

**IN VITRO ASSESSMENT OF ANTIBACTERIAL ACTIVITY OF CRUDE PROPOLIS  
EXTRACTS FROM DIFFERENT REGIONS OF GHANA**

**BY**

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**DECLARATION**

**“I, the undersigned student declare that this project is my own original investigation except where references have been made to other people’s work and were duly cited. This work either in whole or part, has not been previously presented to this university or elsewhere”.**

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**“We hereby declare that the preparation and presentation of this thesis was done in accordance with the guidelines on supervision of thesis laid down by the University of Ghana”.**

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## **DEDICATION**

I dedicate this work to my late father, Mr. Saeed Dude Mohammed, my mother, Fatima Hamidu, my brother Alhaji Usif Smith Seidu, my wife Hamdiya Alhassan and finally to my children, Fatima Danwuob Alhassan, Mufidah Chimbiam Alhassan and Ayman Mingiman Alhassan.



## ABSTRACT

**Background:** Antimicrobial resistance has reached a global dimension and threatens the health and the economy of many nations including developing countries like Ghana. This realization led several researchers to anticipate the forthcoming “end of the antibiotic era”. Studies have shown that medicinal plants and their derivatives still play an important role to cover the basic health needs in resource poor settings. One of such natural products endowed with bioactive compounds used in traditional medicine to treat diseases is propolis.

**Aim:** This study aimed at evaluating the antimicrobial activity of crude propolis extracts from different regions in Ghana against selected multidrug resistant clinical bacterial isolates.

**Methodology:** About 100 g of propolis samples were collected from each selected commercial beekeepers across the (10) ten regions in Ghana. Samples were collected between December 2015 and March 2016. Crude propolis extracts were prepared using petroleum ether (PET), chloroform (CH) and ethyl acetate (EA). Extracts were evaporated using rotary evaporator and dried in a dessicator. A concentration of 64 mg/ml of each crude extract was dissolved in 5% dimethyl sulfoxide (DMSO). Clinical isolates including drug resistant *S. aureus*, *E. coli*, *P. aeruginosa* and their control strains MRSA NCTC 25923, *P. aeruginosa* ATCC 27853 and *E. coli* NCTC 13351 were used in the study. Screening for antimicrobial activity of the propolis extracts were carried out using agar well diffusion method. Active crude propolis extracts were fractionated using chloroform (CH), ethyl acetate (EA) and petroleum ether (PET). The active fractions, chloroform fraction of Northern (CH<sub>NR</sub>), chloroform fraction of Eastern (CH<sub>ER</sub>), Ethyl acetate fraction of Ashanti (EA<sub>AS</sub>), ethyl acetate of Volta (EA<sub>VT</sub>) and ethyl acetate fraction of Northern region (EA<sub>NR</sub>) were also tested. The minimum inhibitory (MIC) and the minimum bactericidal concentration (MBC) of the active fractions were determined using the broth macro dilution method. Screening for phytochemicals was done using standard procedures and HPLC was used to analyze the active fractions.

**Results:** Varying degrees of antimicrobial susceptibility pattern were observed from the screening for antimicrobial activities of propolis collected from the ten (10) region in Ghana. *Pseudomonas aeruginosa* isolated from clinical samples and its control strain *Pseudomonas aeruginosa* ATCC 27853 were more susceptible to Northern (NR), Eastern (ER), Ashanti (AS) and Volta region (VT) crude propolis extract. The average zone of inhibition ranges from 10 to 13mm for *Pseudomonas aeruginosa* (clinical) and 20 to 31mm for *Pseudomonas aeruginosa* ATCC 27853. *S. aureus* (clinical) and its control strain, MRSA ATCC 25923 were also found to be susceptible to NR, ER,

AS and VT region propolis extracts with average zone of inhibition ranging from 19 to 24mm and 20 to 25mm respectively.

Among the twelve propolis fractions tested, CH<sub>NR</sub>, CH<sub>ER</sub>, EA<sub>ER</sub>, EA<sub>VT</sub> and EA<sub>AS</sub> were identified as the active fractions with the average zone of inhibitions ranging from 0 to 22mm and 9 to 31mm against *P. aeruginosa* (clinical isolate) and its control, *P. aeruginosa* (ATTC 27853) respectively, among twelve propolis fractions tested.

At the concentration of 0.01 to 16 mg/ml of each propolis fraction, there were no MIC activities. Activity became evident at the concentration range of 24 to 100 mg/ml. *S. aureus* (clinical isolate) was found to be susceptible to two (2) of the fractions (CH<sub>NR</sub> and EA<sub>VT</sub>) with MICs (MBCs) of 56 (64) and 24 (32) mg/ml respectively whilst its control strain MRSA ATCC 25923 was susceptible to four (4) of the fractions (CH<sub>ER</sub>, EA<sub>ER</sub> and EA<sub>VT</sub> and EA<sub>AS</sub>) with MICs (MBCs) ranging from 24 to 64 mg/ml. EA<sub>VT</sub> exhibited broad spectrum inhibitory and bactericidal effect against all six (6) test isolates (*S. aureus*, MRSA ATCC 25923, *E. coli*, ESBL *E. coli* NCTC 13351, *P. aeruginosa* and *P. aeruginosa* ATTC 27853). The MIC (MBCs) ranges from 24 (24) to 40 (40).

Qualitative phytochemical analysis of active fractions indicates that terpenoids, phenols, flavonoids, alkaloids and saponins were present in all fractions. Moreover, HPLC analysis revealed closely related chromatograms have their active fractions with major peaks and retention time ranging from 26 to 39 minutes.

**Conclusion:** Propolis extracts have inhibitory and bactericidal effects on clinical *S. aureus* and *P. aeruginosa* isolates. The observed antimicrobial activities in fractions can be attributed to the qualitatively identified phytochemicals. The broad spectrum activities of EA<sub>VT</sub> could be due to the activity of major peaks observed at retention time 29 and 35.5 minute.

The EA<sub>VT</sub> fraction demonstrated the highest antimicrobial activities *S. aureus* and *P. aeruginosa*. The observed antimicrobial activities may be due to the presence of alkaloids, phenols, tannins, saponins terpenoids and flavonoids.

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## LIST OF ABBREVIATIONS

% –	Percent
µg	Microgram
ATCC	American Type Culture Collection
BDH	British drug house
CDC	Center for Disease Control
CEO	Chief Executive Officer
CH	Chloroform
CLSI	Clinical and Laboratory Standard Institute
cm	Centimeter
CPMR	Centre for Plant Medicine Research
DMSO	Dimethyl sulfoxide
EA	Ethyl acetate
ESBL	Extended spectrum Beta- lactamases
FLG	Forever Living Ghana
g	Grams
GC	Gas Chromatography
GNDP	Ghana National Drug Programme
HPLC	High Performance Liquid Chromatography
MDR	Multidrug Resistance
MHB	Mueller-Hinton Broth
MIC	Minimum Inhibitory Concentration
Min	minutes
MM	Milliliter
MOFA	Ministry of Local Government
MOLG	Ministry of Local Government
MRSA	Methicillin-Resistance <i>Staphylococcus aureus</i>
NCTC	National collection of Type Cultures
°C	Degree Celsius
PET	Petroleum ether
TLC	Thin Layer Chromatography
UCC	University of Cape Coast

UDS	University for Development Studies
UG	University of Ghana
UNESCO	United Nations Educational, Scientific and Cultural organization
WHO	World Health Organization



## CHAPTER ONE

### 1.0 INTRODUCTION

Antimicrobial resistance trends and their consequences for health and the economy are rapidly changing on the global scene. Drug resistance threatens the success of medical interventions and presents challenges for clinical, therapeutic and public health interventions. These challenges do not only have a local impact, but national and global implications (WHO, 2014a).

Studies have shown that medicinal plants and their derivatives can still cover the basic health needs in a resource poor settings (WHO, 1998). Plant extracts in the form of tincture, infusions, salves, creams and pills are used as part of a holistic treatment plan for addressing the underlying causes of infection (Kennedy *et al.*, 2009; Fabricant, 2009 and Behice, 2012).

One of the derivatives of plants is propolis which is a resin substance mainly collected by the honey bees and mixed with some pollen (Marcucci, 1995). Propolis has been identified as a good candidate for use in the prevention and treatment of bacteria, fungi, parasitic and viral infection (Kujumgiev *et al.*, 1999; Orsi *et al.*, 2005). The source of propolis is important, as its phytochemical content and bioactive characteristics vary by location, based on just what the bees in each area have available to them (Bankova *et al.*, 1992b). Studies have shown that propolis is relatively non-toxic and the use of it by man can be dated back since the ancient times (Ghisalberti, 1979; Burdock, 1998; and Jasprica *et al.*, 2007).

In Ghana, many products such as cough mixtures and herbal concoctions contain honey and propolis as part of their ingredients; nevertheless, there is no data in Ghana to demonstrate its antimicrobial properties (FLG, 2016).

Previous knowledge on propolis emanates from studies conducted in Eastern and Western Europe, China, Egypt and North America. Propolis is still applied in herbal medicines in western part of Africa (Krell, 1996), but very little is known scientifically about its antimicrobial properties (Morris, 2006).

### **1.1 Statement of problem**

Globally, antimicrobial resistance has become an important public health threat and this has been attributable to misuse and wide-scale use of antibiotics antimicrobials. This has limited the therapeutic use of antibiotics to treat and control many types of diseases. The occurrence and spread of multidrug resistant bacteria have become a global problem (WHO, 2014b).

In Ghana, antimicrobial therapy constitutes a major form of treatment for infectious diseases. In a six month study on antimicrobial drug resistance across the southern, middle and northern sectors of Ghana, Opintan *et al.* (2015) observed that varied bacterial species demonstrated different levels of resistance to antimicrobial agents. For example, more than 70% of isolates were resistant to ampicillin, tetracycline, chloramphenicol, and cotrimoxazole. In addition, more than 50% of isolates were found to be resistant to third generation cephalosporins and fluoroquinolones. As a result, the therapeutic use of antimicrobials to treat bacterial diseases is limited.

The problem of antibiotic resistance is further exacerbated by the fact that development of new antibiotics has been unfruitful in the last decade (WHO, 2014b). This depicts a serious situation and highlights the need for alternative methods of dealing with bacterial diseases, which is the focus of this thesis.

### **1.2 Justification**

The study for antimicrobials in bee propolis represent a new area for addressing the antimicrobial resistance problem. The diversity of bioactive constituents available in propolis still remains

largely uninvestigated worldwide. The ability of crude propolis extract to potentiate in- effective antibiotics has not been done although potentiation studies are effective ways of reviving antimicrobial agents to which resistant bacteria were previously susceptible to (Lacmata *et al.*, 2012). Although, some studies on antimicrobial activities of propolis collected in Europe, America, Egypt and Asia revealed antimicrobial activities, there is no published data on the antimicrobial properties and the phytochemical characteristics of Ghanaian propolis despite the emergence and spread of multidrug resistant bacteria in Ghana.

Hence this study seeks to screen for antimicrobial effect of crude propolis extracts on selected multiple drug resistant bacteria.

### **1.3 Hypothesis**

The research hypothesizes that Ghanaian propolis sampled from some geographical locations have antimicrobial properties against multidrug resistant bacteria.

### **1.4 Aim**

This work aimed at evaluating the antimicrobial activity of crude propolis extracts from the ten regions of Ghana against selected multiple drug resistant bacteria.

### **1.5 Specific objectives**

- i. To screen for antimicrobial effect of crude propolis extracts from ten regions of Ghana against selected multidrug resistant pathogens.
- ii. To determine the active fraction of the crude propolis extract.
- iii. To determine the MIC and MBC of active fractions.
- iv. To screen for phytochemicals and characterize the active fractions using HPLC

## CHAPTER TWO

### 2.0 REVIEW OF LITERATURE

#### 2.1 Propolis

Propolis (bee glue) is a resin type substance made up of a complex mixture of several substances mainly collected by the honey bee, *Apis mellifera* from tree sap and leaf buds, exudate and mixed with pollen. Propolis is hard and brittle when dried. However, it becomes soft pliable, gummy and very sticky when heated (Swamy *et al.*, 2013).

The increasing use of propolis in modern traditional medicine have attracted researchers to investigate its chemical composition and antimicrobial properties (Amoros *et al.*, 1992b; Sforcin *et al.*, 2005; Huang *et al.*, 2014).

#### 2.2 Origin and sources of propolis

Plants from which honeybees collect nectar, pollen and exudates (resins, mucilages, gums, lattices) are normally referred by scientific names. However, “propolis” is a bee-oriented term which does not require a botanical name (Crane, 1988).

According to Marcucci (1995), compounds in raw propolis emanate from plant exudate collected by bees, secreted substances from bee metabolism and materials which are introduced during propolis production.

The main source of propolis in temperate countries like New Zealand, North America, Asia and Europe has been confirmed to be the bud exudate of the *Populus* tree species (Marcucci *et al.*, 1998).

Popova *et al.* (2013) identified the mango (*Mangifera indica*) and the neem (*Azadiracta indica*) as the origin of Romanian propolis. *Macaranga* plants have also been found to be the source of

Japanese propolis. Mediterranean and Maltese propolis found to originate from *Cupressus* and *Pinus* plants from Greek propolis. *Acacia paradoxa*, *Araucaria* spp. and *citrus* spp. have also been identified to be origin of Australian, Brazilian and New Zealand propolis respectively (Popova *et al.*, 2009; Melliou and Chinou, 2004; Tran *et al.*, 2012; Bankova *et al.*, 1999; Marcucci *et al.*, 1998; Markham *et al.*, 1996).

In tropical countries, bees use other plant origins for propolis production (Silvana *et al.*, 2009). However, Christov *et al.*, (1998) reported that poplar tree species are widely distributed in Egypt especially at the collection site of propolis. In Tunisia, Martos *et al.* (1997) indicated that the leaf exudate of *Cistus* species is the source of Tunisian propolis.

