The antimicrobial activities of selected local medicinal herbs against *Streptococcus pneumoniae*

By

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DECLARATION

I hereby declare that this dissertation, submitted for the Master of Philosophy in Biomedical Sciences at the University of Malawi, College of Medicine, is the sole result of my own investigation. Acknowledgment has been made to the work and/ contributions made by others.

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ABSTRACT

Streptococcus pneumoniae (S. pnuemoniae) is a major cause of invasive pneumococcal infections. Current treatment involves antibiotic therapy and vaccination. The emergence of antibiotic resistance and vaccine serotype replacement negatively affects the control of pneumococcal infections, necessitating the need for other treatment alternatives. We investigated the antimicrobial activity of local herbs against pneumococcal serotypes 1 and 6A. Dry powdered leaves/stem material (50 g) from 6 plants; *Annona senegalensis (A. senegalensis), Bidens pilosa (B. pilosa), Dichrostachys cinerea (D. cinerea), Erythrina abyssinica (E. abyssinica), Lippia javanica (L. javanica)* and *Trichodesma zeylenicum (T. zeylenicum)*) were extracted using 200 mL of distilled water or methanol. Disc diffusion method was employed for antimicrobial activity testing. The minimum inhibitory concentrations (MIC's) and minimum bactericidal concentrations (MBC's) were determined using agar dilution method. Column chromatography was employed for fractionation of crude extracts. Penicillin and DMSO (1 % in DPBS) were used as positive and negative control, respectively. Water extraction yielded 5.1 % (10.2 g/200 g) of the crude extract material versus 12.1 % (24.3 g/200 g) from methanol. The *Bp* (1.0 mg/mL) and *Ea* (1.0 mg/mL) water extracts showed highest activity against serotype 1 (zone of inhibition (ZI) ~9.0 mm each), while *Bp* and *Dc* (ZI ~11.0 mm) exhibited the highest activity against serotype 6A. The methanol extracts of *B. pilosa* (1.0 mg/mL) and *D. cinerea* (1.0 mg/mL), showed the highest activity against serotype 1 (ZI ~15.6 ± 0.57, MIC<100 μg/mL, MBC ~ 2 mg/mL and ZI ~13.6 ± 0.57 mm, 100 ≤ MIC <512 μg/mL, MBC ~ 4 mg/mL respectively) and serotype 6A (ZI ~24.3 ± 0.00 mm, MIC <100 μg/mL, MBC ~2 mg/mL and ZI ~15 ± 0.00 mm, 100 ≤ MIC<512 μg/mL, MBC ~ 4 mg/mL respectively). The ZI for both serotypes 1 and 6A were 0.00 mm for DMSO (1 %) and 25 ± 0.00 mm for penicillin (25 μg). Column fractionation

was conducted on methanol extracts of *B. pilosa* and *D. cinerea*. Of the 9 fractions from *B. pilosa*, only one fraction showed activity against serotypes 1 and 6A (ZI ~10 mm). Of 11 fractions from *D. cinerea,* only one showed activity against pneumococcal serotypes 1 (ZI ~8 mm) and 6A (ZI ~7 mm). Phytochemical analysis of methanol extracts showed higher content of total polyphenols, flavonoids, 2, 2-Diphenyl-1-Picrylhydrazyl (DDPH), Ferric Reducing Antioxidant Power (FRAP) and total alkaloids compared to water extracts. The Brine Shrimp's lethality analysis for highly active plant (*Bp* and *Dc)* showed no and mild toxicity respectively. We recommend further exploration of these two plants as potential antimicrobials against pneumococcal infections.

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ABBREVIATIONS AND ACRONYMS

ASM	Annual Scientific Meeting
CSF	Cerebrospinal Fluid
CoM	College of Medicine
CoMREC	College of Medicine Research and Ethics Committee
CAM	Complementary Alternative Medicine
CC	Column Chromatography
DCM	Dichloromethane
DMSO	Dimethyl Sulfoxide
DPBS	Dubious Phosphate Buffered Saline
DPPH	2, 2-Diphenyl-1-Picrylhydrazyl
EtOH	Ethanol
EtOAc	Ethyl acetate
FRAP	Ferric Reducing Antioxidant Power
H_2O	Water
H_2SO_4	Sulphuric Acid
HPLC	High Performance Liquid Chromatography
IPD	Invasive Pneumococcal Disease
MeOH	Methanol
MLW	Malawi Liverpool Wellcome Trust
MeOH	Methanol
PCV	Pneumococcal Conjugate Vaccine

PTLC	Preparative Thin Layer Chromatography
RF	Retention Factor
TAC	Total Alkaloid Content
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
TLC	Thin Layer Chromatography
VISR	Vaccine Induced Serotype Replacement
WHO	World Health Organization

CHAPTER I: GENERAL INTRODUCTION

1.1 Introduction to *Streptococcus pneumoniae*

1.1.1 *Streptococcus pneumoniae* biology

The bacteria, *Streptococcus Pneumoniae (S. Pneumoniae)* are lancet-shaped, gram-positive and facultative anaerobic organisms (1). They appear in pairs (diplococci) and may also occur singularly or in short chains (2). *S. Pneumoniae* is part of human nasopharyngeal commensal microbiota and can be isolated in healthy adults and children (3,4). Most pneumococci are encapsulated, their surfaces are composed of complex polysaccharides that surround the bacteria and form a protective barrier (5). *S. pneumoniae* has over 96 unique capsular serotypes, with differences in monosaccharide composition and linkage, as well as other modifications, such as acetylation (6,7). The capsular serotypes are further classified into 46 Serogroups based on antigenic similarities (8,9). For almost all serotypes, all the genes involved in capsule synthesis are located in a region between the genes *dexB* and *aliA* labeled the capsule synthesis (*cps*) locus (10). The complete genome of a representative strain (TIGR4) of *S. pneumoniae* was published for the first time in 2001 (9). *S. pneumoniae* has surface proteins that include hyaluronate lyase (*Hyl*), pneumolysin (*Ply*), two neuraminidases (*NanA* and *NanB*), major autolysin *(LytA*), choline binding protein A (*CbpA*), pneumococcal surface antigen A (*PsaA*) and pneumococcal surface protein A (*PspA*) (11), (Fig. 1).

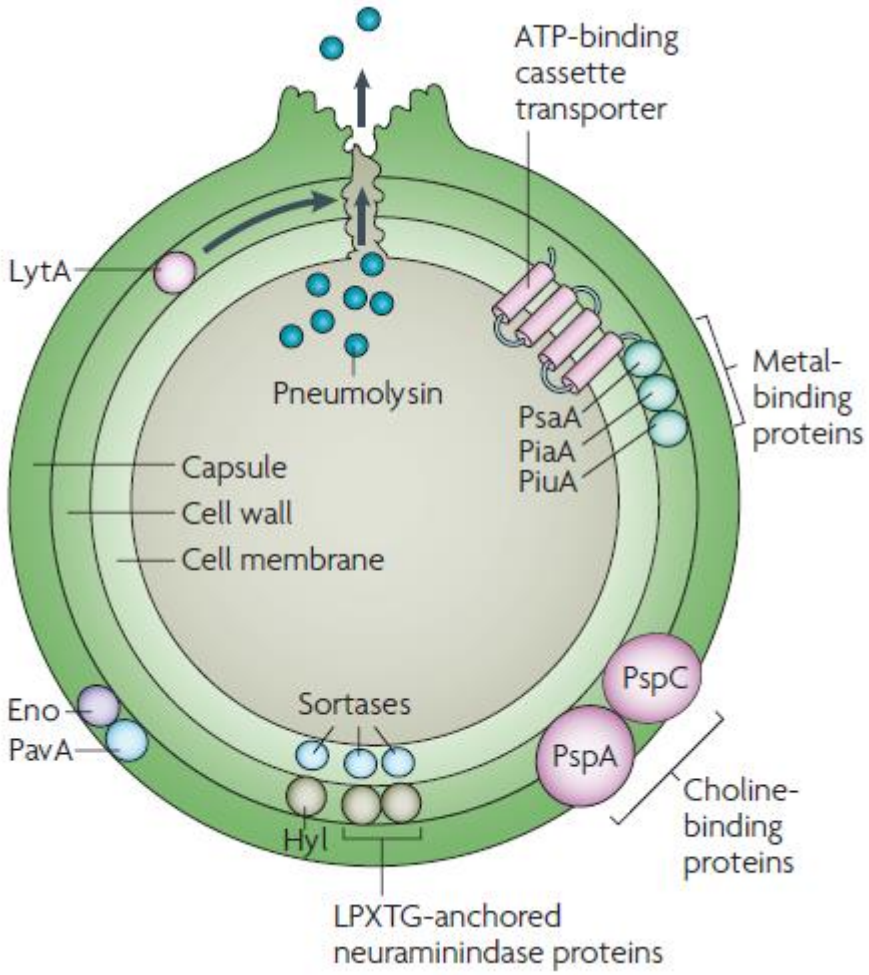

Figure 1: Structure of S. pneumoniae showing virulence factors.

The capsule, the cell wall, choline-binding proteins, pneumococcal surface proteins A and C (PspA and PspC), the LPXTG-anchored neuraminidase proteins, hyaluronate lyase (Hyl), pneumococcal adhesion and virulence A (PavA), enolase (Eno), pneumolysin, autolysin A (LytA) and the metal-binding proteins pneumococcal surface antigen A (PsaA), pneumococcal iron acquisition A (PiaA) and pneumococcal iron uptake A (PiuA) (12–14).

1.1.2 Pathogenicity and virulent factors of *S. pneumoniae*

There are a variety of proteins and toxins that are expressed by *S. pneumoniae* that drive its pathogenesis. One of the major virulence factors include Capsular polysaccharides (15,16). Capsular polysaccharides are antigenic and are used for classifying pneumococci into serotypes (9). The capsule allows the pneumococcus to evade mucociliary clearance, complement deposition and

opsonophagocytosis (5). While over 96 serotypes have been identified, global surveillance has shown that only 20 serotypes cause more than 70–80% of Invasive Pneumococcal Disease (IPD) (8,17). The serotype prevalence may vary by geographic region, patient age and time period of surveillance (18).

Another major determinant of pneumococcal pathogenicity is Pneumococcal surface protein (PspA). This protein has a variable size of 67 to 99 kDa and is located on the cell wall of pneumococci (19). It is a protective antigen that reduces complement mediated clearance and phagocytosis of *S. pneumoniae* (20). PspA has a highly polar electrostatic charge, which results in capsular charge stabilization through the electropositive end of the molecule and in prevention of complement activation through the predominant electronegative part of PspA (21). In addition to the pneumococcal surface protein, there are also other pneumococcal specific proteins like pneumolysin, autolysins, neuraminidase, Pneumococcal Surface Protein C (PspC), Pneumococcal Surface Adhesin A (PsaA), Pneumococcal Surface Adherence and Virulence Factor A *(PavA*) which play a vital role in pneumococcal pathogenesis and colonisation (22–24). Pneumococcal pilus is also another major determinant of pneumococcal pathogenesis. The pilus is encoded by the *rlrA* islet, which encodes three pilus subunit proteins, including *RrgA*, *RrgB* and *RrgC* (25). This pilus plays an important role in adhesion of the organism to humans, and is mediated mainly by RrgA (24). Hyaluronidase, produced by the *S.pneumoniae* also facilitates pneumococcal pathogenesis by degrading connective tissue of the host that leads to the invasion of the organism (26). This observation supports that *S. pneumoniae* strains with higher hyaluronidase activity spread more effectively within the host as indicated by their ability to pass the blood-brain barrier (26).

Pneumococci spreads from person to person by direct respiratory droplets (2). It can progress to invasive pneumococcal disease (IPD) although colonization with *S. pneumoniae* is often

asymptomatic in health individuals (27). After hosts asymptomatic colonization, if the bacterium is not cleared by the immune system, it spreads through horizontal dissemination into the lower airways and other organs and tissues, and becomes pathogenic (14). The migration of *S. pneumoniae* to sterile tissues and organs like the brain and the central nervous system is the main cause of pneumococcal invasive disease (14). The most common manifestations of pneumococcal disease include acute otitis media (infection of the middle-ear space) and pneumonia (infection of the terminal airways) (13). *S. pneumoniae* is also a leading cause of bacterial meningitis in children under the age of five (28). Bacteraemic pneumococcal infection is also common and occurs when the organism spreads directly from its niche in the pharynx into the blood (occult bacteraemia) (13,28). *S. pneumoniae* symptoms may include headache, lethargy, vomiting, irritability, fever, nuchal rigidity, cranial nerve signs, seizures, and coma (28).

1.1.3 Host immune system responses to *S. pneumoniae*

Protection against pneumococcal infections is mediated by opsonin-dependent phagocytosis (29,30). Antibody-initiated complement-dependent opsonisation, which activates the classic complement pathway, is considered the major immune mechanism protecting the host against pneumococcal infections (16,30). Various macrophage receptors are involved in recognition and phagocytosis of *S. pneumoniae*, including Fc receptors, complement receptors, platelet-activating factor receptors, scavenger receptors, and CD14/toll-like receptor (TLR) complexes (31). The mechanism of clearance depends on the interaction of the type-specific antibodies (IgA, IgM, IgG), complement, and neutrophils or phagocytic cells from lung, liver, and spleen (16). In circulation, IgM and IgG antibodies are essential, while on mucosal surfaces IgA antibodies form an important part of protection against *S. pneumoniae* colonization (32). Congenital deficiencies in either immunoglobulin

or complement may therefore result in a predisposition to pneumococcal infection. In elderly populations, aging leads to reduced production of antibodies, immunoglobulin class switching, and cell maturation, which promotes colonization by *S. pneumoniae* (30).

1.1.4 Risk factors for pneumococcal infection

The most common and important risk factor for pneumococcal infection is age (33,34). The incidence of pneumococcal disease is up to 50 times higher in children < 2 years of age and in adults > 65 years of age, than in adolescents (35). Children <2 years of age are at increased risk because of their immature immune system while adults >65 years of age have weakened immune system and potentially other health problems which may increase their likelihood of obtaining pneumococcal infection (36–38). Another risk factor is crowding that happens in hospitals, day-care centres, and prisons which increase horizontal spread of pneumococcal strains (16). Young children are considered to be most important vector for horizontal dissemination of pneumococcal strains within the community because they have the highest frequency of pneumococcal colonisation and the highest crowding index (35). Other risk factors for pneumococcus colonisation are smoking, alcoholism, immunosuppressive conditions like human immunodeficiency virus (HIV) infection, and comorbidities such a chronic obstructive pulmonary disease, diabetes mellitus, chronic liver disease and neurological disease (39,40). For example patients with HIV infection are 10 to 100 times at higher risk for pneumococcal pneumonia and bacteremia than non-HIV infected persons due to the defects in their cellular and humoral immunity (41,42).

The global burden of *S. pneumoniae*

The burden of disease is highest in the youngest and oldest sections of the population in both more and less developed countries (16). In 2017, the incidence and mortality rates of IPD in adults aged ≥ 65 years was 41.8 and 9.56 per 100,000 (43,44).The World Health Organization (WHO) estimates that over 1 million children die of IPD every year, mostly in developing countries (16,45–48). India (68 700 deaths, uncertainty range [UR] 44 600 – 86 100), Nigeria (49 000 deaths, [UR] 32 400 – 59 000), the Democratic Republic of the Congo (14 500 deaths, [UR] 9300 – 18 700), and Pakistan (14 400 deaths, [UR] 9700 – 17 000) had the most and accounted for half of all pneumococcal deaths in HIV-uninfected children aged 1 – 59 months in 2015 (12), (Fig. 2). Case fatality rates can be high for invasive pneumococcal disease, ranging up to 20 % for sepsis and 50 % for meningitis in developing countries (49). In Malawi, *S. Pneumoniae* is one of the most common organisms isolated from blood and Cerebrospinal Fluid (CSF) cultures of children admitted to the hospital, and the case-fatality rate for IPD, pneumonia, septicaemia, and meningitis is approximately 25 % (50).

