The effects of medicinal plant extracts on the microbial, biofilm and autolytic activity of several pathogenic bacteria.

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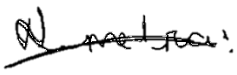

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Abbreviations:

Name:	Abbreviation:
2 (Beta)-mercaptoethanol	BME
Amide (N)-terminal	N-terminal
Ammonium persulfate	APS
Bacilli subtilis	*B. subtilis*
Beta	β
Bicinchoninic acid	BCA
Brachystelma gymnopodum	*B. gymnopodum*
Brain Heart Infusion	BHI
Coccinea rehmannii	*C. rehmannii*
Dimethyl sulfoxide	DMSO
Di-sodium phosphate	Na_2HPO_4
Dithiothreitol	DTT
Doubled distilled water	ddH2O
Et alia	*Et al*
Eulophia hereroensis	*E. hereroensis*
Extracellular polymeric substance	EPS
Fockea angustifolia	*F. angustifolia*
High performance liquid chromatography	HPLC
Hydrochloric acid	HCl
Kilo Daltons	kDa
Lactococcus lactis	*L. lactis*
Lithium Chloride	LiCl
Litre	L or l
Micrococcus luteus	*M. luteus*
Microliter	µl
Micrometre	µm
Millilitre	ml
Millimetre	mm
Millimolar	mM
Molar	M
Molecular weight	Mw

Monopotassium phosphate	KH_2PO_4
N, N, N′, N′-tetramethylethylenediamine	TEMED
Nanometre	nm
N-dinitrophenyl	DNP
Percentage	%
Phosphate Buffered Saline	PBS
Polyacrylamide gel electrophoresis	PAGE
Potassium Chloride	KCl
Quorum-sensing	QS
Random Forest	RF
Relative molecular weight	Mr
Retention time	RT
Revolutions per minute	rpm
Sodium Chloride	NaCl
Sodium dodecyl sulfate	SDS
South Africa	SA
Staphylococcus aureus	*S. aureus*
Staphylococcus simulans	*S. simulans*
Streptococcus equi	*S. equi*
Streptococcus milleri	*S. milleri*
Streptococcus mutans	*S. mutans*
Streptococcus sanguinis	*S. sanguinis*
Streptococcus zooepidemicus	*S. zooepidemicus*
Thin layer chromatography	TLC
Trochomeria macrocarpa	*T. macrocarpa*
Ultraviolet	UV
United Kingdom	UK
United States of America	USA
Volt	V
Volume	Vol
Walleria nutans	*W. nutans*
Weight	Wt
Working reagent	WR

Chapter 1:

Introduction

Summary:

More people are aware of the problems with drugs and are reverting to herbal (plant) and over-the-counter medications. The conclusion made is the urgency to study new plants for antimicrobial activity. Various plant extracts were used in the study to determine the antimicrobial, mode of action and autolytic activity against various microbial species. Techniques used in this study include culturing via streaking method, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), zymography, high performance liquid chromatography (HPLC), autolytic activity assay, minimum inhibition concentration assay, dilution activity assay, Spot-on-lawn assay and Bicinchoninic acid (BCA) assay. The whole cell protein profile was used to show the proteins without any extraction method for comparison with the applied extraction method. With the extraction method implemented, less proteins where seen as the extraction method was specific to the peptidoglycan hydrolases. Sodium dodecyl sulfate (SDS) (4%) extraction method was more effective as an extraction method when compared to lithium chloride (LiCl). Plant extract 4.2 showed antimicrobial activity as seen by the inhibition zone on the agar plate (Figure 27 and Figure 28). Plant extract 4.2 and 5.2 showed decreased autolytic activity (Figure 20-22). The conclusion made was that the presence of the plant extracts decreases growth of the microbial species thus decreases protein production. Markers were identified using HPLC where 1 marker showed antimicrobial activity (Sebacic acid) and another marker showed anti-inflammatory activity (Loxoprofen Sodium) based on previous literature. *S. aureus* 12600 in the presences of extract 4.2 had antimicrobial activity but results were not significant. Extract 5.2 showed biofilm formation. Extract 4.2 showed positive results in most experiments conducted.

Problem Statement:

Antibiotic resistant microbes have proven to be a great challenge for the pharmaceutical industry and for health in general. Researchers are trying to find alternative methods to decrease bacterial infections and resistance to antibiotics. For this reason, plant material has become an interest of study. Plants have medicinal properties as shown by various studies already conduced (1-11). Other studies have also look at peptidoglycan hydrolases as alternative antimicrobial methods as peptidoglycan is abundant in bacteria and can be used as a target (12-16). Peptidoglycan is the substrate of peptidoglycan hydrolases and peptidoglycan is a

very important part of bacterial cell walls (17,18). Peptidoglycan hydrolases can be used as an alternative tool for the management of diseases caused by antibiotic-resistant microorganisms (12).

The conclusion made is the urgency to study alternative antibiotics in the form of peptidoglycan hydrolases and plant materials. Characterization and identification of peptidoglycan hydrolases from selected microbial species and the influence of selected plant extracts on peptidoglycan hydrolases within the cell wall will be studied and the mode of action will be tested.

Introduction:

Literature review:

Eubacteria is differentiated into Gram-negative and Gram-positive bacteria due to the cell wall composition made visible by Gram staining techniques (19). Gram-positive bacteria have a thick peptidoglycan layer whereas Gram-negative bacteria have a thinner peptidoglycan layer and an outer membrane (18-20).

Peptidoglycan (murein) consists of sugar and amino acids and is abundant in bacteria (21,22). The sugar composes of alternating *N*-acetylmuramic acid residue and β-(1,4) linked *N*-acetylglucosamine residue (21,23). A peptide chain is attached to the *N*-acetylmuramic acid and crossed-link to another peptide chain to form a mesh-like layer (21,23). Bacteria use peptidoglycan hydrolases to open the peptidoglycan structure in order to grow and divide (20,24,25).

Peptidoglycan hydrolases are enzymes of different origins and are made of enzyme groups that can assemble, change and/or breakdown the peptidoglycan (26,27). Peptidoglycan is the substrate of peptidoglycan hydrolases and peptidoglycan is an important structure of bacterial cell walls (20). The functions of peptidoglycans include: cell wall regulation, autolysis, separation in cell division, cleaving during sporulation to name a few functions (20,22). Unique hydrolases can increase the pore size in the peptidoglycan for *trans*-envelope complexes for example pili (22).

Peptidoglycan hydrolases are also known as autolysins when their action results in cell lysis (20). Smith *et al*. has reviewed in detail autolysin complement of *Bacillus subtilis (B. subtilis) (28)*. An amount of 35 genes encoding peptidoglycan hydrolases

were identified (28). Comparative analysis on *Lactococcus lactis (L. lactis)* IL1403 found only 5 genes (28). The difference can be due to the lifestyles of both organisms where *B. subtilis* needs autolysins for example growth, separation and sporulation (29). Various types of peptidoglycan hydrolases are required for these processes. *L. lactis* uses autolysins for cell growth, separation and growth turnover (28,30).

Peptidoglycan hydrolases are classified on sources and cleavage site specificity (25). Peptidoglycan hydrolases are separated into various groups namely lysozymes, endolysins, bacteriocins and autolysins regarding sources (25,31-33). Lysozymes represent a natural defence mechanism against bacterial pathogenic in animals and plant and was discovered by Fleming in 1992 (34). Bacteriocins characterized as natural proteinaceous antibiotics that act on closely related species (35). Stressful conditions cause the production of these enzymes that can kill neighbouring cells that are unresisting to their effects (35). Lysis can occur by exposing bacterial cells externally to purified endolysins (36).

Regarding cleavage site, peptidoglycan hydrolases can be classified into many groups. Peptidoglycan amidase cleaves the amide bond between L-alanine and *N*-acetyl muramic acid (23,37-39). Peptidoglycan peptidase can cleave other enzymes such as endopeptidases or carboxypeptidases (23,37-39). *N*-acetylmuramidases, lysozymes and lytic transglycosylases can cleave the glycosidic bond between and *N*-acetyl glucosamine and *N*-acetyl muramic acid (23,37-39). *N*-acetylglucosaminidases hydrolyses the glycosidic bond between neighbouring monosaccharides and *N*-acetylglucosamine (23,37-39). Cleavage specificity is dependent on the catalytic domain of the hydrolase (23,37,39).

The N-terminal domain and the C-terminal domain are constituents of peptidoglycan hydrolases (12,40). The cell wall binding domain (12) is contained within the C-terminal domain. Hydrolase activity is specific for a certain fragment size (small), peptidoglycan type or molecular weight (MW) (18,41).

In 2016, peptidoglycan hydrolase prediction was used as a new class of antibacterial proteins (33). According to this study, alternative antibiotics are a necessity because of the development of resistant bacteria that decreases the effect of antibiotics against infections (33). Due to peptidoglycan hydrolases cleaving properties, it can be used as alternative antibacterial agents (33). Sulfonamide derivatives and tetracycline

antibiotic are examples of compounds that are considered as antibacterial compounds (13). Due to drug resistance in bacteria, antibiotics have shown decreased effects against bacterial infections (13,42). Peptidoglycan hydrolases were proposed as a potential alternative because of multifarious spectrum (16,26,43) bacteriolytic activity and the cell wall target site (24).

The following autolysins are universally present among bacteria that were used in this study. *Staphylococcus simulans (S. simulans)* excretes Lysostaphin a peptidoglycan hydrolase by cleaving the peptidoglycan chain of *Staphylococcus aureus (S. aureus)* (44). *Streptococcus zooepidemicus (S. zooepidemicus) 4881* produces Zoocin A, another peptidoglycan hydrolase, which is similar to lysostaphin that potentially can be an effective use for the control and treatment of infection caused by *S. aureus* (27). *Streptococcus milleri* (*S. milleri*) *NMSCC061* produces Millericin B (peptidoglycan hydrolase) which causes the inhibition of growth of various bacterial species (45). Pneumococcal meningitis infection was treated by muraminidase Cpl-1 (phase lytic enzyme) showing the medicinal value of peptidoglycan hydrolases (46). Bacteriophage endolysin PlyC (aerosolized) was shown to be active against the pathogenic species of *Streptococcus equi* (*S. equi)*. Endolysin PlyV12 and Enterolysin A have anti-enterococcal activity (47,48).

The results of the study showed that peptidoglycan hydrolases were divided based on action site (44). The knowledge gained by this study was used for the development of ‘HyPe’, a computational tool, to identify and classify novel peptidoglycan hydrolases. (33). The RF model showed 99.98 % specificity, 71.12 % sensitivity, 0.80 MCC and 99.55 % accuracy in different peptidoglycan hydrolase classes (33). It was concluded that identification and classification of novel peptidoglycan hydrolases from complete genomic or metagenomic ORFs can be done by the Hype tool (33).

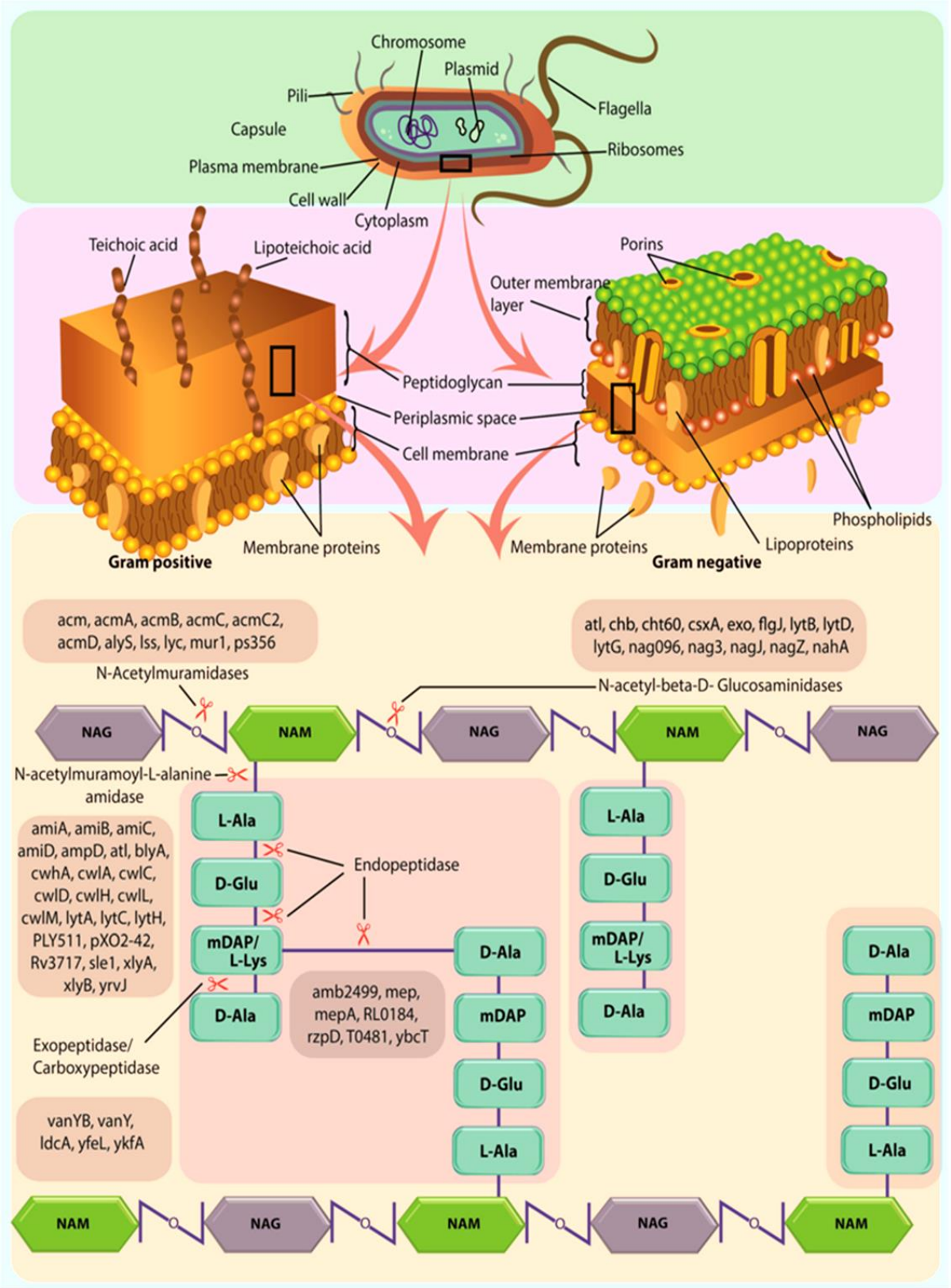


Figure 1: A schematic illustration of a bacterium, the difference between Gram-positive and Gram-negative cell wall structures and the action sites of peptidoglycan hydrolases on the microbial cell wall (1).

Basic concepts and principles:

The basic concepts and principles are outlined in this section. In order to conduct the experiments with the correct technique, these basic concepts are required to understand the reason for the technique. The bacterial cell wall composition, techniques used, and extraction methods are explained.

Bacterial cell wall:

The bacterial cell wall is a major component to the survival of bacterial microorganisms (17,49,50). The cell wall is responsible for the maintenance and protection of cell shape (17,49-51). The rigidity and strength of the cell wall is a result of the peptidoglycan layer (17,49-51). The difference between Gram-positive and Gram-negative microorganisms is a result of the peptidoglycan layer and the effect of Gram-staining (19). Gram-negative bacteria have the outer membrane layer, the peptidoglycan layer and inner phospholipid membrane layer (also known as the cytoplasmic membrane) (17,19,49,50). The outer membrane is composed of lipopolysaccharides, phospholipids, lipoproteins and porins (50). The outer membrane is absent in Gram-positive bacteria and the peptidoglycan layer is thick (19,20,31,50). Now that the cell wall compositions were known, the correct extraction method was used to get the hydrolases out of the cell wall to study. For this reason, LiCl and SDS was used.

Hydrolase extraction:

Lithium Chloride (LiCl) and Sodium dodecyl sulfate (SDS) can be used to extract hydrolases from the cell and cell wall for them to be studied (52-54). LiCl has been used in several studies (45,55,56), but drawbacks in the protein yield has led to the use of SDS. LiCl is a chaotropic agent that reduces the stability of proteins leading to the decreased protein yield (57,58). SDS is used for its ability to solubilize membrane proteins and replacing of membrane proteins that allows for separation of proteins from the cell (59). SDS had an increased protein yield compare to LiCl. The hydrolases had to be visualized in order to see the whole cell protein profile, effects of the lysis and the difference of the lysis in the presence and absence of the plant extracts. For this reason, the SDS-PAGE method was chosen.

SDS-PAGE:

Polyacrylamide gel electrophoresis (PAGE) separates biological macromolecules according to electrophoretic mobility which relies on the conformation, length and charge of the molecule (60-65). SDS ($C_{12}H_{25}NaO_4S$; mW: 288.38) is an anionic detergent which is used in protein samples to denature proteins and for an overall negative charge (60-64). Due to the addition of SDS to PAGE, the process is referred to as SDS-PAGE (60,61,63). Disulfide bonds are reduced by dithiothreitol (DTT) or 2-mercaptoethanol (beta-mercaptoethanol/BME)(60,61,63). A tracking dye is added to track the process of the run. Ammonium persulfate (APS) ($N_2H_8S_2O_8$; mW: 228.2) initiates gel formation and is a source of free radicals. TEMED (N, N, N′, N′-tetramethylethylenediamine) ($C_6H_{16}N_2$; mW: 116.21) improves polymerization and free radicals stabilization (60,61,63). In order to visualize the autolytic activity of the hydrolyses, the enzymes must be able to hydrolyse the cell wall and cannot be harmed by chemicals. For this reason, zymography was the technique used.

Autolysins and zymography:

Autolysis is known as self-digestion through its own enzymes or through another molecule of the same enzyme (14,49,66-69). Autolysis occurs in dying tissue or injured cells and is used in the beverage and food industries (15,70-73).

Zymography was used for hydrolytic enzymes detection (74-76). There are three types of zymography: in-gel zymography, in-situ zymography and in-vivo zymography (75). The samples in-gel zymography are prepared in a standard loading buffer for SDS-PAGE (75,76). The samples are not boiled and don’t contain reducing agent because it would interfere with enzyme refolding (75,76). A substrate is mixed in the stacking gel during preparation and Triton X-100 is used to remove the SDS from the zymogram (75,76). The gels are thereafter incubated in an appropriate digestion buffer at 37°C (75,76). The zymogram gel is stained where the areas of digestion appear as clear bands (75,76). If autolytic activity was being influences in the presence of the plant extract, the composition of the plant extracts must be studied to identify what causes the change. For this reason, chromatography, specifically HPLC, was used.

Chromatography:

Chromatography is used for separating mixtures into individual components (45,77,78). Sample dissolving takes place in the mobile phase which is the solvent

moving through the column and is carried through the stationary phase which is the solvent or substance that stays fixed inside the column (2,45,78,79). The various types of compounds move through the column at different speeds and leading to the separation of the compounds (2,45,77,79). The separation of compounds from the mixture is due to the interaction between the mobile phase and the stationary phase (2,45,77-79). There are different kinds of chromatography techniques namely: column chromatography, thin layer chromatography, ion exchange chromatography, gel filtration chromatography, etc.

High performance liquid chromatography (HPLC):

High performance liquid chromatography (HPLC) is a form of column chromatography (78). Compounds with lower affinity and absorption to the stationary phase will move faster and will elute first (78). Compounds with greater affinity will travel slower and will elute last (78). HPLC allows for a bigger surface area for the interactions between the molecules and stationary phase which allows greater separation of the components with the sample (78). Silica particles fill the column and the solvent is non-polar (78,80). Polar compounds in the sample will stick to polar silica whereas non-polar ones will elute faster through the column (78,80). The rate of movement is the rate of movement of a compound divided by rate of movement of the mobile phase (78). A high pressure pump forces the mobile through the column where the detector detects separated compounds by measuring amounts (78). HPLC has an advantage of speed, efficiency, sensitivity and ease (78).

For a quick detection of compounds, TLC can be used. For more accurate separation, HPLC is better, thus HPLC was used for its speed, efficiency, sensitivity and ease.

The amount of protein that was extracted can influence the results within the experiments, thus it was important to have an adequate amount of protein for accurate results. For this reason, the BCA assay was used.

Bicinchoninic acid (BCA) assay:

BCA assay is used for the quantification of the total protein within a sample and as a colorimetric detection tool (81). This method is constituted of the Biuret reaction (reduction of Cu+2 to Cu+1) and the highly sensitive detection of the cuprous cation (Cu+1) using a reagent that contains bicinchoninic acid (81). The chelation of two molecules of BCA with one cuprous ion forms the purple-coloured product (81,82).

Peptide bond number, cysteine, tryptophan, tyrosine and the macromolecular structure are reported to be responsible for the colour change with BCA (82). BCA assay is not sensitive to the 4% SDS used for the extraction of the peptidoglycan hydrolases and the SDS did not interfere with the results in this assay (82). The Bradford assay is sensitive to SDS and gives inaccurate results (81,82). For the sensitivity reason, BCA assay was used in this study to determine protein concentration.

In order to extract the protein, the biofilm layer, which is a protective mechanism used by bacteria, must be destroyed. Medicinal plants have antibiofilm activity which causes the breakdown of the biofilm giving access to the bacterial cell (83,84).

Biofilm:

A biofilm is an extracellular matrix in which cells stick to each other and to a substrate (83,85,86). Pathogens establish biofilm formation in higher organisms which leads to diseases (85,87). Biofilms may form on living or non-living surfaces and is prevalent in natural, industrial and hospital settings (85,88). The microbial cells in a biofilm are physiologically different from planktonic cells of the same organism (85,88). Microbes form a biofilm in response to many factors for example cellular recognition of attachment sites on a surface, nutritional cues, by exposure of planktonic cells to sub-inhibitory concentrations of antibiotics to name a few (89,90). When a cell switches to the biofilm mode of growth, a phenotypic shift in behaviour occurs in which large suites of genes are differentially regulated (91). Formation of a biofilm begins with the attachment of free-floating micro-organisms to a surface (91). Colonists adhere to the surface through weak, reversible adhesion via van der Waals forces (89,91). If the colonists are not immediately separated from the surface, the colonists may anchor more permanently with the use of cell adhesion structures such as pili (89). Bacteria with increased hydrophobicity have reduced repulsion between the extracellular matrix and the bacterium (86). With colonization, the cells communicate via quorum-sensing (QS) (86).

Biofilms can also be formed on the inert surfaces of implanted devices such as catheters, prosthetic cardiac valves and intrauterine devices (92). New staining techniques are developed to distinguish bacterial cells growing in living animals, for example, from tissues with allergy-inflammations (93). The biofilm on the surface of

teeth is subject to oxidative stress (94) and acid stress (95). Once the biofilm is subjected to acid stress, the competence region is induced, leading to resistance to death by acid (95).

Basic background and information on bacteria and plant species:

Peptidoglycan hydrolases are produced by various number of bacterial species. Literature has shown that *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa,* various species staphylococci and streptococci produce peptidoglycan hydrolases (28,38,44,96). For this reason, the below microbial species were chosen to conduct the experiments. In the section below each microbial species used in this thesis was discussed.

***Streptococcus mutans*:**

The species *Streptococcus mutans* (*S. mutans)* comes from the kingdom Bacteria, phylum Firmicutes, class Bacilli, order Lactobacillales, family Streptococcaceae and genus Streptococcus (97). *S.mutans* is gram-positive coccus found in the oral cavity and contributes to tooth decay (98,99). *S. mutans* must survive harsh environments and exposure to many antimicrobial agents as conditions of the oral cavity changes rapidly (100). *S. mutans* are one of the bacteria species causing tooth decay by using the enzyme glucansucrase (101) to metabolizing sucrose into lactic acid (99). *S. mutans* have receptors to increase adhesion to the surface of the tooth surface (102). Bacteria use sucrose to form polysaccharide (98). However, other sugars digested by *S. mutans* produce lactic acid as a product (103). The combination of acid and plaque causes dental decay (104). *S. mutans* strain used in this study is *S. mutans* ATCC 25175.

***Streptococcus sanguinis*:**

The species *Streptococcus sanguinis (S. sanguinis)* comes from the kingdom Bacteria, phylum Firmicutes, class Bacilli, order Lactobacillales, family Streptococcaceae and genus Streptococcus (97). *S. sanguinis* is a gram-positive anaerobic coccus species and is a member of the Viridans Streptococcus group (97) *S. sanguinis* lives in the oral cavity and plays a role in the formation of plaque (97,99,101). *S. sanguinis* changes the surroundings to make it less hospitable against other Streptococcus strains (97). *S. sanguinis* may gain entrance to the bloodstream through example dental cleanings and surgeries and may colonize the heart valves

and cause bacterial endocarditis (97,105). The complete genomic sequence of *S. sanguinis* was determined at Virginia Commonwealth University (106) in 2007. *S. sanguinis* strain used in this study is *S. sanguinis* ATCC 10556.

***Staphylococcus aureus*:**

The species *Staphylococcus aureus (S. aureus)* comes from the domain Bacteria, kingdom Eubacteria, phylum Firmicutes, class Coccus, order Bacillales, family Staphylococcaceae and genus Staphylococcus (107). *S. aureus* is a gram-positive coccus found in the respiratory tract, nose and skin (107). Infections are caused by pathogenic strains that produce toxins and express cell-surface proteins (107). Staphylococcus was first identified in 1880 Sir Alexander Ogston in pus from a surgical abscess (108). Lower reproductive tract of women serves as a habitat for *S. aureus* (109,110). *S. aureus* can cause different an array of diseases such as minor skin infections (111) to life-threatening diseases (example sepsis). *S. aureus* strains used in this study are *S. aureus* ATCC 12600 and 43300.

Micrococcus luteus:

The species *Micrococcus luteus* (*M. luteus)* comes from the domain Bacteria, kingdom, Eubacteria, phylum Actinobacteria, class Coccus, order Actinomycetales, family Micrococcaceae and genus Micrococcus (112,113). *M. luteus* is a gram-positive to gram-variable coccus bacterium that is nonmotile from the family Micrococcaceae found in soil, water, air and skin (103). Sir Alexander Fleming also discovered *M. luteus*. *M. luteus* is known to have a high G + C ratio (112). *M. luteus* can easily be mistaken for *S. aureus* due to the yellow colony formation on agar plates, thus test must be done to confirm species (112,113). Greenblatt *et al.* has demonstrated on the basis of 16S rRNA analysis that *M. luteus* has survived for at least 34,000 to 170,000 years (113). *M. luteus* was sequenced in 2010 and was shown to have one of the smallest genomes of 2,501,097 bp (112). *M. luteus* was used in this study as cell wall due to the sensitivity of peptidoglycan of other species. *M. luteus* strain used was *M. luteus ATCC* 10240.

Plant extracts:

Many modern drugs originate from medicinal plant extracts (9,10,114).With increasing antibiotic resistance, research has become focused on the medicinal value of plant

extracts. Six potential medicinal plant extracts were included in this study. Unknown samples were designated extract 4.2 and 5.2 to be tested.

Brachystelma gymnopodum:

Brachystelma gymnopodum (B. gymnopodum) (extraction code: BG) ranges from Southern Africa - Zimbabwe, Namibia and Angola to name a few (115). *B. gymnopodum* is an herbaceous perennial plant (115). The original classification was *Ceropegia pygmaea*, but was changed due to morphological characteristics (116,117). *B. gymnopodum* is used as a food source for the native people and extracts of the *Brachystelma* species have bioactive compounds (118).