For propolis to be chemically standardized, the knowledge of plant from which the propolis was produced is very significant (Popravko, 1978). Bee glue can easily be characterized using gas chromatography (GC) thin layer chromatography or high performance liquid chromatography and then compared with its plant source.

There are two types of bees and these are the honeybee (*Apis mellifera*) and the stingless bee (*Melipona scutellaris*). Studies have shown that propolis produced from different bee species influence the antibacterial activity of propolis collected from the same location. Propolis produced by *Melipona* species has better antimicrobial activity than that of *Mellifera* species (Silici and Kutluca, 2005).

### **2.3 Uses and antibacterial properties of propolis**

The phytochemical investigations based on ethnomedicinal and ethnopharmacological information is considered as the right procedure in the discovery of new antimicrobial agents from plants and their products (Duraipandiya *et al.*, 2006).

Propolis is been used for treating pharyngitis, burns, stomach disorder and wounds in many countries (Burdock, 1998). It has also been used in Greece for treatment of abscesses and in Egypt for the embalming of mummies (Pletcher, 2014).

In Ghana, propolis products are sold over-the-counter as cough mixtures, hair pomade, facial cleanser and dermatological creams against skin fungi (FLG, 2016).

Studies have demonstrated that propolis could be effective against aerobic and anaerobic bacteria, as well as cocci and rod-shaped Gram negative and Gram positive bacteria (Bogdanov, 2016). Mirzoeva *et al.*, (1997), Pepeljnjak and Kosale, (2004) have also reported that the antibacterial effect of propolis is bactericidal but activity decreases with storage period.

Study by Amoros *et al.* (1992b) reveals that propolis extract is active against an acyclovir-resistant mutant of HSV-1, adenovirus type 2, vesicular stomatitis virus, poliovirus and HSV-1. Multiple phytochemicals were observed to be more effective against viruses than single chemicals. This explains why crude propolis extract is more effective than its fractions (Amoros *et al.*, 1992a).

Propolis have also been reported to have fungicidal effects against bread and juice spoilage fungi such as *Rhizopus stolonifer* and *Aspergillus species*. Comparisons between propolis extract and nine anti-fungal drugs indicates that propolis extract was as effective as antifungal preparations. In term of synergy, propolis and propylene glycol gave better results against *Scopulariopsis breveicaulis* than the drug alone. Other studies have shown that propolis has an activity against *Candida species*, *Giardia species* and malaria parasites (Wagh, 2013; De Castro *et al.*, 2011; Koc *et al.*, 2007; Miller-Clere *et al.*, 1987).

Propolis is effective against many of intestinal parasites such as *S. mansoni*, *Giardia duodenalis* trophozoites and *Trypanosomas brucei* (Issa, 2007; Freitas *et al.*, 2006).

Clinical study on mice indicates that propolis extract have antioxidant effect and this could be due to its radical scavenging ability which was also influenced by high flavonoids content (Krol *et al.*, 1990).

Because of its antimicrobial effects propolis has been used in pharmaceutical and cosmetic industry (Dobrowolski *et al.*, 1991). It has been found to have effect against gingivitis, stomatitis and cheilitis when used in toothpastes. It is also used as facial cleanser, lotions and ointiments (Marcucci, 1995; Bjorkner, 1994).

Propolis is used as an adjuvant in most vaccine. It stimulates immune response and therefore making vaccines more effective. In an *in vivo* study in mice to determine the effectiveness of swine Suid herpes virus type1 (SuHV-1) vaccine, inclusion of green propolis extract revealed an improved response to cellular immune system against SuHV-1 (Fischer *et al.*, 2007).

Clinical studies on the treatment of a variety of eye diseases have been reported to be effective against keratitis, conjunctivitis and blepharitis (Asafova *et al.*, 2001). 0.3 - 1 % aqueous propolis solutions can be applied. Based on specific propolis fractions, special propolis preparations have been developed, especially and adapted for eye applications (Tikhonov *et al.*, 1998).

#### **2.4 Safety of bee propolis**

Propolis is relatively non-toxic and will not cause side effect for most people when ingested or applied topically (Wander, 1995).

Propolis has been found to have synergistic effect with some antibiotics. However, propolis tincture may cause nausea and vomiting when taking with metronidazole or disulfiram because of its high alcohol content (Eshwar-Shruthi, 2012).

## **2.5 Composition and phytochemical characteristics of propolis**

### **2.5.1 Composition of Propolis**

The constituents of propolis depend on the geographical region, collecting time and plant source and may vary, resulting in differences in intensity of antimicrobial activities (Mohammadzadeh *et al.*, 2007; Katircioglu and Mercan, 2006). These may result in differences in resin colour, which range from dark brown to yellow or transparent (Wagh, 2013). Koo *et al.* (1998) also reported that propolis composition may also vary in terms of quality and quantity, within the same country from region to region, from vegetation to vegetation, from season to season and from hive to hive.

### **2.5.2 Phytochemical characteristics of propolis**

Phytochemicals have been defined as non-nutritive plant chemicals that have health-promoting properties. They are natural bioactive compounds that can protect man from several diseases and help in risk reduction for a variety of chronic conditions (Russo, 2007). Phytochemicals previously with unidentified medicinal applications, have been extensively studied for use as a source of medicine (Krishnaraju *et al.*, 2005).

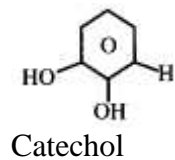
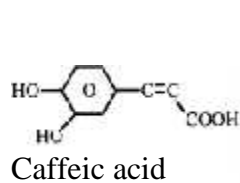
According to Christov *et al.* (1998), preliminary constituents and antimicrobial activities of African propolis carried out in Egypt reveals the presence of phenolic acids, esters of caffeic acid and flavonoids.

With the advent of separation techniques like the HPLC and TLC, more compounds have been isolated from plants and plant products. The development of identification techniques such as

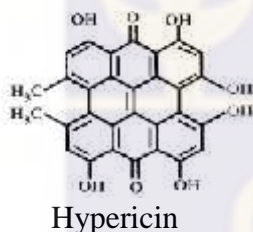
NMR and GC-MS, many bioactive compounds have been identified in propolis. These phytochemicals includes flavonoids, terpenes, phenols and esters (Huang *et al.*, 2014; Alencar *et al.*, 2007; Campo *et al.*, 2008; Maciejewicz *et al.*, 2001).

The figure below illustrates some common classes of bioactive compounds and their subclasses.

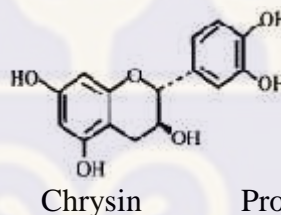
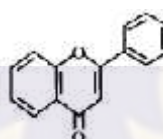
**Simple phenols and phenolic acids**



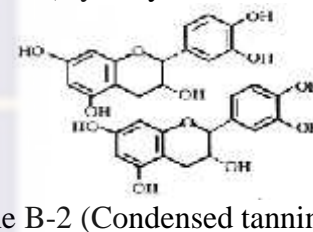
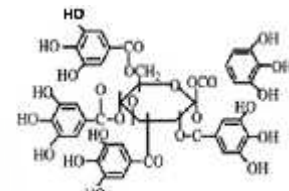
**Quinones and flavonoids**



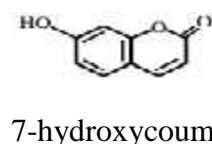
**Flavones**



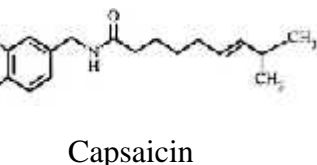
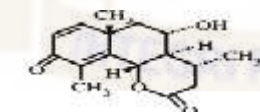
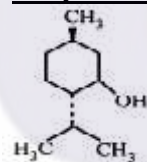
**Tannins**



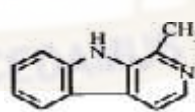
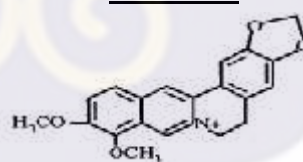
**Coumarins**



**Terpenoids**



**Alkaloids**



**Sugars**

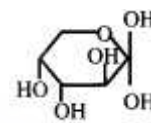


Figure 1: Structure of antimicrobial compounds in plants. Source: Cowan (1999); Silva and Fernandes (2010).

## 2.6 Antimicrobial agents and bacteria resistance

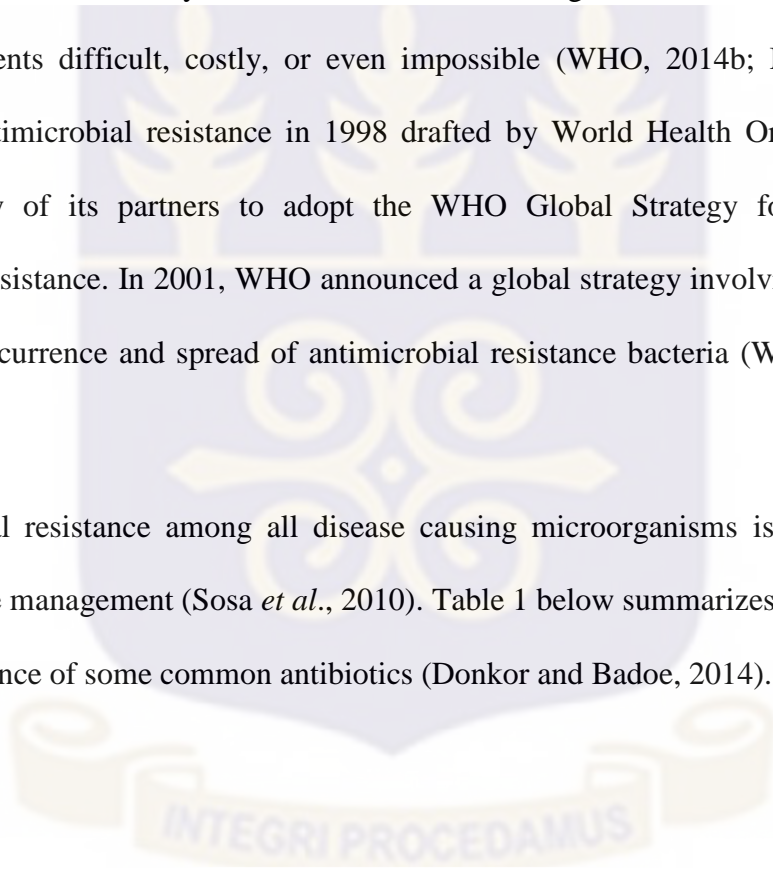
Antimicrobials are chemicals that can kill or inhibit the growth of microorganisms. They are a large group of drugs which consists of anti-viral, anti-fungal anti-parasitic and antibacterial (Agbor *et al.*, 2011). The mechanisms of antimicrobial activities are categorized based on their effectiveness on the bacteria (Jawetz *et al.*, 2010; Agbor *et al.*, 2011). One of the mechanisms of antimicrobial activity is inhibition of cell membrane synthesis. The cytoplasm of all living cells is surrounded by the plasma membrane which is selectively permeable. Cationic antibiotics bind to the cell membrane interfering with the permeability of the cell membrane resulting in cell loss of cellular content and death (Jawetz *et al.*, 2010; Agbor *et al.*, 2011). Another mechanism is the inhibition of protein synthesis. Certain antibiotics inhibit protein synthesis by interfering with the translation in the cell. Examples of these antibiotics includes the Aminoglycoisdes, Tetracyclines, Erythromycins, Lincomycins, and Chloramphenicol (Ochei *et al.*, 2007; Agbor *et al.*, 2011).

Furthermore, to prevent growth and multiplication of the bacterium, some antibiotics inhibit nucleic acid synthesis of bacteria. An example of this antibiotic is Rifampin. It is able to inhibit the growth of bacteria by interfering with activities of DNA dependent RNA polymerase of bacteria resulting in chromosomal mutation and thereby inhibiting bacterial RNA polymerase (Jawetz *et al.*, 2010; Agbor *et al.*, 2011). The tough external layer of bacteria cell wall maintains the shape, size and high internal osmotic pressure of microorganisms. Inhibition of the cell wall formation prevents the cross linking of the polysaccharide chains in the polypeptidoglycan layer of the cell wall leading to cell lysis and death. Examples are Penicillin, Cephalosporins, Vacomycin etc. (Agbor *et al.*, 2011). Finally, the metabolic pathway of sulfonamides and

trimethoprim can be blocked, resulting in interference with folate synthesis, which is required as a cofactor in the biosynthesis of nucleotides, the building blocks of DNA and RNA.

In 1928, Sir Alexander Fleming accidentally discovered penicillin. In the subsequent years, much effort have been made to discover more antibiotics in order to reduce the effect of disease in man (Jorgensen and Turnidge, 1999). However, overuse or abuse of antimicrobial drug in clinical settings, has resulted drug resistance. (Houten and Kumpen, 2000). Antimicrobial drug resistance is defined as reduction of activity of an antimicrobial in treating a disease or condition, making the recovery of patients difficult, costly, or even impossible (WHO, 2014b; MeSH, 2005). The resolution on antimicrobial resistance in 1998 drafted by World Health Organization (WHO) challenged many of its partners to adopt the WHO Global Strategy for Containment of Antimicrobial Resistance. In 2001, WHO announced a global strategy involving all stakeholders to combat the occurrence and spread of antimicrobial resistance bacteria (WHO, 2014b; WHO 2001).

The antimicrobial resistance among all disease causing microorganisms is a global threat to infectious disease management (Sosa *et al.*, 2010). Table 1 below summarizes the mechanisms of action and resistance of some common antibiotics (Donkor and Badoe, 2014).



