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Antimicrobial activity of *Zanthoxylum davyi* and *Ximenia caffra*

by

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A dissertation submitted in partial fulfillment of the requirements for the degree

MAGISTER SCIENTIAE

in

PHARMACOLOGY

in the

FACULTY OF HEALTH SCIENCES

at the

UNIVERSITY OF PRETORIA

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October 2015

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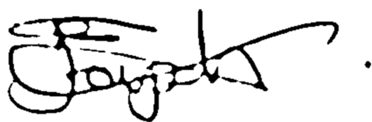
Signature: 

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Acknowledgements

Prof Vanessa Steenkamp, whose guidance, support and encouragement was instrumental in the completion of this study. Without you, this thesis would not have been possible.

Dr Gisela Jooné, for her instruction and advice regarding the microbiology component of this study.

Prof Duncan Cromarty, for his invaluable input and assistance regarding the HPLC component of this study.

Mrs Alet van Tonder, for her patience, guidance and assistance regarding the cell work component of this study.

Dr CJS Edgell at the University of North Carolina for the EA.hy926 cells.

The Department of Microbiology, University of Pretoria, NHLS for providing the microorganisms used in this study.

Dr Norbert Hahn and the South African National Biodiversity Institute, for plant collection.

The Laboratory for Microscopy and Microanalysis for assistance with Scanning Electron Microscopy.

The Department of Physiology for use of the PlasDIC microscope.

The Department of Pharmacology, University of Pretoria, Research Committee of School of Medicine and the National Research Foundation for the financial support in order to be able to conduct and complete this study.

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My friends and colleagues who have supported me and encouraged me throughout my studies, of special mention is Keagile Basetsana Lepule.

Lastly, my parents, for this exceptional opportunity granted to me, their endless faith in my abilities and the opportunity to focus fully on my studies, taking care of all responsibilities, throughout my many years of study.

"Our greatest battles are that with our own minds".

- *Jameson Frank*

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Poster Publications

- Cytotoxicity and phytochemical analysis of extracts of *Zanthoxylum davyi* and *Ximenia caffra*

- Antibacterial and antifungal activity of *Zanthoxylum davyi* and *Ximenia caffra* against planktonic and biofilm species of Staphylococcus aureus and Candida albicans

Awards

- 3rd Prize
  Poster Presentation:
  Antibacterial and antifungal activity of Zanthoxylum davyi and Ximenia caffra against planktonic and biofilm species of Staphylococcus aureus and Candida albicans.
  Wits Pharmacology and Toxicology Congress (31 August- 2 September 2015), South Africa.

- 3rd Prize
  Poster Presentation:
  Antibacterial and antifungal activity of Zanthoxylum davyi and Ximenia caffra against planktonic and biofilm species of Staphylococcus aureus and Candida albicans.
  Faculty Day (18 August 2015), Faculty of Health Sciences, University of Pretoria, South Africa.

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Abstract

Resident skin flora are usually non-pathogenic and prevent colonization of harmful microbes by competing for nutrients and stimulation of the immune system. However, these resident microbes can enter the bloodstream of immunocompromised individuals and cause life-threatening diseases. *Staphylococcus aureus* and *Candida albicans* are examples of such microbes. *S. aureus* is a pathogen responsible for the increased occurrence of nosocomial and community-acquired infections. *C. albicans* causes a wide variety of fungal infections especially in immunocompromised individuals. Due to the increased use of antibiotics, resistant strains have increased in appearance over the past years. As treatment options become limited, the need for novel antimicrobial drugs becomes apparent. Most of the antibacterial drug classes known today are derived from natural products. The aim of this study was to determine the antimicrobial activity of bark extracts of *Z. davyi* and *X. caffra* against planktonic and biofilm forms of *C. albicans* and *S. aureus*, as well as to determine the phytochemistry and cytotoxicity of the crude extracts.

Zanthoxylum davyi and *Ximenia caffra* were chosen for evaluation based on their ethnomedicinal uses. Hot water, methanol and dichloromethane extracts were prepared for each plant. Thin-layer chromatography (TLC) was employed to identify the possible classes of phytochemical compounds present in each extract. High-performance liquid chromatography (HPLC) was used to determine the chemical fingerprints of the plant extracts as well as to determine the identity of phytochemicals via co-chromatography with known standards. The disc diffusion assay was employed as a qualitative crude screening method to identify the extracts which displayed antimicrobial potential against two clinical and one standard strain of *S. aureus* and *X. caffra.* The broth microdilution assay was employed to determine the minimum inhibitory concentration (MIC) of the extracts which displayed antimicrobial activity as determined by the disc diffusion assay. The crude extracts were tested on biofilms of *S. aureus* and *C. albicans* using the biofilm inhibition assay

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with quantification by the crystal violet assay. Anti-biofilm activity was further investigated using scanning electron microscopy (SEM) and drug interactions were determined using the checkerboard assay which allowed for the calculation of the fractional inhibitory concentration index. Cytotoxicity of the crude extracts was assessed using MCF-7 human breast cancer cells and EA.hy926 human umbilical vein cells. Effects on crude cell morphology was visualized using phase contrast and PlasDIC microscopy.

Using TLC phenolic acids, terpenoids and sterols were detected in all extracts. HPLC identified antimicrobial phenolic acids, flavonoids and alkaloids, with ferulic acid, nitidine, quercetin and gallic acid in the highest concentrations. The activity of all extracts were considered to be clinically insignificant against planktonic *S. aureus* and *C. albicans* (>1mg/ml). All extracts displayed a clinically significant ($p < 0.05$) range of concentrations which indicated anti-biofilm activity. Synergism was evident for all extracts when the two plants were combined. From SEM analysis it was evident that the extracts caused notable disintegration of the exopolysaccharide matrix of biofilms.

Although all the extracts displayed poor cytotoxicity, the EA.hy926 cell line was more susceptible to the extracts than the MCF-7 cell line. This low cytotoxicity could be ascribed to the presence of antioxidant compounds detected in all extracts. Signs of apoptosis, such as blebbing, apoptotic bodies and nuclear condensation, was evident in EA.hy926 cells, with visualization using phase contrast and PlasDIC microscopy. At the highest concentrations, signs of necrosis were observed for MCF-7 cells which include swelling and enlargement of cells.

This study provides scientific support for the antibacterial and antifungal activity of *Z. davyi* and *X. caffra*. It was shown that these plants could be used as alternative antimicrobials, especially against biofilms of *S. aureus* and *C. albicans*. To the author's knowledge, this is the first report on the antimicrobial

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activity of the bark extract of *Ximenia caffra*. Prominent antimicrobial potential together with the low cytotoxicity supports the therapeutic potential of these plants. Various antimicrobial phytochemicals were detected in these plant extracts and it would appear as if antimicrobial activity is attributed to their combined activity rather than as a result of a single compound. This study also shows that it could be useful to combine these plants to be used as a single antimicrobial regimen or synergistically with conventional antimicrobials. They could also be used to increase the sensitivity of microorganisms to conventional antimicrobials. Further research regarding isolation of the active compounds is warranted.

Keywords: antimicrobial, biofilms, *Candida albicans*, cytotoxicity, microscopy, phytochemicals, *Staphylococcus aureus*, *Ximenia caffra*, *Zanthoxylum davyi*.

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List of Abbreviations

-	Not determined
°C	Degrees Celsius
µg	Microgram
µg/mL	Microgram per milliliter
µL	Microliter
µm	Micrometer
A431	Epidermoid carcinoma cell line
AIDS	Acquired immune deficiency syndrome
$AlCl_3$	Aluminium trichloride
ANOVA	Analysis of variance
ASC	Ascorbic acid
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
Aur	Aureolysin
Bap	Biofilm associated protein
BIC	Biofilm inhibitory concentration
C. a	*Candida albicans*
C_{18}	Carbon 18
CAF	Caffeic acid
CAT	Catechin
Cdc2	Cell division cycle 2
Cdc25C	Cell division cycle 25C
Cells/mL	Cells per milliliter
CFU	Colony forming units
CFU/mL	Colony forming units per ml
CHE	Chelerythrine
Chk1	Cyclin-dependent kinase 1
Chk2	Cyclin-dependent kinase 2
ClfB	Clumping factor B
Cm	centimeters
CO_2	Carbon dioxide
COL	Colchicine
COU	Coumaric acid

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DCM	Dichloromethane
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DnaK	Chaperone Dna K
drC	Dynein regulatory complex
EA.hy926	Human umbilical vein cell line
Eap	Extracellular adherence protein
Efg1p	Exit from G1 protein
EPS	Exopolysaccharide
ERG11	Ergosterol 11
ERG3	Ergosterol 3
EtOH	Ethanol
FCS	Foetal calf serum
FER	Ferulic acid
fhuD	Iron (III) hydroxamate binding protein D
FIC	Fractional inhibitory concentration
Flo8	Flocculation 8
fnbA	Fibronectin-binding protein A
g	grams
g	gravity
G2/M	Gap2/mitosis
GAL	Gallic acid
Gal4	Galactose responsive transcription factor 4
h	hours
H_2O	Water
H_2SO_4	Sulphuric acid
HBSS	Hank's balanced salt solution
HeLa	Human cervical cancer cell line
HIV	Human immunodeficiency virus
Hla	Human leukocyte antigen
Hlb	Beta toxin
HMWPM	High molecular weight polymeric materials
HPLC	High-performance liquid chromatography

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HT29	Colon carcinoma cell line
HW	Hot water
IC_{50}	50% Inhibitory concentration
INT	Iodonitrotetrazolium chloride
KOH	Potassium hydroxide
M	Molar
MBC	Minimum bactericidal
MCF-7	Human breast cancer cell line
MeOH	Methanol
MFC	Minimum fungicidal concentration
mg/kg	Milligram per kilogram
mg/mL	Milligram per milliliter
MIC	Minimum inhibitory concentration
min	Minutes
mL	Milliliter
mm	Millimetre
mM	Millimolar
MRSA	Methicillin resistant *Staphylococcus aureus*
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
MSSA	Methicillin sensitive *Staphylococcus aureus*
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
N/A	Not applicable
ND	Not determined
NH	National herbarium
NH_3	Ammonia
NIAID	National Institute of Allergy and Infectious Diseases
NIT	Nitidine
nm	Nanometers
NRU	Neutral red uptake
OD520	Optical density at 520 nanometres
pH	Acidity index
PIA	Polysaccharide intercellular adhesion
QS	Quorum sensing
QSI	Quorum sensing inhibitor

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QUE	Quercetin
RES	Resazurin reduction assay
R_f	Retention factor
Rfx2	Regulatory factor x2
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RUT	Rutin
S. a	*Staphylococcus aureus*
S.E.M	Standard error of the mean
SAL	Salicylic acid
SAN	Sanguinarine
SANBI	South African National Biodiversity Institute
SarA	Staphylococcal accessory regulator A
SasG	*Staphylococcus aureus* surface protein G
ScpA	Segregation and condensation protein A
SEM	Scanning electron microscopy
ΣFIC	Sum of the fractional inhibitory concentration
SIN	Sinapic acid
SplaF	Serine protease
SRB	Sulforhodamine B
SspA	Stringent starvation protein A
SspB	Stringent starvation protein B
SstD	*Staphylococcus aureus* lipoprotein (standard data class)
SYR	Syringic acid
TCA	Trichloroacetic acid
TLC	Thin-layer chromatography
UFLC	Ultra-fast liquid chromatography
UK	United Kingdom
US	United States
UV	Ultra-violet
v/v	Volume per volume
v/w	Volume per weight
VAN	Vanillic acid
VCC	Viable colony count

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w/v	Weight per volume
XC	*Ximenia caffra*
XC-DCM-E	*Ximenia caffra* dichloromethane extract
XC-HW-E	*Ximenia caffra* hot water extract
XC-MeOH-E	*Ximenia caffra* methanol extract
ZD	*Zanthoxylum davyi*
ZD-DCM-E	*Zanthoxylum davyi* dichloromethane extract
ZD-HW-E	*Zanthoxylum davyi* hot water extract
ZD-MeOH-E	*Zanthoxylum davyi* methanol extract

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visualized by PlasDIC microscopy at 40 x magnification, E) untreated, F) treated with 100 µg/mL ZD-HW-E, G) treated with 12.5 µg/mL ZD-HW-E, and H treated with 0.78 µg/mL ZD-HW-E. White arrows indicate signs of stress and black arrows indicate signs of apoptosis. 83

Figure 39: MCF-7 cells visualized by phase contrast microscopy at 10 x magnification, A) untreated, B) treated with 100 µg/ml XC-MeOH-E, C) treated with 12.5 µg/ml XC-MeOH-E, D) treated with 0.78 µg/ml XC-MeOH-E, and cells visualized by PlasDIC microscopy at 40 x magnification, E) untreated, F) treated with 100 µg/mL XC-MeOH-E, G) treated with 12.5 µg/mL XC-MeOH-E, and H treated with 0.78 µg/mL XC-MeOH-E. White arrows indicate signs of stress and black arrows indicate signs of apoptosis 84

Figure 40: MCF-7 cells visualized by phase contrast microscopy at 10 x magnification, A) untreated, B) treated with 100 µg/ml XC-DCM-E, C) treated with 12.5 µg/ml XC-DCM-E, D) treated with 0.78 µg/ml XC-DCM-E, and cells visualized by PlasDIC microscopy at 40 x magnification, E) untreated, F) treated with 100 µg/mL XC-DCM-E, G) treated with 12.5 µg/mL XC-DCM-E, and H treated with 0.78 µg/mL XC-DCM-E. White arrows indicate signs of stress and black arrows indicate signs of apoptosis 85

Figure 41: MCF-7 cells visualized by phase contrast microscopy at 10 x magnification, A) untreated, B) treated with 100 µg/ml XC-HW-E, C) treated with 12.5 µg/ml XC-HW-E, D) treated with 0.78 µg/ml XC-HW-E, and cells visualized by PlasDIC microscopy at 40 x magnification, E) untreated, F) treated with 100 µg/mL XC-HW-E, G) treated with 12.5 µg/mL XC-HW-E, and H treated with 0.78 µg/mL XC-HW-E. White arrows indicate signs of stress and black arrows indicate signs of apoptosis 86

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CHAPTER 1: Introduction

1.1 Overview

In humans, microbial cells greatly outnumber somatic cells.[1] Resident skin flora are usually non-pathogenic and prevent colonization of harmful microbes by competing for nutrients and stimulation of the immune system, however; these resident microbes can enter the bloodstream of immunocompromised individuals and cause life-threatening diseases.[2] *Staphylococcus aureus* and *Candida albicans* are medical examples of such microbes. *S. aureus* is a pathogen responsible for the increased occurrence of nosocomial and community-acquired infections.[3] *C. albicans* causes a wide variety of fungal infections especially in immunocompromised individuals.[4] Due to the increased use of antibiotics, resistant strains have increased in appearance over the past 10 years.[5] As treatment options become limited, the need for novel antimicrobial drugs becomes apparent.[6] In the search for such antibiotics, plants have been indicated as valuable sources and drug leads.[7,8]

1.2 Microorganisms

1.2.1 *Staphylococcus aureus*

S. aureus is classified under the kingdom Eubacteria, phylum Firmicutes and belongs to the family Staphylococcaceae.[9] *S. aureus* are round and resemble grapes under a microscope as they typically grow in clusters (Figure 1).[10] This anaerobic, Gram-positive bacterium is part of the normal skin and nasal flora. However, this bacterium can be successfully pathogenic due to nasal carriage and effective immuno-evasive strategies.[11,12] The shift from colonization to invasive pathogen correlates to the expression of genes involved in pathogenesis. Consistent up-regulation of genes such as *drC*, *fnbA*, *fhuD*, *sstD*, and *hla* are important in staphylococcal pathogenesis.[13] *S. aureus* is well recognised for its role in wound infection and sepsis.[14] It can cause a range of illnesses; which ranges from minor skin infections such as acne, boils, and cellulitis to more serious illnesses such as pneumonia, meningitis, endocarditis and bacteraemia. It is also one of the leading causes of nosocomial infections and postsurgical wound infections.[15] Due to the fact that humans are a natural reservoir for *S. aureus*,

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immunodeficient patients are at increased risk for staphylococcal infection.[3] *S. aureus* has the ability to adapt to different environmental conditions.[16] Resistance to antibiotics can be attributed to chromosomal mutations, extra-chromosomal elements acquired from other bacteria (mobile DNA segments, such as plasmids, transposons, and integrons), and efflux pumps.[17] Misuse/overuse of antibiotics has led to an increase in the proportion of methicillin-resistant *S. aureus* (MRSA) infections worldwide. Furthermore methicillin-resistant strains have already become resistant to other antimicrobial agents as well.[3]

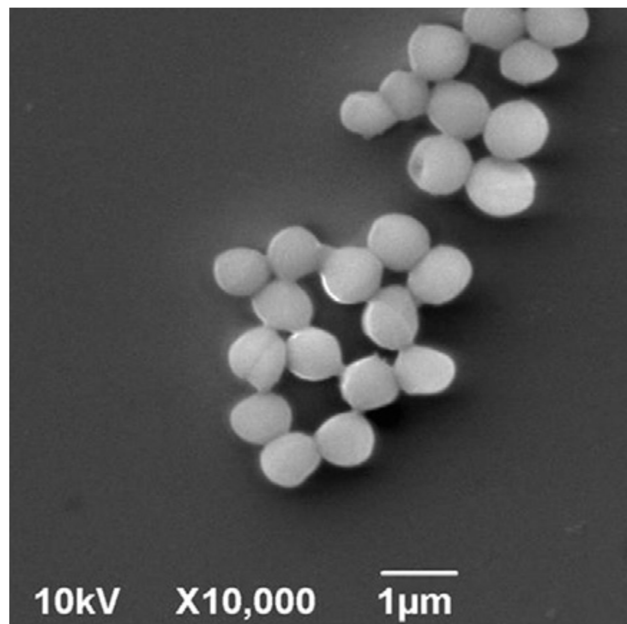

Figure 1: Scanning electron microscopy of *S. aureus* cells.[18]

1.2.2 *Candida albicans*

C. albicans is classified under the kingdom Fungi, phylum Ascomycota and belongs to the family Saccharomycetaceae.[19] It is the most prevalent fungal species in human microbiota and colonizes the gastrointestinal and genito-urinary tracts.[1] *C. albicans* is present in 80% of the human population without causing harmful effects.[20] This fungus can exist either as a commensal or opportunistic pathogen.[21] *C. albicans* is considered a diploid fungus as it grows both yeast cells and filamentous cells.[10,22] Yeast cells (Figure 2A) are important in early infection stages as they have they the ability to disseminate to

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target organs via extravasation.[23] Upon reaching target organs, yeast cells can change morphology to filamentous cells, in response to environmental cues.[10] Filamentous cells (Figure 2B) are required for the establishment of a rooted infection and mortality, which is typical of late-stage infections.[23] When host defence systems are compromised, the mucotaneous surfaces serve as points of entry for such organisms.[24] This explains why patients with cell mediated immunological disorders and severe defects in their phagocytic system, such as those with Acquired Immune Deficiency Syndrome (AIDS) patients, suffer from chronic candida infections of the mucosae and skin.[4] Previous studies have shown that *C. albicans* has surpassed Gram-negative bacilli as the third most commonly isolated bloodstream pathogen in US hospitals.[25]

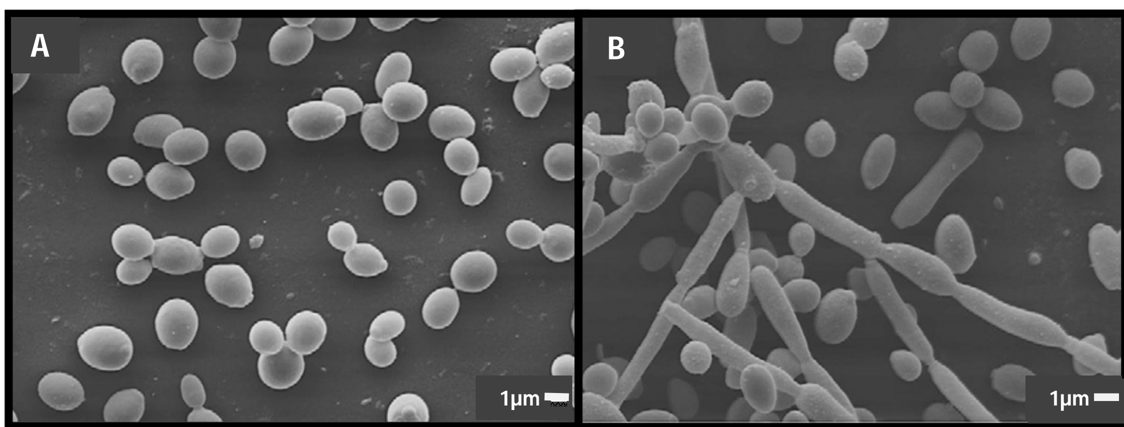

Figure 2: Scanning electron microscopy of A) *C. albicans* yeast cells involved in early infection stages, B) *C. albicans* filamentous cells involved in late infection stages.[26]

Several antifungal drugs are available to treat candidiasis,[27] as described below (1.3.1). Candidiasis describes a number of different disease syndromes which differ in their causes and outcomes.[28,29] Candidiasis includes superficial infections, such as oral thrush and vaginitis, as well as systemic infections and life-threatening diseases.[30] *Candida* infections of the latter category are referred to as candidemia and are mostly limited to severely immunocompromised persons, such as cancer, transplant, and AIDS patients.[30] Repeated therapy for chronic infections has led to an increase in drug resistance over the past years.[31] There are many mechanisms of resistance in *C. albicans*, however, acquired resistance is less common than intrinsic resistance. Intrinsic resistance is found

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naturally among certain fungal strains while acquired resistance is as a result of drug exposure which leads to altered gene expression.[32] Emerging *C. albicans* strains have acquired resistance to azole drugs as well as other commonly used antifungals and this results in treatment failure.[27]

1.2.3 Planktonic microorganisms and biofilms

'Plankton' can be defined as free-floating animals and plants which are distinct from those that are attached.[33] Planktonic microorganisms are single, unattached microorganisms which have properties different from sessile/attached microorganisms of the same species, and are more susceptible to antibiotics (Figure 3).[34] Environmental and other signals can cause phenotypic changes in planktonic microorganisms which increase their hydrophobicity and make them more adherent to surfaces and to each other. These interactions result in the formation of a resistant community structure of microorganisms known as a biofilm.[35] A biofilm is a community of sessile microorganisms embedded in an exopolysaccharide (EPS) matrix. The EPS matrix acts as a barrier which shelters microbes from antibiotics, disinfectants and host defences and this complicates treatment.[36] Biofilm development can be described in three major stages. In the first stage (initial attachment), a planktonic cell will bind reversibly with a surface and if it does not dissociate, it will bind irreversibly mediated by microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). These molecules are important in infection as they enable planktonic cells to bind to host factors such as collagen and fibrinogen. The second stage of biofilm development is maturation. This involves cell division and the production of an EPS matrix which can include host factors, polysaccharides and proteins. The composition of this matrix can vary between different strains of a particular microorganism. The final stage of biofilm development is dispersal. After the accumulation of cells and EPS substance, biofilm cells can revert to planktonic cells and disperse to other areas.[37] Both *S. aureus* and *C. albicans* are capable of forming microbial biofilms.[38] Figure 3 displays the transition process of planktonic cells to a mature biofilm.

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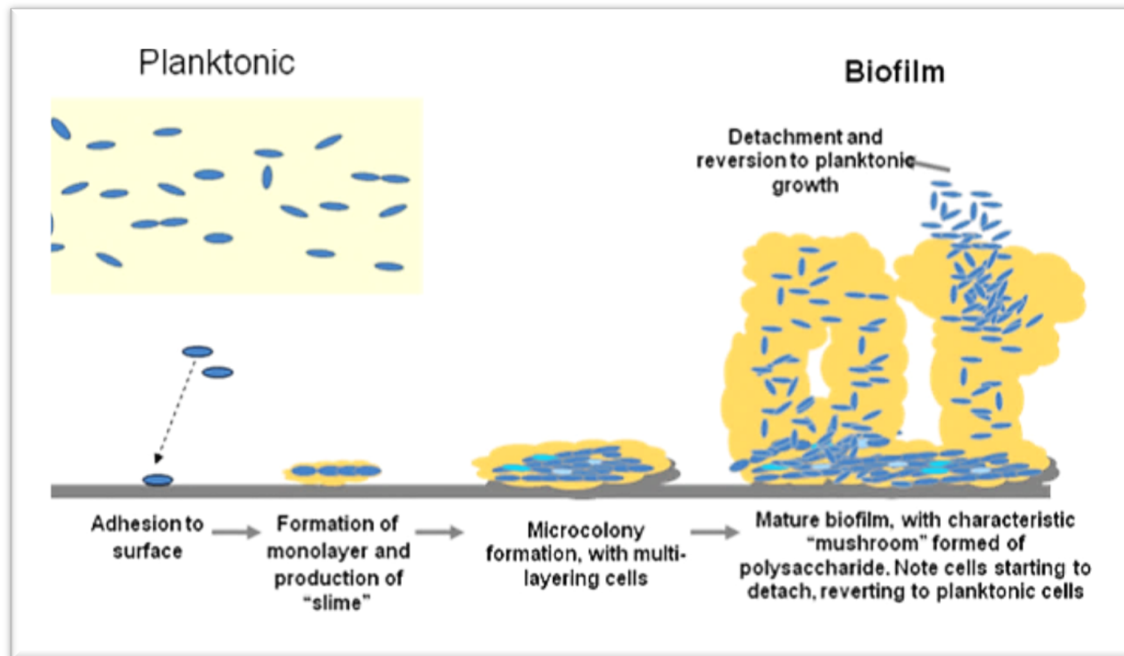

Figure 3: Transition process of planktonic cells into a mature biofilm.[39]

An expanded regulatory network controls biofilm formation in *C. albicans*. *C. albicans* biofilms are dynamic as genes involved in adhesion and metabolism change over time. In addition to the six 'master' regulators of biofilm formation in *C. albicans* mentioned in prior studies,[40-43] three new regulators of biofilm formation (*Flo8*, *Gal4*, and *Rfx2*) are required for its formation *in vitro* and *in vivo*. *Flo8* is required for biofilm at all points while *Gal4* and *Rfx2* are responsible for proper biofilm formation at intermediate time points. Adhesion proteins are differentially expressed over time and biofilm metabolism decreases with time compared to stationary cells.[41-43] While genetics play a vital role in biofilm formation, temperature, cell morphology and cell–cycle growth phase all affect gene expression patterns.[44] Quorum sensing (QS) also regulates biofilm behaviors. QS/cell to cell communication for biofilm formation, produces a variety of virulence factors which are the key causes of multi-drug resistance development and invasive infections.[45]

In *S. aureus* biofilms, polysaccharide intercellular adhesion protein (PIA) plays a vital role in the integrity of biofilms *in vitro* and *in vivo*. Important surface proteins involved in

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attachment and biofilm development include *S. aureus* surface protein (SasG), biofilm-associated protein (Bap), and clumping factor B (ClfB).[37] Secreted proteins such as extracellular adherence protein (Eap), and beta toxin (Hlb) play a role in biofilm maturation. *S. aureus* secretes 10 proteases, including seven serine proteases (SspA and SplA-F), two cysteine proteases (SspB and ScpA), and one metalloprotease (Aur) which mediate dispersal.[37]

Apart from single-species biofilms, biofilms can be made up of multiple species of microorganisms closely associated with each other, allowing for the development of mutually beneficial interactions between species.[38] The medical impact of *C. albicans* and many other microbial species, depends on its ability to thrive as a biofilm.[1] Polymicrobial biofilms can be found throughout the human body and studies suggest that pathogenic phenotypes may emerge as a result of multispecies interactions.[46] It has already been demonstrated that the formation of an *S. aureus*-*C. albicans* biofilm establishes a dynamic relationship between two significant human pathogens and results in the up-regulation of virulence factors.[38] Co-species are more difficult to treat than single species biofilms due to the more complex composition of the EPS matrix.[47]

1.3 Treatment

1.3.1 Conventional therapy

The discovery of antibiotics more than 65 years ago revolutionised medicine. Today we live in a society where antibiotics are prescribed as the first-line of treatment for the majority of ailments encountered by health professionals.[48] Therefore, it is not surprising that we are faced with an ever increasing amount of resistant microorganisms. According to a report by the National Institute of Allergy and Infectious Disease (NIAID), the appearance of resistant strains has increased dramatically over the past 10 years and more than 70% of the bacteria that cause infections are resistant to at least one of the treatment drugs.[5] As a result, people stay longer in hospitals and are forced to use second and third lines of treatment which are often more toxic. In some cases, bacteria are even resistant to last-line drugs such as vancomycin.[5]

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With regard to *S. aureus* infections, vancomycin is typically reserved for patients with life-threatening infections, in areas where community-acquired MRSA infections have been documented. Patients with less severe infections are usually treated with penicillinase-resistant penicillin such as dicloxacillin and nafcillin, or a first-generation cephalosporin such as cefazolin. Clindamycin, co-trimoxazole, linezolid, and minocycline can be used as alternative treatment options for skin and soft tissue infections and in cases of necrotising pneumonias.[49] Established staphylococcal biofilms have been treated with quorum sensing inhibitors (QSI), use of surfactant-like molecules, treatment with enzymes that depolymerize biofilm components, and photodynamic treatment.[50] According to the GERMS-SA Annual Report of 2012, in South Africa, Gauteng was reported to have the most cases of *S. aureus* infections, followed by Kwa-Zulu Natal, the Western Cape and lastly the Free State, between January and July 2012. Drugs of choice include clindamycin, vancomycin and mupirocin.

With regard to *C. albicans*, cross-resistance of fungal species to the different classes of antifungal agents[51] implies that there is an increasing need for the development of new antifungal drugs as well as novel classes of antifungal agents. *C. albicans* biofilms in particular, are resistant to most antifungal agents.[52] GERMS-SA states that in South Africa, the majority of candidemia cases diagnosed were among children and neonates. Of those cases, 50% resulted in death. The epidemiology of candidemia differs between provinces and therefore guides empiric treatment choices. In Gauteng, the empirical drug of choice is amphotericin B due to the high prevalence of azole resistant strains. Where available, caspofungin is also used. In the Western Cape, high dose fluconazole or amphotericin B are both reasonable choices.

Mechanisms of azole resistance include induction of multi-drug pumps, up-regulation of the enzyme target lanosterol 14-α sterol demethylase (encoded by the ERG11 gene), mutations in the ERG11 gene (to prevent binding of azoles to the enzymatic site), as well as the development of by-pass pathways: azoles cause a decrease in ergosterol and build-up of a toxic metabolite; the fungal cells decrease the production of ergosterol

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(therapeutic targets) and replace it with another sterol while also causing a mutation in the gene responsible for producing the toxic metabolite (ERG3).[32]

Echinocandin resistance mechanisms include point mutations and hot-spot mutations (mutations at specific regions) which increases the minimum inhibitory concentration (MIC) of drugs, as well as initiation of the adaptive stress response. Another mechanism of antifungal resistance includes loss of heterozygosity at resistance genes and chromosomal rearrangements which amplify resistance genes.[32] As new antifungal mechanisms evolve, the search for new antifungal drugs becomes a constant challenge.

