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EXTRACTION, GC-MS ANALYSIS AND BIOACTIVITY TEST OF SECONDARY METABOLITES FROM THE CULTURES OF MANGROVE ENDOPHYTIC FUNGI

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MASTER OF PHILOSOPHY

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Graduate Studies in partial fulfillment

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Master of Philosophy
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in the University of Education, Winneba

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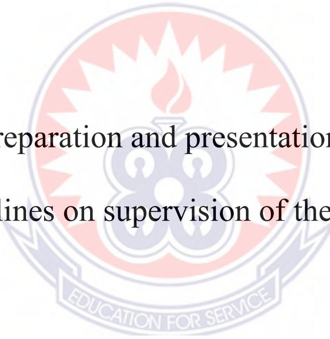
DECLARATION

I, Felix Nelson Akompi, declare that this thesis, with the exception of quotations and references contained in published works which have all been identified and duly acknowledged, is entirely my own original work and it has not been submitted, either in part or whole, for another degree elsewhere.

Signature :……………………………………

Date ………………………………………….

Supervisors' Declaration



I hereby declare that the preparation and presentation of this thesis was supervised in accordance with the guidelines on supervision of thesis as laid down by the University of Education, Winneba.

Supervisor's Name: Dr. E. K. Oppong

Signature :………………………………

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DEDICATION

This research is dedicated to the Akompi and Ankrah families.

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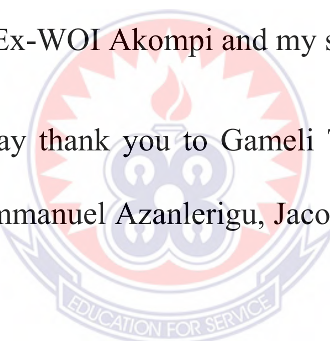

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ABBREVIATIONS

ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
BBMAV	Bark of a Black Mangrove from Akosua Village
BBMS	Bark of a Black Mangrove from Sankor
BWMS	Bark of a White Mangrove from Sankor
DPPH	2,2-diphenyl-1-picrylhydrazyl
EtOAc	Ethyl acetate
FTIR	Fourier-transform infrared spectroscopy
GC-MS	Gas Chromatography- Mass Spectrometry
HLPC	High Liquid Performance Chromatography
LBMS	Leaf of a Black Mangrove from Sankor
MAbs	Monoclonal Antibodies
MBC	Minimum Bactericidal Concentration
MFC	Minimum Fungicidal Concentration
MIC	Minimum Inhibition Concentration
MRI	Magnetic Resonance Imaging
NMR	Nuclear magnetic resonance
RBMAV	Root of Black Mangrove from Akosua Village
TLC	Thin Layer Chromatography
UV-VIS	Ultra-Violet Visible Spectroscopy

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ABSTRACT

Mangrove plants and their associated micro fauna have been a rich source of bioactive molecules, but only a limited antimicrobial screening of this chemo-diversity source has been reported. The unique adaptation of mangroves towards their extreme environment is largely aided by endophytic fungi. Endophytic fungi are underexplored group of microorganisms since only a few plants have been studied with regards to this community. Endophytic fungi live inside the tissues of other organisms, such as mangrove plants and provide protection to them. In return, endophytic fungi support their hosts by fighting off pathogens through the production of antimicrobial compounds. These bioactive compounds are the secondary metabolites which are often produced as waste- or by-products. In this study, the leaves, barks and roots of red, black and white mangroves found along the Ayensu estuary and the Muni lagoon in Winneba were screened for their endophytic fungi in a malt-glucose culture media containing 3%(w/w) malt extract and 6%(w/w) glucose. Five fungi from BBMAV, BWMS, LBMS, RBMAV and BBMS were obtained and cultured in malt-glucose media containing 3%(w/w) malt extract and 6%(w/w) glucose for 8 weeks for their secondary metabolites. The TLC studies and GC-MS analysis of the crude extracts from culture media of the fungal isolates reveal that fungi produced several secondary metabolites. A bioassay on the crude extracts was carried out using human pathogenic microbes Methicillin resistant *Staphylococcus aureus* (NCTC 12493), *Staphylococcus aureus* (NCTC 12973), *E. coli* (NCTC 12241), *S. mutants* (ATCC 700610), *P. aeroginosa* (ATCC 4853), *S. typhi* (ATCC 14028), *K. pneumonia (NCTC 13440)* and *Candida albicans* (ATCC 90028) and two fungi that infest cocoa (*Phytophthora palmivora and Phytophthora megakarya*). The bioassay results indicated that the crude extracts were active against all the test organisms. The crude extracts were tested for antioxidant activity using ABTS and DPPH as scavenging agents. The results indicate that the crude extracts of all the fungal isolates exhibited high antioxidant activity.

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CHAPTER ONE

INTRODUCTION

1.0 Overview

This chapter gives a general introduction to the study. It encompasses the background to the study, statement of the problem, the aim of the study, objectives of the study, as well as the significance of the study. This chapter also looks at the organisation of the report.

1.1 Background to the study

Natural products are chemical compounds or substances isolated from living organisms (Bhat, Nagasampagi & Sivakumar, 2005;Anulika *et al*., 2016). The primary sources of pharmaceuticals and industrial raw materials are these natural products. They come from either animals, plants, or microorganisms. These products are either parts of the organisms or the secondary metabolites produced by the organisms (Stone & Williams, 1992). Natural products from fungi are considered as important sources for novel antibacterial and antifungal compounds because of the abundant fungal species diversity, their rich secondary metabolites and the improvement in their genetic breeding and fermentation processes (Devi *et al*., 2020). The chemistry of these natural products include their biosynthesis, extraction, identification, quantification, structural elucidation, physical and chemical properties and reactions (Anulika *et al*., 2016).

Plants have historically been the main source of compounds used for medicine. However, many research studies are now focusing more on microorganisms living inside the plants and the plants themselves in producing bioactive compounds (Ling *et al*., 2012). Over the years, medicinal plants have been explored for bioactive

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substances with antibacterial, antifungal, anticancer and/or antiviral activities (Chi *et al*., 2019). Paclitaxel, the most famous natural source cancer drug in the world, is derived from the bark of the Pacific yew tree (*Taxus brevifolia*) and is used to treat ovarian, breast, lung, pancreatic and other cancers (Lin *et al.* 2007). Stierle *et al*., (1993), isolated the fungus *Taxomyces andreanae* from *T. brevifolia*, which was found to produce taxol and this discovery provided a more feasible and practical way to mass-produce this compound.

Due to the long history in folklore medicine, medicinal plants have not escaped the attention of today's pharmaceutical chemists. The importance of traditional medicines has been well understood by the pharmaceutical industry since the discovery and successful development of aspirin from the symbolic Willow tree (Mahdi, 2010). For instance, metformin, derived from *Galega officinalis* L., is a commonly used type 2 diabetic drug. Interestingly, a study has shown that metformin can also have potential cytotoxic effects on cancerous cells (Daugan *et al.,* 2016). Taxol, the blockbuster anticancer drug, derived from Taxus brevifolia Nutt, showed significant effect against various types of cancers namely, ovarian, breast, lung cancer, head, and neck tumours (Surapaneni, 2012). One of the richest sources of natural products which is least exploited is the mangroves. In Ghana, most people dwelling in communities around mangroves only use them as source of fuel other than exploiting the mangroves for its diverse medicinal values.

Mangroves are one of the world's dominant coastal ecosystems comprised chiefly of flowering trees and shrubs uniquely adapted to marine and estuarine tidal conditions (Tomlinson, 1986; Duke, 1992; Hogarth, 1999; Saenger, 2002; FAO, 2007;Duke, 2013). Mangrove plants and their associated micro fauna have been rich sources of

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bioactive molecules, although only limited antimicrobial screening of this chemo-diversity source has been reported (Calcul *et al*., 2013). Mangroves are unique for their well-known adaptation towards extreme environmental conditions of high salinity, changes in sea level, high temperatures and anaerobic soils (Shearer *et al.* 2007). Endophytic fungi play important roles in plant adaptation to the environment. Stress conditions caused by a lack of water, confers several benefits to plants, including protection against predators and diseases through the production of toxic substances (Tan & Zou, 2001; Arnold *et al*., 2003; Gunatilaka, 2006; Kusari *et al*., 2012). However, the ecological role of different fungal species is still not entirely clear and may even vary from symbiotic to antagonistic or slightly pathogenic (Schulz & Boyle, 2005).

Endophytic fungi refer to microorganisms colonizing the intercellular and/or intracellular regions of healthy plant tissues at a particular time, the presence of which does not interfere with or cause symptoms in the host (Stone *et al*., 2000; Strobel, 2003; Schulz & Boyle, 2006). Prevalent in nature, these endophyte microbes have been isolated from different plants examined to date (e.g., *Ginkgo biloba* L., *Taxus chinensis* (Pilg.) Rehder, and *Tectona grandis* L. f.), and this association can be obligate or facultative (Nair & Padmavathy, 2014). Some endophytes can produce substances that alter the plant phenotype and thus increase host defenses (Matiello *et al*., 1997; Higgins *et al*., 2014). Other endophytes produce useful natural compounds that can be applied for different industrial purposes (Demain, 2014).

These bioactive compounds are secondary metabolites, which are often produced as waste or by-products. Besides, endophytic fungi also help the host plant in adapting to (extreme) environments, for example by removing harmful heavy metals (Ling et al.,

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2012). The existence of interactions between plants and fungi, especially symbiotic and parasitic interactions, is well known. (Alencar Soares et al., 2017). Several studies have reported that plants colonized by endophytic fungi represent an important repository of microorganisms, including new species (Carvalho *et al*., 2012; Tao *et al*.,2013). Moreover, medicinal plants have been used for the isolation and characterization. Analysis of endophytic fungi that are considered important microbial reservoir for drug discovery with antibiotic activities of immunosuppressants, anticancer agents and biological control agents (Peixoto Neto *et al*., 2004). According to Strobel & Daisy (2003, p. 499), "Torreyanic acid, a selectively cytotoxic quinone dimer (anticancer agent), was isolated from *Pestalotiopsis microspora* (Batista & Peres, 1966; Alencar Soares et al., 2017) strain". This strain was originally obtained as an endophyte associated with the endangered tree *Torreya taxifolia* (Florida torreya) (Kurz, 1938; Lee *et al*., 1996; Strobel *et al*., 2004). The endophytic community encompasses a wide variety of microbial species, constituting a complex microecosystem (El-Shatoury *et al*., 2013).

An increasing number of endophytic fungi with novel metabolites of pharmaceutical importance has been isolated from medicinal plants. These plants may serve as a reservoir of untold numbers of endophytic microorganisms capable of synthesizing bioactive compounds that may act against plant pathogens (Cui *et al*., 2011). The importance of mangroves in the ethnobotany cannot be underestimated as the plants have much therapeutic potential. Mangroves were used in folklore medicines a long time ago and different extracts from various parts of the plants (roots, leaves, fruits, bark, and resin) have shown exciting and significant inhibitory activities in many assays namely antidiabetic, anti-inflammatory, anticancer, antiulcer, antitumor, antiviral, antioxidant and antimicrobial among others (Rajesh *et al*., 2019).

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According to Calabrò (2015), Metabolites are the intermediates and products of metabolism. Metabolites have various functions including provision of energy, structure, signalling, stimulatory and inhibitory effects on enzymes, catalytic activity of their own (usually as a cofactor to an enzyme), defence and interactions with other organisms. The evolving commercial importance of secondary metabolites has in recent years resulted in great interest particularly in the possibility of altering the production of bioactive plant metabolites by means of tissue culture technology (Jian *et al*., 2005).

Secondary metabolites are described as organic compounds which are not directly involved in the normal growth, development or reproduction of an organism (Fraenkel & Gottfried, 1959; Drummond & Rambaut, 2007). Secondary metabolites are typically present in a set of organisms or cells (plants, fungi, bacteria, etc). Some examples of the types of secondary metabolites include: ergot alkaloids, antibiotics, naphthalenes, nucleosides, phenazines, quinolines, terpenoids, flavonoids and coumarins (Miller & Seigler, 2012). According to Thirumurugan *et al*. (2018), a multitude of secondary metabolite secretions are harvested by human kind to improve their health in the areas of antibiotics, enzyme inhibitors, immunomodulators, antitumor agents and growth promoters of animals and plants. Unlike primary metabolites, absence of secondary metabolites does not result in immediate death, but rather in long-term impairment of the organism's survivability, fecundity, or aesthetics, or perhaps with no significant change (Calabrò, 2015). These are often restricted to a narrow set of species within a phylogenetic group (Tiwari & Rana, 2015). These also play important role in plant defence against herbivory (Stamp, 2003) and other interspecies defences (Samuni-Blank *et al*., 2012)

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Secondary metabolites are also used to widen the pyramid of healthy nutrition (pigments and nutraceuticals), enhancing agricultural productivity (pesticides, insecticides, effectors of ecological competition, symbiosis and pheromones), and hence impacting the economics of our society in a certain positive way (Thirumurugan *et al*., 2018). The role of secondary metabolites in the survival of the human race cannot be over emphasized.

1.2 Statement of the Problem

Mangrove plant species are important source of valuable metabolites with their endophytes gaining attention. Extracts from mangrove species and traditional medicines have been known to elicit activity against human, plant and animal pathogens (Saranraj & Sujitha, 2015). Endophytic fungi are a group of fungi that dwell within plants asymptomatically. They represent an important and quantifiable component of fungal biodiversity, affecting plant community biodiversity and structure. Studies have revealed that, these fungi have been found in plants and serve as a potential source of natural products for exploitation in medicine, agriculture and industry (Wacira *et al*., 2016).

The search for novel and bioactive natural products from mangrove endophytic fungi has attracted global attention (Nisa *et al*., 2015). Several studies have been conducted on the endophyte communities of mangrove plants found along the coastlines of the Indian, Pacific and Atlantic Ocean (Onyena & Sam, 2020), but not along the Winneba coast. Most of the communities along the coast where mangroves are found view them as source of fuel other than their exploitation for medicinal use. This study seeks to screen the mangroves along the Winneba coast in Efutu municipality for endophytic fungi and their bioactive compounds.

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1.3 Aims of the Study

The aim of this study is to screen the mangrove plants of Winneba coast for endophytic fungi, culture and extract bioactive compounds.

1.4 Objectives of the study

The following objectives served as guide for the study:

1. screen mangroves along the Winneba coast for endophytic fungi.
2. culture the mangrove endophytic fungi and extract compounds.
3. run GC-MS analysis of the extracts to identify the compounds present in the extracts.
4. test the extracts for bioactivity.

1.5 Significance of the study

Secondary metabolites including antibiotics serve as competitive weapons against other living organisms such as animals, plants, insects, and microorganisms (Keswani *et al*., 2019). They also serve as metal transporting agents, agents for symbiotic relation with other organisms, reproductive agent and agents of communication between organisms (Thirumurugan *et al*., 2018). This study will serve as the basis for further research. In addition, the outcome of this study has great implications for the pharmaceutical industry at the same time enhancing the conservation efforts of mangroves along the Winneba coast and the country at large.

1.6 Organization of the Report

The thesis is organized under five chapters. Chapter one dealt with, the background, the statement of the problem, purpose, significance and organization of the study.

The second chapter reviewed literature related to the study. Under this chapter, the opinions of other researchers who have studied and written on mangroves, mangrove

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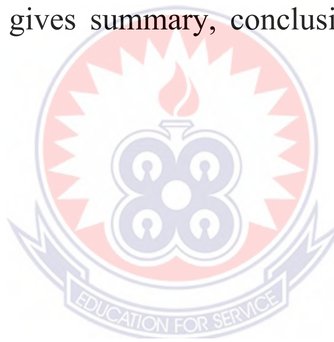
endophytic fungi, threats to mangroves, fungi, fungi as a source of bioactive compounds, endophytic fungi among others were discussed.

The third chapter is the methodology. It includes sample collection and treatment, screening and isolation of mangrove endophytic fungi, fungal culture for extraction of bioactive compounds, endophytic fungi identification, extraction of bioactive compounds, GC-MS analysis of extracts, Antimicrobial and antioxidant activity tests of the extracts.

The fourth chapter took care of analysis and discussion of results obtained from the research.

Finally, the fifth chapter gives summary, conclusions and recommendations of the study.



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CHAPTER TWO

LITERATURE REVIEW

2.0 Overview

This chapter reviews literature related to the study. The opinions, ideas, research works of other researchers and authors who have studied and written on mangroves, fungi and endophytic fungi are discussed. Further reviews are presented on general extraction of natural products, identification and characterization of secondary metabolites.

2.1 Infectious Diseases, Drug Resistance, and Bioactive Compounds

The emergence of new infectious diseases such as H1N1, influenza, SARS and COVID-19 has become a major challenge to human health. Many of these new diseases are related to microorganisms that are becoming more and more drug resistant hence the search for new bioactive compounds to combat them (Bhatia & Narain, 2010). For instance, in South East Asia, signs of infections with *Plasmodium falciparum* (protozoan parasite known to cause malaria) disappear later after the beginning of treatment with the malaria drug (artemisinin). Thus, indicating that the parasite is becoming more resistant to the commonly used medicine, for instance artemisinin, in Thailand (Fairhurst *et al.,* 2012). The resistance to antibiotics is a phenomenon by which a microorganism is no longer affected by the antimicrobial compound to which it was previously sensitive to (WHO 2012).

Bioactive compounds are gaining much attention due to their ability to reduce the incidence of diseases such as cancer and diabetes. One profoundly used antibiotics is penicillin (Holdt & Kraan, 2011).

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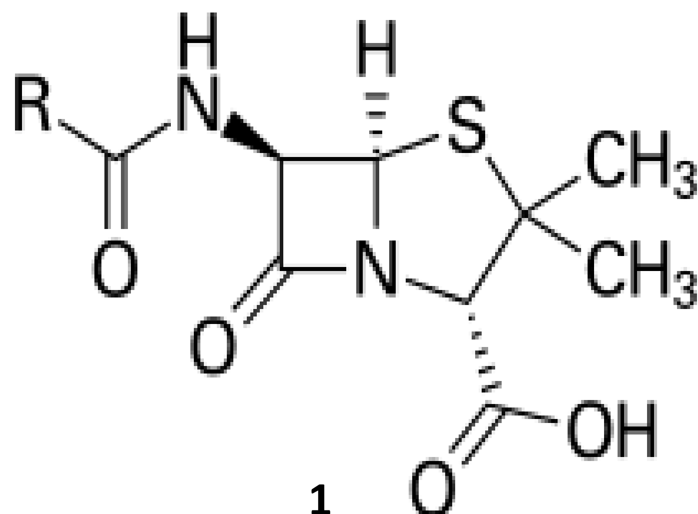
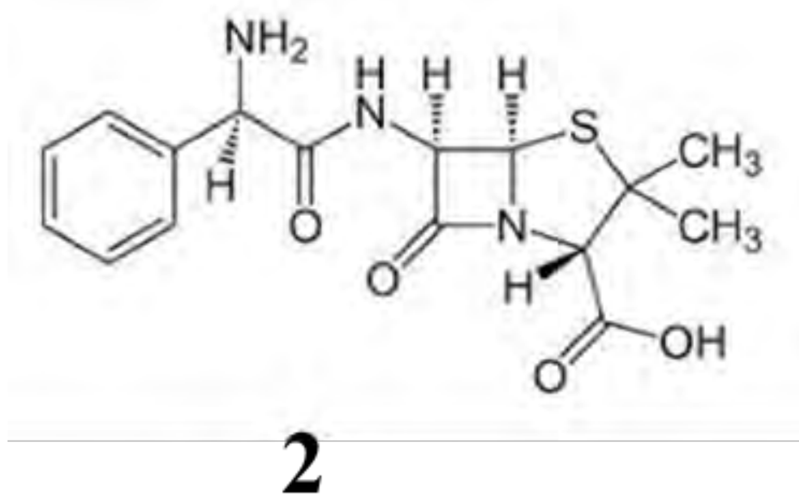


1

Penicillin (**1**) was first discovered by Alexander Fleming, in 1928, produced by a rare mold, *Penicillium notatum* (Derderian, 2007). It was found to be active against Gram-positive bacteria but some semisynthetic penicillin, such as **ampicillin** (**2**), are also effective against Gram-negative bacteria (Behal, 2000). It was widely used for the treatment of infections such as syphilis, pneumonia, diphtheria, bacterial meningitis, and septicemia (Muniz *et al*., 2007).



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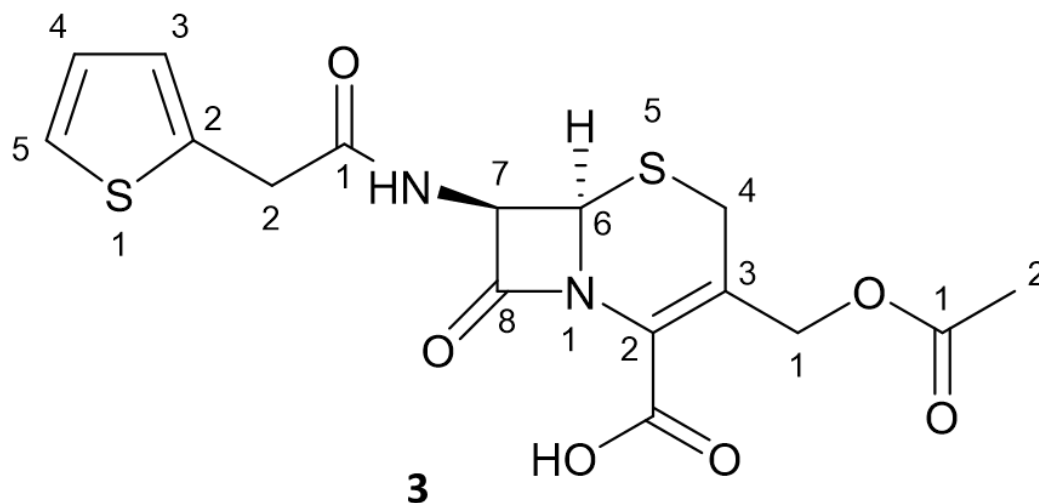
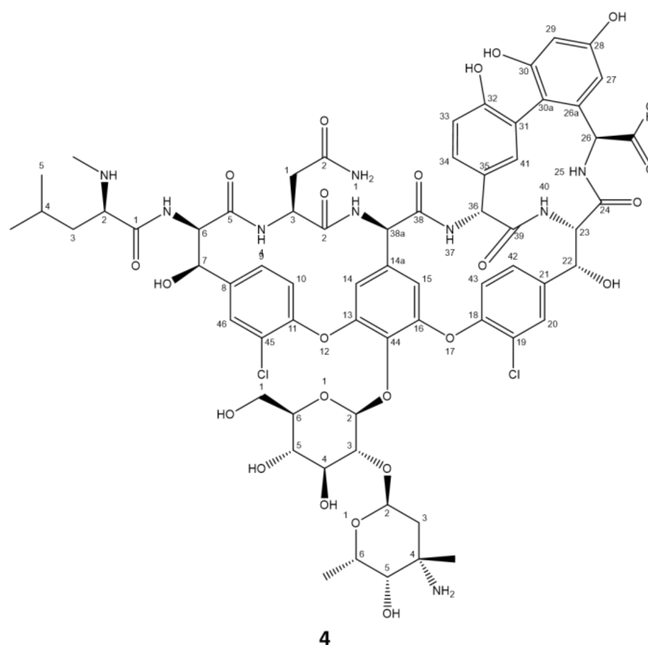
Cephalosporin (**3**) was discovered by Giuseppe Brotzu and was extracted from *Cephalosporium acremonium* and was found to show antibiotic activity against *Staphylococcus aureus*, *Salmonella typhi*, and *Escherichia coli* (Muniz *et al.,* 2007).

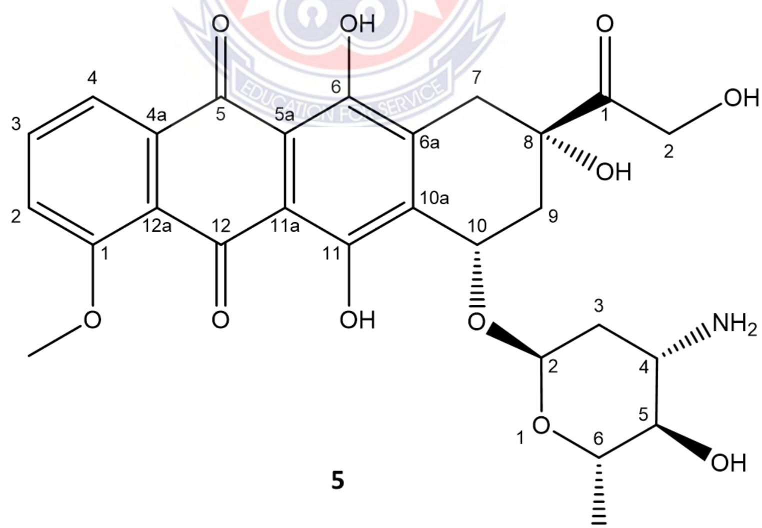


Vancomycin (**4**) was isolated from *Streptomyces orientalis* and found active against most gram-positive organisms, including penicillin-resistant staphylococci (Levine, 2006). However, in 1997, *Staphylococcus aureus* was found resistant towards vancomycin, despite the fact that the compound was the only defence available then (Nicolaou *et al*., 1999).



4

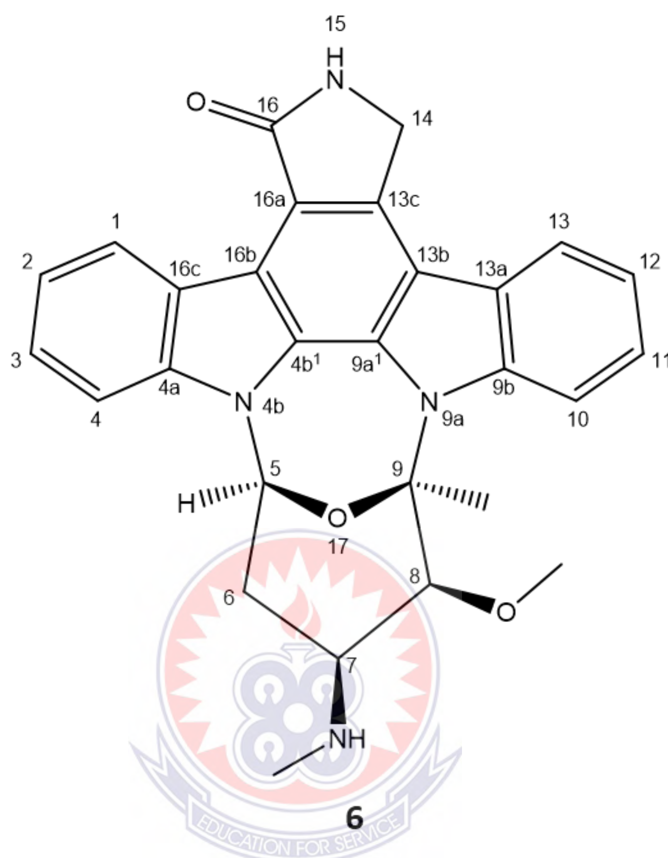
Doxorubicin (**5**) is an anthracycline antineoplastic antibiotic that is potent and widely used in clinical oncology (Yurekli *et al*., 2005; Yu *et al*., 2012). It slows or stops the growth of cancer cells by blocking an enzyme called topo isomerase 2.



