis* were tannis, alkaloids, saponins, steroids, phenols, terpenoids and flavonoids as shown above (table 3.1).

In vitro antioxidant activity carried out in chapter 2 with ethanoic, water and methanolic *Pappea capensis* extracts using the DPPH free radical scavenging method indicated that indeed the plants possess antioxidant. Further testing to determine phytochemicals and phenols also prevailed that different secondary metabolites were eluted on a solvent base. So, the identification and absence or weak isolation of each compound doesn't

mean it's not possessed by the plant that purely depended on extraction by the solvent used. The phytochemical compounds identified from the water and acetone extracts correlated with the study done on *Pappea capensis* stem bark which also identified the same metabolites with an extra cardiac glycoside which was not tested in this study (Karau *et al.,* 2012). The methanolic extract phytochemical analysis identified correlating results with the study of Ngai *et al* that demonstrated the presence of secondary metabolites using methanolic stem bark extracts associated with hypoglycaemic activity (Ngai *et al.,* 2017). It is now evident that the trapping of free radicals was as a result of phytochemical been one of the contributing factors in the antioxidant activity.

Furthermore, the total phenolic contents of *Papea capensis* three extracts was determined by Folin-Ciocalteu method whereby a gallic acid standard curve was constructed to determine the concentration of phenols. All three extracts showed the presence of phenolics with ethanoic and water diluted in the ratio of 1:1. Methanolic extract absorbance was too high to fit in the standard curve during the initial dilution (1:1 dilution). So, it was further diluted to the ratio of 1:10. Then the absorbance value of 0.48 was measured at 750nm, and methanolic extract showed the highest phenolic compounds at 220ug/ml when the absorbance reading was multiplied by ten to determine the final concentration. There is a significant correlation between antioxidant activity and phenolic compounds content identified. This is an indication that phenolic compounds are the major sources of the antioxidant properties of the wood part of this plant. These phenolic compounds association with antioxidant found in this study correlates with the results of previous studies which indicated very strong antioxidant properties in plants possessing high phenolic compounds (Kahkonen *et al.,* 1999; Li *et al.,* 2008). The total

phenolic contents also correlated with the antioxidant activity results reported by Ntsoelinyana and Mashele on *Asparagus laricinus* extract (Ntsoelinyane and Mashele, 2014). These results supported the use of *Pappea capensis* by traditional healers for the treatments of numerous ailments.

3.5 Conclusion:

The antioxidant determined in chapter 2, phytochemical compounds, and total phenolic content were performed in order to meet the necessary requirements for further screening of this plant for safe use and toxicity. Other scientists have further shown that the leaf and stem bark of *Papea capensis* have traditional medicine benefits such as curative effects associated with hypoglycaemic activity due to phytochemicals (Karau *et al.,* 2012; Ngai *et al.,* 2017). The different phytochemical compounds and phenols identified from the wood of *Papea capensis* might possess pharmacological benefits and use in traditional medicinal. More tests such as the antibacterial activity, cytoxicity and many more should be done to ascertain the medical benefits and safety of the woody part of this plant.

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Chapter 4

IN VITRO ANTIBACTERIAL SCREENING OF *PAPPEA CAPENSIS* EXTRACTS USING the *p*-IODONITROTETRAZOLIUM CHLORIDE (INT) ASSAY

4.1 INTRODUCTION

Plants have palyed a vital role in providing necessary metabolites for the treatment of many dieases for many years. Its is therefore important to assess if *Pappea capensis* possesses any of antimicrobial agents. The discovery of the antimicrobial agents (antibiotics) reduced the volume of wide spread different diseases globally (Gertsch., 2009; Talib and Mahasneh., 2010). However, due to non-adherence and failure to control the use of these antibiotics this has led to the development of drug resistant microorganisms (Cowan., 1999). The efficacy of many antimicrobial agents is being threatened by the emergence of the microbial resistant organisms to existing chemotherapeutic treatment (Pareke *et al.,* 2007). Bacterial strains resistant to a lot of antimicrobial agents have made the search for the new and novel drugs to be the highest priority in the sciences fraternity (Alanis., 2005; Sharma *et al.,* 2012); the emergence of methicillin-resistance *Staphylococcus aureus,* drug resistance tuberculosis, multi-drug resistance and extensively resistance tuberculosis to name a few (Ficker *et al.,* 2005). Furthermore, it's been reported that other antimicrobial agents are associated with allergy, hypersensitivity, immune suppression, and side effects (Ahmed *et al.,*1998). Most known novel drugs (Amphotericin B and azole) for the chronic treatment of chronic fungal infection also were clouded by the development of resistant Candida specie (Ficker *et al.,* 2005). Previous studies done on drug resistance proved the need for the development of new and novel antimicrobial agents that would alleviate the emergence and re-emergence of drug resistance organisms globally (John., 1984; Cowan., 1999; Alanis., 2005). Studies have found that plant-derived products represent an attractive source of antimicrobial agents due to their accessibility and affordability, especially for the Sub-

Saharan countries (Ghosh *et al.,* 2008). The use of plants by traditional healers, Sangomas and most people globally pressed for the scientific screening for their toxicity and safety for human consumption (Ghosh *et al.,* 2008; Altemimi *et al.,* 2017). In this study *p*-iodonitrotetrazolium chloride (INT) assay was used to test and screen the inhibitory effects on the different microorganisms mostly isolated from cancer patients.

The following organisms *Staphylococcus aureus* (ATCC 25923) and *Klebsiella pneumoniae* (ATCC700603) representing a Gram-positive and a Gram-negative bacterium, respectively, were used for antimicrobial screening against three extract namely: ethanoic, water and methanolic of *Pappea capensis*. It was also important to include a known antibacterial agent as a positive control for these assays in order to provide direct comparison for the different extracts and to ensure that the assay was performed successfully. Both the gentamycin and vancomycin at 2ug/ml where used for the Gram- negative and Gram-positive organisms.

4.2 Materials and methods

4.2.1. Plant material

The plant material (*Pappea capensis*) was authenticated by Dr Zietzmann Bloemfontein museum and scientists at the National Botanical Gardens in Pretoria, South Africa. The collected bark and wood materials were dried at room temperature and pulverised by a mechanical mill and weighed. It was then stored at room temperature until analysis.

Three 360g wood samples were weighed out for extraction with 1080ml acetone, water or methanol, respectively. Volumes were adapted according to the consistency of the sample. Then the rest of the solvent was added, and solutions allowed to seep out for 24 hours.

Filtering was performed after 24 hours, then the solids were removed from each solution by filtration with a Millipore funnel with medium filter paper (Bright sign nr102) connected to a Millipore vacuum pump. Where needed, samples were centrifuged in 50ml conical tubes.

Removing Solvents: Most extracts contained both aqueous and organic solvents and we employed both Freeze-drying steps with a Virtis Freeze drier to remove aqueous solvent as well as a Rotary evaporate (55°C) to remove organic solvents. After repeated steps of both freeze-drying and vacuum evaporation the samples were moved to pre-weighed containers and the yield determined.

4.2.2 Determination of antibacterial activity of extracts using the p-Iodonitrotetrazolium chloride (INT) assay:

The antibacterial activity of plant extracts was tested on *Staphylococcus aureus* and *Klebsiella pneumoniae,* representatives of Gram-positive and Gram-negative bacteria, respectively. Bacterial cultures were grown on Mueller-Hinton (MH) agar plates at 37˚C. An overnight streak plate was used to inoculate MH broth (Merck, USA) and allowed to grow for 16 hours (log phase) at 37˚C.

Gentamicin sulfate and vancomycin hydrochloride (Sigma, USA) were used as positive controls against *K. pneumoniae* and *S. aureus,* respectively. Antibiotics were dissolved in double distilled water at stock concentrations of 2 mg/mL and filter sterilized (0.2 µm filter). Working concentrations of the antibiotics were prepared in MH broth, depending on the minimum inhibitory concentration (MIC) value.

Fifty µL of MH broth was added to the wells of a sterile 96 well plate and 50 µL of test extracts were added to relevant wells. This was followed by a serial dilution of extracts to achieve the concentration range as indicated in Figures 1 and 2. The cultures were assessed and adjusted to a 0.5 McFarland standard [1.175 % $BaCl_2$ and 1 % H_2SO_4 with absorbance 600nm = 0.08-0.1 to achieve ±1.5 x 10^8 cells/mL]. Fifty µL aliquots of the relevant bacteria was added to each test well. Plates were sealed and incubated at 37˚C for 24 hours.

p-Iodonitrotetrazolium chloride (INT) was prepared at a working concentration of 0.2 mg/mL in ddH_2O and filter sterilized (0.2 µm filter). Fifty µL INT was added to each well and the plates were further incubated for 30 – 60 minutes at 37˚C until a colour change was observed (yellow to pink/purple to indicate the reduction of the dye by viable bacteria). No colour change indicated the inhibition of bacterial growth. Absorbance (abs) was measured at 600 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, USA).

Percentage inhibition was defined as: Percentage inhibition = 1 – (test well abs / mean abs triplicate bacteria only well) x 100

4.3 Results

4.3.1 Determination of antibacterial activity of extracts:

The antibacterial activity of plant extracts was performed in triplicate using the INT assay and a representative of a Gram-positive and Gram-negative bacteria. Figures 4.1 and 4.2 show the results of this assay (refer **to annexure 2**). Gentamicin was used as a positive control for this experiment.

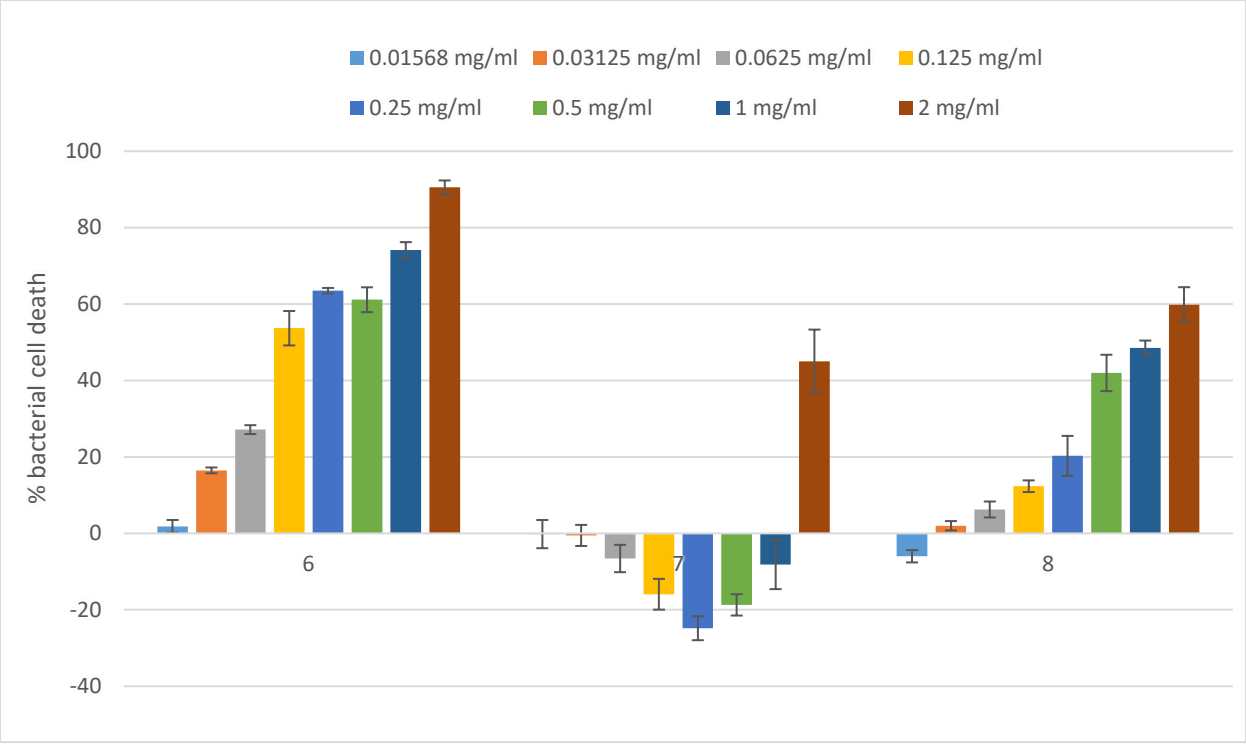

Figure 4.1: Screening for antibacterial activity of six extracts (as indicated) against *S. aureus* for a concentration range of 0.0156 mg/ml – 2 mg/ml. Vancomycin was used as a positive control at its MIC of 2 µg/ml. Error bars indicate standard deviation of quadruplicate values done as a single experiment.

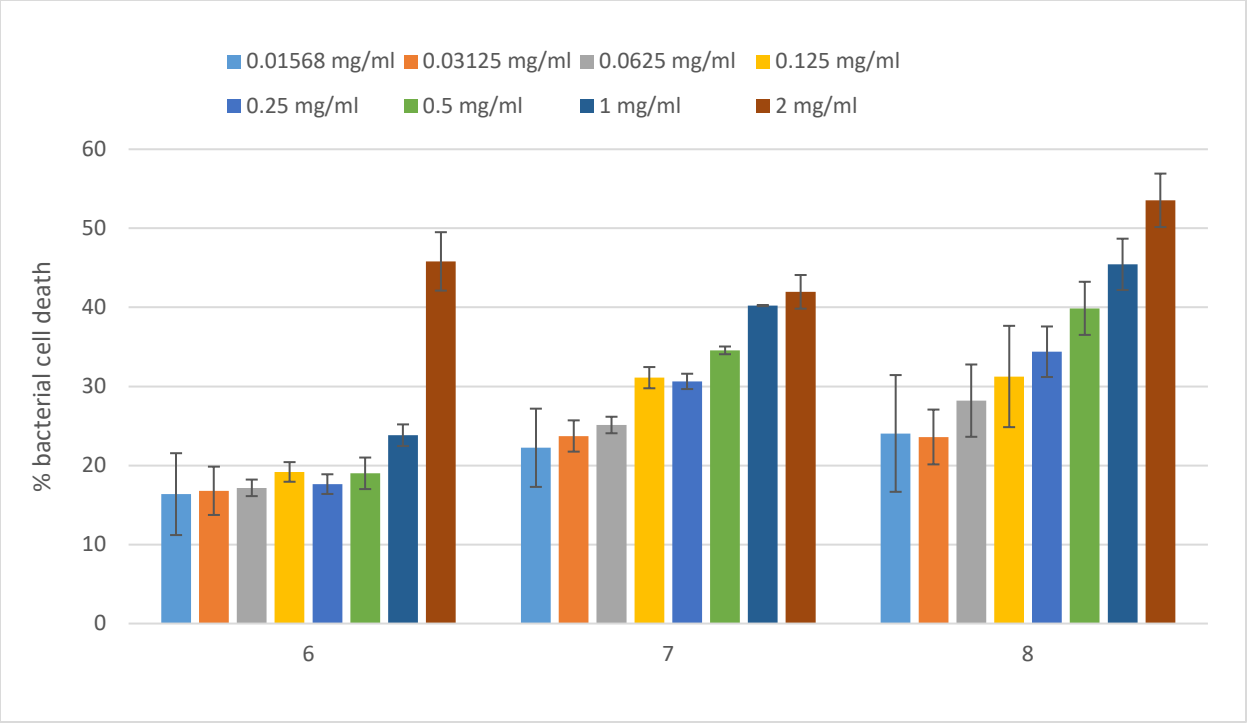

Figure 4.2: Screening for anti-microbial activity of nine extracts against *K. pneumoniae* for a concentration range of 0.0156 mg/ml – 2 mg/ml. Gentamicin was used as a positive control at its MIC of 2 µg/ml. Error bars indicate standard deviation of quadruplicate values done as a single experiment.

4.4 Discussion:

Effective separation of the active compounds purely depends on the type of solvent used in the extraction process (Pareke and Chanda., 2007). Screening of the extract 6 (ethanoic extract) of *Pappea capensis* extracts on *Staphylococcus aureus* (fig. 4.1) showed good antimicrobial activity with 90% bacterial death at 2mg/ml, followed by methanolic extract (no8) which showed 50% bacterial death at 2mg/ml. There was no antimicrobial activity at low concentrations of water extract except for 2mg/ml. The

ethanoic extraction (no6) shows antimicrobial activity against *Klebsiella pneumoniae* with more than 50% bacterial death achieved at 2mg/ml. Similar antimicrobial activity was seen for water extract (no 7) and methanolic extract (8). Fifty percent killing of *K. pneumoniae* will only be achieved by water extract of >2mg/ml and methanolic exceeded the 50% bacterial killing with 2mg/ml. The antibacterial activity showed interesting results with water extract (no 7) indicating Gram specificity, only inhibiting growth of *K. pneumoniae* but not *S. aureus*. This correlates to the results of the study done on Jordanian plants, even though it was the different plants to *Pappea capensis* (Talib and Mahasneh., 2010). It's quite interesting because generally it is expected for the plants extract to be active against Gram positive bacteria as compared to the Gram negatives (Mc Cutchon *et al.,* 1992). The ethanoic extract and methanolic extract results correlates with the research done on Yemen and Jordanian plants where they were found to be active against both Gram positive and Gram negative organsims even though they are different (Al-Fatima *et al.,* 2007; Talib and Mahasneh., 2010). Moreover, methanolic extract (no8) showed less % bacterial cell death against *S. aureus* than *K.* pneumoniae at the lower concentrations. The strength of all extracts was low compared to both controls with an MICs of 2ug/ml to obtain 50% bacterial cell death.

4.5 Conclusion

The antimicrobial screening showed that different extraction methods isolated active compound solvent types. Ethanoic and methanolic extracts showed to have active compounds against Gram positive and negative organism. Both extracts (ethanoic and methanolic) indicated that potential antimicrobial compounds were in the high polarity

fraction. Water is the most usually used solvent by Sangomas and traditional healers to make medicinal mixtures for their patients. However, in this study ethanoic and methanolic extracts produced more antimicrobial activity than the water extract, and this was also reported by other studies (Al-Fatimi *et al.,* 2007; Aliero *et al.,* 2006). The active antimicrobial compounds found in this study need further investigation by purified extracts in order to identify the active compounds associated with antimicrobial activity observed on the three extracts.

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Chapter 5

IN VITRO ANTIBACTERIAL SCREENING OF *PAPPEA CAPENSIS* EXTRACTS USING THE MIDDLEBROTH AND THE *p*-IODONITROTETRAZOLIUM CHLORIDE (INT) ASSAY

5.1. Background

In chapter 4 antimicrobial activity was determined using only two microorganisms namely *Staphyloccous aureus and Klebsiella pneumoniae,* which are Gram positive and Gram negative, respectively. In a scientific world reproducibility across the different methods qualifies the legitimacy of the scientific hypothesis raised. So, increasing the microbial population and comparing reproducibility of the results obtained in chapter 4 will give a good sense for a universal conclusion based on the overall antimicrobial activity of the three *Pappea capensis* extract used in this study. It's also important to check the extracts reactions to the human normal flora organisms.

There are numerous methods for the screening of biological extracts for potential antimicrobial activity (Eloff., 1998 (a); Balajee *et al.,* 2002; Scorzoni *et al.,* 2007). The microbroth dilution susceptibility method in a 96-well microtiter plate (see annexure 1 and chapter 4) has become the preferred method for drug susceptibility testing due to its small sample requirements, cost effectiveness and high-throughput rate (Pauli *et al*., 2005). The *p*-Iodonitrotetrazolium chloride (INT) assay determines the minimum inhibitory concentration (MIC) of biological extracts using INT dye (Eloff, 1998(b)). This dye acts as an electron acceptor and is reduced by viable bacteria to produce a coloured product (Eloff, 1998(b)). The yellow tetrazolium dye is reduced by viable microorganisms to a pink/purple colour. The MIC, for the INT assay, is defined as the lowest concentration that exhibits complete bacterial growth inhibition and prevents the dye from changing colour (Eloff, 1998(b)).

5.2. Materials and Methods

5.2.1 Plant material

The plant material (*Pappea capensis*) was authenticated by Dr Zietzmann Bloemfontein museum and scientists at the National Botanical Gardens in Pretoria, South Africa. The collected bark and wood materials were dried at room temperature and pulverised by a mechanical mill and weighed. It was then stored at room temperature until analysis.

Three 360g wood samples were weighed out for extraction with 1080ml acetone, water or methanol respectively. Volumes were adapted according to the consistency of the sample. Then the rest of the solvent was added, and solutions allowed to seep out for 24 hours.

Filtering was performed after 24 hours, then the solids were removed from each solution by filtration with a Millipore funnel with medium filter paper (Bright sign nr102) connected to a Millipore vacuum pump. Where needed, samples were centrifuged in 50ml conical tubes.

Removing Solvents: Most extracts contained both aqueous and organic solvents and we employed both Freeze-drying steps with a Virtis Freeze drier to remove aqueous solvent as well as a Rotary evaporate (55°C) to remove organic solvents. After repeated steps of both freeze-drying and vacuum evaporation the samples were moved to pre-weighed containers and the yield determined.

5.2.2 Microorganisms, growth conditions and media

The selection criteria of the microorganisms was based on the fact that they are part of the human normal flora. *Staphylococcus epidermidis* resides on the human skin (Fey and Olson., 2010), *Enterococcus faecalis* resides in the gastro intestinal tract (Ryan and Ray., eds. 2004), *Escherichia coli* is a normal flora of the lower gastrointestinal tract (Tenaillon *et al.,*2010) and *Candida albicans* normally found in the digestive tract system (Erdogan and Rao., 2015). The four microorganisms namely *Enterococcus faecalis* (ATCC 29212), *Staphylococcus epidermidis* (ATCC 1228), *Escherichia coli* (ATCC 25922) and *Candida albicans* (clinical strains) were grown in Mueller-Hinton (MH) broth (Merck, USA) for bacterial strains and Malt Extract broth was used for *C. albicans*. One microorganism colony, from an overnight streak plate, was inoculated in the MH broth/Malt Extract broth (10 mL) and allowed to grow for 16 h (log growth phase) at 37 °C in an orbital shaker (150 rpm).

Control drugs

Two antibiotics gentamicin sulfate and vancomycin hydrochloride (Sigma, USA) were used as positive controls against Gram-negative and Gram-positive bacteria, respectively. Furthermore, nystatin was used as positive controls against *C. albicans.* Antibiotics were dissolved in MH broth at stock concentrations of 2 mg/mL and filter sterilized (0.2 µM filter). Working concentrations of the antibiotics were prepared in MH broth, depending on the antibiotics' MIC values.

Plant extracts were dissolved in DMSO at stock concentrations of 100 mg/mL. Working concentrations of 4 mg/mL were prepared in MH broth/Malt Extract broth, and filter-sterilized (0.2 µM filter).