Coccinea rehmannii:

Coccinea rehmannii (C. rehmannii) (extraction code: CR) is found in Southern Angola, Namibia, and Zimbabwe to name a few (115). *C. rehmannii* is an herbaceous, climbing or scrambling plant and has edible roots and fruits and extracts of the Coccinea species have hypoglycaemic and antioxidant properties (8,119).

Eulophia hereroensis:

Eulophia hereroensis (E. hereroensis) (extraction code: EH) ranges from Southern Africa – Zimbabwe, Namibia, and Angola to name a few (115). *E. hereroensis* is not endemic to South Africa and comes from the family Orchidaceae (115). The habitats of *E. hereroensis* include rocky outcrops, granite boulders and sandy soil and anti-inflammatory properties have been identified (6,120).

Fockea angustifolia:

Fockea angustifolia (F. angustifolia) (extraction code: FA) is found in Botswana, Namibia and South Africa (115). Extracts of the roots are used as anthrax treatment (5), while tubers as used as food (121,122).Traditional healers have classified *F. angustifolia* as a medicinal plant (4).

Trochomeria macrocarpa:

Trochomeria macrocarpa (T. macrocarpa) (extraction code: TM) comes from the family Cucurbitaceae which is the pumpkin family (115). The bright red fruit is known to attract birds and traditional healers have claimed appetite supressing and wound healing properties, but medicinal value is still being studied (123).

Walleria nutans:

Walleria nutans (W. nutans) (extraction code: WN) is found in tropical and southern Africa (115). The roots are used in food, but the medicinal properties are unknown (115).

Through the literature review and the discussion of the techniques, microbial species and plant species, a hypothesis was drawn, and experiments were conducted to accept or reject the hypothesis below.

Hypothesis:

The study investigated the following hypothesis:

H_0: Crude extracts from plant materials will have no antimicrobial, antibiofilm, no mode of action or autolytic activity on selected microbial species.

Aims:

The aims of this research project were to:

1. Visualise whole cell protein profile and the effects of the plant extracts on the protein profile with the use of SDS-PAGE.
2. Determine autolytic enzyme activity on microbial species in the presence and absence of selected plant extracts with the use of LiCl extracted and SDS extracted hydrolases via zymography.
3. Determine the activity of crude plant extracts – decreased or increased activity via:
    - Dilution activity assay based on the Minimal Inhibition Concentration results that is used to determine the concentration needed for microbial inhibition.
    - Antimicrobial assay (Spot-on-lawn assay) to visualize inhibition zones.
    - Biofilm activity in the presence and absence of the plant extract in order to gain access into the cell and break the cells defensive mechanism.
4. Determine chemical compounds within the crude extracts via HPLC (High Performance Liquid Chromatography) (Chemistry collaboration) in order to identify possible antimicrobial compounds.

Chapter 2:

Experimental Design and Methods

Experimental Design and Methods:

Methods:

A basic schematic diagram of the methods was shown in Figure 2 below. Details on the methods were described in each section.

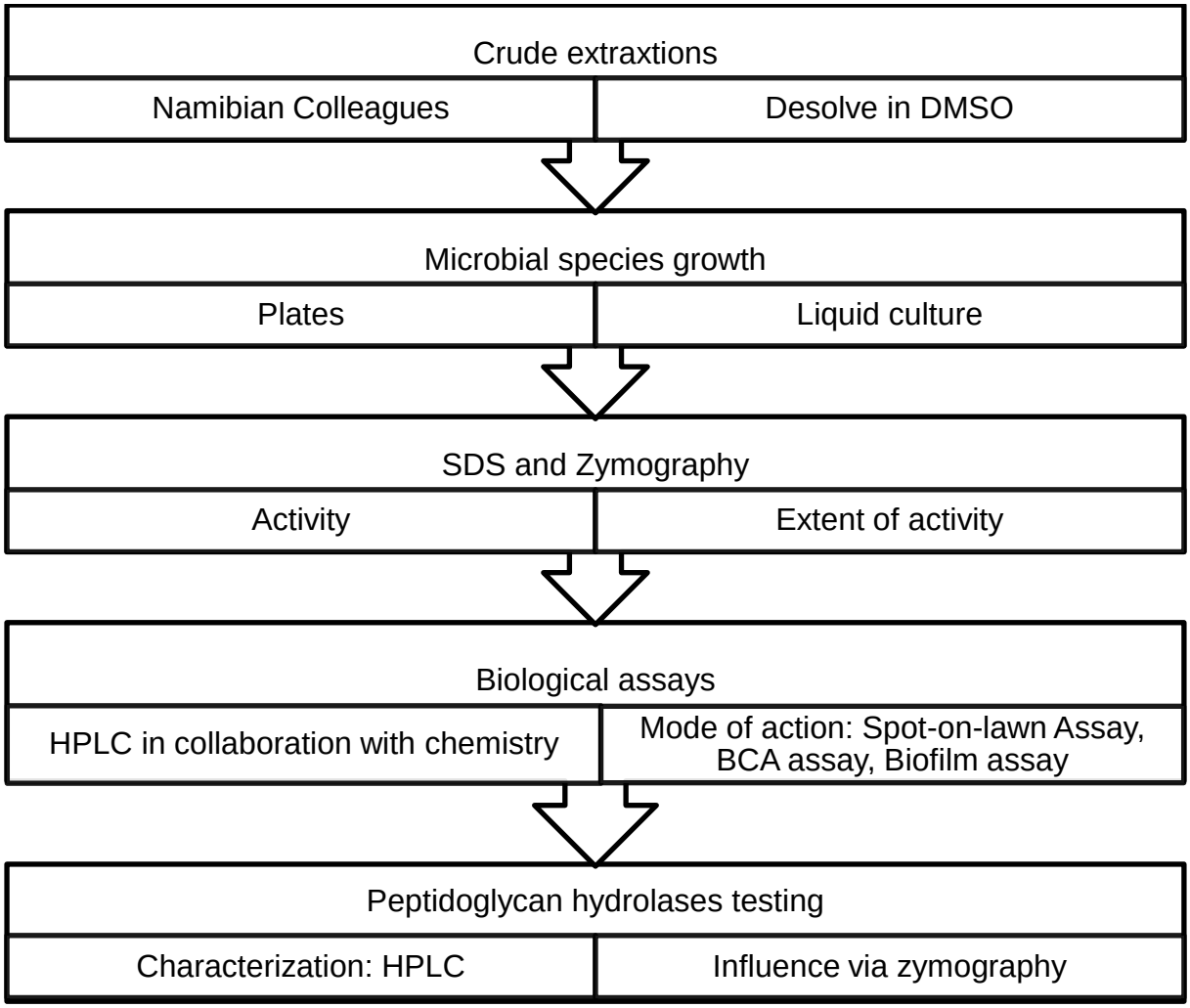


Figure 2: Basic schematic diagram of the experiments that were conducted in the study. Crude extraction was done by Namibian Colleagues and was dissolved in DMSO for the use in experimentation. Microbial species was grown on solid media and liquid media. The total enzyme profile and peptidoglycan hydrolases was tested using SDS and zymography. HPLC was done to identify compounds within the plant extract samples and the mode of action was studied using the spot-on-lawn assay and BCA assay.

Microbial growth:

For any experimentation to have taken place, the microbial species were cultures in the correct manner to insure optimal growth. Microbial species that were used in this study are described in the table below. Culture conditions were also described either in general or specific where applicable.

Table 1: Specific microbial species used for the duration of the study. Microbial species were chosen based on literature as described in Chapter 1.

Microbial Species	**ATCC number**
Streptococcus mutans	25175
Streptococcus sanguinis	10556
Staphylococcus aureus	12600 and 43300
Micrococcus luteus	10240

Plates:

All microbial species were maintained on Brain Heart Infusion (BHI) agar (Biolab, Merck, South Africa (SA), pH =7.4) plates and sub-cultured two weekly onto fresh plates which were incubated at 37^0C overnight. Plates were incubated in a Stuart incubator, SI500.

Small-scale culturing:

All microbial species underwent small-scale culturing. For the growth of all cultures in liquid media, BHI-broth (Oxiod, England) (5 ml) was used in 15 ml blue capped tubes (Cellstar tubes, Greiner bio-one, Germany) making the final concentration of microbial culture 33.33% (vol/vol). Inoculation of microbial species was done aseptically. A control tube consisted of only BHI-broth (100% BHI broth). Tubes were incubated (Stuart, orbital incubator, SI500) for 24 hours at 37°C, shaking at a speed of 220 revolutions per minute (rpm).

Micrococcus luteus (M. luteus) (North-West University) was used as a source for the preparation of crude cell wall material. Crude cell walls were used in zymogram assays as well as plate assays to determine autolytic enzyme activity.

Large-scale culturing:

All microbial species underwent large-scale culturing. In the preparation of large-scale culture material of all microbial cultures, primary overnight cultures (1 ml) were added

to 500 ml of sterile BHI broth (Oxiod, England) in a 1L Erlenmeyer flask (final concentration was 0.2% (vol/vol). Flasks were incubated (Stuart, orbital incubator, SI500) for 24 hours at 25°C for *M. luteus* or 37°C for the other microbial species, in a shaking incubator with a speed of 220 revolutions per minute (rpm).

Preparation of crude plant extracts:

All plant species received from the University of Namibia, was prepared for experimentation. Crude methanolic and ethanolic extracts was prepared by adding 10g of plant material from the roots and leaves to either 100 ml methanol or 100 ml ethanol respectively (Final concentration of 10g/ml). Flasks containing the extracts were kept for 3 days at room temperature with occasional swirling. Following the 3 days maceration contents of the flasks were filtered through Whatman 110 mm filter paper. The solvents were evaporated in a rotary evaporator using round bottom flasks at reduced pressure and at 45°C. Extracts were frozen at -70°C and further dried in a Christ Alpha 2LD plus freeze-drier. After freeze drying, samples were kept at -20˚C until further use. Before use, extracts were dissolved in dimethyl sulfoxide (DMSO).

SDS-PAGE:

After all the preparation was done, the whole cell protein profile was first tested to visualize the total protein extracted from the cell via SDS-PAGE. The first set of experimentation was done in the absence of the plant extracts. Later, the experiments were repeated in the presence of the plant extracts and any differences were noted. All microbial species and plant species were used in the initial testing phase. Based on the results obtained, only selected species were continued with as seen in the results section.

The SDS-PAGE gel preparation was performed as described by Brunelle *et al* (2014) and Berber *et al* (2003) (62,65) using the Laemmli buffer system (1970) (124) with minor modifications. The separating and staking gel components were prepared as described in Table 2. SDS-PAGE sample preparations were done described by I Berber *et al* (65). The electrode buffer contained 0.025M Tris, 0.192M glycine and 0.1% SDS at a pH of 8.5. Polyacrylamide gels at a 12% concentration were prepared. The separating gel contained 1.875 M Tris-HCl pH 8.8 [19.90%], 30% Acrylamide [41.39%], 10% SDS [1%], 10% APS [1%] and TEMED [0.10%]. The stacking gel contained 0.6 M Tris-HCl pH 6.8 [9.93%], 30% acrylamide [1.34%], 10% SDS [0.99%],

10% APS [0.99%] and TEMED [0.20%]. The sample loading buffer contained 0.125 M Tris hydrochloride (pH6.8), 4% (wt/vol) SDS, 0.02% (wt/vol) bromophenol blue, 0.2 M dithiothreitol (DTT), and 20% (vol/vol) glycerol. Samples were solubilized in sample loading buffer in a boiling water bath for 5 minutes prior to loading. Pre-stained protein ladder (PageRuler prestained protein ladder, ThermoScienfific, Lithuania) of 5 µl was included in the gel ranging from 10 to 180 kDa. Electrophoresis occurred at 15mA per gel, until the tracker dye ran off the gel. Following electrophoresis, gels were stained for 30 minutes with Coomassie stain (0.1% Coomassie brilliant blue R250, BioRad, USA, in 50% (vol/vol) methanol, 10% (vol/vol) glacial acetic acid) and destained overnight with 10% vol/vol methanol and 7% vol/vol glacial acetic acid. The gel was visualized with a molecular imager (BioRad, molecular imager, Gel doc™ XR+).

Table 2: Composition of SDS-PAGE gels used in this study. Table outlines both the components of the separating and stacking gel.

Separating gel (12.5%):	Volumes (ml):	Stacking gel (6%):	Volume (ml):
1.875 M Tris-HCl pH 8.8	2	0.6 M Tris-HCl pH 6.8	0.5
Sterile Water	3.68	Sterile Water	3.75
Stock acrylamide (30%)	4.16	Stock acrylamide (30%)	0.675
10% SDS	0.1	10% SDS	0.05
10% APS	0.1	10% APS	0.05
TEMED	0.01	TEMED	0.01

HCl = Hydrochloric acid. SDS = Sodium dodecyl sulfate. APS = Ammonium persulfate. TEMED = tetramethylethylenediamine. % = percentage.

Extraction of crude autolytic enzymes:

To test the autolytic activity, the autolytic enzymes were extracted as described below. The experiments were conducted in the absence of the plant extracts and repeated in the presence of the plant extracts. Any differences were noted. All microbial species and plant species were used in the initial testing phase. Based on the results obtained, only selected species were continued with as seen in the results section.

Small-scale extraction:

Small-scale extraction was performed as described by Antignac *et al* (2007) (14) with a few modifications. All microbial species and plant species were used in the initial

testing phase. Based on the results obtained, only selected species were continued with as seen in the results section. Cultures were inoculated from the plate into 20 ml BHI broth and incubated (Stuart, orbital incubator, SI500) overnight at 37°C while shaking (220 rpm) and grown to an OD_{600} of 1. After incubation, the samples were centrifuged (Eppendorf, MiniSpin plus, Sigma-Aldrich, USA) for 5 minutes at 10 000xg and the resulting pellets resuspended in 50 mM Tris-HCl (pH 7.5) (Melford, UK) and 150 mM NaCl (Merck, SA). After a second centrifugation for 1 minute at 10 000xg, pellets were mixed either in 3 M LiCl (Merck, SA), 0.1% Triton X-100 (Sigma-Aldrich, USA) for LiCl extraction of autolysins, or in 4% (wt/vol) SDS (Melford, UK) for SDS extraction of autolysins. Samples extracted with SDS were left for 30 min at room temperature and LiCl extracted pellets were left at 4°C. Following centrifugation after incubation period, the supernatants containing extracted autolytic enzymes were transferred to Eppendorf tubes and stored at -20°C until further use.

The above mention method was modified and repeated with 5 M LiCl and 4% SDS. Incubation was extended for 2 hours at room temperature for SDS samples and 4°C for LiCl samples with gentle agitation to increase the yield of autolytic enzymes.

Large-scale extraction:

Large-scale extraction was preformed from 500 ml cultures using 5 M LiCl and 4% SDS respectively, dissolved in a renaturation buffer (20 mM Tris, 50 mM NaCl, 20 mM MgCl, 0.5% Triton-X100, pH 7.4 [Sigma-Aldrich, USA]). All microbial species and plant species were used in the initial testing phase. Based on the results obtained, only selected species were continued with as seen in the results section. Briefly, cultures were centrifuged (Heraeus Megafuge 40 Centrifuge, Thermofisher Scientific) at 3488xg for 10 minutes. The resulting pellets were resuspended in 5 M LiCl-renaturation mixture or 4% SDS-renaturation mixture (5 ml) in 15 ml Falcon tubes (Cellstar tubes, Greiner bio-one, Austria). The samples were incubated with gentle agitation (Heto Rotamix, UK) for 24 hours at 4°C for LiCl samples or room temperature for SDS samples. Following centrifugation after the 24-hour incubation period, the supernatants containing extracted autolytic enzymes were transferred to Eppendorf tubes and stored at -20°C until further use.

Preparation of cell wall substrates for autolytic enzyme analysis:

To visualize the autolytic activity, zymography technique was used. For this reason, the cell wall substrate was prepared as described below and used in the zymogram. All microbial species and plant species were used in the initial testing phase. Based on the results obtained, only selected species were continued with as seen in the results section.

Small-scale preparation:

Cell walls of *M. luteus* were prepared from overnight cultures and grown in 20 ml BHI-broth (Oxiod, England) at 37°C with shaking (220 rpm) to an OD_{600} of approximately 0.8. Cells were pelleted at 7700xg for 5 minutes at 4°C (Beckman Avanti™ J-25 centrifuge, JLA 10.500) and resuspended in 3 ml of ddH_2O. The suspensions were placed in a boiling water bath for 30 minutes and centrifuged at 7700xg for 5 minutes. The pellets were washed twice with 50 mM phosphate buffer (pH 7.5), centrifuged at 7700xg for 5 min and resuspended in 1 ml phosphate buffer (1x PBS)(0.8% wt/vol NaCl, MERCK, SA; 0.02% wt/vol KCl, MERCK, Germany; 0.144% wt/vol Na_2HPO_4, MERCK, Germany; 0.024% wt/vol KH_2PO_4, Sigma, USA). This served as the cell wall substrate in zymogram analysis and micro-plate bioassay of the autolytic enzymes.

Large-scale preparation:

Cell walls of *M. luteus* were prepared from overnight cultures and grown in 500 ml BHI-broth (Oxiod, England) at 37°C with shaking (220 rpm) to an OD_{600} of approximately 0.8. Cells were pelleted at 3488xg for 10 minutes at 4°C (Heraeus Megafuge 40 Centrifuge, Thermofisher Scientific) and resuspended in 5 ml of ddH_2O. The suspensions were placed in a boiling water bath for 30 minutes and centrifuged at 7700xg for 5 minutes (Beckman Avanti™ J-25 centrifuge, JLA 10.500). The pellets were washed twice with 50 mM phosphate buffer (pH 7.5), centrifuged at 7700xg for 5 min and resuspended in 3 ml phosphate buffer (1x PBS)(0.8% wt/vol NaCl, MERCK, SA; 0.02% wt/vol KCl, MERCK, Germany; 0.144% wt/vol Na_2HPO_4, MERCK, Germany; 0.024% wt/vol KH_2PO_4, Sigma, USA). This served as the cell wall substrate in zymogram analysis and micro plate bioassay of autolytic enzymes.

Analysis of autolytic enzyme extracts using zymography:

To visualize the autolytic activity on a gel, zymography technique was used. All microbial species and plant species were used in the initial testing phase. Based on

the results obtained, only selected species were continued with as seen in the results section.

Renaturing SDS-PAGE or Zymography was performed according to the method described by Sugai *et al* (1990) (125) with a few modifications. Polyacrylamide SDS gels (12%) containing 500 µl of the crude cell wall substrate, specific to the strain been analysed, was used for the detection of lytic activity. Samples were mixed 1:1 (vol/vol) with sample buffer (2x SDS) (40% Glycerol, 240 mM Tris/HCl pH 6.8, 8% SDS, 0.04% bromophenol blue, 5% beta-mercaptoethanol, 2x dddH2O). Samples were heated for 5 minutes in a boiling water bath prior to loading on the gel. After electrophoresis, gels were soaked for 2 hours in 250 ml dddH2O at room temperature with gentle agitation (50rpm, Stuart, orbital incubator, SI500). The dddH2O was changed every half hour. For the final half hour, the gels were soaked in 250 ml dddH2O and incubated at 37°C (Stuart, orbital incubator, SI500). After the 2-hour incubation, the gels were transferred to 150 ml of renaturation buffer (20 mM Tris, 50 mM NaCl, 20 mM MgCl, 0.5% Triton-X100, pH 7.4) and incubated for 16 hours at 37°C (Stuart, orbital incubator, SI500). Bands with lytic activity were observed as clear in the opaque gel. The gels were stained for 5 minutes in 0.1% (wt/vol) methylene blue, 0.01% Potassium hydroxide (wt/vol) and destained in dddH2O.

Detection of autolytic enzymes in total protein component:

The whole cell protein profile was tested using zymography in the same manner as described above excluding the 4% SDS and/or 5M LiCl extraction of the hydrolytic enzymes. The cell wall was prepared as above and the zymography conditions remained the same. All microbial species and plant species were used in the initial testing phase. Based on the results obtained, only selected species were continued with as seen in the results section.

Liquid Chromatography Mass Spectrometry:

In order to separate the compounds within the crude plant extract to test for possible antimicrobial compounds of sample 4.2 and 5.2, HPLC was performed. Samples 4.2 and 5.2 were prepared by dissolving 5 mg of each in acetonitrile (500 µl) : $ddd H_2O$ (500 µl) (1:1 vol/vol) respectively, giving a concentration of 5mg/ml each. To increase the solubility, samples were sonicated for a duration of 5 minutes after which they were diluted x10 (100 µl of sample in 900 µl solvent). The samples were clarified by

centrifugation for 5 minutes to remove any remaining particles. They were then further diluted x10 (100 µl of sample in 900 µl solvent) and placed into sample vials. Acetonitrile solvent was used as a blank during the HPLC process to eliminate false readings. Formic acid (0.1%) was used as a buffer to facilitate ionization. Samples were analysed via LC/MS using a Waters, Acquity UPLC Synapt G2, binary function system (pump and accumulator for steady flow) for a duration of 30 min. Analysis was performed in both the negative and positive mode and repeated 4 times. For fractionation a C18 reverse column (Kinetex 1.7 µm EVO C18 100 A, LC Column 100 x 2.1 mm, Phenomenex) was used. Data was collected using the MassLynx V4.1 (Waters) software and analysed.

Bioassay for the detection of autolytic activity:

In order to test the autolytic activity in the presence of the plant extracts, the autolytic activity was tested in the absence of the plant extracts. By comparing the results, the activity was determined. A 96 well microtiter plate assay was developed for the detection of autolytic activity from extracted samples. Cell walls and autolytic enzymes were prepared as described above. Reactions were prepared by adding cell wall (100 µl) and LiCl extracted enzymes (100 µl) or cell wall (100 µl) and SDS extracted enzymes (100 µl) respectively. The control consisted of cell wall (100 µl) and phosphate buffer (100 µl). Phosphate buffer of 200 µl was used as the plate blank. All reactions including the controls were done in triplicate (n=3, biological repeats of 3). Plates were incubated at 37^0C for 30min with absorbance readings taken every 3 min at an OD600 (Molecular Devices, SpectraMax, Paradigm, Separations, SA).

Influence of plant extracts on the autolytic activity:

Based on the results obtained of the autolytic activity in the absence of the plant extracts, the assay was modified. The autolytic activity of 4% SDS extracted autolysins in the presence of 4.2 and 5.2 plant extracts was tested using the bioassay described above with minor changes. Cell wall (15 µl, OD_{600} = 0.5), 4% SDS extracted enzymes (20 µl) and phosphate buffer (185 µl) were added to the wells of a microtiter plate. The control consisted of cell wall (15 µl) and phosphate buffer (205 µl). Phosphate buffer of 220 µl was used as the blank. Plates were incubated at 37°C for 30min with absorbance readings taken every 3 min at an OD_{600} (Molecular Devices, SpectraMax, Paradigm, Separations, SA).

Minimum Inhibitory Concentration (MIC) / Double Dilution Assay:

MIC was preformed to determine the minimal inhibition concentration needed for the inhibition of the microbial species. MIC was performed according to the method described by Sultanbawa e*t al.* (2009) (3) with a few modifications. Wells of the 96-well microtiter U-bottom plate (Greinet Bio-one, Cellstar) was filled with BHI (150 µl) (Oxiod, England), overnight culture (2 µl) and plant extract (10 µl). A 6x doubling dilution series was made with phosphate buffer (1x PBS) (10 µl) (0.8% wt/vol NaCl, MERCK, SA; 0.02% wt/vol KCl, MERCK, Germany; 0.144% wt/vol Na_2HPO_4, MERCK, Germany; 0.024% wt/vol KH_2PO_4, Sigma, USA) and plant extract (10 µl). The control consisted of BHI (162 µl) only. Plates were incubated (Stuart, orbital incubator, SI500) overnight at 37°C and analysed for visible microbial growth.

Dilution activity assay:

Based on MIC results, the method was repeated with a few modifications. All microbial species and plant species were used in the initial testing phase. Based on the results obtained, only selected species were continued with as seen in the results section. Wells of the 96-well microtiter F-bottom plate (Greinet Bio-one, Cellstar) was filled with BHI (150 µl) (Oxiod, England), overnight microbial species (2 µl) and plant extract (10 µl). A 1x doubling dilution series was made with phosphate buffer (1x PBS) (10 µl) (0.8% wt/vol NaCl, MERCK, SA; 0.02% wt/vol KCl, MERCK, Germany; 0.144% wt/vol Na2HPO4, MERCK, Germany; 0.024% wt/vol KH2PO4, Sigma, USA) and plant extract (10 µl). The absorbance (OD_{600}) was read (Molecular Devices, SpectraMax, Paradigm, Separations, SA) every hour for a duration of six hours. Plates were incubated (Stuart, orbital incubator, SI500) at 37°C between readings. The controls consisted out of the following: Control 1: BHI and Species, Control 2: No dilution of each extract and BHI, Control 3: No dilution of each extract, BHI and Species, Control 4: Half dilution of each extract and BHI. The difference in controls were designed to exclude external factors and only result in the activity caused in the presence of the extract.

Antimicrobial assay (Spot-on-lawn assay):

The visualize inhibition caused by the plant extracts, A spot-on-lawn assay was performed. Only *S. aureus* species were used based on the results obtained in previous experiments. The antimicrobial activity of the plant extracts was screened

using the spot-on-lawn method (Saguibo 2012, Mukakalisa 2015) with a few modifications. BHI containing petri plates stood for one hour after which 10 µl of plant extracts were spotted onto the plates. Plates were then incubated for 24 hours at 37ºC (Stuart, orbital incubator, SI500). Plates were observed for zones of inhibition.

Bicinchoninic acid (BCA) protein assay:

BCA assay was used to determine the protein concentration. The BCA assay was performed according to the manufacturer's instructions (Pierce BCA Protein Assay Thermo Scientific). Briefly, standard was prepared according to the table provided below (Table 3). The working reagent (WR) was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A: B). A Standard or unknown sample (25 µl) was added to a 96 well micro-titre F-bottom plate (Greinet Bio-one, Cellstar) in triplicate (n=3, triplicate biological repeat). WR (200 µl) was added to each well and the plate was mix for 30 seconds gently by hand. The plate was covered with foil and incubated at 37°C for 30 minutes (Stuart, orbital incubator, SI500). The plate was cooled to room temperature. The absorbance (OD_{562}) was read (Molecular Devices, SpectraMax, Paradigm, Separations, SA).

Table 3: Preparation of diluted Albumin (BSA) standards.

Dilution Scheme for Standard Test Tube Protocol and Microplate Procedure (Working Range = 20-2,000µg/mL)

Vial	Volume of Diluent (µL)	Volume and Source of BSA (µL)	Final BSA Concentration (µg/mL)
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	325	325 of Stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0 = Blank

Preparation of biofilms:

In order to gain access into the cell, the biofilm which is a protection mechanism of bacteria, must be destroyed (83,85,86,126). Only *S. aureus* species were used to study biofilm eradication based on the results obtained from previous experiments.