**Table 1: Mechanism of action and resistance of some common antibiotics**

<b>Antibiotic</b>	<b>Mechanism of action</b>	<b>Mechanism of resistance</b>	<b>Reference</b>
<b>Macrolides</b>	Blocks exit of the growing peptide chain in protein synthesis.	Methylation of 23S rRNA by erm gene; drug efflux mediated by mefF or mefA genes.	Wise, 1999; Walker <i>et al.</i> , 2004; Weisblum, 1995; Leclercq and Courylin, 2002.
<b>Quinolones</b>	Inhibits DNA gyrase.	Mutations in gyrA or parC; drug efflux mediated by pmrA genes.	Walker <i>et al.</i> , 2004; Fukuda and Hiramatsu 1999), Gill <i>et al.</i> , 1999.
<b>Tetracycline</b>	Inhibits codon-anticodon interaction in protein synthesis.	tetM or tetO ribosomal protection protein.	Wise, 1999; Newton, 1995; Connell <i>et al.</i> , 2003.
<b>Chloramphenicol</b>	Inhibits peptidyl transferase.	Inactivation of chloramphenicol by cat gene.	Wise, 1999; Newton, 1995; Nikaido, 2009.
<b>Trimethoprim</b>	Inhibits dihydrofolate reductase.	Mutation in dihydrofolate reductase gene.	Wise, 1999; Newton, 1995; Nikaido, 2009.
<b>Sulfamethoxazole</b>	Inhibits dihydropteroate reductase.	Mutation in dihydropteroate reductase gene	Wise, 1999; Newton, 1995; Nikaido, 2009.
<b>Rifampin</b>	Inhibits bacterial RNA polymerase.	Mutation in RNA polymerase gene.	Wise, 1999; Newton, 1995; Nikaido, 2009; Padayachee and Klugman, 1999.

Source: Donkor and Badoe (2014)

### **2.6.1 Antimicrobial susceptibility testing methods**

Antimicrobial susceptibility testing (AST) is a measure of whether or not a microorganism can grow when it is exposed to a variety of antimicrobials in a laboratory test (Wheat, 2001). In order to treat and control infectious diseases, especially those caused by drug resistant bacteria, it is very important to carry out antimicrobial susceptibility testing so that an effective antimicrobial drug can be prescribed (Agbor *et al.*, 2011). AST methods can either be qualitative or quantitative (Collier, 1995).

### **2.6.2 Qualitative Methods**

Qualitative antimicrobial susceptibility testing is used to indicate whether a microorganism is susceptible, intermediate or resistance to a given antimicrobial drug.

#### **(i) Disc diffusion test**

The disc diffusion method is the most widely used technique because it is convenient, flexible, and easy to perform (Jones, 1992).

#### **(a) Kirby-Bauer test**

This method involves inoculation of the Mueller-Hinton agar plate surface with an adjusted inoculum and then applying a 6 mm disc of paper which have been impregnated with known amount of antibiotic. Plates are incubated at 35 – 37 °C for 16 – 18 hours

The organism is said to be inhibited or killed by the concentration of the antibiotic if there is no grow within the region surrounding the disc. This region is called the zone of inhibition. The zone sizes are measured and compared with standard results chart to show weather the organism is susceptible, resistant or intermediate (Agbor *et al.*, 2011).

**(b) Stoke's comparative method**

The Stoke's method is similar to the Kirby Bauer except that test organisms and its control are tested against the same discs on the same plate. The significant of this method is that any condition that may affect the accuracy of the results is eliminated (Agbor *et al.*, 2011).

**(c) Agar well diffusion method**

Agar well diffusion method is recommended for testing the antimicrobial activity of plants or microbial extracts (CLSI, 2016; Magaldi *et al.*, 2004). The difference between this method and the Kirby-Bauer method is that a hole of diameter of 6 to 8 mm diameter is punched aseptically with sterile cork borer and 20-100 $\mu$ l of desired concentration of the plant extract introduced into the well. The plates are then incubated at 35 – 37 °C for 16 – 18 hours (Balouiri *et al.*, 2016).

**2.6.3 Quantitative Methods**

The technique is used to quantify the amount of antimicrobial agent required to inhibit or to kill a given microorganism. It can be used to measure the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of an antimicrobial agent (Cheesbrough, 2000). To achieve the dilution technique, the following methods are used.

**(ii) Broth dilution methods**

**(a) Macro broth (Tube) dilution test**

The method involves the use of two-fold serial dilution of an antibiotic of the plant extract and a fixed volume of inoculum mixed with the various dilutions. The recommended medium is the Mueller-Hinton broth. For bacterial like Streptococci, Todd – hewit broth can be used (Ochei and Kolhakfor, 2007).

The presence or absence of growth is assessed and the minimum inhibitory concentration (MIC) of the agent is defined as the lowest concentration which inhibits the growth as judged by the naked eye (Woods and Washington, 1995).

**(b) Micro broth dilution tests**

The difference between this method and the macro broth dilution is the volume used in series of wells in a microtitre plate. A total broth volume of about 0.05 to 0.1 ml (50 to 100µl) is used in the microtitre plate (Agbor *et al.*, 2011).

**(c) Agar dilution method**

The procedure is the same as the broth dilution method except that different concentration of the antimicrobial agent are mixed with Mueller – Hinton agar plates. A standardized amount of organism is inoculated. Following 16–18 hours of incubation, the lowest concentration of the agent which has no microbial growth is the MIC (Ochei and Kolhakfor, 2007).

**(d) E-test**

E-test (epsilometer test) kits are commercial available product and is based on the principle of antimicrobial concentration gradient established in an agar medium as a means of determining the susceptibility of an organism (Citron *et al.*, 1991). It consists of plastic strips impregnated with different concentration of antibiotic. Depending on the strip used, the concentration of the antibiotics ranges from 0.002 to 32 mg/ml or 0.064 to 1024mg/ml. After applying to a seeded Mueller – Hinton agar plates and overnight incubation, MICs are identified by the minimum concentration value at the end of the elliptical zone (Baker *et al.*, 1991)

#### **2.6.4. Automated Susceptibility Methods**

The automated susceptibility methods utilize broth culture in an automation machine. Faster results are obtained from this method than conventional agar based tests. It requires less labour and has better reproducibility compared with the manual methods (Ferraro and Jorgensen, 1999).

#### **2.6.5 Factors affecting results of antimicrobial susceptibility tests**

Many factors can affect results of antimicrobial activity in vitro (Jawetz *et al.*, 2010). These factors include pH, depth of medium, inoculum size, medium composition, incubation period and conditions, disc storage, and moisture.

Certain antimicrobial agents (nitrofurantoin) work best at acidic pH range whilst others like aminoglycosides and sulfonamides do better in alkaline pH range (Jawetz *et al.*, 2010).

The depth of medium can also affect the zone of inhibition. The deeper the medium, the longer the antimicrobial diffuses and smaller zones will appear. If the depth of the medium is shallow the zones will appear to be large. The recommended depth of medium for antimicrobial susceptibility testing is 4 ml i.e. approximately 25 ml in 90 mm Petri dish for well diffusion method (Barry *et al.*, 1973).

In addition, the size of inoculum and the composition of the medium plays an important role in determining the susceptibility or resistivity of bacteria. This is because larger population of bacteria tend to resist the action of antibiotics than smaller population. “Sodium polyanetholsulfonate (in blood culture media) and other anionic detergents inhibit aminoglycosides; Para-amino benzoic acid (PABA) in tissue extracts interferes with sulfonamides; Serum proteins can also bind with penicillins in varying degrees, ranging from 40% for methicillin to 98% for dicloxacillin whilst NaCl in a medium facilitate detection of methicillin

resistance in *S. aureus*". In the same vein, "Incubation time can also affect the zone of inhibition. In many instances, microorganisms are not killed but only inhibited upon short exposure to antimicrobial agents. The longer incubation continues, the greater the chance for resistant mutants to emerge or for the least susceptible members of the antimicrobial population to begin multiplying as the drug deteriorates" (Jawetz *et al.*, 2010).

In a separate study, Lalitha, (2005) reported that "medium surface moisture can mar the beauty of zone of inhibition. Because of this, it is recommended that stored plates be dried in a laminar flow or in an incubator at 37 °C just before use".



## CHAPTER THREE

### 3.0. MATERIALS AND METHODS

#### 3.1 Study area, design, sampling and sample preparation

Study design was experimental analytical study carried out on propolis from the ten (10) regions of Ghana. The human population of Ghana is about twenty-seven million (27,000,000) and is considered a middle income country with land area of 238,535 km<sup>2</sup>. The ten region Upper West, Upper East, Northern, Brong Ahafo, Ashanti, Eastern, Western, Greater Accra, Volta And Central Region.



Figure 2: Map of Ghana

The three northern region (Upper East, Upper West and Northern region) are characterized by scattered trees like *Isobertia doka*, *Isobertia dalzielii*, *Daniella* spp., mahogany (*Khaya neqalensis*), *Khaya* spp., ebony (*Diospyros mespilliformis*) *Andropogon* and *Cymbopogon* spp. and *forbes* as well as economic trees such as cashew, dawadawa (*Parkia clappertoniana*) and shea trees (*Vitellaria paradoxa*) (Amoako, 2012). The soil type of these regions are generally gravel

with loamy soil in water logged areas. The regions have a single rainy season with relatively low rain fall ranging from 700 to 1050 mm per annum and dry season beginning from November to March (MOFA, 2015).

The main soil type of Brong Ahafo (BA) region are the forest ochrosols covering the South-Western part of the region; the savanna ochrosols which stretches from wide from the Western and narrows towards the East and the ground water ochrosols laterite (MFOA, 2015). Major staple crops grown in this region includes cassava, yam, cocoyam and plantain. Economic crops such as cocoa, mango, oil palm, coffee, cashew and cotton), Vegetables and fruits are also available in BA (MOFA, 2015; Kyegabador, 2012). The annual average rainfall ranged from 1088 to 1197 mm (SMA, 2008).

The Ashanti region falls within the moist semi-deciduous South-East Ecological Zone of Ghana (Quaye, 2015). The rich loamy soil promotes agriculture in the periphery of the region. Predominant tree species found are *Ceiba pentandra*, Triplochlon, neem (*Azadirachta indica*), cocoa (*Theobroma cacao*), oil palm, coconut and *Celtis* species (KMA, 2006). Food crops grown include maize, plantain, cocoyam, cassava, yam groundnuts and vegetables and the soil type is loamy (KMA, 2006). The region also has two rainfall maxima, beginning from June (214.3 mm) and September (165.2 mm).

The combination of forest and savannah type of soils in the Eastern region have made it suitable for cultivation of different types of crops. Cash crops such as cocoa, cola-nuts, citrus, oil palm and staple food crops like cassava, yam, cocoyam, maize, rice and vegetables are the major crops grown in this region (MOFA, 2015). The region is characterized by double maxima rainfall in June and October (MOLG, 2006).

The Greater Accra region has three broad vegetation zones and these are the shrub land, the grassland and the coastal lands. The grassland consists of dense clusters of small trees and shrubs, which grow, to an average height of five meters. Neem, mango, cassias, avocados, and palms are prominent trees on the Accra landscape. Most of the open spaces in Accra are used for the cultivation of food crops like corn, okra, tomatoes and other vegetables (MOFA, 2006).

Western is the wettest part of Ghana, with rainfall averaging 1,600 mm per annum. The region has two major rainy seasons beginning from May-July and ends in September-October (MOFA, 2006). It is the largest producer of cocoa and timber and rubber in Ghana. Oil Palm, neem, coconut, coffee etc. are also found in this region (WRSDF, 2012).

The soils of Central region is generally laterite, which may be derived from the weathered granite. In the valleys and swampy areas, fine sandy deposits occur extensively. The double maxima rainfall pattern from November (750mm) to January (1000mm) and has made it possible for food crops like rice, maize, cassava, cocoyam and cash crop such as cotton, coffee, cashew, tobacco and tomato to be cultivated in the region. Perennial trees found in this region includes mango (*magnifera indca*, neem (*azadirachta indica*), velvet tamarind (*dialium guineense*), coconut (*Cocos nucifera*) (MOFA, 2015; CCMA, 2015).

Volta region has a savannah ochrosols, sandy coastal soils, tropical grey earth and regolithtic groundwater laterites, topohydric and luthochronic earth as major soil type and they range from heavy clay to sandy loamy (MOFA, 2015). The average mean rainfall ranges from 513.9 to 1099.88 mm which start from March and end in July.

About 100 g of propolis samples were collected from selected commercial beekeepers from the ten (10) regions of Ghana and placed into plastic bags. Samples were collected between October 2015 and March 2016.

Sample preparation was done in accordance to the work of Fabricant and Fansworth, (2001) and Burdock (1998). Propolis was collected by scraping with a knife. The collected propolis was sorted to remove pieces of wood, embalmed insects and other animals. The samples were then air dried in a stainless tray under shed and then pulverised.

### **3.2 Cold Organic extraction**

Chemicals used for propolis extraction include ethyl acetate, chloroform, and petroleum ether. Dimethyl sulphoxide (DMSO) was used as a diluent. All chemicals and solvents used in this experiment were of analytical grade and were purchased from British drug house (BDH) and Fruka.

Preparation of crude propolis extract and preliminary susceptibility testing were carried out at the chemistry and microbiology laboratory of Accra Technical University (ATU).

30 g of each sample was dissolved in chloroform, ethyl acetate and petroleum ether and kept for 72 hours and filtered twice with a Watmann No.1 filter paper. The filtrate was then evaporated in a rotary evaporator and dried in a dessicator.

### **3.3 Clinical and Control bacteria strains**

Archived clinical isolates and control bacteria strains were obtained from the School of Biomedical Sciences and Allied Health, University of Ghana medical School Microbiology Laboratory, Korle Bu. Clinically isolated multidrug resistant *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*) *Pseudomonas aeruginosa* (*P. aeruginosa*) and their corresponding multidrug resistant control

strains (methicillin resistant *Staphylococcus aureus* ATCC 25923, *E. coli* NCTC 13351 and *P. aeruginosa* ATCC 27853) were used in the study.

### **3.4 Antimicrobial Properties of Crude Propolis Extracts using Agar Well Diffusion Method**

#### **3.4.1 Preparation of the extract stock solution**

Preliminary screening reveals appreciable activities at the concentration of 64mg/ml and above. Therefore, 0.64 g of each crude propolis extract was transferred into 10 ml of 5% Dimethyl sulfoxide (DMSO, Daejung, Korea) to make 64 mg/ml of the extract.

