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IN-VITRO EVALUATION OF ANTIBACTERIAL PROPERTIES OF *EUPHORBIA HIRTA* AGAINST SELECTED MULTIDRUG-RESISTANT BACTERIA IN GHANA

BY

JONES GYABENG

(10806233)

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DECLARATION

I, Jones Gyabeng, declare that the work presented in this thesis is the result of my own research work carried out in the Department of Medical Microbiology - University of Ghana, The Centre for Plant Medicinal Research (CPMR) Akuapem-Mampong - Eastern region. The Central Laboratory of Kwame Nkrumah University of Science and Technology under the supervision of Nicholas T.K.D. Dayie (PhD) and Simon K. Attah (PhD)- Department of Medical Microbiology and that all references cited in this work have been duly acknowledged.

20 /06 / 2022

Signature Date

Jones Gyabeng (10806233)

20/06/ 2021

Signature (Supervisor) Date

Nicholas T.K.D. Dayie (PhD)

Department of Medical Microbiology,

University of Ghana Medical School

20 /06/2021

Date

Signature (Co-supervisor)

Simon K. Attah (PhD)

Department of Medical Microbiology,

University of Ghana Medical School

DEDICATION

I dedicate this work to my loving and supportive family, friends, participants, supervisors, collaborators and the Medical Microbiology Department of University of Ghana Medical School. This momentous milestone would not have been achieved without your commitment, support and love.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
AST	Antimicrobial Susceptibility Testing
ATCC	American Type Culture Collection
CF	Color Form
CPMR	Centre for Plant Medicinal Research
DDST	Double-Disc Synergy Test
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
ESBLs	Extended Spectrum B-Lactamases
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GCMS	Gas Chromatography Mass Spectrometer
GCMS	Gas Chromatography Mass Spectrometer
H_2SO_4	Sulfuric Acid
HCl	Hydrochloric Acid
IBC	Inflammatory Breast Cancer
ICU	Intensive Care Units
INT	P-Iodonitrotetrazoliumviolet
L	Litre
M/Z	Mass Charge Ratio
MBC	Minimum bactericidal concentration
MDR	Multidrug-resistance
Mg	Milligram
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration

Ml	Milliliter
Mm	Millimeters
mRNA	Messenger Ribonucleic Acid
MRSA	Methicillin Resistant Staphylococcus Aureus
NCCLS	National Committee for Clinical Laboratory Standards
NCTC	National Collection of Type Cultures
NIST	National Institute of Standard and Technology
PBP	Penicillin- Binding Proteins
PCR	Polymerase Chain Reaction
PMT	Proton Motive Force
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic acid
TLC	Thin layer chromatography
UGMS	University of Ghana Medical School
UTI	Urinary Tract Infection
UV	Ultraviolet
WHO	World Health Organization
µg	Microgram
µl	Microliter

ABSTRACT

Background: Treatment of infections is an important area of public health concern as the prevalence of multidrug-resistant (MDR) bacteria is on the rise. MDR bacteria are associated with high morbidity and mortality worldwide. Medicinal plants including *Euphorbia hirta* have shown effectiveness in the treatment of infections and have been one area of interest worldwide for the treatment of diseases due to their high antimicrobial properties against MDR bacteria. In Ghana, the continuous spread of MDR bacteria has resulted in prolonged illness, increased healthcare costs and heightened fatalities which can suddenly cripple the country's economy. One way to reduce the burden of MDR bacteria is to screen for new classes of antimicrobials from natural products and medicinal plants. Thus, this research aimed to evaluate the antimicrobial properties of *E. hirta* against selected MDR bacteria in Ghana.

Methodology: Five solvents systems **(**methanol, distilled water, ethyl acetate petroleum ether and dichloromethane) with varying polarities were used to extract *E. hirta* via cold and Soxhlet extraction methods. The agar-well diffusion method was used to determine the antimicrobial activity of the various extracts against some selected MDR bacteria. Column chromatographic technique was used to the separate most potent crude extract into fractions and their antimicrobial activity were determined. Fractions that showed antimicrobial activity were further purified using column chromatography. Purified fractions were analyzed for the functional groups of compounds present using gas chromatography mass spectrometer (GCMS). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of crude ethyl acetate extracts and active fraction was determined.

Results: Results from this study showed that, soxhlet maceration process had higher yield than cold maceration but the antimicrobial activity of extracts from both methods were the same. The

Antimicrobial susceptibility test (AST) results revealed that *K. pneumoniae* isolates. recruited in the study were resistant to all extracts used. Furthermore, all test organisms were resistant to dichloromethane and petroleum ether extracts. Out of the 15 test organisms used, methanol and aqueous extracts were potent against 5 test organisms. Phytochemical analysis revealed the presence of phytoconstituents such as reducing sugars, phenolic compounds, saponins, flavonoids, anthracenosides and phytosterols. GC-MS analysis shows that 1,2,3-Benzenetriol is the probable sugars present in the active fraction. MIC and MBC results indicated that ethyl acetate extracts and the active fraction had the same MBC values with 3.13 mg/ml as their lowest MBC concentration. The MIC value recorded for crude ethyl acetate was between 50- 6.25 mg/ml whiles that of the active fraction (CF5) was between 50 -12.5 mg/ml.

Conclusion; Polar extracts of whole *E. hirta* plant have antimicrobial activity with ethyl acetate extracts being the highest. The plant has the potential to be used as an antimicrobial agent. Therefore, in-vivo studies should be conducted using different polar solvents to extract the whole plant of *E. hirta* to exploit its antimicrobial activity in living things.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Plants have been the main source of food, shelter and clothing as they usually form the base of the food chain and food web in an ecosystem. Ethnobotanical studies have provided reliable information on the usefulness of traditional plants worldwide (Hassan, 2012). Currently, research into plant medicine, identifying the active ingredient that plays a role in disease treatment has been an area of much interest (WHO, 2019). In the traditional system of disease treatment, medicinal plants have been documented to have pharmacological properties (Larsen *et al*., 2015). A considerable amount of research into plants to determine their antimicrobial properties to develop new drugs are currently ongoing (WHO, 2019). It is particularly important to produce drugs that will be effective against multidrug-resistant microbes to solve the problems posed by these multidrug-resistant microbes (Saravanan *et al*., 2012).

According to Abah & Egwari (2011), the bioactive compounds of plants can be described on a larger scale as lipids, phytochemicals, pharmaceutics, pigments, flavors, and fragrances. The extracts obtained from plants are extensively used in production industries including pharmaceuticals, cosmetics, food production and processing industries. In October 2017, the World Health Organization stated in their Fact Sheet number 134 that, about 80% of people in rural areas in developing African countries relies on traditional medicines as their first point of contact for their basic health care services (WHO, 2017).

In developing countries like Ghana, the traditional method of disease treatment is high since the ratio of physician to patient is as low as 1:1000. In reality, the low level of physician to patient ratio gives credence to the relevance of traditional methods of disease treatment (Appiah *et al*., 2019). Based on the high level of benefit of the traditional method of disease treatment, the government of Ghana set up the Centre for Plant Medicine Research (CPMR) at Akuapem-Mampong in the Eastern region of Ghana to coordinate and promote various scientific activities that would improve herbal medicines as well as to carry out studies to confirm therapeutic evidence of herbal remedies (Mensah *et al*., 2019). Owing to this, the usage of herbal medicines and the traditional health care system have improved in Ghana (Joshi *et al*., 2020).

Euphorbia hirta, popularly known as the asthma plant, belongs to the family Euphorbiacae. It is an official plant included in the African pharmacopoeia since 1985 (Kumar & Kumar, 2010). It is a small, pantropical plant located along roadsides, pathways, and found abundantly on refuse dumpsites. It is popularly noted for its several medical importance including wound healing, asthma, diarrhoea, cough, athlete's foot, bronchial infection and stomach upset (Kuta *et al.*, 2013). According to Tuhin *et al.* (2017), *E. hirta* possesses several antimicrobial properties including septic, inflammatory, diabetic, plasmodium, bacterial, viral, fungal, convulsion, fertility, aphrodisiac and other characteristics which have been documented previously.

Several studies point to the emergence and wide-spread of multidrug-resistant bacteria (Dayie *et al*., 2015, Opintan *et al*., 2015; Donkor *et al*., 2018). As a result, there is high mortality, morbidity and prolonged duration of infection treatment in hospitals despite a high level of development in healthcare service world-wide (Borquaye *et al*., 2019). Evidence shows that continual use of a particular antimicrobial agent has a direct effect on the rate of resistance against that antimicrobial agent (Saravanan *et al*., 2012). Studies by Donkor *et al*. (2012) and Borquaye *et al.* (2019) stated

that, the rise in antimicrobial resistance is influenced by two main factors; the misuse of the antimicrobial compounds and the evolution of newly modified resistance genes. The inappropriate use of antimicrobials put microorganisms under selective pressure. Resistance strains, on the other hand exploit their resistant genes to evade the antimicrobial agent's effects (Donkor *et al*., 2012; Borquaye *et al.*, 2019).

1.2 Problem Statement

As explained by Richardson (2017), the widespread of antimicrobial agents and antimicrobial resistance were discovered right after the introduction of the first antibiotics. A greater number of pathogenic microorganisms are becoming increasingly resistant to common, potent, and commonly accessible antibiotics. This accounts for the high level of infection-related morbidities and mortalities (Nweneka *et al*., 2009). A six-month nationwide surveillance study by Opintan *et al.* (2015) also reported that bacterial resistance to antimicrobials has reached alarming rates. Other studies conducted by Newman & Opintan (2015), Agyepong *et al*. (2018), and Borquaye *et al*. (2019) also proved the existence of multidrug-resistant strains in Ghana and many African countries.

The widespread of multidrug-resistant microbes is attributed to the inappropriate use of antimicrobials and the evolution of antibiotic resistance genes (Newman *et al*., 2015; Borquaye *et al*., 2019). Other elements contributing to the widespread of antimicrobial resistance are; insufficient infrastructure and resources to carry out surveillance systems in deprived areas, poor infection prevention and control (Iwu-Jaja *et al.*, 2021). The widespread of multidrug-resistant microbes has resulted in crippling economies of developing countries like Ghana's. (Borquaye *et*

al., 2019). In the treatment of infections like gonorrhea, antimicrobial resistance is the main problem (Klausner *et al*., 2021).

Over the years, a number of plant materials have been used in the preparation of drugs; these includes *Cinchona sp.* and *Artemesia annua* which have been used in the preparation of quinine and artemisinin respectively for the treatment of malaria. The brain behind the success in the preparation process of these drugs was based on the laid down information obtained through experimental research studies undertaken to investigate phytochemicals like phenolic acids, polyphenols, phenanthrenes, flavonoids, and terpenoids. None of these components have been accepted as the main agent in the manufacturing process for antimicrobial agents due to the fact that, there is luck of data on the mode of action for the phytoconstituents after purification (Mohammadi *et al.*, 2020).

Furthermore, there is paucity of data in relation to the biological activities of *E. hirta* against multidrug resistant (MDR) bacteria. Also, active fractions and their phytochemical compounds responsible for the antimicrobial activity of *E. hirta* extracts have not been discovered.

1.3 Justification

In 1998, the World Health Organization stated that the main challenge associated with traditional health care is the safe use of medicinal plants. However, according to Magiorakos *et al*. (2011), plant extract medications have reduced toxicity and lower adverse effect than other convention method of disease treatment. Although studies have documented the phytochemical constituents of *E. hirta*, there is paucity of data on the active fractions of crude extracts that show activity against various microorganisms studied (Kuta *et al.*, 2015). Although plant extracts have shown effectiveness against all classes of bacteria, research have shown that the difference in cell wall

arrangement in these two categories of bacteria render plant manufactured products more potent against Gram positive bacteria. That is why Gram negatives are the most common plant pathogens. This suggest that, a mixture of different plant material in manufacturing biological antimicrobial products against gram negatives will yield higher antimicrobial activity than single plant products (Zheng *et al.*, 2013). In-depth knowledge into plants products and their mode of action after conducting a series of experiments will provide knowledge based evidence on the bioactive components of plant extracts that will yield equal benefits in its therapeutics applications (González-Lamothe *et al*., 2009).

Tracing from ancient times, nature has been the main source of antimicrobial agent in the treatment of various ailments which cannot be over emphasized (Saravanan *et al*., 2012). Over the last few years, natural products are used as the main component in preparing nearly half of all newly manufactured drugs either directly or indirectly. These natural products are usually plant products (Newman & Cragg, 2016).

Apart from the great pharmacological properties of plants, they have high dependent ratio for the manufacturing of drugs due to their potency, availability, and lesser side effects compared to other materials (Kunwar & Bussmann, 2008). Despite their extensive traditional history, medicinal plants have had a short-lived research and ethno-pharmacology history over the last 50 years (Yeung *et al*., 2019). The testing of plant extracts against a wide range of diseases to uncover new bioactive components in plants is a remedy for the short history of the ethno-pharmacological capabilities of therapeutic plants (Joshi *et al*., 2020).

It is therefore imperative to establish scientific evidence that *E. hirta* has antimicrobial properties against multidrug-resistant bacteria. This study will also seek to screen for the active fractions and the phytoconstituents of *E. hirta* that show activity against an array of multidrug-resistant bacteria

in Ghana. This will represent a new dimension in dealing with the menace of antibiotic resistance in Ghana.

1.4 Aim

To determine the antibacterial activity of *E. hirta* against some selected multidrug-resistant bacteria.

1.5 Specific Objectives

1. To determine and compare the effectiveness of the crude extracts of *E. hirta* against multidrug-resistant bacteria using different extractants.

2. To determine the fraction of the crude extracts that show antimicrobial activity using chromatographic techniques such as thin layer chromatography (TLC) and column chromatography.

3. To determine the phytochemical constituents of the active fractions of *E. hirta* that show activity.

4. To investigate the functional groups of compounds present in the active fraction.

5. To determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the phytoconstituents identified

CHAPTER TWO

2.0 Literature Review

2.1 Antimicrobials

There are both beneficial and harmful microbes to plants and animals which humans are not exception. The harmful microbes have great negative effect on health and economy. As a remedy to the negative effect imposed by the harmful microbes, a variety of substances that inhibit or kill microbes have emerged (Begum *et al.,* 2021). Compounds or substance that kills, slows or retards the growth of microorganisms is termed as antimicrobials. In the application of antimicrobial agents, antimicrobial chemotherapy is the usage of antimicrobial agents in managing microbial infections whilst antimicrobial prophylaxis is the use of antimicrobial agents in preventing microbial infections. In relation to this, the branch of medicine that studies antimicrobial agents have categorized antimicrobial agents based on the type of microorganisms it work against as; antibiotics, antifungal, antiseptic etc. (Hassan *et al*., 2021).

2.2 Antibiotics

When we talk of drugs, antibiotics first comes in mind. Antibiotics have been used for decades. The name antibiotics was coined from the Greek word antibiosis, which literally means against life. As one's survival may either have positive or negative impact on the lives of others, antibiotics were first considered as substances (chemical compounds) obtained from microbial source that have the ability to kills or prevent other microorganisms from multiplying. These Chemical compounds were of higher concentrations thereby acting as toxic substances to those of lower concentrations (Sengupta *et al*., 2013) Antibiotics have been classified into two broad categories

as bacteriostatic and bactericidal based on their ability to kill or inhibit bacterial (microbial) growth. Whilst bacteriostatic antibiotics inhibit bacterial (microbes) growth, bactericidal antibiotics kills bacterial (microbes) completely (Hassan *et al*., 2021).

2.2.1 Classification and Mechanism of Action of Antibiotics

There are quite a number of antimicrobial agents that are readily available for use worldwide. It is known that all antibiotics have their effect through one of the following; cell-wall synthesis inhibition, inhibition of protein synthesis, destruction of bacterial RNA or DNA (Cunha *et al.*, 2021). This implies that all antibiotics target a specific part of the bacterial cell in other to kill or inhibit its growth. Antibiotics are divided into classes based on how they work as; cell wall synthesis inhibitors, denaturing or cell membrane synthesis inhibitors, protein synthesis inhibitors and folic acid synthesis inhibitors.

2.2.1.1 Cell Wall Synthesis Inhibitors

This group of antibiotics target specific part of the cell walls in other to manifest its complete activity in inhibiting the cell wall synthesis. Antibiotics like the beta-lactams inhibit cell wall synthesis by inhibit peptidoglycan cross linkage. Although Gram positive bacteria have thick (higher) cell wall made of peptidoglycan, the synthesis of this structure is a necessary requirement for the survival of all bacterial cells (Beak *et al*., 2021). The peptidoglycan is made of a cross linking proteins in the form of peptides bonds called the ß-(1-4)-n-acetyl hexamine. Before bacteria can synthesize peptidoglycan, it must activate the penicillin binding proteins (PBP) called trans-peptidase and trans-glycosylase. These PBP are the main target of antibiotics that inhibit cell wall synthesis. Since beta-lactam share similar chemical structure with the D-alanyl D-alanine portion of the cross linked that binds with the penicillin binding proteins (PBPs). The beta lactams

mimic and binds at the PPBs instead. After binding, the PBPs becomes unavailable for bacterial cells to synthesize new peptidoglycan for the cell which eventually leads to cell lysis (Wirtz *et al.*, 2021).

In the case of penicillin, cephalosporin and carbapenems, they inhibit cell wall synthesis by inhibiting the peptide bond formation process which subsequently block the cross-linking unit of peptidoglycan present in the cell wall. Although all antibiotics in this group inhibit cell wall synthesis, they sometimes have different mode of action in performing their activity. For example, the binding of beta lactams and vancomycin to PBPs (Bæk *et al*., 2021). Examples of antibiotics in this group includes penicillin's, cephalosporin's, vancomycin, beta- lactamase inhibitor, carbapenems, azeteronams, polymycin and bacitracin (Wirtz *et al*., 2021).

2.2.1.2 Denaturing / Cell Membrane Synthesis Inhibitors.

Antibiotics in this class inhibit or kills bacteria by denaturing the bacterial cell membrane. The polar nature of the cell membrane makes it easier for the synthesis of macromolecules in the cell membrane. Antibiotics in this class like the daptomycin depolarize cell membranes that depends on calcium. This depolarization prevents the synthesis of macromolecules in the bacterial cell membrane, causing the cell membrane to rupture (Epand *et al*., 2016).

2.2.1.3 Protein Synthesis Inhibitors

Antibiotics in this group includes aminoglycosides (gentamicin) which inhibit the 30s subunits, macrolides, chloramphenicol, clindamycin, linezolid and streptogramins which also inhibit the 50s subunit. Through transcription, DNA is transcribed into RNA and RNA into proteins through translation. As genetic materials transcribe from DNA to messenger RNA (mRNA), the ribosome synthesizes the proteins content of the mRNA in the translation process. The bacterial 70s

ribosome is made up of two smaller subunits, i.e. the 30s subunit and the 50s subunit, which are the main target site for antibiotics that inhibit or kill bacterial cell through the inhibition of protein synthesis (Mccoy *et al*., 2011).

Aminoglycosides also inhibit protein synthesis by targeting the 30s subunits of the ribosomes. Aminoglycosides are positively charged molecules that attract and attach negatively charged particles of the cell membrane. The attached particles in the cell membrane creates larger pores for the entry of the antibiotics. After the entry of the antibiotics, energy in the form of oxygen and active proton motive force (PMT) is required to pass through the cytoplasmic membrane before getting into the bacterial ribosomes. This energy requirement account for the reason why these antibiotics works poorly against anaerobic bacteria's but effective against aerobic organisms. This antibiotic has synergistic effect with other antibiotics that inhibit cell wall synthesis (beta lactams) as it allows penetration for lower dosage of these antibiotics. Aminoglycosides interact with the 16s ribosomal RNA (rRNA) of the 30s subunit closer to the cell membrane through hydrogen bonds formation which eventually leads to the termination of mRNA in the translation process (Arenz & Wilson, 2016).

Tetracycline, another 30s ribosomal subunit inhibitor also prevent the binding of 30s ribosomal subunit thereby interfering with the 16s rRNA to disrupt the binding activity of its transfers RNA. Others like chloramphenicol and macrolides are 50s subunit inhibitors that interfere with peptidyl transferase activity of its 23s ribosomal (rRNA). This action prevents protein synthesis as binding of the transfer (tRNA) to the ribosomal site is ceased. This occurs in chloramphenicol but in macrolides, the action results in detachment of peptide chains at their immature state (Wirtz *et al.*, 2021).

2.2.1.4 Folic Acid Synthesis Inhibitors

Folic acids are needed to enhance metabolism of nucleic acid and amino acids in the bacterial cells. Antibiotics that inhibit folic acid synthesis do so by imitating the bacterial substrate called the tetrahydrofolate which is obligatory in the synthesis of folic acid in the bacterial cell. Sulfonamides also inhibits folic acid synthesis in the bacterial cell or termination the production of nucleic acids (DNA and RNA). This group of antibiotics have synergic activity with trimethoprim since but both drugs have dissimilar step in their biosynthetic pathways of folic acid.

Other classification of antibiotic based on their chemical structure includes; aminoglycoside, monobactams, carbapenems, etc. (Fernández-Villa *et al*., 2019).

2.2.2.1 Mechanism of Resistance

Bacteria use one or combination of the processes bellow in other to form resistance against antibiotics;

Reduction in Antibiotic Uptake: Gram positive bacteria have thick peptidoglycan which prevents the entry of antibiotic due to its rigidity. In Gram negative bacterial, the capsule is made of lipopolysaccharides molecules that confer protection in the cell wall and decreases antibiotics uptake. Although bacteria cells are made of small pores that allow the entry of very minute molecules. Reduction in pore sizes provide resistance by decreasing antibiotics uptake (Uruén *et al.*, 2021).