5

Staurosporine (**6**) was discovered in 1977 from the bacterium *Streptomyces staurosporeus*. It has been shown to possess an array of important biological properties such as antifungal, antihypertensive and platelet aggregation inhibition (Hewavitharana *et al*., 2009). The abilities this compound have can been associated

with their various degrees of bioactivity such as anticancer, antidiabetic and many other properties which are useful in biomedical research and drug development (Strobel & Daisy2003).



2.2 Sources of Bioactive Compounds

Bioactive compounds are naturally occurring metabolites and/or by-products from microorganisms, plants or animals. They are also referred to as secondary metabolites since they are not used by the cell for survival but instead often produced as waste products (Behal, 2000). Plants have historically been the main source of compounds used for medicine. However, many research studies are now focusing on the role of the microorganisms living inside the plants and the plants themselves in producing bioactive compounds.

Microbial secondary metabolites include antibiotics (as mentioned), pigments (astaxanthin), toxins (Conus toxin), enzymes (clavulanic acid) and many more which

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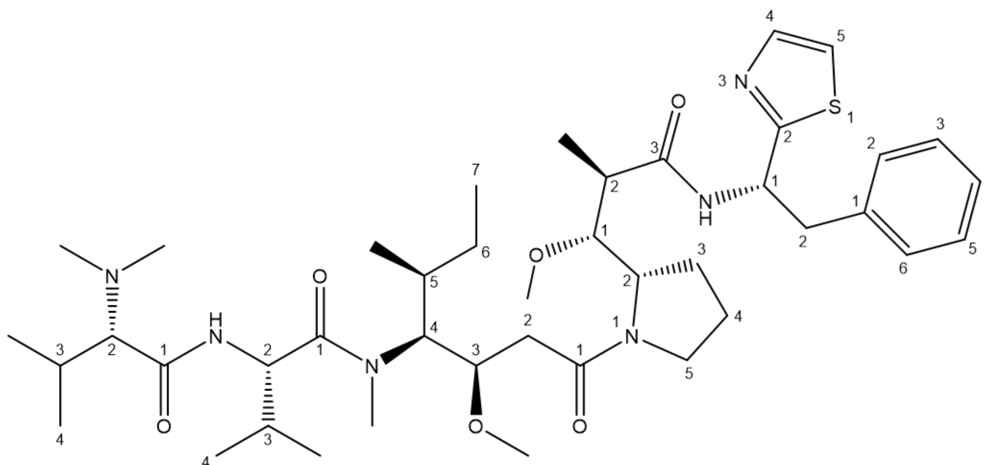
have been of great use to humans, animals and even plants (Demain 1998; Kim & Bhatnagar, 2010). Bioactive compounds have been isolated from microorganisms originating from various terrestrial and marine environments (Strobel & Daisy, 2003; Ortholand & Ganesan, 2004).



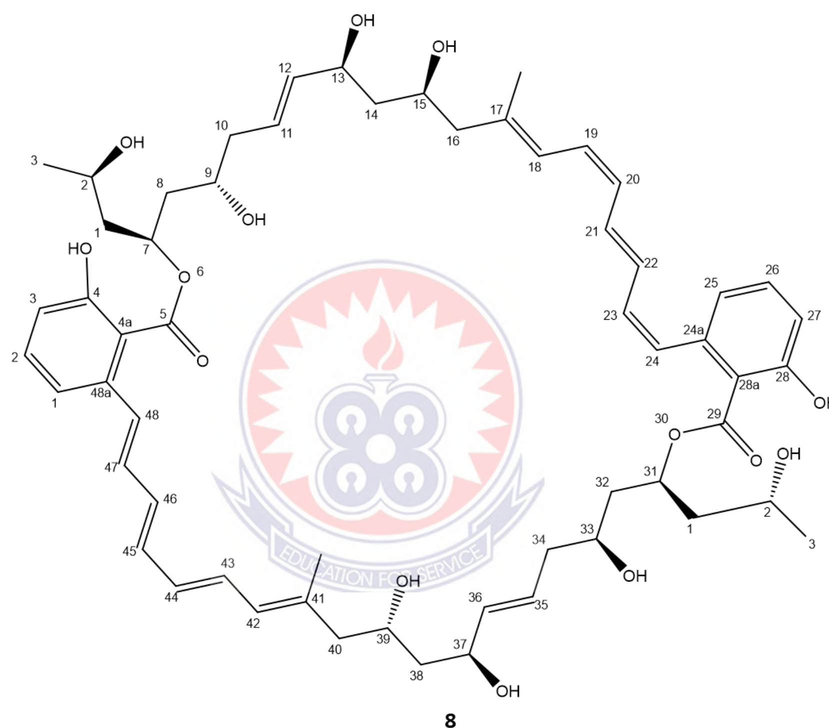
7

Although organisms from the terrestrial environment have been the main source of antibiotics for decades, the marine environment is proving to be the new area of interest with several studies showing marine organisms to be producers of anti-cancer compounds and also compounds which act against infectious diseases and inflammation. Well known examples are **dolastatin** (**7**), a compound produced by marine cyanobacteria (Tan, 2007).

Dolastatin (**7**) is one of the important marine cyanobacterial molecules that was discovered in preclinical testing as anticancer agents. This compound was initially isolated from the sea hare (*Aplysia vaccaria*) (Tan, 2006). **Marinomycin** (**8**) is a polyketide produced by marine actinomycete, *Marinspora* sp with antibacterial and antitumor properties (Lam, 2006 Olano, *et al.,* 2009).

With marine organisms being able to survive in extreme conditions due to their metabolic and physiological capabilities they provide an enormous potential for the

production of unique bioactive compounds that are not present in terrestrial organisms (Rasmussen & Morrissey, 2007). This is not surprising as the marine environment constitutes a large unexplored reservoir with a long evolutionary history and has “produced” organisms with unique biological properties compared to terrestrial ones (Belarbi et al., 2003; Aneiros *et at.,* 2004). However, despite the many successful applications of bioactive compounds from marine organisms, their exploration as sources of bioactive compounds are still underexplored (Kim &Bhatnagar, 2010).



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The discovery of new bioactive compounds requires analysis of previous diversity studies. A knowledge of the types of microorganisms that reside in a certain environment will enable us to design cultivation techniques adapted to capture all the microbial communities present in the environment (Mendes *et al*., 2007).

2.3 Mangroves

A mangrove is a shrub or small tree that grows in coastal saline or brackish water. The term is also used for tropical coastal vegetation consisting of such species

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(Silvestri, *et al.,* 2005). Mangroves occur worldwide in the tropics and subtropics, mainly between latitudes 30° N and 30° S. The total mangrove forest area of the world in 2000 was 137,800 square kilometres (53,200 sq mi), spanning 118 countries and territories (Giri *et al*., 2010).

According to Gong *et al*., (2017), mangrove ecosystems are intertidal wetlands in sub-tropical or tropical temperate coastal zone. They possess a great commercial and ecological value for human. Mangroves play an important role in the environment by providing a wide range of ecological services such as protection against floods and hurricanes, reduction of shoreline and riverbank erosion, and most importantly maintenance of biodiversity (Baig, 2015).

Mangrove remains as an ecosystem of great importance for the ecological balance responsible for the supply of nutrients to the marine environment and forms forests of salt tolerance plant species which harbor a great number of marine microorganisms, with fungi being one of them (Silva *et al*., 2011). These fungi among others also aid with recycling the detritus of mangrove trees, thereby re-generating nutrients and making them available for other organisms again (Aweto, 2012). Recycling of nutrient aids in promoting an ecological balance in the mangrove environment (Bharathidasan & Panneerselvam, 2011).

Mangroves are unique for their well-known adaptation towards extreme environmental conditions of high salinity, changes in sea level, high temperatures and anaerobic soils (Shearer *et al*., 2007). Most of the mangrove genera and families are not closely related, but have common highly developed morphological, biological, physiological and ecological adaptability to extreme environmental conditions (Sari & Rosalina, 2016). Some important characteristics to achieve this kind of adaptability are (a) pneumatophoric roots, (b) stilt roots, (c) salt-excreting leaves, and (d)

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viviparous water-dispersed propagules (Ling *et al*., 2012). The species composition and structure depend on their physiological tolerances and competitive interactions (Kuenzer *et al*., 2011). The differential ability in adapting to high-salinity seawater distinguishes the mangrove species. With that, mangrove species usually have differentiated salt resistance-associated anatomic structures (Lambers & Oliveira, 2019). The pneumatophores, which are typical of black mangroves arise vertically from cable roots and have evolved independently in at least five mangrove families and genera: Laguncularia (Combretaceae), Avicennia (Avicenniaceae), Bruguiera (Rhizophoraceae), Xylocarpus (Meliaceae), and Sonneratia (Sonneratiaceae) (Yanez-Espinosa and Flores, 2011). These specialized roots contain spongy tissue connected to the exterior of the root via small pores called lenticels which allows transportation of oxygen from the atmosphere to the root system (Carrillo-López & Yahia, 2019). During low tide, when the lenticels are exposed to the atmosphere, oxygen is absorbed from the air and transported to and even diffused out of the roots below ground (Shearer *et al*., 2007). This diffusion of oxygen maintains an oxygenated microlayer around the roots that enhances nutrient uptake. The microlayer also avoids toxicity of compounds such as hydrogen sulfide that would otherwise accumulate under such conditions (NOAA 2010; Sun, Wong & Lee 1998; Shi et al., 2005).

The stilt and prop roots, typical of red mangroves, are alternately inundated and exposed by tidal fluctuations, easily entrapping floating debris (Hoff, 2002). Besides, they become hosts for various algae, sponges, and other small plant life and when fully developed, the roots and underlying mud become the habitat of a number of semi-aquatic organisms, such as various mollusks and crustaceans that furnish food for both man and other animals (West, 1976; Acuña-Piedra & Quesada-Román, 2021).

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For salt-excreting leaves, there are special organs or glands found in the leaves which remove salts from the plant tissues. Avicennia and Laguncularia are those mangrove species that have special, salt-secreting glands leading to formation of salt crystals on the leaf surfaces. These crystals would be removed when blown or washed away by the rain. Besides, leaf fall also allows elimination of excess salt in mangroves (NOAA 2010; Sun, Wong & Lee, 1998; Shi *et al*., 2005).

Lastly, the viviparous water-dispersed propagules are an adaptation towards the extreme environment that can be observed in most mangroves. Vivipary is a condition where germination takes place while the offspring is still attached to the parent tree. The offspring has no dormant stage but grows out of the seed coat and the fruit before detaching from the plant (Leck, 2008). Because of this, mangrove propagules are actually seedlings, and not seeds. Hence, vivipary helps mangroves cope with the varying salinities and frequent flooding of their intertidal environment, and increases the likelihood of survival. Since most non-viviparous plants disperse their offspring in the dormant seed stage, vivipary presents a potential problem for dispersal (Naskar & Palit, 2015). However, these species would solve this problem by producing propagules containing substantial nutrient reserves that can float for an extended period. In this way, the propagule can survive for a relatively long time before establishing itself in a suitable location (NOAA 2010; Sun, Wong & Lee, 1998; Shi *et al*., 2005). The commonest types of mangroves found along the coast of Ghana are the red, black and white mangroves (Nabeelah *et al*., 2019).

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Figure 1: Pictures of red, white and black mangroves found in Akosua Village, Winneba

At a glance, one is likely to notice the presence of the red mangrove due to their visible prop roots. They are as well the dominant type of mangroves found in the lagoons and estuaries along the coast of Ghana.

According to Sackey *et al*., (2011), the mangrove of Ghana occupies a narrow, non-continuous coastal area and occurs principally in lagoons which extend from the eastern to the western parts of the country along a coastline of some 550 km. Mangrove products have been traditionally used by the communities in the mangrove areas for a long time. Nevertheless, mangrove areas in Ghana have received practically no attention in terms of rational utilization (Tanner *et al*., 2014). Mangroves are exploited for only fuel (wood), fish, crabs and oysters along the coastal areas of Ghana.

2.4 Fungi

A fungus is any member of the group of eukaryotic organisms that includes microorganisms such as yeasts and molds, as well as the more familiar mushrooms (Singh & Singh, 2020). So far, the known fungi are made up of around 97,000 species (Sime-Ngando, 2012). However, the estimated number of fungi is said to be around

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1,500,000 species, which means that fungi are the second largest guild following insects on earth (Abe & Inaba, 2015).

Fungi as important agents of plant and human diseases, producers of industrial and pharmacological products and even as decomposers have attracted the attention of scientists worldwide (Ling *et al*., 2012). They are heterotrophic, eukaryotic organisms that are unicellular in nature although they appear as multicellular during the vegetative phase (Sag & Kutsal, 2001; Ireland & Bugni, 2004). This means that they lack chlorophyll and thus do not have the ability to photosynthesize their own food. Hence, they obtain their nutrients from substrates by absorption through their tiny thread-like filaments called hyphae that branch in all directions (Ellis *et al*., 2008).

Fungus is referred to as the monophyletic true fungus but Mycologists use the term ‘‘fungi’’ to define all organisms traditionally studied as true fungi, slime molds, water molds. The kingdom of fungi is organized into groups or better known as phyla (Mueller, 2011). The major phyla that have been identified within the true fungi are the Chytridiomycota, Zygomycota, Ascomycota, and Basidiomycota (Lutzoni *et al*., 2004). The three main fungal phyla, Zygomycota, Ascomycota, and Basidiomycota, were said to have evolved from the Chytridiomycota approximately 550 million years ago (Guarro *et al*., 1999).

Chytridiomycota are a phylum of fungi that reproduce through the production of motile spores known as zoospores, typically propelled by a single directed flagellum (James *et al*., 2006). They include unicellular or filamentous forms that produce flagellated cells at some point in their life cycle and which occur in aquatic and terrestrial habitats (Barsanti & Gualtieri, 2005). On the other hand, the Zygomycota comprise a diverse assemblage of taxa that include soil saprobes (Mucorales), symbionts of arthropod guts (Trichomycetes and Harpellales), the widespread

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arbuscular mycorrhizae of plants (Endogonales) and pathogens of animals, plants, amoebae and especially other fungi (Lutzoni *et al*., 2004; Abdel-Azeem, 2010).

Many Ascomycota and Basidiomycota produce complex macroscopic fruiting bodies, such as gilled mushrooms, cup fungi, coral fungi and other forms. Ascomycota constitute by far the largest group of fungi so far known (Money, 2016). A large number of this species are economically important, for instance, *Fusarium* sp., *Colletotrichum* sp., and *Mycosphaerella* sp. The basic characteristic which differentiates Ascomycota from other fungi is the presence of asci inside the ascomata (Haridas *et al*., 2020). Many are free-living saprobes including species which may be cellulose decomposers, chitinolytic, keratinolytic, or coprophilous and others are parasitic forms including species which cause serious plant diseases. Others that are considered symbiotic forms contain species which live in association with insects or algae (lichens) or roots of plants (mycorrhizas) (Guarro *et al*., 1999; Abdel-Azeem 2010). The phylum Basidiomycota consists of three subphyla: Agaricomycotina, Pucciniomycotina and Ustilaginomycotina (Wang *et al*., 2009). The most characteristic feature of basidiomycetes is the formation of basidia (Guarro *et al*., 1999).

2.5 Fungi as Sources of Bioactive Compounds

Fungi are prominent producers of bioactive compounds and have shown some form of biological activity such as antibacterial, antifungal, larvicidal, molluscicidal, antioxidant and free-radical scavenging activities (Doss *et al*., 2010). All these activities have been associated with specific bioactive compounds produced by fungi. The exploration of fungal bioactive secondary metabolites was initiated by the discovery of penicillin in 1928 by Alexander Fleming, which led to an expansion in the field of drug development using microorganisms (Fleming, 1929; Silverman &

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Holladay, 2014). An outstanding group of fungi that produces bioactive compounds is the endophytic fungi.

2.6 Endophytic Fungi

Endophytes refer to a group of fungi that reside in living tissues of plants without causing any adverse effects towards the host plant itself (Yadav, 2018). Several studies have suggested that most fungal communities have become endophytes through invasion of plants via openings made by insects and plant host's stomata (Kaul et al. 2008; Tran *et al*., 2010). The route of entry for these fungal endophytes can be classified as horizontal and vertical transmission. Systemic endophytes are said to transmit vertically via the seeds, while non-systematic endophytes transmit horizontally with host colonization arising from the surrounding environment (Frank *et al*., 2017). Endophytic fungal vertical transmission is described as seed reproduction, which is the same as the reproduction of most plants (Truyens *et al*., 2015).

However, reports on mechanism of endophytic fungal horizontal transmission are still rare (Lemons *et al.,* 2005; Dai *et al*., 2010). Fungal endophytes can be classified into three basic ecological groups which are:

- Mycorrhizal fungi
- Balansiaceous or "grass endophytes"
- Non-balansiaceous

Mycorrhizal fungi are a major functional group of soil organisms that forms a symbiotic relationship with the root cells of higher green plants (Finlay, 2008). The most common mycorrhizal types form with arbuscular mycorrhizal fungi, which penetrate the host cells, but do not modify the external appearance of the root (Bonfante & Genre, 2008). The mycorrhizal fungi occur in most vegetation types and

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have been found to be one of the major constituents of the tropical soil microflora with increased resistance towards pathogens, and even heavy metal stress. Some of the mycorrhizal fungal species reported are *Acaulospora* sp., *Glomus* sp., and *Sclerocystis* sp. (Albert & Sathianesan, 2009). On the other hand, the grass endophytes create a unique group of closely related species whose ecological requirements and adaptations are significantly different from those of other endophytes (Walitang *et al*., 2018). They grow systemically and intercellularly within all above ground grasses, resulting in vertical transmission of the endophytes through the seeds. For instance, the *Neotyphodium* sp. and *Epichloe* sp. are some of the grass endophytes (Eaton *et al*., 2011). Lastly, non-balansiaceous refers to endophytes that mostly belong to the *Ascomycota* of various genera such as *Acremonium, Alternaria, Cladosporium, Coniothyrium, Epicoccum, Fusarium, Geniculosporium, Phoma* and *Pleospora* (Devaraju & Satish, 2010).

Identification of endophytic fungi can be done using microscopic and morphological characters and molecular sequencing analysis (Ravindran *et al*., 2012). Fungal taxonomy has been traditionally based on comparative morphological features, such as ascospore and peridium morphology, glebacolour, odour and other organoleptic characteristics (Lu *et al*., 2011). However, special caution should be taken when identifying closely related or morphologically similar endophytes as their morphological characteristics might be medium dependent and hence, culturing conditions can substantially affect vegetative and sexual compatibility. On the other hand, molecular techniques exhibit higher sensitivity and specificity for microorganism's identification, thus, can be used for classifying microbial strains at diverse hierarchical taxonomic levels. Several studies have shown that genetic methods can be successfully used in the studies of endophytic fungi (Hübler *et al*.,

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2019). Most of the endophytic fungi were detected and identified by comparative analyses of the ribosomal DNA sequences, especially the internal transcribed spacer (ITS) region (ITS 1 and ITS 2) (Huang *et al*., 2011).

Endophytic fungi are an under-explored group of microorganisms as only a few plants have been studied with regards to this endophytic community. However, they are currently gaining attention as they were found to be responsible for a variety of functional benefits to their hosts. Understanding the relationship between the fungi and their host plants will help to understand productivity in ecosystems better in terrestrial as well as in marine environments (Arnold & Lutztoni, 2007). The endophytes play their role in protecting their host plants from diseases or pathogens, promoting plant growth and also enhancing their host resistance to morphological, biochemical changes and unfavorable environmental conditions (Dai *et al*., 2010; Pravabathy & Nachiyar, 2011). In return, host plants are responsible for providing shelter, protection, and even nutrients to the endophytes (Faeth & Fagan, 2002). This symbiotic relationship where both sides benefit from the interaction, explains why plants that are infected with a broad diversity of endophytes exhibit a lower susceptibility to insects and pathogens. Some of the bioactive compounds produced were found to be antifungal and antibacterial and so strongly inhibit the growth of other pathogenic microorganisms invading the host plants (Gao *et al*., 2010).

2.7 Mangrove Endophytic Fungi

The unique mangrove ecosystem adjacent to the coastal waters provides a wide variety of organic substrates and a significant salinity gradient caused by daily changes in the sea level (Shearer *et al*., 2007). This constitutes an ideal environment

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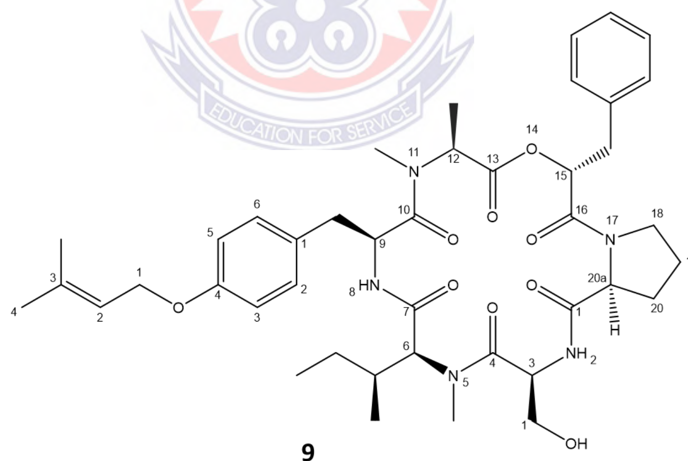
for the bases of trunks and submerged aerating roots of mangrove plants, making mangrove forests an important source for unique endophytic fungi (Xing *et al*., 2011). As mangrove endophytic fungi were found to be partly responsible for the mangrove's ability in adapting to the extreme environment (Silva *et al*., 2011), their bioactive compounds are also of interest. These bioactive compounds are found to be widely distributed in the mangrove environment, making mangroves a potential source for the discovery of new bioactive compounds-producing endophytes (Nag, Bhattacharya and Das, 2012). In a study by Joel and Bhimba, (2012) on bioactive compounds produced by *Hypocrea lixii,* a fungal endophyte isolated from the leaves of mangrove plants was found to possess antioxidant, anticancer and antimicrobial activity. The fungal extract exhibited some antibacterial activity against *Pseudomonas aeruginosa*, a pathogen known for respiratory infections among cystic fibrosis patients.



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Another study by Ebrahim *et al*., (2012) reported two novel compounds, **Pullularins E**(**9**) and **F**(**10**), extracted from the endophytic fungus *Bionectriao chroleuca* which was isolated from the leaves of the mangrove plant *Sonneratia caseolaris.* These compounds showed moderate cytotoxic activity against mouse lymphoma cells (Ebrahim *et al*., 2012).

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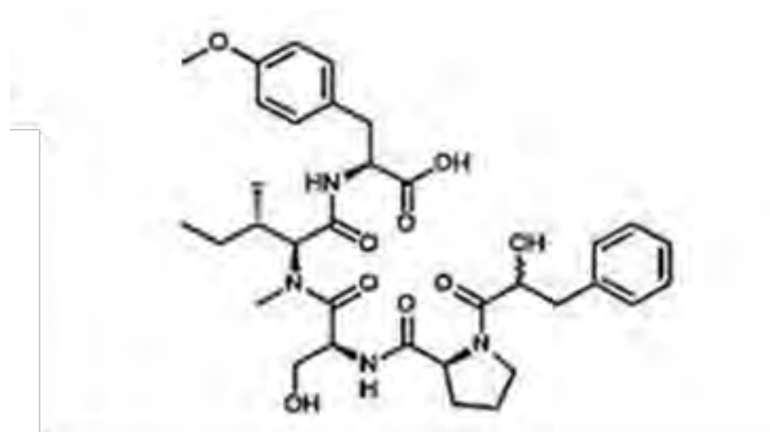

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Gong *et al*., (2017) in their research isolated endophytic fungus *Purpureocillium* sp. A5 from the roots of *Kandelia candel*. This fungus was found to protect the growth of K. *candel* under Cu stress. *Purpureocillium* sp. A5 reduces uptake of Cu in K. *candel* and changes the pH characterization of soil. Furthermore, A5 increased the concentration of Cu complexes in soil and it enhanced the concentration of carbonate-bound Cu, Mn–Fe complexes, Cu and organic-bound Cu in soil (Gong *et al*., 2017). The symbiotic association between the mangroves and their endophytes contribute greatly to the high adaptation of the mangroves to extreme environmental conditions. Most of the endophytes have the potential to produce novel bioactive metabolites, which will undoubtedly boost novel drug discovery (Deshmukh, 2018).

2.8 Antimicrobial Compounds

Antimicrobial compounds can be used not only as drugs but also as food preservatives to control the occurrence of food spoilage and also food-borne diseases during the food production. For instance, biopreservation, a biological method for food preservation where the extension of shelf life and food safety is by the use of natural or controlled microbiota and/or their antimicrobial compounds (Ananou *et al*., 2007).