Infectious diseases remain the second leading cause of death worldwide despite the past success of antibiotic drug discovery,[53] therefore, the discovery of novel antibiotics and antimicrobial compounds is an urgent priority as treatment options for infected patients are becoming extremely limited.[6]

1.3.2 Alternative therapy: herbals

Approximately 25% of all western drugs contain active substances derived from plants.[54] Worldwide, approximately 4 billion people rely on plants as medication.[55] Hong Kong is considered the largest herbal market in the world, importing over $190 million of herbal products per year.[56] In Japan, the system of traditional medicine known as *Kampo*, is successfully used to treat chronic diseases including asthma, hypertension, diabetes mellitus and allergic rhinitis.[57] In the US, the National Cancer Institute has tested 35 000 species of plants for anticancer activity and many have shown promising effects.[58] In South Africa, millions of people rely on traditional medicines to obtain their primary healthcare needs and this is mainly attributed to affordability and accessibility.[59] In various parts of South Africa, a substantial amount of medicinal plants are sold regularly as crude, unprocessed drugs. Despite this large diversity of plants available on traditional markets, only a few indigenous plants have reached the level of being made available for sale in formal markets.[60]

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Approximately 119 pure chemicals derived from plants are used in medicines throughout the world.[55] Most of the antibacterial drug classes known today are derived from natural products.[53] Natural products contain biologically active chemicals and are more likely to have evolved strategies to penetrate cell membranes.[63] They also contain the structural complexity necessary to inhibit microbial targets.[53] There are many approaches to the search for active chemical compounds in plants which can be used to develop new medicines. A common approach is to prepare extracts and screen each extract for pharmacological activity.[61] This broad-screening method led to the discovery of vinca-alkaloids which are used for cancer treatment.[62]

With regard to biofilms, the phytochemicals present in plant extracts have the ability to act as quorum sensing inhibitors.[64] QS molecules are vital for cell-cell communication; a QS molecule can act as a switch turning yeast cells into hyphal cells for formation of biofilms.[64] As analogues, phytochemicals are similar in structure to QS molecules and can prevent the switch from yeast cells to hyphal mode of growth and can thus act as a prophylaxis against biofilm formation.[64] Phytochemicals can inhibit QS by signal degradation, signal sequestration, signal competition and receptor degradation.[64] Therefore, natural food sources play an important role in preventative therapy. Phytochemicals metabolised in the gut lead to the formation of new metabolites which may play a role in up-regulating beneficial flora and decreasing pathogenic flora in the body.[65] Plant extracts have also been shown to act synergistically with antibiotics against microorganisms.[65]

1.3.2.1 Plants as source for secondary metabolites

Natural products can be divided into three main groups, namely, primary metabolites, secondary metabolites and high molecular weight polymeric materials (HMWPM).[66] Primary metabolites such as nucleic acids, amino acids and sugars play an integral role in cellular metabolism and reproduction. HMWPM such as lignins and proteins form part of the cell structure.[66] Secondary metabolites however, are referred to as phytochemicals and xenobiotics which do not play a role in growth or development of the producing organism but are important because of their biological activity in other

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organisms.[67] Main groups of secondary metabolites include: alkaloids, terpenoids, steroids, fatty acid derivatives, polyketides, phenylpropanoids, non-ribozomal polypeptides and enzyme cofactors.[66] These metabolites are responsible for the protection of plants against stresses, external attacks by insects and herbivores, growth regulation, modulation of gene expression and signal transduction.[67] Common groups of phytochemicals associated with antimicrobial activity include alkaloids,[68] amines,[69] flavonoids,[70] phenolic acids[71] and terpenoids.[72]

1.3.2.2 Plants investigated in this study

i) *Zanthoxylum davyi*

Z. davyi (I. Verd.) P.G. Waterman, also known as Forest knobwood, (Figure 4) is a medium to tall tree commonly found in the coastal forests of KwaZulu-Natal and the Transkei, extending through Mpumalanga, Swaziland and Zimbabwe.[68] The *Zanthoxylum* genus which belongs to the family Rutaceae, comprises 250 species.[73] *Z. davyi* is commonly used by the Zulu to treat ailments such as severe coughs and colds, infected wounds, venereal diseases and snakebites.[73] The Vhavenda use the spines to treat infected wounds, leaves for chest pains, stem-bark to treat pleurisy and toothache, and root preparations for mouth ulcers and sore throats.[68] *Z. davyi* also plays an important role in insect control by acting as a fumigant and repellent. It has also been proven to display anti-feedant activity on the beetle, *Tribolium casteneum.*[74] *Z. davyi* has been found to possess anti-human immunodeficiency virus (HIV) properties in that it inhibits the cellular transcription factors necessary for efficient HIV replication.[75] The bark extract of *Z. davyi* has been reported to contain antifungal activity against a standard strain of *C. albicans.*[7] *Z. davyi* (bark) has been shown to exhibit anti-acetylcholinesterase activity which supports its use in the treatment of neurological diseases such as Alzheimer's disease.[76] Other activities reported for the *Zanthoxylum* genus includes larvicidal,[77] analgesic,[78] anti-nociceptive,[79] hepato-protective,[80] anti-proliferative[81] and anticonvulsant.[82, 83]

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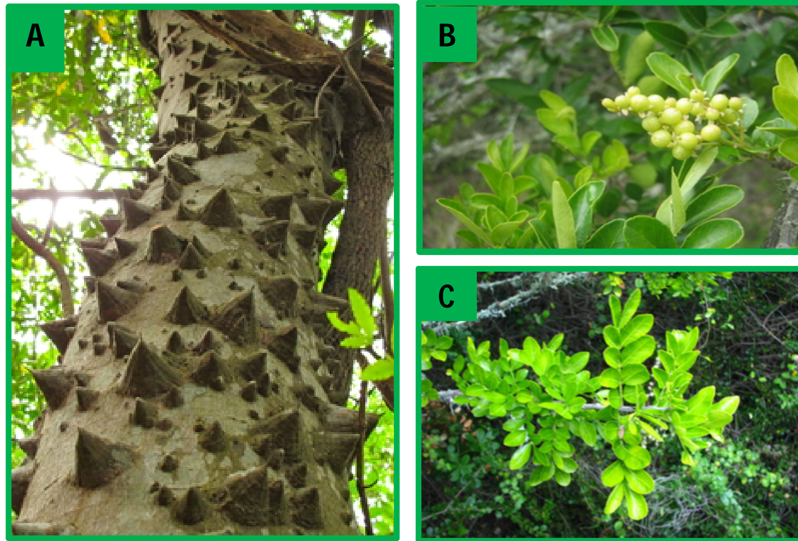

Figure 4: *Z. davyi*; A) Tree trunk showing bark, B) fruit and C) leaves.[84]

ii) *Ximenia caffra*

X. caffra Sond. (Figure 5) is a southern African plant and can be found across Tanzania, Zambia, Zimbabwe, Botswana, Namibia, Mozambique and South Africa.[85] The *Ximenia* genus which belongs to the family Olacaceae, comprises 8 species.[86] *X. caffra* is commonly used to treat inflammation of the eyes, fevers, diarrhoea, vomiting and gynaecological complaints,[73] as well as skin infections and opportunistic diseases in AIDS.[87] Traditionally, root preparations are used as a remedy to treat eye infections, whereas leave preparations are used to treat stomach aches.[88] In South Africa, the fruits are commonly eaten in rural areas and the seed oil is used to soften leather. Leaf powder is used for infertility while cold leaf infusions are used as eyewash for painful eye conditions. The leaves are also taken orally for fever and extracts used as a gargle for tonsillitis. The powdered root is used in soup or porridge as an aphrodisiac. Root infusions are used to treat pelvic and venereal diseases, diarrhoea and haematuria. Systemic sepsis and rheumatism are treated with bark and root preparations.[89] *X. caffra* has been reported to possess; antimicrobial, anticancer and antiviral activities.[8] In Tanzania, *X. caffra* is used for the treatment of irregular menstruation and rheumatism.[90]

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Figure 5: *X. caffra* tree branch showing leaves and fruit.[91]

1.4 Toxicity testing

Plants contain various classes of constituents which are responsible for the biological activities noted. Apart from "beneficial" compounds, plants can contain compounds which are inherently toxic. These may be exploited for the production of cytotoxic or anticancer-drugs.[92] Examples of anticancer plant derived drugs include vinblastine, vinorelbine and more recently, vinflunine, which are derived from vinca-alkaloids.[93] Vinca-alkaloids are effective as chemotherapeutic agents against a wide variety of cancers as they affect multiple cell cycle processes. They have the ability to bind to tubulin, block mitosis and inhibit purine and ribonucleic acid (RNA) synthesis.[93] These cytotoxic drugs not only affect cancer cells, but also healthy cells which can have undesirable side effects (as seen in patients undergoing chemotherapy) and which can even be fatal. An example of such a side effect is bone marrow suppression.[93] Plants are considered safe because they are natural and toxicity testing is not conducted. Novel investigational drugs which are deemed "unsafe" due to cytotoxicity in pre-clinical studies will not receive approval for use in clinical trials and will therefore not be developed further. Cytotoxicity testing not only protects patient safety but also prevents

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sponsors from investing in novel investigational products which may bear no future value.[94]

1.5 Study aim

The aim of this study was to determine the antimicrobial activity of bark extracts of *Z. davyi* and *X. caffra* against planktonic and biofilm forms of *C. albicans* and *S. aureus*, as well as to determine the phytochemistry and cytotoxicity of the crude extracts.

1.6 Study objectives

The objectives of this study were:

- to determine the phytochemical classes present in the crude extracts using thin-layer chromatography (TLC).
- to identify phytochemical compounds present in the crude extracts using high-performance liquid chromatography (HPLC).
- to determine the antimicrobial activity of *Z. davyi* and *X. caffra* crude extracts on planktonic *C. albicans* and *S. aureus*, using the disc diffusion and broth microdilution assays.
- to determine the antimicrobial activity of *Z. davyi* and *X. caffra* crude extracts on biofilms of *C. albicans* and *S. aureus*, using the biofilm inhibition and crystal violet assays.
- to determine ultra-structural changes caused by *Z. davyi* and *X. caffra* crude extracts on biofilms of *C. albicans* and *S. aureus* using scanning electron microscopy.
- to determine synergistic activity between the crude extracts using the checkerboard assay.
- to determine the cytotoxicity of the crude extracts using the Sulforhodamine B (SRB) assay and visualization using phase contrast and PlasDIC microscopy.

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CHAPTER 2: Materials and methods

2.1 Schematic outline of the study

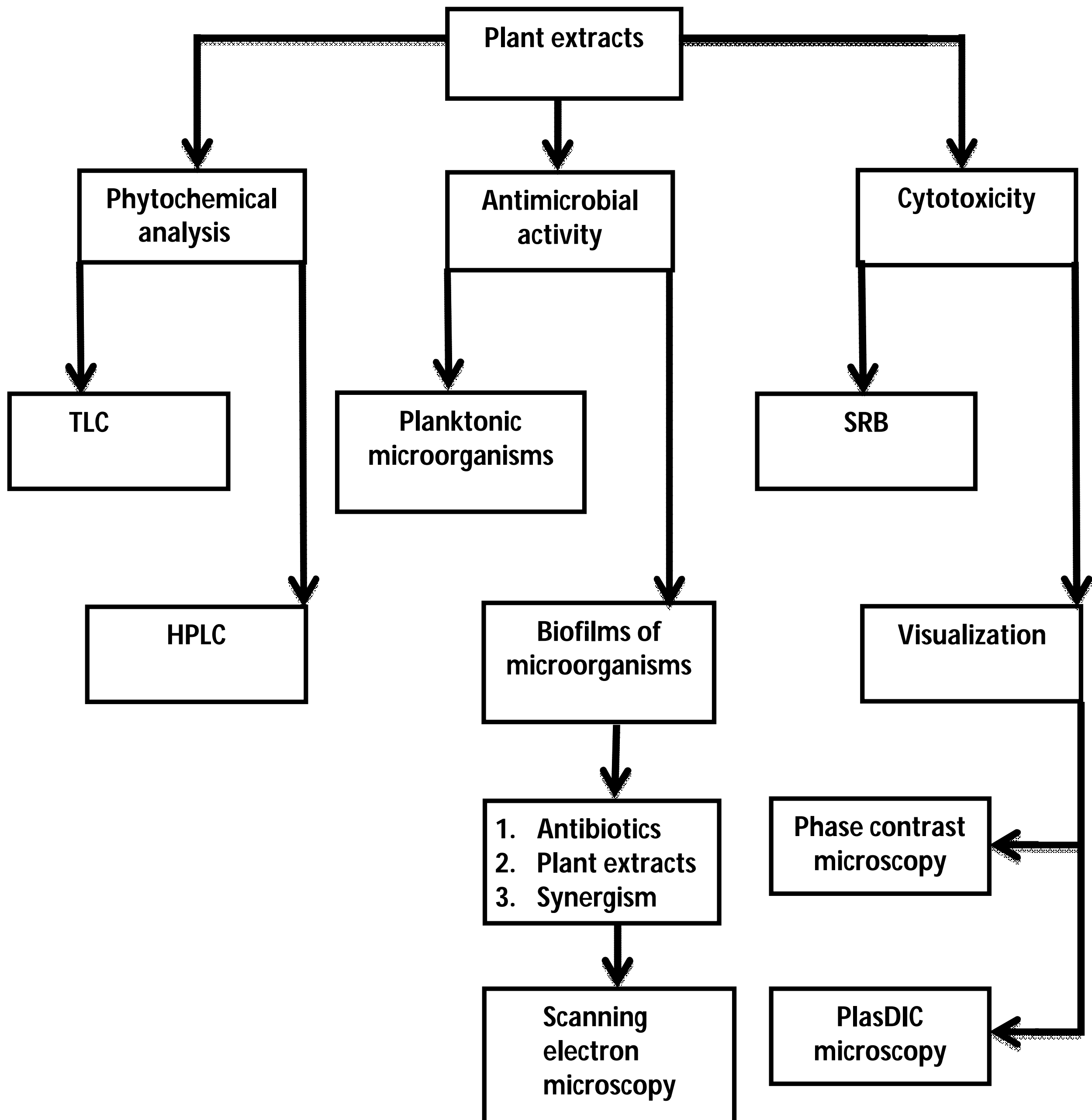

Figure 6: Flow diagram representing the project sequence.

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2.2 Study design

An experimental study design was chosen to investigate the antimicrobial activity of *Z. davyi* and *X. caffra* and to determine the phytochemical compounds which could be responsible for such activity. Cytotoxicity testing was conducted and evaluated using microscopy. Ethical approval was obtained from the University of Pretoria's Ethics Committee to carry out the study (Appendix 1).

2.3 Plant collection and extraction

2.3.1 Plant material

X. caffra (XC) bark (NH 1875) was collected in Venda by a botanist, Dr Norbert Hahn. *Z. davyi* (ZD) bark was provided by Mr K. Baloyi from the South African National Biodiversity Institute (SANBI), Tshwane. Voucher specimens of XC and ZD are deposited at the Soutpansberg's herbarium (Makhado) and SANBI (Tshwane), respectively. The bark was air dried and ground into a fine powder using a yellow-line grinder (Merck). This was stored in a bottle in a dark area in order to retain activity, until the extracts were prepared.

2.3.2 Preparation of crude extracts

Hot water (HW), methanol (MeOH) and dichloromethane (DCM) extracts were prepared for each plant. An exhaustive extraction method was used. A volume of 100 mL of the respective solvent was added to 10 g of plant material. The hot water extract was sonicated in an ultrasonic bath for 30 min. All extracts were then allowed to stir for 1 h using a magnetic stirrer. Preparations were allowed to stand for 24 h at 4°C. The extracts were centrifuged at 1000 *g* for 5 min and the supernatant collected. The hot water extract was filtered through 0.22 µm filters (Millipore) and stored at -18°C until used, to prevent chemical decomposition. As for the non-aqueous extracts, following collection of the supernatant, an additional 100 mL of solvent was added to the surplus plant material and allowed to stir for 2 h. These preparations were then allowed to stand for 24 h at 4°C. The extracts were again centrifuged and the supernatant collected. This process was repeated three times until 300 mL of supernatant was collected in total for

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each non-aqueous extract. These extracts were then filtered using 0.45 µm filters (Millipore) and evaporated to dryness at 40°C. Once dry, the extracts were re-dissolved in 2-3 mL dimethyl-sulphoxide (DMSO) (Merck). These preparations were then diluted to obtain the relevant concentrations so that the final concentration of DMSO in the sample was <0.5%. Yields of all extracts were determined gravimetrically.

2.4 Phytochemical screening

2.4.1 Thin-layer chromatography

Thin-layer chromatography (TLC) was conducted on silica gel (F_{254}) aluminium plates (Merck, Darmstadt, 10 x 10 cm). Extracts were tested for the presence of alkaloids, amines, flavonoids, glycosides, phenolic acids, quinones, sterols and terpenoids, according to the method of Cordier.[95] Approximately 4 µL of crude extract (12 mg/mL) was spotted and developed using specific mobile phases and sprayed with selective visualisation agents as presented in Table 1.[96] The retention factor (R_f) was calculated for specific compounds detected, using the formula:

$$R_f = \frac{\text{Distance travelled by compound}}{\text{Distance travelled by solvent}}$$

2.4.2 High-performance liquid chromatography

In order to determine the chemical fingerprints of the plant extracts, HPLC analysis was conducted. Co-chromatography was performed using standard compounds known to be present in the plant part according to literature, in order to confirm the identity of plant. Standards used: chelerythrine, nitidine, sanguinarine, catechin, quercetin, rutin, caffeic acid, coumaric acid, ferulic acid, gallic acid, salicylic acid, sinapic acid, syringic acid, vanillic acid, and ascorbic acid (Sigma-Aldrich, South Africa).

High-performance liquid chromatography (HPLC) was carried out using a UFLC-LC20AB diode array detector (254 nm- 320 nm) system (Shimadzu) and a C18 column (3.9 mm x 150 mm x 5 µm; Xterra®, Waters). HPLC grade solvents were used in all analyses. The binary mobile phase for polar extracts consisted of A: water (0.1% formic acid) and B:

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water (0.1% formic acid): Acetonitrile (64:36). The binary mobile phase for non-polar extracts consisted of A: water (0.1% formic acid), B: 100% acetonitrile. Gradient profile: 10% solvent B at the start, rising to 45% within 1 min, continuing to rise to 50% at 5 min, rising to 90% at 8 min, remaining at 90% at 10 min, falling back to 10% at 11 min, and remaining at 10% until 15 min. A flow rate of 1.0 mL/min was used with an injection volume of 5 µL and detection was observed at 280 nm. Calibration curves were produced for compounds in the greatest abundance in the extracts in order to determine their relative concentrations.

Table 1: Mobile phases and spray reagents used to detect specific phytochemical constituents by means of TLC.

Phytochemical	Mobile phase	UV visualisation	Spray reagent
Alkaloids	MeOH:DCM (9:1)	254, 366 nm	Dragendorff's reagent
Amines	NH_3:MeOH:DCM (1:13:6)	254, 366 nm	Ninhydrin
Flavonoids	Chloroform:Acetone:Formic acid (15:3:2)	254, 366 nm	1% $AlCl_3$ in EtOH
Glycosides	Xylene:Ethyl-acetate:Formic acid (2.5:1:1)	254, 366 nm	10% KOH in 50% MeOH
Phenolic acids	MeOH:DCM (9:1)	254, 366 nm	Folin-Ciocalteau reagent
Quinones	MeOH:DCM (2:8)	254, 366 nm	H_2SO_4
Sterols/lipids	DCM:MeOH (9:1)	254, 366 nm	85% phosphoric acid:H_2O (1:1)
Terpenoids	Ethyl- acetate:MeOH (9:1)	254, 366 nm	Vanillin-H_2SO_4

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2.5 Determination of antimicrobial activity

2.5.1 Microorganisms

S. aureus (ATCC 12600) and *C. albicans* (ATCC 90028) were used as reference strains and were purchased from the American Type Culture Collection (ATCC). Two clinical strains of both *S. aureus* and *C. albicans* were obtained from the Department of Microbiology, National Health Laboratory Services, Pretoria. Stock cultures of *S. aureus* were maintained on MacConkey agar (Davies Diagnostics, Randburg, South Africa), while *C. albicans* was maintained on Mueller-Hinton agar (Davies diagnostics, Randburg, South Africa). All cultures were kept at 4°C.

2.5.2 Preparation of inocula

Fresh 24 h cultures were used to prepare inoculum in sterile saline (0.85%) and were colorimetrically adjusted (Sherwood colorimeter 254, Sherwood Scientific Ltd, UK) until standard turbidity (0.5 MacFarland) was reached at a wavelength 560 nm. A bacterial suspension with the OD_{520} nm equivalent of a 0.5 MacFarland turbidity standard has a cell density of 1 x 10^8 CFU/mL.[100]

2.5.3 Disc diffusion assay

The disc diffusion assay was performed according to Bauer *et al.*[97] A volume of 200 µL of crude plant extract was added to sterile paper discs (10 mm, Whatman's No. 1) and allowed to dry. Ciprofloxacin and amphotericin B discs (10 µg, Davies Diagnostics, Randburg, South Africa) were used as positive controls for *S. aureus* and *C. albicans*, respectively. Ciprofloxacin was chosen because……clinical strains works….Saline was used as a negative control. 100 µL of the inoculum (0.5 MacFarland) was transferred to the surface of respective agar petri dishes, (MacConkey agar for *S. aureus* and Mueller-Hinton agar for *C. albicans*). Inoculum was spread evenly across the whole surface of the agar plates. A disc containing dried extract at yield concentrations along with the respective control discs were then placed on an inoculated plate and incubated at 37°C for 24 h and 48 h for *S. aureus* and *C. albicans*, respectively. The zones of inhibition (mm) were measured using callipers.

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2.5.4 Broth microdilution assay

The broth microdilution assay[98] was used to determine the MIC's of the extracts which displayed antimicrobial activity as determined by the disc diffusion assay. Serial two-fold dilutions (0.50 µg – 1000 µg) of the crude extracts were made using Mueller-Hinton broth (Davies Diagnostics, Randburg, South Africa). Serial two-fold dilutions of ciprofloxacin and amphotericin B (Sigma-Aldrich, South Africa) were similarly prepared. Ciprofloxacin served as the positive control for *S. aureus*, whereas amphotericin B served as the positive control for *C. albicans*. Wells containing only Mueller-Hinton broth and inoculum served as growth controls. DMSO (5%) was also included as a control. A volume of 80 µl of Mueller-Hinton broth was transferred to the wells of a sterile, flat-bottomed, 96-well microplate (Sigma-Aldrich, South Africa), followed by the addition of 100 µl of inoculum (0.5 MacFarland) and 20 µl of each dilution of crude extract or antibiotic was transferred to the microplate, bringing the final volume of each well to 200 µl. The plates were then incubated at 37°C for 24 h. Thereafter a volume of 30 µl of a 200 µg/mL solution of *p*-iodonitrotetrazolium chloride (INT, Sigma-Aldrich, South Africa) was added to all wells. The plates were allowed to incubate further until maximum colour intensity was achieved (±30 min). Uninhibited microbial growth was indicated by a pink colour, whereas inhibition of microbial growth was indicated by the failure of a well to change colour. The wells that failed to change colour were further investigated to determine the minimum bactericidal concentration (MBC)/minimum fungicidal concentration (MFC) of each crude extract.

2.5.5 Minimum bactericidal/fungicidal concentration

Wells which failed to change colour using the broth microdilution assay were subcultured on agar plates and incubated at 37°C for 24 h. The MBC was determined to be the lowest concentration which, when cultured on agar, showed no colony forming units or microbial growth against *S. aureus*. The MFC was determined to be the lowest concentration which, when cultured on agar, showed no colony forming units or microbial growth against *C. albicans*.[99]

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2.5.6 Growth of biofilms

Biofilms were grown under sterile conditions in 96-well microplates. A volume of 80 µL of Mueller-Hinton broth was added to test wells of a 96-well microplate. *S. aureus* and *C. albicans* (0.5 MacFarland) were diluted to 1 x 10^6 CFU/mL using Mueller-Hinton broth and 100 µL was then transferred to these wells. Final well volume of test wells was 180 µL. Non-test/control wells were prepared as follows: medium control wells = 200 µL broth, saline control wells = 200 µL saline, and growth control wells = 100 µL broth + 100 µL inoculum at 1 x 10^6 CFU/mL. The final volume of non-test wells was 200 µL. The plates were then allowed to incubate at 37°C for 24 h.[100] Presence of growth was confirmed using the crystal violet assay.

2.5.7 Biofilm inhibition assay

The biofilm inhibition assay was used to determine the Biofilm Inhibitory Concentrations (BIC's) of the crude plant extracts. Biofilms were grown as described above (2.5.6). Serial two-fold dilutions of the extracts and antibiotics were prepared using Mueller-Hinton broth. The crude extract concentrations tested were: XC-MeOH-E (0.100 mg/mL – 36.400 mg/mL), XC-DCM-E (0.050 mg/mL – 13.400 mg/mL), XC-HW-E (0.010 mg/mL – 2.850 mg/mL), ZD-MeOH-E (0.030 mg/mL – 8.400 mg/mL), ZD-DCM-E (0.020 mg/mL – 4.200 mg/mL) and ZD-HW-E (0.001 mg/mL – 1.300 mg/mL). Positive controls were diluted as follows: ciprofloxacin (0.001 mg/mL – 0.200 mg/mL) and amphotericin B (0.001 mg/mL – 0.200 mg/mL). A volume of 20 µL of each concentration of the extract or control was transferred to the test wells of a microplate containing either *S. aureus* or *C. albicans* (2.5.6), bringing the final volume of the extract test wells to 200 µL. All plates were allowed to incubate at 37°C for 24 h, followed by the crystal violet assay.

2.5.8 Crystal violet assay

Following incubation (2.5.7) biofilms were detected using the crystal violet assay.[100] After incubation the microplates were emptied and rinsed with distilled water three times to remove loosely attached/planktonic microorganisms and left to dry for 45 min. A volume of 200 µL of 0.1% crystal violet was then added to all the wells of the microplates and plates allowed to incubate in order to stain any adherent

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microorganisms (30 min at room temperature). The plates were then rinsed six times to remove unabsorbed stain and allowed to dry for 24 h. A volume of 200 µL of 95% ethanol was added to the wells and the plates were incubated for 15 min at room temperature to solubilise the stained biofilms.The plates were read at 560 nm using a microplate reader (GLR 1000, Genelabs Diagnostics).[100]

2.5.9 Viable colony count

Viable colony count (VCC) values are used to approximate the actual concentration of viable microorganisms in each of the 0.5 MacFarland solutions. The values obtained were used to calculate the end-point for determining the MBC/MFC of the selected test agents. Inoculum (0.5 MacFarland) were diluted to 1 x 10^6 CFU/mL using Mueller-Hinton broth. This inoculum was further diluted to an optical density of 0.02 using sterile saline. A volume of 10 µL of inoculum was then transferred to specific agar petri dishes and evenly spread across the surface of the petri dish. The petri dishes were incubated at 37°C for 24 h. Following incubation, the number of colonies were manually counted and recorded. Cell concentrations were calculated using the formula:

$$\frac{\text{Number of CFU}}{\text{Volume plated (mL)} \times \text{total dilution used}} = \frac{\text{Number of CFU}}{\text{mL}}$$

The average of triplicate values were used to estimate the actual concentration of bacteria/fungi in each 0.5 MacFarland solution. [100]

2.5.10 Scanning electron microscopy

Scanning electron microscopy (SEM) was employed to examine EPS production. Biofilms were grown on coverslips in sterile, flat bottom, 6-well microplates (Costar, South Africa). The method described in 2.5.6 was adjusted for a 6-well microplate. In this case, 2 mL of Mueller-Hinton broth was added to test wells of a 6-well microplate. *S. aureus* and *C. albicans* (0.5 MacFarland) were diluted to 1 x 10^6 CFU/mL using Mueller-Hinton broth and 2.5 mL was then transferred to these wells. Final well volume of test wells was 4.5 µL. Non-test/control wells were prepared as follows: medium control wells = 5 mL

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broth, saline control wells = 5 mL saline and growth control wells = 2.5 mL broth + 2.5 mL inoculum. Final volume of non-test wells was 5 mL. The plates were then allowed to incubate at 37°C for 24 h. Test wells were then treated with 0.5 mL antibiotic or crude extract and allowed to incubate for a further 24 h at 37°C. Biofilms were rinsed three times using distilled water to remove planktonic microorganisms. The rinsed biofilms were then fixed with 0.1 M cacodylate buffer (pH 7.2, Sigma-Aldrich) at room temperature for 2 h, and air dried for 120 h. Coverslips were then carbon coated and mounted. Scanning electron microscopy was performed using a Jeol (JSM-5800LV, Tokyo, Japan) scanning electron microscope at the Laboratory for Microscopy and Microanalysis, University of Pretoria.[101]

2.6 Determination of synergism

2.6.1 Checkerboard assay

Once the independent BIC's had been assessed for each individual crude extract using the biofilm inhibition assay (2.5.7), synergism between different extracts were determined using the checkerboard assay.[102] Biofilms were grown as described above (2.5.6). Stock solutions (1 mg/mL) of all crude extracts were prepared. *Z. davyi* methanolic extract was combined with *X. caffra* methanolic extract in the following ratios: 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, and 10:90. Biofilms were treated with these preparations as described in the biofilm inhibition assay (2.5.7) followed by quantification using the crystal violet assay (2.5.8). The same procedure was repeated for the hot water and dichloromethane extracts. The BIC's of the combined extracts were then determined.[102]

2.6.2 Sum of the fractional inhibitory concentration index

The sum of the fractional inhibitory concentration index (ΣFIC) is expressed as the interaction of two agents where the concentration of each test agent in combination is expressed as a fraction of the concentration that would produce the same effect when used independently. The ΣFIC is then calculated for each test sample independently as specified in the following equations:

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$$FIC(*i) = \frac{\text{BIC (a) in combination with (b)}}{\text{BIC (a) independently}}$$

$$FIC(*ii) = \frac{\text{BIC (a) in combination with (b)}}{\text{BIC (b) independently}}$$

ΣFIC or FIC index is thus calculated as:

ΣFICI = FIC (*i) + FIC (*ii)[102]

2.7 Determination of cytotoxicity of compounds

2.7.1 Cell lines

MCF-7 human breast cancer cells (ATCC 30-2101) were purchased from the American Type Culture Collection (ATCC). EA.hy926 human umbilical vein cells (ATCC CRL-2922) were a gift from Dr CJS Edgell of the University of North Carolina. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) supplemented with 10% Foetal Calf Serum (FCS, Sigma-Aldrich). Cells were harvested once 80% confluency was reached. The culture medium was discarded and trypsin (10%) added to rinse the cells. The trypsin (10%) was then discarded and a further 15 mL of trypsin was added and cells were allowed to incubate at 37°C for 20 min in an atmosphere of 5% CO_2, until the cells detached from the flask. A volume of 5 mL of DMEM supplemented with 10% FCS was added to neutralize the action of trypsin. The suspension was aspirated, transferred to a 15 mL tube and centrifuged (200 *g*, 5 min). The supernatant was discarded and the pellet re-suspended in 1 mL DMEM supplemented with 5% FCS. Cells were counted using a haemocytometer and the cell suspension was diluted to a concentration of 5×10^4 cells/mL, for use in the sulforhodamine B (SRB) assay.