#### **3.4.2 Preparation of inoculum by direct Colony Suspension Method and inoculation**

Inoculum was prepared by making saline suspension of isolated colonies selected from an 18 to 24 hour culture. Four (4) structurally similar colonies were picked with a sterile inoculating loop and transferred into a bijou bottle containing sterile saline solution. The suspension was adjusted to achieve a turbidity equivalent to a 0.5 McFarland standard, resulting in tube containing approximately  $1 \times 10^6$  Cfu/ml (CLSI, 2016).

Sterile plates of Mueller-Hinton agar were prepared by dispensing approximately 25 ml of the medium into 90 mm diameter Petri dishes such that approximately a depth of 4 mm is obtained. Suspensions of microorganisms containing  $10^6$  cells / ml were inoculated onto plate surface using a sterile swab sticks and with the aid of a cork borer, holes of 6 mm in diameter and 3 mm depth were punched in the agar plates. Each hole was filled with 100 $\mu$ l of the crude propolis extract. The diameters of growth inhibition zones around the holes were measured with the aid of a meter rule after incubation at 37 °C for 18 hours (CLSI, 2016; Dayie *et al.*, 2008; Asiedu-Gyekye *et al.*, 2005).

### **3.5 Determination of Active Fractions of the Crude Propolis Extract**

#### **3.5.1 Extraction of Fractions**

Extraction was carried out at the Centre for Plant Medicine Research (CPMR), Akuapem Mampong. Active fractions of the crude propolis extract were determined by extracting twice with chloroform, ethyl acetate and petroleum ether to separate polar, medium polar and non-polar chemical components respectively. Obtained fractions include Chloroform fraction of Northern (CH<sub>NR</sub>) Chloroform fraction of Eastern (CH<sub>ER</sub>), Chloroform fraction Ashanti (CH<sub>AS</sub>), Chloroform fraction of Volta region (CH<sub>VT</sub>), Ethyl acetate fraction of Northern region (EA<sub>NR</sub>), Ethyl acetate fraction of Eastern (EA<sub>ER</sub>), Ethyl acetate fraction of Ashanti (EA<sub>AS</sub>) Ethyl acetate fraction of Volta region (EA<sub>VT</sub>) Petroleum ether fraction of Northern (PET<sub>NR</sub>), Petroleum ether fraction of Eastern (PET<sub>ER</sub>), Petroleum ether fraction of Ashanti (PET<sub>AS</sub>) and Petroleum ether fraction of Volta (PET<sub>VT</sub>).

#### **3.5.2 Antimicrobial Susceptibility Testing for Active Fractions**

Antimicrobial susceptibility testing for the active fractions was done using agar well diffusion method as described in the screening of the propolis crude extract. Susceptibility testing of active fractions was determined at the Department of Medical Microbiology, University of Ghana Medical School, Korle-Bu; quantitative phytochemical constituents of the most active fraction was carried out at the Department of Clinical Pathology, Noguchi Memorial Institute, Legon-Accra.

### **3.6 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Active Fractions**

#### **3.6.1 Determination of MIC of Active fractions using macro dilution (tube) broth Method**

1 g of propolis fraction was transferred each into 10 ml of 5% Dimethyl sulfoxide (DMSO) to make 100 mg/ml of stock extract. The stock was serially diluted from 64 to 8, and then 0.1 to 0.01 mg/ml.

After 15 minutes of inoculum preparation, 1 ml of the adjusted inoculum was added to each tube containing 1 ml of propolis extract. The mixture was then inoculated into each of the 10 ml of sterile Mueller-Hinton broth tubes and labeled accordingly. The tubes were then incubated at 35-37 °C for 16 - 20 hours.

The lowest concentration of the extract that did not show visible growth of the test organism, as judged by the naked eye, was identified as the MIC.

#### **3.6.2 Determination of MBC of Active fractions**

MBC was determined from the MIC tests by sub-culturing from tubes that showed activities onto Mueller-Hinton agar plates that do not contain test agents. The lowest concentration of the extract that did not show visible growth of the test organism on Mueller-Hinton agar was identified as the MBC.

### **3.7 Determination of Phytochemical Constituents of Active Fraction**

These includes qualitative and quantitative phytochemical tests on the active fractions.

#### **3.7.1 Qualitative Phytochemical Test of active fractions**

Phytochemical screening for bioactive compounds was done using standard procedures described by Sofowora *et al.* (1993), Swamy *et al.* (2013) and Talla *et al.* (2014). Colour change was

physically observed and assigned present (+), present in excess (++) and absent (-) when there is no colour change.

To test for saponins, 1g of the propolis extract was mixed with 5 ml of distilled water, vigorously shaken and allowed to stand. The formation of stable foam indicates the presence of saponins.

Flavonoids were tested by mixing 1g of the extract with 0.5g fragments of magnesium ribbon. Followed by drops of concentrated hydrochloric acid. Development of a pink colour indicates the presence of flavonoids.

The third phytochemical, terpenoid, was determined by transferring 2 ml of chloroform to the active fraction in a test tube, shaken vigorously and then evaporated to dryness over water. 2 ml of concentrated sulphuric acid was added to the mixture and heated for about 2 minutes. Terpenoids said to be present if a greyish colour appears.

Furthermore, test for alkaloids was done by adding 1 ml of 1% of HCl to the active fraction in a test tube. The test tube was heated gently in a water bath and filtered with a filter paper. Few drops of Mayers and Wagner's reagents were added by the side of the test tube. A cloudy yellow colour confirms the presence of alkaloids.

Finally, phenols were tested by adding few drops of 2% iron (III) chloride ( $\text{FeCl}_3$ ) active propolis extract was put in a test tube and treated with a few drops of 2% of iron III chloride ( $\text{FeCl}_3$ ). Blue green or black coloration indicates the presence of phenols.

### **3.7.2 Phytochemical Analysis using HPLC and Chromatographic conditions**

High performance liquid chromatography (HPLC) analysis was conducted using the method described by Coneac *et al.* (2008). Chromatographic analysis for fingerprinting active fractions of crude propolis extract was conducted using HPLC (Agilent 1100 series), controlled by a computer

software equipped with DAD (diode array detector) G1315B, autosampler G1329A, column oven G1316A, pump G1311 and degasser G1322A.

All samples were filtered a Millipore membrane filter with pore size of 0.2  $\mu\text{m}$  and injected in duplicate.

Samples were eluted using a gradient mobile phase consisting of A: 0.1% phosphoric acid in double distilled water and B: HPLC grade methanol (table 8).

### **3.7.2.2 The gradient conditions**

Run Time – 40min, Flow Rate – 1ml/min, Column – C18 (250mm x4.6mm) 5 $\mu\text{m}$ , Column Oven – 40°C, Wavelength – 254nm and Injection Volume – 50  $\mu\text{l}$ .

### **3.8 Data analysis**

Data was entered into Microsoft Excel and IBM SPSS version 20 statistical package for all statistical analysis. Descriptive statistical analysis including mean zone of inhibitions generated for screening antimicrobial susceptibility testing of crude and active fractions of propolis extracts as well as frequencies and proportion of inhibited isolates. The minimum inhibitory concentration and minimum bactericidal concentrations values were presented in tables.

## CHAPTER FOUR

### 4.0 RESULTS

#### 4.1.1 Antimicrobial activity of crude and active fraction of propolis extracts

Figure 3 illustrates results of antimicrobial activity of crude propolis extracts on the selected bacteria isolates. Out of ten (10) regions, 64 mg/ml of crude propolis extracts from Northern (3), Eastern (5), Ashanti (8) and Volta region (10) showed appreciable zones of inhibitions against bacteria. *S. aureus* (clinical) and its control strain MRSA ATCC 25923 were found to be susceptible to NR, ER, AS and VT region crude propolis extracts with the average zone of inhibition ranging from 19 to 24 mm and 20 to 25 respectively.

*Pseudomonas aeruginosa* (clinical) and its control strain *Pseudomonas aeruginosa* ATCC 27853 were also susceptible to NR, ER, AS and VT region propolis extract. The average zone of inhibition ranges from 10 to 13 mm<sup>3</sup> for *Pseudomonas aeruginosa* (clinical) and 20 to 31 mm for *Pseudomonas aeruginosa* ATCC 27853 control strain.

*E. coli* (clinical) and its corresponding control strain ESBL *E. coli* (NCTC 13351) were however resistance to all extracts, except VT which was effective against ESBL *E. coli* NCTC 13351 with the average zone of inhibition 20 mm.

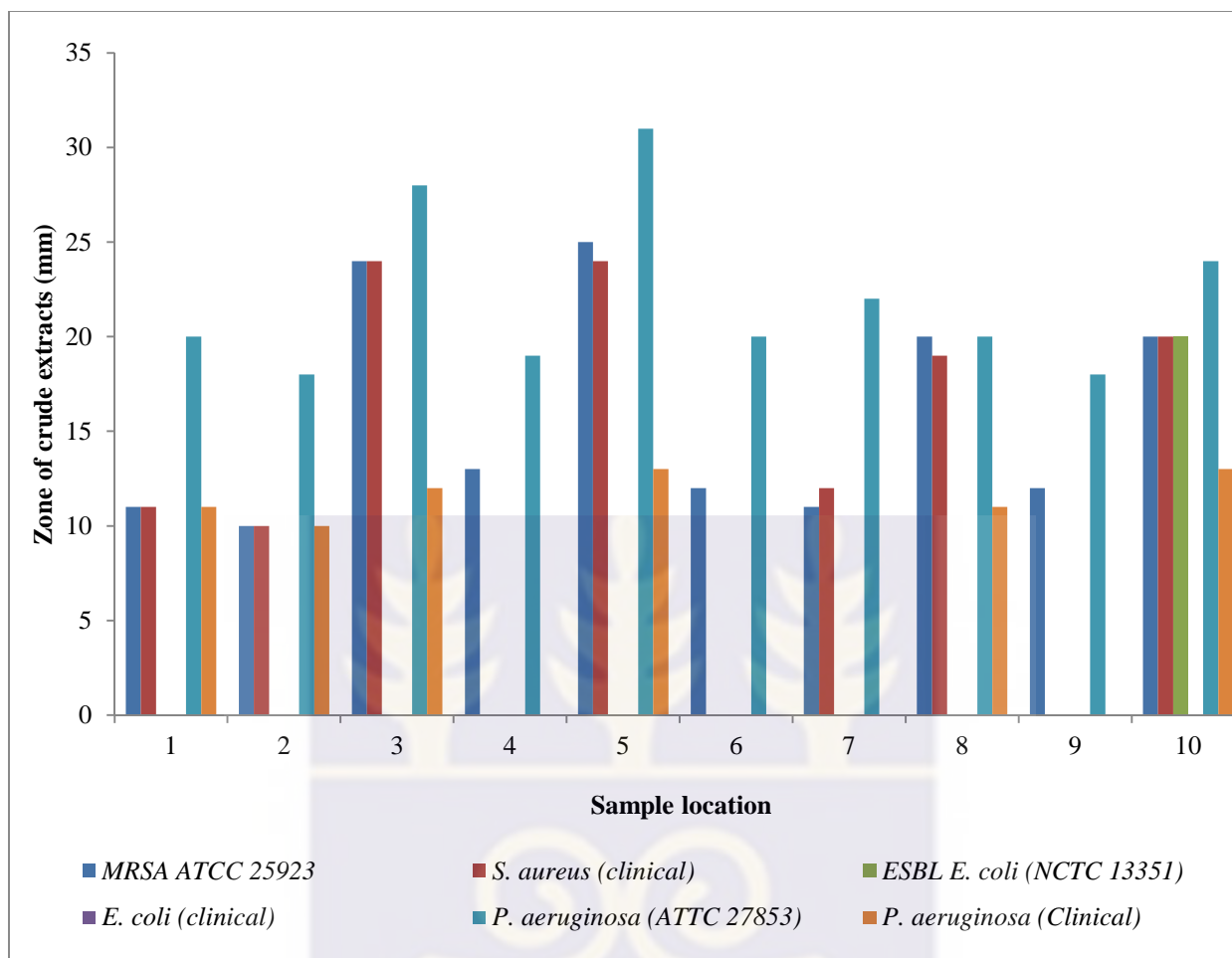


Figure 3: Zone of Crude Propolis Extracts using Agar Well Diffusion Method

**Key:** 1 – Upper West, 2 – Upper East, 3 – Northern, 4 – Brong Ahafo, 5 – Eastern, 6 – Greater Accra, 7 – Western, 8 – Ashanti, 9 – Central and 10 – Volta region.

The antimicrobial susceptibility testing of twelve (12) fractions (CH<sub>NR</sub>, CH<sub>ER</sub>, CH<sub>AS</sub>, CH<sub>VT</sub>, EA<sub>NR</sub>, EA<sub>ER</sub>, EA<sub>AS</sub>, EA<sub>VT</sub>, PET<sub>NR</sub>, PET<sub>ER</sub>, PET<sub>AS</sub> and PET<sub>VT</sub>) was also carried out using the agar well diffusion method to determine the active fraction. Figure 4 shows the average zone of inhibitions of the various fractions (figure 4).