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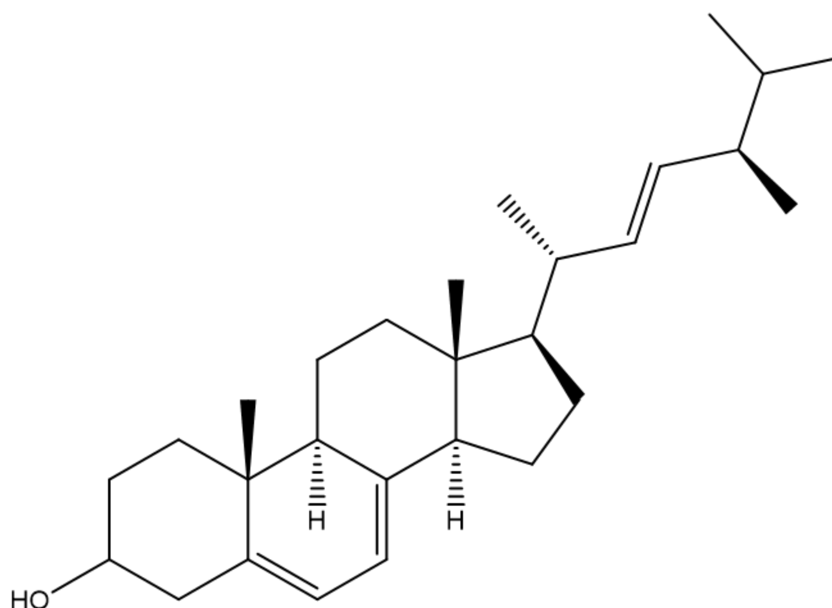

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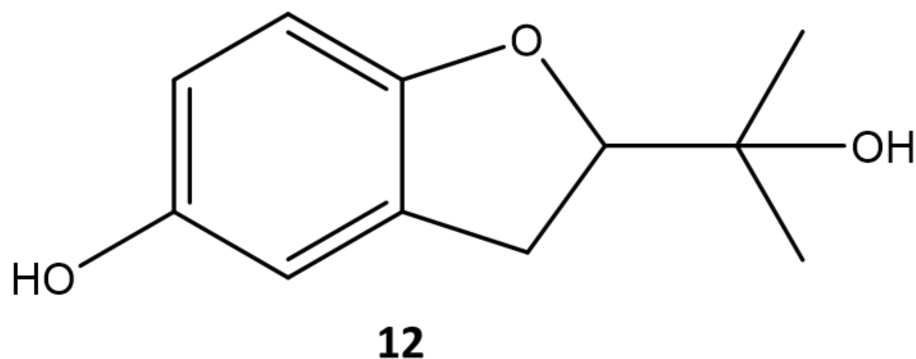
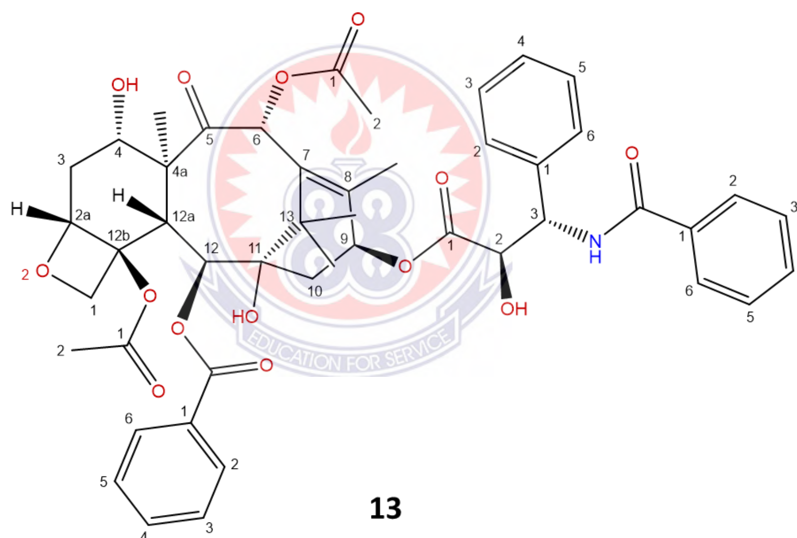
Most of the endophytic antimicrobial compounds belong to some classes of compounds such as alkaloids, peptides, steroids, terpenoids, phenols, quinines, and flavonoids (Premjanu& Jayanthy, 2012). The following are some examples of antimicrobial compounds recently isolated. Besides, two antimicrobial compounds were also extracted from the fungus *Gliomastix murorum,* which was isolated from the Chinese medicinal plant, *Paris polyphylla var. yunnanensis.* These two compounds were identified as **ergosta-5,7,22-trien-3-ol** (**11**) and **2,3-dihydro-5-hydroxy-α,α-dimethyl-2-benzofuranmethanol** (**12**) and found to be active against various test organisms such as *Agrobacterium tumefaciens, Escherichia coli, Pseudomonas lachrymans, Ralstonia solanacearum, Xanthomas vesicatoria, Bacillus subtilis* and *Staphylococcus haemolyticus* (Zhao *et al*., 2012).



2.9 Cytotoxic compounds

Cancer is one of the major causes of the high mortality rate worldwide (WHO 2012). As mentioned earlier, taxol**(13)**, the first billion-dollar anticancer drug, was the first major anticancer product (Schiff & Horwitz 1980: Ling *et al*., 2012).



The alkaloid camptothecin (**Fig. 2a**), an antineoplastic agent isolated from the stems of *Camptotheca acuminate* (**Fig. 2b**) in China, is another famous anticancer compound which is efficient against lung, ovarian and uterian cancer. It was then later found to be produced by *Entrophospora infrequens* (**Fig. 2c**), an endophyte isolated from the medicinal plant *Nothapodytes foetida* (**Fig. 2d;** Premjanu & Jayanthy, 2012), proving once more evidence of the importance of endophytic fungi in the production of bioactive compounds.

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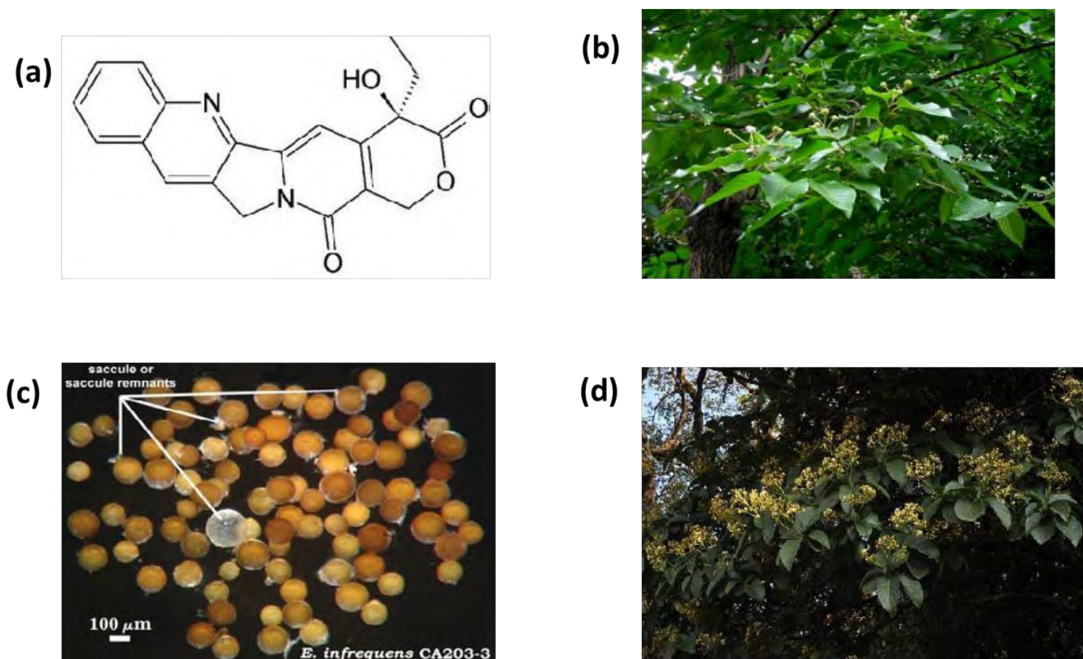

Figure 2: **(a) Camptothecin (b)** ***Camptotheca acuminate*** **(c)** ***Entrophospora infrequens*** **(d)** ***Nothapodytes foetida***

A local study was also undertaken on cytotoxic activity of endophytic fungus. A fungus found to be related to *Phoma sp.* (**Fig. 3b**) was isolated from *Cinnamommollissimum*, a medicinal plant collected at the Universiti Kebangsaan Malaysia Forest Reserve, Selangor, Malaysia. The bioactive compound extracted from this fungus showed maximum cytotoxic activity against murine leukemia cells and was found to be a polyketide named 5-Hydroxyramulosin (**Fig. 3a**, Santiago *et al*., 2012).

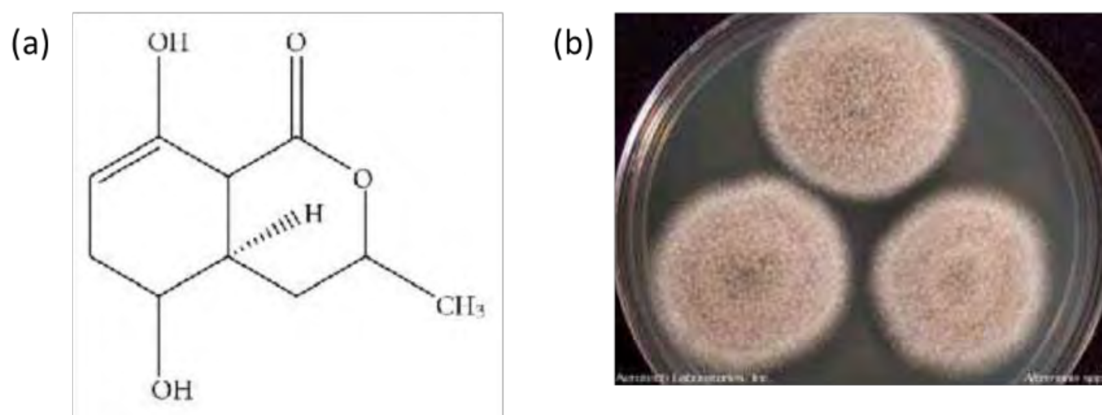

Figure 3: **(a) 5-Hydroxyramulosin** **(b)** ***Phoma sp.***

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A new cytochalasin, cytochalasin H2 (**Fig. 4a**), was extracted from the endophytic fungus *Xylaria* sp. (**Fig. 4b**), which was isolated from leaves of the medicinal plant *Annona squamosa* (**Fig. 4c**). Although it shows weak cytotoxic activity towards HeLa cell lines, cytochalasins are a group of fungal secondary metabolites which have cytotoxic activities that include disruption of actin microfilaments in both non-tumor and tumor cells (Li *et al*., 2012).

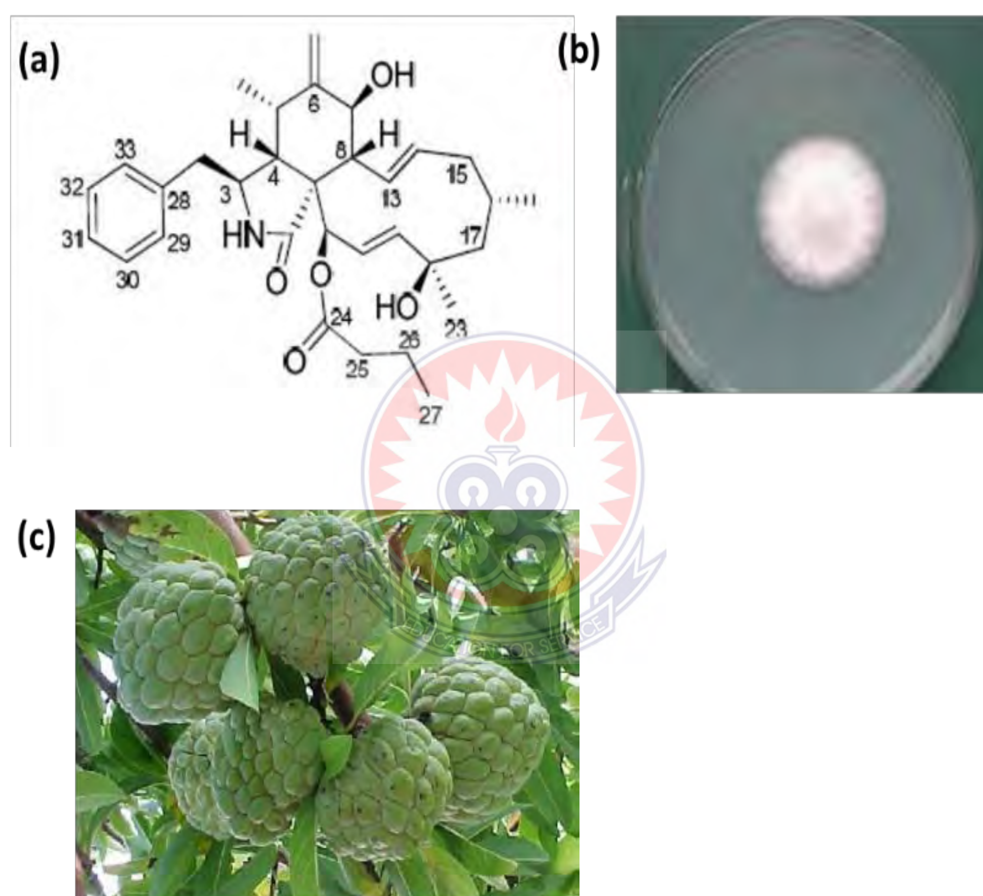

Figure 4: ***(a) Cytochalasin H2 (b) Xylaria sp. (c) Annona squamosa***

Strobel and Daisy proposed in 2003 that endemic plants are good potential sources of novel endophytes and bioactive compounds as they have a long history of growing in areas of great biodiversity. It was also reported that out of the nearly 3,000,000 plant species that exist on the earth, each individual plant is the host to one or more endophytes. Besides, medicinal plants used by indigenous people are also recognized

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as a great source of fungal endophytes as studies reported that these medicinal properties might be mediated by their endophytes (Huang *et al*., 2008; Dai *et al*., 2010). Strobel and Daisy, (2003) also indicated that plants living under unique and extreme environmental conditions, for instance mangrove forests, show great promise as well. This study was focused on endophytic fungi from mangroves and their secondary metabolites.

2.10 General Isolation Extraction of Natural Products

Extraction is the crucial first step in the analysis of medicinal plants (natural products), because it is necessary to extract the desired chemical components from the plant materials or cultures for further separation and characterization (Sasidharan *et al*., 2011). The basic operation included in the steps, such as pre-washing, drying of plant materials or freeze drying, grinding to obtain a homogenous sample and often improving the kinetics of analytic extraction and also increasing the contact of sample surface with the solvent system (Amsath, 2013). In the case of secondary metabolites from fungal culture, crude extract from fungal isolates are extracted using ethyl acetate solvent. This extraction method is particularly useful for extraction of extracellular (excreted by fungi into the medium) and intracellular bioactive compounds (Kumari *et al*., 2018). Proper actions must be taken to assure that potential active constituents are not lost, distorted or destroyed during the preparation of the extract from samples. If the plant was selected on the basis of traditional uses (Fabricant & Farnsworth, 2001), then it is necessary to prepare the extract as described by the traditional healer in order to mimic as closely as possible the traditional 'herbal' drug (Thangaraj, 2016). The selection of solvent system largely depends on the specific nature of the bioactive compound being targeted. Different

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solvent systems are available to extract the bioactive compounds from natural sources. The extraction of hydrophilic compounds uses polar solvents such as methanol, ethanol or ethyl-acetate (Altemimi *et al*., 2017). For extraction of more lipophilic compounds, dichloromethane or a mixture of dichloromethane/methanol in ratio of 1:1 is used. In some instances, extraction with hexane is used to remove chlorophyll (Cosa *et al*., 2006). Ethyl acetate was the solvent used in this study due to its ability to interact with both polar and non-polar compounds.

In addition, plant extracts are also prepared by maceration or percolation of fresh green plants or dried powdered plant material in water and/or organic solvent systems. The other modern extraction techniques include solid-phase micro-extraction, supercritical-fluid extraction, pressurized-liquid extraction, microwave-assisted extraction, solid-phase extraction, and surfactant-mediated techniques, which possess certain advantages (Koparde *et al*., 2017). These are the reduction in organic solvent consumption and in sample degradation, elimination of additional sample clean-up and concentration steps before chromatographic analysis, improvement in extraction efficiency, selectivity, and kinetics of extraction. The ease of automation for these techniques also favours their usage for the extraction of plants materials (Huie, 2002).

2.11 Identification and Characterization

Natural product extracts usually occur as a combination of various type of bioactive compounds or phytochemicals with different polarities, their separation still remains a big challenge for the process of identification and characterization of bioactive compounds (Stephen & Kumar, 2014). It is a common practice in isolation of these bioactive compounds that a number of different separation techniques such as TLC, column chromatography, flash chromatography and HPLC, should be used to obtain

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pure compounds. The isolated compounds are then used for the determination of structure and biological activity (Ingle *et al*., 2017). Preparative TLC is also another approach used to separate pure compounds from crude extracts. Besides that, non-chromatographic techniques such as immunoassay, which use Monoclonal Antibodies (MAbs), phytochemical screening assay, Fourier-transform infrared spectroscopy (FTIR), can also be used to obtain and facilitate the identification of the bioactive compounds (Rasul, 2018). Other means of identifying compounds are discussed as follows;

2.11.1 UV-Visible Spectroscopy

UV-visible spectroscopy can be performed for qualitative analysis and for identification of certain classes of compounds in both pure and biological mixtures (Umamaheswari & Latha, 2018). Preferentially, UV-visible spectroscopy can be used for quantitative analysis because aromatic molecules are powerful chromophores in the UV range. Naturally occurring compounds can be determined by using UV-visible spectroscopy (Kemp, 2017). Moreover, spectroscopic UV-VIS techniques were found to be less selective and give information on the composition of the total polyphenol content. This technique is not time-consuming, and presents reduced cost compared to other techniques (Eberhardt *et al*., 2007).

2.11.2 Nuclear Magnetic Resonance Spectroscopy (NMR)

Nuclear magnetic resonance (NMR) is a physical phenomenon in which nuclei in a strong constant magnetic field are perturbed by a weak oscillating magnetic field (in the near field) and respond by producing an electromagnetic signal with a frequency characteristic of the magnetic field at the nucleus (Pykett, 1982; Murphy & Atala, 2014). This process occurs near resonance, when the oscillation frequency matches

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the intrinsic frequency of the nuclei, which depends on the strength of the static magnetic field, the chemical environment (Olson *et al*., 2000). The magnetic properties of the isotope involved in practical applications static magnetic fields up to ca. 20 tesla, the frequency is similar to VHF and UHF television broadcasts (60–1000 MHz) (De Graaf, 2019). NMR results from specific magnetic properties of certain atomic nuclei. Nuclear magnetic resonance spectroscopy is widely used to determine the structure of organic molecules in solution and study molecular physics and crystals as well as non-crystalline materials (Spiess, 2003). NMR is also routinely used in advanced medical imaging techniques, such as in magnetic resonance imaging (MRI) (Mamin *et al*., 20007).

Nuclear magnetic resonance (NMR) is, at the present time, an established method in a variety of scientific fields such as physics, chemistry, biology, and medicine. However, it took more than 60 years to reach this interdisciplinary status. The discovery of nuclear magnetic resonance was made independently by two groups of prominent scientists, Felix (Bloch *et al*., 1946; Marion, 2013) and Edward (Purcell *et al*., 1946; Marion, 2013) at the end of World War. Nuclear Magnetic Resonance Spectroscopy gives physical, chemical and biological properties of matter. One dimensional technique is routinely used but the complicated structure of the molecules could be achieved through two-dimensional NMR techniques such as COSEY, NOSEY, HMBC and HSQC (Mopper *et al*., 2007). Solid state NMR spectroscopy is used for the determination of molecular structure of solids. Radiolabelled ^{13}C NMR is used to identify the types of carbon present in the compound (de Barros *et al*., 2012). ^{1}H-NMR is used to find out types of hydrogen present in the compound and to find out how the hydrogen atoms are connected (Ingle *et al*., 2017).

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In recent years, NMR has evolved toward more diverse applications. There are number of published structures solved by NMR and has stagnated over the years in comparison with the structures solved by X-ray diffraction (Powell, 2006). NMR can provide other types of information that is hardly amenable by crystallography: dynamics can be investigated by NMR over a wide range of time scales (Mittermaier & Kay, 2009), from slow exchange where the two interconverting species are visible to fast motion using relaxation measurements (Marion, 2013). In the field of drug discovery (Pellecchia *et al*., 2008), chemical shift mapping provides information on which part of the protein is interacting with the ligand and NMR is very powerful at screening or optimizing hints. The ecological niche of NMR is currently not restricted to protein structure determination but covers a wider range of relevant information (Brown & Ladizhansky, 2015).

2.11.3 Mass Spectroscopy

Mass spectrometry is a powerful analytical technique for the identification of unknown compounds, quantification of known compounds and to elucidate the structure of molecules (Ammann, 2007). Through MS spectrum, the molecular weight of samples can be determined. This method is mostly employed for the structural elucidation of organic compounds, for peptide or cyclotides sequencing and for monitoring the existence of previously characterized compounds in complex mixtures with a high specificity by defining both the molecular weight and a diagnostic fragment of the molecule simultaneously (Ingle *et al*., 2017).

2.11.3 X-Ray Crystallography

X-ray crystallography is used to determine the arrangement of atoms within a crystal. In this method, a beam of X-rays strikes the crystal and causes the beam of light to

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spread into many specific directions (Pesyan, 2012). From the angles and intensities of these diffracted beams, a crystallographer can produce a three-dimensional picture of the density of electrons within the crystal. From this electron density, the mean positions of the atoms in the crystal can be determined, as well as their chemical bonds, their disorder, and other valuable information (Shi *et al*., 2013).

In an X-ray diffraction measurement, a crystal is mounted on a goniometer and gradually rotated while being bombarded with X-rays producing a diffraction pattern of regularly spaced spots known as *reflections* (Rhodes, 2010). The two-dimensional images taken at different rotations are converted into a three-dimensional model of the density of electrons within the crystal using the mathematical method of Fourier transforms, combined with chemical data known for the sample (Miao *et al*., 2001). Poor resolution (fuzziness) or even errors may result if the crystals are too small or not uniform enough in their internal makeup. Producing good quality crystals is still recognized as an art (Peach *et al*., 2017).

2.12 Summary of Literature Review

This study's literature review focused on the search for new bioactive compounds in the face of increase emergence of novel diseases and drug resistant microorganisms. The review looked at how drugs like penicillin, cephalosporin, vancomycin, doxorubicin and staurosporine among others were discovered and are now used for the treatment of wide range of ailments. The use of plants (mangroves) and microbes (fungi) as the major sources of bioactive compounds. Modern researchers are now focusing on endophytic fungi (mangrove endophytes) as a rich source of bioactive compounds. It has been revealed in this review that bioactive compounds are obtained either as primary or secondary metabolites.

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Extraction protocols of natural products depending on the source and nature of the target compounds was not left out in this review. Ethyl acetate has been noted to be one of widely used solvent for the extraction of natural products. Finally, this review considered some techniques used in the identification and characterization of compounds which included chromatography, FTIR, IR, UV, NMR and X-ray crystallography.

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CHAPTER THREE

MATERIALS AND METHODS

3.0 Overview

This chapter discusses the study area, sample collection and treatment, preparation of culture media and sterilization procedure. It also discussed sub-culturing of mangrove endophytes, the culturing process, harvesting and solvent extraction of cultures. Concentration of extract, TLC study of the crude extracts and instrumental analysis were equally discussed in this chapter.

3.1 Study Area

This study was carried out in Winneba in the Effutu Municipality of the Central Region of Ghana. Winneba is the capital town of Effutu Municipal District in Central Region of Southern Ghana. Winneba has a population of 55,331. Winneba, traditionally known as Simpa, is a historic fishing port in Ghana, lying on the south coast, 140 kilometres east of Cape Coast. Along its coast are Akosua village and Sankor with mangrove vegetation along their water bodies.

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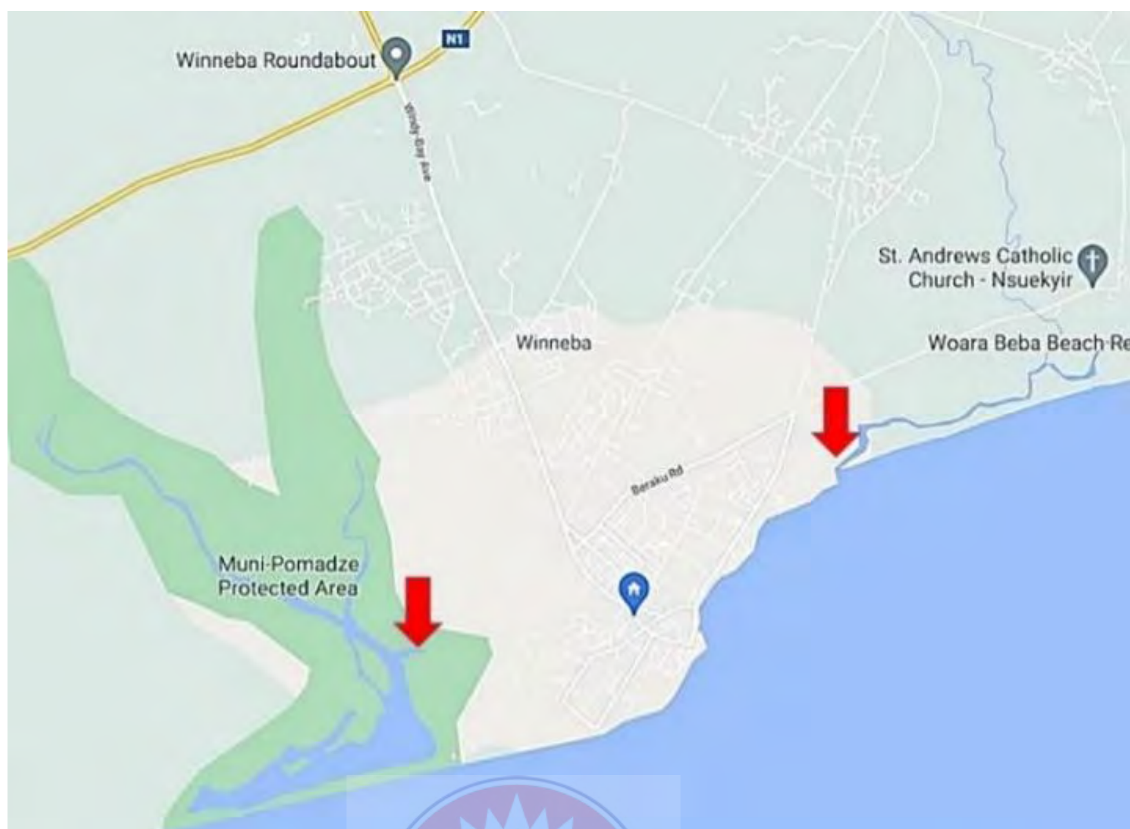

Figure 5: Map of Winneba obtained form Google map

This study focused on the mangroves along the Muni lagoon in Akosua village and the Ayensu estuary in Sankor.