Development of Enzymes that Inactivate Antibiotics: Another mode of antibiotic inactivation within bacteria is the production of enzymes that inactivate antibiotics. These enzymes have the ability to inactivate or render antibiotic inactive. Example is the penicillinase, an enzyme produced by bacteria that inactivate penicillin (Uruén *et al*., 2021).

Efflux Pumps: Efflux pump is a resistant mechanism structure that pump to expel (elute) substances out of the organism's body. This structure is present in organisms like pseudomonas which expel toxic substance out of the organism's body. This structure is sometimes considered as reduction of antibiotic uptake mechanism (Uruén *et al*., 2021).

Mutation: Antibiotics affect a cells by targeting a specific part of the cell which can either be the cell wall, cell membrane, ribosomes, cell proteins etc. Through genetic mutations, microorganisms confer resistance by altering the target sites of the antibiotics. The reduction or alternation of the binding site renders the antibiotics inactive. Mutation in microorganisms sometimes occurs at their receptors where antibiotics have specific ligands for. The alternation in binding sites through genetic mutation marks the binding inability of antibiotics to the bacterial cell. Most resistant bacteria undergo spontaneous mutation or point mutation. Methicillin resistant staphylococcus aureus (MRSA) is one of the most common bacteria to exhibit this form of resistance. Through mutation, bacteria develop structural mechanisms thereby providing resistance against antibiotics (Read & Woods, 2014).

Biofilms: Biofilms are nonliving tissues serving as a protective shelf for microorganisms. When number of microorganisms affect a tissue, they secrete substances that crumps and lay over the surface of the fresh. This are usually seen in wounds that do not respond treatment therapies. The nonliving substances serve as community and harbor number of microorganisms and confer resistance for microbes present by preventing the antibiotics from coming in contact with the microorganism. Not all microorganisms secrete substances in the formation of biofilms, but the most interesting thing is microbial biofilms produced by one microorganisms serve as resistant mechanism for other microorganisms in the dead tissues (Wu *et al*., 2021).

2.3 The Problem of Antibiotic Resistance

Antibiotic resistance has been a major health problem worldwide (Newman *et al*., 2011; Li *et al*., 2017; Borquaye *et al*., 2019; García-Vello1 *et al*., 2020). The rise in antibiotic-resistant levels has been the cause of threat to the health of both humans and animals. It has affected both economic and social development (Li *et al*., 2017). Newman *et al.,* (2015) explained that the main cause of resistance to antimicrobial agents is the misuse of antimicrobials substances which gives a selective advantage for resistant strains over non-resistant strains. As an initiative to control the problem imposed by antimicrobial resistance, the WHO implemented the creation of a taskforce and development of indicators to monitor and evaluate the impact of antimicrobial resistance (WHO, 2019).

Globally, B-lactamase antibiotics are the widely consumed antibiotic. In Ghana , amoxicillin, penicillin, and metronidazole accounts for about 75% of all B-lactamase antibiotics used (Borquaye *et al*., 2019; Ngumba *et al.*, 2020). The B-lactamase; amoxicillin and penicillin work against microorganisms by suppressing the production peptidoglycan in the cell wall resulting in cell growth inhibition leading to cell death. Metronidazole, an antibiotic from the nitroimidazole interferes with DNA synthesis, causing cell death (Rice, 2012). Review of several studies by Newman *et al*. (2014) revealed the development of resistant strains of bacteria in many African countries like Nigeria, Uganda, Zimbabwe, and Ghana. The bacteria have developed resistance to antibiotics like ampicillin, tetracycline, and cotrimoxazole, which have highly been used for decades due to their relatively cheaper prices. In Ghana, antimicrobial resistance has been reported in two (2) teaching hospitals suited at Accra and Kumasi (Newman *et al.,* 2014). Ghana has also recorded the highest resistance rate in microbial isolates from humans, animals, food, and environmental samples (Newman *et al.,* 2014; Li *et al*., 2017).

2.4.1 Medicinal Plants

According to Mack *et al*. (2019), Medicinal plants known variously as herbal medicines, botanical medicines, phototherapy or phytomedicines. These plants are used in whole or their parts are used in making products like medicines, flavors in food, soap and perfumes. He also described medicinal plants as plants with one or more organs constituents of which are useful for therapeutic purposes.

2.4.2 Uses of Medicinal Plants

History of medicinal plants and their antimicrobial purposes can be traced from ancient time. Medicinal plants have served different purposes ranging from traditional to industrial uses. Plants have been the main source for the preparation of medicines before the introduction of Western medicine (Pan *et al*., 2014). According to the WHO on traditional medicines, medicinal plants such as *E. hirta* are relatively freely available, resulting in an increasing demand for their utilization to provide primary health care for about 80% of the rural dwellers who depend on traditional medicines for their primary healthcare (WHO, 2019).In the 1990s, it was documented that there are about 250,000 to 500,000 plant species. With this, 10% serve as food, and over 80,000 species are used for therapeutic purposes (Razzaghi-abyaneh *et al*., 2012). This substantiate that Plants used in the preparation of traditional medicines have the potential to be used in developing new conventional potent drugs. In biological classification, the kingdom plantae are made up of inexhaustible materials acting as ingredients for the treatment of number of diseases (Abah & Egwari, 2011).

2.4.3 Plants as Antimicrobials

The emergence of multidrug resistant (MDR) bacteria over the past years have call for number of researches aimed at investigating plants to discover their antimicrobial properties against these microorganisms. The rise in research into plants derivatives is attributed to the high emergence of multidrug resistance (MDR) bacteria, rendering the currently available drugs ineffective in the treatment of various ailment. In addition to the reasons for the rise in plant research is that, number of plant extracts possess high antimicrobial agents which works against wide range of microorganisms with additional benefits (García-Vello and González-Zorn, 2020). VanEtten (1991) explained that plants produce phyto-anticipins that undergoes constant synthetic process by plants in forming a barrier against microorganisms. In other to respond to external changes, plants produce a substance called phytoalexin which also perform other function in impeding microbial attacks (González-Lamothe *et al*., 2009; García-Vello and González-Zorn, 2020)

2.4.4 The Present Use of Plants as Antimicrobials

Currently, the raw form of plant materials used in the traditional method of treating various ailments have been modified for the preparation of commercial drugs. It is documented that plants serve as the main source for the production of about 50% of western drugs. The use of plants in the production of commercial drugs provide additional advantage in terms of cost, availability and safety with greater therapeutic value as compared to drugs prepared from synthetic sources (Namsa *et al*., 2011).

2.4.5 Euphorbia hirta

In the tropics, *Euphorbia hirta* also known as the asthma-plant, is a weed and a native of India. This hairy weeds grow profusely in refuse dumpsites, grasslands, roadside and walking paths with open spaces (Kumar & Kumar, 2010; Tuhin *et al*., 2017). The plant prefers to grow on acidic, neutral or alkaline soils but shady or dry moist soil does not favor its growth. This is an annual herb that prostrates and grows up to 60 cm long and produces white latex in abundance. The stem bears two simple leaves arranged in opposite pairs. The simple, elliptical, hairy leaves have fine dentate edges and stipules. Each leaf node has axillary cymes with unisexual flowers on stalks without petals. The capsulated fruits have three valves that produces small rectangular red seeds. It releases its ripe seeds through an explosion from the seed capsule. The plant have taproot system for better anchorage and transport substances (Kuta *et al*., 2013; Tuhin *et al*., 2017).

Figure 2.1: Image of *Euphorbia hirta*

2.4.6 Pharmacological Importance of *Euphorbia hirta*

According to Tuhin *et al*. (2017), the leaves of *E. hirta* has been used in the Indian traditional system of disease treatment for decades. It has shown high level of effectiveness against numerous infections caused by pathogenic parasites, bacteria, fungus, viruses and other ailments. Whole plant exhibits high anti-inflammatory, anxiolytic, analgesic, and antipyretic activities from its aqueous extracts. Also, when used in combination with plants that show anti-asthma properties, it helps in controlling respiratory problems for asthma patients. Preparations of the whole plant of *E. hirta* is used in the management of bacterial and fungal infections including tinea pedis commonly called the athlete's foot, dysentery, stomach upsets, warts, scabies, thrush and aphthae (Shih & Cherng, 2012). Titilope *et al.* (2012), in Nigeria, stated that *E. hirta* crude extracts are used in the management of Cellulitis and ear infections. Triterpenes, βamyrin, 24-methylenecycloartenol, and β-Sitosterol are phytoconstituents obtained from the aerial part of *E. hirta* using n-hexane extract. These phytoconstituents showed a higher level of dose-dependent effectiveness for controlling inflammation in mice and rats. In 1995, Mathur and colleagues undertook a study to determine toxic substances in different cell lines. It was revealed after their study that whole plant extract of *E. hirta* showed non-cytotoxic effect with high level of effectiveness as anti-bacterial agents. According to Ubaid *et al*. (2018), Studies conducted by Tona and colleagues to determine the traditional uses plant extracts in the treatment of diarrheal showed that, whole-plant extract of *E. hirta* and seven other extracts have anti-diarrheal activity at an average of 17.39%. In-vitro studies carried out using a whole plant extract of *E. hirta* showed a 60% zone of inhibition of *Plasmodium* parasite growth. Oral administration of *E. hirta* extracts showed significant level of effectiveness against the parasitemia.

Attah *et al.* (2013) also conducted an in-vitro study to determine the anti-filarial activity of *E. hirta* and concluded that *E. hirta* possesses anti-filarial properties with ethyl acetate fraction being the most effective. They also concluded that *E hirta* extract possess low toxicity against monkey kidney cell lines. Tuhin *et al*. (2017) also explained after his study on the in-vivo application of *E. hirta* in wound treatment that oral application of ethanolic extract promoted wound healing.

2.5.1 Extended Spectrum Beta-Lactamase Producers (ESBLs)

The extended spectrum beta-lactamase (ESBLs) are mostly produced by Gram-negative Enterobacteriacea like *E. coli, Klebsiella Pneumoniae, Proteus sp. Pseudomonas Aureginosa and Salmonella sp.* (Riccio *et al.*, 2021; Matloko *et al*., 2021). The extensive use of broad-spectrum antibacterial agents is mostly cited to be the cause of the acquisition of resistant mechanism to beta-lactamases (Al-Hammadi *et al*., 2018). ESBLs are classified into A and B lactamases based on their ability to hydrolyses antibiotics like penicillin, oxyimino-cephalosporins, and monobactams as they produce ESBLs by using genes that were earlier used in beta-lactam production. ESBLs that do not affect cephamycins or carbapenems (Matloko et al., 2021).

Bacteria that produce ESBLs have genes that arise as a result of point mutations at sites of their previous B-lactamase like TEM-1, SHV-1, and CTX-M being the most common among the genotype. Genes like VEB, PER, BEL-1, BES-1, SFO-1, TLA, and IBC are other genotypes with great clinical importance among ESBLs. These are enzymes that are usually mediated by plasmids within their cells (Jamborova *et al*., 2017; Riccio *et al.*, 2021; Matloko *et al.*, 2021). Detailed studies into the genetic makeup of ESBLs shows that these bacteria have genes that confer resistance against some groups of antibiotics. Treatment of infection has been a serious battle between humans and microorganisms that produces extended-spectrum beta-lactamase (Shakya *et al*., 2017; Riccio *et al.*, 2021). ESBLs were first isolated in Germany in 1983 with increasing

reports to date. The prevalence of ESBLs depends on factors like the species themselves, geographic area, health service settings, group of patients infected, and variation among strains. Research into multidrug resistant Gram-negative bacteria that produce ESBLs is an area with much concern (Matloko *et al.*, 2020; Riccio *et al.*, 2021).

2.5.2 Extended Spectrum Beta-Lactamase *Escherichia coli* Producers

Escherichia coli is a Gram-negative, facultative anaerobic, rod-shaped, coliform bacterium. A normal flora in the lower intestine (Mohammadi *et al.*, 2020) but the most prevalent causative agents of UTI with more than 80% of all UTI cases (Abernethy *et al.*, 2015; Fitzpatrick *et al.*, 2016; Widodo *et al.*, 2020). Lipworth *et al.*, (2021) described *E. coli* as the deadliest Gram-negative pathogen which accounts for 18% of all mortality cases among gram negative pathogen. Another condition associated with both ESBL *E. coli* and ESBL *Klebsiella* infection is sepsis. A condition resulting in multiple damage to organ systems, leading to organ failure or death (Al-Hammadi *et al.*, 2018). The widespread of ESBL *E. coli* has complicated treatment of infections caused by *E. coli*. This is due to the high adaptability of resistance mechanisms against routinely used antibiotics for *E. coli* bacteremia infections such as the broad-spectrum cephalosporin. This has led to the widespread and onset of many urinary tract infections (Abernethy *et al.*, 2015; Widodo *et al.*, 2020; Ochien & Atieno, 2021). One advantage possessed by ESBL *E. coli* is the production of extended-spectrum beta-lactamase which serves as resistant mechanisms against beta-lactam antibiotics (Ochien & Atieno, 2021).

2.5.3 ESBL *Klebsiella pneumoniae* Producers

The *ESBL K. pneumoniae* are strain of *K. pneumoniae* that have develop resistance against extended-spectrum beta-lactam antibiotics (Sarojamma & Ramakrishna, 2011; Riwu *et al.*, 2020).

They show resistance to antibiotics like aminoglycoside through their enzymatic activities. This enzymatic activity is peculiar as it is the only mechanism of resistance to aminoglycosides derivatives. This activity occurs in the 16s rRNA in the ribosome leading to its high resistance. The identified 16s methylase genes are armA, rmtB, rmtB, rmtC, rmtD, rmtA and npmA (Daehre *et al*., 2018; Riwu *et al.*, 2020).

ESBL K. pneumoniae are Gram-negative pathogenic bacterium, facultative, lactose fermenters that causes most nosocomial and community-acquired infections. It can be identified on an agar media due to the mucoid nature of its outer-membrane. A leading causative agent for nosocomial infections in the United States (Pfaller *et al*., 2018). Carl Friedlander first described *K. pneumoniae* as bacteremia after its isolation from dead pneumococcal patients in 1882 (Ashurst & Dawson, 2018). In recent years, the emergence and spread of *K. pneumoniae* resistant strains have increasingly been reported in many countries as it has been reported to be one of the most important causative agents for hospital-acquired infections. According to Magill *et al*. (2014) and Kalanuria *et al*. (2014), *Klebsiella spp*. are the major causative agent of pneumonia that arise from poor ventilation in all of the United States, the second causative agent for all Gram-negative *Enterobacteriaceae* bacteremia infections and the third leading cause of all hospital-acquired pneumonia (Martin & Bachman, 2018).

Sarojamma and Ramakrishna explained that the prevalence of ESBL-producing *Klebsiella* is 17% out all cases recorded on ESBL producing organisms in India (Sarojamma & Ramakrishna, 2011; Riwu *et al.*, 2020). Recent studies attest to the fact that ESBL rate is increasing in all parts of the world with *K. pneumoniae* and *E. coli* being the major ESBL-producing bacteria. This opportunistic pathogen account for about 33.3% of all Enterobacteriaceae infection and the third most common cause of nosocomial infections (Magill *et al.*, 2014).

K. pneumoniae cause infections such as urinary tract infections, cystitis, pneumonia, surgical wound infections, endocarditis, and septicemia. They are resistant to the entire beta-lactam class of antibiotics due to the presence of the bla_{KPC} gene that arises as a result point mutation (Martin & Bachman, 2018). The ESBL producing organisms are noted to affect a variety of organisms ranging from humans to plants. In 2017 and 2018, it was reported to affect healthy broilers (Yossapol *et al*., 2017). Hartmann *et al*. (2012) reported its infection in the dairy cow. Germany also recorded the infection of ESBL-producing *K. Pneumoniae* hatcheries in connection to boilers in a farm as of 2017 (Daehre *et al*., 2018). The zoonotic aspect of this pathogen is documented to arise from contact or consumption of contaminated meat (Smet *et al.*, 2010; Riwu *et al.*, 2020).

2.5.4 Methicillin-Resistant *Staphylococcus aureus* (MRSA)

Staphylococcus aureus is a Gram-positive, facultative anaerobe that colonizes epithelial cells causes infections to organs like the skin, bone, lung, heart, brain, and the entire circulatory system (Msed *et al*., 2012; Sharaf *et al*., 2021). The continual use of unprescribed and overuse of antibiotics triggers the emergence of MRSA. MRSA is a specie of *Staphylococcus aureus* that are resistant to methicillin. The gene mecA has a low-affinity to penicillin-binding protein PBP 2 and the newly discovered, mecC providing resistance to methicillin. In classification of infections, MRSA infection was previously considered as hospital-associated as it is usually acquired in hospitals. It was classified as a community-associated MRSA infection as a result of its communicability among individuals in the community. In veterinary medicine, it was also classified as livestock-associated MRSA (Grema *et al.*, 2015; Sharaf *et al.*, 2021).

The World Health Organization categorized the burden of MRSA in antibiotic resistance reports as a rapidly increasing resistant strain, highly bacteremia, and the leading cause of mortality in hospitals (Wangai *et al.*, 2019). In disease epidemiology, MRSA is noted for the high rate of septic

shocks and prolong treatment compared to methicillin-susceptible strains (Otto, 2012; Sharaf *et al.*, 2021). This effect on health is seen as high economic burden associated with increased duration in hospitals.

In the United State, it is reported that *S. aureus* isolates account for more than 60% of all hospital-acquired infections at Intensive Care Units (ICU) (Wangai *et al.*, 2019). In 2014, the World Health Organization following a surveillance study, reported that MRSA infection exceeded 20% in all WHO selected countries put together but more than 80% in some isolated regions. The prevalence of MRSA between and within African countries is believed to be derived from different species (Garoy *et al*., 2019).

Surveillance on MRSA prevalence in 9 African countries shows that the rate of spread is between 12% to 80%, with some individual countries leading with more than 82 % (Falagas *et al*., 2013). The prevalence between the health worker and patients in Uganda is between 31.5 and 42% (Wangai *et al*., 2019), 31 and 82% in Ruanda (Ntirenganya *et al*., 2015; Seni *et al*., 2016), and 50% in Tanzania but with a grate reduction rate of 34 to 24% in southern African between 2011 and 2014 (Dsani *et al.*, 2020; Sharaf *et al.*, 2021).

In 2012, Bagbin undertook a study to determine the angiogram of identified agents of bacterial infections in Ghana and concluded that MRSA was found to be 42.3% and recorded an outbreak of the bacteria infection in the Children's ward of the Korle Bu Teaching Hospital, which led to the temporal closure of the Children's Emergency Ward (Bagbin, 2012).

2.5.5 Salmonella typhi

Salmonella typhi is a gram-negative, bacillus shaped bacterium with flagella associated with blood stream infection. It is the causative agent for typhoid fever. Typhoid fever was first coined by Pierre Louis in 1829 but the causative organism was not known until its discovery in 1880 by Karl

Eberth and later cultured in 1884 by George Gaffky (Mills-robertson *et al.*, 2002; Griffith *et al*.,2019) In other to prevent infections caused by *S. typhi,* scientist started producing drugs and vaccines against this pathogen. Among them is Almroth Wright who developed a vaccine for the diseases. This has been a public health problem ranging from developing to developed countries. In the early 2000s, it was estimated that typhoid fever is associated with 21.7 million illness with 216,000 deaths worldwide. In 2010, the international vaccine institute explained that typhoid fever is accountable for 119 million cases and 12900 deaths in developing countries with low level of sanitation. In relation to this, it was further estimated that *S. typhi* associated infections ranges between 200 to 300 million each year (Joshi *et al*., 2020).

Typhoid fever has been reported in both south and east Asia, west and central Africa with high emergence rate in areas with deprived portable water and lower standard of sanitations. Resistant strains of non-typhoidal salmonella infections have arose in several African countries with increasing frequency over the last generation. Before the introduction of antibiotics, the mortality rate associated with *S. typhi* infection was on a rise with estimated value of about 15% or more, but reduced drastically to 1% after the introduction of antibiotics.

Although chloramphenicol has been the main antibiotics of choice when it comes to the treatment of salmonella infections (Griffith *et al*., 2019). Studies conducted by Mills-Robertson *et al*., (2002) and Arshad *et al*., (2021) point to the rise of resistant strains of *salmonella typhi* against ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole, a common potent antibiotics used in managing typhoid fever. They further explained that the presence of multidrug resistant in *S. typhi* to first line antibiotic may be on a continual rise. It is noted from epidemiological studies that; Salmonella infections are common in Ghana during the rainy season. All this resistance observed in *S. typhi* is associated with plasmid encoded resistance to antibiotics (Arshad *et al*., 2021).

Patience with plasmid encoded resistant strains are susceptible to fluoroquinolones but other studies prove the resistance against fluoroquinolones. In the early 2000s, *S. typhi* was noted as the most prevalent pathogen in hospitals despite its high susceptibility to ciprofloxacin. In diagnosis of this infection, there is positive serological test for the O9 and O12 antigens present in the lipopolysaccharide components of their cell wall (2n 3). The commonly used serological test in most low-income countries is the widal test, which measures antibody titters forming agglutination against the lipopolysaccharide O components of their cell wall and antigen H present in their flagella. Other tests for diagnosis can be done by culturing blood or stool samples. Bone marrow aspirates are samples with high sensitivity when it comes to diagnosis of *S. typhi* (Joshi *et al.*, 2020).