Microbroth dilution method

MH broth/Malt Extract broth (50 µL) was added to all test wells (i.e. plant extracts and antibiotics), except for the highest plant extract and antibiotic concentration wells to which 100 µL of the working concentrations were added. Serial dilutions were prepared for the plant extracts (2 mg/mL to 125 µg/mL) and antibiotics (64 to 0.25 µg/mL). The cultures were assessed and adjusted to a 0.5 McFarland standard (absorbance at 600 nm = 0.08-0.1; equivalent to ~$1.5x10^8$ cells/mL) and 50 µL added to each test well. The following controls were prepared: (i) antibiotic/medium control (50 µL MH broth + 50 µL of highest antibiotic concentration); (ii) plant extract colour control (50 µL MH broth + 50 µL of highest plant extract concentration); (iii) 4% DMSO control (50 µL MH broth + 50 µL 8% DMSO); and (iv) microorganism control (50 µL MH broth + 50 µL microorganism). Plates were sealed with microplate sealing tape and incubated at 37°C for 24 h.

p-Iodonitrotetrazolium chloride (INT) assay. After the incubation period, the absorbance was read at 600 nm. 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT, Sigma) was prepared at a working concentration of 0.2 mg/mL in ddH_20 and filtered (0.2 µM filter). INT (20 µL) was added to each well, and the plates were further incubated

for 60 min at 37 °C until there was a colour change. The absorbance was read at 600 nm. Viable cells reduce the yellow dye to a pink/purple colour, whereas no colour change indicated inhibition of bacterial growth.

5.2.3. Plate Layout (Annexure 3)

1	2	3	4	5	6	7	8	9	10	11	12
Extract 6	Extract 6	Extract 6	Extract 6	Extract 6	Extract 6	Extract 6	Extract 6	Growth	DMSO	Extract	Medium
Extract 7	Extract 7	Extract 7	Extract 7	Extract 7	Extract 7	Extract 7	Extract 7	Growth	DMSO	Extract	Medium

1	2	3	4	5	6	7	8	9	10	11	12
Extract 8	Extract 8	Extract 8	Extract 8	Extract 8	Extract 8	Extract 8	Extract 8	Growth	DMSO	Extract	Medium
Antibiotic	Antibiotic	Antibiotic	Antibiotic	Antibiotic	Antibiotic	Antibiotic	Antibiotic	Growth	DMSO	Extract	Medium

Antibiotic concentration depending on bacteria/yeast:

Bacteria: 0.064 - 0.005 mg/mL

C. albicans: 2 - 0.016 mg/mL

Controls:

Different controls were diluted as follows on the ration of 1:1

- Extract controls = 50 uL medium and 50 uL 4 mg/mL plant; no microorganism (colour control)
- DMSO control = 50 uL 8% DMSO and 50 uL microorganism
- Microbe control = 50 uL medium and 50 uL microorganism
- AB control = 50 uL medium and 50 uL highest [antibiotic]

5.3. Analysis of the results (see annexure 3)

The optical density was measured at 600nm and the results were captured and calculated on Microsoft Excel program.

OD600 nm readings*:*

Absorbance readings of 96-well plates were measured at a wavelength of 600 nm before and after INT addition. OD600 nm readings are summarized in the attached Microsoft Excel file (annexure 3). Percentage inhibition for the plant extracts was calculated for some extracts showed inhibition of growth, however not for those that produced slight pink colouration.

Calculation used to determine percentage inhibition:

(1-(Abs at 600 nm of test extract (absorbance corrected by subtracting the absorbance produced by the extract)/ Abs at 600 nm microbe growth control))*100 (*Tuner et al.,* 2011)

Photos of INT. Photos of the INT treated 96-well plates are included in (annexure 1 and annexure 2 is the Microsoft Excel file).

5.4 Results

Below is an extensive summary of the results obtained per organism on the different plant extracts and compared to the antibiotic controls. Table 5.1 shows the susceptibility results of *Staphylococcus epidermidis* exposed to 2mg/ml of the different plant extract. The control (vancomycin hydrochloride) showed growth inhibition of *Staphylococcus epidermidis* at very low concentration (0.002mg/ml)

S. epidermidis screening:

Table 5.1: MIC values of extracts/antibiotics

	Colour change observation Pink- growth	MIC (mg/mL)
Extract 6	Pink in all wells	No inhibition
Extract 7	Pink in all wells	No inhibition
Extract 8	Lane 1 no colour change	2 mg/mL % inhibition: 93%
Vancomycin hydrochloride	Lane 6 no colour change	0.002 mg/mL

In table 5.2 below, the *E. faecalis* is found to be resistant to all extracts except the methanolic extract which indicated 66% bacterial death at 2mg/m. Two extracts, ethanoic and water, observed colour change in their presence that was slightly pink and which indicated that *E. facilis* was growing even though at low rate. Vancomycin hydrochloride (control) MIC (0.004mg/ml) in this case was not low as in the *Staphylococcus epidermidis.* It can also be seen in the Excel sheet (annexure 2(B)) with the plate containing Extract 8 and Vancomycin that there was a big difference between lane 9 and 10. So, lane 10 containing DMSO was used as growth control as the values looked similar to the plate with extract 6 and 7.

***Enterococcus faecalis* screening**

Table 5.2. MIC values of extract/antibiotics used.

	Colour change observation Pink- growth	MIC (mg/mL)
Extract 6	Pink in all wells (very light)	No inhibition
Extract 7	Pink in all wells (very light)	No inhibition
Extract 8	Lane 1 no colour change	2 mg/mL % inhibition: 66%
Vancomycin hydrochloride	Lane 5 no colour change	0.004 mg/mL

***Escherichia coli* screening**

Table 5.3. MIC values of extracts/antibiotics used.

	Colour change observation Pink- growth	MIC (mg/mL)
Extract 6	Pink in all wells	No inhibition
Extract 7	Pink in all wells	No inhibition
Extract 8	Pink in all wells	No inhibition
Gentamicin	1. Lane 5 no colour change 2. Lane 6 very light pink	1. 0.004 mg/mL (100%) 2. 0.002 mg/mL (93%)

.

Table 5.3 above clearly summarize the results of the three extracts indicating that all wells developed pink as an indication that *E. coli* survived in their presence. Gentamicin inhibited growth of *E. coli* by 93% with 0.002mg/ml and by 100% with 0.004mg/ml of the plants.

All plates were read after 60 min incubation but upon inspection of the *Candida albicans* plate all wells contacting plant extract and the control (Nystatin) didn't have colour change. This indicated that all were able to inhibit growth for that time and the plate was further incubated overnight and all wells turned pink. This simply suggests that *Candida albicans* survived after 60 min, and even the control needed more than 2mg/ml to inhibit its survival.

***Candida albicans* screening**

Table 5.4. MIC values of extracts/antibiotics used.

	Colour change observation Pink- growth	MIC (mg/mL)
Extract 6	Pink in all wells	No inhibition
Extract 7	Pink in all wells	No inhibition
Extract 8	Pink in all wells	No inhibition
Nystatin	Pink in all wells	> 2 mg/mL

Table 5.5 MIC values of antibiotics used.

Microorganism	Positive control	MIC (µg/mL)
Staphylococcus epidermidis	Vancomycin hydrochloride	2
Enterococcus faecalis	Vancomycin hydrochloride	4
Escherichia coli	Gentamicin sulfate	4 (complete clear well)
Candida albicans	Nystatin	> 2 000

5.5 Discussion

In this study the *p*-Iodonitrotetrazolium chloride (INT) assay was used both with middlebroth method to determines the minimum inhibitory concentration (MIC) of biological extracts. The determination of the MICs is considered the gold standard for defining the susceptibility of bacteria to antimicrobials. The MICs are used in diagnostic laboratories to confirm the susceptibility patterns of the different isolates. The *Pappea capensis* extracts (acetonoic and water) were found to possess no antimicrobial activity against the Gram-negative bacteria (*Escherichia coli*), Gram-positive bacteria (*Staphylococcus epidermidis* and *Enterococcus faecalis)* and a medical yeast *Candida albicans* (tab 5.1-5.4). Methanolic extract no 8 showed antimicrobial activity against *Staphylococcus epidermidis* (93% death) and *Enterococcus faecalis* (66% death) at MIC value of 2 mg/mL. However, methanolic extract showed no antimicrobial activity against

Candida albicans. All *Pappea capensis* methanolic extract showed antimicrobial activity at a concentration relatively high compared to gentamycin and vancomycin hydrochloride controls antibiotics (table 5.5). The controls had lower MICs compared to all extracts

5.6 Conclusion

The antimicrobial results obtained from both chapters (4 and 5) clearly indicate that *Pappea capensis* possesses antimicrobial activities which are purely dependent on the type of the solvent and the organism tested. *Staphylococcus aureus* and *Klebsiella pneumoniae* were susceptible to ethanoic and methanolic extracts in chapter 4. Then, in chapter 5 methanolic extract only showed activity against two organisms known as part of the normal flora (*Staphylococcus epidermidis* and *Enterococcus faecalis)* which are both gram positives. It is worth mentioning that in chapter 4 water extract showed antimicrobial activity against the Gram-negative organisms (*Klebsiella pneumoniae*). Sangomas' *Pappea capensis* water extract used is targeting *Klebsiella pneumoniae* as one of the organisms and all Gram-positive organisms tested where resistant towards it. Future analysis should include susceptibility testing on more organisms and further identification of compounds associated with the antimicrobial activity in these extracts.

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Chapter 6

IN VITRO CYTOTOXICITY SCREENING OF *PAPPEA CAPENSIS* EXTRACTS

6.1 Introduction

Cancer continues to deteriorate the lives of both males and females globally. This calls for the need for the discovery of new anticancer drugs with less side effects (WHO 2019). The scientific interest in the use of natural products as potential anti-cancer agents can be dated to 1550 BC during the Ebers papyrus time (Breasted, 1930). Nevertheless, during the early nineteen-sixtys scientific interest focused more on the application of podophyllotoxin and its derivatives as possible anti-cancer agents (Hartwell, 1967). During the late 20th century and beginning of the 21st century has been remarkable and significant scientific and commercial interest in the discovery of new drugs from medicinal plants (Kinghorn *et al*., 2003, Poudyali and Singh, 2019). Natural products play a vital role in the search for discovery of new drugs, with more than 60% of those approved derived from natural plants (Cragg *et al.,* 1997). More than 49% of the tested drugs in the clinical trials as potential anti-cancer agents are derived from natural products or are purely natural products (Newman and Cragg, 2012). The scientific literature supports the rationale behind scientific research done using medicinal plants for the discovery of novel drugs (Jain and Jain 2011).

In this study the three *Papea capensis* plant extracts were used to evaluate cytotoxicity against three cell lines. Cytotoxicity is defined as the ability of an agent to produce a toxic effect on a cell. Cytotoxicity assays was used to test the ability of cells to continue proliferating in the presence of a test compound or substance over a specific time period. Cytotoxicity testing is a popular method used for the screening of pharmaceutical

products and synthetic organic compounds. This is an important initial step when investigating possible new therapies or developing new compounds for the treatment of an ailment, which is the determination of their cytotoxic potential and the determination of their harmful effects.

6.2 Materials and methods

6.2.1. Plant material

The plant material (*Pappea capensis*) was authenticated by Dr Zietsmann Bloemfontein museum and scientists at the National Botanical Gardens in Pretoria, South Africa. The collected bark and wood materials were dried at room temperature and pulverised by a mechanical mill and weighed. It was then stored at room temperature until analysis.

Three 360g wood samples were weighed out for extraction with 1080ml acetone, water or methanol respectively. Volumes were adapted according to the consistency of the sample. Then the rest of the solvent was added, and solutions allowed to seep out for 24 hours.

Filtering was performed after 24 hours, then the solids were removed from each solution by filtration with a Millipore funnel with medium filter paper (Bright sign nr102) connected to a Millipore vacuum pump. Where needed, samples were centrifuged in 50ml conical tubes. Removing Solvents: Most extracts contained both aqueous and organic solvents and we employed both Freeze-drying steps with a Virtis Freeze drier to remove aqueous solvent as well as a Rotary evaporate (55°C) to remove organic solvents. After repeated

steps of both freeze-drying and vacuum evaporation the samples were moved to pre-weighed containers and the yield determined.

6.3. Cytotoxicity evaluation of three plant extracts against three different cell lines:

6.3.1 Sample preparation

Test compounds were reconstituted in dimethyl sulphoxide (DMSO) to give a final concentration of 100 mg/mL. Samples were sonicated if solubility was a problem. Samples were stored at 4°C until required.

6.3.2 *In vitro* anti-cancer screening

The human prostate cancer cell line, PC3; human breast cancer cell line, MCF7 and the African green monkey kidney cell line and Vero were used for cytotoxicity screening. Cells were seeded into 96 well microtiter plates at a density of 4000 cells/well using a volume of 100 µl in each well. The microtiter plates were incubated at 37°C, 5% CO_2, and 100% relative humidity for 24 hours prior to the addition of test compounds to allow for cell attachment. Melphalan 20uM and 40uM were used as positive control drugs.

Cells were treated with the 12.5 µg/mL - 250 µg/mL of each extract. One hundred microliters aliquots of the diluted compound in fresh medium was used to treat cells. Cell lines were incubated at 37°C in a humidified 5% CO_2 for 48 hours.

Treatment medium was aspirated from all wells and replaced with 100 µL of Hoechst 33342 nuclear dye (5 µg/mL) and incubated for 10 minutes at room temperature.

Thereafter, cells were stained with propidium iodide (PI) at 100 µg/mL in order to enumerate the proportion of dead cells within the population. Cells were imaged immediately after addition of PI using the ImageXpress Micro XLS Widefield Microscope (Molecular Devices).

6.4 Data Quantification

Quantification of live and dead cells for the screening assay was performed using the ImageXpress Micro XLS Widefield Microscope (Molecular Devices) and acquired images analysed using the MetaXpress software and Multi-Wavelength Cell Scoring Application Module. Acquired data was transferred to an EXCEL spreadsheet and data was analysed and processed.

6.5 Results

Cytotoxic evaluation of plant extracts 6, 7 and 8

All extracts were screened for cytotoxicity against all three cell lines. Figures 6.1 –6.3 indicate the results of the dual staining cytotoxicity assay.

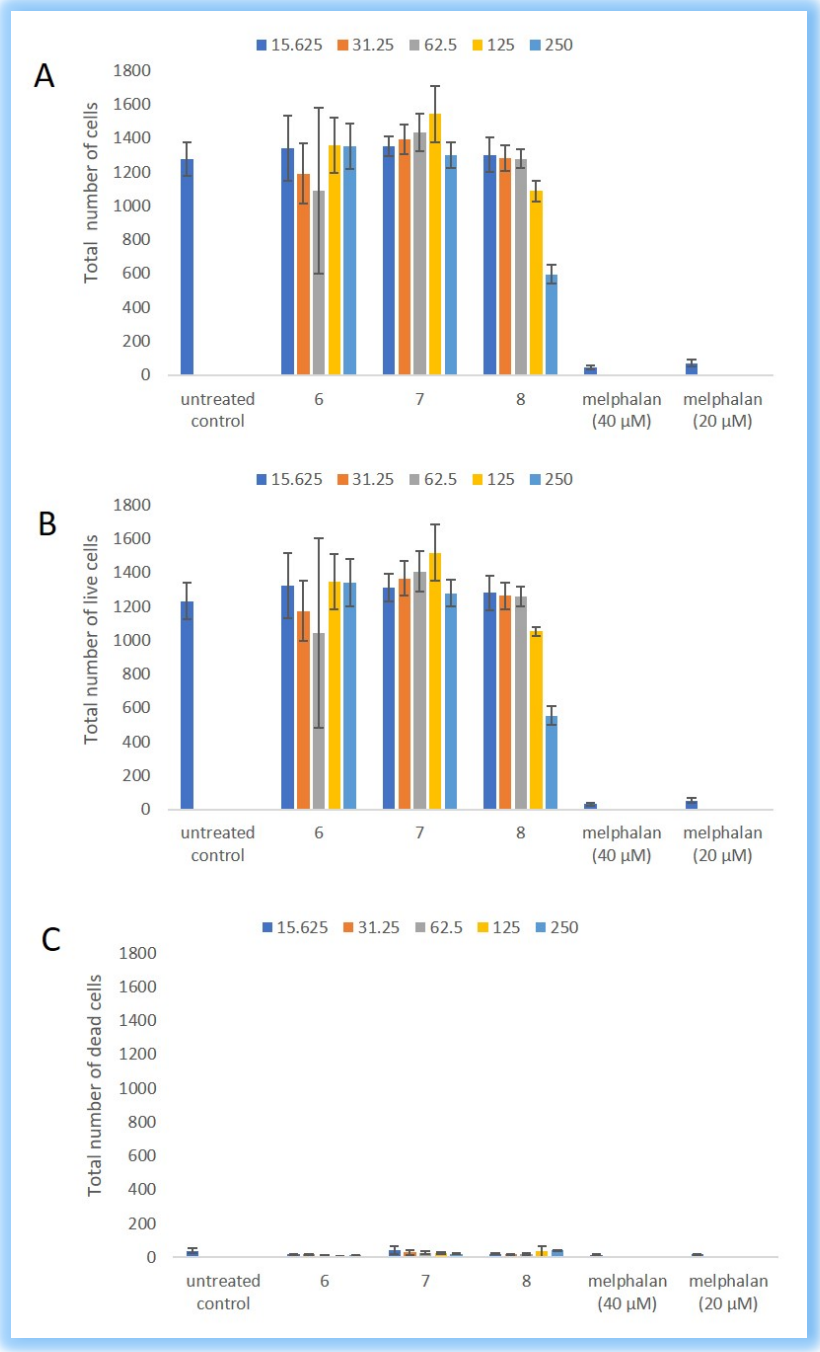

Figure 6.1: Cytotoxic response of Vero cells to treatments (at varying concentrations in µg/mL) with plant extracts 6,7 and 8 as well as melphalan (positive control). A; total cell number, B; total number of live cells, C; total number of dead cells. Error bars indicate standard deviation of quadruplicate values done as a single experiment.

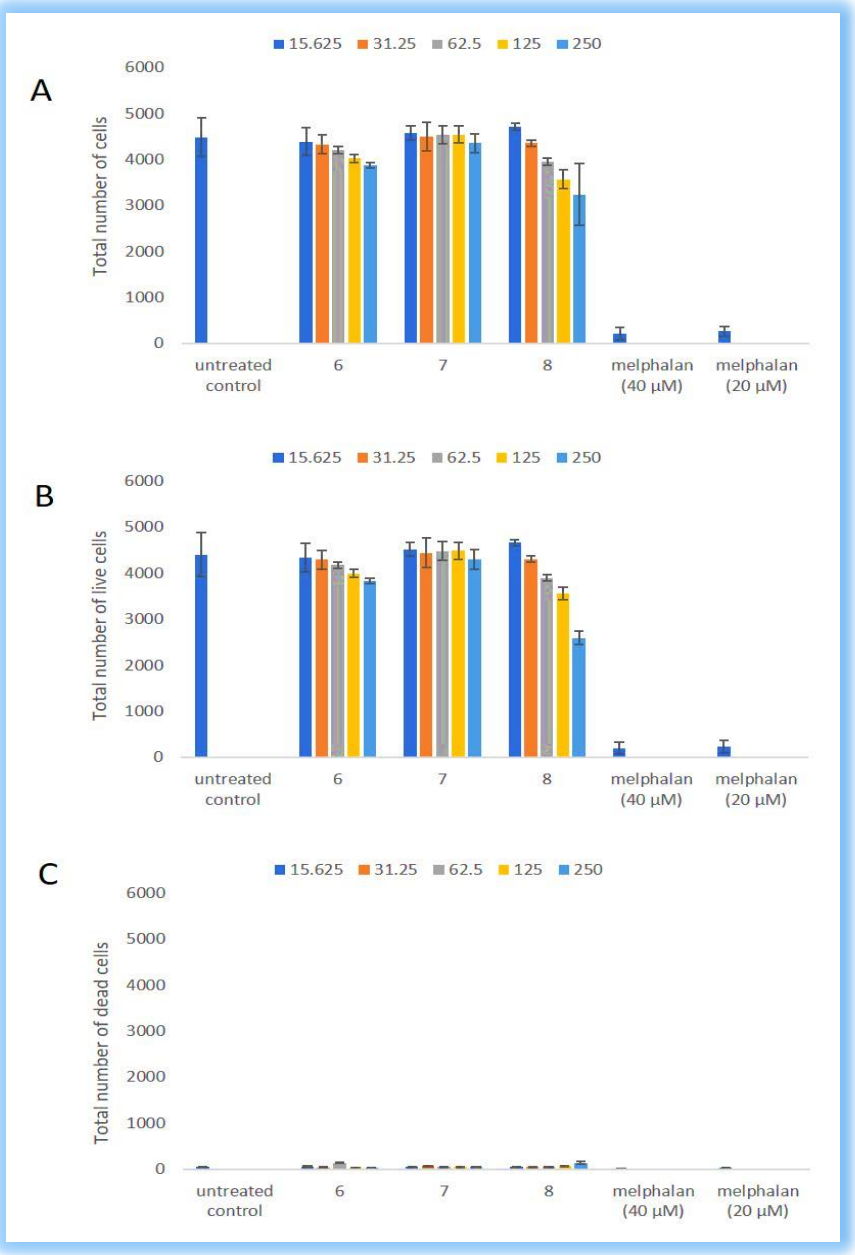

Figure 6.2: Cytotoxic response of MCF7 cells to treatments (at varying concentrations in µg/mL) with plant extracts 6,7 and 8 as well as melphalan (positive control). A; total cell number, B; total number of live cells, C; total number of dead cells. Error bars indicate standard deviation of quadruplicate values done as a single experiment.