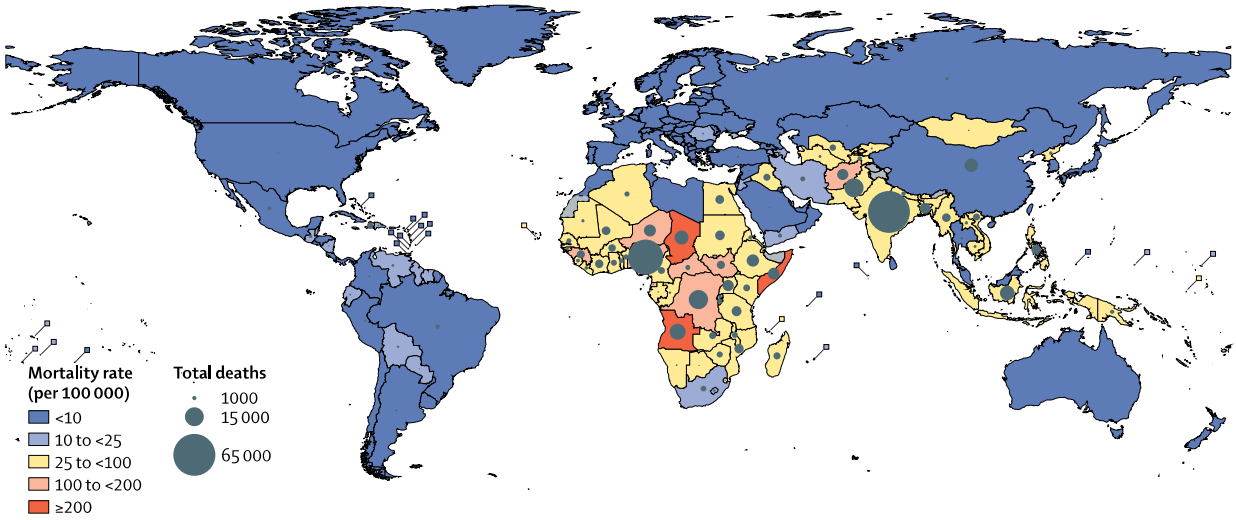

Figure 2: Global burden map of S. pneumoniae.

The figure shows country-specific mortality rates and deaths attributable to pneumococcus in 2015. Mortality rates and deaths in children aged 1 – 59 months are HIV-negative deaths only. Mortality rates are deaths per 100 000 children aged 1 – 59 months (12).

1.1.5 Diagnosis of *S. pneumoniae*

A definitive diagnosis of infection with *S. pneumoniae* generally relies on culture isolation of the organism from sheep blood or chocolate agar (2,35). On an overnight culture plate incubation at 35° C with 5 % CO_2, *S. pneumoniae* colonies appear as small, mucoid and greyish surrounded by a greenish zone of a-haemolysis (51). Susceptibility to optochin (ethylhydrocupreine) and bile solubility are used to differentiate *S. pneumoniae* from the other viridans streptococci (52). Usually pneumococci are optochin susceptible (53). The bile solubility test is considered as very sensitive and specific for identification of pneumococci and is based on the autolysis of *S. pneumoniae* in the presence of the surfactant sodium deoxycholate (51,52). Commercial slide agglutination, coagulation, and DNA probe hybridization tests are alternative methods for rapid identification of *S. pneumoniae* isolates (51). Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is starting to be used in clinical microbiology laboratories for routine bacterial identification

from colonies (54). However, the performance of MALDI-TOF MS for Streptococcus species is generally weak, and further studies are required to improve differentiation between *S. pneumoniae* and closely related viridins species such as *Streptococcus mitis* (*S. mitis)* (20).

The use of Nucleic Acid Amplification Tests (NAAT) for detecting *S. pneumoniae* focuses on multiple genetic targets in blood and respiratory tract samples by detecting the following genes: pneumolysin gene (*ply*), autolysin gene (*lytA*), pneumococcal surface adhesin A gene (*psaA*), *wzg/cpsA*, and the Spn9802 gene fragment (55). More recently, multi-locus sequence typing (MLST) has been adapted to differentiate among streptococcus species (56,57). This approach is called multilocus sequence analysis (MLSA) and is more reliable than previous methods in distinguishing among the streptococcal species but has been used only as a research method (58). Quellung reaction is a more specific method for pneumococcal serotype detection from pure cultures or sputum samples (51–53). The Quellung involves a reaction of the pneumococcus with streptococcal anti-capsular antisera, the pneumococcal capsule becomes visually enhanced, and the bacterial cell appears to be surrounded by a halo (51,52). Multiplex Polymerase Chain Reaction (PCR) improves serotyping in carriage studies and it allows the detection of multiple serotypes from mixed cultures or non-viable specimens (54). Microarray, a genomics-based molecular approach detects *S. pneumoniae* serotypes based on the *cps* gene content of DNA extracts using multiple probes representing all the known capsular polysaccharide synthesis genes (59). Microarray allows rapid detection of multiple serotypes and quantification of the relative abundance of each of the serotype in a sample (60,61). Whole genome sequencing (WGS) by Next generation sequencing (NGS) is one of the latest technologies that has been used for a complete characterisation of *S. pneumoniae* (62). Whole genome sequence data can be used to retrieve many characteristic features for identifying *S. pneumoniae* including the

full length sequences of various targets such as *lytA*, 16S rRNA, *sodA* and *rpoB* (62). WGS also helps in the accurate identification of *S. pneumoniae* by identifying serotypes, serotype variants, antibiotic resistance profile and virulence determinants (63–66).

1.1.6 Current interventions for treating *S. pneumoniae*

1.1.6.1 Antibiotic treatment

Penicillin has been the drug of choice for treatment of pneumococcal disease. Penicillin belongs to a class of beta lactam antibiotics which cause cell autolysis by inhibiting the formation of peptidoglycan cross-links in the bacterial cell wall, through the β-lactam moiety that binds to the bacterial enzyme DD-transpeptidase (9,67). However, due to penicillin resistance on the rise, modified antibiotic regimens have been introduced (68). The most common antibiotics currently recommended for children younger than five years are cotrimoxazole and amoxicillin (69). For very severe pneumonia, parenteral ampicillin (or penicillin) and gentamicin are recommended as a first line treatment and ceftriaxone is normally used as a second line treatment (69,70). For adults diagnosed with community acquired pneumonia, initial outpatient therapy includes a macrolide (azithromycin, clarithromycin, or Erythromycin) or doxycycline (70–72). Macrolides act at the ribosomal level and inhibit proteins synthesis. They bind to the 23S rRNA molecule (in the 50S subunit) of the bacterial ribosome and blocks exit of the growing peptide chain (9). For adults with comorbidities or those who have used antibiotics within the previous three months, Cephalosporins antibiotics such as fluoroquinolones (levofloxacin, gemifloxacin, or moxifloxacin), or an oral beta-lactam antibiotic plus a macrolide are prescribed (68,71–73). Cephalosporins have the same mode of action as other beta-lactam antibiotics such as penicillin (74–76)

a. Limitations with antibiotic treatment

Although there is a new regimen for pneumonia treatment, antibiotic resistance remains a challenge even with the 3rd generation cephalosporins. In 2011, an estimated 31 % of pneumococcal bacteria were resistant to one or more antibiotics (77). Over the past three decades, antimicrobial resistance in *S. pneumoniae* has dramatically increased worldwide (4). Non-susceptibility to penicillin in *S. pneumoniae* was first described in Australia in 1967, and later in New Guinea (1974), South Africa (1977), and Spain (1979) (78). In many countries, currently >40 % of isolates are resistant to penicillin (79). Among the penicillin-resistant *S. pneumoniae* isolates, 60 – 90% are also resistant to chloramphenicol, clindamycin, cotrimoxazole, erythromycin, and tetracycline (79). The highest penicillin and erythromycin resistance proportions worldwide are associated with serotypes 6A, 6B, 9V, 14, 15A, 19F, 19A, and 23F, often referred to as 'paediatric serotypes' (67,78,80). The rates of penicillin resistance range from 20 % in the USA (81) to as high as 44 % in Spain and 58 % in Hungary (82). Although the level of penicillin nonsusceptibility varies by country and usually ranges between 5 % and 50 %, some regions such as South Africa have reported nonsusceptibility as high as 79 % (45). Over the past decade there has been an increase from 28 % to 45 % in the isolation of penicillin-resistant pneumococci causing systemic infections at Chris Hani Baragwanath Hospital in Soweto, South Africa (83). In Malawi, 89 – 96 % of *S. pneumoniae* is resistant to co-trimoxazole in patients receiving treatment in hospitals (84). Strains resistant to other antibiotics including erythromycin, chloramphenicol, trimethoprim-Sulfamethoxazole and tetracycline and multiply resistant strains have also been recently reported (82,85).

b. Mechanisms of antibiotic resistance

The mechanism of penicillin resistance in *S. pneumoniae* involves structural changes in the penicillin targets, the Penicillin-Binding Proteins 1A, 2X, and 2B (78,86,87). Mutations occurring at penicillin binding domains of PBP can decrease their affinity for penicillin, and thereby make the organism resistant (9,86,87). PBP2b and PBP2x are the primary resistance determinants and are spread among pneumococci through intra- or interspecies recombination (9). Mechanisms conferring macrolide resistance include resistance mediated through the erm(B) gene, which results in macrolide–lincosamide–streptogramin B resistance, or through the mef(A) gene, which encodes an antibiotic efflux pump (87). For most commonly used antibiotic classes (beta lactams and macrolides), acquisition of resistance genes by pneumococci results from transformation (incorporation of free DNA from related species in the nasopharyngeal flora) or transfer of genes on conjugative transposons (88). De novo resistance to these antibiotics occurs only rarely in a susceptible population (88). Resistance to quinolones is usually due to mutations in topoisomerases (mainly in the *parC* or *gyrA* sub- units) (86,89). In addition efflux mechanisms also affect the activity of ciprofloxacin and, to a lesser extent levofloxacin (90).

c. Predisposing factors for antibiotic resistance to S. pneumoniae

Several risk factors for increased resistance of *S. pneumoniae* have been identified and include inappropriate treatment with antibiotics and overuse of antibiotics which lead to the development of antibiotic resistance to multiple antibiotic classes through selection pressure (39,84). HIV infection has also been shown to represent a risk factor for infection with drug-resistant pneumococci (45,81). Exposure to specific serotypes and clones, hospitalisation, residence in an urban area, day care attendance, previous exposure to antibiotics also increase the risk for resistance (9,91). Expansion of

resistant clones, as well as serotype replacement favouring resistant serotypes and clones, have also contributed significantly to the dissemination of antibiotic resistance (9). Generally, pneumococcal serotypes 6B, 23F, 14, 9V, 19A and 19F (67) as well as some pneumococcal clones such as ST 63, ST 81, ST 41, ST 75 and ST 185 are relatively more antibiotic resistant (9). The increasing penicillin-resistant pneumococcus has led to the high usage of non-beta lactam antibiotics for treating pneumococcal diseases (39,91). However, increasing incidence of penicillin-resistant *S. pneumoniae* has been paralleled by an increase in resistance to other classes of antimicrobial agents (39,91).

1.1.6.2 Pneumococcal vaccines

A critical role for the capsule is highlighted by the fact that antibodies specific to a capsule type are highly protective against IPD by strains belonging to the same serotype (92,93). As such, development of antibodies against the polysaccharide capsule is the basis of the current vaccines (94). There are two types of pneumococcal vaccine; Pneumococcal Polysaccharide Vaccine (PPV) and Pneumococcal Conjugate Vaccine (PCV) and both vaccines are made from inactivated (killed) bacteria (95).

a. Pneumococcal polysaccharide vaccines (PPV)

The pneumococcal polysaccharide vaccine (PPV) contains long chains of polysaccharide (sugar) molecules that make up the surface capsule of the bacteria (68). The first pneumococcal polysaccharide vaccine, contained 14 serotypes and was licensed in the United States of America (USA) in 1977 (68). In 1983, an improved pneumococcal polysaccharide vaccine was licensed, containing purified polysaccharide from 23 types of pneumococcal bacteria (96). Pneumococcal polysaccharide vaccine (PPV23) contains purified capsular polysaccharide from each of 23 capsular

types of pneumococcus: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V,10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, 33F (95). The PPV23 vaccine is licensed for routine use in adults ≥65 years and people with certain risk factors who are aged 2 to 64 years (68). Pneumococcal capsular polysaccharides are highly antigenic and are therefore able to induce serotype-specific antibodies that enhance opsonization, phagocytosis and killing by host immune response (97). A two-fold or greater increase in serotype-specific antibody develops within three weeks in 80 % or more in healthy young adults (97).

i. Limitations with Pneumococcal polysaccharide vaccines (PPV)

Pneumococcal polysaccharide vaccines activate B-cells but do not elicit T-cell independent immune responses (98). As a result, they do not elicit protective immune responses in children aged less than 2 years (15). In addition, polysaccharide vaccines do not induce mucosal immunity and thus they have little or no impact on nasopharyngeal carriage (97).

b. Pneumococcal conjugate vaccines (PCV)

The first pneumococcal conjugate vaccine, PCV7 (Prevnar 7, Pfizer), was licensed in 2000 in the USA (99). It includes purified capsular polysaccharide of seven serotypes of *S. pneumoniae* (4, 9V, 14, 19F, 23F, 18C, and 6B) conjugated to a nontoxic variant of diphtheria toxin known as CRM197 (100). In 2010, a 13-valent pneumococcal conjugate vaccine (PCV13) was licensed in USA (39) that contained 7 serotypes of *S. pneumoniae* as PCV7 plus serotypes 1, 3, 5, 6A, 7F and 19A which were also conjugated to CRM197 (2,97). All infants and toddlers normally receive doses of PCV13 vaccine, usually given at ages two, four, six, and 12 through 15 months (68). The conjugate vaccine (PCV13) is given as a shot in the muscle (68) . In contrast to the 23-valent non-conjugated vaccine, the

conjugated vaccines activate B- and T-cell leading to immune memory (101). They elicit strong adaptive and booster response; as a result, they are immunogenic in infants under two years of age (97). In addition, PCV elicits mucosal immune responses in immunized hosts due to induction of IgA antibodies against vaccine serotypes (97). The 13-valent pneumococcal conjugate vaccine (PCV13) was introduced in Malawi from November 2011 using a three dose primary series at 6, 10, and 14 weeks of age to reduce *S. pneumoniae*-related diseases (102).

i. Limitations with Pneumococcal conjugate vaccines (PCV)

The pneumococcal conjugate vaccines have significantly reduced the incidence of pneumococcal disease (103). While there has been a reduction in disease due to elimination of vaccine serotypes following the introduction of PCVs, there has been an increase in disease from non-vaccine serotypes and none capsulated *S. pneumoniae* (98,104–107). After the introduction of PCV7, antibiotic-resistant 19A strains emerged and were responsible, in part, for the reformulation to 13 serotypes (103). The most common emerging serotypes depend on the population studied but include 6C, 8, 15A, 22F, and 23A and 23B (96).

1.2 Introduction to traditional herbs and usage

Herbal medicines have been an important source of products world-wide in treating common infectious diseases (108). In Latin America, the WHO Regional Office for the Americas (AMRO/PAHO) reports that 71 % of the population in Chile and 40 % of the population in Colombia have used Traditional Medicine (109). In the US the number of visits to providers of Complementary Alternative Medicine (CAM) now exceeds the number of visits to all primary care physicians and CAM is becoming increasingly popular in many developed countries (109). A US survey reported the

use of at least one of 16 alternative therapies increased from 34 % in 1990 to 42 % in 1997 (109). More than 100 million Europeans rely on traditional or complementary medicine (T/CM) (110). In the USA, US$ 83.1 billion was spent on herbal drugs in 2013 and the market is growing at the rate of 20 % (110). For African countries it is estimated that 80 % of the people have access to herbal medicine only (108,110,111). In Malawi usage of plants for medicinal purposes is an important part of the culture and tradition (112). Most people in Malawi, especially in rural areas, have vast knowledge of a wide variety of herbs that can be used to treat common ailments but they lack some scientific backing (112). To address this scientific gap, a World Bank funded Africa Centre of Excellence in Public Health and Herbal Medicine (ACEPHEM) was established in Malawi at the College of Medicine, Malawi in 2018.