2.6 Antimicrobial Susceptibility Testing (AST)

Techniques for detecting the susceptibility and resistance levels of pathogens to antimicrobial agent are collectively called antimicrobial susceptibility testing. The most commonly used method in checking antimicrobial AST are; disk diffusion method, agar well diffusion, the broth or the agar dilution and the E-test. Some AST methods such as the disk diffusion was developed right after the discovery of the first antibiotics and still in use in laboratories (Pfaller *et al*., 2010; Doerna, 2018). These methods of ASTs detect the susceptibility level of microorganisms phenotypically. The phenotypic method exhibits some advantages compared to other newly developed methods that deals with genotypic constituents of the organism. This advantage includes the opportunity to predict the resistance levels of microbes as well as their susceptibility level. In addition to its advantages, it has the ability to quantitatively measure the level of susceptibility of a pathogenic microbe against the antimicrobial. Meanwhile, these phenotypic method is time consuming (Hsueh *et al*., 2010; Romney *et al.*, 2018; Doerna, 2018; Berinson *et al*., 2021).

Other evolving methods includes the real time PCR, microarray, mass spectrometry, the flow cytometry and the bioluminescent have high sensitivity, reduced time in providing ASTS results and give detailed understanding on their impacts of the antimicrobial agent on the cell of the intended microorganism. The problem associated with this processes are; high cost in purchasing special equipment's, special probe requirement, technically skilled required in their operation and limited microorganism sputum involvement in their data base (Doerna, 2018; Romney *et al.*, 2018; Berinson *et al*., 2021).

The selection of specific antimicrobial susceptibility testing method is influenced by number of factors including cost, level of flexibility, reproducibility, accuracy, automaton, individual's preference and familiarity with the method (Romney *et al.*, 2018). In general, AST has been beneficial in epidemiological studies, discovering antimicrobial agents and predicting the outcome for antimicrobial agents in their therapeutic applications. In giving standard interpretations after antimicrobial susceptibility testing, the clinical and laboratory standard institute(CLSI) of USA and the European committee on antimicrobial susceptibility testing EUCAST publish a standard interpretation for all AST (Doerna, 2018; Romney *et al.*, 2018; Berinson *et al.*, 2021; Bertrand *et al.*, 2021)

2.6.1 Principles of Antimicrobial Susceptibility Testing

2.6.1.1 The Diffusion Method.

In the diffusion method, a known concentration of antimicrobial agent is used to infuse 6 mm in diameter paper disk. The infused disk is placed on an agar medium seeded with the test organism and incubated within a specified period. During the period of incubation, the agar medium first absorb water from the agar medium through diffusion. After the absorption of water in to the agar

medium, the infused antimicrobial compounds (antibiotic) diffuses into the surroundings of the media. The rate of water diffusion is rapid as compared to that of the antimicrobial agent. The difference in diffusion is dependent on factors including; the difference in concentration gradient between the disk and the agar medium, the solubility of the antimicrobial agent and the weight of molecular compounds present in the antimicrobial agent. The rate of diffusion of compounds with higher molecular weight diffuse slower as compared to compounds with lower molecular weight. Each antimicrobial agent has a unique zone of inhibition due to the difference in the rate of diffusion (Berinson *et al*., 2021; Bertrand *et al.*, 2021).

2.6.1.2 The Dilution Method

In this quantitative analysis, different concentration of the antimicrobial agent may be introduced into the broth or agar medium and incubated for about 24 hours. It is expected that the antimicrobial agent (antibiotics) interact directly with the growth of the microorganisms. The minimum inhibitory concentration of an antimicrobial agent is the lowest concentration that can suppress microbial growth after the incubation period. Further comparison of MIC values with a known concentration of those antimicrobial agents obtained from other fluids can be used in checking other responses (Matuschek *et al*., 2014; Berinson *et al*., 2021; Bertrand *et al.*, 2021).

2.7 Role of Chromatography in Science

The application of chromatographic techniques have been one of the fast-growing areas of science due to its numerous application (Coskun & Öztopuz, 2019). When chromatography is compared to other methods of separation of mixtures such as distillation, sublimation, fractional crystallization, partition, chemical separations, fractionation of mixtures of weakly polar molecules and compounds that may be spread across immiscible solvents, it becomes clear that

chromatography has two extremely valuable advantage. It can be used with small amounts of material and the conditions of operation usually cause no change in the components of the mixture being separated (Kilmer, 2010; Mack *et al.*, 2019).

In recent years, chromatography is noted as one of the most essential analytical procedures for the identification and quantification of medication and its metabolites in the medical profession. Several chromatographic approaches have been developed to distinguish medications based on their properties and forms of interactions. Coskun & Öztopuz, 2019; Mack *et al.*, 2019; Pharmacopoeia & Edition, 2019). Chromatography has gained popularity as a potential technique for determining drug-protein binding and examining clinical or pharmaceutical samples (Mack *et al.*, 2019).

In pharmacy, its application includes; pharmaceutical analysis, preparative and analytical procedures for a variety of compounds that can be used in their processes. Many antibiotics have been isolated using chromatography on laboratory and industrial scale for characterization and assays as well as their structural research (Mack *et al.*, 2019).

After the necessary experimental procedures have been thoroughly studied in the preparation of both herbal and other conventional medicines, chromatographic methods are particularly useful in dealing with three types of analytical problems such as; testing for homogeneity of substances susceptible to contamination with chemically similar substances, identification of pharmaceutical substances and preparations, determination of individual components of complex mixtures or substances in dilute solution (Mack *et al.*, 2019; Raj, 2020).

In plant extraction process, homogeneity tests are especially useful for standardizing substances derived from natural sources, such as alkaloids and glycosides, steroids, and lipids.

Chromatography is used in quantitative analysis to isolate the target ingredient in a form that can be determined by a conventional chemical, physical, or biological approach (Mack *et al.*, 2019; Yang *et al.*, 2020). Alkaloids are progressively being isolated, characterized, and estimated using chromatographic techniques (Yang *et al.*, 2020; Raj, 2020).

Several reviews point to the usefulness of chromatography in both qualitative and quantitative analysis of phytochemical compounds and other substances like proteins, peptides, and amino acids (Yakubu *et al*., 2017; Coskun & Öztopuz, 2019; Mack *et al*., 2019; Raj, 2020; Yang *et al.*, 2020).

2.8.1 Thin Layer Chromatography

This is a separation technique used on a microscale to determine the type and number of compounds or ingredients in a mixture. For this reason, this method is only used to select the appropriate solvent system for chromatographic works that involves the use of liquid solvents at the mobile phase. This method is helpful in monitoring chromatographic works, i.e. for separation and combination of elutes based on visualized spots observed on the TLC plates.

In performing of TLC, it has both stationary and mobile phase. The most commonly used stationary phase material is the alumina and the silica. Combination of two solvents are usually used at the mobile phase. Non- polar solvents are used at the initial stage with gradual switch in polarity till the required spots are observed. A small size of the TLC plate is cut with seizers. A thin straight line of about 0.5cm from each end along its length is made and labeled. The plate is then developed in a solvent system at an ambient temperature after spotting the mixture on the TLC plate. After that, the dish is dried in a 90°C oven for about 5 minutes to ensure complete evaporation of the solvent. Plate can be visualized under ultraviolet (UV) light or spraying with

10% or 5% ethanol in sulphuric acid, absolute sulphuric acid or vanillin solution followed by heating in an oven at 100°C for 5 minutes. Changing /switching of solvents can be done repeating the process until the required sports are observed during visualization (Matuschek *et al*., 2014; Raj, 2020)

2.8.2 Column Chromatography

This is one of the most commonly used separation technique in organic chemistry. This has been propagated in disciplines like biology, biochemistry, microbiology and medicine due to its usefulness in separating and collecting a single chemical compound from a mixture of compounds dissolved in a solvent. This technique has been helpful over years in separating both small and large scale of mixture of chemical compounds into individual compound. Column chromatography is useful in isolating active ingredients and metabolites from biological fluid, separating chemical compounds in mixtures, estimating fractions in drug preparation and purification of compounds (Raj, 2020)

The principle of separation of compounds into individual components is based on differential adsorption of compounds as compounds in fluids moving through the column at different rate are collected in separate fractions. For this reason, column chromatography is sometimes called adsorption chromatography. In performing column experiment, TLC is first performed to determine the solvent to use at the mobile phase of the column. Column have stationary and mobile phase. The common elements used at the stationary phase is the alumina or silica gel. These two solid materials are highly preferred due to their varying properties ranging from less expensive, readily available, good adsorption, uniform shape and size ranging from 60 – 200μ in diameter. They are mechanically stable and chemically inert. Other properties like colorless, polar but do

not react with acidic, basic or any other solvents and allow the free flow of mobile phase also increase their chance of use. The mobile phase on the other hand is made up of solvents to use in the column that is determined by the use of TLC. A solvent is selected based on the polarity of the sample. Common examples of solvent used at the mobile phase include ethanol, acetone, water, chloroform, ethyl-acetate, lactic acid, pyridine, etc. The solvent is used in preparing mixture for sample to be introduced in the column. The solvent aid in the separation of individual components in the sample to form separate bands. The individual component is in the solvent mixture separates during the experiment and therefore elute from the column in fractions.

Column chromatography have been classified into four based on the method of separation of compounds as; adsorption column chromatography (ACC), Partition column chromatography, Gel column chromatography and ion exchange column chromatography. In ACC, the components that need to be separated from the mixture are adsorbed on the adsorbent's surface whiles in gel column chromatography, separation occurs in column packed with gel making solvents at the stationary phase held at a fixed position. In partition column chromatography, both the stationary and mobile phase are liquid used in partitioning the column but in an ion exchange column chromatography, it's the stationary phase that is always made of ion exchange resin(Yang *et al.*, 2020).

In undertaking column experiments, one must go through processes like packing of the column, adding of samples, monitoring the samples and isolating the separated compounds. Packing this done before samples will be loaded into the column. This is to ensure complete separation of compounds. The two main method of packing are the dry and slurry method packing. The dry packing is used for micro-scale separations whiles the slurry is used in macro-scales separations. Although the dry method gives better results but the slurry method yields best results. Right after packing using either the dry or the slurry method, the sample is loaded on top of the column. Before

loading the sample, dissolve the sample with few drops of polar solvent. Add the mixture gently using pipette without disrupting the uniform surface of the column after packing. Samples with thin horizontal bands is best for separation. In other to keep a uniform level of column when adding solvents, add small amount of white sand. After adding sand, continually add the eluting solvent whiles collecting the various fractions in few milliliters. Monitor the column by performing TLC on each fraction collected. Color change can be used for taking fractions of colored samples. After collecting all compounds from samples in fractions, another TLC is performed to combine fractions with similar bands. Fractions with different bands will be left behind. Further purify samples by recrystallization (Yang *et al.*, 2020)

2.8.3 Principles of Column Chromatography

The principle of column is based on level of molecules affinity and adsorption. After the introduction of both mobile (solvents to be used) and stationary phase (silica gel or alumina) into the column top, the individual components in the mixture move at a different rate due to the difference in their affinity and adsorption. Components with lower affinity have lower absorption rate to the stationary phase and therefore travels faster than components with higher affinity. Compounds with higher affinity have higher absorption rate at the stationary phase and therefore travels at a slower rate. Chemical components with lower affinity and lower adsorption move faster and elute first in an orderly manner lower to higher affinity compounds. Molecules are adsorbed to the column in an irreversible manner (Yang *et al.*, 2020)

2.9 Gas Chromatography Mass Spectrometer

Gas chromatography mass spectrometer (GCMS) is a common analytical technique use in chemistry, microbiology, biomedical science and other disciplines in order to separate and quantify

chemicals in a mixture. This method involves the fusion of two methods; the gas chromatography which separates chemical of a mixture and the mass spectroscopy which characterize chemical components of a mixture into individual components. The combination of this two methods makes both qualitative and quantitative analysis of a chemical component in a mixture feasible. This implies that, a single individual component of a mixture can be isolated, quantified and evaluated individually (Friesen & Pauli, 2005; Yakubu *et al.*, 2017)

As in other chromatographic techniques, this method has both mobile and stationary phase. The mixture is largely moved toward the stationary phase using an inert gas such as helium in the mobile phase. The stationary phase is located in a tube-like column or stainless steel with varying dimensions. Within the column, the chemical attracts individual components in a selective manner. Compounds in the mixture interact at different rate at both phases. The rate of interaction is specific for each compound as compounds with high interactive power elute the column faster and vice versa. The difference in interaction rate and elution of compounds aid separation of chemical compounds. In other to increase the level of refined components using this process, varying temperature at the stationary phase or varying pressure at the mobile phase can be used (Yakubu *et al.*, 2017)

Compounds that elute the column enters the detector designed to create an electronic signal in the presence of every detectable compounds. The signal's size is determined by the concentration of components present. The stronger the signal, the higher the compound concentration, and the smaller the signal, the lower the compound concentration. The detector is directed to a computer to process signals produced (Yakubu *et al.*, 2017). In the electron ionization director, continuous bombardment of compounds with higher energy (70Ev) beam of electrons break compounds into larger and smaller individual fragments. As molecules are bombarded with higher electrons, other

molecules attached are removed to get individual molecules. This is why GCMS is able to separate individual molecules with charge ions. This single charged molecule are the molecular ions. Some molecular ions are unstable due to the energy imparted on them by the electrons. These unstable ions further break into smaller units.

The charge ions have individual mass but when each mass is divided by a charge, it is termed as the mass charge ratio (M/Z). The mass to charge ratio represent the molecular weight of the fragment since fragments produced by electron ionization have charge of +1. The retention time using this method is the time from introduction of mixture to the time of elution of compounds from the column (Yakubu *et al.*, 2017)

2.9.1 General Principles for Chromatographic Techniques.

Although different chromatographic techniques have different mechanism of retention, they are based on similar principle for the separation of compounds into individual units using both stationary and mobile phase. All chromatographic techniques have fixed stationary as well as a flowing mobile phase. Compounds to be analyzed have affinity for each phase and the distribution of these compounds is regulated by temperature, chemical properties of compounds, nature of stationary and mobile phase. Compounds with higher/larger affinity have greater retention than those with smaller affinity at the stationary phase. This implies that compounds with weak affinity will elute faster than those with strong affinity (Yang *et al.*, 2020; Raj, 2020).

CHAPTER THREE

3.0 Materials and Methods

3.1 Study Design

This was an exploratory study. As simple random sampling technique was used for collecting *E. hirta* plants around the Department of Medical Microbiology, University of Ghana Medical School. The study site was divided into ten sections and a simple random sampling technique was used to collect whole plant of *E. hirta.*

3.2 Study Area

This research was conducted at the Department of Medical Microbiology, University of Ghana Medical School (5° 32' 9.71" N; 0° 13' 23.20" W), Greater Accra Region, where whole plant samples of *Euphorbia hirta* was collected in June 2021. This site was selected because of convenience sampling and the abundance of the *E. hirta* plant that grows profusely around this area. The plant samples were then transported to the Microbiology Laboratory of the Centre for Plant Medicine Research (CPMR), Mampong-Akuapem, Eastern Region for extract preparation and other antimicrobial analysis. The extracts were finally subjected to GCMS analysis at the Central Laboratory of the Kwame Nkrumah University of Science and Technology, Kumasi in the Ashanti Region.

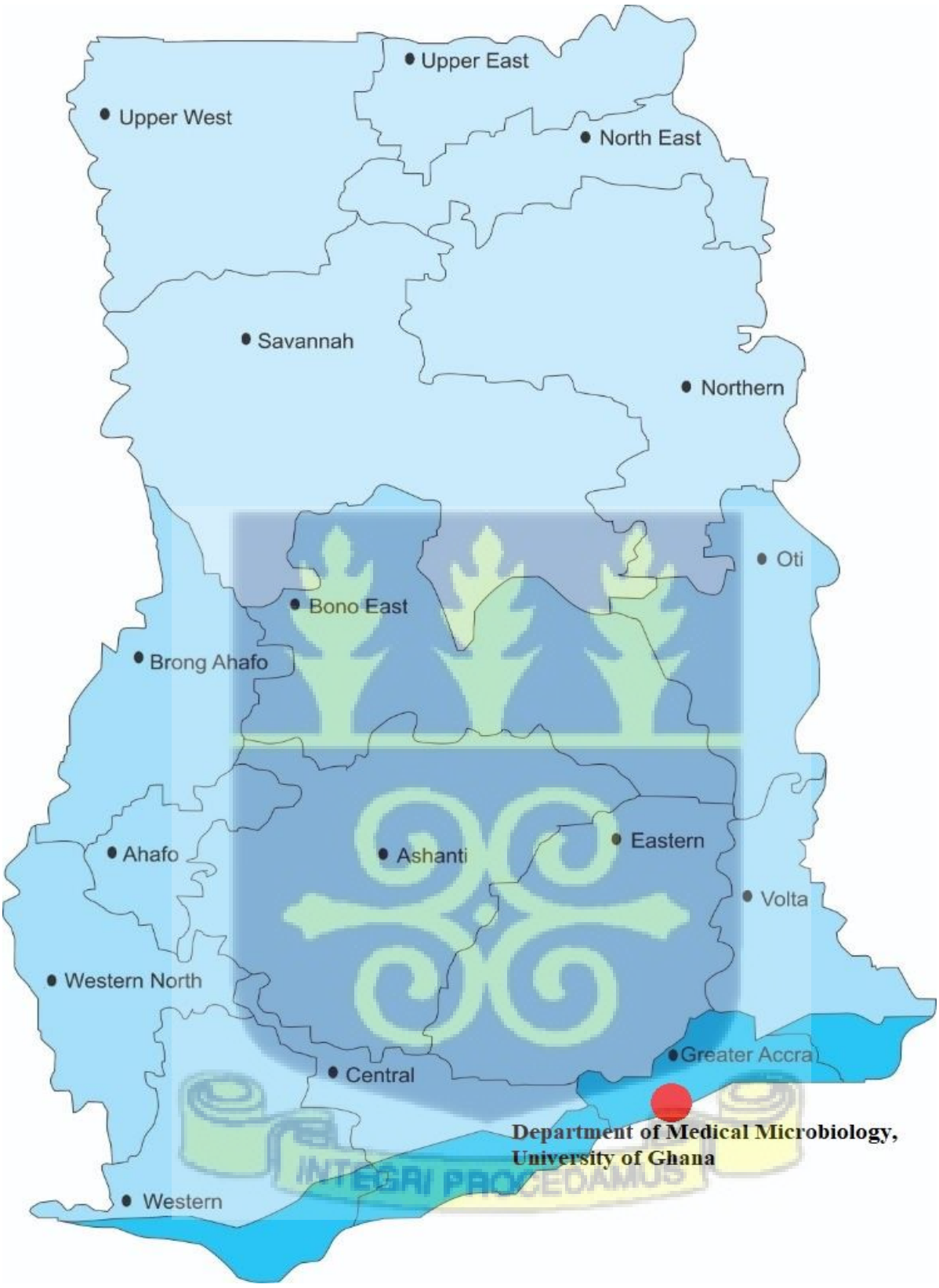

Figure 3.1: Map of Ghana showing the study site

3.3 Media and Reagents

Mueller-Hinton agar, and Mueller Hinton broth were used to culture the organisms. Methanol, ethyl-acetate, dichloromethane, and petroleum ether were the solvents used in the extraction of plant materials. Distilled water was used in the preparation of aqueous extracts.

3.4 Preparation of Plant Extracts

3.4.1 Collection and Identification of plant material

Whole plants of *E. hirta* were collected and identified. The collected plant samples were prepared and submitted for confirmation by a Botanist at the herbarium of the Botany Department of the University of Ghana in Accra, Ghana.

3.4.2 Preparation of Plant Material

The plant specimens were washed thoroughly and dried in aeriated shade for 14 days. The dried specimen was pulverized into fine powder to make the extraction solvents more permeable to the plant cells to facilitate the extraction process (Attah *et al*., 2013).

3.4.3 Extraction of *Euphorbia hirta* using the Cold Maceration Process

This was prepared as documented by Kuta *et al*., (2013) and Attah *et al.*, (2013). In a clean container with a tight-fitting lid, 500 g of the pulverized samples were steeped in one litre (1L) of 80% concentration of methanol. The mixture was covered with the lid, shaken at 30 minutes time intervals for 6 hours, and placed in a dry cool place devoid of light for 72 hours. The mixture was then filtered into another clean container using Whatman No.1 filter paper. The methanol extract was partitioned using petroleum ether, dichloromethane, ethyl acetate and distilled water and the

extracts collected sequentially. The filtrates for each solvent was concentrated in a rotary evaporator at 50°C to obtain a dark slimy substance. The resultant extracts were freeze-dried, weighed and kept in sterile bottle. It was finally stored at 4˚C until needed for antimicrobial susceptibility testing (AST).

3.4.4 Extraction of *Euphorbia hirta* using the Soxhlet Maceration Process

Hundred (100) grams of the pulverized powder was weighed into a muslin bag and extracted for 4 hours in a soxhlet device using petroleum ether, dichloromethane, ethyl acetate and methanol sequentially. At the end of each maceration step, the extracts were sieved using Whatman No. 1 filter paper. In a rotary evaporator, the filtrates were concentrated under reduced pressure. The resultant extracts were freeze-dried, reconstituted into powder, weighed, kept in a sterile universal sample bottle, and stored at 4˚C until needed for AST.