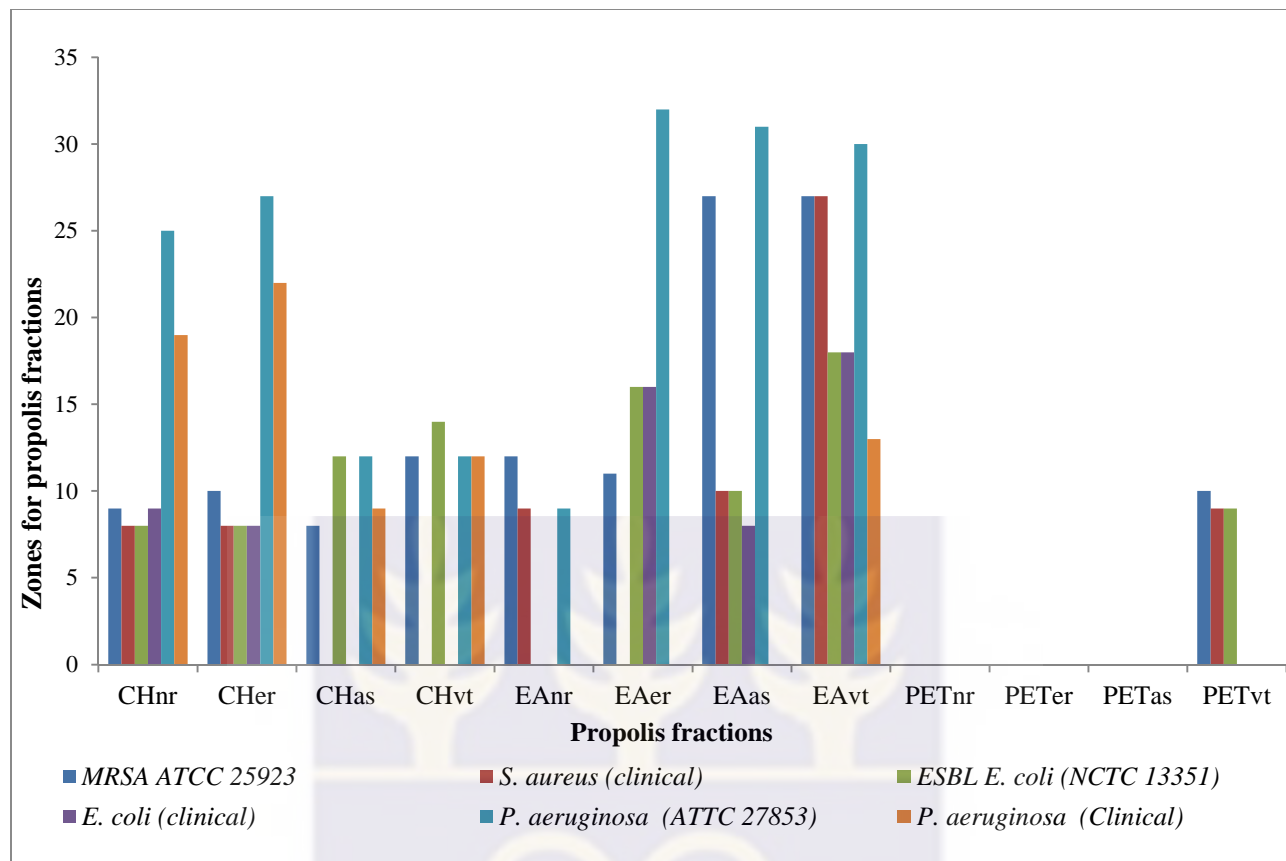


Figure 4: Zone sizes of Fractions

Key: CH<sub>NR</sub> – Chloroform fraction of Northern; CH<sub>ER</sub> – Chloroform fraction of Eastern; CH<sub>AS</sub> – Chloroform fraction Ashanti; CH<sub>VT</sub> – Chloroform fraction of Volta region;

EA<sub>NR</sub> – Ethyl acetate fraction of Northern region; EA<sub>ER</sub> – Ethyl acetate fraction of Eastern; EA<sub>AS</sub> – Ethyl acetate fraction of Ashanti; EA<sub>VT</sub> – Ethyl acetate fraction of Volta region.

PET<sub>NR</sub> – Petroleum ether fraction of Northern; PET<sub>ER</sub> – Petroleum ether fraction of Eastern; PET<sub>AS</sub> – Petroleum ether fraction of Ashanti and PET<sub>VT</sub> – Petroleum ether fraction of Volta.

#### 4.2 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the Active Propolis Fractions

*S. aureus* (clinical) was found to be susceptible to two (2) of the fractions (chloroform fraction of Northern region and ethyl acetate fraction of Volta) with MICs 56 and 24 mg/ml, and MBCs 64 and 32 mg/ml respectively whilst its control strain MRSA ATCC 25923 was susceptible to four (4) of the fractions (chloroform fraction of Eastern region, ethyl acetate fraction of Eastern region,

ethyl acetate fraction of Volta region and ethyl acetate fraction of Ashanti region) with MICs and MBCs ranging from 24 to 64 mg/ml and 100 mg/ml (Table 2) .

EA<sub>VT</sub> was found to have broad spectrum inhibitory and bactericidal activity against all six (6) test isolates (*S. aureus*, MRSA ATCC 25923, *E. coli*, ESBL *E. coli* NCTC 13351, *P. aeruginosa* and *P. aeruginosa* ATTC 27853). The MICs and MBCs of the EA<sub>VT</sub> fraction on the 6 test isolates ranged from 24 mg/ml to 40mg/ ml (Table 2).

At a concentration of 64 mg/ml of CH<sub>NR</sub>, *E. coli* (clinical) and its control strain ESBL *E. coli* (NCTC 13351) were inhibited. Bactericidal activity of CH<sub>NR</sub> and EA<sub>VT</sub> fractions was however observed at the concentration of 100 mg/ml (Table 2).

**Table 2: The MIC and MBC in milligram per milliliter (mg/ml) of the active fractions**

Fractions	MIC (MBC) (mg/ml)					
	MRSA ATCC 25923	<i>S. aureus</i> (clinical)	ESBL <i>E. coli</i> (NCTC 13351)	<i>E. coli</i> (clinical)	<i>P. aeruginosa</i> (ATTC 27853)	<i>P. aeruginosa</i> (Clinical)
CH <sub>NR</sub>	100 (100)	56 (64)	64 (100)	64 (100)	40 (40)	24 (40)
CH <sub>ER</sub>	64 (64)	100 (100)	-	-	32 (32)	32 (40)
EA <sub>ER</sub>	24 (32)	100 (100)	-	-	32 (32)	24 (24)
EA <sub>AS</sub>	32 (32)	100 (100)	-	-	24 (24)	100 (100)
EA <sub>VT</sub>	24 (24)	24 (32)	40 (40)	32 (40)	24 (24)	24 (24)
5% DMSO	-	-	-	-	-	-

– Not effective.

### 4.3 Phytochemical Constituents in the Active Fraction

The qualitative phytochemical analysis of active fractions of propolis extracts is shown in Table 3 below. Phenols, alkaloids and terpenoids were found in in all fractions.

Table 3: Qualitative Phytochemicals Test for active fractions

Fraction	Samples region	Phytochemical				
		Phenols	Flavonoids	Alkaloids	Saponins	Terpenoids
CH <sub>NR</sub>	Northern (NR)	++	-	++	-	+
CH <sub>ER</sub>	Eastern (ER)	+	-	+	+	+
EA <sub>ER</sub>	Eastern (ER)	++	+	++	+	+
EA <sub>AS</sub>	Ashanti (AS)	++	+	++	++	+
EA <sub>VT</sub>	Volta (VT)	++	+	++	++	+

Key: - = Absence; + = Presence and ++ = Present in excess (Talla *et al.*, 2014).

Figure 5 below displays the chromatograms of ethyl acetate fraction of Volta region. Two major peaks were observed at the retention time 27, 29 and 30 and 35.5.

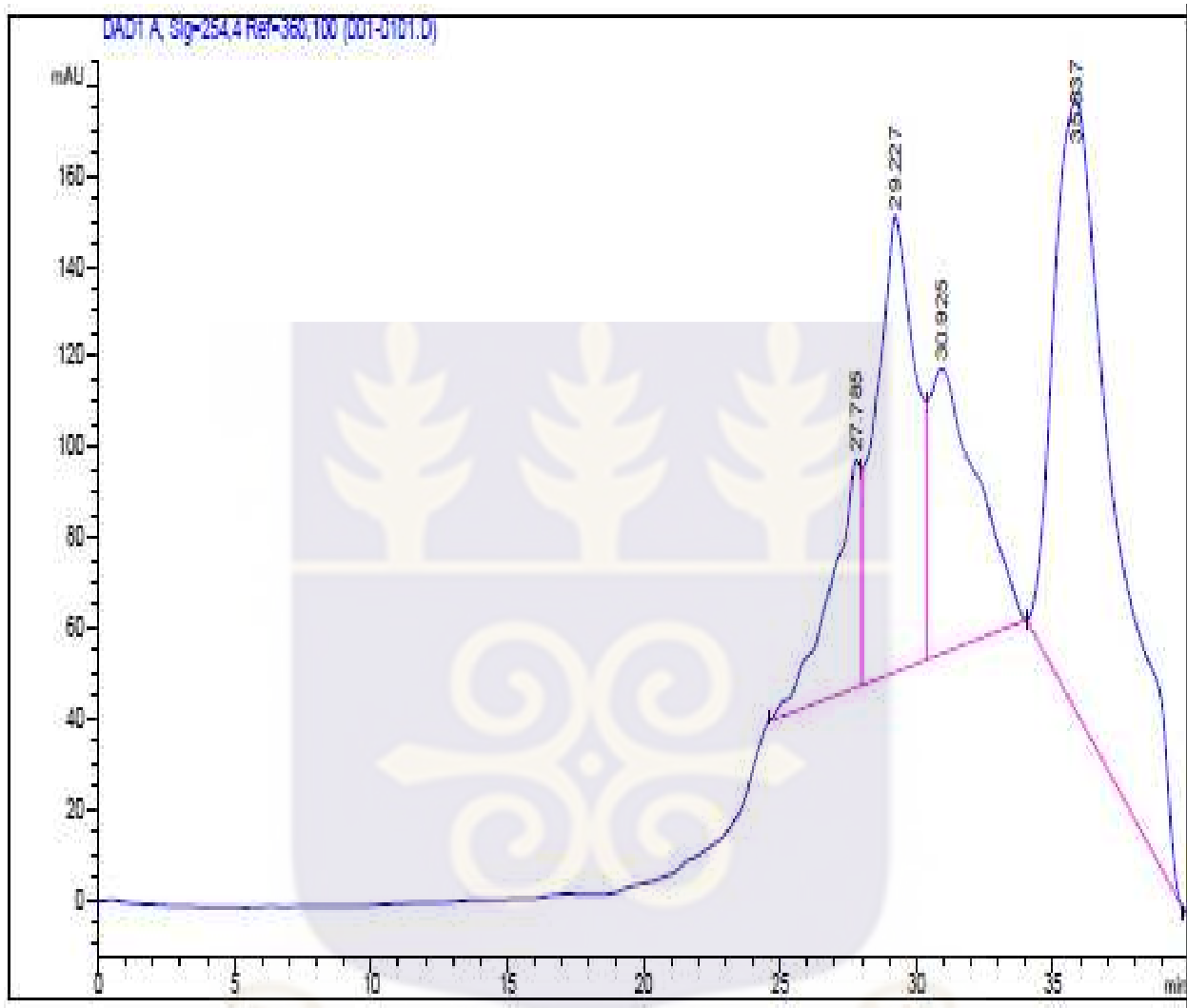


Figure 5: Chromatogram of ethyl acetate fraction of Volta region propolis extract.

Figure 6 below displays the chromatograms of ethyl acetate fraction of Ashanti region propolis fractions. Ten (10) peaks were observed with five (5) major peaks appearing at the retention time 24, 26, 27, 29, and 35.5 minutes.

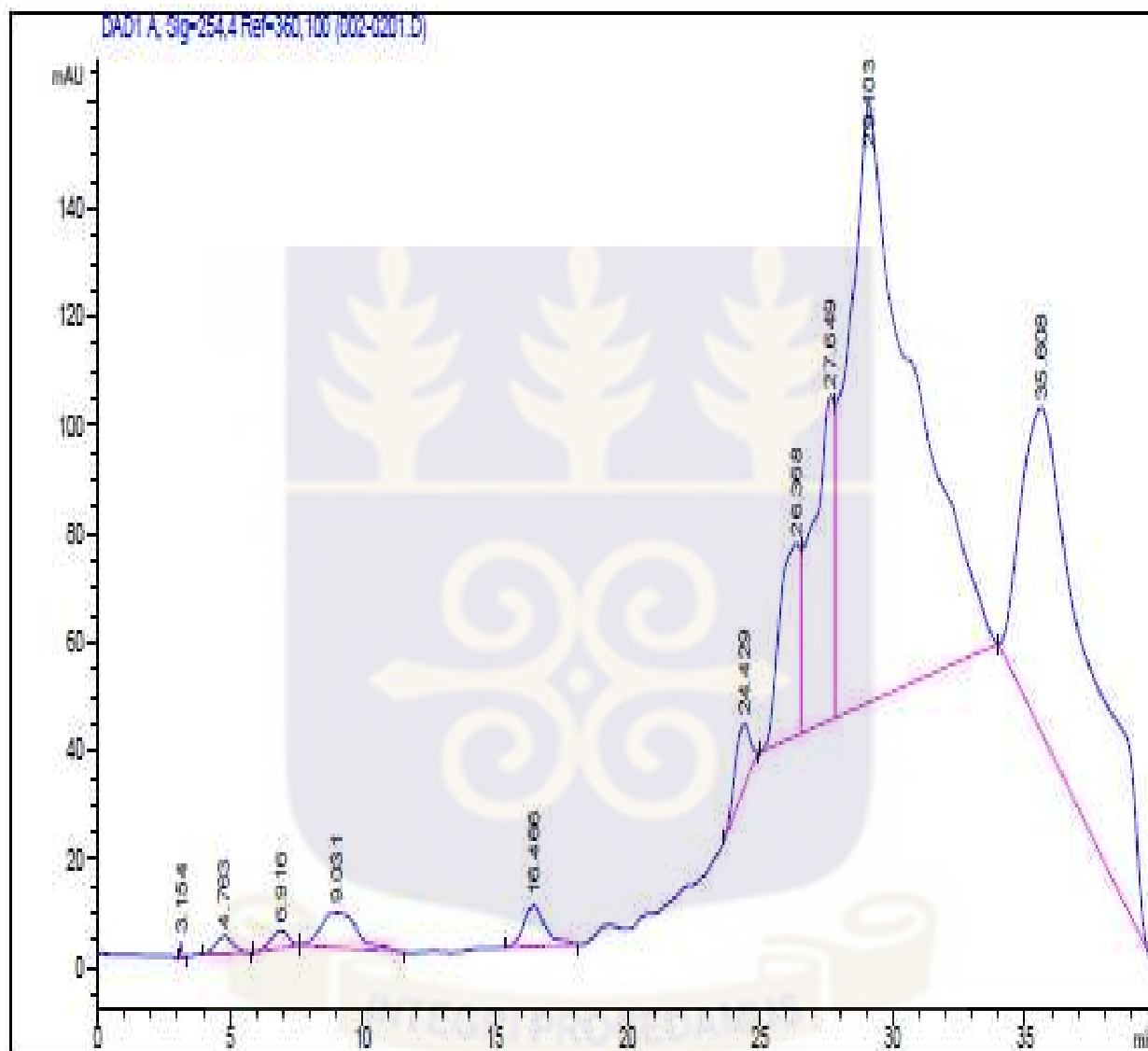


Figure 6: Chromatogram of ethyl acetate fraction of Ashanti region propolis fraction.

