UNIVERSITY OF CAPE COAST

ANTIBACTERIAL, RESISTANCE MODULATION ACTION AND TIME-KILL KINETICS OF CRUDE ALKALOIDAL CONSTITUENTS FROM THREE DIFFERENT MEDICINAL PLANTS

SAMUEL ASIAMAH OBIRI

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BY

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AUGUST, 2023

DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my own original work and that no part of it has been presented for another degree in this University or elsewhere.

Candidate's Signature:……Date ……

Name: Samuel Asiamah Obiri

Supervisors' Declaration

We hereby declare that the preparation and presentation of the thesis was supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast.

Principal Supervisor's Signature:……Date ……

Name: Dr. Isaac Asiamah

Co-Supervisor's Signature:……Date ……

Name: Prof. Yaw Opoku-Boahen

ABSTRACT

Globally, millions of people die from drug-resistant infections. The World Health Organization (WHO) estimates that a resistant variant is 64% more likely than a non-resistant variant to kill an infected victim. As a result, new anti-microbial sources are extensively being explored, and medicinal plants may serve as promising starting point. Alkaloids are a wide group of naturally occurring nitrogenous organic compounds produced by organisms. The study aimed at determining the antibacterial, anti-biofilm properties and the rate of activity of the crude alkaloidal extracts of three medicinal plants; *Occimum gratissimum*, *Zanthozylum zanthoxyloides* and *Phyllanthus fraternus.* Combination therapy has shown promise in overcoming AMR as the active ingredient act in synergy to inhibit the causative microorganism. In the present study, the alkaloids in the three plants showed fairly good *in-vitro* inhibition properties against ten clinical pathogenic strains. Time-kill kinetic results revealed complete bactericidal action of crude alkaloidal constituents from *Z. zanthoxyloides* against *S. aureus* and *S. poona*. Bactericidal action was also revealed by crude alkaloidal constituents from *P. fraternus* against *E. Coli* (ATCC 43888). Modulation of tetracycline (a standard antibiotic) with the crude alkaloidal constituents from *P. fraternus* and *O. gratissimum* resulting in additive effects successfully improved tetracycline's potency against *S. poona* and *Shigella*. LC-ESI-MC analysis revealed four known *Z. zanthoxyloide* alkaloids, two *P. fraternus* alkaloids and one known *O. gratissimum* alkaloid in the crude alkaloid extracts.

KEY WORDS

Bactericidal

Combination Therapy

Minimum Inhibition Concentration

Pseudomolecular Ion

Synergistic Effect

Time Kill Kinetics

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My entire family, dad in particular deserves the utmost gratitude for his support and inspiration during the trying times, as do all of my friends. God bless everyone for their support.

DEDICATION

To my lovely little Sisters Blessing Nana Ama Agyeiwaah-Kwateng Obiri and Janet Obiri.

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LIST OF ABBREVIATIONS

AEOG	Alkaloid Extract of *Occimum gratissimum*
AEPF	Alkaloid Extract of *Phyllantus fraternus*
AEZZ	Alkaloid Extract of *Zanthoxylum zanthoxyloides*
Cipro	Ciprofloxacin
DCM	Dichloromethane
E. coli (10)	*Escherichia coli* ATCC 10455
E. coli (48)	*Escherichia coli* ATCC 10455
Kleb	*Klebsiella pneumoniae*
K. pneumoniae	*Klebsiella pneumoniae*
MHB	Muller Hinton Broth
MeOH	Methanol
MRSA	Methicillin resistance *staph aureus*
OD	Optical Densities
OD_{ave}	Average Optical Density
O. G	*Occimum gratissimum*
P. F	*Phyllanthus fraternus*
pH	Hydrogen Potential
S. aureus	*Staphylococcus aureus*
SD	Standard Deviation
S. lentus	*Staphylococcus lentus*
S. pyogenes	*Streptococcus pyogenes*
S. poona	*Salmonella poona*
S. typhi	*Salmonella typhi*
Tetra	Tetracycline

WHO	World Health Organization
Z. Z	Zanthoxylum zanthoxyloides

CHAPTER ONE

INTRODUCTION

Background to the Study

Plants have long been recognized as an endless source of leads with potential applications in drug discovery and medicine. They are made up of different active constituents, such as tannins, phenols, glycosides, alkaloids, steroids, volatile and fixed oils, flavonoids, resins etc. that are found in different parts, particularly the stem bark, leaves, seeds, fruits, roots, and flowers. (Shehadeh *et al*, 2021; Gupta *et al.*, 2012). The world's largest application of biodiversity is the utilization of plants for medical purposes. Compared to *species* utilized for food, quite a number of plant species are extensively used as medicines. This fact implies that natural products play a key role in the discovery of novel plant leads (Atanasov *et al.*, 2021; Maridass, 2010) and in an effort to find new leads, researchers have, recently, been heavily involved in screening plants used in traditional medicine.

According to the World Health Organization (WHO), about 85% of traditional medicine uses plant extracts, and it is estimated that 80% of people in underdeveloped countries are thought to rely on it for their fundamental medical treatment. This indicates that between 3.5 and 4 billion people on a global scale depend on plants as a source of medications (WHO, 2022). Two relevant examples are galegine, a bioactive substance isolated from *Galega officinalis L*. that was used as a precursor for the synthesis of metformin and other antidiabetic analogues, and papaverine, an alkaloid obtained from *Papaver somniferum L*. that also served as the bedrock for the drug verapamil,

which was used to treat high blood pressure. Most people are also familiar with opium (known to contain morphine and codeine) as the primary source of medications for relieving pain (Cragg & Newman, 2014).

Over the years, medicinal plants have been utilized to alleviate a variety of human diseases and have played a significant role in the provision of quality healthcare in every country at some point (Oluma *et al.*, 2004; Gupta *et al.*, 2012). Recent studies have concentrated on using medicinal plant metabolites as alternatives for currently available drugs in the treatment of diseases in impoverished nations as these easily accessible and have no or transient side effects (Aiyegoro *et al.*, 2007; Ajayi & Akintola, 2010). In most parts of the world, plant-derived medications have long been used in traditional medicine, and there has been growing interest in using their constituents as primary sources of remedies to treat microbial infections (Mohana *et al.*, 2008; Ajayi & Akintola, 2010; Ghaleb *et al.*, 2009). When it comes to the treatment of several infectious diseases, the emergence of numerous antibiotic-resistant microorganisms has become a major problem worldwide. Since 1989, the *Salmonella typhi* strain that causes typhoid fever has reportedly developed resistance towards traditionally recommended antibacterial drugs in a number of endemic regions (Greenwood *et al.*, 2009). Food and water are primarily the means of transmission for this etiologic agent and various traditional antibiotics have also been implicated to be no longer effective against a number of other threatening disease-causing pathogens.

Particularly in underdeveloped nations, infectious diseases continue to be a significant source of morbidity and mortality in humans. Presently, the use of locally produced herbal remedies prepared as hot water infusions, cold water

decoctions, tinctures with alcohol and concoctions with food as an alternative cure for bacterial illnesses is still common in Africa (Oluduro & Omoboye, 2010). The majority of herbal preparations have utilized plant portions like leaves, roots, bark, ripe and unripe fruits etc.

Herbal plants have frequently been explored as a resource for lead identification in medicinal chemistry and drug discovery. For hundreds of years, medicinal plants have played an important role in the treatment and prevention strategies of a number of human diseases worldwide, and once represented the main source for all mankind's medicines (Sofowora *et al.,* 2013). Owing to the fact that folk knowledge and traditional medicine integrate well with the socio-cultural life of the people, most orthodox drugs being expensive to obtain, and others frequently being faked, herbal medicine practitioners are still sought out as a first option in many parts of the world, including Ghana (Amuse *et al.,* 2011). Again, numerous medicinal plants can be used alone or combined with other medicinal plants to produce synergistic effects in treating a variety of diseases.

In order to trace the selection of extracts or constituents with pertinent pharmacological activity when developing novel antibiotics from natural products, there are few issues that need to be resolved, such as the establishment and choice of primary screening assays. The assay should be simple to carry out, rapid, and as economical as possible (McLaughlin *et al.,* 1998; Monteiro *et al.,* 2012). The plant material must first go through a proper extraction process in order to evaluate the biological activity of its constituents. Extraction is the process of selectively isolating the constituents of interest from a matrix using appropriate solvents according to standard protocols; the extraction of active

ingredients can be carried out using several procedures. The possible biologically active components in the crude extract are evaluated through screening. The crude extracts can then be successively fractioned, and these fractions also subjected to additional suitable bio-assay studies (Bobzin, Yang, & Kasten, 2000).

The earliest known life forms are bacteria, they are surprisingly numerous, remarkably diversified and their infections include some of the most prevalent disease conditions in the world. Their infections are regarded among most dangerous ailments in medicine, both now and in the future (Relman, 2002). According to their physical properties, which includes size, shape, and biochemical characteristics, bacteria have been categorized. Genotype analysis has recently taken the lead in classification, especially when using conserved molecules like 16S-ribosomal RNA. As opposed to these, bacteria can also be categorized as harmful (pathogenic) or harmless (non-pathogenic). Normal flora is the term used for non-pathogenic micro-organisms. Unlike normal flora, pathogenic micro-bacteria result in bacterial infections and some *species*, including *Pseudomonas aeruginosa,* are opportunistic pathogens that primarily affect persons with immunosuppression (Entenza *et al.*, 2014).

Antibiotics or antibacterial medications are used to treat infections caused by bacteria. They are chemical compounds that either completely eradicate bacteria (bactericidal agents) or act as bacteriostatic agents to inhibit their growth. These substances' capacity to obstruct vital bacterial cellular processes accounts for their effectiveness against bacterial survival (Sommer & Dantas, 2011). Our society have been profoundly impacted (post-World War II) by the use of modern antibiotic medication, which has also altered medical

practice and patient care. Antibiotics are also used in modern medical operations to prevent infections, during surgery, organ transplants, the care of preterm newborns, and for patient rehabilitation (WHO, 2014). Over the past few decades, usage of antimicrobials has prevented infectious diseases from dominating and becoming the number one healthcare concern among the European nations. However, infectious diseases continue to be the leading causes of death in underdeveloped countries and even the third leading cause of death globally (Ashraf *et al.*, 2020; Nagel *et al.*, 2016).

For a number of years, aatibiotic resistance has made it difficult to treat bacterial infections. The finding of antibiotic resistance was first noted in Flemings' laboratory shortly after the discovery of penicillin in 1928, and this resistance towards penicillin and other antibiotics persisted into the 1950s, when several new medications and penicillin analogues were developed to fight the increasing levels of resistance (Podolsky, 2018; Johnson, 2011). One of the biggest risks to public health worldwide is bacterial resistance to antibiotics, a problem that transcends national boundaries. All types of drug-resistant microorganisms can blithely travel between nations and among humans and animals, and there is no doubt that the issue is worse in underdeveloped countries where there is lack of medicine supply and high abuse of antibacterials (Byarugaba *et al.*, 2004). Antibiotic resistance is considered by the World Health Organization (WHO) as a severe concern which has currently become unpredictable. It is happening everywhere around the world and might have an impact on anyone, at any age and in any nation (WHO, 2014). In light of worries about the growing threat of antibiotic-resistant strains, which are eroding drug efficacies and killing more people, the Infectious Diseases Society of America

(IDSA), operating at the forefront of antibiotic usage, in 2014, urged for diverse researches into the development of new antimicrobial agents in the impending years (Venter, 2014).

Essentially, the difficulty of reducing bacteria-resistant and the subsequent rise in complications and infections-related fatalities should be our main concerns with regards to resistance. Recent years have seen the development of very few truly new antibiotics, and conventional natural antibiotic sources like soil fungi and bacteria may have exhausted most of their supply of antimicrobial leads (Carlos, 2010). As a result, we must explore elsewhere for new antimicrobial sources, and medicinal plants may serve as promising starting point.

The time-kill assay is a convenient technique useful in analysing the dynamics of antimicrobial drugs against microbes to evaluate its activity as a bacteriostat or bactericide over a certain period. Ultimately, when two drugs are combined, a biological assay study can be used to establish whether the selected antibiotic combination is synergistic or not, and whether the combined effect is bacteriostatic or bactericidal (Brennan-Krohn *et. al*., 2019; Gaudereto *et. al*., 2020; Nageeb *et. al*., 2015). Unlike checkerboard bioassays, which exclusively measure growth inhibition, time-kill bioassays, in addition to that, also provide information about the effects of the antibiotics on a microorganism over a specified timeframe. (Gaudereto *et. al*., 2020).

Problem Statement

Longevity of antibiotics is impacted by how bacteria that cause diseases respond to antibiotics used to treat their infections by developing resistance

against them. This natural biological adaptation process and antimicrobial resistance (AMR) is what has affected the effectiveness of antibiotics over the years. Recent increases in antibiotic misuse and inappropriate use have led to an increase in antimicrobial resistance. As a result, many bacterial strains undergo hypermutation, which spreads resistant strains (Chauhan *et al.*, 2013). The negative impacts of microbes' resistance to antimicrobials are already being manifested in every part of the world, and as resistance and virulence rise, so do the expense and burden on society.

At least 50,000 people die from antibiotic-resistant diseases each year in the US and Europe alone, and many more perish in different regions globally. The World Health Organization (WHO) estimates that a resistant variant is 64% more likely than a non-resistant variant to kill an infected victim (WHO, 2018). Because of this, antibiotic resistance is a serious health concern that requires significant attention. Again, most recent figures suggest that, if appropriate measures to combat drug resistant infections are not undertaken, they might cause an additional 10 million deaths worldwide each year by 2050. In order to address the needs of the global public health, the World Health Organization (WHO) is urging political leaders, research institutions and the pharmaceutical companies to collaborate in order to take comprehensive actions against drug-resistant diseases (Suraj & Sapkal, 2015).

Purpose of the Study

Previous evidences linking *Occimum gratissimum, Zanthoxylum zanthoxyloides*, and *Phyllanthus fraternus* to antibacterial activity has significantly attracted the interests of several researchers (Matasyoh *et al.*, 2007;

Mills-Robertson *et al.*, 2017; Mehta *et al.*, 2014). In order to analyze these three medicinal plants therapeutically, the study aimed at determining the antibacterial and the rate of activity (time-kill kinetics) of the crude alkaloidal extracts of these three medicinal plants.

Research Objectives

- To extract the alkaloidal constituents from the crude extracts of *P. fraternus*, *O. gratissimum* and *Z. zanthoxyloides.*
- To evaluate the antibacterial properties of these three crude alkaloids *in -vitro*.
- To enhance the antibacterial potency of tetracycline with the crude alkaloid extract through modulation study.
- To use Time-Kill Kinetics to monitor the activity of the crude alkaloidal extract and the modulated standard drug (tetracycline) in 24 hours using Elisa optical density micro-plate reader.
- To identify the possible alkaloids through LCMS analysis and implicate them as potential constituents involved in the anti-bacterial activity.

Significance of the Study

In the lack of effective primary healthcare systems, traditional treatments involving natural products continue to hold a significant role among rural populations in underdeveloped nations for treating variety of diseases (Ali *et al.*, 2001; Pandey, 2003; Pandey *et al.*, 2010). Even while the development of antibiotic resistance by bacteria cannot be stopped completely, the proper use of more potent antibiotics, particularly those derived from herbal plants, may lower mortality and healthcare expenses (Ahmad and Beg, 2001; Pandey *et al.*,

2010). Bacterial biofilms and efflux pumps have been identified as the primary causes of antimicrobial resistance (AMR) among the various resistance mechanisms (Kvist *et al*., 2008).

To tackle this escalating challenge and assist the health industry in dealing with antibiotic resistance, alternative techniques have been put into place. Investigating the secondary metabolites from herbal plants for their potential antibacterial activity is a promising alternative. In the present study, various techniques were employed to evaluate the antibacterial activity of the crude alkaloid extracts of three medicinal plants; *O. gratissimum, Z. zanthoxyloide and P. fraternus.*

Although the biological effects of *O. gratissimum, Z. zanthoxyloide* and *P. fraternus* have been investigated, there is no comprehensive study available on the crude alkaloidal secondary metabolites. Furthermore, studies on the crude extracts of these three plants report high minimum inhibition concentrations (MICs). Accordingly, in the quest to find plant secondary metabolites with a much better MICs and to tackle the issue of drug resistance, this project conducted the extraction of the crude alkaloids of these plants to demonstrate and investigate their antibacterial properties and their rate of activity (time-kill kinetics).

In drug combination therapy, two ingredients are added for additional therapeutic effect, and one or even both ingredients could be antibiotics. The second ingredient might improve the activity of the first, for example, or in some cases might completely stop or inhibit the activity of a resistance mechanism by a bacterium. On the basis of this theory, drug modulation study (combination

antibiotics) was also performed to enhance the potency of one of the standard drugs used in the study against bacterial strains with growing resistance.

Delimitation

The plant samples were collected from Nkamfoa and Elmina, all in Cape-Coast due to the raining nature of other geographical location where large quantities could be obtained at the time of sample collection. Air drying of samples lasted about three to four weeks before milling and extraction.

Limitation

The quantities of the raw plant materials collected were not enough after the alkaloidal extraction for further antibacterial activity studies to be carried out.

Scope of Study

The study involved plant material collection; whole plant of *Phyllanthus fraternus* from Nkamfoa, Cape-Coast, leaves of *Zanthoxylum zanthoxyloides* from Elmina and leaves of *Ocimum gratissimum* also from Nkamfoa. These plant materials were dried and milled to powder and extracted with Methanol/Dichloromethane (MeOH/DCM) for 72 hours with constant shaking. Extract was filtered and the residue re-macerated with methanol only for another 72 hours with regular shaking. This extract was also filtered and combined with the first filtrate for concentration using a rotary evaporator. Crude alkaloid extract was done using the crude extract obtained after concentration. Phytochemical screening was done to know the type of secondary metabolites present and Thin Later Chromatography (TLC) also performed to know the

number of components of compounds in each crude alkaloid extract. Purification and isolation of the individual components/compounds was followed using flash chromatography. Further purification of the isolated components followed using preparative TLC and repeated to obtain the pure compounds. The isolates were analyzed using liquid chromatography mass spectrometer (LCMS) to know the molecular masses of the individual components isolated. The crude alkaloid extracts were then subjected to biological study to investigate their antibacterial effect on some microorganisms; *Staphylococcus aureus, MRSA, Streptococcus pyogenes, Staphylococcus lentus, Escherichia coli ATCC 10455, Escherichia coli ATCC 43888, Klebsiella pneumoniae, Salmonella poona, Shigella and Salmonella typhi.*

Organization of the Study

This thesis comprises five chapters. The study's introduction, problem description, significance of the study, and explanation of the primary goals and objectives are all provided in Chapter one. Chapter two covers detailed review of literature relevant to the present study with some important information about the three medicinal plants used in the present study, traditional antibacterial reports and some alkaloids isolated and characterized from them. Chapter three consists of materials and methods used in the research work including plant sample collection, extraction, chemical reagents, and media preparations, research instruments and analysis techniques. Chapter four presents the results and discuss the outcomes from the study such as phytochemical screening, minimum inhibition concentration (MIC), time-kill kinetics, modulation study

and molecular masses of purified components. Chapter five gives the summary, conclusion and recommendations for further studies.

Chapter Summary

A general overview of the research has been provided in this chapter. The chapter elaborated the general introduction on alkaloids, bacteria, the motive behind the present study and the importance of this research work.

CHAPTER TWO

LITERATURE REVIEW

Introduction

In this review, a brief and yet comprehensive update of the medicinal uses of *Phyllanthus fraternus*, *Occimum gratissimum* and *Zanthoxylum Zanthoxyloide* is presented. Studies involving the antimicrobial properties of other parts of the plants, drug action of known antibiotics and resistance mechanisms by microorganisms are all discussed.

Review of selected medicinal plants

Occimum gratissimum

O. gratissimum L. belongs to the family lamiaceae; over 250 genera and 6700 species make up this family (Thorne, 1992; Zomlefer, 1994; Mabberley, 1997). In terms of floral structure, the family *Lamiaceae* is regarded as one of the oldest highly evolved plant families (Hedge, 1992). *Scutellarioideae, Chloanthoideae, Pogostemonoideae, Teucrioideae, Nepetoideae, Ajugoideae* , *Viticoideae,* and *Lamioideae* are the eight subfamilies that make up this family (Cantino *et al*., 1992). The plants is a member of the *Ocimeae* tribe, which is made up of 1000 *species* and 35 genera, including the genus *Ocimum*. Primarily found in Africa, South America and Asia are tribe members (Paton & Ryding, 1998; Zomlefer, 1994). The Greek term ‘Okimon,’ which refers to an aromatic herb, is the source of the generic name *Ocimum*, and *gratissimum* meaning most pleasant. *Ocimum gratissimum*, then, is Latin for "the most pleasant scented herb." Clove basil, tree basil, African basil, wild basil and East Indian basil are some of the frequently used English names for *O. gratissimum.*

African native *Ocimum gratissimum L.* is a shrubby plant that produces essential oils and has therapeutic, anthelminthic and antibacterial qualities (Charles & Simon, 1992; Zomlefer, 1994). It is mostly an herb of wasteland and roadside edges, notwithstanding it is also prevalent in pastures. It is primarily spread through its seeds. When growing, it prefers fertile, moist soil, but after flowering, it can withstand harsh climate conditions (Iwu, 1999).

O. gratissimum is commonly referred to as 'Nunum' or 'Onunum' in Ghana. It is a 1 to 4 meter high, heavily branched shrub and its stem is ribbed. The plant is aromatic when crushed, and its opposing pale-green coloured leaves have bluntly serrated edges (Figure 1).

Figure 1: *Ocimum gratissimum L.* leaf branches

Distribution of *O. gratissimum*

Ocimum gratissimum is a geographically spread savannah plant that grows on different soil types at heights between 100 and 2000 meters above sea level, and in regions with annual rainfall around 500 to 1500 mm or even more (FAO, 1986).

Some of its *species* have been found in Malawi, the Uganda, Ivory Coast, Cameroon, Ghana, Sudan, Rwanda, Somalia, Ethiopia, Kenya, Tanzania,

Zimbabwe, Botswana, Zambia, Burundi, Angola, Sierra Leone, Nigeria, Namibia, Guinea and South Africa. It is historically spread across the tropical western, and southern Africa. The *species* has also reportedly been introduced to the West Indies, South America, North Yemen, and the Comoros Island.

Traditional Medicinal Uses

Ocimum gratissimum is a major medicinal herb used extensively throughout sub-Saharan Africa, not just in Ghanaian communities. To alleviate congested nostrils, this plant's potently scented leaves are rubbed between the hands and sniffed (Kokwaro, 1993). The leaves are also used to regulate menstruation, alleviate prolapse of the rectum, treat barrenness, sore eyes, convulsions, fever, ear infections and coughs. They are also used as a tooth gargle, for treating several oral health conditions (Harjula 1980; Watt & Breyer-Brandwijk, 1962; Kokwaro, 1993; FAO, 1986). Additionally, the plant species has been found to have insecticidal and antiseptic qualities (FAO, 1986). As a result, it is utilized in the formulation of perfumes as well as several pharmaceuticals (FAO, 1986). *O. gratissimum* contains a lot of chemotypes such thymol and eugenol. Methyl eugenol was discovered in 1989 to be the predominant component in young *O. gratissimum* plants, as compared to (Z)-ocimene in fully grown ones (Lawrence, 1989; Yusuf *et al*., 1998; Sanda *et al*., 1998).

Antimicrobial Activity of *Ocimum gratissimum*

The effectiveness of *Ocimum gratissimum* against several disease-causing microorganisms has been thoroughly established (Nakamura *et al.*, 1999; Matasyoh *et al.*, 2007). Microbes that can cause disease include bacteria,

fungus, and viruses. The majority of human illnesses are brought on by the bacteria *Klebsiella pneumonia, Staphylococcus aureus, Escherichia coli, Streptococcus pyrogenesis, Salmonella typhi* and *Pseudomonas aeruginosae*, among others. This therefore justified their selection as pathogenic microbes for use in this study.

The following microorganisms were used in this study; *Salmonella typhi, Escherichia coli, Staphylococcus aureus, MRSA, Klebsiella pnuemoniae, Streptococcus pyrogenesis, Shigella, Samonella poona,* and *Staphylococcus lentus. Escherichia coli, Staphylococcus aureus, Samonella poona, Staphylococcus lentus, Salmonella typhi* especially have previously been identified as being resistant to a number of antibiacterials (Matasyoh *et al.*, 2008; Cornelis, 2008; Kvist *et al*., 2008).

Zanthoxylum zanthoxyloides

As described by Matu (2011) *Z. zanthoxyloides* is a tiny spiky tree or shrub (Figure 2A). The maximum height ranges from 4 meters to over 11 meters. The tree or shrub's trunk is frequently fairly short. The quantity of light exposure and the various nutrients present in the surrounding soil affect how coloured the bark is around the tree. The colour spectrum ranges from red to brown to yellow to grey. The spectrum of colour varies from brown to yellow to grey or even to red. Leaves are, alternately, arranged and with the opposite leaflets here and there. The petioles of the main leaves, which are glabrous and have a petiole length of 2 to 5 cm, have leaflets that are rigidly papery, pinnately veined with 10 to 14 pairs of lateral veins that are barely noticeable and fuse near the margin. The leaflets range in shape from obovate to elliptical, with a

base that is cuneate to rounded, an apex that is about 5 to 25 cm long inflorescence with short branches. Flowers are sessile, unisexual, regular, 5-merous, white or greenish (Figure 2C), with somewhat protruding stamens on the male flowers and a higher ovary on the female flowers. Fruits are described as ovoid, brown, glandular-dotted, 1-seeded, dehiscent follicles with a diameter of 5 to 6 mm (Figure 2D). Black to bluish, lustrous, and long-lasting in the fruit is the seed.

Figure 2: Zanthoxylum zanthoxyloides whole plant (A), stem (B), flowers (C) and fruits (D).

Source: Adapted from Matu (2011)

Traditional Medicinal Uses

In Kenya and South Africa *Zanthoxylum species* are used to make pastes that are used to relieve pain and speed up the healing process of wounds and bruises (Kigen *et al.*, 2017; Bodede *et al.*, 2017). In Nigeria, some *Zanthoxylum species* like *Z. zanthoxyloide* are used to treat sickle cell anaemia, rheumatism, venereal disorders, toothache and urinary tract infection (Adesina *et al.*, 2005; Gbadamosi *et al.*, 2015; Ameh *et al.*,

2012). Furthermore, Ugandans consume decoctions prepared from the root-bark of *Z. zanthoxyloides* to treat malaria, gonorrhoea (Supabphol *et al.*, 2014) erectile dysfunction, abdominal pain, toothaches, dysmenorrhea, and elephantiasis (Andima *et al.*, 2020). In Cote D'Ivoire, *Z. zanthoxyloide*s' stem decoction is used to treat oral pathogenic infections and alleviate tooth discomfort (Kiyinlma *et al.*, 2020). Additionally, the leaves of *Z. zanthoxyloides* are used extensively in treating wound by facilitating its healing process while the root bark is used to treat toothaches, swellings and worms as well as inducing lactation after childbirth in Togo (Yaovi, 2018). In Ghana, the stem bark decoction is commonly used to treat malaria (Asase & Oppong-Mensah, 2009). Various portions of the plant are traditionally used to treat hypertension, diabetes, malaria and disorders of the circulatory and respiratory systems in the Central African Republic (Kosh-Komba *et al.*, 2017).

Antibacterial Properties of *Z. zanthoxyloides*

The plant *Z. zanthoxyloides* under examination in the current study has undergone numerous studies to assess its antibacterial and antifungal properties. Numerous published works about the chemical and biological importance of various species of the *Zanthoxylum* genus has also been reported. A study of various metabolites isolated from *Z. leprieurii* and *Z. zanthoxyloide* and their chemical and biological applications was published by Adesina (2005). In the study, the antibacterial properties of the extract from these two plants showed promising activity (Adesina, 2005). In a 2006 analysis of *Z. zanthoxyloides'* root bark, numerous biologically active chemicals were discovered. They displayed promising acetylcholinesterase inhibition, antifungal and antibacterial properties (Queiroz *et al*. 2006). In addition, Agyare *et al.*, (2014)

conducted a preliminary phytochemical screening and examined *Z. zanthoxyloide* extracts for antibacterial qualities. In a trial against multi-drug resistant *Staphylococcus aureus* with six *zanthoxylum species*, Mills-Robertson et al., (2017) found that the majority of the examined plants, including *Z. zanthoxyloides*, shown antibacterial properties. The antimicrobial activities of the aqueous-ethanolic extract of the roots, stem back and leaves of *Z. zanthoxyloides* was also examined in a paper published by Ngane et al. (2000). The outcomes showed that the tested fungi's in vitro growth was generally suppressed. Studies have looked at the effectiveness of essential oils as antibacterial and antifungal agents. One of these investigations was done by Tatsadjieu et al., (2003), who examined Cameroonian plants and found that the essential oils of *Z. zanthoxyloides* showed antibacterial and antifungal action against eight microorganism strains.

Phyllanthus fraternus

The plant *P. fraternus* of the *Phyllanthus* genus is a monoecious, upright, annual herb that grows to a height of 5 to 65 cm belonging to the Euphorbiaceae family and has a wide variety of growth patterns, including pachycaulous succulents, climbers, floating aquatics, annual and perennial shrubs (Burkill 1985; Khatoon *et al.* 2006 & Sen *et al.*, 2011). Its leaves are slender, practically sessile, numerous, alternating, imprecated and simple. In fresh condition, the lower surface is pale green and somewhat glaucous while the upper surface is green and glabrous. The leaves are elliptic-oblong in shape, with an entire margin, an obtuse (rarely sub-acute) apex and rounded bases. They range in size from 7 to 15 by 4 to 9 mm and have only one prominent rib in their leaves (the midrib). They frequently grow in gardens, landfills, and on

the sides of roads (Koffour & Amoateng, 2011). Occasionally referred to as leaf flowers, all Phyllanthus *species*, have a distinctive growth pattern called phyllanthoid branching. It is frequently mistaken for *P. amarus* and *P. niruri*, however, a few things could be used in differentiating the three species. *P. niruri* possesses six incredibly tiny petals, while *P. fraternus* and *P. amarus* have six and five noticeable petals, respectively (Sarin *et al*,. 2014; Sen *et al.*, 2011). Again, the stem of *P. Fraternus* is reddish, as compared to the whitish stems of *P. nuriri* and *P. amarus*. In Twi and Ewe, the common names are "kpavideme" and "bo mma gu w'akyi," respectively (Dokosi, 1998; Burkill, 1985).

Figure 3: *Phyllanthus fraternus* leaf branches

Traditional Medicinal Uses

Herbal practitioners in Ghana treat a wide variety of ailment with decoctions from the plant's leaves including jaundice, malaria, kidney disease, high blood pressure, diabetes, genital-urinary tract infections, stroke, liver disease, intestinal infections, anaemia, hepatocellular cancer, severe abdominal pains, and diarrhea (STEPRI and CSIR, 2007). *Phyllanthus fraternus* was reported to have antiviral, analgesic, pain-relieve and anti-oxidant

properties (Santos *et al.*, 1994; Calixto *et al.*, 1998), and anti-diabetic effects (Okoli *et al.*, 2010; Kushwah *et al.*, 2010). Additionally, *Phyllanthus fraternus* has been reported to decreases oxidative stress, which leads to the development of a number of degenerative disorders include hypertension and cardiovascular issues (Khairunnuur *et al.*, 2010). In Ghana and India, the plant is also used in the treatment of gonorrhea and ulcer (Burkill, 1985; Khan & Khan, 2004 & Chanda *et al.*, 2011). Leave decoctions are consumed to induce labor as well as to treat fever, oedema, and costal pain in Côte d'Ivoire. Fresh roots are also used to treat jaundice, male erectile dysfunction and are also consumed with milk as a galactagogue (Burkill, 1985). It has a variety of uses in Philippines, Malaysia, India for stomach issues, dropsy, and urino-genital infections. The herb is also used to cure other conditions like leprosy, anuria, biliousness, scabies, ringworm, oedematous, swellings, ophthalmia, and conjunctivitis (Oudhia, 2008; Burkill, 1985).

Antibacterial Properties of *P. fraternus*

The crude methanol extract has been reported to suppress microbial activity against the *fungus Aspergillus niger, P. aeruginosa*, and *Salmonella typhi B.* (Mehta *et al.*, 2014). The human fungus *Trichophyton mentagrophytes* and *Trichophyton rubrum*, however, did not respond to the aqueous or ethanol leave extracts (Bapat & Mhapsekar, 2012).

Bio-Active Compounds in Plants

An impressive number of compounds with impressive biological activities be found in plants. Quite a number of them includes fats and oils which are essential food reserves and are commonly deposited in specific

tissues at specific stages in the plant's life cycle. Others, including the waxes and the elements of suberin and cutin act as protective coatings on the outside of the plants. Some also aid in the survival of the species by promoting pollination or acting as a barrier against competing organisms. In addition to these substances, certain plants also biosynthesize chemicals such as rubber that apparently serves no defined purpose in the plant just as the psychoactive ingredient in marijuana, tetrahydrocannabinol (Salisbury & Ross, 1992). These constituents are referred to as bioactive compounds or secondary metabolites as they are not fundamentally necessary for healthy growth and development. Some of these bioactive constituents from plants such as alkaloids, terpenes, and flavonoids have been identified and implicated to have therapeutic qualities (Salisbury & Ross, 1992).

Alkaloids

Alkaloids are a wide group of naturally occurring nitrogenous organic compounds produced by plants. They are often present around the entire plant but occasionally only accumulate in certain parts. They are primarily synthesized by plants as a self-defense strategy against attack by organisms like herbivores and micro-organisms (Levin & York, 1978) and constitutes the greatest single group of secondary metabolites in plants (Harborne & Herbert, 1995; Harborne, 1984). Over the years, hundreds of chemically varied alkaloids have been identified from plant sources. Each of these many alkaloids has a unique pharmacological impact on both people and other animals (Harborne, 1984). By using acid base extraction technique, several alkaloids can be separated from unpurified extracts.

Most alkaloids are also generally basic compounds containing one or even more nitrogen atoms, mostly incorporated in the compounds being a part of a cyclic system and are also usually analogues of amino acids (Harborne & Herbert, 1995; Salisbury & Ross, 1992). Despite being the largest single class of secondary metabolites, only a few plant *species* contain them. Alkaloids may be present in considerable amounts at one phase in a plant's life cycle while being absent entirely from other plant parts at the same time. Potatoes are a common illustration. The plant's edible tuber is free of alkaloids, but its green parts are dangerous because they contain the toxic alkaloid solanine.

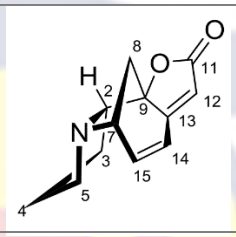
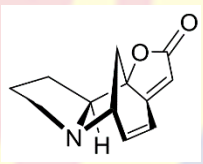
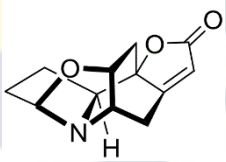
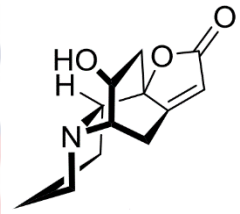
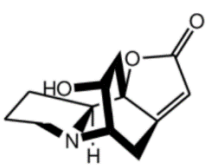
Though, some groups may often be toxic, their tremendous physiological and psychological activities in both humans and other animals make them particularly important (Harborne & Herbert, 1995). A few alkaloids, like nicotine, are liquid at ambient temperature and are often colourless despite the fact that most alkaloids are crystalline. Alkaloids can be detected in fresh fruits of leaves by their bitter taste on the tongue, a straightforward but not always accurate technique used by most herbal practitioners (Harborne & Herbert, 1995). One of the most bitter compounds in this class is the alkaloid quinine (Harborne, 1984). Other alkaloids such as the tropolone alkaloids found in autumn Crocus bulbs are primarily aromatic (Salisbury & Ross, 1992; Harborne, 1984). Strychnine is also a nerve stimulant alkaloid, so are reserpine and morphine which are tranquilizers and narcotics respectively (Ikan, 1992).

Alkaloids' physiological functions and their metabolism importance in the plants that produce them has been debated (Kurek *et al.*, 2019; Salisbury & Ross, 1992). It has been suggested that they serve just as by-products from important metabolic pathways and do not perform any significant

metabolic functions (Levin & York, 1978). However, there are few instances where they are of ecological significance and help the plant survive (Robinson, 1979). For instance, grazing animals and insects that feed on leaves will steer clear of plants that contain certain class of alkaloids known to be toxic. Others such as pyrrolizidine alkaloids (PA) are used by ithomiine and danaid butterflies as precursors for production of sex pheromones (Keeler, 1975).

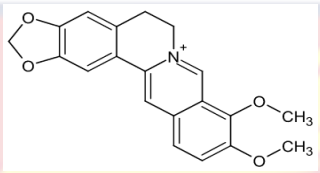
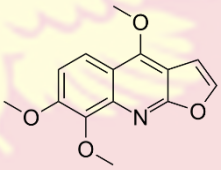
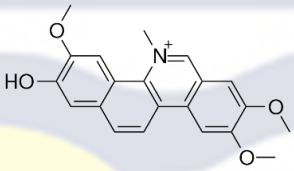
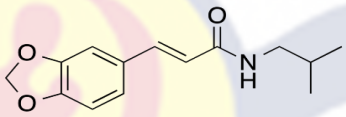
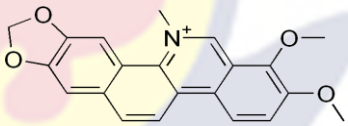
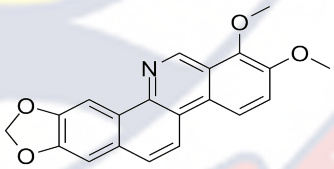
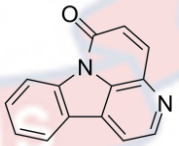
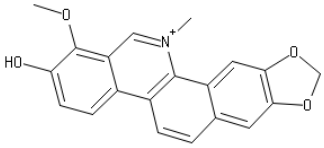
Isolated and Characterized Alkaloids from *P. fraternus*

Table 1: Name and Chemical Structures of Alkaloids Isolated from *Phyllanthus fraternus*

Name	Chemical Structure	Reference
(+)-Allonorsecurinine		Komlaga *et. al.*, 2017
***ent*-Norsecurinine**		Komlaga *et. al.*, 2017
Nirurine		Komlaga *et. al.*, 2017
Bubbialine		Komlaga *et. al.*, 2017
Epibubbialine		Komlaga *et. al.*, 2017

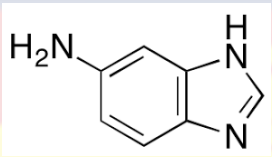
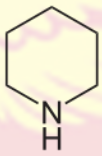
Isolated and Characterized Alkaloids from *Z. zanthoxyloides*

Table 2: Name and Chemical Structures of Alkaloids Isolated from *Z. zanthoxyloides*

Name	Chemical Structure	Reference
Berberine		Tatsadjieu *et. al.*, 2003
Skimmianine		Tatsadjieu *et. al.*, 2003
Fagaronine		Kassim *et. al.*, 2005
Fagaramide		Tatsadjieu *et. al.*, 2003
Chelerythrine		Tatsadjieu *et. al.*, 2003
Norchelerythrine		Queiroz *et. al.*, 2006
Canthin-6-one		Tatsadjieu *et. al.*, 2003
Fagaridine		Tatsadjieu *et. al.*, 2003

Isolated and Characterized Alkaloids from *O. gratissimum*

Table 3: Name and Chemical Structures of Alkaloids Isolated from *O. gratissimum*

Name	Chemical Structure	Reference
Benzimidazol-5-amine		Adeshina *et. al.*, 2018.
Pyrrolizidine derivative		Bageya *et. al.*, 2018

Pathogenic Microbes

Microscopic organisms are tiny, single-celled life forms found almost everywhere on Earth (NIAID, 2009). The group consists of viruses, algae, fungus (yeast and mould), parasites (metazoans and protozoans) and bacteria. Whereas some are pathogenic (opportunistic pathogens), others are not (Tortora *et al.*, 2001). Microbes that cause infectious diseases, therefore, are known as pathogenic microbes. The infections these microorganisms produce may spread from one person to another directly or indirectly, or they may be zoonotic (Pinner *et al.*, 1996). Majority of deaths worldwide are caused by these pathogens, and the gradual increase in pathogenic diseases has also been associated to increased antibiotic resistance (Toone, 2011). Due to this, researchers are working studiously towards finding novel antimicrobials that have low toxicity but high potency against resistant pathogenic microorganisms (Roger & Clire, 1999).

Antimicrobial Resistance (AMR) Mechanism of Action

The following types of antimicrobial resistance mechanisms have frequently observed in reported literature;

Biofilm Formation

Biofilms result from complex microbial interactions that firmly attaches pathogens to biotic or abiotic surfaces. One or more microbial species may be responsible for the formation of this structure. The biofilms themselves form extracellular matrix, within which the cells are encapsulated. Inside this film, the cells interact with each other and their surroundings (Soto, 2013). This polymeric structure shields the cells from antibiotic treatment, phagocytosis, biotoxins, hydrodynamic shear forces and host defence mechanisms (Costerton *et. al.*, 1999). In fact, about 65% of most infectious diseases are considered to be caused by biofilm forming microbes (Potera, 1999).