3.4.5 Preparation of Stock Solution for Antimicrobial Assay

A stock solution of 200 mg/ml concentration was made from methanol, ethyl acetate, dichloromethane, and petroleum ether extracts by dissolving 2g of each extract in 10ml of 5% Dimethyl Sulphoxide (DMSO). The aqueous extracts were made by dissolving 2 g of the aqueous extract in 10 ml of distilled water. The stock solutions were kept in the fridge at 4°C until required for AST.

3.5.1 Test Organisms

Methicillin-Resistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus* (ATCC25922), *Staphylococcus aureus* clinical isolate 1 and 2, *Salmonella typhi* (ATCC29522), *Salmonella typhi*

clinical isolate 1, 2 and 3, ESBL-producing *Klebsiella pneumoniae*, *Klebsiella pneumoniae* (ATCC7000603), *Klebsiella pneumoniae* clinical isolate, *Klebsiella pneumoniae* (NCTC 13438) ESBL-producing *Escherichia coli*, ESBL-producing *Escherichia coli* (NCT C13351), and *Escherichia coli* (ATCC 25922) are the test organisms used for the study. Fifteen micrograms per milliliter (15µg/ml) of ciprofloxacin and 5% DMSO were used as a positive and negative control for all the test organisms respectively.

3.5.2 Confirmation of Bacteria Isolates Identity

The analytical profile index (API) system for bacterial identification in addition to other bacterial identification methods like direct examining of colonial morphology through Gram's reaction, followed by a biochemical test such as indole, catalase, citrate utilization, sugar fermentation was employed in the identification of bacteria isolates (Kuta *et al*., 2013).

3.5.2.1 Gram Stain

A drop of physiological saline was first transferred onto a clean dry slide. A single isolated colony of each test organism was introduced into the drop of physiological saline. The inoculum was spread evenly on the slide, heat-fixed onto the slide and left to cool. A Crystal violet was used to stain the slide for a minute. Acetone alcohol in the ratio of 1:1 was poured onto them and washed with water. Using safranin, they were counter-stained and rinsed with water. The slides were air-dried and examined under the ordinary light microscope using the ×100 objective lens (Fröböse *et al*., 2020)

3.5.2.2 Biochemical Test

Using a sterile wire loop, colonies of bacteria with rod shape after counter stain were inoculated into 5 ml peptone water in a 15 ml tube, citrate slants, Triple Sugar Iron agar slants and motility

test media. The media were then incubated under an aerobic condition at 37˚C for 24 hours and examined for biochemical reactions. Indole test was done after incubating inoculums in peptone water and adding Kovac's reagent in drops. Indole production was indicated by the creation of a pink to red color. Catalase and coagulase tests were performed for *Staphylococcus aureus*. For the catalase test, a single isolated colony of *S. aureus* was placed on a glass slide with hydrogen peroxide and spun about. Oxygen bubbles were produced as a confirmation for catalase production.

In performing the coagulase test, a test tube filled with 1 ml of dilute plasma was inoculated with a bacteria colony. The tube was incubated in an aerobic environment for 35-37 ˚C for 18 to 24 hours. Clots were formed indicating a positive coagulase test. This test results in addition to a positive catalase test and direct microscopic observation of Gram-negative cocci in clusters with golden color confirmed the presence of *staphylococcus aureus*.

3.5.2.3 Phenotypic Screening Test for ESBL Producing *Escherichia coli* and *Klebsiella pneumoniae*

This process was performed using the direct colony suspension method. Using a sterile wire loop, about 3-5 actively growing and well-isolated colonies were transferred to inoculate 10 ml of peptone water in a 15 ml tube. The inoculum's turbidity was regulated to 0.5 McFarlan and finally sown on Mueller-Hinton Agar using swab sticks. ESBL production was detected using the double-disc synergy test (DDST) as suggested by the National Committee for Clinical Laboratory Standards (NCCLS). Cefotaxime and ceftazidime disc alone were placed on the plate after which cefotaxime and ceftazidime together with their respective clavulanic acid were added. After 24 hours of incubation at 37°C, the plates were inspected for antimicrobial activity. It was observed that the zone of inhibition for cefotaxime or ceftazidime in combination with their clavulanic acid

was higher (≥5mm) compared to cefotaxime or ceftazidime without their clavulanic acid. This confirms ESBL production (Shakya *et al.*, 2017).

3.5.3 Standardization of Inoculums

The inoculum of each isolate was prepared using the direct colony suspension method. A sterile wire loop was used to pick three to five isolated colonies of test organism into 5 ml of nutrient broth, which was vortexed into a smooth suspension. The suspension was adjusted to 0.5 McFarland standard by increasing number of inoculums and nutritional broth in too light and too thick suspensions.

3.6 Evaluation of the Antibacterial Activity of Extracts

Mueller-Hinton media were prepared per the manufacterer's directives. The media were dried in the oven at 35°C for 30 minutes. Sterility checks and growth promotion test with control strains was checked on each media. The standardized inoculums were smeared on the surface of the Mueller-Hinton media. Six wells were drilled into the media using a cork borer with a diameter of 6 mm. Eighty microlitres (80 µl) of with different concentration; 200 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml and 12.5 mg/ml was introduced into the wells. Fifteen microlitres per milliliter (15 µg/ml) of ciprofloxacin and 5% DMSO were used as positive control and negative control respectively. The resulting data were recorded after the plates had been incubated for 24 hours. Using a ruler, the inhibitory zones were measured in millimetres (mm). (Abah & Egwari, 2011)

3.6.1 Qualitative Phytochemical Analysis

Phytochemical analysis to determine the bioactive chemical constituents of *E. hirta* was carried out using different qualitative chemical tests as standard procedures as described by Tuhin *et al.*

(2017). The different biochemical components including; tannins, reducing sugars, alkaloids, phenolic compounds, cyanogenic glycosides, Polyuronides, anthraquinones, saponins, and flavonoids were analyzed using the methods stated in Table 3.1.

Table 3.1: Qualitative Phytochemical Analysis

Phytochemical	Methodology	Observation
Alkaloids	A few drops of 25% of ammonia were added to 2 ml of the sample to basify and partition it. The chloroform layer was collected and evaporated to dryness. Two drops of HCl were added and divided into two using one as control. Mayer's reagent was finally added.	The absence of a milky white or yellow precipitate indicates the absence of alkaloids.
Cyanogenic glycosides	Two drops of absolute chloroform were added to 2 ml of the sample. It was then heated in water-bath and the vapor was exposed to picric acid paper.	No color change was observed in picric the acid paper. This demonstrates the absence of cyanogenic glycosides.
Flavonoids	Two millilitres of the sample was evaporated to dryness. A few drops of absolute methanol were added. The sample mixture was divided into two halves, using one as the control. After that, magnesium ribbon and two drops of concentrated HCl were added	An orange or red appearance indicates the presence of flavonoids.
Saponins	Two milliliters of the sample were shaken vigorously with a few drops of distilled water (Just, 1998)	A foam of about 1cm in length persisted for at least 10 minutes indicating the presence of saponins.

Polyuronides	A few drops of acetone were added to 2 ml of the sample.	A precipitate that sticks along the walls of the test tube was formed indicating the presence of Polyuronides.
Triterpenes	After adding two drops of methanol, chloroform, and acetic anhydride to the completely evaporated sample. It is divided into two for control. A concentrated H_2SO_4 was finally added.	A brownish-red color was formed indicating the presence of triterpenes.
Phytosterols	After adding two drops of methanol, chloroform, and acetic anhydride to the completely evaporated sample. It is divided into two for control. A concentrated H_2SO_4 was finally added	There was a formation of green color indicating the presence of phytosteroids.
Reducing sugars	In 2 ml of the sample, a few drops of freshly prepared Fehling's solution were added and heated for 15 minutes.	Brick red color was formed indicating the presence of reducing sugars
Phenolic compounds	In 2 ml of sample, a few drops of 5% ferric chloride were applied.	Blue–black or dark green color was formed indicating the presence of phenolic compounds.
Anthracenosides	In 2 ml of the sample, few drops of 25% ammonia was added.	The red color indicates the presence of anthracenosides

3.7 Collection of Active Fractions

The active fractions of the plant extracts were determined using a column chromatographic technique as described by Adedapo *et al*. (2005). The solvent system for the column was chosen using thin-layer chromatography. It was also used to monitor the column for fractions collection.

3.7.1 Determination of Solvent System

This process was carried out as described by Friesen & Pauli (2005). Using 0.2g of the crude ethyl –acetate extract, the extract was dissolved in 0.5 ml absolute ethyl acetate and used for spotting on a TLC plate coated with silica gel 60 F_{254}, Merck, Germany. The plate was cut at a dimension of 2×6 cm using a pair of scissors and developed in chloroform; methanol, at a volume of 10:1 under ambient temperature. The developed plate was dipped into a 30 ml buffer made of 10% ethanol and 90% H_2SO_4 and dried in an oven at a temperature of 90ºC for 5 minutes until all the solvent is dissolved completely. Direct visualization of the plate for spots was done to select the required solvent system to use. A repeated process was used to monitor the column chromatography for fractions collection using different volume ratios of solvents for the TLC based on solvent mixtures in the column.

3.7.2 Collection of Active Fractions Using Column Chromatography

Five grams (5 g) of crude ethyl acetate extract was dissolved in 1 ml of absolute ethyl –acetate. While stirring, 15g of silica gel powder (TLC grade) was added to the solution until all the samples were adsorbed onto the silica gel with no free-flowing liquid. Following the spread of silica gel on a flat glass slide, the solvent was allowed to evaporate in a fume cupboard. The slightly dried extract was thoroughly dried in an oven at 60^{o}C. One hundred and fifty grams (150 g) of silica gel was dissolved in 1 L of chloroform after packing the base of the column with cotton wool. The

homogeneous mixture (silica gel and chloroform) was then transferred gently into the column whiles beating the sides of the column gently with a pencil. After packing, the homogenous mixture (5 g sample dissolved with ethyl acetate in 15 g silica gel) was gently added. The sides of the column were gently rinsed with 2-3 ml of chloroform without disturbing the surface of the column. Before the application of solvent, a thin layer of white sand was added to keep the column content flat. The effluent solvent was added, commencing with 100% chloroform, while the fractions were collected in a 100 ml container. The fractions were monitored using thin layer chromatography, employing silica gel GF254 at the stationary phase and a fraction of chloroform and methanol as the mobile phase. The plates were developed in 10% ethanol in H_2SO_4. The plates were heated in an oven at 90ºC for about 5 minutes. The plates were visualized for band formation and samples with similar a band (resolution) were combined. Combined samples were labeled as CF1 to CF6. The combined fractions were concentrated at low temperatures until dryness using the rotary evaporator.

3.7.3 Evaluation of the Antibacterial Activity of Ethyl-acetate Extract Fractions

This method was done as described by Bertrand *et al.* (2021). The Mueller Hinton agar plates were prepared according to the manufacterer's directives. Test organisms were prepared under sterile conditions and adjusted to 0.5 McFaland standard as described in 3.4.5. The test organism was inoculated into Mueller Hinton agar medium**.** A 6mm diameter-sized filter papers was infused with 10 µL of 0.1 g/ml extract prepared with 5 percent DMSO. The impregnated sterile papers were placed on the agar medium's surface and incubated at 37°C for 24 hours. The inhibitory zones were measured using a rule through the diameter of the papers.

3.7.4 Determination of Minimum Inhibitory Concentration (MIC).

The MIC was determined using the broth microdilution method in accordance with the CLSI recommendations (CLSI, 2021). A hundred microlitres of sterile nutrient broth was aseptically transferred into the 96 well on the microplates. Starting with the first well, 100 µL of nutrient broth was added, followed by 100 µL of the generated stock solution of most active fraction (CF5A) and crude ethyl acetate extracts. At a concentration of 100 g/ml, the content was mixed thoroughly using the pipette. In other to achieve a two-fold of the original extract after serial dilution, 100 µL of the dilution was put into the nutrient broth of the next well in that row. The step above was repeated until concentrations of 50g/ml, 25g/ml, 12.5g/ml, 6.25g/ml, 3.125g/ml, 1.56g/ml, 0.78g/ml and 0.39g/ml were reached. The same process was repeated using 15µg/mL ciprofloxacin another row of the microplate as a control test. A hundred microlitres (100µL) of the aliquot of the adjusted McFarland standard microbial suspension was transferred into each diluted well. The microplates were sealed for incubation overnight at 37ºC. Following the incubation period, 40µL of 0.2 mg/mL p-iodonitrotetrazolium violet (INT) was added to each well and incubated for 2 hours. The microplates were examined to detect the presence of a red color which suggested that bacterial growth was reduced. The least concentration at which color change was observed and compared to the successive dilutions and taken as MIC values.

3.7.5 Determination of Minimum Bactericidal Concentration (MBC).

Wells with lower concentrations where no growth was observed in them were Subcultured and incubated for 12 hours to determine the MBC. About 1-10 µL aliquot samples from these wells were transferred into a fresh nutrient agar plate, incubated overnight at 37ºC, and examined for the presence or absence of microbial growth. Plates indicating microbial growth were recoded as MBC values.

3.7.6 Gas Chromatography Mass Spectrometer Analysis

In performing GC-MS analysis, a PerkinElmer GC Clarus 580 Gas Chromatograph interfaced to a Mass Spectrometer PerkinElmer (Clarus SQ 8 S) furnished with ZB-5HTMS (5% diphenyl/95% dimethyl poly-siloxane) merged with a capillary column (30 × 0.25 µm ID × 0.25 µm DF) was used. The starting temperature of the oven was set at 100°C (isothermal for 2 mins). Further adjustment was done to raise the temperature rate from 10°C/min to 200°C and from 5°C/min to 280°C. The temperature was maintained at a constant rate for 10 minutes at 280°C. At the GC-MS detection stage, an electron ionizing system was regulated in an electronic mode with 70Ev ionizing energy.

A helium gas (99.99) flowing at a constant rate of 1 ml/min with a 1µl injection volume was the carrier gas used. The temperature for the injector was kept at 250°C, while the ion source was kept at 220°C.

The mass spectra were collected at 70 eV with a 1s scan interval with fragments ranging from 50 to 500 Da. The overall GC/MS running duration was 50 minutes, with a solvent delay of 0 to 3 minutes. Using a Turbo-Mass as the mass detector, a Turbo-Mass version-6.1.0 was employed to handle mass spectra and chromatograms. The final GC-MS interpretation was done using the National Institute of Standards and Technology (NIST) database, which has over 62,000 patterns.

3.8 Ethical Clearance

The ethical clearance with protocol identification number: CSH.Et/M1-P4.6/2021-2022 was issued by the Ethical and Protocol Review Committee of the University of Ghana Medical School (UGMS).

3.9 Statistical Analysis

Results were presented and analyzed to suit the objectives of the study using excel (Microsoft office professional plus 2016) and Minitab 17 version 17.1.0. 0. Comparison of results during the analysis was done using Tukey's and ANOVA range test at a significant level of $P < 0.05$.

CHAPTER FOUR

4.0 Results

4.1 Determination of Yield Using Both Soxhlet and Cold Extraction Method

After conducting the study using the soxhlet and cold maceration process on equal mass of plant sample, there was a significant difference ($P < 0.05$) between the yield for the two maceration processes in all the three extraction solvents (methanol, dichloromethane and petroleum ether) used. Among the three extraction solvents, methanol extracts had the highest yield. In comparing the yield for the two maceration processes, the soxhlet maceration process had the higher yield in the three extraction solvents used (Table 4. 1 and appendix XIV).

Table 4:1: Yield of *E. hirta* Crude Extracts Following the Use of Methanol, Dichloromethane, and Methanol for Soxhlet and Cold Extraction Methods.

	Weight of *E. hirta* Used (mg)		Volume of Solvent Used (ml)		Volume of Solvent After Extraction (ml)		Total Mass of Sample Obtained After Extraction (g)	
Extraction solvent	Soxhlet	Cold	Soxhlet	Cold	Soxhlet	Cold	Soxhlet	Cold
Petroleum Ether	100	100	1000	1000	800	1000	2.4	0.8
Dichloromethane	100	100	1000	1000	700	1000	2.0	0.8
Methanol	100	100	1000	1000	850	950	10	6

4.2 Antimicrobial Activity of the Extracts

The antimicrobial activity of soxhlet and cold maceration process was compared against 15 test organisms (*S. aureus* ATCC 25922, *S. aureus* Clinical Isolate 1, *S. aureus* Clinical Isolate 2, MRSA 744, *K. pneumoniae* ATCC 7000603, *K. pneumoniae* NCTC 13438, K. *pneumoniae* Clinical Isolate, K. *Pneumoniae* ESBL Clinical Isolate. *E. coli* ATCC 25522, *E. coli* ESBL NCTC 13351, *E. coli* ESBL Clinical Isolate, *S. typhi* ATCC 334538, *S. typhi* Clinical Isolate1, *S. typhi* Clinical Isolate 2, and *S. typhi* Clinical Isolate 3), there was no significant difference ($P > 0.05$) in antibacterial activity between the two maceration process using methanol, petroleum ether, or dichloromethane as extraction solvents (Fig 4.1 to 4.3).

Out of the five concentrations; 200 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, and 12.5 mg/ml used, the study revealed that ethyl acetate extracts showed the highest antimicrobial activity compared to methanol and aqueous extracts whilst dichloromethane and petroleum ether extracts showed no zones of inhibition against all the test organisms.

At concentrations of 200 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, and 12.5 mg/ml, ethyl acetate, methanol, and aqueous extracts showed zones of inhibition against *S. aureus* spp. *S. typhi* and *E. coli* spp. The antibacterial activity of the active crude extracts (methanol, aqueous, and ethyl-acetate extracts) increases significantly ($P < 0.05$) with increasing concentration (Fig 4.3 to 4.5).

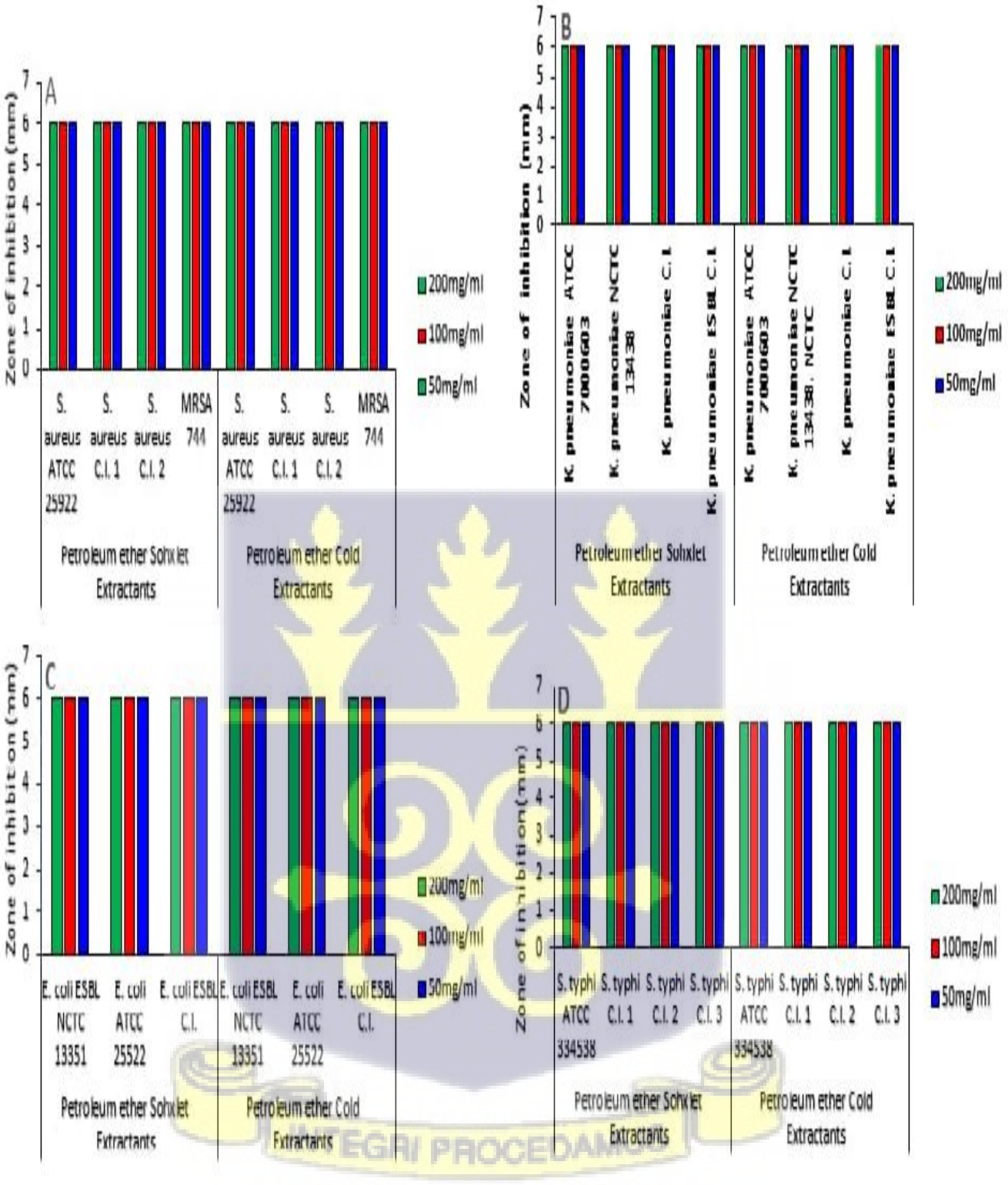

Figure 4.1: Antimicrobial Activity of Petroleum Ether Extracts of *E. hirta* Against Test Organisms