3.2 Materials

All the solvents and reagents used for the study were bought from commercial suppliers. These were used without any additional purification. To culture the fungi, a 1.5kg barley malt extract from Muntons plc, England was purchased and used as the malt; Dextrose monohydrate (100%w/w) was obtained from the pharmacy shop in Winneba. An agar was purchased from a shop in Accra. The following solvents ethyl acetate (EA), chloroform, diethyl ether, petroleum ether, acetic acid and acetone were obtained from MES equipment's, Fregeosco limited and other chemical shops in Circle-Accra, Ghana, each in a 2.5L Winchester bottle. The anisaldehyde reagent (100ml) was also bought from a chemical shop in Circle-Accra. The rest of the

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reagents -sulphuric acid, hexane, ethanol, and methanol -were obtained from the chemistry laboratory of University of Education, Winneba where the solvent extraction took place. The **table 1** below gives the manufacturers and the percentage purity of the solvents used in the study. Several glass plates of measurement 19.5cm by 6cm were purchased from a local shop, prepared and used as TLC plates. These plates were then coated with silica gel (Silica Gel G6) manufactured by BDH. It contained 13% calcium sulphate and was manufactured purposely to be used for TLC studies. A column was also made to be used for column chromatography. The column was 1meter long and 4cm wide.

Table 1: Manufacturers and some percentage purities of the solvents used in the study

Solvent	**Manufacturer**	**Percentage purity**
Ethyl Acetate (EA)	Paskem chemicals	99.5%
Petroleum Ether	BDH	
Chloroform	Fisher Chemicals	99.8%
Diethyl ether	Daejung reagent chemicals	
Acetic acid	BDH	
Acetone	BDH	
Anisaldehyde	LOBA Chemie	98% extra pure
Sulphuric acid	BDH	
Hexane	BDH	98%
Ethanol	Philip Harris	99-99.5%
Methanol	Philip Harris	99-99.5%

Gas chromatography-mass spectrometer (Shimadzu GCMS QP2020) was used to analyze the crude extracts. The analysis was carried out in the chemistry laboratory of the University of Cape Coast. The GC operation parameters used were Column oven Temp: 50.0 C, Injection Temp: 250.0 C, Split ratio: 50.0, Pressure: 84.0 KPa, Column flow: 1.62 mL/min and Linear velocity: 49.2 cm/sec. Column used was Rtx-5ms

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(30×0, 25µm×0.25mm) and *Injection volume* of 1.0µL. These were coupled to mass spectrometer Parameters of Ion source temp: 250^0C, Interface temp: 270^0C and mass scan range: 80 -550 m/z. NIST 14, NIST14s and Wiley 8' libraries were used for the identification of peaks while quantifications were done using area normalization for total ion counts (TIC) and 5 reference ions were selected to improve sensitivity.

Both human infesting microbes and cocoa infesting microbes were used in the study. Eight strains of the human infesting microbes used, were obtained from the School of Basic and Biomedical Sciences, University of Health and Allied Science. These included Methicillin resistant *Staphylococcus aureus* (NCTC 12493), *Staphylococcus aureus* (NCTC 12973), *E. coli* (NCTC 12241), *S. mutants* (ATCC 700610), *P. aeroginosa* (ATCC 4853), *S. typhi* (ATCC 14028), *K. pneumonia (NCTC 13440)* and *Candida albicans* (ATCC 90028). Two fungi that infest cocoa namely, *Phytophthora palmivora and Phytophthora megakarya* were also obtained from Ghana Cocoa Research Institute for the bioassay.

The reagents used for the free radical scavenging activity tests for the crude extracts were Methanol (Sigma Aldrich, analytical grade), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma Aldrich, analytical grade), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Sigma Aldrich, analytical grade), Dimethyl sulfoxide (DMSO) (Sigma Aldrich, analytical grade).

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3.3 Isolation of Mangrove Endophytic Fungi

3.3.1 Sample collection

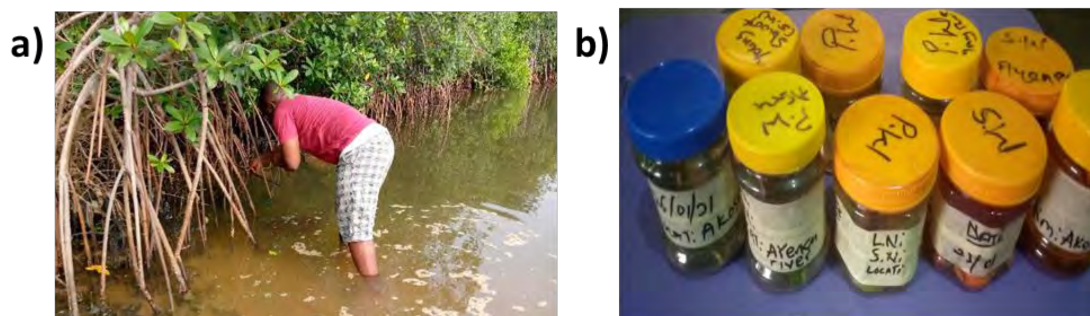

Figure 6: a) collection of samples from a red mangrove b) samples in plastic containers.

Leaves, barks and roots of Red, Black and White mangroves were collected from the mangrove forest along the Ayensu estuary in Sankor and Muni lagoon in Akosua village. The samples were collected into plastic containers with their source water and transported to the culture room.

3.3.2 Sample treatment

One crucial step in the isolation of endophytes is surface sterilization in order to get rid of all the microbes living on the surface of the collected samples. There are several ways to achieve surface sterilization. This was achieved by treating plant tissues with oxidant or general sterilant for a given period, followed by a sterile rinse. Generally, the sterilizing procedure should be optimized for each plant tissue, especially the sterilization time since the sensitivity varies with plant species, age and organs (Zheng *et al*., 2016). Surface sterilization involving the use of a variety of solutions is important to kill the unwanted phylloplane fungal propagules adhering to the surface of the cuticle of the leaves (Gangadevi *et al*., 2008).

The samples collected (bark, root and leaves of black, red and white mangroves) were washed tap water, rinsed with distilled water and then air-dried. The washing of the

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surface of samples is done to remove extraneous matter (Seena & Sridhar, 2004). The samples were surface sterilized by immersing them in 99% ethanol solution for 10 minutes.

3.3.3 Preparation of culture media

The procedure for the preparation of the culture was adopted from Oppong, (2017). The sub-culture and culture media contain 3%$(\frac{w}{v})$ malt extract and 6%$(\frac{w}{v})$ glucose. 30g of the malt extract and 60g of glucose was dissolved in 1000ml of distilled water. This was distributed into nine 250ml conical flasks each containing about 110ml for the sub-culturing. The same media was prepared for the large-scale culturing of the fungi into 2dm^3 flat-bottomed flasks. Three flat-bottomed flasks containing 1dm^3 of the media was used for each of the fungal isolate.

Master culture media were also prepared by dissolving 3g of malt extract and 2g of malt extract agar in 100ml of distilled water (Oppong, 2017). These were prepared to serve as long term static culture of the original fungi isolated and used for the study. About 10mL of the master culture media was placed in twelve (12) 20ml sample tubes with screw covers. The agar was added to the media to set. The master culture media after sterilization were placed on a wooden board slanted at an angle to enable a sideway setting of the media which provided large surface area for fungal growth.

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Figure 7: Master culture media slanted on wooden board to set

3.3.4 Sterilization procedure

A contaminant free instruments and procedure is very essential in the fungal culturing process. The culture media, flask and other materials used in the culturing process were thus sterilized using the Priorclave. The lid of the Priorclave was unlocked and its water tank filled to the required level. The materials to be sterilized were then placed in the machine, locked and set to 121^0C for 30 minutes. It takes the Priorclave about three (3) hours to unlock after the sterilization process is complete within the 30 minutes.

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Figure 8: The Priorclave used to sterilize the culture media and instruments

3.3.5 Fungi sub-culture

The sterilized mangrove plant materials were cut into 1 cm long pieces and placed directly into culture media (3%$(\frac{w}{v})$ malt extract and 6%$(\frac{w}{v})$ glucose) in the 250ml conical flask using sterile scissors. The sub-culture was incubated at a temperature of $25^{o}C$ to $29^{0}C$ for 4 weeks and monitored periodically. They were labelled as follows;

- LBMAV – leaf of a black mangrove from Akosua village.
- BBMAV - bark of a black mangrove from Akosua village.
- RBMAV – root of a black mangrove from Akosua village.

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- LRMAV – leaf of a red mangrove from Akosua village.
- BRMAV – bark of a red mangrove from Akosua village.
- RRMAV – root of a red mangrove from Akosua village.
- LWMAV -leaf of a white mangrove from Akosua village.
- BWMAV – bark of a white mangrove from Akosua village.
- LBMS – leaf of a black mangrove from Sankor.
- BBMS – bark of a black mangrove from Sankor
- RBMS – root of a black mangrove from Sankor
- LWMS - leaf of a white mangrove from Sankor
- BWMS – bark of a white mangrove from Sankor
- LRMS – leaf of a red mangrove from Sankor
- BRMS – bark of a red mangrove from Sankor

After two (2) weeks, some mycelia were observed to be growing on and beneath the surfaces of some of the mangrove pieces in the sub-culture media (**fig. 9**).

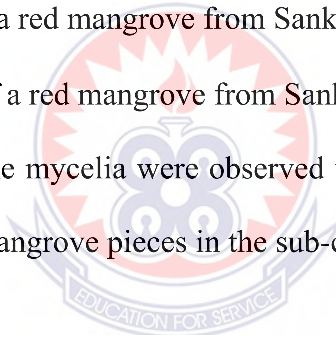

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Figure 9: Two weeks old subculture of mangrove endophytic fungi

3.4 Fungal Culture used in Extracting of Bioactive Compounds

After four weeks, pieces of the mycelia from the sub-culture were cut using sterilized forceps and spatula and placed into sterilized 2dm^3 flat-bottomed flask containing 3%$(\frac{w}{v})$ malt extract and 6%$(\frac{w}{v})$ glucose medium. The process was carried out with burning flame from a Bunsen burner to prevent spores of unwanted fungi from entering and contaminating the culture. The fungal broth cultures were then incubated at 25°C to 29^{0}C for eight weeks before being used for solvent extraction for bioactive compounds (**fig. 10**).

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Figure 10: Eight weeks old culture of mangrove endophytic fungi.

3.5 Harvesting and Solvent Extraction of Culture

The eight weeks old fungal cultures were harvested into 5L gallons. The mycelia were filtered from the broth using muslin cloth. The filtrate (cultured broth) placed in gallons were sent to the chemistry laboratory for the extraction of the secondary metabolites.

The crude extracted from each fungal broth was extracted with ethyl acetate. This extraction method is particularly useful since it extracts both extracellular (excreted by fungi into the medium) and intracellular bioactive compounds (Ling *et al*., 2012). Equal volumes of the broth and ethyl acetate were poured into separating funnels, shook vigorously and allowed to stand for one hour.

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Figure 11: Using separating funnel to collect the ethyl acetate layer from the aqueous layer.

Ethyl acetate is a moderately polar solvent found almost at the middle of the eluotropic series and can extract a wide range of organic compounds. Its use allows for the extraction of most of the compounds produced in the culture media (Tsuda, 2004; Oppong, 2017). After allowing the broth-ethyl acetate mixture to stand for an hour, it separated into two layers namely organic layer (ethyl acetate with metabolites) at the top and the aqueous layer (broth) at the bottom.

The aqueous layer was collected into a beaker and the process repeated three times. The organic layer was concentrated using the rotary evaporator to obtain the crude fungal extracts.

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Figure 12: Crude fungal extract concentrated using rotary evaporator

Dark semi-solids were obtained after the rotary evaporation. In all, five different crude extracts were obtained. The masses of the crude extracts obtained from each fungal broth of the given volumes of the culture media are presented in **table 2** below. TLC and GC-MS analyses were carried out on the crude extracts. Bioassays as well as free radicals scavenging activities were run.

Table 2: Mass of crude extract obtained from fungal broth.

Sample	**LBMS**	**RBMAV**	**BBMS**	**BBMAV**	**BWMS**
Solvent	EtOAc	EtOAc	EtOAc	EtOAc	EtOAc
Vol of culture medium (L)	3.00	2.50	3.00	3.00	3.00
Mass of extracts (g)	1.12	0.89	1.43	1.55	1.62

3.6 Thin-Layer Chromatography (TLC) Study of the Crude Extracts

Crude extracts were spotted on TLC plates made of glass coated with silica gel. This afforded me the opportunity to determine the number of components in each crude extract. Different solvent systems were prepared and used to run the TLC. Three of the solvent systems gave a clear separation on the chromatogram. The solvent systems used were

- Chloroform: methanol: acetic acid (45:3:3)

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- Petroleum ether: ethyl acetate: acetic acid (25:23:3)
- Chloroform: acetone: acetic acid (35:23:3)

The TLC plates were removed from the chromatographic tank and allowed to dry after which they were sprayed with p-anisaldehyde reagent. The sprayed TLC plates were then heated for 15minutes at 105^{0}C – 110^{0}C to enable visualization of the sports chromatograms.

Figure 13: **TLC chromatograms of samples run in chloroform: acetone: acetic acid.**

The spray reagent is good for the detection of phenols, sugars, steroids, and terpenes. The reagent is a freshly prepared solution of 0.5ml p-anisaldehyde in 50ml glacial acetic acid and 1ml 97% sulfuric acid. When this reagent is used, lichen constituents, phenols, terpenes, sugars, and steroids turn violet, blue, red, grey or green respectively.

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3.7 Gas Chromatography – Mass Spectrometry (GC-MS) Analysis

Crude extracts from the fungal cultures were qualitatively and quantitatively analysed using gas chromatography- mass spectrometer at chemistry laboratory of the University of Cape Coast.

Table 3: Temperature program GC-MS analysis

Rate °C/Min	Final temperature	Hold time
-	50	1.0
20	150	0.00
6.0	300	1.0

Total program time: 32 minutes

3.8 Antimicrobial Activity Test

All crude extracts from the fungal broth were screened for their antimicrobial activities. The antimicrobial assay included testing their antibacterial and antifungal activities. The test bacteria and fungi used in this study were selected on the basis of their implication in most infections.

The broth micro-dilution technique was carried out following the methodology described in document M27-A3 published by the Clinical and Laboratory Standards Institute (CLSI) (Watts *et al*., 2008) with minor modifications. 100µl of Mueller Hinton broth were dispensed into all the wells of each of the plates to be used.

100µl each of the extract was then used to prepare well concentrations ranging from 100 – 0.1 mg/mL with wells 11 and 12 serving as positive control (Broth + organism only) and negative control (Broth with no organism) respectively for each microorganism from the columns A- H. These processes were likewise done for voriconazole and chloramphenicol at 30 ug/mL in a separate plate as standard drug controls against all the test bacteria and fungi respectively. Next, 100µl of each of the 0.5 McFarland standardized, test organisms on each column were added after which

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the plates were subjected to incubation at 37 C for 24 to 48 hours for bacterial and fungal strains respectively.

Minimum Inhibitory Concentration (MIC) values were then evaluated by visual analysis by adding Tetrazolium dye after 30 minutes. Each experiment was done thrice and the MICs recorded.

In order to verify if the extract were able to kill the microbial cells (bacteri-/fungi-cidal effect) the plates were also evaluated for Minimum Bactericidal (MBC) and Fungicidal Concentration (MFC).

Aliquots from each well from susceptibility testing assays were transferred to plates containing Nutrient agar, which were incubated at 37ºC for 24 to 48 hour. Results were then evaluated by analyzing the presence or absence of growth in the Nutrient agar or SDA (Bagiu *et al*., 2012).

3.9 Free Radicals Scavenging Activity (ABTS, DPPH) Test

A 300 µL of freshly prepared stock of 0.6 mM of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was dissolved in 100 ml of methanol as a working solution. A 150 µl of this solution was added to 50 µL of the test compound (T) with concentrations of 1, 0.5, 0.25, 0.125, 0.0625, 0.0313, 0.0156 and 0.0078 mg/mL and then incubated for 30 minutes at room temperature. The DPPH radical scavenging activity of the compound was then determined by measurement of the absorbance at 517nm for test compound (T) expressed as a percentage of the absorbance of a control sample (C) of DPPH without the compound. Subsequently, the antioxidant potential against ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) was also determined by adding 10 mL of ABTS and 10 ml of aqueous 2.4 mM potassium persulphate to generate the ABTS free radical to form the stock solution. This stock was further diluted with 50

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ml of methanol as a working solution. A 150 µL of the solution was then added to 50 µL of the prepared compound concentrations, vortexed and incubated at 30°C for 10 min. The absorbance was then recorded at 734 nm. The free radical scavenging activity of the compound as well as Ascorbic acid as a referenced positive control against DPPH and ABTS were therefore evaluated by inputting data into the relation: [22:23] $\% \, Scavenging \, activity = [\frac{C-T}{C}] \times 100\%$.

Where C = concentration of control and T = concentration of test sample.

3.10 Column chromatography

Column chromatography was run BWMS and BBMS to help isolate the various components in the crude extracts. The column was 1m long and 4cm wide. This length and width allowed for proper separation of the components in the concentrates. Acid washed sand was first poured into the column to about 1cm. About 20g of silica gel was mixed with a reasonable amount of the solvent mixture to form a paste and poured into the column and allowed to settle after about one hour. The eluent was run till it was just above the silica gel in the column. 1.0g of the crude extract dissolved with small amount of ethyl acetate was then added and allowed to settle in to the gel followed by another layer of sand. The column was run with chloroform: methanol: acetic acid in the ratio 47:3:3. About 3ml of the eluent was collected into test tubes. TLC was further used to help combine fractions of similar profile. In all 26 isolates were obtained from BWMS and 12 from BBMS.

Figure 14 : column run on BWMS crude extract.

Below is a flow chat illustrating the various steps in volved methodology.

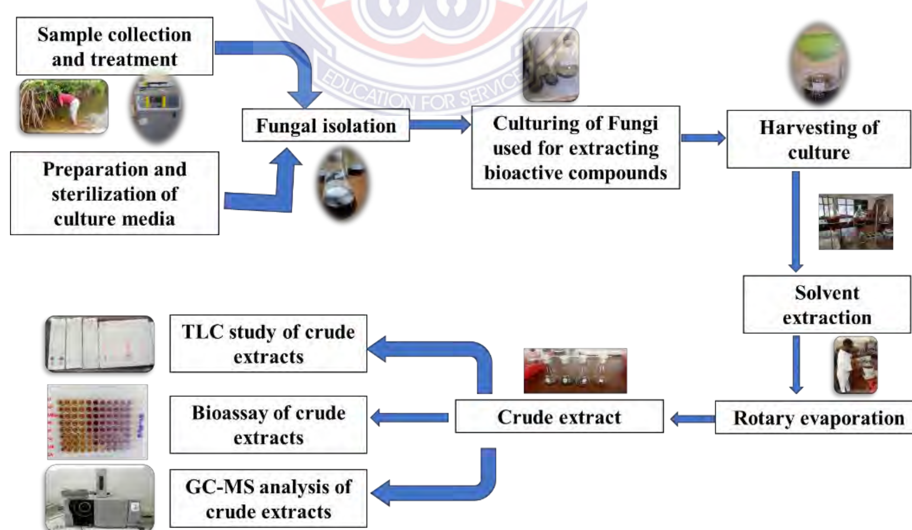

Figure 15 : A flow chat of methodology.

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CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.0 Overview

In this chapter we discuss the results obtained during the various stages of study conducted as well as discussions on them.

4.1 Fungi Isolation and Culture

After two weeks of the initial sub-culture of the leaves, roots and barks in 250ml conical flasks (15 flasks), only five of the sub-cultures were observed to have some fungal growth on and beneath the surface of the media. BBMAV (white) and LBMS (brown) were observed to be growing on the surface of the media while BWMS, RBMAV and BBMS were growing beneath the surface of the media. The fungal growth covered only about 50% of the surface of media after two weeks but covered the entire surface after four weeks of incubation.

Fungal growth in the 2L flat bottomed flasks were observed five days after pieces of the mycelia were transferred from 250ml conical flask sub-culture. After two weeks, the entire surface of media were covered with fungal growth. The mycelia increased in thickness steadily for the first four weeks and stopped. The white color of the mycelia of BBMAV turned yellowish-brown and that of BWMS turned dark-green with more white patches while RBMAV, LBMS and BBMS maintained their appearance after eight weeks of incubation.

4.2 Thin-Layer Chromatography Studies

The TLC studies of the crude extracts of the fungal isolates using different solvent systems produced different colours with the p-anisaldyhide spray reagent. There were slight variations in the degree of separations and colours on the TLC plates with the

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various solvent systems. The colours produced such as violet, blue, red, grey or green indicates the presence of lichen constituents, phenols, terpenes, sugars, and steroids respectively in the crude extracts (Damle & Sharon, 2017).

4.3 Gas Chromatography-Mass Spectrometry Analysis

The GC-MS results of the crude extracts of the fungal broth show that each crude extract contains mixtures of various groups of compounds. A split ratio of fifty (50) was used so as to obtain much clearer peaks. The following are tables of the GC-MS results of each fungal isolate. Chromatograms obtained from the GC-MS analysis of the extracts as well as some structures obtained using CHEMDRAW software are presented in **appendix 1** and **appendix 2** respectively.

4.3.1 Compounds Identified in the Crude Extracts

The tables (**4-8**) below show the compounds identified in the extracts through the GC-MS.

Table 4: Compounds Identified in RBMAV

S/N	Compound name	t_R	Formula	M/Z
1	3-Furanacetic acid, 4-hexyl-2,5-dihydro-2,5-dioxo-	8.3	$C_{12}H_{16}O_5$	240.1
2	Hexadecanoic acid, methyl ester	12.5	$C_{17}H_{34}O_2$	270.3
3	n-Hexadecanoic acid	13.2	$C_{16}H_{32}O_2$	256.2
4	hexadecanoic acid, ethyl ester	13.4	$C_{18}H_{36}O_2$	284.3
5	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	14.7	$C_{19}H_{34}O_2$	294.3
6	11-Octadecenoic acid, methyl ester	14.8	$C_{19}H_{36}O_2$	296.3
7	Methyl stearate	15.1	$C_{19}H_{38}O_2$	298.3
8	9,12-Octadecadienoic acid (Z,Z)-	15.5	$C_{18}H_{32}O_2$	280.2
9	Linoleic acid ethyl ester	15.6	$C_{20}H_{36}O_2$	308.3
10	(E)-9-Octadecenoic acid ethyl ester	15.7	$C_{20}H_{38}O_2$	310.3
11	Octadecanoic acid, ethyl ester	16.0	$C_{20}H_{40}O_2$	312.3
12	2,2,4-Trimethyl-3-(3,8,12,16-tetramethyl-heptadeca-3,7,11,15-tetraenyl)-cyclohexanol	24.1	$C_{30}H_{52}O$	428.4
13	Benzene, 1,2,3-trimethoxy-5-(1-propenyl)-, (E)-	24.9	$C_{12}H_{16}O_3$	208.1
14	Stigmast-4-en-3-one	26.2	$C_{29}H_{48}O$	412
15	Vitamin E	27.8	$C_{29}H_{50}O_2$	430.4

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Table 5: Compounds Identified in LBMS

S/N	Compound name	t_R	Formula	M/Z
1	Phenylethyl Alcohol	5.5		
2	Benzoic acid, 3-methyl-, methyl ester	6.2	$C_9H_{10}O_{21}$	150.1
3	Acetic acid, 2-phenylethyl ester	6.6	$C_{10}H_{12}O_2$	164.1
4	Bicyclo(6.1.0)nonane-9,9-dicarboxylic acid	7.0	$C_{11}H_{16}O_4$	212.1
5	(+)-Limonene oxide	7.4	$C_{10}H_{16}O$	152.1
6	2-Pinen-10-hydroperoxide	7.4	$C_{10}H_{16}O_2$	168.1
7	1-Oxaspiro[3.5]nonan-2-one, 3-methylene-	7.5	$C_9H_{12}O_2$	152.1
8	(3R,6R)-3-Hydroperoxy-3-methyl-6-(prop-1-en-2-yl)cyclohex-1-ene	7.7	$C_{10}H_{16}O_2$	168.1
9	Tetrahydroionyl acetate	7.9	NA	NA
10	Benzeneethanol, 4-hydroxy-	8.1	$C_8H_{10}O_2$	138.1
11	3,6-methanonaphth[2,3-b]oxirene-2,7-dione, octahydro-	8.2	$C_{11}H_{12}O_3$	192.1
12	3-Furanacetic acid, 4-hexyl-2,5-dihydro-2,5-dioxo-	8.3	$C_{12}H_{16}O_5$	240.1
13	3-Oxabicyclo[4.3.0]nonan-2-one, 8-isopropylidene-, cis-	8.7	$C_{11}H_{16}O_2$	180.1
14	Furan, 2,3-dihydro-2,2-dimethyl-3-(1-methylethenyl)-5-(1-methylethyl)-	8.8	$C_{12}H_{20}O$	180.2
15	2H-Pyran-2-one, 5,6-dihydro-4-(2,3-dimethyl-2-buten-1-yl)-	9.0	$C_{11}H_{16}O_2$	180.1
16	Tyrosol, acetate	9.1	$C_{11}H_{15}NO_3$	209.1
17	Dispiro[2.0.2.5]undecane, 8-methylene-	9.3	$C_{12}H_{18}$	162.1
18	(1R,2R,5R,E)-7-Ethylidene-1,2,8,8-tetramethylbicyclo[3.2.1]octane	9.8	$C_{14}H_{24}$	192.2
19	Muurol-5-en-4-one <cis-14-nor->	10.9	NA	NA
20	Acetic acid, 7-hydroxy-1,3,4,5,6,7-hexahydro-2H-naphthalen-4a-ylmethyl ester	11.5	$C_{13}H_{20}O_3$	224.1
21	4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-Z)-	12.4	$C_{23}H_{34}O_2$	342.3
22	Hexadecanoic acid, methyl ester	12.5	$C_{17}H_{34}O_2$	270.3
23	Hexadecanoic acid	13.3	$C_{16}H_{32}O_2$	256.2
24	Methyl octadeca-9,12-dienoate	14.7	$C_{19}H_{34}O_2$	294.3
25	9-octadecenoic acid (z)-, methyl ester	14.8	$C_{19}H_{36}O_2$	296.3
26	9,12-Octadecadienoic acid (Z,Z)-	15.6	$C_{18}H_{32}O_2$	280.2
27	Tricyclo[6.3.0.0e5,7]undecan,1,8-epoxy-2,6,6,9-tetramethyl-	17.2	NA	NA
28	(5z)-5-Ethylidene-4-(2-hydroxyethyl)tetrahydro-2h-pyran-2-one	17.6	$C_9H_{14}O_3$	170.1
29	Phenol,2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-	19.2	$C_{23}H_{32}O_2$	340.2
30	Oxalic acid, di(2-phenylethyl) ester	20.4	$C_{18}H_{18}O_4$	298.1
31	Squalene	24.1	$C_{30}H_{50}$	410.4