The biofilm assay was used as described by Stepanović *et al* (2000) (127) with modifications. BHI-broth (Oxiod, England) (250 µl) was added to the wells of a sterile microtiter plate (Cellstar, Greiner bio-one, Germany). Wells were inoculated with 25 µl

of an overnight culture, incubated (Stuart, orbital incubator, SI500) for 48 hours until it reached an OD_{600} of between 1.4 – 1.8. Wells were emptied and washed with 200 µl sterile 1x PBS (0.8% wt/vol NaCl, MERCK, SA; 0.02% wt/vol KCl, MERCK, Germany; 0.144% wt/vol Na2HPO4, MERCK, Germany; 0.024% wt/vol KH2PO4, Sigma, USA) to remove the un-adhered planktonic cells. The process was repeated twice. The remaining biofilm was heat-fixed for one hour at 60°C (Labotec, EcoTherm Oven). Biofilms were then stained with, 20 µl of 1% (wt/vol) Crystal violet (Judex microscopic satin, England) and shaken briefly and further incubated for 20 minutes at room temperature. The excess crystal violet was extracted with two washes of 250 µl ethyl alcohol (70% (wt/vol)). To quantify the amount of biofilm formation, the absorbance was measured at OD_{600} nm in a spectrophotometer (Molecular Devices, SpectraMax, Paradigm, Separations, SA). Wells that served as negative controls contained BHI-broth only and was processed in the same manner as the test wells. Each test was done in triplicate and the experiment repeated twice.

Influence of plant extracts on biofilm formation:

The assay was performed as described by Stepanović *et al* (2000) (127) with modifications. To the well of a sterile 96-well plate (Cellstar, Greiner bio-one, Germany), BHI-broth (200 µl) (Oxiod, England) and 100 µl of plant extract (5%, 2.5% and 1% concentrations) was added. Test culture (25 µl) was added. Positive control contained the culture in BHI-broth without plant extract. The negative control contained BHI-broth and the plant extract without culture. The samples were incubated (Stuart, orbital incubator, SI500) at 37 °C for 48 hours. The content of each well was removed by gentle overturning the microtiter plate on sterile paper towels. The wells were washed with 250 µl of tripled distilled water ($dddH_2O$) to remove free-floating bacteria. Biofilms formed by adherent cells in the plate was stained with 0.1% (wt/vol) Crystal violet (Judex microscopic satin, England) and the plate contents were incubated at the room temperature for 30 minutes. Excess stain was rinsed off by washing with tripled distilled water ($dddH_2O$). Plates were washed with 200 µl of 70% ethanol. To quantify the amount of biofilm formation, the absorbance was measured at OD_{600} nm in a spectrophotometer (Molecular Devices, SpectraMax, Paradigm, Separations, SA).

Chapter 3:

Results.

Results:

The results in this section was obtained from the experiments done to achieve the rejection or acceptance of the hypothesis. All microbial species and plant species were used unless otherwise noted in the methodology or as seen in the results.

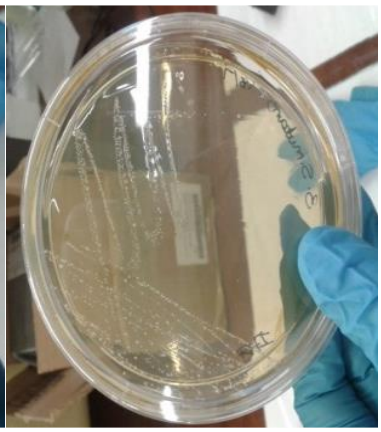
Culturing of microbial species:

For any experimentation to have taken place, the microbial species were cultures in the correct manner to insure optimal growth. The need to culture and propagate the indicator strains in this study was to be achieved in order to study the effects of the plant extracts on the bacteria. Solid cultures were prepared as working stocks during the study (Fig 3). The use of liquid was mainly for activity studies and extraction of substrate and enzyme material.

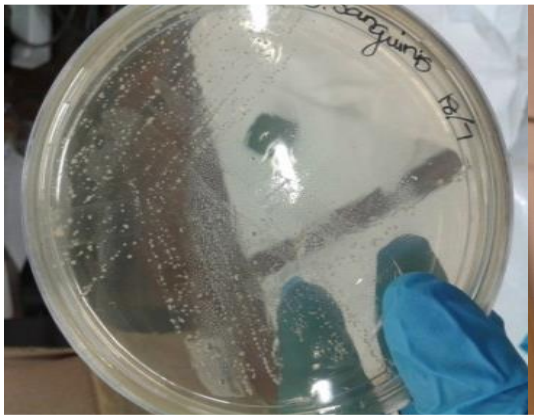
Plates:

S.aureus (12600)

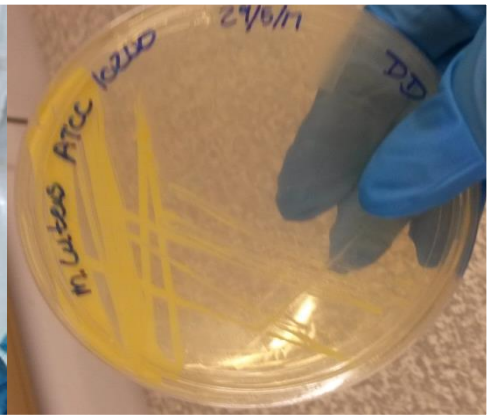
S. aureus (43300)



S. mutans



S. sanguinis



M. luteus

Figure 3: A representation of the growth of microbial species. Microbial culture growth on BHI plates. Plates were prepared under sterile conditions and incubated at 37^0C.

S. aureus (12600), S. aureus (43300), S. sanguinis and *S. mutans* showed growth. After 24 hours *M. luteus, S. aureus (12600)* and *S. aureus (43300)* showed growth and after 48 hours *S. mutans* and *S. sanguinis* showed growth. The control showed no growth. Sub-culturing was done weekly and the results were consistent. This was also the case for liquid cultures. The results show that the growth conditions were optimal for the growth of the microbial species. Streptococcus species requires longer incubation period, and this was the case for *S. mutans* and *S. sanguinis (95,128).*

Plant extracts:

All plant species received from the University of Namibia, was prepared for experimentation in order to use the crude extracts for experiments. The extracts were

used for various experiments and the results are seen in the section. A variety of extraction methods were done by the University of Namibia, Africa. Methanol and ethanol extracted roots and leaves had antimicrobial activity and these samples were sent to the University of Pretoria.

Table 4: Plant extracts name and code as received from the University of Namibia.

Plant extract:	**Code:**
Brachystelma gymnopodum	BG
Coccinea rehmannii	CR
Eulophia hereroensis	EH
Fockea angustifolia	FA
Trochomeria macrocarpa	TM
Walleria nutans	WN

Sample 4.2 and 5.2 were received as dry samples and were dissolved in DMSO resulting in a liquid solution or the dry samples were dissolved in acetonitrile for HPLC testing. In order to use the plant extracts, liquid samples were needed. For this reason, the crude extracts were prepared as described in the methods. The results showed that the extracts were in liquid form and were ready to use in the specific experiments. No contamination was visible and the conditions for storage was optimal.

SDS-PAGE for protein profile:

The aim of the SDS-PAGE was to visualise whole cell protein profile and the effects of the plant extracts on the protein profile. All microbial species and plant species were used in the initial testing phase. Based on the results obtained, only selected species were continued with for the duration of the study. The specified species and plants were named in the figure legends.

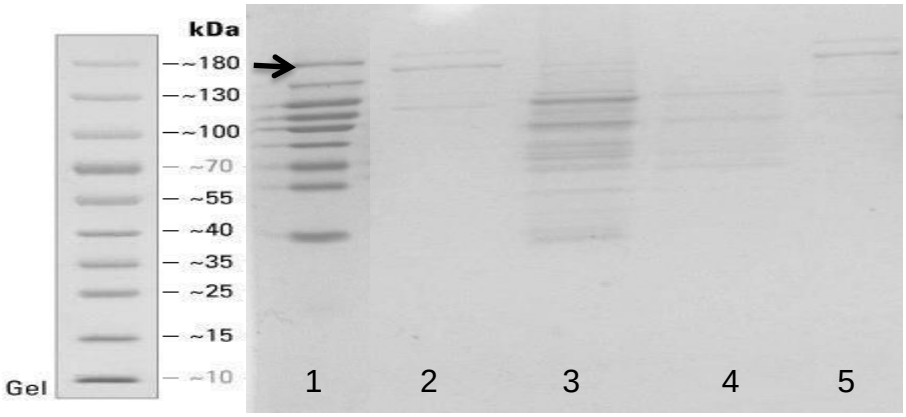


Figure 4: Whole cell protein profile of various small-scale cultured microbial species. 1) Protein marker, 2) *S. mutans, 3) S. aureus 12600, 4*) *S. aureus 43300, 5) S. sanguinis.* Samples of 10 µl from each extraction were loaded onto the gel. Gel stained in Coomassie Brilliant Blue R250. Triplicate biological repeats (n=3). Arrow represented the first protein marker band of 180 kDa used to determine size of various bands compared to the protein marker.

Lane 1 was the protein marker (PageRuler prestained protein ladder, ThermoScienfific, Lithuania). The protein marker was used to determine the size of various bands of interest. For example, the first band of the protein marker was 180 kDa. The first band in Lane 2 – *S. mutans* was roughly 180 kDa. The marker was used through this method to determine the size of a specific band when needed.

Lane 2 was *S. mutans.* There were 3 bands that was seen at ± 180, 130 and 70 kDa when compared with the protein marker. The bands were not very visible when compared to the protein marker. A possible reason was that the protein concentration after the extraction was not high resulting in the low intensity of the bands. After the experiment was repeated, the same result was displayed. Protein bands were only found at the top of the gel showing that the protein extracted from *S. mutans* were large. When compared to lane 3 – *S. aureus (12600)*, *S. mutans* had less bands. This could have been as a result of the lower protein concentration after the extraction process. When compared to lane 4 – *S. aureus (43300), S. mutans* also had less bands. This also could have been as a result of the lower protein concentration after the extraction process. When compared to lane 5 - *S. sanguinis, S. mutans* showed similar results. Both *S. mutans* and *S. sanguinis* only showed bands on the top of the

gel due to the size of the proteins. *S. mutans* and *S. sanguinis* showed similar trends which was to be expected as they are both Streptococcus species.
Lane 3 was *S. aureus (12600)*. There were several faint bands and 2 bright bands at ± 100 and 50 kDa when compared to the protein ladder. When compared to lane 3 – *S. mutans, S. aureus (12600)* had various bands present at higher intensity. The protein concentration after the extraction of *S. aureus (12600)* was more than *S. mutans* resulting in the higher intensity. When compared to lane 4 – *S. aureus (43300)*, *S. aureus (12600)* showed more band with higher intensity. This could have been as a result of the protein concentration differences after the extraction. Both Staphylococcus species were expected to show the same trend. This was not the case as *S. aureus (12600)* showed more bands at higher intensity. Protein concentration differences could have been the main reason for the unexpected result. When compared to lane 5 – *S. sanguinis, S. aureus (12600)* had more bands with higher intensity. This shows the same trend as with *S. mutans*.

Lane 4 was *S. aureus (43300)*. There were several faint bands and 2 brighter bands at ± 100 and 50 kDa when compared to the protein ladder. Comparisons with lane 2 and 3 was already discussed above. When compared to lane 5 – *S. sanguinis, S. aureus (43300)* showed more bands, but also had low intensity. Size and protein concentration could have been the reason for the differences. Lane 5 was *S. sanguinis.* There were 3 bands that can be seen at ± 180, 130 and 70 kDa when compared with the protein marker. Comparisons with the other species were discussed above. Based on the results obtained in the whole cell protein profile of various small-scale cultured microbial species, the experiments were repeated with large-scale cultures microbial species and large-scale extracted proteins.

To determine autolytic enzyme activity on microbial species, autolytic enzymes were extracted with the use of LiCl and SDS. SDS-PAGE was performed to determine the protein profile of the autolytic enzymes after the extraction process. This was used to determine which extracted method yielded better results for the continuation of the experiments. Some autolytic enzymes are anchored proteins, thus extraction methods were preformed to detach the autolytic enzymes (57,58).

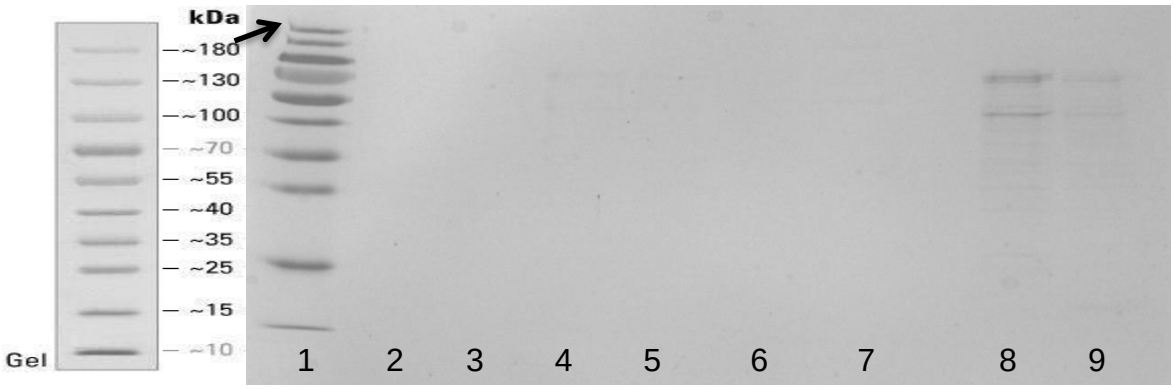


Figure 5: Small-scale extracted proteins - SDS-PAGE gel with LiCl extracted and SDS extracted enzymes. Lane 1) Protein marker, 2) 5M LiCl extracted *S. aureus (12600)*, 3) 5M LiCl extracted *S. aureus (43300)*, 4) 5M LiCl extracted *S. mutans*, 5) 5M LiCl extracted *S. sanguinis.* 6) 4% SDS extracted *S. aureus (12600)*, 7) 4% SDS extracted *S. aureus (43300)*, 8) 4% SDS extracted *S. mutans*, 9) 4% SDS extracted *S. sanguinis.* Samples of 10 µl each were loaded onto the gel. Gel stained in Coomassie Brilliant Blue R250. Biological repeats of 5 was done for this experiment (n=5). Arrow represented the first protein marker band of 180 kDa used to determine size of various bands compared to the protein marker.

The only bands that were visible, were in Lane 1, 8 and 9. Lane 1 was the protein marker (PageRuler prestained protein ladder, ThermoScienfific, Lithuania). The protein marker was used to determine the size of various bands of interest. For example, the first band of the protein marker was 180 kDa. The first visible band in lane 8 – 4% SDS extracted *S. mutans,* was roughly 100 kDa. The marker was used through this method to determine the size of a specific band when needed. Lane 2-5 were the LiCl extracted enzymes. No bands were visible in these lanes. A possible reason could have been that the protein concentration was extremely low or the LiCl didn't extract autolytic enzymes. Lane 6 and 7 were 4% SDS extracted microbial species. Again, no bands were visible. A possible reason could have been that the protein concentration was extremely low or the 4% SDS didn't extract autolytic enzymes from *S. aureus (12600)* or *S. aureus (43300).*

Lane 8 was 4% SDS extracted *S. mutans* enzymes. Two bands were visible with higher intensities and other bands were faintly visible. The intensified bands were roughly 100 kDa and 70 kDa. This showed that 4% SDS did extract autolytic enzymes and the concentration of the extracted proteins were enough to be visible on the gel.

Lane 9 was 4% SDS extracted *S. sanguinis* enzymes. Two bands were also visible with higher intensities and other bands were faintly visible. The intensified bands were roughly 100 kDa and 70 kDa. This showed that 4% SDS did extract autolytic enzymes and the concentration of the extracted proteins were enough to be visible on the gel. *S. mutans* and *S. sanguinis* showed the same trends with the 4% SDS extraction method.

Based on the results obtained, large-scale extraction was first done before either of the methods were discarded. The concentration of the proteins was improved by culturing the microbial species in large-scale. The experiments were repeated and were discussed below.

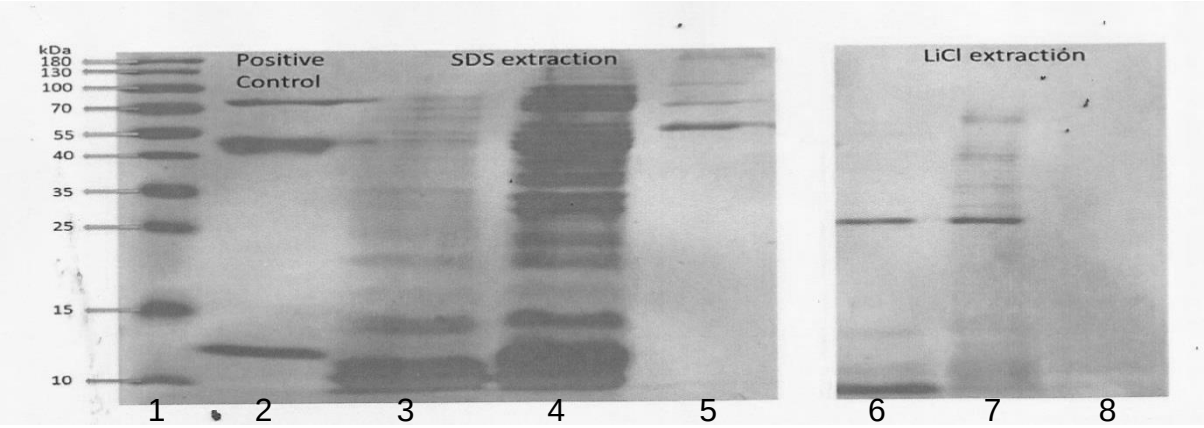


Figure 6: Large-scale extracted proteins - SDS-PAGE gel with LiCl extracted and SDS extracted enzymes. Lane 1) Protein marker, 2) Crude extracted lysozymes used as a positive control, 3) 4% SDS extracted *S. aureus (12600)*, 4) 4% SDS extracted *S. aureus (43300)*, 5) 4% SDS extracted *S. mutans***,** 6) 5M LiCl extracted *S. aureus (12600)*, 7) 5M LiCl extracted *S. aureus (43300)*, 8) 5M LiCl extracted *S. mutans*. Samples of 10 µl each were loaded onto the gel. Gel stained in Coomassie Brilliant Blue R250. Biological repeats of 3 was done for this experiment (n=3).

Lane 1 was the protein marker (PageRuler prestained protein ladder, ThermoScienfific, Lithuania). The protein marker was used to determine the size of various bands of interest. For example, the first band of the protein marker was 180 kDa. The first visible band in lane 2 – crude extracted lysozyme, was roughly 80 kDa. The marker was used through this method to determine the size of a specific band when needed. Lane 2 was crude extracted lysozyme. There were 3 bands visible at ± 80, 50 and 12 kDa when compared with the protein marker. Lane 3 was 4% SDS

extracted *S. aureus (12600)*. There were several bands visible and 3 bright bands at ± 11 – 10 kDa when compared to the protein ladder. Lane 4 was 4% SDS extracted *S. aureus 43300*. Various bands were visible in the gel. *S. aureus (12600)* and *S. aureus (43300)* showed similar trends which was expected as both species were Staphylococcus species. Lane 5 was 4% SDS extracted *S. mutans*. There were 4 bands visible with a bright band at ± 55 kDa. When compared to both *S. aureus* species, *S. mutans* had less bands but still with a high intensity. The 4% SDS extraction method only extracted there 4 proteins from *S. mutans.* Lane 6 was 5M LiCl extracted *S. aureus 12600*. There were 2 bands visible at ± 8 kDa and 25 kDa. When compared to 4% SDS extracted *S. aureus (12600),* the LiCl extracted *S. aureus* was less. Lane 7 was 5M LiCl extracted *S. aureus 43300.* A few bands were visible with a bright band at ± 25 kDa. When compared to 4% SDS extracted *S. aureus (43300),* the LiCl extracted *S. aureus* was less. Lane 8 was 5M LiCl extracted *S. mutans*. No bands were visible. When compared to 4% SDS extracted *S. mutans,* the LiCl extracted *S. mutans* was nothing.

Based on the results obtained, the 4% SDS extraction method was continued with for the duration of the study. The LiCl extracted method didn't extract adequate amounts of enzymes and produced lower yields when compared with the 4% SDS extraction method.

Autolytic extraction (Zymogram):

The aim of the zymogram was to visualise autolytic activity and the effects of the plant extracts on autolytic activity. All microbial species and plant species were used in the initial testing phase. Based on the results obtained, only selected species were continued with for the duration of the study. The specified species and plants were named in the figure legends.

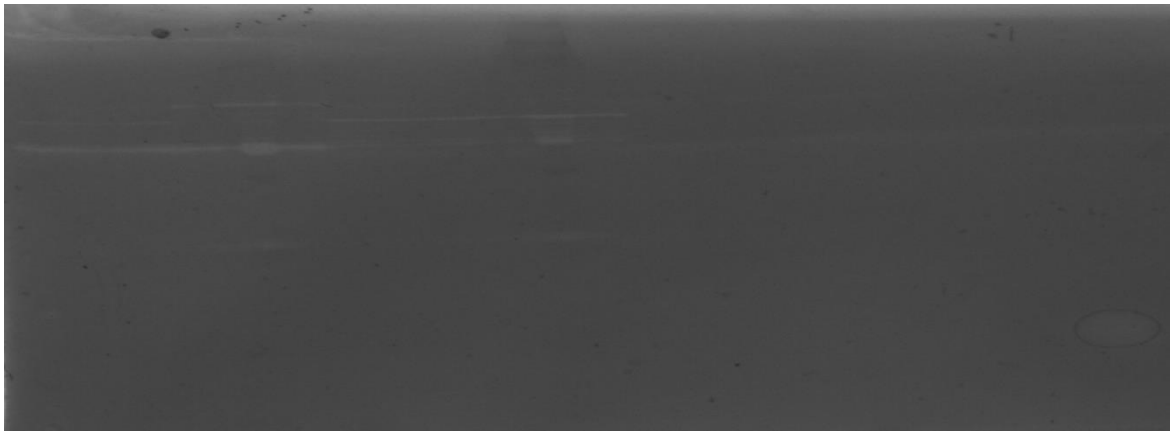


Figure 7: Small-scale extracted enzymes - Zymogram. Lane 1) LiCl extracted enzymes of *S. aureus (12600)*. Lane2) SDS extracted enzymes of *S. aureus (12600)*. Lane 3) LiCl extracted enzymes of *S. aureus (43300)*. Lane 4) SDS extracted enzymes of *S. aureus (43300)*. Lane 5) LiCl extracted enzymes of *S. mutans*. Lane 6) SDS extracted enzymes of *S. mutans*. Lane 7) LiCl extracted enzymes of *S. sanguinis.* Lane 8) SDS extracted enzymes of *S. sanguinis*. Samples of 10 µl each were loaded onto the gel. Samples of 10 µl each were loaded onto the gel. Biological repeats of 11 was done for this experiment (n=11).

All SDS extracted enzymes showed activity as seen by the clear bands on the zymogram, whereas the LiCl extracted enzymes did not as no bands were present. This suggested that the 4% SDS extracted enzymes were more active than the LiCl extracted enzymes or that only the 4% SDS extraction process extracted enzymes. This trend was also seen with previous experiments where only the 4% SDS extracted enzymes showed activity. There seems to be a smear between the SDS extracted enzymes and the bands are very faint. The concentration of the autolytic enzymes after the extraction process was low which explained why the bands were faint or of low intensity.

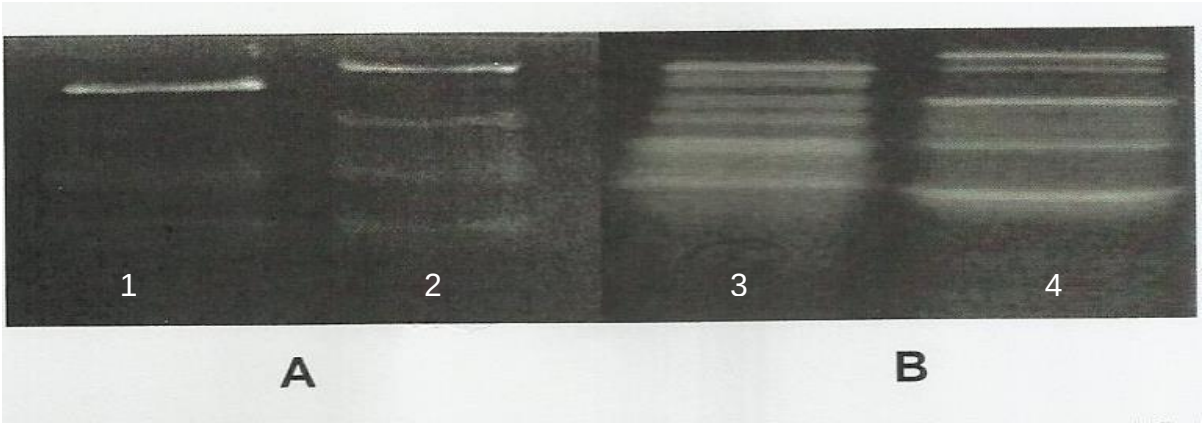


Figure 8: Large-scale 5M LiCl extracted (A) and 4% SDS extracted (B) on *M. luteus* substrate. 1) *S. aureus (12600),* 2) *S. aureus (43300),* 3) *S. aureus (12600),* 4) *S. aureus (43300).* Samples of 10 µl each were loaded onto the gel. Biological repeats of 3 was done for this experiment (n=3).

Only *S. aureus* species were chosen for this part of the experiment as only they showed antimicrobial activity as seen on the Spot-on-lawn assay of the results section below. The experiment was repeated from small-scale to large-scale as the protein yield was low in the small-scale experiments resulting in faint bands. *M. luteus* was used in this study as cell wall due to the sensitivity of peptidoglycan hydrolases of other species. Lane 1 was 5M LiCl extracted *S. aureus (12600)* on *M. luteus* substrate. Only one band was clearly visible which showed that the autolytic enzymes were active and has hydrolysed the cell wall. Lane 2 was 5M LiCl extracted *S. aureus (43300)* on *M. luteus* substrate. There were 4 bands visible on the zymogram which showed that the autolytic enzymes were active and has hydrolysed the cell wall. The first band was clearly visible. Lane 3 was 4% SDS extracted *S. aureus (12600) on M. luteus* substrate. There were several bands visible in the zymogram which showed that the autolytic enzymes were active and has hydrolysed the cell wall.

Lane 4 was 4% SDS extracted *S. aureus (43300)* on *M. luteus* substrate. There were several bands visible on the zymogram which showed that the autolytic enzymes were active and has hydrolysed the cell wall. More bands were visible with 4% SDS extraction method. This trend was seen in previous experiments as the 4% SDS extracted enzymes showed more activity. Comparing LiCl extraction method and 4% SDS extraction method on *M. luteus* as substrate, the 4% SDS extraction method was a better method and was used throughout the rest of the study.