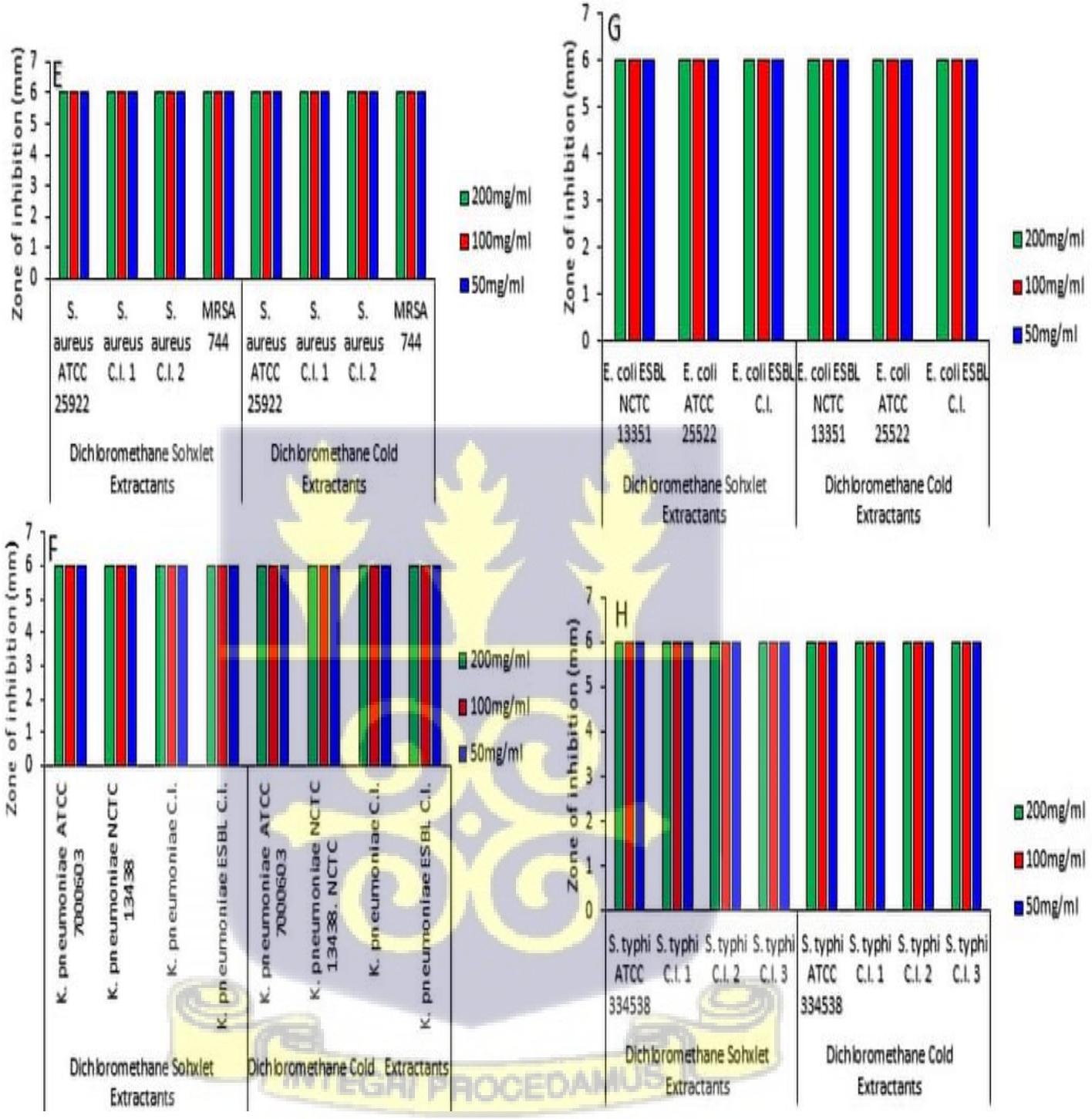

Figure 4.2: Antimicrobial Activity of Dichloromethane Extracts of *E. hirta* Against Test Organisms

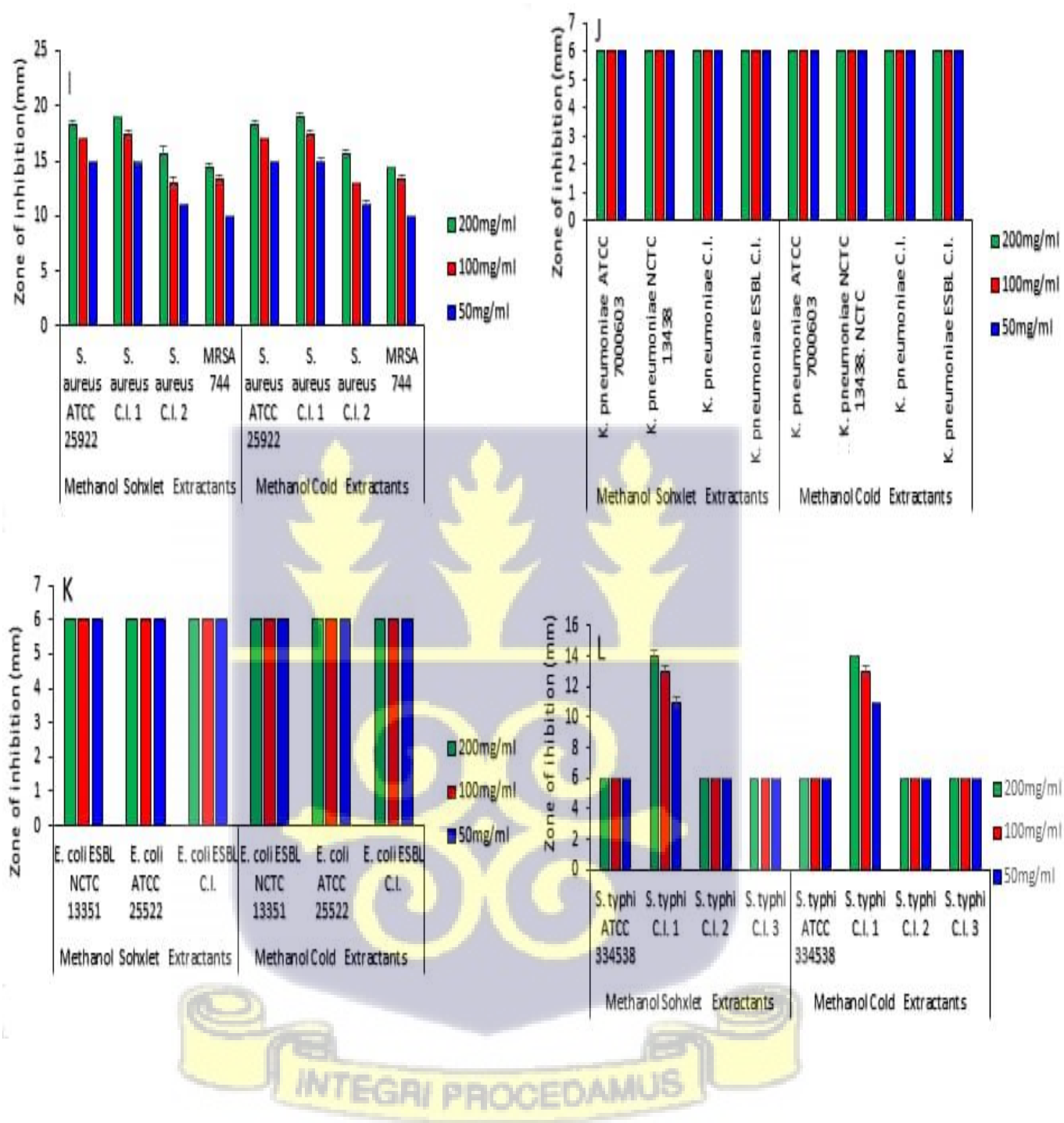

Figure 4.3: Antimicrobial Activity of Methanol Extracts of *E. hirta* Against Test Organisms

Also, methanol and aqueous extracts had significant activity against 5 out of the 15 test organisms. This activity was against *S. aureus* ATCC25922, *S. aureus* clinical isolate 1, *S. aureus* clinical isolate 2, MRSA 744, and *S. typhi* clinical isolate 1 for methanol extracts whiles aqueous extracts showed activity against *S. aureus* clinical isolate 1, *S. aureus* clinical isolate 2, MRSA 744, *S. typhi* clinical isolate 1 and *S. typhi* clinical isolate 2. The zone of inhibition between methanol and aqueous extracts against *S. aureus* clinical isolate 1, *S. aureus* clinical isolate 2, MRSA 744, and *S. aureus* clinical isolate 1 were almost the same (Figure 4.3 and 4.4).

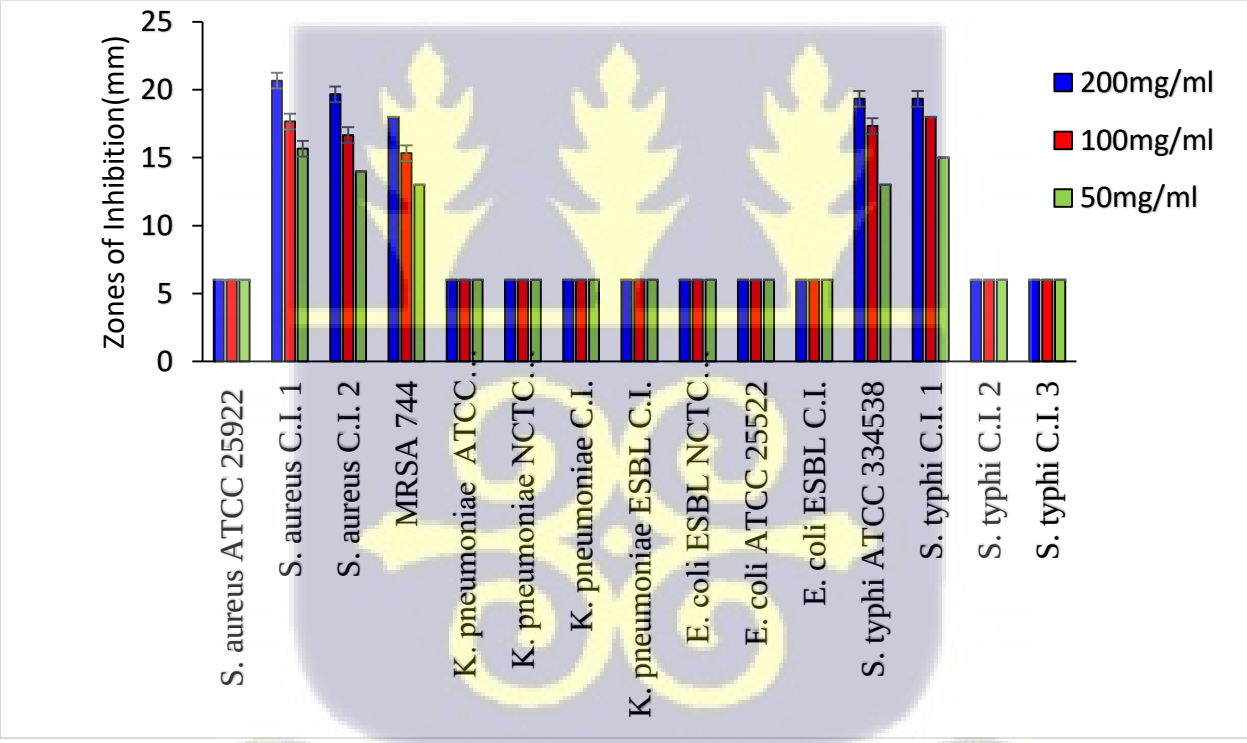

Figure 4.4: Antimicrobial Activity of Aqueous Extracts of *E. hirta* Against Test Organisms

Furthermore, the study revealed that *K. pneumoniae* sp. were resistant to all the *E. hirta* extracts at all concentrations used. Ethyl acetate extracts showed significant activity against 11 test organisms out of the 15 test organisms engaged in the study. However, the interaction between

ethyl acetate and *S. aureus* clinical isolate 1, *S. aureus* ATCC33538 and *S. aureus* clinical isolate 2 showed the highest zones of inhibitions with a mean zone of 23.67±0.33, 23.33 ± 0.33 and 23.33 ± 0.33 respectively. These were higher than the zones of inhibition recorded for all the other extracts (Figure 4.5). The mean zone of inhibition recorded for the interaction between ethyl acetate extracts and *E. coli* ATCC 25522 was 19.33 ± 0.33 mm whiles the mean zone recorded against E. *coli* ESBL NCTC 13351 and *E. coli* ESBL clinical isolate was 19 mm (Figure 4.5).

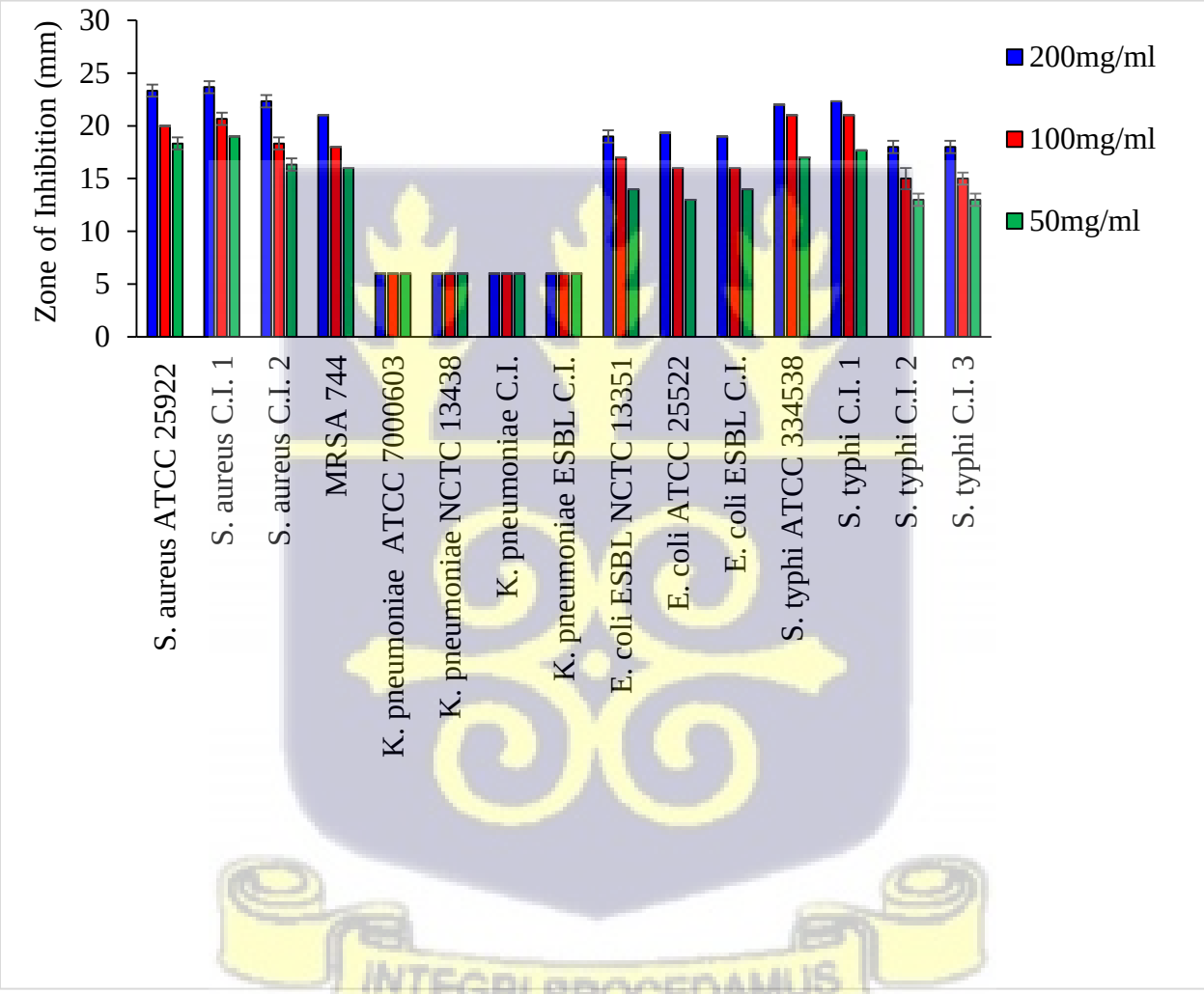

Figure 4.5: Antimicrobial Activity of Ethyl-Acetate Extracts of *E. hirta* Against Test Organisms

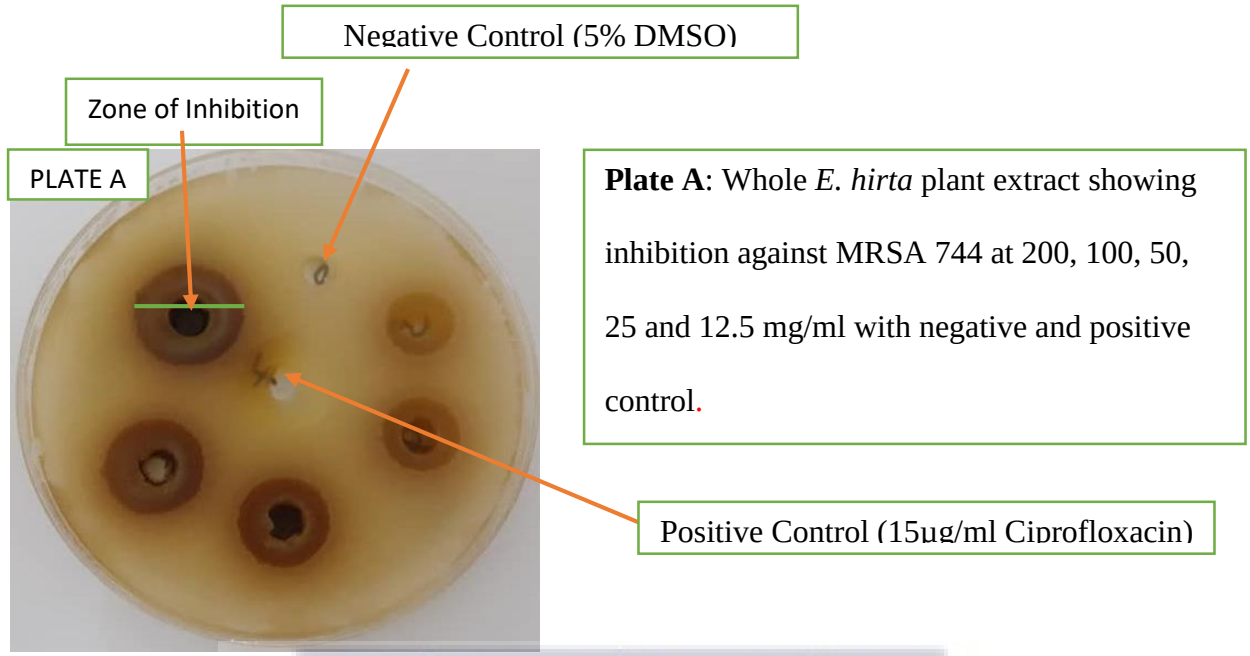

Plate A: Whole *E. hirta* plant extract showing inhibition against MRSA 744 at 200, 100, 50, 25 and 12.5 mg/ml with negative and positive control.

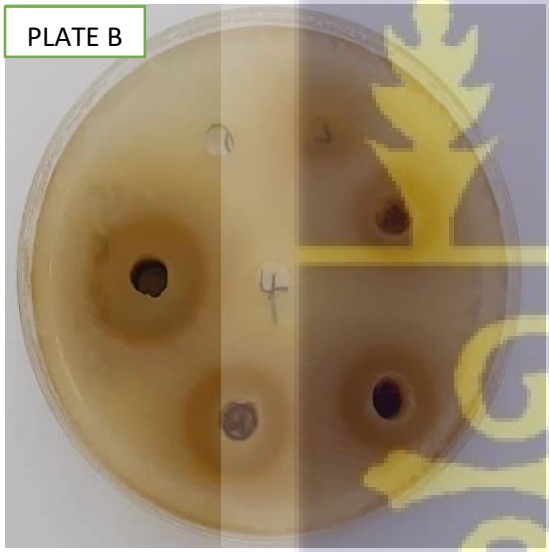

Plate B: Whole *E. hirta* plant extract showing inhibition against *Staphylococcus aureus* ATCC 25922 at 200, 100, 50, 25 and 12.5 mg/ml with negative and positive control.

PLATE C

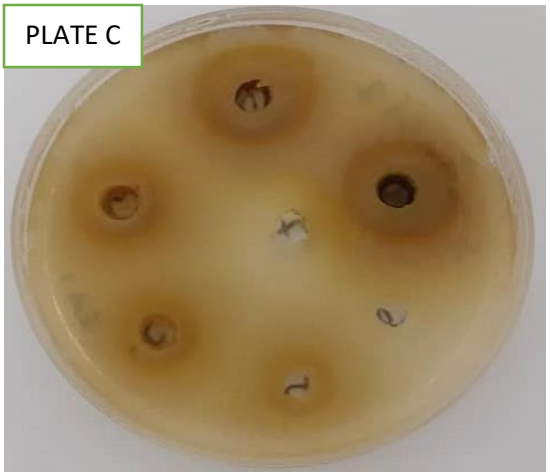

Plate C: Whole *E. hirta* plant extract showing inhibition against *E. coli* ESBL Clinical Isolate at 200, 100, 50, 25 and 12.5 mg/ml with negative and positive control.