2.7.2 Sulforhodamine B assay

This assay was conducted by a modified method of Virchai and Kirtikara.[103] A volume of 100 µL of cell suspension (5×10^4 cells/mL) in 5% DMEM, was placed in each well of a 96-well microplate. Plates were allowed to incubate at 37°C (5% CO_2 atmosphere) for 24 h to allow cells to attach. Stock solutions of the crude plant extracts were prepared in 5% DMEM and serially diluted (1.6 µg/mL – 200 µg/mL). Tamoxifen was used as a positive

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control and was similarly prepared (0.09 µg/mL – 200 µg/mL). A volume of 100 µl of two-fold dilutions of crude plant extract/tamoxifen was added to test wells. Final volume of test wells was 200 µL. Non-test wells were prepared in the following manner: untreated control = 100 µL cell suspension + 100 µL 5% DMEM, vehicle control = 100 µL cell suspension + 100 µL 5% DMEM containing DMSO. Plates were allowed to incubate at 37°C, 5% CO_2 for 72 h. Following incubation, medium was aspirated from the wells and discarded. Cells were washed twice with sterile Hank's balanced salt solution (HBSS), after which they were then fixed by adding 25 µL of 50% (v/v) trichloroacetic acid (TCA) and allowed to incubate at 4°C for 24 h. The TCA solution was aspirated and cells rinsed four times with tap water. Cells were then allowed to dry in an oven (Incotherm) for 1 h at 40°C. Once dry, 100 µl of a 0.057% (w/v) solution of SRB was added to all wells and allowed to incubate for 30 min at room temperature. The SRB solution was then discarded and cells rinsed four times with 1% acetic acid. Plates were dried at room temperature overnight. Once dry, 200 µL of a 10 mM Tris base solution (pH 10.5) was added to all wells and placed on a plate shaker (Thermo Fischer Scientific) for 30 min. The optical density of the plates were read using a microplate reader (BioTek) at a wavelength of 510 nm, with reference wavelength 630 nm.

2.7.3 Gross morphology

Following washing of the cells with sterile HBSS (2.7.2), the gross morphology of the cells was observed using a phase contrast microscope (Zeiss, Oberkochen, Germany) at 10 x magnification. Signs of apoptosis or necrosis were visualized. Apoptosis is characterized by cytoplasmic shrinkage, nuclear condensation, membrane blebbing and the formation of apoptotic bodies/membrane bound vesicles.[104] Necrosis is characterized by swelling and rupturing of cells (cell lysis).[104] PlasDIC was also conducted (40 x magnification). Photos were taken and edited using the software program AxioVision 4.

2.8 Statistical analyses

Preliminary analyses of the various outcome measures were presented using summary statistics such as means, medians and standard deviations of various extracts or concentration level. Additionally, dose-response or concentration profile plots to

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optimally visualise the results were used, where maximum and relative potencies of the extracts and concentration levels were determined. Inferential statistical analyses was based on the application of group comparison tests such as *t*-tests, Wilcoxon rank sum, Analysis of Variance (ANOVA) and Kruskal-Wallis methods depending of the normal assumptions of the data and the size of the replicates/samples. GraphPad Prism® 6 software was used for all the analyses. A *p*-value below 0.05 signified statistical significance. All tests were carried out in at least triplicate, and on three occasions.

Disc diffusion assay: Zones of inhibition were measured using callipers. The average of three measurements was used. Results were reported in mm ± standard error of the mean (S.E.M.). Only complete zones of inhibition were taken into account.

Broth microdilution assay: Minimum inhibitory concentrations were determined visually. The MIC was regarded as the lowest concentration which inhibited microbial growth. Uninhibited microbial growth was indicated by a pink colour, whereas inhibition of microbial growth was indicated by the failure of a well to change colour.

MBC/MFC: These were regarded as the lowest concentration which showed no colony forming units or microbial growth on agar on all occasions after streaking out non-colour MIC-wells.

Crystal violet assay: Quantification of biofilms was determined spectrophotometrically. The Mann-Whitney *U* test was used to analyse the data. This test was used to determine differences between two independent sample groups of data. Groups treated with plant extract were compared to groups treated with standard antibiotics. Statistical significance was accepted if $p < 0.05$.

Viable colony count: The average number of colonies (triplicate tests) were counted and recorded. Values between 5 and 200 colonies were deemed acceptable whilst values outside the range negated the results of the experiment which used the specific 0.5 MacFarland solution.[100]

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Synergy: ΣFIC where a value of ≤ 0.5 is indicative of synergy, values > 0.5–1.0 implies an additive effect, values between 1.0–≤ 4.0 indicate non-interaction, and a value > 4.0 indicates antagonism were used.[102]

SRB assay: Percentage cell survival was assessed against the untreated control using a linear dose-response curve (curve fit) with a bottom constraint of 0% for the sigmoid dose-response (variable slope). The 50% inhibitory concentration (IC_{50}) was extrapolated from this data.

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CHAPTER 3: Results and discussion

3.1 Extract yields

The extract yields of *X. caffra* and *Z. davyi* are presented in Table 2. A higher yield was achieved for the extracts of *X. caffra* as compared to the extracts of *Z. davyi*. The methanol (MeOH) extracts were dark brown in colour and produced the highest yields followed by the hot water (HW) extract (orange in colour). The dichloromethane (DCM) extracts (yellow-green colour) produced the lowest yields. Therefore, it can be deduced that the greater the polarity of the extract, the greater the extract yield and the darker the extract. The differences in extract colours give an indication of the different properties/compounds in the extracts.[105] A yellow colour is indicative of the presence of flavonoids.[76] Previous studies report that darker extracts contain more polyphenolic compounds, associated with increased antioxidant activity.[105] Polyphenols are also associated with antimicrobial activity.[71] MeOH extracts of *Ximenia* species[8] as well as MeOH extracts of the *Zanthoxylum* species[106] have been reported to contain polyphenolic acids. With regard to percentage yield, differences could be attributed to external factors such as temperature, storage, time, preparation and also due to the structural properties of the compounds in the plants.[105]

Table 2: Extract yields of extracts of *X. caffra* and *Z. davyi*.

Plant	Extract	% Yield ± S.E.M.
X. caffra	MeOH	22.48 ± 3.48
	DCM	5.48 ± 2.68
	HW	14.31 ± 1.20
Z. davyi	MeOH	10.09 ± 0.41
	DCM	3.77 ± 1.27
	HW	5.61 ± 0.41
MeOH= Methanol; DCM= Dichloromethane; HW= Hot water (n=3)		

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3.2 Phytochemical analysis

Phytochemical classes detected in the extracts are presented in Table 3 and Figures 7-13. The retention factor (R_f) was calculated for marked compounds on each plate. In this context, TLC is based on the assumption that different compounds in the crude plant extract travel different distances according to how strongly they interact with the stationary phase as compared to the mobile phase.[107] The stationary phase is made up of a polar substance therefore polar compounds will adhere to the stationary phase and non-polar compounds will travel further along the plate. Therefore, TLC gives an indication of the types of compounds present in each extract. Alternatively, if types of compounds present in a sample are known, the polarities and R_f values can be predicted (the lower the polarity, the greater R_f).[107]

Table 3: Phytochemical groups detected in methanol, dichloromethane and hot water extracts of *Z. davyi* and *X. caffra* using thin-layer chromatography.

	EXTRACT					
Phytochemical class	**ZD-MeOH-E**	**ZD-DCM-E**	**ZD-HW-E**	**XC-MeOH-E**	**XC-DCM-E**	**XC-HW-E**
Alkaloids	+	+	+	-	-	-
Amines	+	+	-	-	+	-
Flavonoids	+	+	-	-	+	-
Glycosides	+	+	-	+	+	+
Phenolic acids	+	+	+	+	+	+
Sterols/lipids	+	+	+	+	+	+
Terpenoids	+	+	+	+	+	+
Saponins	-	-	-	-	-	-
Quinones	+	+	-	+	+	-

ZD: *Z. davyi*; XC: *X. caffra*; MeOH: Methanol; DCM: Dichloromethane; HW: Hot water; +: positive (present); -: negative (absent).

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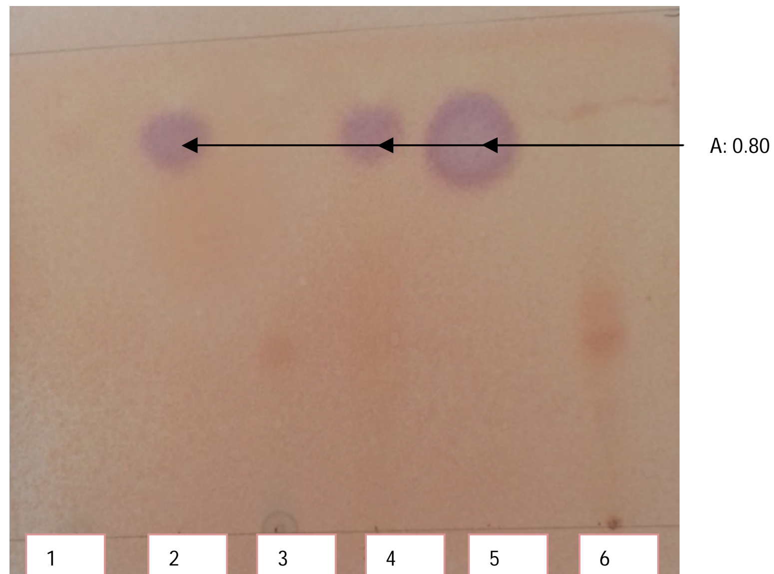

Figure 7: TLC chromatogram of crude extracts of *Z. davyi* and *X. caffra* after development in NH_3:MeOH:DCM (1:13:6) and spraying with ninhydrin. . Lane 1: XC-MeOH-E, Lane 2: XC-DCM-E, Lane 3: XC-HW-E, Lane 4: ZD-MeOH-E, Lane 5: ZD-DCM-E, and Lane 6: ZD-HW-E.

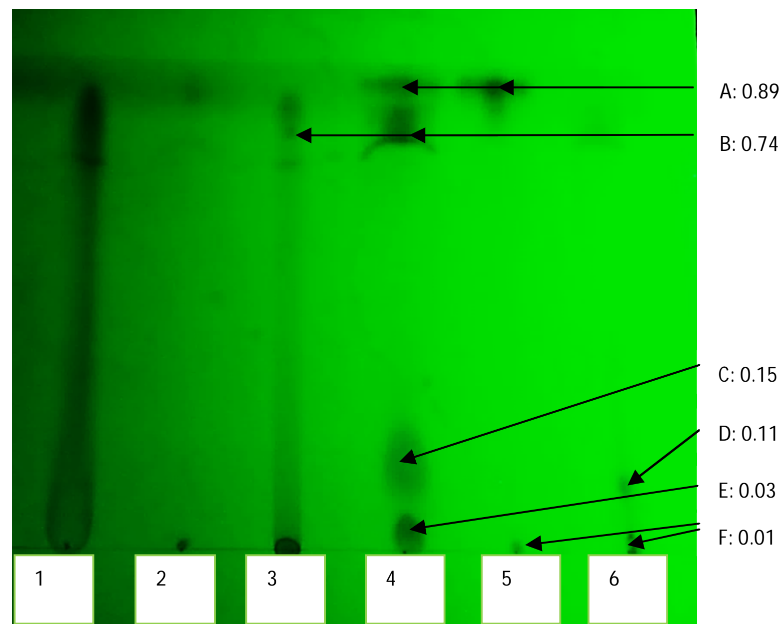

Figure 8: TLC chromatogram of crude extracts of *Z. davyi* and *X. caffra* developed in MeOH:DCM (9:1), visualised under UV at 254 nm to detect alkaloids and phenolic acids. Lane 1: XC-MeOH-E, Lane 2: XC-DCM-E, Lane 3: XC-HW-E, Lane 4: ZD-MeOH-E, Lane 5: ZD-DCM-E, and Lane 6: ZD-HW-E.

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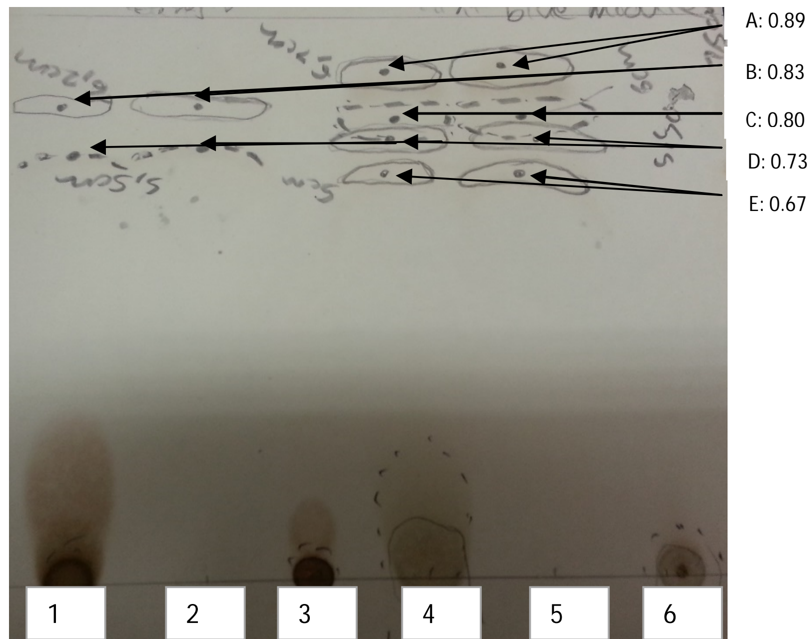

Figure 9: TLC chromatogram of crude extracts of *Z. davyi* and *X. caffra* after development in MeOH:DCM (2:8) and spraying with H_2SO_4 to detect quinones. Dotted lines represent compounds detected at 254 nm and solid lines represent compounds detected at 366 nm. Lane 1: XC-MeOH-E, Lane 2: XC-DCM-E, Lane 3: XC-HW-E, Lane 4: ZD-MeOH-E, Lane 5: ZD-DCM-E, and Lane 6: ZD-HW-E.

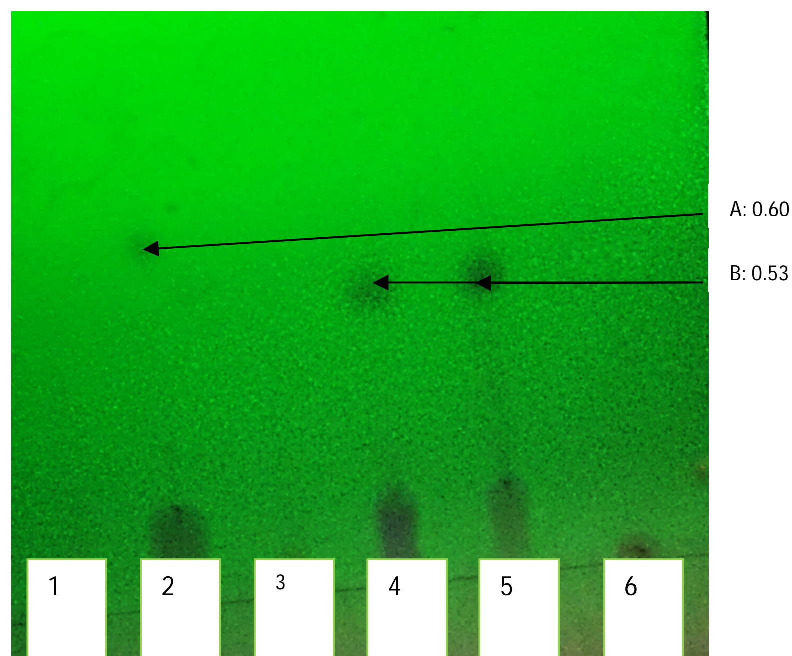

Figure 10: TLC chromatogram of crude extracts of *Z. davyi* and *X. caffra* after development in xylene:ethyl-acetate:formic acid (2.5:1:1), visualised under UV at 254 nm to detect glycosides. Lane 1: XC-MeOH-E, Lane 2: XC-DCM-E, Lane 3: XC-HW-E, Lane 4: ZD-MeOH-E, Lane 5: ZD-DCM-E, and Lane 6: ZD-HW-E.

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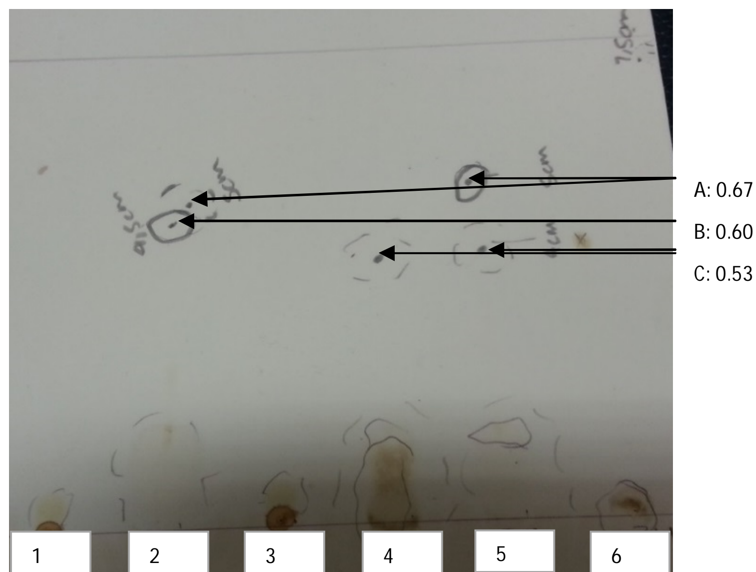

Figure 11: TLC chromatogram of crude extracts of *Z. davyi* and *X. caffra* after development in xylene:ethyl-acetate:formic acid (2.5:1:1) and spraying with 10% KOH in 50% MeOH to detect glycosides. Dotted lines represent compounds detected at 254 nm and solid lines represent compounds detected at 366 nm. . Lane 1: XC-MeOH-E, Lane 2: XC-DCM-E, Lane 3: XC-HW-E, Lane 4: ZD-MeOH-E, Lane 5: ZD-DCM-E, and Lane 6: ZD-HW-E.

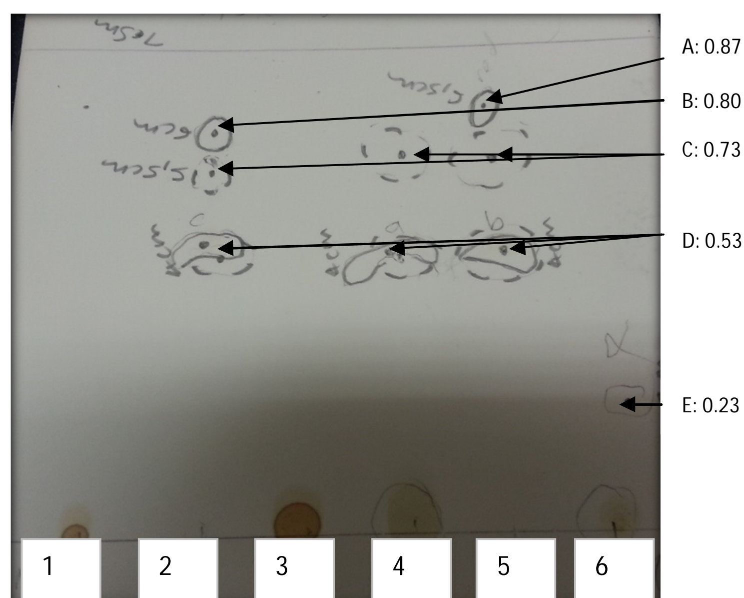

Figure 12: TLC chromatogram of crude extracts of *Z. davyi* and *X. caffra* after development in chloroform:acetone:formic acid (15:3:2) and spraying with 1% $AlCl_3$ in EtOH to detect flavonoids. Dotted lines represent compounds detected at 254 nm and solid lines represent compounds detected at 366 nm. Lane 1: XC-MeOH-E, Lane 2: XC-DCM-E, Lane 3: XC-HW-E, Lane 4: ZD-MeOH-E, Lane 5: ZD-DCM-E, and Lane 6: ZD-HW-E.

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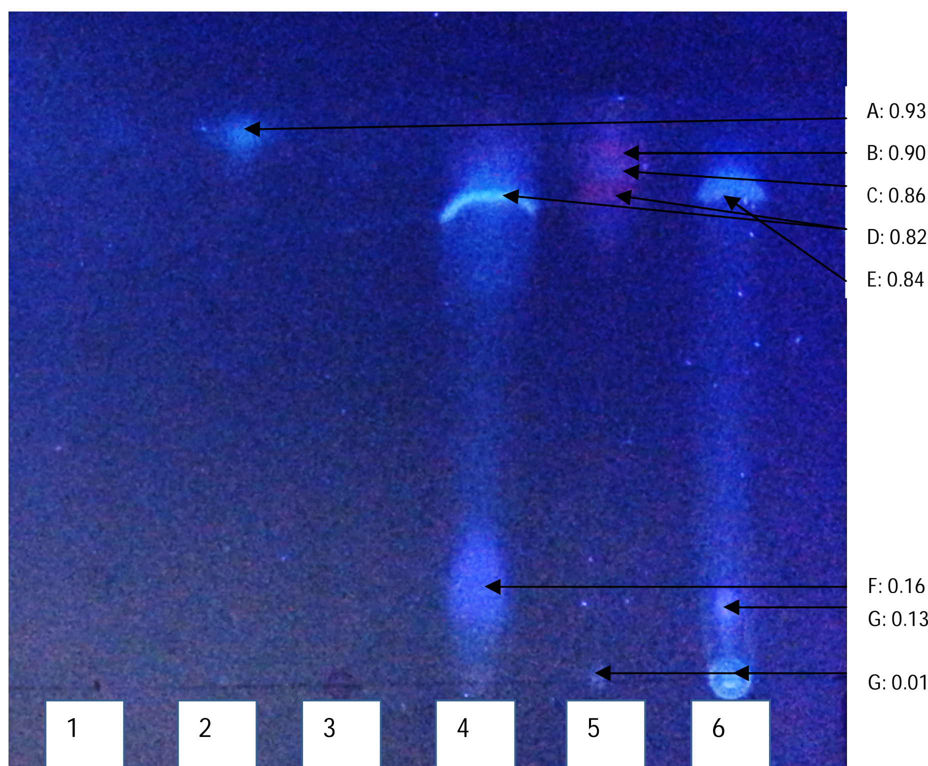

Figure 13: TLC chromatogram of crude extracts of *Z. davyi* and *X. caffra* after development in MeOH:DCM (9:1), visualised under UV 366 nm. Red, purple, violet and blue bands can be seen representing the presence of alkaloids and phenolic acids. Lane 1: XC-MeOH-E, Lane 2: XC-DCM-E, Lane 3: XC-HW-E, Lane 4: ZD-MeOH-E, Lane 5: ZD-DCM-E, and Lane 6: ZD-HW-E.

Saponins, most of which have haemolytic properties,[108] were not present in any of the extracts (Table 3). All *Z. davyi* extracts contained alkaloids (Table 3 and Figures 8 and 13). This is consistent with literature where the *Zanthoxylum* genus and specifically *Z. davyi* have been proven to contain benzophenanthridine alkaloids which have antimicrobial activity.[68,109,110] The benzophenanthridine alkaloids detected in stem-bark extracts of *Z. davyi* include: chelerythrine, dihydrochelerythrine, bocconoline, 6-hydroxydihydrochelerythrine and 6-methoxy-7-demethyldihydrochelerythrine, 4-methoxy-1-methyl-2(1H)-quinolinone as well as the lignin, meso-sesamin.[68]

Phenolic acids, sterols and terpenoids were found to be present in all extracts (Table 3 and Figures 8 and 13). These compounds are associated with antimicrobial activity.[72,111,112] In previous studies phenolic compounds such as vanillic, ferulic and coumaric acid have been isolated and terpenoids were also present in all extracts (Table

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3).[8] Sterols were found in the highest yields in the MeOH extracts of both *Z. davyi* and *X. caffra*. The MeOH extract of *X. caffra* also had the highest yield of terpenoids (Table 3).

Amines were found to be present in the MeOH extract of *Z. davyi* as well as the DCM extracts of both plants (Table 3 and Figure 7). The detection of amines could imply the presence of antimicrobial proteins/enzymes in *X. caffra* and *Z. davyi*.[69] All extracts, with the exception of the HW extract of *Z. davyi*, contained glycosides (Table 3 and Figures 10 and 11). Munodawafa *et al.*[113] found leaf and root extracts of *X. caffra* to contain cardiac glycosides.

The MeOH and DCM extracts of both plants were found to contain quinones (Table 3 and Figure 9). Previous studies support the presence of quinones in both *Zanthoxylum*[114] and *Ximenia* species.[115] Quinones have pharmacological value as they possess anti-haemorrhagic, antioxidant and antimicrobial properties in the body. Vitamin K is a naphthoquinone important in blood clotting, and co-enzyme Q is a ubiquinone important in easing oxidation in tissues.[116] Anthraquinones (such as hypericin) from plants have been shown to have antimicrobial activity.[116]

Previous studies confirm that compounds isolated from the *Ximenia* genus include glycosides, tannins, phenolics, alkaloids, quinones and terpenoids.[115] It has also been reported that leaf extracts of *X. caffra* contain tannins and glycosides, while the root extract contains flavonoids, saponins and coumarins in addition to tannins and glycosides.[113] The *Zanthoxylum* genus has been reported to contain compounds such as benzophenanthridine alkaloids, flavonoids, coumarins, terpenoids, lignans and linear chain fatty acids which concur with the findings obtained via TLC.[109] Chen *et al.*[109] reported that the genus is a rich source of phytochemicals and this may be the reason why this plant is used to treat so many different ailments. The bark of *Z. capense* is traditionally used to make toothbrushes while the powdered root is applied to treat toothache,[89] therefore the different activities (anti-plaque and anti-inflammatory) could be linked to the different parts of the plant, which contain different phytochemicals.

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After qualitating the phytochemical groups present in the plant extracts, HPLC was conducted to quantitate most abundant compounds in the extracts. Co-chromatography confirmed the presence of specific phytochemicals. Standards were also run individually to determine their specific retention times (Table 4).

Table 4: Phytochemical compounds reported to be present in the plant extracts, were analysed using HPLC to determine their retention times.

Phytochemical class	*Phytochemical compound*	*Compound abbreviation*	*Retention time (min)*
Alkaloids	Chelerythrine	CHE	9.883
	Nitidine	NIT	9.502
	Sanguinarine	SAN	8.715
Flavonoids	Catechin	CAT	4.649
	Quercetin	QUE	11.136
	Rutin	RUT	8.373
Phenolic acids	Caffeic acid	CAF	6.316
	Coumaric acid	COU	8.623
	Ferulic acid	FER	8.555
	Gallic acid	GAL	4.426
	Salicylic acid	SAL	10.011
	Sinapic acid	SIN	8.203
	Syringic acid	SYR	5.457
	Vanillic acid	VAN	5.522
Vitamin	Ascorbic acid	ASC	1.480

Ascorbic acid, gallic acid, sinapic acid, sanguinarine and salicylic acid were found in the DCM extract of *X. caffra*, with retention times of 1.488, 4.442, 8.203, 8.733 and 10.043 min, respectively (Figure 14A). Figure 14B displays the HPLC fingerprint for the MeOH extract. It is evident that ascorbic acid, gallic acid, caffeic acid, rutin, sanguinarine and nitidine were present, with retention times of 1.480, 4.355, 6.357, 8.386, 8.744 and

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9.521 min, respectively. The HPLC fingerprint for the HW extract showed the presence of catechin, vanillic acid, and ferulic acid, with retention times 4.669, 5.522 and 8.589 min, respectively (Figure 14C). Phenolic acids were contained in all extracts of *X. caffra*. The DCM and MeOH extracts were found to contain alkaloids and ascorbic acid. The flavonoids, rutin and catechin, were found to be present in the MeOH and HW extracts, respectively.

The DCM extract of *Z. davyi*, indicated the presence of ascorbic acid, syringic acid, sinapic acid, salicylic acid and quercetin, with retention times of 1.467, 5.457, 8.203, 10.011 and 11.136 min, respectively (Figure 15A). The MeOH extract was found to contain rutin, ferulic acid, nitidine and quercetin which eluted at 8.343, 8.574, 9.496 and 11.101 min, respectively (Figure 15B). In the HW extract ascorbic acid, gallic acid, sinapic acid, rutin, sanguinarine, nitidine, chelerythrine and salicylic acid were detected. These compounds had retention times of 1.451, 4.445, 8.249, 8.394, 8.701, 9.514, 9.884 and 10.044 min, respectively (Figure 15C). All extracts of *Z. davyi* contained phenolic acids and flavonoids. The MeOH and HW extracts contained alkaloids. Ascorbic acid was found in the DCM and HW extracts. Ascorbic acid is an essential nutrient in man and is known for its antioxidant activities.[117]

When compared to HPLC, TLC did not detect the presence of flavonoids (rutin) in the HW extract of *Z. davyi*. TLC also failed to detect the presence of alkaloids in the DCM and MeOH extracts of *X. davyi*. The flavonoid catechin was detected in the HW extract of *X. caffra* via HPLC, however, no flavonoids were detected in the same extract via TLC. The ability to detect certain phytochemical classes when using TLC could be ascribed to the concentration/load of extract spotted. Other factors which could have accounted for the discrepancy in the results between TLC and HPLC are environmental factors, such as humidity, temperature, light, fumes and mechanical stress which are known to affect TLC analyses as well as the increased sensitivity of the HPLC methodology.[118]

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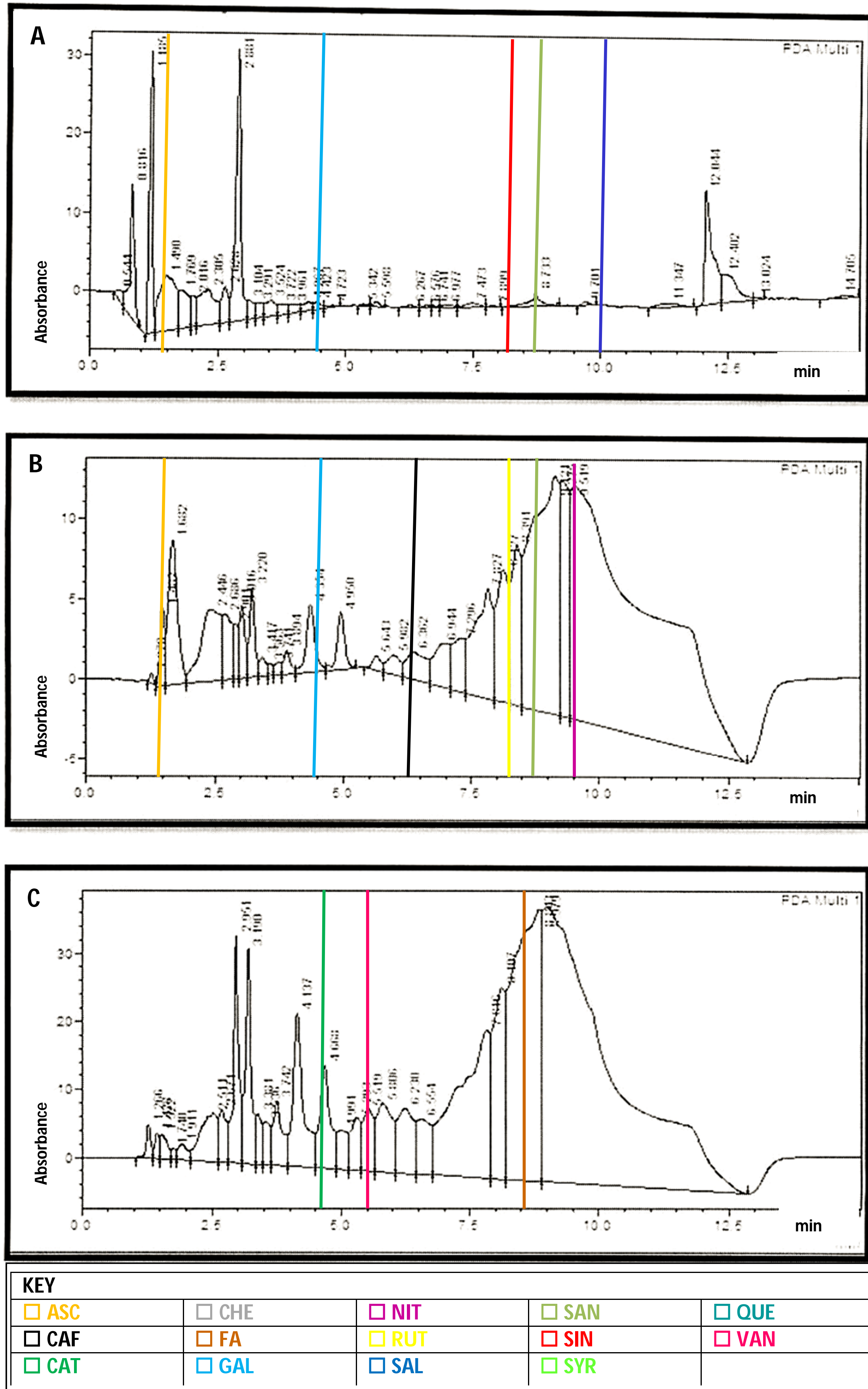

Figure 14: Chromatograms of *X. caffra* extracts, A) XC-DCM-E, B) XC-MeOH-E, and C) XC-HW-E.