Enzymatic-Drug Inactivation

This is one of the most common resistance mechanisms for antibiotics of natural origin such as aminoglycosides. This is observed in enzymatic phosphorylation, acetylation or adenylation and enzymatic hydrolysis by β-lactamases (Egorov *et al.*, 2018). An already-existing cellular enzyme is altered to interact with the antibiotic in a way that the bacteria is no longer harmed. There are genes that code for these enzymes, and if such genes are present on plasmids as an additional genetic component, they will facilitate the development of resistance by the bacterial cell.

Gene Transfer from Other Species by Target Proteins of Lower Susceptibility

The first methicillin-resistant *Staphylococcus aureus* (*MRSA*) strain was discovered in the UK in 1960 (Jevons, 1961; Harkins *et al.*, 2017). The methicillin-resistant penicillin-binding protein PBP-2A or 2, whose expression is primarily stimulated by methicillin and many other β-lactams was found to be present when *MRSA* was produced. According to Brown and Wright, (2005), the gene is located in a huge portion of DNA (30–60 kb) that appears to have originated from a different organism than *S. aureus* and contains additional genes that encode for resistance to macrolides (such as erythromycin, clarithromycin, and roxithromycin) and amino-glycosides (like gentamicin, amikacin, tobramycin). These bacteria are resistant to numerous existing medications with the exception of vancomycin due to the inclusion of a plasmid containing gene that codes for the conventional β-lactamase and tetracycline resistance (Brown & Wright, 2005).

Preventing Drug Access to Target (Efflux Pumps)

Tetracycline was the first medication found whose antibacterial action could be blocked by an active efflux process, and this resistance mechanism can also block drug influx into Gram-negative bacteria (Waters *et al.*, 1983; Levy, 1992). Since the influx of nutrients is also reduced via the latter approach, it might be detrimental to bacterial growth. However, it is also present in some intestinal bacterial species as a "last-resort" defence against the most recent β-lactam antibiotics that can tolerate inactivation by the majority of conventional β-lactamases. Due to the discovery of the multi-drug

efflux pump, the efflux mechanism is now recognised for the crucial role it plays in drug resistance (Piddock, 2006).

The main clinically significant efflux mechanisms in Gram-negative bacteria are usually made up of an outer membrane protein pathway, a periplasmic protein, and a cytoplasmic membrane pump, all belonging to the RND class (Soto, 2013). The two most common efflux pump systems observed in Gram-positive bacteria are MFS (such as Bmr and Blt in *Bacillus subtilis* and NorA in *Staphylococcus aureus*) and the ABC transporters (Li & Nikaido 2009; Soto, 2003).

Target Bypass

Vancomycin, a fermentation byproduct derived from the bacteria, *streptomycetes*, has an unusual mechanism of action (Nambiar *et al.*, 2012). The rationale is that vancomycin attaches itself to a lipid-linked disaccharide pentapeptide substrate, a precursor to cell wall peptidoglycan, as opposed to the enzyme inactivating mechanism by other microbes. Some researchers assumed that it might be challenging to develop resistance to vancomycin as a result of this mechanism. However, due to *enterococci's* natural resistance to β-lactams, aminoglycosides, macrolides (such as erythromycin), and tetracycline as the typical inhabitants of the human digestive tract, resistant strains of vancomycin currently predominate among *enterococci* (Perkins, 1969). It is now common for this vancomycin-resistant strain of enterococci to colonise people in the vicinity of hospitals and other healthcare institutions, leading to infections that are more difficult to treat (Clardy *et al.*, 2006).

Target Protein Mutational Alteration

Even while being completely synthetic, antibacterial agents like fluoroquinolones are unlikely to be rendered inactive by the enzymatic pathways described by Brown and Write, (2005), Nevertheless, bacteria can develop resistance due to mutations that could render the target protein less susceptible towards an antibiotic. For instance, DNA topoisomerases and target protein mutations by some microorganism such as Mycobacterium tuberculosis and Helicobacter pylori are the main causes of fluoroquinolone resistance. This mechanism of resistance occurs as a result of changes to certain genetic make-ups, and it cannot be easily passed to other cells since the inheritor will continue to be drug-susceptible as it would still be producing the unaltered target protein eventually rendering it susceptible (Lambert, 2005). Almost all categories of pathogens are rapidly developing fluoroquinolone resistance through this mechanism (Brown & Wright, 2005).

Bacteria Used in the Study

Bacteria used for the study were based on their WHO priority; involving five (5) high priority, three (3) critical priority and two (2) medium priority pathogens.

Escherichia coli (E. coli)

These bacteria are among the major bacterial *species* that make up the gut flora in mammals' lower intestines. They are however, not only limited to this area; species have also been found in other settings, such as the edges of hot spring. There are hundred strains of this type of bacteria that causes fatal infections to humans (Karch *et al.*, 2005). In general, *E. coli* strains cause a

number of gastrointestinal and extra-intestinal infections, including pneumonia, meningitis, peritonitis, mastitis, and urinary tract infections. Although optimal development is achieved at 37 ^{0}C, the majority of strains are motile and can grow between 18 and 44 ^{0}C (Slack *et al.*, 2007; Cheesbrough, 2007).

Different diseases are inflicted by various *E. coli* strains. For instance, infantile enteritis is caused by enteropathogenic *E. coli* (*EPEC*), mainly in impoverished nations (Slack *et al.*, 2007). Community-acquired and traveler's diarrhoea are caused by enterotoxigenic *E. coli* (*ETEC*) (Slack *et al.*, 2007). While entero-aggregative *E. Coli* (*EAggEC*) is associated with persistent diarrhoea and vomiting, particularly in youngsters, entero-invasive *E. Coli* (*EIEC*) is associated with a condition similar to shigellosis (Cheesbrough, 2007). Haemorrhagic colitis, hemolytic uraemic syndrome, and watery diarrhoea are all inflicted by verocytotoxin-producing *E. coli* (*VTEC*) (Slack *et. al*., 2007). Notwithstanding, the *E. Coli* species are the most frequent causative agents of bacterio-pyogenic liver abscesses (PLA), a biliary disease (Alvarez *et al*., 2001; Rahimian *et al.*, 2004; Chan *et al.*, 2013).

Staphylococcus lentus

The zoonotic, commensal, coagulase-negative, Gram-positive bacterium *Staphylococcus lentus* typically inhabits the skin of various *species* of animals. The pathogen's robust structure is a result of the cocci that make up its clusters (Stepanovi *et al.*, 2005; Issa *et al.*, 2018). According to Stefanovi et al., (2005), it is a member of the *Staphylococcus sciuri* class, which comprises *S. vitulinus, S. lentus,* and *S. sciuri.*

S. lentus is typically a pathogen found in animals and has frequently been found in fowls, dairies, and other food-producing farm animals as well as their products. There have also been reports of disease carriers among those who care for these animals (Deinhofer & Pernthaner, 1995). Similar to other *staphylococci*, *S. lentus* is capable of acquiring genes for antibiotic resistance, such as the erythromycin ribosomal methylase (*erm*) genes that give resistance to the antibacterials macrolide, lincosamide, and streptogramin B (Hauschild et al., 2005).

Streptococcus pyogenes

In the genus *Streptococcus*, *S. pyogenes* is a *species* of aerotolerant, gram-positive bacteria. It thrives best in environments with about 10% carbon dioxide, is a facultative anaerobe, and produces minuscule colonies on agar plates containing blood (Cunningham, *et al.*, 2000).

A major human-specific bacterial pathogen *Streptococcus pyogenes* causes a wide range of symptoms, from benign localised infections to potentially fatal invasive infections (Ibrahim *et al.*, 2016). Acute rheumatic fever and post-streptococcal glomerulonephritis are post-infectious manifestations of ineffective *S. pyogenes* infection treatment (Kanwal & Vaitla, 2022). *S. pyogenes* typically infests the vaginal mucosa, anus, and pharynx. *S. pyogenes* infections are extremely contagious. Atmospheric particulates, hand contact with sneezing, touching infected objects or surfaces, skin contact with contaminated sores, and contaminated fruits and vegetables can all be routes of transmission (Cunningham, *et al.*, 2000).

Salmonella enterica subsp. enterica serotype poona

There are more than 2,500 different serotypes of Salmonella species, and about 50 of these variants can infect people (Jackson *et al.*, 2013). Clinically considered, there are two pathogenic subgroups of *Salmonella species*; typhoidal *Salmonella* and non-typhoidal *Salmonella* (NTS). In immunocompetent people, NTS can result in invasive infections in patients with weakened cellular immunity, pregnant women, children under the age of five, meningitis, osteomyelitis, and aortitis (Chen *et al*, 2013). Ingesting contaminated food and water or coming into contact with animals directly or indirectly are the two ways that humans can contract NTS (Pegues & Miller, 2015).

The majority of *S. Poona* disease reports to date have been linked to gastrointestinal conditions and exposures to foodborne pathogens (Laughlin *et al.*, 2019). Children and adults have both been reported to have invasive *S. Poona* bacteraemia, which is frequently linked to reptile exposure (Fukushima *et al.*, 2020).

Klebsiella pneumoniae

The bacterium, *Klebsiella pneumoniae*, is a member of the *Enterobacteriaceae* family. It belongs to the genus *Klebsiella* and *species pneumoniae*. The natural flora of the mouth, skin, and intestines contain rod-shaped, facultatively anaerobic, Gram-negative, non-motile, encapsulated, lactose-fermenting bacteria that belong to the genera *Klebsiella* (Ryan & Ray, 2004). The *Enterobacteriaceae* genus *Klebsiella* contains *K. pneumoniae*, which is clinically the most significant member. For urinary tract

infections in elderly people, *Klebsiella* comes in second place to *E. coli* (Stamm, 2001). Additionally, it is an opportunistic infectious agent for people who have intestinal pathogenicity, rhinoscleroma, nasal mucosa atrophy, and chronic pulmonary illness (Johnson & Parham, 2016; Gonzales & Murali, 2016). The two main sources of patient infection are though faeces and contact with infected medical equipment (Gonzales & Murali, 2016).

Shigella Sp.

They are gram-negative, non-motile, rod-shaped bacteria and do not produce spores. In the majority of African countries, shigellosis, is responsible for an estimated 10% of all recorded outbreaks of food-borne illness. Animals rarely contract it, and it is commonly identified in waterbodies contaminated by human excrement. *Shigella dysenteriae, shigella boydii, shigella flexneri* and *shigella sonei* are a few other species (Hale, 1996). They are facultative rods of the human digestive system that do not ferment lactose or produce gas and classified based on a combination of biochemical characteristics and antigenic examination (Prescott, 2004). Newborn babies and kids who develop dysentery typically die from it. Although the disease is frequently self-limiting for most adults, this could be attributed to some resistance produced by adults in endemic regions. The disease is notably prevalent in day-care centers and homes for children with disabilities (Oshikoya *et. al.*, 2006). *Shigellosis* also often infects the gastrointestinal tract and is mainly spread from one person to another through the faecal-oral route. according to the United States, Center for Disease Control (2002), sometimes, at daycare centers, epidemics can breakout from contaminated foods handled by infected people.

***Salmonella enterica serovar typhimurium* (*S. typhi*)**

Salmonella bacteria are gram-negative *bacilli*, members of the *Enterobacteriaceae* family, and facultative anaerobes. They may survive on a variety of simple media and can be distinguished from other *species* of the family by their biochemical properties and antigen structure (Slack *et. al.*, 2007). Typhoid fever is caused by *Salmonella Enterica Serovar Typhi*, which was previously given species status as *S. typhi*. The faeces oral route is one way the bacterium might spread. *Salmonella* bacteria infections are mostly linked to sanitation and hygiene, which accounts for their high incidence in developing nations (Cheesbrough, 2007). *Salmonella* bacteria that have been ingested by a host targets the small intestine's epithelial cells before infiltrating the endothelial and into the submucosa connective tissue, where it is subsequently taken up by macrophages (Jawetz & Levinson, 1996).

Staphylococcus aureus

The most prevalent *Staphylococcus* strain that causes infections, *Staphylococcus aureus*, is a sheroidal bacterium that typically resides on a person's skin or in their nose and can cause variety of life-threatening diseases. *Staphylococcus* infections could range from benign skin infections like cellulitis, pimples and boils to more severe ones like toxic shock syndrome (TSS), septicemia, pneumonia, endocarditis, and meningitis (Jameson *et al.*, 2018; Berlon *et al.*, 2014). *S. aureus* is now resistant to a variety of frequently administered antibiotics. *S. aureus* is a commensal organism that may live on surface of the skin, especially in the scalp, underarm and inguinal region (Foster, 2017).

When natural defences are compromised, *S. aureus* might spread to other tissues like the, mucosal lining, resulting in furuncles (boils) and abscess (Knox *et al.*, 2015). One of the causes of mastitis in dairy cows is *Staphylococcus aureus*, and the bacteria is shielded by its thick capsule from the immune system of the cow (Campos *et al.*, 2022).

Structure of bacteria

Bacterial cells are encased and surrounded by a complex rigid layer that protects them and helps maintain their shape and structure. Peptidoglycan (PG), a rigid net-like structural macromolecule that gives the exterior cell wall stiffness and support, is one of the important elements that make up the bacterial cell wall. Also known as a penicillin-binding protein (PBP), the peptidoglycan enzyme DD-transpeptidase, acts to cross-link a single peptidoglycan chain to others similar peptidoglycan chains in order to form the cell wall. In order to compensate for multiple cycles of cell growth and reproduction, the cell wall and therefore the peptidoglycan cross-links are continually biosynthesized and modified throughout a bacterial lifespan.

Bacteria Cell Wall Biosynthesis

The bacteria cell wall is a peptidoglycan structure that is made up of repeating units of N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) (Figure 4) that forms a rigid cell wall that shields the organism from toxic substances. This rigid wall also acts to prevent the cell from expanding abnormally and bursting.

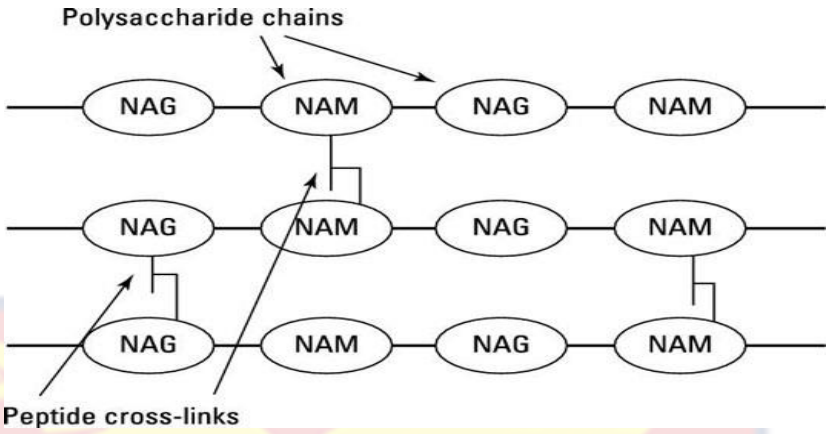


Figure 4: Repeating units of the peptidoglycans NAG and NAM that makes up the bacteria cell wall
Source: Adapted from Yocum *et al.* (1980)

These two peptidoglycans need to be cross-linked by a peptide bond to make the cell wall strong and fixed (Figure 5). The cross-link in forming the bacteria cell well is between the two amino acids; N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) (Figure 5). The organism continuously produces, cross-links and repairs the peptidoglycan chains that make up the cell wall. The transpeptidase enzyme is used to achieve this because it can combine the terminal amino acid groups of two different peptidoglycan chains, forming a robust peptidoglycan structure (Yocum *et al.*, 1980) (Figure 6a).

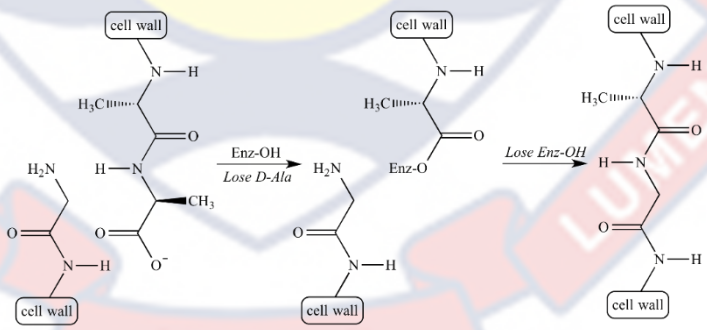


Figure 5: Cross-linking of peptidoglycan monomers by the transpeptidase Enzyme
Source: Adapted from Yocum *et al.* (1980)

Two nucleophilic acyl substitution reactions are involved in the mechanism; the first reaction generates an intermediate ester that reacts to produce a new amide bond which links the peptidoglycan chains (Figure 6b).

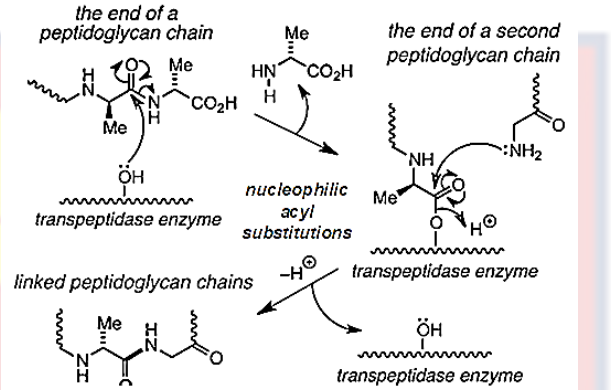


Figure 6: Nucleophilic acyl substitution reactions resulting in cross-linked peptidoglycan bacteria cell wall
Source: Adapted from Yocum *et al.* (1980)

Antibiotics

Antibiotics are used to treat bacterial infections. These are substances that selectively destroys or inactivates the growth of microbes, when used in the proper dosa. They are mostly produced as bioactive secondary metabolites by life forms that live in the soil. Antibiotic toxicity to humans and other animals is typically regarded as low (Ramirez *et al.*, 2020).

One of the most widely used medications is an antibiotic. They are also among the medications that doctors frequently abuse, for instance when they prescribe antibiotics to treat viral respiratory infections (Dawan *et. al.*, 2022). Antibiotic-resistant bacteria have emerged as a consequence of the careless use of antibiotics, posing a huge threat to public health worldwide. Genetic changes in bacteria may be the main factor causing antibiotic resistance, but other factors may also contribute, such as the inappropriate or excessive use of antimicrobial medication, that also undoubtedly creates an environment that is favourable for

the development, emergence, spread, and persistence of resistant microorganisms (Tortora *et. al.*, 2001; Beitha *et al.*, 2008). The choice of antibiotics in treating an infection is dependent on a variety of parameters such as the organism that caused it (Tortora *et. al.*, 2001).

Notwithstanding that some antibiotics may operate as bactericides, when used in clinical settings, especially when given in high doses, on the hand, the majority of antibiotics are bacteriostatic (Milton, 1990). The latter action, while suppressing bacterial replication, uses the immune system (white blood cells) to incapacitate any bacteria that remains after drug action. Tetracycline and other antibiotics like chloramphenicol, for instance, work against bacteria by preventing them from synthesising the proteins needed for certain body parts (Mandell, 1982). As a result, there will be a complete reduction in cell-growth and eventually disruption of cell division (Mandell, 1982 & Milton, 1990). On the other hand, bactericides, such as penicillins, prevent bacteria from forming a cell wall, which inevitably results in cellular breakdown and death (Milton, 1990).

Antibiotics Mode of Action

The mechanism of action of various antibiotics varies. This is as a result of their chemical compositions and affinities to specific areas of bacterium cells. For example, penicillin prevents bacteria from synthesizing their cell walls and therefore will have high affinity into the part of the cell containing the cell wall (Wise & Park, 1965). Although cell walls are absent from human and animal cells, bacteria on the other hand need them for survival and growth.

Sulfonamides prevent bacteria from synthesizing DNA. Although their actions do not completely destroy bacteria, they do prevent their growth and reproduction. This action provides the host's immune system quite enough time to combat bacterial infections.

Penicillin and many other β-lactam antibiotics are classified as bactericides as they effectively kill bacteria by degrading their cell walls. Penicillins, cephalosporins and monobactam, commonly known as β-lactam antibiotics, form the major antibiotic families whose basic structures contain the β-lactam ring. The vast majority of these bactericides function by suppressing the biosynthesis of bacteria's cell walls. The β-lactam ring is the portion of the molecule responsible for the penicillin antibiotic's bactericidal activity. By inhibiting the activity of the enzymes involved in cell wall cross-links formation, it prevents bacteria from synthesizing their cell walls (Wise & Park, 1965). The ring opens upon contact with bacteria and forms an irreversible bond with the enzyme that is responsible for facilitating cross-linking in bacterial cell walls. Water then enters the cell, the osmotic pressure in the cell increases, leading to the cell's eventual rupture.

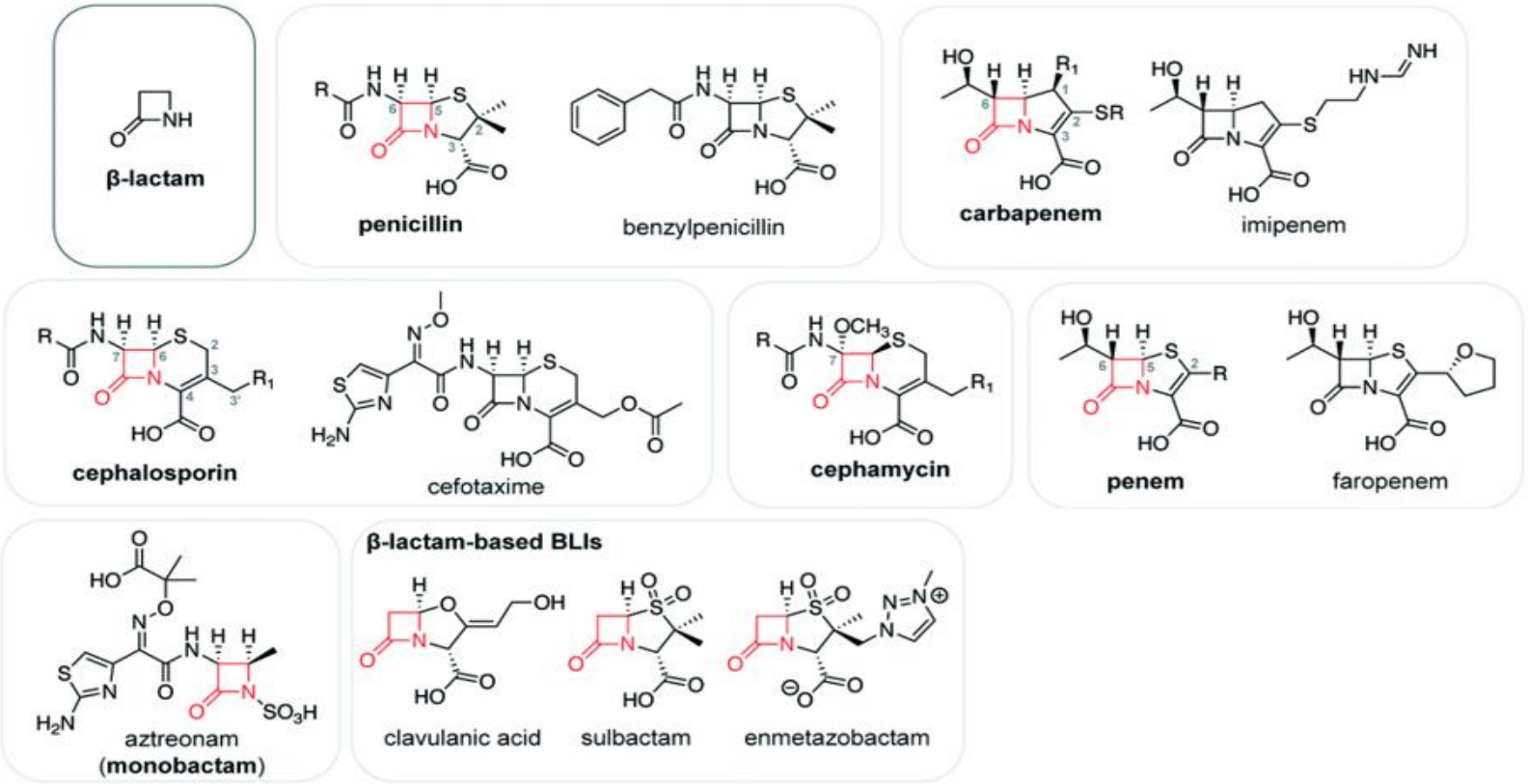


Figure 7: Chemical structures of some β -lactam antibiotics

Source: Tortora *et. al.* (2001)

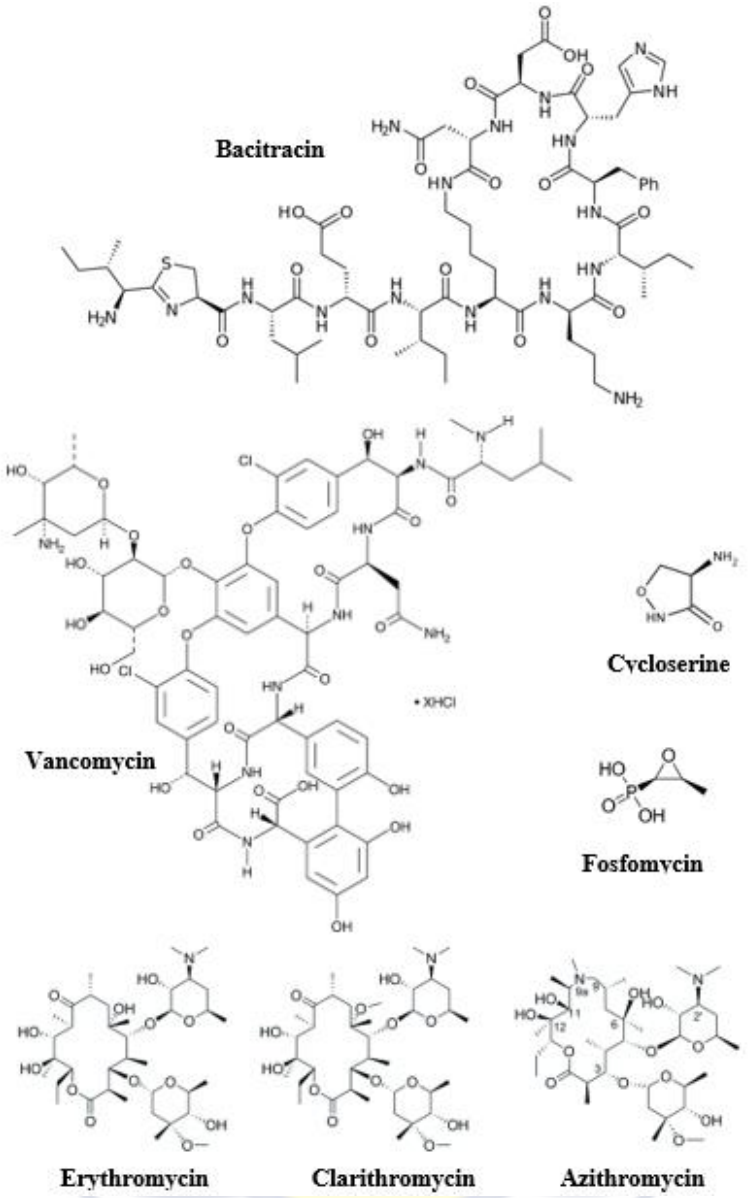


Figure 8: Chemical structures of some non-β-lactam antibiotics

Source: Tortora *et. al.* (2001)

Penicillin Mode of Action

Penicillin (and most beta-lactam antibiotics) act by interfering with the synthesis of the bacteria cell wall. It binds to the transpeptidase enzyme and inhibits the enzyme from cross-linking the peptidoglycan monomers which results in a weak and fragile cell wall (Yocum *et al.*, 1980). This involves mimicking the end of one of the peptidoglycan chains, the beta-lactam ring

opens and a nucleophilic acyl substitution reaction between the transpeptidase enzyme and penicillin results in the formation of an ester that permanently binds the penicillin to the enzyme (Figure 9). Since the bulky penicillin-enzyme complex would hinder a nucleophile from attacking the ester carbonyl, the ester is unable to react with the second peptidoglycan chain (Yocum *et al.,* 1980; Ghooi *et al.,* 1995). The enzyme's inability to form cross links at this point, causes the peptidoglycan wall to gradually disintegrate (Yocum *et al.*, 1980; Sidow *et al.,* 1990). The cell wall wouldn't contain any more peptidoglycan chains and eventually, water enters the cell causing it to burst.

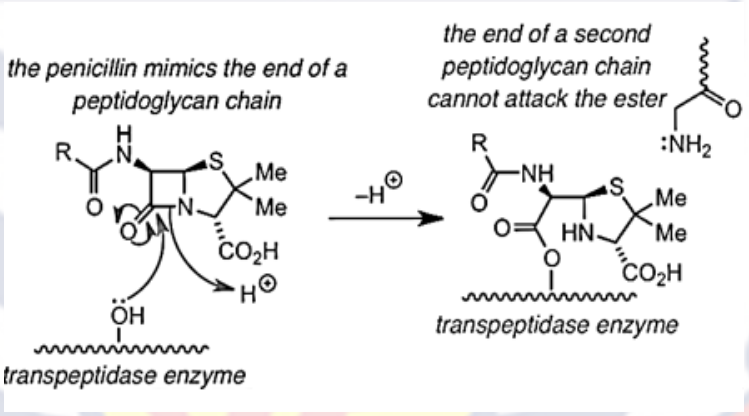


Figure 9: Penicillin irreversibly bound to transpeptidase enzyme

Source: Yocum *et al.,* (1980)

Sulfonamide Antibiotics Mode of Action

Prontosil (a prodrug) is an example of a sulfonamide antibiotic which was found to be antibacterial effective in-vivo but ineffective in-vitro. This is

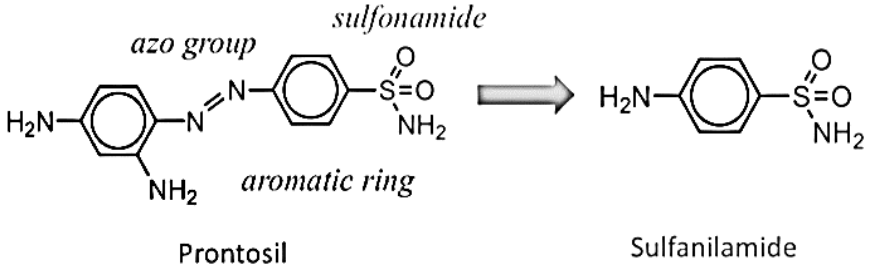


Figure 10: Active Sulfanilamide synthesized from Prontosil
Source: Yocum *et al.* (1980)

because prontosil is converted to the active Sulfanilamide (Figure 10) by enzymes in the small intestines in mammals (Fuller, 1937; Bhat *et al*., 2005).

The structural similarity of sulfanilamide with para-aminobenzoic acid (PABA) makes sulfanilamide a highly potent antibiotic (Figure 11).

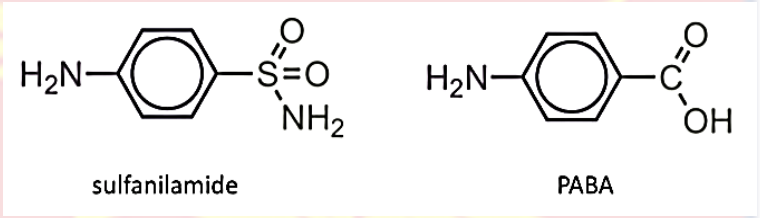


Figure 11: Chemical structure of sulfanilamide and p-aminobenzoic acid
Source: Yocum *et al.* (1980)

Tetra-hydrofolate, which is essential for DNA synthesis in bacteria, can only be produced by the action of PABA. The enzyme DHPS interacts with the PABA molecule at its active site to convert PABA into tetra-hydrofolate (Wegkamp *et al.*, 2007). Sulfanilamide and PABA have structures that are so similar that the DHPS cannot distinguish them from one another. In the active site of DHPS, both compounds assume similar shapes (Engevik *et al.*, 2019). As a result, sulfanilamide can enter and bind to the DHPS active site and inhibit PABA from binding, which will essentially prevent bacteria from synthesizing DNA and tetrahydrofolate (Wegkamp *et al.*, 2007; Zessel *et al.*, 2014; Engevik *et al.*, 2019) (Figure 12). Despite the fact that sulfonamide antibiotics are also very effective antibiotics, penicillins have consistently shown to be the most effective antibiotic and as a result have been the preferred medication for treating the most of infections. This is due to the fact that its action completely induces cell death rather than just acting as the sulfonamide's containment mechanism.

Antimicrobial Assays

There are numerous assays available to analyse a compound's *in vitro* antibacterial activity. These methods include broth-dilution and well-diffusion. The well-diffusion approach involves inoculating agar plates with microorganisms before adding an antibiotic of interest to each well. With the broth dilution bioassay, the drug is initially diluted in broth-filled test tubes before inoculating each tube with microorganisms (Schumacher *et. al.,* 2018; Balouiri et. al., 2016). These techniques have been utilised for a while and has produced quite a few noteworthy results; however, they have certain advantages and disadvantages. Therefore, more advanced screening strategies are being explored, and techniques with quick, repeatable, and easy procedures are being developed. One drug susceptible assay that has the merit of being straightforward, simple, quick and economical is the high-throughput spot culture growth inhibition (HT-SPOTi) assay. It uses small amounts of inhibitory/diagnostic material or drug to produce direct, accurate and rapid results (Danquah *et. al.*, 2016).

High-throughput spot culture growth inhibition (HT-SPOTi) assay

The HT-SPOTi is a type of agar dilution bioassay that involves culturing microorganisms on agar with varying concentrations of potential antibiotics or inhibiting agents over the course of three steps. By making slight adjustments to the growth media and growth conditions, this technique can be modified to work with a wide range of microorganisms. Additionally, it permits the concurrent evaluation of various potential antibiotics or compounds on the same microtitre plate. HT-SPOTi drug sensitivity testing employs the following

steps; selection and culturing the strains of microorganisms to be used (typically, the sub-culturing of the microbes is done twice or more to confirm the viability of the microbial species through continuous growth), preparing stock solutions of the drugs to be tested along with the reference standards (typically 50 mg/mL for new drug testing), and preparing test concentrations using serial dilution technique, inoculation and incubation of prepared microtiter plates (96 well plate). The test's results (MIC) are analysed by examining the plate for visible growth in the test wells compared to the obvious growth in the negative control well where no antimicrobial agent is present and for possible no growth or only minimal growth in the wells of the reference drugs. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) can both be determined using the HT-SPOTi bioassay (Danquah *et. al*., 2016).

Microbial culture

The goal of antimicrobial assay techniques is to detect the susceptibility of prevalent pathogens and clinically significant drug-sensitive reference strains. Use of sensitive strains of microbes or resistant strains, which fall under the categories of pathogenic Gram-positive and Gram-negative bacteria together with pathogenic fungi, is required to detect antimicrobial activity of plant extracts, drugs, or synthetic compounds. The overall bioburden affects how well the extract or drug samples works. To achieve optimal bioburden, standardisation of the inoculums is therefore essential (Cos *et. al.,* 2006; Suss *et al.*, 2018). In general, for most bacteria and fungi, it is recommended to use about 1 x 10^5 CFU/mL and 1 x 10^4 CFU/mL, respectively.

To utilise as stocks for preparing inoculums, Bio-freeze cultures or cultures that were produced overnight have been suggested (Cos *et. al.*, 2006).

Electrospray Ionization Mass Spectrometry (ESI-MS)

The most frequently utilized ionisation method for (bio)pharmaceuticals is the electrospray ionisation (ESI), developed by M. Dole (Dole *et al.*, 1968), a technique which was then utilised by J. B. Fenn for biomolecules for the first time in 1989 (Fenn *et al.*, 1989). In order to create gas phase ions that can enter the mass analyzer, the analyte solution from the LC (Liquid Chromatography) is spritzed (vaporised) via a capillary under a voltage of between 2 and 5 kV. Protonated $[M+H]^+$ or deprotonated $[M-H]^-$ molecular ions that are already in solution are required for ESI. In the last 20 years, liquid chromatography (LC) and electrospray ionisation mass spectrometry (ESI-LC/MS) have since become popular combinations as a versatile workable alternative technique for transforming solution-phase constituents into gas-phase. The ESI's soft nature results in spectra with a high degree of molecular ion intactness and low fragmentation.

An ion produced by ESI fragments into two species: a charged species that is identified as the product ion and a neutral species that is lost for MS detection. The sites and functional groups in a prospective molecule that can be (de)protonated must therefore be thoroughly understood.

In the positive ion mode, the adducts Na^+, K^+, and NH_4^+ are frequently observed, whereas in negative ion mode, the anions Cl^-, and CH_3COO^- are encountered (Cech & Enke, 2001). These typically encountered ions (adducts) result from glassware, additives in the mobile phase, impurities in the

solvent and so forth (Kruve & Kaupmees 2017). In the literature (*Keller et al.*, 2008; Huang *et al.*, 1999) and online, there are many lists of possible adducts. The properties of the analyte determine what types of ions are produced during ESI, as protonated/deprotonated, Na^+, K^+ adducts, etc.

Ions are generated in Electrospray Ionisation MS (ESI-MS) by adding a proton ($[M+H]^+$). The resultant ion produced, however, can be a M+ ion when the analyte molecule is already charged, as in the case of quaternary amine salts.

"Mathematical Charge Deconvolution" is the method of determining the charge and mass of the analyte using the ESI-Mass Spectrum (Covey *et al.*, 1988; Mann *et al.*, 1989; Chapman *et al.*, 1992). As illustrated in (Figure 12), the procedure is simple and predicated on the idea that two consecutive peaks in an ESI-mass spectrum of a single analyte have a charge state difference of one (1). Two mathematical expressions can be written, if the analyte's molecular mass is considered to be M and the measured m/z values for its two nearby charge states, n^+ and $(n + 1)^+$, are m_1 and m_2, respectively. The two equations therefore will be;

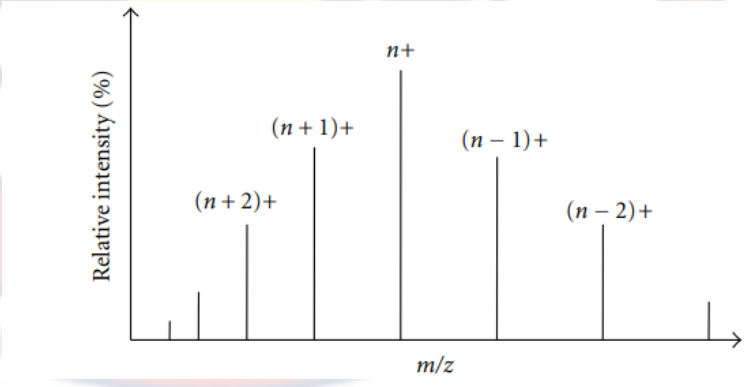


Figure 12: A typical schematic representing the nature of ESI-Mass Spectrum in positive ion mode
Source: Adapted from Chapman *et al.* (1992)

$(M + n)/n = m_1$ and

$[M + (n + 1)]/(n + 1) = m_2$

Since there are only two unknown parameters M and n, a minimum of two equations are required to find out the values of M and n. So, the above two equations can easily be employed to determine the value of n (charge state) and M (deconvoluted mass). One can think the observed multiple peaks as the multiple mass assessment of the same molecule. Making M the subject and equating the two equations give;

$$m_1 n - n = m_2 n + m_2 - n - 1$$

$$m_1 n - m_2 n = m_2 - 1$$

$$n = \frac{m_2 - 1}{m_1 - m_2}$$

Where m is the mass-to-charge and $m_1 > m_2$

By averaging the calculated masses for each peak, one can determine the analyte's molecular mass with more accuracy. The aforementioned method produces more effective results when the analyte generates sequential charge states that differ by unity and when it is a pure analyte (Banerjee & Mazumdar, 2011).

Chapter Summary

As indicated in the review of relevant literature, plants have been used in the treatment of various bacterial infections for many years. Traditionally, these plants in always used due to proximity and affordability. Alkaloids in these plants have also shown to have promising antibacterial properties. The

chapter elaborated on what alkaloids are, their chemical structure and their importance in the treatment of infectious diseases. The chapter also highlighted on bacteria, their cell wall and the mode of action of beta-lactam antibiotics on the cell wall of bacteria.

CHAPTER THREE

MATERIALS AND METHODS

Introduction

Different laboratory equipment and material were involved in the present study. Preliminary phytochemical screen was performed on all three crude extracts. *In-vitro* antibacterial screening, time-kill kinetics and modulation studies were all performed using a 96 micro-titer well plate.