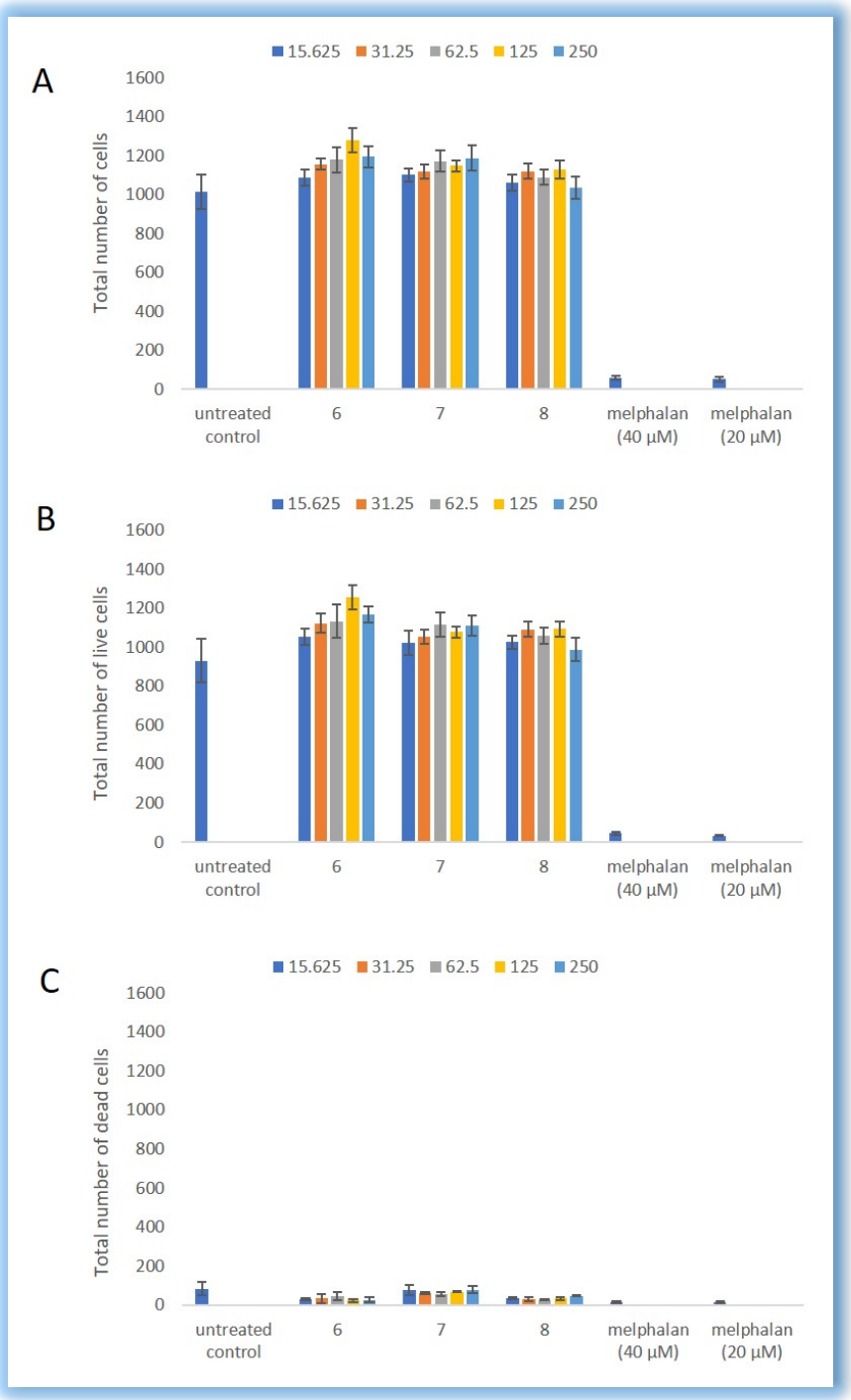

Figure 6.3: Cytotoxic response of PC3 cells to treatments (at varying concentrations in µg/mL) with plant extracts 6,7 and 8 as well as melphalan (positive control). A; total cell number, B; total number of live cells, C; total number of dead cells. Error bars indicate standard deviation of quadruplicate values done as a single experiment.

6.6 Discussion:

Cytotoxicity was determined using the dual staining procedure with a nuclear dye, Hoechst 33342 and propidium iodide (PI). All live cells stained positive with Hoechst 33342 but only dead/dying cells stained positive with PI. Figures 6.1, 6.2 and 6.3 show the results of the cytotoxicity screening against Vero, MCF7 and PC3 cells, respectively. The known chemotherapeutic drug melphalan was used as the positive control for all cell lines. Both the ethanoic (extract 6) and aqueous (extract 7) showed no cytotoxic activity against any of the three cell lines. Methanolic extract 8 showed slight cytotoxicity at a high concentration of 250 µg/mL against Vero and MCF7 cells. This concentration of 250 µg/mL is considered physiologically irrelevant and for this reason, all extracts are considered non-cytotoxic (Gertsch, 2009). Melphalan proved to be an appropriate positive control. Although Panel C in Figures 6.1, 6.2 and 6.3 show a small number of dead cells for melphalan treatment, this corresponds to the very low number of total live cells (Panel B) for all cell lines.

6.7 Conclusion

Through comparing the used control and the only extract showing slight toxicity, it's conclusive to say a high dose of methanolic extract is physiologically inappropriate. All extracts were not active (cytotoxic) enough to suggest *Pappea capensis* can be taken into further anticancer testing. Nonetheless, it's scientifically important to mention that isolation and identification of bioactive compounds on their pure state may indicate otherwise. Further testing using GC-MS may identify volatile compounds present, and their individual biological effect can be determined.

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Chapter 7

IN VITRO GENOTOXICITY SCREENING OF *PAPPEA CAPENSIS* EXTRACTS USING VERO CELLS

7.1 Introduction

Toxic substances which directly interfere with cell viability and produce genetic knockout by damaging the genome and cause formation of mutation are referred to as genotoxic (Mansoori and Gautam., 2014). Genotoxicity refers to the destructive effect that a compound or extract has on the nuclear material of the cell and causes mutations in the cell (Kumari and Singh., 2017). The molecular genotoxic assay such as end-point assays have been used to study and detect DNA damage, can also be employed as indicators for primary damage or analyse the genotoxicity of new compounds (Mortemals and Zeiger., 2000). Micronucleus test, chromosomal test and many other tests are used for considering antimutagenic action of any drug (Radhika and Jyothi., 2019). With regards to hereditary qualities, genotoxicity represents the property of a mixture of operators that damage the hereditary data (Gonzales-Borroto *et al.,* 2013). In attempt to survive the cell prevent enunciation of the genotoxic transformation by either DNA repair mechanisms or programmed cell death (apoptosis) as a result, the harm may not generally settle, prompting mutagenesis (Gonzales-Borroto *et al.,* 2013).

The purpose of genotoxicity test is to decide whether a substrate will impact genetic material or may cause growth (Madle *et al.,*2012). Genotoxicity tests can be characterized as *in-vivo* and *in-vitro* test intended to recognize compounds which incite genetic damage directly or indirectly by the different mechanisms (Madle *et al.,*2012). A feature of a genotoxic compound is the resulting formation of micronuclei after treatment of cells with the genotoxin (Luzhna, *et al.,* 2013). Micronuclei are extra-nuclear bodies that contain

damaged chromosome fragments and/or whole chromosomes that have not been incorporated into the nucleus after cell division. The formation of micronuclei can lead to cell death, genotoxic instability and cancer development (Luzhna, *et al.,* 2013). Previous studies also indicated that different plants and different extraction methods may present different patterns when coming to micronuclei determination (Kahaliw, *et al.,* 2018; Etebari *et al.,* 2012, Radhika and Jyothi., 2019).

7.2 Method and Methods

7.2.1 Plant material

The plant material (*Pappea capensis*) was authenticated by Dr Zietsmann Bloemfontein museum and scientists at the National Botanical Gardens in Pretoria, South Africa. The collected bark and wood materials were dried at room temperature and pulverised by a mechanical mill and weighed. It was then stored at room temperature until analysis.

Three 360g wood samples were weighed out for extraction with 1080ml acetone, water or methanol, respectively. Volumes were adapted according to the consistency of the sample. Then the rest of the solvent was added, and solutions allowed to seep out for 24hours.

Filtering was performed after 24 hours, then the solids were removed from each solution by filtration with a Millipore funnel with medium filter paper (Bright sign nr102) connected to a Millipore vacuum pump. Where needed, samples were centrifuged in 50ml conical tubes.

Removing Solvents: Most extracts contained both aqueous and organic solvents and we employed both Freeze-drying steps with a Virtis Freeze drier to remove aqueous solvent as well as a Rotary evaporate (55°C) to remove organic solvents. After repeated steps of both freeze-drying and vacuum evaporation the samples were moved to pre-weighed containers and the yield determined.

7.3 Genotoxicity evaluation of three plant extracts against Vero cells using micronucleus assay

7.3.1 Sample preparation

The NucRed Live 647 Probe (ThermoFischer Scientific) which is a far red, cell-permeant nuclear stain for live and dead cells was used in the study to determine the genotoxicity of three *Pappea capensis* extracts. The assay was used to determine any existence or the formation of micronuclei.

The Vero cells (African green monkey kidney cells) were seeded at 4000 cells/well and left overnight to attach. Then the cells were treated with the different concentrations of each extract (200, 100, 50, 25 and 12.5) µg/mL. The cells were then treated for 48 hours (incubated at 37˚C). The medium and treatments were aspirated and stained with NucRed as follows: The Prepare NucRed working solution was prepared by adding 2 drops NucRed per mL PBS (+Ca +Mg), then the aspirate medium and treatments. This was followed by adding 100 µL NucRed working solution to each well. Then the plate was incubated for 15 – 30 minutes at 37 °C. Then after that the plates were acquired using the ImageXpress XLS microscope

7.4 Results:

The formation of micronuclei was detected using NucRed nuclear stain and the ImageXpress microscope. Results are shown in figure 1 which clearly indicates the results of this assay shown in bars depicting the number of micronuclei formed in the presence of each extract. It is clear from this result that ethanoic extract (no6) induced the formation of micronuclei while the other two extract shows less formation of micronuclei.

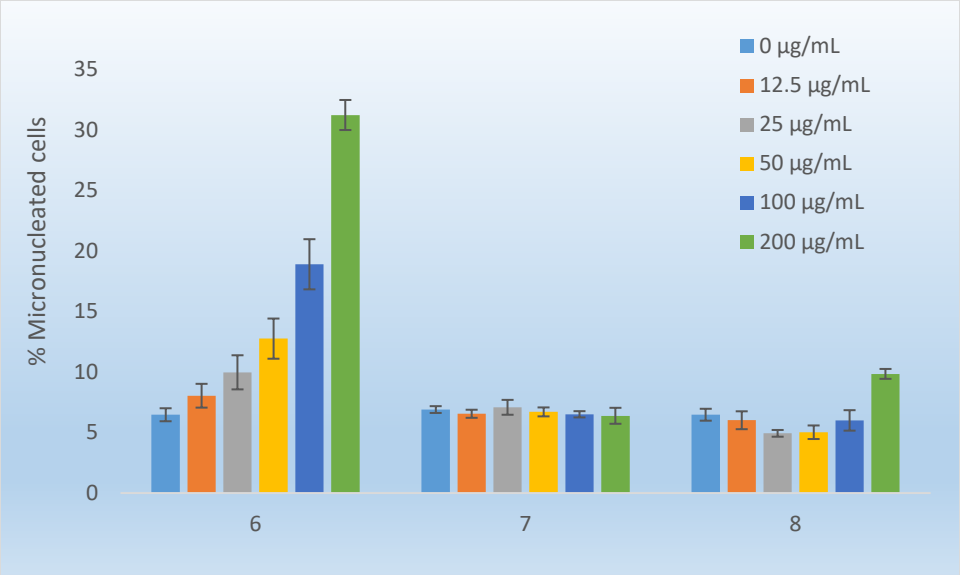

Figure 7.1: Formation of micronuclei in response to treatment of Vero cells with varying concentration (as indicated) of extract. Error bars indicate standard deviation of quadruplicate values.

7.4 Discussion

The NucRed Live 647 probe assay was used determine formation of micronuclei in Vero cells responding to treatment with varying concentrations of the three *Pappea capensis*

extracts. The ethanoic extract no6 could be considered genotoxic as it was found to have induced micronucleated cells by 30%. In the previous studies the aqueous and hydro-alcoholic extracts of *Ecium amoenum* showed to have induced micronuclei (Etebari *et al.,* 2012), and chloroform root extract of *P. stellatum* correlated to the micronuclei response of the Vero cells exposed to ethanoic extract of *Pappea capensis* (Kahaliw, *et al.,* 2018). This ethanoic extract of *Pappea capensis* could be considered genotoxic, however other two couldn't induce micronucleated cells except for methanolic extract that shows partially micronucleated cell activity of 10% at 200ug/ml

7.5 Conclusion

Genotoxins are agents that can possibly interact with the DNA, thus damage its structure and cause mutation which may lead to cancer. Two extracts were considered non-genotoxic and the ethanoic extract could be considered genotoxic. It must also be notified that the micronucleated cells of 30% were determined at the high concentration (200ug/ml). Further tests should be done to predict what mechanism is affected by compounds in the ethanoic extract. The results indicate the potential use of this plant against cancer, despite the moderate genotoxicity at 200ug/ml. This further supports the need for the identification of bioactive compounds using GC-MS chromatography as the *Pappea capensis* extracts show less to no genotoxicity.

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Chapter 8

IN VITRO PRO- AND ANTI-INFLAMMATORY SCREENING OF *PAPPEA CAPENSIS EXTRACTS*

8.1 Introduction

Inflammation is known as the first line of defence against pathogenic effects. It can however contribute to all phases of tumorigenesis, including tumour initiation, promotion and metastasis (Maiorov *et al.,* 2013). Although inflammation is usually associated with a protective or healing response, many chronic diseases are characterised by persistent inflammation, ultimately resulting in tissue dysfunction (Nambiar *et al*., 2011; Courssens and Werb, 2002). Inflammatory cells can moreover secrete reactive oxygen species (ROS) that encourage mutations, lead to the failure to the DNA repair mechanisms, activation of oncogenes and eventually cancer (Chang, 2010). It's of importance to understand that the cell will experience augmented genomic break, increased DNA syntheses due to lose ends, cellular proliferation, pauses in DNA repair as mentioned earlier, inhibition of apoptosis and all that will lead to promotion of angiogenesis which are normally linked with inflammation (Hofseth and Ying, 2006). Throughout chronic inflammation, pro-inflammatory molecules such as cytokines, ROS, and NFkB were secreted creating an environment that will favour exponential development of malignant cells (Sarkar and Fischer, 2006). For this reason, the anti- or pro-inflammatory activity of test samples need to be considered within the context of the disease in cancer as well as the disease progression stage at which intervention was considered, in order to accurately evaluate the potential therapeutic significance. Furthermore, multiple mechanisms may collectively contribute to an inflammatory response; consequently, it is necessary to take into account that a single target specific in vitro model does not assess the total domain of potential therapeutic activity.

Macrophages perform a variety of complex microbicidal functions, including surveillance, chemotaxis, phagocytosis and destruction of pathogenic micro-organisms. Therefore, the development of therapeutics which can non-specifically augment the innate immune response represents a promising strategy to combat classical and emerging infectious agents (Hofseth and Ying., 2006). Furthermore, other diseases such as HIV and ageing which are characterised by a weakened immune response, as well as diseases which evade the classical immune system (cancer) may be targeted through immune modulation (Romay *et al.,*1998; Sidhu *et al.,* 1999; Hebbar *et al.,* 2002).

Macrophages represent a highly heterogeneous group of hematopoietic cells present in almost all tissues, including adipose tissue (Gordon, 1998; Murthuza and Manjunatha, 2018). Depending on the trigger, macrophage responses can be divided into two distinct and mutually exclusive activation programs termed classical and alternative (Gordon, 1998). Classical activation results in a highly inflammatory phenotype and mainly occurs in response to bacterial products such as LPS and IF-γ (Gordon, 1998). These classically activated macrophages produce a myriad of pro-inflammatory signals which can alter the functionality of its surrounding cells (Hayden and Ghosh, 2011). In addition, these activated cells produce various highly reactive oxidants including nitric oxide (NO), a product of the catalysis of arginine by the enzyme inducible nitric oxide synthase (iNOS) (Kiemer and Vollmar, 2001; Murthuza and Manjunatha, 2018).

The mouse macrophage cell line, RAW 264.7, is a well characterised and popular model to investigate the anti-inflammatory potential of test samples (Kiemer and Vollmar, 2001; Murthuza and Manjunatha, 2018). Cells are cultured in multi-well plates and activated by

exposure to LPS which induces the expression of iNOS with concomitant nitric oxide formation (Kiemer and Vollmar, 2001; Sharma *et al.,* 2007; Murthuza and Manjunatha, 2018). Changes in NO production are determined by measuring the levels of nitrate in the culture medium (Kiemer and Vollmar, 2001). Simultaneous evaluation of cell viability (MTT assay) is used to confirm the absence of cytotoxicity of the test sample (Mosmann., 1983; Riss *et al.,* 2011).

8.2 Method and methods

8.2.1 Plant material

The plant material (*Pappea capensis*) was authenticated by Dr Zietsmann Bloemfontein museum and scientists at the National Botanical Gardens in Pretoria, South Africa. The collected bark and wood materials were dried at room temperature and pulverised by a mechanical mill and weighed. It was then stored at room temperature until analysis.

Three 360g wood samples were weighed out for extraction with 1080ml acetone, water or methanol respectively. Volumes were adapted according to the consistency of the sample. Then the rest of the solvent was added, and solutions allowed to seep out for 24hours.

Filtering was performed after 24 hours, then the solids were removed from each solution by filtration with a Millipore funnel with medium filter paper (Bright sign nr102) connected to a Millipore vacuum pump. Where needed, samples were centrifuged in 50ml conical tubes.

Removing Solvents: Most extracts contained both aqueous and organic solvents and we employed both Freeze-drying steps with a Virtis Freeze drier to remove aqueous solvent as well as a Rotary evaporate (55°C) to remove organic solvents. After repeated steps of both freeze-drying and vacuum evaporation the samples were moved to pre-weighed containers and the yield determined.

8.2.2 Sample preparation

Extracts were solubilised in DMSO to a final concentration of 100 000 µg/ml and further diluted into culture medium as indicated in the figures. Resveratrol at 25 uM and 50 uM was used as a positive control to indicate anti-inflammatory activity.

8.2.3 Anti-inflammatory screening protocol

RAW 264.7 cells were seeded into 96-well plates at a density of 25 000 cells per well and allowed to attach overnight. The following day spent culture medium was removed and the samples (diluted in DMEM complete medium) added to give final concentrations of 12.5 and 50 µM (50µl per well at double the desired final concentration). To assess the anti-inflammatory activity, 50ul of LPS containing medium was added to the corresponding wells. Resveratrol, a known inhibitor of iNOS expression served as a positive control. Cells were then returned to the incubator for a further 20hr. To quantify NO production, 50ul of the spent culture medium was transferred to a new 96-well plate and 50ul Griess reagent added. Absorbance was measured at 540nm and the results expressed relative to the appropriate untreated control. To confirm the absence of toxicity as a contributory factor, cell viability was assessed using MTT.

8.2.3 Pro-inflammatory (macrophage activation) screening protocol

The same method as described in section 8.2.2 was carried out for pro-inflammatory analysis, except that LPS was not added to the treatment regime.

To ensure that the effects of the plant extracts were not due to endotoxin contamination, NO production was evaluated in the presence and absence of polymyxin B (PMB) (Cooperstock, 1974).

8.3 Results

The *in vitro* screening results for anti- and pro-inflammatory assays for *Pappea capensis* are indicated in Figures 8.1 and 8. 2, respectively (refer to **annexure** 4). Fig 8.1 shows the results of NO production (A) and (B) indicates the LPS activated macrophages treated with different concentrations of *Pappea capensis* extracts and corresponding cytotoxicity. Resveratrol, a known inhibitor of iNOS expression served as a positive control and LPS was added to the regime. In Fig 8.2, macrophage activation is shown in (A), while (B) clearly shows the response to treatment with different concentrations of *Pappea capensis* extracts and their corresponding cytotoxicity. Also, LPS was added to the regime. Following the observatory of macrophage activation seen with water extract no 7, there was a need to perform a confirmatory test to ensure that the effects of the plant extracts were not due to endotoxin contamination, so NO production was evaluated in the presence and absence of polymyxin B (PMB) as shown in fig 8.3 (refer to **annexure** 4).

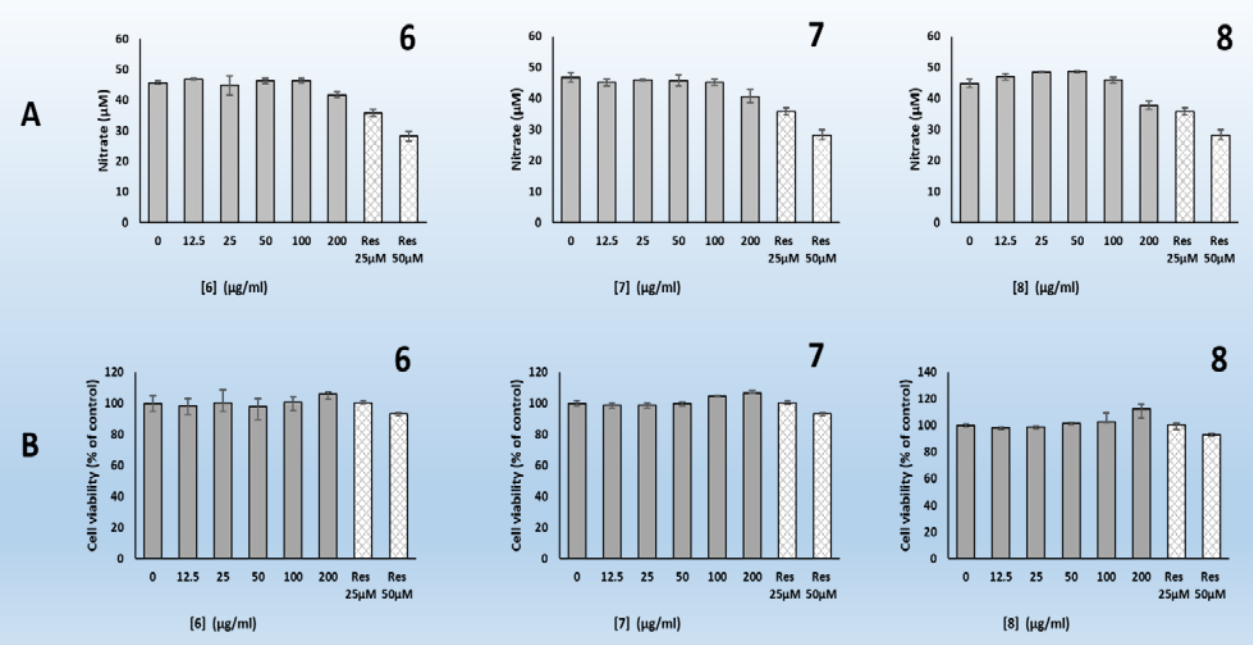

Fig. 8.1: Nitric oxide production (A) in LPS activated macrophages treated with different concentrations of extracts and corresponding cytotoxicity (B).

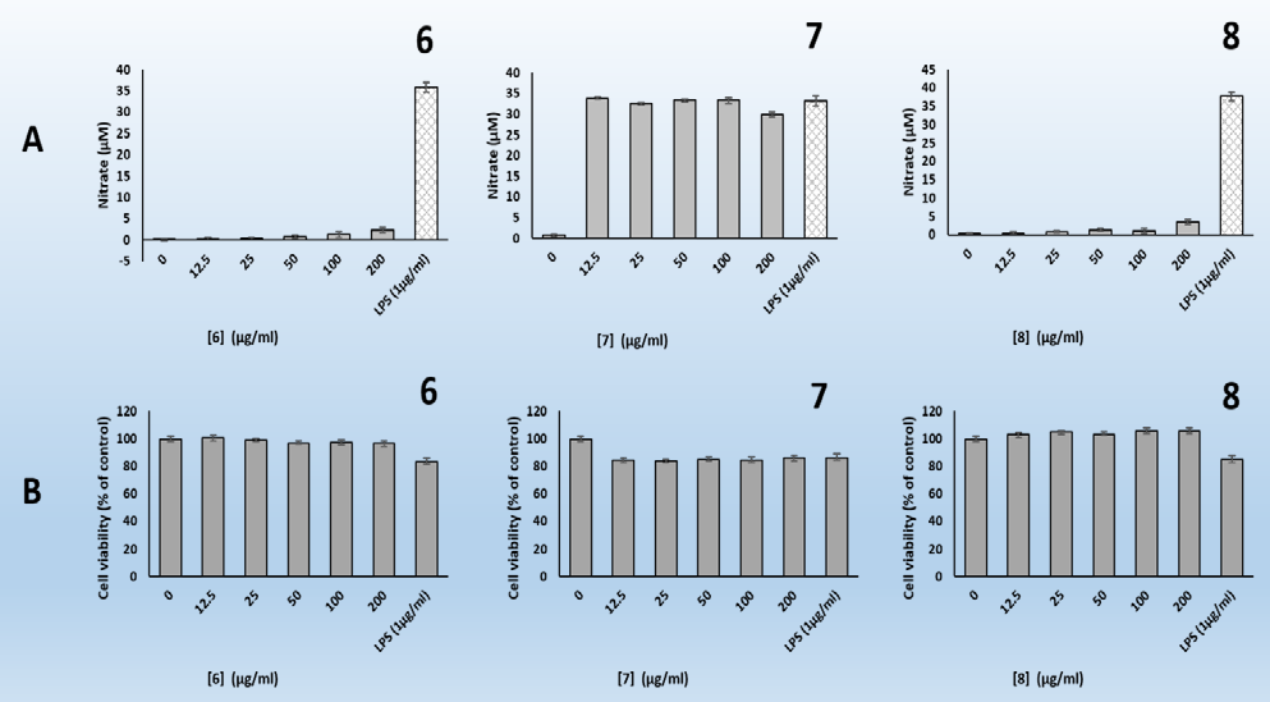

Fig 8. 2: Macrophage activation (A) in response to treatment with different concentrations of extracts 6, 7 and 8 and their corresponding cytotoxicity (B).