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KEY				
☐ ASC	☐ CHE	☐ NIT	☐ SAN	☐ QUE
☐ CAF	☐ FA	☐ RUT	☐ SIN	☐ VAN
☐ CAT	☐ GAL	☐ SAL	☐ SYR	

Figure 15: Chromatograms of *Z. davyi* extracts, A) ZD-DCM-E, B) ZD-MeOH-E, and c) ZD-HW-E.

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Various phenolic acids were detected in the extracts of *Z. davyi* and *X. caffra* (Figures 14 and 15). Polyphenols previously isolated from the leaves *of X. caffra* include gallic acid, quercetin, kaempferol, catechin and their derivatives, with quercetin-rutinoside being most abundant.[119] Ndhlala *et al.*[120] found *X. caffra* to contain 1.2% and about 1% dry weight condensed tannins in peels and pulps, respectively. Gallic acid has been attributed to the antimicrobial activity of several plant extracts and have been shown to have activity against *Salmonella typhimurium* and *S. aureus.*[121] Quercetin has been reported to have properties ranging from including antioxidant, anti-cancer, anti-thrombotic effects, antimicrobial to anti-HIV effects. Catechin-rich extracts have been reported to contain antiviral activity against resistant *herpes simplex* virus (type 1).[122]

HPLC revealed that the phytochemicals of greatest abundance were gallic acid (13.4% in ZD-HW-E), nitidine (33.5% in XC-MeOH-E) and quercetin (26% in ZD-MeOH-E). Calibration curves were generated in order to quantitate these compounds. The concentration of gallic acid in ZD-HW-E was determined as 0.102 mg/mL, nitidine at 0.387 mg/mL in XC-MeOH-E, and quercetin at a concentration of 0.250 mg/mL in ZD-MeOH-E.

3.3 Antimicrobial activity

3.3.1 Planktonic microorganisms

In order to test for antimicrobial activity, the disc diffusion assay was employed as a qualitative crude screening method to identify the extracts which displayed antimicrobial potential against the different microorganisms and strains (2.5.3).

The DCM extracts of both *X. caffra* and *Z. davyi* displayed antimicrobial activity against all microbial strains tested (Table 5). This implies that the activity could be attributed to compounds extracted in non-polar solvents. The MeOH extract of *Z. davyi* also displayed activity against all strains. The HW and MeOH extracts of *X. caffra* displayed activity only against the standard *C. albicans* strain (ATCC10231), whereas the HW extract of *Z. davyi* did not display any activity at all. Activity seen against planktonic species as determined

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by disc diffusion was not considered noteworthy as crude extracts with activity > 1 mg/mL, are not deemed active.[123]

In a previous study using the disc diffusion assay, methanol extracts of *X. caffra*, at a concentration of 100 mg/mL, were reported to produce zones of inhibition of 25 mm, 14 mm, 12 mm, 15 mm and 14.3 mm against *S. aureus*, *Vibrio cholerae*, *Shigella dysenteriae*, *Shigella flexneri* and *Shigella boydi*, respectively.[124] These findings support the results in this study which indicate that *X. caffra* extracts possess activity against *S. aureus*, however, at a concentration not deemed significant (Table 5).

A study conducted by Obi *et al.*[125] found the MeOH, bark extracts of *Z. davyi* to have no activity against *S. aureus* at a concentration of 1 mg/mL. However, *Bacillus cereus*, *Bacillus subtilis* and *Streptococcus pyogenes* produced zones of inhibition of 12 mm, 10 mm and 19 mm at a concentration of 1 mg/mL. With regard to Gram-negative bacteria, *Escherichia coli*, *Shigella spp.* and *Salmonella typhimurium* inhibited bacterial growth of 18 mm, 15 mm and 14 mm, respectively at 1 mg/mL. The authors concluded that *Z. davyi* may be used to combat infections caused by enteric pathogens, which supported its use as an alternative antimicrobial agent. Apart from enteric pathogens, the leaf and bark extracts of *Z. davyi* were found to display antimicrobial activity against *S. aureus, Staphylococcus epidermis, Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli and Micrococcus luteus.*[126]

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Table 5: Screening of plant extracts for antimicrobial activity using the disc diffusion assay.

Plant	Extract	concentration (mg/mL)	C. a. ATCC 90028	C. a. Clinical 1	C. a. clinical 2	S. a. ATCC 12600	S. a. clinical 1	S. a. clinical 2
***X. caffra* (bark)**	MeOH	2.34	23.21 ± 1.85	-	-	-	-	-
	DCM	1.30	19.10 ± 0.48	24.91± 0.54	17.69 ± 0.25	20.50 ± 0.57	20.68 ± 1.21	17.40 ± 0.29
	HW	2.85	19.46 ± 0.84	-	-	-	-	-
***Z. davyi* (bark)**	MeOH	8.40	18.95 ± 0.69	24.90 ± 1.17	24.64 ± 0.55	21.00 ± 1.11	19.17 ± 0.35	18.92 ± 0.86
	DCM	4.20	16.74 ± 1.33	18.47 ± 0.50	21.53 ± 0.42	18.56 ± 0.42	18.13 ± 0.59	15.89 ± 0.31
	HW	1.30	-	-	-	-	-	-
Positive control[a]		**Concentration (mg/mL)**	**C. a. ATCC 90028**	**C. a. clinical 1**	**C. a. clinical 2**	**S. a. ATCC 12600**	**S. a. clinical 1**	**S. a. clinical 2**
Ciprofloxacin		0.010	N/A	N/A	N/A	21.80 ± 1.02	28.28 ± 0.45	28.33 ± 0.59
Amphotericin B		0.010	19.47 ± 0.38	23.32 ± 0.87	21.60 ± 0.40	N/A	N/A	N/A

C. a.: *Candida albicans*; S. a.: *Staphylococcus aureus*; DCM: Dichloromethane; HW: Hot water; MeOH: Methanol; [a]C. a. control: Amphotericin B; [a]S. a. control: Ciprofloxacin; N/A: Not applicable; -: No activity detected. Values reported as mean ± S.E.M. (n=6)

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The variations of the results between studies using the same extracts and microorganisms can be caused by numerous factors. The factors which contribute to the success of this assay need to be taken into account as well as the suitability of the technique.[127] Experimental factors include: ability of the active compounds to diffuse through the agar and type of agar chosen,[127] handling and incubation procedures, timing considerations, volume of agar poured, as well as the interval between seeding the plates and placing the discs on the inoculated plates.[128] Furthermore, Rios *et al.*[127] states that the pH of the compounds in dilutions also plays a major role in the antimicrobial activity of extracts. Other factors may include the ability of the microbial strains to mutate during handling, spontaneous mutations, mutations as a result of temperature change and application of compound.[129] It is recommended that more than one assay be used to determine antimicrobial activity. In this study, the broth microdilution assay was chosen, not only to determine the minimum inhibitory concentrations of the extracts which displayed activity, but also to confirm the results of the disc diffusion assay. An advantage of the broth microdilution assay is that it allows for the testing of both polar and non-polar compounds.[123]

Table 6 displays the MIC values for all the extracts against the strains tested. With the exception of XC-DCM-E all values are > 1 mg/mL therefore the activity of these extracts were considered to be clinically insignificant[123] against planktonic *S. aureus* and *C. albicans* clinical and standard strains. After determining the MIC's of the extracts, the MBC's or MFC's were also determined. The MBC and MFC was regarded as the lowest concentration which showed no colony forming units or microbial growth on agar. Apart from XC-DCM-E the bactericidal/fungicidal concentrations were found to be either equal to, or greater than the inhibitory concentrations determined for the specific extracts (Table 7). It is important to note that XC-DCM-E was the only extract to have an MIC, and MFC < 1 mg/ml on *C. albicans* clinical strains.

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Table 6: Minimum inhibitory concentrations (MIC) of *X. caffra* and *Z. davyi* plant extracts.

		MIC (mg/mL)					
Plant	**Extract**	**C. a. ATCC 90028**	**C. a. clinical 1**	**C. a. clinical 2**	**S. a. ATCC 12600**	**S. a. clinical 1**	**S. a. clinical 2**
***X. caffra* (bark)**	MeOH	23.40	-	-	-	-	-
	DCM	1.60	0.20	0.80	1.60	1.60	1.60
	HW	2.85	-	-	-	-	-
***Z. davyi* (bark)**	MeOH	8.40	4.20	8.40	2.10	8.40	4.20
	DCM	4.20	4.20	4.20	4.20	4.20	4.20
	HW	-	-	-	-	-	-
Control[a]	**Concentration (mg/mL)**	**C. a. ATCC 90028**	**C. a. clinical 1**	**C. a. clinical 2**	**S. a. ATCC 12600**	**S. a. clinical 1**	**S. a. clinical 2**
Ciprofloxacin	0.010	N/A	N/A	N/A	<0.001	<0.001	<0.001
Amphotericin B	0.010	<0.001	0.013	0.050	N/A	N/A	N/A

C. a.: *Candida albicans*; S. a.: *Staphylococcus aureus*; DCM: Dichloromethane; HW: Hot water; MeOH: Methanol; [a]C. a. control = Amphotericin B; [a]S. a. control = Ciprofloxacin; N/A: Not applicable; -: no activity detected (n=3).

Table 7: Minimum fungicidal/bactericidal concentrations of *X. caffra* and *Z. davyi* plant extracts.

		MFC/MBC (mg/mL)					
Plant	**Extract**	**C. a. ATCC 90028**	**C. a. clinical 1**	**C. a. clinical 2**	**S. a. ATCC 12600**	**S. a. clinical 1**	**S. a. clinical 2**
***X. caffra* (bark)**	MeOH	> 23.40	-	-	-	-	-
	DCM	> 1.60	0.40	1.60	> 1.60	> 1.60	> 1.60
	HW	> 2.85	-	-	-	-	-
***Z. davyi* (bark)**	MeOH	> 8.40	8.40	8.40	8.40	> 8.40	> 8.40
	DCM	> 4.20	> 4.20	> 4.20	> 4.20	> 4.20	> 4.20
	HW	-	-	-	-	-	-
Positive control[a]	**Concentration (mg/mL)**	**C. a. ATCC 90028**	**C. a. clinical 1**	**C. a. clinical 2**	**S. a. ATCC 12600**	**S. a. clinical 1**	**S. a. clinical 2**
Ciprofloxacin	0.010	N/A	N/A	N/A	0.003	0.002	< 0.001
Amphotericin B	0.010	0.006	0.025	0.010	N/A	N/A	N/A

C. a.: *Candida albicans*; S. a.: *Staphylococcus aureus*; DCM: Dichloromethane; HW: Hot water; MeOH: Methanol; [a]C. a. control = Amphotericin B; [a]S. a. control = Ciprofloxacin; N/A: Not applicable; -: no activity detected (n=3).

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Antimicrobial activity determined by the broth microdilution assay has been previously reported for *X. caffra* and *Z. davyi*.[7,130] Fabry *et al.*[130] reported that the MIC values of the *X. caffra* (root) extracts which killed/inhibited 90% of *S. aureus* and *Enterococci* was 0.5 mg/mL, whereas activity against *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella* and *Salmonella* were > 1 mg/mL. The MBC of the same root extract required to kill 90% of *S. aureus* was recorded to be 1 mg/mL whereas the MBC's of the other organisms mentioned were > 1 mg/mL. A study conducted by Steenkamp *et al.*[7] found that methanol and water extracts of *X. caffra* (roots) had MIC values of 5.66 mg/mL and 1.29 mg/mL against *S. aureus* which supports the present findings that show activity against *S. aureus* to be > 1 mg/mL. This study also showed that a methanol extract of *Z. davyi* (bark) had MIC values of 1 mg/mL against both *S. aureus* and *S. epidermis*.[7] The MIC of a *Z. davyi* (bark) methanol extract was reported to be 1 mg/mL, whereas the water extract had no activity against a standard planktonic *S. aureus* strain.[7] The latter supports the current results (Tables 5-7). To the best of the author's knowledge, there are no previous studies regarding the antimicrobial activity of *X. caffra* bark extracts.

The results obtained using the broth microdilution assay confirms that the activity on planktonic bacteria/fungi is negligible. Rios *et al.*[127] states that it is a common mistake in many papers to claim positive activity for slight dilutions or excessively high concentrations, whereas, presence of activity in the case of concentrations below 100 µg/mL for extracts and 10 µg/mL for isolated compounds should be considered noteworthy. According to Kuete *et al.*,[131] antibacterial activity of a plant extract is considered to be significant when MIC's are below 100 µg/mL, moderate when 100≤ MIC ≤ 625 µg/mL and weak when MIC's are above 625 µg/mL. It is to be noted that absence of antimicrobial activity as determined by the disc diffusion assay does not necessarily mean that these extracts do not have antimicrobial activity. Inactivity could be noted where compounds do not diffuse into the agar.[123] Furthermore, it is possible that activity was not noted against the microorganisms tested against, but may be active against other microbial species.

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3.3.2 Biofilms

In order to determine the activity of the plant extracts on biofilm species, biofilms of *S. aureus* and *C. albicans* were grown in 96-well microplates. Biofilms were treated with plant extracts and conventional antibiotics. The crystal violet assay was then employed to quantify the biomass of *S. aureus* and *C. albicans* biofilms. Data was processed and represented as percentage biomass relative to untreated control. Biofilm Inhibitory Concentrations (BIC's) were determined from the data. Colonies were counted using the Viable Colony Count (VCC) assay in order to deem the results of the assays valid.

Figure 16A depicts the biomass of *C. albicans* strains treated with XC-MeOH-E. The ATCC strain of *C. albicans* showed a stepwise increase in biomass >100% from 1.10 mg/mL up to 36.40 mg/mL. A decrease in biomass below 45% was noted from 0.10 mg/mL to 0.60 mg/mL. Clinical strain 1 had an increase in biomass >100% from 18.20 mg/mL up to 36.40 mg/mL. A decrease in biomass below 50% was noticeable from 0.10 mg/mL to 9.10 mg/mL. With regard to clinical strain 2, an increase in biomass >100% was evident at 36.40 mg/mL. The biomass decreased to less than 80% for all concentrations below 36.40 mg/mL. Amphotericin B was more effective in inhibiting the biofilm than XC-MeOH-E on all *C. albicans* strains tested ($p < 0.05$).

In Figure 16B, the biomass of *S. aureus* strains treated with XC-MeOH-E can be visualised. There was a stepwise increase in biomass of the ATCC strain of *S. aureus* >100% from 0.60 mg/mL to 36.40 mg/mL. From 0.10 mg/mL to 0.30 mg/mL, the biomass decreased below 80%. For clinical strain 1 a stepwise increase in biomass >100% from 2.30 mg/mL to 36.40 mg/mL was noted. The biomass decreased below 75 % for the rest of the concentrations tested (0.10 mg/mL to 1.10 mg/mL). With regard to clinical strain 2, there was a stepwise increase in biomass >100% from 2.30 mg/mL to 36.40 mg/mL. From 0.10 mg/mL to 1.10 mg/mL, biomass decreased below 75%. The control, ciprofloxacin, had a more pronounced effect on eradicating the biofilm than XC-MeOH-E for all *S. aureus* strains tested ($p < 0.05$).

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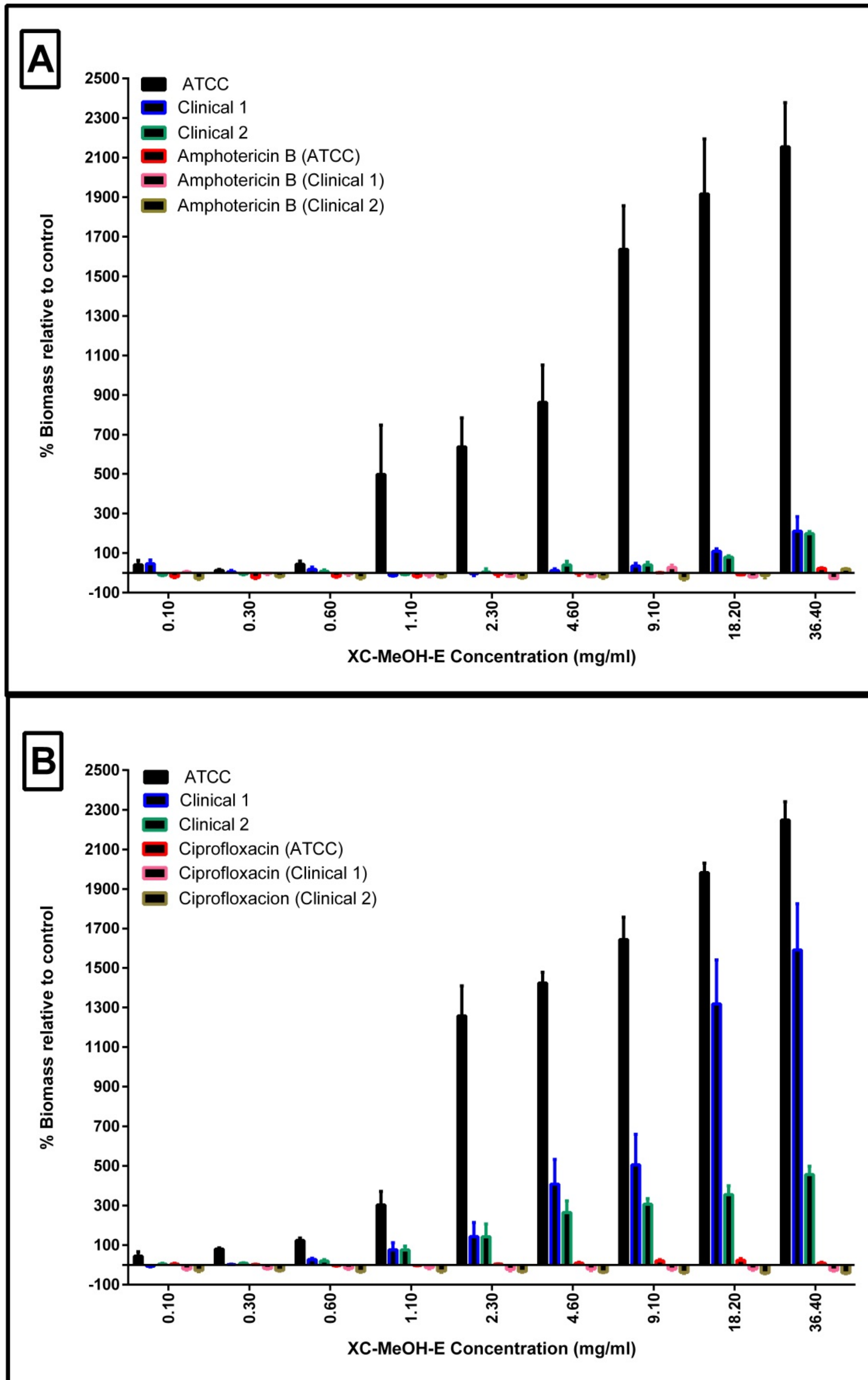

Figure 16: Effect of XC-MeOH-E against biofilms of A) *C. albicans* ATCC and clinical strains, and B) *S. aureus* ATCC and clinical strains. Data is represented as percent biomass relative to untreated control (mean ± S.E.M., n=6).

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The biomass of *C. albicans* strains treated with XC-DCM-E is depicted in Figure 17A. With regard to the ATCC strain, there was a decrease in biomass below 40% at all concentrations tested (0.05 mg/mL to 13.40 mg/mL). As for clinical strain 1, there was a decrease in biomass below 80% at all concentrations tested. With regard to clinical strain 2, there was a decrease in biomass below 10% at concentrations of 13.40 mg/mL, 6.70 mg/mL, 3.35 mg/mL, 1.68 mg/mL and 0.05 mg/mL. At the other concentrations, biomass decreased below 70%. All concentrations of amphotericin B decreased the biomass of the standard and clinical strains of *C. albicans* below 30% which was significantly ($p < 0.05$) better than XC-DCM-E on all strains of *C. albicans.*

XC-DCM-E increased the biomass of the ATCC strain of *S. aureus* >100% at a concentration of 13.40 mg/mL (Figure 17B). At concentrations lower than 13.40 mg/mL, there was a decrease in biomass to under 40%. XC-DCM-E caused a decrease in biomass of clinical strains 1 and 2 below 20% and 30%, respectively, at all concentrations tested. Ciprofloxacin was more effective in eradicating *S. aureus* clinical strain biofilms than XC-DCM-E ($p < 0.05$). There was no significant difference between ciprofloxacin and XC-DCM-E with regard to inhibition in the ATCC strain of *S. aureus* ($p > 0.05$).

When treated with XC-HW-E (Figure 18A) a stepwise decrease in biomass <65% was seen from 0.01 mg/mL to 0.36 mg/mL which was followed by a stepwise increase in the biomass of *C. albicans* ATCC >100% from 0.71 mg/mL up to 2.85 mg/mL. At all concentrations, the biomass of clinical strain 1 decreased below 30% (Figure 18A). A decrease in the biomass of clinical strain 2 was seen below 25% at all concentrations tested (Figure 18A). Amphotericin B was significantly ($p < 0.05$) better at eradicating all strains of *C. albicans* biofilms than XC-HW-E.

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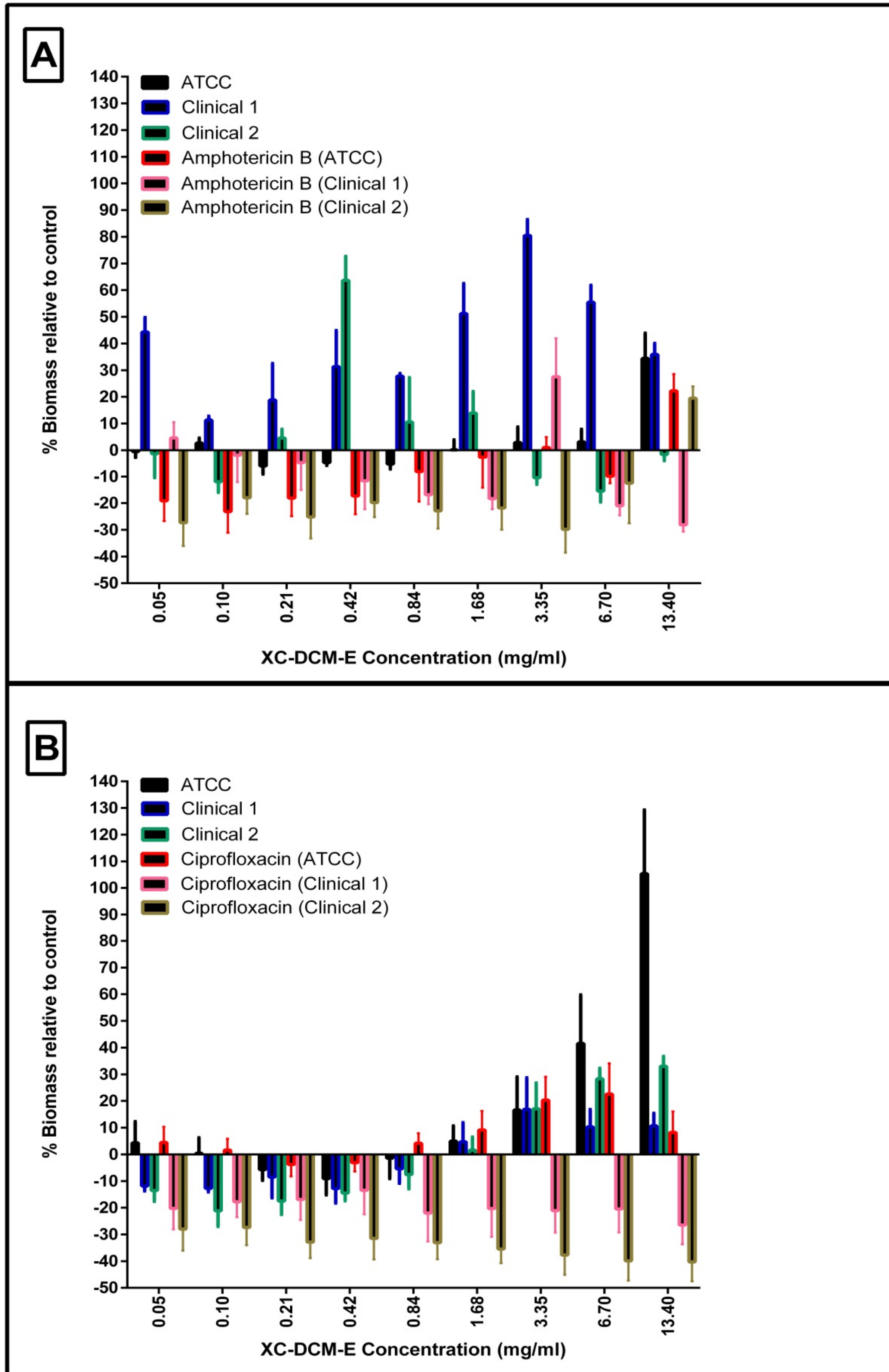

Figure 17: Effect of XC-DCM-E against biofilms of A) *C. albicans* ATCC and clinical strains, and B) *S. aureus* ATCC and clinical strains. Data is represented as percent biomass relative to untreated control (mean ± S.E.M., n=6).

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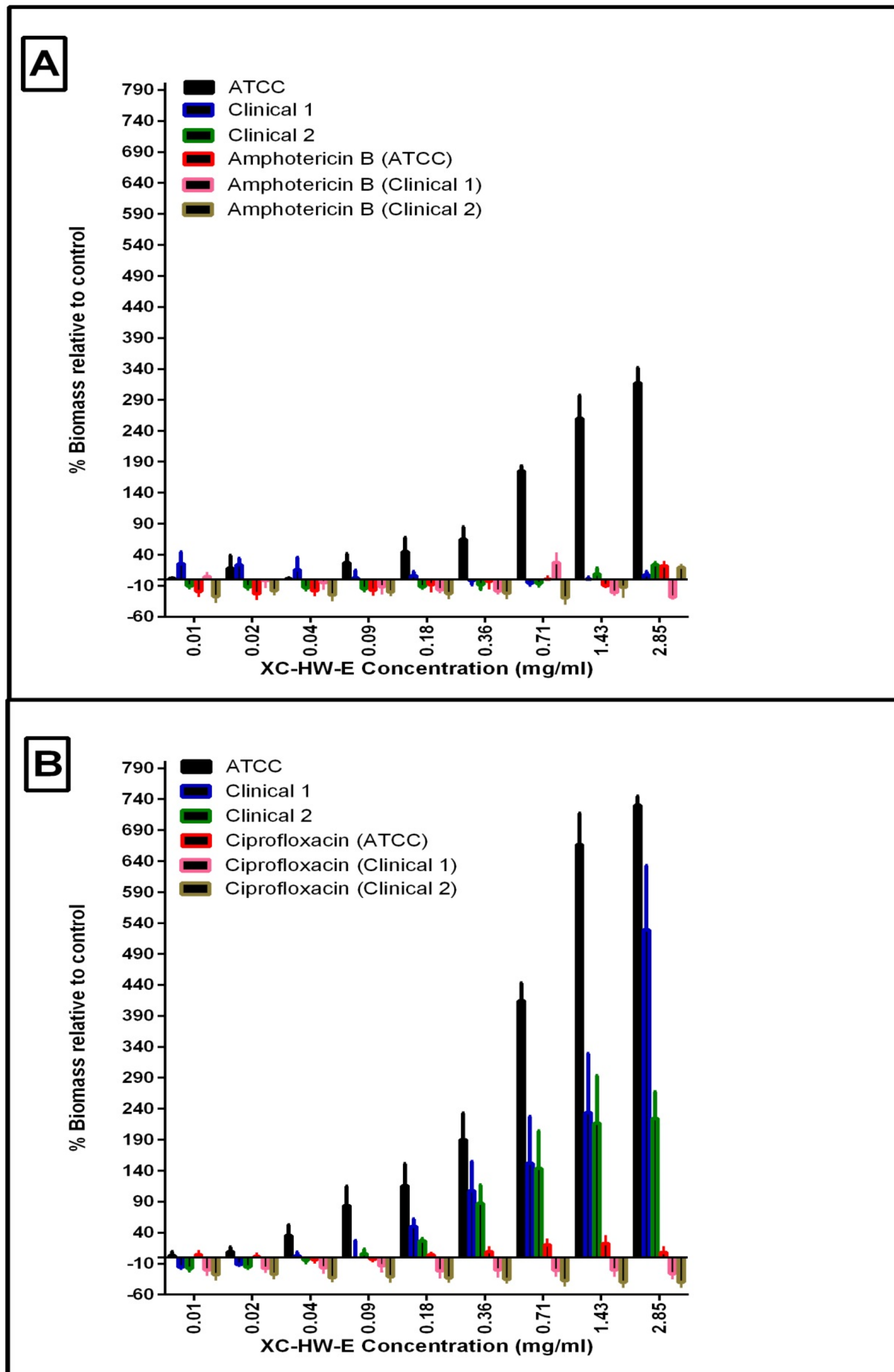

Figure 18: Effect of XC-HW-E against biofilms of A) *C. albicans* ATCC and clinical strains, and B) *S. aureus* ATCC and clinical strains. Data is represented as percent biomass relative to untreated control (mean ± S.E.M., n=6).

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XC-HW-E caused a stepwise increase in biomass of the ATCC strain of *S. aureus* from 0.18 mg/mL to 2.85 mg/mL; however, there was a decrease in biomass below 90% for all concentrations below 0.18 mg/mL (Figure 18B). A stepwise increase in biomass of clinical strain 1 >100% was evident from 0.36 mg/mL to 2.85 mg/mL (Figure 18B). From concentrations 0.01 mg/mL to 0.18 mg/mL, biomass decreased below 50%. An increase in biomass >100% was seen in clinical strain 2 from concentrations 0.71 mg/mL to 2.85 mg/mL. All concentrations below 0.71 mg/mL decreased biomass below 90%. Ciprofloxacin decreased the biomass of all strains below 25% and was found to be significantly ($p < 0.05$) better than XC-HW-E on all strains of *S. aureus* tested.