Equipment

Evaporation was conducted using EcoChyll S (Manufacturer: Ecodyst, Serial number: 7770101, Model number: ECO777, Capacity: 2 L, Apex, USA) rotary evaporator system in *vacuo*. Optical density of media was measured using an Elisa microplate reader (Agilent BioTek synergy Neo2, China). Molecular masses of isolated compounds were determined using LC-ESI-MS, TLC plates were dried before spotting using a hand dryer, Silica gel 60 (70 – 230 mesh ASTM) from Merck (Germany) was used for column chromatography. Thin layer chromatography (TLC) was conducted using Aluminium pre-coated silica gel plates 60 F_{254} of 0.25 mm thickness (Germany). And UV lamp (wavelength 254 nm) was used for spot visualization, Multichannel and single channel micropipettes were used for transferring solutions and media into microplates. Microwave oven (manufacturer: LG Electronics, Weijing, Model number: MS2042DB) was used for heating media. All media, test-tubes and pipette tips were sterilized using Autoclave (Manufacturer: Changsha, Hunan, China, Model: LC-75HD, Serial number: 21L-0123, Vessel volume: 75L). Incubator (Manufacturer: Panasonic Healthcare, Japan, Model number: MIR-154-PE,

Serial number: 12100218) was used in incubating microorganisms. Inoculated broth media in test-tubes were thoroughly mixed using Vortex mixer (Manufacturer: Scientific Industries, USA, Model number: G56E, Serial number: 4.781.487).

Chemicals, Media, Antibiotics and Reagents

Mueller-Hinton broth (MHB) was purchased from Merck, Germany, Mueller-Hinton agar (MHA) was purchased from Oxford Ltd, England. Bacteriological Peptone. Saline, Phosphate buffer saline (PBS) solution, Ciprofloxacin (CIPRO), Tetracycline (TETRA), crystal violet, McFarland solution.

Preparation of Reagents

Reagents for Phytochemical Screening

Wagner's Reagent

This was prepared following the standard protocols as described in literature (Karumi *et. al.*, (2004). Potassium iodide (2 g) and iodine crystals (1.27 g) were dissolved in distilled water (10 ml) in a volumetric flask (100 ml) and the solution topped up to mark with distilled water. The presence of alkaloids was inferred from the formation of large brown amorphous and flocculent precipitate after treating the hydrochloric acid solution (5 ml) of extract with some drops of the reagent.

Mayer's Reagent

This was prepared following the standard protocols as described in the literature (Debiyi & Sofowora, 1978). Mercuric iodide (1.36 g) was dissolved

in distilled water (60 ml) and the resultant solution was added to solution of potassium iodide (5 ml) in distilled water (10 mL). The mixture was topped up to 100 ml with distilled water.

Dragendorff's Reagent

This was prepared following the standard protocols as described in literature by Karumi *et. al.*, (2004). Hydrated bismuth nitrate (4 g) was dissolved in concentrated nitric acid (10 ml) and the resulting solution was slowly added to a solution of potassium iodide (13.47 g) in distilled water (25 ml). Potassium nitrate precipitate was filtered off and the ensuing filtrate topped up to 50 ml with distilled water.

Liebermann-Burchard Reagent

This was prepared following the standard protocols as described in the literature (Sofowaa, 1993). Concentrated H_2SO_4 (5 ml) was cautiously added to acetic anhydride (5 ml). The resulting mixture was slowly added while cooling in ice to absolute ethanol (50 ml).

Salkowski Reagent

This was performed in accordance with the standard protocols documented in the literature (Harborne, 1998; Trease and Evans, 1989). Concentrated H_2SO_4 (5 ml) was cautiously added to distilled water (100 ml). The resulting solution was added to 0.1 M $FeCl_3$ (15 ml).

McFarland Standard

This was prepared following the standard protocols as described in the literature (McFarland, 1907). McFarland standard (0.5) was prepared used and

used for the study. Barium Chloride (1%) solution was prepared by mixing anhydrous $BaCl_2$ (1 g) in distilled water (100 ml). Sulfuric acid solution (1%) was also prepared by adding conc. H_2SO_4 (1 ml) to distilled water (99 ml). The two solutions were combined in appropriate proportion to make a McFarland standard (0.5) by adding $BaCl_2$ (50 µl) to H_2SO_4 (9.95 ml). This was shaken to form a turbid suspension.

General Experimental Methods

Plant Material Selection and Collection

The three plants selected for this study were *Occimum gratissimum, Zanthoxylum zanthoxyloides and Phyllantus fraternus.* All plant materials were collected in the Cape-coast in the Central region in Ghana and their identities authenticated by a taxonomist, at the Botany Department, University of Cape Coast, Ghana and given the voucher numbers; Eup. CC 5152 (*P. fraternus*), Labiate CC 960 a (*O. gratissimum*), Fagara. CC 4226 (*Z. zanthoxyloides*).

Drying and Grinding

The collected plants were washed to remove soil and other debris and then evenly distributed on a large clean plastic sheet in the chemistry lab for air-drying for three – four weeks. A mechanical grinder was used to mill the dried plant materials to about 1 mm particle size to increase the surface area for extraction.

Preparation of Extract for Qualitative Phytochemical Analysis

As described in literature by Soebagio & Rusdiana (2007), plant constituents were extracted with 80 % ethanol for a preliminary qualitative

phytochemical analysis. Dried *O. gratissimum* leaves (80 g), *P. fraternus* whole plant (65 g) and *Z. zanthoxyloide* leaves (95 g) were separately transferred to a 500 ml Erlenmeyer flask and 250 ml 80% ethanol (120:30 v/v) was added. The extract solutions were separately placed over a steam bath and heated for one hour with a 75 mm filtering funnel fitted to the neck of the Erlenmeyer flask. After one hour, the solution was cooled to room temperature and filtered through a whatmann paper. The extract from each plant material was concentrated at 40 ^{0}C under reduced pressure to about 30 ml and was used for qualitative phytochemical analysis.

Qualitative Phytochemical Analysis

Qualitative phytochemical analysis was performed on the ethanolic extracts from the three plants, to determine the class of secondary metabolites present as described by Harborne (1998), Sofowora (1993), Trease and Evans (1989), (Soebagio & Rusdiana, 2007) and Wanyama *et. al.*, (2011). The extracts were screened for the presence of alkaloids, steroids, tannins, terpenoids, saponins, cardiac glycosides and flavonoids using these protocols.

Test for Alkaloids

This was performed in accordance with the standard protocols documented in the literature (Sofowora, 1993; Karumi et. al., 2004). Five (5) mL of 2 M HCl solution was added to 4 mL of the 80% ethanolic solution. This was stirred, heated and filtered. The filtrate from the extract was then divided per three test tubes. Dragendorff's reagent was added to one portion of the test solution. To another portion Mayer's reagent and to the remaining test solution Wagner's reagent. These were then observed for turbidity or colour change.

Screening for Saponins

This was performed in accordance with the standard protocols documented in the literature (Harborne, 1998). Ethanolic solution (2 ml) was added to deionized water (4 ml). This was vigorously shaken and left to stand for about 10 minutes. The presence of saponins was inferred from the formation of thick persistent foam.

Test for Steroids

This was performed in accordance with the standard protocols documented in the literature (Harborne, 1998; Trease and Evans, 1989). Acetic anhydride (2 ml) was added to 2 mL of the 80% ethanolic solution in a test tube and boiled. Upon cooling, H_2SO_4 (2 ml) was added to the resultant mixture. The presence of steroids was confirmed by the development of a brown ring at the interface; with a green upper layer.

Screening for Cardiac Glycosides (Salkowski Test)

This was performed following the standard protocols as described in the literature (Harborne, 1998). Glacial acetic acid (2 ml) containing a drop of $FeCl_3$ solution was transferred into a test tube with ethanolic solution (2 mL). Concentrated H_2SO_4 was cautiously added along the wall of the test tube to form a lower layer. The aglycone moiety of the cardiac glycoside was inferred from the appearance of a reddish-brown colouration at the interface.

Test for Tannins

This was performed following the standard protocols as described in the literature (Debiyi & Sofowaa, 1978; Sofowora, 1993. The extract (0.2 g) was

dissolved in aqueous methanol (5 ml). The resultant mixture was split between 2 test tubes where one portion served as a blank and the other, freshly prepared $FeCl_3$ was added. A greenish or dark blue colouration confirmed the presence of tannins.

Test for Flavonoids

This was performed in accordance with the standard protocols documented in the literature (Harborne, 1998). Three test tubes were each filled with 2 mL of the ethanolic solution. The first test tube served as a blank solution. Magnesium turnings were put in the second test tube followed by the addition of hydrochloric acid (0.5 m). It was observed for change in colour. Concentrated hydrochloric acid (0.5 ml) was added to the third test tube and heated on a water bath for about 5 minutes. The presence of flavonoids and leucoanthocyanins were confirmed by the change in colour in the second and third test tubes with reference to the first test tube.

Methanol/Dichloromethane crude extraction

O. gratissimum (730 g), *P. fraternus* (800 g) and *Z. zanthoxyloide* (820 g) of the plant materials were separately transferred into a 2 L volumetric flask and macerated in mixture of 1.5 L MeOH/DCM (1:1) for 72 hours with regular shaking. The supernatants were filtered through whatmann paper and the 'marc' from each plant was macerated with 1.5 L MeOH for another 72 hours with regular shaking. The separate extracts from each plant were then combined and concentrated at 45 ^{0}C under reduced pressure. The crude extracts obtained were weighed (*O. gratissimum* = 42.70 g, 5.85 *%; P. fraternus* = 58.14 g, 7.23 %; and *Z. zanthoxyloides* = 53.19 g, 6.49 %) and stored in a desiccator until used.

Crude Alkaloidal Extraction

The alkaloidal extraction procedure was carried out as described in literature by Acheampong *et. al.*, (2021). For *P. fraternus* and *Z. zanthoxyloide*, respectively, 38.06 g and 40.11 g of the crude extracts were dissolved in 30 ml 10 % acetic acid solution. For *O. gratissimum*, 22.63 g of the crude extract was dissolved in 30 ml 20 % acetic acid. The resulting solution was defatted with hexane (7×50 ml) until the organic layer was almost colourless. The aqueous layer was then combined and basified with 70 ml aq. NH_3 to a pH between 8 – 9. This solution was then extracted with 5×30 ml of chloroform ($CHCl_3$) until the organic later was almost colourless. The chloroform extracts were then concentrated at 40 ^{0}C under reduced pressure, dried and weighed (*O. gratissimum*, 0.2672 g, 1.18 %; *P. fraternus*, 0.2442 g, 0.64 %; *Z. zanthoxyloide*, 0.5627 g, 1.40 %). The crude alkaloid extracts were named as *AEOG* = Crude alkaloid extract of *O. gratissimum*, *AEPF* = Crude alkaloid extract of *P. fraternus* and *AEZZ* = Crude alkaloid extract of *Z. zanthoxyloide* and stored in a desiccator until used.

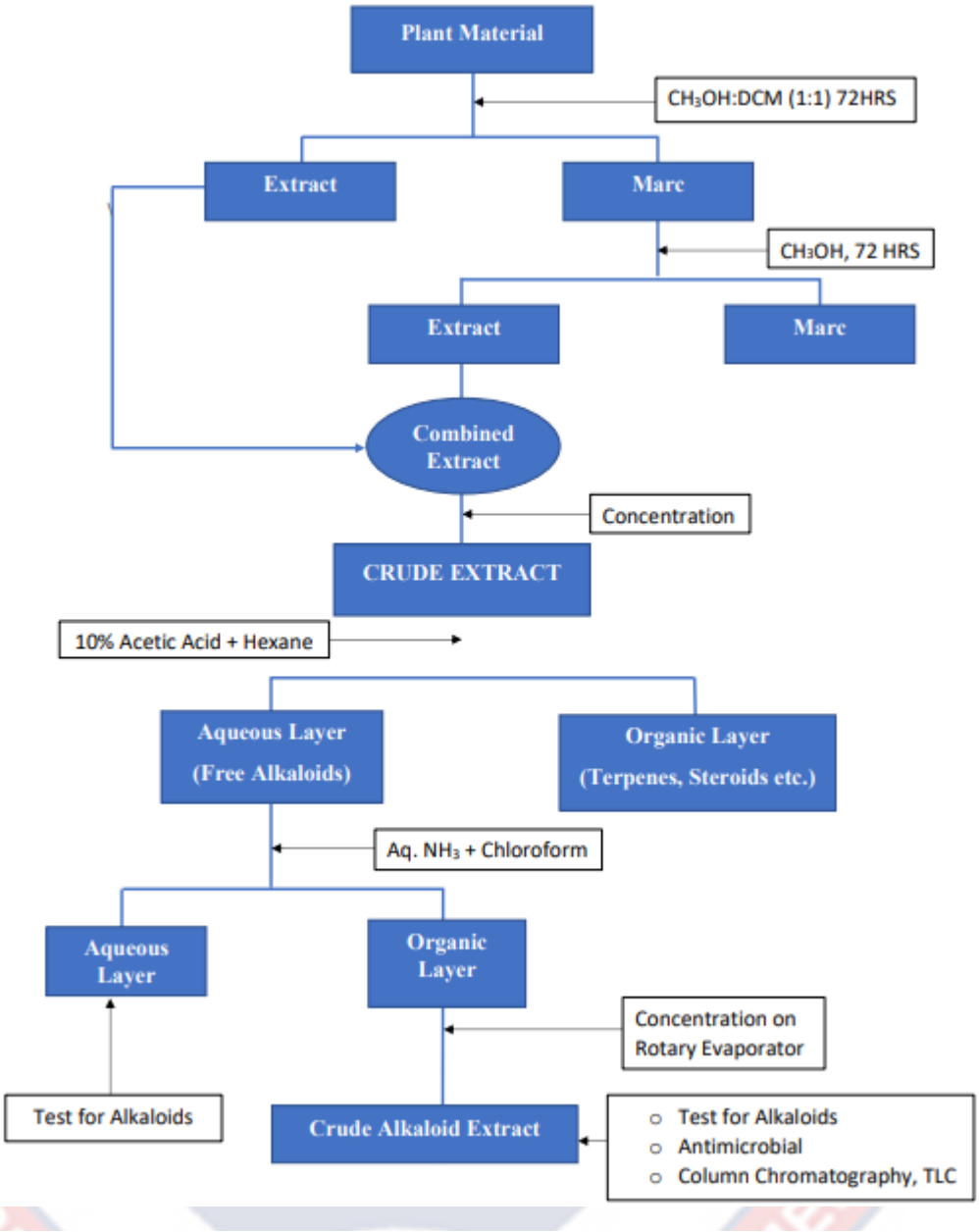


Figure 13: Crude alkaloid extraction flowchart

Biological Assays

The crude and alkaloidal extracts obtained from each of the three herbal plants were tested for their antibacterial properties, rate of killing or inhibition and antibiofilm forming properties.

Microorganisms Used in the Study

The antimicrobial activities of the crude extracts were determined with 10 bacteria comprising;

1. Four Gram-positive, namely; *Staphylococcus aureus, Methicillin-Resistance Staphylococcus Aureus* (*MRSA*), *Streptococcus pyogenes, Staphylococcus lentus* and
2. Six Gram-negative, namely*; Escherichia coli, Klebsiella pneumoniae, Salmonella poona, Shigella, Salmonella typhi.*

All the microorganisms were obtained from the Microbiology Laboratory of the Department of Biomedical Sciences, School of Allied Health, University of Cape-Coast (UCC).

Preparation of Media

Mueller-Hinton Broth Preparation

This was prepared in accordance to the standard protocol described in literature (Muller & Hinton, 1941). Powdered sample (3.8 g) was added to distilled water (100 ml) in 250 ml Erlenmeyer flask. The resulting media was then covered with cotton and then aluminium foil and sterilized by autoclaving at 121 ^{0}C for 15 minutes.

Mueller-Hinton Nutrient Agar Preparation

This was prepared following the protocol described in literature (Muller & Hinton, 1941). Powdered sample (0.8 g) was added to distilled (100 ml). weighed and transferred into a 250 mL Erlenmeyer flask added. The resulting

media was then covered with cotton and aluminium foil and then sterilized by autoclaving at 121 ^{0}C for 15 minutes.

Preparation of Buffered Peptone

This was prepared as described in the literature (Baird *et al.*, 1996). Powdered peptone (2.5 g) was dissolved in distilled water (125 ml) in a 250 mL Erlenmeyer flask, covered with cotton and aluminium foil and sterilized by autoclaving at 121 ^{0}C for 15 minutes.

Preparation of the Inoculum

This was done as indicated in literature (Anokwah *et al.*, 2016). Microorganisms cultured on nutrient agar medium as agar slants in falcon tubes were picked from their parent cultures with a loop, streaked on their respective solidified agar slants. The inoculated plates were then incubated at 37 ^{0}C (overnight) for 18 – 24 h. After incubation, the cultures were diluted with normal saline to a cell count concentration of approximately 1x10^5 CFU/ml, by suspending microorganisms in sterile saline solution (0.9 %) to a concentration equivalent to a McFarland standard (0.5) turbidity. Further dilutions in broth were performed by adding saline-inoculum media (200 µl) to broth solution (1800 µl) to make a final working inoculum media of approximately 5x10^5 CFU/ml.

***In vitro* HT-Spot Culture Growth Inhibition Assay (HT-Spoti)**

The antibacterial properties of the crude alkaloid extracts from the three plants were determined using the high-throughput spot culture growth inhibition (HT-SPOTi) assay as described in literature (Danquah *et al.*, 2016). Each of the

alkaloid crude extracts (0.5 g) was dissolved in distilled water (10 ml) to give stock concentrations of 50 mg/ml using 2 % DMSO.

Two-fold serial dilutions of the extract stocks were carried out in a PCR half-skirted plate to give working concentrations in the range of 3.9 to 500 µg/ml. This was done by adding distilled water (100 µl) to column B to G of the plate. Column A was filled with the original stock (200 µl) of the alkaloid crude extract. Crude alkaloid extract (100 µl) of the stock from column A was added and mixed with the distilled water in column B. The same procedure was done for column C with the solution from column B and continued for the subsequent columns until column G. Column H served as a control for the solvent (DMSO) used. Using a single channel pipette, the crude alkaloid extract dilutions (2 µl) was transferred into corresponding wells in a 96-well plate. Molten agar (196 µl) was dispensed into each well and the plate shaken for about 10 seconds to obtain a uniform distribution of the drug and allowed to solidify. The standardized bacterial suspension (2 µl) was dispensed into each well containing the crude alkaloid extract-media mixture using a single channel pipette. The plates were sealed with parafilm, covered with aluminium foil and incubated at 37 ^{0}C for 24 hours. After 18-24 hours, the plates were visually observed for growth or no growth and the MIC determined. Ciprofloxacin and Tetracycline were used as the standard (control) drugs. All experiments were carried out in duplicates.

Biofilm Study

Tube method (TM) as described by Christensen et al., (1982) was used for qualitative assessment of biofilm production. A loopful inoculum was

inoculated on Tryptone soy broth (TSB) (10 ml) in test tubes. The test tubes were incubated at 37 °C for 24 hours. The test tube contents were discarded and were washed with phosphate buffer saline (1×9 ml) pH 7.2 and then discarded. For biofilm fixation, a volume of 10 ml freshly prepared sodium acetate (2%) was added to each tube for 10 minutes and then discarded. For biofilm staining, 10 ml crystal violet (0.1%) was then added to each tube, and tubes were left at room temperature for 30 minutes after which the stain was discarded. The tubes were then left to dry in an inverted position at room temperature. Biofilm formation was detected by the presence of visible film on the wall and bottom of the tube.

Time-Kill Kinetic Study

Time-kill kinetics of crude alkaloid extract from the three medicinal plants and the extract-drug in combination was carried out in 24 hours following the procedure described by Mojsoska et al., (2017), with slight modifications. In the presence or absence of the crude alkaloid extracts, i*n-vitro* first and last six (6) hours constant MIC time-kill curves were performed in duplicates for the three crude alkaloid extracts that showed activity at MICs less or equal to 250 µg/ml against the test strains. Crude alkaloid extract (2 µl) was dispensed into appropriate wells and Mueller-Hinton nutrient broth (196 µl) added. The standardized bacterial suspension (2 µl) was dispensed into each well containing the extract-media mixture using a single-channel pipette. The optical density of 620 nm at time zero (0) was read using the Elisa micro-plate reader. The plates were then sealed with parafilm, covered with aluminium foil and incubated at 37 °C. For the first 6 hours, the optical density at 30 minutes intervals was taken. Form the last 6 hours (18 – 24 hours) the optical density at

one (1) hour interval was taken. The same test protocol was used to study and monitor the activity of the modulated standard Tetracycline with 'indifference' interaction using the three crude alkaloidal extracts. A blank, comprising the Mueller-Hinton nutrient broth and crude alkaloid extracts, and a negative control experiment, comprising Mueller-Hinton nutrient broth, bacteria and no extract were also run simultaneously.

Modulation Assay

Drug Level Interaction of The Three Alkaloidal Crudes & Tetracycline in Combination

The standard antibiotic (Tetracycline) was modulated using the alkaloid crude extracts from the three plants. Crude alkaloid extracts of the three medicinal plants which showed good activity (MICs ≥ 125 µg/ml) against seven (7) bacteria strains with Tetracycline MICs ≥ 15.6 µg/ml were selected for the study. The following organisms (with their tetracycline MICs) were involved; *Shigella*, 125 µg/ml; *MRSA*, 125 µg/ml; *Escherichia coli* ATCC 43888,15.6 µg/ml; *Staphylococcus aureus*, 15.6 µg/ml; *Klebsiella pneumoniae*, 15.6 µg/ml; *Salmonella poona*, 15.6 µg/ml; and *Staphylococcus lentus*, 15.6 µg/ml.

Synergy Measurement by Checkerboard Analysis

This was performed according the standard protocols described in the literature (Trabelsi *et al.*, 2020). The 8MICs for tetracycline and crude alkaloid extract were both prepared from the 50 mg/ml stock solutions. The volume required to prepare 8MIC concentrations were determined using the dilution formula $C_1V_1 = C_2V_2$.

After preparing the 8MICs for the individual crude alkaloid extracts and tetracycline, two 96-well microtiter plates were used for the synergy checkerboard assay for each pair (i.e. *O. gratissimum*:Tetracycline, *P. fraternus*:Tetracycline and *Z. zanthoxyloides*:Tetracycline). On the first plate (plate 1), different concentration of the respective MICs of the extracts and the standard Tetracycline ranging from 8MIC – 1/16MIC were prepared. This was done by dispensing the crude alkaloid extract (200 µl) in well B1, and Tetracycline (200 µl) into well A2 (Figure 14a). Two-fold serial dilution concentration ranges of 8MIC, 4MIC, 2MIC, MIC, 1/2 MIC, 1/4 MIC, 1/8 MIC and 1/16MIC on each row and column were prepared (as described earlier in HT-SPOTi method) by adding distilled water (100 µl) to rows C1 – H1 and columns A3 – A9 of the plate. The crude alkaloid extract (100 µl) from well B1 was dispensed and mixed with the distilled water in well C1. The same procedure was done for subsequent wells D1 – H1 by dispensing the solution (100 µl) from well C1 into well D1. This same method was used in preparing serial diluted MICs for the antibiotic (tetracycline), from well A3 – A9. Well A1 contained no solution. After preparing the respective concentrations, equal volumes (10 µl) of the MICs from the respective columns and rows were combined (Figure 14). Thus, 8MIC Tetracycline (10 µl) was combined with 8MIC, 4MIC, 2MIC, MIC to 1/16MIC of the crude alkaloid extract solution (10 µl) and the same for 4MIC, 2MIC and all the remaining concentrations of Tetracycline and the crude alkaloid extracts.

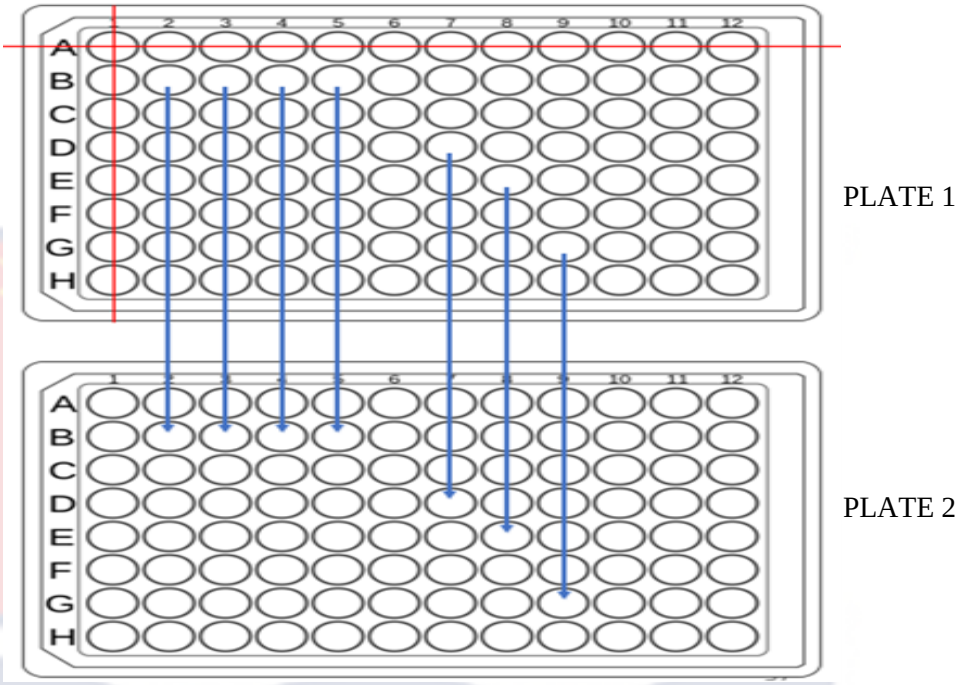


Figure 14: Combined extract and drug mixture from plate 1 are transferred into respective wells on plate 2

The combined tetracycline-extract mixtures (2 µl) were transferred into corresponding wells on the second 96-well plate (plate 2) (Figure 14). Using a multi-channel pipette, molten agar (196 µl) was dispensed into wells containing extract-drug mixture and the plate shaken for about 10 seconds to obtain a uniform distribution of the drug and allowed to solidify. Standardized microbial suspension (2 µl) was then added using a single-channel pipette. The plates were sealed with parafilm, covered with aluminium foil and incubated at 37 °C for 24 hours. After 24 hours, the plates were visually observed for growth or no growth and their FIC indices determined.

FIC Determination

The fractional inhibitory concentration (FIC) index can be used to quantify the interaction of the combined agents. The drug level interaction of

each of the crude alkaloid and tetracycline in combination was quantified by calculating the fractional inhibitory concentration (FIC) index using the formula below (Kohanski *et. al.*, 2010):

$$\frac{A}{MIC\ (A)} + \frac{B}{MIC\ (B)} = FIC\ (A) + FIC\ (B) = FIC\ Index$$

Where A and B are the MIC of each antibiotic in combination (in a single well), and MIC (A) and MIC (B) are the MIC of each drug individually.

A number of different outcomes are possible depending on the chemical compositions of the two antibacterial agents in combination therapy (drug modulation). The net combined drug action could be predicted as additive, antagonistic, or synergistic effect based on the FIC index of the pair (Bonapace *et al.*, 2002; Balouiri, 2016). FIC indices were interpreted using (Table 4).

Table 4: FIC Index Value Interpretation

Type of Interaction	FIC Value
Synergy	< 0.5
Antagonism	> 4
Additive or Indifference	0.5 – 4

Source: Data adapted from Balouiri (2016)

Data analysis

All graphs were plotted in excel and the results were presented as tables. All bioassays were performed in duplicates and their mean determined for analysis.

Chromatographic Analysis

Purification of Components in Crude Alkaloid Extract

Chromatographic Materials and Techniques

Silica gel 60 (70-230 mesh, ASTM, Merck, Germany) was used as the stationary phase material for the fractionation by column chromatography technique. Aluminium pre-coated with silica gel 60 F_{254} (0.25 mm thickness) was used for the analytical thin layer chromatography

Solvents and Reagents

The following solvents were used for the extraction, column chromatography and TLC analysis of the components:

- Dichloromethane (DCM), Petroleum ether (40-60 ℃), Methanol, and Ethyl acetate. All solvents were of analytical grade.

Thin Layer Chromatography (TLC)

Prior to running the column chromatography, thin layer chromatography (TLC) described by Bobbit (1964) was performed to obtain a good solvent system for better separation of the alkaloidal constituents in each of the three crude alkaloid extract. Small quantity (about 10 mg) of each crude alkaloidal extract was dissolved in ethyl acetate (0.5 mL) and the extracts spotted on TLC silica gel plate (G60 F_{254}, 0.25 mm thickness) as the stationary phase using capillary tubes. The plates were developed in 6 ml pre-saturated chromatographic tanks containing solvent systems of different polarities and ratios as the mobile phase; DCM, Methanol, Hexane, Ethyl acetate, Chloroform, DCM- Ethyl acetate (2:4 v/v), Hexane-Ethyl acetate (1.5: 4.5 v/v), Hexane-

Ethyl acetate (1:5 v/v). The spotted TLC plates were then placed in respective chromatographic jars. The developed plates were dried and observed for characteristic fluorescence and quenching of the resolved spots for compounds under UV lamp at 254 nm. Pictures were taken of the plates under the UV lamp. Dragendorff's reagent was also occasionally used in detecting spots.

Column Chromatographic Fractionation of the Crude Alkaloidal Extracts

Figure 15: Flash chromatography setup

A column (0.20 cm x 1.5 cm) was firmly clamped onto a retort stand and packed with 50 g of silica gel (70-230 mesh, Merck, Germany). Chloroform crude alkaloid extract (0.4 g) of each plant was weighed, dissolved in 5 mL DCM and adsorbed onto 3.5 g of diatomaceous earth and further dried by transferring the powdered sample into a round bottom flask and heating it on a rotavap for 10 – 20 minutes at 40 ^{o} C. The adsorbed dried extract was packed gently onto the stationary phase in the column. The surface of the packed column was covered with a wad of cotton wool to prevent disturbances of the surface upon addition of eluent. The elution was done with hexane, hexane-ethyl acetate (5:1 and 5:3) and methanol successively to obtain different

fractions. Based on TLC analysis, the fractions were combined as appropriate after verification and concentrated at 40 ^{o} C in *vacuo* for the HPLC-GCMS analysis. Detailed information for the HPLC-GCMS methods and their mobile phase parameters are included in the relevant sections.

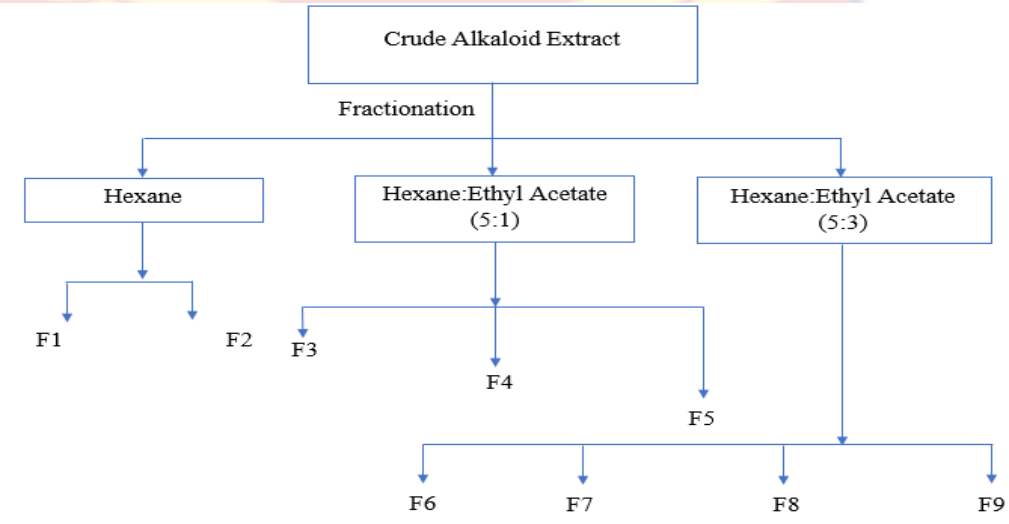


Figure 16: A schematic diagram for the purification and separation of compounds from the crude alkaloid extract of the three medicinal plants

Chromatographic Separation and Purification of the Crude Alkaloids in *O. gratissimum*

Crude alkaloid extract of *O. gratissimum* (0.4 g) was dissolved in 5 ml DCM, absorbed on 3 g of diatomaceous earth and further dried on a rotavap at 40 ^{0}C in *vacuo*. The resulting material was loaded on the prepared column and separation was down by the flash chromatographic technique with 100 % (v) hexane, a mixture of hexane-EtOAc (50:10 v/v and 50:30 v/v) in increasing polarity; Hexane (100 %) < Hexane:EtOAc (50:10 v/v) < Hexane:EtOAc (50:30 v/v). Sixty-three (63) fractions were collected in small test-tubes and combined into nine (9) bulk fractions (F1 – F9) using TLC as a guide to combine fractions

of similar constituents (R_f and spot colour). The combined fractions were concentrated to less than 1 mL, pipetted with micropipette into 1.5 mL HPLC sample vials and placed in a desiccator to further dry them until required for LC-MS analysis.

Chromatographic Separation and Purification of the Crude Alkaloids in *P. fraternus*

Crude alkaloid extract of *P. fraternus* (0.3 g) was dissolved in 4 ml DCM, absorbed on 3 g of diatomaceous earth and further dried on a rotavap at 40 ^{0}C in *vacuo*. The resulting material was loaded on the prepared column and separation was down by the flash chromatographic technique with 100 % (v) hexane, a mixture of hexane-EtOAc (50:10 v/v and 50:30 v/v) in increasing polarity; Hexane (100 %) < Hexane:EtOAc (50:10 v/v) < Hexane:EtOAc (50:30 v/v). Fifty-six (56) fractions were collected in small test-tubes and combined into eight (8) fractions (F1 – F8) using TLC as a guide to combine fractions of similar constituents (R_f and spot colour). The combined fractions were concentrated to less than 1 mL, pipetted with micropipette into 1.5 mL HPLC sample vials and placed in a desiccator to further dry them until required for LC-MS analysis.

Chromatographic Separation and Purification of the Crude Alkaloids in *Z. zanthoxyloides*

Crude alkaloid extract of *Z. zanthoxyloides* (0.5 g) was dissolved in 5 ml DCM, absorbed on 4 g diatomaceous earth and further dried on a rotavap at 40 ^{0}C in *vacuo*. The resulting material was loaded on the prepared column and separation was down by the flash chromatographic technique with 100 % (v)

hexane, a mixture of hexane-EtOAc (50:10 v/v and 50:30 v/v) and 100 % (v) MeOH in increasing polarity; Hexane (100 %) < Hexane:EtOAc (50:10 v/v) < Hexane:EtOAc (50:30 v/v). Seventy-one (71) fractions were collected in small test-tubes and combined into twelve (12) fractions (F1 – F12) using TLC as a guide to combine fractions of similar constituents (R_f and spot colour). The combined fractions were concentrated to less than 1 mL, pipetted with micropipette into 1.5 mL HPLC sample vials and placed in a desiccator to further dry them until required for LC-MS analysis.

LC-ESI-MS Analysis

LC-MS analysis was done at the Pesticides Unit of Ghana Standards Authority, Accra (Ghana).

Preparation of Sample for LC-ESI-MS Analysis

Extracts were purified by a flash column chromatography. Prior to ESI-LC/MS, the combined extracts with similar spots from TLC analysis were evaporated to dryness and dissolved in 1 mL methanol in HPLC sample vials.

LC-ESI-MS Analysis of Extract

Reversed-phase high-performance liquid chromatography with electrospray ionization mass spectrometry was used for the analysis (LC-ESI-MS). An MS QQQ Mass spectrometer (AB Sciex, USA) was used in conjunction with an Agilent 1200 HPLC system (Agilent Technologies, USA) integrated with a binary gradient solvent pump, degasser, autosampler, and column oven. Separations were carried out at 25 °C on a Zorbax SB-C18 column (2.1 50 mm, 1.8-m particles; Agilent Technologies, USA) with a mobile

phase of 0.1% HCOOH in water (solvent A) and methanol:Acetonitrile (v/v) (solvent B), using 2 µl injections. The process was slightly modified from that of Nowacka et al., (2014). The gradient time was 16 minutes, and the flow rate was 100 µl min^{-1}. With a capillary temperature of 300 °C, curtain gas pressure of 30 psi, and nebulizer gas pressure of 60 psi, the ESI worked in the positive ion mode. The collision and curtain gas was nitrogen.

Chapter Summary

This chapter describes in depth the research tools, chemicals, reagents, media and instrumental procedures used to conduct the research and how the results were analyzed.

CHAPTER FOUR

RESULTS AND DISCUSSION

Overview

Phytochemical analysis of the three crude plant extracts have revealed alkaloids, tannins, steroids and glycosides to be present in all three plants. Alkaloids which have been reported in literature to exhibit good antibacterial activity (Harborne, 1984; Harborne, 1984) were successfully extracted and used in an antibacterial study showing promising antibacterial activities. In a modulation study, Tetracycline's antibacterial activity against *S. poona* and *Shigella* was improved using the alkaloid crude extract. LC-ESI-MS analysis on the three crude alkaloids revealed 8 reported alkaloids.

Collection and Preparation of Plant Materials

Plant materials of Z. *zanthoxyloides, O. gratissimum* and *P. fraternus* were obtained as described in chapter 3, from Nkanfua (5.14012° N, 1.24316° W) and Elmina (5.08419° N, 1.35380° W).

Table 5: Dates and locations where plant materials were collected

Plant Material	Part	Location	Date Collected
P. fraternus	Whole plant	Nkanfua	31st March, 2022
Z. zanthoxyloides	Leaves	Elmina	29th March, 2022
O. gratissimum	Leaves	Nkanfua	30th March, 2022

Source: Laboratory data (2022)

Qualitative Phytochemical Analysis

Table 6: Result of Qualitative Phytochemical Analysis on the Three Plant Extracts

Phytochemical	*O. gratissimun* [a (b)]	*P. fraternus* [a (b)]	*Z. zanthoxyloide* [a (b)]
Alkaloid	+ (+)	+ (+)	+ (+)
Triterpenoid	+ (-)	+ (-)	+ (-)
Steroids	+ (-)	+ (-)	+ (-)
Flavonoid	+ (-)	- (-)	- (-)
Tannin	+ (-)	+ (-)	+ (-)
Saponins	- (-)	+ (-)	- (-)
Glycosides	+ (-)	+ (-)	+ (-)

Key: *a* = total crude extract; *b*= crude alkaloid extract; + = detected; - = not detected
Source: Laboratory data (2022)

Phytochemicals also known as secondary metabolites are non-nutritive naturally occurring chemicals in plants which serve as medicine for the protection of human disease (Cheng *et al*., 2002). The three plants were first screened for the class of secondary metabolites they contain. The study discovered the presence of secondary metabolites like, alkaloids, steroids, triterpenoids, tannins, and glycosides in the crude extract of all the three different plants. Flavonoids were absent in both *P. fraternus* and *Z. zanthoxyloide* but present in *O. gratissimum*. Saponins were also absent in *O. gratissimum* and *Z. zanthoxyloides* but present in *P. fraternus* (Table 9).

Studies by Olusola et al. (2020) on methanolic leaf extract of *Z. zanthoxyloides* report the presence of flavonoids and saponins; these compounds were not present in the present studies. However, according to

Olushola-Siedoks et al., (2020) findings, the crude ethanolic leaf extract had no evidence of these two phytochemicals.

According to research by Alexander (2016), saponins were detected in the aqueous leaf extract of *O. gratissimum*. Additionally, according to Hamma et al. (2020), saponins were also present in the aqueous extract but not in the ethanol leaf extract. The methanolic leaf extract is also reported by Akinmoladun et al. (2007) to be free of saponins. Steroids were also detected in the aqueous leaf extracts used in this study. This is, however, contrary to what Hamma et al. (2020) reports for the ethanolic extracts. In the present study, with the exception of saponins, every other phytochemical that was evaluated agreed with the results from these literatures.

Phytochemical screening results on *Phyllanthus fraternus* leaves by Mehta (2013) reports all screened phytochemical as present and flavonoids as absent. Gandhi et al., (2014) also reports flavonoids to be absent in the methanolic, hydromethanolic and petroleum ether leave extracts. The few differences from other studies could possibly be due to different solvents used in the extraction. In contrast to the other studies, Methanol:Dichloromethane (1:1) solvent was used for extractions in this study.

Considering many of these phytochemicals have previously been implicated to be present in other medicinal plants and to exhibit similar antibacterial activity, it is possible that the presence of these phytochemicals greatly contributed to the pharmacological activities that were observed in the study (Agnihotri, *et al.*, 2010).