1.2.1 Complementary alternative medicine (CAM)

Natural products have been successfully employed in the discovery of new drugs and have exerted a far-reaching impact on chemicobiology (113). In fact, about 25 % of the medicines currently prescribed worldwide are higher-plant-derived products (114). Some of the plant derived drugs are currently in use and these include Artemisinin. Artemisinin, from which the artemether component of malaria drug Coartem® is derived, is obtained from the plant sweet wormwood (Artemisia annua) which has been used for over 2,000 years as a Chinese herbal remedy (115). Among anticancer drugs approved in the time frame of about 1940 – 2002, approximately 54 % were derived natural products (113). For instance, the Vince Molecules alkaloids from Catharanthus roseus, and the terpene paclitaxel from Taxus baccata, are among successful anticancer drugs originally derived from plants (113). Taxol isolated as a new compound from *T. Brevifolia* was approved by the US Food and Drug Administration (FDA) for treating ovarian cancer in 1992 (113). *Romaglycate*, based on the plant

Khella, whose active ingredient is Khellanis is used in Egypt to treat kidney stones (116). Some plants have been documented to exhibit antimicrobial activity against bacteria. For example, Clove (*Syzygium aromaticum*), from the Myrtaceae family, is one of the most active antimicrobial herbs against many bacteria including *Listeria monocytogenes, Klebsiella pneumoniae, Staphylococcus aureus, Escherichia coli* and *Salmonella typhimurium* (117). *Achyranthes aspera* Linn of Amaranthaceae family possess antibacterial activity against *Staphylococcus aureus, Streptococcus haemolyticus, Bacillus thyphosus* and *Escherichia coli* (118). The ethyl acetate (EtOAc) leaf extract of *Mentha piperita* exhibits pronounced antibacterial activity against *B. subtilis, P. aeruginosa, S. aureus* and *S. marcescen* (119). Some plants *(Chelidonium majus, Sanguinaria canadensis*, and *Macleaya cordata*) have been documented to work against resistant bacteria like multidrug resistant *S. aureus* (MRSA) (120). Other plants have also been documented to exhibit antiviral properties; a polyphenol-rich extract of the medicinal resurrection plant *Myrothamnus flabellifolia* was reported to inhibit viral (M-MLV and HIV-1) reverse transcriptases (121). Saikosaponins from *Bupleurum* species have shown to inhibit Hepatis B Virus Antigen expression and DNA replication(122,123). Saikosaponins B2 from the same Bupleurum species have also been documented to inhibit Corona virus (HCoV-22E9) at viral attachment and penetration stages (123,124). Aqueous and ethanolic extracts of *Ocimum basilicum* and its component compounds linalool, apigenin, and ursolic acid have been documented to inhibit Enterovirus 71 (123,125). Furthermore, some plants have been documented to exhibit antimicrobial properties against *S. pneumoniae*. For example; an Indian study found that the activities of methanol extracts obtained from *Launaea nudicaulis, Marsilea quadrifolia and Bougainvillaea spectabilis* had promising activity against *S. pnemoniae* and showed a correlation between the traditional uses of these plants and the experimental data against *S. pneumoniae* (126). Another study found that the extracts obtained from fruits, twigs, and root of *Salvadora persica* had

strong activity against *S. pneumoniae* wild type strains (127). Studies in Palestine found that *V. fruticulosum* possessed a potential antibacterial activity against *S. pneumoniae*. Different species of *Verbascum* have been known to contain biologically active compounds such as flavonoids, phenylethanoid, neolignan glycosides, saponins and iridoid glycosides (128).

1.3 Herbal plants under-study

Six plants locally found in Malawi and with history to treat several ailments were chosen**:** *Bidens pilosa* (*B. pilosa*), *Dichrostachys cinerea* (*D. cinerea*), *Lippia javanica* (*L. javanica*), *Annona senegalensis* (*A. senegalensis*), *Erythrina abyssinica* (*E. abyssinica*) and *Trichodesma zeylenicum* (*T. zeylanicum).*

1.3.1 Bidens pilosa (Bp)

Bidens pilosa Linn. var. Radiata (Spanish needles or beggar ticks) belongs to the family Asteraceae and is an annual weed widely distributed throughout the tropical and sub-tropical regions of the world (129,130). It has bright green leaves with serrated prickly edges and produces small yellow flowers (129), (Fig. 3). In many countries of Africa, *B. pilosa* is recorded as a weed and it is likely to occur in all countries, including the Indian Ocean islands. It is reported as a vegetable or potherb, among others, in Sierra Leone, Liberia, Côte d' Ivoire, Benin, Nigeria, Cameroon, Democratic Republic of Congo, Kenya, Uganda, Tanzania, Malawi, Botswana, Zambia, Zimbabwe and Mozambique (131).

Figure 3: Bidens pilosa with flowers (A) and with achenes (B) growing in the forest of Zomba, Malawi

1.3.1.1 Medicinal use of *Bidens pilosa*

Almost all parts of *B. pilosa* (the whole plant, the aerial parts and/or the roots) have been used on an extensive basis, either as food or medicinal components (131,132). It is widely used either in traditional medicine or folk medicine by indigenous people to treat a variety of illnesses including pain, fever, angina, diabetes, edema, rheumatism, diarrhoea, ear and eyes infections (129). It also has a long ethno-medicinal history for treating malaria, skin infections, stomach and liver disorders (130). The in vitro activities of this plant extract have been reported against several foodborne and human pathogenic microorganisms including *Klebsiella*, *Bacillus*, *Pseudomonas*, *Staphylococcus*, *Salmonella* and *E. coli* (131). The pharmaceutical property of the plant is mostly associated with bioactive phytochemical compounds such as sesquiterpene lactones and polyacetylenes, which inhibit the growth of pathogenic microorganisms and the flavonoids, which are considered as effective anti-inflammatory agents (130). Phytochemicals and essential oil of *B. pilosa* have been reported to possess exploitable amount phenolic compounds with free radical scavenging potential (133). Other

bioactive compounds which have been identified from this plant are phenylpropanoid glucosides, diterpenes, and flavone glycosides (130,134).

1.3.2 Dichrostachys cinerea

D. cinerea (L.) Wight & Arn belongs to the Mimosaceae family and it is commonly known as Marabou thorn or Sickle Bush (135). *D. cinerea* is a legume characterized by its two-colored inflorescence native to Africa but can now be found in India, the Caribbean, and parts of Southeast Asia (136). It typically grows up to 7 meters in height and has strong alternate smooth spines, dark grey brown fractures on old branches and stems and bark on young branches (132,137), (Fig. 4). The fruit is edible and the seedpod is mustard brown with 4 small black seeds (138).

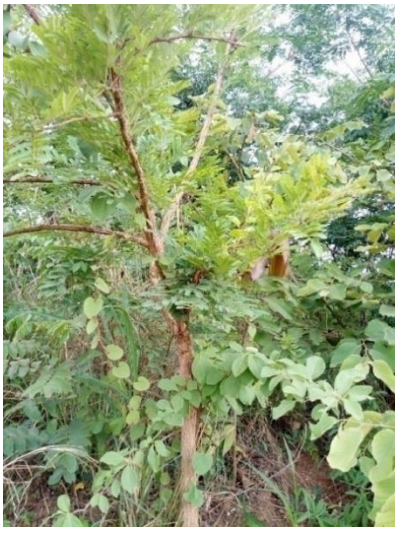

Figure 4: D. cinerea tree growing in Zomba forest, Malawi

D. cinerea is one of the very useful wild medicinal plants used in folk medicine across Africa and Asia (137). The bark, roots, and leaves are used in the treatment of dysentery, headaches, toothaches, elephantiasis, snakebites and scorpion stings, leprosy, syphilis, coughs, epilepsy, gonorrhoea, boils, and sore eyes (136,139). It can also be used as a contraceptive for women, as laxative, and for massage

of fractures (137,140). Bark and root methanol extracts showed antibacterial activity, as well as synergistic effect with antibiotics against multidrug-resistant bacteria (141). Root extracts were demonstrated to be active against *S. aureus, Shigella boydii, Shigella flexneri, E. coli* and *P. aeruginosa* (141). Phytochemical analysis of methanol extracts of the aerial plant parts revealed the presence of flavonoids, tannins, sterols, triterpenes and polyphenols (137,140,142). The flavan-3-ols (–)-mesquitol, oritin, (–)-festidinol and (–)-epicatechin have been isolated from the methanol extracts; the first 3 compounds were found to be advanced glycation end-products inhibitors (143). The new isomer of mesquitol (a main active principle), has shown free-radical scavenging property and a-glucosidase inhibitory activities (142).

1.3.3 Erythrina abyssinica

E. abyssinica is commonly known as "the red hot poker tree" (Fig. 5) and it belongs to the family Leguminosae consisting of 730 genera and over 19,400 species distributed all over the world (144). The genus E*rythrina* comprises of approximately 120 species of which about 30 are found in continental Africa and 6 in Madagascar (144).

Figure 5: Flowering E. abyssinica plant growing in Zomba farms Malawi

The bark is commonly used in traditional medicine to treat snakebites, malaria, sexually transmitted diseases like syphilis and gonorrhoea, amoebiasis, cough, liver inflammation, stomach ache, colic and measles (145). Roasted or powdered bark is applied to burns, ulcers and swellings (145). The pounded flowers are used to treat dysentery and a maceration of flowers is drunk as an abortifacient, also applied externally to treat earache and asthma [3]. The ethyl acetate extract of the stem bark of *E. abyssinica* has shown anti-plasmodial activity against the chloroquine-sensitive (D6) and chloroquine-resistant strains of *Plasmodium falciparum* (144). The plant also has mitogenic effects on cell cultures (146). It has also been shown to have activity against rifampicin resistant *Mycobacterium tuberculosis* (147). Compounds such as Abyssinone IV, Abyssinone V, Abyssinin III, Abyssinone IV, Abyssinone V, 4', 7-Dihydroxy-2', 5'-dimethoxyisoflav-3-ENE 62.0 and Quercetin 5.4 have been isolated from *E. abyssinica* and their structures have been elucidated (148,149).

1.3.4 Lippia javanica

L. javanica also commonly known as lemon bush (150), is widely distributed throughout southern Africa and is particularly abundant in Swaziland and in the northern provinces of South Africa (151). It is also found in Botswana, Malawi, Mozambique, Tanzania, Zambia and Kenya (152,153). *L. javanica* is the largest group of the indigenous Lippia shrubs and it belongs to the family Verbenaceae (153,154). It comprises approximately 32 genera and 840 species (154). The Verbenaceae are often hairy and characteristically the hairs are incrusted with calcium carbonate and/or silicic acid (155). The hairy leaves have conspicuous veins and are highly aromatic, with a strong lemon smell (152). *L. javanica* has small creamy white flowers clustered together in dense, round spikes about 1 cm in

diameter (153), (Fig. 6). In Tanzania, South Africa, Mozambique, Botswana and Malawi, the flowers are mostly produced between February and May but can also be found throughout the year (156).

Figure 6: Javanica stem and leaves growing in Zomba farms, Malawi

The plant has been exploited since pre-historic time by traditional herbalists for the treatment of various ailments including diabetes, coughs, asthma, colds, flu, chest complaints, scabies, scalp infections and malaria (150). The plant is known to possess analgesic, inflammatory, antipyretic, and antispasmodic activities (155). The shrub is claimed to have fever and pain-relieving properties and is used mainly as an infusion to treat a variety of ailments, ranging from bronchial infections to skin disorders (151,153–155,157–162). In Central and South America (Guatemala, Venezuela and Brazil), it is employed as a remedy for colds, grippe, bronchitis, coughs and asthma (161). In Africa, it is used traditionally as a charm for protection against dogs, lightning and crocodiles and for ritual cleansing after contact with a corpse (159). Numerous monoterpenoids have been identified from L. *javanica* including myrcene, caryophyllene, linalool, p-cymene and ipsdienone (152). The presence of substantial levels of alkaloids, flavonoids, terpenes, saponins, phenolics, anthraquinones,

phlobatannins, cardiac glycosides and tannins in the leaf have also been reported (150). It was recently reported that three compounds from *L. javanica* are able to inhibit the HIV-1 reverse transcriptase enzyme (151,153). Traditional medicine practitioners prescribe the synergistic use of *L. javanica* and *Artemisia afra* as a remedy against malaria, as a prophylactic against dysentery and diarrhoea, and for the treatment of fever and influenza (160,163). Scientifically, *L. javanica* has portrayed high activity against pathogens such as *K. pneumoniae* and *Cryptococcus neoformans* which are commonly associated with opportunistic infections in immune-compromised patients (160) and other microorganisms such as *S. aureus*, *S. pneumoniae* and *S. pyogenes* (164).

1.3.5 Trichodesma zeylenicum

Trichodesma zeylenicum (Burm. F.) R.Br. is a densely bristly-hairy annual herbal plant that grows up to 1 meter (165). Leaves are narrowly elliptic, while flowers becomes nodding in terminal 1-sided bracteates inflorescences (166), (Fig. 7). *T. zeylenicum* is extremely unpleasant to handle because it has bulbous-based spiny hairs that break off in the skin when the plant is touched (164). The genus Trichodesma R. Br comprises of ± 45 species known from tropical and subtropical regions of Africa, Asia and Australia (167). It is currently represented by five species and three subspecies in southern Africa, about 15 species occur in Africa south of the Sahara (167). It has various common names like Camel bush, cattle bush and northern blue bell (English) and Herbe cipaye, bourrache sauvage and herbe tourterelle (French) (168).

Figure 7: T. zeylenicum plant growing alongside bush in Blantyre, Malawi

T. zeylenicum leaves are used as remedy for snake bites, as a diuretic and an analgesic (169,170). The roots are chewed or pounded and soaked in water, and the infusion is used as a remedy for tuberculosis, stomach-ache, poisoning and rheumatism (167). The infusion of leaves and roots is used for intestinal worms, coughing, chest complaints, itching and throat pains (171). The aqueous and methanol extracts of *T. zeylenicum* leaves exhibit activity against *S. aureus, P. aeruginosa, S. typhi* and the dermatophyte *T. mentagrophyte* (171). Common compounds isolated from *T. zeylenicum* seed are ricinoleic acid, cyclopropene acid and the pyrrolizidine alkaloids, with low toxic alkaloid supinine as the principal component (172).

1.3.6 Annona senegalensis

A. senegalensis belongs to the family of Annonaceae and is commonly known as 'African custard-apple' (173). It is a small tree or shrub of up to 6m high, from Senegal to Nigeria, in the Cape Verde Islands and across Africa to Sudan (174). The bark is smooth to roughish, silver grey or grey-brown (175). The leaves of this medicinal plant are alternate, simple, oblong, ovate or elliptic, green to bluish

green, mostly lacks hairs on upper surface, with brownish hairs on lower surface (173,175), (Fig. 8). Flowers are up to 3 cm in diameter on 2 cm long stalks, solitary or in groups of 2–4, arising above the leaf axils (173,175). The ripe fruit is yellow in colour and has a sweet edible jelly with pleasant odour (176).