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Table 6: Compounds Identified in BWMS

S/N	Compound name	t_R	Formula	M/Z
1	Phenylethyl Alcohol	5.5	$C_8H_{10}O$	122.1
2	8-Thiabicyclo[3.2.1]oct-2-ene	5.6	$C_7H_{10}S$	126.1
3	Methyl 4-oxo-2-butenoate	5.8	$C_5H_6O_3$	114.0
4	Dehydromevalonic lactone	5.9	$C_6H_8O_2$	112.13
5	1-Dodecanol	6.0	$C_{12}H_{26}O$	186.2
6	Tetrahydrofuran-5-on-2-methanol,.alpha.-[.alpha.-methoxy-(tetrahydrofuran-5-on-2-ylmethoxy)]-	6.2	$C_{11}H_{16}O_7$	260.1
7	5-Hydroxymethylfurfural	6.5	$C_{11}H_{16}O_7$	126.0
8	Benzaldehyde, 4-methoxy-	6.7	$C_8H_8O_2$	136.1
9	Hexyl 3-methylbutanoate	6.8	$C_{11}H_{22}O_2$	186.2
10	2-Methoxy-4-vinylphenol	7.1	$C_9H_{10}O_2$	150.1
11	1-Pentadecene	7.5	$C_{15}H_{30}$	210.2
12	Phenol, 4-propyl-	7.6	$C_9H_{12}O$	136.1
13	Benzeneethanol, 4-hydroxy-	8.0	$C_8H_{10}O_2$	138.1
14	3-Furanacetic acid, 4-hexyl-2,5-dihydro-2,5-dioxo-	8.3	$C_{12}H_{16}O_5$	240.1
15	4a(2H)-Naphthalenol,octahydro-4,8a-dimethyl-,(4.alpha.,4a.alpha.,8a.beta.)-	8.6	$C_{12}H_{22}O$	182.2
16	Benzoic acid, 4-hydroxy-3-methoxy-, methyl ester	8.7	$C_9H_{10}O_4$	182.1
17	Acetamide, n-(p-hydroxyphenethyl)-	9.0	$C_{10}H_{13}NO_2$	179.1
18	Carbamic acid, (2-methylphenyl)-, ethyl ester	9.5	$C_{10}H_{13}NO_2$	179.1
19	2-Pentyl-cyclohexane-1,4-diol	9.7	$C_{11}H_{22}O_2$	186.2
20	4-(4-Hydroxyphenyl)-2-butanone propyleneglycol	9.8	NA	NA
21	1-[1-Propenyl]-8-nonenyl acetate	10.0	$C_{14}H_{24}O_2$	224.2
22	Succinic acid, di(but-3-yn-2-yl) ester	10.3	$C_{12}H_{14}O_4$	222.1
23	Succinic acid, tridec-2-yn-1-yl 2-methylbutyl ester	10.3	$C_{22}H_{38}O_4$	366.3
24	Ethanol, 2-phenoxy-, propanoate	10.6	$C_{11}H_{14}O_3$	194.1
25	1-Nonadecene	11.0	$C_{19}H_{38}$	266.3
26	Cyclohexanecarboxaldehyde, 3,3-dimethyl-5-oxo-	11.2	$C_9H_{14}O_2$	154.1
27	5-(Morpholino)pent-2-en-4-ynal	11.5	$C_9H_{11}NO_2$	165.1
28	Phenol, 3,5-dimethoxy-	11.7	$C_8H_{10}O_3$	154.1
29	9-Octadecenoic acid, 12-(acetyloxy)-, methyl ester, [r-(z)]-	12.5	$C_{21}H_{38}O_4$	354.3
30	3-Isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione	12.9	$C_{11}H_{18}N_2O_2$	210.1
31	Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-	13.1	$C_{11}H_{18}N_2O_2$	210.1

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	(2-methylpropyl)-			
32	2-[(4,6-Dimethyl-2-pyrimidinyl)sulfanyl]ethanol	13.2	$C_8H_{12}N_2OS$	184.1
33	Hexadecanoic acid	13.3	$C_{16}H_{32}O_2$	256.2
34	9,12-Octadecadienoic acid (Z,Z)-	15.5	$C_{18}H_{32}O_2$	280.2
35	Tetradecanoic acid, ethyl ester	16.0	$C_{16}H_{32}O_2$	256.2
36	9,10-Anthracenedione, 1,8-dihydroxy-3-methyl-	18.6	$C_{15}H_{10}O_4$	254.1
37	3-Benzylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione	19.0	$C_{14}H_{16}N_2O_2$	244.1

Table 7: Compounds identified in BBMS

S/N	**Compound name**	**t_R**	**Formula**	**M/Z**
1	Phenylethyl Alcohol	5.4	$C_8H_{10}O$	122.1
2	2',2'-Dimethylspiro{bicyclo[4.1.0]heptane-7,5'-[1,3]dioxane}-4',6'-dione	7.3	$C_{12}H_{16}O_4$	224.1
3	Benzeneethanol, 4-hydroxy-	8.0	$C_8H_{10}O_2$	138.1
4	3-Furanacetic acid, 4-hexyl-2,5-dihydro-2,5-dioxo-	8.3	$C_{12}H_{16}O_5$	240.1
5	Cyclobutane, 3-hexyl-1,1,2-trimethyl-, cis-	8.5	$C_{13}H_{26}$	182.2
6	Phenol, 2,5-bis(1,1-dimethylethyl)-	8.6	$C_{14}H_{22}O$	206.2
7	4a.alpha.(2h)-Naphthol,octahydro-4.alpha.,8a.beta.-dimethyl-	8.7	$C_{12}H_{22}O$	182
8	Piperazine,1-methyl-4-(2,4,6-trimethylphenylsulfonyl)-	8.8	$C_{14}H_{22}N_2O_2S$	282.1
9	Trans-bicyclo[4.4.0]decan-1-ol-3-one	9.7	$C_{10}H_{16}O_2$	168.1
10	12-Methyl-E,E-2,13-octadecadien-1-ol	10.0	NA	NA
11	7-Methyl-oxa-cyclododeca-6,10-dien-2-one	10.3	$C_{12}H_{18}O_2$	194.1
12	Oxacyclotetradecan-2-one	10.5	$C_{13}H_{24}O_2$	212.2
13	2-Methoxy-3-hydrazinyl-pyrazine	10.7	$C_5H_8N_4O$	140.1
14	3,3-Dimethyl-5-oxocyclohexanecarbaldehyde	11.2	$C_9H_{14}O_2$	154.1
15	2(3H)-Benzofuranone, 6-ethenylhexahydro-3,6-dimethyl-7-(1-methylethenyl)-, [3S-(3.alpha.,3a.alpha.,6.alpha.,7.beta.,7a.beta.)]	13.1	$C_{15}H_{22}O_2$	234.2
16	3,5a,9,9-	13.2	$C_{15}H_{26}O_2$	238.2

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	Tetramethyldecahydrobenzo[2,3]cyclohepta[1,2-b]oxiren-3-ol			
17	Koiganal II	14.4	$C_{18}H_{32}O$	264.446
18	Bicyclo[2.2.1]heptane-1-carboxylic acid, 7,7-dimethyl-2-oxo-	14.4	$C_{10}H_{14}O_3$	182.1
19	(E)-2-((8R,8aS)-8,8a-Dimethyl-3,4,6,7,8,8a-hexahydronaphthalen-2(1H)-ylidene)propanal	14.8	$C_{15}H_{22}O$	218.2
20	9,12-Octadecadienoic acid (z,z)-	14.9	$C_{18}H_{32}O_2$	280.2
21	Widdrol hydroxyether	16.1	$C_{15}H_{26}O_2$	238.37
22	Methyl 5,13-docosadienoate	17.3	$C_{23}H_{42}O_2$	350.3
23	(7S)-trans-bicyclo[4.3.0]-3-nonen-7-ol	17.7	$C_9H_{14}O$	138
24	Methyl 5,9-tetracosadienoate	17.9	$C_{25}H_{46}O_2$	378.3
25	(2S,3S,6S)-6-Isopropyl-3-methyl-2-(prop-1-en-2-yl)-3-vinylcyclohexanone	18.8	$C_{15}H_{24}O$	220.2

Table 8: Compounds Identified in BBMAV

S/N	Compound name	t_R	Formula	M/Z
1	3-Furanacetic acid, 4-hexyl-2,5-dihydro-2,5-dioxo-	8.4	$C_{12}H_{16}O_5$	240.1
2	trans-1,10-Dimethyl-trans-9-decalol	8.8	$C_{12}H_{22}O$	182.2
3	7-Methyl-oxa-cyclododeca-6,10-dien-2-one	11.2	$C_{12}H_{18}O_2$	194.1
4	1-Oxetan-2-one, 4,4-diethyl-3-methylene-	11.7	$C_8H_{12}O_2$	140.18
5	Ethyl trans-3-methyltetrazole-5-acrylate	12.4	$C_7H_{10}N_4O_2$	182.1
6	2(3H)-Benzofuranone, 6-ethenylhexahydro-3,6-dimethyl-7-(1-methylethenyl)-, [3S-(3.alpha.,3a.alpha.,6.alpha.,7.beta.,7a.beta.)]	14.6	$C_{15}H_{22}O_2$	234.2
7	3,5a,9,9-tetramethyldecahydrobenzo[2,3]cyclohepta[1,2-b]oxiren-3-ol	14.7	$C_{15}H_{26}O_2$	238.2
8	tricyclo[20.8.0.0e7,16]triacontan, 1(22),7(16)-diepoxy-	16.1	NA	NA
9	1-Methyl-3,6-diazahomoadamantan-9-ol	16.2	$C_{10}H_{18}N_2O$	182.26
10	Koiganal ii	16.6	$C_{18}H_{32}O$	264.446
11	14-.beta.-h-pregna	16.6		
12	4-(5,5-dimethyl-1-oxaspiro[2.5]oct-4-yl)-3-buten-2-one	17.9	$C_{13}H_{20}O_2$	208.1
13	(Z)-9-Octadecen-4-olide	18.5	$C_{18}H_{32}O_2$	280.2
14	Methyl 5,13-docosadienoate	19.1	$C_{23}H_{42}O_2$	350.3
15	Methyl 5,9-tetracosadienoate	19.5	$C_{25}H_{46}O_2$	378.3
16	Spiro[7h-cyclohepta[b]furan-7,2'(5'h)-furan]-2,5'(3h)-dione, octahydro-8-hydroxy-6,8-dimethyl-3-methylene-, [3as-(3a.alpha.,6.	20.7	NA	NA

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Even though majority of the compounds found in the GC-MS study offer little to no information, a select number have been found and studied by prior studies. Ojinnaka et al., 2015 carried out a study of the chemical constituents and bioactivity on the crude extract of the seeds of *Buchholzia coriacea* Engler (*Capparaceae)* an important traditional medicinal plant reported to possess therapeutic potentials. The seeds of the fruits of *Buchholzia coriacea* were extracted with petroleum ether, chloroform and methanol respectively and the crude extracts were analyzed using GC-MS. Among their findings were (Z,Z)-9,12-octadecadienoic acid, n-hexadecanoic acid, methyl ester (Z,Z)-9,12-octadecadienoic acid, 9-octadecanoic acid and methyl ester 3,5-dicyclohexyl-4-hydroxy-benzoic acid which is also present in the crude extracts of the fungal broth. The bioactivity test of their crude extracts showed great potential as antifungal agent than antibacterial agent.

In a study carried out by Obidi *et al*., (2013) on orange oil, cyclohexane; 2-methoxy-4-vinylphenol; 3-furanaceticacid, 4-hexyl-2,5-dihydro-2,5-dioxo; naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl- 1-(1-methylethyl) -, (1S-cis) and n-hexadecanoic acid were discovered to be part of the constituent of the orange oil extract using GC-MS analysis. These also were found in some of the crude extracts of the fungal broth. The bioassay of the orange oils indicates antimicrobial properties and may be applied in local therapies in the treatment of diseases caused by the microorganisms tested.

Geosmin (trans-1, 10-Dimethyl-trans-9-decalol) is an earthl y-smelling sesquiterpene produced by *Penicillium expansum*. It’s a microbial problem that seems to be common in damper vintages. It’s also encountered as a cork taint. It causes both red and white wines to smell of freshly turned earth, beetroot, and mustiness (Goode, 2018).

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Mattheis and Roberts, (1992) identified geosmin [1,10-trans-dimethyl-trans(9)-decalol] as a secondary metabolite from *Penicillium expansum* cultures using gas chromatography-mass spectrometry. Geosmin is also produced by algae (Safferman *et al*., 1967: Juttner & Watson, 2007), bacteria (Collins *et al*., 1970: Mattheis & Roberts, 1992: Yamada *et al*., 2015) and the fungus *Chaetomium globosum* Kunze:Fr (Kikuchi *et al*., 1981: Mattheis & Roberts, 1992: Effmert *et al*., 2012).

Palmitic acid, or hexadecanoic acid is the most common saturated fatty acid found in animals, plants and microorganisms (Gunstone & Harwood, 2007). Its chemical formula is $CH_3(CH_2)_{14}COOH$, and its C:D (the total number of carbon atoms to the number of carbon-carbon double-bonds) is 16:0. It is a major component of the oil from the fruit of oil palms (palm oil), making up to 44% of total fats. Meats, cheeses, butter, and other dairy products also contain palmitic acid, amounting to 50–60% of total fats (Carta *et al*., 2017). Palmitates are the salts and esters of palmitic acid. The palmitate anion is the observed form of palmitic acid at physiologic pH (7.4). Palmitic acid was discovered by Edmond Frémy in 1840, in saponified palm oil (Henderson & Osborne, 2000). This remains the primary industrial route for its production, with the triglycerides (fats) in palm oil being hydrolysed by high temperature water and the resulting mixture fractionally distilled (Anneken *et al*., 2000). Palmitic acid is produced by a wide range of other plants and organisms such as the endophytic fungi isolated from mangroves in this study. It is present in butter, cheese, milk, and meat, as well as cocoa butter, olive oil, soybean oil, and sunflower oil (Faci *et al*., 2021) Karukas contain 44.90% palmitic acid (Purwanto & Munawaroh, 2010). The cetyl ester of palmitic acid (cetyl palmitate) occurs in spermaceti.

Methyl Stearate is an ester used as a nonionic surfactant in various experiments in helping solubilize a variety of chemical species by dissociating aggregates and

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unfolding proteins (Saravanan *et al*., 2016). It is a fatty acid ester, which is used as an emulsifier and stabilizer (Hariani *et al*., 2016). Methyl Stearate is a natural product found in Neolitsea daibuensis (Wong *et al*., 2011), a white crystal or chunky solid and insoluble in water.

Ethyl linoleate is an ethyl ester of a long-chain fatty acid resulting from the formal condensation of the carboxyl group of linoleic acid with the hydroxyl group of ethanol (Sabri *et al*., 2020). It has a role as a plant metabolite and an anti-inflammatory agent (Skanda & Vijayakumar, 2021). Ethyl linoleate is derived from a linoleic acid and it is used as intermediate in vitamin industry (Coakley *et al*., 2003).

Vitamin E is a group of eight fat-soluble compounds that include four tocopherols and four tocotrienols (Niki & Abe, 2019). Vitamin E deficiency, which is rare and usually due to an underlying problem with digesting dietary fat rather than from a diet low in vitamin E, can cause nerve problems (Rafeeq *et al*., 2020). Vitamin E is a fat-soluble antioxidant which may help protect cell membranes from reactive oxygen species (Orucha *et al*., 2011). Yang *et al*., (2021), suggested that vitamin E may be used to treat Alzheimer's Disease and Peyronie's Disease.

Phenethyl alcohol, or 2-phenylethanol, is the organic compound that consists of a phenethyl group attached to OH (Zhu *et al*., 2011). It is a colourless liquid that is slightly soluble in water, but miscible with most organic solvents (Lee & Dordick, 2002). Voon *et al*., (2012), indicates that phenethyl alcohol occurs widely in nature, being found in a variety of essential oils. It has a pleasant floral odor. Phenethyl alcohol has a role as a fragrance, a Saccharomyces cerevisiae metabolite, a plant metabolite, an Aspergillus metabolite and a plant growth retardant (Chang *et al*., 2015). It is a primary alcohol and a member of benzenes. 2-phenylethanol also serves

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as an antimicrobial, antiseptic and disinfectant that is used also as an aromatic essence and preservative in pharmaceutics and perfumery (Kabara, 1997; Kerdudo, 2015).

Methyl benzoate is an organic compound. It is an ester which is a colourless liquid that is poorly soluble in water, but miscible with organic solvents (Santaladchaiyakit & Srijaranai, 2014). Methyl benzoate has a pleasant smell, strongly reminiscent of the fruit of the feijoa tree and it is used in perfumery (León *et al*., 2020). It also finds use as a solvent and as a pesticide used to attract insects such as orchid bees (Bunch *et al*., 2020).

Phenethyl acetate is the ester resulting from the condensation of acetic acid and phenethyl alcohol. Like many esters, it is found in a range of fruits and biological products. It is a colourless liquid with a rose and honey scent and a raspberry-like taste (Januszek *et al*., 2020).

Tetrahydrofurfuryl acetate is an organic chemical compound used in food flavouring and cosmetics (Ravit *et al*., 2019). It has a fruity ethereal flavour and also described as honey, maple, or bread-like (Baines, 2007). Tetrahydrofurfuryl is generally accepted as safe in the USA. Typical levels of use are 2 ppm in drinks, 8 ppm in ice cream, and 20 ppm in baked products and confectionery (Igoe, 2011).

Tyrosol acetate and hydroxytyrosol acetate have wide applications both as functional food components and as nutraceuticals (Guo *et al*., 2021). Tyrosol and hydroxytyrosol derived from virgin olive oil and olives extract, have wide applications both as functional food components and as nutraceuticals (Gambino *et al*., 2018).

Oleic acid is a fatty acid that occurs naturally in various animal and vegetable fats and oils. It is an odorless, colorless oil, although commercial samples may be yellowish

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(Maduabuchi & Awucha, 2020). In chemical terms, oleic acid is classified as a monounsaturated omega-9 fatty acid, abbreviated with a lipid number of 18:1 cis-9 (Chawla *et al*., 2020). The name is derived from the Latin word oleum, which means oil. It is the most common fatty acid in nature (Lobb & Chow, 2007). The salts and esters of oleic acid are called oleates (Cardoso *et al*., 2008).

Docosahexaenoic acid (all cis-4,7,10,13,16,19) methyl ester which is also a component of the crude extract of the mangrove fungal isolate is the methyl ester of one of the omega 3 fatty acids DHA, Docosahexaenoic acid (all cis-4,7,10,13,16,19). DHA is an essential fatty acid found in fish oil (Wijesundera *et al*., 2008). DHA and other essential fatty acids are necessary for synthesis of prostaglandins, which play roles in homeostatic functions and inflammatory responses. DHA methyl ester may be involved in the formation of protein adducts during aging and aging-related diseases (Biswas *et al*., 2013). Docosahexaenoic acid (DHA) is essential for the growth and functional development of the brain in infants (Gibson *et al*., 1996; McNamara & Carlson, 2006). According to Rapoport and Igarashi, (2009), DHA is also required for maintenance of normal brain function in adults. The inclusion of plentiful DHA in the diet improves learning ability, whereas deficiencies of DHA are associated with deficits in learning (He *et al*., 2009).

Squalene is an organic compound. With the formula $(C_5H_8)_6$, it is a triterpene. It is a colourless oil although impure samples appear yellow (Kathirvel, 2021). Squalene was originally obtained from shark liver oil. All plants and animals produce squalene as a biochemical intermediate (Rosales-García *et al*., 2017). According to Pham *et al*., (2015), an estimated 12% of bodily squalene in humans comes from the sebum. Squalene has a role in topical skin lubrication and protection.

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Dodecanol or lauryl alcohol, is an organic compound produced industrially from palm kernel oil or coconut oil. It is a fatty alcohol. Sulfate esters of lauryl alcohol, especially sodium lauryl sulfate, are very widely used as surfactants (Rosen & Kunjappu, 2012). Sodium lauryl sulfate, ammonium lauryl sulfate, and sodium laureth sulfate are all used in shampoos (Bondi *et al*., 2015). Lauryl alcohol is tasteless and colourless with a floral odour (Sharma *et al*., 2018).

Hydroxymethylfurfural (HMF), also 5-furfural, is an organic compound formed by the dehydration of reducing sugars. It is a white low-melting solid which is highly soluble in both water and organic solvents. The molecule consists of a furan ring, containing both aldehyde and alcohol functional groups. HMF can form in sugar-containing food, particularly as a result of heating or cooking. Its formation has been the topic of significant study as HMF was regarded as being potentially carcinogenic to humans. However, so far in vivo genotoxicity was negative. No relevance for humans concerning carcinogenic and genotoxic effects can be derived (Abraham *et al*., 2011). HMF is classified as a food improvement agent and is primarily being used in the food industry as a food additive, as a biomarker as well as a flavoring agent for food products (EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP),(Bampidis *et al*., 2020). It is also produced industrially on a modest scale as a carbon-neutral feedstock for the production of fuels and other chemicals (Kläusli, 2014).

2-Methoxy-4-vinylphenol is an aromatic substance used as a flavoring agent. It is one of the compounds responsible for the natural aroma of buckwheat (Oshomoh & Uzama-Avenbuan, 2020). Some insects such as *Rhynchophorus ferrugineus* use this substance for chemical signaling (Suma *et al*., 2014). Ferulic acid is converted by certain strains of yeast, notably strains used in brewing of wheat beers, such

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as *Torulaspora delbrueckii* to 2-methoxy-4-vinylphenol which gives beers such as Weissbier and Wit their distinctive "clove" flavor. *Saccharomyces cerevisiae* (brewer's yeast) and *Pseudomonas fluorescens* are also able to convert *trans*-ferulic acid into 2-methoxy-4-vinylphenol (Huang *et al*., 1993; Chang & Kang, 2004).

4.4 Determination of Antimicrobial Activities

The Minimum Bactericidal Concentration (MBC), Minimum Fungicidal Concentration (MFC) and Minimum Inhibition Concentration (MIC) analysis were carried out on the crude extracts of the fungal broth. In order to verify if the extract were able to kill the microbial cells (bacteri-/fungi-cidal effect) the plates were also evaluated for MBC and MFC. Briefly, aliquots from each well from susceptibility testing assays were transferred to plates containing Nutrient agar, which were incubated at 37°C for 24/48 h. Results were then evaluated by analyzing the presence or absence of growth in the Nutrient agar or SDA (Bagiu *et al*., 2012). The results of the antimicrobial activity of human pathogenic microbes are presented in **table 9** and that of cocoa infesting fungi in **table 10** below. The abbreviations used to represent the test organisms in the tables are as follows; EC- *E. coli* KP- *Klebsillia pneumonia* MRSA- *Methicilin resistant Staphyloccocus aureus* PA- *Pseudomonas aeruginosa* ST- *Samonella typhii* SA- *Staphylococcus aureus* SM- *Streptococcus mutans* CA- *Candida albicans*.

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Table 9: Results of MBC and MIC of crude extracts on human pathogenic microbes (mg/ml)

ORGANISMS	BWMS			RBMAV			BBMS			LBMS			BBMAV		
	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
EC	3.125	3.125	1.0^{bc}	12.5	12.5	1^{bc}	25	25	1^{bc}	50	50	1^{bc}	1.56	12.5	8.0^{bs}
KP	6.25	3.125	0.5^{bc}	12.5	12.5	1^{bc}	50	50	1^{bc}	50	50	1^{bc}	3.125	6.25	2.0^{bc}
MRSA	6.25	3.125	0.5^{bc}	12.5	12.5	1^{bc}	25	25	1^{bc}	50	50	1^{bc}	3.125	6.25	2.0^{bc}
PA	6.25	3.125	0.5^{bc}	12.5	12.5	1^{bc}	50	50	1^{bc}	50	50	1^{bc}	3.125	6.25	2.0^{bc}
ST	6.25	3.125	0.5^{bc}	12.5	12.5	1^{bc}	50	50	1^{bc}	50	50	1^{bc}	3.125	6.25	2.0^{bc}
SA	3.125	6.25	2.0^{bc}	6.25	12.5	2^{bc}	50	50	1^{bc}	50	50	1^{bc}	1.56	6.25	4.0^{bc}
SM	3.125	6.25	2.0^{bc}	6.25	12.5	2^{bc}	50	50	1^{bc}	50	50	1^{bc}	3.125	6.25	2.0^{bc}
CA	0.78	6.25	8.0^{fs}	6.25	25	4^{fc}	50	50	1^{fc}	50	50	1^{fc}	1.56	6.25	4.0^{fc}

The **MIC** is the lowest concentration of your drug that inhibits bacterial growth so you will have no turbidity in your culture media. But MBC is the lowest concentration that kills bacteria (Belanger & Hancock, 2021). Usually, the concentration which is considered as MBC is higher than the concentration for MIC (Owuama, 2017). Lower MIC and MBC values indicate higher efficacy (Marjorie,1999; Rajeh, *et al*., 2010). From the above table, BWMS recorded the least MIC of 0.78mg/mL against *Candida albicans*, 3.125mg/mL against *E. coli, Staphyloccocus aureus* and *Streptococcus mutans* and 6.25mg/mL against *Klebsillia pneumonia,* Methicilin resistant *Staphyloccocus aureus Pseudomonas aeruginosa* and *Samonella typhii*. The definition of a bactericidal agent is one with the ratio of MBC/MIC ≤ 4, while a bacteriostatic agent has an MBC/MIC ratio of > 4(Azman, *et al*., 2017). The data further confirms stronger bactericidal activity for BWMS against all the organisms

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except *Candida albicans* of which it is bacteriostatic. **Fig. 15** shows the Minimum Inhibition Concentrations of BWMS against the tested organisms.