PLATE D

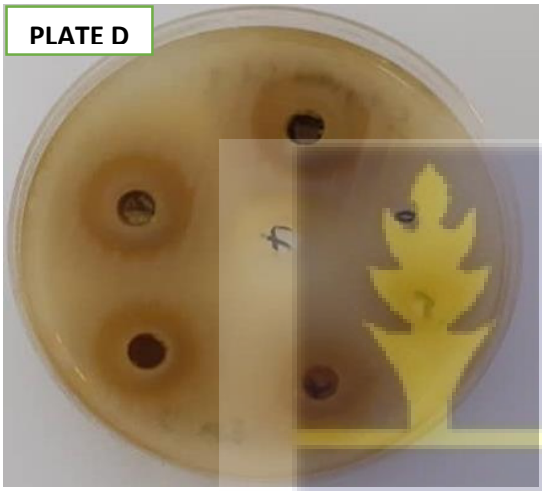

Plate D: Whole *E. hirta* showing inhibition against *Salmonella typhi* ATCC334538 at 200, 100, 50, 25 and 12.5 mg/ml with negative and positive control.

Plate 1: Plates A, B, C, and D Showing the Antimicrobial Susceptibility Test of *E. hirta* extracts Against Some Test Bacteria.

4.3 Phytochemical Components of *E. hirta* Crude Extracts.

The phytochemical analysis of *E. hirta* crude extracts indicated the presence of reducing sugars, phenolic compounds, saponins, flavonoids, anthracenosides, and phytosterols which corroborated previous reports stated by Abubakar (2009) and Kuta1 *et al.* (2013).

Table 4.2: Presence of Phytoconstituents of *Euphorbia hirta* in the Crude Extracts of Various Solvents Systems.

	SOLVENTS			
Phytoconstituents	**Methanol**	**Aqueous**	**Ethyl acetate**	**Dichloromethane**
Reducing Sugars	**+ve**	**+ve**	**+ve**	**+ve**
Phenolic Compounds	**+ve**	**+ve**	**+ve**	**+ve**
Saponins	**-ve**	**-ve**	**+ve**	**-ve**
Polyuronides	**-ve**	**-ve**	**-ve**	**-ve**
Cyanogenic Glycosides	**-ve**	**-ve**	**-ve**	**-ve**
Flavonoids	**+ve**	**+ve**	**+ve**	**-ve**
Triterpenes	**-ve**	**-ve**	**-ve**	**-ve**
Anthracenosides	**-ve**	**+ve**	**-ve**	**+ve**
Phytosterols	**+ve**	**-ve**	**-ve**	**+ve**
Alkaloids	**-ve**	**-ve**	**-ve**	**-ve**

Legend: +ve =Positive, -ve =Negative

4.4 Determination of Fractions Using Column Chromatography

After the column, six fractions were obtained using TLC plates as a guide.

Figure 4.6: Image of TLC Plates Showing Bands Formed from Ethyl Acetate Extracts Fractions

Table 4.3: Fractions Obtained From Crude Ethyl Acetate Extracts of *Euphorbia hirta* Using Column Chromatography.

Fractions Obtained	Fractions of Solvents used
CFIA	Chloroform 100%
CF2A	Chloroform/Methanol 20 :1
CF3A	Chloroform/Methanol 20 :1
CF4A	Chloroform/Methanol 20 : 3
CF5A	Chloroform/Methanol 20 :3 and 6:4
CF6A	Chloroform/Methanol 1:1, 4:6, 3:7 1:9 and 100% methanol

Legend: CF= Combined fraction

4.5 Antimicrobial Activity of Ethyl-Acetate Active Fractions

After conducting the column chromatography on the crude ethyl acetate extracts, six fractions (CF1A, CF2A, CF3A, CF4A, CF5A, and CF6A) were obtained from the crude ethyl acetate extracts. The antimicrobial activity conducted on these fractions revealed that CF5A had the highest zones of inhibition against 11 out of the 15 test organisms used in the study (Figure 4.6).

Smaller zones of inhibitions were recorded for the fractions compared to the crude ethyl acetate extracts but share similar trends in activity against all organisms. This shows that CF5A contained most of the phytoconstituents responsible for the antimicrobial activity as the ethyl acetate crude extracts. The difference among the zones of inhibition for the various fractions was found to be statistically significant ($P < 0.05$). Additionally, all the test organisms were resistant to fraction CF1A but susceptible to the remaining fractions (CF2A. CF3A. CF4A, CF5A, CF6A). The study

also revealed the resistance of *K. pneumoniae* sp. against all fractions obtained from the crude ethyl acetate extracts and its fractions (Figure 4.7).

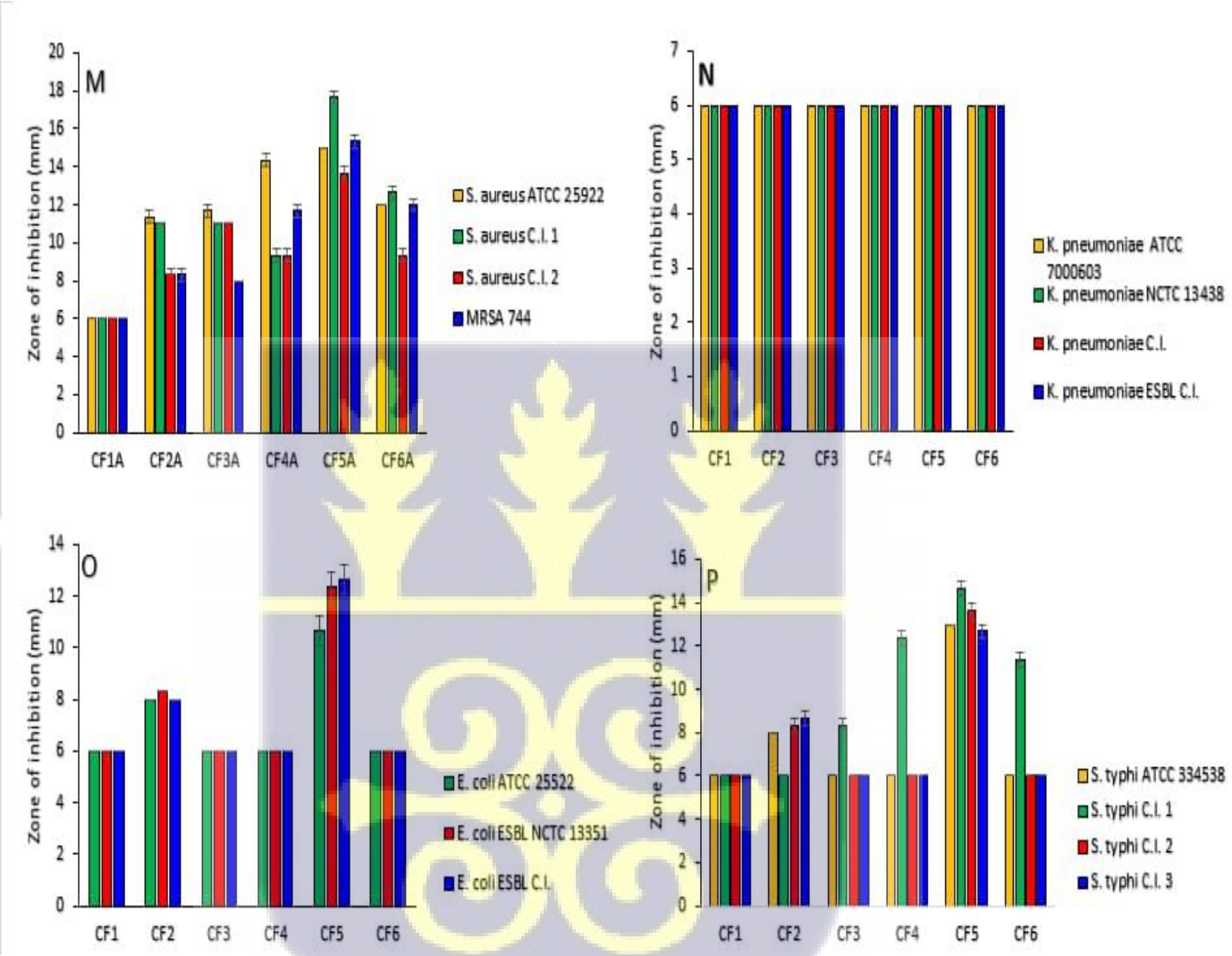

Figure 4.7: Antimicrobial Activity of Ethyl Acetate Extract Fractions of *E. hirta* Against Test Organisms.

4.6 Determination of Possible Compounds Present in the Active Fraction.

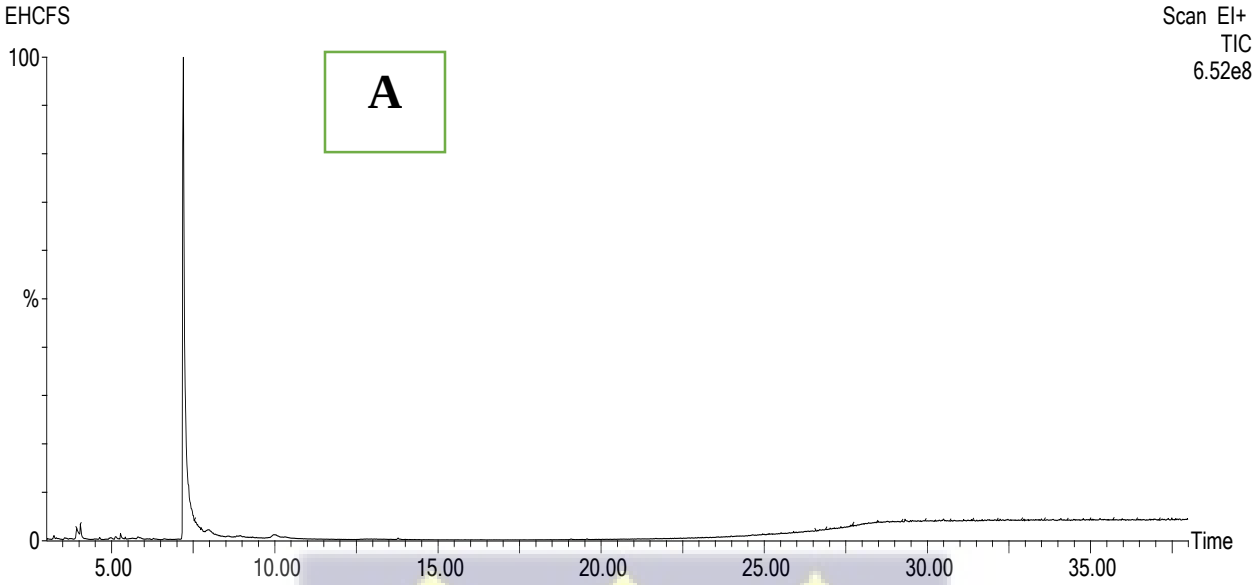

Figure 4.8: Graph Showing Retention Time of Compound Present in the Active Fraction

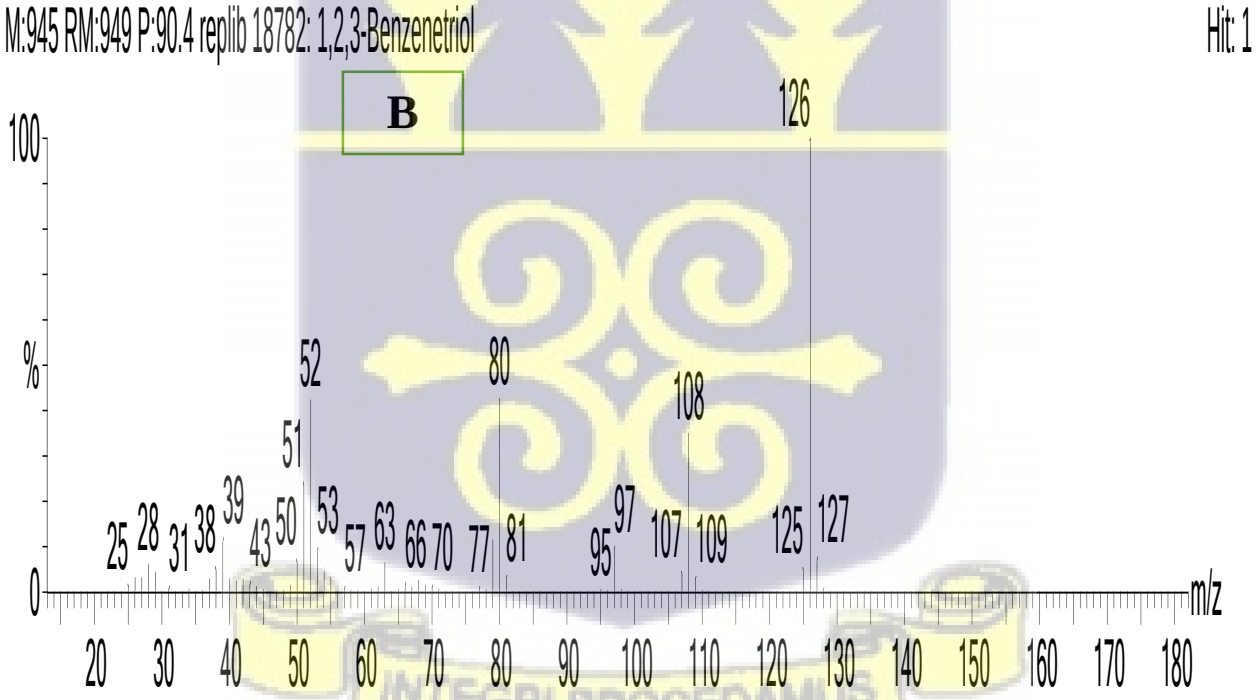

Figure 4.9: Graph Showing the Molecular Weight of Compound Present in the Active Fraction

Table 4.4: Summary of Gas Chromatography Mass Spectrometer results

#	RT	Molecular weight (g/mol)	Compound
1	7.20	126	1,2,3-Benzenetriol

The GC/MS analysis of the fraction CF5A shows a major peak at 7.20 min (**A**) with a corresponding molecular weight of 126 g/mol (**B**). The compound is predicted to be 1,2,3-Benzenetriol according to the National Institute of Standard and Technology (NIST) database.

4.7 The Minimum Inhibition Concentration (MIC) of the Extracts

The MIC for crude ethyl acetate extract and CF5A for their respective susceptible organisms range between 6.25 and 50 mg/ml. At a higher concentration of 50 mg/ml, the active fraction (CF5A) was active to inhibit MRSA 744 and E. *coli* ATCC22522. A concentration of 25 mg/ml of the active fraction (CF5A) was required to inhibit the growth of *S. aureus* clinical isolates 1 and 2, *E. coli* ESBL NCTC 13351, *E. coli* ESBL clinical isolate, *S. typhi* ATCC 334538, *S. typhi* clinical isolate 1, 2 and 3. At a lower concentration of 12.25 mg/ml, the active fraction was potent to inhibit the growth of *S. aureus* ATCC 25922. Comparing this to the crude ethyl acetate extracts, a higher concentration of 50 mg/ml was only active to inhibit MRSA 744 whiles a concentration of 12.25 mg/ml of the crude ethyl acetate extracts was required to inhibit *S. aureus* ATCC 25922, *S. typhi* ATCC 334538, *S. typhi* clinical isolate 1 and E. *coli* ATCC 22522. At a lower concentration of 6.25 mg/ml, the crude ethyl acetate extracts were active to inhibit *S. aureus* clinical isolates 1 and 2, *S. typhi* clinical isolate 1, 2, and E. *coli* ATCC 22522. This implies that with the exception of MRSA 744 which had the same MIC value of 50 mg/ml, the MIC values for the crude ethyl acetate extracts were lower as compared to the active fraction. Statistical analysis of the MIC values

revealed that there is significant difference (P>0.05) between the MIC values recorded for the crude ethyl-acetate extracts and its active fraction(CF5A). The MIC values of *E. hirta* crude ethyl acetate extracts and its active fraction (CF5A) against the 11 susceptible bacteria are shown in **Figure 4.10.**

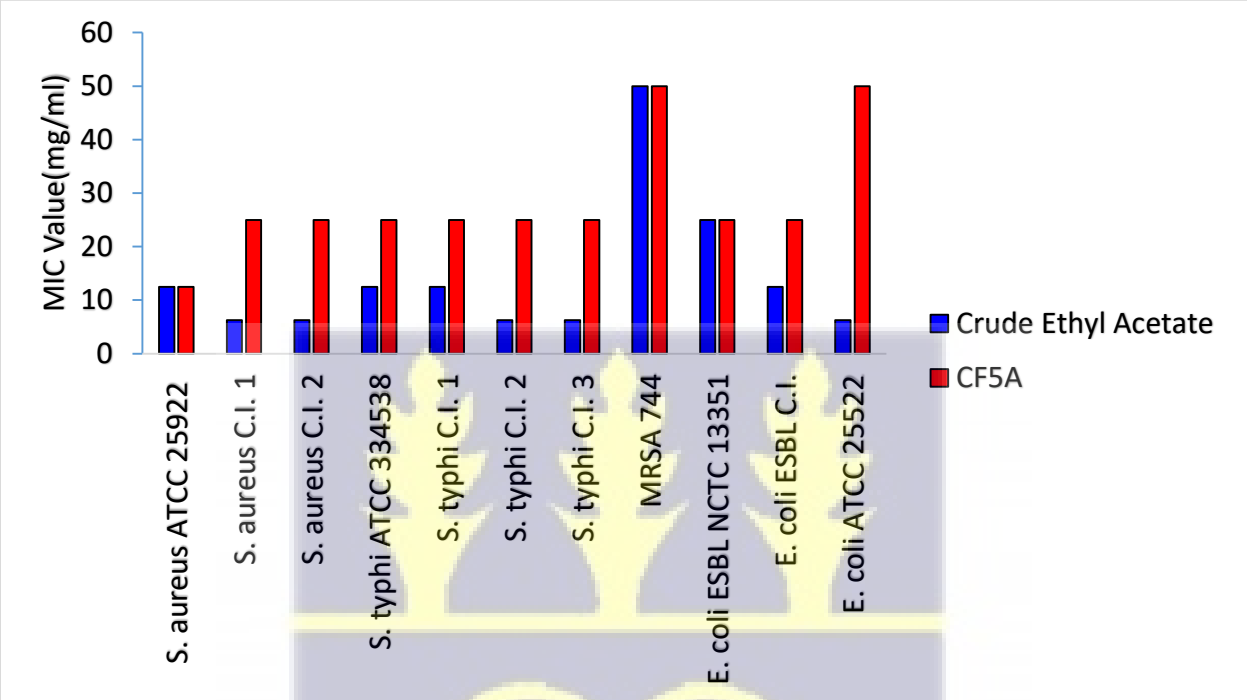

Figure 4.10: Minimum Inhibitory Concentrations of Crude Ethyl Acetate and Active Fraction (CF5A) of *E. hirta* on Test Organisms.

4.8 The Minimum Bactericidal Concentration (MBC) of the Extracts

The MBC for crude ethyl acetate extract and CF5A for their respective susceptible organisms ranged between 0.00 to 6.25 mg/ml. The MBC value recorded for crude ethyl acetate extracts and its active fraction (CF5A) were the same. The extracts had no MBC values against S. *aureus* ATCC 25922 and *S. typhi* ATCC 334538 at a higher concentration of 100 mg/ml. At concertation of 6.25 mg/ml, the extracts showed bactericidal activity against test organisms including; *S. aureus* clinical

isolate 1 and 2, *S. typhi* clinical isolate 1 and 2, E. *coli* ATCC 22522, *E. coli* ESBL NCTC 13351, and *E. coli* ESBL clinical isolate. The lowest MBC value for both extracts was 3.125mg/ml which was recorded against MRSA 744. The MIC values of *E. hirta* crude ethyl acetate extracts and its active fraction (CF5A) against the 11 susceptible bacteria are shown in **Figure 4.11.** Statistically, there is no significant difference ($P<0.05$) between the MIC values for the crude ethyl-acetate extracts and its active fraction(CF5A).