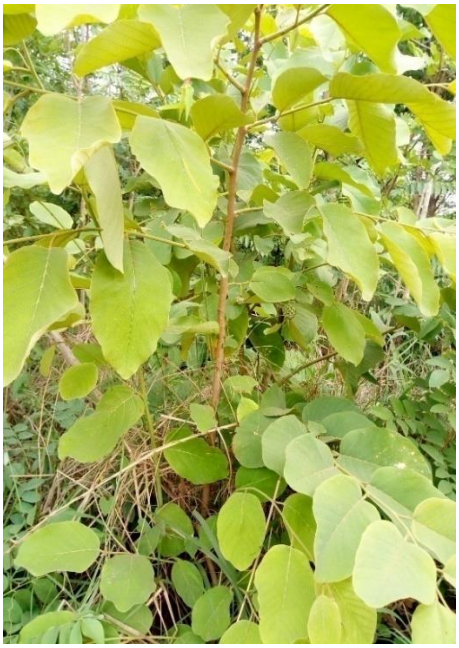

Figure 8: A. senegalensis shrub growing in Zomba forest Malawi

A. senegalensis is one of the multiple medicinal plants widely used for their therapeutic potentials (177). The fruit obtained from this multipurpose plant is widely used locally in the treatment of energy deficiency syndrome known as kwashiorkor and marasmus (173). The leaves and the bark are used in the treatment of diarrhoea, disease of the joints, respiratory diseases, conjunctivitis, wounds, snakebites, trypanosomiasis, jaundice, hemorrhoids, feminine barrenness, convulsions, ovarian cancer, fever, and asthenia (178). The leaves of *A. senegalensis* have also been reported in the treatment of pneumonia, and as a stimulant to improve health (176,179,180). A decoction from the roots is used to stop chest colds, venereal diseases, stomach ache and dizziness(173,181).

Phytochemical constituents of *A. senegalensis* leaf include epicatechin, catechin, rutin, isoquercetrin, anthocyanosides, saponosides, tannins, carotenoids, sterols, triterpenes, alkaloids, and cardiac glycosides (178).

1.4 Problem statement

Current interventions for reducing pneumococcal infections include antibiotic treatment and vaccination with PPV and PCV. Although effective, these approaches have their limitations. For example, the efficacy of antibiotics is often affected by the emergence of antibiotic resistance (3,10,11,14,16). The limitation with PPV is that they do not elicit protective immune responses in children <2 years old. In addition to that, polysaccharide vaccines do not induce mucosal immunity and, thus, have little or no impact on nasopharynx (19). The pneumococcal conjugate vaccines (PCV) are effective in young children; however, they only target a subset of circulating pneumococcal serotypes. This exerts selective pressure which can shift serotype frequencies – a phenomenon termed vaccine induced serotype replacement (VISR) (21,23). Given the above limitations, there is need for alternative interventions such as herbal medicines to combat pneumococcal infections.

1.5 The objectives of the study

1.5.1 Main objective

The main aim of the study was to investigate the antimicrobial activities of selected local Malawian medicinal herbs against *S. Pneumoniae*.

1.5.2 Specific objectives

1. To extract and purify crude herbal extracts.
2. To test antimicrobial activities of crude and purified extracts against *S. Pneumoniae.*

1.6 Structure of the thesis

Chapter 1 of this thesis covers the introduction to *S. pneumonia*e, current interventions for treating *S. pneumoniae* and the existing limitations to the interventions. It further introduces Complementarity Alternative Medicine, the medicinal herbs selected for this study, broad and specific objectives and the problem statement. Chapter 2 of this thesis covers the materials and methods used in the study. Chapter 3 outlines the results for the plants extractions, antimicrobial activity testing, the Minimum Inhibitory and Bactericidal Concentrations. It also provides findings of the chromatographic fractionation of the crude extracts and the antimicrobial activity of the isolated fractions, qualitative and quantitative phytochemical analysis and the Brine Shrimp's lethality test. The last chapter (Chapter 4), provides a general discussion of the findings from this study, outlines major limitations and provides suggestions for future work.

CHAPTER 2: MATERIALS AND METHODS

2.1 Introduction

This was a bioactivity guided isolation study that was conducted at the College of Medicine, Biomedical Sciences Department. Some of the laboratory work was performed at the College of Medicine (plant extraction fractionation) and some at the Malawi Liverpool Wellcome Trust Clinical Research Programme (MLW) (bioactivity testing).

2.2 Plant materials

Ethno-botanical information of medicinal plants was collected on the basis that they are used to treat bacterial infections. The information was compared against the herbs for pneumococcal infections in the natural database from Africa Centre of Excellence in Public Health and Herbal Medicine (ACEPHEM). Six plants were selected (Table 1) and were identified through the national herbarium and botanic gardens of Malawi in Zomba. The samples were collected in sterile polyethylene bags and transported to College of Medicine for storage at 25^{o}C.

Table 1: Local medicinal plants that were selected for testing against S. pneumoniae.

Scientific name, family name, common/English name and location from where the plants were collected are shown below.

Scientific name	Family name	Common/ English name	Location
Dichrostachys cinerea	Mimosaceae	Mpangala/Marabou Thorn	Zomba botanical gardens
Erythrina abyssinica	Leguminosae	Muwale/Mlindimira/ Red hot poker tree	Zomba local farms
Annona senegalensis	Annonaceae	Mpoza/African custard apple	Zomba forest
Bidens pilosa	Asteraceae	Chisoso/Spanish needles/ beggar ticks	Blantyre around QUEENS
Trichodesma zeylenicum	Boraginaceae	Chilungumwamba/ Camel bush/cattle bush,	Zomba botanical gardens
Lippia javanica	Verbenaceae	Bvumbe/Lemon bush	Zomba local farms

2.2 Preparation of extracts from plant material

2.2.1 Plant material selection and processing

The plant material (roots, leaves, barks) were selected and sorted out by removing the damaged and dry materials. The selected plant materials were then washed with distilled water and dried at room temperature for two weeks, followed by grinding using a pestle and mortar.

2.2.2 Extraction of the powdered plant material with water (H_2O) and methanol (MeOH)

Each of the powdered plant material (50 g) was placed into 200 mL of distilled H_2O and MeOH (1:4). The mixture was left to stand for 72 hrs in a glass beaker with intermittent hand-shaking every 18 hrs. The H_2O and MeOH extracts were then filtered using Whatman filter paper No. 1 and the filtrate was

evaporated using a rotor evaporator at 50^{o}C (Fig. 9). The final dried material was stored in labeled sterile screw-capped bottles at -20^{o}C.

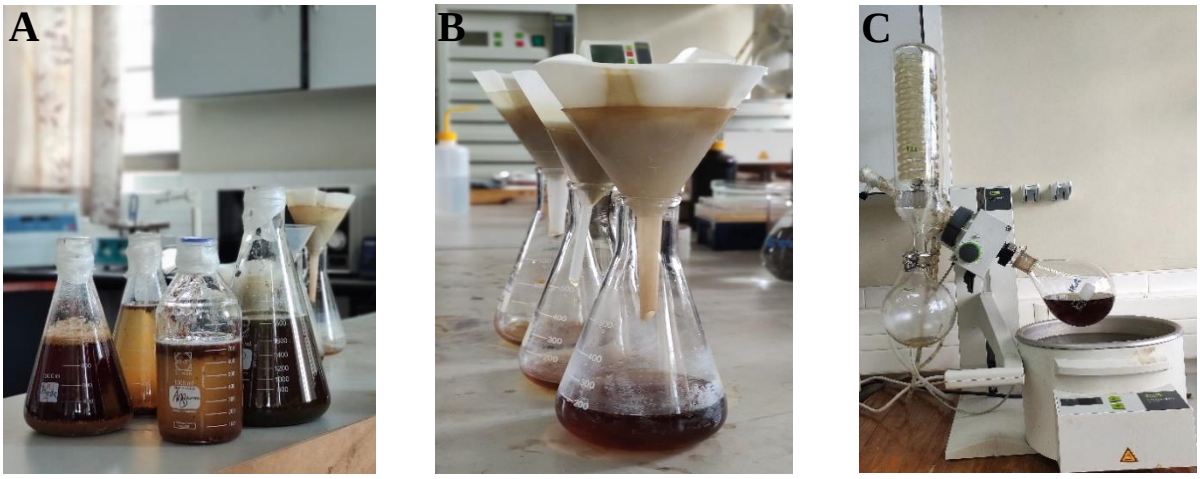

Figure 9: Plant extraction process

Soaking of extracts in water/methanol (A), filtration of the extracts with Whatman filter paper no 1 (B) and extraction with rotor evaporator (C)

2.3 Determination of antimicrobial bioactivity of the crude water and methanol extracts against *S. pneumoniae* serotype 1 and 6A

2.3.1 *S. pneumoniae* serotypes and culture conditions

Two pneumococcal isolates serotype 6A (strain BHBB85)) and serotype 1 (strain BHB8E9) were used to determine the antimicrobial activity of the selected plant extracts. Serotypes 1 and 6A are among the most prevalent IPD serotypes in Malawi and are covered by the current pneumococcal vaccine PCV 13 (182). All serotypes were clinical isolates supplied by the Pneumococcal Carriage of Vulnerable Population in Africa (PCVPA) study at Malawi Liverpool Welcome Trust. The isolates were primarily cultured on sheep blood agar with Gentamicin (SBG) and were then sub-cultured on Sheep Blood Agar media (SBA). McFarland solution was prepared by collecting 3 - 4 overnight SBA

colonies using sterile loop and introducing the colonies into test tubes containing 1 mL of normal saline. The mixture's turbidity was compared with that of 0.5 McFarland solution for inoculum standardization and the density of the suspension was adjusted to approximately 1-2 x 10^8 Colony Forming Unit (CFU)/mL. Inoculation of 100 µL standardized culture solution was made unto sheep blood agar media plates using sterile swabs.

2.3.2 Bioactivity of the crude water and methanol extracts through antimicrobial activity testing against *S. pneumoniae* serotype 6A and 1

In vitro antibacterial activities of water and MeOH extracts of the selected herbal plants against *S. pneumoniae* were determined by standard filter paper discs agar diffusion assay (183). Six plants namely *D. cinerea, B. pilosa, E. abyssinica, L. javanica, T. zeylenicum* and *A. senegalensis* were tested for antimicrobial activity. The plant extract (50 mg) was dissolved separately in 50 mL of 1% DMSO and the resultant solution was serially diluted to 0.001, 0.01, 0.1 and 1 (mg/mL) concentrations. Autoclaved filter paper (Whatman Filter paper no 1) disks (6 mm) were impregnated with 10 µL of the serially diluted solutions. The filter paper discs were air dried in a sterile hood for 24 hrs (183). Impregnated and air dried filter discs were then aseptically deposited on the Sheep blood agar plates inoculated with *S. pneumonia* serotype 6A and 1 and were left at 4°C for 2 hours to allow the diffusion of the extract before incubation for 24 hrs at 37°C in 5 % CO_2 (126). Discs impregnated with 10 µL of 1 % (DMSO in Dulbecco's Phosphate Buffered Saline (DPBS) were used as negative control while commercial penicillin discs were used as positive controls. All tests were performed in triplicate and the antibacterial activity was expressed as the mean diameter (mm) of the zone of inhibition around each disc.

2.3.3 Bioactivity of the crude water and methanol extracts through minimum inhibitory concentration (MIC) determination

The MIC was determined using Mueller Hinton agar (MHA) dilution method (126). The extracts with an inhibition zone of ≥ 10 mm were significant and therefore selected for the determination of MICs. These were: H_2O extracts of B. *pilosa* and *D. cinerea* and MeOH extracts of *B. pilosa, D. cinerea, E. abyssinica, L. javanica* and *A. senegalensis.* The 100 % concentration of each of the extract was serially diluted in two folds to achieve 0.1, 1 , 2, 4, 8 and 16 mg/mL using 1 % DMSO in DPBS (184). The concentrations were made in triplicates. Each dilution (1 mL) was incorporated into 20 mL of melted MHA agar medium and poured into a petri dish. The plates were inoculated with overnight *S. pneumoniae* serotype 6A and 1culture dilutions at a final density 0f 1-2 x 10^8 cfu/mL. The inoculated plates were incubated for 24 hrs at 37^{o}C in 5 % CO_2. Media containing 0.2 mL of 1 % DMSO was used as negative control while plates with only media served as positive control. MIC was determined as the lowest concentration that completely inhibited the growth of the bacteria (185).

2.3.4 Bioactivity of the crude water and methanol extracts through Minimum Bactericidal Concentration (MBC) determination

Minimum bactericidal concentration is defined as the concentration producing 99.9 % reduction in colony forming units (CFU) number in the initial inoculum (186,187). The MBC's were determined by sub-culturing a 10 µL loopful of *S. pneumoniae* serotype 6A and 1 on Sheep Blood Agar from the plates that showed no growth during MIC. The inoculated Sheep Blood Agar plates were incubated for 24 hrs at 37^{o}C in 5 % CO_2. The least concentration at which no growth was observed was noted as MBC. The extracts with an inhibition zone of ≥ 10 mm were significant and therefore selected for

the determination of MBC. These included water extracts of *B. pilosa* and *D. cinerea,* and MeOH extracts of *B. pilosa, D. cinerea, E. abyssinica, L. javanica* and *A. senegalensis*.

2.4 Phytoconstituents analysis of crude water and methanol extracts

The presence or absence of phytochemicals such as flavonoids, phenols, alkaloids, saponins and tannins in the plant was analyzed both qualitatively and quantitatively using standard methods and mass spectrophotometry respectively. This was done for all H_2O and MeOH extracts.

2.4.1.1 Qualitative phytochemical analysis

Both H_2O and MeOH extracts of the six plants were tested for the presence of bioactive compounds using standard methods (165,188–192) with little modifications.

2.4.1.2 Test for flavonoids

The presence of flavonoids was tested using Hydrochloric acid (HCl) test: In this procedure,1 g of extract was dissolved in 2 mL of MeOH. A few drops (5) of concentrated HCl were added to the mixture. A red coloration confirmed the presence of flavonoids (193).

2.4.1.3 Test for phenols

Crude extract was prepared by dissolving 0.5 g of extract in 2 mL distilled H_2O. A few drops of Iron III Chloride ($FeCl_3$) were added to the prepared sample. A blue, dark green, red or purple color was a positive indicator for phenols (194,195).

2.4.1.4 Test for alkaloids

Crude extract (1 g) was dissolved in 2 mL MeOH. 1 mL of HCl was added to the sample. 6 drops of Meyer's reagent were added to the mixture followed by another 6 drops of Wagner's reagent. Positive result was indicated by the formation of a red or brown precipitate (190).

2.4.1.5 Test for saponins

Crude extract (1 g) was mixed with 5 mL of distilled water in a test tube and shaken vigorously. The formation of stable foam was taken as an indication for the presence of saponins (196,197).

2.4.1.6 Test for tannins

5 mL of the active extract was added to 2 mL of 5 % of $FeCl_3$ solution in a test tube. A greenish-black precipitate was indicative of the presence of tannins (189,198).

2.4.1.7 Test for steroids

The MeOH/H_2O extract (1 g) was dissolved in MeOH (1 mL) and mixed with 2 mL of chloroform. Then 2 mL of each of concentrated H_2SO_4 and acetic acid were poured into the mixture. The development of a greenish coloration indicated the presence of steroids (190).

2.4.1.8 Test for glycosides

Liebermann's Test was used. Presence of glycosides was determined by adding 1 mL of chloroform to 1 g of H_2O/MeOH extract. This was followed by additional of 1 mL of acetic acid and the mixture was then cooled and 1 mL Sulphuric acid (H_2SO_4) was added. Green colour showed the entity of aglycone which is a steroidal part of glycosides (190,191).

2.4.1.9 Test for Anthocyanins

In this procedure, 1 g of sample was dissolved in 1 mL of H_2O or MeOH. 1 mL of 2 N HCI was added to the extract solution. A red or purple color was positive for the anthocyanidins after adding ammonia (199).

2.4.1.10 Test for reducing sugars

The MeOH/H_2O extracts (1 g) were dissolved in 1 mL of MeOH/H_2O and made up to 2 mL with distilled H_2O. One drop of each Felling's A and Felling's B solutions were added to the mixture. The color brick red was indicative of presence of reducing sugars (188).

2.4.1.11 Test for Terpenoids

The MeOH/H_2O extract (1 g) was dissolved in 2 mL of MeOH or H_2O. The mixture was then added to 2 mL of chloroform and 3 mL of concentrated H_2SO_4. The presence of terpenoids was indicated by a reddish brown color of interface (200).