Table 7: Qualitative Phytochemical Analysis and Observations

Test	Procedure	Reagent Composition	Observations for *O. G*	Observations for *P. F*	Observations for *Z. Z*
Alkaloids o Wagner's Test:	Acidified solution of extract (2 ml) + (2-3) drops of wagner's reagent	Iodine in potassium iodide	Brown ppt	Brown ppt	Brown ppt
o Mayer's Test:	Acidified solution of extract (2 ml) + (2-3) drops of mayer's reagent	Potassium mercuric iodide	creamy-white ppt	creamy-white ppt	creamy-white ppt
Steroids	2 ml of 80% ethanolic solution of extract + 2 ml acetic anhydride + heat+ cool + H_2SO_4.		Brown-ring at the interface	Brown-ring at the interface	Brown-ring at the interface
Tannins	0.2 g of extract + 5 ml aq. Methanol + $FeCl_3$.	Ferric Chloride	Light-green colour	Dark-green colour	Light-green colour

Key: P.F = *Phyllanthus fraternus*, O. G = *Occimum gratissimum*, Z. Z = *Zanthoxylum zanthoxyloides*
Source: Laboratory data (2022)

Table 7 Continued

Flavonoids ○ Alkaline Reagent Test	2 ml of ethanolic solution of extract + few drops of NaOH solution	Sodium Hydroxide	Intense yellow colour (Disappears upon addition of acid)	No intense yellow colour	No Intense Yellow Colour
	2 ml of ethanolic solution of extract + lead acetate solution	Lead acetate	Yellow ppt	No ppt formed	No Ppt Formed
Saponins	Foam test		< 1 cm layer of foam	2 cm layer of foam	2 cm layer of foam
Glycosides ○ Salkowski's Test	2 ml of 80% ethanolic solution of extract + acetic acid + 2 drops of $FeCl_3$.	Acetic Acid	Deep red colour	Deep red colour	Deep red colour

Key: P.F = *Phyllanthus fraternus*, O. G = *Occimum gratissimum*, Z. Z = *Zanthoxylum zanthoxyloides*
Source: Laboratory data (2022)

Extraction of Plant Material

Methanol/DCM Crude Extract

Cold maceration of the whole plant of *P. fraternus* (800 g), leaves of *Z. zanthoxyloides* (820 g) and the leaves of *O. gratissimum* (730 g) used for the methanol/DCM extraction resulted in *P. fraternus* recording the highest crude % yield (Table 8). Powdered raw material of *P. fraternus* (800 g) yielded 58.14 g crude extract which is 5.85 % yield. Crude extract (53.19 g) was obtained from powdered plant material of *Z. zanthoxyloides* (820 g) of 6.49 % yield. Lastly, powdered plant material of *O. gratissimum* (730 g) produced a 42.70 g crude extract (5.85 % yield).

Table 8: Masses of raw materials, Methanol – DCM crude extract and their Percentage Yields

Plant	RM (g)	CE Mass (g)	% CE (% CAE)
O. gratissimum	730	42.70	5.85 (1.18)
P. fraternus	800	58.14	7.23 (0.64)
Z. zanthoxyloides	820	53.19	6.49 (1.40)

Key**:** RM = Raw Material; CE = Crude Extract; CAE = Crude Alkaloid Extract
Source: Laboratory data (2022)

Test for Alkaloids on crude alkaloid extract

Alkaloids, considered as nitrogenous bases that occur in plants, have marked antibacterial effects on a variety pathogenic bacterium. The three commonly used reagents for alkaloidal test, wagner's, dragendorff's and mayer's reagents used in testing for the presence of alkaloids in all the three crude alkaloid extracts gave a positive test result. This indication of the presence

of alkaloids in all three crude alkaloid extracts also suggested that the plants have potential antimicrobial properties. (Stanley et al., 2007**).** The crude alkaloid extracts were screened for all other phytochemicals in accordance with the same procedure used for the initial screening to ensure that they were no longer present.

Biological Study

Solubility of Extract and Preparation of Stock Solution

The crude alkaloid extract of *O. gratissimum* and *Z. zanthoxyloide* were insoluble in water. DMSO (2 %) was used for dissolution. The crude alkaloid of *P. fraternus* however was soluble in water. 50 µg/ml stock solutions were then prepared for the antibacterial assay.

Micro-bacterial Used in the Study

The antibacterial activity of the three crude alkaloidal extracts were determined using 10 bacteria comprising, four Gram-positive; *Staphylococcus aureus, MRSA, Streptococcus pyogenes, Staphylococcus lentus* and six Gram-negative; *Escherichia coli* (ATCC 10455 & ATCC 43888), *Klebsiella pneumoniae, Salmonella poona, Shigella, Salmonella typhi.*

These 10 sbacteria, also, comprised three (3) critical; *Escherichia coli* ATCC 10455, *Escherichia coli* ATCC 43888 and *Klebsiella pneumoniae*; five (5) *high Staphylococcus aureus, Streptococcus pyogenes, Staphylococcus lentus, Salmonella poona, Salmonella typhi* and two (2) medium; *Shigella, Streptococcus pyogenes*, WHO priority pathogens for research and development of new antibiotics (WHO, 2019).

Sub-Culturing of Microorganisms

Prior to each antibacterial assay study, to guarantee that the bacteria are viable and actively proliferating, sub-culturing of the bacteria was conducted. The growth of sub-cultured micro-orgasms on nutrient agar after 24 hours.

Antibacterial activity of crude alkaloid using the HT-SPOTi assay

The crude alkaloid extract of the three medicinal plants had significant inhibition on the growth of some bacteria (Table 10). Tetracycline and ciprofloxacin were used as the positive controls. All the crude alkaloid extracts showed activity against *Escherichia coli* (ATCC 43888), *Staphylococcus Aureus* and *Shigella* at MICs between 125 – 500 µg/ml, however, no activity was observed against S*taphylococcus pyogenes* and only *Z. zanthoxyloide* extract showed activity against MRSA at the concentrations tested.

Antibacterial Activity of Crude Alkaloid Using the HT-SPOTi Assay

Table 9: MICs for Crude Alkaloidal Extracts of Three Medicinal Plants Against Ten Different Bacteria

Drug/ Extract	Minimum Inhibition Concentration (MIC) (µg/ml)									
	E. coli (10)	*E. coli (43)*	*S. aureus*	*MRSA*	*Kleb*	*Shigella*	*S. typhi*	*S. poona*	*S. lentus*	*S. pyogenes*
AEOG	>500	250	250	>500	500	500	>500	125	500	>500
AEPF	250	125	125	>500	>500	125	250	500	125	>500
AEZZ	>500	250	250	500	500	125	500	125	250	>500
CIPRO	<3.9	<3.9	<3.9	<3.9	<3.9	<3.9	<3.9	<3.9	<3.9	<3.9
TETRA	<3.9	15.6	15.6	125	15.6	125	7.8	15.6	15.6	<3.9

Key: *AEOG* = Crude alkaloid extract *O. gratissimum*; *AEPF* = Crude alkaloid extract *P. fraternus*; *AEZZ* = Crude alkaloid extract *Z. zanthoxyloides*; CIPRO = Ciprofloxacin; TETRA = Tetracycline; *E. coli* (10) = *Escherichia coli* ATCC 10455; E. Coli (43) = *Escherichia coli* ATCC 43888

Source: Laboratory data (2022)

From the antibacterial study (Table 10), for all ten (10) bacteria strains used, the control drug control drug (Ciprofloxacin) showed an MIC <3.9 µg/ml against all of them. However, this was not so for the second standard antibiotic (Tetracycline) as it showed MICs ≥ 125 µg/ml against *Shigella* and MRSA as well as ≥ 15.63 µg/ml against *Staphylococcus aureus, Salmonella poona, Staphylococcus lentus, Klebsiella pneumoniae* and *E. coli* (43).

The alkaloidal extract of *P. fraternus* (Table 10) gave MICs of 125 µg/mL for *Escherichia coli* (ATCC 43888), *Staphylococcus aureus, shigella* and *Staphylococcus lentus.* Against *Salmonella poona,* it inhibited bacteria growth at an MIC of 500 µg/ml. The extract again, showed activity at MICs of 250 µg/ml against *Salmonella typhi* and *Escherichia coli* (ATCC 10455). For *MRSA, Klebsiella pneumoniae* and *Staphylococcus pyogenes, Phyllanthus fraternus* gave MICs > 500 µg/ml.

The crude alkaloid of *O. gratissimum* (*AEOG*), however, showed no activity against five (5) bacteria strains; *Escherichia coli* (ATCC 10455), MRSA, *Salmonella typhi*, *Staphylococcus lentus* and *S. pyogenes* (Table 10).. The extract also showed MICs of 500 µg/ml against *Klebsiella pneumoniae* and *Shigella*, and MICs of 250 µg/ml against *Escherichia coli* (ATCC 43888) and *Staphylococcus aureus*. Of all ten (10) bacteria strains, the lowest MIC recorded for *O. gratissimum*, 125 µg/ml, was observed against *Salmonella poona.*

Crude alkaloid extract of *Z. zanthoxyloides* (*AEZZ)* showed no activity against two (2) bacteria strains; *Escherichia coli* (ATCC 10455) and *Staphylococcus pyogenes* (Table 10). The extract also showed activity against MRSA, *Klebsiella pneumoniae* and *Salmonella typhi* at MIC of 500 µg/ml and

250 µg/ml against three (3) bacteria strains; *Staphylococcus aureus*, *Staphylococcus lentus* and *Escherichia coli* (ATCC 43888). The lowest MIC value obtained for *Z. zanthoxyloide* was 125 µg/ml against *salmonella poona* and *Shigella.*

Biofilm Studies

All ten (10) bacteria strains showed no biofilm forming properties. It is, however, important to note, these bacteria strains may exhibit other antimicrobial resistance properties (Table 11).

Table 10: Biofilm Forming Properties of Strains

Micro-organism	Activity	Micro-organism	Activity
Escherichia coli ATCC10455	-	Salmonella poona	-
Escherichia coli ATCC43888	-	Staphylococcus lentus	-
Salmonella typhi	-	MRSA	-
Shigella	-	Staphylococcus pyogenes	-
Klebsiella pneumoniae	-	Staphylococcus aureus	-

Key: + detected; - not detected
Source: Laboratory data (2022)

Figure 17: Results showing non-biofilm forming properties of test strains

Time-Kill Kinetic Study

Time-kill kinetics of the Three Crude Alkaloid Extracts Against Test Strains

The MIC is commonly described as the *in vitro* threshold concentration used to assess an antibacterial agent's effectiveness against a particular bacterium. Furthermore, the MIC only gives a single point estimate or a glimpse of the antibiotic's overall impact on bacterial growth and bacterial eradication. MIC is also regarded as an "all-or-nothing" concentration impact relation. Although relatively common, this method has a number of drawbacks. Time-kill curves are an alternate method that offers more dynamic data for assessing the relationship between an antibacterial concentration and its potency over time. This method allows for a direct comparison of the MIC finding and antibacterial effects of a potential lead to those of existing conventional antibiotic (Schmidt et al., 2009; Mueller et al., 2004).

Because the crude extracts and drugs (except ciprofloxacin) were coloured, stock solutions and serial diluted solutions were undoubtedly coloured. As a result, in order to obtain the actual optical density of only the bacteria strain, MIC concentrations of the extracts/drug's optical densities were run together as blanks and subtracted from the extract/drug-media-bacteria's optical densities. A graph of optical density (OD_{620}) against time (hrs) was then generated to study the killing (bactericidal) or inhibition (bacteriostatic) rate of the extracts compared to the two positive control drugs in 24-hours.

The growth curve investigation involved monitoring and recording the microplate reader's readings of the bacteria's increasing cell density in the

treated (extract/drug-organism-media) and untreated (negative control) wells. A sigmoidal curve was obtained for the negative control. When compared to the curves obtained for the alkaloid extract and positive controls, deviations from the typical sigmoidal curves were indications of the alkaloidal extracts or standard drug's effect on the bacteria. There are two viable outcomes; a sigmoidal curve with a maximum optical density that is reached at a lower optical density than that of the negative control, or a straight line with no curve at all. While the latter suggests a bactericidal action, the former denotes a bacteriostatic action (Lehtinen et al., 2006; Koch et al., 1970).

As expected, the graph of optical density at 620 nm (OD_{620}) against time (hrs) curve of the negative control showed an increasing regrowth in bacteria concentration (sigmoid curve) from the 0 – 6th h and the 18 – 24th h for all test bacteria, which indicated the increasing number of viable cells (Figure 18). None of the alkaloidal extracts showed activity against *Staphylococcus pyogenes*, and therefore was exempted from the time-kill study. Major findings from the time-kill study focused on the last 6 hours (exponential phase) in the 24 hr time frame (Mouton et al., 2005; Maier & Pepper, 2015). Bacteria time-kill activity in the lag phase (first six 6 hours) were, however, not completely disregarded. From (Figure 18), the average optical density (OD_{ave}) during the last 6 h in the 24 h (showing bacterial growth) recorded for the test strains were as follows; *E. coli (10)*, 0.7481, *E. coli (43)*, 0.7600; *S. aureus*, 1.0040; *S. typhi*, 0.8214; *S. poona*, 0.8286; *S. lentus*, 0.7581; *Kleb pneumoniae*, 1.8390; *Shigella*, 0.6816; *MRSA*, 0.8095 shown in (Figure 18). Table for the Optical Densities (OD) at 620 nm for all ten-negative control in (Appendix A). The test strain *K. pneumoniae* showed the highest OD_{ave}, followed by *S. aureus* and *S. poona*

indicating their rapid growth rate in 24 hrs with *Shigella* showing the slowest growth rate (Figure 18).

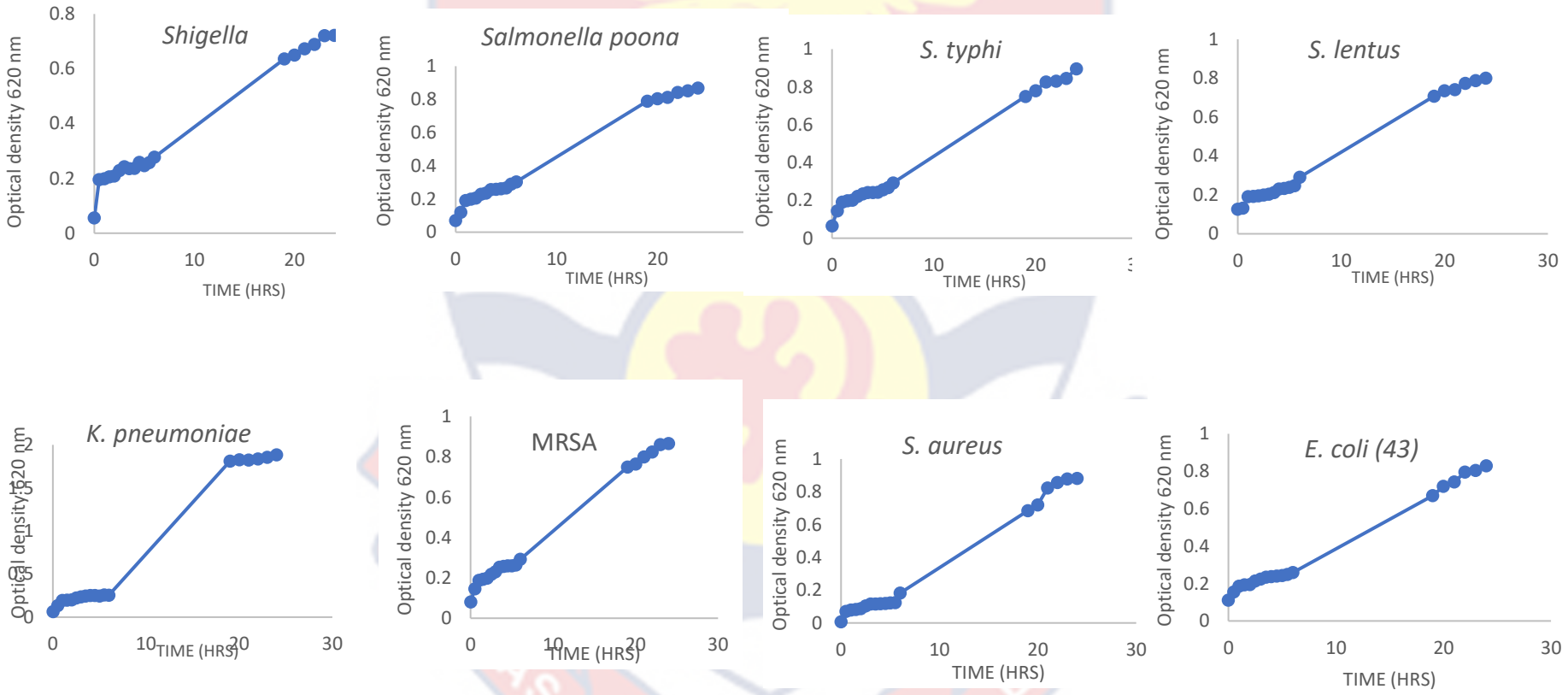


Figure 18: Time-kill kinetic curve showing uninhibited bacteria growth in 24 hours for *Shigella*, *S. poona*, *S. typhi*, *K. pneumoniae*, MRSA, *Staph. lentus*, *E. coli (43) and S. aureus*

Against all test strains, the time-kill kinetic profile of ciprofloxacin showed an unvarying OD during the first and last six hours, ranging from 0.0063 – 0.0255, indicating complete constant reduction in viable cells (Figure 19 – 39). Again, the rate of activity of ciprofloxacin against all test strains, during the entire 24 hr period demonstrated a bactericidal effect, exhibiting zero tolerance for bacteria proliferation; indicating that it is more efficient in killing the test organisms as soon as they were introduced into the wells containing cipro-media mixture. Tetracycline however, acted as bactericidal and/or bacteriostatic agent with different test strains (Figure 19 - 39). All table of results for the OD for each extract/drug vs bacteria from the microplate reader can be found in (Appendix A).

The time-kill kinetics profile of the crude alkaloid extract of *O. gratissimum (AEOG)* against the test strains *S. poona* (Figure 19) and *S. aureus* (Figure 20) showed much better reduction in number of viable cells compared to the standard tetracycline. Against *S. poona*, AEOG showed a better killing rate between 0 – 6 h and 18 – 24 h compared to tetracycline.

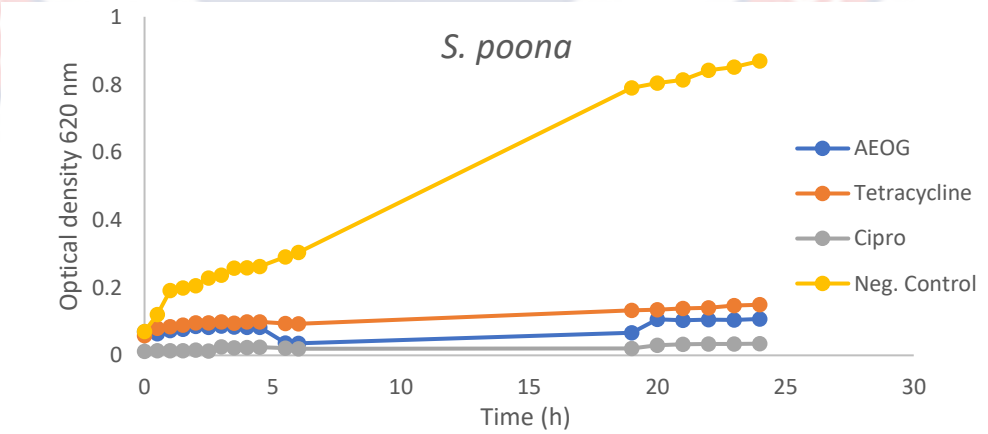


Figure 19: Time-kill kinetics curve for *Salmonella poona* subjected to the minimum inhibition concentrations of AEOG, Tetracycline and Ciprofloxacin

Although both agents (AEGO and Tetracycline) showed bactericidal activity in the 24 h period, *AEOG* showed a better reduction in bacteria growth between the 18 – 24 h with optical density (OD) ranging from 0.0665 – 0.1075 compared to tetracycline with OD ranging from 0.1325 – 0.1495 (Figure 19).

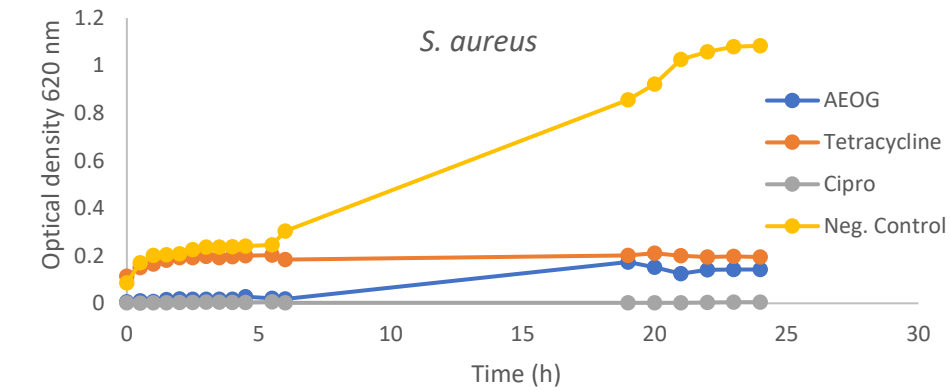


Figure 20: Time-kill kinetics curve for *S. aureus* subjected to the minimum inhibition concentrations of AEOG, Tetracycline and Ciprofloxacin

O. gratissimum's crude alkaloid extract (*AEOG)* vs *S. aureus'* time-kill kinetics profile showed an interesting result (Figure 20). During the first 6 hours, the OD of *AEOG* increased steadily at 0.0055 – 0.0185 with an OD_{ave} of 0.0163 but increased to 0.1735 at the 19th h and maintained an OD_{ave} around this value for the last 6 hrs. Tetracycline however, maintained an average OD of 0.1806 and 0.1995 during the first and last six hours respectively, implying that although the *AEOG* showed a reduction in the number of viable cells than tetracycline against *S. aureus*, it acted as a bacteriostatic agent while tetracycline acted as a bactericidal agent (Figure 20).

Against the bacteria *Shigella* (Figure 21), both *AEOG* and Tetracycline acted as bacteriostatic agents. However, tetracycline showed more potent activity with an OD_{ave} 0.3317 from 18 – 24h compared to *AEOG* (OD_{ave} = 0.4938). For the bacteria *K. pneumoniae* (Figure 22), the crude alkaloid extract

of *O.G* showed poor killing rate (OD_{ave} = 0.610) compared to tetracycline, with an OD_{ave} of 0.1523 from 18 – 24 h.

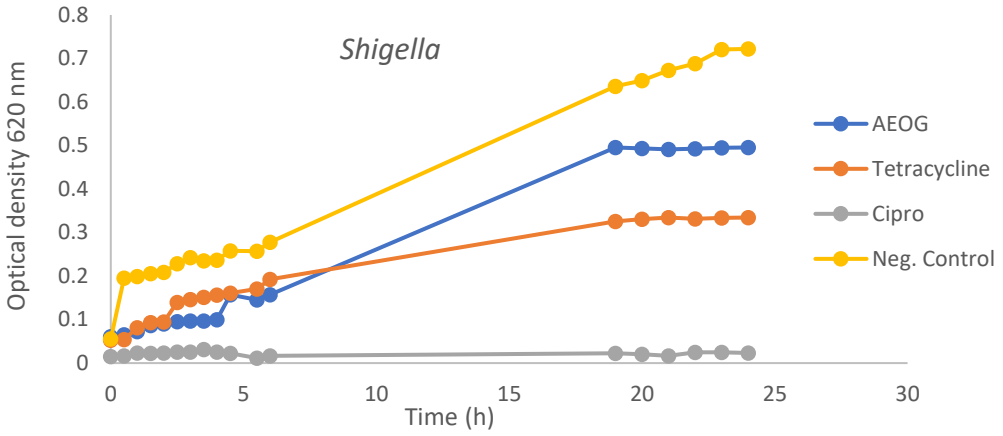


Figure 21: Time-kill kinetics curve for Shigella subjected to the minimum inhibition concentrations of AEOG, Tetracycline and Ciprofloxacin

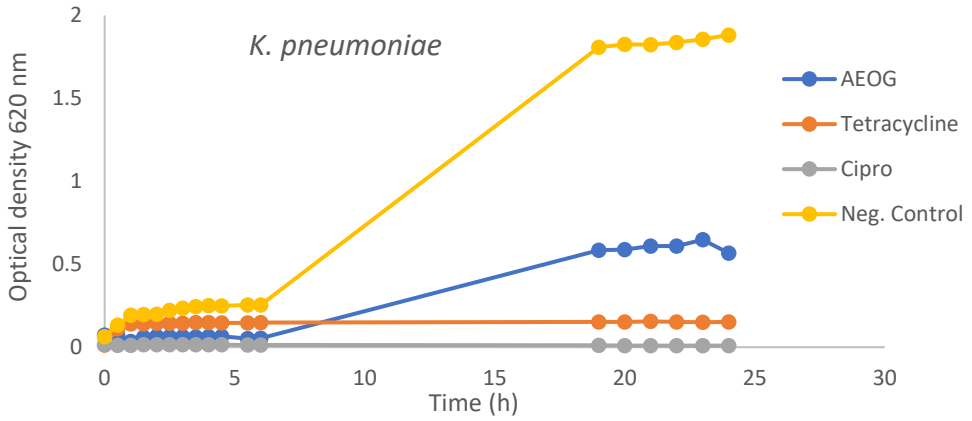


Figure 22: Time-kill kinetics curve for *K. pneumoniae* subjected to the minimum inhibition concentrations of AEOG, Tetracycline and Ciprofloxacin

For the time-kill kinetic profile of tetracycline and *AEOG* against *S. lentus* (Figure 23), both agents showed similar bacteriostatic killing rate from 0 – 6 h and 18 – 24 h. During the first 6 hrs, OD_{ave} of *AEOG* and tetracycline were 0.0304 and 0.0288 respectively. From 18 – 24 h, the OD of both agents increased steadily and maintained an OD_{ave} of 0.1678, for *AEOG* and 0.163 for tetracycline (Figure 23).

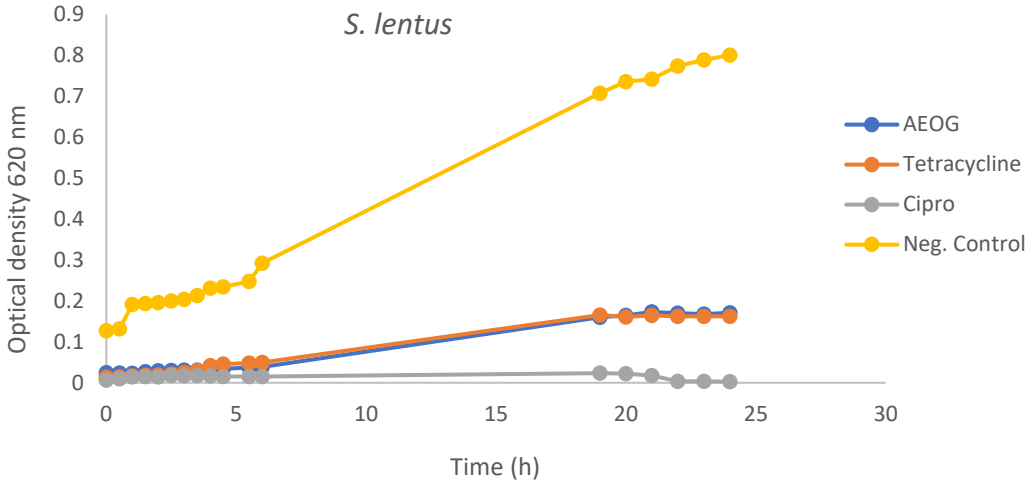


Figure 23: Time-kill kinetics curve for *S. lentus* subjected to the minimum inhibition concentrations of AEOG, Tetracycline and Ciprofloxacin

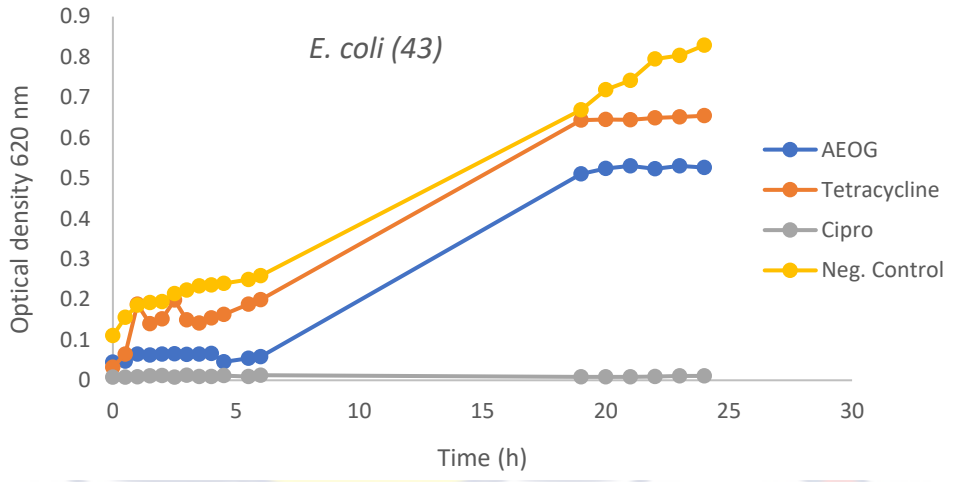


Figure 24: Time-kill kinetics curve for *E. coli* (43) subjected to the minimum inhibition concentrations of AEOG, Tetracycline and Ciprofloxacin

Lastly, both tetracycline and the *AEOG* showed poor antibacterial activity against *E. coli* (43) (Figure 24), however, the crude alkaloidal extract of *O. gratissimum (AEOG)* showed a better activity between the two agents (OD_{ave} = 0.5341 from 18 – 24h), their activity, especially, during last 6 h had high optical density than any recorded against the other five bacteria, indicating that both agents have poor inhibiting rate against *E. coli* (43) (Figure 24).

The time-kill kinetic profile of the crude alkaloid extract of *P. fraternus (AEPF)* against seven (7) test strains was also studied. The results from this study showed an exceptional reduction in number of viable cells against the bacteria *E. coli* (43) (Figure 25) and *E. coli* (10) (Figure 26) compared to the standard tetracycline. Against both bacteria, *P. F's* crude alkaloid extract showed complete bactericidal activity with OD_{ave} of 0.0418 and 0.0517 for the first and last six (6) hours in 24 hours respectively against *E. coli* (43) and an OD_{ave} of 0.0898 and 0.1333 for the first and last six (6) hours in 24 hours respectively against *E. coli* (10).

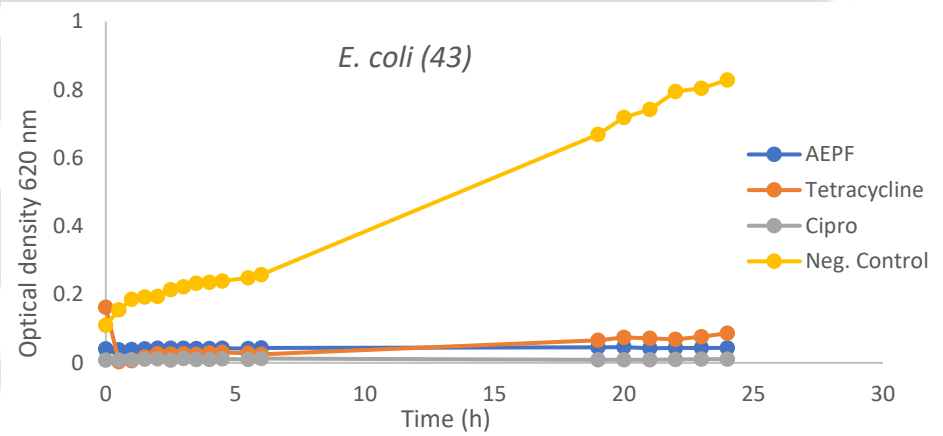


Figure 25: Time-kill kinetics curve for *E. coli* (43) subjected to the minimum inhibition concentrations of AEPF, Tetracycline and Ciprofloxacin

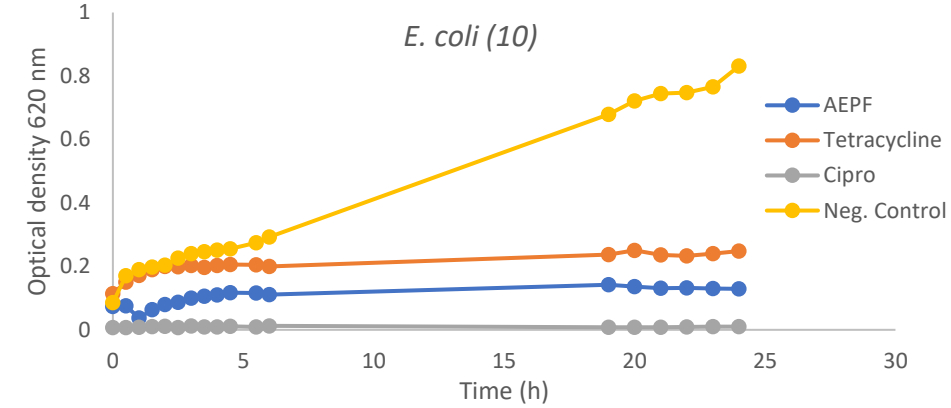


Figure 26: Time-kill kinetics curve for *E. coli* (10) subjected to the minimum inhibition concentrations of AEPF, Tetracycline and Ciprofloxacin

The standard tetracycline also showed complete reduction in bacteria growth against these two bacteria, however, not as effective as the *AEPF* (Figure 25 and 26); against *E. coli* (43) (Figure 25), the OD_{ave} recorded for tetracycline was 0.0337 from 0 – 6 h and 0.0740 from 18 – 24 h. Against *E. coli* (10) the OD_{ave} recorded was 0.1863 from 0 – 6 h and 0.2412 from 18 – 24 h (Figure 26).

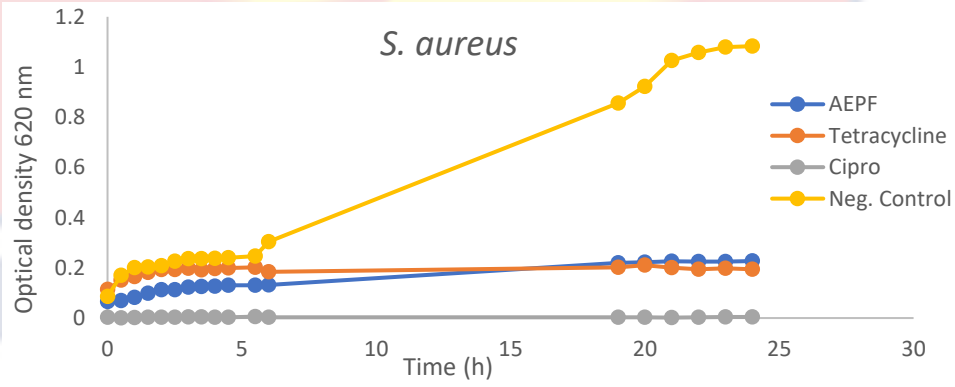


Figure 27: Time-kill kinetics curve for *S. aureus* subjected to the minimum inhibition concentrations of AEPF, Tetracycline and Ciprofloxacin

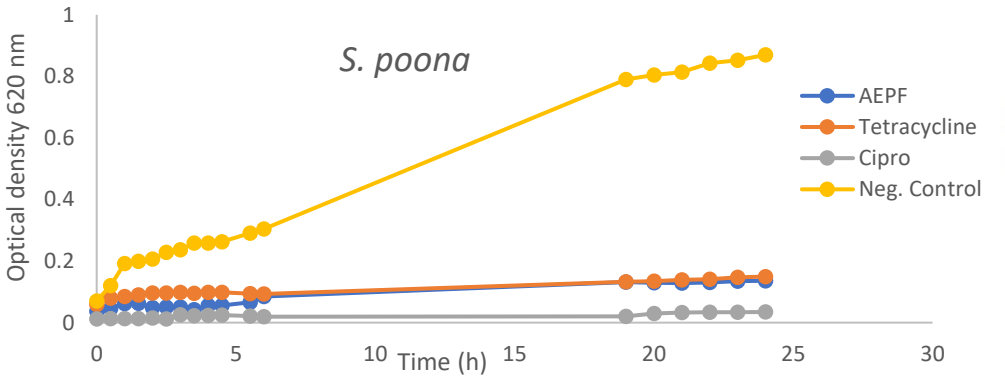


Figure 28: Time-kill kinetics curve for *S. poona* subjected to the minimum inhibition concentrations of AEPF, Tetracycline and Ciprofloxacin

For *S. aureus* (Figure 27) and *S. poona* (Figure 28), both agents showed similar activity from 18 – 24 h. In fact, both agents acted as bactericidal agents against these two bacteria. Against *S. aureus*, the results showed an OD_{ave} of 0.2242 for *AEPF* and 0.2000 for tetracycline and against *S. Poona*, the results showed an OD_{ave} of 0.1320 for *AEPF* and 0.1405 for tetracycline from 18 – 24 h.

The crude alkaloid extract of *P.F* against *Shigella* (Figure 29) also showed much better reduction in the number of viable cells with an OD_{ave} as low as 0.1798 compared to tetracycline with an OD_{ave} of 0.3470 from 18 – 24 h. Tetracycline, however, acted bactericidally whereas the crude alkaloid extract of *P. F* acted bacteriostatically.

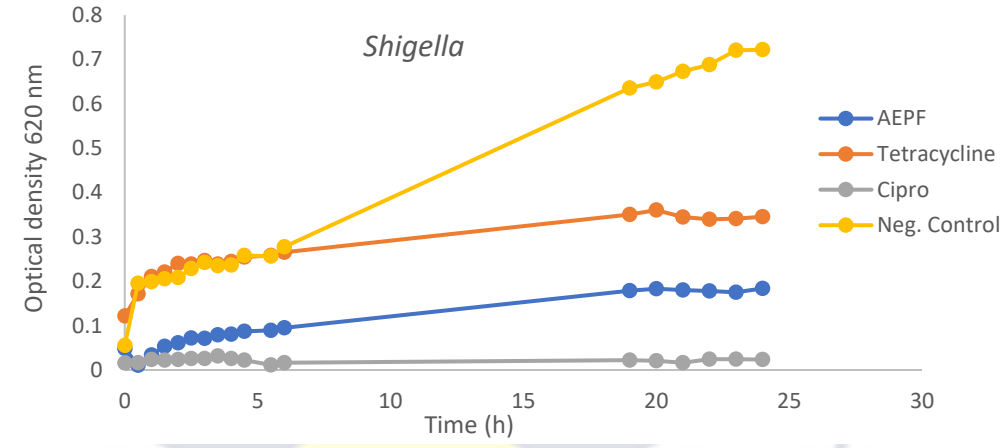


Figure 29: Time-kill kinetics curve for *Shigella* subjected to the minimum inhibition concentrations of AEPF, Tetracycline and Ciprofloxacin

Results obtained from the time-kill curve showed better killing rate of tetracycline against *S. lentus* (Figure 30) compared to the crude alkaloid extract of *P. F.* An OD_{ave} of 0.1005 for tetracycline and 0.2855 for *P. F*, form 18 – 24 h, places tetracycline as a better antibacterial agent ahead of *P. F*, notwithstanding, the crude alkaloid extract of *P. F* also showed a better killing rate against *S. typhi* with an OD_{ave} of 0.1622 compared to 0.2707 for tetracycline, form 18 – 24 h (Figure 31).

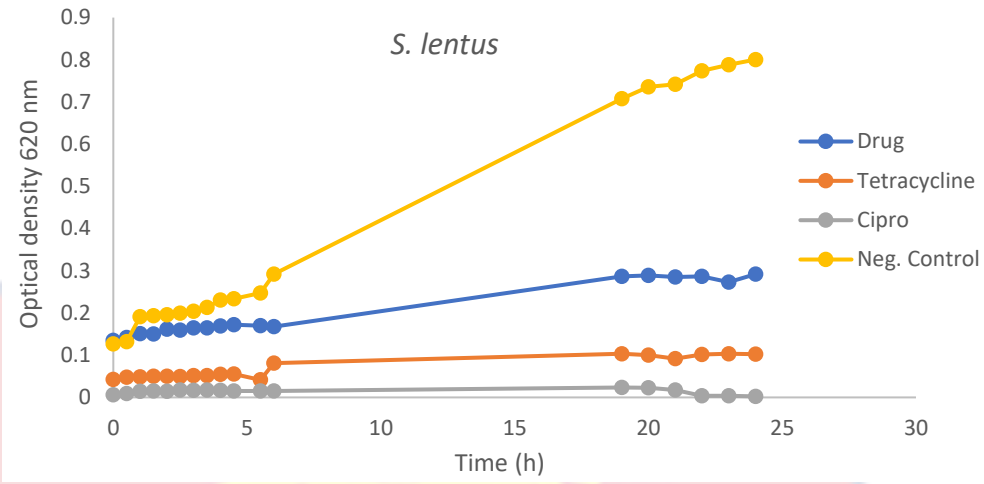


Figure 30: Time-kill kinetics curve for *S. lentus* subjected to the minimum inhibition concentrations of AEPF, Tetracycline and Ciprofloxacin

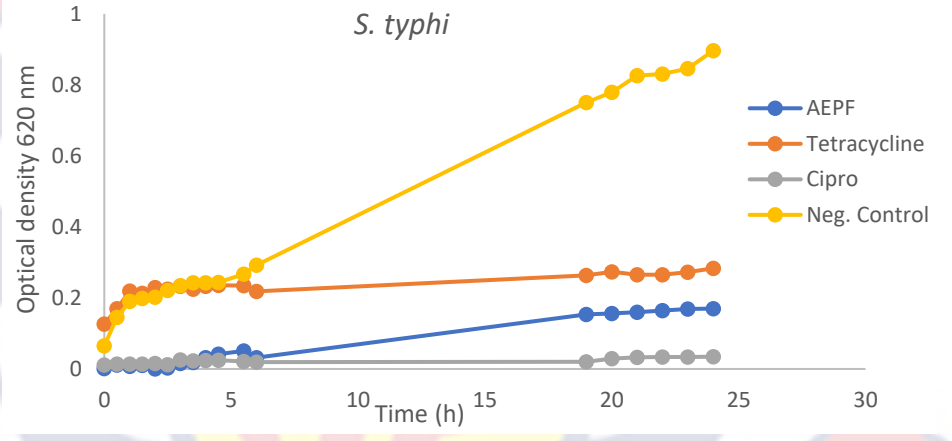


Figure 31: Time-kill kinetics curve for *S. typhi* subjected to the minimum inhibition concentrations of AEPF, Tetracycline and Ciprofloxacin

The last crude alkaloid extract used in the time-kill kinetic study was *Z. zanthoxyloides*. Z. *zanthoxyloides'* crude alkaloid extract (*AEZZ)* time-kill profile showed complete bactericidal action against *S. aureus* (Figure 32) and *S. poona* (Figure 33) far better than tetracycline and almost in the same fashion as the standard ciprofloxacin. The OD_{ave} for tetracycline and the crude alkaloidal extract from 0 – 6h was 0.1806 and 0.0195 respectively and 0.1998 and 0.020 respectively from 19 -24h for *S. aureus* (Figure 32). The OD_{ave} for cipro for the first and last 6 hours was 0.00332 and 0.0032 respectively.