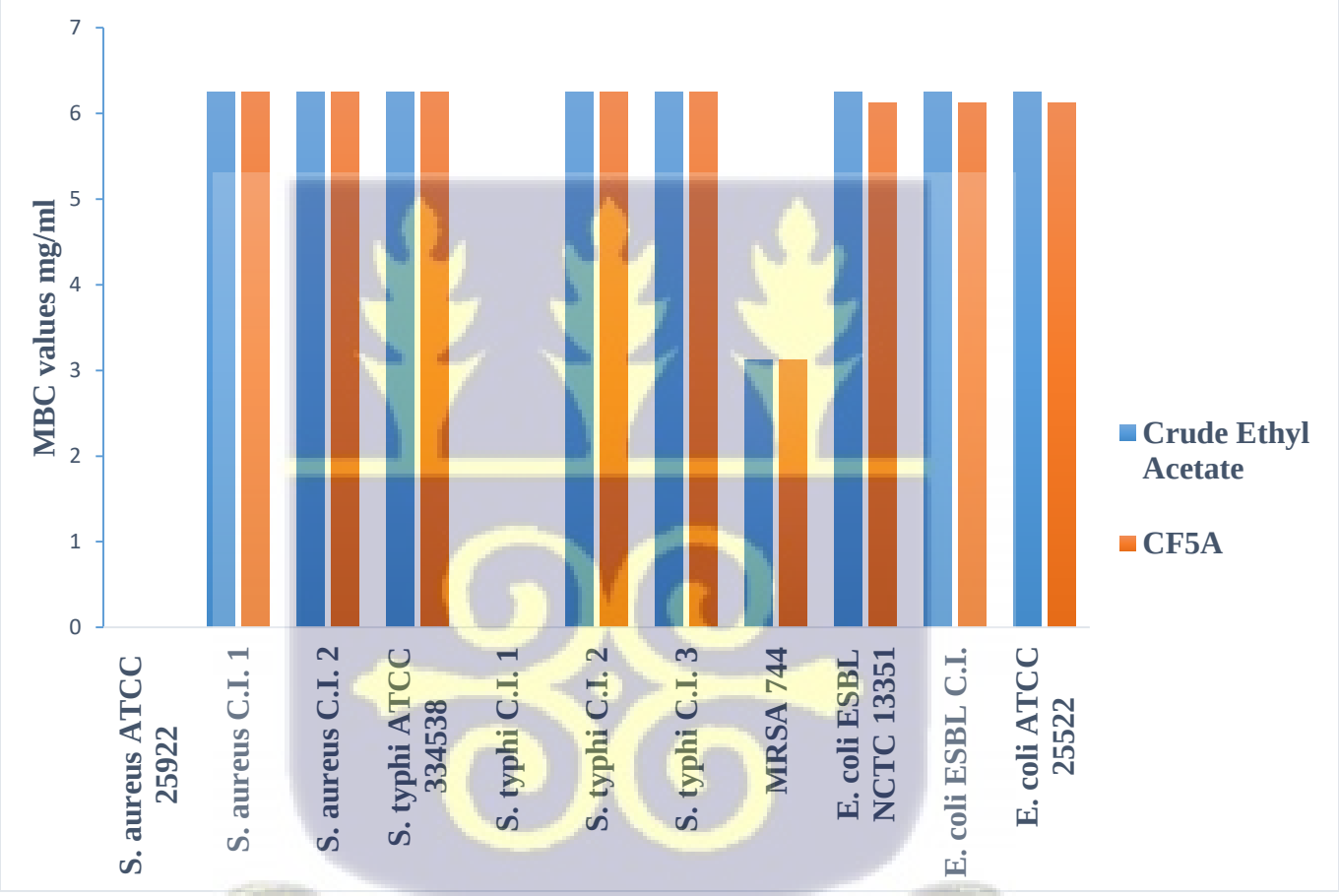

Figure 4.11: Minimum Bactericidal Concentration of Crude Ethyl Acetate and Active Fraction (CF5A) of *E. hirta* against Test Organisms

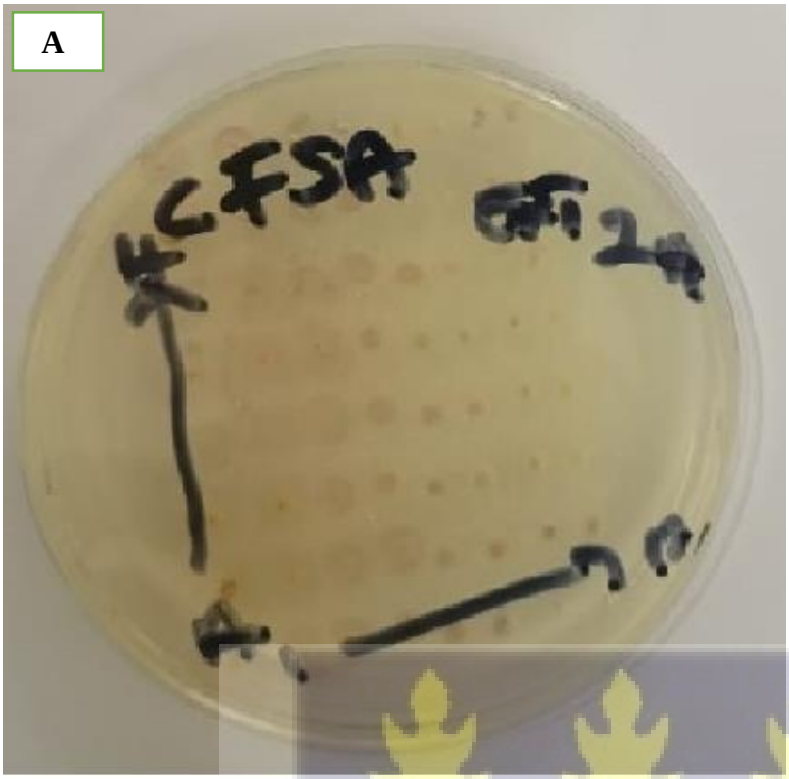

PLATE A: Showing the MBC of CF5A

PLATE B: Showing the MBC of crude ethyl –acetate extracts of *E. hirta*

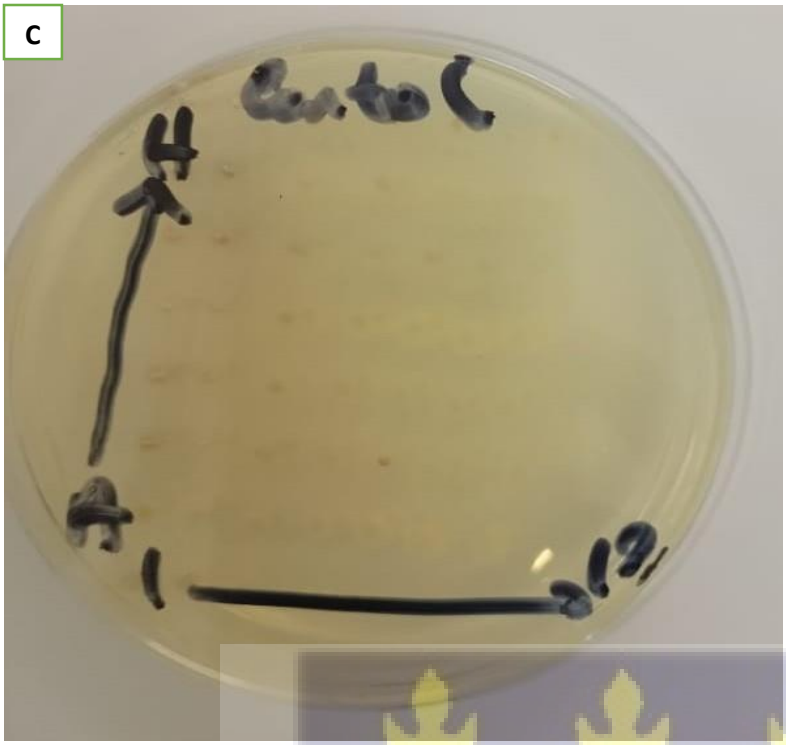

PLATE C: Showing the MBC of 15µg/ml Ciprofloxacin.

Plate 2: Plates A, B, and C Showing the MBC of Crude Ethyl Acetate Extract and Fraction (CF5A) Against Some Test Organisms

CHAPTER FIVE

5.0 Discussion

Many scientists have written about the use of plants as antimicrobial agents in most traditional and other therapeutic settings (Abubakar, 2009; Abu *et al.*, 2010; Rao *et al.*, 2010; Gupta & Gupta, 2019). Several studies have shown that number of plant extracts have antibacterial activity against both Gram-negative and Gram-positive bacteria. This emphasizes the broad-spectrum nature of most medicinal plants.

Results obtained after comparing the yield between the two maceration processes (soxhlet and cold maceration) substantiate that the soxhlet extraction method has a higher yield than that of the cold extraction method. This implies that soxhlet extraction should be considered first when a higher mass of extract is needed for further studies. Choosing soxhlet maceration over cold maceration will help to utilize the resource, reduce cost, and other additional benefits.

Depending on the results gotten on the antimicrobial activity of the two maceration processes (soxhlet and cold maceration), both methods should be considered equally when extracting components for antimicrobial susceptibility testing since extracts obtained using both methods have similar activity with no significant difference.

Although all test organisms recruited in the study were resistant to dichloromethane and petroleum ether extracts, quite a number were susceptible to methanol, aqueous and ethyl acetate extracts. This confirms that polar extracts have high potency than medium and non-polar extracts. This must be a probable reason why polar solvent (water) is mainly used in preparing *E. hirta* plant materials for traditional use. The results obtained in this study were in line with those reported in studies conducted by Gupta & Gupta (2019) in India who found out that polar components of the *E. hirta* plant are effective against *Salmonella typhi* and *Escherichia coli.*

Column chromatographic analysis shows that the most active component of *E. hirta* ethyl- acetate extracts can be obtained using chloroform and methanol at its mobile phase in the fractions 20:3 and 6:4. This shows that the most active components of ethyl acetate extract can be obtained by using chloroform and methanol as the main solvents in the mobile phase. This will serve as a prior knowledge for isolating the most active components of *E. hirta* crude ethyl acetate extracts.

GCMS analysis on the most active fraction predicts the presence of 1,2,3-Benzenetriol. This is sugar that has been reported to have shown antioxidant and antimicrobial activity as stated by Deryabin & Tolmacheva. (2015).

Phytochemical analysis revealed the presence of phytoconstituents such as reducing sugars, phenolic compounds, saponins, flavonoids, anthracenosides and Phytosterols. This support previous suggestions made by Abubakar (2009) and Kuta1 *et al*. (2013) which states that tannins, flavonoids, glycosides and essential oil are some phytochemical components observed in *E. hirta* and have an antibacterial activity that could lead to the killing or growth inhibition of some test organisms. Findings from this study imply that numerous ailments caused by this test organism can be treated with *E. hirta* crude extracts due to the presence of phytochemicals that have antimicrobial activity against such organisms. The therapeutic and traditional use of *E. hirta* in the treatment of ailments like cough, bronchitis, bowel complaints, helminthic infestations, typhoid, uterine problems and abscesses is strongly proven by this study after conducting a series of experiments on the antimicrobial activity of the *E. hirta* against some causative organisms of this infections.

Although crude ethyl acetate extracts and the active fraction had the same bactericidal activity per the MBC results, the MIC value recorded for crude ethyl acetate was between 50- 6.25 mg/ml

whiles the active fraction (CF5) is between 50 -12.5 mg/ml. This publicizes that, components in the active fractions have synergic activity with other components. This can also be a probable reason why *E. hirta* leaves are used in combination with other plants for the treatment of asthma as stated by Saravanan *et al*, (2012).

Studies conducted by Gupta & Gupta (2019) in India attest to the effectiveness of the plant against *Bacillus thuringiensis, Bacillus subtilis, Micrococcus* sp., *Escherichia coli, Klebsiella pneumonia, Proteus mirabilis, Salmonella typhi, Staphylococcus aureus, and Candida albicans.*

Abubakar (2009) and Rao *et al.* (2010) who worked in Nigeria and India respectively, also exploited the effectiveness of *E. hirta* against *Klebsiella pneumoniae* which goes contrary to this study since all *Klebsiella pneumoniae spp.* included in this study were resistant to *E. hirta* extracts. The resistance of *Klebsiella pneumoniae* sp. to all extracts in this study could be due to the strains of *Klebsiella pneumonia* used in this research. Also, the difference observed between this current study and other previous studies may be due to the difference in concentration of extracts and methodology.

Also, Gupta & Gupta (2019) reported the presence of phytoconstituents such as alkaloids, flavonoids, phenolics and tannins in *E. hirta* methanol extracts. The absence of alkaloids in this present study could be due to the difference in time and place of collection of the plant materials. This may account for the variations in the antimicrobial activity recorded in this study since different isolates responded differently to active compounds.

5.1 Limitations

As a result of limited time and resources, compounds present in fractions that showed lower antimicrobial activity were not purified.

Although the above-stated limitations might have little impact on the study results, the results from this study hold the base-line information needed to explore the antimicrobial activity of *E. hirta* against these selected multidrug-resistant bacteria.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.0 Conclusion

It is concluded that the polar components of *E. hirta* extracts contained antimicrobial properties against some strains of MRSA, *E. coli*, *S. typhi* and *S. aureus* whilst the non-polar components had no activity against these same organisms. Additionally, after comparing the yield of extracts obtained from the two maceration processes, the soxhlet extraction method has higher yield as compared to cold. The antimicrobial activity of extracts of extractants using both methods were similar.

Methanol and aqueous extracts demonstrated similar antibacterial activity, with ethyl acetate extracts outperforming both aqueous and methanol extracts. All test organisms were resistant to both dichloromethane and petroleum ether extracts. The presence of phytoconstituents such as reducing sugars, phenolic compounds, flavonoids, anthracenosides, and phytosterols, which are believed to be responsible for the antimicrobial action against the test organisms, is also highlighted in this study.

GCMS results also prove the presence of 1,2,3- Benzenetriol as the sugar component present in the most active component of ethyl acetate extracts.

The MIC and MBC results reveal that the polar components of *E. hirta* exhibit bacteriostatic and bactericidal action against all of the test organisms, with the exception of *S. aureus* ATCC 25922 and *S. aureus* clinical isolate 3, which only displayed the bacteriostatic activity.

In summary, only the polar components of the whole plant of *E. hirta* have better antimicrobial activity against all the test organisms with the exception of *Klebsiella pneumoniae*.

6.1 Recommendations

Further studies to isolate and elucidate pure compounds present in fractions that showed lower antimicrobial activity should be pursued.

Additionally, the efficacy of different extracts from *E. hirta* should be tested against more pathogenic bacteria, fungi, and parasites. In-vivo (animal) experiments should be done to assess the extracts' effectiveness and safety: following this, human experiments can also be done.

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APPENDICES

Appendix 1: Column Chromatography Work Sheet

Solvents used	Fractions collected	Combinations
1. 100% chloroform 500ml+500ml+ 500ml+500ml+ 500ml+500ml+ 500ml+500ml	1. 100ml 2. 100ml 3. 100ml 4. 100ml 5. 100ml 6. 100ml 7. 100ml 8. 100ml 9. 100ml 10. 100ml 11. 100ml 12. 100ml 13. 100ml 14. 100ml 15. 100ml 16. 100ml 17. 100ml 18. 100ml 19. 100ml 20. 100ml 21. 100ml 22. 100ml 23. 100ml 24. 100ml 25. 100ml 26. 100ml	From 42 = F1 43 = F2 44 = F3 45- 46 = F4 47= F5 48 = F6 49 = F7 50 – 52 = F8 53 = F9 54 = F10 55 = F11 56 = F12 57 – 60 = F13 61 - 65= F14 66 - 69 = F15 70 - 74 = F16 75 = F17 76 - 79 = F18 80 - 81 = F19 82 - 84 = F20 85 = F21 86 - 89 = F22 90 - 91 = F23 92 = F24 93 = F25

	27. 100ml	94 - 96 = F26
	28. 100ml	97 - 99 = F27
	29. 100ml	100 = F 28
	30. 100ml	101 - 102 = F29
	31. 100ml	103 = F30
	32. 100ml	104 = F31
	33. 100ml	105 - 109 = F32
	34. 100ml	110 - 112 = F33
	35. 100ml	113 - 114 = F 34
	36. 100ml	115 - 135 = F35
	37. 100ml	137 - 143 = F36
	38. 100ml	144 - 149 = F37
	39. 100ml	150 - 154 = F38
	40. 100ml	155 - 159 = F 39
	41. 100ml	160 - 163 = F40
		1 to 41 = F40
Chloroform/ Methanol	42. 100ml	
20: 1	43. 100ml	
500ml+500ml+	44. 100ml	F40 + F1 = CF1
500ml+500ml+	45. 100ml	F2 = CF2
500ml+500ml+	46. 100ml	F3 = CF3
500ml+200ml	47. 100ml	F4 = CF4
	48. 100ml	F5 + F6 + F7 = CF5
	49. 100ml	F8 = CF6
	50. 100ml	F9 + F10 = CF7
	51. 100ml	F11 + F12 = CF8
	52. 100ml	F13 = CF9
	53. 100ml	F14 + F15 = CF10
	54. 100ml	F16 = CF11
	55. 100ml	F17 + F18 = CF12

	56. 100ml	F19 + F20 + F21 = CF13
	57. 100ml	F22 + F23 + F24+ F25 + F26
	58. 100ml	+ F27 + F28 + F29 = CF14
	59. 100ml	F30 + F31 + F32 + F 33 + F34
	60. 100ml	= CF16
	61. 100ml	F35 + F36 = CF17
	62. 100ml	F38 = CF18
	63. 100ml	F39 = CF19
	64. 100ml	
	65. 100ml	
	66. 100ml	
	67. 100ml	
	68. 100ml	CF1 + CF2 = CF1A
	69. 100ml	
	70. 100ml	CF3 + CF4 + CF5 + CF6 +
	71. 100ml	CF7 + CF8 = CF2A
	72. 100ml	
	73. 100ml	CF9 + CF10 + CF11 = CF3A
	74. 100ml	
	75. 100ml	CF13 = CF4A
	76. 100ml	
	77. 100ml	CF14 + CF15 = CF5A
	78. 100ml	
	79. 100ml	CF16 + CF17 + CF18 + CF19 = CF6A
Chloroform/ Methanol	80. 100ml	
20: 3	81. 100ml	
500ml+500ml+	82. 100ml	
500ml + 500ml +	83. 100ml	
500ml	84. 100ml	

	85. 100ml 86. 100ml 87. 100ml 88. 100ml 89. 100ml 90. 100ml 91. 100ml 92. 100ml 93. 100ml 94. 100ml 95. 100ml 96. 100ml 97. 100ml 98. 100ml 99. 100ml 100. 100ml 101. 100ml 102. 100ml 103. 100ml 104. 100ml	
Chloroform/ Methanol 6: 4 500ml+500ml	105. 100ml 106. 100ml 107. 100ml 108. 100ml 109. 100ml 110. 100ml 111. 100ml 112. 100ml 113. 100ml 114. 100ml	

Chloroform/ Methanol	115.	100ml	
1: 1	116.	100ml	
500ml+500ml	117.	100ml	
	118.	100ml	
	119.	100ml	
	120.	100ml	
	121.	100ml	
	122.	100ml	
	123.	100ml	
	124.	100ml	
	125.	100ml	
Chloroform/Methanol			
4: 6	126.	100ml	
500ml+500ml	127.	100ml	
	128.	100ml	
	129.	100ml	
	130.	100ml	
	131.	100ml	
	132.	100ml	
	133.	100ml	
	134.	100ml	
	135.	100ml	
	136.	100ml	
Chloroform/ Methanol			
3: 7	137.	100ml	
500ml+500ml+	138.	100ml	
300ml	139.	100ml	
	140.	100ml	
	141.	100ml	
	142.	100ml	

	143.	100ml	
	144.	100ml	
	145.	100ml	
	146.	100ml	
	147.	100ml	
	148.	100ml	
Chloroform/ Methanol			
1: 9	149.	100ml	
500ml	150.	100ml	
	151.	100ml	
	152.	100ml	
	153.	100ml	
	154.	100ml	
100 % Methanol			
500ml+500ml	155.	100ml	
	156.	100ml	
	157.	100ml	
	158.	100ml	
	159.	100ml	
	160.	100ml	
	161.	100ml	
	162.	100ml	
	163.	100ml	

Appendix II: Ethical Clearance Form

UNIVERSITY OF GHANA
COLLEGE OF HEALTH SCIENCES
ETHICAL AND PROTOCOL REVIEW COMMITTEE

Ref. No.:..EPRC/SEP/2021..... September 22, 2021

Mr. Jones Gyabeng
Department of Medical Microbiology
University of Ghana Medical School
Korle Bu

ETHICAL CLEARANCE

Protocol Identification Number: ***CHS-Et/M.1 – P4.6/2021-2022***

FWA: 000185779 **IORG: 0005170** **IRB: 00006220**

The College of Health Sciences Ethical and Protocol Review Committee (EPRC) on September 2, 2021 reviewed and approved your research protocol.

Title of Protocol: **"In-Vitro Evaluation of Antimicrobial Properties of Euphorbia Hirta against Multidrug Resistant Bacteria in Ghana"**

Principal Investigator: **Mr. Jones Gyabeng**

This approval requires that you submit six-monthly review report(s) of the study to the Committee and a final full review report to the EPRC at the completion of the study. The Committee may observe, or cause to be observed, procedures and records of the study before, during and after implementation.

Please note that any significant modification(s) to this project/study must be submitted to the Committee for review and approval before its implementation.

You are required to report all serious adverse events related to this study to the EPRC within seven (7) days verbally and fourteen (14) days in writing.

As part of the review process, it is the Committee's duty to review the ethical aspects of any manuscript that may be produced from this study. You will therefore be required to furnish the Committee with any manuscript for publication.

This ethical clearance is valid till September 22, 2022.

Please always quote the protocol identification number in all future correspondence in relation to this protocol.