2.4.2 Quantitative phytochemical analysis of the crude water and methanol extracts

2.4.2.1 Determination of total phenolic content (TPC)

The total phenolic content in the extracts was determined using Folin-Ciocalteu assay (201–204). Briefly, 0.1 g of the powdered extracts was dissolved in MeOH (80:20 v/v) and made up to 20 mL with distilled H_2O and vortexed for 15 seconds. 1 mL of the dissolved extracts was transferred into falcon tubes, diluted 10-fold with distilled H_2O. Then 1 mL of diluted extracts was added into another falcon tubes in which 10-fold diluted folin reagent (5 mL) was added followed by addition of sodium carbonate (I M, 4 mL) to make 20-fold dilution of extract. The mixtures were vortexed for 15 seconds

and allowed to stand for 2 hrs. Then 300 μL of the mixture was added into Generic 8 by 12 plate size and absorbance of mixtures and blank (MeOH) were measured using UV-VIS Spectrophotometer (multiple plate reader, Perkin Elmer 2030- Victor *3) at 765 nm. Gallic acid was used as standard solution with concentrations ranging from 20 to 250 mg/. Total phenolic content was expressed as milligram gallic acid equivalents per gram of dry weight (mg GAE/g DW) based on the calibration curve: $y=0.0057x+0.0872$, $R^2=0.9994$ where y was the absorbance and x was the concentration.

2.4.2.2 Determination of Total Flavonoid Content (TFC)

Total flavonoid content was determined using aluminum chloride colorimetric assay (203,205,206). In brief, 0.1 g of extracts were dissolved in 2 mL MeOH (80:20 v/v). The mixtures were made up to 20 mL with distilled H_2O. 1 mL of the dissolved extracts was transferred into falcon tubes, diluted 10-fold with distilled H_2O. 1 mL of the diluted extracts was added into falcon tubes in which 1ml of 2 % aluminum chloride hexahydrate was added and the mixture was then vortexed for 15 seconds. The mixture was incubated at room temperature (25 ºC) for 60 minutes. Absorbance of the mixture and blank (MeOH) was measured using UV-Vis Spectrophotometer (Multiple plate reader, Perkin Elmer 2030 – Victor X 3) at 415 nm. Calibration curve with quercetin concentration ranging from 0 - 900 ug/mL was used for quantification. Results were expressed as milligram quercetin equivalent per gram of dried weight (mg QE g^{-1} DW) using the following equation based on the calibration curve $Y = 0.0021x+0.1017$ $R^2 = 0.9951$ where Y was the absorbance and X was the concentration.

2.4.2.3 Determination of total alkaloid content (TAC)

The Total Alkaloid Content was estimated spectrophotometrically using Bromocresol green assay (207). The crude extracts (0.1 g) were dissolved in 2 N HCL and then filtered with Whatman Filter

paper no1. Then, 1 mL of the filtrates were transferred into falcon tubes in which phosphate buffer (pH 4.9, 5 mL) and bromocresol green solution (10^{-4} M, 5 mL) were added and vortexed for 15 seconds. The complex formed was extracted using chloroform (5 mL). The absorbance was measured using UV-Vis Spectrophotometer (Multiple plate reader, Perkin Elmer 2030 – Victor X 3) at 415 nm against the blank (MeOH). Calibration curve with caffeine concentration ranging from 0 – 800 ug/mL was used for quantification. Results were expressed as milligram caffeine equivalent per gram of dried weight (mg CE g^{-1} DW) using the calibration equation $Y = 0.0014x+0.0868$, $R^2 = 0.9903$, where Y is the absorbance and x the concentration.

2.4.2.4 Determination of total antioxidant activity

a. Ferric Reducing Antioxidant Power (FRAP) assay

Ferric Reducing Antioxidant Power reagent was prepared by mixing 25 mL acetate buffer, 2.5 mL 4,6-tris-pyridyl-s-triazie (TPTZ) solution and 2.5 mL Iron chloride hexahydrate ($FeCl_3.6H_2O$) solution (10:1:1 by volume) and then warmed at 37^{o}C for 1 minute before (208–210). 0.2 mL of diluted extracts and standard solution were added into falcon tubes in which 6 mL of freshly prepared FRAP reagent was added, vortexed for 10 seconds and incubated at room temperature for 10 minutes. Absorbance of the mixtures and blank (MeOH) solution was measured using UV-Vis Spectrophotometer at 593 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) with concentration ranging from 0 to 500 ug/mL was used as a standard for quantification and results expressed as milligram Trolox equivalent antioxidant capacity (TEAC) per gram of dried weight (mg TEAC/g DW).

b. DPPH (2, 2-Diphenyl-1-Picrylhydrazyl) radical scavenging activity assay

Radical scavenging activity of the MeOH and H_2O extracts was evaluated using DPPH reagent (208–212). Briefly, 0. 1 mL of 10-fold sample extracts each diluted with MeOH and H_2O were mixed with 80% MeOH (80:20 v/v) and then added to DPPH (0.1 mM, 3.9 mL). The mixture was vortexed for 10 seconds and incubated in darkness for 30 minutes at ambient temperature. Absorbance of the mixture and the control (MeOH) was measured using cuvettes on UV-Vis Spectrophotometer at 517 nm. Trolox standards were used for quantification and results expressed as % DPPH free radical scavenging activity according to the following equation: % DPPH = (1- *As* /*Ac*) x100, Whereas *As* is the absorbance of the sample and *Ac* is the absorbance of the control.

2.4.2.5 Statistical analysis for quantifying phytochemicals

All data were presented as means ± standard deviations of analyses in triplicates. The calculations were done in an excel spreadsheet. Graph Pad Prism® software (Version 8.4) was used to obtain a log-sigmoid dose response curve.

2.5 Brine-Shrimp's Lethality Assay (BSLA) for selected crude extracts

The toxicity of herbal extracts was determined by Brine Shrimp's lethality test (213). Brine Shrimp's (*Artemia salina*) eggs were obtained from Physiology Department, University of Malawi, College of Medicine, Malawi.

2.5.1 Preparation of Brine Shrimp's eggs

Artificial sea H_2O was made by dissolving 38 g of Sodium chloride (NaCl) into 1 L of H_2O and adjusting the pH to 8.3 using Sodium bicarbonate ($NaHCO_3$) (214). 500 mL of the artificial sea H_2O

was added to a shallow rectangular and transparent container (150 mm x 5 mm) (215). A plastic divider with several 2 mm holes was clamped in the middle of the dish to make two unequal compartments; the larger compartment which was darkened while the smaller compartment was illuminated (215). Dried Brine-Shrimps' eggs were weighed (0.5 g) and sprinkled into the larger compartment of container (216). The eggs were exposed to a concentrated source of light from a lamp (220–240 V) (216), and incubated for 48 hrs at ambient temperature (217).

2.5.2 Preparation of plant extracts

Plants extracts with antimicrobial activity of ≥ 11 mm zone of inhibition were used for the Brine Shrimp's toxicity test as these extracts exhibited significant Minimum Inhibitory and Bactericidal concentrations. These plants included crude water extracts of *B. Pilosa* and *D. cinirera* and crude MeOH extracts of B. *pilosa, D. cinerea, E. abyssinica L. javanica* and *A senegalensis*. 0.01 g of each test extracts were dissolved in 10 mL of 1 % DMSO in PBS as a stock solution. From the stock solution, two-fold dilution were made to obtain 1000, 500, 250, 125, 63 and 31 µg/mL in 1% DMSO in PBS. Each concentration was tested in triplicates.

2.5.3 Brine Shrimp's lethality test

After 24 hrs of Brine Shrimp's incubation, 200 µL salt H_2O containing 10 live nauplii was added to testing Eppendorf's (218,219). To facilitate easy transfer, nauplii were counted under a magnifying lens (3x) in the stem of the Pasteur pipette against an illuminated background and immediately after nauplii addition to the testing Eppendorf's (at time 0) for any dead brine-shrimp, which would be excluded from percentage mortality calculations. Thereafter, 200 µL of test samples in 1 % DMSO in PBS were added to Eppendorf tubes (in triplicate) for each concentration of concentration of 1000,

500, 250, 125, 63, 31 µg/mL. The negative control consisted of 200 uL of 1 % DMSO in PBS while the positive control consisted of 200 uL MeOH. Dead Brine-Shrimps were then counted after 24 and 48 hours. Larvae were considered dead if they did not exhibit any internal or external movement during 10 seconds of observation (220). The percentage mortality (death) at each dose level and control were determined by using the formula (221):

$$\% \text{ of mortality} = \frac{no.of\ dead\ nauplii}{Total\ number\ of\ nauplii} * 100$$

Samples providing a percentage mortality greater than 50 % were considered toxic (222). Graph Pad Prism® software (Version 8.4) was used to obtain a log-sigmoid dose response curve, from which the LC_{50} and LC_{90} values were determined. The LC_{50} value represented the concentration of a test substance necessary to have a lethal effect on 50 % of the brine-shrimp (221).

2.6 Purification and isolation of herbal active compounds

2.6.1 Chromatographic analysis

2.6.1.1 Thin layer chromatography for determination of column chromatography mobile solvent system

Since the type of the adsorbent, the size of the column, the polarity of the mobile phase as well as the rate of elution all affect the separation, these conditions were optimized to achieve the best separation of the crude extracts. Solvent systems for use as a mobile phase for each crude extract were determined from a prior TLC experiment. The solvent system was determined by using low polar solvent combined with high polar solvent in varying concentrations.

a. Preparation of Thin-layer Plates (TLC)

TLC plates were cleaned through developing in EtOAc:MeOH 2:1 (v/v) in order to move adsorbed contaminants away from the zone of development. Cleaning solvent, 60 mL of EtOAc:MeOH 2:1(v/v) was prepared in a fume hood to cover the bottom of the TLC developing chamber as well as the lower edge of a TLC plate when set inside the chamber. The silica gel plates (20 × 20 cm, Silica gel 60 F254, Merck) were cut using a pair of scissors to fit in the 200 mL developing chamber. Silica gel plates were inserted into the developing chamber with the tops leaning against the chamber walls. The chamber was covered, and the solvent was allowed to move up the plate by capillary action. The TLC plates were removed from chamber (after approximately between 2 and 5 minutes) and were arranged in a standing position within the fume hood until solvent evaporated. The cleaning solvent was discarded from the developing chamber by propping the plates upright in a drying oven at 100°C for 10 - 15 minutes.

b. Preparation of Developing Chambers

A piece of filter paper was cut using a pair of scissors to fit slightly below chamber height, and about half the chamber perimeter in width. This paper acted as a wick to draw solvent up the chamber wall and saturate the chamber with solvent vapors, thus improving reproducibility of separations.

c. Development of TLC Plates

The origin of the TLC plate was lightly marked with a pencil. The crude extracts of *B. pilosa* and *D. cinerea* (0.1 g) were each dissolved in 1 mL MeOH and then spotted on the TLC plate using capillary micropipettes, leaving a 1 cm border on the sides of the plate (19). The bands were air dried in a fume hood. The dried plates were put inside glass TLC chambers each saturated with hexane and DCM in

the following ratios 5:5, 7:3, 8:2 and 9:1 and DCM and MeOH in the following ratios; 8.5:1.5, 9:1, 9.5:0.5 using forceps without the plates touching the wick. The Plates were removed from TLC tank before the solvent front reached the top of the plate. The developed plates were dried in a fume hood before analysis.

d. Visualization of TLC plates

After the plates were dry, bands were visualized under UV light at both 254 nm (short-wave UV) and 365 nm (long-wave UV) (223).

2.6.1.2 Column chromatographic analysis of *Bidens pilosa* and *Dichrostachys cinerea*

The active MeOH extracts of *D. cinerea* and *B. pilo*sa were further purified on silica gel column chromatography. A vertical glass column (40 mm width and 60 mm length) made of borosilicate material was used for the fractionation. The column was rinsed with acetone and then completely dried before packing with 60 g of silica gel material. Silica slurry was prepared with 120 mL hexane (100 %) and was poured into the column to approximately fill 2/3rd of the column with simultaneous draining of the solvent to aid proper packing of the column. The active crude MeOH extracts of *B. pilosa* and *D. cinerea* (0.5 g) was dissolved in hexane (100 %) (1 mL) and the solution was poured down along the sides to form a thin horizontal band of sample at the top without disrupting the silica bed. The extract sticking to the inside surface of the column was gently washed down the column with hexane (100 %). Hexane (100 %) (2 mL) was added to the top of the extract to make the sample meniscus stable. Solvent level (about 6 cm above the silica bed) was maintained to prevent drying of the column. Gradient elution method was followed to separate fractions from the extracts by using solvents from low polarity to high polarity (i.e. from hexane and dichloromethane (DCM), EtOAc

MeOH and ethanol (EtOH) in varying ratios). The flow rate was adjusted to 5 mL/min and 5 mL solvent was collected 50 mL collection tubes for each fraction at the bottom of the column. Fractions with the same retention factors were pooled together.

a. Column chromatography mobile solvent system of Bidens pilosa

The starting elution solvent system was 100 % hexane for the purpose of eluting chloroforms and fatty acids which are non-polar. The following mixture of solvents; hexane: DCM (7:3) and DCM: MeOH (8.5: 1.5) were used as mobile solvent to elute the middle polar and polar compounds respectively. The polarity of the solvent system was then increased with 100 % EtOAc followed by 100 % EtOH as mobile phase for elution of compounds with very high polarity. Finally, the column was washed with 100 % MeOH, followed by drops of acetic acid to elute any remaining polar compounds.

b. Column chromatography mobile solvent system of Dichrostachys cinerea

The starting mobile solvent system was 100 % hexane for elution of nonpolar compounds: chloroforms and the fatty acids in the plant crude extract. Then hexane: DCM (7:3) mixture was used as mobile solvent to elute the middle polar compounds. A higher polar mobile solvent system; EtOAc and DCM in the ratio (9:1) was then used and the strength of polarity of the mobile phase was further increased using ethanol and ethyl acetate in the ratio (9:1) for elution of very polar compounds. The column was then finally washed with 100 % MeOH followed by drops of acetic acid to elute any remaining polar compounds.

2.6.1.3 Thin layer chromatography for pooling column fractions of *Bidens pil*osa and *Dichrostachys cinerea* and antimicrobial activity testing of fractions

Crude MeOH extracts of *B. pilosa* and *D. cinerea* fractions from column chromatography were run on TLC plate using DCM and MeOH (8.5:15) and hexane: DCM (7:3). Fractions with the same retention factor values were pooled and concentrated to dryness using rotary evaporator. Column fractions were tested for antimicrobial activities against *S. pneumoniae* serotype 6A and 1 as previously described. This was performed to identify fractions which were responsible for the antimicrobial activity observed in the extracts.

2.6.2 High Performance Liquid Chromatography of active fraction from *Bidens pilosa*

The *B. pilosa* fraction that showed activity (8 mm zone of inhibition) against *S. pneumoniae* serotype 6A was further analyzed on Agilent 1120 Compact Liquid Chromatography machine. The sample was separated on a Jones c18 column (250 mm* 4.6 mm, 5 uL particle sizes) at 30^{o}C. Sseparation was conducted by eluting with a gradient mobile phase controlled at a flow rate 1 mL/min. The sample was weighted in an analytic balance and diluted at a concentration of 7 mg/mL. The diluted sample was filtered through a 0.45 μM Millipore membrane, and 10 μL was injected in the chromatography machine. Gradient acetonitrile–acidified water with 1% formic acid at pH 2.8 was used as mobile phase. Acetonitrile concentration was gradually increased as follows: 0-min, 10 %; 3 - 7 min, 20 %; 7 - 10 min, 30 %; 10 - 14 min, 40 %; 14 - 18 min, 100 %; and 18 - 20 min, 10 %. The chromatograms were recorded at 254 nm (224).