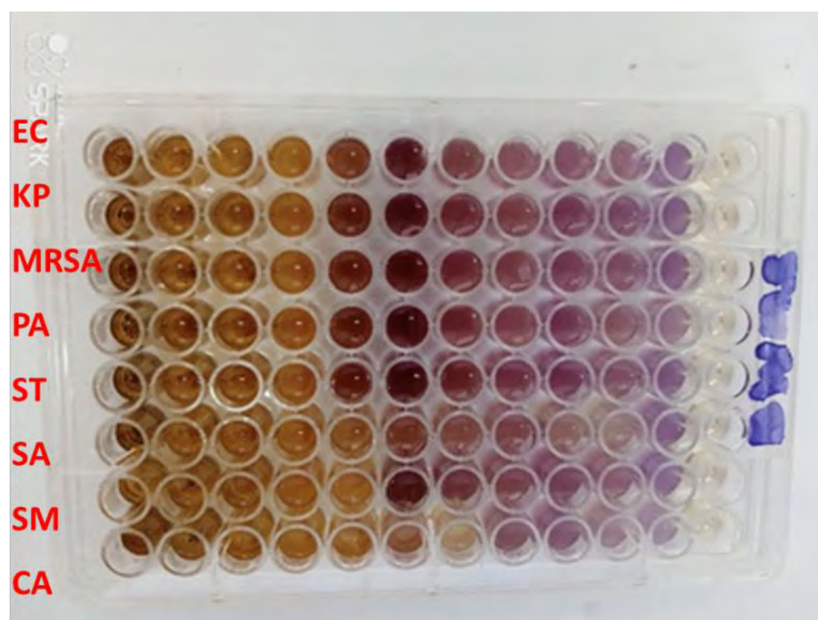

Figure 16: Minimum Inhibition Concentrations of BWMS against test organisms.

RBMAV recorded its least MIC of 6.25mg/mL against *Staphylococcus aureus, Streptococcus mutans* and *Candida albicans* and MIC of 12.5mg/mL against *E. coli, Klebsillia pneumonia,* Methicilin resistant *Staphyloccocus aureus, Pseudomonas aeruginosa* and *Samonella typhii.* However, it exhibited stronger bactericidal activity against all organism.

The MIC and MBC for BBMS are the same for each test organism hence has the same MBC/MIC ratio of 1 indicating a stronger bactericidal activity against all tested organism. It however recorded high MICs of 25mg/mL and 50mg/mL. The MIC is shown in **Fig. 16** below.

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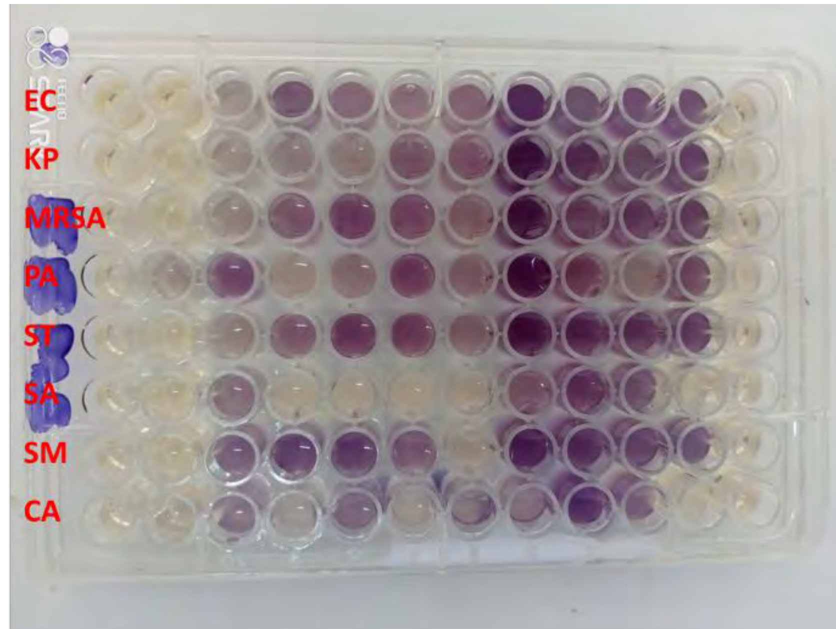

Figure 17: Minimum Inhibition Concentrations of BBMS against test organisms.

LBMS recorded MIC of 50mg/mL against all the test organisms and MBC/MIC ratio of 1. This indicates that the LBMS exhibited a strong bactericidal activity against the organisms at MIC of50mg/mL. BBMAV on the other hand exhibited a strong activity against the test organisms. It recorded a least MIC value of 1.56mg/mL against *E. coli, Staphylococcus aureus* and *Candida albicans* and 3.125mg/mL against *Klebsillia pneumonia,* Methicilin resistant *Staphyloccocus aureus, Samonella typhii* and *Streptococcus mutans*. BBMAV exhibited a stronger bactericidal activity against all tested organism except against *E. coli* of which it is bacteriostatic. The MIC of BBMAV exhibited against the test organisms is shown in **Fig. 18**.

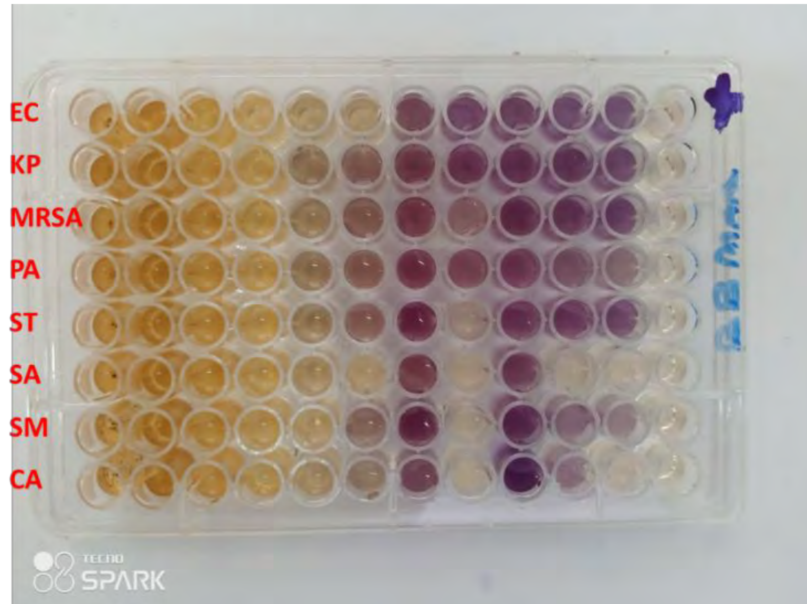

Figure 18: Minimum Inhibition Concentrations of BBMAV against test organisms

Comparing the potencies of the extracts, from the MIC values, it can be deduced that BBMAV has the highest potency with MIC values ranging from 1.56mg/mL to 3.125mg/mL followed by BWMS with MIC values ranging from 0.78mg/mL to 6.25mg/mL. RBMAV followed closely with MIC values ranging from 6.25mg/mL to 12.5mg/mL. BBMS is next with MIC range 25mg/mL to 50mg/mL and LBMS exhibited the least potency with MICs values ranging from 50mg/mL to 100mg/mL.

Black pod disease is a protozoal disease of Cocoa trees (Okeoghene, 2020). This pathogen if left untreated can lower the yields of cocoa trees. Annually, the pathogen can cause a yield loss of up to 1/3 and up to 10% of total trees can be lost completely (Akrofi, et al., 2015). Considerable research have been carried out in recent times aimed at control and the management of *Phytophthora sp*. pathogens. There are several methods available in order to control black pod disease such as cultural, chemical and biological control (Acebo-Guerrero *et al*., 2012).

A study by Opoku *et al*., (2007) that showed a significant reduction in the percentage of the incidence of disease when combined the sanitation and fungicide applied to the pods from the trunk and from the canopy of cocoa in Ghana. This suggests that the application of fungicide on the trunk of cocoa plants could protect the pods from infection and therefore reduce primary and secondary infection both on the trunk and in the canopy (Opoku *et al*., 2007). In addition, the application of 20 ml and 40 ml of fungicide systemic, potassium phosphonate at each injection stage and semi-systemic (metalaxyl) fungicide showed better control compared to copper-based contact fungicides in both locations that were used in the experiment (Guest, *et al*., 1994: Guest, 2007). These copper-based fungicides are not eco-friendly and therefore the need to explore more eco-friendly means to fight these pathogens. The extracts from the mangrove fungi were tested against the *Phytophthora sp.* and the results are presented in **table 10** below.

Table 10: Results of MFC and MIC of crude extracts on Cocoa infesting fungi (mg/ml)

Organism	***Phytophthora palmivora***			***Phytophthora megakarya***		
SAMPLES	**MIC(mg/mL)**	**MFC(mg/mL)**	**MFC/MIC**	**MIC(mg/mL)**	**MFC(mg/mL)**	**MFC/MIC**
BWMS	0.625	12.5	20^{fs}	2.5	2.5	1^{fc}
BBMS	12.5	12.5	1^{fc}	12.5	12.5	1^{fc}
BBMAV	3.125	6.25	2^{fc}	12.5	50	4^{fc}
RBMAV	3.125	3.125	1^{fc}	12.5	25	2^{fc}
LBMS	6.25	12.5	2^{fc}	12.5	12.5	1^{fc}

A lower value of MIC and MFC represents a higher fungal inhibitory activity and fungicidal potential, respectively. Antimicrobial activity is usually regarded as fungicidal if the MFC/MIC ratio is <4 and fungistatic if the ratio is >4 (Okonogi *et al*., 2021). From the data above it could be observed that even though BWMS exhibited a very high activity against *Phytophthora palmivora* with MIC of

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0.625mg/mL but it is fungistatic with MFC/MIC ratio of 20. It was however very active and fungicidal with MIC and MFC/MIC values of 2.5mg/mL and 1 respectively against *Phytophthora megakarya*. BBMS exhibited an activity against *Phytophthora palmivora and Phytophthora megakarya* with MIC and MFC/MIC values of 12.5mg/mL and 1 respectively. BBMAV also recorded MIC and MFC/MIC values of 3.125mg/mL and 2 respectively indicating a high fungicidal activity against *Phytophthora palmivora*. It however showed a comparatively lower activity against *Phytophthora megakarya* with MIC and MFC/MIC values of 12.5mg/mL and 4 respectively.

RBMAV exhibited strong fungicidal activity against *Phytophthora palmivora* with MIC and MFC/MIC values of 3.125mg/mL and 1 respectively and a comparatively lower activity against *Phytophthora megakarya* with MIC and MFC/MIC values of 12.5mg/mL and 2 respectively. LBMS also exhibited a strong fungicidal activity against *Phytophthora palmivora* with MIC and MFC/MIC values of 6.25mg/mL and 2 respectively and a comparatively lower activity against *Phytophthora megakarya* with MIC and MFC/MIC values of 12.5mg/mL and 1 respectively. All the fungal extracts of mangrove are potent against *Phytophthora palmivora and Phytophthora megakarya*.

The MIC of the extracts against *Phytophthora palmivora and Phytophthora megakarya* is shown in **Fig. 19** and **Fig. 20** respectively below.

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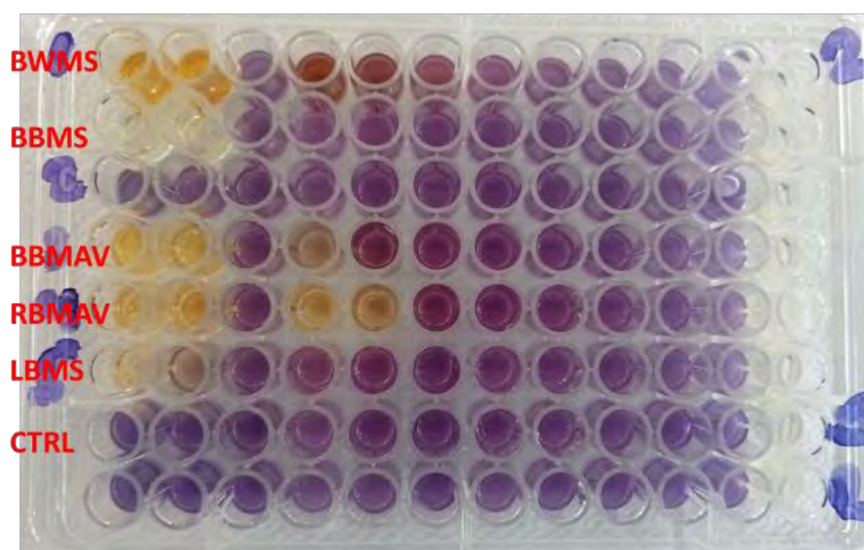

Figure 19: Minimum Inhibition Concentrations of extracts against Phytophthora palmivora

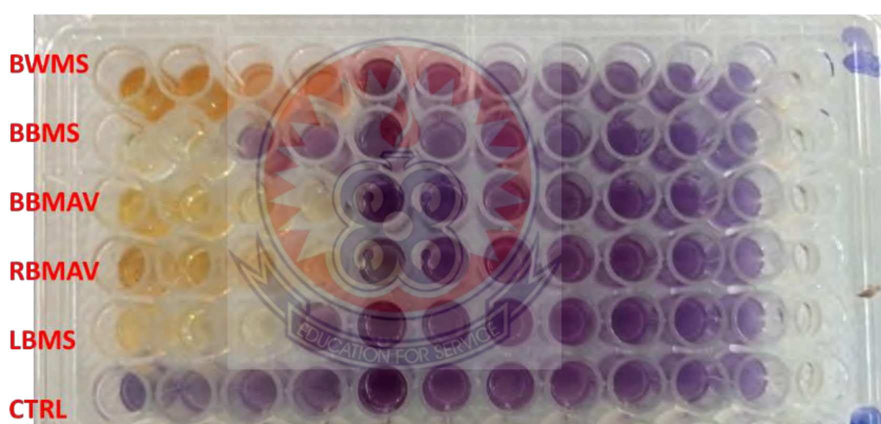

Figure 20: Minimum Inhibition Concentrations of extracts against Phytophthora megakarya

4.5 Determination of Free Radical Scavenging Activities (antioxidant activities)

The antioxidant activities of the extracts from each mangrove fungal broth were tested against DPPH and ABTS by measuring their scavenging potentials for the extracts. The ABTS and DPPH scavenging activities for natural products are widely used methods for the determination of the antioxidant properties of naturally occurring compounds. The ABTS and DPPH scavenging activity of each extract was determined by measuring the absorbance of the concentrations of samples ranging

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from 1, 0.5, 0.25, 0.125, 0.0625, 0.0313, 0.0156 and 0.0078 mg/mL using ascorbic acid as the reference compound. The higher the percentage of scavenging activities of the extracts, the greater the antioxidant potentials (Wu & Ng, 2008). The IC_{50} value was calculated to determine the concentration of the sample required to inhibit 50% of radical. The lower the IC_{50} value, the higher the antioxidant activity of samples (Li *et al*., 2009; Jadid *et al*., 2017). According to Phongpaichit, (2007) as cited by Jadid *et al*., (2017), extracts which possess IC_{50} values ranging from 50 to 100 mg / mL is considered to exhibit intermediate antioxidant activity.

The percentage scavenging activities and the IC_{50} of the extracts at the various concentrations are presented in **tables 11 to 15**

Table 11: Results of Antioxidant Activity of BWMS.

CONC. (mg/mL)	**ABTS activity [IC_{50} = 0.887035±0.00 mg/mL]**			**DPPH activity [IC_{50} = 7.667602±0.00 mg/mL]**		
	EXP1	**EXP2**	**MEAN±SD**	**EXP1**	**EXP2**	**MEAN±SD**
1	66.36	57.01	61.68±3.30	100.00	100.00	100.00±0.00
0.5	51.40	42.06	46.73±3.30	83.04	84.52	83.78±1.05
0.25	34.58	24.30	29.44±3.63	80.51	82.14	81.32±1.16
0.125	1.87	1.40	1.64±0.17	66.07	69.05	67.56±2.10
0.0625	1.40	0.47	0.93±0.33	65.48	68.60	67.04±2.21
0.0313	0.47	0.47	0.47±0.00	65.18	68.60	66.89±2.42
0.0156	0.00	0.00	0.00±0.00	52.38	52.38	52.38±0.00
0.0078	0.00	0.00	0.00±0.00	47.62	50.60	49.11±2.10

BWMS recorded a potent free radical scavenging activity of 61.68% only at a concentration of 1.00 mg/mL against ABTS but 52% to 100% for concentrations

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ranging from 0.0156 to 1.00 mg/mL against DPPH. The IC_{50} of this extract against ABTS and DPPH were 0.887035mg/mL and 7.667602mg/mL respectively.

Table 12: Results of Antioxidant Activity of BBMS

CONC. (mg/mL)	**ABTS activity [IC_{50}=4.919663±0.00 mg/mL]**			**DPPH activity [IC_{50} = 8.120576±0.00 mg/mL]**		
	EXP1	**EXP2**	**MEAN±SD**	**EXP1**	**EXP2**	**MEAN±SD**
1	66.36	71.03	68.69±3.30	100.00	100.00	100.00±0.00
0.5	65.42	70.09	67.76±3.30	95.09	98.07	96.58±2.10
0.25	64.49	67.76	66.12±2.31	94.35	97.62	95.98±2.31
0.125	63.08	63.08	63.08±0.00	82.59	84.23	83.41±1.16
0.0625	62.62	62.62	62.62±0.00	74.55	77.68	76.12±2.21
0.0313	40.19	40.19	40.19±0.00	73.36	76.34	74.85±2.10
0.0156	31.78	31.78	31.78±0.00	49.55	52.68	51.12±2.21
0.0078	22.90	22.90	22.90±0.00	47.02	50.30	48.66±2.31

The data above revealed that BBMS exhibited potent antioxidant activity of 62.62% to 68.69% at concentrations of 0.0625 to 1.00mg/mL and IC_{50} of 4.919663 mg/mL against ABTS while it recorded percent scavenging activities of 76.12% to 100% at same concentrations DPPH. This implies that the extracts of BBMS is more active against DPPH than ABTS.

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Table 13: Results of Antioxidant Activity of BBMAV

CONC. (mg/mL)	ABTS activity [IC_{50} =5.01697±0.00 mg/mL]			DPPH activity [IC_{50} = 8.542313±0.00 mg/mL]		
	EXP1	EXP2	MEAN±SD	EXP1	EXP2	MEAN±SD
1	86.92	81.78	84.35±3.63	79.76	82.74	81.25±2.10
0.5	85.05	80.37	82.71±3.30	72.77	75.89	74.33±2.21
0.25	76.17	71.03	73.60±3.63	69.35	72.32	70.83±2.10
0.125	72.43	67.76	70.09±3.30	63.10	66.07	64.58±2.10
0.0625	72.90	62.15	67.52±7.60	57.44	60.42	58.93±2.10
0.0313	29.44	29.44	29.44±0.00	57.14	60.42	58.78±2.31
0.0156	19.16	19.16	19.16±0.00	56.99	60.27	58.63±2.31
0.0078	18.22	18.22	18.22±0.00	52.83	54.32	53.57±1.05

BBMAV extract exhibited high antioxidant activity of 67.52% to 84.35 at concentrations 0.0625 to 1.00mg/mL against ABTS and 58.93% to 81.25% at same concentrations against DPPH. This indicates that, BBMAV is slightly more active against ABTS than DPPH. However, it showed higher activity against DPPH up to a concentration as low as 0.0078 mg/mL.

Table 14: Results of Antioxidant Activity of LBMS

CONC. (mg/mL)	ABTS activity [IC_{50}=6.468459±0.00 mg/mL]			DPPH activity [IC_{50} = 9.008416±0.00 mg/mL]		
	EXP1	EXP2	MEAN±SD	EXP1	EXP2	MEAN±SD
1	93.46	87.85	90.65±3.97	100.00	100.00	100.00±0.00
0.5	92.06	85.05	88.55±4.96	100.00	100.00	100.00±0.00
0.25	86.92	80.37	83.64±4.63	100.00	100.00	100.00±0.00
0.125	86.92	79.91	83.41±4.96	100.00	100.00	100.00±0.00
0.0625	82.71	65.42	74.07±12.23	70.24	67.26	68.75±2.10
0.0313	82.24	62.62	72.43±13.88	66.37	63.69	65.03±1.89
0.0156	53.74	43.93	48.83±6.94	63.84	66.52	65.18±1.89
0.0078	14.95	4.67	9.81±7.27	60.12	57.14	58.63±2.10

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LBMS recorded high antioxidant activity of 72.43% to 90.65% at concentrations 0.0313 to 1.00mg/mL against ABTS and 68.75% to 100% at same concentrations against DPPH indicating that it has a comparatively higher activity against DPPH than ABTS. LBMS exhibited a 100% scavenging activity at concentration as low as 0.125 mg/mL against DPPH and showed high activity at 0.0078mg/mL with percentage scavenging activity of 58.63%.

Table 15: Results of Antioxidant Activity of RBMAV

CONC. (mg/mL)	**ABTS activity [IC_{50}=3.169513±0.00 mg/mL]**			**DPPH activity [IC_{50} = 12.78498±0.00 mg/mL]**		
	EXP1	**EXP2**	**MEAN±SD**	**EXP1**	**EXP2**	**MEAN±SD**
1	70.56	62.15	66.36±5.95	100.00	100.00	100.00±0.00
0.5	66.36	57.94	62.15±5.95	100.00	100.00	100.00±0.00
0.25	49.07	49.07	49.07±0.00	100.00	100.00	100.00±0.00
0.125	47.66	48.60	48.13±0.66	100.00	100.00	100.00±0.00
0.0625	47.20	47.66	47.43±0.33	93.01	89.88	91.44±2.21
0.0313	20.56	3.27	11.92±12.23	77.83	74.70	76.26±2.21
0.0156	10.28	1.40	5.84±6.28	77.53	74.40	75.97±2.21
0.0078	5.61	0.93	3.27±3.30	74.26	68.75	71.50±3.89

RBMAV exhibited high antioxidant activity of 62.15% to 66.65% only at concentrations of 0.5 to 1.00mg/mL against ABTS but 68.75% to 100% at concentrations of 0.0078 to 1.00mg/mL against DPPH indicating that it has comparatively higher activity against DPPH than ABTS. It exhibited a 100% scavenging activity at concentration as low as 0.125 mg/mL against DPPH and showed some activity at 0.0078mg/mL with percentage scavenging activity of 58.63%.

Generally, the extracts from the mangrove fungi showed comparatively higher free radical scavenging activity against DPPH than ABTS free radicals.

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CHAPTER FIVE

SUMMARY, CONCLUSIONS, RECOMMENDATIONS AND SUGGESTIONS FOR FURTHER RESEARCH

5.0 Overview

This chapter presents a summary of the findings and conclusion of the study. It also includes contributions of the study, recommendations and finally suggestions for further research.

5.1 Summary of Major Findings

Mangrove plants and their associated micro fauna have been a rich source of bioactive molecules, though only limited antimicrobial screening of this chemo-diversity source has been reported. (Calcul *et al*., 2013). The unique adaptation of mangroves towards their extreme environmental conditions of high salinity, changes in sea level, high temperatures and anaerobic soils is largely aided by endophytic fungi (Pramanik *et al*. 2019).

In this study, the leaves, barks and roots of red, black and white mangroves found along the Ayensu estuary and the Muni lagoon in Winneba were screened for their endophytic fungi in a malt-glucose culture media. Five fungi (BBMAV, BWMS, LBMS, RBMAV and BBMS) were isolated and cultured in malt-glucose media for eight weeks for their secondary metabolites. BBMAV and LBMS isolates were observed growing on the surface of the media while BWMS, RBMAV and BBMS grew beneath the surface of the media. LBMS isolate has comparatively highest growth rate and BWMS had the least.

The TLC studies and GC-MS analysis of the crude extracts from the culture media of the fungal isolates revealed that the fungi produced several secondary metabolites.

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The GC-MS results identified 15, 31, 37, 25 and 16 compounds in the crude extracts of RBMAV, LBMS, BWMS, BBMS and BBMAV respectively. Literature reviewed on compounds identified to be present in the crude extracts revealed that most of the compounds have very high antimicrobial and antioxidant properties and are therefore used in pharmaceutical and cosmetic industries.

A bioassay of the crude extracts was carried out on both human pathogenic microbes [(Methicillin resistant *Staphylococcus aureus* (NCTC 12493), *Staphylococcus aureus* (NCTC 12973), *E. coli* (NCTC 12241), *S. mutants* (ATCC 700610), *P. aeroginosa* (ATCC 4853), *S. typhi* (ATCC 14028), *K. pneumonia (NCTC 13440)* and *Candida albicans* (ATCC 90028)] and two fungi that infest cocoa (*Phytophthora palmivora and Phytophthora megakarya*). The bioassay results indicated that the crude extracts were active against all the test organisms at different concentrations.

ABTS and DDPH free radicals scavenging activities were also tested on the crude extracts. The results indicate that the crude extracts of all the fungal isolates exhibited high antioxidant activity.

5.2 Conclusions

Based on the findings of this study, it can be concluded that the endophytic fungi are present in the leaves, bark and roots of mangroves along the Muni lagoon and the Ayensu estuary in Winneba. These mangrove endophytic fungi are able to produce secondary metabolites in malt-glucose static culture media. The secondary metabolites produced by the mangrove fungal isolates have high antimicrobial activities against both human pathogenic microbes Methicillin resistant *Staphylococcus aureus* (NCTC 12493), *Staphylococcus aureus* (NCTC 12973), *E. coli* (NCTC 12241), *S. mutants* (ATCC 700610), *P. aeroginosa* (ATCC 4853), *S.*

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typhi (ATCC 14028), *K. pneumonia (NCTC 13440)* and *Candida albicans* (ATCC 90028) and two fungi that infest cocoa.

It can also be concluded from this study that crude extracts of the mangrove endophytic fungal isolates have high antioxidant activities and can be exploited in the cosmetic and pharmaceutical industries as well as fungicide in the fight against cocoa pod disease.

5.3 Recommendations

Research that is time bound is definitely limited to selective explorations. This study was limited by time and therefore recommends that further studies be carried out on the biomass of the mycelia of the fungal isolates since they are also potential sources bioactive and antioxidant compounds. The aqueous portions of the culture broth obtained from the solvent extraction could be studied since there is the possibility of some secondary metabolites that are more soluble and may remain in the aqueous medium.

Pharmaceutical and cosmetics industries as well as cocoa fungicide manufacturers could explore the crude extracts of the fungal isolates as a potential source of active ingredients for their products.