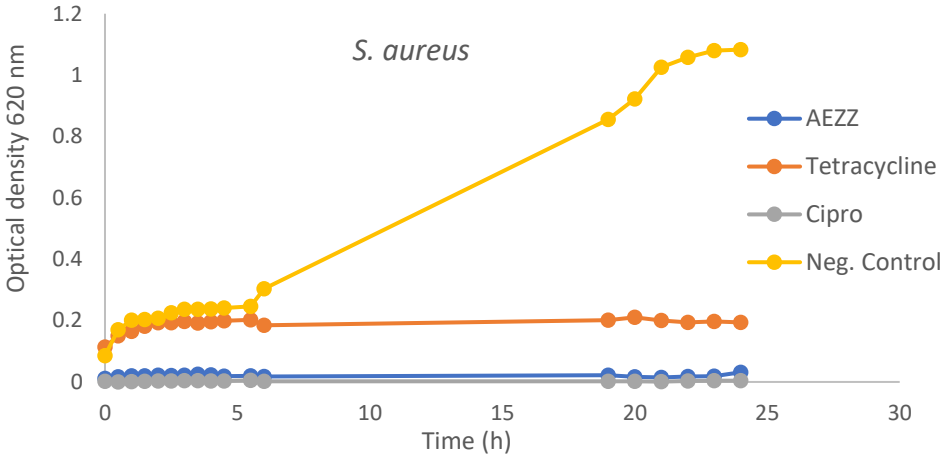


Figure 32: Time-kill kinetics curve for *S. aureus* subjected to the minimum inhibition concentrations of AEZZ, Tetracycline and Ciprofloxacin

For the bacteria *S. poona* also, *AEZZ's* observed activity was completely bactericidal and showed much reduction in viable cell in 24 hours than tetracycline and even ciprofloxacin (Figure 33). An OD_{ave} of 0.0208 was recorded for *AEZZ* compared to 0.1405 for tetracycline from 19 – 24 h. *AEZZ*'s killing rate again was slightly better than that of cipro against *S. poona*.

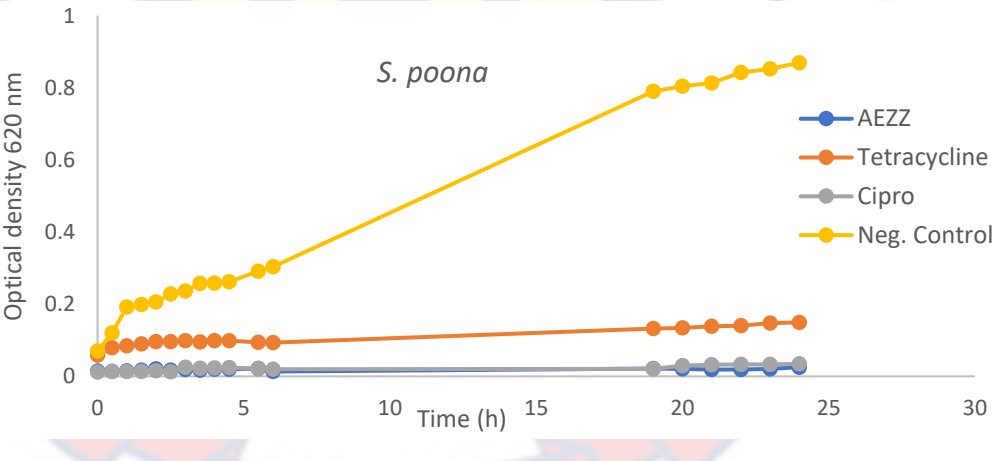


Figure 33: Time-kill kinetics curve for *S. poona* subjected to the minimum inhibition concentrations of AEZZ, Tetracycline and Ciprofloxacin

The OD_{ave} for ciprofloxacin during this time (19 – 24 h) was 0.0307 and 0.0208 for the *AEZZ.* The killing rate of *AEZZ* against *S. lentus* was observed to be almost the same tetracycline's (Figure 34); in both cases, the OD_{ave} was recorded to be 0.092 for *AEZZ* and 0.1005 for tetracycline (19 – 24 h). Although

during the first six (6) the OD_{ave} for the *AEZZ* was slightly higher (0.092) than that of tetracycline (0.0521), this OD was maintained throughout the entire 24 hrs. Making the *AEZZ* a complete bactericidal agent against *S. lentus*.

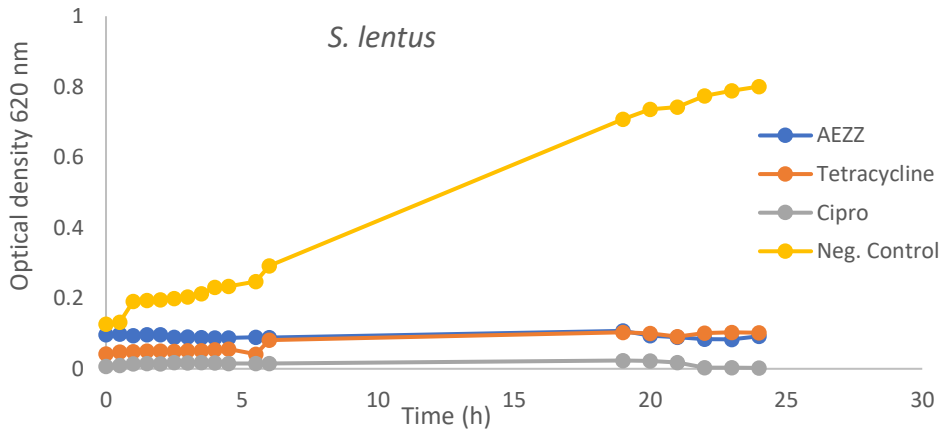


Figure 34: Time-kill kinetics curve for *S. lentus* subjected to the minimum inhibition concentrations of AEZZ, Tetracycline and Ciprofloxacin

The time-kill kinetics profile of both tetracycline and the *AEZZ* showed poor activity against *Shigella* (Figure 35) and *E. coli* (43) (Figure 36). In both cases, the two agents acted bacteriostatically with high optical densities, indicating that they have poor inhibitory action against both bacteria although inhibiting bacteria multiplication to a certain degree. The OD_{ave} for the *AEZZ* and tetracycline was 0.0494 and 0.2259 respectively, from 0 – 6 h and 0.2843 and 0.347 respectively, from 19 – 24 h (Figure 35). *Against E. coli* (43), tetracyclines activity was observed to be far poorer than *AEZZ*. In spite of the fact that both agents seemed to have a poor inhibition activity, the *AEZZ's* activity seemed to be slightly better than that of tetracycline.

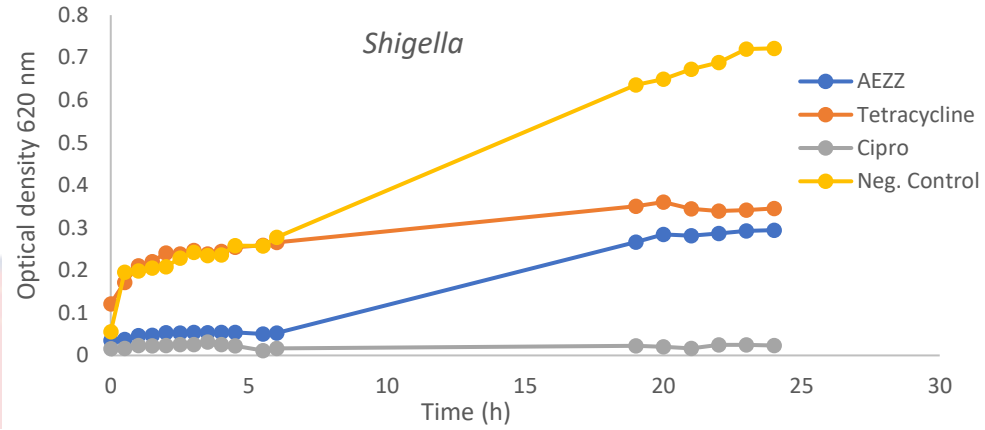


Figure 35: Time-kill kinetics curve for *Shigella* subjected to the minimum inhibition concentrations of AEZZ, Tetracycline and Ciprofloxacin

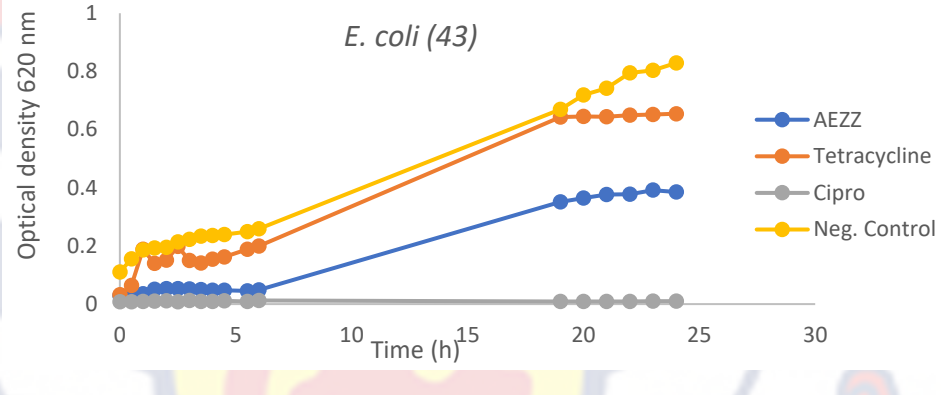


Figure 36: Time-kill kinetics curve for *E. coli* (43) subjected to the minimum inhibition concentrations of AEZZ, Tetracycline and Ciprofloxacin

Results obtained from the time-kill kinetic profile for tetracycline and the *AEZZ* against the three bacteria, MRSA (Figure 37), *K. pneumoniae* (Figure 38) and *S. typhi* (Figure 39) and showed tetracycline as a much better agent than *AEZZ*. Against all bacteria, tetracycline acted bactericidally with an OD_{ave} of 0.0622 against *MRSA*, 0.1292 against *S. typhi* and 0.2382 against *K. pneumoniae* from 19 – 24 h (Figure 38), whereas the OD_{ave} for *AEZZ* against *MRSA* was 0.4363, 0.4382 against *S. typhi* and 0.5208 against *K. peumoniae* during the same period.

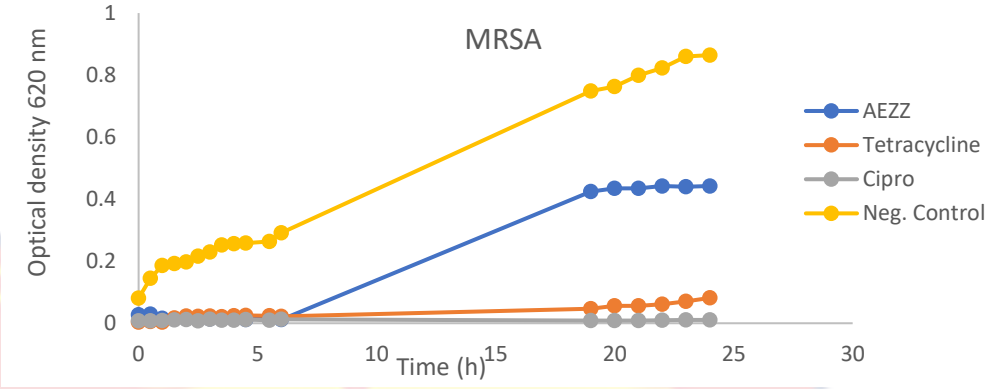


Figure 37: Time-kill kinetics curve for MRSA subjected to the minimum inhibition concentrations of AEZZ, Tetracycline and Ciprofloxacin

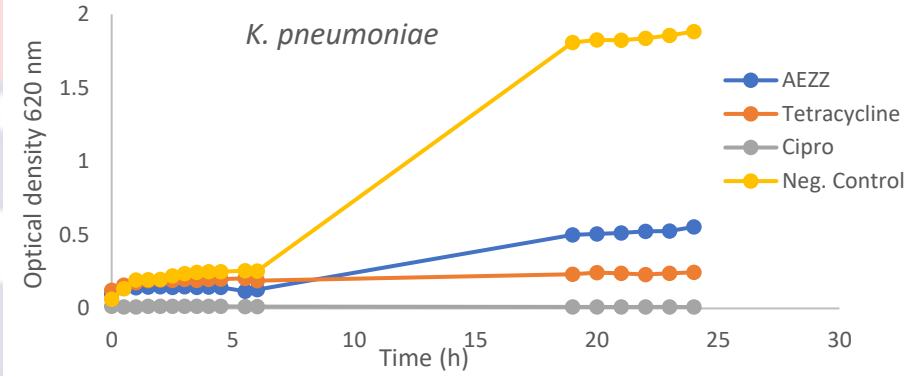


Figure 38: Time-kill kinetics curve for *K. pneumoniae* subjected to the minimum inhibition concentrations of AEZZ, Tetracycline and Ciprofloxacin

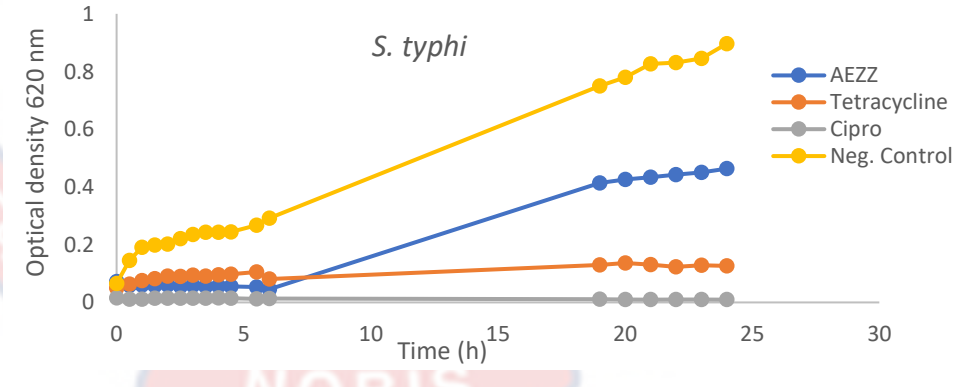


Figure 39: Time-kill kinetics curve for *S. typhi* subjected to the minimum inhibition concentrations of AEZZ, Tetracycline and Ciprofloxacin

Modulation Study Using the Checkerboard Assay

While finding new antimicrobials is a popular approach to tackle bacterial susceptibility in light of the constant advancement of science and the consistent use of antibiotics, this is not the only one. Considering already existing antibiotics and enhancing their potency may be extremely helpful for this purpose. Therefore, by maximising the effectiveness of currently available antibacterial drugs through modulation studies, existing antimicrobials may be optimised to improve their efficacy, reduce resistance, and even with rather lower cytotoxicity. Optimization would require combination with different gents.

A crucial aspect of combination therapy is the fact that drugs involved may have different mechanisms of action that would complement one another (Alalaiwe et al, 2018). A typical instance would be considering a pair in which one of the agents' affiliates has the tendency of either damaging bacterial cell wall in a way that inhibits its recurring biosynthesis, facilitating the influx of the other agent, or affecting certain vital internal biochemical activities of the bacteria. These two chemical agents are, in effect, proactively inhibiting or destroying the bacterial cell by working together.

Example;

For *AEOG* in combination with Tetracycline showing no growth in well D6 against the bacteria *S. poona*; *AEOG*/Tetra Vs *Salmonella Poona* (Figure 40).

[*AEOG* MIC = 125 µg/ml; Tetracycline MIC = 15.6 µg/ml]

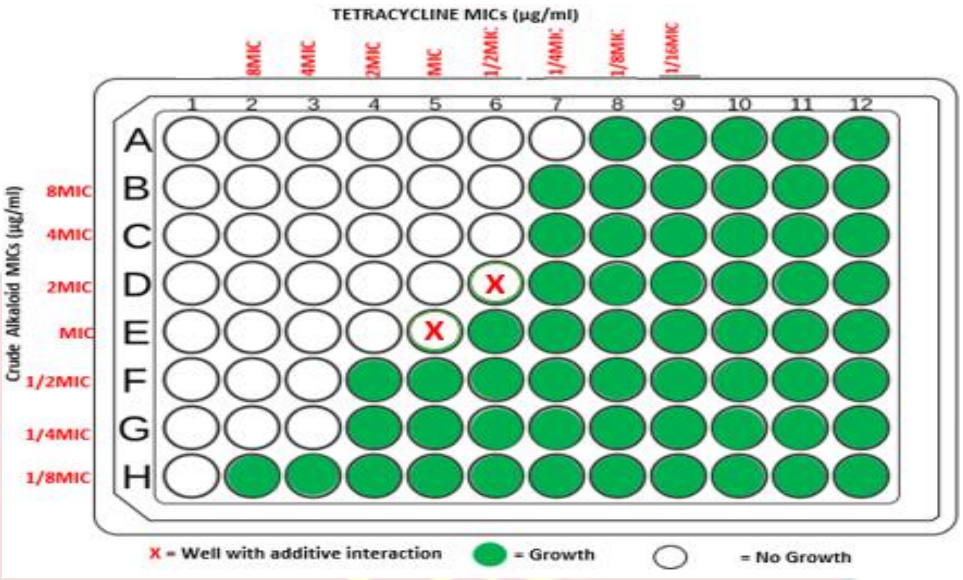


Figure 40: Schematic presentation of checkerboard assay result for Tetracycline in combination with AEOG against the test strain *Salmonella poona*

Using the FIC index equation,

$$\frac{A}{MIC\ (A)} + \frac{B}{MIC\ (B)} = FIC\ (A) + FIC\ (B) = FIC\ Index$$

Where $AEOG = \text{A}$ and $Tetracycline = \text{B}$

$\text{A} =$ MIC of A combination in well D6 $= 2\text{MIC} = 2\ (125\ \mu\text{g/ml})$

$A = 500\ \mu\text{g/ml}$

$\text{B} =$ MIC of B combination in well D6 $= 2\text{MIC} = \frac{1}{2}\ (15.6\ \mu\text{g/ml}) = 7.8$

The equation therefore becomes,

$$FIC\ index\ (well\ D6) = \frac{2(125)}{125} + \frac{1/2(15.6)}{15.6} = 2 + \frac{1}{2} = 2.5$$

FIC indices for all remaining wells were determined using this formula (APPENDIX E).

In the synergistic response, the applied antibiotics work together to produce an effect more potent than if each antibiotic were applied singly (Marr *et. al.*, 2004) compare to the additive effect, where the potency of an antibiotic combination is roughly equal to the combined potencies of each antibiotic singly, and antagonistic effect, where the potency of the combination is less than the combined potencies of each antibiotic (Kohanski *et. al.*, 2010).

Achieving synergism is the primary objective for maximum antibacterial impact at minimal antibiotic dose, following an additive effect. Despite being less potent than synergy, additive drug interactions can nonetheless have considerable positive effect in the discovery of new antibacterial leads.

Drug combinations between the crude alkaloid extract of *O. gratissimum* (*AEOG*) and *P. fraternus* (*AEPF*) in combination with tetracycline resulted in an additive effect against two organisms; *Salmonella poona* and *Shigella* (Table 12). For crude alkaloid extract of *O. gratissimum* (*AEOG)* (MIC; 125 µg/ml) in combination with tetracycline (MIC; 15.6 µg/ml) against *Salmonella poona,* additive effects were observed in wells D6 and E5, with FIC indices 2.5 and 2 respectively. The combination of extract-antibiotic in these wells were in the ratio 2MIC:1/2MIC (well D6) and 1MIC:1MIC (well E5). For crude alkaloid extract *P. fraternus* (*AEPF*) (MIC; 125 µg/ml) in combination with tetracycline (MIC; 125 µg/ml) against *shigella,* the additive effects were observed in wells E7 and F6, with FIC indices 1.25 and 1.0 respectively (Table 12). The combination of extract-antibiotic in these wells were in the ratio 1MIC:1/4MIC (well E7) and 1/2MIC:1/2MIC (well F6).

All the remaining crude extract-Tetracycline combinations resulted in antagonistic effects with FIC indices ranging from 16 – 4.25 (Appendix D). None of the combination could produce a synergistic effect. The table below (Table 12) shows the FIC indices obtained for each crude extract-Tetracycline combination and their interactions as antagonistic, additive or synergistic.

Table 11: Fractional Inhibitory Concentration (FIC) Index: *AEOG, AEPF* in Combination with Tetra Vs *Salmonella poona* and *Shigella*

AEOG-Tetracycline			*AEPF-Tetracycline*		
Salmonella Poona			*Shigella*		
Well No.	FIC	Interpretation	Well No.	FIC	Interpretation
B5	9	A	B8	8.0625	A
C6	4.5	A	C6	4.5	A
D6	2.5	I	E7	1.25	I
E5	2	I	F6	1.0	I
F3	4.5	A	G3	4.25	A

Key; A = Antagonism; I = Additive
Source: Laboratory data (2022)

Time-Kill Kinetics of Modulated Tetracycline with Additive/Indifference Interactions

It is key to know the 'pros' and 'cons' between time-kill and checkerboard assays. Basically, both assays can identify the interactions between two antibiotic agents (additive, synergistic, or antagonistic). The checkerboard method cannot indicate whether a combination of two agents either suppressess (bacteriostat) or induce bacterial cell death (bactericide) as it

operates on the "growth-no growth" method. It only provides details regarding a specific antimicrobial interaction (synergistic, additive or antagonistic). In contrast, time-kill kinetic studies can often identify the nature of interaction and also provide key details on the kinetics of the antimicrobial activities, such as whether it is slow or rapid.

Based on the checkerboard assay results for extract-drug combination with additive effects, time-kill kinetic assay was performed for those combinations to evaluate their interactions as bacteriostatic or bactericidal as well as their rate of action.

Time-kill kinetic curve from tetracycline in combination with the *AEOG* and *AEPF* showed complete bactericidal activity in the study. The results showed similar killing rate as Ciprofloxacin in 24 hours. Tables of the optical densities recorded from the microplate reader and the graphs generated are presented and this discussed accordingly. Each drug combination was run in duplicate. The optical densities for each drug combination and their average optical densities calculated are presented in (Appendix C). The average of each two optical densities was used in the time-kill curves.

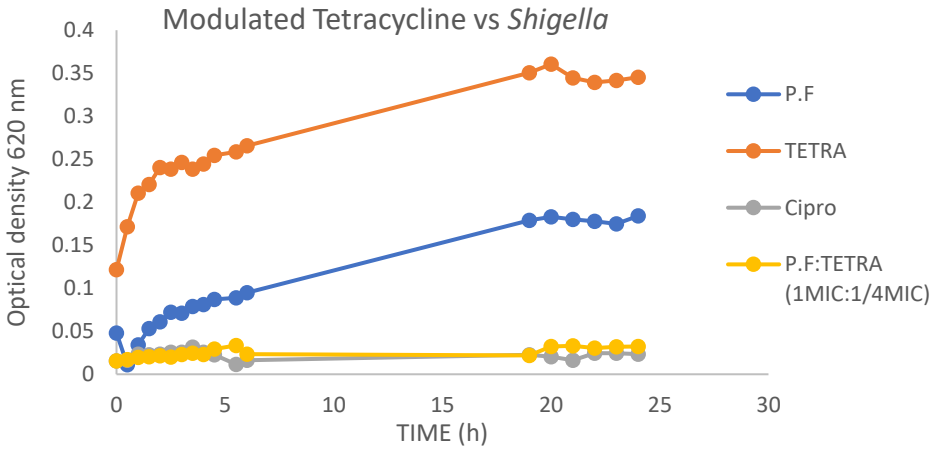


Figure 41: Tetracycline in combination with P.F in the ratio Tetra:P.F (1/4MIC:1MIC) showing bactericidal action (yellow) against the /bacteria *Shigella* similar to ciprofloxacin (grey).

From figure 41, tetracycline used alone against *Shigella* resulted in a bacteriostatic activity and also with poor inhibition property. However, when combined with the crude alkaloid extract of P. F in the ratio 1/4MIC:1MIC (Tetra:*AEPF*), it's activity was enhanced as it not only inhibited bacterial growth but also killed the bacteria right when they were suspended in the drug-media mixture, completely stopping bacteria growth. The improved activity resulted in an OD_{ave} closer to that observed for ciprofloxacin in the earlier study. When the standard tetracycline was used alone against *Shigella* with a MIC of 125 µg/ml, an OD_{ave} of 0.2259 was recorded during the first 6 hours and 0.3470 from 19 – 24 h, however, in combination with the crude alkaloid extract of P. F in a 1/4MIC:2MIC, an OD_{ave} of 0.0226 form 0 – 6 h and 0.0304 from 19 – 24 h. About 10 folds better when combined with AEPF in a 1/4 :2 ratio than when used alone.

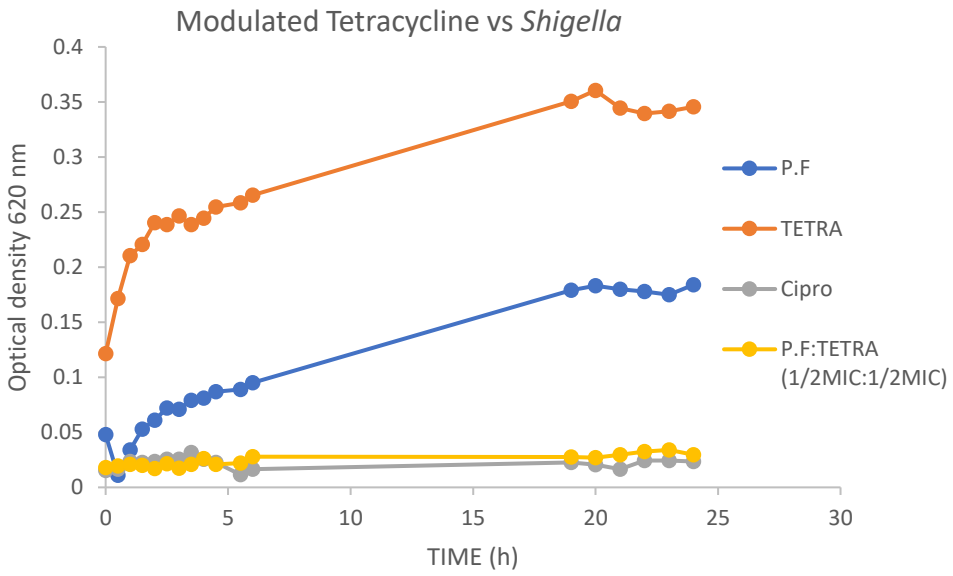


Figure 42: Tetracycline in combination with P.F in the ratio Tetra:AEPF (1/2MIC:1/2MIC) showing bactericidal action (yellow) against the bacteria *Shigella* similar to ciprofloxacin (grey).

Another combination of tetracycline with the crude alkaloid extract that yielded an additive interaction was the combination in the ratio 1/2MIC:1/2MIC (Tetra:*AEPF*) (Figure 42). The time-kill kinetics profile of this combination also showed an improved activity resulting in a complete bactericidal action in 24 h compared to the bacteriostatic activity observed when used alone. The improved activity recorded an OD_{ave} of 0.0210 from 0 – 6 h and 0.0300 from 19 – 24 h. Compared to these $OD_{ave,}$ tetracycline alone from 0 – 6 h and 19 – 24 h recorded 0.2259 and 0.3470 respectively. Results from this combination also was 10 folds better for both the first and last six (6) hours in the 24 hours study, emphasizing that combination therapy sometimes improve drug activity.

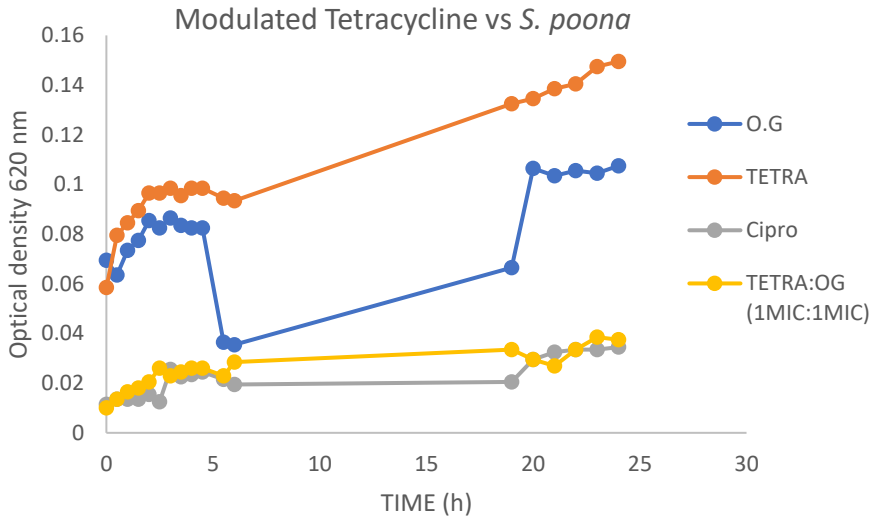


Figure 43: Tetracycline in combination with AEOG in the ratio Tetra:AEOG (1MIC:1MIC) showing bactericidal action (yellow) against the bacteria *S. poona* similar to ciprofloxacin (grey).

The modulation of tetracycline with the *AEOG* (Tetra:*AEOG*) also showed an improved activity against the bacteria *S. poona* in two different combinations; 1/2MIC:2MIC and 1MIC:1MIC. (Figure 43) shows a much better action of tetracycline against *S. poona* when combined with *AEOG* in a 1MIC:1MIC ratio compared to its activity observed in the earlier study when used alone. Although, tetracycline showed a bactericidal activity when used alone against *S. poona*, *AEOG* also showed the same bactericidal property and also had a better killing rate than tetracycline. After combining these two agents in a 1MIC:1MIC ratio, the OD_{ave} of 0.1806 from 0 – 6 h and 0.1995 from 19 – 24 h recorded earlier for tetracycline had changed remarkably to 0.0189 from 0 – 6 h and 0.0333 from 19 – 24 h. About 9.5 folds improvement in its bactericidal activity in the first six (6) hours and 5 folds improvement in the last six (6) hours.

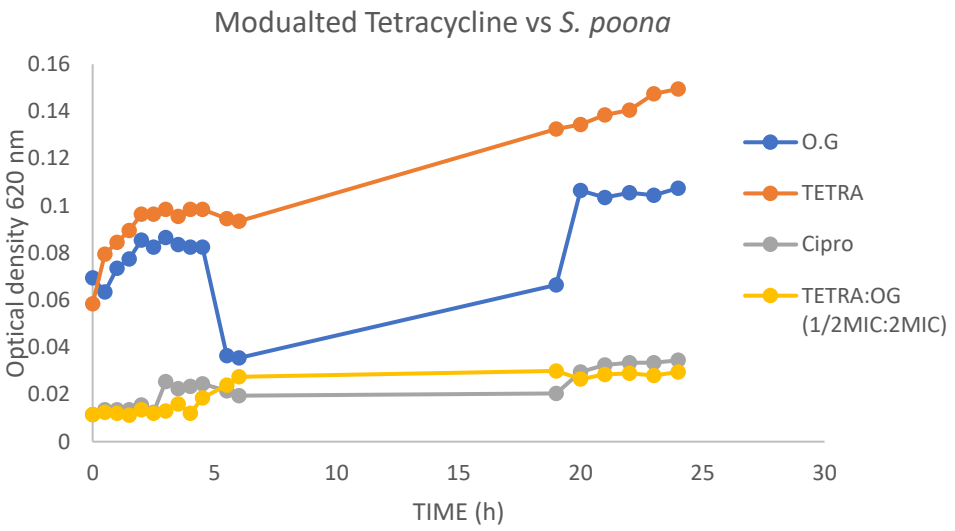


Figure 44: Tetracycline in combination with *O. G* in the ratio Tetra:AEOG (1/2MIC:2MIC) showing bactericidal action (yellow) against the bacteria *S. poona* similar to ciprofloxacin (grey).

The last *AEOG* and tetracycline combination that resulted in an additive interaction was in the ratio 1/2MIC:2MIC (Tetra:*AEOG*) (Figure 44). Just like in the previous observation, the bactericidal action of tetracycline did not change after combining it with *AEOG*. In fact, from the 20 – 24 h, this extract-drug combination recorded smaller optical densities indicating an even much better action than the standard ciprofloxacin. The OD_{ave} from 20 – 24h for ciprofloxacin was 0.0327 and 0.0283 for the modulated standard tetracycline.

Chromatographic Analysis

Thin Layer Chromatography (TLC)

Thin layer chromatography was carried out to establish the chemical profile of the crude alkaloidal extracts. The TLC results showed differences in alkaloid constituents in all three crude alkaloid extracts form all three plants *(AEOG*, 3 spots; *AEZZ*, at least 5 spots; *AEPF*, 3 Spots) under UV-light (245 nm) and when sprayed with Dragendorff's reagent.

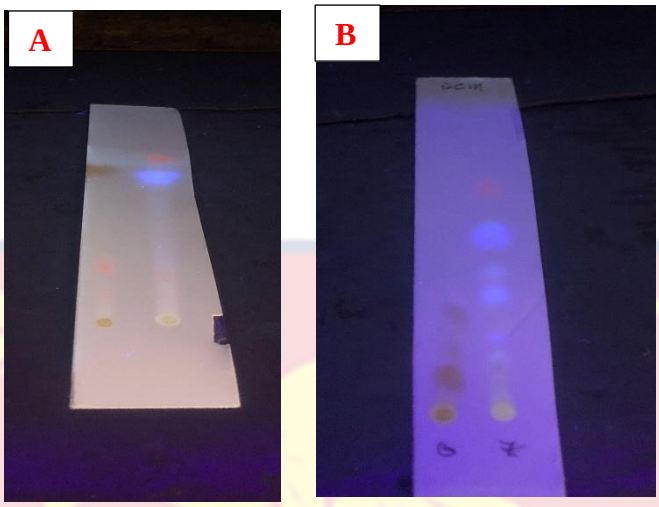


Figure 45: TLC chromatogram for the AEPF whole plant (A), AEOG leaves and AEZZ (B) leaves under UV (365 nm.

Chromatography Fractions of Isolates

Chromatographic fractionation of crude alkaloid extract of *O. gratissimum* for LC-MS Analysis

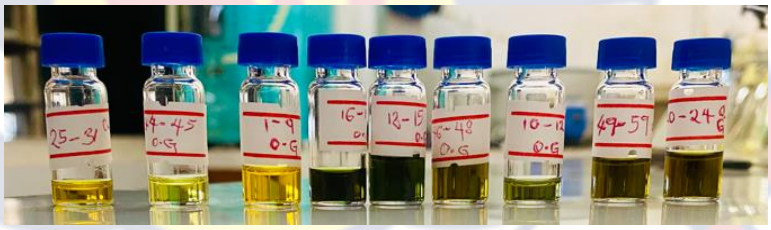


Figure 46: Concentrated fractions of isolates in 1.5 ml HPLC sample vials

Table 12: Masses of *O. gratissimum* Isolated Fractions Obtained from the Column Chromatography

Fraction	F1	F2	F3	F4	F5	F6	F7	F8	F9
Mass /mg	13.5	30	18	29.6	30.1	40.5	27.9	41.8	17.2
TLC Spot(s)	2	3	3	2	1	1	2	2	1

Source: Laboratory data (2022)

Three (3) out of the nine bulk fractions obtained from the column chromatography contained one spot from the thin layer chromatography (TLC) results after viewing the TLC plate under a UV-Lamp and spraying with Dragendorff's reagent; F5, F6 and F9. These fractions were expected to contain single alkaloidal compounds from the LC-MS analysis. Four (4) fractions (F1; F4; F7 and F8) obtained contained two spots and two (2) fractions (F2 and F3) showed three spots. Solvent fractions and their respective masses obtained from the column chromatography after combining the individual fractions based on the TLC results is presented (Figure 47). Due to the polarity of most alkaloids, the increased polarity of EtOAc in the 5:3 (Hexane:EtOAc) ratio yielded quite appreciable mass of fractions compared to hexane alone and the hexane:EtOAc in the 5:1 ratio (Figure 47).

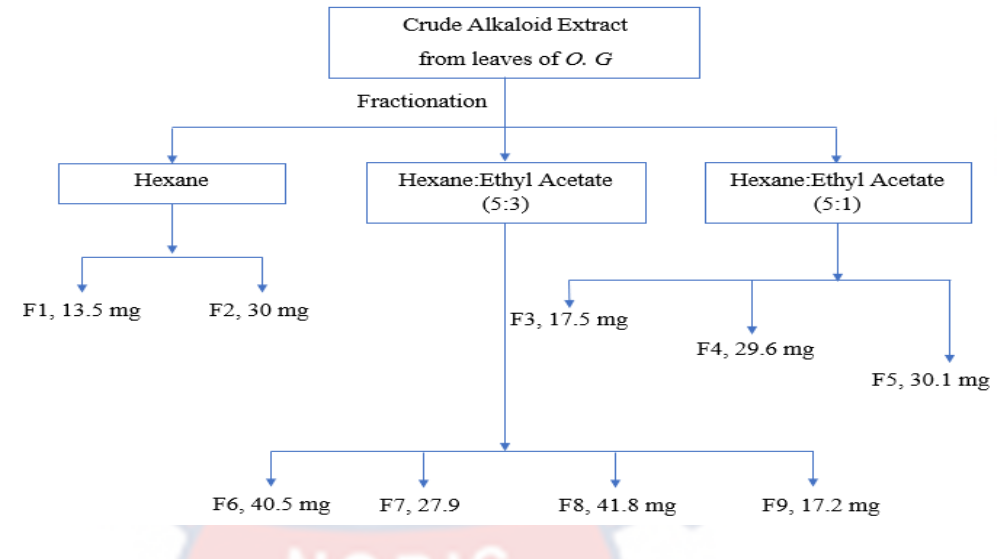


Figure 47: Isolates obtained from column chromatography of AEOG and their masses

Chromatographic fractionation of crude alkaloid extract of *P. fraternus* for LC-MS Analysis

Table 13: Masses of *P. fraternus* Isolated Fractions Obtained from the Column Chromatography

Fraction	F1	F2	F3	F4	F5	F6	F7
Mass /mg	18.2	16.3	24.1	21.2	18.9	22.1	9.1
TLC Spot(s)	1	2	1	1	1	2	1

Source: Laboratory data (2022)

Thin layer chromatography of most of the fractions from *AEPF* obtained from the column chromatography had single spots. Five (5) from the seven (7) fractions showed single spots and two (2) fractions showed two spots from the thin layer chromatography (TLC) results after viewing the TLC plates under a UV-Lamp followed by spraying with Dragendorff's reagent. The mass of the fractions obtained after concentrating the fractions on a rotary evaporator resulted in the Hexane:EtOAc (5:1) with higher yield in mass; F3, 24.1 mg; F4, 21.2 mg and F5, 18.9 mg (Figure 48).

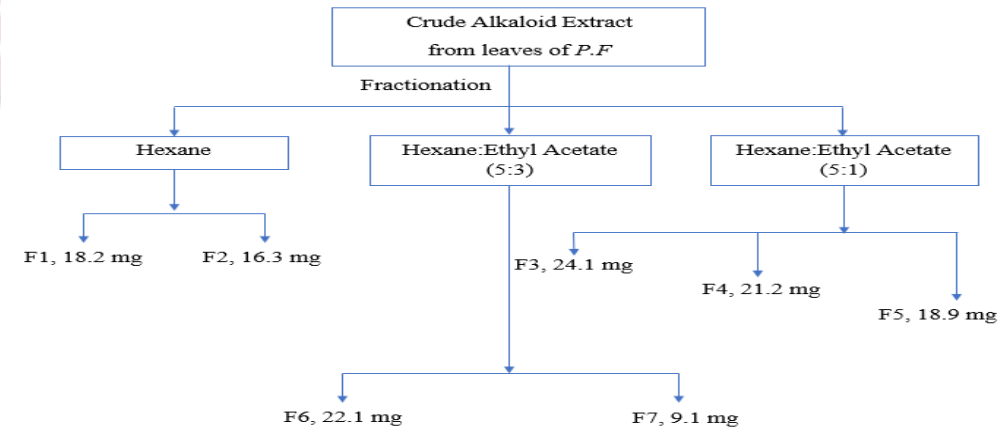


Figure 48: Isolates obtained from column chromatography of AEPF and their masses

Chromatographic fractionation of crude alkaloid extract of *Z. zanthoxyloide* for LC-MS Analysis

Table 14: Masses of *Z. zanthoxyloides* Isolated Fractions Obtained from the Column Chromatography

Fraction	F1	F2	F3	F4	F5	F6
Mass /mg	27.8	29.5	22.2	23.8	17.3	26.6
TLC Spot(s)	1	2	1	1	3	1

Source: Laboratory data (2022)

Table 14 (Continued)

Fraction	F7	F8	F9	F10	F11	F12
Mass /mg	14	11.4	28.2	18.5	33.1	41.8
TLC Spot(s)	4	3	3	3	2	N/A

Source: Laboratory data (2022)

Thin layer chromatography (TLC) results from the *AEZZ* prior to the flash column chromatography showed that the crude alkaloid extract contained at least five or more components. Fractions obtained from the column chromatography resulted in four (4) containing 1 spot, two (2) containing 2 spots, four (4) with 3 spots and one (1) with 4 spots after viewing the TLC plates under a UV-Lamp followed by spraying with Dragendorff's reagent. F7 containing 4 spots had a masses of 27.9 mg. Most of the fractions with high masses were obtained from the methanol and hexane:EtOAc (5:3) fractions (Figure 49).