CHAPTER 3: RESULTS

3.1 Plant extraction yield for water and methanol crude extracts

3.1.1 Water-based plant extraction

The plant materials (Table 2) yielded plant extract products ranging from 7.2 g to 13.6 g respectively. The highest yield of plant extract was obtained from *B. pilosa* (13.6 g) followed by *L. javanica* (12.0 g) and *E. abyssinica* (11.35 g), *T. zeylenicum* and *D. cinerea* with 9.3 g and 8.2 g respectively. *A. senegalensis* had the lowest yield of 7.2 g.

Table 2: Water plant extracts yield and recovery percentage

Plant	Original weight (OW) (g)	Extracted weight (EW)(g)	% recovery ($\frac{EW}{OW} \times 100$)
B. pilosa	200	13.6	6.8
L. javanica	200	12.0	6.0
E. abyssinica	200	11.4	5.6
T. zeylenicum	200	9.3	4.6
D. cinerea	200	8.2	4.1
A. senegalensis	200	7.2	3.6

3.1.2 Methanol-based plant extraction

The plant materials (Table 3) yielded plant extract products ranging from 4.8 to 26.6 g respectively. The highest yield of plant extract was obtained from *B. pilosa* (26.6 g) followed by *L. Javanica* (23.3 g). *E. abyssinica* (17.1 g) and *D. cinerea* and *T. zeylenicum* with 16.0 g and 15.0 g respectively. *A. senegalensis* had the lowest yield of 4.8 g.

Table 3: Methanol plant extracts yield and recovery percentage

Plant	Original weight (OW) (g)	Extracted weight (EW) (g)	% recovery ($\frac{EW}{OW} \times 100$)
B. pilosa	200	26.6	13.3
L. javanica	200	23.3	11.7
E. abyssinica	200	17.1	8.6
D. cinerea	200	16.0	8.0
T. zeylenicum	200	15.0	7.5
A. senegalensis	200	4.8	2.4

3.2 Bioactivity determination of water and methanol crude extracts against *S. pneumoniae* serotype 6A and 1

The bioactivity of the plant samples was tested against both *S. pneumoniae* serotype 6A and 1. This involved antimicrobial activity testing and the determination of minimum inhibitory and minimum bactericidal concentrations.

3.2.1 Antimicrobial activity of water and methanol crude extracts against *S. pneumoniae* serotype 6A and 1

3.2.1.1 Antimicrobial activity of crude water extract

The antimicrobial testing (Table 4) was done using disc diffusion method. Six plants namely: *B. pilosa, D. cinerea, E. abyssinica, L. javanica, T. Zeylenicum* and *A. senegalensis* were tested against *S. pneumoniae* serotype 1 and 6A. The highest zones of inhibitions were observed at the highest concentration of 1 mg/mL against *S. pneumoniae* serotype 6A by *B. pilosa* (11 ± 0.00 mm) and *D. cinerea* (10 ± 0.00 mm). This was followed by *E. abyssinica* (9 ± 0.00 mm) and *L. javanica* (8 ± 0.00 mm). The least inhibition against *S. pneumoniae* serotype 6A was portrayed by *A. senegalensis* (7 ± 0.00 mm) and *T. zeylenicum* (6 ± 0.00 mm). For serotype 1, the H_2O crude extracts generally exhibited lower zones of inhibitions as compared to the zones of inhibition exhibited by the same H_2O crude

extracts against serotype 6A. The largest zone of inhibition against serotype 1 was portrayed by *B. pilosa* and *E. abyssinica* (9 ± 00 mm) each. *D. cinerea, L. javanica* and *A. senegalensis* exhibited 8 ± 0.00 mm each while *T. zeylenicum* had the least inhibition of 7 ± 0.00 mm. Penicillin and 1% DMSO were used as positive and negative controls with ~25.0 mm and 0 mm zones of inhibition respectively.

Table 4: Antimicrobial activity of crude water extracts

Antimicrobial activity against pneumococcal serotypes 1 and 6A was measured by determining the zone of inhibition at different crude water extract concentrations of 0.001, 0.01, 0.1 and 1.0 mg/mL.

Serotype	Plant	Zone of inhibition (mm) (Mean± SD)			
		0.001	**0.01**	**0.1**	**1**
6A	[a] Bp	8.0 ± 0.00	8.6 ± 0.57	9.6 ± 0.57	11.0 ± 0.00
	[b] Dc	6.0 ± 0.00	6.6 ± 1.15	8.6 ± 1.15	10.0 ± 0.00
	[c] Ea	6.0 ± 0.00	6.0 ± 0.00	8.0± 0.00	9.0 ± 0.00
	[d] Lj	6.0 ± 0.00	6.0 ± 0.00	8.0 ± 0.00	8.0 ± 0.00
	[e] As	6.0 ± 0.00	7.0 ± 0.00	7.0 ± 0.00	7.0 ± 0.00
	[f] Tz	6.0 ± 0.00	6.0 ± 0.00	6.0 ± 0.00	6.0 ± 0.00
	[g] PC	25.0 ± 0.00	25.0 ± 0.00	25.5 ± 0.00	25.0 ± 0.00
1	[a] Bp	7.0 ± 0.00	7.6 ± 0.57	8.6 ± 0.57	9.0 ± 0.00
	[c] Ea	6.0 ± 0.00	6.6 ± 0.15	8.6 ± 0.15	9.0 ± 0.00
	[b] Dc	6.0 ± 0.00	6.6 ± 0.57	7.6 ± 0.57	8.0 ± 0.00
	[d] Lj	6.0 ± 0.00	7.0 ± 0.00	7.0 ± 0.00	8.0 ± 0.00
	[e] As	6.0 ± 0.00	7.0 ± 0.00	7.0 ± 0.00	8.0 ± 0.00
	[f] Tz	6.0 ± 0.00	6.0 ± 0.00	6.0 ± 0.00	7.0 ± 0.00
	[g] PC	24.3 ± 1.15	24.6 ± 0.57	25.0 ± 0.00	25.0 ± 0.00

[g] Penicillin was used as positive control while 1% DMSO was used as negative control. [a] Bp: *B. pilosa*, [b] Dc: *D. cinerea*, [c] Ea: *E. abyssinica*, [d] Lj: *L. javanica*, [e] As: *A. senegalensis* [f] Tz: *T. zeylenicum* and [g] PC: Positive control.

3.2.1.2 Antimicrobial activity of crude methanol extract

The antimicrobial testing (Table 5) was performed using disc diffusion method. Six plants: *B. pilosa, D. cinerea, E. abyssinica, L. javanica, T. Zeylenicum* and *A. senegalensis* were tested against *S. pneumoniae* serotype 6A and 1. Crude MeOH extract of *B. pilosa* portrayed the highest zone of inhibition against *S. pneumoniae* serotype 6A with 24.3 ± 0.57 mm zone of inhibition at the highest

concentration of 1 mg/mL. *D. cinerea*, *L. javanica*, *E. abyssinica* and *A. senegalensis* followed with 15 ± 0.00 mm, 11.6 ± 0.57 mm,11 ± 0.00 mm and 10.3 ± 0.57 mm zones of inhibition respectively. The least inhibition against pneumococcal serotype 6A was portrayed by *T. zeylenicum* with a zone of inhibition of 8 ± 0.00 mm. For serotype 1, the highest inhibition was portrayed by *B. pilosa* and *D. cinerea* with 15.6 ± 0.57 mm and 13.6 ± 0.57 mm respectively. *A. senegalensis* and *E. abyssinica* inhibited serotype 1 with 11 ± 0.00 mm and 10.6 ± 0.57 mm respectively. While *L. javanica* and *T. zeylenicum* had the least inhibition of 8 ± 0.00 mm and 7 ± 0.00 mm.

Table 5: Antimicrobial activity of crude methanol extracts

Serotype	**Plant**	**Zone of inhibition (mm) Mean ± SD**			
		0.001	**0.01**	**0.1**	**1**
6A	[a] *Bp*	11± 0.00	14 ± 0.00	16.3 ± 0.57	24.3 ± 0.57
	[b] *Dc*	10.3 ± 0.57	12 ± 0.57	14 ± 0.00	15 ± 0.00
	[c] *Ea*	8.3 ± 0.57	8.6 ± 0.57	9.6 ± 0.57	11 ± 0.00
	[d] *Lj*	8 ± 0.00	9 ± 1.00	10.3 ± 0.57	11.6 ± 0.57
	[e] *As*	8.6 ± 0.57	9 ± 0.57	10 ± 0.00	10.3 ± 0.57
	[f] *Tz*	6.0 ± 0.00	7 ± 0.00	7.6 ± 0.57	8 ± 0.00
	[g] PC	25 ± 0.00	25 ± 0.00	25 ± 0.00	25 ± 0.00
1	[a] *Bp*	10 ± 0.00	12 ± 0.00	13.6 ± 0.57	15.6 ± 0.57
	[b] *Dc*	9 ± 1.11	11 ± 0.11	12.3 ± 0.57	13.6 ± 0.57
	[e] *As*	8.6 ± 0.57	10 ± 0.57	11 ± 0.00	11 ± 0.00
	[c] *Ea*	7.3 ± 0.57	8.3 ± 0.00	9.3 ± 0.57	10.6 ± 0.57
	[d] *Lj*	6 ± 0.00	7 ± 0.00	7.3 ± 0.57	8 ± 0.00
	[f] *Tz*	6 ± 0.00	6 ± 0.00	6.3 ± 0.57	7 ± 0.00
	[g] PC	24.6 ± 0.57	25 ± 0.57	25 ± 0.00	24.3 ± 1.15

The table shows zones of inhibition as a measure of activity at different crude methanol extract concentrations of 0.001, 0.01, 0.1 and 1 mg/mL against *S. pneumoniae* serotypes 1 and 6A. [g] Penicillin was used as positive control with ~25.0

mm zone of inhibition, while DMSO (1 %) was used as negative control with 0.0 mm zone of inhibition. [a] Bp: *B. pilosa*, [b] Dc: *D. cinerea*, [c] Ea: *E. abyssinica*, [d] Lj: *L. javanica*, [e] As: *A. senegalensis* [f] Tz: *T. zeylenicum*, and [g] PC: Positive control

3.2.2 The Minimum Inhibitory Concentration (MIC) of water and methanol extracts against *S. pneumoniae* serotype 6A and 1

The minimum inhibitory concentration was determined using Mueller Hinton agar (MHA) dilution method. MIC was determined as the lowest concentration that completely inhibited the growth of the bacteria. The crude extracts which portrayed antimicrobial activity of >10 mm zone of inhibition were chosen for MICs determination. These were crude H_2O extracts of *B. pilosa* and *D. cinerea* against *S. pneumoniae* serotype 6A and crude MeOH extracts of *B. pilosa*, *D. cinerea, E. abyssinica, L. javanica* and *A. senegalensis* against both *S. pneumoniae* serotype 6A and 1. The MIC interpretation: (—) no growth, (+) growth, (±) variable growth. No growth means the crude extracts completely inhibited the growth of the test organisms, growth means the crude extracts did not inhibit the growth of test organisms and variable growth means the crude extracts were able to suppress the growth of organisms but no complete inhibition.

3.2.2.1 The Minimum Inhibitory Concentration of crude water extracts against *S. pneumoniae* serotype 6A

The MIC (Table 6) determination was performed on crude H_2O extracts of *B. pilosa* and *D. cinerea* against *S. pneumoniae* 6A. The MIC determination of these crude H_2O extracts showed that both *B. pilosa* and *D. cinerea* had an MIC of 16 mg/mL against serotype 6A. Sheep Blood Agar plates with 1% DMSO were used as negative control (+) while plates with Sheep Blood Agar media only were used as positive control (—).

Table 6: Minimum Inhibition Concentration of crude water extracts of B. pilosa and D. cinerea against S. pneumoniae serotype 6A

Serotype	Plant Extract (Water)	MIC at varying plant extract concentrations (mg/ml)					
		16	8	4	2	1	0.1
6A	[a] *Bp*	—	±	±	+	+	+
	[b] *Dc*	—	±	±	+	+	+
	[h] DMSO	+	+	+	+	+	+
	[g] PC	—	—	—	—	—	—

The MICs of water extracts were measured at varying concentrations of 16, 8, 4, 2, 1, and 0.1 mg/mL against serotype 6A. [a] Bp: *B. pilosa*, [b] Dc: *D. cinerea*, [h] 1% DMSO (negative control) and [g] PC: Positive control.

3.2.2.2 The Minimum Inhibitory Concentration of crude methanol extracts against *S. pneumoniae* serotype 6A and 1

The MICs were performed on crude MeOH extracts of *B. pilosa, D. cinerea, E. abyssinica* and *A. senegalensis* against both *S. pneumoniae* serotype 1 and 6A. MIC of *L. javanica was* performed against serotype 6A (Table 7). The MIC determination showed that theMeOH extracts of *B. pilosa* had the lowest MIC of 1 mg/mL against both serotypes. This was followed by *D. cinerea* with 2 mg/mL against both serotypes. *A. senegalensis*, *E. abyssinica* and *L. javanica* portrayed 8 mg/mL as their MICs against serotype 6A and *A. senegalensis* and *E. abyssinica* had a high MIC of 16 mg/mL each against serotype 1. Sheep Blood Agar plates with 1% DMSO were used as negative control (+) while plates with Sheep Blood Agar media only were used as positive control (—).

Table 7: Minimum Inhibition Concentration of crude methanol extracts against S. pneumoniae serotype 6A and 1

Serotype	Plant Extract (Methanol)	MIC at varying plant extracts concentrations (mg/ml)					
		16	8	4	2	1	0.1
6A	[a] *Bp*	—	—	—	—	—	±
	[b] *Dc*	—	—	—	—	±	+
	c Ea	—	—	±	+	+	+
	[d] *Lj*	—	—	±	+	+	+
	[e] *As*	—	—	±	+	+	+
	[h] DMSO	+	+	+	+	+	+
	[g] PC	—	—	—	—	—	+
1	[a] *Bp*	—	—	—	—	—	±
	[b] *Dc*	—	—	—	—	±	+
	[e] *As*	—	±	+	+	+	+
	[c] *Ea*	—	±	+	+	+	+
	[h] DMSO	+	+	+	+	+	+
	[g] PC	—	—	—	—	—	+

The MICs of methanol extracts were measured at varying concentrations of 16, 8, 4, 2, 1, and 0.1 mg/mL against *S. pneumoniae* serotype 6A and 1. 1% DMSO was used as a negative control and plates with test organisms were used as a positive control. [a] Bp: *B. pilosa*, [b] Dc: *D. cinerea*, [c] Ea: *E. abyssinica*, [d] Lj: *L. javanica*, [e] As: *A. senegalensis*, [g] PC: Positive control and [h] DMSO: Negative control

3.2.3 Minimum Bactericidal Concentration (MBC) of water and methanol extracts against *Streptococcus pneumoniae* serotype 6A and 1

Minimum bactericidal concentration is defined as the minimum concentration producing 99.9% reduction in colony forming units (CFU) in the initial inoculum (186,187). The MBC's were determined for extracts with antimicrobial activity of ≥10 mm zone of inhibition. These extracts included H_2O extracts of *B. pilosa* and *D. cinerea* and MeOH extracts of *B. pilosa, D. cinirea, E. abyssinica, L. javanica* and *A. senegalensis*.

3.2.3.1 The minimum bactericidal concentration of crude water and methanol extracts against *S. pneumoniae* serotype 6A and 1

The MBC (Table 8) for H_2O extracts were indeterminate within the concentrations used while MeOH extracts of *B. pilosa* and *D. cinerea* had the lowest MBC of 2 and 4 mg/mL, respectively, and MeOH extracts of *E. abyssinica*, *L. javanica* and *A. senegalensis* had a high MBC of 16 mg/mL against both serotypes 6A and 1.