There are several challenges encounted in this study in terms of running instrumental analysis of samples, carrying out bioassay and antioxidant activity tests and disruption of research setups due to the absence of a graduate research laboratory in the university. It is therefore recommended that the university as matter of urgency should establish a well-resourced graduate research laboratory of international standard to facilitate effective and quality research. The university should in the immediate term consider signing a memorandum of understanding (MOU) with some

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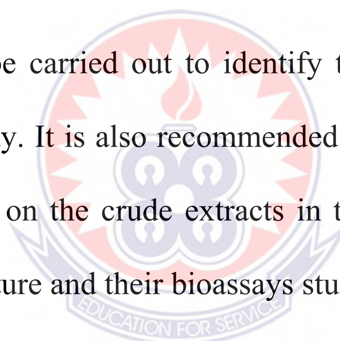
research institutions and universities in the country to facilitate research work by students and faculty members.

5.4 Suggestions for Further Studies

There is a need for a study to be conducted on the same mangrove endophytic fungi isolated with different culture media like potato dextrose broth (PDB) other than the malt-glucose broth to find out if similar or even more active secondary metabolites could be produced. Also, potato dextrose agar or malt extract glucose agar should be used in the isolation of the endophytic fungi from the mangrove plant parts to make the isolation easier. There are mangrove plants located in other parts of the country which should be explored for endophytic fungi and their secondary metabolites.

A further study should be carried out to identify the various mangrove endophytic fungi isolated in this study. It is also recommended that column chromatography and trituration be carried out on the crude extracts in the bid to isolating the individual compounds from the mixture and their bioassays studied as well.



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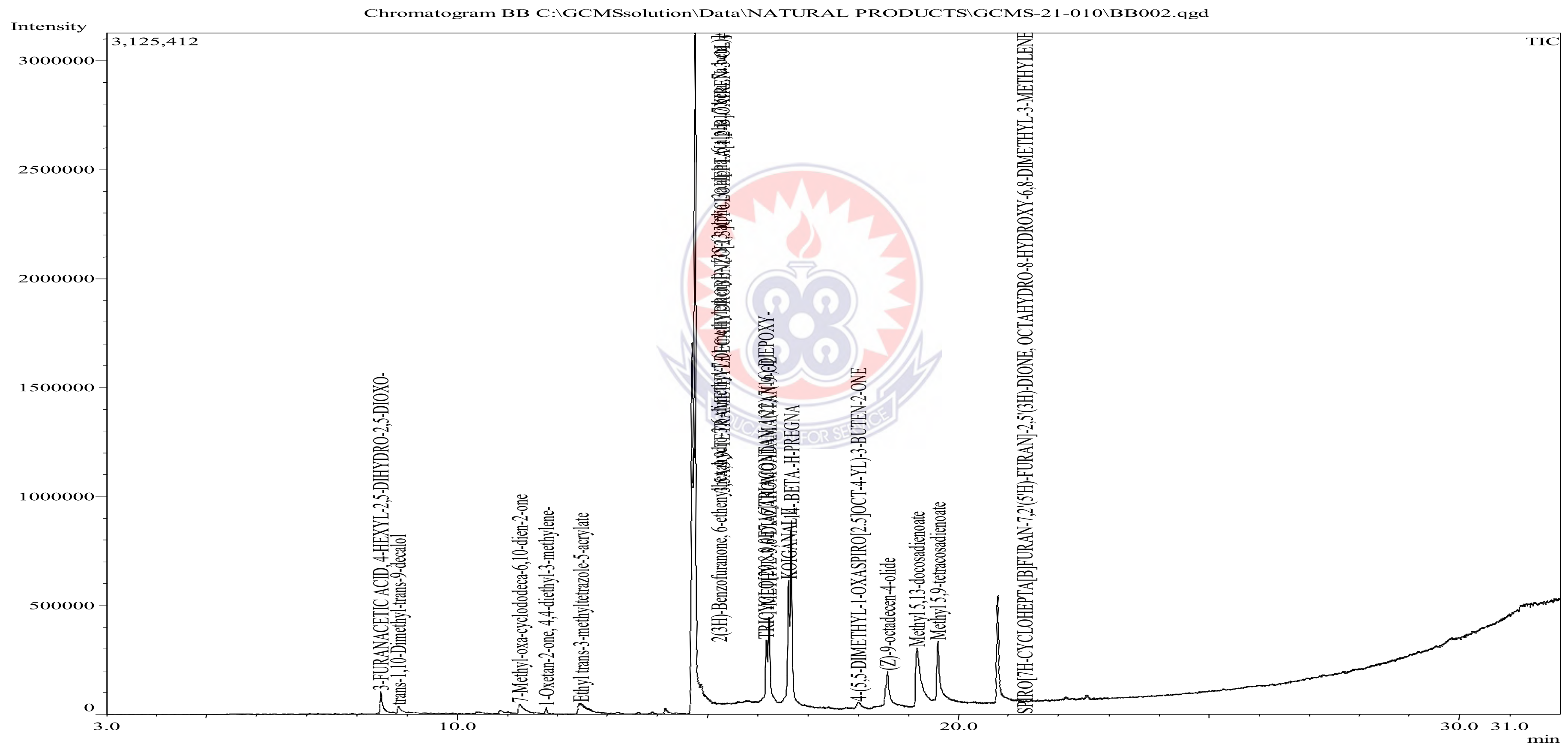
APPENDICES

Appendix A: GC-MS Chromatogram of Crude Extracts



GC-MS Chromatogram of BBMAV

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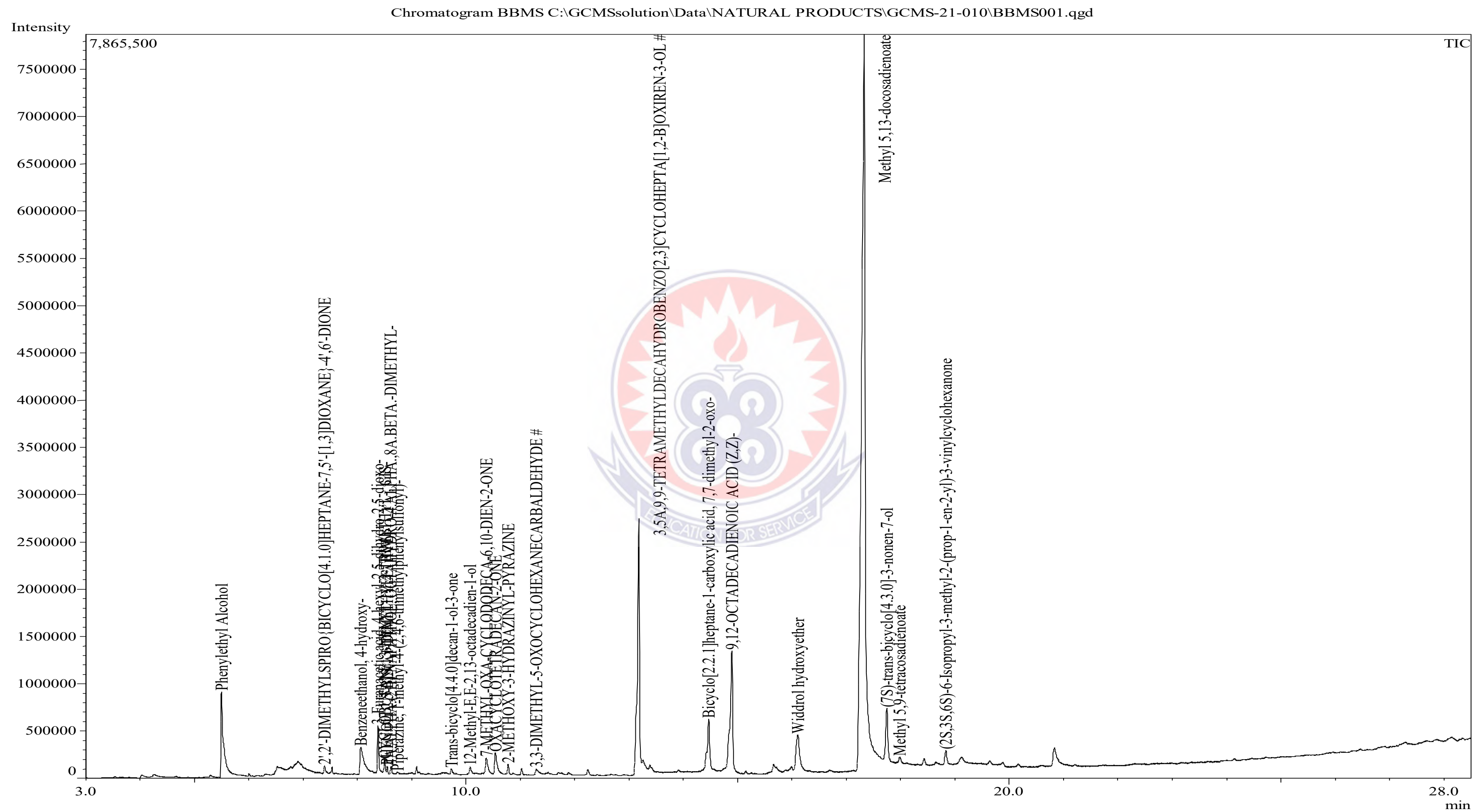
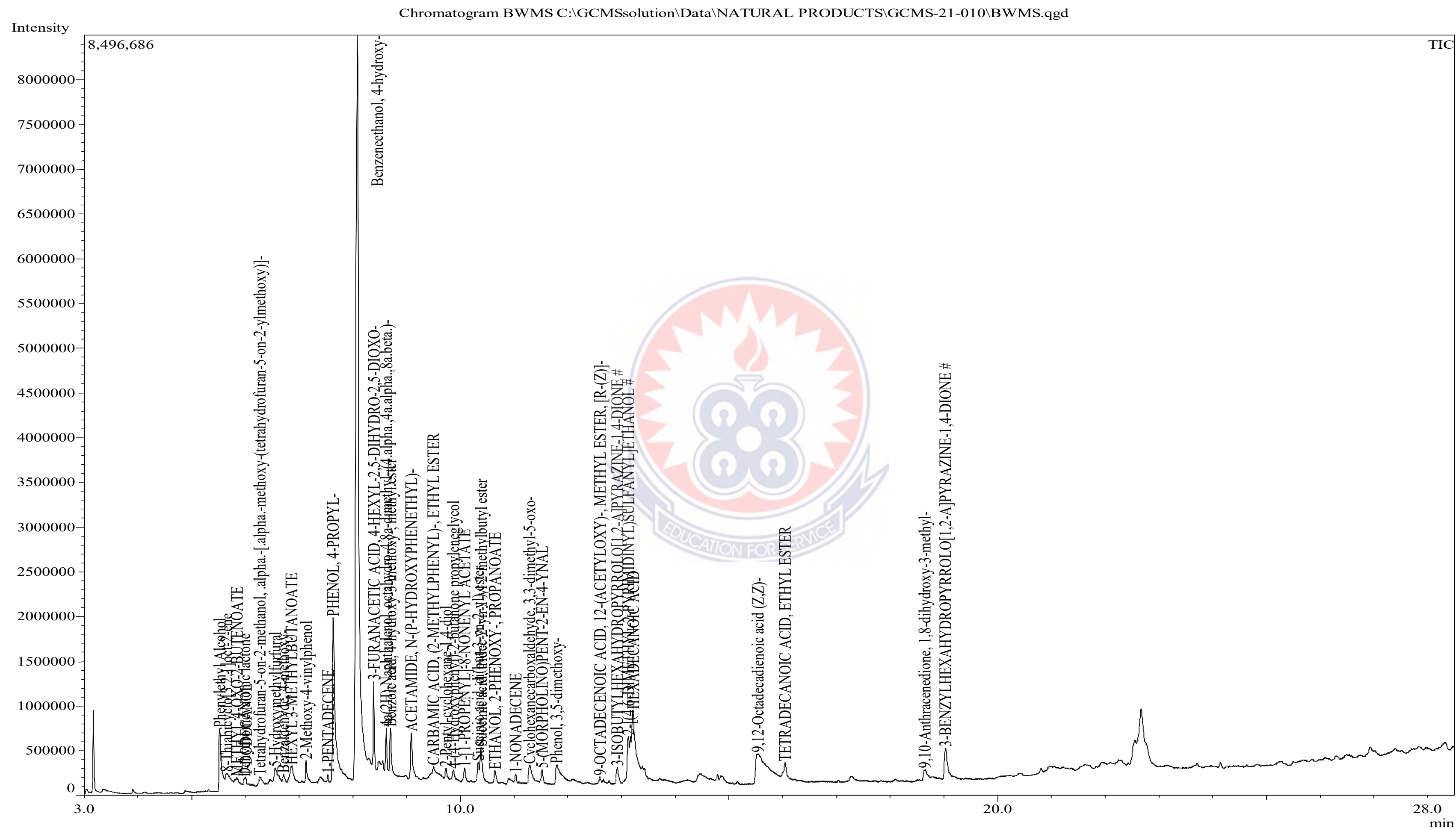


GC-MS Chromatogram of BBMS

University of Education,Winneba http://ir.uew.edu.gh



GC-MS Chromatogram of BWMS

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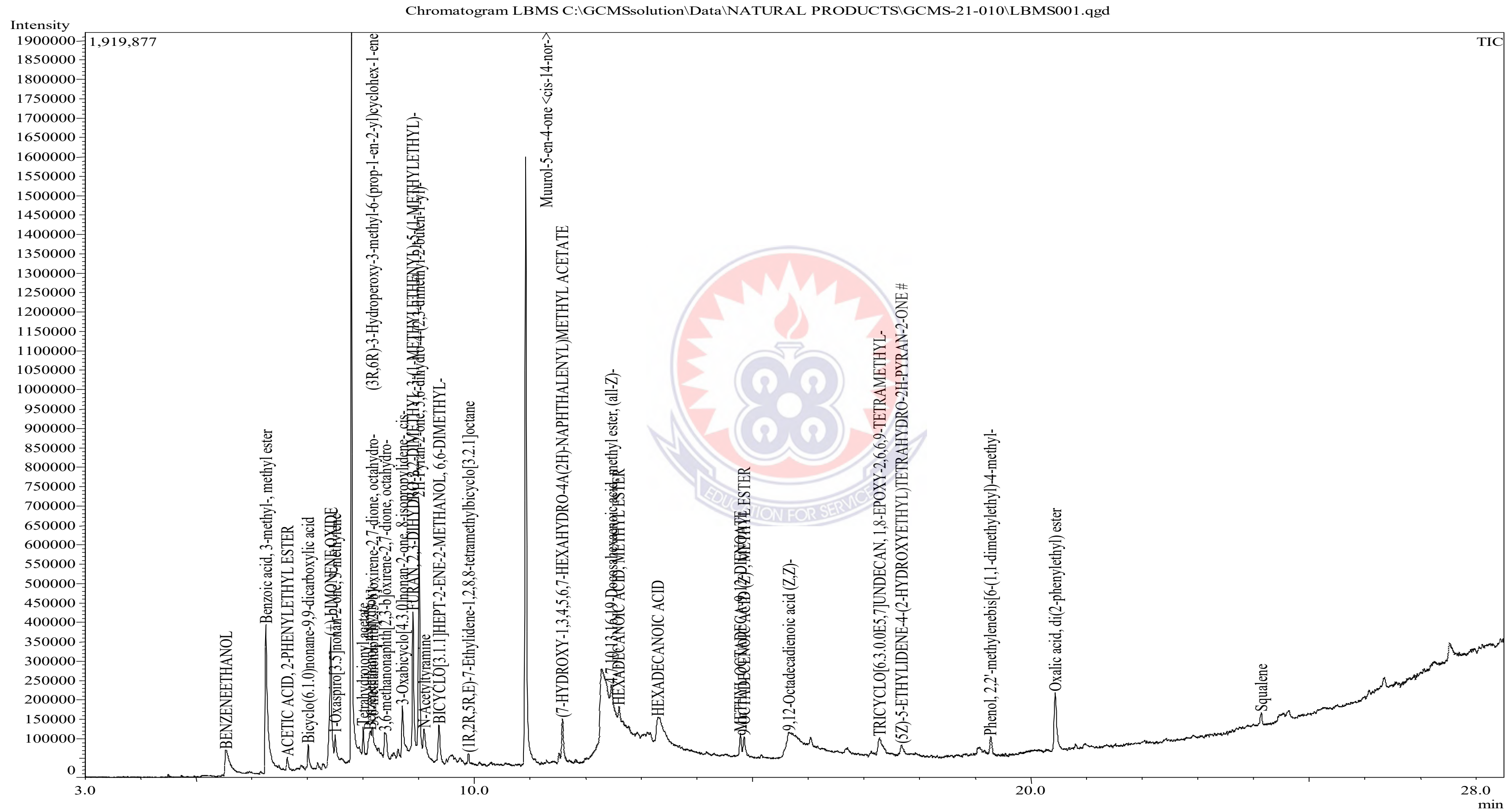

GC-MS Chromatogram of LBMS

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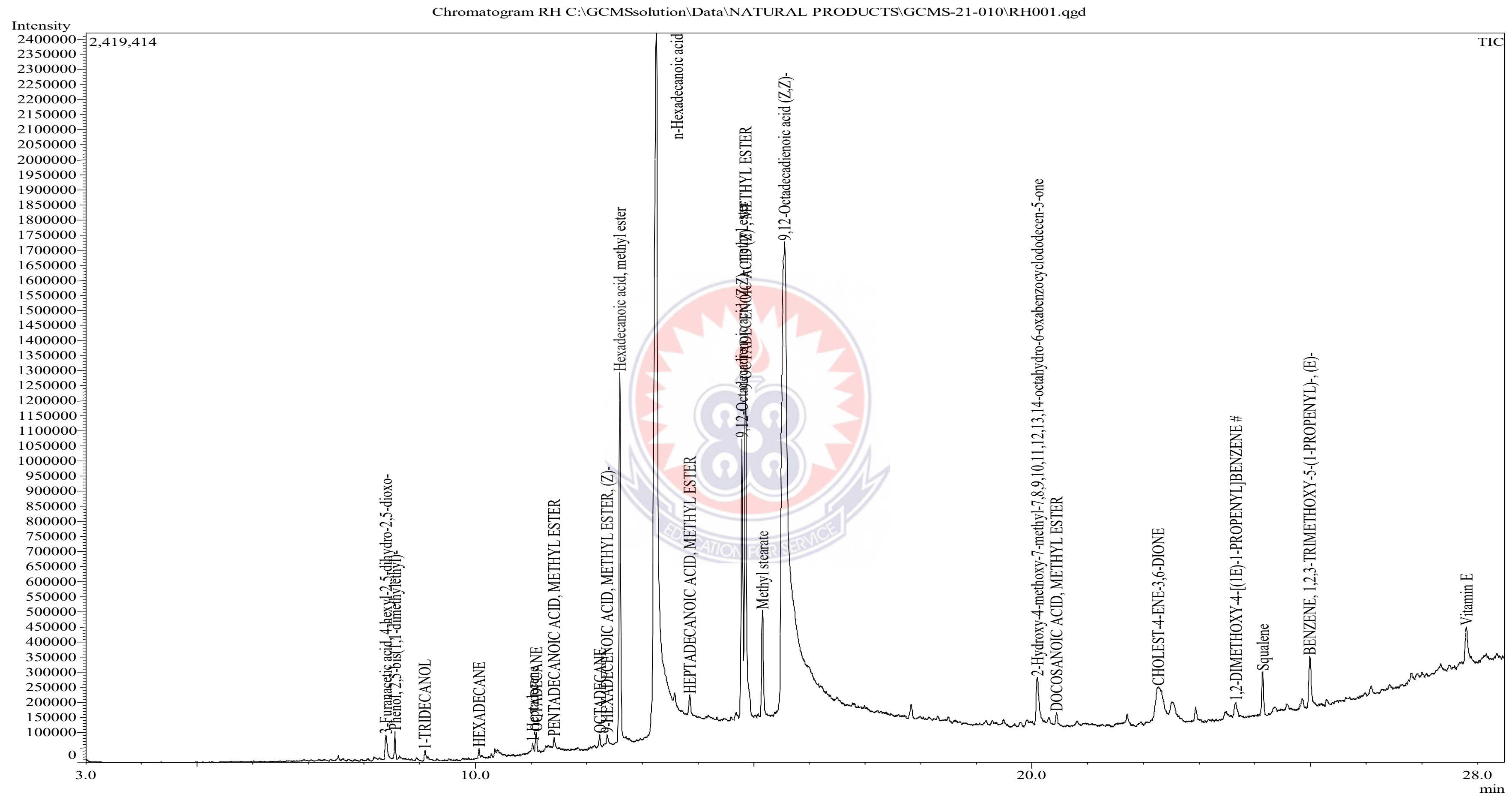

GC-MS Chromatogram of RBMAV

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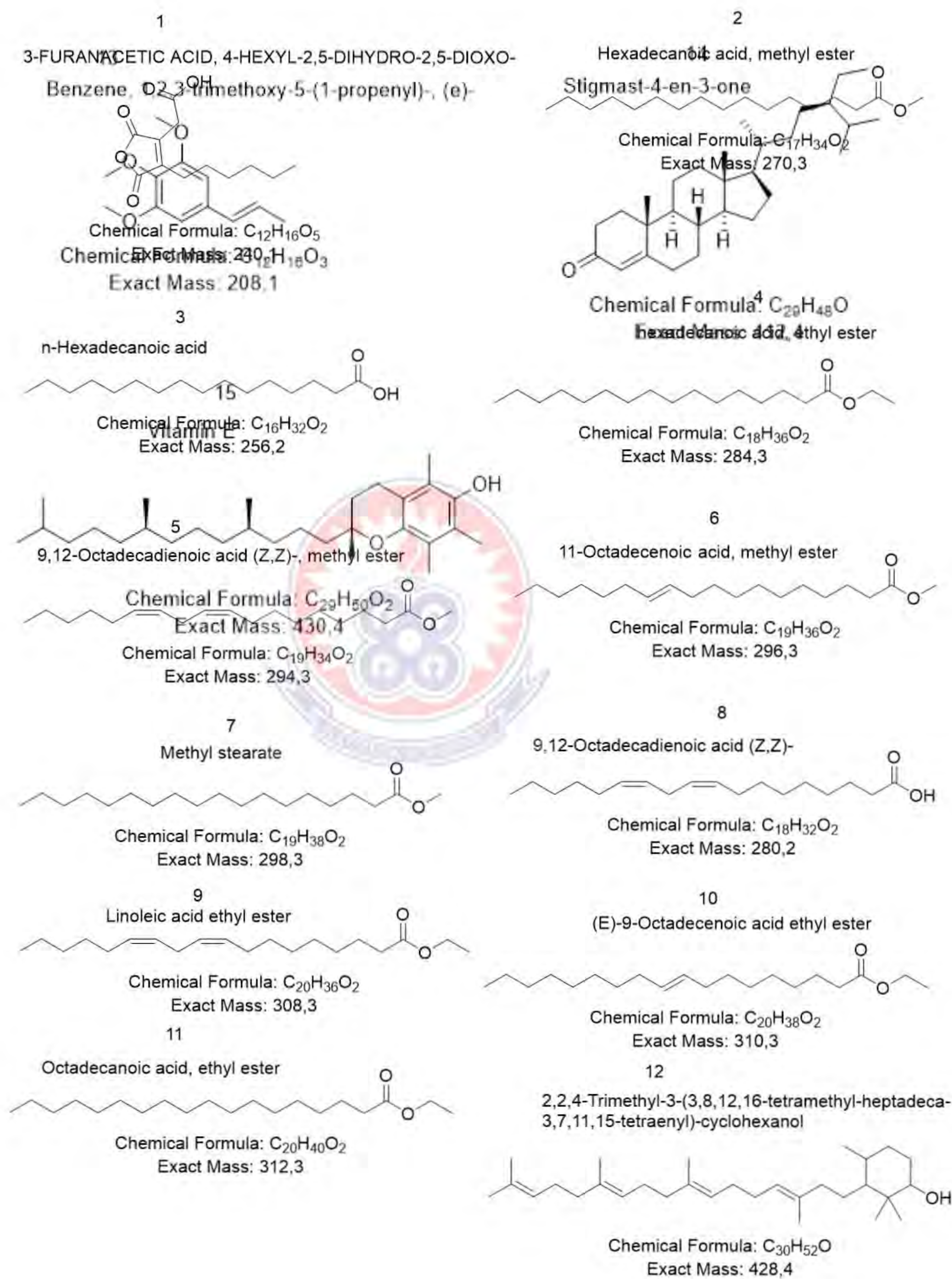
APPENDIX B