A decrease in biomass of *C. albicans* ATCC <100% was evident from 0.03 mg/mL to 4.20 mg/mL; and an increase in biomass >100% at a concentration of 8.40 mg/mL (Figure 19A). Negative inhibition was evident for *C. albicans* clinical strain 1 at concentrations 2.10 mg/mL to 8.40 mg/mL. At concentrations of 0.03 mg/mL to 1.05 mg/mL, a decrease in biomass below 50% was evident (Figure 19A). ZD-MeOH-E caused negative inhibition on clinical strain 2 at most concentrations with the exception of 1.05 mg/mL. Amphotericin B was found to be significantly ($P < 0.05$) better than ZD-MeOH-E at inhibiting the ATCC strain of *C. albicans*; however, there was no significant ($p > 0.05$) difference between ZD-MeOH-E and amphotericin B on the clinical strains.

The biomass of *S. aureus* ATCC strain treated with ZD-MeOH-E resulted in an increase in the biomass of *S. aureus* ATCC >100% from 2.10 mg/mL to 8.40 mg/mL (Figure 19B) with a stepwise decrease in biomass below 70% from 0.03 mg/mL to 1.05 mg/mL. Clinical strains 1 and 2 showed a decrease in biomass below 50% at all concentrations tested. Although ciprofloxacin decreased the biomass of all strains of *S. aureus* below 25%, this was only significantly ($p < 0.05$) better than ZD-MeOH-E in the clinical strains.

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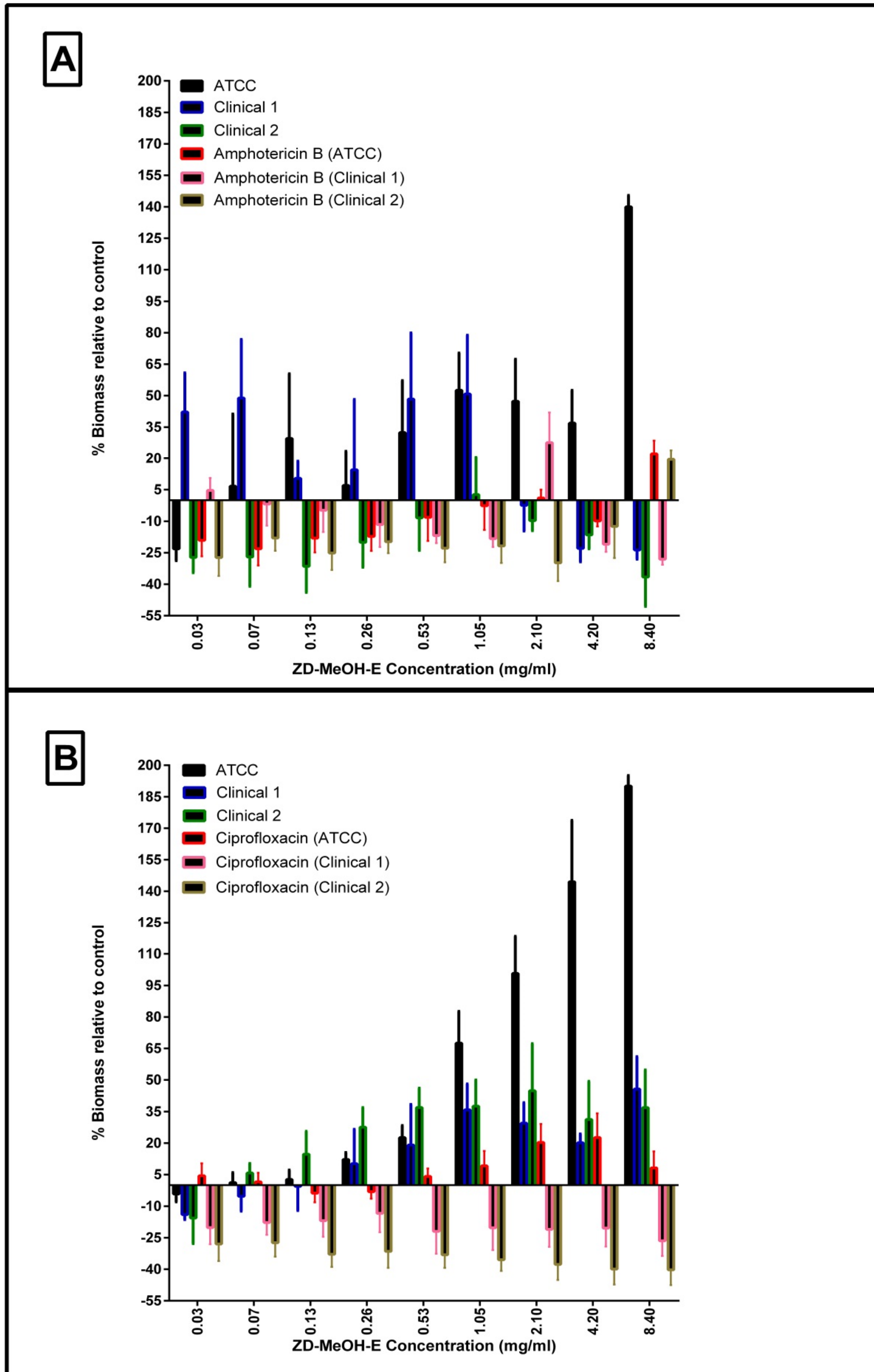

Figure 19: Effect of ZD-MeOH-E against biofilms of A) *C. albicans* ATCC and clinical strains, and B) *S. aureus* ATCC and clinical strains. Data is represented as percent biomass relative to untreated control (mean ± S.E.M., n=6).

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All concentrations of ZD-DCM-E caused a decrease in biomass of the *C. albicans* ATCC strain below 50%, whereas a decrease in biomass below 80% for clinical strain 1 and negative inhibition for clinical strain 2 was evident (Figure 20A). Amphotericin B decreased the biomass of all strains of *C. albicans* at all concentrations below 30% (Figure 21A). Amphotericin B inhibited the growth of ATCC and clinical strain 1 of *C. albicans* biofilms more potently than ZD-DCM-E.

Figure 20B displays the biomass of *S. aureus* strains treated with ZD-DCM-E. ZD-DCM-E caused a stepwise increase in the biomass of *S. aureus* ATCC >100% at concentrations 2.10 mg/mL to 4.20 mg/mL. A stepwise decrease in biomass below 50% was seen from concentrations 0.02 mg/mL to 1.05 mg/mL. The biomass of clinical strain 1 was decreased below 60% at all concentrations whereas the biomass of clinical strain 2 was decreased below 20%. Ciprofloxacin decreased the biomass of all *S. aureus* strains below 25% at all concentrations tested and was found to be significantly ($p < 0.05$) better at decreasing the biomass of all *S. aureus* strains tested.

The biomass of *C. albicans* strains treated with ZD-HW-E is presented in Figure 21A. At all concentrations, ZD-HW-E caused negative inhibition on *C. albicans* ATCC, the biomass of clinical strain 1 decreased below 80%, and the biomass of clinical strain 2 decreased below 55%. Amphotericin B decreased the biomass of all strains of *C. albicans* below 30%, however; it was found to be significantly ($p < 0.05$) superior only on the ATCC strain and clinical strain 1.

In Figure 21B, the biomass of *S. aureus* strains treated with ZD-HW-E. ZD-HW-E caused a stepwise decrease in the biomass of *S. aureus* ATCC below 100% from 0.01 mg/mL to 1.30 mg/mL. Negative inhibition was observed for both clinical strain 1 and clinical strain 2 at all concentrations tested. Ciprofloxacin decreased the biomass of all *S. aureus* strains below 25% at all concentrations and was found to be significantly ($p < 0.05$) effective when compared to ZD-HW-E on the clinical strains.

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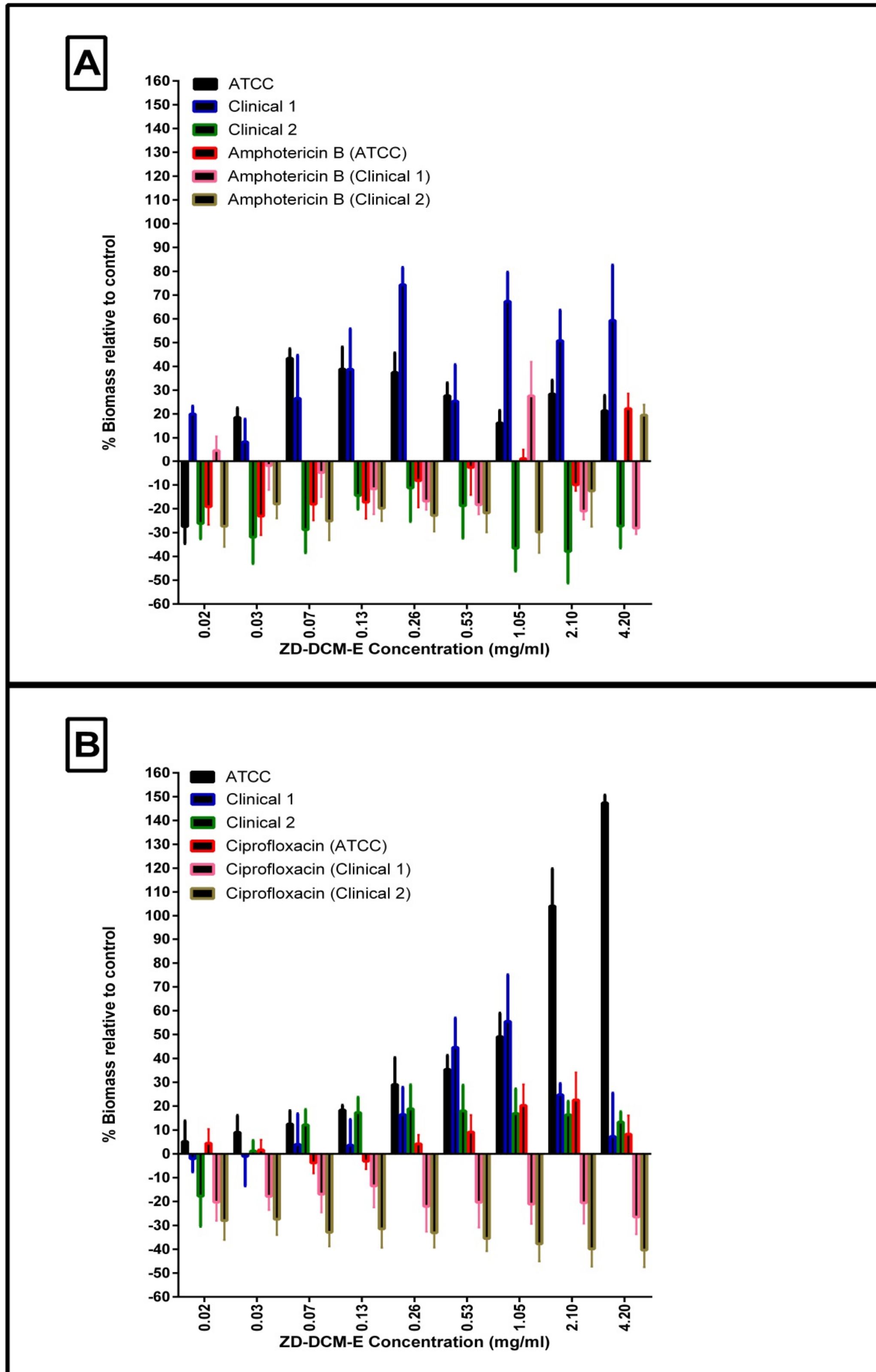

Figure 20: Effect of ZD-DCM-E against biofilms of A) *C. albicans* ATCC and clinical strains, and B) *S. aureus* ATCC and clinical strains. Data is represented as percent biomass relative to untreated control (mean ± S.E.M., n=6).

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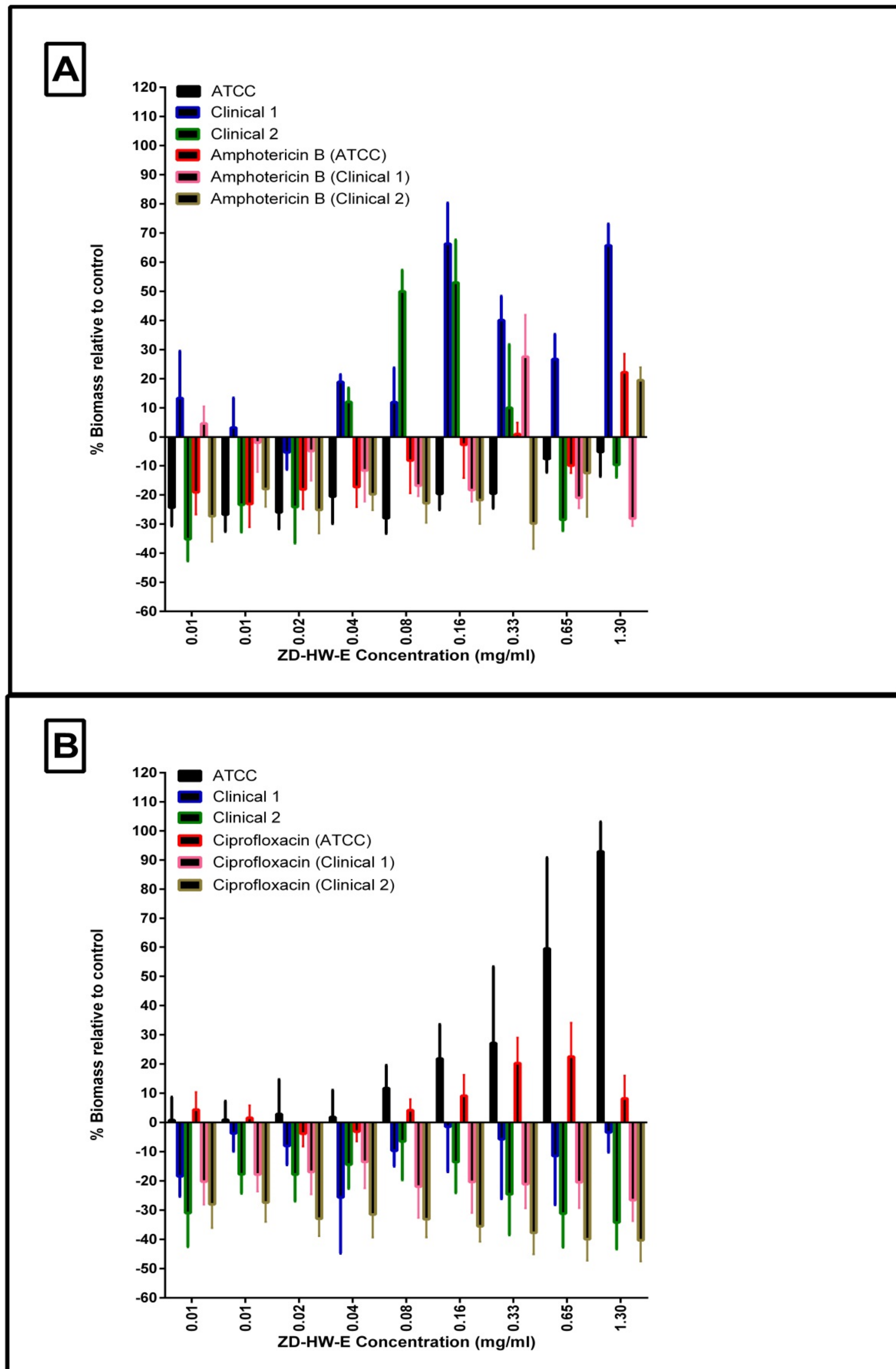

Figure 21: Effect of ZD-HW-E against biofilms of A) *C. albicans* ATCC and clinical strains, and B) *S. aureus* ATCC and clinical strains. Data is represented as percent biomass relative to untreated control (mean ± S.E.M., n=6).

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No studies were found regarding the activity of *X. caffra* and *Z. davyi* plant extracts against microbial biofilms. The majority of the extracts inhibited microbial growth of the clinical strains more significantly ($p < 0.05$) than the standard (ATCC) strains of *C. albicans* and *S. aureus* (with the exception of ZD-HW-E and XC-DCM-E on *C. albicans*). The plant extracts had a greater effect on biofilms as opposed to planktonic microorganisms which is in line with the results of the disc diffusion and broth microdilution assays. All extracts and antibiotics caused biofilm inhibition <0.001 mg/mL. A summary of the biofilm inhibitory concentration (BIC) range for the extracts on the respective strains tested in this study is provided in Table 8.

With regard to the crystal violet assay which was used to determine biomass of extract treated biofilms, a consistent paradoxical drug reaction trend was noted; lower concentrations of plant extracts inhibited biofilm adhesion/growth whereas higher concentrations promoted adhesion/growth. This is consistent with literature where high dose antimicrobials were found to cause paradoxical effects on certain microorganisms.[132,133] An isolate of *C. albicans* has been shown to have paradoxical growth *in vivo* with significantly higher CFU at 20 mg/kg of caspofungin than at 5 mg/kg.[132] Bouza *et al.*[133] reported that echinocandins have paradoxical effects on *Candida* biofilms in particular. The latter was confirmed in *Candida tropicalis* biofilms.[134] The results of this study also indicated that certain extract concentrations, had specific effects on biofilms as opposed to a dose dependent effect.

The MeOH extract of *Z. davyi* was as effective as amphotericin B on both clinical strains of *C. albicans* as well as ciprofloxacin on *S. aureus* ATCC (Figure 19). Compared to amphotericin B, the DCM extract of *Z. davyi* had a similar effect on *C. albicans* clinical strain 2 ($p > 0.05$) (Figure 20A). The HW extract of *Z. davyi* was comparable to, and as efficacious as, amphotericin B on *C. albicans* clinical strain 2 as well as ciprofloxacin on *S. aureus* ATCC (Figure 21). With regard to *X. caffra*, the DCM extract was equally as effective as ciprofloxacin on *S. aureus* ATCC (Figure 17B).

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Table 8: Summary of the biofilm inhibitory concentration (BIC) of plant extracts.

BIC range (mg/mL)							
Plant	**Extract**	**C. a. ATCC 90028**	**C. a. clinical 1**	**C. a. clinical 2**	**S. a. ATCC 12600**	**S. a. clinical 1**	**S. a. clinical 2**
***X. caffra* (bark)**	MeOH	<0.001-0.300	<0.001-1.100	<0.001-1.100	<0.001-0.600	<0.001-18.200	<0.001-18.200
	DCM	<0.001-6.700	<0.001-13.40	<0.001-13.40	<0.001-13.40	<0.001-13.40	<0.001-13.40
	HW	<0.001-0.360	<0.001-2.850	<0.001-2.850	<0.001-0.090	<0.001-0.360	<0.001-0.360
***Z. davyi* (bark)**	MeOH	<0.001-2.100	<0.001-8.400	<0.001-8.400	<0.001-4.200	<0.001-8.400	<0.001-8.400
	DCM	<0.001-1.050	<0.001-4.200	<0.001-4.200	<0.001-4.200	<0.001-4.200	<0.001-4.200
	HW	<0.001-1.300	<0.001-1.300	<0.001-1.300	<0.001-1.300	<0.001-1.300	<0.001-1.300
aPositive control		**BIC (mg/mL)**					
Amphotericin B		<0.001	<0.001	<0.001	N/A	N/A	N/A
Ciprofloxacin		N/A	N/A	N/A	<0.001	<0.001	<0.001

C. a.: *Candida albicans;* S. a.: *Staphylococcus aureus;* DCM: Dichloromethane; HW: Hot water; MeOH: Methanol; aC. a. control = Amphotericin B; aS. a. control = Ciprofloxacin; N/A: Not applicable; -: no activity detected (n=6).

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Other plants of the *Zanthoxylum* species have also been proven to display antimicrobial activities, supporting the anti-biofilm effects observed with regard to *Z. davyi* extracts in the present study. The alkaloid, 8-acetonyldihydroavicine isolated from *Z. caudatum* has been reported to display antibacterial activity.[135] The alkaloid liriodenine, also isolated from *Z. caudatum* is known to possess strong antifungal activity.[135] *Z. capense* has been found to restore the antibiotic activity of antibiotics which have been rendered ineffective by resistant strains including MRSA.[136] The fruit oils of *Z. zanthoxyloides* have been shown to display antimicrobial activities,[137] which are used in wound dressings.[137]

Antineoplastic, anti-trypanosomal, anti-rheumatic, antioxidant, analgesic, moluscicidal and pesticidal activity has been reported for the *Ximenia* genus.[115] Vomifoliol, a compound related to abscisic acid and extracted from *X. caffra* leaves, inhibits *Nisseria gonorrhoea*.[138]

Various factors contribute to the virulence of *S. aureus* such as protein A and hemolysins, which have been associated with inflammatory signalling and cell death in host organisms.[139] *S. aureus* also has an assortment of extracellular toxins and its primary virulence factor is the ability to form biofilms.[140] Possible mechanisms by which the extracts could have inhibited microbial growth are provided. The extracts may have had an effect on the proteins which increased colonisation of *S. aureus* such as clumping factors A and B as well as an array of binding proteins. The antibiotic linezolid has been shown to decrease the secretion of virulence factors such as protein A and alpha- and beta-hemolysins in a dose dependent manner. While the expression of non-toxic exoproteins such as triacylglycerol lipase, glycerol ester hydrolase and DnaK was increased.[135] It is also possible that extract concentration may play a role in decreasing proteins important to biofilm formation such as polysaccharide intercellular protein (PIA),[141] which consists of teichoic acids, extracellular DNA and proteins,[142] and biofilm associated protein (Bap).[143] PIA forms the EPS matrix in which the microbial cells are embedded, whereas Bap is produced during infection and is connected with the persistence of *S. aureus*.[141] Although both Bap and PIA affect intercellular adhesion,[143]

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Bap promotes primary attachment to inert surfaces. The extracts could also have resulted in genetic mutations in *S. aureus* thereby limiting biofilm formation (*sarA* mutants).[144] With regards to *C. albicans*, the extracts may have increased expression of Efg1p, an essential regulator in the morphogenesis of *C. albicans*.[145] This is important as a change in structure is related to the transition from planktonic to biofilm mode of growth.

Since both plant extracts showed promising activity against biofilms, the checkerboard assay was used to determine their combined effect. The ΣFIC, which is expressed as the interaction of two agents where the concentration of each test agent in combination is expressed as a fraction of the concentration that would produce the same effect when used independently,[102] is provided in Table 9.

3.3.3 Synergy

Combination of the MeOH extracts of *X. caffra* and *Z. davyi*, at all ratios, resulted in ΣFIC index of 0.043 for all strains tested (Table 9). The ΣFIC index for the combined DCM extracts were found to be greater than the ΣFIC values for the MeOH combinations on all strains tested (0.070) with the exception of *C. albicans* clinical strain 2. The combination of HW extracts produced ΣFIC values greater than the MeOH and DCM combinations on all strains with the the exception of *C. albicans* ATCC strain (Table 9). Since all values were ≤ 0.5 it was concluded that when the extracts of *X. caffra* were combined with the extracts of *Z. davyi*, the combination displayed synergistic antimicrobial actions. Van Vuuren *et al.*[146] found that when leaf, bark and root extracts of *Croton gratissimus* were combined in a 1:1:1 ratio, antimicrobial activity was either enhanced (lower MIC value) or equivalent to the MIC values independently, for *S. aureus* (ATCC 12600) and *C. albicans* (ATCC 10231).

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Table 9: Sum of the fractional inhibitory concentration index with the BIC*

	ΣFIC					
	C. a. ATCC 90028	**C. a. clinical 1**	**C. a. clinical 2**	**S. a. ATCC 12600**	**S. a. clinical 1**	**S. a. clinical 2**
Combination of MeOH extracts (all ratios)	0.043 (<0.001)	0.043 (<0.001)	0.043 (<0.001)	0.043 (<0.001)	0.043 (<0.001)	0.043 (<0.001)
Combination of DCM extracts (all ratios)	0.070 (<0.001)	0.070 (<0.001)	0.043 (<0.001)	0.070 (<0.001)	0.070 (<0.001)	0.070 (<0.001)
Combination of HW extracts (all ratios)	0.070 (<0.001)	0.200 (<0.001)	0.120 (<0.001)	0.200 (<0.001)	0.200 (<0.001)	0.200 (<0.001)

***BIC is provided in brackets for the various ratios; C. a.: *Candida albicans*; S. a.: *Staphylococcus aureus*; DCM: dichloromethane; HW: hot water; MeOH: methanol, (n=6).**

Synergistic interactions of the plant extracts could be due to the combination of the different antimicrobial phytochemicals present in each plant. It has been suggested that the benefits of phytomedicines are often the result of synergistic actions of multiple active chemicals and that the synergistic effect can be beneficial in eliminating the problematic side effects associated with the predominance of a single xenobiotic compound in the body.[147] The plant extracts in combination could also be used to increase the efficacy of conventional antimicrobial therapy.[148] A flavone and its derivatives have been found to intensify activity synergistically with β-lactams in treating MRSA and MSSA strains.[149]

3.3.4 Scanning electron microscopy

Biofilms were grown on microscopy coverslips, treated with extracts/antibiotics, after which they were fixed with cacodylate buffer, dried and carbon coated for scanning electron microscopy. The method was adapted from microscopy preparation where alcohol dehydration, was used to wash away surface EPS.[100] As EPS production is essential for biofilm formation,[150] it is necessary to preserve it in order to determine the effects of the extracts on an intact biofilm.

When compared to an untreated *C. albicans* biofilm (ATCC 90028) (Figure 22A), amphotericin B caused partial removal of surface EPS, leaving cells partially exposed

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(Figure 22B). XC-MeOH-E resulted in disintegration of surface EPS as well as yeast cells (Figure 22C) whereas, with ZD-MeOH-E, partial removal of surrounding EPS was evident (Figure 22D). With regard to clinical strain 1, amphotericin B caused noticeable disintegration of surface EPS leaving cells partially exposed (Figure 23B) compared to untreated *C. albicans* (Figure 23A). There was no noticeable disintegration/removal of EPS on biofilms treated with XC-MeOH-E (Figure 23C). ZD-MeOH-E caused slight disintegration of EPS on clumps of biofilms (Figure 23D). As for clinical strain 2, amphotericin B partially removed surface EPS (Figure 24B). There was nearly total disintegration of EPS and cells in biofilms treated with XC-MeOH-E (Figure 24C) and ZD-MeOH-E (Figure 24D), respectively.

S. aureus biofilms (ATCC 12600), treated with ciprofloxacin showed distinct breakage in EPS layers resulting in cells being exposed (Figure 25B) when compared to a double layer untreated biofilm (Figure 25A). Biofilms treated with XC-MeOH-E (Figure 25C) and ZD-MeOH-E (Figure 25D) caused total removal of surrounding EPS and a decrease in cell clusters. On clinical strain 1, ciprofloxacin caused the removal of EPS as well as disintegration of cells (Figure 26B), XC-MeOH-E caused disintegration of surface EPS and lysing of cells (Figure 26C), and ZD-MeOH-E caused disintegration of EPS revealing clusters of cells (Figure 26D). When compared to untreated *S. aureus* clinical strain 2 biofilm (Figure 27A), ciprofloxacin caused the removal of the surface layer of EPS and breakage of the biofilm, forming clumps of cells as opposed to a complete biofilm (Figure 27B), XC-MeOH-E caused disintegration of surface EPS and lysing of cells (Figure 27C) whereas ZD-MeOH-E caused removal of total EPS and disintegration of the biofilm, leaving behind a few single exposed cells (Figure 27D).

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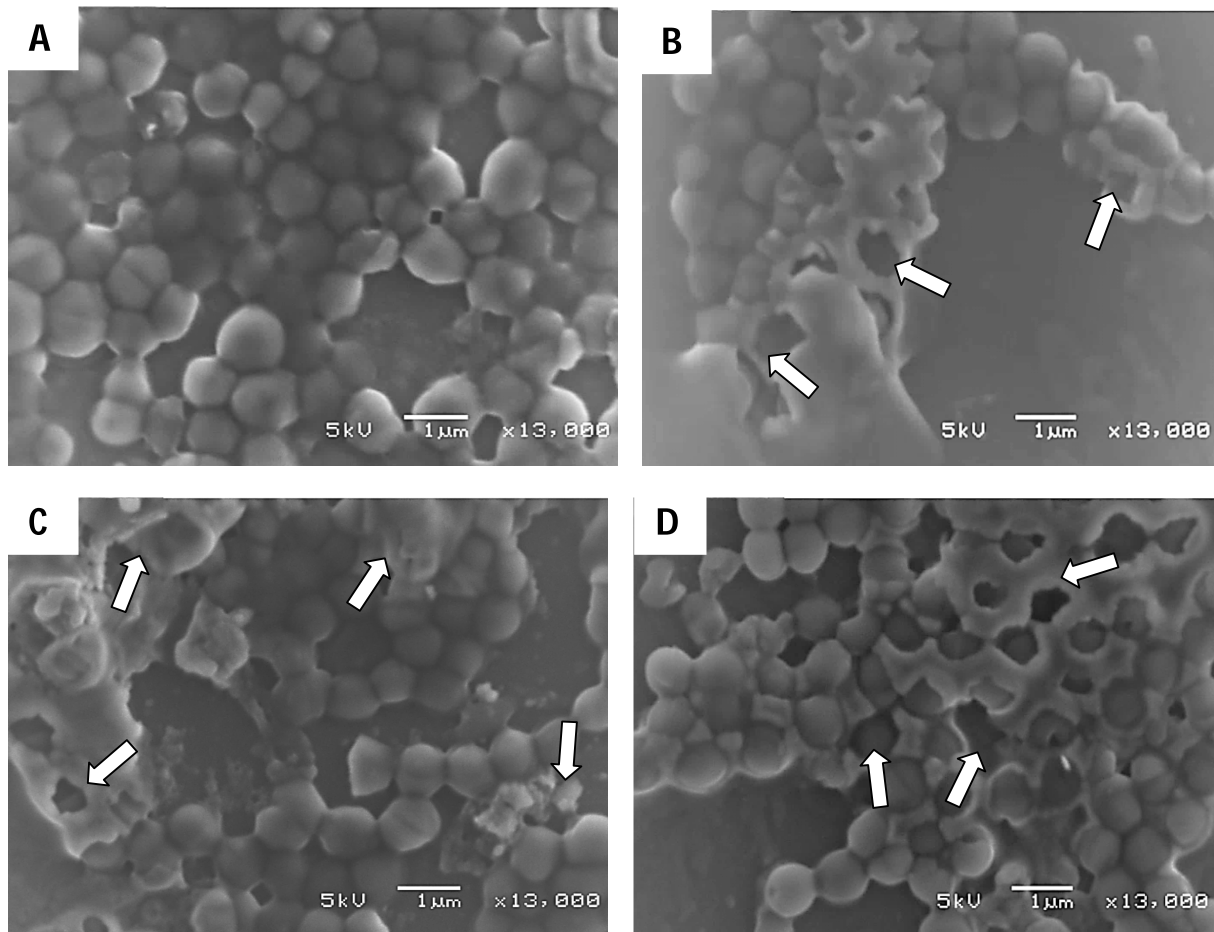

Figure 22: *C. albicans* ATCC 90028 biofilms; A) untreated and B) treated with amphotericin B (1 mg/mL), C) XC-MeOH-E (1 mg/mL) and D) ZD-MeOH-E (1 mg/mL). Arrows indicate disruption of EPS.