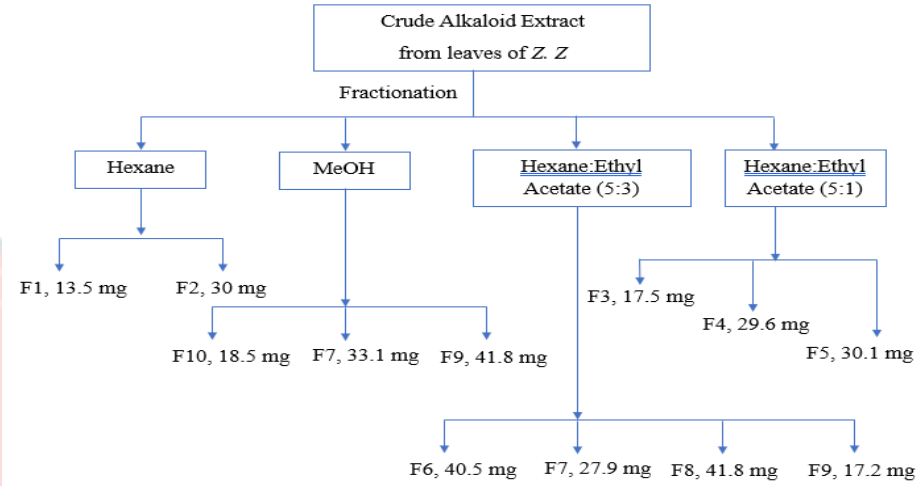


Figure 49: Isolates obtained from column chromatography of AEZZ and their masses

LC/MS Analysis of *Zanthoxylum zanthoxyloide* Isolates

For *Z. zanthoxyloide*, a total of four samples corresponding to the Column Chromatographic Fractions F1, F2, F5 and F7 were analyzed given the sample codes 1662-PES2-22, 1663-PES2-22, 1664-PES2-22, and 1665-PES2-22 respectively. Spectra results showed a total of four (4) compounds for fraction F1, fifteen (15) for F7, and nine (9) for both F2 and F5. Based on the mass spectrum of the samples analyzed, molecular (monoisotopic) masses of a total of 10 alkaloids were determined from their molecular ion $[M + H]^+$ peaks as; 259.1, 488.2, 376.2, 321.2, 405.3, 570.3, 600.4, 139.2, 364.1 and 515.2 Da. Tables for calculated molecular weight from the spectra of each sample analyzed is shown below.

LC/MS Analysis of *Occimum gratissimum* Isolates

A total of four samples corresponding to the Column Chromatographic Fractions F1, F2, F5 and F6 were analyzed given the sample codes, 1668-PES2-22, 1669-PES2-22, 1666-PES2-22 and 1667-PES2-22 respectively. Spectra

results showed a total of nine (9) compounds for F1, five (5) for F2, nine (9) for F5 and seven (7) for F6. Based on the mass spectrum of the samples analyzed, molecular (monoisotopic) masses of a total of 6 alkaloids were determined from their molecular ion $[M+H]^+$ peaks as; 416.2, 418.3, 320.2, 133.3, 696.2 and 402.3 Da. Tables for calculated molecular weight from the spectra of each sample analyzed is included in the appendix.

LC/MS Analysis of *Phyllanthus fraternus* Alkaloid Isolates

For *P. fraternus*, a total of three samples corresponding to the Column Chromatographic Fractions F1, F2, and F6 were analyzed given the sample codes 1670-PES2-22, 1671-PES2-22 and 1672-PES2-22 respectively. Spectra results showed a total of seven (7) compounds for fraction F1 and eleven (11) for both F2 and F6. Based on the mass spectrum of the samples analyzed, molecular (monoisotopic) masses of a total of 7 alkaloids were determined from their molecular ion $[M + H]^+$ peaks as; 416.2, 418.3, 320.2, 133.3, 696.2, 402.3 and 368.3 Da.

Identification of Alkaloids Based on LC-ESI-MS Fragmentation

Molecular mass of isolated and characterized alkaloids form literature were compared with molecular weight determined from the mass spectra. Molecular masses from spectra with similar known alkaloids from literature were selected as possible alkaloids.

LC/MS Spectrum Analysis

***Zanthoxylum zanthoxyloide* Alkaloids Molecular Weight Determination**

From the LC/MS results, for fraction F1, the molecular weight obtained from two spectrum corresponded to two isolated *Z. zanthoxyloide* alkaloids

(Ribalinine; Mol. Wt. 259.30 amu and Skimmianine; Mol Wt. 259.26 amu) reported in literature (Tatsadjieu *et. al.*, 2003). For fraction F2 as well, the spectrum of two (2) compounds had molecular masses corresponding to three (3) reported *Z. Zanthoxyloide* alkaloids; Nor-chelerythrine (Mol. Wt. 333.3), Zanthoamide I (Mol. Wt. 321.4 amu) and 8-Acetonyldihydrochelerythrine (Mol. Wt. 405.42 amu) in the literature (Queiroz *et. al*., 2006). Although the molecular weight obtained from the ESI-LC/MS spectrum may not outrightly imply the exact compounds, the odd molecular weights suggests that they contain nitrogen atoms which make them possible alkaloids.

Structural Identification of Compound A as Zanthoamide 1

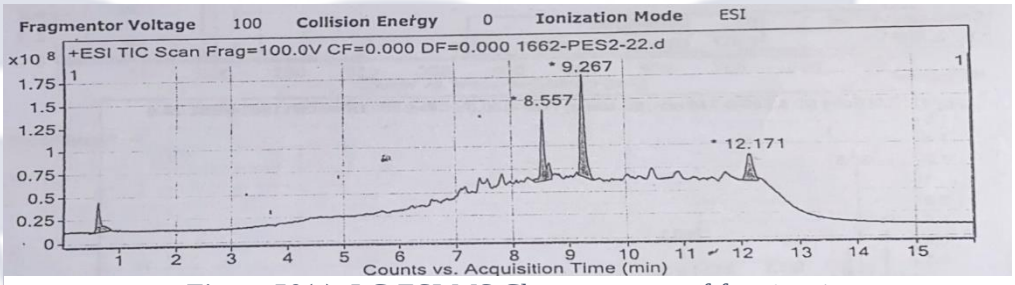


Figure 50(a): LC-ESI-MS Chromatogram of fraction 1

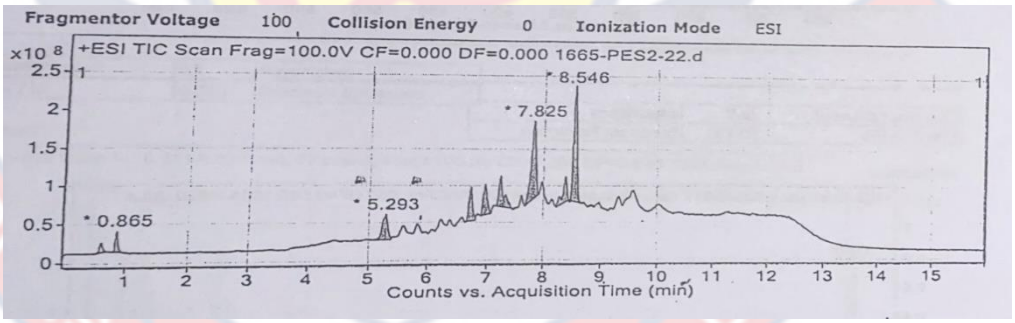


Figure 50(b): LC-ESI-MS Chromatogram of fraction 2

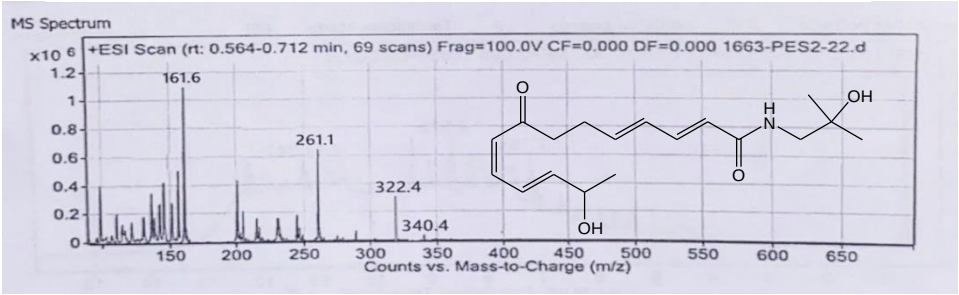


Figure 51: Full Scan LC-ESI-MS Spectrum of Compound A as Zanthoamide I from Fraction 1

Molecular Formula	Exact Mass	Cal. Mol. Weight
$C_{18}H_{27}NO_4$	321.4	321.4

The spectrum showed ions at *m/z* 261.1, 340.4 (weak), 322.4 and 161.6 (most intense). The molecular ion peak $[M+H]^+$ peak was observed at *m/z* 322.4. A strong peak at *m/z* 161.7 was attributed to the doubly charged pseudomolecular ion $[M + 2H]^{2+}$ and a weak peak at *m/z* 340.4 due to the addition of 19 amu (*m/z* 321.4 → *m/z* 340.4) was attributed to a pseudomolecular ion $[M+H_2O+H]^+$. Although strong, the peak at m/z 261.1 could not be assigned to any adduct.

Structural Identification of Compound B as Nor-chelerythrine

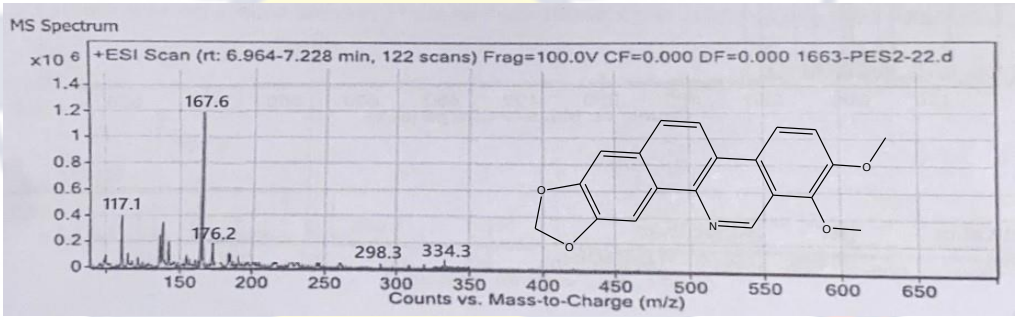


Figure 52: Full Scan LC-ESI-MS Spectrum of Compound B as Nor-chelerythrine from Fraction 1

Molecular Formula	Exact Mass/amu	Cal. Mol. Weight/amu
$C_{14}H_{13}NO_4$	333.337	333.3

The most intense peak $[M + 2H]^{2+}$ was observed at *m/z* 167.6. The m/z 334.3 peak is attributed to the molecular ion peak $[M+H]^+$, implying a molecular weight of 333.4 amu. A doubly charged pseudomolecular ion $[M+H+NH_4]^{2+}$ was also observed at *m/z* 176.2 (*m/z* 333.3 → *m/z* 176.2). Additional peaks at *m/z* 298.3 and 117.1 were also present.

Structural Identification of Compound C as 8-acetonyldihyro-chelerythrine.

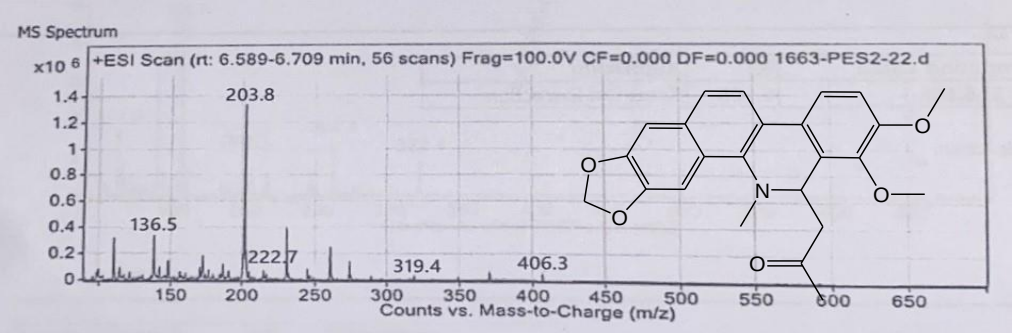


Figure 53: Full Scan LC-ESI-MS Spectrum of Compound C as 8-acetonyldihyro-chelerythrine from fraction 1

Molecular Formula	Exact Mass/amu	Cal. Mol. Weight/amu
$C_{24}H_{23}NO_5$	405.4	405.3

The spectrum showed the protonated molecule $[M+H]^+$ at *m*/z 406.3. The *m/z* 203.8 (strong) and 222.7 (weak) peaks could be attributed to the doubly charged pseudomolecular ions $[M+2H]^{2+}$ and $[M+H+NH_4]^{2+}$ respectively; (*m/z* 405.3 → *m/z* 203.8) for $[M+2H]^{2+}$ and (*m/z* 405.3 → *m/z* 222.7) for $[M+H+NH_4]^{2+}$. Other weak peaks at *m/z* 136.5 and 319.4 were also observed, however, could not be attributed to any adducts.

Structural Identification of Compound D and E as Skimmianine and Ribalinine

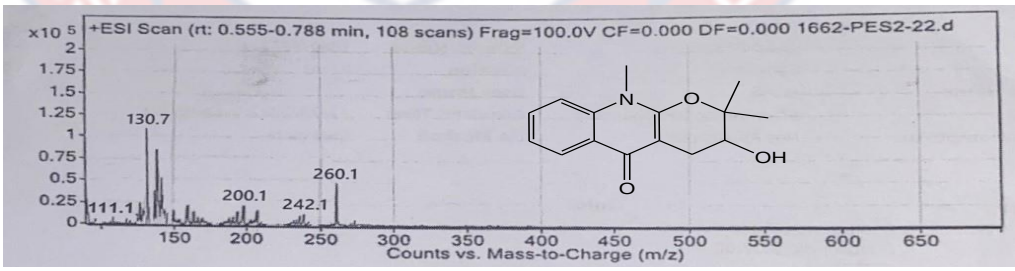


Figure 54: Full Scan LC-ESI-MS Spectrum of Compound D as Skimmianine from Fraction 2

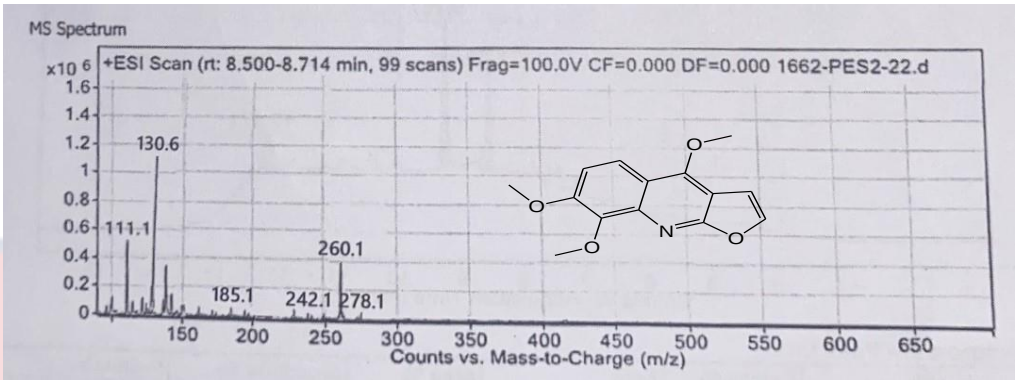


Figure 55: Full Scan LC-ESI-MS Spectrum of Compound E as Ribalinine from Fraction 2

Molecular Formula	Exact Mass/amu	Cal. Mol. Weight/amu
$C_{14}H_{13}NO_4$	259.261	259.1
$C_{15}H_{17}NO_3$	259.30	259.1

The two spectra showed ions at *m/z* 260.1, 242.3 (weak). The most intense peak in both spectra were also similar; *m/z* 130.7 for A and *m/z* 130.6 for B. The presence of a weak peak at 185.1 for B which is absent in spectrum A, and the presence of a prominent *m/z* 200.1 peak in A indicates that the two spectra represent the spectrum of two chemically different compounds. In both spectra, the *m/z* 260.1 is attributed to the $[M + H]^+$ parent molecular ion. The weak *m/z* 242.1 is attributed to the pseudo-molecular ion $[M – H_2O + H]$ by the loss of H_2O and addition of a proton (*m/z* 259.1 → *m/z* 242.1). The most intense peak at *m/z* 130.7 and 130.6 was attributed to the $[M+2H]^{2+}$ adduct. The peak at *m/z* 405.3 → *m/z* 278.1 corresponded to the adduct $[M+H+H_2O]^+$. The low intensity peaks at *m/z* 200.1, 185.1 and 111.1 could not be assigned to any adduct.

Phyllanthus fraternus Alkaloids Molecular Weight Determination

ESI-LC/MS analysis of fraction F2 showed a spectrum for eleven (11) different compounds with the molecular weight determined from two (2) spectra corresponding to two known p*hyllanthus fraternus* alkaloids from literature (Komlaga *et. al.*, 2017); Ent-norsecurinine (Mol. Wt. 203.24 amu) and Epibubbialine (Mol. Wt. 221.25 amu).

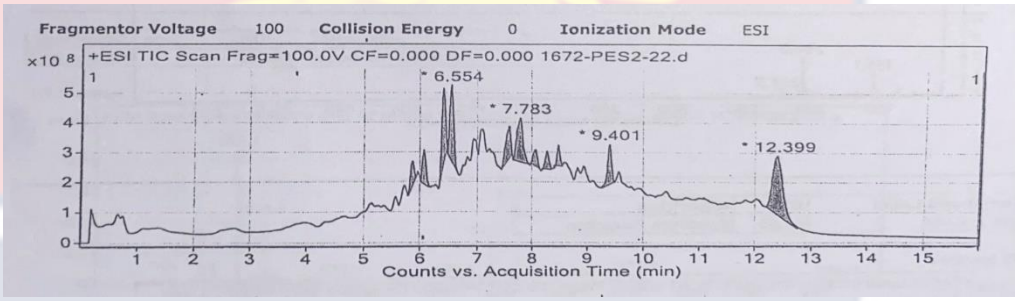


Figure 56: LC-ESI-MS Chromatogram of Fraction 2

Structural Identification of Compound F Ent-norsecurinine

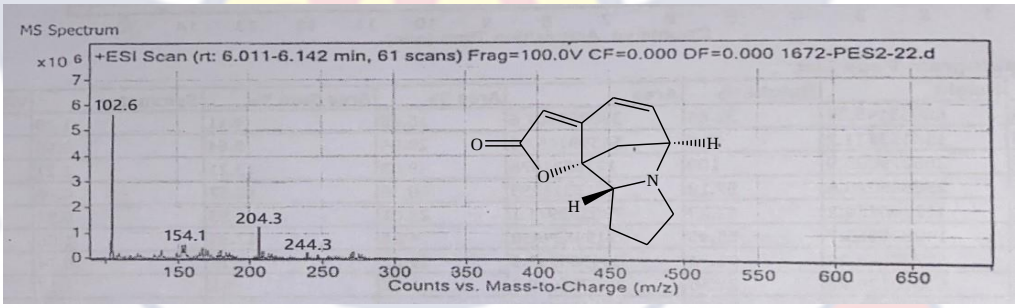


Figure 57: Full Scan LC-ESI-MS Spectrum of Compound F as Ent-norsecurinine

Molecular Formula	Exact Mass/amu	Cal. Mol. Weight/amu
$C_{12}H_{13}NO_2$	203.24	203.3

The spectrum showed ion peaks at *m/z* 244.3 (weak), 154.1 (weak), 204.3 and 102.6 (strong). The molecular ion peak $[M+H]^+$ was attributed to the peak at *m/z* 204.3. Implying a molecular weight 203.3 amu. The peak *m/z* 203.3 → *m/z* 102.6 corresponded to the doubly charged adduct $[M+2H]^{2+}$. The weak

peak at *m/z* 244.3 corresponded to an $[M+Na+H_2O]^+$ adduct. The weak peak at *m/z* 154.1 could however not be assigned to any adduct.

Structural Identification of Compound G as Epibubbialine

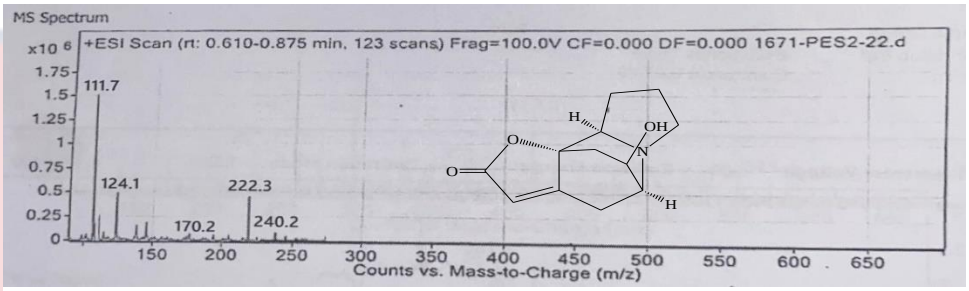


Figure 58: Full Scan LC-ESI-MS Spectrum of Compound G as Epibubbialine

Molecular Formula	Exact Mass/amu	Cal. Mol. Weight/amu
$C_{12}H_{15}NO_3$	221.25	221.3

The spectrum showed ion peaks at *m/z* 240.2 (weak), 170.2 (weak), 204.3, 124.1, 222.3 and 111.7 (strong). The molecular ion peak $[M+H]^+$ was attributed to the peak at m/z 222.3. Implying a molecular weight 221.3 amu. The doubly charged pseudomolecular ion $[M+2H]^{2+}$corresponded to the peak at m/z 111.7. The weak peak at *m/z* 240.2 was attributed to the adduct $[M+H_2O+H]^+$ (*m/z* 221.3 → *m/z* 240.2). No adduct was assigned for the peaks at m/z 1170.2 and 124.1.

***Occimum gratissimum* Alkaloids Molecular Weight Determination**

For ESI-LC/MS analysis, fraction F5 showed spectrum for fifteen (15) different compounds with the molecular weight determined for one (1) spectrum corresponding to one reported *Occimum gratissimum* alkaloids from literature (Adeshina *et. al.*, 2018.); Benimidazol-5-amine (Mol. Wt. 133.3 amu).

Structural Identification of Compound F as Benimidazol-5-amine

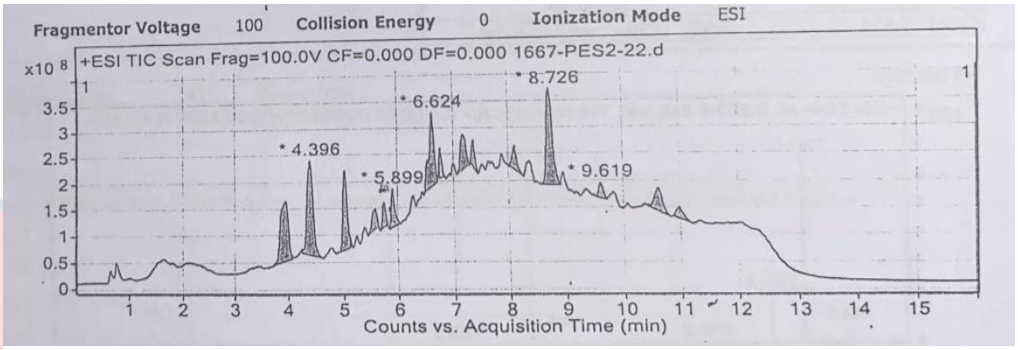


Figure 59: LC-ESI-MS Chromatogram of fraction F3

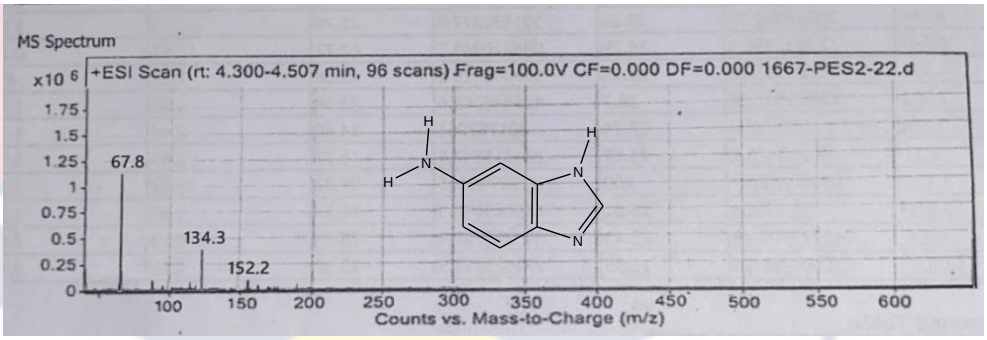


Figure 60: Full Scan LC-ESI-MS Spectrum of Compound H as Benimidazol-5-amine

Molecular Formula	Exact Mass/amu	Cal. Mol. Weight/amu
$C_7H_7N_3$	133.15	133.3

Three prominent peaks were observed from the spectrum; a peak at *m/z* 152.2 and two strong peaks at *m/z* 67.8 and 134.3. The molecular ion peak $[M+H]^+$ was attributed to the peak at *m/z* 134.3. The doubly charged adduct $[M+2H]^{2+}$ corresponded to the peak (*m/z* 133.3 → *m/z* 67.8) and addition of 19 amu by the weak peak at 152.2 was attributed to the adduct $[M+H_2O+H]^+$.

Chemical structures of all possible alkaloids based on the molecular weight determined from the LC-ESI-MS analysis, the nitrogen rule, double bond equivalence (DBE) and molecular formulae were sketched using

Chemdraw and are presented (Figure 61 – 68). Chemical names, molecular formulae and DBE values of all structures are presented in (Appendix E).

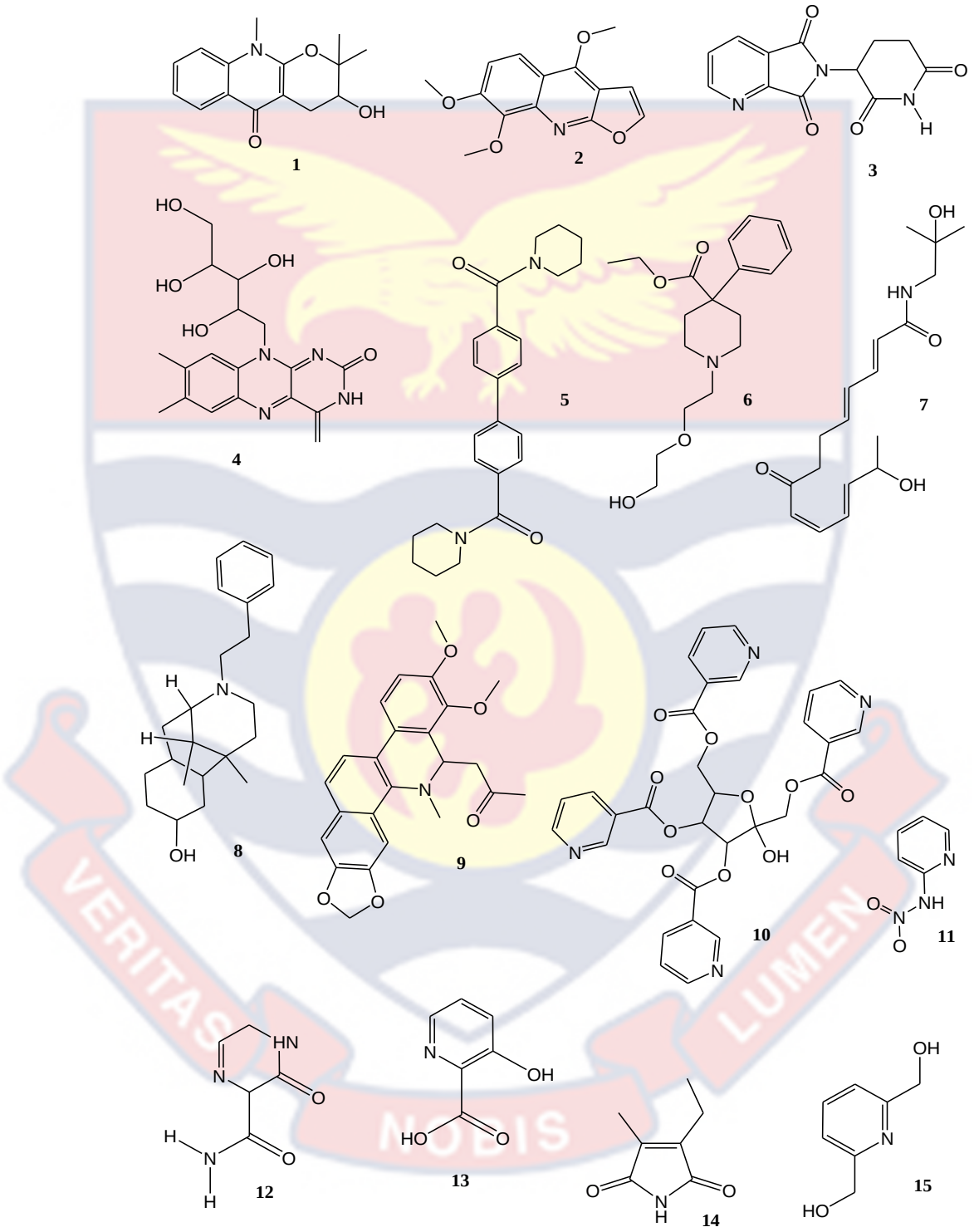


Figure 61: Chemical structure of all possible *Z. zanthoxyloides* alkaloids based on molecular mass, nitrogen rule and DBE calculations

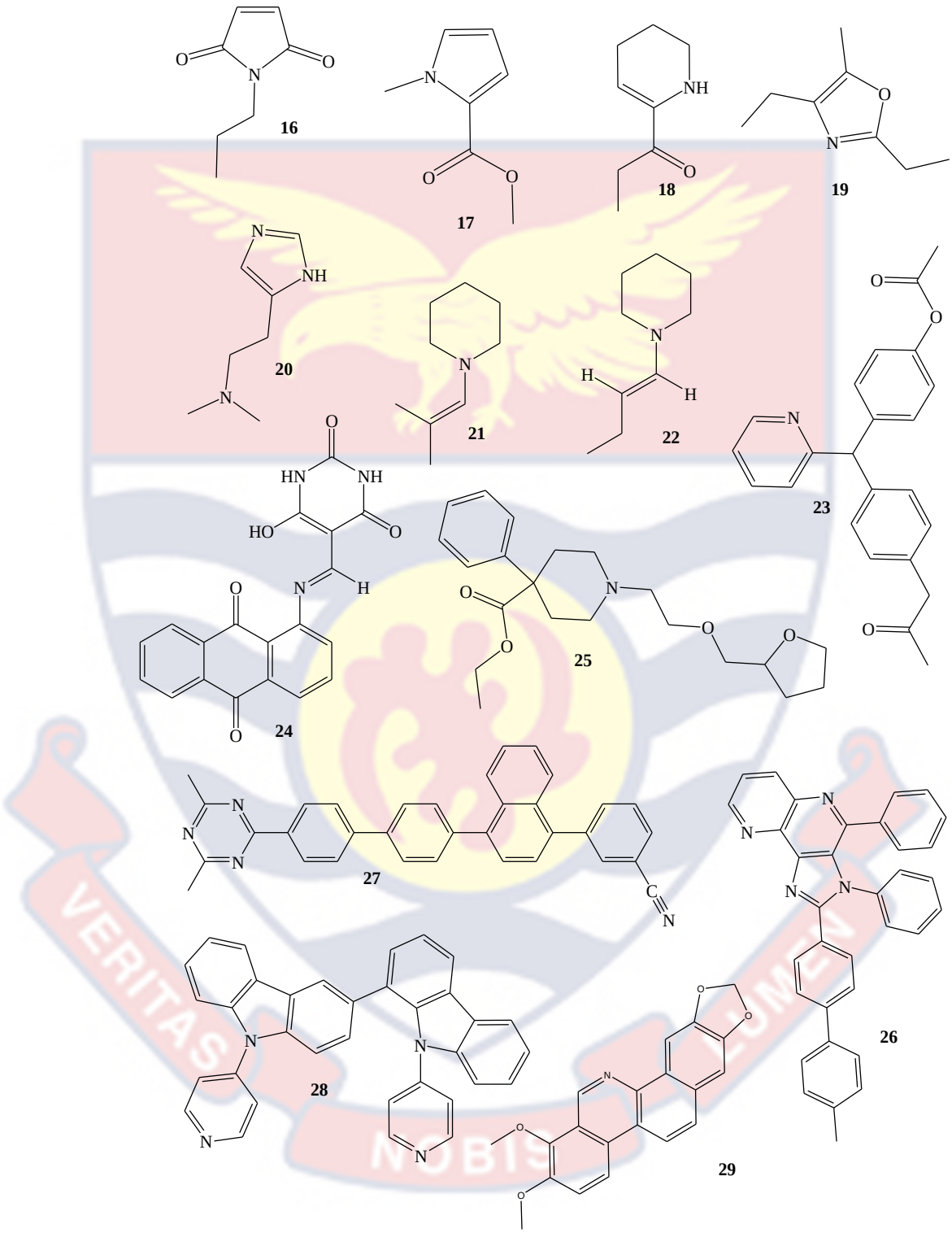


Figure 62: Chemical structure of all possible *Z. zanthoxyloides* alkaloids based on molecular mass, nitrogen rule and DBE calculations

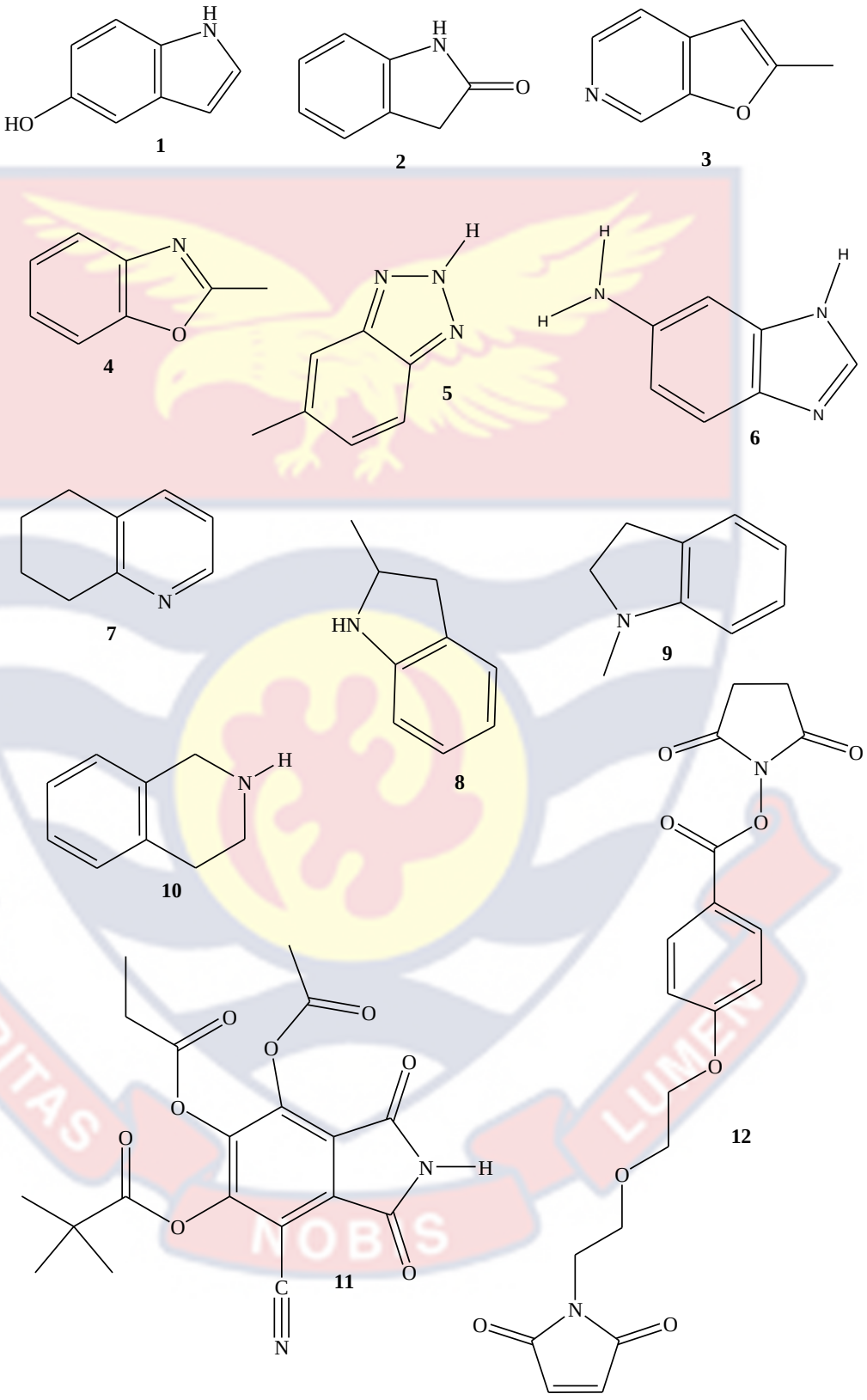


Figure 63: Chemical structure of all possible *Occimum gratissimum* alkaloids based on molecular mass, nitrogen rule and DBE calculations

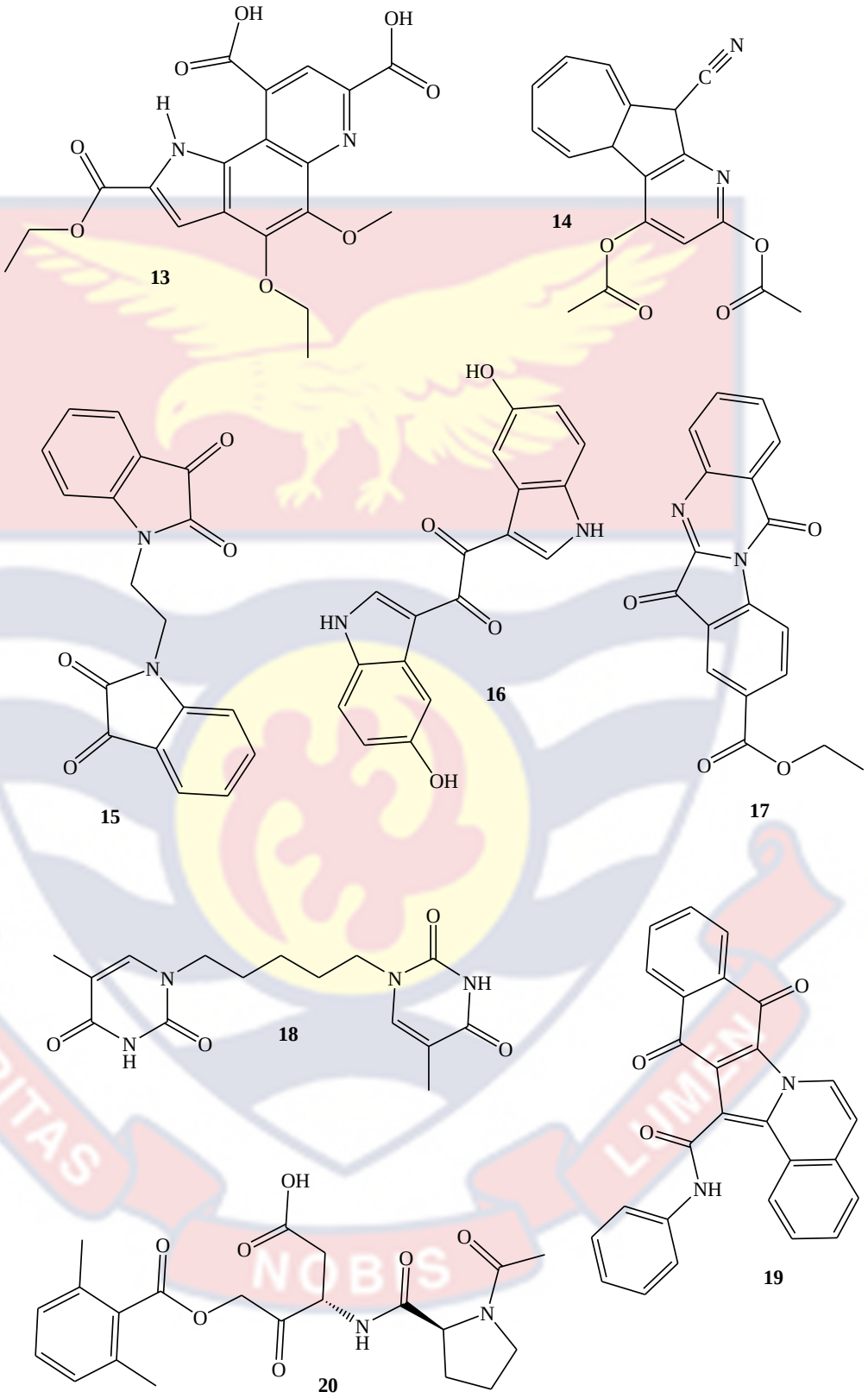


Figure 64: Chemical structure of all possible *Occimum gratissimum* alkaloids based on molecular mass, nitrogen rule and DBE calculations