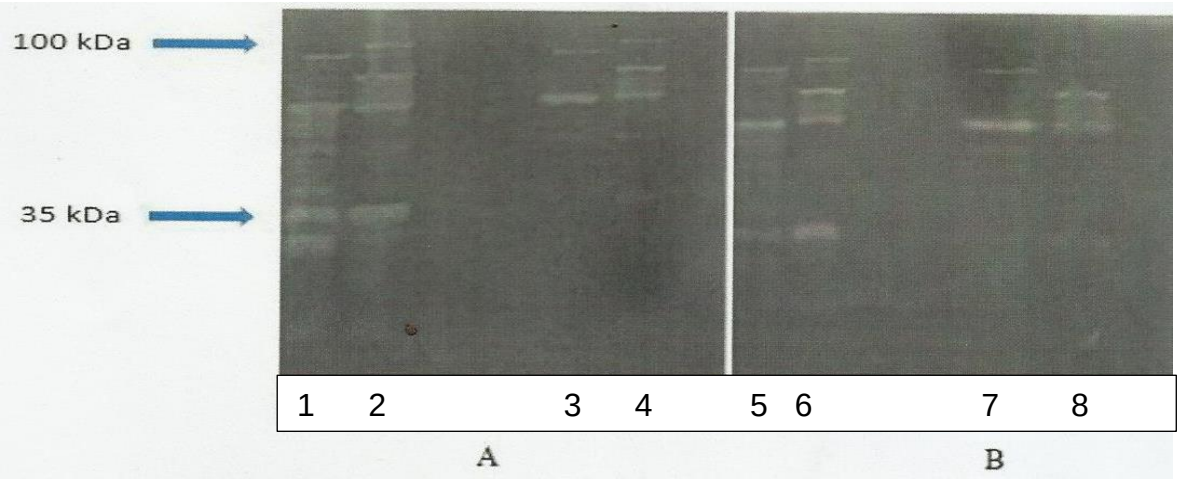


Figure 9: 5M LiCl extracted and 4% SDS extracted on natural substrate. A = Substrate of *S. aureus (43300),* B = Substrate of *S. aureus (12600).* 1) 5M LiCl extracted *S. aureus (12600),* 2) 5M LiCl extracted *S. aureus (43300),* 3) 4% SDS extracted *S. aureus (12600),* 4) 4% SDS extracted *S. aureus (43300).* 5) 5M LiCl extracted *S. aureus (12600),* 6) 5M LiCl extracted *S. aureus (43300),* 7) 4% SDS extracted *S. aureus (12600),* 8) 4% SDS extracted *S. aureus (43300).* Samples of 10 µl each were loaded onto the gel. Biological repeats of 3 was done for this experiment (n=3).

Lane 1 was 5M LiCl extracted *S. aureus (12600)* on *S. aureus (43300)* substrate. Several bands were visible on the zymogram. This showed that there was autolytic activity although it was not the natural substrate. Lane 2 was 5M LiCl extracted *S. aureus (43300)* on *S. aureus (43300)* substrate. Few bands were visible on the zymogram. Comparing lane 1 and 2 showed that with 5M LiCl extraction method, more autolytic activity was present on alternative substrate and not natural substrate. This was unexpected as the substrate was not the natural substrate, thus less activity was expected. Lane 3 was 4% SDS extracted *S. aureus (12600)* on *S. aureus (43300)* substrate. Only 2 bands were visible on the zymogram at ± 100 kDa and ± 70 kDa. This showed that there was autolytic activity although it was not the natural substrate. Lane 4 was 4% SDS extracted *S. aureus (43300)* on *S. aureus (43300)* substrate. There were 3 bands that were clearly visible on the zymogram. This showed that there was autolytic activity. Comparing lane 3 and 4 showed that 4% SDS extraction was better with natural substrate. Comparing the LiCl and 4% SDS extraction methods on the substrate *S. aureus (43300)*, 5M LiCl extracted enzymes showed more activity.

Lane 5 was 5M LiCl extracted *S. aureus (12600)* on *S. aureus (12600)* substrate. There are 3 bands that are clearly visible on the zymogram. This showed that there was autolytic activity. Lane 6 was 5M LiCl extracted *S. aureus (43300)* on *S. aureus (12600)* substrate. There are 4 bands that are clearly visible on the zymogram. This showed that there was autolytic activity although it was not the natural substrate. Comparing lane 5 and 6 showed that with 5M LiCl extraction method, more autolytic activity was present on natural substrate. This was expected as the substrate was natural substrate, thus more activity was expected. Lane 7 was 4% SDS extracted *S. aureus (12600)* on *S. aureus (12600)* substrate. Only 2 bands are clearly visible on the zymogram. This showed that there was autolytic activity. Lane 8 was 4% SDS extracted *S. aureus (43300)* on *S. aureus (12600*) substrate. There were 2 faint bands on the zymogram. This showed that there was autolytic activity although it was not the natural substrate. Comparing lane 7 and 8 showed that 4% SDS extraction was better with natural substrate. Comparing the LiCl and 4% SDS extraction methods on the substrate *S. aureus (12600),* 5M LiCl extracted enzymes showed more activity.

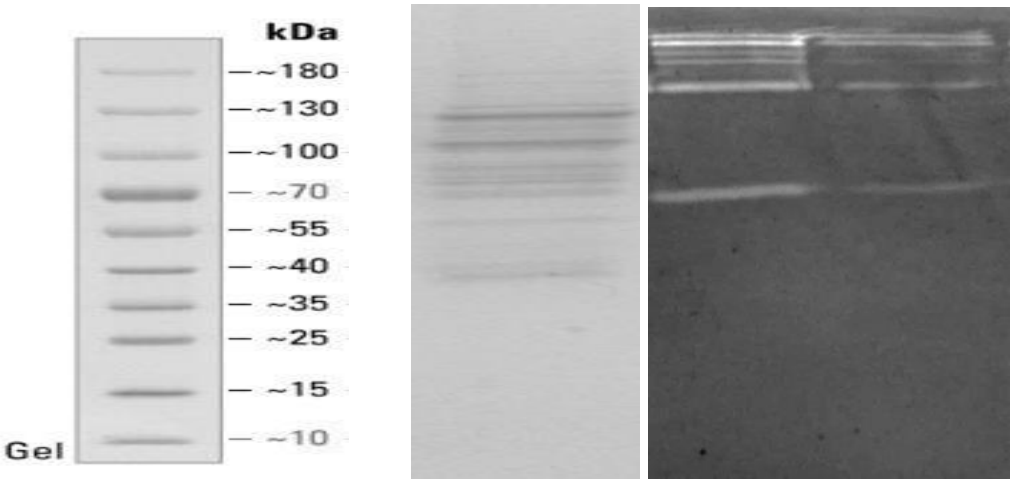


Figure 10: Autolytic activity in the presence of selected plant extract. 1) Prestained protein marker. 2) SDS-PAGE of protein profile of *S. aureus (12600).* 3) Autolytic activity via zymogram in the presence of plant extract on *S. aureus (12600). 4)* Autolytic activity via zymogram on *S. aureus (12600)* in the absence of the plant extract. Sample loaded (10 µl). Biological repeat of 3 (n=3).

Lane 1 was the prestained protein marker (PageRuler prestained protein ladder, ThermoScienfific, Lithuania). The protein marker was used to determine the size of

various bands of interest. Lane 2 was the SDS-PAGE gel of *S. aureus (12600).* Several bands are visible on the gel. Lane 3 showed the autolytic activity in the presence of plant extracts on *S. aureus (12600)*. Lane 4 showed the autolytic activity on *S. aureus (12600)* in the absence of the plant extract. More bands were visible in the presence of the selected plant extract suggesting the that plant extract increased autolytic activity.

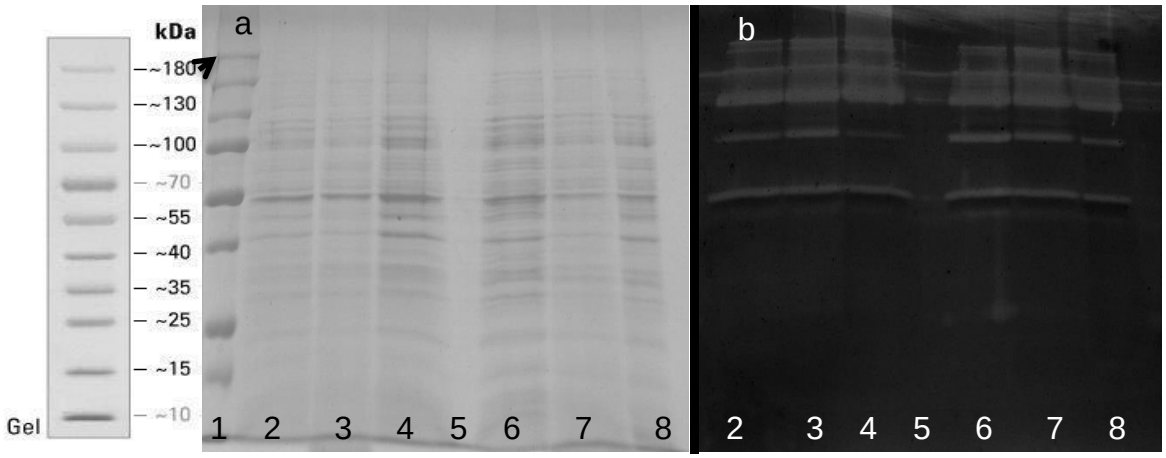


Figure 11: The whole cell protein profile in the presence and absence of plant extract 4.2 and 5.2. a) SDS – PAGE and b) Zymogram of the whole cell protein profile in the presence and absence of extract 4.2 and extract 5.2. Lane 1) Prestained protein marker. Lane 2) *S. aureus (12600)* in the absence of extract 4.2 and extract 5.2. Lane 3) *S. aureus (12600)* in the presence of extract 4.2. Lane 4) *S. aureus (12600)* in the presence of extract 5.2. Lane 5) Open lane to differentiate between *S. aureus* strains. Lane 6) *S. aureus (43300)* in the absence of extract 4.2 and extract 5.2. Lane 7) *S. aureus (43300)* in the presence of extract 4.2. Lane 8) *S. aureus (43300)* in the presence of extract 5.2. Sample loaded of 5 µl for protein marker and 10 µl for tested samples. Biological repeats of 3 (n=3). Arrow represented the 180 kDa mark of the protein marker.

The whole cell protein profile in the presence of plant extract 4.2 and 5.2 was seen above. Lane 1 was the prestained protein marker (PageRuler prestained protein ladder, ThermoScienfific, Lithuania). The protein marker was used to determine the size of various bands of interest. Lane 2a showed the protein profile of *S. aureus (12600)* in the absence of extract 4.2 and extract 5.2 whereas 2b showed the autolytic activity of *S. aureus (12600)* in the absence of extract 4.2 and extract 5.2. Many bands were seen in the SDS-PAGE gel (a) with thicker band ranging from 40 – 55 kDa. Many

bands were also seen in the zymogram (b). Lane 3a showed *S. aureus (12600)* in the presence of extract 4.2 whereas 3b showed the autolytic activity of *S. aureus (12600)* in the presence of extract 4.2. Many bands were seen in the SDS-PAGE gel (a) with thicker band ranging from 40 – 55 kDa. Many bands were also seen in the zymogram (b). Lane 4a showed *S. aureus (12600)* in the presence of extract 5.2 whereas 4b showed the autolytic activity of *S. aureus (12600)* in the presence of extract 5.2. Many bands were seen in the SDS-PAGE gel (a) with thicker band ranging from 40 – 70 kDa. Many bands were also seen in the zymogram (b). More bands were seen in the presence of extract 5.2. Lane 5 was an open lane to differentiate between *S. aureus* strains.

Lane 6a showed *S. aureus (43300)* in the absence of extract 4.2 and 5.2 whereas 6b shows the autolytic activity of *S. aureus (43300)* in the absence of extract 4.2 and 5.2. Many bands were seen in the SDS-PAGE gel (a) with thicker band ranging from 40 – 55 kDa. Many bands were also seen in the zymogram (b). Lane 7a showed *S. aureus (43300)* in the presence of extract 4.2 whereas 7b showed the autolytic activity of *S. aureus (43300)* in the presence of extract 4.2. Many bands were seen in the SDS-PAGE gel (a) with thicker band ranging from 40 – 55 kDa. Many bands were also seen in the zymogram (b). Lane 8a showed *S. aureus (43300)* in the presence of extract 5.2 whereas 8b showed the autolytic activity of *S. aureus (43300)* in the presence of extract 5.2. Many bands were seen in the SDS gel (a) with thicker band ranging from 40 – 70 kDa. Many bands were also seen in the zymogram (b). More bands were seen in the presence of extract 5.2 suggesting that plant extract 5.2 had autolytic activity. No extraction method was used.

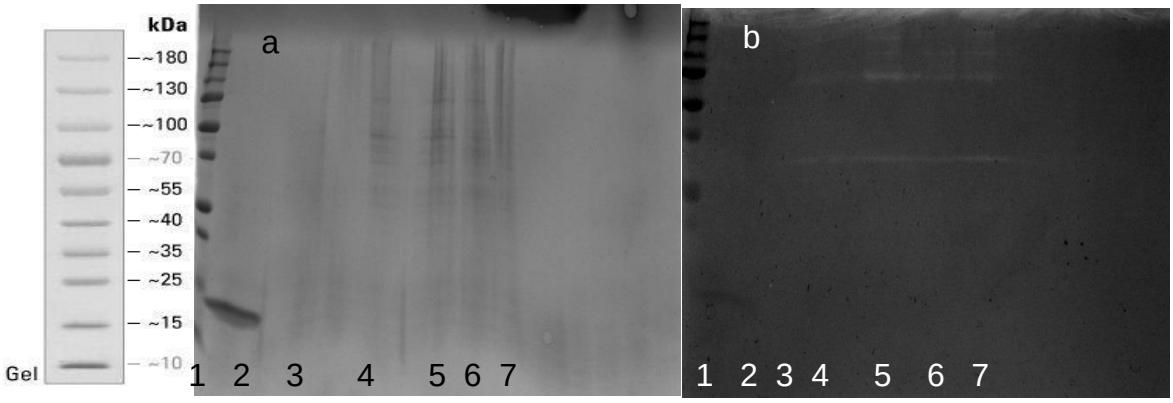


Figure 12: Small-scale SDS (4%) extracted peptidoglycan hydrolase activity with *M. luteus* cell wall. a) SDS – PAGE and b) Zymogram. Lane 1) Prestained protein marker. Lane 2) Positive control lysozyme. Lane 3) Open lane. Lane 4) *S. aureus (12600)* in the presence of extract 4.2. Lane 5) *S. aureus (12600)* in the presence of extract 5.2. Lane 6) *S. aureus (43300)* in the presence of extract 4.2. Lane 7) *S. aureus (43300*) in the presence of extract 5.2. Sample loaded of 5 µl for protein marker and 10 µl for tested samples. Biological repeats of 3 (n=3).

Peptidoglycan hydrolases was extracted using 4% SDS as previously shown after the bacteria was grown in the absence or presence of extract 4.2 and 5.2. Lane 1 was the prestained protein marker (PageRuler prestained protein ladder, ThermoScienfific, Lithuania). The protein marker was used to determine the size of various bands of interest. Lane 2a was the positive control of crude extracted lysozyme. Only one band is seen at ±14 kDa. Lane 2b showed a very faint band of the lysozyme. Lane 3 was an open lane. Lane 4a showed *S. aureus (12600)* in the presence of extract 4.2 whereas 4b shows the autolytic activity of *S. aureus (12600)* in the presence of extract 4.2. Smears were seen in the SDS-PAGE gel (a) and faint bands were also seen in the zymogram (b). Lane 5a showed *S. aureus (12600)* in the presence of extract 5.2 whereas 5b showed the autolytic activity of *S. aureus (12600)* in the presence of extract 5.2. Smears were seen in the SDS-PAGE gel (a) and faint bands were also seen in the zymogram (b). More bands were seen in the presence of extract 5.2 suggesting that plant extract 5.2 had better autolytic activity when compared to plant extract 4.2.

Lane 6a showed *S. aureus (43300)* in the presence of extract 4.2 whereas 6b showed the autolytic activity of *S. aureus (43300)* in the presence of extract 4.2. Smears were seen in the SDS-PAGE gel (a) and faint bands were also seen in the zymogram (b).

Lane 7a showed *S. aureus (43300)* in the presence of extract 5.2 whereas 7b showed the autolytic activity of *S. aureus (43300)* in the presence of extract 5.2. Smears were seen in the SDS-PAGE gel (a) and faint bands were also seen in the zymogram (b). More bands were seen in the presence of extract 5.2 suggesting that plant extract 5.2 had better autolytic activity when compared to plant extract 4.2.

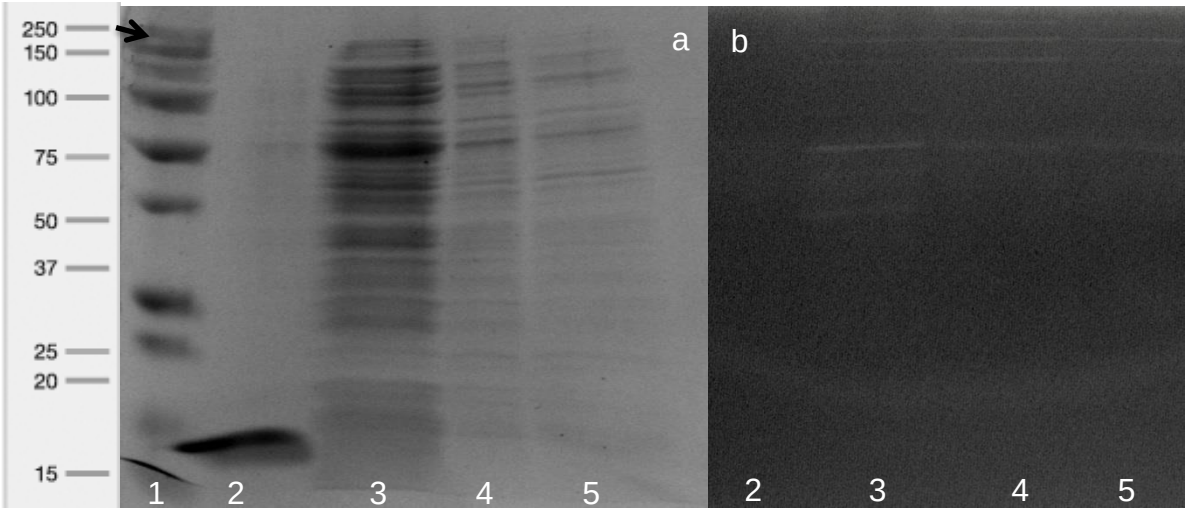


Figure 13: Large-scale SDS (4%) extracted peptidoglycan hydrolase activity with *M. luteus* cell wall. a) SDS – PAGE and b) Zymogram of the whole cell protein profile in the presence and absence of extract 4.2 and extract 5.2. Lane 1) Prestained protein marker. Lane 2) Positive control, Crude extracted lysozyme. Lane 3) *S. aureus (43300)* in the absence of extract 4.2 and 5.2. Lane 4) *S. aureus (43300)* in the presence of extract 4.2. Lane 5) *S. aureus (43300)* in the presence of extract 5.2. Sample loaded of 5 µl for protein marker and 10 µl for tested samples. Biological repeats of 3 (n=3).

Lane 1 was the prestained protein marker (BioRad, Precision Plus, All Blue). The protein marker was used to determine the size of various bands of interest. Lane 2 was the positive control of crude extracted lysozyme. Only one band is seen at approximately 13 kDa on the SDS-PAGE gel (Figure 13a). No band is seen on lane 2 of Figure 13b suggesting that there was no or minimal autolytic activity. Lane 3 was *S. aureus (43300)* in the absence of plant extract 4.2 and 5.2. Many bands were seen in Figure 13a. Fewer bands were seen on the zymogram (Figure 13b) suggesting that there was little autolytic activity. Lane 4 was *S. aureus (43300)* in the presence of extract 4.2. Fewer bands were seen when compared to the absence of the plant

extract (Figure 13a). Brighter bands were seen between 50 – 100 kDa. Few bands are seen in Figure 13b suggesting that autolytic activity was less. Lane 5 was *S. aureus (43300)* in the presence of extract 5.2. Fewer bands were seen when compared to the absence of the plant extract (Figure 13a). Brighter bands were seen between 50 – 100 kDa. Few bands were seen in Figure 13b suggesting that autolytic activity was less. Less bands were present in the presence of the plant extract at large-scale extraction when compared to the absence of the plant extracts suggesting a decreased autolytic activity or decreased protein concentration in the presence of the plant extracts. In the presence of the plant extracts, extract 4.2 and extract 5.2 showed similar bands.

High Performance Liquid Chromatography (HPLC) and Mass/Spec:

The aim of HPLC was to determine chemical compounds within the crude extracts in order to identify possible autolytic and antimicrobial compounds. Based on the results obtained, the plant extracts showed autolytic activity and antimicrobial activity. Crude extracts have multiple compounds within the mixture that could have accounted for the results presented. It is for this reason; the compounds were identified individually to find a compound with possible autolytic or antimicrobial properties. Only plant extracts 4.2 and 5.2 were used for HPLC analyses.

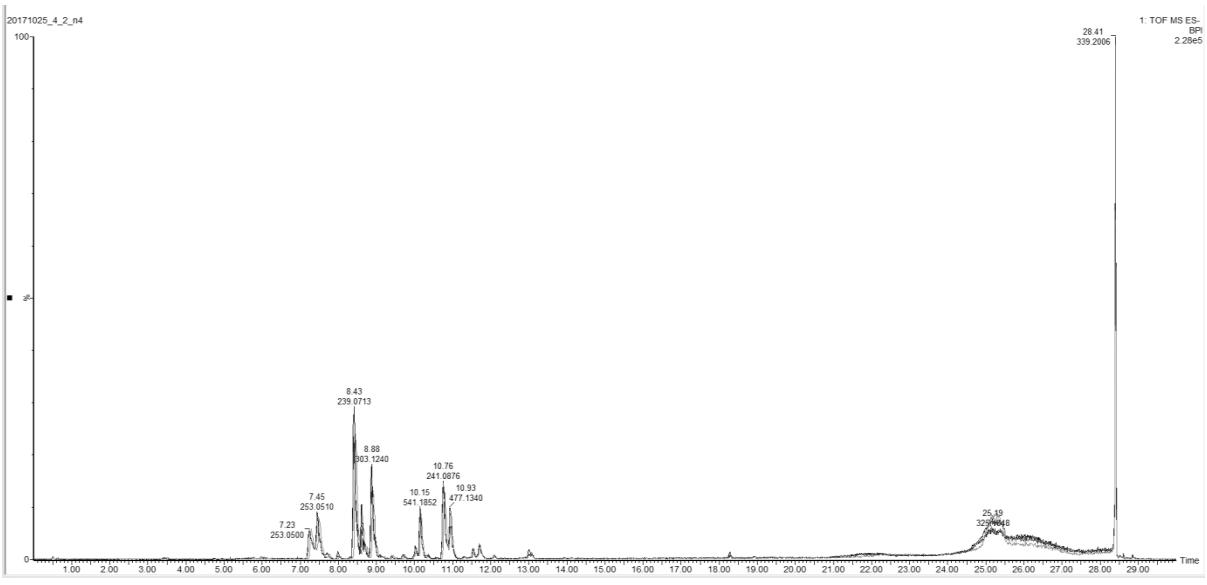


Figure 14: Schematic representation of sample 4.2 run in a negative mode chromatogram. Various peaks on the figure shows individual compounds.

Detected chromatographic peaks of sample 4.2 in negative mode showed that multiple peaks were obtained with various masses as shown in Figure 14. The peaks ranged from early to late run. The last peak at the end of the run (± 29 minutes) was due to the washing solvent and was discarded. Individual compounds were detected from 7 minutes to approximately 13 minutes and again at 25 minutes. By using the masses and times with specific programs and databased, the compounds of interest were identified as shown below.

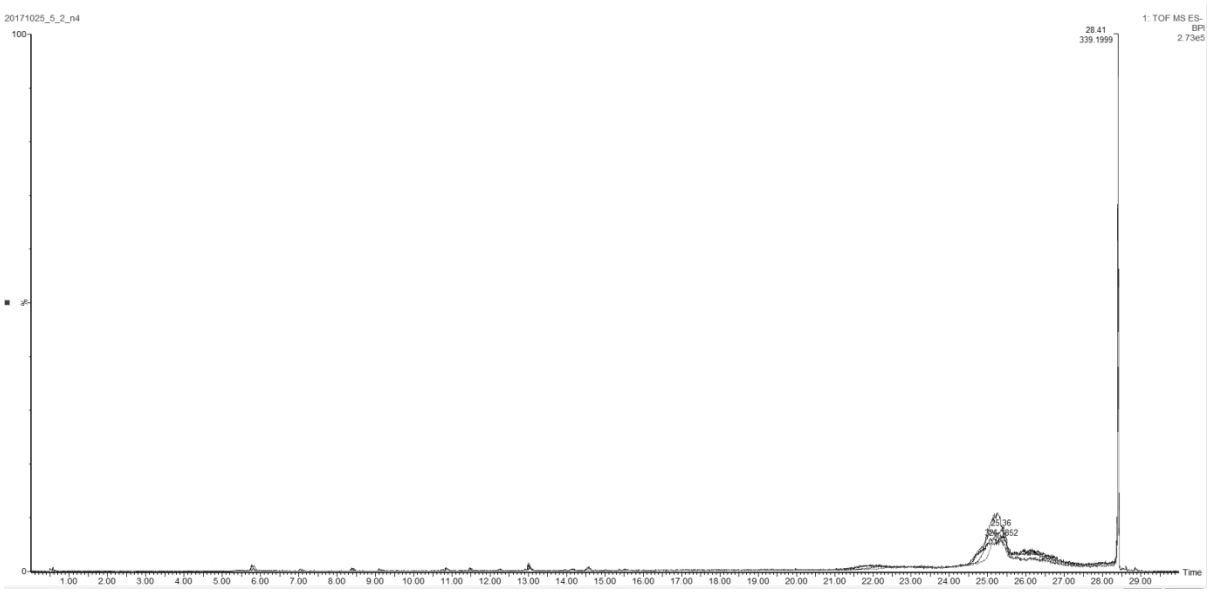


Figure 15: Schematic representation of sample 5.2 run in a negative mode chromatogram. Various peaks on the figure shows individual compounds.

Detected chromatographic peaks of sample 5.2 in negative mode showed that multiple peaks were obtained with various masses to the end of the run as shown in Figure 15. The last peak at the end of the run (± 29 minutes) was due to the washing solvent and was discarded. Individual compounds were detected from approximately 25 minutes. By using the masses and times with specific programs and databased, the compounds of interest were identified as shown below. Comparing extract 4.2 and 5.2 showed that extract 4.2 had individual compounds detected in throughout the duration of 30 minutes whereas extract 5.2 only showed compounds towards the end of the run.

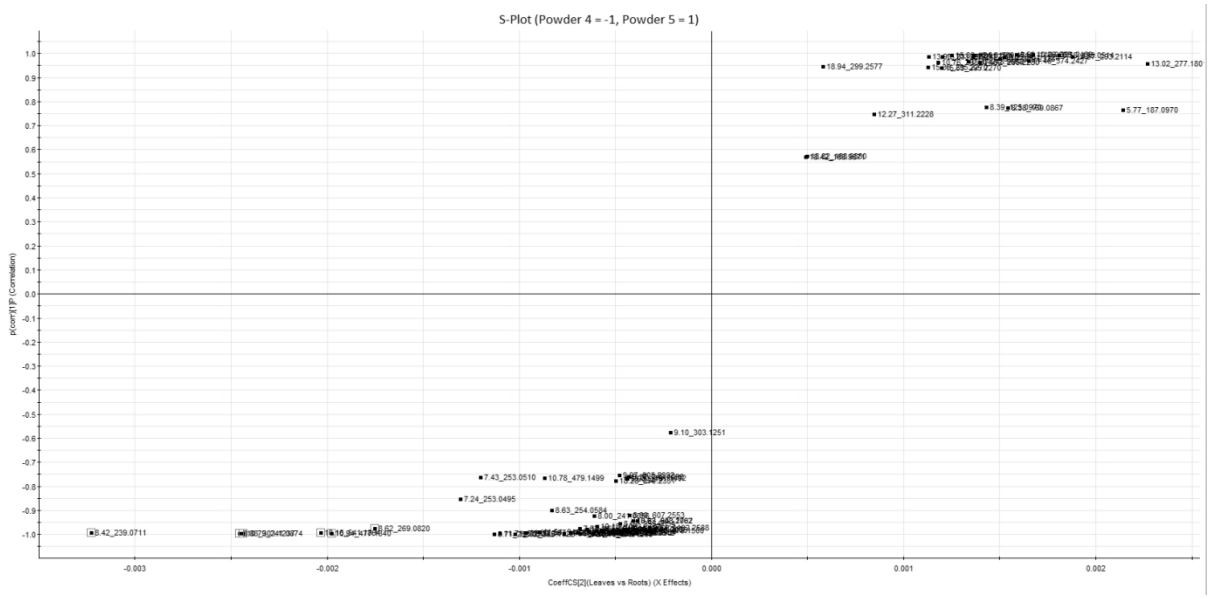


Figure 16: Schematic representation of the S-plots of sample 4.2 vs 5.2 run in the negative mode. The S-plot shows the difference of mass peaks between the samples. The far end points were selected as markers as it showed individuality of the compounds.