Figure 7 below also illustrates chloroform fraction of Northern region with major peaks at retention time 26th and 35.5th minutes

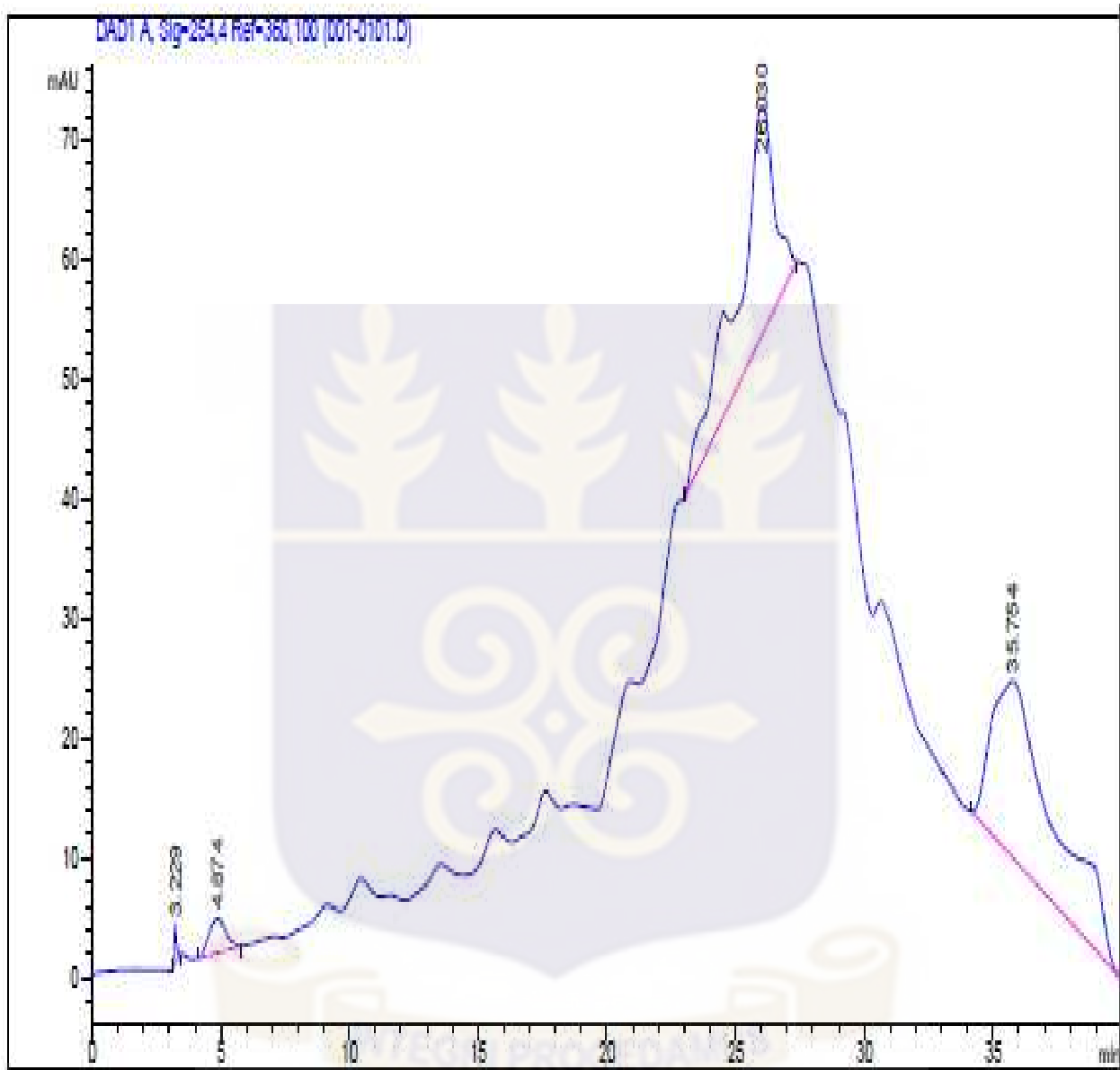


Figure 7: Chromatogram of chloroform fraction of Northern region propolis fraction.

Figure 8 below reveals 10 peaks in the Eastern region propolis fraction. However major peaks appeared at the retention time 24, 25, 26, 27, and 29 and 35.5 minutes.

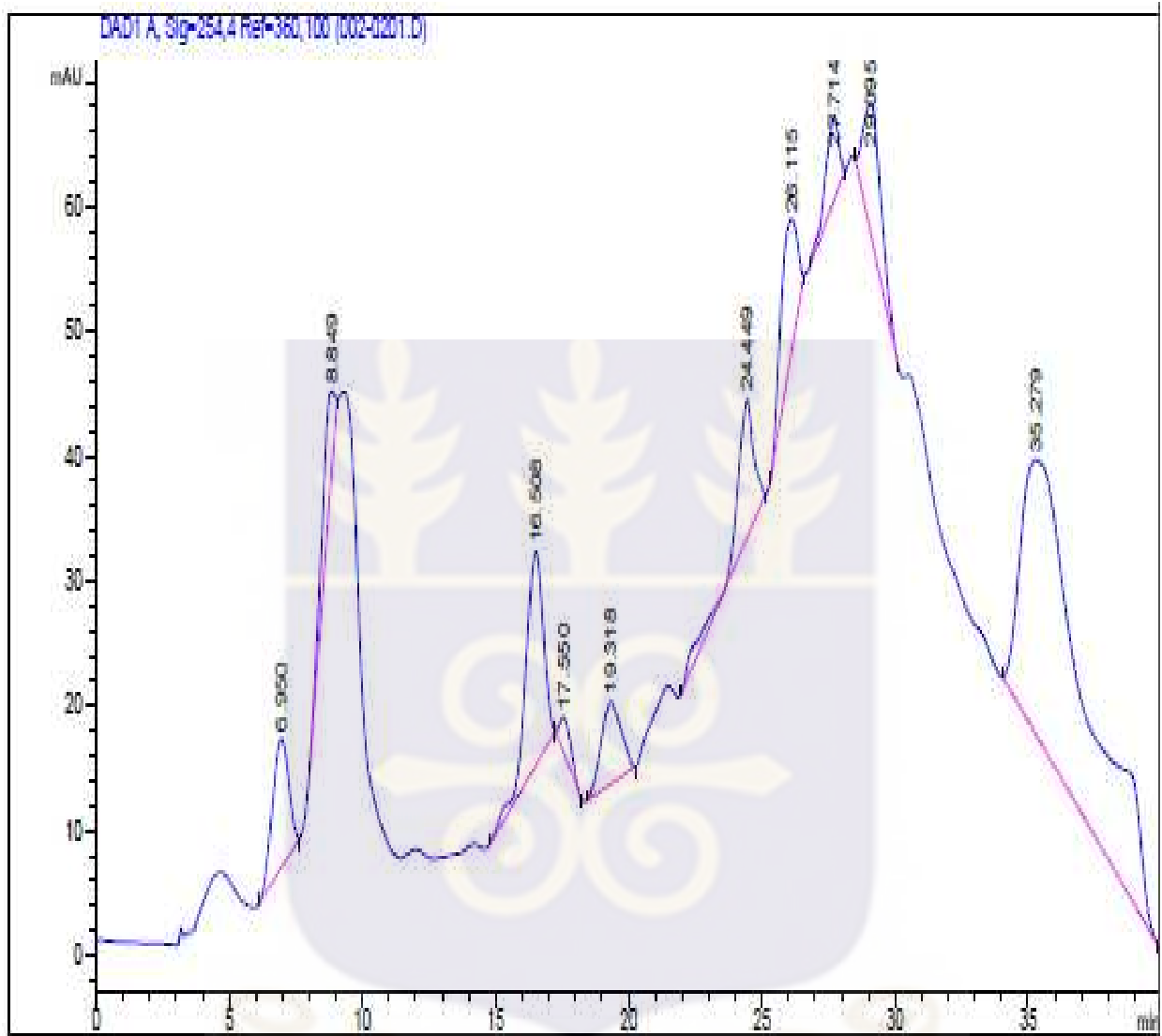


Figure 8: Chromatogram of ethyl acetate fraction of Eastern region propolis fraction.

Finally, figure 9 below reveals 9 peaks in the Eastern propolis fraction. However major peaks appeared at the retention time 24, 25, 26, 27, and 29 and 35.5 minutes

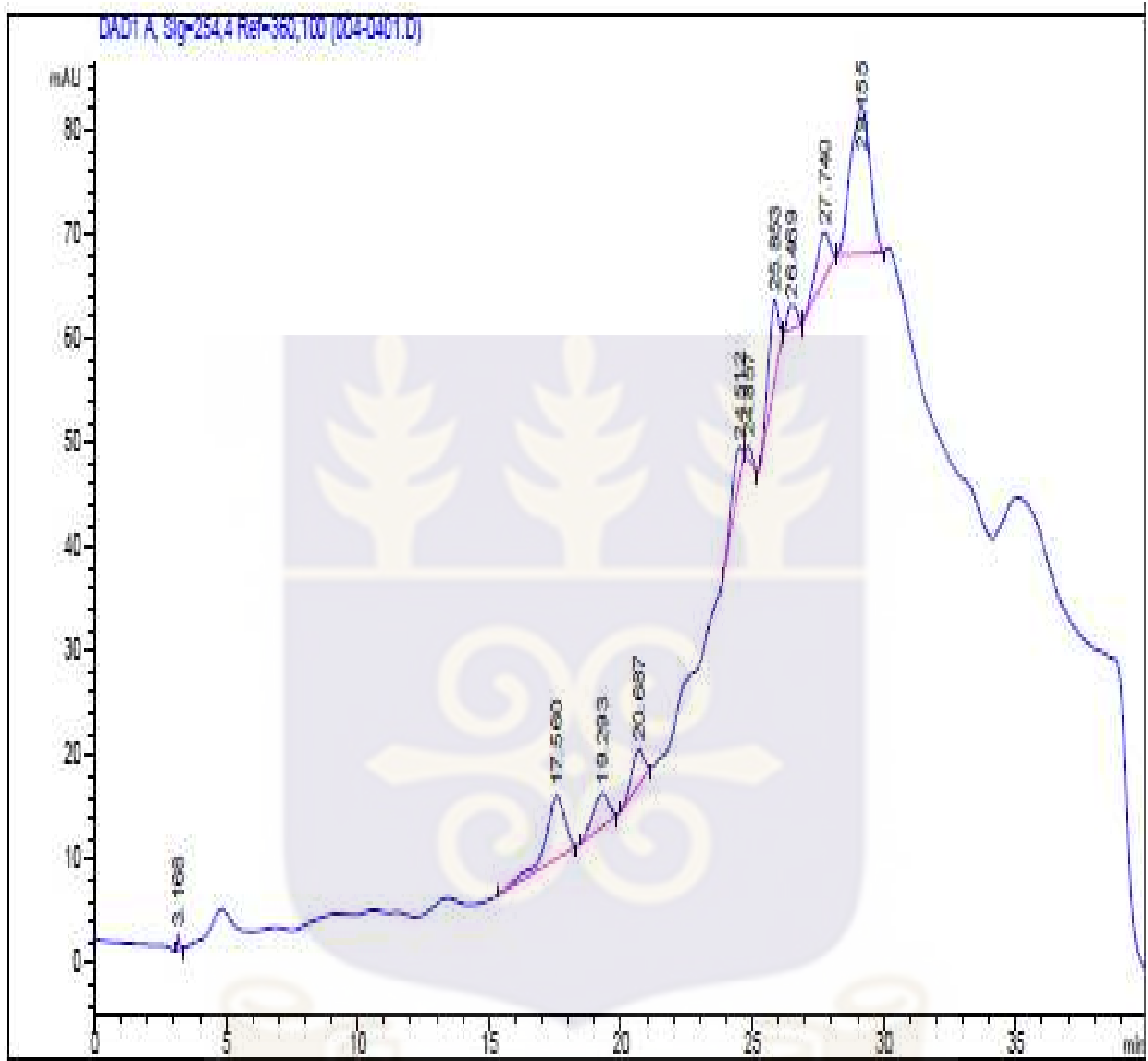


Figure 9: Chromatogram of chloroform fraction of Eastern propolis fraction.

## CHAPTER FIVE

### 5.0 DISCUSSION

The search for antimicrobials in natural products is promising. Antimicrobial activities exhibited by propolis extract highlights the need for alternative means of combating antimicrobial resistance. Crude propolis extracts as their well fractions showed varied zone of inhibitions from an *in vitro* assessment of antibacterial activity of propolis extract from different regions of Ghana.