Table 8: MBC results of selected water and methanol extracts

	Sp	Extracts	MIC (mg/ml)	MBC (mg/ml)
Water	S6A	[a] *Bp*	16	–
		[b] *Dc*	16	–
Methanol	S6A	[a] *Bp*	1	2
		[b] *Dc*	2	4
		[c] *Ea*	8	16
		[d] *Lj*	8	16
		[e] *As*	8	16
	S1	[a] *Bp*	2	4
		[b] *Dc*	4	8
		[e] *As*	16	16
		[c] *Ea*	16	16

The table shows MIC's and MBC's of selected crude water and methanol extracts. The MBC's results were interpreted as the minimum concentration that produces 99.9% reduction in the number of colony forming units (cfu) in the initial inoculum. [a] Bp: *B. pilosa*, [b] Dc: *D. cinerea*, [c] Ea: *E. abyssinica*, [d] Lj: *L. javanica*, and [e] As: *A. senegalensis*.

3.3 Phytochemical analysis of the crude extracts

3.3.1 Qualitative phytochemical analysis of crude extracts

3.3.1.1 Qualitative phytochemical analysis of crude water extract

The crude water extracts were tested for the presence of various phytochemicals using standard methods (Table 9). These phytochemicals included flavonoids, phenols, alkaloids, tannins, saponins, glycosides, steroids, terpenoids, reducing sugars and anthocyanins. The phytochemical analysis for

H_2O extracts showed that the rest of the extracts did not contain flavonoids and phenols except for *A. senegalensis* and *E. abyssinica*. All the crude H_2O extracts tested positive for alkaloids and saponins except for *A. senegalensis*. The H_2O extracts did not contain glycosides, steroids, terpenoids, reducing sugars and anthocyanins except for *E. abyssinica* for reducing sugars.

Table 9: Qualitative Phytochemical analysis of crude water extract

Phytochemical analysis	**Result**					
	[a] Bp	[b] Dc	[c] Ea	[d] Lj	[e] As	[f] Tz
Flavonoids	-	-	+	-	+	-
Phenols	-	-	+	-	+	-
Tannins	-	-	+	-	-	-
Alkaloids	+	+	+	+	+	+
Saponins	+	+	+	+	-	+
Glycosides	-	-	-	-	-	-
Steroids	-	-	-	-	-	-
Terpenoids	-	-	-	-	-	-
Reducing sugars	-	-	+	-	-	-
Anthocyanins	-	-	-	-	-	-

The presence of a phytochemical compound was indicated by (+) while the absence of a phytochemical was presented as (—). [a] Bp: *B. pilosa*, [b] Dc: *D. cinerea*, [c] Ea: *E. abyssinica*, [d] Lj: *L. javanica*, [e] As: *A. senegalensis* and [f] Tz: *T. zeylenicum*.

The absence/presence of phytochemicals was interpreted based on physical properties of chemical reactions (Fig. 10). The presence of alkaloids (A) was shown by a brown precipitate at the bottom of the test tubes (the test tubes contained plant extract, hydrochloric acid, Meyer's and Wagner's reagents). A positive test for tannins (B) was indicated by color change to black (The test tubes contained a reaction between plant extracts and ferric chloride). The presence of saponins (C) was confirmed by the formation of foam (The test tubes contained plant extracts and distilled H_2O)

Figure 10: An illustration of qualitative phytochemical analysis of crude water extracts

Test for alkaloids (A), test for tannins (B) and test for saponins (C)

3.3.1.2 Qualitative phytochemical analysis of crude methanol extracts

The qualitative phytochemical analysis of crude MeOH extracts showed that all extracts contained phenols, flavonoids and tannins using the standard methods. Only *E. abyssinica* showed the presence of alkaloids (Table 10).

Table 10: Qualitative phytochemical analysis of crude methanol extracts

Phytochemical analysis	**Result**					
	[a] Bp	[b] Dc	[c] Ea	[d] Lj	[e] As	[f] Tz
Flavonoids	+	+	+	+	+	+
Phenols	+	+	+	+	+	+
Tannins	+	+	+	+	+	+
Alkaloids	-	-	+	-	-	-
Saponins	+	-	-	+	+	+
Glycosides	+	+	-	+	-	-
Steroids	+	+	-	+	-	-
Terpenoids	-	-	-	-	-	+
Reducing sugars	+	-	+	-	-	-
Anthocyanins	-	-	+	-	+	-

The presence of a phytochemical compound was indicated by (+) while the absence of a phytochemical was presented as (—), [a] Bp: *B. pilosa*, [b] Dc: *D. cinerea*, [c] Ea: *E. abyssinica*, [d] Lj: *L. javanica*, [e] As: *A. senegalensis* and [f] Tz: *T. zeylenicum*.

Saponins were present in *B. pilosa*, *L. javanica*, *A. senegalensis* and *T. zeylenicum*. Glycosides and steroids were present in *B. pilosa*, *D. cinerea* and *L. javanica*. *T. zeylenicum* was the only MeOH

extract that showed presence of terpenoids. Reducing sugars were present in *B. pilosa* and *E. abyssinica.* Anthocyanins were present in *E. abyssinica* and *A. senegalensis.*

3.3.2 Quantitative phytochemical analysis

Quantitative phytochemical analysis was performed on both crude water and methanol extracts using UV-VIS Spectrophotometer. The following phytochemical compounds were quantified: Total Phenolics Content (TPC), Total Flavonoid content (TFC), 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) radical scavenging activity, Ferric Reducing Antioxidant Power (FRAP) and Total Alkaloid Content.

3.3.2.1 Quantitative phytochemical analysis of crude water extracts

The quantitative phytochemical analysis was first performed on H_2O extracts of *B. pilosa*, *D. cinerea*, *E. abyssinica*, *L. javanica*, *A. senegalensis* and *T. zeylenicum* (Table 11). The Total Phenolic Content (TPC) analysis showed the following concentrations from the highest to the lowest: *B. pilosa* (744.68 ± 1.01), *E. abyssinica* (569.24 ± 0.88), *D. cinerea* (411.34 ± 1.0) *L. javanica* (261.63 ± 2.67), *T. zeylenicum* (206.08 ± 1.01) and *A. senegalensis (*112.51 ± 1.01) mg GAE/100 g, dw. Total Flavonoids Content (TFC) showed that the highest content was obtained from *E. abyssinica* (109.91 ± 0.59) followed by *D. cinerea* (108.25 ± 0.36), *T. zeylenicum* (107.98 ± 0.62), *B. pilosa* (89.04 ± 0.72) and *A. senegalensis* (72.21 ± 0.85) while the lowest TFC was observed from *L. javanica* with TFC of 54.29 ± 1.5 TFC in dry matter (mg QE/100 g). Percentage scavenging activity presented by DPPH (2, 2-Diphenyl-1-Picrylhydrazyl) showed that the highest activity was observed from H_2O extracts of *D. cinerea* (45.46 ± 0.32 %) followed by *B. pilosa* (41.93 ± 0.00 %). Water extract of *E. abyssinica* and *L. javanica* had % scavenging activity of 39.43 ± 0.30 % and 31.56 ± 0.20 % respectively while H_2O extract of *A. senegalensis* and *T. zeylenicum* had 29.30 ± 0.145 % and 24.40 ± 0.30 % scavenging

activity respectively. The Ferric Reducing Antioxidant Power (FRAP) determination ofH_2O extracts showed the following concentrations from the highest to the lowest: *D. cinerea* (391.44 ± 0.99), *B. pilosa* (342.47 ± 0.19), *E. abyssinica* (299.48 ± 1.72), *L. javanica* (283.39 ± 2.63), *A. senegalensis* 283.39 ± 0.99 and *T. zeylenicum* (220.11 ± 0.09) AA in dry matter (mg TEAC/100 g). Total Alkaloid Content of crude H_2O extracts showed the highest TAC from E. *abyssinica (*821.09 ± 2.25) followed by *B. pilosa* (666.35 ± 0.00) and *D. cinerea (*534.61 ± 0.06*), L. javanica (*524.95 ± 1.29), *A. senegalensis* (165.22 ± 0.30) and *T. zeylenicum* (95.60 ± 0.54) TAC in dry matter (mg CE/100 g).

Table 11: Quantitative phytochemical analysis of crude water extracts

Water extract	[i] **TPC in Dry matter (mg GAE/100 g)**	[j] **TFC in dry matter (mg QE/100 g)**	[k] **DPPH %**	[l] **AA in dry matter (mg TEAC/100 g)**	[m] **TAC in dry matter (mg CE/100 g)**
[a] Bp	744.67 ± 1.01	89.03 ± 0.72	41.93 ± 0.05	342.47 ± 0.19	666.34 ± 0.42
[b] Dc	411.34 ± 1.01	108.24 ± 0.36	45.46 ± 0.32	391.43 ± 0.99	534.61 ± 0.06
[c] Ea	569.23 ± 0.88	109.91 ± 0.59	39.43 ± 0.30	299.48 ± 1.72	821.09 ± 2.25
[d] Lj	261.63 ± 2.67	54.28 ± 1.50	31.56 ± 0.20	283.39 ± 2.63	524.95 ± 1.29
[e] As	112.51 ± 1.01	72.21 ± 0.85	29.30 ± 0.14	283.39 ± 0.99	165.22 ± 0.30
[f] Tz	206.08 ± 1.01	107.92 ± 0.62	24.40 ± 0.30	220.11 ± 0.09	95.59 ± 0.54

Quantitative phytochemical analysis of water extracts. The tests were done in triplicates and standard deviation was used to generate the results. [a] Bp: *B. pilosa*, [b] Dc: *D. cinerea*, [c] Ea: *E. abyssinica*, [d] Lj: *L. javanica*, [e] As: *A. senegalensis* and [f] Tz: *T. zeylenicum*, [i] TPC: Total Phenolic Content, [j] TFC: Total Flavonoid Content, [k] DPPH: 2, 2-Diphenyl-1-Picrylhydrazyl) [l] FRAP: Ferric Reducing Antioxidant Power and [m] TAC: Total Alkaloid Content.

3.3.2.2 Quantitative phytochemical analysis of methanol extracts

The quantitative phytochemical analysis was performed on MeOH extracts of *B. pilosa*, *D. cinirea*, *E. abyssinica*, *L. javanica*, *A. senegalensis* and *T. zeylenicum* (Table 12). Quantification of Total Phenolic Content (TPC) of the extracts showed the following concentrations from the highest to the lowest: *B. pilosa (*2025.38 ± 1.01), *D. cinerea* (1416.61 ± 1.01), *E. abyssinica* (1113.68 ± 0.50), *L. javanica* (825.38 ± 1.01), *A. senegalensis* (649.94 ± 1.01) and *T. zeylenicum* (615.44 ± 1.75) mg

GAE/100 g, dw. The Total Flavonoid Content (TFC) of the extracts showed the highest TFC from *B. pilosa (*322.85 ± 0.27) followed by D. *cinerea* (236.42 ± 1.22) and *E. abyssinica (*186.74 ± 0.82) TFC in dry matter (mg QE/100 g). These were followed *A. senegalensis, T. zeylenicum* and *L. javanica* with Total Flavonoids Content of 151.26 ± 0.47, 145.86 ± 0.49 and 143.88 ± 0.82 TFC in dry matter (mg QE/100 g) respectively. DPPH (2, 2-Diphenyl-1-Picrylhydrazyl) scavenging activity of methanol extracts showed that *D. cinerea* recorded the highest % scavenging activity of 55.05 ± 0.60 % followed by *B. pilosa* (52.42 ± 0.40 %) , *E. abyssinica* (44.67 ± 0.15 %) and *L. javanica* (40.26 ± 0.20 %) While *T. zeylenicum* and *A. senegalensis* had 39.60 ± 0.43 % and 38.53 ± 0.28 % scavenging activity respectively. The Ferric Reducing Antioxidant Power (FRAP) results showed that *D. cinerea* had a higher FRAP of 567.87 ± 0.99 AA in dry matter (mg TEAC/100g. This was followed by *B. pilosa* and *E abyssinica* with 497. 701 ± 0.09 and 393.74 ± 0.99 AA in dry matter (mg TEAC/100 g) respectively. While *A. senegalensis*, *L. javanica* and *T. zeylenicum* had a FRAP of 361.55 ± 0.00, 348.91 ± 0.99 and 314.43 ± 1.99 AA in dry matter (mg TEAC/100 g) respectively. The Total Alkaloid Content determination of the extracts showed the following TAC from highest to the lowest: *B. pilosa* (3734.98 ± 1.22), *E. abyssinica* (2249.72 ± 1.10), *D. cinerea (*1892.13 ± 1.06), *L. javanica* (524.95 ± 1.29), *A. senegalensis* (490.90 ± 1.97) and *T. zeylenicum* (169.67 ± 1.93) TAC in dry matter (mg CE/100 g).

Table 12: Quantitative Phytochemical analysis of crude methanol extracts

Methanol extract	[i] TPC in Dry matter (mg GAE/100 g)	[j] TFC in dry matter (mg QE/100 g)	[k] DPPH %	[l] AA in dry matter (mg TEAC/100 g)	[m] TAC in dry matter (mg CE/100 g)
[a] BP	2025.38 ± 1.01	322.84 ± 0.27	52.42 ± 0.40	497.70± 0.09	3734.97 ± 1.22
[b] Dc	1416.60 ± 1.01	236.42 ± 1.22	55.05 ± 0.60	567.87 ± 0.99	1892.13 ± 1.06
[c] Ea	1113.68 ± 0.50	186.73 ± 0.82	44.66 ± 0.15	393.73 ± 0.99	2249.72 ± 1.10
[d] Lj	825.38 ± 1.01	143.88 ± 0.82	40.26 ± 0.20	348.90 ± 0.99	1465.94 ± 1.27
[e] As	649.94 ± 1.01	151.26 ± 0.47	38.53 ± 0.28	361.55 ± 0.00	490.90 ± 1.97
[f] Tz	615.43 ±1.75	145.86 ± 0.49	39.60 ± 0.43	314.42 ± 1.99	169.66 ± 1.93

The tests were done in triplicates and standard deviation was used to generate the results. [a] Bp: *B. pilosa*, [b] Dc: *D. cinerea*, [c] Ea: *E. abyssinica*, [d] Lj: *L. javanica*, [e] As: *A. senegalensis* and [f] Tz: *T. zeylenicum*, [i] TPC: Total Phenolic Content, [j] TFC: Total Flavonoid Content, [k] DPPH: 2, 2-Diphenyl-1-Picrylhydrazyl), [l] FRAP: Ferric Reducing Antioxidant Power and [m] TAC: Total Alkaloid Content.

3.4 Brine Shrimp's lethality assay

The Brine Shrimps lethality analysis was performed on crude extracts with antimicrobial activity ≥ 11 mm: water extract of *B. pilosa* and MeOH extracts of *B. pilosa, D. cinerea, E. abyssinica, L. javanica* and *A. senegalensis* (Table 13).

Table 13: The percent mortality and the lethality dose of extracts required to kill 50 and 90 % of the population of Brine Shrimps

Plant Extract	% mortality at 48 hrs Concentration µg/mL						% Mortality of same Extract (mean ± SD)	[n] LC_{50} (µg/mL)	[o] LC_{90} (µg/mL)
	0.03	**0.06**	**0.12**	**0.25**	**0.5**	**1**			
[a] *Bp* (H_2O)	0	0	0	3	7	10	3.33 ± 4.27	4498.52	8073.14
[z] *As* (MeOH)	0	0	3	3	7	16	4.83 ± 6.04	3121.19	5594.76
[x] *Ea* (MeOH)	0	7	7	10	17	27	11.3 ± 9.43	2019.71	3813.35
[y] *Lj* (MeOH)	7	10	13	13	20	33	16.9 ± 9.38	1660.18	3206.79
[v] *Bp* (MeOH)	0	0	7	10	27	47	15.16 ± 18.47	1061.39	1886.79
[w] *Dc* (MeOH)	13	17	23	30	40	60	30.5 ± 17.3	751.33	1660.87

[n] LC_{50}: lethal dose concentration required to kill 50 % of the Brine Shrimps population, [o] LC_{90}: lethal dose concentration required to kill 90% of the brine shrimp population, [a] Bp (H_2O): water extract of *B. pilosa*, and MeOH extracts of; [z] As: *A. senegalensis*, [x] Ea: *E. abyssinica*, [y] Lj: *L. javanica*, [v] Bp: *B. pilosa* and [w] Dc: *D. cinerea*

The percent mortality and the lethality dose of extracts required to kill 50 and 90 percent of the population of Brine Shrimps for these crude extracts was recorded. The mean ± SD for H_2O extract of *B. pilosa,* MeOH extracts of *B. pilosa, D. cinerea, E. abyssinica, L. javanica* and *A. senegalensis* was found to be 15.16 ± 18.47, 3.33 ± 4.27, 30.50 ± 17.30, 11.30 ± 9.43, 16.00 ± 9.38 and 4.83 ± 6.04 respectively. Of the six plant extracts tested, 5 (83 %) showed an LC_{50} values greater than 1000 µg/mL while one extract showed LC_{50} value of less than 1000 µg/mL. The extracts with LC_{50} values of greater than 1000 µg/mL were H_2O extract of *B. pilosa*, MeOH extracts of *B. pilosa, E. abyssinica, L. javanica* and *A. senegalensis* with 4498.52,1061.39, 2019.71, 1660.18 and 3121.19 µg/mL respectively. MeOH extract of *D. cinerea* had an LC_{50} value of less than 1000 µg/mL of 751.33 µg/mL. Overall, there was an increase in percent mortality of larvae (nauplii) with the increase of the doses of the extracts.