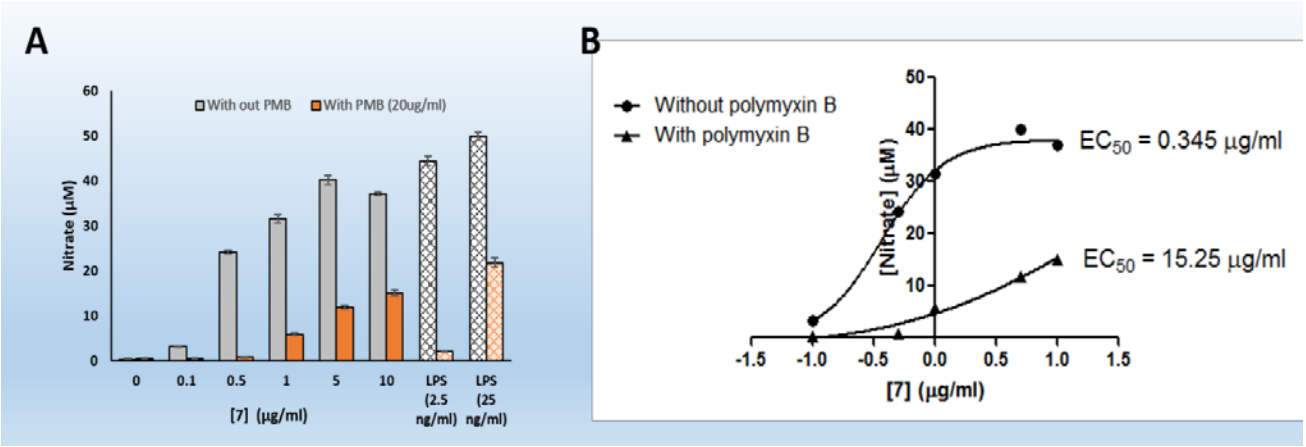

Fig.8.3: Determination of endotoxin contamination using polymyxin B (PMB).

8.4 Discussion and Conclusion

The anti-inflammatory potential of all *Pappea capensis* extracts were determined using *in vitro* model RAW 264.7. It was found that all extracts produced high NO at low concentration and slightly reduced at 200ug/ml as compared to the Res (positive control). In 2007 Sharma *et al* explained the role of NO as a signalling molecule that is considered as a pro-inflammatory mediator that induces inflammation (Sharma *et al.,* 2007). The very low anti-inflammatory activity showed at 200ug/ml by the three extracts cannot be considered physiologically active.

The ethanoic and methanolic extracts were not found to induce nitrate production during macrophage activation as shown in fig 8.2 (A). It is interesting to find that the water extract which was not found to have anti-inflammatory activities was found to have elevated fluctuating none concentration dependent NO production. Water extracts results correlates with the study of Ligacheva *et al* where they found that NO production by mouse macrophages on *Betula leaves* contributed to the signalling of molecules to macrophage activation (Ligacheva *et al.,* 2014). None of the extracts were cytotoxic against RAW 264.7 macrophages cells as indicated in fig 8.1 (B) and fig 8.2 (B).

A confirmatory test to water extract no 7 (Fig 8.2 A) was done in order to ascertain whether this pro-inflammatory activity is as a result of possible endotoxin contamination of the extract with using PMB. From figure 8.3, it is evident that PMB counteracts the pro-

inflammatory effect suggesting that this activity is possibly a result of endotoxins in the extract, either from the solvent of extraction or as part of the plant. The determination of endotoxin as a co-factor perpetrating pro-inflammatory activity correlates with the study of Cooperstock, who clearly showed that polymyxin B can be used to inactivate endotoxin produced by organisms (Cooperstock, 1974). More extensive analysis could be conducted to determine exact active compounds associated with pro-inflammatory effect because the solvent in this case used was water. The low anti-inflammatory effect seen of the three extracts at 200ug/ml are not physiologically active. Therefore, the extracts do not possess anti-inflammatory properties. Possible toxin identified in figure 8.3 (B) could be identified by compound separation using GC-MS, but water cannot be a contributing factor harbouring a toxin. The water extract appeared to be less safe for human use.

8.5 References

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Chapter 9

GC-MS ANALYSIS OF VOLATILES PRESENT IN *PAPPEA CAPENSIS* EXTRACTS

9.1 introduction

Most people have entrusted plants with having qualities of an important source of medicine for decades. Globally, people have treated and healed sick ones with herbal derived remedies, and that expanded through the generations. Traditional medicine is collective of knowledge and skills, and performs based on the theories, beliefs and original practices of diverse cultures that are used to maintain health, to diagnose, prevent and treat diseases (Home page on the internet, World Health Organization, available from url http:// www.who.int / medicines/ areas / traditional/ definitions /en/). Different kinds of traditional medicine and other medical practices known as complementary or alternative medicine are increasingly used world-wide. Previous study has shown that herbs can provide remedial actions and have historically been used as popular folk medicines (Sathyaprabha *et al.,* 2010). A good cultural practice is of the Chinese people. Wherever in the world they are, they enthusiastically participate in the import and export of their medical system (Aneesh *et al.,* 2009). It is a sad fact that currently generations are moving away from nature. And, owing to our unmanageable life styles, we experience new and re-emerging diseases. Nonetheless, the fact is that our rich nature comprises remedy for all illnesses, including cancer. Research has shown that *in vitro* screening methods could play a critical role in identifying the elect crude plant extracts with potentially valuable properties for additional chemical and pharmacological investigations (Mathekaga and Meyer*.,* 1986). Phytochemistry has grabbed research attention in recent years as a distinct discipline. Many studies done on new drugs development focused on plant biochemistry and natural product organic chemistry. It is uneasy with the massive variety

of organic substances that are expounded with and accrued by plants and deals with the biochemical structures of these substances, their biosynthesis, turn over and metabolism, their usual spreading and their genetic function (Harborne., 1986). Phytochemicals are the basic compounds extracted from plants and are classified as primary or secondary constituents, depending on their role in plant metabolism.

Primary elements include the common sugars, purines and pyrimidines of nucleic acids, amino acids, proteins, chlorophylls, etc. Secondary elements are the remaining plant compounds such as phenolics, alkaloids and terpenes (Liu, 2004). These secondary compounds have been found to provide protection to the plants, but recent research demonstrates that these compounds' isolates from the plant source also provide protection again microorganism in humans. A factual dietary role for phytochemicals is becoming more possible every day as research reveals more of their extraordinary benefits (Hamburger and Hostettmann, 1991). With the speedy developments in the scientific fields, there were a number of drastic developments in analytical techniques including: Gas Chromatography Mass Spectroscopy (GC-MS), NMR, TLC, and UV that were powerful tools for separation, identification and structural determination of phytochemicals (Roberts and Xia, 1995).

In this study, GC-MS, a hyphenated system which is a very compatible, and the most commonly used technique for the identification and quantification purpose will be used on the three *Pappea capensis* extracts. The unknown organic compounds in the wood extracts of *Pappea capensis* were determined by interpretation and by matching the spectra with reference spectra as done in the previous studies (Kirtikar and Basu, 1918).

The plant, *Pappea capensis* contained similar constituents like: alkaloids, flavonoids, triterpenoids and sterols, same as in other studies (Bhogaonkar and Devankar, 2006). This indigenous plant has been a very integral part of the life of many tribes in India as they had identified and had been using it for many past centuries. The rhizome paste is being used as a remedy for headache by the Bhilla tribe of Maharashtra (Kamble *et al.,* 2010). And, the rhizome is reported to be used for the treatment of blood dysentery by tribal rehabitants of Amarkantak plateau, Madhya Pradesh, India.

The aim of this study is to determine the organic compounds present in the *Pappea capensis wood* extracts with the aid of GC-MS Technique, which may provide an insight in its use in tradition medicine.

9.2 Gas chromatography-mass spectrometry (GC/MS)

9.2.1 Method and material

The plant material (*Pappea capensis*) was authenticated by Dr Zietsmann Bloemfontein museum and scientists at the National Botanical Gardens in Pretoria, South Africa. The collected bark and wood materials were dried at room temperature and pulverised by a mechanical mill and weighed. It was then stored at room temperature until analysis.

Three 360g wood samples were weighed out for extraction with 1080ml acetone, water or methanol respectively. Volumes were adapted according to the consistency of the

sample. Then the rest of the solvent was added, and solutions allowed to seep out for 24hours.

Filtering was performed after 24 hours, then the solids were removed from each solution by filtration with a Millipore funnel with medium filter paper (Bright sign nr102) connected to a Millipore vacuum pump. Where needed, samples were centrifuged in 50ml conical tubes.

Removing Solvents: Most extracts contained both aqueous and organic solvents and we employed both Freeze-drying steps with a Virtis Freeze drier to remove aqueous solvent as well as a Rotary evaporate (55°C) to remove organic solvents. After repeated steps of both freeze-drying and vacuum evaporation the samples were moved to pre-weighed containers and the yield determined. The methods and extractions procedures were done at Synexa Life Sciences

9.2.2 Sample procedure

One milliliter of dichloromethane was added to 500 mg of methanolic, aqueous and ethanoic wood extracts and sonicated overnight. One microliter was injected onto the GC-MS system in splitless mode. GC/MS analysis was carried out on a Shimadzu 2010 QB gas chromatograph with a MSD detector equipped with an HP-5 fused silica capillary Column (30m x 0.25 mm x 25m film thickness). The aqueous plant extract was injected *via*, an all glass injector working in split mode with Helium as the carrier gas at a flow rate of 1 mL min^{-1}. The oven temperature was programmed as follows: Injector temperature 200 ^{0}C, Ion source 200 ^{0}C and Interphase 200 ^{0}C. Column temperature was raised to

45°C (3 min hold at 45°C, 4°C min), then gradually increased to 150°C (3 min hold at 150°C, 4°C min) then raised to 250°C and a 15 min hold. A split ratio of 1:5 was used (Ajayi *et al*., 2011).

9.3 Identification of chemical constituents

Compound identification was accomplished by comparing the retention times with those of authentic compounds or the spectral data obtained from the Wiley Library and National Institute of Standards and Technologies Library, as well as with data published in the literature.

9.3 Results

9.3.1 Percentage yield

From the three batches of approximately 500 mg sonicated of powdered wood, mean percentage yields of 8.2% of methanolic extract, 4.6% aqueous extract and 4.23% ethanoic extracts were obtained. Methanol extracts gave the highest percentage yield. Most of the compounds were polar in nature

9.3.2 Bioactive compounds present in the extracts

The bioactive compounds present in methanol, aqueous and acetone extracts obtained from *Pappea capensis* are shown in Tables 9.1 - 9.3. Their identification and characterization were based on their elution order in a HP-5 fused silica capillary column (30m x 0.25 mm x 25m film thickness), the elution time, library identification name and the amounts at which these bioactive compounds were also presented. Based on

abundance, the top three major compounds present in the methanolic extracts were 2-propenoic acid, dodecyl ester (24.48%), 4-ethylbenzaldehyde (18.33%) and 1,3-Di-tert-butylbenzene (10.62%).

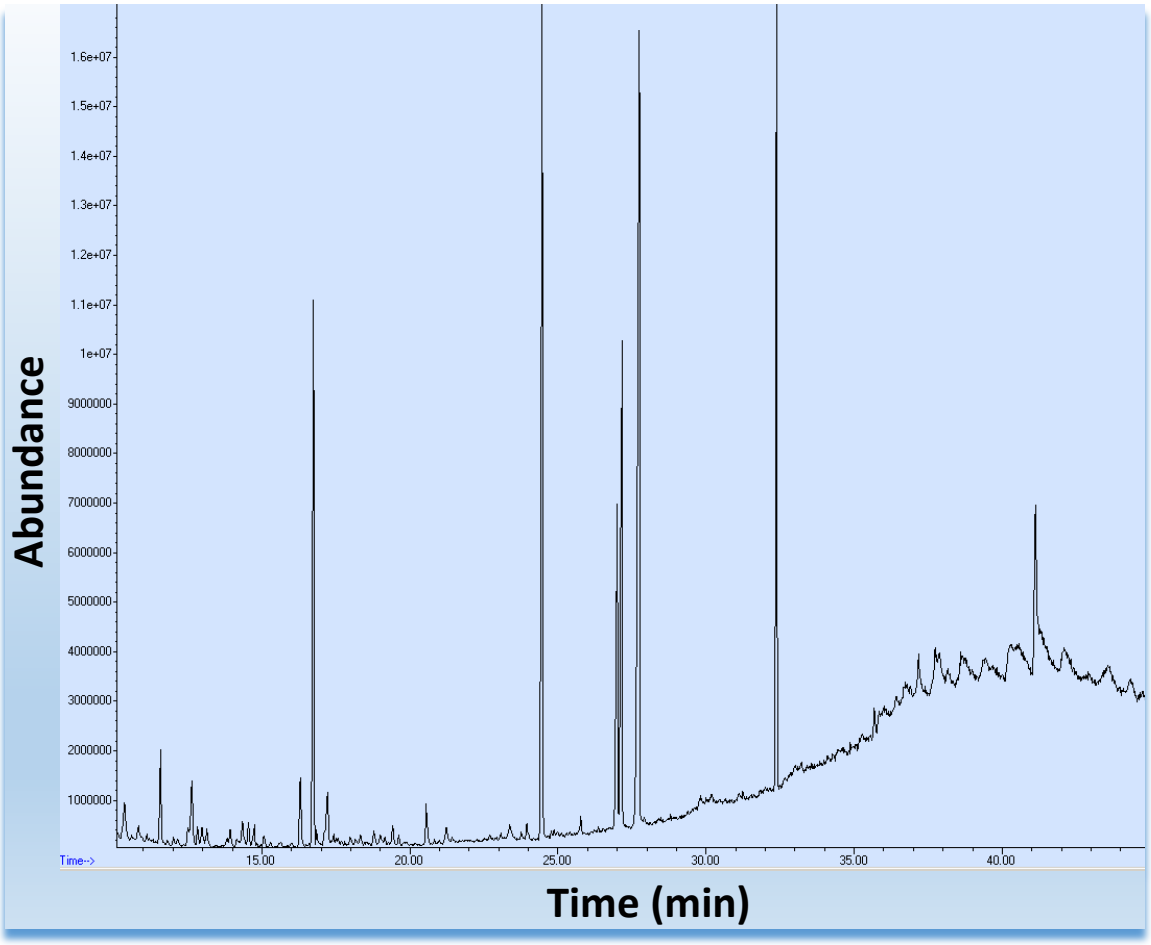
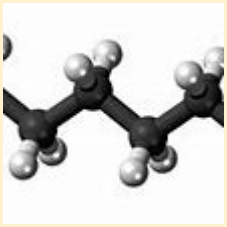
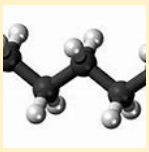
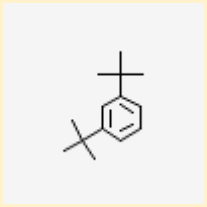
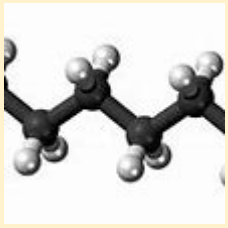
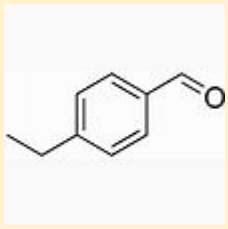
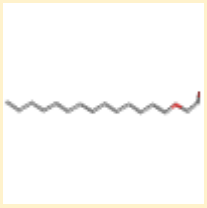
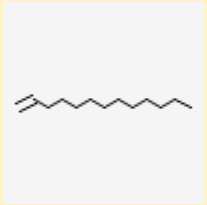
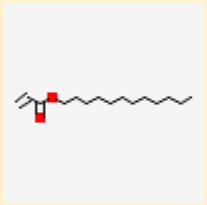
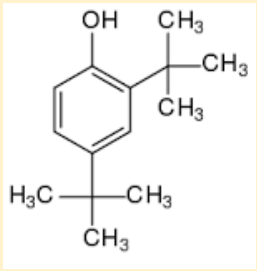
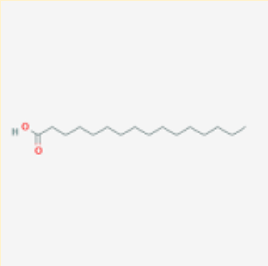


Fig 9.1: A typical chromatogram of a 500 mg methanolic wood extract.

Table 9.1: Tentative identification and area percentage (%) of volatiles present in the methanolic wood extract.

		NB: Take note that all the compounds are tentatively reported as we had to rely solely on the NIST 95 and WILEY275 libraries for compound matches as we were not in possession of standards to confirm.	
		The concentration of the compounds is reported in terms of area percent	
		500 mg methanolic wood extract	
Retention Time	**Library identification**	**Area Percent (%) and structure**	**Biological activity**
11.58	Dodecane	1.98	Promoter of skin carcinogenesis for ultraviolet radiation, did not produce an increase in mutations when tested in the Ames *Salmonella typhimurium* assay with and without of metabolic activation. However, Dodecane was able to enhance mutagenesis induced by methylazoxymethanol acetate at the ouabain resistance locus. Dodecane is a liquid alkane hydrocarbon with the chemical formula $CH_{10}CH_3$, an oily liquid of the paraffin series. It has 355 isomers https://toxnet.nlm.nih.gov/cgi-bin/sis/search/a?dbs+hsdb:@term+@DOCNO+5133
12.64	Pentadecane	1.79 	May be harmful by inhalation, ingestion, or skin absorption during industrial use. https://toxnet.nlm.nih.gov › cgi-bin › sis › search
16.30	Tetradecane	1.69 	Tetradecane is an alkane hydrocarbon with the chemical formula $CH_{12}CH_3$. Tetradecane has 1858 structural isomers https://toxnet.nlm.nih.gov › cgi-bin › sis › search

16.74	1,3-Di-tert-butylbenzene	10.62 	1, 3-Di-tert-butylbenzene is an alkylbenzene. 1, 3-Di-tert-butylbenzene belongs to the class of organic compounds known as phenylpropanes. These are organic compounds containing a phenylpropane moiety https://pubchem.ncbi.nlm.nih.gov › compound › 1_3-Di-tert-butylbenzene
17.22	Docosane	1.79	It has a role as a plant metabolite. From ChEBI. N-docosane is a solid. Insoluble in water. Docosane is a straight-chain alkane with 22 carbon atoms. Docosane is a component of petroleum products and may be released into the environment through the processing and combustion of petroleum products containing this chemical. Docosane is a component of various plant parts and tissues https://toxnet.nlm.nih.gov › cgi-bin › sis › search
20.57	Hexadecane	1.02 	It has a role as a plant metabolite, a volatile oil component and a non-polar solvent. from ChEBI. N-hexadecane is a colorless liquid. (NTP, 1992) from CAMEO Chemicals. https://pubchem.ncbi.nlm.nih.gov › compound › Hexadecane
24.47	4-Ethylbenzaldehyde	18.33 	**4**-Ethylbenzaldehyde has been used in the synthesis of 4, 4'-diaminotriphenylmethanes under microwave irradiation, which is useful for parallel library syntheses. https://www.sigmaaldrich.com › catalog › product › aldrich
27.01	2-Tetradecyloxyethanol	8.64 	2-(Dodecyloxy)ethanol It is used as a detergent, and medically as a local anesthetic, and as a sclerosing agent for the treatment of ESOPHAGEAL AND GASTRIC VARICES and VARICOSE VEINS.