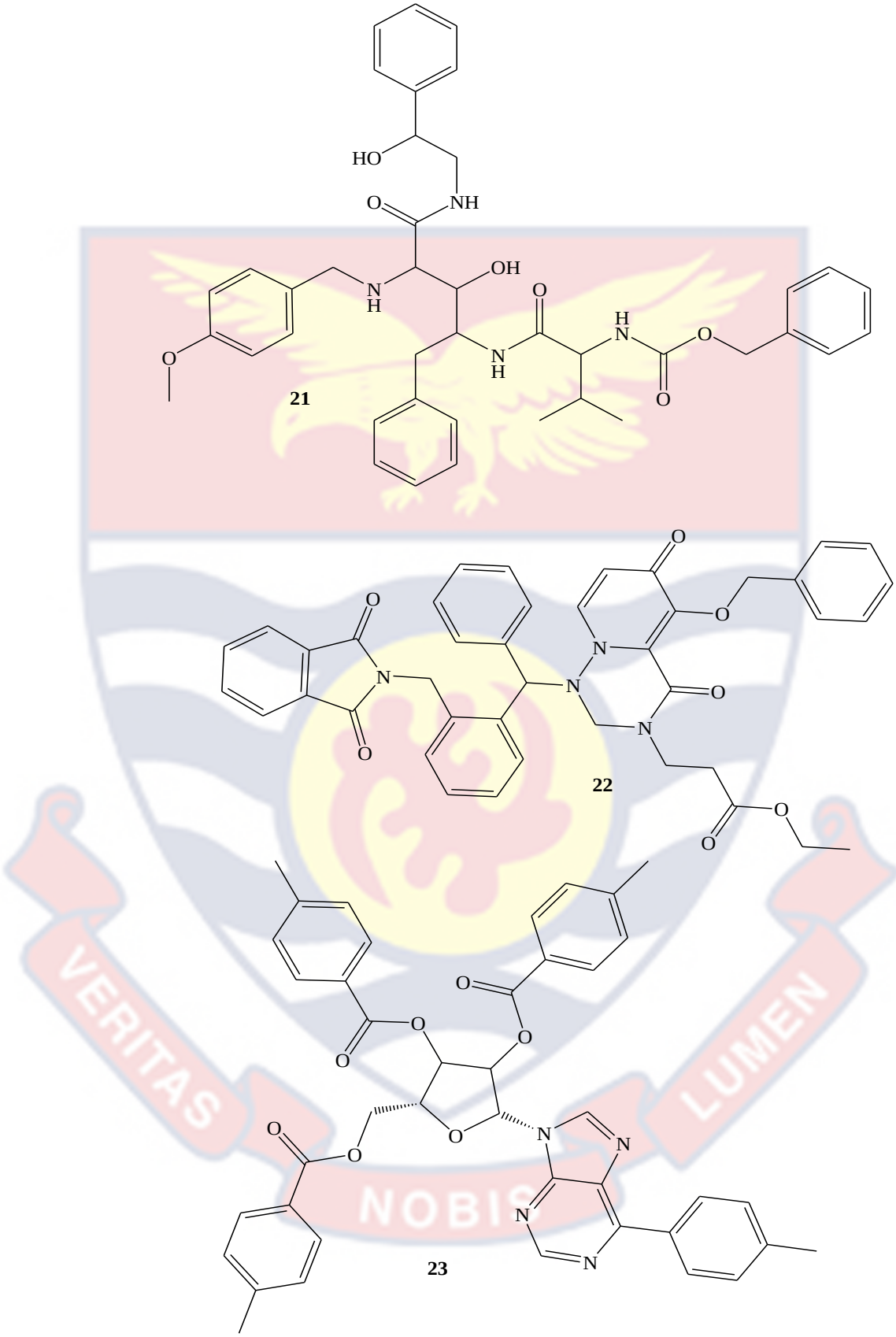


Figure 65: Chemical structure of all possible *Occimum gratissimum* alkaloids based on molecular mass, nitrogen rule and DBE calculations

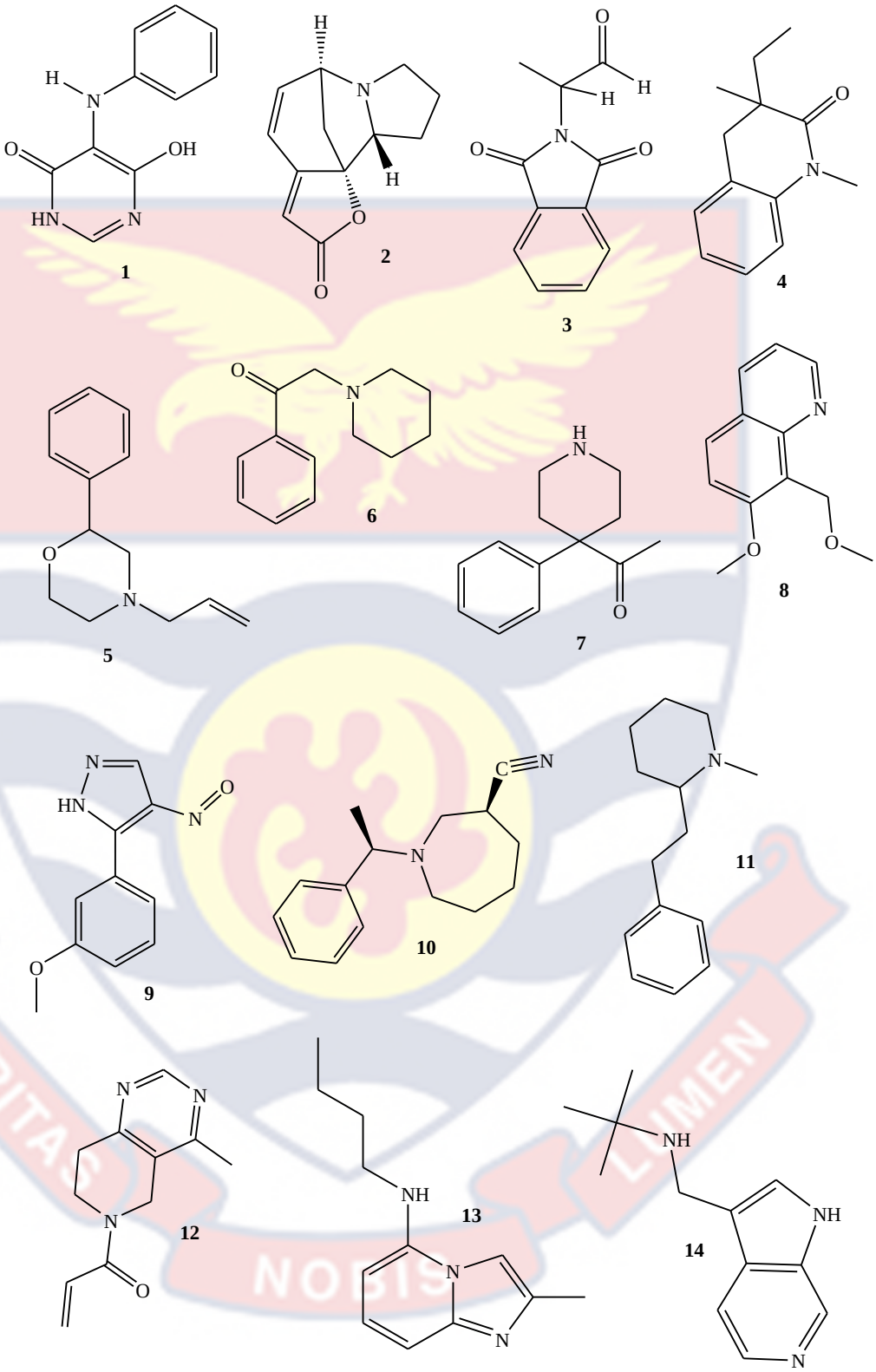


Figure 66: Chemical structure of all possible *Phyllanthus fraternus* alkaloids based on molecular mass, nitrogen rule and DBE calculations

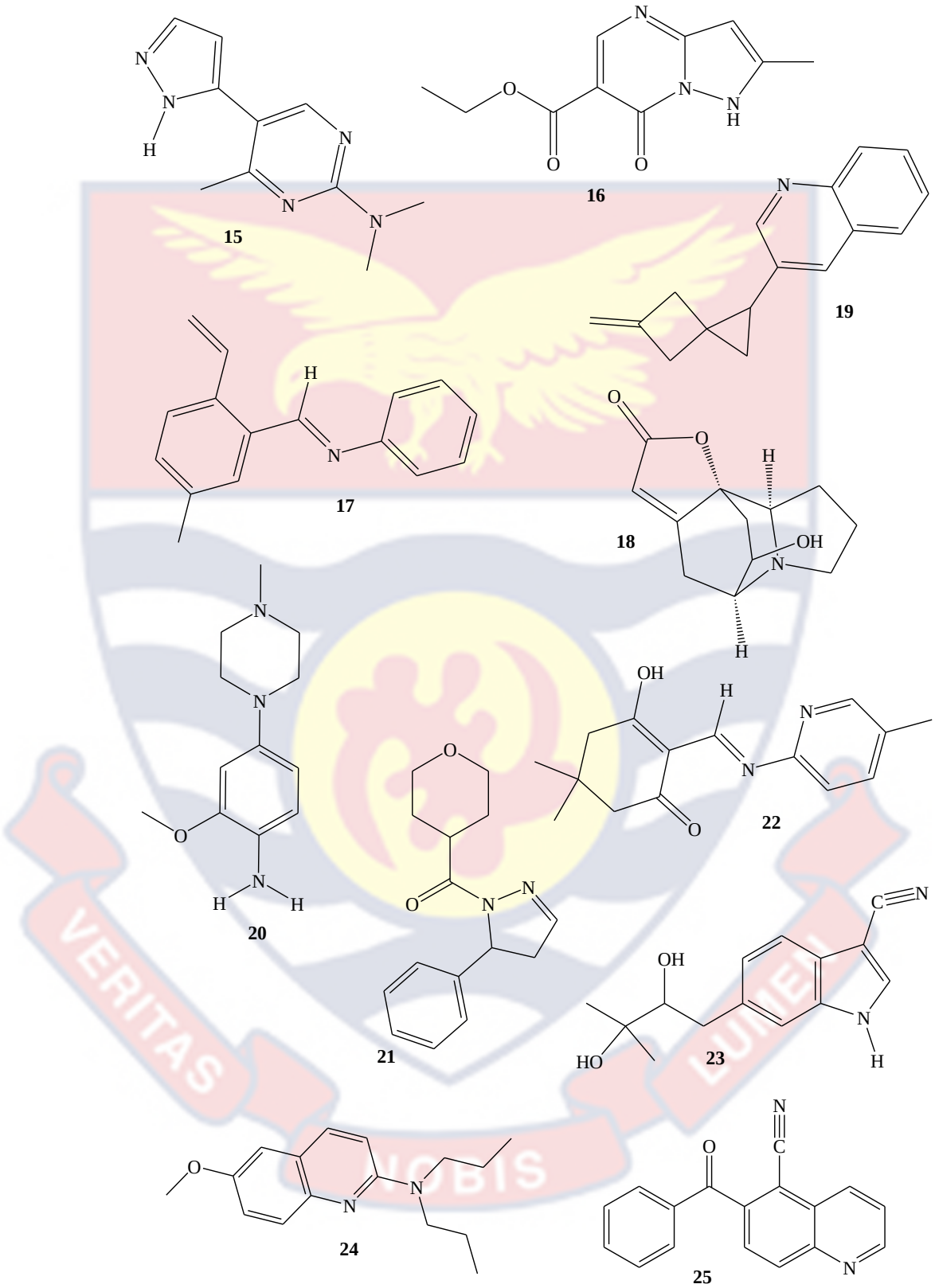


Figure 67: Chemical structure of all possible *Phyllanthus fraternus* alkaloids based on molecular mass, nitrogen rule and DBE calculations

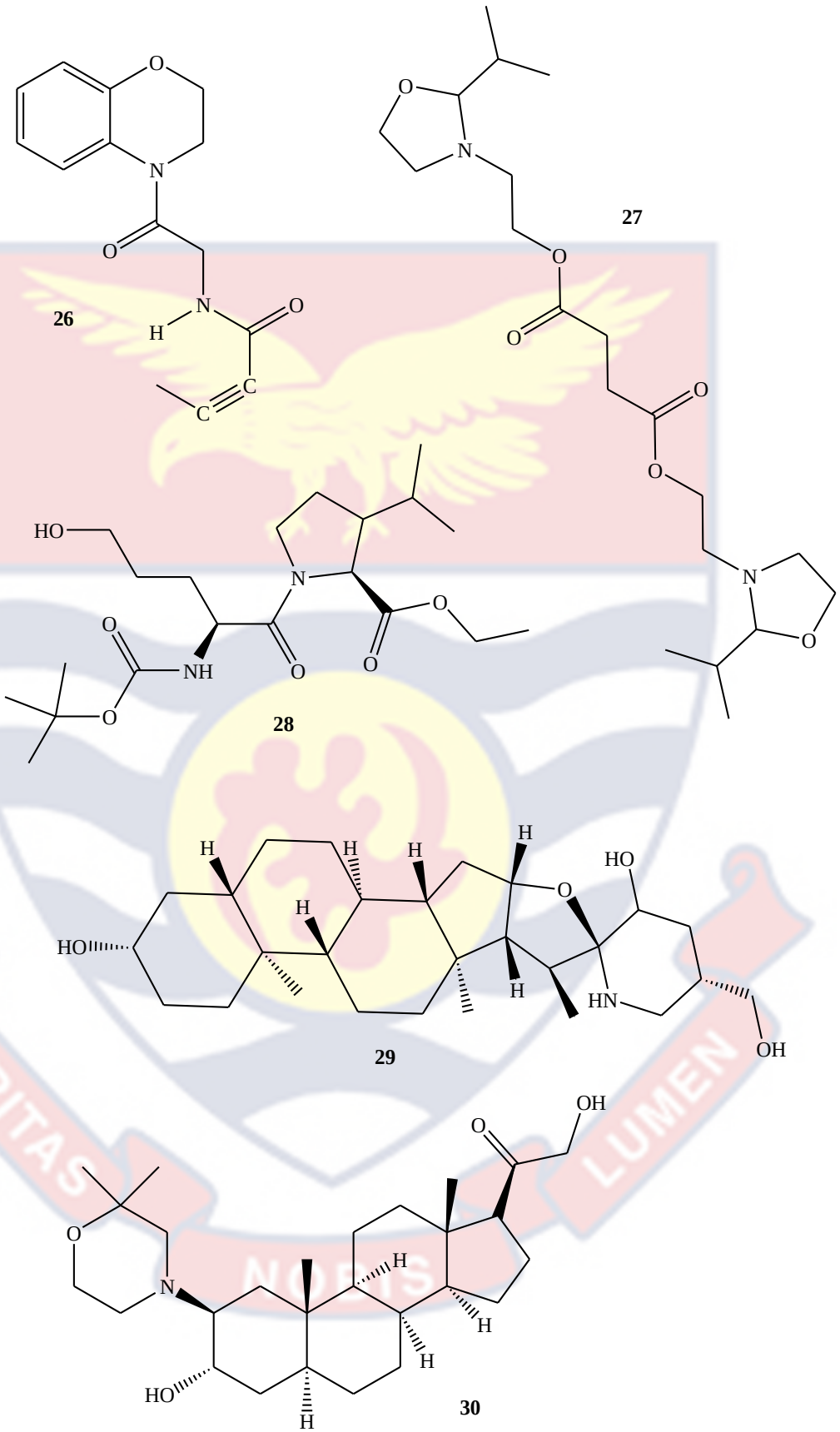


Figure 68: Chemical structure of all possible *Phyllanthus fraternus* alkaloids based on molecular mass, nitrogen rule and DBE calculations

Chapter Summary

This chapter presents the main results in the present study. All the findings from the research have been presented and discussed in this chapter. Results including phytochemical screening, test for alkaloids, minimum inhibition concentration (MIC), time-kill kinetics and molecular masses of purified and isolated alkaloids have all been discussed.

CHAPTER FIVE

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Overview

The study aimed at investigating the leaves of *Occimum gratissimum*, *Phyllanthus fraternu*s (whole plant) and leaves of *Zanthoxylum zanthoxyloide* for their secondary metabolites, extraction of their alkaloids, as well as the antibacterial properties of the crude alkaloids, *in-vitro*, using the HT-SPOTi assay. Again, due to bacteria resistance, the standard Tetracycline was modulated with the crude alkaloids from the three plants to improve its activity.

Summary

In traditional herbal medicine *O. gratissimum, P. fraternus* and *Z. zanthoxyloide* are used in the treatment of diverse infections. The qualitative phytochemical analyses revealed that the crude extract of *O. gratissimum* (leaves), *P. fraternus* (whole plant) and *Z. zanthoxyloide* (leaves) all contain alkaloids, triterpenoids, steroids, tannins and glycosides. These phytochemicals are known to have established antimicrobial activities and multiple pharmacological effects and hence the use of these plants in traditional medicine may be rationalized by the presence of these compounds.

In-vitro antibacterial studies of the three crude alkaloid extract showed fairly good inhibition properties against most of the test bacteria used in the study, especially, *S. aureus* and *E. coli (43), Shigella* and *Staph lentus.* Ciprofloxacin showed complete bacteria growth inhibition towards all bacteria involved in the study, *in-vitro*. Tetracycline however showed greater inhibition against most of the bacteria, but performed poorly against *Shigella.*

Conclusion

In conclusion, the extraction of the crude constituents in the three medicinal plants revealed that they all contain Alkaloids, Steroid, Tannins, Glycosides and Flavonoids which are important class of secondary metabolites with reported medicinal properties.

In-vitro antibacterial studies of the three crude alkaloid extract showed fairly good inhibition properties against most of the test bacteria used in the study, especially, *S. aureus* and *E. coli (43), Shigella* and *Staph lentus.*

Time-kill kinetic study to monitor crude alkaloids as *in-vitro* bactericides or bacteriostats revealed the *AEZZ* as by far, a more potent bacteriostatic agent against *S. poona, S. aureus* and *S. lentus* compared to Tetracycline, in 24 hours. The *AEPF* also exhibited a much more potent activity (bacteriostatic) against the bacteria *E. coli* (43), *E. coli* (10) and *S. aureus* compared to Tetracycline.

For Tetracycline, which showed poor *in-vitro* activity (MIC; 125 µg/ml) against *Shigella* when used alone, its antibacterial potency was successfully optimized in the modulation study with the crude alkaloid of *P. fraternus* and *O. gratissimum* showing complete bactericidal activities.

LC-ESI-MS analysis revealed that the *Zanthoxylum zanthoxyloide* fractions contained four known alkaloids; Zanthoamide I (**A**), Nor-chelerythrine (**B)**, 8-Acetonyldihydrochelerythrine (**C**), Skimmianine (**D)** and Ribalinine (**E**). *Phyllanthus fraternus* alkaloid fractions revealed two reported alkaloids from literature; Entonorsercurinine (**F**) and Epibubbialine (**G**). *Occimum*

gratissimum alkaloid fractions revealed one reported alkaloid from literature; Benzimidazol-5-amine (**H**).

Recommendations

1. The individual components of these crude alkaloids could be isolated and tested for their individual antibacterial activity.
2. It has frequently been proven that the effectiveness of an antibiotic agent can be rescued by combining it with other biochemically active compounds (Jenkins & Cooper, 2012; Uzair *et al.*, 2017). Actions involving combining these alkaloids with other existing antimicrobials that are ineffective or no more thought to be potent against the two bacteria; *Salmonella Poona* and *Shigella* can be investigated to improve their potency.
3. Owing to the fact that some alkaloids are poisonous, toxicity studies of these alkaloid extracts should be considered in future work, because a typical example being MRSA infections require the application of a topical as the infections are on the skin.
4. Another very important future step would be determining if the alkaloids extracts can be used to inhibit these bacteria growth *in -vivo.*

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APPENDICES

APPENDIX A

TIME-KILL KINETICS TABLES

Table A1: Optical densities of *AEPF*, Tetra, Cipro and Tetracycline in Combination with *AEPF* Against *Shigella* Used for the Time-Kill Kinetic Curve

Time/h	$OD_{P.F}$	OD_{Tetra}	OD_{Cipro}	$OD_{Tetra:PF}$
0	0.048	0.1215	0.0155	0.0155
0.5	0.011	0.1715	0.0165	0.017
1	0.034	0.2105	0.0235	0.0195
1.5	0.053	0.2205	0.0225	0.0205
2	0.061	0.2405	0.0235	0.0215
2.5	0.072	0.2385	0.0255	0.02
3	0.071	0.2465	0.0255	0.023
3.5	0.079	0.2385	0.0315	0.0245
4	0.081	0.2445	0.0255	0.023
4.5	0.087	0.2545	0.0225	0.0295
5.5	0.089	0.2585	0.0115	0.0335
6	0.095	0.2655	0.0165	0.0235
19	0.179	0.3505	0.0225	0.022
20	0.183	0.3605	0.0205	0.0325
21	0.18	0.3445	0.0165	0.033
22	0.178	0.3395	0.0245	0.0305
23	0.175	0.3415	0.0245	0.032
24	0.184	0.3455	0.0235	0.0325

P.F MIC = 125 µg/mL; Tetracycline MIC = 125 µg/mL; Cipro MIC = 3.9 µg/mL; Tetra:*P.F* = 1/4MIC:1MIC

Table A2: Optical densities of *AEPF*, Tetra, Cipro and Tetracycline in Combination with *AEPF* Against *Shigella* Used for the Time-Kill Kinetic Curve

Time/h	$OD_{P.F}$	OD_{Tetra}	OD_{Cipro}	$OD_{Tetra:PF}$
0	0.048	0.1215	0.0155	0.018
0.5	0.011	0.1715	0.0165	0.0195
1	0.034	0.2105	0.0235	0.021
1.5	0.053	0.2205	0.0225	0.02
2	0.061	0.2405	0.0235	0.017
2.5	0.072	0.2385	0.0255	0.02
3	0.071	0.2465	0.0255	0.0175
3.5	0.079	0.2385	0.0315	0.021
4	0.081	0.2445	0.0255	0.026

4.5	0.087	0.2545	0.0225	0.021
5.5	0.089	0.2585	0.0115	0.022
6	0.095	0.2655	0.0165	0.028
19	0.179	0.3505	0.0225	0.0275
20	0.183	0.3605	0.0205	0.027
21	0.18	0.3445	0.0165	0.0295
22	0.178	0.3395	0.0245	0.0325
23	0.175	0.3415	0.0245	0.034
24	0.184	0.3455	0.0235	0.0295

P.F MIC = 125 µg/mL; Tetracycline MIC = 125 µg/mL; Cipro MIC = 3.9 µg/mL; Tetra:*P.F* = 1/4MIC:1MIC

Table A3: Optical densities of *AEOG*, Tetra, Cipro and Tetracycline in Combination with *AEOG* Against *S. Poona* Used for the Time-Kill Kinetic Curve

Time/h	$OD_{O.G}$	OD_{Tetra}	OD_{Cipro}	$OD_{Tetra:O.G}$
0	0.0695	0.0585	0.0115	0.0101
0.5	0.0635	0.0795	0.0135	0.0135
1	0.0735	0.0845	0.0135	0.0165
1.5	0.0775	0.0895	0.0135	0.018
2	0.0855	0.0965	0.0155	0.0205
2.5	0.0825	0.0965	0.0125	0.026
3	0.0865	0.0985	0.0255	0.023
3.5	0.0835	0.0955	0.0225	0.0245
4	0.0825	0.0985	0.0235	0.026
4.5	0.0825	0.0985	0.0245	0.026
5.5	0.0365	0.0945	0.0215	0.023
6	0.0355	0.0935	0.0195	0.0285
19	0.0665	0.1325	0.0205	0.0335
20	0.1065	0.1345	0.0295	0.0295
21	0.1035	0.1385	0.0325	0.027
22	0.1055	0.1405	0.0335	0.0335
23	0.1045	0.1475	0.0335	0.0385
24	0.1075	0.1495	0.0345	0.0375

O.G MIC = 125 µg/mL; Tetracycline MIC = 15.6 µg/mL; Cipro MIC = 3.9 µg/mL; Tetra:*O.G* = 1MIC:1MIC

Table A4: Microplate Reader's Recorded Optical Densities for the blanks (The Crude Alkaloid Extract, Drug Solutions, MHB)

Solution	OD1	OD2	OD_{ave} ± SD
O. Gratissimum	0.321	0.323	0.322 ± 0.001
Z. Zanthoxyloide	0.236	0.24	0.238 ± 0.003
P. Fraternus	0.211	0.215	0.213 ± 0.003
Tetracycline	0.281	0.285	0.283 ± 0.003

Ciprofloxacin	0.003	0.003	0.003 ± 0.000
MHB	0.181	0.178	0.1795 ± 0.002

Table A5: OD for Crude Alkaloidal Extract of P.F against *E. coli* (43) at 125 µg/ml MIC

Time/hr	Broth-Extract OD	Broth-Tetra OD	Broth-Cipro OD	Neg. Control
0	0.041	0.1625	0.0075	0.1105
0.5	0.038	0.0035	0.0075	0.1555
1	0.039	0.0065	0.0085	0.186
2	0.041	0.0175	0.0105	0.1925
2.5	0.043	0.0265	0.0115	0.1945
3	0.043	0.0255	0.0075	0.214
3.5	0.043	0.0265	0.0125	0.223
4	0.042	0.0245	0.0095	0.2335
4.5	0.042	0.0285	0.0095	0.236
5	0.043	0.0305	0.0115	0.2395
5.5	0.042	0.0275	0.0095	0.249
6	0.044	0.0245	0.0125	0.2585
19	0.045	0.0665	0.0085	0.669
20	0.046	0.0745	0.0085	0.719
21	0.043	0.0715	0.0085	0.742
22	0.044	0.0685	0.0095	0.795
23	0.044	0.0765	0.0105	0.804
24	0.044	0.0865	0.0105	0.829

Table A6: OD for Crude Alkaloidal Extract of P.F against *E. coli* (10) at 250 µg/ml MIC

Time/hr	Broth-Extract OD	Broth-Tetra OD	Broth-Cipro OD	Neg. Control
0	0.074	0.1145	0.0075	0.0855
0.5	0.076	0.1505	0.0075	0.1705
1	0.037	0.1715	0.0085	0.1895
2	0.064	0.1895	0.0105	0.198
2.5	0.08	0.1995	0.0115	0.204
3	0.087	0.1985	0.0075	0.2255
3.5	0.1	0.2025	0.0125	0.2405
4	0.106	0.1965	0.0095	0.246
5	0.117	0.2055	0.0115	0.255
5.5	0.116	0.2045	0.0095	0.274
6	0.111	0.1995	0.0125	0.293
19	0.142	0.2375	0.0085	0.679
20	0.136	0.2505	0.0085	0.721
21	0.131	0.2365	0.0085	0.7445
22	0.132	0.2335	0.0095	0.7475
23	0.13	0.2405	0.0105	0.7655
24	0.129	0.2485	0.0105	0.831

Table A7: OD for Crude Alkaloidal Extract of P.F against *S. aureus* at 125 µg/ml MIC

Time/hr	Broth-Extract OD	Broth-Tetra OD	Broth-Cipro OD	Neg. Control
0	0.065	0.1135	0.0025	0.0855
0.5	0.069	0.1505	0.0005	0.1705
1	0.082	0.1645	0.0015	0.201
2	0.098	0.1815	0.0025	0.2035
2.5	0.113	0.1925	0.0035	0.208
3	0.113	0.1925	0.0035	0.2255
3.5	0.123	0.1975	0.0045	0.2365
4	0.125	0.1915	0.0045	0.2365
4.5	0.127	0.1965	0.0035	0.238
5	0.131	0.1995	0.0035	0.2405
5.5	0.131	0.2025	0.0055	0.246
6	0.132	0.1845	0.0025	0.304
19	0.22	0.2015	0.0025	0.8555
20	0.222	0.2105	0.0025	0.922
21	0.226	0.2005	0.0015	1.0255
22	0.225	0.1945	0.0035	1.0575
23	0.225	0.1975	0.0045	1.0795
24	0.227	0.1945	0.0045	1.083

Table A8: OD for Crude Alkaloidal Extract of P.F against *Shigella* at 125 µg/ml MIC

Time/hr	Broth-Extract OD	Broth-Tetra OD	Broth-Cipro OD	Neg. Control
0	0.048	0.1215	0.0155	0.0555
0.5	0.011	0.1715	0.0165	0.1955
1	0.034	0.2105	0.0235	0.199
2	0.053	0.2205	0.0225	0.2055
2.5	0.061	0.2405	0.0235	0.2085
3	0.072	0.2385	0.0255	0.2285
3.5	0.071	0.2465	0.0255	0.2425
4	0.079	0.2385	0.0315	0.235
4.5	0.081	0.2445	0.0255	0.2365
5	0.087	0.2545	0.0225	0.258
5.5	0.089	0.2585	0.0115	0.257
6	0.095	0.2655	0.0165	0.2775
19	0.179	0.3505	0.0225	0.636
20	0.183	0.3605	0.0205	0.6495
21	0.18	0.3445	0.0165	0.673
22	0.178	0.3395	0.0245	0.6885
23	0.175	0.3415	0.0245	0.7205
24	0.184	0.3455	0.0235	0.722

Table A9: OD for Crude Alkaloidal Extract of P.F against *S. typhi* at 250 µg/ml MIC

Time/hr	Broth-Extract OD	Broth-Tetra OD	Broth-Cipro OD	Neg. Control
0	0.001	0.1265	0.0115	0.0655
0.5	0.011	0.1695	0.0135	0.1455
1	0.008	0.2195	0.0135	0.1905
2	0.01	0.2135	0.0135	0.1985
2.5	0	0.2295	0.0155	0.202
3	0.003	0.2245	0.0125	0.221
3.5	0.016	0.2325	0.0255	0.235
4	0.018	0.2245	0.0225	0.2425
4.5	0.032	0.2325	0.0235	0.2425
5	0.042	0.2355	0.0245	0.244
5.5	0.0512	0.2345	0.0215	0.2675
6	0.032	0.2185	0.0195	0.292
19	0.154	0.2635	0.0205	0.7505
20	0.156	0.2735	0.0295	0.7795
21	0.16	0.2655	0.0325	0.826
22	0.164	0.2655	0.0335	0.8305
23	0.169	0.2725	0.0335	0.8455
24	0.17	0.2835	0.0345	0.8965

Table A10: OD for Crude Alkaloidal Extract of P.F against *S. poona* at 250 µg/ml MIC

Time/hr	Broth-Extract OD	Broth-Tetra OD	Broth-Cipro OD	Neg. Control
0	0.037	0.0585	0.0115	0.0705
0.5	0.045	0.0795	0.0135	0.1205
1	0.062	0.0845	0.0135	0.192
2	0.063	0.0895	0.0135	0.199
2.5	0.048	0.0965	0.0155	0.206
3	0.05	0.0965	0.0125	0.2285
3.5	0.05	0.0985	0.0255	0.2365
4	0.042	0.0955	0.0225	0.258
4.5	0.056	0.0985	0.0235	0.2585
5	0.056	0.0985	0.0245	0.2625
5.5	0.066	0.0945	0.0215	0.291
6	0.085	0.0935	0.0195	0.304
19	0.132	0.1325	0.0205	0.79
20	0.13	0.1345	0.0295	0.804
21	0.128	0.1385	0.0325	0.8135
22	0.131	0.1405	0.0335	0.8425
23	0.135	0.1475	0.0335	0.852
24	0.136	0.1495	0.0345	0.8695

Table A11: OD for Crude Alkaloidal Extract of P.F against *S. lentus* at 500 µg/ml MIC

Time/hr	Broth-Extract OD	Broth-Tetra OD	Broth-Cipro OD	Neg. Control
0	0.135	0.0425	0.0065	0.127
0.5	0.142	0.0475	0.0095	0.132
1	0.151	0.0485	0.0145	0.1915
2	0.15	0.0505	0.0155	0.1935
2.5	0.162	0.0505	0.0145	0.196
3	0.159	0.0495	0.0175	0.1995
3.5	0.165	0.0515	0.0165	0.204
4	0.165	0.0515	0.0175	0.213
4.5	0.169	0.0545	0.0165	0.231
5	0.172	0.0555	0.0155	0.234
5.5	0.17	0.0415	0.0155	0.2475
6	0.168	0.0815	0.0155	0.292
19	0.287	0.1035	0.0235	0.7075
20	0.289	0.1005	0.0225	0.736
21	0.285	0.0915	0.0175	0.742
22	0.287	0.1015	0.0035	0.774
23	0.273	0.1035	0.0035	0.7885
24	0.292	0.1025	0.0025	0.8005

Table A12: OD for Crude Alkaloidal Extract of O.G against *E. coli* (43) at 500 µg/ml MIC

Time/hr	Broth-Extract OD	Broth-Tetra OD	Broth-Cipro OD	Neg. Control
0	0.0445	0.0325	0.0075	0.1105
0.5	0.0475	0.0645	0.0075	0.1555
1	0.0645	0.1885	0.0085	0.186
2	0.0625	0.1395	0.0105	0.1925
2.5	0.0645	0.1515	0.0115	0.1945
3	0.0655	0.1985	0.0075	0.214
3.5	0.0635	0.1495	0.0125	0.223
4	0.0645	0.1415	0.0095	0.2335
4.5	0.0665	0.1545	0.0095	0.236
5	0.0455	0.1625	0.0115	0.2395
5.5	0.0545	0.1885	0.0095	0.249
6	0.0585	0.1995	0.0125	0.2585
19	0.5105	0.6435	0.0085	0.669
20	0.5245	0.6455	0.0085	0.719
21	0.5305	0.6445	0.0085	0.742
22	0.5235	0.6495	0.0095	0.795
23	0.5305	0.6515	0.0105	0.804
24	0.5265	0.6545	0.0105	0.829

Table A13: OD for Crude Alkaloidal Extract of O.G against *S. aureus* at 250 µg/ml MIC

Time/hr	Broth-Extract OD	Broth-Tetra OD	Broth-Cipro OD	Neg. Control
0	0.0055	0.1135	0.0025	0.0855
0.5	0.0105	0.1505	0.0005	0.1705
1	0.0075	0.1645	0.0015	0.201
2	0.0155	0.1815	0.0025	0.2035
2.5	0.0185	0.1925	0.0035	0.208
3	0.0175	0.1925	0.0035	0.2255
3.5	0.0175	0.1975	0.0045	0.2365
4	0.0175	0.1915	0.0045	0.2365
4.5	0.0175	0.1965	0.0035	0.238
5	0.0275	0.1995	0.0035	0.2405
5.5	0.0215	0.2025	0.0055	0.246
6	0.0185	0.1845	0.0025	0.304
19	0.1735	0.2015	0.0025	0.8555
20	0.1515	0.2105	0.0025	0.922
21	0.1245	0.2005	0.0015	1.0255
22	0.1405	0.1945	0.0035	1.0575
23	0.1425	0.1975	0.0045	1.0795
24	0.1425	0.1945	0.0045	1.083

Table A14: OD for Crude Alkaloidal Extract of O.G against *Kleb* Pneumonia at 500 µg/ml MIC

Time/hr	Broth-Extract OD	Broth-Tetra OD	Broth-Cipro OD	Neg. Control
0	0.0725	0.0125	0.0155	0.063
0.5	0.0725	0.1125	0.0115	0.134
1	0.0355	0.1405	0.0115	0.1925
2	0.0595	0.1415	0.0145	0.1965
2.5	0.0655	0.1445	0.0145	0.1985
3	0.0635	0.1455	0.0145	0.2215
3.5	0.0645	0.1455	0.0145	0.236
4	0.0625	0.1485	0.0145	0.245
4.5	0.0645	0.1485	0.0155	0.25
5	0.0665	0.1465	0.0145	0.2495
5.5	0.0525	0.1465	0.0125	0.2555
6	0.0545	0.1485	0.0135	0.254
19	0.5855	0.1515	0.0115	1.809
20	0.5885	0.1525	0.0105	1.826
21	0.6105	0.1555	0.0095	1.8245
22	0.6105	0.1525	0.0105	1.8365
23	0.6475	0.1505	0.0105	1.856
24	0.5665	0.1515	0.0095	1.8825

Table A15: OD for Crude Alkaloidal Extract of O.G against *Shigella* at 500 µg/ml MIC

Time/hr	Broth-Extract OD	Broth-Tetra OD	Broth-Cipro OD	Neg. Control
0	0.0605	0.0525	0.0155	0.0555
0.5	0.0655	0.0545	0.0165	0.1955
1	0.0725	0.0815	0.0235	0.199
2	0.0865	0.0935	0.0225	0.2055
2.5	0.0905	0.0945	0.0235	0.2085
3	0.0955	0.1395	0.0255	0.2285
3.5	0.0965	0.1465	0.0255	0.2425
4	0.0965	0.1515	0.0315	0.235
4.5	0.0995	0.1565	0.0255	0.2365
5	0.1575	0.1605	0.0225	0.258
5.5	0.1455	0.1705	0.0115	0.257
6	0.1575	0.1925	0.0165	0.2775
19	0.4955	0.3255	0.0225	0.636
20	0.4935	0.3305	0.0205	0.6495
21	0.4915	0.3345	0.0165	0.673
22	0.4925	0.3315	0.0245	0.6885
23	0.4945	0.3335	0.0245	0.7205
24	0.4955	0.3345	0.0235	0.722

Table A16: OD for Crude Alkaloidal Extract of O.G against *S. Poona* at 125 µg/ml MIC

Time/hr	Broth-Extract OD	Broth-Tetra OD	Broth-Cipro OD	Neg. Control
0	0.0695	0.0585	0.0115	0.0705
0.5	0.0635	0.0795	0.0135	0.1205
1	0.0735	0.0845	0.0135	0.192
2	0.0775	0.0895	0.0135	0.199
2.5	0.0855	0.0965	0.0155	0.206
3	0.0825	0.0965	0.0125	0.2285
3.5	0.0865	0.0985	0.0255	0.2365
4	0.0835	0.0955	0.0225	0.258
4.5	0.0825	0.0985	0.0235	0.2585
5	0.0825	0.0985	0.0245	0.2625
5.5	0.0365	0.0945	0.0215	0.291
6	0.0355	0.0935	0.0195	0.304
19	0.0665	0.1325	0.0205	0.79
20	0.1065	0.1345	0.0295	0.804
21	0.1035	0.1385	0.0325	0.8135
22	0.1055	0.1405	0.0335	0.8425
23	0.1045	0.1475	0.0335	0.852
24	0.1075	0.1495	0.0345	0.8695

Table A17: OD for Crude Alkaloidal Extract of O.G against *S. lentus* at 500 µg/ml MIC

Time/hr	Broth-Extract OD	Broth-Tetra OD	Broth-Cipro OD	Neg. Control
0	0.025	0.0135	0.0065	0.127
0.5	0.024	0.0145	0.0095	0.132
1	0.023	0.0165	0.0145	0.1915
2	0.027	0.0185	0.0155	0.1935
2.5	0.029	0.0195	0.0145	0.196
3	0.03	0.0215	0.0175	0.1995
3.5	0.031	0.0255	0.0165	0.204
4	0.031	0.0305	0.0175	0.213
4.5	0.035	0.0415	0.0165	0.231
5	0.034	0.0455	0.0155	0.234
5.5	0.037	0.0485	0.0155	0.2475
6	0.039	0.0495	0.0155	0.292
19	0.16	0.1655	0.0235	0.7075
20	0.165	0.1605	0.0225	0.736
21	0.173	0.1645	0.0175	0.742
22	0.17	0.1625	0.0035	0.774
23	0.168	0.1625	0.0035	0.7885
24	0.171	0.1625	0.0025	0.8005

Table A18: OD for Crude Alkaloidal Extract of Z.Z against *E. coli* (43) at 250 µg/ml MIC