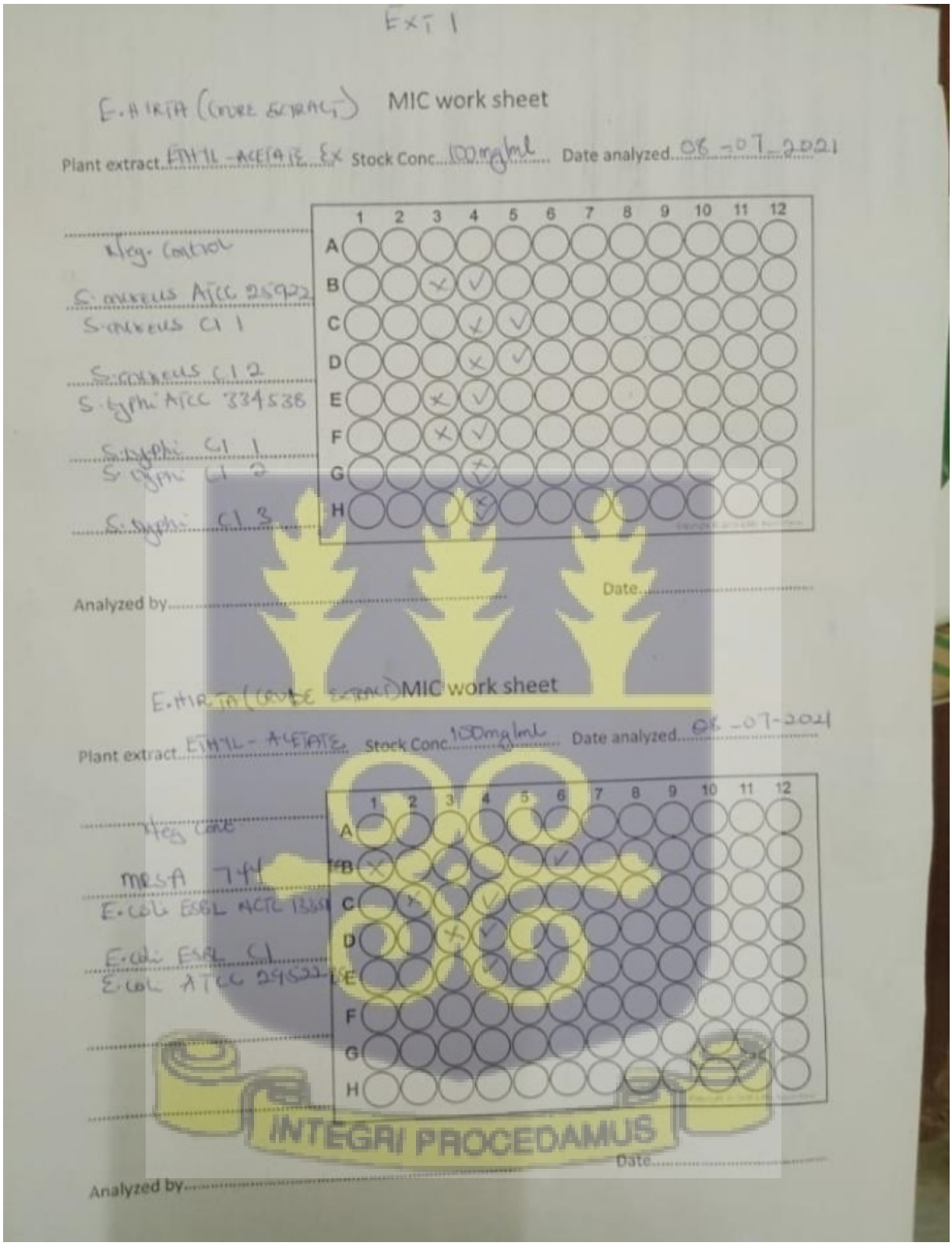
Signed: .....

Professor Andrew Anthony Adjei
Chair, Ethical and Protocol Review Committee

cc: Provost, CHS
Dean, UGMS
Head, Medical Microbiology

• P. O. Box KB 52, Korle Bu, Accra, Ghana. **Telephone:** +233 (0) 302 665103/4 • **Fax:** +233 (0) 302 660762
• **Email:** admin.chs@ug.edu.gh /provost.chs@ug.edu.gh • **Website:** www.chs.ug.edu.gh

Appendix III: MIC Work Sheet for Crude Ethyl Acetate Extracts



Appendix IV: MIC Work Sheet for Crude Ethyl Acetate Extract

Appendix V: Data Showing Antimicrobial Activity of Methanol Cold Extracts

Concentration/organisms	200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	5% DMSO	Ciprofloxacin 15µg/ml
S. aureus ATCC 25955	18.33 ± .33	17.00 ± .00	15.00 ± .00	13.33 ± .33	11.00 ± .00	6.00 ± .00	21.00 ± .00
S. aureus C. I 1	19.00 ± .00	17.33 ± .33	15.00 ± .00	12.00 ± .00	10.33± .33	6.00 ± .00	20.00 ± .33
S. aureus C. I 2	15.67 ± .33	13.00 ± .00	11.00 ± .00	9.00 ± .00	8.00 ± .00	6.00 ± .00	21.00 ± .00
MRSA 744	14.33 ± .33	13.33 ± .33	10.00 ± .00	9.00 ± .00	8.00 ± .00	6.00 ± .00	22.33 ± .33
K. pneumoniae ATCC 7000603	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	19.00 ± .00
K. pneumoniae NTCC 13438	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00
K. pneumoniae C.I	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	24.33 ± .00
K. pneumoniae ESBL NCTC 13438	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00
E. coli ATCC 29522	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	30.00 ± .00
E. coli ESBL NCTC 13351	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00
E. coli ESBL C. I	6.00 ±.00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	24.00 ± .33
S. typhi ATCC 33438	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	33.33 ± .00
S. typhi C. I 1	13.67 ± .33	12.67 ± .33	10.67 ± .33	9.00 ± .000	7.00 ± .00	6.00 ± .00	21.00 ± .00
S. typhi C. I 2	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	31.67 ± .33

S. typhi C. I 3	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	30.33 ± .33

(Data are Mean ± SEM) Diameter of well = 6mm

Appendix VI: Data Showing Antimicrobial Activity of Methanol Soxhlet Extracts

concentration/organisms	200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	5% DMSO	Ciprofloxacin 15µg/ml
S. aureus ATCC 25955	18.00 ± .33	17.00 ± .00	15.00 ± 00	12.33 ± .33	11.00 ± 00	6.00 ± .00	21.00 ± .00
S. aureus C. I 1	19.33 ± 00	17.33 ± .33	15.33 ± 33	12.68 ± .33	10.33 ± 00	6.00 ± .00	20.33 ± .33
S. aureus C. I 2	15.68 ±.33	13.00 ± 00	11.00 ± 33	8.00 ± 00	7.00 ± 00	6.00 ± .00	21.00 ± .00
MRSA 744	14.33 ±.00	13.33 ±.33	10.00 ±.00	9.00 ±.00	8.00 ±.00	6.00 ± .00	22.33 ± .33
K. pneumoniae ATCC 7000603	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	19.00 ± .00
K. pneumoniae NTCC 13438	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00
K. pneumoniae C.I	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	24.33 ± .58
K. pneumoniae ESBL NCTC 13438	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00
E. coli ATCC 29522	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	31.00 ± .33
E. coli ESBL NCTC 13351	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00
E. coli ESBL C. I	6.00 ±.00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	24.00 ± .33
S. typhi ATCC 33438	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	33.33 ± .33

S. typhi C. I. 1	16.00± .33	15.67 ± .33	13.67 ± .33	10.00 ± .00	7.00 ± .00	6.00 ± .00	21.33 ± .00
S. typhi C. I. 2	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	31.33 ± .58
S. typhi C. I. 3	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	31.00 ± .00

(Data are Mean ± SEM) Diameter of well = 6mm

Appendix VII: Data Showing Antimicrobial Activity of Petroleum Ether Soxhlet Extracts

concentration/organisms	200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	5%DMSO	Ciprofloxacin 15µg/ml
S. aureus ATCC 25955	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	21.00 ± .00
S. aureus C. I 1	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	20.00 ± .33
S. aureus C. I 2	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	21.00 ± .00
MRSA 744	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	22.00 ± .00
K. pneumoniae ATCC 7000603	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	20.00 ± .33
K. pneumoniae NTCC 13438	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00
K. pneumoniae C.I	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	22.33 ± .00
K. pneumoniae ESBL NCTC 13438	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00
E. coli ATCC 29522	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	30.00 ± .58
E. coli ESBL NCTC 13351	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00

E. coli ESBL C. I	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	24.00 ± .00
S. typhi ATCC 33438	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	33.33 ± .33
S. typhi C. I1	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	23.00 ± .00
S. typhi C. I2	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	31.00± .33
S. typhi C. I3	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	30.33 ± 00

(Data are Mean ± SEM) Diameter of well = 6mm

Appendix VIII: Data Showing Antimicrobial Activity of Petroleum Ether Cold Extracts

concentration/organisms	200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	5%DMSO	Ciprofloxacin 15µg/ml
S. aureus ATCC 25955	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	21.00 ± .58
S. aureus C. I 1	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	19.67 ± .33
S. aureus C. I 2	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	21.00 ± .58
MRSA 744	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	22.33 ± .67
K. pneumoniae ATCC 7000603	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	19.00 ± .58
K. pneumoniae NTCC 13438	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00
K. pneumoniae C.I	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	24.33 ± .67
K. pneumoniae ESBL NCTC 13438	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00
E. coli ATCC 29522	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	30.00 ± .58

E. coli ESBL NCTC 13351	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00
E. coli ESBL C. I	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	24.00 ± .58
S. typhi ATCC 33438	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	34.33 ± .33
S. typhi C. I1	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	22.00 ± .58
S. typhi C. I2	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	31.67 ± .33
S. typhi C. I3	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	29.33 ± .33

(Data are Mean ± SEM) Diameter of well = 6mm

Appendix IX: Data Showing Antimicrobial Activity of Dichloromethane Soxhlet Extracts

concentration/organisms	200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	5%DMSO	Ciprofloxacin 15µg/ml
S. aureus ATCC 25955	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	21.00 ± .58
S. aureus C. I 1	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	19.67 ± .33
S. aureus C. I 2	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	21.00 ± .58
MRSA 744	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	22.33 ± .67
K. pneumoniae ATCC 7000603	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	19.00 ± .58
K. pneumoniae NTCC 13438	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00
K. pneumoniae C.I	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	24.33 ± .67
K. pneumoniae ESBL NCTC 13438	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00

E. coli ATCC 29522	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	30.00 ± .58
E. coli ESBL NCTC 13351	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00
E. coli ESBL C. I	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	24.00 ± .58
S. typhi ATCC 33438	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	34.33 ± .33
S. typhi C. I1	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	22.00 ± .58
S. typhi C. I2	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	31.67 ± .33
S. typhi C. I3	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	29.33 ± .33

(Data are Mean ± SEM) Diameter of well = 6mm

Appendix X: Data showing antimicrobial activity of Dichloromethane cold extracts

concentration/organisms	200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	5% DMSO	Ciprofloxacin 15µg/ml
S. aureus ATCC 25955	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	21.00 ± .58
S. aureus C. I 1	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	19.67 ± .33
S. aureus C. I 2	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	21.00 ± .58
MRSA 744	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	22.33 ± .67
K. pneumoniae ATCC 7000603	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	19.00 ± .58
K. pneumoniae NTCC 13438	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00
K. pneumoniae C.I	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	24.33 ± .67

K. pneumoniae ESBL NCTC 13438	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00
E. coli ATCC 29522	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	30.00 ± .58
E. coli ESBL NCTC 13351	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00
E. coli ESBL C. I	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	24.00 ± .58
S. typhi ATCC 33438	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	34.33 ± .33
S. typhi C. I1	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	22.00 ± .58
S. typhi C. I2	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	31.67 ± .33
S. typhi C. I3	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	29.33 ± .33

(Data are Mean ± SEM) Diameter of well = 6mm

Appendix XI: Data Showing Antimicrobial Activity of Aqueous Extracts

concentration/organisms	200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	5% DMSO	Ciprofloxacin 15µg/ml
S. aureus ATCC 25955	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	21.00 ± .00
S. aureus C. I 1	20.67 ± .33	17.67 ± .33	15.67 ± .33	12.67 ± .33	11.00 ± .00	6.00 ± .00	19.00 ± .33
S. aureus C. I 2	19.67 ± .33	16.67 ± .33	14.00 ± .00	13.00 ± .00	11.00 ± .00	6.00 ± .00	21.00 ± .00
MRSA 744	18.00 ± .00	15.33 ± .33	13.00 ± .00	12.00 ± .00	7.33 ± .00	6.00 ± .00	22.33 ± .33
K. pneumoniae ATCC 7000603	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	20.00 ± .33
K. pneumoniae NTCC 13438	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00

K. pneumoniae C.I	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	23.00 ± .33
K. pneumoniae ESBL NCTC 13438	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00
E. coli ATCC 29522	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	29.00 ± .00
E. coli ESBL NCTC 13351	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00
E. coli ESBL C. I	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	24.00 ± .00
S. typhi ATCC 33438	19.33 ± .33	17.33 ± .33	13 ± .00	10.33 ± .33	7.00 ± .00	6.00 ± .00	32.33 ± .33
S. typhi C. I 1	19.33 ± .33	18.00 ± .00	15 ± .00	13.00 ± .00	7.00 ± .00	6.00 ± .00	22.00 ± .00
S. typhi C. I 2	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	30.00 ± .00
S. typhi C. I 3	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	30.00 ± .33

(Data are Mean ± SEM) Diameter of well = 6mm

Appendix XII: Data Showing Antimicrobial Activity of Ethyl Acetate Extracts

concentration/organisms	200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	5% DMSO	Ciprofloxacin 15µg/ml
S. aureus ATCC 25955	23.33 ± .33	20.00 ± .00	18.33 ± .33	15.67 ± .33	13.00 ± .00	6.00 ± .00	21.00 ± .00
S. aureus C. I. 1	22.33 ± .33	21.00 ± .33	17.67 ± .00	16.00 ± .00	13.67 ± .33	6.00 ± .00	19.00 ± .33
S. aureus C. I. 2	22.33 ± .33	18.33 ± .33	16.33 ± .33	14.00 ± .00	12.00 ± .00	6.00 ± .00	21.00 ± .00
MRSA 744	21.00 ± .00	18.00 ± .00	16.00 ± .00	14.00 ± .00	12.00 ± .00	6.00 ± .00	22.00 ± .00
K. pneumoniae ATCC 7000603	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	20.00 ± .33

K. pneumoniae NTCC 13438	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00
K. pneumoniae C.I	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	24.00 ± .33
K. pneumoniae ESBL NCTC 13438	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00
E. coli ATCC 29522	19.33 ± .33	16.00 ± .00	13.00 ± .00	12.00 ± .00	10.67 ± .33	6.00 ± .00	31.00 ± .00
E. coli ESBL NCTC 13351	19.00 ± .00	17.00 ± .00	14.00 ± .00	12.00 ± .00	11.00 ± .00	6.00 ± .00	6.00 ± .00
E. coli ESBL C. I.	19.00 ± .00	16.00 ± .00	14.00 ± .00	12.67 ± .33	11.67 ± .33	6.00 ± .00	24.00 ± .00
S. typhi ATCC 33438	22.00 ± .00	21.00 ± .00	17.00 ± .00	14.33 ± .33	12.33 ± .33	6.00 ± .00	33.33 ± .33
S. typhi C. I. 1	23.67 ± .33	20.67 ± .57	19.00 ± .33	17.00 ± .00	15.33 ± .33	6.00 ± .00	22.00 ± .00
S. typhi C. I. 2	18.00 ± .33	15.00 ± .33	13.00 ± .00	11.67 ± .33	8.67 ± .00	6.00 ± .00	31.00 ± .33
S. typhi C. I. 3	18.00 ± .00	15.00 ± .00	13.00 ± .00	11.67 ±.33	8.67 ± .33	6.00 ± .00	30.00 ± .00

(Data are Mean ± SEM) Diameter of well = 6mm

Appendix XIII: Data Showing Antimicrobial Activity of Fractions Obtained from Column Chromatography

Organism/fraction	CF1A	CF2A	CF3A	CF4A	CF5A	CF6A	5%DMSO	Ciprofloxacin 15µg/ml
S. aureus ATCC 25955	6.00 ± .00	11.33 ± .33	11.67 ± .33	14.33 ± .33	15.00 ± .00	12.00 ± .00	6.00 ± .00	21.33 ± .00
S. aureus C. I 1	6.00 ± .00	11.00 ± .00	11.00 ± .00	9.33 ± .33	17.67 ± .33	12.67 ± .33	6.00 ± .00	20.33 ± .33

S. aureus C. I 2	6.00 ± .00	8.33 ± .33	11.00 ± .00	11.67 ± .33	13.67 ± .33	9.33 ± .33	6.00 ± .00	21.00 ± .00
MRSA 744	6.00 ± .00	8.33 ± .33	8.00 ± .00	13.67 ± .33	15.33 ± .33	12.00 ± .33	6.00 ± .00	22.33 ± .00
K. pneumoniae ATCC 7000603	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	21.00 ± .33
K. pneumoniae NTCC 13438	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00
K. pneumoniae C.I	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	23.33 ± .00
K. pneumoniae ESBL NCTC 13438	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00
E. coli ATCC 29522	6.00 ± .00	8.00 ± .00	6.00 ± .00	6.00 ± .00	10.67 ± .58	6.00 ± .00	6.00 ± .00	30.00 ± .33
E. coli ESBL NCTC 13351	6.00 ± .00	8.33 ± .58	6.00 ± .00	6.00 ± .00	12.33 ± .58	6.00 ± .00	6.00 ± .00	6.00 ± .00
E. coli ESBL C. I.	6.00 ± .00	8.00 ± .58	6.00 ± .00	12.33 ± .58	12.67 ± .58	6.00 ± .00	6.00 ± .00	24.33 ± .00
S. typhi ATCC 33438	6.00 ± .00	8.00 ± .00	6.00 ± .00	6.00 ± .00	13.00 ± .00	6.00 ± .00	6.00 ± .00	31.33 ± .33
S. typhi C. I. 1	6.00 ± .00	6.00 ± .00	6.00 ± .00	6.00 ± .00	14.67 ± .33	11.33 ± .33	6.00 ± .00	22.00 ± .00
S. typhi C. I. 2	6.00 ± .00	8.33 ± .33	6.00 ± .00	6.00 ± .00	13.67 ± .33	6.00 ± .00	6.00 ± .00	32.00 ± .00
S. typhi C. I. 3	6.00 ± .00	8.67 ± .33	6.00 ± .00	6.00 ± .00	12.67 ± .33	6.00 ± .00	6.00 ± .00	30.00 ± .33

Appendix XIV: Data Analysis of the Yield of *E. Hirta* Crude Extracts using Soxhlet and Cold Extraction Methods

Method

Null hypothesis All means are equal
Alternative hypothesis At least one mean is different
Significance level $\alpha = 0.05$
Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	2	Soxhlet, Cold

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	1	7.935	7.935	0.55	0.501
Error	4	58.113	14.528		
Total	5	66.048			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
3.81161	12.01%	0.00%	0.00%

Means

Factor	N	Mean	StDev	95% CI
Soxhlet	3	4.83	4.48	(-1.28, 10.94)
Cold	3	2.53	3.00	(-3.58, 8.64)

Pooled StDev = 3.81161

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
Soxhlet	3	4.83	A
Cold	3	2.53	A

Means that do not share a letter are significantly different.

Appendix XV: Analysis of Ethyl-Acetate Active Fractions

Method

Null hypothesis All means are equal

Alternative hypothesis At least one mean is different

Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	6	CF1A, CF2A, CF3A, CF4A, CF5A, CF6A

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	5	271.3	54.267	8.47	0.000
Error	84	538.4	6.410		
Total	89	809.7			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.53170	33.51%	29.55%	23.67%

Means

Factor	N	Mean	StDev	95% CI
CF1A	15	6.000	0.000	(4.700, 7.300)
CF2A	15	7.889	1.707	(6.589, 9.189)
CF3A	15	7.333	2.153	(6.033, 8.633)
CF4A	15	7.800	2.870	(6.500, 9.100)
CF5A	15	11.69	3.88	(10.39, 12.99)
CF6A	15	7.822	2.754	(6.522, 9.122)

Pooled StDev = 2.53170

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
CF5A	15	11.69	A
CF2A	15	7.889	B
CF6A	15	7.822	B
CF4A	15	7.800	B
CF3A	15	7.333	B
CF1A	15	6.000	B

Means that do not share a letter are significantly different.

Appendix XVI: Data Analysis on the MIC Values for Crude Ethyl Acetate Extracts and Active Fractions

Method

Null hypothesis All means are equal
Alternative hypothesis At least one mean is different
Significance level $\alpha = 0.05$
Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	2	Crude Ethyl Acetate, CF5A

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	1	1110	1109.7	7.39	0.013
Error	20	3004	150.2		
Total	21	4114			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
12.2561	26.97%	23.32%	11.64%

Means

Factor	N	Mean	StDev	95% CI
Crude Ethyl Acetate	11	14.20	13.14	(6.50, 21.91)
CF5A	11	28.41	11.31	(20.70, 36.12)

Pooled StDev = 12.2561

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
CF5A	11	28.41	A
Crude Ethyl Acetate	11	14.20	B

Means that do not share a letter are significantly different.

Appendix XVII: Data Analysis on the MBC Value for Ethyl Acetate Crude Extracts and Active Fractions Method

Null hypothesis	All means are equal
Alternative hypothesis	At least one mean is different
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	2	Crude Ethyl Acetate, CF5A

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	1	0.006	0.00639	0.00	0.975
Error	20	130.361	6.51804		
Total	21	130.367			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.55305	0.00%	0.00%	0.00%

Means

Factor	N	Mean	StDev	95% CI
Crude Ethyl Acetate	11	4.830	2.563	(3.224, 6.435)
CF5A	11	4.795	2.543	(3.190, 6.401)

Pooled StDev = 2.55305

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
Crude Ethyl Acetate	11	4.830	A
CF5A	11	4.795	A

Means that do not share a letter are significantly different.