Different masses that were collected together, show the similarities between the extracts. These compounds are shared between the extracts and was not a good indicator of unique compounds and identity. For this reason, the compounds that were separate were chosen to identify as they would show unique properties of the extracts.

Table 5: Identification of individual compounds of interest of extract 4.2 in the negative mode based on the S-plot results.

	Ret. Time	m/z	Elemental Composition (mDa, i-FIT)	Database	ID
1	0.5804	195.0514	(1) 0.9, 0.1, C6H11O7	Golm Metabolome Database (Online)	(1) Hexonic acid
2	0.4982	112.9865	(2) -1.0, 4.2, C4HO4	PubChem (Online)	(1) 3-Carboxy-2-propynoate
3	10.8606	331.2489	(2) 0.5, 1.3, C18H35O5	KEGG (Online)	(1) 9,10,18-Trihydroxyoctadecanoic acid
4	9.1003	329.2328	(1) 0.0, 0.2, C18H33O5	KEGG (Online)	(1) (11E)-9,10,13-Trihydroxy-11-octadecenoic acid
5	14.5742	293.2114	(2) -0.3, 1.9, C18H29O3	KEGG (Online)	(1) 8-(5-Hexyl-2-furyl)octanoic acid
6	13.0199	277.1801	(1) 2.1, 0.3, C15H26O3Na	PubChem (Online)	Sodium 3-hydroxy-3-methyl-6-(2,2,3-trimethylcyclopentyl)hexanoate
7	7.0508	201.1125	(2) -0.2, 1.0, C10H17O4	Golm Metabolome Database (Online)	(1) Sebacic acid

Ret. Tim = retention time, m/z = mass, ID = identification

The retention time, mass, elemental composition (mDA, i-FIT), formula and ID were shown. Marker 1 had a retention time of 0.5804, mass of 195.0514, elemental composition of 0.9, 0.1, C6H11O7, formula of C6H11O7 and was identified via the

Golm Metabolome Database as Hexonic acid. Marker 2 had a retention time of 0.4982, mass of 112.9865, elemental composition of -1.0, 4.2, C4HO4, formula of C4HO4 and was identified via the PubChem Database as 3-Carboxy-2-propynoate. Marker 3 had a retention time of 10.8606, mass of 331.2489, elemental composition of 0.5, 1.3, C18H35O5, formula of C18H35O5 and was identified via the KEGG Database as 9,10,18-trihydroxyoctadecanoic acid. Marker 4 had a retention time of 9.1003, mass of 329.2328, elemental composition of 0.0, 0.2, C18H33O5, formula of C18H33O5 and was identified via the KEGG Database as 9,10,13-trihydroxy-11-octadecenoic acid. Marker 5 had a retention time of 14.57424, mass of 293.2114, elemental composition of -0.3, 1.9, C18H29O3, formula of C18H29O3 and was identified via the KEGG as 8-(5-hexyl-3-furyl)octanoic acid. Marker 6 had a retention time of 13.0199, mass of 277.1801, elemental composition of 2.1, 0.3, C15H26O3Na, formula of C15H26O3Na and was identified via the PubChem Database as sodium-3-hydroxy-3-methyl-6-(2,2,3-trimethylcyclopentyl)hexanoate. Marker 7 had a retention time of 7.0508, mass of 201.115, elemental composition of -0.2, 1.0, C10H17O4, formula of C10H17O4 and was identified via the Golm Metabolome Database as Sebacic acid. Research was done on each of the compounds and was found that Sebacic acid has antimicrobial activity (2,129-132).

Table 6: Identification of individual compounds of interest of extract 5.2 in the negative mode based on the S-plot results.

	Ret. Time	m/z	Elemental Composition (mDa, i-FIT)	Database	ID
1	10.1571	541.1864	(3) 0.2, 2.2, C32H29O8	NIST (Online)	(1) 9,9',10,10'-Tetramethoxy-5,5',6,6',7,7',8,8'-octahydro-2,2'-bianthracene-1,1',4,4'-tetrone
2	10.9447	477.1340	(2) 0.2, 2.2, C30H21O6	PubChem (Online)	(1) Bis[2-(4-biphenylyl)-2-oxoethyl] oxalate
3	8.8776	303.1237	(1) 2.9, 0.3, C15H20O5Na	KEGG (Online)	Sodium 2-(4-[(2-oxocyclopentyl)methyl]phenyl)propanoate hydrate (1:1:2)
4	8.6223	269.0820	(1) 3.0, 0.1, C14H14O4Na	PubChem (Online)	(1) Sodium (2Z,4E)-5-(2,4-dimethoxyphenyl)-3-methyl-2,4-pentadienoate
5	10.7662	241.0874	(1) 3.3, 0.3, C13H14O3Na	PubChem (Online)	(1) Sodium [(5S,6S)-5-hydroxy-6,7,8,9-tetrahydro-5H-benzo[7]annulen-6-yl]acetate
6	8.4169	239.0711	(1) 2.7, 0.2, C13H12O3Na	PubChem (Online)	(1) Sodium (5-hydroxy-5,7,8,9-tetrahydro-6H-benzo[7]annulen-6-ylidene)acetate

Ret. Tim = retention time, m/z = mass, ID = identification

The retention time, mass, elemental composition (mDA, i-FIT), formula and ID were shown. Marker 1 had a retention time of 10.1571, mass of 541.1864, elemental composition of 0.2, 2.2 C32H29O8, formula of C32H29O8 and was identified via the NIST Database as 9,9',10,10'-tetramethoxy-5,5',6,6',7,7',8,8'-octahydro-2,2'-bianthracene-1,1',4,4'-tetrone. Marker 2 had a retention time of 10.9447, mass of

477.1340, elemental composition of 0.2, 2,2 C30H21O6, formula of C30H21O6 and was identified via the PubChem Database as bis[2-(4-biphenylyl)-2-oxoethyl]oxalate. Marker 3 had a retention time of 8.8776, mass of 303.1237, elemental composition of 2.9, 0.3, C15H20O5Na, formula of C15H20O5Na and was identified via the KEGG Database as sodium-2-{4-[2-oxocyclopentyl)methyl]phenyl}propanoate hydrate (1:1:2). Marker 4 had a retention time of 8.6223, mass of 269.0820, elemental composition of3.0, 0.1, C14H14O4Na, formula of C14H14O4Na and was identified via the PubChem Database as sodium (2Z,4E)-5-(2,4-dimethoxyphenyl)-3-methyl-2,4-pentadienoate. Marker 5 had a retention time of 10.7862, mass of 241.0874, elemental composition of 3.2, 0.3, C13H14O3Na, formula of C13H14O3Na and was identified via the PubChem as sodium-[(5S,6S)-5-hydroxy-6,7,8,9-tetrahydro-5H-benzo[7]annulen-6-yl]acetate. Marker 6 had a retention time of 8.4169, mass of 239.0711, elemental composition of 2.7, 0.2, C13H12O3Na, formula of C13H12O3Na and was identified via the PubChem Database as sodium (5-hydroxy-5,7,8,9-tetrahydro-6H-benzo[7]annulen-6-ylidene)acetate. Marker 3 was identified to be Loxoprofen which was the dihydrated form of loxoprofen sodium. Oral administration converts the prodrug Loxoprofen into the active metabolite. Sodium 2-[4-(2-oxocyclopentylmethyl)phenyl]propionate dihydrate (CS-600) was investigated as a new anti-inflammatory agent for the inhibition of prostaglandin (PG) synthesis in vivo and in vitro by Matsuda et al 1984 (107) and CS-600 was an effective inhibitor of inflammatory prostaglandins production in vivo (1,107,108). This compound has been shown to have anti-inflammatory properties which was the causative agent activity of extract 5.2.

The HPLC analyses was also run in a positive mode and the results were used to identify compound with possible autolytic and antimicrobial properties.

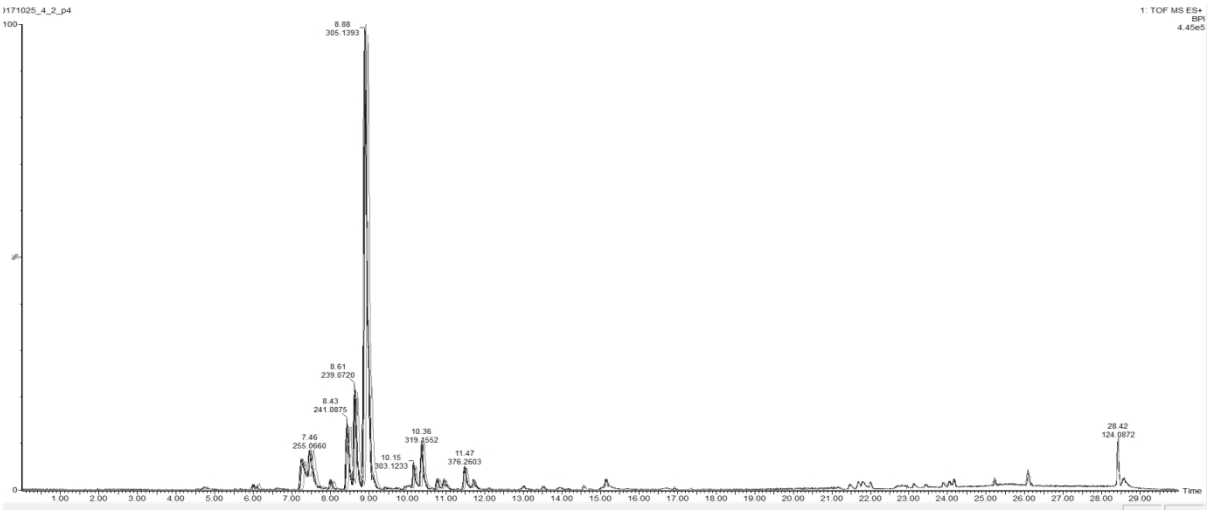


Figure 17: Schematic representation of sample 4.2 run in a positive mode chromatogram. Various peaks on the figure shows individual compounds.

The detected chromatographic peaks of sample 4.2 in positive mode showed that multiple peaks were obtained with various masses. The largest peak was seen at ± 9 minutes. The peaks ranged from early to late run. Individual compounds were detected from 7 minutes to approximately 12 minutes and again at 28.5 minutes. By using the masses and times with specific programs and databased, the compounds of interest were identified as shown below.

Figure 18: Schematic representation of sample 5.2 run in a positive mode chromatogram. Various peaks on the figure shows individual compounds.

Detected chromatographic peaks of sample 5.2 in the positive mode showed that multiple peaks were obtained with various masses as shown in Figure 18. The peaks ranged from early to late run. The last peak at the end of the run (± 29 minutes) was

due to the washing solvent and was discarded. Individual compounds were detected from 6 minutes and onwards. By using the masses and times with specific programs and databased, the compounds of interest were identified as shown below.

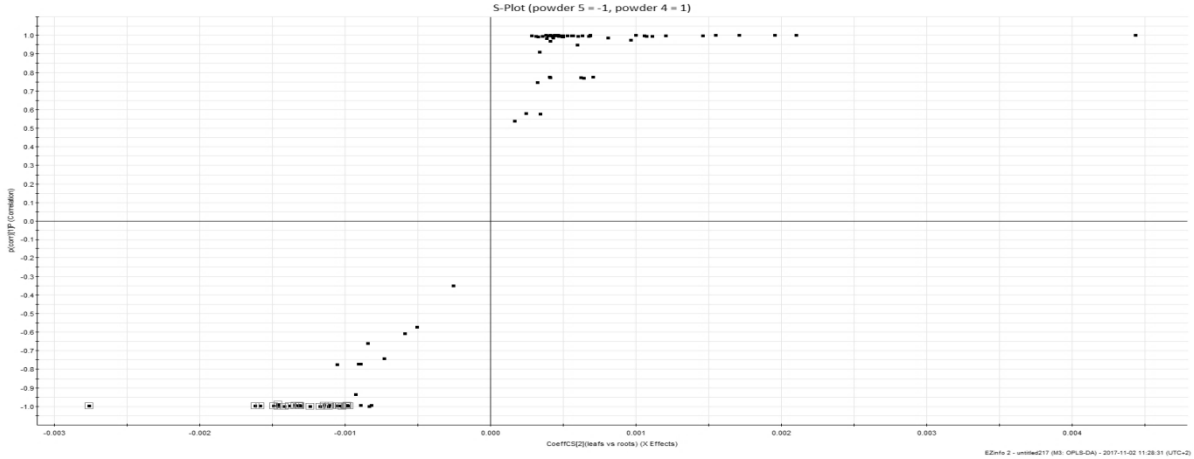


Figure 19: Schematic representation of the S-plots of sample 4.2 vs 5.2 run in the positive mode. The S-plot shows the difference of mass peaks between the samples. The far end points were selected as markers as it showed individuality of the compounds.

Different masses that were collected together, show the similarities between the extracts. These compounds are shared between the extracts and was not a good indicator of unique compounds and identity. For this reason, the compounds that sere separate were chosen to identify as they would show unique properties of the extracts.

Table 7: Identification of individual compounds of interest of extract 4.2 in the positive mode based on the S-plot results.

	m/z	Elemental Composition (mDa, i-FIT)	Database	ID
1	167.0710	(3) 0.2, 7.5, C9H11O3	Golm Metabolome Database (Online)	(1) 3-(2-Hydroxyphenyl)propanoic acid
2	305.1393	(1) -0.9, 0.4, C18H17N4O	NIST (Online)	(1) 2-Phenyl-6-(1-phenylethyl)-6,7-dihydro-5H-imidazo[1,5-b][1,2,4]triazol-5-one
3	271.0977	(1) -0.7, 1.9, C17H11N4	NIST (Online)	(1) Tricyclo[7.2.2.0~2,7~]trideca-2,4,6,12-tetraene-10,10,11,11-tetracarbonitrile
4	273.1129	(1) 0.2, 0.2, C16H17O4	KEGG (Online)	(1) (1R,3S)-9-Methoxy-1,3-dimethyl-3,4-dihydro-1H-benzo[g]isochromene-5,10-dione
5	255.0657	(1) 0.0, 0.0, C15H11O4	Golm Metabolome Database (Online)	(1) 5,7-Dihydroxy-2-phenyl-4H-chromen-4-one
6	255.0660	(1) 0.3, 0.1, C15H11O4	Golm Metabolome Database (Online)	(1) 5,7-Dihydroxy-2-phenyl-4H-chromen-4-one
7	239.0715	(3) 0.7, 1.9, C15H11O3	Golm Metabolome Database (Online)	(1) 3-Hydroxy-2-phenyl-4H-chromen-4-one
8	303.1233	(2) 4.9, 1.6, C14H24O3PS	PubChem (Online)	(1) Dibutyl [2-(2-thienyl)vinyl]phosphonate
9	319.1542	(5) 2.3, 3.2, C14H19N6O3	NIST (Online)	(1) N,N'-(9-Propionyl-9H-purine-2,6-diyl)dipropanamide
10	241.0871	(1) -2.4, 0.7, C14H14N2P	PubChem (Online)	1,1'-(Phenylphosphinediyl)bis(1H-pyrrole)
11	211.0763	(1) 0.4, 0.0, C14H11O2	KEGG (Online)	(1) Benzil
12	295.0947	(1) 0.4, 0.5, C14H11N6O2	NIST (Online)	(1) 1-(2,4-Diamino-6-pteridinyl)-2-phenyl-1,2-ethanedione
13	327.1209	(1) 1.7, 0.0, C14H19N2O7	PubChem (Online)	[3,6,9,15-Tetraazabicyclo[9.3.1]pentadeca-1(15),11,13-trien-6-ylmethyl]phosphonic acid

Ret. Tim = retention time, m/z = mass, ID = identification

The mass, elemental composition (mDA, i-FIT), formula and ID were shown. Marker 1 had a mass of 167.0710, elemental composition of 02.7,5, C9H11O3, formula of C9H11O3 and was identified via the Golm Metabolome Database as 3-(2-hydroxyphenyl)propanoic acid. Marker 2 had mass of 305.1393, elemental composition of -0.9, 0.4, C18H17N4O, formula of C18H17N4O and was identified via the NIST as 2-phenyl-6-(1-phenylethyl)-6,7-dihydro-5H-imidazo[1,5-b][1,2,4]triazol-5-one. Marker 3 had a mass of 271.0977, elemental composition of -0.7, 1.9, C17H11N4, formula of C17H11N4 and was identified via the NIST Database as tricycle[7.2.2.0~2, 7~]trideca-2,4,6,12-tetraene-10,10,11,11-tetracarbonitrile.

Marker 4 had a mass of 273.1129, elemental composition of 0.2, 0.2, C16H17O4, formula of C16H17O4 and was identified via the KEGG Database as (1R,3S)-9-methoxy-1,3-dimethyl-3,4-dihydro-1Hbenzo[g]isochromene-5,10-dione. Marker 5 had a mass of 255.0657, elemental composition of 0.0, 0.0, C15H11O4, formula of C15H11O4 and was identified via the Golm Metabolome Database as 5,7-dihydroxy-2-phenyl-4H-chromen-4-one. Marker 6 had a mass of 255.0660, elemental composition of 0.7, 1.9 C15H11O4, formula of C15H11O4 and was identified via the Golm Metabolome Database as 5,7-dihydroxy-2-phenyl-4H-chromen-4-one. Marker 7 had a mass of 239.0715, elemental composition of 0.7, 1.9, C15H11O3, formula of C15H11O3 and was identified via the Golm Metabolome Database as 3-hydroxy-2-phenyl-4H-chromen-4-one. Marker 8 had mass of 303.1233, elemental composition of

4.9, 1.6, C14H24O3PS, formula of C14H24O3PS and was identified via the PubChem Database as dibutyl[2-(2-thirnyl)vinyl]phosphate.

Marker 9 had a mass of 319.1542, elemental composition of 2.3, 3.2, C14H19N6O3, formula of C14H19N6O3 and was identified via the NIST Database as N,N'-(9-9propionyl-9H-purine-2,6-diyl)dipropanamide. Marker 10 had a mass of 241.0871, elemental composition of -2.4, 0.7, C14H14N2P, formula of C14H14N2P and was identified via the PubChem Database as 1,1''-(phenylphosphinediyl)bis(1H-pyrrole). Marker 11 had a mass of 211.0763, elemental composition of 0.4, 0.0, C14H11O2, formula of C14H11O2 and was identified via the KEGG as benzil. Marker 12 had a mass of 295.0947, elemental composition of 0.4, 0.0, C14H11N6O2, formula of C14H11N6O2 and was identified via the NIST as 1-(2,4-diamino-6-pteridinyl)-2-phenyl-1,2-ethanedione. Marker 13 had a mass of 327.1209, elemental composition of 1.7, 0.0, C14H19N2O7, formula of C14H19N2O7 and was identified via the PubChem Database as [3,6,9,15-tetraazabicyclo[9.3.1]pentadeca-1(15),11,13-trien-6-ylmethyl]phosphonic acid.

Comparing the negative and positive mode of extract 4.2 showed that extract 4.2 in the positive mode had more compounds that was not shared with extract 5.2 in the positive mode.

Table 8: Identification of individual compounds of interest of extract 5.2 in the positive mode based on the S-plot results.

	Ret. Time	m/z	Elemental Composition (mDa, i-FIT)	Database	ID
1	15.1844	149.0238	(3) -3.4, 9.2, C5H9O3S	Golm Metabolome Database (Online)	(1) 4-(Methylsulfanyl)-2-oxobutanoic acid
2	14.9462	674.5045	(5) -2.3, 3.3, C34H68N5O8	PubChem (Online)	2-Methyl-2-propanyl (4-aminobutyl)(2,2-dimethyl-9,14-bis{[(2-methyl-2-propanyl)oxy]carbonyl}-4-oxo-3-oxa-5,9,14-triazaheptadecan-17-yl)carbamate
3	14.0050	660.4896	(5) -1.5, 2.5, C33H68N5O8	PubChem (Online)	2-Methyl-2-propanyl (3-aminopropyl)(2,2-dimethyl-9,14-bis{[(2-methyl-2-propanyl)oxy]carbonyl}-4-oxo-3-oxa-5,9,14-triazaheptadecan-17-yl)carbamate
4	13.9977	572.4375	(7) -1.2, 4.4, C29H58N5O6	PubChem (Online)	Methyl N-[11-(dimethylamino)undecanoyl]-L-seryl-L-lysyl-L-leucinate
5	13.0176	558.4209	(7) -2.2, 3.0, C28H56N5O6	PubChem (Online)	(1) Methyl N-(12-aminododecanoyl)-L-seryl-L-lysyl-L-leucinate
6	14.9776	542.4271	(6) -4.0, 3.0, C27H57N7O2P	PubChem (Online)	N,N,N',N',N'',N''-Hexabutyl-N'''-(3,5-dioxo-2,3,4,5-tetrahydro-1,2,4-triazin-6-yl)phosphorimidic triamide
7	14.9933	498.4009	(8) -2.4, 6.0, C27H48N8	PubChem (Online)	(1) N~6~-(4-Aminocyclohexyl)-9-cyclopentyl-N~2~-{1-[4-(dimethylamino)butyl]-4-piperidinyl}-9H-purine-2,6-diamine
8	13.9646	484.3847	(2) -2.9, 1.2, C26H46N8	PubChem (Online)	N~6~-(4-Aminocyclohexyl)-9-cyclopentyl-N~2~-{1-[3-(dimethylamino)propyl]-4-piperidinyl}-9H-purine-2,6-diamine
9	12.9442	470.3684	(7) -2.2, 5.5, C24H48N5O4	PubChem (Online)	2-Methyl-2-propanyl [14-(3-cyanopropyl)-2,2-dimethyl-4-oxo-3-oxa-5,9,14-triazaheptadecan-17-yl]carbamate
10	13.9476	440.3589	(5) -1.2, 2.8, C23H46N5O3	PubChem (Online)	(1) N-(1-{[6-({3-[(4-Aminobutyl)amino]propyl}amino)-6-oxohexyl]amino}-1-oxo-2-propanyl)cyclohexanecarboxamide
11	11.4835	393.2859	(1) 0.7, 0.4, C20H41O7	PubChem (Online)	(1) 2-(Dodecyloxy)ethyl beta-D-galactopyranoside
12	11.4849	376.2597	(1) -3.3, 0.2, C20H35N5P	PubChem (Online)	N,N-Dimethyl-4-[(tri-1-pyrrolidinylphosphoranylidene)amino]aniline
13	13.8892	335.2791	(1) -0.6, 1.2, C18H39O5	NIST (Online)	(1) 3,6,9,12-Tetraoxadocosan-1-ol
14	9.9359	279.0935	(1) -0.4, 0.1, C18H16OP	MassBank (Online)	(1) Triphenylphosphine oxide
15	7.7120	200.1074	(1) -0.1, 0.0, C13H14NO	NIST (Online)	(1) 3-Methoxy-N-phenylaniline
16	15.1771	301.1420	(1) -1.0, 0.5, C12H22N4O3P	PubChem (Online)	[3,6,9,15-Tetraazabicyclo[9.3.1]pentadeca-1(15),11,13-trien-6-ylmethyl]phosphonic acid
17	8.7251	228.1501	(1) -3.3, 0.4, C11H22N3S	NIST (Online)	(1) 2,4-Dimethyl-1,2,4-triazaspiro[4.7]dodecane-3-thione
18	8.7236	233.1052	(2) 1.3, 0.9, C11H13N4O2	KEGG (Online)	(1) Ethyl 2-(1-phthalazinyl)hydrazinecarboxylate

Ret. Tim = retention time, m/z = mass, ID = identification

The retention time, mass, elemental composition (mDA, i-FIT), formula and ID were shown. Marker 1 had a retention time of 15.1844, mass of 149.0238, elemental composition of -3.4, 9.2, C5H9O3S, formula of C5H9O3S and was identified via the Golm Metabolome Database as 4-(methylsulfanyl)-2-oxobutanoic acid. Marker 2 had a retention time of 14.9462, mass of 674.5045, elemental composition of -2.3, 3.3, C34H68N5O8, formula of C34H68N5O8 and was identified via the PubChem Database as 2-methyl-2-propanyl (4-aminobutyl)(2,2-dimethyl-9,14{[(2-methyl-2-propanyl)oxy]carbonyl}-4-oxo-3-oxa-5,9,14-triazaheptadecan-17-yl)carbonate.

Marker 3 had a retention time of 14.0050, mass of 660.4896, elemental composition of -1.5, 2,5, C33H66N5O8, formula of C33H66N5O8 and was identified via the PubChem Database as 2-methyl-2-propanyl (3-aminobutyl)(2,2-dimethyl-9,14{[(2-methyl-2-propanyl)oxy]carbonyl}-4-oxo-3-oxa-5,9,14-triazaheptadecan-17-yl)carbonate. Marker 4 had a retention time of 13.9977, mass of 572.4375, elemental composition of -1.2, 4.4, C29H58N5O6, formula of C29H58N5O6 and was identified via the PubChem Database as methyl N-[11-(dimethylamino)undecanoyl]-L-seryl-L-lysyl-L-leucinate. Marker 5 had a retention time of 13.0176, mass of 558.4209, elemental composition of -2.2,3.0, C28H56N5O6 formula of C28H56N5O6 and was identified via PubChem Database methyl N-[12-aminodecanoyl)-L-seryl-L-lysyl-L-leucinate. Marker 6 had a retention time of 14.69776, mass of 542.4271, elemental composition of -0.4. 3.0, C27H57N7O2P, formula of C27H57N7O2P and was identified via the PubChem Database as N,N,N’,N’,N’’,N’’-hexabutyl-N’’’-(3,5-dioxo-2,3,4,5-tetrahrydro-1,2,4-triazin-6-yl) phosphorimidic triamide.