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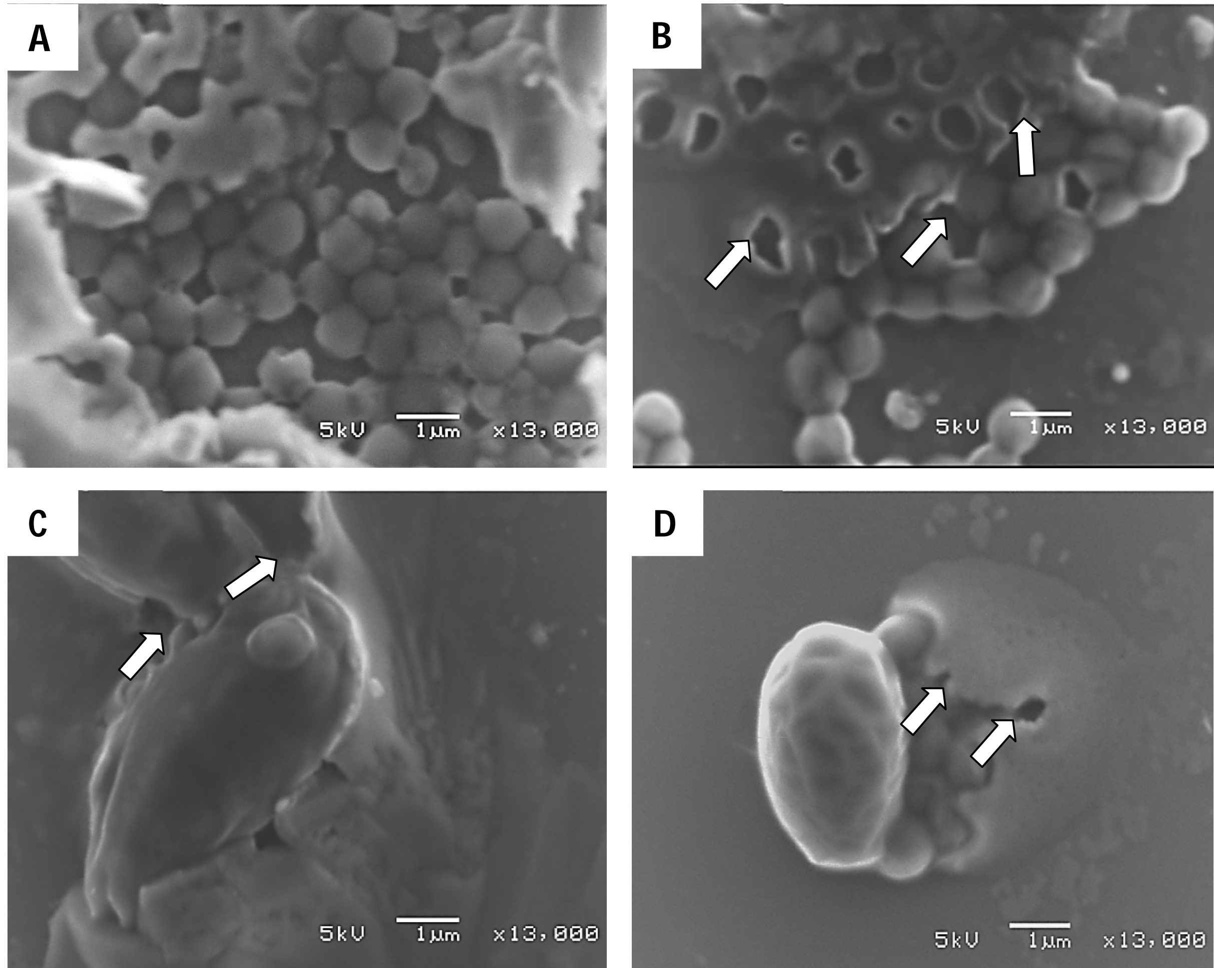

Figure 23: *C. albicans* clinical strain 1 biofilms; A) untreated and B) treated with amphotericin B (1 mg/mL), C) XC-MeOH-E (1 mg/mL) and D) ZD-MeOH-E (1 mg/mL). Arrows indicate disintegration of surface EPS matrix.

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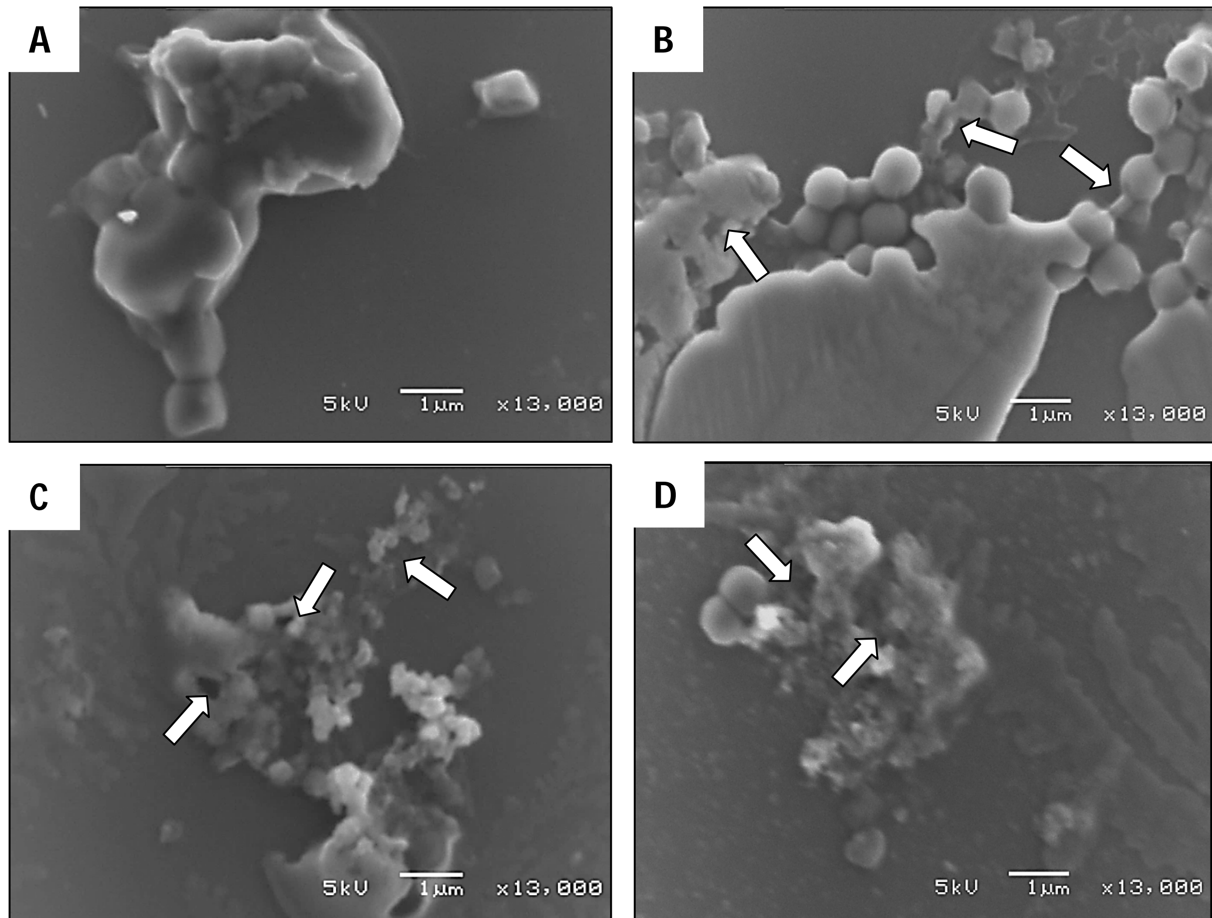

Figure 24: *C. albicans* clinical strain 2 biofilms; A) untreated and B) treated with amphotericin B (1 mg/mL), C) XC-MeOH-E (1 mg/mL) and D) ZD-MeOH-E (1 mg/mL). Arrows indicate substantial disintegration of surface EPS matrix by arrows.

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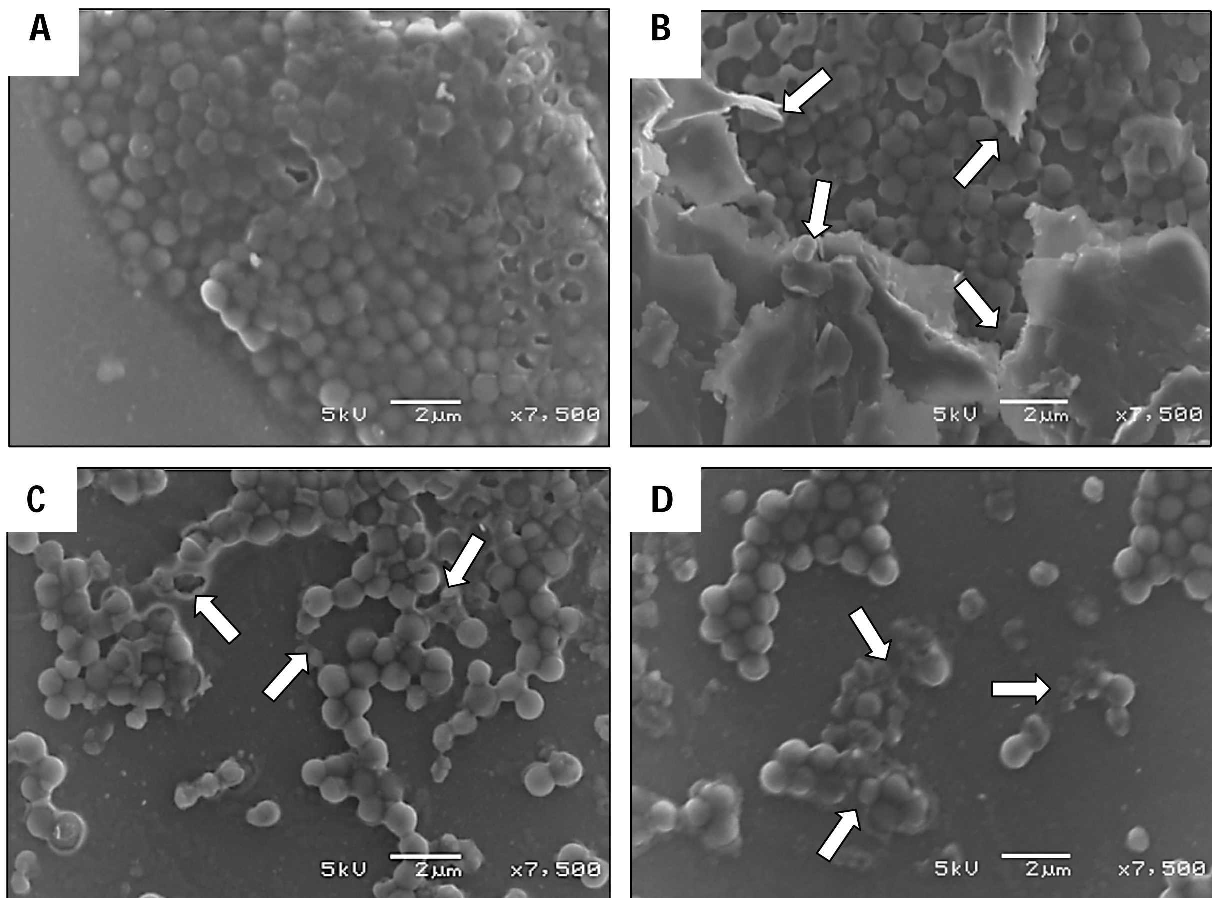

Figure 25: *S. aureus* ATCC 12600 biofilms; A) untreated double layer biofilm and B) treated with ciprofloxacin (1 mg/mL), C) XC-MeOH-E (1 mg/mL) and D) ZD-MeOH-E (1 mg/mL). Arrows indicate disintegration of EPS matrix.

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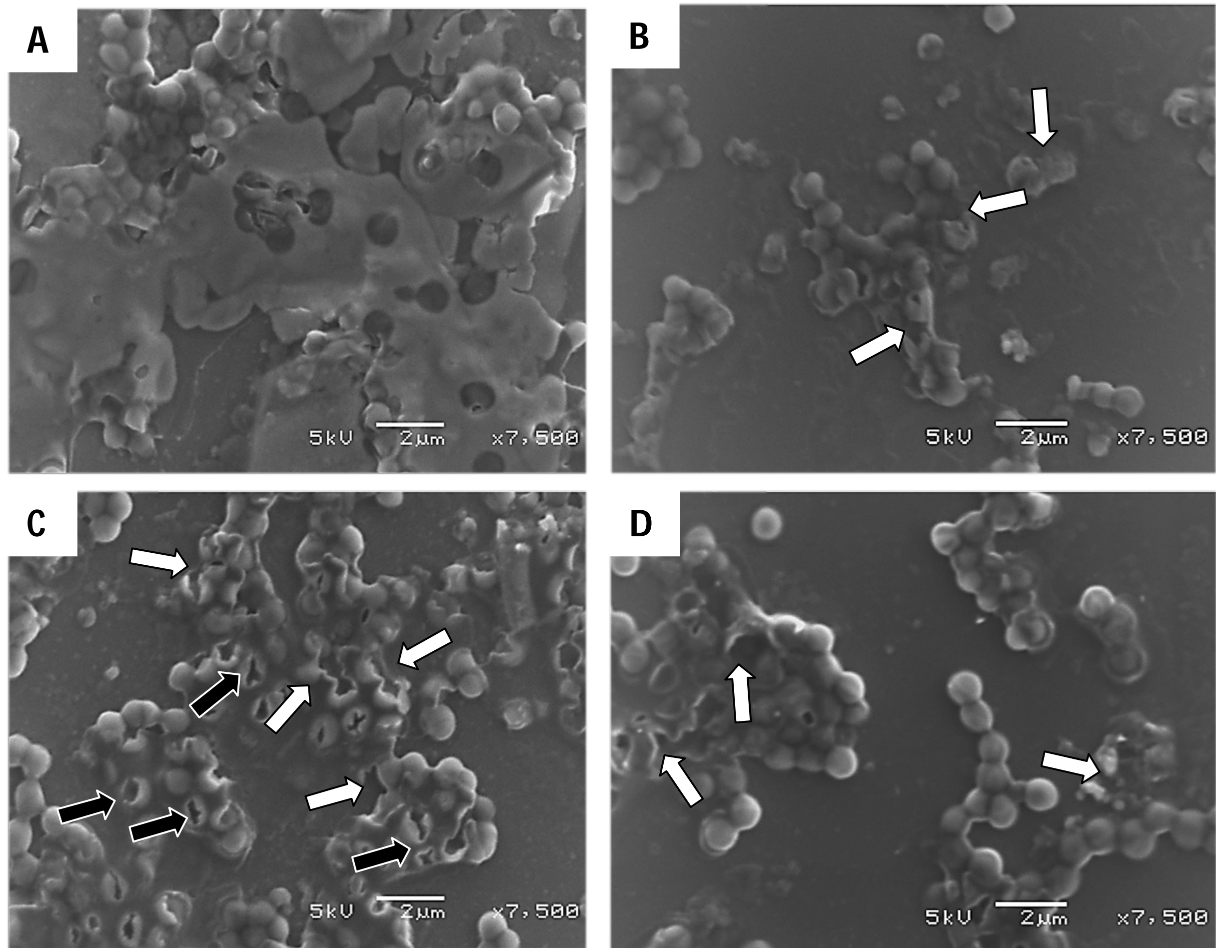

Figure 26: *S. aureus* clinical strain 1 biofilms; A) untreated and B) treated with ciprofloxacin (1 mg/mL), C) XC-MeOH-E (1 mg/mL) and D) ZD-MeOH-E (1 mg/mL). The white arrows indicate disintegration of surface EPS and the black arrows, lysing of cells.

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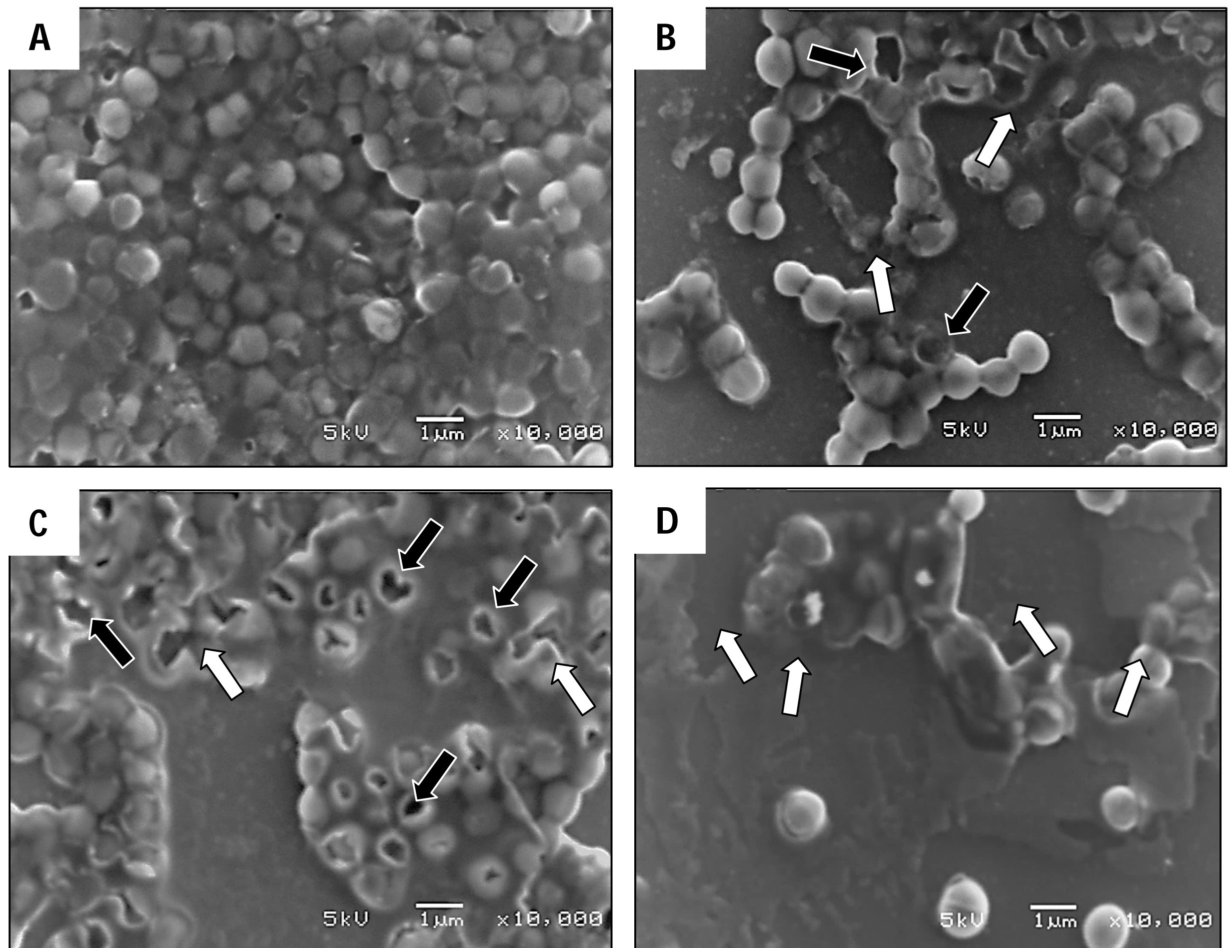

Figure 27: *S. aureus* clinical strain 2 biofilms; A) untreated and B) treated with ciprofloxacin (1 mg/mL), C) XC-MeOH-E (1 mg/mL) and D) ZD-MeOH-E (1 mg/mL). The white arrows indicate disintegration of surface EPS and the black arrows, lysing of cells.

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Production of EPS is central to biofilm development (Figure 3) therefore the disintegration of EPS leads to the disintegration of biofilms. Slight disintegration of EPS leads to partial removal of biofilms, whereas, complete disintegration leads to fatal removal/breakdown of biofilms (Figures 22-27). The disintegration seen in Figures 22-29, could be attributed to factors/phytochemicals which have the ability to inhibit/breakdown EPS.[151] Curcumin has been reported to significantly decrease initial cell adhesion in *C. albicans* biofilms.[152] Phytochemicals may also play a role in the interruption of quorum-sensing (QS), which is vital for cell to cell communication.[152] Quercetin has been shown to sensitize resistant *C. albicans* to antifungal agents and to induce apoptosis by modulating QS systems.[45] At high concentrations, quercetin was reported to be effective against a wide range of microorganisms such as *S. aureus*, *Aspergillus flavus* and *Aspergillus parasiticus*.[45] Quercetin has also been ascribed to have antiviral activity, including anti-HIV activity, possibly due to its ability to inhibit the enzyme reverse transcriptase.[153] Quinones have the ability to form complexes with nucleophillic amino acids in proteins, which results in protein inactivation.[116] This could explain the disintegration of the EPS matrix. Other microbial targets could be surface-exposed adhesins and membrane bound enzymes. Quinones also have the ability to render substrates unavailable to a microorganism.[116]

The plant extracts may also cause cell cycle defects which in turn cause substances such as glucose and intercellular adhesion proteins to become limited in the cells/decreasing their production, thus decreasing the production of EPS.[154] Jäger *et al.*[154] have found that nutrient limitation has an effect on biofilm stability and showed that glucose-limiting conditions results in the disintegration of *S. epidermis* biofilms. Nutrient concentration can have profound effects on biofilms. High concentrations of leucine and peptones have been found to cause transition of cells from biofilm mode to planktonic mode.[155] Similarly, the phytochemicals present in the plant extracts could play a role in the re-planktonisation of pre-grown biofilms, as visualised in Figure 27D.

The phytochemicals detected using HPLC, could be responsible for the anti-biofilm activity visualized in scanning electron microscopy. Tesaki *et al.*[156] found that methanol

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extracts containing sinapic acid had antibacterial activity against *Escherichia coli*, *Salmonella enteritidis* and *S. aureus* and that activity was attributed to the specific structure of the phenolic acid. Plants sprayed with salicylic acid were found to stimulate pathogenesis-related proteins and increase antiviral ability.[157] It is believed that the presence of ferulic acid plays a role in wheat fungal resistance.[158] The antimicrobial activity (against *Salmonella typhimurium* and *S. aureus*) of several extracts of the plant *Caesalpinia mimosoides*, has been attributed to gallic acid.[121] Caffeic acid has been shown to hinder the production of aflatoxin in the fungus Aspergillus *flavus*.[159] Caffeic acid (as a food additive) is used to inhibit *Clostridium botulinum*.[160] Dicaffeoylquinic acids and dicaffeoyltartaric acids have been proven to be potent anti-HIV type 1 virus selective enzyme inhibitors.[160] Caffeic acid has antifungal and antibacterial activity against a wide range of microorganisms including *Escherichia coli*, *Pseudomonas fluorescens*, *S. aureus*, *C. albicans*, *Trichophyton rubrum* and *Bacillus subtilis*.[160] The antimicrobial potential of vanillic acid is evident from its use in curing acne and chicken pox-pustules,[161] and activity against probiotic and pathogenic bacteria.[162] Phenolic compounds have a wide array of antimicrobial mechanisms and these could contribute to the anti-biofilm effects observed in the present study.

Another group of phytochemicals which have been ascribed to the antimicrobial activity of *Z. davyi* are alkaloids.[68,109,110] Nitidine, sanguinarine and chelerythrine, detected via HPLC (Figures 14 and 15), are bioactive benzo[c]phenanthridine alkaloids and are known for their biological activities, which include regulation of inflammatory diseases, antimalarial, anti-cancer, antioxidant, antimicrobial and bacteriostatic activity.[163] Benzo[c]phenanthridine alkaloids are widely found in high plant families such as Papaveraceae, Fumariaceae and Rutaceae. Benzo[c]phenanthridine alkaloids are abundant in the *Zanthoxylum* species which belong to the family Rutaceae[164] and are most probably responsible for the antimicrobial activity noted in this study. Nitidine, first isolated from *Z. nitidum* as well as 8-acetonyldihydronitidine, isolated from *Z. tetraspermum* bark, have strong antibacterial and antifungal activity.[165,166] Nitidine, has been found to inhibit the growth of *Clostridium sporogenes*, *Streptococcus pyogenes* and *Clostridium bacteria*.[163] Chelerythrine has antimicrobial activity against *C. albicans* and

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S. aureus.[167] The antibacterial activity of sanguinarine and chelerythrine is reported to be dependent on the iminium bond in the molecule.[167]

As for the other phytochemicals detected in these plants in this study, many groups of flavonoids have known antifungal, antibacterial and antiviral activity.[70] In particular, catechin was revealed to have antimicrobial activity at nanomolar levels and was proven to have better activity than antibiotics such as vancomycin and tetracycline.[168] Several terpenoids have been found to inhibit microbial oxygen uptake and oxidative phosphorylation.[72] This could explain the cessation of the production of EPS which hold microbial cells together in a biofilm and account for the anti-biofilm activity found in this study. As regards terpenoid structure, it has been shown that carbonylation increases bacteriostatic activity whereas it is postulated that the free hydroxyl group on terpene alcohols could be key to their antimicrobial activity.[72]

Plant sterols (long-chain unsaturated fatty acids) also display antibacterial activity and are the key ingredients of antimicrobial food additives and some antibacterial herbs.[111] Zheng *et al.*[111] discovered that long chain unsaturated linolenic acids inhibited bacterial enoyl-acyl carrier protein reductase which is essential for bacterial fatty acid synthesis. Lauric acid has been shown to display anti-MSSA and anti-MRSA activity.[69] Since antimicrobial action is mediated by the inhibition of fatty acid synthesis, sterols could play an important role as antimicrobials and could account for anti-biofilm activities.

Apart from phytochemical activities, other mechanisms of anti-biofilm activity have been reported for *S. aureus.* A mixture of D-Amino acids; D-leucine, D-methionine, D-tyrosine, and D-tryptophan, *was found to* prevent formation of *S. aureus* and *P. aeruginosa* biofilms.[169] The aforementioned amino-acids are produced by a number of bacteria and may be a widespread signal for biofilm disassembly.[169] Anti-biofilm polysaccharides produced by *E. coli* have been shown to inhibit biofilm formation of the following bacteria: *P. aeruginosa*, *Klebsiella pneumonia*, *S. aureus* and *S. enterococcus.*[170] This production of anti-biofilm polysaccharides acts as an auto-regulatory process to control biofilm architecture.[170]

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The results obtained from this study indicated that the plant extracts have significant activity against biofilms of *S. aureus* and *C. albicans*. This is ascribed to the presence of phytochemicals with antimicrobial activity detected in the study confirmed by TLC and HPLC. The results are also supported by previous reports where both the *Zanthoxylum* and *Ximenia* genus have been described as having antimicrobial activities. [70,99,100,115]

3.4 Cytotoxcity

The SRB assay was used to determine the cytotoxicity of extracts of *Z. davyi* and *X. caffra*. Although the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) assay has been regarded as the gold standard for cytotoxicity testing, it has been reported that different test compounds, such as glycolysis inhibitors, can cause interference with the MTT assay.[171] Different plant extracts, antioxidants and other plant compounds also interfere with the MTT assay.[172] After comparison of the Neutral Red Uptake (NRU) assay, the Resazurin reduction assay (RES), the SRB assay as well as the MTT assay, it was concluded that the SRB assay performed best overall, having had the lowest variability, providing the most reproducible results and displaying no interference with the compounds tested.[171] The RES assay should not be used when assessing plant extracts for cytotoxicity due to potential interference between samples and substrates. A study conducted by Cordier *et al.*[172] showed that the RES assay failed to predict IC_{50} values where cell density exceeded 50%. A graphic representation of the dose response curve of the plants against the cell lines tested is shown in Figures 28 and 29. All graphs indicated a dose-response effect.

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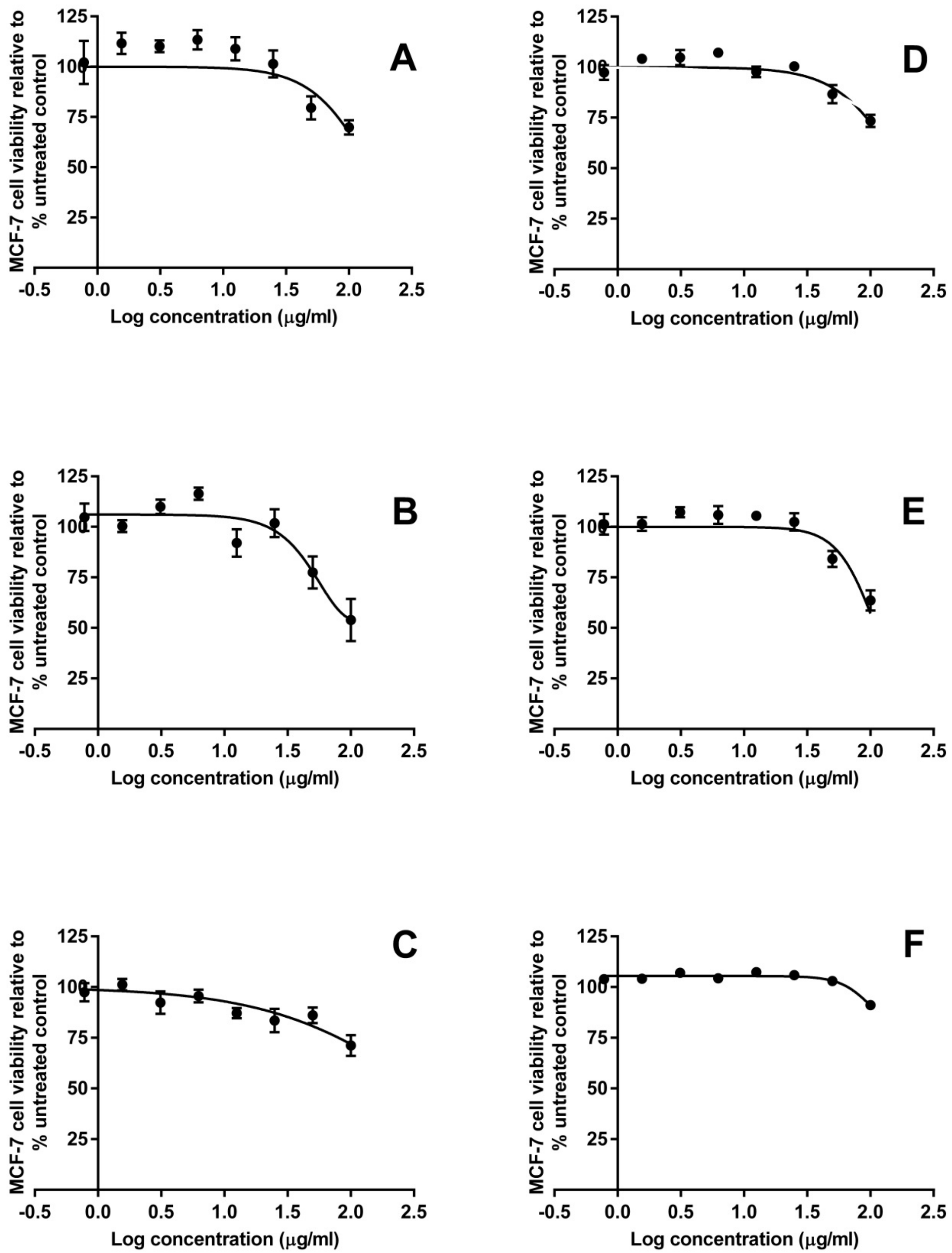

Figure 28: Growth inhibition of MCF-7 cell lines when exposed to A) cells treated with XC-MeOH-E, B) XC-DCM-E, C) XC-HW-E, D) ZD-MeOH-E, E) ZD-DCM-E and F) ZD-HW-E.