This study revealed that the antimicrobial activities of propolis extract vary by geographic location and also just what the bees have available to them. Out of ten regions, Northern, Ashanti, Eastern and Volta region propolis extracts showed activities. Different vegetation of entomophilous plants were observed at the environs of hives of the four regions. In the Northern region (University for Development studies apiary) mainly cashew tree (*Anacardium pulsatilla*) and neem tree (*Azadirachta indica*), in the Ashanti region (Kwame Nkrumah University of Science Technology apiary) is mainly consists of coral vine (*Antigonon leptopus*), mango tree (*Mangifera indica*), lime (*Citrus aurantifolia*), sweet orange (*Citrus sinensis*), in the Eastern region (Kokrantumi-Koforidua apiary) mainly comprised of cocoa (*Theobroma cacao*) and *Azadirachta indica* and in the Volta region (Akachi Abor) *Mangifera indica*, *Azadirachta indica*, velvet tamarind (*Dalium guineense*), coconut tree (*Cocos nucifera*).

This variability in antimicrobial activities by geographical location has been supported by Kumar *et al.* (2008) and Bankova *et al.* (1992b). For example, 200 mg/ml Indian crude ethanolic propolis extract against *S. aureus* and *E. coli* showed 12.9 mm and 13.6 mm respectively which was lower than the zone of inhibition exhibited by 64 mg/ml of crude propolis extract from Volta region of Ghana against *S. aureus* and *E. coli* (20 mm and 20 mm respectively). In separate studies, 1 mg/ml of crude ethanolic Libyan propolis extract was effective against MRSA NCTC 25923 (19 mm), *S.*

*aureus* (26 mm) and *E. coli* (16 mm). It is reported in Brazil that 1 mg/ml ethanolic extracts of Brazilian and Cameroonian propolis were effective against *E. coli* (26mm), *Pseudomonas aeruginosa* (32.5mm) and *S. aureus* (21.43 mm) respectively (Tall *et al.*, 2014; Antonio *et al.*, 2011; Bankova *et al.*, 1998).

Susceptibility tests on fractions reveal that PET fractions were not effective against all test isolates. Chloroform fraction of Northern (CH<sub>NR</sub>), Chloroform fraction of Eastern (CH<sub>ER</sub>), Ethyl acetate fraction of Eastern (EA<sub>ER</sub>), Ethyl acetate fraction of Ashanti (EA<sub>VT</sub>) and Ethyl acetate fraction of Volta region (EA<sub>AS</sub>) showed appreciable activities against selected bacteria. Greater activities were seen in CH and EA fractions against *P. aeruginosa* and its control *P. aeruginosa* (ATTC 27853).

Consonant with this study, Silvana *et al.* (2009) reported that the antimicrobial activity of different chemical fractions of propolis extracted with hexane, chloroform, ethyl acetate and ethanol investigated by several authors from northeastern Brazil have significant antimicrobial activities.

The MIC and MBC of the active propolis fractions determined on selected bacteria also showed inhibitory and bactericidal activities. *S. aureus* and *P. aeruginosa* (clinical) and *P. aeruginosa* (ATTC 27853) were also observed to be susceptible to all fractions.

Clinically isolated *E. coli* and its control strain ESBL *E. coli* NCTC 13351 were susceptible to HC<sub>NR</sub> and EA<sub>VT</sub>. The MICs (MBCs) of HC<sub>NR</sub> and EA<sub>VT</sub> for the clinical isolate and its control strain range from 32 (40) to 64 (100) respectively.

Investigations by Sawaya *et al.*, (2004) and Silvana *et al.*, (2009) indicate that the tube dilution method gives more reproducible results in the determination of the antibacterial activity of propolis extracts or natural products with poor water solubility than the agar well diffusion method. This

explains why results of broth dilution method were better than the agar well diffusion method used in this research.

EA<sub>VT</sub> was found to have broad spectrum inhibitory and bactericidal activity against all six (6) test isolates (*S. aureus*, MRSA ATCC 25923, *E. coli*, ESBL *E. coli* NCTC 13351, *P. aeruginosa* and *P. aeruginosa* ATTC 27853).

Authors from different countries have identified varying values of MIC and MBC. MICs (MBCs) of ethyl acetate fraction of Egyptian propolis determined against *S. aureus* and *E. coli* was found to be 0.75 (0.75) and 0.85 (0.85) respectively (EI Fadaly and EI Badrawy., 2001); The MICs (MBCs) of ethanolic fraction of Brazilian of propolis was identified to be 0.4 (0.8) and 0.8 (1.6) respectively (Machado *et al.*, 2016).

MICs (MBCs) of Methanolic of Serbian propolis tested against MRSA 25923 and *E. coli* was also identified to be 16 (16) and 13.6 (13.6) respectively (Ristivojevi *et al.* (2016).

It is evident that the MICs of Egyptian, Brazilian and Serbian propolis were lower than the MICs and MBCs tested in this research, it is reported that antimicrobial activity of propolis extract vary by location.

Consistent with findings of Kalia *et al.* (2013), terpenoids, flavonoids, alkaloids, phenols, tannins and saponins are the main phytochemicals present in propolis extract.

HPLC fingerprint was further carried out on the active fractions following the qualitative analysis to determine the characteristics of compound varying number of chromatograms (figure 5-9). Some of the peaks were observed to have closely related retention time and common in most fractions. The two fractions of chloroform extracts (CH<sub>NR</sub> and CH<sub>ER</sub>) have closely related

chromatograms (fig. 4 and 5). The same trend was observed in ethyl acetate fraction of Eastern, Ashanti and Volta (EA<sub>ER</sub>, EA<sub>ER</sub> and EA<sub>VT</sub>) region.

Major peaks were identified at 26 minutes in CH<sub>NR</sub>, 29 minutes in CH<sub>ER</sub> and was also seen in EA<sub>ER</sub>, EA<sub>AS</sub> and EA<sub>VT</sub>. Another peak was also observed at 35 minute in CH<sub>NR</sub>, CH<sub>ER</sub>, EA<sub>ER</sub>, EA<sub>AS</sub>, and EA<sub>VT</sub>.

From the chromatograms, EA<sub>VT</sub> had the highest peak area at 29 and 35.5 minute. Studies have shown that phenols have the retention time of 35.5 minutes and the subclass was found to be Pinobanksin hexanoate (Nina *et al.*, 2015; Falcão *et al.* (2013 and Pellati *et al.*, 2011).

Using Agilent 1100 HPLC apparatus with same conditions, Coneac *et al.* (2008) observed a number of chromatograms in “cold” and “hot” propolis extract of Croatian propolis. The retention time of peaks in Croatian propolis ranged from 5 minutes to 39 minutes whilst the retention time of Romanian propolis ranged from 0.2 seconds to 1 minute (Medi -Šari *et al.*, 2009).

The chromatograms were different from the ones identified in this study, suggesting that different compounds were isolated in Ghanaian propolis.

## 5.1 LIMITATION

Most bee farmers intend to produce honey and not propolis, making it difficult to collect large quantity of propolis at a time.

The HPLC used did not have a library to illustrate the characteristic structures of isolated compounds.

## CHAPTER SIX

### 6.0 CONCLUSION AND RECOMMENDATION

#### 6.1 CONCLUSION

- Significant inhibitory and bactericidal effect of crude propolis extracts and their fractions from Northern, Eastern, Ashanti and Volta region were observed particularly on bacteria (*S. aureus* and *P. aeruginosa*) previously highly resistant to some antibiotics.
- Phytochemical analysis of active fractions revealed the presence of flavonoids, alkaloids, phenols, terpenoids and saponins in all fractions which are known to have antibacterial properties.
- The broad spectrum antimicrobial activities of EA<sub>VT</sub> could be due to the activity of pinobanksin hexanoate at retention time 35.5 minute.

#### 6.2 RECOMMENDATION

- Further works on synergistic effect of propolis fractions and ineffective antimicrobial on the selected bacteria would help in potentiating the ineffective drugs.
- Isolated propolis fractions should be analyzed using nuclear magnetic resonance (NMR) to identify compounds responsible for antimicrobial activities.
- Antimicrobial susceptibility testing of propolis extract should be investigated on other pathogenic bacteria, fungi, parasites and viruses.
- It imperative that other flowering plants and trees such as the mango, the palm, neem and cashew found at where bee hives are located be screened for antimicrobial activities.
- For new compounds to be isolated, it is important that other solvents be used to fractionate crude propolis extract.

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## APPENDICES

### Appendix 1: Preparation of 5% dimethyl sulfoxide (DMSO)

5ml of DMSO (Daejung, 3047-4405, Korea) was transferred into 95ml of sterile distilled water.

The solution was stored at 2-8 °C.

### Appendix 2: Preparation of 0.5 McFarland Standard

90ml of 1% sulfuric acid (0.18M H<sub>2</sub>SO<sub>4</sub>, BDH, England) was transferred into a 100ml volumetric flask. With the of a volumetric pipette, add 0.5ml of 1.175% anhydrous barium chloride (BaCl<sub>2</sub>, BDH, England) was added drop wise to the 1% sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) while constantly swirling the flask. The volume was topped up to 100ml with 1% H<sub>2</sub>SO<sub>4</sub> the solution was swirled for 3 minutes to mix, while examining visually, until the solution appears homogeneous and free of clumps. This is equivalent to a suspension containing approximately  $1 \times 10^8$  colony-forming units (CFU)/ml. The optical density (0.08 to 0.10) of the 0.5 McFarland standard at a wavelength of 625nm was determined.

### Appendix 3: Preparation of Mueller- Hinton Agar (BIOMARK)

Formula	gm/litre
Beef, dehydrated infusion from	300.0
Casein acid hydrolysate	17.5
Starch	1.5
Agar	17.0
pH 7.3 ± 0.2	

#### Directions

38 g of the medium was added to 1 litre of distilled water. It was boiled to dissolve the constituents completely and sterilized by autoclaving at 121°C for 15 minutes.

**Appendix 4: Preparation of Mueller Hinton broth (BIOMARK)**

<b>Formula</b>	<b>gm/litre</b>
Beef, dehydrated infusion from	300.0
Casein hydrolysate	17.5
Starch	1.5
pH 7.3 ± 0.1	

**Directions**

21g of the medium was suspended in 1 litre of distilled water and heated while swirling until it dissolves completely. The medium was then dispensed into screw-capped test tubes and sterilized by autoclaving at 121°C for 15 minutes.

**LIST OF TABEES****Table 4: Propolis sample location within each region**

	<b>REGION</b>	<b>LOCATION</b>
<b>1.</b>	UPPER EAT	NAVERONGO
<b>2.</b>	UPPERWEST	WA
<b>3.</b>	NORTHERN	UDS
<b>4.</b>	BRONG AHAFO	SENE
<b>5.</b>	EATERN	KOKKRANTUMI
<b>6.</b>	ACCRA	DZORWULU
<b>7.</b>	WESTERN	ASANKRAGUA
<b>8.</b>	ASHANTI	KNUST
<b>9.</b>	CENTRAL	UCC
<b>10.</b>	VOLTA	ABOR

**Table 5: Percentage yield of crude and fraction of propolis extracted**

Propolis sample origin	Weight (g) of raw taken for each solvent	Percentage fraction extracted with solvents (%)		
		Chloroform fraction	Ethyl acetate fraction	Petroleum ether fraction
1. UER	30.00	14.0	16.7	12.0
2. UWR	30.00	16.0	23.3	10.0
3. NR	30.00	29.70	52.50	9.90
4. BA	30.00	16.3	19.3	12.7
5. ER	30.00	28.30	68.50	11.70
6. AC	30.00	16.0	16.7	16.7
7. WR	30.00	22.7	16.7	14.0
8. AS	30.00	30.40	40.40	13.10
9. CR	30.00	14.7	16.7	12.0
10. VT	30.00	32.70	51.00	16.30

$$\% \text{ yield} = \frac{\text{Weight of propolis extracted}}{\text{Weight of raw propolis}} \times 100$$

**Table 6: Zone sizes of control antibiotics analyzed**

	MRSA (NCTC 12493)	<i>S. aureus</i> (Clinical)	<i>E. coli</i> NCTC 13351	<i>E. coli</i> (Clinical)	<i>P. aeruginosa</i> ATCC 27853	<i>P. aeruginosa</i> (Clinical)
Chloramphenicol (30µg)	30	30	28	25	30	15
Penicillin (10 µg)	0	0	0	0	0	0
Tetracycline (30 µg)	30	30	30	25	20	19

**Table 7: Screening for crude propolis extracts activity using agar well diffusion method**

Sample location		Average zone of inhibition (mm)					
Code	Sample	MRSA (ATCC 25923)	<i>S. aureus</i> (clinical)	ESBL <i>E. coli</i> (NCTC 13351)	<i>E. coli</i> (clinical)	<i>P. aeruginosa</i> (ATTC 27853)	<i>P. aeruginosa</i> (Clinical)
1	UER	11	11	0	0	20	11
2	UWR	10	10	0	0	18	10
3	NR	24	24	0	0	28	12
4	BA	13	0	0	0	19	0
5	ER	25	24	0	0	31	13
6	AC	12	0	0	0	20	0
7	WR	11	12	0	0	22	0
8	AS	20	19	0	0	20	11
9	CR	12	0	0	0	18	0
10	VT	20	20	20	0	24	13

**Table 8: Zone sizes of Active Fractions of the Crude Propolis Extract**

FRACTION	MRSA (NCTC 12493)	<i>S. aureus</i> (clinical)	ESBL <i>E. coli</i> (NCTC13351)	<i>E. coli</i> (clinical)	<i>P. aeruginosa</i> (ATTC 27853)	<i>P. aeruginosa</i> (Clinical)
CH <sub>NR</sub>	9	8	8	9	27	24
CH <sub>ER</sub>	10	8	8	8	27	25
CH <sub>AS</sub>	8	0	12	0	12	9
CH <sub>VT</sub>	12	0	14	0	12	12
EA <sub>NR</sub>	12	9	0	0	9	0
EA <sub>ER</sub>	11	0	16	16	32	0
EA <sub>AS</sub>	27	10	10	8	31	0
EA <sub>VT</sub>	27	27	18	18	30	13
PET <sub>NR</sub>	0	0	0	0	0	0
PET <sub>ER</sub>	0	0	0	0	0	0
PET <sub>AS</sub>	0	0	0	0	0	0
PET <sub>VT</sub>	10	9	9	0	0	0