27.15	1-Tridecene	10.39 	1-Tridecene is found in fats and oils. 1-Tridecene is a constituent of coconut (Cocos nucifera) flesh. Also present in heated oils of peanut, sunflower and butter.
27.75	2-Propenoic acid, dodecyl ester	24.48 	Acrylic acid (2-propenoic acid) is a highly reactive carboxylic acid that can react with itself to form polyacrylic acid, which is used as an absorbent in hygiene products. It also can react with alcohols to form acrylates (esters) that are used in a wide range of polymers.Oct 22, 2007 https://www.acs.org › content › acs › molecule-of-the-week › archive › acryl...
32.39	2,4-Di-tert-butylphenol	13.98 	2,4-di-tert-butylphenol is a member of the class of phenols carrying two tert-butyl substituents at positions 2 and 4. It has a role as a bacterial metabolite, an antioxidant and a marine metabolite. It is an alkylbenzene and a member of phenols. ttps://pubchem.ncbi.nlm.nih.gov › compound › 2_4-Di-tert-butylphenol
41.12	Hexadecanoic acid	3.53 	Palmitic acid (also known as hexadecanoic acid) is a fatty acid that is found naturally in animals and plants and also can be created in laboratory settings. Palmitic acid is widely used in a variety of applications, including personal care products and cosmetics. https://www.chemicalsafetyfacts.org › palmitic-acid

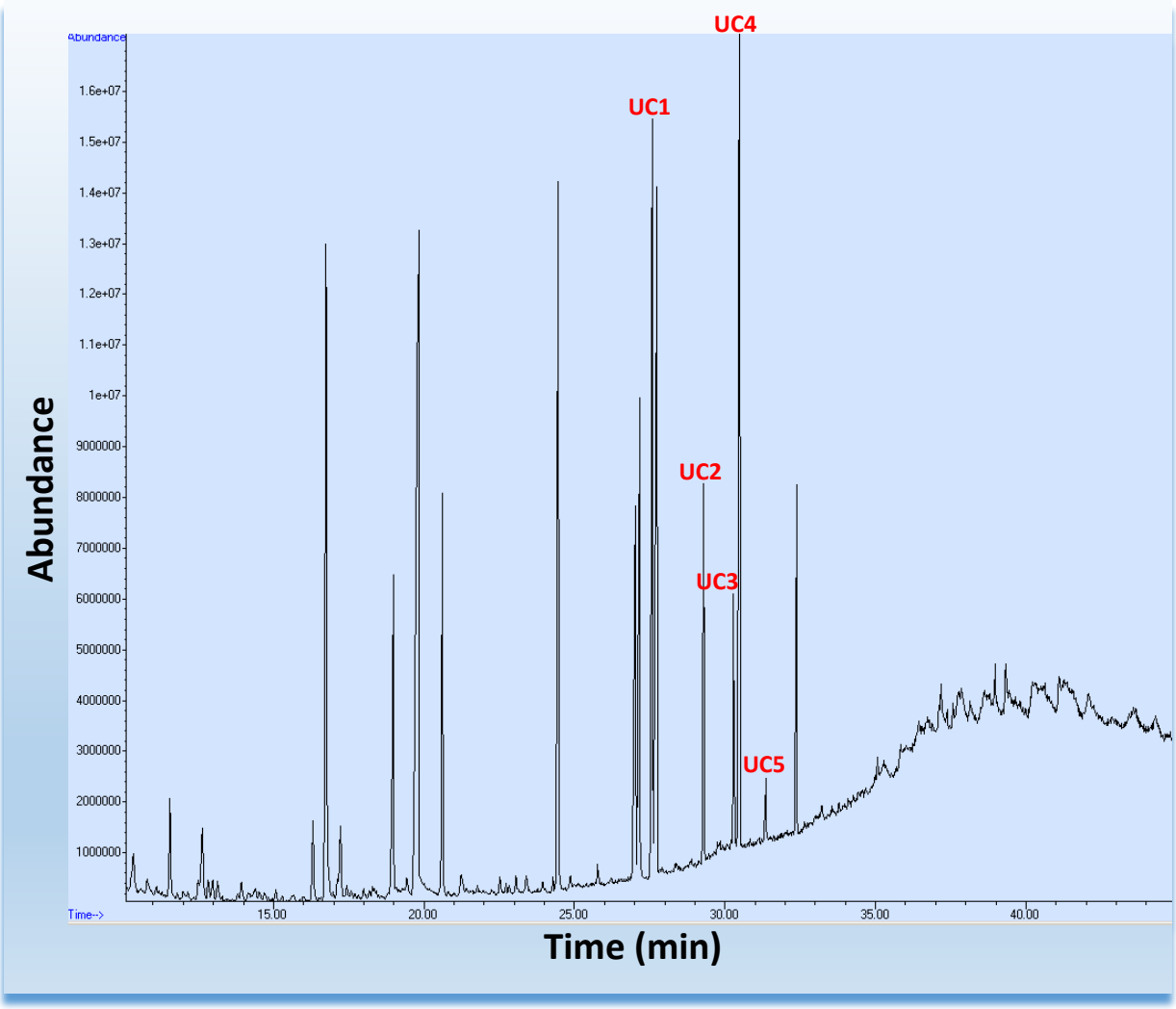
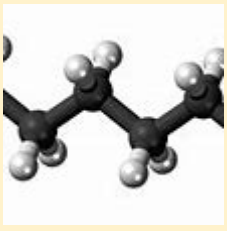
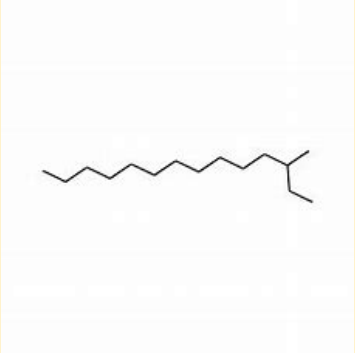
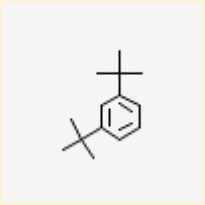
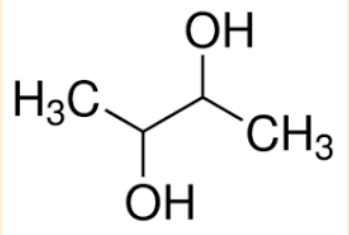
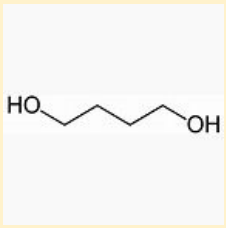
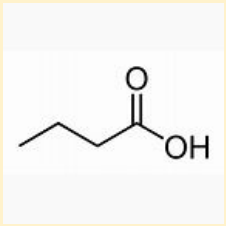
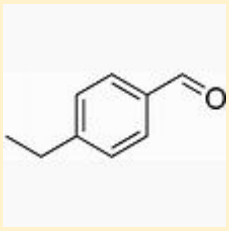


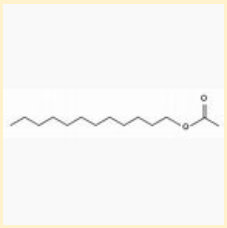
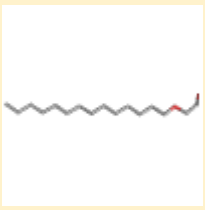
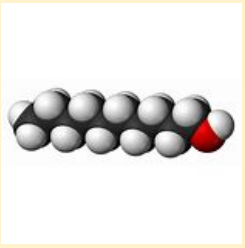
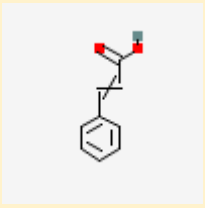
Fig 9.2: Chromatogram of a 500 mg aqueous wood extract. UC = unidentified compound.

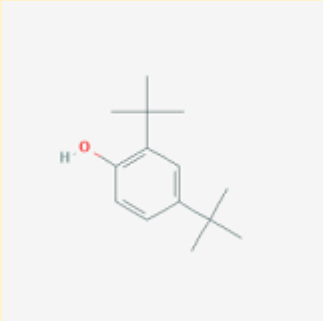
Table 9.2: Tentative identification and area percentage (%) of volatiles present in the aqueous wood extract.

NB: Take note that all the compounds are tentatively reported as we had to rely solely on the NIST 95 and WILEY275 libraries for compound matches as we were not in possession of standards to confirm.			
The concentration of the compounds is reported in terms of area percent			
500 mg aqueous wood extract			
Retention Time	**Library identification**	**Area Percent (%) and structure**	**Biological activity**
11.56	Dodecane	1.33	Dodecane has been shown to be a promoter of skin carcinogenesis for ultraviolet radiation. Dodecane, did not produce an increase in mutations when tested in the Ames Salmonella typhimurium assay with and without of metabolic activation. However, Dodecane was able to enhance mutagenesis induced by methylazoxymethanol acetate at the ouabain resistance locus. Dodecane is a liquid alkane hydrocarbon with the chemical formula $CH_{10}CH_3$, an oily liquid of the paraffin series. It has 355 isomers https://toxnet.nlm.nih.gov/cgi-bin/sis/search/a?dbs+hsdb:@term+@DOCNO+5133
12.64	Pentadecane	1.30 	May be harmful by inhalation, ingestion, or skin absorption during industrial use. https://toxnet.nlm.nih.gov › cgi-bin › sis › search

16.31	Tetradecane	1.23 	Tetradecane is an alkane hydrocarbon with the chemical formula $CH_{12}CH_3$. Tetradecane has 1858 structural isomers https://toxnet.nlm.nih.gov › cgi-bin › sis › search
16.75	1,3-Ditertiarybutylbenzene	9.06 	1,3-Di-tert-butylbenzene is an alkylbenzene. 1, 3-Di-tert-butylbenzene belongs to the class of organic compounds known as phenylpropanes. These are organic compounds containing a phenylpropane moiety https://pubchem.ncbi.nlm.nih.gov › compound › 1_3-Di-tert-butylbenzene
17.24	Docosane	1.32	It has a role as a plant metabolite. From ChEBI. N-docosane is a solid. Insoluble in water. Docosane is a straight-chain alkane with 22 carbon atoms. Docosane is a component of petroleum products and may be released into the environment through the processing and combustion of petroleum products containing this chemical. Docosane is a component of various plant parts and tissues https://toxnet.nlm.nih.gov › cgi-bin › sis › search
18.98	(R or S)-2,3-Butanediol	4.01 	The acid is of considerable commercial importance as a raw material in the manufacture of esters of lower alcohols for use as flavouring agents; its anhydride is used to make cellulose butyrate, a useful plastic. Butyric acid is manufactured by catalyzed air oxidation of butanal (butyraldehyde).

			https://www.webmd.com/vitamins/ai/ingredientmono-21/butanediol-bd
19.82	(R or S)-2,3-Butanediol	15.05 	The acid is of considerable commercial importance as a raw material in the manufacture of esters of lower alcohols for use as flavouring agents; its anhydride is used to make cellulose butyrate, a useful plastic. Butyric acid is manufactured by catalyzed air oxidation of butanal (butyraldehyde). https://www.webmd.com/vitamins/ai/ingredientmono-21/butanediol-bd
20.61	Butanoic acid	3.67 	Butyric is a chemical compound, an acid with a lot of biological effect including antitumor activity. Its anticancer activity is linked to its ability to enhance programme cell death in cancer cells by activating the mitochondrial pathway of apoptosis https://www.britannica.com › science › butyric-acid
21.24	HENEICOSANE	0.42	Heneicosaneis a solid n-alkane containing 21 carbon atoms (C21). Solid n-alkanes (paraffin waxes) are used in a variety of applications: as feeds for cracking them to gasoline blendstock materials, oxidation, and chlorination reactions. n-Heneicosane - National Library of Medicine HSDB Database
24.46	4-Ethylbenzaldehyde	8.21 	Ethylbenzaldehyde has been used in the synthesis of 4,4′-diaminotriphenylmethanes under microwave irradiation, which is useful for parallel library syntheses. https://www.sigmaaldrich.com › catalog › product › aldrich

25.79	LAURYL ACETATE	0.37 	Dodecyl acetate or lauryl acetate, $CH_3COO(CH_2)_{11}CH_3$, is the dodecyl ester of acetic acid. It has a floral odor and is useful as a perfume additive https://pubchem.ncbi.nlm.nih.gov › compound › lauryl_ acetate
27.02	2-Tetradecyloxyethanol	5.96 	2-(Dodecyloxy)ethanol It is used as a detergent, and medically as a local anesthetic, and as a sclerosing agent for the treatment of esophageal, gastric varices and varicose veins https://pubchem.ncbi.nlm.nih.gov › compound › 2-_Dodecyloxy_ethanol
27.15	1-DODECANOL	6.69 	Dodecanol is used to make surfactants, lubricating oils, pharmaceuticals, in the formation of monolithic polymers and as a flavor enhancing food additive. In cosmetics, dodecanol is used as an emollient. It is also the precursor to dodecanal, an important fragrance. https://pubchem.ncbi.nlm.nih.gov/compound/1-Dodecanol
27.59	unidentified compound 1	9.01	
27.73	2-Propenoic acid, dodecyl ester	10.14 	Uses of polypropenoates (polyacrylates) The polymers derived from the esters of propenoic acid are used as a base in many paints and varnishes. The polymers of ethyl and butyl propenoates are used in water-based emulsion paints, as is the co-polymer of butyl propenoate and methyl (2-methylpropenoate). www.essentialchemicalindustry.org › polymers › polypropenoic-acid

29.29	unidentified compound 2	4.02	
30.30	unidentified compound 3	2.91	
30.49	unidentified compound 4	10.21	
31.35	unidentified compound 5	0.81	
32.37	2,4-Di-tert-butylphenol	3.41 	2,4-di-tert-butylphenol is a member of the class of phenols carrying two tert-butyl substituents at positions 2 and 4. It has a role as a bacterial metabolite, an antioxidant and a marine metabolite. It is an alkylbenzene and a member of phenols. https://pubchem.ncbi.nlm.nih.gov › compound › 2_4-Di-tert-butylphenol

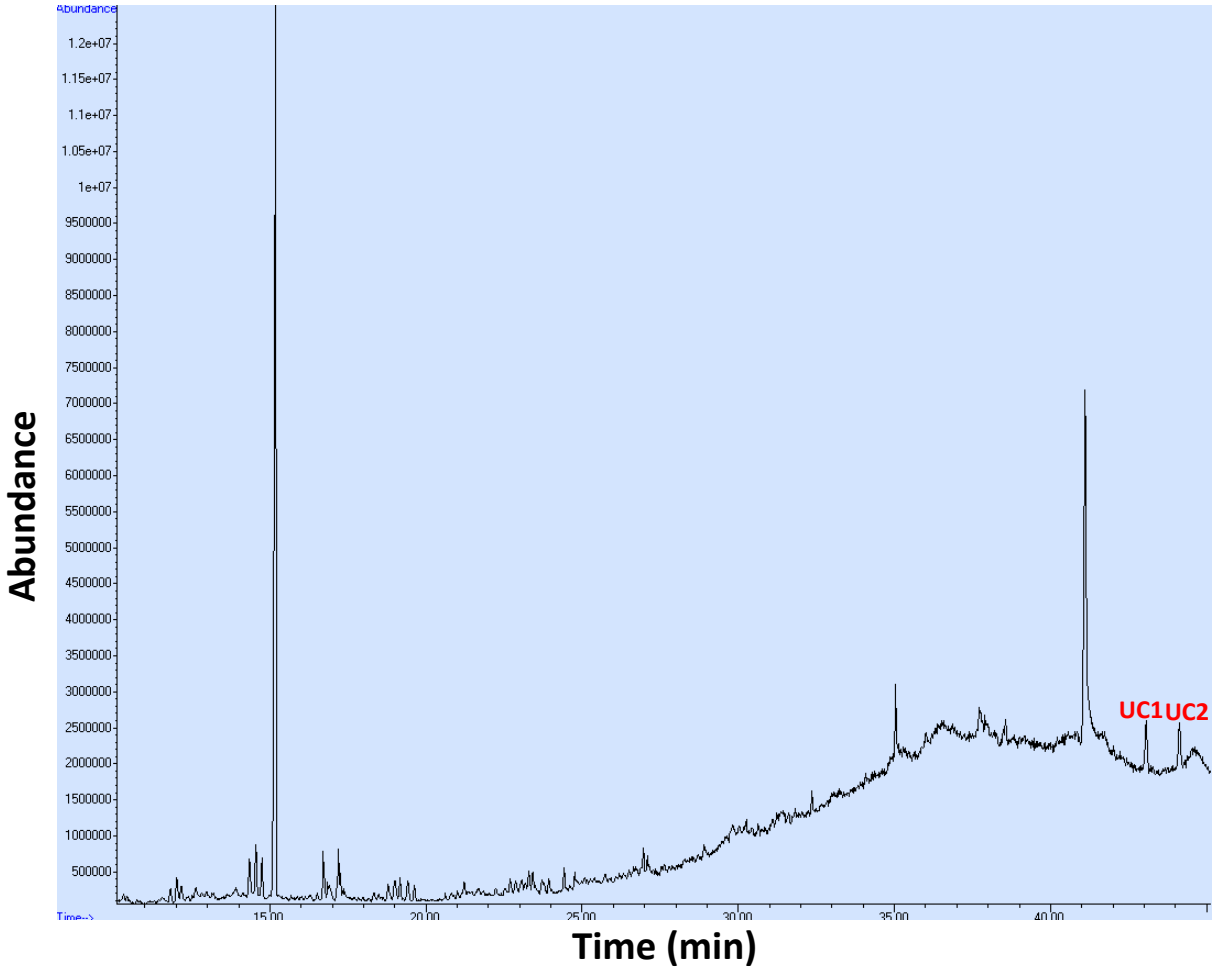
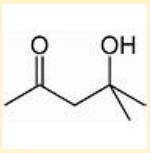
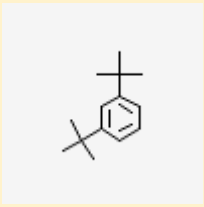
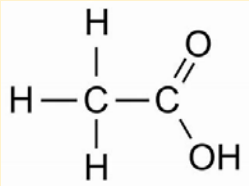
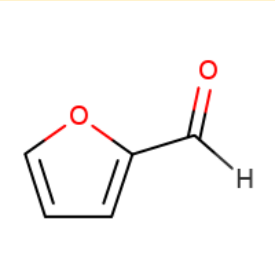
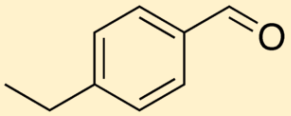
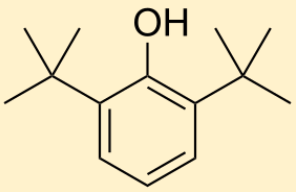
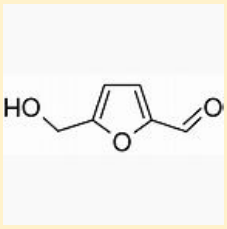
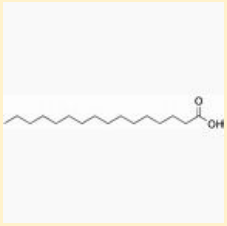


Fig 9.3: Chromatogram of a 500 mg ethanoic wood extract. UC = unidentified compound.

Table 9.3: Tentative identification and area percentage (%) of volatiles present in the ethanoic wood extract.

NB: Take note that all the compounds are tentatively reported as we had to rely solely on the NIST 95 and WILEY275 libraries for compound matches as we were not in possession of standards to confirm.			
The concentration of the compounds is reported in terms of area percent			
500 mg ethanoic wood extract			
Retention Time (min)	**Library identification**	**Area Percent (%) and structure**	**Biological activity**
15.18	4-HYDROXY-4-METHYL-2-PENTANONE	20.36 	Diacetone alcohol is a chemical compound with the formula CH3C(O)CH2C(OH)(CH3)2, sometimes called DAA. This liquid is a common synthetic intermediate used for the preparation of other compounds, and is also used as a solvent. Diacetone Alcohol is a ketone. http://www.merckmillipore.com/ZA/en/product/4-Hydroxy-4-methyl-2-pentanone,MDA_CHEM-804512
16.73	1,3-Di-tert-butylbenzene	1.21 	1,3-Di-tert-butylbenzene is an alkylbenzene. 1, 3-Di-tert-butylbenzene belongs to the class of organic compounds known as phenylpropanes. These are organic compounds containing a phenylpropane moiety https://pubchem.ncbi.nlm.nih.gov› compound › 1_3-Di-tert-butylbenzene
16.85	Acetic acid	0.31	It was discovered that not only does acetic acid kill planktonic bacteria but it also eradicates bacteria growing in biofilms. Acetic acid is a liquid at ambient pressure and

			temperature, and therefore any compositions comprising acetic acid are liquid or wet compositions. https://www.ncbi.nlm.nih.gov › pmc › articles › PMC4486441
17.21	Furfural	1.12 	Furfural is an organic compound with the formula C_4H_3OCHO. It is a colorless liquid, although commercial samples are often brown. It consists of an aldehyde group attached to the 2-position of furan. It is a product of the dehydration of sugars, as occur in a variety of agricultural by products, including corncobs, oat, wheat bran, and sawdust. The name furfural comes from the Latin word furfur, meaning bran, referring to its usual source https://pubchem.ncbi.nlm.nih.gov/compound/2-Furaldehyde
24.43	4-Ethylbenzaldehyde	0.61 	4-Ethylbenzaldehyde is found in alcoholic beverages. 4-Ethylbenzaldehyde is present in roasted chicken, cider, tea and roasted peanuts. 4-Ethylbenzaldehyde is a flavouring ingredients. https://pubchem.ncbi.nlm.nih.gov/compound/4-ethylbenzaldehyde
26.97	Benzothiazole	0.83 	The benzothiazole, in the family of heterocyclic compounds has assumed special significance in synthetic chemistry, pharmaceutical chemistry as well as in clinical applications because of its anti-tumor properties https://pubchem.ncbi.nlm.nih.gov/compound/benzothiazole

32.37	2,4-Di-tert-butylphenol	1.58 	2,4-di-tert-butylphenol is a member of the class of phenols carrying two tert-butyl substituents at positions 2 and 4. It has a role as a bacterial metabolite, an antioxidant and a marine metabolite. It is an alkylbenzene and a member of phenols. https://pubchem.ncbi.nlm.nih.gov › compound › 2_4-Di-tert-butylphenol
35.05	5-(Hydroxymethyl)furfural	4.39 	http://en.wikipedia.org/wiki/Hydroxymethylfurfural The molecule consists of a furan ring, containing both aldehyde and alcohol functional groups. https://pubchem.ncbi.nlm.nih.gov/compound/5-hydroxymethylfurfural
41.11	Hexadecanoic acid	12.95 	Hexadecanoic acid is a straight-chain, sixteen-carbon, saturated long-chain fatty acid. It has a role as an (prostaglandin-E2 9-reductase) inhibitor, a plant metabolite, a Daphnia magna metabolite and an algal metabolite. It is a long-chain fatty acid and a straight-chain saturated fatty acid. It is a conjugate acid of a hexadecanoate A common saturated fatty acid found in fats and waxes including olive oil, palm oil, and body lipids. https://pubchem.ncbi.nlm.nih.gov/compound/Palmitic-acid
43.07	unidentified compound 1	1.55	
44.13	unidentified compound 2	1.57	

The aqueous extracts contained (R or S)-2, 3-butanediol (15.05%), followed by 3-Di-tert-butylbenzene (9.06%) and 4-ethylbenzaldehyde (8.21%). The ethanoic extract had 4-hydroxy-4-methyl-2-pentanone (20.36%), hexadecanoic acid (12.95%) and 5-(hydroxymethyl)-furfural (4.39%) as the three major compounds. The GC chromatograms of the three extracts presented in Figures 9.1-9.3 showed the retention time in the column and the detected peaks which correspond to bioactive compounds present in the extract.

9.4 Discussion

Plants have almost unlimited ability to produce a lot of constituents. Different plant extracts were obtained from the wood of *Pappea capensis* through chronological extraction with solvents of increasing polarity namely: acetone, methanol and water. GC-MS analysis of the acetone, water and methanol extracts revealed the presence of different bioactive compounds. A total of 41 compounds were identified in *Pappea capensis* from the three extracts of which 7 were unidentified compounds. Dodecance, pentadecance, tetradecane, docosane and 2-tetradecyloxyethanol were all present in both the methanolic and aqueous extracts in various quantities. Five and 2 unidentified compounds were identified from the aqueous and ethanoic extracts respectively. The three bioactive compounds (1, 3-Di-tert-butylbenzene, 4-ethybenzaldehyde and 2, 4-Di-tert-butylphenol) were present in all extracts (acetone, methanol and water) but in different quantities. The three extracts didn't reveal a common major compound in them.

However, GC-MS revealed some of the volatiles which are biologically active compounds. In previous studies these volatiles were reported to possess pharmacological activities which may contribute to the healing effect of this plant. 2, 4di-tert-butyl phenol from the leaves of *Pereskia bleo* (Kunth) were proved to exhibit antifungal and antioxidant activity, and had cytotoxicity against the human cancer cell lines; KB, MCF-7, CasKi, HCT 116, A549; normal human cell line MRC-5, HeLa and H9c2 cell lines (Song *et al.,* 2018; Malek *et al.,* 2009; Varsha I et *al.,* 2015). The leaves of *Pereskia bleo* are used traditionally in Malaysia for the treatment of cancer (Varsha *et al.,* 2015). Hexadoconic acid extracted from the marine algal was found to possess selective cytotoxic activity to human leukemia cells and no cytotoxicity activity to normal human dermal fibroblast (DF) cells (Harada *et al.,* 2002). Furthermore, Hexadoconic acid in the same study was found to induce apoptosis in the human leukemic cell line (Harada *et al.,* 2002).