3.5 Purification of crude extracts and isolation of active compounds

3.5.1 Thin Layer Chromatography (TLC)

TLC was performed on two active crude MeOH extracts of *B. pilosa* and *D. cinerea* to determine solvent separating system that would be used for column separation. *B. pilosa* separated well with DCM and MeOH (8.5:1.5) while *D. cinerea* separated well with Then hexane and DCM (7:3).

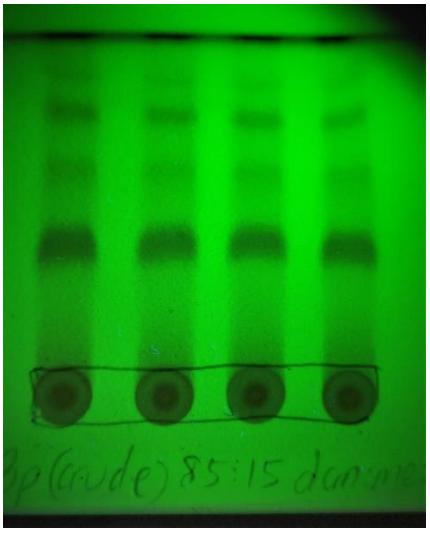

Figure 11: Prior TLC for crude methanol extract of Bidens Pilosa.

TLC for crude MeOH extract of B. pilosa using DCM and MeOH (8.5:1.5) as mobile solvent system.

3.5.2 Column chromatography of crude methanol extracts from *Bidens pilosa* and *Dichrostachys cinerea*

3.5.2.1 Column chromatography of crude methanol extract from *Bidens pilosa*

Column chromatography of crude MeOH extract from *B. pilosa* (Fig. 12) eluted a total of 14 fractions which were sequentially collected with the following mobile solvent systems: hexane (100 %), hexane and DCM (7:3), DCM and MeOH (8.5:1.5), EtOAc (100 %), EtOH (100 %) and 100 % MeOH followed by 5 drops of acetic acid. The 14 fractions contained non-polar, middle polar and very polar

compounds based on the properties of the mobile separating solvent systems stated in the methods (9).

Figure 12: Column chromatography of crude methanol extract of Bidens pilosa

3.5.2.2 Column chromatography of crude methanol extract of *Dichrostachys cinerea*

Column chromatography for crude MeOH extract of *D. cinerea* (Fig. 13) eluted a total of 18 fractions by using the following mobile solvent phases in the following sequence: hexane (100 %), hexane and DCM (7:3), ethyl acetate and DCM (9:1), EtOH and EtOAc (9:1) and then MeOH (100 %) followed by 5 drops of acetic acid. The solvents ranged from non-polar, middle polar to very polar to achieve separation across all polarities.

Figure 13: Column Chromatography of crude methanol extract of Dichrostachys cinerea

3.5.3 Thin Layer chromatography for column fractions of crude methanol extracts of *Bidens pilosa* and *Dichrostachys cinerea*

Column fractions for both extracts were spotted on a separate TLC plate. Fractions with same retention factor values were combined. Crude methanol extract of *B. pilosa* had a total of 14 fractions which were pooled to 9 fractions while *D. cinerea* had a total of 18 column fractions which generated 11pooled fractions from TLC analysis.

3.5.4 Antimicrobial activity for *Bidens pilosa* and *Dichrostachys cinerea* column fractions

Fraction 5 of *B. pilosa* exhibited antimicrobial activity of 8 mm zone of inhibition against both serotypes 6A and 1. *D. cinerea* fraction 10 showed varying activity for the pneumococcal serotypes with 7 mm zone of inhibition against serotype 6A, and 5 mm zone of inhibition against serotype 1. Penicillin and 1 % DMSO were used as positive and negative controls with ~25.0 mm and 0 mm zones of inhibition respectively.

3.5.5 High Performance Liquid Chromatography (HPLC) of *Bidens pilosa's* active fraction

The HPLC analysis produced 3 peaks between 4 and 8 minutes with mobile solvent of acetonitrile and 1% formic acid - water: 2:8 and 3:7. The fractions were further tested for antimicrobial activity against both *S. pneumoniae serotype* 6A and 1. However no activity was observed from these fractions.

CHAPTER 4: DISCUSSION AND CONCLUSION

4.1 Yield of plant extractions

Higher yields were given by methanol extracts ($p < 0.05$). This difference may be attributable to the higher solubility of extractable bioactive components such as carbohydrates and proteins in MeOH than in H_2O (225). Typically, high polarity solvent like MeOH is widely used to extract various plant components due to its compatibility and efficacy towards solubilization of plant compounds (226). The significant difference between MeOH and H_2O extraction yields could also be associated to the different chemical nature of compounds in the plant materials (227). The high range difference observed between MeOH extract of *A. senegalensis* as compared to the other MeOH extracts and generally low yield from H_2O extract of the same could be attributed to plant degradation and co-precipitation with starches during extraction which is common in *A. senegalensis* and results in low yield (228). The overall low yield from both MeOH and H_2O extracts could be attributed to the extraction methods used. The grinding of plant extracts used in this study results in coarse sample particles which means reduced surface area contact with solvents and therefore low yield as compared to powdered samples which produce homogenized and fine particles leading to increased surface area contact with extraction solvents and more efficient extraction to occur (229).

4.2 Bioactivity of plant extracts against *S. pneumoniae* and purification of the plant extracts

The high zones of inhibition of *B. pilosa* against both serotypes 6A and 1 (24.3 and 15.6 mm) concurs with a study reported by Garima Singh *et al* (130) who found that methanolic extract of *B. pilosa* showed zone of inhibition of 18.2 mm against *E. coli* and 15 mm against *S. aureus*. The reason for the increased activity from *B. pilosa* shown by high zones of inhibition and reduced MIC and MBC

concentrations could be due to the several (201) compounds that have been identified from *B. pilosa* comprising of 70 aliphatics, 60 flavonoids, 25 terpenoids, 19 phenylpropanoids, 13 aromatics, 8 porphyrins, and 6 other compounds ((230) and that this complex phytochemical composition reflect the diverse bioactivities observed in this study. Methanolic extract of *D. cinerea* exhibited antimicrobial inhibitory effect of 15 mm zone of inhibition with MIC of 2 mg/mL and MBC of 4 mg/mL) against *S. pneumoniae* serotype 6A and 13.6 mm zone of inhibition with MIC of 2 mg/mL and MBC of 8 mg/mL against *S. pneumoniae* serotype 1 which is comparable to standard known antibiotics penicillin (50 μg/disc). The inhibitory activities exhibited by *D. cinerea* indicated could be attributed to the presence of tannins, alkaloids, flavonoids, terpenoids and essential oils which have been identified from the plant (231). For example, a study conducted on evaluation of antimicrobial activities of *D. cinerea* found that tannins isolated from this plant exhibited antibacterial activity against *S. aureus* 12.0 ± 0.02, *S. boydii* 18.0 ± 0.1, *S. flexneri* 15.0 ± 0.05, *E. coli* 18.0 ± 0.3 and *P. aeruginosa* 16.0 ± 0.1 (232). The difference in activity between MeOH extracts of *B. pilosa* and *D. cinerea* could be associated with differences in the nature of chemical compounds constituents of each plant.

The active MeOH extracts (*B. pilosa* and *D. cinerea*) were fractionated using column chromatography to separate active compounds. The thin layer chromatography results showed DCM and MeOH (8.5:1.5) as a working mobile solvent system for *B. pilosa* and DCM and hexene (7:3) as a working mobile solvent system for *D. cinerea*. The active compounds responsible for bioactivity observed from both *B. pilosa* and *D. cinerea* were polar compounds as the initial plant extraction was carried out using a polar solvent (MeOH) and the crude MeOH extracts from both plants were further fractionated on column chromatography using polar solvents: DCM, EtOAc and EtOH. A study by

Zolobo found that polar extracts are best separated by polar solvents for compound elution (233). The HPLC fractions obtained from *B. pilosa* did not show any activity**.** The reduced activity of the column fractions and lack of activity from HPLC fractions could be attributed to low concentration of the active component (s) in the active fraction of *B. pilosa*.

4.3 Phytochemical analysis

4.3.1 Qualitative phytochemical analysis

Phytochemical analysis of the active extracts demonstrated the presence of common phytoconstituents like phenols, tannins, glycosides, flavonoids and alkaloids. MeOH extracts showed presence of more phytochemicals as compared to H_2O extracts and the presence of these phytochemical compounds might be responsible for the overall increased bioactivity portrayed by MeOH extracts as compared to H_2O. The phytochemicals extracted were similar to those extracted from other studies that worked on the hydro-alcoholic extracts of the plant (234–237). These phytochemical compounds are known to have antibacterial, anti-inflammatory, and antifungal properties and therefore their presence in the studied plants presents the plants as potential antimicrobial agents as described in many previous studies (238,239).The presence of diverse phytochemicals portrayed by MeOH extracts with strong presence of polar phytoconstituents such as tannins and flavonoids can be attributed to ability of MeOH to extract vast phytochemicals as compared to H_2O. Dhawan and Gupta also reported that MeOH as an extraction solvent worked best for the extraction of various active phytochemicals (240). Alkaloids on the other hand, were weakly or moderately present in MeOH extracts than in H_2O extracts. A plausible explanation for this could be because alkaloids are highly soluble in H_2O and are known to exist in large proportions in the seeds whereas in this study the plant parts used were leaves, stem bark and whole stem and roots (241). MeOH extracts of *B. pilosa* and *D. cinerea*

presented with a complex variety of phytochemicals as compared to the rest of the extracts and had therefore increased bioactivity properties.

4.3.2 Quantitative phytochemical analysis

The present study indicated that MeOH extracts *B. pilosa, D. cinerea* and *E. abyssinica*, contains considerably high total polyphenols, flavonoids, DDPH, FRAP and TAC as compared to other MeOH extracts and H_2O extracts. The antioxidant and biological activities portrayed by these plants might be due to the synergistic actions of bioactive compounds present in them. The mechanism of action of plant phenols is through membrane disruption of bacteria through either direct interaction with the cell membrane or interfering with intracellular processes (242). Flavonoids have been shown to exhibit their actions through effects on membrane permeability, and by inhibition of membrane-bound enzymes such as the ATPase and phospholipase A2 (243). Alkaloids intercalate between DNA strands causing cytotoxic effect (244). DPPH and FRAP are responsible for antioxidant activities through radical scavenging effects (245). In this study MeOH extract of *B. pilosa* and *D. cinerea* exhibited considerably high antibiotic activities against *S. pneumoniae* serotype 6A and 1 attributable to the high amounts of phytochemicals extracted from these plant materials. Surprisingly, MeOH extract of *E. abyssinica, L. javanica, A. senegalensis* and *T. zeylenicum* had moderate to low microbial activity despite presence of phytochemicals. Their low activity may be attributed to the variations in the active constituents possibly related to seasonal or geographical setting and ability of phytoconstituents to mask the activity of other phytoconstituents (246). It is still unclear which components are playing vital roles for the extract's bioactivity properties. Therefore, further studies are still needed to elucidate mechanistic way how the plant extracts contribute to the bioactivity property. On the other hand, the low or no activity observed from most of the H_2O extracts could be due to inability of H_2O

as a solvent to extract polar compounds from plant materials that would be able to cause appreciable amount of bioactivity.

4.4 Brine shrimp's lethality testing

The cytotoxicity potential of drugs on the basis of brine shrimp lethality test was classified in various studies such that LC_{50} value above 1000 μg/mL is considered as nontoxic, LC_{50} value between 500 - 1000 μg/mL is classified as weakly cytotoxic, 100 - 500 μg/mL reveals moderate toxicity and 0 - 100 μg/mL is a predictor of strong cytotoxicity (213,247,248).

In the present study, the Brine Shrimp's test results indicate that 83.3% of the plant extracts tested had LC_{50} values above 1000 μg/mL which suggests that they are practically not toxic. This implies that these plants are safe for therapeutic use. For example, aqueous extracts of B. *pilosa* shows to be nontoxic with LC_{50} value of 4498.525 μg/mL at 48 hours. The findings support the traditional medicinal use of the B. *pilosa* without any significant toxicity to humans (249,250). MeOH extracts of *A. senegalensis, E. abyssinica, L. javanica* and *B. pilosa* were also not toxic against brine shrimps with LC_{50} values off greater than 1000 μg/mL of 3121.198, 2019.717, 1660.186 and 1061.39 μg/mL respectively. MeOH extract of *D. cinerea* exhibited a weak cytotoxic activity of 751.33 μg/mL against the Brine Shrimps. However, the observed weak toxic activity of *D. cinerea* may be due to the presence of documented cytotoxic compounds such as Triterpenoid 1 and flavone 4 (251). Further experiments to isolate active compounds in this plant would be recommended to ascertain its toxic effects. Although, Brine Shrimp's lethality assay is inadequate in determining the mechanism of action of the bioactive substances in the plant, it is very useful tool in providing a preliminary

screening that can be supported by a more specific bioassay, once the active compound has been isolated.

4.5 Limitations of the study

We faced various challenges that included inadequate availability of extraction solvents and lack of expertise to use equipment like mass spectroscopy and High Liquid Chromatography. We managed to source the required solvents from other departments within and outside College of Medicine. We also attended various trainings in plant extraction and separation methods that included the use of equipment such as mass spectrophotometry and the High-Performance Liquid Chromatography to acquire expertise and technical skills for handling such equipment. We also sought technical support and expertise from the laboratory technologists that operate the equipment.

4.6 Conclusion

In conclusion, this study has shown the pharmacological basis of the six medicinal plants that are locally used in Malawi to treat suspected pneumococcal and other infections. Of particular importance are MeOH extracts of *B. pilosa* and *D. cinerea* which have exhibited significant *in vitro* antimicrobial activity against *S. pneumonia* serotypes 6A and 1. The potential of these extracts as antimicrobial agents is invaluable where there is resistance to routine antibiotics for treating pneumococcal infections and the emergency of vaccine serotype replacement to the current rolled out pneumococcal vaccines. In addition, *B. pilosa* had no cytotoxic effect against Brine Shrimps suggesting that the extract can be a potential source for the isolation of safe and effective antibacterial compounds. There are several strategies that are available for the discovery of antibiotics drugs, but identification of active traditionally used plants extracts exhibiting IC_{50} values of greater than 1000 μg/mL are

important first steps to discovering plant derived drugs. Further characterisation of the active fractions, through Gas Chromatography/Mass Spectrophotometry (GC/MS), Proton Nuclear Magnetic Resonance (NMR) and X-ray crystallography may be employed to identify and isolate active compounds and to determine mechanism of action of the active compounds responsible for the bioactivity of MeOH extracts of *B. pilosa* and *D. cinerea* that has been observed in this study.

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