Time/hr	Broth-Extract OD	Broth-Tetra OD	Broth-Cipro OD	Neg. Control
0	0.0285	0.0325	0.0075	0.1105
0.5	0.0365	0.0645	0.0075	0.1555
1	0.0355	0.1885	0.0085	0.186
2	0.0505	0.1395	0.0105	0.1925
2.5	0.0535	0.1515	0.0115	0.1945
3	0.0525	0.1985	0.0075	0.214
3.5	0.0515	0.1495	0.0125	0.223
4	0.0495	0.1415	0.0095	0.2335
4.5	0.0475	0.1545	0.0095	0.236
5	0.0475	0.1625	0.0115	0.2395
5.5	0.0455	0.1885	0.0095	0.249
6	0.0485	0.1995	0.0125	0.2585
19	0.3515	0.6435	0.0085	0.669
20	0.3645	0.6455	0.0085	0.719
21	0.3765	0.6445	0.0085	0.742
22	0.3775	0.6495	0.0095	0.795
23	0.3915	0.6515	0.0105	0.804
24	0.3855	0.6545	0.0105	0.829

Table A19: OD for Crude Alkaloidal Extract of Z.Z against *S. aureus* at 250 µg/ml MIC

Time/hr	Broth-Extract OD	Broth-Tetra OD	Broth-Cipro OD	Neg. Control
0	0.0115	0.1135	0.0025	0.0855
0.5	0.0165	0.1505	0.0005	0.1705
1	0.0195	0.1645	0.0015	0.201
2	0.0195	0.1815	0.0025	0.2035
2.5	0.0215	0.1925	0.0035	0.208
3	0.0205	0.1925	0.0035	0.2255
3.5	0.0215	0.1975	0.0045	0.2365
4	0.0255	0.1915	0.0045	0.2365
4.5	0.0225	0.1965	0.0035	0.238
5	0.0185	0.1995	0.0035	0.2405
5.5	0.0195	0.2025	0.0055	0.246
6	0.0175	0.1845	0.0025	0.304
19	0.0215	0.2015	0.0025	0.8555
20	0.0165	0.2105	0.0025	0.922
21	0.0145	0.2005	0.0015	1.0255
22	0.0175	0.1945	0.0035	1.0575
23	0.0185	0.1975	0.0045	1.0795
24	0.0315	0.1945	0.0045	1.083

Table A20: OD for Crude Alkaloidal Extract of Z.Z against *MRSA* at 500 µg/ml MIC

Time/hr	Broth-Extract OD	Broth-Tetra OD	Broth-Cipro OD	Neg. Control
0	0.0275	0.0035	0.0075	0.0805
0.5	0.0295	0.0055	0.0075	0.145
1	0.0155	0.0035	0.0085	0.1865
2	0.0125	0.0165	0.0105	0.192
2.5	0.0135	0.0225	0.0115	0.1975
3	0.0125	0.0215	0.0075	0.2165
3.5	0.0135	0.0235	0.0125	0.2295
4	0.0125	0.0205	0.0095	0.2525
4.5	0.0125	0.0245	0.0095	0.256
5	0.0115	0.0255	0.0115	0.2585
5.5	0.0165	0.0245	0.0095	0.263
6	0.0115	0.0215	0.0125	0.291
19	0.4245	0.0465	0.0085	0.7485
20	0.4345	0.0565	0.0085	0.763
21	0.4345	0.0565	0.0085	0.799
22	0.4425	0.0615	0.0095	0.8225
23	0.4395	0.0705	0.0105	0.86
24	0.4425	0.0815	0.0105	0.8645

Table A21: OD for Crude Alkaloidal Extract of Z.Z against *Kleb* at 500 µg/ml MIC

Time/hr	Broth-Extract OD	Broth-Tetra OD	Broth-Cipro OD	Neg. Control
0	0.1005	0.1235	0.0155	0.063
0.5	0.1375	0.1595	0.0115	0.134
1	0.1415	0.1765	0.0115	0.1925
2	0.1455	0.1885	0.0145	0.1965
2.5	0.1485	0.1935	0.0145	0.1985
3	0.1435	0.1915	0.0145	0.2215
3.5	0.1475	0.1975	0.0145	0.236
4	0.1435	0.1915	0.0145	0.245
4.5	0.1445	0.1975	0.0155	0.25
5	0.1425	0.1995	0.0145	0.2495
5.5	0.1175	0.2045	0.0125	0.2555
6	0.1285	0.1885	0.0135	0.254
19	0.4995	0.2335	0.0115	1.809
20	0.5065	0.2425	0.0105	1.826
21	0.5135	0.2385	0.0095	1.8245
22	0.5245	0.2305	0.0105	1.8365
23	0.5265	0.2385	0.0105	1.856
24	0.5545	0.2455	0.0095	1.8825

Table A22: OD for Crude Alkaloidal Extract of Z.Z against *Shigella* at 125 µg/ml MIC

Time/hr	Broth-Extract OD	Broth-Tetra OD	Broth-Cipro OD	Neg. Control
0	0.0355	0.1215	0.0155	0.0555
0.5	0.0375	0.1715	0.0165	0.1955
1	0.0465	0.2105	0.0235	0.199
2	0.0475	0.2205	0.0225	0.2055
2.5	0.0535	0.2405	0.0235	0.2085
3	0.0525	0.2385	0.0255	0.2285
3.5	0.0545	0.2465	0.0255	0.2425
4	0.0535	0.2385	0.0315	0.235
4.5	0.0545	0.2445	0.0255	0.2365
5	0.0545	0.2545	0.0225	0.258
5.5	0.0505	0.2585	0.0115	0.257
6	0.0525	0.2655	0.0165	0.2775
19	0.2665	0.3505	0.0225	0.636
20	0.2845	0.3605	0.0205	0.6495
21	0.2815	0.3445	0.0165	0.673
22	0.2865	0.3395	0.0245	0.6885
23	0.2925	0.3415	0.0245	0.7205
24	0.2945	0.3455	0.0235	0.722

Table A23: OD for Crude Alkaloidal Extract of Z.Z against *S. typhi* at 500 µg/ml MIC

Time/hr	Broth-Extract OD	Broth-Tetra OD	Broth-Cipro OD	Neg. Control
0	0.0725	0.0495	0.0155	0.0655
0.5	0.0585	0.0635	0.0115	0.1455
1	0.0595	0.0755	0.0115	0.1905
2	0.0575	0.0825	0.0145	0.1985
2.5	0.0595	0.0905	0.0145	0.202
3	0.0555	0.0895	0.0145	0.221
3.5	0.0585	0.0945	0.0145	0.235
4	0.0555	0.0905	0.0145	0.2425
4.5	0.0565	0.0955	0.0155	0.2425
5	0.0555	0.0975	0.0145	0.244
5.5	0.0535	0.1055	0.0125	0.2675
6	0.0455	0.0805	0.0135	0.292
19	0.4135	0.1295	0.0115	0.7505
20	0.4255	0.1365	0.0105	0.7795
21	0.4335	0.1305	0.0095	0.826
22	0.4425	0.1235	0.0105	0.8305
23	0.4505	0.1285	0.0105	0.8455
24	0.4635	0.1265	0.0095	0.8965

Table A24: OD for Crude Alkaloidal Extract of Z.Z against *S. poona* at 125 µg/ml MIC

Time/hr	Broth-Extract OD	Broth-Tetra OD	Broth-Cipro OD	Neg. Control
0	0.0145	0.0585	0.0115	0.0705
0.5	0.0135	0.0795	0.0135	0.1205
1	0.0155	0.0845	0.0135	0.192
2	0.0175	0.0895	0.0135	0.199
2.5	0.0205	0.0965	0.0155	0.206
3	0.0175	0.0965	0.0125	0.2285
3.5	0.0185	0.0985	0.0255	0.2365
4	0.0175	0.0955	0.0225	0.258
4.5	0.0195	0.0985	0.0235	0.2585
5	0.0195	0.0985	0.0245	0.2625
5.5	0.0215	0.0945	0.0215	0.291
6	0.0135	0.0935	0.0195	0.304
19	0.0215	0.1325	0.0205	0.79
20	0.0205	0.1345	0.0295	0.804
21	0.0185	0.1385	0.0325	0.8135
22	0.0185	0.1405	0.0335	0.8425
23	0.0205	0.1475	0.0335	0.852
24	0.0255	0.1495	0.0345	0.8695

Table A25: OD for Crude Alkaloidal Extract of Z.Z against *S. lentus* at 250 µg/ml MIC

Time/hr	Broth-Extract OD	Broth-Tetra OD	Broth-Cipro OD	Neg. Control
0	0.0965	0.0425	0.0065	0.127
0.5	0.0985	0.0475	0.0095	0.132
1	0.0945	0.0485	0.0145	0.1915
2	0.0965	0.0505	0.0155	0.1935
2.5	0.0965	0.0505	0.0145	0.196
3	0.0895	0.0495	0.0175	0.1995
3.5	0.0905	0.0515	0.0165	0.204
4	0.0885	0.0515	0.0175	0.213
4.5	0.0875	0.0545	0.0165	0.231
5	0.0875	0.0555	0.0155	0.234
5.5	0.0895	0.0415	0.0155	0.2475
6	0.0885	0.0815	0.0155	0.292
19	0.1075	0.1035	0.0235	0.7075
20	0.0945	0.1005	0.0225	0.736
21	0.0895	0.0915	0.0175	0.742
22	0.0845	0.1015	0.0035	0.774
23	0.0835	0.1035	0.0035	0.7885
24	0.0925	0.1025	0.0025	0.8005

APPENDIX B
NEGATIVE CONTROL GRAPH TABLES

Table B1: Neg. control OD for E. Coli (10) at time 0 – 6 h and 19 – 24 h

Time/hr	OD1	OD2	OD_{ave} – Blank OD
0	0.25	0.28	0.0855
0.5	0.3	0.4	0.1705
1	0.368	0.37	0.1895
1.5	0.377	0.378	0.198
2	0.385	0.382	0.204
2.5	0.401	0.409	0.2255
3	0.416	0.424	0.2405
3.5	0.43	0.421	0.246
4	0.432	0.43	0.2515
4.5	0.435	0.434	0.255
5	0.439	0.438	0.259
5.5	0.441	0.466	0.274
6	0.461	0.484	0.293
19	0.822	0.895	0.679
20	0.817	0.984	0.721
21	0.863	0.985	0.7445
22	0.868	0.986	0.7475
23	0.882	1.008	0.7655
24	0.922	1.099	0.831

Table B2: Neg. control OD for E. Coli (43) at time 0 – 6 h and 19 – 24 h

Time/hr	OD1	OD2	OD_{ave} – Blank OD
0	0.27	0.31	0.1105
0.5	0.32	0.35	0.1555
1	0.369	0.362	0.186
1.5	0.375	0.369	0.1925
2	0.376	0.372	0.1945
2.5	0.396	0.391	0.214
3	0.406	0.399	0.223
3.5	0.415	0.411	0.2335
4	0.417	0.414	0.236
4.5	0.419	0.419	0.2395
5	0.422	0.421	0.242
5.5	0.429	0.428	0.249
6	0.43	0.446	0.2585
19	0.851	0.846	0.669
20	0.898	0.899	0.719
21	0.883	0.96	0.742
22	0.962	0.987	0.795

23	0.967	1	0.804
24	0.996	1.021	0.829

Table A7: Neg. control OD for S. Aureus at time 0 – 6 h and 19 – 24 h

Time/hr	OD1	OD2	OD_{ave} – Blank OD
0	0.182	0.191	0.007
0.5	0.19	0.194	0.071
1	0.2	0.201	0.0795
1.5	0.202	0.204	0.082
2	0.206	0.209	0.0865
2.5	0.224	0.226	0.104
3	0.234	0.238	0.115
3.5	0.234	0.238	0.115
4	0.237	0.238	0.1165
4.5	0.241	0.239	0.119
5	0.243	0.242	0.1215
5.5	0.249	0.242	0.1245
6	0.304	0.303	0.1825
19	0.809	0.801	0.684
20	0.842	0.841	0.7205
21	0.931	0.959	0.824
22	0.952	1.002	0.856
23	0.963	1.035	0.878
24	0.965	1.04	0.8815

Table A8: Neg. control OD for S. Typhi at time 0 – 6 h and 19 – 24 h

Time/hr	OD1	OD2	OD_{ave} – Blank OD
0	0.25	0.24	0.0655
0.5	0.3	0.35	0.1455
1	0.377	0.363	0.1905
1.5	0.385	0.371	0.1985
2	0.388	0.375	0.202
2.5	0.406	0.395	0.221
3	0.418	0.411	0.235
3.5	0.426	0.418	0.2425
4	0.428	0.416	0.2425
4.5	0.433	0.414	0.244
5	0.438	0.435	0.257
5.5	0.452	0.442	0.2675
6	0.484	0.459	0.292
19	0.923	0.937	0.7505
20	0.934	0.984	0.7795
21	1.016	0.995	0.826
22	1.013	1.007	0.8305

23	1.038	1.012	0.8455
24	1.063	1.089	0.8965

Table A9: Neg. control OD for S. Poona at time 0 – 6 h and 19 – 24 h

Time/hr	OD1	OD2	OD_{ave} – Blank OD
0	0.25	0.25	0.0705
0.5	0.3	0.3	0.1205
1	0.373	0.37	0.192
1.5	0.377	0.38	0.199
2	0.382	0.389	0.206
2.5	0.405	0.411	0.2285
3	0.416	0.416	0.2365
3.5	0.445	0.43	0.258
4	0.444	0.432	0.2585
4.5	0.441	0.443	0.2625
5	0.444	0.448	0.2665
5.5	0.453	0.488	0.291
6	0.482	0.485	0.304
19	0.993	0.946	0.79
20	1.012	0.955	0.804
21	1.009	0.977	0.8135
22	1.05	0.994	0.8425
23	1.057	1.006	0.852
24	1.081	1.017	0.8695

Table A10: Neg. control OD for S. Lentus at time 0 – 6 h and 19 – 24 h

Time/hr	OD1	OD2	OD_{ave} – Blank OD
0	0.305	0.308	0.127
0.5	0.311	0.312	0.132
1	0.371	0.371	0.1915
1.5	0.375	0.371	0.1935
2	0.376	0.375	0.196
2.5	0.382	0.376	0.1995
3	0.395	0.372	0.204
3.5	0.392	0.393	0.213
4	0.411	0.41	0.231
4.5	0.415	0.412	0.234
5	0.422	0.415	0.239
5.5	0.428	0.426	0.2475
6	0.476	0.467	0.292
19	0.929	0.845	0.7075
20	0.938	0.893	0.736
21	0.879	0.964	0.742
22	0.89	1.017	0.774
23	0.898	1.038	0.7885

24	0.916	1.044	0.8005

Table A11: Neg. control OD for Shigella at time 0 – 6 h and 19 – 24 h

Time/hr	OD1	OD2	OD_{ave} – Blank OD
0	0.22	0.25	0.0555
0.5	0.36	0.39	0.1955
1	0.38	0.377	0.199
1.5	0.386	0.384	0.2055
2	0.389	0.387	0.2085
2.5	0.407	0.409	0.2285
3	0.419	0.425	0.2425
3.5	0.418	0.411	0.235
4	0.416	0.416	0.2365
4.5	0.438	0.437	0.258
5	0.431	0.421	0.2465
5.5	0.433	0.44	0.257
6	0.456	0.458	0.2775
19	0.797	0.834	0.636
20	0.809	0.849	0.6495
21	0.839	0.866	0.673
22	0.848	0.888	0.6885
23	0.875	0.925	0.7205
24	0.872	0.931	0.722

Table A12: Neg. control OD for Shigella at time 0 – 6 h and 19 – 24 h

Time/hr	OD1	OD2	OD_{ave} – Blank OD
0	0.262	0.258	0.0805
0.5	0.325	0.324	0.145
1	0.368	0.364	0.1865
1.5	0.373	0.37	0.192
2	0.378	0.376	0.1975
2.5	0.396	0.396	0.2165
3	0.41	0.408	0.2295
3.5	0.431	0.433	0.2525
4	0.439	0.432	0.256
4.5	0.434	0.442	0.2585
5	0.441	0.436	0.259
5.5	0.439	0.446	0.263
6	0.463	0.478	0.291
19	0.893	0.963	0.7485
20	0.907	0.978	0.763
21	0.94	1.017	0.799
22	0.964	1.04	0.8225

23	0.992	1.087	0.86
24	0.992	1.096	0.8645

Table A13: Neg. control OD for Kleb Pneumonia at time 0 – 6 h and 19 – 24 h

Time/hr	OD1	OD2	OD_{ave} – Blank OD
0	0.254	0.231	0.063
0.5	0.312	0.315	0.134
1	0.377	0.367	0.1925
1.5	0.379	0.373	0.1965
2	0.38	0.376	0.1985
2.5	0.403	0.399	0.2215
3	0.413	0.418	0.236
3.5	0.429	0.42	0.245
4	0.433	0.426	0.25
4.5	0.431	0.427	0.2495
5	0.42	0.426	0.2435
5.5	0.434	0.436	0.2555
6	0.43	0.437	0.254
19	0.989	2.988	1.809
20	1.008	3.003	1.826
21	0.999	3.009	1.8245
22	1.014	3.018	1.8365
23	1.032	3.039	1.856
24	1.06	3.064	1.8825

APPENDIX C

MODULATION OPTICAL DENSITIES

Table C1: Optical Density of Tetracycline in combination with *O. gratissimum* (1/2MIC:2MIC) against *S. poona* from 0 – 6 h and 19 – 24 h.

Time/hr	OD1	OD2	Ave. OD	Time/hr	OD1	OD2	Ave. OD
0	0.011	0.012	0.0115	5	0.021	0.024	0.0225
0.5	0.012	0.013	0.0125	5.5	0.025	0.023	0.024
1	0.015	0.009	0.012	6	0.027	0.028	0.0275
1.5	0.013	0.0094	0.0112	19	0.031	0.029	0.03
2	0.012	0.015	0.0135	20	0.025	0.028	0.0265
2.5	0.011	0.013	0.012	21	0.026	0.031	0.0285
3	0.013	0.013	0.013	22	0.024	0.034	0.029
3.5	0.015	0.017	0.016	23	0.025	0.031	0.028
4	0.012	0.012	0.012	24	0.026	0.033	0.0295
4.5	0.018	0.019	0.0185				

O.G MIC = 125 µg/ml; Tetra = 15.6 µg/ml; Tetra:*O.G* = 1/2MIC:2MIC

Table C2: Optical Density of Tetracycline in combination with *O. gratissimum* (1MIC:1MIC) against *S. Poona* from 0 – 6 h and 19 – 24 h.

Time/hr	OD1	OD2	Ave. OD	Time/hr	OD1	OD2	Ave. OD
0	0.01	0.0101	0.01005	5	0.028	0.023	0.0255
0.5	0.012	0.015	0.0135	5.5	0.022	0.024	0.023
1	0.015	0.018	0.0165	6	0.027	0.03	0.0285
1.5	0.017	0.019	0.018	19	0.032	0.035	0.0335
2	0.02	0.021	0.0205	20	0.03	0.029	0.0295
2.5	0.024	0.028	0.026	21	0.028	0.026	0.027
3	0.021	0.025	0.023	22	0.031	0.036	0.0335
3.5	0.023	0.026	0.0245	23	0.036	0.041	0.0385
4	0.029	0.023	0.026	24	0.037	0.038	0.0375
4.5	0.027	0.025	0.026				

O.G MIC = 125 µg/ml; Tetra = 15.6 µg/ml; Tetra:*O.G* = 1MIC:1MIC

Table C3: Optical Density of Tetracycline in combination with *P. fraternus* (1MIC:1/4MIC) against *Shigella* from 0 – 6 h and 19 – 24 h.

Time/hr	OD1	OD2	Ave. OD	Time/hr	OD1	OD2	Ave. OD
0	0.016	0.015	0.0155	5	0.021	0.025	0.023
0.5	0.017	0.017	0.017	5.5	0.032	0.035	0.0335
1	0.019	0.02	0.0195	6	0.024	0.023	0.0235
1.5	0.021	0.02	0.0205	19	0.02	0.024	0.022
2	0.021	0.022	0.0215	20	0.031	0.034	0.0325
2.5	0.019	0.021	0.02	21	0.032	0.034	0.033
3	0.021	0.025	0.023	22	0.03	0.031	0.0305
3.5	0.023	0.026	0.0245	23	0.031	0.033	0.032
4	0.025	0.021	0.023	24	0.033	0.032	0.0325
4.5	0.027	0.032	0.0295				

P. F MIC = 125 µg/ml; Tetra = 125 µg/ml; Tetra:*P. F* = 1/4MIC:1MIC

Table C4: *P. fraternus* (1/2MIC:1/2MIC) against *Shigella* from 0 – 6 h and 19 – 24 h.

Time/hr	OD1	OD2	Ave. OD	Time/hr	OD1	OD2	Ave. OD
0	0.017	0.019	0.018	5	0.025	0.019	0.022
0.5	0.018	0.021	0.0195	5.5	0.021	0.023	0.022
1	0.02	0.022	0.021	6	0.029	0.027	0.028
1.5	0.02	0.02	0.02	19	0.026	0.029	0.0275
2	0.018	0.016	0.017	20	0.029	0.025	0.027
2.5	0.022	0.021	0.0215	21	0.028	0.031	0.0295
3	0.017	0.018	0.0175	22	0.032	0.033	0.0325
3.5	0.021	0.021	0.021	23	0.033	0.035	0.034
4	0.027	0.025	0.026	24	0.032	0.027	0.0295
4.5	0.023	0.019	0.021				

P. F MIC = 125 µg/ml; Tetra = 125 µg/ml; Tetra:*P. F* = 1/2MIC:1/2MIC

APPENDIX D

MODULATION CALCULATIONS

Extract and Tetracycline Concentrations Used for Synergy Checkerboard Assay

The volume required to prepare 8MIC concentrations were determined using the dilution formula $C_1V_1 = C_2V_2$.

e.g., For bacteria *Salmonella poona*,

Tetracycline MIC was determined to be 15.6 µg/ml and the crude alkaloid extract of *O. gratissimum*, 125 µg/ml.

Stock solution concentration for both = 50 mg/ml

For Tetracycline;

8MIC = 8 × 15.6 µg/ml = 124.8 µg/ml = 125 µg/ml (approx.) = 0.125 mg/ml

Now, from $C_1V_1 = C_2V_2$, the volume needed to prepare 0.125 mg/ml in 10 ml will be,

$$V_1 = \frac{C_2V_2}{C_1} = \frac{0.125\ mg/ml\ \times 10\ ml}{50\ mg/ml} = 0.025\ ml = 25\ \mu L$$

Hence, 0.025 ml of the stock solution was taken with a micropipette and diluted to 10 ml to prepare the 8MIC concentration.

Similarly, for the crude alkaloidal extract of *O. gratissimum;*

8MIC = 8 × 125 µg/ml = 1000 µg/ml = 1 mg/ml

Again, from $C_1V_1 = C_2V_2$, the volume needed to prepare 1 mg/ml in 5 ml will be,

$$V_2 = \frac{C_1V_1}{C_2} = \frac{1\ mg/ml\ \times 5\ ml}{50\ mg/ml} = 0.1\ ml = 100\ \mu L$$

Hence, 0.1 ml of the stock solution was taken with a micropipette and diluted to 5 ml to prepare the 8MIC concentration.

The 8MICs for *AEPF* and *AEOG*'s were also calculated using this method (Appendix C). Two-folds serial dilution of the 8MIC stock solutions were used to prepare 4MIC to 1/16MIC.

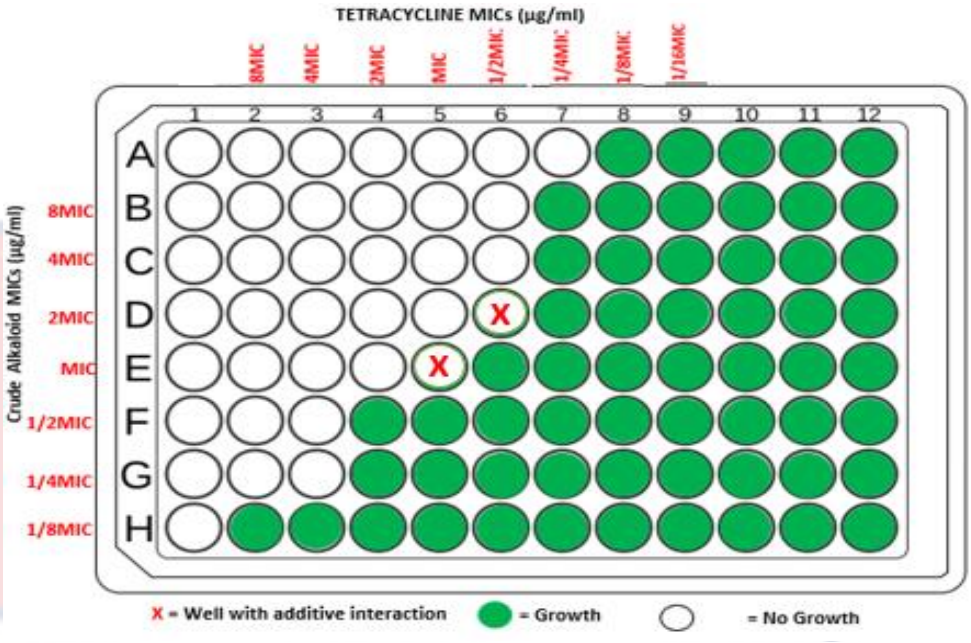

Figure 1: Schematic presentation of checkerboard assay result for Tetracycline in combination with AEOG against the test strain *Salmonella poona*

For *AEOG* in combination with Tetracycline showing no growth in well D6 against the bacteria *S. Poona*; *AEOG*/Tetra Vs *Salmonella Poona* **(Figure 1)**

[*AEOG* MIC = 125 µg/ml; Tetracycline MIC = 15.6 µg/ml]

Using the FIC index equation,

$$\frac{A}{MIC\ (A)} + \frac{B}{MIC\ (B)} = FIC\ (A) + FIC\ (B) = FIC\ Index$$

Where $AEOG = \text{A}$ and $Tetracycline = \text{B}$

$\text{A} =$ MIC of A combination in well D6 $= 2\text{MIC} = 2\ (125\ \mu\text{g/ml})$

$A = 500\ \mu\text{g/ml}$

$\text{B} =$ MIC of B combination in well D6 $= 2\text{MIC} = \frac{1}{2}\ (15.6\ \mu\text{g/ml}) = 7.8$

The equation therefore becomes,

$$FIC\ index\ (well\ D6) = \frac{2(125)}{125} + \frac{1/2(15.6)}{15.6} = 2 + \frac{1}{2} = 2.5$$

For well E8, *PF*-Tetracycline vs *Shigella*

Using the FIC index equation,

$$\frac{A}{MIC\ (A)} + \frac{B}{MIC\ (B)} = FIC\ (A) + FIC\ (B) = FIC\ Index$$

Where $AEPF = \text{A}$ and $Tetracycline = \text{B}$

$\text{A} = \text{MIC of A combination in well E8} = 1\text{MIC} = 1\ (125\ \mu\text{g/ml})$

$A = 125\ \mu\text{g/ml}$

$$\text{B} = \text{MIC of B combination in well D6} = \frac{1}{8}\text{MIC} = \frac{1}{8}\ (125\ \mu\text{g/ml})$$
$$= 15.625\ \mu\text{g/ml}$$

The equation therefore becomes,

$$FIC\ index\ (well\ D6) = \frac{1(125)}{125} + \frac{\frac{1}{8(125)}}{125} = 1 + \frac{1}{8} = 1.125$$

e.g., For bacteria *Shigella*,

Tetracycline MIC was determined to be 125 µg/ml and the crude alkaloid extract of *P. fraternus* = 125 µg/ml.

Stock solution concentration for both = 50 mg/ml

For Tetracycline;

8MIC = 8 × 125 µg/ml = 1000 µg/ml = 1 mg/ml

Now, from $C_1V_1 = C_2V_2$, the volume needed to prepare 1 mg/ml in 5 ml will be,

$$V_1 = \frac{C_2V_2}{C_1} = \frac{1\ mg/ml\ \times 5\ ml}{50\ mg/ml} = 0.1\ ml = 100\ \mu\text{l}$$

Hence, 0.1 ml of the stock solution was taken with a micropipette and diluted to 5 ml to prepare the 8MIC concentration.

Similarly, for the crude alkaloidal extract of *P. fraternus;*

8MIC = 8 × 125 µg/ml = 1000 µg/ml = 1 mg/ml

Again, from $C_1V_1 = C_2V_2$, the volume needed to prepare 1 mg/ml in 5 ml will be,

$$V_2 = \frac{C_1V_1}{C_2} = \frac{1\ mg/ml\ \times 5\ ml}{50\ mg/ml} = 0.1\ ml = 100\ \mu\text{L}$$

Hence, 0.1 ml of the stock solution was taken with a micropipette and diluted to 5 ml to prepare the 8MIC concentration.

Table D1: Fractional Inhibitory Concentration (FIC) Index: *AEPF* in Combination with Tetracycline Vs *Shigella* and *Staph aureus*

	AEPF-Tetracycline			***AEPF-Tetracycline***	
	Shigella			*Staph Aureus*	
Well No.	FIC	Interpretation	Well No.	FIC	Interpretation
B8	8.0625	A	B7	8.25	A
C6	4.5	A	C7	4.25	A
E7	1.25	I	D4	4	A
F6	1.0	I	E2	9	A
G3	4.25	A			

A = Antagonism; I = Additive

Table D2: Fractional Inhibitory Concentration (FIC) Index: *AEZZ* in Combination with Tetracycline Vs *Shigella* and *Salmonella Poona*

	AEZZ-Tetracycline			***AEZZ-Tetracycline***	
	Shigella			***Salmonella Poona***	
Well No.	FIC	Interpretation	Well No.	FIC	Interpretation
B2	16	A	B5	9	A
C3	8	A	C6	4.5	A
D2	10	A			

A = Antagonism

APPENDIX E

NAMES AND MOLECULAR FORMULA OF ALL POSSIBLE ALKALOIDS BASED ON MOLECULAR MASS, NITROGEN RULE AND DBE CALCULATIONS

Table E1: Possible *Z. zanthoxyloide* Alkaloids

No.	Compound Name	DBE	MF	Exact MW	Calculated MW
1	Ribalinine	8	$C_{15}H_{17}NO_3$	259.30	259.1
2	Skimmianine	9	$C_{14}H_{13}NO_4$	259.26	
3	6-(2,6-Dioxopiperidin-3-yl)-5H-pyrrolo[3,4-b]pyridine-5,7(6H)-dione	10	$C_{12}H_9N_3O_4$	259.21	
4	Riboflavine	10	$C_{17}H_{20}N_4O_6$	376.36	376.2
5	Piperidine, 1,1'-([1,1'-biphenyl]-4,4'-diyldicarbonyl)bis	12	$C_{24}H_{28}N_2O_2$	376.49	
6	Etoxeridine	6	$C_{18}H_{27}NO_4$	321.4	321.2
7	Zanthoamide I	6	$C_{18}H_{27}NO_4$	321.20	
8	Phenazocine	10	$C_{22}H_{27}NO$	321.46	
9	8-Acetonyldihydrochelerythrine	13	$C_{21}H_{19}NO_4$	405.3	405.3
10	Nicofuranose	21	$C_{30}H_{24}N_4O_{10}$	600.4	600.4
11	2-Pyridinamine, N-nitro-	5	$C_5H_5N_3O_2$	139.1121	139.2
12	Pyrazinecarboxamide, 3,4-dihydro-3-oxo-	5	$C_5H_5N_3O_2$	139.1121	
13	3-Hydroxypicolinic acid	5	$C_5H_5N_3O_2$	139.1088	139.2

14	1H-Pyrrole-2,5-dione, 3-ethyl-4-methyl-	4	$C_7H_9NO_2$	139.1519	139.2
15	2,6-Pyridinedimethanol	4		139.1519	
16	N-n-Propylmaleimide	4		139.1519	
17	Methyl 1-methylpyrrole-2-carboxylate	4		139.1519	
18	2-Propionyl-1,4,5,6-tetrahydropyridine	3	$C_8H_{13}NO$	139.1949	139.2
19	2,4-Diethyl-5-methyloxazole	3		139.1949	
20	1H-Imidazole-4-ethanamine, N,N-dimethyl-	3	$C_7H_{13}N_3$	139.1982	139.2
21	Piperidine, 1-(2-methyl-1-propenyl)-	2	$C_9H_{17}N$	139.2380	
22	Piperidine, 1-(1-butenyl)	2	$C_9H_{17}N$	139.2380	
23	Bisacodyl	14	$C_{22}H_{19}NO_4$	361.3906	361.1
24	5-(((9,10-dioxo-9,10-dihydroanthracen-1-yl)amino)methylene) pyrimidine-2,4,6(1h,3h,5h)-trione	16	$C_{19}H_{11}N_3O_5$	361.5505 361.4751	
25	Furethidine	7	$C_{21}H_{31}NO_4$		
26	2-[4-(4-methylphenyl)phenyl]-3,4-diphenylimidazo[4,5-c][1,5] naphthyridine	25	$C_{34}H_{24}N_4$	488.6	488.2
27	3-[4-[4-[4-(4,6-Dimethyl-1,3,5-triazin-2-yl)phenyl]phenyl]naphthalen-1-yl]benzonitrile	25	$C_{34}H_{24}N_4$	488.6	488.2
28	9-pyridin-4-yl-1-(9-pyridin-4-yl-7,8-dihydrocarbazol-3-yl)carbazole	25			
29	Nor-chelerythrine	14	$C_{20}H_{15}NO_4$	333.3	333.3

Table E2: Possible *Occimum Gratissimum* Alkaloids Based on Molecular Mass, Nitrogen Rule and DBE Calculations

No.	Compound Name	DBE	MF	Exact MW	Calculated MW
1	1H-Indol-5-ol	6	C_8H_7NO	133.15	133.2
2	2H-Indol-2-one, 1,3-dihydro-	6		133.15	
3	Furo[2,3-c]pyridine, 2-methyl-	6		133.15	
4	Benzoxazole, 2-methyl	6		133.15	
5	1H-Benzotriazole, 5-methyl	5	$C_7H_7N_3$	133.15	133.2
6	Benzimidazole-5-amine	5	$C_7H_7N_3$	133.15	
7	Quinoline, 5,6,7,8-tetrahydro	5	$C_9H_{11}N$	133.1903	133.2
8	2-Methylindoline	5		133.1903	
9	1H-Indole, 2,3-dihydro-1-methyl	5		133.1903	
10	Isoquinoline, 1,2,3,4-tetrahydro	5		133.1903	
11	(7-acetyloxy-4-cyano-1,3-dioxo-6-propanoyloxyisoindol-5-yl) 2,2-dimethylpropanoate	12	$C_{19}H_{18}N_2O_8$	402.35	402.3
12	(2,5-dioxopyrrolidin-1-yl) 4-[2-[2-(2,5-dioxopyrrol-1-yl)ethoxy]ethoxy]benzoate	12		402.36	
13		12		402.36	

	4-ethoxy-2-ethoxycarbonyl-5-methoxy-1H-pyrrolo[2,3-f]quinoline-7,9-dicarboxylic acid				
14	2,4-Diacetoxy-10-cyanoazuleno(2,1-b)pyridine	14	$C_{18}H_{12}N_2O_4$	320.29	320.2
15	1-[2-(2,3-dioxoindol-1-yl)ethyl]indole-2,3-dione	14		320.27	
16	1,2-bis(5-hydroxy-1H-indol-3-yl)ethane-1,2-dione	14	$C_{18}H_{12}N_2O_4$	320.25	
17	Ethyl 6,12-dioxoindolo[2,1-b]quinazoline-8-carboxylate	14		320.3	
18	1,1'-(1,5-pentanediyl)bis[thymine]	8	$C_{15}H_{20}N_4O_4$	320.34	320.2
19	1-Phenylcarbamoyl-2,3-phthaloyl-7,8-benzopyrrocoline	21	$C_{27}H_{16}N_2O_3$	416.42	416.2
20	(3*S*)-3-[[(2*S*)-1-acetylpyrrolidine-2-carbonyl]amino]-5-(2,6-dimethylbenzoyl)oxy-4-oxopentanoic acid	10	$C_{21}H_{26}N_2O_7$	418.44	418.3
21	benzyl N-[(2S)-1-[[(2S,3R,4R)-3-hydroxy-5-[(2-hydroxy-2-phenylethyl)amino]-4-[(4-methoxyphenyl)methylamino]-5-oxo-1-phenylpentan-2-yl]amino]-3-methyl-1-oxobutan-2-yl]carbamate	19	$C_{40}H_{48}N_4O_7$	696.8	696.2
22	ethyl 3-[1-[[2-[(1,3-dioxoisoindol-2-yl)methyl]phenyl]-phenylmethyl]-4,6-dioxo-5-phenylmethoxy-2H-pyrido[2,1-f][1,2,4]triazin-3-yl]propanoate	26	$C_{41}H_{36}N_4O_7$	696.7	
23	[(2R,5R)-3,4-bis[(4-methylbenzoyl)oxy]-5-[6-(4-methylphenyl)purin-9-yl]oxolan-2-yl]methyl 4-methylbenzoate	26	$C_{41}H_{36}N_4O_7$	696.7	

Table E3: Possible *Phyllanthus fraternus* Alkaloids Based on Molecular Mass, Nitrogen Rule and DBE Calculations

No.	Compound Name	DBE	MF	Exact MW	Calculated MW
1	5-anilino-4-hydroxy-1H-pyrimidin-6-one	8	$C_{10}H_9N_3O_2$	203.20	203.2
2	Ent-norsecurinine	7	$C_{12}H_{13}NO_2$	203.24	
3	Phthalimide, N-(1-formylethyl)-	8	$C_{11}H_9NO_3$	203.19	
4	(3S)-3-ethyl-1,3-dimethyl-4H-quinolin-2-one	6	$C_{13}H_{17}NO$	203.28	
5	2-phenyl-4-allyl-tetrahydro-1,4-oxazine	6	$C_{13}H_{17}NO$	203.28	
6	a-Pyrrolidinopropiophenone	6	$C_{13}H_{17}NO$	203.28	
7	4-Acetyl-4-phenylpiperidine	6	$C_{13}H_{17}NO$	203.28	
8	7-methoxy-8-(methoxymethyl)quinoline	7	$C_{12}H_{13}NO_2$	203.24	
9	5-(3-methoxyphenyl)-4-nitroso-1H-pyrazole	8	$C_{10}H_9N_3O_2$	203.2	203.2
10	1-[(1R)-1-phenylethyl]azepane	5	$C_{14}H_{21}N$	203.32	
11	1-Methyl-2-Phenethylpiperidine	5	$C_{14}H_{21}N$	203.32	
12	1-(4-methyl-7,8-dihydro-5H-pyrido[4,3-d]pyrimidin-6-yl)prop-2-en-1-one	14	$C_{11}H_{13}N_3O$	203.24	
13	N-butyl-2-methylimidazo[1,2-a]pyridin-5-amine	8	$C_{12}H_{17}N_3$	203.280	
14	2-methyl-N-(1H-pyrrolo[2,3-c]pyridin-3-ylmethyl)propan-2-amine	6	$C_{12}H_{17}N_3$	203.280	
15	N,N,4-trimethyl-5-(1H-pyrazol-5-yl)pyrimidin-2-amine	7	$C_{10}H_{13}N_5$	203.2	
16	Ethyl-2-methyl-7-oxo-4,7-dihydropyrazolo[1,5-a]pyrimidine-6-carboxylate	7	$C_{10}H_{11}N_3O_3$	221.21	221.3
17	1-(2-ethenyl-5-methylphenyl)-N-phenylmethanimine	10	$C_{16}H_{15}N$	221.3	
18	Bubbialine	6	$C_{12}H_{15}NO_3$	221.25	
19	3-(5-methylidenespiro[2.3]hexan-2-yl)quinoline	10	$C_{16}H_{15}N$	221.300	
20	2-methoxy-4-(4-methylpiperazin-1-yl)aniline	5	$C_{12}H_{19}N_3O$	221.3	

21	oxan-4-yl-(3-phenyl-3,4-dihydropyrazol-2-yl)methanone	8	$C_{15}H_{18}N_2O_2$	258.320	258.2
22	3-hydroxy-5,5-dimethyl-2-[(5-methylpyridin-2-yl)iminomethyl]cyclohex-2-en-1-one	8			
23	2-[6-[(2S)-2,3-dihydroxy-3-methylbutyl]-1H-indol-3-yl]acetonitrile	8			
24	6-methoxy-N,N-dipropylquinolin-2-amine	7	$C_{16}H_{22}N_2O$	258.36	258.2
25	6-benzoylquinoline-5-carbonitrile	14	$C_{17}H_{10}N_2O$	258.27	
26	N-[2-(2,3-dihydro-1,4-benzoxazin-4-yl)-2-oxoethyl]but-2-ynamide	9	$C_{14}H_{14}N_2O_3$	258.27	
27	Bis(2-(2-isopropyloxazolidin-3-yl)ethyl) succinate	4	$C_{20}H_{36}N_2O_6$	400.5	400.0
28	Ethyl (2S)-1-[(2S)-5-hydroxy-2-[(2-methylpropan-2-yl)oxycarbonylamino]pentanoyl]-3-propan-2-ylpyrrolidine-2-carboxylate	4			
29	Esculeogenin A	6	$C_{27}H_{45}NO_4$	447.6	447.2
30	3alpha,21-Dihydroxy-2beta-(2,2-dimethylmorpholino)-5alpha-pregn-20-one	6	$C_{27}H_{45}NO_4$	447.6	
31	N/A	N/A	N/A	N/A	502.4
32	N/A	N/A	N/A	N/A	

APPENDIX F

PHOTOS