In addition, 4-ethylbenzaldehyde was found to have antibacterial activity against gram-positive bacteria *Staphylococcus aureus*. Studies have also proven that alkanes and aldehydes have been found to be carcinogenic, and even have activation pathways that lead to the formation of cancer. Other studies revealed that butanoic acid possesses anti-cancer properties by inducing apoptosis (Fulda, 2008). Other compounds identified are used as surfactants, detergents, agricultural by-products and flavourings as indicated in table 9.1-9.3 above.

In vitro antioxidant activity was carried out witth the three extracts indicated in the existence of antioxidants and phenols from *Pappea capensis*. So, those results indicated the need to further test any possible compounds associated with this evidently clear trapping of this radicals by the three extracts of *Pappea capensis.*

These results were in collaboration with the previous studies which used the same plant and had shown that the leaf and stem bark carried medicinal benefits such as curative effects associated with hypoglycaemic activity due to phytochemicals (Karau *et al.,* 2012; Ngai *et al.,* 2017). The antimicrobial screening of this plant indicated that microorganisms were killed based on the compounds eluted per extraction methods as each could have isolated different active compounds. Aqueous extract was found to be an only extraction technique mostly used Sangomas and traditional healers to make medicinal mixtures for their patients. However, in this study it was found that ethanoic and methanolic extracts have more antimicrobial activity than water extract which correlated with other studies (Al-Fatimi *et al.,* 2007; Aliero *et al.,* 2006). The active antimicrobial compounds found in this study need further investigation with purified extracts in order to identify the active compounds associated with antimicrobial activity observed in the three extracts.

Upon screening for the cytotoxity and genotoxicity all extracts were not found to possess any activity. Other bioactive compounds such as hexadonoic acid correlated with the results of Haranda, *et al.*, which indicated that hexadoconic acid was also found to be selectively cytotoxic to human leukemic cells but no cytotoxic to normal HDF cells *in vitro* (Haranda, *et al.,* 2002)

The GC-MS analysis also identified alkanes and aldehydes which are poisonous. This correlated with the confirmatory test results which indicated a possibility of toxin from the water extract no. 7 (Fig 8.2 A) for its false pro-inflammatory activity caused by endotoxin contamination of the extract which can be attributed to the plant itself (Cooperstock,

1974). It is evident in table 9.2 that water extracts eluted the majority of compounds, of which most are alkanes and aldehydes as part of the plant.

9.5 Conclusion

The GC-MS identification of seven (7) unknown bioactive chemical compounds, regardless of their amounts, may perhaps be significant as therapeutic agents and a source of the biological activities claimed between traditional healers. Furthermore, many diverse chemical compounds were identified and some quantified, which makes it a holistic plant for use in traditional medicine and for aesthetic value between the local communities in Limpopo. More instrumental analysis is required to carry out bioassay guided fractionation of the three extracts to determine the purified compounds and identify ones that are biologically active.

Medicinal plants, which form the pillar of traditional medicine, recently have been the focus for very intense pharmacological studies, as a result of the acknowledgement of the value of medicinal plants as probable sources of new compounds of therapeutic significance and a new direction for the discovery of bioactive compounds that will pave the way in drug development. Thus, the identification of the bioactive compound in *Pappea capensis* was done through GC-MS analysis which showed the presence of 41 compounds. Among the identified compounds, 4-ethylbenzaldehyde, 2, 4-di-ter-butyl phenol, acetic acid and butanoic acid have a role in antioxidant, antimicrobial, antitumor and antifungal effects. In conclusion, this study has shown that the *Pappea capensis* may serve as a new possible

source of remedies due to the presence of these phytochemicals, bioactive compounds and biological activities.

9.6 References

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https://www.webmd.com/vitamins/ai/ingredientmono-21/butanediol-bd

https://toxnet.nlm.nih.gov › cgi-bin › sis › https://toxnet.nlm.nih.gov/cgi-bin/sis/search/a?dbs+hsdb:@term+@DOCNO+5133searc

https://pubchem.ncbi.nlm.nih.gov › compound › 1_3-Di-tert-butylbenzene

https://www.sigmaaldrich.com › catalog › product › aldrich

https://pubchem.ncbi.nlm.nih.gov › compound › lauryl_ acetate

https://www.sigmaaldrich.com › catalog › product › aldrich

https://www.webmd.com/vitamins/ai/ingredientmono-21/butanediol-bd

https://pubchem.ncbi.nlm.nih.gov › compound › 1_3-Di-tert-butylbenzene

ttps://pubchem.ncbi.nlm.nih.gov › compound › 2_4-Di-tert-butylphenol

https://www.acs.org › content › acs › molecule-of-the-week › archive › acryl...

https://pubchem.ncbi.nlm.nih.gov › compound › 1_3-Di-tert-butylbenzene

https://pubchem.ncbi.nlm.nih.gov › compound › Hexadecane

https://toxnet.nlm.nih.gov/cgi-bin/sis/search/a?dbs+hsdb:@term+@DOCNO+5133

www.essentialchemicalindustry.org › polymers › polypropenoic-acid

https://pubchem.ncbi.nlm.nih.gov/compound/1-Dodecanol

https://pubchem.ncbi.nlm.nih.gov › compound › lauryl_ acetate

https://toxnet.nlm.nih.gov › cgi-bin › sis › search

https://pubchem.ncbi.nlm.nih.gov › compound › 2_4-Di-tert-butylphenol

https://www.acs.org › content › acs › molecule-of-the-week › archive › acryl...

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https://pubchem.ncbi.nlm.nih.gov › compound › Hexadecane

https://pubchem.ncbi.nlm.nih.gov › compound › 1_3-Di-tert-butylbenzene

https://toxnet.nlm.nih.gov/cgi-bin/sis/search/a?dbs+hsdb:@term+@DOCNO+5133

https://toxnet.nlm.nih.gov › cgi-bin › sis › search

Chapter 10

OVERVIEW OF THE PHYTOCHEMICALS AND BIOLOGICAL ACTIVITY OF *PAPPEA CAPENSIS TREE* FOR THE TREATMENT OF CANCER

10.1 Introduction

Globally, cancer is a prominent complex, extensively spread lethal disease characterized by less controllable growth and affecting different organs owed to spreading of abnormal cells. Cancer remains one of the leading morbidity and mortality factors globally. Amongst the non-communicable diseases, cancer is the second leading cause of deaths following cardiovascular disease (WHO, 2005; Mathers and Loncar, 2006; Lopez *et al.,* 2006, Hoyert *et al.,* 2006). Cancer is causing a quarter of deaths globally more than the well-known deadly diseases (AIDS, tuberculosis, and malaria together) (Sener and Grey, 2005). Cancer is the leading cause of death in North America, Australia, New Zealand and Western Europe compared to other parts of the world (Parkin *et al.,* 2001). In the United States of America, a quarter of deaths are attributed to cancer (Jemal *et al.,* 2007). Over two decades the number of deaths due to cancer are projected to increase from 7.1 million in 2002 to 11.5 million by 2030 globally (Mathers and Loncar, 2006).

The choice of therapy for cancer depended upon the location, the grade of the tumour and the stage of the patient. So, the basic treatment for cancer for many years has been relying on chemotherapy, radiation, immunotherapy, monoclonal antibody therapy and many more. During the progression of cancer, the cells lose many of the regulatory functions present in the normal cells. They divide continuously while normal cells don't. That makes cancer cells susceptible to chemotherapeutic drugs. More than five decades of systemic drugs discovery and development have resulted in a large collection of useful chemotherapeutic agents. However, chemotherapeutic agents are not devoid of their intrinsic problems such as different kinds of toxicities. Several types of toxicities which

include 5-fuorouracil, a common therapeutic agent known to cause myelotoxicity, cardiotoxicity and is widely documented in few cases as a vasospatic agent (Macdonald, 1999; Rastogi *et al.*, 1993*)*. Another universally used chemotherapeutic agent, doxorubicin causes cardiac toxicity, renal toxicity and myelotoxicity (Aviles *et al.,* 1993; Leo *et al.,* 1997; Gibaud *et al.,* 1994). Another well-known chemodrug, bleomycin, is said to cause pulmonary toxicity and cutaneous toxicity (Parvinen *et al.,* 1983*;* Karam *et al.,* 1995; Cohem *et al.,* 1973). Likewise, cyclophosphamide, a drug identified to treat many lignant conditions, has been described to cause bladder toxicity in the form of hemorrhagic, immunosuppression, alopecia, and at high doses it causes cardiotoxicity (Fraiser *et al.,* 1991). The toxicity associated with the chemotherapeutic drugs generally created significant problems in the management of cancer, using allopathy or established medicine.

In combatting the alarming toxicity of the current anti-cancer agents there is an increase in the interest for the discovery of new drugs with less toxicity, genotoxicity and high scavenging activity. Research have shown that plant-derived agents possess medical benefits to different conditions and could play a better role in the control and treatment of cancer. Science should go back to the roots and reverse the slow diminishing of indigenous culture, in favour of western European-derived culture, which is an accelerating process among indigenous people around the world (Prance, 1994). Subsequently, the traditional knowledge that forms the basis of use of medicinal plants is in danger of being lost and warrants rigorous scientific investigation. The antigenicity and toxicity of the many plant extracts in the treatment of cancer persists.

10.2 Overall study approach and results

The current study was encouraged by case reports describing astonishing improvement of patients who have been critically ill due to progressive prostate cancer, the doctors around Mokopane area claimed so, but had no explanation for the improvement. Mrs RR Matabane (the traditional healer) confirmed that some doctors asked patients what they have used, these patients reported that they had been treated with an extract from a wood of a medicinal plant. This plant material was offered to us for an initial analysis for anti-cancer activity. After characterisation by a botanist (Dr PC Zietsman), the plant was found to be *Pappea capensis* (PC Zietsman & A Makhoahle 5448 the specimen is housed at the herbarium of the National Museum, Bloemfontein (NMB)). Currently, the investigation of these claims, the efficacy of the use of *Pappea capensis*, as well as further investigation of its therapeutic potential has received less scientific investigation. The aim of the study was to prepare crude plant extracts of *Pappea capensis*, broadly establish the bioactivity and describe the phytochemical properties of *Pappea capensis* water extract

So, in-*vitro* antioxidant activity was carried out with ethanoic, water and methanolic *Pappea capensis* wood extracts by the DPPH free radical scavenging method. The DPPH free scavenging activity of the three extracts of *Pappea capensis* displayed antioxidant comparable to the used control. These results correlated with the results of previous studies which showed that the presence phytochemicals and phenolics was responsible for the trapping of free radicals. So, in this study free radicals were evidently trapped. Henceforth, further investigation to establish the existence of phytochemicals and phenolic compounds as the contributing factor was necessary.

The positive identification of antioxidant, phytochemical compounds and total phenolic content paved the way for further screening of the toxicity of the plant. It's important for traditional healers to also know the safe use of their medicinal plants because the high toxicity level of the current anti-cancer agents triggered the attention of researchers globally in seeking and developing of the new drugs that will meet the necessary requirements for safe use with less or no toxicity. In this study the different phytochemical compounds and phenols identified from the wood of *Papea capensis* might possess pharmacological benefits which could be used in traditional medicines. Previous studies on a similar plant using the leaf and the bark have shown that *Pappea capensis* possesses medicinal benefits. That study had shown that the leaf and stem bark of *Pappea capensis* possess curative effects associated with hypoglycaemic activity. This is due to phytochemicals which have been scientifically proven to play a critical role in plants used for traditional medicine (Karau *et al.,* 2012; Ngai *et al.,* 2017). The extracts were then subjected to further tests such as the antibacterial activity, cytotoxicity. And many more tests should be done to ascertain the medical benefits and safety of the woody part of this plant.

The sensitivity testing of the plants plays a critical role to ascertain the safe use of the plant extracts. For a drug/extract to be regarded as safe, it important that during its use it specifically kills microorganisms found during and along the progression of cancer cells but pose less to no threat to the normal flora. The normal flora indirectly provided protection by owning its environment from invaders and this simply indicates the need to

include microorganisms, also known as normal flora, and see how they react in the presence of the different extracts. In this study, the antimicrobial screening showed that different extraction methods isolated active compound which were dependent of the solvent type used. The ethanoic and methanolic extracts were shown to have active compounds against Gram positive and Gram-negative organisms. Both extracts (ethanoic and methanolic) indicated that potential antimicrobial compounds were in the high polarity fraction. Water was found to be the only solvent mostly used by Sangomas and traditional healers to make medicinal mixtures for their patients. However, this study proved otherwise, whereby ethanoic and methanolic extracts produced more antimicrobial activity than the water extract. And these results correlated with results of other studies (Al-Fatimi *et al.,* 2007; Aliero *et al.,* 2006). The active antimicrobial compounds found in this study need further investigation with purified extracts in order to identify the active compounds associated with antimicrobial activity observed in the three extracts.

Then antimicrobial activity was extended by including other organisms. The antimicrobial results obtained from both chapters (4 and 5) clearly indicate that *Pappea capensis* possesses antimicrobial activities which are solvent-based on compounds eluted that specifically inhibit different organisms. *Staphylococcus aureus* and *Klebsiella pneumoniae* were found to be susceptible to ethanoic and methanolic extracts in chapter 4. However, in chapter 5 methanolic extract showed broad antimicrobial activity against two organisms known as part of the normal flora (*Staphylococcus epidermidis* and *Enterococcus faecalis)* which are both gram positives. It is worth mentioning that in

chapter 4 water extract showed antimicrobial activity against the Gram-negative organisms (*Klebsiella pneumoniae*). Sangomas and traditional healers' use of *Pappea capensis* water extract as their common extraction method was followed in this study. Aqueous extracts possess antimicrobial activity against *Klebsiella pneumoniae*, although no antimicrobial activity was observed against all Gram-positive organisms. Future analysis should include susceptibility testing on more organisms and further identification of compounds associated with the antimicrobial activity in these extracts

The *Pappea capensis* plant's extracts were screened for cytotoxity against the three cell lines (vero cells, MCF7 and PC). Upon comparing the used control and the only extract showing slight toxicity, it's conclusive to say a high dose of methanolic extract is physiologically inappropriate. All extracts are not active (cytotoxic) enough to suggest that *Pappea capensis* can be taken into further anti-cancer testing. Nonetheless, it's scientifically important to mention that isolation and identification of bioactive compounds in their pure state may indicate otherwise. Further testing using GC-MS may possibly indicate volatiles present and their individual biological effect must be tested.

The extracts were further screened for genotoxicity. Genotoxins are agents that can possibly intact with the DNA, thus damage its structure and cause mutation which may lead to cancer. Two extracts were considered none genotoxic and ethanoic extract could be considered genotoxic. It must also be notified that the micronucleated cells of 30% were determined at the high concentration (200ug/ml) of ethanoic extract. Further tests

should be done to predict what mechanism is affected by compounds in the ethanoic extract. The results indicate the potential use of this plant against cancer, despite the moderate genotoxicity at 200ug/ml. This further supports the need for the identification of bioactive compounds using GC-MS chromatography as the *Pappea capensis* extracts show less to no genotoxicity.

The *in vitro* screening results for anti- and pro-inflammatory assays for *Pappea capensis* are indicated in Figures 8.1 and 8. 2, respectively. It is shown in Fig 8.1 that NO production (A) and (B) indicates the LPS activated macrophages treated with different concentrations of *Pappea capensis* extracts and corresponding cytotoxicity. Resveratrol, a known inhibitor of iNOS expression served as a positive control and LPS was added to the regime. In Fig 8.2 macrophage activation as shown in (A), while (B) clearly shows the response to treatment with different concentrations of *Pappea capensis* extracts and their corresponding cytotoxicity. Also, LPS was added to the regime. There was observable macrophage activation in the presence of water extract no. 7. So, a confirmatory test was done to ascertain if this effect is owed to plant constituents or due to endotoxin contamination. Then, NO production was be evaluated in the presence and absence of polymyxin B (PMB) as shown in fig 8.3. It can be concluded that PMB counteracts the pro-inflammatory effect suggesting that this activity is possibly a result of endotoxins in the extract, either from the solvent of extraction or as part of the plant. The determination of endotoxin as a co-factor perpetrating pro-inflammatory activity correlates with the study of Cooperstock who clearly showed that polymyxin B can be used to inactivate endotoxin produced by organisms (Cooperstock, 1974). More extensive analysis could be

conducted to determine exact active compounds associated with the pro-inflammatory effect because the solvent used in this case was water. The low anti-inflammatory effects seen of the three extracts at 200ug/ml are not physiologically active. Therefore, the extracts do not possess anti-inflammatory effects. A possible toxin identified in figure 8.3 (B) could be identified by compound separation using GC-MS.

The GC-MS, a hyphenated system which is a very compatible and the most commonly used technique for the identification and quantification purpose was used on the three *Pappea capensis* extracts. Thus, the identification analysis revealed the presence of 41 compounds. Among the identified compounds, 4-ethylbenzaldehyde, 2, 4-di-ter-butyl phenol, acetic acid and butanoic acid have a role in antioxidant, antimicrobial, antitumor, antifungal effects. The GC-MS analysis also identified alkanes and aldehydes which corelates with possible poisonous compounds identified in fig 8.2A. Then it can be concluded that the pro-inflammatory activity was indeed caused by a toxin or poisonous compounds from the plant itself (Cooperstock, 1974). It is evident in table 9.2 that water extracts eluted the majority of compounds of which most are alkanes and aldehydes as part of the plant.

Furthermore, the GC-MS identified that seven (7) unknown bioactive chemical compounds, regardless of their amounts, may perhaps be significant therapeutic agents and a source of the biological activities claimed between traditional healers. Furthermore, many diverse chemical compounds were identified and some quantified, which makes it a holistic plant for use in traditional medicine and for aesthetic value between the local communities in Limpopo. More scientific tests are needed to carry out bioassay-guided

fractionation of the three extracts to determine the purified compounds and identify the ones that are biologically active.

10.3 Holistic conclusion

Medicinal plants, being the only source that healers and Sangomas rely on for treatment of their patients have received tremendous attention in drug therapy, discovery and development. Recently, intense focus has been witnessed in pharmacological studies, as a result of the acknowledgement of the value of medicinal plants as probable sources of new compounds of therapeutic significance, and a new direction for the discovery of bioactive compounds that will pave the way in drug development. In conclusion, this study has shown that the *Pappea capensis* may serve as a new possible source of remedies due to the presence of identified phytochemicals, bioactive compounds and biological activities.

10.4 Limitations of the study

- Due to limited resources, it was not possible to expand the study to a large bacterial population and a number of tumor cell lines
- It was not possible to further the study by testing for active isolated compounds due to financial constraints and the fact that the plant is collected in the Limpopo Province

8.6. Recommendations for the future research

Based on the results obtained from the study, future studies using the same plant should be conducted as outlined below:

- Do activity-guided fractionation, isolation and identification of compounds. This study paved a way and contributed to the knowledge based on the discovery and development of new anti-cancer drugs
- Further tests should be employed as there is no single method that can provide a comprehensive profile of the oxidant, phenolics and anti-inflammatory, cytotoxicity and genotoxity of the plants. It is important that different methods are used to further screen the inflammatory enzymes and other parameters that qualify for drug developments according to regulation
- Isolation, identification and naming of the 7 unknown compounds identified in this study is important
- Individual toxicity of these 7 compounds is an area that must be investigated and fully understand their role in influencing the results obtained

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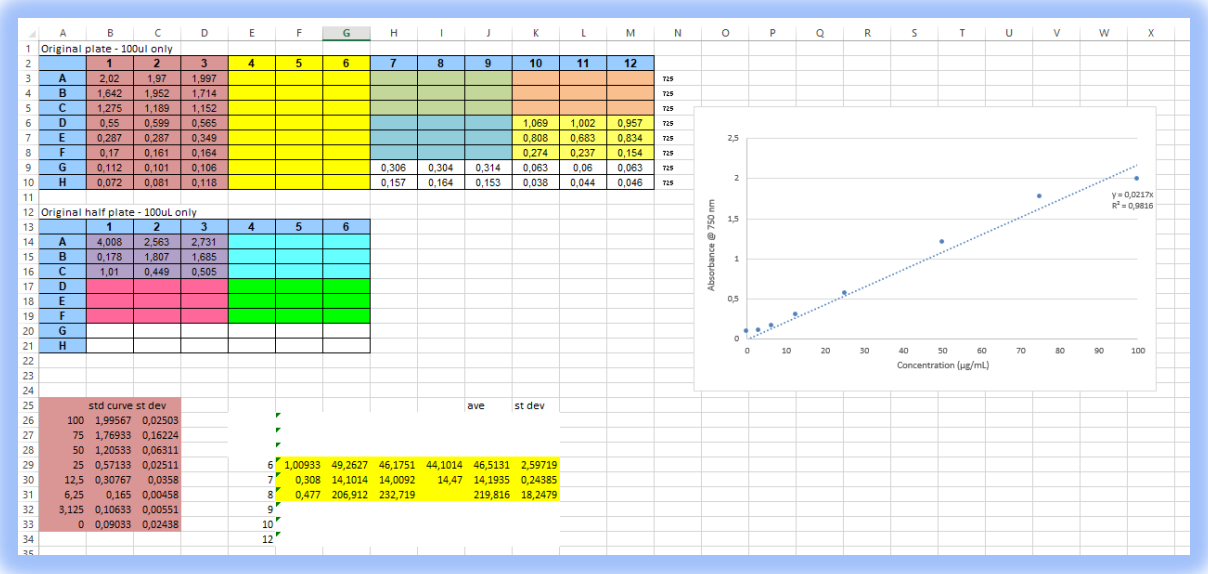
Annexure 1

Chapter 3 related raw data

***IN VITRO PHYTOCHEMICAL ANALYSIS AND* TOTAL PHENOLICS SCREENING FROM *PAPPEA CAPENSIS* EXTRACTS USING FOLIN-CIOCALTEU (FC) ASSAY**

Total phenolics FC readings



Original plate - 100ul only

	1	2	3	4	5	6	7	8	9	10	11	12
A	2,02	1,97	1,997									
B	1,642	1,952	1,714									
C	1,275	1,189	1,152									
D	0,55	0,599	0,565							1,069	1,002	0,957
E	0,287	0,287	0,349							0,808	0,683	0,834
F	0,17	0,161	0,164							0,274	0,237	0,154
G	0,112	0,101	0,106				0,306	0,304	0,314	0,063	0,06	0,063
H	0,072	0,081	0,118				0,157	0,164	0,153	0,038	0,044	0,046

Original half plate - 100uL only

	1	2	3	4	5	6
A	4,008	2,563	2,731			
B	0,178	1,807	1,685			
C	1,01	0,449	0,505			
D						
E						
F						
G						
H						

	std curve	st dev					ave	st dev
100	1,99567	0,02503						
75	1,76933	0,16224						
50	1,20533	0,06311						
25	0,57133	0,02511	6	1,00933	49,2627	46,1751	44,1014	46,5131	2,59719
12,5	0,30767	0,0358	7	0,308	14,1014	14,0092	14,47	14,1935	0,24385
6,25	0,165	0,00458	8	0,477	206,912	232,719		219,816	18,2479
3,125	0,10633	0,00551	9						
0	0,09033	0,02438	10						
			12						