– Not effective

**Table 9: Gradient Time programme**

Time (min)	% B	% A
0.1	10	90
10	20	80
15	35	65
20	60	40
25	80	20
30	80	20
35	80	20
40	10	90

**LIST OF PALTES**



Plate 1: Raw propolis

Plate 2: Preparing the crude extracts

Plate 3: Filtration of extracts

Plate 4: Drying the extracts in a dessicator

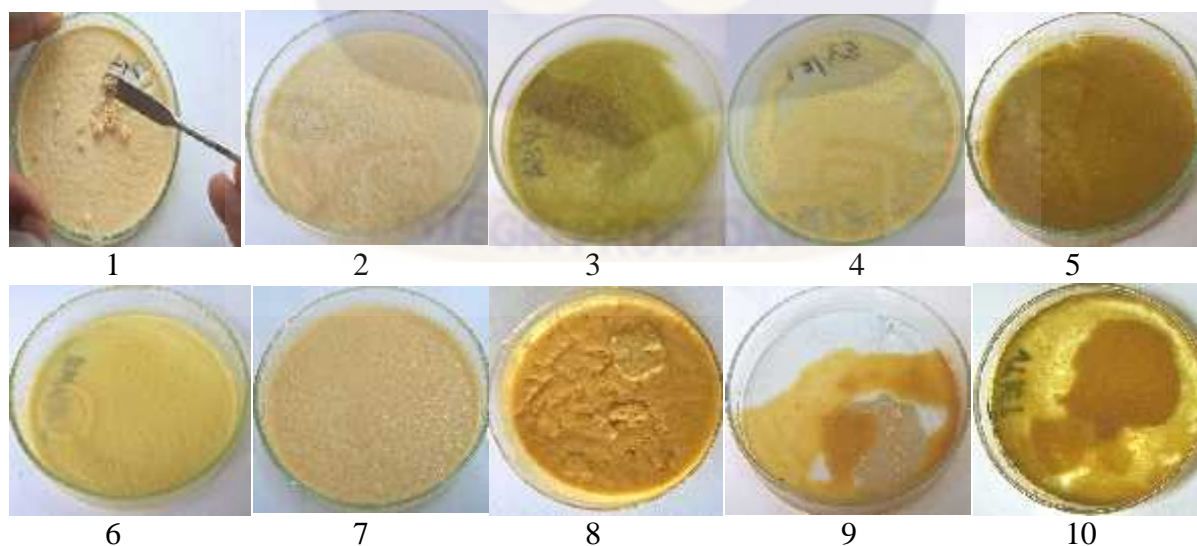


Plate 5: Crude propolis extracts; 1-Upper East; 2-Upper West; 3-Northern; 4-Brong Ahafo; 5-Eastern; 6-Greater Accra; 7-Western; 8-Ashanti; 9-Central and 10-Volta.

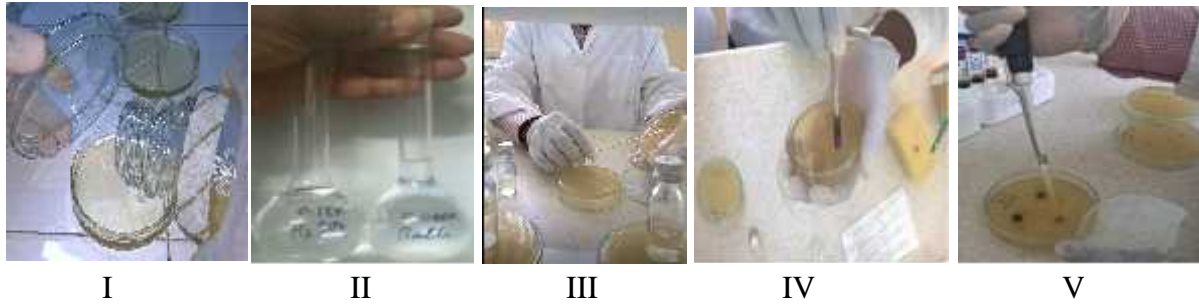


Plate 6: I-Mueller-Hinton agar; II-0.5 McFarland Standard reagents; III-Inoculating the adjusted inoculum on Mueller-Hinton agar with the aid of sterile swab stick (b ttger 94249, Germany); Making a well with the aid of sterile cork borer; Dispensing an extract into the well.



A) *S. aureus* (clinical)

B) MRSA NCTC 25923



C) *P. aeruginosa* (clinical)

D) *P. aeruginosa* ATCC 27853



E) *E. coli* (clinical)

F) *E. coli* ESBLNCTC13351

Plate 7: Zone sizes of 10 crude extracts against; A) *S. aureus* (clinical) and B) its control strain

C) *P. aeruginosa* (clinical) and D) its control strain (*P. aeruginosa* ATCC 27853); *E. coli* (clinical) and its control strain (*E. coli* ESBLNCTC13351).



Plate 8a: Chloroform fraction    b: Ethyl acetate fraction    c: Petroleum ether fraction



Plate 9: Zone sizes of petroleum ether, ethyl acetate and chloroform against *S. aureus* (clinical).



Plate 10: Zone sizes of petroleum ether, ethyl acetate and chloroform against MRSA (NCTC 25923).



Plate 11: Zone sizes of petroleum ether, ethyl acetate and chloroform against *E. coli* (clinical).



Plate 12: Zone sizes of petroleum ether, ethyl acetate and chloroform against *E. coli* ESBL NCTC 13351.



Plate 13: Zone sizes of petroleum ether, ethyl acetate and chloroform against *P. aeruginosa* (clinical).



Plate 14: Zone sizes of petroleum ether, ethyl acetate and chloroform fraction against *P. aeruginosa* ATCC 27853.

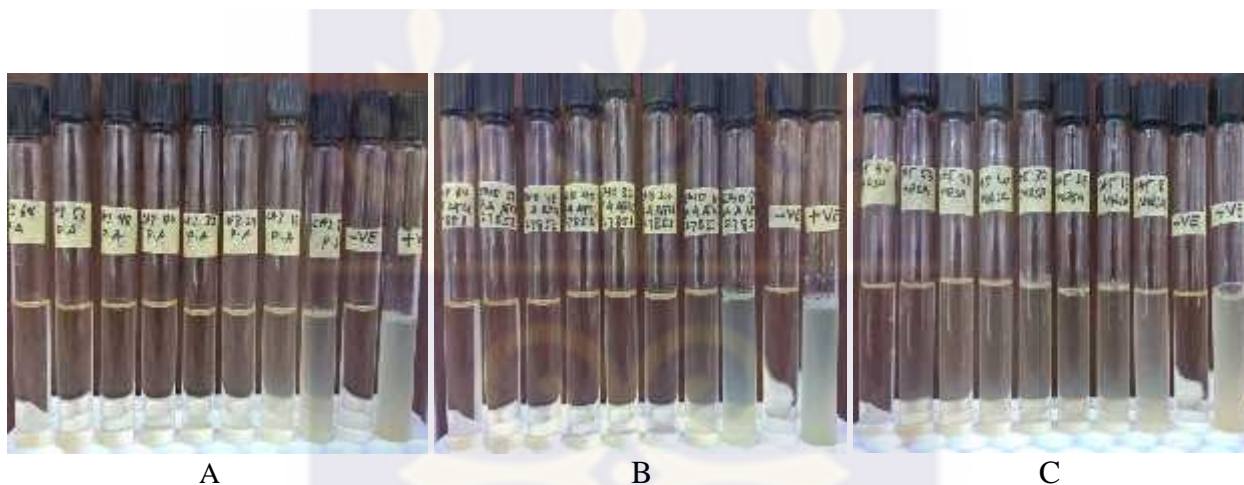


Plate 15: MIC of fractions against selected bacteria; A: MIC of CH<sub>NR</sub> against PA (24 mg/ml), B: MIC of EA<sub>VT</sub> against PA ATCC 27853 (24 mg/ml), C: MIC of CH<sub>ER</sub> against MRSA ATCC 25923 (64 mg/ml)

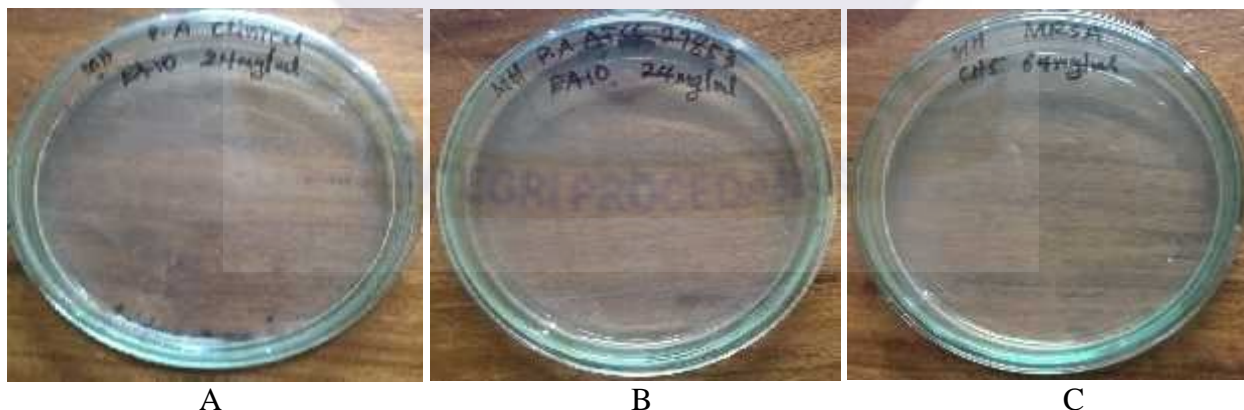


Plate 16: MBC of fractions against selected bacteria; A: MIC of CH<sub>NR</sub> against PA (24 mg/ml), B: MIC of EA<sub>VT</sub> against PA ATCC 27853 (24 mg/ml), C: MIC of CH<sub>ER</sub> against MRSA (64 mg/ml).

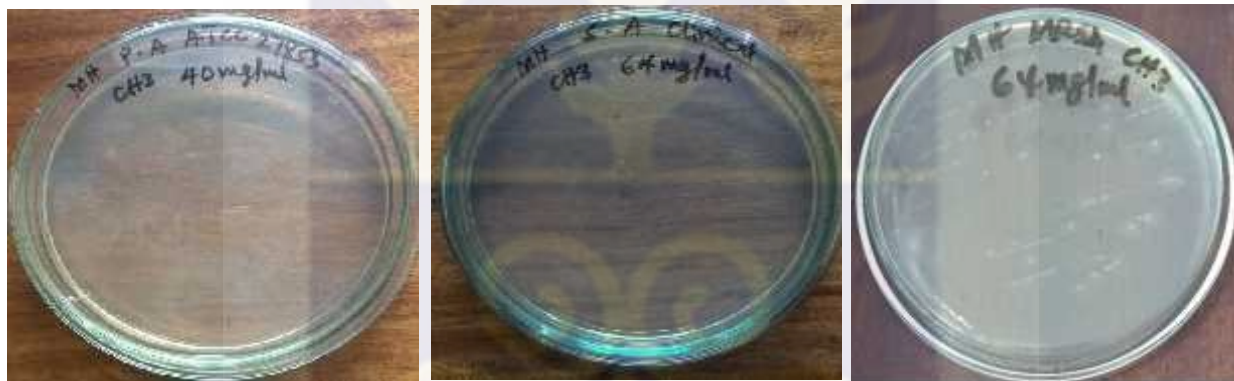


CH<sub>NR</sub> MIC of PA is 40  
A

CH<sub>NR</sub> MIC of PA is 40  
B

CH<sub>NR</sub> MIC of MRSA is -  
C

Plate 17: MIC of fractions against selected bacteria; A: MIC of CH<sub>NR</sub> against PA (40 mg/ml), B: MIC of EA<sub>NR</sub> against PA (40 mg/ml), C: CH<sub>ER</sub> has no MIC against MRSA.



A

B

C

Plate 18: MBC of fractions against selected bacteria; A: MIC of CH<sub>NR</sub> against PA (24 mg/ml), B: MIC of EA<sub>VT</sub> against PA ATCC 27853 (24 mg/ml), C: MIC of CH<sub>ER</sub> against MRSA (64 mg/ml).

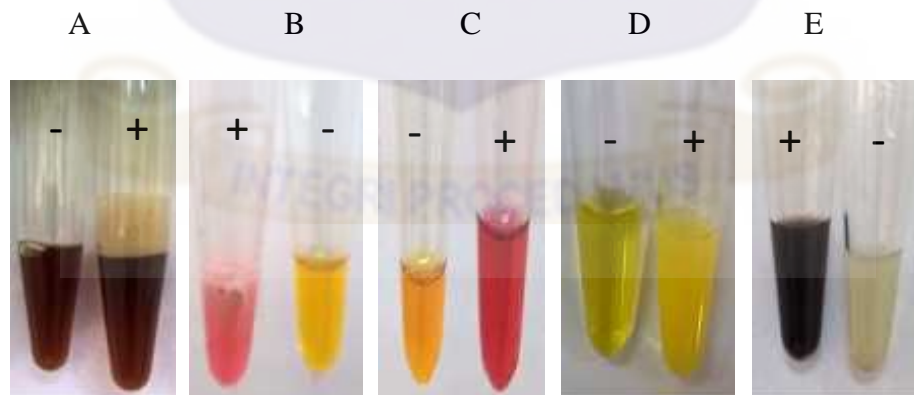


Plate 19: Qualitative Phytochemicals Screening of Active Fractions: Test for: A) Saponins; B) Flavonoids; C) Terpenes; D) Alkaloids; E) Phenols.