Marker 7 had a retention time of 14.9933, mass of 498.4009, elemental composition of -2.4, 6.0, C27H48N9, formula of C27H48N9 and was identified via the PubChem Database as N~6~-(4-aminocyclohexyl)-9-cyclopentyl-N~2~-{1-[4-(dimethylamino)-butyl]-4-piperidynyl}-9H-purine-2,6-diamine. Marker 8 had a retention time of 13.9646, mass of 484.4847, elemental composition of –2.9, 1.2, C26H46N9, formula of C26H46N9 and was identified via the PubChem Database as N~6~-(4-aminocyclohexyl)-9-cyclopentyl-N~2~-{1-[3-(dimethylamino) propyl]-4-piperidynyl}-9H-purine-2,6-diamine. Marker 9 had a retention time of 12.9442, mass of 470.3684, elemental composition of -2.2, 5.5, C24H48N5O4, formula of C24H48N5O4 and was identified via the PubChem Database as 2-methyl-2-propanyl[14-(3-cyanopropyl)-2,2-

dimethyl-4-oxo-3-oxa-5,9,14-triazaheptadecan-17-yl) carbomate. Marker 10 had a retention time of 13.9476, mass of 440.3589, elemental composition of -1.2. 2.8, C23H46N5O3, formula of C23H46N5O3 and was identified via the PubChem Database as N-(1-{[6-({3-[(4-aminobutyl)amino)-6-oxohexyl]amino}-1-oxo-2-propan-yl)cyclohexane-carbox-amide. Marker 11 had a retention time of 11.4835, mass of 393.2859, elemental composition of -0.7, 0.4 C20H41O7 formula of C20H41O7 and was identified via PubChem Database as 2-(dodecyloxy)ethyl beta-D-galactopyranoside. Marker 12 had a retention time of 11.4849, mass of 376.2597, elemental composition of -3.3, 0.2, C20H35N5P, formula of C20H35N5P and was identified via the PubChem Database as N,N-dimethyl-4-[(tri-1-pyrrolidinyl-phosphoranylidene)amino]aniline.

Marker 13 had a retention time of 13.8892, mass of 335.2791, elemental composition of -0.6, 1.2, C18H39O5, formula of C18H39O5 and was identified via the NIST Database as 3,6,9,12-tetraoxadocosan-1-ol. Marker 14 had a retention time of 9.9359, mass of 279.0935, elemental composition of -0.4, 0.1, C18H16OP, formula of C18H16OP and was identified via the MassBank Database as triphenylphosphine oxide. Marker 15 had a retention time of 7.7120, mass of 200.1074, elemental composition of -0.1, 0.0, C13H14NO, formula of C13H14NO and was identified via NIST as 3-methoxy-N-phenylaniline. Marker 16 had a retention time of 15.1771, mass of 301.1420, elemental composition of -1.0, 0.5, C12H22N4O3P, formula of C12H22N4O3P and was identified via the PubChem Database as [3,6,9,15-tetraazabicyclo- [9.3.1]pentadeca-1(15),11,13-trien-6-ylmethyl]phosphonic acid. Marker 17 had a retention time of 8.7251, mass of 2281501, elemental composition of -3.3, 0.4, C11H22N3S, formula of C11H22N3S and was identified via the NIST Database as 2,4-dimethyl-1,2,4-triazaspirol[4.7]dodecane-3-thione. Marker 18 had a retention time of 8.7236, mass of 233.1052, elemental composition of 1.3, 0.9 C11H13N4O2, formula of C11H13N4O2 and was identified via the KEGG Database as ethyl 2-(phthalazinyl)hydrazinecarboxylate.

Comparing the negative and positive mode of extract 5.2 showed that extract 5.2 in the positive mode had more compounds that was not shared with extract 4.2 in the positive mode. Basic information on the individual compound were shown in the discussion section below.

Autolytic activity of LiCl and SDS extracted cell wall hydrolases:

The aim of this section was to determine the activity of crude plant extracts via autolytic activity assay over a period. The increased or decreased activity was tested with extracted autolytic enzymes.

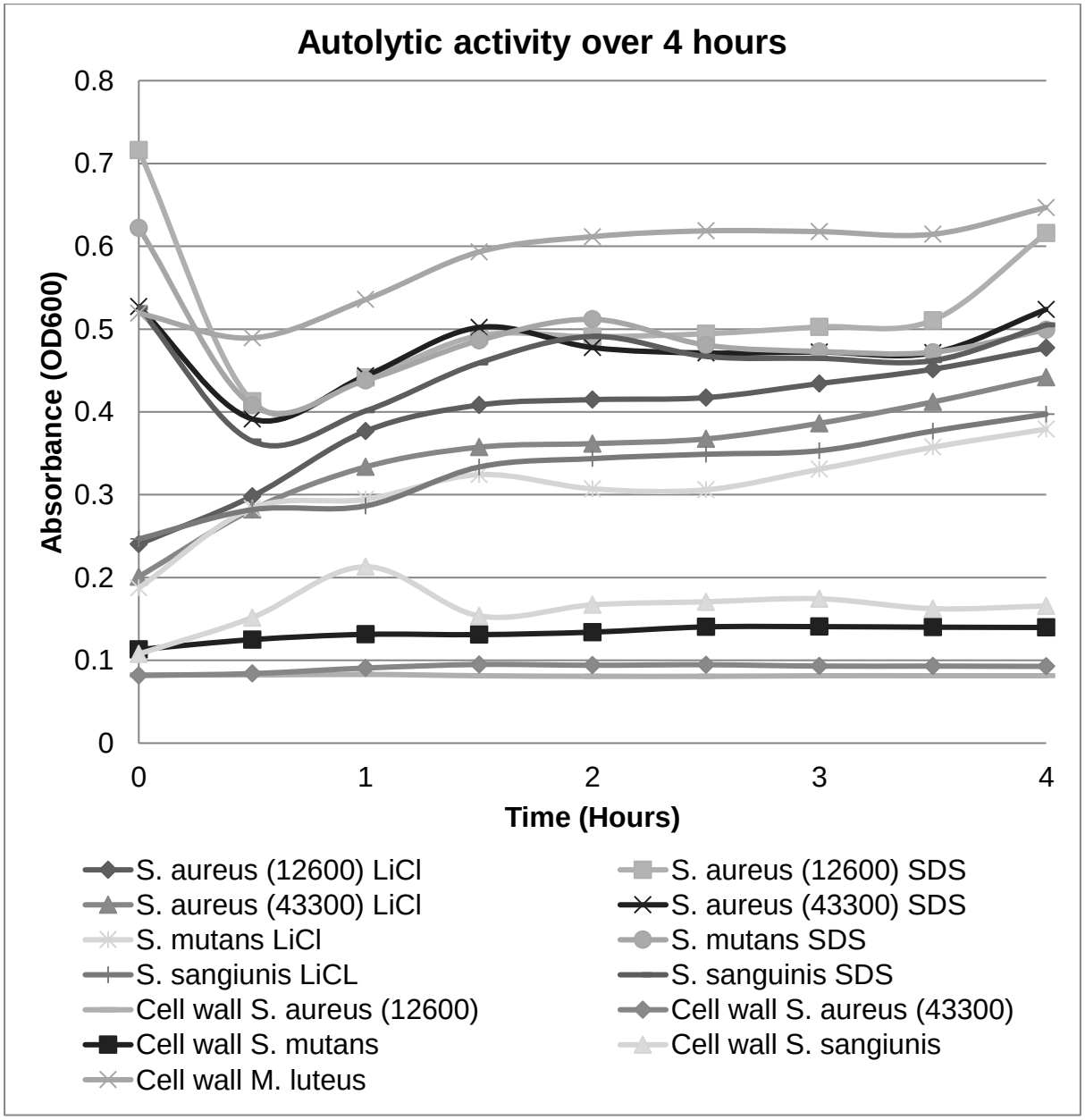


Figure 20: Autolytic activity over a period of 4 hours measured at OD_{600}. Every half an hour the OD_{600} was measured. Each line is described by the legends at the bottom of the figure. Biological repeats of 3 (n=3).

The cell wall of the microbial species had a lower OD_{600} value when compared to the cell wall with enzyme of that microbial specie. The cell wall of *S. aureus (12600)* had a constant OD_{600}. The cell wall of *S. aureus (43300)* showed a slight increase and then

became constant. The cell wall of *S. mutans* followed the same pattern as *S. aureus (43300).* The cell wall of *S. sanguinis* showed an increase where it had the highest value at 1 hour. Thereafter there was a decrease between 1- 3 hours followed by a slight increase until 4 hours. The cell wall of *M. luteus* showed an increase and then became constant with a slight decrease towards the end. All the SDS extracted studies showed a decrease initially from 0 – 0.5 hours into the study. Thereafter an increased was observed followed by a decrease. All LiCl extracted enzymes showed fluctuation in OD_{600} values that lead to an increase in the end.

The expected results were a decrease in absorbance as the enzymes are cleaving the cells. Peptidoglycan was a macromolecule and the intact substrate was not soluble. As soon as the substrate was broken up (autolytic activity), the molecule became soluble, thus decreasing the absorbance. Absorbance fluctuation and increase in absorbance is not the expected results.

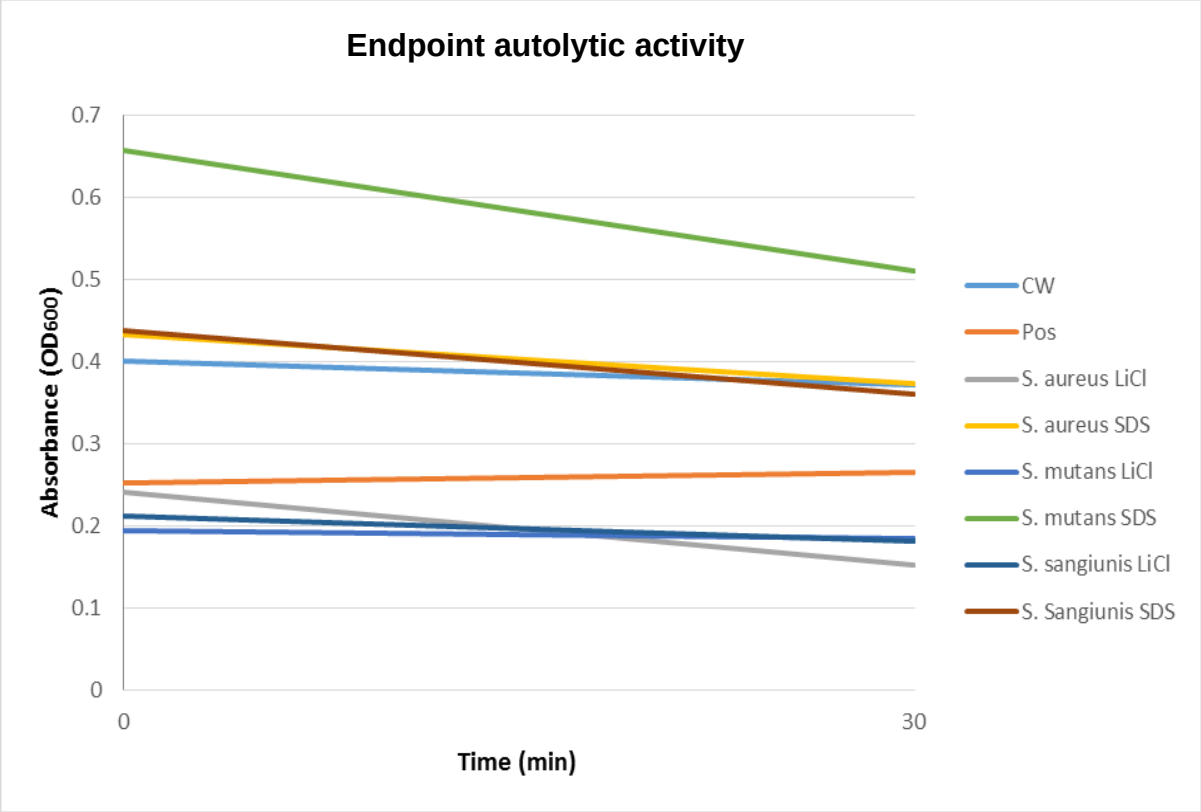


Figure 21: Activity study of 30 min including 5M LiCl or 4% SDS extracted autolytic enzymes. Total volume 200 µl. Enzyme (20 µl), cell wall (80 µl), phosphate buffer (100 µl) and 5M LiCl (20µl) or 4% SDS (20µl). Biological repeats of 3 (n= 3).

Only one *S. aureus* species was used in this experiment and that was *S. aureus (43300).* Cell wall (CW) control consisted of cell wall (100 µl) and phosphate buffer (100 µl) and showed no increase in absorbance. This was the expected result. The positive control (Pos) showed slight increase. *S. aureus* LiCl and SDS extracted samples showed a decrease in absorbance. This was the expected result. *S. mutans* LiCl sample showed a stable absorbance, whereas *S. mutans* SDS sample showed a decrease in absorbance. The decrease in absorbance was the expected result. *S. sanguinis* LiCl and SDS extracted samples showed a decrease in absorbance. This was the expected result. Both extraction methods showed autolytic activity, but SDS extraction method showed a higher decline, thus increased autolytic activity. For this reason, 4% SDS extraction method was used to test the autolytic activity in the presence of the plant extracts.

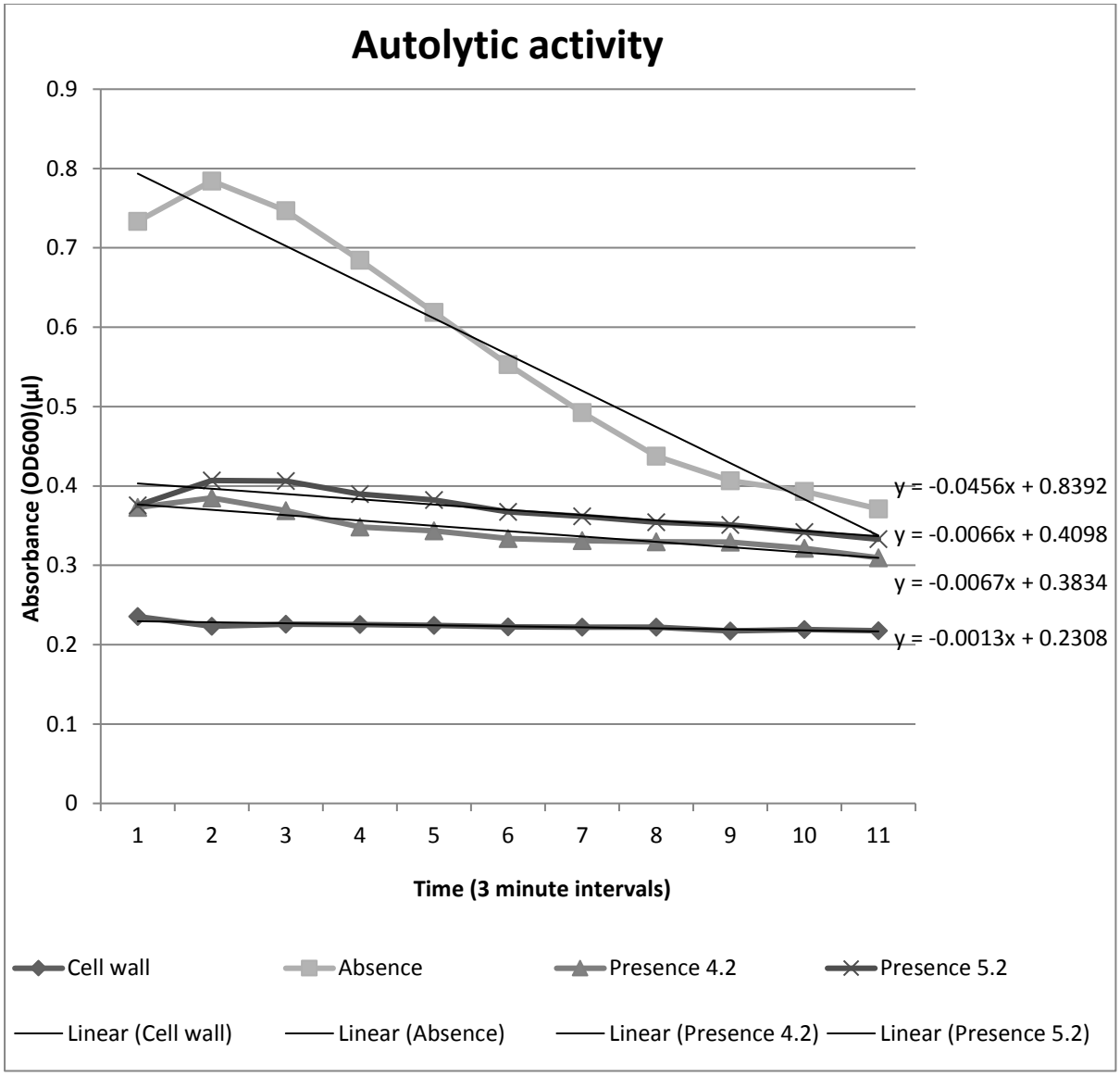


Figure 22: Autolytic activity of 4% SDS extracted peptidoglycan hydrolases in the absence and presence of extract 4.2 and 5.2. Biological repeats of 3 (n=3). Line of best fit shown to determine decline rate.

The cell wall had a relative constant absorbance. Using the best line of fit, the rate of decrease for cell wall control was 0.0013 µl/min. The absorbance in the absence of the plant extracts showed a slight increase initially. At 3 minutes (reading 2), the absorbance decreased until 30 minutes (reading 11). Using the best line of fit, the rate of decrease in the absence of plant extract was 0.0456 µl/min. The absorbance in the presence of the plant extract 4.2 showed a slight increase initially. At 3 minutes (reading 2), the absorbance decreased until 30 minutes (reading 11). Using the best line of fit, the rate of decrease in the presence of extract 4.2 was 0.0067 µl/min. The absorbance in the presence of the plant extract 5.2 showed a slight increase initially.

At 3 minutes (reading 2), the absorbance decreased until 30 minutes (reading 11). Using the best line of fit, the rate of decrease in the presence of extract 5.2 was 0.0066 µl/min. The absence of plant extracts showed a greater decrease in absorbance than in the presence of plant extracts suggesting that the plant extracts had minimal autolytic activity when compared to the absence of the plant extracts.

Minimum Inhibitory Concentration (MIC) / Double Dilution Assay:

The aim of this section was to determine the activity of crude plant extracts via the dilution activity assay based on the MIC results that was used to determine the concentration needed for microbial inhibition.

Table 9: MIC of plant extracts on microbial species.

Extract:	**No growth:**	**Growth**
BG	No dilution (1)	½ dilution
CR	No dilution (1)	½ dilution
EH	No dilution (1)	½ dilution
FA	No dilution (1)	½ dilution
TM	No dilution (1)	½ dilution
WN	No dilution (1)	½ dilution

BG = *Brachystelma gymnopodum*, CR = *Coccinea rehmannii*, EH = *Eulophia hereroensis*, FA = *Fockea angustifolia*, TM = *Trochomeria macrocarpa*, WN = *Walleria nutans.*

Table 9 shows that no growth was present in any sample at no dilution of the plant extracts. Growth was present once the plant extracts were diluted to half the relative concentration of the plant extracts (0.058 µl/µl) suggesting that the plant extracts have no antimicrobial activity once diluted.

Dilution activity assay:

The aim of this section was to determine the activity of crude plant extracts via the dilution activity assay based on the MIC results. Each microbial species was tested on their own.

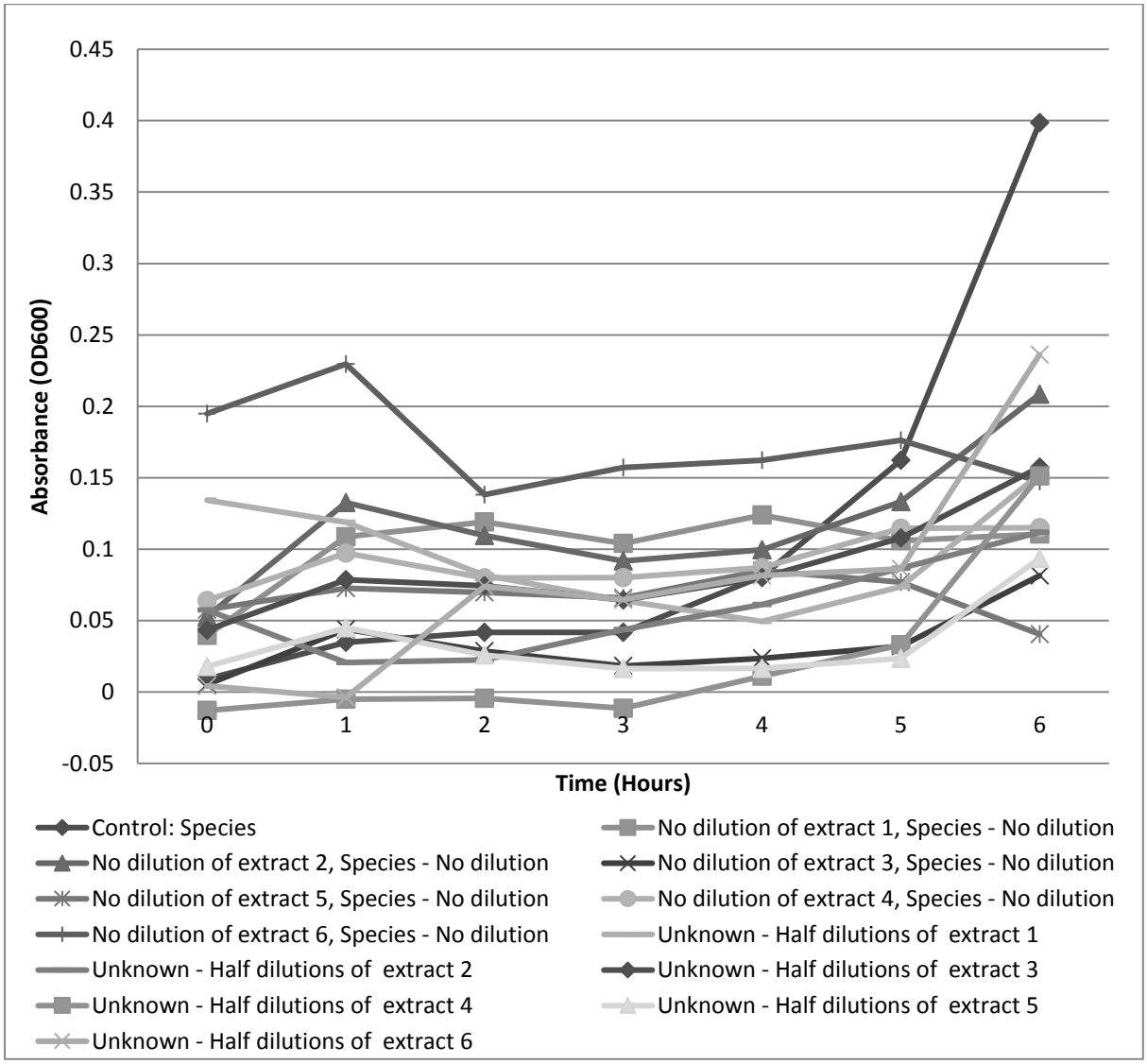


Figure 23: Dilution activity assay of *S. aureus (12600)* in the presence of various plant extracts. BHI broth (150 µ), microbial specie (2 µl), plant extract (10 µl) and for dilution PBS (1x) (10 µl). Biological repeats of 3 (n=3).

The activity assay with full concentration (0.062 µl/µl) and ½ dilution (0.058 µl/µl) of plant extracts against *S. aureus (12600)* was shown. The results of the controls consisted out of the following: Control: Species with BHI showed an increase in absorbance throughout the study with a stable absorbance at ± 2-3 hours. Control: No dilution of each extract 1, Species – No dilution showed an increase in absorbance

until hour 2, thereafter a fluctuation in absorbance until hour 6. Control: No dilution of each extract 2, Species – No dilution showed an increase in absorbance until hour 1, thereafter a decrease in absorbance until hour 3 followed by an increase in absorbance until hour 6. Control: No dilution of each extract 3, Species – No dilution showed a decrease in absorbance until hour 3, thereafter an increase in absorbance until hour 6. Control: No dilution of each extract 4, Species – No dilution showed an increase in absorbance until hour 1, thereafter a decrease in absorbance until hour 2 followed by an increase in absorbance until hour 6. Control: No dilution of each extract 5, Species – No dilution showed an increase in absorbance until hour 1, thereafter a decrease in absorbance until hour 3 followed by an increase in absorbance until hour 6. Control: No dilution of each extract 6, Species – No dilution showed an increase in absorbance until hour 1, thereafter a decrease in absorbance until hour 2 followed by an increase in absorbance until hour 5 and a decrease in absorbance at hour 6.

Unknown of half dilution of extract 1, species – control half dilution of extract 1 showed a decrease in absorbance until hour 4, followed by an increase in absorbance until hour 6. Unknown of half dilution of extract 2, species – control half dilution of extract 2 showed a decrease until hour 1, thereafter an increase in absorbance until hour 6. Unknown of half dilution of extract 3, species – control half dilution of extract 3 showed an increase of absorbance until hour 1, followed by a decrease in absorbance until hour 3, thereafter an increase in absorbance until hour 6. Unknown of half dilution of extract 4, species – control half dilution of extract 4 showed an increase in absorbance until hour 2, thereafter a decrease in absorbance until hour 3, followed by an increase in absorbance until hour 6. Unknown of half dilution of extract 5, species – control half dilution of extract 5 showed an increase in absorbance until hour 1, thereafter a decrease in absorbance until hour 3, followed by an increase in absorbance until hour 6. Unknown of half dilution of extract 6, species – control half dilution of extract 6 showed a decrease in absorbance until hour 1, followed by fluctuation in absorbance until hour 6.

The expected results were a gradual increase in absorbance as the activity assay and MIC had shown that the microbial species continued to grow. The results obtained were not expected as there is fluctuation among the data. In the dilution assay, it is noticeable that due to the fluctuation in absorbance, growth was present in the MIC

assay at ½ concentrations (1x dilution series) (0.058 µl/µl). A possible reason is that the microbial species may overcome the extract's activity shown by the fluctuation.