PLATE 1 Standard curve

	1	2	3	4	5	6	7	8	9	10	11	12
A	2,02	1,97	1,997									
B	1,642	1,952	1,714									
C	1,275	1,189	1,152									
D	0,55	0,599	0,565							1,069	1,002	0,957
E	0,287	0,287	0,349							0,808	0,683	0,834
F	0,17	0,161	0,164							0,274	0,237	0,154
G	0,112	0,101	0,106				0,306	0,304	0,314	0,063	0,06	0,063
H	0,072	0,081	0,118				0,157	0,164	0,153	0,038	0,044	0,046

Extract 6

Extract 7

First half of plate 1

Standard curve

	1	2	3
A	2,02	1,97	1,997
B	1,642	1,962	1,714
C	1,275	1,189	1,152
D	0,55	0,599	0,565
E	0,287	0,287	0,349
F	0,17	0,161	0,164
G	0,112	0,101	0,106
H	0,072	0,081	0,118

Second half of plate 1 Absorbance readings at 750nm

	1	2	3	
A	4,008	2,563	2,731	1:1
B	0,178	1,807	1,685	1:2
C	1,01	0,449	0,505	1:10

Extract 8

Extract 7

	7	8	9	10	11	12	
1:1				1,069	1,002	0,957	1:1
1:2				0,808	0,683	0,834	1:2
1:10				0,274	0,237	0,154	1:10
1:1	0,306	0,304	0,314	0,063	0,06	0,063	1:10
1:2	0,157	0,164	0,153	0,038	0,044	0,046	

Extract 6

Annexure 2

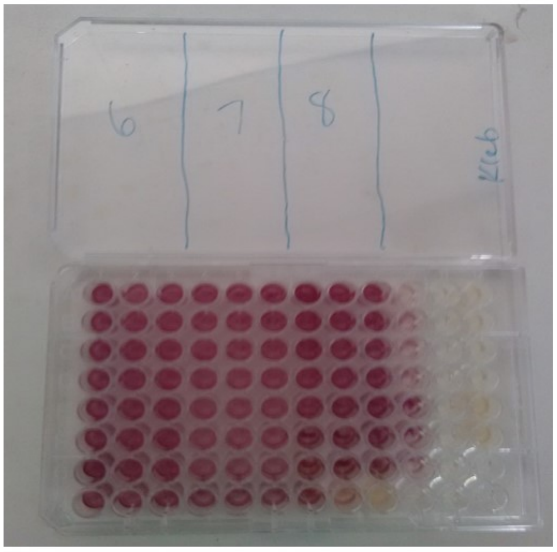
Chapter 4 related raw data

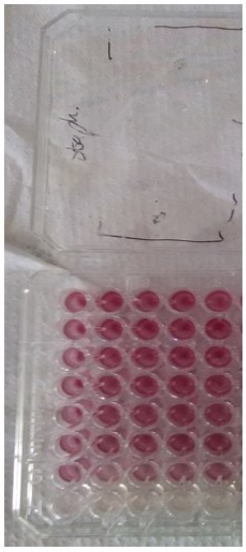
IN VITRO ANTIBACTERIAL SCREENING OF _PAPPEA CAPENSIS_ EXTRACTS

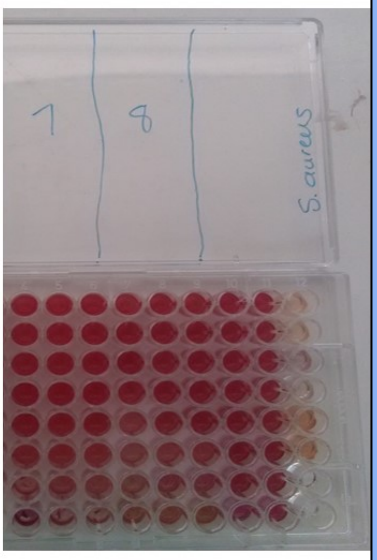
USING THE _p_-IODONITROTETRAZOLIUM CHLORIDE (INT) ASSAY

ANX2(A): Microbial broth plate results related to Fig 4.1 and 4.2

K. pneumoniae *S. aureus*







	extract			extract			extract			GC	PC	
0.0156												
0.03125												
0.0625												
0.125												
0.25												
0.5												
1												
2												

PC - refers to respective positive control. Note these were only tested at their MIC values

GC - refers to growth control which only contained medium and respective micro-organism

All extracts were tested at the concentrations indicated in the table above.

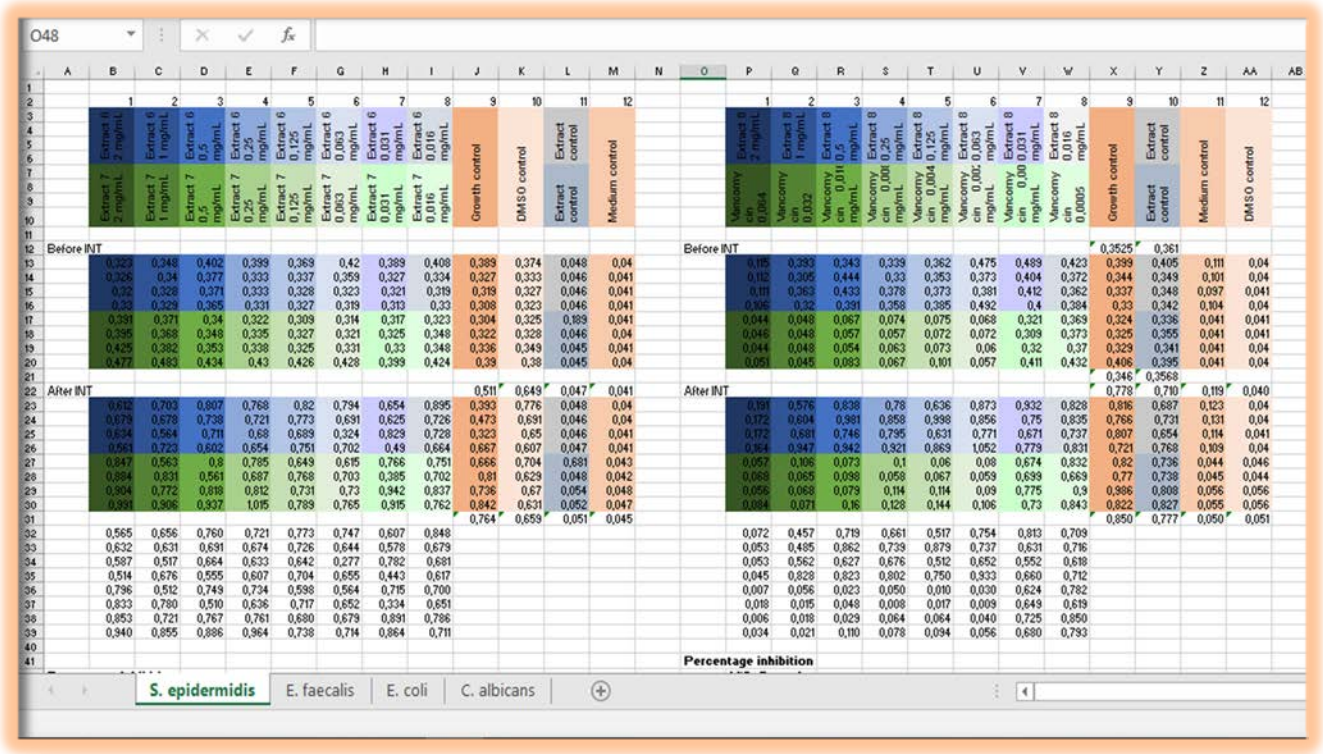
Screening for antibacterial activity of extracts 6, 7 and 8 against *K. pneumoniae* and *S. aureus* at a concentration range of 0.0156 mg/ml – 2 mg/ml.

Annexure 3

Chapter 5 related raw data

IN VITRO ANTIBACTERIAL SCREENING OF _PAPPEA CAPENSIS_ EXTRACTS USING THE MIDDLEBROTH AND THE _p_-IODONITROTETRAZOLIUM CHLORIDE (INT) ASSAY

ANX3 (A): *Staphylococcus epidermidis*

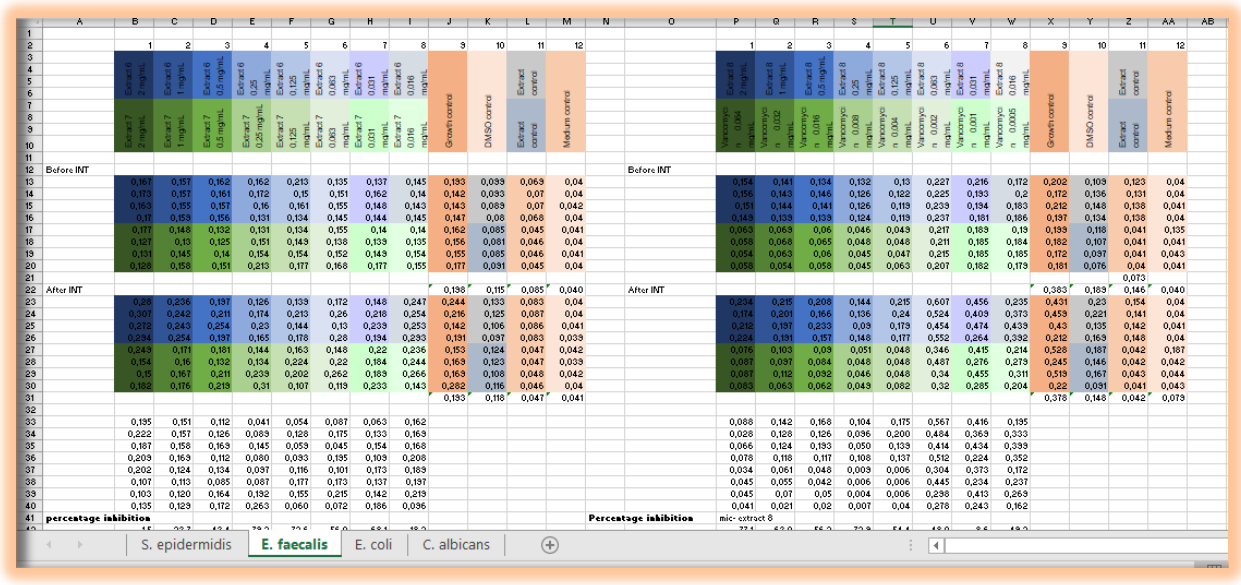


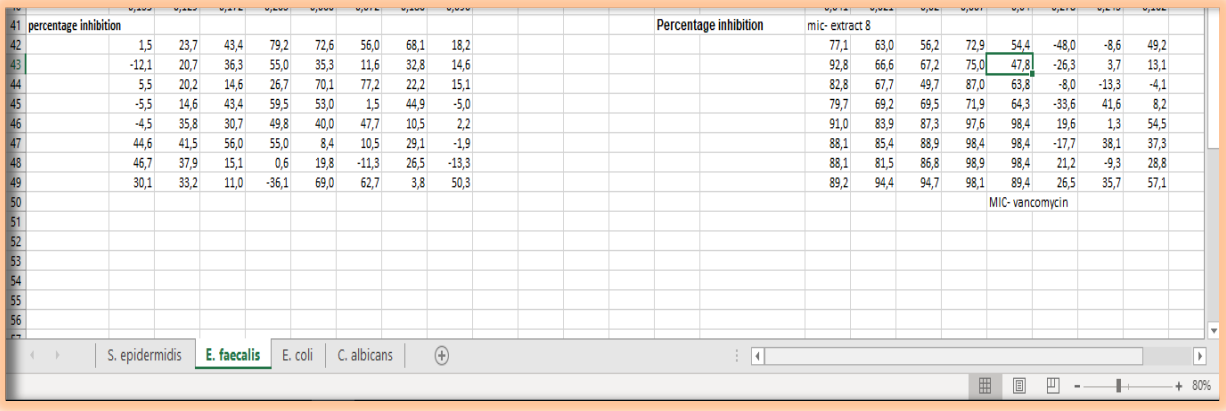
Percentage inhibition								
-11	-28	-49	-41	-51	-46	-19	-66
-24	-24	-35	-32	-42	-26	-13	-33
-15	-1	-30	-24	-26	46	-53	-33
-1	-32	-9	-19	-38	-28	13	-21
-4	33	2	4	22	26	6	8
-9	-2	33	17	6	15	56	15
-12	6	0	0	11	11	-17	-3
-23	-12	-16	-26	3	7	-13	7

Extract 6	No inhibition
Extract 7	No inhibition

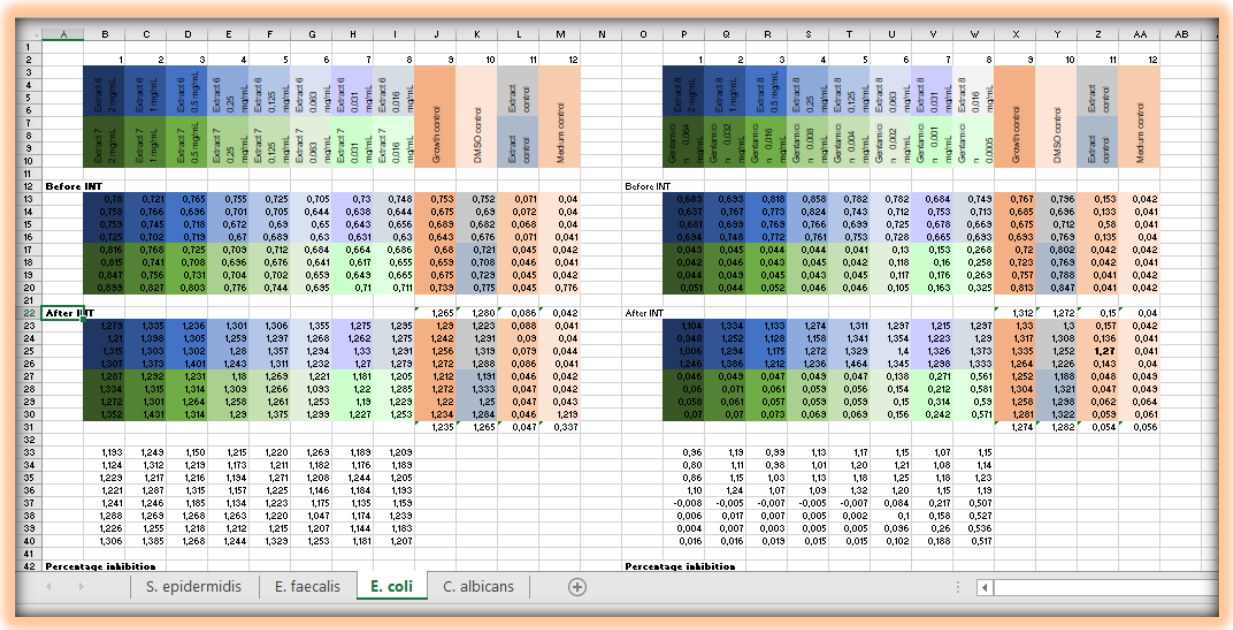
Percentage inhibition								
MIC - Extract 8								
90,772	41,254	7,5563	15,016	33,537	3,0547	-4,534	8,8424
93,215	37,653	-10,84	4,9839	-13,02	5,2412	18,875	7,9421
93,215	27,749	19,389	13,087	34,18	16,174	29,035	20,547
94,244	-6,463	-5,82	-3,119	3,5691	-19,97	15,145	8,4566
99,176	93,408	97,293	94,114	98,823	96,469	26,545	7,9459
97,881	98,234	94,35	99,058	97,999	98,941	23,602	27,134
99,294	97,881	96,586	92,466	92,466	95,291	14,656	-0,059
95,998	97,528	87,051	90,818	88,935	93,408	19,953	6,651
					MIC - Vancomycin		
Extract 8	2 mg/mL						
Vancomycin	0,002 mg/mL						

ANX3 (B): *Enterococcus faecalis*



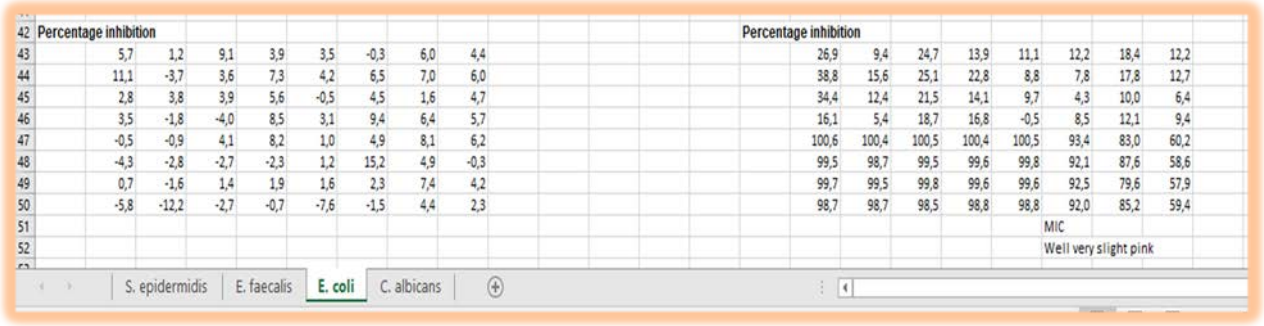


ANX3 (C) *Escherichia coli*



	1	2	3	4	5	6	7	8	9	10	11	12
	Extract 6 2 mg/mL	Extract 6 1 mg/mL	Extract 6 0.5 mg/mL	Extract 6 0.25 mg/mL	Extract 6 0.125 mg/mL	Extract 6 0.063 mg/mL	Extract 6 0.031 mg/mL	Extract 6 0.016 mg/mL	Growth control	DMSO control	Extract control	Medium control
	Extract 7 2 mg/mL	Extract 7 1 mg/mL	Extract 7 0.5 mg/mL	Extract 7 0.25 mg/mL	Extract 7 0.125 mg/mL	Extract 7 0.063 mg/mL	Extract 7 0.031 mg/mL	Extract 7 0.016 mg/mL			Extract control	
Before INT	0,78	0,721	0,765	0,755	0,725	0,705	0,73	0,748	0,753	0,752	0,071	0,04
	0,758	0,766	0,696	0,701	0,705	0,644	0,638	0,644	0,675	0,69	0,072	0,04
	0,759	0,745	0,716	0,672	0,68	0,65	0,643	0,656	0,683	0,682	0,068	0,04
	0,725	0,702	0,715	0,67	0,683	0,63	0,631	0,63	0,643	0,676	0,071	0,041
	0,816	0,768	0,725	0,709	0,712	0,684	0,664	0,686	0,68	0,721	0,045	0,042
	0,815	0,741	0,708	0,696	0,676	0,641	0,617	0,655	0,659	0,708	0,046	0,041
	0,847	0,756	0,731	0,704	0,702	0,659	0,643	0,665	0,675	0,723	0,045	0,042
	0,839	0,827	0,803	0,776	0,744	0,695	0,71	0,711	0,739	0,775	0,045	0,776
After INT									1,265	1,280	0,086	0,042
	1,279	1,335	1,236	1,301	1,306	1,355	1,275	1,295	1,29	1,223	0,088	0,041
	1,21	1,336	1,305	1,253	1,297	1,268	1,262	1,275	1,242	1,231	0,09	0,04
	1,315	1,303	1,302	1,28	1,357	1,294	1,33	1,291	1,256	1,319	0,073	0,044
	1,307	1,373	1,401	1,243	1,311	1,232	1,27	1,279	1,272	1,288	0,086	0,041
	1,287	1,232	1,231	1,18	1,263	1,221	1,181	1,205	1,212	1,191	0,046	0,042
	1,334	1,315	1,314	1,309	1,266	1,093	1,22	1,285	1,272	1,333	0,047	0,042
	1,272	1,301	1,264	1,258	1,261	1,253	1,19	1,229	1,22	1,25	0,047	0,043
	1,352	1,431	1,314	1,29	1,375	1,299	1,227	1,253	1,234	1,284	0,046	1,219
									1,235	1,265	0,047	0,337
	1,193	1,243	1,150	1,215	1,220	1,269	1,183	1,209				
	1,124	1,312	1,213	1,173	1,211	1,182	1,176	1,183				
	1,223	1,217	1,216	1,194	1,211	1,208	1,244	1,205				
	1,221	1,287	1,315	1,157	1,225	1,146	1,184	1,193				
	1,241	1,246	1,185	1,134	1,223	1,175	1,135	1,153				
	1,288	1,269	1,268	1,263	1,220	1,047	1,174	1,239				
	1,226	1,255	1,218	1,212	1,215	1,207	1,144	1,183				
	1,306	1,385	1,268	1,244	1,329	1,253	1,181	1,207				
Percentage inhibition												