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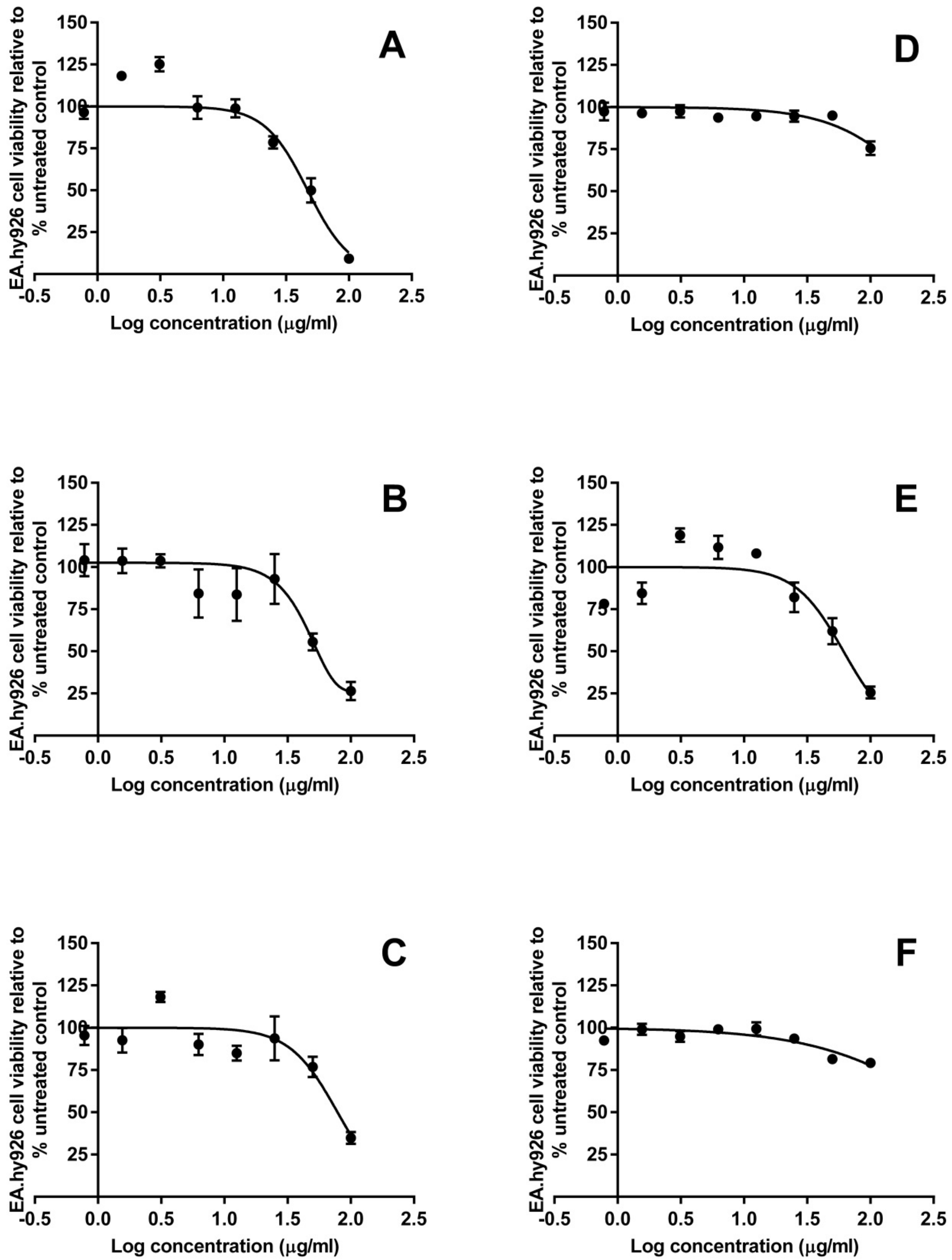

Figure 29: Growth inhibition of EA.hy926 cell lines when exposed to A) cells treated with XC-MeOH-E, B) XC-DCM-E, C) XC-HW-E, D) ZD-MeOH-E, E) ZD-DCM-E and F) ZD-HW-E.

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The MeOH and DCM extracts of *Z. davyi* had an IC_{50} >100 µg/mL towards MCF-7 cells, and the MeOH and HW extracts had an IC_{50} >100 µg/mL in EA.hy926 cells (Table 10). The cytotoxic activity of ZD-HW-E in MCF-7 cells could be attributed to the high percentage of gallic acid (13.4 %) in the extracts.[173] Gallic acid has been proven to have anticancer effects on prostate cancer cells by blocking growth at the G2/M phase, activating Chk1 and Chk2 and by inhibiting Cdc25C and Cdc2.[117] This compound has also been shown to have synergistic effects with the anticancer drug doxorubicin on prostate cancer cells.[173] *In vivo*, gallic acid and cisplatin have been shown to increase apoptosis in tumour cells compared to cisplatin alone.[174] Additionally, gallic acid also has antioxidant effects due to its strong reducing power and weak metal chelating ability.[117] This is important for inactivating reactive oxygen species (ROS) involved in cancer and other activities.[117] The presence of benzophenanthridines could be responsible for the cytotoxicity of the *Z. davyi* extracts noted.[175] *Zanthoxylum* species have been noted for their cytotoxic, trypanocidal, anti-leishmanial and anti-mycobacterial metabolites.[175] *Z. buesgenii* and *Z. nitidium* have been found to contain cytotoxic benzophenanthridine and furoquinoline alkaloids.[175] Lignans isolated from *Z. alatum* and *Z. planispinum* are reported to contain cytotoxic potential.[175]

Table 10: Half-maximal inhibitory (IC_{50}) concentrations of plant extracts on MCF-7 and EA.hy926 cell lines.

	IC_{50} (µg/mL)	
PLANT EXTRACT	**MCF-7***	**EA.hy926***
ZD-MeOH-E	>100	>100
ZD-DCM-E	>100	61.27 ± 0.05
ZD-HW-E	52.27 ± 0.13	>100
XC-MeOH-E	>100	46.99 ± 0.03
XC-DCM-E	>100	44.52 ± 0.06
XC-HW-E	>100	78.56 ± 0.05
Positive control Tamoxifen	4.01 ± 1.06	2.47 ± 1.50
***mean ± S.E.M. (n=6)**		

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All extracts of *X. caffra* showed greater cytotoxicity towards the EA.hy926 cells than MCF-7 cells (Table 10). This is evident from the IC_{50} values >100 µg/mL which were in MCF-7 cells. In previous studies, *X. caffra* exhibited cytotoxicity against HeLa (human cervical cancer), HT29 (colon carcinoma) and A431 (epidermoid carcinoma) cells at a concentration of 100 µg/mL.[176] IC_{50} values for *X. caffra* bark and leaf extracts were >100 µg/mL in Vero cells, which supports the current findings.[177] With regards to another *Ximenia* species, *X. americana* has been reported to display antineoplastic properties which has been attributed to the presence of ribosome inactivating proteins such as riproximin and ricin,[178] possibly explaining the mechanism of cytotoxicity of the *Ximenia* extracts noted in EA.hy926 cells.

Overall, all extracts displayed low cytotoxicity when compared to the positive control, tamoxifen, and had a greater effect on the EA.hy926 cell line than the MCF-7 cell line (Table 10). Since flavonoids are reported to have cytotoxic properties,[179] and since they were present in all extracts of *Z. davyi* and *X. caffra*, they could have contributed to the cytotoxicity noted. Other phytochemicals detected in the extracts which have anti-cancer properties are; salicylic acid,[180] caffeic acid,[181] quercetin,[153] rutin,[182] and the alkaloids; nitidine, sanguinarine, and chelerythrine.[163]

Following the SRB assay the effects of cytotoxicity was visualised using phase contrast and PlasDIC microscopy, in order to distinguish morphologically between apoptosis and necrosis; two methods of cell death. Apoptosis is a biologically important process as a lack thereof leads to uncontrolled cell proliferation.[183] In apoptosis, cells actively participate in the cell death process, the cells fragments into apoptotic bodies while DNA breaks down into pieces of varying length. As this process is taking place, proteins and energy (in the form of ATP), which is required for the cells metabolic processes and functioning, are continually produced. Therefore each apoptotic body (bleb) contains functional organelles.[104] During necrosis, cells relinquish metabolic function, no proteins or energy is produced, organelles are non-functional, the integrity of the cell membrane is lost and blebs contain no organelles. Cells rupture and the released contents induce inflammation in the affected tissues.[104]

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Figure 30A and E displays untreated EA.hy926 cells. Only a few cells indicate apoptosis which is normal in cell growth and death cycles. Therefore we do expect to see some cell death in untreated cells. In Figure 30B and D, it can be clearly seen that with an increase in concentration of ZD-MeOH-E, there is an increase in apoptosis which corroborates with the results of the SRB assay. At 100 µg/mL, most of the cells are round due to cytoskeletal breakdown (Figure 30B and F). Dark nuclear and organelle condensation is visible as well as cell blebbing. At 12.5 µg/mL, there is minimal apoptosis. Although cell blebbing is evident, the majority of cells are elongated which is an indication of stress (Figures 30C and G). Apart from the extracts, another factor which can cause stressed cells is the lack of space to grow in a surface with such a high cell density. At 0.78 µg/mL, the EA.hy926 cells were comparable to the untreated cells in morphology (Figure 30D and H). A similar trend was noticed for EA.hy926 cells treated with ZD-DCM-E, ZD-HW-E, XC-MeOH-E, XC-DCM-E and XC-HW-E (Figures 31-35).

A multiplicity of cells is evident in untreated MCF-7 cells (Figure 36A and E) compared to untreated EA.hy926 cells (Figure 30A and E). Cell growth seems almost irrepressible and only a few rounded cells are evident, however, no nuclear condensation is visible and cells appear swollen and necrotic (Figure 36F) as opposed to the apoptotic EA.hy926 cells (Figure 31F). It is evident that with an increase in concentration of ZD-MeOH-E, there is an increase in the amount of swollen cells and thus necrosis, which corroborates with the results of the SRB assay (Figure 36F and H). At 100 µg/mL, the cells were either highly stressed (elongated) or rounded as the cell's organelles had swollen and become non-functional (Figure 36B and F). At 12.5 µg/mL, necrosis took place but to a lesser extent compared to 100 µg/mL, however, cells were still swollen (Figure 36C and G). At 0.78 µg/mL, the treated cells are comparable to untreated MCF-7 cells (Figure 36D and H). A similar trend is evident for MCF-7 cells treated with ZD-DCM-E, ZD-HW-E, XC-MeOH-E, XC-DCM-E and XC-HW-E (Figures 37-41).

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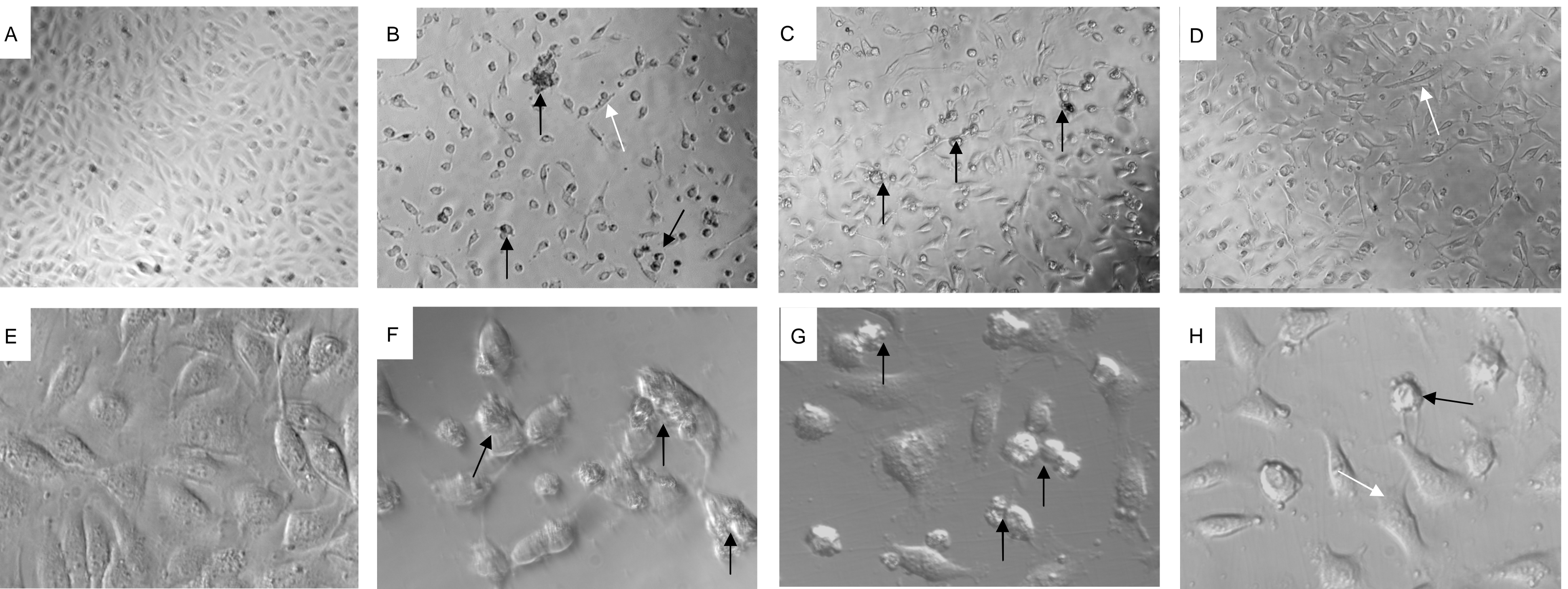

Figure 30: EA.hy926 visualized by phase contrast microscopy at 10 x magnification, A) untreated, B) treated with 100 µg/ml ZD-MeOH-E, C) treated with 12.5 µg/ml ZD-MeOH-E, D) treated with 0.78 µg/ml ZD-MeOH-E, and cells visualized by PlasDIC microscopy at 40 x magnification, E) untreated, F) treated with 100 µg/mL ZD-MeOH-E, G) treated with 12.5 µg/mL ZD-MeOH-E, and H treated with 0.78 µg/mL ZD-MeOH-E. White arrows indicate signs of stress and black arrows indicate signs of apoptosis.

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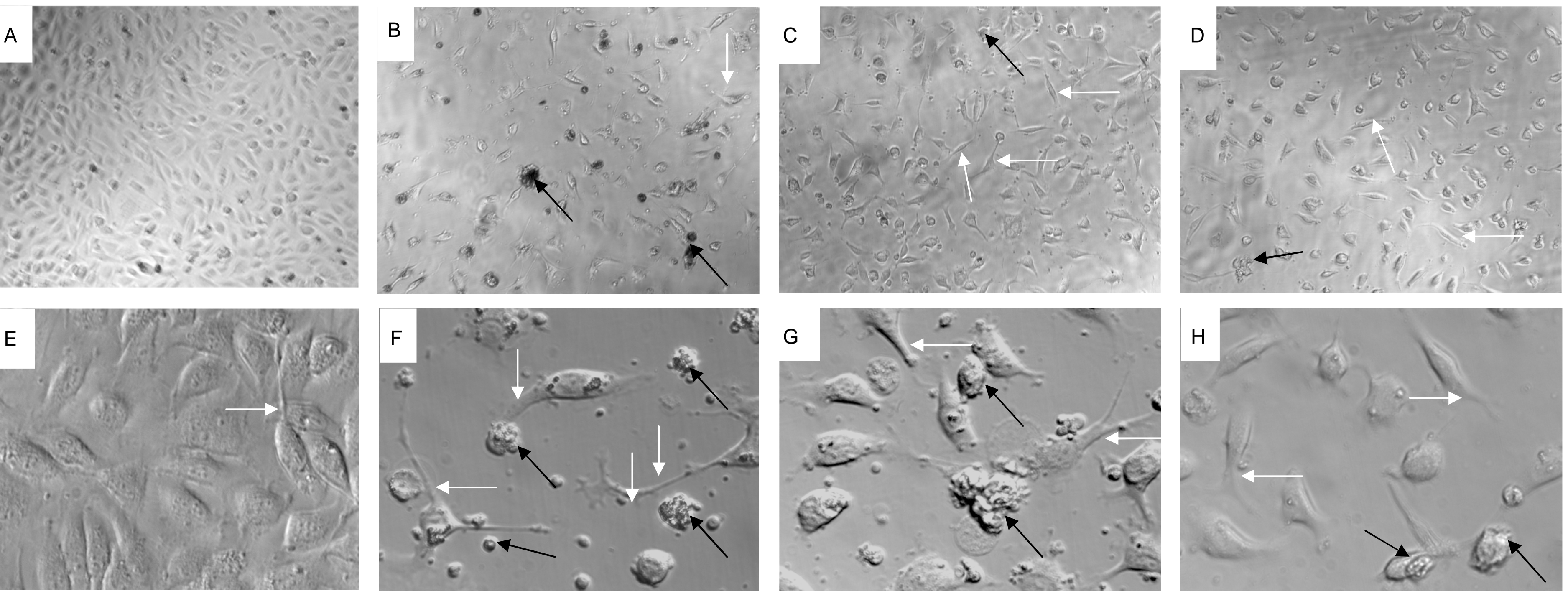

Figure 31: EA.hy926 visualized by phase contrast microscopy at 10 x magnification, A) untreated, B) treated with 100 µg/ml ZD-DCM-E, C) treated with 12.5 µg/ml ZD-DCM-E, D) treated with 0.78 µg/ml ZD-DCM-E, and cells visualized by PlasDIC microscopy at 40 x magnification, E) untreated, F) treated with 100 µg/mL ZD-DCM-E, G) treated with 12.5 µg/mL ZD-DCM-E, and H treated with 0.78 µg/mL ZD-DCM-E. White arrows indicate signs of stress and black arrows indicate signs of apoptosis.

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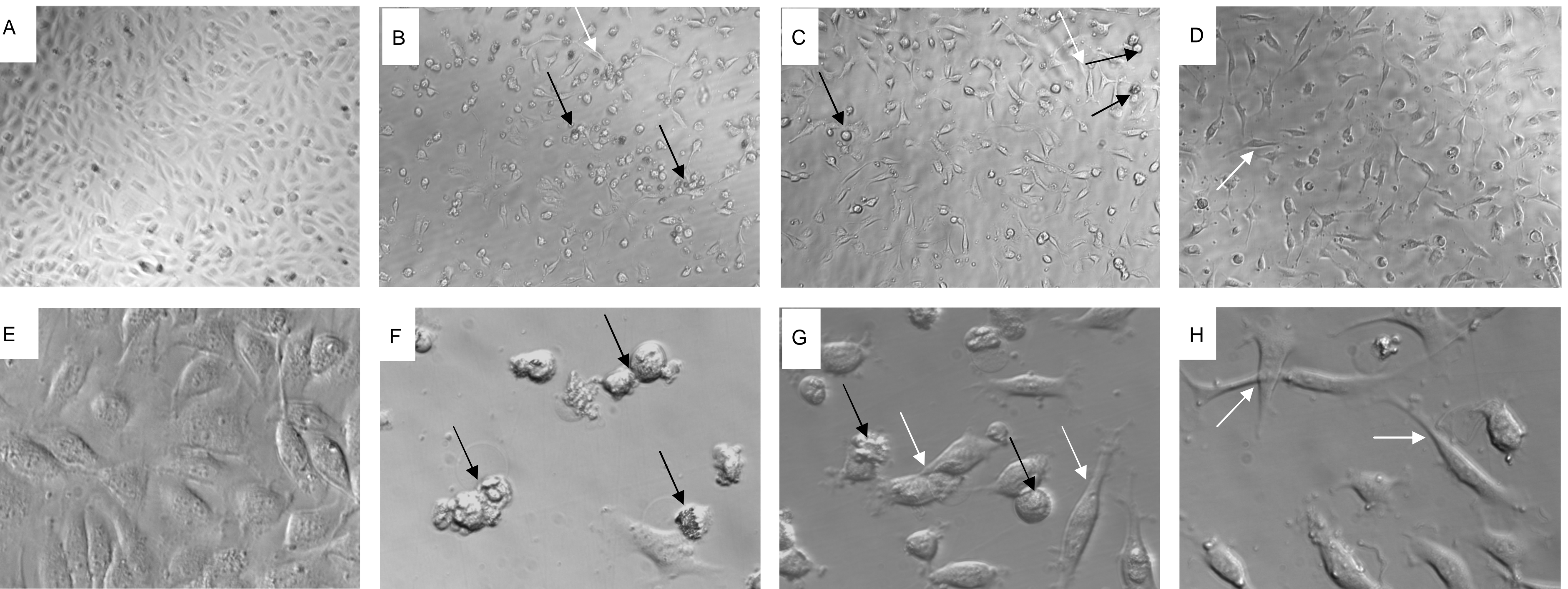

Figure 32: EA.hy926 visualized by phase contrast microscopy at 10 x magnification, A) untreated, B) treated with 100 µg/ml ZD-HW-E, C) treated with 12.5 µg/ml ZD-HW-E, D) treated with 0.78 µg/ml ZD-HW-E, and cells visualized by PlasDIC microscopy at 40 x magnification, E) untreated, F) treated with 100 µg/mL ZD-HW-E, G) treated with 12.5 µg/mL ZD-HW-E, and H treated with 0.78 µg/mL ZD-HW-E. White arrows indicate signs of stress and black arrows indicate signs of apoptosis.

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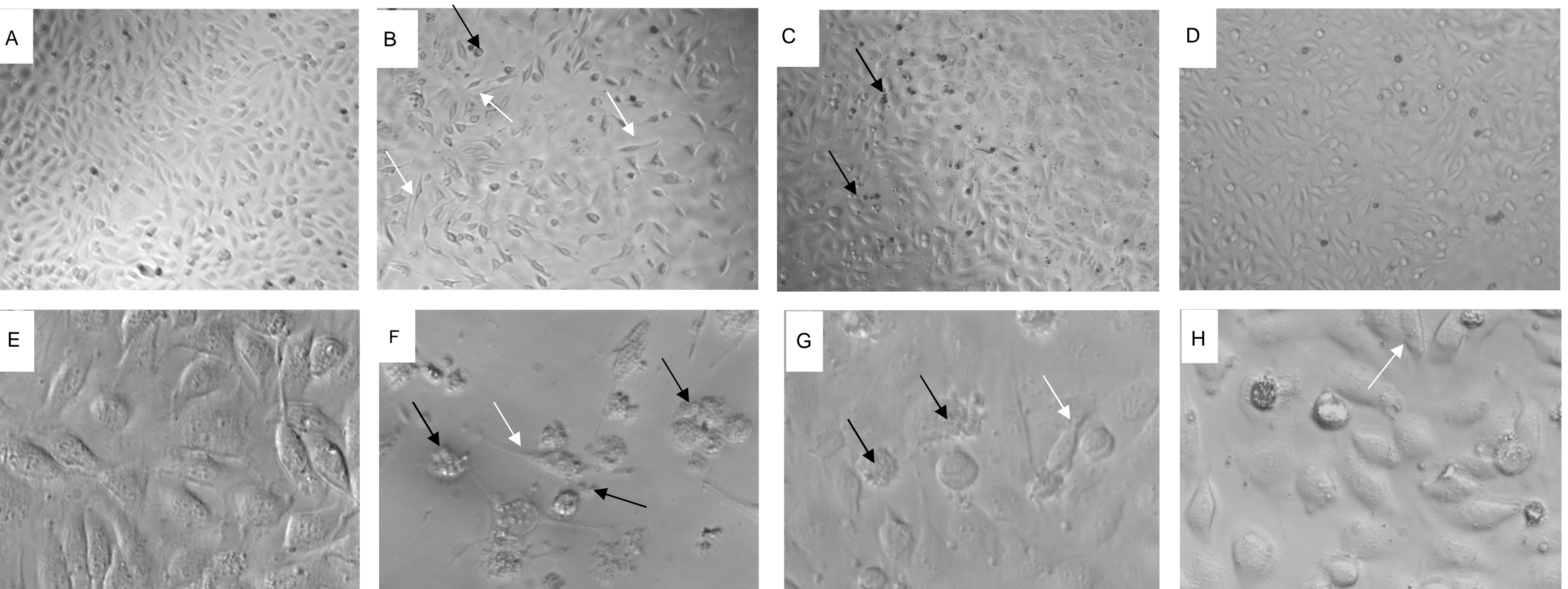

Figure 33: EA.hy926 visualized by phase contrast microscopy at 10 x magnification, A) untreated, B) treated with 100 µg/ml XC-MeOH-E, C) treated with 12.5 µg/ml XC-MeOH-E, D) treated with 0.78 µg/ml XC-MeOH-E, and cells visualized by PlasDIC microscopy at 40 x magnification, E) untreated, F) treated with 100 µg/mL XC-MeOH-E, G) treated with 12.5 µg/mL XC-MeOH-E, and H treated with 0.78 µg/mL XC-MeOH-E. White arrows indicate signs of stress and black arrows indicate signs of apoptosis.

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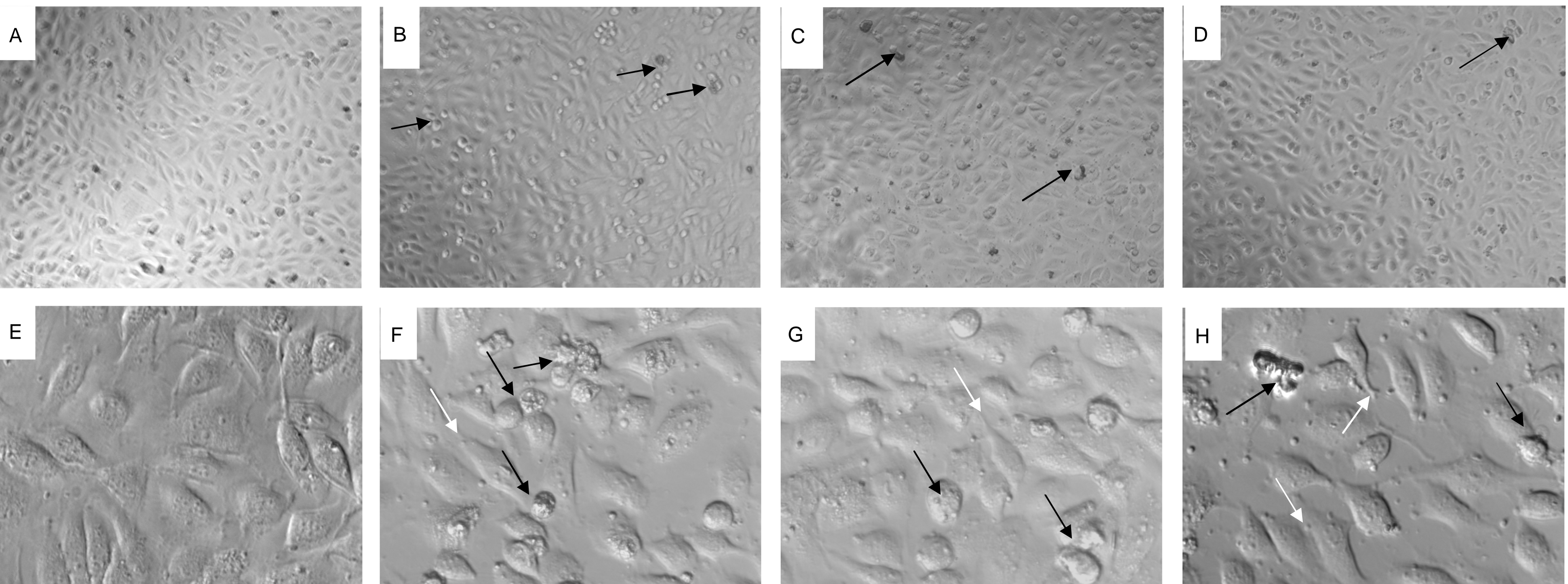

Figure 34: EA.hy926 visualized by phase contrast microscopy at 10 x magnification, A) untreated, B) treated with 100 µg/ml XC-DCM-E, C) treated with 12.5 µg/ml XC-DCM-E, D) treated with 0.78 µg/ml XC-DCM-E, and cells visualized by PlasDIC microscopy at 40 x magnification, E) untreated, F) treated with 100 µg/mL XC-DCM-E, G) treated with 12.5 µg/mL XC-DCM-E, and H treated with 0.78 µg/mL XC-DCM-E. White arrows indicate signs of stress and black arrows indicate signs of apoptosis.

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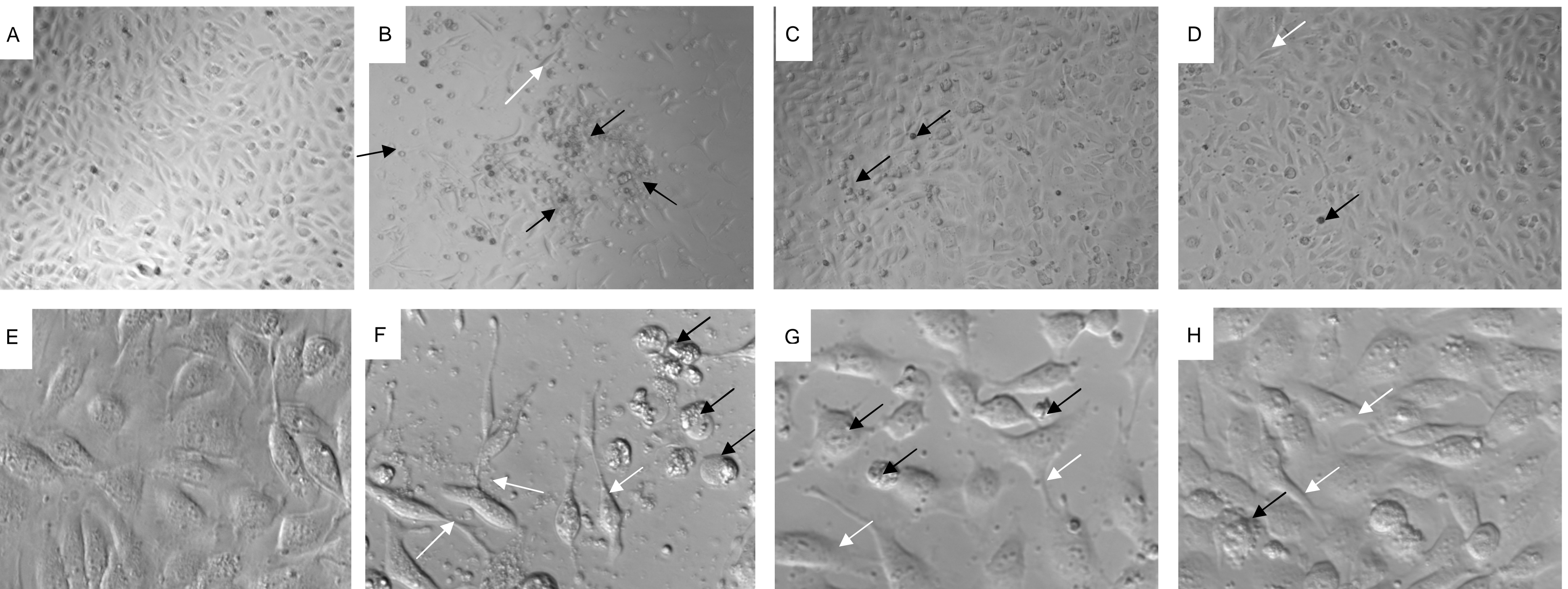

Figure 35: EA.hy926 visualized by phase contrast microscopy at 10 x magnification, A) untreated, B) treated with 100 µg/ml XC-HW-E, C) treated with 12.5 µg/ml XC-HW-E, D) treated with 0.78 µg/ml XC-HW-E, and cells visualized by PlasDIC microscopy at 40 x magnification, E) untreated, F) treated with 100 µg/mL XC-HW-E, G) treated with 12.5 µg/mL XC-HW-E, and H treated with 0.78 µg/mL XC-HW-E. White arrows indicate signs of stress and black arrows indicate signs of apoptosis.