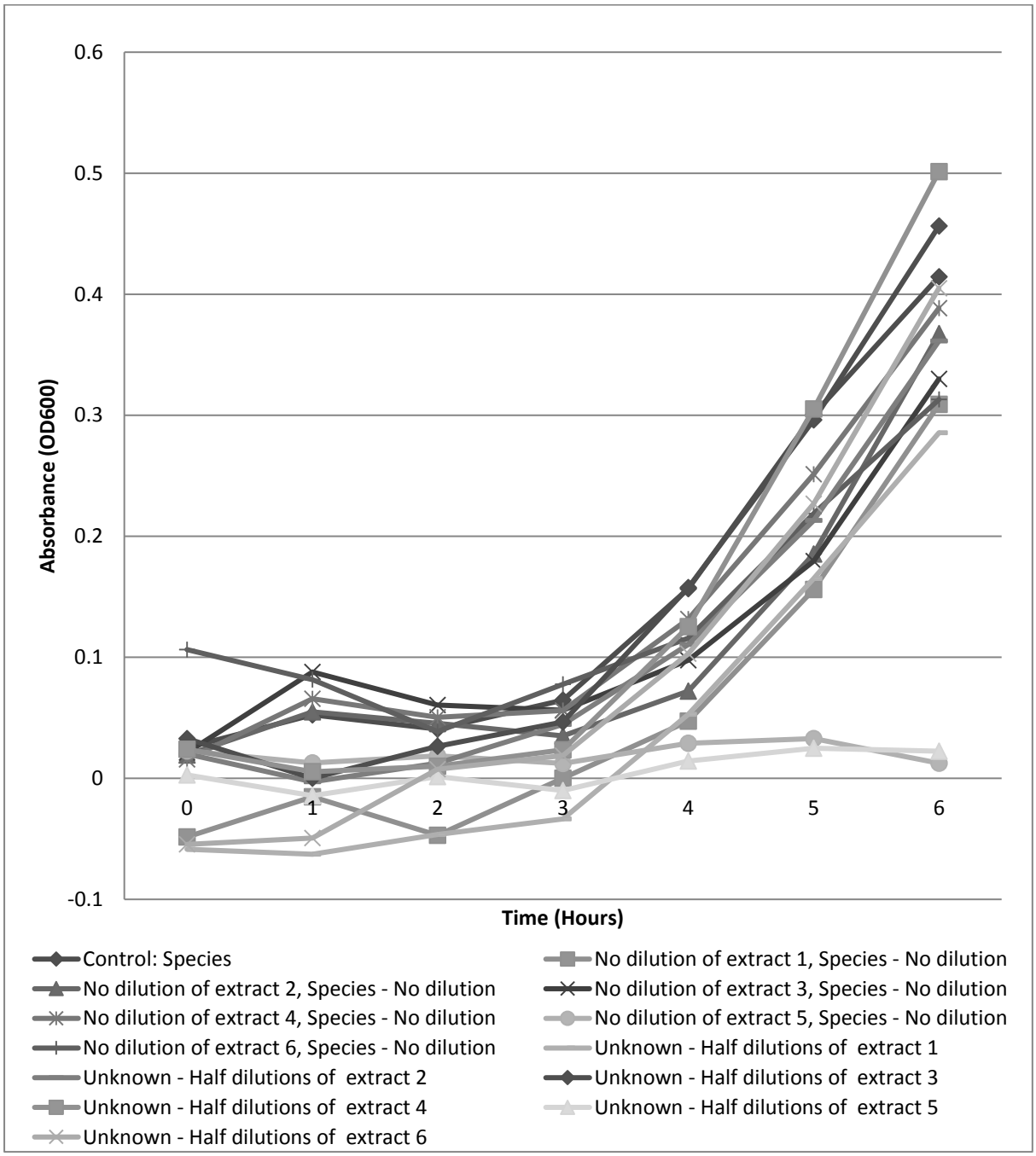


Figure 24: Dilution activity assay of *S. aureus (43300)* in the presence of various plant extracts. BHI broth (150 µ), microbial specie (2 µl), plant extract (10 µl) and for dilution PBS (1x) (10 µl). Biological repeats of 3 (n=3).

The activity assay with full concentration (0.062 µl/µl) and ½ dilution (0.058 µl/µl) of plant extracts against *S. aureus (43300)* was shown. The results of the controls consisted out of the following: Control: Species with BHI showed an increase in

absorbance until hour 1, followed by a decrease in absorbance until hour 2, thereafter an increase in absorbance until hour 6. Control: No dilution of each extract 1, Species – No dilution showed an increase in absorbance until hour 6. Control: No dilution of each extract 2, Species – No dilution showed an increase in absorbance until hour 1, thereafter a decrease in absorbance until hour 3 followed by an increase in absorbance until hour 6. Control: No dilution of each extract 3, Species – No dilution showed an increase in absorbance until hour 1, thereafter a decrease in absorbance until hour 3 followed by an increase in absorbance until hour 6. Control: No dilution of each extract 4, Species – No dilution showed an increase in absorbance until hour 1, thereafter a decrease in absorbance until hour 2 followed by an increase in absorbance until hour 6. Control: No dilution of each extract 5, Species – No dilution showed a decrease in absorbance until hour 1, followed by a fluctuation in absorbance until hour 6. Control: No dilution of each extract 6, Species – No dilution showed a decrease in absorbance until hour 2, followed by an increase in absorbance until hour 6.

Unknown of half dilution of extract 1, species – control half dilution of extract 1 showed a decrease in absorbance until hour 1, followed by an increase in absorbance until hour 6. Unknown of half dilution of extract 2, species – control half dilution of extract 2 showed a decrease until hour 1, thereafter an increase in absorbance until hour 6. Unknown of half dilution of extract 3, species – control half dilution of extract 3 showed an increase of absorbance until hour 1, followed by a decrease in absorbance until hour 2, thereafter an increase in absorbance until hour 6. Unknown of half dilution of extract 4, species – control half dilution of extract 4 showed a decrease in absorbance until hour 1, thereafter an increase in absorbance until hour 6. Unknown of half dilution of extract 5, species – control half dilution of extract 5 showed a fluctuation in absorbance hour 3, thereafter an increase in absorbance until hour 6. Unknown of half dilution of extract 6, species – control half dilution of extract 6 showed an increase in absorbance until hour 3, followed by a decrease in absorbance until hour 4, thereafter an increase in absorbance until hour 6.

The expected results were a gradual increase in absorbance as the activity assay and MIC had shown that the microbial species continued to grow. The results obtained were not expected as there is fluctuation among the data. In the dilution assay, it is noticeable that due to the fluctuation in absorbance, growth was present in the MIC

assay at ½ concentrations (1x dilution series) (0.058 µl/µl). A possible reason is that the microbial species may overcome the extract's activity shown by the fluctuation.

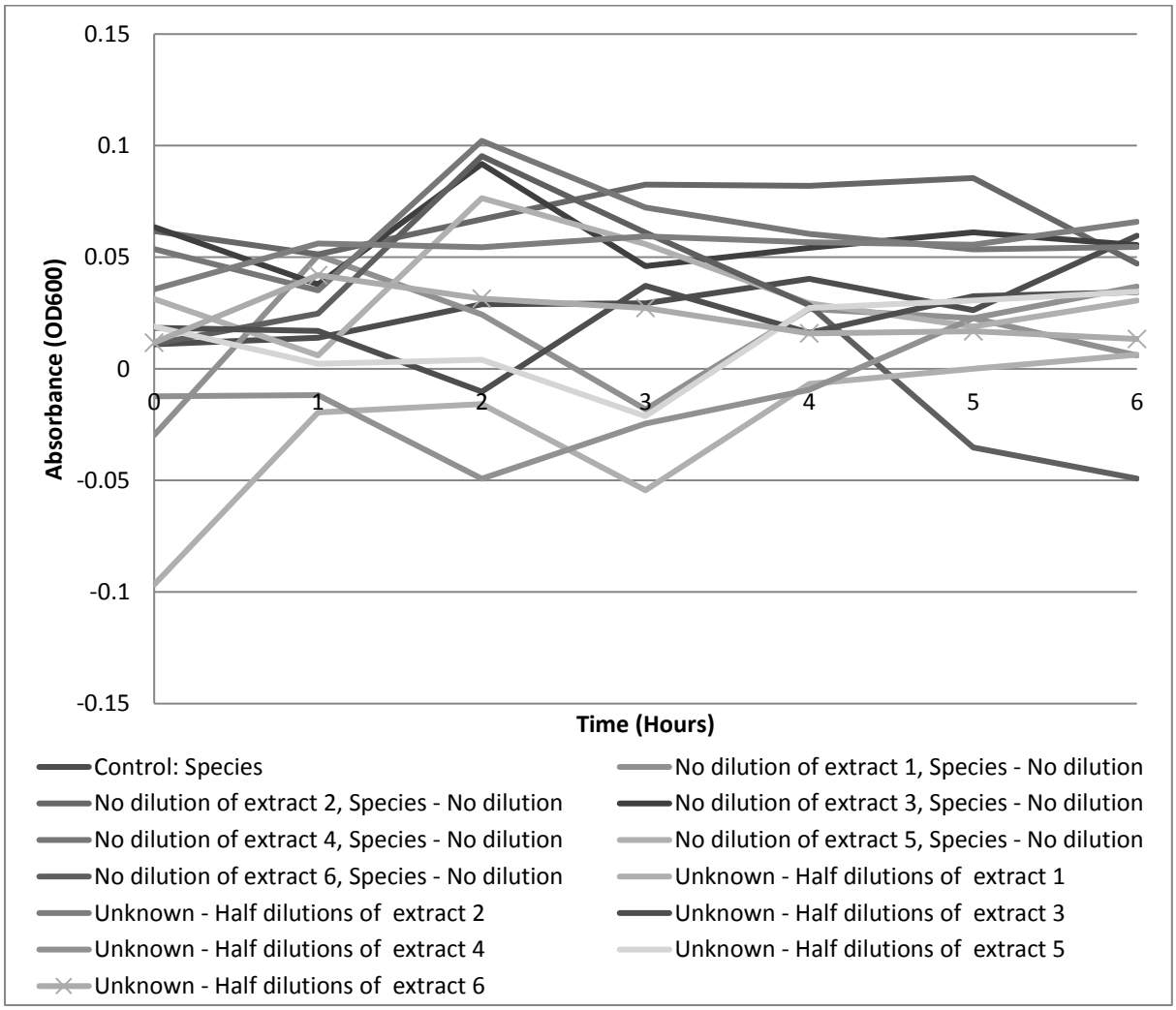


Figure 25: Dilution activity assay of *S. mutans* in the presence of various plant extracts. BHI broth (150 µ), microbial specie (2 µl), plant extract (10 µl) and for dilution PBS (1x) (10 µl). Biological repeats of 3 (n=3).

The activity assay with full concentration (0.062 µl/µl) and ½ dilution (0.058 µl/µl) of plant extracts against *S. mutans* was shown. The results of the controls consisted out of the following: Control: Species with BHI showed an increase in absorbance until hour 4, followed by a decrease in absorbance until hour 5, thereafter an increase in absorbance until hour 6. Control: No dilution of each extract 1, Species – No dilution showed a fluctuation in absorbance until hour 6. Control: No dilution of each extract 2, Species – No dilution showed a decrease in absorbance until hour 1, thereafter an increase in absorbance until hour 5 followed by a decrease in absorbance until hour

6. Control: No dilution of each extract 3, Species – No dilution showed a fluctuation in absorbance until hour 6. Control: No dilution of each extract 4, Species – No dilution showed a fluctuation in absorbance until hour 6. Control: No dilution of each extract 5, Species – No dilution showed a decrease in absorbance until hour 1, followed by an increase in absorbance until hour 2, followed by a decrease in absorbance until hour 5, thereafter an increase in absorbance until hour 6. Control: No dilution of each extract 6, Species – No dilution showed a fluctuation in absorbance until hour 2, followed by a decrease in absorbance until hour 6.

Unknown of half dilution of extract 1, species – control half dilution of extract 1 showed an increase in absorbance until hour 6. Unknown of half dilution of extract 2, species – control half dilution of extract 2 showed an increase in absorbance until hour 1, followed by fluctuation in absorbance until hour 6. Unknown of half dilution of extract 3, species – control half dilution of extract 3 showed a decrease in absorbance until hour 2, thereafter an increase and fluctuation in absorbance until hour 6. Unknown of half dilution of extract 4, species – control half dilution of extract 4 showed a decrease in absorbance until hour 2, thereafter an increase in absorbance until hour 6. Unknown of half dilution of extract 5, species – control half dilution of extract 5 showed an increase in absorbance hour 2, thereafter a decrease in absorbance until hour 3, followed by an increase in absorbance until hour 6. Unknown of half dilution of extract 6, species – control half dilution of extract 6 showed a decrease in absorbance until hour 6.

The expected results were a gradual increase in absorbance as the activity assay and MIC had shown that the microbial species continued to grow. The results obtained were not expected as there is fluctuation among the data. In the dilution assay, it is noticeable that due to the fluctuation in absorbance, growth was present in the MIC assay at ½ concentrations (1x dilution series) (0.058 µl/µl). A possible reason is that the microbial species may overcome the extract's activity shown by the fluctuation.

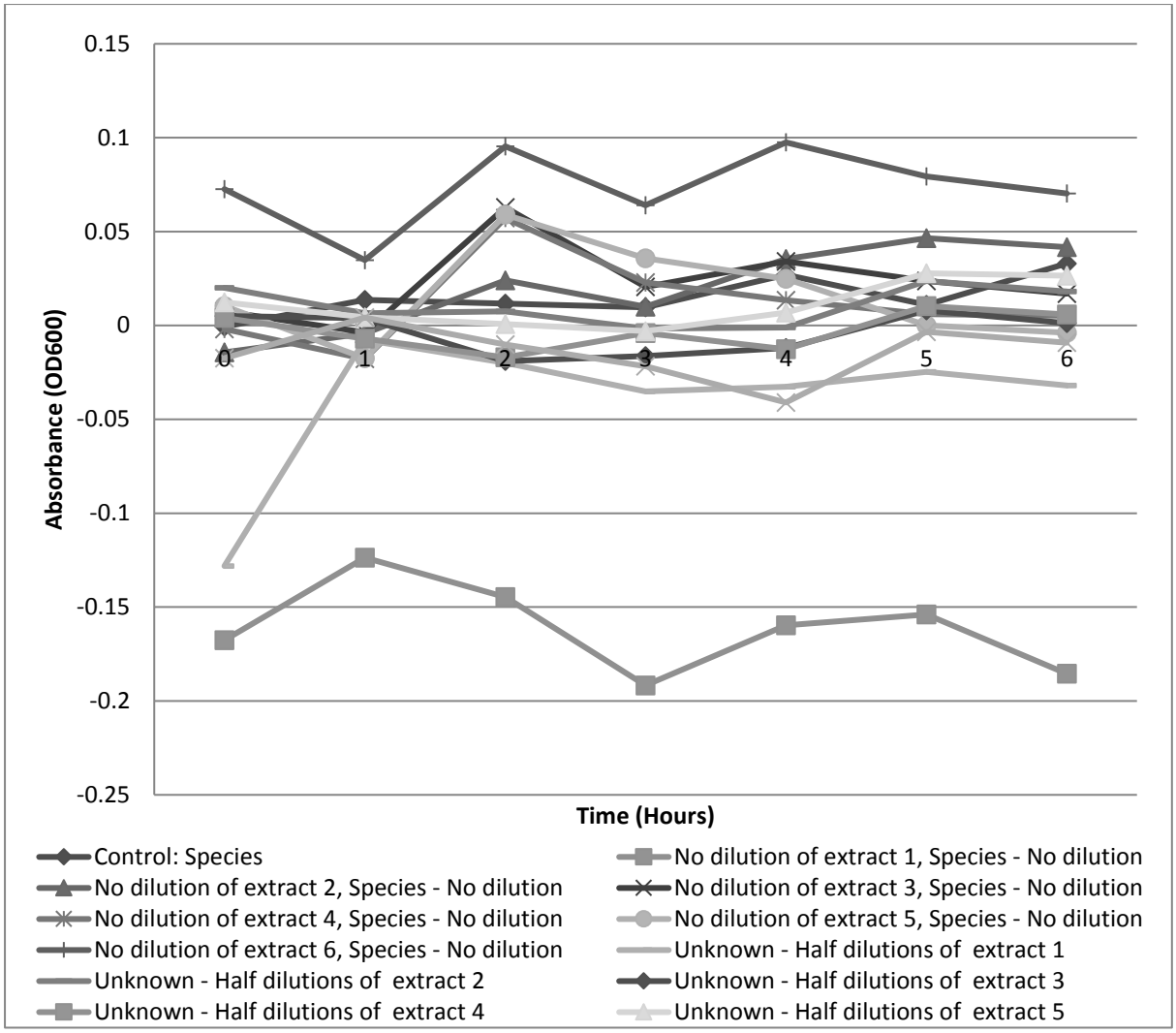


Figure 26: Dilution activity assay of *S. sanguinis* in the presence of various plant extracts. BHI broth (150 µ), microbial specie (2 µl), plant extract (10 µl) and for dilution PBS (1x) (10 µl). Biological repeats of 3 (n=3).

The activity assay with full concentration (0.062 µl/µl) and ½ dilution (0.058 µl/µl) of plant extracts against *S. sanguinis* was shown. The results of the controls consisted out of the following: Control: Species with BHI showed an increase in absorbance until hour 1, followed by a decrease in absorbance until hour 5, thereafter an increase in absorbance until hour 6. Control: No dilution of each extract 1, Species – No dilution showed a fluctuation in absorbance until hour 6. Control: No dilution of each extract 2, Species – No dilution showed fluctuation in absorbance until hour 6. Control: No dilution of each extract 3, Species – No dilution showed a fluctuation in absorbance until hour 6. Control: No dilution of each extract 4, Species – No dilution showed a decrease in absorbance until hour 1, followed by an increase until hour 2, thereafter a

decrease in absorbance until hour 6. Control: No dilution of each extract 5, Species – No dilution showed fluctuation in absorbance until hour 6. Control: No dilution of each extract 6, Species – No dilution showed a fluctuation in absorbance until hour 4, followed by a decrease in absorbance until hour 6.

Unknown of half dilution of extract 1, species – control half dilution of extract 1 showed fluctuation in absorbance until hour 6. Unknown of half dilution of extract 2, species – control half dilution of extract 2 showed fluctuations in absorbance until hour 6. Unknown of half dilution of extract 3, species – control half dilution of extract 3 showed a decrease in absorbance until hour 2, thereafter an increase in absorbance until hour 5, thereafter a decrease in absorbance until hour 6. Unknown of half dilution of extract 4, species – control half dilution of extract 4 showed a decrease in absorbance until hour 4, thereafter an increase in absorbance until hour 5, followed by a decrease in absorbance until hour 6. Unknown of half dilution of extract 5, species – control half dilution of extract 5 showed a decrease in absorbance until hour 3, followed by an increase in absorbance until hour 6. Unknown of half dilution of extract 6, species – control half dilution of extract 6 showed an increase in absorbance until hour 1, followed by a decrease in absorbance until hour 6.

The expected results were a gradual increase in absorbance as the activity assay and MIC had shown that the microbial species continued to grow. The results obtained were not expected as there is fluctuation among the data. In the dilution assay, it is noticeable that due to the fluctuation in absorbance, growth was present in the MIC assay at ½ concentrations (1x dilution series) (0.058 µl/µl). A possible reason is that the microbial species may overcome the extract's activity shown by the fluctuation.

Antimicrobial assay (Spot-on-lawn assay):

The aim of this section was to determine the activity of crude plant extracts on the microbial species via an antimicrobial assay (Spot-on-lawn assay) to visualize inhibition zones. Only *S. aureus s*pecies were used for this experiment based on the results obtained in all the experiments.

Figure 27: Inhibition zone of *S. aureus (12600)* with extract 4.2 and 5.2. Red arrow displays the inhibition zone. Biological repeats of 3 (n=3).

Only extracts 4.2 showed an inhibition zone on *S. aureus (12600)*, when compared to extract 5.2.

Figure 28: Inhibition zone of *S. aureus* (43300) with extract 4.2 and 5.2. Red arrow displays the inhibition zone. Biological repeats of 3 (n=3).

Only extracts 4.2 showed an inhibition zone on *S. aureus (43300)*, when compared to extract 5.2. Extract 4.2 showed inhibition zones on the lawn of *S. aureus (12600)* of 1.33 cm and on the lawn of *S. aureus 43300* of 0.67 cm. Extract 5.2 showed no zones of inhibition on the selected microbial species suggesting that only extract 4.2 had microbial inhibition properties.

BCA Assay:

Based on the results obtain by all the experiments, the protein concentration in the presence and absence of the plant extracts were tested to determine the influence of the extracts on the protein concentration of microbial species.

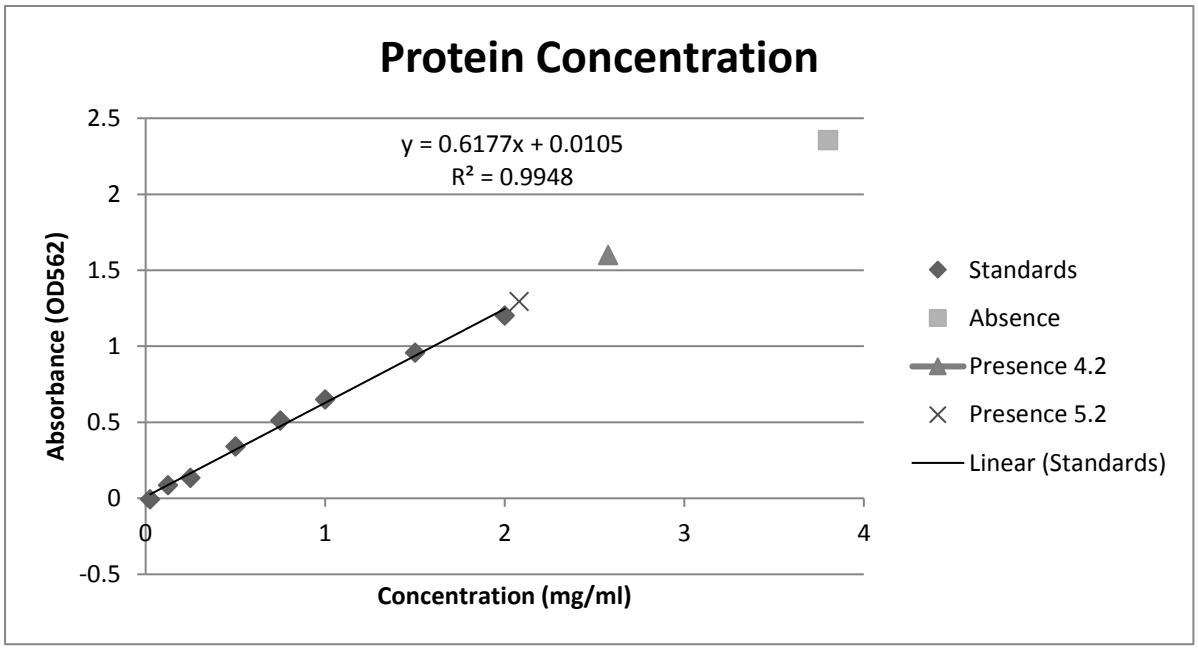


Figure 29: BCA assay standards vs absorbance to determine protein concentration in the absence and presence of plant extract 4.2 and 5.2. Biological repeats of 3 (n=3). Best line of fit used to determine protein concentration.

Only *S. aureus (43300)* was used based on the Spot-on-lawn results. The standards were plotted, and a best line of fit is displayed on the graph. Using the equation y = 0.6177x + 0.0105, the unknown concentration of *S. aureus (43300)* in the absence of plant extract 4.2 and 5.2 with an OD_{562} of 2.356 was 3.797 mg/ml ~ 3.80 mg/ml. The unknown concentration of *S. aureus (43300)* with an OD_{562} of 1.602 in the presence of extract 4.2 was 2.576 mg/ml ~ 2.58 mg/ml. The unknown concentration of *S. aureus (43300)* with an OD_{562} of 1.296 in the presence of extract 5.2 was 2.081 mg/ml ~ 2.08

mg/ml. The difference between the absence of plant extract and the presence of extract 4.2 was 1.22 mg/ml and in the presence of extract 5.2 is 1.72 mg/ml.

Biofilm formation and Biofilm eradication:

The aim of this section was to determine the activity of crude plant extracts on microbial species via biofilm activity in the presence and absence of the plant extract in order to gain access into the cell and break the cells defensive mechanism. Only *S. aureus* species were used based on the results of all the experiments.

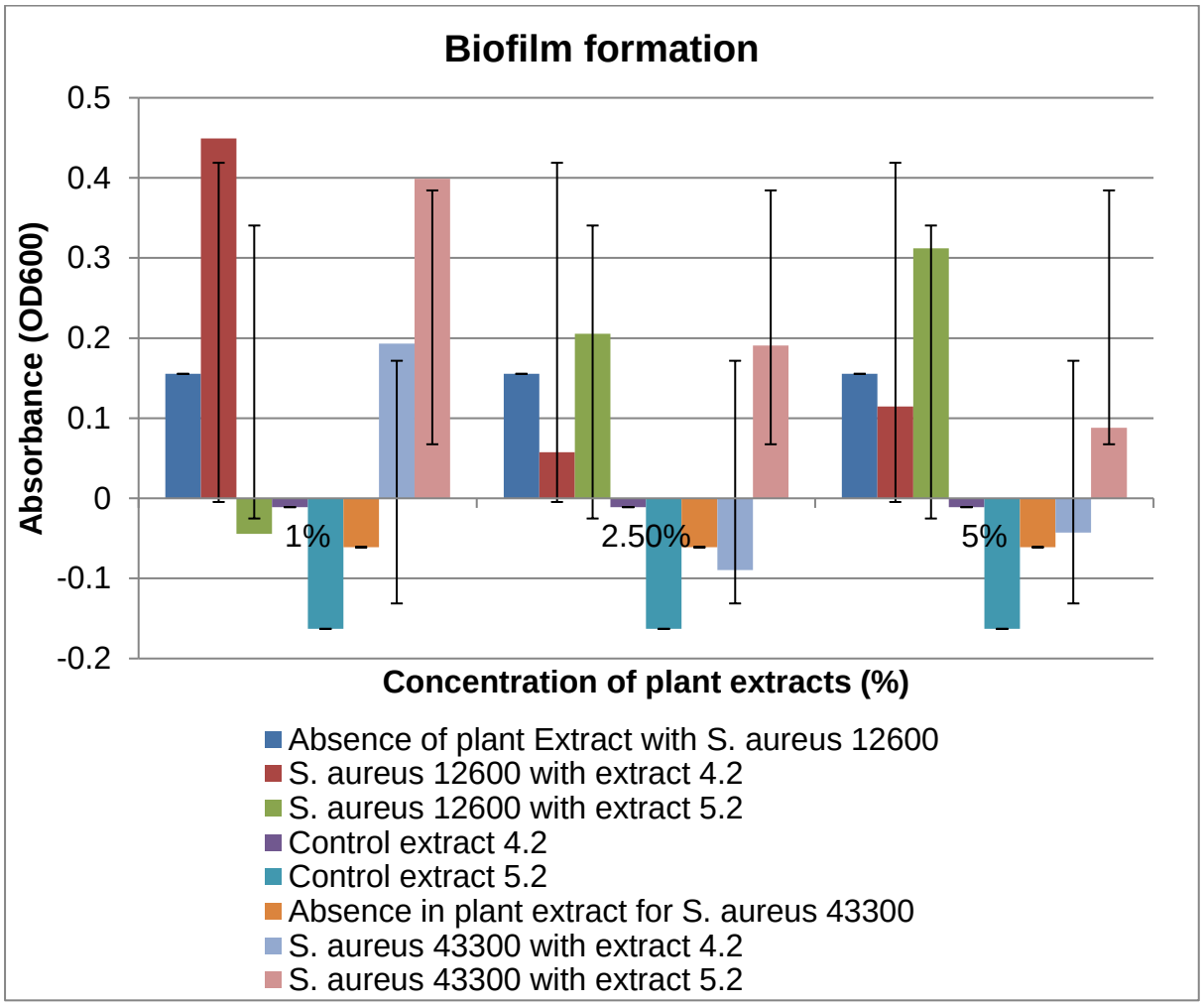


Figure 30: Biofilm and biofilm eradication in the absence and presence of plant extracts. Biological repeats of 3 (n=3). Error bars as standard deviations.

At 1% of plant extract, *S. aureus (12600)* in the absence of plant extracts, showed biofilm formation. *S. aureus (12600)* in the presence of extract 4.2 showed an increased biofilm formation. *S. aureus (12600)* in the presence of extract 5.2 showed no biofilm formation, thus biofilm eradication (antibiofilm) activity as it showed negative

absorbance. *S. aureus (43300)* in the absence of plant extracts showed no biofilm formation. *S. aureus (43300)* in the presence of extract 4.2 showed an increased biofilm formation. *S. aureus (43300)* in the presence of extract 5.2 showed increased biofilm formation. In the control of extract 4.2, no biofilm was formed. In the control of extract 5.2, no biofilm was formed.