CHEMDRAW Structure of Compounds

Structures of compounds Identified in RBMAV



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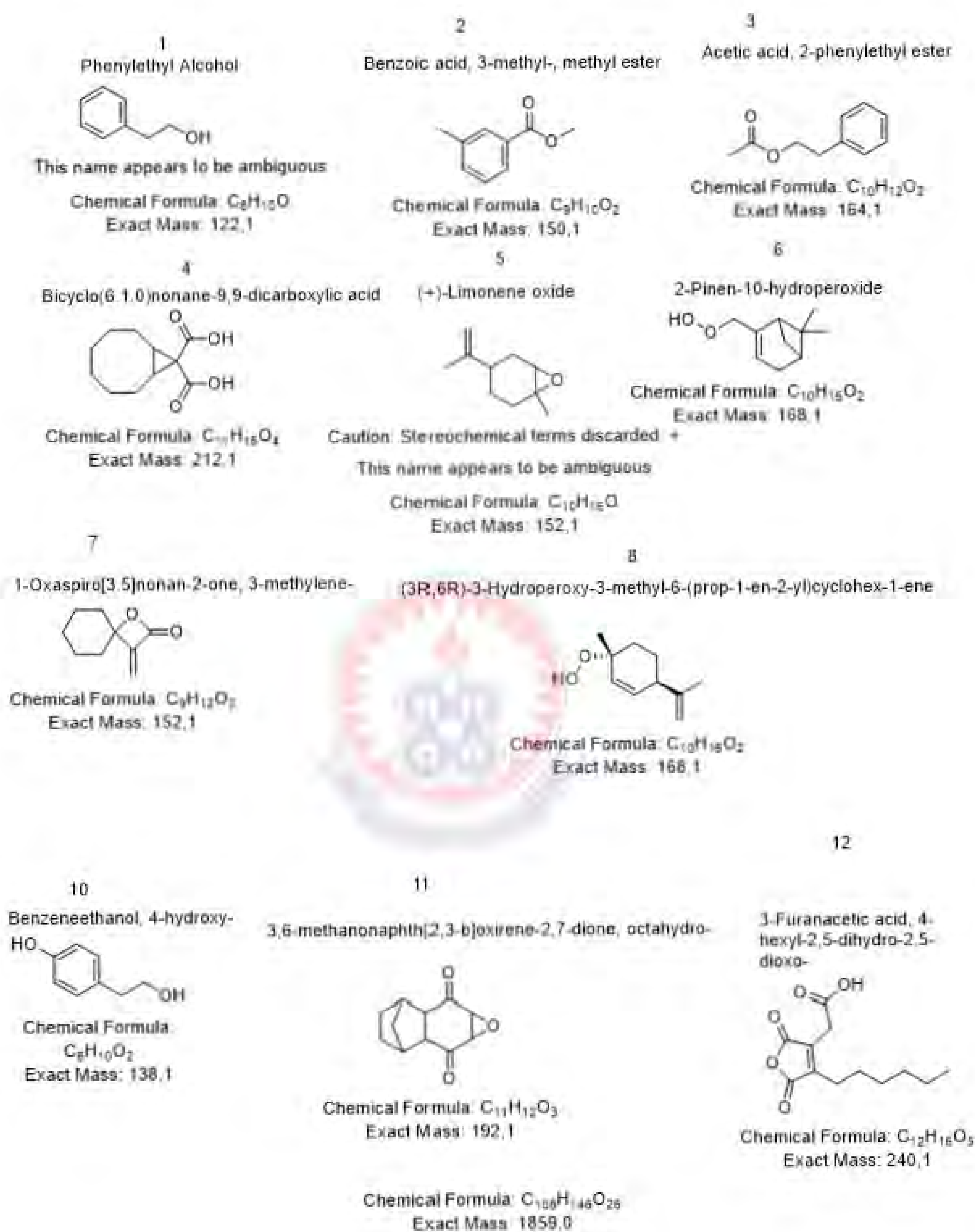
Structures of compounds identified in LBMS



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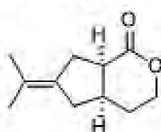
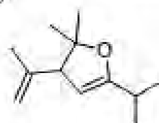
13
3-Oxabicyclo[4.3.0]nonan-2-one, 8-isopropylidene-, cis-



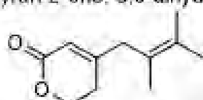
Chemical Formula: $C_{11}H_{16}O_2$
Exact Mass: 180,1

14
Furan, 2,3-dihydro-2,2-dimethyl-3-(1-methylethenyl)-5-(1-methylethyl)-



Chemical Formula: $C_{12}H_{20}O$
Exact Mass: 180,2

15
2H-Pyran-2-one, 5,6-dihydro-4-(2,3-dimethyl-2-buten-1-yl)-



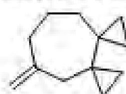
Chemical Formula: $C_{11}H_{16}O_2$
Exact Mass: 180,1

16
Tyrosol, acetate

This name appears to be ambiguous

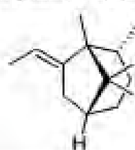
Chemical Formula: $C_{11}H_{15}NO_3$
Exact Mass: 209,1

17
Dispiro[2.0.2.5]undecane, 8-methylene-



Chemical Formula:
$C_{12}H_{18}$
Exact Mass: 162,1

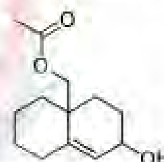
18
(1R,2R,5R,E)-7-Ethylidene-1,2,8,8-tetramethylbicyclo[3.2.1]octane



Chemical Formula: $C_{14}H_{24}$
Exact Mass: 192,2

19
Muurol-5-en-4-one <cis-14-nor->

20
Acetic acid, 7-hydroxy-1,3,4,5,6,7-hexahydro-2H-naphthalen-4a-ylmethyl ester



Chemical Formula: $C_{13}H_{20}O_3$
Exact Mass: 224,1

21
4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-*Z*)-

Chemical Formula: $C_{23}H_{34}O_2$
Exact Mass: 342,3

22
Hexadecanoic acid, methyl ester

Chemical Formula: $C_{17}H_{34}O_2$
Exact Mass: 270,3

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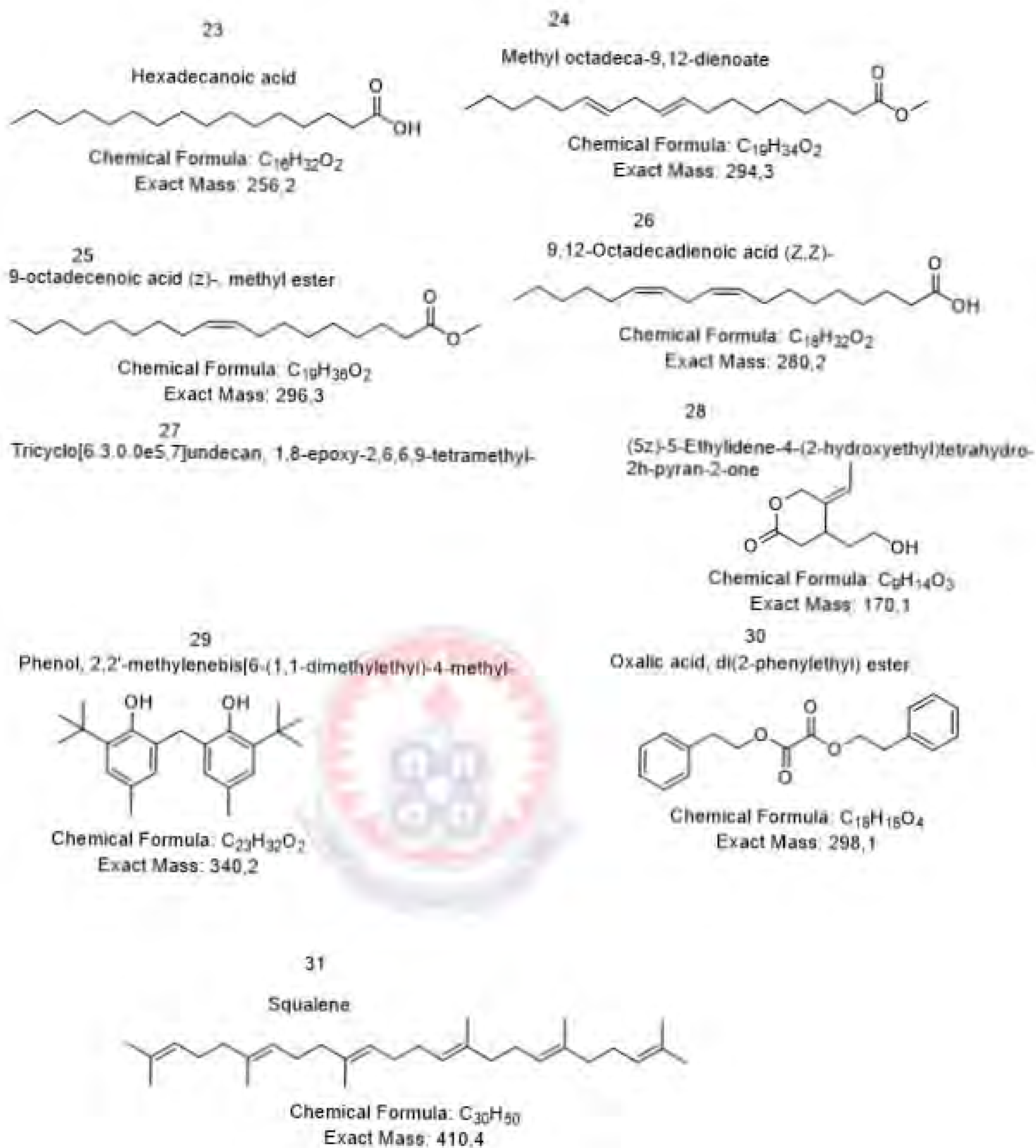

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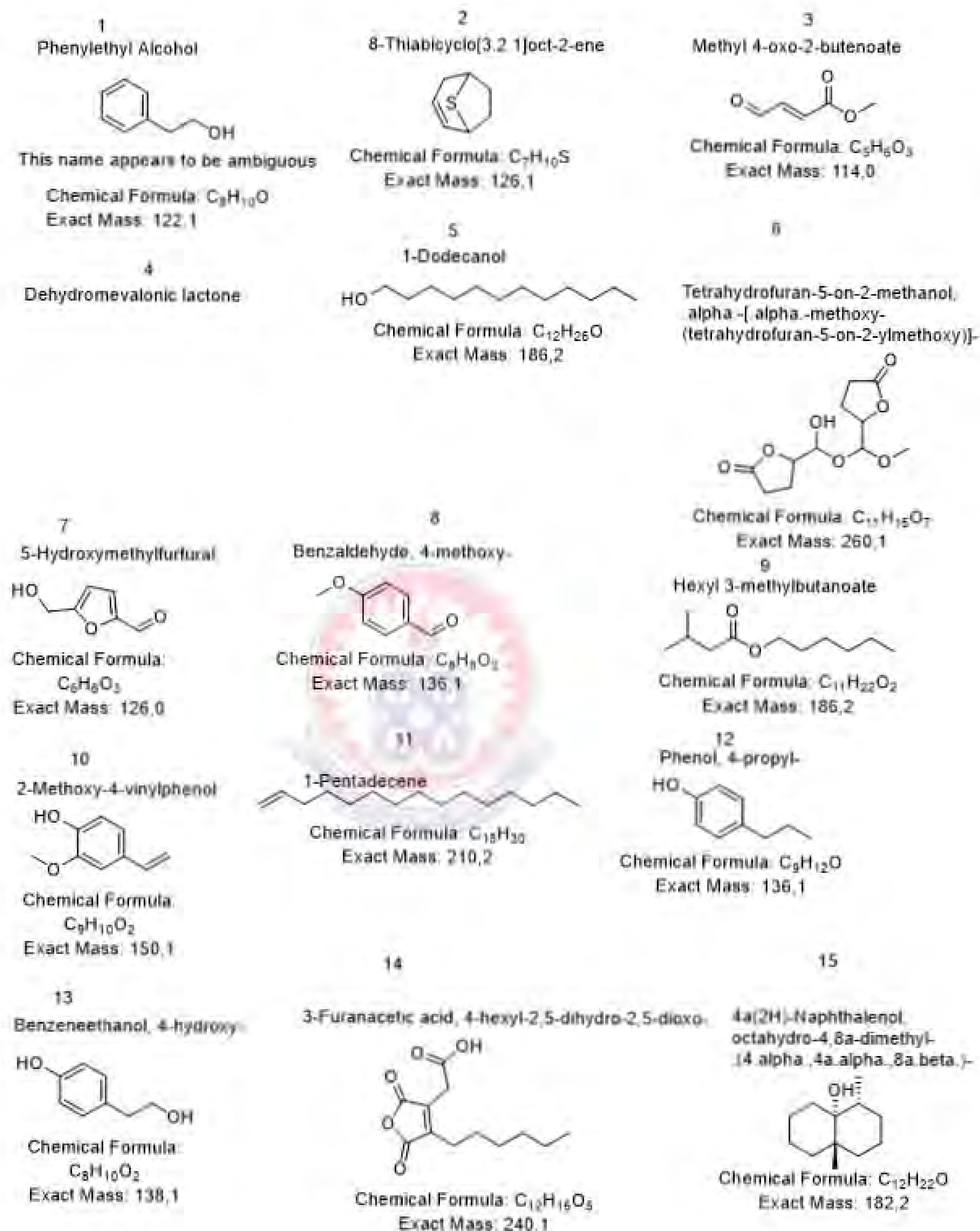
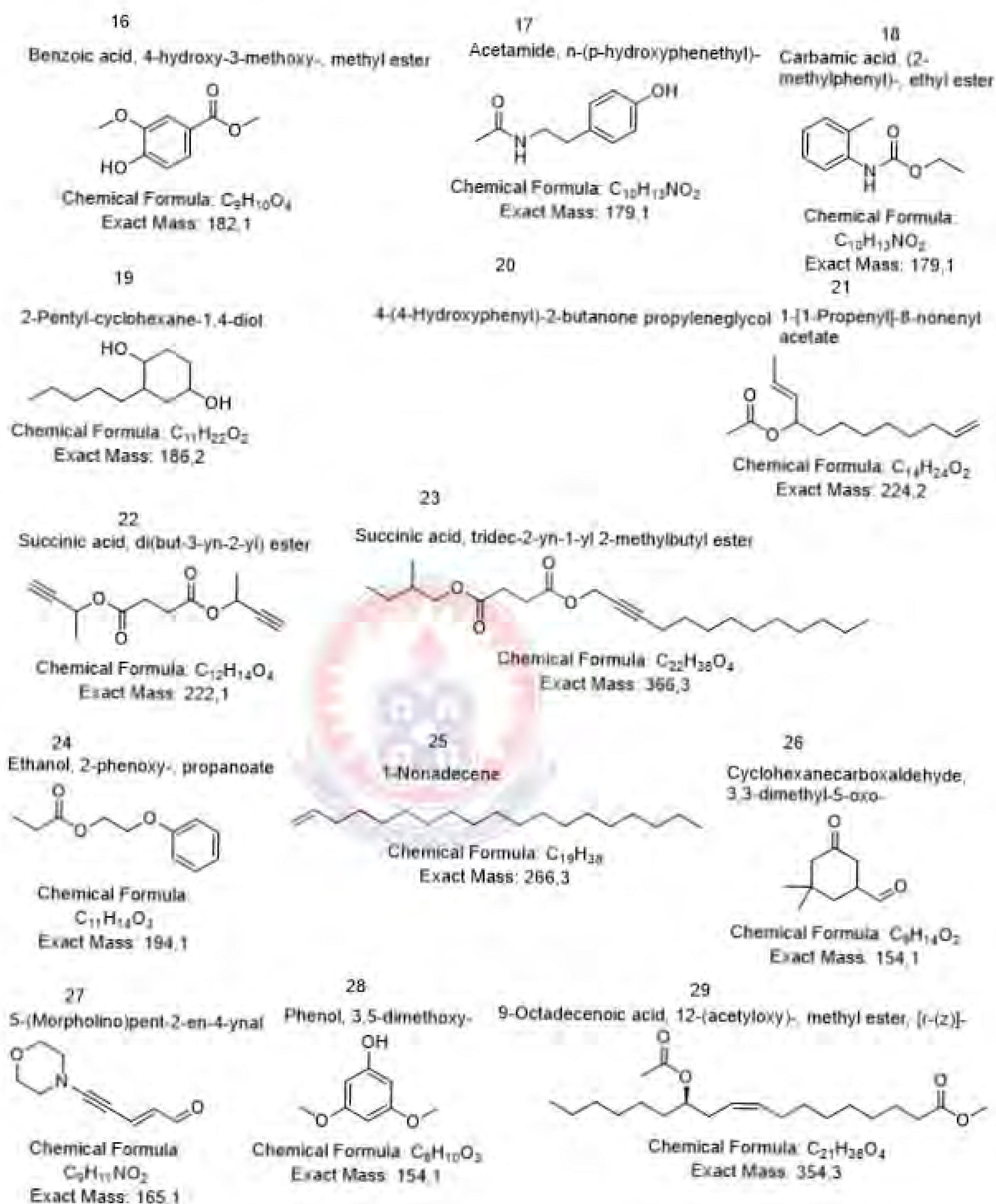
Structures of compounds identified in BWMS




16
Benzoic acid, 4-hydroxy-3-methoxy-, methyl ester
Chemical Formula: C9H10O4
Exact Mass: 182.1
17
Acetamide, n-(p-hydroxyphenethyl)-
Chemical Formula: C10H13NO2
Exact Mass: 179.1
18
Carbamic acid, (2-methylphenyl)-, ethyl ester
Chemical Formula: C10H13NO2
Exact Mass: 179.1
19
2-Pentyl-cyclohexane-1,4-diol
Chemical Formula: C11H22O2
Exact Mass: 186.2
20
4-(4-Hydroxyphenyl)-2-butanone propyleneglycol
21
1-[1-Propenyl]-8-nonenyl acetate
Chemical Formula: C14H24O2
Exact Mass: 224.2
22
Succinic acid, di(but-3-yn-2-yl) ester
Chemical Formula: C12H14O4
Exact Mass: 222.1
23
Succinic acid, tridec-2-yn-1-yl 2-methylbutyl ester
Chemical Formula: C22H38O4
Exact Mass: 366.3
24
Ethanol, 2-phenoxy-, propanoate
Chemical Formula: C11H14O3
Exact Mass: 194.1
25
1-Nonadecene
Chemical Formula: C19H38
Exact Mass: 266.3
26
Cyclohexanecarboxaldehyde, 3,3-dimethyl-5-oxo-
Chemical Formula: C9H14O2
Exact Mass: 154.1
27
5-(Morpholino)pent-2-en-4-ynal
Chemical Formula: C9H11NO2
Exact Mass: 165.1
28
Phenol, 3,5-dimethoxy-
Chemical Formula: C8H10O3
Exact Mass: 154.1
29
9-Octadecenoic acid, 12-(acetyloxy)-, methyl ester, [r-(z)]-
Chemical Formula: C21H38O4
Exact Mass: 354.3

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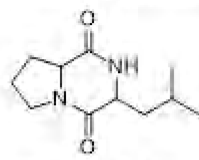
30

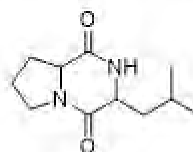
3-Isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione



Chemical Formula: $C_{11}H_{18}N_2O_2$
Exact Mass: 210,1

31

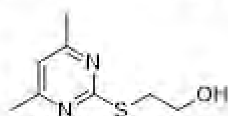
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-



Chemical Formula: $C_{11}H_{18}N_2O_2$
Exact Mass: 210,1

32

2-[(4,6-Dimethyl-2-pyrimidinyl)sulfanyl]ethanol



Chemical Formula: $C_8H_{12}N_2OS$
Exact Mass: 184,1

33

Hexadecanoic acid

Chemical Formula: $C_{16}H_{32}O_2$
Exact Mass: 256,2

34

9,12-Octadecadienoic acid (Z,Z)-

Chemical Formula: $C_{18}H_{32}O_2$
Exact Mass: 280,2

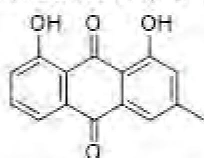
35

Tetradecanoic acid, ethyl ester

Chemical Formula: $C_{16}H_{32}O_2$
Exact Mass: 256,2

36

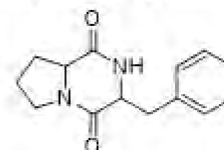
9,10-Anthracenedione, 1,8-dihydroxy-3-methyl-



Chemical Formula: $C_{15}H_{10}O_4$
Exact Mass: 254,1

37

3-Benzylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione



Chemical Formula: $C_{14}H_{16}N_2O_2$
Exact Mass: 244,1

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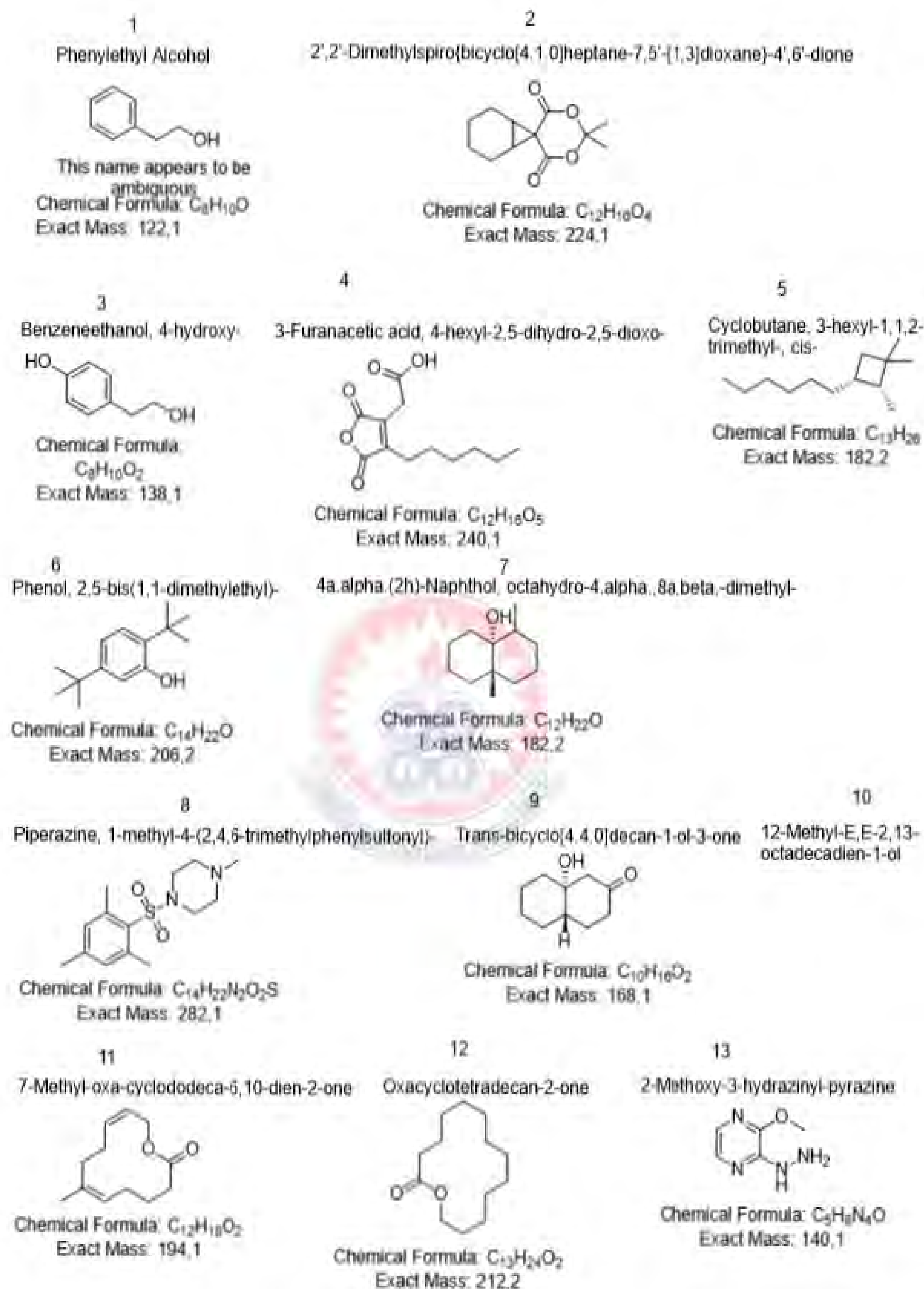
Structures of compounds in BBMS



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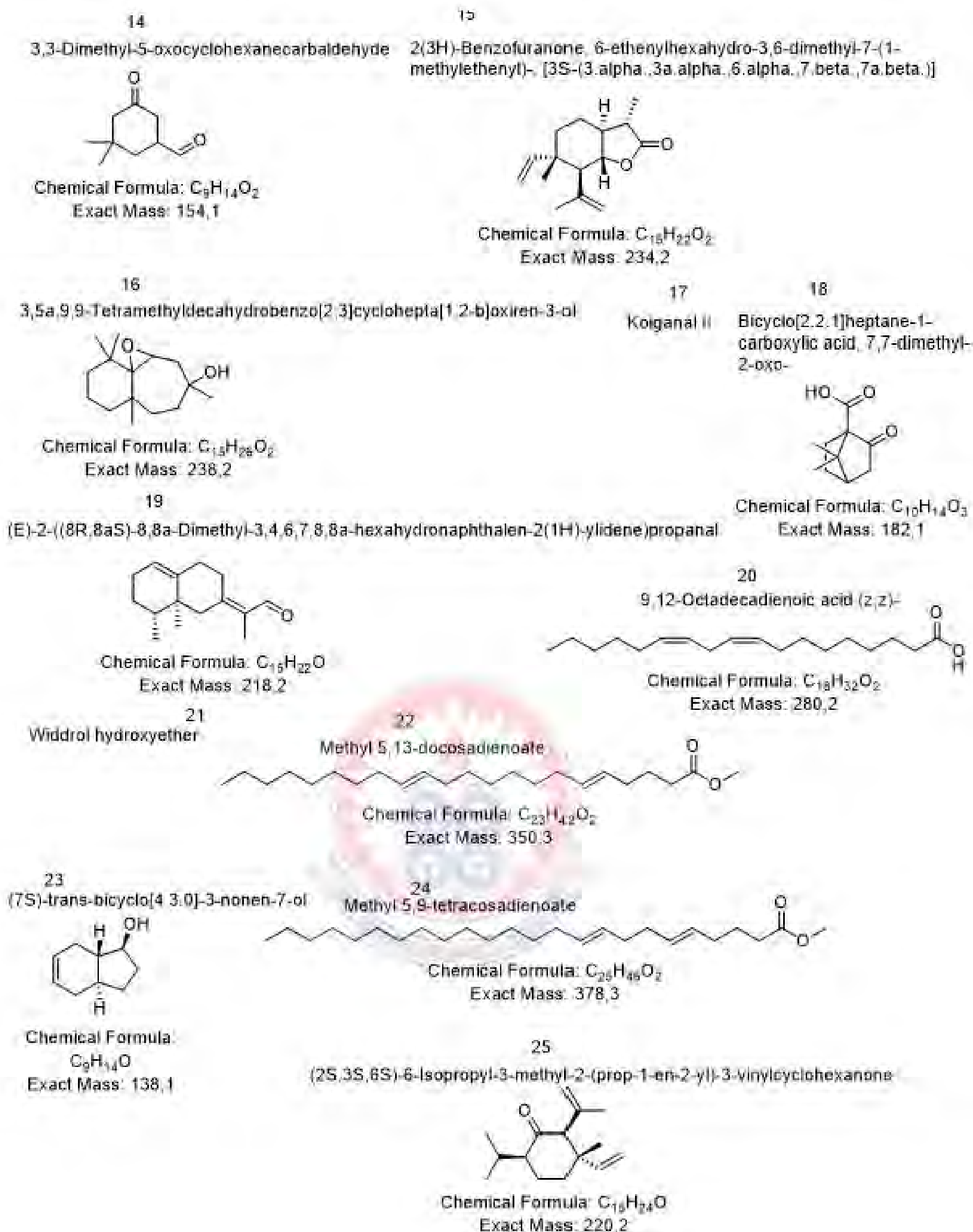

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Structures of compounds in BBMAV