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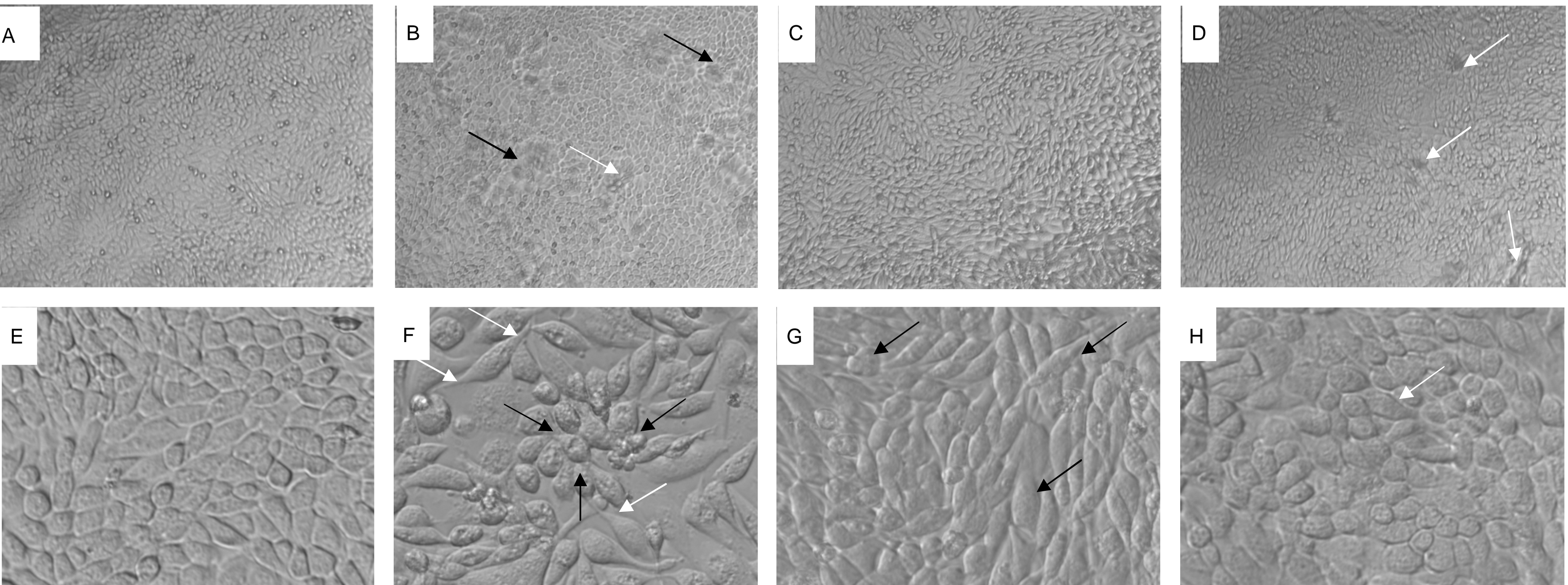

Figure 36: MCF-7 cells visualized by phase contrast microscopy at 10 x magnification, A) untreated, B) treated with 100 µg/ml ZD-MeOH-E, C) treated with 12.5 µg/ml ZD-MeOH-E, D) treated with 0.78 µg/ml ZD-MeOH-E, and cells visualized by PlasDIC microscopy at 40 x magnification, E) untreated, F) treated with 100 µg/mL ZD-MeOH-E, G) treated with 12.5 µg/mL ZD-MeOH-E, and H treated with 0.78 µg/mL ZD-MeOH-E. White arrows indicate signs of stress and black arrows indicate signs of necrosis.

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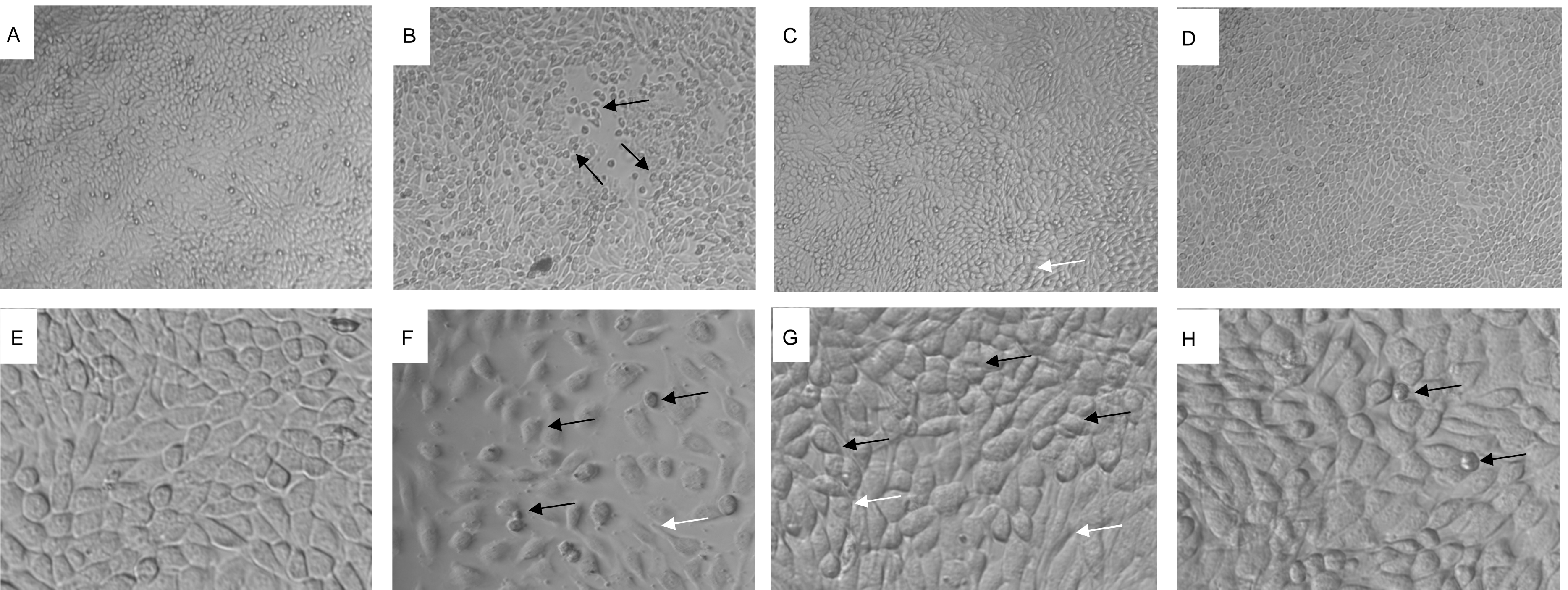

Figure 37: MCF-7 cells visualized by phase contrast microscopy at 10 x magnification, A) untreated, B) treated with 100 µg/ml ZD-DCM-E, C) treated with 12.5 µg/ml ZD-DCM-E, D) treated with 0.78 µg/ml ZD-DCM-E, and cells visualized by PlasDIC microscopy at 40 x magnification, E) untreated, F) treated with 100 µg/mL ZD-DCM-E, G) treated with 12.5 µg/mL ZD-DCM-E, and H treated with 0.78 µg/mL ZD-DCM-E. White arrows indicate signs of stress and black arrows indicate signs of necrosis.

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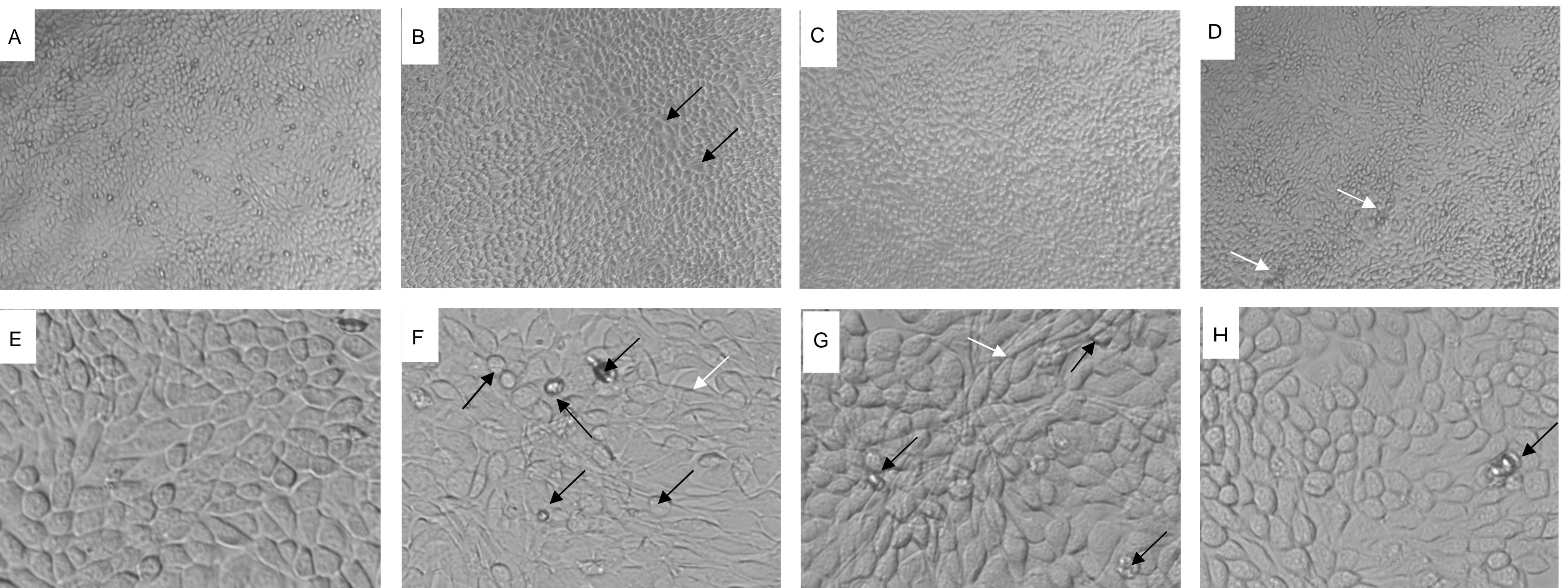

Figure 38: MCF-7 cells visualized by phase contrast microscopy at 10 x magnification, A) untreated, B) treated with 100 µg/ml ZD-HW-E, C) treated with 12.5 µg/ml ZD-HW-E, D) treated with 0.78 µg/ml ZD-HW-E, and cells visualized by PlasDIC microscopy at 40 x magnification, E) untreated, F) treated with 100 µg/mL ZD-HW-E, G) treated with 12.5 µg/mL ZD-HW-E, and H treated with 0.78 µg/mL ZD-HW-E. White arrows indicate signs of stress and black arrows indicate signs of necrosis

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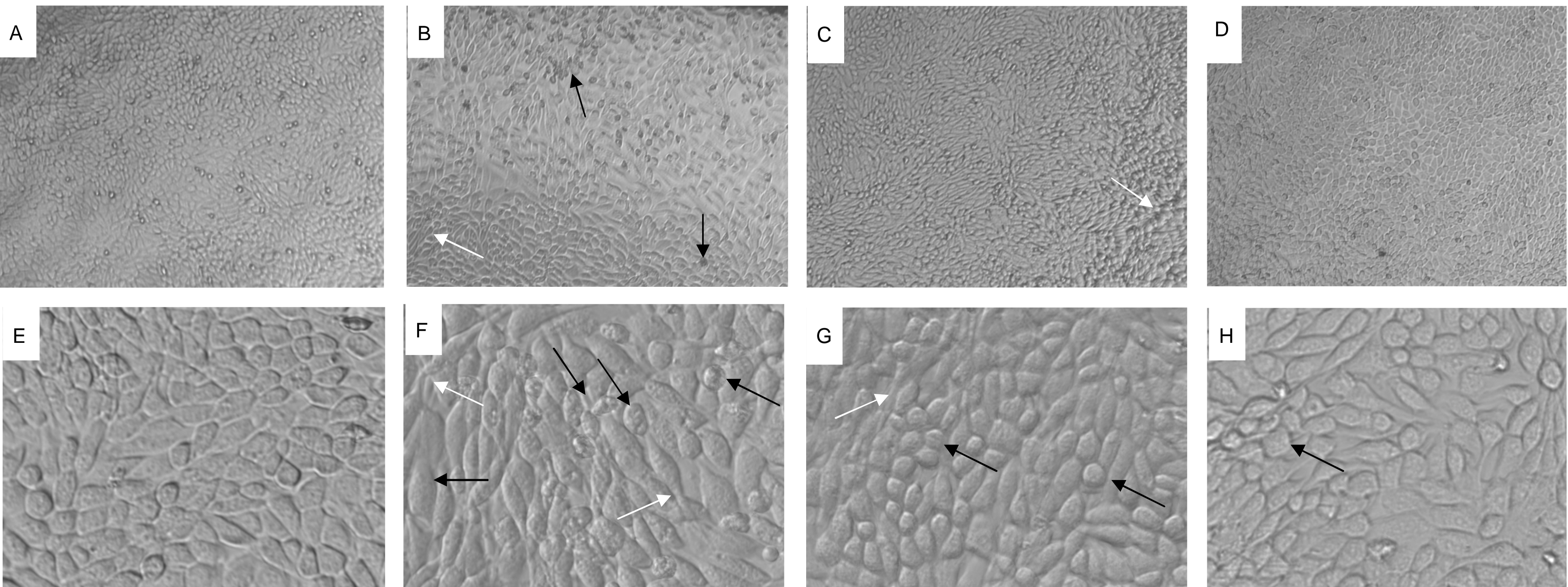

Figure 39: MCF-7 cells visualized by phase contrast microscopy at 10 x magnification, A) untreated, B) treated with 100 µg/ml XC-MeOH-E, C) treated with 12.5 µg/ml XC-MeOH-E, D) treated with 0.78 µg/ml XC-MeOH-E, and cells visualized by PlasDIC microscopy at 40 x magnification, E) untreated, F) treated with 100 µg/mL XC-MeOH-E, G) treated with 12.5 µg/mL XC-MeOH-E, and H treated with 0.78 µg/mL XC-MeOH-E. White arrows indicate signs of stress and black arrows indicate signs of necrosis

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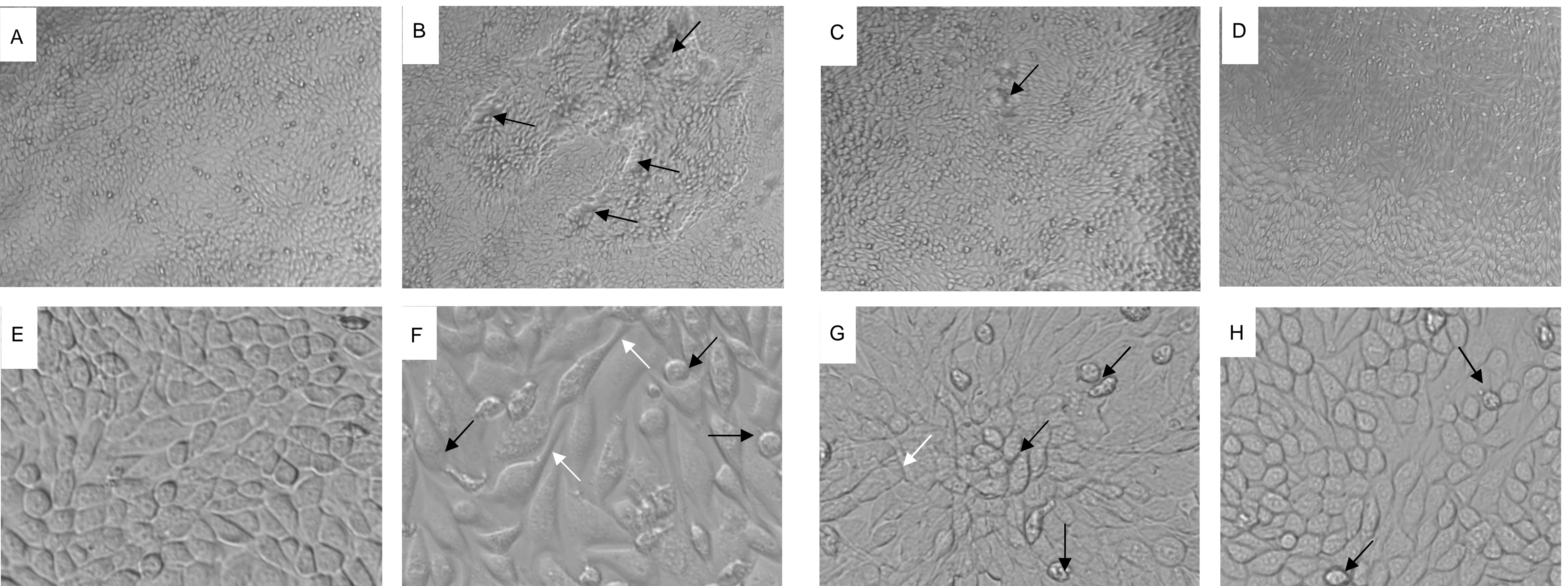

Figure 40: MCF-7 cells visualized by phase contrast microscopy at 10 x magnification, A) untreated, B) treated with 100 µg/ml XC-DCM-E, C) treated with 12.5 µg/ml XC-DCM-E, D) treated with 0.78 µg/ml XC-DCM-E, and cells visualized by PlasDIC microscopy at 40 x magnification, E) untreated, F) treated with 100 µg/mL XC-DCM-E, G) treated with 12.5 µg/mL XC-DCM-E, and H treated with 0.78 µg/mL XC-DCM-E. White arrows indicate signs of stress and black arrows indicate signs of necrosis.

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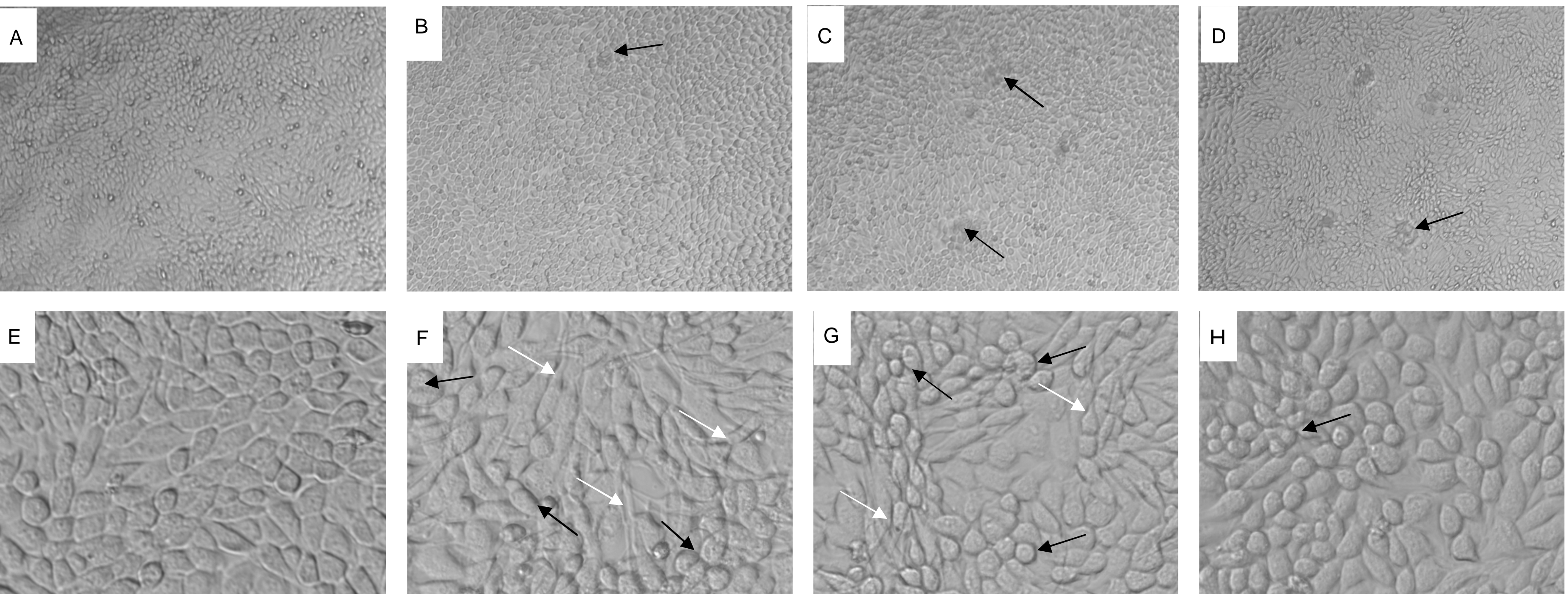

Figure 41: MCF-7 cells visualized by phase contrast microscopy at 10 x magnification, A) untreated, B) treated with 100 µg/ml XC-HW-E, C) treated with 12.5 µg/ml XC-HW-E, D) treated with 0.78 µg/ml XC-HW-E, and cells visualized by PlasDIC microscopy at 40 x magnification, E) untreated, F) treated with 100 µg/mL XC-HW-E, G) treated with 12.5 µg/mL XC-HW-E, and H treated with 0.78 µg/mL XC-HW-E. White arrows indicate signs of stress and black arrows indicate signs of necrosis

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CHAPTER 4: Conclusion

Phytochemical screening of extracts of *X. caffra* and *Z. davyi* indicated the presence of phenols, sterols and terpenoids in all extracts. Quinones were present in all extracts except the HW extracts. ZD-MeOH-E and ZD-DCM-E contained 8 out of 9 classes of phytochemicals tested. The DCM extract of *X. caffra* contained 7 out of the 9 classes of phytochemicals tested. HPLC confirmed the presence of various phytochemical classes and identified the compounds ascorbic acid, salicylic acid, caffeic acid, gallic acid, rutin, quercetin, nitidine and sanguinarine. Gallic acid (13.4%), nitidine (33.5%) and quercetin (26%) were found in the greatest abundance in ZD-HW-E, XC-MeOH-E and ZD-MeOH-E, respectively. Previous studies confirm that compounds isolated from the *Ximenia* genus include glycosides, tannins, phenolics, alkaloids, quinones and terpenoids.[115] It has also been reported that leaf extracts of *X. caffra* contain tannins and glycosides, while the root extract contains flavonoids, saponins and coumarins in addition to tannins and glycosides.[113] The *Zanthoxylum* genus has been reported to contain compounds such as benzophenanthridine alkaloids, flavonoids, coumarins, terpenoids, lignans and linear chain fatty acids which concur with the findings obtained via TLC.[109]

The extracts of *Z. davyi* and *X. caffra* displayed both antibacterial and antifungal activity against planktonic and biofilm preparations of *S. aureus* and *C. albicans*, respectively. The activity against` planktonic microorganisms was insignificant (MIC >1.0 mg/mL) and was therefore not investigated further as the extract was not deemed a suitable lead. Anti-biofilm activity was significant and comparable to conventional antibiotics (ciprofloxacin and amphotericin B). A paradoxical effect was observed when biofilms were treated with the plant extracts. This is consistent with literature where antimicrobials were found to cause paradoxical effects on certain microorganisms.[132,133] When extracts of *Z. davyi* were combined with extracts of *X. caffra*, a synergistic effect was apparent and greater anti-biofilm efficiency was achieved ($\sum FIC < 0.5$). This implicates that the combination of *Z. davyi* extracts with *X. caffra* extracts would have more pronounced antimicrobial effects than a single extract in isolation.

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Scanning electron microscopy indicated that the extracts were efficient in degrading the exopolysaccharide matrix vital to biofilms. Antimicrobial activity is ascribed to the phytochemicals found to be present in the extracts as these phytochemicals were reported in literature to possess antimicrobial activity. The mechanism of anti-biofilm action of phytochemicals could be due to their role in interfering with quorum sensing systems (vital for cell-to-cell communication) due their specific chemical structures, their ability to re-sensitize microorganisms to antimicrobials or their ability to interfere with the cell cycle and limit nutrients and proteins vital to EPS production. Quinones in particular have been reported to interact with nucleophilic amino acids resulting in protein inactivation. Other antimicrobial activities including the anti-viral activity of *Z. davyi* reported in previous studies could be attributed to compounds like quercetin.

With regard to safety, low cytotoxicity was observed for all extracts, with a dose dependent relationship between extract concentration and percentage cell death. Phase contrast and PlasDIC microscopy confirmed these results where an increase in concentration of plant extract, showed an increase in cell death. Apoptosis was noticeably induced in EA.hy926 human umbilical vein cells whereas necrosis was observed in MCF-7 breast cancer cells. Therefore high concentrations of extracts caused cell death via a controlled process in non-cancerous cells. In all, the cell extracts seemed to induce apoptosis (programmed cell death) in EA.hy926 cells while necrosis (cytolysis) was induced in MCF-7 cancer cells. Low cytotoxicity could be attributed to the presence of antioxidant compounds in the extracts; phenolic acids, flavonoids, terpenoids and quinones.

This study is the first to determine the effect of *Z. davyi* and *X. caffra* on biofilms of *S. aureus* and *C. albicans*, as well as to determine the effect of these plants on microbial morphology. This study provides scientific support for the antimicrobial activities of *Z. davyi* and *X. caffra*, especially against biofilms which is the way in which microbes are present in the body. Accompanied with the latter, the extracts showed negligible cytotoxicity. Due to the findings, investigations into the mechanism of action and isolation of active compounds responsible for the antimicrobial activity is warranted.

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CHAPTER 5: Limitations of the study

Phytochemical analyses detected a range of phytochemicals using MeOH, DCM and HW as extract solvents. Other solvents may have been used which could have provided a greater yield and extracted phytochemicals not detected in the present study. The detection of phytochemical classes by TLC is variable and depends on the concentration/load spotted, therefore, a greater concentration of the extract spotted could increase the sensitivity of the method and result in further compounds being detected. Alternatively, HPLC-MS/MS could have been used which is much more sensitive and able to detect trace quantities of phytochemicals.

Although the antimicrobial activity against planktonic microorganisms was deemed insignificant, there could be significant activity against other microorganisms not tested in this study. A wider spectrum of microorganisms could have been investigated. Anti-biofilm activity was assessed by the crystal violet assay which depends on the quantitation of biomass. Although it is widely accepted as a means of assessing biofilms, the various rinsing steps could wash away parts of the biofilm giving a false indication of antimicrobial activity which may account for negative inhibition. With regard to the BIC's, testing a broader range of concentrations may have different effects on biofilms than those seen in the present study. Combinations of different plant parts could also be explored with regard to synergy testing.

As for cytotoxicity, extracts of *X. caffra* and *Z. davyi* may be toxic to cell lines not tested in this study. Different solvents could also affect cell viability and could be investigated for a more comprehensive overview. The effect of each extract on cytotoxicity was examined in this study. Combinations of the extracts could also be investigated. The synergistic antimicrobial effect of the combinations of extracts tested could result in greater toxicity to the cell lines tested. Although antimicrobial activity was confirmed and different phytochemicals detected, the specific compounds which have antimicrobial activity were not isolated and could have provided valuable information.

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Summary

This study aimed to determine the antimicrobial activity of extracts of *X. caffra* and *Z. davyi*. The phytochemicals present in the plant extracts were also investigated and the phytochemical profile of the plants determined.

A range of antimicrobial phytochemicals were detected which may account for antifungal and antibacterial activity noted in this study. Although activity against planktonic microorganisms were deemed insignificant, anti-biofilm activity was prominent. Phenolic compounds have a wide array of antimicrobial mechanisms and these could contribute to the anti-biofilm effects observed in the present study. Since antimicrobial action is mediated by the inhibition of fatty acid synthesis, sterols could also play an important role as antimicrobials and could account for anti-biofilm activities. Extracts of *X. caffra* and *Z. davyi* were efficient in disintegrating biofilms of *S. aureus* and *C. albicans,* in a dose-dependent manner. Several terpenoids have been found to inhibit microbial oxygen uptake and oxidative phosphorylation. This could explain the cessation of the production of EPS which hold microbial cells together in a biofilm and account for the anti-biofilm activity found. This study is the first to determine the effect of *Z. davyi* and *X. caffra* on biofilms of *S. aureus* and *C. albicans*, as well as to determine the effect of these plants on microbial morphology.

When extracts of *X. caffra* were combined with extracts of *Z. davyi*, synergism was evident. Synergistic interactions of the plant extracts could be due to the combination of the different antimicrobial phytochemicals present in each plant. Literature suggests that the benefits of phytomedicines are often the result of synergistic actions of multiple active chemicals.

While the extracts induced apoptosis in EA.hy926 cells and necrosis in MCF-7 cells, they were not toxic to the cell lines tested against. Low cytotoxicity could be due to the presence of antioxidant phytochemicals such as gallic acid and quercetin. The cytotoxicity noted in this study could be attributed to the flavonoids detected, which

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have been reported to have cytotoxic activities. Other phytochemicals detected, which could account for the anti-cancer activity observed, include salicylic acid, caffeic acid, quercetin, rutin, and the alkaloids; nitidine, sanguinarine, and chelerythrine.

The results obtained from this study indicated that the plant extracts have significant activity against biofilms of *S. aureus* and *C. albicans*. This is ascribed to the presence of phytochemicals with antimicrobial activity detected in the study confirmed by TLC and HPLC. The results are also supported by previous reports where both the *Zanthoxylum* and *Ximenia* genus have been described as having antimicrobial activities. Apart from antimicrobial activity, the results obtained from cytotoxicity testing indicated negligible toxicity on the cell lines tested. Since plants are frequently considered safe and toxicity testing is commonly overlooked, this study is particularly meaningful and recognizes the need for cytotoxicity testing. This is significant as regulations regarding Complementary and Alternative Medicine in South Africa (CAMs) have become stricter and no new complementary medicines will be allowed on the market unless registered and consequently safe for public use.[184] This study provides scientific support for the antibacterial and antifungal activity of *Z. davyi* and *X. caffra*. Isolation of the active compounds is warranted, as these plants could serve as alternative antimicrobials in the search for novel antimicrobial drugs.

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Appendix 1

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 13/04/2011 and Expires 13/04/2014.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

19/09/2013

Approval Certificate
New Application

Ethics Reference No.: 369/2013

Title: Antimicrobial activity of *Zanthoxylum davyi* and *Ximenia caffra. Dept:Pharmacology. Cell : 083 456 6681*

Dear Ms Jayshri Rangasamy

The **New Application** as supported by documents specified in your cover letter for your research received on the 28/08/2013, was approved by the Faculty of Health Sciences Research Ethics Committee on the 18/09/2013.

Please note the following about your ethics approval:
- Ethics Approval is valid for -2 years.
- Please remember to use your protocol number (369/2013) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

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