At 2.5% of plant extract, *S. aureus (12600)* in the absence of plant extracts, showed biofilm formation. *S. aureus (12600)* in the presence of extract 4.2 showed a decrease biofilm formation thus biofilm eradication (antibiofilm) activity. *S. aureus (12600)* in the presence of extract 5.2 showed increased biofilm formation. *S. aureus (43300)* in the absence of plant extracts showed no biofilm formation. *S. aureus (43300)* in the presence of extract 4.2 showed no biofilm formation. *S. aureus (43300)* in the presence of extract 5.2 showed biofilm formation. In the control of extract 4.2, no biofilm was formed. In the control of extract 5.2, no biofilm was formed.

At 5% of plant extract, *S. aureus (12600)* in the absence of plant extracts, showed biofilm formation. *S. aureus (12600)* in the presence of extract 4.2 showed decreased biofilm formation (antibiofilm activity). *S. aureus (12600)* in the presence of extract 5.2 showed increased biofilm formation. *S. aureus (43300)* in the absence of plant extracts showed no biofilm formation. *S. aureus (43300)* in the presence of extract 4.2 showed no biofilm formation. *S. aureus (43300)* in the presence of extract 5.2 showed biofilm formation. In the control of extract 4.2, no biofilm was formed. In the control of extract 5.2, no biofilm was formed.

In the presence of the plant extracts, it was expected to show a decrease in biofilm formation (antibiofilm or biofilm eradication) when compared to the control in the absence of the plant extracts. This was a case only for *S. aureus (12600)* in the presence of extract 4.2. *S. aureus (12600)* in the presence of extract 5.2 showed biofilm formation and *S. aureus (43300)* in the presence of the plant extracts showed biofilm formation.

Chapter 4:

Discussion.

Discussion:

The hypothesis, H_0: Crude extracts from plant materials will have no antimicrobial, antibiofilm, no mode of action or autolytic activity on selected microbial species, was rejected due to the results obtained and the discussion below.

Plant extracts preparation:

The plant extractions and preparations were done by the University of Namibia. Since the ethanol and methanol extraction methods showed to have antimicrobial activity, a possible reason for the results obtained, is that the active compounds within the plant roots or leaves might be of polar nature. Since not much was known regarding the plant species and the chemical compounds within the plant root or leaves, more investigation was needed to make an accurate conclusion. Possible troubleshooting was that the plant materials might have been harmed during extraction, thus loss of compounds could have skewed results and environmental changes could have led to unexpected results.

SDS-PAGE for protein profile:

Various bands were expected on the gel. Small-scale extraction was expected to have thinner bands when compared to large-scale extraction. On the gel of the small-scale extraction, there were several faint bands as expected (Figure 4). The large-scale extraction showed bright bands as expected due higher protein concentration present from a large-scale extraction. LiCl was expected to show more bands as LiCl was used to extract tightly bound proteins and was more specific to cell wall proteins (133,134). LiCl extraction showed fewer bands when compared to SDS extraction (Figure 5). LiCl extraction method was said to target hydrophobic bound proteins (96), but because LiCl was a chaotropic agent it caused irreversible damage to proteins (57,58). Therefore, it was a possible reason for the decreased number of visible bands. The solution was to use an extraction solvent that was just as adequate as LiCl, but less harsh on proteins. SDS solubilises the cell wall and was used to extract proteins from the cell wall and the cytoplasm (133,134). SDS extraction methods was said to target hydrophobic and cysteine-bond proteins (96). The possible reason for more bands in the SDS extracted samples was due to the SDS activity as mentioned above. It was also possible that protein loss or damage had occurred or protein was absorbed to the plastic of the Falcon tubes during the extraction process (135). Protein

concentration differences was also a reason for the difference in the amount and number of bands on the gel. The minimal weight of detectible protein using Coomassie stay was 100 ng (136) which explained the absence of bands due to the low concentration. A solution was to add the same concentration of protein into the wells.

Extracts 4.2 and 5.2 showed unclear bands when the extraction method was used (Figure 12). A possible reason was that the extracts were not properly dissolved in DMSO resulting in low protein concentration. A possible solution was to dissolve the extracts in stronger solution or to conduct a protein extraction method on the extracts. The extracts were crude extracts; thus, purification of the extracts was also a possible solution. Protein concentration was too low, thus large-scale production was needed to increase protein concentration.

Autolytic extraction (Zymogram):

The expected results of a zymogram were oblique bands against a dark background if the enzyme was renatured and active in cutting the substrate. Better results were expected using the natural substrate which each microbial species than compared to using an alternative substrate of *M. luteus*. This was the case. More bands were seen across the zymogram as shown in Figure 9. *S. aureus* against *M. luteus* display the same results as seen in literature (137), where the hydrolytic bands correlate to the 5 forms of the AtlA hydrolase. The results showed that hydrolytic activity was higher in the presence of the natural substrate and led to the idea of substrate specificity.

The zymogram with the whole cell protein profile in the absence and presence of extract 4.2 and 5.2 (Figure 11b) showed multiple bands ranging from thin and thick bands. No extraction method was used thus the whole protein profile was seen. Multiple bands were expected in this case. Figure 12 showed smears on the SDS-PAGE expect the protein marker and positive control. A possible reason was that the protein concentration was too low as definite bands are not present or separation of proteins was not adequate. When looking at the zymogram however, autolytic activity was seen very faintly by the clear zones on the dark background showing that even in low protein concentrations; the enzymes were still active and cleaved the cell wall. In the presence of the plant extract 4.2 and extract 5.2, more autolytic activity was present (more clear bands, Figure 12b).

Large-scale production of *S. aureus (43300)* on the SDS – PAGE gel and zymogram showed that in the absence of the plant extract, more proteins are extracted (Figure 13a). There were fewer proteins in the presence of the plant extract 4.2 and 5.2 as less and fainter bands were seen. The plant extracts had an influence on the microbial species as the production of the proteins was less in the presence of the plant extracts when compared to the absence of the plant extracts. The conclusion made was that the presence of the plant extracts decreased growth of the microbial species thus decreased protein production.

High Performance Liquid Chromatography (HPLC) and Mass/Spec:

The expected result for HPLC was separated, well defined peaks. In most cases the results obtained were expected. Many separate peaks were shown, and the masses associated with each peak. Markers of extract 4.2 in the negative mode were as follows: Marker 1 was identified via the Golm Metabolome Database as Hexonic acid. Synonyms for Hexonic acid were 2,3,4,5,6-pentahydroxy-hexanoic acid, aldonic acid, altronic acid and glyconic acid to name a few. Hexonic acid had a melting point of 131 °C. No antimicrobial properties had been linked to this compound. Marker 2 was identified via the PubChem Database as 3-Carboxy-2-propynoate. Synonyms for marker 2 were 2-butynedioic acid, 3-carboxyprop-2-ynoate and acetylene-dicarboxylate to name a few. Marker 2 had a boiling point of ±362.4°C. No antimicrobial properties had been linked to this compound. Marker 3 was identified via the KEGG Database as 9,10,18-trihydroxyoctadecanoic acid. Synonyms for marker 3 were 9,10,18-Trihydroxystearic acid, octadecanoic acid and 9,10,18-trihydroxy to name a few. No antimicrobial properties had been linked to this compound, but this compound was linked to the biosynthesis of cutin acid C18 family.

Marker 4 was identified via the KEGG Database as 9, 10, 13-trihydroxy-11-octadecenoic acid. A synonym for this marker was (11E)-9,10,13-Trihydroxy-11-octadecenoic acid. This acid was a trihydroxyoctadecenoic acid metabolite of linoleic acid and was involved in the regulation of prostaglandin synthesis. It has a density of ±1.1 g/cm^3 and boiling point of ± 543.4°C. No antimicrobial properties had been linked to this compound. Marker 5 was identified via the KEGG as 8-(5-hexyl-3-furyl)octanoic acid. A synonym for this marker was 2-Furanoctanoic acid. It had a density of ±1.0 g/cm^3 and boiling point of ±426.7°C at 760 mmHg. No antimicrobial properties had been linked to this compound. Marker 6 was identified via the PubChem Database as

sodium-3-hydroxy-3-methyl-6-(2,2,3-trimethylcyclopentyl)-hexanoate. No antimicrobial properties had been linked to this compound.

Marker 7 was identified via the Golm Metabolome Database as Sebacic acid. A synonym for sebacic acid was 1,8-octanedicarboxylic acid. Sebacic acid had a density of 1.209 g/cm^3 and boiling point of 294.4 °C at 100 mmHg. Sebacic acid was a saturated, long straight chain naturally occurring dicarboxylic acid. Sebaceus was Latin for tallow candle and refers to its use in candle manufacturing. A derivative of Castrol oil was sebacic acid. Carnitine-acylcarnitine translocase deficiency and acyl-CoA dehydrogenase deficiency was associated with sebacic acid. Sebacic acid was used as a monomer for lubricants, nylon 610 and plasticizers also as an intermediate for antiseptics, aromatic compounds and painting materials. Sebacic acid was used as a treatment for brain cancer (129). Sebacic acid was broken down into succinyl CoA and acetyl-CoA used in the Krebs' cycle (130). Sebacic acid had activity against *Staphylococcus* and *B. subtilis* species (138). A positive influence was seen on baby's skin with sebacic acid (131). Sebacic acid decreased hyperglycaemia, thus used as an alternative dietary source of energy (132). Sebacic acid served for anaerobic bacterial strains identification (2). Due to its use as an antiseptic, antimicrobial activity in extract 4.2 might have been due to sebacic acid.

Markers of extract 5.2 in negative mode were as follows: Marker 1 was identified via the NIST Database as 9,9',10,10'-tetramethoxy-5,5',6,6',7,7',8,8'-octahydro-2,2'-bi-anthracene-1,1',4,4'-tetrone. On its own, there was minimal information regarding this marker. A derivative 1-(1,2,3,4,5,6,7,8-Octahydro-2,3,8,8tetramethyl-2-naphthalenyl)-ethanone was used in fragrances. No antimicrobial activity had been linked to this compound. Marker 2 was identified via the PubChem Database as bis[2-(4-biphenylyl)-2-oxoethyl]oxalate. Minimal information on this compound was known. No antimicrobial activity had been linked to this compound.

Marker 3 was identified via the KEGG Database as sodium-2-{4-[2-oxocyclopentyl)methyl]-phenyl}-propanoate hydrate (1:1:2). Synonyms were Benzeneacetic acid, α-methyl-4-[(2-oxocyclopentyl)methyl]-, sodium salt, hydrate (1:1:2), Loxoprofen Sodium and Sodium 2-{4-[(2-oxocyclopentyl)methyl]phenyl}-propanoate hydrate (1:1:2) to name a few. Sodium 2-[4-(2-oxocyclopentyl-methyl)phenyl]propionate dihydrate (CS-600) was investigated as a new anti-

inflammatory agent for the inhibition of prostaglandin (PG) synthesis in vivo and in vitro by Matsuda *et al* 1984 (139). In rats, a decrease in the level of urinary PGE_2 and PGF_{2a} was caused by CS-600. Matsuda *et al* concluded that CS-600 was a new phenylpropionate anti-inflammatory agent with analgesic, antipyretic activities and weak gastrointestinal ulcerogenicity (139) and CS-600 was an effective inhibitor of inflammatory prostaglandins production in vivo (1,139,140). This compound showed to have anti-inflammatory properties which might have been the causative agent activity of extract 5.2.

Marker 4 was identified via the PubChem Database as sodium (2Z,4E)-5-(2,4-dimethoxyphenyl)-3-methyl-2,4-pentadienoate. Minimal information was known on this compound. No antimicrobial activity had been linked to this compound. Marker 5 was identified via the PubChem as sodium-[(5S,6S)-5-hydroxy-6,7,8,9-tetrahydro-5H-benzo[7]annulen-6-yl]acetate. Synonyms were 5H-Benzocyclo-heptene-5,6-diol-6,7,8,9-tetrahydro-,5-acetate,trans, trans-6,7,8,9-Tetrahydro-5H-benzocycloheptene-5,6-diol-5-acetate and 1,2-trans-1-Acetoxy-2-hydroxybenzo-suberan. No antimicrobial activity had been linked to this compound. Marker 6 was identified via the PubChem Database as sodium (5-hydroxy-5,7,8,9-tetrahydro-6H-benzo[7]annulen-6-ylidene)-acetate. Synonyms for this compound were Acetic acid, 2-(5,7,8,9-tetrahydro-5-hydroxy-6H-benzocyclohepten-6-ylidene)- and sodium salt. No antimicrobial activity had been linked to this compound.

Markers of extract 4.2 in positive mode were as follows: Marker 1 was identified via the Golm Metabolome Database as 3-(2-hydroxyphenyl)propanoic acid. Synonyms were Benzenepropanoic acid, 2-hydroxy, melilotic acid and CN1RK42MAD to name a few. It was slightly soluble (in water) and was a weakly acidic compound. It was found in food and had a melting point of 82 - 83 °C. It was involved in enzyme reactions, but no antimicrobial activity had been linked to this compound. Marker 2 was identified via the NIST as 2-phenyl-6-(1-phenylethyl)-6,7-dihydro-5H-imidazo[1,5-b][1,2,4]triazol-5-one. Minimal information on this compound was found. No antimicrobial activity had been linked to this compound. Marker 3 was identified via the NIST Database as tricycle[7.2.2.0~2,7~]trideca-2,4,6,12-tetraene-10,10,11,11-tetracarbonitrile. Minimal information on this compound was found. No antimicrobial activity had been linked to this compound.

Marker 4 was identified via the KEGG Database as (1R,3S)-9-methoxy-1,3-dimethyl-3,4-dihydro-1Hbenzo[g]isochromene-5,10-dione. Synonyms were Eleutherin and (1R,3S)-9-Methoxy-1,3-dimethyl-3,4-dihydro-1H-benzo[g]iso-chromene-5,10-dione to name a few. It had a density of ±1.3 g/cm^3 and boiling point of ±465.3°C at 760 mmHg. No antimicrobial activity had been linked to this compound. Marker 5 was identified via the Golm Metabolome Database as 5,7-dihydroxy-2-phenyl-4H-chromen-4-one. Synonyms were O948D0K9BQ, 4H-1-Benzopyran-4-one and 3,7-dihydroxy-2-phenyl- to name a few. No antimicrobial activity had been linked to this compound. Marker 6 was identified via the Golm Metabolome Database as 5,7-dihydroxy-2-phenyl-4H-chromen-4-one. No antimicrobial activity had been linked to this compound.

Marker 7 was identified via the Golm Metabolome Database as 3-hydroxy-2-phenyl-4H-chromen-4-one. Synonyms were 3-hydroxy-2-phenylchromen-4-one, 3-hydroxy-2-phenylchromone and 3-hydroxyflavanol to name a few. No antimicrobial activity had been linked to this compound. Marker 8 was identified via the PubChem Database as dibutyl[2-(2-thirnyl)vinyl]phosphate. Minimal information on this compound was found. No antimicrobial activity had been linked to this compound. Marker 9 was identified via the NIST Database as N,N'-(9-9propionyl-9H-purine-2,6-diyl)dipropanamide. Minimal information on this compound was found. No antimicrobial activity had been linked to this compound. Marker 10 was identified via the PubChem Database as 1,1''-(phenylphosphinediyl)bis(1H-pyrrole). Minimal information on this compound was found. No antimicrobial activity had been linked to this compound.

Marker 11 had a mass of 211.0763, elemental composition of 0.4, 0.0, C14H11O2, formula of C14H11O2 and was identified via the KEGG as benzil. Synonyms were 1,2-diphenylethane-1,2-dione, 1,2-Ethanedione and 1,2-diphenyl to name a few. It had a density of 1.23 g/cm3 and melting point of 94.0 to 96.0 °C. It was an organic compound and was used as the building block in organic synthesis. No antimicrobial activity had been linked to this compound. Marker 12 was identified via the NIST as 1-(2,4-diamino-6-pteridinyl)-2-phenyl-1,2-ethanedione. Minimal information on this compound was found. No antimicrobial activity had been linked to this compound. Marker 13 was identified via the PubChem Database as [3,6,9,15-tetraazabicyclo[9.3.1]pentadeca-1(15),11,13-trien-6-yl-methyl]phosphonic acid. This compound was used as a diagnostic compound for the targeting of a chemokine receptor, but no antimicrobial activity had been linked to this compound.

Markers of extract 5.2 in positive mode were as follows: Marker 1 was identified via the Golm Metabolome Database as 4-(methylsulfanyl)-2-oxobutanoic acid. Synonyms were 4-Methylthio-2-oxobutanoic acid, 2-keto-4-methylthiobutyric acid, 4-methylthio-2-oxobutanoate, 2-Oxo-4-thiomethy-lbutyric acid and 2-oxo-4-methylthiobutanoate to name a few. This compound was a member of the class fatty acids. It was slightly soluble and was a weak acid. It was found in all living species and had been involved with several metabolic disorders. It was used as a food flavouring agent, but no antimicrobial activity had been linked to this compound.

Marker 2 was identified via the PubChem Database as 2-methyl-2-propanyl (4-aminobutyl)(2,2-dimethyl-9,14{[(2-methyl-2-propanyl)oxy]carbonyl}-4-oxo-3-oxa-5,9,-14-triazaheptadecan-17-yl)carbo-nate. Minimal information was known on this compound. No antimicrobial activity had been linked to this compound. Marker 3 was identified via the PubChem Database as 2-methyl-2-propanyl (3-aminobutyl)(2,2-dimethyl-9,14{[(2-methyl-2-propanyl)oxy]carbonyl}-4-oxo-3-oxa-5,9,14-triazahepta-decan-17-yl)carbonate. Minimal information was known on this compound. No antimicrobial activity had been linked to this compound.

Marker 4 was identified via the PubChem Database as methyl N-[11-(dimethylamino)undecanoyl]-L-seryl-L-lysyl-L-leucinate. Minimal information was known on this compound. No antimicrobial activity had been linked to this compound. Marker 5 was identified via PubChem Database methyl N-[12-aminodecanoyl)-L-seryl-L-lysyl-L-leucinate. Synonyms were L-Leucine, N-(12-amino-1-oxododecyl)-L-seryl-L-lysyl-, methyl ester, hydrochloride (1:1) and methyl N-(12-aminododecanoyl)-L-seryl-L-lysyl-L-leucinate hydrochloride (1:1) to name a few. Minimal information was known on this compound. No antimicrobial activity had been linked to this compound.

Marker 6 was identified via the PubChem Database as N,N,N’,N’,N’’,N’’-hexabutyl-N’’’-(3,5-dioxo-2,3,4,5-tetra-hrydro-1,2,4-triazin-6-yl) phosphorimidic triamide. Minimal information was known on this compound. No antimicrobial activity had been linked to this compound. Marker 7 was identified via the PubChem Database as N~6~-(4-aminocyclohexyl)-9-cyclopentyl-N~2~-{1-[4-(dimethylamino)-butyl]-4-piperidynyl}-9H-purine-2,6-diamine. Minimal information was known on this compound. No antimicrobial activity had been linked to this compound. Marker 8 was identified via the PubChem Database as N~6~-(4-aminocyclohexyl)-9-cyclopentyl-N~2~-{1-[3-

(dimethylamino)propyl]-4-piperidynyl}-9H-purine-2,6-diamine. Minimal information was known on this compound. No antimicrobial activity had been linked to this compound. Marker 9 was identified via the PubChem Database as 2-methyl-2-propanyl[14-(3-cyanopropyl)-2,2-dimethyl-4-oxo-3-oxa-5,9,14-triazahepta-decan-17-yl) carbomate. Minimal information was known on this compound. No antimicrobial activity had been linked to this compound.

Marker 10 was identified via the PubChem Database as N-(1-{[6-({3-[(4-aminobutyl)amino)-6-oxohexyl]amino} -1-oxo-2-propanyl)cyclohexane carboxamide. Minimal information was known on this compound. No antimicrobial activity had been linked to this compound. Marker 11 was identified via PubChem Database as 2-(dodecyloxy)ethyl beta-D-galactopyranoside. Minimal information was known on this compound. No antimicrobial activity had been linked to this compound. Marker 12 was identified via the PubChem Database as N,N-dimethyl-4-[(tri-1-pyrrolidinylphospho-ranylidene)-amino]aniline. Synonyms were 1,4-Benzenediamine, N1,N1-dimethyl-N4-(tri-1-pyrrolidinylphosphoranylidene) and pyrrolidinylphosphoranylidene)amino]aniline to name a few. It had a density of ±1.2 g/cm^3 and boiling point of ±513.6°C at 760 mmHg. No antimicrobial activity had been linked to this compound. Marker 13 was identified via the NIST Database as 3,6,9,12-tetraoxadocosan-1-ol. A synonym was 2-(2-(2-(2-decyloxy-ethoxy)-ethoxy)-ethoxy)-ethanol. It had a density of 1.0±0.1 g/cm^3 and boiling point of 423.2±30.0 °C at 760 mmHg. No antimicrobial activity had been linked to this compound.

Marker 14 was identified via the MassBank Database as triphenylphosphine oxide. A synonym was triphenylphosphine oxide. It had a density of 1.212g/cm^3 and melting point of 154 to 158 °C. It was an organo-phosphorus compound and a side product in reactions involving triphenylphosphine. No antimicrobial activity had been linked to this compound. Marker 15 was identified via NIST as 3-methoxy-N-phenylaniline. Minimal information was known on this compound. No antimicrobial activity had been linked to this compound. Marker 16 and was identified via the PubChem Database as [3,6,9,15-tetraazabicyclo-[9.3.1]pentadeca-1(15),11,13-trien-6-ylmethyl]phosphonic acid. No information was known on this compound. No antimicrobial activity had been linked to this compound. Marker 17 was identified via the NIST Database as 2,4-dimethyl-1,2,4-triazaspirol[4.7]dodecane-3-thione. Minimal information was known on this compound. No antimicrobial activity had been linked to this compound.

Marker 18 was identified via the KEGG Database as ethyl 2-(phthalazinyl)hydrazinecarboxylate. Minimal information was known on this compound. No antimicrobial activity had been linked to this compound. A possible reason for inaccurate results could have been due to sample preparation errors. A solution was to prepare all samples aseptically and the use of pure, high grade chemicals. Column and equipment calibration errors could have also skewed results. A solution was to calibrate the column regularly and keep it clean. Only one compound within extract 4.2 and 5.2 showed antimicrobial and anti-inflammatory results and could have been the reason for the results obtained. Further investigation was needed to conclude if these compounds were the only reason for the results seen.

Autolytic activity of LiCl and SDS extracted cell wall hydrolases:

The expected results were a decrease in absorbance as the enzymes were cleaving the cells. Peptidoglycan was a macromolecule and the intact substrate was not soluble. As soon as the substrate was broken up (autolytic activity), the molecule became soluble, thus decreasing the absorbance. Absorbance fluctuation and increase in absorbance was not the expected results. A possible reason was that the microbial species overcame the hydrolase activity and continued to grow as there was a decrease in absorbance in the beginning of the study as shown in Figure 20 and Figure 21. A solution was to increase the hydrolase concentration. Another possible reason for the results obtained was errors in the experiment for example inadequate temperature control within the incubator. A solution was to add a thermometer inside to incubator to check the consistence of temperature. The time study was also decreased to the minimal time for enzyme activity.

The expected results were a decrease in the absorbance as the peptidoglycan hydrolases lysed the cell. The cell wall was expected to remain the same absorbance as it was not treated with enzyme. In the presence of the plant extract, a greater decrease was expected, however this was not the case. In the presence of the plant extracts, the decrease in absorbance was less than in the absence of the plant extracts (Figure 22). A possible reason was that the enzymes were more active in the absence of plant extracts or the plant extracts were inactivating the enzymes, thus causing the enzymes not to cleave the cell wall. A possible solution was to increase the enzyme concentration in the presence of the plant extracts. The cell wall had relative constant absorbance as expected.

Minimum Inhibitory Concentration (MIC) / Double Dilution Assay:

The expected results were inhibition of growth as seen from literature. All the plant extracts showed growth within the first dilution series. A possible reason for this result was that the extract concentration was too low resulting in the partial inhibition of the microbial specie growth. The microbial species overcame the extract's inhibition activity as there was no growth without the dilution of the extracts suggesting that the extracts must not be diluted. A solution to this problem was to increase the concentration of the extracts. Since the active compounds of the plant species had not yet been identified, only arbitrary MIC was determined. A solution was to identify the active compounds and purify the compound for experimentation.

Dilution activity assay:

The expected results were a gradual increase in absorbance as the activity assay and MIC had shown that the microbial species continued to grow. The results obtained were not to be expected as there was fluctuation among the data. *S. aureus (43300)* was the closest to the expected results. In the dilution assay, it was noticeable that due to the fluctuation in absorbance, growth was present in the MIC assay at ½ concentrations (1x dilution series). A possible reason was that the microbial species overcame the extract's activity shown by the fluctuation. A solution to this problem was to increase the concentration of the extracts. Identification and purification of active compounds was also a possible solution.

Antimicrobial assay (Spot-on-lawn assay):

Extract 4.2 showed antimicrobial activity as seen by the inhibition zone on the agar plate. Extract 5.2 showed no inhibition zone. The extracts were crude extractions that resulted in the inactivity as seen by the lack of inhibition zones of extract 5.2. The extracts were to be purified to obtain possible activity. The DMSO used as the dissolving agent was insufficient in dissolving the extract, thus stronger dissolving agents might be tested.

BCA assay:

The unknown concentration of *S. aureus (43300)* in the absence of plant extract 4.2 and 5.2 with an OD_{562} of 2.356 was 3.797 mg/ml ~ 3.80 mg/ml. The unknown concentration of *S. aureus (43300)* with an OD_{562} of 1.602 in the presence of extract 4.2 was 2.576 mg/ml ~ 2.58 mg/ml. The unknown concentration of *S. aureus (43300)*

with an OD_{562} of 1.296 in the presence of extract 5.2 was 2.081 mg/ml ~ 2.08 mg/ml. As the results showed, there was a decrease in protein concentration. This result explained the decrease autolytic activity as seen in the zymogram (Figure 13). BCA was not sensitive to the 4% SDS extraction method, thus BCA assay was used for protein concentration.

Biofilm and biofilm eradication:

In the presence of the plant extracts, it was expected to show a decrease in biofilm formation (antibiofilm or biofilm eradication) when compared to the control in the absence of the plant extracts. This was a case only for *S. aureus (12600)* in the presence of extract 4.2. *S. aureus (12600)* in the presence of extract 5.2 showed biofilm formation and *S. aureus (43300)* in the presence of the plant extracts showed biofilm formation. A possible reason was that the presence of the plant extracts did not influence the biofilm or the formation of biofilm. Another possible reason was that the plant extract concentration was too low to influence the biofilm or that the microbial species did not have enough stress to produce biofilm. A possible solution was the increase the plant concentration or to increase the stress component to be able to increase the biofilm formation.

Conclusion:

H_0: Crude extracts from plant materials did have antimicrobial, antibiofilm, mode of action or autolytic activity on selected microbial species, thus the hypothesis was rejected. H_0 was rejected as extract 4.2 showed antimicrobial activity as seen in the spot-on-lawn assay. H_0 was rejected as there was a decrease in autolytic bands in the presence of plant extracts. H_0 was rejected as the profiles of zymograms in the absence and presence of plant extracts were different. H_0 was rejected as antimicrobial activity, biofilm activity and hydrolase activity was shown. H_0 was rejected as there was a decrease and increased interaction between the peptidoglycan hydrolases and the compounds within the plant extracts.

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Chapter 5:

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