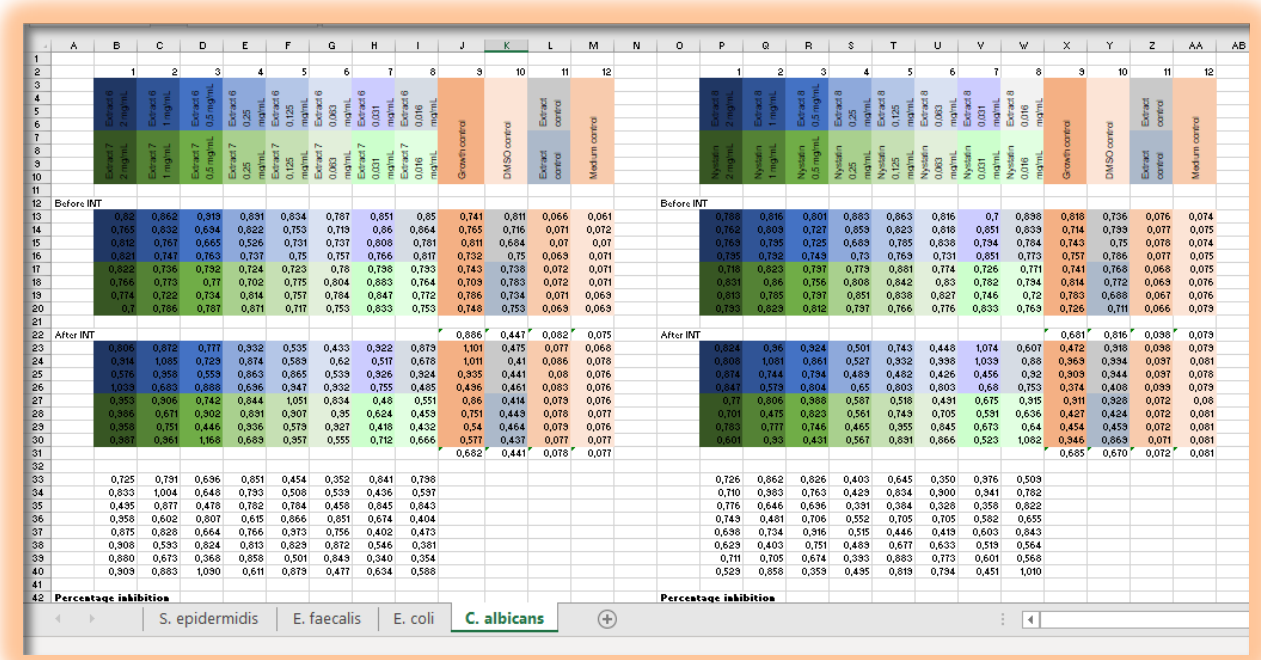
	1	2	3	4	5	6	7	8	9	10	11	12
	Extract 8 2 mg/mL	Extract 8 1 mg/mL	Extract 8 0.5 mg/mL	Extract 8 0.25 mg/mL	Extract 8 0.125 mg/mL	Extract 8 0.063 mg/mL	Extract 8 0.031 mg/mL	Extract 8 0.016 mg/mL	Growth control	DMSO control	Extract control	Medium control
	Gentamicin 0.004 mg/mL	Gentamicin 0.002 mg/mL	Gentamicin 0.016 mg/mL	Gentamicin 0.008 mg/mL	Gentamicin 0.004 mg/mL	Gentamicin 0.002 mg/mL	Gentamicin 0.001 mg/mL	Gentamicin 0.0005 mg/mL				
Before INT	0,683	0,633	0,818	0,858	0,782	0,782	0,684	0,743	0,767	0,796	0,153	0,042
	0,637	0,767	0,773	0,824	0,743	0,712	0,753	0,713	0,685	0,696	0,133	0,041
	0,631	0,633	0,763	0,766	0,689	0,725	0,678	0,663	0,675	0,712	0,58	0,041
	0,634	0,748	0,712	0,761	0,753	0,728	0,665	0,633	0,693	0,769	0,135	0,04
	0,043	0,045	0,044	0,044	0,041	0,13	0,153	0,268	0,72	0,802	0,042	0,042
	0,042	0,046	0,043	0,045	0,042	0,118	0,16	0,258	0,723	0,769	0,042	0,041
	0,044	0,043	0,045	0,043	0,045	0,117	0,176	0,269	0,757	0,788	0,041	0,042
	0,051	0,044	0,052	0,046	0,046	0,105	0,163	0,325	0,813	0,847	0,041	0,042
After INT									1,312	1,272	0,15	0,04
	1,104	1,334	1,133	1,274	1,311	1,297	1,215	1,297	1,33	1,3	0,157	0,042
	0,348	1,252	1,128	1,158	1,341	1,354	1,223	1,23	1,317	1,308	0,136	0,041
	1,006	1,294	1,115	1,272	1,329	1,4	1,326	1,373	1,335	1,252	**1,27**	0,041
	1,246	1,386	1,212	1,236	1,464	1,345	1,238	1,333	1,264	1,226	0,143	0,04
	0,046	0,043	0,047	0,043	0,047	0,138	0,211	0,561	1,252	1,188	0,048	0,043
	0,06	0,071	0,061	0,053	0,056	0,154	0,212	0,581	1,304	1,321	0,047	0,043
	0,058	0,061	0,057	0,053	0,053	0,15	0,314	0,59	1,258	1,236	0,062	0,064
	0,07	0,07	0,073	0,069	0,069	0,156	0,242	0,571	1,281	1,322	0,059	0,061
									1,274	1,282	0,054	0,056
	0,96	1,19	0,99	1,13	1,17	1,15	1,07	1,15				
	0,80	1,11	0,98	1,01	1,20	1,21	1,08	1,14				
	0,86	1,15	1,03	1,13	1,18	1,25	1,18	1,23				
	1,10	1,24	1,07	1,09	1,32	1,20	1,15	1,19				
	-0,008	-0,005	-0,007	-0,005	-0,007	0,084	0,217	0,507				
	0,006	0,017	0,007	0,005	0,002	0,1	0,158	0,527				
	0,004	0,007	0,003	0,005	0,005	0,096	0,26	0,536				
	0,016	0,016	0,019	0,015	0,015	0,102	0,188	0,517				
Percentage inhibition												



	1	2	3	4	5	6	7	8	9	10	11	12
Percentage inhibition												
	5,7	1,2	9,1	3,9	3,5	-0,3	6,0	4,4				
	11,1	-3,7	3,6	7,3	4,2	6,5	7,0	6,0				
	2,8	3,8	3,9	5,6	-0,5	4,5	1,6	4,7				
	3,5	-1,8	-4,0	8,5	3,1	9,4	6,4	5,7				
	-0,5	-0,9	4,1	8,2	1,0	4,9	8,1	6,2				
	-4,3	-2,8	-2,7	-2,3	1,2	15,2	4,9	-0,3				
	0,7	-1,6	1,4	1,9	1,6	2,3	7,4	4,2				
	-5,8	-12,2	-2,7	-0,7	-7,6	-1,5	4,4	2,3				
											MIC	
											Well very slight pink	

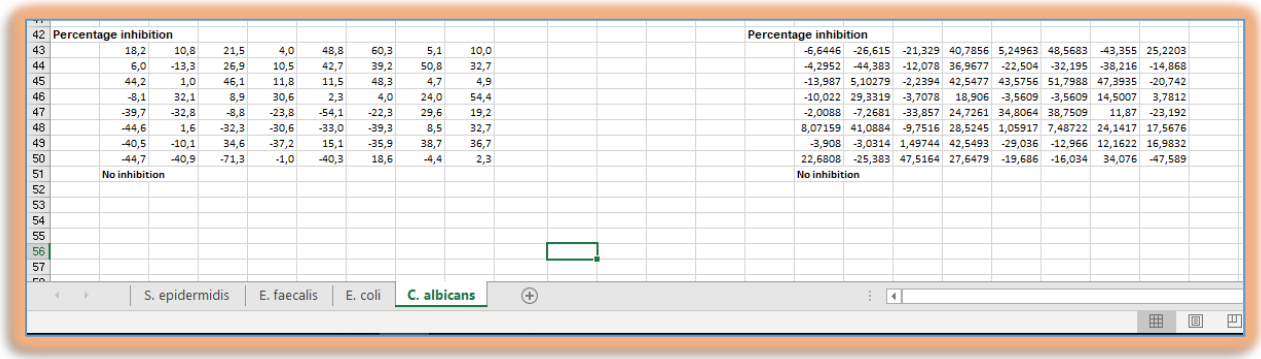
	1	2	3	4	5	6	7	8	9	10	11	12
Percentage inhibition												
	26,9	9,4	24,7	13,9	11,1	12,2	18,4	12,2				
	38,8	15,6	25,1	22,8	8,8	7,8	17,8	12,7				
	34,4	12,4	21,5	14,1	9,7	4,3	10,0	6,4				
	16,1	5,4	18,7	16,8	-0,5	8,5	12,1	9,4				
	100,6	100,4	100,5	100,4	100,5	93,4	83,0	60,2				
	99,5	98,7	99,5	99,6	99,8	92,1	87,6	58,6				
	99,7	99,5	99,8	99,6	99,6	92,5	79,6	57,9				
	98,7	98,7	98,5	98,8	98,8	92,0	85,2	59,4				

ANX3 (D): *Candida albicans*



	1	2	3	4	5	6	7	8	9	10	11	12
	Extract 6 2 mg/mL	Extract 6 1 mg/mL	Extract 6 0.5 mg/mL	Extract 6 0.25 mg/mL	Extract 6 0.125 mg/mL	Extract 6 0.063 mg/mL	Extract 6 0.031 mg/mL	Extract 6 0.016 mg/mL	Growth control	DMSO control	Extract control	Medium control
	Extract 7 2 mg/mL	Extract 7 1 mg/mL	Extract 7 0.5 mg/mL	Extract 7 0.25 mg/mL	Extract 7 0.125 mg/mL	Extract 7 0.063 mg/mL	Extract 7 0.031 mg/mL	Extract 7 0.016 mg/mL			Extract control	
Before INT												
	0,82	0,862	0,919	0,891	0,834	0,787	0,851	0,85	0,741	0,811	0,066	0,061
	0,765	0,832	0,634	0,822	0,753	0,719	0,86	0,864	0,765	0,716	0,071	0,072
	0,812	0,767	0,665	0,526	0,731	0,737	0,808	0,781	0,811	0,684	0,07	0,07
	0,821	0,747	0,763	0,737	0,75	0,757	0,766	0,817	0,732	0,75	0,063	0,071
	0,822	0,736	0,792	0,724	0,723	0,78	0,798	0,793	0,743	0,738	0,072	0,071
	0,766	0,773	0,77	0,702	0,715	0,804	0,883	0,764	0,709	0,783	0,072	0,071
	0,774	0,722	0,734	0,814	0,757	0,784	0,847	0,772	0,786	0,734	0,071	0,069
	0,7	0,786	0,787	0,871	0,717	0,753	0,833	0,753	0,748	0,753	0,063	0,063
After INT									0,686	0,447	0,082	0,075
	0,806	0,872	0,777	0,932	0,535	0,433	0,322	0,879	1,101	0,475	0,077	0,068
	0,914	1,085	0,723	0,874	0,589	0,62	0,517	0,678	1,011	0,41	0,086	0,078
	0,576	0,358	0,553	0,863	0,865	0,539	0,326	0,324	0,935	0,441	0,08	0,076
	1,033	0,683	0,888	0,636	0,347	0,332	0,755	0,485	0,496	0,461	0,083	0,076
	0,953	0,906	0,742	0,844	1,051	0,834	0,48	0,551	0,86	0,414	0,079	0,076
	0,386	0,611	0,902	0,831	0,907	0,95	0,624	0,459	0,751	0,443	0,078	0,077
	0,358	0,751	0,446	0,936	0,579	0,327	0,418	0,432	0,54	0,464	0,079	0,076
	0,387	0,361	1,168	0,683	0,357	0,555	0,712	0,666	0,577	0,437	0,077	0,077
									0,682	0,441	0,078	0,077
	0,725	0,731	0,696	0,851	0,454	0,352	0,841	0,798				
	0,833	1,004	0,648	0,793	0,508	0,533	0,436	0,597				
	0,435	0,877	0,478	0,782	0,784	0,458	0,845	0,843				
	0,358	0,602	0,807	0,615	0,866	0,851	0,674	0,404				
	0,875	0,828	0,664	0,766	0,973	0,756	0,402	0,473				
	0,908	0,593	0,824	0,813	0,829	0,872	0,546	0,381				
	0,880	0,673	0,368	0,858	0,501	0,849	0,340	0,354				
	0,909	0,883	1,090	0,611	0,879	0,477	0,634	0,588				
Percentage inhibition												

	1	2	3	4	5	6	7	8	9	10	11	12
	Extract 8 2 mg/mL	Extract 8 1 mg/mL	Extract 8 0.5 mg/mL	Extract 8 0.25 mg/mL	Extract 8 0.125 mg/mL	Extract 8 0.063 mg/mL	Extract 8 0.031 mg/mL	Extract 8 0.016 mg/mL	Growth control	DMSO control	Extract control	Medium control
	Nystatin 2 mg/mL	Nystatin 1 mg/mL	Nystatin 0.5 mg/mL	Nystatin 0.25 mg/mL	Nystatin 0.125 mg/mL	Nystatin 0.063 mg/mL	Nystatin 0.031 mg/mL	Nystatin 0.016 mg/mL			Extract control	
Before INT												
	0,788	0,816	0,801	0,883	0,863	0,816	0,7	0,838	0,818	0,736	0,076	0,074
	0,762	0,803	0,727	0,859	0,823	0,818	0,851	0,833	0,714	0,739	0,077	0,075
	0,763	0,735	0,725	0,689	0,785	0,838	0,794	0,784	0,743	0,75	0,078	0,074
	0,735	0,732	0,743	0,73	0,763	0,731	0,851	0,773	0,757	0,786	0,077	0,075
	0,718	0,823	0,737	0,779	0,881	0,774	0,726	0,771	0,741	0,768	0,068	0,075
	0,831	0,86	0,756	0,808	0,842	0,83	0,782	0,794	0,814	0,772	0,069	0,076
	0,813	0,785	0,737	0,851	0,838	0,827	0,746	0,72	0,783	0,688	0,067	0,076
	0,793	0,823	0,812	0,737	0,766	0,776	0,833	0,763	0,726	0,711	0,066	0,073
After INT									0,681	0,816	0,038	0,079
	0,824	0,96	0,924	0,501	0,743	0,448	1,074	0,607	0,472	0,918	0,038	0,079
	0,808	1,081	0,861	0,527	0,932	0,938	1,039	0,88	0,969	0,934	0,037	0,081
	0,874	0,744	0,734	0,483	0,482	0,426	0,456	0,32	0,909	0,944	0,037	0,078
	0,847	0,573	0,804	0,65	0,803	0,803	0,68	0,753	0,374	0,408	0,039	0,079
	0,77	0,806	0,988	0,587	0,518	0,431	0,675	0,915	0,911	0,928	0,072	0,08
	0,701	0,475	0,823	0,561	0,743	0,705	0,531	0,636	0,427	0,424	0,072	0,081
	0,783	0,777	0,746	0,465	0,355	0,845	0,673	0,64	0,454	0,459	0,072	0,081
	0,601	0,33	0,431	0,567	0,831	0,866	0,523	1,082	0,946	0,869	0,071	0,081
									0,685	0,670	0,072	0,081
	0,726	0,862	0,826	0,403	0,645	0,350	0,376	0,509				
	0,710	0,383	0,763	0,429	0,834	0,300	0,941	0,782				
	0,776	0,646	0,636	0,331	0,384	0,328	0,358	0,822				
	0,743	0,481	0,706	0,552	0,705	0,705	0,582	0,655				
	0,638	0,734	0,316	0,515	0,446	0,419	0,603	0,843				
	0,629	0,403	0,751	0,489	0,677	0,633	0,519	0,564				
	0,711	0,705	0,674	0,393	0,883	0,773	0,601	0,568				
	0,529	0,858	0,359	0,495	0,819	0,794	0,451	1,010				
Percentage inhibition												



Percentage inhibition																								
	18,2	10,8	21,5	4,0	48,8	60,3	5,1	10,0					-6,6446	-26,615	-21,329	40,7856	5,24963	48,5683	-43,355	25,2203				
	6,0	-13,3	26,9	10,5	42,7	39,2	50,8	32,7					-4,2952	-44,383	-12,078	36,9677	-22,504	-32,195	-38,216	-14,868				
	44,2	1,0	46,1	11,8	11,5	48,3	4,7	4,9					-13,987	5,10279	-2,2394	42,5477	43,5756	51,7988	47,3935	-20,742				
	-8,1	32,1	8,9	30,6	2,3	4,0	24,0	54,4					-10,022	29,3319	-3,7078	18,906	-3,5609	-3,5609	14,5007	3,7812				
	-39,7	-32,8	-8,8	-23,8	-54,1	-22,3	29,6	19,2					-2,0088	-7,2681	-33,857	24,7261	34,8064	38,7509	11,87	-23,192				
	-44,6	1,6	-32,3	-30,6	-33,0	-39,3	8,5	32,7					8,07159	41,0884	-9,7516	28,5245	1,05917	7,48722	24,1417	17,5676				
	-40,5	-10,1	34,6	-37,2	15,1	-35,9	38,7	36,7					-3,908	-3,0314	1,49744	42,5493	-29,036	-12,966	12,1622	16,9832				
	-44,7	-40,9	-71,3	-1,0	-40,3	18,6	-4,4	2,3					22,6808	-25,383	47,5164	27,6479	-19,686	-16,034	34,076	-47,589				
	No inhibition												No inhibition											

Annexure 4

Chapter 8 related raw data

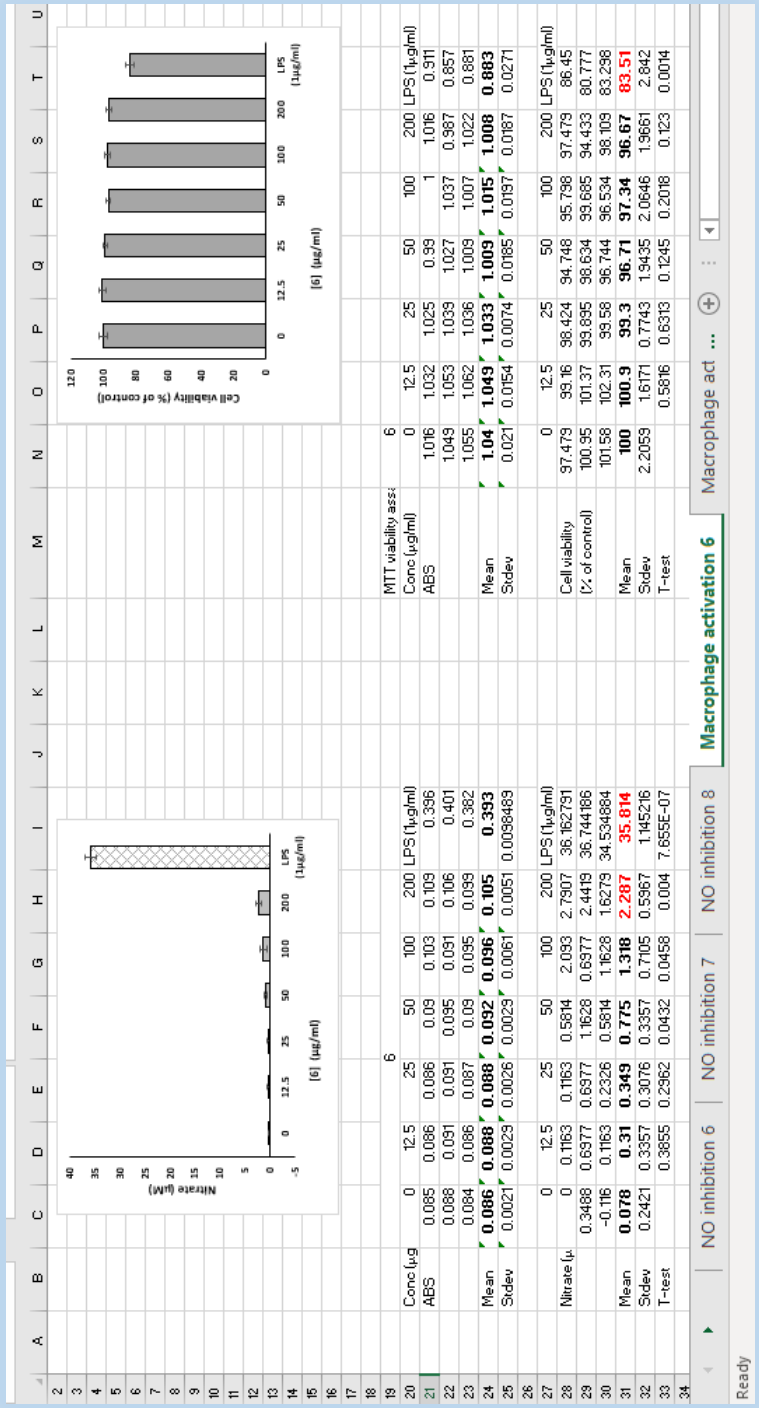
IN VITRO PRO- AND ANTI-INFLAMMATORY SCREENING OF *PAPPEA CAPENSIS EXTRACTS*

NO inhibition 6 (Extract no 6/ Ethanoic extract)

NO inhibition 7 (Extract no 7/ Aqueous (water) extract)

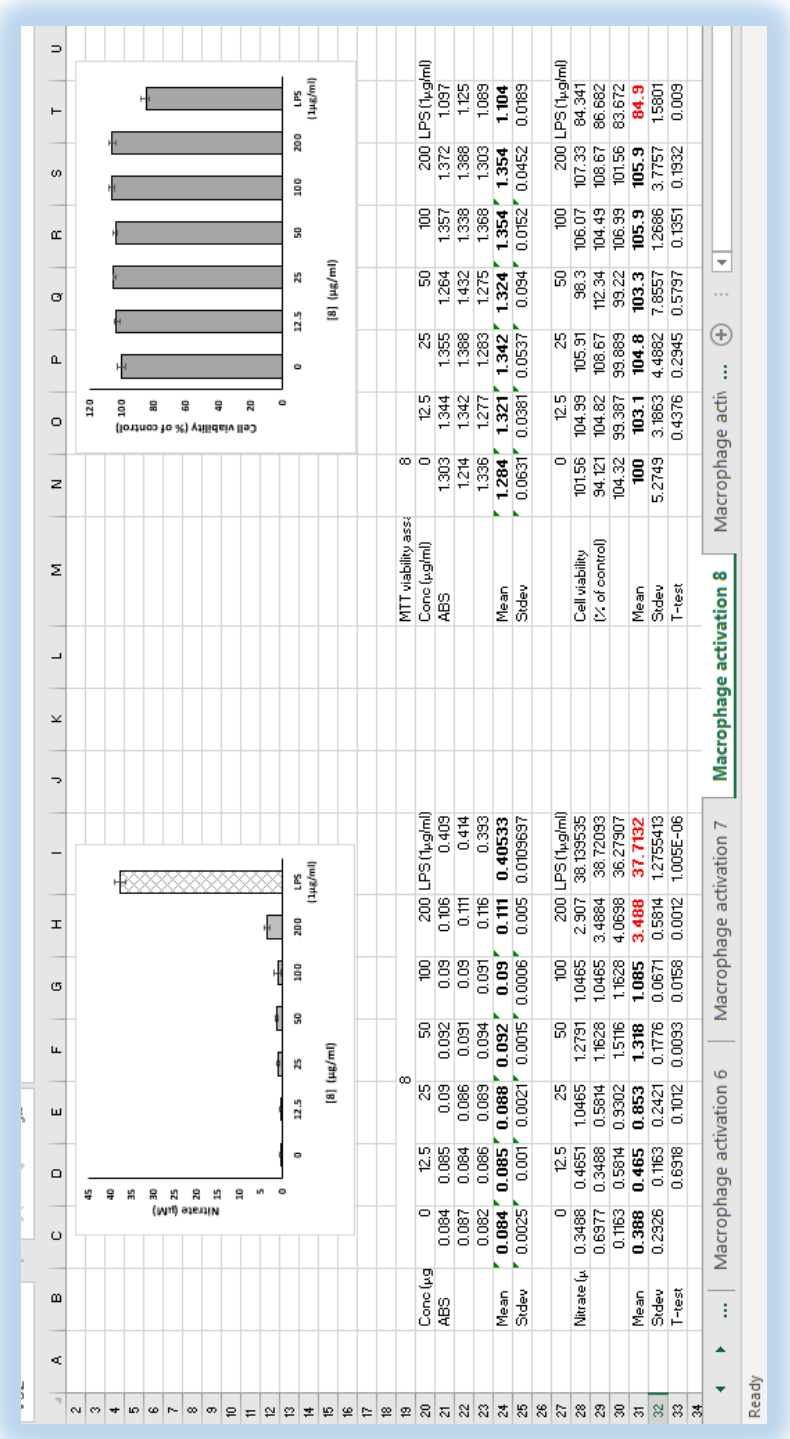
NO inhibition 8 (Extract no 8/ methanolic extract)

Macrophage activation 6 (Extract no 6/ Ethanoic extract)



Macrophage activation 7 (Extract no 7/ Aquoues (water) extract)

Macrophage activation 8 (Extract no 8/ methanolic extract)



Macrophage activation 7 (Extract no 7/ Aquoues (water) extract with and without PMB)